in the sense that they all contain fungus-like symbionts in the renal sac (*4*); aquarium culture should show whether these microorganisms are metabolically associated with any of the solid deposits in the renal sac. The production of oxalate by many fungi (*13*) raises the possibility that the weddellite crystals in *Molgula* are a metabolic product of the renal sac symbionts, rather than of *Molgula* itself.

Although the role of the renal sac remains unclear, our results do indicate that in *M. manhattensis* the chemical activities of the sac are not limited to the production and accumulation of uric acid. The contents and metabolic activities of the renal sac are thus unexpectedly complex and suggest the need for further scrutiny of the organ's role in the biology of the Molgulidae.

MARY BETH SAFFO
Department of Botany, University of California, Berkeley 94720
HEINZ A. LOWENSTAM
Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena 91125

References and Notes

1. P.-J. van Beneden, *Mem. Acad. R. Belg.* **20**, 3 (1847); H. de Lacaze-Duthiers, *Arch. Zool. Exp. Gen.* **3**, 304 (1874).
2. I. Goodbody, *J. Exp. Biol.* **42**, 299 (1965).
3. J. R. Nolfi, thesis, University of California, Berkeley (1965); *Comp. Biochem. Physiol.* **35**, 827 (1970).
4. M. B. Saffo, thesis, Stanford University (1976); *J. Morphol.* **155**, 287 (1978).
5. The NaClO-resistant residue also includes an assemblage of fine white material whose infrared absorption bands (1642 and 1324 cm^{-1}) match those of weddellite. Scanning electron micrographs, however, show not only dipyramidal microcrystals in this fraction, but an array of other particles as well. Thus, the composition of some constituents of this material remains to be determined.
6. R. D. Lewis, H. A. Lowenstam, G. R. Rossman, *Arch. Pathol.* **98**, 149 (1974).
7. H. A. Lowenstam, *Science* **162**, 1129 (1968).
8. Infrared absorption spectra are routinely employed by one of us (H.A.L.) to detect and characterize x-ray-amorphous compounds, which may occur together with crystalline material or form crystal nuclei. We used infrared spectrophotometry here to be sure that both amorphous and crystalline material would be detected.
9. M. Azéma, *Ann. Inst. Oceanogr. Monaco* **17**, 1 (1937); A. Nørrevang, *Proc. 6th Int. Congr. Electron Microsc.* (1966), p. 669.
10. F. Gaill, *C. R. Acad. Sci.* **279**, 351 (1974); ——— and P. Lasserre, *ibid.* **285**, 1343 (1977); M. Miller and R. D. Packer, *Comp. Biochem. Physiol. A* **57**, 423 (1977).
11. B. T. Murphy and L. N. Pyrah, *Br. J. Urol.* **34**, 129 (1962).
12. E. L. Prien and E. L. Prien, Jr., *Am. J. Med.* **45**, 654 (1968); K. Lonsdale and D. J. Sutor, *Sov. Phys. Crystallogr.* **16**, 1060 (1972).
13. J. Foster, *Chemical Activities of Fungi* (Academic Press, New York, 1949); W. C. Graustein, K. Cromack, Jr., P. Sollins, *Science* **198**, 1252 (1977).
14. We thank M. Dekkers and J. Muramoto for technical assistance and W. Mays for editorial advice. S. Weiner took the scanning electron micrographs shown in Fig. 1. A portion of this work was included in a doctoral thesis submitted to Stanford University (M.B.S.). We acknowledge the support of NIH training grant GM 365-14 (M.B.S.), a Sigma Xi grant-in-aid (M.B.S.), a Miller research fellowship (M.B.S.), and NSF grant DES 76-03725 (H.A.L.). California Institute of Technology contribution No. 3030.

24 January 1978; revised 28 March 1978

Reversed-Phase, High-Pressure Liquid Chromatography of Peptides and Proteins with Ion-Pairing Reagents

Abstract. *Reversed-phase, high-pressure liquid chromatography has been successfully applied to the analysis of peptides and proteins by the addition of hydrophilic (for example, phosphoric acid) or hydrophobic (for example, hexanesulfonic acid) ion-pairing reagents, or both, to the mobile phase. Examples described included proteins such as insulin, glucagon, and 1-24 ACTH pentaacetate (ACTH is adrenocorticotrophic hormone).*

