Evidence for Two Lipoic Acid Residues per Lipoate Acetyltransferase Chain in the Pyruvate Dehydrogenase Multienzyme Complex of *Escherichia coli*

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The reaction of two maleimides, *N*-ethylmaleimide and bis-(*N*-maleimidomethyl) ether, with the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* in the presence of the substrate, pyruvate, was examined. In both cases, the reaction was demonstrated to be almost exclusively with the lipoate acetyltransferase component, and evidence is presented to show that the most likely sites of reaction are the lipoic acid residues covalently bound to this component. With both reagents the stoichiometry of the reaction was measured: 2 mol of reagent reacted with each polypeptide chain of lipoate acetyltransferase, implying that each chain bears two functionally active lipoic acid residues. This observation can be reconciled with previous determinations of the lipoic acid content of the complex by allowing for the variability of the subunit polypeptide-chain ratio that can be demonstrated for this multimeric enzyme.

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

The complex is a multimeric structure composed of three 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 acetyltransferase component, E2, has a key role in the structure and the activity of the complex. It is probably composed of 24 polypeptide chains and forms the core of the complex to which enzymes E1 and E3 are non-covalently bound. The cofactor lipoic acid is covalently attached by an amide bond to a lysine residue in the E2 component. This cofactor carries the acyl group produced by the decarboxylation of pyruvate by enzyme E1 whence it is transferred to CoA at the active site on E2, thereby forming the product acetyl-CoA. This transfer reaction generates the reduced form of the lipoic acid whose reoxidation is brought about by the lipoamide dehydrogenase component, E3. Thus the lipoic acid is thought to rotate among the catalytic centres of the three enzymes in the complex as part of the mechanism, and studies of the mobility of spin-labelled lipoic acid are consistent with this view (Ambrose & Perham, 1976).

In order to describe the mechanism of the enzyme more precisely, it is essential to know the amount of lipoic acid in the complex. Initial estimates indicated 1 lipoyl residue per acetyltransferase component (assumed mol.wt. 35000) (Koike *et al.*, 1963), although a revised determination of one residue per

polypeptide chain of E2 (assumed mol.wt. 70000) has since been reported (Eley *et al.*, 1972). Brown & Perham (1976) have devised a method for the specific modification of the lipoic acid in the *E. coli* complex with *N*-ethylmaleimide, and their preliminary studies indicated the incorporation of 2 mol of this reagent per acetyltransferase chain (assumed mol.wt. 80000).

In the present paper, we describe the use of two maleimides, *N*-ethyl[2,3-¹⁴C]maleimide and the bifunctional bis-(*N*-maleimidomethyl) ether, to modify the lipoic acid of the pyruvate dehydrogenase complex of *E. coli*. The incorporation of the former reagent was measured direct whereas the incorporation of the bismaleimide was measured by titration of the free unchanged maleimide group with [3-¹⁴C]-cysteine. Since we are now able to determine accurately the stoichiometry of the enzymes in the complex (Bates *et al.*, 1975), we can calculate that 2 mol of maleimide react per chain of E2. Assuming that the site of maleimide reaction is enzyme-bound lipoic acid, we infer that each E2 chain bears two functionally active lipoic acid residues.

Materials and Methods

Reagents

Pyruvate (monosodium salt), NAD⁺ (free acid, grade 2) and CoA (grade 1) were obtained from C. F. Boehringer und Soehne, Mannheim, W. Germany; thiamin pyrophosphate was from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; DL-dihydrolipoamide was a kind gift of G. Hale (Department of Biochemistry, University of Cambridge, Cambridge, U.K.) and *N*-ethyl[2,3-¹⁴C]maleimide (CFA.293) and DL-[3-¹⁴C]cysteine hydrochloride (CFA.332) were from The Radiochemical Centre, Amersham,

Bucks., U.K. Bis-(*N*-maleimidomethyl) ether was the kind gift of Dr. S. Simon (State University of New York, Stony Brook, NY, U.S.A.).

