

## Enzyme Resolution in Starch Gel Electrophoresis

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In the relatively short time since its introduction, electrophoresis in starch and polyacrylamide gels has become extremely valuable for the study of proteins. Visualization of separated bands by appropriate staining has been generally adequate for examination and photography of the results; clearing of interzones with minimal loss of the dye-protein complex usually permits observation of fine structure.

Efforts to quantitate the patterns have not been entirely successful; the problems have been discussed by Pert and Pinteric (1) and by Ornstein (2). Price *et al.* (3) note that devices presently available for recording the density of individual bands (and it might be added, photography as reproduced in the journal) are less sensitive than the naked eye. Even so, the literature contains many excellent photographs of complex, well-resolved protein patterns.

Since staining is so effective for proteins, it is logical that most work on visualizing enzyme bands in gel electrophoresis has used analogous procedures. Development of a color, precipitate, or bleached area on the gel surface has been most used for the purpose (4-9). These usually provide only qualitative results and suffer other shortcomings. To provide more quantitative data the gel may first be cut into slices of various lengths and the enzyme removed and assayed (10-13). As Pert and Pinteric have noted (1): "Starch gels may be cut into 1 mm slices for special studies (enzymes, radioactivity, etc.) but this is too time-consuming, and the value of the high-resolution electrophoresis is largely lost." Some investigators have used 1 cm (14), 2 mm (15), or  $\frac{1}{16}$  in (11) slices, possibly compromising to reduce the manipulation needed for 1 mm slices. Although in some instances valuable information is obtained, much is missed when larger slices are taken. Even so, Vincent (15) noted that, when 2 mm slices of gel were cut, the electrophoresis band of alka-

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line phosphatase showed discrete peaks that were not evident when the gel was stained as a whole.

Disc electrophoresis on polyacrylamide gels may appear to provide greater resolving power for proteins because of its usually narrower (minimum  $50 \mu$ ) bands (2), but if disc electrophoresis columns, with their closer resolution and shorter total length, are sliced into 1 mm (12), 1.27 mm (27), or 2 mm (3) sections for enzyme assay, the relative loss in resolution becomes excessive. Slices of the order of  $50 \mu$  or less would be needed to avoid loss of the resolution if disc electrophoresis columns are assayed for enzymes by this procedure.

Maizel (16) has described a mechanical fractionator for polyacrylamide gel electropherograms to eliminate manual slicing when quantitative data are required. He has automatically obtained fractions equivalent to 1.7–1.8 mm of gel length for radioactivity assay. He shows quantitatively similar fractionations of labeled type 2 adenovirus proteins, made both by hand (1.6 mm) and automatic sectioning, with eight separated bands over a 100 mm long gel. The 1.7 mm equivalent slice thickness seems excessively thick for exploitation of the fine detail available in polyacrylamide gel electrophoresis; under his conditions Maizel found a  $\pm 10\%$  variability in fraction size. Reducing equivalent slice thickness by changing operating conditions would probably increase this considerably.

Probably because of its laborious nature, close slicing of gel electrophoresis slabs for enzyme assay is reported only rarely, but studies at this laboratory have demonstrated that the increased resolution of enzyme bands obtained in this way provides information not obtained by staining methods. Because many isozymes of clinical interest are so widely separated, a staining assay is generally adequate and has become routine for many of them. Since enzyme levels can be measured over at least  $10^4$  in concentration range when eluted from the gel, by adjustment of incubation times and concentrations, more flexibility is present when using the slicing technique.

We have examined the degree of resolution attainable by using various procedures in the gel electrophoretic examination of the  $\alpha$ -glucosidase of honey. The variables examined included direct gel staining, use of transfer paper, and the effect of slice thickness, distance of migration, and the use of horizontal or vertical migration.

### METHODS

The earlier electrophoretic work was carried out in a horizontal tray with paper bridges to the buffer solutions as described by Smithies (17).

