Effect of Neutral Salts on Enzyme Activity and Structure*

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ABSTRACT: When trypsin, α-chymotrypsin, renal acylase, wheat germ lipase, estradiol-17β dehydrogenase, β-amylase, and β-galactosidase utilize uncharged substrates, neutral salts inhibit their activity in an order of increasing effectiveness for anions: $CH_3COO^- < Cl^- < Br^- \cong NO_3^- < I^- < ClO_4^- \cong SCN^-$. This order is essentially similar to the order in which these anions disrupt the structure of diverse macromolecules and inhibit the activity of various enzymes utilizing charged substrates. Neutral salts, at concentrations which only partly inhibit the activity of β-amylase, increase the rate of reaction of its sulfhydryl groups with 5,5′-dithiobis-(2-nitrobenzoic acid) in an order of increasing effectiveness for anions: $CH_3COO^- < Cl^- < ClO_4^- < SCN^-$, which is similar to the inhibition order. These salts increase the reaction rate of β-amylase sulfhydryl groups to a degree which exceeds their effects on the reaction rate of the sulfhydryl groups of reduced glutathione for which the order of effectiveness is also different. These observations provide additional evidence that salts alter enzyme activity by altering the organized structure of the protein macromolecule.

It has been recently demonstrated (Warren *et al.*, 1966) that neutral salts at high concentrations inhibit the activity of widely different enzymes in an order of increasing effectiveness for anions: $Ac^- < Cl^- < NO_3^- < Br^- < I^- < SCN^- < ClO_4^-$ and an order of increasing effectiveness for cations: $(CH_3)_4N^+ < Cs^+ < K^+ < Na^+ < Li^+$. Because the orders are similar to those in which these ions disrupt the structure of diverse macromolecules as determined by physical methods (von Hippel and Wong, 1964), it seems plausible that the observed inhibition may result, at least in part, from disruption of organized enzyme structure. Although the orders of effectiveness for inhibition of activity and disruption of structure of myosin were similar, the ranges were different in that activity was generally completely lost at salt concentrations lower than those at which optical rotatory dispersion indicated a definitive decrease in helix content (Warren *et al.*, 1966; Tonomura *et al.*, 1962).

This investigation was carried out for two reasons. First, to evaluate the anion series on a group of enzymes which uses uncharged substrates to establish the order of effectiveness and allow a general comparison of magnitude of inhibition with the previously studied group which utilized charged substrates. We considered that when the substrate is uncharged, electrostatic shielding effects at the active site would be minimized and effects on activity more clearly related to structural change. Second, to examine the possibility that an indication of conformational change can be obtained at a salt concentration where partial activity remains. Using the parameter of sulfhydryl group reaction rates of sweet potato β-amylase precisely this result was obtained.

Experimental Section

Materials. Reagent grade salts, purchased from the Mallinckrodt Co. or G. Frederick Smith Co. ($NaClO_4$), were used without further purification. PNPA,[1] estradiol-17β and its 3-methyl ether, estrone sulfate, Tris base, maleic acid, $NADP^+$, BAPNA, PNPG, GSH, EDTA, and guanidine hydrochloride were from the Sigma Co. The 3,5-nitrosalicylic acid was from Eastman Organic Chemicals Co. Ellman's reagent (DTNB) was obtained from the Aldrich Chemical Co. Distilled, deionized water was used in the preparation of all solutions.

Estradiol 3-sulfate was prepared by reduction of estrone 3-sulfate with sodium borohydride (Warren and Salhanick, 1961) and purified by Celite chromatography (Siiteri *et al.*, 1963). After hydrolysis, chromatography in the benzene–formamide system of Zaffaroni (1953) revealed <0.5% estrone.

Enzymes and Assays. Sweet potato β-amylase (two times recrystallized) and trypsin (containing <0.35% chymotrypsin) were obtained from the Mann Co. The α-chymotrypsin (bovine pancreas, three times crystallized, lyophilized, salt free), porcine renal acylase, bovine liver β-galactosidase, and wheat germ lipase

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[1] Abbreviations used in this work: PNPA, *p*-nitrophenyl acetate; BAPNA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride; PNPG, *p*-nitrophenyl β-D-galactopyranoside; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; GSH, reduced glutathione; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) or Ellman's reagent; Ac, acetate; $NADP^+$, nicotinamide-adenine dinucleotide phosphate; ATPase, adenosine triphosphatase.

