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Intramolecular Coupling of Active Sites in the Pyruvate Dehydrogenase Multienzyme Complex of *Escherichia coli*

By MICHAEL J. DANSON, ELIZABETH A. HOOPER and RICHARD N. PERHAM
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

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The intramolecular passage of substrate between the component enzymes of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* was examined. A series of partly reassembled complexes, varying only in their E1 (pyruvate decarboxylase, EC 1.2.4.1) content, was incubated with pyruvate in the absence of CoA, conditions under which the lipoic acid residues covalently bound to the E2 (lipoate acetyltransferase, EC 2.3.1.12) chains of the complex become reductively acetylated, and the reaction then ceases. The fraction of E2 chains thus acetylated was estimated by specific reaction of the thiol groups in the acetyl-lipoic acid moieties with *N*-ethyl[2,3-^{14}C]maleimide. The simplest interpretation of the results was that a single E1 dimer is capable of catalysing the rapid acetylation of 8–12 E2 chains, in good agreement with the results of Bates, Danson, Hale, Hooper & Perham [(1977) *Nature* (*London*) **268**, 313–316]. This novel functional connexion of active sites must be brought about by transacetylation reactions between lipoic acid residues of neighbouring E2 chains in the enzyme complex. There was also a slow transacylation process between the rapidly acetylated lipoic acid residues and those that did not react in the initial, faster phase. This interaction was not investigated in detail, since it is too slow to be of kinetic significance in the normal enzymic reaction.

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

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

The complex is composed of multiple copies 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). The acetyltransferase component is probably composed of 24 polypeptide chains and forms the structural core of the complex to which enzymes E1 and E3 are non-covalently bound [for reviews, see Reed (1974) and Perham (1975)]. In the overall complex reaction, the substrate is carried by a lipoic acid residue which is covalently linked by an amide bond to a lysine residue in each chain of the E2 component (Nawa *et al.*, 1960). This lipoyl-lysine moiety is thought to rotate among the catalytic centres of the three component enzymes of the complex (Green & Oda, 1961; Koike *et al.*, 1963) and studies of the mobility of spin-labelled lipoic acid residues support this view (Ambrose & Perham, 1976; Grande *et al.*, 1976).

However, modifications to this simple scheme are necessary in view of the observation that each E2 chain bears at least three lipoic acid residues (Hale & Perham, 1978), of which two become reductively acetylated in the presence of pyruvate (Danson & Perham, 1976). In a recent study (Bates *et al.*, 1977) we demonstrated that the lipoyllysine 'swinging arms' can participate in subunit interactions additional to those normally envisaged in the reaction mechanism. By examining the fate of [2-^{14}C]pyruvate decarboxylated within the complex in the absence of CoA, it was found that a single E1 dimer can bring about the acetylation of multiple copies (probably 12) of the E2 chains in the core. A possible explanation is that each E1 dimer interacts directly with a small number of E2 chains and that this is followed by the transfer of acetyl groups between lipoic acid residues bound to neighbouring E2 chains in the complex. We referred to these internal transfer reactions as 'transacetylation' and the E2 chains were said to be 'serviced' by the E1 dimers in the complex (Bates *et al.*, 1977).

The discovery of such novel interactions in this multienzyme complex warrants further supporting evidence. We decided to use the same basic procedure of incubating a series of E1 deficient complexes with pyruvate in the absence of CoA (Bates *et al.*, 1977), but to measure the degree of acetylation in each complex by reaction of the acetyldihydrolipoic acid residues so generated with *N*-ethyl[2,3-^{14}C]-maleimide. This chemical modification will also inactivate those E2 chains that bear acetylated dihydrolipoic acid residues (Brown & Perham, 1976).

Thus determination of the fraction of the E2 core inactivated will provide a further, independent measurement of the degree of lipoyl-group acetylation. The results that we describe in this paper confirm and extend our previous observations (Bates *et al.*, 1977).