Later a horizontal tray was used with both ends dipping at 45° into 1.5 liter intermediate electrolyte vessels, in turn connected by agar bridges to electrode vessels. Finally a vertical apparatus was employed which also provided direct gel-to-liquid contact at both ends, which allowed much more constant current and voltage conditions. Tsuyuki (18) has also described such direct contact. All apparatus was constructed of Plexiglas at this laboratory.

Starch prepared according to Smithies (17) was used (Connaught Medical Research Laboratories, Toronto, Canada) with pH 8.9 borate buffer (0.044 *M* boric acid and 0.016 *M* NaOH), twice the strength recommended by the manufacturers for serum protein separations.

Electrophoresis was generally at about 4.0 vols/cm, and 3 ma/cm of width for 16–18 hr at 2–4°C in a refrigerator. The starch slab was 12 × 28 cm and 9 mm thick. After migration, gels were sliced lengthwise by a taut-wire device as needed, with slab thickness determined by the number of 2.3 mm plastic side strips removed from the tray before slicing. One slab slice was stained in the usual way for protein with amido black or, later, nigrosine, which gave better detection for the faint bands from the  $\alpha$ -glucosidase preparation.

A second slab of the desired thickness was carefully aligned on a glass or plastic plate and the area of interest cut into 1.0 mm slices by an array of 50 razor blades cemented 1 mm apart in a brass block. Elapsed time before this cutting was minimized to reduce diffusion. Each slice was transferred to a 10 ml beaker and frozen 4–48 hr in a covered tray. After thawing, 2.0 ml buffer (0.05 *M* phosphate, pH 5.9) was added and the slice disintegrated against the bottom of the beaker by a metal disc with intersecting grooves in its lower face, fixed to a rod. After another overnight freezing the extract was recovered by filtration. The filter apparatus was made by cutting off the sides of a porcelain Büchner funnel, size 4/0, flush with the surface of the perforated plate. The funnel was connected by a short piece of flexible tubing to a filtering bell (Fisher Filtrator) within which test tubes were successively placed to receive filtrate. Circles (15–17 mm) of filter paper (S. and S. 589) as needed covered the perforations, and the filtration was accomplished by lowering the filter surface into the beaker and finally pressing the gel particles against the bottom to obtain as complete extraction as possible without washing. Rinsing of the apparatus between slices was not needed. Details of the extraction are given because the per cent recovery appears to be superior to previous procedures (see later). Extracts or portions thereof were incubated with equal volumes of substrate (10% sucrose and 10  $\mu$ g/ml Fungizone (E. R. Squibb & Sons brand of amphotericin B) in the above buffer) and held at 37° for 0.5–150 hr as required. Total

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hydrolysis was always less than 5% of the available substrate. The incubation was stopped by a 2 min immersion of the tubes in a boiling water bath, followed by cooling. Glucose was determined in the substrate or a portion thereof by a specific glucose oxidase reagent, in which glucosidase activity was inhibited by tris (19). The sensitivity of the reagent is considerably improved by omitting the acid color stabilization and determining the absorbance at 450 m $\mu$  of each sample just 60 min after reagent addition. Using a self-filling cell in the photometer, 120 samples per hour were easily determined. Activity is expressed as mg invert sugar (glucose  $\times$  2) produced per hour at 37°. These units may be converted to EC (Enzyme Commission) units by multiplying by 0.0488.

### RESULTS AND DISCUSSION

In a study of enzymes and proteins of honey, crude preparations from various sources, made by dialysis of diluted honey and concentration by treatment in the sac with solid carboxymethylcellulose (Aquacide I), were subjected to starch gel electrophoresis. Details of sample preparation and results are described elsewhere (20). When enzyme-revealing reagents were applied directly to the gel, as described by Hunter and Burstone (4), only the general area of activity was revealed. The relatively low degree of activity shown by enzyme preparations under study (honey "invertase" or  $\alpha$ -glucosidase) required such a long incubation time that diffusion of the products reduced the value of the results. A single, diffuse area of activity was found 30–50 mm toward the anode from the origin. A piece of filter paper dipped in substrate was placed in contact with another gel slab for 4 hr and protected from evaporation. It was then dried and sprayed with triphenyltetrazolium reagent (21) for reducing sugars, which showed color development in the same area. Again only a single broad area of activity was found, but it was much larger than would be expected from a single species migrating this distance.

