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Purification of the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus* and resolution of its four component polypeptides

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1. The pyruvate dehydrogenase complex was purified from *Bacillus stearothermophilus* in high yield. The specific activity (about 40nkat/mg of protein) was substantially lower than that of the pyruvate dehydrogenase complex from *Escherichia coli* (about 570nkat/mg of protein) measured at 30°C under the same conditions. 2. The relative molecular masses of the four types of polypeptide chain in the complex were estimated by means of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to be 57000, 54000, 42000 and 36000 respectively. These polypeptide chains showed no evidence of seriously anomalous behaviour during tests of electrophoretic mobility. 3. The enzyme complex was resolved into its constituent proteins by means of gel filtration on Sepharose CL-6B in the presence of 2M-KI, followed by chromatography on hydroxyapatite in the presence of 8M-urea. These harsh conditions were necessary to cause suitable dissociation of the enzyme complex. 4. The amino-acid compositions of the four constituent proteins after resolution were determined and their chain ratios were measured for several preparations of the complex. Some variability was noted between preparations but all samples contained a significant molar excess of the chains thought to contribute the pyruvate decarboxylase (EC 1.2.4.1) activity. 5. From the relative molecular masses and chain ratios of the four constituent proteins, it was calculated that the empirical unit must be repeated at least 50 times to make up the assembled complex. This conclusion is fully consistent with the demonstration by means of electron microscopy of apparent icosahedral symmetry for the *Bacillus stearothermophilus* complex, implying a 60-fold repeat. The structure stands in sharp contrast with the octahedral symmetry (24-fold repeat) of the *Escherichia coli* enzyme.

The pyruvate dehydrogenase multienzyme complex catalyses the overall reaction:

$$\text{Pyruvate} + \text{NAD}^+ + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{NADH} + \text{H}^+ + \text{CO}_2$$

The complex is a multimeric structure composed of different types of polypeptide chain responsible for the three constituent enzymic activities: pyruvate decarboxylase (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12) and lipoamide dehydrogenase (E3) (EC 1.6.4.3) [for reviews, see Reed (1974) and Perham (1975)].

The bacterial enzyme most thoroughly investigated is that from *Escherichia coli*. Methods are available for the separation of its component enzymes and their reconstitution to form the enzyme complex (Reed & Oliver, 1968; Reed, 1974). The E2 component is thought to contain 24 identical polypeptide chains arranged with octahedral symmetry (Reed & Oliver, 1968; Eley *et al.*, 1972; Danson *et al.*, 1979) and acts as a structural core to which the E1 and E3 components (each dimers of identical polypeptide chains) can bind with variable stoicheiometries (Vogel *et al.*, 1972; Bates *et al.*, 1977; Hale & Perham, 1979 and references therein).

The pyruvate dehydrogenase complex from mammalian sources, notably heart and kidney, broadly resembles the *E. coli* enzyme but differs from it in several important respects (Reed, 1974; Koike & Koike, 1976). It contains four different types of polypeptide chain, the E1 activity of the complex

Abbreviation used: SDS, sodium dodecyl sulphate.

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now reposing in two chains (E1α and E1β). The separated E2 component has the appearance in the electron microscope of a pentagonal dodecahedron. The most likely structure for this component, which again forms the complex core, is 60 identical polypeptide chains arranged with icosahedral symmetry (Reed, 1974).

We have recently demonstrated (Henderson *et al.*, 1979) that the pyruvate dehydrogenase complex of *Bacillus stearothermophilus* bears a striking resemblance to the mammalian enzyme in terms of its morphology, subunit composition and molecular weight, sharply contrasting with the enzyme complex from *E. coli*. In the present paper we describe the purification of the *B. stearothermophilus* complex in high yield and the separation of the component polypeptide chains. We characterize these in terms of their amino acid compositions and molecular weights and demonstrate that the native complex, as isolated, can have modest variations in polypeptide chain stoicheiometry, a property it shares with the *E. coli* enzyme.

Materials and methods

Bacteria

Bacillus stearothermophilus, strain NCA 1503, was obtained as a frozen cell paste from the Microbiological Research Establishment, Porton Down, Wilts., U.K. The organism had been grown in a medium based on that described by Sargeant *et al.* (1971) and was harvested in the exponential phase of growth. It was stored at −15°C.

Chemicals

Protamine sulphate (ex salmon roe) was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K., or from ICN Pharmaceuticals, Cleveland, OH, U.S.A. Yeast RNA was from BDH, Poole, Dorset, U.K., and phenylmethanesulphonyl fluoride was a product of Sigma Chemical Co., St. Louis, MO, U.S.A. Coenzyme A (grade I) and NAD^+ (free acid, grade II) were obtained from C. F. Boehringer & Son, Mannheim, West Germany. All reagents used in the preparation and resolution of the pyruvate dehydrogenase complex were of analytical grade. Urea was recrystallized twice from hot 96% ethanol. For use in molecular weight determinations, acrylamide specially purified for electrophoresis was purchased from BDH and *NN'*-methylenebisacrylamide was recrystallized from acetone. Dihydrolipoamide was synthesized and kindly provided by Dr. G. Hale (Department of Biochemistry, University of Cambridge). Radioactive reagents were products of The Radiochemical Centre, Amersham, Bucks., U.K. Hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Enzymes

Glyceraldehyde 3-phosphate dehydrogenase was purchased from Boehringer, lysozyme chloride (hen egg-white) from BDH and ovine serum albumin and ovalbumin from Sigma. Pancreatic ribonuclease A and trypsin were products of the Worthington Biochemical Corp., Freehold, NJ, U.S.A. Pyruvate dehydrogenase complex from *E. coli* was purified as described elsewhere (Danson *et al.*, 1978).

Enzyme assays

Pyruvate dehydrogenase complex activity was assayed at 30°C in the direction of NAD^+ reduction, as described by Danson *et al.* (1978). The method of Reed & Willms (1966), which uses exogenous dihydrolipoamide as substrate, was used to assay lipoamide dehydrogenase activity.

