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GLUTAMATE DEHYDROGENASE - A DISSOCIABLE ENZYME

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LIST OF ABBREVIATIONS

Glutamate dehydrogenase is the trivial name used throughout this thesis for L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3, according to the Report of the Commission for Enzymes of the International Union of Biochemistry.

ADP	adenosine 5′–diphosphate
ATP	adenosine 5′–triphosphate
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetate
EQN	equation
GDH	glutamate dehydrogenase
GDP	guanosine 5′-triphosphate
glu	sodium glutamate
GTP	guanosine 5′–triphosphate
mol.wt.	molecular weight
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
$NAD(P)^+$	oxidized nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
2–OG	sodium 2-oxoglutarate
Tris	Tris (hydroxymethyl) aminomethane

1. INTRODUCTION

The quaternary structure of proteins has gained considerable importance since the finding that allosteric properties are largely confined to proteins containing subunits (MONOD et al., 1965; KOSHLAND et al., 1966; KIRTLEY and KOSHLAND, 1967).

Glutamate dehydrogenase is one of the most intensively studied allosteric proteins. It possesses the highest molecular weight in the group of the pyridine nucleotide dehydrogenases (SUND, 1968a). A value of 2×10^6 was reported for the associated native enzyme isolated from beef liver (SUND, 1964a, 1968b). However, higher molecular weights are possible as suggested by the work of EISENBERG and TOMKINS (1968), and that of DESSEN and PANTALONI (1969). The enzyme dissociates upon dilution, and in the limit of $c \rightarrow 0$, the molecular weight of the enzymically active molecule is 316000 (EISENBERG and TOMKINS, 1968). In the presence of urea or long-chain alkyl sulphate, or at extreme pH values, dissociation into the constituent polypeptide chains occurs, with loss of enzymic activity. The polypeptide chains have a molecular weight of 50000–52000; end-group analysis, as well as amino acid analysis indicate that they are identical (JIRGENSONS, 1961; FISHER et al., 1962b; MARLER and TANFORD, 1964; APPELLA and TOMKINS, 1966).

Ever since the physical measurements of OLSON and ANFINSEN (1952) on the enzyme purified and crystallised by them from bovine liver, 1×10^6 had been assumed to be the molecular weight of the enzyme at concentrations above 3–4 mg/ml. The associated particle was assumed to be in a fully reversible association-dissociation equilibrium with its subunits of molecular weights 0.5×10^6 and 0.25×10^6 (see also FRIEDEN, 1958). More recently, FRIEDEN and COLMAN (1967) reported a molecular weight of 400000 for the monomer[1], which they considered to be composed of 8 identical chains of 50000. On the basis of diffusion, sedimentation and viscosity data, SUND (1964a, b; 1968a) proposed a scheme according to which the fully associated prolate particle of 2×10^6 dissociates by transverse cleavages to 1×10^6, 0.5×10^6 and 0.25×10^6 molecular weight subunits.

Spectrophotometric studies of Fisher and coworkers (FISHER et al., 1962b, c; CROSS and FISHER, 1965; CROSS and FISHER, 1966) have shown that the peptide chains of the glutamate dehydrogenase molecule are held together by tyrosyl carboxylate hydrogen bonds, and that the rupture of these bonds in any manner will cause dissociation of the protein molecule into peptide chains.

The association-disociation equilibrium can be displaced into one direction or another by a great variety of compounds (among which are nucleotides, hormones, inorganic ions and even the coenzymes of the reaction) which also

[1] In the glutamate dehydrogenase literature, the term 'monomer' refers to the smallest subunit which possesses catalytic activity.

act as inhibitors or activators of the catalytic reaction (FRIEDEN, 1959, 1963d; TOMKINS and YIELDING, 1961; YIELDING and TOMKINS, 1962; WOLFF, 1962). Contrarily to earlier views (FRIEDEN, 1959; TOMKINS et al., 1963), according to which only the polymeric form was considered to be enzymically active in the glutamate dehydrogenase reaction, there is no direct connection between particle size and enzyme activity. By reacting with functional groups of the enzyme and/or altering its conformation, the allosteric effectors influence the association-dissociation equilibrium as well as the catalytic reaction. Both phenomena result from the same cause (FISHER et al., 1962a; FRIEDEN, 1963d; SUND, 1964a; TOMKINS et al., 1965). The existence of multiple active forms of glutamate dehydrogenase has been demonstrated by immunological techniques (TOMKINS et al., 1963). These authors postulated earlier (TOMKINS et al., 1961) that the monomeric form of glutamate dehydrogenase had alanine dehydrogenase activity. Factors which caused inhibition or activation of the glutamate dehydrogenase reaction caused respectively, activation or inhibition of the alanine dehydrogenase activity with alanine or pyruvate plus NH_4^+ as substrates (see also ANDERSON and REYNOLDS, 1966).

The displacement of the association-dissociation equilibrium by various effectors has been studied also under conditions of decreased stability of the enzyme, such as low protein concentration and low ionic strength. GRISOLIA (1964) suggested that inactivation of glutamate dehydrogenase in the presence of NADPH could be used as a sensitive and simple method for studying conformational changes. INAGAKI (1959) was among the first investigators who pointed out that NADH accelerated the urea-induced inactivation of the enzyme. The NADPH-inactivation was studied among others by GRISOLIA and coworkers (1962), by FRIEDEN (1963a), and by DI PRISCO and STRECKER (1966).

As GRISOLIA et al. (1962) pointed out, the NADPH-inactivation of the enzyme changed markedly with increasing salt concentration; at low ionic strengths, increasing NADPH concentrations appeared to be less effective. Preparations preincubated with NADPH were less stable to denaturation by urea and to digestion by trypsin. The authors stated that the inactivating effect of NADPH was not due to aggregational changes. The chicken-liver enzyme, which shows no change in aggregation with concentration (FRIEDEN, 1962b) is also less stable in the presence of NADPH. FRIEDEN (1963a) pointed out that the major product of the NADPH-induced inactivation of the enzyme in dilute Tris buffer (pH 8) at 25° was material of low molecular weight, which aggregated and finally became insoluble. In the presence of NADPH, activity was lost long before any turbidity was observed in the solution.

The work of BITENSKY et al., (1965) showed that at low ionic strength, glutamate dehydrogenase undergoes a denaturation reaction resulting in a loss of both glutamate and alanine dehydrogenase activities, a decrease in protein fluorescence, and the appearance of about 114 thiol groups per molecule of 10^6 molecular weight. DI PRISCO and STRECKER (1966) studied the effect of ionic compounds on the stability of glutamate dehydrogenase at pH 8 and higher. These authors showed that alkaline phosphate and ammonium salts

were among the best protective agents against inactivation at alkaline pH. A scheme of inactivation was proposed according to which a stable active form of the enzyme is converted into another active but unstable form. This conversion is promoted by increasing pH and prevented by ADP.

HENDERSON and HENDERSON (1969) found that D_2O protected the enzyme against denaturation caused by dilution at low ionic strengths.

It is known that glutamate dehydrogenases from animal sources, which utilize both NAD and NADP as coenzymes, are strongly affected by purine nucleotides (FRIEDEN, 1965). In contrast, glutamate dehydrogenases from non-animal sources (*Fusarium oxysporum*, *Neurospora crassa*, etc.) are specific for either NAD or NADP (SANWAL, 1961; SANWAL and LATA, 1962) and are generally not influenced by purine nucleotides. FRIEDEN (1965) suggested that the role of purine nucleotides in the glutamate dehydrogenase reaction is to control the rate of utilization of one coenzyme relative to the other.

In contrast to the isolated enzyme, glutamate dehydrogenase from rat liver reacts only with NADP within the mitochondrion (TAGER and PAPA, 1965). KLINGENBERG and SLENCZKA (1959) and later BORST (1962) suggested that the NADP specificity may explain why the glutamate dehydrogenase pathway of glutamate oxidation is suppressed by the transamination pathway in rat-liver mitochondria; the rate-limiting reaction in the oxidation of glutamate by glutamate dehydrognease would be the rate of reoxidation of NADPH.

The first part of this work was initiated by the publication of several reports concerning the different behaviour of NAD and NADP in the reactions catalyzed by bovine liver glutamate dehydrogenase (FRIEDEN, 1959, 1963b, 1965), especially with regard to the allosteric action of ATP. Subsequently, our interest became concentrated on enzyme stability, the means of influencing it, and its relation to the state of aggregation of the protein. The analysis of the molecular species composition of an associating system has been applied to glutamate dehydrogenase. The results of this analysis have led to the finding of a linear relationship between the rate of inactivation and the amount of monomer present at the corresponding enzyme concentration. The mechanism of inactivation suggested by this finding and by the results of experiments in which inactivation was followed in the presence or absence of ligands was confirmed by the results of sucrose density gradient centrifugation experiments.

2. MATERIALS AND METHODS

2.1. Enzyme preparations and other materials

Crystalline beef-liver glutamate dehydrogenase was obtained from Boehringer und Soehne (Mannheim) as a suspension in 50% glycerol. Alcohol dehydrogenase and catalase, used as markers in the sucrose gradient experiments, were also products of Boehringer und Soehne.

NAD^+ and $NADP^+$ were obtained from Sigma Chemical Co. or from Boehringer und Soehne. Sigma NADH and NADPH were dissolved on the day of the experiment in 0.05 M Tris-HCl (pH 8). ATP was obtained from Sigma, while ADP, GDP and GTP were from Boehringer, as were sodium glutamate, 2-oxoglutarate and most of the other compounds.

The pH of all solutions was adjusted to pH 8. All solutions were stored at 4°, except for the stock solutions of phosphate buffers. Care was taken to prepare fresh solutions of all the reagents at regular time intervals.

2.2. The assay system

The commercial preparation of GDH was diluted prior to the experiment in 10 mM sodium phosphate buffer – 0.1 mM EDTA (pH 8). Under these conditions, the dilute enzyme was stable on ice for several hours. Except where otherwise indicated, the assay mixtures contained 0.1 mM coenzyme, and either 5 mM sodium glutamate (oxidative deamination) or 5 mM sodium 2-oxoglutarate and 50 mM NH_4 Cl (reductive amination). The reaction took place at 25° in 10 mM Tris-HCl – 0.1 mM EDTA (pH 8) in a total volume of 3 ml. After the assay mixtures had been equilibrated at 25° for at least 10 minutes, the reaction was initiated by the addition of enzyme. Absorbance changes due to $NAD(P)^+$ reduction or NAD(P)H oxidation were recorded in cuvettes of 1 cm light-path with the aid of a Photovolt Corp. recorder Model 43 attached to a Zeiss spectrophotometer PMQ II. An extinction coefficient of 6.22 mM^{-1} cm^{-1} was used at 340 nm, and of 3.33 mM^{-1} cm^{-1} at 366 nm. Performing rate measurements at 366 nm enabled us to maintain a constant slit opening even when high concentrations of the reduced pyridine nucleotides were present.

Enzyme concentrations were adjusted so that initial velocities could easily be estimated from the tangent to the earliest part of the curve. The protein concentration of enzyme solutions was estimated spectrophotometrically at 280 nm using an absorption coefficient of 0.97 for a 1 mg/ml glutamate dehydrogenase solution (Olson and Anfinsen, 1952). It is known that the specific activity of glutamate dehydrogenase remains constant over a wide range of enzyme concentrations (Fisher, 1962a; Frieden and Colman, 1967). In comparing effects obtained in different experiments, it was assumed that the rates calculated per mg protein were independent of enzyme concentration.

2.3. INACTIVATION EXPERIMENTS

For the purpose of studying inactivation, the desired enzyme concentration was obtained by diluting the original glycerol suspension in 10 mM Tris-HCl buffer (pH 8) containing 0.1 mM EDTA. Incubation was carried out at 25°, except for the experiments where the effect of temperature was studied. In the latter case, the water bath in which the samples were incubated was adjusted to the desired temperature by means of a Tisch-KRYOMAT, Messgeräte-Werk-Lauda (West Germany). The dilute Tris buffer was freshly prepared on the day of the experiment from a 1 M stock solution which was made once a week or once in two weeks and was kept in the refrigerator. The pH of the buffer was adjusted with an accuracy of $\pm$ 0.01 pH units with dilute HCl by means of a Radiometer Titrator 11 (Copenhagen) at the temperature of incubation. Whenever the effect of some compound upon inactivation was studied, the enzyme was diluted into a solution of the buffer which contained that compound. Equal aliquots were removed at given time intervals and added to 3-ml assay mixtures containing 0.1 mM NAD^+ and 5 mM sodium glutamate in 10 mM Tris-HCl (pH 8) at 25°. In working with various dilutions of enzyme, care was taken to add a constant amount of protein to the assay mixtures. In order to eliminate possible effects due to the presence of various amounts of glycerol, the effect of glycerol on the inactivation of a low concentration of enzyme was tested. Similar rates of inactivation were found over the 8fold glycerol concentration range of 0.25 to 2.0% (Fig. 2.1).

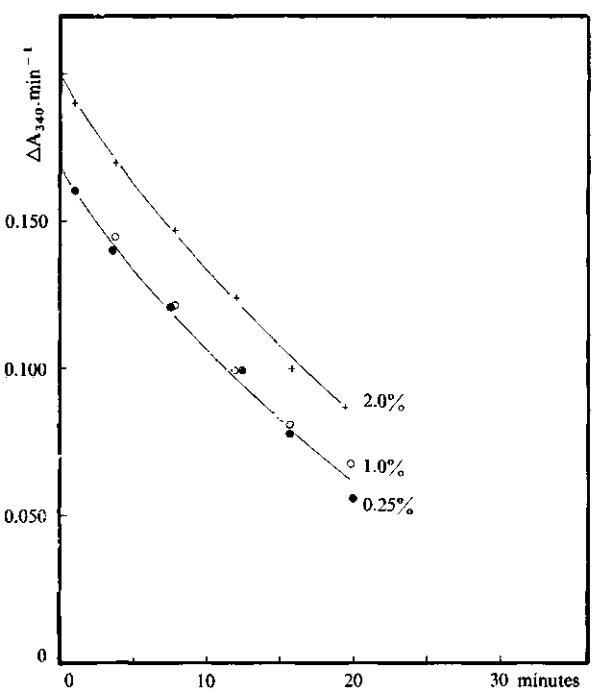

FIG. 2.1. Effect of glycerol on the time course of inactivation of GDH. The figures represent percent glycerol. Enzyme concentration ~0.1 mg/ml. Aliquots removed at the indicated time intervals were added to assay mixtures containing 0.1 mM NAD^+ and 5 mM sodium glutamate in 10 mM Tris-HCl (pH 8).

2.4. Spectral measurements

Difference spectra between stable and inactivated enzyme were recorded on a Cary 14 spectrophotometer at 25° in 1-cm light path quartz cuvettes. Enzyme dialysed overnight against 0.1 M sodium phosphate buffer (pH 8) was used.

The reference compartment contained stable enzyme (in 0.1 M sodium phosphate-0.1 mM EDTA, pH 8), while the sample compartment contained enzyme diluted in 10 mM Tris-HCl (pH 8) with 0.1 mM EDTA. The difference spectrum recorded immediately after dilution of the dialysed enzyme was taken as base line and was subtracted from the difference spectrum recorded at the end of 45 minutes of inactivation, thus correcting for possible pipetting errors. The slide wires 0–0.5, 0.5–1.0 and 0–0.1, 0.1–0.2 were alternatively used depending on enzyme concentration.

Corrections for light scattering were made by the method of Leach and Scheraga (1960).

2.5. Sucrose density gradients

The method of Martin and Ames (1961) was used, with slight modifications. Linear gradients were prepared by mixing 4% and 20% or 9% and 25% (weight per volume) stock solutions of cold sucrose in 0.1 M sodium phosphate buffer (pH 8) containing 0.1 mM EDTA. Volumes of 1 ml of the low, respectively, high concentration of sucrose were added to each mixing chamber. To assure linearity of the gradients, care was taken that the fluid levels in the two chambers were equal during emptying. In initial experiments, the linearity was tested by measuring the index of refraction of the collected fractions by means of an Abbé refractometer, or by mixing dichlorophenolindophenol with the concentrated sucrose solution and measuring the absorbance at 600 nm.

The gradients were prepared 4–6 hours before the run and were stored in the refrigerator. To start a run, 0.10 or 0.15 ml of the substance to be studied was layered on top of the preformed gradients in the cold. Centrifugation was carried out at 3–4° in the 3 × 3 ml precooled swing-out bucket rotor of the MSE Super Speed 50 centrifuge, at 37000 r.p.m. for 17 hours. At the end of the run, a hole was punched in the bottom of the tubes by means of the MSE fractionator, and fractions of 2 drops (about 0.027 ml) were collected for activity measurements from a total of 150–155 drops per tube.

The assay system contained 5 mM 2-oxoglutarate, 50 mM NH_4Cl and 0.1 mM NADH in 10 mM Tris-HCl-0.1 mM EDTA (pH 8) at 25°. The reaction was followed as absorbance decrease at 340 nm. The enzyme fractions were added to the cuvettes by means of micropipettes (Goldbrand, W. Germany). Pipetting errors were eliminated by using the same micropipette for sampling throughout the experiment.

Fractions of 5 drops or larger were collected for protein determination by the method of Murphy and Kies (1960). Calibration curves were perfectly linear with both bovine serum albumin and glutamate dehydrogenase as standards,

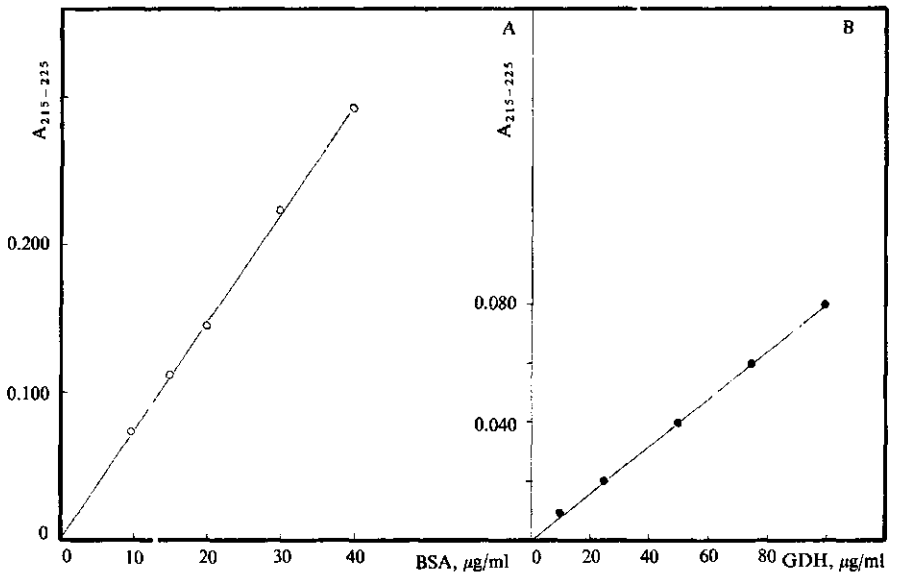

FIG. 2.2. Calibration curves for protein by the method of MURPHY and KIES (1960). A – for BSA; B – for GDH. Dilutions were made in 0.1 M phosphate buffer-0.1 mM EDTA (pH 8).

diluted in 0.1 M sodium phosphate buffer (pH 8) containing 0.1 mM EDTA (see Fig. 2.2.).