TABLE I: Inhibition of Enzymes by Neutral Salts.[a]

Enzyme[b]	Act. as % of Control in 2.0 M KAc	2.0 M KCl	2.0 M KBr	2.0 M KNO_3	1.0 M[c] KI	1.0 M[c] KSCN	1.0 M[c] $NaClO_4$	Charged Substrate
(1) Myosin	<18	<14	<8	<10	0	0	..	Yes
(2) Lactate D	...	65	27	32	3	1	..	Yes
(3) Fumarase	75	40	60	...	3	1	..	Yes
(4) Estradiol D	41	27	10	15	8	2	..	Yes (?)
(5) T-BAEE	82	62	56	55	50	35	..	Yes
(6) T-BAPNA	30	11	5	9	5	2	..	Yes
(7) T-PNPA	...	170	135	120	90	84	90	No
(8) C-PNPA	...	178	100	89	92	15	10	No
(9) Acylase	...	70	40	45	39	10	33	No
(10) Lipase	...	108	83	100	80	46	67	No
(11) β-Amylase[d]	65	59	40	40	..	2	11	No
(12) β-Galactosidase	34	20	19	17	16	12	11	No

[a] For experimental conditions used with the first five enzymes see Warren *et al.* (1966). For conditions with other enzymes see text. All assays were at pH 8.0 except β-amylase, which was at pH 7.0. [b] Myosin, myosin ATPase; lactate D, lactate dehydrogenase; fumarase, fumarase converting fumarate to malate; estradiol D, estradiol-17β dehydrogenase with estradiol-17β as substrate; T-BAEE, trypsin using BAEE as substrate; T-BAPNA, trypsin with BAPNA as substrate; T-PNPA, trypsin with PNPA as substrate; C-PNPA, α-chymotrypsin with PNPA as substrate; acylase, porcine renal enzyme with PNPA as substrate; lipase, wheat germ lipase with PNPA as substrate; β-amylase, sweet potato enzyme with starch as substrate; β-galactosidase, liver enzyme with PNPG as substrate. [c] For myosin and T-BAEE indicated 1.0 M salts were actually 1.2 M. [d] With β-amylase sodium salts were used.

were obtained from the Sigma Co. Estradiol-17β dehydrogenase was prepared from human placenta by the method of Jarabek *et al.* (1962) utilizing only the first six steps of their purification scheme.

The assay of β-amylase was carried out at 25° in 5.0 ml of 0.05 M Tris–maleate buffer, pH 7.0, containing 0.65% soluble starch. The reaction was initiated with 2.2 μg of enzyme and aliquots were taken at 3 and 6 min for determination of liberated maltose by the method of Dahlquist (1962). The quantity of enzyme used liberated 1.60 μmoles of maltose/min in the absence of salts. With β-amylase sodium salts were used for inhibition studies.

The other enzymes were assayed at 25° in 0.05 M Tris–hydrochloride buffer, pH 8.0, with and without potassium salts (except perchlorate where solubility factors required use of the sodium salt). All assay solutions had a final volume of 3.0 ml. Initial velocities recorded originally at several and finally at two substrate levels allowed determination of rates for substrate-saturated enzyme.

Trypsin (1.00 mg), acylase (0.250 mg), and lipase (0.500 mg) were assayed utilizing 2.0 and 1.0 μmoles of PNPA added in 0.1 ml of isopropyl alcohol by determination of liberated *p*-nitrophenol spectrophotometrically at 400 mμ. In the absence of salts these amounts of substrate-saturated enzymes generated 16.5, 48.0, and 32.4 mμmoles of product/min, respectively. Trypsin (0.250 mg) was also assayed with 0.4 and 0.2 μmole of BAPNA as substrate in an otherwise identical solution. The reaction was followed spectrophotometrically at 380 mμ. In the absence of salts, this amount of substrate-saturated enzyme generated 505 mμmoles of product/min. The α-chymotrypsin (0.200 mg) was assayed spectrophotometrically with 0.6 and 0.3 μmole of PNPA added in 0.3 ml of isopropyl alcohol. In the absence of salts the substrate-saturated enzyme generated 27.0 mμmoles of product/min. The β-galactosidase (2.0 mg) was assayed spectrophotometrically with 6.0 and 3.0 μmoles of PNPG by determination of liberated *p*-nitrophenol at 400 mμ. In the absence of added salts the substrate-saturated enzyme generated 59.5 mμmoles of product/min. Estradiol-17β dehydrogenase (0.108 mg) was assayed with 1.0 μmole of $NADP^+$ and either 40 μg of estradiol-17β (30 mμmoles of product/min without salts) or 50 μg of estradiol 3-methyl ether (40.5 mμmoles of product/min without salts) or 100 μg of estradiol 3-sulfate (13.7 mμmoles of product/min without salts) added in 0.1 ml of propylene glycol. These conditions were shown to be essentially saturating since initial velocities in the absence and presence of salts did not change when both cofactor and substrate concentrations were altered 40%.

