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Selective Inactivation of the Transacylase Components of the 2-Oxo Acid Dehydrogenase Multienzyme Complexes of *Escherichia coli*

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1. The reaction of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* with maleimides was examined. In the absence of substrates, the complex showed little or no reaction with *N*-ethylmaleimide. However, in the presence of pyruvate and *N*-ethylmaleimide, inhibition of the pyruvate dehydrogenase complex was rapid. Modification of the enzyme was restricted to the transacetylase component and the inactivation was proportional to the extent of modification. The lipoamide dehydrogenase activity of the complex was unaffected by the treatment. The simplest explanation is that the lipoyl groups on the transacetylase are reductively acetylated by following the initial stages of the normal catalytic cycle, but are thereby made susceptible to modification. Attempts to characterize the reaction product strongly support this conclusion. 2. Similarly, in the presence of *N*-ethylmaleimide and NADH, much of the pyruvate dehydrogenase activity was lost within seconds, whereas the lipoamide dehydrogenase activity of the complex disappeared more slowly: the initial site of the reaction with the complex was found to be in the lipoyl transacetylase component. The simplest interpretation of these experiments is that NADH reduces the covalently bound lipoyl groups on the transacetylase by means of the associated lipoamide dehydrogenase component, thereby rendering them susceptible to modification. However, the dependence of the rate and extent of inactivation on NADH concentration was complex and it proved impossible to inhibit the pyruvate dehydrogenase activity completely without unacceptable modification of the other component enzymes. 3. The catalytic reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) by NADH in the presence of the pyruvate dehydrogenase complex was demonstrated. A new mechanism for this reaction is proposed in which NADH causes reduction of the enzyme-bound lipoic acid by means of the associated lipoamide dehydrogenase component and the dihydrolipoamide is then oxidized back to the disulphide form by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). 4. A maleimide with a relatively bulky *N*-substituent, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, was an effective replacement for *N*-ethylmaleimide in these reactions with the pyruvate dehydrogenase complex. 5. The 2-oxoglutarate dehydrogenase complex of *E. coli* behaved very similarly to the pyruvate dehydrogenase complex, in accord with the generally accepted mechanisms of the two enzymes. 6. The treatment of the 2-oxo acid dehydrogenase complexes with maleimides in the presence of the appropriate 2-oxo acid substrate provides a simple method for selectively inhibiting the transacylase components and for introducing reporter groups on to the lipoyl groups covalently bound to those components.

The pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multienzyme complexes of *Escherichia coli* are each multimeric structures (about 30nm across) that comprise three different types of polypeptide chain responsible for the three component enzymic activities of the complexes [for reviews see Reed & Oliver (1968) and Reed (1974)]. These three enzymes, in order of their participation in the commonly accepted mechanism, are a decarboxylase (E1), a lipoyl transacylase (E2) and lipoamide dehydrogenase (E3). Enzymes E1 and E2 are specific for their respective complexes and cannot be interchanged, whereas enzyme E3 from either complex can be used to assemble active complexes (Pettit & Reed, 1967). The available chemical evidence suggests, in fact, that the E3 components of the two complexes are identical (Brown & Perham, 1973; J. P. Brown, R. A. Harrison & R. N. Perham, unpublished work).

Disulphide bridges play an essential part in the catalytic mechanism of the complexes. The cofactor lipoic acid, covalently bound to a lysine residue

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in enzyme E2, carries the acyl group by a thiol ester linkage and shuttles between oxidized and reduced forms, and the oxidation of the reduced form is brought about by the lipoamide dehydrogenase component, at whose active site is to be found a disulphide bridge that is alternately reduced and oxidized as part of the mechanism.

The disulphide bridge of the free lipoamide dehydrogenase component has provided a convenient starting point for chemical studies of the active site of this enzyme (Brown & Perham, 1972, 1974; Burleigh & Williams, 1972). However, it is important also to consider the properties of the component enzymes when assembled into the intact complexes. In the present paper we describe the reaction of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase multienzyme complexes with various maleimides and with Nbs_2 [5,5'-dithiobis-(2-nitrobenzoic acid)]. We show that the substrates, pyruvate, 2-oxoglutarate and NADH, can potentiate selective reaction of the enzyme-bound lipoic acid with the maleimides and we propose a new mechanism for the catalytic reduction of Nbs_2 by NADH brought about by both multienzyme complexes, a reaction first described for the 2-oxoglutarate dehydrogenase complex of ox heart by Erfle & Sauer (1968). These observations can all be interpreted in terms of the generally accepted mechanism of the intact complexes (Reed & Oliver, 1968) and demonstrate at a structural level the co-ordination of successive reactions catalysed by the component enzymes.

