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The Reaction of 2,4,6-Trinitrobenzenesulphonic Acid with Amino Acids. Peptides and Proteins

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1. The kinetics of the reaction of 2,4,6-trinitrobenzenesulphonic acid with various amino acids, peptides and proteins were studied by spectrophotometry. 2. The reaction of the α- and ϵ-amino groups in simple amino acids was found to be second-order, and the unprotonated amino group was shown to be the reactive species. 3. By allowing for the concentration of unreactive $-NH_3^+$ group, intrinsic reactivities for the free amino groups were derived and shown to be correlated with the basicities. 4. The SH group of *N*-acetylcysteine was found to be more reactive to 2,4,6-trinitrobenzenesulphonic acid than most amino groups. 5. The reactions of insulin, chymotrypsinogen and ribonuclease with 2,4,6-trinitrobenzenesulphonic acid were analysed in terms of three exponential rate curves, each referring to one or more amino groups of the proteins. 6. The reaction of lysozyme with 2,4,6-trinitrobenzenesulphonic acid was found to display an acceleration effect. 7. From the reaction of 2,4,6-trinitrobenzenesulphonic acid with glutamate dehydrogenase at several enzyme concentrations, it was possible to discern two sets of amino groups of different reactivity, and to show that the number of groups in each set was decreased by aggregation of the enzyme.

The chemical modification of proteins has been widely used in establishing the functional importance of amino acid side chains in enzyme activity (Fraenkel-Conrat, 1959; Tsou, 1962; Sri Ram, Bier & Maurer, 1962). Some reactions have also formed the basis of structural studies in differentiating between exposed and buried side chains (Timasheff & Gorbunoff, 1967).

The reaction of TNBS* with amino groups was first studied for analytical purposes (Okuyama & Satake, 1960; Satake, Okuyama, Ohashi & Shinoda, 1960). The TNP derivatives of amino acids and peptides were characterized by paper chromatography and spectroscopy. No reaction was observed with the side chains of histidine, tyrosine, threonine and serine after treatment for 3 days at elevated temperatures. No study of SH reactivity was recorded.

This favourable specificity of TNBS prompted its use in the modification of cytochrome *c* (Takemori *et al.* 1962), haemoglobin (Shinoda, 1965) and xanthine oxidase (Greenlee & Handler, 1964). The SH groups of cysteine and mercaptoethanol were observed to react with TNBS (Kotaki, Harada & Yagi, 1964). Goldfarb (1966*b*) described the reaction of TNBS with human serum albumin and analysed its course in terms of three classes of amino groups of different reactivities. The same reagent was used to establish the functional role of amino groups in some natural trypsin inhibitors (Haynes, Osuga & Feeney, 1967) with a mathematical procedure suggested by Ray & Koshland (1961).

To evaluate the potential of TNBS as a reagent for studies of protein structure and function we examined quantitatively the reactivities of a variety of amino acids and peptides. We also extended the mathematical treatment for analysing the reaction of proteins and selected insulin and several enzymes (lysozyme, ribonuclease, chymotrypsinogen and glutamate dehydrogenase) as examples to illustrate the generality of the method. The functional interpretation of these modifications requires a detailed set of investigations for each particular enzyme system, and has already been reported in a preliminary form for glutamate dehydrogenase (Freedman & Radda, 1968).

* Abbreviations: TNBS, 2,4,6-trinitrobenzenesulphonic acid; TNP, trinitrophenyl.

MATERIALS AND METHODS

TNBS tetrahydrate was obtained from British Drug Houses Ltd. (Poole, Dorset). The sodium salt dihydrate (Pierce Chemical Co., Rockford, Ill., U.S.A.) was also used.

Glycine, DL-alanine, DL-phenylalanine, DL-tyrosine, L-lysine monohydrochloride, L-cysteine and *N*-acetyl-DL-cysteine were British Drug Houses Ltd. biochemical

reagents. DL-Phenylglycine was obtained from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.), and substituted DL-phenylglycines (*p*-methyl, *p*-methoxy, *p*-fluoro, *m*-chloro and *m*-fluoro) were a gift from Mr J. H. C. Nayler of Beecham Research Laboratories (Betchworth, Surrey). Glycine amide hydrochloride, glycyl-L-tyrosine, glycylglycyl-L-tyrosine and *N*-α-acetyl-L-lysine amide hydrochloride were grade I from Cyclo Chemical Corp. (Los Angeles, Calif., U.S.A.). *N*-ε-Acetyl-L-lysine was obtained from Ralph N. Emanuel Ltd. (London, S.E. 1).

