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PYRUVATE DEHYDROGENASE COMPLEX FROM *AZOTOBACTER VINELANDII*

(with a summary in Dutch)

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CONTENTS

LIST OF ABBREVIATIONS

A	absorbance
ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
ATCC	American type culture collection
ATP	adenosine 5′-triphosphate
BSA	bovine serum albumin
BV	benzyl viologen
CoA	co-enzyme A
CoASAc	acetyl co-enzyme A
cyclic AMP	adenosine-3′:5′-monophosphate
$D_{20,w}$	diffusion coefficient in water at 20°
DCIP	2,6-dichlorophenol indophenol, oxidized form
DEAE-	diethyl amino ethyl-
DTNB	5,5-dithio-bis(-2-nitro)benzoic acid
DTT	*dithiothreitol* (CLELAND's reagent)
EPR	electron paramagnetic resonance
FAD	flavin adenine dinucleotide, oxidized form
FMN	flavin adenine mononucleotide, oxidized form
GDP	guanosine 5′-diphosphate
GSH	glutathione, reduced form
GTP	guanosine 5′-triphosphate
K_D	dissociation constant
KGDH	α-keto glutarate dehydrogenase complex
K_I	dissociation constant of enzyme-inhibitor complex
K_M	Michaelis constant
lipoamide S_2	lipoamide, oxidized form
lipoamide$(SH)_2$	lipoamide, recuced form
lipoamide(SH)-S-acetyl	S-acetyldihydrolipoamide
$lipS_2$	lipoic acid, oxidized form
$lip(SH)_2$	lipoic acid, reduced form
lip(SH)-S-acetyl	S-acetyldihydrolipoic acid
LTA	dihydrolipoyl transacetylase component
M	molecular weight
M_{app}	apparent molecular weight
Mg^{2+}.TPP	complex between Mg^{2+} and TPP
Mw	weight average molecular weight
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate), oxidized form
NAD(P)H	nicotinamide adenine dinucleotide (phosphate), reduced form
NEM	N-ethylmaleimide
PDC	pyruvate dehydrogenase complex
PDH	pyruvate dehydrogenase component
PEG	poly-ethylene glycol
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
PTA	phosphotransacetylase
RNA	ribonucleic acid
r.p.m.	revolutions per minute
$s_{20,w}$	sedimentation coefficient in water at 20°
SDS	sodium dodecylsulphate, sodium laurylsulphate
TCA	trichloroacetic acid

TPP	thiamine pyrophosphate
Tris	tri(hydroxymethyl) amino methane
v_o	initial velocity
V	maximum velocity

LIST OF ENZYMES USED THROUGHOUT THIS THESIS

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.

EC number	Systematic name	Trivial name
1.2.4.1.	pyruvate: lipoate oxidoreductase (acceptor acylating)	pyruvate dehydrogenase complex
4.1.1.1.	pyruvate decarboxylase (2-oxoacid carboxy-lyase)	pyruvate dehydrogenase component
2.3.1.12	CoASAc: dihydrolipoate S-acetyltransferase	dihydrolipoyl transacetylase
1.6.4.3.	NADH: lipoamide oxido reductase	lipoamide dehydrogenase
2.3.1.8.	CoASAc: orthophosphate acetyltransferase	phosphotransacetylase

1. INTRODUCTION

Enzyme systems, catalyzing a CoA- and NAD^+-linked oxidative decarboxylation of pyruvate, are called pyruvate dehydrogenase complexes (PDC). They are performing this aerobic oxidation of pyruvate (described by Equation 5) via a complex sequence, consisting of at least four reactions (Equations 1 to 4) with the aid of three enzymes; thus they are multi-enzyme complexes (REED and Cox, 1966).

$$H^+ + pyruvate + E_1\text{-}Mg^{2+}.TPP \longrightarrow CO_2 + E_1\text{-}Mg^{2+}.TPP\text{-}hydroxyethyl \quad (1)$$

$$E_1\text{-}Mg^{2+}.TPP\text{-}hydroxyethyl + E_2\text{-}lipS_2 \longrightarrow E_1\text{-}Mg^{2+}.TPP + E_2\text{-}lip(SH)\text{-}S\text{-}acetyl \quad (2)$$

$$E_2\text{-}lip(SH)\text{-}S\text{-}acetyl + CoA \rightleftharpoons E_2\text{-}lip(SH)_2 + CoASAc \quad (3)$$

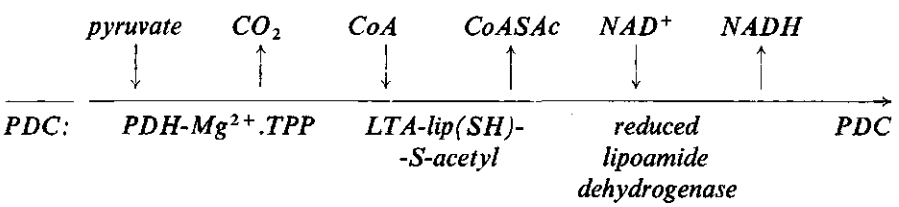
$$E_2\text{-}lip(SH)_2 + NAD^+ \overset{E_3.FAD}{\rightleftharpoons} E_2\text{-}lipS_2 + NADH + H^+ \quad (4)$$

Sum:

$$pyruvate + NAD^+ + CoA \xrightarrow[Mg^{2+},TPP,lipS_2]{E_1,E_2,E_3.FAD,} CoASAc + CO_2 + NADH \quad (5)$$

In these Equations, E_1 is the pyruvate dehydrogenase component (PDH), which catalyzes the Mg^{2+}.TPP-dependent decarboxylation and the dehydrogenation of hydroxyethyl-TPP with subsequent reductive acetylation of lipoic acid covalently bound to E_2 by amide linkage to a lysine ε-amino group; E_2 is the dihydrolipoyltransacetylase (LTA) and catalyzes the transfer of acetyl groups from acetyl dihydrolipoic acid to CoA; E_3.FAD is lipoamide dehydrogenase, which catalyzes the dehydrogenation of protein-bound dihydrolipoic acid by NAD^+ resulting in generation of the oxidized lipoic acid. The cycle of transformations involving the interaction (possibly by rotation) of the protein-bound lipoyl moiety with PDH and lipoamide dehydrogenase is, according to REED and OLIVER (1968), schematically represented in FIG. 1.1.

CLELAND (1963) suggested that the overall reaction (Equation 5) proceeds via a three-site ping-pong mechanism, viz. products are released before all substrates have added to the enzyme. This can be presented graphically as:



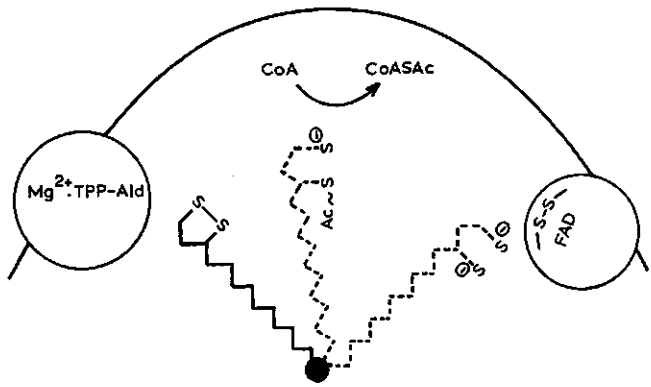


FIG. 1.1.

The individual components of the complex can be located on basis of the reactions described by Equations 6 to 8 respectively (partial reactions).

$$pyruvate + H_2O + 2\,Fe(CN)_6^{3-} \xrightarrow{PDH,\ Mg^{2+}.TPP} acetate + CO_2 + 2\,Fe(CN)_6^{4-} + 2\,H^+ \quad (6)$$

$$CoASAc + lipoamide(SH)_2 \xrightarrow{LTA} lipoamide\,(SH)\text{-}S\text{-}acetyl + CoA \quad (7)$$

$$H^+ + NADH + lipoamideS_2 \xleftrightarrow{lipoamide\ dehydrogenase} NAD^+ + lipoamide(SH)_2 \quad (8)$$

These multi-enzyme complexes have been isolated as functional units (with molecular weights in the millions) from a number of sources, like pigeon breast (JAGANNATHAN and SCHWEET, 1952), *Mycobacterium tuberculosis* (GOLDMAN, 1958), *E.coli* (KOIKE et al., 1960a; VOGEL et al., 1971; ELEY et al., 1972), pig heart (HAYAKAWA et al., 1966b), beef kidney (ISHIKAWA et al., 1966; LINN et al., 1972), beef heart (LINN et al., 1972), *Neurospora* (HARDING et al., 1970; WIELAND et al., 1972a) and *Hansenula miso* (HIRABAYASHI et al., 1972). The major part of the investigations, concerning the pure enzyme, have been performed with the *E. coli* complex and some with complexes from mammalian origin. Those complexes have been separated into the three compiling components (KOIKE et al., 1963; HAYAKAWA et al., 1969) and after mixing of the three pure enzymes, reconstitution of overall activity together with reassociation has been observed.

Biochemical and electron microscopic studies suggest, that *E. coli* PDC contains a '*core*' of LTA, consisting of 24 identical polypeptide chains, which are linked together by non-covalent bonds. One molecule of lipoic acid is co-

valently bound to each chain (REED and OLIVER, 1968; ELEY et al., 1972). Twenty four identical monomeric chains of PDH and twelve monomeric chains of lipoamide dehydrogenase are thought to be distributed in a regular manner along the edges of the transacetylase cube; isolated from the complex, the physiological active species of PDH and of lipoamide dehydrogenase are dimers (cf. VOGEL and HENNING, 1971; MASSEY, 1963). The cubical structure of the transacetylase can be deduced from the electron micrographs, where tetrads (12–14 nm on a side) and images, having the appearance of two parallel rows of subunits with a length of 17–20 nm, are visible (WILLMS et al., 1967). After the other two components have been joined, non-covalently, a polyhedral structure is observed with a diameter of about 30 nm (REED and OLIVER, 1968). The exact stoechiometry of the components in the bacterial complex does not seem to be solved unequivocally yet, since VOGEL et al. (1972a,b) have shown PDC from *E.coli* K-12 not to be a unique entity in that the amount of the PDH component, attached to the transacetylase, can vary. The '*core*' complex, from which this excess PDH has been removed, consists of 16 chains of the PDH, 16 chains of LTA and 16 chains of lipoamide dehydrogenase.

The appearance of the mammalian transacetylase '*core*' in the electron micrographs is not a cube; this enzyme seems to be an isometric particle, of *pentagonal dodecahedral* symmetry, with a diameter of 21–24 nm (ISHIKAWA et al., 1966; REED and COX, 1966; HAYAKAWA et al., 1969; JUNGER and REINAUER, 1972). Attachment of the other components results in an isometric particle with a diameter of 40–45 nm. The transacetylase '*core*' would be consisting of 60, possibly identical, polypeptide chains, each containing a lipoyl moiety (BARRERA et al., 1972). Beef kidney and beef heart PDH contains, contrary to *E.coli*, two nonidentical subunits (ROCHE and REED, 1972). Sixty pyruvate dehydrogenase units ($\alpha\beta$) or 30 units ($\alpha_2\beta_2$), but only 10–12 flavoprotein chains are attached to the transacetylase '*core*' (HAYAKAWA et al., 1969; LINN et al., 1972; BARRERA et al., 1972). In addition 5 units of a kinase, pyruvate dehydrogenase kinase, and up to 5 units phosphatase are found associated with the '*core*' (LINN et al., 1969a; LINN et al., 1972; BARRERA et al., 1972). They appeared to be regulatory subunits (see below) and are characteristic for all complexes originating from mammals and *Neurospora* (LINN et al., 1969a,b; WIELAND and VON JAGOW-WESTERMANN, 1969; WIELAND et al., 1972a). *E.coli* PDC is reported to be devoid of the latter enzymes (SCHWARTZ and REED, 1970a).

DIETRICH and HENNING (1970) have shown that the synthesis of the complex in *E.coli* K-12 is subject to regulation; depending on the carbon source used for growth, the amount of PDC produced can vary by a factor of about 10. Pyruvate is the metabolite causing induction. In the mechanism of regulation of the activity of the pyruvate dehydrogenase complexes, differences are observed between those from mammalian and bacterial sources. In both cases the activity is inhibited by the products of pyruvate oxidation: CoASAc and NADH (GARLAND, 1964; GARLAND and RANDLE, 1964; HANSEN and HENNING, 1966; SCHWARTZ and REED, 1968). The inhibition by CoASAc is reversed by CoA in

the mammalian system and by pyruvate in the *E.coli* system. NADH inhibition is exerted through action on the flavoprotein component, lipoamide dehydrogenase, of the complex and is competitive with respect to NAD^+. When only substrates are present, the kinetic properties of the mammalian PDC are consistent with the patterns predicted for three-site ping-pong mechanisms, while most of the observed product inhibition patterns also agree (CLELAND, 1973; TSAI et al., 1973).

ATKINSON (1968) reasoned that the PDC from *E.coli* is subject to regulation by the energy charge (a parameter measuring the mole fraction of phosphoanhydride bounds in the adenylate pool) and also to the concentration of feedback modifiers. This type of control is generally thought to modulate the biosynthetic and biodegradative pathways that use or replenish ATP. The PDH, which catalyzes the first step of the reaction of the PDC, seems to be the main site for the regulation by energy charge.

E.coli PDC shows, contrary to the mammalian complexes, a sigmoidal kinetic behaviour with respect to variations in the pyruvate concentration (SCHWARTZ et al., 1968; BISSWANGER and HENNING, 1971). Such kinetics are a common feature of a large number of regulatory enzymes and compatible with various theories (MONOD et al., 1965; KOSHLAND et al., 1966; FERDINAND, 1966; NICHOL et al., 1967; RABIN, 1967).

The relative importance of the inhibition by the products NADH and CoASAc in regulating the activity of the mammalian complex, remains to be determined, since LINN et al. (1969a,b) and WIELAND and VON JAGOW-WESTERMANN (1969) described that PDC activity from different sources (beef heart, pig heart, beef liver, beef kidney) is controlled by phosphorylation and dephosphorylation (cf. the interconversions of phosphorylase a and phosphorylase b and those of the glucose 6-phosphate-dependent and -independent forms of glycogen synthetase; KREBS, 1972). Phosphorylation and concomitant inactivation is catalyzed by an ATP-specific kinase, which action can be weakly (competitively) inhibited by ADP (LINN et al., 1969b; HUCHO et al., 1972; ROCHE and REED, 1972). This effect is exerted more pronounced by the kidney than by the heart kinase.The target of the phosphorylation is the PDH component of the enzyme complex; more precisely the α subunit, probably by esterification of one seryl residue (LINN et al., 1972). The PDH does not decarboxylate anymore. Separation of the PDH from the LTA results in enhancement of the K_M of the kinase for PDH, thus defacilitating the phosphorylation (HUCHO et al., 1972). Reactivation by dephosphorylation is catalyzed by a phosphatase in the presence of 10 mM Mg^{2+}, a value about ten times the concentration required for the action of the kinase.

HUCHO et al. (1972) have suggested that a change in the magnesium concentration, produced by a change in the ATP/ADP ratio, may be even more effective than the ATP/ADP ratio itself in regulating the PDC activity. Contrary to these authors, SIESS and WIELAND (1972) are claiming the involvement of Ca^{2+} too in the action of PDH phosphatase from pig heart muscle. Pyruvate protects the PDC against inactivation by inhibiting the kinase (LINN et al.,

1969b; WIELAND and VON JAGOW-WESTERMANN, 1969; HUCHO et al., 1972). The latter phenomenon is more pronounced with the bovine heart complex than with the bovine kidney complex. In intact rat liver mitochondria and isolated rat liver during perfusion, pyruvate causes, dependent on the concentration in a sigmoidal way, a shift of the inactive to the active form of the PDC (PORTENHAUSER and WIELAND, 1972; PATZELT et al., 1973). Maximal activity is not achieved, however.

The regulatory mechanism of mammalian PDC is of special importance for the regulation of gluconeogenesis and the synthesis of fat. Recent investigations have revealed that pyruvate oxidation might represent the rate-limiting step in conversion of carbohydrate to fat (WIELAND et al., 1972b; TAYLOR et al., 1973; BERGER and HOMMES, 1973) in liver and adipose tissue. The multiple regulatory controls of PDC in intact mitochondria by fatty acids and hormones are mostly interpreted in terms of alteration of the degree of phosphorylation of the enzyme protein, thus producing a change in the steady state levels of active PDC.

In adipose tissue, liver and heart mitochondria pyruvate oxidation is inhibited by fatty acids (WIELAND et al., 1971, 1972b; STUCKI et al., 1972; TAYLOR et al., 1973 and PATZELT et al., 1973). Improvement of the ATP/ADP ratio in the mitochondrial matrix results in conversion of active PDC into the inactive form. Oligomycine or 2,4-dinitrophenol counteracts this effect. The fatty acids behave antagonistic with respect to pyruvate in their regulation of PDC-interconversion. The way of action of the short- and medium chain fatty acids differs from that of the long chain ones and their influence can be shown to vary with the source of the mitochondria (STUCKI et al., 1972). It could be argued that part of the inhibition is caused by the products of fatty acid oxidation viz. NADH and CoASAc (REED, 1969; WIELAND et al., 1972b).

The hormonal regulation of PDC in intact mitochondria by the antilipolytic acting insulin and the lipolytic acting epinephrine is not clearly understood yet. According to, for instance, COORE et al. (1971), JUNGAS (1971), WIELAND et al. (1972b), TAYLOR et al. (1973) and SICA and CUATRECASAS (1973) insulin activates PDC in mitochondria of adipose tissue upon incubation. Bicarbonate ions, glucose and fructose were shown stimulatory to this process; insulin's effect is probably mediated by changes in the concentration of intramitochondrial metabolites. This view is supported by the observation of the latter authors, that insulin causes, in comparison with a control experiment, a more rapid fall in enzyme activity in the initial period, followed by a rise. The total activity increased, thus the effect does not result simply from conversion of inactive to active PDC. The effects of insulin on PDC may be mediated by changes in protein synthesis, for they are blocked in the presence of cyclohexiimide and puromycin.

Epinephrine activates PDC activity in adipose tissue in a biphasic way (JUNGAS, 1971; TAYLOR et al., 1973; SICA and CUATRECASAS, 1973) by converting the inactive into the active form, but without increasing the total amount of the enzyme. Conflicting views are present in the literature; COORE et al. (1971) and JUNGAS (1971) found the effects of epinephrine and insulin antagonistic, but SICA and

CUATRECASAS (1973) did not conclude this. Although exogenous cyclic AMP apparently elevates the PDC activity, while it is generally accepted that epinephrine and insulin regulate, however in different mode of action, the intracellular levels of cyclic AMP, it has not been possible to demonstrate a direct effect on the PDC-, PDH-kinase- or PDH-phosphatase activities in homogenates or on the purified enzymes by this compound (JUNGAS, 1971; HUCHO et al., 1972; SIESS and WIELAND, 1972).

Concerning the oxidative decarboxylation of pyruvate in microbial systems, variations in mechanism have been observed. Except the lipoic acid-dependent, and consequently NAD^+-dependent, system (as described already), it is worthwhile to mention some of the '*clastic*' reactions, involving the direct formation of CoASAc and subsequent conversion to acetylphosphate. Under anaerobic conditions, in many *Enterobacteriaceae*, pyruvate is fermented according to Equations 9 and 10 (CHANTRENNE and LIPMANN, 1950).

$$pyruvate + CoA \xleftarrow{E\text{-}Mg^{2+}.\ TPP} CoASAc + formate \qquad (9)$$

$$CoASAc + phosphate \longleftrightarrow acetylphosphate + CoA \qquad (10)$$

Reaction (9) is catalyzed by the, so-called, pyruvate formate-lyase (CHASE and RABINOWITZ, 1968) and is completely inhibited under aerobic conditions by inactivation of the enzyme (HENNING, 1963). The purpose of the reaction is thought to be the formation of ATP. The pyruvate formate-lyase from *E.coli* and its regulation has been studied in more detail by KNAPPE et al. (1969) and it turned out to be a single enzyme, which is only in the active form when being alkylated with S-adenosyl methionine in the presence of a second protein which itself requires an activation by ferrous ions and a dithiol. Inactivation is caused by reoxidation after dealkylation of the enzyme. There are still many open questions concerning the relation between this covalent modification and the enzyme activity. Reaction (10) is catalyzed by the enzyme phosphotransacetylase (PTA) and will actually produce the '*phosphoroclastic*' reaction, although in general it is the result of the two reactions together being referred to as '*phosphoroclastic*'. Furthermore, the pyruvate formate-lyase reaction was demonstrated to be present in some *Clostridia* species, where the main function of the enzyme was proposed to be the supply of one-carbon units (THAUER et al., 1972). Contrary to the *E.coli* enzyme, the *Clostridial* enzymes are essentially reversible in their action.

Alternatively, in some species, the '*phosphoroclastic*' reaction does not give rise to electron-transfer to CO_2, producing the low potential donor formate, but the electrons are transferred via the primary acceptor ferredoxin to protons leading to formation of molecular hydrogen. As shown by MORTENSON et al. (1962) the '*phosphoroclastic*' cleavage of pyruvate plays an important role in the

process of nitrogen fixation of anaerobically grown organisms by supplying reducing equivalents and energy. A similar process has until now not been discovered for strictly aerobic nitrogen fixing bacteria, like *Azotobacter vinelandii*, where NADPH has been proposed to act as electron donor for the nitrogenase (BENEMANN et al., 1971).

During our joined efforts to investigate, how reducing equivalents and energy are supplied in the nitrogen fixing process of the obligatory aerobic *Azotobacter vinelandii* and how the apparently equivalent processes of nitrate assimilation and nitrogen fixation are interrelated (cf. BECKING, 1962), the involvement of pyridine nucleotide transhydrogenase (VAN DEN BROEK and VEEGER, 1968; VAN DEN BROEK et al., 1971a,b,c,d,e) and the PDC have been studied. Information concerning the way of action of the PDC from *Azotobacter vinelandii* was of importance. This in order to answer the then question how, for a basically anaerobic process like nitrogen fixation in this stricktly aerobic organism (DALTON and POSTGATE, 1969), energy and reducing-equivalents are supplied. Some of the ideas have been summarized two years ago by VEEGER et al. (1972a) in the following scheme (FIG. 1.2). Although the present view is not in accordance with this scheme and actually indicating a much more complex membrane-process (HAAKER et al., 1974), the proposed interrelation between PDC and nitrogen reduction is still not excluded.

The work reported in this thesis concerns the purification, partial characterization and regulatory properties of the PDC from *Azotobacter vinelandii*.

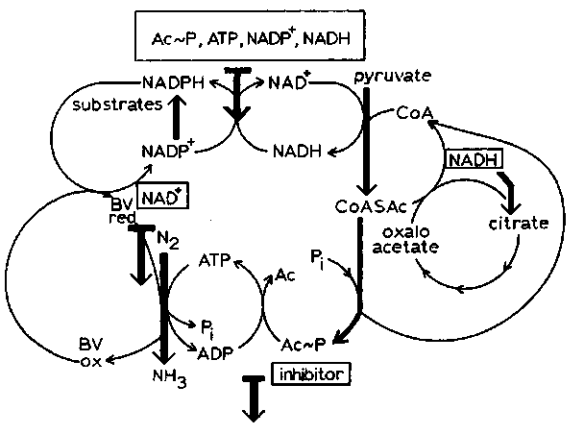


FIG. 1.2.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. *Bacteria*

Large-scale production of *Azotobacter vinelandii* (ATCC 478) was kindly performed, either by the Royal Yeast and Fermentation Industries (Delft, The Netherlands), or by Diosynth BV (Oss, The Netherlands), on a nitrogen free medium according to PANDIT-HOVENKAMP (1966). A 3000 litres fermentor filled with 2500 litres of growth medium was inoculated with a 2% inoculum and the cells were allowed to grow for 20 hours at 30° under aeration (0.5 litre air/litre medium/min.) and stirring (maximal 100 r.p.m.). After 20 hours (end of the logarithmic phase), the suspension was cooled to 10–15° and the cells were collected in a Sharples-centrifuge at a speed of 180 litres per hour. The yield (8.5 kg wet weight) was stored, unwashed, at –20°.

When experiments were performed with extracts prepared from freshly grown cells of either *Azotobacter vinelandii* ATCC 478 and/or strain OP, cells were grown for 24 hours on a rotary shaker in 5 litres flasks (final volume 1 litre) under air and harvested by centrifugation.

2.1.2. *Enzymes*

PDC from *Azotobacter vinelandii* (ATCC 478) was purified as described in CHAPTER 3 and stored in 0.05 M potassium phosphate buffer (pH 7.0), in the frozen state at –20° (protein concentration 40–70 mg/ml). When phosphate had to be replaced by another buffer, the enzyme was dialyzed overnight (at 4°) against two times 2 litres of the proper buffer before use. If the enzyme had to be diluted, this was performed just before the experiment and as late as possible. PDC from beef heart was partially purified according to LINN et al. (1972). Pure lipoamide dehydrogenase from *Azotobacter vinelandii* (ATCC 478) was prepared according to the method of MASSEY et al. (1960) and was kindly provided by Mr. J. S. SANTEMA. Acetate-activating enzyme (acetate thiokinase) was (partially) purified from bovine heart according to the method of BEINERT et al. (1953). Glucose-6-phosphate dehydrogenase, creatine-phosphokinase, myokinase, hexokinase, catalase, phosphotransacetylase isolated from *Clostridium kluyveri*, carnitine-acetyltransferase and citrate-synthase were purchased from Boehringer and Soehne.

2.1.3. *Reagents*

NAD^+, NADH, $NADP^+$, NADPH, thio-NAD^+, FAD, FMN, ATP, ADP, AMP, lipoic acid, ovalbumine, bovine serumalbumine, TPP, CoASAc, DTT (CLELANDS reagent), potassium pyruvate, bromo-pyruvic acid, N-ethylmaleimide, 2-heptyl-4-hydroxyquinoline-N-oxide were obtained from Sigma Chemical Co. Creatine-phosphate, CoA, glucose-6-phosphate, GTP, GDP and

acetylphosphate were purchased from Boehringer and Soehne. N- (1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl) maleimide was from Synvar Associates; $K_3Fe(CN)_6$ and oxaloacetate from the British Drug House; sodium dithionite from Merck; Sepharose 6- and 4B, Sephadex G-25 and DEAE Sephadex from Pharmacia (Uppsala); protamine sulphate and DEAE-cellulose from Serva; argon from Loos and Co. Calcium phosphate gel (SWINGLE and TISELIUS) was prepared as described earlier (VAN DEN BROEK, 1971). Reduced lipoamide, synthesized according to the method of REED et al. (1959), was a gift from Dr. J. VISSER. DTNB was a gift from Dr. G. E. J. STAAL. All other chemicals used were Analytical Grade and solutions were made up in bidistilled water.

2.2. METHODS

All changes from the conditions, presented below, are mentioned in the text or legends.

2.2.1. *The enzymatic assays*

2.2.1.1. The standard assay of PDC at 25°: the overall activity was measured according to SCHWARTZ and REED (1970a) in 0.05 M potassium phosphate (pH 7.0) buffer containing 5 mM pyruvate, 0.5 mM TPP, 5 mM $MgCl_2$, 0.13 mM CoA, 1.5 mM DTT and 0.6 mM NAD^+ in a final volume of 1 or 3 ml. After temperature equilibrium, the reaction was started by the addition of the diluted enzyme; diluted in either 0.05 M phosphate buffer plus 1 % ovalbumine (pH 7.0) or in 0.05 M Tris-HCl plus 1 % ovalbumine (pH 7.5). The absorbance change at 340 nm due to the formation of NADH ($\varepsilon = 6{,}220\ M^{-1}.cm^{-1}$.) was followed with a Zeiss spectrophotometer PMQ II in combination with a Honeywell Elektronic 16 high-speed recorder. The activity was calculated from the initial phase of absorbance changes (0.1 per min.) in the kinetical experiments. Due to the strong inhibition of the reaction by product, increasing with time, 'real' initial velocities were approached by performing the measurements on the Aminco-Chance dual wavelength instrument (380–345 nm), using the 5–20% transmission scale after calibration.

When rather crude preparations are assayed, due to the strong NADH oxidase activity, the reactions were performed either in the presence of 30 μM HQNO plus 0.1 mM KCN or anaerobically in 3 ml Thünberg cuvettes sealed with 'Suba seal' rubber stoppers after flushing (argon). One unit of activity is defined as the amount of enzyme required to produce 1 μmole of NADH per min. The specific activity is defined as units per mg of protein.

