Enzyme-like Catalysis by Polymers: Disulphide Cleavage effected by Hydrophobically Alkylated Polyethyleneimine Derivatives acting *via* a Complexation Step

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Polyethyleneimine, benzylated to the extent of 10% of its nitrogen atoms, cleaves the disulphide link of Ellman's reagent. The reaction exhibits a binding step, characteristic of the action of enzymes, followed by a bond-forming step between sulphur and nitrogen. The polymer molecule (M_w 240 000) possesses from 1 to 2 amino groups in 800 which are rapidly sulphenylated by the substrate as deduced from titration and kinetic equilibrium techniques; this is consistent with *ca.* 1—2% of the benzyl groups providing a binding site close to a reactive amine. The benzyl polymer exhibits a remarkable 10^6-fold catalytic enhancement over the corresponding efficiency of a simple amine nucleophile of similar pK_a. The cleavage reaction, namely reaction of bound substrate with adjacent amine, possesses a first-order rate constant with an effective molarity of 26M compared with the corresponding intermolecular process.

RECENT work, mainly from the laboratories of Klotz [1] and Hine,[2] has shown that polyethyleneimine and its derivatives possess enzyme-like qualities in their catalysis of a number of simple reactions including α-dedeuteriation of aldehydes, hydrolysis of aryl sulphates, and hydrolysis of 4-nitrophenyl carboxylate esters.

Our recent work [3] indicates that disulphide cleavage by primary and secondary amines proceeds by nucleophilic attack and proton transfer occurs in a step after rate-determining attack of the amine [reaction (1)]. Bond formation between nitrogen and sulphur is only half complete in the transition-state of the rate-limiting step as judged from the Brønsted exponent for a plot of the

$$\begin{array}{ccc} R_2NH + R'S\text{–}SR' & \rightleftharpoons & R_2\overset{+}{N}H\text{–}SR' + \bar{S}R' \\ & & \updownarrow \\ & & R_2NSR' + H^+ \end{array} \quad (1)$$

logarithm of the rate constant *versus* the pK_a of the amine.[3] Under certain conditions the full amount of

[1] (*a*) H. C. Kiefer, W. I. Congdon, I. S. Scarpa, and I. M. Klotz, *Proc. Nat. Acad. Sci. U.S.A.*, 1972, **69**, 2155; (*b*) I. M. Klotz, G. P. Royer, and I. S. Scarpa, *ibid.*, 1971, **68**, 263.

[2] (*a*) J. Hine, F. E. Rogers, and R. E. Notari, *J. Amer. Chem. Soc.*, 1968, **90**, 3279; (*b*) J. Hine, E. F. Glod, R. E. Notari, F. E. Rogers, and F. C. Schmalstieg, *ibid.*, 1973, **95**, 2537.

[3] H. Al-Rawi, K. A. Stacey, R. H. Weatherhead, and A. Williams, *J.C.S. Perkin II*, 1978, 663.

thiol is not released because the back reaction involving attack of thiolate on protonated sulphenamide is significant. Since the thiolate leaving group has a pK_a in the region of that for the attacking amine, proton transfer, as in acid catalysis, may not assist cleavage of the disulphide by the amine. Moreover, there is no evidence for acceleration of disulphide cleavage by supernucleophiles: for example, hydrazine, a typical nucleophile known to exhibit the ' α-effect ' has the reactivity in disulphide cleavage expected from its pK_a in comparison with simple amines. For these reasons disulphide cleavage is a useful tool with which to test the possibility of complexation in enzyme-like polymer catalysts because ' chemical ' forms of catalysis such as nucleophilic, proton transfer, or electrophilic may not contribute to any observed rate enhancement.[4] Explanations are thus narrowed down to complexation and microscopic medium effects. Disulphide cleavage is an important reaction in its own right both theoretically [5] and in regard to its controlling role in protein biosynthesis.[6]

We report here that *N*-benzylpolyethyleneimine cleaves a disulphide (Ellman's reagent) [7,8] *via* a complexation step followed by nucleophilic attack on the sulphur. The system is intriguing because an apparent ' burst ' of thiol is produced on adding substrate to polymer followed by a much slower steady release of product. This is contrary to what might be expected because the sulphenylation of an active nitrogen ought to be irreversible thus allowing no active amine to be regenerated to participate in the later phase of the catalysis. Further, the thiolate released on addition of a large excess of polymer catalyst is *less* than the theoretical amount by *ca.* 50%. It is the purpose of this work to examine the sulphenylation reaction of *N*-benzylpolyethyleneimine in order to understand the mechanism of the process.

EXPERIMENTAL

Materials.—Polyethyleneimine (PEI) was obtained from B.D.H. as a 50% solution in water (Polymin P) and this was dialysed against distilled water for several days to remove any low molecular weight polymer. Polyethyleneimine treated in this manner has been shown to have $\bar{M}_n$ 110 000 and $\bar{M}_w$ 1 200 000.[9] An experiment to determine the limiting viscosity by the method of Van der Berg [9] using an Ubbelohde [10] suspended level viscometer gave a value for $[\eta]$ of 18.6 ml g^{-1}; the Mark–Houwink equation (2) [9] gives a value for $\bar{M}_w$ 240 000 for our material.

$$[\eta] = K_v M^{\nu} \qquad (2)$$

All concentrations of PEI and its modified material (merlarity) were determined from the nitrogen content using a Kjeldahl digestion apparatus (Markham modification) [11a] with prior digestion of the polymer with a copper sulphate catalyst.

