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Polypeptide-Chain Stoicheiometry and Lipoic Acid Content of the Pyruvate Dehydrogenase Complex of *Escherichia coli*

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The pyruvate dehydrogenase multienzyme complex was isolated from *Escherichia coli* grown in the presence of [^{35}S]sulphate. The three component enzymes were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the molar ratios of the three polypeptide chains were determined by measurement of the radioactivity in each band. The chain ratio of lipoamide dehydrogenase to lipoate acetyltransferase approached unity, but there was a molar excess of chains of the pyruvate decarboxylase component. The ^{35}S-labelled complex was also used in a new determination of the total lipoic acid content. It was found that each polypeptide chain of the lipoate acetyltransferase component appears to bear at least three lipoyl groups.

The pyruvate dehydrogenase multienzyme complex of *Escherichia coli* catalyses the overall reaction:

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

It is a multimeric structure somewhat larger than a ribosome and contains three different types of polypeptide chain responsible for the three constituent enzyme activities; these are: a 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 E2 component forms the structural core of the complex and is probably composed of 24 polypeptide chains (Reed & Oliver, 1968; Reed, 1974). It also contains covalently bound lipoic acid, which plays an essential part in the reaction mechanism (Nawa *et al.*, 1960; Koike *et al.*, 1960; Danson & Perham, 1976; Bates *et al.*, 1977).

The stoicheiometric ratio of the three components has been a matter of controversy. Direct measurements in this laboratory on chemically radiolabelled complex have shown that the E3/E2 chain ratio in the native complex, as isolated, is about 1:1, whereas there is always a molar excess of E1 chains compared with E2 chains (Bates *et al.*, 1975*a*; also see the Appendix). Reassembly experiments have further indicated that the limiting chain ratio (E1/E2/E3) is 2:1:1 (Perham & Hooper, 1977; Bates *et al.*, 1977) (chain ratios are all relative to E2 as unity, because E2 forms the structural core of the complex). These results for the native complex are in excellent accord with those of Vogel *et al.* (1972), but do not agree with the findings of Reed and co-workers, who have used fundamentally different techniques and report that the polypeptide-chain ratio (E1/E2/E3) in the native complex is 1:1:0.5 (Eley *et al.*, 1972; Reed *et al.*, 1975). These lower ratios were calculated by an indirect method, from the measured molecular weight of the complex and of the E2 core and from the flavin content of the complex (Eley *et al.*, 1972) (FAD is non-covalently bound to the E3 component). Large molecular weights are notoriously difficult to measure and a comparatively small error in the molecular weight of the complex would have a large effect on the calculated E1/E2 chain ratio. In the more recent experiments (Reed *et al.*, 1975), a radiolabelling technique was used that permitted the measurement of chain ratios in artificially reassembled complexes. It was reported that E3 subunits initially bound to the E2 core are displaced on the addition of the E1 component; however, we have found no evidence for this phenomenon in comparable reassembly experiments (Perham & Hooper, 1977; Bates *et al.*, 1977). The discrepancies between measurements in different laboratories may be caused by systematic errors in the different methods used, or may be a genuine reflection of different preparations of the complex.

In the present paper we report a new approach to the problem by measuring the radioactivity in each component enzyme of the complex intrinsically radiolabelled with ^{35}S by growth of the bacteria on [^{35}S]sulphate. The results are in full agreement with our previous findings (Bates *et al.*, 1975*a*).

Another property of the complex about which there has been disagreement in the literature is the

Abbreviations used: E1, pyruvate decarboxylase (EC 1.2.4.1); E2, lipoate acetyltransferase (EC 2.3.1.12); E3, lipoamide dehydrogenase (EC 1.6.4.3).

lipoic acid content. Values between 5.5 and 12.3 nmol of lipoic acid/mg of complex have been reported by Reed and co-workers (Eley *et al.*, 1972; Suzuki & Reed, 1963), but the results have been interpreted in terms of one lipoyl group per E2 chain. However, experiments in our laboratory have demonstrated that there are two lipoyl groups per E2 chain, which become reductively acetylated during the normal enzyme reaction (Danson & Perham, 1976; Bates *et al.*, 1977; Danson *et al.*, 1978*a*). This result has been confirmed independently by others (Collins & Reed, 1977; Speckhard *et al.*, 1977). From a study of the ^{35}S-labelled complex, we show here that each polypeptide chain of the E2 component probably bears at least three lipoic acid residues.

