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Anomalous Behaviour of Yeast Isocitrate Dehydrogenase During Isoelectric Focusing

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Isoelectric focusing of yeast isocitrate dehydrogenase apparently reveals a number of 'isoenzymes'. These have isoelectric points near pH5.5 in crude material, but during purification the mean isoelectric point progressively rises to pH7.0 and the band pattern changes. The shift in isoelectric point during purification is apparently genuine, since it is also manifested in the electrophoretic and chromatographic properties of the enzyme. The multiple forms, however, are an artifact, generated by exposure of the enzyme to Ampholine, since their activities vary with the protein/Ampholine ratio and they cannot be observed in any system from which Ampholine is excluded. There are no detectable isoenzymes of yeast isocitrate dehydrogenase.

Since the introduction of Ampholine by Vesterberg (1969) isoelectric focusing has found increasing favour both as a test for homogeneity of proteins and as a small-scale purification technique. In many cases enzymes that were formerly considered to be homogeneous have been shown by isoelectric focusing to consist of a number of closely related sub-forms. The present paper describes one case in which these sub-forms were generated by the Ampholine itself from an enzyme which was initially homogeneous. These results must throw doubt on the individuality of other sub-forms exposed by isoelectric focusing, and on the accuracy of isoelectric points determined by this method. Nevertheless the technique correctly revealed changes in isoelectric point during the purification of yeast isocitrate dehydrogenase, and explained the baffling failure of AMP elution when applied to crude enzyme samples.

Materials and Methods

Yeast isocitrate dehydrogenase was prepared and assayed as described in the preceding paper (Illingworth, 1972). Ampholine was obtained from LKB Produkter AB, Bromma 1, Sweden. Isoelectric focusing was carried out according to the manufacturer's instructions, by using an LKB 8101 column (110ml capacity). No differences were apparent between runs at 15°C and those at 10°C, nor when glycerol rather than sucrose was used to stabilize the gradient. The columns were loaded by using a gradient mixer, and enzyme activity was initially distributed evenly along the length of the apparatus. Mercaptoethanol (2ml/litre) was added to both solutions immediately before loading. Separations took 40–50h. The finishing voltage was 800–1000V at about 1mA current.

All polyacrylamide gels contained *NN'*-methylenebisacrylamide as cross-linking agent (5% of total monomer) and 5mM-tetramethylethylenediamine as accelerator. After degassing for 5min polymerization was initiated with 1mM-$K_2S_2O_8$. The gel time was about 15min at 25°C. Separations were carried out at 15°C in a water-cooled apparatus, on 13cm × 0.6cm gels. Enzyme activity was revealed by soaking the gels in the following mixture: 5ml of Nitro Blue Tetrazolium (2mg/ml); 0.1ml of phenazine methosulphate (5mg/ml); 25ml of tris–HCl (40mM; pH7.6, containing 4mM-$MgCl_2$); 2ml of trisodium DL-isocitrate (100mM); 2ml of NAD^+ (10mM). Protein bands were stained with 1% Naphthalene Black in 7% (v/v) acetic acid. This stain cannot be used in the presence of Ampholine, which itself reacts with the stain.

Most conventional electrophoresis runs were performed in 4% (w/v) acrylamide gels, though 3 and 5% gels were also used when it was important to demonstrate sample homogeneity. Two types of discontinuous buffer systems were employed: (*a*) gel buffer 0.1M-tris–HCl, pH8.1, cathode buffer 0.1M-tris–glycine, pH9, running pH about 9.5; and (*b*) gel buffer 0.1M-imidazole–HCl, pH7.0, cathode buffer 0.1M-tris–glycylglycine, pH8.2, running pH about 8.3. Samples were normally loaded in 50μl of the gel buffer and contained a trace of sucrose so that they could be layered beneath the cathode solution at the start of the separation. The current used was normally 2mA/gel initially and 3mA/gel after 30min.

