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Analytical Techniques for Cell Fractions

XXIV. Isoelectric Point Standards for Two-Dimensional Electrophoresis[1,2]

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We have investigated the two-dimensional electrophoretic behavior of a number of proteins throughout the course of carbamylation induced by heating in urea solution. Stepwise charge changes occur which are apparently commensurate with the number of free amino groups in the protein. Characteristic changes also occur in the sodium dodecyl sulfate (SDS) electrophoretic mobility (SDS molecular weight) of some heavily carbamylated proteins, implying altered structure or SDS binding. A mixture of all carbamylation intermediates of a protein provides a simple solution to the problem of internal isoelectric point standardization in the two-dimensional electrophoretic technique of P. H. O'Farrell (1975, *J. Biol. Chem.* **250**, 4007–4021).

When a protein is heated in a solution of urea, its amino groups are progressively carbamylated through the production of cyanate (2). Since the loss of a free amino group below pH ~8.5 results in a unit change in the charge of a molecule (and hence a shift in isoelectric point), the products of this reaction appear as a row of spots at roughly constant molecular weight when analyzed by high-resolution, two-dimensional electrophoresis [isoelectric focusing in urea followed by SDS[3] electrophoresis (1,3,4)]. By heating a protein for various lengths of time, the extent of carbamylation can be varied to obtain a uniform series of spots. We blend a number of partially carbamylated samples plus the original uncarbamylated protein and thus obtain a uniform series of spots ranging from native to completely modified molecules. We call such a complete series a "carbamylation train."

If the native protein used has a fairly basic isoelectric point (pH 7–8), its carbamylation train can span a large fraction of the pH range accessible in isoelectric focusing experiments and can serve as a regular series of p*I* markers. As would be expected, the number of spots appears to be 1 plus the number of free amino groups in the protein (number of spots = number of charge shifts + 1). Contrary to our expectation, some unusual effects occur in the highly carbamylated forms of some (but not all) proteins, indicating specific alterations in the molecular properties that affect SDS electrophoretic mobility.

MATERIALS AND METHODS

Rabbit muscle creatine phosphokinase (CPK; EC 2.7.3.2) and bovine erythrocyte carbonic anhydrase (CA; EC 4.2.1.1) were ob-

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[3] Abbreviations used: SDS, sodium dodecyl sulfate; CPK, creatine phosphokinase; CA, carbonic anhydrase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

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TABLE 1

COMPOSITION OF TYPICAL CARBAMYLATION BLENDS[a]

	Minutes at 95°C												
	0	2	4	6	8	10	15	20	25	30	45	60	90
Hb	2	0	1	2	1	2	1	2	0	1	0	2	0
CPK	1	0	1	1	1	2	2	0	0	1	0	1	0

[a] The table shows the relative amounts of the aliquots heated for various times which must be mixed to achieve a uniform carbamylation train (all spots at about equal intensity) for human hemoglobin (Hb) and rabbit muscle creatine phosphokinase (CPK). Some time points are unused for these two proteins, but are required in other cases and should be performed when first trying a new protein.

tained from Worthington. Rabbit muscle soluble proteins were extracted from psoas muscle in 4 vol of buffered saline by treatment of the muscle in a Waring Blendor and removal of the myofibrils by centrifugation for 1 h at 100,000*g*. Human hemoglobin was prepared as an unfractionated red cell lysate.

Carbamylation was performed by heating proteins (about 5 mg/ml) in 8 M urea (without mercaptoethanol) at 95°C for various periods (generally 0, 2, 4, 8, 10, 15, 20, 30, 45, 60, and 90 min). Two-dimensional gels of each fraction were run using the ISO-DALT apparatus (3,4), and a uniform blend of the different carbamylation species was prepared. This blend, showing all spots at about equal intensity, was used as the standard mix for each protein. Table 1 shows the composition of two typical blends.

Gels are shown with the acid end (of the isoelectric focusing dimension) to the left, i.e., with pH running in accordance with the Cartesian convention. Carbamylation standards are added directly to the protein samples applied to the first-dimension isoelectric focusing gels.