Materials and Methods

Reagents

Iodoacetic acid and Nbs_2 were from BDH Chemicals Ltd., Poole, Dorset, U.K. *N*-Ethylmaleimide was from Sigma Chemical Co., St. Louis, MO, U.S.A., *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide from Ralph N. Emanuel Ltd., Alperton, Middx., U.K., and 2-mercaptoethanol from Eastman-Kodak Ltd., Kirkby, Lancs., U.K. Iodoacetic acid was purified by recrystallization from *n*-heptane.

NAD^+ (free acid, grade 2), NADH (disodium salt, grade 2) and CoA (grade 1) were from C. F. Boehringer und Soehne, Mannheim, W. Germany. Thiamin pyrophosphate was from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and DL-dihydrolipoamide was a kind gift of Dr. M. Riley (Department of Biochemistry, University of Cambridge, U.K.).

N-Ethyl[2,3-^{14}C]maleimide (CFA.293), specific radioactivity 4.8mCi/mmol, dissolved in pentane, was from The Radiochemical Centre, Amersham, Bucks., U.K. The pentane was removed in a gentle stream of N_2, and unlabelled *N*-ethylmaleimide was added to lower the specific radioactivity to the required value. The maleimide was dissolved in ethanol before use.

Enzymes

The pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes of *E. coli* were purified as described by Reed & Mukherjee (1969). The pyruvate dehydrogenase complex was prepared from a mutant of *E. coli* K12 constitutive for pyruvate dehydrogenase kindly provided by Professor H. L. Kornberg and had a specific activity of about 15units/mg in the pyruvate dehydrogenase assay and about 30units/mg in the lipoamide dehydrogenase assay. The 2-oxoglutarate dehydrogenase complex was prepared from *E. coli* (Crookes strain, grown on pyruvate) and had a specific activity of about 15units/mg in the 2-oxoglutarate dehydrogenase assay and about 110units/mg in the lipoamide dehydrogenase assay.

Lipoamide dehydrogenase and the transacetylase–lipoamide dehydrogenase sub-complex were prepared from pyruvate dehydrogenase complex by slight modification (Harrison, 1974) of the methods of Koike *et al.* (1963).

Enzyme assays

The overall activities of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (hereafter called the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities) were assayed by a slight modification of the method of Reed & Mukherjee (1969). Assays were carried out at 30°C in a final volume of 1ml of potassium phosphate buffer (50mM, pH8.0) containing NAD^+ (2.5mM), thiamin pyrophosphate (0.2mM), $MgCl_2$ (1.0mM), CoA (0.13mM), cysteine (2.6mM) and sodium pyruvate (or sodium 2-oxoglutarate) (2.0mM). The assay was started with enzyme and the reaction followed by the increase in E_{340}.

The lipoamide dehydrogenase activity of the enzyme complexes was assayed at 30°C in potassium phosphate buffer (50mM, pH8.0) containing NAD^+ (2.5mM), thiamin pyrophosphate (0.2mM) and dihydrolipoamide (0.25mM). The assay (in a final volume of 1ml) was started with enzyme and the reaction followed by the increase in E_{340} as before.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Samples of protein were run in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (Shapiro & Maizel, 1969) as described by Perham & Thomas (1971). Radiolabelled proteins

in the gels were located by radioautography: the gels were sliced longitudinally, placed on a sheet of chromatography paper (Whatman 3 MM), covered on the top side with a thin plastic film and dried *in vacuo* on an electrically heated block. The dried gels were then placed in contact with Kodak Blue Brand X-ray film and left in the dark for several weeks before the film was developed.

Radioactivity measurements

The radioactivity in ^{14}C-labelled proteins present as Coomassie Blue-stained bands in polyacrylamide gels was measured essentially as described by Tishler & Epstein (1968). The bands were excised from the gel, the gel slices placed in scintillation vials and then dried *in vacuo*. The dried gels were dissolved by incubation overnight at 40°C with 0.1ml of 100-vol. H_2O_2. Scintillant [3.0ml of toluene/Triton X-100 (2:1, v/v) containing 2,5-diphenyloxazole (5g/l)] was added and the clear samples were counted for radioactivity in a Nuclear–Chicago Unilux II scintillation counter. The counting efficiency was about 75% and blank gel slices put through the entire procedure were counted for background radioactivity.

Radiolabelled proteins adsorbed on glass-fibre filters (Whatman) were counted for radioactivity in the same counter by using toluene containing 2,5-diphenyloxazole (5g/l) as scintillant.

Results

Inhibition by maleimides in the presence of NADH

Pyruvate dehydrogenase complex (1mg/ml) incubated at 0°C in potassium phosphate buffer (0.1M, pH7.0) containing EDTA (1mg/ml) and *N*-ethylmaleimide (10mM) lost neither its pyruvate dehydrogenase nor its lipoamide dehydrogenase activity over a period of 1h. However, in the presence of NADH (2mM) there was a rapid loss of about half the pyruvate dehydrogenase activity and a much slower loss of lipoamide dehydrogenase activity (Fig. 1). The initial loss of the overall activity took place without effect on the individual activity of the lipoamide dehydrogenase component, suggesting that the initial site of reaction was not in that component.