Alcohol dehydrogenase (mol.wt. 150000) and catalase (mol. wt. 250000) were used as markers for molecular weight determinations (MARTIN and AMES, 1961). Alcohol dehydrogenase activity was assayed by following the increase in absorption at 340 nm in a 3-ml reaction mixture containing 0.1 ml absolute ethanol, 0.4 mM NAD^+ and 0.1 M glycine brought to pH 9.6 with NaOH. Catalase activity was assayed by following the decrease in absorption at 240 nm of a 3-ml reaction mixture containing 0.3 ml of a 0.1 M sodium phosphate solution brought to pH 7.5 with KH_2PO_4, and about 3 μl of a 3% H_2O_2 solution (see MARTIN and AMES, 1961).

3. EFFECT OF 2-OXOGLUTARATE ON THE CATALYTIC PROPERTIES OF GLUTAMATE DEHYDROGENASE

3.1. RELATION BETWEEN THE CONCENTRATION OF 2-OXOGLUTARATE AND THE EFFECT OF ATP

The effect of ATP on the reductive amination of 2-oxoglutarate was studied at various concentrations of NADH, at a low (1.6 mM) and a high (50 mM) 2-oxoglutarate concentration.

As seen in Fig. 3.1, the normal MICHAELIS-MENTEN kinetics shown by curve 1 is not followed by the 3 other lines. The deviation caused by NADH inhibition is apparent at 50 mM 2-oxoglutarate in the absence of ATP (curve 2), but also at 1.6 mM 2-oxoglutarate in the presence of ATP (curve 3). ATP enhances NADH inhibition at both low and high 2-oxoglutarate concentrations. With low concentrations of coenzyme, ATP inhibits the reaction at low concentrations of 2-oxoglutarate; however, it slightly but reproducibly stimulates the reaction at 50 mM 2-oxoglutarate.

A different behaviour was observed when NADPH was used as coenzyme instead of NADH. At 50 mM 2-oxoglutarate, the double reciprocal plot of initial rate against NADPH concentration is a straight line, as shown by line 3 in Fig. 3.2B. At low 2-oxoglutarate concentrations, high levels of NADPH are inhibitory, as illustrated by curves 1 and 2.

As in the case of NADH oxidation, NADPH inhibition depends on the

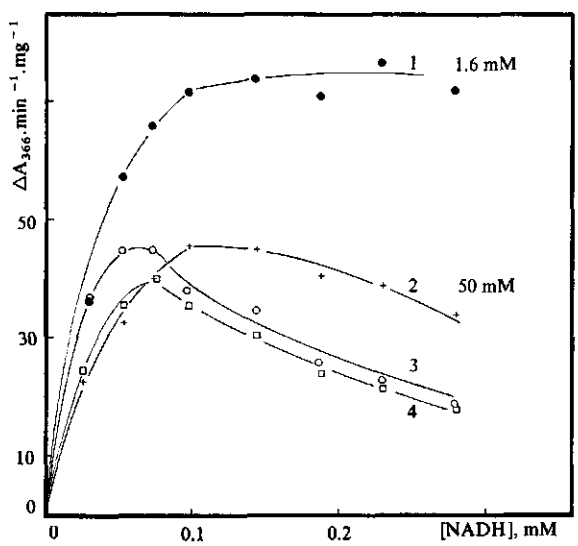

FIG. 3.1. Effect of ATP on the reductive amination of 2-oxoglutarate with various concentrations of NADH. Curve 1- with 1.6 mM 2-OG; curve 2- with 50 mM 2-OG; curve 3- like 1, but with 0.1 mM ATP; curve 4- like 2, but with 0.1 mM ATP. The amount of enzyme in the reaction mixtures varied between 1.2 and 2.5 μg.

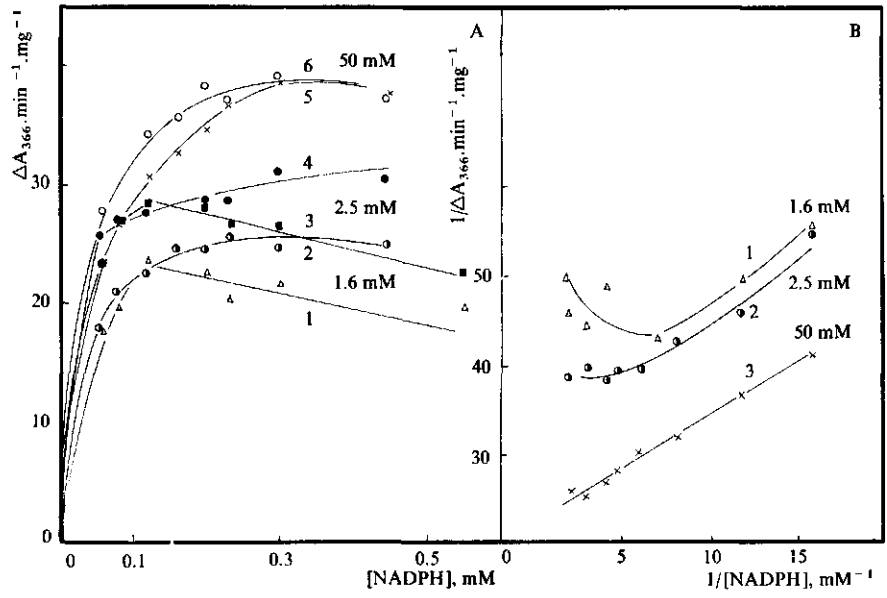

FIG. 3.2. Effect of ATP and of [2-OG] on the reductive amination of 2-oxoglutarate with various [NADPH]. A. Effect of ATP. Curve 1- with 1.6 mM 2-OG; curve 2- idem, with 0.1 mM ATP; curve 3- with 2.5 mM 2-OG; curve 4- idem, with 0.1 mM ATP; curve 5- with 50 mM 2-OG; curve 6- idem, with 0.1 mM ATP. B. Effect of [2-OG]: double reciprocal plot of the control lines (without ATP) from Part A. Curve 1- with 1.6 mM 2-OG; curve 2- with 2.5 mM 2-OG; curve 3- with 50 mM 2-OG. The amount of enzyme in the reaction mixtures was 2.5 μg.

concentration of the substrate 2-oxoglutarate, but an opposite effect is noted: NADPH inhibition increases as 2-oxoglutarate concentration decreases (cf. curves 5,3 and 1, Fig. 3.2A).

ATP activates NADPH oxidation, but the extent of activation diminishes at high concentrations of 2-oxoglutarate (cf. curves 6–5 and 2–1, Fig. 3.2A).

3.2. EFFECT OF 2-OXOGLUTARATE ON THE OXIDATIVE DEAMINATION OF GLUTAMATE

The behaviour of 2-oxoglutarate in the reaction of reductive amination suggested that this compound may have an additional effect on the enzyme apart from that of a usual substrate or product. This assumption was tested by studying the effect of 2-oxoglutarate when added to the assay system of the glutamate deamination reaction.

Usually one would expect 2-oxoglutarate to exhibit product inhibition in the glutamate oxidation reaction. However, the results illustrated in Fig. 3.3 show that in the low 2-oxoglutarate concentration range (see Insert), the reaction of $NADP^+$ reduction is slightly activated, or at least is uninhibited, in contrast to the NAD^+ reduction.

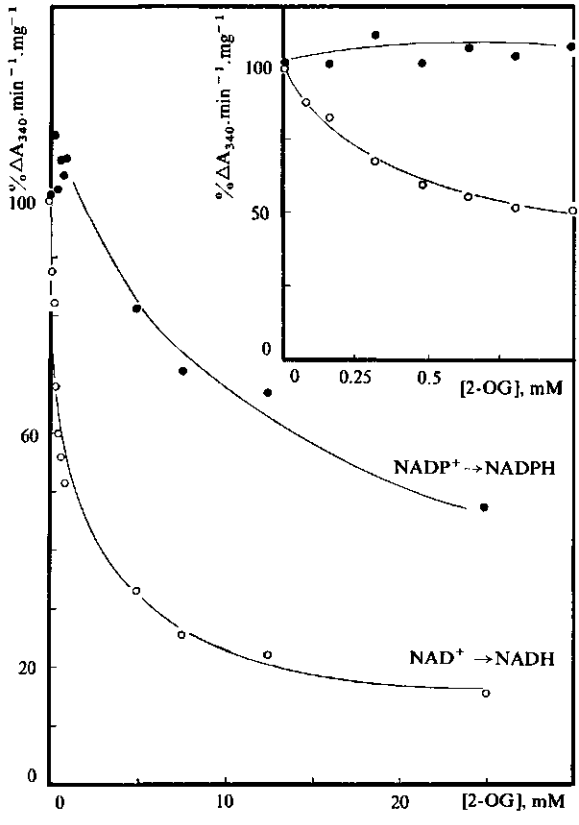

FIG. 3.3. Effect of 2-oxoglutarate on glutamate oxidative deamination. The upper curve represents an experiment with 0.1 mM $NADP^+$ and 50 mM glutamate; the lower curve represents a similar experiment with 0.1 mM NAD^+ instead of $NADP^+$. The amount of enzyme in the reaction mixture was 24 μg in the $NAD^+ \rightarrow$ NADH reaction, and 38 μg in the $NADP^+ \rightarrow$ NADPH reaction. The insert represents a similar experiment at an enlarged scale of the 0–1 mM [2-OG] range.

The effect was reproducible under these conditions, but when the concentration of glutamate was reduced from 50 mM (as in the experiment illustrated in Fig. 3.3) to 10 mM, the initial rise in $NADP^+$ reduction rate was no longer observed. Fig. 3.3 shows also that the decline in activity caused by increasing 2-oxoglutarate concentrations tends to level off at high concentrations of the latter.

The nature of the inhibition exhibited by 2-oxoglutarate in the glutamate deamination reaction was studied in the experiments illustrated in Fig. 3.4. At 3 mM, 2-oxoglutarate appears to be a competitive inhibitor of glutamate in the NAD^+ reduction reaction, as opposed to the cooperative type of inhibition observed in the case of $NADP^+$ reduction. At 20 mM, a type of inhibition which may be described as cooperative noncompetitive-like was observed with both coenzymes. The non-linearity of the plots with 20 mM 2-oxoglutarate may be open to discussion, but since a similar distribution of experimental points recurred in 2–3 experiments, the possibility of cooperative effects cannot be excluded (see also Section 3.3).

The cooperative type of inhibition shown by 2-oxoglutarate in the glutamate

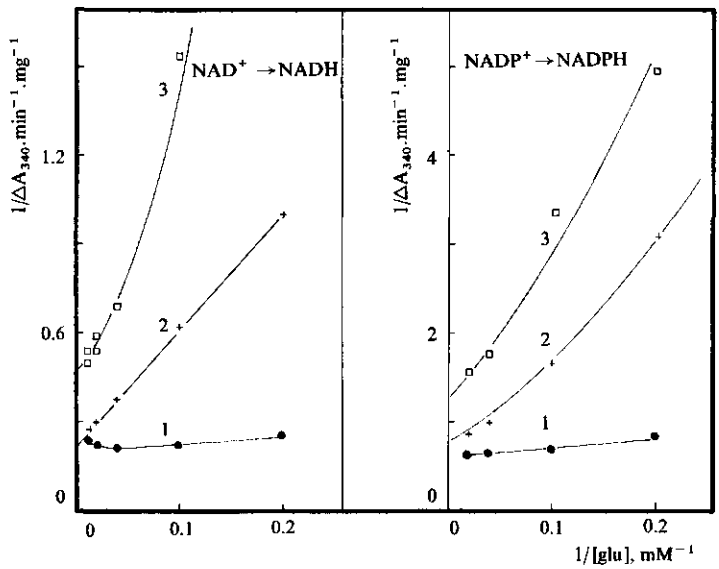

FIG. 3.4. Effect of 2-oxoglutarate on the $1/v$ vs. 1/[glu] plot. The concentration of both oxidized coenzymes was 0.1 mM. Curve 1- control; curve 2- with 3 mM 2-OG; curve 3- with 20 mM 2-OG. The amount of enzyme in the reaction mixture was 48 μg in the $NAD^+ \rightarrow NADH$ reaction, and 100 μg in the $NADP^+ \rightarrow NADPH$ reaction.

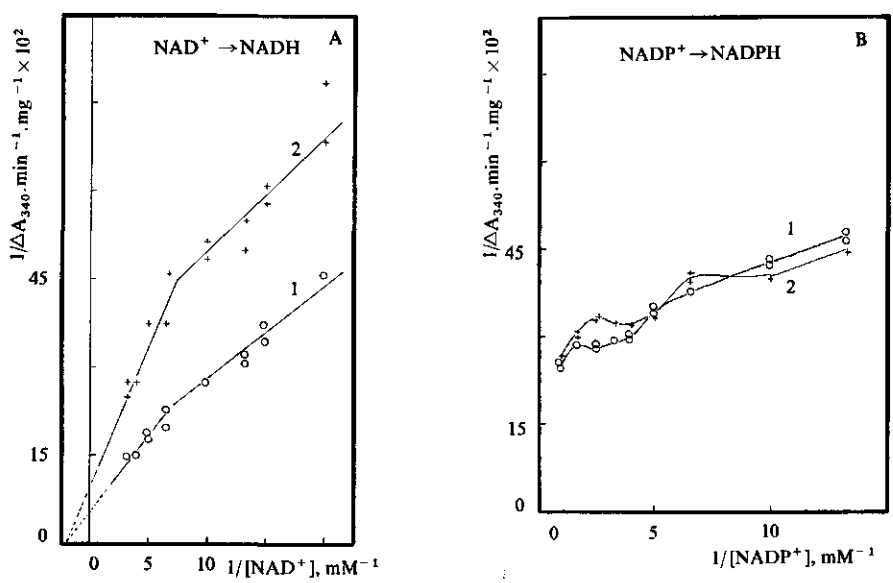

FIG. 3.5. Effect of 2-oxoglutarate on glutamate oxidation with various concentrations of coenzyme. A. Curve 1- control; curve 2- with 3.75 mM 2-OG. The amount of enzyme in the reaction mixture varied between 10 and 50 μg. B. Curve 1- control; curve 2- with 1 mM 2-OG. The amount of enzyme in the reaction mixture varied between 25 and 75 μg. The concentration of glutamate was 50 mM.

deamination reaction with $NADP^+$ as coenzyme was found also when glutamate acted as inhibitor of NADPH oxidation. Also in this case, slight activation of NADPH- as opposed to NADH-oxidation by increasing glutamate concentrations was obtained at 50 mM 2-oxoglutarate. At a concentration of 10 mM 2-oxoglutarate, increasing glutamate levels caused inhibition with both coenzymes. Plotting $1/v$ against glutamate concentration by the method of DIXON (DIXON and WEBB, 1964) at 2 concentrations of 2-oxoglutarate, the 2 lines (referring to 10 and 50 mM 2-oxoglutarate) were found to cross in the first quadrant. This may be explained by the existence of 2 different forms of the enzyme at low and at high 2-oxoglutarate concentrations.

The experiments presented above were all performed with 0.1 mM coenzyme. The next step was to vary $NAD(P)^+$ concentration while maintaining the 2-oxoglutarate concentration constant. As seen in Fig. 3.5A, 2-oxoglutarate showed noncompetitive inhibition in the high coenzyme concentration range.

The effect of 2-oxoglutarate on $NADP^+$ reduction at varying concentrations of the coenzyme is shown in Fig. 3.5B. The results of these experiments are in disagreement with those reported by others (OLSON and ANFINSEN, 1953; FRIEDEN, 1959a; DI PRISCO et al., 1965) where linear LINEWEAVER-BURK plots were obtained in experiments with $NADP^+$. On the other hand, DALZIEL and ENGEL (1968) also report about plots with 'rather sharp discontinuities, which apparently consist of several linear portions with different slopes'.

The influence of varying glutamate concentration upon NAD^+ reduction was investigated in the experiment illustrated in Fig. 3.6. Note the increase in the biphasicity of the line as the concentration of glutamate increases from 5 through 10 to 25 mM.

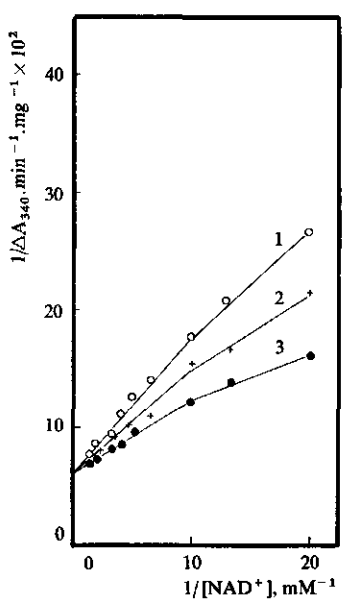

FIG. 3.6. Effect of substrate concentration on the activating effect of high NAD^+ concentrations in the glutamate oxidation reaction. Curve 1- with 5 mM glu; curve 2- with 10 mM glu; curve 3- with 25 mM glu. The points represent averages from duplo measurements.

3.3. Discussion

On the basis of kinetic, as well as sedimentation and fluorescence studies, Frieden (1959a; 1962a; 1963a, b; 1965) postulated 3 types of specific binding sites on the enzyme to explain the effect of coenzymes and purine nucleotides on the activity of beef-liver glutamate dehydrogenase. These are *a.* an active site, which binds oxidized and reduced nicotinamide adenine nucleotides, *b.* a binding site which is highly specific for purine nucleotides, and *c.* a nonactive site which binds NADH but not the other coenzymes. Postulation of the latter site was based mainly on the observation that at high concentrations, NADH inhibits its own oxidation.

The specificity of the purine nucleotide site is dependent on the presence or absence of coenzymes. GTP and GDP are uncompetitive inhibitors, i.e., they bind more strongly to the enzyme-coenzyme complex than to the free enzyme, while ADP is an uncompetitive activator, i.e., it binds more strongly to the free enzyme than to the enzyme-coenzyme complex (Frieden, 1963b). ATP was reported to have no effect or cause slight activation with NAD^+, $NADP^+$ and NADPH as coenzymes of the reaction, but inhibit NADH oxidation except at very low coenzyme concentrations (Frieden, 1959a). In a later work (Frieden and Colman, 1967), activation by ATP with NADH as the coenzyme was reported at pH 7 in contrast to ATP-inhibition at pH 8.

Unlike the well-studied effect of coenzyme binding on the properties of the nucleotide-binding site, the influence of substrates (2-oxoglutarate and glutamate) on nucleotide binding has seldom been investigated (Kun and Achmatowicz, 1965; Iwatsubo et al., 1966). The latter authors showed by protein fluorescence studies that the dissociation constant of the enzyme-NADH complex is larger in the absence of the substrates than in their presence, and is about 5 times smaller in the presence of 2-oxoglutarate than in the presence of glutamate.

The results described in the previous chapter are presented in a concise form in the table on the following page.

According to Figs. 3.1 and 3.2, ATP-inhibition or activation of the reductive amination can be influenced by varying the concentration of 2-oxoglutarate. A similar effect is shown for glutamate and its influence on the 'second NAD^+ site' by the results in Fig. 3.6. According to Frieden (1959a), the biphasicity of the $1/v$ vs. $1/NAD^+$ plot is due to the presence of a second, noncatalytic, site for NAD^+, which would explain the activation caused by high NAD^+ concentrations. As seen in the figure, decreasing the concentration of glutamate may lead to linearization of the biphasic plot.