Solutions for enzyme purification and resolution

Protamine sulphate was suspended in water immediately before use at a concentration of 20g/litre. The pH was adjusted to 6.2 with 10% (w/v) KOH and any insoluble material was removed by low-speed centrifugation at room temperature. Batches of protamine that gave alkaline solutions when suspended, or that contained more than about 10% of insoluble material, were not used. Yeast RNA solutions (10g/litre) were prepared in a similar fashion.

Solutions of phenylmethanesulphonyl fluoride were prepared as follows. Approximately one-tenth of the final volume of buffer was heated to 90°C. It was then stirred rapidly during the addition dropwise of the appropriate amount of a concentrated solution of the reagent in acetone, made up freshly. Whenever possible, solutions were prepared immediately before use, to avoid degradation (James, 1978).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Protein samples were normally analysed using phosphate-buffered 5% (w/v) polyacrylamide gels (Shapiro *et al.*, 1967), although occasional use was made of the Tris/glycine system of Laemmli (1970).

For the determination of the molecular weights of the component polypeptide chains of the enzyme complex, the following procedure was adopted. Gels were prepared by the dilution of a stock solution of acrylamide and bisacrylamide to give a range of final concentrations (T) of acrylamide, where T = (g of acrylamide + g of bisacrylamide)/100ml of solution (Hjerten, 1962). The cross-linking of the gels was constant at C = 2.60, where C = 100 × (g of bisacrylamide/100ml of solution)/T. Each slab gel (1.5mm thick) was run at a constant voltage of 50V until the tracker dye had migrated 10cm. The gels were fixed, stained with Coomassie Brilliant Blue,

and then scanned using the thin-layer attachment of a Joyce-Loebl Chromoscan microdensitometer. Mobilities of protein bands relative to myoglobin were measured from the traces.

Amino-acid analysis

Protein samples of approximately 60μg were hydrolysed in evacuated acid-washed tubes at 105°C, after the addition of 0.2ml of 6M-HCl (Aristar) containing 5mM-2-mercaptoethanol. Amino acids in the hydrolysates were estimated by using a Rank Hilger Chromaspek analyser, interfaced with a Digico Micro 16V computer. Norleucine was added to all samples as an internal standard.

To obtain the complete amino acid composition of a protein, samples were hydrolysed for 24, 48 and 72h. Values for each amino acid at each time point expressed relative to the sum of the values for aspartic acid, glutamic acid, glycine, alanine, phenylalanine and lysine for that time point, all of which were found by inspection to be invariant with time. Serine and threonine values were extrapolated to zero time of hydrolysis, whereas the highest values measured for valine and isoleucine (usually at 72h hydrolysis) were taken for these amino acids. Half-cystine was quantified as *S*-carboxymethylcysteine after hydrolysis of *S*-carboxymethylated protein. No special procedures were adopted for the accurate determination of tryptophan in these studies.

Protein estimation

The protein concentration in crude extracts or solutions of high nucleic acid content was determined by the method of Lowry *et al.* (1951) or that of Warburg & Christian (1941). Amino-acid analysis was normally used for accurate determination of protein concentration in pure solutions.

Reduction and S-carboxymethylation of protein

Samples of protein (0.05–2.0mg) were reduced and carboxymethylated by a modification of the method of Gibbons & Perham (1970). To the freeze-dried protein was added 0.5ml of 0.2M-Tris/HCl buffer, pH8.2, saturated at room temperature with guanidine hydrochloride, and the solution was bubbled with N_2. After addition of 10μl of 0.1M-dithiothreitol, the tube was covered and placed in a boiling-water bath for 5min. The sample was cooled and flushed with N_2, and a further 10μl of 0.1M-dithiothreitol was added. After 30min at room temperature the protein was incubated with 15μl of 0.5M-iodoacetic acid for 1h in the dark, during which time the solution was flushed once more with N_2. The reaction was stopped by addition of 2-mercaptoethanol, and the modified protein was dialysed exhaustively against 1mM-EDTA, pH7.0.

Detection of glycoprotein in polyacrylamide gels

SDS/polyacrylamide gels were stained for glycoprotein by oxidation with performic acid and treatment with Schiff's reagent, as described by Zacharius *et al.* (1969).

Determination of polypeptide chain ratios

Samples (0.5mg) of pyruvate dehydrogenase complex were modified with methyl [1-^{14}C]acetimidate under denaturing conditions by the method of Bates *et al.* (1975), as modified by Hale *et al.* (1979). By estimating the lysine released during acid hydrolysis of the amidinated protein (Hale *et al.*, 1979), this method was shown to cause effectively complete modification of the lysine residues of the complex after only 10min exposure to the imidoester. Modified protein was analysed using SDS/15% (w/v) polyacrylamide gels with Tris/glycine buffer.

Labelled methyl acetimidate was synthesized from [1-^{14}C]acetonitrile and was stored desiccated at −15°C.

Results

Purification of the pyruvate dehydrogenase multienzyme complex

The pyruvate dehydrogenase complex was purified from *B. stearothermophilus* by the following method, which is based on that of Reed & Mukherjee (1969) for the enzyme from *E. coli*. All operations were carried out between 0 and 5°C.

(*a*) *Preparation of cell-free extract.* Frozen cell paste (500g) was suspended in 1 litre of 20mM-sodium phosphate buffer, pH7, containing 3mM-EDTA and 0.15mM-phenylmethanesulphonyl fluoride at 4°C, using the liquidizer of a Kenwood food-mixer. The suspension was stirred for 2h with 2.5g of lysozyme chloride, and then portions (75ml) in beakers immersed in ice were sonicated for 30s each with a Dawes Soniprobe (type 7532A) at an output of 110W. This treatment was shown in preliminary experiments to give maximal release of enzyme activity. Cell debris was removed by centrifugation for 1h at 19000rev./min (53700*g*) in the type-19 rotor of a Beckman ultracentrifuge. The pH of the supernatant was adjusted to 6.2 with 1% (v/v) acetic acid to give a cell-free extract containing about 20mg of protein/ml.