Insulin was obtained recrystallized as the citrate from Novo Terapeutisk Laboratorium (Copenhagen, Denmark). Lysozyme (hen's egg) was obtained in a crystalline form from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex) and from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Glutamate dehydrogenase from ox liver [L-glutamate–NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] was obtained as a suspension in ammonium sulphate solution from C.F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Ribonuclease A and α-chymotrypsinogen A (bovine pancreas, six times crystallized) were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Phosphate buffers were made up in twice-distilled water to a total ionic strength of 0·1, and all solutions were made up quantitatively in such buffers. TNBS and its sodium salt were stored as the solid in darkened bottles at room temperature, under which conditions they were quite stable, but the solutions (which are liable to become hydrolysed to picric acid) were stored in the dark at 4°, and were not used more than 36 hr. after being made up.

The ammonium sulphate suspension of glutamate dehydrogenase was dialysed against 0·1 M-phosphate buffer, pH 7·7, for 24 hr. at 4° before use. Concentrations of enzymes were estimated by their extinction at 280 mμ by using the published values for the extinction coefficients (Olson & Anfinsen, 1952; Chervenka & Wilcox, 1956; Uber & Ells, 1941). The concentrations of insulin and lysozyme were estimated by weight.

Spectra were recorded on a Cary 14 recording spectrophotometer. Kinetic spectrophotometry was performed by monitoring the extinction of the –NH–TNP group at 340 mμ either in the Cary 14 or in a Hilger and Watts Uvispek fitted with a Gilford automatic cuvette changer.

The pH of solutions was determined in an E.I.L. direct-reading pH-meter and pK_a values were determined in a Radiometer (Copenhagen, Denmark) TTT1b Automatic Titrating pH-meter.

All the kinetic runs were performed at 25±0·1° and, unless otherwise stated, the pH was 7·4.

When the TNBS concentration in a kinetic run exceeded 2 mM, cells of 0·5 cm. or 0·2 cm. path length were used to follow the reaction because of the high background extinction.

Analysis of the data. Kinetic analysis of the reaction of TNBS with amino acids was facilitated by the use of pseudo-first-order conditions, in this case a considerable excess of TNBS. The concentrations of amino acids were 50 μM, and TNBS was used at 10–150-fold excess. Where the excess of TNBS was 40-fold or greater, the entire reaction was assumed to occur under pseudo-first-order conditions, but, when the excess was smaller than this, the data taken were only those in which the TNBS concentration had diminished by $\leqslant 2{\cdot}5\%$.

Under these circumstances:

$$[\text{–NH–TNP}]_t = [\text{–NH}_2]_{t=0}\,(1-e^{-kt})$$

where k is a pseudo-first-order rate constant, so that:

$$\Delta E_t = \Delta E_\infty\,(1-e^{-kt})$$

where ΔE_t and ΔE_∞ are the changes in extinction at 340 mμ at time t and time ∞ respectively. Values of ΔE were taken since the unchanged TNBS had a small extinction at the wavelength monitored (ϵ_{340} 600). Then k is given by the gradient of $\ln[\Delta E_\infty/(\Delta E_\infty-\Delta E_t)]$ versus t. From a series of values of k at various concentrations of TNBS the second-order rate constant k_2 was derived. Values of k and k_2 quoted are in units of min.$^{-1}$ and l.mole^{-1}min.$^{-1}$ respectively.

The value of ΔE_∞ in any given run was determined by extrapolation of the exponential kinetic curve, rather than by direct observation, which would have required long periods of scanning with the possibility of undesired side reactions. From the properties of the exponential curve, if at times t_a, t_b and t_c, where:

$$t_b-t_a = t_c-t_b$$

the extinction increments are ΔE_a, ΔE_b and ΔE_c, then:

$$\Delta E_\infty = \frac{\Delta E_b{}^2-\Delta E_a.\Delta E_c}{2\,\Delta E_b-(\Delta E_a+\Delta E_c)}$$

For a given set of data, different combinations of t_a, t_b and t_c gave consistent values for ΔE_∞ (±1–2%) if t_b-t_a was of the order of the half-time of the reaction.

Analysis of the reaction of TNBS with the heterogeneously reactive amino groups of proteins was by a sequential method similar to that of Goldfarb (1966*b*). Considerable excess of TNBS was taken (defined as [TNBS]/[–NH_2]) and the concentration of TNBS could always be assumed to be constant. With insulin and lysozyme, ΔE_∞ was directly observed, but in the reactions of ribonuclease, chymotrypsinogen and glutamate dehydrogenase it was obtained by extrapolation, as for the amino acids, only the data of the later part of the reaction, which, by inspection, was simply exponential, being used. This assumption was supported by the derivation of a consistent value of ΔE_∞ and by the good linearity of the semi-logarithmic plot. With the values of ΔE_∞ thus found, plots of $\ln[\Delta E_\infty/(\Delta E_\infty-\Delta E_t)]$ versus t could again be constructed, and the pseudo-first-order rate constants for the slowest-reacting group or groups were determined from the linear portions of the graphs. By extrapolation of these portions it was possible to estimate the change in extinction due to the reaction of these slowest groups and thus to suggest the number of groups in the slowest 'set'.