Isotachophoresis (Haglund, 1970) experiments were run in 3% acrylamide gels containing 0.1M-tris–HCl, pH8.1. Enzyme samples in 2mM-potassium phosphate, pH6.5, were mixed with an equal volume of 40% (w/v) Ampholine (pI range 7–9) and 0.1ml of

this mixture was layered on to each gel. The cathode solution was 0.2M-NaOH. Mercaptoethanol was carefully excluded from these experiments, since it ran as a tight band and gave rise to a spurious tetrazolium stain. Bromothymol Blue was used as a leading marker and ε-Dnp-lysine was used as a subsidiary trailing marker. Haemoglobin (which runs between Bromothymol Blue and Dnp-lysine) and cytochrome *c* (which runs at the rear of the ampholytes immediately in front of the OH^- ions) were also used as markers in early experiments, but the use of haem compounds was abandoned after they were found to inhibit the tetrazolium activity stain. No loss of definition was observed at high power dissipations and so separations were normally performed at 10mA/gel (final voltage of about 1000V). Water cooling was essential under these conditions. Each experiment lasted about 1h, in which period Bromothymol Blue moved about 10cm and horse heart cytochrome *c* moved about 4.5cm. The isocitrate dehydrogenase bands were clustered around Dnp-lysine, which travelled about 6cm. Excellent separations of lactate dehydrogenase isoenzymes were obtained when this system was initially tested.

Results and Discussion

Figs. 1–3 show the focusing behaviour of the enzyme during purification. A rather large enzyme load was used in the experiment shown in Fig. 3 so as to obtain reliable protein measurements. With lower loading the single large peak near pH7 splits into two components, as shown in Figs. 5 and 6, and the 'acidic' bands become more pronounced. There is an obvious shift in isoelectric point during purification, of nearly 2 pH units. (The values for effluent volumes are intended only as an approximate guide, since these values were estimated from the elution time and the peristaltic-pump speed.)

The band pattern changed when material from one experiment was subsequently re-focused. Fig. 4 shows one result taken from a series of re-focusing experiments on the main peak from the experiment shown in Fig. 3. In general re-focusing introduced additional acidic peaks, especially when the protein load was small. Enzyme samples were stored frozen, without removal of the old Ampholine, until they were re-run. If these old ampholytes were partially removed by gel filtration into 5mM-potassium phosphate buffer (pH6.8) before the second focusing experiment then