The difference between the behaviour of NADH and NADPH at high concentrations (see Figs. 3.1 and 3.2) is probably explainable in terms of the lower affinity of the enzyme for NADPH. Higher concentrations of NADPH are required to achieve the same effect, e.g., inhibition of its own oxidation (see also Eisenkraft and Veeger, 1967). In this respect, it is worthwhile mentioning the spectral work of Fisher and Cross (1966) and of Pantaloni and Iwatsubo (1967) which showed that NADH binds to the enzyme by means of both the adenine and the nicotinamide groups. In the case of NADPH, only

SUMMARIZING TABLE OF THE RESULTS PRESENTED IN FIGS. 3.1–3.6

Reaction measured	1/v vs. 1/[coenzyme] plot	1/v vs. 1/ [glu] plot	Effect of 2-OG
$NAD^+ \rightarrow NADH$	– biphasic, due to NAD^+-activation at high [NAD^+] – biphasicity promoted at 1 mM 2-OG – biphasicity diminished at low [glu]	– slight inhibition at high [glu] – competitive inhibition at 3 mM 2-OG – cooperative noncompetitive inhibition at 20 mM 2-OG	competitive at low [2-OG]; noncompetitive--cooperative at high [2-OG]
$NADP^+ \rightarrow NADPH$	nonlinear, consisting of several portions with different slopes	– linear – cooperative competitive-like inhibition at 3 mM 2-OG – cooperative noncompetitive--like inhibition at 20 mM 2-OG	cooperative
$NADH \rightarrow NAD^+$	– biphasic, due to NADH-inhibition at high [NADH] – biphasicity promoted at 50 mM 2-OG, and at both 1.6 mM and 50 mM 2-OG in the presence of ATP – ATP-inhibition of the reaction is diminished at low [NADH] and at high [2-OG]		high concentrations promote NADH-inhibition and counteract ATP-inhibition
$NADPH \rightarrow NADP^+$	– biphasic at low [2-OG], linear at 50 mM 2-OG – ATP-activation of the reaction is diminished at high [2-OG]		high concentrations promote both NADPH-inhibition and ATP-activation

the absorption band of nicotinamide is visible in the difference spectrum.

The effect of 2-oxoglutarate on glutamate deamination differs depending on the concentration of glutamate, and depending upon which of the 2 nucleotides is utilised as cofactor. Kinetic plots show that 2-oxoglutarate is not merely a competitive inhibitor of glutamate, and suggest that cooperative effects are involved. Recent work performed on D-amino-acid oxidase (KOSTER and VEEGER, 1968) shows that the observed substrate inhibition is due to conformations with different activities having different affinities for the substrates and effectors. Activity cannot be related to the ligand saturation function (MONOD et al., 1965). Only the state functions can be related to activity, while keeping in mind that in two-substrate reactions, v_{max} and K_m are dependent upon the concentration of both substrates. As conformations with different activities may show different affinities for various reagents, one substrate can act as an allosteric effector and affect as such the kinetic parameters of the second substrate. The result will be different according to the kinetic mechanism operating, i.e., modified enzyme mechanism, ordered mechanism or random mechanism (KOSTER and VEEGER, 1968; DALZIEL, 1968).

Kinetic studies of FRIEDEN (1959a) and FISHER (1960) support a sequential mechanism for glutamate dehydrogenase, the order of substrate addition being reduced coenzyme, ammonium ion and 2-oxoglutarate. In experiments of equilibrium dialysis with tritium-labeled 2-oxoglutarate, FISHER (1960) could detect no binding of 2-oxoglutarate to the enzyme, either in the presence or absence of NAD^+. Fluorimetric results indicated the possible formation of an enzyme-NADPH-2-oxoglutarate complex. Formation of such an abortive complex in which the binding of NH_4^+ is sterically prevented would account, in FISHER's view, for the inhibition of the reaction by high 2-oxoglutarate concentrations. In the following chapters of this dissertation, evidence shall be brought in favour of the binding of 2-oxoglutarate to the free enzyme. This raises the question whether a sequential mechanism for glutamate dehydrogenase is obligatory. Assuming validity of the compulsory order mechanism, the inhibition exhibited by 2-oxoglutarate in the glutamate deamination reaction (see non-linear plots in Fig. 3.4) may be due to the formation of ternary complexes of the type enzyme-NAD^+-2-oxoglutarate or enzyme-$NADP^+$-2-oxoglutarate (cf. FROMM and NELSON, 1962).

The regulatory function of the pyridine nucleotides (coenzymes) and of the purine nucleotides (allosteric effectors) has often been mentioned in discussing the role of glutamate dehydrogenase in metabolic control. The experiments described above demonstrate that markedly different results are obtained when the concentration of the substrates is varied. This is consistent with the idea that conformations with different affinities for the 2 substrates (and for the same substrate at different concentrations) play a role in the catalytic mechanism. In this connection it is interesting to mention the work of PAPA et al. (1967) who stressed the importance of 2-oxoglutarate concentration in the function of the enzyme in isolated mitochondrial particles and found stimulation of glutamate oxidation by removal of 2-oxoglutarate.

4. RELATION BETWEEN STABILITY OF GLUTAMATE DEHYDROGENASE AND ITS STATE OF ASSOCIATION

4.1. VARIATION OF INACTIVATION RATE WITH ENZYME CONCENTRATION

The decline in activity of the enzyme was tested by diluting the original glycerol suspension in 10 mM Tris-HCl – 0.1 mM EDTA (pH 8), incubating the diluted enzyme at 25°, and taking aliquots for assay at various time intervals (see METHODS). In view of the well-known ability of glutamate dehydrogenase to dissociate upon dilution, the relationship between rate of inactivation and enzyme concentration was examined (Fig. 4.1).

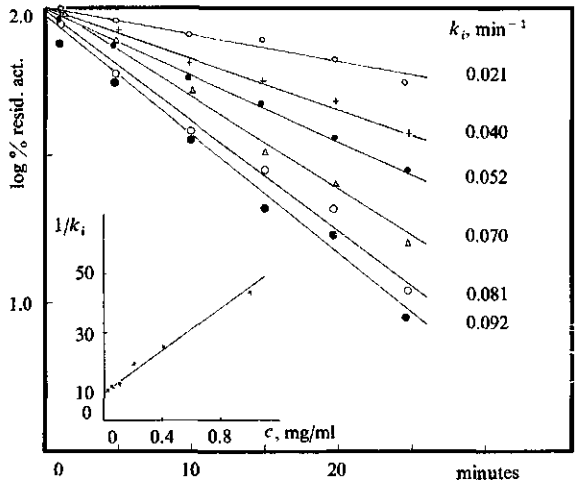

FIG. 4.1. Effect of enzyme concentration on the rate of inactivation of GDH. Initial rates at various time intervals after inactivation started were recalculated as per cent of the extrapolated catalytic activity at zero time, and the logarithms were plotted against time. (The activity at zero time was obtained by taking the average of the intercepts of the straight lines in the plot $\log \Delta A_{340}.\text{min}^{-1}$ vs. time). Enzyme concentrations corresponding to the inactivation constants in the figure are in descending order: 1 mg/ml; 0.4 mg/ml; 0.2 mg/ml; 0.1 mg/ml; 0.05 mg/ml and 0.025 mg/ml. In the insert, reciprocals of the rate constants of inactivation are set out against enzyme concentration.

Pseudo-first order rate constants can be calculated from the lines in Fig. 4.1. In the insert, reciprocals of these constants are set out against enzyme concentration. It is noteworthy that the value of the inactivation constant, which is a measure of the rate of inactivation, reaches a finite value at infinite dilution (see Insert). This suggested a possible relationship between the relative amount of monomer and the rate of inactivation of the coenzyme. As the monomer is

the smallest catalytic subunit and the predominant molecular species at high dilutions (EISENBERG and TOMKINS, 1968; SUND and BURCHARD, 1968), the possibility of dimer dissociation being the limiting step in inactivation seemed remote. In order to test the predicted relationship, it was necessary to find a method by means of which the types of molecular species present in a polymerizing system can be determined.

4.2. APPLICATION OF STEINER'S METHOD TO GLUTAMATE DEHYDROGENASE

The formulae derived by STEINER (1952) make it possible to calculate the consecutive association constants and the types of polymer species present in a system from the concentration dependence of molecular weight. While molar concentrations are used in the calculations of STEINER, preference has been given to weight concentrations (see also FUJITA, 1962) with the result that the association constants are expressed in l/g instead of in l/mole.

When a monomer A_1 associates to form a series of polymers, A_2(dimer), A_3(trimer), etc., then assuming 1. that the system attains chemical equilibrium very rapidly, and 2. that the activity coefficients of the solutes are unity, the concentrations in mg/ml of A_1 and A_2, denoted by c_1 and c_2, are related by the equation

$$c_2 = K_2 c_1^2 \tag{1}$$

where K_2 is the equilibrium constant for the formation of A_2 from A_1. Similarly,

$$c_3 = K_2 K_3 c_1^3 \tag{2}$$

where c_3 is the concentration of the trimer, and K_3 is the equilibrium constant for the reaction $A_1 + A_2 \rightleftharpoons A_3$.

The dimensions of K_2 and K_3 are l/g and they are related to the respective molar association constants (K'_2 and K'_3) by the following equations

$$K'_2 = K_2 M_1/2 \tag{3}$$

$$K'_3 = 2K_3 M_1/3 \tag{4}$$

where M_1 is the molecular weight of the monomer.

The weight average molecular weight M_w of A_1, A_2,...A_i is given by

$$M_w = \sum_1^n i\, c_i M_i / \sum_1^n i\, c_i \tag{5}$$

where c_i and M_i are the concentration and molecular weight respectively, of species i. Substituting in Eqn. (5) the above relations for c_2, c_3, etc., [Eqns. (1) and (2)], one obtains

$$M_w c/M_1 c_1 = 1 + 2K_2 c_1 + 3K_2 K_3 c_1^2 + \ldots \text{etc.} \tag{6}$$

In this way, the problem of calculating the equilibrium constants K_2 and K_3 is reduced to measuring molecular weight as a function of c. The weight fraction of monomer (c_1/c) can be evaluated using the formula derived by STEINER (1952).

We shall present a derivation of this formula in a few steps (see FUJITA, 1962).

Equation (5) can be written as

$$M_w c = M_1(c_1 + 2c_2 + 3c_3) \tag{7}$$

The total concentration of mono-, di- and trimer is

$$c = c_1 + c_2 + c_3 \tag{8}$$

Substituting Eqns. (1) and (2) into Eqn. (8) and differentiating with respect to c_1, we get

$$c_1(dc/dc_1) = c_1 + 2K_2c_1^2 + 3K_2K_3c_1^3 = c_1 + 2c_2 + 3c_3 \tag{9}$$

Comparison of Eqn. (9) with Eqn. (7) leads to

$$(M_1/M_w)dc/c = dc_1/c_1 \tag{10}$$

The weight fraction of monomer can be defined as

$$c_1/c = x \tag{11}$$

Logarithmic differentiation of (11) gives

$$dc_1/c_1 = dc/c + dx/x \tag{12}$$

Combining (10) and (12) leads to

$$dx/x = [(M_1/M_w) - 1](dc/c) \tag{13}$$

and after integration

$$\ln x = \int^c [(M_1/M_w) - 1](dc/c) + k \tag{14}$$

Combination of (1), (2), (7) and (11) gives

$$1 = x + (x^2K_2) + (x^3K_2K_3)c^2 \tag{15}$$

which shows that when $c \to 0$, x approaches 1. This condition applied to Eqn. (14) leads to the Steiner relation

$$\ln c_1/c = {}_0\int^c [(M_1/M_w) - 1]c\, dc \tag{16}$$

which means that graphic integration of the plot $[(M_1/M_w) - 1]/c$ *vs.* c provides $\ln c_1/c$, and thus c_1, at every c (Fig. 4.2).

FIG. 4.2. STEINER-type of plot for calculation of the relative amounts of monomer at various [enzyme].

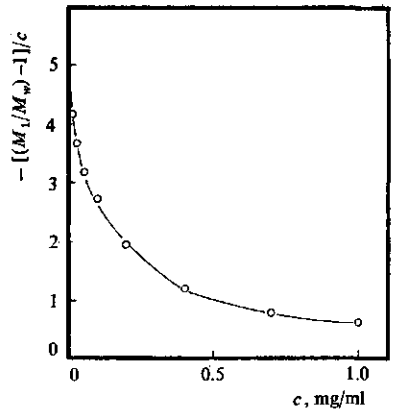

TABLE 4.1. Apparent weight-average molecular weight of GDH as a function of enzyme concentration (from data of EISENBERG and TOMKINS, 1968). Light scattering experiments were performed at 25° in 0.2 M phosphate buffer-10^{-3} M EDTA (pH 7).

Enzyme concn. (mg/ml)	Molecular weight
1.0	837000
0.4	607000
0.2	514000
0.1	431000
0.05	373000
0.025	345000
0.0125	334000

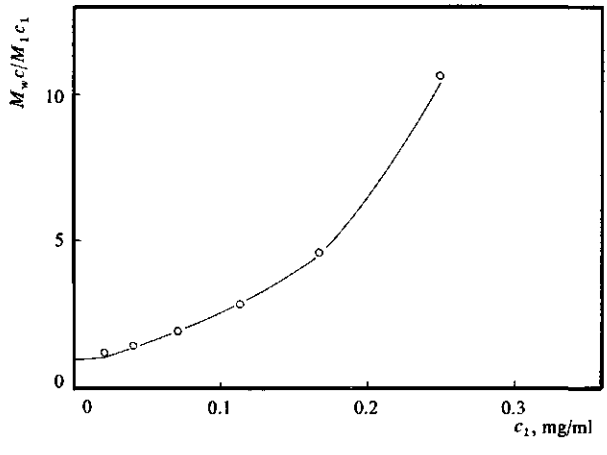

FIG. 4.3. Plot for calculation of the dimerization constant K_2 (see text).

In our calculations we used the $M_w(c)$ data obtained by EISENBERG and TOMKINS (1968) from light scattering experiments, which yielded an average molecular weight of 310000–316000 for the monomer (Table 4.1).

The equilibrium constants needed for determination of the molecular species composition at various concentrations of enzyme were obtained from plots of $M_w c/M_1 c_1$ against c_1 (for K_2) and $M_w c/M_1 c_1 - 2K_2 c_1$ against c_1^2 (for K_3) at *lim* $c \to 0$, as shown by Eqn. (6). These plots are presented in Figs. 4.3 and 4.4.

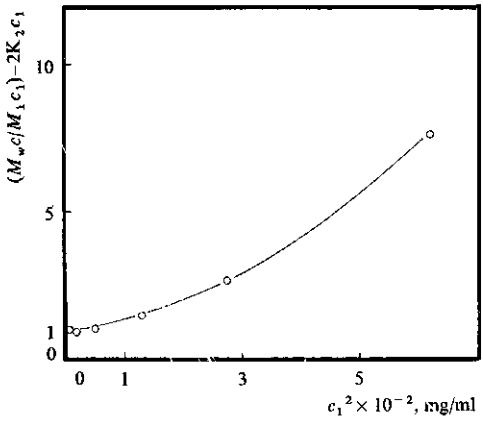

FIG. 4.4. Plot for calculation of the trimerization constant K_3 (see text).

The difficulty encountered in determination of K_2 from the slope of the curve in Fig. 4.3 was eliminated by plotting $(M_w c/M_1 c_1)-1]/c_1$ vs. c_1, following slight rearrangement of Eqn. (6). In this plot (see Fig. 4.5), K_2 is 1/2 the value of the intercept, while K_3 is obtained from the slope at *lim* $c \to 0$, assuming that both K_2 and K_3 are independent of c.

Values of 5 l/g were obtained for K_2 and 1.7 l/g for K_3. The corresponding values for K_2' and K_3' are 7.8×10^5 l/mole and 3.5×10^5 l/mole, respectively.

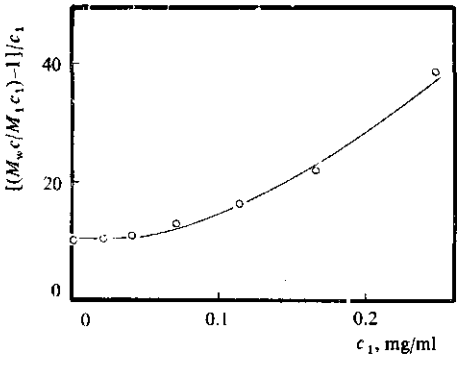

FIG. 4.5. Plot for calculation of both K_2 and K_3 (see text).

The value of K_2 obtained by means of plots derived from Eqn. (6) was tested and confirmed by means of 2 additional methods. ADAMS and WILLIAMS (1964) proved the validity of the 2 following relations:

$$\lim_{c \to 0} [(M_1/M_{w(c)}) - 1] = -K_2 \tag{17}$$

$$\lim_{c \to 0} dM_{w(c)}/dc = K_2 M_1 \tag{18}$$

Eqn. (17) says that the intercept of the curve in Fig. 4.2 equals the numerical value of K_2. According to Eqn. (18), K_2 can be obtained from the slope of the tangent at *lim* $c \to 0$ to the experimental curve of M_w against enzyme concentration (EISENBERG and TOMKINS, 1968).

In principle it is possible to determine all the equilibrium constants up to K_i (see Eqn. (6)), but in practice it is difficult to determine constants higher than K_3 because of the limited accuracy in the measurement of $M_w(c)$, especially at very low concentrations of protein. Moreover, the error introduced in the determination of c_1 is accumulated in the subsequent steps of calculation. Due to this difficulty, there is no certainty with regard to the value of K_3.

4.3. RELATION BETWEEN MONOMER CONCENTRATION AND RATE OF INACTIVATION

Determination of the di- and trimerization constants for glutamate dehydrogenase at 25° added a quantitative dimension to the experimental data presented in Fig. 4.1. As noted previously, a relationship was sought between the relative amounts of monomer at a given concentration and the rate of enzyme inactivation at that concentration. Table 4.2 lists the results of calculations based on data from Figs. 4.2 and 4.5 and Eqns. (1) and (2).

TABLE 4.2. Change in molecular species composition with enzyme concentration. Based on data from EISENBERG and TOMKINS (1968) and calculated as in Figs. 4.2–4.5.

Total concn. (mg/ml)	Monomer mg/ml	%	Dimer mg/ml	%	Trimer mg/ml	%
1.0	0.25	25	0.31	31	0.127	12.7
0.4	0.17	42	0.14	35	0.038	9.5
0.2	0.11	55	0.066	33	0.013	6.5
0.1	0.07	70	0.026	26	0.003	3.0
0.05	0.04	80	0.009	17	–	–
0.025	0.02	88	0.003	11	–	–

It is noteworthy that only di- and monomer species are found at concentrations of 0.05 and 0.025 mg/ml enzyme, and that the monomer obviously predominates at the 3 lowest concentrations investigated (see above). Above 0.1 mg/ml, the curve in Fig. 4.5 shows an upward curvature indicating the presence of polymers higher than the trimer (see Eqn. (6)).

When the rate constants of inactivation obtained from the lines in Fig. 4.1 are plotted against the relative amounts of mono-, di-, and trimer at the corres-

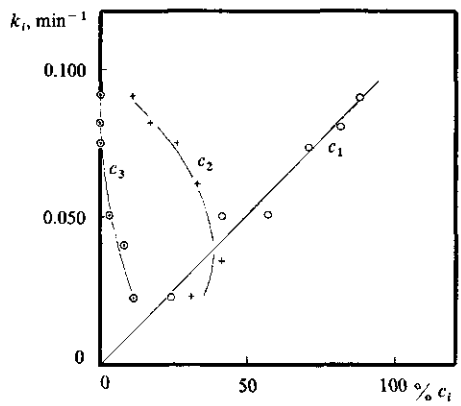

FIG. 4.6. Relationship between the rate constants of inactivation and the molecular species composition at various [enzyme]. Rate constants of inactivation calculated from the lines in Fig. 4.1 are set out against the relative amounts of monomer (c_1), dimer (c_2) and trimer (c_3) calculated by the method of Steiner (see Table 4.2 and text).

ponding total concentrations, a straight line relationship obtains for monomer variation (see Fig. 4.6). This confirms our preliminary assumption that the monomer is the 'active species' in the inactivation process of glutamate dehydrogenase.