Then, by subtracting the increment in extinction due to the slowest-reacting set from the total, data for the faster-reacting sets were obtained, from which a further logarithmic plots could be constructed. Thus the number of groups in each set, and the corresponding rate constants, could be derived. The disadvantage of this sequential method is that it may lead to a cumulative error in the rate constant ascribed to the most reactive set of groups. The errors in the measured rate constants are between 5 and 10%. The errors in the rate constants found in proteins are likely to be greater, particularly in the highest rate constants, for the reasons outlined above.

This mathematical analysis is based on two assumptions: (1) that the reaction for all groups is pseudo-first-order; (2) that the reaction is followed until only a single set of groups is reacting, i.e. until the reaction course can be described by a single exponential. Both these assumptions were borne out by the linearity of the semi-logarithmic plot over a considerable extent of reaction (see Fig. 8). The best fit to the complete reaction curve is defined by the accuracy of the semi-logarithmic plots. The error in these plots limits the accuracy of both the reactivity and the number of groups described to each kinetic set. This error is 5–10% for the slowest set but increases for each successive set up to about 25%.

RESULTS

α-Amino groups of amino acids

Pseudo-first-order rate constants at 25° and pH 7·4 in 0·1 M-phosphate buffer were obtained by following the reaction of amino acids with various excesses of TNBS. The dependence of these rates on TNBS concentration, for several amino acids, showed the reaction to be first-order in TNBS (Fig. 1). Deviations from linearity were observed at large TNBS excess, particularly with aromatic amino acids. This was probably a result of complex-formation between the two reactants. The second-order constants were derived from the dependence in the linear range (Table 1). The amino acids were chosen so that the importance of steric and electronic effects could be discerned. Phenylglycine derivatives were particularly suitable for observing electronic effects.

The comparison of the measured second-order rate constants was only meaningful when differences in unprotonated amino group concentrations had been allowed for. That this was important was shown by the pH-dependence of the reaction with DL-phenylglycine (Fig. 2). The reactivities of free amino groups were obtained by dividing the observed second-order rate constants by the fraction of amino acid with an unprotonated amino group. The 'intrinsic' reactivities derived in this way, together with the measured pK_a values, are summarized in Table 1.

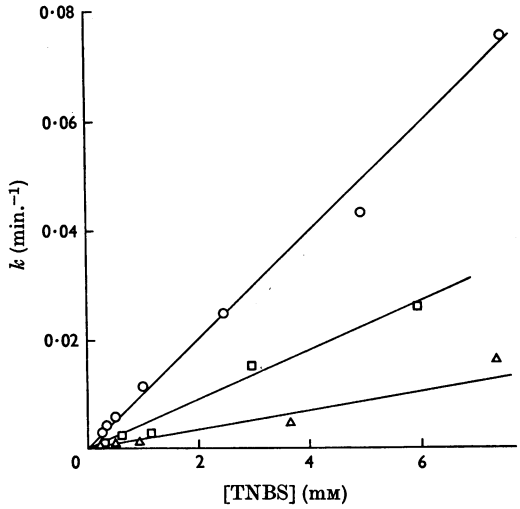

Fig. 1. Linear dependence of k on [TNBS]. ○, Tyrosine; □, glycine; △, alanine. Concentrations of amino acid were about 50 μM, so that TNBS was in 10–150-fold excess.

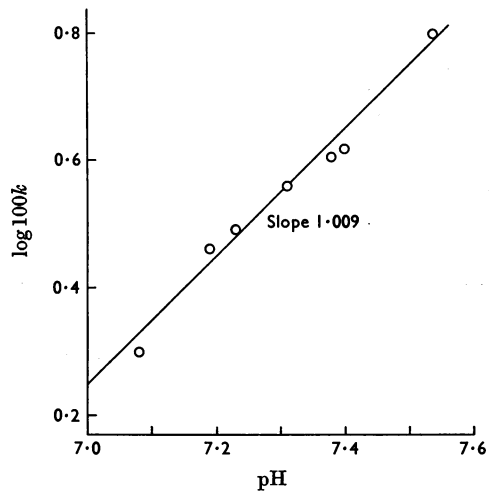

Fig. 2. Variation of k with pH. The concn. of phenylglycine was 47·4 μM and that of TNBS 4·98 mM in the reaction mixtures; 0·1 M-phosphate buffers of various pH values were used.

Other amino acids and peptides

To make these model studies more comparable with situations found in proteins, it was important to have some knowledge of the behaviour of ε-amino groups and of α-amino groups in amides and peptides.

It was expected that the reaction of TNBS with lysine would be complicated by the presence of two reacting groups, giving a curve comprising the sum of two distinct exponential curves. It was found, however, by plotting $\ln[\Delta E_\infty/(\Delta E_\infty - \Delta E_t)]$ versus t, that the reaction could be described by a single rate constant. The total change in extinction observed showed that both amino groups of lysine reacted (Table 2).

The effect of the ionized carboxyl group on the reactivity of α-amino groups was studied by comparing the reaction of some amides and peptides (Table 2).

