An Ion-Exchange Reagent for Use in the Chromatography of Large Peptides

By J. FEITELSON* AND S. M. PARTRIDGE

*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge,
and Department of Scientific and Industrial Research*

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In attempts to determine the amino acid sequence in proteins, two major problems arise which are as yet unsolved. The first is the development of methods for the specific cleavage of the protein chain into a limited number of fragments of a size that is not too great for sequence analysis by the methods introduced by Sanger and his colleagues (Sanger, 1945; Sanger & Tuppy, 1951). The second is to devise methods suitable for the isolation and purification of the chain fragments and, not less important, to provide rigid tests for the chemical homogeneity of the peptides isolated. Digestion with proteolytic enzymes provides a possible solution to the first of these problems. However, since the cleavage of the various susceptible bonds proceeds at very different rates, the reaction cannot be brought wholly to completion; thus some of the chains are degraded to a less extent than others and this gives rise to a great increase in the complexity of the mixture to be separated.

A successful fractionation procedure must be suitable for the separation of peptides containing some 10 to 30 residues and must have a very high resolving power; it must also be able to deal with quantities of the order of 50 mg. of each peptide. With these considerations in mind, it was thought probable that elution chromatography would provide the only method of fractionation with sufficient resolving power and which at the same time would be capable of handling the amount of material required. To avoid difficulties due to irreversible adsorption, preference was given to the investigation of ion-exchange reagents rather than to molecular adsorbents such as charcoal.

Conventional ion-exchange resins are unsuitable for the purpose since peptides of this size diffuse only slowly within the gel-like structure of the resin particles. The position is improved somewhat if resins having a very low degree of cross-linking are used; but here a difficulty is met with in the very high degree of swelling and shrinking suffered by such resins as the pH or ionic strength of the ambient solution is varied. However, it seemed possible that the disadvantages of swelling and

shrinkage could be avoided if a resin with a very low degree of cross-linkage were spread as a thin film on the surface of an inert extender. Such a resin would be particularly suitable for the chromatography of large peptides since the very great surface presented by the layers of resin, and their extreme thinness, would both tend to reduce the effects of slow diffusion which must always be encountered in attempts to separate substances of high molecular weight.

In the preparation of a resin of this kind, the choice of the extender will be important. The powder used must be sufficiently inert to permit chemical operations such as sulphonation to be carried out on the coated particles and also to ensure complete insolubility in the common buffers; the particles of extender must present a large and irregular surface to 'key' with the resin, and the properties of the powder should be such as will ensure uniform packing in columns. These considerations led to the choice of a commercial preparation of diatomaceous earth. A composite resin, prepared by coating diatomaceous earth with sulphonated polystyrene, has already been prepared in this laboratory by Boardman (1955). This material was intended for the separation of proteins and differed from the reagent described here in that the resin used for coating was highly cross-linked; this prevented the ingress of protein molecules so that adsorption was at the surface only.

In discussing the effect of alterations in the degree of cross-linking of ion-exchange resins it should be pointed out that considerable use has already been made of this principle both in attempts to improve the separation of amino acids and to extend the range to peptides with up to five or six residues. In 1950, Partridge, Brimley & Pepper introduced a lightly cross-linked sulphonated polystyrene resin (4-5% nominal divinylbenzene content) for the separation of amino acids by displacement chromatography, and sulphonated polystyrene resins with a similar degree of cross-linking were later used by Moore & Stein (1954), and Campbell, Jacobs, Work & Kressman (1955) to improve the performance of elution chromatograms. Dowmont & Fruton (1952) showed that a number of

* Present address: Department of Physical Chemistry, The Hebrew University, Jerusalem, Israel.

simple peptides may be satisfactorily chromatographed with a sulphonated-polystyrene resin cross-linked with 4 % of divinylbenzene, and similar resins have been used recently by Askonas, Campbell, Godin & Work (1955) and Thompson (1955), both of whom report excellent separations obtained with complex mixtures of small peptides.

EXPERIMENTAL

Materials

Styrene. The commercial material contained a polymerization inhibitor which was removed by extracting four times with 6 % (w/v) NaOH, followed by washing with water until the aqueous phase was no longer alkaline. The styrene was then dried over anhydrous Na_2SO_4 and distilled under a reduced pressure of N_2 .