Materials and Methods

Reagents

Pyruvate (monosodium salt), NAD^+ (free acid, grade 2) and CoASH (grade 1) were obtained from C. F. Boehringer und Soehne, Mannheim, W. Germany; thiamin pyrophosphate was from Koch–Light Laboratories, Colnbrook, Bucks., U.K.; and *N*-ethyl[2,3-^{14}C]maleimide (CFA.293) was from The Radiochemical Centre, Amersham, Bucks., U.K. DL-Dihydrolipoamide was prepared by the reduction of lipoamide with $NaBH_4$ by the method of Reed *et al.* (1958). All other reagents were of analytical-reagent grade.

Enzyme

Pyruvate dehydrogenase multienzyme complex was purified from a pyruvate dehydrogenase constitutive mutant of *Escherichia coli* K12 essentially as described by Reed & Mukherjee (1969). The mutant organism was kindly provided by Professor H. L. Kornberg of this Department.

Enzyme assays

The overall activity of the pyruvate dehydrogenase complex was assayed spectrophotometrically at 340nm and 30°C by a slight modification of the method of Reed & Mukherjee (1969). Assay mixtures contained 50mM-potassium phosphate, pH8.0, 2.5mM-NAD^+, 0.2mM-thiamin pyrophosphate, 1.0mM-$MgCl_2$, 0.13mM-CoASH, 2.6mM-cysteine hydrochloride and 2.0mM-sodium pyruvate. The final pH of the mixture is pH7.0. The assay, in a final volume of 1ml, was started with enzyme, and the reaction followed by the increase in A_{340}.

The lipoamide dehydrogenase activity of the complex was assayed at 30°C in 50mM-potassium phosphate, pH8.0, 2.5mM-NAD^+, 0.2mM-thiamin pyrophosphate, 1.0mM-$MgCl_2$ and 0.4mM-dihydrolipoamide. The assay, the final pH of which is pH7.5, was started with enzyme and the reaction followed by the increase in A_{340}.

Preparation of partly reassembled pyruvate dehydrogenase complexes

A series of partly reassembled complexes, varying only in their E1 content, was prepared as described by Bates *et al.* (1977). Pyruvate dehydrogenase complex was dissociated into free E1 and E2–E3 subcomplex at pH10 (Reed & Willms, 1966) and these were resolved by gel filtration on Sepharose 6B (Coggins *et al.*, 1976). The reassembled complexes were then prepared by adding increasing amounts of E1 component to a constant amount (0.6mg) of E2–E3 subcomplex in 50mM-sodium phosphate (pH7.0)/2mM-EDTA. The mixtures were incubated overnight at 4°C and any unbound E1 component was then separated from assembled complex by gel filtration on Sepharose 6B in the same buffer. The stoicheiometries of the polypeptide chains in these pyruvate dehydrogenase complexes were determined by the radioamidination method of Bates *et al.* (1975).

Modification of the reassembled pyruvate dehydrogenase complexes with N-ethyl[2,3-^{14}C]maleimide

The lipoic acid residues of the partly reassembled complexes were specifically modified with *N*-ethyl-[2,3-^{14}C]maleimide in the presence of pyruvate as described by Brown & Perham (1976) and Danson & Perham (1976). Each complex was incubated at 0°C in 0.3ml of 20mM-sodium phosphate buffer, pH7.0, containing 1.6mM-pyruvate, 0.4mM-thiamin pyrophosphate, 4.8mM-$MgCl_2$, 2mM-EDTA and 0.8mM-NAD^+. To each was added 0.9mM-*N*-ethyl[2,3-^{14}C]-maleimide and the enzymic activities of the whole complex and of the associated E3 component were followed with time. When the whole-complex activity had been inhibited more than 90% (at approx. 60min), the modification was stopped by the addition of 2-mercaptoethanol to a final concentration of 0.2M. Four samples (50μl) were taken from each incubation mixture and to these in turn were added 1mg of unmodified pyruvate dehydrogenase complex as a carrier protein followed by 1ml of ice-cold 12.5% trichloroacetic acid. The precipitated proteins were collected on glass microfibre filters (Whatman GF/C) and were washed with 25ml of 12.5% trichloroacetic acid and then with 6ml of acetone. The dry filters were placed in vials with 4ml of toluene containing 2,5-diphenyloxazole (5g/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 quenched standards (Amersham/Searle, Des Plaines, IL, U.S.A.).