2.2.1.2. For the assay of the PDH component of the complex, two procedures of measuring the oxidative decarboxylation of pyruvate with ferricyanide as electron acceptor were used. The first one, only and exclusively for comparison of the PDH measurements in pure PDC with the literature data (CHAPTER 3). Ferrocyanide produced was colorimetrically determined at 540 nm as prussian

blue in a discontinuous assay (REED and WILLMS, 1965) after incubating the enzyme at 30° (30 min.) in the presence of 0.1 M phosphate buffer (pH 6.0) containing 0.13 mM TPP, 0.2 mM $MgCl_2$, 33 mM pyruvate and 16 mM ferricyanide in a volume of 1.4 ml. The reaction was terminated with 1 ml TCA (10%, w/v) and after centrifugation (for 5 min. in a clinical centrifuge), 0.1–0.2 ml aliquots of the supernatant were added to 2.4 ml of a mixture containing 10 mM ferricyanide and 4% TCA. After addition of 1 ml SDS (4%, w/v) and 0.5 ml of a ferri(ammonium)sulphate-SDS reagent (prepared according to REED and WILLMS, 1965) the colour was allowed to develop for 30 min. A standard curve is prepared with ferricyanide.

Secondly, the decrease of the ferricyanide can be continuously followed at 420 nm ($\varepsilon = 1{,}030\ M^{-1}cm^{-1}$) at 25° with a Zeiss spectrophotometer and a recorder with scale-expander (see 2.2.1.1.). The assay mixture contained 30 mM Tricine buffer (pH 7.5) or 50 mM Tris-HCl (pH 7.5), 5 mM pyruvate, 10 mM $MgCl_2$, 0.1 mM TPP and 1 mM ferricyanide. The reaction was started by the addition of the diluted enzyme (see 2.2.1.1.) to the assay mixture. One unit of activity is (for both methods) the amount of enzyme required to reduce 2 μmoles of ferricyanide per min. Specific activity is expressed as units per mg of protein.

2.2.1.3. The LTA component of the PDC is assayed by colorimetric determination of the S-acetyldihydrolipoamide (produced by the LTA from CoASAc and dihydrolipoamide) as the ferric acethydroxamate-complex (REED and WIILMS, 1965). CoASAc is generated from 10 mM acetylphosphate and 0.1 mM CoA with the aid of 5 units PTA from *Clostridium kluyveri* during incubation of the enzyme (30 min. at 30°) in 0.01 M Tris-HCl (pH 7.1) containing 10 mM dihydrolipoamide in a volume of 1 ml. After the reaction had been terminated by the addition of 0.1 ml N HCl excess acetylphosphate is destroyed by boiling during 5 min. (*au bain-marie*). Back at room temperature, 0.5 ml of a neutral hydroxylamine solution is added. After a reaction-period of 10 min. the solution was centrifuged (in a clinical centrifuge) for another 10 min. and the acetyl groups, that have reacted with hydroxylamine, are coloured with 1.5 ml of an acid ferrichloride solution. Both, the neutral hydroxylamine- and the acid ferrichloride solution are prepared as described by REED and WILLMS (1965). A standard curve is prepared with synthetic acethydroxamic acid. A unit of activity is defined as the amount of enzyme required to produce 1 μmole S-acetyldihydrolipoamide per min. Specific activity is expressed as units per mg of protein.

2.2.1.4. The standard lipoamide dehydrogenase assay was performed (as described by VAN DEN BROEK, 1971) in 0.8 M tri-sodium citrate-H_3PO_4 buffer (pH 6.5), in a final volume of 2.5 ml, containing 0.1% BSA, 1 mM EDTA, 0.8 mM lipoic acid, 0.1 mM NAD^+ and 0.1 mM NADH. Activities are expressed as μmoles NADH oxidized per mg per min.

2.2.1.5. PTA was measured according to a modification of the method of BERGMEYER (SHIMUZU et al., 1969) at 25° by recording the change at 233 nm, due to the formation of CoASAc and the removal of CoA ($\varepsilon = 4{,}440\ M^{-1}cm^{-1}$), with a Zeiss spectrophotometer plus recorder (see 2.2.1.1.). To the thermostated assay-mixture, consisting of 0.1 M Tris-HCl buffer (pH 7.4), 2 mM DTT, 0.4 mM CoA, 8 mM acetyl phosphate and 1 mM ammonium chloride, the diluted enzyme (see 2.2.1.1.) was added. A unit of activity is defined as the amount of enzyme required to produce 1 μmole CoASAc per min. Specific activity is expressed as units per mg of protein.

2 2.1.6. Acetate-kinase can be measured according to ROSE (1955). Better results were obtained on measuring, continuously, ATP formation from acetyl phosphate and ADP at 340 nm, due to the formation of NADPH ($\varepsilon = 6{,}220\ M^{-1}cm^{-1}$), with the aid of hexokinase plus glucose and glucose 6-phosphate dehydrogenase plus $NADP^+$ in our case. To a spectrophotometer cuvette thermostated at 25° is added: 3 mM acetylphosphate, 0.7 mM ADP, 50 mM Tris-HCl (pH 7.5), 10 mM glucose, 10 mM $MgCl_2$, 0.1 mM $NADP^+$, 0.4 unit hexokinase and 0.4 unit glucose 6-phosphate dehydrogenase; final volume 3 ml. The reaction is started by adding the enzyme in a proper dilution. In the presence of NADPH oxidase activity (in rather crude preparations) the reactions were performed anaerobically (see 2.2.1.1.).

2.2.2. *Determination of concentrations*

Protein concentration was determined by the biuret method of GORNALL et al. (1949) or by the micro-biuret method of ITZHAKI and GILL (1964).

The absorbance at 340 nm was a measure for the concentration of freshly prepared NADH and NADPH ($\varepsilon = 6{,}220\ M^{-1}cm^{-1}$). The concentration of NAD^+ was either enzymatically determined with ethanol and alcohol dehydrogenase or was assayed as the pyridine nucleotide cyanide complex ($\varepsilon = 6{,}000\ M^{-1}cm^{-1}$ at 327 nm).

The flavin content of the complexes was determined according to BEINERT and PAGE (1957), by measuring the absorbancy of neutralized TCA-extracts at 450 nm ($\varepsilon = 10{,}300\ M^{-1}cm^{-1}$) before and after reduction with dithionite.

Acetate was determined enzymatically according to BERGMEYER and MOELLERING (1966) with acetate thiokinase (cf. 2.1.2.) instead of acetate kinase, citrate synthase and malate dehydrogenase.

The concentration of pyruvate was determined with lactate dehydrogenase according to BERGMEYER (1970). Small concentrations of acetate and pyruvate were measured on the Amino-Chance dual wavelength instrument using the 5–10% transmission scale after calibration (340–380 nm).

Oxygen consumption was measured at 30° on the Gilson Oxygraph Model KM equipped with a Clark electrode.

The CoA concentration was calculated from SH-group measurements according to ELLMAN (1959) and the CoASAc concentration with a system containing oxaloacetate, citrate synthase and DTNB ($\varepsilon = 13{,}600\ M^{-1}cm^{-1}$

at 412 nm, pH 8.0). The CoA regenerating systems used, consisted either of 0.2 mM oxaloacetate plus 3.5 enzyme units of citrate synthase or 20 mM carnitine plus 2 enzyme units of carnitine acetyltransferase.

ATP was measured enzymatically and with the aid of an Aminco-Chance dual wavelength spectrophotometer after calibration (340–380 nm) essentially as described for acetate kinase (2.2.1.6.).

Desired values of the energy charge of the adenylate pool were obtained with the aid of myokinase after mixing of the proper concentrations of AMP, ADP and ATP. A K (equilibrium constant) of 2.26 (10 mM $MgCl_2$, pH 7.4, 25°) has been used for the reaction $2\ ADP \rightleftarrows ATP + AMP$ according to BERGMEYER (1970).

2.2.3. *Anaerobic ATP synthesis*

Anaerobic ATP synthesis from pyruvate was performed by incubating at 30° in flasks, closed with 'Suba seal' rubber stoppers and gassed with argon, containing a standard reaction mixture consisting of 50 mM pyruvate, 0.15 mM CoA, 2 mM DTT, 0.5 mM TPP, 0.5 mM NAD^+, 2 mM EDTA, 4 mM $MgCl_2$, 2 mM ADP, 5 mM phosphate, 5 mM glucose, 1.5 enzyme units hexokinase and 50 mM Tris-HCl (pH 7.5).

2.2.4. *Inactivation experiments*

For the purpose of studying inactivation, the enzyme (specific activity = 8) was incubated at 28° aerobically (slow stirring) or anaerobically (flushing with argon) in a thermostated flask (20 ml) at a protein concentration of 0.5–1 mg ml^{-1} in the presence of 0.05 M Tris-HCl (pH 7.1) containing either 2 mM Mg^{2+}, 0.1 mM TPP, 0.1 mM pyruvate and 1 mM sulfhydryl reagent or 0.1 mM NAD(P)H and 1 mM sulfhydryl reagent. Whenever the effect of some compound upon inactivation was studied, it was added in a concentrated form, not changing the volume significantly. The reactions were started by the addition of the enzyme. At the times indicated, 0.01–0.1 ml aliquots were withdrawn from the incubation-mixture and either the overall reaction or the partial reactions were measured directly. The overall assay was performed in 0.05 M Tris-HCl (pH 7.1) and in the absence of DTT or GSH.

2.2.5. *Absorption spectrophotometry*

Absorption spectra were recorded on a Cary model 14 recording spectrophotometer, thermostated at 25° in cells with a 1 cm light path with the 0–1 absorbance indicating slidewire. When necessary, the spectra were recorded versus buffer with enough glycogen added to correct for most of the loss of light by scattering. This can be achieved by compensating the absorption of the enzyme at 700 nm with the blank, before recording of the spectra.

2.2.6. *Fluorescence*

Fluorescence emission- and exitation spectra were recorded on a Hitachi Perkin-Elmer MPF-24 spectrofluorimeter, equipped with a thermostated cuvette

holder. Anaerobic conditions were obtained by evacuation and refilling of the cuvettes with oxygen-free nitrogen. This procedure was repeated at least five times.

2.2.7. *Electron spin resonance spectra*

Electron spin resonance spectra were recorded with a Varian E-3 X-band (9.45 GHz) spectrometer in a aquaous flat cell (quartz) at room temperature. The EPR-spectra of the spin labeled protein were analysed by comparing both, the line shape and the amplitude with those of the free maleimide spin label (known concentration) dissolved in water-glycerol mixtures of different viscosities and temperatures as mentioned by GRANDE et al. (1972). Assignment of the rotational correlation times (τ_c) was done according to the semi-empirical method developed by MENSCH and MEIER (EPR-atlas, to be published).

2.2.8. *Electron microscopy*

Electron micrographs of some preparations were kindly taken by Drs. H. VAN HEERIKHUIZEN and Mr. J. F. L. VAN BREEMEN, Laboratorium voor Structuurchemie, University, Groningen.

2.2.9. *Ultracentrifugation*

Sedimentation- and diffusion patterns were obtained using a MSE analytical ultracentrifuge. Sedimentation velocity runs were performed in 10 and 20 mm double sector cells at 20° and at various rotor speeds (33,000–45,000 r.p.m.). Sedimentation coefficients (s) were determined grafically or calculated according to the relation of SVEDBERG and PEDERSON (1940) and corrected to standard conditions (cf. ELIAS, 1961). Scans were made at at least seven different times. The diffusion coefficient was calculated from the equation

$$D = (A/H)^2/4\pi t$$

where A is the area under the Schlieren peak and H is the maximum ordinate of the peak at time t. Values of $(A/H)^2$ are plotted with respect to t and the diffusion coefficient is evaluated (cf. ELIAS, 1961); corrections were performed to standard conditions.

The molecular weight of PDC can be calculated from

$$M = RT s_{20,w}/D_{20,w}(1 - \bar{v}\zeta)$$

where $\bar{v}$ is partial specific volume (0.75 was assumed) and ζ is the density of the solvent. Sedimentation equilibrium experiments were carried out at a rotor speed of 4600 r.p.m. in the double sector synthetic boundary cell at 6°.

The sedimentation- and diffusion measurements were kindly performed by Dr. J. VISSER and Ir. D. VERDUIN.

Sucrose density gradient centrifugation was performed with a MSE 50 superspeed centrifuge at 4° in a 3 × 3 ml swing-out bucket rotor at 28,000–37,000 r.p.m. for 20 hours. Linear gradients from 10–35% and 15–40% sucrose in

either 0.05 M potassium phosphate buffer (pH 7.0) or 0.05 M Tris-HCl (pH 7.5) were used. Fractions were collected with the aid of the MSE fractionator.

2.2.10. *Light-scattering*

Light scattering data were obtained with a Cenco-TNO apparatus at room temperature. Measurements were kindly performed by Mr. VAN MARKWIJK (N.I.Z.O., Ede, The Netherlands). The molecular weight has been calculated according to the methods of ZIMM (1948) and YANG (1957) originating with the relation:

$$\frac{Kc}{R_\theta} = \left\{ 1 + \frac{16\pi^2 R_G{}^2}{3\lambda^2} \sin^2 \frac{\theta}{2} + 2Bc \right\} \times \frac{1}{M} \text{ (TANFORD, 1961).}$$

The optical constant K, defined as

$$K = \frac{2\pi^2 n_0^2 (dn/dc)^2}{N\lambda^4} \text{ cm}^2 \text{ g}^{-2} \text{ and}$$

$$R_\theta \quad \frac{i_\theta r^2}{I_0 (1 + \cos^2\theta)}$$

dn/dc = refractive increment = 0.192 $\text{cm}^3\ \text{g}^{-1}$ for PDC in 0.05 M phosphate buffer (pH 7.0).
n_o = index of refraction of the solvent.
λ = wavelength of the light used, 546 nm.
i_θ/I_o = the ratio of the light scattered under the angle θ and of the incident beam. Benzene was used as a standard.
R_G = the z-average radius of gyration.
r = distance from the sample to the photocell.

In the case of heterogeneous samples, M is to be replaced by Mw.
The constant K amounts 3.06×10^{-7} cm^2g^{-2}. The samples were filtered before use with a 100 nm filter and enzyme solutions centrifuged before dilution. Values obtained were corrected for contributions of the solvent.

3. PURIFICATION AND SOME PROPERTIES OF THE *AZOTOBACTER* PYRUVATE DEHYDROGENASE COMPLEX

3.1. INTRODUCTION

It is generally accepted that PDC is responsible for the aerobic oxidation of pyruvate via a complex sequence of at least four reactions and catalyzed by three enzymes; PDH, LTA and lipoamide dehydrogenase. The complex has been isolated from a number of sources, for instance pig heart (HAYAKAWA et al., 1966b), beef heart (LINN et al., 1972), beef kidney (ISHIKAWA et al., 1966; LINN et al., 1972), *E. coli* (KOIKE et al., 1960a; VOGEL et al., 1971; ELEY et al., 1972), *Neurospora crassa* (HARDING et al., 1970). These multi-enzyme complexes are functional units with molecular weights in the millions (4–9 million daltons), but the complex of *E. coli* seems to be somewhat smaller than those isolated from mammals. Each type of complex has been separated into the three compiling components (KOIKE et al., 1963; HAYAKAWA et al., 1969) and, together with reassociation, after mixing of the three enzymes reconstitution of the overall activity has been reported. Investigations of LINN et al., (1969a,b), WIELAND and VON JAGOW-WESTERMANN (1969) and WIELAND et al. (1972a) have revealed that the complexes originating from mammals and *Neurospora* in addition contain a kinase and a phosphatase, apparently as regulatory subunits of the PDC. PDC of *E. coli* was found to be devoid of the latter activities (SCHWARTZ and REED, 1970a).

Biochemical and electron microscopic studies suggest, that *E. coli* PDC contains a '*core*' of LTA consisting of 24 identical polypeptide chains (M:65,000–70,000 daltons), which are linked together by non-covalent bonds. One molecule of lipoic acid is covalently bound to each chain (REED and OLIVER, 1968; ELEY et al., 1972). Twenty four identical PDH units (M:96,000 daltons) and 12 units of lipoamide dehydrogenase (M:56,000 daltons) are thought to be distributed in a regular manner along the edges of the transacetylase cube in non-covalent interaction. However, the exact stoechiometry of the components in the bacterial complex does not seem to be solved unequivocally yet, since VOGEL et al. (1972a,b) have shown PDC from *E. coli* K-12 not to be a unique entity in that the amount of the PDH component can vary. The '*core*' complex from which this excess PDH had been removed, consists of 16 chains of the PDH (M:100,000 daltons), 16 chains of LTA (M:80,000 daltons) and 16 chains of lipoamide dehydrogenase. In other words, a large difference between the complexes of *E. coli* Crookes and K-12 can be noticed.

Except for the higher molecular weights and the presence of a kinase and a phosphatase, the mammalian PDC's are also accounting for non-identity of the PDH units (two types are demonstrated) and a different stoechiometry of the compiling components (ROCHE and REED, 1972; LINN et al., 1972). The appear-

ance of the mammalian transacetylase in the electron micrographs also differs from that of the *E. coli* enzyme (ISHIKAWA et al., 1966).

The occurrence of PDC in *Azotobacter*, which bacterium has no other pyruvate '*oxidizing*' system (HAAKER, unpublished results), offers an opportunity to investigate whether the bacterial PDC's are truely a group separate from the mammalian system. In this CHAPTER the purification of the complex from *Azotobacter vinelandii* is described and a start is made to disclose its characteristics.

3.2. RESULTS

3.2.1. *Purification of the complex; the 'pure' complex*

Like most procedures concerning the isolation and purification of PDC, the one for *Azotobacter* is also very similar with that developed and improved by REED and WILLMS (1965) and ELEY et al. (1972) for *E. coli* Crookes strain. The group of HENNING is using a different and very elegant procedure, which is not satisfactorily used in our case (VOGEL et al., 1972b). The purification of *Azotobacter* PDC will be described below in more detail in a four-step scheme and will be referred to as method 1a. All steps were performed at 4°, unless otherwise stated.

Step 1: Preparation of the cell-free extract:

500 g (wet weight) frozen cellpaste were washed after being thawed, two times with 1 litre deionized water and two times with 1 litre phosphate buffer 0.05 M (pH 7.0). The addition of EDTA to the latter buffer is not advisable in view of the occurrence of considerable cell lysis, especially at higher concentrations of the chelator. The washed cellpaste was suspended in the same buffer using 2 to 3 ml for 1 g of cells. The cell suspension is in portions of 40 ml for 4 min. treated in a 100 Watt ultrasonic disintegrator, MSE London, and then centrifugated at 2100 $\times$ *g* in a MSE Mistral rotor for 45 min. The resulting cell-free extract (protein concentration 35–40 mg ml^{-1}) is diluted with 0.05 M phosphate buffer (pH 7.0) to a protein concentration of 20 mg ml^{-1}. The specific activity of PDC in this crude extract varies between 0.05 and 0.25 depending on the batch of bacteria used.

Step 2: Fractionation of the PDC from the pH 6.1 cell-free extract:

The diluted cell-free extract is brought to pH 6.1 under continuous stirring with a 1% acetic acid solution. This extract is fractionated with a clear solution of 2% (w/v) protaminesulphate of the same pH. The latter solution must be kept at room temperature and in order to get reproducible results it is important to use the proper protaminesulphate (cf. MATERIALS). Furthermore, the effectiveness of this procedure is rather dependent on the batch of bacteria used. Thus, before every large scale purification a pilot run is advisable. For that, the

2% protaminesulphate solution (6% of the volume) is added dropwise under stirring to a sample of the extract. The solution is allowed to stir for another 20 min. and centrifuged at 18,000 × *g* in a MSE 18 rotor for 30 min. The precipitate is discarded and the activity of the supernatant is tested. This procedure is repeated with 3% of the volume protaminesulphate until the PDC just has precipitated. Usually, this will occur after the third addition, although sometimes four or even five additions are necessary. As soon as it is known from the pilot run how PDC precipitates during this procedure, scaling up is possible. The precipitate containing the bulk of the PDC activity is suspended in 200 ml 0.05 M phosphate buffer (pH 7.0) and stirred slowly overnight to extract PDC from the sediment. The next day 20 ml of a neutralized 1% (w/v) RNA solution is added slowly, precipitating excess protaminesulphate. Stirring is continued for another two hours and then centrifuged for 45 min. at 36,000 × *g*. The clear yellow-brownish supernatant contains the PDC, which is collected by the addition of solid ammoniumsulphate to 50% saturation. The suspension is allowed to stand for 20 min. and centrifuged at 10,000 × *g*. The precipitate is dissolved in 35 ml 0.05 M phosphate buffer (pH 7.0) and dialyzed against 2 litres of the same buffer (two times).

Step 3: Separation of the PDC from the KGDH by isoelectric precipitation:

In the steps already described, PDC and KGDH are purified simultaneously and at this stage both the specific activities and the total activities have about the same values. The complexes are separated by acid fractionation at this stage and not later, for instance the ultracentrifugation step, as described by REED and WILLMS (1965), because better results are obtained in this way.

The dialyzed solution of step 2 is diluted with 0.05 M phosphate buffer (pH 7.0) to a protein concentration of 5 mg ml^{-1} and the pH is carefully adjusted with 1% acetic acid under stirring until a precipitate appears; usually at pH 5.6 and occasionally slightly lower. The solution is stirred for 5 min. and centrifuged at 30,000 × *g* for 10 min. The precipitate can be discarded; KGDH from *Azotobacter* inactivates upon precipitating it isoelectrically. The pH of the supernatant is further adjusted to 4.9 and after stirring for 5 min. and centrifugation the precipitate is solubilized in about 30 ml 0.05 M phosphate buffer (pH 7.0). Insoluble material is removed by centrifugation at 30,000 × *g* for min.

Step 4: Ultracentrifugation:

The clear solution of step 3 is subjected to differential centrifugation in a MSE 50 preparative ultracentrifuge with an angle rotor. After 30 min. at 144,000 × *g* the brown pellet is discarded and the centrifugation is resumed for another $4^1/_2$ hours. The sedimented PDC is carefully collected, avoiding the brown heart of the pellet. The precipitate is homogenized in a POTTER-ELVEHJEM using a small volume 0.05 M phosphate buffer (pH 7.0). Insoluble material is removed

by centrifugation at 30,000 × *g* for 20 min. If the ultimate specific activity of the enzyme complex is still too low, the ultracentrifugation is repeated after proper dilution of the enzyme.

Alternatively, a fractionation with solid ammoniumsulphate increases the specific activity somewhat; the enzyme is found to precipitate (not very sharply) between 30% and 50% saturation. After dialysis the complex is stored in the frozen state; up to 90% of the activity is recovered after a few months of storage. Upon storage at 4° a gradual decrease in activity is observed, especially at lower protein concentrations. For instance, after 24 hours at 4° at a protein concentration of about 1 mg ml^{-1}, not more than 40% of the original activity can be measured; the exact value depends on the age of the preparation. The activity cannot be stabilized by the addition of Mg^{2+} plus TPP or thiols (HARDING et al., 1970). Stabilization of the dilute PDC solutions can only be achieved by the addition of ovalbumine or BSA (1%, w/v).

In TABLE 3.1 the procedure is summarized. Once a specific activity of about 10 is obtained, no further enhancement of activity can be achieved. Various column procedures have been tried, involving adsorption chromatography (Ca-phosphate gel), ion exchange (DEAE-cellulose, DEAE-Sephadex) and gelfiltration (Sepharose 4B- and 6B). The complex easily dissociates at high purity now (cf. 3.2.3.) and thus these preparations were considered to be the purest and most stable possible. After subjecting the complex to SDS-gel electrophoresis (10% gels) four major bands were observed (not shown).

The purification procedure can be modified slightly by replacing the isoelectric fractionation, separating the PDC from KGDH, by a PEG (*Mw* 6000) fractionation at 4° exactly according to ELEY et al. (1972). This method (1b) seems to be satisfactory as well in our case and although the final specific activity is not enhanced, the recovery is somewhat higher (about 50%).

Sometimes a new purification procedure, according to a description by FRIED and CHUN (1971) and modified in co-operation with Dr. J. KRUL, is used (method 2). The crude extract of step 1 is adjusted with 1% acetic acid to pH 5.5 and fractionated by means of accumulative saturation at 4° with PEG (*Mw*

TABLE 3.1. Purification of PDC and PTA (cf. 3.2.3.) of *Azotobacter* by method 1[a].

	PDC					PTA		
Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Total activity (units)	Specific activity (units/mg) protein)	Yield (%)
1	2250	45,000	6750	0.15	100	42,000	0.85	100
2	40	2000	4500	2.25	67	23,000	1.5	55
3	30	750	3000	4	45	7200	9.5	17
4	6	320	2550	6–10	40	2250	7	5.5

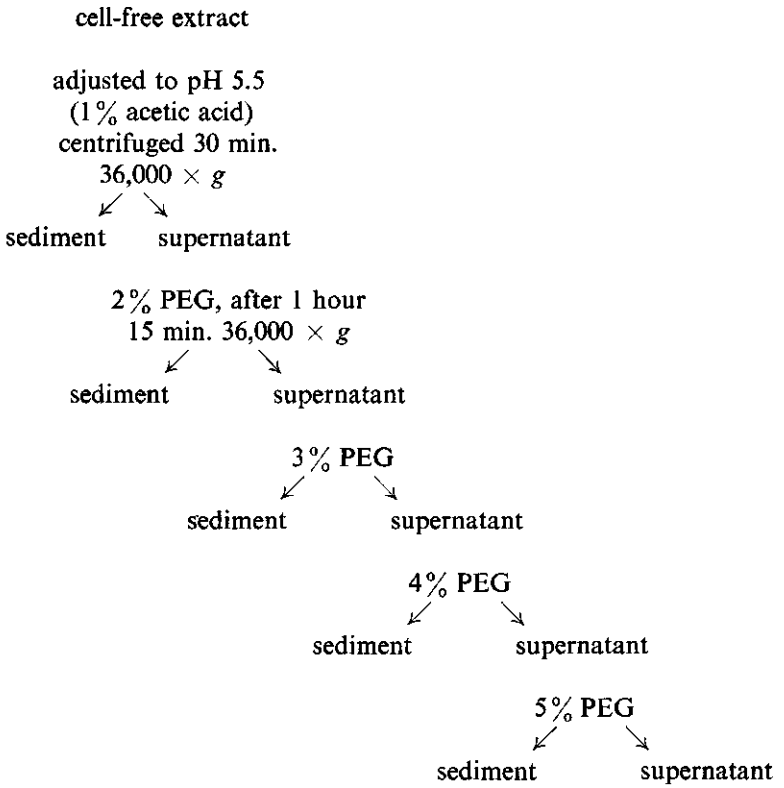


FIG. 3.1. Fractionation-scheme of PDC with PEG (*Mw* 6000) from crude extracts at 4° and pH 5.5.

6000). This is followed by chromatography on Sepharose 6B and subsequent concentration of the pooled active fractions by ultracentrifugation ($4^1/_2$ hours at 144,000 × *g*). In FIG. 3.1 the fractionation scheme is presented. The percentage of PEG saturation as indicated is achieved by dropwise addition of the proper volume of a 50% (w/v) PEG solution in 0.05 M phosphate buffer (pH 7.0). After each addition the solution is allowed to stand for one hour. The sediments obtained at 4% and 5% saturation contain 60% of the activity and are combined after they were solubilized. Hundred mg of the combined sediments are brought on a column of Sepharose 6B (2 × 80 cm), which is eluted with 0.05 M phosphate buffer (pH 7.0). The results of this alternative purification procedure are shown in TABLE 3.2. It is seen, that the extend of purification and the recovery are about the same as obtained by method 1. However, the second PEG method is less suitable for the preparation of large amounts of PDC. Rechromatography of the purified preparations on Sepharose after concentration will not further improve the specific activity and leads to considerable

TABLE 3.2. Purification of PDC by method 2.

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)
cell free extract	50	1075	226	0.21	100
combined sediments of 4 and 5% PEG-saturation	6	99	142	1.44	63
pooled active fractions after Sepharose column	22	20	120	6	53
ultra-centrifugation	2	14	114	8	50

loss of activity. Replacement of the Sepharose column procedure by Ca-phosphate gel is not satisfactory (cf. VOGEL et al., 1972b).

Mostly the purification of PDC was performed according to method 1a,b. The purified enzyme has an absorption spectrum as shown in FIG. 3.2. Due to the flavoprotein component of the complex a maximum occurs at 455 nm with shoulders at about 430 nm and 485 nm, just as reported for pure lipoamide dehydrogenase from *Azotobacter* (VAN DEN BROEK, 1971). The spectrum is tentatively corrected for most of the light-scattering as described in METHODS.