RESULTS

In the case of the human hemoglobin β-chain, carbamylation yields progessively more acidic forms until an end point is reached (Fig. 1). This end point appears to coincide with the blockage of all 12 free amino groups [11 lysines + 1 α-amino group (5)], since it occurs at the 12th shift position. The α-chain spots cannot be reliably counted because the native molecule is too basic to focus on these gels.

Figure 2 shows the trains produced by carbamylation of carbonic anhydrase and creatine phosphokinase. In these cases, the number of shifts cannot be counted very accurately due to resolution-limiting effects at the acid end of the trains. Nevertheless, best values of 20 $\pm$ 1 and 36 $\pm$ 4 lysines can be determined for CA and CPK, respectively, by comparing a number of gels. These results agree fairly well with published values: 19 lysines in bovine erythrocyte CA-B (6) and 30.5 to 33 in rabbit muscle CPK (7–9).

It is evident from a comparison of hemoglobin (Hb), CA, and CPK that proteins differ in the form of their carbamylation trains, particularly at the acid (left) end. The differences are shown more clearly in Fig. 3, which shows the result of carbamylating the soluble proteins of unfractionated rabbit muscle. There appear to be at least three types of train: those that run horizontally as a single row of spots from beginning to end (Hb, aldolase); those that appear to streak upward toward some higher SDS molecular weight value when approaching the acid end (CPK, albumin); and those that undergo stepwise SDS molecular weight shifts as carbamylation proceeds (D in Fig. 3). These anomalous changes in SDS molecular weight cannot be due to protein degradation, since they are shifts toward higher values; nor can they be due simply to mass added in the carbamylation reaction, since some proteins show no shift at all. Hence, it seems reasonable to suppose that the changes are due to alterations in SDS binding with increasing molecular negative charge.

The use of HB and CPK carbamylation trains as standards in the two-dimensional analysis of complex protein mixtures is shown in Fig. 4 and 5. Hb is useful when the pH range is wide (3–10), if SDS–slab gels of sufficiently low porosity are used (Hb runs with the dye front on gels of less than

