Elastase. II. Optical Properties and the Effects of Sodium Dodecyl Sulfate*

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ABSTRACT: The optical properties of elastase were determined with ultraviolet, infrared, circular dichroism, and optical rotatory dispersion spectroscopic techniques. The enzyme has little, if any, periodic structure. The circular dichroism spectrum, for example, has a major, negative extremum at 198 nm ($[\theta] = -9900$ deg cm^2 dmole^{-1}) which is representative of unordered polypeptides or proteins with aperiodic structures. A reversible inhibitor of elastase such as sodium fluoride does not cause any changes in the circular dichroism or optical rotatory dispersion spectrum of elastase. When the inhibitor dinitrophenylbromobutanone reacts irreversibly with elastase to form a covalent complex, the rotational strengths of the optically active transitions are reduced but their frequencies remain the same as in native elastase. It was observed that treatment of elastase and other enzymes such as trypsin, chymotrypsin, and pepsin with the anionic detergent, sodium dodecyl sulfate, caused a substantial change in their circular dichroism spectra, attributable to an apparent increased amount of α helix in their structures (negative ellipticity extrema at 207 ± 2 nm, and 218 ± 2 nm). Subsequent removal of the detergent by exhaustive dialysis led to a further change in the circular dichroism spectra of these enzymes (and also of that of lysozyme), which suggests the presence of an appreciable amount of β structure. The conclusions based on circular dichroism spectra are supported by changes in the infrared amide I frequencies of films of the proteins. These results indicate that great caution must be exercised in the interpretation of protein conformations in the presence of detergents or after detergents have been employed in extraction procedures.

One of the first observations on the ability of a pancreas extract to dissolve elastic tissue was made in 1890 by Ewald. Only in relatively recent times has an interest been rekindled in this proteolytic enzyme, chiefly as a result of its purification by Balo and Banga (1950) and Lewis *et al.* (1956). A major impetus was provided by the studies of Hartley and his coworkers at Cambridge which revealed a remarkable similarity in amino acid composition and sequence between elastase and two other proteolytic enzymes of the pancreas, chymotrypsin and trypsin (Brown *et al.*, 1967). This finding later led to the postulation by Bender and Marshall (1968) that the mechanism of the catalytic action of elastase is similar to that of chymotrypsin, involving the same amino acid residues (histidine, serine) found in homologous positions along the sequences of the two enzymes.

Progress in the investigations by the Cambridge group has been so rapid during the last few years that the crystal structure of elastase and the complete amino acid sequence were deduced almost simultaneously (Shotton and Hartley, 1970; Shotton and Watson, 1970). Very little was known or published about the solution properties of elastase when these significant advances were made. In this paper we report on the optical properties of elastase as determined by circular dichroism, optical rotatory dispersion, ultraviolet, and infrared spectroscopy.

Experimental Section

Materials. Sodium dodecyl sulfate was purchased from Fisher Scientific Company. Bovine pepsin (twice crystallized), trypsin (twice crystallized), chmyotrypsin (three-times crystallized), and ribonuclease A (three-times crystallized) were supplied by Worthington Biochemicals Corporation, hen egg-white lysozyme (three-times crystallized) by Sigma Chemical Corporation, and bovine hemoglobin (A.R.) by Pentex Incorporated. Guanidine hydrochloride (Eastman Organic Chemicals) was crystallized from methanol–benzene and then crystallized twice from methanol (Nozaki and Tanford, 1967). Guanidine sulfate (Eastman Organic Chemicals) was recrystallized from 50% methanol.

Methods. Circular dichroism and optical rotatory dispersion measurements were obtained with a Cary 60 recording spectropolarimeter fitted with a Model 6001 circular dichroism attachment, and with a Jasco ORD/UV/CD-5 recording spectropolarimeter fitted with attachments for temperature-controlled runs. Infrared spectra were run on a Perkin-Elmer Model 521 recording infrared spectrometer. Ultraviolet spectra were obtained from a Cary 15 double-beam recording spectrophotometer.

In the calculation of mean residue ellipticity and rotation values, the following mean residue weights were employed: elastase, 108; trypsin, 107; chymotrypsin, 104; pepsin, 103; lysozyme, 112; ribonuclease, 110; hemoglobin, 118. Protein concentrations varied from 0.01 to 0.1%. In the experiments with sodium dodecyl sulfate, solid detergent was added to protein solutions to give a concentration of 0.2–2% sodium dodecyl sulfate, and spectra were determined after the solution stood at least 1 hr at room temperature. Dialysis was performed at room temperature (24 hr, 3 changes of 2 l. of water) since a precipitate forms at 3° in the presence of detergent. The solutions for experiments with the various salts were prepared in analogous fashion. Temperature effects were studied in jacketed cells through which an ethylene glycol–water mixture was circulated from a thermostatically controlled bath. Temperatures were measured inside the cells with a thermistor probe.

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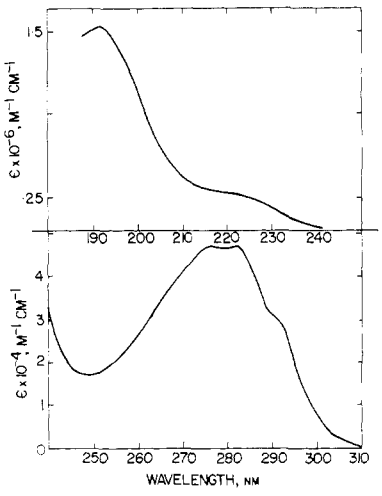

FIGURE 1: The ultraviolet absorption spectra of elastase in aqueous solution.

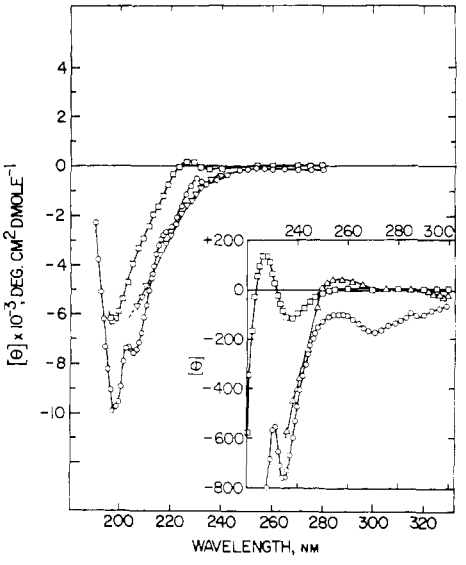

FIGURE 3: The effect of temperature on the circular dichroism spectrum of elastase. The spectra at 25° (○—○) and 77° (△—△) were run on a Jasco instrument after 15-min temperature equilibration. The cooled spectrum (□—□) was obtained on a Cary 60 instrument after overnight cooling; elastase concentration, 0.01%.