(*b*) *Precipitation with protamine.* The cell-free extract was stirred in ice while 0.05vol. of 2% (w/v) protamine were added dropwise. The mixture was stirred at 2°C for a further 30min before being centrifuged for 20min at 10000rev./min (16300*g*) in the GS-A rotor of the Sorvall centrifuge. The pellet was discarded and the supernatant was treated similarly with a further 0.03vol. of 2% (w/v) protamine. This process was repeated stepwise until

less than 10% of the starting enzyme activity remained in the supernatant, at which point one more addition of 0.03 vol. of protamine was made. This final precipitate was found to contain a significant proportion of the pyruvate dehydrogenase complex, which is inhibited by these concentrations of protamine. Three successive treatments with 0.03 vol. of protamine were normally sufficient and the remaining supernatant was then discarded. Precipitates containing pyruvate dehydrogenase complex activity were pooled and resuspended by stirring for 10h at 4°C in 100ml of 100mM-sodium phosphate, pH 7.0, containing 5mM-EDTA and 0.5mM-phenylmethanesulphonyl fluoride. The yellow supernatant ('protamine eluate') obtained after centrifugation of this suspension for 10min at 10000rev./min (16300*g*) in the Sorvall GS-A rotor was diluted with distilled water to a protein concentration of 5mg/ml.

(*c*) *Ultracentrifugation.* To the diluted protamine eluate was added 5ml of 1% (w/v) yeast RNA solution for every g of protein and the mixture was stirred in ice for 4h. This treatment was designed to remove excess protamine. The solution was clarified by centrifugation for 20min at 25000rev./min (104000*g*) in the Beckman SW27 rotor, and ribonuclease was added to the supernatant to a final concentration of 5mg/litre. After incubation for 2h at 2°C, the enzyme solution was centrifuged at 42000rev./min (195000*g*) in the Beckman type 42.1 rotor for 3h. The bright-yellow pellets were pooled, and stirred in 20ml of 50mM-sodium phosphate buffer, pH 7, containing 5mM-EDTA, 0.15mM-phenylmethanesulphonyl fluoride and ribonuclease (5mg/litre) for 10h or until they were completely resuspended, after which the solution was clarified by centrifugation for 10min at 20000rev./min (66400*g*) in the Beckman SW27 rotor. The supernatant was yellow-brown in colour, and is referred to as the '42K centrifuge pellet'.

(*d*) *Gel filtration and isoelectric precipitation.* The '42K centrifuge pellet' was further purified by gel filtration on a column of Sepharose 6B (Fig. 1). Fractions containing the peak of enzyme activity at the exclusion volume of the column were pooled as the 'Sepharose 6B peak'. The solution was stirred on ice while 0.1 vol. of 100mM-sodium acetate were added and the pH was lowered slowly to 5.2 with 1% (v/v) acetic acid. The white precipitate was removed by centrifugation and discarded. The pH of the supernatant was then brought to 4.65 with 1% acetic acid and the heavy yellow precipitate (an isoelectric precipitate) was collected by centrifugation for 10min at 9000rev./min (9800*g*) in the Sorvall SS-34 rotor. It was redissolved by stirring overnight with 6ml of 50mM-sodium phosphate buffer, pH 7, containing 2mM-EDTA, 0.15mM-phenylmethanesulphonyl fluoride and NaN_3 (0.2g/litre), and the pH of this solution was adjusted if necessary to pH 7.0. The solution of pure enzyme complex was frozen immediately and stored at −15°C. Under these conditions, no degradation or loss of activity were apparent even after several months.

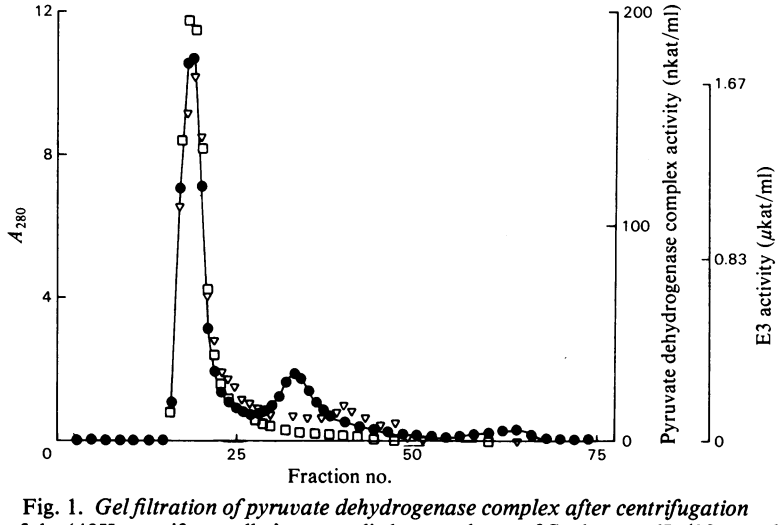

Fig. 1. *Gel filtration of pyruvate dehydrogenase complex after centrifugation*

A portion (20ml) of the '42K centrifuge pellet' was applied to a column of Sepharose 6B (98cm × 3cm) equilibrated with 50mM-sodium phosphate buffer, pH 7.0, containing 2mM-EDTA, 0.15mM-phenylmethanesulphonyl fluoride and NaN_3 (0.2g/litre). Fractions (10ml) were collected. ●, A_{280}; □, pyruvate dehydrogenase activity; ▽, E3 activity. For other details see the text.

The above procedure allows the preparation of 200mg of pyruvate dehydrogenase complex from 500g of cell paste. The recovery of enzyme activity varied from 25 to 40% of that measured in the cell-free extract, and the purification relative to total protein was nearly 500-fold. A summary of the main steps in the purification is provided in Table 1. The final specific activity (about 40nkat/mg of protein) is substantially lower than that of *E. coli* pyruvate dehydrogenase complex (about 570nkat/mg of protein) measured under the same conditions (Hale & Perham, 1979).

