the United States Rubber Company, the Monsanto Chemical Company, and the National Science Foundation for fellowships awarded to him during this research.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, INDIANA UNIVERSITY, BLOOMINGTON, INDIANA]

Changes in the Optical Rotation of Proteins after Cleavage of the Disulfide Bonds[1,2]

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RECEIVED JULY 15, 1960

Increase in dextrorotation of proteins after cleavage of the dithio bonds has been attributed, by other authors, chiefly to changes in conformation, and particularly to the formation of helices from randomly coiled peptide chains. Since the conversion of cystine into cysteic acid or cysteine is accompanied by a large increase in dextrorotation, the influence of the cleavage of dithio bonds in proteins on the optical rotation was investigated. In agreement with earlier observations on the oxidative fission of disulfide bonds in proteins, the present study revealed a similar increase in dextrorotation after reduction with thioglycol and after sulfitolysis, provided that simultaneous changes in the secondary structure were prevented by stabilization of the native state or by complete denaturation prior to the fission of the dithio bonds. It is concluded that the levorotation of cystinyl residues contributes essentially to the levorotation of those proteins which are rich in cystine and that fission of the disulfide bonds is responsible for most of the decrease in levorotation which accompanies reduction or sulfitolysis of these proteins.

It is well known that the levorotation shown by most of the native proteins increases on denaturation. The extent of this change in rotation depends on the protein and on the denaturing agent. An increase in levorotation also occurs when synthetic L-polypeptides are transferred from a non-polar solvent to a more polar solvent.[3] This has been attributed to unfolding of the peptide chains and to the conversion of α-helices into random coils caused by the fission of intramolecular hydrogen bonds. An analogous interpretation has been applied also to the increase in dextrorotation observed after cleavage of the dithio bonds in proteins.[4] Indeed, one could imagine that the disulfide linkages of proteins maintain the peptide chain in a strained configuration and that cleavage of the SS-bonds would allow the peptide chains to assume the α-helix conformation. However, when the dithio groups of serum albumin were oxidized by performic acid to the corresponding sulfonic acids, the increase in dextrorotation was of the order of magnitude calculated for the conversion of cystine into cysteic acid residues. Accordingly, Turner, *et al.*,[5] attributed the change in optical rotation after oxidation chiefly to the cleavage of the cystinyl residues rather than to configurational changes.

Since a similar increase in dextrorotation is observed when cystine is cleaved by reduction or sulfitolysis, we investigated the changes in rotation of several proteins exposed to these reactions. In contrast to the oxidation, which is usually performed in concentrated formic acid, reduction and sulfitolysis can be carried out in aqueous solutions under mild conditions. Reduction was achieved with thioglycol at *p*H 7–8 at room temperature. The reaction with sulfite was performed either in the presence or in the absence of an oxidizing agent. Sodium sulfite alone reacts with free cystine and also with peptide-bound cystinyl residues to form one equivalent of cysteine-S-sulfonic acid and one equivalent of cysteine (reaction 1); in the presence of sodium tetrathionate or another oxidant, the cysteine formed in reaction 1 is reoxidized and converted into a second molecule of cysteine-S-sulfonic acid[6] (reaction 2).

$$RSSR + SO_3^{--} \longrightarrow RSSO_3^- + RS^- \qquad (1)$$

$$RS^- + SO_3^{--} \xrightarrow{[O]} RSSO_3^- \qquad (2)$$

Experimental

Material and Methods.—All the proteins used were in crystalline form. Ovalbumin, bovine serum albumin and pepsin were obtained from the Nutritional Biochemicals Corporation, lysozyme from Sigma Chem. Co., γ-globulin from Armour Laboratories and chymotrypsin from Pentex Incorp. Dr. Marsh of Eli Lilly & Co. kindly supplied the insulin. Urea, sodium sulfite, sodium sulfate and LiBr were reagent grade chemicals, guanidine hydrochloride was recrystallized from methanol–ether. The detergent solutions were prepared from sodium dodecylsulfate (K. & K. Laboratories). Sodium tetrathionate was prepared by the oxidation of sodium thiosulfate with iodine.[7] Glutathione was oxidized according to Mason.[8] Sodium cysteine-S-sulfonate was prepared from cystine and sodium sulfite according to Clarke.[9]

Reduction of Proteins by Thioglycol.—100 or 200 mg. protein were dissolved in a small amount of water in a 10 ml. volumetric flask. Urea, guanidine hydrochloride, LiBr or sodium dodecylsulfate (SDS) was then added to give a final concentration of 8 *M* urea, 5 *M* guanidine-HCl, 6 to 12 *M* LiBr or 0.1 to 0.2 *M* SDS, respectively. The *p*H of the solution was maintained between 7 and 8 by the addition of phosphate or tris-(hydroxymethyl)-aminomethane buffer (=tris). When guanidine hydrochloride was used, the sample was kept at 50° for 3 hr. in order to accomplish maximum denaturation. After the addition of 0.1–0.2 ml. of thioglycol, the flask was filled to the mark with distilled

(1) This work has been supported by research grants from the National Science Foundation (G-5468) and the U. S. Public Health Service (RG 1852) and by contracts of Indiana University with the Atomic Energy Commission (AT-11-1-209) and the Office of Naval Research (3104-00).