Another gel slab was cut serially into 1 mm slices and processed as described under "Methods." Figure 1a shows the results; subsequent migrations over greater lengths with other gels yielded the diagrams given in Fig. 1b,c. Additional migration did not improve resolution. Differing distances of migration in these exploratory runs were not produced by increasing time, but resulted from uncontrolled variation in slab thickness, buffer concentration, and the variability in voltage and current associated with the use of paper wicks.

When enzyme concentration applied to the gel was reduced, less overlap of components was evident. This increase of resolution was limited; when incubation times in analyses were correspondingly lengthened, microbiological action often intervened. After testing several com-

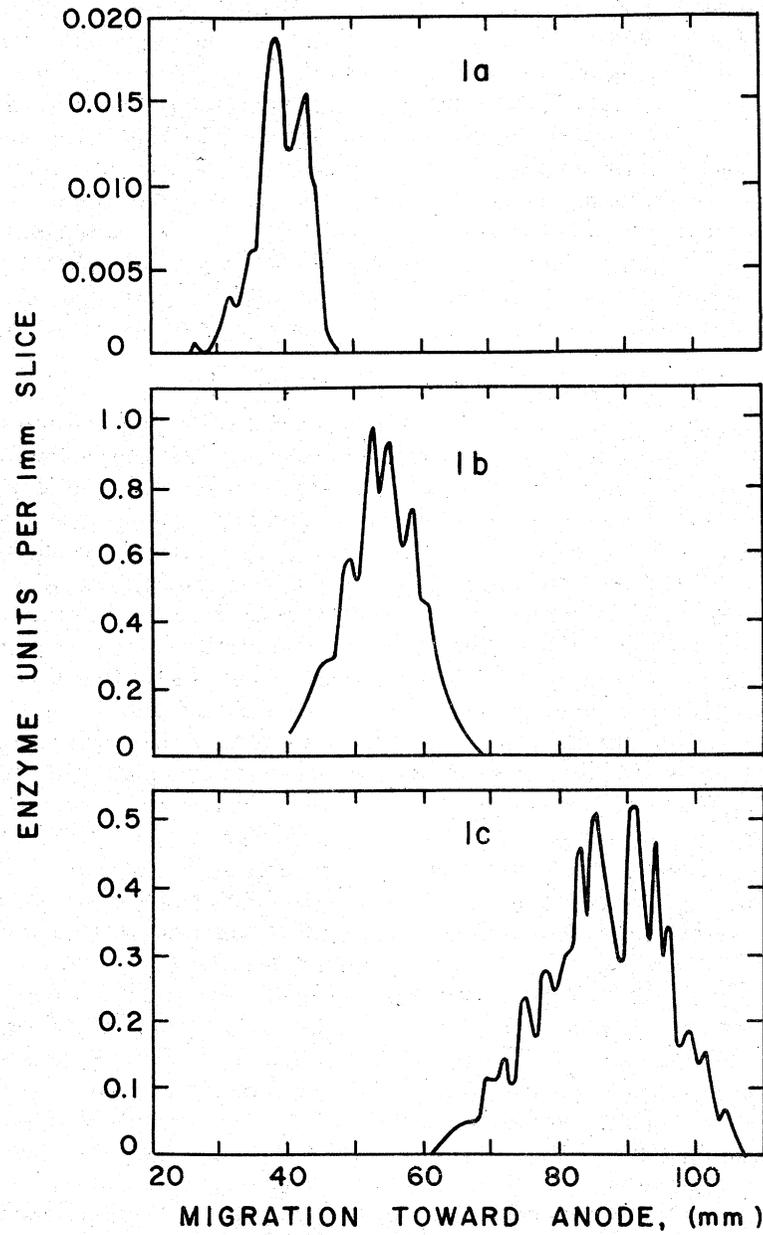


FIG. 1. Effect of increased migration distance on resolution of honey  $\alpha$ -glucosidase (horizontal migration; see text for details). Enzyme preparation from goldenrod-aster honey.

