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Chemical Modification of Glutamate Dehydrogenase by 2,4,6-Trinitrobenzenesulphonic Acid

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1. Modification with 2,4,6-trinitrobenzenesulphonic acid was studied for its effect on the structure, activity and response to regulatory effectors of ox liver glutamate dehydrogenase. 2. The modification affected amino groups only, and the relative reactivities of the amino groups of the enzyme are described. 3. A biphasic inactivation of the enzyme was observed and analysis of the course of inactivation and of modification showed that the rapid reaction of one amino group/subunit leads to loss of 80% of the enzymic activity. 4. NADH retarded the inactivation by 2,4,6-trinitrobenzenesulphonic acid, the protection increasing with NADH concentration. This, together with the previous observation, suggests that the rapidly reacting group is essential for the activity of the enzyme. 5. The effects of modification on the optical-rotatory-dispersion and sedimentation behaviour of the enzyme were studied. 6. The enzyme's response to the allosteric effector GTP was rapidly lost on modification, whereas its response to ADP was unaffected. Comparison of the inactivation and desensitization suggests that the reactive amino group is essential for both activity and GTP response, and that only a completely unmodified enzyme oligomer responds fully to GTP. 7. The merits of chemical-modification studies of large enzymes are discussed critically in connexion with the interpretation of these results.

Chemical modification of proteins with concurrent observation of functional changes has been widely used to implicate particular functional groups in the activity of enzymes and other proteins (Sri Ram, Bier & Maurer, 1962; Singer, 1967). The major problems in deriving useful information from such studies are in finding reagents of sufficient selectivity and in showing that their effect on function is a primary result of a specific chemical reaction rather than a consequence of generalized structural change in the protein. Ray & Koshland (1961) have shown that the functional role of particular groups can be understood from information correlating the rate of loss of function to the rate of modification of those groups; their treatment has been generalized by Tsou (1962).

In the modification of amino side chains, specificity is difficult to obtain (Cohen, 1968) since other nucleophilic side chains also react, but it has been shown that TNBS* at neutral pH will modify only thiol and amino groups (Kotaki, Harada & Yagi, 1964; Freedman & Radda, 1968*a*).

GDH has been widely studied with respect to physical properties (Cross & Fisher, 1965, and references cited therein; Bayley & Radda, 1966; Eisenberg & Tomkins, 1968; Sund & Burchard, 1968) and kinetic parameters (Frieden, 1963*a*,*b*; Dalziel & Engel, 1968). The activity of the enzyme is affected by a large number of molecules, particularly purine nucleotides (Frieden, 1963*a*; Frieden & Colman, 1967) and some hormones (Tomkins & Yielding, 1961). In some cases these effects have been correlated with conformational changes in the protein (Bayley & Radda, 1966; Dodd & Radda, 1967, 1969). Modification of GDH with acetic anhydride showed that amino groups are particularly important in both the activity and the regulation of the enzyme (Colman & Frieden, 1966*a*,*b*). Because of the difficulties involved in following the rate of acetylation no attempt was made to apply the procedure of Ray & Koshland (1961).

By using TNBS we hoped to modify these groups in a mild and easily quantifiable manner, in order to establish if the group or groups modified could be described as being at the active site, or as being involved in the conformational changes associated with the regulatory properties of GDH. A preliminary account of part of this work has appeared (Freedman & Radda, 1968*b*).

* Abbreviations; GDH, L-glutamate–NAD(P) oxidoreductase (deaminating), EC 1.4.1.3; TNBS, 2,4,6-trinitrobenzenesulphonic acid; TNP, 2,4,6-trinitrophenyl.

MATERIALS AND METHODS

TNBS sodium salt dihydrate was obtained from the Pierce Chemical Co. (Rockford, Ill., U.S.A.). Papain was obtained

from Sigma Chemical Co. (St Louis, Mo., U.S.A.) as a suspension in sodium acetate, and leucine aminopeptidase was supplied by Seravac Laboratories Ltd. (Maidenhead, Berks.) as a suspension in $(NH_4)_2SO_4$ solution. Sephadex G-25 (medium grade) was obtained from Pharmacia (Uppsala, Sweden). Sodium dodecyl sulphate was obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Phenylmercuric acetate was a microanalytical reagent from British Drug Houses Ltd. (Poole, Dorset), and HCl for protein hydrolysis was AristaR grade from the same source. Sodium phosphate, sodium acetate and tris–HCl buffers were made up with AnalaR reagents from British Drug Houses Ltd.; the NH_4Cl and EDTA for enzymic assays were also AnalaR grade. L-Lysine monohydrochloride and L-cysteine were both British Drug Houses Ltd. biochemical reagents and the Folin–Ciocalteu reagent was also supplied by British Drug Houses Ltd. All other compounds and ox liver GDH were obtained from the sources described by Dodd & Radda (1969).