The isolation of peptides and proteins, frequently present in only trace amounts in biological systems, has enlarged our understanding of the molecular biology of many complex biochemical and endocrine pathways. When high-pressure liquid chromatography (HPLC) (*1*) was introduced as an analytical tool, it was anticipated that this technique would allow the rapid but selective separation of natural polypeptides (*2*). The early applications of HPLC to the analysis of underivatized peptides and proteins were not entirely successful, however, with the use of either liquid-solid or liquid-liquid reversed-phase systems. Poor resolution was frequently observed to be associated with peak broadening and long retention times (*3, 4*). These features are not altogether unexpected when one considers the complex ionic equilibria that these amphoteric compounds can undergo. In a recent publication (*5*) we offered a solution to the problems of poor resolution and reproducibility by suggesting that hydrophilic ion-pairing reagents may be used with reversed-phase systems. We show here that these reagents do in fact make it possible to analyze and purify a wide range of underivatized peptides and proteins by HPLC.

Ion-pair partition chromatography or paired-ion chromatography has recently been applied to a variety of substances (*6–9*). The use of ion-pairing reagents such as the PIC reagents [tetrabutyl ammonium phosphate and heptanesulfonic acid (Waters Associates)] has resulted in increased retention times for highly polar molecules by the formation of hydrophobic ion-pair complexes (*10*). The hydrophilic ion-pairing reagents such as H_3PO_4 (*5*), perchlorates (*7, 8*), methylsulfonates (*7*), and picrates (*6*) have greatly extended the scope and potential of reversed-phase, ion-pair partition chromatography. When used alone or in combination with hydrophobic ion-pairing reagents, hydrophilic ion-pairing reagents result in marked alterations in retention times, allowing the resolution of complex mixtures (*5–7*). On a reversed-phase chromatographic support, increased polarity due to the formation of

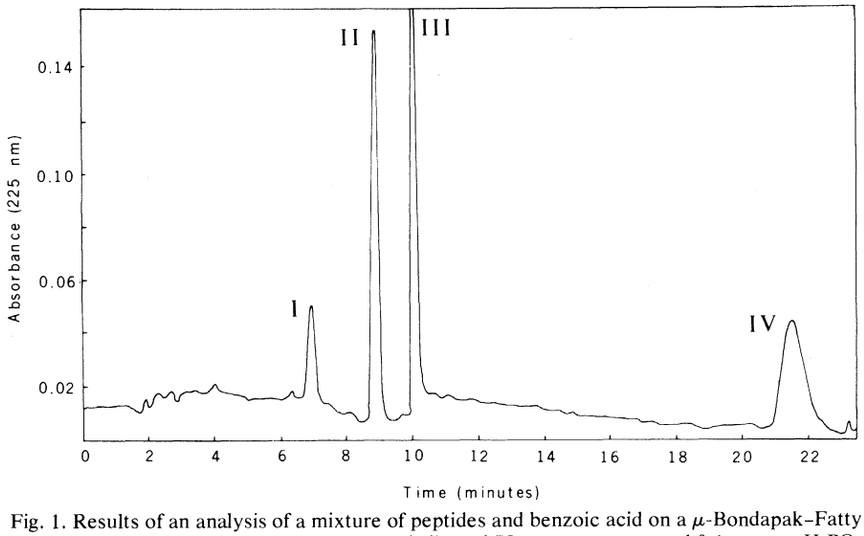

Fig. 1. Results of an analysis of a mixture of peptides and benzoic acid on a μ-Bondapak–Fatty acid analysis column with 25 percent acetonitrile and 75 percent water, and 0.1 percent H_3PO_4 as the mobile phase. Peak I corresponds to Met-Arg-Phe-Ala, peak II to Leu-Trp-Met-Arg, peak III to benzoic acid, and peak IV to Leu-Trp-Met-Arg-Phe. One nanogram of each component, made up in the mobile phase, was loaded in a volume of 25 μl; the flow rate was 1.5 ml/min, the pressure 144 atm, and the temperature 22°C. All the HPLC was carried out with a Waters Associates system as detailed in (*5*). The μ-Bondapak–Fatty acid analysis and μ-Bondapak-C_{18} columns (10-μm particles, 30 cm long and 4 mm in inside diameter) were purchased prepacked from Waters Associates. All other apparatus, materials, and methods were as outlined in (*5*).

0036-8075/78/0609-1168$00.50/0

hydrophilic ion-paired complexes results in a decreased retention time for the material. Considerable flexibility in the degree of retention and possibly elution order can thus be achieved with a judicious choice of counter ion.