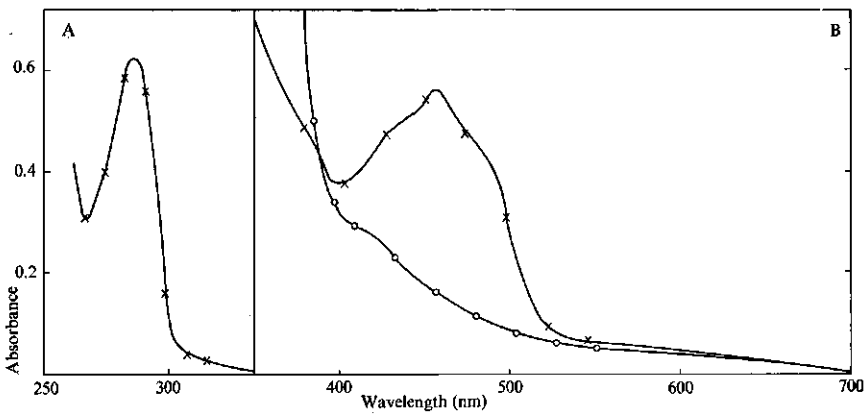


FIG. 3.2. Absorption spectrum of *Azotobacter* PDC. (A) ultraviolet (B) visible light. Measurements were done in 1 cm cuvettes using a Cary model 14 recording spectrophotometer, with the 0–1 absorbance indicating slidewire. Temperature: 25°. Protein concentrations (A) 0.41mg ml^{-1} in 0.05 M potassium phosphate buffer (pH 7.0), (B) 22.4 mg ml^{-1}. Spectrum recorded versus buffer with enough glycogen added to correct for most of the loss of light by scattering (see METHODS and text). Symbols: (×—×), untreated PDC; (○—○), after reduction by the addition of a small amount of solid dithionite.

For instance, A_{455} amounted 0.642 before its correction for scattering. On assuming Rayleigh scattering, its contribution to the total absorbance may be represented in terms of turbidity according to:

$$\Delta A_{turbidity} = \left\{ \frac{16\pi}{3 \times 2.303} \right\} c\, M\, K = 0.103$$

(cf. TANFORD, 1961). For M and K see 3.2.4 and 2.2.10; the refractive index increment has been taken independent of wavelength. This contribution had to be substracted from A_{455}, originally observed, thus producing a value that is very near to the one actually presented in the spectrum. Furthermore, from the spectrum a ratio of A_{280}/A_{455} of about 60 can be obtained, a value in reasonable agreement with the 50 as calculated from the results of DENNERT and HÖGLUND (1970) for PDC from *E. coli* K-12. A number of preparations, with a high specific activity, had an additional band in the spectrum with a maximum at 415 nm, due to the presence of a brown contaminant nearly inseparable from the enzyme complex.

The flavin content of most PDC preparations, determined according to BEINERT and PAGE (1957), is found to vary between 1.6 and 1.8 nmoles flavin per mg protein and the flavin can be identified as FAD according to the method of Dr. S. G. MAYHEW (personal communication), involving conversion of the FAD into FMN followed by a fluorimetric titration with apo-flavodoxine. From the difference in A_{455}, before and after reduction with dithionite (FIG. 3.2.), a flavin content of 1.6 nmoles FAD per mg protein can be calculated (ε = 10,300 $M^{-1}cm^{-1}$), which value may be employed to calculate the minimal molecular weight for PDC from *Azotobacter*; $M_{minimal}$ = 620,000 daltons. The FAD content of the *Azotobacter* complex is lower than the values of 2.6–2.8 nmoles FAD per mg protein reported for *E. coli* Crookes (KOIKE et al., 1960a) and of 4.4 nmoles FAD per mg protein reported by VOGEL et al., (1972a) for PDC from *E. coli* K-12. However, after correction of A_{455} (as given by VOGEL and from which value the FAD content was calculated) for light-scattering, the actual value is 2.9 nmoles FAD per mg protein.

3.2.2. *Reactions catalyzed by the Azotobacter PDC*

Azotobacter PDC also catalyzes, in addition to the overall reaction, the partial reactions as outlined in the introduction. However, preparations purified by either method 1a,b or 2 are catalyzing one extra reaction (BRESTERS et al., 1972) and are actually capable to convert CoASAc into acetylphosphate in the presence of inorganic phosphate. Phosphotransacetylase activity (PTA) is demonstrated to be responsible for the latter phenomenon. Although VAN DEN BROEK (1971) was able to show the existence of a close correlation between pyridine nucleotide transhydrogenase and PDC in *Azotobacter*, the purified PDC is devoid of any transhydrogenase activity. Thus, the overall reaction can be written as:

$$H^+ + pyruvate + LTA\text{-}lipS_2 \xrightarrow[Mg^{2+}.\ TPP]{PDH} CO_2 + LTA\text{-}lip(SH)\text{-}S\text{-}acetyl$$

$$LTA\text{-}lip(SH)\text{-}S\text{-}acetyl + CoA \xrightarrow{LTA} LTA\text{-}lip(SH)_2 + CoASAc$$

$$LTA\text{-}lip(SH)_2 + NAD^+ \xrightarrow[lipoamide\ dehydrogenase]{} LTA\text{-}lipS_2 + NADH + H^+$$

$$CoASAc + P_i \xrightarrow{PTA} CoA + acetylphosphate$$

Sum:
$$pyruvate + NAD^+ + P_i \xrightarrow[Mg^{2+}.\ TPP,\ CoA]{PDH,\ LTA,\ PTA,\ lipoamide\ dehydrogenase} acetylphosphate + CO_2 + NADH$$

In TABLE 3.3 a comparison is presented between the various specific activities of the compiling enzymes in purified PDC from different sources. When necessary and possible the data given from the literature are expressed in the units as described in METHODS. The data in the TABLE show, that for *Azotobacter* PDC, the specific activities of the overall- and of the lipoamide dehydrogenase reactions are significantly lower in comparison with the corresponding ones from *E. coli*. In this context it must be mentioned, that it is not possible to raise the overall activity of the complex by the addition of pure lipoamide dehydrogenase isolated from either *Azotobacter* or pig heart. On the other hand, compared with pig heart PDC, the specific activity of the *Azotobacter* complex overall reaction is much higher; in the pig heart complex there seems to exist a different ratio between the overall activity and the activities of the partial reactions. However, much higher specific activities of the overall reaction were reported recently for other mammalian PDC's, for instance 9 for the complex purified from bovine kidney and bovine heart (LINN et al., 1972). It is important to notice in the TABLE, that only the PDC from *Azotobacter* contains PTA activity. The varying amount of PTA may be due to inactivation occurring upon dilution or during storage.

Since crude extracts of *Azotobacter* cells also contain a soluble acetate kinase (cf. ROSE et al., 1954), which was found not to be associated with PDC, the significance of the PTA is probably its involvement in the synthesis of ATP. FIG. 3.3 shows that with a crude extract, under anaerobic conditions, indeed ATP synthesis is demonstrable by the combined action of the PTA-containing

TABLE 3.3. The specific activities of the compiling enzymes of '*pure*' PDC from *Azotobacter* in comparison with figures derived from the literature for different sources.

source	specific activity *Azotobacter*	*E. coli* Crookes	enzyme from *E. coli* K-12	Pig heart
activity				
PDC	6–10	4–6[1] 12–35[2,3]	25–35[7]	1.6[4]
PDH	0.09–0.14	0.20[1]	0.15[7]	*0.085[4]
LTA	2.4–3.6	2.2[1]	3–3.7[7]	1.34[5]
lipoamide dehydrogenase	2.3–2.9	4.7[8]	4.7[9]	1.75[6]
PTA	10–2	not present[1]	no report	irrelevant

* measured at pH 6.5
[1] ref KOIKE et al., 1960[a]
[2] ref SCHWARTZ and REED, 1970[a]
[3] ref ELEY et al., 1972
[4] ref KANZAKI et al., 1969
[5] ref HAYAKAWA et al., 1969
[6] derived from (5) and VISSER, 1970
[7] ref MALDONADO et al., 1972
[8] derived from (1), KOIKE et al., 1960[b] and VAN DEN BROEK, 1971
[9] derived from (7) plus (8)

PDC and acetate kinase. The process is found to be linear over a period of at least 30 min. and is much less efficient under conditions where no ATP-trapping system (glucose plus hexokinase) is added. In the absence of this system the reaction comes to an early stop due to ineffective removal of the strong feedback

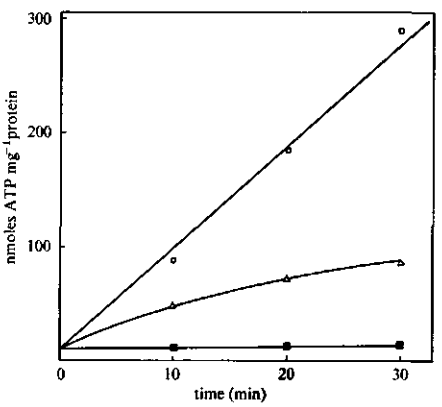


FIG. 3.3. Anaerobic ATP synthesis from pyruvate. The reaction was started by adding 8.6 mg crude extract to 2.0 ml standard reaction mixture as described under METHODS. At the times indicated samples were taken and the reaction was stopped by addition of $HClO_4$ (final conc. 4%); (○—○), standard condition; (△—△), standard condition minus glucose and hexokinase; (■—■), standard condition minus pyruvate.

inhibitor of PDC, CoASAc. The piling of CoASAc is due to the fact that in the PTA- and acetate kinase reactions the equilibria lye in the directions of respectively CoASAc and acetylphosphate. In addition, the overall reaction of PDC is under control of the energy charge of the adenylate pool, ATP being inhibitory (CHAPTER 4).

It was demonstrated by HAAKER et al (1972) that this substrate-bound phosphorylation in the presence of an ATP-trapping system, can be measured at atmospheric oxygen tension, whenever the oxidative phosphorylation system is active. Anaerobically performed, equal amounts of NADH and ATP are to be expected. In FIG. 3.4 an illustration is presented. The drifting at the very end of the curve is caused by the action of transhydrogenase present in the crude extract. It must be mentioned, however, that other ratio's between NADH and ATP also can be obtained by the action of secundary reactions, depending somewhat on the type of preparation used.

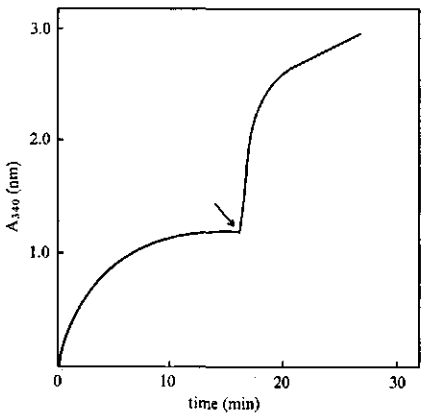


FIG. 3.4. Stoechiometry of NADH production and ATP formation in *Azotobacter* PDC. The incubation mixture contained: 50 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM EDTA, 2.5 mM pyruvate, 0.5 mM TPP, 0.25 mM NAD^+, 0.1 mM CoA, 2.5 mM DTT, 5 mM P_i (pH 7.5), 0.5 mM ADP, 10 mM glucose and 0.5 units hexokinase. Final volume 3.0 ml. After flushing with argon, the reaction was started by the addition of crude PDC. NADH production was followed at 340 nm. At the time indicated by the arrow 0.5 units glucose 6-phosphate dehydrogenase and 1 mM $NADP^+$ were added.

3.2.3. *The association between PDC and PTA*

It was shown in the previous section, that PTA activity is present in varying amounts in purified PDC from *Azotobacter*. Although the activities of the complex and of PTA are connected in a meaningful sequence, this observation does not provide the necessary arguments that PTA is associated with or is an integral part of the total complex, thus leading to a composition of the *Azotobacter* complex quite different from those of other sources. Additional evidence is needed and it is the aim of this section to present some.

TABLE 3.4. Distribution of different enzyme activities in cell fractions of *Azotobacter vinelandii.*

Fraction	% activity				
	Transhydro-genase	PTA	PDC	acetate kinase	lipoamide dehydrogenase
crude extract	100	100	100	+	100
large particles	4	0	15	−	8
supernatant	10	40	14	+	5
fluffy layer	73	25	50	+	60
small particles	17	0	20	−	30

+ is present
− is absent

At first the distribution of the different enzyme activities in the cellular fractions of *Azotobacter vinelandii* was studied. In TABLE 3.4 the data are presented. The fractions were prepared by differential centrifugation of a sonically prepared cell-free extract (JONES and REDFEARN, 1966), viz. large particles, 30 min. 35,000 × *g*; supernatant, 2/3 of the top layer after centrifugation for 90 min. at 144,000 × *g*; fluffy layer, rest fluid; small particles, homogenized sediment after 90 min. at 144,000 × *g*. The particulate nature of PDC and transhydrogenase can be derived from the TABLE and although the correlation between PDC and lipoamide dehydrogenase is not particularly good, these data don't provide evidence that a tight complex between PTA and PDC exists physiologically. Twenty five percent of the total activity is found in the fluffy layer, where both the highest total activity and specific activity of PDC are present (cf. VAN DEN BROEK, 1971). On the other hand, it must be noticed that the recovery of the total PTA activity is only 65% after centrifugation compared with crude extract standing at 4° during the time of the experiment. Recombination of the resulting cell fractions does not improve the recovery, from which can be concuded that the PTA activity disappeared in an apparent irreversible way. In this context it is useful to recall the observation of LINN et al. (1972), that the PDH phosphatase, which may be called to be an integral part of the mammalian PDC, appeais only to be loosely associated. It can be separated from the overall activity, for instance, by centrifugation (HUCHO et al., 1972) resulting in pure PDC preparations containing variable amounts of the phosphatase. It can be concluded from TABLE 3.4 that the PTA itself is not a very large molecule, since the highest activity is present in the supernatant. In addition, the fact that nearly all PTA precipitates at neutral pH from crude extracts by saturating with 40% ammoniumsulphate or less, might point to a hydrophobic nature of the molecule. For this reason, PTA could be easy copurified, aspecifically, during the fractionation of PDC.

In TABLE 3.1 the figures are presented, accounting for the behaviour of the PTA activity during purification of PDC according to method 1. After the isoelectric precipitation only 10% of the activity can be recovered in the pH 4.9

supernatant; serious losses have occurred, possibly due to acid-denaturation. After ultracentrifugation 10% of the activity remains in the supernatant (specific activity 10). Moreover, after repeating the ultracentrifugation step, the isolated PDC still contains considerable PTA activity. This result contrasts the expected behaviour of a smaller, non-interacting protein (cf. separation PDC and PEP synthetase in *E. coli*; CHULAVATNATOL and ATKINSON, 1973). Even when the purification is supplemented with chromatography on Ca-phosphate gel, Sepharose and/or fractionation wit ammoniumsulphate, no separation can be achieved without totally destroying the overall activity of the PDC.

FIG. 3.5 shows the elution pattern of a stable preparation, with a specific overall activity of 6, after subjecting it to chromatography on Sepharose 4B. Partial dissociation of PTA from the complex also leads to dissociation of LTA and even lipoamide dehydrogenase activity as can be concluded from the difference in elution patterns with respect to that of the overall PDC activity. This phenomenon is accompanied by a drop in the specific activity and considerable loss of overall activity. Recombination does not lead to improvement. The same behaviour is observed, when these preparations are brought on 15–40% sucrose density gradients and subjected to sedimentation for at least 16 hours (74,000 × *g*). In view of these observations, showing the copurification of PTA with a large enzyme complex (cf. 3.2.4.) during several steps and the fact that the

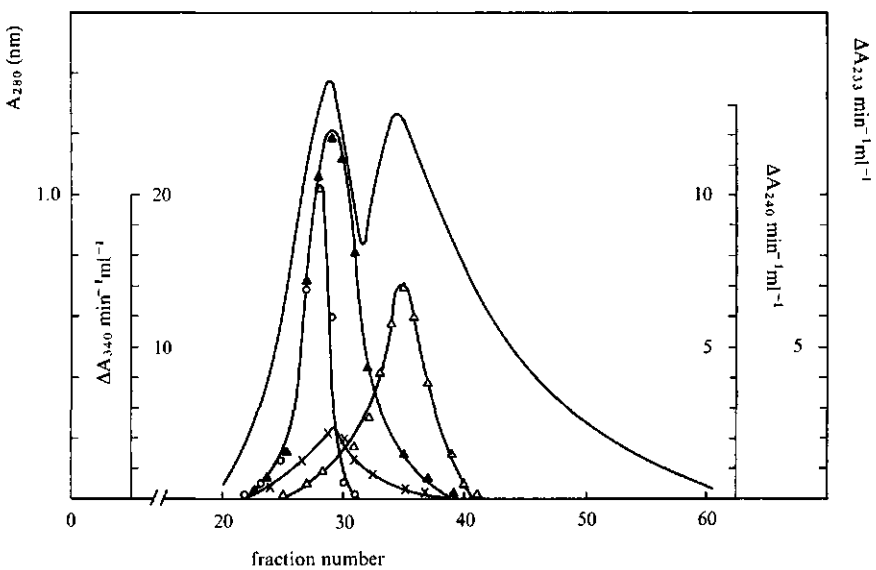


FIG. 3.5. Elution pattern obtained upon chromatography of *Azotobacter* PDC (specific activity 6 μmoles NADH $min.^{-1}mg^{-1}$) at 4° on Sepharose 4B. 80 mg of enzyme was applied to a column of 2 × 80 cm. Elution was performed with 0.1 M potassium phosphate buffer (pH 7.0) at a flow rate of 9 ml per hour. Fractions of 5 ml were collected; (—), A_{280} nm; (○—○), PDC activity measured at 340 nm; (▲—▲), LTA activity measured at 240 nm according to SCHWARTZ and REED (1969); (△—△), PTA activity measured at 233 nm; (+—+), lipoamide dehydrogenase activity measured at 340 nm.

molecular weights of most PTA's, purified until now, are smaller than 90,000 daltons (cf. RADO and HOCH, 1973), the idea of an interaction between PDC and PTA does fit.

3.2.4. *The molecular weight of PDC*

Sedimentation velocity studies were performed with the purified PDC, at a number of protein concentrations and with different preparations. In FIG. 3.6 a typical run is shown. Although not with absolute certainty these pictures suggest, that the preparations used are rather homogeneous under these conditions. However, at protein concentrations lower than about 0.3 mg ml^{-1}, mostly slower sedimenting components are observed at the meniscus. The sedimentation coefficients ($s_{20,w}$) usually found are between 19 and 20 *S* (SVEDBERG) and almost independent of the protein concentration in the region 0.3–5 mg ml^{-1}. In TABLE 3.5 some values are listed. Below a protein concentration of 0.3 mg ml^{-1} a tendency for a lower $s_{20,w}$ is observed, probably caused by some dissociation of the complex (just as reported by KOLB and WIELAND (1972) for

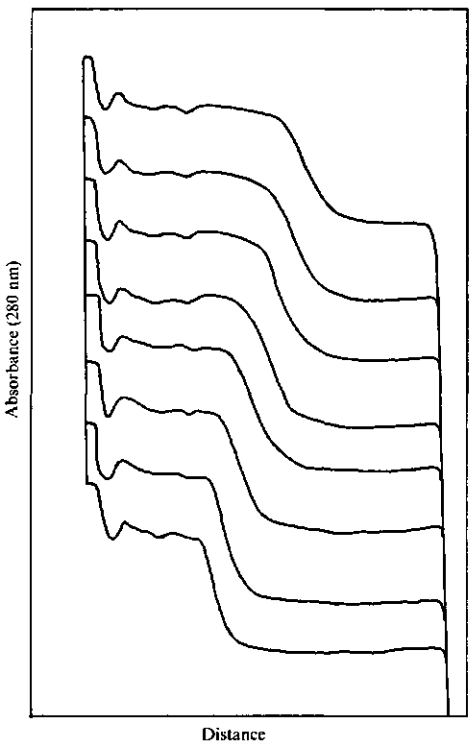


FIG. 3.6. Ultracentrifugal sedimentation patterns with *Azotobacter* PDC (0.3 mg ml^{-1}) in 0.05 M phosphate buffer (pH 7.0). Rotor speed 33,230 r.p.m.; temperature 20° (± 0.1). Ultraviolet scans have been taken at 420, 780, 1140, 1500, 1860, 2220, 2580 and 2940 seconds after reaching rotor speed.

TABLE 3.5. Some sedimentation coefficients ($s_{20,w}$) at different protein concentrations.

Protein concentration (mg/ml)	$s_{20,w}$	optical system used
4.6	19	Schlieren
0.62	19.2	u v
0.41	19.6	u v
0.31	19.8	u v
0.22	17.2	u v
0.16	16.8	u v

the pig heart enzyme). Compared with the $s_{20,w}$ values reported for PDC from the *E. coli* strains and mammals, measured at about the same protein concentrations, a large difference exists.

In TABLE 3.6 $s_{20,w}$ values of different PDC's are listed together with their molecular weights. The M_{app} of *Azotobacter* PDC was calculated by combining sedimentation and diffusion measurements (see Methods) both performed at a protein concentration of 0.4 mg ml^{-1}. The diffusion coefficient ($D_{20,w}$) was found to be 1.96×10^{-7} cm^2sec^{-1} under these conditions. For *Azotobacter* PDC, M_{app} (= 970,000 daltons) is much lower than reported for all other PDC's known yet. Thus, the enzyme is either smaller or it is nearly totally degradated during the purification procedure.

It must be emphasized, that if the preparations are heterogeneous, the molecular weight average calculated, is not necessary the weight average molecular weight (Mw), although $s_{20,w}$ and $D_{20,w}$ are.

Attempts to estimate the Mw by using the sedimentation equilibrium method were unsuccessful. A Mw of about 600,000 daltons was found. However, in the graphs of ln c against r^2 obtained at equilibrium after a centrifugation period of 76 hours, the presence of low molecular weight components is visible. Tests after the run show, that most of the enzyme activity had disappeared and the value obtained for Mw is doubtful.

TABLE 3.6. Sedimentation coefficients ($s_{20,w}$) and molecular weights of the bacterial PDC's.

Source	$s_{20,w} \times 10^{13}$ (sec.)	Mw or M_{app} (daltons)
E. coli Crookes	63	4,600,000[1,2]
E. coli K-12	53[3]	3,000,000[3]
	56[4]	3,750,000[4]
Azotobacter vinelandii	19.5	970,000[5]
		1,000,000–1,200,000[6]

[1] ref KOIKE et al., 1960[a]
[2] ref ELEY et al., 1972 (Mw determined from sedimentation-equilibrium studies)
[3] ref DENNERT and HÖGLUND, 1970 (Mw determined from light-scattering studies)
[4] ref VOGEL et al., 1972[b] (M_{app} determined from $s^{\circ}_{20,w}$ and $D^{\circ}_{20,w}$)
[5] M_{app} determined from $s_{20,w}$ and $D_{20,w}$
[6] Mw determined from light-scattering studies

In order to get more information about the molecular weight and possibly about the configuration of the *Azotobacter* PDC, light-scattering experiments were performed. A exact mathematical treatment for this technique is available and the measurements are relatively rapid, so dissociation and concomitant inactivation will get a minimal change. The preparations used were stable during the experiments and had a specific activity of about 6; PTA activity was present (specific activity 3.5). The data obtained at the different protein concentrations (see legend FIG. 3.7) and the distribution of the scattering at different angles were plotted according to the method of ZIMM (1948) and extra-

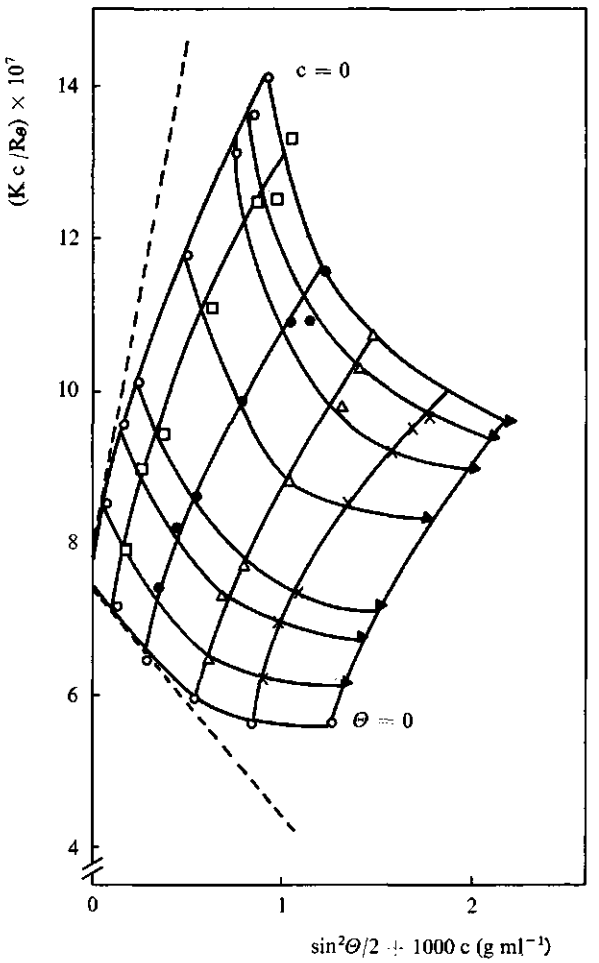


FIG. 3.7. ZIMM plot of angular light-scattering data obtained with *Azotobacter* PDC (specific activity 6 μmoles NADH min.$^{-1}$mg^{-1}) in 0.05 M phosphate buffer (pH 7.0) at room temperature. Experimental points (▲, ×, △, ●, □) measured at different scattering angles (30–150°) and at protein concentrations of respectively 0.07; 0.13; 0.3; 0.85 and 1.4 mg ml^{-1}.

polation to zero concentration and zero angle was made (FIG. 3.7). The intercept $(Kc/R_\theta)_{c\to o,\theta\to o}$ must be equal to the reciprocal of the *Mw*. From the curvature in the ZIMM plot at $\theta\to 0$, a strong dependence on the concentration (indicative for dissociation-association phenomena) is seen, making the double extrapolation uncertain; a *Mw* of 1,200,000 daltons is found. To check this value for the molecular weight of PDC from *Azotobacter*, the results were also plotted according to the method of YANG (1957). By plotting $Kc/\sin^2\theta/2$ against $1/\sin^2\theta/2$ a serie of straight lines must be obtained (FIG. 3.8). The slope of the

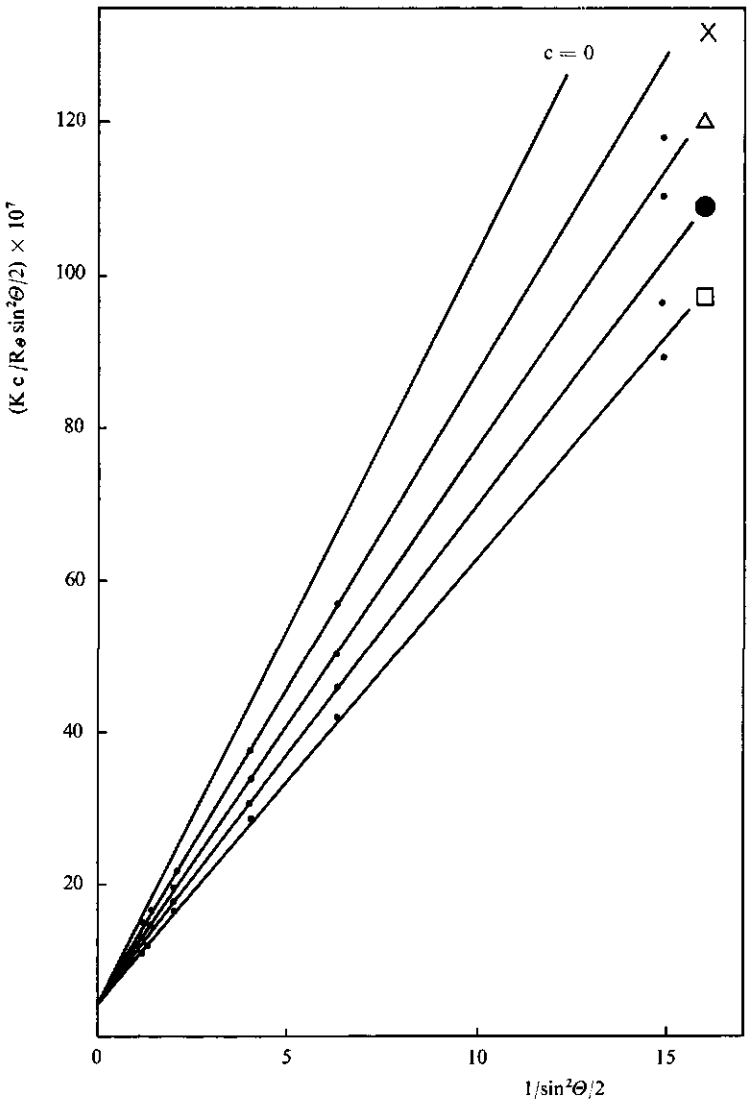


FIG. 3.8. YANG plot of the experimental points (×, △, •, □) from FIG. 3.7.

line at zero concentration equals the reciprocal of the *Mw*; in fact the higher angle values of the ZIMM plot are extrapolated now.