The relative incorporation of radiolabel per chain of E2 component in each complex was calculated from the measured E2:E3 chain ratio and the enzymic activity of the E3 component.

Samples of each partly assembled complex were taken before and after the modification with *N*-ethyl[2,3-^{14}C]maleimide, and were mixed with a 10-fold molar excess of E1 component in 20mM-sodium phosphate buffer, pH7.0, containing 2mM-

EDTA and 0.1M-2-mercaptoethanol. After an incubation of 1h at 0°C these complexes were assayed for whole-complex and E3 enzyme activities. The gain in whole-complex activity for each sample allowed us to calculate the fraction of the E2 core inactivated by the treatment with pyruvate and *N*-ethylmaleimide when the E1 content was below its maximal value.

Treatment of the partly reassembled complexes with *N*-ethyl[2,3-^{14}C]maleimide was also carried out in the absence of pyruvate to allow for any non-specific incorporation of label and its effects on the enzymic activities.

Measurement of the rates of incorporation of acetyl groups from [2-^{14}C]pyruvate into the partly reassembled pyruvate dehydrogenase complexes

Samples of the reassembled complexes were incubated at 0°C with 0.2mM-[2^{14}C]pyruvate in 0.22ml of 50mM-sodium phosphate buffer, pH7.0, containing 0.7mM-thiamin pyrophosphate, 10mM-$MgCl_2$ and 1mM-NAD^+. At various time intervals, 25μl of the incubation mixture was removed and mixed with 100μl of rabbit immunoglobulin G (10mg/ml in 20mM-sodium phosphate buffer, pH7.0), followed immediately by 1ml of ice-cold 12.5% trichloroacetic acid. The shortest incubation period that could be attained by this method was 20s. The precipitated proteins were collected on glass microfibre filters (Whatman GF/C) and were washed with 25ml of 12.5% trichloroacetic acid, 6ml of acetone containing 50mM-HCl and finally 6ml of diethyl ether. The dry filters were placed in vials with 4ml of toluene containing 2,5-diphenyloxazole (5g/l) and counted for radioactivity in a Nuclear Chicago Unilux II-A scintillation counter.

The relative incorporation of radiolabel per chain of E2 component in each complex was calculated from the measured E2:E3 molar chain ratio and the enzymic activity of the E3 component.

Results

The reassembly of E1 component with E2–E3 subcomplex followed precisely the same pattern as that described by Bates *et al.* (1977). The E1 component added became bound to the subcomplex with no apparent displacement of enzyme E3 during the assembly process. In the present experiments E1 component was added to give an E1:E2 chain ratio of no higher than 1.5:1. The overall complex activities of the reassembled multienzymes varied directly with their E1 contents, whereas the E3 enzymic activity remained constant throughout (Bates *et al.*, 1977).

Treatment of each of the reassembled pyruvate dehydrogenase complexes with *N*-ethyl[2,3-^{14}C]-maleimide in the presence of pyruvate produced a pseudo-first-order loss of whole-complex activity without any loss in the activity of the lipoamide dehydrogenase (E3) component (Brown & Perham, 1976). Consistent with our observations that the *N*-ethylmaleimide reacts specifically with the lipoic acid residues on the E2 component (Ambrose & Perham, 1976; Brown & Perham, 1976; Danson & Perham, 1976), there were no significant differences between the rates of inactivation of the different complexes. In addition, less than 10% of the overall complex activities was lost on treatment with the *N*-ethylmaleimide in the absence of pyruvate.

The incorporation of radiolabel as a function of the E1:E2 polypeptide-chain ratio is shown in Fig. 1. The maximum incorporation corresponded to the modification of 2.0 (±0.2) lipoic acid residues per E2 chain.

