GENETIC VARIATION IN PROTEINS: COMPARISON OF ONE-DIMENSIONAL AND TWO-DIMENSIONAL GEL ELECTROPHORESIS

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ABSTRACT

Two proteins with known characteristics on one-dimensional gels were studied by two-dimensional electrophoresis to compare the sensitivities of the two methods in detecting genetic variation. Two-dimensional electrophoresis was found to be less sensitive than several types of one-dimensional gels in distinguishing variants of both proteins. Denaturation of proteins in urea in the two-dimensional method makes it possible to distinguish closely related proteins that differ from each other by units of charge. Many more types of variation in protein sequences can be distinguished on one-dimensional gels in the absence of denaturants. The estimates of heterozygosity based on two-dimensional gels are lower than those based on other methods, at least in part, because of the limited types of sequence differences that can be detected on two-dimensional gels. The application of two-dimensional electrophoresis to the measurement of genetic variation and to the detection of new mutations should be made carefully, in view of the limited sensitivity of the method in finding differences in sequence.

TWO-DIMENSIONAL gel electrophoresis, as originally described by O'FARRELL (1975), has recently been applied to the measurement of genetic variation. The results have shown consistently less variability than one-dimensional electrophoretic surveys of enzyme variation in humans (WALTON, STYER and GRUENSTEIN 1979; MCCONKEY, TAYLOR and PHAN 1979; SMITH, RACINE and LANGLEY 1980), mice (RACINE and LANGLEY 1980; AQUADRO and AVISE 1981) and Drosophila (LEIGH BROWN and LANGLEY 1979). The two-dimensional method has the advantage of separating a large number of proteins from each other so that they can be visualized on a single gel. The authors who have used the two-dimensional method to assess the level of genic heterozygosity, presenting their results as "new estimates" (MCCONKEY, TAYLOR and PHAN 1979) or "reevaluation" of the level of heterozygosity (LEIGH BROWN and LANGLEY 1979), have assumed that two-dimensional gels have the same power to resolve differences in protein sequence as the more commonly used one-dimensional

gels, and that the only difference between the methods was the type and number of proteins visualized by each.

There have been some attempts at direct comparison of the same proteins by both one- and two-dimensional electrophoresis. α-Glycerophosphate dehydrogenase (α-GPDH) from ten species of Drosophila was examined on two-dimensional gels and compared to starch gel electrophoresis (LEIGH BROWN and LANGLEY 1979). Only major charge differences were detected on two-dimensional gels, whereas more variants were seen on starch. The authors concluded that "it is premature to make a direct comparison between the level of heterozygosity observed with 2D electrophoresis and that observed with standard gel techniques." A second study examined two to five variants for each of five proteins (WANNER, NEEL and MEISLER 1982). Several of the variants were not separated from the "common" form when studied by two-dimensional gels, and separation of variants from each other was not examined. A second gel with a higher pH range in the first dimension was necessary for distinguishing some of the variants. Neither were the criteria for original recognition of the variants given, nor was the basis for the difference between the methods explained. Both studies have produced evidence that two-dimensional gels are less sensitive for the detection of genetic variants in proteins than standard gels.

Two-dimensional electrophoresis has the potential to permit study of a larger number of proteins and of a set of proteins that are not soluble under the conditions of standard one-dimensional gels. Because of this, two-dimensional gels may become widely used in the assessment of genetic variation, mutation rates and comparison of taxa. Therefore, it seemed important to compare the sensitivities of the two techniques using proteins with known variants and that could be visualized on both types of gels. This paper describes such a comparison, using α-GPDH from different species of Drosophila and human hemoglobins of known sequence. There is a clear difference in the sensitivities of the two types of methods, and an explanation for the difference is offered.

MATERIALS AND METHODS

Five human hemoglobin mutants of known sequence, Hb Hope, Hb Pyrgos, Hb Lufkin, Hb J-Baltimore and Hb J-Bangkok, as well as Hb A, were examined. Each of the mutant hemoglobins has a substitution of aspartic acid for glycine in the beta subunit. The positions of the substitutions are given in Table 1. Red blood cell lysates, all of which were heterozygous with Hb A, were diluted 300-fold in solution A of O'FARRELL (1975); 5 μl were used for each gel. Each of the mutant hemoglobins was examined on a two-dimensional gel by itself and in all pairwise combinations with the other hemoglobins.