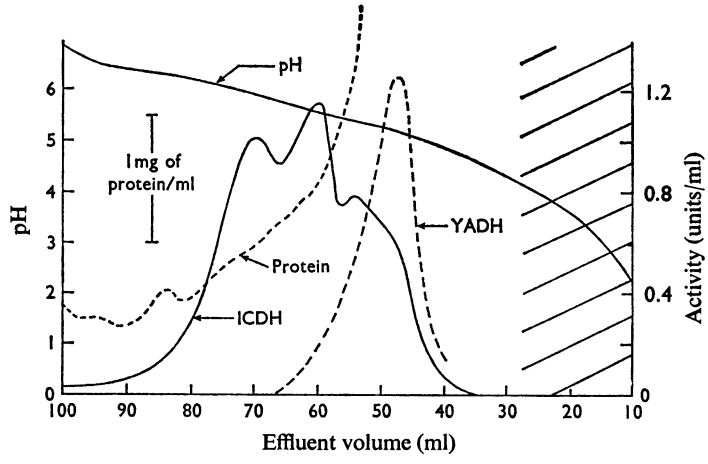

Fig. 1. *Isoelectric focusing of crude yeast isocitrate dehydrogenase*

The enzyme sample was clarified with calcium phosphate and precipitated with ammonium sulphate before transfer into 10mM-potassium phosphate, pH6.8, by gel filtration. A portion (20ml) of this solution (approx. 200mg of total protein, 30 units of isocitrate dehydrogenase) was mixed with Ampholine and the precipitate was removed by centrifugation before loading. More precipitate formed during focusing, and this is indicated by cross-hatching on the diagram. The Ampholine used was a mixture of equal proportions of pI range 3–6 with pI range 5–8, average concentration 1% (w/v), starting pH5.5. The protein curve was estimated from the E_{280}. The entirely normal behaviour observed for yeast alcohol dehydrogenase during the same run is included for comparison. Abbreviations: YADH, yeast alcohol dehydrogenase; ICDH, isocitrate dehydrogenase. The alcohol dehydrogenase activities have been multiplied by 0.003 in order to plot them on the same activity scale as isocitrate dehydrogenase.

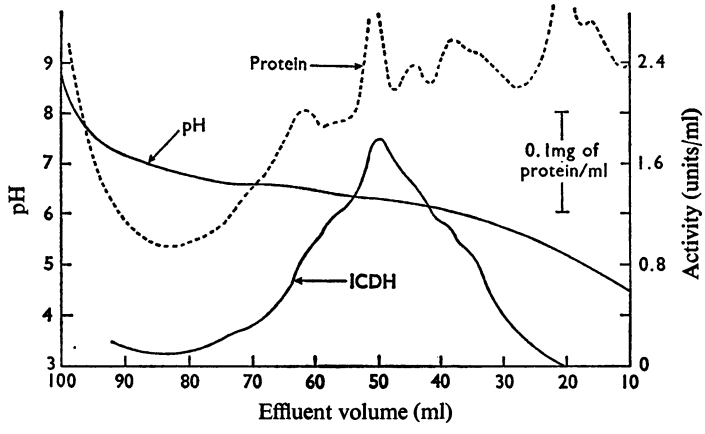

Fig. 2. *Isoelectric focusing of yeast isocitrate dehydrogenase after chromatography on DEAE-cellulose*

The enzyme was focused immediately after elution from DEAE-cellulose. The load was 60 units of enzyme (approx. 30mg of protein) in 20ml of 50mM-triethanolammonium chloride–5mM-cysteine, pH7.5. The Ampholine concentration was 1% (w/v), pI range 5–8. The protein concentration was estimated from the E_{280}. Other details are given in the text. For abbreviation see Fig. 1.

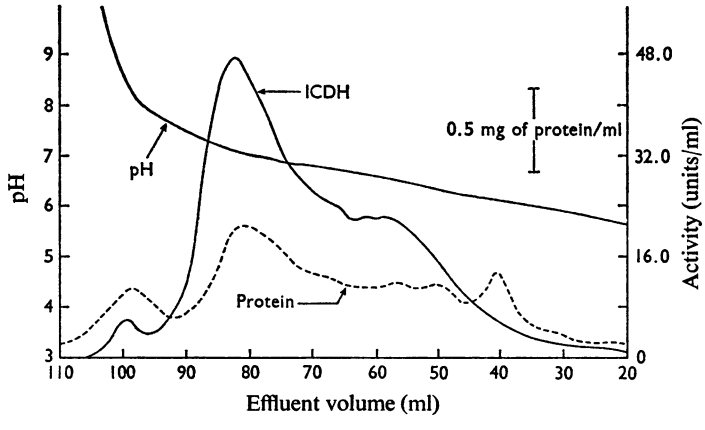

Fig. 3. *Isoelectric focusing of pure yeast isocitrate dehydrogenase*

After AMP elution the nucleotide was removed by gel filtration. The enzyme (1200 units) was loaded in 40ml of 5mM-potassium phosphate buffer, pH6.8. The ampholine concentration was 1% (w/v), pI range 5–8. The protein concentration was estimated from the E_{280}. Other details are given in the text. For abbreviation see Fig. 1.

the changes on re-focusing were much smaller and the acidic bands did not increase in intensity.