The films for infrared determinations were prepared by draining a droplet of protein solution onto a silver chloride disk and evaporating *in vacuo* over calcium chloride at room temperature. The process was repeated, if necessary, until enough protein was deposited to give satisfactory absorption heights on the direct scale of the infrared spectrometer.

Spectral perturbation studies using glycerol were carried out on $\sim 10^{-5}$ M solutions according to the procedure of Laskowski and his coworkers (1965) in order to determine the number of exposed tyrosine and tryptophan residues in elastase.

Results and Discussion

Ultraviolet Absorption. The ultraviolet spectrum in Figure 1 of elastase purified by DEAE- and CM-cellulose column chromatography (Gertler and Hofmann, 1967) shows the typical absorption maxima near 290, 280, 220, and 190 nm expected of tryptophan- and tyrosine-containing proteins (Wetlaufer, 1962). The absorption values at the 190-, 220-, 276-, 282-, and 290-nm maxima relative to that at the 250-nm trough are 90.8, 16.2, 2.66, 2.68, and 1.74, respectively. A characteristic feature of the elastase spectrum is the essentially equal magnitudes of the extinction coefficients for the 276-nm and 282-nm maxima. Less pure elastase preparations have characteristically lower A_{282}/A_{250} ratios. The extinction coefficient at 282 nm is 4.7×10^4 M^{-1} cm^{-1} for chromatographically purified elastase and 4.2×10^4 M^{-1} cm^{-1} for a commercial electrophoretically purified preparation (Worthington ESFF71A; no special drying precautions were taken during weighing). This compares to reported extinction coefficients for elastase of 4.75×10^4 (Naughton and Sanger, 1961), 4.85×10^4 (Kaplan and Dugas, 1969), and 5.5×10^4 M^{-1} cm^{-1} (Wasi and Hofmann, 1968). A value of 5.29×10^4 M^{-1} cm^{-1} is calculated from the tryptophan, tyrosine, and disulfide content of elastase using extinction coefficients of 5550, 1340, and 150 M^{-1} cm^{-1} for *N*-acetyl-L-tryptophan ethyl ester, *N*-acetyl-L-tyrosine ethyl ester, and the disulfide bonds, respectively (Wetlaufer, 1962). Little or no hypochromicity is apparent from the observed mean residue extinction coefficient of 6.7×10^3 M^{-1} cm^{-1} (*i.e.*, $(1.6 \times 10^6)/240$) at 190 nm; it does not appear that a large contribution to the overall enzyme conformation is made by an α-helical structure since an ϵ_{190} value of about $3\text{–}4 \times 10^3$ M^{-1} cm^{-1} (Rosenheck and Doty, 1961) would then be expected. Amino acid side-chain chromophores that absorb near 200 nm (mainly phenylalanine) could obscure a hypochromic effect, but because elastase has only 3 phenylalanines out of a total of about 240 residues, its far-ultraviolet spectrum probably represents mainly absorption by peptide bonds.

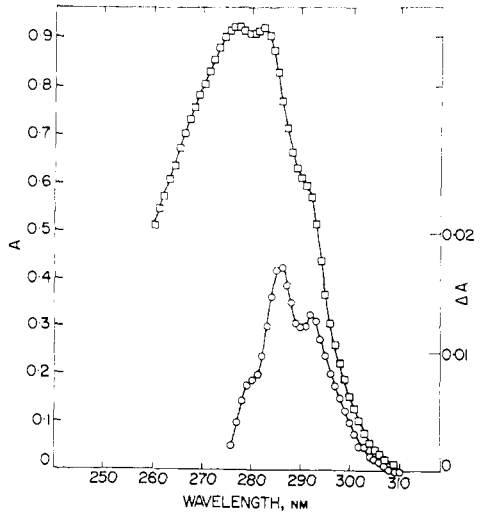

FIGURE 2: The ultraviolet absorption spectra of elastase in 20% glycerol, □—□, the absorption difference (solvent perturbation) spectrum of elastase (2×10^{-5} M), ○—○, caused by the presence of 20% glycerol (v/v) was determined using four cylindrical, optically matched 1-cm path-length cells.

The solvent perturbation difference spectrum of elastase produced by 20% glycerol (Figure 2) allows the calculation (Herskovitz and Sorensen, 1968) of values of 0.33 and 0.45 for the apparent exposed fraction of tryptophans and tyrosines in this solvent system, respectively. Of the 7 tryptophan and 10 tyrosine residues in elastase, about 2 tryptophans and 4–5 tyrosines are therefore on the average sufficiently exposed to experience spectral perturbations by glycerol.

TABLE I: Effect of Sodium Dodecyl Sulfate on the Mean Residue Ellipticity of Elastase and Other Proteins.