Molecular weights of the component polypeptide chains of the pyruvate dehydrogenase complex

The purified pyruvate dehydrogenase complex of *B. stearothermophilus* contains four different types of polypeptide chain, as revealed by SDS/polyacrylamide gel electrophoresis (Fig. 2). They are referred to as band 1, band 2, band 3 and band 4 in descending order of apparent molecular weight. We have previously shown (Henderson *et al.*, 1979) that band 1 is derived from the lipoate acetyltransferase (E2) component, and band 2 from lipoamide dehydrogenase (E3), whereas bands 3 and 4 very probably represent subunits of the pyruvate decarboxylase (E1) component.

The experiments we describe here were carried out to provide a reliable estimate of the polypeptide chain molecular weight of each of these components. The technique of electrophoresis in SDS/polyacrylamide gels was chosen for its convenience and for the fact that determinations may be carried out on native complex, without the need for prior resolution of the component polypeptides. However, in view of reports that factors such as intrinsic charge (Panyim & Chalkley, 1971), glycosylation (Bretscher, 1971), abnormal conformation (Furthmayr & Timpl, 1971; Tung & Knight, 1971) or asymmetric distribution of SDS along the polypeptide chain (Lehtovaara, 1978) may cause erroneous estimation of molecular weight, the empirical criteria suggested by Banker & Cotman (1972) were used to check for anomalous behaviour of the polypeptides of the pyruvate dehydrogenase complex in this system.

Samples of the *Bacillus* enzyme complex and its individual components (see below) were run in parallel with seven reference proteins in a series of

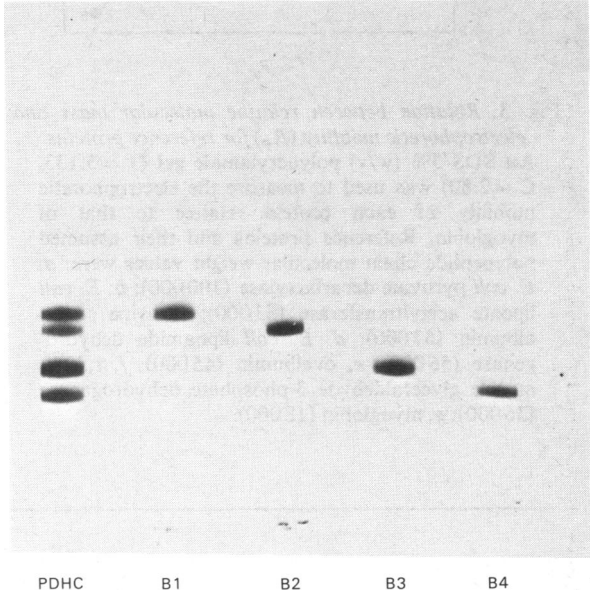

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of the pyruvate dehydrogenase complex and its resolved components*

Samples of the purified enzyme complex and its resolved components were analysed by electrophoresis in SDS/5% (w/v) polyacrylamide gels. PDHC, pyruvate dehydrogenase complex; B1, lipoate acetyltransferase (band 1); B2, lipoamide dehydrogenase (band 2); B3, band 3; B4, band 4. For other details see the text.

Table 1. *Purification of the pyruvate dehydrogenase complex from Bacillus stearothermophilus*

The pyruvate dehydrogenase complex from 500g of *B. stearothermophilus* cell paste was isolated as described in the text. The recovery in this particular preparation was 41% of the pyruvate dehydrogenase activity measured in the cell-free extract, and the purification relative to total protein was 480-fold.

	Total pyruvate dehydrogenase complex activity (μkat)	Specific activity (nkat/mg of protein)
Cell-free extract	20.9	0.08
Protamine eluate	8.69	1.8
'42K' centrifuge pellet	12.0	18
Sepharose 6B peak	9.14	37
Isoelectric precipitate	8.47	40

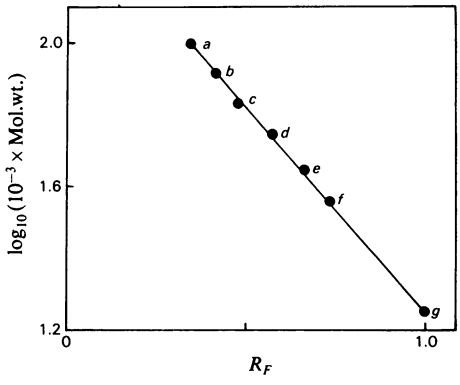

Fig. 3. *Relation between relative molecular mass and electrophoretic mobility (R_F) for reference proteins*
An SDS/5% (w/v) polyacrylamide gel (T = 5.133, C = 2.60) was used to measure the electrophoretic mobility of each protein relative to that of myoglobin. Reference proteins and their assumed polypeptide chain molecular weight values were: *a*, *E. coli* pyruvate decarboxylase (100000); *b*; *E. coli* lipoate acetyltransferase (83000); *c*, ovine serum albumin (67000); *d*, *E. coli* lipoamide dehydrogenase (56000); *e*, ovalbumin (45000); *f*, rabbit-muscle glyceraldehyde 3-phosphate dehydrogenase (36000); *g*, myoglobin (18000).

slab gels of varying acrylamide concentration. The reference proteins (and their assumed subunit molecular weights) were pyruvate dehydrogenase complex from *E. coli* (100000, 83000, 56500), ovine serum albumin (67000), ovalbumin (45000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36000) and myoglobin (18000). Their behaviour in SDS/polyacrylamide gels had previously been shown to be normal (Banker & Cotman, 1972; Vogel, 1977). The mobility of each protein, measured in several tracks of the gel, was expressed relative to myoglobin, and a linear relationship between this relative mobility (R_F) and the logarithm of the molecular weight was observed at all acrylamide concentrations tested (Fig. 3). Relative mobilities could be measured in this fashion with a standard error that was less than 1% of the mean.