The linear pattern of monomer variation was reproducible despite variations in the absolute value of the inactivation constants. As seen in Fig. 4.6, the monomer line extrapolates to the origin of the coordinates suggesting that there is no inactivation when the monomer concentration is very small, i.e., at very high enzyme concentrations. Examination of Fig. 4.1 seems to confirm this. From the lines in Fig. 4.6 one can also see that the highest rates of inactivation are recorded in dilute enzyme solutions, where the monomer fractions are the largest, while the di- and trimer concentrations are relatively low or absent.

4.4. THE LIMITING STEP IN THE PROCESS OF INACTIVATION

The results of the analysis presented above suggest that dissociation of higher molecular species into the monomer precedes inactivation of the latter. In other words, we assume that a rapid equilibrium is established between dimer and monomer in the lower range of enzyme concentrations, and that inactivation of the monomer is the limiting step.

In order to test this assumption it had to be shown that the rate constant of the dissociation of dimer into monomer is larger than that of monomer inactivation. This proved indeed to be the case. The results of an experiment in which an enzyme solution of 0.1 mg/ml enzyme underwent inactivation were used for the calculations. Allowing for the (uncertain) value of 3% trimer (Table 4.2), only dimer and monomer are present at this concentration. Since the specific activity of glutamate dehydrogenase was shown to remain constant over a wide range of enzyme concentrations (FRIEDEN and COLMAN, 1967; FISHER, 1962a) suggesting that mono- and dimer have similar catalytic activities, it may be assumed that the decline in activity after a given time is proportional to the decline in the concentration of total active enzyme. As the ratio of

TABLE 4.3. Relative amounts of monomer and dimer in an enzyme undergoing inactivation. For conditions and method, see text.

Min. of inactivation	% residual activity	Active enzyme (mg/ml)	c_1	c_2
0	100	0.100	0.071	0.029
1	88	0.088	0.064	0.024
10	47	0.047	0.039	0.008
15	30	0.030	0.026	0.004
20	21	0.021	0.019	0.002
25	14	0.014	0.013	0.001

monomer to dimer does not change in the course of inactivation, the mono- and dimer distributions at the 'new' concentrations were calculated by the method described above (Table 4.3).

This is based on the following representation of the process of inactivation: $M_2 \rightleftharpoons 2M \rightarrow$ inactive, which implies that mono- and dimer concentrations decrease by the same amount during inactivation. Semilogarithmic plots of dimer and monomer concentrations were constructed against time, and first-order constants were calculated from the resulting straight lines (Fig. 4.7).

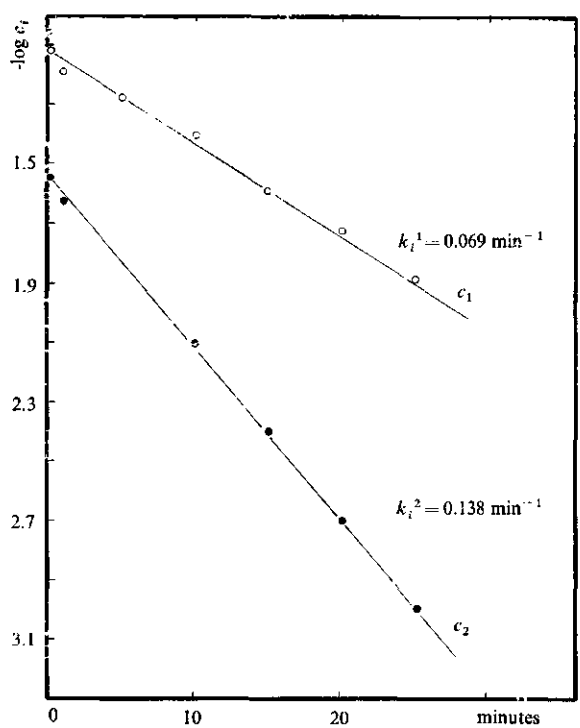

FIG. 4.7. Pseudo-first order plot for concentration of enzymically active monomer (c_1) and dimer (c_2) at various stages of inactivation (see Table 4.3).

As seen in Fig. 4.7, the rate of dimer dissociation (0.138 min^{-1}) is much more rapid than the rate of inactivation of the monomer (0.069 min^{-1}). Note the agreement between the latter value and the k_i for the 0.1 mg/ml enzyme in Fig. 4.1.

The significantly lower value of 0.01 min^{-1} was found for the constant calculated in a similar way for the monomer inactivation in a 1 mg/ml enzyme solution. This is not surprising if one conceives that higher polymers are present aside from the dimer at this concentration, and that the continuous removal of active monomer in the course of inactivation is accompanied by dissociation of higher molecular weight species.

4.5. Discussion

In his studies of enzyme inactivation, Frieden (1963a) mentioned that enzyme stability increases with increasing concentration and added that this was 'presumably in connection with the reversible association-dissociation reaction'.

Bitensky and coworkers (1965) concluded form their experiments that only the form of the enzyme which has enhanced alanine dehydrogenase activity (and has virtually no glutamate dehydrogenase activity) is the precursor of the denatured form of the protein. It will be remembered that these authors accepted the following scheme relating structure and catalytic properties of glutamate dehydrogenase (Tomkins et al., 1963).

$$\text{POLYMER} \rightleftharpoons \text{MONOMER } X \rightleftharpoons \text{MONOMER } Y$$

where monomer X catalyzes the glutamate dehydrogenase reaction, and monomer Y has increased alanine dehydrogenase activity. Unlike monomer X, monomer Y is not capable of aggregating to form higher molecular weight polymers as its concentration is increased. Both glutamate and alanine dehydrogenase activities are lost during denaturation of GDH at low ionic strengths.

Recent work on inactivation of glutamate dehydrogenase in the presence of D_2O (Henderson and Henderson, 1969) shows that several allosteric transitions take place in the enzyme molecule. According to these authors, the active polymer is in equilibrium with an active monomer which in its turn is in equilibrium with an inactive monomer. The observation that D_2O stabilizes the polymer and at the same time reduces inactivation supports this scheme.

The results of the experiments described in Chapter 4 confirm that the monomer is the species involved in inactivation. A more precise description was obtained by calculating the molecular species distribution in GDH undergoing inactivation. With regard to the use of Eisenberg and Tomkins' data (1968) in the calculation of molecular species distribution, one may question whether it is correct to extrapolate molecular weight determinations made in 0.2 M phosphate buffer (pH 7) to enzyme which was diluted in 10 mM Tris-HCl (pH 8). As a matter of fact, determination of molecular weight with an unstable enzyme would be difficult to interpret, as there is probably more than one kind

of monomer in an inactivating system (see Section 5.4). With reference to the discrepancy in pH, FRIEDEN (1962c) found no significant differences between the sedimentation patterns of glutamate dehydrogenase in phosphate and Tris-acetate buffers; an essentially constant sedimentation coefficient was determined in the pH range 6–10 in Tris-acetate buffer.

It is more difficult to find justification for the use of the above data considering the discrepancy in ionic strength between these experiments and those of EISENBERG and TOMKINS (1968). In taking the molecular weight distribution determined in 0.2 M phosphate buffer as a basis of calculation, it has been assumed that it holds at least for the state of the enzyme at time zero (i.e., just prior to inactivation) under these conditions. However, in the calculations presented in Section 4.4, the same method was applied to enzyme undergoing inactivation during periods of time up to 30 minutes. The argument in this case is that the data were applied to low enzyme concentrations (the highest being 0.1 mg/ml, Table 4.3) where the non-ideality term is less marked.This is due to the fact that the second virial coefficient is multiplied by the concentration factor, as seen in the following relation

$$1/M_{app} = 1/M_w + 2\overline{B}c$$

Even if $\overline{B}$ is large, the deviation of the real molecular weight from the apparent molecular weight, which is the one determined experimentally, will be less significant if c is very small (see also SCHACHMAN, 1959).

While keeping in mind the above remarks, it seems nevertheless justified to conclude that the monomer is the molecular species involved in inactivation of glutamate dehydrogenase at low ionic strengths. The linear relationship between the rate of inactivation and the amount of monomer, as well as the fact that inactivation of the monomer is the rate-limiting step in inactivation are experimental findings supporting this conclusion.

5. PROPERTIES OF THE INACTIVATED ENZYME

5.1. Effect of various ligands upon stability

Low concentrations of glutamate dehydrogenase underwent a gradual decline in catalytic activity following incubation at 25° in a medium of low ionic strength (10 mM Tris-HCl-0.1 mM EDTA, pH 8). The effect of several compounds was tested by adding them to the buffer solution in which the enzyme was incubated. At first, catalytic activity was the main criterion for characterizing the stability of the enzyme after various periods of incubation (Eisenkraft and Veeger, 1968).

Most of the experiments described below were carried out at an enzyme concentration of 1 mg/ml*. This gave a convenient decline in activity within 30 minutes for the study of both protective and inactivation-promoting agents.

First to be tested for their effect on enzyme stability were the substrates and coenzymes of the reactions catalysed by glutamate dehydrogenase. The behaviour of glutamate and 2-oxoglutarate is illustrated in Fig. 5.1.

The stronger protective effect of 2-oxoglutarate as compared to that of glutamate is evident. In subsequent experiments, similar results were obtained with one tenth this concentration of 2-oxoglutarate (see Fig. 5.2).

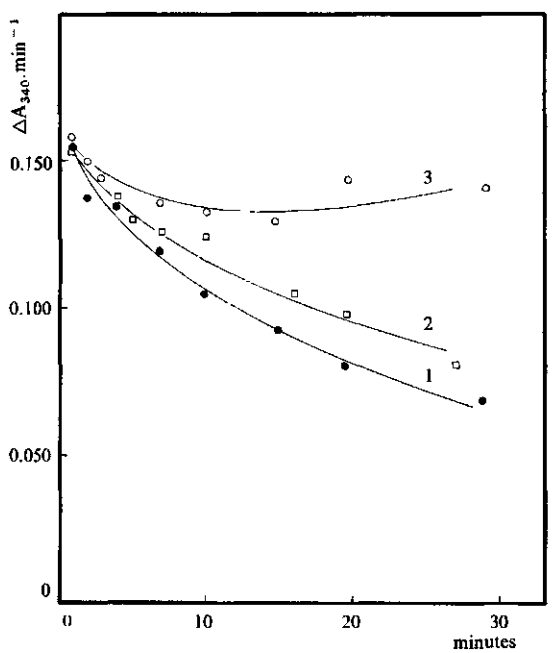

Fig. 5.1. Effect of substrates on the rate of inactivation of GDH (oxidative deamination). Curve 1- control; curve 2- with 2.5 mM glu; curve 3- with 1 mM 2-OG. Enzyme concentration – 1 mg/ml.

* Taking 50000 as the equivalent weight of the enzyme per coenzyme binding site, a solution of 1 mg/ml is 20 μM.

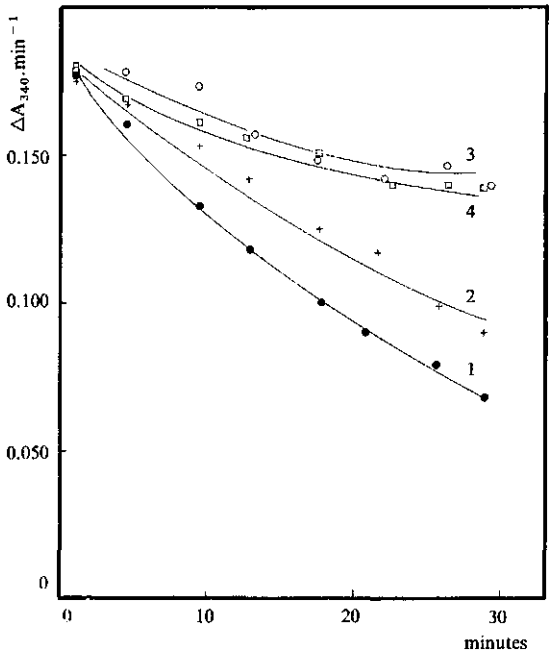

FIG. 5.2. Effect of substrates on the rate of inactivation of GDH. Curve 1- control; curve 2- with 2.5 mM NH_4Cl; curve 3- with 0.1 mM 2-OG; curve 4- with 2.5 mM NH_4Cl plus 0.1 mM 2-OG. Enzyme concentration – 1 mg/ml.

A comparison of Figs. 5.1 and 5.2 reveals a certain resemblance between ammonium chloride and sodium glutamate with regard to their effect on enzyme stability. Fig. 5.2 also shows that no synergistic action is achieved by a combination of 2.5 mM NH_4Cl and 0.1 mM 2-oxoglutarate.

It is known (DI PRISCO and STRECKER, 1966) that higher concentrations of the substrates and other ionic compounds offer protection against inactivation in aqueous solution. The reason for presenting these results has been to show that ionic effects alone do no explain the ensuing stabilization.

An additional example is offered by the curves in Fig. 5.3. The partially protective effect of 2-oxoglutarate at 0.1 mM is again apparent. At a concentration of 0.2 mM, the coenzyme NADPH appears to be only slightly protective; when 0.2 mM NADPH is added to the enzyme together with 0.1 mM 2-oxoglutarate, the protective effect of the latter is abolished.

With regard to their effect on the stability of glutamate dehydrogenase, NADH and NADPH exibit a remarkable behaviour. Generally speaking, one may state that very low, substoichiometric concentrations (assuming one binding site per subunit of 50000 molecular weight) promote inactivation, while saturating concentrations prevent it. NADH is more efficient than NADPH in protecting the enzyme: 0.1 mM NADH is sufficient for full stabilization, as compared to 0.3–0.4 mM NADPH. At the very high concentrations required to demonstrate stabilization with NADPH, the inhibition caused by this compound in the assay becomes apparent (Fig. 5.4).

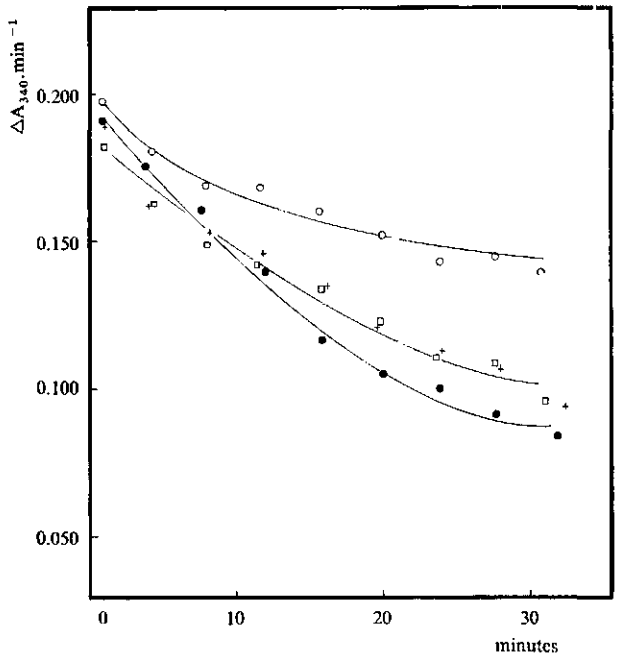

FIG. 5.3. Effect of 0.2 mM NADPH on the stability of GDH. Dots- control experiment; open circles- with 0.1 mM 2-OG; crosses- with 0.2 mM NADPH; squares- with 0.2 mM NADPH plus 0.1 mM 2-OG. Enzyme concentration – 1 mg/ml.

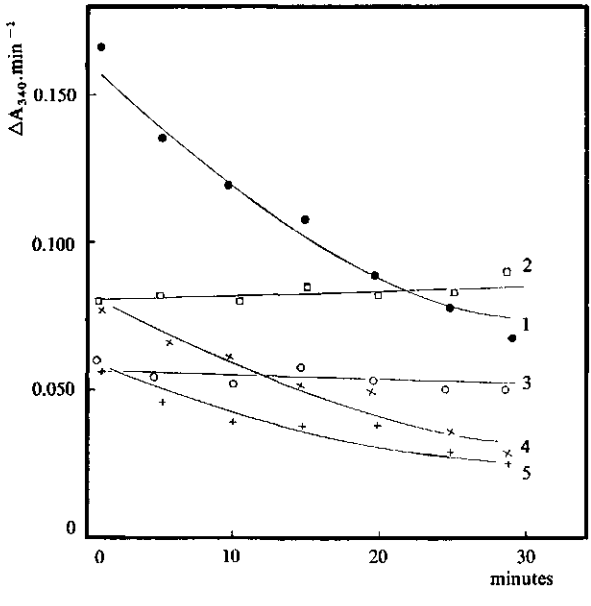

FIG. 5.4. Stabilizing effect of saturating [NADH] and [NADPH]. Curve 1- control; curve 2- enzyme incubated with 0.4 mM NADH; curve 3- enzyme incubated with 0.4 mM NADPH; curves 4 and 5- like 1, but with NADH and NADPH respectively added to the assay at the same concentrations as obtained in the assay mixtures of experiments 2 and 3 after dilution of the enzyme (5 μM). Enzyme concentration – 1 mg/ml.

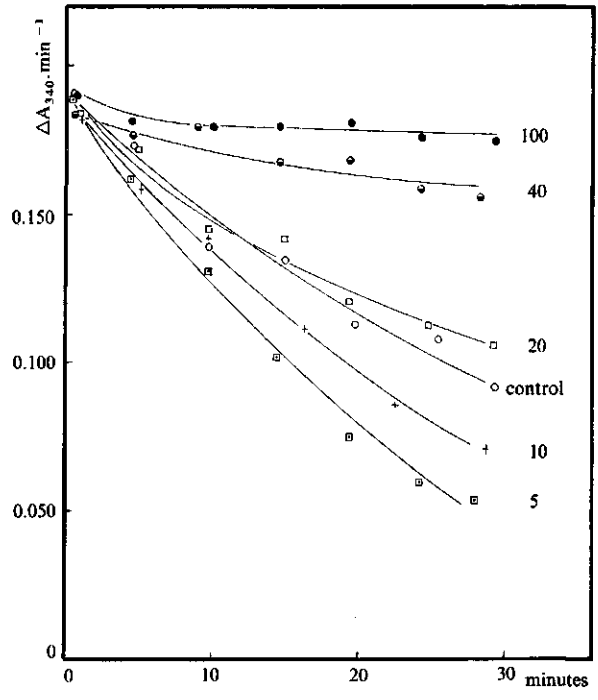

FIG. 5.5. Effect of various concentrations of NADH on the stability of GDH. The figures represent micromolar concentrations of NADH.

At the same time, one can see that in the presence of the coenzymes, there is no decline in activity during the period of the experiment.

Below the concentration of about 20μM, both NADH and NADPH promote inactivation of the enzyme. This is illustrated for NADH in Fig. 5.5, where one can also see that a concentration of 0.1 mM NADH offers full protection against inactivation.

Among the purine nucleotides, ADP has been recognised as the most effective protective agent (DI PRISCO and STRECKER, 1966). In several experiments in which various ADP concentrations were used, full stabilization was achieved at a concentration as low as 20 μM. Moreover, at the substoichiometric concentration of 10 μM, ADP can still offer significant protection (Fig. 5.6).

ATP is considerably less efficient than ADP. Very slight protection is observed at a concentration of 10 μM, and a concentration of at least 100 μM is required for full stabilization. However, the possibility of smal lcontamination with ADP cannot be eliminated. As expected, no inactivation takes place in the presence of 0.1 mM ADP plus 0.1 mM 2-oxoglutarate.