There is no change in the 'isoenzyme' pattern when the enzyme is merely stored, either in Ampholine or phosphate buffer. Fig. 5 shows two parallel experiments, one with enzyme aged at 4°C for 13 days in 4mM-potassium phosphate buffer, pH6.5, and the other with the same batch of enzyme stored for 13 days in 1% Ampholine (pI range 5–8). Both results are identical with those from fresh enzyme (see Fig. 6). All stored samples contained 2ml of mercaptoethanol/litre.

Figs. 6 and 7 show the effect of increased Ampholine concentration more clearly. Except for the ampholyte content these two experiments were duplicates run simultaneously on two identical focusing columns. Again the acidic peaks are intensified at high Ampholine/protein ratios.

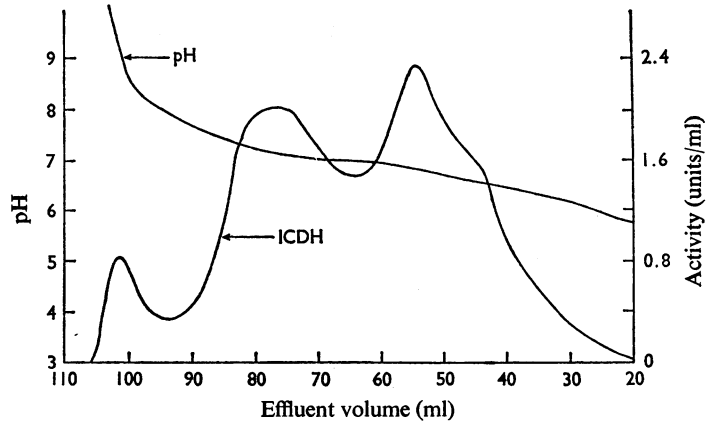

Fig. 4. *Re-focusing of pure yeast isocitrate dehydrogenase*

Enzyme (100 units) from the main peak in Fig. 3 was immediately re-focused in 1% Ampholine, pI range 5–8, without removal of the old ampholytes (N.B. the enzyme sample volume was only 5ml, so the absolute amount of old Ampholine transferred to the re-focusing experiment was small). Other details are given in the text. Similar results were observed in the other experiments of this series where the enzyme was stored frozen before re-focusing. For abbreviation see Fig. 1.

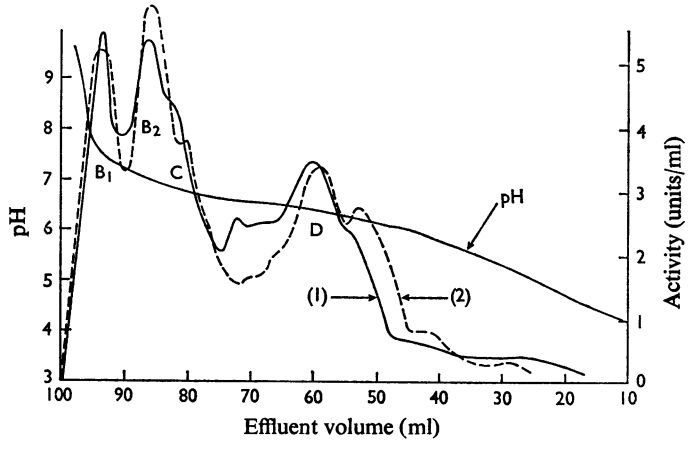

Fig. 5. *Effect of prolonged storage in Ampholine on the focusing behaviour of yeast isocitrate dehydrogenase*

Curve (1) shows the results observed with enzyme stored for 13 days in 4mM-potassium phosphate buffer, pH 6.5, and curve (2) shows the pattern produced by enzyme stored for the same period in 1% Ampholine, pI range 5–8. The two experiments were run simultaneously in two identical columns. The Ampholine concentration was 1%, consisting of equal proportions of pI range 3–6 and pI range 6–8 mixed together. Only one curve is shown for the pH profile because in this respect the results from the two columns were indistinguishable. In each case 200 units of enzyme were loaded per column. Other details are given in the text.