The inhibition was examined further by using ^{14}C-labelled *N*-ethylmaleimide. Pyruvate dehydrogenase complex (2mg/ml) was incubated at 0°C with NADH (0.05mM) and *N*-ethyl[2,3-^{14}C]maleimide (20mM, 1.3mCi/mmol) in potassium phosphate buffer (0.1M, pH7.0) containing EDTA (1mg/ml). After 30s, the pyruvate dehydrogenase activity was found to be inhibited by approx. 80% and the reaction was stopped by adding 2-mercaptoethanol to a final concentration of 0.1M. A sample of the treated complex was then examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The usual pattern of three bands was observed,

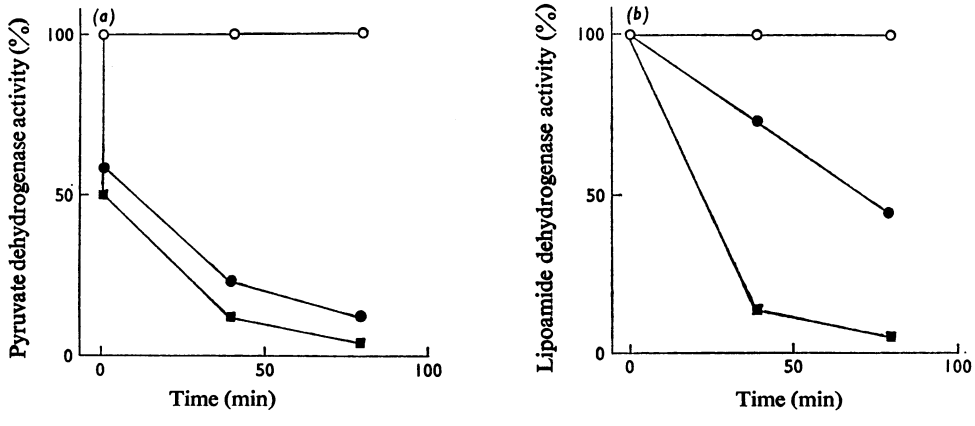
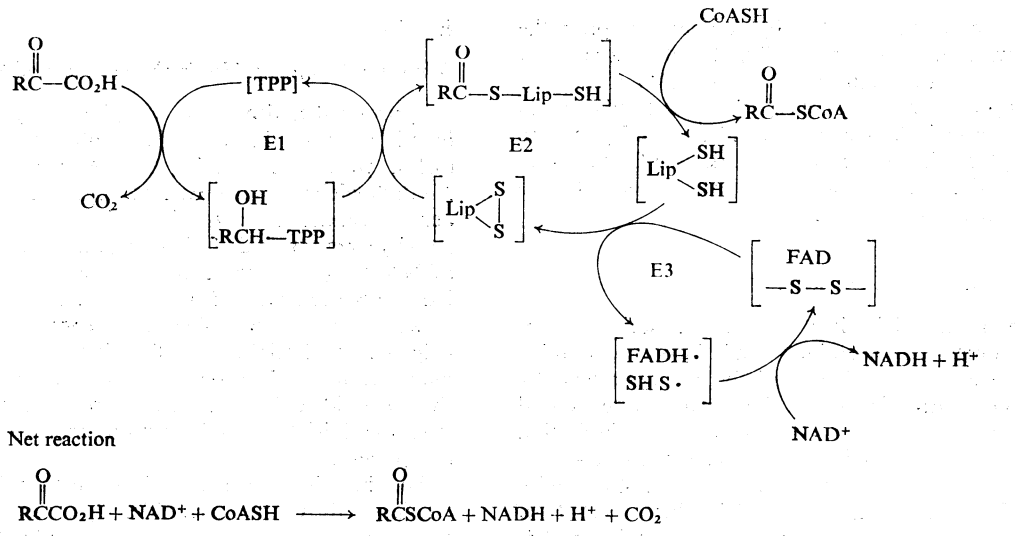


Fig. 1. *Effect of N-ethylmaleimide on the enzymic activities of the pyruvate dehydrogenase complex of E. coli in the presence of NADH*

Pyruvate dehydrogenase complex (1mg/ml) was incubated with NADH (2mM) in potassium phosphate buffer (0.1M, pH7.0) at 0°C in the absence (○) or presence of *N*-ethylmaleimide (●, 4mM; ■, 20mM). Samples were assayed for pyruvate dehydrogenase (*a*) and lipoamide dehydrogenase (*b*) activities after various times. Activities are expressed as percentages of the initial activity. For other details, see the text.