All proteins tested exhibited a linear relation between relative mobility (R_F) and acrylamide concentration (T) in the gel (Fig. 4). This fits the equation first proposed by Ferguson (1964):

$$\log M = \log M_0 - k_R T$$

where M is the electrophoretic mobility in a gel of acrylamide concentration T (Hjerten, 1962), M_0, the

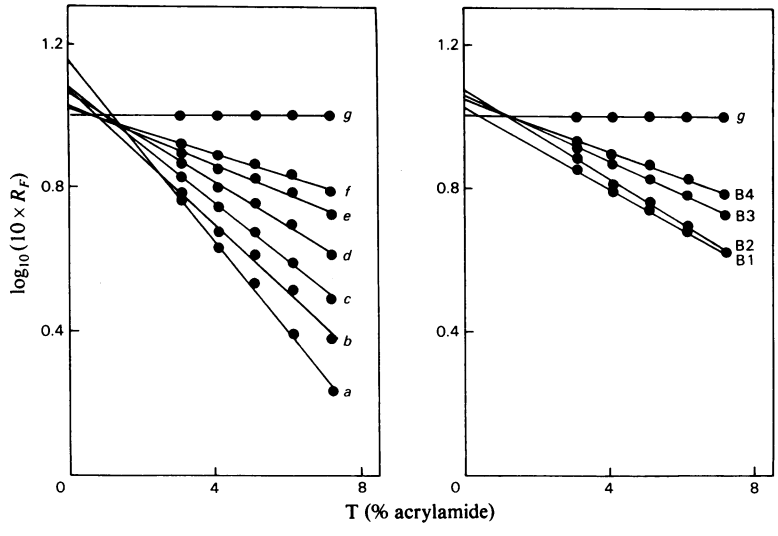

Fig. 4. *Ferguson plots for reference proteins and the components of the pyruvate dehydrogenase complex*
The electrophoretic mobility (R_F) relative to myoglobin was measured at a series of acrylamide concentrations (T). Reference proteins (*a*–*g*) and components of the multienzyme complex (B1–B4) are identified in the legends to Figs. 2 and 3. Values of the intercept and gradient for each line were calculated by linear regression analysis. Correlation coefficients ranged from 0.986 to 0.997. The reference and *B. stearothermophilus* proteins were studied simultaneously as mixtures on the same gels but the results are displayed separately for clarity. For other details, see the text.

'free electrophoretic mobility', is the mobility in the absence of sieving gel, and k_R, the 'retardation coefficient', represents the sieving effect of the gel on the migration of the protein–detergent complex. As expected from other reports and from the generally accepted physico-chemical basis of this technique, values of M_0 (intercept) did not vary widely for different protein–SDS complexes, whereas k_R (slope) increased markedly with molecular weight (Fig. 4), showing a linear relationship for the reference proteins tested (Fig. 5).

A plot of free electrophoretic mobility, M_0, against retardation coefficient, k_R, allows the behaviour of proteins under test to be compared directly with that of reference proteins, without the need for a knowledge of their molecular weights. Banker & Cotman (1972) showed that proteins whose molecular weights were incorrectly estimated by SDS/polyacrylamide-gel electrophoresis behaved abnormally in this plot. We therefore tested the relationship between log M_0 and k_R for all proteins under study (Fig. 6). It was linear within experimental error for the reference proteins, and all four polypeptide chains of the *B. stearothermophilus* pyruvate dehydrogenase complex lay on, or very close to, the line. This suggests that the molecular weights of these components will be reliably estimated by SDS/polyacrylamide-gel electrophoresis.

Values for the subunit molecular weights were extracted from the data presented in two different ways. Estimates were first obtained by interpolation in plots of R_F against logarithm of molecular weight (for example, Fig. 3) at all gel concentrations. Alternatively, interpolation in the plot of Fig. 5 was used. The values obtained by these two methods did not differ significantly, and the polypeptide chain molecular weights were taken as 57000 (band 1), 54000 (band 2), 42000 (band 3) and 36000 (band 4).

Despite the apparently normal migration of the components of the pyruvate dehydrogenase complex, the behaviour of band 1 was in one way slightly unusual. Whereas in Fig. 4 the intersection of the majority of Ferguson plots is around T = 2, that of band 1 tends to an intersection with the line for band 2 at around T = 10. Indeed, in SDS/15% (w/v) polyacrylamide gels run in the Tris/glycine system (not shown), the order of these two bands is reversed. The reason for this is unknown. Samples of pyruvate dehydrogenase complex run on SDS/polyacrylamide gels did not stain for glycoprotein.

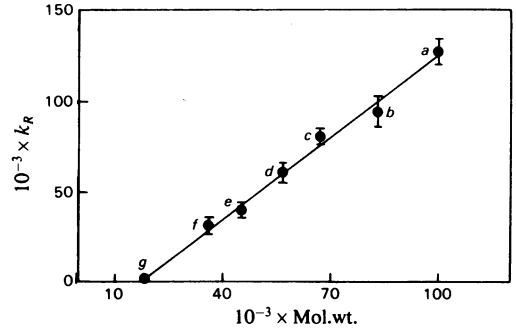

Fig. 5. *Relation between retardation coefficient (k_R) and relative molecular mass for reference proteins*
Values for the retardation coefficients (k_R) of reference proteins (see legend to Fig. 3) were obtained from the slopes of the Ferguson plots shown in Fig. 4. Bars indicate the S.E.M. for each value. For other details, see the text.

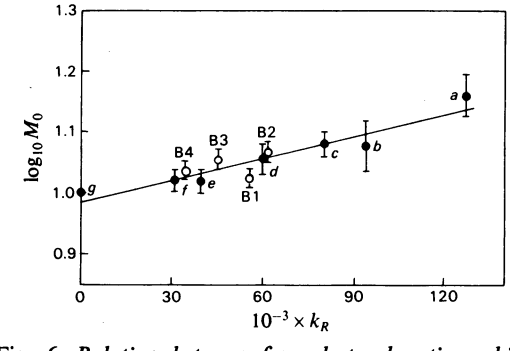

Fig. 6. *Relation between free electrophoretic mobility (M_0) and retardation coefficient (k_R)*
The proteins are identified in the legends to Figs. 2 and 3. Bars indicate the S.E.M. for the values of M_0. Errors in the values of k_R were of similar magnitude. For other details, see the text.