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pounds, Fungizone at 5 ppm was found adequate for at least 150 hr incubations, without interfering with enzyme activity or the sugar determination.

In Fig. 2 are shown results of electrophoresis of the enzyme preparation at three concentrations on the same gel. Eight to ten distinct bands of activity within 30 mm are evident. The irregularities on the slow side of the diagram in Fig. 2a are seen to be significant when 2b and 2c are examined.

These results were obtained with horizontal disposition of the gel slab and insertion of the sample on filter paper (Whatman 3 MM) into a slit cut just to accept the paper. Amounts of enzyme that could be applied were thus limited to about 0.02 ml; use of a preformed slot yielded rather wider bands with poorer resolution.

Subsequent use of vertical cells with preformed slots allowed larger amounts to be introduced without sacrifice of the narrow starting zone, as noted by Smithies (22). Six slots,  $2 \times 14$  mm and 6 mm deep, were spaced across the 12 cm slab width. Each slot was filled with an average of 0.111 ml sample. When the expected resolution was not consistently obtained, it was traced to the excessive depth of the 1 mm wide gel slices that was used in an effort to increase enzyme content of filtrates. The disposition of enzyme bands in contiguous 2.3 mm layers of a gel slab was found to differ enough to reduce band resolution considerably when two such layers were taken in one slice. When starch gels are stained for protein, only that near the surface is seen, so that fine detail is more easily revealed. Bloemendal (23) has commented on variation in zone patterns in different layers of the starch strip. By using only minimum thickness gel slabs (2.3 mm) for slicing, a much more reproducible and better resolved enzyme pattern was obtained. Improvement of resolution by use of the tris-containing discontinuous buffer system of Poulik (24) was not possible because the enzyme under study is inhibited by tris.

The general slicing and extraction procedure described here is not new; Smithies (17) proposed freezing (one time) of the gel to liberate proteins, followed by centrifugation. His procedure has been described as not quantitative (18). Bloemendal (23) also noted that quantitative recovery is not obtained this way. While we make no claim that the procedure described here is quantitative, examination of the results of several experiments shows (Table 1) that an average of 88% recovery of enzyme activity (ranging between 79 and 103%) was obtained. In the eleven experiments in Table 1, enzyme concentration was high enough that only 1 to 2 hr incubation was required in assay of the slices. Where extended incubation time was required, recovery fell to 40-60%. Quan-