Despite the complexity of these results the reproducibility of duplicate experiments was excellent. There is no reason to suppose that these changes result from small random variations in experimental technique. In all cases there was a good recovery of activity from the column, ranging from about 66% at the lowest enzyme loads (30–40 units total) to well over 90% when the enzyme load was high (over 1000 units). When samples were stored before focusing the storage losses were always less than 10%.

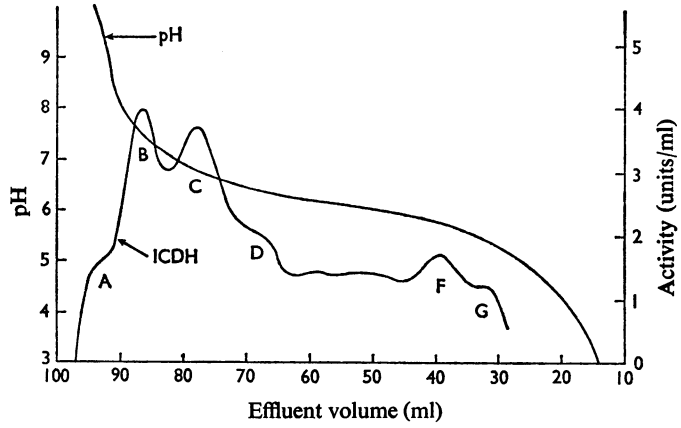

Fig. 6. *Isoelectric focusing of freshly prepared yeast isocitrate dehydrogenase at moderate concentration*

The column was loaded with 150 units of freshly prepared pure enzyme. The Ampholine concentration was 1%, pI range 5–8. Other details are given in the text. The results of this experiment should be compared with Figs. 5 and 7. Equivalent peaks have been labelled with the same letters in Figs. 5, 6 and 7 to facilitate this comparison. For abbreviation see Fig. 1.

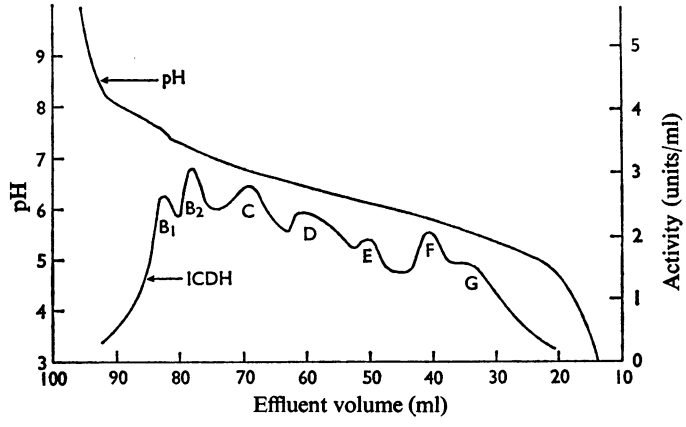

Fig. 7. *Effect of increased Ampholine concentration on the focusing behaviour of yeast isocitrate dehydrogenase*

This experiment was identical with that shown in Fig. 6, except that the Ampholine concentration was 3% rather than 1%. For abbreviation see Fig. 1.

With crude samples, the protein load was severely limited by precipitation problems, and in consequence crude enzyme was necessarily focused at a high Ampholine/enzyme ratio. Nevertheless this was not the cause of the differences between the isoelectric points of crude and pure enzyme. In other experiments (not illustrated) pure material was focused at concentrations comparable with those used for crude samples, but the mean pI never fell below 6.8. Moreover, crude enzyme has a higher electrophoretic mobility than either pure enzyme or 'post-DEAE-cellulose' material, and in contrast to the DEAE-cellulose eluate crude enzyme is not retarded on CM-cellulose even at pH 6.0. [Details of CM-cellulose chromatography are given in the preceding paper (Illingworth, 1972).]