Table 1. *Reactivities of simple amino acids towards 2,4,6-trinitrobenzenesulphonic acid*

Amino acid	Second-order rate constant (k_2) (l.mole^{-1}min.$^{-1}$)	pK_a of amino group	Intrinsic second-order rate constant (k_1) (l.mole^{-1}min.$^{-1}$)
Glycine	4·3	9·60*	680
Alanine	1·8	9·69*	360
Phenylalanine	7·1	9·13*	390
Tyrosine	9·8	9·11*	510
DL-Phenylglycine	10·6	8·91†	350
DL-*p*-Methylphenylglycine	12·0	8·96†	450
DL-*p*-Methoxyphenylglycine	11·1	9·06†	520
DL-*p*-Fluorophenylglycine	10·8	8·84†	310
DL-*m*-Fluorophenylglycine	12·7	8·55†	190
DL-*m*-Chlorophenylglycine	16·2	8·48†	210

* Edsall (1943).
† G. R. Penzer (unpublished work).

Table 2. *Reactivities of other small molecules towards 2,4,6-trinitrobenzenesulphonic acid*

Compound	Second-order rate constant (k_2) (l.mole^{-1}min.$^{-1}$)	pK_a of amino group	Intrinsic second-order rate constant (k_1) (l.mole^{-1}min.$^{-1}$)
L-Lysine	2·3	α 8·95*	90
		ϵ 10·53*	3100
ϵ-*N*-Acetyl-L-lysine	1·9	9·63	320
α-*N*-Acetyl-L-lysine amide	~4	10·5	~5000
Glycine amide	18	7·93*	90
Glycyl-L-tyrosine	17	8·40*	180
Glycylglycyl-L-tyrosine	18	7·62	50
N-Acetyl-DL-cysteine	26	(SH) ~10·0†	~10000

* Edsall (1943).
† Cecil (1963).

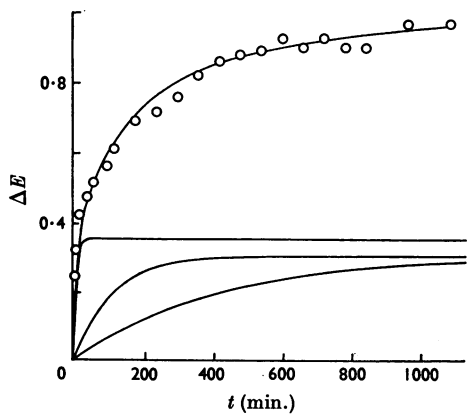

Fig. 3. Analysis of the reaction of insulin with TNBS. The uppermost line is the reaction course predicted on the basis of the exponential contributions represented by the lower lines. ○, Experimental points. The concn. of insulin was 31 μM and that of TNBS 4·74 mM.

By observing the SH wave of cysteine in a polarograph we were able to show qualitatively that the SH group reacted rapidly with excess of TNBS. The reaction of TNBS with *N*-acetylcysteine was observed spectrophotometrically. The spectral changes were small but similar to those found for the reaction with amino groups (cf. Kotaki *et al.* 1964). The measured second-order rate constant and an intrinsic reactivity (assuming that only the anion of SH reacts) are shown in Table 2.

Proteins

Insulin. The reaction of insulin was studied with 31 μM-insulin and 4·6 mM-TNBS so that the molar excess of TNBS per amino group (there are three in insulin) was 49·5. Thus the reaction course could be treated as pseudo-first-order. The reaction went to completion only after 18 hr. To convert data for extinction at 340 mμ into molarities, or in this case to number of residues reacting, it was necessary to

Table 3. *Reactivities of groups in proteins to 2,4,6-trinitrobenzenesulphonic acid*

Protein	Concn. (mg./ml.)	No. of groups in kinetic set	Second-order rate constant (l.mole^{-1}min.$^{-1}$)
Insulin	0·18	1	0·53
		1	2·2
		1	28
Ribonuclease A at pH 7·6	0·52	6	0·60
		1	5·0
		1	30
α-Chymotrypsinogen A at pH 7·6	0·72	9	0·63
		3	7·9
		1	45
Glutamate dehydrogenase	0·15	20	0·6
		4	11
	1·1	13	0·4
		2	12
Lysozyme	0·22	7	

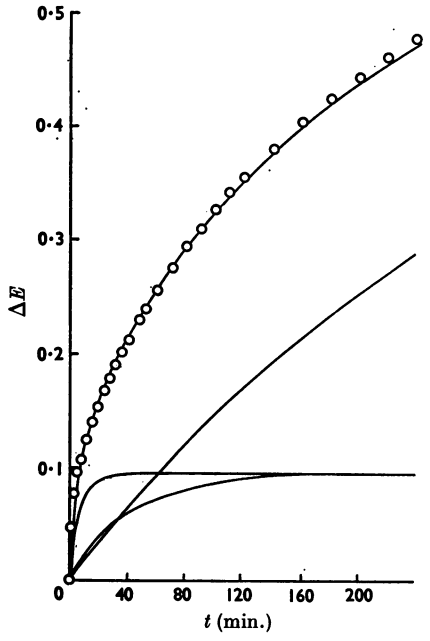

Fig. 4. Analysis of the reaction of ribonuclease with TNBS. The lines and points have the same meaning as in Fig. 3. The concn. of ribonuclease was 40·6 μM and that of TNBS 5·04 mM. The pH was 7·6.

