31. C. Flurer, C. Borra, S. Beale, M. Novotny, *ibid.* **60**, 1826 (1988).
32. T. Tsuda, *ibid.* **59**, 521 (1987).
33. S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, *ibid.* **56**, 111 (1984).
34. J. H. Knox, *Chromatographia* **26**, 329 (1989).
35. R. A. Wallingford and A. G. Ewing, in *Advances in Chromatography: Biotechnological Applications and Methods*, J. C. Giddings, E. Grushka, P. R. Brown, Eds. (Dekker, New York, 1989), vol. 29, pp. 1–76.
36. K. A. Tweeten and T. N. Tweeten, *J. Chromatogr.* **359**, 111 (1986).
37. R. C. Williams, F. J. Vasta-Russell, J. L. Glajch, K. Golebiowski, *ibid.* **371**, 63 (1986).
38. D. J. Burke *et al.*, *ibid.* **353**, 425 (1986).
39. R. W. Slingsby and C. A. Pohl, *ibid.* **458**, 241 (1988).
40. T. C. Pinkerton and K. A. Koeplinger, *ibid.* **458**, 129 (1988).
41. I. M. Chaiken, *ibid.* **488**, 145 (1989).
42. A. Figueroa, C. Corradini, B. Feibush, B. L. Karger, *ibid.* **371**, 335 (1986).
43. E. Gil-Av, in *The Science of Chromatography*, F. Bruner, Ed. (Elsevier, Amsterdam, 1985), pp. 111–130.
44. D. W. Armstrong, T. J. Ward, R. D. Armstrong, T. E. Beesley, *Science* **232**, 1132 (1986).
45. G. Schill, I. W. Wainer, S. Barkan, *J. Chromatogr.* **365**, 73 (1986).
46. J. G. Dorsey, *Adv. Chromatogr.* **27**, 167 (1987).
47. E. S. Yeung, Eds., *Detectors for Liquid Chromatography* (Wiley-Interscience, New York, 1986).
48. M. D. Oates and J. W. Jorgenson, *Anal. Chem.* **61**, 432 (1989).
49. S. C. Beale *et al.*, *Talanta* **36**, 321 (1988).
50. Y.-F. Cheng and N. J. Dovichi, *Science* **242**, 562 (1988).
51. L. A. Haff, *Chromatography* **2**, 25 (1987).
52. M. Novotny, S. R. Springston, P. A. Peaden, J. C. Fjeldsted, M. L. Lee, *Anal. Chem.* **53**, 407A (1981).
53. F. Erni, W. Steuer, M. Schindler, G. Schill, paper presented at the 12th International Symposium on Column Liquid Chromatography, Washington, DC, 19 to 24 June 1988.
54. R. W. Gale, J. L. Fulton, R. D. Smith, *Anal. Chem.* **59**, 1977 (1987).
55. K. E. Markides and M. L. Lee, *Supercritical Fluid Chromatography Applications* (Brigham Young Univ. Press, Provo, UT, eds. 1 and 2, 1988 and 1989).
56. T. L. Chester and D. P. Innis, *J. High Resolut. Chromatogr. Chromatogr. Commun.* **9**, 209 (1986).
57. J. Kuei, G. R. Her, V. N. Reinhold, *Anal. Biochem.* **172**, 228 (1988).
58. P. A. David and M. Novotny, *J. Chromatogr.* **461**, 111 (1989).
59. I. L. Davies *et al.*, *Anal. Chem.* **60**, 683A (1988).
60. E. C. Huang, B. J. Jackson, K. E. Markides, M. L. Lee, *ibid.*, p. 2715.
61. K. H. Shafer, S. L. Pentoney, Jr., P. R. Griffiths, *ibid.* **58**, 58 (1986).
62. S. B. French and M. Novotny, *ibid.*, p. 164.
63. M. W. Raynor *et al.*, paper presented at the 10th International Symposium on Capillary Chromatography, Riva del Garda, Italy, 22 to 25 May 1989.
64. H. Alborn and G. Stenhagen, *J. Chromatogr.* **323**, 46 (1985).
65. E. D. Lee, J. D. Henion, T. R. Covey, *J. Microcolumn Seps.* **1**, 14 (1989).
66. M. A. Moseley *et al.*, *Anal. Chem.* **61**, 1577 (1989).

Microcolumn Separations and the Analysis of Single Cells

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Capillary zone electrophoresis and open tubular liquid chromatography are two examples of an emerging area of analytical instrumentation known as microcolumn separations. The high resolution and small sample requirements of these methods make them suitable for the quantitative, multicomponent chemical analysis of single cells. Appropriate instrumentation for the analysis of nanoliter and subnanoliter samples is discussed. Data from the analysis of individual neurons are presented, including amino acid and neurotransmitter content.

IN THE DEVELOPMENT OF ANALYTICAL INSTRUMENTATION, miniaturization is often a fruitful endeavor. During the past 20 years, numerous scientific advances arising from the development of microelectrodes, microsensors, and ion, electron, and light microprobes have been made. Two distinct benefits are associated with the miniaturization of analytical methods. The first is a change in the properties of the analytical tool, which often can be turned to advantage. The second is that the miniaturized instrument allows analysis of smaller samples and with higher spatial resolution.

Capillary electrophoresis (CE) (*1–3*) and open tubular liquid chromatography (OTLC) (*4*) are two examples of miniaturized, or microcolumn, separation methods that have analytical advantages over their conventional counterparts. In CE the separation of compounds is based on the different mobilities of molecules in an electric field. The separation takes place inside a capillary tube, typically with an inner diameter (ID) of 5 to 100 μm and a length of 10 to 100 cm. The capillary tube may be filled with only a buffer, as in capillary zone electrophoresis (CZE) (*2*), or with a gel, as in capillary gel electrophoresis (*5*). In both types of CE, the small dimensions of the capillary allow the rapid dissipation of Joule heat, which in turn allows potentials as high as 30 kV to be applied across the capillary. The strong electric field makes possible rapid, high-resolution separations. More than 1 million theoretical plates have been achieved in the separation of proteins by CZE (*6*).

