sheep homozygous for Hb-B to the stress of severe experimental anaemia has been reinvestigated.

2. In animals subjected to extreme blood loss the Hb-A was replaced entirely by a new haemoglobin variant (Hb-C), whereas the production of Hb-B apparently was not affected. Under conditions of moderate blood loss, the replacement of Hb-A by Hb-C was partial. Hb-C was detectable in fairly constant concentrations in the blood of heterozygous sheep during the recovery phase of the anaemia.

3. Hb-C differs from Hb-A and from Hb-B in electrophoretic mobility and in its behaviour in DEAE-cellulose chromatography. Haemoglobins A, B, C and foetal haemoglobin seem to share the same α-polypeptide chain, but the non-α-chains of each of the four haemoglobin types are distinctly different.

4. The affinity of Hb-C for molecular oxygen was identical with that of Hb-A and therefore different from that of Hb-B. No changes in oxygen affinities of total erythrocyte haemolysates of a heterozygous AB sheep and a homozygous B sheep were observed during the experimental anaemia.

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Effect of Cofactors, Oestrogens and Magnesium Ions on the Activity and Stability of Human Glutamate Dehydrogenase

By J. C. WARREN, D. O. CARR and S. GRISOLIA

Department of Biochemistry, University of Kansas School of Medicine, Kansas City 3, Kans., U.S.A.

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It is well known that glutamate dehydrogenase [L-glutamate–NAD(P) oxidoreductase, EC 1.4.1.3] undergoes aggregation and deaggregation under a variety of conditions, including changes in concentration (Olson & Anfinsen, 1952). It has been claimed (Tomkins, Yielding & Curran, 1961) that with aggregation there are reciprocal changes in the rates of oxidation of glutamate and other amino acids (such as alanine) that are substrates for this enzyme. Other investigators believe that in the range of 0·1–1·0 mg./ml. (e.g. completely deaggregated to essentially fully aggregated enzyme)

there is no change in the activity with glutamate (Fisher, Cross & McGregor, 1962). Diethylstilboestrol, and to a smaller extent certain natural steroid hormones, at micromolar concentrations, will change the activity of this enzyme. Further, at much higher concentrations diethylstilboestrol appears to affect aggregation. It was found in this Laboratory that the ox-liver enzyme is remarkably unstable in the presence of NADPH and that diethylstilboestrol potentiates remarkably the NADPH-induced inactivation (Grisolia, Fernandez, Amelunxen & Quijada, 1962). Most studies with glutamate dehydrogenase have been carried out with the ox-liver enzyme. Little is known about the behaviour of human glutamate dehydrogenase.

This paper delineates the effects of diethylstilboestrol, certain natural steroids and various cofactors on the stability and activity of glutamate dehydrogenase of both human and bovine origin. It demonstrates that the designation of this enzyme as an important component in the mechanism of hormonal action, except possibly by influencing the NADPH effects, may have been grossly overstressed. It further reports that Mg^{2+} ions in physiological concentrations markedly increase stability. Some of the biological implications of these findings are discussed.

MATERIALS AND METHODS

The following trivial names are used in this paper: oestradiol-17β for oestra-1,3,5(10)-triene-3,17β-diol; oestradiol-17α for oestra-1,3,5(10)-triene-3,17α-diol; testosterone for 17β-hydroxyandrost-4-en-3-one; epitestosterone for 17α-hydroxyandrost-4-en-3-one; progesterone for pregn-4-ene-3,20-dione; cortisol for 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; dehydroepiandrosterone for 3β-hydroxyandrost-5-en-17-one.

Diethylstilboestrol, steroids, crystalline ox-liver glutamate dehydrogenase, NAD^+, NADH, $NADP^+$, NADPH, ATP and ADP were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Protein determinations were done by a biuret method (Rodwell, Towne & Grisolia, 1957).

The buffers were prepared according to Gomori (1955) and pH values were checked on a Beckman model G pH-meter at 25°. All preincubations, unless otherwise specified, were at 37°. Estimation of thiol groups was by the method of Carter (1959); titrations were done in duplicate.

The binding of NADH and NADPH to the enzyme was measured by fluorimetric enhancement as described by Velick (1958). Acetone-dried powders were prepared by a standard procedure (Grisolia, 1955).

Methods of assay. Measurements of activity were carried out in a Beckman model DU spectrophotometer at 30° as described by Grisolia *et al.* (1962). For clarity, the components of the assays used are indicated and assays are referred to by number. One unit is defined as the amount of enzyme that will generate 1 μmole of NADH/min. under the conditions described in assay no. 1. This definition conforms to the recommendation of the Commission on Enzymes of the International Union of Biochemistry, but it is about 2000 times larger than the unit that has been commonly used in the past for this enzyme. In all cases the final volume of the assay solution was 3·0 ml. and the reaction was started by the addition of enzyme.

Assay no. 1: 130 μmoles of potassium phosphate buffer, pH 7·6, 50 μmoles of L-glutamate and 2·0 μmoles of NAD (or, when indicated, NADP) were used.

Assay no. 2: 100 μmoles of potassium phosphate buffer, pH 7·6, 0·2 μmole of NADH (or, when indicated, NADPH), 100 μmoles of α-oxoglutarate and 300 μmoles of ammonium chloride were used.

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

Purification of human-liver enzyme. A human liver weighing 1400 g. was obtained from a 35-year-old healthy man immediately after sudden death. Purification of the enzyme was effected by a modification of the method of Kubo *et al.* (1957) as follows. The liver was diced with scissors and homogenized for 5 min. in a 4 l. Waring Blendor with 3 vol. of deionized water. The homogenate was heated at 45° for 10 min., cooled and then centrifuged. (Unless specified otherwise, centrifugations were for 20 min. at 4000g.) To each litre of supernatant fluid, 150 g. of sodium sulphate was added. The mixture was heated at 35° and then centrifuged. The precipitate was taken up in 3 vol. of 0·1 M-potassium phosphate buffer–1 mM-EDTA, pH 7·6. The solution was heated at 55° for 5 min., centrifuged and the precipitate discarded. To the supernatant fluid, which had a volume of 1050 ml., 131 g. of sodium sulphate was added. After stirring, the mixture was centrifuged. (From here on all centrifugations were at 20000g.) The precipitate was dissolved in 100 ml. of 0·1 M-potassium phosphate buffer–1 mM-EDTA, pH 6·4. At this point the activity of the enzyme was 1·6 units/mg. Sodium sulphate was added to a concentration of approx. 12% and the solution was allowed to stand at room temperature. A precipitate collected after 21 hr. was of low specific activity but precipitates collected at 88 and 136 hr. revealed specific activities of 2·2 and 1·9 units/mg. respectively. These were combined and taken up in 20 ml. of water, and sodium sulphate was added to a concentration of 10%. From this solution crystals of high specific activity were harvested, yielding a total of 183 mg. of crystalline material with a specific activity of 4·0 units/mg. The overall recovery was about 30%.

