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Desensitization of Glutamate Dehydrogenase by Reaction of Tyrosine Residues

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1. The reaction of glutamate dehydrogenase with *N*-acetylimidazole and with tetranitromethane leads to modification of tyrosine residues. 2. Modification of 1 tyrosine residue/subunit does not affect the enzymic activity but decreases the response of the enzyme to the allosteric inhibitor, GTP. 3. The physical properties of the enzyme (sedimentation coefficient and optical rotatory dispersion) remain unaltered. 4. GTP partially protects against desensitization. 5. The diminished responses of the modified enzymes to GTP are also detected by using the fluorescence of 1-anilinonaphthalene-8-sulphonate as a conformational probe. 6. Difficulties that generally arise in chemical modifications from inhomogeneous distributions of products are discussed.

One of the common features of allosteric models is the requirement for an effector-binding site that is distinct from the active site (Monod, Wyman & Changeux, 1965; Stadtman, 1966; Koshland, Némethy & Filmer, 1966). It has been suggested on kinetic grounds (Frieden, 1963) that the inhibition of ox liver GDH* by GTP involves a separate nucleotide-binding site. In the ADH activity of GDH, GTP in contrast is an activator (Tomkins, Yielding, Curran, Summers & Bitensky, 1965).

One of the best ways to demonstrate a separate inhibitor-binding site is by selective chemical modification of amino acid side chains such that the regulatory properties of the enzyme are lost without any change in the catalytic properties. This has been achieved for a number of enzymes (Monod, Changeux & Jacob, 1963; Pontremoli, Grazi & Accorsi, 1966; Chapman, Sanner & Pihl, 1969) and is generally known as desensitization.

The reaction of GDH with methylmercuric chloride decreased the sensitivity of the enzyme to inhibition by several inhibitors (Bitensky, Yielding & Tomkins, 1965). Modification with 1-fluoro-2,4-dinitrobenzene (di Prisco, 1967) showed similar effects.

The experiments described here demonstrate a desensitization of GDH to the effector GTP. It seemed reasonable to examine the role of aromatic amino acid side chains in GDH, as the interaction of these with the aromatic ring of GTP may be partly responsible for the binding. A number of chemical modifications of tyrosine residues in proteins have been described: iodination (Ostwald, 1910), diazo coupling (Horinishi, Hachimori, Kurihara & Shibata, 1964; Sokolovsky & Vallee, 1966), reaction with 2,4,6-trifluoro-1,3,5-triazine (Kurihara, Horinishi & Shibata, 1963), acetylation by NAIm (Riordan, Wacker & Vallee, 1965) and nitration by TNM (Sokolovsky, Riordan & Vallee, 1966). Of these reactions the last two seemed particularly suitable for our experiments because of the relatively good specificity of the reactions, the mild conditions required for them, the ease of measurement of the extent of reaction spectrophotometrically and the possibility of further reactions on the modified enzymes, e.g. reversal of acetylation (Riordan *et al.* 1965) or reduction of the nitrotyrosine group (Sokolovsky, Riordan & Vallee, 1967). A preliminary account of some of this work has been given (Radda, 1969).

* Abbreviations: GDH, L-glutamate–NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3); NAIm, *N*-acetylimidazole; TNM, tetranitromethane; ANS, 1-anilinonaphthalene-8-sulphonate; GDH activity, activity of GDH in the α-oxoglutarate–L-glutamate conversion; ADH activity, activity of GDH in the pyruvate–L-alanine conversion.

MATERIALS AND METHODS

GDH [as an $(NH_4)_2SO_4$ suspension or a glycerol suspension], α-oxoglutaric acid, NADH (disodium salt) and sodium pyruvate were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). GTP (type IIS, sodium salt) was a product of Sigma Chemical Co. (St Louis, Mo., U.S.A.). NAIm was obtained from Pierce Chemical Co. (Rockford, Ill., U.S.A.). The reagent was dissolved in benzene, dried (over Na_2SO_4), recrystallized and stored over silica gel in a desiccator. TNM was obtained from R. N. Emanuel Ltd. (Wembley, Middlesex).

Tris buffers were made up by using the Laboratory Reagent tris (British Drug Houses Ltd., Poole, Dorset) and adjusting the pH with HCl. Phosphate buffer was made up by using the AnalaR sodium salts (British Drug Houses Ltd.). The pH values of solutions were determined with an E.I.L. direct-reading pH-meter.

NADH solutions were made up in 50mM-tris–HCl buffer, pH9·0. Concentrations of NADH and GTP were checked spectrophotometrically.

GDH solutions were prepared by dialysis of the glycerol or $(NH_4)_2SO_4$ suspension supplied against the appropriate buffer for 18hr. at 4°, removal of any denatured protein by centrifugation and dilution to the required concentration with buffer. Concentrations were determined by using the published value of extinction at 280nm. (Olson & Anfinsen, 1952).