GDH was freed from its suspension in $(NH_4)_2SO_4$ solution either by dialysis against two changes of 0·1M-phosphate buffer, pH7·6, at 4°, or by centrifugation of the suspension followed by dissolution of the pellet in 0·1M-phosphate buffer, pH7·6, and gel filtration of this solution through Sephadex G-25. It was shown that after this treatment no NH_3 was present in the solution to interfere with the TNBS reaction. The concentration of native GDH was determined by measuring its extinction at 280nm. (Olson & Anfinsen, 1952). Modified GDH was determined by the Folin method (Lowry, Rosebrough, Farr & Randall, 1951), with an unmodified GDH sample as the standard. The modification by TNBS does not affect tyrosine residues, nor the extinction at 750nm.

NADH, ADP and GTP were determined by measuring their extinctions at appropriate wavelengths (Dodd & Radda, 1969). NADH was made up in double-distilled water. All other compounds were determined by weight, and, unless otherwise mentioned, were made up quantitatively in 0·1M-phosphate buffer, pH7·6. The buffers were made up in twice-distilled water.

Solutions of TNBS sodium salt were stored in the dark at 2° to minimize hydrolysis, and were not used more than 36hr. after having been made up. Solutions of NADH, GTP and ADP were made up freshly each day.

pH determinations were made with an E.I.L. direct-reading pH-meter. The u.v. and visible spectra were recorded on a Cary 14 recording spectrophotometer.

The course of reaction of TNBS with the enzyme was followed by monitoring the extinction of the TNP-amino group at 340nm. in a Hilger and Watts Uvispek spectrophotometer fitted with a Gilford scale expansion and automatic cuvette-change attachment. This instrument could present a full-scale deflexion of from 0·1 to 1·0 E unit at any background extinction up to 3·0 E units. The extent of modification was determined by using Goldfarb's (1966) value for the extinction coefficient of the TNP-amino group at neutral pH. Modification reactions were carried out with enzyme concentrations in the range 0·1–1·5mg./ml. and TNBS concentrations in the range 0·1–10mM. The reaction conditions were always 25·0±0·2° and pH7·6. When the modification was performed to study changes that could not be monitored continuously, samples of the reaction mixture (2–3ml.) were removed and filtered on Sephadex G-25 columns (1·5cm. × 15cm.) that had previously been equilibrated with 0·1M-phosphate buffer, pH7·6. This stopped the reaction and separated the protein from any other components (e.g. NADH) present in the incubation mixture.

Protein samples were hydrolysed with 6M-HCl at 108° for 24hr. and also by the enzymic method of Hill & Schmidt (1962), which employs papain and leucine aminopeptidase consecutively. Prolidase was omitted from the second stage of this digestion. The hydrolysates were analysed on a Locarte automatic amino acid analyser, which was calibrated by standard mixtures of amino acids. The elution procedure was essentially that of Spackman, Stein & Moore (1958).

The thiol content of protein samples was determined by polarographic titration with phenylmercuric acetate. A sample of protein was denatured by 1% (w/v) sodium dodecyl sulphate and then deoxygenated in the presence of Na_2SO_3 (50mM) with KCl (30mM) as supporting electrolyte. This solution was then titrated with standard phenylmercuric acetate solution (0·5mM) in a polarograph fitted with an automatic titrating attachment. Samples (10μl.) of titrant were added, the mixture was agitated by a stream of N_2 and then the concentration of unchanged mercurial was determined by measurement of the diffusion current at −0·6v. The theory of the method has been outlined by Cecil & Snow (1962).

ϵ-TNP-lysine was synthesized by a method similar to that of Okuyama & Satake (1960) modified to employ TNBS rather than picryl chloride. The product gave a single yellow spot (R_F0·70) on ascending paper chromatography in butan-1-ol–acetic acid–water (12:3:5, by vol.) and gave a positive ninhydrin test. In the amino acid analyser some unchanged lysine (approx. 5%) could be detected. *S*-TNP-cysteine was synthesized by preparing *NS*-bis-TNP-cysteine by the method of Kotaki *et al.* (1964) and then submitting it to acid hydrolysis, which cleaves the α-TNP-amino group to picric acid and amino group but does not affect the *S*-TNP group. The products of this hydrolysis were analysed on the amino acid analyser and showed a very acidic component absorbing at 440nm. that gave no ninhydrin colour, and a considerably retarded acid that is yellow and gives a positive ninhydrin test.

The activity of enzyme preparations was determined in the direction of oxidation of NADH, by following the loss of extinction at 340nm. in a Hilger and Watts Uvispek spectrophotometer with scale expanded to give full-scale deflexion of 0·1 E unit. The solution for the assay of GDH activity was as described by Dodd & Radda (1969). Alanine dehydrogenase activity was assayed by the method of Anderson, Anderson & Churchich (1966) with 0·1M-tris buffer, pH9·0. The quantity of enzyme required to give a good activity in this assay is about 100-fold that required in the GDH assay.

