STUDIES ON THE STRUCTURE AND FUNCTION OF PYRUVATE DEHYDROGENASE COMPLEXES

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RONNEY DE ABREU

STUDIES ON THE STRUCTURE AND FUNCTION OF PYRUVATE DEHYDROGENASE COMPLEXES

(*with a summary in Dutch*)

Proefschrift

ter verkrijging van de graad
van doctor in de landbouwwetenschappen,
op gezag van de Rector Magnificus,
dr. H.C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
op woensdag 13 december 1978
des namiddags te vier uur in de Aula
van de Landbouwhogeschool te Wageningen

ISN 104201-03

STELLINGEN

1

Bij de kwantificering van het aantal ingebouwde acetylgroepen in het pyruvaat dehydrogenase complex, dient rekening te worden gehouden met het deacetylerend effekt van zuurstof.

Barrera, C.M., Namihera, G., Hamilton, L., Munk, P., Eley, M.H., Linn, T.C. and Reed, L.H. (1972), Arch.Biochem.Biophys. *148*, 343.
Dit proefschrift.

2

Perham en medewerkers hebben bij de bepaling van de verhouding van de enzym-componenten in het pyruvaat dehydrogenase complex van *E.coli* onvoldoende rekening gehouden met de slechts gedeeltelijke ontvouwing van de lipoyltransacetylase "core" onder de voor hun experimenten geldende omstandigheden.

Bates, D.L., Harrison, R.A. and Perham, R.N. (1975), FEBS Lett., *60*, 427.
Perham, R.N. and Hooper, E.A. (1977), FEBS Lett. *73*, 137.
Koike, M., Reed, L.J. and Caroll, W.R. (1963), J.Biol.Chem. *238*, 30.

3

Gezien de afmetingen, verdient het aanbeveling om de ijzer-zwavel klusters in hydrogenase te bestuderen op hun eventuele clathraat funktie.

Adman, E.T., Sieker, L.C. and Jensen, L.H. (1973), J.Biol.Chem. *248*, 3987.

4

Bij onderzoek naar het verband tussen abnormale enzymaktiviteit en maligne afwijking, dient men zich bewust te zijn van de verschillen in de ontwikkelingsstadia van de diverse celtypen.

Zimmer, J., Khalifa, A.S. and Lightbody, J.J. (1975), Cancer Research, *35*, 68.

Smyth, J.F. and Harrap, K.R. (1975), Br.J.Cancer *31*, 544.
Dinescu-Romalo, G, Mihai, C. and Vlad, L. (1977) Rev.roum.Biochim. *14*, 161.
Huang, A.T., Logue, G.L. and Engelbracht, H.L. (1976), Br.J.Haemalology, *34*, 631.
Tritsch, G.L. and Minowada, J. (1978) J.Natl.Cancer.Inst. *60*, 1301.

5

De door Erbes, Burris en Orme-Johnson veronderstelde aanwezigheid van slechts één 4Fe/4S kluster in hydrogenase van *P.elsdenii*, is niet in overeenstemming met hun E.S.R. spectra.

Erbes, D.C., Burris, R.M. and Orme-Johnson, W.H. (1975), PNAS, *72*, 4795.

6

De problemen van minderheidsgroepen kunnen alleen worden opgelost, indien men deze groepen betrekt bij de besluitvorming.

7

Het is wenselijk, dat het aantal wetenschappelijke tijdschriften op biochemisch gebied sterk wordt verminderd in het belang van de overzichtelijkheid en het niveau.

8

In verschillende homeopatische preparaten die op de markt worden gebracht als aktieve-enzympreparaten, blijken er geen aantoonbare enzymaktiviteiten te zijn. Het is daarom gewenst dat de overheid strengere maatregelen tegen deze misstanden treft.

9

De kreatie van een nieuw gerecht schept evenveel vreugde als de ontdekking van een nieuw enzym.

Proefschrift van R.A. De Abreu 13 december 1978.

San de na doengroe, ini wantin
a sa kon na krien.
(Wat nu nog duister is, komt eens
aan het licht).

Surinaamse odo

Aan Rigoni
Aan mijn ouders

VOORWOORD

Bij het verschijnen van dit proefschrift wil ik graag mijn dank uitbrengen aan allen die op enigerlei wijze hebben medegewerkt aan het tot standkomen ervan.

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Tenslotte dank ik alle medewerkers en oud medewerkers voor hun hulp en adviezen tijdens mijn verblijf op Biochemie.

LIST OF ABBREVIATIONS

acetyl-CoA, CoASAc	acetyl coenzyme A
A.vinelandii	Azotobacter vinelandii
anti-lipDH A.v.	antibody against lipoamide dehydrogenase from *A.vinelandii*
anti-4.PDC A.v.	antibody against 4-component dehydrogenase complex from *A.vinelandii*
anti-PDC E.c.	antibody against pyruvate dehydrogenase complex from *E.coli*
anti-sub E.c.	antibody against subcomplex from *E.coli*
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CoA	coenzyme A
dis/min	desintegrations per minute
DTBP	dimethyl 3,3'-dithiobispropionimidate
E.coli	Escherichia coli
E.D.T.A.	ethylene diamine tetra acetate
FAD	flavine adenine dinucleotide, oxidized form
$FADH_2$	flavine adenine dinucleotide, reduced form
M	molar
mol.wt.	molecular weight
NAD^+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form.

NEM	N-ethyl maleimide
TPP	thiamine pyrophosphate
tricine	N-[tris(hydroxymethyl)-methyl]-glycine
tris	tris[hydroxymethyl]aminomethane

LIST OF ENZYMES

In this thesis the non-systematic names of enzymes are used. This list includes the trivial and the systematic names of the investigated enzymes; also is included the enzyme number according to the Report of the Commission for Enzymes of the International Union of Biochemistry.

E.C. number	systematic name	Trivial name
1.2.4.1	pyruvate: lipoate oxido reductase (acceptor acylating)	pyruvate dehydrogenase complex
4.1.1.1	pyruvate decarboxylase (2-oxoacid carboxy-lyase)	pyruvate dehydrogenase
2.3.1.12	CoASAc: dihydrolipoate S-acetyltransferase	dihydrolipoyl transacetylase
1.6.4.3	NADH: lipoamide oxido reductase	lipoamide dehydrogenase

1. GENERAL INTRODUCTION

In all living aerobic organisms pyruvate dehydrogenase complexes play a very important role in metabolic processes. The active unit is a cluster of enzymes, joined together by protein-protein interactions, having the ability to convert pyruvate, by means of sequential steps, into coenzyme A. These properties and the fact that they are multi-enzyme complexes with several co-factors, make them worthwhile to study in order to obtain new insights in the catalytic mechanism and physico-chemical interactions.

This thesis deals with pyruvate dehydrogenase complexes from *Azotobacter vinelandii* and *Escherichia coli*.

In chapter 2 a review of recent literature on regulation and molecular organization of pyruvate dehydrogenase complexes is given.

In chapter 3 a method is given to obtain a 3-component complex from the originally 4-component complex of *A.vinelandii* by affinity chromatography. Both complexes are compared by means of their overall activity, partial activities, sedimentation coefficients, diffusion coefficients and flavin contents.

In chapter 4 studies with radio-active substrate and N-ethylmaleimide are presented. The lipoyl groups of the multi-enzyme complexes from *A.vinelandii* and *E.coli* are specifically labelled in order to determine the stoichiometry of the multi-enzyme complexes.

In chapter 5 studies with bifunctional reagents on the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* are described. These studies were performed in an attempt to elucidate protein-protein interactions in both complexes.

In chapter 6, immunochemical studies on the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* are presented in an attempt to obtain insight in the structural organization of the enzyme components in these complexes.

The major goal of this investigation was originally to study the major differences between the mechanisms of action and of the physico-chemical interaction within the 4-component complex from *A.vinelandii* and the 3-component complex from *E.coli*. This problem was very much simplified by the observation

made during this investigation, that the 4-component complex from *A.vinelandii* can be converted into a 3-component complex with properties alike that of the *E.coli* complex.

ERRATA

Page 47, text of Figures

2.3 and 3.3 are from ..... *should read* 2.2 and 3.3 are from .....

Page 48

Figure 7: a spot between 1.1 and 1.2 (spot 1a) should be drawn (see Figure 6).

Text of Figure 7: to the text should be added spot 1.1a and 3.2 are from unknown products.

Page 75

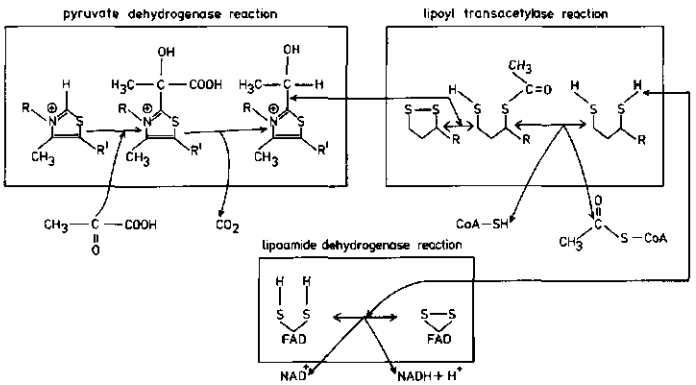
5.5 DISCUS *should read* 6.5 DISCUSSION.

2. THE PYRUVATE DEHYDROGENASE COMPLEXES

(a Review from the Literature)

2.1 Mechanism

Pyruvate dehydrogenase complexes have been isolated from a great number of aerobic organisms [1-17]. These multienzyme complexes catalyse the conversion of pyruvate to acetyl-coenzyme A in a reaction sequence in which five cofactors are involved; TPP, lipoic acid, coenzyme A, FAD and NAD^+. The reaction sequence has been extensively studied [18-20] and it can be summarized as follows:



The complexes consist of three different enzyme components; pyruvate dehydrogenase, lipoyltransacetylase and lipoamide dehydrogenase. The decarboxylation of pyruvate to hydroxyethyl-TPP and CO_2 is catalysed by the pyruvate dehydrogenase component. The hydroxyethyl group is then transfered to lipoic acid bound to the lipoyltransacetylase component. In the course of the transfer reaction the hydroxyethyl group is oxidized to an acetyl group with the concomitant reduction of the lipoate to dihydrolipoate. The next step, the transfer of the acetyl group from the thiolgroup of dihydrolipoate to the thiol group of coenzyme A, is also catalyzed by the lipoyltransacetylase component. The recycling of dihydrolipoate to lipoate is catalyzed by the lipoamide dehydrogenase component - to which FAD is bound - using NAD^+ as hydrogen acceptor. Different enzyme systems exist for the conversion of pyruvate to acetyl-CoA. Under anaerobic condi-

tions pyruvate is converted, in microorganisms into acetyl-CoA by the so-called phosphoroclastic reaction. In *E.coli* the phosphoroclastic reaction is catalyzed by pyruvate formate-lyase, a single enzyme [79,80]:

$$pyruvate + CoA \xrightarrow{E\text{-}Mg^{2+}TPP} Acetyl\text{-}CoA + formate.$$

Extensive studies have been performed on pyruvate dehydrogenase complexes of mammalian tissue and *Escherichia coli*. In our laboratory we have concentrated our studies on pyruvate dehydrogenase complexes from *Azotobacter vinelandii* and *Escherichia coli*. Hence, in next sections I will review some results of recent research reported on pyruvate dehydrogenase complexes from mammalian tissue, *E.coli* and *A.vinelandii*.

2.2. Regulation

Pyruvate dehydrogenase complexes play an important role in metabolic processes. The formation of acetyl-CoA from pyruvate links glycolysis with metabolic pathways such as lipogenesis, ketogenesis and the citric acid cycle. In animals, acetyl-CoA cannot be used as a source for carbohydrate synthesis and thus metabolism of pyruvate represents net loss of carbohydrate reserves. Regulation of this irreversible step is of critical importance for the general energy balance of animals.

Regulation of mammalian pyruvate dehydrogenases complexes occurs by two types of mechanisms.

Firstly, the complex is regulated by phosphorylation and dephosphorylation of the pyruvate dehydrogenase component [3,7,13,21-29]. Phosphorylation and inactivation of pyruvate dehydrogenase is catalysed by a protein kinase in the presence of Mg ATP^{2-}; dephoshorylation and activation of pyruvate dehydrogenase is catalysed by a phosphatase in the presence of Mg^{2+} and Ca^{2+}. It is shown that the pyruvate dehydrogenase component from mammalian complexes consists of two non-identical polypeptide chains; an α- and a β-chain [6,26-28]. The α-subunit probably binds TPP and Mg^{2+} and catalyses the decarboxylation of pyruvate. It is also observed that this subunit is phosphorylated and dephosphorylated [7,21,26,29,34-36]. The β-subunit probably catalyses the subsequent reductive acetylation of the oxidized lipoic acid moiety on the transacetylase [37].

Second, the end products of the total reaction, NADH and acetyl-CoA are competitive feedback inhibitors of the active form of pyruvate dehydrogenase

[29,38-40]. Phosphorylation and inactivation is facilated by high ratios of [ATP]/[ADP], [NADH]/[NAD^+], and [acetyl-CoA]/[CoASH] and is inhibited by pyruvate [17,41]. Cate and Roche [29] have proposed that reduction - by NADH - or acetylation - by acetyl CoA or pyruvate - of the lipoic acid moiety of the lipoyltransacetylase component of the complex is required for the stimulation of the pyruvate dehydrogenase kinase activity.

Effects of mono- and divalent ions have been studied on the phosphatase and the protein kinase in a number of animal tissues. Studies on the phosphatase showed that the enzyme needs a dual requirement for both Mg^{2+} and Ca^{2+} to obtain a maximal activity. In experiments where only Mg^{2+} is used and Ca^{2+} is omitted, only half of the maximum activation was observed [7,21,23,34,42-45,47]. The major effect of Ca^{2+} is a lowering of the K_m-value of the phosphatase for its substrate: the phosphorylated pyruvate dehydrogenase complex, as well as a lowering of the K_m-value for Mg^{2+} [34,42]. Mn^{2+} appears to have a similar effect as Ca^{2+}, while the effect of Ca^{2+} is inhibited by Ni^{2+} and Zn^{2+} [34]. Monovalent ions have no effect on the phosphatase activity [46]. Regulation of the pyruvate dehydrogenase kinase is more complex [34]. The enzyme is inhibited by Ca^{2+} and by high concentrations of Mg^{2+}. The monovalent ions K^+ or NH_4^+ are required for the inhibition of the kinase by ADP [34,46]. Denton *et al* [34] observed that the inhibition by ADP is mainly competitive with respect to ATP, but that there is an additional non-competitive element in the inhibition. In addition, the kinase is inhibited by pyruvate, TPP and pyrophosphate [7,32,34, 36] and activated by acetoin [44]. TPP and pyrophosphate are uncompetitive inhibitors with respect to ATP.

Various studies have been performed to explain the effect of fatty acids on the interconversion of active to inactive pyruvate dehydrogenase [17,48-55]. It has been suggested that the inhibition of pyruvate oxidation by fatty acids occurs by way of changes in the intramitochondrial [ATP]/[ADP], [NADH]/[NAD^+] and [acetyl-CoA]/[CoASH] ratios. Wieland and co-workers [48,49] and Walajtys *et al* [50,51] have provided some evidence that the pyruvate dehydrogenase activity is regulated primarilary as a result of changes in the mitochondrial [ATP]/[ADP] ratio. Other laboratories [52-56], however, have presented data that large alterations in pyruvate dehydrogenase interconversion by fatty acids need not necessarily involve changes in the [ATP]/[ADP]ratio. Changes in the [NADH]/[NAD^+] and [acetyl-CoA]/[CoASH] ratios were supposed to be of primary importance in the regulation of the interconversion of pyruvate dehydrogenase between its active and inactive forms [53].

Studies on pyruvate dehydrogenase complexes from bacteria, have revealed great differences in the regulation of these complexes to those of animal tissue. The bacterial pyruvate dehydrogenase complexes are not regulated by phosphorylation or dephosphorylation through a kinase or phosphatase [57,61]. The complexes of *Escherichia coli* and *A.vinelandii* are mainly regulated by the [acetyl-CoA]/[CoASH], [NADH]/[NAD^+] ratios and by the value of the energy charge [58-61]. In both complexes pyruvate shows a homotropic cooperative effect. Acetyl-CoA is a competitive inhibitor with respect to pyruvate. The inhibition by acetyl-CoA can be reversed by positive effectors like AMP, GMP, ADP, GDP and phosphate. The regulatory site for all these effectors including acetyl-CoA is supposed to be on the pyruvate dehydrogenase component [60,61]. In our laboratory, Bresters *et al* [61] observed a strong activation of the *A.vinelandii* complex by sulphate. Bisswanger [60] showed that the inactive complex from *E.coli* is activated more efficiently by TPP than by pyruvate. In both complexes NADH turned out to be a competitive inhibitor with respect to NAD^+. The site of inhibition by NADH occurs by means of overreduction by two extra reducing equivalents of the FAD of the lipoamide dehydrogenase component [81]. Bresters *et al* [61] showed that also NADPH is a weak competitive inhibitor with respect to NAD^+.

2.3. Molecular organisation

The composition and organisation of mammalian pyruvate dehydrogenase complexes have been studied in detail [6,13,26,30-33]. The molecular weight of mammalian pyruvate dehydrogenase complexes is about 10^7. The complexes of bovine heart and kidney consist of a "core" of approximately sixty identical lipoyltransacetylase units, each containing one moiety of covalently bound lipoate, to which the pyruvate dehydrogenase and lipoamide dehydrogenase components are bound [6,30,31]. The amount of covalently bound lipoate is in view of the lipoate content of transacetylase from *E.coli* [3,36,70] as will be discussed later, questionable.

The molecular weight of the transacetylase "core" is about 3×10^6 and that of the subunits is approximately 70000. The lipoamide dehydrogenase component is a dimer and consists of two identical polypeptide chains with a molecular weight of approximately 53000. When isolated, the pyruvate dehydrogenase component has a molecular weight of bout 154000 and appears to be a tetramer of subunit structure $\alpha_2\beta_2$. The molecular weight of the α-subunit is approximately 41000 and of the β-subunit is approximately 36000 [6,26-28]. Bovine

heart pyruvate dehydrogenase complex appears to consist of 60 transacetylase chains, 30 pyruvate dehydrogenase tetramers and 10-12 lipoamide dehydrogenase chains [26]. In the bovine kidney complex the transacetylase "core", although of the same size as the bovine heart complex, appears to be able to bind only 20 pyruvate dehydrogenase tetramers [33]. In addition to these components, 5 molecules each of pyruvate dehydrogenase kinase and phosphatase are associated with the mammalian complexes [26]. The kinase is tightly bound to the transacetylase "core" of the complex [13]. The phosphatase is less tightly bound than the kinase and can be separated from the other components of the complex by high-speed centrifugation [7,21,23].

Reed and co-workers [3,4,20,30,62-66] have performed extensive studies on the pyruvate dehydrogenase complex from *E.coli* Crookes. The studies showed [3,20] that the *E.coli* complex has a molecular weight of about 4.6×10^6. Biochemical and electron-microscopical of analysis suggests that the complex consists of a lipoyltransacetylase core with a molecular weight of about 1 million - composed of identical polypeptide chains with molecular weight of 65000 to 70000 -, which is organized in a cube-like structure to which pyruvate dehydrogenase dimers are distributed symmetrically on the edges of the cube and lipoamide dehydrogenase dimers are located on the six faces of the cube. It was proposed [11] that the complex contains 12 pyruvate dehydrogenase dimers each with molecular weights of about 183000 and 6 lipoamide dehydrogenase dimers each with molecular weights of about 112000. The pyruvate dehydrogenase dimer as well as the lipoamide dehydrogenase dimer consists of identical polypeptide chains. Vogel *et al* [67,68] isolated a "core" complex from *E.coli* K12 after removing excess of pyruvate dehydrogenase. It was proposed that this complex has a molecular weight of about 3.8×10^6 and consists of 16 pyruvate dehydrogenase chains, 16 transacetylase chains and 16 lipoamide dehydrogenase chains. In addition Perham and co-workers [69-71] presented evidence that the complex from *E.coli* K12 generally shows a chain-stoichiometry of 2:1:1 for the pyruvate dehydrogenase, lipoyltransacetylase and lipoamide dehydrogenase respectively. Hence several conflicting versions concerning the number of polypeptide chains for the intact pyruvate dehydrogenase from *E.coli* are current.

The FAD contents usually found are in agreement with the number of 12 lipoamide dehydrogenase chains per mole of complex with a molecular weight of 4.6×10^6. In cases where different chain stoichiometries were proposed, the FAD content was either not determined [82] or deviating values were found [68].

In addition an ambiguity is observed in the determination of the number

of lipoyl groups per mole pyruvate dehydrogenase complex. Koike *et al* [3] found a content of 11-12 nmol lipoic acid per mg complex from *E.coli* Crookes, which suggests that there are 51-55 lipoyl groups present per mole complex based on a molecular weight of 4.6×10^6. Later, however, the same group reported [63], a lipoic acid content of 5.2 - 5.7 nmol per mg complex from *E.coli* Crookes which indicates the presence of 24-26 lipoyl groups per mole complex. Recently Danson and Perham [70] measured two lipoyl groups present per transacetylase chain for the *E.coli* K12 complex. Thus number reported by Koike [3] suggests the presence of 24 lipoyltransacetylase chains in the *E.coli* Crookes; that reported by Eley *et al* [63] on the other hand, suggests 12 chains.