Acetylation of tyrosine residues in GDH was carried out by the method Riordan *et al.* (1965) used for modification of other proteins and peptides. A 60-fold excess of NAIm with respect to the subunit of GDH was allowed to react at room temperature (approx. 20°) for 1hr. in 10mM-tris–HCl buffer, pH7·5, containing 0·1M-NaCl. After removal of the excess of NAIm by gel filtration (Sephadex G-25), the extent of acetylation of tyrosine residues was determined by the change in extinction at 278nm. (Riordan *et al.* 1965). GDH concentrations used for modification ranged from 0·8 to 0·3mg./ml. The concentration of modified GDH after gel filtration was measured by two methods: by total elution of the enzyme and by use of the 'amide chromophore' technique, in which the difference between the extinctions at 215 and 225nm. is proportional to protein concentration (Waddell, 1956).

Nitration of GDH was initially performed by the method that Riordan, Sokolovsky & Vallee (1967) used for carboxypeptidase modification [i.e. TNM dissolved in aq. 95% (v/v) ethanol was added to a solution of the enzyme in 50mM-tris–HCl buffer, pH8·0, containing 0·1M-NaCl, at room temperature], but this led to rapid loss of about 20% of the GDH activity. Later experiments were performed with GDH in 0·1M-sodium phosphate buffer, in which the activity was unaffected. At this lower pH (pH7·6) the rate of nitration was less than at pH8·0, since the phenolate anion is the reactive species (Sokolovsky *et al.* 1966). Protein concentrations used for modification were between 0·6 and 1·0mg./ml. The extent of nitration was followed by the change in extinction at 280, 380 and 428nm. after removal of excess of reagent and nitroformate ion by gel filtration (Sephadex G-25).

Reduction of nitrated tyrosine residues in GDH was carried out by addition of 0·05ml. of a solution of $Na_2S_2O_4$ (20mg./ml.) in 0·1M-sodium phosphate buffer, pH7·6, to 2ml. of the nitrated enzyme.

The GDH activity of the enzyme was measured as described by Dodd & Radda (1969). The ADH activity of the enzyme was measured as described by Anderson, Anderson & Churchich (1966) except that the solutions were made up in 10mM-tris–HCl buffer, pH9·0, containing 0·1M-NaCl. The reactions were initiated by the addition of 0·6μg. of enzyme (GDH activity) or 20μg. of enzyme (ADH activity). NADH oxidation was followed spectrophotometrically as described by Dodd & Radda (1969).

Sedimentation coefficients were determined as described by Dodd & Radda (1969).

Optical rotatory dispersion was measured with a Bendix–Ericsson Polarmatic 62 recording spectropolarimeter with slit widths of 0·5mm. (entrance) and 0·4mm. (exit) at 25°. The results were analysed in terms of the Moffitt–Yang equation as described by Bayley & Radda (1966).

Amino acid analyses were performed essentially by the procedure of Spackman, Stein & Moore (1958) with a Locarte automatic analyser.

Fluorescence measurements were made on a spectrofluorimeter constructed in this laboratory (Dodd & Radda, 1969).

RESULTS

Stability of GDH in the various buffers

GDH in 10mM-tris–hydrochloric acid buffer, pH7·5, lost 70% of its GDH activity on standing for 1hr. at 0°. On inclusion of 0·1M-sodium chloride in the buffer, full enzymic activity was retained over a period of at least 30hr. at 0°, so that all the acetylation experiments were performed in this tris–hydrochloric acid–sodium chloride buffer (referred to as the standard tris buffer, pH7·5). The specific GDH activity of the enzyme in the standard tris buffer, pH7·5, is close to that in 0·1M-sodium phosphate buffer, pH7·6. There was no change in GDH activity of the enzyme during incubation in 50mM-tris–hydrochloric acid buffer, pH8·0, containing 0·1M-sodium chloride, for 18hr. at 0°.

Acetylation

The extent of *O*-acetylation of GDH varied with the concentration of enzyme used for reaction (Fig. 1). The maximum error in the determination of extent of acetylation is ±2 tyrosine residues/subunit, and arises from the small change in extinction at 278nm. on acetylation of GDH (2·2%/tyrosine residue modified/subunit).

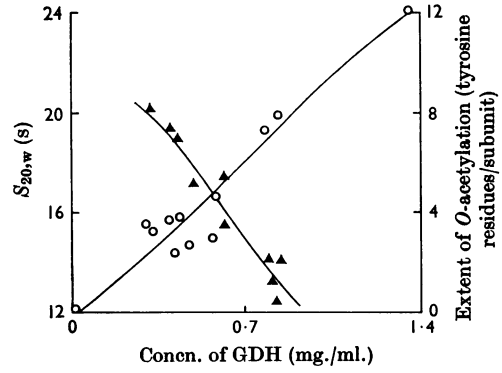

Fig. 1. Concentration-dependence of extent of acetylation and $S_{20,w}$ of GDH. ▲, Extent of acetylation by 60-fold molar excess of NAIm; ○, $S_{20,w}$ of native GDH. Experiments were performed in the standard tris buffer, pH7·5.