To determine the response of the enzyme to activators and inhibitors the enzyme was assayed by the standard method except for the presence of GTP (1–10μM) or ADP (0·1–1mM). In the protection studies TNBS and GDH were incubated together in the presence of other molecules, e.g. NADH, GTP. In such cases the activity was assayed directly if the final concentration of the 'protecting' species would be insignificant in the assay mixture, but otherwise it was necessary to remove samples and free the enzyme from the small molecules by gel filtration. In the former case it was shown that the same activities were obtained when the mixture was submitted to gel filtration as without this step.

Kinetic sedimentation studies were performed at 20° on a Spinco model E analytical ultracentrifuge at about 50000rev./min. with schlieren optics. The optical-rotatory-dispersion curves for both native and modified protein were determined in a Bendix–Ericsson recording spectropolarimeter (Polarmatic 62).

RESULTS

Analysis of the modification reaction. In a previous paper (Freedman & Radda, 1968*a*) we analysed the course of reaction of TNBS with GDH in terms of 'sets' of reacting groups, four reactive groups/subunit (mol.wt. approx. 52000 according to Appella & Tomkins, 1966; Marler & Tanford, 1964) and about 20 less reactive groups/subunit. We have attempted to refine this analysis by detailed observation of the earliest part of the reaction course, using low concentrations of TNBS (0·1–1mM) and correcting for the slowly reactive set. This analysis shows that among the 'reactive set' one group is particularly reactive. By the method of sequential corrections previously outlined, we can obtain an approximate value for the rate constant of modification of this group at pH7·6 at 25° of 100–2001.mole^{-1} min.$^{-1}$. The method does not allow a more accurate evaluation. By allowing the enzyme to react with TNBS at concentrations equal to the concentration of enzyme subunits we attempted to obtain the second-order rate constant for the fastest-reacting group directly. A value of 200–3001.mole^{-1}min.$^{-1}$ was obtained. The two or three moderately reactive groups/subunit have a rate constant of 20–251.mole^{-1}min.$^{-1}$ under these conditions. These values are slightly higher than those obtained before (Freedman & Radda, 1968*a*) because of the shift of pH from 7·4 to 7·6.

The known specificity of TNBS and the observation of the low reactivity of thiol groups in this enzyme (Hellerman, Schellenberg & Reiss, 1958; Colman & Frieden, 1966*b*) led us to assume that only amino groups were modified by this procedure. This assumption has been tested by a number of methods.

(1) ϵ-TNP-lysine and *S*-TNP-cysteine were synthesized as described above. Fig. 1 shows the spectra of ϵ-TNP-lysine and of the trinitrophenylated enzyme; the spectrum of *S*-TNP-cysteine (Kotaki *et al.* 1964) shows a much less pronounced shoulder at 420nm. The major difference between the two spectra shown in Fig. 1 is the result of protein absorption at about 280nm.

(2) We determined the thiol content of samples of GDH treated with 0·5mM-TNBS for various times by polarographic titration with phenylmercuric acetate. Fig. 2 shows a typical titration. In Table 1 are given the number of thiol groups titrating/enzyme subunit as a function of time of reaction for samples modified simply and for samples modified in the presence of NADH or of α-oxoglutaric acid. There is no difference between the sets, nor is there any significant loss of thiol groups with time of reaction. The readings give a mean of $4{\cdot}5 \pm 0{\cdot}5$ thiol groups/subunit.

(3) The positive identification of ϵ-TNP-lysine in modified protein samples was more difficult to obtain. Although we confirmed the finding (Kotaki & Satake, 1964) that ϵ-TNP-lysine was stable to 24hr. hydrolysis in 6M-hydrochloric acid at 108° in a sealed evacuated tube, the yellow colour of the modified protein was lost during such hydrolysis. Further, if a sample of synthetic ϵ-TNP-lysine (1mg.) and unmodified GDH (2·5mg.) were hydrolysed together, the ϵ-TNP-lysine was hydrolysed. Presumably the decomposition products of the protein catalyse this hydrolysis. The modified protein could be hydrolysed by total enzymic

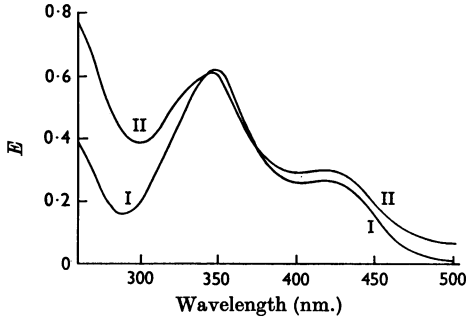

Fig. 1. Spectra of ϵ-TNP-lysine and of TNP-GDH. Curve I, ϵ-TNP-lysine (52μM); curve II, modified GDH (approx. 0·2mg./ml.). The protein was incubated for 1hr. with 2·5mM-TNBS at pH7·6. Both samples were in 0·1M-phosphate buffer, pH7·6.