The *E.coli* Crookes complex has an FAD content of 2.4 - 2.8 nmol/mg protein [3,72]. Which is consistent with the presence of 6 lipoamide dehydrogenase dimers, each having one FAD per polypeptide chain.

In our laboratory studies were performed on the composition and the interaction of the enzyme components of the pyruvate dehydrogenase complex from *A.vinelandii* [14,73-76]. The *A.vinelandii* complex has a molecular weight of $1.0 - 1.2 \times 10^6$ and a FAD content of approximately 1.6 - 1.8 nmol/mg protein [73]. This indicates that the complex contains 1 molecule of lipoamide dehydrogenase dimer with a molecular weight of 112000. Furthermore, the complex from *A.vinelandii* contains pyruvate dehydrogenase showing identical polypeptide chains with molecular weight 89000 and a lipoyltransacetylase showing two non-identical chains: of molecular weights approximately 59000 and 82000 each [73]. Mn^{2+}-binding studies suggest that there might be eight pyruvate dehydrogenase molecules in the complex [74].

The complexes from *A.vinelandii* and *E.coli* were also studied by means of specific labeling of the lipoyl moieties with spinlabels and fluorescent labels in order to have a clear insight into the interaction of the enzyme components with each other [75-77]. These studies have provided the first physico-chemical evidence consistent with the hypothesis that the lipoyl arm rotates among the catalytic centres of the enzymes as proposed by Koike *et al* [4]. However, binding studies with thiochrome diphosphate - a fluorescent competitive inhibitor of TPP - to the complexes from *E.coli* and *A.vinelandii* indicate that the TPP binding site of pyruvate dehydrogenase is at least 4 nm from the flavine component of lipoamide dehydrogenase [76,78]. Likewise, studies with several fluorescent sulfhydryl labels, which were specifically bound to the lipoyl moiety of the lipoyltransacetylase component of both complexes, indicate that the active sites of all the component enzymes in the pyruvate dehydrogenase complexes

from *E.coli* and *A.vinelandii* are at least 4 nm apart [76,77].

In the next chapters data will be reported from studies performed, to obtain more insight into the chain stoichiometry, structure and function of the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* Crookes.

2.4. REFERENCES

1. Jagannathan, V. and Schweet, R.S. (1952) J.Biol.Chem. *196*, 551.
2. Goldman, D.S. (1958) Biochim.Biophys.Acta. *27*, 513.
3. Koike, M., Reed, L.J. and Caroll, W.R. (1960) J.Biol.Chem. *235*, 1924.
4. Koike, M., Reed, L.J. and Carroll, W.R. (1963) J.Biol.Chem. *238*, 30.
5. Hayakawa, T., Hirashima, M., Ide, S., Hamada, M. Okabe, K. and Koike, M. (1966) J.Biol.Chem. *241*, 4964.
6. Ishikawa, E., Oliver, R.M. and Reed, L.J. (1966) Proc.Natl.Acad.Sci. USA *56*, 534.
7. Linn, T.C., Pettit, F.H. and Reed, L.J. (1969) Proc.Natl.Acad.Sci. USA *62*, 234.
8. Harding, R.W., Carline, D.F. and Wagner, R.P. (1970) Arch.Biochem.Biophys. *138*, 653.
9. Severin, S.A., Khailova, L.S. and Gamazkova, V.S. (1970) Abstracts of the 8th Internatl. Congress of Biochemistry, Symposium no. *3,4*, 117.
10. Vogel, O. and Henning, U. (1971) Eur.J.Biochem. *18*, 103.
11. Eley, M.H., Namihira, G., Hamilton, L., Munk, P. and Reed, L.J. (1972) Arch.Biochem.Biophys. *152*, 655.
12. Hirabayashi, T. and Harada, T. (1972) J.Biol.Chem. *71*, 797.
13. Linn, T.C., Pelley, J.W., Pettit, F.M., Hucho, F., Randall, D.D. and Reed, L.J. (1972) Arch.Biochem.Biophys. *148*, 327.
14. Bresters, T.W., De Abreu, R.A., de Kok, A., Visser, J. and Veeger, C (1975) Eur.J.Biochem. *59*, 335.
15. Reid, E.E., Thompson, P., Lyttle, C.R. and Dennis, D.T. (1977) Plant Physiol. *59*, 842.
16. Roche, T.E. and Cate, R.L. (1977) Arch.Biochem.Biophys. *183*, 644.
17. Olson, M.S., Dennis, S.C., Routh, C.A. and Debuysere, M.S. (1978) Arch. Biochem.Biophys. *187*, 121.
18. Gunsalus, I.C. (1954) The Mechanism of Enzyme Action (W.B. McElroy and H.B. Glass eds., John Hopkins Press, Baltimore), 545.
19. Reed, L.J. (1960) The Enzymes (P.B. Boyer, H.Lardy and K. Myrback eds., Acad. Press, New York) *3*, 195.

20. Reed, L.J. and Cox, D.J. (1966) Ann.Rev.Biochem. *35*, 57.
21. Linn, T.C., Pettit, F.M., Hucho, F. and Reed, L.J. (1969) Proc.Natl. Acad.Sci. USA *64*, 227.
22. Wieland, O.H. and von Jagow-Westermann, B. (1969) Febs Lett. *3*, 271.
23. Wieland, O.H. and Siess, E.A. (1970) Proc.Natl.Acad.Sci. USA *65*, 947.
24. Siess, E.A., Witmann, J. and Wieland O.H. (1971) Z.Physiol.Chem. *352*, 447.
25. Wieland, O.H., Hartmann, U. and Siess, E.A. (1972) Febs Lett. *27*, 240.
26. Barrera, C.R., Namihira, G., Hamilton, L., Munk, P., Eley, M. and Reed, L.J. (1972) Arch.Biochem.Biophys. *148*, 343.
27. Khailova, L.S., Feigina, M.M., Georgui, S. and Severin, S.E. (1972) Biokhimiya *37*, 1312.
28. Severin, S.E. and Feigina, M.M. (1977) Adv.Enzyme Regul. *15*, 1.
29. Cate. R.L. and Roche, T.E. (1978) J.Biol.Chem. *253*, 496.
30. Reed, L.J. and Oliver, R.M. (1968) Brookhaven Symp.Biol. *21*, 397.
31. Hayakawa, T., Kanzaki, T., Kitamura, T., Fukuyoshi, Y., Sakurai, Y., Koike, K., Suetmatsu, T. and Koike, M. (1969) J.Biol.Chem. *244*, 3660.
32. Roche, T.E. and Reed, L.J. (1972) Biochem.Biophys.Res.Commun. *48*, 840.
33. Reed, L.J. (1974) Accounts of Chem.Res. *7*, 40.
34. Denton, R.M., Randle, P.J. and Martin B.R. (1972) Biochem.J. *128*, 161.
35. Siess, E.A. and Wieland O.H. (1972) Eur.J.Biochem. *26*, 96.
36. Hucho, F., Randall, D.D., Roche, T.E., Burgett, M.W., Pelley, J.W. and Reed, L.J. (1972) Arch.Biochem.Biophys. *151*, 328.
37. Denton, R.M., Randle, P.J., Bridges, B.J., Cooper, R.H., Kerbey, A.L., Pask, H.T., Severson, D.L., Stansbie, D. and Whitehouse, S. (1975) Mol. Cell.Biochem. *9*, 27.
38. Garland, P.B., Newsholme, E.A. and Randle, P.J. (1964) Biochem.J. *93*, 409.
39. Bremer, J. (1969) Eur.J.Biochem. *8*, 535.
40. Tsai, C.S., Burgett, M.W. and Reed, L.J. (1973) J.Biol.Chem. *248*, 8348.
41. Kerbey, A.L., Radcliff, P.M. and Randle, P.J. (1977) Biochem.J. *164*, 509.
42. Pettit, F.H., Roche, T.E. and Reed, L.J. (1972) Biochem.Biophys.Res.Commun. *49*, 563.
43. Wieland, O.H., Siess, E.A., Weiss, L., Löffler, G., Patzelt, C., Portenhauser, R., Hartmann, V. and Schirmann, A. (1973) Symp.Soc.Exp.Biol. *27*, 371.
44. Cooper, R.H., Randle, P.J. and Denton, R.M. (1974) Biochem.J. *143*, 625.
45. Randle, P.J., Denton, R.M., Pask, H.T. and Severson, D. (1974) Biochem. Soc.Symp. *39*, 75.

3. TRANSFORMATION OF THE 4-COMPONENT PYRUVATE DEHYDROGENASE COMPLEX FROM *AZOTOBACTER VINELANDII* INTO A 3-COMPONENT COMPLEX

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1. Introduction

Blue dextran-Sepharose has been used with success as a matrix for affinity chromatography of proteins which contain the so-called dinucleotide fold [1–4]. In some other proteins the affinity seems to be due to ionic or $\pi-\pi$ interactions [1,5]. This matrix seems very suitable for affinity chromatography of keto-acid dehydrogenase complexes because their activities are NAD^+-dependent and regulated by a variety of nucleotides.

In contrast to other bacterial keto-acid dehydrogenase complexes, the pyruvate dehydrogenase complex from *Azotobacter vinelandii* contains a fourth component with transacetylase activity [6]. We have called this component, low molecular weight lipoyl-transacetylase, to differentiate it from the transacetylase usually present, here called, high molecular weight lipoyl-transacetylase. This fourth component is co-purified in a constant ratio to the other components of the complex and is an active constituent of the complex, i.e., it does accept the acetyl group at relatively high pyruvate concentrations [7,8]. Crosslinking experiments [8] with diimidates have shown that the low molecular weight lipoyl-transacetylase interacts with the pyruvate dehydrogenase component of the complex.

Here we present results describing the behaviour of the pyruvate dehydrogenase complexes from *Az. vinelandii* and *Escherichia coli* Crookes on blue dextrane–Sepharose 4B columns. It is shown that the 4-component complex from *Az. vinelandii* binds strongly to the column through its low molecular weight lipoyl-transacetylase. Upon elution with 0.6 M KCl, a 'normal' 3-component complex is obtained. Some of the properties of this complex are described.

2. Materials and methods

The pyruvate dehydrogenase complex from *Az. vinelandii* (ATCC 478) was prepared according to Bresters et al [6]. The pyruvate dehydrogenase complex from *E. coli* Crookes was prepared as described by Eley et al. [9]. Both complexes were purified and stored in the presence of EDTA and *p*-phenylmethane sulfonylfluoride. Sepharose 4B and blue dextran were obtained from Pharmacia. Blue dextran was covalently coupled to activated Sepharose 4B using the procedure described by Ryan and Vestling [10]. A blue dextran-Sepharose 4B column (dimension 1.0 × 5 cm, void vol. approx. 8 ml) was used. The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.0. About 15 mg pyruvate dehydrogenase complex in 0.5 ml was applied to the column. The column was washed with 50 mM phosphate buffer, pH 7.0, until protein absorbance and enzyme activity were no longer detectable in the effluents (usually 80 ml) and then eluted with 0.6 M potassium chloride in 50 mM phosphate buffer pH 7.0.

Pyruvate-NAD^+ reductase (overall) activity, pyruvate-$K_3Fe(CN)_6$ reductase (pyruvate dehydrogenase) activity, reduced lipoate-CoA transacetylase activity and lipoamide dehydrogenase activity were assayed as described earlier [6,11–13].

Sedimentation and diffusion experiments were performed with an MSE analytical ultracentrifuge.

The sedimentation runs were carried out in 50 mM potassium phosphate buffer, pH 7.0, at 20°C. Scans were made with time intervals of 360 s with rotor speeds of 30 000 rev/min and 35 000 rev/min for sedimentation experiments and time intervals of 900 s after reaching equilibrium with a rotor speed of 2650 rev/min for diffusion experiments. The sedimentation and diffusion coefficients were calculated according to Elias [14]. The molecular weights were calculated from sedimentation coefficients and diffusion coefficients assuming partial specific vol. 0.75 $ml.g^{-1}$.

Sodium dodecylsulphate–polyacrylamide gel electrophoresis was performed according to Laemmli [15].

3. Results and discussion

3.1. *Behaviour of the complexes from* Az. vinelandii *and* E.coli *on the blue dextran–Sepharose 4B column*

The complex from *Az. vinelandii* binds strongly to the blue dextran-Sepharose 4B column, equilibrated with 50 mM potassium phosphate buffer pH 7.0. It elutes as a sharp peak with 0.6 M potassium chloride (fig.1). A 9.1 mg yield is obtained from 15 mg pyruvate dehydrogenase complex. The complex eluted from the column consists of pyruvate dehydrogenase, high molecular weight lipoyl-transacetylase and lipoamide dehydrogenase as revealed by sodium dodecylsulphate–gel electrophoresis. In none of the fractions could the low molecular weight lipoyl-transacetylase be detected. The low molecular weight lipoyl-transacetylase remains bound to the column even after elution with 2.5 M KCl. A fraction containing pyruvate dehydrogenase and low molecular weight lipoyl-transacetylase was eluted when 5% Triton X-100 was applied to the column after elution with 0.6 M potassium chloride. In this fraction only transacetylase activity could be measured. Apparently the 4-component complex is bound to the blue dextran through the low molecular weight lipoyl-transacetylase component. At high salt concentrations interactions between this component and other components of the complex are weakened. Because no appreciable elution was seen with nucleotides such as NAD^+, CoA or AMP, binding is probably not due to the presence of a dinucleotide fold in the low-molecular weight lipoyl-transacetylase but rather to an ionic or $\pi-\pi$ type of interaction with the dye. The low molecular weight lipoyl-transacetylase has a very high content of aromatic amino acid residues as can be concluded from its ultraviolet absorption. Upon

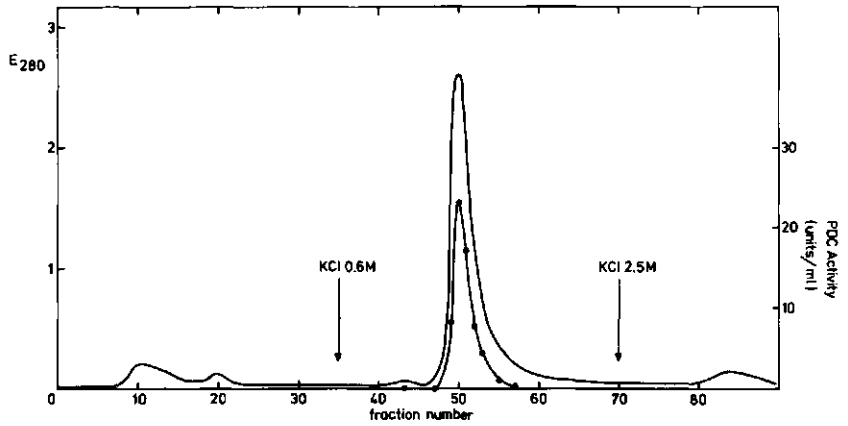

Fig.1. Elution pattern of *Az. vinelandii* pyruvate dehydrogenase complex (15 mg) on a blue dextran–Sepharose 4B column. The complex was eluted as described in Materials and methods. Fractions of 2.5 ml were collected at an elution rate of 30 ml/h. (——) Absorption at 280 nm; (–•–•–) Pyruvate-NAD^+ reductase activity in μmol $NADH.min^{-1}.ml^{-1}$.

rechromatographing the eluted complex, after dialysis against 50 mM potassium phosphate buffer pH 7.0, under identical conditions, more than 90% of the activity appears in the void volume. The pyruvate dehydrogenase complex from *E. coli* Crookes also elutes in the void volume, even in buffers of low ionic strength. A small part of the complex, about 10% of the activity is bound and not eluted below a concentration of 0.6 M KCl. A protein with mol. wt 63 000, often found in the complex isolated according to Eley et al. [9] was removed by this procedure, but the complex in the void still contained this protein.

3.2. *Properties of the three component complex from* Az. vinelandii

The values for the overall activities and the partial activities of the 3-component complex and of the original 4-component complex, are given in table 1.

The removal of the low molecular weight lipoyl-transacetylase and part of the pyruvate dehydrogenase apparently has no effect on the overall reaction. Partial removal of the pyruvate dehydrogenase from the *E. coli* complex was also reported not to influence the overall activity [16]. In this case there is a clear connection between the 'excess' pyruvate dehydrogenase and the low molecular weight lipoyl-transacetylase. It may well be that such a connection also exists in the *E. coli* complex, but has hitherto escaped attention.

The slight increase in lipoyl-transacetylase activity of the 3-component complex indicates that the contribution of the low molecular weight lipoyl-transacetylase to the total activity is probably very low. The total activity in the Triton X-100 eluate is only 5.5 units. No inactivation of the transacetylase has been observed during the time period necessary for the experiment. Because we found that the low molecular weight lipoyl-transacetylase is rapidly acetylated [7] this suggests that the physiological acetyl acceptor might be different from CoA or alternatively that the enzyme reacts poorly with CoASAc in the assay. The FAD content of the 3-component complex (table 2) has increased and is comparable with that of the *E. coli* complex [12,17]. A 3-fold increase in lipoamide dehydrogenase activity of the 3-component complex is found which is much larger than the increase in FAD-content. A further 4-fold increase is found in the pure enzyme [18]. This again indicates a functional association of the low molecular weight lipoyl-transacetylase with the complex. This could be due to an increased accessibility of the substrate lipoamide, which is of no significance for the overall reaction. Alkonyi et al. [19] observed that the unresolved lipoamide dehydrogenase of the pigeon breast-muscle pyruvate dehydrogenase complex is completely inactive in the NADH: lipoamide reductase assay, in contrast to the resolved enzyme.

From the sedimentation and diffusion coefficients, mol. wt 760 000 was calculated for the 3-component complex, which is in good agreement with the molecular weight calculated from the FAD content assuming lipoamide dehydrogenase to be a dimer.

Table 1
Partial and overall activities of 4- and 3-component complexes from *Az. vinelandii*, before and after blue dextran–Sepharose chromatography, respectively

Enzyme	4-Component complex			3-Component complex		
	(Units/mg protein)	(Units/μmol FAD)	Total units	Units/mg protein	Units/μmol FAD	Total units
Pyruvate-NAD^+ reductase	7.0	4100	105	12.0	4450	109
Pyruvate-$Fe(CN)_6$ reductase	0.12	70	1.8	0.13	40	1.2
Lipoyl-trans-acetylase	3.4	2000	51	7.4	2700	67
Lipoamide dehydrogenase	1.9	1100	28.5	9.5	3500	86.5

Table 2
Comparison of some physical constants of the two pyruvate dehydrogenase complexes from *Az. vinelandii*

Physical constants	4-Component pyruvate dehydrogenase complex from *Az. vinelandii*	3-Component pyruvate dehydrogenase complex from *Az. vinelandii*
$s_{20,w}$	19 S [6] (protein conc. > 0.4 mg/ml) 16 S (protein conc. < 0.2 mg/ml)	15 S (conc. 0.7 mg protein/ml) 14 S (conc. 0.2 mg/ml)
$D_{20,w}$	1.96×10^{-7} cm^2.s^{-1} [6]	1.90×10^{-7} cm^2.s^{-1}
Mol. wt (from s- and D-values and $\bar{v}$ 0.75)	970 000 [6]	760 000 (15 S)
FAD content	1.77 nmol FAD/mg protein	2.6–2.8 nmol/mg protein
Mol. wt (calculated from FAD-content)[a]	1 130 000	740 000

[a] It was assumed that lipoamide dehydrogenase is present as a dimer in the complex

Other properties of the 3-component complex, such as the Hill coefficient for the overall reaction, the stimulation by AMP and the inhibition by CoASAc were not different from the original complex [20].

The results indicate that the low molecular weight lipoyl-transacetylase and part of the pyruvate dehydrogenase form a separate entity within the complex, the function of which will now be investigated.

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References

[1] Thompson, S. T., Cass, K. H. and Stellwagen, E. (1975) Proc. Natl. Acad. Sci. USA 72, 669–672.
[2] Buehner, M. Ford, G. C., Moras, D., Olsen, K. W. and Rossmann, M. G. (1973) Proc. Natl. Acad. Sci. USA 70, 3052–3054.
[3] Bryant, T. N., Watson, H. C. and Wendell, P. L. (1974) Nature 245, 14–17.
[4] Drenth, J., Hol W. S. T., Jansonius, J. N. and Koekoek, R. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 107–116.
[5] Jankowski, W. J., Von Münchhausen, W., Suikowski, E. and Carter, W. A. (1976) Biochemistry 15, 5182–5187.
[6] Bresters, T. W., De Abreu, R. A., De Kok, A., Visser, T. and Veeger, C. (1975) Eur. J. Biochem. 59, 335–345.
[7] De Abreu, R. A., De Kok, A., De Graaf-Hess, A. C. and Veeger, C. (1977) submitted.
[8] De Abreu, R. A. (1976) Abst. 175, 10th Int. Cong. Biochem., Hamburg.
[9] Eley, M. H., Genshin, N., Hamilton, L., Munk, P. and Reed, L. J. (1972) Arch. Biochem. Biophys. 152, 655–669.
[10] Ryan, L. D. and Vestling C. S. (1974) Arch. Biochem. Biophys. 160, 279–284.
[11] Schwartz, E. R. and Reed, L. J. (1970) Biochemistry 9, 1434–1439.
[12] Reed, L. J. and Willms, C. R. (1965) Meth. Enzymol. IX, 247–265.
[13] Massey, V. and Veeger, C. (1961) Biochim. Biophys. Acta 48, 33–47.
[14] Elias, H. G. (1961) Ultrazentrifugen-Methoden, 2 völlig rev. Aufl., Beckman Instruments G.m.b.H., München.
[15] Laemmli, U. K. (1970) Nature (London) 227, 680–685.
[16] Vogel, O., Hoehn, B. and Henning, U. (1972) Eur. J. Biochem. 30, 354–360.
[17] Speckhard, D. C. and Frey, P. A. (1975) Biochem. Biophys. Res. Commun. 62, 614–620.
[18] Veeger, C., Fehrmann, H., Visser, A. J. W. G., Grande, H. J., Müller, F. and Santema, J. (1975 in: Reactivity of Flavins (Yagi, K. ed) p. 119, University of Tokyo Press.
[19] Alkonyi, I., Bolygo, E., Gyocsi, L. and Szabo, D. (1976) Eur. J. Biochem. 66, 551–557.
[20] Bresters, T. W., De Kok, A. and Veeger, C. (1975) Eur. J. Biochem. 59, 347–353.