Divinylbenzene. The commercial material (supplied as nominally 50 %) was treated with NaOH as described above for styrene but was not distilled. The dry product was assumed to contain 50 % of divinylbenzene.

Celite 545. The preparation, as received from the manufacturers (Johns-Manville Co., London), was made water-repellent by treatment with dichlorodimethylsilane according to the method of Howard & Martin (1950).

Fraction B of oxidized insulin. This was prepared according to the procedure of Sanger (1949) from a commercial preparation of crystallized insulin (Boots Pure Drug Co. Ltd.).

Mixture of peptides from fraction B of oxidized insulin. The phenylalanyl chain of insulin was hydrolysed with trypsin according to the method of Sanger & Tuppy (1951). The trypsin used was a crystalline preparation from Armour Laboratories, but no attempt was made to purify it by further recrystallization. The hydrolysis mixture, after heating for 2 min. at 100° , was acidified to pH 4 with acetic acid. The light precipitate which formed was removed by centrifuging and the solution containing the mixture of peptides was then freeze-dried for storage until use.

Methods

Preparation of the ion-exchange reagent. Styrene (10 ml.), divinylbenzene (commercial 50%, 0.25 ml.) and CHCl_3 (50 ml.) were mixed together and benzoyl peroxide (0.16 g.) was added. The silane-treated Celite (10 g.) was placed in a Büchner flask fitted with a tap-funnel and brought under low vacuum. The mixture of monomers and solvent was then introduced through the funnel and the whole contents of the flask were shaken thoroughly. In order to control the thickness and structure of the coating layer, the excess of the reaction mixture was removed by centrifuging under controlled conditions; this was carried out in the following way.

After standing for 1 hr. at 0° , the mixture was transferred quantitatively to a weighed filter tube (diameter 3 cm.) fitted with a sintered-glass plate. The filter was fitted into a centrifuge tube over a thick pad of cotton wool and centrifuged at approximately 140 g for 18 min. By weighing the filter tube it was shown that the Celite had retained 0.65–0.70 g. of monomer solution/g. of Celite. Most of the CHCl_3 was now evaporated by drawing dry N_2 through the filter until the weight of liquid retained by the Celite had fallen to 0.3 g./g. The wetted Celite, which was a free-flowing powder,

was now transferred to a thick-walled glass ampoule, cooled in solid CO_2 and sealed. Polymerization was allowed to take place at 60° for 24 hr. and then continued at 80° for a further 6 days.

Sulphonation. The resin-coated Celite (10 g.) was sulphonated with 50 ml. of H_2SO_4 and 25 mg. of Ag_2SO_4 (as catalyst) by heating the mixture on a water bath, with occasional shaking, for 1 hr. After cooling, the mixture was gradually diluted first with 50 % (v/v) H_2SO_4 and then with water. It was then poured into water and washed by decantation. When free from acid the product was passed through a 50 mesh/in. screen by sifting in the wet condition and assisted by a jet of water; the few coarse particles and agglomerates were discarded. The material was stored under 1 % (w/v) Na_2CO_3 solution until required for use.

Determination of the exchange capacity showed that 1 ml. of the wet reagent (measured by packing as a column in a filtration tube) exchanged 0.11 m-equiv. of Na^+ ions. When stained with basic fuchsin, microscopic observation of the particles revealed that the coating of the diatoms was fairly even and the preparation was free from large impervious aggregates. A high proportion of the vacuoles in the diatom skeletons were filled with swollen resin.

Chromatography of a mixture of peptides prepared by tryptic digestion of the 'B' chain of insulin. The buffer was prepared by mixing 0.05 M solutions of NaH_2PO_4 and Na_2HPO_4 in the volume ratio 27:73 and had pH 6.44 (glass electrode at 20°). The ion-exchange reagent described above was suspended in the buffer solution and after standing for 45 min. the cloudy supernatant was poured off. This procedure was repeated 2–3 times until the supernatant was clear. A chromatographic filtration tube 18 mm. in diameter was filled to a height of approximately 10 cm. with a slurry of buffer and resin (2:1, v/v) and left to settle and drain. Buffer was then passed through the column at a pressure head of 1 m. to consolidate the layer; in this way the filtration tube was filled in stages to a height of 39 cm.