Polyethyleneimine benzylated to the extent of 10% of its total nitrogen atoms (BPEI) was prepared by adding the appropriate amount of benzyl chloride slowly, with stirring, to dialysed polyethyleneimine solution. The reaction was checked by analysis for chloride ions by titration using a silver electrode: the sample to be titrated was diluted by 20% with acetone and adjusted to pH 2 with nitric acid. A Radiometer potentiometric titrator was employed with an automatic burette containing 0.025 M-$AgNO_3$ in 0.025M-HNO_3 and a silver electrode. Suitable control determinations were carried out to correct for any halide present in the solutions as background. These analyses were kindly undertaken by Mr. G. Powell of the Microanalytical Laboratory of the University of Kent. The limiting viscosity of the product material was $[\eta]$ 13.4 ml g^{-1} which represents a large shrinkage of the polymer compared to the native PEI and is presumably due to the aggregation of the hydrophobic pendant groups on the polymer.

Tetramethylethylenediamine (TEMED) was obtained from B.D.H. and Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] from Aldrich. Glutathione was from the Sigma Chemical Company and benzyl chloride was distilled from bench grade material. Glass distilled water was used throughout the investigation.

Methods.—Kinetics were measured using Pye–Unicam SP 600UV or SP 800 recording spectrophotometers. The pH of the solutions was measured before and after each run using a Pye–Dynacap pH-meter or a Radiometer PHM 62 digital instrument calibrated with E.I.L. standard buffers to 0.02 pH unit tolerance. At high polymer concentration (>0.2M$_i$) no buffer was necessary. The silica cell containing the appropriate polymer solution (2.5 ml) was placed in the thermostatted cell holder for 10—15 min before a portion (10—100λ) of a stock solution of Ellman's reagent (*ca.* 10mM) in acetonitrile was introduced on the flattened tip of a glass rod. Solutions were degassed and stored under nitrogen and prior to recording the trace of the absorbance, the cell was flushed (not bubbled) with nitrogen and sealed tightly with a Teflon stopper. This procedure was necessary, especially at alkaline pH, to prevent oxidation of the product thiol and hence spurious kinetics. The optical density (converted from transmittance *via* a linear–logarithmic recorder in the case of the SP 600UV) was recorded as a function of time using a Servoscribe potentiometric recorder within 10 s of the starting time. The cleavage of the disulphide bond was followed by observing the increase in absorbance at 410 nm where the product, 5-mercapto-2-nitrobenzoic acid, absorbs strongly.

Assay of the Released Thiol.—In order to determine exact concentrations of thiol produced from given amounts of Ellman's reagent it was necessary to determine the extinction coefficient at 410 nm at various pH values. Several test tubes with 0.5M-TEMED buffer (2.5 ml) at pH between 7 and 10 were prepared and the solutions flushed with nitrogen as above. Each tube was then charged with 40λ of glutathione (1mM) and 40λ of Ellman's reagent (10 mM) both from stock solutions in acetonitrile. The solutions were

[4] W. P. Jencks, in ' Current Aspects of Biochemical Energetics,' eds. N. O. Kaplan and E. P. Kennedy, Academic Press, New York, 1966, p. 273.

[5] (*a*) E. Ciuffarin and A. Fava, *Progr. Phys. Org. Chem.*, 1968, **6**, 81; (*b*) R. E. Davis, *Survey Progr. Chem.*, 1964, **2**, 189.

[6] P.C. Jocelyn, ' Biochemistry of the SH Group,' Academic Press, New York, 1972.

[7] H. Deakin, M. G. Ord, and L. A. Stocken, *Biochem. J.*, 1963, **89**, 296.

[8] G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70.

[9] J. W. A. Van der Berg, C. J. Bloys van Treslong, and A. Polderman, *Rec. Trav. chim.*, 1972, **92**, 3.

[10] J. M. G. Cowie, ' Polymers,' Intertext Books, Aylesbury, 1972, p. 166.

swirled and kept at room temperature for 15 min to obtain the maximum absorbance change which was measured at 410 nm on an SP 500 machine. Small corrections were needed for the liberation of thiol by the buffer itself during the reaction although these only accounted for *ca.* 2% of the final value. Below pH 8 the extinction coefficient was constant (Table 1) at *ca.* 13 000 and rose to 23 000 above

TABLE 1

Extinction coefficients of Ellman's reagent cleaved in stoicheiometry 1 : 1 with glutathione as a function of pH [a]

pH	$10^{-4}\varepsilon$
6.9	1.29
7.5	1.29
7.9	1.35
8.7	1.62
8.9	1.62
9.5	1.9
10.1	2.02
10.4	2.23
10.7	2.26

[a] 35° 0.5M-TEMED buffer (2.5 ml) at the given pH, 1 mM-glutathione (40λ) and 10mM-Ellman's reagent (40λ).

pH 10.4. These values are consistent with those derived from the literature for single pH values [7] and at pH 8.8, the value used for the majority of this work, the extinction coefficient is 16 000. The values are independent of the nucleophile used to cleave the disulphide link (amine or thiol) and the presence of even 1M_i-BPEI * does not alter the extinction coefficients of the liberated thiol. For the work requiring a very accurate knowledge of thiol concentration the extinction coefficient of the cleaved Ellman's reagent was measured using the spectrophotometer being used for the reaction in question.