4. DETERMINATION OF THE CHAIN STOICHIOMETRIES FROM THE NUMBER OF REACTIVE SULFHYDRYL GROUPS IN THE PYRUVATE DEHYDROGENASE COMPLEXES OF *AZOTOBACTER VINELANDII* AND *ESCHERICHIA COLI*

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The pyruvate dehydrogenase complex from *Azotobacter vinelandii* incubated with 0.05–0.7 mM [2-^{14}C]pyruvate, magnesium chloride and thiamine pyrophosphate under anaerobic conditions at 0 °C, incorporates four [^{14}C]acetyl groups per mole FAD which are bound to the high-molecular-weight lipoyl transacetylase. With 10 mM pyruvate, the low-molecular-weight lipoyl transacetylase is also labelled; to this enzyme 3–4 [^{14}C]acetyl groups are bound per mole FAD. This enzyme is not labelled when pyruvate is used in concentrations lower than 0.7 mM. The complex of *A. vinelandii* pre-labelled with non-radioactive *N*-ethylmaleimide did not incorporate label onto low-molecular-weight lipoyl transacetylase; a maximum of four acetyl groups are bound to the high-molecular weight lipoyl transacetylase.

Maximum incorporation of four [^{14}C]acetyl groups per mole FAD is found, within five seconds, on the lipoyl transacetylase component when the *Escherichia coli* complex is labelled under anaerobic conditions, also in the presence of 10 mM pyruvate.

In both complexes all incorporated acetyl groups are bound to sulfhydryl groups since they can be completely removed with hydroxylamine, by performic acid oxidation and by reaction with coenzyme A and arsenite. Under aerobic conditions a slow deacetylation is observed, which is blocked in the presence of *N*-ethylmaleimide.

The multi-enzyme complex from *A. vinelandii* pre-labelled with non-radioactive *N*-ethylmaleimide and then labelled with *N*-ethyl [2,3-^{14}C]maleimide in the presence of pyruvate, magnesium chloride and thiamine pyrophosphate incorporates under anaerobic conditions at 0 °C, even at 10 mM pyruvate, a maximum of four *N*-ethyl[^{14}C]maleimide groups per mole of FAD. The label is almost exclusively bound to the high-molecular-weight lipoyl transacetylase of the *A. vinelandii* complex.

The *E. coli* complex binds only 2–3 *N*-ethyl[^{14}C]maleimide groups per mole of FAD under these conditions. Direct labelling and correction for labelling without pyruvate yields 4–5 *N*-ethyl-[^{14}C]maleimide per mole FAD.

The low-molecular-weight lipoyl transacetylase can be removed by chromatography of the complex from *A. vinelandii* on a blue-dextran–Sepharose 4B column [De Abreu *et al.* (1977) *FEBS Lett. 82*, 89–92]. The complex eluted from the column, consisting of the other three enzyme components, binds a maximum of four [^{14}C]acetyl groups per mole of FAD, even with 10 mM pyruvate.

It is concluded that the lipoyl/FAD ratio is four in the complexes as isolated by us from both sources. The interpretation of this ratio with respect to the stoichiometries of the different enzyme components will be discussed.

The oxidative decarboxylation of pyruvate and the transfer of the formed acetyl groups to coenzyme A is catalyzed through the following sequence of reactions [2]:

$$\text{Pyruvate} + \text{thiamine-}P_2 \overset{Mg^{2+}}{\rightleftharpoons} CO_2 + \text{hydroxyethyl-thiamine-}P_2 \quad (1)$$

$$\text{Hydroxyethyl-thiamine-}P_2 + \text{lip-(S)}_2 \rightleftharpoons \text{thiamine-}P_2 + \text{Ac-}S\text{-lip-SH} \quad (2)$$

$$\text{Ac-}S\text{-lip-SH} + \text{CoA} \rightleftharpoons \text{lip-(SH)}_2 + \text{Ac-CoA} \quad (3)$$

$$\text{Lip-(SH)}_2 + NAD^+ \overset{FAD}{\rightleftharpoons} \text{lip-(S)}_2 + NADH + H^+ \quad (4)$$

tion of four acetyl groups/FAD (Fig. 1) is observed within 10–20 s; under these conditions hardly any further incorporation is observed after a 40-min incubation period.

The multi-enzyme complex of *E. coli* reacts even faster; within 5 s four acetyl groups/FAD (Fig. 1) are incorporated upon incubation with 0.05–1 mM pyruvate. The complex, further purified on calcium phosphate gel by the method of Speckhard and Frey [5], yielded the same number of acetyl groups per FAD, both in Tricine and phosphate buffer. When the complexes are incubated with 10 mM [2-^{14}C]pyruvate under anaerobic conditions at 0 °C, 7–8 acetyl groups/FAD are bound to the enzyme complex of *A. vinelandii* within 20–30 s but the number bound to the *E. coli* complex remains four.

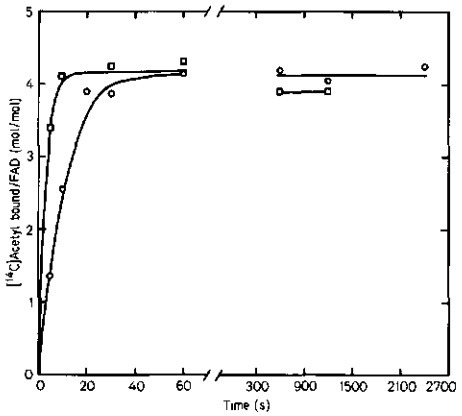

Fig. 1. *Time-dependent incorporation of [^{14}C]acetyl groups under anaerobic conditions at 0 °C, using [2-^{14}C]pyruvate as substrate.* The incubations were performed as described in Methods. (□—□) Pyruvate dehydrogenase complex from *E. coli* (6.2 μM of FAD) was labelled with 50 μM [2-^{14}C]pyruvate. (○—○) The pyruvate dehydrogenase complex from *A. vinelandii* (3.1 μM of FAD) was labelled (in the presence of Mg^{2+} and thiamine pyrophosphate) with 50 μM [2-^{14}C]pyruvate

All acetyl groups incorporated upon incubation with [^{14}C]pyruvate can be removed by treatment with hydroxylamine and by performic acid oxidation according to Barrera *et al.* [17]. Only a small part of the radioactivity (about 25%) in both complexes could be removed upon the addition of 5 mM coenzyme A. When a coenzyme A-regenerating system, consisting of citrate synthetase (2.8 U) and oxaloacetate (10 mM) was added, or phosphotransacetylase and phosphate, a further decrease in radioactivity was observed. Only in the presence of 5 mM arsenite was an almost complete deacetylation found (Table 1).

The labelled *A. vinelandii* complex was electrophoresed on dodecylsulphate/polyacrylamide gels. The distribution of label on the gels is given in Table 2. Although the data are not quantitative, since some loss of label occurs during electrophoresis, label is found on the high-molecular-weight lipoyl transacetylase after incubation with 0.3 mM [^{14}C]pyruvate, whereas label on both transacetylases is found after incubation with 10 mM [^{14}C]pyruvate (Table 2).

After pre-labelling the *A. vinelandii* complex with non-radioactive *N*-ethylmaleimide followed by incubation of the enzyme with either 0.3 mM [^{14}C]-pyruvate or with 10 mM [^{14}C]pyruvate, four acetyl groups per FAD are incorporated at both concentra-

Table 1. *Incorporation of [2-^{14}C]pyruvate under different experimental conditions into the pyruvate dehydrogenase complexes from* A. vinelandii *and* E. coli
MalNEt = *N*-ethylmaleimide

Experimental conditions	Acetyl groups bound	
	A. vinelandii	*E. coli*
	mol/mol FAD	
Incubation with 16-fold excess of [2-^{14}C]pyruvate to FAD, anaerobic, 0 °C	4.3±0.4 (from 28 exps)	4.0±0.3 (from 12 exps)
Incubation with 10 mM [2-^{14}C]pyruvate, anaerobic, 0 °C	7.4±0.7 (from 24 exps)	3.8±0.6 (from 9 exps)
Preincubation with MalNEt, then incubated with 16-fold excess of [2-^{14}C]pyruvate to FAD, anaerobic, 0 °C	3.8±0.2 (from 2 exps)	
Preincubation with MalNEt, then incubated with 10 mM [2-^{14}C]pyruvate, anaerobic, 0 °C	4.4±0.3 (from 2 exps)	
After performic acid		0.08–0.10
After M hydroxylamine		<0.00
After 5 mM coenzyme A and 5 mM arsenite		0.7

Table 2. *Distribution of [^{14}C]acetyl groups on the subunits of the pyruvate dehydrogenase complex from* A. vinelandii *after labelling with [2-^{14}C]-pyruvate*
Results of two experiments are given

Polypeptide chain	Labelling with					
	0.3 mM pyruvate (spec. act. 8.2 Ci/mol)			10 mM pyruvate (spec. act. 0.243 Ci/mol)		
	[^{14}C]acetyl	FAD	[^{14}C]acetyl/FAD	[^{14}C]acetyl	FAD	[^{14}C]acetyl/FAD
	dis./min	pmol	mol/mol	dis./min	pmol	mol/mol
Pyruvate dehydrogenase	188	76.8	0.14	<0.00	442	<0.00
	0	128	0.00	101	361	0.52
High-M_r transacetylase	2259	76.8	1.6	309	442	1.3
	5565	128	2.4	639	361	3.3
Low-M_r transacetylase	366	76.8	0.27	475	442	2.0
	<0	128	<0.0	343	361	1.8
Front	3089	76.8	2.2	534	442	2.3
	1814	128	0.80	898	361	4.7

Table 3. *Reaction of pyruvate dehydrogenase complex from* A. vinelandii, *from the blue-dextran – Sepharose column, with [2-^{14}C]pyruvate*
^{14}C values were corrected for the blank

[2-^{14}C]Pyruvate		FAD content	^{14}C	[^{14}C]Acetyl bound	[^{14}C]Acetyl/FAD
Concn	spec. act.				
mM	Ci/mol	pmol	dis./min	pmol	mol/mol
0.3	8.2	70	5000	287.2	4.1
			5347	306.4	4.4
10	1.61	60	799	226	3.8
			767	217	3.6
			754	213	3.6

tions of pyruvate (Table 1). The three-component complex of *A. vinelandii*, obtained after chromatography on a blue-dextran – Sepharose 4B column, incubated either with 0.3 mM [^{14}C]pyruvate or with 10 mM [^{14}C]pyruvate, binds in both cases four acetyl groups per FAD (Table 3).

Incorporation of acetyl groups into the multienzyme complexes of both organisms was also studied under aerobic conditions at 0 °C with [2-^{14}C]pyruvate. Under these conditions loss of label (Fig. 2) is observed. No difference is observed between experiments in which enzyme is labelled from the beginning under aerobic conditions and those experiments in which enzyme was labelled for 1 min under anaerobic conditions and then left to stand aerobically. When *N*-ethylmaleimide was added after acetylation, loss of label under aerobic conditions was no longer observed.

Labelling with N-*Ethyl[2,3-^{14}C]maleimide*

The pyruvate dehydrogenase complexes from *A. vinelandii* and from *E. coli* were pre-labelled with 1 mM *N*-ethylmaleimide for 1 h at 0 °C. The prelabelled enzyme from *A. vinelandii* was incubated with 1 mM *N*-ethyl[2,3-^{14}C]maleimide, 5 mM Mg^{2+}, 0.5 mM thiamine pyrophosphate and either 0.3 mM pyruvate or with 10 mM pyruvate. After separation of the components on 7.5% polyacrylamide gels, 75% of the total label is found on the high-molecular-weight lipoyl transacetylase (approx. 4 mol/FAD); a small, but reproducible part is found on the pyruvate dehydrogenase and the lipoamide dehydrogenase (Table 4). When the *A. vinelandii* complex is incubated according to the conditions of Danson and Perham [8] with 1 mM *N*-ethyl [2,3-^{14}C]maleimide, 1 mM pyruvate, 1 mM NAD^+, 5 mM Mg^{2+} and 0.5 mM thiamine pyrophosphate for 20 min at 0 °C (rest activity 5%), we find 6.2 ± 0.8 groups bound per FAD in the presence of pyruvate and 2.3 ± 0.3 groups bound in the absence of pyruvate. Thus under these conditions also four *N*-ethyl[2,3-^{14}C]maleimide groups are bound per mole FAD. The pyruvate dehydrogenase complex from *E. coli* binds 2–3 *N*-ethylmaleimides per mole FAD when the complex was pre-treated with *N*-ethylmaleimide. When incubated according to the method of Danson and Perham, *i.e.* aerobically and in the

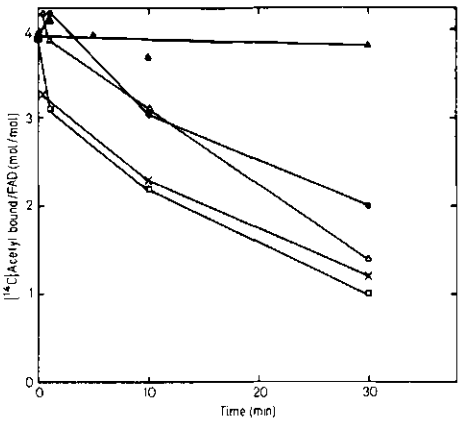

Fig. 2. *Time-dependent loss of [^{14}C]acetyl groups under aerobic conditions at 0 °C, using [2-^{14}C]pyruvate as substrate.* For conditions see Methods. (× ×) Complex of *E. coli* (25 μM of FAD) was incubated, under aerobic conditions, with 0.2 mM [2-^{14}C]pyruvate. (□—□) Complex of *E. coli* (25 μM of FAD) was incubated for 1 min with 0.2 mM [2-^{14}C]pyruvate under anaerobic conditions, then it was incubated under aerobic conditions. (△ –△) Complex of *A. vinelandii* (11.6 μM of FAD) was incubated, under aerobic conditions, with 0.2 mM [2-^{14}C]pyruvate. (● –●) Complex of *A. vinelandii* (11.6 μM of FAD) was incubated for 1 min with 0.2 mM [2-^{14}C]pyruvate under anaerobic conditions, then it was incubated under aerobic conditions. (▲– ▲) Complex of *E. coli* (25 μM of FAD) was incubated for 1 min with 0.2 mM [2-^{14}C]pyruvate under anaerobic conditions, then incubated for 5 min in the presence of 1 mM *N*-ethylmaleimide, then it was incubated under aerobic conditions

Table 4. *Results from incubation of* N-*ethylmaleimide-pretreated pyruvate dehydrogenase complex from* A. vinelandii *with* N-*ethyl-[2,3-^{14}C]maleimide (spec. act. 3.7 Ci/mol), after electrophoresis on 7.5% dodecylsulphate-polyacrylamide gels*
Values were corrected for the blank

Polypeptide chain	*N*-Ethyl[^{14}C]maleimide/FAD with	
	0.05 mM pyruvate	10 mM pyruvate
	mol/mol	
Pyruvate dehydrogenase	0.8	0.8
	0.8	0.8
High-M_r transacetylase	4.6	3.1
	3.9	3.9
Low M_r transacetylase	0.0	0.0
	0.0	0.0
Lipoamide dehydrogenase	0.7	0.7
	0.6	0.9

presence of 1 mM NAD^+ for 5 min at 0 °C (rest activity 5%), 6.2 ± 0.5 groups are bound per mole FAD in the presence of pyruvate and 0.9 ± 0.1 group is bound in the absence of pyruvate, which means five *N*-ethylmaleimides bound per FAD. 4 – 5 groups per FAD were also bound when the complex was incubated under anaerobic conditions in the absence of NAD^+.

DISCUSSION

It is generally accepted that the oxidative decarboxylation of pyruvate by the pyruvate dehydrogenase complex in the absence of coenzyme A and NAD^+ leads to the formation of an *S*-acetyl-dihydrolipoyl group, covalently attached to the lipoyltransacetylase component of the complex. It seems thus possible to determine the number of lipoyl groups by reaction with [2-^{14}C]pyruvate or by reaction with labelled SH reagents. Both methods were applied here. Barrera *et al.* [17] found a good agreement between the number of radioactive acetyl groups and the number of lipoyl transacetylase chains in the kidney and heart complexes from pig, assuming 1 lipoyl group per chain. They showed furthermore a high degree of specificity of the radioactive label towards the lipoyl moiety and claimed a similar specificity for the *E. coli* complex. Since the *E. coli* lipoyl transacetylase contains only one half-cystine [4, 18], other S-S groups acting as acceptor are not present. Secondary transacetylation reactions cannot be excluded but these have to be considered unlikely, because the dihydrolipoyl group thus formed would transfer its reducing equivalents to the lipoamide dehydrogenase component. Reduction of the flavin under anaerobic conditions in the presence of pyruvate, Mg^{2+} and thiamine pyrophosphate was only observed under conditions that favor deacetylation such as the presence of coenzyme A, dithiothreitol, hydroxylamine or the presence of phosphotransacetylase and catalytic amounts of coenzyme A which are found in impure preparations of the complexes (unpublished observations).

Under our conditions, *i.e.* low pyruvate concentrations (50 μM) and anaerobic conditions, even a high excess of coenzyme A removed only a small amount of [^{14}C]acetyl groups. Apparently an equilibrium exists between acetyl-CoA and the *S*-acetyl-dihydrolipoyl moiety, which can be shifted in the direction of acetyl-CoA by the presence of arsenite.

Only indirect evidence that the [^{14}C]acetyl groups are exclusively bound to the lipoyl moiety can be given. The *S*-acetyl bond is relatively stable under acidic conditions, but difficulties arise at higher temperature and pH as used in sodium dodecylsulphate gel electrophoresis. Although variable amounts of radioactivity are found at the front of the gels (Table 2), the label is specifically bound to the transacetylase components.

The specificity of *N*-ethylmaleimide analogues for the lipoyl transacetylase component has been demonstrated by Grande *et al.* [19, 20] and Brown and Perham [21] and is confirmed in this report. The demonstration that nearly a 1 : 1 ratio is obtained by using both labelled substrate and SH reagent confirms the

Table 5. *Polypeptide chain ratios calculated from different lipoyl contents of transacetylase in the pyruvate dehydrogenase complexes from* E. coli *and* A. vinelandii, *based on a lipoyl/FAD ratio of 4*

The number of pyruvate dehydrogenase chains is calculated by difference, using for the *E. coli* complex a minimum molecular weight, based on flavin content, of 385000 ± 25000 and chain weights of 100000, 83000 and 56000 for pyruvate dehydrogenase, lipoyl transacetylase and lipoamide dehydrogenase, respectively [23]. A minimum molecular weight of 370000 ± 14000 was used for the three-component complex of *A. vinelandii* and 570000 ± 20000 for the four-component complex based on flavin content and molecular weight determination [1]. Chain weights used were 89000, 80000, 63000 and 56000 for pyruvate dehydrogenase, high-molecular-weight lipoyl transacetylase, low-molecular-weight lipoyl transacetylase and lipoamide dehydrogenase, respectively [3]. In stating the number of lipoyl residues per chain it was assumed that the low-molecular-weight transacetylase contains the same amount of lipoyl residues as the high-molecular-weight form. In the case of the four-component *A. vinelandii* complex the number of transacetylase chains per lipoamide dehydrogenase applies to the high-molecular-weight form as well as to the low-molecular-weight form

Lipoyl residues per chain (assumed)	Transacetylase chains per lipoamide dehydrogenase chain (calculated)	Calculated number of pyruvate dehydrogenase chains per lipoamide dehydrogenase chain		
		E. coli complex	*A. vinelandii*	
			3-component complex	4-component complex
1	4	−0.3 − 0.2	−0.2 − 0	−0.9 − −0.4
2	2	1.4 − 2.0	1.6 − 1.9	2.4 − 2.8
4	1	2.2 − 2.7	2.4 − 2.8	3.9 − 4.4

idea that only the lipoyl moiety of the high-molecular-weight *A. vinelandii* transacetylase is labelled at low pyruvate concentrations. The inhibition by *N*-ethylmaleimide of the aerobic deacetylation reaction can be understood considering that *N*-ethylmaleimide reacts with the free −SH of the *S*-acetyl-dihydrolipoyl moiety, thus blocking the reversal of the reductive acetylation reaction. At low pyruvate concentrations the reversed reaction leads to hydroxyethyl-thiamin pyrophosphate formation which is oxidized to thiamine pyrophosphate and acetate [3].