In OTLC the separation takes place inside a capillary tube with an ID of 1 to 50 μm and a typical length of 1 m or more. The stationary phase is attached to the inner wall of the capillary instead of to particles packed into the column as in conventional high-performance liquid chromatography (HPLC). Open tubular columns, if their ID is small enough, have considerably greater resolving power than packed-bed columns (*7, 8*). The theory for OTLC predicts that, given certain time and pressure constraints, the optimum ID is approximately 2 μm. Such an OTLC column would generate over 1 million theoretical plates for a well-retained compound with an analysis time of less than 1 hour (*9*). Microcolumns have also been of interest because the inherently low volumetric flow rates facilitate the coupling of the separation to other analytical techniques such as mass spectroscopy.

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Decreasing the dimensions of the separation column not only provides the analytical advantages discussed above but also decreases the sample volume requirements. Injection volumes in CZE and OTLC are on the order of picoliters to nanoliters. The fact that separations can be achieved on nanoliter and even picoliter volumes suggests a number of interesting applications of these separation methods to microanalysis, including the analysis of single cells. We and others have recently begun to explore this possibility (*3, 10–12*). This article describes the methodology that we have developed for analyzing single cells and recent, relevant advances in microcolumn technology.

In order to appreciate the potential advance in single cell analysis that the microcolumn separation techniques offer, we review what has been done before in this area. It is now possible to analyze metals and some inorganic compounds at the single cell level, with the use of tools as diverse as ion-selective microelectrodes (*13*), secondary ion mass spectrometry (*14*), and fluorescence microscopy (*15*). However, the analysis of trace organic compounds in single cells remains a formidable analytical challenge. The difficulty arises from the complexity of the cell and the limited amount of sample available. A substance present at a concentration of 1 μM in a large cell (volume, 1 nl) yields only 1 fmol available for the analysis. The cell will also contain many compounds, some in high concentrations, that may interfere with the measurement of the analyte or analytes. Any method chosen for the analysis of single cells must therefore be suitable for dealing with the complexity of the cellular contents and must have the sensitivity required for small samples. The ideal method should also make it possible to determine a wide variety of compounds in one analysis, even if their concentrations vary over a wide range, give qualitative and quantitative information, and be nondestructive to the cell.

The interest in the chemistry of single cells is such that, in spite of the difficulties discussed above, a number of analytical methods have been developed for them [for complete reviews, see (*16, 17*)]. Representative examples of methods used include micro–thin-layer chromatography (TLC) (*18*), gas chromatography–mass spectroscopy (GC-MS) (*19*), HPLC with amperometric detection (*20*), and enzymatic radiolabeling (*21*). Although all of these methods provide good information, they have important limitations. Micro-TLC is not sensitive enough to permit the analysis of individual cells and therefore requires the pooling of four to six giant cells. Also the method does not lend itself very well to quantification. Because of a lack of sensitivity, GC-MS of individual cells is limited to using the mass spectrometer in the selected ion–monitoring mode. This limitation means that only a few compounds can be measured in one run, and those compounds must be selected before the experiment. As a result of this limitation, the possibility of discovering a new compound is virtually precluded. Also this method often requires sample derivatization in order to improve the volatility of analytes, especially for many molecules of biological interest. HPLC with amperometric detection is also limited by sensitivity and can only be used for cells that contain high levels of analyte. Enzymatic radiolabeling is sensitive, but it requires that the analyte be selected before the experiment. In addition, the specificity of the method is limited by cross-reactivities.

Microcolumn separation methods, with their small volume requirements and high resolution capabilities, have the potential to be considerably more effective in the analysis of single cells than the methods just discussed. In order to tap this potential, however, a number of difficulties must be overcome. The first problem is the development of appropriate injection and sample-handling techniques for nanoliter and subnanoliter volumes. The second is the development of reliable columns that can fulfill the theoretical promise of the techniques. Another challenge facing researchers in microcolumn separation techniques is detection. The small injection volumes, although quite useful for certain applications, require that detectors be sensitive to femtomole and attomole amounts of analyte.

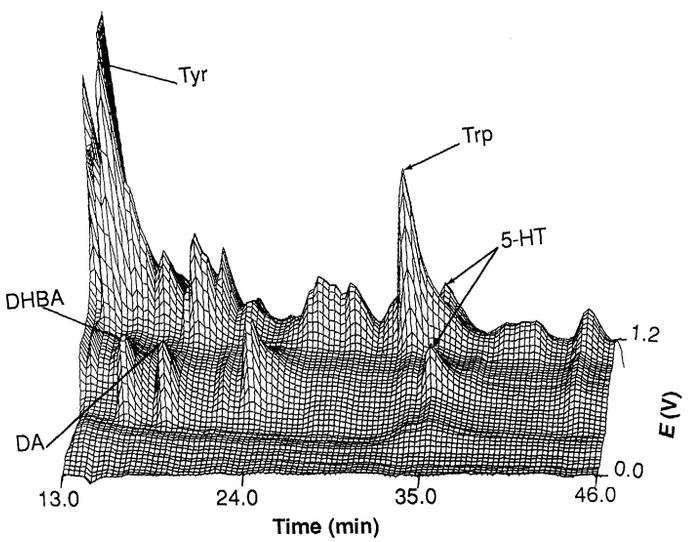

Fig. 1. Data from cell F1 [cell designation according to the map in (*49*)] obtained with an OTLC column (ID, 19 μm) with voltammetric detection. Each line running parallel with the time axis represents a chromatogram obtained at the voltage indicated on the potential axis, and each line parallel with the potential axis represents a voltammogram obtained at the time indicated. Abbreviations: Tyr, tyrosine; DHBA, 3,4-dihydroxybenzylamine; DA, dopamine; Trp, tryptophan; and 5-HT, 5-hydroxytryptamine (serotonin). The detector was scanned from 0.0 to +1.2 V versus an Ag/AgCl reference electrode. The mobile phase was 0.21 mM dimethyloctylamine and 0.62 mM sodium octyl sulfate dissolved in 0.1M citrate buffer, adjusted to pH 3.1 with NaOH. The data are shown beginning just after the elution of the unretained peak. [Adapted from (*11*) with permission of the American Chemical Society, copyright 1989]

Instrumentation for Microcolumn Analysis of Single Cells

Sample preparation and injection. In considering possible methods of preparing low-volume samples, one needs to examine the physical consequences of working on such a small scale. In transferring liquids, gravity becomes inconsequential, whereas surface tension becomes the dominant force to consider. The possible rapid evaporation of the sample may also be problematic. Finally, in the smaller glassware that is used for microanalysis, the ratio of surface area to volume is relatively high, so that the possible adsorption of reagents and analytes becomes a greater concern.