Scheme 1. *Reaction mechanism of the 2-oxo acid dehydrogenase multienzyme complexes of E. coli (after Reed & Oliver, 1968)*
E1 (a decarboxylase), E2 (a lipoyl transacylase) and E3 (lipoamide dehydrogenase) represent the three enzymic activities that make up the complex. The other abbreviations are: TPP, thiamin pyrophosphate; lip<S, lipoic acid. For other details, see the text.

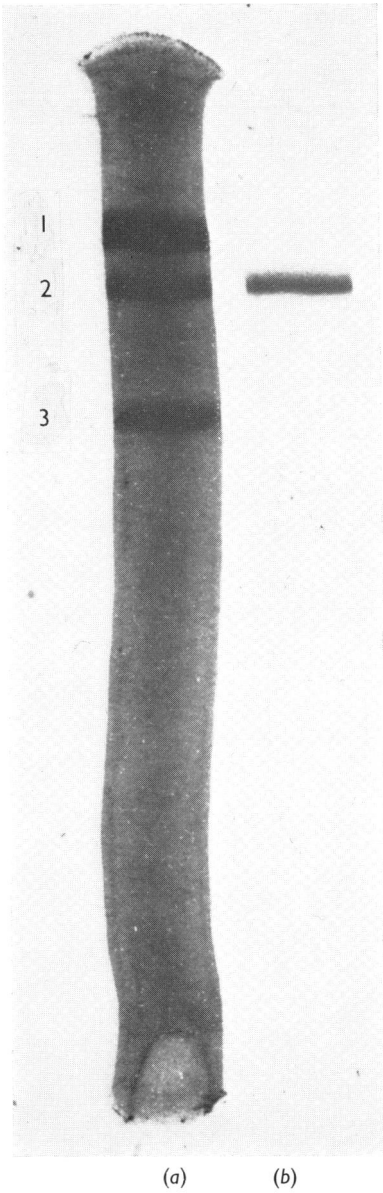
that with an apparent subunit mol.wt. of 100000 being identified as the polypeptide chain of E1, that with an apparent subunit mol.wt. of 80000 as the chain of E2 and that with an apparent subunit mol.wt. of 56000 as the chain of E3 (Perham & Thomas, 1971). Measurement of the radioactivity in the three bands showed that 83% of the label was in the transacetylase component (E2), suggesting that this modification caused loss of the pyruvate dehydrogenase activity of the complex.

These results can be interpreted in terms of the commonly accepted reaction scheme for the complex (Scheme 1). In what is merely a reversal of the normal forward reaction, NADH reduces the lipoic acid on the transacetylase component by means of the lipoamide dehydrogenase component; the dihydrolipoyl moiety then reacts with *N*-ethylmaleimide.

Inhibition by maleimides in the presence of 2-oxo acid

Pyruvate dehydrogenase complex incubated separately with either pyruvate or *N*-ethylmaleimide lost little pyruvate dehydrogenase activity. However, the activity was rapidly lost in the presence of both, whereas the lipoamide dehydrogenase activity remained unaffected. The inactivation was found to follow pseudo-first-order kinetics (Fig. 2) and it was observed that concentrations of pyruvate as low as 10 μM would lead to almost complete inactivation.

As with the NADH-dependent inactivation, it was likely that the site of reaction was other than in the lipoamide dehydrogenase component, since its activity was unaffected. This was confirmed as before by repeating the experiment with *N*-ethyl[2,3-^{14}C]maleimide. Pyruvate dehydrogenase complex (1.5mg/ml) was incubated at 0°C in 50mM-potassium phosphate buffer, pH7.0, containing thiamin pyrophosphate (0.2mM), $MgCl_2$ (2mM), sodium pyruvate (2mM) and *N*-ethyl[2,3-^{14}C]maleimide (2.4mCi/mmol; 1mM). A control incubation was also set up from which only pyruvate was omitted. After various time-intervals up to 40min, samples were withdrawn and the free maleimide was destroyed by addition of 2-mercaptoethanol to a final concentration of 0.1M. The pyruvate dehydrogenase activity in each sample was assayed and the enzyme was then precipitated by adding trichloroacetic acid (70mg/ml). The protein precipitates were filtered on to glass-fibre filters, washed with fresh trichloroacetic acid and counted for radioactivity. The results are shown in Fig. 3. Incorporation of ^{14}C, i.e. reaction of the maleimide, ran in parallel with the loss of pyruvate dehydrogenase activity and there was little incorporation of label in the absence of pyruvate. Examination of the inactivated radiolabelled complex by means of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that more than 90% of the label was associated with the transacetylase (E2 component) as determined



EXPLANATION OF PLATE 1

Location of the site of reaction of N-ethyl[2,3-^{14}*C*]*maleimide with the pyruvate dehydrogenase multienzyme complex of E. coli in the presence of pyruvate*

The incubation conditions were those given in the legend to Fig. 3. (*a*) A sodium dodecyl sulphate/polyacrylamide-gel separation of the inactivated enzyme complex was dried down on Whatman 3MM paper after staining; (*b*) a radioautograph of the gel shown in (*a*) is shown. The bands are: 1, pyruvate decarboxylase, E1; 2, lipoyl transacetylase, E2; 3, lipoamide dehydrogenase, E3.