There is less evidence that the acetyl binding sites on the low-molecular-weight transacetylase are also lipoyl groups. The experiments with *N*-ethylmaleimide could indicate that the acetyl group is bound to a pre-existing −SH group, which makes the presence of an additional electron acceptor necessary. Thus it seems more likely that prelabelling an −SH group with *N*-ethylmaleimide prevents indirectly the reductive acetylation of a lipoyl group or an S-S bridge on this component. This has little or no effect on the overall activity, probably because the contribution to the total transacetylase activity of the low-molecular-weight enzyme is of the order of 10% [1].

It seems therefore reasonable to conclude that the lipoyl content of the high-molecular-weight lipoyl transacetylase in the complexes of *A. vinelandii* as well as the transacetylase of *E. coli* is four per molecule of FAD. This value is in good agreement with the data of Koike *et al.* [2], who found contents of 11 − 12 nmol of [$^{35}S_2$]lipoic acid per mg complex and 2.6 − 2.8 nmol of FAD per mg complex. Eley *et al.* [4] found, however, lipoic acid contents of 5.2 − 5.7 nmol [$^{35}S_2$]lipoic acid per mg of complex by the method of Koike *et al.* [2], without indicating the cause of the difference. Furthermore Ikeda *et al.* [22] recently reported a [^{14}C]acetyl/FAD ratio of 4 − 5 in the *E. coli* Crookes complex.

Several possibilities arise when a chain stoichiometry lipoyltransacetylase/lipoamide dehydrogenase is to be calculated from the lipoyl content. Table 5 summarizes these possibilities. It is clear that the minimum number of lipoyl residues per chain is two. A value of two lipoyl groups per transacetylase chain was recently reported by Danson and Perham [8] for the *E. coli* K 12 complex. These authors based this number on the chain stoichiometry pyruvate dehydrogenase : lipoyltransacetylase : lipoamide dehydrogenase of 1.7 : 1 : 1 found by radioactive labelling of lysine residues. Such a chain stoichiometry leads to a minimum molecular weight of 306000, which differs considerably from that of 385000 ± 25000, which can be calculated from the range of FAD contents (2.4 − 2.8 nmol FAD/mg protein) found by us and others [4, 5, 7]. It seems unlikely that the difference is due to the different strain of *E. coli* used. Speckhard and and Frey [5] found no difference in flavin content; Bates *et al.* [24] found no difference in chain stoichiometries of the complexes of the Crookes strain and of the K 12 strain. A difference in purification procedure cannot be excluded but seems unlikely, because *E. coli* complexes at different stages of purity gave identical results.

Without having determined directly the number of pyruvate dehydrogenase chains and knowing that the variability in the number of these chains, is between 1 and 2 [6, 24, 25], no choice can be made between the values of two and four lipoyl residues per chain. We arrive therefore either at a chain stoichiometry pyruvate dehydrogenase : lipoyltransacetylase : lipoamide dehydrogenase of 1.4 − 2.0 : 2 : 1 in agreement with the group of Reed, assuming two lipoyl groups per chain lipoyltransacetylase chain or of 2.2 − 2.7 : 1 : 1, in agreement with Perham's group, assuming a lipoyl content of four per chain. Since direct estimates [6, 24, 25] vary be-

tween 1 and 2 pyruvate dehydrogenase chains the ratio of 1.4 – 2.0 : 2 : 1 seems more likely.

The incorporation of four *N*-ethylmaleimide groups per mole of FAD into the three-component complex from *A. vinelandii* is consistent with the incorporation of 3 – 4 spin labels per mole of FAD we reported earlier from experiments with spin label *N*-ethylmaleimide, derivatives which are like fluorescence labels presumably bound to the high-molecular-weight transacetylase [19]. Here again either two or four lipoyl residues could be present per chain. With the four-component complex the calculations are based on the assumption that the low-molecular-weight lipoyltransacetylase, with 3 – 4 acetyl binding sites per FAD, shows a similar distribution of these sites per chain as its high-molecular-weight counterpart. Assuming four acetyl binding sites on both transacetylase chains, 3.9 – 4.4 pyruvate dehydrogenase chains can be accomodated in the four-component complex. This value seems to be in good agreement with the number of Mn^{2+} binding sites reported earlier [19]. However, it is difficult to estimate accurately the actual number of binding sites from a non-linear Scatchard plot which shows at least three types of Mn^{2+} binding sites. As is evident from the calculations of Table 5 a loss of pyruvate dehydrogenase is anticipated when converting the four-component complex into a three-component complex such as is performed upon blue-dextran – Sepharose chromatography. A loss of one third of the pyruvate dehydrogenase activity is found indeed [1], but on this basis no decision between either two or four lipoyl residues per chain can be made. Further experiments, such as Mn^{2+} binding studies with the three-component complex and direct determinations of chain ratios by sodium dodecylsulphate gel electrophoresis of the pure components will be performed to settle this problem.

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REFERENCES

1. De Abreu, R. A., De Kok, A. & Veeger, C. (1977) *FEBS Lett. 82*, 89 – 92.
2. Koike, M. & Reed, L. J. (1960) *J. Biol. Chem. 235*, 1931 – 1938.
3. Bresters, T. W., De Abreu, R. A., De Kok, A., Visser, J. & Veeger, C. (1975) *Eur. J. Biochem. 59*, 335 – 345.
4. Eley, M. H., Genshin, N., Hamilton, L., Munk, P. & Reed, L. J. (1972) *Arch. Biochem. Biophys. 152*, 655 – 669.
5. Speckhard, D. C. & Frey, P. A. (1975) *Biochem. Biophys. Res. Commun. 62*, 614 – 620.
6. Vogel, O., Hoëhn, B. & Henning, U. (1972) *Proc. Natl Acad. Sci. U.S.A. 69*, 1615 – 1619.
7. Koike, M., Reed, L. J. & Carroll, W. R. (1963) *J. Biol. Chem. 238*, 30 – 39.
8. Danson, M. J. & Perham, R. N. (1976) *Biochem. J. 159*, 677 – 682.
9. Thompson, S. Th., Cass, K. H. & Stellwagen, E. (1975) *Proc. Natl Acad. Sci. U.S.A. 72*, 669 – 672.
10. Schwartz, E. R. & Reed, L. J. (1970) *Biochemistry, 9*, 1434 – 1439.
11. Reed, L. J. & Willms, C. R. (1965) *Methods Enzymol. 9*, 247 – 265
12. Massey, V. & Veeger, C. (1960) *Biochim. Biophys. Acta, 40*, 184 – 185.
13. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680 – 685.
14. Nellis, L. P. & Bamburg, J. R. (1976) *Anal. Biochem. 73*, 522 – 531.
15. Wassink, J. H. & Mayhew, S. G. (1975) *Anal. Biochem. 68*, 609 – 616.
16. Bergmeyer, H. U. (1970) *Methoden der Enzymatischen Analyse II*, p. 1407, Verlag Chemie, Weinheim.
17. Barrera, C. M., Namihera, G., Hamilton, L., Munk, P., Eley, M. H., Linn, T. C. & Reed, L. J. (1972) *Arch. Biochem. Biophys. 148*, 343 – 358.
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24. Bates, D. L., Harrison, R. A. & Perham, R. N. (1975) *FEBS Lett. 60*, 427 – 430.
25. Reed, L. J., Petit, F. H., Eley, M. H., Hamilton, L., Collins, J. H. & Oliver, R. M. (1975) *Proc. Natl Acad. Sci. U.S.A. 72*, 3068 – 3072.

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REFERENCES

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4. Eley, M. H., Genshin, N., Hamilton, L., Munk, P. & Reed, L. J. (1972) *Arch. Biochem. Biophys. 152*, 655 – 669.
5. Speckhard, D. C. & Frey, P. A. (1975) *Biochem. Biophys. Res. Commun. 62*, 614 – 620.
6. Vogel, O., Hoëhn, B. & Henning, U. (1972) *Proc. Natl Acad. Sci. U.S.A. 69*, 1615 – 1619.
7. Koike, M., Reed, L. J. & Caroll, W. R. (1963) *J. Biol. Chem. 238*, 30 – 39.
8. Danson, M. J. & Perham, R. N. (1976) *Biochem. J. 159*, 677 – 682.
9. Thompson, S. Th., Cass, K. H. & Stellwagen, E. (1975) *Proc. Natl Acad. Sci. U.S.A. 72*, 669 – 672.
10. Schwartz, E. R. & Reed, L. J. (1970) *Biochemistry, 9*, 1434 – 1439.
11. Reed, L. J. & Willms, C. R. (1965) *Methods Enzymol. 9*, 247 – 265
12. Massey, V. & Veeger, C. (1960) *Biochim. Biophys. Acta, 40*, 184 – 185.
13. Laemmli, U. K. (1970) *Nature (Lond.) 227*, 680 – 685.
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16. Bergmeyer, H. U. (1970) *Methoden der Enzymatischen Analyse II*, p. 1407, Verlag Chemie, Weinheim.
17. Barrera, C. M., Namihera, G., Hamilton, L., Munk, P., Eley, M. H., Linn, T. C. & Reed, L. J. (1972) *Arch. Biochem. Biophys. 148*, 343 – 358.
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22. Speckhard, D. C., Ikeda, B. H., Wong, S. S. & Frey, P. A. (1977) *Biochem. Biophys. Res. Commun. 77*, 708 – 713.
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5. CROSSLINKING STUDIES WITH THE PYRUVATE DEHYDROGENASE COMPLEXES FROM *AZOTOBACTER VINELANDII* AND *ESCHERICHIA COLI*

5.1. SUMMARY

The spatial arrangements of the individual components of the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* have been studied by crosslinking the whole complex with diimido esters of different chain lengths and with dimethyl - 3,3'-dithiobispropionimidate, a bisalkylimidate containing a cleavable disulfide linkage between the two reactive groups. The results indicate for the *A.vinelandii* complex distances of less than 0.5 nm between the lysine groups of the peptide chains of lipoamide dehydrogenase and high-molecular weight transacetylase and of 0.5 - 0.9 nm between the lysine groups of the peptide chains of pyruvate dehydrogenase and low-molecular-weight transacetylase. No interaction between the other chains was observed.

After removal of the low-molecular-weight transacetylase from the complex from *A.vinelandii* [1], the resulting three-component complex showed distances between the peptide chains of the transacetylase and those of either the pyruvate dehydrogenase or lipoamide dehydrogenase of less than 0.5 nm, identical with those observed for the *E.coli* complex. Interaction between pyruvate dehydrogenase and lipoamide dehydrogenase was observed, but only in the three component complex from *A.vinelandii*.

With diimidates of longer chain lengths, such as dodecyl diimidate and with dimethyl - 3,3'-dithiobispropionimidate a pyruvate dehydrogenase dimer was formed upon reaction with the two *A.vinelandii* complexes. In the *E.coli* complex crosslinking between two pyruvate dehydrogenase monomers was only observed with dodecyl diimidate under non-dissociating conditions, *i.e.* 50 mM phosphate buffer pH 8.0. In the presence of pyruvate, Mg^{2+} and TPP extensive crosslinking with diimidates takes place between the transacetylase chains of the *E.coli* complex and also between the high-molecular-weight transacetylase chains of the *A.vinelandii* complex. This indicates a conformational change of the transacetylase core induced by reaction of substrates with the pyruvate dehydrogenase component, exposing lysyl residues previously hidden.

Crosslinking with N,N'-o-phenyl-dimaleimide of the 4-component *A.vinelandii*

complex, pre-treated with N-ethylmaleimide, in the presence of pyruvate, Mg^{2+} and TPP shows a close positioning of the SH-groups generated by reductive acetylation of the transacetylase isoenzymes (0.6 nm). Although during crosslinking of the *E.coli* complex, under identical conditions, a large decrease of lipoyl transacetylase monomers was observed no homologous dimer formation took place. Instead a dimer was observed, consisting of pyruvate dehydrogenase and transacetylase as well as formation of polymers which could not be identified. This indicates that SH-groups on the pyruvate dehydrogenase are exposed in the presence of pyruvate, Mg^{2+} and TPP.

5.2. INTRODUCTION

Previous experiments have shown that the pyruvate dehydrogenase complex from *Azotobacter vinelandii* consists of 4 components, *e.g.* pyruvate dehydrogenase (mol.wt. 89000), lipoamide dehydrogenase (mol.wt. 56000) and two transacetylase isoenzymes with molecular weights of 80000 and 63000 respectively [2]. Incorporation of acetyl-groups into the complex upon incubation with 2-^{14}C-pyruvate indicated that the two transacetylase isoenzymes react in non-identical ways [3]. Removal of the low-mol.wt. lipoyltransacetylase [1] leads to structural changes of the remaining components as indicated by physical and catalytic properties.

The usefullness of bifunctional reagents for topological studies on interacting protein systems is well documented [4-9]. In this study diimidates of different chain lengths were used in order to evaluate interaction between the components of the *A.vinelandii* complexes. These reagents react with amino groups forming amidine groups. Since the amidines formed in the reaction between the imidoesters and primary amines carry a formal positive charge (the pK_a of the amidines is considerably higher than that of the ε-amino groups of lysine), extensive reaction with lysine residues can be carried out without any change in the net charge of the protein [4]. Furthermore bifunctional SH-reagents, o- and p-N,N'-phenyldimaleimides were used to study the interaction between the lipoyl groups of both transacetylases after reduction either by pyruvate, Mg^{2+} and TPP or by NADH. The results were compared with those obtained with the *E.coli* complex, which has only one transacetylase component.

5.3 MATERIALS AND METHODS

5.3.1. Enzyme preparations

The preparation of pyruvate dehydrogenase complex from *Azotobacter vinelandii* (ATCC478) as well as methods for the determination of the enzyme activity have been described [2]. The specific activities of the different batches used, varied between 11-12 μmol NADH.min^{-1}.mg protein^{-1}. The pyruvate dehydrogenase complex from *Escherichia coli* Crookes was prepared according to Eley *et al* [10] and was pure on sodium dodecylsulphate gels. The specific activity was 22 μmol NADH.min^{-1}. mg protein^{-1}.

5.3.2. Crosslinking with diimidates

The dimethylimidates were prepared from their dinitriles according to Davies and Stark [4]. The reagents were stored desiccated above calciumoxyde at 4°C.

Crosslinking experiments with diimido esters were performed in a reaction mixture (total volume 200 μl) consisting of 100 μg complex and 200 μg diimidate in 0.2 M triethanolamine buffer (pH 8.5) which was allowed to stand at room temperature. In some cases, 50 mM phosphate buffer pH 8.0 or 50 mM Tricine buffer pH 8.0 was used. At certain times a solution was added containing 2% sodium dodecylsulphate, 2% 2-mercaptoethanol, 0.05% bromphenol blue and 25% glycerol in water. The mixture was immediately heated for 5 minutes at 100°C, and its composition analysed by polyacrylamide gel electrophoresis. Since diimidates are unstable in water, solutions were prepared just before use. When the cleavable crosslinker was used, the experiments were performed according to Wang *et al* [7].

5.3.3. Crosslinking with phenyldimaleimides

Crosslinking experiments with phenyl-N,N'-dimaleimides were performed by adding 5 μl of a freshly prepared solution of o-or p-phenyl-N,N'-dimaleimide (0.1 M) in dimethylformamide to 200 μl solution containing 400 μg of complex in 50 mM sodium phosphate buffer (pH 7.4). After incubation at room temperature for the time required, the reaction was stopped as described for the diimidates. When the complex was crosslinked through reaction with groups spe-

cifically generated by reaction with pyruvate, Mg^{2+} and TPP or with NADH, it was prior to the addition of substrates non-specifically labelled with N-ethylmaleimide using a 50-fold excess of the reagent (based on FAD content), during 10 minutes at room temperature [*cf. ref.* 11]. Excess of sulfhydryl-reagent was removed by Sephadex G-10 filtration. This pre-treated complex was then crosslinked under the same conditions as described above but in the presence of either 5 mM Mg^{2+}, 0.5 mM TPP and 5 mM pyruvate or 1 mM NADH. As a blank, pretreated complex was crosslinked with dimaleimide without addition of pyruvate or NADH.

5.3.4. Gel Electrophoresis and Molecular Weight determination

In the analysis by polyacrylamide gel electrophoresis, approximately 50 µg of crosslinked protein was brought on 5% acrylamide gels containing 0.1% sodium dodecylsulphate, according to the procedure of Shapiro *et al* [12] and Weber and Osborn [13]. In some experiments the methods of Laemmli were used [14].

After electrophoresis the gels were fixed and stained with a solution containing 0.25% coomassie blue, 50% methanol and 7.5% acetic acid and destained in a solution of 5% methanol and 7.5% acetic acid.

The gels were scanned at 540 nm using a Beckman spectrophotometer model G 2400 equipped with a Gilford gel-scanner type 3AG and a Kipp & Zonen recorder type BD8. When the analysis was performed on polyacrylamide slabgels, approximately 100 µg of crosslinked protein was brought on the gels and the electrophoresis was done according to Wang *et al* [7].

The molecular weights of the crosslinked products were determined from the recorder scans using markers as well as the location of non-crosslinked components of the complexes. The gels were also calibrated by using bovine serum albumine, crosslinked with malonyldiimidate, yielding a perfectly straight line for the monomer, dimer, trimer and tetramer forms when R_f *vs* ln mol.wt. was plotted. By measuring the mol.wt., the low-molecular weight transacetylase seems to have a mol.wt. of 63000 rather than of 59000 as reported earlier [2]. For the *E.coli* complex the monomer mol.wts. of lipoamide dehydrogenase, transacetylase and pyruvate dehydrogenase were respectively 56000, 80000 and 90000.

5.3.5. Materials

The dinitriles were obtained from Merck and from Fluka A.G. The phenyldimaleimides and dimethyl dithiobispropionimidate were from Aldrich. The marker proteins were from Boehringer. All other chemicals used were of reagent grade and were obtained from common suppliers.

5.4. RESULTS

5.4.1 Crosslinking experiments with dimethylimidates

5.4.1.1 Crosslinking of the *A.vinelandii* complex.

When pyruvate dehydrogenase complex from *A.vinelandii* reacts under crosslinking with dimethylmalonylimidate, four new products appear with time. The scan patterns of the dodecylsulphate polyacrylamide gels (Fig. 1), show that at first the high-mol-weight transacetylase and the lipoamide dehydrogenase bands decrease in intensity, while three new products with mol.wts. of 105000, 140000 and 163000 appear. At longer times of exposure to the reagent, the pyruvate dehydrogenase and the low-mol-weight transacetylase are also clearly lower in intensity while products with mol.wts. of 152000 and > 220000 appear.

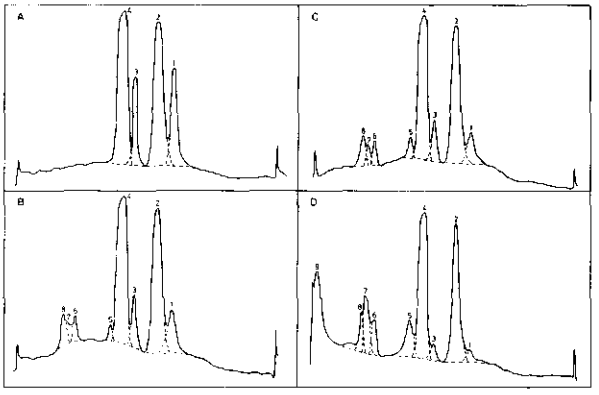
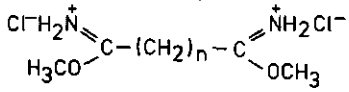


Figure 1

Scan patterns from polyacrylamide gels of 4-component pyruvate dehydrogenase complex from *A.vinelandii* before (A) and after (B,C,D) crosslinking with dimethyl malonylimidate. B after 40 minutes, C after 90 minutes and D after 120 minutes.
1. lipoamide dehydrogenase, 2. low.-mol.wt. transacetylase, 3. high mol. wt. transacetylase, 4. pyruvate dehydrogenase, 5. 105000 band, 6. 140000 band, 7. 152000 band, 8. 163000 band and 9. higher polymers with mol.wt. > 220000.

The complex was also crosslinked with diimidates of longer chain lengths with the general structure;



: dimethyladipimidate (n=4; 0.9 nm) dimethylpimelimidate (n=5; 1.0 nm); dimethylsuberimidate (n=6; 1.1 nm); dimethyldodecylimidate (n=10; 1.5 nm). The length of the molecules was calculated using bond lengths and angles from Pauling [15] and considering the fully extended configuration of the crosslinkers. In experiments with these reagents (Fig. 2) the same crosslinked products appear as found from crosslinking with dimethylmalonylimidate (CH_2; 0.5 nm). With longer diimidates, however, also a crosslinked product of 180000 is formed and the selectivity in the rate of appearance is not longer present so all crosslinked products are already formed in short periods of reaction. In addition, the longer the chain length of the diimidate, the more polymers with mol.wts > 220000 are formed.