α-GPDH from Drosophila was examined from crude homogenates of flies. *Drosophila pseudoobscura, D. lebanonensis, D. funebris, D. willistoni, D. hydei, D. busckii* and three strains of *D. melanogaster* were examined. They were chosen as representatives of six of the nine isoelectric focusing classes obtained in a previous study of α-GPDH (COYNE *et al.* 1979). The *D. melanogaster* 112 strain, which formed a tenth mobility class by isoelectric focusing (J. RAMSHAW, unpublished results) was also added. Pure α-GPDH from *D. melanogaster* 104 was used as a standard marker on some of the gels.

Two-dimensional polyacrylamide gel electrophoresis was performed according to O'FARRELL (1975), in a manner similar to that used by WALTON, STYER and GRUENSTEIN (1979), MCCONKEY, TAYLOR and PHAN (1979), RACINE and LANGLEY (1980), AQUADRO and AVISE (1981) and LEIGH BROWN and LANGLEY (1979). The gels were stained with Coomassie blue according to FAIRBANKS, STECK and WALLACH (1971), dried and photographed.

TABLE 1

Electrophoretic mobilities of five human hemoglobins

Name	Position of Substitution[a]	Classification	
		One-dimensional[b]	Two-dimensional[c]
Hope	136	1.00	I
Pyrgos	83	1.14	I
Lufkin	29	1.16	I
J-Baltimore	16	1.17	I
J-Bangkok	56	1.17	I

[a] All have substitution of aspartic acid for glycine in the beta subunit.
[b] According to RAMSHAW, COYNE and LEWONTIN (1979).
[c] The mobility of the beta subunit of Hb Hope is taken as class I.

RESULTS

The patterns obtained with the hemoglobins by two-dimensional electrophoresis consisted of one or two major spots, the globins, and a few minor spots on gels with more concentrated samples (Figure 1). The alpha and beta globins of hemoglobin A overlapped, producing a single spot (Figure 1C). Two spots were visible on each of the gels of the five mutant hemoglobins. One spot corresponded to the alpha subunit and the unmutated beta subunit, whereas the other, having the same molecular weight but located toward the acidic end of the gel, represented the beta subunit containing the substitution of aspartic acid for glycine. All gels with the hemoglobin variants, including all combinations of pairs of samples, had the same two-spot pattern (Figure 1). Therefore, for the five hemoglobins examined, the mutated beta chain was in the same location on each of the two-dimensional gels.

The same hemoglobin variants had been examined previously (RAMSHAW, COYNE and LEWONTIN 1979) by one-dimensional polyacrylamide gel electrophoresis and formed four mobility classes on a single gel (Table 1). Two of the sequence variants, J-Baltimore and J-Bangkok, could not be distinguished from each other with any of the three gel conditions employed in that study, involving two acrylamide concentrations and two pHs. Therefore, five proteins known to be different in sequence were indistinguishable on two-dimensional gels but formed four distinct entities on one-dimensional gels.

α-GPDH was identified by comparing the patterns obtained with (1) purified *D. melanogaster* 104 α-GPDH alone, (2) *D. melanogaster* 104 fly homogenate alone and (3) a combination of the two (Figure 2). The enzyme appeared on the lower half of the gel with a molecular weight of 30,000 and an isoelectric point of about 6.8. α-GPDH was identified in the other species as having the same molecular weight and the same shape as the purified *D. melanogaster* 104 protein. Few other proteins appeared in this area of the gel with the same mobility in the SDS dimension. The identification of α-GPDH in two species, *D. funebris* and *D. busckii*, was not certain, as there were two spots with the same molecular weight as the purified protein, either of which could have represented the α-GPDH. The shapes of the spots were different, and the one that resembled