Partial purification of glutamate dehydrogenase from human uterus. Portions (1 g.) of acetone-dried powders from three human uteri obtained from women in the menstrual age were extracted for 20 min. at room temperature with 15 ml. of 0·15 M-potassium phosphate buffer, pH 7·3, and then centrifuged. The activities in these extracts were determined by using assay no. 1. Control measurements without glutamate were carried out. The approximate specific activity of the extracts was 1 milliunit/mg. Assuming complete extraction, the concentration of glutamate dehydrogenase in the uteri tested has been calculated to be 32, 40 and 40 milliunits/g. wet wt. The acetone-dried powders from the three uteri were then combined and extracted in 300 ml. of water for 5 min. Sodium sulphate was added to a concentration of 5%, the mixture was heated at 58° for 5 min., cooled, centrifuged, and the precipitate discarded. To the supernatant fluid sodium sulphate was added to a final

concentration of 20%, and the mixture heated to 35° and allowed to stand at room temperature for 1 hr. The precipitate that formed was taken up in 3 vol. of 0·1M-potassium phosphate buffer–1 mM-EDTA, pH 6·4 (the specific activity at this point was 15·4 milliunits/mg.), heated at 55° for 5 min., centrifuged and the precipitate discarded. Sodium sulphate was added to the supernatant fluid (12·5 g./100 ml.) and within a few hours a precipitate formed. This precipitate, which contained most of the activity, was suspended in 20% sodium sulphate and stored as such. The activity was 22 milliunits/mg.

RESULTS

Human-liver enzyme

The crystalline human-liver enzyme was photographed by using phase-contrast microscopy at approx. 1200 × magnification. The small needle-like crystals are shown in Fig. 1.

The enzyme was examined in the Spinco model E analytical ultracentrifuge in 0·05M-tris–hydrochloric acid buffer, pH 8·0, at 4°. At concentrations of 8 and 4 mg./ml. a single sharp peak was observed (Fig. 2). At 2 mg./ml. a single peak was observed but it displayed a shoulder, suggesting that some deaggregation occurs with dilution.

Stability of the enzyme

Because of previous demonstrations that inactivation of the ox-liver enzyme by NADH, NADPH and diethylstilboestrol was dependent on both enzyme and salt concentration as well as on pH (Grisolia *et al.* 1962), it seemed pertinent to examine enzyme stability at physiological concentrations in a solution resembling intracellular fluid. From our measurements, an initial concentration of 0·5 mg. of fresh liver/ml. seems reasonable. Accordingly, stability studies were carried out at an enzyme concentration of 0·5 mg./ml. at pH 7·3 in two solutions. A phosphate–chloride–Mg^{2+} ion buffer which resembled intracellular fluid had the following constituents/ml.: 75 μmoles of potassium phosphate buffer, pH 7·3, 15 μmoles of magnesium sulphate and 5 μmoles of sodium chloride. A phosphate–chloride buffer had the following constituents/ml.: 90 μmoles of potassium phosphate buffer, pH 7·3, and 5 μmoles of sodium chloride. NADPH and diethylstilboestrol did inactivate the human-liver enzyme in both solutions but the inactivation was significantly less in the presence of Mg^{2+} ions (Table 1). Similar results were obtained with the ox-liver enzyme (Table 2).

It is obvious that Mg^{2+} ions in physiological concentrations markedly protect the enzyme against inactivation by NADH and NADPH, with or without diethylstilboestrol. Magnesium chloride yielded results similar to those seen with magnesium sulphate.

The effect of variation in the Mg^{2+} ion concentration is shown in Fig. 3. These data show that maximal effect is seen at the physiological intracellular concentration (about 0·02M). In previous

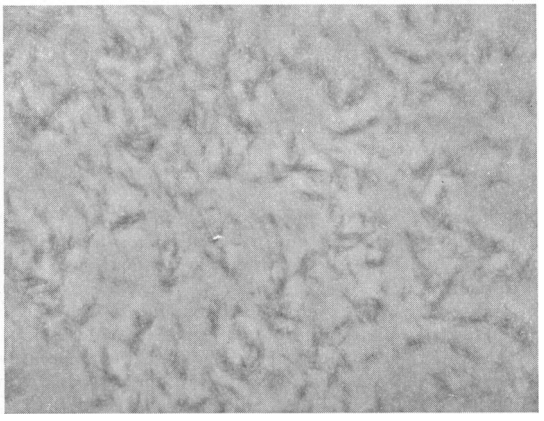

Fig. 1. Crystalline human-liver enzyme photographed by using phase-contrast microscopy at approx. 1200 ×.