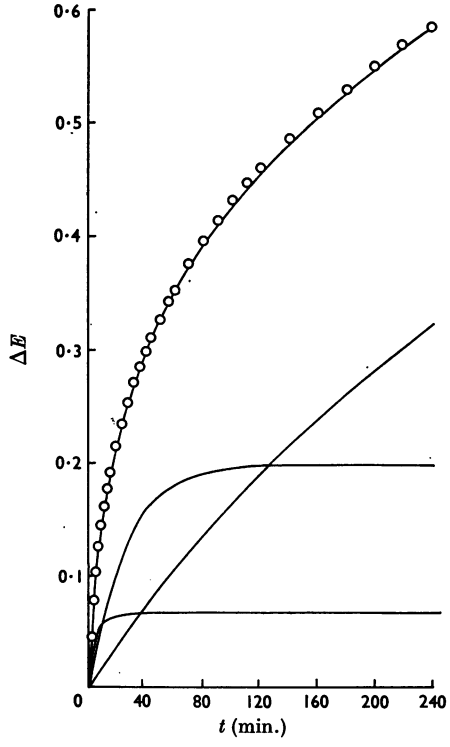

Fig. 5. Analysis of the reaction of chymotrypsinogen with TNBS. The lines and points have the same meaning as in Fig. 3. The concn. of chymotrypsinogen was 28·9 μM and that of TNBS 5·04 mM. The pH was 7·6.

assume extinction coefficients. Okuyama & Satake (1960) prepared the pure mono-TNP derivative of the oxidized insulin A-chain and the bis-TNP derivative of the oxidized B-chain and found values of 1·02 and 1·98 × 10^{4}l.mole^{-1}cm.$^{-1}$ respectively. Goldfarb's (1966*a*) data are more appropriate since they refer to the unacidified reaction product at neutral or alkaline pH. His values for ε-amino-TNP-lysine and α-amino-TNP-oligopeptides were

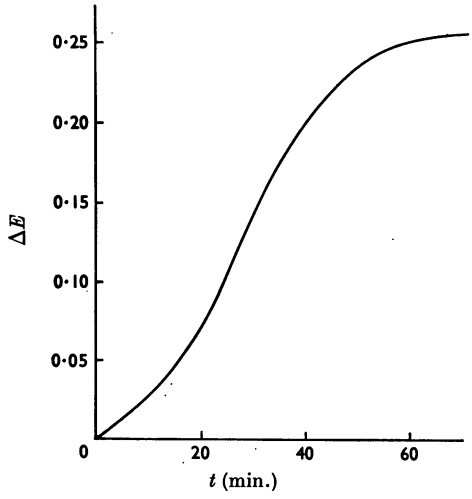

Fig. 6. Reaction of lysozyme with TNBS. The concn. of lysozyme was 15·2 μM and that of TNBS 5·36 mM.

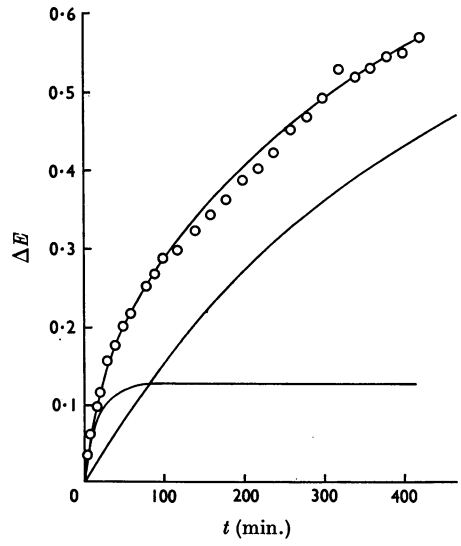

Fig. 7. Analysis of the reaction of glutamate dehydrogenase with TNBS. The lines and points have the same meaning as in Fig. 3. The concn. of glutamate dehydrogenase was 0·15 mg./ml. and that of TNBS 4·77 mM.

$1{\cdot}15 \times 10^4$ and $1{\cdot}00 \times 10^4$ l.mole^{-1}cm.$^{-1}$ respectively. Assuming these extinction coefficients it was possible to analyse the entire reaction curve for insulin by the method described in the Materials and Methods section.

The data were adequately explained by three distinct reactions with rate constants summarized in Table 3. Fig. 3 shows the predicted reaction curve for each residue, together with the composite predicted curve, in comparison with the experimental observations.

