STUDIES ON LIPOAMIDE DEHYDROGENASE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS DR.IR. F. HELLINGA,
HOOGLERAAR IN DE CULTUURTECHNIEK,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
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DOOR

JACOB VISSER

GEBOREN TE HAARLEMMERMEER
13 JULI 1941

Voor Guusta, Han, Gijs en Cees,
met wie ik zoveel goede uren deelde.

VOORWOORD

Bij het voltooien van dit proefschrift wil ik niet nalaten te onderstrepen dat ik de waarde van een dergelijk rapport maar zeer betrekkelijk acht. Het huidige karakter van de research vergt de medewerking van een groot aantal personen en het koppelen van één naam aan een dissertatie is derhalve een onjuiste weergave van de werkelijkheid. In dit geval overtreft het aantal mensen zelfs het aantal letters in het woord "doctor" en dat maakt dat het "één man-één letter" principe hier onmogelijk is.

In de loop van de periode 1965-1970 hebben meegewerkt: GUUSTA KOK, HAN KALSE, KLAAS HITMAN, JILLERT SANTEMA, CEES DE RANITZ, JACQUES RUITER, GIJS VAN DEDEM, JAN GEELEN, JACQUES WOUTERS VAN DEN OUDENWEYER en BAS VAN MARKWIJK. In meer of minder belangrijke mate is dit onderzoek ook het hunne geweest en zij verdienen meer dan dank.

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CONTENTS

LIST OF ABBREVIATIONS

Lipoamide dehydrogenase is the trivial name used throughout this thesis for NADH: lipoamide oxido reductase, EC 1.6.4.3, according to the Report of the Commission for Enzymes of the International Union of Biochemistry.

AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
$APNAD^+$	3-acetylpyridine nicotinamide adenine dinucleotide
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CT	charge transfer
$D_{20,w}$	diffusion coefficient in water at 20° at a finite protein concentration
DCIP	2,6-dichlorophenol indophenol
EDTA	ethylene diaminetetra acetate
EPR	electron spin resonance
FAD	flavin adenine dinucleotide, oxidised form
$FADH_2$	flavin adenine dinucleotide, fully reduced form
F-8bromoAD	flavin 8-bromo adenine dinucleotide
FMN	flavin mononucleotide
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
K_{ass}	association constant of enzyme-substrate (prosthetic group) complex
K_{diss}	dissociation constant of enzyme-substrate (prosthetic group) complex
K_m	Michaelis constant
K_i	dissociation constant of enzyme-inhibitor complex
α-KGDC	α-ketoglutarate dehydrogenase complex
L-B	Lineweaver-Burk plot
$lipS_2$	lipoic acid (oxidised form)
$lip(SH)_2NH_2$	lipoamide (reduced form)
$lipS_2(NH_2)$	lipoamide (oxidised form)
M_{app}	apparent molecular weight
mol.wt.	molecular weight

$NAD(P)^+$	oxidised nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
$PANAD^+$	pyridine aldehyde nicotinamide adenine dinucleotide
PCMB	para-chloromercuribenzoate
PDC	pyruvate dehydrogenase complex
$s_{20,w}$	sedimentation coefficient in water at 20°
TCA	trichloro-acetic acid
Tris	Tri(hydroxymethyl) amino methane
V_{max}	maximum velocity at infinite substrate concentration.

1. INTRODUCTION

Lipoamide dehydrogenase (EC. 1.6.4.3) is found abundantly in nature and it has been isolated from a number of micro-organisms, higher plants and animals. The enzyme isolated from pig heart, originally known as Straub diaphorase (STRAUB, 1939), was later found to be identical with lipoamide dehydrogenase (MASSEY, 1958, 1960[a]; SEARLS and SANADI, 1960, 1961). The metabolic function of this enzyme is strongly associsted with its occurrence in multi-enzyme complexes. The oxidative decarboxylation reactions of pyruvate and α-ketoglutarate occurring in animal tissues and micro-organisms are catalysed by specific enzyme complexes. Such complexes which have a molecular weight of several millions have been isolated from various mammalian sources (SANADI et al., 1952; HAYAKAWA et al., 1964, 1967) as well as from Escherichia coli (KOIKE et al., 1960[a]). Much is known about the architecture of these complexes, due to the work of REED and collaborators who separated the individual components of the E. coli complexes and were also able to reconstitute the system (KOIKE et al., 1963; MUKHERJEE et al., 1965). The pyruvate dehydrogenase complex of E. coli consists of 12 molecules pyruvate decarboxylase, 6 molecules lipoamide dehydrogenase and 24 lipoyl reductase transacetylase subunits, the latter creating the specific binding sites for both other enzymes (REED and COX, 1966). Studies concerning the relative amounts of α-ketoglutarate decarboxylase, lipoyl reductase-transsuccinylase and lipoamide dehydrogenase in the α-kg complexes and the electron microscopic aspects of the association have not yet been published.

Though mammalian enzymes have always been isolated from mitochondria, JACOBI and ÖHLERS (1968) localised the enzyme in spinach chloroplasts where probably, as in mitochondria, it is present as part of a higher organised enzyme complex.

Preparations of lipoamide dehydrogenase from pig- or beef heart which were homogeneous by other physical criteria, have been separated into 5 or 6 active flavoprotein bands on starch gel electrophoresis (ATKINSON et al., 1962). LUSTY (1963) by chromatography on DEAE-cellulose affirmed this for beef liver enzyme prepared according to MASSEY (1960[b]) or under mild conditions using phospholipase A. Similar results were obtained by STEIN et al. (1965[a,b]) and MILLARD et al. (1969) for the pig heart enzyme.

LUSTY and SINGER (1964) distinguished between three different fractions of lipoamide dehydrogenase in mitochondria, a free form (± 25 %) and two complex-bound fractions. The free enzyme resulted in three bands upon electrophoresis. A similar distinction has been made by HAYAKAWA et al. (1968). However, upon electrophoresis the free enzyme consisted of two bands each one corresponding with either the enzyme derived from the α-ketoglutarate complex or from the pyruvate dehydrogenase complex both moving as a single band. The two forms are different in their circular dichroism spectrum and with respect to the NADH induced stimulation of the diaphorase activity (SAKURAI et al., 1969). COHN et al. (1968) identified the multiple bands (5-6) as they occur in Straub diaphorase. The slowest two (or three) originate from the α-ketoglutarate complex, the other three from the pyruvate dehydrogenase complex. In contrast to the enzyme from pig or beef, the human liver enzyme (IDE et al., 1967) and the Escherichia coli enzyme (PETTIT and REED, 1967; COHN et al., 1968) are reported to be homogeneous. Recently WILSON (1969) suggested that proteolytic activities in the Keilin and Hartree preparation are responsible for these multiple enzyme patterns of the pig heart enzyme.

Lipoamide dehydrogenase has a fairly broad substrate specificity. MASSEY (1963) suggests that a negative charge on the substrate hinders the approach to the enzyme as uncharged derivatives as lipoamide and lipoanilide have higher turnover numbers than lipoic acid itself. Under physiological conditions the lipoic acid is protein-bound and NAWA et al. (1960) proved with the Escherichia coli system that the carboxyl group is linked to an ε-amino lysine group of the protein. Studies with lipoyl derivatives indicated that a greater distance between the carboxyl group and the dithiolane ring results in an increase of the turnover number (MASSEY, 1960[b]; GOEDDE et al., 1963). These last authors also synthesised lipoyl derivatives which were inactive but inhibitory with respect to lipoic acid and some without affinity for the enzyme.

Although SANADI and SEARLS (1957) initially reported the mammalian enzyme to be stereospecific, more precise data have shown that both optical isomers are active but that the rate with which the (+) stereo-isomer reacts is much greater (SANADI et al., 1959; MASSEY, 1960[b]). The enzyme from spinach differs from the mammalian and bacterial species in that the (-) isomer is much more reactive (BASU and BURMA, 1959, 1960) though in the latter case no absolute stereospecificity is observed (MATTHEWS and REED, 1963).

The $-COHN_2$ side group on position 3 of the pyridinium moiety of NAD^+ is important as all modifications with the exception of the related thioamide ($-CSNH_2$) are much less reactive (MASSEY, 1963). Stereospecificity of the NAD^+ reduction reaction is absent (MAHLER and CORDES, 1966, pg. 357) though it seems unlikely that the rates are equal.

Lipoamide dehydrogenase is also able to catalyse oxidation of NADH by artificial electron acceptors, such as 2,6-dichlorophenol indophenol and ferricyanide (SAVAGE, 1957; MASSEY, 1960) or menadione (LEVINE et al., 1960; MISAKA and NAKANISHI, 1963). The transhydrogenase activity has been demonstrated by WEBER and KAPLAN (1957). Several kinetic data of this reaction have been published by MASSEY et al. (1960).

Crystals of lipoamide dehydrogenase are only reported for yeast enzymes (MISAKA et al., 1965; MISAKA and NAKANISHI, 1965). The molecular weights of the enzyme from different sources are in agreement (cf. Chapter III), and in all cases two FAD groups per dimer are found (MASSEY et al., 1962; KOIKE et al., 1963; MATTHEWS and REED, 1963). The enzymes from all sources are not only very similar in physical properties but they have many other factors in common as well. Some of the differences will be outlined in the following. The amino acid composition is only known for the pig heart enzyme (MASSEY, 1963) and the Escherichia coli B enzyme (WILLIAMS et al., 1967). The distribution is very similar except for the half-cystine residues in the Escherichia coli enzyme which is half the amount of the pig heart enzyme and an increased number of proline residues which might have implications for the helical properties. The number of helix-promoting amino acids and the difference between helix- and non-helix-promoting amino acids is larger in the Escherichia coli enzyme (cf. HAVSTEEN, 1966), though no actual helical data is known for the Escherichia coli enzyme. The pig heart enzyme contains 5 to 6 -SH groups per flavin and two more under substrate reduced flavin conditions (PALMER and MASSEY, 1962; VEEGER and MASSEY, 1962). Three -SH groups per flavin molecule are detected in the yeast enzyme (MISAKA, 1966). A disulfide bridge in combination with the flavin part is known to operate in the catalytic centre. Addition of NADH results in a 2-electron reduction product, which is called the semiquinone form, and red in color. However, this species is EPR inactive.

Dithiol inhibitors as Cd^{2+} or arsenite in combination with NADH (under anaerobic conditions) stimulate the diaphorase activity while the lipoate and the transhydrogenase activities decrease (SEARLS et al., 1961; MASSEY and VEEGER, 1960, 1961). The red flavin semiquinone spectrum changes simultaneously into that of the colourless fully reduced enzyme (MASSEY and VEEGER, 1961) which in the presence of NAD^+ ultimately results in a fully reduced flavin -NAD^+ charge transfer complex with a green broad band at 720 nm (MASSEY and PALMER, 1962). Mercurials and Cu^{2+} are also known to stimulate the diaphorase activity and destabilise the semiquinone (VEEGER and MASSEY, 1960, 1962; CASOLA et al., 1966[a,b]). MISAKA and NAKANISHI (1963) found no stimulation of diaphorase activity by iodoacetamide. The PCMB-modified yeast enzyme has altered pyridine nucleotide binding properties in the menadione reduction reaction; the K_m for NADH increases while the affinity for NAD^+ decreases (MISAKA and NAKANISHI, 1963). KREGER (1968) reported semiquinone destabilisation by quinine optochinin hydrochlorides for the E. coli and the pig heart enzyme. Mercurials as well as these compounds probably interfere with the NAD^+ binding site, thus preventing development of the charge transfer band. The differences between the enzyme species are only quantitative. The diaphorase activity of the E. coli enzyme is stimulated more by quinine than the pig heart enzyme (KREGER, 1968). The arsenite concentration necessary to produce the reduced flavin spectrum of the baker's yeast enzyme is a tenfold increase over the usual concentration (WREN and MASSEY, 1966) and monothiols are better protectors against arsenite in the case of the spinach enzyme than in the pig heart enzyme (MATTHEWS and REED, 1963).

The addition of large amounts of NADH under anaerobic conditions results, even in the presence of NAD^+ in a partially 4-electron reduced flavin (VEEGER, 1966). This equilibrium between the stable 2-electron reduced state is shifted to the 4-electron reduced state by lowering the pH or the temperature. This phenomenon is also of importance in the assay. It is known from the work of MASSEY and VEEGER (1961) that the lag period in the oxidation reaction of NADH with lipoamide is overcome by NAD^+. The differences in substrate inhibition patterns for the bacterial and mammalian enzymes studied so far are striking (KOIKE et al., 1960[b]; LUSTY, 1963; IDE et al., 1967). A strong NADH inhibition at concentrations higher than 6 µM is observed for the E. coli enzyme (NOTANI and GUNSAKUS, 1959). The bacterial catalytic centre is easily overreduced, even by reduced lipoamide (KOIKE et al., 1960; WILLIAMS, 1965). With the pig heart enzyme 4-electron reduced flavin states are

never observed on addition of reduced lipoamide, which might indicate that the oxidised lipoamide is able to stabilise the semiquinoid state as is NAD^+. Another possibility might be a difference in redoxpotential of the bound flavin in the two different sources as no actual data is known for the redoxpotential of the enzyme-bound flavin. The redox-potentials of the NAD^+/NADH and $lipS_2/lip(SH)_2$ couples are not very different, -0.32 and -0.29 V, respectively. The pH-optima of the different enzymes only show slight differences: bacterial, spinach and yeast enzyme have a higher pH optimum in the NADH reduction reaction, which is caused by a different 2-4-electron reduced state equilibrium (GOLDMAN, 1959; BASU and BIRMA, 1959; KAWAHARA et al., 1968; IDE et al., 1967).

The structural differences between lipoamide dehydrogenase from different genetic origin are probably larger than generally thought of, e.g. HAYAKAWA et al. (1967) found no immunochemical relationship between the E. coli enzyme and the human liver enzyme with respect to the antibodies prepared against the pig heart enzyme.

The yeast enzyme is sensitive to trace metals in the presence of reduced substrate reversibly (MISAKA et al., 1965) but it is less sensitive when the flavin is in the oxidised state. Moreover, the lipoate and ferricyanide activities are only partially inhibited while the DCIP-activity is almost unstimulated (WREN and MASSEY, 1966). The yeast enzyme is considerably more active with $APNAD^+$ and $K_3Fe(CN)_6$ as electron acceptors while the V_{max} value of the transhydrogenase differs from the lipoamide dehydrogenase reaction ($lip(SH)_2NH_2$ —$APNAD^+$). This is in contrast to the pig heart enzyme where the same rate-limiting step exists (VEEGER, thesis 1960).

MASSEY and VEEGER (1961) proposed a "ping pong bi bi" mechanism for the catalytic action of the pig heart lipoamide dehydrogenase. This was based on the series of parallel lines obtained in the Lineweaver--Burk plots when donor and acceptor concentrations were varied. The same mechanism is proposed for the yeast enzyme though WREN and MASSEY (1966) do not explain the convergent lines obtained in the reverse reaction with NADH as donor and $lipS_2$ or $lipS_2(NH_2)$ as acceptor. Recent results with other flavoproteins favour ternary complex mechanisms (KOSTER and VEEGER, 1968: ZEYLEMAKER et al., 1969; STAAL and VEEGER,

1969), which led us to reinvestigate the kinetic mechanism of this enzyme. Rapid reaction kinetics as measured with the stopped-flow technique indicate a high reaction rate between enzyme and pyridine nucleotides. NADH reacts with the enzyme within the dead-time (< 3 msec) of the stopped--flow apparatus (MASSEY and GIBSON, 1963) also in the presence of NAD^+ (VEEGER and MASSEY, 1961) to form another intermediate. Reduced lipoamide reacts slower.

Another interesting aspect of this enzyme is dealt with in this thesis, namely the importance of the flavin binding site and the flavin compound for the maintainance of the tertiary structure. Because this thesis is based to a large extent on papers which are already published every chapter has its own specific introduction. Therefore in this general introduction the emphasis is laid on differences between lipoamide dehydrogenases, from different genetical sources and on some of their enzymological aspects.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Enzymes

The purification method used for lipoamide dehydrogenase from pig heart has been described elsewhere (MASSEY, 1960[b]; MASSEY et al., 1960). This procedure is rather a general one and has been basically used for lipoamide dehydrogenase from various sources (CHANNING et al., 1962; KOIKE et al., 1963; WREN and MASSEY, 1965; MISAKA and NAKANISHI, 1963; BASU and BURMA, 1960; MATTHEWS and REED, 1963). Apoenzyme preparations of bacterial lipoamide dehydrogenase have been reported before (KOIKE et al., 1962; WILLIAMS, 1965). The removal of the flavin without denaturation of the apoenzyme has met with more difficulties in the case of the pig heart enzyme (VEEGER et al., 1966; KALSE and VEEGER, 1968). The procedure is basically the method used by STRITTMATTER (1961) for cytochrome b_5 reductase, viz. an acid ammonium sulphate treatment.

Procedure: 2-3 mgs of enzyme from a concentrated stock solution made up in 0.03 M sodium phosphate pH 7.2 with 3 mM EDTA, are diluted with a cold 1 M Tris-HCl buffer (pH 8.1) to a final volume of 1 ml; next 1 ml KBr is added and the mixture is kept on ice in a 25 ml beaker. In the meantime the saturated ammonium sulphate solution is stirred at room temperature ($\pm$ 20°) while the pH is checked before use. The most critical point is the pH of the solution (KALSE and VEEGER, 1968); if too low the protein easily denatures, if too high there is no efficient removal of the flavin. It is better to prepare a fresh solution once a fortnight. 1 ml of this saturated ammonium sulphate is added dropwise within 20 seconds, whirling slightly. This addition results in a slight turbidity. After 40 seconds an additional 4 ml ammonium sulphate is added which precipitates the apoenzyme. Immediately the solution is centrifuged for 6 minutes at 18,000 g (temperature 5°).

The yellow supernatant is decanted after which the centrifuge tubes are wiped out carefully with absorbant paper. Adding 1-2 drops EDTA to the precipitate, the apoenzyme is dissolved in 0.6 ml 0.3 M sodium phosphate buffer pH 7.6 which is kept between 20 and 25°. When the apoenzyme is solubilised, the solution is immediately placed on ice and diluted with 0.4 ml of a cold 0.03 M sodium phosphate buffer pH 7.2 containing 3 mM EDTA to prevent buffer crystallisation. The

apoenzyme is kept on ice. It is critical to use a buffer of a high ionic strength to solubilise the apoenzyme. The temperature of this buffer is important as well; no differences however were found varying the pH from 7.2 to 7.6.

In case the precipitate has still a yellow colour it is dissolved in half its original volume of Tris buffer and KBr after which a second $(NH_4)_2SO_4$-treatment is carried out.

The individual preparations which are obtained may vary rather. The flavin content amounts approximately 5 % or less some of which is not enzyme-bound. The rest activity with $lipS_2$ is 0.3-3 % while the DCIP-activity varies between 80-300 % of the original activity of the holoenzyme.

2.1.2. Reagents

NAD^+, NADPH, $NADP^+$, 5'-AMP, 3,5'-cyclicAMP, ADP, ATP, GDP, GTP, adenine, adenosine, FAD, FMN, riboflavin, lipoic acid, bovine serum albumin and ribonuclease were obtained from Sigma Chemical Co; NADH, alcohol dehydrogenase and lactate dehydrogenase from Boehringen and Söhne; DCIP from the British Drug House. Ovalbumin, acrylamide and N,N'-methylenebisacrylamide were purchased from Kochlight, blue dextran 2000 and Sephadex G-25 and G-200 from Pharmacia. Amido Schwartz was obtained from Hartman-Leddon Co, Philadelphia and ammonium persulphate and N,N,N'N'-tetramethylethylenediamine (TMED) from E.C. Company, Philadelphia.

The 3-methyl-, 3-carboxymethyl- and 2-morpholinoFAD which were synthesised via their monophosphate esters (CHASSY and MCCORMICK, 1965; FÖRY and HEMMERICH, 1967) by condensation with 5-phosphomorpholidate (MOFFATT and KHORANA, 1958) were a gift from Professor P. Hemmerich, University of Konstanz.

F8-bromoAD was synthesised by Professor D.B. McCormick (1969) while the FMN-derivatives were synthesised by Professor P. Hemmerich.

Lipoamide and reduced lipoamide were synthesised according to REED et al. (1959).

2.2. METHODS

2.2.1. The enzymatic assay of lipoamide dehydrogenase

The enzyme activity of the overall reaction:

$$NADH + H^+ + lipS_2 \longrightarrow NAD^+ + lip(SH)_2 \qquad (1)$$

is assayed spectrophotometrically by recording the decrease in extinction at 340 nm. The diaphorase activity is also determined spectrophotometrically recording the reduction of DCIP at 600 nm in the reaction:

$$NADH + H^+ + DCIP \longrightarrow NAD^+ + DCIPH_2 \qquad (2).$$

The specific activity with lipoate can be calculated from

$$\frac{\Delta E}{\text{addition (ml)}} \times \frac{\text{dilution factor}}{\text{protein conc.}}$$

The activity with DCIP is obtained the same way but multiplied by a factor 100 (SAVAGE, 1957).

Procedure: a) lipoate activity. To a spectrophotometer cuvette which is thermostated at 25° is added: H_2O to a final volume of 3 ml; 2.5 ml citrate buffer (1 M, pH 5.65); 0.1 ml bovine serum albumin (2 % w/v in EDTA, 0.03 M); 0.1 ml lipoate (0.02 M); 0.03 ml NAD^+ (0.01 M solution in bidest) and 0.03 ml NADH (0.01 M in water, freshly prepared every day and kept on ice). The reaction is started by adding enzyme in an appropriate dilution giving an initial change in extinction at 340 nm, not exceeding 0.2 per minute.

b) DCIP-activity. To a spectrophotometer cuvette which is thermostated at 25°, is added: H_2O to a final volume of 2.8 ml; 0.5 ml sodium phosphate buffer (0.3 M, pH 7.2); 0-1 ml bovine serum albumin (2 % w/v in EDTA, 0.03 M); 0.12 ml DCIP (1 mM in bidest); 0.03 ml NADH (0.01 M). The reaction is started by adding that amount of enzyme which gives an initial change inextinction at 600 nm not exceeding 0.25 per minute. The activities are all based upon the initial rate.

Activities are generally expressed in % activity with respect to those of the pure holoenzyme, e.g. lipoate activity ± 20 μmoles $mg^{-1}min^{-1}$ and DCIP-activity 0.7-0,8 μmoles $mg^{-1}min^{-1}$. In the kinetic experiments the specific activities have been expressed in μmoles NADH formed or disappearing per minute.

2.2.2. Gel-filtration

Sephadex G-200 columns were calibrated using alcohol dehydrogenase, ovalbumin and bovine serum albumin as standard proteins. Void volumes were determined with blue dextran-2000. The effective pore radius, $\bar{r}$, within the gel was calculated according to the procedure of ACKERS (1964). Stokes radii and frictional coefficients of lipoamide dehydrogenase were calculated from the elution volume as determined by activity measurements (SIEGEL and MONTY, 1966). The buffer used for elution was a 0.05 M Tris-HCl buffer (pH 7.5) containing 0.1 M KCl and 0.3 mM EDTA, unless otherwise stated. The experiments were performed at low temperatures, 4° to 8°. Fractions of 3 ml were collected with an LKB fraction collector. The absorption pattern of the elution diagram was registered with a 8300 A Uvicord II at 280 nm. Fractions collected in experiments with the apoenzyme were incubated with FAD for 1 hr at room temperature (cf. ref. KALSE and VEEGER, 1968) before the activities with lipoate were measured. In the experiment with the recombined enzyme, the temperature of the column did not exceed 4°. The fractions were allowed to stand for 1 hr at room temperature before measuring the activity.

2.2.3. Ultracentrifugation

Sedimentation and diffusion patterns were obtained using an M.S.E. analytical ultracentrifuge. Molecular weights were determined using the Svedberg relationship and the approach to equilibrium method at different speeds, as used by TRAUTMAN (1956). Preparations of the apoenzyme were extensively dialysed against 0.1 M or 0.16 M sodium phosphate buffer (pH 7.6) containing 0.3 mM EDTA.

2.2.4. Sucrose gradients

The sucrose gradients were prepared with a simple gradient device, consisting of two communicating legs equipped with a rotating magnetic stirrer. The gradient was formed at 4° by mixing one volume sucrose solution (50 grams with 100 ml buffer or water) with one volume of sucrose solution containing 20 grams with either 100 ml buffer or water. The buffer used was a 30 mM sodium phosphate buffer pH 7.2 with 0.3 mM EDTA. In the cases indicated 0.1 mM FAD was added. The total gradient was 2.3 ml, while the samples layered on the top of the gradient varied from 0.2 to 0.4 ml.

The experiments were performed with a M.S.E. 50 superspeed ultra-

centrifuge using generally a speed of 45,000 rpm at 4^{o} for at least 20 hours. Samples were collected in the cold from the bottom of the tube by means of a M.S.E. tube piercer; the fractions consisted of 7 to 10 drops which were spectrophotometrically analysed for enzyme activity and refractometrically to determine the exact gradient shape. A Zeiss Abbe-refractometer was used for this purpose.

In some cases the protein fluorescence was used as an analysing tool for which a Hitachi Perkin Elmer MP2A fluorospectrophotometer was used.

2.2.5. Light-scattering

Light-scattering data was 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 relation:

$$R = \frac{i}{I_o} = \frac{Kc}{1/M + 2Bc + 3Cc^2 + \ldots.} \quad cm^{-1} \qquad \text{(TANFORD, 1961)}$$

in which the optical constant K is defined as

$$K = \frac{2^2 \, n_o^2 \, (dn/dc)^2}{N \, \lambda^4} \; cm^2 \, g^{-2}$$

λ = wavelength of the light used

n_o = index of refraction of the solvent

dn/dc = refractive increment = 0.176 $cm^3 \, g^{-1}$ for lipoamide dehydrogenase in 0.03 M sodium phosphate buffer, pH 7.2 with 0.3 mM EDTA

$\frac{i}{I_o}$ = ratio of the intensities of the light scattered under the angle and of the incident beam. Benzene was used as a standard. The constant K amounts 2.57×10^{-7} $cm^2 \, g^{-2}$.

The samples were filtered before use with a 100 mμ filter and the values were corrected for contributions of the solvent.

2.2.6. Fluorescence polarisation

Fluorescence polarisation measurements were performed with a modified Zeiss spectrofluorimeter. A thermostated cuvette holder is placed behind the exit slit of the monochromator, the holder having a polariser in front of it and an analyser behind. Polariser and analyser are in a horizontal plane and at right angles to one another. By rotating the analyser the electric vector of the exciting light is

either perpendicular to the plane formed by the direction of propagation of the exciting light and the direction of observation (V) or in the plane (H). As detector a phototube (IP 28) was used with a Zeiss FL 56 filter in front of it. Usually 396 nm was chosen as excitation wavelength for FAD and other flavins. The polarisation of the fluorescent light emitted is defined by

$$p = \frac{I_{vV} - I_{hV}}{I_{vV} + I_{hV}}$$

in which I_{vV} = vertical polarised emission vector obtained under influence of the vertical excitation vector

I_{hV} = horizontal emission vector obtained by excitation with the vertical excitation vector.

The method of BAYLEY and RADDA (1966) has been used to calculate the fractions of FAD or flavin analogue bound to the apoenzyme assuming that the polarisation of enzyme-bound analogue is the same as that of FAD itself. The fractions of flavin which are bound enables one to calculate the association constants. The number of binding sites is calculated using the equation derived by KLOTZ (1946)

$$\frac{S}{Dx} = \frac{1}{n} + \frac{K}{nD(1-x)}$$

in which S = total enzyme concentration

D = total ligand concentration

x = fraction ligand bound

n = number of binding sites

K = dissociation constant of the enzyme-ligand complex.

2.2.7. Fluorescence

The fluorescence measurements were performed with a Hitachi Perkin Elmer MP 2A fluorospectrophotometer which was equipped with a thermostated cuvette holder. The excitation and emission spectra are corrected for scatter of the solvent, and variations in light intensity of the 150 W Xenon-light source.

2.2.8. Recombination

Recombination of apoenzyme with FAD or its derivatives was performed mostly in 0.13 M sodium phosphate buffer (pH 7.6) which contained 0.3 mM EDTA. Samples were withdrawn at the times and temperatures indicated.

Preincubation experiments with FMN and FMN-analogues were performed on ice unless otherwise stated. The experimental conditions have been described in the text.

2.2.9. Serology

Antisera from rabbits were prepared by intraveneous injections and by a combination of intraveneous and intramuscular injections, the latter together with Freund's adjuvant incomplete. After a month intramuscular injections were left out of the immunisation scheme as they had no or little effect on the antiserum titers. Weekly 1.5-2 mg protein of either apoenzyme or DCIP-active enzyme and 2-3 mg of either holoenzyme or Cu^{2+}-modified enzyme, all prepared from one enzyme stock, were injected. After a month blood samples were collected from the ear veins at regular intervals. The γ-globulins of normal sera and antisera were prepared according to KEKWICK (1940).

The antisera titers were determined with the micro-precipitation reaction under paraffin oil (VAN SLOGTEREN, 1954) by observing this reaction after one hour incubation at 37°. The Ouchterlony agar double-diffusion test (OUCHTERLONY, 1962) was used to test the homogeneity and the relationships of the antisera. These tests were performed in the cold room.

2.2.10. Polyacrylamide disc electrophoresis

The acrylophore was from Pleuger; the power supply from Shandon. Stock solutions were made and gel polymerisation performed as described by ORNSTEIN and DAVIS (1962) and DAVIS (1964). The system consisted of a fine-pore gel (8 %) and a small layer of large-pore gel on top (4 %). The electrophoresis buffer used was a 5 mM Tris-glycine buffer (pH 8.3-8-5) and 3.5 mA per tube was applied during 3 hrs at 4° C. The amount of protein applicated varied between 30 and 60 micrograms.

Staining and fixation of the separated bands was performed in a 1 % Amido Schwarz solution in 5 % TCA during 3 hrs while* destaining occurred electrically in a 5-7 % acetic acid solution. The gels were analysed with a Photovolt model 520-A densitometer with reduced slit width.

To obtain a better resolution of protein bands in gels containing 8 M urea, these gels were made in three layers, a 4 % large-pore on top with a 7 % and a 10 % fine-pore gel beneath (VAN DORT, unpublished results).

* L.C. van Loon, unpublished results

3. PROTEIN ASSOCIATION-DISSOCIATION AND THE INFLUENCE ON CATALYTIC PROPERTIES

3.1. INTRODUCTION

NADH: lipoamide oxidoreductase, E.C. 1.6.4.3 is widespread in nature and has been isolated from a number of animals and plants, e.g. pig heart (STRAUB, 1939, SAVAGE, 1957, MASSEY, 1961), dog fish liver (CHANNING et al., 1962), Escherichia coli (HAGER and GUNSALUS, 1963; KOIKE et al., 1963), Mycobacterium tuberculosis (GOLDMAN, 1960), Saccharomyces species (WREN and MASSEY, 1965; MISAKA and NAKANISHI, 1963) and Spinacea oleracea (BASU and BURMA, 1960; MATTHEWS and REED, 1963). Originally many preparations were found to have a high diaphorase or menadione activity (STRAUB, 1939; SAVAGE, 1957; MISAKA and NAKANISHI, 1963) which could be lowered drastically by adding EDTA during the purification. Traces of metal ions, especially Cu^{2+}, are considered responsible for the modification of activity and spectrum (VEEGER and MASSEY, 1962; MISAKA et al., 1965). The physiological function consists of catalzing the oxidation of protein amide-linked dihydrolipoic acid by NAD^+, the lipoic acid having been reduced during the oxidative decarboxylation of pyruvate or α-oxoglutarate (HAGER and GUNSALUS, 1953; SEARLS and SANADI, 1960). SAVAGE (1957) reported for the pig heart enzyme a molecular weight of 81,000 based on sedimentation and diffussion coefficients. MASSEY et al. (1962) calculated molecular weights on the basis of sedimentation and diffusion values or with Archibald's approach to equilibrium method, the values varying from 98,000 to 114,000. Approximately the same molecular weight has been determined for lipoamide dehydrogenase from other sources (WREN and MASSEY, 1965; MATTHEWS and REED, 1963; MASSEY, 1963; KOIKE et al.,1962; MISAKA, 1966).

The enzyme contains 2 moles of FAD per 100,000 grams of protein. A disulfide vicinal to the flavin was demonstrated to be involved in the catalysis as well as FAD itself (MASSEY and VEEGER, 1960, 1961; SEARLS and SANADI, 1960). The oxidised enzyme is stable in 6.5 M urea, but reduction under these conditions with NADH or dithionite denatures the enzyme. This results in a FAD-free protein with half the molecular weight (41,000-48,000). MASSEY et al. (1962) proposed a model for the enzyme structure, the main features of which are two polypeptide chains held together by the active-centre disulfide bridges close to the two FAD's. However the data presented in this study and the preceding

one (KALSE and VEEGER, 1968) do suggest an alternative model, namely a monomer-dimer system in equilibrium. Preliminary data of this study has been published (VEEGER et al., 1968).

3.2. RESULTS

3.2.1. Gel filtration with Sephadex G-200 columns

The results from the gelfiltration experiments are given in Table I. The distribution coefficient for alcohol dehydrogenase was used to determine the effective pore radius of the Sephadex gel. The reliability of this value is supported by the Stokes radii which have been found for other proteins, e.g. bovine serum albumin, and ovalbumin (HABEEB, 1966). The r-values were found to vary between 180 Å and 200 Å for different columns and at different temperatures. To calculate the other physical constants which are derived from the Stokes radius, one must assume that the molecule does not expand or contract under these conditions. The data show that the apoenzyme differs considerably from the holoenzyme with respect to its physical properties. Though the distribution coefficients are more a function of the Stokes radius than of the molecular weight, the relation between $(K_d)^{1/3}$ and the $(\text{mol.wt.})^{1/2}$ is approximately valid. Using lactate dehydrogenase, alcohol dehydrogenase, bovine serum albumin, ovalbumin and ribonuclease for calibration of the column, the values for the mol.wt. of lipoamide dehydrogenase and its apoenzyme were found to be approximately 109,900 and 62,500, respectively.

3.2.2. Ultracentrifugation

The sedimentation coefficients for the apoenzyme and the enzyme reconstituted on ice are given in Table II. Ultracentrifugation studies, however, are rather limited due to the low protein concentrations of the apoenzyme preparations (2.5-3 mg/ml). The sedimentation coefficients at this concentration are much lower compared to the value of the holoenzyme. The values for the reconstituted DCIP-enzyme are very similar to those of the apoenzyme and suggest approximately the same dimensions. On the basis of diffusion coefficients as obtained with Sephadex-G 200 and with the analytical ultracentrifuge, estimations of the mol.wts of apoenzyme and the DCIP-enzyme have been made. They are compared, in Table III, with values for holoenzyme and Cu^{2+}-modified lipoamide dehydrogenase.

The data clearly indicate a mol.wt. of approx. 52,000 for both

the apoenzyme and the DCIP-enzyme. Additional evidence comes from the Trautman plot for the apoenzyme which is shown in Fig. 1. The non--linearity of this plot is partially due to residual holoenzyme left in the apoenzyme preparation. The mol.wt. determined by this method was calculated to be 54,4000, again half that of the holoenzyme. Further evidence that the mol.wt. of apoenzyme is different from that of the native holoenzyme comes from incubating apoenzyme with FAD at 25°. This restores the lipoic acid activity and results in a sedimentation peak with a sedimentation coefficient identical to that of the holoenzyme itself.

3.2.3. Monomerigation under anaerobic conditions

MASSEY et al. (1962) reported denaturation of lipoamide dehydrogenase when studied under anaerobic conditions in a 0.03 M sodium phosphate buffer which contained 6.5 M urea and excess NADH. Anaerobic denaturation under the influence of NADH and dithionite resulted, as in the former case, in a flavin-free protein. In both cases the molecular weight proved to be approximately half that of the holoenzyme. The apoenzyme is more easily solubilised at high ionic strength, therefore three mgs of native lipoamide dehydrogenase were dissolved in 0.15 M sodium phosphate buffer (pH 7.2) which contained 0.3 mM EDTA and 6.5 M urea. The enzyme was made anaerobic in a Thunberg cell after which excess of NADH was added. The solution was kept anaerobic at 7° for 30 minutes. After opening the Thunberg tube, the solution was poured immediately over a Sephadex G-25 column (10 cm height) which was equilibrated with 0.15 M sodium phosphate buffer (pH 7.2) and 0.3 mM EDTA. On the Sephadex G-25 column, two fluorescent bands separated: one containing the holoenzyme and apoenzyme both migrating through the void volume, the second one containing FAD which had been liberated. The activities before and after FAD addition are shown in Table IV.

The return of a relatively high DCIP-activity on ice after adding FAD as well as the increase in lipoate dehydrogenase activity after elevating the temperature are phenomena characteristic for the presence of apoenzyme, e.g. monomerisation (cf. ref. KALSE and VEEGER, 1968). Monomerisation coupled with an increase in DCIP-activity also takes place under anaerobic conditions upon addition of NADH. It is known from previous studies with this enzyme (cf.ref. MASSEY et al., 1961) that anaerobic addition of NADH to lipoamide dehydrogenase results in the formation of a semiquinone by the uptake of two reducing equivalents, with a very small fraction of totally (4-equivalent) reduced enzyme

Fig. 1. Trautman plot of the apoenzyme of lipoamide dehydrogenase at 4°. 0.1 M sodium phosphate buffer (pH 7.6) which contained 0.3 mM EDTA was used. Rotor speeds, □---□, 21 000 rev./min; o—o, 30 700 rev./min; ●—●, 40 500 rev./min. The protein concentration was 2.0 mg/ml; activities with oxidised lipoate and with DCIP were 3 % and 25 %, respectively.

Fig 1

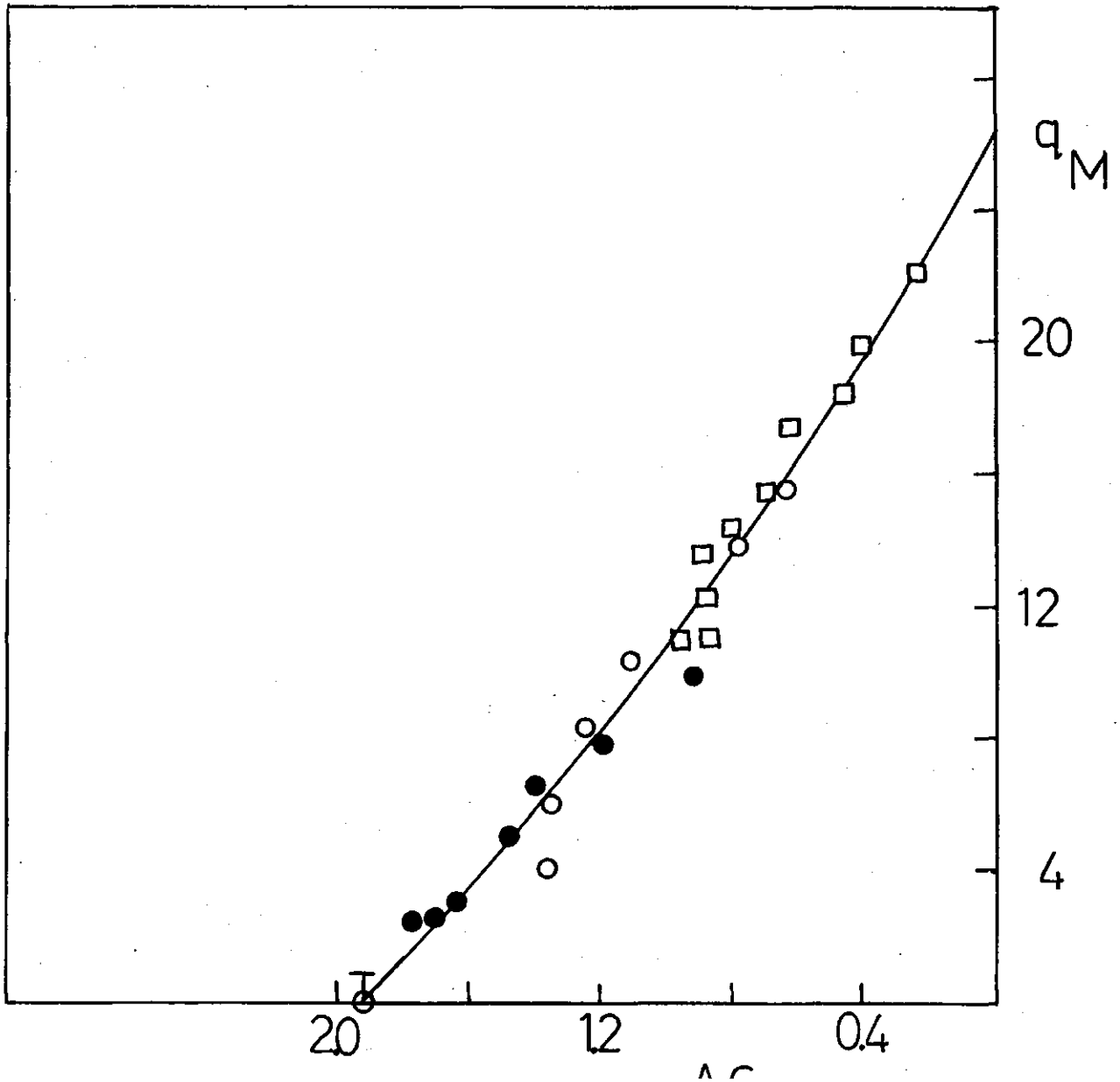
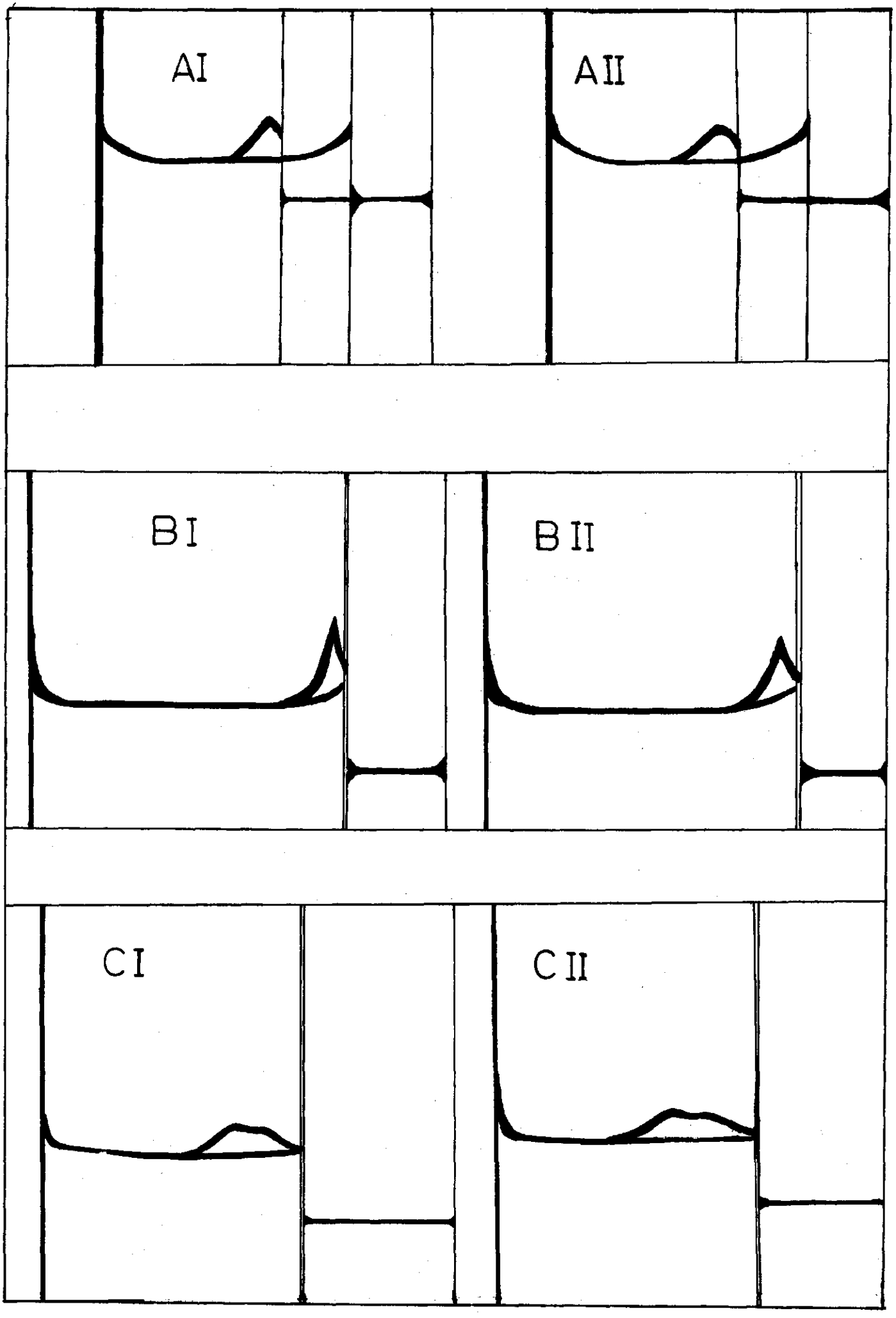


Fig. 2. Sedimentation patterns of lipoamide dehydrogenase apoenzyme, recombined DCIP-active enzyme and anaerobically frozen enzyme. A. Apoenzym (2.6 mg/ml) in 0.15 M sodium phosphate (pH 7.2) with 0.3 mM EDTA. Temperat 19.2°; rotor speed, 58 400 rev./min; pictures taken at 13 min (I) and 17 m (II). B. Recombined DCIP-enzyme (2.6 mg/ml) in 0.16 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Temperature, 3°; rotor speed, 55 100 rev min; pictures taken it at 9 min (I) and 14 min (II). C. Anaerobically froz (6 mg/ml) in 0.03 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Temperature, 8°; rotor speed, 49 600 rev./min; pictures taken at 48 min (I and 60 min (II). Sedimentation is from right to left.

Fig 2



present at 25°. The latter fraction increases by lowering the temperature to 0°; concomitantly the activity of the preparation with DCIP increases.

In Table V the results summarised have been obtained by freezing holoenzyme with a tenfold molar excess of NADH under nitrogen during 5 days. Under these conditions the DCIP-activities are much higher than those obtained at 0° under reducing conditions. The preparations became colourless during the freezing process and are practically non-fluorescent. As can be concluded from Table V the differences in activities between the different treatments are only slight.

It is of importance to known if the four-electron reduced state is coupled to a monomer structure and permits the dimer to dissociate to give an enzyme comparable with the DCIP-active monomer, or whether it may remain a dimer but with another conformation resulting in a high activity with DCIP, comparable with that of the Cu^{2+}-modified enzyme. Therefore, higher protein concentrations (5 mg/ml) were also frozen under anaerobic conditions with an excess of NADH. After reoxidation on ice, the preparations were analysed with the analytical ultracentrifuge. The sedimentation patterns showed two components in contrast to the oxidised or the oxidised frozen enzyme. This presumably represents a monomer-dimer equilibrium as judged from the observation that upon incubation at 25°, the DCIP-activity which is originally high declines, accompanied by an increase of the $lipS_2$--activity. In Table VI a survey of the results under different conditions is given.

Fig. 2 gives the sedimentation patterns of the frozen reduced enzyme, apoenzyme and reconstituted DCIP-active enzyme.

3.2.4. Light-scattering

Light scattering has been used to demonstrate that the native holoenzyme dissociates upon dilution, even at a neutral pH. In Fig. 3 the molecular weight has been plotted versus the protein concentration. M_{app} values differ slightly in different experiments between 95,000 and 100,000. The M_{app} value decreases below enzyme concentrations of 0.1 mg/ml which is in accordance with the protein concentration at which the lipoate activity declines (cf. VEEGER et al., 1969). Extrapolation to zero concentration, although rather tentative, results in a monomer molecular weight of 48,000-50,000. Amounts of monomer and dimer present can be calculated according to STEINER (1952) using

Fig. 3. Relation between molecular weight (M_{app}) and concentration of lipoamide dehydrogenase. Enzyme (A280/A455=4.9) was dialysed against sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA, ●●● 30 mM, XXX 100 mM, ■■■ 100 mM with 1 mM $(NH_4)_2SO_4$ or against sodium phosphate pH 8.2 containing 0.3 mM EDTA, 30 mM (▲▲▲). Light-scattering measurements were done 20 to 30 min after diluting the enzyme at 20°. The corresponding lipoate activities were determined in the case of the 30 mM phosphate buffer pH 7.2 (ΔΔΔ) and pH 8.2 (000).

Fig.3

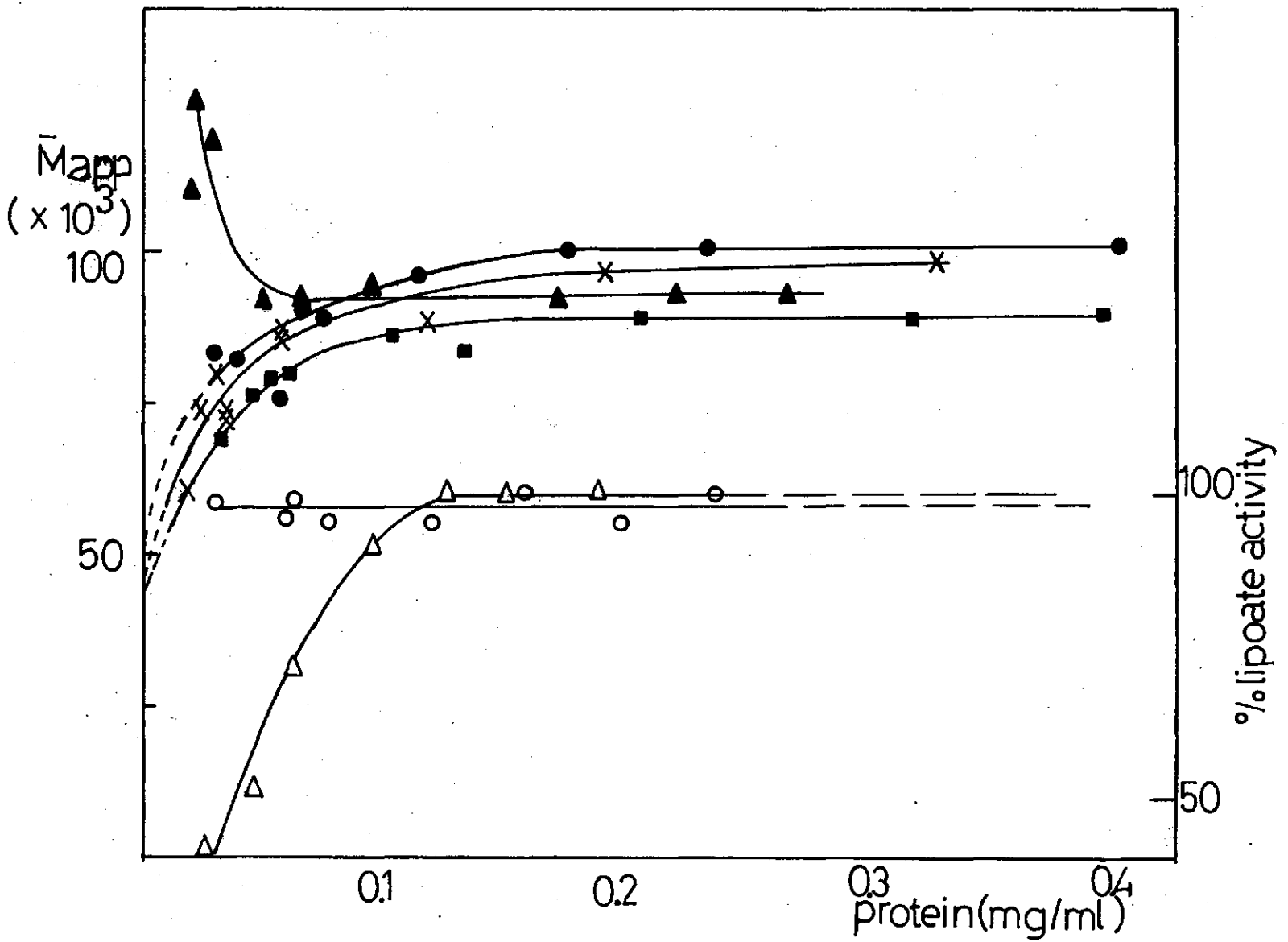
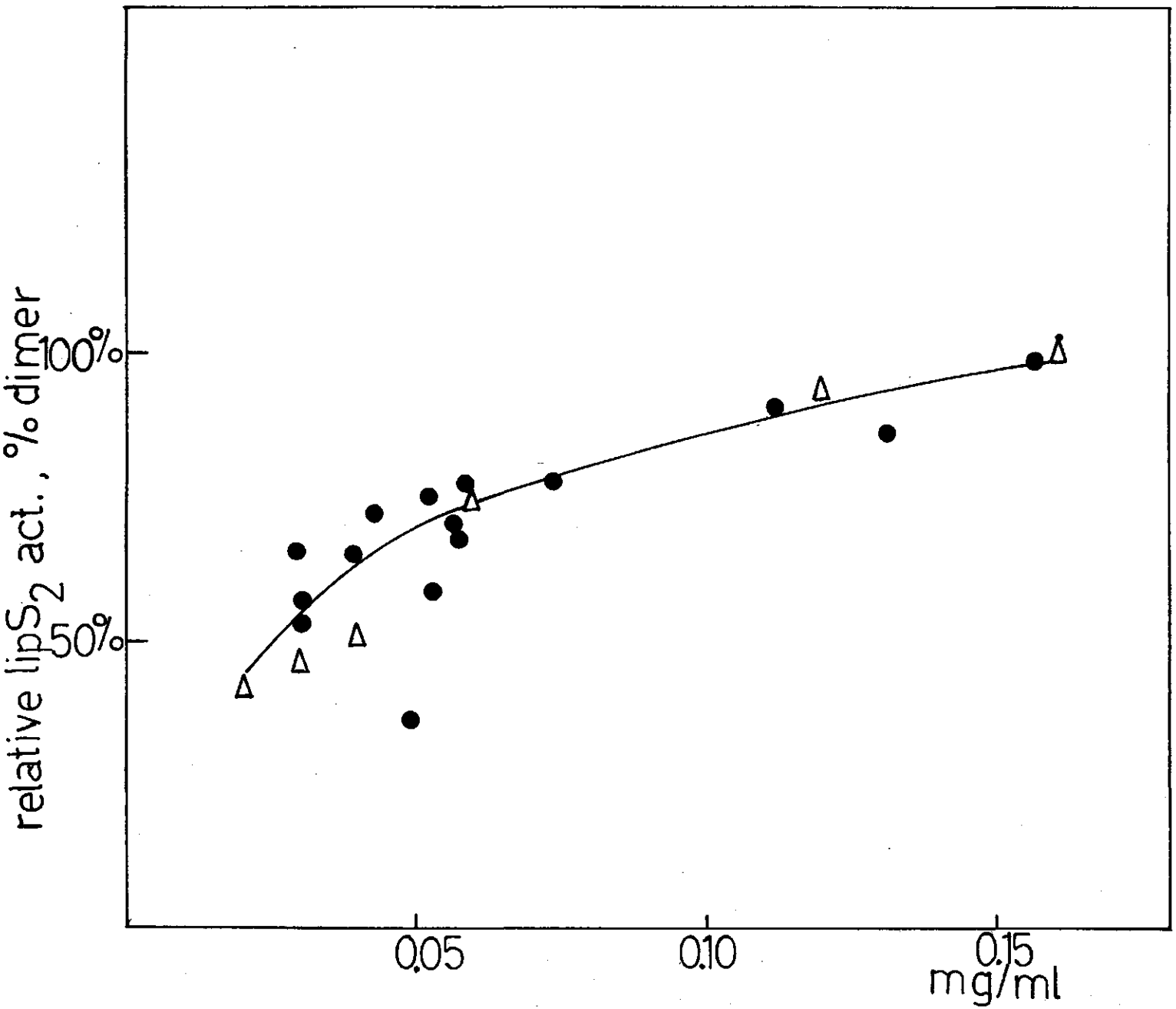

$\bar{M}_{app}$
($\times 10^3$)
100
50
100
50
% lipoate activity
0.1
0.2
0.3
0.4
protein(mg/ml)

Fig. 4. Relation between the percentage of dimer present as calculated wit
the Steiner method (see text) and the percentage of lipoate activity. The
experimental data from the different plots in Fig. 3 were used to calculat
the percentage of dimer while the lipoate activities correspond also with
Fig. 3. ΔΔΔ, % lipoate activity; ●●●, % dimer from M_{app}.