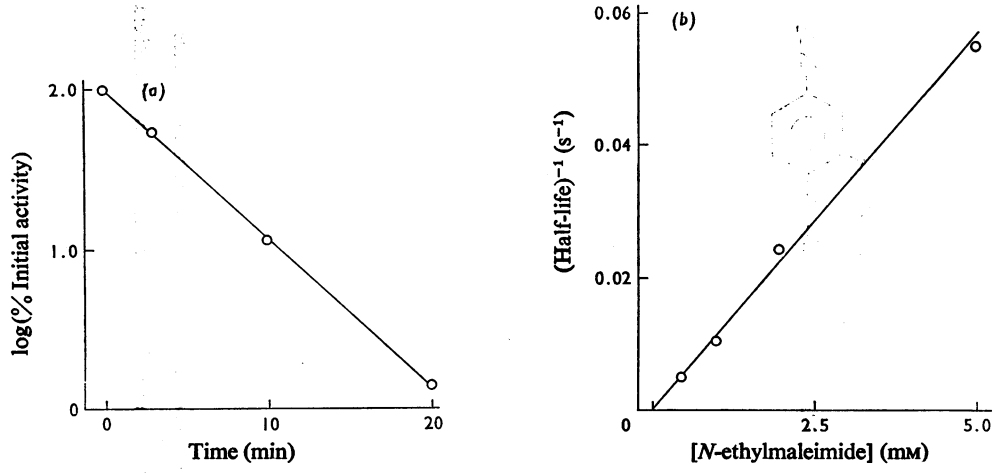


Fig. 2. *Effect of N-ethylmaleimide on the pyruvate dehydrogenase activity of the pyruvate dehydrogenase multienzyme complex of E. coli in the presence of pyruvate*

(*a*) Pyruvate dehydrogenase complex (1 mg/ml) was incubated with *N*-ethylmaleimide (0.5 mM) and pyruvate (0.25 mM) in potassium phosphate buffer (0.1 M, pH 7.0) at 0°C. Enzymic activity was measured after various times. (*b*) The half-life of the inactivation is shown as a function of the concentration of *N*-ethylmaleimide. For other details, see the text.

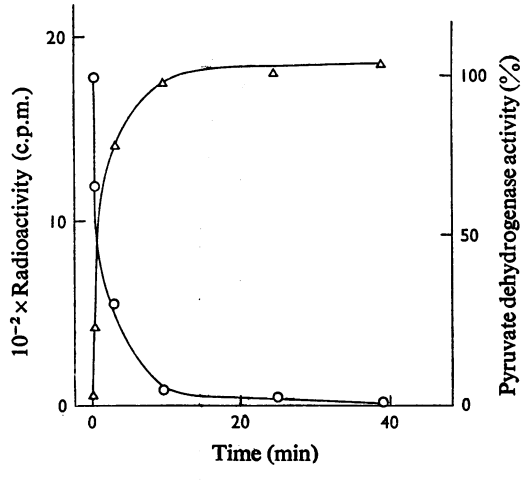


Fig. 3. *Incorporation of radioactivity and loss of pyruvate dehydrogenase activity when the pyruvate dehydrogenase multienzyme complex of E. coli* (1.5 *mg/ml*) *was incubated with N-ethyl*[2,3-^{14}*C*]*maleimide* (2.4 *mCi/mmol*) *and pyruvate* (2 *mM*) *in potassium phosphate buffer* (0.1 M, *pH* 7.0) *at* 0°*C*

△, Incorporation of ^{14}C into the enzyme; ○, pyruvate dehydrogenase activity. For other details, see the text.