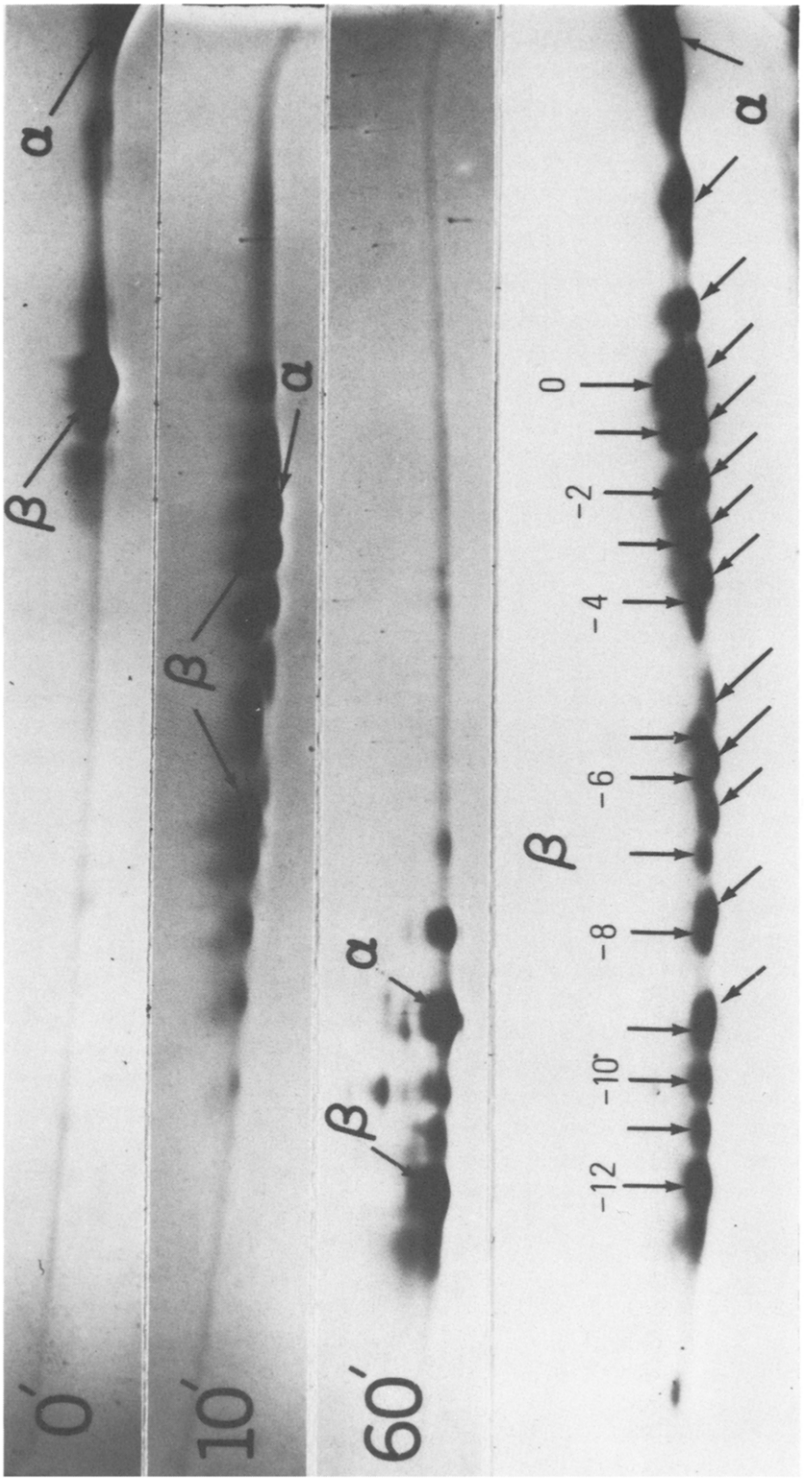

FIG. 1. Sections of two-dimensional gels showing the range of carbamylation products produced by heating human hemoglobin in 8 M urea at 95°C for 0, 10, or 60 min, and the complete train (lower panel) produced by mixing variously heated aliquots according to the recipe in Table 1. The upper row of spots in each panel is the β-chain and the lower row is the more basic α-chain (the two differ by five amino acid residues in length). Numbers on the β-chain arrows indicate the net excess (negative) charge of the molecule relative to the native form. Upward pointing α-chain arrows are not numbered since the α-chain O-spot is not visible. First-dimension gels contained 3.5–10 ampholines (LKB) only and second dimension slabs were 12% acrylamide to 17% acrylamide + 3.8 M urea gradients.