Fig. 1 also shows the increase in $S_{20,w}$ with increasing enzyme concentration, reflecting the concentration-dependent aggregation of the oligomer of GDH. It would appear that fewer tyrosine residues are available for reaction with NAIm as aggregation of the oligomer becomes significant.

Enzyme activity. Up to 6 tyrosine residues/subunit could be acetylated with no significant change in GDH activity (Table 1). The K_m for substrates of native and acetylated enzyme (approx. 1 tyrosine residue/subunit acetylated) are shown in Table 2. The Lineweaver–Burk plot for NH_4^+ indicated that a very high concentration of ammonium chloride would be required to saturate the enzyme in the ADH assay (the concentration of enzyme is about 50 times that in the GDH assay). No experimentally significant differences in the K_m values for substrates could be observed between native and modified enzyme.

GTP response of native and acetylated enzyme. Acetylation of GDH by NAIm caused a marked decline in the response of the enzyme to GTP in both GDH and ADH assays (curves 1 and 2 of Figs. 2*a* and 2*b*). The concentrations of GTP required to cause 50% inhibition of GDH activity were: for native enzyme, 0·4 μM; for acetylated enzyme, 1·8 μM. Neither the GDH activity nor the response of the enzyme to GTP in the GDH assay was affected by further modification of up to 6 tyrosine residues/subunit.

Protection against loss of GTP response on acetylation. Figs. 2(*a*) and 2(*b*) show the results of experiments in which the coenzyme (NADH) and GTP were included in the acetylation mixtures. After reaction with NAIm for 1 hr. samples from each of the acetylation mixtures were withdrawn and quenched by dilution for assay. At these decreased concentrations the NADH and GTP have no effect on the assay procedures, as shown by control experiments where the modifiers were removed by gel filtration before assay. Inclusion of 50 μM-NADH afforded little (not more than 6%) protection against the loss of GTP response. GTP at

Table 1. *Enzymic activity as a function of the extent of O-acetylation of GDH*

GDH and ADH activities are referred to native enzyme activities as 100%.

Extent of *O*-acetylation (tyrosine residues/subunit)	GDH activity (%)	ADH activity (%)
0·3	100·2	98·0
0·5	102·7	
1·0	100·0	
1·3	104·6	
2·3	110·0	
3·3	102·7	
4·2	108·0	
5·1	105·0	
5·6	98·5	
7·1	91·8	
7·3	83·1	64·4
8·2	85·6	

Table 2. *K_m values for substrates of native and acetylated GDH*

K_m values in the GDH activity assay were determined over a tenfold range of substrate concentration. For α-oxoglutarate the NH_4Cl and NADH concentrations were 50 mM and 100 μM respectively; for NH_4^+ the α-oxoglutarate and NADH concentrations were 5 mM and 100 μM respectively; for NADH the NH_4Cl and α-oxoglutarate concentrations were 50 mM and 5 mM respectively. K_m values in the ADH activity assay were determined over a sixfold range of substrate concentration. For pyruvate the NH_4Cl and NADH concentrations were 150 mM and 100 μM respectively; for NH_4^+ the pyruvate and NADH concentrations were 40 mM and 100 μM respectively; for NADH the NH_4Cl and pyruvate concentrations were 150 mM and 40 mM respectively. The K_m values shown are the means of two determinations, the variation being less than 10%. The extent of acetylation in the modified enzyme was approx. 1 tyrosine residue/subunit for both GDH and ADH activity assays.

	GDH activity		
	K_m (α-oxoglutarate) (mM)	K_m (NH_4^+) (mM)	K_m (NADH) (μM)
Native GDH	1·05	32	83
Modified GDH	1·05	32	83

	ADH activity		
	K_m (pyruvate) (mM)	K_m (NH_4^+) (M)	K_m (NADH) (μM)
Native GDH	67	⩾1	30
Modified GDH	67	⩾1	30