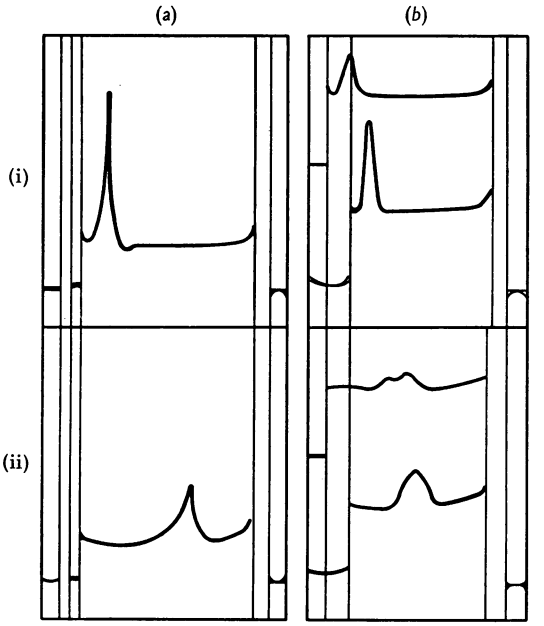

Fig. 2. Sedimentation diagrams indicating the behaviour of crystalline human-liver glutamate dehydrogenase in 0·05M-tris–HCl buffer, pH 8. The average rotor temperature was 4°. Tracings represent pictures taken 8 min. (i) and 32 min. (ii) after reaching speed (59780 rev./min.). In (*a*) the concentration was 8 mg./ml. In (*b*) the concentrations were 4 mg./ml. (upper curves) and 2 mg./ml. (lower curves).

experiments the effect of Mg^{2+} ions was missed owing to the fact that only extracellular (e.g. serum) concentrations were tested (Grisolia *et al.* 1962).

Effects of ADP and ATP on the instability induced by NADPH and diethylstilboestrol were studied with the human-liver enzyme in phosphate–chloride–Mg^{2+} ion buffer. Results are shown in Table 3. Clearly ADP and ATP protect the human-liver enzyme from inactivation, as previously reported for the ox-liver enzyme (Grisolia *et al.* 1962).

The partially purified uterine enzyme was incubated at physiological concentrations (44 milliunits/ml.) in phosphate–chloride buffer and phosphate–chloride–Mg^{2+} ion buffer. The results, shown in Table 4, indicate that Mg^{2+} ions protect against the instability induced by NADPH and diethylstilboestrol. The activity of the control incubations in this experiment actually increased as compared with that at zero time. This is a poorly understood phenomenon occasionally noted on preincubation of crude samples, but it may reflect non-specific activation (Grisolia, 1964).

Table 1. *Effect of* Mg^{2+} *ions on the stability of human-liver glutamate dehydrogenase*

Each tube contained, in 1·0 ml. final volume, 0·5 mg. (2·0 units) of glutamate dehydrogenase in phosphate–chloride buffer at 37° and the additions indicated. When used, 0·3 μmole of NADPH or NADH was added to each tube. Final concentrations of diethylstilboestrol were as shown. Activity was determined by using assay no. 1; 0·5 mg. of enzyme protein catalysed the reduction of 2·0 μmoles of NAD^+/min. and equals 100% activity. Values in parentheses are for preincubation in phosphate–chloride–Mg^{2+} ion buffer.

Cofactor	Concn. of diethylstilboestrol (μM)	Residual activity (%) At 60 min.	At 120 min.
None	0	98 (97)	98 (96)
None	1·0	95 (100)	94 (99)
None	5·0	87 (88)	83 (92)
None	10·0	84 (99)	76 (98)
NADPH	0	51 (76)	40 (65)
NADPH	1·0	44 (70)	31 (59)
NADPH	10·0	24 (36)	13 (18)
NADH	0	89 (93)	84 (94)
NADH	1·0	84 (95)	79 (91)
NADH	10·0	77 (78)	71 (75)

Table 2. *Effect of* Mg^{2+} *ions on the stability of ox-liver glutamate dehydrogenase*

The conditions used were as described in Table 1 except that the ox-liver enzyme was used. Activity was determined by using assay no. 1; 0·5 mg. of enzyme catalysed the reduction of 2·0 μmoles of NAD^+/min. and equals 100% activity. Values in parentheses are for preincubation in phosphate–chloride–Mg^{2+} ion buffer.

Cofactor	Concn. of diethylstilboestrol (μM)	Residual activity (%) At 60 min.	At 120 min.
None	0	100 (95)	97 (94)
None	1·0	95 (93)	90 (94)
None	10·0	80 (90)	77 (87)
NADPH	0	67 (82)	50 (72)
NADPH	1·0	57 (72)	40 (55)
NADPH	10·0	23 (70)	15 (49)

Mechanism of action of Mg^{2+} *ions*

To elucidate the mechanism by which Mg^{2+} ions protect the enzyme from inactivation, investigations were made with human-liver enzyme as to (*a*) the effect of Mg^{2+} ions on initial velocity, (*b*) the effect of Mg^{2+} ions when added to the reaction at equilibrium and (*c*) the effect of Mg^{2+} ions on the binding of cofactors by the enzyme.

Effects on assay. The enzyme utilized both NAD^+ and $NADP^+$ as cofactor. Under the conditions of assay no. 1, with 5 μg. of enzyme, the rate with NAD^+ was 2·6 times that with $NADP^+$. Initial velocities at high NAD^+ concentrations were greater than expected (Fig. 4). Similar results with NAD^+ and ox-liver enzyme have been obtained previously (Olson & Anfinsen, 1952; Frieden, 1959). The latter worker originally attributed this finding to the presence of both an active and an activating site for NAD^+ on the enzyme molecule, but more

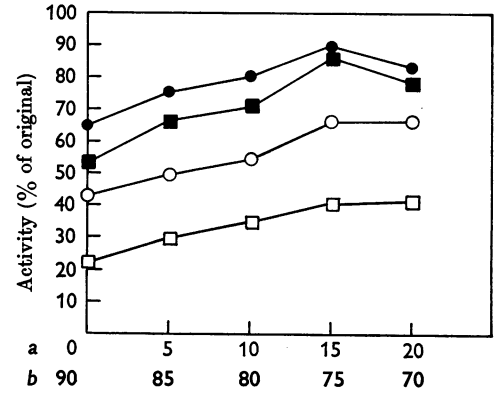

Fig. 3. Effect of increasing concentration of Mg^{2+} ions on the stability of ox-liver glutamate dehydrogenase. Each point represents the average of two determinations. Each tube contained 0·5 mg. of glutamate dehydrogenase and 0·3 μmole of NADPH/ml. of solution at pH 7·3 containing the indicated μmoles/ml. of (*a*) magnesium chloride and (*b*) potassium phosphate buffer. Activity was evaluated by assay no. 1; 0·5 mg. of enzyme protein catalysed the reduction of 2·0 μmoles of NAD^+/min. and equals 100% activity. ● and ○, At 60 min.; ■ and □, at 120 min.; ● and ■, diethylstilboestrol absent; ○ and □, diethylstilboestrol (10 μM) present.

recently has negated this interpretation (Frieden, 1963*b*). With $NADP^+$ no such cofactor activation was seen and the K_m for $NADP^+$ was 75 μM, which is comparable with the value determined for the ox-liver enzyme (Frieden, 1959).