We have found that transfer of samples, addition of reagents, and even injections onto the separation column can be accomplished with the use of techniques similar to those developed for the injection of substances into single cells (*22, 23*). The basic tool is a microsyringe, which consists of a glass micropipette, drawn to appropriate dimensions for a particular job and connected to a controlled pressure source. Two types of pressure source, one hydraulic and the other pneumatic, have been used. The pneumatic microsyringe is considerably more accurate than the hydraulic, and, once calibrated, it can be used to accurately and reproducibly dispense volumes less than 1 nl. One can also use the pneumatic microsyringe to accurately inject small samples onto separation columns by inserting the micropipette tip into the inlet end of the column and applying pressure for a controlled length of time (*24*).

The ability to inject samples of such limited volumes is a crucial step in the ability to perform microanalysis. Most previous injection methods for CE and OTLC required the use of a larger amount of sample than was actually injected onto the column.

Another approach to microinjection has been described and used with CZE by Wallingford and Ewing (*25*). This microinjector allows a probe to be inserted into a cell; sample is then removed and introduced directly onto the capillary by electromigration. This type of microinjection allows samples to be taken directly from an intact cell, without destroying the cell, which is important because it may allow changes in intracellular concentration to be observed directly as a function of time. Also this method of sampling should limit the possible artifactual changes in concentrations of substances normally associated with the delay between sample collection and analysis. One limitation of this method of sample injection is that it precludes any precolumn sample preparation such as derivatization of analytes.

Columns. The preparation of OTLC columns with IDs less than 10 μm that perform as well as theory predicts is difficult. The major problem has been the development of a method that allows a uniform layer of stationary phase to be attached to the column. Two different methods are used to prepare OTLC columns. One is to coat a polymeric stationary phase onto the inner surface of the capillary by static (*26*), dynamic (*27*), or precipitation coating (*7*). The other method is to chemically bond a monolayer of the stationary phase to the roughened inner wall of the capillary (*28*). The roughening is necessary to allow enough stationary phase to be incorporated into the column for effective chromatography. The chromatography columns used in this work were roughened by a chemical etching procedure; then octadecysilane, a common nonpolar stationary phase, was bonded to the wall.

The columns used for CZE are almost always prepared from fused silica tubing. When CZE is being used for the analysis of compounds that do not adsorb to the wall, minimal preparation of the capillary is required. Unfortunately, bases, such as amines, and large compounds with amine functional groups, such as proteins, often have kinetically slow adsorption-desorption interactions with the wall. The interactions result in broad, tailed bands and limit the resolution of the technique. Two methods are currently being explored to weaken or eliminate adsorption. One approach is to coat the inner wall of the capillary with a polymer that is inert to the analyte. The greatest success in limiting protein-capillary wall interactions has been obtained with hydrophilic polymers (*29*). The other approach is to manipulate the buffer medium in such a way that interactions are minimized (*30*).

Detection. A number of detection schemes, including those based on ultraviolet absorption (*31*), fluorescence (*32*), electrochemical reactions (*33, 34*), potentiometric electrodes (*35*), photoionization (*36*), indirect fluorescence (*37*), thermo-optical effects (*38*), conductivity (*39*), and mass spectroscopy (*40–42*), have been used with microcolumns. The most sensitive, and hence the most appropriate for the analysis of tiny samples, are fluorescence and electrochemical detection.

The electrochemical detector that we have used is based on inserting a carbon-fiber microelectrode, which acts as the working or sensing electrode, into the outlet end of the column (*33, 43*). The electrode is cylindrical in shape and has a length of 0.1 to 1 mm and a diameter of 5 to 10 μm. This configuration results in an electrochemical cell, which consists of an annular region just a few micrometers thick, between the wall of the column and the electrode. The electrode can be operated either amperometrically or voltammetrically. In the amperometric mode, the potential on the electrode is held constant while the current due to the oxidation or reduction of eluting species is monitored. The electrochemical cell is so thin that a high percentage of molecules entering the cell are oxidized or reduced and hence give a signal. Because a microelectrode is used, the noise in the detector is low, usually well below 100 fA. Using this detector, we have obtained detection limits as low as $10^{-10}M$, or 1 amol, based on a 10-nl injection volume (*44*). In the voltammetric mode the potential on the electrode is scanned, which allows voltammograms to be obtained on the solutes as they elute from the column (*45, 46*). The minimum detectable quantity for the detector in the voltammetric mode is approximately 100-fold greater than in the amperometric mode. The increased information that this mode provides is often worth the loss of sensitivity.

Another detection method that we have used in the analysis of single cells is laser-induced fluorescence (LIF). In this method, a laser beam is focused to a 5-μm spot inside the capillary column, which results in a tiny, intensely illuminated region inside the column that acts as the detection cell. The fluorescence that is produced as excitable eluents pass through the beam is collected to generate a signal. The detection limit of this system is $2 \times 10^{-9}M$ for naphthalene-2,3-dicarboxyaldehyde (NDA)–labeled phenylalanine when a 10-mW He-Cd laser is used (*47, 48*). Cheng and Dovichi have shown that, with the use of an improved optical design, a more powerful laser, and a fluorophore with a higher quantum yield (fluorescein isothiocyanate–labeled amino acids), detection limits as low as $5 \times 10^{-12}M$ may be obtained (*32*).

Analysis of Single Cells

The samples we have used are neurons from the land snail *Helix aspersa*. These cells were chosen because they are part of a heterogeneous cell population and, as such, should be distinguishable by chemical analysis. Moreover, the ganglia of gastropod mollusks contain large neurons, several with diameters larger than 100 μm, which can be reproducibly found in the same location from specimen to specimen. Electrophysiological and pharmacological studies of these identifiable neurons have indicated that they are indeed the same from specimen to specimen. On the basis of such studies, maps have been published that allow the identification of the neurons (*49*). Although we have used *Helix* neurons exclusively in this work, the analytical concepts that are presented are applicable to other cell types.