Under suitable conditions an amphoteric molecule, such as a peptide or protein, can undergo either hydrophilic or hydrophobic ion-pairing, often with a dramatic change in the polarity of the resulting complex (*5*). A typical separation achieved with hydrophilic ion-pairing is shown in Fig. 1 where the excellent resolution of two tetrapeptides, Met-Arg-Phe-Ala and Leu-Trp-Met-Arg, and a pentapeptide, Leu-Trp-Met-Arg-Phe, is shown. Prior to the addition of H_3PO_4 to the mobile phase, the retention time of the tetrapeptide Leu-Trp-Met-Arg, chromatographed under similar conditions, was too long for convenient analysis (retention time, 40.6 minutes) and lacked reproducibility.

Figure 2 shows the resolution of three separate peptides related to angiotensin II, Val-Tyr-Ile-His-Pro-Phe, Val-Ile-His-Pro-Phe, and Tyr-Ile-His-Pro-Phe. The samples represent crude mixtures obtained from solid-phase peptide synthesis; Val-Ile-His-Pro-Phe represents a failure peptide caused by an incomplete coupling of tyrosine during the synthesis (*11*). The retention times obtained indicate that very good resolution of similar peptides is possible. This technique can thus be used to detect minor deletion products from peptide synthesis.

The marked success of the preceding separations on analytical systems led us to adapt the process to the preparative level. Figure 3 illustrates the advantages of preparative reversed-phase HPLC in the purification of a synthetic pentapeptide Ac-Ser-Thr-Ile-Glu(OBzl-*p*-NO_2)-Arg(NO_2)OH (*12*). Figure 3A shows that initial attempts at the purification by chromatography on Sephadex LH-20 gave a separation that was unsatisfactory and slow. The material from the column was pooled (cross-hatched area, Fig. 3A), and a sample was analyzed by reversed-phase HPLC (Fig. 3B). In this case the separation was rapid, being complete in less than 7 minutes, with good resolution. The bulk of the residue from the chromatogram in Fig. 3A was then chromatographed preparatively, the purified material (cross-hatched area, Fig. 3C) was collected and then subjected to an analytical separation. The purity of the sample is evident from the single large peak (Fig. 3D). The results show that there is no significant loss of resolution with the use of the preparative system, an observation confirmed by