Materials and Methods

Materials

Yeast RNA was obtained from BDH Chemicals, Poole, Dorset, U.K., and protamine sulphate (from salmon roe) was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Cysteic acid and methionine sulphone were prepared by oxidation of cysteine and methionine with performic acid (Hirs, 1956). Both were shown to be pure by amino acid analysis. Sodium [^{35}S]sulphate, carrier-free (SJS1), was from The Radiochemical Centre, Amersham, Bucks., U.K. Silica-coated plastic sheets (Polygram Sil G, 0.25 mm-thick silica-gel layer on 20 cm × 20 cm plastic sheets), manufactured by Machery–Nagel and Co., Düren, Germany, were supplied by Camlab, Cambridge, U.K.

Growth of bacteria

The organism used was a mutant of *Escherichia coli* K12 constitutive for the production of pyruvate dehydrogenase complex, kindly supplied by Professor H. L. Kornberg of this Department. It also requires thiamin, tryptophan, arginine and histidine for growth. The cells were cultured aerobically in six 2-litre flasks, each containing 1 litre of the following medium: 14 mM-KH_2PO_4, 36 mM-Na_2HPO_4, 50 mM-NH_4Cl, 0.4 mM-$CaCl_2$, 0.32 mM-$MgCl_2$, 0.01 mM-$FeCl_3$, 0.02 mM-$MnCl_2$, 0.54 mM-tryptophan, 1.28 mM-arginine, 0.73 mM-histidine, 0.40 μM-thiamin, 100 mM-glucose, 0.15 mM-Na_2SO_4. To one flask was added 20 mCi of carrier-free sodium [^{35}S]sulphate. The contents of the six flasks were pooled before harvesting of the bacteria. To ensure a high incorporation of label, the total sulphate concentration in the growth medium was just above the minimum (0.08 mM) required for maximal cell growth. Control experiments in which cells grown in the presence of 0.1 mM- and 0.3 mM-sulphate were compared revealed no significant difference in the pyruvate dehydrogenase activity.

The cells were harvested by centrifugation (8000*g*, 5 min) at the end of exponential growth. This and all subsequent purification steps were carried out near 4°C.

Purification of ^{35}S-labelled pyruvate dehydrogenase complex

The procedure of Reed & Mukherjee (1969) was followed as far as possible, although some modifications were necessary to scale down the method for the small quantity of highly radioactive bacteria. The cell paste (10–14 g) was suspended in 20 ml of 20 mM-potassium phosphate buffer, pH 7.0. This and all other buffers used contained EDTA (1 g/l) and NaN_3 (0.2 g/l). The suspension, packed in ice, was sonicated for 8 min with a Dawe Soniprobe (type 3532A) fitted with a titanium microtip and operating at minimum intensity. In control experiments these conditions were sufficient to release at least 95% of the available enzyme activity from the cells. The cell extract was processed by the method of Reed & Mukherjee (1969) except that the final step, isoelectric precipitation, was omitted. Instead, the partially purified complex was gel-filtered on a column (490 mm × 16 mm) of Sepharose 6B in 20 mM-sodium phosphate buffer, pH 7.0, at 4°C. Only the purest fraction from this column (as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) was used for the experiments described below. The specific enzyme activity of the ^{35}S-labelled pyruvate dehydrogenase complex was 34 μmol of NADH/min per mg of protein, and its specific radioactivity was approx. 1.1×10^7 d.p.m./mg. Protein concentrations were estimated by A_{280}, assuming $A^{1\%}_{1cm} = 9.87$ (Harrison, 1974).

The complex was resolved into the E1 component and the E2·E3 subcomplex by gel filtration on Sepharose 6B in ethanolamine/phosphate buffer, pH 10.0, as described by Coggins *et al.* (1976).