A *Mw* of 1,000,000 daltons is found. From the common intercept on the ordinate, the *Z*-average radius of gyration (R_G = 49 nm) could be calculated, but due its uncertainty, it is of no use to fit this value with a model. However, assuming a partial specific volume of 0.75 ml g^{-1} for *Azotobacter* PDC, with the formula derived by SVEDBERG and PEDERSSEN (1940), the frictional ratio of the molecule can be calculated from the molecular weight and $s^\circ_{20,w}$; f/f_{min} is found to be 1.5. This value is about equal with the one reported by REED and COX (1966) for PDC from *E. coli* Crookes and is higher than published for that of the K-12 enzyme by DENNERT and HÖGLUND (1970). Thus, a more open structure than a sphere can be expected.

In electron micrographs of the complex from *Azotobacter* only low molecular weight protein is visible. The typical structures of PDC as observed with the *E. coli* complex are not present. Only in preparations, characterized by transacetylase-, PTA- and overall activity, which still contain traces of transhydrogenase, tetrad-like structures are visible (FIG. 3.9), very similar to the structure of the LTA component of *E. coli* PDC (REED and OLIVER, 1968) and with about the same dimensions (12–14 nm on a side). The micrographs show, that there exists a tendency to polymerize into regular structures, suggestive for a reconstitution of the LTA (cf. WILLMS et al., 1967). Since only small amounts of transhydrogenase are present in these purified PDC preparations, no ladder-like structures can be seen in the micrographs (cf. VAN DEN BROEK, 1971b).

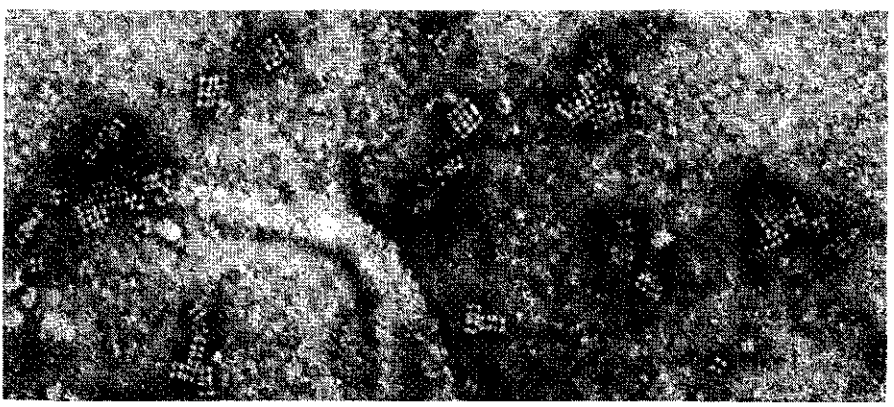


FIG. 3.9. Electron micrograph of structures found in preparations characterized by transacetylase-, PTA- and PDC activity. Traces of transhydrogenase were also present. Negatively stained with uranyl acetate. 260000$\frac{1}{4}\times$.

3.3. Discussion

Although the purification-method used here is essentially similar with that of Reed and Willms (1965), is the ultimate maximal specific activity of *Azotobacter* PDC much lower than that of *E. coli* Crookes -and K-12 (Koike et al., 1960a; Schwartz and Reed, 1970a; Eley et al., 1972; Dennert and Höglund, 1970; Vogel et al., 1971). On the other hand, it is difficult to explain, that the specific activity of the PDC from *E. coli* Crookes, during several years, has increased from 6 to 35 units $min.^{-1}mg^{-1}$ (Koike et al., 1960a; Eley et al., 1972), whereas, as can be simply seen from the flavin content per mg protein, the extend of the purity has not.

The lower specific activity of the *Azotobacter* enzyme is accompanied by a lower specific activity of the lipoamide dehydrogenase component and a lower flavin content per mg protein too. As derived from the constant ratio between the PDH activity and the overall activity, in a large number of preparations, it is not likely that purified *Azotobacter* PDC still contains '*excess*' PDH as described by Vogel et al. (1972a) for PDC from *E. coli* K-12. The question arises whether the complex of *Azotobacter* truely contains less of the flavoprotein or that its low content is due to substancial dissociation during the purification procedure. Other results, especially the light-scattering- and the electron microscopic studies, together with the behaviour of the '*pure*' complex upon chromatography (Sepharose columns), are suggestive for the latter possibility by showing its lability.

Mammalian PDC is also suspected to be deficient in flavoprotein for that reason (Barrera et al., 1972), a view supported by the fact, that with preparations of this complex from the same source, large differences in sedimentation coefficients have been observed (Ishikawa et al., 1966; Junger and Reinauer, 1972). The addition of pure, uncomplexed, lipoamide dehydrogenase from the same organism will not improve the activity and the same is true for the *Azotobacter* complex when it is supplemented with pure *Azotobacter* lipoamide dehydrogenase. The possibility of rearrangements after dissociation has to be considered. In this context, it is useful to recall the observation of Schwartz and Reed (1969); treatment of the transacetylase with increasing amounts of N-acetylimidazole results in dissociation and partial inactivation of its reaction. The partial inactivated transacetylase was separated into two classes (7 *S* and 11–15 *S*) with the same specific activity. Both classes retained the capacity to combine with PDH and lipoamide dehydrogenase resulting in complexes with overall activity. The molecular weights of these rearranged complexes were found to be 500,000 and 4,000,000 daltons respectively.

Purified *Azotobacter* PDC is shown to be strongly associated with varying amounts of PTA activity, inseparable from the complex without total destruction, while the PTA activity itself is extreme labile when PDC is no longer present. Although Koike et al. (1960a) did not find PTA activity in their preparations, there are also positive indications in the literature pointing at a close association in the *E. coli* case. Gounaris and Hager (1961) have reported

about the inseparability of PTA and LTA in a mutant strain of *E. coli*. They were only able to separate PTA from LTA of the wild type enzyme after heat-treatment. Earlier GOLDMAN (1958) reported about the presence of PTA activity in his PDC preparations from *Mycobacterium tuberculosis*. It has been shown that PTA is mainly involved in the generation of ATP via the '*phosphoroclastic*' cleavage of pyruvate into CoASAc and acetylphosphate with the subsequent formation of ATP from acetylphosphate and ADP by acetate kinase in anaerobes (cf. for instance MORTENSON et al., 1962 and PELROY and WHITELEY, 1972). Experiments on the regulation of PTA from aerobically grown and PDC-containing *Bacillus subtilis* are also consistent with this idea (RADO and HOCH, 1973). An amphibolic role of the acetate kinase-PTA system, however, is shown for aerobically grown *Aerobacter aerogenes* (BROWN et al., 1972) and the regulation of pure PTA from aerobically grown *E. coli* B by pyruvate and NADH (SUZUKI, 1969) is suggestive for the same, in spite of the high K_M of the enzyme with respect to acetylphosphate.

Although, it is very difficult to estimate a precise molecular weight for a multi-enzyme complex, that shows, at least extensively purified, a tendency to dissociate, some attempts were made. The result, a molecular weight of 1,000,000–1,200,000 daltons, suggest that the PDC from *Azotobacter* is the smallest one known yet. Nearest in molecular weight is the enzyme isolated from *E. coli* K-12, which is, in the hands of DENNERT and HÖGLUND (1970), very labile too. The suggestion made by VOGEL et al. (1972b), that this lability could be introduced by the acid fractionation step in the purification procedure, turned out to be by no means valuable in the *Azotobacter* case, for complexes purified according to method 1b are behaving similarly. *Active Enzyme Centrifugation* might be the method to be applied; the sedimentation coefficient of the enzyme during catalysis can be estimated in crude extracts and after purification (SCHMITT et al., 1971). Unfortunately, we are not equipped for this. The minimal molecular weight of about 600,000 daltons, derived from the flavin content per mg protein, does not permit a conclusion as to whether the flavoprotein is present in the complex in a monomeric- or dimeric form (REED and OLIVER, 1968). On the other hand, the value of 1,000,000–1,200,000 daltons for the molecular weight suggests that two flavins are present in the isolated complex.

Concerning the electron micrographs, it is rather peculiar that the tetrad-like structures are only visible in less pure (transhydrogenase-containing) preparations and not in the purest enzyme preparations. May be some complex-formation between the transhydrogenase and the transacetylase is responsible for these structures (cf. VAN DEN BROEK, 1971). It is clear, that the low flavin content and the relatively small molecular weight are main features of *Azotobacter* PDC compared with the enzymes from *E. coli* and mammals.

4. REGULATION OF THE PYRUVATE DEHYDROGENASE COMPLEX FROM *AZOTOBACTER VINELANDII*

4.1. INTRODUCTION

In the mechanism of regulation of the activity of the pyruvate dehydrogenase complex differences are observed between those from mammals and bacteria. In both cases the activity is inhibited by the products of pyruvate oxidation: CoASAc and NADH (GARLAND, 1964; GARLAND and RANDLE, 1964; HANSEN and HENNING, 1966; SCHWARTZ et al., 1968). The inhibition by CoASAc is reversed by CoA in the mammalian system and by pyruvate in the *E. coli* system. NADH inhibition is exerted through action on the flavoprotein component, lipoamide dehydrogenase, of the complex and is competitive with respect to NAD^+.

The relative importance of product inhibition for the mammalian system remains questionable, since LINN and co-workers (1969a,b) and WIELAND and VON JAGOW-WESTERMANN (1969) described the regulation by a phosphorylation and dephosphorylation process. Phosphorylation and concomitant inactivation is catalyzed by an ATP-specific kinase. The activity is restored by the action of a phosphatase. Kinase and phosphatase are both Mg^{2+}-dependent. By combined action the activity of the PDC can be regulated by the ATP/ADP ratio.

In *E. coli* such a type of regulation of PDC (SCHWARTZ and REED, 1970a) does not exist. SHEN et al. (1968) and SHEN and ATKINSON (1970) reasoned that the PDC of *E. coli* is subject to regulation by the energy charge (a parameter measuring the mole fraction of phosphoanhydride bonds in the adenylate pool) and also to the concentration of negative feedback effector(s). This type of control is generally thought to modulate the biosynthetic and biodegradative pathways that use or replenish ATP respectively (ATKINSON, 1968).

The enzyme pyruvate dehydrogenase which catalyzes the first step of the PDC seems to be the main site for the regulation by energy charge. In this CHAPTER, the regulatory properties of the *Azotobacter* PDC are described and discussed in comparison with data in the literature.

4.2. RESULTS

4.2.1. *Effect of Mg^{2+}. TPP on the activity*

The activities of purified PDC preparations from *E. coli* (HAYAKAWA et al., 1966a), mammals (KANZAKI et al., 1969) and the yeast *Hansenula miso* (HIRABAYASHI and HARADA, 1972) have been shown to be partially dependent on Mg^{2+} or Ca^{2+} as cofactor, whereas for the activity of PDC from *Neurospora crassa* Mg^{2+} is not required (HARDING et al., 1970). Purified PDC from *Azoto-*

bacter still contains small amounts of Mg^{2+} resulting in a low basic activity, when no metal was added to the reaction mixture. EDTA inhibits this activity.

Frequently Mn^{2+} can be substituted for Mg^{2+} in Mg^{2+}-dependent reactions. From the influence of the metal concentration on the overall activity of PDC, measured in 0.05 M Tris-HCl (pH 7.6) under standard conditions, the K_M's for Mg^{2+} and Mn^{2+} were found to be 100 μM (FIG. 4.1) and 10 μM respectively. Ca^{2+} acts as a competitive inhibitor with respect to Mg^{2+} (K_I = 10 μM) and does not induce a low activity in the absence of Mg^{2+}.

The K_M value obtained upon varying the concentration of Mg^{2+}.TPP complex instead of Mg^{2+}, is found to be 25 μM. In these experiments, performed in 0.05 M Tris-HCl (pH 7.5) under standard conditions, the concentration of TPP was varied in the presence of 10 mM Mg^{2+}. This is permitted, because the K_D of the Mg^{2+}.TPP complex is reported to be 0.41 mM (POULSEN and WEDDING, 1970). At higher pyruvate concentrations (10 mM), the K_M for Mg^{2+}.TPP can be even shown to decrease to 10 μM.

In view of the lower K_M for Mg^{2+}.TPP compared with the K_M for Mg^{2+} and the fact that the Mg^{2+} concentrations used were smaller than the K_D for Mg^{2+}.TPP, it must be concluded that the K_M for Mg^{2+} as determined in this way (FIG. 4.1) does not reflect affinity between enzyme and Mg^{2+}. After con-

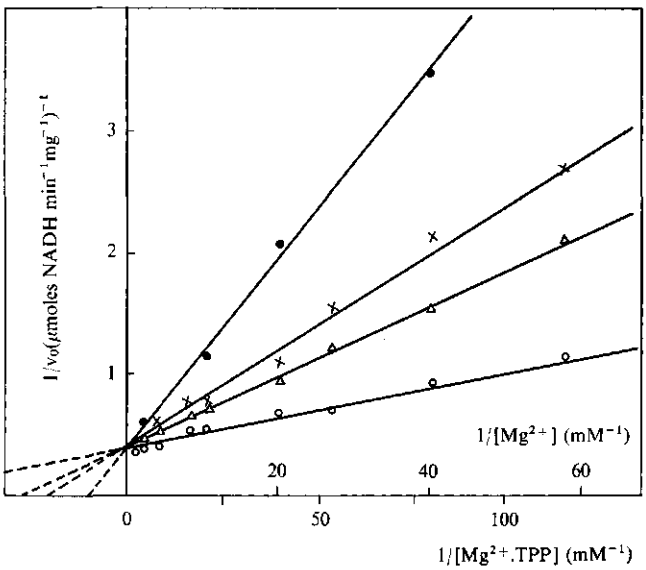


FIG. 4.1. Effect of Mg^{2+} and Mg^{2+}.TPP on the overall activity of *Azotobacter* PDC in the absence or presence of CoASAc and AMP respectively. Reaction mixture as described under METHODS in the presence of 5 mM pyruvate, 0.5 mM TPP and 0.05 M Tris-HCl. The final pH was 7.6. The Mg^{2+} concentrations are indicated in the FIG. The concentrations Mg^{2+}.TPP are calculated using a K_D of 4.07 × 10^{-4} M for the Mg^{2+}.TPP complex according to POULSEN and WEDDING (1970). Temperature: 25°.
Symbols: (×—×), no effector present; (△—△), in the presence of 0.1 mM AMP; (○—○), in the presence of 1 mM AMP; (●—●), in the presence of 10 μM CoASAc.

version of the Mg^{2+} concentrations into Mg^{2+}.TPP, under these conditions, a K_M for Mg^{2+}.TPP can be found of 50μM, in good agreement with the previous value for Mg^{2+}.TPP.

The dependence of the activity on the Mg^{2+}.TPP concentration is greatly influenced by the presence of AMP or the reaction product CoASAc (FIG. 4.1). Sulphate and in less extend phosphate are tending, like AMP, to enhance the affinity of the enzyme for Mg^{2+}.TPP. CoASAc decreases the affinity and behaves as a competitive inhibitor (K_I = 10 μM).

4.2.2. *Dependence of rate on pyruvate concentration*

In the studies on the dependence of the overall activity of PDC on variations in the pyruvate concentration, NAD^+ is used as final electron acceptor. The enzyme which catalyzes the first reaction (PDH) utilizes the lipoyl moiety, covalently bound to the second enzyme, as oxidant in this reaction. Alternatively, ferricyanide can replace the protein-bound lipoyl moiety in accepting the electrons and acetate is produced. Study of the PDH-catalyzed first step is possible under the latter conditions.

FIG. 4.2 shows the kinetics of the overall reaction with different pyruvate concentrations. Pyruvate exerts a positive homotropic effect. AMP and sulphate stimulate the activity at lower pyruvate concentrations. In contrast, CoASAc

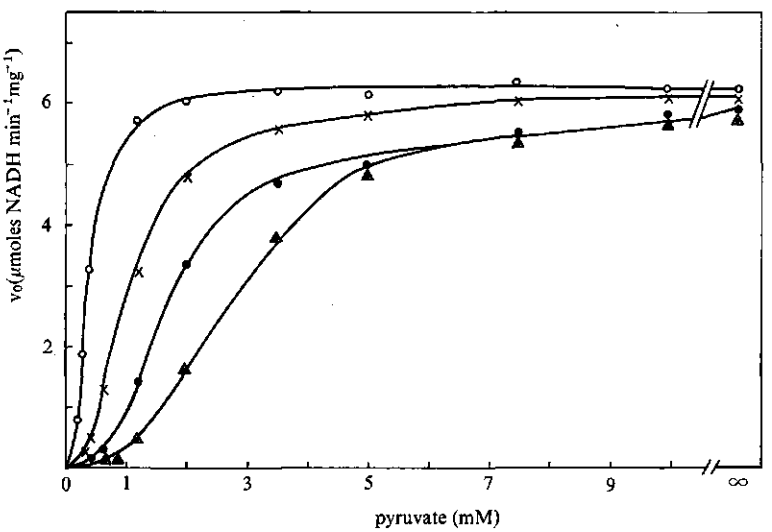


FIG. 4.2. Dependence of the rate of the overall reaction on the pyruvate concentration in the presence or absence of effectors. Reaction mixture as described under METHODS in the presence of 5 mM Mg^{2+}, 0.5 mM TPP and 0.05 M Tris-HCl, final pH = 7.5, at 27°. Pyruvate is added as indicated. The production of NADH is measured in 3 ml cuvettes on the Aminco-Chance dual-wavelength spectrophotometer (380–345 nm) using the 5–20% transmission scale. Standardization was performed with a NADH-solution of known concentration.
Symbols: (●—●), no additions; (▲—▲), in the presence of 10 μM CoASAc; (○—○), in the presence of 0.1 mM AMP; (×—×), in the presence of 0.1 mM sulphate.

inhibits competitively as can be concluded from V, which inhibition can be reversed (not shown) by the positive effectors. $S_{0.5} = 1.9$ mM for pyruvate, as found under the conditions used. It is convenient in allosteric proteins to use the $S_{0.5}$ at half saturation, since a simple K_M cannot be calculated for curves which deviate from MICHAELIS-MENTEN kinetics (KOSHLAND et al., 1966).

In FIG. 4.3 a HILL-plot (log $\{v_0/(V-v_0)\}$ versus log $[S]$) of the same results is presented. Maximum velocities were extrapolated from the 1/velocity versus 1/[pyruvate] curves. The HILL-coefficients (h) as calculated from the plot are above 2 in all cases ($h = 2.6$–2.7), so it must be concluded that the cooperative effect exerted by pyruvate is not influenced by the presence of the effectors, despite the apparent change of the shape in the velocity versus pyruvate curves. It must be mentioned, that the shapes of the curves are not dependent on the protein concentration used in the experiments.

The stimulatory effects as observed with AMP and sulphate at low pyruvate concentrations are also being exerted at decreasing efficiencies by for instance ADP, GMP, GDP, phosphate, arsenate, maleate, fumarate, malonate. However, to achieve maximum effects, much higher concentrations are needed. These anions are also effective in reversing the inhibitory action of CoASAc. ATP is slightly inhibitory at a relative high concentration (5 mM). GTP, chloride and acetate are without any effect. In TABLE 4.1 the influence of some of the strongest effectors is summarized (see Legend). CoASAc behaves as a

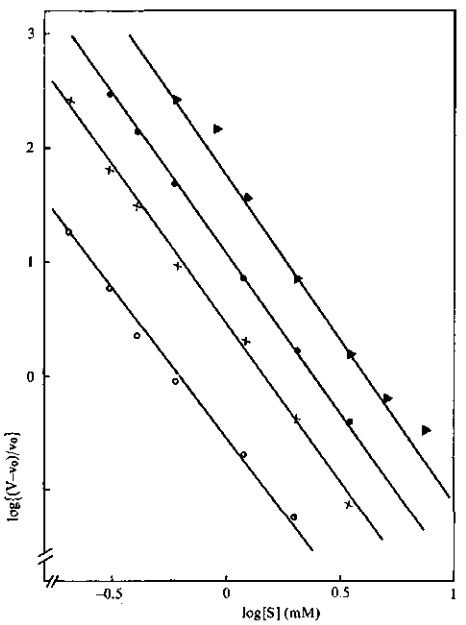


FIG. 4.3. HILL-plots of the data presented in FIG. 4.2. The same symbols were used; details, see text.

TABLE 4.1. Influence of the effector concentration on the overall reaction.
$E_{0,5}$, the concentration of effector required for half-maximal effect, was determined from a curve of reaction rate as a function of the effector concentration at the pyruvate and Mg^{2+}.TPP concentrations indicated. All curves were hyperbolic.

effector	type of action (+/−)*	pyruvate (mM)	Mg^{2+}.TPP (mM)	$E_{0,5}$ (μM)
AMP	+	2	0.5	60
ADP	+	2	0.5	160
SO_4^{2-}	+	0.4	0.4	140
CoASAc	−	0.16	0.4	8

* + = stimulation
  − = inhibition

very strong competitive inhibitor with respect to Mg^{2+}.TPP-pyruvate ($I_{0.5}$ = 8 μM).

In contrast with the observation of SHEN and ATKINSON (1970) for PDC from *E. coli*, the stimulatory effect of AMP or sulphate on the initial velocity at the

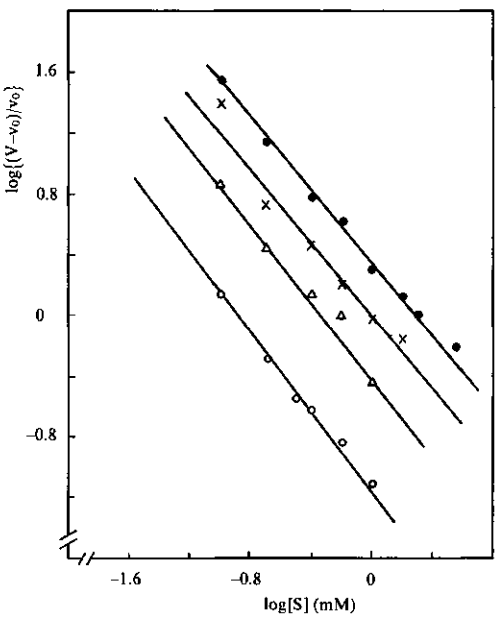


FIG. 4.4. HILL-plots of the ferricyanide-linked PDH activity. The activity was measured in Tris-HCl as described under METHODS with the whole complex. The pyruvate concentrations were as indicated. V was obtained from LINEWEAVER-BURK plots.
Symbols: (△—△), no effector present; (×—×), in the presence of 10 μM CoASAc; (●—●), in the presence of 20 μM CoASAc; (○—○), in the presence of 1 mM AMP.

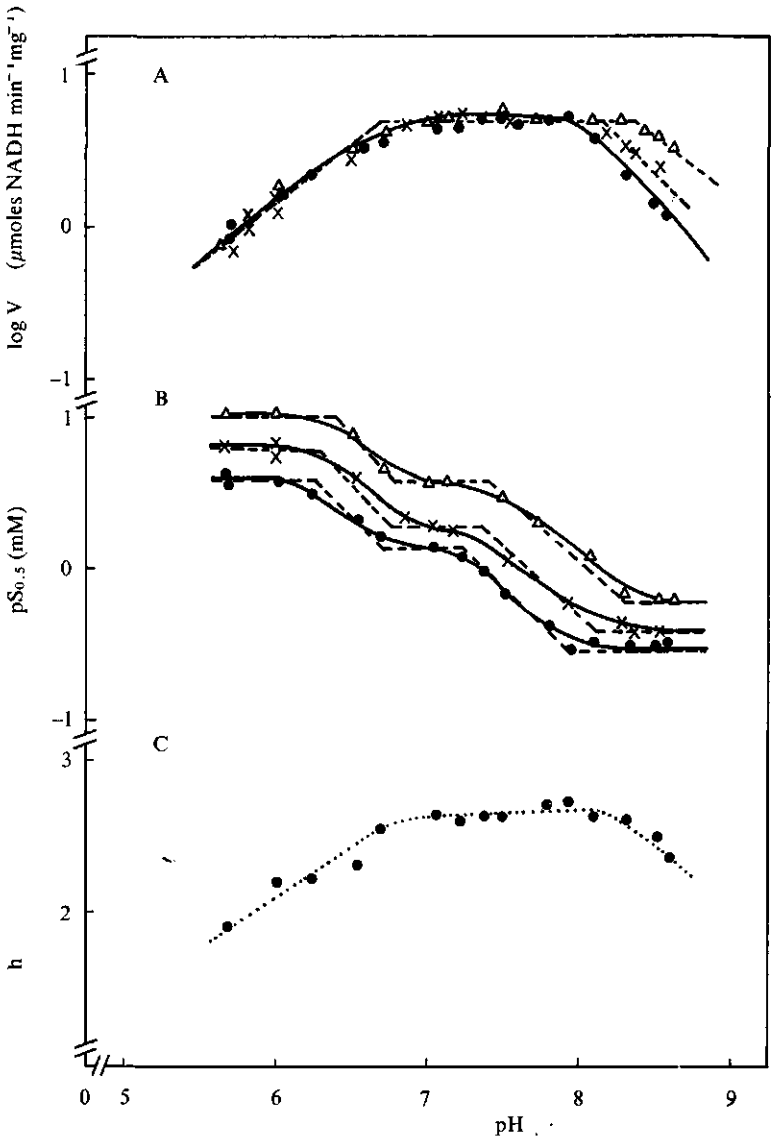


FIG. 4.5. Dependence of log V (A), $pS_{0.5}$ (B) and h (C) for pyruvate on the pH. Each point was derived from plots as shown in FIG. 4.2. under the conditions described. Measurements were performed in 50 mM Tris-acetate '*buffer*'. Details are given in the text.
Symbols: (●—●), no additions; (×—×), in the presence of 0.1 mM sulphate; (△—△), in the presence of 0.1 mM AMP.

lower pyruvate concentrations is not dependent on the presence of sulfhydryl compounds like DTT in the reaction mixture. In the absence of DTT, however, V is about 20% lower either with or without the effectors.

In using the alternative electron acceptor ferricyanide, to locate the influence of the effectors on the first enzyme of the complex, the effects of AMP and CoASAc are still present and compete with each other or with pyruvate. It is necessary in this context to mention that the ferricyanide assay is much less sensitive and more difficult to handle than that measuring the overall reaction. Flow of electrons to ferricyanide via the lipoyl moieties of the second enzyme will not occur in this assay as can be concluded from experiments after the lipoyl moieties had been blocked with NEM (cf. CHAPTER 5). Some of the results were plotted according to HILL (FIG. 4.4) and the coefficients derived (h) are 1.3 ($\pm$ 0.2). The tendency of these effects suggests, that at least part of the characteristics are also present in the ferricyanide-linked PDH (partial) reaction. On the other hand one could question if, although consistent, such a deviation from $h = 1.0$ is significant (see CHAPTER 5).

4.2.3. *pH influence of rate-dependence on pyruvate concentration*

To study the influence of the pH, the velocities were measured at varying pyruvate concentrations and at different pH's. The experiments were performed in Tris-acetate '*buffer*'. Before and after the reaction, the pH of each cuvette was checked. Corrections were made for irreversible destruction of the enzyme by a change of pH. This was tested by exposing the enzyme to the range of pH values and measuring of the activity after readjustment of the pH to a standard value (pH 7.4). At pH-values, more extreme than used here, the enzyme nearly completely denatures.