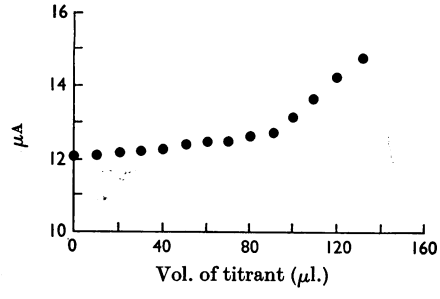

Fig. 2. Determination of thiol content of GDH by polarographic titration. The solution titrated was 1ml. of GDH (0·54mg./ml.), 0·5ml. of sodium dodecyl sulphate (1%), 0·3ml. of KCl (0·2M) and 0·2ml. of Na_2SO_3 (0·5M). Titrant (phenylmercuric acetate, 0·5mM) was added in 10μl. portions.

Table 1. *Titratable thiol content of native and modified GDH*

GDH (approx. 1mg./ml.) was incubated with 0·5mM-TNBS. Samples were removed and gel-filtered and the protein concentration was determined. Thiol content was determined by polarographic titration (see the text). All values are means of two determinations.

Time of modification (min.)	Titratable SH groups/subunit
0	4·5±0·4 (4 samples)
5	5·3
15	4·4
15*	4·4
20	4·8
25	5·3
25†	5·1
50	3·8
50*	4·3
60	4·2
75	4·8
75†	4·6
120	3·7
120*	3·9
180	4·0

* Incubation with TNBS in the presence of 185μM-NADH.

†Incubation with TNBS in the presence of 9·3mM-α-oxoglutarate.

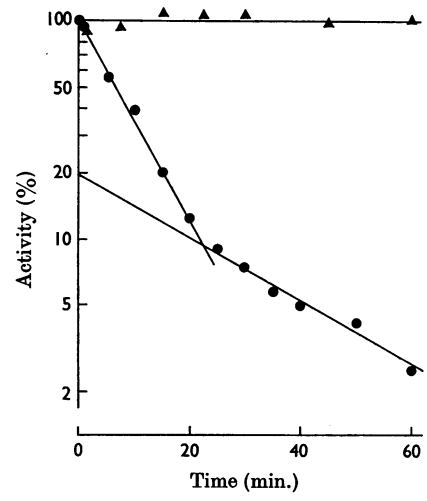

Fig. 3. Inactivation of GDH on incubation with TNBS. The enzyme (approx. 0·2mg./ml.) was maintained at 25° and samples were withdrawn at intervals and assayed for GDH activity. ▲, No addition; ●, 1·00mM-TNBS present in the incubation mixture.

digestion (Hill & Schmidt, 1962) without loss of the modified group. Amino acid mixtures were analysed by the standard elution procedures for the Locarte amino acid analyser, namely successive elutions with sodium citrate buffers, pH 3·25, 4·25 and 6·65, on a 23cm. × 1cm. column, or single elution with citrate buffer, pH 5·28, on a 7cm. × 1cm. column. Under these conditions both ε-TNP-lysine and *S*-TNP-cysteine had retention times very close to that of ammonia, and it was not possible to resolve these species when a standard amino acid mixture was taken together with the synthetic TNP derivatives. Further, no unusual amino acid constituents could be detected in the enzymic digests of modified GDH. Finally the digested modified enzyme mixture was submitted to paper chromatography and the yellow component was shown to have the same mobility as synthetic ε-TNP-lysine in three solvent systems, namely butan-1-ol saturated with water (R_F 0·18), butan-1-ol–acetic acid–water (12:3:5, by vol.) (R_F 0·70) and aq. ammonia (sp.gr. 0·88)–phenol (saturated with water) (1:200, v/v) (R_F 0·79).

Effect of modification on the activity of GDH. When GDH (0·1–1·2mg./ml.) was incubated with TNBS (1–3mM) in phosphate buffer, pH 7·6, the activity of the enzyme decreased rapidly (Fig. 3). The enzyme alone was quite stable under the incubation conditions. Very little enzyme was taken for the assay so that the concentration of TNBS in the assay mixture was less than 10μM. Such a concentration of TNBS included in an assay of unmodified GDH had no effect on the activity, and gel filtration on Sephadex G-25 of modified enzyme did not alter its activity. The inactivation is thus irreversible and due to the modification reaction. Fig. 3 shows that the inactivation is biphasic, since the reaction was carried out under pseudo-first-order conditions.

