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Glutamate-Dehydrogenase Inactivation by Reduced Nicotinamide–Adenine Dinucleotide Phosphate

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There has been considerable interest in the study of glutamate dehydrogenase [L-glutamate–NAD(P) oxidoreductase, EC 1.4.1.3], an enzyme that is readily disaggregated and aggregated (Olson & Anfinsen, 1952; Frieden, 1959*a*). Recent studies have indicated that crystalline glutamate dehydrogenase catalyses both leucine (Struck & Sizer, 1960) and alanine (Tomkins, Yielding & Curran, 1961) deamination, although at a slower rate, and that increased aggregation stimulates and inhibits the glutamate- and alanine-dehydrogenase activities respectively. It has been reported also that diethylstilboestrol and some steroids influence the activity, probably owing to aggregational effects (Yielding & Tomkins, 1960).

Contemporaneously with the demonstration by Inagaki (1959) that glutamate dehydrogenase was less stable to heat in the presence of reduced nicotinamide–adenine dinucleotide, it was shown that enzyme instability induced by substrates and cofactors is a fairly general phenomenon (Grisolia & Joyce, 1959). Such a finding might have important consequences in biology, particularly since it occurs at physiological concentrations and conditions (Tucker & Grisolia, 1962).

Glutamate dehydrogenase was selected for further study because many of its properties are known and because it plays a crucial role in nitrogen metabolism. Glutamate dehydrogenase is remarkably unstable to reduced nicotinamide–adenine dinucleotide phosphate (Grisolia, Grady, Fernandez & Tucker, 1961; Frieden, 1961); these experiments were carried out at low salt concentrations. However, as the salt concentration increases, the enzyme becomes more stable to NADPH. It appears then that additional knowledge of the stability of this important enzyme is needed, particularly as it is affected by ionic strength.

This paper describes the effects of various salts, of adenosine 5′-diphosphate, of adenosine 5′-triphosphate, of pH and of temperature on enzyme stability to NADPH. It also shows that there is unmasking of SH groups during inactivation together with a change in rotatory power. Although induced inactivation of crystalline glutamate dehydrogenase by NADPH does not yield preparations with grossly changed aggregational properties, aggregation may be related to stability since reagents such as diethylstilboestrol are very effective in potentiating this inactivation. However, activity measurements may be misleading if induced changes in stability by reagents are not taken into consideration. Some of the possible biological implications of these findings are discussed in this paper.

MATERIALS AND METHODS

Diethylstilboestrol was a gift from O. K. Behrens of Eli Lilly Co., Indianapolis, Ind., U.S.A. Crystalline ox-liver glutamate dehydrogenase, NAD^+, NADH, $NADP^+$, NADPH, ATP and ADP were obtained from Sigma Chemical Company, St Louis, Mo., U.S.A. Enzyme samples (Boehringer) from California Foundation were also tested and found to behave similarly. In all experiments the enzyme (kept in saturated sodium sulphate at pH 7·0 and at 0–4°) was centrifuged for 10 min. at 20 000*g* before use and the protein precipitate taken up in water. There were about 10 μmoles of sodium sulphate/mg. of protein. All other materials were commercial products. Antibody titres were measured by a standard technique (Boyd, 1956). Protein measurements were by standard procedures (Lowry, Rosebrough, Farr & Randall, 1951; Rodwell, Towne & Grisolia, 1957). All incubations, unless otherwise specified, were carried out at 37°.

The buffers utilized (except Ringer phosphate) were prepared according to Gomori (1955). Ringer phosphate was prepared as described by Cohen (1957). All pH values were determined in a Beckman model G pH meter at 25°. After addition of the components used in the incubation systems no change in pH could be detected.

Methods of assay. Assays were performed at 30° in a Beckman DU spectrophotometer with cells of 1·0 cm. light-path. Unless specified otherwise, measurements were carried out at 340 mμ and the rate during the first 3 min. was recorded at 30 sec. intervals and used for calculation of activity (Caughey, Smiley & Hellerman, 1957). For clarity the assays will be referred to by number in the paper. In all cases the final volume was 3 ml.

In all experiments appropriate portions (usually 20, 4 and 250 μl. for assays nos. 1, 2 and 3 respectively) were withdrawn from the incubation mixtures and tested. Unless recorded, the theoretical activity at zero time before incubation was unaffected by the experimental conditions used.

Assay no. 1. This is essentially the method recommended by Strecker (1955), except that 100 μmoles of DL-glutamate were used and the NAD^+ was increased from 0·3 μmole to 2 μmoles. DL-Glutamate was employed to decrease the velocity of the reaction (Caughey *et al.* 1957), and the rate was one-fifth of that found with L-glutamate. Consistent results were obtained under the conditions outlined. However, there was always a disproportionate increase in activity compared with lower NAD^+ concentrations, in agreement with the findings of Olson & Anfinsen (1953). For example, we found consistently some 600 units and some 2000 units/mg. of protein with 0·3 and 2 μmoles of NAD^+ respectively.