Enzyme assay

The activity of the pyruvate dehydrogenase complex was assayed spectrophotometrically in the direction of NAD^+ reduction by the method of Reed & Mukherjee (1969), as described by Danson & Perham (1976).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate was carried out as described by Perham & Thomas (1971). Radioactivity in the stained protein bands was measured by the method of Tishler & Epstein (1968) as described by Bates *et al.* (1975*a*). The

liquid-scintillation-counter channel settings and quench correction for ^{35}S were identical with those used for ^{14}C (Bates *et al.*, 1975*a*).

Hydrolysis of proteins and amino acid analysis

Unlabelled lipoic acid (10 μg) was added to samples of ^{35}S-labelled pyruvate dehydrogenase complex before chemical modification or hydrolysis as a carrier to protect against oxidation of small amounts of [^{35}S]lipoic acid (Wagner *et al.*, 1956). Reduction and carboxymethylation or performic acid oxidation were carried out, when required, as described respectively by Gibbons & Perham (1970) and Hirs (1956). Protein samples (0.1–0.5 mg) were hydrolysed for various times with 6M-HCl in sealed evacuated tubes at 105°C. Radiolabelled *S*-carboxymethylcysteine and methionine were separated on the long column of a Beckman 120C amino acid analyser and eluted with the standard buffer system of Spackman *et al.* (1958). The amino acids were quantified with ninhydrin in the usual way.

Electrophoresis

Acid hydrolysates of protein were dried down *in vacuo* and redissolved in 10 μl of 50mM-NH_3 solution. Samples were applied to the centre of a silica-coated plastic sheet (100mm × 100mm) and electrophoresed in one dimension at pH 2.0 for 12min at 50V/cm as described by Bates *et al.* (1975*b*). Amino acids were detected by staining the electrophoretogram with the ninhydrin/cadmium reagent of Heilmann *et al.* (1957), and radiolabelled compounds were detected by radioautography. The radioautograms were scanned with a Joyce–Loebl microdensitometer (mark IIIc), but the amount of radioactivity was quantified by liquid-scintillation counting. The silica plate was cut into small pieces (about 10mm × 10mm), which were placed in plastic scintillation vials to which was added 0.3ml of 50mM-NH_3 solution and 3ml of scintillant (toluene/Triton X-100, 2:1, v/v, containing 5g of 2,5-diphenyloxazole/litre). Control experiments established that all of the radioactivity was solubilized under these conditions.

Extraction of [^{35}S]lipoic acid into toluene

Samples of ^{35}S-labelled enzyme (0.1 mg), to which had been added 10 μg of unlabelled lipoic acid, were hydrolysed in 0.2ml of 6M-HCl for various times. The hydrolysate was diluted to 5ml with water and 0.5ml of this solution was transferred to a stoppered test tube. To this was added 2.7ml of toluene containing 2,5-diphenyloxazole (5g/litre). The liquids were vortex-mixed for 30s and then centrifuged for 1min at 300*g*. The upper layer was carefully removed to a scintillation vial by means of a Pasteur pipette. This process was repeated several times, and finally the whole of the aqueous layer was transferred to another vial containing 4ml of toluene/Triton scintillant.

Results

Specific radioactivity of cysteine and methionine

A sample of ^{35}S-labelled protein (0.4mg) discarded during the enzyme purification was reduced and *S*-carboxymethylated and then hydrolysed with 6M-HCl for 24h. The hydrolysate was evaporated to dryness and subjected to amino acid analysis, samples of the effluent from the amino acid analyser being assayed for radioactivity by liquid-scintillation counting. As expected, only *S*-carboxymethylcysteine and methionine were found to be radiolabelled and their specific radioactivities were identical within the limits of experimental error (±3%). The absolute value, corrected for decay since the cells were grown, was 26.5±0.8mCi/mmol, which agrees well with the nominal specific radioactivity of the [^{35}S]sulphate (22mCi/mmol) on which the cells were originally grown.