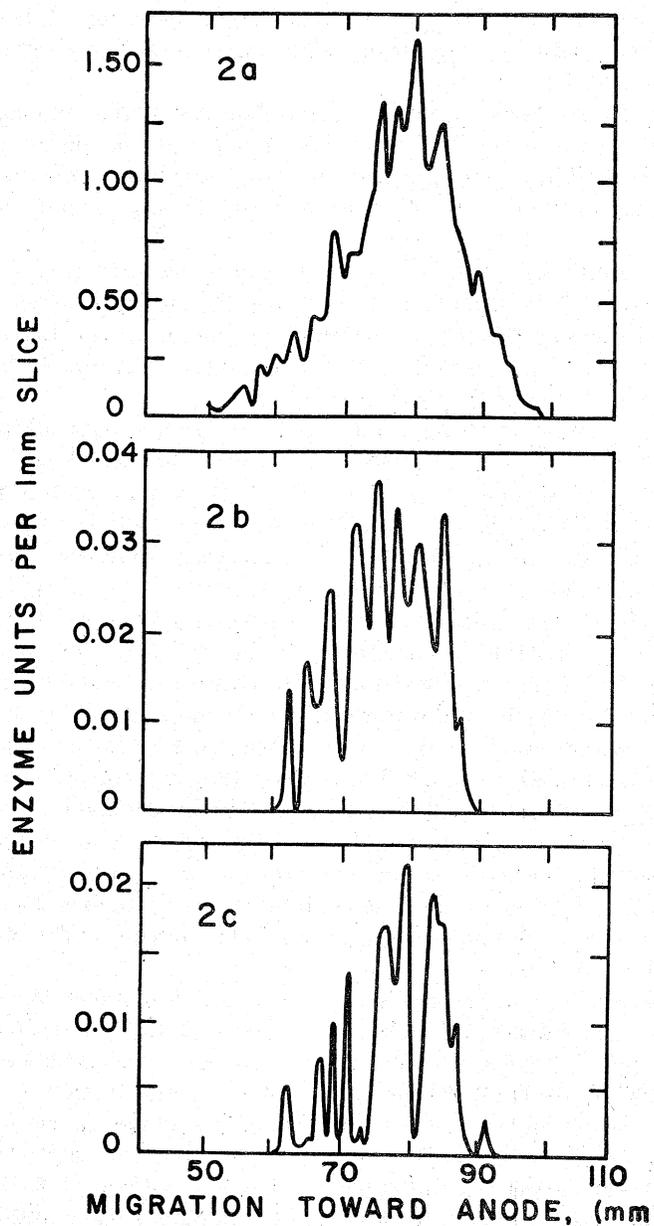


FIG. 2. Effect of dilution of original preparation of honey  $\alpha$ -glucosidase on resolution. Sample applied on paper (Whatman 3 MM). Concentration: (2a) 695 u/ml; (2b) 35 u/ml; (2c) 23 u/ml. All on same gel, horizontal migration.

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 TABLE 1  
 RECOVERY OF  $\alpha$ -GLUCOSIDASE FROM STARCH GEL SLICES AFTER ELECTROPHORESIS

Enzyme applied, <sup>a</sup> units	Depth of slab analyzed <sup>b</sup>	No. and size (mm) of slices	Enzyme recovered from slices, <sup>c</sup> units	Conversion factor <sup>d</sup>	Enzyme recovered, units	Recovery, %
58.0	2	51-1 mm	25.3	2	50.6	88
69.1	1	1-50	15.0	4	60.0	87
32.0	1	1-120	6.46	4	25.9	81
16.0	1	1-120	3.64	4	14.6	91
10.7	1	1-120	2.27	4	9.08	85
8.0	1	1-120	1.59	4	6.35	79
6.45	1	1-120	1.47	4	5.90	92
2.46	4	24-2	2.54	1	2.54	103
20.2	1	46-1	4.89	4	19.50	97
11.1	4	48-1	8.60	1	8.60	77
3.69	4	36-1	3.53	1	3.53	95

<sup>a</sup> Calculated from average 0.111 ml placed in slot (5 experiments).

<sup>b</sup> In units of 2.3 mm thickness.

<sup>c</sup> Summation of assays of individual slices indicated.