Enzyme

The organism used in this work was a mutant of *Escherichia coli* K12 constitutive for pyruvate dehydrogenase, kindly provided by Professor H. L. Kornberg of this Department. Cells were grown on MRE-162 medium containing 1% glycerol and were obtained as a deep-frozen homogenate from the Microbiological Research Establishment, Porton Down, Wilts., U.K. The pyruvate dehydrogenase complex was purified from this homogenate essentially as described by Reed & Mukherjee (1969). Two separate preparations of enzyme were used, one for the experiments with *N*-ethylmaleimide and the other for those with bis-(*N*-maleimidomethyl) ether.

Enzyme assays

The overall activity of the pyruvate dehydrogenase complex was assayed spectrophotometrically at 340 nm and 30°C by a slight modification of the method of Reed & Mukherjee (1969). Assay mixtures contained 50 mM-potassium phosphate, pH 8.0, 2.5 mM-NAD⁺, 0.2 mM-thiamin pyrophosphate, 1.0 mM-MgCl₂, 0.13 mM-CoA, 2.6 mM-cysteine hydrochloride and 2.0 mM-sodium pyruvate. The assay, in a final volume of 1 ml, was started with enzyme, and the reaction followed by the increase in E_{340} .

The lipoamide dehydrogenase activity of the complex was assayed at 30°C in 50 mM-potassium phosphate, pH 8.0, 2.5 mM-NAD⁺, 0.2 mM-thiamin pyrophosphate, 1.0 mM-MgCl₂ and 0.4 mM-dihydrolipoamide. Again, the assay was started with enzyme and the reaction followed by the increase in E_{340} .

Modification of pyruvate dehydrogenase complex with maleimides

Inhibition with maleimides, *N*-ethyl[2,3-¹⁴C]maleimide and bis-(*N*-maleimidomethyl) ether, was carried out in the presence of pyruvate as described by Brown & Perham (1976).

(i) *N*-Ethyl[2,3-¹⁴C]maleimide. Pyruvate dehydrogenase complex (3 mg/ml) was incubated at 0°C with 0.3 mM-*N*-ethyl[2,3-¹⁴C]maleimide in 50 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-sodium pyruvate, 0.5 mM-thiamin pyrophosphate, 5 mM-MgCl₂ and 1 mM-NAD⁺. When whole complex activity had been inhibited more than 95%, modification was stopped by the addition of 2-mercaptoethanol to a final concentration of 10 mM. Enzyme was then separated from unbound radioactive label by gel filtration on a column (22 cm × 1 cm) of Sephadex G-25 equilibrated with 10 mM-*N*-ethylmorpholine/acetic acid buffer, pH 8.0, and the fractions containing protein were pooled.

(ii) Bis-(*N*-maleimidomethyl) ether. Modification of

enzyme (3 mg/ml) was carried out with 0.3 mM-bis-(*N*-maleimidomethyl) ether as described for *N*-ethylmaleimide. However, before the addition of 2-mercaptoethanol, the bismaleimide was allowed to react completely by treatment with a fourfold molar excess of DL-[3-¹⁴C]cysteine hydrochloride. The enzyme was again separated from unbound radioactive label by gel filtration on Sephadex G-25.

Protein concentration

The approximate protein concentrations of the two modified enzyme preparations were estimated spectrophotometrically, assuming $E_{280}^{1\%} = 9.87$ (Harrison, 1974). Accurate protein concentrations were determined by amino acid analysis with a Beckman 120C analyser. Samples of protein were hydrolysed for 48 h at 105°C with 6M-HCl containing 0.03% (v/v) 2-mercaptoethanol in sealed evacuated tubes. The quantities of the amino acids aspartic acid, glutamic acid, glycine, alanine, isoleucine and phenylalanine were used in the calculation of the protein concentrations, as the sum of their amounts per mg of pyruvate dehydrogenase complex is independent of the stoichiometry of the three component enzymes (Harrison, 1974).