The following simple scheme was used for the analysis of individual cells from *Helix* (*10, 11*). The cell was removed from the ganglia by microdissection techniques (*16, 50*) and was then transferred to a microvial by means of a small pipette. (The microvial consisted of a capillary that was melted closed at one end and could hold a total volume of 500 nl.) A calibrated microsyringe was then used to add a small amount (1 to 2 nl) of an internal standard, in this case, 3,4-dihydroxybenzylamine (DHBA). The internal standard allowed losses due to sample adsorption, incomplete transfer of sample from the vial to the column, and varying injection volumes to be taken into account. After the addition of the internal standard, the cell was homogenized and centrifuged, and then the supernatant was removed and injected into an OTLC column. The detector for this work was a carbon-fiber microelectrode operated in the voltammetric mode.

An example of data that have been obtained with this method is shown in Fig. 1 (*11*). We identified the peaks by matching their retention times and voltammetric maxima with standards. The use of voltammetric detection not only gives added qualitative information, that is, voltammograms, which is useful in identifying peaks, but it also improves resolution. An example of the improved resolution that results from the combination of chromatography and voltammetry is seen with the dopamine and DHBA peaks. These compounds were resolved in their voltammograms from several

Table 1. Measured amounts of identified compounds in *Helix aspersa* neurons. The compound abbreviations are the same as in Fig. 1. The values are the mean ± standard deviation of five analyses; ND, not detected. [From (*11*)]

Compound	Measured amount per cell (fmol)		
	D2	E4	F1
Tyr	340 ± 98	550 ± 420	490 ± 140
DA	ND	6.2 ± 1.9	71 ± 6.2
Trp	160 ± 20	59 ± 22	89 ± 24
5-HT	ND*	30 ± 14	43 ± 28

*One run of cell D2 contained 2.4 fmol of serotonin (the other runs had no detectable serotonin). This occurrence was presumably due to contamination.

unknown compounds that coeluted with them.

This method was used to analyze 15 cells (*11*), five each of cells designated F1, E4, and D2 in the map of Kerkut *et al.* (*49*). The four compounds that were identified were quantified in each cell (Table 1). The amounts of the neurotransmitter compounds that were observed in the cell are comparable to what has been observed in other neurons by other methods (*51*). Dopamine and serotonin were found to coexist in two of the neurons and to be below detectable limits in cell D2. This result is consistent with a number of studies on individual neurons that have indicated that neurotransmitter compounds may coexist in certain cells (*51, 52*). The coexistence of dopamine and serotonin in this case may be due to contamination of the samples, although this does not seem likely, given the results for cell D2. In addition, histochemical tests indicate that *Helix* neurons in the region of F1 and E4 can take up both 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan, the precursors to dopamine and serotonin, respectively, and convert them to the corresponding neurotransmitter compound (*53*). The same study demonstrated, somewhat ambiguously, that the cells in this region naturally contained both dopamine and serotonin. Although the biological significance of these findings is not clear, these results indicate the type of information that can be obtained when one is analyzing individual cells.

The relative standard deviations of the method are comparable and in most cases lower than what has been reported before for other methods of single cell analysis (*18, 19, 51*). The variability that is observed may be due to analytical error or biological variability. One likely source of analytical error is contamination of the samples from exogenous tissue. It is difficult to extract an intact cell without any additional, smaller cells adhering to it (*50*). Another possible source of error, especially in the case of tyrosine, which eluted close to several large peaks, is the incomplete separation from other compounds. Finally, the use of an internal standard to account for losses of analyte due to adsorption is limited because its use assumes that all analytes and the internal standard have the same affinity for the surfaces they encounter. Obviously this assumption may result in error. One way around this problem is to use several internal standards, each one closely related to the analytes of most interest. The ultimate internal standard would be isotopically labeled compounds that could be separated on column or distinguished by the detector. The high resolution needed to separate isotopically labeled compounds on column has been demonstrated with OTLC and CE (*7, 54*). Biological factors that may contribute to the observed variability include differences in the size of the cell (the amounts were reported per cell, so a slight change in the diameter of the cell would result in a considerable change in the volume and hence in the amount of the compound in the cell) and differences in the state of the neuron or snail at the time of dissection.

The quantitative results indicate that cells can be distinguished on the basis of their chemical contents as determined chromatographically. This idea can be more graphically illustrated with the data shown in Fig. 2 (*11*). As the data show, a given cell type has a reproducible chromatogram, including many unknown peaks. A comparison of the data from two different cell types shows that they have noticeably different chromatographic profiles. The most striking difference between the cells is the presence of the large peak labeled "6" in cell E4 and its absence in cell D2. The apparently high concentration and uneven distribution of the compound suggests some important function. An attempt to identify the peak by comparing its retention time and voltammetric wave with several derivatives of the aromatic amino acids yielded no match. The difficulty in identifying the peak exemplifies a major gap in the instrumentation available for microcolumns; that is, interfaces to methods that can give detailed qualitative information on compounds, such as MS, are not yet well developed.

Amino Acid Profiling of Single Cells

The experiment described above, while interesting, has limitations. The most obvious limit is that only the relatively few compounds that can be oxidized or reduced at the electrode can be detected. The method must be applicable to a wider variety of compounds to be truly useful. In new work in our laboratory, we have begun to extend the applicability of the method through the