Fig.4



$$M_w = \frac{c_1 M_1 + 2c_2 M_1}{c}$$

With the aid of this method (for details cf. EISENKRAFT, 1969) association constants of $3\text{-}6 \times 10^6$ l mole^{-1} can be calculated.

The relative lipoate activity and the percentage of dimer present (see Fig. 4) are fairly well in agreement which is indicative of the existence of a dimer - monomer equilibrium in which the dimer is the lipoate-active species.

Similar results were obtained in 30 mM as well as in 100 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA. Adding 10 mM $(NH_4)_2SO_4$ to the 30 mM buffer system results in a lowering of the M_{app} value as measured in the dimer region (88,000-90,000) which might be due to a different optical constant K.

The monomer-dimer equilibrium is pH-dependent as shown in Fig. 3; in 30 mM sodium phosphate buffer pH 8.2 there is no decrease in molecular weight upon dilution but rather the reverse, a slight increase. The lipoate activity remains constant over the whole range.

The dissociation of the holoenzyme is not completely understood. For instance it is not known whether the enzyme derived from α-KGDC behaves identical in this respect as the one from PDC. The enzyme preparation described was stored in a concentration of 10 mg/ml during four months at -14° which resulted in a partial loss of activity. Dilution of the enzyme did not result in a further loss of the lipoate activity. The electrophoretic pattern obtained was the same as known for the frozen enzyme conformation (cf. Chapter 8) though conclusions are hard to draw as no electrophoretic pattern was made of the original dissociating holoenzyme.

3.2.5. Sucrose gradients*

A preliminary experiment in which apoenzyme was centrifuged in a sucrose gradient with a saturating concentration of flavin resulted in a partial separation of lipoate activity and DCIP-activity. Previously the meaning of both activities has been discussed in connection with the dimerisation reaction, viz. the dimer structure having a high lipoate activity, the monomer active with DCIP.

In Fig. 5a the distribution over a sucrose gradient of equal amounts of apoenzyme, recombined enzyme and holoenzyme from the same sample is compared. The temperature during the run was kept at approxim. 5° to prevent apoenzyme denaturation as well as total dimerisation.

*Footnote: this part of the work has been fulfilled in collaboration with ir. Cees de Ranitz.

In the case of the apoenzyme, the gradient shape was determined first before adding excess FAD to the samples. The activities with DCIP were measured after 10 min. incubation on ice. From Fig. 5a it is obvious that the apoenzyme is normally symmetrically distributed. The curve of the lipoate activity is slightly asymmetrical caused by a dimer rest-activity superimposed on a partial lipoate activity caused by a dimerisation in the apoenzyme fractions after addition of flavin. The lipoate activities were remeasured after elevating the temperature to 22°; the position of the band is as expected identical to the original DCIP-activity curve. The band position of the apoenzyme is completely different from the holoenzyme which is used as a reference. These results suggest that the apoenzyme has another density than the holoenzyme as centrifugation for 24 hrs 45.000 rpm is enough to assume the system to be in equilibrium. Moreover, it is clear that the apoenzyme itself does not associate without FAD. In a sucrose gradient to which FAD is added there exists a monomer-dimer equilibrium (Fig. 5b); the DCIP-active monomer is partially converted into dimer even at low temperatures. Although there is no exact identity between the positions of the apoenzyme and the FAD-apoenzyme monomer complex the lipoate activity in the flavin containing gradient corresponds to the holoenzyme position. This dimer activity is asymmetrical and skewed to the monomer side due to the equilibrium.

The experiments as described above using a sucrose gradient with flavin were performed in bi-distilled water (pH 5,8). Using 30 mM sodium phosphate buffer (pH 7.2) results to a large extent in a protein association during running in the presence of FAD, even at low temperatures. Presumably this is due to a pH-effect as the pH-optimum for return of the lipoate activity is near to pH 7.2 (see Chapter V) in combination with ionic strength.

Another remarkable point is that the DCIP-activity which is still stimulated, sometimes greatly overlaps or even coincides with the lipoate activity while only this DCIP-activity curve is skewed to the monomer side (Fig. 6). This is strongly suggestive for the existence of a dimer conformation which still has a high artificial activity with DCIP in between the DCIP-active monomer and the ultimate dimer structure fully active with lipoate (cf. Chapter V).

Preincubation of apoenzyme with FAD and subsequent centrifugation of the recombination mixture on a FAD-gradient at 5° also separates the lipoate - and DCIP - activities. The position of the lipoate activity corresponds to the holoenzyme position, the DCIP-activity to the monomer

position (Fig. 5b,c).

The holoenzyme was also studied in a gradient containing 100 μM FMN; the lipoate activity was found back in the region corresponding to the dimer and no indication was obtained that a FMN change for FAD occurs in the holoenzyme. An apoenzyme which was regaining its lipoate activity very fast upon recombination was kept for 4 hrs at 25° with FAD before running it in a FMN containing gradient. The monomer DCIP-activity was hard to detect but the lowered lipoate activity (cf. Chapter V) proved to be due to a dimer. Although no quantitative data is available this indicates at least that part of the recombined enzyme regains its original properties (cf. Chapter V).

In Fig. 7 the results are given for the apoenzyme distribution after recombination during 16 hrs with FAD or FMN at room temperature. Excess of flavin was removed by sieving the recombination mixtures on a short Sephadex G-25 column (8 cm height, ∅ 0.5 cm). The fluorescent samples were centrifuged on sucrose gradients which did not contain any flavin. The fractions were analysed for fluorescence properties and lipoate activity. In the case of the FAD-recombined enzyme the protein fluorescence coincides completely with the flavin fluorescence and also the lipoate activity. This indicates that the flavin is firmly bound to the protein after such a long preincubation time (cf. binding forces of flavin binding in Chapter V). The FMN apoenzyme recombination mixture exhibits an intensive protein--fluorescence with a maximum not corresponding with the density of the FAD enzyme but coinciding with the monomer position. Moreover, the fluorescence excitation spectra show a double peak identical to the patterns obtained with the apoenzyme. No lipoate activity is present under the maximum of the protein fluorescence while incubation with excess of FAD in that region partially restores the $lipS_2$-activity. This means that the FMN is reversibly bound and split off during the run to be distributed over the whole gradient. In the dimer region there is a weak detectable flavin fluorescence corresponding with a low lipoate activity. As the relative 520 nm flavin fluorescence emission intensities upon 280, 360 and 450 nm excitation are practically the same as in the FAD experiment we assume that this fluorescence is due to FAD which was left in the apoenzyme. Moreover, no lipoate activity with FMN has been found.

Fig. 5. Distribution of apoenzyme, recombined enzyme and holoenzyme over sucrose density gradients. Condition: 22 hrs at 5° at 45 000 rpm. A. Apoenzyme (0.15 mg) distribution over a sucrose gradient assayed by adding to the fractions excess FAD on ice during 10 min before determining the DCIP--activity (●●●) and lipoate activity (+++). The lipoate activity was remeasured after elevating the temperature (ΔΔΔ) during 30 min. B. Apoenzyme (0.15 mg) distribution over a buffered sucrose gradient containing 100 μM FAD assayed by measuring DCIP-activity (●●●) and lipoate activity (+++). C. Holoenzyme (0.07 mg) distribution over a buffered sucrose gradient as measured by the lipoate activity (+++).

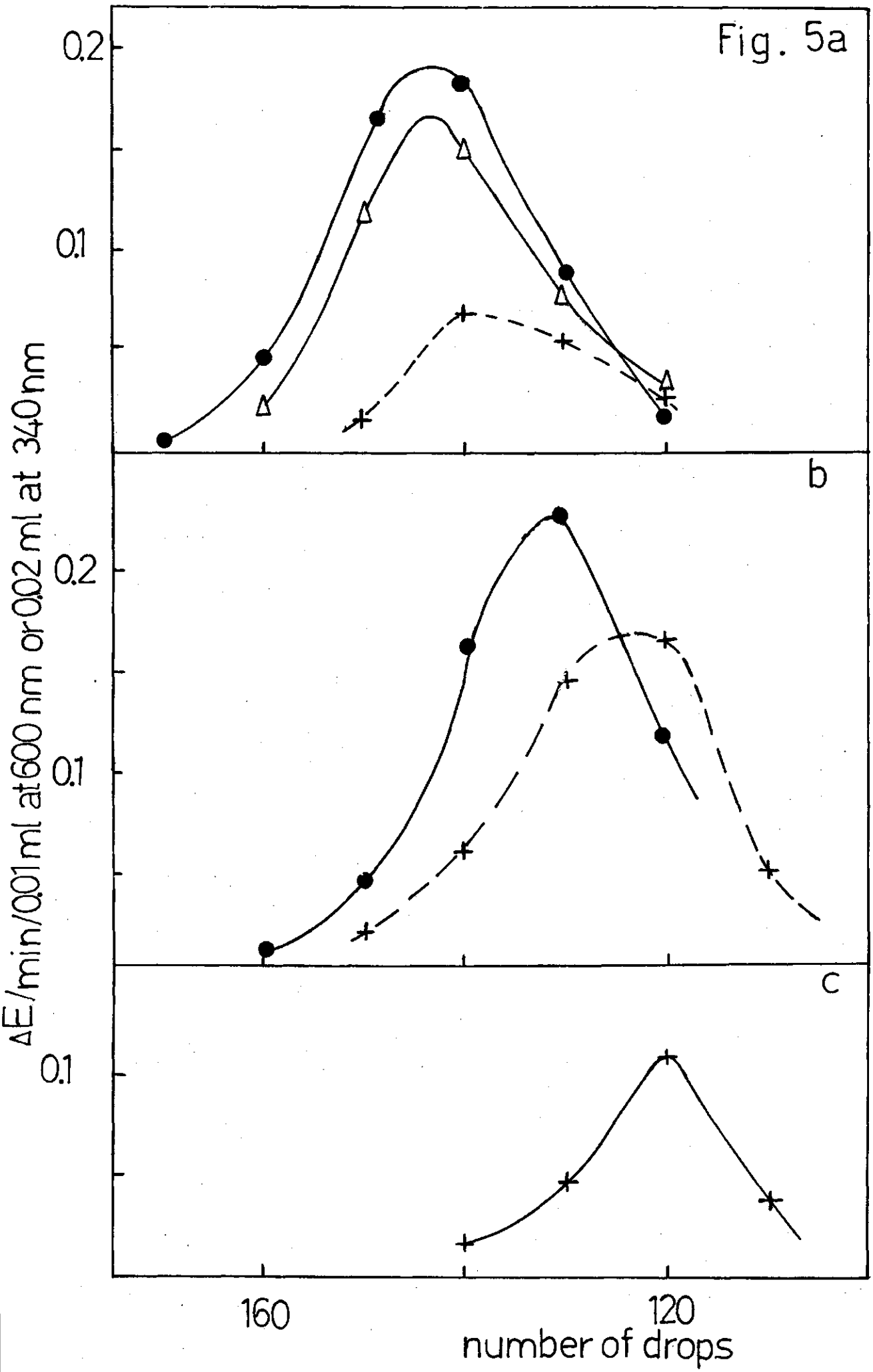


Fig. 5a
0.2
0.1
b
0.2
0.1
c
0.1
ΔE/min/0.01 ml at 600 nm or 0.02 ml at 340 nm
160
120
number of drops

Fig. 6. Distribution of recombined enzyme and holoenzyme over a buffered sucrose gradient. Apoenzyme (0.2 mg) was centrifuged for 20 hrs on a sucr gradient containing 100 µM FAD, 30 mM sodium phosphate buffer pH 7.2 and 0.3 mM EDTA. Temperature approx. 7°. From the recombined system the lipoa activity (ΔΔΔ) and the DCIP-activity (●●●) were measured. From the holoenzyme (0.2 mg) the $lipS_2$-activity was determined (+++).

Fig6

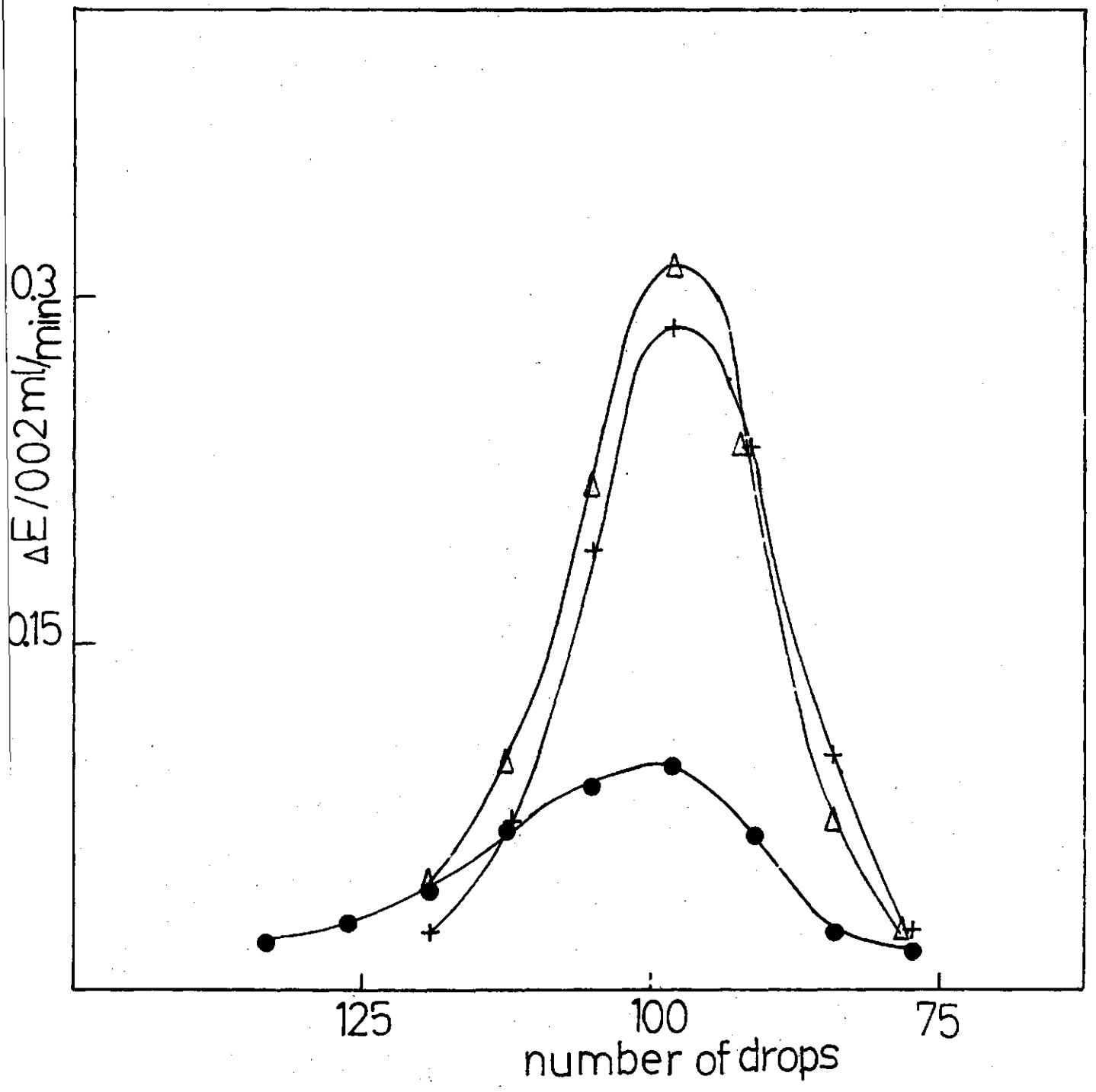


Fig. 7. Distribution of FMN- and FAD recombined enzyme over a buffered sucrose gradient after removing excess of flavin by Sephadex-G25 sieving. A. Apoenzyme (0.17 mg) was preincubated with 100 μM FAD during 20 hrs at room temperature, and run for 20 hrs at 45 000 rpm(5°). The fractions were analysed for protein fluorescence at 350 nm emission wavelength (●●●), flavin fluorescence emission at 520 nm (ooo) and lipoate activity (+++). B. Apoenzyme (ooo) was preincubated with 100 μM FMN during 20 hrs and run under the same conditions as A. Lipoate activity (+++) determined after addition of excess flavin to the samples and incubation at room temperature

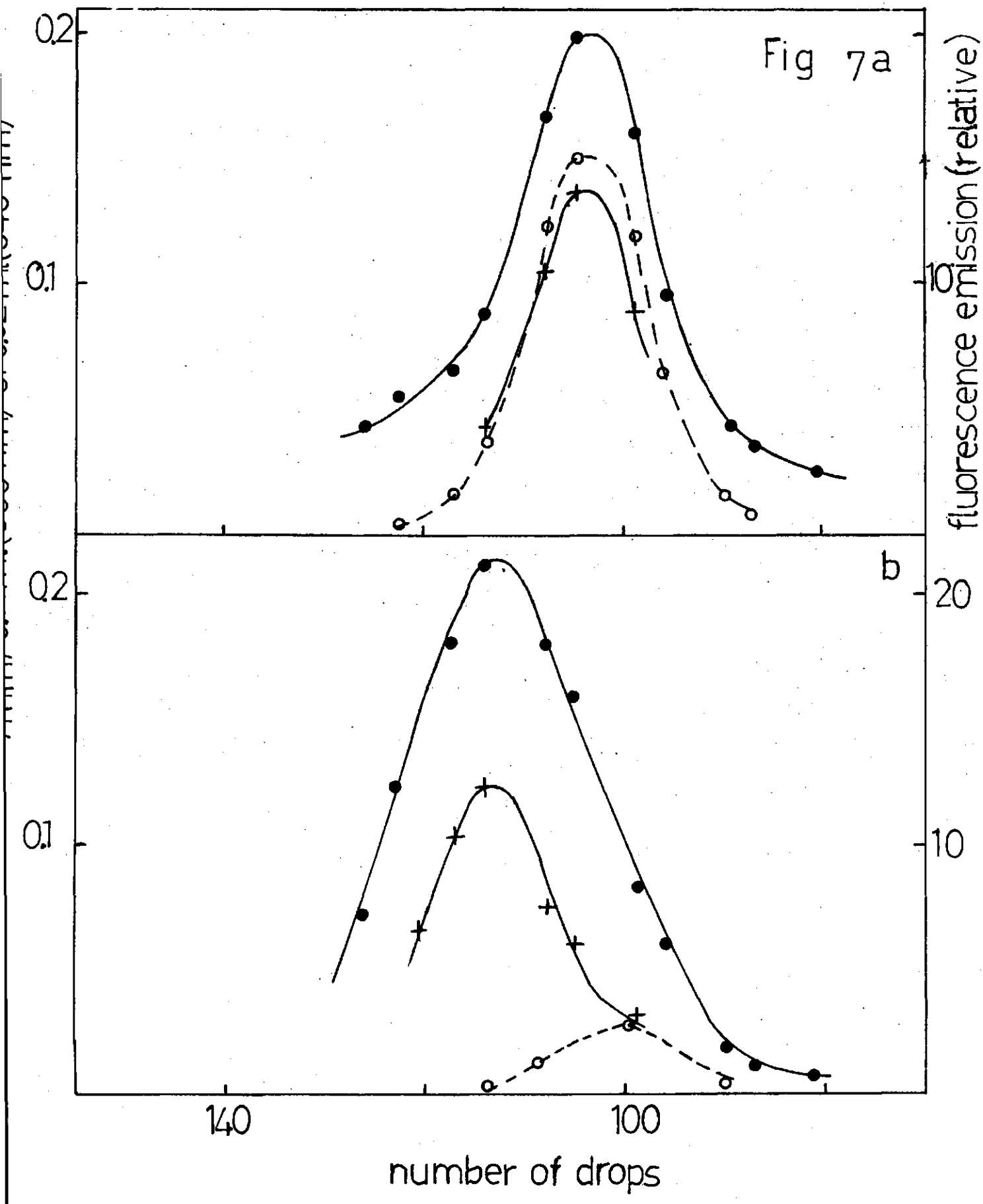
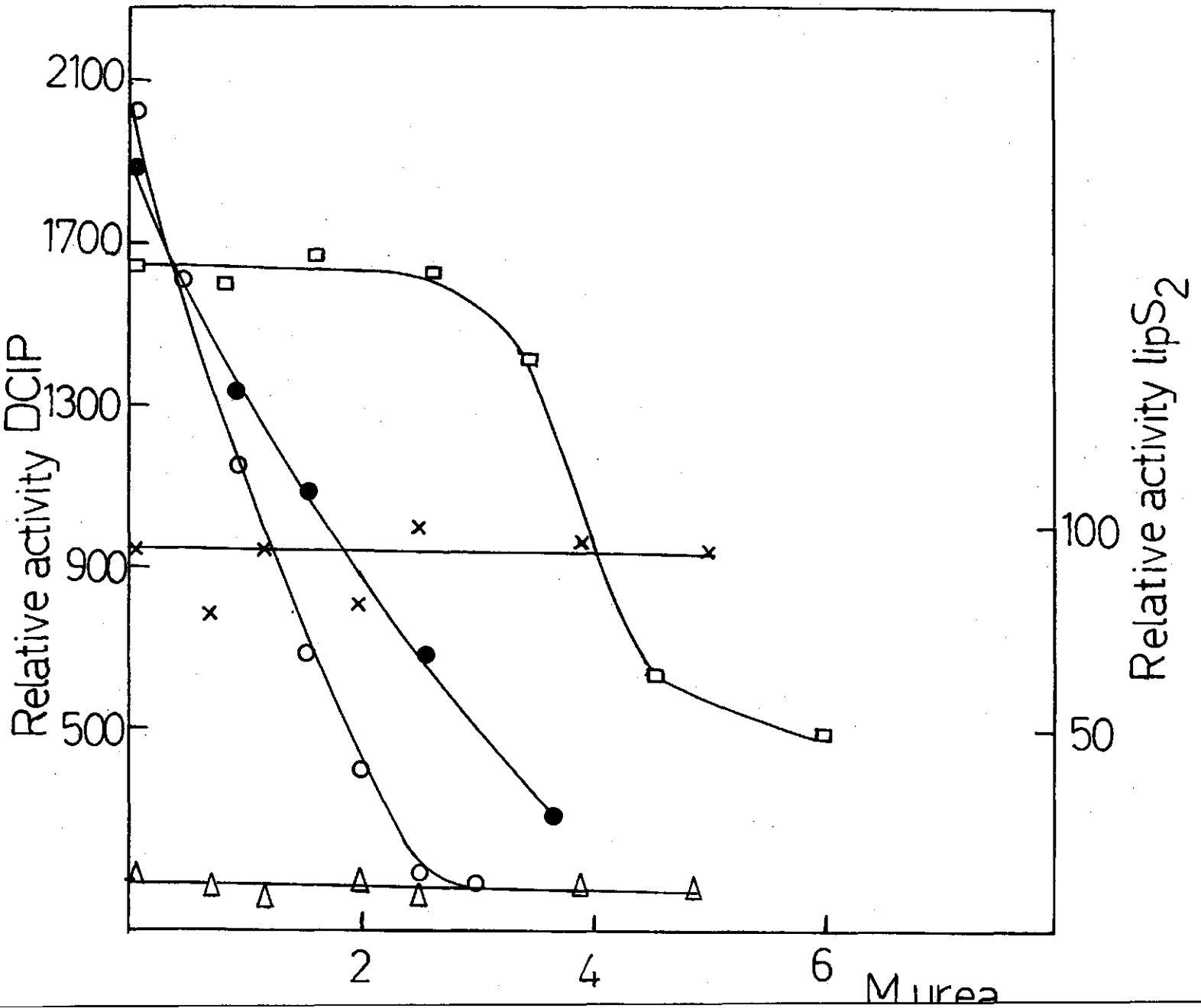


Fig 7a
0.2
0.1
10
fluorescence emission (relative)
b
0.2
0.1
20
10
140
100
number of drops

Fig. 8. Influence of urea on the different conformations of lipoamide dehydrogenase. The DCIP-activities of the different forms of enzyme were measured, O—O, DCIP-enzyme (monomer); ●—●, frozen enzyme; □—□, Cu^{2+}-modifi enzyme; Δ—Δ, native holoenzyme (with DCIP). X—X, activity with oxidised lipoate of native holoenzyme. The different enzymes were incubated with ur for 10 min on ice before samples were withdrawn and the activity measured described under METHODS. The protein concentrations varied between 0.3 and 0.5 mg/ml. The reconstituted DCIP-active enzyme and the frozen enzyme were allowed to stand for 15 min on ice after thawing before use.

Fig.8


2100
1700
1300
900
500
Relative activity DCIP
100
50
Relative activity lipS2
2
4
6
M urea

3.2.6. Sensitivity of the different enzyme conformations to urea

It is known that incubation of the normal oxidised lipoamide dehydrogenase with 6.5 M urea only results in a small decrease in activity with lipoic acid and with DCIP. The Cu^{2+}-modified enzyme, however, is less stable; 10 minutes incubation with a concentration of 4 M urea results in a 60 % decrease of activity.

The apoenzyme itself is very sensitive to urea; a concentration of 1 M largely prevents recombination with FAD as can be concluded from the small increase in fluorescence polarisation and the small enhancement of the DCIP-activity. This explains the relatively small restoration of DCIP-activity after reductive urea-treatment. The reconstituted DCIP-enzyme is also urea sensitive though much less than the apoenzyme itself.

The frozen enzyme which has high DCIP-activity behaves in a similar way as the DCIP-active enzyme. In Fig. 8 the results of urea--treatment upon the forms of lipoamide dehydrogenase are given.

3.3. DISCUSSION

The sedimentation coefficients for the holoenzyme and for the Cu^{2+}-modified enzyme are identical. An $s_{20,w}$ value of 5.8 S was calculated from experiments performed in 0.1 M sodium phosphate buffer (pH 7.2). Since the protein concentration dependency of the sedimentation constants is very small for this enzyme, the discrepancy between this value and those reported previously (SAVAGE, 1957; MASSEY et al., 1962) is rather small. The values as described here are similar to those for the E.coli enzyme (KOIKE et al., 1962). The differences in values for the mol.wts reported in the literature are mainly due to differences in diffusion coefficients. SAVAGE found a $D_{20,w}$ value of $6.08 \times 10^{-7} cm^2 sec^{-1}$ while MASSEY reported $4.63 \times 10^{-7} cm^2 sec^{-1}$.

The diffusion coefficients based on present Sephadex G-200 experiments are 5.07 and $5.19 \times 10^{-7} cm^2 sec^{-1}$ for the holoenzyme and the Cu^{2+}-modified enzyme, respectively. These values are more in agreement with those of Massey and practically identical with the values reported for the E.coli enzyme.

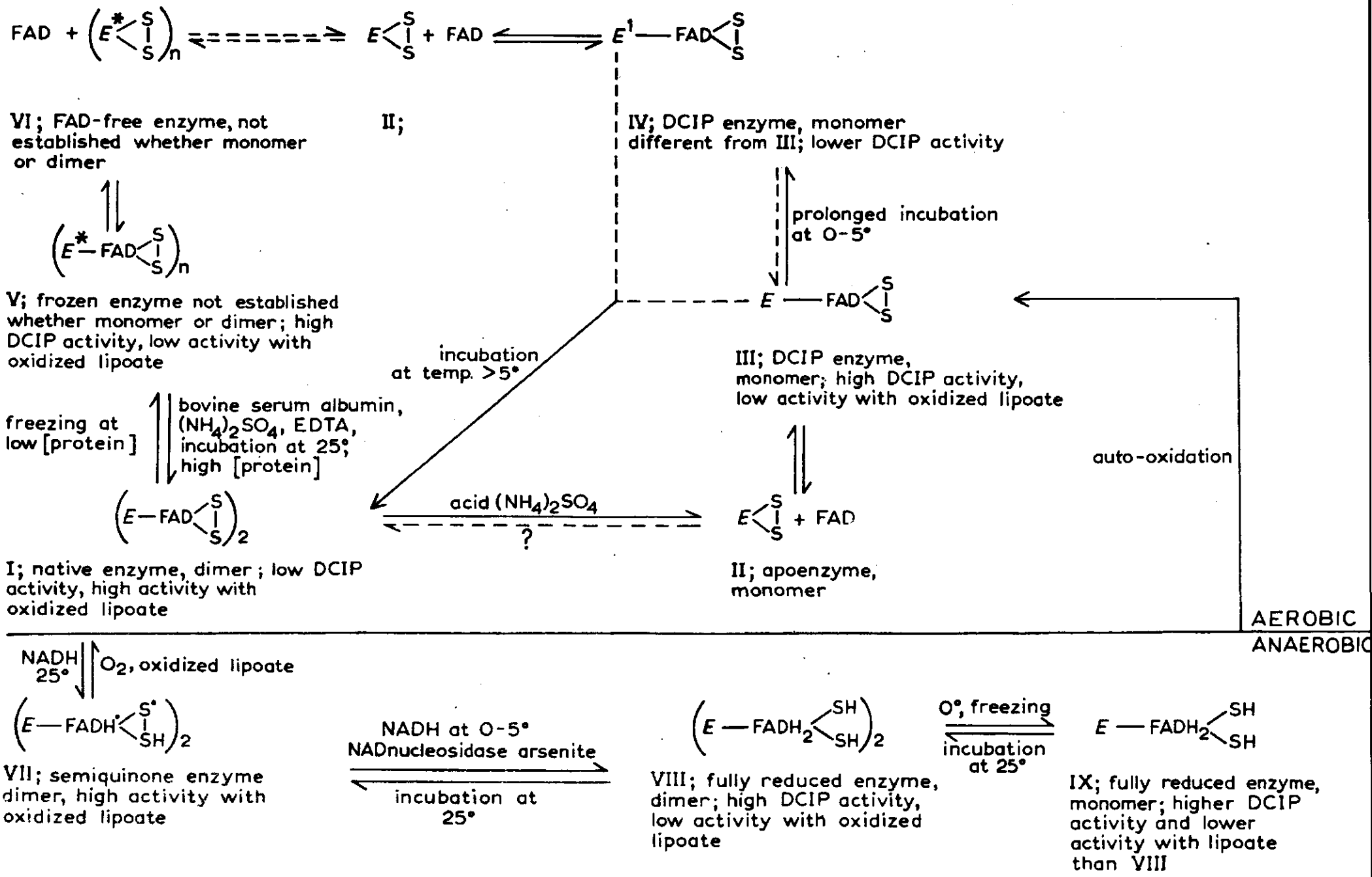
Several independent methods, viz. gel filtration, sedimentation and diffusion, and approach to equilibrium centrifugation, give ample evidence for a mol.wt. of the apoenzyme of approximately 52,000. This value is in fair agreement with the value Massey described for the inactive, urea-treated protein (cf. MASSEY et al., 1962). As has already

been marked in this paper, it is possible to demonstrate the presence of apoenzyme under the anaerobic conditions used by Massey. One must take care to choose a buffer with a high ionic strength and not to expose too long the formed apoenzyme to the 6.5 M urea. Therefore the apoenzyme can be made both under anaerobic and under aerobic conditions (cf. ref. KALSE and VEEGER, 1968).

The present results indicate that the model described by Massey for lipoamide dehydrogenase in which two disulfide bridges are considered responsible for the association of the two polypeptide chains, is rather unlikely. The consequence of such a model would be that it must be assumed that either the disulfide bridges must be opened to form sulfhydryl groups under aerobic, acid conditions which is very unlikely, or that an exchange must occur in which the interpeptide S-S bridges are replaced by two intrapeptide S-S bridges. If an interpeptide S-S bridge is replaced by an intrapeptide one by acid-$(NH)_2SO_4$, it is difficult to understand, why the apoenzyme does not show a mol.wt. equal to that of the holoenzyme, since after preparation it is dissolved in neutral buffer, in which a reversed exchange to the original interpeptide bridges could be expected in that case. But even accepting this possibility one needs the binding of FAD to the apoenzyme plus elevated temperature for the sulphur exchange.

Although this mechanism cannot be completely excluded, it is in my opinion more logical to explain the results by a simpler mechanism i.e. the association, without sulphur exchange, of two molecules of DCIP--active monomer to the $lipS_2$-active dimer.

It accounts for the observed second order rate of return of the $lipS_2$-activity (cf. ref. KALSE and VEEGER, 1968), which is much more difficult to envisage in case of an exchange of S-S bridges. Further arguments can be found in Table VI, where it is shown that in the presence of a large excess of arsenite monomer is still present. In the case of an interpeptide S-S bridge this would be impossible since arsenite would connect the reduced peptides. The most important argument in favour of an association is the observation that the FAD containing monomer is very active with DCIP (KALSE and VEEGER, 1968). This phenomenon cannot be explained at all by the original model of MASSEY et al. (1962). The results are summarised in Scheme I.



Scheme I. Relation between conformations and activities.

In a previous paper (cf. ref. KALSE and VEEGER, 1968) evidence for the existence of conformations II, III and IV, with their activities and mol.wts has also been given and does not need discussion.

A few remarks need to be given in connection with the mol.wts of conformation V and VI. No actual evidence indicates that freezing of the oxidised holoenzyme leads to a monomer with DCIP-activity. However, the strong dependence of the formation of these forms on the protein concentration as well as the similar values of the association constants for FAD-binding by the apoenzymes prepared by acid $(NH_4)_2SO_4$ and freezing and the urea sensitivity support this. As a counter argument the results with Cu^{2+}-modified enzyme might be taken, indicating that this protein is a dimer. However, there is the possibility, that at the very low protein concentrations of the assay (1-5 µg/ml) dissociation does occur.

The present results show that under reducing conditions freezing causes monomerisation. This is accompanied by an increase in DCIP-activity in comparison with reduction at 0° without freezing. It is possible that the DCIP-activity of the enzyme reduced by NADH at 0° in the absence or presence of arsenite (cf. ref. MASSEY and VEEGER, 1960) reflects the presence of monomer. Experiments in this laboratory have shown that the conversion of VII into VIII by lowering the temperature in the presence of an excess of NADH, as can be followed by the appearance of the $FADH_2$-NAD^+ charge transfer band at 700 mµ (cf. ref. VEEGER and MASSEY, 1962) is slow and takes several hours. On raising the temperature to 25° the reverse reaction is much faster. This indicates that this process is directed by conformational changes rather than shifts in equilibrium.

STEIN and CZERLINSKY (1967) have reported that addition of NADH to lipoamide dehydrogenase results in a S-shaped titration-curve. Our own results (cf. ref. MASSEY et al., 1962) have never shown such a relationship. It is possible, that the presence of DCIP-active enzyme like the frozen enzyme, is responsible for this. A gradual conversion at 25° into the native enzyme during the titration can lead to such a result, because it can be expected that the frozen enzyme, as the Cu^2-modified enzyme will show a spectral response at 450 and 530 mµ which is different from that of the native enzyme.

Table I

Gelfiltration on Sephadex - G 200 columns. Column height approx. 80 cm ∅ 12 mm. The buffer used for elution was 0.05 M Tris - HCl (pH 7.5) which contained 0.1 M KCl and 0.3 mM EDTA. For the apoenzyme a 0.15 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA was used. Temperature was between 4 and 8°, except in the experiment with DCIP-enzyme. The r-values of the columns were based on the determined K_d-value of alcohol dehydrogenase and its known Stokes radius, 46 Å (ref. ACKERS, 1964; SIEGEL and MONTY, 1966). The $f/_{fo}$ - and $D_{20,w}$-values were calculated according to ref. SIEGEL and MONTY (1966), assuming a mol.wt. of either 104,000 for native lipoamide dehydrogenase and Cu^{2+}-modified enzyme or 52,000 for the apoenzyme and reconstituted DCIP-enzyme (cf. Table III).

Preparation	No of experiments	Stokes radius (Å)	$f/_{fo}$	$D_{20,w}$ $cm^2/sec \times 10^7$
Lipoamide dehydrogenase	6	42	1.35	5.07
Cu^{2+}-modified enzyme	2	41	1.32	5.19
Apoenzyme	3	31	1.25	6.86
Reconstituted DCIP-enzyme	1	33	1.33	6.45

Table II

Sedimentation coefficients of the apoenzyme of lipoamide dehydrogenase and the enzyme recombined on ice. The apoenzyme and the reconstituted enzyme were extensively dialysed against 0.1 M or 0.15 M sodium phosphate buffers which contained 0.3 mM EDTA (pH 7.2). Rotor speed 55,000 rpm, temperature as indicated.

Preparation	Protein concentration	Temperature o	$s_{20,w}$
	1.2 mg/ml	12	4.0
Apoenzyme	2.3 mg/ml	7.8	4.4
Apoenzyme	2.6 mg/ml	19.5	4.2
Reconstituted	2.6 mg/ml	3	3.8
DCIP-enzyme	2.6 mg/ml	6.8	3.7

Table III

Molecular weight of lipoamide dehydrogenase holoenzyme Cu^{2+}-modified enzyme, apoenzyme and DCIP--enzyme. The values for diffusion coefficients and sedimentation coefficients are also given; for the apoenzyme and the DCIP-enzyme the same buffer and the same temperature were used for both diffusion and sedimentation (see Table II). $\bar{V}$ is assumed to be 0.73. $D_{20,w}$ for Sephadex G-200 was calculated according to ref. SIEGEL and MONTY (1966); $D_{20,w}$ ultracentrifuge was calculated according to ref. ELIAS (1961).

Preparation	$D_{20,w}$ $cm^2/sec \times 10^7$ Sephadex G-200	$D_{20,w}$ $cm^2/sec \times 10^7$ Ultracentrifuge	$s_{20,w}$	$M_{s,D}$ D-Sephadex	$M_{s,D}$ D-ultracentrifuge
Holoenzyme	5.07	-	5.8	102.800	
	-	4.63*	5.3*		102.800*
	-	6.08**	5.7**		84.200**
Cu^{2+}-modified enzyme	5.19	-	5.8	105.600	
Apoenzyme	6.86	6.92***	4.2	55,000	54,500
Reconstituted DCIP-enzyme	6.45	-	3.8	44,300	

* ref. MASSEY et al.,(1962)

** ref. SAVAGE (1957)

*** still uncertain value

Table IV

Apoenzyme formed on ice under anaerobic conditions in 6.5 M urea. The percentage of the original activities with lipoic acid and DCIP are given. The experiment was carried out as described in the text.

	$LipS_2$ - act.	DCIP - act.
Experiment 1		
Holoenzyme	100	100
Column fraction	8	24
Recombination during 6 minutes on ice with FAD	5	267
Experiment 2		
Holoenzyme	100	100
Enzyme fraction	1	not determined
Recombination during 4 $^{1}/_{2}$ hours on ice with FAD	0.1	136
30 minutes at 25°	22	not determined

Table V

Influence of freezing on reduced lipoamide dehydrogenase. Holoenzyme (0.27mg/ml) anaerobically frozen in 0.03 M phosphate buffer (pH 7.2) in the presence of a tenfold molar excess of NADH for five days. Activities were determined immediately after thawing and again after incubating for 30 minutes at 25° and expressed as percent activity of the original holoenzyme.

Sample	+ EDTA 0.3 mM		+ EDTA 0.3 mM, trace NADase		- EDTA		- EDTA, trace NADase	
	DCIP	$lipS_2$	DCIP	$lipS_2$	DCIP	$lipS_2$	DCIP	$lipS_2$
	%		%		%		%	
After thawing	1600	17	1950	21	2150	21	2100	16
30' at 25 %	480	36	580	49	670	47	590	54

Table VI

Sedimentation coefficients of lipoamide dehydrogenase under different conditions. Holoenzyme (4-5 mg/ml) anaerobically frozen in 0.03 M phosphate buffer (pH 7.2) and 3 x 10^{-4} M EDTA with a threefold molar excess of NADH and the additions given in the Table. After thawing the samples were oxidised by oxigen.

	Addition	Temperature o	$s_{t,c}$	$s_{20,w}$
Lipoamide dehydrogenase (4-5 mg/ml) + NADH	trace NADase	1.7	3.5 - 1.9	6.1 - 3.3
	none	8.3	4.1 - 2.3	5.7 - 3.2
	sodium arsenite (10^{-2}M) + trace NADase	5.7	3.6 - 2.2	5.5 - 3.4
	none	5.9	4.0 - 1.8	6.1 - 2.8
	NAD^+ ($2x10^{-3}$)	7.0	3.9 - 2.2	5.8 - 3.3

4. APOENZYME AND RECOMBINATION PROPERTIES WITH FLAVINS AND OTHER NUCLEOTIDES

4.1. INTRODUCTION

The recombination of the apoenzyme of lipoamide dehydrogenase with FAD was studied and some of the characteristics of this process delineated in a preceding paper (KALSE and VEEGER, 1968). These included changes in polarisation and intensity of fluorescence as well as activities on both the artificial substrate, DCIP and the more natural substrate, lipoic acid. One of the features of the apoenzyme was the almost instantaneous binding of the FAD deduced from a rapid increase in polarisation. To obtain more detailed information about the sequence of the reactions following the attachement of the flavin, recombination with analogues of FAD has now been examined. The 3-methyl- and 3-carboxymethylFAD, compounds alkylated at the 3-imino position in the isoalloxazine system of the flavin-adenine dinucleotide, were used for this purpose.

4.2. RESULTS

4.2.1. Restoration of activities

The effectiviness of 3-methyl- and 3-carboxymethylFAD relative to FAD in restoring the diaphorase activity can be seen from the data in Fig. 1. In comparison to activities with FAD, those obtained with the 3-methyl-analogue are reasonably high, whereas those with the 3-carboxymethyl-derivative low. For example at 10 μM concentration, the 3-carboxymethylFAD shows only 5 %, but the 3-methyl-derivative 60 % of the activity restored by FAD. A similar experiment with 2-morpholinoFAD showed this compound to be inactive*. In Table I the maximum values of the specific activities in the diaphorase and lipoate dehydrogenase reactions are given after incubation with FAD and its 3-substituted-analogues. As seen previously (KALSE and VEEGER, 1968), the maximal diaphorase activity is obtained in the first few minutes after incubating the apoenzyme with FAD on ice. Again both the 3-methyl- and the 3-carboxymethylFAD's partially restore the DCIP--activity; both analogues are less coenzymatically active than FAD;

* The 2-morpholinoFAD as well as 3-alkyl-derivatives are essentially inactive with D-amino acid apoenzyme (ref. CHASSY and MCCORMICK, 1965; and KOSTER, unpublished results).

Fig. 1. Effect of coenzyme concentration on the restoration of diaphorase activity. The solutions contained varying amounts of FAD (○), 3-methylFAD (△), or 3-carboxymethylFAD (□) with 5 μM apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6). After 5 min incubation at 0°, samples were withdrawn and activities measured with DCIP.

Fig.1

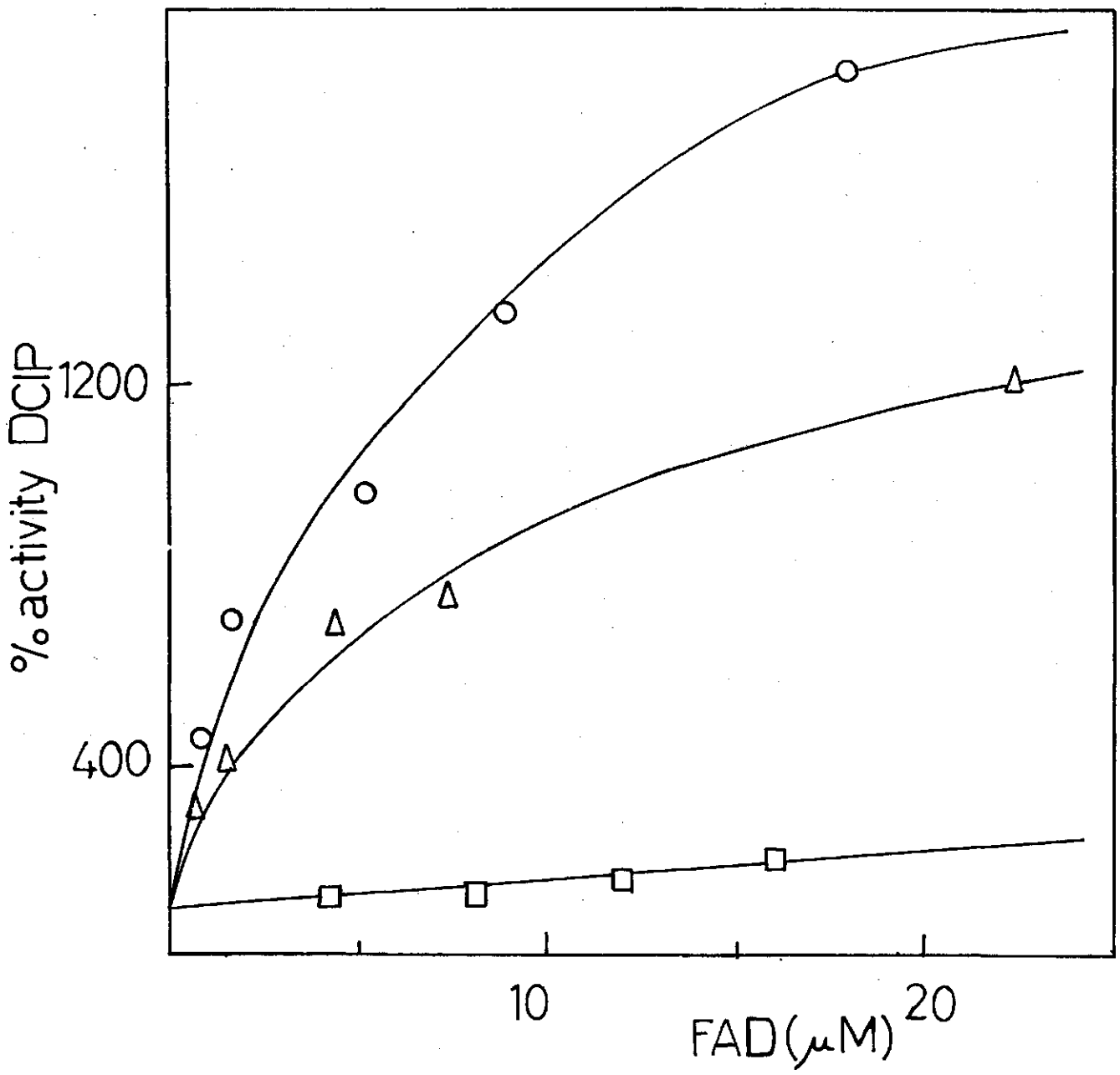
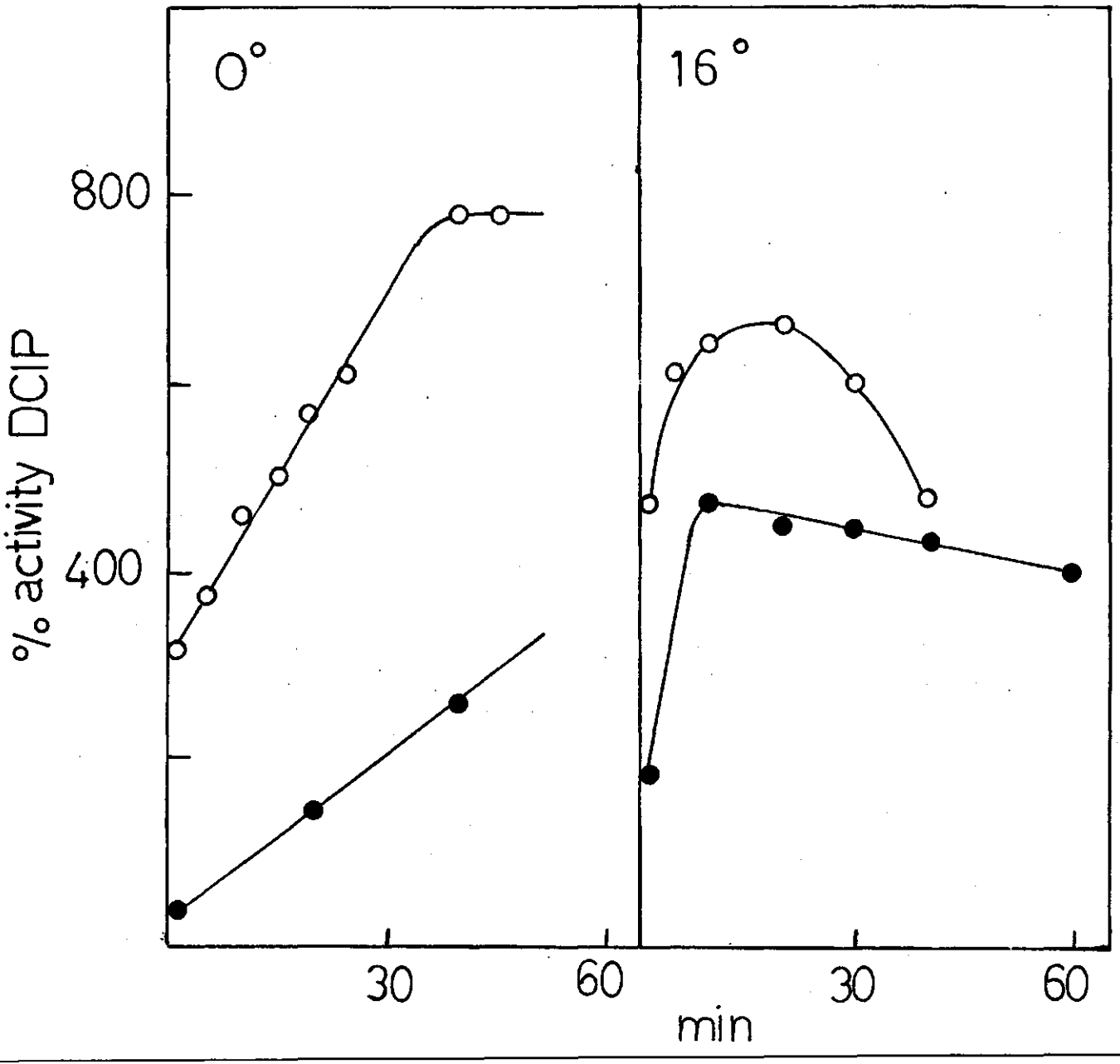


Fig. 2. Restoration of diaphorase activity as affected by time and tempera
ture of incubation of apoenzyme with 3-methyl- (O) or 3-carboxymethylFAD (
Solutions contained 14 μM each of flavin and apoenzyme, 0.3 mM EDTA and
0.13 M sodium phosphate buffer (pH 7.6).

Fig.2



the 3-carboxymethylFAD is least active.

The amount of lipS_2-activity restored after raising the temperature, indicates that the analogues are coenzymatically less functional than FAD. The 3-methylFAD restores only 15 % of the activity obtained with the same amount of FAD, the 3-carboxy--methylFAD is essentially inactive.

The recombination with the derivatives is strongly temperature dependent as is the case with FAD itself. Fig. 2 shows the results obtained at two different temperatures. Over the temperature range from 0° to 25°, a practically linear increase in DCIP-activity to a plateau is found in the presence of 3-carboxymethylFAD. The properties of 3-methylFAD are in between those of FAD and the 3-carboxymethylFAD: the increase of activity with DCIP is still fairly linear at 0°, while at higher temperatures it shows similarities with the behaviour of FAD (cf. ref. KALSE and VEEGER, 1968). Both the analogues have in common with FAD that the DCIP-activities reach a plateau followed by a decrease; the decline in activity is faster at higher temperatures.

Activation energies for the temperature-dependent increase of the DCIP-activity upon recombination of apoenzyme with either 3-methyl- or 3-carboxymethylFAD, have approximately the same values (5500 cal.-$mole^{-1}$). These values are obtained from the slopes of the Arrhenius plots (Fig. 3).

4.2.2. Fluorescence changes

The fluorescence - pH profiles of FAD and its 3-alkyl-derivatives are given in Fig. 4. As with FAD, both 3-carboxymethyl- and 3-methyl--FAD exhibit maxima for fluorescence intensity near pH 2.5. In every case the fluorescence decreases toward neutral pH with a midpoint at pH 3.5 indicative of the pK_a for protonation of the 6-amino group of adenosine (LONG, 1961). Relatively low fluorescence is seen from mildly acid to slightly alkaline solutions in which these flavins are intramolecularly complexed (CHASSY and MCCORMICK, 1965). Thus the FAD analogues like FAD are presented as internally complexed species to the apoenzyme. The fact that the fluorescence characteristics of the derivatives are very similar to those of FAD, justifies the assumptions made for the calculation of the association constants i.e. show the same increase in fluorescence upon binding to the protein.

The patterns for change in polarisation and intensity of fluorescence for the binding of the methylFAD at 20° are given in Fig. 5. The

Fig. 3. Arrhenius plots of the rates of restoration of DCIP-activity with the 3-methyl- (O) and 3-carboxymethylFAD (●). Solutions contained 14 μM ea of flavin and apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6). Activities with DCIP were measured after 5 min at the given temperatures and corrected for the residual activity of the holoenzyme.