Resolution of the component polypeptide chains of the pyruvate dehydrogenase complex

The method most commonly used to resolve the pyruvate dehydrogenase complex of *E. coli* is that of Reed & Willms (1966). At pH 10, this complex dissociates to E1 and a subcomplex of E2–E3, and these can then be separated by gel filtration (Coggins *et al.*, 1976). The E2–E3 subcomplex is resolved into the E2 and E3 components by chromatography on a column of hydroxyapatite in the presence of 6M-urea. However, when the pyruvate dehydrogenase complex of *B. stearothermophilus* was subjected to these treatments, we found it to be intact and active after the first step, and still intact after the second. Its extreme stability therefore necessitated the development of harsher techniques to promote dissociation, which we describe here.

A sample (45mg) of *B. stearothermophilus* pyruvate dehydrogenase complex was treated with 2M-KI and gel-filtered on a column of Sepharose CL-6B, as described previously (Henderson *et al.*,

1979). Proteins in the column effluent were analysed by SDS/polyacrylamide-gel electrophoresis. Of the four peaks of protein eluted, the first contained essentially pure lipoate acetyltransferase (E2, band 1), while the second consisted mainly of band 3. Lipoamide dehydrogenase (E3, band 2) contaminated with bands 3 and 4 was found in the third peak, and was still active. The fourth peak consisted for the most part of extremely pure band-4 protein. In addition to these protein peaks, a fifth peak of A_{280} at the inclusion limit of the column was characterized as FAD by its colour, low molecular weight and A_{450}.

The second step in the resolution procedure we developed is chromatography on a column of hydroxyapatite. Fractions from the Sepharose CL-6B column that contained significant amounts of more than one type of polypeptide chain were pooled, concentrated by ultrafiltration to a final volume of 10ml (protein concentration about 2.5mg/ml) and then dialysed against 10mM-sodium phosphate buffer, pH7.0, containing 1mM-EDTA, 0.4mM-phenylmethanesulphonyl fluoride and 5mM-mercaptoethanol. The protein solution was saturated with urea at room temperature and stirred for 1h, at which point it was applied to a freshly-poured hydroxyapatite column equilibrated in 6mM-sodium phosphate buffer, pH7, containing 8M-urea and 0.5mM-phenylmethanesulphonyl fluoride. The proteins were eluted by a stepwise increase of phosphate concentration in a series of buffers containing 8M-urea, 0.5mM-phenylmethanesulphonyl fluoride and 2mM-mercaptoethanol (Fig. 7). After addition of EDTA to a final concentration of 2mM to all fractions, their A_{280} was measured. Fractions in each of the three well-separated protein peaks were dialysed against 20mM-sodium phosphate buffer, pH7, containing 2mM-EDTA, 2mM-mercaptoethanol and NaN_3 (0.2g/litre). SDS/polyacrylamide gel electrophoresis showed that, in order of elution, the peaks contained predominantly band 4, band 3 and band 2.

If necessary, the individual peaks could be further purified by rechromatography on hydroxyapatite under identical conditions. For lipoamide dehydrogenase (E3, band 2), the chromatography was preceded by treatment with 2% (w/w) trypsin for 5min at room temperature, the proteinase action being stopped by adding soya-bean trypsin inhibitor. The purified polypeptides were stored at 4°C in 20mM-sodium phosphate buffer, pH7.0, containing 2mM-EDTA, 5mM-mercaptoethanol and NaN_3 (0.2g/litre), for several weeks without any apparent proteolytic degradation.

Highly purified preparations of each of the four components of the *Bacillus* pyruvate dehydrogenase complex were obtained by this resolution procedure, as judged from the SDS/polyacrylamide gels shown in Fig. 2. Levels of cross-contamination were negligible, and lipoamide dehydrogenase prepared in this fashion had an E3 specific activity of 1.78μkat/mg of protein. The resolved components were completely indistinguishable on SDS/polyacrylamide gels from the bands of the unresolved pyruvate dehydrogenase complex, suggesting that no major degradation of the subunits took place during resolution.

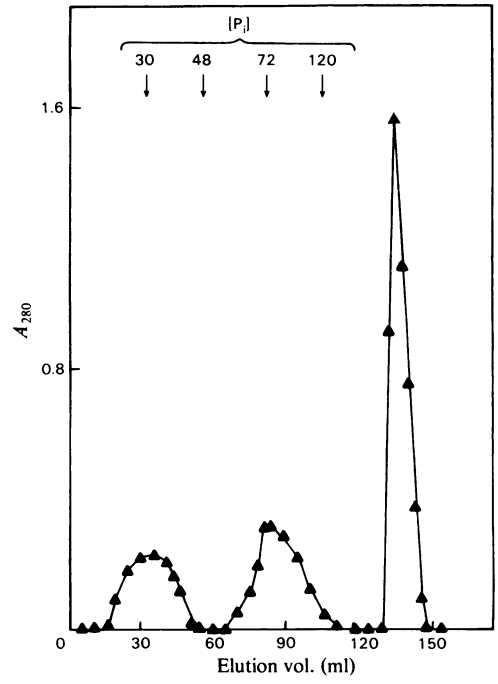

Fig. 7. *Chromatography of components of the pyruvate dehydrogenase complex on hydroxyapatite in the presence of urea*

The solution of protein was saturated with urea and applied to a column (5cm × 2cm) of freshly-poured hydroxyapatite equilibrated with 6mM-sodium phosphate buffer, pH7.0, containing 8M-urea and 0.5mM-phenylmethanesulphonyl fluoride. Proteins were eluted by stepwise application of 25ml portions of buffer, pH7.0, containing successively 7mM-, 30mM-, 48mM-, 72mM- and 120mM-sodium phosphate as indicated by the arrows. All elution buffers contained in addition 8M-urea, 0.5mM-phenylmethanesulphonyl fluoride and 2mM-mercaptoethanol.

Characterization of the uncomplexed lipoamide dehydrogenase activity

During the purification of the pyruvate dehydrogenase complex (Fig. 1), lipoamide dehydrogenase (E3) activity associated with proteins of lower molecular weight than the intact complex was

consistently detected. The nature of the polypeptide responsible for this catalytic activity was investigated.