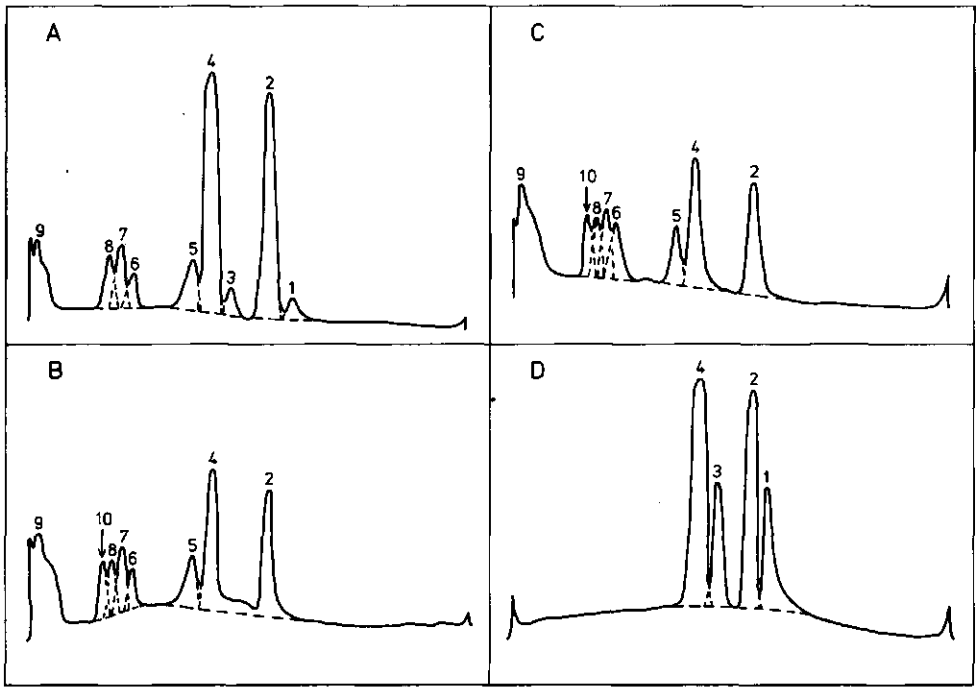


Figure 2

Scan patterns from polyacrylamide gels of 4-component pyruvate dehydrogenase complex from *A.vinelandii* after crosslinking during 2 hours with: A. dimethylmalonyldiimidate, B. dimethylsuberimidate, C. dimethyldodecylimidate. The scan pattern of the 4-component complex before crosslinking is presented in D. The numbers 1-9 correspond with those in Fig. 1. 10. is the extra band of 180000 formed when the complex is crosslinked with longer diimidates.

The complex has after 3 hours of reaction with dimethylmalonylimidate still a high overall activity of 88% of the activity of the unreacted complex. The total percentage of the crosslinked products is about 33% but no unreacted lipoamide dehydrogenase and high-mol.wt. transacetylase are left. Crosslinking the complex with longer diimidoesters results in lower overall activity but in the case of dimethyldodecylimidate the activity is still 40% of the overall activity, while 63% of the total proteins is crosslinked. When the complex is crosslinked with dimethylimidates in 50 mM phosphate buffer at either pH 7.4 or pH 8.0 smaller amounts of the same crosslinked products appear as compared with triethanolamine buffer. The overall activities after crosslinking in phosphate buffer are with all diimidates very high: 100% for dimethyl malonylimidate to 80% for dimethyldodecylimidate. With dimethylmalonylimidate no crosslinked higher polymers (>220000) and with dimethyldodecylimidate only a very small amount of crosslinked higher polymers is formed.

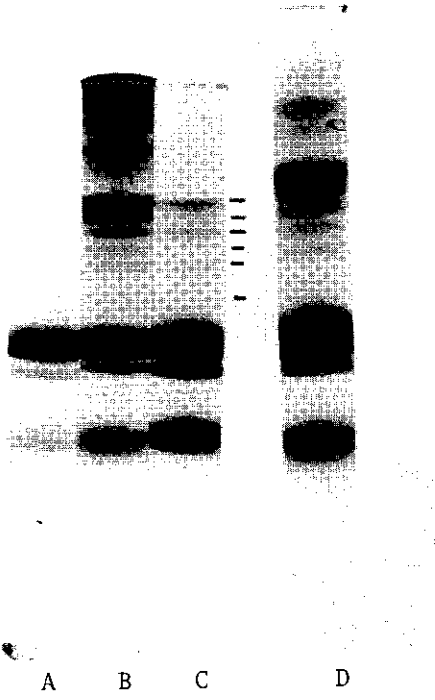


Figure 3
Gel patterns of 3-component pyruvate dehydrogenase complex from *A.vinelandii* before (A) and after crosslinking for 30 minutes with: B. dimethyl-3,3'-dithiobispropionimidate, C. dimethylmalonylimidate, D. dimethyldodecylimidate. From the bottom to the top the arrows indicate the following crosslinked products: 113000, 133000, 144000, 158000, 170000 and 182000.

Three-component complex from *A.vinelandii*, isolated as described earlier [1] was crosslinked with dimethylmalonylimidate, dimethyldodecylimidate and the cleavable crosslinker dimethyl-3,3'-dithiobispropionimidate. Crosslinking with dimethylmalonylimidate yields peaks with mol.wts. of 133000, 158000, 170000 and 182000. Upon crosslinking with dimethyldodecylimidate and dimethyl-3,3'-dithiobispropionimidate also crosslinked products with mol.wts. of 113000 and 144000 and higher polymers are observed (Fig. 3).

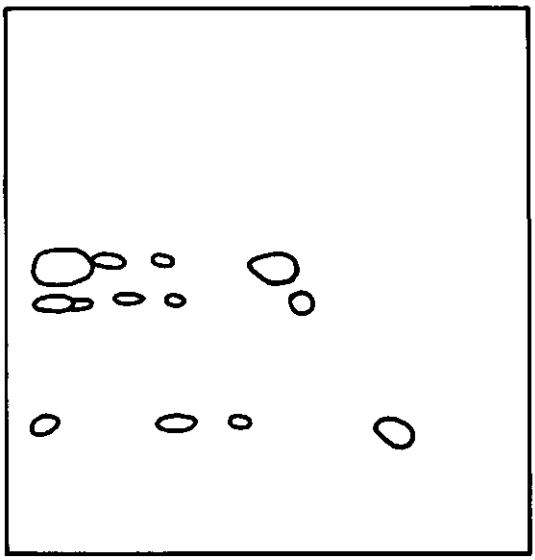


Figure 4
Two-dimensional gel patterns of crosslinked 3-component pyruvate dehydrogenase complex from *A.vinelandii* with DTBP.
The direction of elctrophoresis is from left (cathode) to right (anode) for the first direction and from top (cathode) to bottom (anode) in the second direction.
The electrophoresis in the first direction was performed after incubation without 2-mercaptoethanol.
The electrophoresis in the second direction was performed after incubation with 2-mercaptoethanol to obtain a cleavage of the crosslinked bands.

After electrophoresis in one direction, the crosslinked products - formed with dimethyl-3,3'-dithiobispropionimidate - can be split by reductive cleavage during an initial phase of separation in the second direction on slabgels (Fig. 4). A schematic composite diagram - drawn from the slabgel - can be used for the identification of the crosslinked products (Fig. 5).

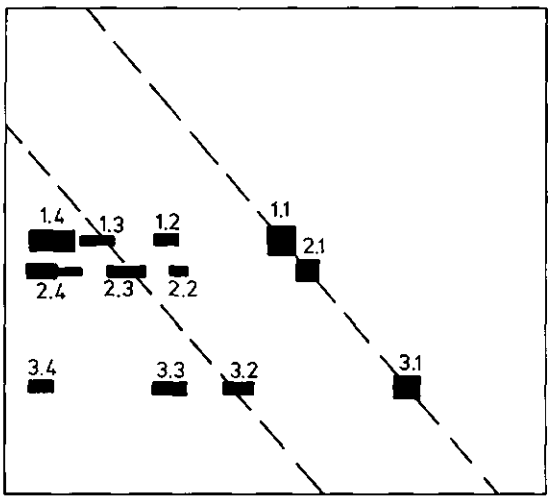


Figure 5
A schematic composite diagram of the crosslinking patterns of figure 4.
The spots on the dotted diagonals are from monomers and dimers of the enzyme components. Off diagonal spots are from crosslinking between different enzyme components.
1.2 and 3.3 are derived from crosslink product 144000. 2.3 and 3.3 are from crosslink product 133000. The bands 1.4, 2.4 and 3.4 are from polymers.

From the diagram it appears that the three bands with mol.wts. of 113000, 158000 and 182000 are made up of lipoamide dehydrogenase dimers, transacetylase dimers and pyruvate dehydrogenase dimers, while the three bands with mol.wts. of 133000, 144000 and 170000 consist of the combinations lipoamide dehydrogenase/transacetylase, lipoamide dehydrogenase/pyruvate dehydrogenase and pyruvate dehydrogenase/transacetylase. The higher polymers (>220000) are mainly formed by crosslinked polymers of pyruvate dehydrogenase and transacetylase and by the totally crosslinked complex.

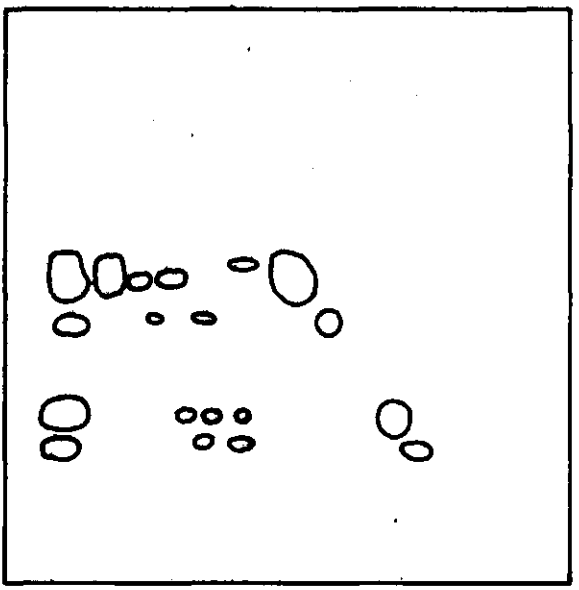


Figure 6
Two-dimensional gel patterns of crosslinked 4-component pyruvate dehydrogenase complex from *A.vinelandii*.
The electrophoresis was performed as described in fig. 4.

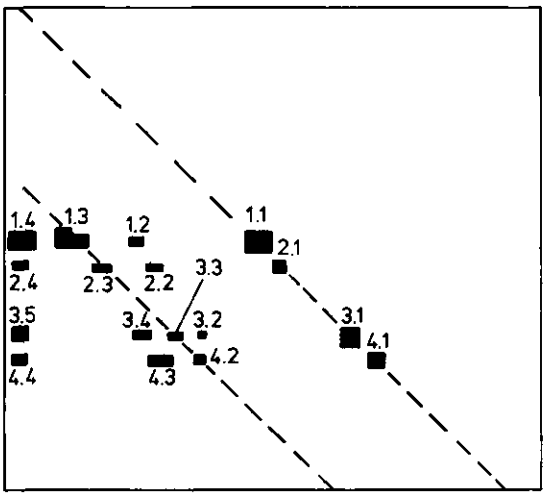


Figure 7
A schematic composite diagram of the crosslinked patterns of figure 6. The spots on the dotted diagonals are from monomers and dimers of the enzyme components. Off-diagonal spots are from crosslinking between different enzyme components. 1.2 and 3.4 are derived from crosslink product 141000, 2.2 and 4.3 are derived from crosslink product 133000. The 1.3, 2.3, 3.3 and 4.2 bands are from dimers. The 1.4, 2.4, 3.5 and 4.4 bands are from high polymers.

The two-dimensional gel pattern of the crosslinked four-component complex is shown in Figure 6. From the schematic composite diagram (Fig. 7) the bands with mol.wts. of 105000, 163000 and 180000 seem to be due to lipoamide dehydrogenase dimers, transacetylase dimers and pyruvate dehydrogenase dimers. The assignment of the 140000 and 152000 bands is more difficult. The 140000 band is mainly formed from crosslinked high-mol.wt. - transacetylase/lipoamide dehydrogenase. However, some slight crosslinking between high-mol.wt-transacetylase/low-mol.wt.transacetylase seems to have occured. The 152000 band is formed from crosslinked pyruvate dehydrogenase/low-mol.wt. transacetylase.

5.4.1.2. Crosslinking of the *E.coli* complex.

The *E.coli* complex reacts faster with diimidates than the *A.vinelandii* complex. After 30 min. reaction under standard conditions, the concentration of dimeric species decreases and higher polymers are formed. The crosslinking pattern is rather similar to that of the three-component *A.vinelandii* complex. With malonyldiimidate four bands were found with mol.wts. 106000 (lipoamide dehydrogenase dimer), 142000, 162000 (transacetylase dimer), 172000 (transacetylase/pyruvate dehydrogenase). The band at 142000 is difficult to assign. Both the combinations transacetylase/lipoamide dehydrogenase (expected mol. wt. 135000) and pyruvate dehydrogenase/lipoamide dehydrogenase (expected mol. wt. 146000) are possible, especially since all bands appear at about the same time.

No change in the pattern was found when dodecyldiimidate was used. The *E.coli* complex is less stable than the *A.vinelandii* enzyme in 0.2 M triethanolamine buffer, pH 8.5. In fact, this buffer has been used at a higher pH to dissociate the pyruvate dehydrogenase from the complex [16]. The decline in overall activity, but not in the partial activities as observed under these conditions, points to changes in interchain contacts of the pyruvate dehydrogenase with other components of the complex occurring in the course of the experiment, thus influencing the outcome of the crosslinking experiments. Experiments were therefore repeated under conditions where the enzyme complex was stable during the course of the incubations, usually 30 min. These conditions, incubation in 50 mM phosphate or tricine buffer pH 8.0, are less favourable for crosslinking. With dodecyldiimidate small amounts of the same bands as found in triethanolamine buffer were obtained without polymer formation under these conditions, but in addition a relatively strong band with mol.wt. 178000 appeared,

which can be ascribed to a pyruvate dehydrogenase dimer.

5.4.1.3. Crosslinking with diimidates in the presence of substrates

Crosslinking of the 4-component complex from *A.vinelandii* with malonyldiimidate in the presence of 5 mM pyruvate, 5 mM Mg^{2+} and 0.5 mM TPP (phosphate buffer pH 8.0) leads to quite different patterns on the gels, compared with those obtained after crosslinking in the presence of Mg^{2+} and TPP alone. The high-mol.wt. transacetylase component disappears rapidly (Table I).

Table I

The effect of pyruvate on the crosslinking of the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* with malonyldiimidate.
The crosslinking was performed as described in methods. The incubation mixture contained 5 mM $MgCl_2$ and 0.5 mM TPP in 50 mM phosphate buffer, pH 8.0 and when present 5 mM pyruvate. The amount of transacetylase was calculated from the area in the gel scanning patterns.

time min	*A.vinelandii* % decrease of high mol.wt. transacetylase		*E.coli* % decrease of transacetylase	
	without pyruvate	with pyruvate	without pyruvate	with pyruvate
0	0		0	
5	-2	57	6	46
30			16	73
40	29	78		

Within 5 min of incubation 57% of the high-mol.wt. transacetylase is converted into polymer, while the other bands are hardly affected. Although the band of dimeric high-mol.wt. transacetylase is present, formation of high-mol. wt. material (>220000) is quite extensive and already apparent within 5 min of incubation. The amount of high-mol.wt. transacetylase dimer does increase in the presence of pyruvate, but not in the amount expected from the decrease in monomer concentration. Thus it seems that the high-mol.wt. transacetylase "core" crosslinks as one entity. The presence of substrate had no effect on the low-mol.wt. transacetylase. Similar results are obtained with the *E.coli* complex (Table I), but in this case only high-mol.wt. material (>220000) is formed. Dodecyldiimidate gives results identical with those obtained with malonyldiimidate. Crosslinking in the presence of NADH has no additional effects.

5.4.2 Crosslinking studies with N,N'-phenyldimaleimides

In Table II the results are summarized from experiments in which prelabelled 4 component complex from *A.vinelandii* was crosslinked with ortho-N,N'-phenyldimaleimide either in the presence or in the absence of substrate. Crosslinking experiments of prelabelled complex in the absence of substrate still gives a small amount of crosslinking; based on density-ratio only 2-5% of the optical density was from the crosslinked products. These crosslinked products have mol.wts. of 178000 - pyruvate dehydrogenase dimer -, 163000 - high-mol.wt. transacetylase dimer -, 140000 - probably a combination of high-mol.wt. transacetylase with lipoamide dehydrogenase - and 105000 - lipoamide dimer. During a reaction time of 3 hours in the absence of substrate, the activity of the crosslinked complex drops to 55% of the initial activity.

When prelabelled complex is crosslinked in the presence of 5 mM pyruvate, 5 mM Mg^{2+} and 0.5 mM TPP the overall activity disappears within 15 min. After 30 min. of incubation the bands at mol.wts. 105000, 140000 and 178000 have about the same size as those observed after incubation for 3 hours in the absence of pyruvate. Furthermore the 163000 band increases 4 times in magnitude, while in addition two extra bands appear; at 120000 low-mol.wt.-transacetylase dimer- and at 144000 - combination low-mol.wt. transacetylase/high-mol.wt. transacetylase - (Table II).

Table II

Relative contribution in optical density ratios of pyruvate dehydrogenase, high-mol.wt. transacetylase, low-mol.wt. transacetylase, lipoamide dehydrogenase and crosslinked products in crosslinking experiments of 4-component pyruvate dehydrogenase complex from *A.vinelandii* with o-N,N'-phenyldimaleimide after prelabelling with N-ethylmaleimide.
Column A : in the presence of 5 mM pyruvate, 5 mM $MgCl_2$ and 0.5 mM TPP.
Column B : in the absence of pyruvate.
Column (A-B) : increase of protein bands in the presence of substrate in comparison to those in the absence of substrate.

Component	Optical Density Ratio (in %) A	B	(A-B)
lipoamide dehydrogenase	11	12	-1
low-mol.wt. lipoyl transacetylase	26	38	-12
high-mol.wt. lipoyl transacetylase	5	12	-7
pyruvate dehydrogenase	39	40	-1
105.000 band	3	2	1
122.000 band	9	not observed	9
140.000 band	2	1	1
144.000 band	9	not observed	9
163.000 band	8	2	6
178.000 band	2	2	0

No further increase nor additional bands were observed after longer incubation times. Crosslinking of prelabelled complex either in the presence of NADH or in the presence of pyruvate, Mg^{2+}, TPP plus NADH gives similar results as in the presence of pyruvate, Mg^{2+} and TPP. Using p-phenyl-N,N'-dimaleimide instead of o-phenyl-N,N'-dimaleimide does not affect the patterns.

Crosslinking of the pretreated *E.coli* complex with the bifunctional reagent o-phenyl-N,N'-dimaleimide yields quite different results. Under non-specific conditions, but in the presence of Mg^{2+} and TPP, only the lipoamide dehydrogenase component reacts. In 110 min 50% of the monomer disappears. Although some lipoamide dehydrogenase dimer is present, this represents only a few percent of total amount of protein crosslinked. The rest is presumably present as polymer in the spacer gel.

In the presence of Mg^{2+}, TPP and pyruvate a large decrease (65% in 110 min) of the transacetylase monomer occurs. In addition a new band with mol.wt. 173000 - probably a mixed dimer of pyruvate dehydrogenase and transacetylase together with material above mol.wt. 220000 was found. The 173000 mol.wt. species was maximally present after 30 min and declined after longer reaction times.

When an enzyme preparation was used which was prepared in the presence of phenylmethanesulfonylfluoride and EDTA but stored in the absence of these protease inhibitors two transacetylase bands were present after a few weeks of storage. Upon specific crosslinking such a preparation two new bands were observed in the gel pattern instead of one. This further supports the idea that the crosslinked band is due to a mixed dimer of pyruvate dehydrogenase and transacetylase. However the decrease in transacetylase monomer is in no way accounted for by the increase in 173000 material and must therefore have been converted into higher polymers.

5.5. DISCUSSION

The results of the experiments with diimidoesters can be used to calculate the distances of the individual components of the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli*. From the crosslinking experiments of the four-component complex with malonyldiimidate (0.5 nm), it can be concluded that the high-mol.wt. transacetylase and lipoamide dehydrogenase contain lysyl groups which are located within a distance of 0.5 nm from each other. The fact that even after prolonged times of reaction no evidence is obtained for the formation of the product lipoamide dimer/high-mol.wt. transacetylase, indicates that probably two groups are responsible for the pattern observed. One lysine of the lipoamide dehydrogenase peptide chain crosslinked to another lysine of either a lipoamide dehydrogenase peptide chain or of a high-mol.wt. transacetylase chain. Similarly the lysine of the high-mol.wt. transacetylase chain is linked either homologously to transacetylase or heterologously to lipoamide dehydrogenase. From these results it is obvious that in this complex lipoamide dehydrogenase exists in the dimeric form, a result to be expected from the work of van Muiswinkel-Voetberg and Veeger showing that the monomer is inactive [21]. The formation of dimeric lipoyltransacetylase is in agreement with the core structure as the dimaleimide experiments show.