During the studies of stability described above, the amount of Mg^{2+} ions added to the assay medium with the enzyme itself was 0·75 μmole or less. This small quantity was shown to have no demonstrable effect on initial velocity when added to the constituents of assay no. 1.

By using assay no. 1 with $NADP^+$ as cofactor and 5 μg. of enzyme, magnesium sulphate at concentrations of 8·3 and 16·6 mM had little or no effect on initial velocity (velocity was essentially equal to that in control assays without Mg^{2+} ions). By using the same assay with NAD^+ as cofactor, 8·3 mM-Mg^{2+} ion inhibited the initial rate by 24 %. Initial velocities were determined at several NAD^+ concentrations with and without 8·3 mM-Mg^{2+} ion in the assay medium. A double-reciprocal plot of NAD^+ concentration against initial velocity is shown in Fig. 4. The plot is compatible with a competitive or partially competitive inhibition of NAD^+ by Mg^{2+} ions and in both instances activation of the enzyme by the cofactor is seen. By using assay no. 2 with 1·2 μg. of enzyme, the initial velocity in the presence of 1·75 mM-Mg^{2+} ion was inhibited 28 % with NADH and 45 % with NADPH.

Thus Mg^{2+} ions inhibit the oxidation of both cofactors and the reduction of NAD^+ but have no effect on the reduction of $NADP^+$.

Effect of Mg^{2+} *ions at equilibrium.* In several experiments cuvettes containing the constituents of assay no. 1 (except that 1 μmole of NAD^+ or $NADP^+$ was added) were prepared, the reaction started with 50 μg. of enzyme and the cuvette sealed. Equilibrium was reached in about 1·5 hr. with approx. 52 % of the NAD^+ and 45 % of the $NADP^+$ in the reduced form. Controls containing 50 μg. of enzyme and the constituents of assay no. 2 (NADH or NADPH) except for ammonium chloride were also prepared. The addition of Mg^{2+} ions where NAD^+ was cofactor caused a progressive decrease in extinction. Similar additions of Mg^{2+} ions to controls containing NADH but no ammonium chloride caused no change. Additions of Mg^{2+} ions to cuvettes where $NADP^+$ was cofactor caused a progressive increase in extinction. Also, the addition of Mg^{2+} ions to control incubations containing NADPH but no ammonium chloride caused no change. These findings are illustrated in Fig. 5.

The results indicate that Mg^{2+} ions affect the apparent equilibrium. Whether this is due to a direct effect on the active site and/or a modifier site or to coenzyme–Mg^{2+} ion complex-formation re-

Table 3. *Effect of adenosine 5′-diphosphate, adenosine 5′-triphosphate, reduced nicotinamide–adenine dinucleotide phosphate and diethylstilboestrol on the stability of human-liver glutamate dehydrogenase*

Conditions were as described in Table 1 with a final volume of 1·0 ml. When used, 0·3 μmole of NADPH and 1 μmole of ADP or ATP were added. The final diethylstilboestrol concentration was 20 μM. Activity was determined by using assay no. 1; 0·5 mg. of enzyme catalyses the reduction of 2·0 μmoles of NAD^+/min. and equals 100 % activity.

Additions			Residual activity (%)	
NADPH	Diethylstilboestrol	ADP or ATP	At 60 min.	At 120 min.
0	0	0	100	90
+	0	0	81	62
+	0	ATP	95	94
+	0	ADP	105	106
+	+	0	52	20
+	+	ATP	82	75
+	+	ADP	105	100

Table 4. *Effect of* Mg^{2+} *ions on the stability of human-uterine glutamate dehydrogenase*

Each tube contained 2 mg. (44 milliunits) of the uterine glutamate-dehydrogenase preparation in 1·0 ml. of phosphate–chloride buffer with the additions shown. Activity was determined by using assay no. 1; 2·0 mg. of enzyme protein catalysed the reduction of 0·09 μmole of NAD^+/min. and equals 100 % activity. Values in parentheses represent preincubation in phosphate–chloride–Mg^{2+} ion buffer.

Additions	Residual activity (%)	
	At 60 min.	At 120 min.
0	112 (114)	112 (112)
NADPH (0·3 mM)	91 (98)	84 (97)
NADPH (0·3 mM) + diethylstilboestrol (10 μM)	67 (79)	47 (64)

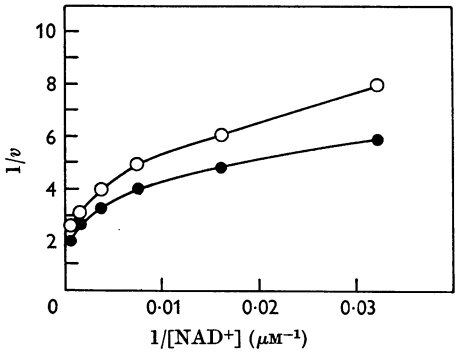

Fig. 4. Double-reciprocal plot for NAD^+ in the presence and absence of Mg^{2+} ions. The results were obtained at 30° by using assay no. 1 and 5 μg. of human-liver enzyme, with (○) and without (●) 8·4 mM-magnesium sulphate.

mains to be determined. Other kinetic studies have shown no effect of Mg^{2+} ions on initial velocities with $NADP^+$. Therefore, the experiments suggest a decrease in the availability of NADPH after the addition of Mg^{2+} ions at equilibrium.

Effect of Mg^{2+} *ions on cofactor binding.* Attempts were made to study the binding of NAD^+ and $NADP^+$ by change in protein fluorescence in an Aminco–Bowman spectrophotofluorimeter (American Instrument Co. Inc., Silver Springs, Md., U.S.A.) with the human-liver enzyme at a concentration of 20 μg./ml., activation at 300 mμ and quantitation of emission at 350 mμ, as described by Frieden (1963*a*). In all cases, the fluorescence decreased at such a rapid rate (even without the addition of cofactor) that no determinations could be made. Attempts to protect the human-liver enzyme, and the bovine enzyme, with high salt concentration and glycerol were unsuccessful.