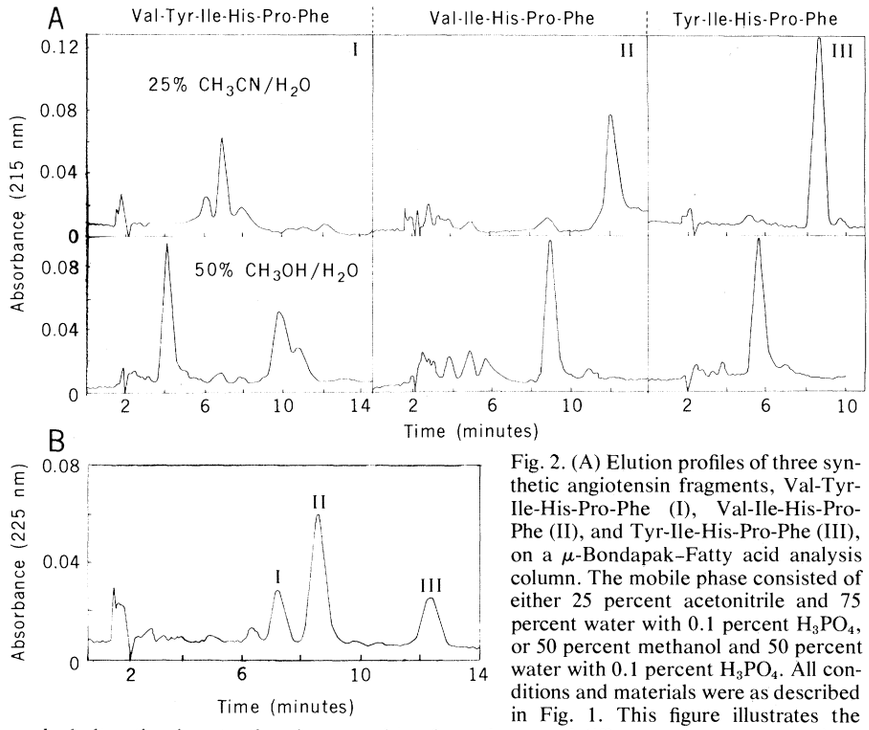

Fig. 2. (A) Elution profiles of three synthetic angiotensin fragments, Val-Tyr-Ile-His-Pro-Phe (I), Val-Ile-His-Pro-Phe (II), and Tyr-Ile-His-Pro-Phe (III), on a μ-Bondapak–Fatty acid analysis column. The mobile phase consisted of either 25 percent acetonitrile and 75 percent water with 0.1 percent H_3PO_4, or 50 percent methanol and 50 percent water with 0.1 percent H_3PO_4. All conditions and materials were as described in Fig. 1. This figure illustrates the marked alteration in retention times resulting from the use of different solvent compositions. (B) The elution profile of a mixture of the same three angiotensin fragments on a μ-Bondapak–Fatty acid analysis column. The mobile phase was 25 percent acetonitrile and 75 percent water, with 0.1 percent H_3PO_4.

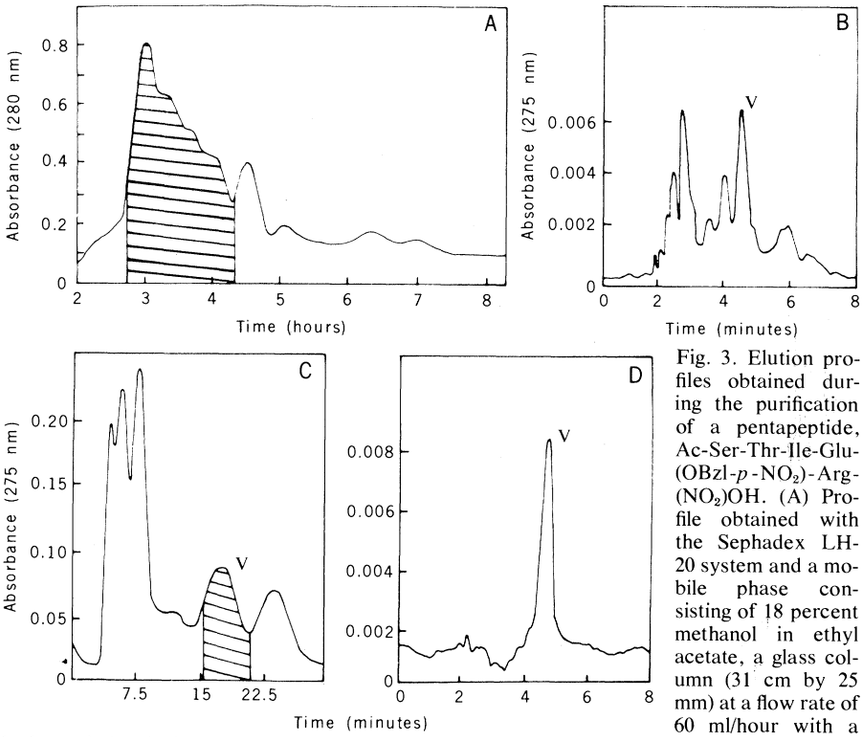

Fig. 3. Elution profiles obtained during the purification of a pentapeptide, Ac-Ser-Thr-Ile-Glu-(OBzl-*p*-NO_2)-Arg-(NO_2)OH. (A) Profile obtained with the Sephadex LH-20 system and a mobile phase consisting of 18 percent methanol in ethyl acetate, a glass column (31 cm by 25 mm) at a flow rate of 60 ml/hour with a hydrostatic head of 1 m; 10 ml of the sample was loaded and a Uvicord ultraviolet detector at 280 nm was used. (B and D) Profiles obtained from a μ-Bondapak–Fatty acid analysis column (30 cm long and 4 mm in inside diameter), with a mobile phase consisting of 50 percent methanol and 50 percent water, with 1 percent acetic acid. This column had a plate number of 2100. Other conditions were as in Fig. 1. (C) Profile obtained for the pentapeptide chromatographed on the preparative column. The preparative HPLC system consisted of two columns (60 cm long and 7 mm in inside diameter) connected in series to the Waters pumps. The columns were packed with silanized Bondapak Phenyl-Porasil B (37 to 50 μm; Waters Associates) by the tap-fill method (*1*). The mobile phase consisted of 40 percent methanol and 60 percent water, with 1 percent acetic acid. Samples (2 ml) made up in the mobile phase were loaded; the flow rate was 5 ml/min, and the required pressure was 13.7 atm. The two columns had a plate number of 520, which, although significantly lower than the plate number for the analytical column, made no significant difference to the separation obtained.

other studies (*13*). In this case, where the protected peptide being analyzed has no free amino groups, hydrophilic ion-pairing is not applicable. Instead, acetic acid was used to suppress the ionization of the terminal carboxyl group, making the peptides more nonpolar to increase retention (*14*).