Protein	Mean Residue Ellipticity	
	Position (nm) of max, min	$[\theta] \times 10^{-3}$ deg cm^2 dmole^{-1}
Elastase, native[a]	289; 270; 234 219; 205; 198; 187	−0.112; −0.173; −0.762; −2.7; −7.6; −9.9; 0
Elastase + sodium dodecyl sulfate	220; 206; 198; 190	−9.2; −14.45; 0; +11.25
Elastase, sodium dodecyl sulfate, dialyzed	216; 202; 191	−7.49; 0; +11.92
Chymotrypsin, native	230; 201; 190	−3.15; −7.86; 0
Chymotrypsin + sodium dodecyl sulfate	221; 206; 196	−6.22; −10.81; 0
Chymotrypsin, sodium dodecyl sulfate, dialyzed	216; 201; 192	−6.84; 0; +9.22
Trypsin, native	218; 206; 193	−3.6; −5.1; −5.8
Trypsin + sodium dodecyl sulfate	218; 207; 197	−7.64; −10.08; 0
Trypsin, sodium dodecyl sulfate, dialyzed	220; 203; 195; 191	−6.7; 0; +8.57; +9.3
Pepsin, native[a]	212; 201; 195	−6.6; 0; +18.29
Pepsin + sodium dodecyl sulfate	219; 207; 199; 191	−7.72; −9.35; 0; +10.37
Pepsin, sodium dodecyl sulfate, dialyzed	217; 205; 195	−6.4; 0; +10.36
Lysozyme, native[a]	227; 207; 200; 193	−7.43; −11.42: 0; +11.75
Lysozyme + sodium dodecyl sulfate	222; 206; 200; 191	−8.32; −12.2; 0; +15.08
Lysozyme, sodium dodecyl sulfate, dialyzed	217; 202; 194	−9.98; 0; +17.46
Ribonuclease, native[a]	215; 210; 199; 193	−9.49; −10.2; 0; +4.22
Ribonuclease + sodium dodecyl sulfate	222; 215; 205; 195	−6.03; −6.71; −10.46; 0
Ribonuclease, sodium dodecyl sulfate, dialyzed[b]	231; 219; 206	−2.15; 0; +2.88
Methemoglobin, native	221; 209; 202; 194	−17.53; −16.24; 0; +35.93
Methemoglobin + sodium dodecyl sulfate	220; 207; 201; 192	−14.1; −17.2; 0; +13.9 (?)
Methemoglobin, sodium dodecyl sulfate, dialyzed	220; 208; 202; 193	−14.89; −15.06; 0; +33.28
Phosvitin, pH 1.8[c]	215; 206; 194	−11.8; 0; +14.6
Lactoglobulin (40% β)[d]	216; 204; 193	−6.0; 0; +9.2
Lactoglobulin (random chain)	204;	−11.8
Lactoglobulin (70%, α helix)	220; 208; 205; 194	−22.0; −21.0; 0; +40.0
α Helix (poly-L-lysine)[e]	222; 207; 190; −192	−30.4; −28.5; +55.0
Unordered (poly-L-lysine)[d]	238; 217; 202	−0.2; +2.4; −26.0
Antiparallel β-(poly-L-lysine)[f]	217; 197; 190	−8.7; +29.6; +15.6
Antiparallel β-(poly-L-lysine)[g]	216	−6.3
Antiparallel β-(poly-L-lysine)[h]	217	−14.3

[a] Jirgensons (1970). [b] Solution became turbid during dialysis. [c] Taborsky (1968). [d] Timasheff *et al.* (1967a,b). [e] Holzwarth and Doty (1965). [f] In sodium dodecyl sulfate; Holzwarth and Doty (1965). [g] In H_2O; Li and Spector (1969). [h] In H_2O; Sarkar and Doty (1966).

Circular Dichroism. The circular dichroism spectrum of elastase below 310 nm (Figure 3) consists of 6 negative extrema and shoulders with the positions and ellipticities shown in Table I. Resolution of the region below 250 nm into component Gaussian curves gives 3 minor and 1 major negative bands centered at about 233, 224, 208 (minor components), and 200 nm (major component). These peak positions may be related, respectively, to far-ultraviolet aromatic side-chain absorptions near 230 nm (Beychok and Fasman, 1964), to transitions of the peptide bond near 220 nm and 210 nm if a small amount of α helix (Holzwarth and Doty, 1965) is present in elastase, and to the major 200 nm to peptide-bond absorption (Timasheff *et al.*, 1967a,b).

The inset in Figure 3 of the near-ultraviolet region more clearly illustrates the negative 272-nm ellipticity minimum and suggests the presence of a second (in the 285- to 295-nm region) optically active aromatic transition. The former is probably caused by tyrosine side chains (Simmons and Glazer, 1967) together with some contribution from disulfide bonds (Coleman and Blout, 1967) and the weak shoulder near 280 nm may be due to tryptophan transitions (Beychok, 1967).

Effect of Temperature. Heating elastase to 77° causes a loss of both the 230-nm shoulder and the extremum at 272 nm in the circular dichroism spectrum. There is also an indication (Figure 3) of a small positive extremum near 250 nm. Subsequent cooling (24 hr, 4°) of a solution heated for 1 hr at 77° does not reestablish the original circular dichroic characteristics (Figure 3). Instead, the aromatic region (270–290 nm) is devoid of any circular dichroism bands, the ellipticity of the 235-nm transition is reduced by about 85% to −115 deg cm^2 dmole^{-1} and a small positive extremum ($[\theta]$ +137 deg cm^2 dmole^{-1}) centered at 227 nm appears. Therefore, it seems likely that heat treatment followed by cooling leaves elastase in a random chain form, as the latter parameters correlate qualitatively with those observed for randomly coiled polyglutamic acid (Yang, 1967a,b), polylysine (Timasheff *et al.*, 1967a,b); and the highly charged protein, phosvitin (see Table I).

If the ellipticity at 272 nm is determined as a function of temperature over the range of 25–80°, the profile in Figure 4 gives a midpoint "melting" temperature (T_m) of 44°; no further change occurs above 60°. Thermally denatured, cooled elastase is completely inactive toward the ester substrate *p*-nitrophenyl Boc-L-alaninate.

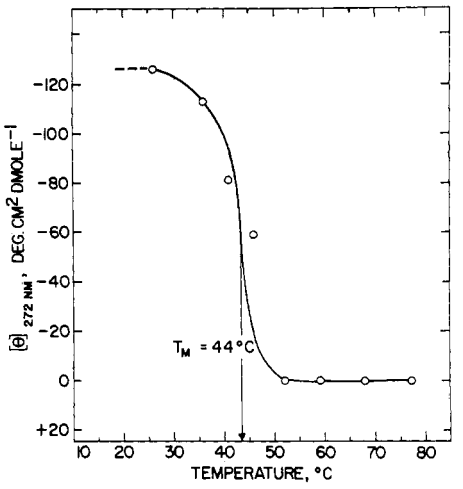

FIGURE 4: The effect of temperature on the ellipticity of elastase at 272 nm; elastase concentration, 0.1%, 1-cm cell.

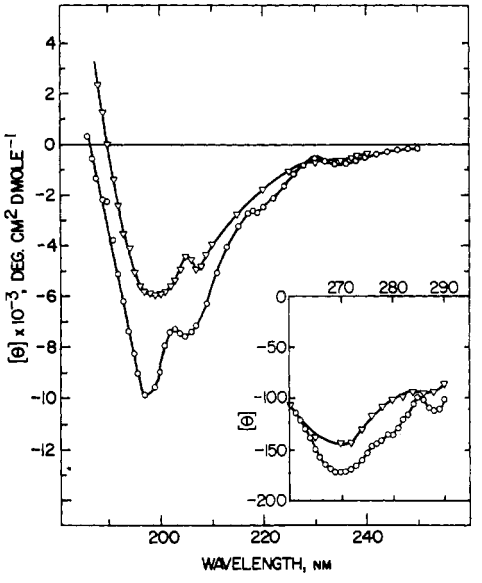

FIGURE 5: Circular dichroism spectra of elastase and DPB-elastase. The concentration of DPB-elastase was calculated from $A_{282\ nm}$ and the optical factor of 0.454 for elastase multiplied by 0.845, the latter to correct for DPB absorption; protein concentrations, 0.07% (in water).