Assay no. 2. Potassium phosphate buffer, pH 7·6 (130 μmoles), 50 μmoles of L-glutamate and 2 μmoles of NAD^+ were used.

Assay no. 3. Potassium phosphate buffer, pH 7·6 (100 μmoles), 0·2 μmole of NADH, 100 μmoles of sodium pyruvate and 300 μmoles of ammonium chloride were used.

For estimation of SH groups samples containing 0·5 mg. of glutamate dehydrogenase/ml. in 0·05 M-tris–HCl, pH 7·2, were incubated for 15 min. with and without 0·1 mM-NADPH. The controls remained active but there was about 56% inactivation in the presence of NADPH. Samples (4 ml.) were then titrated by the method of Benesch, Lardy & Benesch (1955). In each experiment duplicate or triplicate titrations were carried out.

RESULTS

Influence of aggregation on rate. Glutamate dehydrogenase dissociates at concentrations of 2·5 mg./ml. or lower (Olson & Anfinsen, 1952). When the product of enzyme concentration and time at the lower enzyme concentrations is compared with that obtained at the highest, there is a disproportionate increase in glutamate-dehydrogenase activity, and similar results were obtained when NAD^+ was replaced by $NADP^+$ (Table 1). Moreover, increased protein concentration diminishes the specific activity for alanine deamination, confirming that aggregation influences catalytic activity. On the other hand, within the range of protein concentration used (from essentially complete disaggregation to complete aggregation) there is only a twofold change in activity, indicating either that the reagents in the assay influence aggregation and therefore activity, or that activity is not fully dependent on aggregation.

Influence of concentration on enzyme stability. The stability of glutamate dehydrogenase at several concentrations with and without NADPH was determined. Although the approximate concentration of the enzyme in liver is 0·5 mg./g. (Strecker, 1955), it is not known whether the enzyme is uniformly distributed. There is increased stability with higher enzyme concentrations, but this is not entirely due to aggregation since the stability at 1·5 mg./ml. (80 % disaggregation) is nearly equal to that at 5 mg./ml., at which concentration the enzyme is entirely aggregated (Frieden, 1959*a*). At all concentrations of protein tested, however, the enzyme becomes markedly unstable in the

Table 1. *Effect of enzyme concentration on glutamate- and alanine-dehydrogenase activity*

For Expt. 1 assay no. 1 was used, except that 5 μmoles of NAD^+ were employed and the increase in *E* was measured at 386 mμ. For Expt. 2 assay no. 3 was used. For brevity only one measured value of *E* is shown.

Expt. no.	Glutamate dehydrogenase used (mg. of protein)	Time required for ΔE 0·250* (min.)	Enzyme × time (mg. × min.)
1	0·5	17·7	8·85
1	1·5	4·7	7·05
1	10·0	0·55	5·5
2	0·25	4·15	1·04
2	0·5	2·27	1·13
2	1·5	1·0	1·5
2	9·0	0·21	1·9

* *E* at 386 mμ of NADH is about 0·1 of that at 340 mμ.

presence of NADPH (Table 2). Additions of bovine albumin up to 6 mg./ml. had no influence on enzyme stability to NADPH under the conditions of Table 2.

Effect of salt on enzyme stability. Increased salt concentration results in increased enzyme stability with or without NADPH, and K^+, Na^+ and NH_4^+ ions are equally effective (Table 3). The protection is mainly due to ionic strength and not to specific anion effects (Table 4).

As shown above, enzyme stability increases progressively with increasing salt concentrations. However, at high salt concentrations, an increase

Table 2. *Influence of reduced nicotinamide–adenine dinucleotide phosphate on stability at several enzyme concentrations*

Glutamate dehydrogenase was diluted in 0·05 M-tris–HCl, pH 7·2, to the indicated protein concentrations, and to contain, when used, 0·5 mM-NADPH. Assay no. 1 was used. Although not illustrated here there was also decreased enzyme stability at 0·1, 0·3 and 1·0 mM-NADPH, and the inactivation increased with time of incubation, as ascertained by sampling at 5, 10, 15 and 30 min. 1·0 mg. of enzyme protein catalysed the reduction of 1 μmole of NAD^+/min. and equals 100% activity.