Potentiometric Titration.—A Radiometer REC 61/REA 160 recording titration system coupled with a pH-meter PHM 62 and an autoburette ABU 11 was employed to measure the amount of polymer protonated at different pH values.

Analysis of the Kinetic Data.—The data for π and k were analysed in terms of an hyperbolic function by the use of the least squares fit devised by Wilkinson.[11b] This was carried out with a Basic program run on the Kent On-line System through the University of Kent Computer Centre; the programme is a modification of the algorithm reported by Williams.[12]

RESULTS

N-Benzyl polyethyleneimine (10%) causes the release of thiol from Ellman's reagent by a mechanism involving at least two steps as evidenced by the fast exponential increase in absorbance followed by a slower increase (Figure 1). Figure 1 also illustrates the kinetics for release of thiol catalysed by the buffer without polymer and that for release of thiol by the same concentration of a simple amine at the same pH and possessing a pK_a similar to that for the polymer. A plot of ΔA_t (see Figure 1) *versus* time on two cycle semilogarithmic graph paper gives a linear relationship corresponding to a first-order rate constant k.

* We define M_i (merlarity) as the concentration of monomer residues in moles per litre; molarity is given the symbol M.

[11] (*a*) R. Markham, *Biochem. J.*, 1942, **36**, 790; (*b*) G. N. Wilkinson, *ibid.*, 1961, **80**, 324.

Variation of Polymer Concentration.—The ' burst ' at zero time (π or ΔA_0) varies with polymer concentration and this is recorded in Table 2 and illustrated in Figure 2; at high

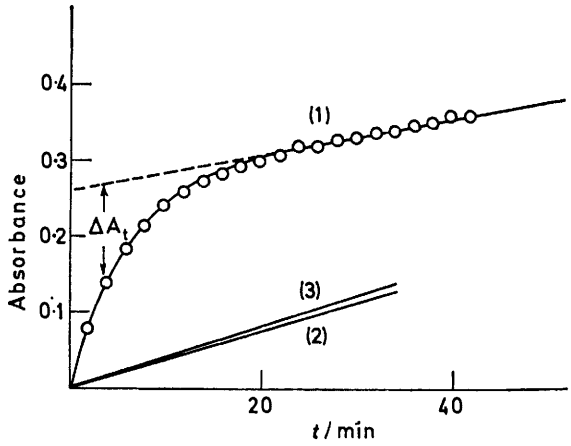


FIGURE 1 (1) Reaction between Ellman's reagent (40 μM) and BPEI (10% nitrogen benzylated, 0.037M_i) in 0.5M-TEMED buffer at pH 8.8; (2) reaction without BPEI; (3) reaction with a primary amine of pK_a 7.49 at 0.037M; the value of ΔA_t at zero time is π

concentrations of polymer π becomes constant. These observations are under conditions where polymer merlarity exceeds that of substrate and these are counter to those

TABLE 2

Variation of ' initial ' kinetic parameters with the concentration of BPEI (10%) at a constant Ellman's reagent concentration [a]

[BPEI]/M_i	10^3k/s^{-1} [b,c]	$10^5\pi$/M [d]
0.93	3.5	4.5
0.47	3.6	4.3
0.37	3.9	4.4
0.19	4.0	4.4
0.093	4.6	3.0
0.037	3.7	2.0
0.019	2.8	1.4
0.0093	2.8	1.1
0.0037	0.43	0.6

[a] 35°, [S] = 80 μM, pH 8.8, 0.5M-TEMED. [b] Corrected for background rate constant. [c] $K = 1.3 \pm 0.4 \times 10^{-2}M_i$; [e] $k_{max.} = 5.2 \pm 0.6 \times 10^{-3}\ s^{-1}$. [d] $K = 11 \pm 2mM_i$; [e] $\pi_0 = 43 \pm 5$ μM. [e] These values are considered to be equal within the experimental error and their average is 12 mM_i.

usually prevailing in enzyme substrate studies where [catalyst] ≪ [substrate].[12] Figure 2 indicates that saturation is complete at *ca.* 0.1M_i-polymer and the data fit equation (3a) which is analogous to that derived by Bender [13] for the case where substrate is in excess of the enzyme.

$$\pi_0^{1/2} = \pi^{1/2} + K^{1/2}[P] \qquad (3a)$$

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \qquad (3b)$$

$$\downarrow$$

$$P_1$$

Equation (3a) is a special case of Bender's [13] equation for three-step enzyme processes where $k_2 \gg k_3$ (*i.e.* the

[12] A. Williams, ' The Chemistry of Enzyme Action,' McGraw-Hill, London, 1969.