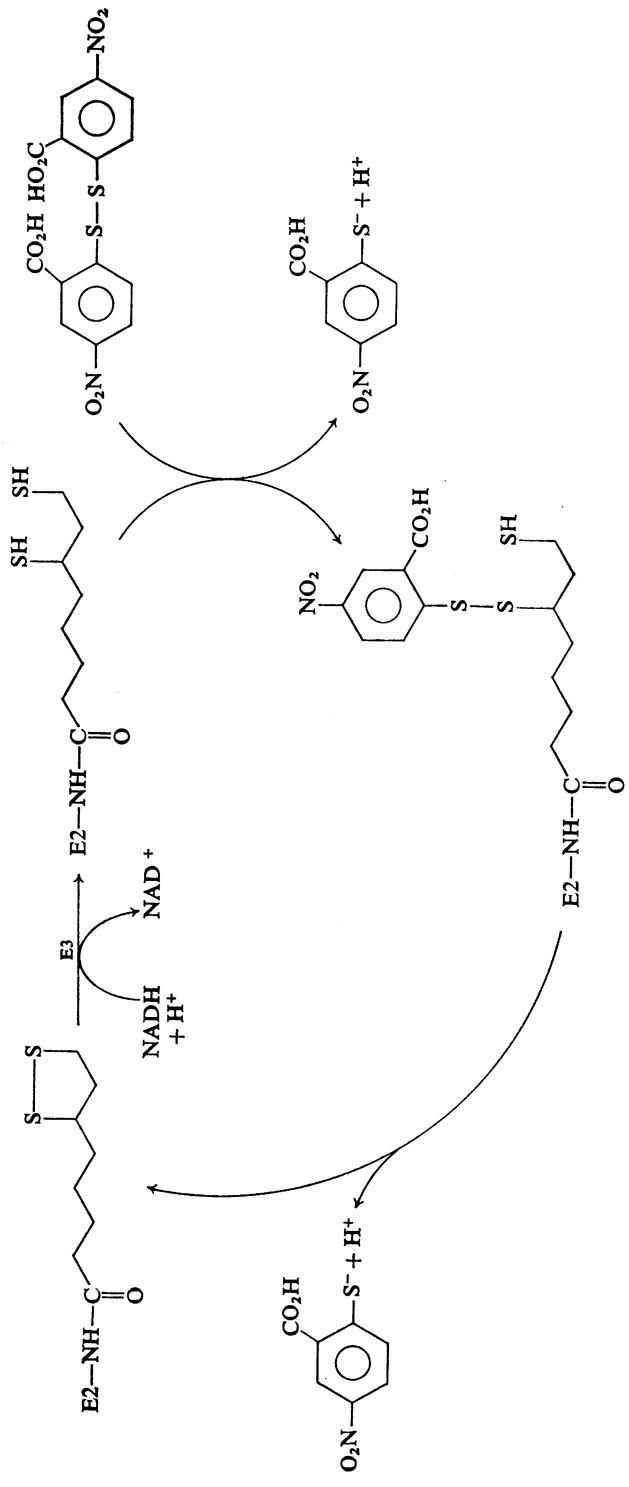
by scintillation counting of the radioactivity of the stained protein bands. This result was confirmed by radioautography of the polyacrylamide gels (Plate 1). The specificity of the incorporation is high.

The more general nature of this inhibition was demonstrated by showing that *N*-(4-dimethyl-3,5-dinitrophenyl)maleimide, a maleimide with a relatively bulky *N*-substituent (Witter & Tuppy, 1960), was an effective replacement for *N*-ethylmaleimide in these reactions. Indeed, in experiments carried out under identical conditions, inhibition of complex activity was more rapid in the presence of the aromatic maleimide than in the presence of *N*-ethylmaleimide. These experiments have not been pursued further owing to the lack of radiolabelled *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, but there is no reason to believe that its specificity is any different from that of *N*-ethylmaleimide.

The 2-oxoglutarate dehydrogenase complex is functionally very similar to the pyruvate dehydrogenase complex (Reed & Oliver, 1968). It was therefore expected that the 2-oxoglutarate dehydrogenase complex would behave similarly in the inhibition experiments described above and no significant differences were noticed. However, the substrate specificity of the enzymes was underlined by the observation that pyruvate could not potentiate inhibition of the 2-oxoglutarate dehydrogenase complex and that 2-oxoglutarate could not potentiate inhibition of the pyruvate dehydrogenase complex.

Attempts to determine more precisely the site of maleimide inhibition

The most plausible explanation for the inhibition of the 2-oxo acid dehydrogenase complexes caused



Scheme 2. ***Proposed reaction mechanism for the catalytic reduction of Nbs_2 with NADH in the presence of the 2-oxo acid dehydrogenase multienzyme complexes***

The intermediate mixed disulphide is shown for convenience as involving the *S*-6 of the dihydrolipoamide, but there is no evidence to exclude the alternative mixed disulphide involving *S*-8.

by maleimides in the presence of the 2-oxo acid substrates is modification of the free thiol group of the thiol ester intermediate of lipoic acid bound to enzyme E2 (Scheme 1). This thiol group appears in the enzyme only in the presence of substrate and will persist in the absence of CoA. Reaction with the maleimide will then cause irreversible inhibition of the pyruvate dehydrogenase activity without necessarily affecting the lipoamide dehydrogenase activity measured with exogenous dihydrolipoamide.