Concn. of enzyme protein (mg./ml.)	Residual activity at 15 min. of incubation (%)	
	No NADPH	With NADPH
0·5	90	60
2·5	100	82
5·0	100	89

Table 3. *Effect of K^+, Na^+ and NH_4^+ ions on the stability of glutamate dehydrogenase to reduced nicotinamide–adenine dinucleotide phosphate*

Each tube contained 0·5 mg. of glutamate dehydrogenase in 0·05 M-tris–HCl, pH 7·2, with the indicated additions, in 1 ml. final volume. When used, 0·1 μmole of NADPH was added per tube. Assay no. 2 was used. No difference was noted with K^+ and NH_4^+ ions and from pH 7·15 to 7·95 when stability to NADPH in the presence of 130 μmoles of NaCl or NH_4Cl was compared. 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100% activity.

Expt. no.	Salt addition (μmoles)	Residual activity at 15 min. (%)			
		KCl		NaCl	
		No NADPH	With NADPH	No NADPH	With NADPH
1	None	49	11	58	9
1	4	63	23	68	19
1	20	84	46	87	48
1	60	100	75	90	79
				NH_4Cl	
2	4	85	43	75	37
2	8	98	51	98	39
2	12	100	55	87	44
2	20	100*	68*	100†	55†
2	60	97‡	88‡	100§	79§

* With 6 μmoles of NH_4Cl. † With 6 μmoles of KCl.
‡ With 20 μmoles of NH_4Cl. § With 20 μmoles of KCl.

Table 4. *Effect of anions on the inactivation of glutamate dehydrogenase by reduced nicotinamide–adenine dinucleotide phosphate*

Conditions were as described for experiments of Table 3 except where indicated. 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100% activity.

Reagent present during incubation (μmoles)	Residual activity (%)	
	No NADPH	With NADPH
KCl (20·0)	100	65
Potassium acetate (20·0)	94	51
K_2SO_4 (6·7)	93	64
Potassium phosphate buffer, pH 7·2 (8·0)	89	60

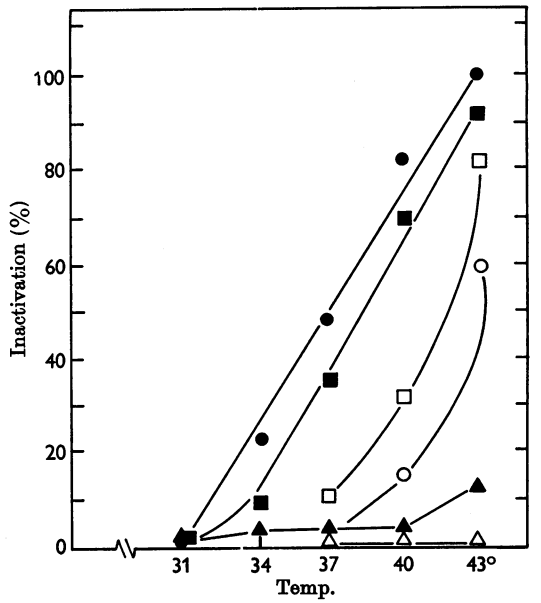

Fig. 1. Effect of temperature, salt concentration and NADPH on glutamate-dehydrogenase stability. The various mixtures, incubated at 37° for 10 min., consisted of 0·05 M-tris–HCl, pH 7·2, with the following amounts of salt and NADPH in a total of 1 ml. Assay conditions no. 2 were employed; 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100% activity. ▲, No NADPH, 20 mM-KCl; ●, 0·1 mM-NADPH, 20 mM-KCl; ■, 0·5 mM-NADPH, 20 mM-KCl; △, no NADPH, 140 mM-KCl; ○, 0·1 mM-NADPH, 140 mM-KCl; □, 0·5 mM-NADPH, 140 mM-KCl.

in the incubation time, temperature or pH increases NADPH-induced enzyme instability; some of these points are considered in more detail.

Effect of temperature and of concentration of NADPH. Although glutamate dehydrogenase is less stable at high temperatures in the presence of NADH (Inagaki, 1959), at the concentrations and temperature used in the present work NADH has no destructive effect on glutamate dehydrogenase except as demonstrated below. As shown in Fig. 1, increasing the temperature decreases stability, particularly in the presence of NADPH, and salt protection against NADPH inactivation decreases with increased temperature. NADPH in increasing concentrations may become less destructive, as is illustrated in Fig. 2. The marked influence of salt concentration on enzyme stability to NADPH is exemplified in Fig. 3, which shows that at 20 mM-K^+ ions, NADPH is markedly more destructive at 0·1 than at 0·5 and 1·0 mM, but at 80 mM-K^+ ions the inactivation by 0·1 mM-NADPH decreases, with 0·5 mM it decreases slightly and with 1·0 mM there is increased inactivation. These effects are even more pronounced at 140 mM-K^+ ions.

Effect of ATP and ADP. ADP prevents disaggregation (Frieden, 1959*b*). Under the conditions of Table 5, both ADP and ATP protect glutamate dehydrogenase against the destructive effect of NADPH. However, addition of ADP up to 2 mM does not reactivate NADPH-inactivated preparations.