Effect of Inhibitors. Elastase is known to be reversibly inhibited by sodium chloride (Lewis *et al.*, 1956). Sodium fluoride, a salt more suitable for circular dichroism investigation because of its higher transparency in the ultraviolet, was found to have a similar effect, *e.g.*, a concentration of 0.2 M NaF in an assay solution at pH 6.7 inhibits the hydrolysis of NBA[1] by 57%. Dinitrophenylbromobutanone on the other hand is an irreversible inhibitor that reacts covalently with elastase (Visser and Blout, 1969). The circular dichroism pattern in the presence of NaF is virtually identical with that for an aqueous elastase solution lacking NaF; the quite marked inhibitory effect of inorganic salts on elastase activity is thus not reflected in changes of the enzyme's dichroic

[1] The abbreviations used are: NBA, *p*-nitrophenyl *tert*-Boc-L-alaninate; DPBB, 1-bromo-4-(2,4-dinitrophenyl)butan-2-on; DPB-elastase, dinitrophenylbutanonyl-elastase; Gdn·HCl, guanidine hydrochloride.

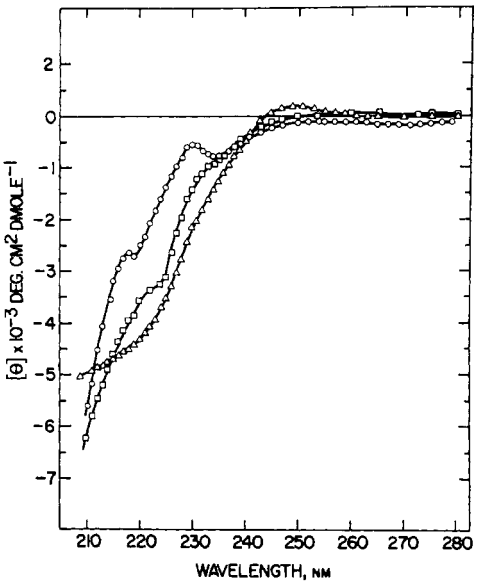

FIGURE 6: The effect of pH on the circular dichroism of elastase. The spectra were obtained 1 hr after preparation of the solutions. pH 7, 0.025% elastase (O—O). pH 2.5, 0.025% elastase in 0.05 M citrate (□—□). pH 13, 0.025% elastase in 0.1 N NaOH (Δ—Δ).

properties. In contradistinction is the reduced ellipticity of dinitrophenylbutanonyl-elastase (Figure 5). At both the 205–207-nm shoulder and the 197–199-nm extremum the values are only about 65% of native elastase (−4900 *vs.* −7600, and −6700 *vs.* −10,000 deg cm² dmole⁻¹, respectively). The wavelength positions of the inflection and extremum, however, are the same for inhibited and native elastase.

Effect of pH. Elastase is unusually stable over the wide range of pH 4–12 (Lewis *et al.*, 1956). Below pH 4 a conformational change, apparently caused by the protonation of carboxyls, has been observed by fluorescence measurements (Wasi and Hofmann, 1968). The circular dichroism spectrum of the enzyme in citrate, pH 2.45, also differs considerably from that at neutral pH (Figure 6). The major negative extremum at pH 2.45 (not shown in the figure) occurs at 205 nm (*vs.* 198 nm at neutral pH). No 230-nm shoulder is observed but a slight plateau appears around 222 nm and the region above 250 nm has no circular dichroism, *i.e.*, the native enzyme's low-intensity bands disappear at pH 2.45. Elastase has no enzymic activity at this low pH, but upon assaying a preparation kept for 3.5 hr at pH 2.45, about 70% of the esterase activity toward NBA is regained at pH 6.5; thus, it seems that the conformational change caused by acid is apparently partially reversible.

Even more drastic changes in the circular dichroism characteristics are observed above pH 12 (Figure 6). Not only are the negative ellipticity bands in the aromatic region absent in 0.1 N NaOH, but a new small positive one appears around 250 nm ([θ] +200 deg cm² dmole⁻¹). The ellipticity at shorter wavelengths rapidly becomes more negative until the high noise level due to ultraviolet absorption by the hydroxide ions prevents further measurements. At this high pH the enzyme is completely and irreversibly inactivated, since no hydrolysis of NBA occurs when the enzyme solution is brought to pH 6.5.

Optical Rotatory Dispersion. The observed optical rotatory dispersion spectrum of elastase agrees satisfactorily with one calculated from circular dichroism data with the aid of the Kronig–Kramers transform, and a computer program

TABLE II: Effect of Sodium Dodecyl Sulfate on the Mean Residue Rotation of Elastase.

Protein	Mean Residue Rotation	
	Position (nm)	$[m] \times 10^{-3}$ deg cm^2 dmole^{-1}
Elastase, native	235; 216; 206; 193	−2.44; −3.82; 0; +15.4
Elastase + sodium dodecyl sulfate	232; 218; 213; 198	−5.29; 0; +1.37; +20.65
Elastase, sodium dodecyl sulfate, dialyzed	229; 221; 211; 191	−2.62 0; +12.43; 0
Phosvitin, pH 1.8[a]	230; 220; 203	−3.9; 0; +19.5
Phosvitin, pH 7.9	206; 196	−9.9; 0
α Helix (poly-L-lysine)[b]	232; 222; 198	−18.2; 0; +73.0
Unordered (polyglutamic acid)[c]	238; 228; 205; 197; 189	—; +; −15.0; 0; −17.0
β Form (silk fibroin)[c]	229; 220; 205; 190	−5.0; 0; +24.0; −17.0
β Form (poly-L-lysine)[b]	230; 220; 205	−6.3; 0; +23.0

[a] Taborsky (1968). [b] In H_2O; Sarkar and Doty (1966). [c] Yang (1967a,b).

written for integration by summation of areas for 1-nm intervals according to Simpson's parabolic rule. Like the circular dichroism results, the optical rotatory dispersion data for native elastase indicate the absence of any substantial amount of α-helical structure, for which a much deeper trough at 233 nm would be expected (Table II).