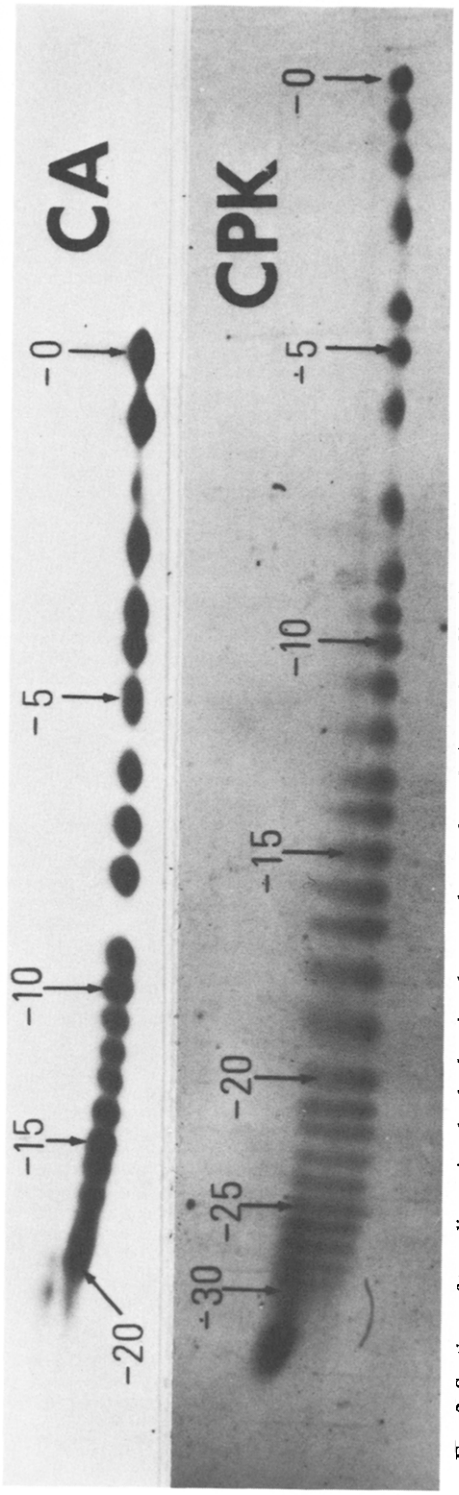

FIG. 2. Sections of two-dimensional gels showing the complete carbamylation trains of bovine erythrocyte carbonic anhydrase (CA) and rabbit muscle creatine phosphokinase (CPK). The first-dimensional focusing gel contained two-thirds 5–7 ampholines and one-third 3.5–10 ampholines, and the second-dimension slabs were as in Fig. 1. Material focusing between CA spots −1 and −2 is a minor contaminant.

~15% acrylamide). For work in which the 5–7 pH range is expanded, or when high-porosity slab gels are used, CPK is more useful. Other proteins will ultimately be found that are more useful in particular situations, and there should be no problem with their use since accurate conversion graphs can be constructed to relate various trains. A conversion graph relating Hb and CPK is shown in Fig. 6.

DISCUSSION

Mild carbamylation has been used previously to calibrate the size of single charge shifts in various proteins on two-dimensional gels (10). By extending the course of modification to apparent completion, a charge train may be constructed which in some cases allows counting of all the free amino groups of a protein. Since the succession of single charge modifications involved is equivalent to titrating the protein from its native to its complete carbamylated p*I*, considerable information about the other buffering groups of the protein (histidines, particularly) is also potentially available.

The anomalous changes in SDS electrophoretic mobility (SDS molecular weight) at the acid end of several carbamylation trains (CPK; glyceraldehyde-3-phosphate dehydrogenase, G3PDH) are reminiscent of those observed in heavily glycosylated proteins (11,12). Similar anomalous changes are also obtained if acetic anhydride rather than cyanate is used as blocking reagent for the amino groups (data not shown). The direction of the shift is the same as that observed by Banker and Cotman in one-dimensional SDS electrophoresis when soluble proteins were extensively maleylated (13). In their studies, Banker and Cotman concluded that both free SDS electrophoretic mobility (M_0) and retardation coefficient (K_r) were altered by maleylation. This suggests that the shape of the SDS–protein micelle, as well as the amount of SDS bound, is affected. We have