A solution of the mixed peptides in buffer (0.3–0.5 ml., containing 3–5 mg. of peptides) was then applied carefully to the top of the column and the liquid level allowed to sink to the surface of the resin. The column was then carefully covered with buffer and elution was allowed to continue at room temperature under a pressure head of 6 cm., the rate of flow being 2 ml./hr. Fractions (1 ml.) of the effluent solution were collected with the aid of an automatic fraction collector at half-hourly intervals.

Analysis of the effluent solution. Procedures based on reaction with ninhydrin are extremely sensitive for amino acids and lower peptides, but are often insensitive, or fail altogether, with higher peptides. The converse is true with colorimetric methods based on the biuret reaction. Since the molecular-weight spectrum in the mixture was wide (Sanger & Tuppy, 1951), it was considered advisable to employ both types of procedure for the analysis of the effluent fractions; accordingly, alternate fractions were estimated by the ninhydrin procedure of Moore & Stein (1951) and the modified biuret method of Lowry, Rosebrough, Farr & Randall (1951).

Separation of the peptide of peak E from buffer salts

(a) **Ion-exchange method.** The fractions forming peak E (Fig. 1) were combined and the solution (50 ml.) was passed through a mixed-bed column containing Dowex 50 (Dow Chemical Company, Michigan, U.S.A.) and De-acidite G

(The Permutit Co. Ltd., London). To reduce surface adsorption of the peptide, the resins chosen were in the form of large beads, 15–30 mesh/in.; the column was prepared by packing a filtration tube (diameter 10 mm.) with a slurry prepared from the mixture of Dowex 50 (H^+ form; 1.1 g. dry wt.) and De-acidite G (OH^- form; 1.2 g. dry wt.). The buffered solution was allowed to flow through the column at 20 ml./hr. The column was then washed with 50 ml. of water and the washings were added to the desalted filtrate containing the peptide. Recovery of peptide was 75–85% in different experiments.

(b) *Charcoal method.* A mixture of charcoal ('Activated charcoal', British Drug Houses Ltd.) and Celite 545 (1:8, w/w) was saturated with 1% (w/v) ethanolic solution of stearic acid (Porath, 1954*a, b*) and washed with 50% (v/v) propanol–water mixture, followed by water. The mixture was then packed into a filtration tube (about 1.0 g. of mixture for each mg. of peptide) and the solution of peptide in buffer allowed to filter through. After washing the column with water, the peptide was displaced with 50% (v/v) propanol–water. The solvent was then removed by evaporation.

RESULTS

In their study of the reaction mixture resulting from the hydrolysis of the 'B' chain of insulin with enzymes Sanger & Tuppy (1951) showed that the only bonds split to any extent by trypsin are those involving the carboxyl groups of the arginine and lysine residues. From a knowledge of the amino acid sequence in the 'B' chain as determined by Sanger & Tuppy the mixture of peptides resulting from the action of trypsin would be expected to contain three major components: a polypeptide containing 22 residues terminating in arginine, a heptapeptide terminating in lysine, and free alanine, which represents the C-terminal residue in the chain.

Two-dimensional chromatograms of the reaction mixture carried out by Sanger & Tuppy showed three ninhydrin-reacting spots in the hydrolysate, i.e. alanine and two other spots which were designated *Bt 2* and *Bt 3*. *Bt 2* was shown to be the expected heptapeptide and *Bt 3* was of similar amino acid composition to *Bt 2* but contained 'tyrosine X' instead of tyrosine. No free-moving spot which could be ascribed to a peptide with 22 residues was found. *Bt 2* was contaminated with small quantities of other ninhydrin-reacting substances which did not appear to be an integral part of the peptide. In the experiments of Sanger & Tuppy, and also in the present work, only a small amount of material having the same amino acid composition as the original 'B' chain precipitated after acidification of the reaction mixture. The major part of the material, representing the 22 residues in the 'B' chain which are left after removal of *Bt 2* and alanine, remains in the filtrate, but fails to migrate on the two-dimensional paper chromatograms. It was first assumed that this material was present as a single poly-

peptide with 22 residues, but, as is described later, amino acid analysis of the peptide after isolation by the new procedure showed it to contain the expected amino acids with the exception of arginine. It can be inferred that, under the conditions used in these experiments, trypsin splits the chain at another point in addition to those established by Sanger & Tuppy, and this results in the loss of the terminal arginine residue or, more probably, a short terminal sequence containing arginine.