Different peptides and proteins exhibit a wide range of polarities, and consequently a single set of mobile phase conditions would not be expected to achieve chromatographic analysis of all molecules on reversed-phase HPLC. As indicated above, ionic suppression can be utilized for peptides that have their NH_2-terminals protected. Other workers (*15*) have also noted similar results. We have found, however, that a careful choice of ion-pairing reagents can extend this approach considerably and can assist the rapid chromatographic resolution of a number of diverse peptides and proteins. With very polar peptides and proteins—for example, acyl carrier protein—which contain free amino groups and are poorly retained on reversed-phase supports, hydrophobic ion-pairing with the use of a hydrophobic counter ion (heptanesulfonic acid or sodium dodecyl sulfate) can be used to increase the retention time. The addition of polar counter ions can be used in the converse situation with hydrophobic peptides (for example, linear antamanid), which are strongly retained without ion-pairing. Similar manipulations of free carboxyl groups can be achieved with the use of suitable cationic counter ions. A combination of both ionic suppression and ion-pairing has been used successfully for a wide range of peptides and proteins, including the series shown in Table 1. Although it may be possible to elute a particular peptide from the reversed-phase support with a simple eluant, good resolution was generally impossible because of extensive tailing. Upon the addition of a small concentration of H_3PO_4 (0.1 percent), a dramatic improvement in peak shape and resolution occurred. The proteins listed in Table 1 do not elute from reversed-phase columns unless an ion-pairing reagent is added to the eluant. In addition, the reagent improves peak symmetry and plate count (generally ranging from 1000 to 5000 plates per meter). Figure 4 shows the elution profile for a sample of porcine insulin analyzed by an acetonitrile gradient on a reversed-phase column. The gradient system is particularly useful for the detection of impurities which only elute at higher concentrations of the organic component in the eluant.

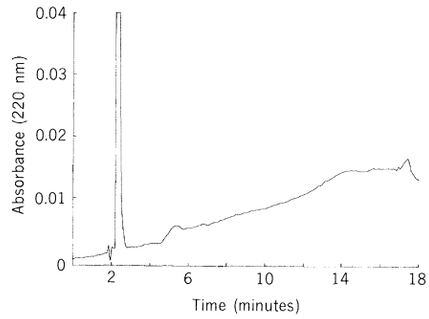

Fig. 4. Results of an analysis of a sample of porcine insulin (10 μg) on a μ-Bondapak–Fatty acid analysis column with a 10-minute linear gradient of 5 to 20 percent acetonitrile and 95 to 80 percent water containing 0.1 percent H_3PO_4. The gradient was started at the time of injection of the sample.

Table 1. Examples of reversed-phase HPLC analysis of several peptides and proteins.

Sample	Eluant*	Type of ion-pairing with amino groups	Ion-pairing reagent	Plate counts (plates per meter)	Retention time (min)
Leu-Trp-Met-Arg-Phe†	50% CH_3OH				40.6
Leu-Trp-Met-Arg-Phe†	50% CH_3OH	Hydrophilic	0.1% H_3PO_4	2700	7.3
Leu-Trp-Met-Arg-Phe†	50% CH_3OH	Hydrophobic	5 m*M* sodium hexane sulfonate, *p*H 6.5	6100	13.6
Linear antamanid†	60% CH_3OH				100
Linear antamanid†	60% CH_3OH	Hydrophilic	0.1% H_3PO_4 + 0.1*M* KH_2PO_4	4500	2.4
Porcine insulin‡	60% CH_3OH	Hydrophilic	0.1% H_3PO_4	1100	6.0‖
ACTH 1-24 pentaacetate§	40% CH_3OH	Hydrophilic	0.1% H_3PO_4	800	2.1‖
Glucagon§	40% CH_3OH	Hydrophilic	0.1% H_3PO_4	2000	4.6‖
Acyl carrier protein†	5% CH_3CN	Hydrophilic	0.1% H_3PO_4	1300	1.8‖
Acyl carrier protein†	30% CH_3CN	Hydrophobic	5 m*M* sodium hexane sulfonate, *p*H 6.5	3400	4.2

*Expressed as a percentage of the organic component; in all cases the other solvent was water. †Column used was μ-Bondapak–Fatty acid analysis. ‡Column used was μ-Bondapak-C_{18}. §Column used was Bondapak-C_{18}-Corasil. ‖In the absence of an ion-pairing reagent, the sample was retained indefinitely.