Stoicheiometry of polypeptide chains in ^{35}S-labelled pyruvate dehydrogenase complex

If the subunit molecular weights and amino acid compositions of each component of the complex are known and the incorporation of ^{35}S radioactivity into each is measured, the molar ratio of polypeptide chains can readily be calculated (Bates *et al.*, 1975*a*), since for any two components i and j:

$$\frac{X_i}{X_j}=\frac{D_iS_j}{S_iD_j}$$

where

X_i = amount of component i (mol)

D_i = radioactivity (in d.p.m.) in component i

S_i = number of sulphur atoms per molecule of component i

Two different preparations of ^{35}S-labelled complex were used. Electrophoresis on sodium dodecyl sulphate/polyacrylamide gels demonstrated the presence of the three component enzymes. One such gel was cut transversely into uniform slices and the radioactivity in each slice was measured. As expected, radioactivity was associated only with the three protein bands (Fig. 1). Many such gels were run for each preparation of ^{35}S-labelled complex, the stained protein bands were cut out with a scalpel and the radioactivity in each band was determined. To calculate the sulphur content of the E2 component it was assumed that each chain contains three lipoyl

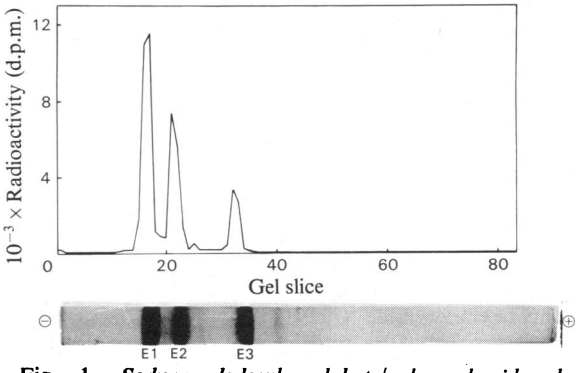

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of ^{35}S-labelled pyruvate dehydrogenase complex (approx. 15 μg)*

The gel was cut transversely into sections 0.8mm thick by using a device of multiple razor blades, and the measured radioactivity in each section is plotted above the gel. The polypeptide-chain stoicheiometry of this preparation, determined from 26 replicate gels, was 1.53:1:0.90 (E1/E2/E3). For other details, see the text.

Table 1. *Stoicheiometry of polypeptide chains in ^{35}S-labelled pyruvate dehydrogenase complex of E. coli*

Two different preparations of ^{35}S-labelled pyruvate dehydrogenase were made from separate cultures of the same strain of *E. coli* K12. The three polypeptide chains were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1). Measurement of the ratio of radioactivity incorporated into each protein band enabled the chain stoicheiometry to be calculated as described in the text, assuming three lipoyl groups per E2 chain and the subunit molecular weights and amino acid compositions reported by various workers ([a,b,c] in Table 2).

Sample	Number of gels run	Ratios of ^{35}S d.p.m. (S.D. in parentheses) (E1/E2/E3)	Chain stoicheiometry (E1/E2/E3)
1	4	1.68:1:0.53 (0.03) (0.03)	1.62:1:1.06[a] 1.39:1:0.88[b] 1.59:1:1.04[c]
2	26	1.59:1:0.45 (0.10) (0.05)	1.53:1:0.90[a] 1.32:1:0.75[b] 1.50:1:0.89[c]

groups (see the following sections) and that the specific radioactivity of the two sulphur atoms in each lipoic acid was the same as those in the cysteine and methionine residues (which had been shown to be identical).

The results are presented in Table 1. They have been calculated in three ways, by using in turn the amino acid analyses and subunit molecular weights of the component chains determined in this laboratory and elsewhere (Table 2). Chain ratios are expressed relative to E2 as unity because this component is thought to be the structural core of the complex (Reed & Oliver, 1968). The results of the three sets of calculations are in reasonable agreement and confirm previous conclusions (Vogel *et al.*, 1972; Bates *et al.*, 1975*a*) that there is a molar excess of E1 chains compared with E2 chains in the native complex, whereas the chain ratio of E3/E2 approaches unity. If the results were to be calculated by assuming only two lipoyl groups per E2 chain, the calculated E1/E2 and E3/E2 ratios would be about 8% smaller.