Effect of pH. It is impossible to ascertain the effect of NADPH at acid pH because it is spontaneously destroyed, but the results shown in Table 8 (as well as more extensive experiments not

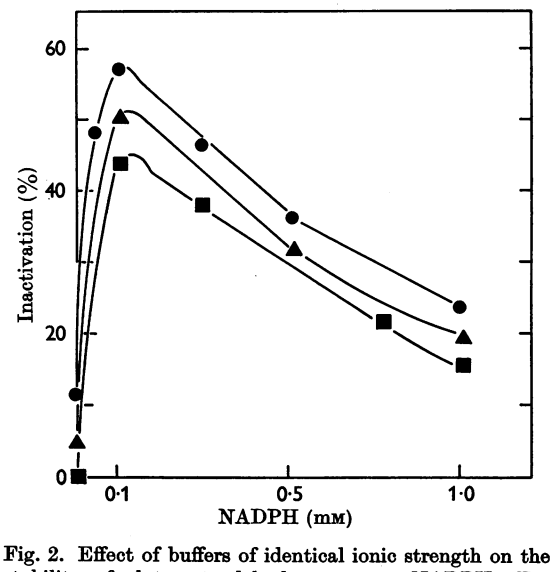

Fig. 2. Effect of buffers of identical ionic strength on the stability of glutamate dehydrogenase to NADPH. For each buffer three concentrations of NADPH (0·1, 0·5 and 1·0 mM) were employed and each tube contained 0·5 mg. of glutamate dehydrogenase in a total of 1 ml. (pH 7·2). Incubation was at 37° for 15 min. Assay conditions no. 2 were employed; 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100% activity. ●, 50 μmoles of tris–HCl and 20 μmoles of KCl; ▲, 30 μmoles of potassium phosphate buffer; ■, 30 μmoles of sodium phosphate buffer.

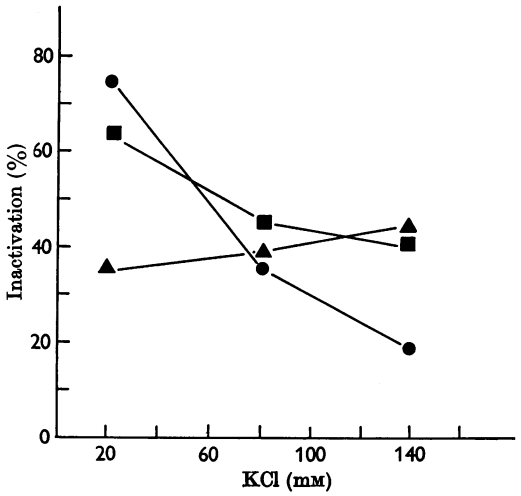

Fig. 3. Effect of salt concentration on the stability of glutamate dehydrogenase to NADPH. The incubation mixtures consisted of 0·05 M-tris–HCl, pH 7·2, three concentrations of NADPH (●, 0·1 mM; ■, 0·5 mM; ▲, 1·0 mM), three concentrations of KCl (20, 80 and 140 mM) for each NADPH concentration, and 0·5 mg. of glutamate dehydrogenase in a total of 1 ml. Incubation was at 37° for 15 min. Assay conditions no. 2 were employed; 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100% activity.

Table 5. *Effect of adenosine 5′-diphosphate, adenosine 5′-triphosphate and reduced nicotinamide–adenine dinucleotide phosphate on the stability of glutamate dehydrogenase*

The reaction mixture contained 0·5 mg. of glutamate dehydrogenase in 75 mM-sodium phosphate–75 mM-NaCl, pH 7·3, and the indicated additions. Portions (4 μl.) were tested under conditions of assay no. 2. 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and this equals 100% activity.

Reagent present (and concentration, mM) during preincubation	Residual activity at 60 min. (%)	
	No NADPH	With NADPH
None	96	63
ADP (0·5)	90	96
(2·0)	100	96
ATP (0·5)	95	100
(2·0)	97	97

presented; 0·05 M tris-HCl, 0·1 and 0·5 mM-NADPH) leave little doubt that inactivation occurs more readily at alkaline pH.

The acid products of NADPH are not active and the salts and concentrations of salt used throughout this paper do not inactivate NADPH as ascertained by spectrophotometric measurements.

Effect of NADPH destruction and reoxidation. A sample of NADPH was boiled for 10 min. in 0·1 N-hydrochloric acid. After neutralization, a portion incubated with the protein under the usual conditions did not destroy the enzyme. Also, $NADP^+$ formed by enzymic oxidation of NADPH did not decrease the stability of glutamate dehydrogenase.

Studies with diethylstilboestrol. The activity of glutamate dehydrogenase is affected by hormones, probably because of aggregation. Only experiments obtained with diethylstilboestrol are presented in this paper, but preliminary experiments indicate that similar effects occur with natural steroid hormones.