Modification of the lipoic acid residues of native complex with *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate selectively inactivates the E2 component (Brown & Perham, 1976). Therefore, in partly reassembled complexes, only those E2 chains that bear acetylated dihydrolipoic acid residues will be modified and inactivated by the maleimide. After modification of these partly acetylated complexes, it was possible to add back a molar excess of enzyme E1 to produce fully assembled multienzyme complexes (see the Materials and Methods section). The

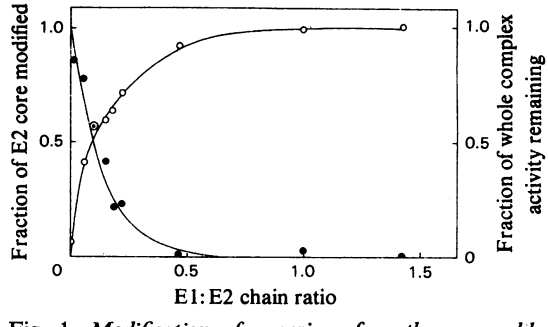

Fig. 1. *Modification of a series of partly reassembled pyruvate dehydrogenase complexes with N-ethyl*[2,3-^{14}C]-*maleimide in the presence of pyruvate*

Treatment with *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate was carried out as described in the text. The E1:E2 polypeptide-chain ratios were determined by the radioamidination method of Bates *et al.* (1975). ○, Fraction of the E2 core modified by the maleimide (data are the mean of four replicate samples); ●, overall complex enzymic activity recovered when the fully inhibited E1-deficient complexes were reassembled with an excess of E1 component (the activity is expressed as a fraction of the activity recovered before modification with *N*-ethylmaleimide and the data are the means of two replicate assays).

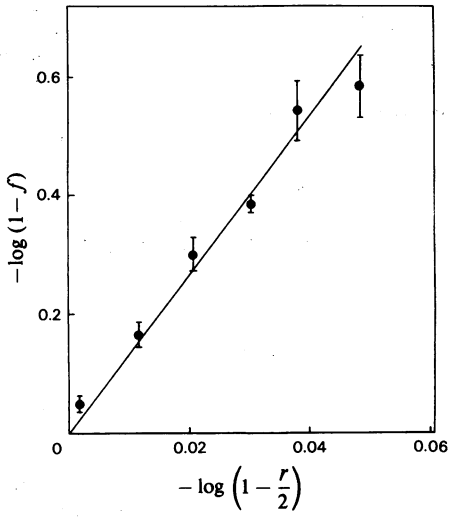

Fig. 2. *Relation between the fraction of E2 component modified by N-ethyl*[2,3-^{14}C]*maleimide in the presence of pyruvate and the E1:E2 polypeptide-chain ratio*
The data from Fig. 1 are plotted in the form of the equation:

$$-\log(1-f)=n\left[-\log\left(1-\frac{r}{2}\right)\right]$$

where f is the fraction of E2 chains modified with *N*-ethyl[2,3-^{14}C]maleimide [means ± S.E.M. of the six individual values calculated from (○) and (●) experimental points in Fig. 1], r is the E1:E2 polypeptide-chain ratio and n is the number of E2 chains that can be 'serviced' by a single E1 dimer.

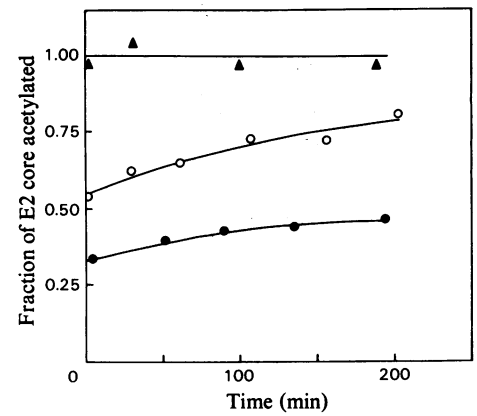

Fig. 3. *Time course of the incorporation of acetyl groups from* [2-^{14}C]*pyruvate into the E2 component of three partly reassembled pyruvate dehydrogenase complexes*
The acetylation with [2-^{14}C]pyruvate was carried out as described in the text. The shortest incubation time was 20s. The E1:E2 polypeptide chain ratios of the complexes, determined by the radioamidination method of Bates *et al.* (1975), were 0.07:1 (●), 0.15:1 (○) and 1.59:1 (▲).

overall complex activities of these reassembled complexes should then be inverse measures of the extents of modification of the lipoic acid residues. These activities are also shown in Fig. 1 and, as expected, they describe a curve which is an approximate inverse of that for the incorporation of the *N*-ethylmaleimide.