Ribonuclease and chymotrypsinogen. The reaction of 40 μM-ribonuclease and 29 μM-chymotrypsinogen with 5 mM-TNBS was analysed in terms of the three exponential curves shown in Figs. 4 and 5. The rate constants and numbers of amino groups in each set are shown in Table 3. For each protein we used an extinction coefficient of $1{\cdot}15 \times 10^4$ l.mole^{-1}cm.$^{-1}$ for the –NH–TNP derivative, and the numbers of amino groups shown in Table 3 were corrected to the nearest integer. This resulted in a slightly worse fit of the experimental data on the theoretical curves. The fit was easily improved if non-integral numbers of amino groups were allowed.

Lysozyme. In contrast, lysozyme, which contains six ϵ-amino groups and one α-amino group, exhibited a different reaction course. The curve shown in Fig. 6 is one of four almost identical curves obtained by using two different preparations of lysozyme. The reaction mixtures were 15 μM in lysozyme and 5·36 mM in TNBS, giving a 51-fold excess of TNBS per amino group.

The reaction showed an acceleration at about 15 min. and appeared to reach a maximum rate at about 25 min. It was completed after 70 min. From the overall change in extinction, the known

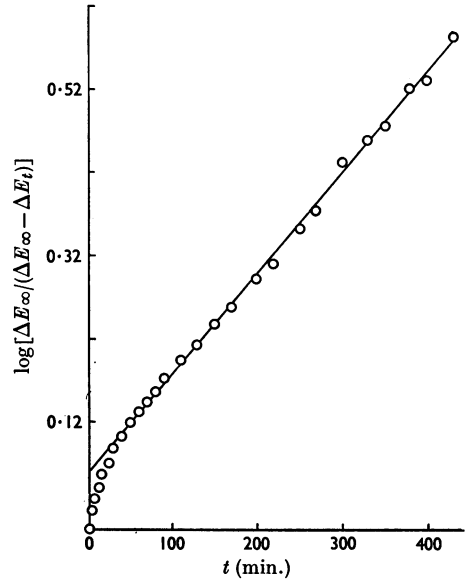

Fig. 8. Derivation of the rate constant for the slower kinetic set in glutamate dehydrogenase. The conditions were as in Fig. 7. $\Delta E\infty$ was derived by exponential extrapolation of the values of ΔE_t from t 80 min. onwards.

concentration of lysozyme and the assumed values of extinction coefficients, it was clear that all seven amino groups had reacted.

Glutamate dehydrogenase. Glutamate dehydrogenase undergoes reversible aggregation as a function of enzyme concentration (Olson & Anfinsen, 1952; Frieden, 1963). Because of this its reaction with TNBS was observed at several enzyme concentrations. Fig. 7 records the reaction curve for a mixture containing 0·15mg. of the enzyme/ml. and 4·77mM-TNBS. At this dilution the enzyme was almost entirely monomeric, i.e. in the form of the 400000mol.wt. unit (Colman & Frieden, 1966*b*). From the data after about 200min. a value of E_∞ was calculated, and thus it was possible to construct the semi-logarithmic plot shown in Fig. 8. The linearity of this plot from 50min. onwards implied that the reaction during this period could be described by a single rate constant. Initially a more rapid reaction (or reactions) also occurred, which was completed in 50min. The rate constant for the slow reaction could be derived from the slope of the line in Fig. 8.

The difference between the observed reaction course and that predicted on the basis of the slow reaction alone could adequately be explained by one further pseudo-first-order process. From the two rate constants and the extinction changes assignable to each reaction, the number of amino groups in the two classes of reactivity was derived (Table 3).

The predicted line of Fig. 7 comprises the reaction of four amino groups with k 0·052min.$^{-1}$ and 20 groups with k 0·0027min.$^{-1}$. It assumes ϵ $1{\cdot}15 \times 10^4$l.mole^{-1}cm.$^{-1}$ for all the –NH–TNP groups.

The interference of SH groups with the above analysis is considered in the Discussion section.

DISCUSSION

In agreement with Goldfarb's (1966*a*) results, the reaction of TNBS with amino acids and peptides is of second-order. The pH-dependence shows that only the unprotonated amino group is the reactive species. The reaction is not as specific as previously implied (Okuyama & Satake, 1960), as the SH group of cysteine reacts faster than most amino groups. However, the –S–TNP group is unstable at alkaline pH and has a much lower extinction coefficient at 340mμ than the –NH–TNP group (cf. Kotaki *et al.* 1964).