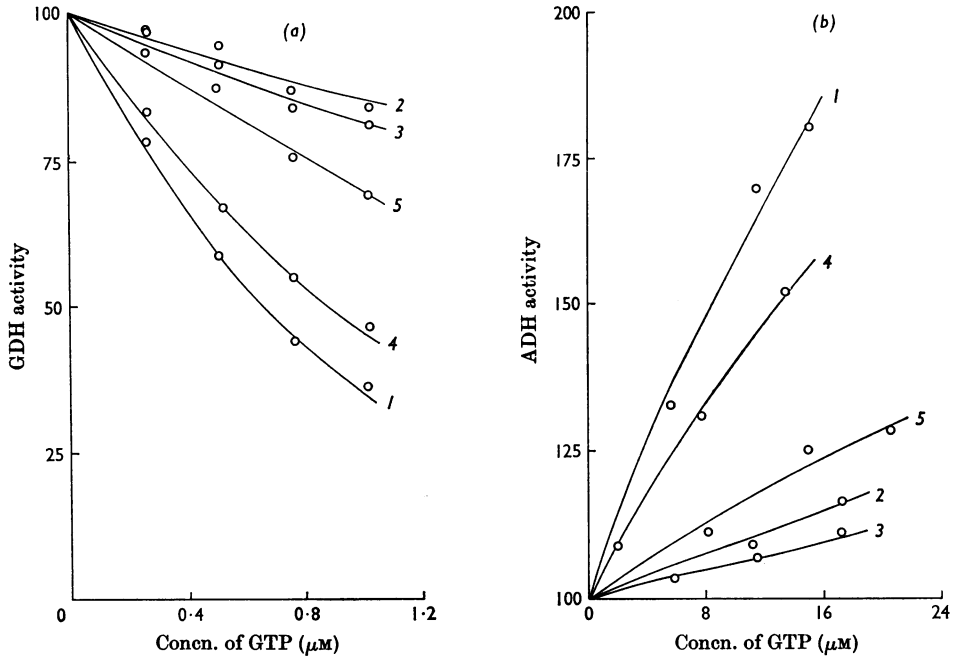

Fig. 2. Response of native and acetylated GDH to GTP. (*a*) GDH activity; (*b*) ADH activity. Curves *1*, native enzyme; curves *2*, acetylated enzyme (approx. 1 tyrosine residue/subunit acetylated); curves *3*, enzyme acetylated in the presence of 50μM-NADH; curves *4*, enzyme acetylated in the presence of 50μM-NADH and 50μM-GTP; curves *5*, enzyme acetylated in the presence of 50μM-GTP.

50μM gave 24% protection; this protection could be increased to about 50% by raising the GTP concentration in the acetylation mixture to 200μM. Inclusion of 50μM-NADH and 50μM-GTP in the acetylation mixture led to good (not less than 75%) protection. The activities of the variously modified enzymes in Figs. 2(*a*) and 2(*b*) in the absence of added GTP were, within experimental error, identical with those of the native enzyme.

The effects of inclusion of NADH or GTP or both in the acetylation mixtures were broadly similar in the assays of GDH and ADH activities. Quantitative evaluation of these effects was difficult because of the inaccuracies in the measurement of the rate of the small ADH activities.

Physical properties of native and acetylated GDH

Sedimentation coefficients. $S_{20,w}$ values were determined at various extents of modification (Table 3). The results, compared with the $S_{20,w}$ values for native enzyme in Fig. 1, show that there was no significant change in the apparent molecular weight on modification.

Optical rotatory dispersion. Optical-rotatory-dispersion curves were obtained over the range 400–260nm. The Moffitt–Yang parameters for native and acetylated enzyme (approx. 1 tyrosine residue/subunit acetylated) were: $a_0 = 65$, $b_0 = -203$. The difference between these parameters and those obtained by Bayley & Radda (1966) may well be due to the different buffers and pH values employed in the two sets of measurements.

Fluorescence titrations. ANS can be used as a fluorescent probe for detecting the conformational change in GDH brought about by GTP and NADH (Dodd & Radda, 1967). Addition of 100μM-GTP

Table 3. $S_{20,w}$ *values for acetylated GDH at various extents of modification*

$S_{20,w}$ values were determined in 10mM-tris–HCl buffer, pH7·5, containing 0·1M-NaCl.

Concn. of acetylated enzyme (mg./ml.)	Extent of *O*-acetylation (tyrosine residues/subunit)	$S_{20,w}$ (S)
0·29	5·1	16·1
0·32	2·3	15·8
0·43	1·3	17·3
0·53	2·3	17·1

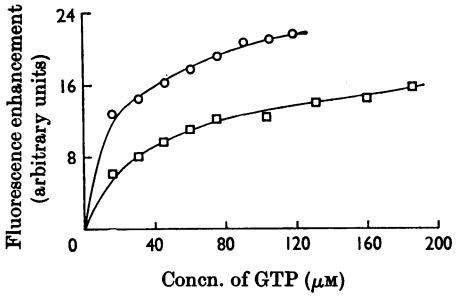

Fig. 3. Enhancement of fluorescence of ANS by GTP. ○, Native GDH; □, monoacetylated GDH. Enzyme was present at 0·46mg./ml., ANS at 25μM and NADH at 133μM. Titrations were performed in the standard tris buffer, pH7·5. The excitation and emission wavelengths were 410 and 550nm. respectively.