Lipoic acid content of pyruvate dehydrogenase complex

The availability of ^{35}S-labelled pyruvate dehydrogenase complex made it possible to determine the total lipoic acid content in a simple and direct way. Two methods were used, and both relied on the quantitative separation of lipoic acid from cysteine

Table 2. *Molecular weight and sulphur content of polypeptide chains in pyruvate dehydrogenase complex of E. coli*

Component	Mol.wt.	Sulphur atoms per molecule: Cysteine	Methionine	Lipoic acid (assumed)	Total	Reference
E1[a]	100000	7.1	21.5	0	28.6	Harrison (1974)
E2	80000	2.3	19.3	6	27.6	Harrison (1974)
E3	56000	4.5	9.3	0	13.8	Harrison (1974)
E1[b]	96000	6.9	20.8	0	27.7	Eley *et al.* (1972)
E2	70000	1.1	15.9	6	23.0	Eley *et al.* (1972)
E3	56000	4.5	9.3	0	13.8	Harrison (1974)
E1[c]	100000	5.1	20.1	0	25.2	Vogel & Henning (1971)
E2	83000	1.0	16.8	6	23.8	Vogel *et al.* (1971)
E3	56500	1.9	10.2	0	12.1	Vogel & Henning (1973); Vogel (1977)

[a,b,c]These three sets of analyses for sulphur content were used to calculate the polypeptide-chain ratios in Table 1.

and methionine in an acid hydrolysate of the complex. From the amino acid composition of each component and the polypeptide-chain stoicheiometry of the complex, the number of [^{35}S]lipoyl groups per E2 chain can then be calculated.

(*a*) *Performic acid oxidation and electrophoresis.* Samples of ^{35}S-labelled pyruvate dehydrogenase complex (0.1 mg) were oxidized with performic acid, hydrolysed in 6M-HCl for various times and submitted to electrophoresis on silica-gel thin-layer plates at pH 2.0 (Fig. 2). Three major radioactive spots were observed on the radioautogram, two of which had the same mobility as unlabelled markers of methionine sulphone and cysteic acid. The third component migrated towards the positive electrode more rapidly than cysteic acid and therefore was presumed to be 6,8-disulpho-octanoic acid, the product formed by performic acid oxidation of lipoic acid (Nawa *et al.*, 1960). It proved very difficult to locate the position of an authentic marker sample of this compound. Although it could be detected before electrophoresis as a yellow spot on a blue background after spraying the thin-layer plate with a 0.4% (w/v) solution of Bromophenol Blue in ethanol, severe streaking occurred when it was at a sufficiently high loading to permit detection after electrophoresis.

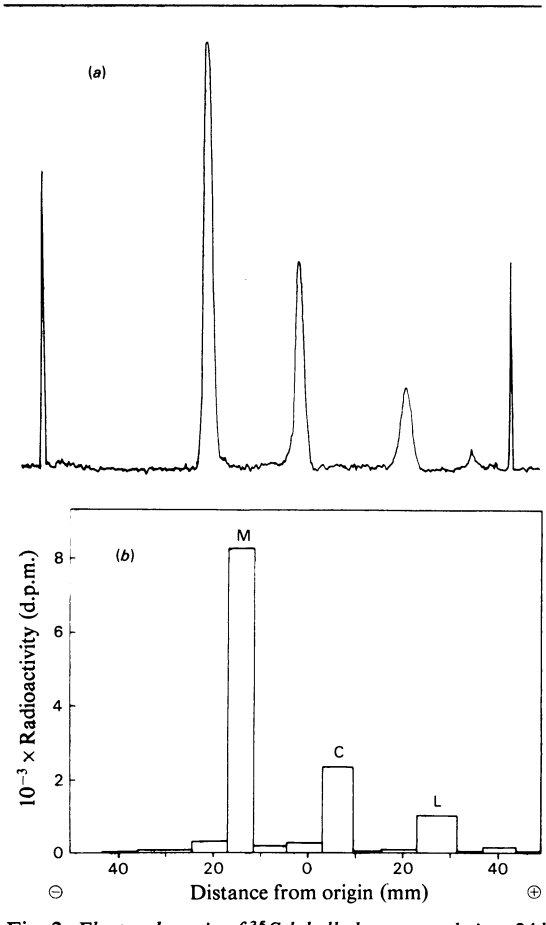

Fig. 2. *Electrophoresis of ^{35}S-labelled compounds in a 24h hydrolysate of pyruvate dehydrogenase complex after performic acid oxidation*