As shown in Table 6, diethylstilboestrol markedly affects enzymic activity at several protein concentrations, in confirmation of the results of Yielding & Tomkins (1960). Since substrates are necessarily present during the assay of activity and since the aggregation induced by diethylstilboestrol appears to depend on the presence of cofactors, although this requirement has been minimized (Yielding & Tomkins, 1960), the stability of glutamate dehydrogenase with and without diethylstilboestrol in the presence and in the absence of cofactors was tested. In 75 mM-sodium phosphate–75mM-sodium chloride, pH 7·3, and at physiological temperature and concentration of NADPH (Glock & McLean, 1955), the enzyme is rapidly inactivated and diethylstilboestrol potentiates the effect of NADPH even at very low concentrations (Table 7).

As illustrated in Table 8, inactivation of glutamate dehydrogenase by NADPH increases as the pH is raised, and potentiation of this inactivation occurs with increasing concentrations of diethylstilboestrol. Inactivation is also very rapid in Ringer phosphate.

Under the conditions of Table 9, high concentrations of diethylstilboestrol showed little effect on either glutamate- or alanine-dehydrogenase activities; in the presence of NADPH and diethylstilboestrol both activities were rapidly destroyed, indicating that aggregation is not directly responsible for the stability effects. Frieden (1959*a*) has shown disaggregation of glutamate dehydrogenase by NADH. Table 10 shows that, whereas NADH is ineffective, the combination of NADH and diethylstilboestrol is destructive. Other experiments, not shown, failed to indicate a potentiation for either protection or destruction of enzymic activity with NAD^+. This is of interest since NAD^+ and NADH have higher affinities and afford higher velocities for glutamate dehydrogenase than $NADP^+$ or NADPH.

Titration of thiol content of controls and of NADPH-inactivated preparations. Although many physical parameters for glutamate dehydrogenase are well known, the SH content of the enzyme has not been studied until recently (Hellerman, Schellenberg & Reiss, 1958). Because of turbidity

Table 6. *Effect of diethylstilboestrol on glutamate- and on alanine-dehydrogenase activities of crystalline glutamate dehydrogenase*

Glutamate- and alanine-dehydrogenase activities were measured by assays no. 1 and no. 3 respectively; 5, 25, 62 and 125 μg. of protein were used for experiments of Table columns I, II, III and IV respectively. Diethylstilboestrol was added as indicated. For glutamate dehydrogenase 100% activity equals 0·005 and 0·025 μmole of NAD^+ reduction/min. for the experiments of columns I and II respectively. For alanine dehydrogenase 100% activity equals 0·0062 and 0·0125 μmole of NADH oxidation/min. for the experiments of columns III and IV respectively.

Concn. of diethylstilboestrol (μM)	Enzyme activity (%)			
	Glutamate dehydrogenase		Alanine dehydrogenase	
	I	II	III	IV
0	100	100	100	100
0·8	100	100	113	104
1·6	97	96	132	116
8·0	2	26	141	157
16·0	0	4	—	—

Table 7. *Effect of reduced nicotinamide–adenine dinucleotide phosphate and diethylstilboestrol on glutamate-dehydrogenase stability*

Conditions were as described for experiments of Table 5. Figures in parentheses are data obtained from other experiments with 0·3 mM-NADPH. 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100% activity.

Concn. present during incubation (μM)		Residual activity (%)	
NADPH	Diethylstilboestrol	At 60 min.	At 120 min.
0	0	96	95
0	0·025	93	89
0	0·25	95	88
0	0·5	98	94
0	2·5	98	95
0	5·0	97	95
100	0	62 (44)	47 (26)
100	0·025	62 (40)	44 (22)
100	0·25	58 (35)	38 (17)
100	0·5	50 (30)	26 (14)
100	2·5	40 (14)	13 (0)
100	5·0	20 (8)	7 (0)

Table 8. *Effect of* pH, *reduced nicotinamide–adenine dinucleotide phosphate and diethylstilboestrol on glutamate-dehydrogenase stability*

Each tube contained, in 1 ml., 0·6 mg. of crystalline ox-liver glutamate dehydrogenase in 75 mM-NaCl–75 mM-phosphate buffer at the indicated pH values, and diethylstilboestrol was added as recorded. Ringer phosphate was used for the experiments at pH 7·55. 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100% activity.