After acid hydrolysis of the complex inhibited by treatment with ^{14}C-labelled maleimide in the presence of substrate, the expected radiolabelled product would therefore be *S*^{8}-[(1,2-dicarboxy)-ethyl]dihydrolipoic acid. This presented a considerable synthetic problem and we attempted to make model compounds as follows. Dihydrolipoamide was incubated with *N*-ethyl[2,3-^{14}C]maleimide at two molar ratios (0.5 and 40mol/mol) in 0.1M-NH_4HCO_3, pH8.0, at 0°C. After 30min, the samples were freeze-dried and hydrolysed with 6M-HCl in sealed evacuated tubes for 24h at 105°C. Samples of the inactive radiolabelled pyruvate dehydrogenase complex were similarly hydrolysed. The products of hydrolysis were separated by paper electrophoresis at pH6.5 and radioautographs prepared. Multiple anionic bands were observed for all samples, but those of the protein hydrolysate closely resembled those found for the dihydrolipoamide reaction products. The radioactive compounds could be distinguished from the single band of *S*^{8}-[(1,2-dicarboxy)ethyl]cysteine, similarly prepared by hydrolysis of the reaction product of cysteine with *N*-ethyl[2,3-^{14}C]maleimide. The reasons for the production of multiple bands with the dihydrolipoamide adducts are unclear and merit further investigation, but the close resemblance of the products of hydrolysis of the inactivated complex and the model compounds provides further evidence for the mechanism of inhibition proposed above.

This conclusion was strengthened by the results of chromatography of the acid hydrolysates on a Beckman 120C amino acid analyser by using the conventional 0.2M-sodium citrate buffer, pH3.25. The cysteine derivative was eluted 37min after application to the 50cm column, just before aspartic acid, in exactly the position reported by Guidotti & Konigsberg (1963). The dihydrolipoamide derivatives were unretarded and emerged 29min after application, which is consistent with their having no amino groups. The radiolabelled products from the protein hydrolysate were also eluted 29min after application.

Reaction of the dehydrogenase complexes with 5,5′-dithiobis-(2-nitrobenzoic acid) and NADH

Nbs_2 [5,5′-dithiobis-(2-nitrobenzoic acid)] is catalytically reduced by NADH in the presence of the 2-oxoglutarate complex of ox heart (Erfle & Sauer, 1968). The pyruvate dehydrogenase complex of *E. coli* was observed to be capable of the same reaction when assayed in 0.1M-potassium borate buffer, pH8.5, release of Nbs^- (thionitrobenzoate anion) being followed spectrophotometrically at 412nm. The concentrations of substrates for half-maximal velocity were about 0.2mM for Nbs_2 and 0.02mM for NADH. The maximum specific activity, about 0.2unit/mg of complex, is about one-hundredth that of the pyruvate dehydrogenase activity.

This catalytic activity of the enzyme was completely inhibited by preincubation of the complex with the substrate, pyruvate. The free lipoamide dehydrogenase component did not catalyse the reaction, in accord with the experiments of Erfle & Sauer (1968) with the ox heart enzyme, but the lipoyl transacetylase–lipoamide dehydrogenase sub-complex showed full activity. As expected, in these reactions the 2-oxoglutarate dehydrogenase complex behaved similarly in all respects to the pyruvate dehydrogenase complex.

To explain these reactions of the 2-oxoglutarate dehydrogenase complex of ox heart, Erfle & Sauer (1968) advanced a mechanism in which the lipoamide dehydrogenase component catalytically reduced a mixed disulphide formed by reaction of 1mol of dihydrolipoamide with 2mol of Nbs_2. We think the mechanism proposed in Scheme 2 is more likely than that of Erfle & Sauer (1968) on several grounds. First, it does not demand a novel reaction to be catalysed by lipoamide dehydrogenase. Secondly, the cyclization to re-form the oxidized lipoic acid from the mixed disulphide form carrying one Nbs group can be observed with free dihydrolipoamide in solution. It was found by titration in the spectrophotometer in 0.1M-Tris/HCl buffer, pH8.0, that 1mol of dihydrolipoamide reacted with 1mol of Nbs_2 to give 2mol of Nbs^-. Thirdly, an intra-chain disulphide bridge can be formed between two cysteine residues in the active site of glyceraldehyde 3-phosphate dehydrogenase by just such a mechanism in the reaction with Nbs_2 (Wassarman & Major, 1969).

Discussion

The pyruvate dehydrogenase multienzyme complex of *E. coli* was unaffected by treatment at 0°C with maleimides in the absence of substrates; the enzymic activity was unchanged and little or no ^{14}C was incorporated when *N*-ethyl[2,3-^{14}C]maleimide was used. Clearly, no functional groups, such as thiol groups, capable of reaction with maleimides are accessible to the reagent in the native enzyme structure.