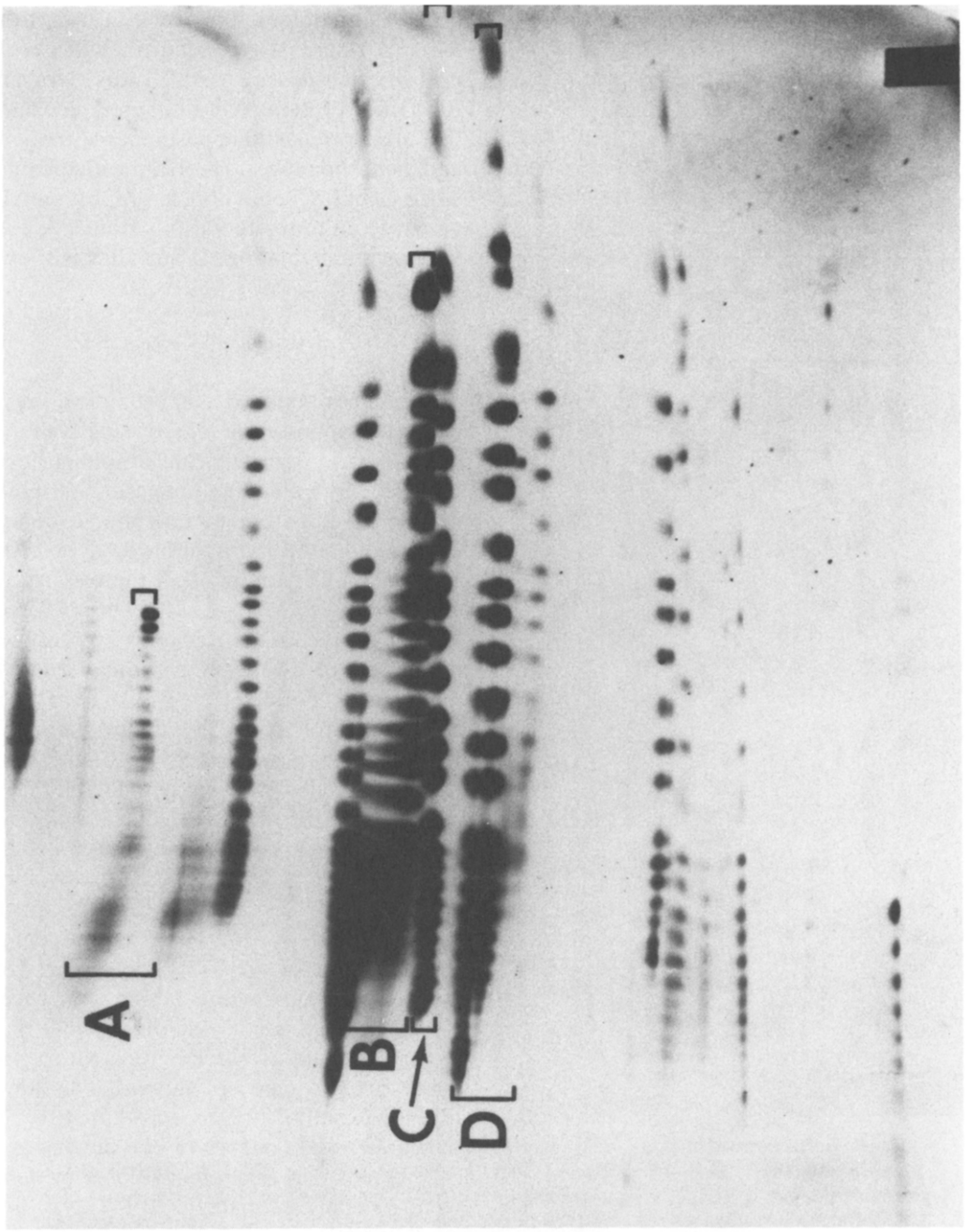

FIG. 3. Carbamylation trains of the soluble proteins of rabbit muscle: A, albumin; B, CPK; C, aldolase; D, glyceraldehyde-3-phosphate dehydrogenase. Note particularly the contrast between aldolase's horizontal single row of spots and the stepwise shifts in SDS mobility which occur in G3PDH over the same range of modification [both begin as single rows off the right (basic) end of the gel]. Procedures used to identify these and other muscle proteins will be described elsewhere.