Effect of Sodium Dodecyl Sulfate. Attempts to bring about the unfolding of elastase without resorting to the use of heat denaturation or a reagent, such as guanidine chloride, with undesirable absorption characteristics led to an investigation of the effect of sodium dodecyl sulfate on the optical properties of elastase. The circular dichroism and optical rotatory dispersion patterns are shown in Figures 7 and 8, respectively. These data reveal that the general form of the optical activity spectra for native elastase is drastically changed by the addition of an anionic detergent. A more detailed examination shows that in the presence of sodium dodecyl sulfate a positive ellipticity maximum at 190 nm, a 199-nm crossover, a negative extremum at 206 nm, and a pronounced shoulder at 220 nm occur in the circular dichroism spectrum. Qualitatively, the wavelength frequencies of these transitions in the presence of sodium dodecyl sulfate correspond with those in circular dichroism spectra of some synthetic polypeptides (Sarkar and Doty, 1966) and the protein β-lactoglobulin A in acidic methanol (Timasheff *et al.*, 1967a,b) having large amounts of α helix. The corresponding optical rotatory dispersion spectrum (Figure 8) with a 233-nm trough and a positive shoulder near 212 nm also suggests that some α-helical conformation is present in sodium dodecyl sulfate treated elastase. Comparison of the magnitudes of the ellipticities and rotations of elastase in sodium dodecyl sulfate with the literature values for fully helical

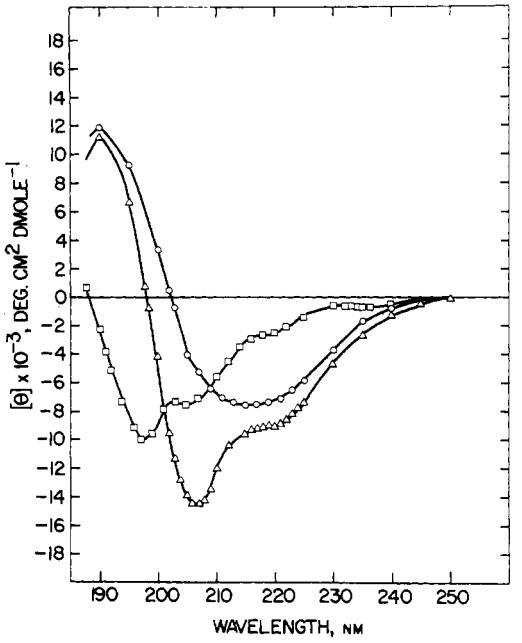

FIGURE 7: The effect of sodium dodecyl sulfate treatment and subsequent dialysis on the circular dichroism spectrum of elastase. Solid sodium dodecyl sulfate was added to 0.025% elastase and then circular dichroism spectra were obtained after 1 hr. Similar results were obtained with 0.2% and 2% sodium dodecyl sulfate: elastase (□—□) plus sodium dodecyl sulfate (△—△) and subsequent dialysis (○—○).

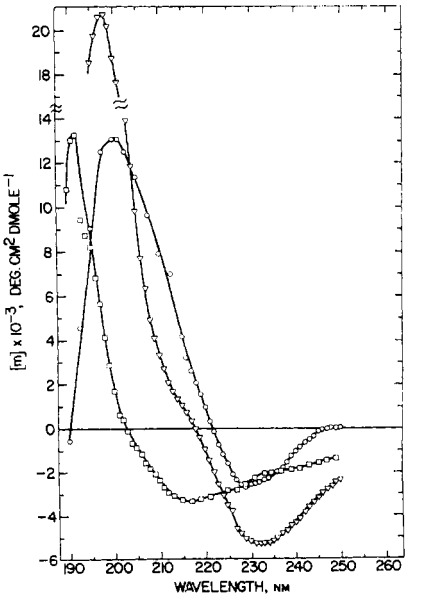

FIGURE 8: The effect of sodium dodecyl sulfate treatment and subsequent dialysis on the optical rotatory dispersion of elastase. Conditions were the same as in Figure 7: elastase (□—□) plus sodium dodecyl sulfate (△—△) and subsequent dialysis (○—○).

TABLE III: Effect of Sodium Dodecyl Sulfate on the Amide Infrared Frequencies of Elastase and Other Proteins.

Protein	Amide I (cm^{-1})[a]	Amide II (cm^{-1})[a]
Elastase, native	1635	1515
Elastase + sodium dodecyl sulfate	1650	1525
Elastase, sodium dodecyl sulfate, dialyzed	1622	1512
Chymotrypsin, native	1635 (1637)	1520
Chymotrypsin + sodium dodecyl sulfate	1657	1540
Chymotrypsin, sodium dodecyl sulfate, dialyzed	1627	1527
Trypsin, native	1635	1535, 1517
Trypsin + sodium dodecyl sulfate	1655	1530
Trypsin, sodium dodecyl sulfate, dialyzed	1650, 1625	1530
Pepsin, native	1640	1533
Pepsin + sodium dodecyl sulfate	1655	1530
Pepsin, sodium dodecyl sulfate, dialyzed	1627	1525
Lysozyme, native	1655 (1650)	1567
Lysoyzme + sodium dodecyl sulfate	1655	1575
Lysozyme, sodium dodecyl sulfate, dialyzed	1655, 1637	1533
Ribonuclease, native	1640 (1640)	1565
Ribonuclease + sodium dodecyl sulfate	1650	1570
Ribonuclease, sodium dodecyl sulfate dialyzed	1650, 1625	1520
Methemoglobin, native	1655	1540
Methemoglobin + sodium dodecyl sulfate	1655	1540
Methemoglobin, sodium dodecyl sulfate, dialyzed	1655	1540
β-Lactoglobulin A, native[b]	(1632)	
β-Lactoglobulin A + methanol	(1649)	
β-Lactoglobulin A + alkali	(1656)	
Bovine carbonic anhydrase, native[b]	(1637)	
Bovine carbonic anhydrase, pH 1.8	(1646)	
Insulin, fresh, pD 2.4[b]	(1654)	
Insulin, pD 12.5	(1644)	
Insulin, pD 2.4, heated	(1633, 1658)	
Mitochondrial structural protein[c]	(1625)	
α Helix (poly-γ-methylglutamate)[d]	(1650)	(1545)
Unordered polyserine[d]	(1656)	(1535)
Antiparallel β form (polyglycine I)[d]	(1632)	(1540, 1550)
Antiparallel β form (polylysine)[e]	(1625)	

[a] Values in parentheses are from the literature. [b] Timasheff *et al.* (1967a,b). [c] Wallach *et al.* (1969). [d] Miyazawa (1967). [e] Timasheff *et al.* (1967a,b).

structures (Table I) appears to indicate that the enzyme has taken up between 30 and 40% of a helical structure.