When the slow phase is extrapolated to zero time, a value is obtained for the activity of the enzyme after the rapid step of the inactivation only. This value derived from four such plots is 22±2% and inactivation to 22% is complete in 13min. under these conditions. With identical solutions the extent of modification of GDH after 13min., as determined by the change in extinction at 340nm., is equivalent to 1·2±0·2 amino groups/subunit. The biphasic curve can be analysed to yield pseudo-first-order rate constants for the slow and rapid phases of inactivation, and hence second-order rate constants. From six runs at a variety of TNBS concentrations (0·5–1·5mM) the slow inactivation was found to proceed at 28·2±5·2l.mole^{-1}min.$^{-1}$ and the rapid inactivation at 153±43l.mole^{-1}min.$^{-1}$ At a concentration of TNBS such that the reaction was throughout pseudo-first-order the time-course of inactivation of GDH did not depend on enzyme concentration over the range 0·1–1·2mg./ml.

With a lower concentration of TNBS the inactivation proceeded less rapidly and the 'rapid' phase of inactivation was extended over a period of about

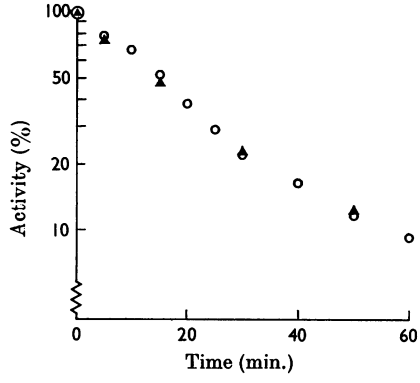

Fig. 4. Comparison of the loss of GDH and alanine dehydrogenase activities. In both cases TNBS was 0·5mM. ○, GDH activity, assayed on a sample modified as described in Fig. 3; ▲, alanine dehydrogenase activity of gel-filtered samples from an incubation mixture in which the enzyme was present at 1·9mg./ml.

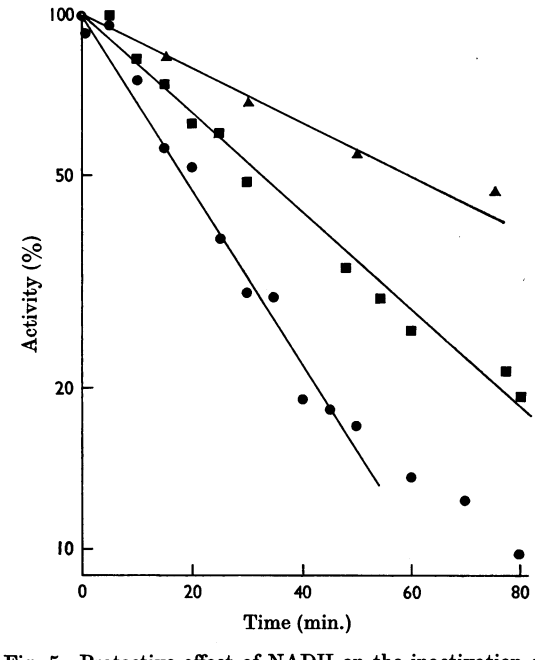

Fig. 5. Protective effect of NADH on the inactivation of GDH by TNBS. In each mixture TNBS was present at 0·5mM and GDH at approx. 0·3mg./ml. ●, No addition; ■, NADH (40μM) present in the incubation mixture; ▲, NADH (100μM) present in the incubation mixture.

50min. In most of the studies described below the concentration of TNBS was 0·5mM.

The alanine dehydrogenase activity of the enzyme was lost on incubation with TNBS (Fig. 4), and

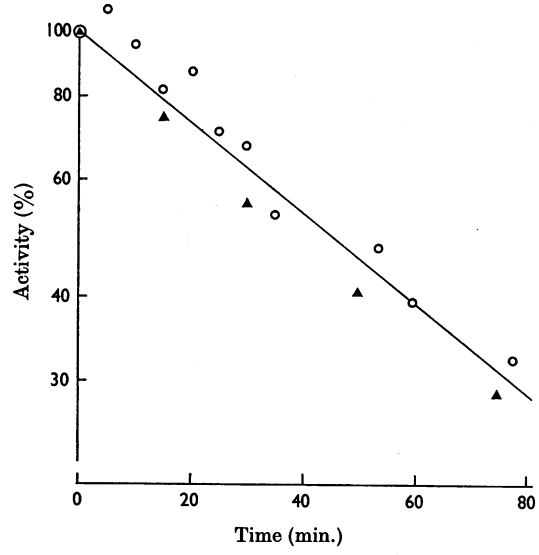

Fig. 6. Comparison of the protective effect of NADH on the inactivation of the GDH and alanine dehydrogenase activities by TNBS. In both cases TNBS was present at 0·5mM and NADH at 100μM in the incubation mixture. ○, GDH activity determined directly, GDH concentration 0·3mg./ml. in the reaction mixture; ▲, alanine dehydrogenase activity of gel-filtered samples from an incubation mixture in which GDH was present at 1·4mg./ml.

clearly both activities were lost at the same rate. The measured slope of this plot does not correspond to the rate of the 'rapid' inactivation since the apparent rate shown here compounds both the inactivation processes.