Working with 0.5 mg/ml enzyme at pH 7.5, HENDERSON and HENDERSON (1969) observed enhancement of inactivation in the presence of GTP. No effect on the rate of inactivation was seen with GDP or GTP in these experiments, neither alone nor in combination with a saturating NADH concentration (Fig. 5.7).

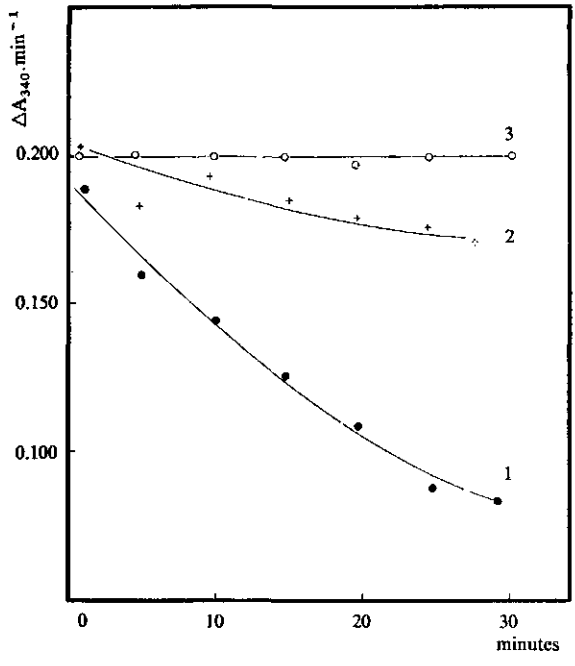

FIG. 5.6. Effect of ADP on the stability of GDH. Curve 1-control; curve 2- with 10 μM ADP; curve 3 – with 20μM ADP. The [enzyme] was 20μM (based on a 50000 mol. wt. binding unit.)

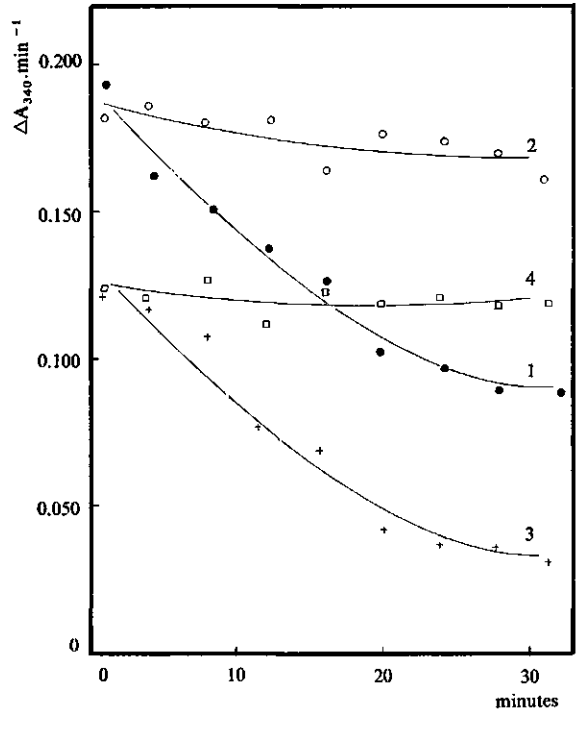

Fig. 5.7. Effect of NADH and GTP on the stability of GDH. Curve 1-control; curve 2- with 0.2 mM NADH; curve 3- with 10 μM GTP; curve 4- with 0.2 mM NADH plus 10 μM GTP.

While NADH does not inhibit the catalytic reaction at this concentration, GTP strongly inhibits even at the very low final concentration obtained after dilution in the assay mixture.

Urea is a well-known hydrogen-bond breaking and denaturing agent. At low concentrations, such as 1 and 10 mM, urea had no effect on the enzyme. However, at a concentration of 1 M, 60% of the original activity was lost by the 10th minute of incubation. At 3 M urea, 85% of the original activity was lost by the 5th minute of incubation. The experiments that follow were performed with 0.5 M urea, which caused a measurable decline in activity at 25° within 30 minutes of incubation.

The effect of various ligands and combinations of ligands on stability is illustrated in Fig. 5.8. Special emphasis is laid on the ability of ADP, and to a lesser extent, of 2-oxoglutarate to counteract, the destructive effect of urea and of NADPH. It is surprising that the protective effect of 2-oxoglutarate, which abolishes the inactivation-promoting effect of 5–10 μM NADPH, should be completely masked in the presence of 0.2 mM NADPH, which in itself has a slight protective effect (see Fig. 5.3).

When a partially inactivated enzyme was supplied with stabilizing agents such as ADP or saturating concentrations of NADH, no further inactivation took place. In the case of 0.1 mM ADP, almost full recovery of the initial activity was observed at the end of 60 minutes if ADP was added in the tenth

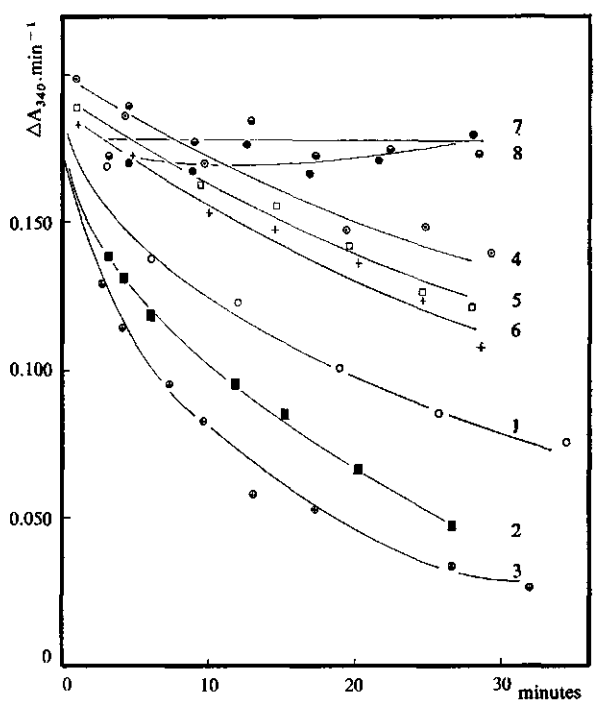

FIG. 5.8. Effect of various ligands and combinations of ligands upon enzyme stability. Curve 1- control experiment; curve 2- with 0.5 M urea; 3- with 10 μM NADPH; curve 4- with 0.1 mM 2-OG; curve 5- with 0.5 M urea and 0.1 mM 2-OG; curve 6- with 10 μM NADPH and 0.1 mM 2-OG; curve 7- with 10 μM NADPH and 0.1 mM ADP; curve 8- with 0.5 M urea and 0.1 mM ADP.

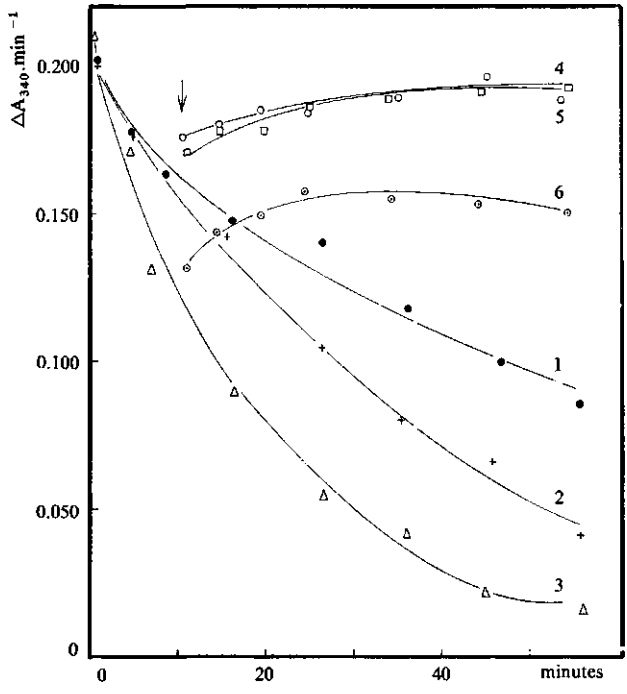

FIG. 5.9. Addition of ADP to a partially inactivated enzyme. Curve 1-control; curve 2- with 0.5 M urea; curve 3- with 10 μM NADPH; curve 4- with 0.1 mM ADP added to the control at minute 10; curve 5- with 0.1 mM ADP added to the enzyme incubated with 0.5 M urea; curve 6- with 0.1 mM ADP added to the enzyme incubated with 10 μM NADPH. The arrow represents the time of ADP addition. Protein concentration 0.8 mg/ml.

minute of incubation; this occurred both in the control and in the experiment in which inactivation took place in the presence of 0.5 M urea (Fig. 5.9).

Addition of ADP to enzyme which had been inactivated in the presence of 10 μM NADPH led to only partial regain of activity. At first this result was attributed to the greater extent of inactivation of the enzyme after 10 minutes' incubation with NADPH as compared to the control. However, similar results were obtained in another experiment in which ADP was added after 5 minutes, corresponding to the level of activity reached by the control after 10 minutes. This suggests that ADP is less efficient in reactivating the NADPH-inactivated enzyme.

At a concentration of 0.1 mM, NADH also prevented further inactivation when added to the enzyme at the end of 10 minutes' incubation. This held also for enzyme incubated in the presence of 0.5 M urea and 10 μM NADPH. However, no return to the original level of catalytic activity was achieved under these conditions. A similar behaviour was exhibited by 2-oxoglutarate at a concentration of 0.5 M, while GDP at 0.1 mM neither slowed down nor accelerated the rate of inactivation.

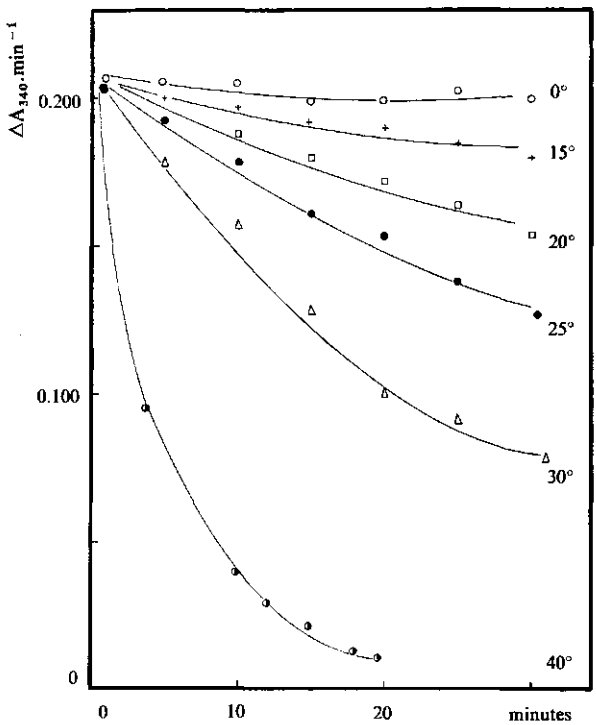

FIG. 5.10. Effect of temperature on the stability of GDH. Enzyme at a concentration of 1 mg/ml was incubated at the indicated temperatures and assayed at 25° at various time intervals.

5.2. EFFECT OF TEMPERATURE UPON STABILITY

In the experiments described below, the decline in activity of a 1 mg/ml enzyme solution was followed at several carefully controlled temperatures. Aliquots were removed from the water bath at various time intervals and assayed for activity at 25°. The results of such an experiment are illustrated in Fig. 5.10.

In an attempt to calculate activation energies characteristic of the process of inactivation, the pseudo-first order rate constants obtained at various temperatures were plotted against the reciprocal absolute temperatures. The plots were linear in the range of 8–25°. Below 8° large inaccuracy was introduced by the low rates of inactivation. Similar difficulties arose when working with relatively concentrated enzyme solutions (in the range of 1 mg/ml) or under conditions where the enzyme was stable even at 25° (e.g., in the presence of ADP).

Most experiments were carried out with an enzyme concentration of 0.1 mg/ml, with or without 10 μM NADPH (Fig. 5.11).

The 2 lines, which were fitted by the method of the least squares, appear to be parallel. Energies of activation calculated from the slopes of the lines are in the range of 20000 cal.mole^{-1}. While the slopes are equal, there is a difference in intercept or rate constant.

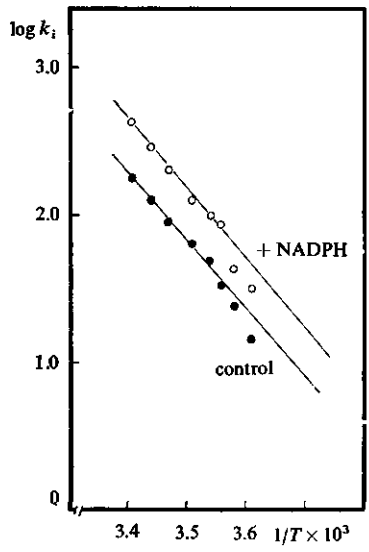

FIG. 5.11. Arrhenius plots of enzyme inactivation. Enzyme concentration -0.1 mg/ml. The concentration of NADPH (upper line) was 10 μM. The points represent averages from 3 experiments. Pseudo-first order rate constants of inactivation were determined as described in the legend to Fig. 4.1, except that the values of k_i refer to periods of 20 minutes.

The entropy of activation can be calculated from

$$k_i = kT/h.e^{\Delta S^*/R}.e^{-\Delta H^*/RT} \qquad (1)$$

where

k = Boltzmann's constant
h = Planck's constant
k_i = rate constant at T

Hence

$$ln\ k_i = ln\ kT/h + \Delta S^*/R + \Delta H^*/RT \qquad (2)$$

Similar equations can be written for both control- and NADPH-lines in Fig. 5.11. Subtraction of one from the other yields

$$ln\ k^1{}_i - ln\ k^2{}_i = (\Delta S^{*1} - \Delta S^{*2})/R = \Delta\Delta S^*/R \qquad (3)$$

The value calculated for $\Delta\Delta S^*$ is 1.4 e.u. This small increase in entropy is a consequence of the presence of 10 μM NADPH during inactivation of the enzyme.

5.3. SPECTRAL PROPERTIES OF THE INACTIVATED ENZYME

In experiments on inactivation with NADPH and urea, attention was attracted to the obvious difference in the turbidity of the solutions after 30 minutes' incubation with either 0.5 M urea or with NADPH at substoichiometric concentrations. The control enzyme solution was less turbid than the urea-inactivated enzyme, but was slightly more turbid than the NADPH-inactivated enzyme. These visual observations were tested by recording UV- spectra of the protein at various stages of inactivation. The increase in absorption at wavelengths where the protein does not absorb indicated light-scattering contribution. This was greatly enhanced in the presence of 0.5 M urea, and was totally

abolished in the presence of 0.1 mM ADP. It was slightly greater in the control than in the experiment with 10 μM NADPH.

FRIEDEN (1963a) reported that in the presence of NADPH, catalytic activity was lost long before any turbidity appeared in the solution. In the present experiments, the pronounced turbidity caused by inactivation in the presence of urea was accompanied by a comparatively small decline in enzymic activity. This, like the difference in response to reactivation (see Section 5.1) between NADPH- and urea-inactivated enzymes, suggests that different products form as a result of inactivation in the 2 cases.

The technique of difference spectra was used in an attempt to find spectral changes accompanying inactivation of the enzyme. No thermostatted tandem arrangement of cells was available, as is usually referred to in work on difference spectra (FISHER and CROSS, 1965). When experiments were carried out at several concentrations of enzyme, no clear relationship was found between the magnitude of the recorded change and enzyme concentration. UV-spectra were recorded with both stable enzyme, diluted in 0.1 M phosphate - EDTA (pH 8), and enzyme diluted in 10 mM Tris – EDTA (pH 8). Both spectra were recorded twice: immediately after dilution in the respective buffers, and at the end of 45 minutes (during which the cuvettes were shielded from light). While the spectrum of the stable enzyme remained unchanged during the period of the experiment, a considerable increase in absorption occurred in the enzyme undergoing inactivation: the 280 nm protein peak was no longer seen clearly due to the disappearance of the descending part of the peak at about 260 nm. Instead of this, an ascending limb characterized the spectrum of the inactivated enzyme. This was also present in the difference spectrum of stable vs. unstable enzyme after correction for the light-scattering contribution (see Fig. 5.12).

The light-scattering effect may be represented (DOTY and GEIDUSCHEK, 1953a) in terms of the turbidity τ which is $\tau = K.\lambda^{-n}$ where K and n are constants. According to this equation, a log-log plot of the experimental data should be linear in the wavelength region where the effect is due to scattering and should depart from linearity in the region of absorption. Extrapolation of the linear

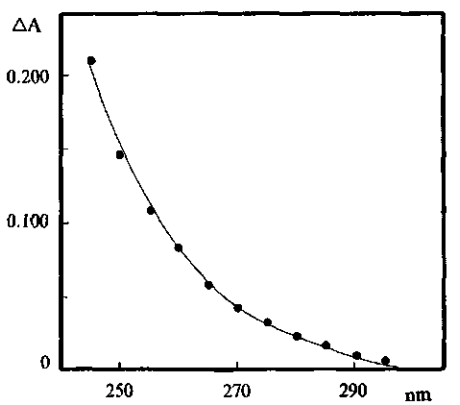

FIG. 5.12. Difference spectrum between stable enzyme and enzyme inactivated by incubation in dilute Tris buffer (45 min/ 25 °), corrected for light scattering. Enzyme concentration -0.05 mg/ml. For other details, see text.

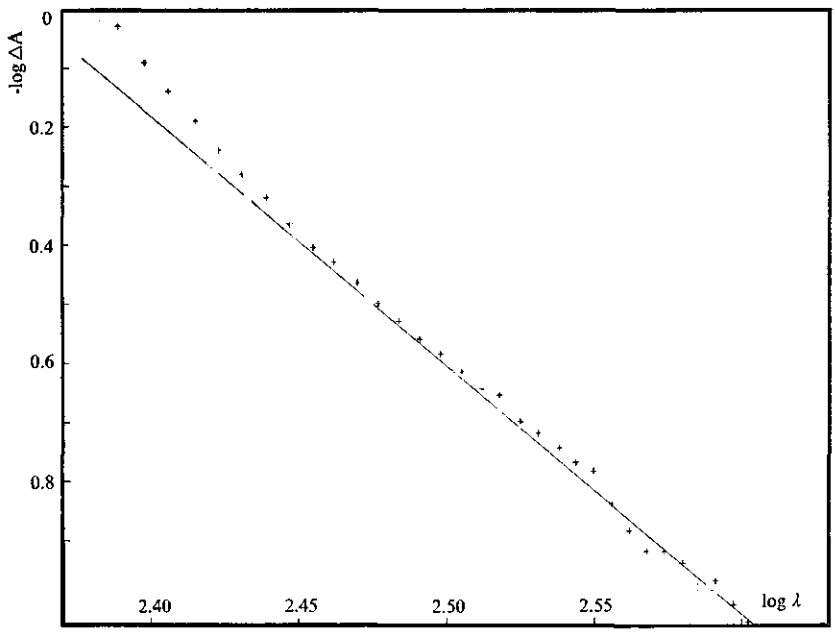

FIG. 5.13. Log-log plot of absorbance vs. wavelength for the experiment illustrated in Fig. 5.12. Correction method for light scattering contribution after LEACH and SCHERAGA (1960).

portion due to scattering into the absorption region indicates the scattering contribution, which may be subtracted from the total optical density in order to obtain the absorption effect. This forms the basis of the method of LEACH and SCHERAGA (1960) for eliminating the contribution of light-scattering to the UV-spectra of proteins.