Stoichiometry of the polypeptide chains

The stoichiometry of the polypeptide chains in the pyruvate dehydrogenase complex was determined by the amidination method of Bates *et al.* (1975).

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). Location of radioactively labelled protein in the gels by radioautography and the measurement of the radioactivity in the three protein bands were performed as described by Brown & Perham (1976).

Specific radioactivities of *N*-ethyl[2,3-¹⁴C]maleimide and DL-[3-¹⁴C]cysteine

(i) *N*-Ethylmaleimide. *N*-Ethyl[2,3-¹⁴C]maleimide was converted into *S*-[(1,2-dicarboxy)ethyl]cysteine and the product quantified by amino acid analysis in the following way.

The maleimide was allowed to react with a sixfold molar excess of cysteine hydrochloride in 0.2M-*N*-ethylmorpholine/acetic acid buffer, pH 8.0, at 0°C. After 15 min, the reaction mixture was freeze-dried and hydrolysed in 6M-HCl containing 0.03% (v/v) 2-mercaptoethanol in sealed evacuated tubes for 72 h at 105°C. The hydrolysed sample was then subjected to chromatography on a Beckman 120C amino acid analyser, and the effluent from the column was collected. The radioactivity in the eluted *S*-[(1,2-dicarboxy)ethyl]cysteine peak was measured as

described below. The colour constant for this cysteine derivative was taken as 90% of the value for aspartic acid (D. G. Smyth & W. H. Konigsberg, personal communication).

(ii) DL-[3-¹⁴C]Cysteine. DL-[3-¹⁴C]Cysteine was converted into *S*-carboxymethyl[3-¹⁴C]cysteine, and the product quantified by amino acid analysis by the following method.

DL-[3-¹⁴C]Cysteine was allowed to react, at 0°C under N₂, with a 10-fold molar excess of iodoacetic acid in 0.1M-*N*-ethylmorpholine/acetic acid buffer, pH 8.0, containing 2mM-dithiothreitol. After incubation for 1 h in the dark, the sample was freeze-dried and chromatographed on the Beckman 120C amino acid analyser. The effluent from the column was collected and the radioactivity in the *S*-carboxymethylcysteine peak measured as described below.

Radioactivity measurements

Samples were added to 3.0ml of toluene/Triton (2:1, v/v) containing 2,5-diphenyloxazole (5 g/l) and counted for radioactivity in a Nuclear-Chicago Unilux II-A scintillation counter. Corrections for quenching of the samples were calculated from a calibration curve constructed by using a series of sealed quenched standards (Amersham/Searle, Des Plaines, IL, U.S.A.).

Results

Modification with *N*-ethyl[2,3-¹⁴C]maleimide

Treatment of the pyruvate dehydrogenase complex with *N*-ethylmaleimide in the presence of pyruvate produced a rapid loss of whole complex activity, whereas the lipoamide dehydrogenase activity (E3) remained completely unaffected (Fig. 1). At the time of addition of 2-mercaptoethanol to stop the modification, only 3% of the initial overall activity remained. As reported by Brown & Perham (1976), the inactivation followed pseudo-first-order kinetics.

The reaction mixture was gel-filtered on Sephadex G-25 and the protein concentration and incorporation of radioactivity into the enzyme were determined. The ratio of the three types of polypeptide chain in the complex was measured by means of the amidination technique (Bates *et al.*, 1975) in a separate experiment with untreated enzyme. The results are given in Table 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the inactivated enzyme, followed by scintillation counting of the radioactivity in the stained protein bands, demonstrated that 91% (±2%) of the radioactive label had been incorporated into the lipoate acetyltransferase component, E2. Radioautography of the polyacrylamide gels before and after fixing and staining confirmed this specificity of incorporation.