Samples were applied to the centre of a silica-gel thin-layer sheet and electrophoresed at pH 2.0. A radioautograph of the electrophoretogram was scanned with a densitometer (*a*). The silica sheet was cut into slices and the radioactivity in each was measured and plotted on the same scale (*b*). Abbreviations used: M, methionine sulphone; C, cysteic acid; L, 6,8-disulpho-octanoic acid (presumed, the oxidation product of lipoic acid). For other details, see the text.

However, when a sample of separated pyruvate decarboxylase (E1), which is thought not to contain lipoic acid, was subjected to the same procedure of oxidation, hydrolysis and electrophoresis, only two radiolabelled compounds, corresponding to methionine sulphone and cysteic acid, were detected, which strengthens the identification of the third radioactive spot from samples of intact complex as 6,8-disulpho-octanoic acid.

The ^{35}S-labelled pyruvate dehydrogenase complex used in this experiment had a chain stoicheiometry of 1.53:1:0.90 (E1/E2/E3) (Table 1). According to the amino acid analyses of Harrison (1974), this implies a total of 60.6 methionine residues and 17.2 cysteine residues in the complex per E2 chain (Table 2). The mean ratio of ^{35}S in the three compounds separated by electrophoresis was found to be 60.6:16.2 (±1.0):6.8 (±0.6) (methionine sulphone/cysteic acid/6,8-disulpho-octanoic acid). These results are the averages (±S.D.) of nine determinations over a range of hydrolysis times (7, 12 and 24h). The observed ratio of methionine sulphone to cysteic acid agrees with that predicted within experimental error. However, since each lipoic acid residue contains two sulphur atoms, the results indicate that there are 3.4 lipoyl groups per E2 chain. Recalculation from the amino acid analyses and subunit molecular weights reported by Reed and co-workers (values and references in Table 2) gives 2.8 lipoyl groups per E2 chain, whereas from the data of Henning and co-workers (values and references in Table 2) the result is 3.1 lipoyl groups per E2 chain.

(*b*) *Extraction with toluene.* An alternative method for determining the lipoic acid content of ^{35}S-labelled pyruvate dehydrogenase complex was based on the extraction of lipoic acid from an acid hydrolysate into an organic solvent. It has been shown that benzene would be suitable for this purpose (Mitra *et al.*, 1965), but a more convenient procedure was to use a toluene-based scintillant. The organic extract could then simply be transferred to a scintillation vial and the radioactivity determined direct. The partition coefficient of lipoic acid between toluene

and 0.24M-HCl was found to be 220 (±5), indicating that quantitative extraction by this system should be possible.

Samples (0.1 mg) of ^{35}S-labelled pyruvate dehydrogenase complex containing 10 μg of carrier unlabelled lipoic acid were hydrolysed in 6M-HCl for various times (24, 48 or 72h). After dilution of the acid to 0.24M-HCl, the hydrolysates were extracted several times with the toluene scintillant and the radioactivity in each extract was determined. At the end of the experiment the total radioactivity remaining in the aqueous layer was also measured. A typical set of results is shown in Fig. 3.

Although most of the extracted radiolabel was found in the first extract, the radioactivity in the organic phase was still just above background even after ten cycles of extraction. In a control experiment using [^{35}S]cysteine plus [^{35}S]methionine (prepared from an acid hydrolysate of protein from *E. coli* grown on [^{35}S]sulphate), a small amount (0.9%) of the total radioactivity was found in the combined first five extracts, but thereafter no more radioactivity was extracted. Therefore, in the calculations that follow, it is assumed that all of the radioactivity extracted from hydrolysates of the pyruvate dehydrogenase complex was due to [^{35}S]lipoic acid.