From the data presented in Fig. 1, it is clear that a single E1 chain can catalyse the reductive acetylation of the lipoic acids attached to more than one E2 polypeptide chain. Assuming non-co-operative binding of E1 dimers to identical sites on the E2–E3 subcomplex (Henning *et al.*, 1972; Perham & Hooper, 1977) we have shown previously (Bates *et al.*, 1977) that:

$$\log(1-f)=n\log\left(1-\frac{r}{2}\right) \qquad (1)$$

where f is the fraction of E2 chains whose lipoic acid residues have become reductively acetylated, r is the E1:E2 polypeptide-chain ratio and n is the number of E2 chains that can be 'serviced' by a single E1 dimer. Plotting of the experimental data from Fig. 1 in the form of eqn. (1) gives a good straight line of gradient $n=13\pm2$ (Fig. 2).

This value of $n=13$ is in excellent agreement with that obtained in our earlier studies of the acetylation by [2-^{14}C]pyruvate of the lipoic acid residues of partly reassembled complexes ($n=12\pm1$; Bates *et al.*, 1977). However, a time course of the reductive acetylation by [2-^{14}C]pyruvate revealed that, in addition to the rapid acetylation process (complete in less than 20s) for which $n \simeq 12$, there is also a very slow transacylation between the rapidly serviced lipoic acid residues and those not acetylated in this period. The time courses for the acetylation by [2-^{14}C]pyruvate of three partly reassembled complexes, with E1:E2 chain ratios of 0.07:1, 0.15:1 and 1.59:1 respectively, are shown in Fig. 3. In the complexes with low E1:E2 chain ratios, where the E2 core was not fully acetylated in the rapid process, a second, slow increase in the incorporation of [^{14}C]acetyl groups took place. However, when the whole of the core was rapidly acetylated, no further incorporation of radiolabel into the complex was observed.

In the time taken for the modification of the acetylated dihydrolipoic acid residues with *N*-ethyl-[2,3-^{14}C]maleimide (approx. 60min), the extent of the slow transacetylations may become significant,

which would result in an overestimation of the value of n. At present, it is not possible to give an exact correction to the value of n, because we do not have sufficient data on the relationship between the rapid and slow transacetylation processes. A correction based on the rate of incorporation of [2-^{14}C]pyruvate in the absence of maleimide (see Fig. 3) would lower the value of n to $\geqslant 8$. However, the simultaneous modification of the acetyldihydrolipoic acid residues with *N*-ethylmaleimide will decrease the rate and extent of the slow transacetylation. Thus it is reasonable to conclude that the value of n determined in our experiments implies that between 8 and 12 E2 chains are serviced by a single E1 dimer.

Discussion

The experiments described in this paper were undertaken in an attempt to provide further evidence for the 'transacetylation' reactions that we have reported for the natural substrate, pyruvate (Bates *et al.*, 1977). In that sense, the most important conclusion to be drawn from the present data is the further demonstration that any E1 dimer of the pyruvate dehydrogenase complex can catalyse the reductive acetylation of a large fraction of the E2 core. Again, we have tried to analyse this interaction quantitatively and find that 8–12 E2 polypeptides can be 'serviced' with substrate by a given E1 dimer, as measured by subsequent reaction with N-ethylmaleimide. It has previously been argued on grounds of symmetry that the value of n must be 1, 2, 3, 4, 6, 8, 12 or 24 (Bates *et al.*, 1977). The evidence available to us at present suggests that $n=12$.

To account for the large physical distances involved in such interactions, we again have to invoke the model that any E1 enzyme can reductively acetylate the lipoic acid residues of a small number of E2 chains by direct transfer of the acetyl moiety between these two components. This is then followed by the continued transfer of acetyl groups between the neighbouring E2 chains in the core to give the observed extent of acetylation. In a previous paper (Bates *et al.*, 1977), rapid migration of an E1 dimer from one E2 binding site to another was ruled out on the grounds that this would result in the acetylation of all 24 E2 chains in the core of the complex if the binding sites remained equivalent. We make the same assumption in the interpretation of the present experiments.