Fractions from the Sepharose 6B column (Fig. 1) containing E3 activity were concentrated by ultrafiltration to about 10mg of protein/ml, and treated with 2% (w/w) trypsin before chromatography on hydroxyapatite, as described in the preceding section. The protein eluted by 120mM-sodium phosphate buffer was yellow, had an E3 specific activity of 1.42μkat/mg of protein, and on SDS/polyacrylamide-gel electrophoresis showed only one major band, indistinguishable on 5 and 7.5% gels from band 2 of the pyruvate dehydrogenase complex. The two are very probably identical proteins.

Amino acid compositions of the component polypeptides of the Bacillus pyruvate dehydrogenase complex

Samples of the four components of the complex were hydrolysed for 24, 48 and 72h, as described in the Materials and methods section. Their amino acid compositions were calculated on the basis of the measured subunit molecular weights, and are shown in Table 2. Tryptophan was not detected in all samples as no special procedures were adopted for its protection during hydrolysis. The figures quoted thus represent minimum estimates for this amino acid.

Tryptic 'fingerprints' (not shown) of components that had been reduced and *S*-carboxymethylated with iodo[2-^{14}C]acetic acid were subjected to radioautography. The 'maps' for bands 1, 2, 3 and 4 were found, respectively, to contain 6–7, 5, 2 and 3–5 radioactive peptides, each presumed to contain one or more residues of carboxymethylcysteine. These values are in good agreement with those obtained for the half-cystine contents by amino acid analysis (Table 2). The patterns of ninhydrin-staining peptides in these maps did not suggest any extensive sequence homology between the components of the pyruvate dehydrogenase complex.

Polypeptide chain stoicheiometry of the pyruvate dehydrogenase complex of Bacillus stearothermophilus

The radioamidination method (Bates *et al.*, 1975; Hale *et al.*, 1979) was used in conjunction with the subunit molecular weights and amino acid compositions reported above to measure the molar chain ratios of the purified enzyme (see the Materials and (methods section).

In Table 3 are listed the values obtained for the stoicheiometry of three different preparations of pyruvate dehydrogenase complex. The molar content of each polypeptide chain is expressed relative to that of the lipoate acetyltransferase (E2, band 1) chain, since it is known that this component forms the structural core of the complex, to which the other enzymes bind (Henderson *et al.*, 1979). It is apparent from these results that the relative amounts of the four components in the purified complex can

Table 2. *Amino-acid compositions of components of the pyruvate dehydrogenase complex*

Values are expressed as mol amino acid/mol of polypeptide chain. The assumed relative molecular masses of polypeptide chains were: band 1: 57000; band 2: 54000; band 3: 42000; band 4: 36000. The S.E.M. for three to twelve determinations is quoted for each residue except tryptophan. CMCys, carboxymethylcysteine.

Amino acid	Composition (mol/mol of chain) of Band 1	Band 2	Band 3	Band 4
Asx	46.4 (±0.4)	43.9 (±0.3)	29.2 (±0.8)	25.3 (±0.4)
Thr	30.4 (±3.5)	28.0 (±0.6)	19.4 (±1.7)	15.5 (±0.3)
Ser	16.1 (±3.6)	17.1 (±0.9)	15.3 (±0.5)	14.6 (±0.5)
Glx	60.7 (±0.7)	48.9 (±0.3)	58.0 (±1.2)	38.7 (±0.7)
Pro	33.9 (±1.7)	17.8 (±0.7)	18.7 (±1.0)	17.7 (±0.4)
Gly	42.1 (±1.0)	60.8 (±0.9)	30.1 (±0.4)	30.3 (±0.4)
Ala	67.3 (±1.4)	68.5 (±0.3)	38.2 (±0.6)	33.8 (±0.4)
Val	44.3 (±1.0)	48.0 (±1.7)	23.4 (±0.7)	32.5 (±0.6)
Met	7.5 (±0.5)	3.9 (±0.2)	5.2 (±0.3)	5.6 (±0.3)
Ile	37.6 (±1.5)	41.1 (±0.5)	25.8 (±0.4)	28.8 (±0.3)
Leu	41.6 (±1.0)	37.0 (±0.3)	29.0 (±0.2)	27.0 (±0.1)
Tyr	7.3 (±0.3)	11.7 (±0.2)	11.3 (±0.5)	8.6 (±0.3)
Phe	14.3 (±0.3)	14.2 (±0.4)	20.6 (±1.3)	14.8 (±0.3)
His	10.6 (±1.3)	7.1 (±0.4)	5.4 (±0.2)	4.9 (±0.2)
Lys	44.1 (±1.0)	34.0 (±1.1)	25.5 (±0.4)	13.9 (±0.3)
Arg	17.5 (±2.4)	13.4 (±1.3)	14.9 (±2.1)	14.0 (±1.5)
CMCys	7.1 (±0.8)	4.6 (±0.3)	2.0 (±0.5)	3.4 (±0.4)
Trp	4.1	3.1	2.8	1.4

Table 3. *Polypeptide-chain stoicheiometries of different preparations of the pyruvate dehydrogenase complex*
Samples of pyruvate dehydrogenase complex were modified with methyl [1-^{14}C]acetimidate and analysed on SDS/polyacrylamide gels, as described in the Materials and methods section. In these experiments, the average background radioactivity in the gels represented 5% of the radioactivity incorporated into band 1. By using the polypeptide chain molecular weight and lysine content previously determined for each band, the molar polypeptide chain stoicheiometries were expressed relative to band 1 as unity. Results are expressed ± S.E.M. of *n* determinations. Minimum molecular weights (i.e. g of protein/mol of band-1 polypeptide) were calculated.

	Relative amount of					
Preparation	Band 1	Band 2	Band 3	Band 4	*n*	Minimum mol.wt.
A	1	0.45 (±0.06)	1.31 (±0.05)	1.30 (±0.09)	6	$1.83 (\pm 0.08) \times 10^5$
B	1	0.96 (±0.05)	1.75 (±0.09)	1.93 (±0.07)	9	$2.52 (\pm 0.09) \times 10^5$
C	1	0.83 (±0.03)	1.52 (±0.07)	2.03 (±0.09)	9	$2.39 (\pm 0.08) \times 10^5$

vary quite widely between preparations. This conclusion was supported by densitometric scanning of SDS/polyacrylamide gels stained with Coomassie Blue (not shown). The maximum proportions measured by means of the radioactive amidination method for each component were 1.0 (band 1):1.0 (band 2):1.7 (band 3):2.0 (band 4), though these were not observed with the same preparation of enzyme complex in each case.

