

## The Separation of Whale Myoglobins with Two-Dimensional Electrophoresis

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*Five myoglobins (sperm whale, Sei whale, Hubbs' beaked whale, pilot whale, and Amazon River dolphin) were examined using two-dimensional electrophoresis. Previous reports indicated that none of these proteins could be separated by using denaturing (in the presence of 8–9 M urea) isoelectric focusing. This result is confirmed in the present study. However, all the proteins could be separated by using denaturing nonequilibrium pH-gradient electrophoresis in the first dimension. Additionally, all the myoglobins have characteristic mobilities in the second dimension (sodium dodecyl sulfate), but these mobilities do not correspond to the molecular weights of the proteins. We conclude that two-dimensional electrophoresis can be more sensitive to differences in primary protein structure than previous studies indicate and that the assessment seems to be incorrect that this technique can separate only proteins that have a unit charge difference.*

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**KEY WORDS:** two-dimensional electrophoresis; isoelectric focusing; nonequilibrium pH-gradient electrophoresis; sodium dodecyl sulfate gel electrophoresis; genetic variation; myoglobin.

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## INTRODUCTION

Two-dimensional electrophoresis is a powerful technique for simultaneously separating many polypeptides (O'Farrell, 1975). The technique accomplishes this separation by using isoelectric focusing to separate proteins by charge in the first dimension and sodium dodecyl sulfate gel electrophoresis to separate by molecular weight in the second dimension. Two-dimensional electrophoresis is increasingly being used to assess both qualitative and quantitative genetic variation (Klose, 1982; Klose *et al.*, 1983; Rosenblum *et al.*, 1983, 1984; Anderson *et al.*, 1985; Bahrman *et al.*, 1985; Jungblut and Klose, 1985; Neel *et al.*, 1985; Coulthart, 1986; Hanash *et al.*, 1986a,b; Leonardi *et al.*, 1987), to screen for mutants (Lee *et al.*, 1980; Marshall *et al.*, 1983; Neel *et al.*, 1984; Kelley *et al.*, 1985; Giometti *et al.*, 1987), and in systematic studies (Ohnishi *et al.*, 1983a,b; Lee and Pak, 1986; Takai and Kanda, 1986; Spicer, 1988). The advantage of this technique over standard one-dimensional electrophoresis is that it enables a researcher to simultaneously examine a large number of loci for genetic markers, mutations, or genetic variation.

However, some workers have suggested that two-dimensional electrophoresis may not be an appropriate technique for genetic surveys because it does not adequately separate proteins that differ in primary sequence (McLellan *et al.*, 1983; McLellan and Inouye, 1986). Studies examining the ability of isoelectric focusing (the first and primary dimension for separating similar proteins with two-dimensional electrophoresis) to detect genetic variation have been somewhat contradictory. Some studies have reported poor resolution (Ramshaw and Eanes, 1978), some have reported resolution approximately equal to that of standard one-dimensional electrophoresis (Basset *et al.*, 1978; Coyne *et al.*, 1979; McLellan and Inouye, 1986), and others have reported even better resolution (Powell, 1975). Comparable studies examining two-dimensional electrophoresis are similarly at odds. Some have indicated that it does not adequately detect genetic variants (Leigh Brown and Langley, 1979; McLellan *et al.*, 1983), while others have shown that it performs about equally as well as standard techniques (Lee *et al.*, 1980; Hickey, 1981; Wanner *et al.*, 1982). Consequently, more studies are needed to resolve this area of dispute (Singh and Coulthart, 1982).

The purpose of this study was to reinvestigate the results of a recent study by McLellan and Inouye (1986). They examined the ability of denaturing (the kind used in two-dimensional electrophoresis) and nondenaturing isoelectric focusing to separate several myoglobin protein sequences. Their results indicated that under denaturing conditions the technique performed poorly and that only unit charge differences could be detected. The present study shows that the isoelectric focusing technique used by McLellan and Inouye (1986) is not comparable to the usual two-dimensional electrophoresis methodology employed to separate proteins that have such a basic isoelectric point

and that the assessment seems to be incorrect that only unit charge substitutions are detected.