	Residual activity (%)											
	pH 7·0		pH 7·45		pH 7·55		pH 7·7		pH 7·9		pH 8·5	
Time (min.)	30	60	30	60	30	60	10	30	15	30	0*	15
Concn. of diethylstilboestrol (μM)	Experiments without NADPH											
—	95	94	99	100	100	90	100	100	100	98	100	95
2·5	97	92	94	92	90	90	100	100	100	100	100	94
5·0	94	91	100	94	91	87	100	100	100	100	100	73
7·5	97	91	96	91	90	90	100	100	100	98	100	73
	Experiments with 0·1 mM-NADPH present											
—	86	76	77	60	62	46	67	29	22	9	100	0
2·5	73	56	51	40	29	10	29	13	19	0	86	0
5·0	66	53	31	20	20	0	28	6	9	0	84	0
7·5	59	47	21	9	7	0	20	0	8	0	62	0

* Assayed immediately after mixing, before incubation.

Table 9. *Effect of diethylstilboestrol on glutamate- and alanine-dehydrogenase stability with and without reduced nicotinamide–adenine dinucleotide phosphate*

Incubation was carried out for 10 min. under the conditions described for Table 3, except where indicated. Portions containing 0·012 and 0·25 mg. of protein were tested for glutamate- and alanine-dehydrogenase activities by assays no. 1 and no. 3 respectively. For glutamate dehydrogenase 0·5 mg. of enzyme protein catalysed the reduction of 0·5 μmole of NAD^+/min. and equals 100% activity. For alanine dehydrogenase 0·5 mg. of enzyme protein catalysed the oxidation of 0·05 μmole of NADH/min. and equals 100% activity. Similar results were obtained in 75 mM-sodium phosphate–75 mM-NaCl, pH 7·7.

Concn. present during incubation (μM)		Residual activity (%)	
NADPH	Diethylstilboestrol	Glutamate dehydrogenase	Alanine dehydrogenase
0	0	78	74
0	0·6	78	79
0	1·2	78	75
0	2·4	78	76
0	12	69	100
0	24	47	106
100	0	50	54
100	0·6	37	32
100	1·2	28	26
100	2·4	20	13
100	12	0	8
100	24	0	8

we were unable to compare the SH content of active and inactive enzyme preparations by spectrophotometric analysis (Boyer, 1954). However, by argentometric titration we found an increase in titratable SH groups after NADPH-induced enzyme inactivation.

The results from five separate experiments showed 31 ± 1 moles of SH/mole for the control preparations and 42 ± 1 moles of SH/mole for the NADPH-treated preparations. When the amperometric titration was carried out in three separate experiments in the presence of urea as a denaturing agent (Carter, 1959), 92 moles of SH/mole were found for both the control and NADPH-inactivated enzyme. These findings are in fair agreement with those of Hellerman *et al.* (1958), except that our figures are slightly lower, probably reflecting the influence of salt (Benesch *et al.* 1955).

Under the conditions described above we found 31 SH groups for the enzyme incubated with 20 μM-diethylstilboestrol. However, when incubated with 0·1 mM-NADPH and 20 μM-diethylstilboestrol there were 71 SH groups/mole, or an increase of 29 groups over the sample inactivated with NADPH only.

These results confirm that a part of the SH content of the enzyme is masked (Hellerman *et al.* 1958), and that the inactivation by NADPH results in partial unmasking of SH groups. It was not possible to reactivate NADPH-inactivated samples by addition of cysteine nor did the addition of this reagent prevent the inactivation by NADPH.

Measurements of optical rotation. Our measurements were handicapped by the extremely low rotatory power of the native enzyme and by slight

turbidity occasionally encountered after NADPH-induced inactivation. However, with the aid of a Rudolph precision polarimeter, we found the $[\alpha]_{5890}$ for the native enzyme to be +6·3. Although there was no change in the presence of diethylstilboestrol, after inactivation (64 %) by incubation with diethylstilboestrol and NADPH the $[\alpha]_{5890}$ was −2·2. When the enzyme was denatured in 8 M-urea the $[\alpha]_{5890}$ was −88. These findings agree with those of Jirgensons (1961), who has shown that glutamate-dehydrogenase rotation changes from *dextro-* to *laevo*-rotatory with denaturing agents such as sodium decyl sulphate.

Table 10. *Effect of diethylstilboestrol, of reduced nicotinamide–adenine dinucleotide phosphate and of reduced nicotinamide–adenine dinucleotide on glutamate-dehydrogenase stability*

Conditions were identical to those of Table 3. The coenzyme, when used, was at 0·1 mM. The incubation period was 15 min. 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100 % activity.

Concn. of diethylstilboestrol (μM)	Residual activity (%)		
	No coenzyme	NADPH	NADH*
—	100	51	100
1·2	100	48	100
12·0	100	0	65
25·0	93	0	46

* Similar findings were obtained with Ringer phosphate and at 0·1–0·3 mM-NADH.