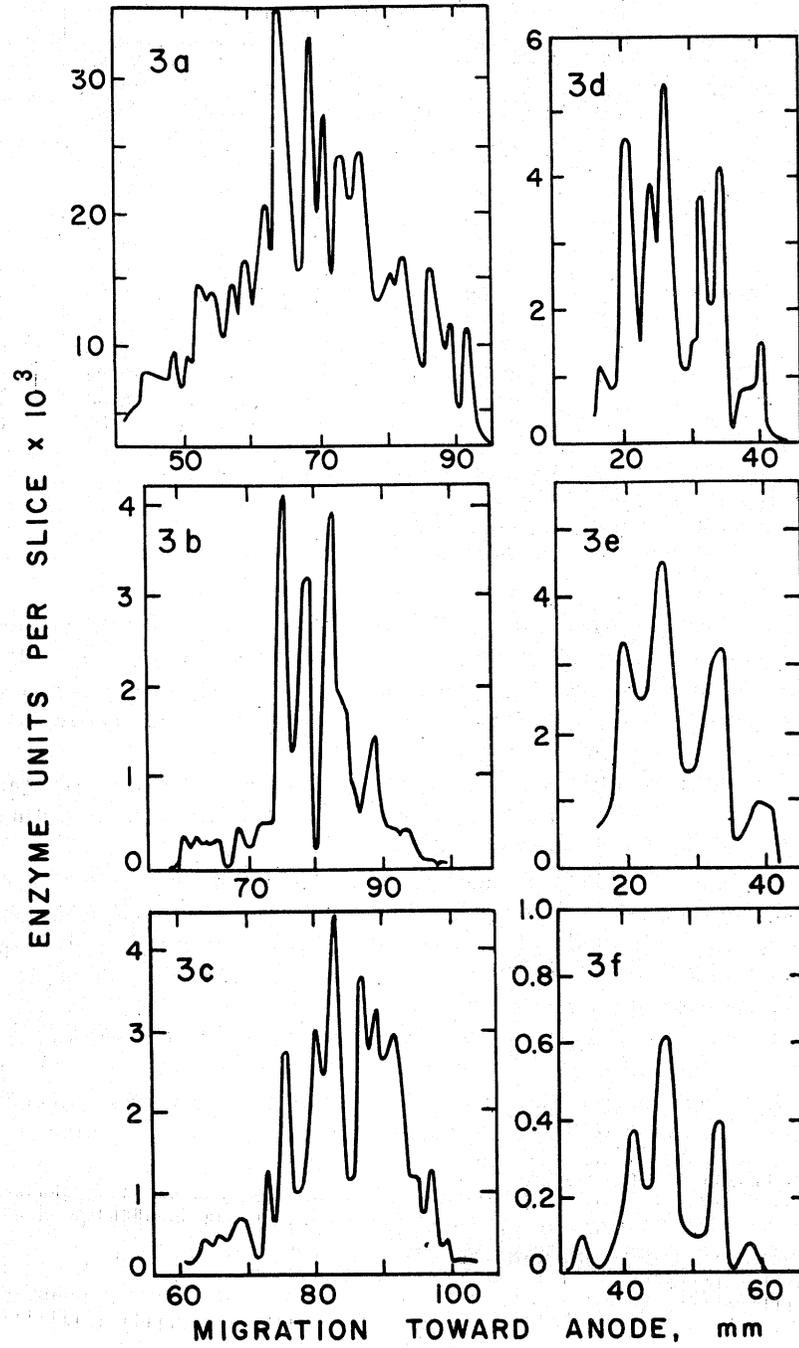
<sup>d</sup> To convert to amount in entire gel.

titative data on the charts in Figure 3 are not given because of uncertainties as to amounts of enzyme actually placed in the gel. The electrophoretic patterns for the  $\alpha$ -glucosidase from a given honey preparation are generally reproducible in numbers of peaks, but their relative heights may vary somewhat.

Examples of starch gel electropherograms of  $\alpha$ -glucosidase preparations from several honeys are in Fig. 3a-d. Discussion of these and other results will be found elsewhere (20).

In Fig. 3e is shown a diagram constructed from the data plotted in 3d (obtained with 1 mm slices), but with pairs of adjacent values averaged and plotted so that it corresponds to an electrophoresis diagram with 2 mm slices. Figure 3f is an electrophoretic run of the same preparation as that in 3d, except that 2 mm slices were actually cut and analyzed. The similarity of 3e and 3f is obvious. Each of the four peaks in the 2 mm diagrams (Fig. 3e,f) is seen to be resolved into two in the 1 mm diagram (Fig. 3d). Further reduction in slice width was not carried out, so that there is no reason to conclude that the 1 mm slice is optimal for starch gel.

In Figure 4 are seen the patterns of Fig. 3f and 3d with the individual data points shown. In Fig. 4a (2 mm slices), the peaks A, B, C, D each contain only one point, which is nearly inevitable when actual detail is finer than the resolution available. In 4b (1 mm slices), peaks A, B, and C are each resolved into two, and peak D is partially resolved. For