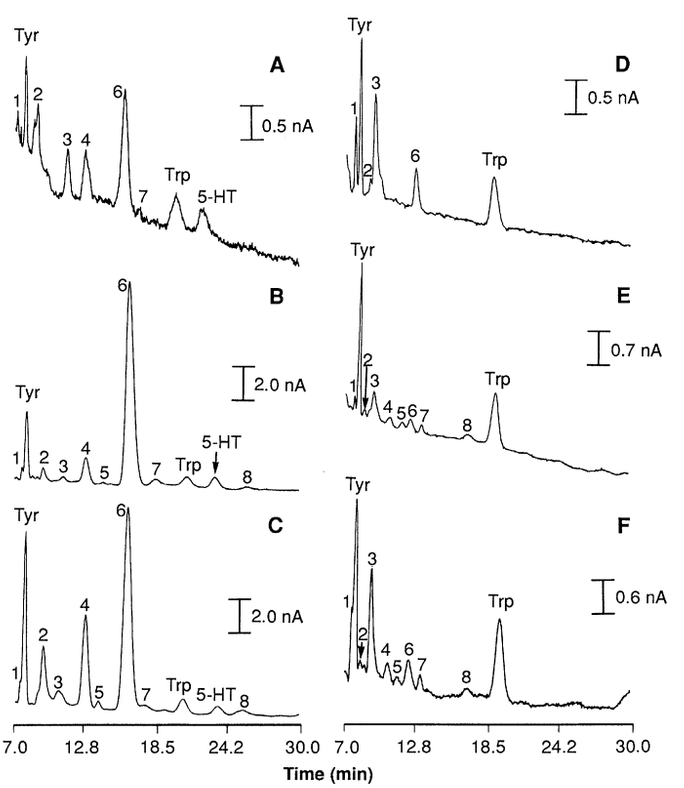

Fig. 2. (**A–C**) Data from cell E4 obtained from three different specimens with the use of OTLC with voltammetric detection and the same conditions as in Fig. 1 except that the column had an ID of 15 μm. The data represent a "slice" taken from the combined chromatographic and voltammetric data at 1.0 V; the plots were constructed with a computer. The numbered peaks represent unknowns that appeared reproducibly in each cell. The abbreviations for identified peaks are the same as for Fig. 1. Dopamine and DHBA do not appear in the chromatogram because they oxidize at a lower potential. (**D–F**) Same as in (A) to (C) except that the data are from cell D2 for three different specimens. The peak numbers do not correspond to peaks with the same number in (A), (B), and (C). [Adapted from (*11*) with permission of the American Chemical Society, copyright 1989]

use of derivatizing reagents. An example of such a reagent is the recently developed NDA, which reacts specifically with primary amines in the presence of cyanide ion to form a fluorescent and oxidizable product (*55*).

The procedure for using NDA in an analysis is the same as described above until after the cell is centrifuged. (The internal standard used to account for losses of sample was changed from DHBA to normetanephrine for this set of experiments.) Instead of the supernatant being injected directly, it is transferred to another vial, where an excess of NDA and appropriate amounts of other necessary reagents are added with a microsyringe, together with a second internal standard, norleucine, which is necessary to account for variability in the extent of the tagging reaction. The total volume of the reaction mixture is approximately 20 nl, of which roughly 25% is injected onto the column. It should be stressed that two internal standards, one to account for losses of sample and the other to account for reaction completeness, are necessary for quantitative work. The voltammograms of all the derivatives are similar because they are due to the same NDA moiety; therefore, the advantages of using the detector voltammetrically are negated and the more sensitive amperometric mode is favored.

The use of NDA as a precolumn tag allowed the identification and quantification of 17 amino acids in the cells. An example of the chromatograms that were obtained is shown in Fig. 3. The method as described did not allow the measurement of lysine, dopamine, and serotonin; work with standards indicates that the NDA derivatives of these compounds are lost during the sample workup. The losses may be due to precipitation, adsorption to the glassware, or, in the case of dopamine, oxidation at the high *p*H required for the reaction.

Resolution of all the amino acids in a reasonable time required the use of gradient elution. The use of a gradient with electrochemical detection deserves some comment. A common perception among those familiar with electrochemical detection is that it is difficult or even impossible to use it with gradient elution. No special precautions were taken in the chromatogram shown, yet the gradient caused little effect on the detector. In fact, the detection limit for NDA-tagged asparagine was 36 amol despite the wide gradient that was used (*56*).

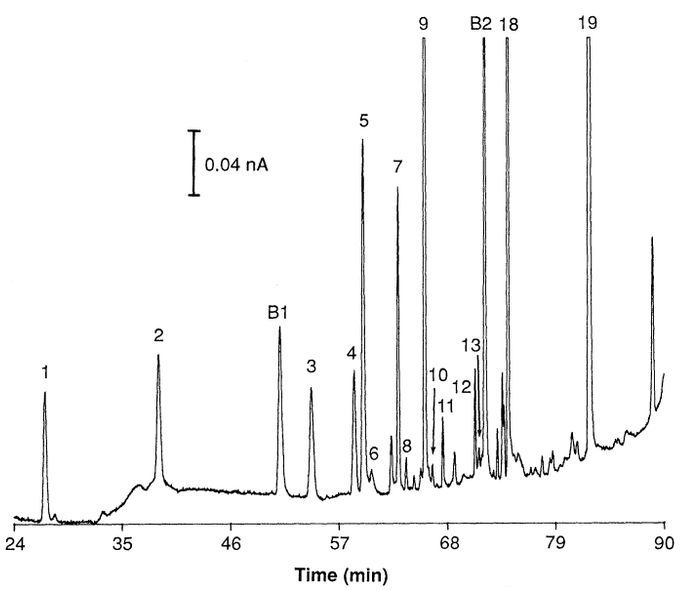

Fig. 3. Chromatogram of NDA-tagged amino acids from cell F1. Peak numbers 1 to 17 correspond to those listed in Table 2. Peaks 14 to 17 appear between B2 and peak 18. B1 and B2 are present in blanks, and peaks 18 and 19 are the internal standards norleucine and normetanephrine, respectively. All unlabeled peaks are unknowns found in the cell. Mobile phase A was 3% tetrahydrofuran in 0.05*M*, *p*H 7.0, sodium phosphate buffer; mobile phase B was acetonitrile. A linear gradient was used as follows: 100 to 89% A and 11% B in 38 min, and then to 47% A and 53% B in 80 min.

The method was used to quantitatively analyze seven cells, five of cell E4 and one each of F1 and D2. The resulting amino acid profiles are shown in Table 2. Reasonable mean values and standard deviations were observed for the amino acids. Note the agreement between the results obtained with the first method (Table 1) and with this method for the amount of tyrosine and tryptophan in cell E4. (A comparison between methods for the other cells is not as meaningful because of a lack of repetitive measurements on the other cells by the derivatization method.) The relative amounts of amino acids are in good agreement with the amino acid profiles obtained by micro-TLC by Osborne *et al.* on similar neurons in *Helix pomatia* (*18*). (Only relative amounts can be compared because the micro-TLC method did not allow absolute quantification.) The high value of alanine is not surprising because of its role in metabolism as a latent source of energy after its conversion to pyruvate (*57*).