Salts had no significant effect on the absorbancy of the *p*-nitrophenol, *p*-nitroaniline, or maltose products. The pH of each activity and sulfhydryl assay solution was checked after recording was completed. None differed more than 0.05 unit from stated pH.

Sulfhydryl Reaction Rates. DTNB (Ellman, 1959)

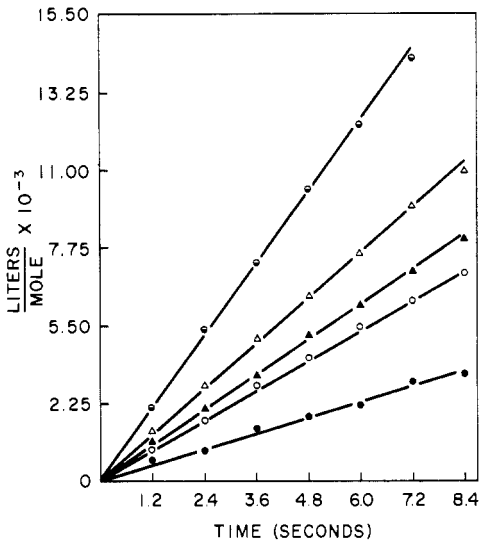

FIGURE 1: Second-order plots for the reaction of DTNB with GSH in the absence and presence of 2.0 M sodium salts. Reaction: A + B → x, with [A] = [B] = 0.20 μmole in 3.0-ml volume of 0.05 M Tris–maleate buffer at 25°. The pH of each reaction mixture was adjusted to 7.0 immediately prior to assay and checked on completion: ●–●, no salt; O–O, NaSCN; ▲–▲, $NaClO_4$; Δ–Δ, $NaNO_3$; ⊖–⊖, NaCl and NaAc. Points are averages of three determinations. The ordinate represents: [x]/[A] [A−x].

was recrystallized from dimethylformamide before use. Reactions were carried out at 25° in 3.0 ml of solution containing 10 μmoles of EDTA, 150 μmoles of Tris–maleate buffer (pH 7.0), DTNB, and 0.20 μmole of GSH or 2.25 mg of β-amylase with and without 2.0 M sodium salts. Reaction of sulfhydryl groups was followed at 412 mμ with a thermostatically controlled DU-2 spectrophotometer attached to a Sargent linear-log recorder at a chart speed of 5 in./min. Corrections were made for changes in time zero OD produced by addition of the initiating substance. Reactions were initially carried out under nitrogen but later under air as no pronounced differences were observed. Absence of pronounced salt effects on the absorbancy of the DTNB–sulfhydryl product was demonstrated by allowing complete reaction to occur and then adding aliquots to solutions containing salts. The effect of NaCl and $NaClO_4$ on the pK of the GSH sulfhydryl group were determined by the method of Benesch and Benesch (1955). This experiment was carried out in Tris–hydrochloride buffer because the high absorbancy of maleic acid at 231 and 237 mμ prohibited its use.

Results

The effect of the anion series on the activity of trypsin, chymotrypsin, acylase, lipase, β-amylase, β-galactosidase, and those enzymes previously reported (Warren *et al.*, 1966) expressed as per cent of a control in the absence of added salts is shown in Table I. Stabilizing Ca^{2+} was purposely omitted from the trypsin assays to avoid possible ion competition. Acetate ion is not included in those cases where PNPA is substrate because it is a product inhibitor. The anions can be arranged in an order of increasing effectiveness in the inhibition of enzymes using uncharged substrates: $Ac^- < Cl^- < Br^- \cong NO_3^- < I^- < ClO_4^- \cong SCN^-$. This order is generally similar to that established with charged substrates except that with uncharged substrates SCN^- appears somewhat more effective than ClO_4^-. The inhibition seen with acylase, β-amylase, and β-galactosidase is of an order of magnitude similar to the range observed with the group of enzymes using charged substrates, while that seen with trypsin, α-chymotrypsin, and lipase (all using PNPA as substrate) is clearly less impressive. The most striking example of this difference is trypsin using PNPA as substrate compared to the same enzyme using BAPNA under identical conditions.