Lysyl groups of pyruvate dehydrogenase and low-mol.wt. transacetylase are also at close distance (0.5 - 0.9 nm), for they can be linked together with dimethyladipimidate (0.9 nm). On the other hand, even with dimethyl dodecyldiimidate, neither crosslinking can be found of high-mol.wt. transacetylase with either pyruvate dehydrogenase or with low-mol.wt. transacetylase nor is crosslinking observed of lipoamide dehydrogenase with either pyruvate dehydrogenase or low-mol.wt. transacetylase. Thus assuming that the amino

groups are randomly distributed on the surface of the subunits, we conclude that two pairs of heterologous chains are in interaction in the 4-component complex, *e.g.* lipoamide dehydrogenase/high-mol.wt. transacetylase as well as low-mol.wt. transacetylase/pyruvate dehydrogenase. In addition interactions between homologous chains exist, due to the presence of the individual enzymes in at least dimeric structure. A complete rearrangement of these interactions occur after removal of the low-mol.wt. transacetylase and some pyruvate dehydrogenase in the conversion into the 3-component complex, making interactions possible between transacetylase and both pyruvate dehydrogenase and lipoamide dehydrogenase. Crosslinking between pyruvate dehydrogenase and lipoamide dehydrogenase is also observed, in contrast with the *E.coli* complex. Lysyl groups of pyruvate dehydrogenase are at close distance (0.5 - 1.5 nm) to lysyl groups of high-mol.wt. transacetylase and of lipoamide dehydrogenase, because they can be linked together with dimethyl malonylimidate, dimethyl dodecylimidate and dimethyl-3,3'-dithiobispropionimidate. So, a rearrangement of the remaining components of the complex is induced when the low-mol.wt. transacetylase is removed from the complex by chromatography on blue-dextran Sepharose 4B, a conclusion which is in agreement with the observation that the partial activities as well as the overall activity are affected in opposite ways by this treatment [1].

The loss of activity observed, when the complex is crosslinked with longer diimidates in triethanolamine buffer, could be due to the formation of high-mol. wt. polymers. The results from the crosslinking in 50 mM phosphate buffer instead of triethanolamine buffer support this hypothesis. The same crosslinked products and only a very small amount of crosslinked higher polymers are formed while the activity remains very high.

The formation of a pyruvate dehydrogenase dimer when the *E.coli* complex is crosslinked with diimidates is in contrast with observations of Coggins *et al.* [17] who reported that pyruvate dehydrogenase does not form a dimer when the *E.coli* complex or the resolved enzyme was crosslinked with diimidates. This was thought to be due to monomerization at the high pH used for crosslinking. It may well be that under the different conditions used here, more dimer is present, thus leading to crosslinking between pyruvate dehydrogenase subunits. However the results with the *E.coli* complex and the 3-component *A.vinelandii* complex point to the same similarities *e.g.* dimeric homologous structures of the three component enzymes within the complex as well as short-range heterologous interactions between the chains of the component enzymes.

The transacetylase peptide chains crosslink quite extensively, to a "core", when the complexes of *A.vinelandii* and *E.coli* are crosslinked in the presence of substrate. These results point to considerable structural changes upon the addition of substrates, either Mg^{2+}, TPP, pyruvate or NADH, bringing lysyl groups on individual transacetylase chains within a distance of 0.5 nm. This is of interest with respect to our earlier conclusion [18] that the sigmoidal shape of the pyruvate saturation curve of the overall activity of the *A.vinelandii* complex is not based on a conformation change of the pyruvate dehydrogenase component upon binding of pyruvate, but rather on a change in subunit interactions in or with other components of the complex. Extensive structural changes in the reacted complex can also explain the failure to observe energy-transfer from thiochrome diphosphate to a fluorescent lipoyl derivative or from the latter to FAD [23,24].

The results of specific crosslinking with dimaleimide of the *A.vinelandii* 4-component complex show that under these conditions two lipoyl-moieties bound either to low- or to high-mol.wt. transacetylase approach each other to a distance of 0.6 nm or less, for they can be linked to form homologous or heterologous dimers with o-phenyl-N,N'-dimaleimide (chain length 0.58 nm). On the other hand this observation is in contrast with the observation that no radioactive N-ethylmaleimide could be incorporated into the low- mol.wt. transacetylase after pretreatment with NEM [3]. Maleimides, coupled to SH groups are reported to react slowly with NH_2 groups [19]. Crosslinking by such a mechanism seems unlikely here, because at pH 7.4 such a reaction takes several hours for completion. After 30 min no further changes (apart from the formation of higher polymers) are observed with both complexes. Rapid linking of the two transacetylase isoenzymes can be achieved by reductive acetylation (pyruvate, Mg^{2+}, TPP) or by reduction with NADH. These results with the dimaleimides do not contrast in our opinion the results obtained with crosslinking by diimidates. The high- and low-mol.wt. enzymes are located at a distance larger than 1.5 nm from each other in the unreacted complex. However after reaction with pyruvate or NADH transacetylase of *E.coli* reacts as a core in the presence of dimaleimide. Similarly the high-mol.wt. transacetylase of *A.vinelandii* reacts as a core which is however not the case with the low-mol.wt. form. However, as judged from the results with dimaleimide, lipoyl groups of both transacetylases of the 4-component *A.vinelandii* complex approach each other within about 0.5 nm.

Thus each transacetylase has a specific environment in the complex. One might consider the possibility that the low-mol.wt. transacetylase is a product

of proteolytic attack. In a very specific way only one group of transacetylase subunits could be transformed in a stoichiometric way [3] into another group. However, when the complex is isolated in the absence of protease inhibitor during the purification procedure, the high-mol.wt. transacetylase is degraded and many new bands are formed but not at the position of the low-mol.wt. transacetylase, an observation which rules out the possibility. In addition it was found upon scanning the gels at 280 nm prior to staining, that the area of the low-mol.wt. transacetylase was 10-11 times that of the high-mol.wt. transacetylase, which indicates that over 90% of degradation cannot be explained by the observed stoichiometry and mol.wt. of the complex [3]. Proteolytic attack on a single chain of mol.wt. 143000, yielding both types of transacetylases can not be excluded, but seems unlikely in view of the identical compositions of the 3-component *A.vinelandii* complex and the *E.coli* complex.

Thus it can be concluded that in the 4-component *A.vinelandii* complex the original transacetylase core consists of two types of transacetylase subunits, one (low-mol.wt.) associated with the pyruvate dehydrogenase and one (high-mol. wt.) with the lipoamide dehydrogenase at least in the unreacted complex. The differences in topology in this form may reflect differences in function, *e.g.* acetyl groups transfer and transfer of reducing equivalents; our radioactive experiments with 2-^{14}C pyruvate point also to such a functional difference [3].

With the *E.coli* complex no transacetylase dimers are formed. With only one SH-group per transacetylase chain specifically generated by reductive acetylation of the lipoyl moiety, no crosslinking beyond the dimeric stage would be expected. Crosslinking with lipoamide dehydrogenase dimer can be excluded, while formation of the mixed transacetylase/pyruvate dehydrogenase dimer cannot account for the large decrease in transacetylase, especially in view of the chain ratios [3]. Indirectly therefore, these results point to the formation by pyruvate conversion of more than one SH-group on each transacetylase chain, in agreement with results obtained by Danson and Perham [20] and with our radioactive experiments with 2-^{14}C-pyruvate [3].

Crosslinking between a transacetylase and a pyruvate dehydrogenase chain as observed upon treatment with dimaleimide indicates that a SH-group on the pyruvate dehydrogenase is exposed in the presence of pyruvate. The overall activity of the *E.coli* complex decreases rapidly in the presence of pyruvate, Mg^{2+} and TPP; this inactivation can be prevented, but not reversed, by thiols such as dithiotreitol and CoASH. Since the partial reactions are not affected, an SH-group seems to participate in acetyl transfer from pyruvate dehydrogenase

to transacetylase. The *A.vinelandii* complex is less sensitive to such inactivation.

The observation that in the *E.coli* complex pre-treated with N-ethylmaleimide, lipoamide dehydrogenase crosslinks in the presence of Mg^{2+} and TPP but in the absence of pyruvate under influence of dimaleimide to higher polymers, casts some doubt on the morphological structure of this complex as proposed by Reed *et al* [22], at least during catalysis. The pretreatment has the purpose to prevent reaction of SH-groups of lipoamide dehydrogenase with the dimaleimide and thus aspecific crosslinking. Nevertheless the binding of Mg^{2+} and TPP to the pyruvate dehydrogenase triggers a conformational change in the lipoamide dehydrogenase. It is very likely that this triggering is mediated via the transacetylase since no short range interaction between pyruvate dehydrogenase and lipoamide dehydrogenase could be observed. The result is a reaction of previously hidden SH-groups with the alkylating agent resulting in the formation of dimers and higher polymers. Thus it seems obvious that during catalysis lipoamide dehydrogenase is functioning in a core-like structure.

5.6. REFERENCES

1. De Abreu, R.A., De Kok, A. and Veeger, C. (1977), FEBS Lett. *82*, 89-92.
2. Bresters, T.W., De Abreu, R.A., De Kok, A., Visser, J., and Veeger, C. (1975), Eur.J.Biochem. *59*, 335-345.
3. De Abreu, R.A., De Kok, A., De Graaf-Hess, A.C. and Veeger, C. (1977), Eur.J.Biochem. *81*, 357-364.
4. Davies, G.A. and Stark, G.R. (1970), Proc.Nat.Acad.Sci.U.S. *66*, 651-656.
5. Wold, F. (1972), Meth.in Enzymol. *25*, 623-651.
6. Slobin, L.I. (1972), J.Mol.Biol. *64*, 297-303.
7. Wang, K. and Richard, R.M. (1974), J.Biol.Chem. *249*, 8005-8010.
8. Lutter, T.C., Ortlanderl, F. and Fasold, H. (1974), FEBS Lett. *48*, 288-292.
9. Kornberg, R.D. and Thomas, J.O. 1974), Science *184*, 865-868.
10. Eley, N.H., Namihira, G., Hamilton. L., Munk, P. and Reed, L.J. (1972), Arch.Biochem.Biophys. *152*, 655-669.
11. Grande, H.J., Bresters, W.T., De Abreu, R.A., De Kok, A. and Veeger, C. (1975), Eur.J.Biochem. *59*, 355-363.
12. Shapiro, A.L., Vinuela, E. and Maizel, J.V. (1967), Biochem.Biophys.Res. Commun. *28*, 815-820.
13. Weber, K. and Osborn, M. (1969), J.Biol.Chem. *244*, 4406-4412.
14. Laemmli, U.K. (1970), Nature (Long.) *227*, 680-685.
15. Pauling, L. (1960), Nature of the Chemical Bond, 3rd ed. Ithaca, N.Y. Cornell University Press.
16. Koike, M., Reed, L.J., Caroll, W.R. (1963), J.Biol.Chem. *238*, 30-39.
17. Coggins, J.A., Hooper, E.A. and Perham, R.N. (1976), Biochemistry *15*, 2527-2532.
18. Bresters, T.W., De Kok, A. and Veeger, C. (1975), Eur.J.Biochem. *59*, 347-353.
19. Wu, C.W., Yarbrough, L.R. and Wu, R.Y.H. (1976), Biochemistry *15*, 2863-2868.
20. Danson, M.J. and Perham, R.N. (1976), Biochem.J. *159*, 677-682.
21. Van Muiswinkel-Voetberg, H. Visser, J. and Veeger, C. (1973) Eur.J.Biochem. *33*, 265-270.
22. Reed, L.J. and Cox, D.L. (1966) Anal.Rev.Biochem. *35*, 37.

23. Scouten, W.H., De Graaf-Hess, A.C., De Kok, A., Grande, H.J., Visser, A.J.W.G. and Veeger, C., (1978) Eur.J.Biochem *84*, 17.
24. Moe, O.A., Lerner, D.A. and Hammes, G.G. (1974) Biochemistry *13*, 2552.

6. IMMUNOCHEMICAL STUDIES ON THE PYRUVATE DEHYDROGENASE COMPLEXES FROM *AZOTOBACTER VINELANDII* AND *ESCHERICHIA COLI*

6.1. SUMMARY

The pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* were studied in their non-reacting state with antisera, prepared from administration of the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli*, lipoamide dehydrogenase from *A.vinelandii* and lipoamide dehydrogenase/lipoyltransacetylase subcomplex from *E.coli* in New Zeeland rabbits.

The results of the immunochemical studies with the *A.vinelandii* complex indicate that pyruvate dehydrogenase is situated at the surface of the 3-component complex. Likewise, pyruvate dehydrogenase and low-mol.wt. lipoyltransacetylase are situated at the surface of the 4-component complex. Lipoamide dehydrogenase seems to be partly buried within both pyruvate dehydrogenase complexes from *A.vinelandii*, although this component is still accessible to anti-lipoamide dehydrogenase. The high-mol.wt. lipoyltransacetylase is situated at the inside of the complex.

The immunochemical studies also show that the molecular organization of lipoamide dehydrogenase and of the high-mol.wt. lipoyltransacetylase is different in both complexes.

Pyruvate dehydrogenase and probably lipoamide dehydrogenase are located at the surface of the complex from *E.coli*, while lipoyltransacetylase seems to be buried inside the complex.

No cross-reaction is observed in experiments where antigen of *A.vinelandii* is used against antiserum of *E.coli* and *vice versa*.

6.2. INTRODUCTION

Detailed biochemical and electronmicroscopical studies have been performed with pyruvate dehydrogenase complex from *Escherichia coli* Crookes, in order to design a model for the macromolecular organization of this multienzyme complex. Reed and co-workers (1-3) have given a model, using data obtained from

biochemical and electronmicroscopical studies. It was proposed that the multienzyme complex consists of a "core" of transacetylase chains, linked together by non-covalent bonds. The polypeptide chains of pyruvate dehydrogenase and lipoamide dehydrogenase are thought to be distributed in a regular manner along the edges of the transacetylase cube.

Up to now, no topological model for the pyruvate dehydrogenase complex from *A.vinelandii* has been designed. In earlier experiments, in our laboratory the enzyme complexes of *E.coli* and *A.vinelandii* with crosslinking reagents (chapter 5) and fluorescence- and spinlabels were studied [4]. From these experiments, the information obtained was however not decisive to design a model. Thus we tried to obtain supplementary data from studies with antibodies against the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* and some of their constituents.

Antibodies are known to combine specifically with antigen and are used in a large variety of qualitative and quantitative techniques. The structure and function of several enzymes [5-8] and even large protein complexes like ribosomes [9] and collagen [10-13] have been studied. In our experiments we used antisera from rabbits, after administration with one of the pyruvate dehydrogenase complexes, with lipoamide dehydrogenase from *A.vinelandii* or with subcomplex from *E.coli*. Subcomplex, obtained by dissociation of pyruvate dehydrogenase complex at alkaline pH, consists of lipoyltransacetylase and lipoamide dehydrogenase [3]. The reactions with antisera were observed, using the pyruvate dehydrogenase complexes, lipoamide dehydrogenase (*A.vinelandii*), pyruvate dehydrogenase (*E.coli*) and subcomplex (*E.coli*) as antigens.

The immunoprecipitation technique was used in double diffusion experiments. The employment of concentration gradients in immunodiffusion experiments permits the establishment of more or less narrow zones in the agar medium, where the ratio of the reactants is favourable for precipitation. Outside this zone, aggregation does not take place due to either excess or lack of antibody. Test material containing two or more precipitating systems induces the formation of a precipitation spectrum, in which each line corresponds to a specific pair of antigen and antibody.

In the present study conclusions concerned with the topology of the complexes obtained by this technique will be reported.

6.3. MATERIALS AND METHODS

6.3.1. Enzymes

The pyruvate dehydrogenase complexes from *E.coli* Crookes and *A.vinelandii* (ATCC 478) were purified as described earlier [14-17].

Pyruvate dehydrogenase and subcomplex- containing lipoyltransacetylase and lipoamide dehydrogenase - from *E.coli* Crookes were prepared from purified pyruvate dehydrogenase complex according to Eley *et al* [16]. However, ethanolamine phosphate buffer pH 9.7 instead of pH 9.5 was used. The higher pH was used to obtain a better dissocation.

Lipoamide dehydrogenase from *A.vinelandii* was prepared according to Massey *et al* [18] and was kindly provided by Mr. J.S. Santema.

Pyruvate-NAD^+ reductase (overall) activity, pyruvate-DCIP-reductase(pyruvate dehydrogenase) activity, reduced lipoate-CoA transacetylase activity and lipoamide dehydrogenase activity were measured as described earlier [19-22].

Protein concentrations were measured according to Lowry *et al* [23]. Bovine serum albumine was used as a standard.

The concentrations of FAD bound to the multi-enzyme complexes were measured as described [24].

Lipoamide dehydrogenase from *A.vinelandii* is electrophoretically pure. Purified pyruvate dehydrogenase from *E.coli* appears to have also no protein band on SDS-polyacrylamide gels. The enzyme shows no overall activity, lipoamide dehydrogenase activity or lipoyltransacetylase activity. Subcomplex from *E.coli* consists of lipoyltransacetylase and lipoamide dehydrogenase. Except these two enzymes, it still contains a small amount of pyruvate dehydrogenase and thus exhibits a weak overall activity and pyruvate dehydrogenase activity (Table I). A mixture (w/w) of dehydrogenase and subcomplex reconsitutes overall activity.

Table I The specific activities of pyruvate dehydrogenase complex, pyruvate dehydrogenase, subcomplex and "reconstituted" complex from *E.coli*.

enzyme	overall	Specific activities pyruvate dehydrogenase	lipoyl transacetylase	lipoamide dehydrogenase
Pyruvate dehydrogenase complex	16	0.20	3.50	4.21
Pyruvate dehydrogenase	0.00	0.00	0.00	0.00
Subcomplex	0.55	0.004	8.75	0.71
Reconstituted complex PDH:subcomplex (1 : 2)	6.12	--	--	--

6.3.2. Antibody production

The antigen dissolved in 1 ml of 10 mM potassium phosphate buffer pH 7,0 containing 0.15 M NaCl, was injected into New Zeeland rabbits subcutaneously; injections were always given at four distinct sites of the animal body. The injections were immediately followed by subcutaneous injections of 1 ml incomplete Freud's adjuvant. The pyruvate dehydrogenase complexes were injected at a concentration of 5 mg/ml, while lipoamide dehydrogenase from *A.vinelandii* and subcomplex from *E.coli* were injected at a concentration of 2 mg/ml. The first administration was followed by subsequent administrations as summarized in Table II.

2-3 ml of blood was taken in order to test the antibody activity after each injection. Approximately 10 days after the final administration, blood was withdrawn from immunized and non-immunized rabbits (Table II). The blood was allowed to clot for 1-2 hours at 25°C and centrifuged to collect the serum. The pellet was resuspended in 10 mM potassium phosphate buffer pH 7.0, containing 0.15 M NaCl. The volume of buffer added, was the same as that of the serum. The suspension was again centrifuged. Supernatant and serum were mixed and stored in small portions at -20°C.

These antibody preparations were used for immunochemical studies. Non-immunized serum was used as a control.

Table II Administrations of antigen and withdrawal of blood.

Time of injection (days)	Antigen injected			
	Pyruvate dehydrogenase complex of *E.coli*	Subcomplex *E.coli*	4-Component pyruvate dehydrogenase complex *A.vinelandii*	Lipoamide dehydrogenase *A.vinelandii*
0	1 ml (5 mg/ml) + 1 ml Freud's Adjuvant subcutaneous	1 ml (2mg/ml) + 1 ml Freud's Adjuvant subcutaneous	1 ml (5 mg/ml) + 1 ml Freud's Adjuvant subcutaneous	1 ml (2 mg/ml) + 1 ml Freud's Adjuvant subcutaneous
7		1 ml (2 mg/ml) + 1 ml Freud's Adjuvant subcutaneous		1 ml (2 mg/ml) + 1 ml Freud's Adjuvant subcutaneous
14	1 ml (5 mg/ml) + 1 ml Freud's Adjuvant subcutaneous	1 ml (1 mg/ml) interveneous	1 ml (5 mg/ml) + 1 ml Freud's Adjuvant subcutaneous	1 ml (1 mg/ml) interveneous
21		3 ml blood		3 ml blood
23		1 ml (1 mg/ml) interveneous		1 ml (1 mg/ml) interveneous
29	3 ml blood		3 ml blood	
34		2 ml blood		2 ml blood
36		1 ml (1 mg/ml) interveneous		10 ml blood
40	3 ml blood		3 ml blood	
40	0.5 ml (4 mg/ml) interveneous		0.5 ml (5 mg/ml) interveneous	
47	3 ml blood		3 ml blood	
48	15 ml blood	3 ml blood	15 ml blood	
55		15 ml blood		

6.3.3. Double diffusion tests

Immunodiffusion studies in agar were performed according to Ouchterlony [25] at 25°C. Petri dishes (8.5 cm diameter) were layered with a solution of 1% agar agar and 0.15 M NaCl in 10 mM potassium phosphate buffer pH 7.0. When the gel became solid, one centre and six outer wells were made to the agar layer. The appropriate wells were filled with antiserum and antigen and diffusion was allowed to proceed. After antigen-antibody precipitation bands were observed, unchanged protein was dissolved by extensive washing during two days. The precipitation bands were stained for 30 min in a solution containing per liter distilled water; 5g of amidoblack, 50 g $HgCl_2$ and 50 ml acetic acid. After staining the bands, the dishes were washed with 2% (v/v) acetic acid in water until the background was colourless.