Preliminary experiments with columns packed with the new ion-exchange reagent showed that the monoaminomonocarboxylic acids were unadsorbed above pH 3 but the higher peptides from oxidized insulin were strongly adsorbed at pH 6–7 in spite of their very low isoelectric points. In the present work we were particularly interested in the large polypeptide thought to contain 22 residues, and a number of trials showed that it could be isolated from the other components of the mixture by elution chromatography with the new resin and 0.05M phosphate buffer of pH 6.4. Fig. 1 shows a chromatogram obtained from the mixture of peptides by elution at this pH, in which the peptide concentration in the eluate was estimated by means of the biuret reaction (Lowry *et al.* 1951). The chromatogram shows six main peaks. Peaks *A*, *B*, *C* and *D* gave relatively strong reactions with the ninhydrin reagent of Moore & Stein (1951) and were therefore due to free amino acids or small peptides; peak *E* gave only a faint ninhydrin reaction, suggesting a compound of high molecular weight. Peak *F* was a displacement peak produced during the buffer change to pH 8.7 and represented material which was irreversibly adsorbed at pH 6.4.

The chromatogram was accurately reproducible from experiment to experiment, both as regards the height and position of the peaks. The pattern suggested that the mixture may have contained more minor components than would be inferred from the description of Sanger & Tuppy, but this may have been due to the use of a less highly purified enzyme preparation. As would be expected, peak *A* contained alanine and small quantities of other amino acids. Peaks *B*, *C* and *D* were not investigated in detail, but the relative colour yields from the ninhydrin and biuret reactions suggested that they were peptides of intermediate molecular weight. The fractions of peak *E*, in which the main interest of this work lies, were collected together and the peptide was freed from buffer salts in preparation for further analysis.

Separation of the peptide from buffer salts

Two methods of separating the peptide from buffer salts were investigated. The first involved the use of commercial ion-exchange resins in bead form in the belief that the molecular weight of the peptide

would be too high to permit any but surface adsorption. This proved to be the case, and it was found that the dilute solution of the peptide resulting from collection of the fractions of peak *E* could be efficiently desalted, without undue loss, by passage through a mixed-bed column containing a mixture of Dowex 50 and De-acidite G (both resins 15- to 30-mesh/in.). The weight of mixed resin used was in each case fixed at the minimum required to remove the buffer salts, to avoid unnecessary losses by surface adsorption. Since, however, this method of desalting would not be satisfactory for peptides of lower molecular weight, an alternative procedure, which may be more generally useful, was investigated. This was based on the adsorption of the peptide from salt solution by charcoal pretreated with stearic acid (cf. Porath, 1954*b*), and subsequent desorption of the peptide with propanol-water mixture. Both methods gave recoveries of peptide in the range 75–85 %.

Amino acid constitution of the peptide of peak E

The peptide was hydrolysed for 18 hr. in a closed tube with 6*N* hydrochloric acid at 103°. Two-dimensional paper chromatography showed the presence of aspartic acid, glutamic acid, cysteic acid, glycine, serine, alanine, valine, leucine, histidine, phenylalanine and tyrosine; of these the spots due to leucine and valine were very heavy. The amino acids threonine, lysine, proline and arginine, which are present in the 'B' chain of insulin, were not observed in the peptide. As already pointed out, the amino acid constitution of the peptide accords

with that given by Sanger & Tuppy (1951), except that in the present experiments arginine was not observed.