Gel filtration of the modified complex on a column (12cm×0.6cm) of Sephadex G-75 equilibrated with

Fig. 1. Effects of *N*-ethylmaleimide and of bis-(*N*-maleimidomethyl) ether on the activities *E. coli* of pyruvate dehydrogenase complex in the presence of pyruvate

Treatment with *N*-ethyl[2,3-¹⁴C]maleimide and with bis-(*N*-maleimidomethyl) ether in the presence of pyruvate was carried out as described in the text. Overall complex activity: ○, with 0.3 mM-*N*-ethyl[2,3-¹⁴C]maleimide; △, with 0.3 mM-bis-(*N*-maleimidomethyl) ether. Lipoamide dehydrogenase activity: ●, with 0.3 mM-*N*-ethyl[2,3-¹⁴C]maleimide; ▲, with 0.3 mM-bis-(*N*-maleimidomethyl) ether.

50mM-sodium phosphate buffer, pH 7.0, containing 0.1% (w/v) sodium dodecyl sulphate, demonstrated that all the radioactive label was firmly associated with the protein and that none was merely trapped within the structure of the multienzyme complex. Neither boiling in 1% (w/v) sodium dodecyl sulphate with 1% (v/v) 2-mercaptoethanol nor incubation in 25% (w/v) trichloroacetic acid at 30°C for 4 h removed the radioactivity from the protein, as judged by subsequent gel filtration on Sephadex G-75 as above. It was thus concluded that the *N*-ethylmaleimide had reacted with a covalently attached moiety of the acetyltransferase component and not with a dissociable cofactor of the complex.

The specific radioactivity of the *N*-ethyl[2,3-¹⁴C]maleimide, determined as the *S*-[(1,2-dicarboxy)ethyl]cysteine derivative, was 9.0 (±2%) μCi/μmol. Thus, from the results in Table 1, we calculate that 2.03 (±0.15) mol of *N*-ethylmaleimide was incorporated per acetyltransferase chain (subunit mol.wt. 80000).

Several observations, similar to those made by Brown & Perham (1976), support the conclusion that the site of reaction of *N*-ethylmaleimide in the acetyltransferase is the lipoic acid.

(1) Treatment of the complex with *N*-ethylmale-

Table 1. *Measurement of the substrate-induced reaction of N-ethylmaleimide and bis-(N-maleimidomethyl) ether with the pyruvate dehydrogenase multienzyme complex of E. coli*

The multienzyme complex was treated with the appropriate maleimide (0.3 mM) in the presence of pyruvate (1.0 mM) in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.5 mM-thiamin pyrophosphate, 5 mM-MgCl₂ and 1.0 mM-NAD⁺, at 0°C. The overall pyruvate dehydrogenase activity was 97% inhibited when the reaction was terminated. For other experimental details, see the text. Results are means $\pm$ S.D. of five determinations.

	<i>N</i> -Ethylmaleimide	Bis-(<i>N</i> -maleimidomethyl) ether
Protein concentration (mg/ml)	0.95 ($\pm 5\%$)	0.58 ($\pm 7\%$)
Specific radioactivity of reagent (d.p.m./ μ mol)		
(1) <i>N</i> -Ethyl[2,3- ¹⁴ C]maleimide	20.0×10^6 ($\pm 2\%$)	—
(2) DL-[3- ¹⁴ C]Cysteine	—	12.9×10^6 ($\pm 7\%$)
Counts incorporated into protein (d.p.m./ml)	145000 ($\pm 1\%$)	53170 ($\pm 1\%$)
Percentage of total radioactivity incorporated into lipoate acetyl-transferase component	91% ($\pm 2\%$)	90% ($\pm 5\%$)
Polypeptide-chain stoichiometry		
E ₁	1.69 ($\pm 2\%$)	1.71 ($\pm 0.6\%$)
E ₂	1.0	1.0
E ₃	1.03 ($\pm 1\%$)	0.96 ($\pm 1\%$)
Calculated number of lipoic acid residues per E ₂ polypeptide chain	2.03 (± 0.15)	2.14 (± 0.21)

imide in the absence of pyruvate resulted in no detectable loss of either whole complex or lipoamide dehydrogenase enzymic activities. Moreover, the incorporation of the radioactive label into the acetyl-transferase under these conditions was less than 5% of that observed in the presence of the substrate.