An essential aspect of the model proposed to account for the observed extents of acetylation is that an E1 dimer can catalyse the direct acetylation of a number of E2 chains. Support for this type of multiple interaction has been obtained by McMinn & Ottaway (1977) during their investigation of the mechanism and kinetics of the 2-oxoglutarate dehydrogenase system from pig heart. Computer-fitted intial-velocity studies of this complex indicated that the sequence of events in the catalytic mechanism appeared to be random after the release of CO_2. These authors suggested that a potential ability of each subunit in the multienzyme complex to react with more than one subunit of another type would give the reaction within the complex this semi-random character. Thus McMinn & Ottaway (1977) go on to suggest that, when the complex as a whole is not working at its maximum rate, it is not necessary that the oxidized lipoate chain that accepts an acyl group from a particular E1 component is the same as that which accepted the previous group. Although one cannot extrapolate directly from the pig heart 2-oxoglutarate complex to the *E. coli* pyruvate dehydrogenase complex, it is significant for the present discussion that independent kinetic evidence for such interactions has been found in a 2-oxo acid dehydrogenase complex.

Our present model, therefore, takes the simplest assumption that a single E1 dimer can cause the acetylation of two lipoic acid residues on each of 12 E2 chains (if $n=12$; Bates *et al.*, 1977; and the present paper). However, it should be pointed out that our evidence cannot exclude an alternative formulation, that a single E1 dimer can cause the acetylation of a single lipoic acid residue on each of the 24 E2 chains. Since in the native complex two lipoic acid residues on each E2 chain become acetylated (Danson & Perham, 1976; Speckhard *et al.*, 1977) the latter model would imply two types of E1–E2 interaction, each of which can lead to the acetylation of 24 lipoic acid residues (one on each E2 chain) by a rapid intramolecular transacetylation process. These two networks of lipoic acid residues could not of course be rapidly coupled, since n would then have to equal 24.

Since the completion of the experiments we describe here, Collins & Reed (1977) have also published evidence for a network of interacting lipoyl moieties in the *E. coli* pyruvate dehydrogenase complex. These authors reported that all the lipoic acid residues of the E2 core could be reductively acetylated by pyruvate in reconstituted subcomplexes containing either one or three E1 dimers per complex molecule. However, the 'transacetylation' reactions that we have been investigating are those that occur at a kinetically competent rate in the catalytic mechanism of the enzyme. Pulsed-quenched-flow experiments on the rate of acetylation of the lipoic acid residues in partly reassembled complexes have indicated that the rate of the internal transacetylations is equal to, or greater than, the rate at which any lipoic acid receives an acetyl group by direct transfer from an E1 component (M. J. Danson, A. R. Fersht & R. N. Perham, unpublished work). In contrast, full acetylation of the E2 core reported by Collins & Reed (1977)

required an incubation period of up to 30min at a pyruvate concentration of 0.25mM. Given that the specific activity of native complex is at least 25 μmol/min per mg (Bates *et al.*, 1977; Collins & Reed, 1977) and that the overall complex activity is directly proportional to the E1 content of the complex (Bates *et al.*, 1977), one would expect even the partly reassembled complex with only one E1 dimer to have a catalytic-centre activity at 30°C of not less than 2 μmol of pyruvate utilized/s per μmol of E2 chain at this concentration of substrate (approx. $0.5 \times K_m$). This activity is much greater than would be predicted from the requirement of 30min to bring about full acetylation of the E2 core, implying that the actual number of E2 chains 'serviced' at a kinetically competent rate by an E1 dimer will be considerably less than 24. Thus, although the two sets of experiments are in good qualitative agreement, the results of Collins & Reed (1977) ought not to be compared quantitatively with our observations. As we have shown (Fig. 3), there is a slow transacetylation process after the rapid acetylation which would lead to higher values of n if longer periods of incubation are used. However, we have not concentrated further on this slow phase for the reason that it is obviously not fast enough to be important in the catalytic mechanism of the multienzyme complex.

Collins & Reed (1977) have also described a similar interaction of the lipoic acid residues in the 2-oxoglutarate dehydrogenase complex of *E. coli*. Thus it is likely that these transacylation reactions are widespread among the 2-oxo acid dehydrogenase complexes and that they play an important part in the catalytic mechanism.

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