DISCUSSION

Since the peptide of peak *E* does not contain arginine its complete identity cannot be established. However, the absence of threonine, lysine and proline clearly shows that the peptide was not contaminated with unchanged fraction *B*, and all that is in doubt is the length of the terminal sequence containing arginine, which was unexpectedly missing. From the sequence found by Sanger & Tuppy (1951), together with the amino acid analysis reported here, it is nevertheless clear that the peptide contains not more than 21 residues and not less than 16. This identification is sufficient for the present purpose since what we desire to establish in this preliminary work is that a peptide of this order of size moves as a narrow and symmetrical peak on the new chromatogram and has a distribution ratio in the useful range. The great difference in adsorption behaviour between amino acids and small peptides on the one hand and the peptide of peak *E* on the other suggests that the method will prove to be of high resolving power.

Since polypeptides are ampholytes, ion-exchange chromatography is possible both with anion-exchange and with cation-exchange resins. There are, however, certain advantages to be gained by limiting the choice of resins to those containing highly ionized acidic or basic groups, since these are effective over the whole pH range and exhibit little

Fig. 1. Chromatogram showing separation of the mixture of peptides from trypsin digestion of the 'B' chain of oxidized insulin. Buffer, 0.05*M* phosphate, pH 6.45; column, 39 cm. $\times$ 18 mm.; flow rate, 2 ml./hr. Fractions (1 ml.) were taken at 30 min. intervals and analysed by means of the modified biuret method of Lowry *et al.* (1951).

buffering capacity. The present work has been based on the use of sulphonated polystyrene resins as these have additional advantages in stability and ease of preparation.

To ensure the maximum rapidity of diffusion when dealing with peptides in the high-molecular-weight range, it was considered necessary to reduce the degree of cross-linking of the resin to the minimum compatible with stability; from preliminary experiments this limit appeared to be in the range provided by divinylbenzene contents of the order of 0.5–1.0 %. The greatest difficulty met with in early experiments lay in securing adequate adhesion of the resin film to the surface of the extender, and the procedure finally adopted was a modification of that described by Boardman (1955). The Celite was first treated with dichlorodimethylsilane to make it water-repellent. The particles of extender were then coated with a mixture of styrene, divinylbenzene and polymerization catalyst in a volatile solvent, and after removal of solvent the mixture was polymerized on the surface of the particles by raising the temperature. Finally sulphonation was carried out by treating the coated powder with concentrated sulphuric acid in the presence of a catalyst.

With some preparations it was found that there was a tendency for the resin film to break away from its support during use. This appeared to be due to the swelling or shrinkage of the resin film that accompanies changes in pH or salt concentration. Such volume changes are much more marked with resins of a low degree of cross-linking, which may take up as much as 10–15 times their weight of water, than they are with less porous resins of the conventional type. The diatom skeletons, of which Celite is composed, are plate-like structures, many of which are pierced with a regular arrangement of holes of a size great enough to allow the penetration of resin. Advantage was taken of this to secure a firm 'key' for the resin film; but it was found that if too great a proportion of resin were applied to the particles, the structures tended to break down when the sulphonated product was allowed to swell in water. Accordingly, the thickness of the resin coat was regulated by applying the mixture of monomers as a solution in an inert solvent. Part of the solvent was then removed before carrying out the polymerization reaction.

The effect of varying the degree of cross-linkage of sulphonated polystyrene resins has been studied by several workers (cf. Partridge *et al.* 1950; Hale, Packham & Pepper, 1953). Since the rate of adsorption of large organic cations is considerably influenced by the degree of resin cross-linkage, it seems probable that a mechanism of exchange governed by particle diffusion may be rate-determining (for the theory see Boyd, Adamson & Myers, 1947, and Glueckauf, 1955). To improve the rate of

diffusion with high-molecular-weight substances the present exchange reagent was prepared so that the resin films spread on the diatom structures presented only a short path of diffusion, and at the same time the low degree of cross-linking facilitated the exchange.