As seen in Fig. 5.13, the wavelength region in which there is a departure from linearity corresponds to the region of absorption of phenylalanine (DOTY and GEIDUSCHEK, 1953a; DONOVAN et al., 1958) and cystine (see below). However, several points should be stressed. Extrapolation to the absorbing region becomes uncertain (DOTY and GEIDUSCHEK, 1953a) when τ is very high due to high concentration and high molecular weight because in this case, the refractive index increment is no longer a constant. In the above equation, K represents a quantity which depends on the size and shape of the solute particle, its refractive index and the refractive index of the suspending medium. The value of n depends on the dimensions of the solute particles; it does not exceed 4 when none of them is large compared with the wavelength of the incident light. In these experiments, values between 4.0 and 4.5 were found at all enzyme concentrations.

It is generally assumed that a difference spectrum arises from a change in the environment of a chromophore (DOTY and GEIDUSCHEK, 1953a). Detailed spec-

trophotometric studies (CROSS and FISHER, 1965, 1966) have shown that no phenylalanyl, tryptophanyl or tyrosyl residues undergo a change in environment in the course of the dilution-dependent dissociation of glutamate dehydrogenase into the monomer. Conversely, the splitting of the monomer into the peptide chains leads to a change in the environment of tyrosyl residues (FISHER et al., 1962b). Solvent-perturbation studies (CROSS and FISHER, 1966) point to the absence of large proportions of phenylalanine on the surface, while there are probably large concentrations in the anhydrous interior suggesting possible involvement in hydrophobic bonding. In the light of the above difference spectrum, it may be assumed that some of these phenylalanyl residues come into a more polar environment during inactivation.

As YANARI and BOVEY (1960) pointed out, the quantitative interpretation of difference spectra can often be misleading because of the varying contributions of spectral shift, spectral broadening and overall increase in absorption in different cases. The maxima in a difference spectrum occur near the inflection points of the absorption spectra and therefore slight spectral broadening can yield a large difference spectrum, even though the λ_{max} is practically unchanged (YANARI and BOVEY, 1960). According to DONOVAN et al. (1958), the difference spectrum shows peaks at 247, 253, 259, 265 and 268 nm if there is a change in the charge of the carboxyl group of phenylalanine. Although no such peaks have been found but only an ascending limb (see Fig. 5.12), one cannot be sure that they are not masked, among others, by cystine which is known to absorb in the 220–250 nm region, probably overlapping the CO-NH band (DOTY and GEIDUSCHEK, 1953a). Changes in the environment of cystine residues have been reported to accompany inactivation of glutamate dehydrogenase (BITENSKY et al., 1965).

CROSS and FISHER measured the phenylalanine peaks in the 250–270 nm range not as the height from the base line but as the deviation from the generalized absorption in this area, 'as these spectral peaks are often imposed on high slopes of differential absorption from spectral contributions in the far UV region' (CROSS and FISHER, 1966). SEGAL (1963) found an ascending curve at 246 nm for the difference spectrum between 2 bovine serum albumins at pH 8.7, one of which was heated for 20 minutes at 100°. A similar increase was found by him after alkaline treatment and was attributed to ionization of side chain groups. The author states that rupture of hydrogen bonds between peptide chains, which probably takes place during heat denaturation, has a much smaller effect on the spectrum and is easily masked by ionization of OH- groups on side chains.

While keeping in mind the limitations of the above experiments, the difference spectrum between stable and unstable enzyme suggests a change in the environment of phenylalanine and/or cystine residues taking place as a result of inactivation. This is consistent with the idea of fragments (probably not the peptide chains) liberated from the active monomer in the course of inactivation. The increased turbidity of the enzyme solutions under certain conditions is probably due to aggregation of these fragments.

5.4. SUCROSE GRADIENT CENTRIFUGATION STUDIES

5.4.1. *Introduction.*

MARTIN and AMES adapted sucrose gradient centrifugation, which had been used previously in the determination of sedimentation constants of viruses, mitochondria, microsomes and ribosomes, to relatively low molecular weight substances (MARTIN and AMES, 1961). The authors also gave a theoretical characterisation of the system and demonstrated applicability of the technique to the determination of sedimentation constants of enzymes in multicomponent solutions. Sedimentation constants were determined for 3 enzymes of the histidine biosynthesis pathway in *Salmonella typhimurium* (MARTIN and AMES, 1961).

Subsequently the method has gained widespread use. TSUTSUI and MARKS (1962) demonstrated that the sedimentation of glucose 6-phosphate dehydrogenase in sucrose density gradients is affected by NADP concentration. These authors postulated that either the monomer-dimer equilibrium was affected or interconvertible conformational states were formed with no difference in the state of aggregation. Studies on the same enzyme from human erythrocytes (KIRKMAN and HENDRICKSON, 1962) made it possible to differentiate between a monomer and dimer with no bound NADP and a dimer with bound NADP.

CODDINGTON and FINCHAM (1965) studied hybrid enzyme formation in glutamate dehydrogenase mutants of *Neurospora crassa* by the method of sucrose gradient centrifugation. This technique made it possible to confirm previous evidence according to which the hybrid molecules are of the same size as the wild type and pure mutant enzyme varieties.

NAD-specific and NADP-specific glutamate dehydrogenases from *Thiobacillus novellus* were characterized by LEJOHN et al. (1968). Apart from kinetic and other methods, sucrose gradient centrifugation was used. Similar molecular weights (about 120000) were determined for both enzymes.

LEJOHN and JACKSON (1968) reported that the NAD-specific glutamate dehydrogenase from *Blastocladiella emersonii* dissociates in sucrose density gradients into subunits or monomers with consequent loss of enzymic activity. Stabilizing allosteric ligands such as AMP, ADP and NAD^+ prevented this inactivation. The molecular weight of the protein was estimated to be about 230000.

To the author's knowledge no sucrose gradient centrifugation has been applied as yet to beef liver glutamate dehydrogenase, the molecular weight of which considerably exceeds that of the enzymes isolated from other sources. The choice of this method was dictated by the desire to study the molecular properties of the enzyme at various stages and under various conditions of inactivation. Centrifugation in the cold and preparation of the sucrose solutions in a 'stabilizing environment' (0.1 M phosphate buffer) made it possible to retard considerably the progress of inactivation after layering on the gradients.

In the first series of experiments, 4% and 20% sucrose solutions were used for preparing the gradients, according to the technique of MARTIN and AMES (1961). In order to facilitate comparison with previous experiments (see Section

5.1), the enzyme concentration was chosen at 1 mg/ml. In the long runs (17 hours, 3–4°), the enzyme sedimented quite close to the bottom of the tube in the 4–20% gradients. In contrast, it appeared in the vicinity of the meniscus in short runs (4 hours, 22°). The best results were obtained in long runs (17 hours, 3–4°) with 9–25% sucrose gradients.

5.4.2. *Sedimentation in 4–20% sucrose gradients.*

The sedimentation pattern of glutamate dehydrogenase in preformed sucrose gradients was studied under a variety of conditions. In order to detect changes in the sedimentation of the ezyme after 30 minutes of inactivation (see Section 5.1.), parallel runs were carried out with the same concentration of stable enzyme (1 mg/ml). This was obtained by diluting the original enzyme suspension in 0.1 M phosphate buffer - 0.1 mM EDTA (pH 8) followed immediately by layering on top of one of the 3 precooled gradients. When used, markers were layered on the same gradient with the stable enzyme, and never together with the sample of inactivated enzyme. For the sake of simplicity, enzyme diluted prior to the experiment in 0.1 M phosphate buffer-EDTA will be called below 'stable enzyme', in contrast to enzyme inactivated by incubation for 30 minutes at 25° in 10 mM Tris-HCl-0.1 mM EDTA (pH 8) which will be referred to as 'unstable enzyme'.

A slight shift towards the meniscus as well as decreased enzymic activity were observed in the unstable enzyme as compared to the stable one. When inactivation took place in the presence of 0.1 mM 2-oxoglutarate, these changes took place to a smaller extent (Fig. 5.14).

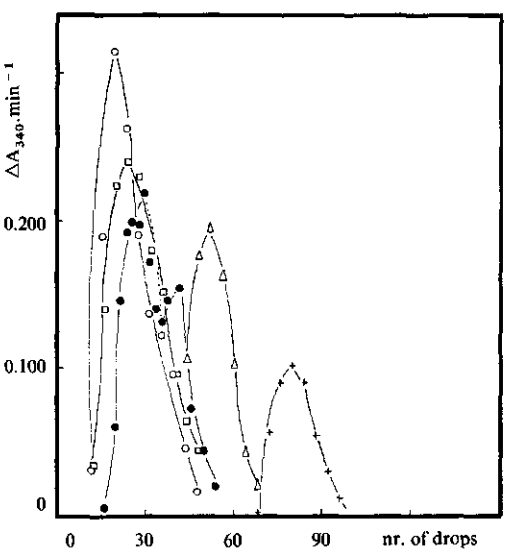

FIG. 5.14. Sedimentation pattern of GDH and markers in 4–20% sucrose density gradients. Open circles – stable enzyme; dots – unstable enzyme; squares – with 0.1 mM 2-OG added to the incubation mixture of the unstable enzyme; triangles – catalase; crosses – alcohol dehydrogenase. Prior to the run, all 3 enzymes (stable GDH and 2 markers) were layered on one of the tubes, while the same amount (100 μg) of unstable enzyme and unstable enzyme with 2-OG, respectively, was layered on the remaining 2 tubes. Activities (measured in 4-drop fractions, except for the peak region of the unstable enzyme where 2-drop fractions were collected) are expressed in $\Delta A_{340}.min^{-1}$. 10 μl^{-1} for GDH (reductive amination) and alcohol dehydrogenase, and in $\Delta A_{240}.min^{-1}.2\ \mu l^{-1}$ for catalase.

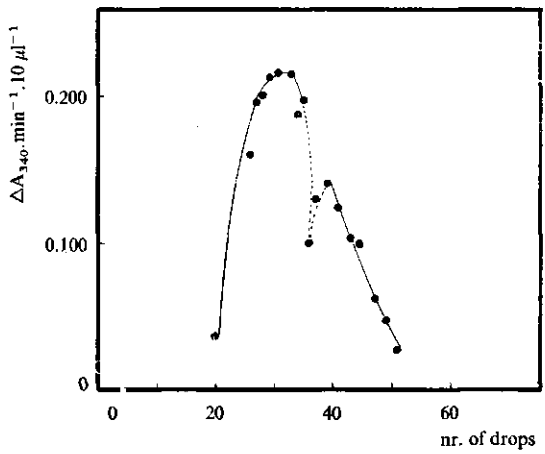

FIG. 5.15. Sedimentation pattern of the control (unstable enzyme) from Fig. 5.14 on a slightly enlarged scale.

Noteworthy is the irregular sedimentation curve of the unstable enzyme. The appearance of a second peak is clearly seen in the experiment illustrated in Fig. 5.15, indicating lower molecular weight components.

An identical sedimentation pattern to that of the stable enzyme was found when ADP at a concentration of 10 μM was added to the incubation mixture.

At this stage of the work it became evident that the 4–20% gradients did not offer optimal conditions for the sedimentation of beef liver glutamate dehydrogenase, a polydisperse system containing species of various molecular weights. As the protein was found very close to the bottom of the tube at the end of the run, small variations in sedimentation under different conditions might have been overlooked.

5.4.3. *Sedimentation in 9–25% sucrose gradients*

Better resolution was obtained by this method, as seen in Fig. 5.16 (cf. Fig. 5.14). It should be noted that inactivation took place here at 35° unlike in all the other experiments where the temperature of incubation was set at 25°.

The appearance of a shoulder in the stable enzyme (Fig. 5.17) was at first attributed to experimental errors. However, as perfectly regular sedimentation patterns were found with alcohol dehydrogenase and catalase under similar conditions, the shoulder was attributed to the polydisperse character of the glutamate dehydrogenase solutions. In protein determinations a single peak was found (Fig. 5.18). However, due to the very low amounts of protein, larger fractions had to be collected. Similarly, larger fractions of sedimented stable enzyme were collected in the experiments where 4–20% gradients were used; this may be one reason for the lack of visible side-peaks in the stable enzyme in those experiments, as opposed to the unstable enzyme where smaller fractions were used in the determinations.

The results shown in Fig. 5.18A in conjunction with the calibration curve for glutamate dehydrogenase (Fig. 2.2B) provide a yield of about 65% protein in the inactivation experiment of Fig. 5.18B. In the case of a stable enzyme, a

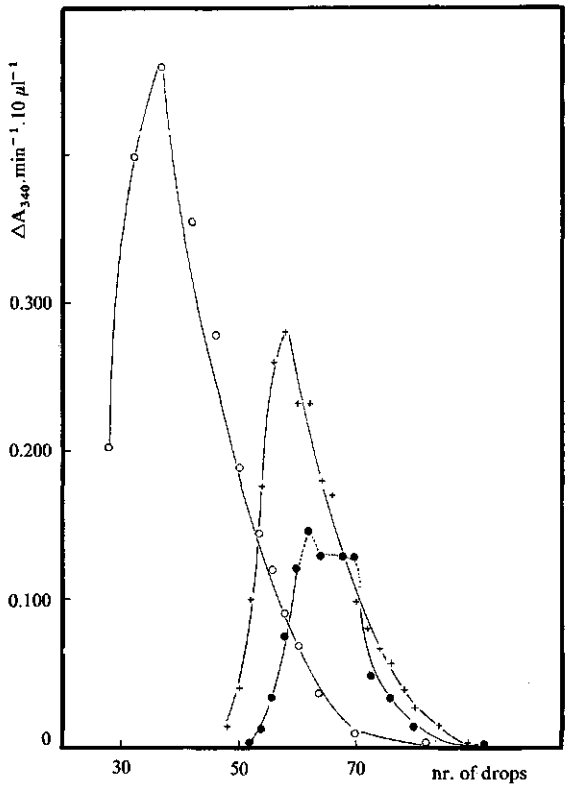

FIG. 5.16. Sedimentation of GDH in 9–25% sucrose density gradients. Dots- unstable enzyme (control experiment); open circles-unstable enzyme preincubated with 0.1 mM ADP; crosses-unstable enzyme preincubated with 0.1 mM 2-OG. The temperature of incubation was 35°; 150 μg protein was layered on each tube, and fractions of 2 drops were collected at the end of the run.

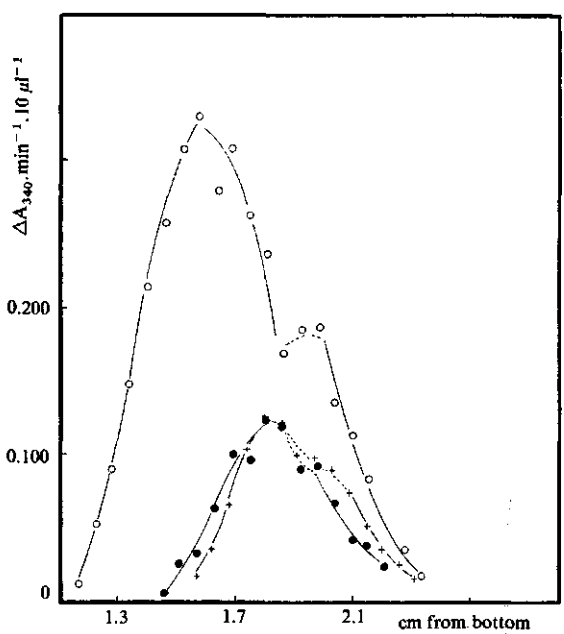

FIG. 5.17. Sedimentation of GDH in 9–25% sucrose density gradients. Open circles represent stable enzyme, crosses and dots represent unstable enzyme in duplo. The amount of protein was 100 μg per tube.

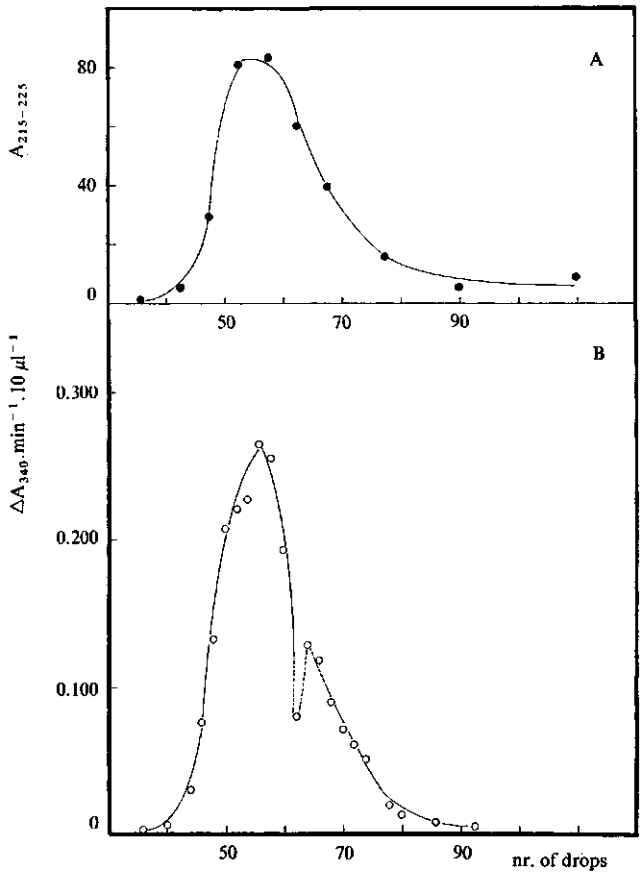

FIG. 5.18. Parallel activity assay and protein determination in an unstable enzyme. The amount of protein was 150 μg per tube. In part A, where protein readings are recorded, the points are represented in the middle of the 5-drop fractions. Fractions of 2 drops were collected for activity assay (part B).

yield of about 85% was calculated. It seems reasonable to assume that part of the total protein is not determined by this method due to the excessive dilution undergone in the gradient, i.e., almost 10fold in the peak. Consequently, one must assume that the dissociation-association equilibirum, which sets in upon dilution in both stable and unstable enzymes, is responsible for the additional peaks in the sedimentation pattern. The significantly lower yield obtained for the unstable enzyme points to liberation of protein fragments during inactivation, with the result that lower enzymic activity is recorded; the specific activity is practically unchanged.

It appeared that the exact degree of inactivation was to some extent an experimental hazard despite the precautions taken to work under controlled conditions. This was not an entirely new observation: when catalytic activity was the only criterion for the progress of inactivation (Section 5.1), variations as large as 20% in the degree of inactivation in different experiments were not uncommon. Differences in the position of sedimentation of the controls in different experiments were accompanied by differences in enzymic activity,

reflecting various degrees of inactivation prior to the run. As a matter of fact, a delay of several minutes between the end of incubation at 25° and layering of the sample inevitably led to some extra inactivation before stabilization on the gradient could set in. When sucrose was added to an unstable enzyme and aliquots were assayed for periods up to 2 hours, activity was maintained at the level prevalent prior to sucrose addition. However, some inactivation may have occurred due to the high dilution undergone by the enzyme during the run.

The effect of NADH, NADPH and urea upon inactivation of the enzyme was discussed in Section 5.1 using catalytic activity as the sole criterion. Their effect on the sedimentation of the enzyme is illustrated in Figs. 5.19 and 5.20.

The sedimentation curve of enzyme inactivated in the presence of substoichiometric concentrations of NADH shows a shift of the residual activity towards lower molecular weight fractions. In contrast, enzyme incubated with 0.2 mM NADH is more active and at the same time sediments nearer to the bottom of the tube than the unstable enzyme, especially when NADH is added also to the solutions forming the gradients (Fig. 5.19A).