Table 11. *Effect of reduced nicotinamide–adenine dinucleotide phosphate, trypsin and urea on the stability of glutamate dehydrogenase*

Tubes contained 0·5 mg. of crystalline glutamate dehydrogenase in 0·05 M-tris–HCl, pH 7·2, and the indicated additions in a total volume of 1 ml. Values in parentheses were obtained when tris was replaced by 75 mM-NaCl–75 mM-potassium phosphate buffer, pH 7·4; assay no. 2 was used for the results in parentheses; 0·5 mg. of enzyme protein catalysed the reduction of 2·5 μmoles of NAD^+/min. and equals 100 % activity. Assay no. 1 was used for all other results; 0·5 mg. of enzyme protein catalysed the reduction of 0·5 μmole of NAD^+/min. and equals 100 % activity.

Time (min.) ...	Residual activity (%)		
	15	30	
Reagent present during incubation			
None	83	60	(100)
0·2 M-Urea	60	50	(100)
0·4 M-Urea	60	30	(100)
1·0 M-Urea	26	10	(100)
0·1 mM-NADPH	40	26	(60)
0·1 mM-NADPH and 0·2 M-urea	16	10	(26)
0·1 mM-NADPH and 0·4 M-urea	6	0	(0)
0·1 mM-NADPH and 1·0 M-urea	0	—	(0)
5 μM-Trypsin	76	40	(100)
10 μM-Trypsin	43	20	(45)
100 μM-Trypsin	0	—	(0)
0·1 mM-NADPH and 5 μM-trypsin	6	0	(0)
0·1 mM-NADPH and 10 μM-trypsin	0	—	(0)

Stability of glutamate dehydrogenase to urea, trypsin and NADPH. Low concentrations of urea are destructive for the enzyme, and they are more destructive in the presence of NADPH (Table 11). Inactivation by trypsin is also markedly stimulated by NADPH. These experiments extend and confirm preliminary findings (Grisolia *et al.* 1961). Increased proteolysis was demonstrated as follows: samples containing 1 mg. of protein/ml. in 0·05 M-tris–HCl, pH 7·2, without and with NADPH (0·1 mM, when used, yielding 86 % inactivation), were incubated for 30 and 60 min. with and without the addition of 5 μM-trypsin. Perchloric acid (10 %, 0·1 ml.) was added and the samples were centrifuged. Acid-soluble products were then measured (Lowry *et al.* 1951). The equivalents of 0·031 and 0·207 mg. of protein were hydrolysed in the controls without and with trypsin respectively, whereas 0·034 and 0·39 mg. of protein were hydrolysed in the experimental tubes without and with trypsin. Similar results were obtained with 10 μM-trypsin.

Antigenicity experiments. Antigenic differences between the control and the NADPH-inactivated preparation might exist. Rabbits were injected biweekly for a series of five injections with 0·5 mg. of protein for both control and NADPH-inactivated enzyme preparations. Preimmunization titres of sera were negative, as was expected; after the series of injections with both active and inactive enzyme, titres were very low and no differences were observed. 'Booster' injections of the active enzyme increased the precipitin titres. However, no differences in precipitin titres were observed when the anti-active serum was treated with both inactive and active enzymes, and there was no enzymic inhibition of the active enzyme.

DISCUSSION

The stability of liver glutamate dehydrogenase at physiological concentrations is markedly decreased by concentrations of NADPH of the same order as those present in liver (Glock & McLean, 1955). As shown above, ionic strength affects stability to NADPH; consequently the comparison of cofactor-induced enzyme inactivation and kinetic constants (Caravaca & Grisolia, 1960) may be misleading for glutamate dehydrogenase

(Frieden, 1961). Since at low salt concentrations there is more inactivation with lower concentrations of NADPH than with higher concentrations, the enzyme must change to a relatively more stable conformation, perhaps owing to buttressing (Koshland, 1960).

Although it is difficult to interpret the effects of aggregation on activity because association probably occurs during assay, as shown, the NADPH-induced inactivation phenomenon is not likely to be related directly to aggregation. Inactivation of triose phosphate dehydrogenase by NADH results in losses of SH groups, disulphide formation and in NADH utilization with formation of NADH–X (Chaykin, Meinhart & Krebs, 1956; Tucker & Grisolia, 1962). We have not detected NADPH utilization or derivative formation from NADPH; however, glutamate-dehydrogenase inactivation by NADPH results in the appearance of additional titratable SH groups. The potentiation by NADPH of urea and trypsin denaturation and proteolysis suggests that the change in conformation induced by the reagent facilitates inactivation. Although it is too early to generalize and present a mechanism or theory for substrate inactivation of enzymes, this phenomenon may be related to enzyme-protein turnover (Grisolia *et al.* 1961).

The interpretation of activity measurements with concentrations of enzyme and reagents that are entirely out of proportion to their tissue concentration may be misleading. Addition of reagents to physiological concentrations of enzyme followed by assay of a portion (with considerable dilution of reagents) may be a better approximation to effects *in vivo*.