Binding of NADH and NADPH to the human and ox enzymes was studied by fluorimetric titration of the enzyme as described by Velick (1958) by using a Farrand photofluorimeter (Farrand Optical Co. Inc., New York, N.Y., U.S.A.). The results obtained were plotted as described by Tomkins, Yielding & Curran (1962). Unfortunately, fluorimetric measurements with the instrumentation so far available, in spite of all precautions, are neither constant nor reproducible by more than a few per cent. Therefore the plots must be interpreted with a great deal of caution. Several runs were made with the human enzyme and some variations were seen (e.g. Fig. 6). With phosphate–chloride buffer, K_D of NADH was calculated as 1·2 μM and K_D of NADPH was 1·3 μM. With phosphate–chloride–Mg^{2+} ion buffer K_D of NADH was 1·7 μM and K_D of NADPH was 1·7 μM. The calculated binding sites on the human enzyme were $16/10^6$ g. in both buffers. Similar plots with the bovine enzyme revealed in phosphate–chloride buffer that K_D of NADH was 0·98 μM and K_D of NADPH was 0·86 μM, whereas in phosphate–chloride–Mg^{2+} ion buffer K_D of NADH was 3·7 μM and K_D of NADPH was 2·4 μM. These values are comparable with those reported by others for the bovine enzyme (Tomkins *et al.* 1962), and suggest that Mg^{2+} ions act in some way to decrease association of cofactor and enzyme without changing the number of binding sites. The Mg^{2+} ions had no effect on the fluorescence of NADH and NADPH in the absence of enzyme. Nor could an indication of complex-formation in the absence of enzyme be found by study of absorption spectra.

Mechanism of cofactor and diethylstilboestrol inactivation

Several experiments were conducted to elucidate the mechanism by which cofactors and diethylstilboestrol inactivate the human enzyme.

Titration of thiol groups. An attempt was first made to compare thiol groups in the active and inactivated enzyme by spectrophotometric analysis with *p*-chloromercuribenzoate (Boyer, 1954), but this was prevented by turbidity, as previously noted with the ox-liver enzyme (Grisolia *et al.* 1962). However, the argentometric-titration tech-

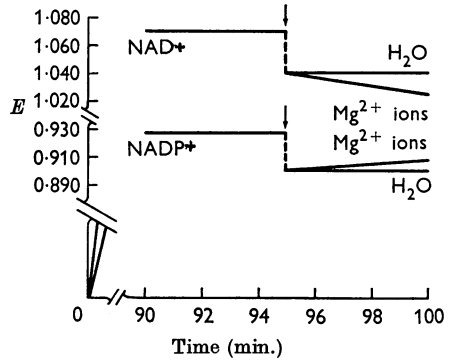

Fig. 5. Effect of Mg^{2+} ions on the apparent equilibrium. Cuvettes (3 ml. capacity) contained the constituents of assay no. 1 except that the amount of NAD^+ or $NADP^+$ added was 1·0 μmole. At zero time, 50 μg. of enzyme was added and cuvette sealed. Arrows indicate time of addition of 0·1 ml. of water or 0·5 M-magnesium sulphate (a similar result was obtained with 0·5 M-magnesium chloride). The broken line indicates the immediate decrease in extinction due to dilution.

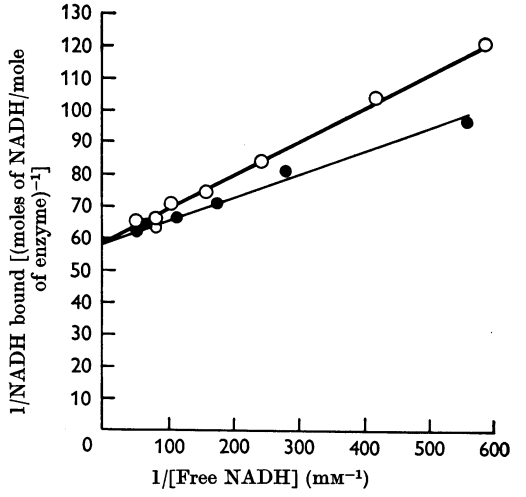

Fig. 6. Effect of Mg^{2+} ions on NADH binding by human-liver glutamate dehydrogenase. The enzyme was at a concentration of 1·0 mg./ml. ○, Incubation in phosphate–chloride–Mg^{2+} ion buffer; ●, incubation in phosphate–chloride buffer.

nique of Carter (1959) was successful. The results from duplicate titrations revealed 27 moles of SH/10^6 g. of enzyme for control preparations, 35 moles of SH/10^6 g. of enzyme after inactivation with 0·3 mM-NADPH and 68 moles of SH/10^6 g. of enzyme after inactivation with 0·3 mM-NADPH and 10 μM-diethylstilboestrol. After denaturation with urea, the enzyme contained 105 moles of SH/10^6 g. Thus inactivation with NADPH and diethylstilboestrol results in the unmasking of thiol groups, confirming the results of Grisolia *et al.* (1962) with bovine enzyme.

Generation of 'NADPH-X' during enzyme inactivation. 'NADH-X' is the hydrated product formed from NADH as described by Chaykin, Meinhart & Krebs (1956). It has been well established that inactivation of triose phosphate dehydrogenase in the presence of NADH is accompanied by the formation of NADH-X (Amelunxen & Grisolia, 1962). More recently, the formation of NADPH-X from NADPH under mild conditions (e.g. neutral pH at 37°) has been demonstrated (B. Regueiro, R. Amelunxen & S. Grisolia, unpublished work).