The large amounts of amino acids in cell D2 were due to the large size of this particular specimen. The same sources of variability that were discussed for the previous experiment apply in this case as well. Because the amino acids are associated with the metabolic pathway, possible changes in the chemical composition of the cell during sample preparation is an important possible source of error. Further work in this area would require the application of sample preparation methods that limit such metabolic changes.

The use of derivatizing reagents in conjunction with sensitive detectors has a number of advantages. It allows the best detectors to be used with any compound that can be appropriately derivatized rather than requiring that a new type of detector be developed for each type of analysis that is performed. Also, derivatization allows a particular class of compounds to be the target of analysis, in this case primary amines and, in particular, amino acids. This advantage gives the method additional selectivity and provides qualitative information; that is, the detected compounds are primary amines or are intrinsically electroactive.

Capillary Zone Electrophoresis of Single Cells

Miniaturized forms of gel electrophoresis have been used to determine proteins and nucleotides in single cells for over 20 years (*58, 59*). The advent of CE techniques promises to bring significant improvements to such analyses, including ease of use, improved reliability, improved quantitation, speed of analysis, and the possibility of sampling from intact cells. This last advantage has already been demonstrated by Ewing's group in the analysis of the intracellular contents of the giant dopamine neuron in *Planoribus corneus*, in which the previously mentioned microinjector was used with an electrochemical detector (*12*).

We have begun to analyze single *Helix* neurons by CZE with LIF detection. In our work, a preparation and injection scheme similar to that described for the OTLC separation of NDA–amino acids was used. The major difference was that no attempt at quantification has been made, so internal standards were not used. Also no attempt has been made to resolve any particular class of compounds. An example of an electropherogram that was obtained on the NDA-derivatized contents of a cell is shown in Fig. 4. Some of the peaks have been tentatively identified on the basis of their migration times as NDA-labeled amino acids. The electropherogram shows many unidentified peaks in addition to the amino acids. In this case, we allowed most of the NDA–amino acid peaks to go off scale in order

to demonstrate that there is ample signal to measure not only them but also many less concentrated substances as well.

In this preparation only a fraction of the cell contents, roughly 20%, was actually injected onto the column, which suggests a number of interesting possibilities. First, multiple runs can be obtained on one cell. A series of runs could be made under identical conditions to determine the precision of the measurement method. Runs could also be performed under different conditions, each optimized for a particular class of compounds. The latter possibility would allow a vast amount of information to be generated on one cell. Because only a fraction of the cell was used to obtain the data shown in Fig. 4, a considerably smaller cell could be used for the analysis. The work we have performed thus far has focused on the large neurons of *Helix*, primarily because of the convenience of working with them. In many cases, however, it would be desirable to analyze smaller cells. For example, typical mammalian neurons have a 50- to 100-fold smaller volume than the neurons used in this work. The good signal-to-noise ratio obtained on the fraction of cell E4 implies that it should be possible to analyze smaller cells.

Future Prospects

The work done thus far can be used in an evaluation of the status of microcolumn separations and of the improvements that are required to further the analysis of single cells. Perhaps the most desired improvement in microcolumn instrumentation is the need for detectors that can give qualitative information. Such detectors would facilitate the identification of unknowns and the confirmation of known peaks in the chromatograms. The coupling of microcolumns and MS appears to be the most likely way of obtaining such information, and a number of research groups are working on appropriate interfaces (*40–42*). Detection limits as low as 54 fmol for peptides have been demonstrated for a mass spectrometer in full scan mode (150 to 1500 daltons) with a coaxial, continuous-flow, fast-atom-bombardment interface (*42*).

More sophisticated sampling and sample handling procedures would greatly improve the ease, speed, and quality of analysis on nanoliter and smaller samples. The goal of any microanalysis should be to reduce sample handling in order to minimize losses, lessen the possibility of contamination, and limit artifactual changes in cell composition. Another important step would be to allow sampling from intact cells. A step toward these goals has already been taken with the development of a microinjector for CZE (*25*). However, considerable improvement is necessary to reach the full potential of the technique. As working with small-volume samples becomes more routine, other possibilities, such as fraction collection from the microcolumns, will become feasible (*60*).

In principle, the methods that have been described, especially CZE with LIF, would be applicable to larger molecules such as peptides and proteins; however, the advantages of this method have yet to be demonstrated with single cells. One problem that can be anticipated is losses of the larger molecules as a result of adsorption to the glassware. Another problem is that detection of such compounds is often more problematic than the detection of small molecules; that is, it is difficult to use precolumn tagging with proteins because, with the large number of functional groups, more than one tagged species is formed (*9*).

Other microcolumn methods, such as capillary gel electrophoresis (*5*), packed microcolumn LC (*61*), and micellar electrokinetic capillary chromatography (*62*), should also be useful in the analysis of small biological samples. The separation method of choice will depend largely on the type of compound that is to be measured and on the choice of methods that are available in the future. For example, packed microcolumn LC columns may supplant OTLC as the chromatographic method of choice simply because the packed columns are easier to prepare (*61*). The electrophoretic methods are usually preferred for the separation of large biopolymers, whereas smaller molecules, especially neutrals, are often best resolved by the use of a chromatographic method. Commercial availability of instruments may also determine which methods are used most often. Several commercial CE instruments are now available, but the capillary chromatographic methods are being developed more slowly. The commercial availability of microseparation methods is a welcome development. It may soon be possible to buy all of the instruments needed for single cell analysis from commercial sources without the need to build in-house versions.

Table 2. Amino acid profiles of individual neurons of *Helix aspersa*. The values for E4 are the mean ± standard deviation (n = 5); the values for D2 and F1 are the values obtained in a single analysis of each cell.