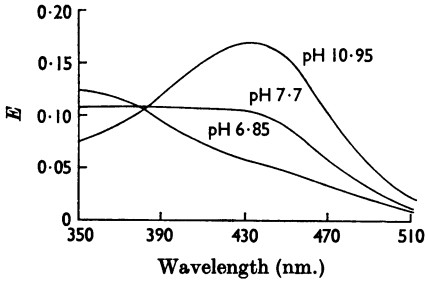

Fig. 4. Spectrum of nitrated GDH at different pH values. The solution contained 0·19mg. of modified GDH (approx. 13 tyrosine residues/subunit nitrated)/ml. of 0·1M-sodium phosphate buffer.

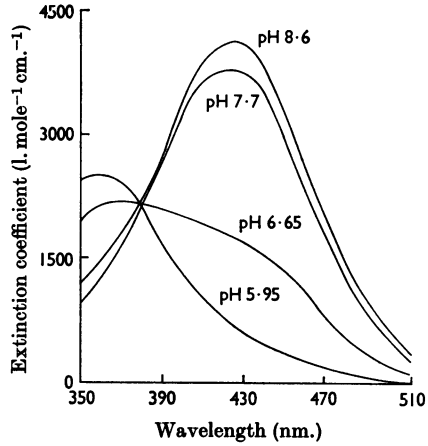

Fig. 5. Spectrum of 3-nitrotyrosine at different pH values. The 3-nitrotyrosine was dissolved in 0·1M-sodium phosphate buffer.

to native GDH (0·46mg./ml.) and ANS (25μM) in the presence of 130μM-NADH led to an approximately twofold enhancement of the ANS fluorescence (shown in Fig. 3 as an increase of 20 units). When a similar titration was performed with modified enzyme (approx. 1 tyrosine residue/chain acetylated) the fluorescence enhancement occurred only at higher concentrations of added GTP (Fig. 3). The $s_{0.5}$ values for GTP (Dodd & Radda, 1969) were 15μM (native enzyme) and 45μM (acetylated enzyme).

Nitration and subsequent reduction of GDH

For the reaction of TNM with model substrates (e.g. *N*-acetyltyrosine) the extent of reaction can be measured by: (*a*) the increase in extinction at 350nm., as nitroformate anion is produced; (*b*) the increase in extinction at 428nm., representing the production of 3-nitrotyrosine; (*c*) the rate of proton release. When TNM reacts with proteins, methods (*a*) and (*c*) are unsatisfactory because of general base-catalysed breakdown of TNM (Sokolovsky *et al.* 1966). After removal of TNM and nitroformate anions by gel filtration, the extent of nitration has been followed by the extinction at 428nm. (Sokolovsky *et al.* 1966).

This method proved unsatisfactory for GDH. The absorption spectrum of nitrated GDH at pH7·7 (approx. 13 tyrosine residues/subunit nitrated) is shown in Fig. 4. There is absorption over a wide part of the visible region, but no well-defined peak at 430nm., in contrast with the spectrum of 3-nitrotyrosine at this pH (Fig. 5). The spectrum of nitrated GDH is more like that of 3-nitrotyrosine at pH6·65. By plotting the ratio of the extinctions at 428 and 380nm. (the latter wavelength representing the isosbestic point for the nitrotyrosine–nitrotyrosine anion system) against pH, titration curves were obtained. The pK_a values of the phenolic group of 3-nitrotyrosine in the three cases were: free 3-nitrotyrosine, pK_a 6·9; nitrated GDH (approx. 1 tyrosine residue/subunit nitrated), pK_a 8·0; nitrated GDH (approx. 13 tyrosine residues/subunit nitrated), pK_a 7·7.

Since the nitration reaction is carried out at a pH very close to the pK_a of the nitrotyrosine in nitrated GDH, the extinction at 428nm. is a poor measure of the extent of GDH modification if the extinction coefficient of 3-nitrotyrosine at pH7·6 is used as a standard. The extinction at 380nm. is a more reliable measure of protein modification.

The extent of reaction of GDH in these studies was therefore estimated from the extinctions at 280 and 380nm. after removal of nitroformate anion and excess of TNM by gel filtration by using the known extinction coefficients of tyrosine and

3-nitrotyrosine (Sokolovsky *et al.* 1966) to solve the simultaneous equations. This method is not completely satisfactory since the extinction coefficient of 3-nitrotyrosine at 280nm. is itself slightly pH-dependent (at pH 9·8, 7·65, 6·63 the extinction coefficients are 4750, 4400 and 5190 l.mole^{-1}cm.$^{-1}$ respectively), but except at very large extents of modification the error in determination of extent of reaction by the method described should not exceed 10%.