Fig.3

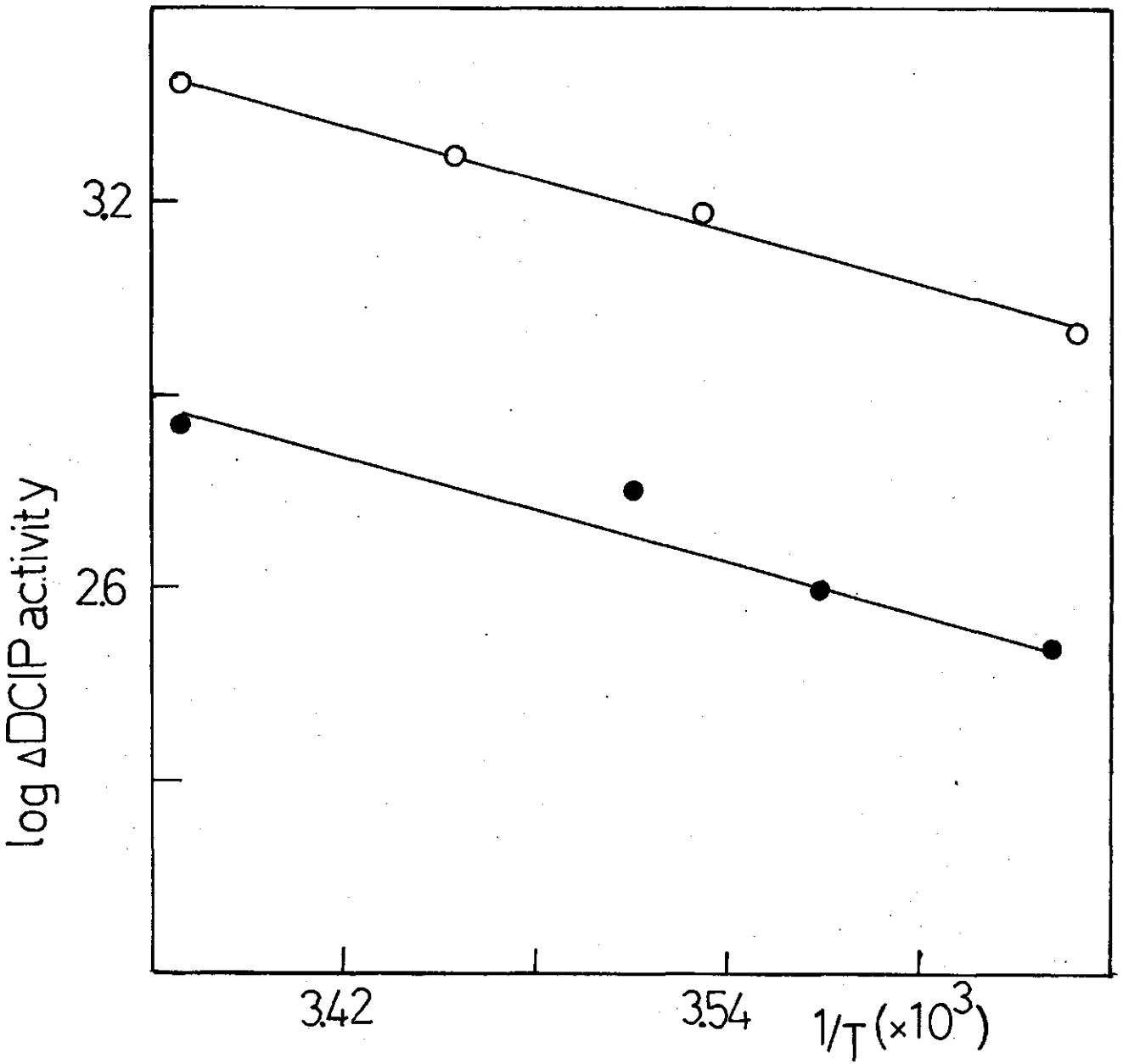
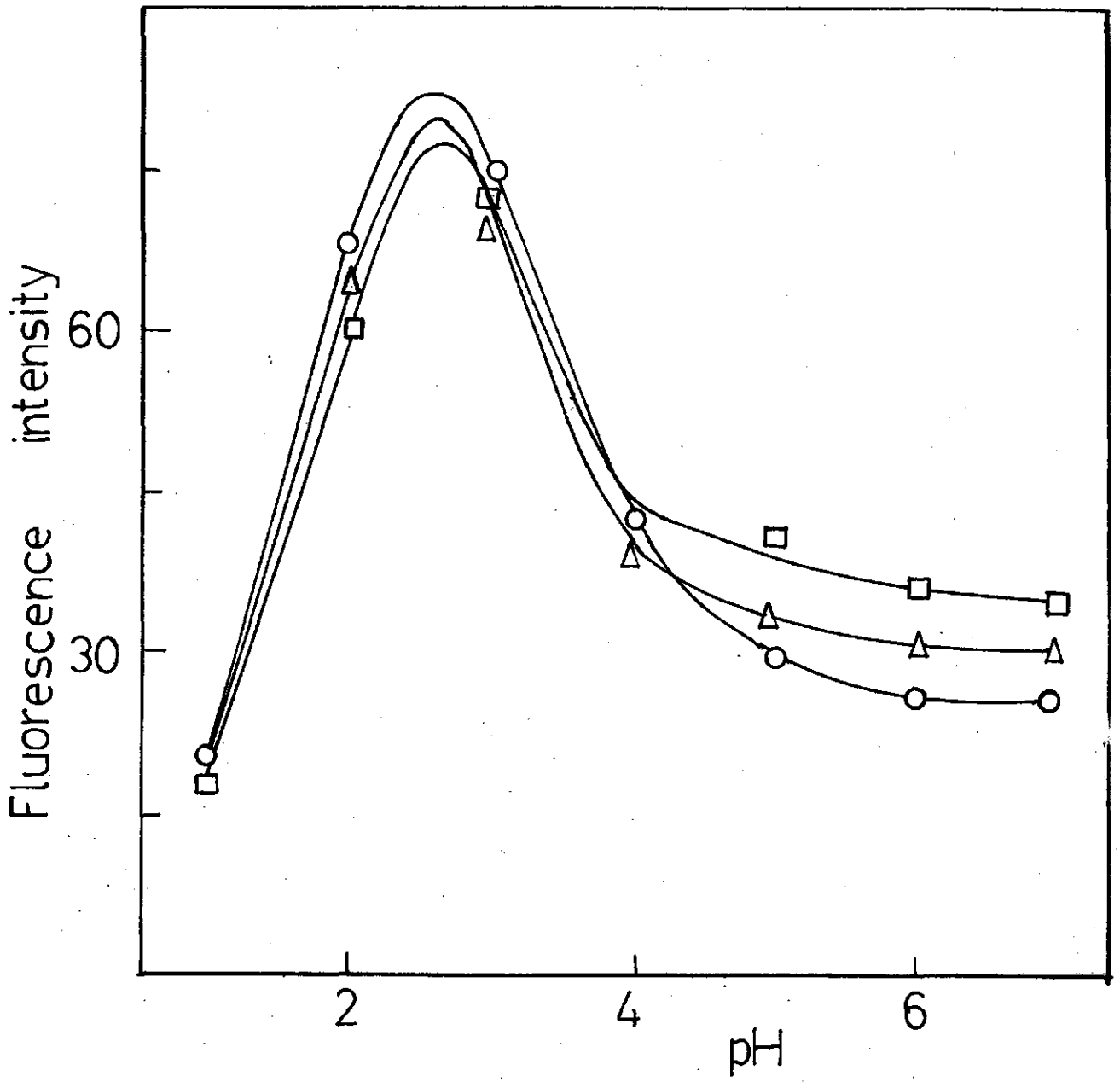


Fig. 4. Effect of pH on the fluorescence intensity of FAD and its 3-alkyl analogues. Solutions were 10 μM in FAD (○), 3-methylFAD (△), or 3-carboxymethylFAD (□) with 0.1 M buffers of KCl-HCl (pH 1); glycine-HCl (pH 2 and 3); sodium acetate (pH 4 and 5); sodium phosphate (pH 6 and 7).

Fig.4



polarisation increases rapidly within the first minute; then keeps increasing, but at a much slower rate. The intensity also increases rapidly in a way comparable to the changes in polarisation. The recombination with 3-methylFAD is very similar to that of FAD over the whole range studied; however, the polarisations are somewhat less. The relatively small increase in polarisation which characterises the 3-carboxymethylFAD system, indicates that the affinity of this derivative for the protein is lower than FAD or the 3-methyl-analogue. This low affinity of 3-carboxymethylFAD, in combination with a lower activity of saturating concentrations for the activity with DCIP provides an explanation for the shape of the saturation curve of Fig. 1.

The fluorescence polarisation as well as the intensities of the free flavin-analogues were determined. The 3-carboxymethyl- and the 3-methylFAD do not differ from FAD with respect to polarisation. All the p values found were between 0.028 and 0.032 and are practically temperature independent. A sample of apoenzyme (DCIP-activity 80 %, $lipS_2$-activity less than 3 %) was reconstituted with excess 3-methyl-FAD, precipitated with ammonium sulphate, and extensively dialyzed against 0.03 M sodium phosphate buffer (pH 7.2) which contained 0.3 mM EDTA. The polarisation of fluorescence of the FAD bound to this reconstituted enzyme was 0.37 determined at 10°. This is in fair agreement with values obtained for the FAD-containing lipoamide dehydrogenase (0.38). This reconstituted enzyme showed a $lipS_2$-activity of 17 % and a DCIP-acivity of 330 %, indicating that the physiological activity is also partially restored. The value of 0.37 was used for the polarisation of both the bound 3-methylFAD and 3-carboxymethylFAD in the calculations. The association constants are average values from at least four independent experiments using different flavin : apoenzyme ratio's. Using the method of KLOTZ (1946) it was calculated that 1.0 - 1.2 moles of flavin-analogues were bound per 50,000 grams of apoenzyme, a value which is close to that obtained with FAD (cf. ref. KALSE and VEEGER, 1968).

The absorption spectrum of the purified 3-methylFAD containing enzyme has maxima around 454 mμ and 350 mμ and minima around 410 mμ and 330 mμ. Since it shows a shoulder between 465 and 485 mμ it is not significantly different from that of the FAD enzyme.

Fig. 6 shows that the sedimentation patterns of reconstituted 3-methylFAD-containing lipoamide dehydrogenase maintain a very sharp peak which is asymmetrical and skewed to the side of the meniscus. The

Fig. 5. Change in intensity (○) and polarisation (●) of fluorescence upon binding of FAD (B) and its 3-methyl analogue (A) to lipoamide dehydrogenas apoprotein. Solutions contained 2.5 μM each of flavin and apoenzyme, 0.3 m EDTA and 0.13 M sodium phosphate buffer (pH 7.6) at 20°.

Fig.5

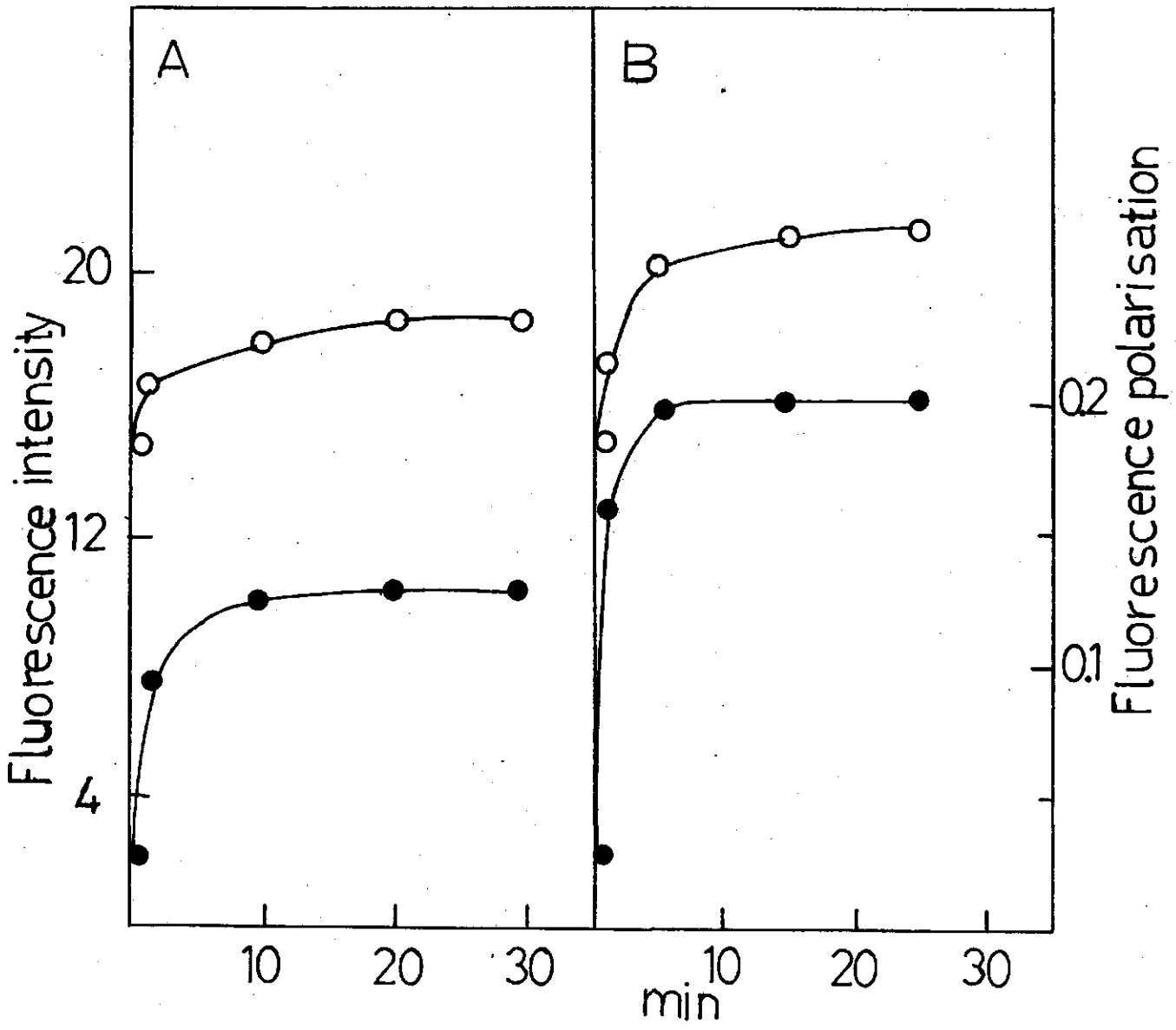
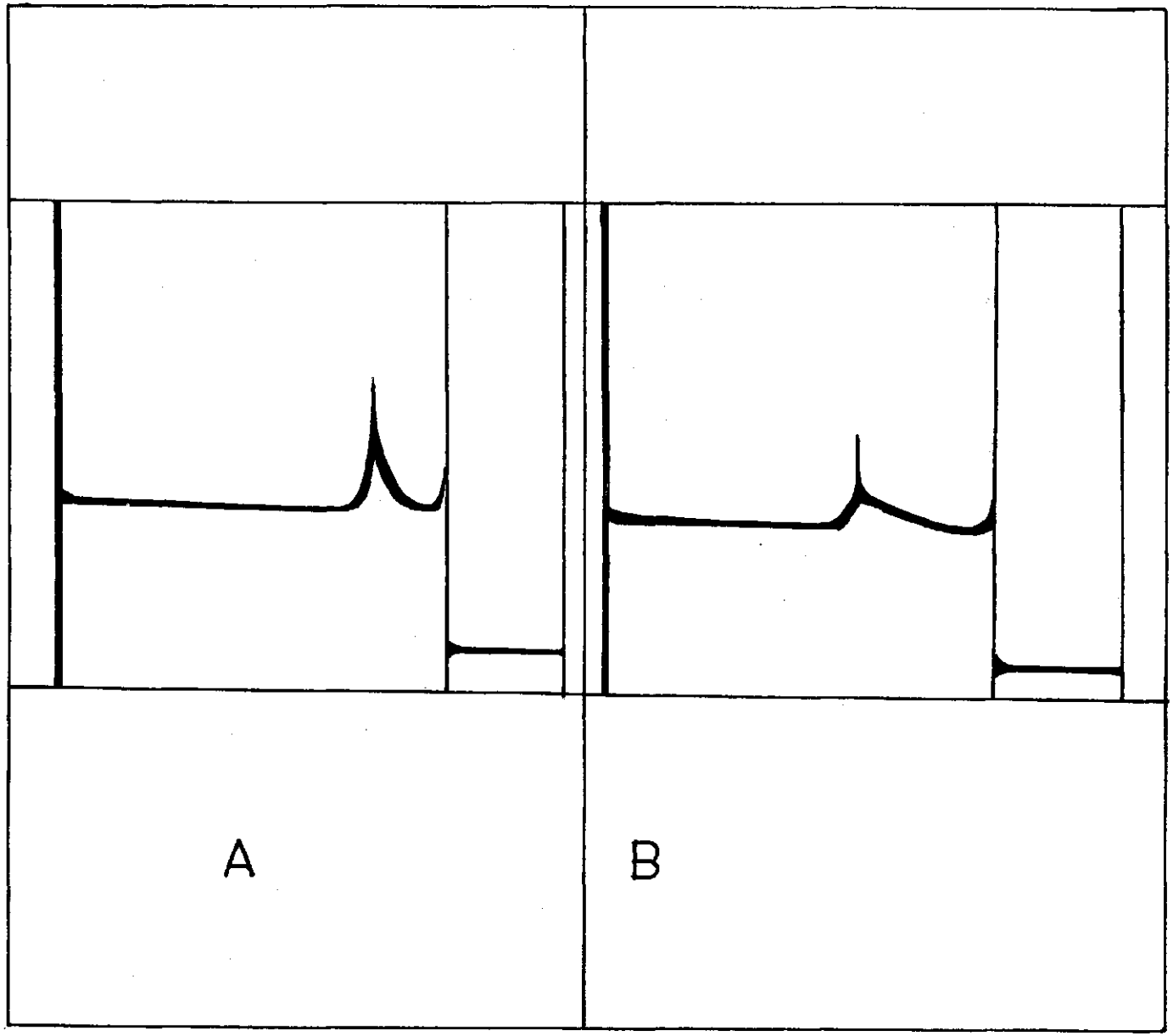

A
B
Fluorescence intensity
20
12
4
0.2
0.1
Fluorescence polarisation
10
20
30
10
20
30
min

Fig. 6. Sedimentation of 3-methylFAD reconstituted lipoamide dehydrogenase in 0.03 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Rotor speed: 55 300 rev./min; temperature 12°. Photographs taken after 24 min (A) and 39 min (B). Sedimentation direction, from right to left.

Fig.6



rather low ionic strength may be partially responsible for this effect. Sedimentation coefficients were calculated from the top of the peak. At 7°, 12°, and 17° the respective values are 4.1, 4.9, and 5.2 S. An average $s_{20,w}$ value of 6.0 S can be calculated from these results. Extrapolation to zero protein concentration was not possible, as there was insufficient material available.

4.3. DISCUSSION

The present finding that the apoenzyme of lipoamide dehydrogenase is able to recombine with FAD-analogues is of real significance for studying flavin-protein interactions. Up to now, FAD-analogues have been tested for their coenzymatic functions almost exclusively on the apoenzyme of D-amino acid oxidase. However the results now obtained emphasise that one cannot appraise coenzyme function on the basis of only one enzyme.

Replacement of FAD by the two 3-alkyl-derivatives in the lipoamide dehydrogenase resulted in reasonably active compexes with the artificial substrate DCIP. As to physical properties, the 3-methyl- as well as the 3-carboxymethylFAD do not differ very much from FAD, e.g. the redoxpotentials are the same (MCCORMICK, 1969). Spectral and fluorescence behaviour are also practically the same. Apparently the equilibria between the internally quenched complexes of the 3-alkyl--analogues and their open fluorescent forms, evidenced by fluorescence--pH profiles, are similar to that of FAD (MCCORMICK, 1969). This suggests that replacement of the 3-imino hydrogen of FAD by a substituent which is larger or negatively charged does not markedly influence the ability to form an intramolecular non-fluorescent complex.

The increase in fluorescence polarisation upon addition of the 3-alkyl-analogues, as with FAD, reflects the binding process. Moreover, since the unbound flavins are less fluorescent than when bound, the increase in their intensities must reflect an opening of their intramolecular complexes upon association with the enzyme. The analogues cannot fully replace FAD. This is not surprising as the association constants have been shown to be different. From the midpoints of the saturation curves (see Fig. 1) one can only calculate the association constants approximately, as the maximal obtainable velocities are uncertain. These are assumed for the DCIP-activity to be 2000 % and 1300 % of the value of holoenzyme for the FAD and the 3-methyl-derivative,

respectively. However the latter value is quite uncertain, since saturating concentrations of the methyl-derivative were not applied. The fact that 3-substituted flavins are able to bind to form an active enzyme indicates that the 3-position is not involved primarily in the binding to the protein as has been proposed previously (THEORELL and NYGAARD, 1954) for other flavoproteins (cf. ref. VEEGER et al., 1966).

Table I

Extent of restoration of diaphorase and lipoamide dehydrogenase activities with FAD and its 2-alkyl-analogues. Solutions contained 13 μM each of flavin and apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6). Activities with DCIP were measured after incubating 5 minutes at 0°; those with $lipS_2$ were measured after 30 minutes incubation at room temperature. Activities are expressed in comparison with the holoenzyme.

	Specific activities	
	DCIP % act.	$LipS_2$ % act.
None	170	9.9
FAD	1600	69.2
3-MethylFAD	1100	20.2
3-CarboxymethylFAD	780	10.6

Table II

Association constants of FAD and its 3-alkyl-derivatives with diaphorase at different temperatures. Solutions contained varying amounts of flavin with 1.9 μM apoenzyme, 0.3 mM EDTA and 0.13 M sodium phosphate buffer (pH 7.6) at the temperatures indicated. Fluorescence polarisations were measured and values for K_{ass} calculated as described in the text.

	$K_{ass} \times 10^{-5}$	
	10°	20°
FAD (Table II ref. KALSE and VEEGER, 1968)	3.3	2.0
3-MethylFAD	1.1	1.1
3-CarboxymethylFAD	0.3	-

5. APOENZYME STRUCTURE AND MORE FLAVIN BINDING ASPECTS

5.1. INTRODUCTION

The apoenzyme of lipoamide dehydrogenase can be prepared under both aerobic and anaerobic conditions (KALSE and VEEGER, 1968; VISSER and VEEGER, 1968). It has a mol.wt of 52,000, half that of the holoenzyme. In previous studies (KALSE and VEEGER, 1968; VISSER and VEEGER, 1968[a]; VISSER et al., 1968) the recombination process in particular has been investigated. This process depends on at least three reactions, i.e.

1) binding of the flavin molecule with the protein which leads to opening of the intramolecular complex between the isoalloxazine ring and adenine moiety of FAD and the formation of a DCIP-active monomer;
2) the transition to a complex which is less active with DCIP and which can be trapped at low temperatures;
3) the temperature-dependent dimerisation which determines the return of the lipoate activity.

It has been shown in our laboratory that the apoenzyme used in our studies differs from that prepared with guanidine-HCl as described by BRADY and BEYCHOK (1968,1969). Recently STRITTMATTER (1967) showed that several distinct conformations of cytochrome b_5 aporeductase exist. Evidence will be presented here for the apoenzyme and the DCIP-active enzyme of lipoamide dehydrogenase existing in several forms. The recombination process is in fact a continuous change in protein structure ending in the dimeric holoenzyme. Binding studies of flavin derivatives and other molecules to the apoenzyme of lipoamide dehydrogenase demonstrate that interactions of the protein and the flavin are based on multiple binding forces as has already been postulated by several authors (THEORELL and NYGAARD, 1954; THEORELL, 1958; WALAAS and WALAAS, 1956) for the binding mechanism of FAD in flavoproteins.

5.2. RESULTS

Acidification of a holoenzyme preparation with a saturated ammonium sulphate solution (pH 1.5) precipitates the apoenzyme. As this method is rather severe and in fact based upon more or less reversible stages of protein denaturation it is not surprising that the properties of different apoenzyme preparations may vary considerably. For instance in the case when the acid ammonium sulphate solution is added quickly

without complete and intensive mixing, the rest activity with lipoate of the precipate will be rather high (20-25 %); in other preparations the lipoate activity will be low while the DCIP-activity is stimulated 600 to 800 %. Combined with the previous observation (KALSE and VEEGER, 1968), that the FAD monomer has a high DCIP-activity this suggests that monomerisation occurs before FAD dissociation. In general the rest--activities with lipoate and DCIP as compared with the holoenzyme are 4-8 % and 150-200 %, respectively. However, the milder one treats the preparation, the better the return of lipoate activity.

5.2.1. Stability of the apoenzyme

The ability of the apoenzyme to recombine after ageing on ice for prolonged times is given in Table I. The main effect of standing concerns the return of the lipoate activity e.g. the dimerisation, although the maximum activity with DCIP also diminishes. Furthermore the DCIP-activity of an aged apoenzyme decreases less after elevating the temperature than with a freshly made preparation. These observations indicate that although the structure of the apoenzyme gradually alters the FAD-binding site is much less affected. Sometimes a small increase (10 to 15 %) in recombination ability with FAD is noticed during the first hour. On standing on ice in concentrated (1-2 mg/ml) solution this is observed by means of fluorescence - polarisation and DCIP--activity measurements. A process of refolding of a fraction of the apoenzyme, comparable with the observations made by STRITTMATTER (1967), with cytochrome b_5 reductase, might be responsible for this phenomenon.

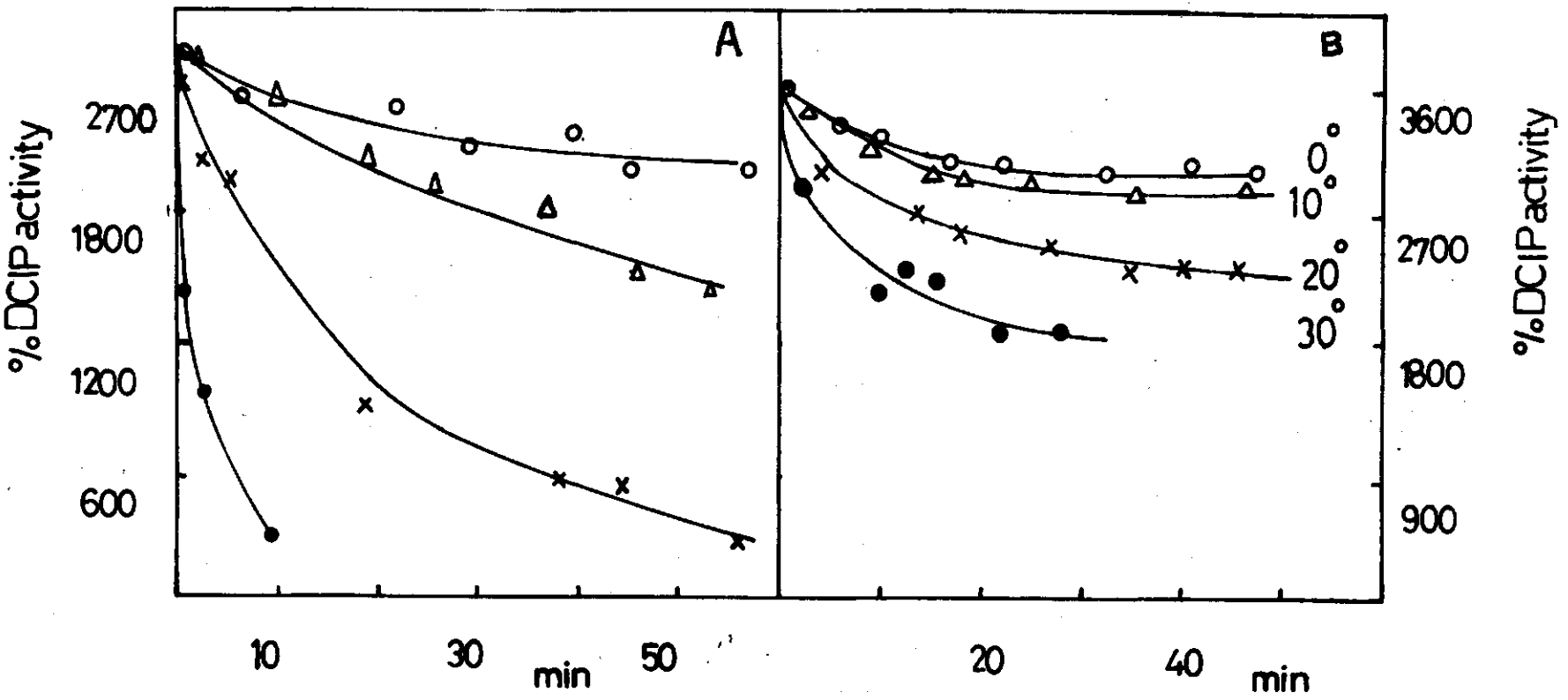
Dialysis of the apoenzyme for 2-3 hours against sodium phosphate buffers (30 mM or 150 mM pH 7.5) always results in apoenzyme preparations which are upon addition of FAD 20-40 % less active with lipoate and DCIP.

In contrast with the holoenzyme the apoenzyme is thermo-labile (Fig. 1A). The inactivation which is reflected in the loss of restoration of the DCIP-activity is temperature-dependent and promoted by low concentrations of urea. At 5° the half time of inactivation at zero urea concentration is approximately 300 min. while the values for apoenzyme incubated with 2 M and 3 M urea are about 15 min. and 2 min., respectively.

The inactivation is also dependent on the protein concentration. At 0.4 mg/ml the apoenzyme after an initial decline in restoration capacity is almost completely stable at 0°-5° for at least one hour. However, at 0.1 and 0.05 mg/ml the inactivation reaches 20 % and 40 %

Fig. 1. A. Stability of lipoamide dehydrogenase apoenzyme under different conditions. Stability of apoenzyme incubated at different concentrations at 5° in 50 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA. A sample of the incubation mixture was added to the assay cuvette, to whic 5 μM FAD was added and the DCIP-reaction was measured at 25°. ○○○, 0.1 mg/ ΔΔΔ, 0.05 mg/ml; xxx, 0.1 mg/ml in the presence of 2 M urea; ●●●, 0.1 mg/m in the presence of 3 M urea. B. The stability of the apoenzyme (0.4 mg/ml) at different incubation temperatures, incubated and assayed under the conditions of Fig. 1A.

Fig. 1


A
B
%DCIP activity
2700
1800
1200
600
3600
2700
1800
900
%DCIP activity
0°
10°
20°
30°
10
30
min
50
20
40
min

respectively after standing at 5° (Fig. 1B) for 50 minutes.

The apoenzyme withstands freezing at - 14° for several days. Prolonged freezing promotes irreversible structural changes in the apo-protein reflected in a proportional lowering of the restoration of both the DCIP and lipoate activities. In general the results vary somewhat but the tendency is that both bovine serum albumin and $(NH_4)_2SO_4$ protect which also protect the holoenzyme against inactivation during freezing. Apoenzyme which is frozen for 1 month after recombination with FAD on ice in the presence of urea (1-6 M concentration range), has lost all its activity in contrast to the control which still shows considerable activity.

The recombination process was mainly studied in sodium phosphate. The pH-profile is curved with an optimum value between pH 7.2 and 7.5; increase of ionic strength accelerates the pH-dependent return of the lipoate activity, but the maximal activities at low and high ionic strength only differ slightly.

The urea-sensitivity of the DCIP-activity of the reconstituted enzyme has been mentioned previously (VISSER and VEEGER, 1968[a]). The apoenzyme itself is also very urea-sensitive as demonstrated in Fig. 1A. However some features need more attention. In Table II and Fig. 2A the influence of urea on the FAD binding as measured by fluorescence--polarisation has been surveyed. The apoenzyme incubated for a short time with urea, even in concentrations as low as 2 M, is not able to bind the FAD effectively as can be concluded from the lowered polarisation of fluorescence of the flavin. However, if the flavin is added prior to the urea addition, the polarisation of the recombined enzyme decreases slightly and rather slowly. This means that the binding of the flavin rapidly induces a protein conformational change which largely protects the flavin binding site itself against unfolding. Although the flavin-protein interaction is little affected, the function of the catalytic centre is more easily disrupted as the activities with DCIP are strongly influenced. At 2 M urea this activity is about 70 % inhibited (cf. ref. VISSER and VEEGER, 1968[a]; Fig.), which indicates that the protein does undergo conformational changes. The results of Table II and Fig. 1 seem to be contradictory as the half times are different. However, the techniques used are quite different. In the fluorescence-polarisation experiments the amount of FAD is limited. The stability experiments contain an excess of FAD while upon measuring, the samples are diluted in the assay which drastically lowers the urea concentration.

Addition of PCMB to the apoenzyme decreases the FAD-binding capacity. In Fig. 2B the K_{ass} value is given for flavin binding to the apoenzyme which is incubated with different amounts of PCMB. The influence of PCMB on the restoration of the DCIP-activity is also shown. An amount of 1 mole PCMB per mole of apoenzyme incubated for approximately 30 minutes results in 35 % inhibition of the DCIP--activitiy while amounts of 2 and 4 moles PCMB/mole protein results in 60 % and 90 % inhibition, respectively. These values fairly well agree with the percentage of lipoate activity which returns upon elevating the temperature under these conditions. This behaviour is not very different from that of the holoenzyme. Binding of 1 and 2 moles of phenylmercuric acetate per mole of enzyme gives almost the same loss of lipoate activity (CASOLA and MASSEY, 1966), which has been ascribed by PALMER and MASSEY (1962) and by VEEGER and MASSEY (1962) to two fast reacting SH group.

The addition of 50 µM L-cysteïne to a recombination system without PCMB containing 6.4 µM apoenzyme and 100 µM FAD at 20° completely prevents the return of any lipoate activity (cf. ref. CASOLA et al., 1966). On the other hand, addition of dithiothreitol does not stimulate the return of the lipoate activity as has been stated by BRADY and BEYCHOK (1968).

5.2.2. Apoenzyme fluorescence

The apoenzyme shows a strongly enhanced protein fluorescence (280 nm excitation) as compared with the holoenzyme. At the emission maximum (330 nm) the increase is approximately 20-fold. The position of this maximum indicates a rather non-polar environment for the tryptophan groups (TEALE, 1960). In the holoenzyme the protein fluorescence is mainly quenched due to binding of the prosthetic group and the connected energy-transfer to the flavin (VEEGER et al., 1969) though influence of other factors like protein conformational changes cannot be ruled out. The excitation spectrum (Fig. 3A) of the apoenzyme shows a double maximum, at 282-284 and 290 nm, while the holoenzyme shows only one peak at 287 nm. Lipoamide dehydrogenase contains 7 tyrosyl, 2 tryptophyl and 13 phenylalanyl residues per mole of flavin (MASSEY, in BOYER et al., 1963). According to several authors (WEBER, 1960; COWGILL, 1964; KONEV, 1967) in protein fluorescence the contributions of the tyrosyl residues are generally small compared with those of the tryptophan residues. However, studies with the holoenzyme

Fig. 2. A. Influence of urea on the binding of FAD. Apoenzyme (5 μM) and FAD (5 μM) were incubated at 10° in 30 mM sodium phosphate buffer with 0.3 mM EDTA; the apoenzyme was preincubated during 5 min with various urea concentrations. After 30 min recombination, the K_{ass} values were calculate from the fluorescence polarisation as previously described (ref. 1). B. Influence of PCMB on the binding of FAD 000, as Fig. 2A with various PCMB concentrations relative to the monomer concentrations. ▲▲▲, 6 μM apoenzyme preincubated with the relative amounts of PCMB indicated, during 30 min. Samples were withdrawn and the DCIP-activity determined in an assay cuvett to which 4 μM FAD was added.

Fig. 2

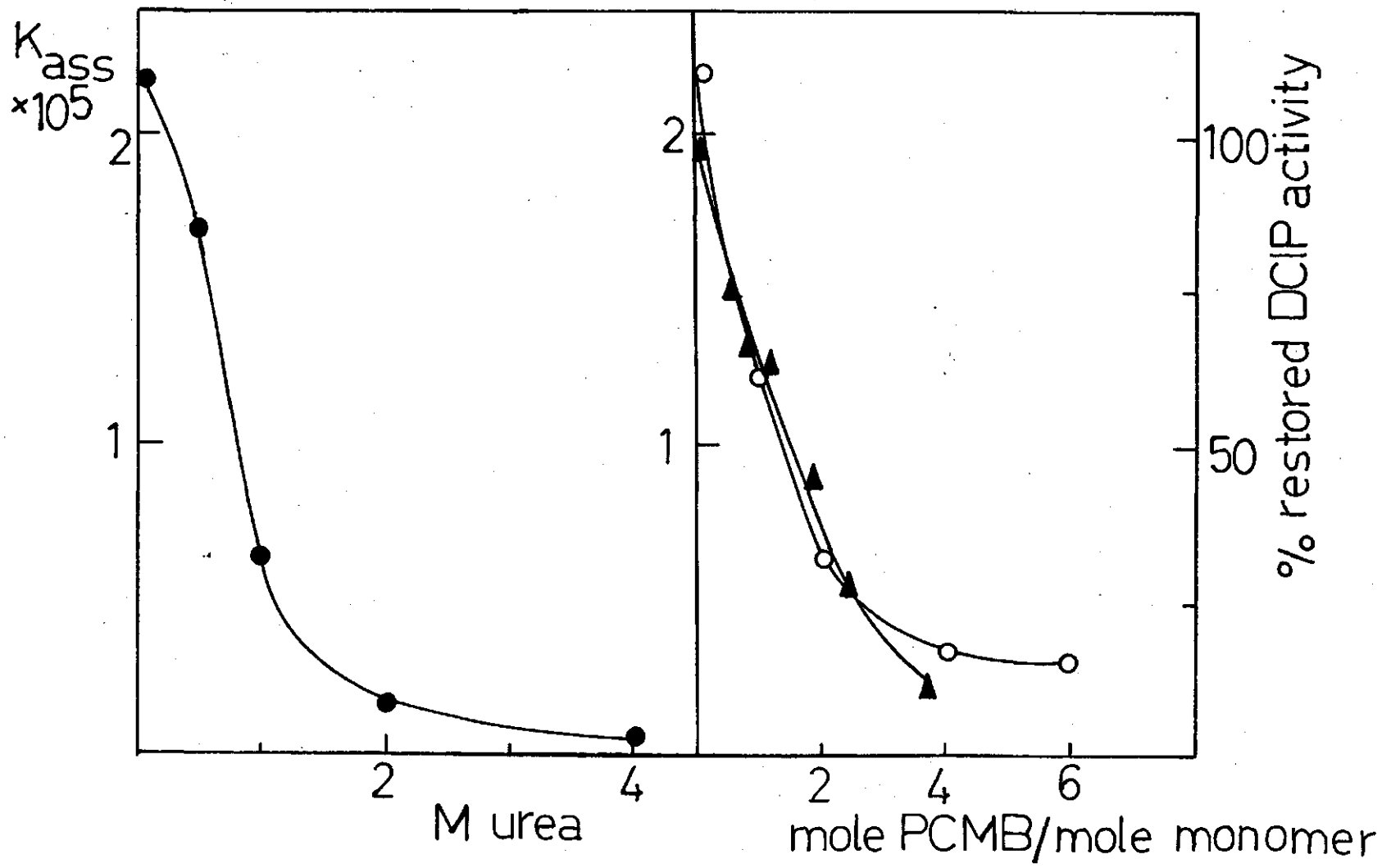
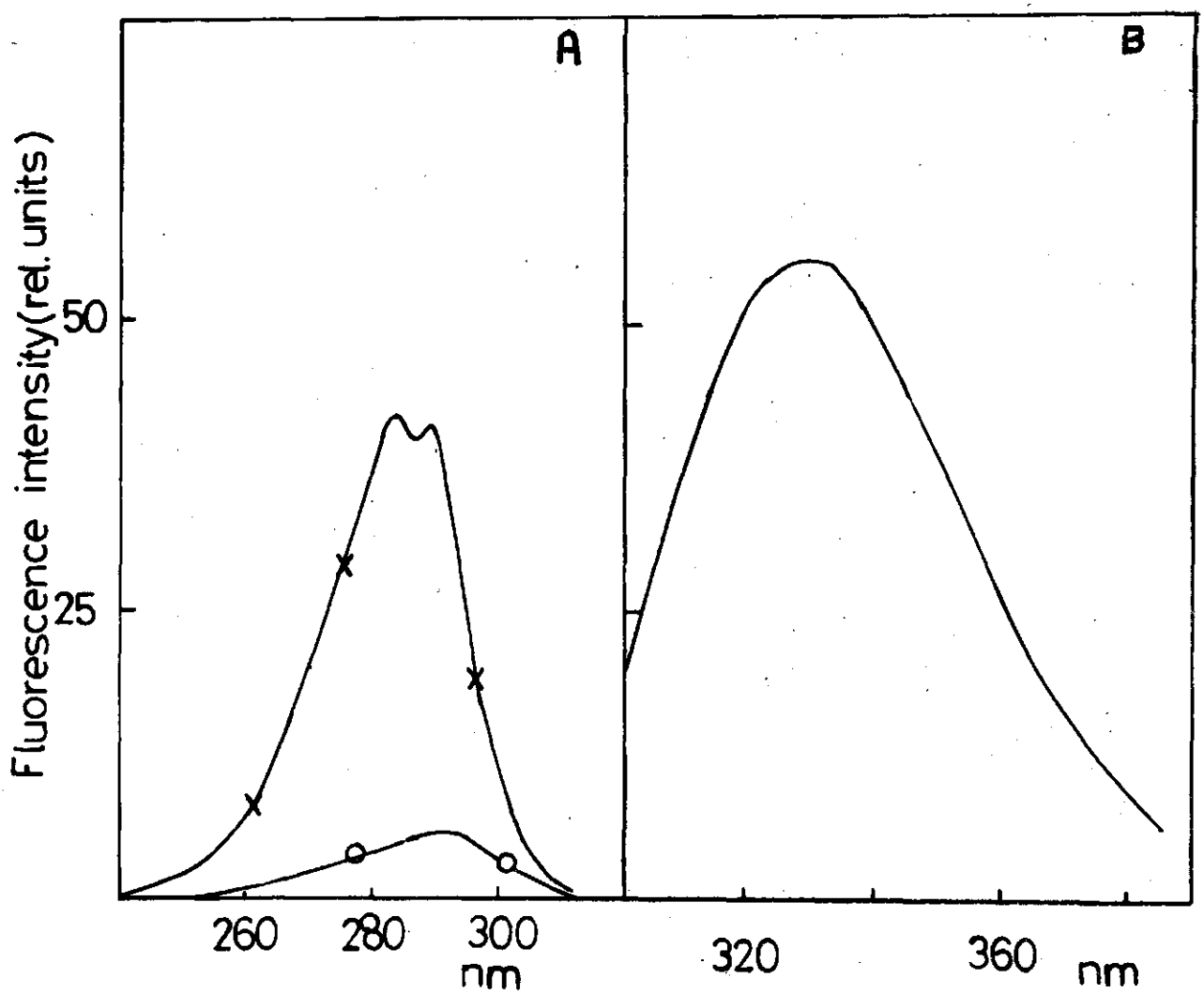

K_{ass} ×10^5
2
1
2
4
M urea
2
1
2
4
6
mole PCMB/mole monomer
100
50
% restored DCIP activity

Fig. 3. A. Fluorescence of lipoamide dehydrogenase apoenzyme. Excitation spectrum in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA. Emission wavelength 330 nm; excitation slit 4 nm; emission slit 3 nm; XXX apoenzyme 0.04 mg/ml; 000 holoenzyme 0.33 mg/ml. B. Fluorescence emission spectrum of a 0.1 mg/ml apoenzyme solution; excitation wavelength 280 nm; excitation and emission slits 5 nm. Temperature 5°. The spectra are not corrected for lamp spectrum and photomultiplier sensitivity.

Fig. 3


A
B
Fluorescence intensity(rel. units)
50
25
260
280
300
nm
320
360
nm

show (VEEGER et al., 1969) that the tyrosyl contibution to fluorescence with respect to that of the tryptophans is relatively large. Difference fluorescence spectroscopy (WEBER and YOUNG, 1964; SUELTER, 1967) shows that in the apoenzyme the contribution of the tyrosyl fluorescence with respect to that of tryptophyl residues is relatively small. Urea (4 M), shifts the emission wavelength to 340-345 nm (cf. ref. TEALE, 1960) which indicates a change from apolar to polar surroundings of the tryptophyl residues. On addition of urea the excitation spectrum is quenched and the 282-284 nm maximum is reduced to a shoulder at urea concentrations > 4 M. The tyrosyl fluorescence contribution is affected by 4 M urea to the same extent as the tryptophan fluorescence.

The absorption spectrum of the apoenzyme shows a maximum at 277 nm with two shoulders at 282 and 292 nm. The main peak is probably due to tyrosine absorbance while it is likely that the shoulders belong to the tryptophan groups though a contribution of S-S bridges cannot be excluded (STEINER and EDELHOCH, 1961; LEHRER and FASMAN, 1967).

The apoenzyme fluorescence studied as a function of temperature shows discontinuities around 12° and 17° when the temperature is increased from 5° to 25°. However, this process seems to be irreversible as upon lowering the temperature no return to the original emission level occurs.

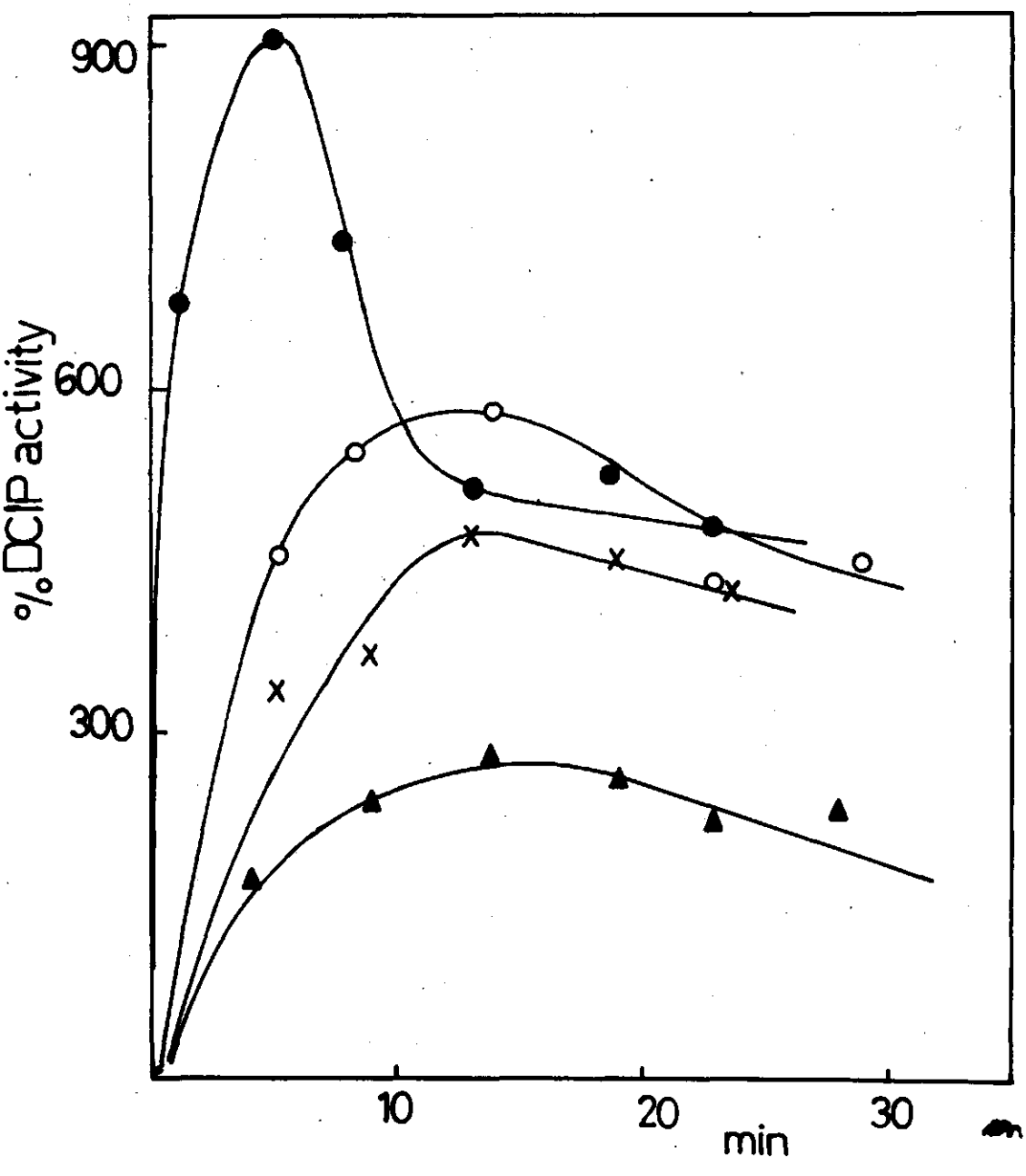
5.2.3. Influence of halogenide-ions on the recombination

As KBr is used in the preparation procedure of the apoenzyme, its influence, if any, on the recombination process is of importance. In Fig. 4 the influence which potassium halogenides have on the recombination process on ice with respect to the DCIP-activity is given. The order of magnitude in which the potassium salts are interfering with the level of the DCIP-activity is KBr < KCl < KI. A second point of interest is the shift in the time necessary for the DCIP-activity to reach its lower maximum value i.e. from approximately 5-8 min. to 11-15 min.

At higher flavin concentration (60 μM) the difference in activity between the untreated and the salt-treated recombination systems disappears although there is still a distinct difference in the time at which the maximum of the DCIP-activity is reached. The return of the lipoate activity is diminished in the presence of low flavin concentrations. The DCIP-activity remains high which indicates that denaturation is not important. Furthermore none of the potassium halogenides (60 mM) interferes with the return of the lipoate activity

Fig. 4. Influence of potassium halogenides on the recombination of lipoami dehydrogenase apoenzyme with FAD. Apoenzyme (8 μM final concentration) was preincubated on ice in 30 mM phosphate buffer pH 7.2 containing 0.3 mM EDT ●●●, no addition; ○○○, 60 mM KBr; XXX, 60 mM KCl and ▲▲▲, 60 mM KI. After 10 min FAD was added (10 μM final concentration) and samples withdrawn to measure the DCIP-activity at the times indicated.

Fig. 4



when a saturating concentration (100 μM) of flavin is used.

In Table III the influence is summarized of several halogenides on the polarisation of a recombination mixture as well as on its DCIP-activity. Bromides and iodides are well-known flavin fluorescence quenchers (cf. ref. WEBER, 1948) which causes an increase of the flavin polarisation as the life-time of the excited state declines. The polarisation in the systems with bromide is enlarged while the DCIP--activities are less than in the control. On the other hand, iodides cause a markedly reduced DCIP-activity while the polarisation is only slightly higher than in the control. Thus due to interaction of the flavin with the halogenides, especially I-ions, the flavin binding is partially prevented; nevertheless the polarisation is increased and actually reaches almost the same value as that of the control. The increase in fluorescence polarisation is not due to binding of the flavin to an inactive form of the enzyme, since this cannot explain the observation that in the presence of halogenides the maximum activity can be obtained at saturating concentrations of FAD.

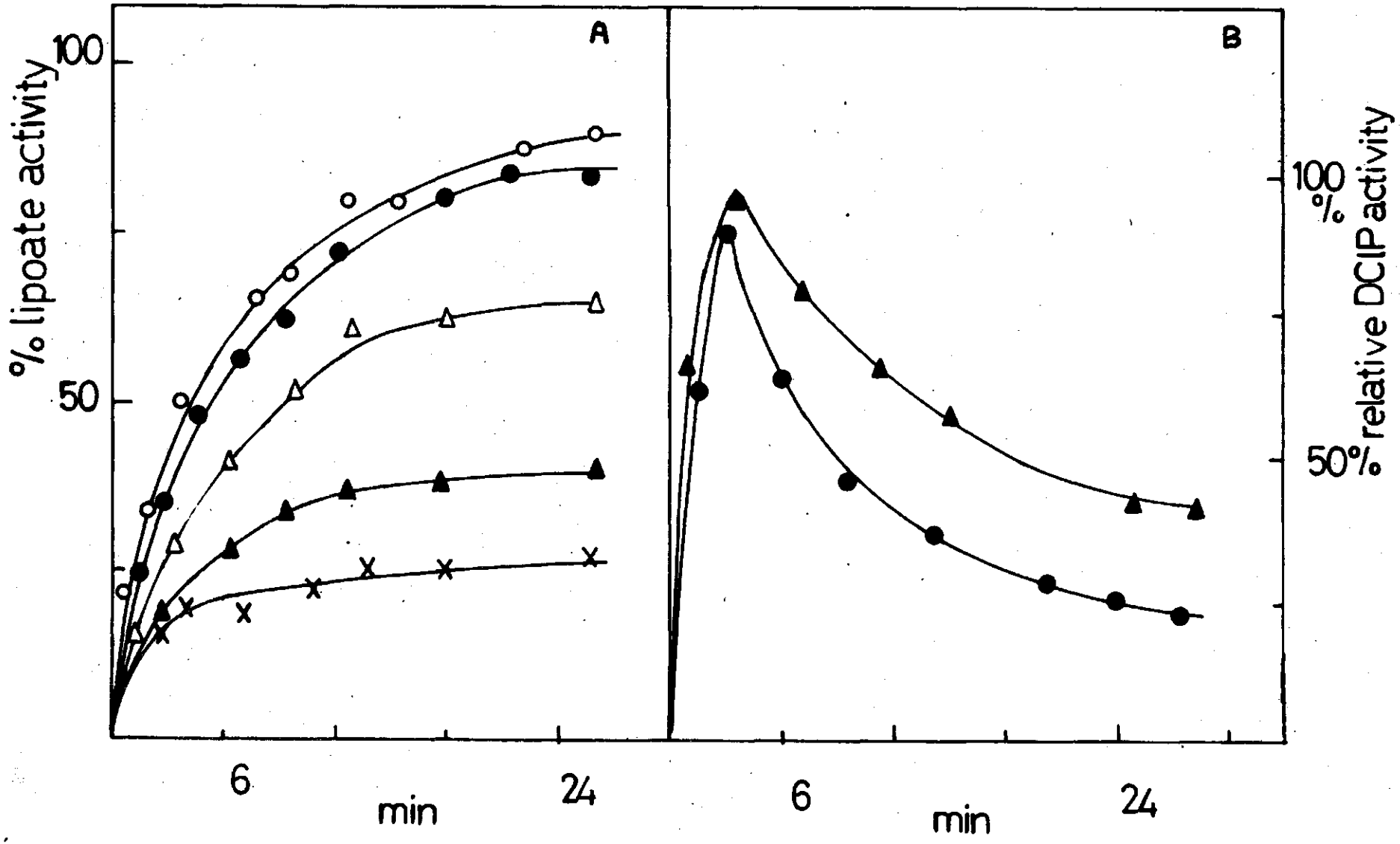
5.2.4. Effect of protein concentration on the binding with FAD

In previous papers (KALSE and VEEGER, 1968; VISSER and VEEGER, 1968[a]) evidence was presented that the return of the lipoate activity is due to a dimerisation reaction as the apoenzyme has half the mol.wt. of the holoenzyme, while futhermore the return of this activity fits a second order reaction rate. The dimerisation is promoted by increase of temperature which probably causes an increase in hydrophobic interactions (cf. ref. SCHERAGA et al., 1962). Fig. 5A demonstrates the return of the lipoate activity at 25° using different apoenzyme concentrations with excess of flavin. The influence of the protein concentration on the dimerisation is clear; the lipoate activities in this experiment reach constant levels within 30 minutes. The activities with DCIP are still high at that time especially in the case of the lowest protein concentrations (Fig. 5B). It is therefore unlikely that the levelling off of the lipoate activity is caused by denaturation of unreacted apoenzyme but rather due to an equilibrium between the lipoate--inactive monomer and dimer.

Assuming an equilibrium between these species and using the level of the lipoate activities to calculate the actual fractions monomer and the fully active dimer, one might calculate the association constants at the different protein concentrations. The association constants obtained vary with the protein concentration from 3.6×10^6 l mole^{-1} to

Fig.5 A. The influence of the apoenzyme concentration on the return of the lipoate activity. The different protein concentrations were incubated with excess FAD (100 μM) at 25° in 100 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA, and the activity measured after different incubation times ○○○, 0.83 mg/ml; ●●●, 0.42 mg/ml; ΔΔΔ, 0.21 mg/ml; ▲▲▲, 0.10 mg/ml; XXX, 0.05 mg/ml. B. The influence of the apoenzyme concentration on the decrease of the DCIP-activity. Conditions as in Fig. 5A. ▲▲▲, 0.1 mg/ml; ●●●, 0.42 mg/ml.

Fig. 5


A
B
% lipoate activity
100
50
6
min
24
6
min
24
100
%
50%
relative DCIP activity

2.5×10^5 l mole^{-1} which suggests a more complicated system than a pure monomer-dimer equilibrium. One of the reasons for this discrepancy might be the changes of the apoenzyme properties upon extreme dilution (cf. apoenzyme-stability).

5.2.5. The binding of flavin analogues

It has been shown (VISSER et al., 1968) that FAD and some of its derivatives, viz. 3-methylFAD and 3-carboxymethylFAD can be bound to the apoenzyme; only FAD and 3-methylFAD are able to give a lipoate active enzyme, all compounds give DCIP-active complexes. It was thus of interest to study the binding of other substituted flavins, in order to obtain information about the different parts of the molecule involved.