In a continuation of these studies sodium dodecyl sulfate containing elastase solutions were dialyzed extensively against distilled water at room temperature and the optical properties reinvestigated. The data for the dialyzed solution in Figures 7 and 8 clearly are not consonant with the expected circular dichroism and optical rotatory dispersion patterns of random chain polypeptides. Rather, the observed crossover at 202 nm, together with the almost symmetrical negative ellipticity band centered around 216 nm in the circular dichroism spectrum, suggests that a substantial contribution to the optical properties is made by a structure related to the β conformation observed in synthetic polypeptides like poly-L-lysine (Sarkar and Doty, 1966) or poly-*O*-acetyl-L-serine (Stevens *et al.*, 1968) and also seen in two soluble proteins, β-lactoglobulin A (131) (Visser, 1969) and the enzyme carbonic anhydrase (Timasheff *et al.*, 1967a,b). The circular dichroism results after dialysis are corroborated by the corresponding optical rotatory dispersion data (shallow trough at 229 nm, crossover at 221 nm, peak at 201 nm) in Figure 8, which also agree qualitatively with the presence of a β-type structure (see Table II). We have calculated the β content, based on the best available information (Townend *et al.*, 1966; Yang, 1967a,b), to be at least 50%.

Assays with the ester substrate NBA indicated that elastase is irreversibly inactivated by sodium dodecyl sulfate, even after its apparent removal by dialysis.

Infrared Absorption. It has been convincingly demonstrated that conclusions about the solution structure of proteins based on circular dichroism and/or optical rotatory dispersion measurements can be put on a much firmer footing if substantiated by results from infrared spectroscopy (Timasheff *et al.*, 1967a,b). The latter technique records vibrational instead of electronic transitions involved in circular dichroism and optical rotatory dispersion. Thus, if compatible interpretations follow from the two techniques with their different theoretical bases, structure assignments can be made with greater confidence. The vibrational frequencies of the C=O (amide I, carbonyl stretching vibration) and NH (amide II,

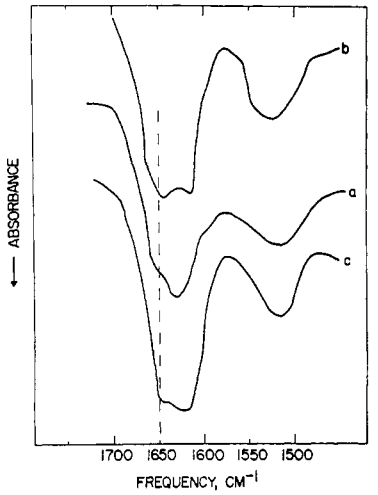

FIGURE 9: The effect of sodium dodecyl sulfate treatment and subsequent dialysis on the infrared spectrum of elastase. Treated elastase solutions (0.025) were evaporated on silver chloride disks and dried *in vacuo*. The sodium dodecyl sulfate concentration was 0.2 to 2%: (a) native elastase; (b) elastase treated with sodium dodecyl sulfate; (c) elastase treated with sodium dodecyl sulfate and subsequently dialyzed.

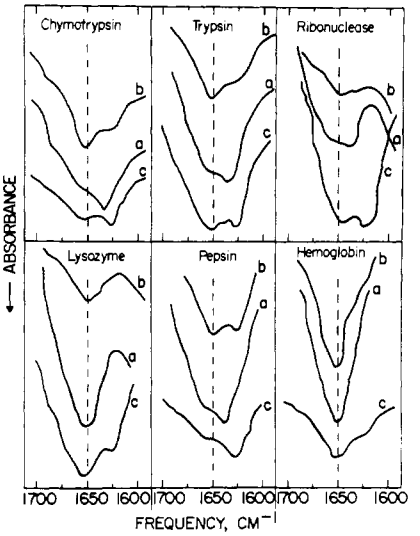

FIGURE 10: The effect of sodium dodecyl sulfate treatment and subsequent dialysis on the infrared spectra of several proteins. The protein solutions were evaporated on silver chloride disks *in vacuo*.

mixture of NH de-formation and CN stretching frequencies) moieties of the peptide-bond CONH are of major interest in polypeptide and protein infrared spectra. Previous workers have verified that the amide absorption frequencies of some polypeptides and proteins in aqueous solution agree closely with those obtained from films; the results for dry films of elastase, elastase in the presence of sodium dodecyl sulfate, and elastase to which sodium dodecyl sulfate was added and then dialyzed away are presented in Figure 9 and Table III.

The major amide I band at 1635 cm^{-1} in films of native elastase is largely shifted to 1650 cm^{-1} in the presence of sodium dodecyl sulfate and to 1622–1625 cm^{-1} after sodium dodecyl sulfate has been removed by dialysis. The corresponding amide II (NH) frequencies are 1515, 1525, and 1512 cm^{-1}. A weak shoulder near 1680 cm^{-1} was present in many films of sodium dodecyl sulfate treated, dialyzed preparations. From the literature data on amide I frequencies of polypeptides and proteins known to contain α-helical or β structure (Table III), it appears the frequency shifts observed with elastase suggest some α helix and/or random coil in the presence of sodium dodecyl sulfate (1650 cm^{-1}). On the other hand, a type of β structure (1622–1632 cm^{-1}) is indicated to be present in elastase after removal of the detergent; this structure may be related to the antiparallel pleated sheet with its characteristic 1680-cm^{-1} shoulder (Miyazawa and Blout, 1961; Timasheff *et al.*, 1967a,b).