Peak (Fig. 3)	Amino acid	Measured amount in cell (fmol)		
		E4	D2	F1
1	Asp	500 ± 100	560	300
2	Glu	1,300 ± 440	6,000	430
3	Asn	300 ± 130	940	500
4	Ser	950 ± 370	2,300	570
5	Gln	1,900 ± 1,100	14,000	930
6	His	320 ± 55	1,000	110
7	Gly	870 ± 300	2,400	510
8	Thr	290 ± 24	920	140
9	Ala	4,200 ± 2,400	25,000	2,100
10	Arg	39 ± 14	56	73
11	Tyr	260 ± 100	500	80
12	Val	200 ± 63	950	150
13	Met	120 ± 57	210	48
14	Trp	69 ± 28	130	19
15	Ile	170 ± 46	860	110
16	Phe	380 ± 160	1,300	290
17	Leu	250 ± 150	870	160

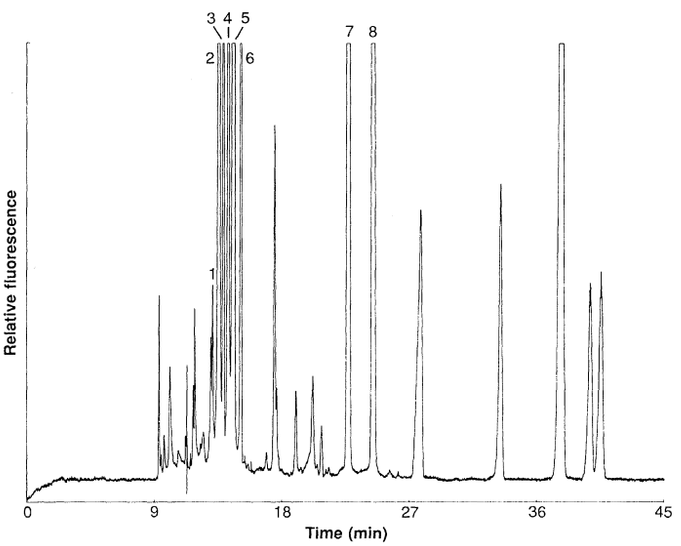

Fig. 4. CZE-LIF run of NDA derivatives of an E4 cell. The numbered peaks correspond to NDA-labeled amino acids as follows: 1, Trp; 2, Gln, His, Ile, Leu, Met, Phe; 3, Asn, Thr, Tyr, Val; 4, Ser; 5, Ala; 6, Gly; 7, Glu; and 8, Asp. Conditions of the run were as follows: capillary had an ID of 25 μm and was 104.5 cm long; detection was done 79.5 cm from the injection end; buffer was 0.01*M* borate, 0.04*M* KCl, *p*H 9.5; the applied potential was −25 kV (−8 μA). Injection was made at the grounded end of the capillary.

Conclusions

The methods described in this article complement other techniques that have been used in the chemical analysis of single cells. For example, although microcolumn separation methods can be used to map cells, they will not compete with the immunohistochemical methods in terms of speed for mapping a large group of cells. Also, microcolumn methods cannot provide the time resolution that is possible with implantable probes, such as ion-selective or voltammetric electrodes (*63*). Microcolumns can provide extremely detailed chemical information, including quantification, on a large number of compounds within the analyzed cell. Unlike many of the other methods, they allow for the possibility of discovering unexpected compounds within the cells.

In conclusion, it is appropriate to quote A. J. P. Martin, a pioneer in the field of chemical separations, who said in 1962 (*64*), "The appetite of the chemist to work on a small scale will grow as it becomes more possible. He will be able to analyze and experiment on single cells. There is obviously an almost limitless field in making and using apparatus for measuring various physical properties on small objects."