[13] M. L. Bender, ' Mechanisms of Homogeneous Catalysis,' Wiley–Interscience, New York, 1971, p. 417.

enzyme is not regenerated at a significant rate). In the present case the disulphide is cleaved spontaneously but this is relatively slow compared with sulphenylation of the polymer and in any case this complication is eliminated

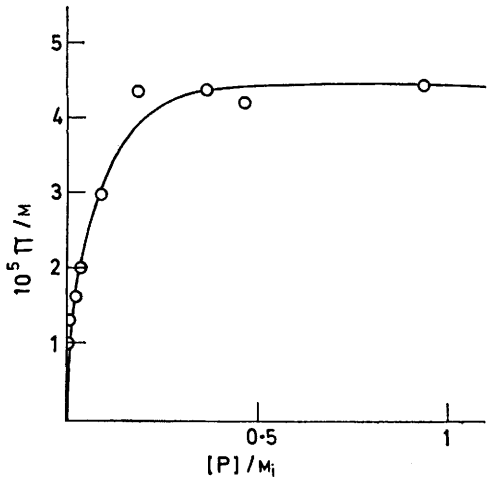


FIGURE 2 Variation of the initial ' burst ' (π) with [BPEI]. Conditions are Ellman's reagent at 80 μM, pH 8.8, 35°, 0.5M-TEMED buffer; the line is theoretical from Table 2

because π is obtained by extrapolation. It should be stressed that the second part of the two-phase progress curve does not correspond to k_3 as is usual in enzyme kinetics; this phase is not a regeneration of the active polymer.

The complication due to the ' spontaneous ' reaction also arises in studies on the rate constant for sulphenylation (see below); it is, however, small compared with the overall sulphenylation rate and the effect is eliminated by using as an infinity the extrapolation of the last phase of the reaction (see Figure 1 and above).

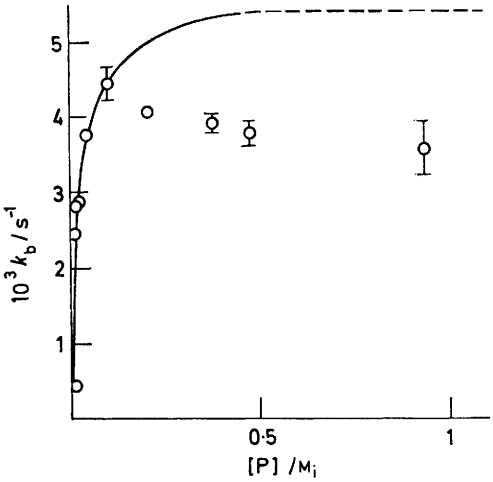


FIGURE 3 Variation of k with BPEI (10%) concentration; conditions are as in Figure 2; the line is theoretical (assuming no polymer inhibition) from parameters in Table 2. The dotted line represents $k_{max.}$ estimated from the data at lower values of polymer concentration

The value of π and K are 43 μM and 11 mMi respectively at pH 8.8 with substrate at 80 μM and 35°.

Variation of the first-order rate constant (corrected for background rate constant) with polymer concentration is illustrated in Figure 3. Whereas at low concentration a Michaelis–Menten type equation is obeyed ($k_{max.}$ and K are 5.2×10^{-3} s^{-1} and 11 mMi respectively), at higher concentrations of *ca.* 0.1Mi-polymer the rate constant begins to diminish and reaches a limiting value at 3.5×10^{-3} s^{-1} with substrate at 80μM, 35°, and pH 8.8.

The above data indicate that even at concentration levels of apparent saturation the polymer does not release the full amount of thiol in the ' burst ' reaction (43 μM as opposed to a theoretical 80μM).

Variation of Substrate Concentration.—The effect of altering the concentration of the substrate on the various kinetic parameters for the initial reaction for a constant concentration of polymer is shown in Table 3. The first-order rate constant, the amount of thiol liberated in the initial ' burst ', and the speed of the second phase of the reaction are all observed to increase with substrate concentration. The value of k and π both reach limiting values as the substrate concentration is increased. The former parameter fits a Michaelis–Menten rate law while the latter fits an equation similar to (3). Both equations yield a value for a dissociation constant which is identical within experimental error (see Table 3).

Effect of pH *and Added Salt.*—The results of the potentiometric titration of BPEI (10%) are shown in Figure 4. It becomes more difficult to protonate the polymer as the charge on the species increases and at pH 6 only one amine in three is protonated. This behaviour may be expected

TABLE 3

Cleavage of Ellman's reagent with BPEI (10%) at a variety of substrate concentrations [a]

10^5[S]/M [BPEI] 0.93Mi	10^3k/s^{-1} [b,c]	$10^5\pi$/M
3.3	2.8	2.2
4.0	3.1	2.8
6.7	3.5	4.1
7.9	3.2	4.0
10.0	3.7	5.5
10.0	3.6	5.7
15.0	3.9	6.0
20.0	4.4	6.4
[BPEI] 0.037Mi		
4.0	2.6	1.3
8.0	3.1	2.2
10.0	3.3	2.3
15.7	3.4	3.2
19.6	3.7	3.2