In Fig. 5.20 one can see that following incubation of the enzyme with 5 μM NADPH, activity is considerably lowered, but in contrast to the urea-inactivated enzyme, the residual activity is not associated with lower molecular weight components. A comparison of Figs. 5.20A and B confirms the idea that in the case of NADPH, loss of enzymic activity precedes the appearance of turbidity,

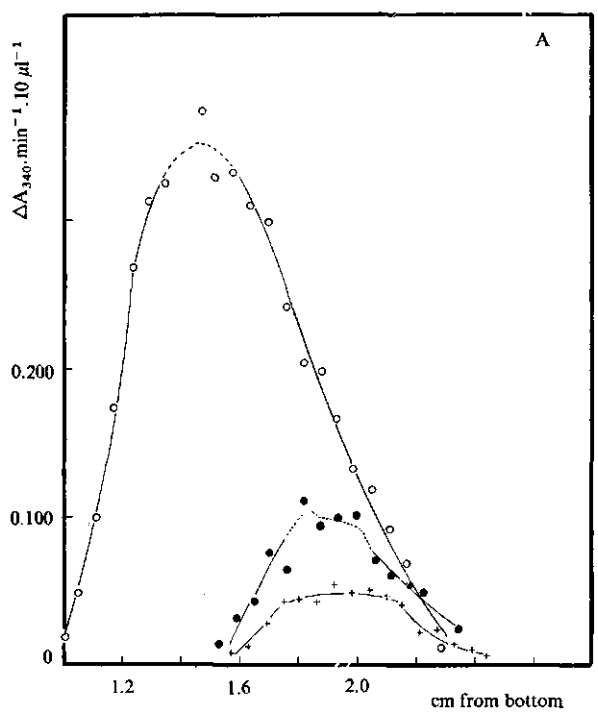

FIG. 5.19. Effect of NADH on the sedimentation of GDH in 9–25% sucrose gradients. 150 µg of protein was layered on each tube. The dots represent unstable enzyme, the crosses-enzyme preincubated with 5 µM NADH, and the open circles-enzyme preincubated with 0.2 mM NADH. Part A-NADH not present in the gradients.

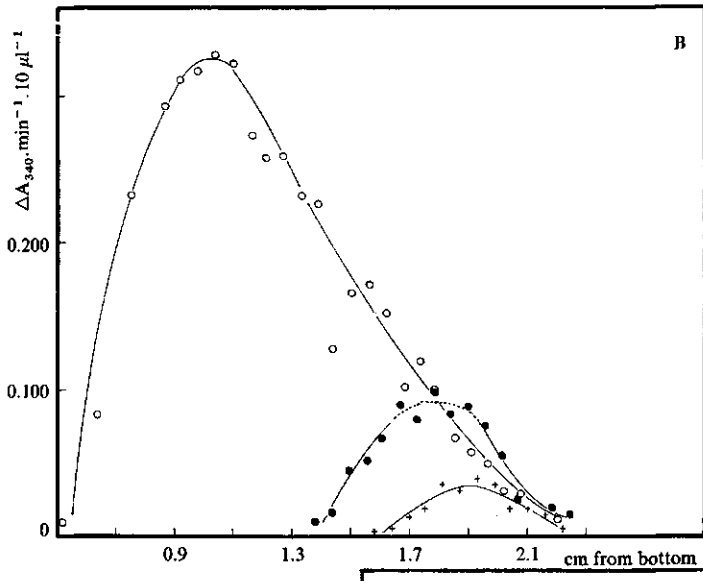

FIG. 5.19. Part B- NADH was added both to the incubation mixture of the enzyme (except for the control) and to the sucrose solutions prior to preparation of the gradient.

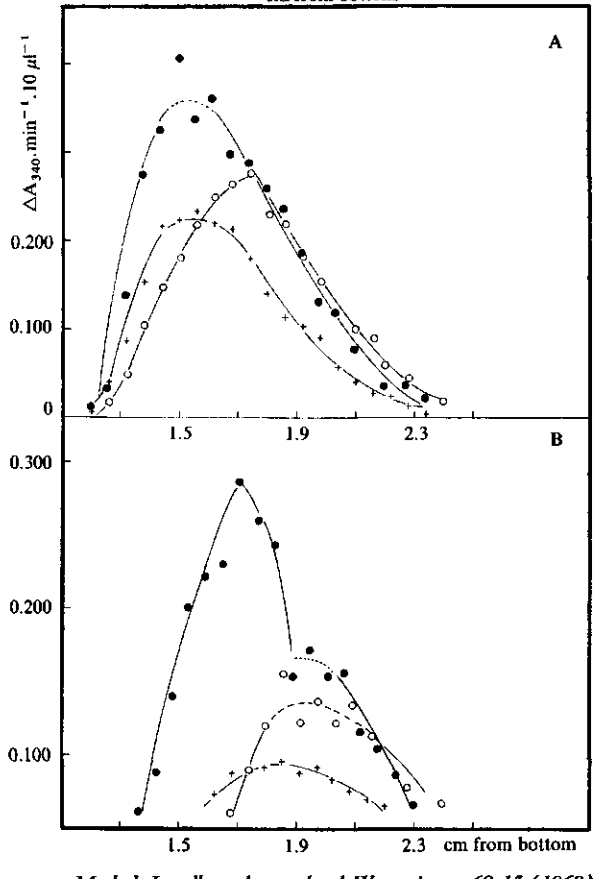

FIG. 5.20. Effect of 5 μM NADPH (crosses) and 0.5 M urea (open circles) on the sedimentation of unstable enzyme. Dots–control experiment. Other conditions like in Fig. 5.19.

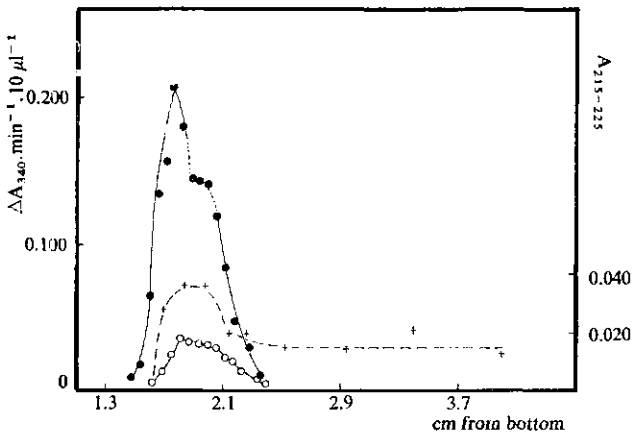

FIG. 5.21. Parallel activity (open circles) and protein (crosses) determinations in enzyme inactivated in the presence of 5 μM NADPH. The dots represent activity of the unstable enzyme (control). Only 46% of the amount of protein layered on the tube (150 μg) was determined under these conditions. Note the inactive protein in the regions near the meniscus.

assuming that the latter is due to aggregation of liberated protein fragments. Protein determinations in an experiment with enzyme incubated with 5 μM NADPH (Fig. 5.21) show that a significant amount of protein is distributed in the upper layers of the gradient pointing to 'solubilization'. One may assume that favourable conditions for aggregation of the fragments (resulting in turbidity) are created in the case of urea; no protein determinations could be made in this case due to the high absorbance of this compound in the UV- range.

When unstable enzyme, after 10 minutes' incubation at 25°, was layered on top of a gradient containing 0.1 mM ADP, the result shown in Fig. 5.22 was obtained. The position of curve 3 in this figure corresponds to the position of

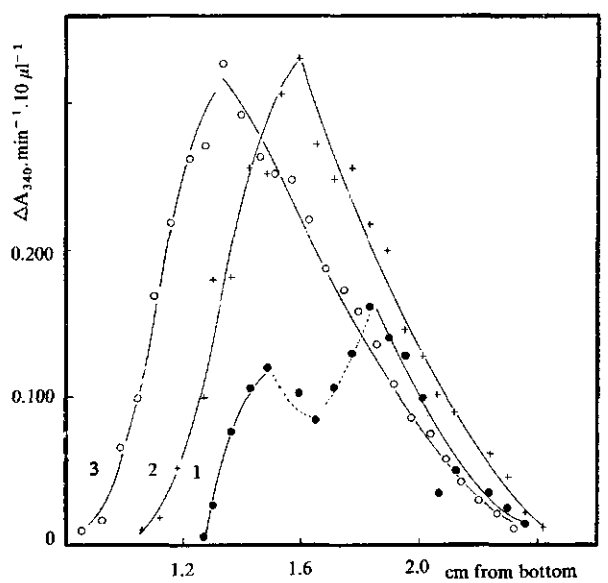

FIG. 5.22. 'Reactivation' of unstable enzyme (after 10 min/25°) by centrifugation in a sucrose gradient containing 0.1 mM ADP. Dots represent enzyme incubated for 30 min/25°, while crosses represent enzyme inactivated for 10 min/25°. Open circles represent enzyme inactivated for 10 min/25° and layered on a gradient containing 0.1 mM ADP. The amount of protein was 150 μg per tube.

stable enzyme or of unstable enzyme incubated with 0.1 mM ADP, in other experiments.

Accepting molecular weights of 150000 for alcohol dehydrogenase and 250000 for catalase, and using the approximation (MARTIN and AMES, 1961):

$$R = \left(\frac{\text{mol.wt. of unknown}}{\text{mol.wt. of marker}} \right)^{2/3}$$

where R is the ratio of the distance from the meniscus travelled by the unknown to the distance from the meniscus travelled by the marker, the following molecular weights were estimated (Table 5.1).

TABLE 5.1. Molecular weight estimates by the method of sucrose gradient centrifugation. Alcohol dehydrogenase, of molecular weight 150000, was used as standard. In the case of the unstable enzyme, ligands were added both to the enzyme during incubation at 25° and to the preformed gradients. In the case of the stable enzyme, ADP was present only in the gradient. For other experimental conditions and method of calculation, see text.

	Molecular weight
Stable enzyme — main peak	440000
— shoulder	340000
Stable enzyme + 0.1 mM ADP	499000
Unstable enzyme — main peak	360000 ± 20000
— shoulder	317000 ± 10000
Unstable enzyme + 0.5 M urea	317000 ± 10000
+ 0.2 mM NADH	499000
+ 0.1 mM ADP or 0.1 mM 2-OG	440000

The molecular weights listed in Table 5.1 are comparable to the values determined by light scattering at the corresponding protein concentrations (EISENBERG and TOMKINS, 1968). In one experiment with stable enzyme accompanied by marker in which parallel protein determinations were made, a value of 440000 was estimated as compared to 460000, reported by the above authors. Obviously, higher molecular species than the monomer are present at this concentration.

5.5. DISCUSSION

The results of experiments in which the effect of various ligands on glutamate dehydrogenase was studied show no clear relationship between the effect on catalytic activity and the effect on stability. An obvious exception is ADP, which is both an activator of the catalytic reaction and a potent protective agent. Contrarily, GDP and GTP, which are strong inhibitors of the reaction, do not affect the rate of inactivation. Substoichiometric concentrations of

NADH and NADPH accelerate inactivation, while saturating concentrations stabilize the enzyme. The substrate 2-oxoglutarate is more protective than the substrates glutamate and ammonium ion.

In glutamate dehydrogenase, like in most other dehydrogenases, the molecular weights of the polypeptide chains approximate the equivalent weights of the coenzyme binding sites (BAYLEY and RADDA, 1966; PFLEIDERER et al., 1966). In the case of glutamate dehydrogenase, this probably holds also for the purine nucleotide binding sites (PANTALONI and IWATSUBO, 1967). Consequently, it seems reasonable to assume that each polypeptide chain should be enzymically active, provided that the individual polypeptide chain can assume the same conformation as it has in the associated molecule (SUND, 1968). However, no catalytically active subunit of molecular weight 50000 has been reported for beef liver glutamate dehydrogenase. The smallest enzymically active subunit is probably the monomer of about 300000 mol.wt., which consists of 6 polypeptide chains (REITHEL, 1963; EISENBERG and Tomkins, 1968). Electron microscope studies of HORNE and GREVILLE (1963) with a dilute glutamate dehydrogenase solution (0.2 mg/ml) revealed particles the predominant shape of which was an equilateral triangle. These particles were no longer visible if the enzyme was treated with sodium dodecyl sulphate before addition of the phosphotungstate. At high magnifications, structures showing a three-fold-symmetry arrangement, as well as U- and V-shapes could also be seen. The different shapes were explained by the authors on the assumption that the subunit of glutamate dehydrogenase approximates a tetrahedron formed from 6 bars, each representing a peptide chain.

In this respect, glutamate dehydrogenase appears to be an exception as most allosteric enzymes have been reported to be composed of 4 identical subunits or of 2 identical pairs, like in hemoglobin (MONOD et al., 1965; KOSHLAND et al., 1966).

In the previous chapter it was shown that the rate limiting step in the process of inactivation of a polydisperse system like glutamate dehydrogenase at 1 mg/ml (probably a mixture of mono-, di-, tri- and tetramers) is inactivation of the monomer rather than dissociation of higher molecular weight species into the monomer. An attempt shall be made here to probe into the nature of this process, which has been arbitrarily coined 'inactivation of the monomer'.

The higher efficiency of saturating NADH- as compared to NADPH concentrations in stabilizing the enzyme is understandable in the light of the greater affinity of the enzyme for NADH as compared to NADPH (see Section 3.3). The same explanation is valid for the higher efficiency of substoichiometric NADPH concentrations in accelerating inactivation, assuming that low amounts of reduced coenzyme produce a labile form of the monomer in which not all the available sites are occupied. This requires recognition of destabilizing interactions between subunits which contain bound NADH (or NADPH) and free subunits, while stabilizing interactions would operate between subunits which bind the reduced coenzyme. The occurrence of negative homotropic interactions in NAD binding to glutamate dehydrogenase has been recognised

(DALZIEL and ENGEL, 1968). In the experiments presented above (Section 5.1) addition of excess NADH to an enzyme which had been partially inactivated with low concentrations of NADH prevented further loss of catalytic activity. Possibly a labile monomer with only a few sites bonded to NADH was converted into a fully saturated and stable monomer. Another possibility is that NADH stabilizes whatever 'whole' monomers are left after liberation of protein fragments from the molecule. HENDERSON and HENDERSON (1969) speak of inactive subunits of unknown molecular weight which are liberated from the monomer during denaturation.

Admitting the lack of spectral changes due to breaking of tyrosyl-H bonds during inactivation (see Section 5.3), the above-mentioned fragments are not the 50000 mol.wt. polypeptide chains which constitute the active monomer. It is conceivable that the 30 minutes' exposure of a dilute enzyme to low ionic strength at 25° is a relatively mild procedure compared to the drastic treatments with guanidine-HCl, 6 M urea or sodium alkyl sulphate usually employed for obtaining the polypeptide chains (e.g., APPELLA and TOMKINS, 1966). Interesting in this respect is the comparison between the value of 99600 cal.mole^{-1} reported for the ΔH of chymotrypsinogen inactivation at pH 2.0–3.0 (DOTY and GEIDUSCHEK, 1953b) and the value of 20000 cal.mole^{-1} calculated from the slopes in Fig. 5.11. Side-chain groups on the enzyme, not involved in holding the chains together, but having a function in the spatial arrangement of the chains within the monomer, may be more susceptible than the tyrosyl -H bonds to the kind of treatment applied in this work. One may have difficulties in detecting unequivocal spectral changes in such a case, or in relating the magnitude of such changes to enzyme concentration (Section 5.3).

With regard to the effect of ADP, HENDERSON and HENDERSON (1969) report that no reactivation could be detected if ADP was added to the enzyme after partial denaturation. This is in contradiction with the results shown in Figs. 5.9 and 5.22. The possibility of a conformational change induced by ADP is more likely than an association effect, at least at the concentration of 10 μM (Fig. 5.6). In contrast to the experiments with substoichiometric NADPH concentrations, no destabilizing interactions between liganded and free subunits operate in the case of 10 μM ADP, suggesting that the subunits have different conformations in the 2 cases. This idea is consistent with KOSHLAND's induced-fit theory (KOSHLAND et al., 1962). Nevertheless, it cannot be excluded that a shuttling of the ADP molecules between the subunits takes place in such a manner that the relaxation time needed for conversion of the stable conformation into the unstable one following dissociation of ADP is longer than the relaxation time of the shuttling process.

Several experiments demonstrate the protective effect of 2-oxoglutarate (Section 5.1), and the results of sucrose gradient centrifugation bear out the assumption that this substrate induces a change in the enzyme molecule which counteracts inactivation (Fig. 5.16). Protection against inactivation is sufficient evidence against the previously reported lack of binding of 2-oxoglutarate to the free enzyme (FISHER, 1960). It is recalled that protection by 2-oxoglutarate

against inactivation of glutamate dehydrogenase by 4-(iodoacetamido)-salicylic acid was reported by MALCOLM and RADDA (1968).

It should be mentioned that the quantitative differences between parts A and B of Figs. 5.19 and 5.20 are due not only to the various degrees of inactivation undergone by the control in the 2 cases, but also to the fact that in the experiments presented in Figs. 5.19B and 5.20B, the effectors had been added both to the enzyme during inactivation and to the sucrose solutions prior to preparation of the gradients. This procedure prevented dissociation of the effector from the enzyme in the course of centrifugation.

While the results presented in Chapter 4 point to the role played by the state of aggregation in the process of inactivation, the results of sucrose gradient centrifugation experiments indicate the possibility of conformational changes. The role of conformational changes becomes apparent when there is a change in activity without significant change in the relative sedimentation coefficient. The formula suggested by MARTIN and AMES (1961), and used in the previous section for molecular weight estimations, is based on the assumption of a partial specific volume of roughly 0.725 cm^3/g for all proteins. This assumption results in less than 3% error in the estimation of $S_{20,w}$ for most proteins (MARTIN and AMES, 1961). Admitting that many proteins are essentially spherical molecules, a crude estimation of the molecular weight can be obtained from the sedimentation constant alone (SCHACHMAN, 1959). However, if this is not the case, the contribution of the frictional coefficient may become important. While an estimate of sedimentation constants can be obtained by the method of sucrose gradient centrifugation, information on frictional coefficients must be gained from diffusion data. Similar difficulties to those encountered in making determinations of molecular weights in an unstable enzyme (see Section 4.5) would appear in frictional coefficient measurements.

Noteworthy is the similarity between the positions of the lighter fractions in the sedimentation patterns of various experiments as opposed to the differences observed between the heavier fractions under different conditions of inactivation. This finding, as well as the results of the 'reactivation' experiments with ADP (Fig. 5.22) suggest that changes in the state of association do take place during inactivation. On the basis of the available evidence, it appears that the process of inactivation is characterised by a combination of aggregational and conformational changes. Higher molecular weight species dissociate into the monomer. The active monomer which participates in the association-dissociation equilibrium is gradually converted into a less active conformation, which eventually breaks down into inactive fragments. The shoulder found in the sedimentation (activity) curve of the unstable enzyme suggests that there may be 2 forms of monomer with very similar sedimentation rates (see also HENDERSON and HENDERSON, 1969). Aggregation of the liberated fragments is probably responsible for the increased turbidity accompanying inactivation, especially in the presence of urea. In the presence of substoichiometric NADPH concentrations, the greatest part of the liberated protein becomes soluble. ADP reverses the equilibrium by reactivating the inactive monomer.

However, since ADP cannot associate the inactive fragments, 'reactivation' does not occur when ADP is added at later stages of inactivation. In the presence of stabilizing ligands (ADP, saturating NADH concentrations, and to some extent, 2-oxoglutarate), conversion of the active monomer into the inactive form is prevented, with the result that inactivation does not take place.