In FIG. 4.5 (A, B) the relationship between log V and pH, and $pS_{0.5}$ and pH with and without effectors is given. V was extracted from reciprocal plots and $S_{0.5}$ values were derived from the HILL-plots. These curves must be interpreted according to DIXON and WEBB (1964). The two bends, at pH 6.7 and pH 8.0 respectively, in the curve log V versus pH are both representing pK values in the enzyme-substrate complex (ES) and we will refer to them as K_1, which is the ionization constant of ES affecting the acid side of the pH curve, and K_2 affecting the alkaline side. Below pK_1^{ES}, log V decreases with decreasing pH (slope $\sim$ 0.8) and just like between the two pK values, no influence of the effectors on V is observed. Above pK_2^{ES}, log V decreases with increasing pH (slope = 1) and moreover AMP and sulphate are bringing about a slight shift of this pK towards a higher pH, thus preventing deprotonation of ES. Upon increasing the AMP concentration to 2 mM (not shown), K_2^{ES} even seems to disappear.

Because, the pH-dependent equation for the velocity of the PDC is,

$$v_0 = \frac{\dfrac{V}{1 + \dfrac{[H^+]}{K_1^{ES}} + \dfrac{K_2^{ES}}{[H^+]}}}{1 + \dfrac{S_{0.5}}{[S]}\left\{\dfrac{1 + \dfrac{[H^+]}{K_1^{E}} + \dfrac{K_2^{E}}{[H^+]}}{1 + \dfrac{[H^+]}{K_1^{ES}} + \dfrac{K_2^{ES}}{[H^+]}}\right\}} \qquad (1)$$

where K_1^E and K_2^E are ionization constants, affecting the acid- and alkaline side of the pH curve of the free enzyme (E) respectively, it is clear that every pK value in V must be also found expressed in $S_{0.5}$. From analysis of the $pS_{0.5}$ versus pH graphs, except the pK^{ES} values, two pK's at pH 6.2 and at pH 7.2, evidently due to ionizations in E were obtained. Approximately, the slopes of the straight-line sections are integrals (0 and –1). AMP and sulphate equally tend to enhance the affinity of the PDC for pyruvate in the whole pH-range studied. In line with the data already presented, the effectors are also producing a shift in alkaline direction of pK_2^{ES} and the same can be observed for pK_1^E and pK_2^E. In addition, it is of interest to mention, that upon measuring the ferricyanide-linked PDH (partial) reaction, the positive effectors AMP and sulphate were also enhancing V at about pH 8.5 and on the other hand, at pH 7.5, such an enhancement is not observed (cf. 4.2.2).

In FIG. 4.5 (C) the values of h at the different pH's are given. There is a general tendency pointing at a decline of h upon lowering the pH. This h versus pH graph indicates, that h is determined by ionizations in the ES-complex (cf. log V versus pH). Most of the coefficients are above 2, suggesting the involvement of interactions between more than two subunits, either identical or not. Furthermore by these results, the multi-site hypothesis as proposed by ATKINSON et al. (1965) explaining the sigmoidal rate curve as a function of the substrate concentration for NAD^+-dependent isocitrate dehydrogenase, can ruled out to be operative in our case;
From equation (1), see above, can be derived

$$\left\{\frac{v_0}{(V - v_0)}\right\} = \left\{\frac{1 + \dfrac{[H^+]}{K_1^{ES}} + \dfrac{K_2^{ES}}{[H^+]}}{1 + \dfrac{[H^+]}{K_1^{E}} + \dfrac{K_2^{E}}{[H^+]}}\right\}\frac{[S]}{S_{0.5}} \qquad (2)$$

According to ATKINSON et al. (1965), for an enzyme with h interacting sites (assuming the concentration of ES-complexes that contain fewer than h molecules of substrate is negligibly small) equation (2) must be modified to

$$\left\{\frac{v_0}{(V-v_0)}\right\} = \left\{\frac{1+\frac{[H^+]}{K_1^{ES}}+\frac{K_2^{ES}}{[H^+]}}{1+\frac{[H^+]}{K_1^{E}}+\frac{K_2^{E}}{[H^+]}}\right\}\frac{([S])^h}{S_{0.5}} \tag{3}$$

Therefore,

$$\log\left\{\frac{v_0}{(V-v_0)}\right\} = \log\left\{\frac{1+\frac{[H^+]}{K_1^{ES}}+\frac{K_2^{ES}}{[H^+]}}{1+\frac{[H^+]}{K_1^{E}}+\frac{K_2^{E}}{[H^+]}}\right\} +$$

$$+ \text{h} \log([S]) - \log(S_{0.5}) \tag{4}$$

Equation (4) says that h and pH must be independent of each other upon using this theory.

4.2.4. *Control of the PDC-catalyzed reaction by [CoASAc]/[CoA] ratio*

Apart from the allosteric control of the PDC-catalyzed reaction by the pyruvate concentration, the strong competitive inhibitory effect of CoASAc was already mentioned. These experiments were performed in the presence of saturating amounts of CoA and NAD^+. In FIG. 4.6 the inhibitory effect of CoASAc in the absence of phosphate is plotted. $I_{0.5}$ was found to be 8 μM at the low pyruvate concentration used. From the inserted HILL-plot it can be seen that there is no cooperativity in the CoASAc binding ($h = 1$), just as reported by SHEN and ATKINSON (1970) for the *E. coli* case.

However, in the presence of phosphate the inhibition is apparently cooperative, partly by the effectivity of the phosphate anion in reversing the CoASAc inhibition (cf. 4.2.2) and partly by the presence of PTA activity in the PDC preparations. This activity reversibly converts CoASAc to acetylphosphate in the presence of phosphate (cf. CHAPTER 3). For that reason the preparations of the complex are contamined with a CoA-regenerating system.

FIG. 4.7 shows that the dependency of the overall reaction from the CoA concentration is also apparently cooperative, originating from the combined responses of the PDC and PTA to the CoASAc/CoA ratio. The curve without further additions as well as the fact, that the measurements were performed on a

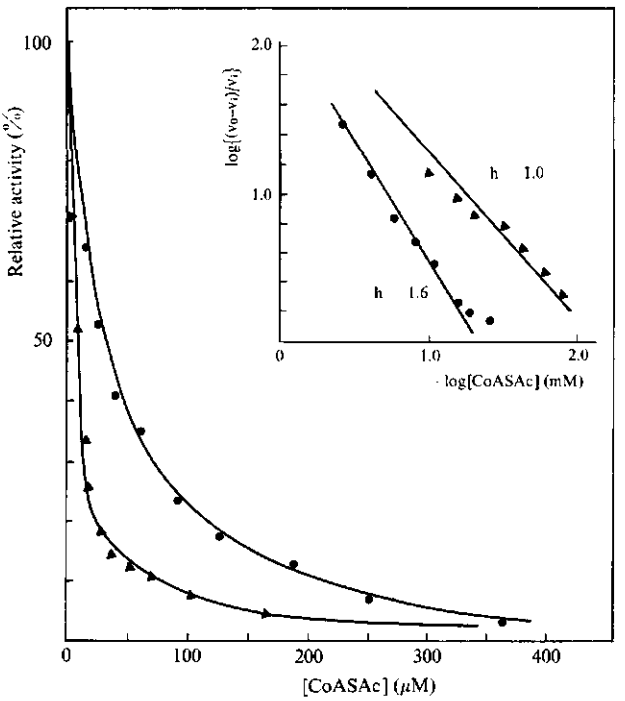


FIG. 4.6. Effect of CoASAc on the overall activity of *Azotobacter* PDC in the presence and absence of phosphate. Conditions as described under METHODS in the presence of 2 mM NAD^+, 0.16 mM pyruvate, 0.11 mM and 2 mM reduced glutathione. The concentrations of CoASAc were as indicated.
Symbols: (▲—▲), in 50 mM Tricine-KOH buffer (pH 7.0); (●—●), in 50 mM potassium phosphate buffer (pH 7.0).
Insert: HILL-plots derived from these results.

Zeiss PMQ II spectrophotometer with the aid of a recorder, are indicating clearly that no initial velocities were measured and that the strong inhibitor CoASAc is already produced in a appreciable amount. This observation points at possible regulation, viz. CoASAc is at this low CoA concentration much slower converted into acetylphosphate and CoA than it is produced from pyruvate, which leads to a high [CoASAc]/[CoA] ratio. The slow conversion is due to the unfavourable K_M of PTA for CoASAc and the low CoASAc concentration.

It is also indicated by the FIG., that a CoA-regenerating system increases the rate and gives a hyperbolic saturating curve. This observation is independent of the regenerating system used. Moreover, an experiment without a CoA-regenerating system performed on the 5–20% transmission scale of the Aminco-Chance dual wavelength spectrophotometer to insure initial velocities gives an identical result. It was found, from the inserted reciprocal plot, that the K_M for

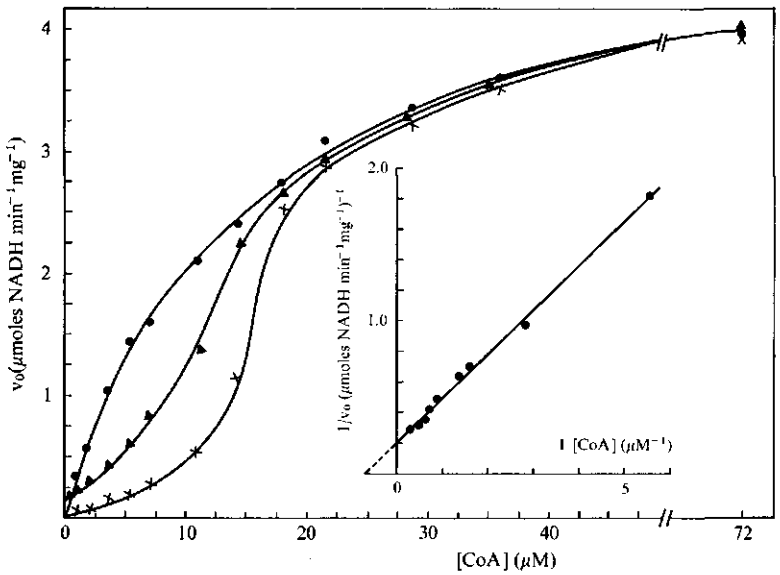


FIG. 4.7. Influence of CoA on the overall activity of *Azotobacter* PDC. Reaction mixture as mentioned under METHODS in the presence of 5 mM pyruvate, 2 mM NAD^+ and 2 mM reduced glutathione. Regenerating systemx used: either 20 mM carnitine plus 2 units carnitine acetyl-transferase or 0.2 mM oxaloacetate plus 3.5 units citrate synthase. The CoA concentrations were indicated.
Symbols: (+—+), no regenerating system present; (▲—▲), no regenerating system but in the presence of 12 μM CoASAc; (●—●), in the presence of a regenerating system.
Insert: reciprocal plot derived from v_0 versus CoA curve in the presence of a regenerating system.

CoA is 14 μM and this value is independent of the concentration of pyruvate used in the experiments.

Furthermore we observed enhancement rather than inhibition by the addition of CoASAc at zero CoA concentration in the presence of phosphate. Not shown in the FIG. is the observation that in the absence of phosphate higher concentrations of CoA are necessary to produce comparable rates. CoASAc is more inhibitory now.

4.2.5. *Influence of varying concentration of NAD^+; inhibition by NADH*

By variation of the concentration of NAD^+ attention is focussed on the third enzyme of the complex, the well known lipoamide dehydrogenase. Through this enzyme NAD^+ performs its role as electron acceptor of the overall reaction. A lot of studies have been performed to elucidate the reaction mechanism of this enzyme, for example VEEGER (1960), VISSER (1970), VAN MUISWINKEL-VOETBERG (1972) and VEEGER et al. (1972a).

FIG. 4.8 gives a plot of the overall activity against the concentration of NAD^+ under varying conditions. These experiments were performed on a Zeiss PMQ II

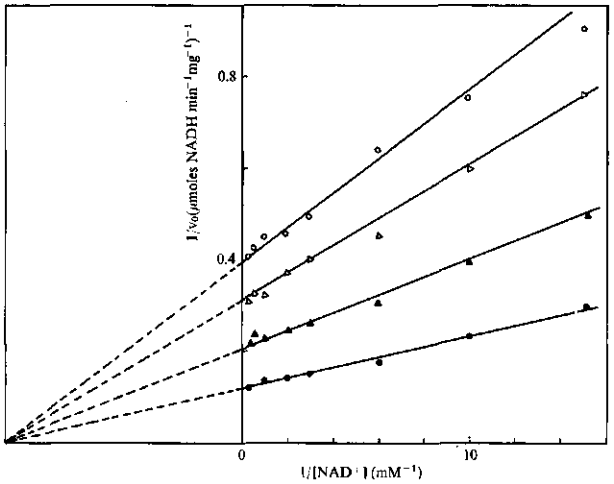


FIG. 4.8. Dependence of rate of NADH production on different conditions. LINEWEAVER-BURK plots of the rate measured in the presence of 4 mM pyruvate, 2 mM Mg^{2+}, 1.5 mM DTT, 0.1 mM CoA, 0.5 mM TPP and buffer pH 7.5. The enzymes: carnitine acetyl-transferase and citrate synthase were dialyzed against a large volume of 0.05 M Tris-HCl (pH 7.5) before use.
Symbols: no regenerating system present and 50 mM Tris-HCl (○—○) or 50 mM potassium phosphate buffer (△—△); in the presence of a CoA regenerating system either 30 mM carnitine plus 4 units carnitine acetyl-transferase or 0.2 mM oxaloacetate plus 2.5 units citrate synthase and 50 mM Tris-HCl (●—●) or 50 mM potassium phosphate buffer (▲—▲). Temperature: 25°.

spectrophotometer equipped with a fast-response recorder. To make sure that real initial velocities were measured, some of the experiments were done in the presence of an external CoA-regenerating system, thus opposing the inhibition by CoASAc. Little difference is in this respect observed between the two systems employed for this purpose: *carnitine acetyl-transferase plus carnitine* or *citrate synthase plus oxaloacetate.* The stimulation of the activity, compared with the measurements in Tris-HCl, suggest that the activity observed in the presence of phosphate and in the absence of a CoA-regenerating system is mainly due to diminished inhibition of the '*initial*' rate by CoASAc. The fact that phosphate inhibits in the presence of a CoA-regenerating system might be caused by the lower K_I for NADH under these conditions (see further below) and consequently the velocities have been measured less initial.

The K_M for NAD^+ remains unchanged under all conditions employed and was found to be 0.1 mM. This value is about the same as the one reported by VAN DEN BROEK (1971) for pure lipoamide dehydrogenase (0.18 mM) from the same organism. The pH of the experiments was 7.5 and it can be demonstrated (data not shown) that the K_M for the NAD^+-binding decreases at the higher pH values.

HANSEN and HENNING (1966) have shown for PDC from *E. coli* that NADH

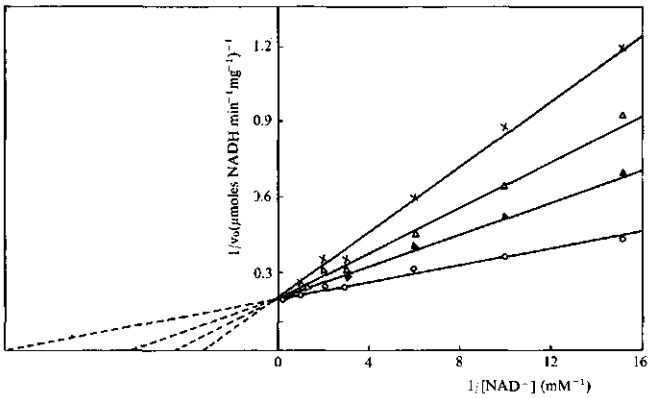


FIG. 4.9. Effect of NADH on the reduction of NAD^+ in the overall reaction of *Azotobacter* PDC. Conditions as described under METHODS in the presence of 5 mM pyruvate, 0.13 mM CoA, 2 mM DTT and 50 mM Tris-HCl (pH 7.5). The CoA regenerating systems were used exactly as described for FIG. 4.8. The NAD^+ concentrations were as indicated.
Symbols: (○—○), no NADH; (▲—▲), 30 μM NADH; (×—×), 130 μM NADH; (△—△), 30 μM NADH plus 50 mM potassium phosphate buffer (pH 7.5) instead of Tris-HCl.

is a competitive inhibitor with respect to NAD^+. The amount of inhibition by NADH in our system is shown in FIG. 4.9 and depends somewhat on the buffer used. Assuming pure competitive inhibition a $K_I = 40$ μM could be found in the absence of phosphate and $K_I = 20$ μM in its presence; the latter value is slightly higher than that previously reported by BRESTERS et al. (1972) and in good agreement with the value found for pure lipoamide dehydrogenase from pig heart (VISSER, J., personal communication). In addition, it turned out that, for PDC from *Azotobacter*, NADPH is a weak competitive inhibitor with respect to NAD^+ ($K_I = 450$ μM in the presence of phosphate; see also CHAPTER 5).

Furthermore, VEEGER (1960) showed that phosphate is a competitive inhibitor with respect to NAD^+ for pure lipoamide dehydrogenase from pig heart. Thus, increased affinity for NADH in the presence of phosphate could be expected due to competitive action of the phosphate ion. On the other hand, for the complexed lipoamide dehydrogenase from *Azotobacter* PDC, such competitive action would become visible only in the presence of NADH, since the data of FIG. 4.8 show non-competitive inhibition by phosphate. Moreover, TSAI et al. (1973) have observed differences in kinetic mechanism between pure lipoamide dehydrogenase and the enzyme bound to PDC; both were isolated from bovine kidney (see DISCUSSION).

In order to evaluate the results of the next section, it is necessary here to mention the weak competitive inhibition of PDC by high concentrations (2 mM or higher) ATP with respect to NAD^+ (not shown). K_I was calculated to be 1.4 mM.

4.2.6. *Response to variation in energy charge*

The energy charge of the adenylate pool is, according to a hypothesis (ATKINSON, 1968), a quantitative parameter for the energy condition of the whole cell. It is defined as:

$$\varepsilon = \tfrac{1}{2}\left\{\frac{2\,[ATP] + [ADP]}{[ATP] + [ADP] + [AMP]}\right\} \qquad \text{and}$$

ε varies between 0 and 1. This hypothesis is useful because individual adenine nucleotides cannot act independently within the cell.

It was shown (4.2.2) that AMP and ADP stimulate the activity of PDC at low pyruvate concentrations, however not specifically. ATP is mainly a weak inhibitor at low NAD^+ concentrations (4.2.5), competitive with respect to this nucleotide.

Bearing in mind the remarkable response of yeast citrate synthetase (ATKINSON, 1968) on variations in energy charge, whereas all three the nucleotides individually are inhibitory, the response of *Azotobacter* PDC to variations in energy charge has been investigated. PDC isolated from *E. coli* already was reported to be sensitive to this parameter (SHEN and ATKINSON, 1970).

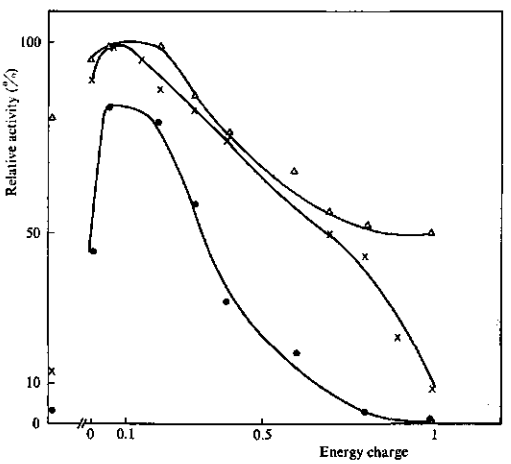


FIG. 4.10. Effect of energy charge on *Azotobacter* PDC. The experiments were carried out in the presence of 10 mM Mg^{2+}, 1.5 mM DTT, 0.1 mM CoA, 0.5 mM TPP, 50 mM Tris-HCl (pH 7.5), 1 mM NAD^+ and pyruvate as indicated. Desired values of the energy charge were obtained with varying amounts of ATP, ADP and AMP (total concentration 5 mM) and 3.5 units myokinase, dialyzed against a large volume of 0.05 M Tris-HCl (pH7.5). Temperature: 25°. The activity of the PDC without added nucleotides is indicated at the ordinate. The values are expressed in % of the highest activity obtained in these experiments (7 μmoles NADH $min.^{-1}mg^{-1}$.).
The values at the abscissa give the relative rates without effector.
Symbols: (×—×), 0.5 mM pyruvate; (●—●), 0.5 mM pyruvate plus 0.1 mM CoASAc; (△—△), 5 mM pyruvate.

FIG. 4.10 shows the results. Desired values of the adenylate energy charge were established by the addition of appropriate amounts of the three nucleotides together with myokinase plus Mg^{2+}.
Since myokinase catalyzes the equilibrium,

$2\ ADP \rightleftharpoons ATP + AMP$, it is possible with the known equilibrium constant of this reaction ($K = 2.26$; cf. METHODS) to calculate the concentration of the nucleotides individually and from them the energy charge parameter. At the lower pyruvate concentration the activity reaches an optimum before decreasing. However, there is only little inhibition at maximum charge compared with the activity without added nucleotides. In experiments where the Mg^{2+} concentration is decreased from 10 to 1 mM (data not shown), the decrease of the curve at high energy charge is even sharper, probably due to chelation by ATP of the metal necessary for the reaction. Lowering of the total concentration nucleotides does not affect the results significantly. Also seen from the FIG. is the influence of the negative effector CoASAc.

For the reason that at the low pyruvate concentration these experiments can be simply explained by the change in affinity for pyruvate (cf. 4.2.2), the experiment was also performed at a higher pyruvate concentration. This curve shows a slight increase at low energy charge and a less sharp decrease at the higher energy charges. Nevertheless a marked inhibition at high energy charge is observed as compared with the activity without added nucleotides. The same curve has been obtained by VEEGER et al. (1972a) on measuring the energy charge at low pyruvate concentration and in the presence of 1 mM sulphate (cf. 4.2.2), which condition was introduced by using undialyzed myokinase.

4.2.7. *About modification of the activity by phosphorylation and dephosphorylation*

In contrast to the mammalian PDC's and the PDC from *Neurospora crassa* (WIELAND et al., 1972a) no regulation by phosphorylation and dephosphorylation of PDC from *E. coli* was observed (SCHWARTZ and REED, 1970a). Experiments with preparations of the complex from *Azotobacter* at different stages of purification gave no evidence for the existance of such regulation. In these incubations an ATP-regenerating system was used in order to eliminate the influence of ATP-ase activities. The system is shown to be effective with a purified PDC preparation isolated from beef heart as is illustrated in FIG. 4.11.

This interconversion of active mammalian PDC in an inactive form probably also occurs *in vivo*. Recently, the proportions of active (dephospho) and inactive (phospho) forms of PDC and the corresponding adenine nucleotide contents have been determined in isolated rat liver mitochondria (WIELAND and PORTENHAUSER, 1974). On plotting the ratios of [ATP]/[ADP] against the active/inactive enzyme ratios a straight correlation was obtained. A drop of [ATP]/[ADP] from 1 to values below 0.4 is accompanied by an eight-fold increase in PDC activity. However, as outlined by HUCHO (1974), to accomplish metabolic regulation by this mechanism one has to postulate that the interconverting enzymes (PDH-kinase and PDH-phosphatase) themselves are subject to regulation, viz. are under the control of the energy charge. Thus, in view

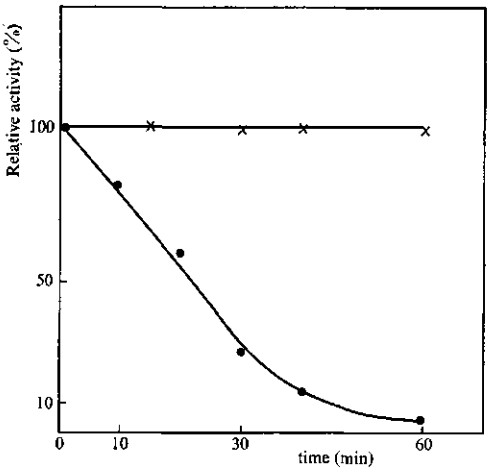


FIG. 4.11. Time course of the effect of ATP on the activity of crude *Azotobacter* PDC and partly purified bovine heart PDC. Incubations were done at 25° with 1 mM ATP, 2 mM Mg^{2+} and a ATP regenerating system of 20 mM creatine phosphate and 0.75 mg creatine-kinase in a volume of 2 ml. At the time indicated aliquots were removed and anaerobically assayed for activity. The results are corrected for blanco decrease of activity with time.
Symbols: (×—×), *Azotobacter* PDC; (●—●), bovine heart PDC.

of the results presented in 4.2.6, one even would not expect regulation of the bacterial PDC's by covalent modification (phosphorylation-dephosphorylation).

4.3. DISCUSSION

The results presented indicate, that like PDC from *E. coli*, the *Azotobacter* enzyme shows a real sigmoidal rate curve as a function of the pyruvate concentration (SCHWARTZ et al., 1968; BISSWANGER and HENNING, 1971). Although such a behaviour is compatible with various theories, from the dependence of $pS_{0.5}$- and of log V on the pH some conclusions can be derived (see CHAPTER 6).

Compared with data from the literature, the K_M of the complex with respect to Mg^{2+}.TPP is in accordance with the value (8 μM) for cauliflower KGDH (POULSEN and WEDDING, 1970; WEDDING and BLACK, 1971), while for the mammalian PDC a K_D for the enzyme-Mg^{2+}.TPP complex of 5 μM has been reported, which value increases to 60 μM after phosphorylation (ROCHE and REED, 1972). The PDH-component of the mammalian PDC's consists of two non-identical subunits and the kinetics with respect to pyruvate is hyperbolic. This and the fact that only one of the subunits can be phosphorylated, suggests that the function of the two chains is different (BARRERA et al., 1972). On the other hand, in the intact mitochondrion a sigmoidal dependence of the amount of

active PDC on the concentration of pyruvate has been observed (PORTENHAUSER and WIELAND, 1972; CHAPTER 1).

For purified PDC from mammalian origin, different K_M's with respect to pyruvate have been reported, like 1 mM and 0.4 mM for the pig heart enzyme (BREMER, 1969; KANZAKI et al., 1969) and 44 μM and 35 μM for respectively bovine kidney- and bovine heart PDC (HUCHO et al., 1972). Potato (CROMPTON and LATIES, 1971) – and *Neurospora crassa* PDC (HARDING et al., 1970) have a K_M for pyruvate of 0.2 mM and in view of their regulation, the latter complexes belong to the same category as the mammalian (mitochondrial) systems, viz. in exerting hyperbolic kinetics with respect to pyruvate and absence of stimulation by AMP, whereas the inhibitory action of CoASAc does not occur at the level of the first enzyme of the complex. The activity of the PDC from *Neurospora crassa* is also regulated by phosphorylation and dephosphorylation (WIELAND et al., 1972a).

E. coli PDC has been extensively investigated and from the sigmoidal response to pyruvate a $S_{0.5}$ of 0.5 mM (SHEN and ATKINSON, 1970; SCHWARTZ et al., 1968) and 0.2 mM (BISSWANGER and HENNING, 1971) is derived; values only slightly lower than for the *Azotobacter* PDC. Furthermore, the complex from *E. coli* is inhibited non-competively by GTP and competively by CoASAc with respect to pyruvate (SCHWARTZ and REED, 1970a). The latter inhibition can be reversed by phospho-enolpyruvate, nucleosides monophosphate and to a less extend by ADP and phosphate. In the presence of phospho-enolpyruvate or CoASAc, the HILL-coefficient for the pyruvate binding decreases from 1.8 to 1, on measuring the PDH activity with ferricyanide as electron acceptor, while the affinity for pyruvate increases or decreases respectively. AMP does not influence the HILL-coefficient (SCHWARTZ et al., 1968).

The effect of sulphate has not been described for PDC, but it is of interest to know that sulphate inhibits the interaction between CoASAc and pyruvate carboxylase from chicken liver (SCRUTTON and FUNG, 1972). Moreover, sulphate is reported to overcome the inhibitory action of phosphate on pyruvate kinase from *Azotobacter* (LIAO and ATKINSON, 1971). It is clear from the data presented in this CHAPTER that PDC from *Azotobacter* is neither stimulated by phospho-enolpyruvate nor that CoASAc and the other effectors are influencing the HILL-coefficient for the pyruvate binding.

The results of SCHWARTZ et al. (1968) with *E. coli* PDC are opposed by those of BISSWANGER and HENNING (1971), who found an increased HILL-coefficient for the pyruvate binding in the presence of CoASAc ($h = 2.8$) on assaying the overall activity. These authors also observed, under certain conditions, activation by CoASAc of inactivated isolated PDH. By the combined activating- and inhibitory effects of CoASAc (presumably by acting at different sites) negative cooperativity is produced in the kinetics under these conditions, which must be taken as evidence for the 'KOSHLAND' model (CONWAY and KOSHLAND, 1968).