The effects of the anion series on estradiol-17β dehydrogenase purified from human placenta are shown in Table II. At pH 8.0, estradiol sulfate is anionic, the 3-methyl ether is uncharged, and estradiol-17β is predominantly uncharged as its pK is 9.3. The order of effectiveness of the anion series is similar in all cases and the degree of inhibition seen with estradiol 3-sulfate and estradiol 3-methyl ether are generally similar.

TABLE II: Inhibition of Estradiol-17β Dehydrogenase by Neutral Salts at pH 8.0.[a]

Salt and Concn (M)		Substrate and Act.		
		Estradiol-17β	Estradiol 3-Sulfate	Estradiol 3-Methyl Ether
NaAc	1.0	82	100	100
	2.0	45	71	57
NaCl	1.0	39	75	33
NaBr	1.0	24	32	20
$NaNO_3$	1.0	25	32	19
$NaClO_4$	0.5	18	10	21
NaSCN	0.5	18	10	18

[a] Assays initiated by addition of 0.108 mg of enzyme to a solution containing 1.0 μmole of $NADP^+$, 150 μmoles of Tris–hydrochloride buffer, pH 8.0, indicated substrate, and salts at molarities shown at 25° having a final volume of 3.0 ml. Activities expressed as per cent of control to which no salt was added. The quantity of enzyme used generated 30.0 mμmoles of NADPH/min with estradiol-17β, 13.7 mμmoles/min with estradiol 3-sulfate, and 40.5 mμmoles/min with estradiol 3-methyl ether as substrates in the absence of added salts.

Sulfhydryl Reaction Rates of Glutathione. Second-order plots for the reaction of 0.20 μmole of GSH with 0.20 μmole of DTNB are shown in Figure 1. In the absence of added salts (*i.e.*, control solution) k = 3.75 × 10^2 l./mole sec. Salts increase the reaction rate. Ratios of the second-order rate constants in the presence of 2.0 M salts to those in the control solution are: NaSCN, 1.8; $NaClO_4$, 2.2; $NaNO_3$, 2.9; NaAc and NaCl, 4.5. Rates in the control solution at pH 7–9.0 indicate that the anionic form of the sulfhydryl group (S^-) is reactive. To evaluate the possibility that the observed salt activation was due to changes in apparent pK of GSH sulfhydryl groups, titration using the method of Benesch and Benesch (1955) was carried out with and without 2.0 M $NaClO_4$. The pK in the control solution was 9.0, while in 2.0 M $NaClO_4$ the pK was 8.75. Similar studies at pH 7.0 revealed the number of total sulfhydryl groups of glutathione existing as S^- to be 1.2 and 2.8 times the control solution in 2.0 M NaCl and 2.0 M $NaClO_4$, respectively.

Sulfhydryl Reaction Rates of β-Amylase. In 3.0 ml of a solution of 0.835 saturated guanidine hydrochloride (recrystallized from aqueous alcohol before use) containing 10 μmoles of EDTA and 150 μmoles of Tris-maleate buffer, pH 7.0, 2.25 mg of β-amylase gave an OD increase (after addition of 3.15 μmoles of DTNB) equivalent to 0.20 μmole of GSH and the reaction was complete within 2 min. Salts and saturated urea did not make more SH groups accessible to DTNB than did guanidine. These data indicate the presence of 0.089 μmole of SH/mg of protein. This value agrees with the 0.08 μmole of SH/mg indicated by amino acid analysis (French, 1960) and the 0.084 μmole of SH/mg as determined with *p*-mercuribenzoate (Thoma *et al.*, 1965). While it is less than the SH content determined with iodoacetate by Englard *et al.* (1951), this difference probably reflects reagent specificity and conditions. EDTA was added to reduce instability of the colored reaction product. In its presence the color resulting from reaction of 0.20 μmole of GSH with 3.15 μmoles of DTNB was stable in the absence of salts and decreased in absorbancy <2%/20 min in all salts except NaBr and $NaNO_3$. The more rapid decrease in absorbancy in the presence of these salts precluded their use in study of reaction rates with the enzyme.