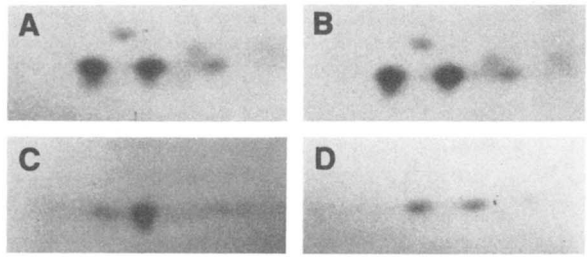

FIGURE 1.—Two-dimensional gels of human hemoglobins, A, Hb A, Hb Hope and Hb Lufkin. B, Hb A, Hb Hope and Hb J-Bangkok. C, Hb A. D, Hb Lufkin. The anode is on the left.

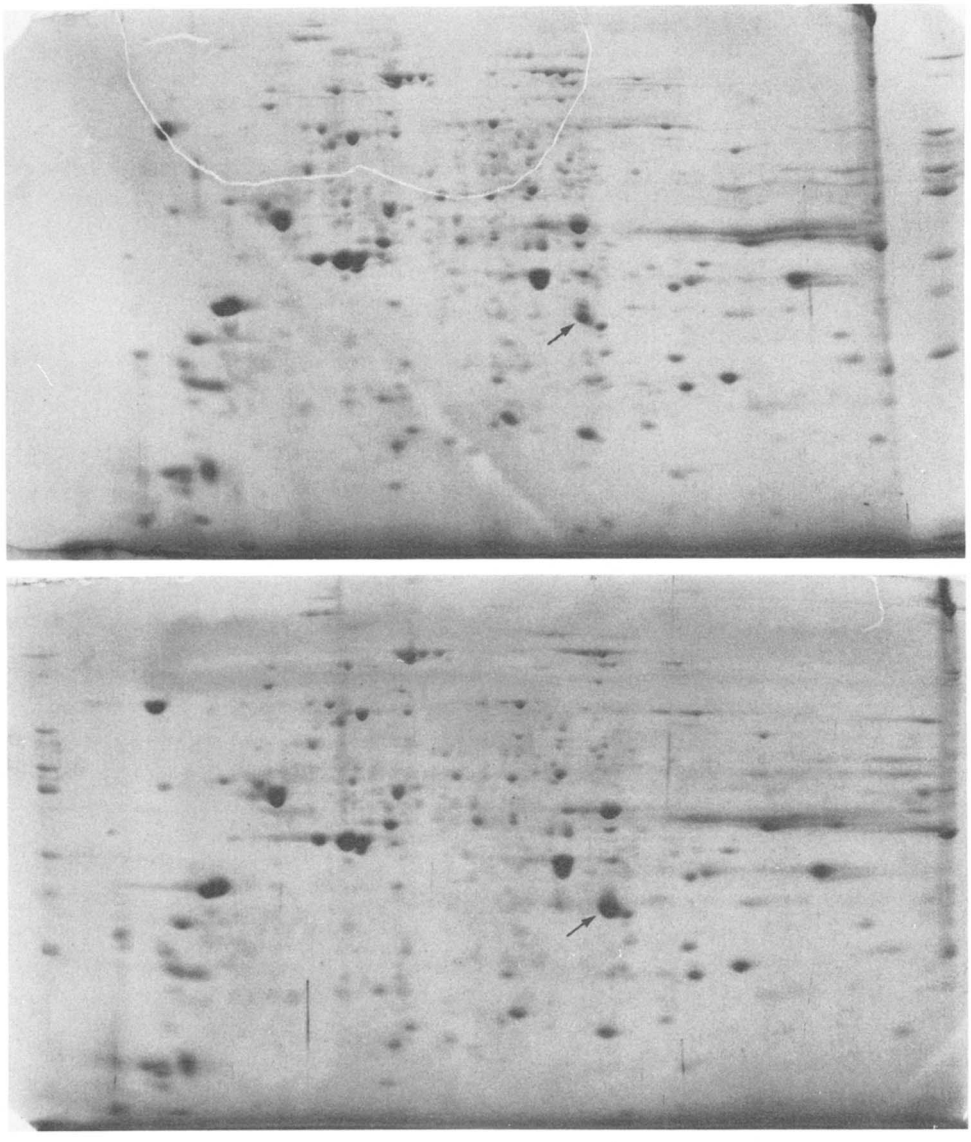

FIGURE 2.—Two-dimensional gels of Drosophila. Upper gel, *D. melanogaster* 104. Lower gel, *D. melanogaster* 104 and 4 μg of pure α-GPDH from *D. melanogaster* 104. The arrows indicate α-GPDH.

in shape the known α-GPDH in other species was scored as α-GPDH in these species.

The mixtures of four species, *D. willistoni, D. hydei, D. busckii* and *D. melanogaster* 104, with the pure protein from the *D. melanogaster* 104 strain, produced gels that were indistinguishable from those with the fly homogenates alone, indicating that α-GPDH from these species was identical in mobility on the two-dimensional gels. Three species, *D. lebanonensis, D. funebris* and *D. pseudoobscura* produced spots indistinguishable from each other but shifted toward the basic end of the gel from the α-GPDH of *D. melanogaster* 104. *D. melanogaster* strains 108 and 112 α-GPDH were shifted toward the acidic end of the gel in relation to that of the 104 strain; each of them appeared in a different position. Thus, the nine α-GPDH variants examined produced at least four distinct mobility classes on two-dimensional gels. Due to the difficulty in positively identifying α-GPDH in two species, there may be five or six distinct mobility classes among the nine variants.