Thus the differences between enzyme samples at various stages of purification appear to be genuine, but the 'isoenzymes' are probably artifacts. This was confirmed as follows.

(1) A batch of pure enzyme was refocused three times, yielding two 'isoenzyme' bands with pI values

of 6.7 and 5.8. The reaction kinetics of these two samples were compared with each other and with pure, non-focused enzyme, by using an improved version of the automatic method described by Illingworth & Tipton (1969). No significant differences were found [see the preceding paper (Illingworth, 1972) for values].

(2) Most of the ampholytes and sucrose were removed from the bands labelled 'B_1' and 'D' in the experiment shown in Fig. 5 by passage through Sephadex G-25 in 4mM-potassium phosphate buffer, pH6.5, containing 1ml of mercaptoethanol/litre and the products were concentrated by ultrafiltration. These samples were then subjected to polyacrylamide-gel electrophoresis in 4% gels by using both buffer systems described above, with staining for both protein and enzyme activity on duplicate gels. No differences in mobility were found between the 'isoenzymes'. All of the gels displayed a single activity band which exactly corresponded to the single protein band, and in each electrophoretic system both 'isoenzymes' showed exactly the same mobility as pure unfocused enzyme. An intense Amido Black stain overlaid the Bromothymol Blue leading marker in cases where the sample had been previously focused. This was attributed to traces of Ampholine, which were not completely removed from protein samples by gel filtration.

(3) No evidence for the existence of isoenzymes was uncovered during an extended series of conventional electrophoresis experiments on non-focused enzyme at various stages of purification. These runs were carried out in a variety of gel and buffer systems and showed that (*a*) mobility was independent of enzyme concentration; (*b*) crude enzyme had a higher mobility than either 'post-DEAE-cellulose' material or pure enzyme (which were indistinguishable); (*c*) in all samples mobility with respect to Bromothymol Blue fell with decreasing ionic strength.

Spurious 'isoenzymes' were not confined to focusing experiments. Several bands could be seen after isotachophoresis on polyacrylamide gels. Once again the pattern varies with the Ampholine/enzyme ratio. Maximally sharp bands (and a minimum number of 'isoenzymes') were observed when about 0.1 unit of enzyme was loaded in 0.1ml of 20% Ampholine per gel. This may well reflect the titration of a minor Ampholine component by the enzyme. No significant differences emerged between the mobilities of crude and pure enzyme in this system. However, the very high Ampholine/enzyme ratio in these experiments may have been responsible for a preponderance of acidic 'isoenzymes', which obscured the authentic differences.

A recent report (Pejaudier *et al.*, 1971) describes a similar generation of 'isoenzymes' during isoelectric focusing of caeruloplasmin. These authors suggested that damage occurred at the isoelectric end point, since in their case there was again no damage on storage in Ampholine. Kaplan & Foster (1970) also reported that spurious bands could be generated in albumin samples by exposure to Ampholine, and these workers also found that the effect was most serious at high Ampholine/protein ratios.

In the present case 'isoenzymes' were generated even during isotachophoresis when the ionic strength was high and when the system was nowhere near its isoelectric end point. This indicates that the artifact depends solely on the passage of electric current through Ampholine–protein mixtures. This suggests the following explanation, that commercial Ampholine samples may contain a small percentage of an acidic 'sticky' component, which has a marked tendency to adsorb on to other polyelectrolytes. This minor component would normally be concealed beneath a layer of adsorbed ordinary Ampholine molecules and would not be able to react with added proteins when these were simply stored in Ampholine. The protective layer would presumably consist mainly of basic ampholytes, and during electrophoresis these might be stripped from the offending minor component, allowing it to adsorb on to suitable protein molecules.

These experiments indicate the need for extreme caution when isoelectric focusing is the sole evidence for microheterogeneity. Even when a single band is observed its isoelectric point might well be seriously in error. In all cases it is necessary to know that the band can be successfully re-focused and that its intensity does not change with the Ampholine/protein ratio.

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