Protection by substrates. Incubation of the enzyme with TNBS in the presence of NADH (10–200μM) effectively lowered the rate of inactivation of the enzyme (Fig. 5). The alanine dehydrogenase activity was also lost less rapidly in the presence of NADH (Fig. 6). The protection against inactivation produced by NADH is the result of a small decrease in the extent of modification in the presence of the coenzyme, as shown by the results in Table 2.

α-Oxoglutarate (1–10mM) also retarded the inactivation of the enzyme by TNBS. Whereas the initial apparent rate of inactivation was halved by the presence of approx. 100μM-NADH, about 2mM-α-oxoglutarate was required to produce the same effect. In this case it was found from studies of the changes in extinction at 340nm. that the modification of the enzyme was almost entirely inhibited (Table 2). It seemed that all the amino groups of the enzyme were protected by α-oxoglutarate whereas NADH protected only one of the amino groups of the 'reactive' set.

Table 2. *Activity and extent of modification of GDH after incubation in various conditions*

GDH (approx. 1 mg./ml.) was incubated with the reagents given and at various times samples were removed and gel-filtered. The filtered samples were assayed for activity as described in the text, were assayed for protein, and the extinction at 340nm. was used to estimate the content of ε-TNP-lysine.

Incubation mixture	Incubation time (min.)	Amino groups reacted/ subunit	Activity (%)
0·5mM-TNBS	15	1·8	56
	50	3·2	26
	120	5·4	24
0·5mM-TNBS+ 185μM-NADH	15	1·1	100
	50	2·3	78
	120	3·7	67
0·5mM-TNBS+ 9·9mM-α-oxo-glutarate	10	0·6	98
	45	1·4	55
	75	1·8	43

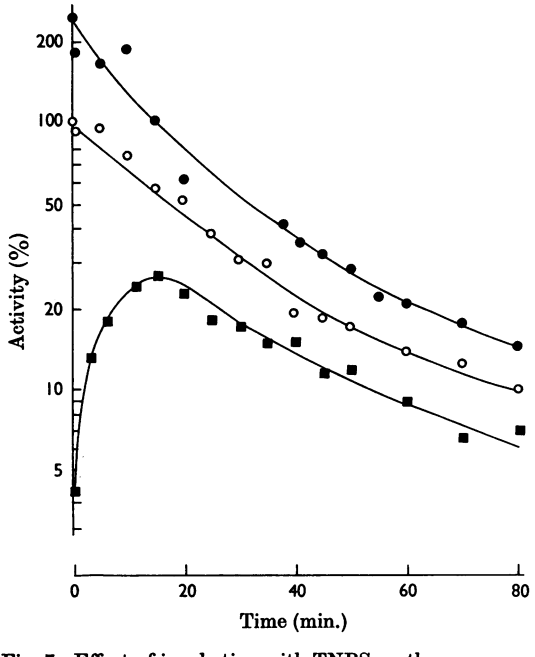

Fig. 7. Effect of incubation with TNBS on the response of GDH to activators and inhibitors. GDH (0·3mg./ml.) was incubated at 25° with TNBS at 0·5mM. ○, Standard assay, no effector present; ●, assay in the presence of 500μM-ADP; ■, assay in the presence of 10μM-GTP.

NAD^+ (0·1mM), GTP (0·1mM) and ADP (0·5mM) do not protect against inactivation when included in the incubation mixture.

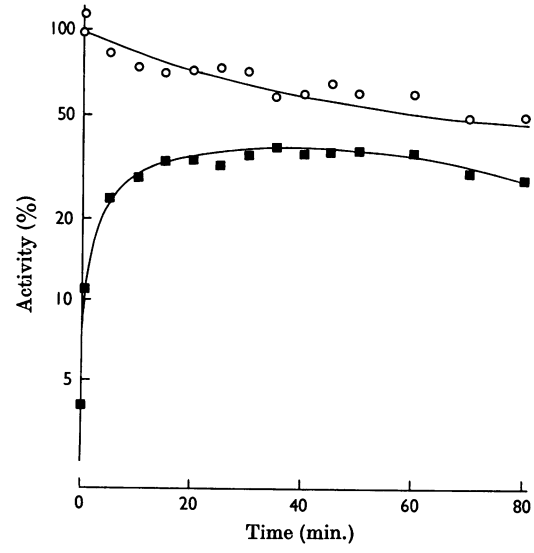

Fig. 8. Effect of NADH on the desensitization of GDH to GTP. Modification conditions were as described in Fig. 7 except that NADH (200μM) was also present. ○, Standard assay; ■, GTP assay as in Fig. 7.