## MATERIALS AND METHODS

**Proteins.** The five myoglobins (Table I) were kindly provided by Dr. Tracy McLellan, Department of Biological Sciences, University of California at Santa Barbara. They were obtained by Dr. Tracy McLellan from Dr. F. R. N. Gurd, Department of Chemistry, Indiana University (McLellan, 1984). The lyophilized protein was dissolved at a concentration of 0.125 mg/ml in a 9 M urea, 2% Nonidet P-40 detergent, 2% mercaptoethanol, and 2% pH 9–11 ampholyte (LKB) mixture. The amount of sample loaded onto a gel ranged from 10 to 16  $\mu$ l. This resulted in a total loading of 1.25–2.00  $\mu$ g of protein per gel. Because samples were coelectrophoresed, 0.75–1.00  $\mu$ g of each myoglobin sequence was loaded per gel. Pure samples of myoglobin ranging from 0.625 to 1.875  $\mu$ g (5–15  $\mu$ l) were electrophoresed to examine the purity of the proteins.

**Charge Standards.** To align the gels properly, for accurate determination of the relative positions of the myoglobins, charge standards were applied (Anderson and Hickman, 1979; Hickman *et al.*, 1980). The carbamylated protein used was glyceraldehyde-3-phosphate dehydrogenase (Pharmacia). This was reconstituted from its dehydrated form by adding a 1:1 mixture of water and 9 M urea, 2% Nonidet P-40 detergent, 2% mercaptoethanol, and 2% pH 9–11 ampholyte (LKB) mixture. Between 2 and 6  $\mu$ l of charge standard was added per gel.

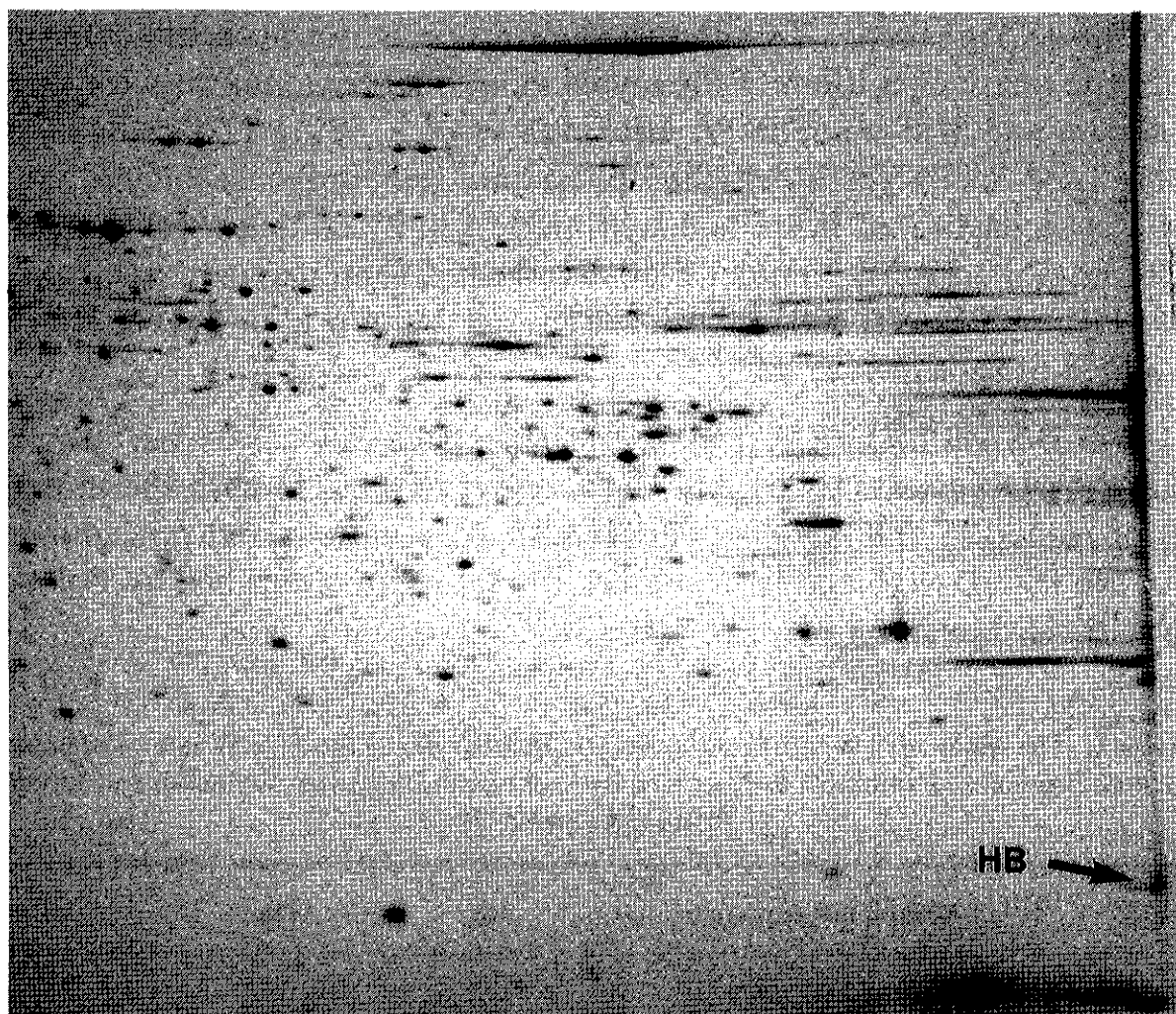
**Two-Dimensional Electrophoresis.** Electrophoresis was performed as outlined by O'Farrell (1975) and O'Farrell *et al.* (1977) with the modifications of Anderson and Anderson (1978a,b). All solutions and procedures for use of the ISO-DALT and BASO-DALT systems are described by Tollaksen *et al.* (1984).