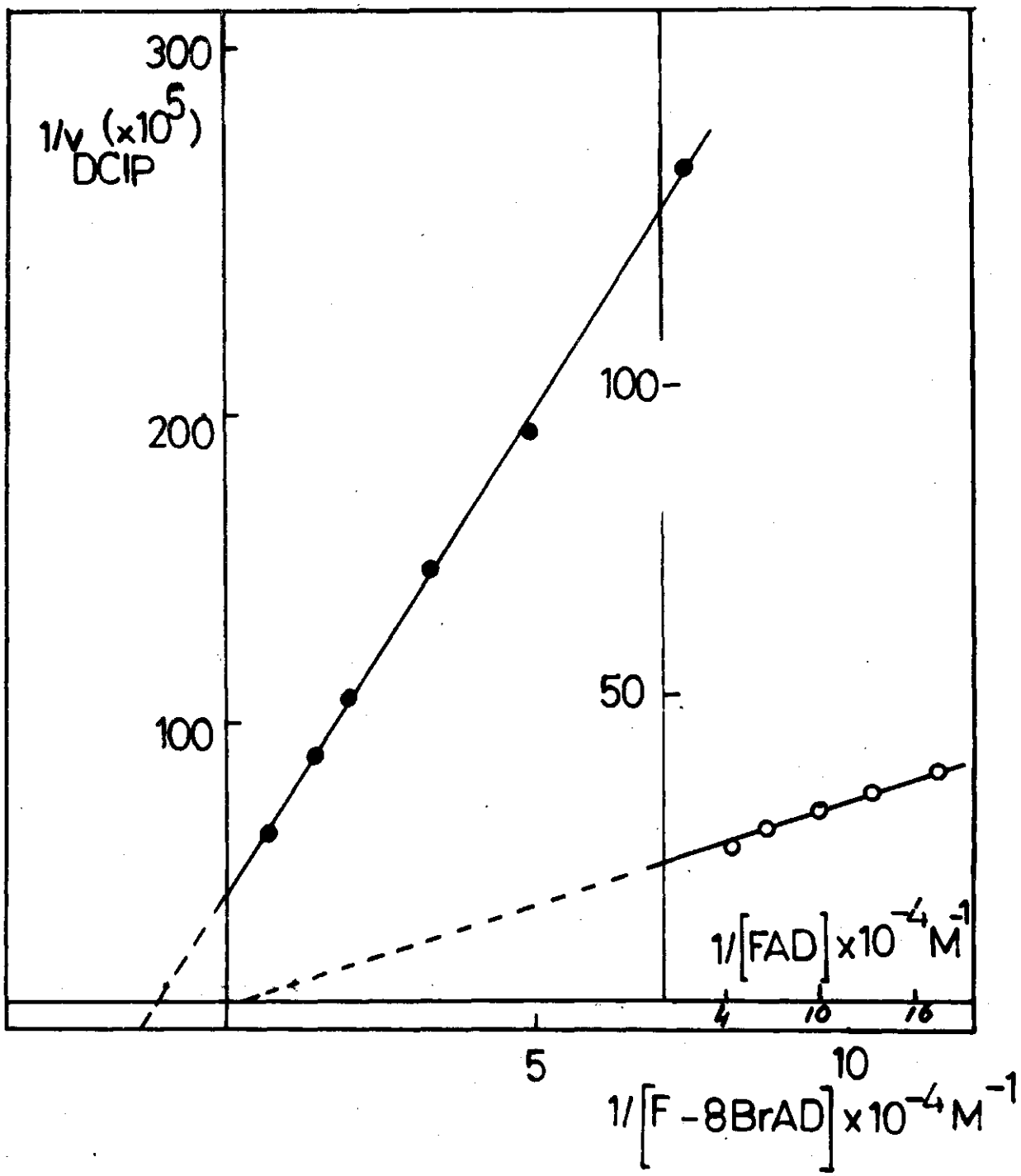
F8-bromoAD which is substituted in the adenine moiety restores the DCIP-activity upon incubation with the apoenzyme. The K_{ass} value for flavin binding of 1.5×10^4 l.mole^{-1} after 20 minutes incubation on ice was calculated from the Lineweaver-Burk plot of the restored DCIP-activities obtained at varying flavin concentrations (Fig. 6). A V_{max} value of 2400 was found, which is 55 % of the V_{max} obtained with FAD. In contrast to the FAD-enzyme itself, the F8-bromoAD-containing enzyme shows in comparison with the free flavin compound a quenched flavin fluorescence. This cannot be due to differences in intramolecular complex formation of the free flavins, since the fluorescence spectra of FAD and F8-bromoAD have the same pH-dependency (MCCORMICK). It is possible that the bromo group functions as a dynamic quencher of the flavin fluorescence, which explains the rather high value of the fluorescence polarisation of apoenzyme-bound F8-bromoAD ($p=0.4$; $\lambda_{ex}=396$ nm) as calculated from standard calibration curves and the estimated K_{ass}.

The bromo-compound does not restore any lipoate activity but interferes with the FAD-induced return of this activity. Addition at 25° of F8-bromoAD (60 µmoles) 2 minutes before that of FAD (30 µmoles) to 8 µmoles of apoenzyme, almost completely prevents the restoration of the lipoate activity. This behaviour will be discussed together with the FMN binding which behaves similarly. It is of interest to note that F8-bromoAD restores the activity when added to D-amino acid oxidase apoenzyme (KOSTER and VEEGER, unpublished results).

FMN can be bound by the lipoamide dehydrogenase apoenzyme though it restores neither DCIP nor lipoate activity. Although the restoration

Fig. 6. L-B plot of the apoenzyme recombination with F8-bromoAD and FAD. The apoenzyme (3.6 μM) was incubated on ice in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA during 20 min with the concentrations of flavin indicated after which the DCIP-activity was determined.

Fig. 6


300
200
100
1/v (x10^5)
DCIP
100
50
1/[FAD] x10^-4 M^-1
4
10
16
5
10
1/[F-8BrAD] x10^-4 M^-1

of activities fails, the binding of this compound can be demonstrated by equilibrium dialysis (Fig. 7) and by interference with the FAD--apoenzyme recombination process. The spectrum of the oxidised enzyme does not show any shoulders around the 445 nm maximum. The amount of FMN bound was calculated to be 0.9 mole per 52,000 grams of apoenzyme. The enzyme is reduced very slowly by NADH and causes a slight increase of absorbance above 500 nm. The slow rate of reduction has no catalytic significance and might be due to photoreduction. When FAD and FMN are added together to the apoenzyme, the DCIP-activity when measured after 20 minutes incubation on ice does not differ significantly from that of the control with FAD alone or is even slightly higher. However, preincubation with FMN shows inhibition of the restoration of the DCIP--activity with respect to FAD (Figs. 7 and 8), Remarkable is the deviation from linearity in the L-B plots at the lower FAD concentrations which is due to the presence of FMN, since it is not observed in the presence of NADH, a compound competitively inhibiting the FAD binding. Although the last part of the FMN inhibition curve is drawn for competitive inhibition it is difficult to distinguish between competitive and noncompetitive inhibition from these measurements at different times of incubation. It is also not possible to ascertain whether the V_{max} values found after different times of incubation with FAD are identical or different. The V_{max} values of different apoenzyme preparations are not always identical (cf. Figs. 7 and 8) but generally amount to 2500-3000 % of the value of the holoenzyme. It is of interest to note that the amcunt of inhibition by FMN is dependent on the preincubation time and increases considerably. The K_{ass} values for FMN as calculated from these plots show an increase from 0.1-0.3 x 10^5 $l.mole^{-1}$ after 30 minutes preincubation time to 2 x 10^5 $l.mole^{-1}$ after several hours of preincubation. The values obtained for binding of FAD are similar to those measured by fluorescence polarisation (cf. ref. KALSE and VEEGER, 1968). The competition between both flavins indicate that the same protein binding site is involved. Moreover, addition of different FMN concentrations, give L-B plots with approximately the same V_{max} value (Fig. 7) which underlines the competitive character.

One might argue that the slow increase in K_{ass} is due to a slow reaction of FMN with the apoenzyme. However, in that case one would expect a slow dissociation of FMN apoenzyme complex, but upon incubation with an excess of FAD no increase in rate with time is found.

The increase of the flavin association, a phenomenon most likely due to protein conformational changes is perceptible upon exchange of

Fig. 7. Effect of different FMN concentrations on the inhibition of the FAD binding to the apoenzyme. Apoenzyme was preincubated on ice in 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA with FMN (90 μ and incubated with the FAD concentrations given during 5 min (ΔΔΔ) and 15 min (▲▲▲). Also preincubated with FMN (9 μM) followed by incubation with FAD during 15 min (●●●). Control with FAD after 5 min (ooo) and 15 min (X

Fig. 7

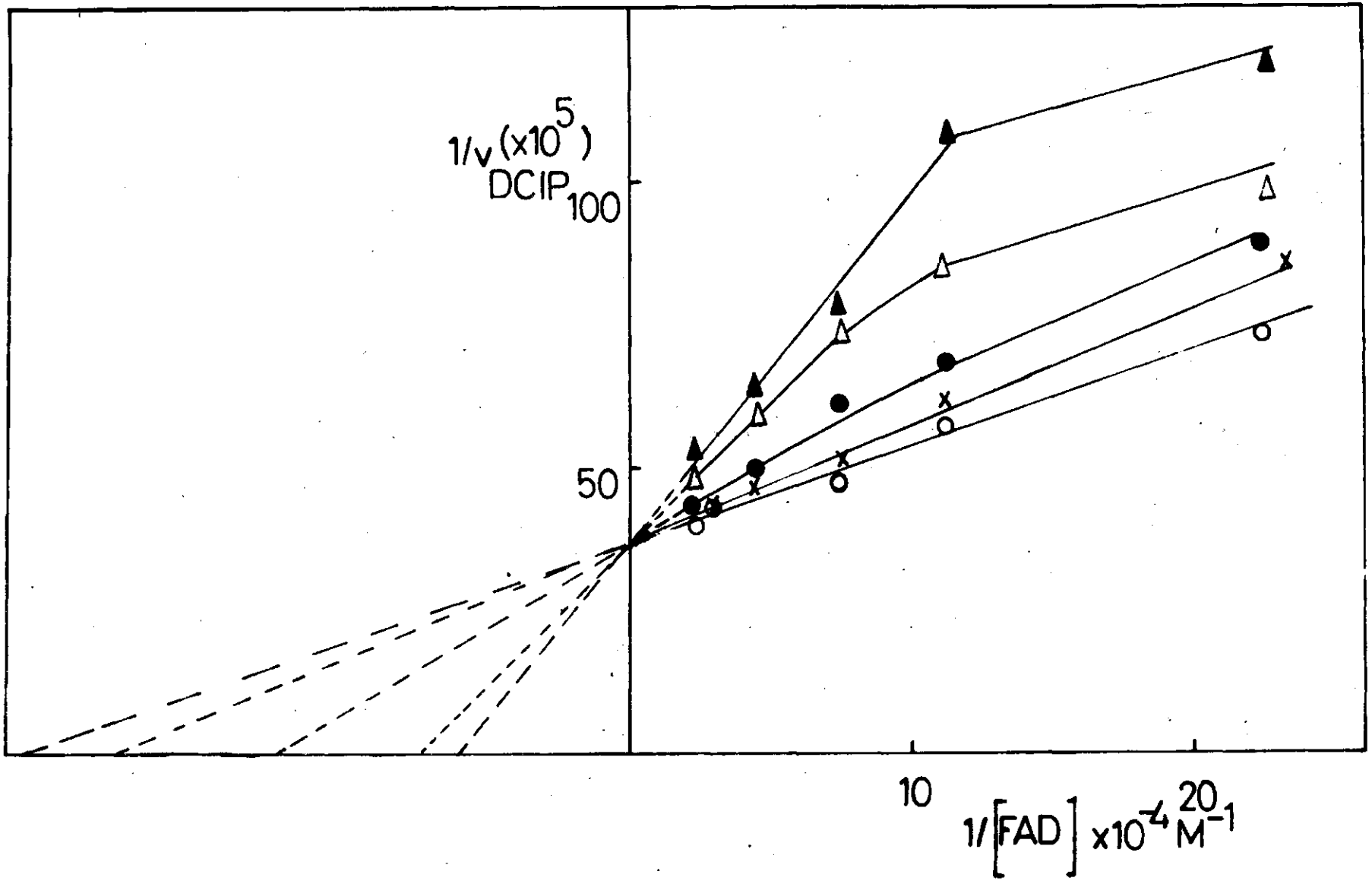


Fig. 8. Effect of different times of preincubation with FMN on the inhibition of FAD binding to the apoenzyme. The results are expressed in a L-B plot. 5 μM of the pretreated apoenzyme was incubated on ice in 30 mM phosphate pH 7.2 containing 0.3 mM EDTA, during 20 min with the FAD concentrations given, before the DCIP-activity was determined. The apoenzyme was pretreated on ice with FMN (95 μM) during 2 hrs (o●o); during 30 min (XXX); without addition (000) and with 1.1 mM NADH (■■■) during 2 min.

Fig.8

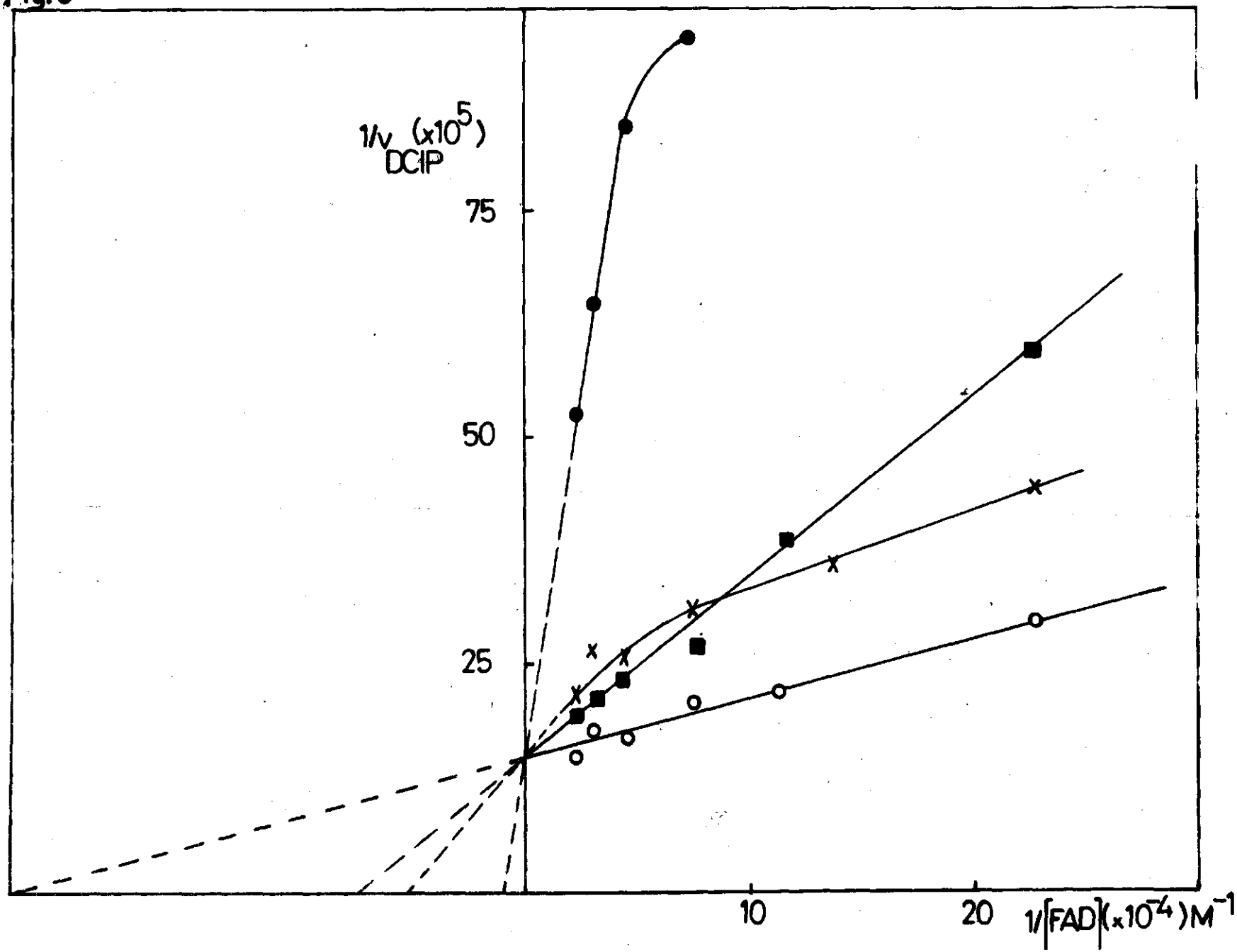
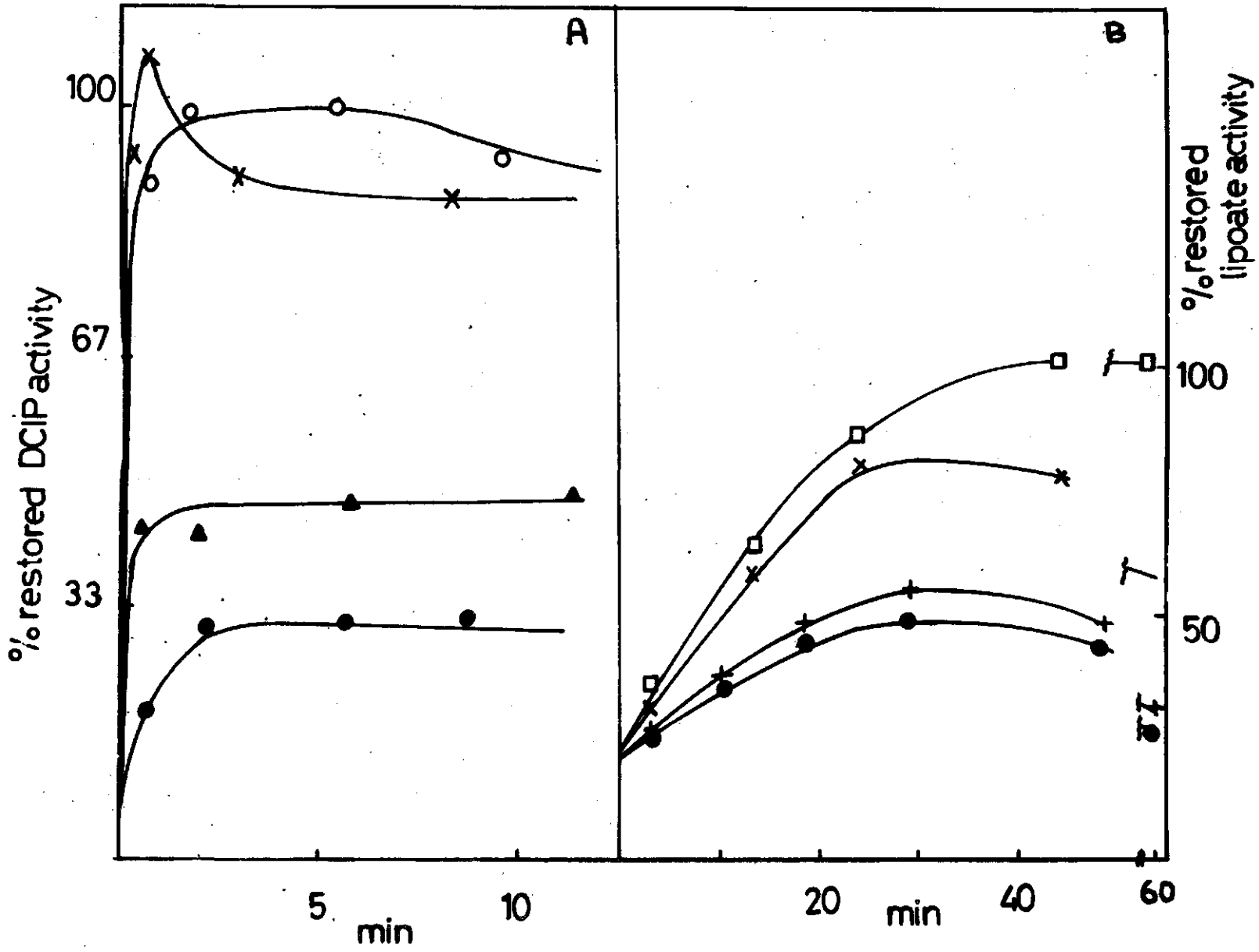


Fig. 9. A. The influence of FMN preincubation with lipoamide dehydrogenase apoenzyme on the restoration of activity with DCIP by FAD. Apoenzyme (12 μ was incubated on ice with FMN (200 μM) in 0,03 M sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA. Samples were withdrawn at 5(oo), 30(xx), 90(▲▲) and 180(●●) minutes, incubated with 100 μM FAD on ice and the DCIP-activit determined with time. B. Influence of FMN on the restoration of the activi with lipoate. Recombination of 9 μM apoenzyme of lipoamide dehydrogenase a 20° with 20 μM FAD in 0.03 M sodium phosphate buffer (pH 7.2) with 0.03 mM EDTA. □ □ apoenzyme not preincubated with FMN: xx apoenzyme preincubated during 30' minutes with 10 μM FMN; ++ with 50 μM; ●● with 90 μM FMN.

Fig. 9


A
B
100
67
33
% restored DCIP activity
% restored lipoate activity
100
50
5
10
min
20
40
60
min

an aspecific flavin compound as FMN for FAD. In order to demonstrate that the binding of FMN to the apoenzyme leads to a series of time--dependent conformational changes, the experiments given in Fig. 9 were performed. At different times FAD was added to a recombining apoenzyme-FMN system and the DCIP-activities belonging to the FAD--containing enzyme were followed. It is clear that both the maximum velocity and the time to reach the maximum are dependent on the time of preincubation with FMN. The maximum activity is always obtained between 2 and 5 min. after FAD addition; after preincubation with FMN for 30 minutes on ice the maximum appears almost immediately (within 1 min.) after the addition of FAD and then diminishes with time as has been found in three independent experiments. Longer periods of preincubation with FMN lead to much lower maximum values of the DCIP-activity. Part of the flavin still exchanges very fast as can be concluded from this rapid restoration of part of the DCIP-activity.

Preincubation with FMN at higher temperatures decreases the amount of lipoate activity restored after FAD addition. The amount of lipoate activity restored, is also dependent on the FMN concentration. Remarkable is the slow decrease of the lipoate activity in the presence of FMN even after full restoration of the lipoate activity.

Addition of FMN to the dimerising apoenzyme-FAD system at 20° diminishes the restored lipoate activity, even after reaching full activity. The dimerising system becomes less sensitive to FMN upon prolonged incubation at 25° though even after one night a 15-20 % decline of activity is observed. Similar effects have been found in the presence of F8-bromoAD. The holoenzyme itself is not FMN-sensitive. Incubation on ice of low holoenzyme concentrations, less than 0.1 mg/ml, leads due to dissociation of the enzyme to a 60-70 % inactivation of the lipoate activity (VEEGER et al., 1969) but addition of FMN does not affect this process as shown in Table IV.

The binding of FAD by apoenzyme as followed by the rate of increase of flavin fluorescence (ref. KALSE and VEEGER, 1968) occurs in a few msecs as shown by preliminary experiments with the stopped-flow fluorescence technique*. Therefore we propose the following scheme for the recombination process:

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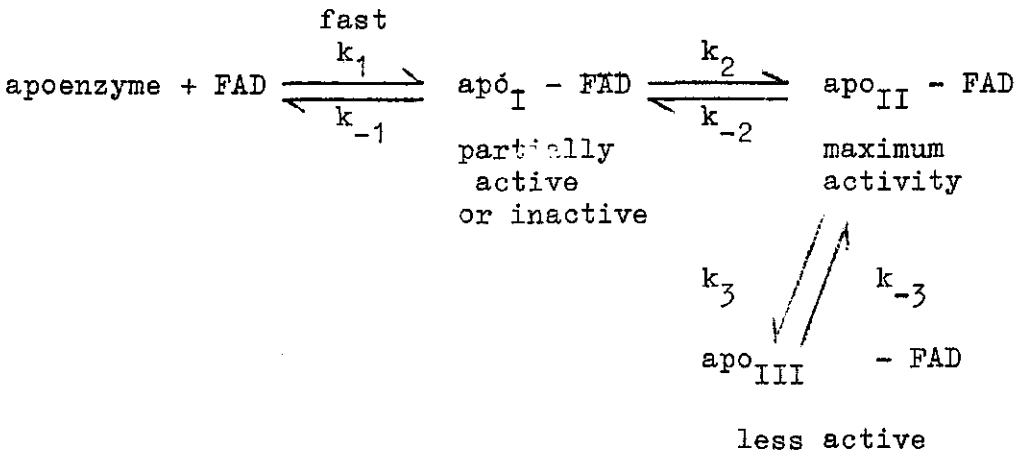
* The stopped-flow experiments were kindly performed by Dr. J.F. Koster



Scheme I

Thus the association constant measured at the maximum development of apo_{II} - FAD is actually a combination of several rate constants. The Lineweaver-Burk plots of Fig. 8 were determined after 20 min. incubation and thus after the apo_{III} - FAD complex is formed. It is possible that binding of FMN to the apoenzyme occurs in a similar though slower process as schematically indicated. However, Fig. 7 suggests that the K_{ass} value of FAD binding diminishes slightly with time. We could not definitely prove that this is a real effect. Preincubation with FMN for a short period leads to a process of FAD binding not distinct from the control or slightly higher in activity. However, after preincubation with FMN for 30 minutes the $apoenzyme_{II}$ conformation is mainly present. The optimum DCIP-activity is, due to the rapid exchange, reached very quickly upon FAD addition.

The binding properties of other FMN-analogues were also studied. None of the flavin mononucleotide derivatives restored any catalytic activity, neither did a mixture of 5'-AMP and FMN. Lineweaver-Burk plots for FAD binding with apoenzymes preincubated with 3(N)-methylFMN, 3(N)-carboxymethylFMN, 2-$NHCH_2CH_2OH$FMN and 2-NHphenylFMN show non-competitive inhibition patterns, which are dependent on the preincubation time with the analogue as is the case with FMN itself. The 3-substituted derivatives are better inhibitors than the 2-substituted compounds. IsoFMN behaves similarly to FMN; the plots deviate from linearity showing either a break or are curved. 6, 7, 8, 9 tetrahydroFMN shows a decreased affinity for the apoenzyme with respect to FMN, which suggests multiple binding forces existing between the isoalloxazine moiety and the protein.

In Table V the influence of these flavin derivatives on the dimerisation reaction is summarised. 3(N)-methylFMN and 3(N)-carboxymethylFAD are even better inhibitors than FMN. The influence of the

2-substitud derivatives depends on the kind of substituent. Substitution of sulphur for oxygen on position 2 (2-thioFMN) decreases the inhibitory properties as compared with those of FMN; since the 2-thio derivative was not completely pure, this conclusion has to be made with restrictions.

NADH and NAD^+, preincubated with apoenzyme, show a weak competitive inhibition with respect to the FAD binding as concluded from the lowered DCIP-activity. K_{ass} values for NADH and NAD^+ are 10^3 $l.mole^{-1}$ and 3×10^2 $l.mole^{-1}$, respectively. The binding of NADH to the apoenzyme can also be followed by the increase in polarisation of the NADH fluorescence working under anaerobic conditions. NADPH in the concentration tested (1 mM) did not interfere with the FAD binding as judged on the basis of the DCIP-activity.

The influence of ADP on the FAD binding results in a decreased polarisation of the flavin fluorescence. Other nucleotides tested like GTP, GDP and ATP have no effect on the DCIP-activity up to a concentration of 1 mM, but the compounds affect markedly the return of the lipoate activity as does ADP.

The effect of NAD^+ on the lipoate activity is rather peculiar. It behaves competitively with respect to the FAD binding but stimulates the rate of the dimerisation reaction at elevated temperature in concentration up to 0.5 mM. Pyrophosphate, adenosine and adenine also affect negatively the return of the lipoate activity; $NADP^+$. NADPH, 5'AMP, 3'-5'-AMP have very little or no effect. The other substrate, lipoate also has a stimu stimulating effect on the dimerisation.

5.3. DISCUSSION

The apoenzyme of lipoamide dehydrogenase has to be dissolved in buffers of high ionic strength; removal of the flavin completely alters the solubility properties and promotes the hydrophobic character of the protein. Physical properties such as mol.wt. and frictional coefficients also change (KALSE and VEEGER, 1968; VISSER and VEEGER, 1968[a]). The helix content increases from about 30 % in the holoenzyme to about 60 % in the apoenzyme as found by VOETBERG and VEEGER (unpublished results), in contrast with the results of BRADY and BEYCHOK (1968). Since the latter authors refer to a high degree of denaturation during the preparation of the enzyme, we suggest that their apoenzyme preparation, has reached further stages of denaturation than our preparation. Several indications for subsequent alterations in the apoenzyme tertiary structure could explain the variations occurring in recombination

ability. Supporting evidence for this comes from the effect of freezing, ageing and urea on the properties of the apoenzyme.

Unfolding occurs in the presence of urea as concluded from the shifts of the protein fluorescence emission maximum towards longer wavelengths. The position of the tyrosine fluorescence (304 nm emission maximum) is not influenced by urea; the difference spectra (280-292 nm excitation) indicate that the relative contribution of tyrosine fluorescence is hardly altered by 4 M urea. The tryptophan fluorescence is quenched by urea (cf. ref. WEBER, 1960) while the difference between the spectra excited at 292 and 297 nm becomes less.

The quenching of the protein fluorescence which occurs upon addition of the flavin to the apoenzyme occurs in a similar way as the increase in flavin fluorescence intensity and polarisation i.e. an initially rapid phase is followed by a slower gradual increase. The time-dependence of the DCIP-activity upon addition of flavin to apoenzyme (cf. ref. KALSE and VEEGER, 1968; Fig. 2) can be explained by either differences in V_{max} values of distinct species or a decrease in the value of the K_{ass} of FAD after a protein conformational change induced by the bound FAD itself. On the basis of our results we favour the latter possibility.

It is doubtless that the bound flavin molecule plays the most important role in the stabilisation of the tertiary protein structure. The slow time-dependent increase in the affinity of the apoenzyme for bound FMN and FMN analogues is an example of the Koshland induced fit theory (KOSHLAND Jr., 1958). Using circular dichroism, BRADY and BEYCHOK (1969) distinguish between two conformations of apoenzyme, one identical with the holoenzyme spectrum. FMN has a stabilizing effect on this native apoenzyme conformation. On binding, the small flavin molecule introduces a series of protein conformational changes which lead to an increase in the binding forces of the nucleotide, ultimately leading to the original holoenzyme structure. These structural changes already concern the monomer as the urea - stability experiments indicate. The flavin fluorescence polarisation of a recombined apoenzyme-FAD system to which urea is added is higher than the value of the same mixture to which urea was added before addition of the flavin. Moreover, the influence of flavin concentration on the effects of the potassium halogenides indicates the importance of the flavin for protein conformational changes. A reasonable explanation for the deviation from linearity at low FAD concentrations as observed in Fig. 8 is the existence of different apoenzyme-FMN species with different affinities

for the flavin. Only at higher concentrations FAD is able to displace FMN in the complex with the greatest affinity. Furthermore it must be kept in mind that the results show that the DCIP-activity measured after 20 minutes incubation with FAD is a resultant of several processes (cf. ref. KALSE and VEEGER, 1968). The decrease in FMN--sensitivity of the lipoate activity with time suggests that even after reaching maximum activity the protein conformation undergoes small changes without much influence on the catalytic centre. But apparently a fraction of the recombination mixture maintains a more labile structure since the holoenzyme as isolated is not FMN-sensitive at all.

Restoration of the DCIP-activity is confined to FAD and some of its derivatives, 3(N)-methylFAD and 3-(N)-carboxymethylFAD, F8-bromoAD; substitution of a bulky group on position 2 does not restore activity (2-morpholinoFAD). Introduction of side-chains in the FMN nucleus give derivatives which affect the FAD binding noncompetitively. 3(N)--derivatives are fairly good inhibitors (cf. 3(N)-FAD derivatives); 3-methylFMN is even slighter better than FMN itself. Changes on position 2 of nucleus affect the inhibitory properties to some extent. IsoFMN and FMN have similar inhibitory action but the introduction of hydrogen in the nucleus (tetrahydroFMN) decreases the inhibition. Unpublished observations show that the reduced flavin has a lower affinity for the apoenzyme as it partially dissociates from the holo-enzyme if held in the cold under anaerobic conditions in the presence of NADH, with (cf. ref. MASSEY et al., 1962) or without urea, followed by gel filtration or electrophoresis.

Flavin binding to several non-metallo apoenzyme appears to occur in different ways. In D-amino acid oxidase which has its FAD losely bound, neither 3(N) nor 2- substituted derivatives restore any activity (CHASSY and MCCORMICK, 1965; MCCORMICK et al., 1964) whereas F8-bromoAD partially restores the activity. The binding of FMN is noncompetitive with respect to FAD. Both the FMN and the AMP-moiety are of importance for the FAD binding to the apoenzyme (YAGI and OZAWA, 1960. In the L-amino acid oxidase the flavin is firmly bound while upon substrate reduction this enzyme is more stable (WELLNER and MEISTER, 1960) in contrast to lipoamide dehydrogenase (MASSEY et al., 1962) and glutathion reductase (SCOTT et al., 1963; ICEN, 1967). In the latter two enzymes the flavin binding shows similarities in the influence of FMN on the FAD binding. The holoenzyme of glutathione reductase is not FMN-sensitive but the reconstituted enzyme produced from apoenzyme and FAD is sensitive

after reaching the maximum activity (STAAL et al., 1969).

The importance of the polarity of the flavin surroundings for the catalytic properties was emphasized earlier (VEEGER and MASSEY, 1962); a shift to 450 nm is always concurrent with a stimulated DCIP--activity in lipoamide dehydrogenase. No exact information is yet available concerning the groups of the protein involved in the binding process. The quenching of the tryptophan fluorescence and the energy transfer between protein and flavin (cf. ref. VEEGER et al., 1969) is no indication for a direct tryptophan-flavin interaction as energy transfer can occur over large distances (ref. WEBER and TEALE, 1959). However, it is known that flavin-indole complexes are easily formed (HARBURY and FOLEY, 1958), by charge-transfer complex formation (MULLIKEN, 1952; KARREMAN, 1962); such complexes, however, have only been found with the oxidised, neutral form of the isoalloxazine ring (WILSON, 1966). The primary interaction in flavin-indole complexes is envisaged between the rings themselves; the strength of the interaction should be relatively independent of changes in side chain but dependent on modifications in electronic structure of the ring system. These results are suggestive as they have similarities with our own observations; the 3-iminoposition can be altered but ring modifications diminish the affinity of the protein for the flavin compound.

Recently DE KOK, SPENCER and WEBER (1968) have suggested a sulfydryl group in the neighbourhood of the flavin to be responsible for the dynamic quenching of the flavin fluorescence.

The refolding processes vary among different apoenzyme preparations. Accepting the idea that apoenzyme formation is a reversible denaturation process, one can expect to find some lesions in the protein structure which are only slowly and partially restored or not restored at all. Another questionable point in this respect is the homogeneity of the apoenzyme population.

While this paper was in progress, SWOBODA (1969) has found interactions between glucose oxidase apoenzyme and adenine nucleotides but not between the isoalloxazine moiety and the apoenzyme.

Table I

The effect of ageing on the recombination properties of lipoamide dehydrogenase apoenzyme. Apoenzyme (1 mg/ml) was stored on ice in 30 mM phosphate buffer pH 7.2. At the times indicated samples of 0.1 mg were incubated in a volume of 0.3 ml containing 400 µM FAD and 30 mM sodium phosphate buffer pH 7.2. After 5 min. incubation on ice the DCIP-activity was determined after which the samples were incubated at 20° and both the lipoate and the DCIP-activity determined. In the control the rest activity was determined without preincubation with FAD.

Incubation time of apoenzyme	5' on ice	1 hr at 20°	
	% DCIP activity	% DCIP activity	% lipoate activity
Control	-	170	3
1 hr	1240	340	66
50 hr	1175	400	35
75 hr	-	450	23
100 hr	955	935	20
175 hr	850	650	15

Table II

Influence of urea on the binding of FAD. 2.6 μM apoenzyme was incubated with a limited amount of FAD (1 μM) at 10° in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA with or without pretreatment with urea. The polarisation of the flavin fluorescence was measured as previously described (ref. KALSE and VEEGER, 1968) with the exception that the excitation wavelength was 450 nm.

Treatment	Time recombination min.	p
A. Control	2	0.206
	6	0.232
B. 0.5 M urea added after 10 incubation with FAD	16	0.220
C. As B but with 2 M urea	6	0.156
	several hrs	0.134
D. Apoenzyme preincubated with 2 M urea during 2 min.; then FAD was added	6	0.080
	30	0.065

Table III

The influence of halogen ions on the fluorescence and DCIP--activity of a recombining system. The apoenzyme was previously dialysed at a concentration of 1.5 mg/ml against 50 mM sodium phosphate buffer containing 0.3 mM EDTA. 3.8 μM of apoenzyme was reacting at 10° with the same flavin concentration. The halogenides were present in 60 mM concentration.

Halogenide	Recombination time (min)	DCIP-activity after 5' %	p	p(free flavin)
KBr	5	665	0.110	0.050
	30		0.120	
NaBr	5	405	0.110	0.045
	30		0.120	
LiBr	5	405	0.110	0.050
	30		0.120	
KI	5	175	0.100	0.050
	30		0.105	
NaI	5	155	0.095	0.055
	30		0.100	
KCl	5	500	0.080	0.030
	30		0.085	
NaCl	5	480	0.080	0.030
	30		0.095	
Control	5	780	0.090	0.030
	30		0.095	

Table IV

Stability of the holoenzyme in the presence of FMN. Enzyme, 13 mg/ml and 200 units/mg DCIP-activity was dialysed overnight at 0° against 0.03 M sodium phosphate buffer (pH 7.2) containing 0.1 m M EDTA. After dialysis the enzyme was diluted with the same buffer and the DCIP-activity measured at the time indicated, in the absence and presence of 0.1 mM FMN.

Time after dilution	Enzyme concentration			
	0.02 mg/ml FMN		0.05 mg/ml FMN	
	-	+	-	+
0	1000	-	1300	-
30 min.	1500	-	1360	-
90 min.	1450	1650	1450	1650
48 hr	1800	1750	2000	2050

Table V

Influence of FMN, FMN-derivatives and other nucleotides on the return of the lipoate activity. The flavin derivatives were preincubated on ice in 100 μM concentration with 7.3 μM apoenzyme for 30 and 60 min. after which 100 μM FAD was added while the temperature was raised to 20°. Lipoate activities were determined after one hour incubation. Medium: phosphate buffer pH 7.2 containing 0.3 mM EDTA. The nucleotides were incubated on ice in 100 μM concentration with 6.4 μM apoenzyme; after 30 min. preincubation 100 μM FAD was added. The lipoate activity was determined after 30 min.incubation at 20°.

Flavin derivate	% lipoate activity 30 min. preincubation	% lipoate activity 60 min. preincubation
FAD	100	100
FMN	62	43
IsoFMN	68	50
TetrahydroFMN	81	67
3(N)- methylFMN	48	32
2-thioFMN	92	65
2-$NHCH_2CH_2OH$FMN	66	38
2-NHphenylFMN	102	70
3(N)-carboxymethylFAD	50	24
Nucleotides added		% lipoate activity
FAD		100
ATP		74
ADP		62
5'AMP		98
3'5'-cyclAMP		91
Adenosine		84
Adenine		72
NAD^+		115
$NADP^+$		94
GTP		83
GDP		74
Pyrophosphate		63

6. THE CATALYTIC MECHANISM OF LIPOAMIDE DEHYDROGENASE

6.1. INTRODUCTION

The essential role of NAD^+ in the catalytic mechanism of lipoamide dehydrogenase was discovered by MASSEY and VEEGER (1961) for the pig heart enzyme and confirmed for the proteins from other species (MASSEY, 1963). MASSEY and VEEGER concluded from their studies that apart from a role in the catalytic reaction, NAD^+ is bound to an unknown site Y in order to prevent the enzyme against being converted by NADH into the inactive four--equivalent reduced enzyme. The enzyme is only able to exert its physiological function by shuttling between the oxidised and the two-equivalent reduced forms of the enzyme. It was thought that one equivalent reduces the flavin and the second opens a reactive S-S bridge, forming a SH group and a S-radical. The reaction pathway of the overall reaction with reduced lipoamide ($lip(SH)_2NH_2$) as donor is summarised in the following "ping pong bi bi" mechanism (CLELAND, 1963):

(1) $FAD\text{-}enz\text{-}S_2 + lip(SH)_2NH_2 \rightleftharpoons FAD\text{-}enz\text{-}(SH)_2 + lipS_2NH_2$

(2) $FAD\text{-}enz\text{-}(SH)_2 \rightleftharpoons FADH^{\cdot}\text{-}enz\text{-}S^{\cdot}SH$

(3) $FADH^{\cdot}\text{-}enz\text{-}S^{\cdot}SH + NAD^+ \rightleftharpoons FADH^{\cdot}\text{-}enz\text{-}S^{\cdot}S(NAD) + H^+$

(4) $FADH^{\cdot}\text{-}enz\text{-}S^{\cdot}S(NAD) \rightleftharpoons FAD\text{-}enz\text{-}SH\ S(NAD)$

(5) $FAD\text{-}enz\text{-}SH\ S\ (NAD) \rightleftharpoons FAD\text{-}enz\text{-}S_2 + NADH$

The slow side reaction to the 4-equivalent reduced enzyme is:

(6) $FADH^{\cdot}\text{-}enz\text{-}S^{\cdot}SH + NADH + H^+ \rightleftharpoons FADH_2enz(SH)_2 + NAD^+$.

Studies of MASSEY et al. (1960 and 1963) have shown that the rate of formation of the so-called semiquinone form of the enzyme ($FADH^{\cdot}\text{-}enz\text{-}S^{\cdot}SH$) accounts for the rate of the overall reaction, while other studies show that a complex between NAD^+ and the 2-electron reduced enzyme might be involved as well (VEEGER and MASSEY, 1963).

One of the features of the proposed mechanism is the occurrence of NAD^+ bound to a SH-group. In later studies however, it was demonstrated that after conversion of the 2-electron reduced enzyme into the $FAD\text{-}enz\text{-}S_2 = AsO^-$ form by the addition of AsO_3^{3-}, the addition of NADH results in the formation of the 4-equivalent reduced enzyme via the formation of intermediates with similar spectral characteristics as those of the 2-electron reduced enzyme (VEEGER et al., 1966). This observation suggests that instead

of by an attack on the S-S bridge NADH reduces the enzyme by direct reaction with the FAD.

Studies with other flavoproteins (KOSTER and VEEGER, 1968; ZEYLEMAKER et al., 1969; STAAL and VEEGER, 1969) i.e. succinate dehydrogenase, D-amino acid oxidase and glutathione reductase, show that instead of the postulated ping pong mechanisms, Theorell-Chance or ordered bi bi mechanisms are operating. With these enzymes the oxidised product (i.e. fumarate, pyruvate and $NADP^+$) acts as competitive inhibitor of the donating substrate, while furthermore these compounds show spectrally visible complexes with the oxidised enzyme (VEEGER et al., 1966; STAAL and VEEGER, unpublished results). Especially the occurrence of the complex between $NADP^+$ and glutathione reductase (STAAL and VEEGER, unpublished results) is of interest for the closely related enzyme lipoamide dehydrogenase, as well as the existence of two $NADP^+$ complexes with transhydrogenase (VAN DEN BROEK and VEEGER, 1969) from Azotobacter vinelandii. STEIN and CZERLINSKI (1967) have mentioned that at high NAD^+ concentrations multi-complexes with lipoamide dehydrogenase are formed, while furthermore the pyridine nucleotide is a competitive inhibitor with respect to NADH in the reduction of lipoic acid derivatives with a K_i = 0.2 mM (STEIN and CZERLINSKI, 1967).

In our laboratory it has been shown by VOETBERG (VISSER et al., 1969) that two different NAD^+-lipoamide dehydrogenase complexes exist. One binding site has a K_D of 35 μM while the overall K_D amounts to 90-110 M. At saturating NAD^+ concentrations, the difference spectrum of the enzyme results in two positive maxima at 507 and 387 nm and four negative maxima at 477, 450, 430 and 370 nm. The 450 nm maximum only arises at high NAD^+ concentrations (> 0.1 mM). The differences between the two complexes are better resolved at a low titration temperature.

6.2. RESULTS

6.2.1. Kinetics

In contrast with the results previously reported (MASSEY et al., 1960), the series of L-B plots at varying acceptor and donor concentrations in the reaction of NAD^+ with $lip(SH)_2NH_2$ is not parallel but convergent instead and intersects at one point in the third quadrant (Fig. 1). This type of kinetics is known from studies with several other flavoproteins viz. D-amino acid oxidase (KOSTER and VEEGER, 1968), succinate dehydrogenase

(ZEYLEMAKER et al., 1969 and ZEYLEMAKER, 1969) and glutathione reductase (STAAL and VEEGER, 1969) and points to an "ordered bi bi" mechanism (CLELAND, 1963).

Furthermore the occurrence of a ternary complex between both substrates can be derived from the spectral data. The rate of formation of the NAD^+-complex is extremely high. At 1 mM NAD^+ at 25°, the complex at 507 nm is formed completely within 3-5 msec., which is much faster than the rate of reduction by $lip(SH)_2NH_2$. This can account for the postulation that NAD^+ is the first substrate; nevertheless also $lip(SH)_2NH_2$ can react directly with the oxidised enzyme (MASSEY et al., 1960).

In the following scheme which summarizes these results, the state of reduction of the flavin is left open.

(1) $E + NAD^+ \rightleftharpoons ENAD^+$

(2) $ENAD^+ + lip(SH)_2NH_2 \rightleftharpoons ENAD^+lip(SH)_2NH_2$

(3) $ENAD^+lip(SH)_2NH_2 \rightleftharpoons E'NAD^+ + lipS_2(NH)_2$

(4) $E'NAD^+ \rightleftharpoons E + NADH + H^+$

In such a mechanism it can be expected that the product NADH inhibits competitively with respect to NAD^+ and noncompetitively with respect to $lip(SH)_2NH_2$. At high NAD^+ concentrations the L-B plot in the presence of NADH is non-linear and the inhibition tends to become competitive; at low NAD^+ concentrations the L-B plot is linear and the inhibition non-competitive (Fig. 2). These experiments were performed at a relatively high $lip(SH)_2NH_2$ concentrations. In the L-B plots of Fig. 1 a tendency to deviate from linearity exists at low concentrations of the variable substrate, especially at low concentrations of the second substrate. At lower temperatures (10-15°) this deviation from linearity is less than at 25° and inhibition by NADH is also better competitive with respect to NAD^+.

The inhibition pattern of the second product, $lipS_2(NH_2)$ is more complex (Fig. 3). In an ordered bi bi mechanism $lipS_2(NH_2)$ is expected to be a noncompetitive inhibitor with respect to $lip(SH)_2NH_2$ at all NAD^+ concentrations. However, at high NAD^+ concentrations, the L-B plot of the inhibited reaction intersects that of the not-inhibited reaction in the first quadrant. No activation of the reaction was found compared with the control. Upon lowering the NAD^+ concentration, the inhibition pattern shifts to a noncompetitive type of inhibition via an approximately competitive picture. Inhibition by NADH tends to become competitive with respect

Fig. 1. Kinetic characteristics of the reduction of NAD^+ by $lip(SH)_2NH_2$. A. L-B plots obtained with $lip(SH)_2NH_2$ as variable substrate at different NAD^+ levels at 25°. The cuvettes contained sodium phosphate buffer (50 mM pH 7.6), 0.067 % (w/v) bovine serum albumin, 0.3 mM EDTA and $lip(SH)_2NH_2$ as indicated. NAD^+ concentrations: 800 µM (xxx), 600 µM (■■■), 400 µM (□□□), 300 µM (▲▲▲), 250 µM (ΔΔΔ), 150 µM (●●●) and 100 µM (000). The dotted L-B plot (⊙⊙⊙) is obtained by extrapolating to infinite NAD^+ concentration. The reaction was started by adding 1.25 µg of enzyme (A280 nm A455 nm=5.2) to a total volume of 3 ml. Enzyme activities are expressed i Δ absorbancy 340 nm/min/mg protein. B. L-B plots with NAD^+ as variable substrate at different $lip(SH)_2NH_2$ levels: 400 µM (●●●), 333 µM (000), 267 µM (ΔΔΔ), 200 µM (▼▼▼) and 167 µM (□□□). The dotted L-B plot (⊙⊙⊙) is obtained by extrapolating to infinite $lip(SH)_2NH_2$ concentration. Conditio as in Fig. 1A.

Fig 1

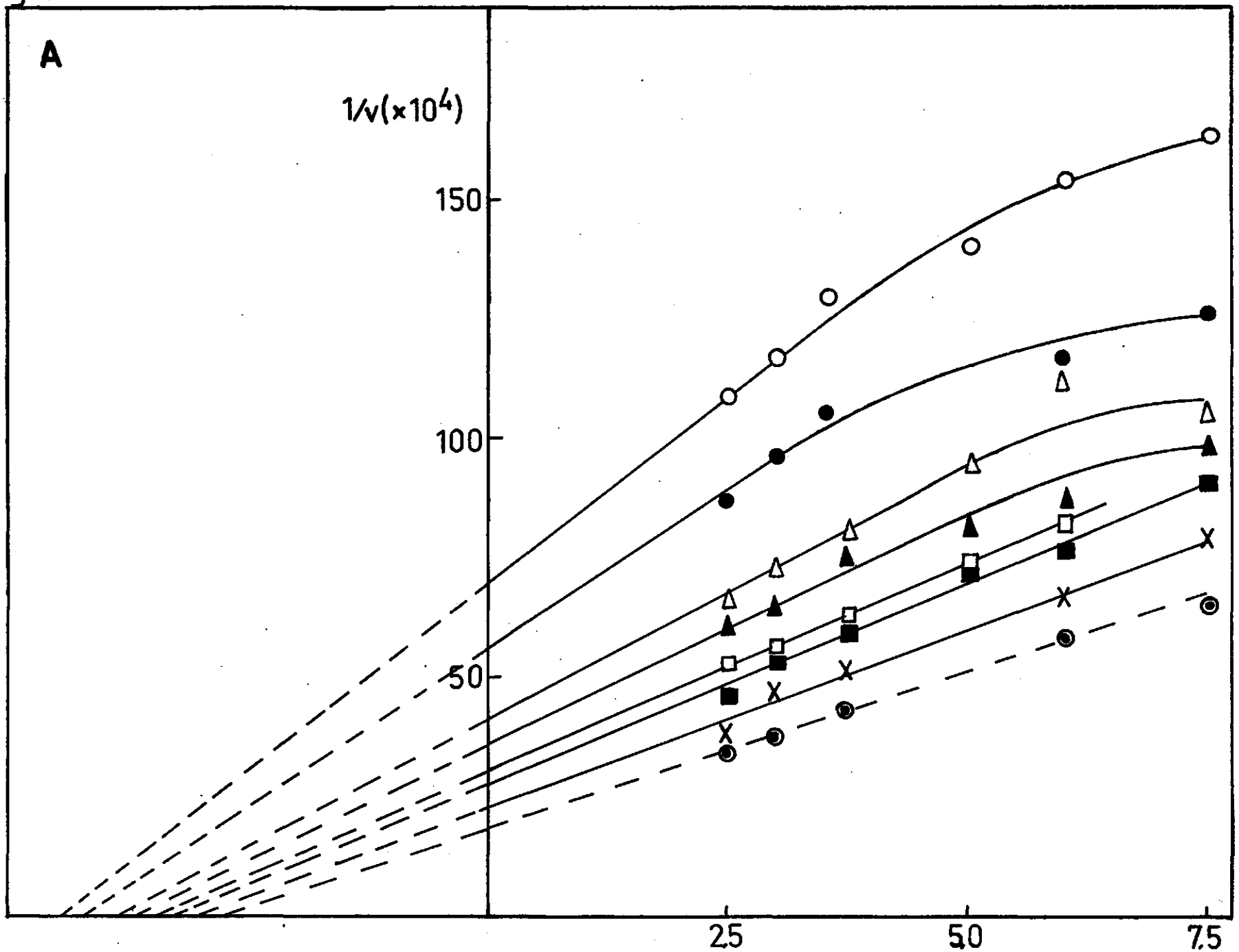


Fig.1

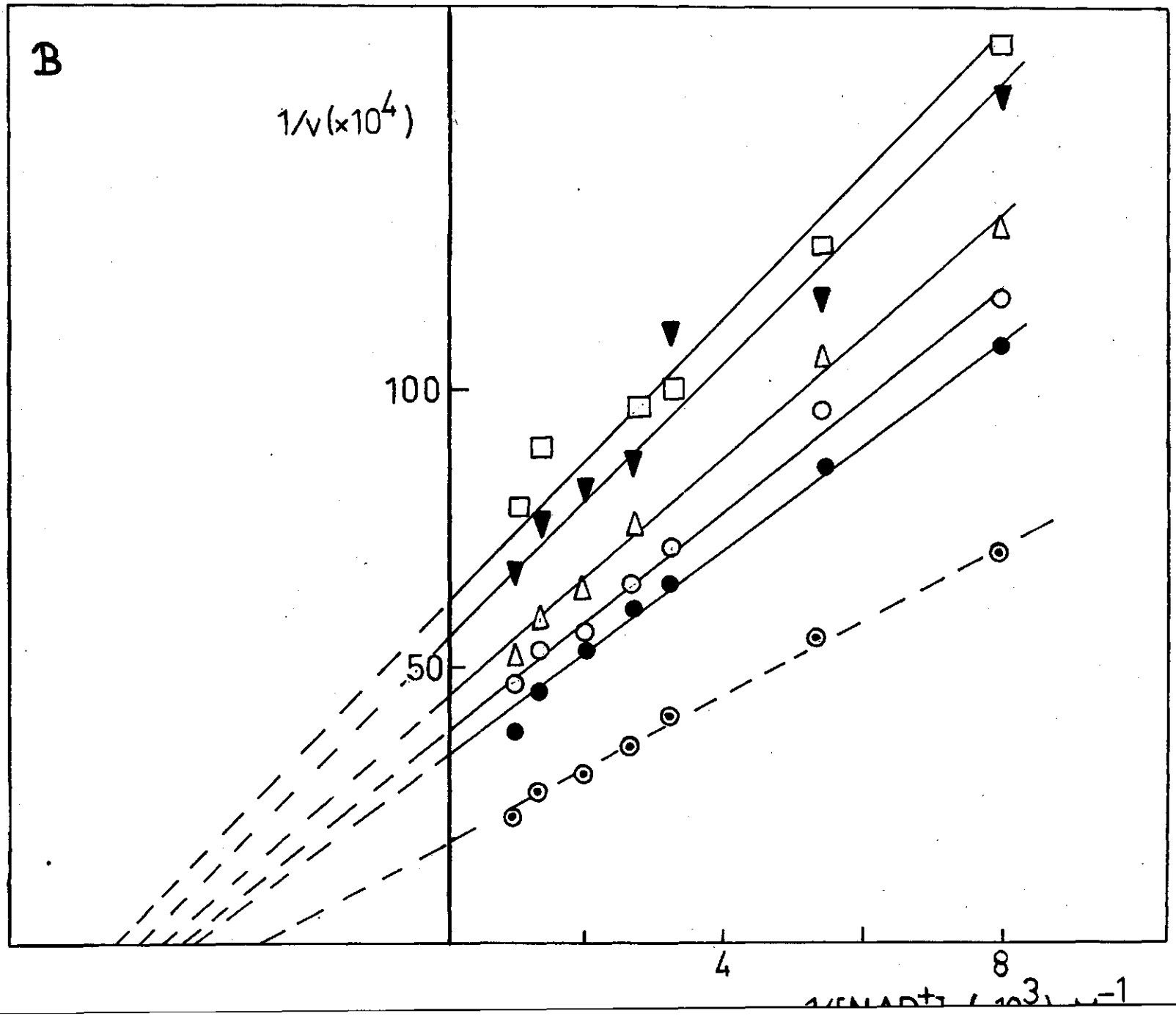


Fig. 2. Inhibition by NADH of the reduction of NAD^+ by $lip(SH)_2NH_2$ at a relatively high $lip(SH)_2NH_2$ concentration (333 μM). Conditions as in Fig. L-B plot without (○○○) and with (●●●) 0.1 mM NADH added to the reaction cuvette.