The intrinsic reactivity of amino acids and small peptides with TNBS follows the same order as the basicity of their amino groups. This is shown by the surprisingly good correlation between pK_a values and the logarithms of the intrinsic second-order rate constants (Fig. 9). In this system

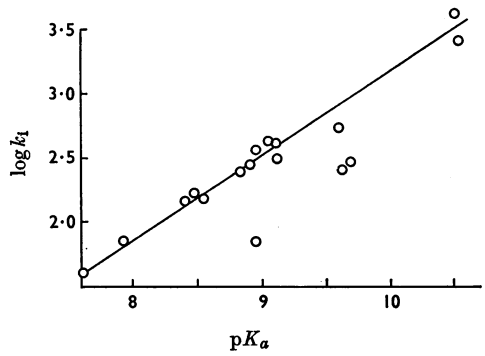

Fig. 9. Correlation of pK_a and nucleophilicity of the amino groups in model compounds. The three most deviating points are for the α-amino groups of alanine, lysine and ϵ-*N*-acetyl-lysine.

therefore the nucleophilicity of the amino group parallels its basicity. As one might have suspected, reactivities are somewhat less susceptible to effects of structural changes than are acidities (i.e. the slope of the line in Fig. 9 is less than 1). A quantitative estimate of electronic effects on reactivity may be obtained by using substituted phenylglycines and analysing the results in terms of the Hammett (1940) equation. Because, in this system, there can be no direct conjugation between the aromatic ring and the site of reaction in the transition state, modified Hammett parameters, σ_n (Wells, 1963), are used. Reasonable correlations of σ_n with pK_a and with logk_1 are obtained, giving ρ values of $-1{\cdot}03$ and $-0{\cdot}76$ respectively. (The correlation coefficients are 0·975 and 0·962 respectively.)

Because of the opposing effects of increasing protonation and increasing intrinsic reactivity, the range of observed reactivities at pH 7·4 is relatively small. This explains the observation of a single rate process for the reaction of both amino groups of lysine, as a small difference in rates would not be detected by the mathematical analysis.

An unexpected feature of the reaction is the decrease in intrinsic reactivities on converting the carboxyl group of an amino acid into an amide group or a peptide linkage. This suggests that abolition of the repulsion between the ionized carboxyl group and the negatively charged sulphonate anion is insignificant in comparison with the decrease in nucleophilicity of the α-amino group. In contrast, preliminary investigations suggest that neither the pK_a nor the reactivity of the ϵ-amino group of α-*N*-acetyl-lysine amide differs greatly from that in lysine itself.

The range of observed reactivities for proteins is an order of magnitude larger than that found for the

models. For insulin it has been shown (Li, 1956) that only the ϵ-amino group reacts with 2,4-dinitrobenzenesulphonic acid over a period of 9 days. It is therefore reasonable to assume that it is this group which is also most reactive towards TNBS.

Results with chymotrypsinogen and ribonuclease show that even when there are more amino groups than in insulin we can distinguish three classes of reactivities. It is unlikely that our method would detect more than three different rate constants unless there was one of greatly differing magnitude. On the basis of the model experiments one might conclude that groups that are in the 1–201. $mole^{-1}$ $min.^{-1}$ range of reactivities are available for reaction and the differences are a result of differing pK_a values caused by electronic effects. For instance, it has been suggested by Hirs (1962) that the preferential reactivity of lysine-41 in ribonuclease is caused by the effect on its pK_a of adjacent cationic centres. This is probably the group that reacts fastest with TNBS. Because it is likely from the crystallographic data that all the lysine residues in ribonuclease (cf. Wyckoff *et al.* 1967) and chymotrypsinogen (cf. Matthews, Sigler, Henderson & Blow, 1967) are exposed, the low reactivity of the last class of amino groups is probably due to masking by the phosphate anions that tend to cluster around the amino groups.

The observed acceleration effect for lysozyme can be explained if it is assumed that reaction of the fast-reacting groups in the molecule facilitates the reaction of the other groups, perhaps through an unfolding of the protein.

The interpretation of the data for an enzyme as complex as glutamate dehydrogenase is less certain. The first problem arises from the presence of SH groups. There are five (Sund & Akeson, 1964) or eight (Appella & Tomkins, 1966) half-cystine residues for each polypeptide sub-unit of glutamate dehydrogenase of mol.wt. 50000. This contains 30–32 lysine residues and one *N*-terminal amino group. Because of the relatively few half-cystine residues and because the –S–TNP derivatives contribute less to the extinction at 340 mμ, even if all the SH groups react they will not affect our mathematical analysis significantly. There is evidence, however, that in the native enzyme not all SH groups are reactive. Not more than two SH groups per polypeptide chain react with mercurials (Hellerman, Schellenberg & Reiss, 1958), and even these react relatively slowly (P. M. Bayley & G. K. Radda, unpublished work). *N*-(*N*-Acetyl-4-sulphamoylphenyl)maleimide reacts with only one SH group per chain (Pfleiderer, Holbrook, Nowicki & Jeckel, 1966). All this evidence suggests that neglect of the possibility of SH-group reaction can only introduce a possible error of 1 into the number of amino groups assigned to a given kinetic 'set'.