(2) On chromatography of the acid hydrolysate of the modified protein on the amino acid analyser, the radioactive products were eluted well before aspartic acid, in the region expected for S⁸-(1,2-dicarboxy)ethyl]lipoic acid (Brown & Perham, 1976). Unlabelled S-[(1,2-dicarboxy)ethyl]cysteine, prepared from *N*-ethylmaleimide and cysteine as described above, was chromatographed simultaneously with the protein; less than 5% of the radioactivity from the protein was eluted coincidentally with this cysteine derivative. Brewer & Riehm (1967) report that *N*-ethylmaleimide may slowly react with lysine and histidine residues under relatively vigorous conditions (pH 7.0, 25°C, 0.1 M-*N*-ethylmaleimide). The products of such reactions yield *N*-[(1,2-dicarboxy)ethyl]-lysine and 1- or 3-[(1,2-dicarboxy)ethyl]histidines respectively on acid hydrolysis and their elution positions on amino acid analysis are well characterized (Brewer & Riehm, 1967). No radioactivity from the modified complex coincided with the known positions of these derivatives.

(3) Further evidence for the selective modification of the lipoic acids by maleimides comes from spin-label studies of the enzyme complex modified with 4-maleimido-2,2,6,6-tetramethylpiperidinoxyl (Ambrose & Perham, 1976). The introduction of this maleimide into the complex in the presence of

pyruvate is directly competitive with *N*-ethylmaleimide, and the e.s.r. (electron-spin-resonance) spectra show the product to be a highly mobile moiety, as expected for a lipoyl-lysine residue. The spectra strongly suggest that there is no modification of groups other than the lipoic acid.

Modification with bis-(N-maleimidomethyl) ether

Treatment of *E. coli* pyruvate dehydrogenase complex with bis-(*N*-maleimidomethyl) ether in the presence of pyruvate resulted in a rapid loss of overall complex activity with little effect on the lipoamide dehydrogenase component (Fig. 1). Again, the inactivation was a pseudo-first-order process and, as might reasonably be expected, the rate of loss of activity produced by the bismaleimide was approximately twice that observed with equimolar amounts of the monofunctional *N*-ethylmaleimide. The incorporation of bis-(*N*-maleimidomethyl) ether into the complex was measured by reaction of the free maleimide group of the reagent with [3-¹⁴C]cysteine, followed by estimation of the protein concentration and scintillation counting, as described above. These results are also given in Table 1.

Scintillation counting of the radioactivity in the three component enzymes after separating them by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis demonstrated that 90% ($\pm 5\%$) of the radioactive label incorporated into the complex resided in the acetyltransferase. This observation was confirmed by radioautography of the polyacrylamide gels. Knowing the ratio of polypeptide chains in the complex, it was calculated that 2.14 (± 0.21) mol of

bis-(*N*-maleimidomethyl) ether were incorporated per acetyltransferase chain (subunit mol.wt. 80000).

It should be pointed out that the acid treatment used for fixing the polyacrylamide gels after electrophoresis [immersion overnight in 25% (w/v) trichloroacetic acid] was sufficient to cause hydrolysis of the incorporated bismaleimide such that all the radioactivity was lost from the protein band in this process. Therefore to locate the site(s) of incorporation of [^{14}C]cysteine, the polyacrylamide gels were sliced longitudinally immediately after electrophoresis; one half was stained for protein and the other sliced laterally into 1 mm pieces for scintillation counting.