Reactions were then initiated by addition of 2.25 mg of β-amylase to 3.0 ml of solutions at pH 7.0 containing 3.15 μmoles of DTNB. This marked excess of reagent allowed data to be evaluated with pseudo-first-order plots as shown in Figure 2. It can be seen that 2.0 M sodium salts increase the reaction rate in an order of increasing effectiveness: $Ac^- < Cl^- < ClO_4^- < SCN^-$. The former two ions increase the rate approximately eightfold while ClO_4^- increases it 62-fold and SCN^- increases it 212-fold.

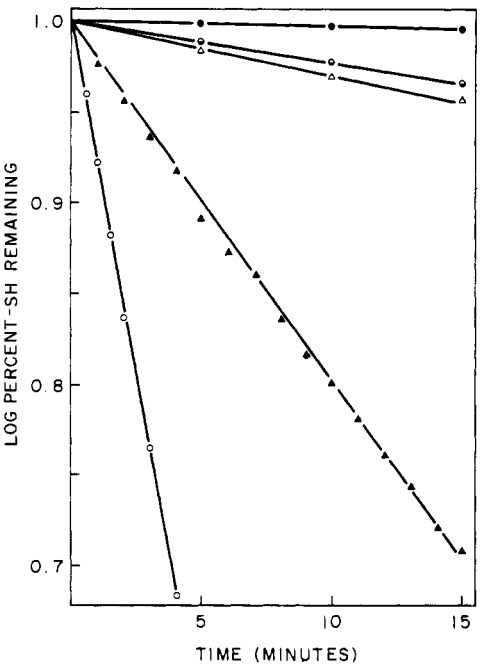

FIGURE 2: First-order plots for the reaction of 3.15 μmoles of DTNB with 2.25 mg of β-amylase in the presence and absence of 2.0 M salts. Reactions were carried out at 25° in 3.0-ml total volume of 0.05 M Tris-maleate buffer. The pH of each reaction was adjusted to 7.0 immediately prior to assay and checked on completion: ●–●, no salt; ○–○, NaSCN; ▲–▲, $NaClO_4$; Δ–Δ, NaCl; ◒–◒, NaAc. Points are averages of three determinations.

Discussion

It has been previously suggested (Warren *et al.*, 1966) that enzyme inhibition induced by neutral salts at high concentrations results from structural changes in the enzyme protein. Such structural changes could be mediated through changes in solvent structure or could result from direct effects on the protein molecule. Mediation of salt-induced changes in macromolecular structure *via* change in the organized structure of water has been suggested by Klotz (1965). Jencks (1965) has challenged this interpretation on the basis of insensitivity of protein disruption to the nature of the alkali cation (as compared, *e.g.*, with the halide anions). Robinson and Jencks (1965) have presented evidence that salt effects are due to a direct action on peptide and amide groups or possibly relocation of excluded ions at the polar–nonpolar surface which would account for cation insensitivity. Indeed, this same insensitivity to the nature of the alkali cation has been noted in studies on activity (Warren *et al.*, 1966). While cations can be ordered, differences are not of the magnitude seen with a series of anions, which accounts for the use of the anion series in this study. With β-amylase, *e.g.*, activity in the presence of 2.0 M KAc, KNO_3, and KSCN is not clearly different from that seen with the sodium salts.

Although Robinson and Jencks (1965) tended to exclude electrostatic effects, it is to be noted that the

order of anion effectiveness in structure disruption and enzyme inhibition generally parallels the order in which anions are bound by albumin (Bancroft, 1915) and myosin (Brahms and Brezner, 1961). Further, a similar order is found when dilute salt solutions inhibit certain enzymes (Massey, 1953; Fridovich, 1963) supposedly by binding to a charged group.

If activity is to be used as a parameter for evaluation of the anion series on enzyme structure, other factors not clearly related to structural change must be considered. Notable among these are shielding effects on the approach of a charged substrate to a charged site and alteration of the nature of the substrate (*i.e.*, association of ion and substrate or change in substrate pK) by ions present in solution. While the first of these might be expected to be a simple function of ionic strength (at least at low salt concentrations) and be overcome by substrate saturation, we thought to minimize both by using uncharged substrates.

The salt-induced inhibition of β-amylase, β-galactosidase, acylase, and estradiol-17β dehydrogenase using uncharged substrates is generally within the range of the charged substrate group. That of chymotrypsin, trypsin, and lipase using PNPA as substrate is considerably less, and activation is seen in several cases. PNPA may be the common denominator. In the presence of 2-propanol (which was routinely used in assays with PNPA as substrate) the rate of liberation of *p*-nitrophenol from PNPA, in the absence of enzyme, is increased by lysine methyl ester (Stewart and Ouellet, 1959) and even 2.0 M salts, demonstrating that this reaction requires little in terms of an organized active site. Possibly salts do disrupt the classic active sites of these enzymes but in doing so expose previously buried residues which activate liberation of *p*-nitrophenol in a nonspecific manner similar to that described above.