Fig.2

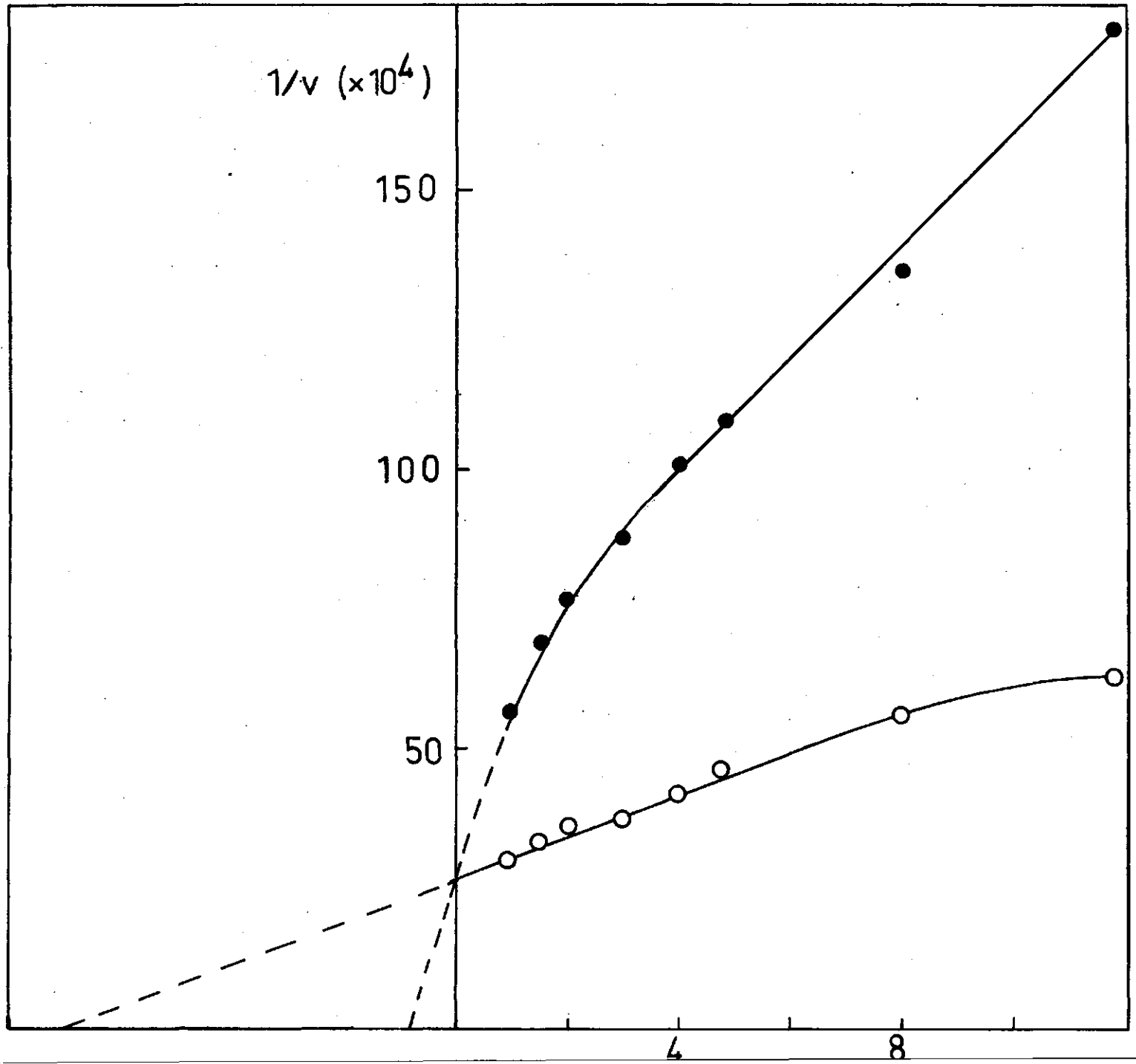
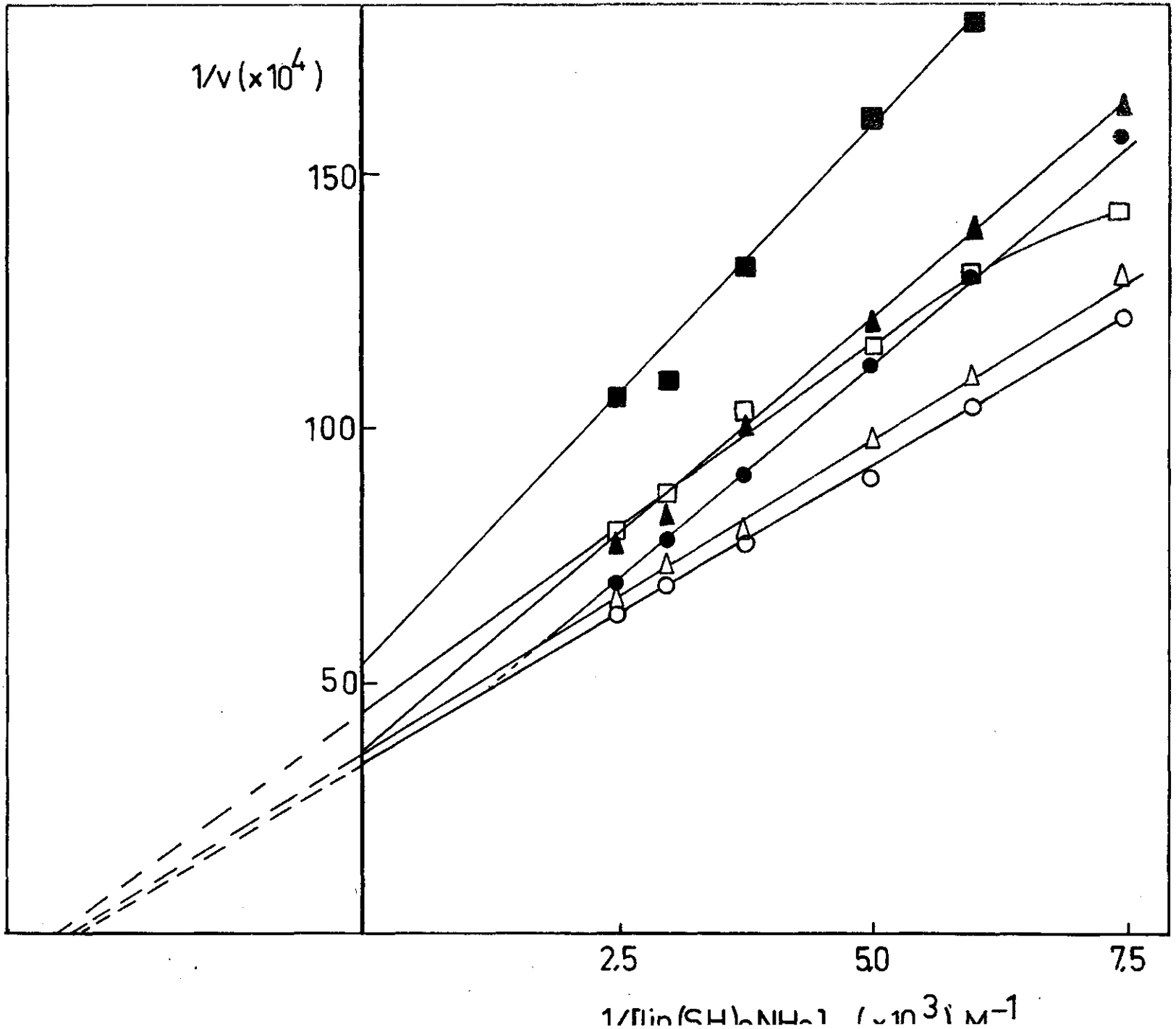


Fig. 3. Inhibition patterns of 0.5 mM $lipS_2(NH_2)$ with respect to $lip(SH)_2N$ at three different NAD^+ levels. 667 μM NAD^+ without (000) and with (●●●) inhibitor; 500 μM NAD^+ without (ΔΔΔ) and with (▲▲▲) inhibitor; 250 μM NAD^+ without (□□□) and with inhibitor (■■■). Conditions as in Fig. 1.

Fig.3



to $lip(SH)_2NH_2$ at low NAD^+ concentrations, whereas it is noncompetitive at high NAD^+ concentrations.

A situation in which both products inhibit competitively points according to the terminology of CLELAND (1963) to a "rapid equilibrium random bi bi mechanism". However, ultimately the $lipS_2(NH_2)$ inhibition becomes noncompetitive with respect to $lip(SH)_2NH_2$, which rules out this possibility. On the other hand, it is clear that the inhibitory effect of both products NADH and $lipS_2(NH_2)$ is dependent on the concentrations of both substrates. In combination with the non-linearity of the plots the most likely explanation seems to be a "preferred order" mechanism (FERDINAND, 1966). In our case this means a reversal of the postulated binding sequence at low NAD^+ and high $lip(SH)_2NH_2$ concentrations.

The substrate saturation curves of NAD^+ and $lip(SH)_2NH_2$ are not hyperbolic but more or less biphasic (cf. ZEYLEMAKER et al., 1969) as shown for NAD^+ in Fig. 4A. The Hill coefficient of NAD^+ depends on the reduced lipoamide level. At infinite $lip(SH)_2NH_2$ the slope is 1.0-1.1 while at finite concentrations the coefficient is 0.7-0.8 in the lower NAD^+ concentration range and tends to become 1.0 at higher concentrations (Fig. 4B). In the reverse situation, the Hill coefficient of $lip(SH)_2NH_2$ at infinite NAD^+ concentration is 1.0 as well. There are two possible ways of explaining why the Hill coefficient is 0.7-0.8 for NAD^+. The first one is that both NAD^+ binding sites are involved in the catalytic process but that the complexes differ in turnover number. It could be visualised that the specific binding to the high affinity form leads to a lower turnover than in case both NAD^+ sites are saturated.

The second alternative is the Ferdinand model assuming a shift in binding sequence of both substrates. This model explains why at a relatively high $lip(SH)_2NH_2$ level the NAD^+ concentration necessary to approach a Hill coefficient of 1 is larger than at a low $lip(SH)_2NH_2$ level. NAD^+ takes over as first substrate at a higher concentration in the first case. Moreover, this model explains why at infinite $lip(SH)_2NH_2$ a coefficient of 1 is obtained for NAD^+ as under those conditions a normal NAD^+ saturation pattern is expected since $lip(SH)_2NH_2$ acts as first substrate.

The relation between the Hill coefficient and different substrate concentrations has not been studied extensively in case of the product inhibitors. The product inhibitor, $lipS_2(NH_2)$behaves peculiarly and has an interaction coefficient larger than 1.0 which tends to increase with decreasing NAD^+ concentrations (Fig. 5A). The increase of the Hill coefficient from

Fig. 4. A. NAD^+ saturation of lipoamide dehydrogenase at different $lip(SH)_2NH_2$ levels. Catalytic activities were measured at 25°. NAD^+ concentrations were varied as indicated with ΔΔΔ, 330 μM $lip(SH)_2NH_2$ and 000 100 μM $lip(SH)_2NH_2$, respectively. B. Hill plots of NAD^+ at different $lip(SH)_2NH_2$ concentrations. The data used are those of Fig. 4A with ●●●, 100 μM and ▲▲▲, 330 μM $lip(SH)_2NH_2$, respectively. The V_m values used have been obtained from the L-B plot. V_{max} values 208 and 454 units, respectively.

Fig 4

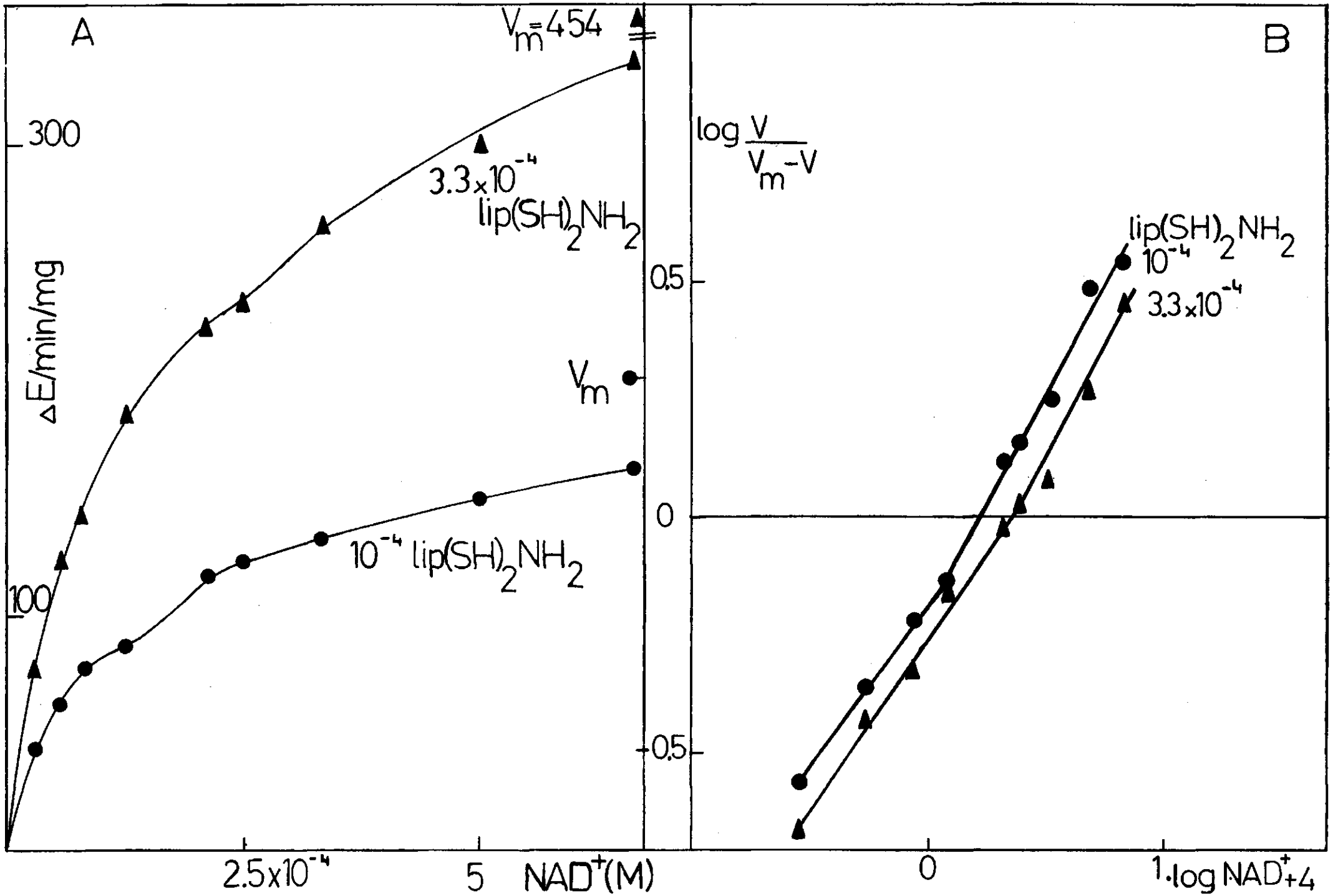
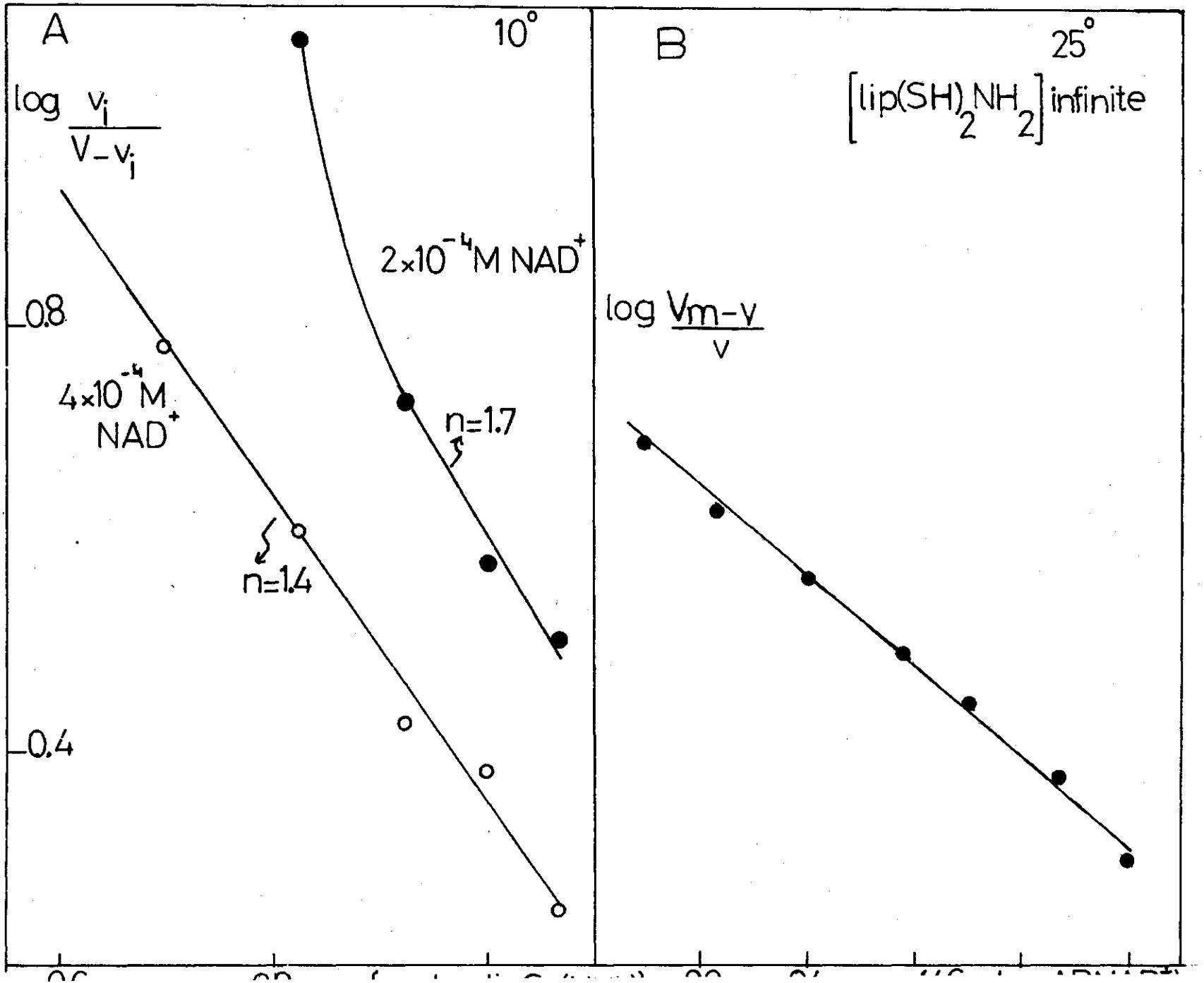

A
V_m=454
300
100
3.3×10^{-4} lip$(SH)_2NH_2$
10^{-4} lip$(SH)_2NH_2$
V_m
ΔE/min/mg
2.5×10^{-4}
5
NAD^+(M)
B
log $\frac{V}{V_m-V}$
lip$(SH)_2NH_2$
10^{-4}
3.3×10^{-4}
0.5
0
-0.5
0
1.log NAD^++4

Fig. 5. A. Hill plots of the $lipS_2(NH_2)$ inhibition. The product inhibition has been measured at 10° with 200 µM $lip(SH)_2NH_2$ at NAD^+ levels of 400 µM (●●●) and 200 µM (●●●), respectively. B. Hill plot of $APNAD^+$ at infinite $lip(SH)_2NH_2$ concentration. Data from Fig. 6.

Fig.5


A
10°
log $\frac{v_i}{V-v_i}$
2×10⁻⁴M NAD⁺
0.8
4×10⁻⁴M NAD⁺
n=1.7
n=1.4
0.4
B
25°
[lip(SH)2NH2] infinite
log $\frac{Vm-v}{v}$

1.4 to 1.7 on lowering the NAD^+ concentration suggests that either two binding sites are available for $lipS_2(NH_2)$ or that the inhibitory function is a complex one. Though there is in fact no direct argument against the first suggestion and even some support for it from the 4-electron reduction in the Escherichia coli enzyme by $lip(SH)_2NH_2$ (KOIKE et al., 1960b), the second suggestion seems a sound one. $LipS_2(NH_2)$ acts as a regular product inhibitor but from the spectral data mentioned before it is known that abortive complexes of the type $E\text{-}(NAD^+)_2\text{-}(lipS_2(NH_2))_x$ also exist. For certain sequences the combination of both types of inhibition results in a rate equation which is a quadratic function of the inhibitor concentration. However, a definite conclusion requires more work.

The preferred order mechanism complicates the calculation of the velocity constants but some of the kinetic parameters have been calculated for conditions under which one of the two pathways dominates, viz. at relatively high NAD^+ concentrations when the Hill coefficient tends to become 1.0. For a mechanism in which a ternary complex operates the following rate equation is derived for the steady state with $[P] = 0$ (ALBERTY, 1958).

$$\frac{E_0}{v} = \frac{1}{k_3} + \frac{1}{k_4} + \frac{1}{k_1 a} + \frac{1 + k_{-2}/k_3}{k_2 b} + \frac{k_{-1}\,(1 + k_{-2}/k_3)}{k_1 k_2 ab}$$

This equation fits to a mechanism schematically indicated as:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EAB$$

$$EAB \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EC + D$$

$$EC \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + C$$

The velocity constants which can be calculated according to this simplified mechanism are k_{+1}, k_{-1}, $(1/k_{+3} + 1/k_{+4})$ and $(1 + k_{-2}/k_{+3})/k_{+2}$.

$(1/k_3 + 1/k_4)$ is identical with $1/V_{max}$. From a 1/v vs $1/S_I$ plot maximal velocities can be extrapolated at infinite $[S_I]$ but at different finite levels of S_{II}. From the L-B plot of $1/V_{max}$ (S_I =) vs $1/S_{II}$ the V_{max} can be calculated (Fig. 1B). $1/k_1$ is derived from the slope of the L-B plot 1/v vs $1/NAD^+$ at infinite $lip(SH)_2NH_2$ concentration. Under those conditions one would expect $lip(SH)_2NH_2$ to be the first substrate. However, the V_{max} values used are obtained by extrapolation to infinite $lip(SH)_2NH_2$ under conditions where NAD^+ is the first substrate.

$(1 + k_{-2}/k_3)/k_2$ is obtained from the slope of the L-B plot 1/v vs $1/lip(SH)_2NH_2$ at infinite NAD^+ concentrations (Fig. 1A). The term k_{-1} is obtained by substituting experimental data in the rate equation in combination with the parameters calculated above.

A similar set of velocity constants can be obtained from the reverse reaction *viz.*, k_{+4}, k_{-4}, $(1/k_{-1} + 1/k_{-2})$ and $(1 + k_3/k_{-2})/k_{-3}$. By combining these data all eight rate constants in this mechanism can be calculated. In our case the reverse reaction has been studied at a different pH which makes a combination of these data rather impossible.

A survey of the calculations in comparison with the data from the literature, is given in Table I.

When 3-acetylpyridine NAD^+ ($APNAD^+$) is used as electron acceptor instead of NAD^+ with either $lip(SH)_2NH_2$ or NADH as donor, the rate-limiting step according to the reaction mechanism of MASSEY and VEEGER (1961) is the reoxidation of the flavin semiquinone by $APNAD^+$. Explained in terms of the ordered bi bi mechanism it means a rate-limiting formation of APNADH. The velocity is almost independent of the $lip(SH)_2NH_2$ concentration (MASSEY and VEEGER, 1961; MASSEY et al., 1960), except at very high levels of $APNAD^+$ but dependent on the $APNAD^+$ concentration (Fig. 6A); at different $lip(SH)_2NH_2$ levels approximately the same 1/v vs $1/[APNAD^+]$ plot is found, but deviations from linearity occur at lower coenzyme concentrations (Fig. 6B).

Addition of $lipS_2(NH_2)$ as a product inhibitor results in a series of convergent 1/v vs $1/[lip(SH)_2NH_2]$ plots. The same pattern as observed with NAD^+ as acceptor returns in the 1/v vs $1/[lip(SH)_2NH_2]$ plots; at high $APNAD^+$ concentrations the plot with inhibitor intersects the control curve in the first quadrant, upon lowering the acceptor concentration the inhibition pattern changes via competitive towards noncompetitive. The rate of the inhibited reactions is dependent on the $lip(SH)_2NH_2$ concentration (Fig. 6B). For this reason the noncompetitive inhibitory effect of $lipS_2(NH_2)$ with respect to $APNAD^+$ is particularly clear at low donor concentrations. As the

donor concentration increases, the inhibition diminishes. It can be argued whether the inhibition becomes competitive with respect to $APNAD^+$ or remains noncompetitive at high donor concentrations. Both types of inhibition are in agreement with the proposed preferred order mechanism i.e. noncompetitive inhibition in case at high $APNAD^+$ concentration the ordered bi bi mechanism with $APNAD^+$ as first substrate is operating and competitive inhibition in case at high $lip(SH)_2NH_2$ concentration the donor is working as first substrate. In agreement is the tendency of the inhibition to be competitive with respect to acceptor at low concentrations of it, at all donor concentrations (Fig. 6B). However, a "ping pong bi bi" mechanism cannot be excluded under these conditions. Since $lipS_2(NH_2)$ inhibits competitively in a non-linear relationship (intersection in the first quadrant) with respect to $lip(SH)_2NH_2$ at high NAD^+ concentration in the physiological reaction, the ternary complex mechanism is also operating. At low NAD^+ concentration the inhibition is noncompetitive.

Similar calculations as for NAD^+ can be made with $APNAD^+$ as acceptor (Table I). The molar extinction coefficient (365 nm) of 9.1×10^3 $cm^2 mmol^{-1}$ in the reaction of $lip(SH)_2NH_2$ with $APNAD^+$ has been taken from SIEGEL et al. (1959). The calculation of the different parameters meets more difficulties than in the reaction with NAD^+. $Lip(SH)_2NH_2$ is acting as first substrate except at very high $APNAD^+$ concentrations. In Fig. 6A this can be seen from the slight dependence on the $lip(SH)_2NH_2$ concentration in the $1/v$ vs $1/lip(SH)_2NH_2$ plot at the two highest $APNAD^+$ concentrations. Moreover, the inhibition curve of $lipS_2(NH_2)$ intersects under those conditions with the control curve in the first quadrant. However, it is clearly understood that even under these conditions there is still a considerable simultaneous contribution of both pathways. The extrapolation for the situation where $APNAD^+$ is the first substrate is only based upon 2 or 3 figures; the steepness of the slope is probably underestimated. Therefore the value of k_{+1} in Table I has to be taken with restrictions. The term $(1+k_{-2}/k_3)/k_2$ cannot ce calculated so that k_{-1} and K_{diss} $(APNAD^+)$ are also unknown. The Hill plot of $APNAD^+$ at infinite $lip(SH)_2NH_2$ has a slope of 0.9-1.0 which is explained by the fact that $lip(SH)_2NH_2$ is acting as first substrate under these conditions (Fig. 5B).

From experiments with pyridinealdehydeNAD^+ the V_{max} value calculated per flavin moiety from the slightly bent L-B plot amounts 530 moles/min while the K_m is 1.7 mM (Fig. 7). $LipS_2(NH_2)$ inhibited noncompetitively with respect to $PANAD^+$ under the limited conditions used.

Fig. 6. Inhibition characteristics of $lipS_2(NH_2)$ on the reduction of NAD^+ by $lip(SH)_2NH_2$. Left hand side: L-B plots 1/v vs $1/lip(SH)_2NH_2$ with and without 0.33 mM $lipS_2(NH_2)$ as inhibitor at different $APNAD^+$ levels. The $APNAD^+$ concentrations, respectively without and with inhibitor are: 500 μM curves 1 and 2; 400 μM, curves 3 and 4; 300 μM, curves 5 and 6; 200 μM, curves 7 and 8; 150 μM, curves 9 and 10; 100 μM, curves 11 and 12; 75 μM, curves 13 and 14; 50 μM, curve 15. The reaction was started at 25° by addition of 10 μg of enzyme under the conditions as in Fig. 1. Right hand side: L-B plots of 1/v vs $1/APNAD^+$ with and without 0.33 mM $lipS_2(NH_2)$ at different $lip(SH)_2NH_2$ concentrations; (000), without inhibitor at differen $lip(SH)_2NH_2$ levels. The $lip(SH)_2NH_2$ concentrations used with inhibitor are 333 μM, (000); 267 μM, (ΔΔΔ); 200 μM, (+++); 167 μM, (000); 133 μM, (▼▼▼) and 100 μM, (■■■).

Fig. 6

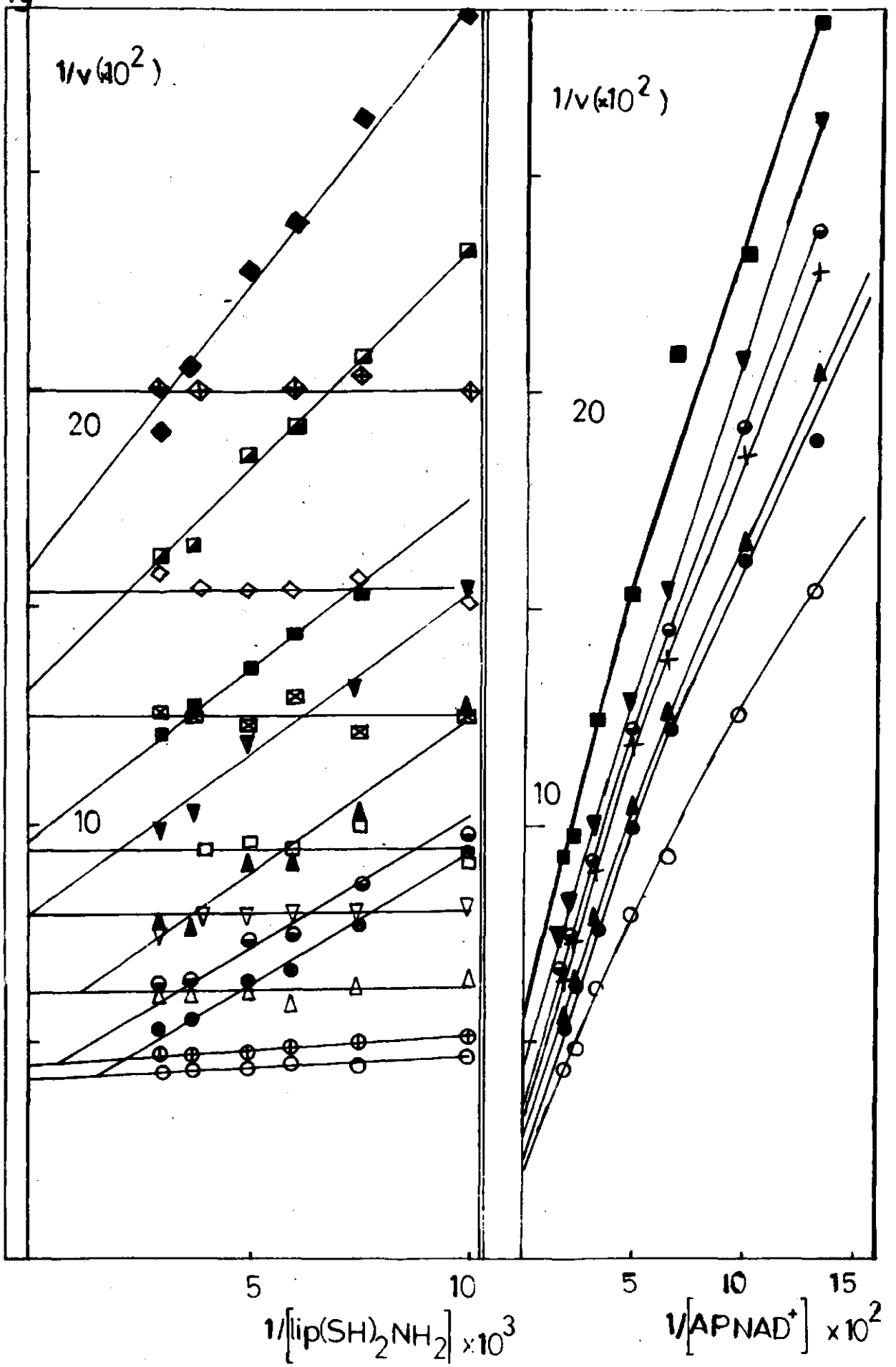
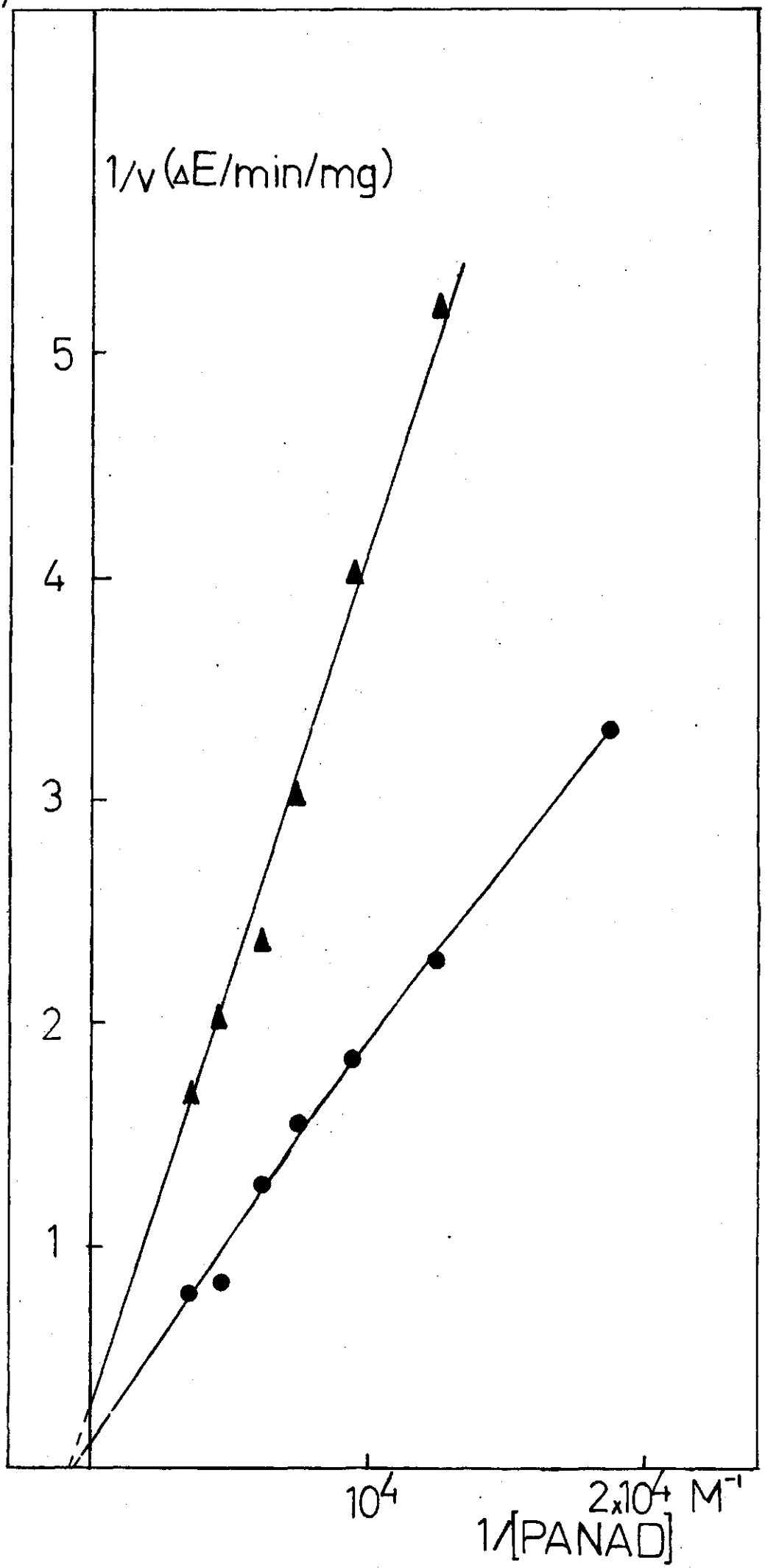


Fig. 7. L-B plots with pyridine aldehydeNAD$^+$ as variable substrate at a constant lip$(SH)_2NH_2$ concentration (167 μM). Conditions as in Fig. 1. (●●○), without product inhibition; ▲▲△, with lipS_2NH_2 present (333 μM).

Fig.7



6.2.2. Temperature-dependent kinetics

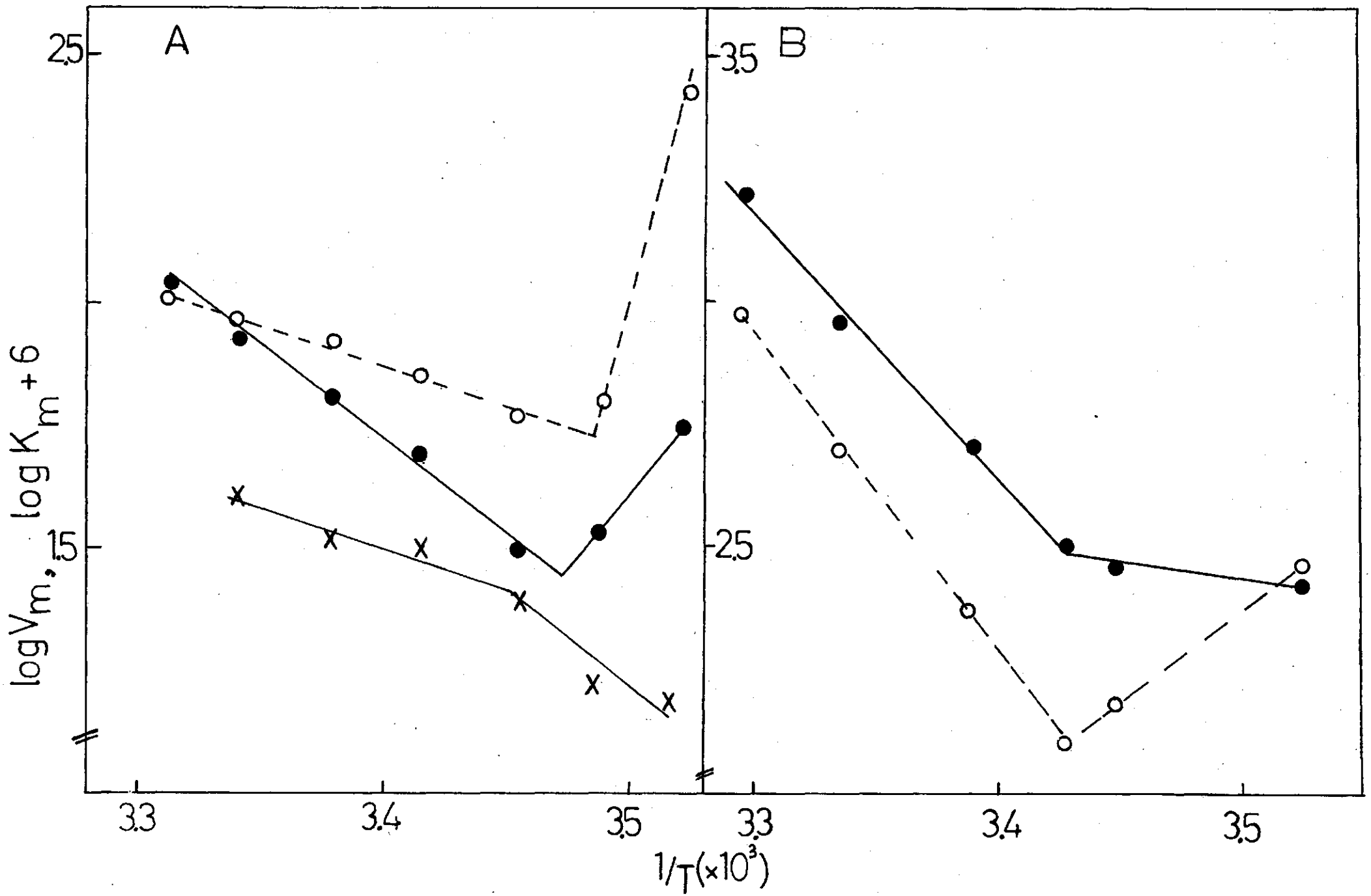
The influence of temperature on the reaction velocity is shown in Fig. 8. In these Arrhenius plots made in the presence and absence of $lipS_2(NH_2)$, the V_{max} values are determined at infinite $APNAD^+$ concentration, though at a finite $lip(SH)_2NH_2$ concentration. Though a specific kinetic effect cannot be excluded, these results are possibly better explained by assuming two enzyme conformations in temperature-dependent equilibrium of which the low temperature form has the highest activity. The inactivation observed upon increasing the temperature means that the lowering of activity due to the shift in equilibrium exceeds the influence of the temperature on the rate--limiting velocity constant. The conformational transition is complete at 14-15°. In the presence of $lipS_2(NH_2)$ a lower activity is observed below 14-15° indicating a shift in equilibrium towards the high-temperature form.

Spectral data, show that due to the presence of $lipS_2(NH_2)$ the NAD^+--complex at 5° resembles the complex at 25° though they differ in magnitude (VOETBERG in VISSER et al., 1969). Below 15° the enzyme is in the low temperature form in the absence of $lipS_2(NH_2)$, of which MASSEY and VEEGER (1961) have shown that the enzyme is reduced by $lip(SH)_2NH_2$ by two equivalents, in contrast with the four equivalents taken up by reduction with NADH. However, after reducing the enzyme with $lip(SH)_2NH_2$ under the conditions of the spectrophometric experiment (MASSEY and VEEGER, 1961) the medium contains $lipS_2(NH_2)$, which could maintain the enzyme in the high temperature form. Since it was shown (MASSEY and VEEGER, 1961) that removal of NAD^+ even at 25° leads to four equivalents taken up from NADH, it cannot be excluded that $lipS_2(NH_2)$ exerts a similar effect in preventing the enzyme against overreduction by $lip(SH)_2NH_2$. Thus the reduction of $APNAD^+$ below 15° could occur by a shuttling between the two and four equivalent reduced enzyme--forms with a higher activity than observed upon oxidation of the two equivalent reduced high temperature form. In agreement with this idea is the shape of the Arrhenius plot of the reduction of NAD^+ by $lip(SH)_2NH_2$ at infinite concentrations of both substrates; because NAD^+ keeps the enzyme in the high temperature form, this plot shows a similar break at 18° as observed with $APNAD^+$ in the presence of $lipS_2(NH_2)$. However,it must be kept in mind that the latter experiment was carried out with finite $lipS_2(NH_2)$. The inactivation energies calculated are 13,700 cal/mole for $APNAD^+$ and 3200 and 24,800 cal/mole for the two parts of the NAD^+ plot.

About the origin of the break at 18° one can only speculate, but in view of the results with $APNAD^+$, it is most likely that a conformational

Fig. 8. A. Arrhenius plot of the oxidation of $lip(SH)_2NH_2$ (167 μM) by APNA (extrapolated to infinite concentration): ○○○, without inhibitor; XXX, in the presence of 330 μM $lipS_2(NH_2)$; ●●●, variation of log K_m for $APNAD^+$ at saturating $lip(SH)_2NH_2$ concentration. B. Arrhenius plot of the oxidation of $lip(SH)_2NH_2$ by NAD^+. The reaction velocity at each temperature has been obtained by extrapolating to infinite concentrations (●●●); ○○○, variation of log K_m for NAD^+ at infinite concentration of $lip(SH)_2NH_2$.

Fig.8



transition area exists below 18°. This idea is supported by the observation that the difference spectrum of the abortive NAD·lipS$_2$(NH$_2$) complex at 5° has the same shape as that at 25°, but is less in magnitude, which indicates a slightly different structure around the catalytic site. Furthermore it was shown (MASSEY and VEEGER, 1961) that prolonged incubation at 0° even in the presence of 20 μM NAD$^+$ shifts the reduction state of the NADH-reduced enzyme reversibly towards the four equivalent-reduced state, in a process of slow and gradual changes.

Of interest are the concomitant temperature-dependent changes of the K_m-values of NAD$^+$ and APNAD$^+$ (Fig. 8): the K_m values have a minimum at 18° and increase below and above this temperature. This indicates that although a difference in the mechanism of reduction exists at low temperatures, the binding characteristics of the nucleotides are very much alike. The difference in mechanism at low temperature is probably due to the inability of APNAD$^+$ to bind, in contrast with NAD$^+$, to the regulatory site. The present evidence points to a role of a regulatory site, which upon binding NAD$^+$, keeps the enzyme in its high temperature conformation. As pointed out this is the complex with the highest affinity, which by changing the environment around the flavin, exerts its influence.

Table II summarizes the temperature-dependency of the kinetic parameters as calculated from the data in Fig. 8. The Arrhenius plots of the individual rate constants and of their combinations present additional information in the analysis of the temperature-dependent conformational changes next to the V_{max} values. It is suggestive in Fig. 9 that k_{+1} and k_{-1} are temperature-dependent in a different way; the overall effect is shown in the Arrhenius plot of the dissiciation constant of the enzyme-NAD$^+$ complex. The parameter $(1 + k_{-2}/k_3)/k_{+2}$ is also of importance. In this case a transition hardly occurs in the Arrhenius plot.

The protein conformational changes seem to be reflected mainly in the NAD$^+$-NADH binding site but less in the formation of the ternary complex. It is possible that the changes are occurring in the oxidised enzyme as well as in the reduced enzyme although there is no evidence as far as the reduced enzyme is concerned. NAD$^+$ is bound more tightly to the high-temperature form; the K_{diss} (NADH) behaviour is unknown as k_{-4} cannot be calculated from these data. However, the next paragraphs indicate that the low-temperature form has the highest affinity for NADH.

In the usual assay procedure 0.8 M citrate buffer pH 5.65 is used. It is known that under these conditions anaerobic addition of NADH to the

Fig. 9. Arrhenius plots of different sets of kinertic parameters as calculated from the experimental data of Fig. 8. ○○○, log k_{+1} -7; ●●●, log k_{-1} -4; ΔΔΔ, log K_{diss} (NAD^+) +5; ▲▲▲, log $(1+k_{-2}/k_3)/k_{+2}$ +8.

Fig.9

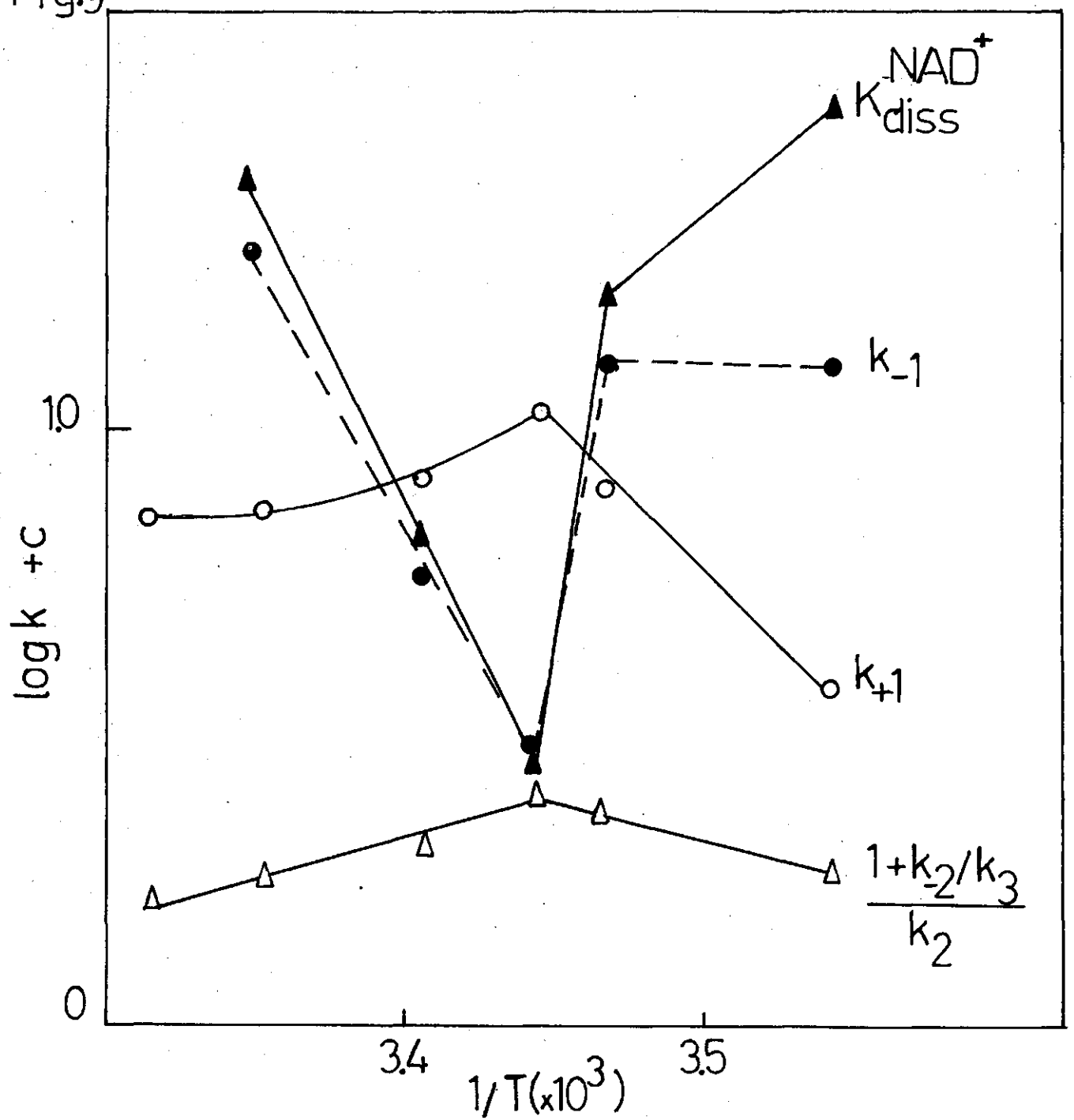
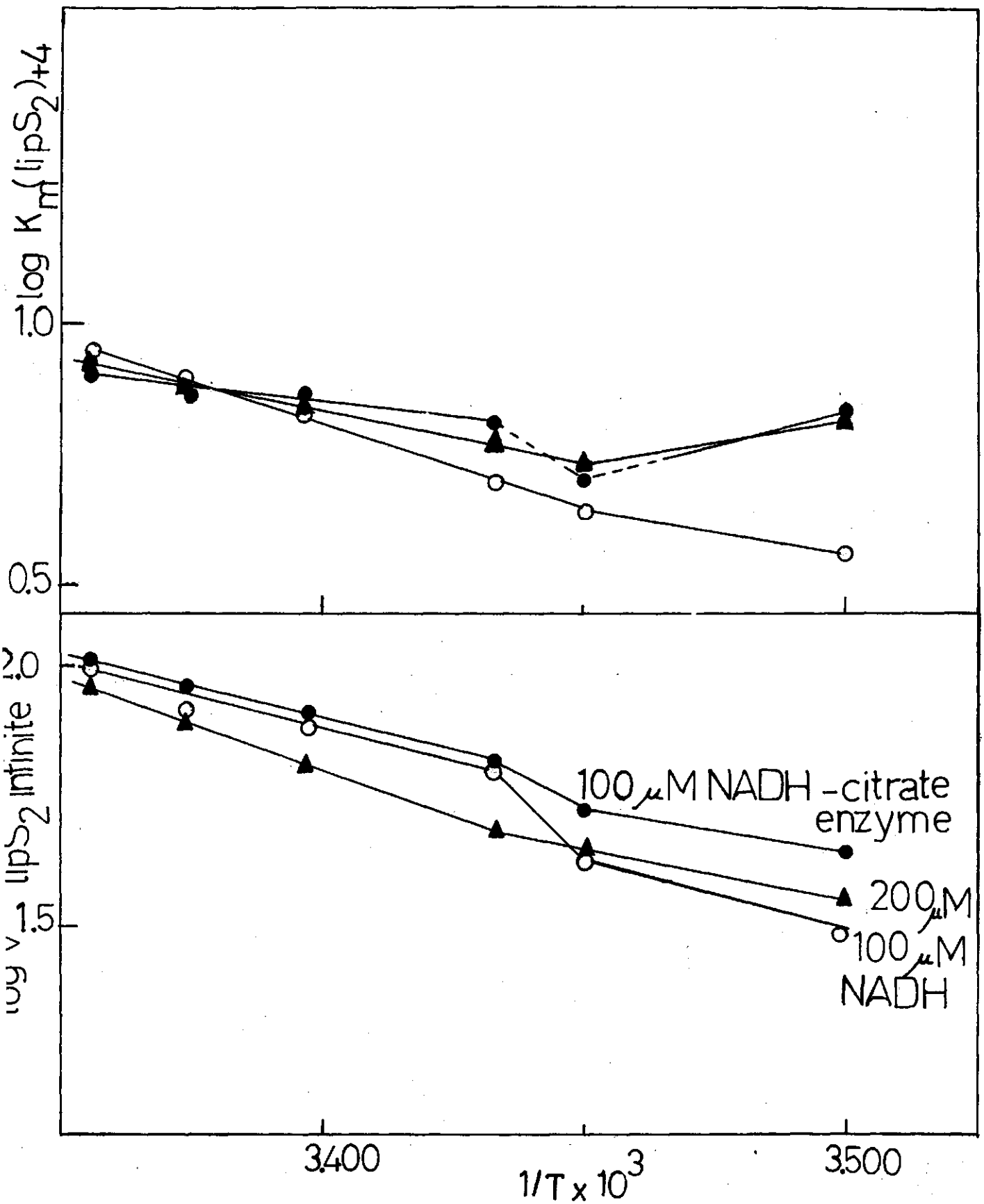


Fig. 10. A. Arrhenius plot of the K_m ($lipS_2$) values under different conditions. ●●●, enzyme (0.3 mg/ml) solution in 0.8 M sodium phosphate citrat (pH 5.65) containing 0.3 mM EDTA; K_m values are obtained by varying the lipoate concentration at a constant level of NADH (100 μM) in the normal assay procedure. With an enzyme solution in 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA the normal assay procedure was also follow at two different NADH concentrations, 100 μM (○○○) and 200 μM (▲▲▲). B. Arrhenius plots of the V_{max} ($lipS_2 \longrightarrow$ ○○) values of the same systems. ▲▲▲, phosphate enzyme, 200 μM NADH, ○○○, phosphate enzyme 100 μM NADH and ●●●, citrate enzyme, 100 μM NADH.