SUMMARY

Evidence is presented in favour of a regulatory role for 2-oxoglutarate in both oxidative deamination and reductive amination reactions catalysed by glutamate dehydrogenase. In the reaction of NADH oxidation/2-oxoglutarate reduction, the inhibitory effect of ATP is counteracted by high concentrations of 2-oxoglutarate. The inhibition of its own oxidation by high concentrations of NADH is enhanced by 2-oxoglutarate. This is unlike the inhibition at high concentrations of NADPH which occurs only at low levels of 2-oxoglutarate; at 50 mM 2-oxoglutarate, the double reciprocal plot of initial rate against coenzyme concentration provides a straight line. The reaction of reductive amination of 2-oxoglutarate with NADPH as coenzyme is slightly activated by ATP; the degree of activation is inversely related to the concentration of 2-oxoglutarate.

The double reciprocal plot of initial rate vs. $NADP^+$ concentration in the deamination reaction is not a straight line, as can be found in many literature reports, but shows several discontinuities, especially at high $NADP^+$ concentrations. The activating effect exhibited by NAD^+ at high concentrations is enhanced by 2-oxoglutarate and counteracted by high glutamate concentrations. 2-oxoglutarate shows a cooperative type of inhibition in the glutamate deamination reaction with 0.1 mM $NADP^+$ as coenzyme and 50 mM glutamate as substrate. Under similar conditions, competitive inhibition is exhibited in the NAD^+ reduction reaction, but noncompetitive inhibition is observed in the higher NAD^+ concentration range.

Inactivation of the enzyme at low protein concentration and low ionic strength (10 mM Tris-HCl-0.1 mM EDTA (pH 8), 25°) is promoted by substoichiometric concentrations of NADH and NADPH but counteracted at saturating concentrations. Higher concentrations of NADPH than of NADH are required in order to achieve full stabilization of the enzyme. The difference between inactivation in the presence of low concentrations of NADPH and of urea appears probably in the intial stages of the reaction. Substoichiometric concentrations of ADP are sufficient to hold the enzyme in a stable form, so that only very little inactivation takes place under these conditions. The substrate 2-oxoglutarate has a considerably stronger protective effect in the inactivation reaction than the 2 other substrates of the catalytic reaction, glutamate and ammonium ion. Addition of ADP to a partially inactivated enzyme at early stages of inactivation leads to almost full restoration of the initial catalytic activity. When added at a more advanced stage, both ADP and saturated concentrations of NADH prevent further inactivation; activity is maintained at the level prevalent prior to addition of the effectors.

At low temperatures and high enzyme concentrations, the rate of inactivation is diminished. A study of the effect of temperature on inactivation of a 0.1 mg/ml enzyme solution made it possible to calculate an activation energy

of 20000 cal.mole^{-1} from the Arrhenius plot of inactivation. An identical value was determined for the process of inactivation of the enzyme in the presence of 10 μM NADPH. However, the entropy of inactivation (ΔS^*) was increased in the latter case by 1.4 e.u. The small energy changes which characterize inactivation of the enzyme under the above conditions are consistent with the idea of conformational transitions accompanying inactivation. The results of sucrose density gradient centrifugation studies bring additional support to this representation. Inactivated enzyme sediments closer to the meniscus than stable enzyme, or enzyme inactivated in the presence of stabilizing agents. A second peak or shoulder often accompanies the main peak of inactivated enzyme. In the presence of 0.5 M urea, only this shoulder appears in the sedimentation pattern. In both cases, the residual activity is associated with lower molecular weight components.

A study of the relationship between inactivation and enzyme concentration revealed that the value of the pseudo-first order inactivation constants, which is a measure of the rate of inactivation, tends to reach a finite value at infinite dilution. The amount of monomer, which is the predominant molecular species at high dilutions, has been calculated from $M_w(c)$ data by the method of STEINER. This method made it possible to calculate the association constants for the dimerization and trimerization reactions, by means of which the relative amounts of di- and trimer at various enzyme concentrations could also be determined. The finding of a linear relationship between the rate constants of inactivation at various concentrations of enzyme and the relative amount of monomer present at the corresponding concentrations led to the conclusion that the monomer is the active species in the inactivation process. Application of the above method to an inactivating enzyme provided evidence according to which the appearance of a different form of the monomer constitutes the limiting step in the inactivation process. This intermediary stage is probably followed by fragmentation of the monomer into smaller inactive subunits.

SAMENVATTING

Experimentele aanwijzingen zijn verkregen voor een regulerende rol van 2-oxoglutaraat in zowel de oxidatieve deaminering- als de reduktieve aminering-reakties gekatalyseerd door glutamaat dehydrogenase. Het remmende effekt van ATP in de NADH oxidatie/2-oxoglutaraat reduktie reaktie wordt door hoge 2-oxoglutaraat konsentraties opgeheven. De NADH remming van de NADH oxidatie reaktie wordt gestimuleerd door 2-oxoglutaraat. Daarentegen remmen hoge NADPH konsentraties slechts bij lage [2-oxoglutaraat]; bij 50 mM 2-oxoglutaraat wordt er een rechte lijn verkregen in de grafiek waarin $1/v$ tegen 1/[NADPH] (Lineweaver-Burk plot) is uitgezet. De reaktie van reduktieve aminering van 2-oxoglutaraat met het coenzym NADPH wordt geaktiveerd door ATP. De mate van aktivering is omgekeerd evenredig met de konsentratie van 2-oxoglutaraat.

In tegenstelling tot wat er in de literatuur vermeld staat, geeft de Lineweaver-Burk plot met $NADP^+$ geen rechte lijn. Vooral bij hoge $NADP^+$ konsentraties wordt er een onregelmatig verloop waargenomen. Het aktiverende effekt van hoge konsentraties NAD^+ wordt versterkt door 2-oxoglutaraat en door hoge [glutamaat]. 2-Oxoglutaraat vertoont een koöperatieve remming in de glutamaat deamineringreaktie met 0.1 mM $NADP^+$ als coenzym en 50 mM glutamaat als substraat. Onder soortgelijke omstandigheden wordt er in de NAD^+ reduktie reaktie een kompetitieve remming gevonden. Bij hoge NAD^+ konsentraties wordt non-kompetitieve remming waargenomen.

Inaktivering van het enzym bij lage eiwit konsentraties en lage ionsterkte (10 mM Tris-HCl-0.1 mM EDTA (pH 8), 25°) wordt gestimuleerd door substoichiometrische konsentraties van NADH en NADPH. Verzadigende konsentraties van NADH en NADPH voorkomen inaktivering. Om volledige stabilisatie van het enzym te bewerkstelligen heeft men meer NADPH nodig dan NADH. Substoichiometrische konsentraties van ADP zijn voldoende om het enzym in een stabiele vorm te behouden met als gevolg dat er onder deze omstandigheden slechts een geringe inaktivering plaats vindt. Het substraat 2-oxoglutaraat beschermt het eiwit aanzienlijk meer dan de 2 andere substraten van de katalytische reaktie, glutamaat en NH_4^+. Toevoeging van ADP in de eerste stadia van de inaktivering heeft als gevolg een vrijwel volledig herstel van de oorspronkelijke katalytische aktiviteit. Door toevoeging van ADP of verzadigende NADH konsentraties in latere stadia kan men verdere inaktivering voorkomen, m.a.w. de aktiviteit blijft op het peil van vóór de toevoeging.

De snelheid van inaktivering wordt minder bij lage temperaturen en hoge eiwit konsentraties. Het effekt van temperatuur op de inaktivering van 0.1 mg/ml enzym werd nagegaan, en een waarde van 20000 $cal.mole^{-1}$ voor de aktiveringsenergie werd berekend uit de Arrhenius plot. Wanneer de inaktivering geschiedde in aanwezigheid van 10 μM NADPH, een gelijke waarde voor de aktiveringsenergie werd gevonden, terwijl de entropie (ΔS^*) 1.4 e.u. hoger

lag. De betrekkelijk kleine energieveranderingen welke het inaktiveringsproces typeren komen overeen met de voorstelling dat conformatie veranderingen bij de inaktivering betrokken zijn. De resultaten van centrifugeringsproeven in sucrose dichtheidsgradienten ondersteunen deze voorstelling. Geïnaktiveerd enzym sedimenteert dichter bij de meniscus dan het stabiele enzym of het enzym waaraan stabiliserende effektoren werden toegevoegd tijdens de inaktivering. Dikwijls wordt er een schouder op de piek van het geïnaktiveerde enzym waargenomen. In aanwezigheid van 0.5 M ureum gaat deze schouder overheersen in het sedimentatie patroon.

Onderzoekingen over de relatie tussen inaktivering en enzym konsentratie hebben aangetoond dat de waarde van de inaktiveringskonstante van de pseudo-eerste orde reaktie, welke een mate is voor de snelheid van inaktivering, een limiet nadert in de lage konsentratiegebied. Bij grote verdunning vindt men hoofdzakelijk monomeer. De hoeveelheid monomeer werd berekend uit $M_w(c)$ gegevens m.b.v. de methode van Steiner. Deze methode maakte het mogelijk de associatiekonstanten van de di- en trimerisatie reakties te berekenen, waarmee de relatieve hoeveelheden van di- en trimeer bij verschillende enzym konsentraties konden worden bepaald. Er werd een lineair verband gevonden tussen de inaktiveringskonstanten bij verschillende enzym konsentraties en de relatieve hoeveelheid monomeer bij de respektievelijke eiwit konsentraties. Dit feit leidt tot de conclusie dat de inaktivering het monomeer als intermediair heeft. Door toepassing van de bovenstaande methode op geïnaktiveerd enzym blijkt dat de beperkende stap in het inaktiveringsproces de overgang is van het aktieve monomeer naar een minder aktieve vorm. Deze tussenstap wordt waarschijnlijk gevolgd door fragmentering van het monomeer in kleinere inaktieve subeenheden.

REFERENCES

ADAMS, E. T. and WILLIAMS, J. W., J. Am. Chem. Soc., **86** (1964) 3454.
ANDERSON, B. M. and REYNOLDS, M. L., J. Biol. Chem., **241** (1966) 1688.
APPELLA, E. and TOMKINS, G. M., J. Mol. Biol., **18** (1966) 77.
BAYLEY, P. M. and RADDA, G. K., Biochem. J., **98** (1966) 105.
BITENSKY, M. W., YIELDING, K. L. and TOMKINS, G. M., J. Biol. Chem., **240** (1965) 663; 1077.
BORST, P., Biochim. Biophys. Acta, **57** (1962) 256.
CHURCHICH, J. E. and WOLD, F., Biochemistry, **2** (1963) 781.
CODDINGTON, A. and FINCHAM, J. R. S., J. Mol. Biol., **12** (1965) 152.
CROSS, D. G. and FISHER, H. F., Arch. Biochem. Biopphys., **110** (1965) 222.
CROSS, D. G. and FISHER, H. F., Biochemistry, **5** (1966) 880.
DALZIEL, K., FEBS Letters, **1** (1968) 346.
DALZIEL, K. and P. C. ENGEL, FEBS Letters, **1** (1968) 349.
DESSEN, P. and PANTALONI, D., Eur. J. Biochem., **8** (1969) 292.
DI PRISCO, G., ARFIN, S. M. and STRECKER, H. J., J. Biol. Chem., **240** (1965) 1611.
DI PRISCO, G. and STRECKER, H. J., Biochim. Biophys. Acta, **122** (1966) 413.
DI PRISCO, G., Biochem. Biophys. Res. Communn., **26** (1967) 148.
DIXON, M. and WEBB, E. C., Enzymes, 2nd ed, Longmans, London, 1964, p. 329.
DONOVAN, J. W., LASKOWSKI, M. and SCHERAGA, H. A., Biochim. Biophys. Acta, **29** (1958) 455.
DOTY, P. and GEIDUSCHEK, E. P., in The Proteins, vol. IA, ed. by H. Neurath and K. Bailey, Ac. Press, New York, 1953a, p. 393.
DOTY, P. and GEIDUSCHEK, E. P., in The Proteins, vol. IA, ed. by H. Neurath and K. Bailey, Ac. Press, New York, 1953b, p. 406.
EISENBERG, H. and TOMKINS, G. M., J. Mol. Biol., **31** (1968) 37.
EISENKRAFT, B. and VEEGER, C., Abstr. 4th Meeting Federation Eur. Biochem. Soc., 1967, Universitetsforlaget, Oslo, 1967, p. 69.
EISENKRAFT, B. and VEEGER, C., Biochim. Biophys. Acta, **167** (1968) 227.
FISHER, H. F., J. Biol. Chem., **235** (1960) 1830.
FISHER, H. F., CROSS, D. G. and MCGREGOR, L. L., Nature, **196** (1962a) 895.
FISHER, H. F., MCGREGOR, L. L., and CROSS, D. G., Biochim. Biophys. Acta, **65** (1962b) 175.
FISHER, H. F., MCGREGOR, L. L. and POWER, U., Biochem. Biophys. Res. Communn., **8** (1962c) 402.
FISHER, H. F. and CROSS, D. G., Biochem. Biophys. Res. Communn., **20** (1965) 120.
FISHER, H. F. and CROSS, D. G., Science, **153** (1966) 414.
FRIEDEN, C., Biochim. Biophys. Acta, **27** (1958) 431.
FRIEDEN, C., J. Biol. Chem., **234** (1959a) 809; 815.
FRIEDEN, C., J. Biol. Chem., **234** (1959b) 2891.
FRIEDEN, C., Biochim. Biophys. Acta, **59** (1962a) 484.
FRIEDEN, C., Biochim. Biophys. Acta, **62** (1962b) 421.
FRIEDEN, C., J. Biol. Chem., **237** (1962c) 2396.
FRIEDEN, C., J. Biol. Chem., **238** (1963a) 146.
FRIEDEN, C., J. Biol. Chem., **238** (1963b) 3286.
FRIEDEN, C., Biochem. Biophys. Res. Commun., **10** (1963c) 410.
FRIEDEN, C., in The Enzymes, 2nd ed., vol. 7, ed. by P. D. Boyer, H. Lardy and K. Myrbäck, Ac. Press, New York, 1963d, p. 3.
FRIEDEN, C., J. Biol. Chem., **240** (1965) 2028.
FRIEDEN, C. and COLMAN, R. F., J. Biol. Chem., **242** (1967) 1705.
FROMM, H. J. and NELSON, D. R., J. Biol. Chem., **237** (1962) 215.
FUJITA, H., in Mathematical Theory of Sedimentation Analysis, Ac. Press, New York, 1962, p. 199.

GRISOLIA, S., FERNANDEZ, M., AMELUNXEN, R. and QUIJADA, C. L., Biochem. J., **85** (1962) 568.
GRISOLIA, S., Physiol. Revs., **44** (1964) 657.
HENDERSON, R. F. and HENDERSON, T. R., Arch. Biochem. Biophys., **129** (1969) 86.
HORNE, R. W. and GREVILLE, G. D., J. Mol. Biol., **6** (1963) 506.
INAGAKI, M., J. Biochem. (Tokyo), **46** (1959) 1001.
IWATSUBO, M., LÉCUYER, B., di FRANCO, A. and PANTALONI, D., C.R. Acad. Sci. Paris, **263** (1966) 558.
JIRGENSONS, B., J. Am. Chem. Soc., **83** (1961) 3161.
KIRTLEY, M. E. and KOSHLAND, D. E., J. Biol. Chem., **242** (1967) 4192.
KLINGENBERG, M. and SLENCZKA, W., Biochem. Z., **331** (1959) 486.
KOSHLAND, D. E., NÉMETHY, G. and FILMER, D., Biochemistry, **5** (1966) 365.
KOSHLAND, D. E., Jr., YANKEELOV, J. A., Jr., and THOMA, J. A., Federation Proc., **21** (1962) 1031.
KUN, E. and ACHMATOWICZ, B., J. Biol. Chem., **240** (1965) 2619.
LEACH, S. J. and SCHERAGA, H. A., J. Am. Chem. Soc., **82** (1960) 4790.
LÉJOHN, H. B., SUZUKI, I. and WRIGHT, J. A., J. Biol. Chem., **243** (1968) 118.
LÉJOHN, H. B. and JACKSON, S., J. Biol. Chem., **243** (1968) 3447.
MALCOLM, A. D. B. and RADDA, G. K., Nature, **219** (1968) 947.
MARLER, E. and TANFORD, C., J. Biol. Chem., **239** (1964) 4217.
MARTIN, R. G. and AMES, B. N., J. Biol. Chem., **236** (1961) 1372.
MONOD, J., WYMAN, J. and CHANGEUX, J.-P., J. Mol. Biol., **12** (1965) 88.
MURPHY, J. B. and KIES, M. W., Biochim. Biophys. Acta, **45** (1960) 382.
OLSON, J. A. and ANFINSEN, C. B., J. Biol. Chem., **197** (1952) 67.
OLSON, J. A. and ANFINSEN, C. B., J. Biol. Chem., **202** (1953) 841.
PANTALONI, D. and IWATSUBO, M. I., Biochim. Biophys. Acta, **132** (1967) 217.
PAPA, S., TAGER, J. M., FRANCAVILLA, A., de HAAN, E. J. and QUALIARELLO, E., Biochim. Biophys. Acta, **131** (1967) 14.
PENZER, G. R., J. Chem. Educ., **45** (1968) 692.
PFLEIDERER, G., HOLBROOK, J. J., NOWICKI, L. and JECKEL, R., Biochem. Z., **346** (1966) 297.
REITHEL, F. J., Adv. Prot. Chem., **18** (1963) 124.
ROGERS, K. S., HELLERMAN, L. and THOMPSON, T. E., J. Biol. Chem., **240** (1965) 198.
SANWAL, B. D., Arch. Biochem. Biophys., **93** (1961) 377.
SANWAL, B. D. and LATA, M., Arch. Biochem. Biophys., **98** (1962) 420.
SCHACHMAN, H. K., in Ultracentrifugation in Biochemistry, Ac. Press, New York, 1959, p. 205.
SCHACHMAN, H. K., in Methods in Enzymology, vol. IV, ed. by S. P. Collowick and N. O. Kaplan, Ac. Press, New York, 1957, p.32.
SEGAL, L., Acta biol. med. germ., **10** (1963) 488.
STEINER, R. F., Arch. Biochem. Biophys., **39** (1952) 333,
STRECKER, H. J., Arch. Biochem. Biophys., **46** (1953) 128.
SUND, H., in Mechanismen enzymatischer Reaktionen (14. Colloquium der Gesellschaft für Physiologische Chemie, 1963), Springer Verlag, Berlin, 1964a, p.318.
SUND, H., Angew. Chem., **76** (1964b) 954.
SUND, H., in Biological oxidations, ed. by T. P. Singer, J. Wiley and Sons, New York, 1968a, p.641.
SUND, H. and Burchard, W., Eur. J. Biochem., **6** (1968b) 202.
TAGER, J. M. and PAPA, S., Biochim. Biophys., Acta, **99** (1965) 570.
TOMKINS, G. M., YIELDING, K. L. and CURRAN, J. F., Proc. Natl. Acad. Sci., **47** (1961) 270.
TOMKINS, G. M., YIELDING, K. L., TALAL, N. and CURRAN, J. F., Cold Spring Harbor Symp. Quant. Biol., **28** (1963) 461.
TOMKINS, G. M., YIELDING, K. L., CURRAN, J. F., SUMMERS, M. R. and BITENSKY, M. W., J. Biol. Chem., **240** (1965) 3793.
TSUTSUI, E. A. and MARKS, P. A., Biochem. Biophys. Res. Communn., **8** (1962) 338.
YANARI, S. and BOVEY, F. A., J. Biol. Chem., **235** (1960) 2818.
WOLFF, J., J. Biol. Chem., **237** (1962) 230.