The accuracy of the spectrophotometric method of measuring the extent of reaction was checked by amino acid analyses of native and nitrated GDH, together with a standard mixture of synthetic tyrosine and 3-nitrotyrosine. Spectrophotometric measurements indicated that the nitrated GDH should have an average of 13 tyrosine residues/subunit nitrated and hence 3 tyrosine residues/subunit unmodified. The amino acid analysis showed that the proportions of all amino acid residues except tyrosine did not change on modification. The modified enzyme showed a new amino acid that was eluted 11min. after phenylalanine at exactly the position corresponding to 3-nitrotyrosine. The area under the 3-nitrotyrosine peak was difficult to measure accurately because of peak asymmetry; approx. 12 nitrotyrosine residues/subunit were found; 3 tyrosine residues/subunit had not been modified.

Enzymic activity of nitrated and aminated GDH. The GDH activities and K_m values for substrates of native and modified GDH are shown in Table 4. Although the GDH activity did not change significantly on modification, there was a considerable fall in ADH activity (Table 4). The effect of small changes in the K_m values for substrates should be much more marked in the assay for ADH activity than for GDH activity, since in the former the substrate concentrations are well below saturating conditions, although this has been used as the standard assay for ADH activity (Anderson *et al.* 1966).

GTP response of modified enzymes. The modified enzymes had a greatly diminished response to GTP (in both GDH and ADH assays). Fig. 6 shows the results for GDH activity. The ethanol used to dissolve the TNM was not responsible for this desensitization, since neither the GDH activity nor the GTP response of the enzyme was affected by ethanol in the concentrations used for modification reactions over a period of 2hr.

Kinetics of loss of GTP response on nitration. Several experiments were performed in which the nitration of GDH was followed spectrophotometrically. At known time-intervals samples of the reaction mixture were withdrawn, quenched by dilution and assayed for activity and GTP response. At these lowered concentrations TNM had no effect on the assay procedures for GDH activity. Nitroformate anion did not interfere with the assays as shown by its removal (gel filtration) in a control experiment. Fig. 7 shows the GTP response of the GDH activity as a function of time of nitration.

A sample of the reaction mixture at time T (Fig. 7) was removed and analysed for extent of reaction by the spectrophotometric procedure described. An average of $1 \pm 0{\cdot}2$ tyrosine residues/subunit had been nitrated. Because the pH at which nitration of GDH can be carried out is close to the pK_a of the nitrotyrosine residues in the modified enzyme, the continuous spectrophotometric method of following the rate of nitration is

Table 4. *GDH and ADH activities and K_m values for native, nitrated and aminated GDH*

The K_m values were determined as described in Table 2, except that GDH assays were performed in 0·1 M-sodium phosphate buffer, pH 7·6. In the ADH assays the concentration range covered was only four- to five-fold because of the difficulty of measuring very small rates of NADH oxidation for the modified enzymes. The nitrated and aminated GDH were modified to an extent of $1{\cdot}0 \pm 0{\cdot}2$ tyrosine residues/subunit.

	GDH activity			
	Activity (% of native enzyme)	K_m (α-oxoglutarate) (mM)	K_m (NH_4^+) (mM)	K_m (NADH) (μM)
Native GDH	100·0	0·62	29	44
Nitrated GDH	110·0	0·75	36	38·5
Aminated GDH	96·0	0·81	37·5	42·5

	ADH activity		
	Activity (% of native enzyme)	K_m (pyruvate) (mM)	K_m (NH_4^+) (M)
Native GDH	100·0	53	≥ 1
Nitrated GDH	38·0	57	≥ 1
Aminated GDH	27·0	57	≥ 1

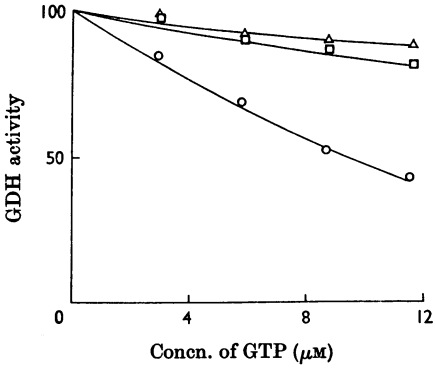

Fig. 6. GTP response of native GDH, nitrated GDH and aminated GDH in the GDH assay. ○, Native enzyme; □, nitrated enzyme; △, aminated enzyme. The extent of modification was $1 \pm 0{\cdot}2$ tyrosine residues/subunit. Assays were performed in 0·1 M-sodium phosphate buffer, pH 7·6.