Fig.10


log K_m(lipS$_2$)+4
1.0
0.5
log v_{∞} lipS$_2$ infinite
2.0
1.5
100 μM NADH - citrate enzyme
200 μM
100 μM NADH
3.400
3.500
1/T x 10^3

enzyme results in a complete reduction of the flavin (VEEGER, unpublished results). The two-four equivalent reduction equilibrium is strongly influenced by the pH. At pH 5.65 there is no lipoate activity unless NAD^+ is present.

At a higher pH (e.g. pH 6.3), addition of NAD^+ is still stimulating but not necessary to give an initial reaction which is explained by the fact that the two-four electron reduction equilibrium is shifted to the semiquinone side.

In Fig. 10 the Arrhenius plots are shown of the reaction with NADH and lipoate as substrates at pH 5.65 in 0.8 M citrate buffer in the presence of 100 µM NAD^+. The V_{max} values are based upon extrapolation to infinite concentration at two different NADH levels. With 100 µM NADH the protein conformational transition is thought to occur within a relatively small temperature area. The plot with 200 µM NADH demonstrates the complexity of the NAD^+ : NADH ratio. In contrast to temperatures (> 5°) no or less substrate inhibition is observed. The substrate inhibition at higher temperatures is indicative for a higher affinity of NADH to the low--temperature form.

The log K_m ($lipS_2$) vs 1/T plot also shows a breakage point, indicating a change in affinity for this substrate. In this respect the changes in the K_m ($lipS_2$) values remind to the results with NAD^+ in the reverse reaction (cf. Fig. 8) and seems to be inherent in the oxidised substrate. The high-temperature form has a higher affinity for $lipS_2$. Citrate has a marked influence on the enzyme conformation since the activity remains high at low-temperature.

6.3. DISCUSSION

6.3.1. Kinetics

The kinetic patterns of lipoamide dehydrogenase are complicated for at least two reasons.

1. The pH optimum of the reaction $lip(SH)_2NH_2 + NAD^+ \longrightarrow lipS_2NH_2 + NADH^+ + H^+$ differs from that of the reverse reaction. This is due to the proton concentration which is of importance when the reaction is reversed.
2. In the reverse reaction, especially the one with NADH and $lipS_2$ assayed at pH 5.65, the presence of NAD^+ is obligatory. The pH is closely related to the two-four electron reduction state of the flavin which equilibrium can be shifted by NAD^+ to the semiquinone state. Kinetic patterns are there-

fore complicated because NAD^+ is not only activating but also acts as product inhibitor. Moreover, product inhibition studies with $lip(SH)_2$ or $lip(SH)_2NH_2$ are excluded.

The preferred order mechanism gives a quite satisfactionary explanation of the kinetic phenomena observed at 25°. The Hill coefficients for both substrates tend to a value of 1 at infinite concentrations which is in agreement with such a mechanism. As discussed before it is hard to distinguish on the basis of the Hill coefficients of NAD^+ at finite $lip(SH)_2NH_2$ whether n-values < 1 are based on the preferred order mechanism or on a negative cooperativity between the two NAD^+ binding sites. A similar problem arises with the n-values between 1.4 and 1.7 found for the inhibition of $lipS_2(NH_2)$. Two lipoyl-binding sites cannot be excluded though the combination of a normal product inhibition and an abortive ternary complex inhibition can explain these data as well. As for the kinetic parameters, the V_{max}-values calculated per mole of flavin of 20,500 moles/min. is less than that of the value given by MASSEY et al. (1960). This discrepancy has not to be a real one since the extrapolations to infinite concentrations in the L-B plots are different as a consequence of the different models used which might extensively influence the slopes as well as the intercept on the Y-axis.

The K_m (NAD^+) and K_m ($lip(SH)_2NH_2$) of 2.5×10^{-4} M and 3.6×10^{-4} M respectively are in good agreement with the values in the literature of 2.0×10^{-4} M and 3.0×10^{-4} M.

NAD^+ acts by binding to a regulatory site as demonstrated with difference spectroscopy (VOETBERG, in VISSER et al., 1969) with an affinity which is higher than the affinity for the catalytic site ($K_{diss} \approx$ 35 µM while the overall $K_{diss} \approx$ 110 µM). NAD^+-analogues cannot replace NAD^+ as activator (VEEGER, 1960). In the transhydrogenase reaction of lipoamide dehydrogenase with NADH and $APNAD^+$ VEEGER (1960) mentions a strong product inhibition of NAD^+ which is competitive with respect to NADH and which has a K_i value of 18 µM. These results suggest that NAD^+ acts on the regulatory site. The term regulatory site is not fully justified as it is likely from the results with the transhydrogenase reaction that this site is catalytically active as well. Nevertheless the term "regulatory" site is maintained as it indicates that binding of NAD^+ to this site is preventing the 4-electron reduction of the flavin.

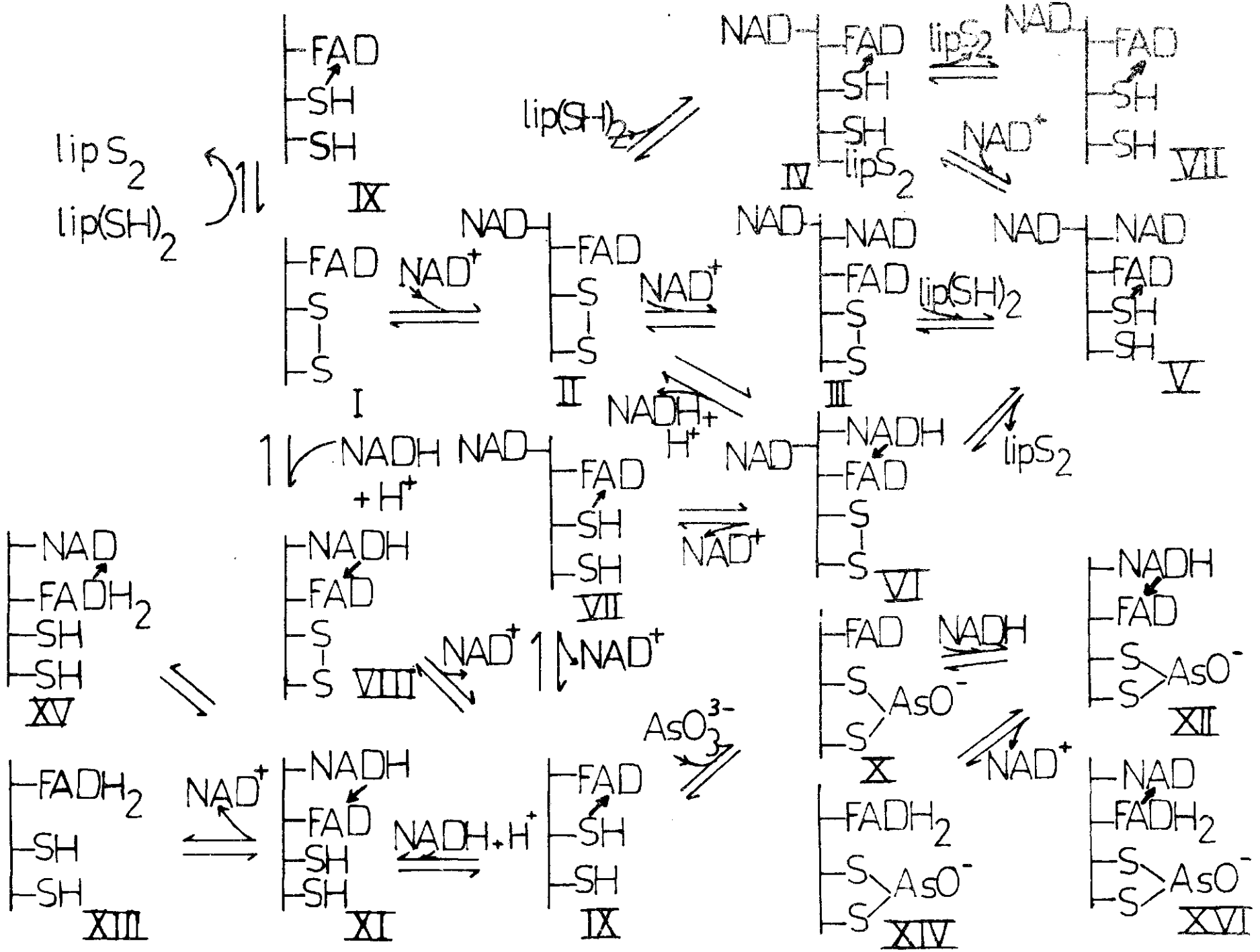
Moreover, VOETBERG (1969, unpublished results) could not demonstrate more than one spectral complex between $APNAD^+$ and lipoamide dehydrogenase per

mole of flavin which is suggestive that $APNAD^+$ binding to regulatory site is not of importance in the transhydrogenase reaction or in the lipoamide dehydrogenase reaction with $lip(SH)_2NH_2$ and $APNAD^+$. A competitive effect of NAD^+ is explicable when the active centre is also modified on NAD^+ binding to the regulatory site and made unaccessible for $APNAD^+$. Another interesting hypothesis could be that the regulatory site becomes the NADH binding site which is supported by the fact that NAD^+ inhibits competitively with a K_i-value of 18 μM corresponding with the K_{diss} (NAD^+) of the regulatory site. Flavin reduction to the four electron reduced state could occur using the catalytic site as well as the regulatory site to transfer reducting equivalents. From the 720 nm green CT band between four electron reduced enzyme and NAD^+ it can be concluded that NAD^+ still has an affinity for one of the two binding sites upon reduction (MASSEY and PALMER, 1962).

A competition between NAD^+ and NADH for the regulatory site occurs with the similar binding site between $NADP^+$ and NADPH in Azotobacter transhydrogenase (VAN DEN BROEK and VEEGER, 1969). This theory, in which the NAD^+ binding sites have an outspoken different affinity while the affinities for NADH, though this is speculative, are presumably not that very different from each other (cf. K_{diss} NADH in the $lip(SH)_2NH_2 \longrightarrow NAD^+$ reaction $\approx$ 13-15 μM), gives rise to an oxido-reduction system which can be regulated by the NADH/NAD^+ ratio. This is very attractive from the point of vieuw of the activity of the pyruvate and α-oxoglutarate dehydrogenase complexes in which lipoamide dehydrogenase exerts its function. Moreover, it is worthwhile to study both enzymes individually as differences in reduction properties have been mentioned (SAKURAI et al., 1969).

$LipS_2(NH_2)$ forms a ternary complex with NAD^+ bound to the catalytic site since no spectral shifts are observed in the absence or at low concentration of the coenzyme (VOETBERG, in VISSER et al., 1969). This is actually an abortive complex. With $lip(SH)_2NH_2$ a linear noncompetitive L-B plot is to be expected (FROMM and NELSON, 1962), in case NAD^+ is the first substrate. When $lip(SH)_2NH_2$ is the first substrate such an abortive complex cannot be formed and noncompetitive inhibition by $lipS_2(NH_2)$ is expected and observed; NADH will inhibit competitively. The peculiar type of inhibition kinetics at high NAD^+ (Figs 3 and 6) is probably due to noncompetitive inhibition of the abortive complex with respect to $lip(SH)_2NH_2$ and in addition a change in mechanism towards this donor acting as the first substrate.

Our present knowledge is summarised in scheme I.


lipS2
lip(SH)2
FAD
SH
SH
IX
lip(SH)2
NAD
FAD
SH
SH
lipS2
IV
lipS2
NAD
FAD
SH
SH
VII
NAD+
FAD
S
S
I
NAD+
NAD
FAD
S
S
II
NAD+
NAD
NAD
FAD
S
S
III
lip(SH)2
NAD
NAD
FAD
SH
SH
V
NADH
+H+
NADH+
H+
NAD
FAD
SH
SH
VII
NAD
NADH
FAD
S
S
VI
lipS2
NAD+
NAD
FADH2
SH
SH
XV
NADH
FAD
S
S
VIII
NAD+
NAD+
FAD
S
AsO-
S
X
NADH
NADH
FAD
S
AsO-
S
XII
FADH2
SH
SH
XIII
NAD+
NADH
FAD
SH
SH
XI
NADH+H+
AsO3 3-
FAD
SH
SH
IX
NAD+
FADH2
S
AsO-
S
XIV
NAD
FADH2
S
AsO-
S
XVI

I Oxidised enzyme present at low NAD^+ concentration (< 5 μM).

II Oxidised enzyme present at NAD^+ concentrations > 10 μM difference spectrum without 450 nm maximum NAD^+ bound at regulatory site.

III NAD-bound to catalytic site with lower affinity than to regulatory site; spectral characteristics as observed in difference spectrum at saturating NAD^+ concentrations.

IV Oxidised enzyme directly reacting with $lip(SH)_2$, at low concentrations. One SH group in either charge-transfer interaction with FAD (cf. SEARLS et al., 1961) or covalently linked to the C(4)-α atom; characterised by its 530 nm band. This complex which is EPR inactive and diamagnetic was postulated to be an interaction between $FADH^{\cdot}$- semiquinone and a $S^{\cdot}$- radical i.e. the so called semiquinone form (MASSEY and VEEGER, 1961).

V Ternary complex between enzyme, acceptor and donor. Spectral characteristics unknown.

VI Complex between NADH and FAD, which could be formed either by charge-transfer interaction or by covalent linkage between C(4) of NADH and N(5) of the isoalloxazine ring (HEMMERICH et al., 1965). In the latter case the flavin has a $FADH_2$--like structure, spectral band at 600 nm. The spectral characteristics of intermediate like IV are gradually shifted towards a 600 nm band by the addition of NAD^+. The absorbance at 440 nm shifts towards 450 nm without change in magnitude.

VII Spectral characteristics as IV. In the case of intermediate VIIa this could lead to NADH formation according to a "ping pong bi bi" mechanism.

III Produced at very low NAD^+ concentrations, spectral characteristics of VI.

IX Spectral characteristics of IV, can also be produced by direct reduction with $lip(SH)_2$ (cf. MASSEY and VEEGER, 1961). At low NAD^+ or at low temperature is this the complex which starts the sequence to four equivalent reduction. With NAD^+ bound the regulatory site (VII), high concentrations of NADH do not give over-reduction.

X Arsenite enzyme; its formation is counteracted by NAD^+ (VEEGER, unpublished results).

XI This reaction was demonstrated with the Cu^{2+}-modified enzyme (VEEGER and MASSEY, 1962), which has no stable form VII, lacks lipoate activity and shows increased activity with 2,6-dichlorophenol indophenol. The Cu^{2+}-modified enzyme needs NAD^+ for full activity (VISSER and VEEGER, 1968[b]).

Spectral characteristics of VI.

XII This intermediate was shown to be present upon reacting the arsenite enzyme with excess NADH (MASSEY and VEEGER, 1961).

III, XIV Fully reduced enzyme.

XV, XVI Slow side reactions, leading to charge-transfer complex between NAD^+ and $FADH_2$, with band at 720 nm. In the scheme the binding of NAD^+ is assumed to occur at the catalytic site, but binding at the regulatory site is as well possible.

Kinetic pathway II ⟶ III ⟶ V ⟶ VI ⟶ II; at low NAD^+ concentrations II ⟶ IV ⟶ V ⟶ II.

6.3.2. Temperature-dependent kinetics

MASSEY et al. (1966) mentioned a number of enzymes which show a discontinuous Arrhenius plot, among them several flavoproteins. Protein conformational changes are generally thought to be responsible for this phenomenon. A careful kinetic analysis of these temperature-dependent conformational changes in D- and L- amino acid oxidase has been given by KOSTER (1969). In those two enzymes there is evidence for the existence of two conformations with different activities but the same activation energy which enables one to calculate the allosteric equilibrium constant. In the case of D-amino acid oxidase there is additional evidence for such changes in conformation from fluorescence and sedimentation velocity data (MASSEY et al., 1966) and from the temperature-dependency of the dimerisation constant of the apoenzyme (HENN and ACKERS, 1969). Studies with succinate dehydrogenase (ZEYLEMAKER et al., 1969 and ZEYLEMAKER, 1969) and with glutathione reductase (STAAL and VEEGER, 1969) emphasize the importance of donor and acceptor concentration and activating ions as Na^+ on the equilibrium of the conformations.

A breakage point of 22° as mentioned by MASSEY et al. (1966) for lipoamide dehydrogenase does not correspond with our results. At infinite $APNAD^+$, NAD^+ and $lip(SH)_2NH_2$ concentrations the breakage occurs at 15° and 18°, respectively.

At infinite lipoate concentration at different NADH levels, the transition occurs between 16.5° and 18°. Especially in this reaction performed at a low pH, the NADH substrate inhibition is considerable; moreover NAD^+ is obligatory to prevent a lag period in the activity determination. As a consequence the NAD^+/NADH ratio is of importance as not only the two-four electron reduced equilibrium is influenced but as a secondary effect the high-low temperature equilibrium of the enzyme conformations is shifted.

The importance of this nucleotide ratio is clear from Table III where a survey is given of the activation energies and the transition temperatures measured under different NAD^+/NADH conditions at a finite lipoate concentration.

Additional evidence for the conformational changes occurring in the oxidised protein comes from the fluorescence measurements of VOETBERG (VEEGER et al., 1969) where a breakage in the efficiency of the energy transfer from the protein to the chromophore occurs, i.e. changes in the interaction of the aromatic amino acids and the flavin is observed.

Table I

The relation between kinetic parameters calculated with NAD^+, $APNAD^+$ and $lip(SH)_2NH_2$ as substrates at 25°. The figures are based upon flavin content of the enzyme and expressed per catalytic centre.

These studies		NAD+ Literature data	
V_{max}	= 20,500	V_{max}	= 33,000 (MASSEY, 1963)
NAD^+ / $lip(SH)_2NH_2$	infinite concentration		
$K_m(NAD^+)$	= 2.5×10^{-4} M	$K_m(NAD^+)$	= 2.0×10^{-4} M
$K_m(lip(SH)_2NH_2)$	= 3.6×10^{-4} M	$K_m(lip(SH)_2NH_2)$	= 3.0×10^{-4} M
k_{+1}	= 8×10^{7} $M^{-1}sec^{-1}$		
k_{-1}	= 1.3×10^{4} sec^{-1}		
$\frac{1 + k_{-2}/k_3}{k_2}$	= 1.75×10^{-8} M sec		
$K_{diss}(NAD^+)$	= 1.1×10^{-4} M		
		$APNAD^+$	
V_{max}	= 1090	V_{max}	= 2000 (MASSEY, 1963)
$APNAD^+$ / $lip(SH)_2NH_2$	infinite concentration		
$K_m(APNAD^+)$	= 7×10^{-5} M	$K_m(APNAD^+)$	= 8.6×10^{-5} M
k_{+1}	= 1.5×10^{7} $M^{-1}sec^{-1}$		

Table II

The relation between kinetic parameters calculated with NAD^+ and $lip(SH)_2NH_2$ as substrates at different temperatures. The rate constants have been calculated on the basis of the mechanism described in the text and based on the data used in Fig. 8.

Temp. °o	V_{max} (sec^{-1})	k_{+1} ($secM^{-1}$) ($x10^7$)	$\frac{1+k_{-2}/k_3}{k_2}$ (M sec)$_{x10^{-8}}$	k_{-1} (sec^{-1}) $x10^4$	$K_D^{(NAD^+)}$ (M) ($x10^{-5}$)
5	10,700	3.7	1.8	1.3	35.0
13.5	11,700	8.0	2.2	1.4	17.0
17	13,400	11.0	2,5	0.3	2.7
22	20,600	8.3	2.0	0.6	6.8
31	37,200	7.4	1.8	2.0	27.0
33	67,900	7.1	1.6	*	*

* due to the inaccuracy of the extrapolation, these values cannot be calculated.

Table III

Influence of different NAD^+/NADH ratios on the activation energy of the reaction NADH $\longrightarrow$ $lipS_2$ and the transition temperature calculated from the Arrhenius plot. Lipoate (670 µM) and NADH as indicated were used as substrate while NAD^+ was added as activator in concentrations indicated. NADH was not completely free from NAD^+ 0,05 µmole of enzyme was added to each cuvette and the velocities reached after 1 minute have been measured. Activation energies are expressed in cal/mole.

NAD^+(µM)	NADH (µM)				
	330	100		33	
	Δ E	Δ E	T break	Δ E	T break
0	-	high ± 3000 low 16,450	25°	high 8250 low 23,000	18°
33	9500	high 5600 low 13,000	22°	high 8050 low 11,100	19°
100	5500	-		-	
330	7100	9700		high 7700 low 12,000	18°

7. IMMUNOCHEMICAL RELATIONSHIP BETWEEN DIFFERENT ENZYME CONFORMATIONS*

7.1. INTRODUCTION

Antisera prepared against enzymes are well known to have an effect on the catalytic activity; most enzymes are partially or completely inhibited by their antibodies (CINADER, 1963). In the past much attention has been paid to influences of structural changes on antibody specifity. Most studies however are concerned with chemically modified proteins e.g. iodo-, nitro- or diazo-compounds (MAURER, 1963). Association-dissociation phenomena and localised conformational changes (except the influence of substrate or inhibitors) did have less attention.

Antisera against lipoamide dehydrogenase from pig heart and Saccharomyces oviformis have been prepared (STEIN et al., 1965b; HAYAKAWA et al., 1967 and MISAKA, 1966). Lipoamide dehydrogenase enzyme preparations obtained from different sources, e.g. Escherichia coli, pig heart, human liver, are reacting differently against these antisera, indicating that there are differences in antigenic properties (HAYAKAWA et al., 1967). However, multiple enzyme forms isolated from one source -pig heart - were immunochemically equivalent as studied by agar gel diffusion (STEIN et al., 1965). In the present study antisera have been prepared against four distinct conformations of the pig heart enzyme, viz. the holoenzyme, the Cu^{2+}-modified enzyme, the apoenzyme and the reconstituted DCIP-active enzyme.

7.2. RESULTS

7.2.1. Formation of the antibodies

In Table I a survey of the serum titers obtained at different times after the first injection is given. Administration was continued up to 9 weeks after the first blood samples were taken. The antiserum concentration formed against the dimer conformations e.g. the holoenzyme and Cu^{2+}-treated enzyme increases slowly and reaches a constant level. The amount of antisera formed against the DCIP-active enzyme and apoenzyme is difficult to determine as these forms of the enzyme are labile, easily resulting in aspecific flocculation. This can be concluded from the

* Footnote: This part of the study has been performed in cooperation with Mr. Jan Geelen.

observation that all the precipitation reactions of these enzyme forms with the normal sera were negative. On the other hand, the reactions with the two monomer conformations were positive but this might be due to denaturation of the enzyme protein.

The Ouchterlony double-diffusion test was performed in the cold to prevent the apoenzyme denaturing and to delay the DCIP-active enzyme dimerising (KALSE and VEEGER, 1968). In Fig. 1 the different precipitation lines obtained after one day have been drawn schematically. In Table II the time-dependent changes in precipitation patterns are summarised. The antisera against Cu^{2+}-modified enzyme and apoenzyme form with their homologous antigens only one precipitation line. Both the DCIP-active recombined enzyme and the apoenzyme give three lines against this Cu^{2+}-modified enzyme antiserum. As the residual activities with $lipS_2$ and DCIP of the apoenzyme are very low, the presence of either dimer and/or recombined monomer cannot be the reason for the multiple patterns.

The apoenzyme itself contains an antigenic site returning in all the other conformations as they react positively with the apoenzyme antiserum forming only one precipitation line. However, it remains questionable whether this is an antiserum against the native apoenzyme as the apoenzyme is very labile at the blood temperature of the rabbit.

The antiserum against the DCIP-active enzyme cannot be expected to be homogeneous because the enzyme will contain under these conditions monomer, lipoate active dimer and apoenzyme. Even with its homologous antigen it reacts with two precipitation bands. On the other hand it shows with Cu^{2+}-treated enzyme one band. Remarkable is that the DCIP--active enzyme and the apoenzyme react very similar with all four antisera underlining that they are closely related.

The antiserum against the normal holoenzyme probably contains two components as even the reaction with the Cu^{2+}-modified enzyme eventually results in two precipitation lines. On the other hand, one has to be careful with the interpretation of bands which develop after such a long time.

7.2.2. The influence of antibodies on the catalytic activities

A considerable influence of the antibodies on the enzymatic activities of lipoamide dehydrogenase has been observed (HAYAKAWA et al., 1967; MISAKA, 1966). Therefore, the influence of the four antisera on the

Fig. 1. Agar gel-diffusion patterns obtained after one day with different antisera-antigen combinations. The antisera were dissolved in 0.9 % sodium chloride in protein concentrations of approx. 50 mg/ml and used undiluted. The enzyme concentrations were kept constant (1 mg/ml). To prevent dimerisation of the DCIP-active enzyme, the temperature was maintained at 4°. Th antisera are indicated by (a), DCIP-active enzyme antiserum; (b), holoenzyme antiserum; (c) apoenzyme antiserum; (d) Cu^{2+}-modified enzyme. The enzyme conformations are indicated by figures: (1) holoenzyme; (2) apoenzyme (3) Cu^{2+}-modified enzyme; (4) DCIP-active enzyme.

Fig.1

1 holoenzyme

2 apoenzyme

3 Cu^{2+}-enzyme

4 DCIP-enzyme

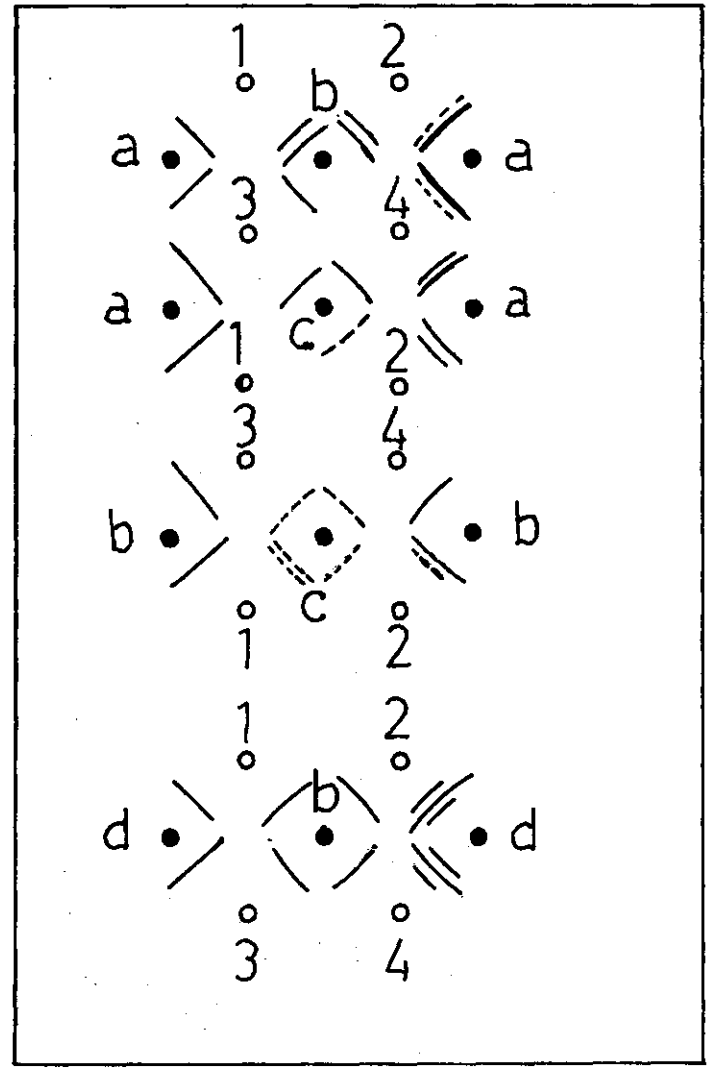
Antisera

a DCIP-enzyme

b holoenzyme

c apoenzyme

d Cu^{2+}-enzyme



activities of the different conformations has been examined to establish the degree of their relationship. In Fig. 2 the inhibition of the catalytic activities has been given, caused by increasing amounts of antiserum. As the antibody concentrations are unknown and probably different in the four antisera, the enzyme concentrations were kept constant, viz. 0.5 mg/ml.

The antisera against the dimer conformations of lipoamide dehydrogenase have their strongest inhibitory action on the Cu^{2+}-treated enzyme. Although the antiserum against the Cu^{2+}-treated enzyme inhibits the catalytic properties of its homologous antigen more specifically, it is never complete. The DCIP-activity and the $lipS_2$-activity of the holoenzyme are inhibited by this antiserum to the same extent; the degree of inhibition is less than obtained in the case of a homologous holoenzyme/antibody combination.

The DCIP-active monomer is inhibited more by its homologous antiserum than the other enzyme conformations. Interesting is the influence of this antiserum on the DCIP-activity of the holoenzyme. The inhibition is even a larger one than with the Cu^{2+}-treated enzyme, while there is only a small inhibition of the $lipS_2$-activity.

The apoenzyme antiserum interferes slightly with the $lipS_2$-activity of the holoenzyme and the DCIP-activity of the Cu^{2+}-modified enzyme. The DCIP-activity of the holoenzyme and that of the DCIP-active monomer are unaffected or slightly activated. This antiserum, incubated for 25 minutes on ice with the apoenzyme, does not significantly interfere with the recombination process with FAD. Similarly with the other antisera suggesting that the FAD binding site itself is not the antigenic determinant in the apoprotein structure. Addition of FAD immediately results in an equally active complex if compared with a control experiment in which bovine serum albumin was used instead of the antiserum. However, this is only one possibility. Another might be that the antibody-antigen complex in the case of the apoenzyme is either very slowly formed on ice or not formed at all. In the preceding it was already stated that the apoenzyme antiserum is made against a thermolabile antigen. The apoenzyme kept on ice is structurally different from the 37° C apoenzyme, especially in dilution (cf. Chapter V).

Fig. 2. Relative inactivation of the catalytic activities of different enzyme conformations by addition of increasing antibody concentrations. 0.5 ml solutions of holoenzyme or Cu^{2+}-modified enzyme in 0.1 M sodium phosphate buffer (pH 8) containing 0.5 mg were incubated during 20 min at 25° with the amounts of antisera indicated. The apoenzyme and the DCIP-act monomer were incubated for the same time on ice in the same amounts. Activities are expressed in % activity: OOO, DCIP-activity Cu^{2+}-modified enzyme; @@@, DCIP-activity holoenzyme; ΔΔΔ, $lipS_2$-activity holoenzyme; XXX DCIP-activity DCIP-active monomer. 2A, antiserum holoenzyme; 2B, antiserum Cu^{2+}-enzyme; 2C, antiserum DCIP-monomer and 2D, antiserum apoenzyme.

Fig. 2

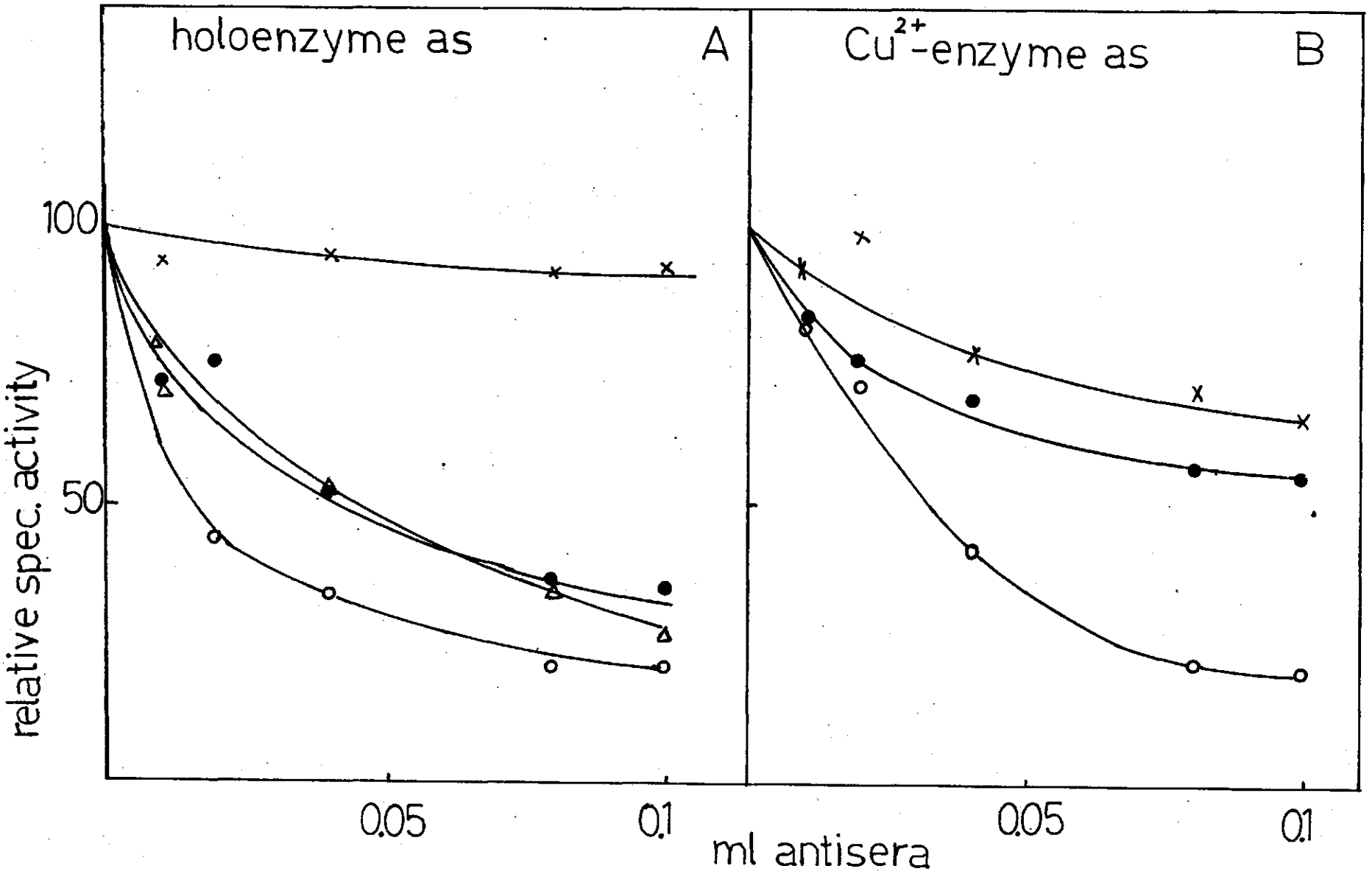

holoenzyme as
A
Cu2+-enzyme as
B
100
50
relative spec. activity
0.05
0.1
0.05
0.1
ml antisera

Fig.2

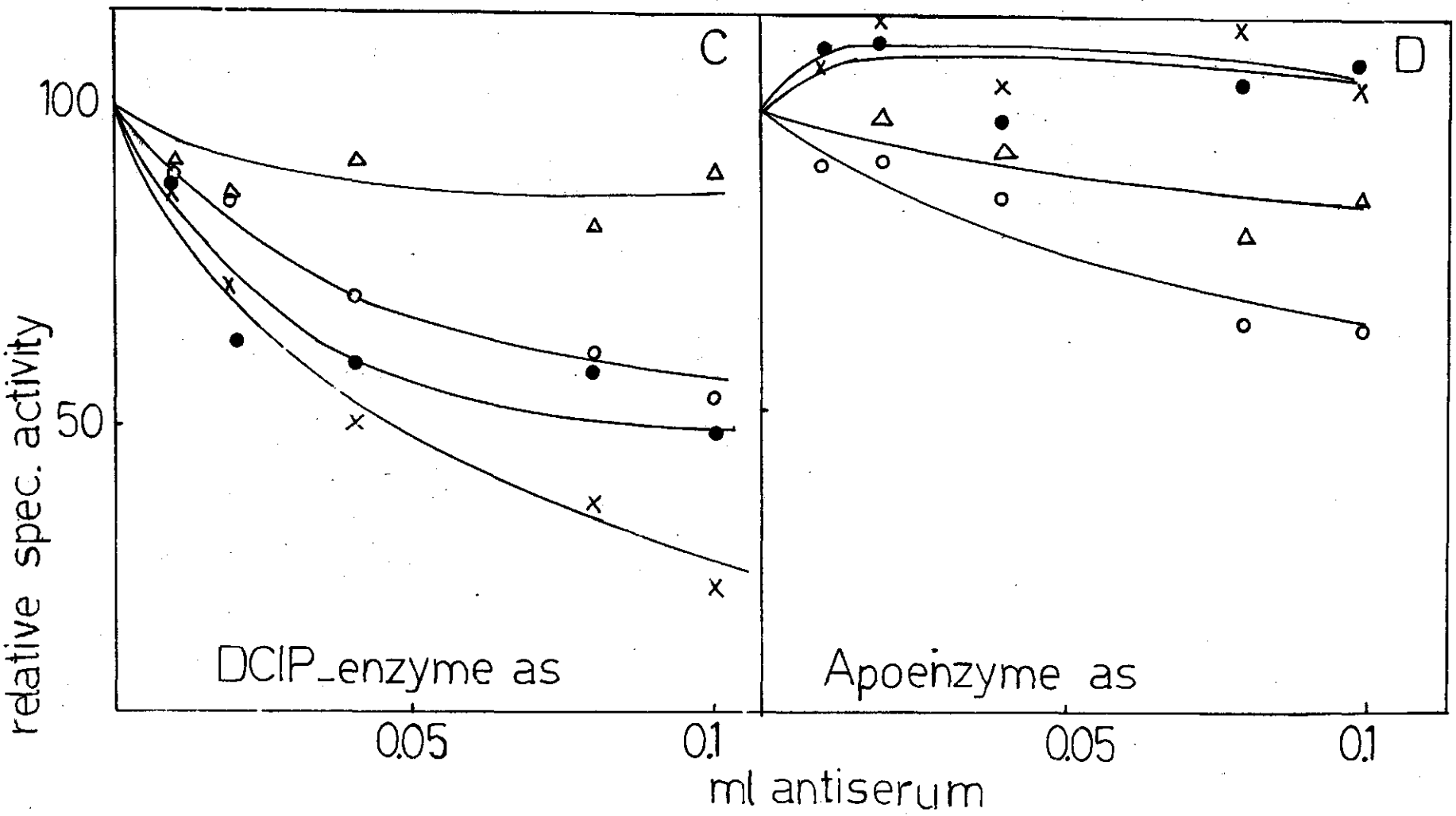
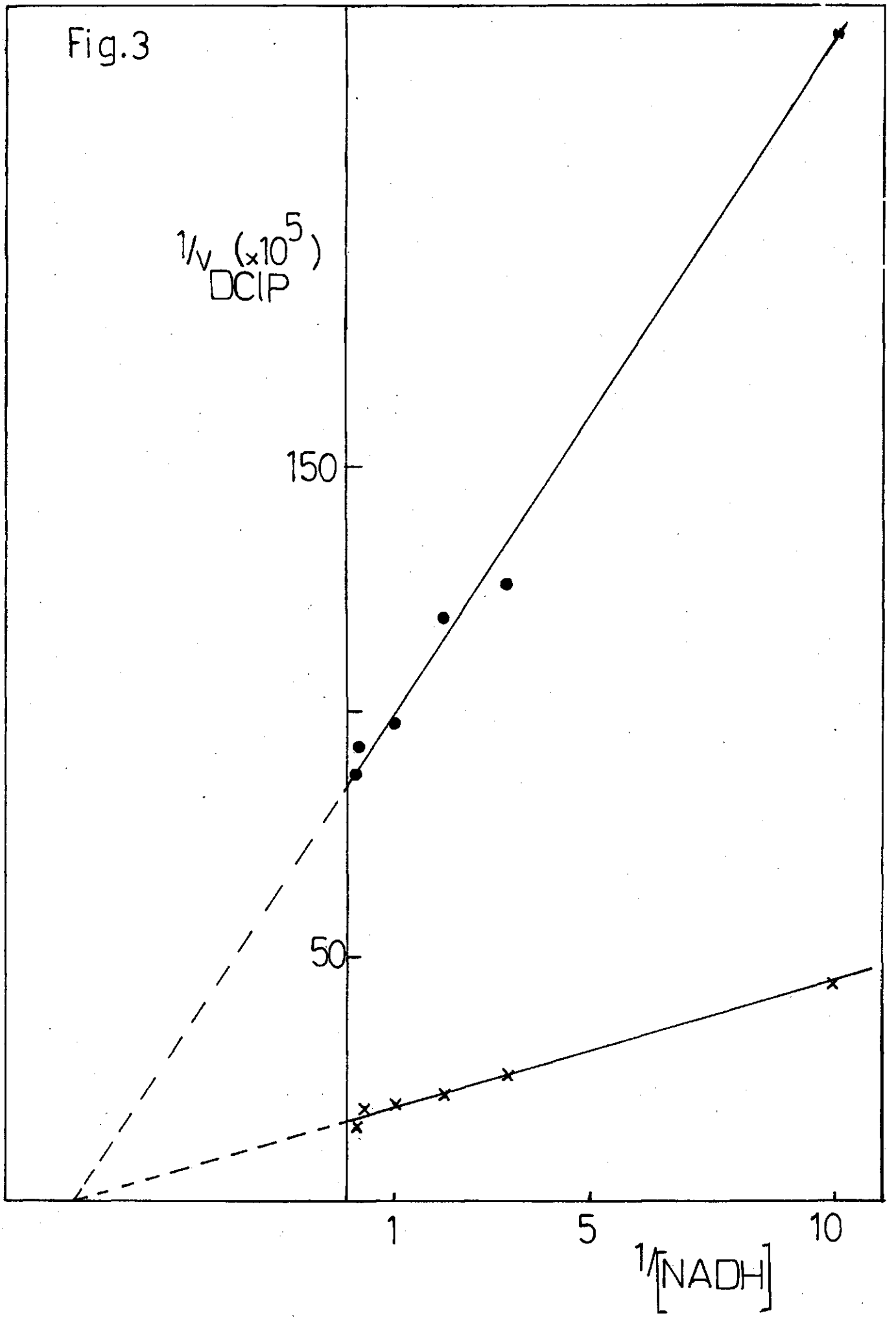

C
D
100
50
relative spec. activity
DCIP_enzyme as
Apoenzyme as
0.05
0.1
0.05
0.1
ml antiserum

Fig. 3. Lineweaver-Burk plots for the uninhibited and the homologeous inhibited Cu^{2+}-modified enzyme. 0.6 µM Cu^{2+}-modified enzyme was incubated in the cuvette with 0.05 ml antiserum during 2 min at 25°. The DCIP-reacti was started by adding NADH (100 µM).

Fig.3



7.2.3. Nucleotide protection against inactivation by antisera

STEIN et al. (1965b) and MISAKA (1966) refer to a slight protection of NADH against the inactivation of the enzyme activity by antibodies. Table III summarises the protective action of NADH on the inactivation of the holoenzyme by different antisera. A similar influence was found on the Cu^{2+}-modified enzyme. The DCIP-active monomer antiserum loses almost all its inhibitory influence on the latter form. The activity of the DCIP-monomer is strongly influenced by NADH as this compound interferes with the FAD binding site (cf. Chapter V). Therefore it is impossible to distinguish between this interference and an additional protective action against the antisera.

Lineweaver-Burke plots to determine the kind of inhibition caused by the antisera reveal in the case of the Cu^{2+}-modified enzyme that the inactivation is due to a lowering of the enzyme concentration by the binding with the homologous antiserum as can be derived from the nearly identical K_m-value of 17 μM (Fig. 3). In other words, part of the enzyme is taken away and is inactivated.

7.3. DISCUSSION

The double-diffusion test gives insufficient information about the heterogeneity of the antisera obtained, especially since no cross-reactions after antiserum saturation with heterologous antigens have been performed. The DCIP-active enzyme and the apoenzyme form identical precipitation patterns with all the antisera; these patterns are multiple, the apoenzyme antiserum excepted in which case a single precipitation line occurs. These data indicate that the antigenic properties of the monomer conformations are related to each other more than to the dimer forms.

As to the reason for these multiple lines, there is a lack of evidence. We can only mention several hypothetical causes. The monomer conformations react with the antiserum of the Cu^{2+}-modified enzyme with three precipitation lines, both dimers however only with one. Differences in number and localisation of antigenic determinants in monomer and dimer or complexes with different ratios of antigen-antibody could be reasons. Another possibility is that we are dealing with a Liesegang phenomenon, produced by so-called unbalanced immuno-systems. In these systems the initial precipitation line interferes further with the

diffusion of both components giving rise to more precipitation lines as explained by VAN OSS and HIRSCH-AYALON (1959).

All the four tested enzyme conformations react with the apoenzyme antiserum forming one precipitation line; presumably the antigenic site in the apoenzyme returns in all other conformations.

The DCIP-active monomer consists of a mixture of conformations, i.e. a small amount of dimer as well as apoenzyme. Heterogeneity of this antiserum is therefore not astonishing.

As for the holoenzyme antiserum, generally one line has been found (STEIN et al., 1965; HAYAKAWA et al., 1967; MISAKA, 1966) though STEIN et al. (1965) reported multiple patterns on cellulose acetate. MISAKA (1966) found a combination of two antibody molecules with one enzyme molecule in the equivalence zone. A reason for a double-line precipitation pattern with our antiserum even in the homologous case may be caused by different ratios of antibody-antigen. Another point we want to mention is the rather prolonged immunisation program. Heterogeneity of antibodies is known to be stimulated by continued immunisation (CINADER, 1963).

The maximal inhibition of the catalytic activities varies between 70 % and 80 %, in good agreement with values reported in the literature (STEIN et al.,1965; HAYAKAWA et al., 1967; MISAKA, 1966). CINADER (1963) explains this incomplete inhibition in terms of heterogeneity of the antibodies; ARNON and SHAPIRA (1967a,b) could prove recently for the papain-antipapain system that this explanation holds. If less than 100 % inhibiting, the antibodies are either inactive or only partially acting on the catalytic site itself.

The antigenic determinant of the apoenzyme is not the catalytic site itself as the homologous antiserum and also the other antisera do not prevent the FAD binding. In this respect the observations of KISTNER (1958, 1960) are of interest in old yellow enzyme the FAD binding site is also not the antigenic site. The apoenzyme antiserum only slightly interferes with the catalytic activities of the stable dimer conformations and even enhances the activity of the reconstituted monomer, probably by stabilising this conformation. This indicates that the antigenic sites, i.e. tertiary structure, of the apoenzyme are different from those in the other conformations. NADH largely prevents the FAD binding process (cf. Chapter V).

It remains questionable if the antiserum of the apoenzyme has been prepared against the native apoenzyme structure as the blood

temperature of the rabbit might have been promoting denaturation of the apoenzyme. From the specific antiserum prepared against the reconstituted DCIP-active enzyme, which is also a temperature-labile conformation, we known that the immunological information is carried over reasonably fast.

The antisera obtained against the active lipoamide dehydrogenase conformations are generally inhibiting optimally the homologous combinations, the normal anti-holoenzyme excluded in which case the Cu^{2+}--modified enzyme is inhibited more. This general aspect emphasises the importance of the tertiary of the protein conformation. Monomer and dimer are known to be different in hydrodynamic properties, e.g. frictional coefficient (VISSER and VEEGER, 1968[a]). The reconstituted enzyme does exist in several conformations. The DCIP-active form obtained after a approx. 20 min. recombination of apoenzyme and FAD was used as the main antigen (Chapter V). The main chemical difference between the holoenzyme and the Cu^{2+}-modified enzyme is that in the latter two sulfhydryl groups are oxidised per flavin moiety.

The protection of NADH against inactivation by antibodies is clear from Table III in the case of the native holoenzyme. As for the apoenzyme which had to be measured as reconstituted enzyme, it is impossible to detect any influence as NADH itself complicates this pattern too much by competing with FAD for the flavin binding site. The Cu^{2+}-modified enzyme is protected as well by NADH though not completely.

The Cu^{2+}-modified enzyme and the DCIP-active monomer have in common that FAD is bound less apolar than is the case in the native holoenzyme as can be derived from spectral data; their catalytic site will therefore be more exposed which apparently is reflected by a stimulated DCIP-activity. The results with NADH as protectant suggest that the antigenic determinant of the active monomer borders the catalytic area closer than in the dimer. A change in the state of reduction of the flavin in the Cu^{2+}-modified enzyme by NADH (this conformation is known to be quite easily reduced to the 4-equivalent reduced state) abolishes the affinity of the antigenic site either completely (DCIP-enzyme antiserum) or partially (homologous and holoenzyme antiserum).

Table I

Antiserum concentrations. Enzyme concentrations used in this test were 1 mg/ml for all conformations. The antisera, prepared as described in the Methods were diluted in 0.1 M sodium phosphate buffer (pH 8.0). The dilutions were used in the microprecipitation reaction. The figures in the colomns correspond with the highest dilution which still gives a positive reaction. Observations of the precipitate were done after one hour reaction at 37°.

Number of weeks After first injection	Dilution of antiserum			
	Holoenzyme	Cu^{2+}-modified enzyme	Apoenzyme	DCIP-active enzyme
4	2	4	-	-
6 ½	4	8	-	-
8	8	16	16*	32*
9	16	32	-	32*

* aspecific flocculation

Table II

Development of agar gel-diffusion patterns with time. Enzyme concentrations were kept constant (1 mg/ml), the concentrated antisera (see Methods) were used undiluted. Temperature 4°. Figures represent the number of precipitation lines (cf. Fig. 1).

Days		Antisera			
		Holo-as	Cu^{2+}-enzyme-as	DCIP-as	Apo-as
1	Holoenzyme	1(+1)*	1	1	-
	Cu^{2+}-enzyme	1	1	1	1
	DCIP-enzyme	1(+1)*	3	2	1
	Apoenzyme	1	3	2	1*
2	Holoenzyme	2	1	1(+2)*	1
	Cu^{2+}-enzyme	1	1	1	1
	DCIP-enzyme	2	3	2	1
	Apoenzyme	2	3	2	1
3	Holoenzyme	2	1	3	1
	Cu^{2+}-enzyme	2	1	1	1
	DCIP-enzyme	2	3	2	1
	Apoenzyme	2	3	2	1

* weak undeveloped band

Table III

Protective effect of NADH on the inactivation of different form of lipoamide dehydrogenase by different antisera. 0.5 mg holoenzyme or Cu^{2+}-modified enzyme was incubated with either 0.1 ml undiluted antiserum or 0.1 ml bovine serum albumin (2 %) in the control experiments for 2 min. at 25° with or without 0.1 ml NADH (10^{-2} M). Then the activities were measured. Apoenzyme (0.5 mg) was incubated on ice for 10 min. with or without 0.1 ml NADH (10^{-2} M) and with 0.1 ml antiserum. FAD (0.02 ml 10^{-4} M) was added 2 min. before measuring the activities. The activities are expressed as relative activities.

	Holoenzyme		Apoenzyme		Cu^{2+}-enzyme	
	$lipS_2$-act.(%)		DCIP-act.(%)			
	+NADH	-NADH	+NADH	-NADH	+NADH	-NADH
B.S.A.	110	100	20	100	93	100
Holo-as	92	59	9	90	40	31
Cu^{2+}-enzyme as	110	86	16	79	41	12
Apoenzyme as	113	82	8	84	112	90
DCIP-as	not checked	not checked	19	52	96	51

8. MISCELLANEOUS ASPECTS OF THE Cu^{2+}-MODIFIED ENZYME AND THE FROZEN HOLO-ENZYME

8.1. INTRODUCTION

Pig heart lipoamide dehydrogenase is modified by Cu^{2+}-ions (VEEGER and MASSEY, 1960) in a catalytic way under aerobic conditions (CASOLA et al., 1966). The Cu^{2+}-modified enzyme shows a 20 to 30-fold stimulated reduction rate of 2,6-dichlorophenol indophenol and a diminished rate of reduction of lipoate by NADH. This phenomenon has been ascribed to oxidation of sulfhydryl groups since VEEGER and MASSEY determined 1.8-2.3-SH groups less in the Cu^{2+}-treated enzyme than in the native enzyme. Amperometric titrations (PALMER and MASSEY, 1962) resulted in 6 -SH groups and one S-S bridge per mole of flavin in the native enzyme. CASOLA et al. (1966) found variations in -SH content of the Cu^{2+}-modified enzyme dependent on the time of incubation and, even after removal of the cupric ions, dependent on the age of the modified enzyme. The disappearence of -SH groups resulted in a proportionally increased disulfide content. The oxidisation process is rather complex and only initially reversible. The mechanism of the copper catalysed oxidation of cysteine to cystine is well known (CAVALLINI et al., 1969).

The rate of increase of the DCIP-activity depends on conditions as for instance on the phosphate buffer concentration (VEEGER and MASSEY, 1960) while the subsequent decrease in $lipS_2$-activity is pH-dependent (CASOLA et al., 1966).

CASOLA and MASSEY (1966) observed an activating effect upon the DCIP--activity after treatment of the native enzyme with another sulfhydryl reagent viz., phenylmercuric acetate.

The Cu^{2+}-modified enzyme which has a more polar flavin environment, is easily reduced by NADH to the four equivalent reduced state in which one disulfide bridge is reduced to 2-SH groups and FAD is converted into $FADH_2$. The green charge transfer band at 720 nm due to NAD^+ interaction with $FADH_2$ (VEEGER and MASSEY, 1962) is also formed. The reduction state induced by excess $lip(SH)_2NH_2$ as concluded from the decrease of the 530 nm band, is different from the semiquinone intermediate of the native enzyme as formed by two equivalent reduction. Blocking of the sulfhydryl groups by PCMB initially stimulates the DCIP-activity and induces a rapid four equivalent reduction state of the enzyme, however without a NAD^+ charge transfer band

at 720 nm. This suggests that a -SH group which is easily accessible, plays a role in one of the NAD^+ binding sites.

KALSE and VEEGER (1968) observed an interesting effect on the activities of lipoamide dehydrogenase which had been frozen in protein concentrations less than 1 mg/ml. Apart from a many-fold stimulated DCIP-activity and a diminished lipoate activity this frozen enzyme also shows spectral shifts in the flavin region from 454 nm to 450 nm as well as a promoted anaerobic reduction by NADH to the four equivalent reduced state. This suggests at least a similarity in protein conformation of Cu^{2+}-treated and frozen enzymes.