PDC constitutes the link between glycolysis (or its physiological equivalent) and the citric acid cycle and apart from oxidation of the fatty acids, this provides the main pathway by which CoASAc can be generated from any metabolite.

Thus, PDC has a key-function and it is not surprising that its activity must be also controlled in a second line. The apparent cooperative effect, when the overall reaction is followed by varying CoA concentrations in the presence of phosphate, is due to a subtle mechanism of product inhibition by CoASAc and subsequent conversion of CoASAc into acetylphosphate; the combined responses of PDC and PTA to the [CoASAc]/[CoA] ratio is the origin of this phenomenon (cf. CHAPTER 3). Moreover, the *Azotobacter* PDC is shown to be inhibited by NADH, competitively with respect to NAD^+.

From the kinetic studies with the mammalian PDC's, which are regulated in the first line by phosphorylation and dephosphorylation, important differences with the bacterial complexes are obvious (BREMER, 1969; WIELAND et al., 1969b; KANZAKI et al., 1969 and TSAI et al., 1973). CoASAc inhibits the activity competitive with respect to CoA, viz. the target is the second enzyme of the complex now. Most values found for K_M and K_I agree fairly well with those published by GARLAND and RANDLE (1964), 7 and 13 μM respectively. Therefore, these PDC's are not very sensitive to a shift in the [CoASAc]/[CoA] ratio, while BLASS and LEWIS (1973) were not able at all to detect consistent inhibition by CoASAc on partially purified PDC from ox brain. NADH behaves as a strong inhibitor of the activity of the complex, competitive with respect to NAD^+; for the rat kidney complex K_M is 10 μM and K_I is 2 μM (BREMER, 1969). Both CoASAc and NADH are inhibiting un-competitively with respect to pyruvate (TSAI et al., 1973) and as pointed out by CLELAND (1973) these results are consistent with the derived rate equations when a three-site ping-pong mechanism accounts for the overall reaction. However, TSAI et al. (1973) have observed anomalous inhibition patterns (non-competitive rather than un-competitive), when respectively the NAD^+ concentration is varied in the presence of CoASAc or the CoA concentration in the presence of NADH. The explanation assumes these substances to influence protein-protein interactions between the transacetylase and lipoamide dehydrogenase after they are bound. Also of interest is the observation (TSAI et al., 1973) that pure lipoamide dehydrogenase is not inhibited by CoA, while the complexed flavoprotein is inhibited non-competitively by CoA with respect to NADH and lipoamide (cf. 4.2.5). With NAD^+ and dihydrolipoamide the same can be noticed. This may mean that indeed the flavoprotein is bound in the monomeric form in the complex (REED and OLIVER, 1968). In addition, for pure lipoamide dehydrogenase from pig heart (still containing a multiplicity of isoenzymes), VISSER (1970) has demonstrated that a ping-pong mechanism is not the correct model for the reaction and a ternary complex mechanism has been proposed instead.

The response of the *Azotobacter* PDC to variations in the energy charge is even more sharply than for the *E. coli* complex (ATKINSON, 1968; SHEN and ATKINSON, 1970) and according to a R-system. The same type of curves have been obtained for pyruvate kinase from *Azotobacter* (LIAO and ATKINSON, 1971) and they are shaped quite differently from those observed for the regulatory enzymes of glycolysis and citric acid cycle. The latter are constant over most of the energy charge range; at values above 0.8, the beginning of the physiological

range in a lot of organisms (CHAPMAN et al., 1971, they drop rather sharply. Regulation by energy charge of A*zotobacter* PDC occurs over a larger range, especially in the presence of the negative effector CoASAc. At high pyruvate concentrations the response to variations in the energy charge seems to be less sensitive, but inhibition is observed at a high value of the energy charge compared with the activity without added nucleotides.

5. ON THE INACTIVATION OF PDC FROM *AZOTOBACTER VINELANDII* BY SULFHYDRYL REAGENTS

5.1. INTRODUCTION

Purified PDC from *Azotobacter* has been shown to catalyze the CoA- and NAD^+-linked oxidative decarboxylation of pyruvate and the partial reactions characteristic for the compiling components. The involvement of protein-bound lipoic acid in the reaction sequence is therefore highly probable. Apart from the amount of lipoic acid present in the complex, the question remains to be solved how this lipoyl moiety participates in the overall reaction of a complex in which movement of the individual enzymes is probably restricted.

Possible mechanisms, operating either individually or in combination, are: One or more of the component-enzymes change in conformation during the reaction by which the bound prosthetic groups are brought into *juxtaposition*. The attached lipoyl moiety provides a flexible arm permitting rotation between the prosthetic groups of the other two enzymes. Interaction between several lipoyl moieties is necessary for acetyl- and electron transfer (KOIKE et al., 1963; REED and OLIVER, 1968). The latter proposal could account for the apparent excess bound lipoic acid generally found in PDC's (ELEY et al., 1972; BARRERA et al., 1972).

Active-site-directed inactivation- and labeling experiments thus may be helpful tools in the approach of these problems. However, reacting PDC contains several sulfhydryl groups as candidates for chemical modification. The PDH component of the PDC from *E. coli* is reported to contain at least eight sulfhydryl groups per 192,000–200,000 daltons (SCHWARTZ and REED, 1970b; VOGEL and HENNING, 1971; ELEY et al., 1972). Modification of two sulfhydryl groups per molecule of isolated '*dimer*', nearly completely destroys the activity, without affecting the state of aggregation or the ability to combine with the transacetylase (SCHWARTZ and REED, 1970b). Mg^{2+}.TPP partially protects the enzyme against inactivation and exposes two nonessential, until then, hidden sulfhydryl groups to modification while pyruvate exerts no influence.

Secondly, the LTA component of the *E. coli* complex which, according to the frequencies of amino acid residues per 70,000–80,000 daltons, essentially seems to be devoid of half-cystine (HENNEY et al., 1967; VOGEL et al., 1971: ELEY et al., 1972); however at each residue one molecule of lipoic acid is covalently bound resulting in one or two sulfhydryl groups during the overall reaction. This lipoic acid is reported not to be required in any of the partial reactions (KOIKE and REED, 1960). Thirdly lipoamide dehydrogenase; it has been reported that the enzymes from *E. coli* and pig heart contain a S-S bridge. A view supported by the isolation of an active centre peptide (WILLIAMS and ARSCOTT, 1972; BROWN and PERHAM, 1974) and consistent with the reaction scheme deduced from

sulfhydryl group titration data after reduction of the enzyme with NADH (MASSEY and VEEGER, 1960). Thus, it can be concluded that the action of sulfhydryl reagents on PDC may be very complex, as actually shown by MALDONADO et al. (1972) in their studies on *E. coli* PDC with bromopyruvate.

It is the aim of this CHAPTER to report on some data concerning the lipoyl-site-directed inactivation of the overall activity by sulphydryl reagents and the subsequent selective incorporation of a paramagnetic maleimide spinlabel. From these data, the amount of protein-bound lipoic acid per molecule FAD could be estimated in *Azotobacter* PDC. Part of these studies were performed in co-operation with DRS. H. J. GRANDE from our laboratory.

5.2. RESULTS

5.2.1. *Pyruvate-dependent inactivation of the overall reaction by sulfhydryl reagents and oxygen*

On accepting the proposed sequence for the overall reaction (REED and COX, 1966; CHAPTER 1), one sulfhydryl group, originating from the lipoyl moiety, will be generated during reaction of the complex with pyruvate plus Mg^{2+}.TPP in the absence of CoA. It thus might be expected that the subsequent addition of one of the relatively rapid alkylating agents NEM or bromopyruvate will result in an irreversible block and complete loss of the overall activity. In FIG. 5.1. this is illustrated and it can be seen that upon incubating PDC anaerobically in the presence of 0.1 mM pyruvate and 1 mM sulfhydryl reagent, the activity is completely destroyed within a few minutes. The process is dependent on Mg^{2+}.TPP, for without added Mg^{2+}.TPP the inactivation proceeds much slower. It has to be mentioned, that the pure complex still might contain a small amount of Mg^{2+}.TPP, tightly bound to it, for some overall activity can be measured when the enzyme, at the protein concentrations used, was assayed in the absence of Mg^{2+}.TPP.

Once inactivation has occurred, no reactivation can be achieved by adding 2 mM or higher concentration of DTT, indicating that the modified groups are not attainable for the action of this dithiol. In this respect it must be noticed, that unmodified PDC is not able to catalyze a DTT-dependent reduction of NAD^+ in the absence of externally added oxidized lipoic acid. In experiments in the absence of pyruvate, but in the presence of NEM, not more than 20% inactivation is observed after 30–40 min., probably due to reaction of NEM with other sulfhydryl groups than the ones specifically generated in the lipoyl moieties. Although somewhat slower, iodoacetic acid can be demonstrated (not shown) to react similarly as NEM in the presence and in the absence of pyruvate.

The reaction of PDC with bromopyruvate may occur as the result of attack on different groups affecting the activity, since this compound appreciably inactivates in a Mg^{2+}.TPP-dependent process. Moreover, especially during the early stages of the latter inactivation process, partial reactivation of the enzyme is observed now upon incubating the removed aliquot with DTT prior to the

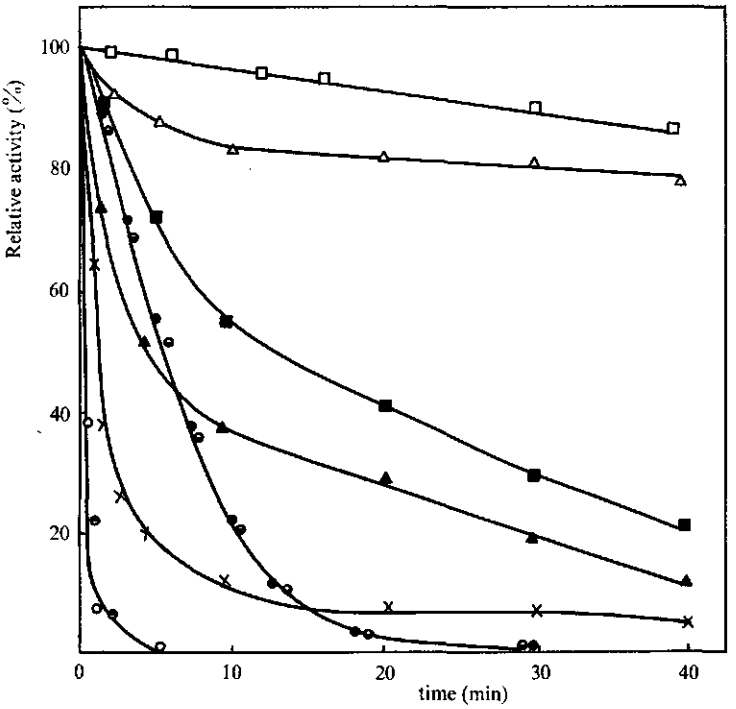


FIG. 5.1. Pyruvate-dependent inactivation of the overall reaction by sulfhydryl reagents. The incubations were anaerobically performed as described in METHODS. At the times indicated, 0.01 ml aliquots were withdrawn and assayed for overall activity.
Symbols: (○—○), complete mixture with NEM; (◓—◓), complete mixture with bromopyruvate; (●—●), without Mg^{2+}.TPP plus NEM; (◒—◒), without Mg^{2+}.TPP plus bromopyruvate; (×—×), bromopyruvate without pyruvate; (■—■), bromopyruvate without both pyruvate and Mg^{2+}.TPP; (▲—▲), bromopyruvate without pyruvate, while prior to the assay the removed aliquots were incubated for 10 min. with 2 mM DTT; (△—△), NEM without pyruvate; (□—□), without both sulfhydryl reagents and pyruvate.

assay. These results suggest the Mg^{2+}.TPP-dependent formation of some catalytically inactive complex between bromopyruvate and the enzyme before it irreversibly inactivates. The latter can involve alkylation of any of a number of susceptible groups in the proximity of the active site. Therefore bromopyruvate cannot be taken as active-site-directed inhibitor towards PDC (cf. MALDONADO et al., 1972).

It turned out (FIG. 5.2) that in the absence of a sulfhydryl reagent, but still in the presence of 0.1 mM pyruvate plus Mg^{2+}.TPP, aerobically also an inactivation is observed at slower rate. Within about 5 min. a final level of 70% of the original activity is reached. Omission of Mg^{2+}.TPP reduced the inactivation rate by 90% (not shown). At higher pyruvate concentrations the extend of inactivation becomes more complete. Exclusion of air largely prevents this inactivation process. No reactivation can be achieved by using DTT, but the

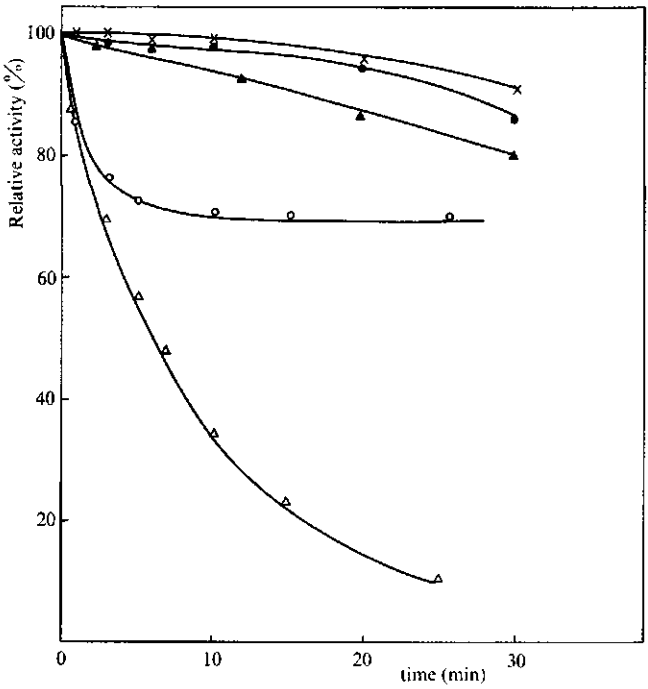


FIG. 5.2. Pyruvate-dependent inactivation of the overall reaction by oxygen. The incubations were performed as described in METHODS in the absence of sulfhydryl reagents, with the following pyruvate concentrations: (○—○), 0.1 mM aerobically; (△—△), 2 mM aerobically; (●—●), 0.1 mM anaerobically; (▲—▲), 2 mM anaerobically; (×—×), no pyruvate aerobically.

addition of 2 mM DTT when added at any stage during the inactivation prevents further inactivation.

5.2.2. *Pyruvate 'oxidase' activity of PDC from Azotobacter vinelandii*

During pyruvate plus Mg^{2+}.TPP-dependent inactivation of the overall reaction under aerobic conditions, disappearance of pyruvate with concomitant oxygen consumption can be demonstrated and addition of catalase after the reaction has stopped, causes a small increase in the final oxygen level due to hydrogen peroxide produced (FIG. 5.3). On the other hand, when catalase was present from the very start, more oxygen apparently is consumed under the conditions used and, after deproteination, an analysis of the concentration pyruvate left and acetate produced revealed that all pyruvate has been converted into acetate. Acetaldehyde is shown not to be produced. Under anaerobic conditions no pyruvate disappeared. From these data we concluded that this pyruvate '*oxidase*' (partial) activity catalyzes:

$$pyruvate + O_2 + H_2O \xrightarrow{Mg^{2+}\,.\,TPP} H_2O_2 + CO_2 + acetate$$

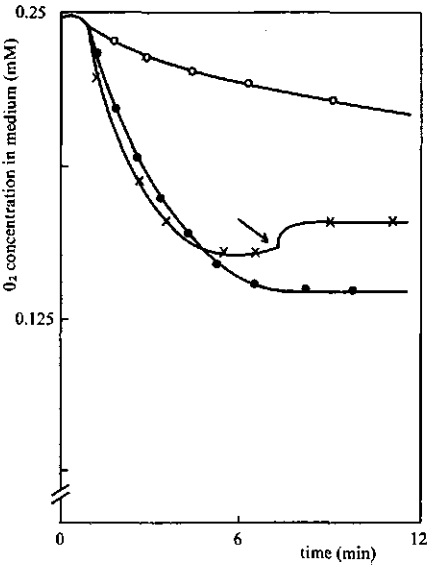


FIG. 5.3. The oxidation of pyruvate by oxygen. The oxygen consumption was measured as described in METHODS in a medium of 0.05 M Tris-HCl (pH 7.1) containing 0.1 mM TPP, 5 mM mg^{2+}, 100 units catalase and 0.26 mM pyruvate. The reaction was started by the addition of PDC (final protein concentration 0.75 mg ml^{-1}).
When the oxygen consumption has stopped the solution was deproteinated by adding $HClO_4$ (5% final concentration) with subsequent centrifugation and after neuterization (solid Tris-base) the concentrations pyruvate left and acetate produced were measured according to METHODS.
Symbols: (●—●), complete; (×—×), catalase only added at the arrow; (○—○), without TPP.

Thus, in the presence of catalase, the stoechiometry of the reaction must be:

$$2\ pyruvate + 2O_2 + 2H_2O \xrightarrow[catalase]{Mg^{2+}.\ TPP} 2H_2O_2 + 2CO_2 + 2\ acetate$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Sum:

$$2\ pyruvate + O_2 \xrightarrow{Mg^{2+}.\ TPP,\ catalase} 2CO_2 + 2\ acetate$$

In agreement with this prediction, oxygen consumption stops at about the proper concentration. In the absence of catalase, the produced peroxide will co-oxidize pyruvate, nonenzymatically, according to:

$$H_2O_2 + pyruvate \longrightarrow CO_2 + acetate + H_2O$$

The rate of the latter well-known reaction is greatly enhanced by the presence of Mg^{2+}. Consequently, nearly the same stoechiometry would be expected.

In view of the following observations, the PDH component of the complex seems to be responsible for the direct (Mg^{2+}.TPP-dependent) oxidation of pyruvate with oxygen as electron acceptor.

1. FIG. 5.4 shows that the reciprocal plot derived from a curve of v_0 versus pyruvate shows a linear dependence, viz. pyruvate does not exert a positive homotropic effect (cf. CHAPTER 4). V is found to be 0.2 μmoles O_2 $min.^{-1}mg^{-1}$ and K_M for pyruvate is 0.3 mM. These values were both comparable with those found by the ferricyanide-dependent oxidation of pyruvate as catalyzed by the PDH-component.
2. Added ferricyanide (1 mM) halves the '*oxidase*' reaction rate by competing for the electrons.
3. CoASAc inhibits the '*oxidase*' competitively with respect to pyruvate and the positive effectors AMP and sulphate, which are stimulating the '*oxidase*' activity at low pyruvate concentrations (pH 7.1), were also active in reversing the inhibition by CoASAc. At about pH 8.5, AMP (0.2 mM) also enhances V by 50% (cf. CHAPTER 4).
4. Participation of flavin in this reaction can be largely ruled out, since under anaerobic conditions no quenching of its fluorescence has been observed

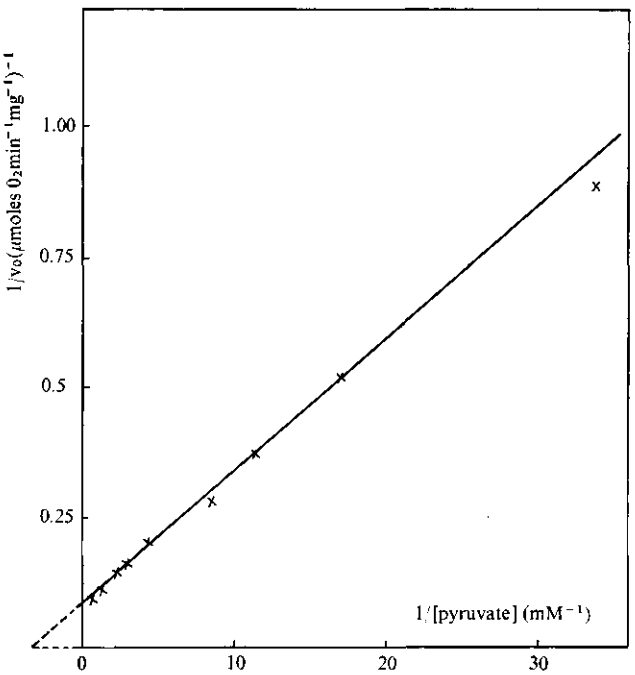


FIG. 5.4. Reciprocal plot of the dependence of PDC '*oxidase*' velocity on the pyruvate concentration. The oxygen consumption was measured according to METHODS in a medium of 0.05 M Tris-HCl (pH 7.4) containing 0.1 mM Mg^{2+}.TPP, 100 units catalase and pyruvate as indicated. The reaction was started by adding the enzyme.

upon incubation of PDC with pyruvate plus Mg^{2+}.TPP in the absence of CoA. Arsenite (10 mM) is not inhibitory.

Finally, it is worth mentioning that JAGANNATHAN and SCHWEET (1952) with PDC isolated from pigeon breast and GOLDMAN (1958) with PDC from *Mycobacterium tuberculosis* also have observed the oxidation of pyruvate with oxygen as electron acceptor.

5.2.3. *Behaviour of the partial activities during inactivation*

In an attempt to provide evidence for the target(s) of the inactivation of the overall reaction by either sulfhydryl reagents or oxygen, the behaviour of the partial reactions was investigated. It can be seen from TABLE 5.1, that the inactivation of the overall reaction by the combined action of NEM and pyruvate is not accompanied by a comparable drop in the activity of one of the compiling enzymes, suggesting mainly an attack of the inhibitor at the protein-bound lipoyl moiety. This compound is reported not to be essential for or to be required for the partial reactions of PDC from *E. coli* (KOIKE and REED, 1960) and its involvement in the '*oxidase*' activity, exerted by PDC from *Azotobacter*, can be ruled out. On the other hand, bromopyruvate decreases the overall activity in a more complex way as can be concluded from the residual activities of PDH and the '*oxidase*' after incubation with bromopyruvate in the absence of pyruvate. The presence of pyruvate rather seems to protect the latter activities from inactivation and therefore it appears likely that bromopyruvate also reacts via the normal sequence of steps of the substrate. Partially inactivated '*oxidase*' (by bromopyruvate) reactivates for about 50% upon addition of 2 mM DTT (data not shown; see also FIG. 5.1). By action of bromopyruvate upon the lipoamide dehydrogenase, this activity is about 25–30% reduced, independently of the presence of pyruvate.

TABLE 5.1. Effects of NEM, bromopyruvate and oxygen on different activities of the PDC. PDC was aerobically incubated under the conditions as described in METHODS without stirring. The pyruvate concentration was increased to 2 mM. The activities of the partial reactions were assayed as described in METHODS.

	Percentage of residual activity									
	+NEM +pyruvate		+bromo-pyruvate +pyruvate		−sulfhydryl reagents +pyruvate		+NEM −pyruvate		+bromo-pyruvate −pyruvate	
	1 min.	10 min.	1 min.	10 min.	1 min.	10 min.	1 min.	10 min.	1 min.	10 min.
PDC	7	0	10	0	85	35	95	85	56	15
PDH	95	90	71	35	97	80	98	85	50	20
pyruvate '*oxidase*'	98	97	75	32	98	82	98	88	52	15
LTA	93	96	95	93	98	96	95	95	93	95
lipoamide dehydrogenase	99	93	92	75	100	99	99	94	91	72

Furthermore, these experiments show that pyruvate (in the presence of Mg^{2+}.TPP) makes PDC from *Azotobacter* sensitive to inactivation by oxygen without reducing the activities of the individual components. The above data plus our observation that catalase does not influence the pyruvate-dependent inactivation of the overall activity by oxygen, indicate that the '*oxidase*' activity by itself is not responsible for this phenomenon. In view of the observed pyruvate accumulation when *Azotobacter* grows in the presence of excess oxygen (DILWORTH, 1962), PDC seems to be equally sensitive to oxygen in the intact cell.

5.2.4. *Reduced pyridine nucleotide-dependent inactivation of the overall reaction by NEM*

Reduced, protein-bound, lipoic acid generated after transacetylation is reoxidized by transfer of the reducing equivalents to the disulfide and FAD of lipoamide dehydrogenase. Stopped-flow studies with pig heart lipoamide dehydrogenase (VEEGER et al., 1972b) have shown that the two-equivalent-reduced enzyme-NAD^+ complex rather than the red-intermediate (also two-equivalent reduced but without NAD^+ bound to the enzyme), which was initially proposed as such (MASSEY et al., 1960), is the catalytically active intermediate in the reduction of NAD^+ by reduced lipoamide. The way of reduction of pure oxidized lipoamide dehydrogenase from *Azotobacter* by reduced lipoamide or NADH differs slightly from the pattern observed with the pig heart enzyme (VEEGER et al., 1972a); with NADH mainly the reduced enzyme-NAD^+ complex is formed, with reduced lipoamide the red intermediate.

Thus, attempts were made to reduce the protein-bound lipoyl moiety via the reduced flavoprotein and to block the overall reaction irreversibly by subsequent reaction with NEM. In TABLE 5.2 data are listed about the inactivation of the overall activity upon incubation of PDC with NEM and NADH and compared with the lipoamide dehydrogenase activity. The other partial reactions remain unaffected (data not shown). It can be seen that the overall activity has become

TABLE 5.2. Effect of NEM in the presence of NADH on both the overall- and the lipoamide dehydrogenase activity.
PDC was anaerobically incubated under the conditions as described in METHODS. Between the first and second addition 10 sec. passed.

	Percentage of residual activity			
	overall reaction		lipoamide dehydrogenase	
addition (order)	1 min.	10 min.	1 min.	10 min.
NEM + NADH	10	0	88	60
NADH + NEM	88	50	98	92
NEM — NADH	93	85	99	94
NADH — NEM	100	98	100	98

zero, when the lipoamide dehydrogenase activity is still high. Addition of NAD^+ (0.1 mM) does not influence the rate of the inactivation-process. The sequence of the additions is important, since addition of NEM after that of NADH does not lead to a higher degree of inactivation compared with the effect of NEM alone (cf. DISCUSSION).

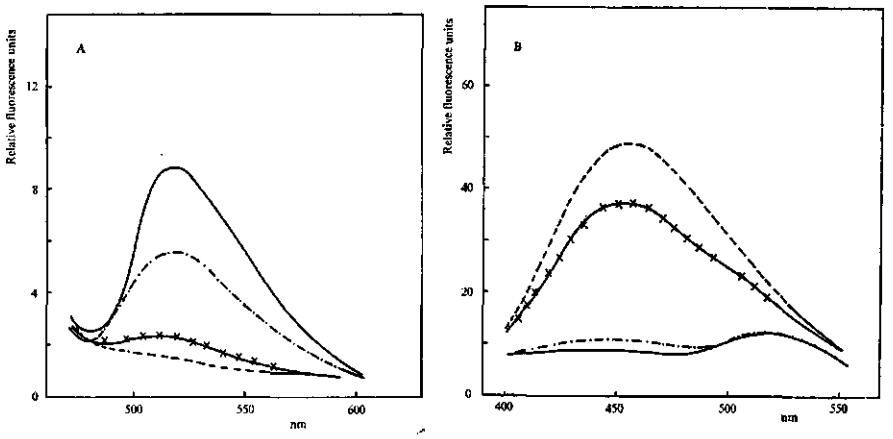


FIG. 5.5. Quenching of the FAD fluorescence of PDC by the addition of NADPH under anaerobic conditions. Enzyme 2.5 mg ml^{-1}; NADPH 0.1 mM in 0.05 M tris-HCl (pH 7.1); temperature 25°. Excitation wavelengths: (A) 460 nm, (B) 360 nm. (A) after air had been admitted, the FAD fluorescence intensity increases; (B) the NADPH fluorescence decreases. Symbols: (———), no NADPH added; (— — —), NADPH present; (+ — +), 2 min. after air-admission; (●—●), 5 min. after air-admission.