Effect of Detergent on Other Proteins. Elastase is very closely related to chymotrypsin and trypsin. Hence it was of interest to determine what the effect of sodium dodecyl sulfate would be on the solution properties of the latter two proteases. This investigation was extended to include lysozyme, ribonuclease, pepsin, and hemoglobin in order to establish whether the phenomenon is a general one. Tables I and III contain the magnitudes and frequencies of the major transitions observed by circular dichroism and infrared spectroscopy, and Figure 10 shows the amide I region of the infrared spectra. The most significant feature of the circular dichroism data in Table I is the fact that chymotrypsin, trypsin, pepsin, and lysozyme assume conformations after sodium dodecyl sulfate is dialyzed away that are characterized in all four enzymes by the appearance of a single, new circular dichroism minimum near 217 nm which is absent or concealed in their native spectra. In this respect their behavior is similar to that of elastase (*vide supra*). Comparison of the circular dichroism spectra of chymotrypsin in Figure 11 to those of elastase in Figure 7 provides a good illustration of the parallel nature of the detergent-induced transformations in the two enzymes. In the presence of sodium dodecyl sulfate, pepsin, trypsin, elastase, and chymotrypsin all apparently assume a more α-helical structure as judged from the emergence of two prominent circular dichroism minima near 207 nm and 220 nm, but the lysozyme circular dichroism spectrum in sodium dodecyl sulfate (Table I) is not much different

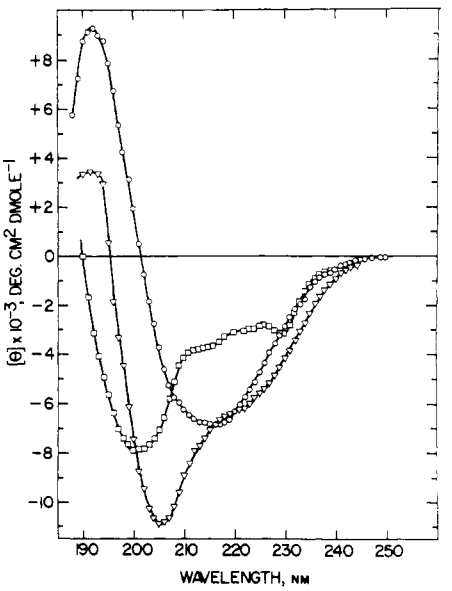

FIGURE 11: The effect of sodium dodecyl sulfate treatment and subsequent dialysis on the circular dichroism spectrum of chymotrypsin: chymotrypsin concentration = 0.05%; sodium dodecyl sulfate concentration = 1.1%; chymotrypsin (□—□) plus sodium dodecyl sulfate (△—△) and subsequent dialysis (○—○).

from the native form. It should be noted that native lysozyme has a relatively high α-helix content—about 30%—as deduced from optical rotatory dispersion calculations (Yang, 1967a,b) and crystallographic observations (Blake *et al.*, 1967). Also, native hemoglobin, which has an even higher α-helical content of ~70% (Beychok, 1967), shows very little change in the rotatory strength of its $n \rightarrow \pi^*$ (221 nm) and $\pi \rightarrow \pi^*$ (208 nm) transitions in the presence of 1% sodium dodecyl sulfate, or after the detergent has been dialyzed away (Table I).

An interesting feature of the circular dichroism spectrum of trypsin after sodium dodecyl sulfate has been dialyzed away is that it contains two apparent positive maxima below 200 nm, *viz.*, at 195 and 190 nm (Table I). This observation may be an example of the splitting of the $\pi \rightarrow \pi^*$ transition of an antiparallel β-pleated-sheet structure into 2 components near 195 nm as predicted by Pysh (1966) on theoretical grounds.

Ribonuclease behaved differently from the other enzymes. During dialysis to remove sodium dodecyl sulfate the protein solution became increasingly turbid; the circular dichroism data obtained for this (aggregated?) solution in Table I do not appear to agree with a transition to a β-type structure similar to that of the other proteins; the amide I infrared frequency at 1625 cm^{-1} of a film prepared from the turbid solution is, on the other hand, not incompatible with such a transition.

The infrared data for the proteins other than hemoglobin are qualitatively similar, and typically involve the appearance of a new peak or enhancement of a shoulder near 1650 cm^{-1} in the presence of detergent; after dialysis a new peak or an intensified existing peak or shoulder is found in the region 1625 $\pm$ 3 cm^{-1}, often accompanied by the emergence of a diffuse shoulder in the vicinity of 1680 cm^{-1}. These changes are most apparent in the infrared spectra of chymotrypsin (Figure 10). It should be noted also in Figure 10 that the presence of sodium dodecyl sulfate does not alter the position of the amide I band of hemoglobin and lysozyme, the only two proteins investigated with high α-helix content in their native state (*cf.* also their related circular dichroism behavior above).

All the enzymes were inactive after the detergent treatment and subsequent dialysis, except for ribonuclease which regained most of its activity during an assay based on incubation in the presence of its natural substrate, ribonucleic acid (Kalnitzky *et al.*, 1959).

Conclusions

The data presented indicate that elastase in its native state is a globular protein with little evidence from ultraviolet, circular dichroism, optical rotatory dispersion, and infrared measurements for the presence of α helix. This conclusion is in agreement with the features of the crystal structure recently deduced by X-ray diffraction (Shotton and Watson, 1970) which shows that only 12 out of 240 amino acids (or 5%) are present in a short α-helical segment at the carboxyl-terminal end of elastase. No gross conformational changes could be detected by circular dichroism measurements of elastase in the presence of 0.2 M sodium fluoride, even though such a salt concentration inhibits enzyme activity by almost 60%. Similar results were obtained with 5 M guanidine sulfate; a decrease in the activity of elastase in the presence of high salt concentrations is therefore not necessarily due to extensive conformational change (Visser, 1969). (In guanidine chloride, however, the enzyme does undergo a large conformational change and is also inhibited.) If full enzymatic activity is retained in the elastase crystals required for X-ray diffraction work (crystallized from 0.1 M to 0.2 M salt solutions—Shotton and Watson, 1970), the inhibitory effect of inorganic anions certainly cannot be ascribed to any major conformational change.

Elastase loses enzymic activity at extreme pH values and, not surprisingly, this is accompanied by substantial changes in conformation. The appearance of a small positive circular dichroism extremum at 250 nm at low or high pH distinguishes the conformation in these media from a more usual random chain structure with a positive extremum near 220 nm which is observed after heat treatment of elastase.

The results obtained using solvent perturbation spectroscopy to ascertain the relative exposure of tryptophan and tyrosine residues agree reasonably well with conclusions based on the crystal structure of elastase (Shotton and Hartley, 1970) in which 2 of the 7 tryptophans and 9 out of 10 tyrosines are thought to be completely exposed to solvent; the corresponding numbers from our glycerol solution study are 3 tryptophans and 5–6 tyrosines.