The results show that hydrophilic ion-pairing with H_3PO_4 is a useful method, when used in conjunction with ionic suppression and hydrophobic ion-pairing, for manipulating the retention times of peptides and proteins. Furthermore, hydrophilic ion-pairing allows the use of an eluant which contains a significantly lower concentration of organic solvents, thus reducing the risk of denaturation associated with high concentrations of these solvents conventionally used in reversed-phase HPLC (*4*, *16*).

W. S. HANCOCK*, C. A. BISHOP
R. L. PRESTIDGE, D. R. K. HARDING
Department of Chemistry, Biochemistry, and Biophysics, Massey University, Palmerston North, New Zealand
M. T. W. HEARN*
Immunopathology Group, Medical Research Council of New Zealand, Medical School, Post Office Box 913, Dunedin, New Zealand

References and Notes

1. An alternative term for this technique is high-performance liquid chromatography, which emphasizes that efficient separations are achieved by the use of small particle-size packings and thin liquid films.
2. L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography* (Wiley-Interscience, New York, 1974).
3. L. R. Pickart and M. M. Thaler, *Prep. Biochem.* **5**, 397 (1975); I. Shechter, *Anal. Biochem.* **58**, 30 (1974).
4. W. S. Hancock, C. A. Bishop, M. T. W. Hearn, *FEBS Lett.* **72**, 139 (1976); E. J. Kikta and E. Grushka, *J. Chromatogr.* **135**, 367 (1977); J. J. Hansen, T. Greibrokk, B. L. Currie, K. N. Johansson, K. Folkers, *ibid.*, p. 155; K. Krummen and R. W. Frei, *ibid.* **132**, 429 (1977); R. Burgus and J. Rivier, in *Peptides 1976, Proceedings of the Fourteenth European Peptide Symposium* (Brussels, Belgium, 1976), pp. 85–94.
5. W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding, M. T. W. Hearn, *J. Chromatogr.*, in press.
6. W. Santi, J. M. Huen, R. W. Frei, *ibid.* **115**, 423 (1975).
7. B. A. Persson and P. O. Lagerstrom, *ibid.* **122**, 305 (1976).
8. B. L. Karger, S. C. Su, S. Marchese, B. A. Persson, *J. Chromatogr. Sci.* **12**, 678 (1974).
9. J. H. Knox and J. Jurand, *J. Chromatogr.* **110**, 103 (1975).
10. E. Fitzgerald, *Anal. Chem.* **48**, 1734 (1976); "Bulletin D61" (Waters Associates, Framingham, Mass., 1976).
11. R. B. Merrifield, *J. Am. Chem. Soc.* **85**, 2149 (1963); W. S. Hancock, unpublished results.
12. Nomenclature and abbreviations follow the tentative rules of the International Union of Pure and Applied Chemistry–International Union of Biochemistry Commission on Biological Nomenclature, *J. Biol. Chem.* **241**, 1491 (1966); *ibid.* **242**, 555 (1967).
13. J. N. Done, *J. Chromatogr.* **125**, 43 (1976).
14. C. Horvath, W. Melander, I. Molnar, *ibid.*, p. 129.
15. T. F. Gabriel, J. Michalewsky, J. Meienhofer, *ibid.* **129**, 287 (1976); T. F. Gabriel, M. H. Jimenez, A. M. Felix, J. Michalewsky, J. Meienhofer, *Int. J. Pept. Protein Res.* **9**, 129 (1977).
16. The application of the technique reported here to peptide mapping (W. S. Hancock, C. A. Bishop, R. L. Prestidge, D. R. K. Harding, M. T. W. Hearn, *Anal. Biochem.*, in press) and the analysis of isoforms of natural polypeptides and glycoproteins is currently under investigation.
17. We thank J. E. Battersby for expert technical assistance. Supported by University Grants Committee (New Zealand) grants 72/74 and 73/94, Medical Research Council (New Zealand) grant 74/126, National Heart Foundation of New Zealand award 102, and Lottery Distribution Committee grant 20/12508.

* To whom correspondence should be addressed.

7 November 1977; revised 24 January 1978