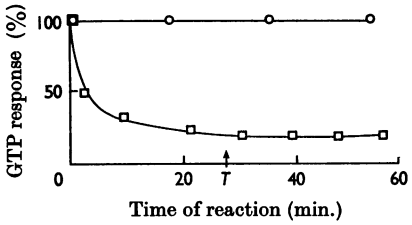

Fig. 7. GTP response as a function of time of nitration of GDH. □, Reaction mixture; ○, control. Samples of GDH (1 mg./ml.) that had been allowed to react with TNM (300 μM) were assayed by the standard method. The GTP response is defined by:

$$\text{GTP response at time } t = \frac{[1-(\text{GTP})_t/(\text{Act})_t]}{[1-(\text{GTP})_0/(\text{Act})_0]} \times 100\%$$

where $(\text{GTP})_t$ and $(\text{Act})_t$ represent the GDH activities in the presence and absence of GTP respectively. The GTP concentration used for the assay was 11·3 μM.

not very reliable, i.e. the method of Freedman & Radda (1968) for analysing the course of reaction is not applicable. Hence no direct evidence for a uniquely reactive tyrosine residue in the enzyme could be obtained.

Protection against loss of GTP response on nitration. Kinetically we have observed that GDH is less susceptible to GTP inhibition in phosphate buffer than in tris–hydrochloric acid buffer (compare Figs. 2*a* and 6). Because of this, protection experiments against loss of GTP response by inclusion of GTP in the nitration mixture were performed in 50 mM-tris–hydrochloric acid buffer, pH 8·0 (containing 0·1 M-sodium chloride). GTP (100 μM) afforded 20–50% protection against loss of GTP response in

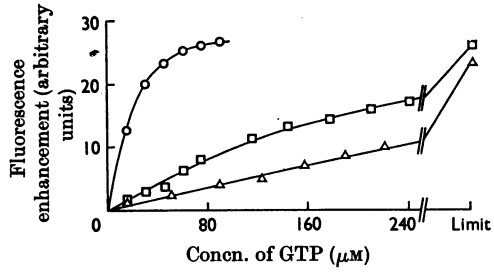

Fig. 8. Enhancement of fluorescence of ANS by GTP. ○, Native GDH; □, nitrated GDH; △, aminated GDH. Enzyme was present at 0·5 mg./ml. Titrations were performed as described in Fig. 3 except that 0·1 M-sodium phosphate buffer, pH 7·6, was used. The extent of modification was $1 \pm 0{\cdot}2$ tyrosine residues/subunit. Limiting fluorescence enhancements for the modified enzymes were obtained by the addition of crystalline GTP.

different experiments. NADH was found to react with TNM under the conditions of nitration, and could not therefore be included in nitration reaction mixtures.

Physical properties of native and modified enzymes

Optical rotatory dispersion. Curves obtained over the same wavelength range as for acetylated enzyme were analysed in terms of the Moffitt–Yang equation to give the parameters: native GDH, $a_0 = 100$, $b_0 = -208$; nitrated GDH, $a_0 = 100$, $b_0 = -208$; aminated GDH, $a_0 = 25$, $b_0 = -192$. The enzymes were modified to the extent of $1 \pm 0{\cdot}2$ tyrosine residues/subunit; experiments were performed in 0·1 M-sodium phosphate buffer, pH 7·6.

Sedimentation coefficients. The $S_{20,w}$ values at enzyme concentrations of 0·9 mg./ml. were: native GDH, 21·5 S; nitrated GDH, 21·0 S; aminated GDH, 20·6 S. The extents of modification and conditions of experiment were as described for optical rotatory dispersion above.

Fluorescence titrations. Fig. 8 shows the results of fluorescence titrations performed as described for acetylated enzyme, but in 0·1 M-sodium phosphate buffer, pH 7·6. The $s_{0.5}$ values for GTP were: native GDH, 15 μM; nitrated GDH, 150 μM; aminated enzyme, approx. 300 μM. The concentration of enzymes used for the titrations was 0·5 mg./ml. and the degree of modification was $1 \pm 0{\cdot}2$ tyrosine residues/subunit.

DISCUSSION

The modifications of GDH (acetylation and nitration) lead to desensitization to the allosteric inhibitor, GTP, without any marked changes in enzymic activity.

O-acetylation by NAIm of approx. 1 tyrosine residue/subunit diminishes the GTP response (Fig.

2), but no change in the activity or K_m values for the substrates (Table 2), sedimentation behaviour (Table 3) and optical-rotatory-dispersion properties of the enzyme can be detected. Nitration by TNM, again of 1 tyrosine residue/subunit, slightly alters the K_m values (by a maximum of about 20%; Table 4), but does not affect the overall physical properties of GDH. In this case, and also when the nitro group is reduced, the extent of desensitization is consistently higher than that obtained by acetylation (compare Figs. 2*a* and 6).