**Table I.** The Molecular Weight and Isoelectric Points (*pI*) of the Five Whale Myoglobin Protein Sequences Examined, as Reported by McLellan (1984) and McLellan and Inouye (1986), Respectively

Myoglobin sequence	MW	<i>pI</i>
Sei whale ( <i>Balanoptera borealis</i> )	17,745	7.6
Sperm whale ( <i>Physeter catodon</i> )	17,816	7.7
Hubbs' beaked whale ( <i>Mesoplodon carlhubbsi</i> )	17,787	7.8
Amazon river dolphin ( <i>Inia geoffrensis</i> )	17,701	7.9
Pilot whale ( <i>Globicephala malaena</i> )	17,715	7.9

The first-dimension isoelectric focusing and nonequilibrium *pH*-gradient electrophoresis were performed using 2% *pH* 3–10 ampholytes (Biolyte), 4% Nonidet P-40, 4% acrylamide, 9 M urea. Two other isoelectric focusing mixtures were also used: 1% *pH* 3–10 ampholytes (Biolyte), 1% *pH* 5–7 ampholytes (Biolyte), 4% Nonidet P-40, 4% acrylamide, 9 M urea, and 3.6% *pH* 5–8 ampholytes (Pharmalyte), 0.2% *pH* 3–10 ampholytes (Biolyte), 0.2% *pH* 3–10 ampholytes (Pharmalyte), 4% Nonidet P-40, 4% acrylamide, 9 M urea. The isoelectric focusing was run with the upper reservoir containing 0.001 M phosphoric acid and the lower reservoir containing 0.02 M sodium hydroxide. These were reversed when performing nonequilibrium *pH*-gradient electrophoresis. This dimension was run in tube gels 25 cm long. The isoelectric focusing was at 30,000 V-hr for an overall run time of 23 hr. The nonequilibrium *pH*-gradient electrophoresis was run at 17,500 V-hr for 17.5 hr.

The second dimension consisted of 9–17% polyacrylamide sodium dodecyl sulfate (SDS) gels poured using computer-controlled stepping pumps



**Fig. 1.** Two-dimensional electrophoretic gel of mouse liver coelectrophoresed with Hubbs' beaked whale myoglobin. Notice how the protein spots surrounding the myoglobin sequence are markedly streaked. The high molecular weight proteins are at the top of the gel, and the basic proteins are at the right. HB, Hubbs' beaked whale.

and run at 100–150 V (0.6 amp) overnight. These gels measured 20 × 25 cm.