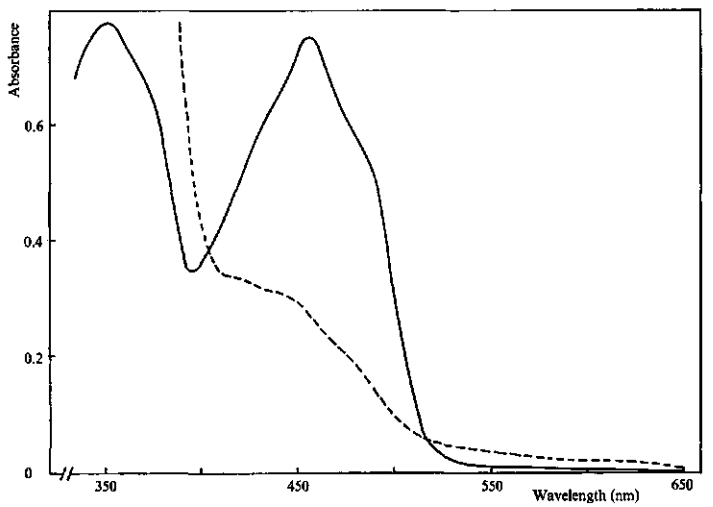
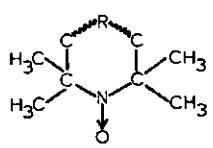


FIG. 5.6. Absorption spectrum of *Azotobacter* lipoamide dehydrogenase, before and after reduction with 1 mM NADPH under aerobic conditions. The enzyme is dissolved in 0.05 M phosphate buffer (pH 7.0) at an enzyme-FAD concentration of 70 μM. Temperature 20°.

Surprisingly the same results are obtained by using NADPH instead of NADH; in contrast with the pig heart enzyme, the FAD of the *Azotobacter* lipoamide dehydrogenase can be reduced by NADPH, either when the enzyme is in the pure state or bound in the PDC. In FIG. 5.5 this is demonstrated by showing the total quenching of the FAD fluorescence from PDC upon addition of NADPH under anaerobic conditions. After air was admitted a relatively slow reoxidation can be observed from the increase of its fluorescence emission with concomitant decrease in the NADPH fluorescence. In addition, FIG. 5.6 shows the visible absorption spectrum of pure lipoamide dehydrogenase from *Azotobacter*, before and after reduction with 1 mM NADPH under aerobic conditions. NEM does not exert any influence on the visible absorption spectrum in the presence or absence of either NADH or NADPH. Attempts to use $NADP^+$ as an electron acceptor in the overall reaction or to use NADPH as an electron donor in the lipoamide dehydrogenase catalyzed reduction of oxidized lipoate or DCIP failed. Thus only oxygen can be used as electron acceptor with NADPH as donor.

5.2.5. *Selective introduction of maleimide spin label in PDC*

The previous data concerning the pyruvate- and NAD(P)H-dependent inactivation of the overall reaction by NEM, without influencing the partial reactions, indicates that the protein-bound lipoyl moiety is the target. To obtain evidence about the involvement of this lipoyl moiety and its content, the paramagnetic maleimide spin label (*N-(1-oxyl-2,2,5,5-tetra methyl-3-pyrrolidinyl) maleimide*) was used. Like most spin labels, the maleimide spin label is based on the nitroxide radical, having the general formula:



The odd-electron is localized almost entirely on the nitrogen and oxygen atoms; the four methyl groups attached to the tertiary carbon atoms are necessary to decrease the chemical reactivity of the free radical and the group *R* (maleimide) serves to direct the free radical to the appropriate target (HALMILTON and MCCONNELL, 1968; MCCONNELL and MCFARLAND, 1970).

The maleimide spin label behaves like NEM in the specific inactivation of the overall activity upon incubation in the presence of either pyruvate or NAD(P)H. In FIG. 5.7, the EPR spectrum of the unbound, freely rotating label is shown. Three lines of equal amplitude due to the hyperfine interaction with the nitrogen nucleus are visible. It is known that the EPR spectrum of the nitroxide radical is strongly dependent on its rotational motion, so a completely immobilized species can be expected upon binding to a large complex like PDC. A feature of such a spectrum is the appearance of broad lines with different amplitudes and an increase in distance (gauss) between the two outer peaks. In a control experiment pure PDC was incubated at room temperature with excess spin label (6

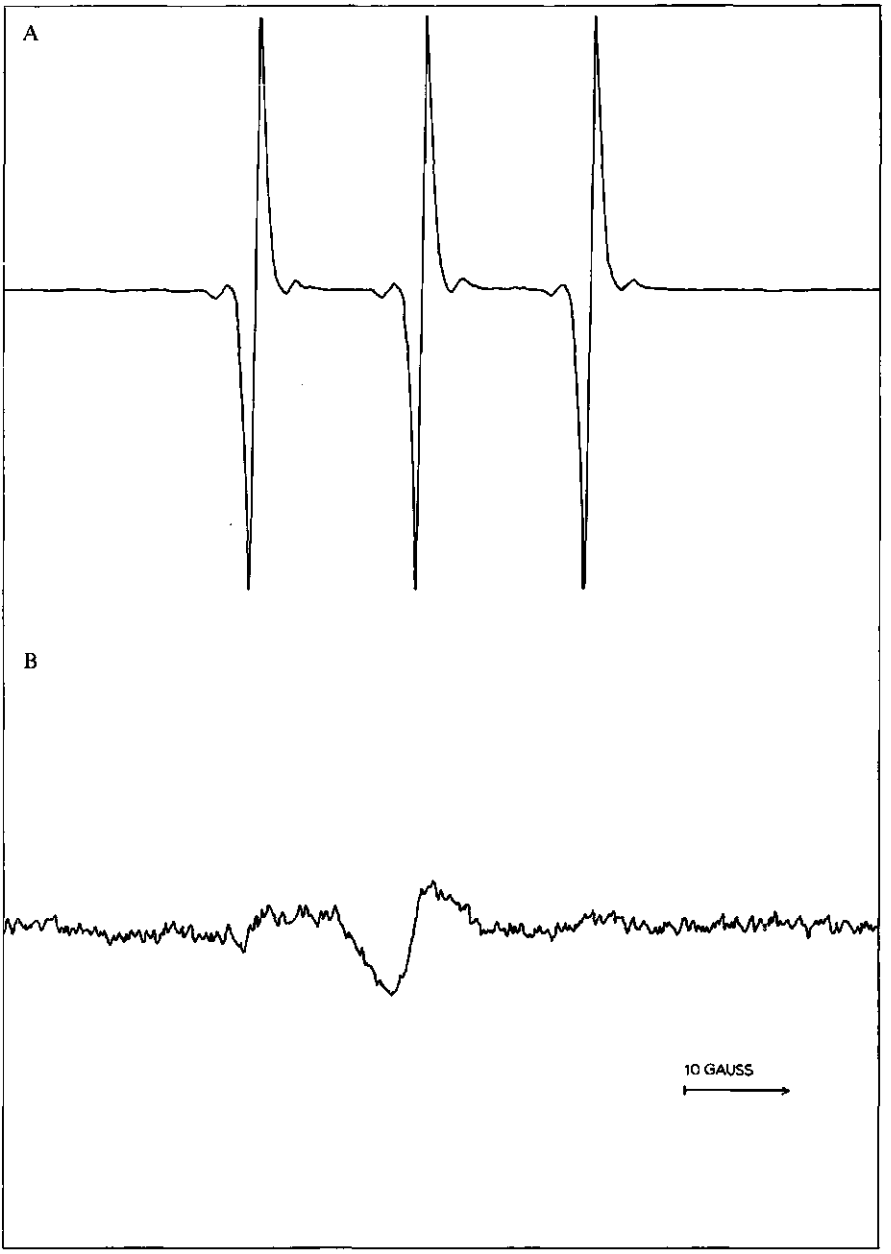


FIG. 5.7. EPR spectra of the maleimide spin label. (A) 27 μM label in 0.05 M Tris-HCl (pH 7.1). (B) the label aspecifically bound to PDC (protein concentration 3 mg ml^{-1}). Binding is the result of incubating 9 mg PDC with 90 nmoles spin label in 0.05 M Tris-HCl (pH 7.1) in a volume of 0.3 ml during 3 min. at 20°. The reaction was stopped by eluting the mixture through a Sephadex G25 column with 0.05 M Tris-HCl (pH 7.1).
EPR conditions: temperature, 20°; scanning rate, 6 gauss min^{-1}; time constant, 3 sec.; frequency, 9458 GHz; modulation amplitude, 1 gauss; gain, (A) 10^5 (B) 10^6.

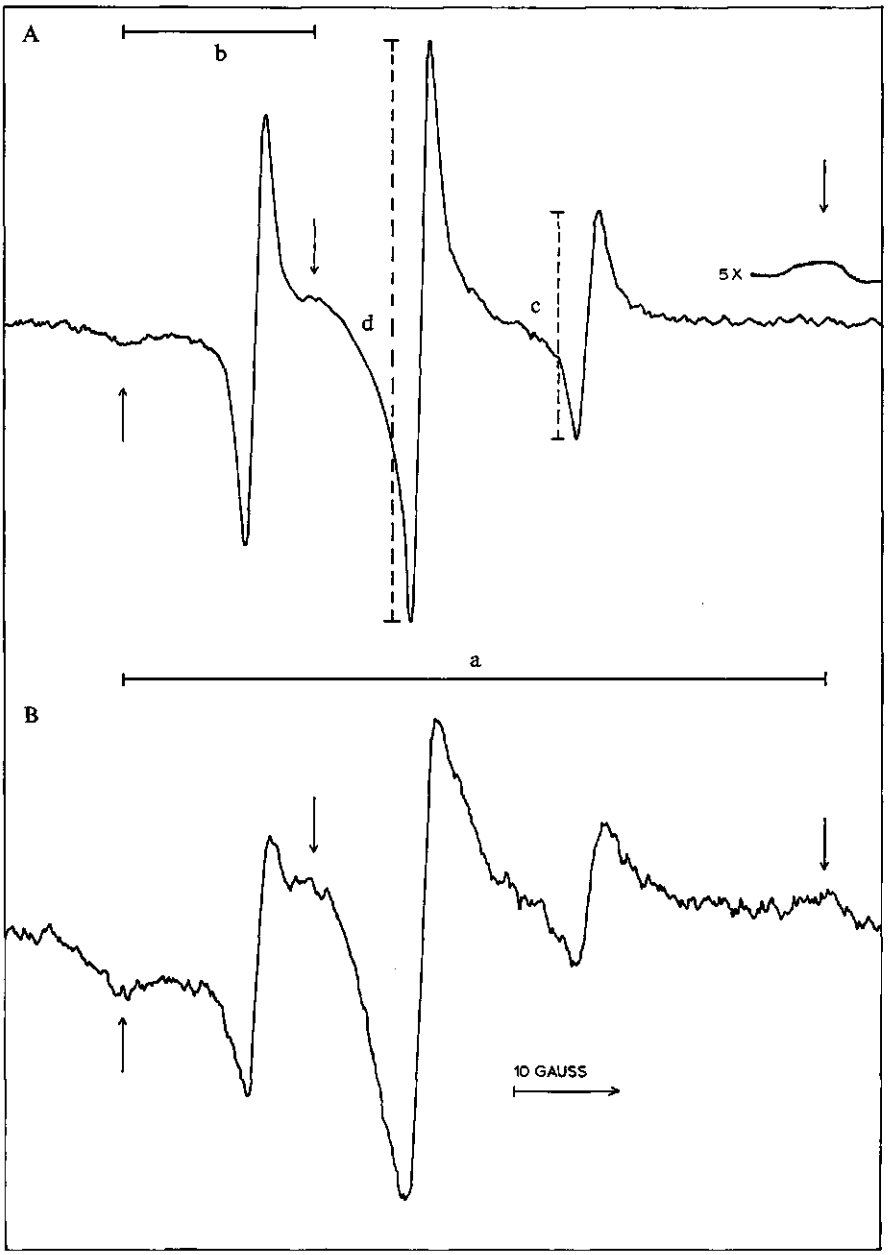


FIG. 5.8. EPR spectra of labeled PDC. (A) labeled as described under FIG. 5.7. in the presence of 0.1 mM pyruvate plus 0.1 mM Mg^{2+}.TPP. (B) labeled in the presence of 0.1 mM NADH; this reaction was started by the addition of NADH.

moles of the label per mole FAD) in the absence of either pyruvate plus Mg^{2+}.TPP or of NAD(P)H. The reaction was stopped by pouring the mixture on a Sephadex G25 column. Such a control experiment was essential in order to have an impression about amount and properties of aspecific incorporation. The EPR spectrum of the eluted protein fraction (FIG. 5.7) shows a superposition of semi-mobilized and immobilized components. The short reaction period of 3 min. is necessary in order to prevent labeling of ε-NH_2 groups and hydrolysis of the spin label (BOEYENS and MCCONNELL, 1966).

Labeling of PDC in the presence of pyruvate plus Mg^{2+}.TPP leads to much more incorporation (FIG. 5.8). Although this spectrum shows some immobile species present, mainly a semi-mobile species is visible. Its mobility is underlined by the calculated rotational correlation time as derived from the ratio c/d according to the semi-empirical method of MENSCH and MEIER; τ_c is 0.5 nsec. Further analysis of the spectrum shows a small amount of, at least, two minor components to be present, strongly immobilized. Their τ_c's were determined to be respectively 30 and 75 nsec. from the distances a and b (MENSCH and MEIER; GRANDE et al., 1972). The latter values are not accurate because of the presence of the semi-mobile species at about the same field.

On the other hand, labeling of the PDC followed by reduction with NAD(P)H results even more clearly in a superimposed spectrum (FIG. 5.8). Much more of the immobilized species is observed with respect to the semi-mobile one, which makes accurate determination of τ_c more difficult (τ_c is 1.1 nsec.). The τ_c's of the immobilized components were found to be 30 and 70 nsec. indicating that the same groups are labeled. In the presence of NAD(P)H less of the label appeared to be bound in the semi-mobile configuration compared with the amount incorporated in the presence of pyruvate during an incubation period of 3 min. This can be concluded from the differences in gain used for recording of the spectra in FIG. 5.8.

From the amount of maleimide spin label incorporated during pyruvate-dependent reaction in a known quantity of PDC, it can be estimated that two molecules of the semi-mobile label are bound per molecule FAD, independent of aerobic or anaerobic conditions during the experiment. The latter favours the conclusion that in the presence of pyruvate the inactivation of PDC with NEM or maleimide spin label is much faster than the inactivation with oxygen (cf. 5.2.1 and 5.2.3).

Moreover, from spin-spin interactions between two unlike spin labels (for instance the maleimide spin label and Mn^{2+}), producing pertubation of the line width of the resonance lines of the nitroxide radical, it is possible to calculate their distance. Unfortunately, addition of either Mn^{2+}.TPP (0.1 mM) or free Mn^{2+} (0.1 mM) to the labeled PDC failed to influence the amplitude or width of the signals. Therefore, it can be concluded that the binding- site of these cofactors to the PDH component of the complex (cf. CHAPTER 4) is not within about 2 nm from the maleimide label. Only addition of pyruvate, in the presence of either Mn^{2+}.TPP or of Mg^{2+}.TPP, decreases slowly the amplitude (50% in 30 min.) of the semi-mobile species, without influencing its mobility.

The latter observation excludes a conformational change upon pyruvate-binding as a possible explanation for this phenomenon. Presumably, the pyruvate-Mg^{2+}.TPP-enzyme complex or some derivative chemically reduces the bound nitroxide label with consequently a loss of paramagnetism.

5.3. Discussion

From the pyruvate plus Mg^{2+}.TPP-dependent inactivation of the overall activity (and not the partial activities) by NEM and subsequently the incorporation of a spin label, which nearly completely conserves its rotational mobility, it can be concluded that *Azotobacter* PDC, like PDC from *E. coli*, also contains an apparent excess of two lipoyl moieties per molecule FAD (Eley et al., 1972). For mammalian PDC this excess has been reported even to be larger (Barrera et al., 1972).

The extreme mobility of the lipoyl moieties as concluded from the spin label experiments is surprising. From the fact that, contrary to unbound lipoic acid (Cleland, 1964), the protein-bound lipoic acid cannot be reduced by DTT (no DTT-dependent reduction of NAD^+ could be measured), one could conclude that they were hidden, at least in the oxidized form. Another explanation is that the redox potential and/or the polarizability of the protein-bound lipoyl moieties are lower than of the unbound couple and therefore attempts to reduce them with DTT were not successful. The idea that the lipoyl moiety, provides a flexible arm of 1.4 nm which permits rotation between the prosthetic groups of the two other enzymes and is driven by the change in net charge on it (-lipS_2 $\rightarrow$ -lip-(S^-)-S-acetyl $\rightarrow$ -lip-(S^-)$_2$), is supported by the following observations: In PDC from *E. coli*, the activities of PDH, LTA and lipoamide dehydrogenase are unaffected by enzymatic removal of the lipoyl moieties (Koike and Reed, 1960). The equality of the line widths of the unbound spin label with those of the semi-mobile (protein-bound) label strongly suggests that indeed the lipoyl moieties are located hydrophilically (Lassmann et al., 1973). The absence of spin-spin interactions, when Mn^{2+} is bound to the PDH component of the spin-labeled PDC, suggests their distance to be at least 2 nm. Thus the proposed length of the flexible arm alone, will be not enough to account for the catalysis. Therefore additional factors i.e. conformational changes must be involved (cf. 5.2.4).

The extreme mobility of the lipoyl moieties, together with the apparent excess of them, probably is responsible for the pyruvate plus Mg^{2+}.TPP-dependent inactivation of the overall activity by oxygen. S-S bridge-formation due to oxidation of two protein-bound lip(SH)-S-acetyl species can be imagined to occur. Like protein-bound oxidized lipoic acid, such a S-S bridge could be chemically insensitive for the action of DTT. Alternatively, in view of the results recently obtained by Stokes and Stumpf (1974) who investigated the nonenzymatic acylation of DTT by CoASAc, reoxidation of protein-bound lip(SH)-S-acetyl with consequently transacetylation by migration of the acetyl group to any

other vicinal group that is exclusively important to or is participating in the overall reaction, can also account for this irreversible inactivation.

Although the mechanism of inactivation may differ, still another report about inactivation of an enzyme by pyruvate plus oxygen exists; the '*clastic*' pyruvate dehydrogenase from the strictly anaerobic bacterium *Peptostreptococcus elsdenii* (PEEL and WATKINSON, 1965). The complete inactivation of pyruvate formate-lyase from *E. coli* without pyruvate occurs aerobically (HENNING, 1963; KNAPPE et al., 1969) and this control of the activity by oxygen could explain inhibiton- or delay of growth on shifting the growing organism from anaerobic to aerobic conditions. Growth of the strictly aerobic *Azotobacter* is inhibited at high oxygen tensions. This is widely assumed to be related to the known oxygen sensitivity of the nitrogenase (DALTON and POSTGATE, 1969) and not to for instance inactivation of PDC. However, responses to excessive aeration are also found with other, not nitrogen-fixing, aerobes (cf. HARRISON, 1972) and therefore a simplified view cannot usefully be applied.

The distinct hyperbolic response on varying the pyruvate concentrations of the '*oxidase*' activity, catalyzed by the PDH component of PDC from *Azotobacter*, strengthens our doubts about the significance of the HILL-coefficient derived from the kinetics using ferricyanide as electron acceptor (cf. 4.2.2).

The partial reactivation with DTT of both the '*oxidase*'- and PDH activities after Mg^{2+}.TPP-dependent inactivation by bromopyruvate in the absence of pyruvate, especially in the early stages of the process, suggests involvement of at least two effects. Prior to irreversible inactivation by bromopyruvate of PDC, PDH and '*oxidase*', we suggest interaction between the Mg^{2+}.TPP-bromopyruvate-enzyme complex and another (probably sulfhydryl) group, a view acceptable from our pH-dependent kinetics (cf. 4.2.3 and see also CHAPTER 6). Contrary to the results of MALDONADO et al. (1972) with *E. coli* PDC, no significant Mg^{2+}.TPP-independent inactivation by bromopyruvate has been observed.

Pure *Azotobacter* lipoamide dehydrogenase forms a two-equivalent-reduced enzyme-NAD^+ complex in the presence of excess NADH (VEEGER et al., 1972a) and from the absorption spectrum presented, it is clear that addition of excess NADPH (over FAD) leads to a two-equivalent-reduced enzyme as may be concluded from the changes in the 400–500 nm region and the presence of a flat band beyond 500 nm. NEM does not influence its development. This two-equivalent-reduced form is shown to be operative in reducing lipoyl moieties of the transacetylase component in the whole complex as illustrated by the appearance of semi-mobile species after incubation of PDC with the spin label and NAD(P)H. However, less label appeared to be bound compared with the amount incorporated in the presence of pyruvate. The importance of the sequence of addition of NEM and NAD(P)H for both labeling and accomplishment of inactivation of the overall activity, indicates that in the period (10 sec.) between reduction of the flavoprotein in the complex by NAD(P)H and addition of the sulfhydryl reagent, a rapid conformational change must proceed. The EPR spectra of pure lipoamide dehydrogenase from either pig heart or from

Azotobacter, after they were labeled under the conditions used, were showing the presence of some strongly immobile species (GRANDE et al., 1972; GRANDE, personal communication). This is to be expected from the report of MATTHEWS and WILLIAMS (1973) about the existence of ten half cystines per mole of enzyme-bound FAD in pig heart lipoamide dehydrogenase. Finally, against functioning of the two-equivalent-reduced form of lipoamide dehydrogenase in labeling the lipoyl moieties argues the observation of STEIN and STEIN (personal communication, 1972), that the two-equivalent-reduced enzyme from pig heart is not stable in the presence of the sulfhydryl reagent iodoacetamide.

6. PYRUVATE DEHYDROGENASE COMPLEX FROM *AZOTOBACTER VINELANDII*

6.1. COMPOSITION OF PDC FROM *AZOTOBACTER VINELANDII*

The results described in the previous CHAPTERS indicate, that the composition, the size and the shape of catalytically active PDC from *Azotobacter vinelandii* differs from similar multi-enzyme complexes isolated from either mammals or from *E. coli* (BARRERA et al., 1972; ELEY et al., 1972; VOGEL et al., 1972a,b). It is not certain yet whether the PTA, that has been found closely associated with PDC from *Azotobacter*, can be called part of the complex or is in fact a purification-artefact. On the other hand, we were not able to isolate active PDC completely devoid of the latter activity (cf. CHAPTER 3). In accordance, SDS-gel electrophoresis shows the existence of four major bands. Until conclusive evidence will be presented, this has to be kept in mind in proposing any model about the composition of this complex.

In an extension of the studies presented in this thesis, Drs. R. DE ABREU of our laboratory (personal communication) recently estimated that these SDS-bands have molecular weights of 90,000; 80,000; 56,000 and 50,000 daltons and in view of the data from the literature (VOGEL et al., 1972b) they probably correspond to PDH, LTA, lipoamide dehydrogenase and PTA respectively. Furthermore, interpretation of the flavin content per mg protein points at a minimal molecular weight of 550,000–620,000 daltons and the latter value agrees with that found for a catalytically-inactive subunit. Data from both the light-scattering- and the sedimentation-diffusion experiments (CHAPTER 3) indicate, that the molecular weight of the '*pure*' active enzyme-complex is about 1,000,000–1,200,000 daltons. In addition, the spin labeling experiments (CHAPTER 5) show that *Azotobacter* PDC contains two lipoyl moieties per molecule of FAD. The mobility of the labeled lipoyl moieties is consistent with an open structure.

From the above arguments and the fact that the typical structures, as observed in the electron micrographs of PDC from *E. coli*, are not present, it appears that the models concerning the arrangement of the compiling components in the enzyme-complex as designed by REED and OLIVER (1968) will have to be revised for the *Azotobacter* complex. Therefore, we conclude that the minimal composition of the pure, intact, *Azotobacter* complex is: Four identical LTA units (M = 80,000 daltons), each containing one molecule of lipoic acid covalently bound, are distributed around one **dimeric** lipoamide dehydrogenase (M = 112,000 daltons) in non-covalent interaction. Next, from these data we calculate that six to eight identical PDH units (M = 90,000 daltons) and consequently four to zero PTA units (M = 50,000 daltons) are joined to this sub-complex in a regular manner (non-covalently). It is possible that the intact complex in crude extracts contains eight PDH units and no PTA and that some

PDH is removed upon purification (cf. VOGEL et al., 1972a,b). The vacant positions were filled up with PTA, thus producing a stable and active PDC containing one enzyme more than that of *E. coli*.

The suggestion of REED and OLIVER (1968), that within the complex lipoamide dehydrogenase is bound in a monomeric form, conflicts with the following observations:

1. Lipoamide dehydrogenase purified from PDC of different sources is a dimer (cf. VAN DEN BROEK, 1971).
2. Fluorescence- and spectral characteristics are identical in either free- or complexed lipoamide dehydrogenase from *Azotobacter*; cf. its reduction by NAD(P)H (CHAPTERS 3 and 5; VEEGER et al., 1972a).
3. Monomeric lipoamide dehydrogenase from pig heart is not a physiological active species (VAN MUISWINKEL-VOETBERG, 1972).

Concerning the amount of PDH in the PDC from *Azotobacter*, Mn^{2+}-titration data (Drs. H. J. GRANDE, personal communication) indicate that eight-ten ions of Mn^{2+} are tightly bound per molecule pure complex. Two of these ions are bound at least an order of magnitude stronger than the other ions. The latter six-eight ions have about the same affinity for the complex. Although not proving, these data suggest that six-eight units of PDH are present in intact PDC. Until now, no role has been attributed to the strongly bound metal ions. The ideas are summarized in FIG. 6.1.

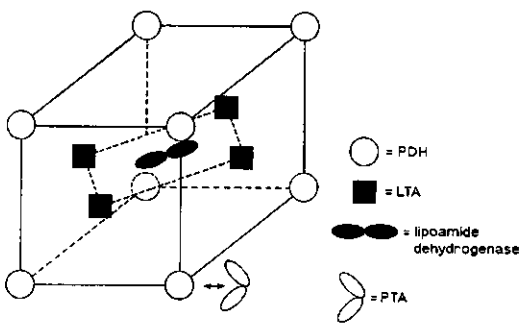


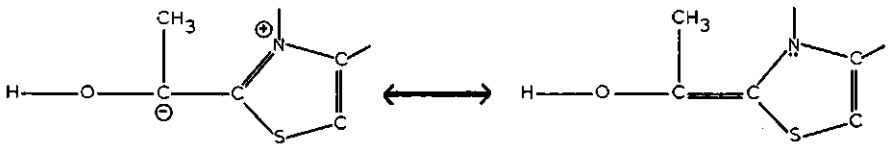
FIG. 6.1.

6.2. REACTION-SEQUENCE DURING THE FIRST STEP(S)

The reaction-sequence in pyruvate oxidation by PDC, according to REED and COX (1966), has been given in CHAPTER 1. For mammalian PDC the overall reaction is assumed to proceed via a ping-pong mechanism (CLELAND, 1963, 1973; TSAI et al., 1973), which is rather unlikely for the bacterial PDC's where sigmoidal kinetics with respect to variations in the pyruvate concentration have been observed (SCHWARTZ et al., 1968; BISSWANGER and HENNING, 1971; CHAPTER 4 and section 6.3).

Since BRESLOW (1957) demonstrated the occurrence of carbanion formation in thiazolium salts and HOLZER et al. (1960) isolated α-hydroxyethylTPP after

incubation of TPP with pyruvate and pyruvate oxidase from yeast, the events during the first step(s) of the overall reaction are accepted to involve: ionization of PDH-Mg^{2+}.TPP to the carbanion and to this form the carbonyl group of pyruvate is added. After decarboxylation the following crucial carbanion-enamine intermediate is formed after rearrangements,



Nucleophilic attack of this compound by protein-bound oxidized lipoic acid, producing an energy-rich thiol ester of reduced lipoic acid, is visualised to complete the first step(s) (cf. JENCKS, 1969). SANADI (1963) proposed – without experimental evidence – that the ferricyanide-linked PDH reaction proceeds by oxidation of the protonated carbanion intermediate (α-hydroxyethylTPP) to the acyl-compound, which hydrolyses.

Our data permit an extension of these views. From the pH-influence on the rate of the overall reaction in relation to the pyruvate concentration (CHAPTER 4) it can be concluded that either a (unknown) group ($pK = 8.0$) is developed during catalysis or that this group is involved in catalysis without being developed. In the latter case, before pyruvate-binding, this group must be located at a site remote from the active centre. This unknown group is part of the PDC-pyruvate complex and is fully active only in the protonated form; deprotonation, caused by increase of pH, leads to diminished activity. Moreover, the positive effectors (AMP and sulphate) influence deprotonation after they are bound as can be concluded from the distinct shift in pK_2^{ES} observed in both the $pS_{0.5}$- and log V versus pH curves. With the observation, that these positive effectors are (at pH 8.5) equally enhancing V of both, the ferricyanide- and the oxygen-linked PDH activities, it is suggested that the unknown group (*-XH*) must be located in the first enzyme of the PDC (CHAPTERS 4 and 5).