6.3.4. Antibody-enzyme preincubation

In inactivation experiments, the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* were incubated with anti-4 PDC A.v. and anti-PDC. E.c. Inactivation of the complexes was measured following the overall and partial activities. The overall and partial activities were measured after antigen-antibody formation, using various amounts of antibody. The pyruvate dehydrogenase complexes and antisera were dialysed against 50 mM potassium phosphate buffer pH 7.0 prior to incubation. The incubations were performed in a mixture containing: 20 µl pyruvate dehydrogenase complex (1.5 mg/ml), varying amounts of antiserum and 50 mM potassium phosphate buffer pH 7.0. The incubations were performed at room temperature. In control experiments, non-immunized serum was used as blank. After certain incubation periods, aliquots of the mixture were withdrawn and the enzyme activities were measured with an Aminco-DW-2a UV/vis Spectrophotometer.

6.3.5. Materials

Agar Noble and Freud's incomplete adjuvant were bought from Difco Laboratories; sodium pyruvate, magnesium chloride and thiamine pyrophosphate were obtained from Merck; coenzyme A, NAD^+ and bovine serum albumine were from Boehringer. Other biochemicals were from Sigma.

6.4. RESULTS

6.4.1. Immunodiffusion experiments with anti-4-component pyruvate dehydrogenase from A.vinelandii

The Ouchterlony patterns show that anti-4.PDC.Av. does not react with pyruvate dehydrogenase complex, subcomplex and pyruvate dehydrogenase from *E.coli*. In immunodiffusion experiments with the 4-component complex and the 3-component complex from *A.vinelandii* two and one precipitation lines occur respectively (Fig. 1a). An experiment in which the 4- and 3-component complexes reacted with anti-4.PDC. A.v. as well as anti-LipDH. showed that the weak precipitation band of lipoamide is not identical with the two lines from the 4-component complex, while the precipitation band of the 3-component complex is identical with the line located closer to the outer well. A precipitate formed after a two hours incubation of 20 µl 4-component pyruvate dehydrogenase complex from *A.vinelandii* (30 µg) with 80 µl anti-4.PDC. A.v.. After centrifugation the precipitate was washed three times with 1% sodium dodecylsulphate.

When enzyme at a much lower concentration is used, the complexes seem to dissociate and 4-component complex now gives four bands indicating dissociation in the serum. The 3-component complex gives two bands (Fig. 1b).

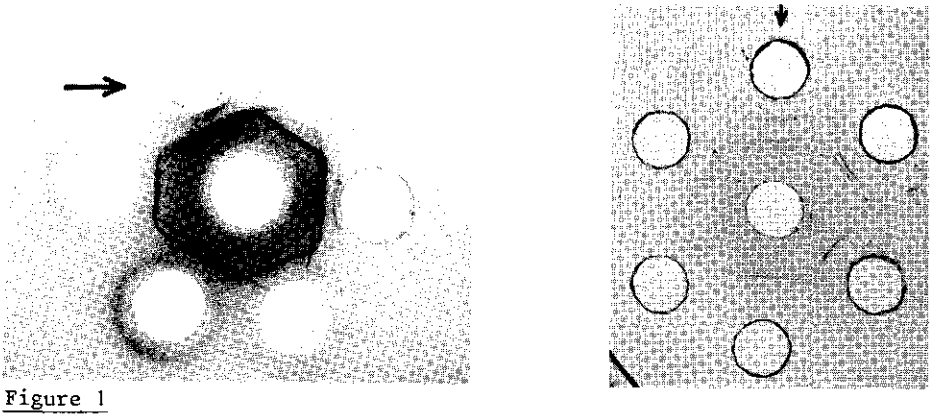


Figure 1

Double diffusion tests with 20 µl anti-4.PDC.A.v. against antigens from *A.vinelandii*.

a. Clockwise from ↓ : 10 µl 4-component PDC (2 mg/ml), 20 µl 3-component PDC (1.3 mg/ml), 10 µl 4-component PDC (0.5 mg/ml), 20 µl 3-component PDC (0.33 mg/ml), 10 µl 4-component PDC (0.25 mg/ml) and 20 µl 3-component PDC (0.16 mg/ml).

b. Clockwise from ↓ : 10 µl 4-component PDC (0.10 mg/ml), 20 µl 3-component PDC (0.08 mg/ml), 10 µl LipDH (0.50 mg/ml), 10 µl 4-component PDC (0.05 mg/ml), 20 µl 3-component PDC (0.04 mg/ml) and 10 µl LipDH (0.1 mg/ml).

Furthermore it is clear that the outer band obtained by the 3-component complex is identical with the precipitation band of lipoamide dehydrogenase. This leads to the conclusion that the outer of the two middle bands obtained with the 4-component complex belongs to lipoamide dehydrogenase.
The precipitate was then heated with 2-mercaptoethanol and sodium dodecylsulphate in Tris HCl buffer and was electrophoresed according to Leammli [27]. On the gel the protein bands of pyruvate dehydrogenase, and low-mol.wt. lipoyltransacetylase and of the heavy antibody chain were observed. This indicated that the two precipitation lines from the 4-component complex with anti-4.PDC.A.v. belong to pyruvate dehydrogenase and low-mol.wt. transacetylase.

6.4.2. Immunodiffusion experiments with anti-pyruvate dehydrogenase complex from E.coli

In immunodiffusion experiments with anti-PDC.E.c. precipitation bands are observed when pyruvate dehydrogenase complex, subcomplex and pyruvate dehydrogenase from *E.coli* are used as antigen (Fig. 2a). Pyruvate dehydrogenase complex reacts with antiserum to give two precipitation bands. Subcomplex and pyruvate dehydrogenase each give one band upon immunodiffusion against anti-PDC. E.c.. From the complete fusion of the arcs it is apparent that the precipitation band with the pyruvate dehydrogenase complex, that is closest to the well containing complex, is also obtained by pyruvate dehydrogenase as antigen; the precipitation line that is closest to the well containing antibody is also obtained by subcomplex.

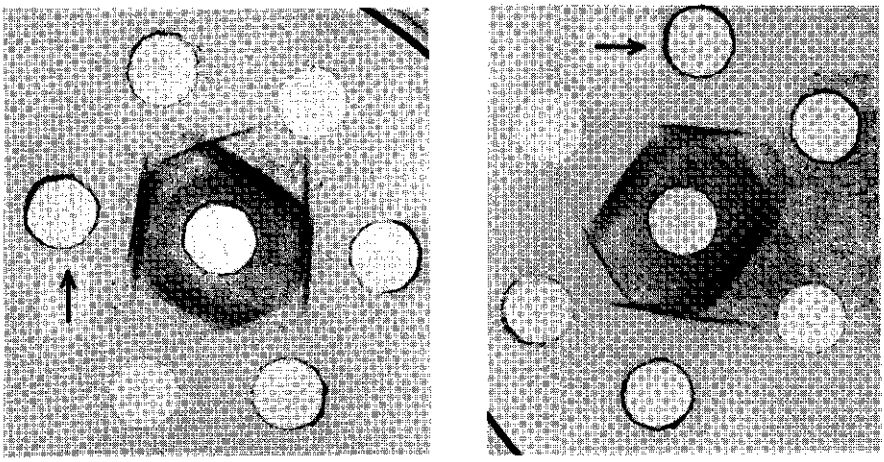


Figure 2

Double diffusion tests with 20 µl anti-PDC.E.c. and 10 µl antigens from *E.coli*.
Clockwise from ↓ : PDC (2 mg/ml), subcomplex (1 mg/ml), PDH (0.5 mg/ml), PDC (2 mg/ml), subcomplex (1 mg/ml) and PDH (0.5 mg/ml).
a. "pure" PDC is used.
b. "impure" PDC is used.

An "impure" pyruvate dehydrogenase complex from *E.coli*, eluting from the calcium phosphate column before the "pure" multi-enzyme complex [26], was also used in these studies. This complex consists of four components and resembles the 4-component complex from *A.vinelandii* due to the presence of a component with a mol.wt. of 65000 and a FAD content of 1.8 µmol/mg complex. This "impure" enzyme complex reacts with the antiserum against the "pure" *E.coli* complex to give two precipitation bands. It is remarkable that the outer band of this "impure" complex is identical with the inner band of pure complex and that the inner band seems to be identical to the outer band of "pure" complex (Fig. 2b).

Both pyruvate dehydrogenase complex and lipoamide dehydrogenase from *A.vinelandii* do not precipitate with anti-PDC.E.c..

6.4.3. Immunodiffusion experiments with anti-lipoamide dehydrogenase from A.vinelandii

Anti-LipDH.A.v. does not react with the pyruvate dehydrogenase complex, the subcomplex or the pyruvate dehydrogenase from *E.coli*.

Immunodiffusion experiments with the 4-component complex, the 3-component complex and lipoamide dehydrogenase from *A.vinelandii* showed the appearance of

two bands with the 4-component complex and of one band with the 3-component complex and with lipoamide dehydrogenase (Fig. 3).

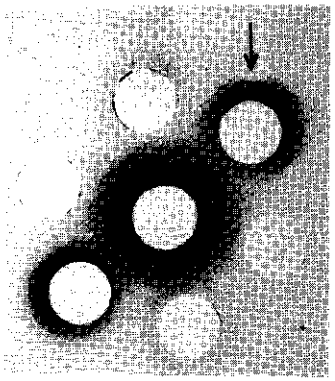


Figure 3

Double diffusion tests with anti-LipDH A.v. against antigens from *A.vinelandii* 20 µl antiserum was used. Clockwise from ▼ : Alternatively 10 µl 4-component PDC (2 mg/ml), 10 µl 3-component PDC (2 mg/ml) and 10 µl LipDH (0.5 mg/ml)

The precipitation band caused by the 4-component complex and located on the outside is identical with that precipitated by free lipoamide dehydrogenase. In addition the band caused by the 4-component complex and located on the inside is identical with the band of the 3-component complex.

6.4.4. Immunodiffusion experiments with anti-subcomplex from E.coli

The experiments with anti-sub.E.c. showed comparable results as observed with anti-PDC.E.c. With lipoamide dehydrogenase and both complexes from *A.vinelandii* no precipitation bands were obtained.

Pyruvate dehydrogenase and subcomplex from *E.coli* give each one precipitation band. The bands are not identical for they intersect (Fig. 4a). The intact pyruvate dehydrogenase complex from *E.coli* gives two precipitation bands, the outer band is identical with the band caused by pyruvate dehydrogenase and the inner band is identical with the band caused by the subcomplex (Fig. 4a).

In experiments with "impure" pyruvate dehydrogenase complex with anti-sub. E.c., the same remarkable patterns were observed as in experiments with anti-PDC.E.c. (Fig.,4b). It also appeared that the outside band of "impure" complex is identical with the inside band of "pure" complex while the inside band of "impure" complex is identical with the outside band of pure complex.

6.4.5. Control experiments

In control experiments all enzymes were tested with non-immunized serum. No precipitation lines were observed in any of these experiments. To check whether the complement-factor of the immune system was involved in the formation of the precipitation bands, parallel experiments were performed with antisera which had been heated during 30 min at 56°C. At this temperature the complement system denaturates. These experiments show no precipitation lines. The results from experiments with several enzyme preparations are reproducible.

Except for the dilution experiments of the *A.vinelandii* complexes reacting with Anti-4-PDC.a.v., no other precipitation bands could be observed, upon varying the enzyme concentrations.

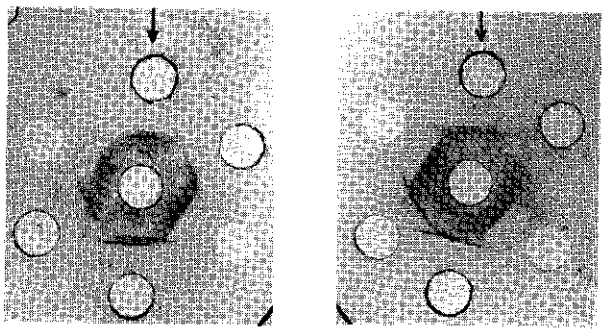


Figure 4

Double diffusion tests with anti-subcomplex from *E.coli* against PDC, subcomplex and PDH from *E.coli*. The conditions were the same as given in Figure 2, only anti-sub.E.c. is used instead of PDC. E.c.
a. "pure" PDC is used.
b. "impure" PDC is used.

6.4.6. The effect of antigen-antibody formation on the overall activities.

Incubations of the 3-component and 4-component complexes from *A.vinelandii* with anti-PDC.E.c. do not affect the overall activity. Also no decrease in activity was observed when pyruvate dehydrogenase complex from *E.coli* was incubated with anti-4.PDC.A.v..

In experiments where the 3-component and 4-component complexes from *A.vinelandii* were incubated with anti-4.PDC.A.v., the activities of both complexes drop to respectively 35% and 65% of the initial activity when incubated with 80 µl antiserum after 2 hours (Figs. 5a, 5b). In both experiments approximately the same amount of antigen was used, so it seems that the overall activity of

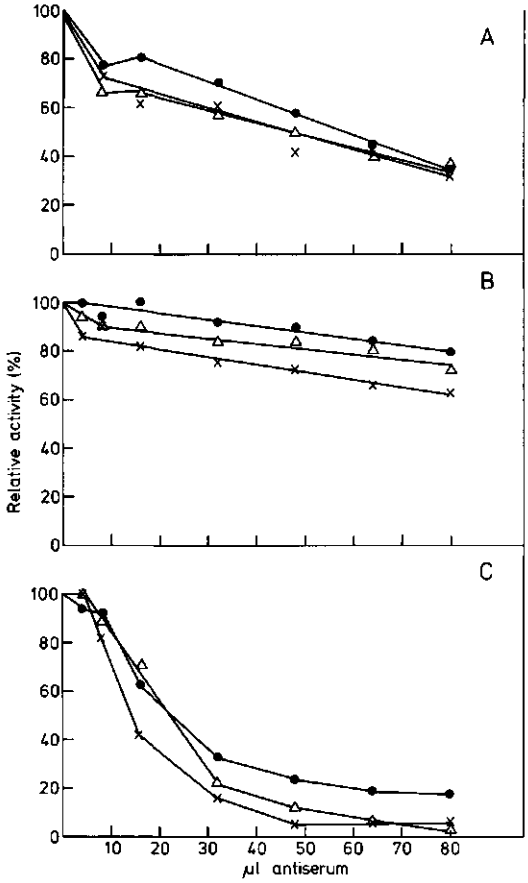


Figure 5

Decrease in overall activity after incubation of the pyruvate dehydrogenase complexes with their antibodies. The values are corrected for the blanks.

A. 3-component complex from *A.vinelandii*.

B. 4-component complex from *A.vinelandii*.

C. Complex from *E.coli*.

The activities were measured after incubation for 0 minutes (.), 60 minutes (Δ) and 90 minutes (x).

3-component complex is more sensitive to antigen-antibody formation than that of the 4-component complex. Furthermore the decline in activity of the 3-component complex is almost instantaneous.

With pyruvate dehydrogenase complex from *E.coli* an instantaneous decline in overall activity to a value of approximately 5% of the initial activity (Fig. 5c) was observed, when it was incubated with anti-PDC.E.c..

6.4.7. The effect of antigen-antibody formation on the partial activities

The decrease in pyruvate dehydrogenase activity, after antigen-antibody formation, is given in Fig. 6. The pyruvate dehydrogenase activity of 4-component of *A.vinelandii* drops slowly to about 70% of the original activity (Fig. 6b), upon incubation with 4-C.PDC.A.v. after 90 min. Hardly any decrease of pyruvate dehydrogenase activity was measured in experiments where 3-component complex from *A.vinelandii* was incubated with the same antibody (Fig. 6a). A slow decrease to about 50% in pyruvate dehydrogenase activity was observed in antigen-antibody formation of pyruvate dehydrogenase complex from *E.coli* with anti-PDC. E.c. after 90 min. (Fig. 6c).

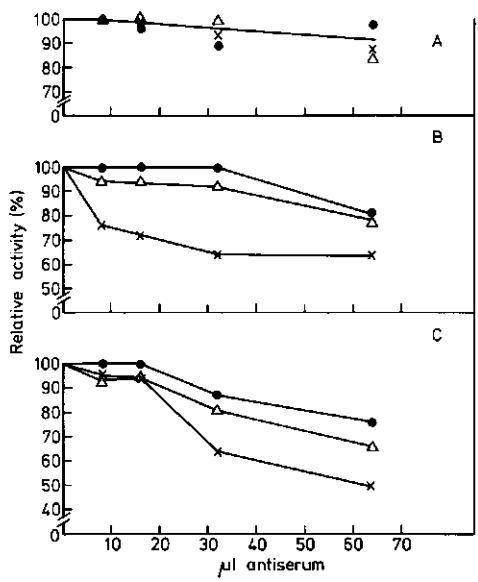


Figure 6

Decrease in pyruvate dehydrogenase activity after incubation of the pyruvate dehydrogenase complexes with their antibody. The values are corrected for the blanks.
A. 3-component complex from *A.vinelandii*.
B. 4-component complex from *A.vinelandii*.
C. complex from *E.coli*.
The activities were measured after incubation for 0 minutes (.), 30 minutes (Δ) and 90 minutes (x).

The results of the effect of antibody-antigen formation on the lipoamide dehydrogenase activity of the complexes from *A.vinelandii* show a decrease in activity (Fig. 7a,b) with the 3-component complex to about 55% and with the 4-component complex to about 75% of the original activity after incubation with antiserum for 90 min. The decrease of lipoamide dehydrogenase activity in experiments of pyruvate dehydrogenase complex from *E.coli* with anti-PDC.E.c. (Fig. 7c) is comparable with the observed decrease in pyruvate dehydrogenase activity (Fig. 6c) *e.g.* declining to 50%.

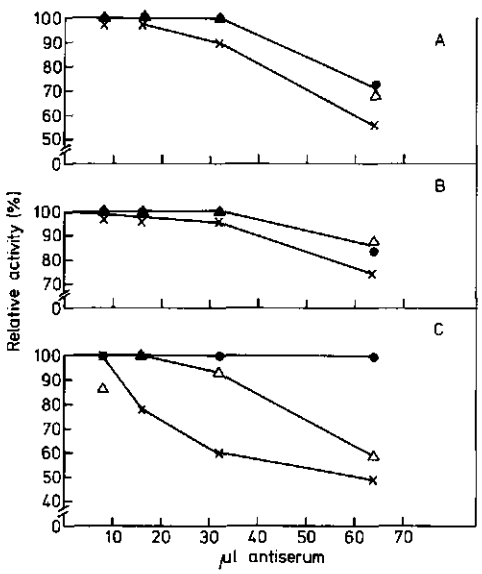


Figure 7

Decrease in lipoamide dehydrogenase activity after incubation of the pyruvate dehydrogenase complexes with their antibodies.
A, B and C stand for respectively 3-component complex from *A. vinelandii*, 4-component complex from *A.vinelandii* and *E.coli* complex.
The same incubation times and corresponding symbols are used as given in Figure 6.

We did not succeed to develop a good measurement for the lipoyltransacetylase activity after antigen-antibody formation. We observed higher lipoyltransacetylase activities upon addition of antiserum. The quite high increase of lipoyltransacetylase activity was observed with both a-specific and specific antisera.

5.5. DISCUS

It must be emphasized that the information obtained in the present study is concerned with the topology of the different complexes in a non-reacting state. It has been shown [chapter 5], that during catalysis major conformational changes occur.

The results of this immunological study show that the 4-component pyruvate dehydrogenase complex from *A.vinelandii* reacts, at higher concentrations, with anti-4-PDC.A.v. to give two bands. On polyacrylamide gels it is observed that these bands are from immunoprecipitation of pyruvate dehydrogenase and low-mol. wt. lipoyltransacetylase with anti-4.PDC.A.v.. This must mean that both components are located at the surface of the 4-component complex.

At higher concentrations the 3-component pyruvate dehydrogenase complex from *A.vinelandii* shows one band in immunodiffusion experiments with anti-4-PDC.A.v.. The precipitation band shows a pattern of identity with one of the two precipitation bands obtained in antigen-antibody formation of 4-component complex with anti-4-PDC.A.v.. Thus it can be concluded that the precipitation bands originate from one antigen having a set of identical determinant groups in both complexes with respect to anti-4.PDC.A.v.. Since the 3-component complex does not contain low-mol.wt. lipoyltransacetylase, it is obvious that pyruvate dehydrogenase is also located at the surface of the 3-component complex from *A.vinelandii*. In view of the fact that the 3-component *A.vinelandii* complex shows two precipitation bands upon dilution, it seems appropriate to conclude that it dissociates alike the *E.coli* complex into pyruvate dehydrogenase and subcomplex (high-mol.wt. lipoyltransacetylase plus lipoamide dehydrogenase). The observation that the second precipitation band is caused by lipoamide dehydrogenase as antigen points to the fact that in this subcomplex, this flavoprotein is located at the outside of a core of lipoyltransacetylase chains. Although this seems to be supported by the fact that the dissociated 4-component complex gives four bands, which shows that the high-mol.wt. lipoyltransacetylase antibody is present in the serum, several observations argue against this idea. The structural differences between the two complexes [chapter 5] reflect themselves in a large difference in lipoamide dehydrogenase activity [15]. In addition the fact that on a weight basis the amount of lipoyltransacetylase is three times that of lipoamide dehydrogenase, makes protection of the transacetylase by the flavoprotein rather improbable. Thus we conclude that in the 3-component complex the antigenic determination groups are hidden due to a conformational change.