An advantage of this acid hydrolysis of the bismaleimide was that it permitted the determination of the stoichiometry of the complex both before and after the modification process. Native and modified enzymes were amidinated by the method of Bates *et al.* (1975) and the proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After the gels had been fixed in trichloroacetic acid, the radioactivity in the protein bands was due entirely to the incorporated [^{14}C]amidino groups. Thus the stoichiometry remained completely unchanged during the inactivation, and no cross-linking of the proteins in the complex by the bismaleimide had taken place.

The dependence on the presence of pyruvate of the incorporation of bis-(*N*-maleimidomethyl) ether into the lipoate acetyltransferase was observed to be the same as that with *N*-ethylmaleimide. This, and the similarity of the kinetics of incorporation to those observed with other maleimides (Ambrose & Perham, 1976; Brown & Perham, 1976), point to the specific reaction of the bismaleimide with the lipoic acid residues of the acetyltransferase.

Discussion

The interpretation of the present results turns on the specificity of the reaction of maleimides with enzyme-bound *S*⁶-acetyldihydrolipoamide formed by the pyruvate dehydrogenase complex in the presence of pyruvate and absence of CoA. All the evidence presented here and in previous experiments (Brown & Perham, 1976; Ambrose & Perham, 1976) is compatible with the view that this reaction is highly specific under the conditions used. The second important factor is an accurate knowledge of the stoichiometry of the three types of polypeptide chain that comprise the complex. For this investigation we have used the amidination method introduced by Bates *et al.* (1975) and results have been obtained with a high level of internal consistency. On the basis of these methods, therefore, the results summarized in Table 1 imply that each lipoate acetyltransferase

chain carries two functionally active lipoic acid residues.

It is necessary to consider our conclusion in the light of previous work from other laboratories. First, on the question of polypeptide chain stoichiometry, it has been proposed that the pyruvate dehydrogenase complex of *E. coli* (Crookes strain) consists of 24 chains of pyruvate decarboxylase, 24 chains of lipoate acetyltransferase and 12 chains of lipoamide dehydrogenase (Eley *et al.*, 1972), although an increased content of enzyme E1 at the expense of a decreased content of enzyme E3 has been reported (Reed *et al.*, 1975). These calculations have depended, in part, on assays of the amount of coenzyme (e.g. FAD) bound per mg of multienzyme complex as a measure of the polypeptide chain responsible for binding it (Eley *et al.*, 1972). On the other hand, direct measurements of the three component proteins by gel scanning after separating them by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis have indicated that the chain stoichiometry (E1/E2/E3) in the complex is 1:1:1, although occasionally higher ratios of E1/E2 (up to 1.3:1) were found (Vogel *et al.*, 1972). Our own results, which are also direct measurements of the component enzyme proteins and not of cofactors, are more consistent with these findings of Vogel *et al.* (1972), as explained elsewhere (Bates *et al.*, 1975). It should be added that we have used subunit mol.wts. of 100000, 80000 and 56000 for enzymes E1, E2 and E3 respectively, as reported by Perham & Thomas (1971) and Vogel *et al.* (1972), whereas Eley *et al.* (1972) have determined values of 96000, 70000 and 56000 for the same enzymes. These differences are small enough to be disregarded for our present purposes and they tend to cancel out in the calculation of chain ratios.

Several attempts to measure the lipoic acid content of the pyruvate dehydrogenase complex have been reported. Koike *et al.* (1963) originally proposed a value of one lipoic acid residue per acetyltransferase component, assuming then a subunit mol.wt. of 35000. However, in revising the subunit mol.wt. of enzyme E2 upwards to 70000, Eley *et al.* (1972) have revised downwards their estimate of the lipoic acid content, to one residue per E2 chain. Their most recent value, of approx. 5.5 nmol of lipoic acid per mg of complex (Eley *et al.*, 1972), implies 1 μmol of lipoic acid per 180 mg of protein. This does not differ greatly from our own values of 1 μmol of lipoic acid per 152 mg of protein, calculated from the results in Table 1. Since variability in the E1 content of the pyruvate dehydrogenase complex is now well documented (Vogel *et al.*, 1972; Bates *et al.*, 1975) and can be accounted for in terms of symmetry-imposed limits for the assembled complex (Perham, 1975; Bates *et al.*, 1975), it seems likely that variability in the subunit composition of the complexes in use in different laboratories will be sufficient to explain the