The possibility that inactivation of human-liver glutamate dehydrogenase is accompanied by formation of NADPH-X was investigated. NADPH-X shows an extinction that is marked at 290 mμ and negligible at 340 mμ, whereas NADPH has a much higher extinction at 340 mμ than at 290 mμ. The extinction of solutions of 0·3 mM-NADPH in phosphate–chloride buffer at 290 and 340 mμ in both the presence and absence of enzyme at a concentration of 0·5 mg./ml. at 37° was followed over a 2 hr. period. (Whenever there was turbidity arising from protein denaturation the samples were centrifuged and corrections for protein lost were made by using biuret determinations on portions of the supernatant fluid.) The $E_{290\,m\mu}/E_{340\,m\mu}$ extinction ratios of control samples containing NADPH, in the absence of enzyme, were 0·42 at zero time, 0·56 at 60 min. and 0·80 at 120 min. The ratios in the presence of enzyme were 0·42, 0·49 and 0·76 at 0, 60 and 120 min. respectively. Thus no differences in formation of NADPH-X due to enzyme inactivation were seen. (The activity decreased to 30 % and 20 % respectively at 60 and 120 min.)

Differences in inactivation with reduced nicotinamide–adenine dinucleotide and reduced nicotinamide–adenine dinucleotide phosphate. Changes in relative glutamate-dehydrogenase and alanine-dehydrogenase stability of the human-liver enzyme in phosphate–chloride buffer in the presence of cofactor and diethylstilboestrol are shown in Table 5. In the presence of diethylstilboestrol or NADH or both, the alanine-dehydrogenase activity decreased to a smaller extent than did glutamate-dehydrogenase activity; indeed, in some instances it appeared to be activated. In the presence of NADPH alone or with diethylstilboestrol alanine-dehydrogenase activity decreased to a greater extent than did glutamate-dehydrogenase activity.

The relative changes in glutamate-dehydrogenase and alanine-dehydrogenase stability of the ox-liver enzyme in 0·05 M-tris–hydrochloric acid buffer, pH 7·2, with and without 0·3 mM-NADH were studied (Table 6). NADH at low salt concentration actually protects the enzyme. In previous studies with the ox-liver enzyme (Grisolia *et al.* 1962), NADPH always inactivated the enzyme under these conditions.

Table 5. *Effect of reduced nicotinamide–adenine dinucleotide, reduced nicotinamide–adenine dinucleotide phosphate and diethylstilboestrol on the stability of the glutamate-dehydrogenase and alanine-dehydrogenase activities of the human-liver enzyme*

Incubation was in phosphate–chloride buffer with the additions as shown. When added, the final concentration of NADH was 0·3 mM, that of NADPH was 0·1 mM and that of diethylstilboestrol was 10 μM. The enzyme concentration was 0·5 mg./ml. Free values are for glutamate-dehydrogenase activity by 5·0 μg. of enzyme and assay no. 1. Values in parentheses are for alanine-dehydrogenase activity by using 25 μg. of enzyme and assay no. 3; for alanine dehydrogenase 0·5 mg. of enzyme catalysed the oxidation of 0·04 μmole of NADH/min. and equals 100 % activity.

Additions	Residual activity (%) At 60 min.	At 120 min.
None	95 (100)	89 (89)
NADPH	93 (87)	84 (68)
NADH	99 (107)	100 (110)
Diethylstilboestrol	92 (98)	91 (102)
NADPH + diethylstilboestrol	39 (23)	26 (2)
NADH + diethylstilboestrol	64 (95)	59 (88)

Table 6. *Effect of reduced nicotinamide–adenine dinucleotide on the stability of glutamate-dehydrogenase and alanine-dehydrogenase activities of the ox-liver enzyme*

Incubation was in 0·05 M-tris–HCl buffer, pH 7·2. The concentration of NADH, when added, was 0·3 mM. The enzyme concentration was 0·5 mg./ml. The conditions of assay were as in Table 5. Free values are for glutamate-dehydrogenase activity. Values in parentheses are for alanine-dehydrogenase activity.

Addition	Residual activity (%) At 60 min.	At 120 min.
None	31 (49)	7 (16)
None	36 (54)	14 (24)
NADH	91 (86)	89 (86)
NADH	98 (90)	92 (84)

Effects of natural steroid hormones and diethylstilboestrol

Because it has been previously suggested that glutamate dehydrogenase may play a role in the mechanism of hormone action, the effects of natural steroids on activity and stability were evaluated.

Effects on activity. The effects of the presence of diethylstilboestrol and natural steroid hormones on the activity of human-liver glutamate dehydrogenase are shown in Table 7. Oestradiol-17β, a natural oestrogen of high biological activity, inhibits whereas its 17α-isomer, which is at best a weak oestrogen, lacks inhibitory effect. On the other hand, all steroid concentrations used markedly exceed physiological steroid concentrations.

Possible antagonistic effects of oestrogenic and androgenic compounds on activity were evaluated (Table 8). In all instances the effects of compounds whose biological activities are in general antagonistic are synergistic in this specific experiment.

Effects on stability of the enzyme. The effects of natural steroids on the stability of both human-liver and -uterine enzymes are shown in Table 9. It is obvious that in a buffer resembling intracellular fluid, natural steroids, even at 10 μM, have little or no effect on the stability of the enzyme.

Effects of steroids on stability of the human-liver enzyme in the presence of NADH and NADPH are shown in Table 10. With NADPH only oestradiol-17β and possibly dehydroepiandrosterone caused a decrease in stability, and even this was minimal. With NADH only oestradiol-17β in phosphate–chloride buffer decreased stability. Actually, several of the Δ^4-3-ketones seemed to protect the enzyme against NADH inactivation. All these effects were absent with 1 μM-steroid concentrations.

The same absence of impressive steroid effects is seen with the ox-liver enzyme (Table 11). Further, similar experiments with the human-uterine enzyme also revealed lack of significant steroid effect.

DISCUSSION

The findings presented above demonstrate similarities in the kinetic and molecular behaviour of human and bovine glutamate dehydrogenase. These findings confirm and extend previous studies with the ox-liver enzyme (Grisolia *et al.* 1962). That such similarities should be expected is indicated by the work of Kubo, Iwatsubo, Hiroshi & Soyama (1959). The observations of Kubo *et al.* (1957), with light-scattering techniques, indicate that deaggregation of the human-liver enzyme occurs with decreasing concentration between 3·0

Table 7. *Effects of steroids and diethylstilboestrol on the activity of human-liver glutamate dehydrogenase*

The results were obtained by using assay no. 1, with 1 μmole of cofactor and 12·5 μg. of enzyme; 12·5 μg. of the enzyme catalysed the reduction of 0·05 μmole of NAD^+ or 0·02 μmole of $NADP^+$/min. and equals 100% activity. Steroids, when present, were 40 μM and were added in 0·05 ml. of propylene glycol. Activities with NAD^+ and $NADP^+$ are expressed as percentages of a control to which only propylene glycol was added.