There is a range in sensitivity of several electrophoretic methods which have been used to examine α-GPDH previously (Table 2). Two-dimensional gels are clearly less sensitive than four of the one-dimensional methods. The mobility classes on two-dimensional gels correspond to the major classes found by isoelectric focusing in the absence of denaturants, but the small differences

TABLE 2

Electrophoretic mobilities of α-GPDH as determined by different techniques

	Starch (pH 7.1)[a]	Cellulose acetate[b]	Starch (pH 8.6)[c]	PAG[c]	IEF[c]	Two-dimensional
C. pseudoobscura	100	1	1	1000	1a	I
D. lebanonensis	[d]	[d]	2	2000	1b	I
D. funebris	100	2	3	2001	1c	I[e]
D. willistoni	104	4	5	3000	2b	II
D. hydei	104	4	7	4100	2c	II
D. melanogaster 104	104	4	7	4110	2c	II
D. busckii	106	5	7	4130	2b	II[e]
D. melanogaster 108	108	7	10	7000	3b	III
D. melanogaster 112	112[f]	8[f]	11[f]	8000	4[f]	IV
No. examined	8	8	9	9	9	9
No. distinguished	5	6	7	9	7	4[e]

The classifications for the techniques in the first five columns are taken from the original references, where larger numbers indicate greater mobility toward the anode. For two-dimensional gels the classifications are also in order of increasing mobility toward the anode.

[a] LAKOVAARA, SAURA and LANKINEN 1977.

[b] COLLIER 1977.

[c] COYNE *et al.* 1979.

[d] Not examined.

[e] *D. funebris* and *D. busckii* may have α-GPDHs with mobilities distinct from those assigned. The lack of a specific identification for the enzyme made positive identification impossible.

[f] Unpublished results by J. RAMSHAW show that this form is distinct from 71 other species (COYNE *et al.* 1979) and is anodal to all other forms.