In the presence of NADH and *N*-ethylmaleimide, however, much of the pyruvate dehydrogenase activity was lost within seconds, whereas the lipoamide dehydrogenase activity disappeared more slowly (Fig. 1). Use of *N*-ethyl[2,3-^{14}C]maleimide showed that the transacetylase (E2) component was the principal site of reaction and therefore presumably of the inhibition. The simplest interpretation of these experiments is that the NADH reduces the lipoyl groups on the transacetylase by means of the lipoamide dehydrogenase component (E3) of the complex, thereby making them susceptible to modification (Scheme 1). A similar conclusion was reached by Erfle & Sauer (1968) in their report of the reaction of Nbs_2 with the 2-oxoglutarate dehydrogenase complex of ox heart, although these workers did not determine which component carried the site(s) of reaction. However, there are drawbacks to this as a method of selective inactivation of the transacetylase component of the pyruvate dehydrogenase multienzyme complex. The reaction with *N*-ethylmaleimide was not restricted absolutely to the transacetylase component and the dependence of the rate and extent of inactivation on NADH concentration was complex. In particular, it proved impossible to inhibit the pyruvate dehydrogenase activity completely without unacceptable modification of the other component enzymes.

The inactivation of the pyruvate dehydrogenase complex in the presence of pyruvate and *N*-ethylmaleimide did not suffer from any of these drawbacks. The kinetics were straightforward (Fig. 2); the incorporation of ^{14}C-labelled *N*-ethylmaleimide was proportional to the observed inactivation (Fig. 3) and the reaction was restricted exclusively to the transacetylase component (Plate 1). The simplest explanation is that the lipoyl group is reductively acylated as in the normal catalytic cycle (Scheme 1), but then undergoes modification. In effect, the enzyme brings about its own inhibition and ensures the specificity of the reaction. Our attempts to characterize the reaction product, without being absolutely conclusive, strongly support the proposal that it is the lipoyl groups on the transacetylase component that become modified.

The method of reacting the lipoic acid with *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate offers potentially a simple method of determining the functionally active lipoic acid content of the multienzyme complex. However, the calculation demands knowledge of the stoicheiometry of the three component enzymes in the complex. This matter is controversial, fundamentally different subunit structures for the pyruvate dehydrogenase complex having been proposed (Eley *et al.*, 1972; Vogel *et al.*, 1972). We have recently devised a novel method for measuring the ratio of the polypeptide chains and shown that in different preparations of the complex the ratio does, in fact, vary (Bates *et al.*, 1975; Perham, 1975). It should therefore now be possible to determine the lipoic acid content of the multienzyme complex by these methods. A preliminary analysis of the experiments reported in the present paper suggests that 2 mol of *N*-ethyl[2,3-^{14}C]-maleimide are incorporated per chain (subunit mol.wt. 80000) of lipoyl transacetylase for complete inhibition of the pyruvate dehydrogenase activity, which implies the presence of two lipoic acid residues per transacetylase chain. This would be in accord with the early estimates of Reed and his collaborators (Koike *et al.*, 1963), although a revised lipoic acid content of one residue per transacetylase chain has been reported (Eley *et al.*, 1972). The ability of a single lipoic acid residue to span the distance between the active sites of the E1 and E3 components, as measured by fluorescence energy-transfer experiments, has also been called into question (Moe *et al.*, 1974). However, it must be emphasized that our experiments will need careful repetition in the light of the improvement in our knowledge of stoicheiometry (Bates *et al.*, 1975) before firm quantitative conclusions can be drawn.

These experiments also provide a convenient method for the selective introduction of reporter groups on to the lipoic acid. The treatment with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide showed that maleimides with bulky *N*-substituents could be successfully employed and the use of 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl, a maleimide carrying a nitroxide spin label, has permitted exploration of the mobility of the lipoyl residues in the intact pyruvate dehydrogenase complex (Ambrose & Perham, 1976). Other uses will obviously suggest themselves.

The catalytic reduction of Nbs_2 by NADH in the presence of the pyruvate dehydrogenase multienzyme complex was an expected activity in view of the work by Erfle & Sauer (1968) on the catalysis of the same reaction by the 2-oxoglutarate dehydrogenase multienzyme complex of ox heart. However, the mechanism that we propose (Scheme 2) differs from theirs and has the attractions, we think, of taking account of the stoicheiometry of the reaction of dihydrolipoamide and Nbs_2 in free solution and not demanding a novel reaction catalysed by lipoamide dehydrogenase.

The 2-oxoglutarate dehydrogenase multienzyme complex of *E. coli* behaved very similarly to the pyruvate dehydrogenase complex in all these reactions, but the substrate specificity for pyruvate and 2-oxoglutarate was absolute. Since the inactivation of the enzyme complexes in the presence of 2-oxo acid and *N*-ethylmaleimide is a function of the decarboxylase component and its interaction with enzyme-bound lipoic acid, these experiments

have in fact demonstrated the high specificity of the decarboxylase components of the two enzymes. There seems little doubt that in terms of mechanism, the principal (if not the only) difference between the two complexes is one of specificity.

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