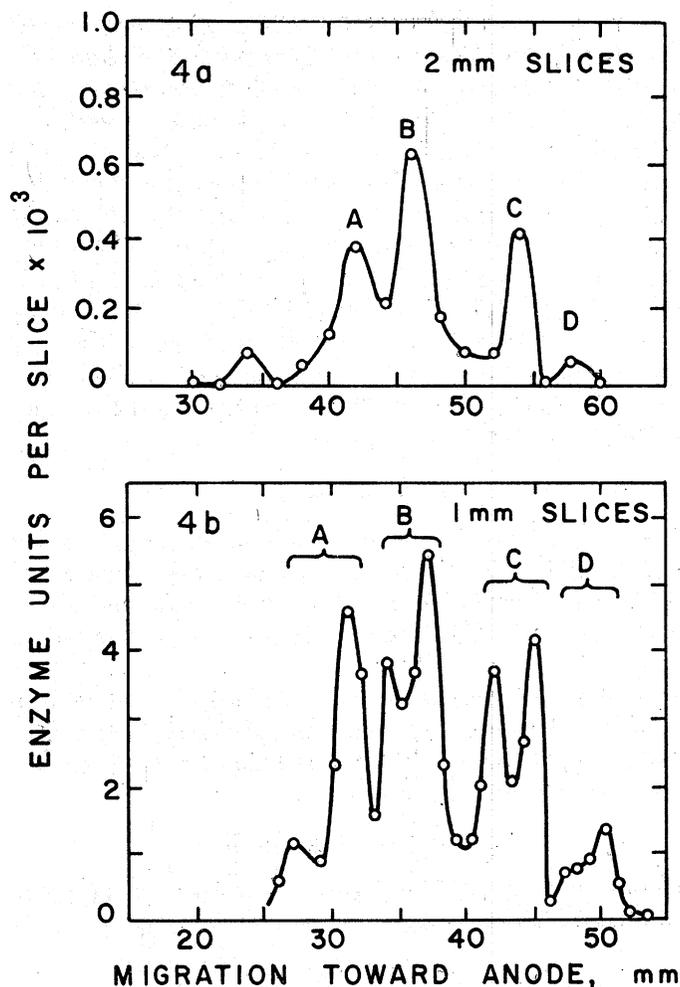


FIG. 4. Electropherogram of  $\alpha$ -glucosidase preparation from stores from sugar-fed bees, showing individual data points: (4a) gel sliced at 2 mm intervals; (4b) gel sliced at 1 mm intervals.

peaks B and C, two adjacent data points fix the valley between the peaks. For the patterns in Fig. 4b to be in error in showing the peak splitting, both these pairs of adjacent values would have to be erroneous with such

FIG. 3. Electropherograms of various honey  $\alpha$ -glucosidase preparations: (3a) clover honey; (3b) mixture of wing-stem (*Actinomeris alternifolia*) and *Lespedeza* honeys; (3c) *Lespedeza* honey; (3d, 3e, 3f) stores from sugar-fed bees. (3a-d) 1 mm slices from gel; (3e) data from 3d averaged at 2 mm intervals; (3f) 2 mm slices from gel. All vertical electrophoresis, sample in preformed slot.

error far in excess of the  $\pm 10\%$  variation seen for enzyme extractability (Table 1). We conclude then that the finer resolution afforded by 1 mm slicing is real and is not an apparent effect caused by uncontrolled variation in the degree of extraction of adjacent gel slices.

One comparison of procedures is available using these enzyme preparations. Starch block electrophoresis with elution of enzyme from 1 cm portions was carried out by Dr. E. T. Reese (Q. M. Research and Engineering Center, Natick, Mass.), using procedures applied to fungal carbohydrases at that laboratory (14, 25, 26). They used a 100 cm block; many published diagrams from that laboratory, with 1 cm resolution, cover 25–50 cm and show 4–8 partially resolved peaks. The diagram for honey  $\alpha$ -glucosidase thus obtained, however, had a single asymmetric peak, 8 cm wide, centered at 12 cm (corrected) toward the anode, with no indication of structure within the band.

#### SUMMARY

1. Improved resolution of enzyme bands in starch gel electrophoresis obtained by assaying extracts of 1 mm slices is well worth the increased manipulative requirements, for research purposes. Resolution is also improved by limiting slab thickness to about 2 mm or less before cutting the slices.

2. Starch gel electrophoresis of honey  $\alpha$ -glucosidase preparations show up to ten distinct bands in a 30 mm zone of activity when processed in this manner; only one activity zone was found by direct staining of the gel by chromogenic substrate reagents.

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