[a] 35°, pH 8.8, 0.5M-TEMED buffer. [b] Corrected for background rate constant. [c] [BPEI] 0.93Mi, K 22 ± 8 μM;[e] $k_{max.}$ 4.5 ± 0.3 10^{-3} s^{-1};[f] [BPEI]0.03Mi, K 22 ± 9 μM;[e] $k_{max.}$ 4.0 ± 0.4 10^{-3} s^{-1}.[f] [d] [BPEI] 0.93Mi, K 32 ± 4 μM;[e] π_0 89 ± 7 μM; [BPEI] 0.037Mi, K 37 ± 7 μM;[e] π_0 47 ± 5 μM. [e] The experimental error in these determinations means that these values of K are essentially indistinguishable and the average is K 28μM. [f] These values of $k_{max.}$ should be the same (within experimental error) as $k_{max.}$ obtained from the variation of polymer concentration (see Table 2); the latter determination is subject to considerable error due to the incursion of polymer inhibition.

to modify the reaction with Ellman's reagent in three ways: (a) as the concentration of free amine decreases the pseudo-first-order rate constant for a given total amount of amine should also decrease, (b) as the polymer is protonated there will be competition between the increase in its ability to bind with anionic reagent and its lower binding ability due to the inevitable extension of the polymer brought about by protonation, and (c) the uncharged amines at low pH will

have a lower intrinsic nucleophilicity for attack than at high pH due to their decreasing effective pK_a. Table 4 illustrates the effect of changing pH on the value of k and π.

TABLE 4

Dependence of k and π on pH for a constant Ellman's reagent and BPEI (10%) concentration [a]

pH	$10^3k/s^{-1}$ [b]	$10^5\pi$/M
9.8	5.6	3.6
9.1	4.0	4.0
8.9	3.2	3.3
8.8	3.2	3.3
8.6	3.0	3.3
8.5	2.4	
8.35	1.5	4.0
8.25	1.6	3.6
8.1	1.4	3.6
7.84	1.0	2.7

[a] [BPEI] 0.93M_i, [S] = 80 μM, 35°. Corrected for background rate constant.

Keeping the pH constant while increasing the concentration of KCl effectively decreased k and π (see Table 5).

TABLE 5

Effect of added KCl on the cleavage of Ellman's reagent by BPEI (10%) [a]

[KCl]/M	$10^3k/s^{-1}$ [b]	$10^5\pi$/M
0	3.9	4.3
0.2	2.84	2.8
0.5	1.74	2.4
0.66	1.7	2.0
0.8	1.76	1.6

[a] [BPEI] 0.47M_i, [S] 80 μM, pH 9.00, 35°. [b] Corrected for background rate constant.

Increasing the concentration of KCl in a 1M_i solution of BPEI (10%) at pH 8.5 causes the pH to rise to a value of 9.2 due presumably to an increase in the effective pK_a of the polymer.

To obtain an estimate of the 'pK_a' of the polymer the Henderson-Hasselbalch equation (4) may be employed where n' is assumed to be unity and α is defined as the

$$pK_a = pH + n'\log[(1 - \alpha)/\alpha] \quad (4)$$

fraction of free amine. Reading from Figure 4 the value of α at pH 8.73 is 0.945 hence the pK_a is 7.49 (35°).

Second Phase of the Cleavage Reaction.—The rate of cleavage after the initial reaction was complete was calculated from the gradient of the first part of the second phase of the reaction. This was an accurate procedure because the rates for the two phases were very different. The concentration of substrate in this region is given approximately by $[S_0] - \pi$. We have shown that Ellman's reagent spontaneously cleaves in buffers as a function of pH (as well as any possible reaction due to the attack of the buffer species).[3] The spontaneous rate was determined for the experiments in hand from the reaction in a buffer solution (TEMED) with an identical concentration of substrate. Data for the observed rate and the calculated spontaneous rate for the second phase are given in Table 6 and the close agreement indicates that this reaction involves no interaction with the polymer.

Stoicheiometry of the Reaction.—Studies with thiol reactions over long periods of time are fraught with difficulties of oxidation and since the second phase is very slow its investigation is subject to some error. Liberation of thiol was followed to completion over a period typically of two days using an SP 800 instrument making special attempts to ensure that oxygen had been thoroughly purged from the cell. It was noted that more thiol was eventually produced than could be explained by a simple 1 : 1 reaction

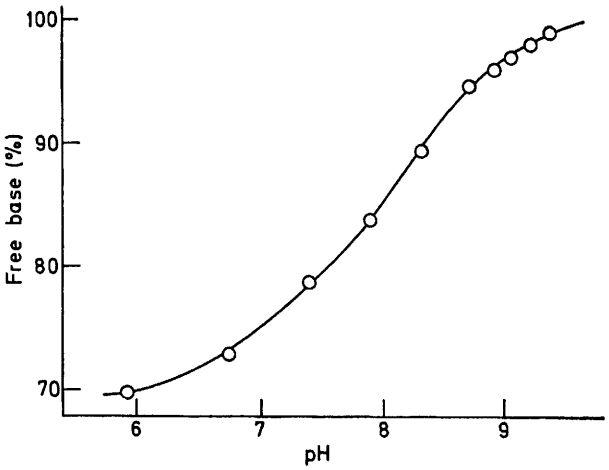


FIGURE 4 Potentiometric titration of BPEI (10%) with HCl at 35°

of Ellman's reagent with nucleophile. Table 7 indicates the overall stoicheiometry of the reaction where T is the