Of the 30–32 amino groups of glutamate dehydrogenase, 24 react at low enzyme concentrations, where the enzyme is almost completely dissociated into the 400000 unit (Colman & Frieden, 1966*a*,*b*; Bayley & Radda, 1966). This number compares reasonably with the 25·6 groups per polypeptide sub-unit found to react with ninhydrin (Colman & Frieden, 1966*b*). It is also clear that association of the enzyme (by increasing protein concentration) leads to a diminution of the number of reactive amino groups in each of the two kinetic sets, giving only 15 groups capable of reacting with TNBS (Table 3).

It is clear that the method is applicable to a variety of systems. For structural studies it is essential that the anion effect is elucidated and that intrinsic reactivities for amino groups are derived from pH-dependence. The structural integrity of the protein throughout the reaction course should be checked by independent physical measurements. To implicate a group in a functional role (cf. Haynes *et al.* 1967) it is essential (as in all chemical modifications) to minimize the extent of reaction; hence, in this case, the overall analysis is required only as a means of relating reactivity to loss of functionality at an early stage (Ray & Koshland, 1961; Freedman & Radda, 1968). The unequivocal identification of a modified group requires isolation of the product.

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REFERENCES

Appella, E. & Tomkins, G. M. (1966). *J. molec. Biol.* **18**, 77.

Bayley, P. M. & Radda, G. K. (1966). *Biochem. J.* **98**, 105.

Cecil, R. (1963). In *The Proteins*, vol. 1, p. 392. Ed. by Neurath, H. New York: Academic Press Inc.

Chervenka, C. H. & Wilcox, P. E. (1956). *J. biol. Chem.* **222**, 621.

Colman, R. F. & Frieden, C. (1966*a*). *J. biol. Chem.* **241**, 3652.

Colman, R. F. & Frieden, C. (1966*b*). *J. biol. Chem.* **241**, 3661.

Edsall, J. T. (1943). In *Proteins, Amino Acids and Peptides*, p. 84. Ed. by Cohn, E. J. & Edsall, J. T. New York: Reinhold Publishing Corp.

Fraenkel-Conrat, H. (1959). In *The Enzymes*, vol. 1, p. 589. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Freedman, R. B. & Radda, G. K. (1968). *Biochem. J.* **108**, 5P.

Frieden, C. (1963). In *The Enzymes*, vol. 7, p. 1. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.

Goldfarb, A. R. (1966*a*). *Biochemistry*, **5**, 2570.
Goldfarb, A. R. (1966*b*). *Biochemistry*, **5**, 2574.
Greenlee, L. & Handler, P. (1964). *J. biol. Chem.* **239**, 1096.
Hammett, L. P. (1940). *Physical Organic Chemistry*, p. 184. New York: McGraw–Hill Book Co. Inc.
Haynes, R., Osuga, D. T. & Feeney, R. E. (1967). *Biochemistry*, **6**, 541.
Hellerman, L., Schellenberg, K. A. & Reiss, O. K. (1958). *J. biol. Chem.* **233**, 1468.
Hirs, C. H. W. (1962). *Brookhaven Symp. Biol.* **15**, 154.
Kotaki, A., Harada, M. & Yagi, K. (1964). *J. Biochem., Tokyo*, **55**, 553.
Li, C. H. (1956). *Nature, Lond.*, **178**, 1402.
Matthews, B. W., Sigler, P. B., Henderson, R. & Blow, D. M. (1967). *Nature, Lond.*, **214**, 652.
Okuyama, T. & Satake, K. (1960). *J. Biochem., Tokyo*, **47**, 454.
Olson, J. A. & Anfinsen, C. B. (1952). *J. biol. Chem.* **197**, 67.
Pfleiderer, G., Holbrook, J. J., Nowicki, L. & Jeckel, R. (1966). *Biochem. Z.* **346**, 297.
Ray, W. J., jun. & Koshland, D. E., jun. (1961). *J. biol. Chem.* **236**, 1973.
Satake, K., Okuyama, T., Ohashi, M. & Shinoda, T. (1960). *J. Biochem., Tokyo*, **47**, 654.
Shinoda, T. (1965). *Biochim. biophys. Acta*, **97**, 382.
Sri Ram, J., Bier, M. & Maurer, P. H. (1962). *Advanc. Enzymol.* **24**, 105.
Sund, H. & Akeson, A. (1964). *Biochem. Z.* **340**, 421.
Takemori, S., Wada, K., Ando, K., Hosokawa, M., Sekuzu, I. & Okunuki, K. (1962). *J. Biochem., Tokyo*, **52**, 28.
Timasheff, S. N. & Gorbunoff, M. J. (1967). *Annu. Rev. Biochem.* **36**, 13.
Tsou, C.-L. (1962). *Sci. sin.* **11**, 1535.
Uber, F. M. & Ells, V. R. (1941). *J. biol. Chem.* **141**, 229.
Wells, P. R. (1963). *Chem. Rev.* **63**, 171.
Wyckoff, H. W., Hardman, K. D., Allewell, N. M., Inagami, T., Johnson, L. N. & Richards, F. M. (1967). *J. biol. Chem.* **242**, 3984.