The group (*-XH*) does not react readily with NEM or iodoacetate, but it would be the first target for Mg^{2+}.TPP-dependent inactivation of PDC, PDH and the '*oxidase*' by bromopyruvate. Likely candidates for *-XH* are sulfhydryl- and histidine groups. Although the evidence is not conclusive yet, the partial reactivation upon addition of DTT after inactivation by bromopyruvate suggests the involvement of a sulfhydryl group in the overall activity and the PDH-catalyzed partial reactions. As pointed out in CHAPTER 5, the pyruvate-dependent inactivation of the overall reaction in the presence of oxygen is not exerted at this level. Furthermore, inhibition of the overall and PDH activities by CoASAc, competitive with respect to Mg^{2+}.TPP-pyruvate, could be due to the induction of deprotonation of *-XH*, either directly or indirectly, after the CoASAc is bound.

The mechanism of participation in catalysis of *-XH* is tentatively represented in FIG. 6.2. The Mg^{2+}.TPP-acyl compound easily hydrolyses, thus producing

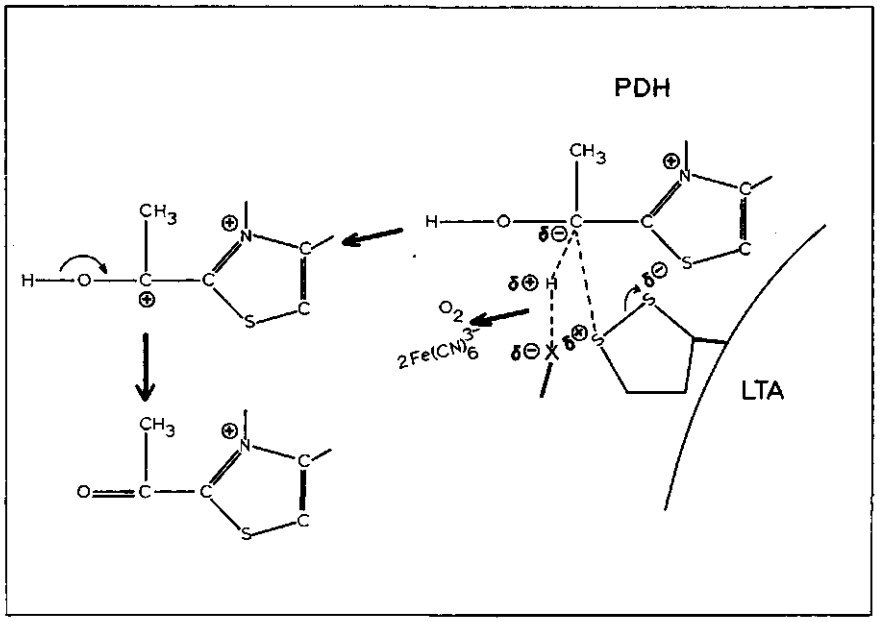


FIG. 6.2.

acetate. FIG. 6.2 illustrates the function of the unknown group (protonated form) firstly in stabilizing the carbanion intermediate and secondly in the transport of reducing equivalents to either oxygen and ferricyanide or by preparing the reaction by protein-bound oxidized lipoic acid.

6.3. REGULATION-MODEL FOR PDC FROM *AZOTOBACTER VINELANDII*

Like PDC from *E. coli* (SCHWARTZ et al., 1968; BISSWANGER and HENNING, 1971) the *Azotobacter* enzyme shows a behaviour with respect to the variation of the rate with the pyruvate concentration that is a common feature of a large number of regulatory enzymes and compatible with various theories. Among them the allosteric or concerted transition model of MONOD et al. (1965) and the non-concerted transition model of KOSHLAND et al (1966); both theories are leading to similar predictions in most respects and in many experimental situations a distinction between them is not meaningful. Apart from these isomeric models, cooperativity can be explained by polymerization (NICHOL et al., 1967). Although the theories mentioned are actually based on differences in binding-parameters and we have dealt with kinetical data in CHAPTER 4, DALZIEL (1968) has shown this not to be of influence. In addition, the sigmoid rate curve as a function of the substrate concentration of PDC from *Azotobacter* cannot be explained by the multi-site hypothesis, proposed by ATKINSON et al. (1965), as outlined in section 4.2.3.

To interprete the '*allosteric control*' of PDC, the following considerations are of importance:

1. The enzyme is a multi-enzyme complex of which the exact stoechiometry of the compiling components, the shape and its symmetry are unknown yet, but with a twofold rotation axis which is a prerequisite in the theory of MONOD et al. (1965). However, as argued below, pyruvate-binding did not cause cooperativity and therefore the latter theory cannot be fitted.
2. Pyruvate, and not Mg^{2+}.TPP, exhibits sigmoidal kinetics in the overall reaction, but not in the ferricyanide-linked PDH- and in the '*oxidase*' reaction. Although pyruvate promotes Mg^{2+}.TPP-binding to the enzyme (K_M for Mg^{2+}.TPP is somewhat lower at very high pyruvate concentrations), the response of the reaction velocity with respect to pyruvate cannot be explained by this phenomenon. No difference between the HILL-coefficients, found in either the overall reaction or the partial (PDH-catalyzed) reactions, would be expected then. A HILL-coefficient of above 2, as found in the overall reaction, suggests the involvement in the cooperative interactions of more than two subunits within the complex. The positive- and negative effector(s), which were shown to compete with each other or with Mg^{2+}.TPP-pyruvate, are hardly influencing the HILL-coefficients, apparently of the pyruvate-binding. Curves of the reaction rate as a function of the effector concentration, measured at low pyruvate concentration, were hyperbolic. According to the 'KOSHLAND' model, sigmoidal binding of ligands and sigmoidal kinetics are explained by changes in subunit interactions caused by binding, which induces a conformational change. The concomitant change in free energy of binding are now favouring cooperativity. From the lower HILL-coefficients for the pyruvate-binding, found in the PDH- and '*oxidase*' reactions (h is about 1.3 and 1.0, respectively) it must be concluded that cooperativity is only exerted in the overall reaction and cannot be the consequence of pyruvate-binding. Protein-bound lipoic acid has been demonstrated not to be involved in the latter reactions (cf. CHAPTER 5).
3. The importance of the sequence of the additions during NAD(P)H-dependent inactivation of the overall reaction by NEM (CHAPTER 5) indicates, that reduction of the flavoprotein component in the whole complex causes rapidly a conformational change within the PDC resulting in less accessibility of the lipoyl moieties (bound to LTA) for the action of NEM and/or NAD(P)H.

Therefore, we conclude that the observed cooperativity in the overall reaction of PDC from *Azotobacter vinelandii* must be caused by:

1. Transfer of the carbanion intermediate (of α-hydroxyethylTPP), via the unknown group (-*XH*, *pK* is 8.0) located at each of the PDH components of the complex, to the four lipoyl moieties (bound to LTA).
2. Interaction, which occurs after transacetylation to CoA, between lipoamide dehydrogenase and LTA. In accordance, the curve of h versus pH (CHAPTER 4) shows the same tendency like that of log V versus pH and the NEM-experiments (CHAPTER 5) show interaction between lipoamide dehydrogenase and LTA. Furthermore, it is possible that the state of reduction of the flavoprotein contributes to this cooperativity. Lipoamide dehydrogenase has a high catalytic

centre activity. Consequently at low pyruvate concentration and in contrast to the situation at high pyruvate concentration, in steady state catalysis the flavoprotein will be mainly in its oxidized form. Since reduction leads to changes in conformational interaction (cf. CHAPTER 5), it seems reasonable to relate the cooperativity also with the state of reduction of the flavin.

SUMMARY

The isolation and some alternatives for purification of PDC from *Azotobacter vinelandii* are described (CHAPTER 3). Ultimate extent and recovery seem to be limited by the lability of the enzyme: sensitivity to shearing forces. Moreover, sedimentation-velocity runs and light-scattering experiments show dissociation of the complex at low protein concentrations (< 0.3 mg.ml^{-1}).

The maximal specific activity achieved (10 units.mg^{-1}) is much lower than that reported for pure PDC from *E. coli*. PTA-activity was found to be closely associated with PDC from *Azotobacter*. The question, whether or not this PTA may be called part of the complex or that its presence in the complex is due to a purification-artefact, could not be answered unequivocally. Each attempt to improve the specific activity by removing the PTA also leads to dissociation of LTA and lipoamide dehydrogenase from the complex, with concomitant loss of activity. It was shown, that connection of PDC and PTA may be meaningful, for in the presence of a non-rate limiting amount of acetate kinase substrate-bound phosphorylation could be demonstrated, due to combined actions.

After correction for light-scattering the absorption spectrum of pure PDC shows a maximum at 455 nm and shoulders at about 430 and 485 nm, exactly as for pure lipoamide dehydrogenase. The flavin content of most PDC preparations varied between 1.6 and 1.8 nmoles FAD per mg protein, pointing at a minimal molecular weight of 550,000–620,000 daltons. Data from light-scattering studies and sedimentation-diffusion experiments indicate, that the actual molecular weight of the pure active multi-enzyme complex is 1,000,000–1,200,000 daltons. From molecular weight and $s_{20,w}$ (19.5 *S*), a frictional ratio of 1.5 could be calculated, thus a more open structure than a solid sphere is possible. In electron micrographs, the typical structures of PDC as observed with the *E. coli* complex, are not present. In preparations, that in addition to PDC- and PTA activity contain some pyridine nucleotide transhydrogenase, tetrad-like structures (12–14 nm on a side) are visible.

The overall activity of the complex (CHAPTER 4) was shown to be dependent on the Mg^{2+}.TPP concentration ($K_M = 25$–50 μM). The value $K_M = 0.1$ mM as found for Mg^{2+} does not reflect affinity between enzyme and Mg^{2+}. AMP and sulphate enhance the affinity of the enzyme for Mg^{2+}.TPP; CoASAc inhibits competitively ($K_I = 10$ μM).

The kinetics of the overall reaction with different pyruvate concentrations show a sigmoidal response ($S_{0.5} = 1.9$ mM under the conditions used). AMP and sulphate stimulate the activity at low pyruvate concentrations. In contrast, CoASAc behaves as a strong competitive inhibitor with respect to Mg^{2+}.TPP-pyruvate ($I_{0.5} = 8$ μM), which inhibition is reversed by AMP and sulphate. The HILL-coefficients (h), apparently for the pyruvate-binding, as calculated from the plots were above 2 ($h = 2.6$–2.7) and not influenced by the presence of the effectors. The influence of effectors was also exerted on the ferricyanide-linked

(partial)PDH reaction. However cooperativity seems to be nearly completely absent in this reaction ($h = 1.3$).

The rate dependence of the overall reaction on the pyruvate concentration studied at different pH's allowed analysis of $pS_{0.5}$, log V and h versus pH profiles. Due to ionizations, two pK's (at pH's 6.7 and 8.0) in the free enzyme and two pK's (at pH's 6.7 and 8.0) in the enzyme-Mg^{2+}-TPP-pyruvate complex (*ES*-complex) are present; the slopes of the straight-line sections in the curves are approximately integrals (1,0 and −1). Protonation of PDC causes an increase in affinity between enzyme and pyruvate; AMP and sulphate also enhance the affinity of the PDC for pyruvate in de whole pH-range studied. Deprotonation of the *ES*-complex upon increasing the pH, which leads to diminished activity, is largely prevented by AMP and sulphate by shifting pK_2^{ES} (8.0) towards a higher value. These phenomena are also present in the ferricyanide-linked PDH reaction. The HILL-coefficient for the overall reaction depends in a similar way on the pH as log$V$, viz.; at least partially, h is determined by ionizations within the subunits of the *ES*-complex.

Apart from the allosteric control, which was based on measurements of initial (overall) reaction rates, the active proportion of PDC from *Azotobacter* is controlled by the [CoASAc]/[CoA] ratio in a second line. Due to PTA activity, in the presence of phosphate CoASAc is reversibly converted into acetylphosphate and CoA, thus abolishing part of the inhibitory action of CoASAc.

A K_M for NAD^+ in the overall reaction of 0.1 mM is found (in good correspondence with the value found for pure lipoamide dehydrogenase from *Azotobacter*). Phosphate inhibits non-competitively, NADH inhibits competitively ($K_I = 40\ \mu M$) with respect to NAD^+. In the presence of phosphate however the affinity of the enzyme for NADH increases ($K_I = 20\ \mu M$).

The activity of PDC from *Azotobacter* was shown not to be modified by phosphorylation and dephosphorylation, but instead controlled by the energy charge of the adenylate pool (especially in the presence of CoASAc) over a large range according to a R-system; ATP being inhibitory at high charge.

In CHAPTER 5 the rapid irreversible inactivation of PDC by sulfhydryl reagents is described. Under anaerobic conditions, sulfhydryl groups (originating from the protein-bound oxidized lipoyl moieties) are generated during reaction with either pyruvate plus Mg^{2+}.TPP in the absence of CoA or with NAD(P)H; it was shown that lipoamide dehydrogenase from *Azotobacter*, either unbound or complexed, forms a two-equivalent-reduced enzyme with excess NADPH.

The sulfhydryl groups developed are blocked by NEM, iodoacetic acid and bromopyruvate. The overall activity disappears completely within a reaction period of 10 min., without affecting the partial activities of the complex at comparable rates. To accomplish blocking of the LTA-bound lipoyl moieties by NEM via the reduced flavoprotein, the addition of this reagent prior to NAD(P)H turned out to be important. Reduction of the flavin leads to a rapid conformational change within the PDC, resulting in less accessibility of the lipoyl moieties for the action of NEM.

Instead of NEM, maleimide spin label can be selectively introduced in the

complex in a either pyruvate plus Mg^{2+}.TPP- or a NAD(P)H-dependent process. Mainly a semi-mobile species is visible in the EPR spectrum ($\tau_c = 0.5$ nsec) pointing at a high mobility of the lipoyl moieties. Two of them are found per molecule FAD in the presence of Mg^{2+}.TPP-pyruvate.

Furthermore, in the absence of pyruvate, bromopyruvate slowly inactivates the overall activity in a Mg^{2+}.TPP process. During the early stages of the latter process, partial reactivation of the PDC can be observed upon incubating with DTT prior to the assay. Two processes are involved. The first enzyme (PDH) is the main target.

Aerobically, in the absence of a sulfhydryl reagent, but in the presence of pyruvate plus Mg^{2+}.TPP, also a slow irreversible inactivation of the overall reaction (without affecting the partial activities) is observed. Most probably, S-S bridge formation due to oxidation of two protein-bound lip(SH)-S-acetyl species occurs. Although not responsible for the latter phenomenon it was discovered that the PDH component is also able to use oxygen instead of ferricyanide as electron acceptor in its partial reaction, catalyzing:

$$pyruvate + O_2 + H_2O \xrightarrow{Mg^{2+}.TPP} H_2O_2 + CO_2 + acetate$$

A HILL-coefficient of 1 for the dependence on the pyruvate concentration is found in this reaction.

In accordance with these studies, composition and catalytical behaviour of PDC from *Azotobacter vinelandii* are schematically given in CHAPTER 6. In the *Azotobacter* complex, four identical LTA units ($M = 80{,}000$ daltons) each containing one molecule of covalently bound lipoic acid, are distributed around one dimeric lipoamide dehydrogenase unit ($M = 112{,}000$ daltons) in non-covalent interaction. It is calculated that six to eight identical PDH units ($M = 90{,}000$ daltons) and consequently four to zero PTA units ($M = 50{,}000$ daltons) are joined to this sub-complex in a regular manner. Moreover, it is suggested that the presence of PTA in the complex actually is due to a purification-artefact and that the intact complex contains eight PDH units.

The reaction-sequence for the oxidation of pyruvate by PDC has been extended. From the pH-influence on the rate of the overall activity and of the PDH-catalyzed partial reactions, in relation to the pyruvate and AMP concentration (CHAPTER 4 and 5), and from the inactivation experiments (CHAPTER 5), participation of a protonated (unknown) group ($pK = 8.0$), probably a sulfhydryl group, in catalysis is obvious. It has a function in stabilizing the carbanion intermediate (α-hydroxyethyl TPP) and the transfer of reducing equivalents to either oxygen and ferricyanide or by preparing the reaction with protein-bound oxidizing lipoic acid. It is concluded from the present studies that since the allosteric control of PDC from *Azotobacter* cannot be the consequence of pyruvate-binding, it must be caused by the transfer reaction via this unknown group – which is located at each of the PDH components – to the four lipoyl moieties and by interaction of LTA-bound reduced lipoic acid with lipoamide dehydrogenase. It seems that the state of reduction of the flavin is related to the cooperativity.

SAMENVATTING

In het 3e hoofdstuk worden verschillende methoden beschreven om het multienzym complex PDC uit de bacterie *Azotobacter vinelandii* te zuiveren. De uiteindelijke omvang van de zuiverheid en de opbrengst lijkt beperkt te worden door de geringe stabiliteit van het enzym, vooral onder invloed van schuifkrachten. Bovendien tonen ultracentrifuge- en lichtverstrooiingsexperimenten aan, dat het enzym complex bij lage eiwitconcentraties (< 0.3 mg ml^{-1}) dissocieert.

De specifieke activiteit, die maximaal bereikt kon worden (10 eenheden mg^{-1}), is veel lager dan de gepubliceerde waarde voor zuiver PDC uit *E. coli*. Nauw verbonden met het PDC uit *Azotobacter* werd PTA activiteit aangetroffen. Het bleef echter de vraag of dit PTA deel van het complex uitmaakt of dat het aanwezig is in het complex als gevolg van *'zuivering'*. Bij elke poging om de specifieke activiteit van het PDC op te voeren door het PTA te verwijderen, dissocieren ook de componenten LTA en lipoamide dehydrogenase van het complex. Dit gaat gepaard met fors activiteitsverlies. Aantoonbaar was, dat de bundeling van PDC en PTA doelmatig zou kunnen zijn, want in aanwezigheid van overmaat acetaat kinase kan oxydatie van substraat nu ook leiden tot fosforylering.

Net als het absorptie spectrum van lipoamide dehydrogenase heeft dat van zuiver PDC, na correctie voor lichtverstrooiing, een maximum bij 455 nm en schouders bij ongeveer 430 en 485 nm. Het flavine gehalte van de meeste PDC preparaten varieerde tussen 1,6 en 1,8 nmoles FAD per mg eiwit, hetgeen duidt op een minimaal molecuulgewicht voor PDC van 550.000–620.000 daltons. De gegevens uit de lichtverstrooiingsstudies en de sedimentatie-diffusie experimenten wijzen erop, dat in werkelijkheid het molecuulgewicht van het actieve, zuivere multi-enzym complex 1.000.000–1.200.000 daltons bedraagt. Uit dit molecuulgewicht en $s_{20,w}$ (19,5 *S*) werd een frictieverhouding van 1,5 berekend. Dit betekent, dat de structuur minder compact is dan die van een massieve bol. Electronenmicroscopische opnamen laten niet die kenmerkende structuren zien als opnamen van het *E. coli* complex dat doen. In preparaten, die behalve PDC- en PTA activiteit ook nog wat pyridine nucleotide transhydrogenase bevatten, zijn vierkantige structuren (12–14 nm per zijde) zichtbaar.

De overall activiteit van het complex (Hoofdstuk 4) is afhankelijk van de Mg^{2+}.TPP concentratie (K_M = 25–50 μM). De waarde K_M = 0,1 mM zoals voor Mg^{2+} gevonden werd, stelt geen affiniteit tussen het enzym en Mg^{2+} voor. AMP en sulfaat verhogen de affiniteit van het enzym voor Mg^{2+}.TPP; CoASAc remt competitief (K_I = 10 μM). De kinetiek van de overall reactie bij verschillende pyruvaat concentraties verloopt sigmoïdaal ($S_{0.5}$ = 1,9 mM onder de gebruikte condities). AMP en sulfaat stimuleren de activiteit bij lage pyruvaat concentraties. In tegenstelling hiermee gedraagt CoASAc zich als een sterke competitieve remmer ten opzichte van Mg^{2+}.TPP-pyruvaat ($I_{0,5}$ = 8 μM), welke

remming ongedaan gemaakt kan worden met AMP en sulfaat. De HILL-coëfficiënten (h) schijnen betrekking te hebben op de pyruvaat-binding en liggen boven de 2 ($h = 2{,}6$–$2{,}7$), zoals berekend uit de grafieken. De aanwezigheid van effector oefent geen invloed op ze uit. In de met ferricyanide te meten PDH (deel) reactie bleken de effectoren evenzeer werkzaam. In deze reactie is de coöperativiteit echter bijna volledig afwezig ($h = 1{,}3$).

Omdat de relatie tussen de snelheid van de overall reactie en de pyruvaat concentratie bestudeerd was bij verschillende pH's, was het mogelijk verband te leggen tussen de pH en respectievelijk $pS_{0,5}$, log V of h. Twee pK's (bij pH's 6,7 en 8,0) zijn gevonden in het vrije enzym en ook twee (bij pH's 6,7 en 8,0) in het enzym-Mg^{2+}.TPP-pyruvaat complex (*ES*-complex). Ze worden veroorzaakt door ionisaties. De hellingen van de rechte stukken in de grafieken zijn bij benadering gehele getallen (1,0 en –1). Protonering van PDC veroorzaakt een toename in affiniteit tussen enzym en pyruvaat; in het hele pH-gebied vergroten AMP en sulfaat ook de affiniteit van PDC voor pyruvaat. Deprotonering van het *ES*-complex bij toenemende pH leidt tot lagere activiteit en kan grotendeels tegengegaan worden door AMP en sulfaat; deze effectoren verschuiven pK_2^{ES} (8,0) naar hogere waarden. Dit doet zich ook voor in de met ferricyanide te meten PDH reactie. De in de overall reactie gemeten HILL-coëfficiënt hangt van de pH af als log V, d.w.z. dat h, tenminste gedeeltelijk, bepaald wordt door ionisaties binnen de subeenheden van het *ES*-complex.

De hoeveelheid actief PDC in *Azotobacter* wordt, naast geregeld te worden door allosterie, ook nog beheerst door de verhouding [CoASAc]/[CoA]. Door PTA activiteit wordt CoASAc in aanwezigheid van fosfaat reversibel omgezet in acetylfosfaat en CoA, waardoor de remmende werking van CoASAc gedeeltelijk teniet gedaan wordt.

Een K_M van 0,1 mM is voor NAD^+ in de overall reactie gevonden (komt goed overeen met de gevonden waarde voor zuiver lipoamide dehydrogenase uit *Azotobacter*). Fosfaat remt non-competitief, NADH remt competitief (K_I = 40 μM) ten opzichte van NAD^+. In aanwezigheid van fosfaat neemt de affiniteit van het enzym voor NADH zelfs nog toe (K_I = 20 μM).

De activiteit van PDC uit *Azotobacter* werd niet gemodificeerd door fosforylering en defosforylering, maar was in plaats daarvan over een groot gebied (speciaal in aanwezigheid van CoASAc) onderworpen aan de invloed van de energie lading van het adenylaat depot en wel volgens een R-systeem, waarbij ATP remmend werkt bij hoge energie lading.

In hoofdstuk 5 wordt de snelle- en irreversibele inactivering van PDC door sulfhydryl reagentia beschreven. Onder anaerobe condities ontstaan er sulfhydryl groepen (afkomstig van de aan eiwit gebonden geoxydeerde lipoïnezuurresten) tijdens de reactie met pyruvaat plus Mg^{2+}.TPP en zonder CoA of tijdens reactie met NAD(P)H; aangetoond kon worden dat, zowel vrij als in PDC verankerd, lipoamide dehydrogenase uit *Azotobacter* een twee-equivalenten gereduceerd enzym met overmaat NADPH vormt.

De *'ontwikkelde'* sulfhydryl groepen worden geblokkeerd met NEM, joodazijnzuur en broompyruvaat. De overall activiteit verdwijnt helemaal binnen

een reactieperiode van 10 min., zonder dat de deelactiviteiten van het complex met vergelijkbare snelheden aangetast worden. Om via het gereduceerde flavoproteine de aan LTA gebonden lipoïnezuurresten met NEM te kunnen blokkeren, bleek toevoeging vòòr NAD(P)H van dit reagens belangrijk. Reductie van het flavine leidt tot een snelle conformatieverandering binnen het PDC, waardoor de lipoinezuurresten minder toegankelijk worden voor de werking van NEM.

Inplaats van NEM kan, afhankelijk van pyruvaat plus Mg^{2+}.TPP of van NAD(P)H, selectief een maleimide spin label in het complex ingebracht worden. In het EPR spectrum is nu hoofdzakelijk een semi-mobiele specie te zien (τ_c = 0,5 nsec.), hetgeen wijst op een grote mobiliteit van de lipoïnezuurresten. Er worden twee labels ingebracht per molecuul FAD met Mg^{2+}.TPP-pyruvaat.

Verder inactiveert broompyruvaat, in afwezigheid van pyruvaat, zelf langzaam de overall activiteit in een Mg^{2+}.TPP afhankelijk proces. Gedurende de eerste stadia van dit laatste proces valt het PDC gedeeltelijk weer te reactiveren door, voorafgaande aan de activiteitsbepaling, het complex met DTT te incuberen. Er zijn twee processen mee gemoeid. Hoofdzakelijk is het eerste enzym (PDH) het doelwit.

Zonder sulfhydryl reagentia bleek pyruvaat plus Mg^{2+}.TPP, onder aerobe condities, ook nog in staat langzaam de overall activiteit irreversibel te inactiveren (zonder de deelactiviteiten aan te tasten). Waarschijnlijk treedt er vorming van een S-S brug op door oxydatie van twee eiwit-gebonden lip(SH)-S-acetyl species. Ofschoon niet verantwoordelijk voor genoemd verschijnsel, werd ontdekt, dat de PDH component ook zuurstof in plaats van ferricyanide als electronenacceptor kan gebruiken in de deelreactie, volgens:

$$pyruvaat + O_2 + H_2O \xrightarrow{Mg^{2+}\,.\,TPP} H_2O_2 + CO_2 + acetaat.$$

Ten opzichte van de pyruvaat concentratie wordt een HILL-coëfficiënt van 1 gevonden in deze reactie.

In Hoofdstuk 6 worden in kort bestek de samenstelling en de katalytische mogelijkheden van het PDC uit *Azotobacter* uit de doeken gedaan, althans voor zover te rijmen met deze studies. In het *Azotobacter* complex vinden we vier gelijke LTA eenheden (M = 80.000 daltons), die elk een molecuul covalent gebonden lipoïnezuur bevatten, verspreid om een *dimere* lipoamide dehydrogenase eenheid (M = 112.000 daltons), waarbij hun interactie niet-covalent is. Berekend kon worden, dat zes tot acht gelijke PDH eenheden (M = 90.000 daltons) en dientengevolge vier tot nul PTA eenheden (M = 50.000 daltons) op regelmatige wijze aan dit sub-complex toe te voegen zijn. Bovendien wordt er gesuggereerd, dat uiteindelijk het PTA in het complex aanwezig is als gevolg van *'zuivering'* en zou het *'intacte'* complex acht PDH eenheden bevatten.

Er is uitbreiding gegeven aan de reactie-volgorde bij de oxydatie van pyruvaat door PDC. Uit de invloed, die de pH heeft op de relatie tussen snelheid en pyruvaat- of AMP concentratie van zowel de overall reactie als van de door PDH gekatalyseerde deelreacties (Hoofdstukken 4 en 5) en uit de inactiverings-

experimenten (Hoofdstuk 5), blijkt de deelname in de katalyse van een geprotoneerde (onbekende) groep (pK = 8,0); waarschijnlijk een sulfhydryl groep. Deze groep heeft zowel een taak in het stabiliseren van het carbanion intermediair (α-hydroxyethyl TPP) en in het overdragen van reductie-equivalenten aan zuurstof of ferricyanide, als in het voorbereiden van de reactie met het eiwitgebonden geoxydeerde lipoïnezuur. Verder kan uit de voorliggende studies geconcludeerd worden, dat de allosterische regeling van de activiteit van het PDC uit *Azotobacter* niet voort vloeit uit de pyruvaat-binding, maar veroorzaakt moet worden door de *overdrachtsreactie* via de onbekende groep, waarvan er op elke PDH één aanwezig is, naar de vier lipoinezuurresten en door interactie van aan LTA gebonden gereduceerd lipoïnezuur met lipoamide dehydrogenase. Het lijkt er op, dat de reductie-toestand van het flavine gekoppeld is aan de coöperativiteit.

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