(2) Presented in part at the 137th A.C.S. meeting in Cleveland, Ohio, April 1960.

(3) P. Doty, A. M. Holtzer, J. H. Bradbury and E. R. Blout, THIS JOURNAL, **76**, 4493 (1954); P. Doty, J. H. Bradbury and A. M. Holtzer, *ibid.*, **78**, 947 (1956); E. R. Blout and M. Idelson, *ibid.*, **78**, 497 (1956).

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(5) J. E. Turner, R. T. Bottle and F. Haurowitz, *ibid.*, **80**, 4117 (1958); J. E. Turner, M. Kennedy and F. Haurowitz, "Sulfur in Proteins," ed. by R. Benesch, *et al.*, Academic Press, Inc., New York and London, 1959, p. 10. The statement in the former paper that the excess of performic acid was reduced by ethanol is erroneous.

(6) J. L. Bailey and R. D. Cole, *J. Biol. Chem.*, **234**, 1733 (1959).

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(8) H. L. Mason, *J. Biol. Chem.*, **90**, 409 (1931).

(9) H. T. Clarke, *ibid.*, **97**, 235 (1932).

TABLE I

$[\alpha]_{SS}$ VALUES OF CYSTINE BEFORE AND AFTER REDUCTION OR SULFITOLYSIS IN PHOSPHATE *p*H 7.5

Cystine	−64°
Cysteine (pure)	− 3°
Cystine + thioglycol	− 4°
Cysteine-S-sulfonate (synth.)	−37°
Cystine + sulfite + tetrathionate	−42°
Cystine + sulfite	−22°

water. The specific rotation $[\alpha]_D$ was determined after keeping the solutions at room temperature for 3–4 hr. In a control run, the optical rotation of the untreated protein was determined in the absence of thioglycol.

$RSSO_3H$, respectively. Consequently, the equivalent rotation of cystine is equal to its molar rotation, whereas the equivalent rotations of cysteine or cysteine-S-sulfonic acid are twice the value of the corresponding molar rotations since each cysteine molecule gives two molecules of cysteine or cystine-S-sulfonate or one molecular of each of these according to reaction 1.

Table I shows that the rotation of an aqueous solution of cysteine produced from cystine by the action of thioglycol without further purification is almost identical with that of pure cysteine. Likewise, the optical rotation of cysteine-S-sulfonate,

TABLE II

CHANGES IN THE EQUIVALENT ROTATION, $[\alpha]_{SS}$, OF PROTEINS AFTER REDUCTION WITH THIOGLYCOL[a]

Solvent →	A			B			C			D		
	SDS			8 *M* Urea			5 *M* Gu			6–12 *M* LiBr		
Substance	Oxid.	Reduc.	Δ	Oxid.	Reduc.	Δ	Oxid.	Reduc.	Δ	Oxid.	Reduc.	Δ
Cystine	− 64	− 4	+60									
Oxid. glutathione	− 72	− 16	+56									
Chymotrypsin	−502	−440	+62									
Bovine serum albumin	−256	−192	+64				−398	−353	+45	−237	−200	+37
Bovine-γ-globulin							−720	−673	+47	−413	−346	+67
Lysozyme	−159	− 81	+78	−126	−234	−108	−246	−222	+24	−108	− 24	+84
Insulin	−125	− 44	+81	−167	−171	− 4	−109	−130	−21	Insoluble		
Ribonuclease[10]				−407	−346	+ 61				−192	−128	+64

[a] Abbreviations and notes: SDS = sodium dodecylsulfate; Gu = guanidine-HCl; oxid. and reduc. = values of the equivalent rotation, $[\alpha]_{SS}$, before and after treatment with thioglycol, respectively; Δ = change in $[\alpha]_{SS}$ after reduction. The values recorded for insulin in column A were obtained at *p*H 8–9; those of ribonuclease were calculated from the data of Harrington and Sela.[10]

Reaction with Sodium Sulfite.—The sulfitolysis of proteins was performed according to Bailey and Cole.[6] Three 100 mg. samples of each of the proteins were examined in parallel runs. All three samples were dissolved in buffer solutions containing either guanidine-HCl, urea or SDS. To one of the samples 1.5 ml. of 1 *M* sodium sulfite solution was added, to the second, which served as a blank, the equivalent amount of sulfate. In the third sample, oxidative sulfitolysis was brought about by the alternate addition of 1 *M* solutions of sodium sulfite and sodium tetrathionate. The three samples were prepared simultaneously and kept in a thermostat at 37° for 1–2 hr. in the experiments with urea or SDS; in those with guanidine-HCl, the samples were allowed to stand for 3–4 hr. at 50°.