TABLE 6

Comparison of rates at the second phase of reaction of Ellman's reagent with BPEI (10%) with the spontaneous rate [a]

$10^5[S_0]$/M	10^9 rate/ 1 mol^{-1} s^{-1} [b]	$10^5([S_0] - \pi)$/M [c]	10^9 spontaneous rate mol l^{-1} s^{-1} [d]
[BPEI] 0.93M_i			
3.33	0.74	1.1	0.66
4.00	1.5	1.2	0.72
8.00	2.4	4.0	2.4
10.0	2.8	4.4	2.6
15.7	3.1	9.3	5.6
[BPEI] 0.037M_i			
4	2.7	2.7	1.6
8	3.8	5.8	3.5
10	5.3	7.5	4.5
15.7	7.3	13	7.5
19.6	9.3	16	9.8

[a] pH 8.8, 35°, 0.5M-TEMED buffer. [b] Rate of the first part of the second phase. [c] Concentration of substrate in the initial part of the second phase. [d] Spontaneous rate from the release of thiol from substrate at $[S_0] - \pi$ concentration.

TABLE 7

Stoicheiometry of the reaction of Ellman's reagent with BPEI (10%) [a,b]

[BPEI]/ M_i	10^5T_s/ M	$10^5\pi$/ M	10^5T/ M	$(T - \pi)/(T_s - \pi)$	pH
0.93	8	4.12	10.2	1.5	8.8
0.93	8	4.0	9.6	1.4	7.08
0.93	8	4.8	9.6	1.5	9.24
0	8		11.8	1.5	8.9

[a] 0.5M-TEMED buffer, 35°. [b] See text for details of nomenclature.

total thiol concentration of thiol produced and T_s is the amount from a 1 : 1 reaction. It is noted that the ratio

$(T - \pi) : (T_s - \pi)$ is remarkably constant at 1.5 and represents the ratio of the observed thiol production from substrate after the burst $(T - \pi)$ to the total theoretical amount of thiol after the burst has occurred $(T_s - \pi)$.

DISCUSSION

The data from this study for benzylpolyethyleneimine may be easily accommodated by the mechanism involving complexation of substrate with polymer followed by an intramolecular reaction to give sulphenamide [reaction (5)]. The attainment of a concentration of thiol

$$P + S \underset{}{\overset{K}{\rightleftharpoons}} PS \xrightarrow{k_{max.}} \overset{+}{\text{N}}\text{H–SR} + \bar{\text{S}}\text{R} \qquad (5)$$
$$\text{N–SR} + \text{H}^+$$

less than the theoretical maximum in the initial release at saturation of substrate by polymer (Figure 2) is explained by the presence of a significant reverse reaction of thiolate ion (a powerful nucleophile) attacking sulphur bearing an ammonium leaving group. This phenomenon has been observed in simple model systems recently [3] and has been used as an assay for sulphenamides.[14] It is noted that the standard method for sulphenamide degradation involves treatment with concentrated HCl which protonates the nitrogen leaving a halide ion (a comparatively weak nucleophile in aqueous systems) to attack the sulphur to give sulphenyl chloride. With model systems involving amine attack on disulphides it is possible to force the equilibrium completely to the right by increasing the amine concentration.[3] Owing to the complexation equilibrium it is not possible to drive the substrate completely to thiol because at saturation the forward rate constant becomes independent of polymer concentration ($k_{max.}$).

The turnover rate corresponds closely with the spontaneous reaction calculated for the system (Table 6) indicating that the substrate first reacts with the polymer to set up an equilibrium and once this is attained the residual disulphide decomposes *via* its spontaneous mechanism. The final amount of thiol released is not known very accurately because of experimental difficulties due to long time periods but it is certainly greater than that expected from a 1 : 1 reaction. We can explain this simply because it is known that sulphenic acids decompose in water to yield sulphinic acid and thiol;[15] thus one mol. equiv. of disulphide yields one thiol and one sulphenic acid by reaction with water and the latter by disproportionation yields half a thiol and half a sulphinic acid making a final release of 1.5 mol. equiv. of thiol in good agreement with the results of Table 7.

$$\text{RS–SR} \xrightarrow{\text{H}_2\text{O}} \text{RSOH} + \text{RSH} \qquad (6)$$
$$\downarrow$$
$$\tfrac{1}{2}\text{RSH} + \tfrac{1}{2}\text{RSO}_2\text{H}$$