As indicated, we found effects similar to the potentiation by diethylstilboestrol of NADPH- and NADH-induced enzyme inactivation with natural steroid hormones. Although these effects relate to hormonal action *in vivo*, Maxwell & Topper (1961) have shown that a number of steroid effects on aldehyde dehydrogenase depend on substrate concentration and on pH. We have shown above that glutamate-dehydrogenase inactivation by NADPH and its potentiation by diethylstilboestrol are markedly dependent on pH and produce a change from *dextro-* to *laevo-*rotation. It is possible therefore that hormones, in conjunction with substrates and cofactors, may influence protein conformation.

SUMMARY

1. A disproportionate increase in glutamate-dehydrogenase activity with increasing concentration of the enzyme has been found, together with a disproportionate decrease in the alanine-dehydrogenase activity of crystalline glutamate dehydrogenase, demonstrating that aggregation influences activity.

2. Rapid induced inactivation of glutamate dehydrogenase by NADPH, which is markedly dependent on salt concentration, pH and temperature, occurs at nearly physiological conditions. The preparations inactivated by NADPH exhibit less stability to denaturation by urea and digestion by trypsin.

3. The inactivation by NADPH is remarkably potentiated by diethylstilboestrol and there is parallel inactivation of the crystalline glutamate dehydrogenase for both glutamate- and alanine-dehydrogenase activities.

4. An increase in titratable SH groups and a change in rotatory power from *dextro* to *laevo* follows inactivation of glutamate dehydrogenase by NADPH.

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Specificity and Properties of Wheat-Germ Esterase

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Singer & Hofstee (1948*a*, *b*) studied an enzyme from wheat germ, 'wheat-germ lipase', that hydrolysed glyceryl and aliphatic esters. Gowron, Grelecki & Duggan (1953) investigated the hydrolysis of aromatic esters by the same enzyme. Jansen, Nutting & Balls (1948) showed that certain plant enzymes, including that from wheat germ, would hydrolyse glyceryl, aromatic and choline esters. Since acetates were cleaved more rapidly than esters of higher acid homologues, they called these enzymes acetylesterases. Plant acetylesterases were inhibited by organophosphorus compounds (Jansen *et al.* 1948; Mounter, Tuck, Alexander & Dien, 1957). It is apparent that this hydrolytic enzyme of wheat germ has several points of similarity to other esterases, cholinesterases and lipases. However, in a number of specificity studies true maximal velocities of enzymic reactions have not been obtained and many experiments with hydrolytic enzymes have been limited by the use of readily soluble substrates. The kinetics of certain esterases and lipases in heterogeneous systems have been discussed by Aldridge (1954). Desnuelle (1961) has studied the properties of highly purified pancreatic lipase and the kinetics of hydrolysis of emulsions of sparingly soluble esters. Additional data for the establishment of distinctions between esterases and lipases are desirable. In the present investigation, the rates of hydrolysis of a number of esters by wheat-germ esterase have been studied.

EXPERIMENTAL

Freeze-dried wheat-germ 'esterase' was obtained from: Mann Research Laboratories, lot no. C1804 (preparation 1); Nutritional Biochemicals Corp., no lot no. (preparation 2); Worthington Biochemical Corp., lot no. LP 5528 (preparation 3). Rates of hydrolysis of esters were determined manometrically at 38° (Mounter & Whittaker, 1950; Mounter, 1951). All determinations were accompanied by controls for non-enzymic hydrolysis.

Most of the esters used as substrates were obtained commercially and were of analytical grade. 3,3-Dimethylbutyl acetate was prepared from 3,3-dimethylbutanol given by Dr V. P. Whittaker. When the substrate concentration exceeded the solubility limit of the ester, the material was usually added to the Warburg flask as an emulsion in a 1% solution of gum acacia (1 g./100 ml. of 25 mM-$NaHCO_3$). The emulsion was prepared by subjecting the suspension to ultrasonic treatment in a Sonoblast ultrasonic cleanser for 5 min.

RESULTS

Preliminary studies were made of the effect of substrate concentration on the rates of hydrolysis of a soluble and a partially soluble ester, with and without emulsification in gum acacia. Fig. 1 shows that the presence of the gum had no effect on the rate of hydrolysis of triacetin; also the maximal rate of hydrolysis of *n*-pentyl acetate was only attained at concentrations exceeding the solubility limit. Direct addition of the sparingly soluble esters to the Warburg flasks produced the same rate of hydrolysis as the emulsified substrate but the latter form of addition was preferred because it permitted the handling of small amounts of volatile substances more readily. After the direct addition of an excess of ester to the enzyme, the substrate became emulsified on shaking in the presence of the protein, although some ester separated as an oily layer at the highest concentrations. There was no evidence of any distinct change in solubility in the presence of the protein. In all cases, the initial rate