Addition	Activity (%) With NAD^+	Activity (%) With $NADP^+$
Diethylstilboestrol	2	1
Oestradiol-17β	77	67
Oestradiol-17α	95	100
Testosterone	85	100
Epitestosterone	98	100

Table 8. *Effects of mixtures of sex hormones on the activity of human-liver glutamate dehydrogenase*

The conditions of assay were as described in Table 7; 12·5 μg. of the enzyme catalysed the reduction of 0·05 μmole of NAD^+ or 0·02 μmole of $NADP^+$/min. and equals 100% activity. The additions and final concentrations were as shown. Activities are expressed as percentages of a control to which a similar quantity of propylene glycol was added.

Addition	Activity (%) With NAD^+	Activity (%) With $NADP^+$
Diethylstilboestrol (10 μM)	20	10
Diethylstilboestrol (10 μM) + testosterone (50 μM)	11	6
Oestradiol-17β (10 μM)	87	73
Oestradiol-17β (10 μM) + testosterone (50 μM)	82	61
Diethylstilboestrol (1 μM)	—	82
Diethylstilboestrol (1 μM) + testosterone (50 μM)	—	65

Table 9. *Effects of natural steroids on the stability of human glutamate dehydrogenases*

Each tube contained 0·5 mg. of glutamate dehydrogenase from human liver or 2·0 mg. prepared from uterus, together with the steroid listed at a final concentration of 10 μM. Activity was determined by assay no. 1; activity of 100% is defined in Tables 1 and 4. Free values are for incubation in phosphate–chloride buffer. Values in parentheses are for incubation in phosphate–chloride–Mg^{2+} ion buffer.

Steroid	Residual activity (%) at 120 min. With liver enzyme	With uterine enzyme
None	99 (95)	— (99)
Dehydroepiandrosterone	93 (92)	— (100)
Oestradiol-17β	91 (97)	— (99)
Testosterone	102 (100)	— (108)
Progesterone	103 (90)	— (102)
Cortisol	90 (95)	— (99)

Table 10. *Effect of natural steroids on the stability of human-liver glutamate dehydrogenase in the presence of reduced nicotinamide–adenine dinucleotide or reduced nicotinamide–adenine dinucleotide phosphate*

Each tube contained 0·5 mg. of human-liver enzyme in 1 ml. Cofactors as shown were at a final concentration of 0·3 mM, steroids at a final concentration of 10 μM. Activity of 100% is defined in Table 1. Free values are for incubation in phosphate–chloride buffer. Values in parentheses are for incubation in phosphate–chloride–Mg^{2+} ion buffer.

Cofactor	Steroid	Residual activity (%) At 60 min.	Residual activity (%) At 120 min.
None	None	95 (94)	97 (98)
NADPH	None	62 (71)	51 (61)
NADPH	Dehydroepiandrosterone	46 (80)	34 (70)
NADPH	Oestradiol-17β	54 (66)	37 (53)
NADPH	Testosterone	57 (67)	48 (51)
NADPH	Progesterone	60 (67)	48 (56)
NADPH	Cortisol	54 (75)	46 (64)
NADH	None	81 (86)	74 (79)
NADH	Dehydroepiandrosterone	85 (96)	87 (95)
NADH	Oestradiol-17β	67 (96)	51 (93)
NADH	Testosterone	93 (100)	88 (96)
NADH	Progesterone	95 (88)	91 (83)
NADH	Cortisol	93 (93)	89 (89)

Table 11. *Effect of natural steroids on the stability of ox-liver glutamate dehydrogenase in the presence of reduced nicotinamide–adenine dinucleotide*

Conditions were as described in Table 10 except that ox-liver enzyme was used. Free values are for incubation in phosphate–chloride buffer. Values in parentheses are for incubation in phosphate–chloride–Mg^{2+} ion buffer.

Cofactor	Steroid	Residual activity (%) At 60 min.	Residual activity (%) At 120 min.
None	None	98 (95)	97 (94)
NADPH	None	67 (82)	50 (72)
NADPH	Oestradiol-17β	68 (88)	60 (77)
NADPH	Dehydroepiandrosterone	72 (79)	54 (76)
NADPH	Testosterone	73 (79)	61 (69)
NADPH	Progesterone	62 (80)	55 (69)
NADPH	Cortisol	77 (82)	73 (78)

and 0·1 mg./ml. The present ultracentrifugation results are in agreement with their findings.

The stability of the human-liver enzyme under physiological conditions is decreased by physiological concentrations (Glock & McLean, 1955) of NADPH and to a lesser extent by NADH. Further, diethylstilboestrol markedly potentiates the cofactor effect. ADP and ATP protect the human-liver enzyme from inactivation by NADPH both alone and in the presence of diethylstilboestrol.

It has been claimed that inactivation of glutamate-dehydrogenase activity by diethylstilboestrol results in a concomitant increase of alanine-dehydrogenase activity, originally interpreted as being an obligatory result of changes in the aggregational state of the enzyme. It was stated that 'the degree of association or dissociation directly determines initial velocities or, alternatively, that reaction velocities are a measure of the state of association or dissociation of the enzyme molecule' (Frieden, 1959). This concept was extended by others to interpret steroid-hormone effects, and it was suggested that change in enzyme structure induced by steroids was responsible for the action of hormones as inhibitors of glutamate-dehydrogenase activity and activators of alanine-dehydrogenase activity (Yielding & Tomkins, 1960). The dissociated enzyme was said to catalyse the alanine-dehydrogenase reaction whereas the tetramer was active as glutamate dehydrogenase (Tomkins *et al.* 1961). These conclusions were based on studies of activity and aggregation performed with dissimilar enzyme concentrations.

Grisolia *et al.* (1962), observing rather limited changes in activity with glutamate as substrate over a wide range in enzyme concentration, suggested that activity could not be fully dependent on aggregation. They pointed out that otherwise there should be no measurable activity at low enzyme concentrations (e.g. in the range commonly used for spectrophotometric measurements of activity) unless there was aggregation by the components of

the assay. It remains to be determined, however, if there is a different time factor involved in changes of aggregation and activity.

