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APPENDIX

Amidination of Pyruvate Dehydrogenase Complex of *Escherichia coli* under Denaturing Conditions

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In a previous paper (Bates *et al.*, 1975) it was shown that the polypeptide-chain ratios in a mixture or complex of proteins could easily be determined by treating the protein with a large excess of methyl [$1\text{-}^{14}\text{C}$]acetimidate under strongly denaturing conditions, e.g. in 5M-guanidine hydrochloride. The chains were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and their ratios determined from the measured radioactivity in each band. It was necessary to know the lysine content of each chain and to assume that each chain was modified to the same extent (preferably at or near completion). In view of the rigour of the experimental conditions this assumption seemed reasonable, and tests with artificial mixtures of proteins were consistent with it.

To measure the extent of modification we have now carried out further experiments. A sample (3mg) of pyruvate dehydrogenase complex from *E. coli* was treated for 5h with unlabelled methyl acetimidate (0.1M) in the presence of 5M-guanidine hydrochloride, as described by Bates *et al.* (1975), except that the pH of the reaction mixture was 10.0, as recommended by Browne & Kent (1975a,b). Samples of the amidinated protein, after dialysis against water, were hydrolysed with 6M-HCl (containing 4mM-2-mercaptoethanol) in sealed evacuated tubes at 105°C for times ranging from 6 to 72h. Samples of untreated complex were hydrolysed similarly.

The amount of lysine in each hydrolysate was measured by means of a Rank Chromaspek amino

acid analyser. The lysine value in the control samples was at its maximum and independent of the time of hydrolysis between 12 h and 48 h, and this value was used in subsequent calculations. As expected, the amount of lysine in the hydrolysates of the amidinated protein increased with the time of hydrolysis, owing to the breakdown of the modified lysine residues (Reynolds, 1968) following a pseudo-first-order reaction with a rate constant of $8.4 \times 10^{-3} \text{ h}^{-1}$ (a half-life of 83 h). Extrapolation of the first-order plot to zero time of hydrolysis showed that the modification of lysine residues under the conditions used for amidination was 92% ($\pm 1\%$) complete. Identical results were obtained when methyl $[1-^{14}\text{C}]$ -acetimidate was substituted for the unlabelled imido ester.

By means of the radioamidination technique (Bates *et al.*, 1975), the polypeptide chain stoichiometry of the complex used was found to be 1.36:1:0.93 (E1/E2/E3). Assuming the lysine contents of each chain quoted by Bates *et al.* (1975), the total amount (mol) of lysine in the complex per mol of E2 chain was approx. 172. However, as shown in the main paper (Hale & Perham, 1979), each E2 chain of the complex bears at least three lipoyl groups, presumably all attached in amide linkage to lysine residues (Nawa *et al.*, 1960), which will serve to diminish the extent of amidination measured by back-hydrolysis. The corrected extent of amidination of potentially available lysine residues was therefore approx. 94%.

The almost complete modification of lysine residues in the complex observed in these experiments

supports the assumption that each chain is modified to the same extent and strengthens belief in the reliability of the radioamidination method of determining polypeptide chain ratios. As a routine, we now use and recommend amidination at pH 10, rather than pH 8.5 (Bates *et al.*, 1975), to take account of the conclusion of Browne & Kent (1975*a,b*) that quantitative amidination at pH 8.5 is impossible. However, in many trial experiments, no difference has been found in chain ratios measured at pH 8.5 or pH 10 for the treatment with methyl $[1-^{14}\text{C}]$ -acetimidate, although the extent of amidination at pH 8.5 could have been less.

Since at least three lysine residues in each E2 chain are probably lipoylated, appropriately lower values for the free lysine content of this chain should be used in future calculations of the polypeptide-chain ratios determined by the radioamidination method. The effect is to lower published E1/E2 and E3/E2 ratios by approx. 5%.

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