REFERENCES AND NOTES

1. M. J. Gordon, X. Huang, S. L. Pentoney, Jr., R. N. Zare, *Science* **242**, 224 (1988).
2. J. W. Jorgenson and K. D. Lukacs, *ibid.* **222**, 266 (1983).
3. A. G. Ewing, R. A. Wallingford, T. M. Olefirowicz, *Anal. Chem.* **61**, 292A (1989).
4. D. Ishii and T. Takeuchi, in *Microcolumn Separations*, M. V. Novotny and D. Ishii, Eds. (Elsevier, New York, 1985), pp. 3–19.
5. B. L. Karger and A. S. Cohen, *J. Chromatogr.* **397**, 409 (1987).
6. Y. Walbroehl and J. W. Jorgenson, *J. Microcolumn Sep.* **1**, 41 (1989).
7. P. R. Dluzneski and J. W. Jorgenson, *J. High Resolut. Chromatogr. Chromatogr. Commun.* **11**, 332 (1988).
8. J. H. Knox and M. T. Gilbert, *J. Chromatogr.* **186**, 405 (1979).
9. J. W. Jorgenson and E. J. Guthrie, *ibid.* **255**, 335 (1983).
10. R. T. Kennedy, R. L. St. Claire, J. G. White, J. W. Jorgenson, *Mikrochim. Acta* **II**, 37 (1987).
11. R. T. Kennedy and J. W. Jorgenson, *Anal. Chem.* **61**, 436 (1989).
12. R. A. Wallingford and A. G. Ewing, *ibid.* **60**, 1972 (1988).
13. M. Lavallee, O. F. Schanne, N. C. Herbert, *Glass Microelectrodes* (Wiley, New York, 1969).
14. R. W. Linton *et al.*, *Trace Elem. Med.* **4**, 99 (1987).
15. D. W. Tank, M. Sugimori, J. A. Connor, R. R. Llinás, *Science* **242**, 773 (1988).
16. N. N. Osborne, *Biochem. Soc. Trans.* **9**, 583 (1981).
17. E. Giacobini, in *Biochemistry of Characterized Neurons*, N. N. Osborne, Ed. (Pergamon, New York, 1978), pp. 3–15.
18. N. N. Osborne, A. C. Szczepaniak, V. Neuhoff, *Int. J. Neurosci.* **5**, 125 (1973).
19. D. J. McAdoo, in *Biochemistry of Characterized Neurons*, N. N. Osborne, Ed. (Pergamon, New York, 1978), pp. 19–45.
20. C. M. Lent, R. L. Meuller, D. A. Haycock, *J. Neurochem.* **41**, 481 (1983).
21. R. M. McCaman, D. Weinrich, H. Borys, *ibid.* **21**, 473 (1973).
22. D. W. Corson and A. Fein, *Biophys. J.* **44**, 299 (1983).
23. M. Graessmann and A. Graessmann, *Methods Enzymol.* **101**, 482 (1983).
24. R. T. Kennedy and J. W. Jorgenson, *Anal. Chem.* **60**, 1521 (1988).
25. R. A. Wallingford and A. G. Ewing, *ibid.* **59**, 678 (1987).
26. J. Bouche and M. Verzele, *J. Gas Chromatogr.* **6**, 501 (1968).
27. G. Dykstra and J. d. Goey, in *Gas Chromatography 1958*, D. H. Desty, Ed. (Butterworths, Washington, DC, 1958), pp. 56–68.
28. R. L. St. Claire, thesis, University of North Carolina, Chapel Hill (1986).
29. S. Hjerten, *J. Chromatogr.* **347**, 191 (1985).
30. H. Lauer and D. McManigill, *Anal. Chem.* **58**, 166 (1986).
31. D. Ishii, K. Asai, K. Hibi, T. Jonokuchi, M. Nagaya, *J. Chromatogr.* **144**, 157 (1977).
32. Y.-F. Cheng and N. J. Dovichi, *Science* **242**, 562 (1988).
33. R. L. St. Claire and J. W. Jorgenson, *J. Chromatogr. Sci.* **23**, 186 (1985).
34. R. A. Wallingford and A. G. Ewing, *Anal. Chem.* **59**, 1762 (1987).
35. A. Manz and W. Simon, *J. Chromatogr. Sci.* **21**, 326 (1983).
36. J. S. M. de Wit and J. W. Jorgenson, *J. Chromatogr.* **411**, 201 (1987).
37. W. G. Kuhr and E. S. Yeung, *Anal. Chem.* **60**, 2642 (1988).
38. M. Yu and N. J. Dovichi, *ibid.* **61**, 37 (1989).
39. X. Huang, T. J. Pang, M. J. Gordon, R. N. Zare, *ibid.* **59**, 2747 (1987).
40. R. D. Smith and C. J. Barinaga, *ibid.* **60**, 1948 (1988).
41. E. Lee, W. Muck, J. D. Henion, P. R. Covey, *J. Chromatogr.* **458**, 313 (1988).
42. M. A. Moseley *et al.*, *Anal. Chem.* **61**, 1577 (1989).
43. L. A. Knecht, E. J. Guthrie, J. W. Jorgenson, *ibid.* **56**, 479 (1984).
44. R. T. Kennedy, thesis, University of North Carolina, Chapel Hill (1988).
45. J. G. White and J. W. Jorgenson, *Anal. Chem.* **58**, 2992 (1986).
46. ———, *ibid.*, p. 293.
47. E. J. Guthrie, J. W. Jorgenson, P. R. Dluzneski, *J. Chromatogr. Sci.* **22**, 171 (1984).
48. B. Nickerson and J. W. Jorgenson, *J. High Resolut. Chromatogr. Chromatogr. Commun.* **11**, 878 (1988).
49. G. A. Kerkut, J. D. C. Lambert, D. Gayton, R. J. Walker, *Comp. Biochem. Physiol. A* **50**, 1 (1975).
50. N. N. Osborne, *Microchemical Analysis of Nervous Tissue* (Pergamon, Oxford, 1974).
51. M. J. Brownstein, J. M. Saaverda, J. Axelrod, G. H. Zeman, D. O. Carpenter, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4662 (1974).
52. V. Chan-Palay and S. Palay, *Coexistence of Neuroactive Substances in Neurons* (Wiley, New York, 1984).
53. G. A. Kerkut, G. B. Sedden, R. J. Walker, *Comp. Biochem. Physiol.* **23**, 159 (1967).
54. M. M. Bushey and J. W. Jorgenson, *Anal. Chem.* **61**, 491 (1989).
55. P. de Montigny *et al.*, *ibid.* **59**, 1096 (1987).
56. M. D. Oates and J. W. Jorgenson, *ibid.* **61**, 432 (1989).
57. M. S. Gardiner in *McGraw-Hill Series in Organismic Biology*, M. S. Fuller and P. Licht, Eds. (McGraw-Hill, New York, 1972), p. 396.
58. H. Hyden, K. Bjurstam, B. McEwen, *Anal. Biochem.* **17**, 1 (1966).
59. D. L. Wilson, in *Biochemistry of Characterized Neurons*, N. N. Osborne, Ed. (Pergamon, New York, 1978), pp. 261–281.
60. D. J. Rose and J. W. Jorgenson, *J. Chromatogr.* **438**, 23 (1988).
61. M. Novotny, *Anal. Chem.* **60**, 500A (1988).
62. S. Terabe, K. Otsuka, T. Ando, *ibid.* **57**, 834 (1985).
63. A. Meuleumans, B. Poulain, G. Baux, L. Tauc, D. Henze, *ibid.* **58**, 2091 (1986).
64. A. J. P. Martin, in *Gas Chromatography 1962*, M. van Swaay, Ed. (Butterworths, Washington, DC, 1962), p. xxxi.

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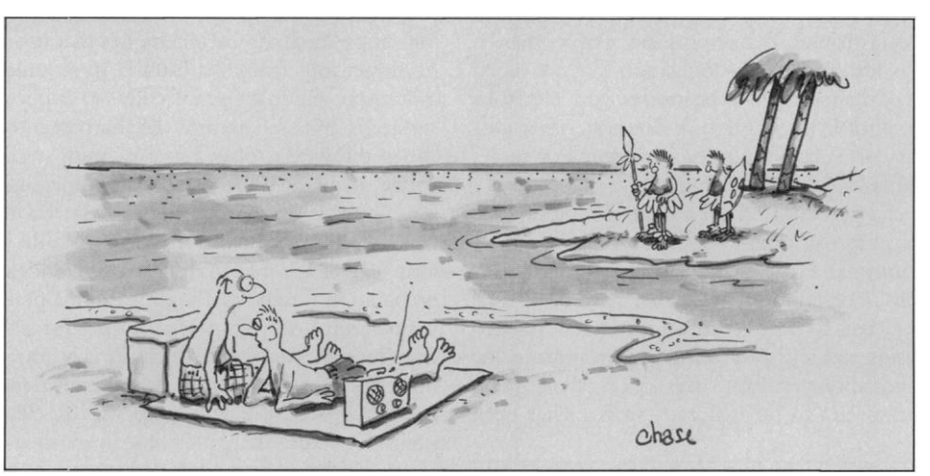

"I guess this strengthens the continental drift theory!"