Effect of the modification on the regulatory response of GDH. When samples of the GDH and TNBS reaction mixture were assayed in the presence of the inhibitor GTP (10μM) or the activator ADP (500μM) we obtained the results shown in Fig. 7. The activity found by assay in the presence of ADP was lost at about the same rate as that found by assay in the absence of effectors, i.e. the activating effect was hardly altered. In contrast, the activity found by assay in the presence of GTP initially rose and then fell off parallel to the simple activity. Thus modification with TNBS significantly decreases the sensitivity of the enzyme to GTP. Two points are noteworthy: (i) the loss of GTP response was very rapid, being well within the time corresponding to the rapid inactivation; (ii) the GTP response was not lost entirely. Thus, whereas initially the activity of the enzyme was decreased 20-fold by 10μM-GTP, from 20min. onwards the inhibition was only two-fold.

The loss of response to GTP could not be prevented by incubation in the presence of either GTP (0·1mM) or ADP (0·5mM). NADH (200μM) retarded the inactivation of the enzyme but had minimal effect on the loss of GTP response (Fig. 8). Thus by carrying out the incubation in the presence of 200μM-NADH it was possible to decrease the sensitivity of the enzyme to GTP with little effect on the actual activity. NADH, GTP and ADP can react with TNBS, but the reaction is very slow under these

Table 3. *Activity and sedimentation coefficients of GDH samples modified in the absence and presence of NADH*

GDH (approx. 1·5mg./ml.) was incubated with 0·5mM-TNBS with or without NADH (0·1mM). Samples were removed, the protein content was determined, the samples were assayed for activity and the sedimentation coefficients were determined as described in the Materials and Methods section. Final protein concentrations were approx. 0·6mg./ml. $S_{20,w}$ values in parentheses correspond to minor peaks in the schlieren trace.

Reaction Time (min.)	TNBS only		TNBS+NADH	
	Activity (%)	$S_{20,w}$ (s)	Activity (%)	$S_{20,w}$ (s)
0	100	21·5	100	21·6
5	50	12·6, (16·3)	97	13·3
15	53	12·4, 17·0	84	11·7, 21·2
30	25	(12·2), 23·9	69	(15·2), 24·2
50	26	24·5	56	26·4
75	10	27·5	47	26·9

conditions and was certainly negligible in the time-period under consideration.

The modification with TNBS altered the effects of regulators on the alanine dehydrogenase activity comparably with its alteration of their effects on the GDH activity. Thus the stimulation of the alanine dehydrogenase activity by GTP was rapidly lost on incubation with TNBS, and this loss of response was not prevented by either GTP or NADH alone.

The results describing the effects on activity and sensitivity in this and the previous sections were derived partly from direct assays on diluted incubation mixtures and partly from assays on samples that had been removed from such mixtures and separated by gel filtration on Sephadex G-25. There were no significant differences between results obtained in these two ways. The results obtained with the separated samples are less reliable since they include errors from the estimation of protein as well as the uncertainty in the assay itself.

Physical studies on the modified enzyme. Optical-rotatory-dispersion curves were determined on the native enzyme and on a sample that had been allowed to react with 0·5mM-TNBS for 1hr. The curves obtained were practically identical and the Moffitt parameters (Bayley & Radda, 1966) were: for the native enzyme, $a_0 = 48$, $b_0 = -189$; for the trinitrophenylated enzyme, $a_0 = 64$, $b_0 = -187$. The concentrations of the solutions studied were 0·5–0·6mg./ml.

In contrast, striking changes in the sedimentation behaviour were brought about by the modification. Two schlieren peaks were seen soon after the modification commenced and the relative sizes of the peaks altered with time of modification. We evaluated the 'apparent' sedimentation coefficient for the species present in enzyme samples modified for various times with TNBS in the absence and presence of NADH, and these are tabulated in Table 3 together with the activities of these samples. The $S_{20,w}$ values corresponding to minor peaks and spikes are given in parentheses in Table 3. Table 3 shows clearly that the changes in sedimentation behaviour produced by modification with TNBS were also found in samples modified in the presence of NADH, although the activities of these samples differed markedly.

DISCUSSION

Before the role of amino acid side chains in the functions of GDH can be assessed from modification experiments, it is essential to characterize the chemical and physical changes brought about by the reaction with TNBS. The chemical data, together with our earlier model studies (Freedman & Radda, 1968*a*), suggest that only amino groups are modified by this procedure, and that most of these groups only react fairly slowly. The rate of the rapid inactivation of the enzyme (approx. 150l.mole^{-1} min.$^{-1}$) is of the same order of magnitude as the rapid phase of modification. The rapid stage of inactivation is complete within a time that corresponds to a change in extinction at 340nm. equivalent to the reaction of approx. 1·2amino groups/subunit.