Support for this conclusion is presented in chapter 5 where it is shown that, in contrast to the enzyme in the 3-component complex, high-mol.wt. lipoyltransacetylase is not in short-range interaction with pyruvate dehydrogenase in the 4-component complex.

The immunoprecipitation of the undissociated 4-component and 3-component complexes with anti-lipDH.A.v. indicates that lipoamide dehydrogenase, bound in the complexes, has determinants which are accessible to anti-lipDH.A.v., but not to anti-4.PDC.A.v.. These observations seem to contradict. However, when it is supposed that lipoamide dehydrogenase is located partly buried under the surface of both complexes, it will not give a precipitation reaction with anti-4.PDC.A.v.. The latter anti-serum contains, as shown in Fig. 1b, antibodies against all four enzymes. The antibodies against pyruvate dehydrogenase and low-mol.wt. transacetylase react first since these antigens are located at the surface of the complex.

Surprisingly, the immunodiffusion experiments of the 4-component complex with anti-lipDH.A.v. show two precipitation bands. One of the precipitation bands is identical with that of the 3-component complex with anti-lipDH.A.v. while the other precipitation band is identical with that of lipoamide dehydrogenase with anti-lipDH.A.v.. The following explanation seems plausable. The 4-component complex, contains one molecule of dimeric lipoamide dehydrogenase [24]. One of these chains is due to short range contact with high-mol.wt. lipoyltransacetylase [chapter 5] in a structure or conformation, alike in the 3-component complex. The other chain is due to long-range influence of the low-mol.wt. lipoyltransacetylase [chapter 5] in a conformation resembling the free enzyme. Removal of the low-mol.wt. lipoyltransacetylase - as it occurs in the conversion of 4-component complex into 3-component complex - brings the second lipoamide dehydrogenase chain in short range contact with the high-mol.wt. transacetylase, thus changing structure- conformation [chapter 5] and activity [15]. In agreement with this conclusion following fluorescence emission studies of the 3- and 4-component complexes of *A.vinelandii* show that each complex has two types of flavin fluorescence emittors. However, the anistropy of the emission as well as the fluorescence lifetimes of the two emittors are different in the two types of complexes [26]. In contrast the flavin fluorescence emission of free *A.vinelandii* lipoamide dehydrogenase is determined by only one emitting species [28].

Immuno-diffusion experiments with the pyruvate dehydrogenase complex from *E.coli* and anti-PDC.E.c. show that the multi-enzyme complex is precipitated in

two bands. One of the precipitation bands is formed from antigen-antibody formation of pyruvate dehydrogenase and anti-PDC.E.c., the other precipitation band is originated from determinant sites on the complex which are also reactive in immunoprecipitation of the subcomplex from *E.coli* with anti-PDC.E.c. thus either lipoyltransacetylase or lipoamide dehydrogenase. In experiments of the complex from *E.coli* with anti-sub.E.c. also two bands are formed, one of the precipitation bands is from antigen-antibody formation of pyruvate dehydrogenase with anti-sub.E.c., the other band is from antigen-antibody formation by identical determinant sites present on the pyruvate dehydrogenase complex and the subcomplex. It is obvious that the presence of a small amount of pyruvate dehydrogenase in the subcomplex is responsible for immuno-precipitation by pyruvate dehydrogenase. The remarkable observation of the inversion of the positions of the two precipitation lines of the *E.coli* complex and the "impure" complex can in our opinion be best explained by a decrease in mobility due to the presence of the fourth component, indicating interaction with the antigen.

Whether lipoamide dehydrogenase or lipoyltransacetylase is responsible for antigen-antibody formation with anti-PDC.E.c. and anti-sub.E.c., is not clear. Sodium dodecylsulphate electrophoresis of immunoprecipitates of pyruvate dehydrogenase complex from *E.coli* with anti-PDC.E.c. revealed a pyruvate dehydrogenase band and a heavy chain α-globuline band. Neither a lipoyltransacetylase band nor a lipoamide band could be observed. In view of the many similarities with the immunoprecipitation studies with the 3-component *A.vinelandii* complex, it seems likely to us to expect that the second precipitation band of the *E.coli* complex is caused by lipoamide dehydrogenase.

No direct relation was found between immunoprecipitation and inactivation. In general the overall activity decrease with respect to time course and extent different from those of the partial activities. Thus it seems that the transfer of acetyl groups, in the overall reaction, rather than the activities of the individual enzyme components is inhibited by antigen-antibody formation. In addition, it can be seen that the overall activity of the 4-component complex is less affected than that of the 3-component complex, which is in agreement with the conclusions drawn above *e.g.* the structure of 4-component complex is tighter and more closed [cf. chapter 5].

No cross-reaction was observed in inactivation and immunodiffusion experiments. Since the pyruvate dehydrogenase component is located at the surface of the complexes, it can be concluded that the pyruvate dehydrogenase components of the complexes from the two organisms are serologically different.

6.6. REFERENCES

1. Willms, C.R., Oliver, R.M., Henney, H.R., Mukherjee, B.B. and Reed, L.J. (1967) J.Biol.Chem. *242*, 889.
2. Reed, L.J. and Oliver, R.M. (1968) Brookhaven Symp.Biol., *21*, 397.
3. Reed, L.J. (1969) Current Topics in Cellular Regulation, Vol. I (Acad. Press, New York) 233.
4. Grande, H.J., van Telgen, H.J. and Veeger, C. (1976) Eur.J.Biochem. *59*, 355.
5. Cinader, B. (1963) Annals of The New York Academy of Sciences, *103*, 493.
6. Margoliash, E., Reichlin, M. and Nisoneff, A. (1967) Conformation of biopolymers (Acad.Press., New York).
7. Weir, D.M. editor (1973) Handbook of Experimental Immunology (Blackwell, Oxford).
8. Benjamin, E., Michaeli, D. and Young, J.B. (1972) Current topic in Microbiology and Immunology, *58*, 85.
9. Stöffler, G. (1974) Ribosomes (Cold Spring Harbor Laboratory).
10. Beil, W., Furthmayer, H. and Timpl, R. (1972) Immunochemistry, *9*, 779.
11. Adelmann, B.C. (1973) Immunology, *24*, 871.
12. Chidlow, J.W., Bourne, L.J. and Barley, A.J. (1974) Immunology, *27*, 665.
13. Nowack, H., Hahn, E. and Timpl, R. (1975) Immunology, *29*, 621.
14. Bresters, T.W., De Abreu, R.A., De Kok, A., Visser, J. and Veeger, C. (1975) Eur.J.Biochem. *81*, 357.
15. De Abreu, R.A., De Kok, A. and Veeger, C. (1977) FEBS Lett. *82*, 89.
16. Eley, M.H., Genshin, N., Hamilton, L., Munk, P. and Reed, L.J. (1972) Arch.Biochem.Biophys. *152*, 655.
17. Speckhard, D.C. and Frey, P.A. (1975), Biochem.Biophys.Res.Commun. *62*, 614.
18. Massey, V., Gibson, Q.H. and Veeger, C. (1960) Biochem.J., *77*, 341.
19. Schwartz, E.R. and Reed, L.J. (1970) Biochemistry, *9*, 1434.
20. Alkonyi, I., Bolygo, E., Gyocsi, L. and Szabo, D. (1976) Eur.J.Biochem. *66*, 551.
21. Reed, L.J. and Willms, C.R. (1975) Methods in Enzymol. *9*, 247.
22. Massey, V. and Veeger, C. (1960) Biochim.Biophys.Acta. *40*, 184.
23. Lowry, O.H., Rosebrough, N.J., Far, A.L. and Randall, R.J. (1951), J.Biol. Chem. *193*, 265.
24. De Abreu, R.A., De Kok, A., De Graaff-Hess, A.C.and Veeger, C. (1977) Eur.J.Biochem. *81*, 357.
25. Ouchterlony, O (1949) Acta Pathol.Microbiol.Scan. *26*, 507.

26. Veeger, C., Visser, A., Eweg, J., Grande, H., De Abreu, R., de Graaf-Hess, A. and Müller, F., Proc. of the 6th Symposium on Flavins and Flavoproteins (K. Yagi and T. Yamano eds.), Japanese Scientific Soc. Press, to be published.
27. Leammli, U.K. (1970), Nature *227*, 680.
28. Veeger, C., Visser, A.J.W.G., Krul, J., Grande, H.J., De Abreu, R.A. and De Kok, A. (1976) Flavins and Flavoproteins (T.P. Singer ed.), 500.

SUMMARY

The aim of the present investigation was to obtain more information of the structure and function of the pyruvate dehydrogenase complexes from *Azotobacter vinelandii* and *Escherichia coli*.

In chapter 2 a survey is given of the recent literature on pyruvate dehydrogenase complexes.

In chapter 3 results are presented, describing the behaviour of the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* Crookes on blue dextran Sepharose 4B columns. It is shown that the 4-component pyruvate dehydrogenase complex from *A.vinelandii* binds strongly to the column through its low-mol.wt. transacetylase while the pyruvate dehydrogenase complex from *E.coli* Crookes does not bind. The pyruvate dehydrogenase complex from *A.vinelandii* can be eluted with 0.6 M potassium chloride as an active 3-component complex. Properties of the 3-component complex, such as the Hill coefficient of the overall reaction, the stimulation by AMP and the inhibition by acetyl-CoA are not different from the original complex. The 3-component complex, however, shows a 3-fold increase of lipoamide dehydrogenase activity which is much larger than the increase in FAD content.

In chapter 4, the results from studies with radioactive pyruvate and radioactive N-ethylmaleimide are presented. The results from these studies show that maximally four radioactive groups per mole FAD are incorporated into the high-mol.wt. lipoyltransacetylase component of the pyruvate dehydrogenase complex from *A.vinelandii* and to the lipoyltransacetylase component of the pyruvate dehydrogenase complex from *E.coli* when the complexes are incubated with [2-^{14}C] pyruvate, magnesium chloride and TPP or with N-ethyl [2,3-^{14}C] maleimide in the presence of pyruvate, magnesium chloride and TPP under anaerobic conditions. With 10 mM [2-^{14}C] pyruvate, the low-mol.wt. transacetylase is also labelled; to this enzyme three to four [^{14}C] acetylgroups are bound. The 3-component pyruvate dehydrogenase complex from *A.vinelandii*, eluted by chromatography from a blue dextran-Sepharose 4B column, binds a maximum of four [^{14}C] acetyl groups per mole of FAD. From these results it is concluded, that four lipoyl groups per mole of FAD are present in the complexes from both sources. Furthermore possible

stoichiometries of the different enzyme components are discussed.

In chapter 5, the crosslinking studies of bifunctional reagents with the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* are described. The results from these studies show that lipoamide dehydrogenase and high-mol. wt. transacetylase, as well as that low-mol.wt. transacetylase and pyruvate dehydrogenase are at close distances in the 4-component complex from *A.vinelandii*. In the 3-component pyruvate dehydrogenase complex from *A.vinelandii* and the pyruvate dehydrogenase complex from *E.coli*, all three components seem to be organized at close distances to one another. Crosslinking with diimidates in the presence of pyruvate, Mg^{2+} and TPP show a conformational change of the transacetylase core of the pyruvate dehydrogenase complex from *A.vinelandii*. In the complex from *E.coli* no crosslinking was observed under these conditions between the lipoyltransacetylase monomers but rather close positioning was observed of lipoyl groups from the lipoyltransacetylase component and pyruvate dehydrogenase component which indicate that SH-groups are exposed on the pyruvate dehydrogenase in the presence of pyruvate Mg^{2+} and TPP.

In chapter 6 results are presented of immuno-chemical studies on the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli*. The results of the immunochemical studies indicate that the pyruvate dehydrogenase component is located at the surface of both complexes. In addition, the low-mol.wt. lipoyltransacetylase is also located at the surface of the 4-component pyruvate dehydrogenase complex from *A.vinelandii*. The low-mol.wt. lipoyltransacetylase seems to protect the 4-component complex against inactivation by its antiserum. Furthermore it is shown that the pyruvate dehydrogenase complexes from *A.vinelandii* and *E.coli* are serologically different.

The results from the studies presented in this thesis seem to fit with the generally accepted model of the pyruvate dehydrogenase complex from *E.coli* presented by Reed and coworkers (chapter 2). The pyruvate dehydrogenase complex from *A.vinelandii* seems to consist of a "core" of high-molecular weight transacetylase chains to which a lipoamide dehydrogenase dimer is bound. At the surface of the complex pyruvate dehydrogenase components and low-molecular weight transacetylase are bound.

It is apparent that more experiments are needed to be performed, to obtain a more detailed model of especially the pyruvate dehydrogenase complex from *A.vinelandii*.

SAMENVATTING

Dit proefschrift handelt over studies die zijn verricht aan de pyruvaat dehydrogenase complexen van *Azotobacter vinelandii* en *Escherichia coli*. Het doel van het onderzoek was meer inzicht te verkrijgen in de structuur en functie van deze multi-enzym complexen.

Hoofdstuk 1 bevat een algemene inleiding tot het onderzoek. In dit hoofdstuk wordt tevens in het kort een motivatie gegeven voor het onderzoek.

In hoofdstuk 2 wordt een literatuuroverzicht gegeven van studies aan de pyruvaat dehydrogenase complexen van eukaryoten en prokaryoten. Daarbij wordt samengevat wat er bekend is over het mechanisme, de regulatie en de structuur van deze eiwitcomplexen.

In hoofdstuk 3 wordt het gedrag beschreven van de pyruvaat dehydrogenase complexen van *A.vinelandii* en *E.coli* op blue dextran-Sepharose 4B kolommen. Het blijkt dat het oorspronkelijke 4-componenten pyruvaat dehydrogenase complex van *A.vinelandii* via de laag-moleculaire lipoyltransacetylase component goed bindt op deze kolommen, terwijl het pyruvaat dehydrogenase complex van *E.coli* hoegenaamd niet bindt aan dit materiaal. Het pyruvaat dehydrogenase complex van *A.vinelandii* kon uiteindelijk als een actief 3-componenten complex van deze kolom worden geëlueerd met 0.6 M kalium chloride als elutievloeistof. In dit complex ontbreekt de laag-moleculaire lipoyltransacetylase component, die onder deze omstandigheid met een deel van de pyruvaat dehydrogenase component op de kolom gebonden blijft. Het 3-componenten complex blijkt in vergelijking tot het 4-componenten complex een veel hogere lipoamide dehydrogenase activiteit te bezitten. Andere eigenschappen zoals de Hill coëfficient van de totale reactie, de stimulering door AMP en de remming door acetyl-CoA, verschillen niet van die van het 4-componenten complex.

Hoofdstuk 4 handelt over specifieke inbouw van [^{14}C] acetyl groepen en N-ethyl [2,3-^{14}C] maleimide op de lipoylresten van de pyruvaat dehydrogenase complexen van *A.vinelandii* en *E.coli*. Daarbij blijkt, dat bij incubatie met [2-^{14}C] pyruvaat, magnesium chloride en thiamine pyrofosfaat maximaal vier acetyl groepen per molecuul gebonden FAD kunnen worden ingebouwd op de lipoyltransacetylase componenten van de pyruvaat dehydrogenase complexen van *A.vinelandii*

en *E.coli*. Bij het 4-componenten complex van *A.vinelandii* vindt uitsluitend inbouw op de hoog-moleculaire lipoyltransacetylase plaats indien concentraties lager dan 0.7 mM worden gebruikt. Extra inbouw tot 3-4 groepen per mol. FAD vindt op de laag-moleculaire lipoyltransacetylase plaats bij incubatie met 10 mM pyruvaat. De experimenten met N-ethyl [2,3-^{14}C] maleimide in aanwezigheid van pyruvaat, magnesiumchloride en thiamine pyrofosfaat tonen aan dat ook van deze radioactieve label maximaal vier groepen worden ingebouwd per mol FAD. Uit deze gegevens wordt geconcludeerd, dat vier acetyl bindingsplaatsen per mol gebonden FAD op de respectievelijke transacetylase componenten van de pyruvaat dehydrogenase complexen van *A.vinelandii* en *E.coli* aanwezig zijn.

In hoofdstuk 5 worden de studies van bifunctionele reagentia met de pyruvaat dehydrogenase complexen van *A.vinelandii* en *E.coli* beschreven. Uit de resultaten van de crosslinking experimenten met diimidaten blijkt, dat hoog-moleculaire lipoyltransacetylase en lipoamide dehydrogenase dicht bij elkaar zitten in het 4-componenten complex van *A.vinelandii*. Hetzelfde geldt voor pyruvaat dehydrogenase en laag-moleculaire lipoyltransacetylase, die ook dicht bij elkaar gestapeld zitten in het 4-componenten complex van *A.vinelandii*. In het 3-componenten complex van *A.vinelandii* en het complex van *E.coli* zitten alle drie enzym componenten dicht bij elkaar. Verder blijkt het pyruvaat dehydrogenase complex van *A.vinelandii* in aanwezigheid van pyruvaat, Mg^{2+} en thiamine pyrofosfaat een conformatieverandering te ondergaan, die wordt geïnduceerd door pyruvaat. Onder deze omstandigheden vertoont het pyruvaat dehydrogenase complex van *E.coli* deze conformatieverandering niet. Hier blijkt echter, dat de lipoylresten van de lipoyltransacetylase dicht bij SH-groepen zitten, die vrij komen op de pyruvaat dehydrogenase component bij incubatie met pyruvaat, magnesium chloride en thiamine pyrofosfaat.

Tenslotte worden in hoofdstuk 6 voorlopige immunochemische experimenten aan pyruvaat dehydrogenase complexen van *A.vinelandii* en *E.coli* behandeld. De resultaten van deze studies tonen aan, dat pyruvaat dehydrogenase en laag-moleculaire lipoyltransacetylase aan de buitenkant van het pyruvaat dehydrogenase complex van *A.vinelandii* gelocaliseerd zijn. De lipoamide dehydrogenase component ligt gedeeltelijk verborgen in het complex. Ook in het complex van *E.coli* blijkt pyruvaat dehydrogenase aan de buitenkant te zitten. Verder is aangetoond dat er geen precipitatie waar te nemen is bij experimenten waarbij antigen van *E.coli* wordt gebruikt tegen antilichaam van *A.vinelandii* of omgekeerd. We veronderstellen een serologisch verschil tussen de pyruvaat dehydrogenase componenten van de pyruvaat dehydrogenase complexen van de beide bacte-

ria.

De resultaten van de studies beschreven in de hoofdstukken van dit proefschrift komen overeen met het model van Reed en zijn medewerkers voor het pyruvaat dehydrogenase complex van *E.coli* (hoofdstuk 2). Het complex van *A.vinelandii* bestaat waarschijnlijk ook uit een kern van hoog-moleculaire transacetylase ketens, waaraan de lipoamide dimer gebonden is. Aan de buitenkant van het complex zijn dan pyruvaat dehydrogenase en laag-moleculaire lipoyltransacetylase in onderlinge interactie gebonden.

CURRICULUM VITAE

Ronney Alphonso De Abreu, werd op 26 september 1937 te Paramaribo in Suriname geboren.

In 1953 behaalde hij het mulo-B diploma, waarna hij een studie begon voor chemisch analist. Het diploma voor chemisch analist II^A van de KNCV werd door hem behaald in oktober 1959.

Vanaf september 1954 tot september 1967 heeft hij als analist gewerkt op de Keuringsdienst van Waren en de Pharmaceutische dienst te Paramaribo, Apotheek van Gorp te Tilburg en de Keuringsdienst van Waren te Nijmegen.

In de periode 1965-1968 volgde hij de avond HBS-opleiding op het Craneveldt Lyseum te Nijmegen. Op 26 juli 1968 werd deze studie afgesloten met het behalen van het HBS-B diploma.

Een jaar eerder, in september 1967, schreef hij zich als scheikunde student in aan de Katholieke Universiteit van Nijmegen. Op 1 juni 1970 werd het Kandidaatsexamen (S2) afgelegd. Voor de doctoraal studie koos hij als hoofdvak biochemie (prof. H. Bloemendal) en de bijvakken biofysische chemie (prof. G.A.J. van Os) en de capita: instrumentele methoden, chemische technologie, kwantumchemie en reactiekinetiek en katalyse.

Het onderzoek voor dit proefschrift werd verricht op de afdeling Biochemie van de Landbouwhogeschool, waar hij van 16 mei 1973 tot 15 mei 1974 in dienst was van de stichting Z.W.O. en van 16 mei 1974 tot 1 augustus 1978 van de Landbouwhogeschool

Vanaf 1 augustus 1973 doceert hij chemie aan de avondopleiding van de O.L.A.N. te Arnhem.

Met ingang van 1 september 1978 is hij in dienst getreden van de Katholieke Universiteit van Nijmegen. Hij werkt daar als biochemicus op het laboratorium voor Kindergeneeskunde van het Radboudziekenhuis.