Experimental evidence that the aggregated and deaggregated enzymes have identical catalytic activity with glutamate as substrate was presented in studies where activity and aggregation were analysed with similar conditions and enzyme concentrations (Fisher *et al.* 1962). It now appears that, although inhibitors may induce configurational changes in the enzyme that prevent association of sub-units, the extent of association in itself has no effect on enzymic activity (Frieden, 1963*b*).

The inactivation by NADPH and its potentiation by diethylstilboestrol results in a progressive increase in the number of titratable thiol groups and decreases activity with both glutamate and alanine as substrate. Thus it differs from that which occurs with NADH and diethylstilboestrol, where reciprocal changes in activity with these substrates may be seen.

The natural steroid hormones, as shown, have little effect on the activity of the human enzyme. Although oestradiol-17β did inhibit initial velocity whereas its biologically less active 17α-isomer did not, the hormone concentrations necessary to demonstrate clear-cut effects were 500–5000 times those found *in vivo* (Jensen & Jacobson, 1960). Even demonstrated differences in intracellular steroid distribution (Westphal, 1961) fail by far to account for differences of this magnitude. Further, though oestrogenic and androgenic substances are generally antagonistic in their biological effects (one important exception being in nitrogen retention and anabolism), they are synergistic in their inhibitory effect on the activity of human-liver glutamate dehydrogenase.

The effects of natural steroids on the stability of both human-liver and uterine enzyme at physiological concentrations and conditions are unimpressive even with steroid concentrations markedly exceeding those *in vivo*. It should be fairly obvious that the inhibition and instability observed with any enzyme markedly diluted in a solution almost free of salts and with the steroid added in a concentration 1000 times that found *in vivo* may be of a general chemical nature rather than of physiological significance. To conclude that hormone effects are due to changes in aggregation, with the attractive feature of reciprocal changes in glutamate and alanine oxidation, seems grossly incorrect.

In the presence of NADPH, oestradiol-17β and dehydroepiandrosterone at high concentrations did decrease stability but this was minimized by 0·015M-Mg^{2+} ion. However, as shown previously, and further exemplified in the present work, the potentiation of cofactor effects on stability by natural steroids and particularly by diethylstilboestrol may have physiological significance. Indeed, although the cofactor requirement has been generally overlooked or minimized (Yielding & Tomkins, 1960; Tomkins *et al.* 1961), previous work from this Laboratory (Grisolia *et al.* 1962) suggests that this may be the more important aspect of the effects of diethylstilboestrol and related reagents on glutamate dehydrogenase. Aggregation effects may remain as a model for studies of diethylstilboestrol at rather high concentrations.

The effect of Mg^{2+} ions is of much interest. Intracellular Mg^{2+} ions are effective in changing both kinetic and stability properties. Though the complicated interactions of substrate, cofactor and modifier (e.g. metal ion) may require much additional investigation and clarification, they possibly offer new dimensions to the evaluation of substrate- and hormone-induced changes in activity and stability.

SUMMARY

1. The stability of crystalline human-liver glutamate dehydrogenase and purified human-uterine glutamate dehydrogenase is markedly decreased by NADPH at physiological concentrations. This effect is potentiated by diethylstilboestrol and associated with an increase in titratable thiol groups.

2. Magnesium ions at intracellular concentrations protect the enzyme (both human and ox) against the NADPH-, NADH- and diethylstilboestrol-induced instability; protection is almost nil at extracellular concentrations.

3. Magnesium ions affect the velocity with NAD^+, NADH and NADPH but not with $NADP^+$. They change apparent equilibrium and cofactor dissociation constants but not the number of binding sites.

4. In a buffer resembling intracellular fluid, the stability of human-liver and -uterine glutamate dehydrogenase at physiological concentrations is not significantly affected by 10μM-oestradiol-17β, -dehydroepiandrosterone, -testosterone, -progesterone or -cortisol. Even in the presence of physiological concentrations of NADPH, natural steroids exert only minimal effects not comparable with those of diethylstilboestrol.

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Peptidase Activity in Shoot Tips of the Tea Plant (*Camellia sinensis* L.)

BY G. W. SANDERSON AND G. R. ROBERTS
Tea Research Institute of Ceylon, Talawakelle, Ceylon

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The shoot tips of tea plants (*Camellia sinensis* L.) comprising the buds, the first two leaves and the included stems are collectively called the 'flush' in the tea industry. The flush of the tea plant is important commercially because it is the portion of the plant used in the manufacture of tea.

The first step in tea manufacture after the plucking of the flush is a process called 'withering'. In orthodox withering the flush is spread in a thin layer on tats where it is stored until the moisture content has been suitably decreased. In Ceylon, under average conditions, the moisture content of the flush is decreased from 78 to 55 % in about 18 hr. The loss of moisture, called 'physical withering', is of importance in preparing the flush for subsequent manufacturing processes (cf. Eden, 1958; Keegel, 1958; Harler, 1963), but chemical changes also occur within the tissues of the flush during this storage period and these apparently affect the quality of the final product (Trinick & Choudhury, 1963). This latter aspect of withering, called 'chemical withering', is only poorly understood at the present time and it is the subject of current investigation.

An increase in the soluble nitrogen content of tea shoot tips during withering was reported by Evans (1929), and the observation that there was a loss of protein during the withering period was also reported (Roberts, 1939). However, it remained for Roberts & Wood (1951), using paper chromatography, to show definitely that free amino acids were formed.

Bhatia (1962*a*, *b*, 1963), using quantitative methods, has studied the increase of individual amino acids during withering. He has applied his techniques in comparing orthodox and faster new withering processes for their effect on chemical withering (Bhatia, 1962*a*, *b*, 1963).

The object of our investigation was to establish whether the changes in the concentration of free amino acids in tea shoot tips during withering was enzymic or not, and, if these changes were enzymic, to characterize the enzyme system involved. The results of our investigation are reported below.

MATERIALS AND METHODS

Source of tea shoot tips. The tea shoot tips (flush) used in the present investigation were collected from tea plants grown in a field near the Laboratory (4500 ft. elevation).