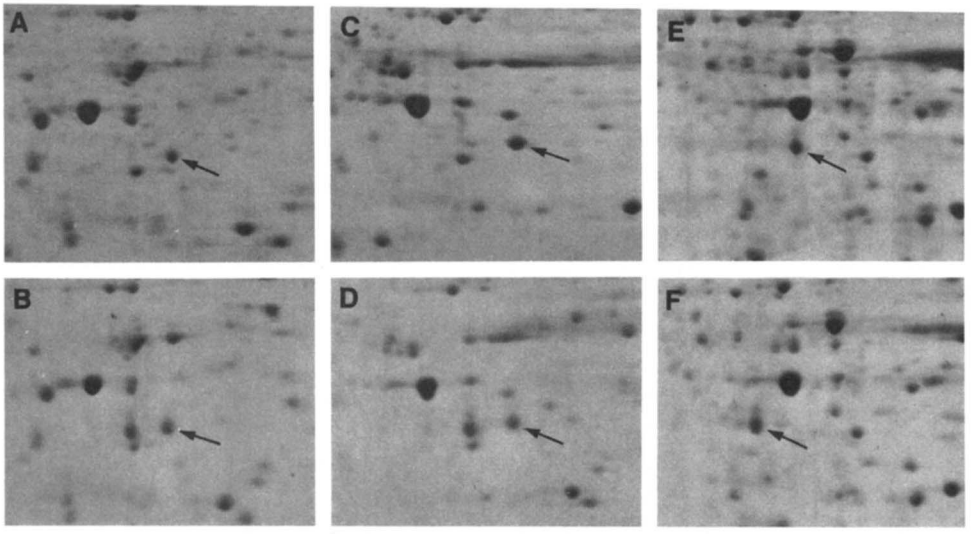

FIGURE 3.—Sections of two-dimensional gels of Drosophila. A, *D. lebanonensis*. B, *D. lebanonensis* with 4 μg of pure α-GPDH from *D. melanogaster* 104. C, *D. pseudoobscura*. D, *D. pseudoobscura* with 4 μg of pure α-GPDH from *D. melanogaster* 104. E, *D. melanogaster* 108. F, *D. melanogaster* 112. Arrows indicate the α-GPDH spots.

within each class were not distinguished, even using the comparison to the purified protein standard.

DISCUSSION

With the two proteins examined here, two-dimensional gels distinguished fewer variants than have been separated by one-dimensional electrophoresis. The mutant hemoglobins could not be distinguished from each other, and fewer than half as many α-GPDH classes were seen on two-dimensional gels as on the most sensitive one-dimensional electrophoretic technique. A direct comparison of the same hemoglobin mutants on starch gels is not available. However, examination of hemoglobin mutants on starch gels at pH 8.6 distinguished nine of 12 sequences in the +2 charge class from each other (FUERST and FERRELL 1980). If these hemoglobins were to behave the same way as the five examined here, then these mutants would presumably not be separated from each other on two-dimensional gels.

The biochemical basis for separation of proteins differs between the two electrophoretic methods. Charge appears to be the major determinant of separation of genetic variants in both types of gels. Amino acid substitutions of one major charge type—acidic, basic or neutral—for one of the other charge types have been hypothesized to be the major cause of separation of proteins differing by one or a few residues in sequence (MARSHALL and BROWN 1975). In addition to unit charge change substitutions, one-dimensional gels have been shown to separate proteins within the same nominal major charge class (FUERST and FERRELL 1980; RAMSHAW, COYNE and LEWONTIN 1979; RAMSHAW and EANES

1978). Separation within a charge class may be due to differential ionization of substituted residues or the "partial charges" carried by some amino acids (JOHNSON 1974). Differences between proteins have often been divided into two classes: unit charge change substitutions and conformational differences (LEIGH BROWN and LANGLEY 1979). Minor charge differences can be important in determining electrophoretic differences without influencing conformation of the molecule. Alternatively, changes in conformation can influence the interaction of charged or polar groups with each other and, thus, cause minor charge differences, so that categories of charge and conformational variation may not be independent.

The first step in the O'FARRELL (1975) method is the treatment of extracts with 8 or 9 M urea, mercaptoethanol or dithiothreitol, and sometimes SDS. This treatment serves to solubilize hydrophobic proteins and to dissociate noncovalently bound subunits; it also completely denatures (unfolds) polypeptides (TANFORD 1968). Denaturation in urea, therefore, results in the loss of the site-specific influences on ionization, so that the only differences in ionization are the major charge change substitutions. Unit charge changes, therefore, should be detectable on two-dimensional gels, as has been demonstrated previously (STEINBERG *et al.* 1977), but all proteins in one major charge class should appear identical, as we have found with the hemoglobins examined here.