Dissociation Constant of the Polymer–Substrate Complex.—The observation that the dissociation constant (see Table 3) obtained from analysis by the 'Bender' equation (3) for variation of substrate concentration equals that for variation of substrate concentration in the Michaelis–Menten law (k) indicates that the polymer is undergoing sulphenylation by a complexation step [equation (5)] which has a dissociation constant K equal to that from the two methods (28 μM). Variation of polymer concentration with constant substrate gives a dissociation constant (12 mM$_i$, 35°, pH 8.8) from the two methods for π and k. The latter parameter will be discussed later as it does not fit the Michaelis–Menten rate law over the whole of its concentration range in polymer. Since the polymer concentration is expressed in merlarity the dissociation constant from the polymer concentration variation does not equal that for change in substrate concentration. Converting the merlarity to molarity in polymer molecules yields a value $12 \times 10^{-3} \times 43/240\,000 = 2.2$ μM; this result should equal that obtained from the substrate concentration studies (28 μM) if one polymer reacted with one substrate molecule only; the discrepancy indicates that 12.7 molecules of substrate may react with one polymer molecule. Alternatively, we may say that the polymer molecule possesses on average 12.7 active amine sites.

The titration experiments (Table 3) indicate that in a polymer solution at 0.037M$_i$ there are 47 μM active amine sites. The concentration range of the polymer is such that $\pi \propto [P]$ (see Figure 2) and this leads us to estimate that there are $47 \times 10^{-6}/0.037$ molar sites per 1M$_i$ polymer concentration (1.27mM/M$_i$). This means that there are on average $1.27 \times 10^{-3} \times 2.4 \times 10^5/43 = 7.1$ active sites per polymer molecule. This value comes close to that obtained by the entirely different, and unconnected, method described above and in view of the experimental difficulties involved in this work we believe that this is remarkably good agreement. An alternative way of expressing these results is in terms of substrate molecules reacted per number of monomer subunits; this approach is probably more meaningful than the use of polymer molarity (whether from number or weight average) because of the wide polymer distribution. Using this method the comparison of dissociation constants gives one substrate molecule reacted per 429 monomer units and the titration method gives one in 787.

Thus of a total of 240 000/43 (5 581) amino groups in the polymer molecule of which there are 1 256 primary groups (allowing for benzylation occurring with equal chance on primary, secondary, and tertiary amines and a primary amine content of 25% of the total in native polyethyleneimine) [16] only 7—13 are actively involved in forming a covalent link to sulphur.

The observation that $k_{max.}$ values obtained by the two different methods (variation of [P] or [S], see Tables 2 and 3) are identical within experimental error is further

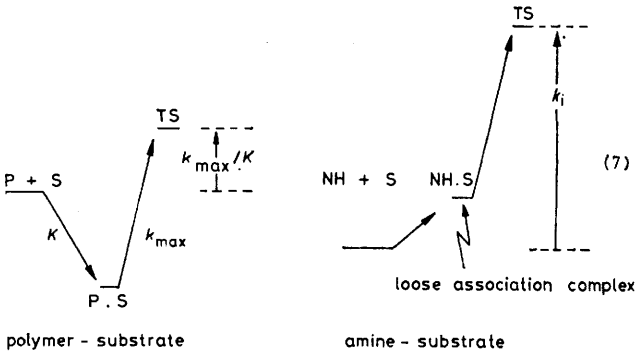
[14] O. Foss, *Acta Chem. Scand.*, 1947, **1**, 307.
[15] N. Kharasch, S. J. Potempa, and H. L. Wehrmeister, *Chem. Rev.*, 1946, **39**, 269.
[16] T. W. Johnson and I. M. Klotz, *Macromolecules*, 1974, **7**, 149.

confirmatory evidence that the mechanism [equation (5)] holds and that our estimates of the numbers of active amines per polymer molecule are valid.

The proportion of active amines may not be such a surprise as it first seems because the number of hydrophobic groups in BPEI is 10% of the total amines and these reside at secondary, tertiary, and quaternary nitrogen.[16] We think it is most likely that benzyl groups are providing hydrophobic binding sites for the substrate which then reacts with a conveniently located primary amine. The sulphenylation reaction probably has a high steric requirement so that the structures attendant on the secondary nitrogen functions in the polymer are not likely to be active. If only one benzyl group is involved per binding site then the correct combination of binding and reactive group comes between 1 in 40 to 1 in 80, that is, roughly 1% of the benzyl groups is providing a catalytic site. The assumption of one benzyl group per site is probably naive and a more realistic proposal is that groups of benzyl functions aggregate in the polymer to give hydrophobic regions which act as binding sites. Although it is almost certain that the backbone of the polymer also provides hydrophobic sites the chance that a binding site occurs with a reactive primary amine could be relatively small. Moreover, not all the primary and secondary amines in the polymer will be on the surface of the molecule and hence available for sulphenylation.