It has been shown that GTP and NADH together induce a conformational change in GDH (Bayley & Radda, 1966) and that this change can be followed by using the fluorescence properties of ANS as a probe (Dodd & Radda, 1967, 1969). The kinetic observation is that the modified enzymes are less sensitive to GTP. The fluorescence titrations with ANS as the probe (Figs. 3 and 8) indicate that this decreased response of modified enzyme is associated with an increased value of $s_{0.5}$ for GTP (Dodd & Radda, 1969), i.e. higher concentrations of the allosteric ligand are required to bring about the conformational change. The total enhancement of ANS fluorescence is the same for native and modified enzymes, suggesting that the same allosteric range is being covered.

The dissociation constant of the enzyme–GTP complex for native GDH is 20 μM, whereas that for mono-*O*-acetylated GDH is 60 μM (N. C. Price & G. K. Radda, unpublished work). In addition, GTP partially protects against desensitization by NAIm. The observed dissociation constant of GTP can be used to calculate the degree of protection expected if GTP completely prevented reaction of the tyrosine residue near the binding site, e.g. 200 μM-GTP should give 92% protection, whereas the observed protection is 57%. Since GTP alone does not affect the conformation of GDH (Bayley & Radda, 1966; Dodd & Radda, 1969) the protection experiments suggest that the tyrosine residue forms part of the GTP-binding site. The higher protection in the presence of GTP and NADH (Fig. 2) may be a result of stronger GTP binding in the presence of NADH (Colman & Frieden, 1966), or a result of the conformational change in the enzyme brought about by these ligands.

The general features of modification by TNM are similar to those observed in acetylation. Although there is no detectable change in the physical properties of the modified enzyme, the small changes in K_m values suggest that some small structural rearrangement has occurred. This could be a result of the ionization of the nitrotyrosine residue in the modified enzyme, since its pK_a is close to 8·0. The pK_a is considerably higher than the pK_a of free 3-nitrotyrosine (6·9), and the shift in pK_a in nitrated GDH is in the opposite direction from that of mononitro-carboxypeptidase (Riordan *et al.* 1967). Clearly the nitrotyrosine residue in nitrated GDH is in a less polar environment than would be expected for a totally exposed residue.

As in all chemical modifications, one has to be aware of two problems. (i) In acetylation we cannot entirely exclude the possibility that some amino groups have reacted. Using the ninhydrin method described by Spies (1957), we were unable to detect changes in the number of free amino groups on modification, but for an enzyme as large as GDH the method is not accurate enough to detect small amounts of *N*-acetylation. Reactions of the amino groups of GDH, however, in acetylation with acetic anhydride (Colman & Frieden, 1966), in imine formation with pyridoxal phosphate (Anderson *et al.* 1966) and in trinitrophenylation (Freedman & Radda, 1969) all lead to a rapid loss of enzyme activity. In addition, it is unlikely that two such different reactions as acetylation and nitration should give similar results if reactions occurred with groups other than tyrosine. (ii) As we have not been able to demonstrate that the tyrosine residue near the GTP-binding site is uniquely reactive, the possibility of inhomogeneous modification must be considered. Assuming that the enzyme has x groups of a particular kind/molecule, and that these react with a pseudo-first-order rate constant k, the distribution of enzymes with n groups modified after time t can be calculated (Malcolm & Radda, 1968):

$$\frac{[E_n]}{[E]} = \frac{x!}{(x-n)!n!}(1-e^{-kt})^n\, e^{-(x-n)kt}$$

where $[E_n]/[E]$ is the fraction of enzyme with n groups modified.

Table 5. *Distribution of modified enzymes for large numbers of equally reactive groups*

$[E_n]/[E]$ (the fraction of total enzyme with n groups modified) was calculated by the method of Malcolm & Radda (1968). x is the number of equally reactive groups. In both cases the average degree of modification is 1 group/subunit.

		$[E_n]/[E]$				
x	n ...	0	1	2	3	4 or more
16		0·355	0·375	0·185	0·058	0·02
7		0·345	0·395	0·195	0·068	0·01

Table 5 shows the distribution of modified enzymes, where the average degree of modification is 1 tyrosine residue/subunit, for the two cases: where $x = 16$, i.e. all 16 tyrosine residues are equally reactive, and where $x = 7$, i.e. 7 equally reactive and 9 non-reactive tyrosine residues/subunit. The latter is reasonable from solvent perturbation studies of GDH (Cross & Fisher, 1966). The distribution of products is not significantly different in the two cases.

This shows that for unequivocal interpretation of modification experiments it is necessary to show the presence of particularly reactive residues by the kinetic method (Freedman & Radda, 1968) or by peptide 'mapping'. The tryptic digest of GDH contains 57 peptides (Appella & Tomkins, 1966), so that it would be impossible to detect changes in one peptide after the reactions we have studied, where radioactive labelling cannot be used. But our main conclusions are supported by the observations that further modification of tyrosine residues (even up to 6 or 7 tyrosine residues/subunit in acetylation) does not lead to further desensitization.

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