8.2. RESULTS

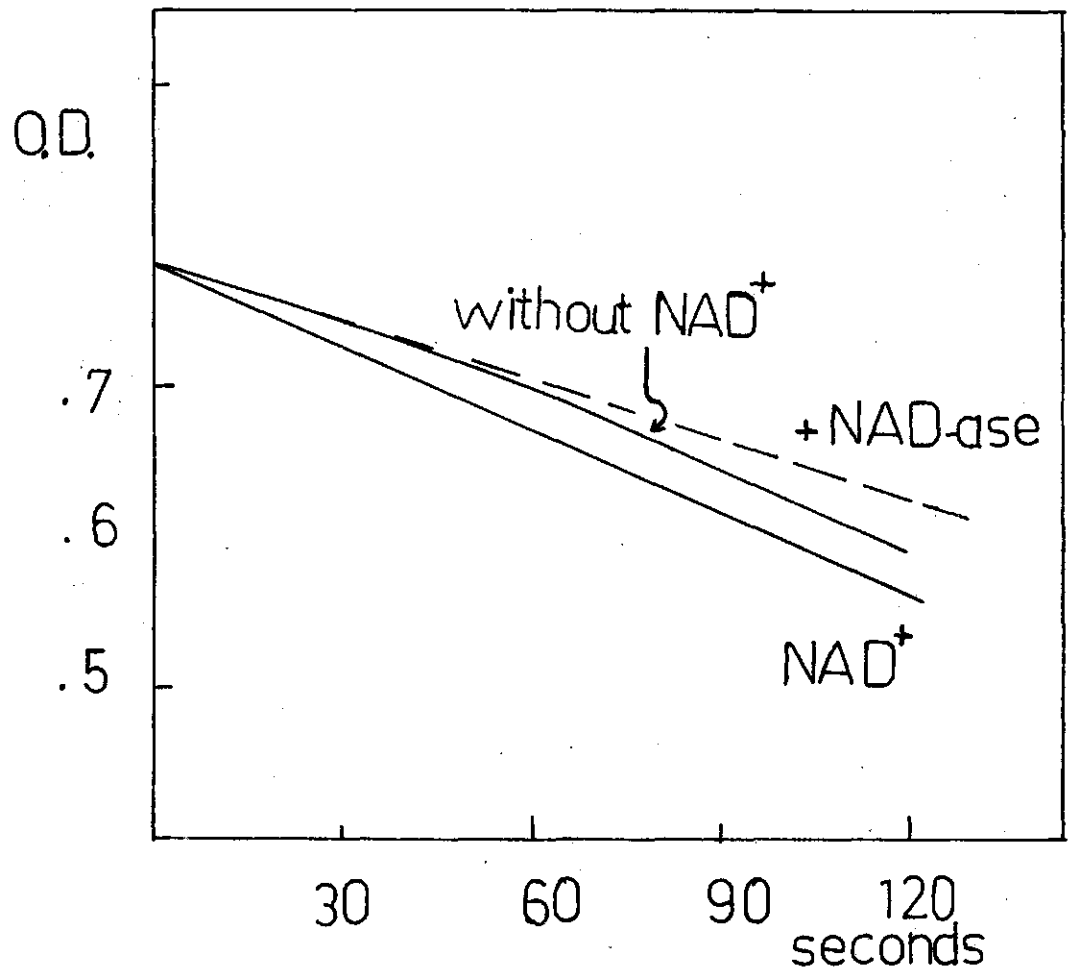
8.2.1. Influence of NAD^+ and phosphate buffer concentration on the DCIP--activity of the Cu^{2+}-modified enzyme

CASOLA et al. (1966) did not mention an activation of the Cu^{2+}-modified enzyme in the DCIP-assay although they have found this with the phenylmercuric acetate-treated enzyme (cf. CASOLA and MASSEY, 1966). However, a similar activation can be observed within the first minute of the assay of the Cu^{2+}-modified enzyme as shown in Fig. 1. (VISSER and VEEGER, 1968b). Phosphate buffer as well as NAD^+ influence this stimulating effect in a rather complex way (Table I). High phosphate concentrations (e.g. 0.2 M) inhibit the reaction in the absence as well as in the presence of NAD^+. The phosphate inhibition has been found to be competitive (VEEGER and MASSEY, 1962) with respect to NAD^+ in the $lip(SH)_2NH_2$ oxidation catalysed by the native enzyme. With the Cu^{2+}-enzyme it is clear from Table I that using low phosphate concentrations (0.02-0.10 M) in combination with for instance 40 µM NAD^+ the linearity of the reaction in the cuvette is promoted. Increasing the phosphate concentration also requires enlargement of the NAD^+ concentration to maintain linearity. High NAD^+ concentrations inhibit the reaction by a regular product inhibition.

Preincubation of the enzyme with NAD^+ also revokes linearity in the velocity with a rate equal to that ultimately reached in the control. When Cu^{2+}-enzyme was kept on ice for prolonged times (30 hrs or longer in a dilution of 0.2-1 mg/ml there was a tendency to linearity in the assay. This effect was slowly shifted in the opposite direction by increasing the temperature of the sample.

Fig. 1. Activation effect of NAD^+ on the DCIP-activity of the Cu^{2+}-modifie enzyme. Assay conditions as described under methods. The reactions were pe formed without and with 50 μM NAD^+ in the cuvette and in the presence of a trace of NAD-ase without addition of NAD^+.

Fig.1



8.2.2. Recombination

The apoenzyme of the Cu^{2+}-modified enzyme has been prepared the same way as that of the holoenzyme. The residual activity with DCIP is higher than in the case of the normal enzyme and amounts 10 to 20 percent of the activity of the original Cu^{2+}-enzyme. This is due to 5-10 % residual flavin which is mainly protein-bound and the fact that the recombined apoenzyme initially has a 1,5 to 2 times stimulated DCIP-activity over that of the Cu^{2+}-modified enzyme. The recombination process is more difficult to analyse on the basis of activities as one parameter fails viz. the return of the lipoate activity. In Fig. 2 the changes in DCIP-activity and fluorescence properties upon recombination are given. The way the flavin fluorescence intensity (I_{vv}) and the fluorescence polarisation increase is not different from the normal recombination system. The DCIP-activity also goes through a maximum in the course of this process and declines though to a less extent than in the normal case.

The flavin binding constant is slightly higher than those observed with the normal apoenzyme (KALSE and VEEGER, 1968). The saturation curve based upon DCIP-activity is shown in Fig. 3 from which a K_{ass} value of 50 μM has been calculated. Similar values were found with the fluorescence polarisation technique as shown in Table II. A temperature-dependency is hardly observed from the data presented here but there is a tendency that the flavin binding constant decreases upon enlarging the amount of flavin. Although a similar behaviour is not observable in the saturation curve (Fig. 3) such an effect could be due to an inhomogeneity of the population of apoenzyme molecules resulting in different binding constants (cf. Chapter V) or to the inaccuracy of the fluorescence polarisation method at low concentrations.

For the Cu^{2+}-modified apoenzyme it has not been proved that this enzyme has half the original molecular weight but there are indirect indications for a similar association-dissociation behaviour*. The DCIP-activity behaves similarly to that in the normal case, as it decreases faster at elevated temperatures. Moreover, there is another very remarkable difference

* Footnote: Upon prolonged incubation with Cu^{2+}-ions species of higher molecular weight are observed (e.g. tetramers) probably due to intramolecular disulfide bridge formation. LITTLE and O'BRIEN (1967) observed dimerisation of yeast cytochrome c on thiol oxidation by weak oxidants as Cu^{2+}-ions.

Fig. 2. Recombination of Cu^{2+}-modified apoenzyme with FAD. Apoenzyme (4 μM) was recombined with an equal amount of flavin in 30 mM sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA at 10°. The fluorescence intensity (▲▲▲) and the polarisation (X-X) were measured. Samples were withdrawn at the tim indicated and the DCIP-activity (●●●)determined.

Fig.2

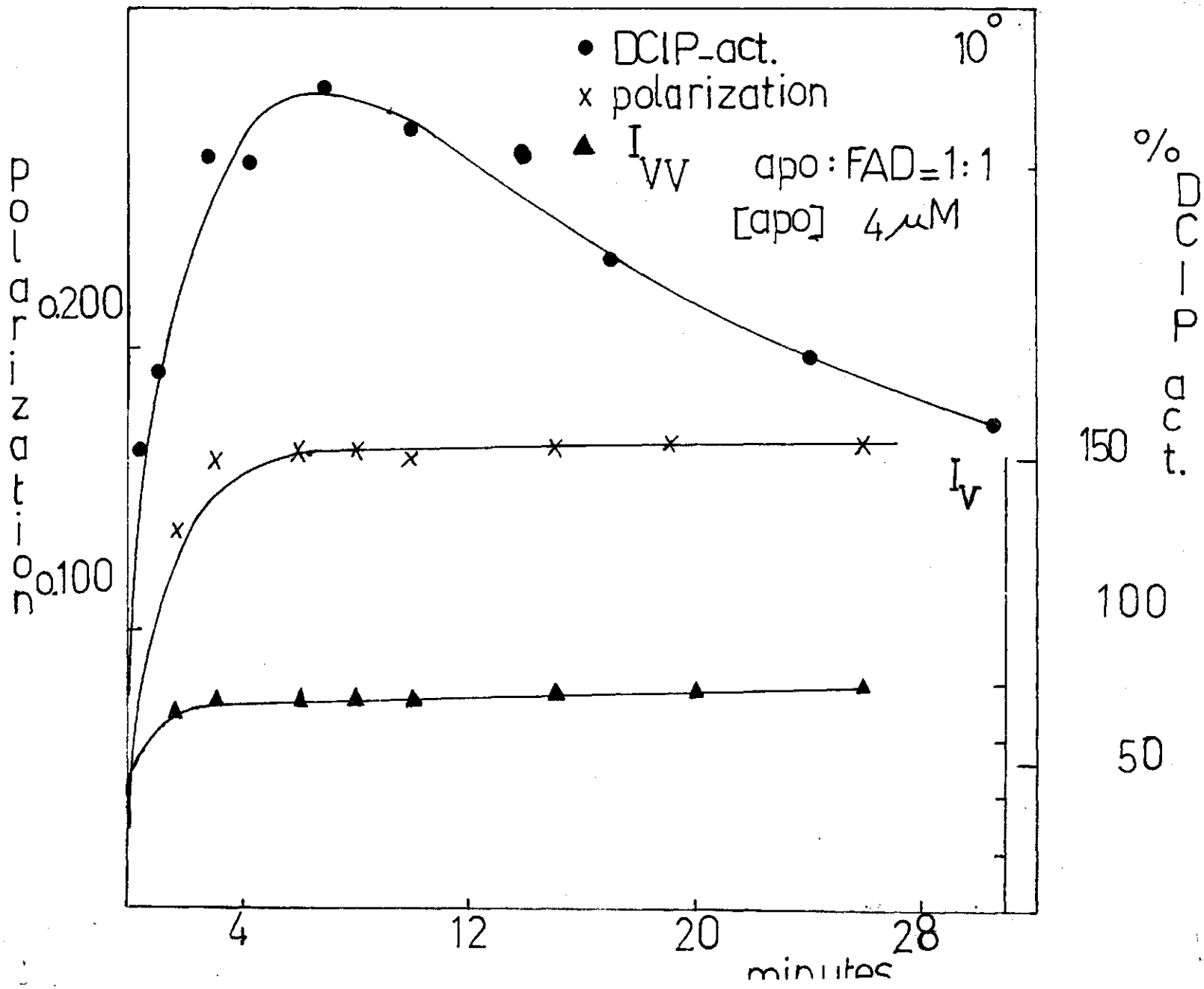
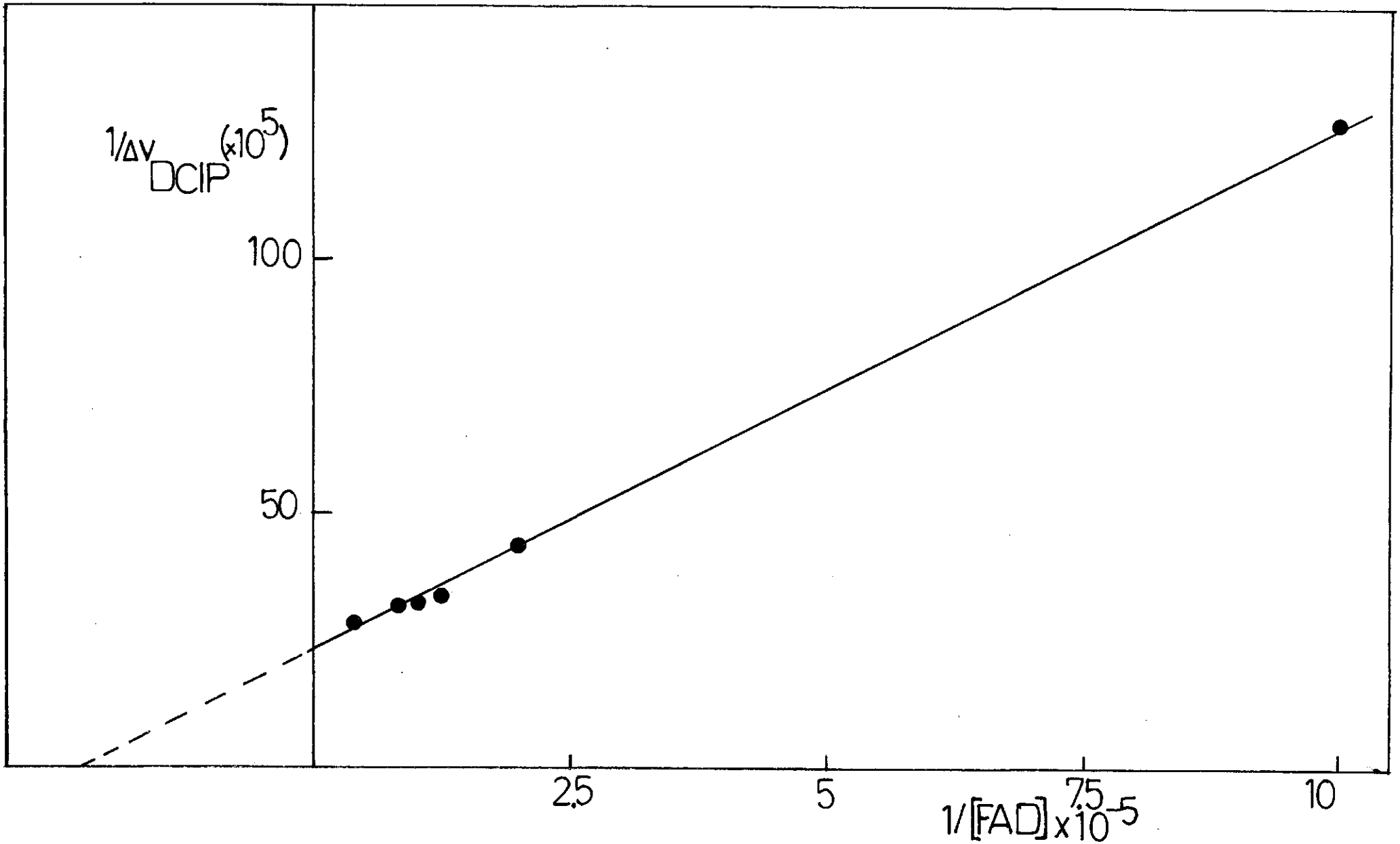

• DCIP-act.
x polarization
▲ I_{VV}
10°
apo : FAD = 1:1
[apo] 4 μM
I_V
polarization
0.200
0.100
% DCIP act.
150
100
50
4
12
20
28
minutes

Fig. 3. L-B plot of the recombination of Cu^{2+}-modified apoenzyme with FAD
Apoenzyme (0.2 mg/ml) was incubated on ice with different amounts of
flavin in 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA. Th
DCIP-activities were measured after 15 min. incubation.

Fig.3



between the reconstituted enzyme and the original Cu^{2+}-enzyme. The DCIP--activity of the reconstituted enzyme is linear in the assay or even declines caused by FAD dissociation in the cuvette. However, the activation effect during 30-60 seconds as observed in the original Cu^{2+}-enzyme progressively returns upon recombination, and faster at elevated temperatures (15^{o}-25^{o}). This strongly suggests that this parameter belongs to the dimer structure.

8.2.3. Freezing effects on the Cu^{2+}-modified enzyme

The native enzyme is sensitive to freezing at low protein concentrations (<1 mg/ml), as will be discussed in section 8.3. The frozen enzyme has a highly stimulated, linear DCIP-activity in the assay. The Cu^{2+}-modified enzyme undergoes similar structural changes when frozen in dilution (< 1 mg/ml). Freezing results in a 1.5 to 2.5-fold stimulated DCIP-activity while the activation effect is lost. BSA, and ammonium sulphate proved to be effective in a (partial) maintainance of the original structure since they protected against loss of the activation effect in the assay. Addition of these compounds does not completely prevent a stimulation of the activity. Addition of lipoate has a similar effect as on the native holoenzyme (cf. 8.3) since in the concentration range of 0.5 mM to 10 mM the DCIP-activity is either largely reduced or absent after thawing.

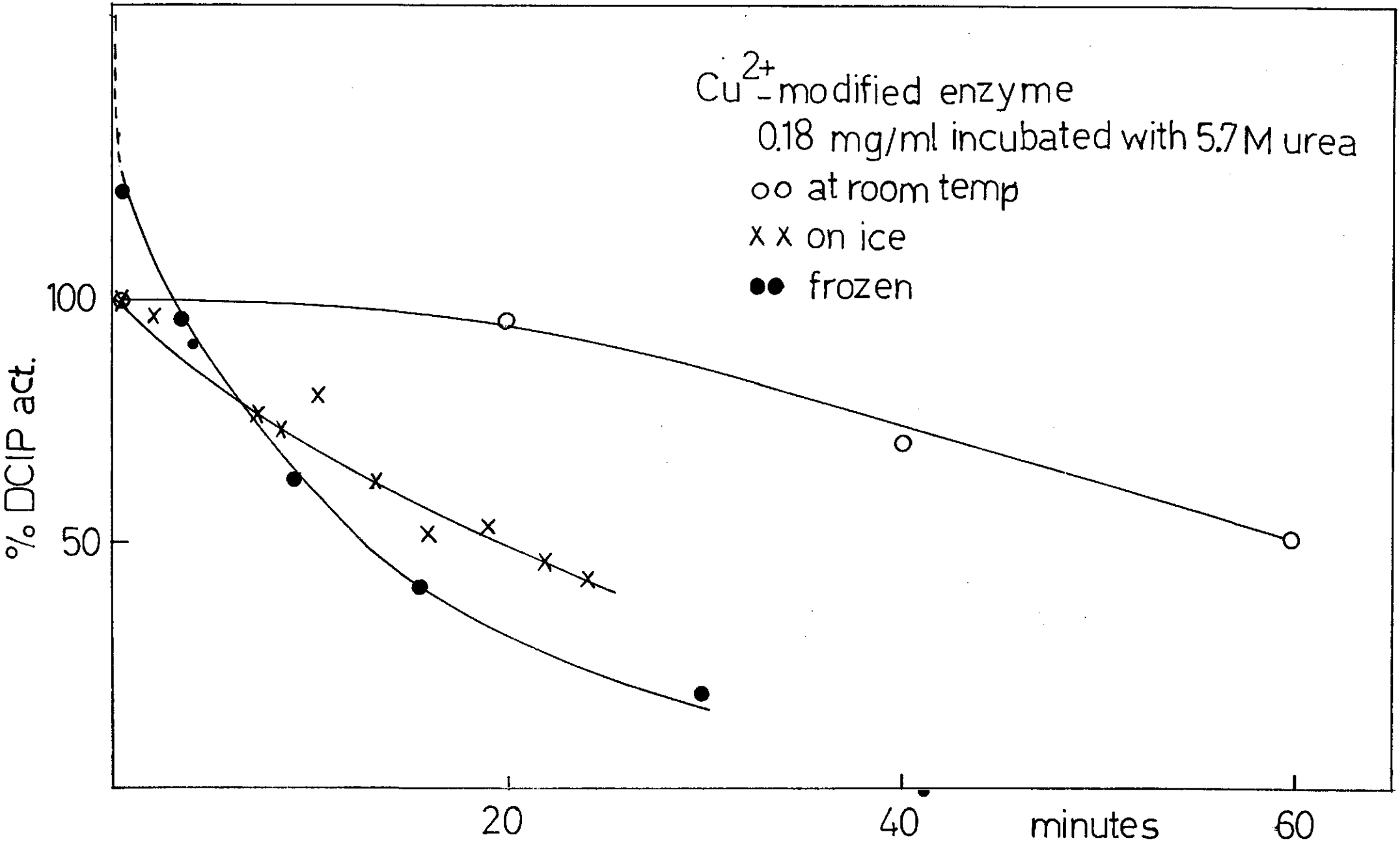
In some preparations the DCIP-activity was not stimulated after freezing but lowered for unclarified reasons. A reason could be that denaturation has occurred as was observed with the native holoenzyme after relatively long freezing periods. Another possibility is that this is correlated to the inability of the enzyme to dissociate since the lipoate activity of these preparations is not inactivated in extreme protein dilutions (VOETBERG and VEEGER, unpublished results).

8.2.4. The influence of urea

As mentioned before (VISSER and VEEGER, 1968a) the Cu^{2+}-modified enzyme is less stabile in urea concentrations > 4 M than the native enzyme. The native frozen enzyme and the recombined enzyme have been shown to become even more easily inactivated by urea. The frozen Cu^{2+}-modified enzyme behaves similarly and is as easily disrupted as the native frozen enzyme. In Fig. 4 the inactivation patterns of the Cu^{2+}-modified enzyme at room temperature and on ice are compared with the frozen enzyme. The Cu^{2+}-enzyme kept on ice is faster inactivated than if kept at room temperature which clearly

Fig. 4. Influence of urea on the activities of different Cu^{2+}-modified enzyme conformations. Cu^{2+}-enzyme (0.18 mg/ml) was incubated with 5.7 M urea on ice (XXX) and at 20° (000). Moreover, Cu^{2+}-enzyme was frozen overnight in the same concentration. Immediately after thawing urea was added. The DCIP-activities were measured at the times indicated and are expressed in relative units with respect to the original prepared Cu^{2+}-enzyme. Conditions: 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA.

Fig.4


Cu^{2+}-modified enzyme
0.18 mg/ml incubated with 5.7 M urea
oo at room temp
x x on ice
●● frozen
% DCIP act.
100
50
20
40
minutes
60

indicates conformational changes upon lowering the temperature. E.g. the half times for the inactivation reactions with 5.7 M urea at a protein concentration of 0.18 mg/ml are 15-20 min. on ice and 60 min. at room temperature while it is less than 10 min. for the frozen Cu^{2+}-enzyme. The inactivation of the diluted (0.09 mg/ml) enzyme proceeds faster than at 0.18 mg/ml concentration, where the decrease is even limited during the first 20 minutes incubation at room temperature.

It is of importance to make clear that although the catalytic activity drops with 30-40 %, as observed in 4 M urea, the flavin fluorescence polarisation remains almost constant. When the denaturation proceeds, the polarisation declines too. These results remind to those obtained when the apoenzyme is treated with urea before or just after addition of flavin (cf. Chapter V). It is evident that the flavin binding site is less urea-sensitive if occupied by FAD in comparison with other parts of the protein molecule.

8.2.5. Temperature-dependent protein conformations

There are some indications for the existence of temperature-dependent protein conformations of the Cu^{2+}-modified enzyme as is the case with the native holoenzyme. The urea-sensitivity on ice exceeds the one at room temperature which as a phenomenon observed before with several holoenzyme preparations at low protein concentration (0.05 mg/ml). This must be due to a low-temperature enzyme conformation which is more labile. Moreover, DE KOK (1970) observed a non-linear relationship in the Arrhenius plot of the flavin fluorescence lifetimes of the excited state with native as well as with Cu^{2+}-modified enzyme. In Fig. 5 the L-B plots are given measured with a Cu^{2+}-modified enzyme by varying the concentration of NADH. The V_{max} values are calculated at infinite NADH concentration but at a finite acceptor (DCIP) concentration. The slopes of the L-B plots are almost equal at high temperatures but there is a sudden change between 21° and 23.5°. Below this transition area the L-B plots are about parallel again over the temperature range measured (14°-21°). The Arrhenius plots of K_m and V_{max} values are given in Fig. 6. The log K_m (NADH) vs 1/T plot shows a sharp transition which is in agreement with the transition temperature found with a Cu^{2+}-treated enzyme by DE KOK (1970) for the flavin fluorescence lifetime of the excited state. The transition temperature is higher than in the case of the holoenzyme (cf. Chapter VI) which might be due to the influence of the substrate on the equilibrium between the conformations. NAD^+ has more affinity

Fig. 5. L-B plots of the Cu^{2+}-modified enzyme at different temperatures. Cu^{2+}-enzyme (0.2 mg/ml) was solved in 30 mM sodium phosphate buffer (pH 7 containing 0.3 mM EDTA and 0.5 % BSA. The concentration of NADH was varie using the usual assay conditions. The symbols indicate the different temperatures: 000, 33°; ●●●, 30.5°; ΔΔΔ, 28°; ▲▲▲, 25,5°; XXX, 23.5°; ∇ ∇ ∇, 2 ▼▼▼, 18°; □□□, 16.5°; ■■■, 14°. At 21° two possible curves are drawn.

Fig.5

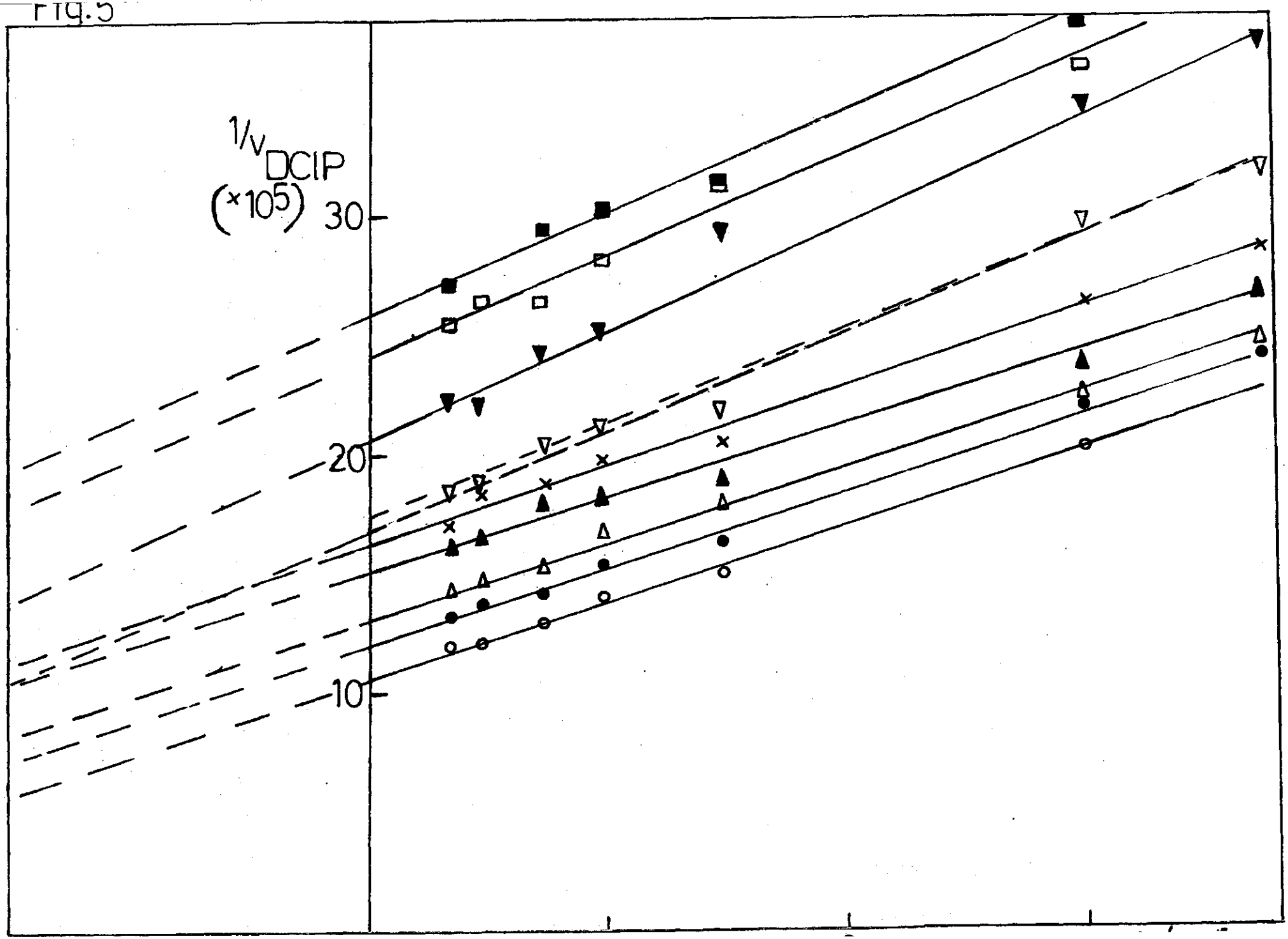
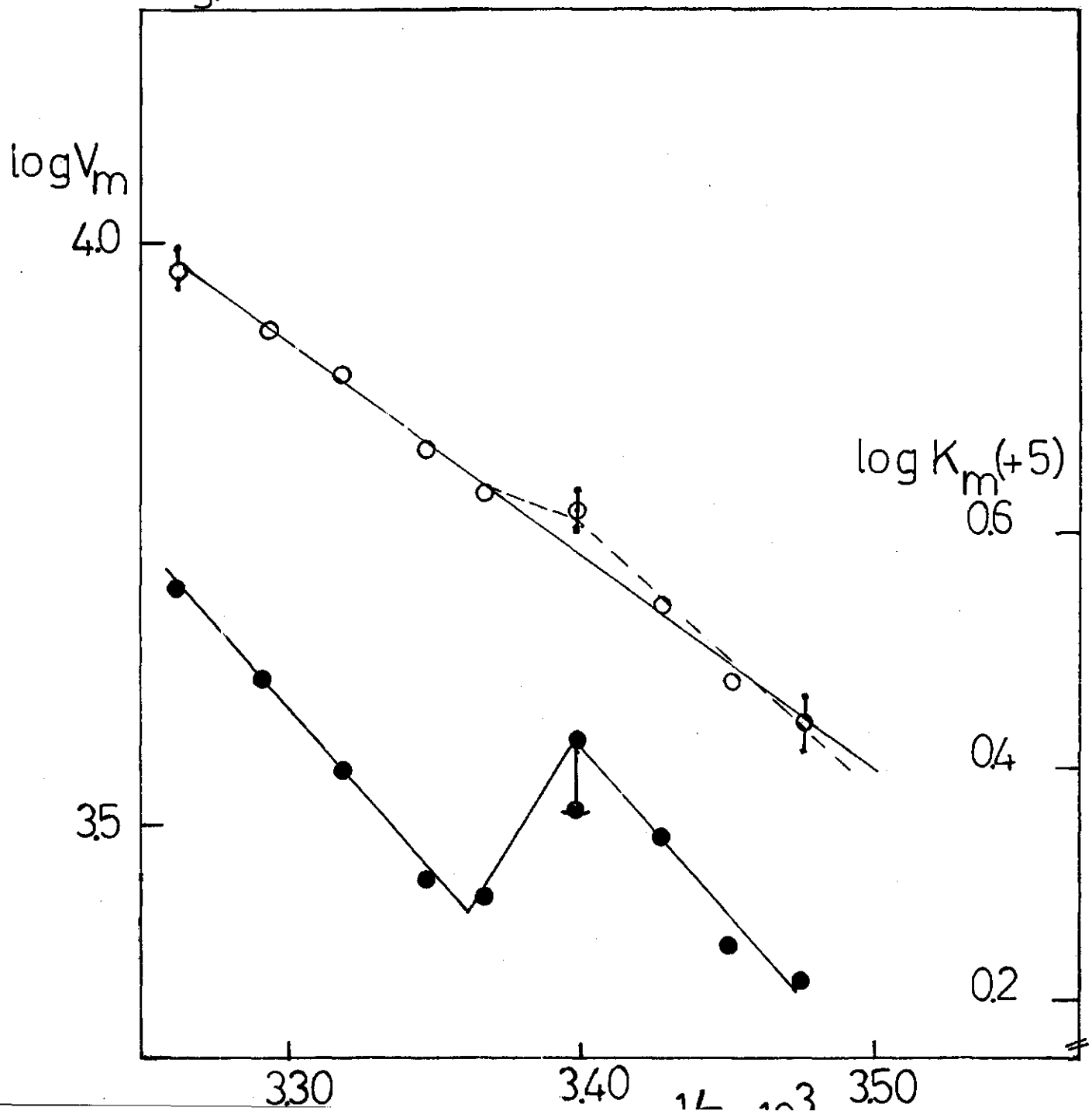


Fig. 6. Arrhenius plot of the reaction velocity of the Cu^{2+}-modified enzy
The V_{max} values are obtained by extrapolating the L-B plots shown in Fig.
(000). Moreover, a spreading of 5 % was taken into account which has only
been indicated at three experimental figures. The log K_m (NADH) values ar
also plotted vs 1/T (000). At 21° two figures are given each one correspo
ing with one of the two possible L-B plots given in Fig. 5 for this tempe
rature as dotted lines.

Fig.6



for the high-temperature form which probably lowers the transition temperature.

From the log V_{max} vs 1/T plot a breakage is hardly observable. The L-B plots were not constructed using the least-square method and therefore the spreading not determined. However, in Fig. 6 a spreading of $\pm$ 5 % of the V_{max} value at 21° is indicated. Taking into consideration that the experimental data at the other temperatures also have the same inaccuracy, it could be doubtful whether a deviation from linearity occurs. However, the same phenomenon has been found in other experiments. Therefore, it is likely that the deviation is a real one. The activation energy of the left hand part of the Arrhenius plot amounts 8000 cal/mole.

8.3. THE FROZEN ENZYME

8.3.1. Influence of ionic strength and pH of the buffer

The freezing experiments described by KALSE and VEEGER (1968) were performed in 15 µM sodium phosphate buffer pH 7.2 without EDTA. The results obtained in the following experiments were not always as reproducable as one should like for reasons which are not well understood. The freezing effects viz. the increase of the DCIP-activity and the decrease of the lipoate activity, are not always easily induced. For some preparations several days of freezing are necessary, others are altered within 14 hrs. No correlation is found between the purity-factor (E280/E455 ratio) and the sensitivity for freezing.

In Table III the influence is shown of freezing an enzyme overnight in sodium phosphate buffers of different pH values. At 30 mM buffer concentration the DCIP-activity rises and the lipoate activity falls to approx. the same degree at all pH values. Elevating the temperature stimulates the lipoate activity with only slight variations.

The influence of the sodium phosphate buffer concentration is not observed in all cases. In several cases buffer variations from 12 mM to 180 mM (pH 7.2) hardly show differences in their effect on lipoate- and DCIP-activity levels. However, in case the enzyme is less easily converted to the frozen enzyme conformation, high phosphate concentrations promote this conversion. For both cases an example is given in Table III.

8.3.2. The influence of BSA, ammonium sulphate, and lipoate on the freezing process

Although the protective effect of BSA was not tested in relation to different EDTA levels or purity factors of the enzyme, the protection was found effective in a concentration range of 0.025 % to 1 % when EDTA was absent in the enzyme.

Lipoate (ox.) added during the freezing procedure has a remarkable effect on both activities measured after thawing. When the $lipS_2$ concentration exceeds 0.2-0.3 mM the DCIP-activity is not stimulated any more after thawing but both activities have almost disappeared in an irreversible way; the lipoate activity does not return by elevating the temperature.

Though the reason for the lipoate-induced inactivation is unknown, a postulation might be an irreversible sulphur exchange between lipoate and the catalytic disulfide bridge. The lipoate concentration which is effective amounts 0.2-0,3 mM. Such values are of the same order of magnitude as the K_m values for $lipS_2$ which might be indicative for the involvement of the catalytic centre though I am fully aware that it is difficult to correlate K_m values and binding constants. The involvement of other sulfhydryl groups in the frozen enzyme is not to be excluded since in our laboratory NORDHOLT (unpublished results) observed a drastic decrease of the amount of -SH groups in a frozen enzyme which did not restore its lipoate activity after thawing. This is indicative for the great reactivity of these groups (induced by freezing) to become oxidised. In this respect it is worthwhile to remind the species of higher molecular weight observed with the Cu^{2+}-modified enzyme which was treated for prolonged times with Cu^{2+}-ions. Bovine liver rhodanese is known to dimerise under mild oxidising conditions using reduced lipoate (VOLINI et al., 1967).

The influence of $(NH_4)_2SO_4$ on the freezing process is known from the work of KALSE and VEEGER (1968) who found a protection of the lipoate activity by a 2 % solution of this salt. However, this salt is only protective against freezing during a limited period of time. This period may vary from 2 to 6 days freezing depending on the preparation one uses. Initially the lipoate activity of the enzyme is stabilised in the presence of for instance 50 mM $(NH_4)_2SO_4$. Varying the sodium phosphate buffer concentration in a range from 15 to 90 mM at pH 7.2 did not influence this pattern. In the control experiments without $(NH_4)_2SO_4$ the lipoate activity drops to approx. 20 %. Continuation of the freezing procedure results also in a partial loss of the lipoate activity and a stimulation of the DCIP-activity

of the $(NH_4)_2SO_4$ containing samples; especially between 70 and 100 mM phosphate buffer concentration but almost not at a 30 mM buffer concentration. In the control experiments prolonged freezing leads to a further decrease of the lipoate activity. The DCIP-activity may become either unproportionally stimulated or ultimately largely inactivated.

Though the effective concentration of $(NH_4)_2SO_4$ is high it is nevertheless likely that the protective effect is not due to an ionic strength effect but is specifically bound to the ionic species used. This will be discussed in the next section.

8.3.3. The protective influence of specific cations

In Table IV is shown the protection of the lipoate activity induced by several ammonium salts in a 30 mM sodium phosphate buffer (pH 7.2) with 0.3 mM EDTA as well as the influence of some sulphates. The main effect is due to the NH_4^+-ion as the DCIP-activity is completely or partially (in the case of NH_4Cl) repressed. In combination with multi-valent anions the lipoate activity is better protected. In other experiments NH_4Cl was found better protective with respect to the lipoate activity (cf. Table V). The NH_4^+-ions in the case of NH_4Cl can not prevent the conformational change by which the lipoate-activity partially disappears but they still prevent the rise of the DCIP-activity. This can only be explained in case of the existence of an intermediate form between the native and the DCIP-stimulated enzyme.

As NH_4^+-ions were more specific in their action than the anion it was of interest to find out whether other alkali-ions were also protecting against modifications of the protein structure. In Table V a survey is given of the influence of several alkali-chlorides and it is shown that lithium- and cesium-ions are even better protecting against the modifications in the enzyme structure. On the other hand it is clear that sodium chloride promotes loss of both activities in the enzyme modification while the return of the lipoate activity is slower compared with the other salts and as the DCIP-activity is also lower than in the control. The whole system becomes less reversible. A lower concentration of cations diminishes the protection-level (but not the sequence) and as a consequence the DCIP--activities are slightly stimulated even in the presence of Li^+, Cs^+ or $(NH_4)^+$. Here again the period over which the enzyme is frozen will be of importance as we saw before with $(NH_4)_2SO_4$. The irreversible changes induced by Na^+ and Mg^{2+} are less, at the lower salt concentration. This influence is expected to diminish when the salt concentration is lowered.

To establish the concentration of LiCl which still is effective in maintaining the lipoate activity, a range of Li^+-ion concentrations was tested. Moreover, two freezing times were introduced. The results are summarised in Table VI. It is clear that the protection is better after 2 days of freezing instead of a week. In the former case a rather sharp concentration treshold is observed. It was surprising that a combination of Na^+- and K^+-ions is also protective though individually they are not. The ratios Na^+/K^+ and Na^+/Li^+ are identical $51 \times 10^{-3}/7.5 \times 10^{-3} = 6.8$ and $51 \times 10^{-3}/8 \times 10^{-3} = 6.4$.

However, there is a difference between the potassium- and the lithium--ions as high concentrations of K^+-ions (0.1 M KCl) are not protective in contrast to 0.1 M LiCl (cf. Table V).

There is a strong tendency that a combination of potassium and sodium phosphate buffer is not only protecting against freezing but is enhancing the specific lipoate activity as well.

8.3.4. Influence of protein concentration

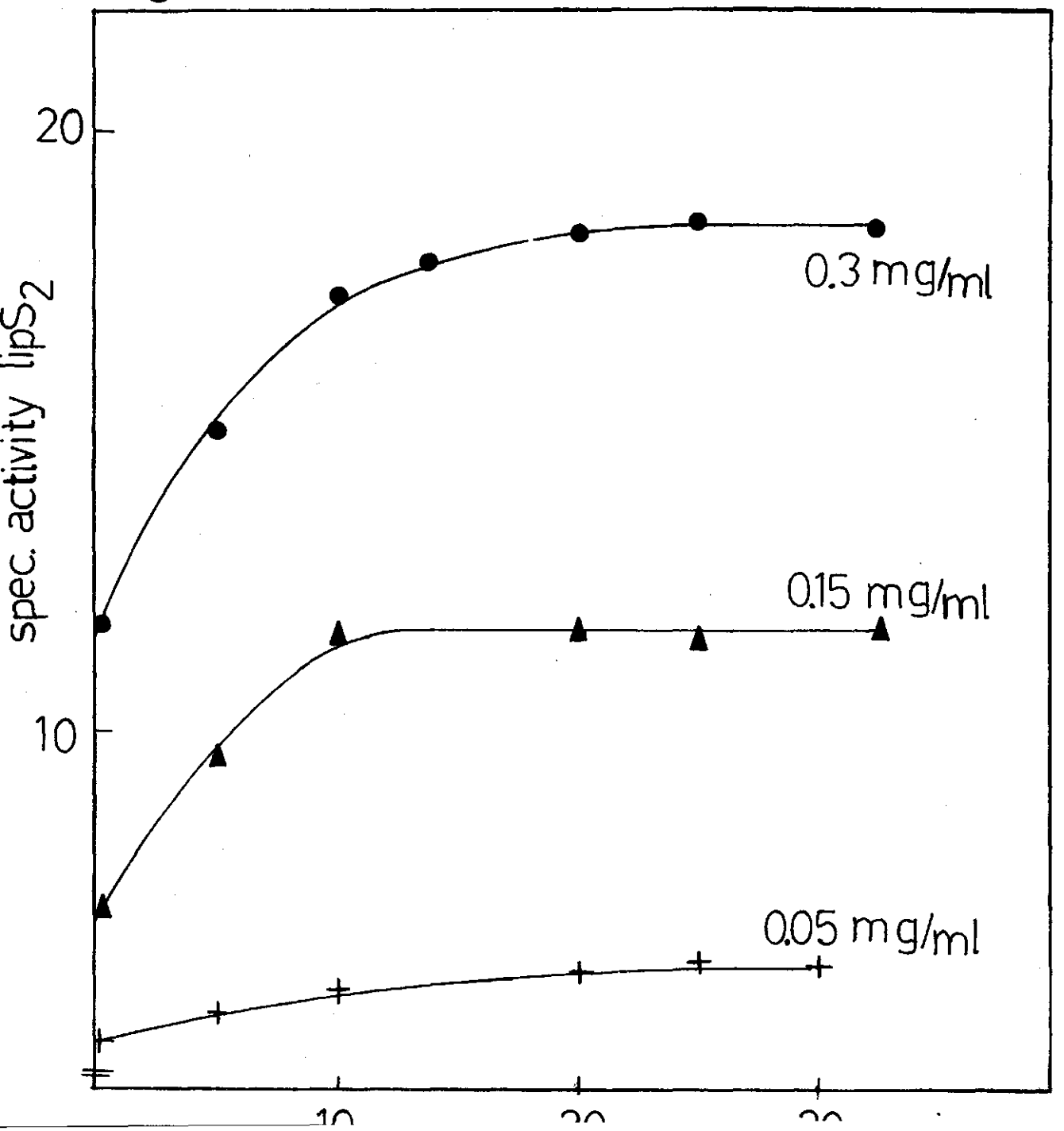
The stimulation of the DCIP-activity and the inactivation of the lipoate activity are dependent on the protein concentration and they are better observed when the freezing time is limited; e.g. protein concentrations of 2 - 1.5 - 1 - 0.75 - 0.50 and 0.25 mg/ml have DCIP-activation of 1500 - 1400 - 2250 - 2000 - 2650 and 3500 respectively, after a three days freezing period.

In another experiment the enzyme was frozen for a prolonged time in the presence of FAD as to keep the enzyme saturated with flavin which dissociates easier from the frozen enzyme (cf. KALSE and VEEGER, 1968). The reversibility of the lipoate activity by elevating the temperature is only partial when the enzyme is exposed to freezing for a long time. In Fig. 7 it is shown that the return of the lipoate activity is protein concentration-dependent though this result could also be explained by a faster irreversible denaturation during the freezing of lower protein concentrations. The DCIP--activity is linear in the assay after freezing for relatively short periods (1-5 days). This is in contrast to the DCIP-activity of the recombined native enzyme directly after recombination suggesting that the flavin is more tightly bound in the frozen enzyme.

It was attractive to think of a monomerisation as a result of freezing at low protein concentrations. This could account for the shifts in catalytic activities. To determine the order of changes in DCIP- and lipoate activities

Fig. 7. Influence of the protein concentration on the restoration of the lipoate activity after freezing. Enzyme (A280/A455=5.8; $lipS_2$-act. 32) wa frozen during 4 days at -14° with 100 µM FAD at three different protein concentrations: ●●●, 0.3 mg/ml; ▲▲▲, 0.15 mg/ml and +++, 0.05 mg/ml. The samples were thawed and incubated at 20°. The restoration of the lipoate activity was followed during 30 min.

Fig.7



several samples of one enzyme were frozen with FAD (100 μM) and thawed at different temperatures while both activities were determined. The results for the decline of the DCIP-activity are shown in Figs 8 and 9.

The rate of the conversion of the DCIP-activity is characterised by

$$\frac{d\ (\text{spec.activity})_t - (\text{spec.activity}_{t\to\infty})}{dt} = \frac{d\ \Delta\ \text{spec.activity}}{dt}$$

The specific activity $t \to \infty$ corresponds with the original DCIP-activity of the native enzyme. From the relation between Δ spec.activity and t one obtains information concerning the order of the reaction. A second order reaction follows the equation

$$t = \frac{1}{k.\ \Delta\ \text{spec.activity}} - C.$$

The result of such a plot is shown in Fig. 8. It is obvious that at 0° the DCIP-activity declines according to a second order reaction. At 7° and 12° the first part of the experimental data still fit a second order reaction, but there is, progressively with time, deviation. At higher temperatures the total plot is curved.

In Fig. 9 log Δ spec.activity is plotted vs t which gives a straight line in case one deals with a first order reaction which follows the equation

$$t = \frac{1}{k} \ln\ \Delta\ \text{spec.activity} + C$$

The changes at low temperatures can not be described by a first order reaction because of the second order reaction (Fig. 8) but at higher temperatures the experimental data fit. To explain these results one must assume that a dimerisation reaction is measured at low temperatures which is followed by an intramolecular conformational change. At higher temperatures the dimerisation reaction is not rate-limiting any more and the intramolecular rearrangement in the formed dimer is observed. These data confirm the suggestions (Chapter III) that a DCIP-active dimer exists in between the lipoate active dimer and the DCIP-active monomer.

The lipoate activity has been found to be a more difficult parameter under the conditions used since no complete return of the lipoate level is obtained. Protein denaturation or formation of a partial active enzyme are possibilities.

Fig. 8. Data fitting plot for a second order decline of the DCIP-activity of a frozen enzyme at different temperatures. The 1/Δ specific DCIP-activi is plotted vs time (see text). Samples of enzyme (0.4 mg/ml) were frozen i 30 mM sodium phosphate buffer containing 0.3 mM EDTA and 100 μM FAD during 36 hrs. The samples were thawed at the temperatures indicated and the decline of the DCIP-activity followed for at least 30 min.

Fig.8

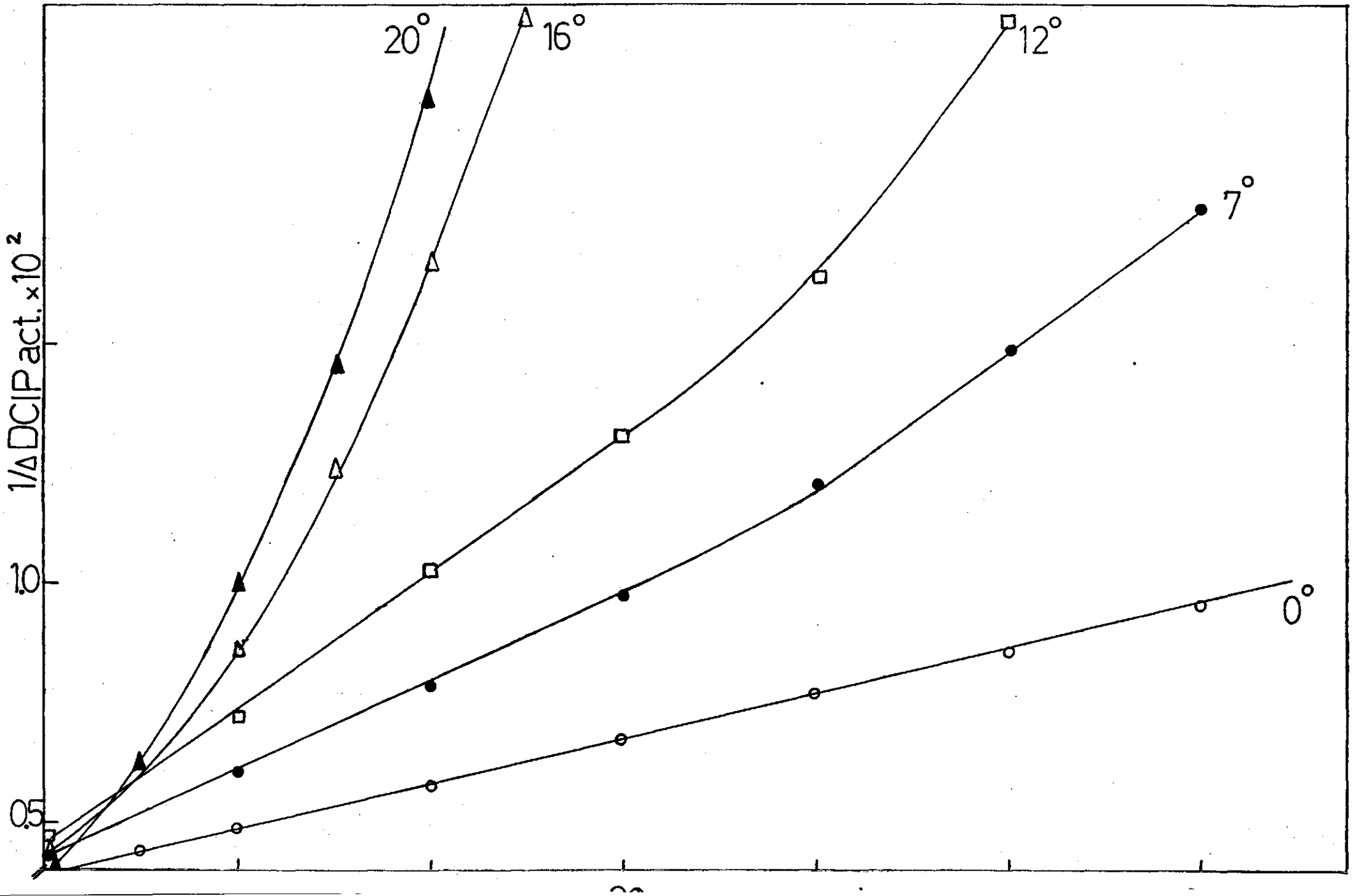
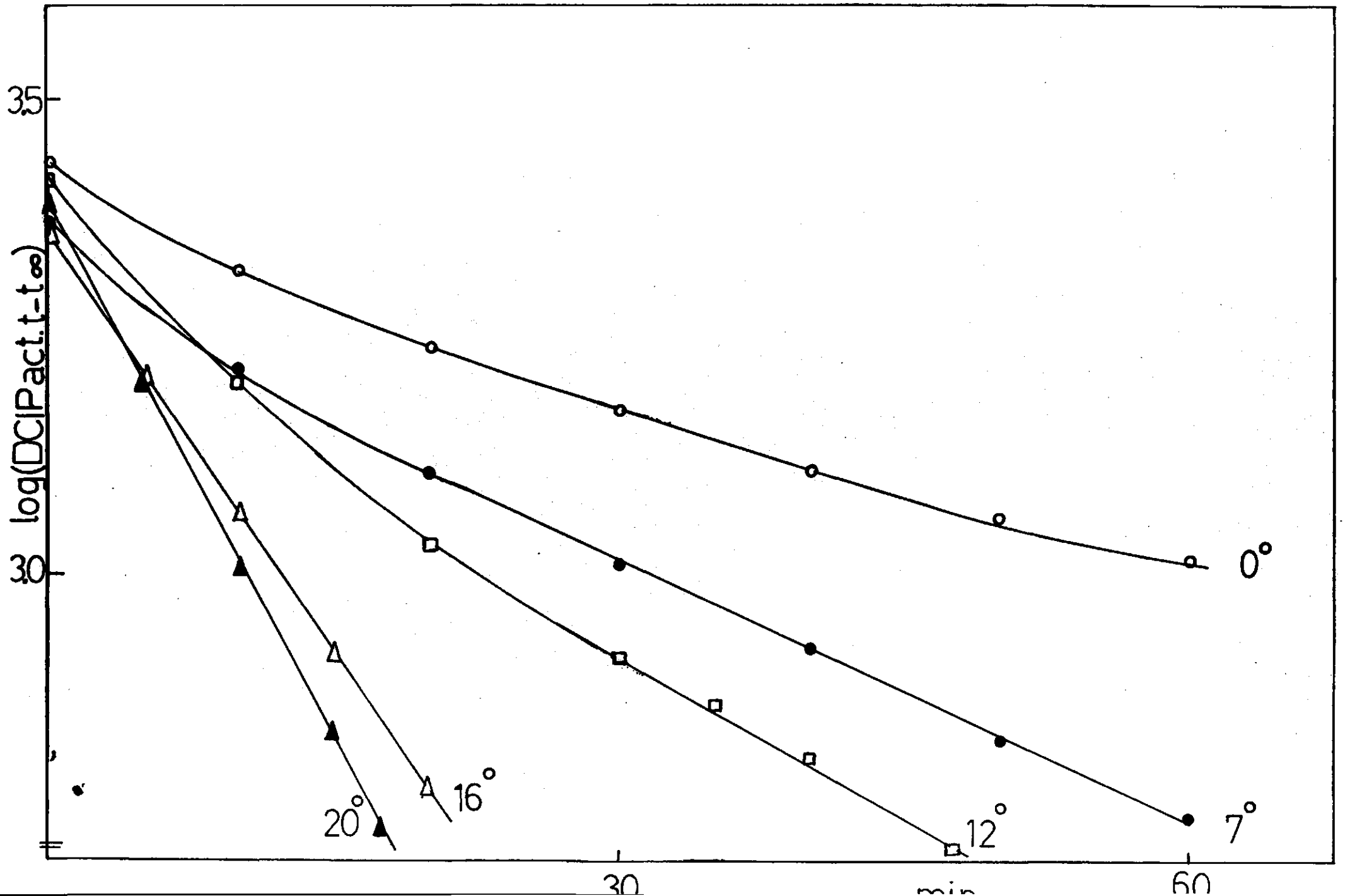


Fig.9



8.4. ELECTROPHORETIC BEHAVIOUR OF DIFFERENT ENZYME CONFORMATIONS

Lipoamide dehydrogenase from pig heart has no electrophoretical homogeneity (ATKINSON et al., 1962; STEIN et al., 1965a, b; COHN et al., 1968; MILLARD et al., 1969). Generally five fluorescent, active bands are reported, sometimes accompanied by a small sixth one. The patterns obtained in our experiments, are in agreement with the literature. Electropherograms of the holoenzyme are shown in Fig. 10A, B. The relative amounts of the components vary to some extent in different enzyme preparations although the A280/A455 ratio is about the same in both preparations, viz., 5.4 and 5.6, respectively. The specific activities are almost the same. The results from COHN et al. (1968) make it very likely that band 1 and 2 are derived from α-KGDC, band 3, 4 and 5 from PDC. Differences in composition of individual preparations of lipoamide dehydrogenase, which itself consists of a mixture derived from both multienzyme complexes, could occur when extraction from one of the complexes is not always effective to the same degree. Another explanation might be differences in the relative amounts of complex in individual hogs. Moreover, a partial purification of enzyme from either α-KGDC (HIRASHIMA et al., 1967) and PDC (HAYAKAWA et al., 1966) resulted in either a relative increase of the intensities of bands 1 and 2, or bands 3, 4 and 5, respectively.

Cu^{2+}-modified enzyme was made from the native holoenzyme. Part of the enzyme, after the oxidation reaction has been stopped, was dialysed during 2 hrs and part of it overnight. From the densitometer scanning patterns, shown in Fig. 11A and B, it is clear that the intensities of the bands 1 and 2 diminish. The relative mobilities remain constant. The changes in migration patterns indicate that conformational changes in the protein influence the surface charges which causes a shift in the relative amounts of the individual bands. Though the pK-values of the sulfhydryl groups involved in the disulfide formation are unknown, the likely range according to BENESCH and BENESCH (1955) for sulfhydryl groups in macromolecules is from 8.3 - 8.6. This would mean a change in netto charge as negative charges are neutralised. On the other hand, the results are not consistent with such changes which means that in spite of charge effects from sulfhydryl groups the conformational changes dominate.