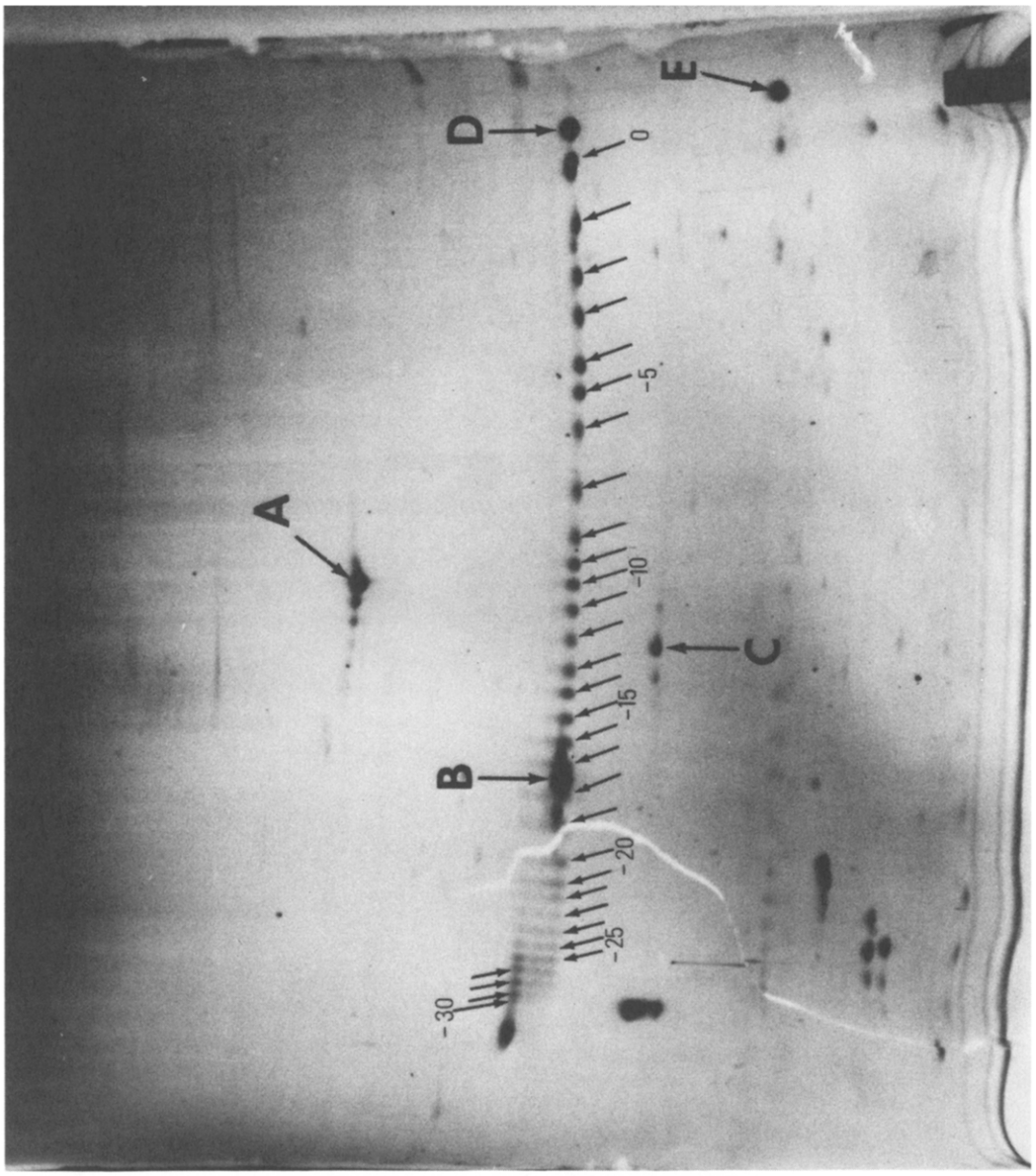

FIG. 4. Unfractionated human muscle coelectrophoresed in two dimensions with the CPK carbamylation train standards. A is albumin (p*I* = −10 CPK); B is muscle actin (p*I* = −17.5 CPK); C is an unknown protein at −12.1 CPK; D is human muscle CPK (main spot at ~+0.8 relative to rabbit CPK); and E is an unknown protein with a p*I* too basic to measure with CPK. Gels used were same as for Fig. 2.

not determined these detailed characteristics, but the variability we observed in the effect of complete carbamylation on various proteins is also evidence for a complex rather than a simple mechanism. By carefully analyzing the effect in a two-dimensional system, accurate data on mobility vs charge could be obtained.