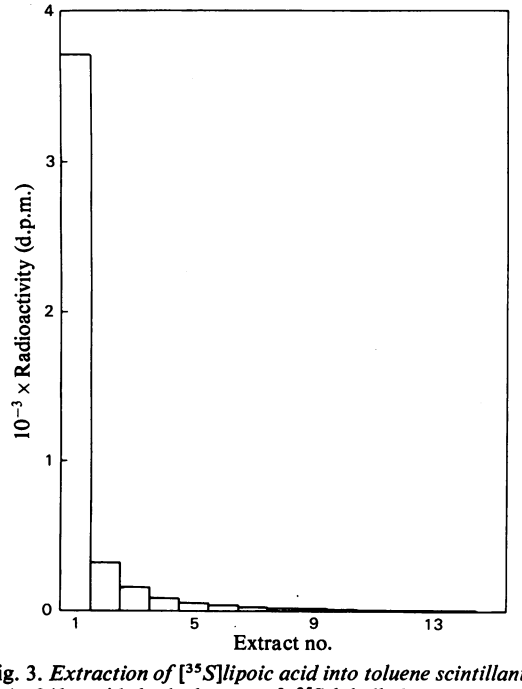

Fig. 3. *Extraction of [^{35}S]lipoic acid into toluene scintillant*
A 24h acid hydrolysate of ^{35}S-labelled pyruvate dehydrogenase complex was shaken with toluene scintillant. The radioactivity appearing in the organic layer at each extraction is plotted as a function of the number of cycles of extraction. The amount of radioactivity remaining in the aqueous layer at the end of the experiment was 48200d.p.m.

The mean total radioactivity in the first ten extracts as a fraction of that remaining in the aqueous layer (after correction for quenching) was 0.096±0.004. The complex used in this experiment had a polypeptide-chain stoicheiometry (E1/E2/E3) of 1.53:1:0.90 (see Table 1). On the basis of amino acid analyses and subunit molecular weights for each component determined in our laboratory (Table 2), this implies that there are 3.7 lipoyl groups per E2 chain. On the basis of data reported by Reed and colleagues and Henning and colleagues (for references, see Table 2), the result is 3.1 and 3.2 lipoyl groups per E2 chain respectively.

Discussion

The availability of pyruvate dehydrogenase complex from *E. coli* that is intrinsically radiolabelled has made it possible to answer two outstanding questions about the structure of the complex. There has been a continuing controversy about its polypeptide-chain stoicheiometry (Eley *et al.*, 1972; Vogel *et al.*, 1972; Bates *et al.*, 1975*a*). The method described here for measuring chain ratios is perhaps the simplest and most direct yet proposed. It does depend on the quantitative separation of each component by gel electrophoresis and would fail if any of them were proteolytically degraded. However, there was no evidence for any degradation of the enzyme preparations used here (Fig. 1). To calculate stoicheiometries it is necessary to know the amino acid composition and subunit molecular weight of each component, but fortunately there is reasonably good agreement between various workers about these properties (Table 2). Furthermore, we must assume that there is no 'isotope effect' in the biosynthesis of cysteine, methionine or lipoic acid that results in gross differences in their specific radioactivities. It was shown that the specific radioactivities of cysteine and methionine were identical. Any variation in that of lipoic acid would have a negligible effect on the calculated chain stoicheiometries.

The results obtained here are in excellent agreement with previous conclusions (Vogel *et al.*, 1972; Bates *et al.*, 1975*a*) that the E3/E2 chain ratio approaches unity, but that there exists a molar excess of E1 chains compared with E2 chains in the native complex.

There have been several measurements of the lipoic acid content of the complex. Chemical modification in the presence of the substrate, pyruvate, indicated that two lipoyl residues per E2 chain can become reductively acetylated during the normal enzymic reaction (Danson & Perham, 1976). This result has been confirmed by other measurements in