discrepancies. For example, if the complex has the chain stoichiometry (E1/E2/E3) of 2:1:1 and each E2 chain carries two lipoic acid residues, the lipoic acid content would be one residue per 170 mg of protein. If the chain stoichiometry fell to 1:1:1, the lipoic acid content would be one residue per 120 mg of protein.

When we turn to the FAD content of the complex, it is again possible to explain the various answers given in the literature by invoking variability of the polypeptide chain stoichiometry in the different enzyme preparations used. Thus Speckhard & Frey (1975) and Eley *et al.* (1972) give values of approx. 2.4–2.8 nmol of FAD per mg of protein, whereas Vogel *et al.* (1972) report a value of approx. 4.4 nmol of FAD per mg of complex. The former value (1 μ mol per 370 mg of protein) would be reasonably consistent with one FAD per E3 chain in a complex that had chain stoichiometry (E1/E2/E3) of 2:1:1, whereas the latter value (1 μ mol FAD per 230 mg of protein) is that expected for a complex with chain stoichiometry of 1:1:1. Since this was the chain stoichiometry actually determined by Vogel *et al.* (1972) for the preparation of the complex in question, that lends support to their value, there being no disagreement that one chain of E3 is capable of binding very strongly 1 mol of FAD (Koike *et al.*, 1963; Williams, 1965; Vogel *et al.*, 1972). Any loss of E3 component would, of course, further distort the values for any particular preparation of pyruvate dehydrogenase complex.

An important conclusion of Eley *et al.* (1972) is that the FAD content of the complex is half that of the lipoic acid content. This might be regarded as evidence that the molar chain ratio of E2/E3 is 1:0.5. However, it is equally consistent with the results of our present work that imply two lipoic acid residues per E2 chain and a chain ratio for E2/E3 of 1:1 determined by different (but direct) methods in two separate laboratories (Vogel *et al.*, 1972; Bates *et al.*, 1975). In this connexion, it may well be functionally significant that the E2/E3 subcomplex is capable of binding a molar excess of E1 chains (Vogel *et al.*, 1972; Bates *et al.*, 1975) and that the limiting chain stoichiometry appears to be 2:1:1 (Bates *et al.*, 1975; Perham, 1975). This would imply an active-site stoichiometry of 2:2:1, i.e. equivalence of active sites on E1 and E2, the molecular meaning of which in terms of polypeptide-chain arrangement now needs to be investigated.

The experiments of Reed and his colleagues have largely been carried out with *E. coli* (Crookes strain) (Eley *et al.*, 1972; Reed *et al.*, 1975), those of Vogel *et al.* (1972) with regulatory mutants of *E. coli* K12, whereas Speckhard & Frey (1975) and our own laboratory (Bates *et al.*, 1975) have used *E. coli* Crookes strain and *E. coli* K-12. However, the evidence available to us at present suggests that there are no differences of structural principle between these

enzymes from different sources (Speckhard & Frey, 1975; Bates *et al.*, 1975). The discrepancies in subunit composition are best accounted for by losses of subunits during purification of the complex or by the actual presence in the cell of multienzyme complexes in different stages of assembly before extraction (Perham, 1975). The covalent attachment of lipoic acid to the E2 component is catalysed by an enzyme (Reed *et al.*, 1958), and the results of our present investigation indicate that all potential lipoic acid-binding sites on the E2 component have been filled. The experiments of Grande *et al.* (1975), in which lipoic acid was found attached to two different polypeptide chains identified as lipoate transacetylase components in the pyruvate dehydrogenase complex of *Azotobacter vinelandii*, emphasize the need for a fresh look at the details of the reaction mechanism of these enzymes.

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