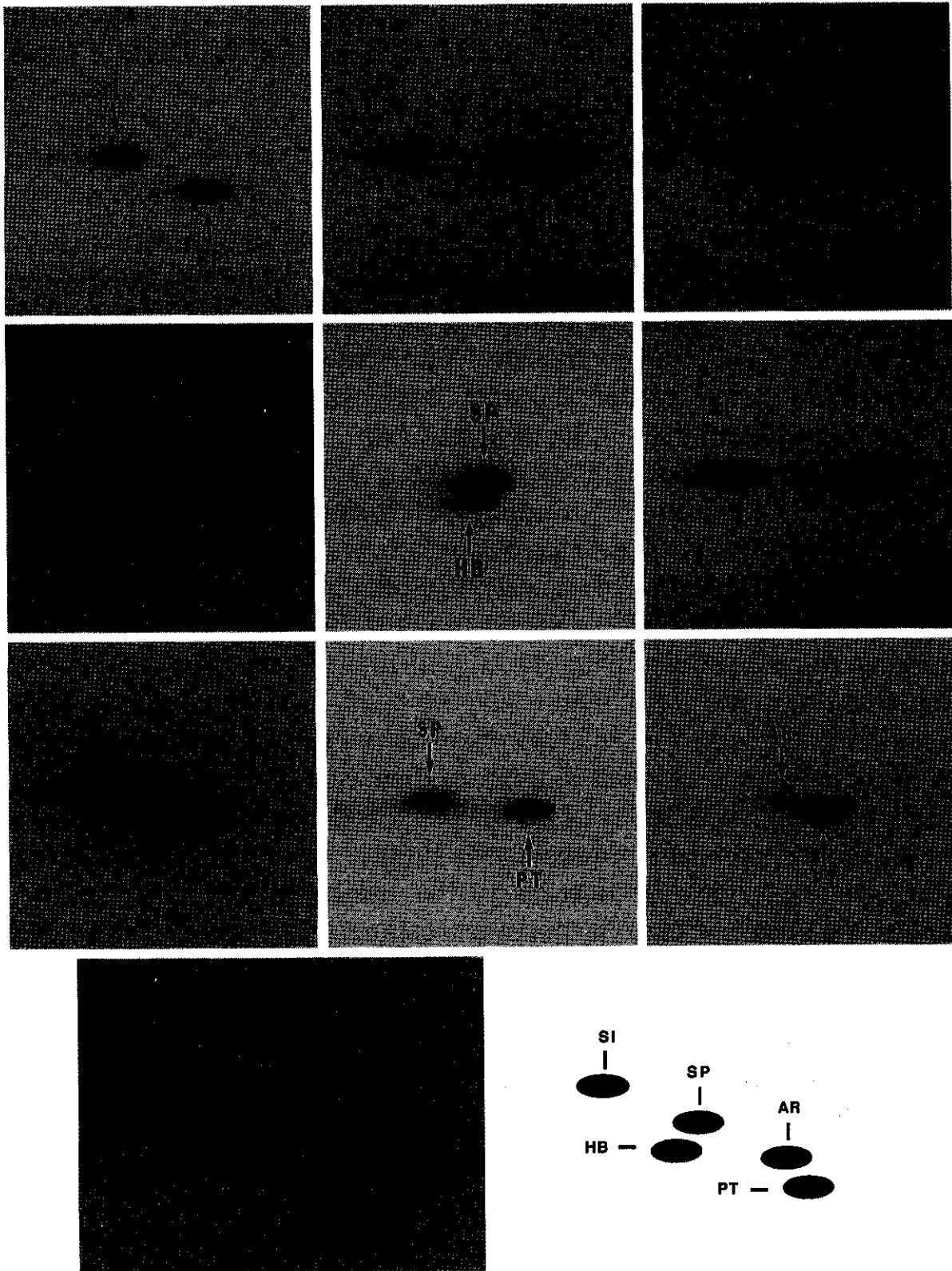
Gels were stained overnight in a solution of 0.125% Coomassie brilliant blue (Serva blue R), 2.5% phosphoric acid, and 50% ethanol and destained several times in 20% ethanol.