In addition to the direct influences of sequence on charge, there are several other causes of electrophoretic differences in proteins in native conformation. Differential affinity for the gel matrix (SWALLOW *et al.* 1975; MARTINUIK and HIRSCHHORN 1980) or for a component of the electrophoresis buffer (HARRIS *et al.* 1968) causes separation of variants under some conditions but not others. Variation in affinity for cofactors may also influence electrophoretic behavior of some genetic variants (JOHNSON 1978). Changes in sequence influencing the association of subunits allow the separation of variants by molecular weight differences between forms in which different numbers of subunits are found together (SMITHIES 1965; COBBS 1976). Unless unit charge change substitutions accompany affinity and subunit differences, these types of differences would not be expected to be found by two-dimensional electrophoresis, as the peptides are examined as separated subunits, and denaturation should remove any differences in affinity. Few variable proteins have been fully characterized according to affinity and subunit differences, so that the extent of their importance in electrophoretic surveys is not known.

The method of comparison of samples to assess variation differs between the two types of electrophoresis. One-dimensional gels are usually slab gels; adjacent lanes can be compared, and small mobility differences can be scored unambiguously and repeatedly. The identification of genetic variants from two-dimensional gels, on the other hand, requires the comparison of independent gels. The variability in patterns between gels has been identified as a major problem in the application of two-dimensional electrophoresis to genetic screening (CLARK 1981). Significant broadening of bands during separation in the second dimension (O'FARRELL 1975) may obscure small separations made in the first dimension. A purified protein standard can be added to mark the region of

the gel that is of interest, but even then there may be some ambiguity in comparing separate gels. Mixed samples, labeled with different isotopes, can also be used (McCONKEY, TAYLOR and PHAN 1979; WALTON, STYER and GRUENSTEIN 1979), but this approach limits the type of samples that can be examined to those that can be grown on radioactive media and limits the number of comparisons that can be made, because all samples must be examined in pairwise combinations.

There is a potential for the modification of the conditions of two-dimensional electrophoresis to increase its sensitivity in detecting small differences in protein sequence (EDWARDS and HOPKINSON 1980). Examination of extracts on two gels with different pH ranges in the first dimension has increased the number of variants detected as well as the number of proteins visualized (WANNER, NEEL and MEISLER 1982). Using a narrower pH range for the first dimension might result in finding more variation than on a gel with broad pH range. The use of a first dimension gel without denaturants might increase the number of variants found in a particular protein, although the total number of proteins visualized might decrease.

Two-dimensional gels have proven to be an important technique in many areas of biological research. Because most proteins can be clearly separated from each other, the identification of the presence or absence of a certain peptide can be made unambiguously. Large differences in quantity of certain proteins can also be compared between samples. The two-dimensional method has been used successfully to identify genetic variants in some proteins (COMINGS 1979; ZANNIS, JUST and BRESLOW 1981) and was instrumental in clarifying the basis for the polymorphism of human apolipoprotein E. Posttranslational modifications of the protein produce multiple molecular forms, which can be distinguished most clearly on two-dimensional gels (ZANNIS, JUST and BRESLOW 1981; UTERMAN, STEINMETZ and WEBER 1982).

Nevertheless, the sensitivities of two-dimensional and standard one-dimensional gels in distinguishing genetic variants are not the same. There are also differences in the amount of variability in the different fractions of proteins examined by the two methods (EDWARDS and HOPKINSON 1980; KLOSE and FELLER 1981; SINGH and COULTHART 1982). The application of the two-dimensional method to the detection of new mutants, to general screening for genetic defects and to quantification of genic heterozygosity must be done with an awareness of the sensitivity of the method. Direct comparison of different samples of proteins measured by the different techniques is inappropriate. The claim that two-dimensional gels reveal a new class of mostly invariant proteins, typical of the entire genome, seems to be based on an inappropriate comparison of both different samples of proteins and of methods that differ in their sensitivity.

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