When amino acids are separated by chromatography on an ion-exchange column their distribution between the resin and buffer solution phases is a function of their degree of ionization, and this is in turn determined by the pH of the surrounding liquid. The cation concentration inside the resin will be influenced by the electrostatic potential in the vicinity of the fixed-resin charges. In the cation exchanger this means that, assuming a constant ratio of H^+ to other cations in the two phases, the H^+ concentration also will be higher in the resin phase. As the electrostatic potential increases with charge density it is evident that this effect will increase with the increasing degree of cross-linkage. In other words, to maintain a certain pH inside the resin matrix it will be necessary to employ a more acidic buffer for a highly swollen, lightly cross-linked ion-exchanger. This effect becomes apparent when comparing the separation of aspartic acid and alanine with a commercial highly cross-linked sulphonated-polystyrene resin (Moore & Stein, 1951) with a similar separation carried out with the present resin. Distribution coefficients in the useful range were obtained at pH 3.42 in the experiments of Moore & Stein, but it was necessary to reduce the pH to 2.7 before a useful degree of adsorption was obtained with the new resin.

With substances of higher molecular weight another factor has to be considered, namely, the contribution of other than coulombic forces. These forces—van der Waals interactions and hydrogen-bond formation—are not greatly influenced by the pH or ionic strength of the buffer; the bonds are of short range, and although individually weak they are collectively strong. The contribution of short-range forces assumes a much greater importance with the higher peptides than with amino acids and lower peptides; thus in the experiments described here adsorption of the large acidic peptide from the 'B' chain of insulin was affected from buffers of pH 6.4, in which it carries a strong net negative charge.

SUMMARY

1. An ion-exchange reagent designed specifically for the chromatographic separation of large peptides is described.

2. The reagent was prepared by applying a thin coating of a lightly cross-linked styrene polymer to the siliceous diatom skeletons which comprise a selected commercial filter-aid. The film of resin was

sulphonated by reaction with sulphuric acid in the presence of catalyst.

3. The performance of the new reagent was tested in relation to the isolation of a large acidic peptide from the products of trypsin digestion of the 'B' chain of oxidized insulin.

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The Biosynthesis of Ergothioneine and Histidine by *Claviceps purpurea*

1. THE INCORPORATION OF [2-¹⁴C]ACETATE

By H. HEATH AND JENNIFER WILDY

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

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Although ergothioneine occurs in blood (Hunter & Eagles, 1927), semen (Mann & Leone, 1953) and in various mammalian tissues, principally liver and brain (Melville, Horner & Lubschez, 1954), it would appear to be exogenous in origin, as no biosynthesis has been demonstrated in animals. Melville, Horner, Otken & Ludwig (1955) and Melville, Otken & Kovalenko (1955) studied the incorporation of ¹⁴C-labelled histidine, formaldehyde, glycine and methionine by the rat and chicken and ³⁵S-labelled methionine and cystine by guinea pig, rat, pig and human, and in no case was there any demonstrable incorporation of isotope into the isolated ergothioneine. The earlier work of Heath, Rimington, Glover, Mann & Leone (1953) on the utilization of [³⁵S]methionine for this biosynthesis by the mature pig has not, after fuller investigation by these workers, been confirmed; the evidence now available is quite conclusive that, under the feeding conditions of the experiment, the seminal ergothioneine of the pig was not derived from the sulphur of methionine (Heath, Rimington & Mann, 1957).

In order to investigate the biosynthetic pathway of ergothioneine we decided to study the culture of ergot (*Claviceps purpurea*), from which ergothio-

neine was originally isolated by Tanret (1909). Much work on the artificial culture of this fungus has been carried out, principally to see whether the important pharmacological alkaloids could be formed. Nutritional requirements for submerged culture have been established by Tyler & Schwarting (1952). Although alkaloids are not formed in artificial culture by the standard strain of *C. purpurea*, Abe (1948) and Stoll, Brack, Kobel, Hofmann & Brunner (1954) have isolated two strains from *Agropyrum* and *Pennisetum* respectively, which produce some alkaloids (agroclavine and penniclavine) but not the principal naturally occurring alkaloids of ergot (ergotamine, etc.). This paper describes the isolation and degradation of ergothioneine from a culture of *C. purpurea* grown on a medium containing [2-¹⁴C]acetate; a relationship between the biosynthesis of histidine and ergothioneine has been established. Ames & Mitchell (1955), Levy & Coon (1951, 1954), Ehrensward, Reio, Saluste & Stjernholm (1951) and Westley & Ciethaml (1956) have studied the biosynthesis of histidine by micro-organisms and it would appear that the five-carbon chain is not derived from glutamate or other derivatives of the citric acid