## RESULTS

A total of 128 two-dimensional electrophoretic gels was run for this study. None of the five whale myoglobins could be separated using any of the three denaturing isoelectric focusing conditions (see Materials and Methods) in the first dimension. This confirms the results presented by McLellan and Inouye (1986) with the qualification that they used a slightly narrower and more basic pH range of ampholines. However, as Fig. 1 shows, these proteins are so basic that they barely migrated onto the gel with the pH range of the ampholytes that are ordinarily used in the first dimension of two-dimensional electrophoresis. This indicated that it would be more appropriate to run them by using nonequilibrium pH-gradient electrophoresis (O'Farrell *et al.*, 1977).

When run using denaturing nonequilibrium pH-gradient electrophoresis in the first dimension, all five whale myoglobin proteins can clearly be distinguished (Fig. 2). The relative relationships of the protein sequences in the first dimension correspond roughly to their isoelectric points (Table I) as given by McLellan and Inouye (1986). Two exceptions were noted, however. The Hubbs' beaked whale sequence was slightly more acidic than the sperm whale sequence, which is contrary to the results given by McLellan and Inouye (1986) (see Table I). In the other instance, the Amazon River dolphin sequence was more acidic than the pilot whale sequence. The latter two proteins were not separable with nondenaturing isoelectric focusing by McLellan and Inouye (1986), and consequently they were given the same isoelectric point. The observation that the isoelectric point of a protein may differ between native and denaturing gels has been noted before (Lee *et al.*, 1980).

The second dimension also resolved differences among these proteins, so that each sequence could be separated by the SDS dimension alone (Fig. 2). These differences did not correspond to the known molecular weights of these myoglobins. For example, the Sei whale sequence migrated to a higher molecular weight position on the gels than did the sperm and Hubbs' beaked whale sequences, although the latter protein sequences have a greater molecular weight. The same is true for the Amazon River dolphin and pilot whale myoglobins. The position on the gels would indicate that the Amazon River dolphin sequence has a greater molecular weight than the pilot whale



**Fig. 2.** Sections of two-dimensional electrophoretic gels showing all possible combinations of coelectrophoresis of the five myoglobins together with an exaggerated diagram indicating the position of each myoglobin protein sequence relative to the others. The high molecular weight proteins are at the top, and basic proteins are at the left. AR, Amazon River dolphin; HB, Hubbs' beaked whale; PT, pilot whale; SI, Sei whale; SP, sperm whale.

sequence, but the opposite is actually the case. The pilot whale myoglobin is slightly heavier than the Amazon River dolphin myoglobin.

## DISCUSSION

The myoglobins examined here have previously been studied by using both one-dimensional electrophoresis and isoelectric focusing (denaturing and nondenaturing). The one-dimensional procedure separated all of the myoglobins studied here, but under only one of the five electrophoretic conditions used (McLellan, 1984). Under the other four buffer conditions the five myoglobins resolved into only three or four mobility classes. With the nondenaturing isoelectric focusing these five myoglobins separated into four mobility classes, and with the denaturing isoelectric focusing these protein sequences resolved into only a single mobility class. From these observations, McLellan and Inouye (1986) concluded that isoelectric focusing was no better than one-dimensional electrophoresis in resolving protein differences. They also supported the conclusion of McLellan *et al.* (1983) that under the denaturing conditions used in two-dimensional electrophoresis, only proteins that differ by a unit charge substitution are separable.