We imagine that the polymer is not acting as a rigid species as is commonly found in enzyme catalysis where the substrate binds at a relatively rigid active site on the surface of the macromolecule. It may be that in our case substrate is binding deep in the polymer molecule rather than on the surface. Increasing the benzyl group content of the polymer reduces the number of active sites and this may be due to two causes: (a) removal of primary and secondary amines as nucleophiles and (b) destruction of the hydrophobic aggregation in the polymer through increased steric requirements. The latter phenomenon has been observed in the catalytic activity of alkylated polysoaps which reaches a maximum as alkylation is increased and then decreases.[17]

Catalytic Efficiency.—Since we are sure of a simple mechanism for the ' burst ' activity of BPEI we are in a position to compare the reactivity with simple amines of similar pK_a which do not possess a binding capability. The model reaction involves collison of reagent and amine to yield a reaction complex followed by nucleophilic attack, proton transfer, and, finally, expulsion of products from the association complex. We may picture the whole process in terms of a ground-state for reactants and a transition-state for the covalent reaction. The other energy levels are not significant because the proton transfer succeeds the rate-limiting step while the association step is very weak (all bimolecular processes must involve ' association ' [18] and it is axiomatic in recent approaches to enzyme catalysis [19,20] that strong association produces a more efficient reaction). The polymer reaction involves a strongly favourable associative step [see equation (7)]. The bimolecular rate constant for the model (k_i) measures the energy difference between ground (reactants separated) and transition state of the rate-limiting step. The same quantity for the polymer is given by the relation $k_{max.}/K$ and this has



the value 5.2×10^{-3} s^{-1}/28 μM = 186 l mol^{-1} s^{-1}. We use here the value of $k_{max.}$ for an infinite concentration of polymer (omitting the inhibition of the reaction as the polymer concentration increases) and the value of K determined from substrate variation studies. At the pH of this study (8.8) 94.5% of the polymer is present as free amine (see Figure 4) of which 28.2% is tertiary and quaternary and will not react (this allows for the fact that 10% benzylation has occurred and we assume this is equally on primary, secondary, and tertiary amine residues). We assume that the composition of the polyethyleneimine starting material has 25% primary, 50% secondary, and 25% tertiary amine. Thus the fraction of total amine theoretically available is 0.945 (1 − 0.282) = 0.679. The overall rate constant for cleavage of disulphide from free amine form of the polymer is therefore 186/0.679 = 274 l mol^{-1} s^{-1} and this refers to polymer and substrate reacting from their respective ground states. The effective enhancement for the rate constant for the bimolecular attack of polymer compared with an amine of similar pK_a (we find a simple amine of pK_a 7.49 has k_i 2×10^{-4} l mol^{-1} s^{-1} at 35°) [3] is thus $274/2 \times 10^{-4} = 1\,370\,000$-fold.* This enhancement must be tempered with the knowledge that we cannot ascribe a single pK_a to the amino groups of the polymer; the pK_a of 7.49 is only a sort of average. We should point out, however, that since the β_{nuc} for attack of simple amines on Ellman's reagent is not large [3] we are allowed a certain latitude in our choice of pK_a.

The enormous acceleration is provided by the introduction of complexing ability into the amine which is of the order of that enjoyed by the more specific enzymes. Less specific enzymes such as papain or chymotrypsin

* Since the polymer reaction does not proceed completely to products at pH 8.8 the value is smaller than this.

[17] T. Kunitake, personal communication.

[18] M. Eigen, *Angew. Chem. Internat. Edn.*, 1964, **3**, 1.

[19] H. Gutfreund, *Adv. Catalysis*, 1957, **9**, 284.

[20] (*a*) W. P. Jencks, *Adv. Enzymol.*, 1975, **43**, 219; (*b*) W. P. Jencks and M. I. Page, Proc. 8th FEBS Meeting, Amsterdam, 1972.

have dissociation constants for the complex with a typical substrate of the order of 1mM.[12,21,22] A further point of interest is the intra-complex reaction with respect to the bimolecular model; the effective molarity (5.2×10^{-3} $s^{-1}/2 \times 10^{-4}$ l mol^{-1} $s^{-1} = 26$) is relatively small for a nucleophilic attack and is in the region expected for weak bond formation in the transition state [23] consistent with the Brønsted data for amine attack.[3] However, it is unlikely that the association complex is a 'tight' one with precisely controlled stereochemistry favouring reaction. It could be that the low effectively molarity comes from a 'loose' complex where the reactants have not 'lost' the entropy that normally occurs upon covalent bond formation or the formation of a 'tight' complex. Another possibility is unfavourable steric effects on the intracomplex reaction reducing the effective molarity.

The provision of hydrophobic sites to bind substrate is very important for catalysis but amine attack on disulphides involves the formation of a zwitterionic transition state from a neutral ground state. A completely hydrophobic environment would therefore retard the intracomplex reaction. We should also point out that Ellman's reagent possesses two negative charges at pH 8.8 and these must be accommodated in the hydrophobic binding site.

The inhibition observed in the rate constant (k) as polymer concentration exceeds $0.1M_i$ is probably due to progressive aggregation between polymer molecules; this may not necessarily alter the number of the active sites in the solution but it may alter their reactivity for reasons stated above. Both reactivity and numbers of sites are altered on changing the pH although there seems to be a plateau for both figures near pH 9. It would be very difficult to isolate the precise cause of these variations which could be due to conformational changes, aggregation, or merely protonation of reactive amines.

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[22] E. C. Lucas and A. Williams, *Biochemistry*, 1969, **8**, 5125.
[23] M. I. Page, *Chem. Soc. Rev.*, 1973, **2**, 295.