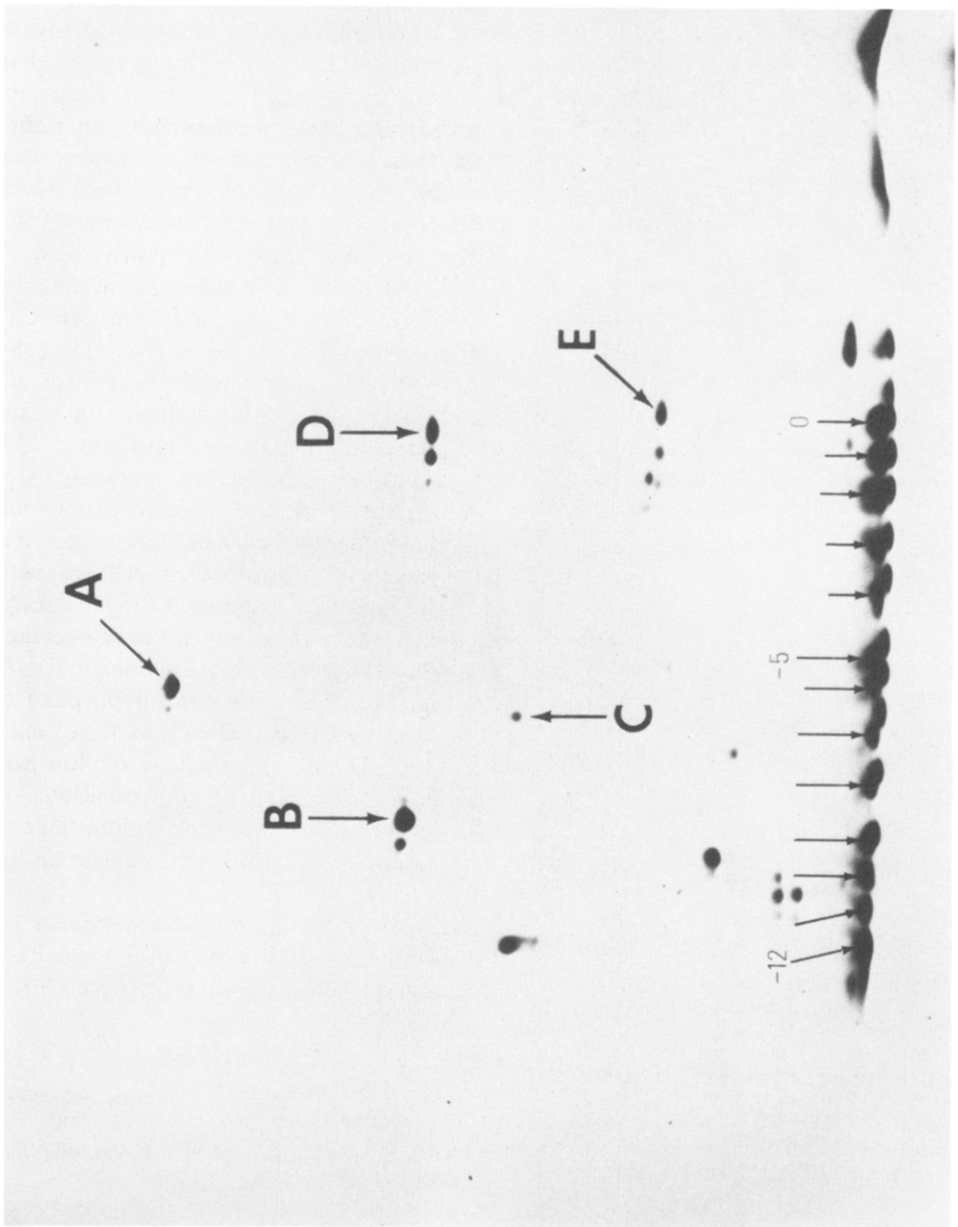

FIG. 5. The same solubilized human muscle as in Fig. 4, but run using wide range ampholines (pH 3.5–10) and with the Hb carbamylation standards. Labels refer to the same spots as in Fig. 4, but p*I* measurements relative to Hb are: A, −6.1 Hb; B, −8.8 Hb; C, −6.7 Hb; D, −0.4 Hb; and E, approximately +0.1 Hb.

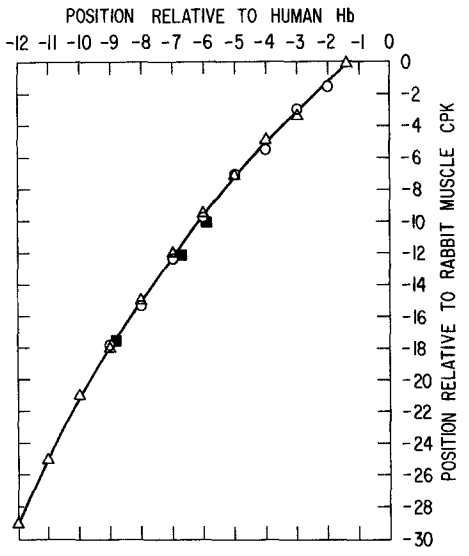

FIG. 6. Standard curve relating Hb and CPK carbamylation train standards. This was obtained directly from gels of mixed Hb and CPK trains (two gels, △ and ○) and checked using values from Figs. 4 and 5 (■). Values were read using a ruler perpendicular to the top of the slab and interpolating between spots by eye.