It was calculated from these polypeptide chain stoicheiometry values that the minimum molecular weight, that is to say the weight of protein associated on average with each lipoate acetyltransferase (band 1) chain, ranged from 1.83×10^5 to 2.52×10^5 g of protein/mol of E2 component.

Discussion

We have developed a method for purifying the pyruvate dehydrogenase complex from *B. stearothermophilus* that yields 100-mg quantities of pure enzyme rapidly and reproducibly (Table 1). This is the only Gram-positive bacterial source to have been studied in such detail thus far. The complex is remarkable in that it contains four types of polypeptide chain and is based on icosahedral symmetry, closely resembling the complex from mammalian (mitochondrial) sources in these properties (Henderson *et al.*, 1979). This may have implications for the evolutionary origin of the mitochondrion (Henderson *et al.*, 1979).

A careful study by means of SDS/polyacrylamide-gel electrophoresis (Figs. 3–6) indicated that the subunit molecular weights of the *B. stearothermophilus* pyruvate dehydrogenase complex are 57000, 54000, 42000 and 36000, as we reported earlier (Henderson *et al.*, 1979). These values may be compared with those of 74000, 55000, 41000 and 36000, obtained for the pig heart enzyme (Hamada *et al.*, 1975). It may well be that the polypeptide chains of highest relative molecular mass in *B. stearothermophilus* and mammalian complexes differ in some significant way. However, the largest polypeptide of the ox-heart complex was judged from sedimentation equilibrium studies in guanidine hydrochloride to have a relative molecular mass of 51000, whereas SDS/polyacrylamide-gel electrophoresis indicated a value of 74000 (Barrera *et al.*, 1972). This discrepancy remains to be explained, as do certain features of the migration of band 1 (E2) of the *B. stearothermophilus* complex in SDS/polyacrylamide gels (e.g. Fig. 4). These points obviously merit further investigation.

The conventional methods used to resolve the *E. coli* pyruvate dehydrogenase complex into its component enzymes proved ineffective when applied to the complex from *B. stearothermophilus*. Other experiments demonstrated the extreme resistance of the enzyme complex to heat and to denaturing solvents (C. E. Henderson, P. Lazo & R. N. Perham, unpublished work). We therefore had to resort to the rather harsh conditions of gel filtration in the presence of 2M-KI to effect a resolution, followed by chromatography on hydroxyapatite in the presence of 8M-urea (Fig. 7). Although the polypeptide chains we obtained by this technique were judged pure by analysis on SDS/polyacrylamide gels (Fig. 2), our attempts to reconstitute active enzyme complexes from the separated components have not been successful.

Obtaining pure polypeptide chains did allow us to determine the amino acid composition of each component (Table 2). From this, and assuming the relative molecular masses of the polypeptide chains derived from SDS/polyacrylamide-gel electrophoresis, we could measure polypeptide chain ratios by means of the radioactive amidination method (Table 3). It is clear that these ratios vary somewhat between the three different preparations of the complex. In particular, preparation A contained substantially less than a stoicheiometric amount of E3 (band 2), whereas bands 3 and 4, which are likely to be the E1α and E1β polypeptide chains (Henderson *et al.*, 1979), were both present in substantial

molar excess, all compared with the E2 chain (band 1) taken as unity. Comparable variability in the polypeptide chain ratios of the purified pyruvate dehydrogenase complex from *E. coli* has also been observed (Vogel *et al.*, 1972; Bates *et al.*, 1975; Hale & Perham, 1979). It remains to be determined whether such variation occurs *in vivo* or reflects a loss of subunits bound to the E2 core during purification of the complex (Danson & Perham, 1976). Differences in E1:E2 chain ratios for the pyruvate dehydrogenase complex from ox heart compared with that from ox kidney (Reed, 1974) show that similar variation is also to be found between enzymes from different tissues of a eukaryotic organism.

Inspection of the results in Table 3 suggests that the *B. stearothermophilus* complex can achieve polypeptide-chain binding proportions (bands 1:2:3:4) of at least 1.0:1.0:1.7:2.0. It is noteworthy that the corresponding chain proportions (E2:E3:E1α:E1β) of the pig heart enzyme have been found to be approx. 1:0.5:2:2 (Koike & Koike, 1976; Sugden & Randle, 1978), although somewhat different values have been reported (see Reed, 1974) for the ox heart complex (1:0.16:1:1) and different values again for the ox kidney complex (1:0.16:0.67:0.67). The correspondence between the mammalian and *B. stearothermophilus* enzymes that we noted earlier (Henderson *et al.*, 1979) therefore extends in part to the polypeptide chain ratios in the complexes.

The relative molecular mass of the pyruvate dehydrogenase complex in preparation A (Table 3) has been estimated to be approx. 9.6×10^6 (C. E. Henderson & A. J. Rowe, unpublished work). This implies that the structure of minimum relative molecular mass 1.8×10^5 (Table 3) must be repeated about 52 times in the intact complex. The only symmetry that can reasonably account for this large a multienzyme complex is octahedral (24-fold repeat) or icosahedral (60-fold repeat) point group symmetry. An electron microscopic analysis of the enzyme from *B. stearothermophilus* strongly indicated that the symmetry is icosahedral and not octahedral (Henderson *et al.*, 1979). The evidence we have now obtained concerning polypeptide chain ratios supports that conclusion. However, Koike & Koike (1976) and Sugden & Randle (1978) have suggested that the similar E2 core of the mammalian complex contains 24 polypeptide chains, whereas Reed (1974) proposes 60 polypeptide chains arranged with icosahedral symmetry. Our analysis of the *B. stearothermophilus* enzyme appears to favour the latter model.

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