On the basis of early optical rotatory dispersion investigations of proteins, Jirgensons (1962, 1963, 1966) postulated that an α-helical structure with its intramolecular hydrogen bonds would not have a high affinity for a nonpolar detergent "tail," but that the hydrophobic interior of globular proteins (without much helical structure) may be an attractive site in which nonpolar tails can become buried, subsequently leading to conformational changes. It was, for example, shown later that the optical rotatory dispersion of lysozyme near 233 nm is not affected by sodium dodecyl sulfate (Glazer and Simmons, 1965) and the present circular dichroism and infrared results with detergent-treated hemoglobin and lysozyme are also in agreement with the postulate that the conformation of proteins with substantial α-helical content is not altered by sodium dodecyl sulfate.

In a comprehensive review of protein denaturation, Tanford (1968) pointed out that many proteins denatured with detergent still have a compact structure with a large degree of order, although the new conformation is often quite different from that of the native protein. The low concentration of detergent required (in the present study a concentration of 0.2% or 7×10^{-3} M sodium dodecyl sulfate was sufficient to bring about a large change in the conformation) is in sharp contrast to the high concentrations of Gdn·HCl (5 M) needed for denaturation. This fact suggests that the binding affinity of elastase for sodium dodecyl sulfate is very high, whereas Gdn·HCl binding may be relatively unspecific. The transformation of elastase and other proteins into more ordered structures in the presence of sodium dodecyl sulfate reported here clearly demonstrates that a detergent treatment of proteins does not invariably cause a randomization of structure (or that a true representation of the original structure is obtained after detergent removal—*vide infra*) as is often assumed.

The way detergents bring about the observed conformational changes to more ordered structures is not clear. Tanford (1968) suggested that proteins are partially unfolded by the detergent after which existing ordered regions or isolated hydrophobic side chains can interact with the hydrophobic tails of detergent molecules to form micelle-like ordered regions. Recent nuclear magnetic resonance studies on the broadening of the proton resonance peaks of sodium dodecyl sulfate when it binds to a fully deuterated protein (phycocyanin) suggest that the structure of this protein also becomes

more ordered when it interacts with moderate amounts of detergent (Rosenberg *et al.*, 1969). The broadening of the detergent proton resonances and their upfield chemical shift were interpreted as a demonstration that it is the hydrocarbon tail of dodecyl sulfate and not its ionized head that interacts with the protein. Simmons and Glazer (1967) showed that sodium dodecyl sulfate eliminates the tyrosine ellipticity band (at 275 nm) of ribonuclease, but concluded that this is due to the disruption of *electrostatic* interactions. If the latter interpretation is correct, it may help to explain why the ribonuclease behavior does not adhere to the general pattern observed for the other proteins in the present investigation.

Some time ago Astbury (1943) and Palmer and Galvin (1943) reported that the X-ray diffraction patterns of denatured globular proteins, in particular that of steam-stretched egg albumin fibers obtained from an alkylbenzenesulfonate detergent solution, gave X-ray spacings identical with those of β-keratin. The more recent finding of extended, β-type conformations in soluble globular proteins indicates that this conformation occurs more often in proteins than had previously been thought. Varying amounts of β structure have been detected by different physical methods in native lysozyme (X-ray, Blake *et al.*, 1967), chymotrypsin (X-ray, Sigler *et al.*, 1968), ribonuclease (X-ray, Kartha *et al.*, 1967), subtilisin (X-ray, Wright *et al.*, 1969), carbonic anhydrase (optical rotatory dispersion, Myers and Edsall, 1965), carboxypeptidase (X-ray, Lipscomb *et al.*, 1968), mitochondrial structural protein (infrared, Wallach *et al.*, 1969), and β-lactoglobulin (infrared and circular dichroism, Timasheff *et al.*, 1967a,b). The conversion described above of native elastase, chymotrypsin, trypsin, pepsin, and lysozyme is a good *in vitro* illustration of the ease with which globular proteins can assume a β conformation. It is not possible to decide unequivocally what type of β structure is assumed in most cases, but the circular dichroism spectrum of treated trypsin suggests that this enzyme assumes an antiparallel-chain pleated-sheet structure. The ultraviolet circular dichroism maximum in the 195-nm region is split into two components at 190 and 195 nm (Table I) as predicted from theoretical considerations (Pysh, 1966). This type of splitting has thus far only been reported in two other instances, *viz.*, for the β form of polylysine and insulin heated in acid solution (Timasheff *et al.*, 1967a,b). The infrared spectrum of treated trypsin confirms this assignment. The amide I absorption is a doublet with maxima at 1625 cm^{-1} and 1650 cm^{-1}, and there is a discernible shoulder near 1690 cm^{-1} (Table III and Figure 10).

Any calculation of the extent of β structure in solution is a hazardous procedure because of the sensitivity of β form rotational strengths to solvent environment (Sarkar and Doty, 1966). The figure of about 50% β form derived above for the proteins may be incorrect by a factor of two, since any small residual amount of α helix (below 10%) would still give the typical 216–217 nm negative circular dichroism minimum, but with an increased ellipticity due to the much higher rotational strength of the amide transition of an α helix (Timasheff *et al.*, 1967a,b; Li and Spector, 1969). There can, however, be no doubt about the qualitative similarities of the observed spectral characteristics of these treated proteins to those of known β structures.

There are some reports in the literature on similar transconformations in other soluble proteins (Jirgensons, 1963, 1966; Taborsky, 1968). The circular dichroism and optical rotatory dispersion data for two of these, phosvitin and β-lactoglobulin (Tables I and II), indicate that their ellipticities and rotations, as well as the band positions, are very similar to those of the *in vitro* induced β conformation in the proteins investigated here. Previous infrared investigations have shown that it is also easy to interconvert α-helical and β conformations in highly charged synthetic polypeptides (Blout and Lenormant, 1957; Lenormant *et al.*, 1958).

The molecular basis for the facile conversion into a β conformation of the proteins we have studied is obscure. Although several possible explanations such as the distribution and content of "non-helix-forming" amino acids (Bloom *et al.*, 1962) have been considered, it does not appear profitable to discuss them here since all the experimental evidence is not definitive. It is nevertheless worth considering that similar transconformations could occur naturally in membranes or at the lipid–water interfaces of cells during metabolic processes. Certain membrane-bound enzymes may depend for their activity on the presence of "detergents;" the activation of β-hydroxybutyrate dehydrogenase by phospholipids (Jurtshuk *et al.*, 1963) is perhaps a suggestive example. The notion that the reported *in vitro* effect of sodium dodecyl sulfate upon protein conformation may have some *in vivo* counterpart would be strengthened if it proves possible to regenerate fully active enzymes after detergent treatment.

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