Alternatively, salt effects on trypsin, as exemplified by 2.0 M KCl, may result from summation of: (a) structural stabilization of the protein (Green and Neurath, 1953) and (b) some unique substrate-mediated inhibition (see above) observed when the substrate is charged. That KCl at this concentration could possibly order trypsin structure is suggested by its structure-enhancing effect on ribonuclease as determined by physical methods (von Hippel and Wong, 1964) and the increased activity coefficients of acetyltetraglycine ethyl ester still present at 2.0 M KCl concentration (Robinson and Jencks, 1965).

Actually, variables in substrate size and type of bond broken as well as possible pH effects, and variation in deacylation rate, which has been indicated to be rate controlling (Neurath and Schwert, 1950), preclude complete explanation at this time. Nevertheless, these observations, as do earlier ones with myosin (Warren *et al.*, 1966), indicate that while salt-induced structural changes in an enzyme are not necessarily equally reflected with all substrates, anionic order of effectiveness is the same whether substrate is charged or uncharged.

Although Tonomura *et al.* (1962) found demonstrable decreases in helical content and reduced viscosity of myosin at concentrations of KI and KSCN which almost completely inhibit ATPase activity, KCl and LiBr at concentrations which completely inhibited activity (3.0 and 2.0 M) caused no detectable change in reduced viscosity or helical content as evaluated by the $-b_0$ term of Moffitt and Yang (1956). It is, nevertheless, possible that structure changes of a degree too small to be detected did occur, and two observations indicate that this is the case. First, Tonomura *et al.* (1962) demonstrated that, when the reciprocal transition temperature ($1/T_{tr}$) is plotted *vs.* LiBr concentration, a straight line of positive slope is obtained which satisfies the Schellman equation (Schellman, 1955). Therefore, even minimal concentrations of LiBr would be expected to cause a decrease (even if not clearly measureable with available techniques) in T_{tr} which is generally accepted as a disruption of structure. Second, Kominz *et al.* (1965) using heavy meromyosin, the fragment with ATPase activity, were able to demonstrate, using the conformation-dependent Cotton effect (Simmons *et al.*, 1961), a decrease in helical content in the presence of 0.5 M KCl as compared to 0.05 M KCl, and these are ranges where activity remains. It appears that the larger light meromyosin fragment with its greater helical content obscures this finding when intact myosin is used. Thus, conformation changes probably do occur in the course of inhibition of myosin and their demonstration appears to be a matter of sensitivity.

In this investigation we hoped to evaluate the effects of the inhibition–disruption anion series on another enzyme by yet another parameter. While physical methods such as optical rotatory dispersion and viscosity are satisfactory to delineate changes in ordered structure, another reliable and powerful technique is amino acid modification (Yankeelov and Koshland, 1965). The increased reactivity of the SH groups of β-amylase in the presence of 2.0 M salts (and guanidine hydrochloride, a known structure-disrupting agent) could only result from either a change in their local environment or induced accessibility of buried groups. To evaluate the former factor, GSH was chosen as a model compound. Because of the rapidity of its reaction with DTNB, available instrumentation forced study at pH 7.0 rather than the classic 8.0 (Ellman, 1959). Salts did increase reaction rates up to 4.5 times that seen in their absence but these increases did not parallel effects on pK alone and their order of effectiveness: $SCN^- < ClO_4^- < NO_3^- < Cl^- \cong Ac^-$ is essentially opposite to that seen with β-amylase. As judged by salt effects on GSH, changes in local environment are not of a great enough magnitude or of appropriate order to account for salt-induced enhancement of the reaction rate of the SH groups of β-amylase.

It appears that the responsible mechanism is structure disruption of the protein with resulting accessibility of groups which in the absence of salts are buried and hence nonreactive. Thoma *et al.* (1965) have presented evidence that the reactive SH groups of β-amylase are near the active site. Further, Banaszak *et al.* (1963) have correlated residue reactivity with exposure as determined by three-dimensional crystalline structure of sperm whale metmyoglobin. Thus with another parame-

ter, the inhibition of enzyme activity by neutral salts is associated with and probably mediated by a disruption of enzyme structure demonstrable at salt concentrations where partial activity remains.

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