this laboratory (Bates *et al.*, 1977; Danson *et al.*, 1978*a*) and elsewhere (Collins & Reed, 1977; Speckhard *et al.*, 1977), although it should be noted that both the latter authors assumed in their calculations a structural model different from that which we have proposed. Previous direct measurements of the total lipoic acid content of the complex by incorporation of [^{35}S]lipoic acid have given conflicting results. Thus values of 8.5nmol/mg (Koike *et al.*, 1960), 12.3nmol/mg (Suzuki & Reed, 1963) and 5.5nmol/mg (Eley *et al.*, 1972) have been obtained. For comparison, the values that we have determined were 12.7nmol/mg (by performic acid oxidation and electrophoresis) and 13.8nmol/mg (by organic-solvent extraction). The major advantage of our methods is that only the ratio of radioactivity in lipoic acid to the sulphur-containing amino acids is measured and it is unnecessary to know its absolute specific radioactivity. The wide variation in the results taken from the literature may reflect, in part, variations in the subunit stoicheiometry of different enzyme preparations (Danson & Perham, 1976) or difficulties in ensuring complete incorporation of [^{35}S]lipoic acid (Collins & Reed, 1977).

It may seem that there is an element of circular argument in our calculation of polypeptide-chain stoicheiometry, which must adopt a value for the lipoic acid content because it contributes to the ^{35}S content of the E2 chain, yet measurement of the lipoic acid content also requires prior knowledge of the chain stoicheiometry. However, it can easily be seen that the same results are obtained if an arbitrary value for the lipoyl content is first assumed (say one or two lipoyl groups per E2 chain) and the approximate chain stoicheiometry thus calculated is then used to determine the actual lipoyl content. The effect of varying the assumed lipoyl content from two to three lipoyl groups per E2 chain on the calculated chain ratio is small, since this variation in lipoic acid content of the E2 chain is small compared with its total sulphur content.

The conclusion that we have reached is that there are at least three lipoyl groups per E2 chain, although we are not bound to accept that the number must be integral. Partial incorporation of lipoic acid at a susceptible lysine residue by the relevant activating enzyme (Leach, 1970) is possible. The assumptions that we have made must be considered carefully. It is conceivable that another sulphur-containing cofactor, or breakdown product of cysteine or methionine, may have behaved as lipoic acid during electrophoresis and extraction into organic solvent. Thiamin can be discounted, since it is an essential vitamin for the strain of bacteria used and is added to the growth medium. Therefore, whether or not the purified complex contains its cofactor thiamin pyrophosphate (non-covalently bound), the thiamin will not be radiolabelled. It is unlikely that CoA will remain bound to enzyme purified by the method that we describe, which includes gel filtration. Moreover, taurine, the ^{35}S-containing product of the performic acid oxidation and acid hydrolysis of ^{35}S-labelled CoA, would be electrophoretically separated from the measured compounds at pH2. The results obtained from control experiments argue against the possibility of contamination by breakdown products of the amino acids. The possible existence of a cofactor very similar to lipoic acid, however, cannot be completely discounted, for another biologically important compound containing the dithiolane ring, asparagusic acid, has been detected in other systems (Yanagawa & Egami, 1976).

Several observations in the literature suggest that the physical distance between the active sites of E1 and E3 is significantly greater than the 2.8nm that can be spanned by a single lipoic acid residue. These include small-angle X-ray-scattering studies (Durchschlag, 1975) and measurements of fluorescence energy transfer between suitable probes in the active sites of the various component enzymes (Moe *et al.*, 1974; Shepherd & Hammes, 1977). It has been shown that the lipoic acid residues on the E2 core that can be acetylated by the action of E1 are connected up by extensive intramolecular transacetylation reactions in a novel form of active-site coupling (Bates *et al.*, 1977; Danson *et al.*, 1978*a*). These reactions are kinetically competent in the normal mechanism of the enzyme (Danson *et. al.*, 1978*b*). However, since only two lipoic acid residues per E2 polypeptide chain can be acetylated in this way (Danson & Perham, 1976; Bates *et al.*, 1977; Collins & Reed, 1977; Speckhard *et al.*, 1977), the third lipoyl group that we describe here may act to pass reducing equivalents to the active site of E3, thereby spanning the gap between E1 and E3 suggested by other measurements. Further elucidation of the possible role of three lipoic acid residues in the enzymic reaction must await additional chemical modification studies and a reinvestigation of the amino acid sequence of lipoate acetyltransferase (E2).

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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-^{14}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 (1975*a*,*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