The results here indicate that these conclusions were not general with respect to all two-dimensional electrophoretic conditions. With the standard pH range commonly employed for two-dimensional electrophoresis, the myoglobin proteins are not separable. However, as Fig. 1 shows, this is not a proper electrophoretic condition, because proteins with such a basic isoelectric point would not be scored in a genetic survey. The reason for this is that the ampholines available for separating basic proteins do not produce high-resolution pH gradients (O'Farrell *et al.*, 1977; McLellan and Inouye, 1986). Under more appropriate conditions [nonequilibrium pH-gradient electrophoresis (O'Farrell *et al.*, 1977)] all of these proteins were separated (Fig. 2). Although these five proteins differ by 8–17 amino acids of a total of 153, they all have the same unit charge (McLellan, 1984). It was thought that the denaturants commonly used would eliminate the subtle differences that allow separation of proteins that differ by less than a unit charge. However, the results presented here indicate that the presence of a denaturant fails to eliminate some of these minor differences. It seems that the partial or complete denaturation of the proteins by urea may have even created additional charge differences by exposing previously unexposed parts of the molecules (Tracy McLellan, personal communication). In fact, two-dimensional electrophoresis with denaturing nonequilibrium pH-gradient electrophoresis performed better than nondenaturing isoelectric focusing in the study of McLellan and Inouye (1986) and the same as the most successful one-dimensional system studied by McLellan (1984). Consequently, the

observation that two-dimensional electrophoresis in the presence of a denaturant can separate only proteins that differ by a unit charge substitution seems to be incorrect.

Another rarely mentioned aspect of the separation involved in two-dimensional electrophoresis concerns the second dimension. All five myoglobins had characteristic mobilities in the presence of SDS. This dimension traditionally has been thought to separate proteins by their molecular weight, although it recently has become apparent that some proteins do not follow this general rule (Andrews, 1981). It is now known that a change in a single amino acid can be detected (De Jong *et al.*, 1978; Noel *et al.*, 1979; Vandekerckhove *et al.*, 1980; Wilson *et al.*, 1983; Vlasuk *et al.*, 1984; Takahashi *et al.*, 1987), and even the carbamylation of the amino group can change the mobility of some proteins in this dimension (Anderson and Hickman, 1979). Consequently, electrophoresis in the presence of SDS is probably much more sensitive to changes in the primary sequence of proteins than was previously realized. This observation is borne out in many two-dimensional electrophoretic studies of genetic variation that reveal genetic variants in the SDS dimension (Rosenblum *et al.*, 1983, 1984; Neel *et al.*, 1985; Hanash *et al.*, 1986a,b). Although deletions in a protein have been shown to be detectable in the SDS dimension of two-dimensional electrophoresis (Kelley *et al.*, 1985), deletions are unlikely to cause all of the observed variants that have been noted. The most probable explanation is that many amino acid substitutions produce detectable differences in the SDS dimension.

A view that has been recently emphasized is that no technique under any one condition is ideal for separating all proteins (Coyne *et al.*, 1979; Coyne, 1982). However, this view has not yet become standard for isoelectric focusing and two-dimensional electrophoresis. Comparative studies have all assumed that there is only one way to perform these techniques. But these techniques are no different from any other—different conditions produce different results. Simply changing the size of the isoelectric focusing gel changes the resolving power of the technique. The first-dimension gels run in this study were considerably longer than those in other studies examining the ability of isoelectric focusing to separate proteins (Basset *et al.*, 1978; Ramshaw and Eanes, 1978; Coyne *et al.*, 1979; McLellan and Inouye, 1986), and yet the gels run in the present study are smaller than those in some other studies. Young (1984) demonstrated that running longer gels (about twice as long as those in this study) increased the resolution dramatically.

An additional area of concern is the ampholines that are used. This is the critical element in separating polypeptides with isoelectric focusing. It is well known that different manufacturers use different protocols for producing ampholines (Righetti, 1983), and different products can give dramatically different results (Tollaksen *et al.*, 1981). However, the comparative studies

have seemingly used only LKB products (Basset *et al.*, 1978; McLellan and Inouye, 1986), and some studies have not indicated what product was used (Ramshaw and Eanes, 1978; Coyne *et al.*, 1979; McLellan *et al.*, 1983). With the advent of immobilized pH-gradient isoelectric focusing, an attempt to generalize about the ability of isoelectric focusing to separate proteins becomes even more difficult. An initial study by Whitney *et al.* (1985) indicated that these gradients can dramatically outperform other electrophoretic techniques in separating mouse hemoglobins that differ only by neutral amino acid substitutions. Consequently, generalizations about the ability of isoelectric focusing and two-dimensional electrophoresis to separate proteins must be assessed with caution, because rapid advances in the technology will enable different systems to produce very different results.

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