The finding that inactivation is prevented by the substrates of reaction, coupled with the observations on the stoicheiometry of inactivation, allows us to conclude that the group modified is essential for the activity and is probably at the active site. It is noteworthy that the time-course of inactivation is unaltered over a range of enzyme concentration in which the further aggregation of the enzyme oligomer becomes significant. The specific activity of the enzyme is known to be independent of concentration (Fisher, Cross & McGregor, 1962), so that this finding is consistent with the view that the environment of the active site is hardly altered by the aggregation. The loss of alanine dehydrogenase activity occurs at the same rate as the loss of GDH activity and is equally protected by NADH, so that the rapidly modified group is essential for the activity of the enzyme towards both substrates.

This interpretation requires that there are no major physical changes in the enzyme on modification of the rapidly reacting group. This is supported by the optical-rotatory-dispersion data, which show no difference between the native and modified enzyme in the Moffitt b_0 parameter. The changes in the sedimentation behaviour of the enzyme on

modification appear to result from reaction at sites that cannot be protected by NADH, as identical changes still occur when little of the activity is lost (Table 3).

We interpret the changes in sedimentation as being mainly due to the slowing down of the aggregation equilibrium that occurs with this enzyme (Eisenberg & Tomkins, 1968; Sund & Burchard, 1968). In the concentration range 0·1–2mg./ml. the sedimentation coefficient increases with concentration from about 12 to 27s (Sund & Burchard, 1968; Olson & Anfinsen, 1952) and only a single schlieren peak is observed. The existence of intermediate apparent $S_{20,w}$ values shows that the equilibration between oligomer and aggregate is a rapid process. The system is thus of Class IV (Nichol, Bethune, Kegeles & Hess, 1964); the theory of such systems has been discussed by Gilbert (1959). Thus the changes in sedimentation behaviour observed on modification of GDH by TNBS do not require that new or substantially altered protein species appear, merely that the rates of interconversion are altered. At high extents of modification the position of equilibrium may also be altered to favour the aggregate because of the decrease of positively charged side chains. The effects of modification on activity were studied with enzyme in the concentration range 0·1–1·5mg./ml., whereas the sedimentation studies could only be performed on samples in which the protein concentration was greater than 0·6mg./ml., so that the effects on the aggregation equilibrium may be less significant at the lower concentrations.

The slow stage of the inactivation has not been studied in much detail because of the very large number of differently modified species that would be present. Modification studies of this kind can present useful information only at small extents of modification.

The modification reaction has no effect on the sensitivity of the enzyme to ADP, but greatly decreases its sensitivity to GTP in both the GDH and alanine dehydrogenase reactions. This supports the view that the modes of action of the two effectors are different (Dodd & Radda, 1967, 1969). GTP, in the presence of NADH, is thought to induce a conformational change in the enzyme, so that the modification could have affected either its ability to undergo the conformational change or its ability to bind GTP. Since the sensitivity was lost within the time that corresponds to modification of one group/subunit, the loss of response is likely to be mainly due to the same modification process that causes inactivation.

A problem in this interpretation is that NADH should protect against both inactivation and desensitization to the same extent, and this is not observed (Fig. 8). This finding may be rationalized as follows. The more rapid loss of GTP response compared with loss of activity suggests that, although the subunits of a given oligomer can function separately in catalysis, the GTP response requires that all the subunits should be unmodified. This prediction has been tested with a more specific reagent (Malcolm & Radda, 1968). It follows from this that protection by NADH against loss of GTP response will only be about one-sixth as effective as against loss of activity. NADH at concentrations up to 200μM decreases the initial rate of loss of activity by up to 60%, so that by this theory it should decrease the rate of loss of GTP response by up to about 10%. Since 'GTP response' is a complex quantity involving both the activity in the simple assay and the activity when assayed in the presence of GTP, it is liable to considerable uncertainty. The rates of loss of GTP response at a number of NADH concentrations show no real trend and show an error of about 20%. Hence a 10% change in actual rate of loss of GTP response would be undetected.

These modification studies suggest that a particular amino group, probably at the active site of GDH, is involved in both the activity and regulation of this enzyme. Nevertheless, even with a reagent specific for a given type of residue, problems arise when the protein is large enough to contain a number of such residues and it is pertinent to mention these. Because of the large number of residues attacked it was not possible to obtain accurate rate constants for the modification of the most reactive residues. In addition, the inactivation is biphasic, showing that the small activity of the enzyme modified at the most reactive residue is further decreased by reaction at other sites. This non-specific reaction has provided further problems in its effect on the sedimentation behaviour. These complications have made it impossible to associate the properties of 'partially modified enzyme' with those of a defined chemically modified species. Such problems occur in all studies using chemical modification unless the modifying reagent is site-specific (Baker, 1967) or the modified protein species are subsequently resolved and studied separately (Hirs, 1962; Ettinger & Hirs, 1968).

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