As a method of assigning isoelectric points to proteins in two-dimensional systems, we believe that carbamylation trains will prove more reproducible and more convenient than either direct pH measurement or use of scattered standard proteins mixtures such as viral preparations. Even under the best generally achieved conditions, direct pH measurement is reproducible to within only ~0.1 pH unit between different laboratories,[4] and the difficulty of making nondestructive determinations on 1.5-mm-diameter tube gels at a lengthwise resolution of ~0.5 mm introduces substantial additional error. Since single charge shifts in many large polypeptides are on the order of 0.01 pH unit, the use of such shifts clearly affords better resolution than direct pH measurement, as well as superior reproducibility. All protein standards share the advantage of permanent presence in the finished two-dimensional gel. However, the charge trains have the unique feature of easy countability, thus yielding a ready-made isoelectric point coordinate system.

The numbering system employed for carbamylation train standards runs from low to high numbers (left to right) in the same sense as the pH values and the standard Cartesian x-coordinate. The predominant, or rightmost, native form is more unambiguous than the completely carbamylated (left-most) form for many proteins (including CPK), and so we have chosen its spot as zero. A carbamylation train thus extends in the negative direction from zero and counts directly the charge added to the native molecule. When convenient chemical procedures are developed for generating stepwise increases in positive charges on proteins, then such series will extend positively from zero.

When the isoelectric point of each individual member of a carbamylation train has been precisely determined under the experimental conditions employed for isoelectric focusing, such trains will probably replace pH measurements for first-dimensional calibration. However, the central purpose of both internal standards such as those used here, and pH measurements, is to allow precise intercomparison of spot positions on different gels. For this purpose, internal carbamylation train standards appear to be superior.

High-resolution calibration standards for the second or SDS dimension have been developed and described elsewhere (14).

[4] J. V. Kilmartin, personal communication.

REFERENCES

1. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
2. Bobb, D., and Hofstee, B. H. J. (1971) *Anal. Biochem.* **40**, 209–217.
3. Anderson, N. G., and Anderson, N. L. (1978) *Anal. Biochem.* **85**, 331–340.
4. Anderson, N. L., and Anderson, N. G. (1978) *Anal. Biochem.* **85**, 341–354.
5. Braunitzer, G., Gehring-Muller, R., Hilschmann, N., Hilse, K., Hobom, G., Rudloff, V., and

Wittmann-Liebold, B. (1961) *Hoppe-Seyler's Z. Physiol. Chem.* **325**, 283–286.
6. Nyman, P. O., and Lindskog, S. (1964) *Biochim. Biophys. Acta* **85**, 141–151.
7. Naltmann, E. A., Mahowald, T. A., and Kuby, S. A. (1962) *J. Biol. Chem.* **237**, 1146–1154.
8. Eppenberger, H. M., Dawson, D. M., and Kaplan, N. D. (1967) *J. Biol. Chem.* **242**, 204–209.
9. Yue, R. H., Palinieri, R. H., Olson, O. E., and Kuby, S. A. (1967) *Biochemistry* **6**, 3204.
10. Steinberg, R. A., O'Farrell, P. H., Friedrich, U., and Coffino, P. (1977) *Cell* **10**, 381–391.
11. Anderson, N. L., and Anderson, N. G. (1977) *Proc. Nat. Acad. Sci. USA* **74**, 5421–5425.
12. Jones, P. P. (1977) *J. Exp. Med.* **146**, 1261–1279.
13. Banker, G. A., and Cotman, C. W. (1972) *J. Biol. Chem.* **247**, 5856–5861.
14. Anderson, N. G., and Anderson, N. L. (1979) *Mitt. Behring Inst.*, in press.