The frozen DCIP-active enzyme has also a characteristically changed

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* This part of the work was done in collaboration with Mrs. J. Wouters van den Oudeweijer and J. Ruiter.

electropherogram (Fig. 12). The three bands derived from PDC are more diffuse and less fluorescent than the α-KGDC bands, indicating that lipoamide dehydrogenase derived from the pyruvate complex is converted more readily into the DCIP-active enzyme and loses its FAD more easily than the α-KGDC enzyme. Incubation with excess FAD at room temperature results, though not always, in a return of the original electrophoresis pattern and of the lipoate activity. Freezing for prolonged times also changes the first two peaks, results which are consistent with the observation that freezing effects are complex as the lipoate activity has not completely vanished after short periods of freezing while a slower and incomplete return of the lipoate activity, even in the presence of FAD, results from prolonged freezing. The main conclusion which can be derived from these experiments is that lipoamide dehydrogenase obtained from α-KGDC differs from the enzyme extracted from PDC.

As some of the conformational changes are so nicely reflected in the electrophoretic patterns, the components in the apoenzyme are of real importance. One of the interesting aspects is whether in the apoenzyme the two separated polypeptide chains are genetically different or not. In this respect references can be given to other flavoproteins: a) luciferase, a dimeric enzyme as many of the flavoproteins, has two polypeptide chains which differ from each other (FRIEDLAND and HASTINGS, 1967); b) L-amino acid oxidase, a dimer, which has three main electrophoretic components built up from αα, αβ en ββ chain combinations (DE KOK and RAWITCH, 1969).

A scanning pattern of an apoenzyme, freshly prepared is given in Fig. 13 together with the same aged apoenzyme which remained for 4 hrs at room temperature. With the native apoenzyme there are many diffuse bands found which suggests, as already mentioned in Chapter 5, that the apoenzyme population is inhomogeneous and consists of polypeptide chains in different conformations. In this respect it is of interest that VEEGER et al. (1969) have shown that the apoenzyme has a high helix content.

Recombination experiments with FAD (see Fig. 14) clearly indicate the rise of a main peak and some smaller shoulders, depending on the recombination temperature. The apoenzyme recombined during 2 hrs with FMN at room temperature (Fig. 13B), shows a pattern which is almost non-fluorescent and similar to the native apoenzyme. FMN seems to be protective with respect to the maintainance of the native apoenzyme structure (cf. patterns of the aged apoenzyme at 20° as shown in Fig. 13C). It is evident from this pattern that the native enzyme structure is not induced by FMN as is the case with

Fig. 10 Densitometer patterns of native lipoamide dehydrogenase. A. Electrophoresis of 40 µgs of enzyme (A280/A455=5.3; $lipS_2$-activity 40, DCIP--activity 100) under the buffer conditions described in the METHODS durin 2 1/2 hrs at 3.5 mA/tube. B. Electrophoresis of 50 µgs of enzyme (A280/A45 5.6; $lipS_2$-activity 30, DCIP-activity 200).

Fig 10

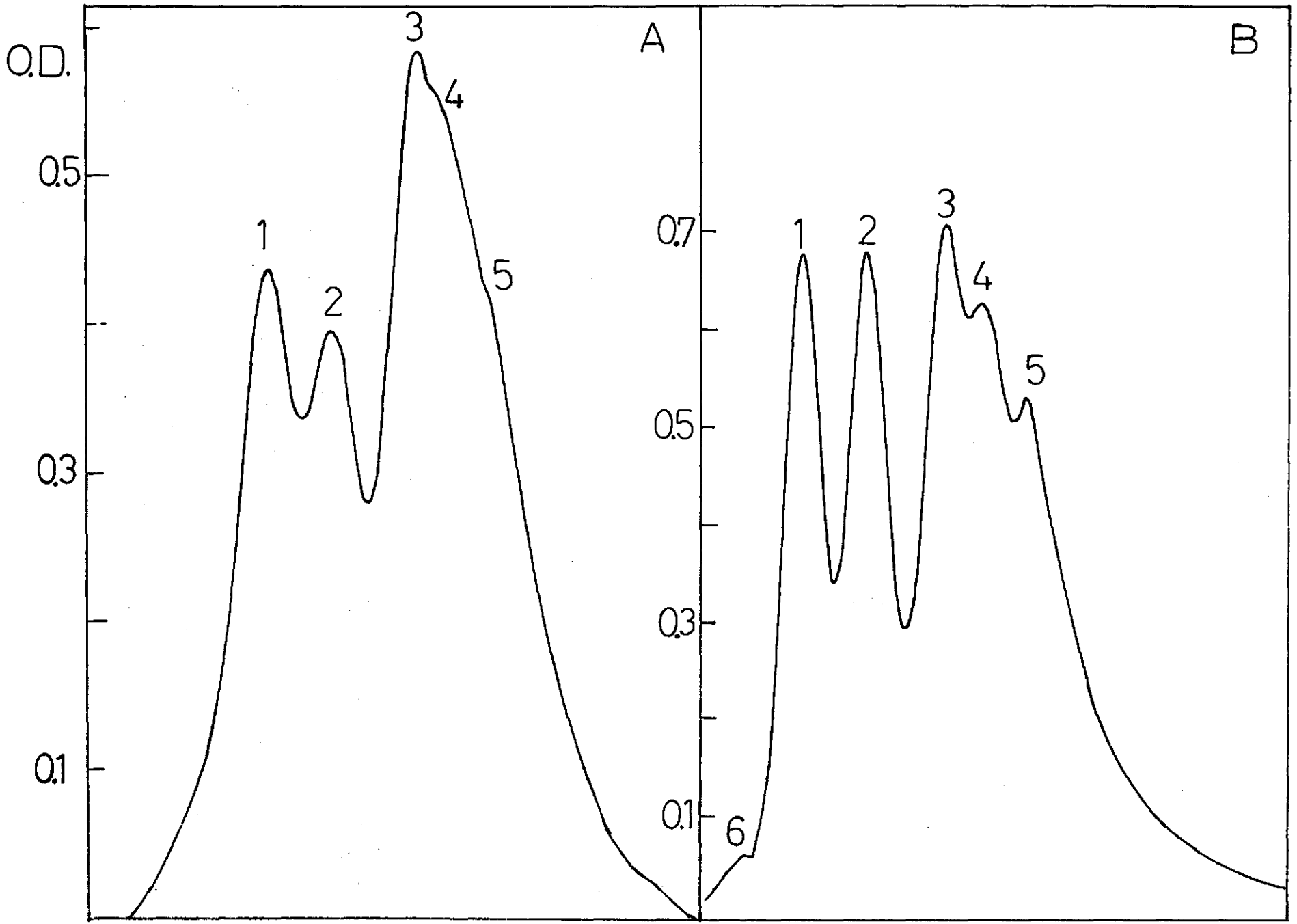


Fig. 11. Densitometer patterns of Cu^{2+}-modified enzyme. After preparation the Cu^{2+}-enzyme was dialysed for 2 hrs against 30 mM sodium phosphate (pH 7.2) with 0.3 mM EDTA (A) and overnight (B). From stock dilutions of 0.5 mg/ml 40 μgs were taken for the electrophoresis. DCIP-activity of sample A and B 2600 and 2200, respectively. To the stock solution of (B) 10^{-3} M NAD^{+} was added before electrophoresis and after 5-10 min incubation a sample was taken (C).

Fig.11

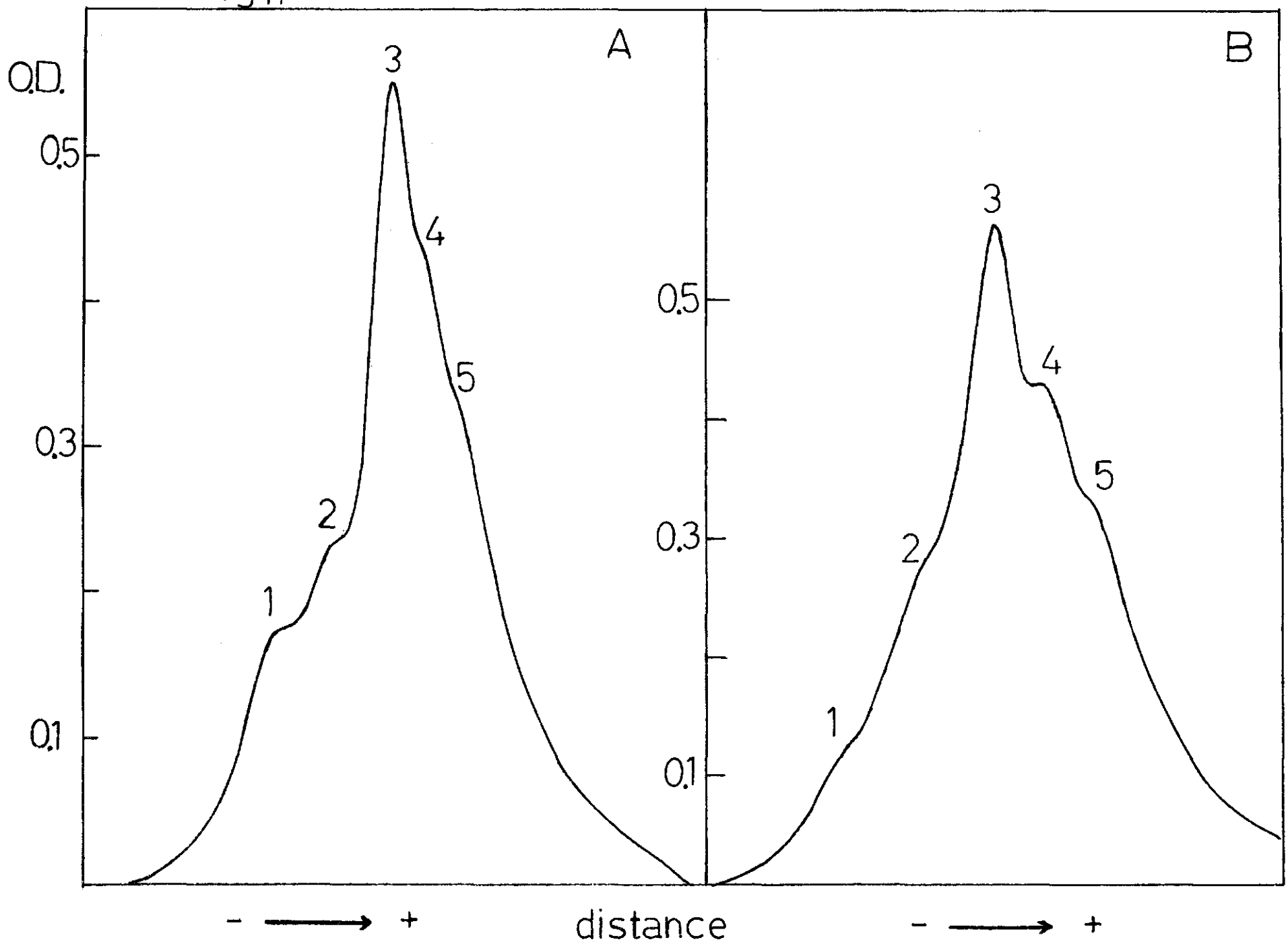


Fig. 12. Densitometer pattern of the frozen holoenzyme. Enzyme (0.3 mg/ml, $lipS_2$-activity 30; DCIP-activity 200) was frozen overnight. A sample was taken (50 µg) for the electrophoresis after which the activities were determined ($lipS_2$-activity 11; DCIP-activity 1100).

Fig.12

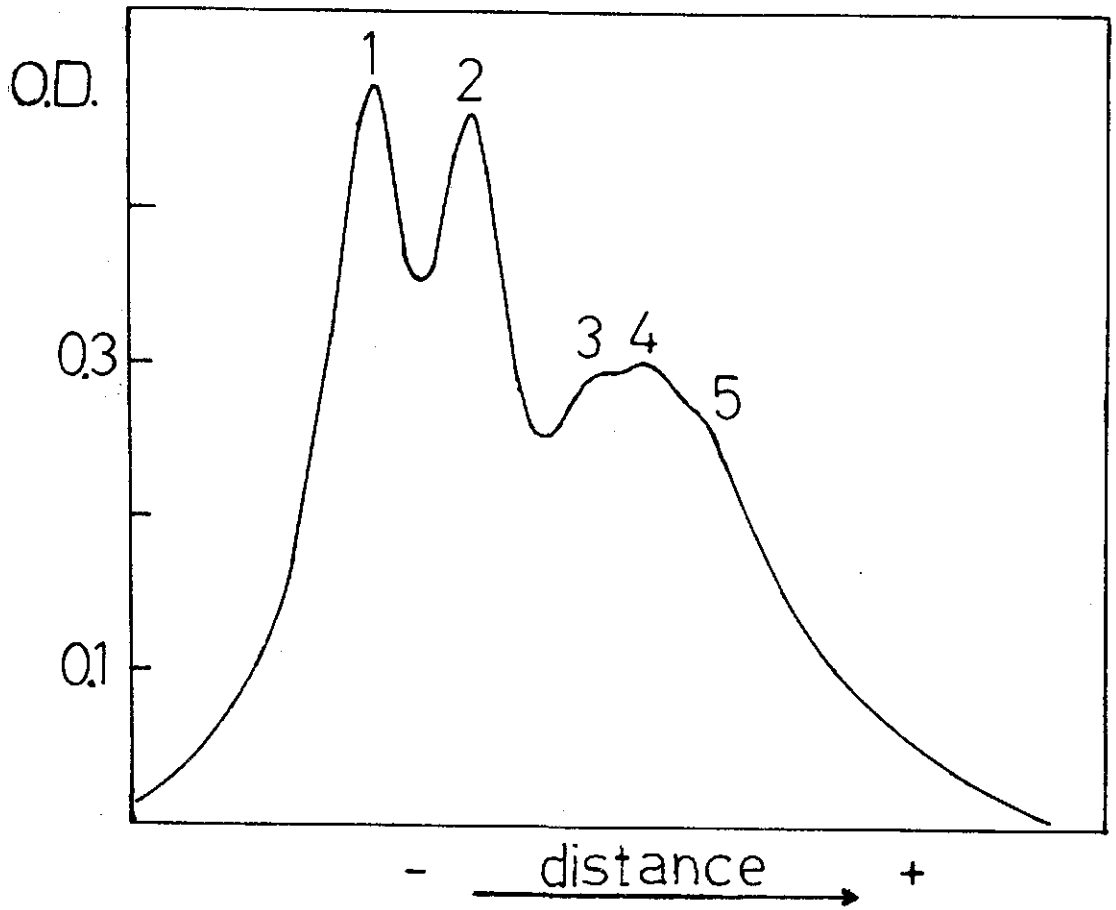


Fig. 13. Densitometer patterns of the electrophoresis of apoenzyme. The apoenzyme (1 mg/ml stock solution; DCIP-activity 200) was immediately use (A) or kept at 20° during 4 hrs (C) in an amount of 40 g. Moreover, apoenzyme was incubated with FMN ($5x10^{-4}$ M) during 2 hrs at 20° before electrophoresis (B).

Fig 13

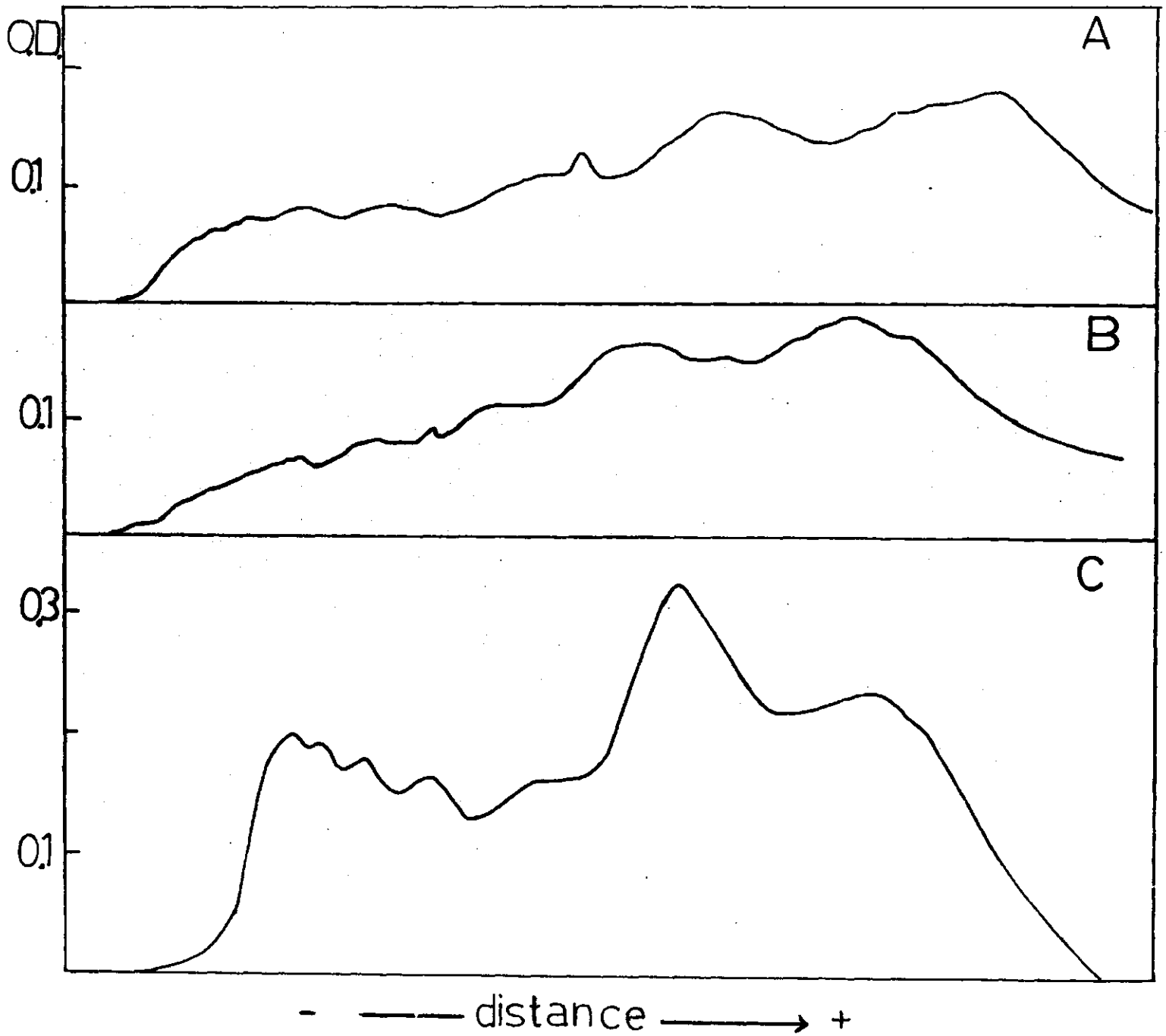


Fig. 14. Densitometer patterns of recombined enzyme. Apoenzyme (1 mg/ml) was incubated with FAD (1.5×10^{-4} M) during 2 hrs at 0° (upper part) and a 20° after which samples were taken for the electrophoresis. The DCIP--activities were 3300 and 1250, respectively.

Fig 14

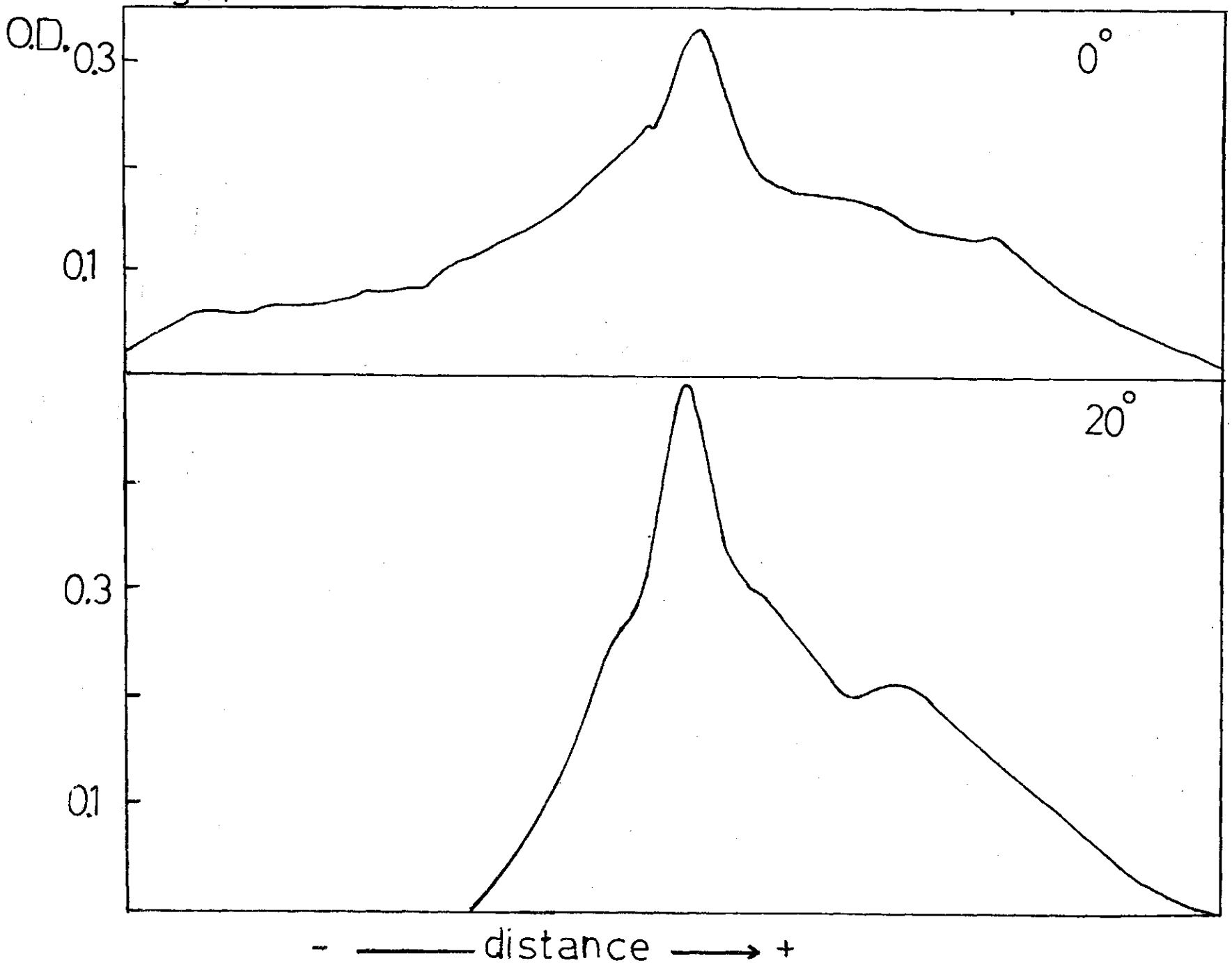
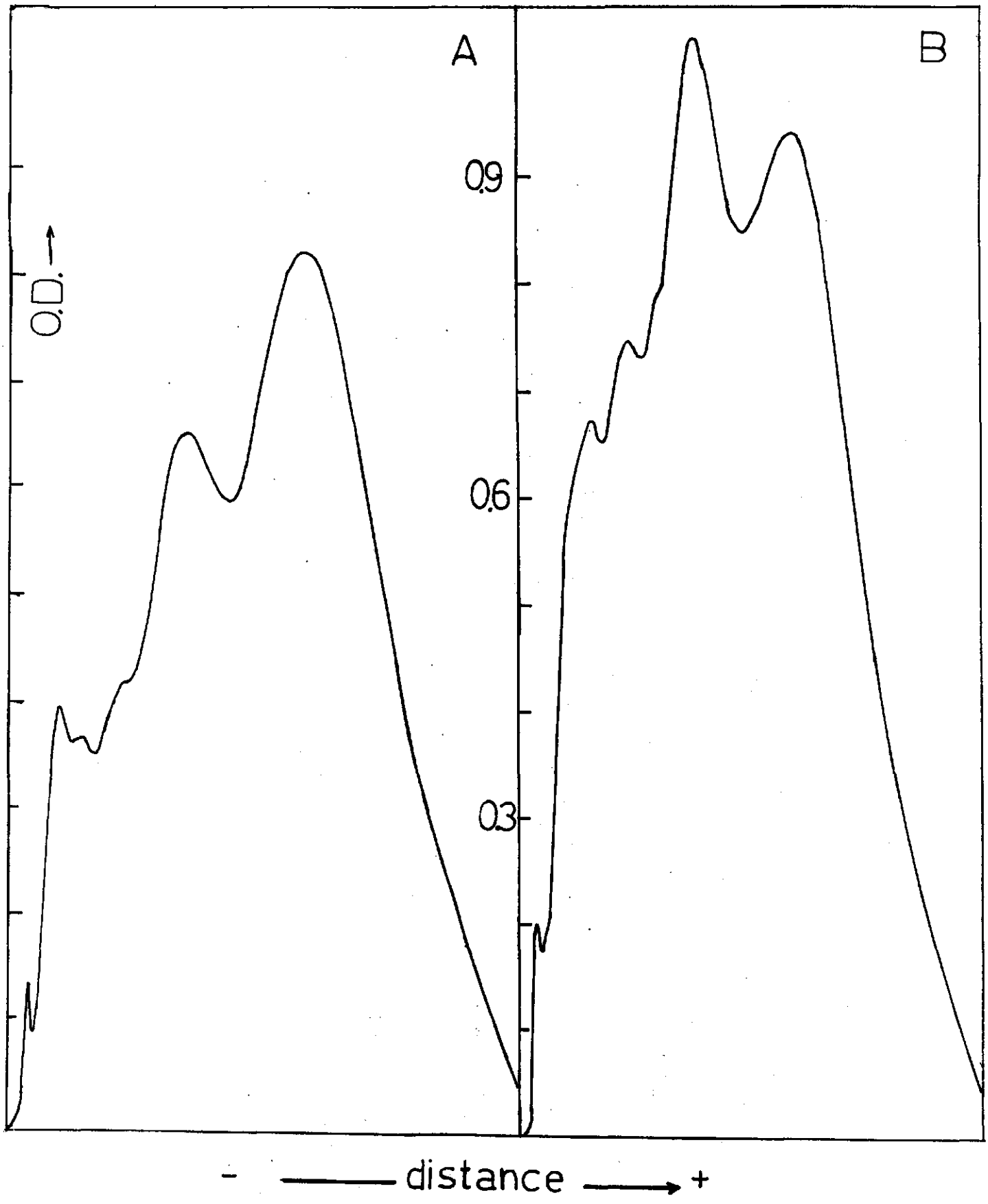


Fig. 15. Densitometer patterns of apoenzyme (B) and NADH-reduced holoenzyme (A) in 8 M urea. The gels were also made with 8 M urea and consisted of 4 % (0.2 ml) -7 % (0.4 ml) and 10 % (1 ml) gels. Time of electrophoresis was prolonged for at least one hour.

Fig.15



the apoenzyme prepared in guanidine HCl as shown by BRADY and BEYCHOK (1969).

The apoenzyme is easily disrupted in urea, the results indicate six distinct bands. These bands have also been observed with the holoenzyme held anaerobically in 8 M urea after addition of NADH (Fig. 15A, B).

8.5. DISCUSSION

It is necessary to realise in all discussion about the Cu^{2+}-modified enzyme that this enzyme represents an inhomogeneous population of molecules since the number of oxidised -SH groups will slightly vary among individual enzyme molecules. Both native lipoamide dehydrogenase and Cu^{2+}-modified enzyme are stimulated by NAD^+ in their respective reactions, NADH $\longrightarrow$ $lipS_2$ and NADH $\longrightarrow$ DCIP. Monomer conformations are not stimulated. So it is very attractive to assume that this second NAD^+ binding site is created in the dimeric enzyme.

Though the apoenzyme of the Cu^{2+}-treated enzyme denatures easier, the recombination process is essentially not different from that of the native enzyme. The behaviour of this system justifies the assumption that Cu^{2+}--modified apoenzyme is a monomer as well. The recombined Cu^{2+}-enzyme is initially more active with DCIP (1.5-2 times) than the original enzyme. The argument of the accelerated return of the non-linearity in the assay at higher incubation temperatures is also in favor of a dimerisation process.

The oxidised holoenzyme is stable, even in 8 M urea according to MASSEY et al. (1962) though not after freezing. In extreme dilution an inactivation can also be observed on ice but less at room temperature, which might be due to dissociation as in this form the enzyme is more susceptible for urea. Though no data are available about helical properties of the Cu^{2+}--enzyme, the tertiary structure is more easily disrupted by urea. The concentration of urea at which the catalytic activity is attacked varies between 4 and 5 M urea, depending on the individual enzyme preparation. Temperature--dependent conformational changes are obvious since this urea-sensitivity is promoted on ice. The structure of the Cu^{2+}-modified enzyme is very similar to the native holoenzyme on the basis of the criteria used. Most of the specific characteristics are conserved, e.g. the DCIP-activity is stimulated by freezing diluted Cu^{2+}-modified enzyme and the original structure is protected by $(NH_4)_2SO_4$. Moreover, the frozen Cu^{2+}-enzyme is as sensitive as the frozen native holoenzyme with respect to low concentrations urea. The Arrhenius plot suggests temperature-dependent conformations of the Cu^{2+}-enzyme.

The frozen enzyme shares some features with the Cu^{2+}- modified enzyme viz. a highly stimulated DCIP-activity, the correlated blue shift of the 545 nm flavin band and the easy overreduction by NADH.

I must disappoint those who expect explanations for the phenonological observations done with the freezing experiments. The water structure and the influence of ions on it are extensively surveyed by KAVANAU (1964) and still so badly understood that this recently led HOLTZER and EMERSON (1969) to the statement that "arguments based upon the concept of water structure are sufficiently ambiguous so that the change in a given physical property that accompanies dissolution of a solute in water can always be "explained" whether that change is positive or negative". Since we are dealing in this system with an even greater complexity as the water also contains large protein molecules and is frozen, the hope for a real explanation is reduced extensively. The results of DROST-HANSEN (1967) who studied water-ice interfaces and preferential incorporation of cations and anions in the ice lattice are of in interest, i.e. for NH_4Cl(10^{-3}-10^{7} M) solutions the ice becomes positively charged while large freezing potentials are observed between ice and remaining solution but for KCl and NaCl (10^{-3}-10^{-5} M) the reverse occurs. The protein is partially concentrated during the freezing process and is incorporated in the ice lattice in a relatively late stage of freezing. Moreover, in the remaining solution many things as ion composition and ionic strength as well as the pH may have changed. Next to that nothing is known about the behaviour of the enzyme in the ice-water interface as a consequence of the freezing potentials.

The protective influence of the different cations is clearly demonstrated ($Li^+ > Cs^+ > NH_4^+ > K^+ > Rb^+ > Na^+$. I have no explanation for the ratios Li^+/Na^+ and K^+/Na^+ which are necessary to give protection.

Studying the catalytic properties of the protein after freezing makes clear that the freezing process has short-time effects in a stimulation of the DCIP-activity and a partial diminishing of the lipoate activity. In addition there is a long-time effect in a partial denaturation of the protein as the lipoate activity is badly regained after elevating the temperature even in the presence of excess of flavin. The second order decline of the DCIP-activity on ice and the approch to a first order reaction at elevated temperatures is in agreement with a model in which a dimerisation occurs which is rate-limiting at low temperatures followed by a dimer intermediate with a lower DCIP- activity.

Polyacrylamide disc-electrophoresis showed that the influence of freezing was mainly limited to the three faster migrating bands. Moreover, the flavin fluorescence became less intensive in these bands indicating that there is a difference in FAD dissociation pattern of the different components upon freezing. Though further investigations are required it is attractive to assume that the two bands derived from α-KGDC which or not or less changed, represent the lipoate level (20-30 %) which is initially maintained during freezing.

Table I

Influence of phosphate buffer concentration and NAD^+ on the linearity in the Cu^{2+}-enzyme assay. Cu^{2+}-enzyme was diluted in 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA to 0.4 mg/ml. The cuvettes contained different amounts of sodium phosphate and no NAD^+ or 40 μM NAD^+. The activities were determined at 10°.

Sodium phosphate (M)	NAD^+		$-NAD^+$	
	DCIP-activity		DCIP-activity	
	min.	max	min.	max
0.024	2500	2500	2700	2900
0.048	3000	3000	2100	2850
0.072	2900	2900	2300	2900
0.0.6	3400	3600	1700	2700
0.120	3000	3400	1500	2350
0.145	2600	3250	1700	2350
0.168	2500	3150	1450	1900
0.192	2350	2350	1000	1700

Table II

K_{ass} values for flavin binding to the Cu^{2+}-modified apoenzyme. Apoenzyme, 3.6 μM was recombined at the temperatures indicated with different amounts of FAD. Conditions: 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA. The fluorescence polarisation values used were those obtained in the plateau region (cf. Fig. 2).

Apoenzyme (μM)	FAD/apoenzyme	K_{ass} ($\times 10^5$) $l.mole^{-1}$	Temperature °C
3.6	1:1	1.7-2.0	5
3.6	2:1	0.6	5
3.6	1:2	1.5-2.0	5
3.6	1:1	1.8-2.2	10
3.6	1:2	2.0-3.0	10
3.6	1:1	2.0-2.3	15
3.6	2:1	1.0	15
3.6	1:1	2.0-2.2	23

Table III

Freezing of diluted holoenzyme under different conditions of pH and ionic strength. A. Enzyme (A280/A455=5.9; $lipS_2$-activity 29; DCIP--activity 250) was dialysed against 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA. The enzyme was frozen during 20 hrs at -14° in 0.46 mg/ml protein concentration in a volume of 0.5 ml. The DCIP- and the lipoate activities were determined immediately after thawing on ice. B. Enzyme (A280/A455=5.4; $lipS_2$-activity 32) was frozen during 4 days in a 0.27 mg/ml protein concentration after dialysis against 30 mM sodium phosphate buffer.

A	0.03 M sodium phosphate		
pH	DCIP-act.	$lipS_2$-act.	$lipS_2$-act. restored (1 hr 20°)
7.6	2700	10.8	21.4
7.2	2600	9.3	21.0
6.9	2750	9.6	26.0
6.6	3000	9.0	21.8
6.3	2750	10.8	22.8
5.8	3000	10.6	25.2

	A		B	
PO_4-concentration (M)	DCIP-act.	$lipS_2$-act	DCIP-act.	$lipS_2$-act.
0.012	2300	11.3		
0.015			550	30
0.020	2650	10.4	250	34
0.030	2700	11.5		
0.040			300	30
0.045	2450	9.8		
0.065	2050	9.3	350	27
0.090			700	25
0.095	2450	9.3		
0.110			1150	25
0.125	2300	9.2	2050	16
0.150	2800	8.9		
0.185	3000	9.6		

Table IV

The influence of NH_4^+-ions and SO_4^{2-}-ions on the freezing effect of diluted lipoamide dehydrogenase. Enzyme (A280/A455=5.9); $lipS_2$-act. 29; DCIP-act. 250) was dialysed against 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA. The enzyme (0.46 mg/ml) was frozen during 20 hrs at -14° with 0.1 M of the salts indicated. The DCIP- and $lipS_2$-activities were determined immediately after thawing on ice. The restoration of the lipoate activity was determined after standing for 1 hr at 20°.

Salt added (0.1 M)	Spec. act. DCIP	Spec. act. $lipS_2$	1 hr 20° $lipS_2$
NH_4Cl	750	12.9	21
$(NH_4)_2SO_4$	350	27.6	27
$(NH_4)\ HCO_3$	150	28.5	28
$(NH_4)_3$ molybdate	300	27.5	25.5
Na_2SO_4	2400	12.0	25.2
K_2SO_4	2300	12.4	25.1
Control	2600	9.3	21.0

Table V

Influence of alkali-ions on the freezing effects in lipoamide dehydrogenase. A. The enzyme (A280/A455=4.9; $lipS_2$-act. 42; DCIP-act. 300) was dialysed against 30 mM sodium phosphate buffer (pH 7.2) containing 0.3 mM EDTA. The enzyme (0.12 mg/ml) was frozen during 3 days with 0.1 M of the salts indicated and activities determined immediately after thawing. B. The enzyme (0.18 mg/ml) was frozen during 2 1/2 days with 0.1 M and 0.05 M of the salts indicated. Activities determined immediately after thawing and after standing for 1 1/2 hr at 25°.

A

Salt (0.1 M)	pH	DCIP-act.	$lipS_2$-act.
LiCl	7.5	1250	27.3
CsCl	7.2	1350	24.1
NH_4Cl	7.2	1650	19.5
Control	7.2	5350	11.5
KCl	7.2	4800	10.3
$RbNO_3$	7.2	3700	5.5
NaCl	7.2	1450	3.7

B

Salt	0.1 M				0.05 M			
	0 hr		1 1/2 hr 25°		0 hr		1 1/2 hr 25°	
	DCIP-act.	$lipS_2$-act.	DCIP-act.	$lipS_2$-act.	DCIP-act.	$lipS_2$-act.	DCIP-act.	$lipS_2$-act.
LiCl	440	44.0	260	37.2	1410	31.1	225	38.5
CsCl	380	34.4	370	36.6	2300	31.7	490	29.4
NH_4Cl	330	27.5	370	34.1	1610	31.9	370	41.0
Control	3025	23.4			3025	23.4		
KCl	2170	22.4	330	36.0	3590	18.7	620	39.1
NaCl	1240	6.9	370	24.2	1480	13.2	540	24.2
$MgCl_2$	1210	12.7	270	19.2	2850	14.9	330	35.2

Table VI

The influence of Li^+/Na^+ and K^+/Na^+ ratios on the protection of the lipoate activity of lipoamide dehydrogenase during freezing 0,18 mg enzyme (f=4.9 sp.act. DCIP 300) was frozen after dialysis against 30 mM sodium phosphate buffer pH 7.2 containing 0,3 mM EDTA and subsequent addition of different concentrations of potassium phosphate and lithium phosphate. The Na^+ ion concentration is 5.1×10^{-2} in all cases.

	2 days freezing		7 days freezing	
	DCIP-act.	$lipS_2$-act.	DCIP-act.	$lipS_2$-act.
Li^+ concentration (M^{-1})				
0	3100	13.2	-	-
2×10^{-3}	3000	12.9	2950	8.8
4×10^{-3}	2750	12.7	3500	9.4
5×10^{-3}	3200	13.8	3700	9.6
6×10^{-3}	3050	17.6	3530	11.6
7×10^{-3}	2500	20.9	3850	11.0
8×10^{-3}	450	35.2	3450	11.0
9×10^{-3}	300	39.3	3150	13.2
10×10^{-3}	250	39.7	3150	19.3(?)
40×10^{-3}	200	37.1	2100	15.7
K^+ concentration (M^{-1})				
0	3500	25.0		
0.3×10^{-3}	1900	23.4		
1.5×10^{-3}	2600	25.3		
3.0×10^{-3}	4200	22.0		
4.5×10^{-3}	4100	27.5		
6.0×10^{-3}	3000	35.8		
7.5×10^{-3}	400	44.8		
9.0×10^{-3}	300	48.1		
15.0×10^{-3}	300	42.6		
30.0×10^{-3}	350	48.4		

SUMMARY

Gel-filtration, ultracentrifugation and sucrose density gradient centrifugation demonstrated differences in physico-chemical properties of holoenzyme and apoenzyme of lipoamide dehydrogenase. The native apoenzyme has a mol.wt. of approx. 52,000 which is half that of the native holoenzyme. The DCIP-active enzyme formed directly after reconstitution of apoenzyme with FAD, is still a monomer. Light-scattering experiments with holoenzyme at low protein concentrations (< 0.1 mg/ml) indicated a dissociation of the enzyme related to a decrease in lipoate activity. These results were not consistent with previously proposed models for the enzyme structure. The idea of two interchain disulfide bridges, which moreover take part in the catalysis, is replaced by a real protein association-dissociation model in which the two catalytically important disulfide bridges are intrachain ones. The dimerisation constants derived from light-scattering data and from the return of the lipoate activity in recombination studies at relatively high apoenzyme concentrations agree with each other ($3\text{-}6 \times 10^{6}$ l mole^{-1}).

The influence of urea on the catalytic activities of different conformations of the enzyme has been studied. Recombined DCIP-active enzyme, and both the frozen holoenzyme and frozen Cu^{2+}-modified are very urea-sensitive while the Cu^{2+}-modified enzyme is sensitive at concentrations > 4 M. The holoenzyme is very stable in 8 M urea except when the protein concentration is low (0.1 mg/ml). The apoenzyme is thermo-labile and also urea-sensitive; the stability is protein concentration-dependent. The apoenzyme population is inhomogeneous as indicated electrophoretically.

The FAD binding to the apoenzyme induces a series of protein conformational changes, before dimerisation occurs.

The K_{ass} (FAD) value for the protein ($2\text{-}3 \times 10^{5}$ l mole^{-1}) is considerably lower than in the holoenzyme. The dimerisation is dependent on pH (7.2-7.5) and ionic strength (0.2 M) and is promoted by elevated temperatures. The return of the lipoate activity is due to dimerisation though even complete restoration of this activity does not prove that the native holoenzyme structure is regained. The formed dimer still has initially a high DCIP-activity and it is partially sensitive to urea- and FMN-treatment over a period of at least 24 hrs all in contrast to the holoenzyme.

Some of the flavin derivatives studied are able to restore the monomer-activity with DCIP viz. F8-BrAD (K_{ass} $1.5x10^4$ l $mole^{-1}$), 3-methyl-FAD (K_{ass} $1.1x10^5$ l $mole^{-1}$) and 3-carboxymethylFAD (K_{ass} $3x10^4$ l $mole^{-1}$). Only 3-methylFAD partially restores the lipoate activity. The flavin binding is due to multiple binding forces as shown by the interference of related compounds with the binding site. FMN which is a competitive inhibitor of the FAD binding induces, like FAD, protein conformational changes as the K_i value increases with time. FMN-derivatives generally show a noncompetitive inhibition pattern with respect to the DCIP-activity. Parts other than the isoalloxazine moiety of the flavin molecule viz. the adenine part and pyrophosphate, are also involved in the FAD binding: NADH, NAD^+, ADP, adenine, ATP and pyrophosphate all inhibit the flavin binding. All nuclectides which inhibit the flavin binding affect the dimerisation except NAD^+ which promotes the return of the lipoate activity.

The "ping pong bi bi" mechanism previously proposed to be the reaction mechanism of lipoamide dehydrogenase is not the correct model. The involvement of a ternary complex has been proposed instead. As deviations from linearity are observed in the L-B plots with $lip(SH)_2NH_2$ and NAD^+ as variable substrates depending on the level of the second substrate a prefered order mechanism is postulated according to FERDINAND (1966). NAD^+ is the substrate which is prefered as first one (K_D $NAD^+ \approx$ 110 µM at 25°). A second binding site with a higher affinity for NAD^+ than the catalytical important one ($K_D \approx$ 20-30 µM) prevents 4-electron reduction of the flavin by NADH. It is likely that this site is the NADH binding site in the transhydrogenase reaction. The product inhibition by $lipS_2(NH_2)$ with respect to $lip(SH)_2NH_2$ is complicated and the type of inhibition is dependent on the level of both substrates. Under conditions where the mechanism has become random, the inhibition of $lipS_2(NH_2)$ is competitive. At lower NAD^+ levels this inhibition is noncompetitive with respect to $lip(SH)_2NH_2$.

Kinetical evidence is presented in the case of the native holoenzyme and the Cu^{2+}-modified enzyme that the oxidised form exists in different temperature-dependent conformations. Analysis of the sets of rate constants revealed that this conformational change is clearly reflected in the NAD^+/NADH binding properties.

Antisera against four different structures of lipoamide dehydrogenase have been succesfully prepared, viz. against the native holoenzyme and the Cu^{2+}-modified enzyme (both dimers) and against the apoenzyme and the reconstituted, DCIP-active enzyme (both monomers). The inhibition of the

enzyme activity proved to be the most sensitive method to detect a reaction between antibodies and the different antigenic structures. The homologous combinations are the most specifically inhibited but always less than 100 %. The antiserum against the apoenzyme does almost not interfere with the activities of the different enzyme conformations. The antisera against the dimeric enzyme forms are closer to each other than to the one formed against the DCIP-active monomer. Inhibition patterns of the homologous Cu^{2+}-enzyme/antiserum combination are noncompetitive.

The Cu^{2+}-modified enzyme has many structural properties in common with the holoenzyme. NAD^+ in low concentrations activates the DCIP-activity and promotes the linearity in the assay which is generally non-linear. The recombination phenomena are very similar and the K_{ass} value of FAD almost identical ($2\text{-}5 \times 10^5$ l mole^{-1}) to the normal case. The non-linearity of the assay is probably a property of the dimer structure.

Freezing of diluted holoenzyme promotes the DCIP-activity while the lipoate activity drops. These changes are protein concentration-dependent. Li^+, Cs^+ and NH_4^+-ions are protecting in 0.1 M concentration against these changes but Na^+, K^+ and Rb^+ do not. However, a combination of K^+ and Na^+ (Na^+/K^+ ratio 7) is as protective as the Na^+/Li^+combination of the same ratio. When the frozen enzyme is thawed and the decrease of the DCIP-activity and the return of the lipoate activity are followed, the structural changes induced by freezing pass at least through one intermediate as the decrease of the DCIP-activity follows a second-order reaction rate on ice. The order of the reaction decreases and approaches 1 at elevated temperatures. The freezing process is very complicated indeed. Results with polyacrylamide electrophoresis strongly favour the existence of two different lipoamide dehydrogenases isolated in a mixture from the pyruvate dehydrogenase complex and the α-ketoglutarate dehydrogenase complex. Freezing of the enzyme changes mainly the electrophoretic patterns of lipoamide dehydrogenase obtained from the pyruvate dehydrogenase complex.

SAMENVATTING

Gel-filtratie, ultracentrifugeren en centrifugeren op sucrose gradienten toonden verschillen in fysisch-chemische eigenschappen aan tussen het holoenzym en het apoenzym van lipoïnezuur dehydrogenase. Het natieve apoenzym bezit een moleculair gewicht van 52,000, de helft van dat van het holoenzym. Het DCIP-actieve enzym dat ontstaat direct na recombinatie van het apoenzym met het FAD, is nog steeds een monomeer. Lichtverstrooiingsexperimenten met het holoenzym uitgevoerd bij lage eiwitconcentraties (< 0.1 mg/ml) tonen aan dat het enzym dissocieert. Dit gaat gekoppeld aan een achteruitgang van de lipoïnezuur activiteit. Deze resultaten waren niet in overeenstemming met voorheen voorgestelde modellen betreffende de enzym structuur. Het idee dat de twee disulfide bruggen welke tevens bij de catalytische reactie betrokken zijn, de twee polypeptide ketens onderling verbinden is vervangen door een model dat berust op een werkelijk associatie-dissociatie evenwicht van het eiwit terwijl de twee disulfide bruggen ieder in een keten voorkomen. De dimerisatie constante berekend uit de lichtverstrooiingsgegevens en die welke uit de terugkeer van de lipoïnezuur activiteit bij recombinatie experimenten met hoge concentraties apoenzym is berekend, komen goed met elkaar overeen ($3\text{-}6\times10^{6}$ l mole^{-1}).

De invloed van ureum op de catalytische activiteit van verschillende enzym conformaties is eveneens nagegaan. De activiteiten van het gerecombineerde enzym met zijn hoge DCIP-activitei en van de bevroren conformaties van het verdunde holoenzym en het Cu^{2+}-enzym zijn zeer ureum-gevoelig. Het niet bevroren Cu^{2+}-enzym is gevoelig by ureum concentraties > 4 M, terwijl het holoenzym zijn volledige lipoïnezuur activiteit behoudt als de eiwitconcentratie niet te laag is (> 0.15 mg/ml). Het apoenzym is thermolabiel en eveneens gevoelig voor ureum; de stabiliteit is afhankelijk van de eiwitconcentratie. Het apoenzym bezit een inhomogene molecuul populatie zoals met electrophoresis is aangetoond.

De binding van FAD aan het apoenzym induceert een serie conformatie veranderingen in het eiwit alvorens de dimerisatie reactie plaats vindt. De initieële associatie constante (K_{ass} FAD) bedraagt $2\text{-}3\times10^{5}$ l mole^{-1} bij recombinatie maar deze is lager dan in het holoenzym waar het FAD zelfs niet door dialyse is te verwijderen. De dimerisatie is afhankelijk van pH (7.2-7.5) en ionaire sterkte (0.2 M) en wordt bevorderd door verhoging van de temperatuur. De terugkeer van de lipoïnezuur activiteit is het gevolg van de dimerisatie hoewel zelfs een volledige terugkeer van

deze activiteit niet bewijst dat de oorspronkelijke natieve holoenzym structuur wordt teruggekregen. Het gevormde dimeer bezit aanvankelijk nog een verhoogde DCIP-activiteit. Bovendien is het volledig actieve enzym gevoelig voor ureum en voor FMN gedurende tenminste 24 uur in tegenstelling tot het natieve enzym.

Sommige derivaten van FAD zijn in staat de DCIP-activiteiten van het monomeer ten dele te herstellen zoals F8-BrAD (K_{ass} $1.5x10^4$ l $mole^{-1}$), 3-methylFAD (K_{ass} $1.1x10^5$ l $mole^{-1}$) en 3-carboxymethylFAD (K_{ass} $3x10^4$ l $mole^{-1}$). 3-MethylFAD is als enige in staat een deel van de lipoïnezuuractiviteit te herstellen. De binding van het flavine molecuul berust op multipele bindingskrachten hetgeen blijkt uit studies over de interacties tussen aan FAD verwante moleculen en de FAD bindingsplaats. FMN is een competitieve inhibitor van de FAD binding en induceert evenals FAD zelf eiwit conformatie-veranderingen wat blijkt uit het toenemen van de K_i-waarde met de tijd. Derivaten van FMN vertonen in het algemeen noncompetitieve remmingsbeelden als men de DCIP-activiteit beschouwt. Behalve het isoalloxazine gedeelte van het FAD zijn ook het adenine gedeelte en het pyrofosfaat betrokken bij de flavine binding: NADH, NAD^+, ADP, adenine, ATP en pyrofosfaat remmen allen de flavine binding. Deze nucleotiden remmen ook de dimerisatie met uitzondering van NAD^+. Deze verbinding bevordert de terugkeer van de lipoïnezuur activiteit.

Het "ping pong bi bi" mechanisme dat vroeger is voorgesteld als reactie mechanisme vòor lipoïnezuur dehydrogenase is niet correct gebleken voor dit enzym. Er is nu een mechanisme voorgesteld waarin een ternair complex optreedt. De afwijkingen van lineaire kinetische L-B patronen bij variaties van de beide substraten, $lip(SH)_2NH_2$ en NAD^+, afhankelijk van het niveau van het tweede substraat, hebben geleid tot de postulatie van een "prefered order" mechanisme volgens FERDINAND (1966). NAD^+ is het eerste substraat (K_{diss} (NAD^+) = 110 µM bij 25°). Er is nog een tweede NAD^+ bindingsplaats met een grotere affiniteit voor NAD^+ dan de catalytisch belangrijke (K_{diss} (NAD^+) = 20-30 µM) en deze, indien bezet door NAD^+, voorkomt de 4-electronen reductie van het flavine door NADH. In de transhydrogenase reactie van het enzym is het waarschijnlijk juist deze bindingsplaats die als NADH bindingsplaats fungeert. De productremming van $lipS_2(NH_2)$ ten opzichte van $lip(SH)_2NH_2$ is gecompliceerd en afhankelijk van de concentratie niveau's van beide substraten. Onder de condities waarbij het mechanisme random wordt, is de $lipS_2(NH_2)$ remming competitief. Bij verdere vermindering van het NAD^+

niveau wordt de remming noncompetief ten opzichte van $lip(SH)_2NH_2$.

Op kinetische gronden is aangetoond voor zowel het natieve holoenzym als voor het Cu^{2+}-gemodificeerde enzym dat in geoxideerde toestand verschillende temperatuur-afhankelijke eiwitconformaties bestaan. Analyse van de verschillende snelheidsconstanten leidde tot de conclusie dat deze conformatieverandering zich duidelijk aftekent in de NAD^+/NADH bindingseigenschappen.

Er zijn vier antisera bereid, nl. tegen het natieve holoenzym, het Cu^{2+}-gemodificeerde enzym (beiden bezitten een dimeer structuur), het gereconstitueerde DCIP-actieve enzym en tegen het apoenzym. De beide laatsgenoemde hebben een monomeer structuur. De remming van de enzymatische activiteit is de meest gevoelige methode om reacties tussen antibodies en de verschillende antigenen te meten. Bij de homologe combinaties treedt het hoogste remmingspercentage op maar dit is altijd minder dan 100 %. Het antiserum gevormd tegen het apoenzym veroorzaakt geen activiteitsremming van de verschillende enzymconformaties. De antisera gevormd tegen de dimeer vormen van lipoïnezuur dehydrogenase zijn onderling meer verwant dan aan het antiserum gevormd tegen het DCIP--actieve monomeer. De remming van het Cu^{2+}-enzym antiserum is noncompetitief in de homologe combinatie.

Het Cu^{2+}-gemodificeerde enzym heeft vele structurele eigenschappen gemeen met het holoenzym. NAD^+, in lage concentraties, activeert de DCIP-activiteit en leidt tot lineariteit in de activiteitsbepaling. De recombinatie kenmerken lijken sterk op die van het normale holoenzym en ook de K_{ass} (FAD) waarde is ongeveer gelijk ($2\text{-}5 \times 10^5$ l mol^{-1}). De non- - lineariteit in de DCIP-activiteitsbepaling van het Cu^{2+}-enzym is waarschijnlijk een eigenschap welke gebonden is aan de dimeer structuur.

Bevriezen van verdund holoenzym (< 1 mg/ml) bevordert de DCIP--activiteit sterk terwijl de lipoïnezuur-activiteit vermindert. Deze veranderingen zijn eiwitconcentratie afhankelijk. Li^+, Cs^+ en NH_4^+ ionen beschermen in een concentratie van 0.1 M tegen deze veranderingen dit in tegenstelling tot Na^+, K^+ en Rb^+. Echter een combinatie van K^+ en Na^+ ionen in de Na^+/K^+ verhouding 7 beschermt even goed als eenzelfde Na^+/Li^+ verhouding. De structurele veranderingen geïnduceerd door het bevriezen verlopen minstens via één meetbaar intermediair aangezien de DCIP-activiteit bij 0° afneemt volgens een tweede orden reactie, wanneer het bevroren

enzym ontdooid wordt en de afname van de DCIP-activiteit, respectievelijk toename van de lipoïnezuur activiteit wordt gevolgd in de tijd. De orde van de reactie neemt af tot een waarde naderend tot 1 bij verhoging van de temperatuur. Het bevriezingseffect is zeer gecompliceerd, temeer daar experimenten met polyacrylamide gel-electrophorese aanwijzingen hebben gegeven voor het bestaan van twee verschillende lipoïnezuur dehydrogenases. Deze beide enzymen worden als mengsel geïsoleerd maar zijn afkomstig uit twee verschillende multi-enzym complexen, het pyruvaat dehydrogenase complex en het α-ketoglutaarzuur dehydrogenase complex. Bevriezen van het enzym verandert voornamelijk de electrophoretische patronen van het lipoïnezuur dehydrogenase uit het pyruvaat dehydrogenase complex.

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