Determination of $[\alpha]_D$.—The optical rotation was measured to ± 0.05° in a Rudolph polarimeter with sodium light at temperatures varying from 20 to 25°. The influence of the temperature changes on $[\alpha]_D$ was too small to be of significance.

Results and Discussion

An increase in dextrorotation was observed earlier after oxidation[5] and is observed now after reduction or reaction with sulfite. The increment depends on the disulfide content of the protein.

Cystine itself has an extremely high levorotation of −285° in formic acid and of −265° in phosphate buffer. This high levorotation value is almost completely lost after conversion of cystine either into cysteine ($[\alpha]_D$ = −13° in buffer) or into cysteic acid ($[\alpha]_D$ = + 5.5° in formic acid). Sulfitolysis leads to the formation of one molecule of cysteine and one of cysteine-S-sulfonic acid, the $[\alpha]_D$ of which is −87° in H_2O or −70° in SDS solution. Oxidative sulfitolysis yields two molecules of cysteine-S-sulfonic acid.

For better comparison of cystine with its derivatives and also with proteins, we use the rotation per equivalent of dithio bonds and designate it as "equivalent rotation" or $[\alpha]_{SS}$, which is equivalent to one mole of RSSR or two moles of RSH or obtained by the reaction of a solution of cystine with sulfite and tetrathionate is very close to that of a preparation of pure cysteine-S-sulfonate. When cysteine is exposed to sulfite in the absence of tetrathionate or iodosobenzoate, a mixture of equivalent concentrations of cysteine and cysteine-S-sulfonate is formed (reaction 1). The value of −22° found after this reaction is in satisfactory agreement with the calculated mean value of (− 37 − 3)/2 = −20°.

The equivalent rotation of proteins was calculated from the molecular weight and the number of disulfide bonds per molecule. Example: lysozyme in 5 *M* guanidine hydrochloride has an $[\alpha]_D$ value of −82°; assuming a molecular weight of 15,000, we obtain a molar rotation of 15 × −82° = −1230°; since lysozyme contains 5 dithio bonds, the equivalent rotation, $[\alpha]_{SS}$, is equal to −1230/5 = −246° (see Table II).

The $[\alpha]_{SS}$ values of several proteins in different solutions and the changes in equivalent rotation after reduction with thioglycol (= Δ) are presented in Table II. The reduction of cystine in a solution of buffer or sodium dodecylsulfate causes a decrease in levorotation from −64 to −4°, *i.e.* a change in equivalent rotation by + 60°. Similar values of Δ are found after the reduction of oxidized glutathione, chymotrypsin and other proteins.

Only small changes in the optical rotation were observed when cysteine was used as a reducing agent for proteins. This is not suprising since the decrease in levorotation of the protein is then compensated by the increase in levorotation which accompanies the conversion of cysteine into cystine.

(10) W. F. Harrington and M. Sela, *Biochem. Biophys. Acta*, **31**, 427 (1959).

Thus the equivalent rotation of bovine serum albumin in buffer and sodium dodecyl sulfate decreased after the addition of thioglycol by 64° but after reduction by L-cysteine only by 20° (after correction for the optical rotation of the added L-cysteine).

Table II demonstrates that Δ, the change in rotation, depends to a high extent on the composition of the solution in which the reaction is performed. After reduction in urea solution (Table II, B) we found in some instances (*e.g.*, insulin) no significant change, in others (*e.g.* lysozyme) an increase in levorotation. Similar irregular results were obtained with sulfite in urea solutions. One explanation of this phenomenon might be that the protein is only partly denatured by urea as long as disulfide bonds are present and that the fission of the disulfide bonds is accompanied by further unfolding and complete denaturation. The increase in dextrorotation on cleavage of the SS-bonds may then be compensated or even overbalanced by the increase in levorotation caused by unfolding. Complications of this type were avoided in those experiments (Table II, column C) in which the proteins before treatment with thioglycol were almost completely denatured by 5 *M* guanidine hydrochloride at 50°. In other experiments, denaturation during the reduction was prevented by 0.1 molar detergent solution (Table II, column A) or by concentrated LiBr solution (Table II, column D). Lithium bromide, according to Harrington and Schellman,[11] stabilizes the folded structure of proteins by increasing the strength of intrachain hydrogen bonds. In most of the experiments shown in Table II, column A, C and D, an increase in dextrorotation was observed after reduction. Diverging results obtained in urea lead to the conclusion that urea in the absence of thioglycol cannot bring about complete unfolding and that further unfolding takes place after cleavage of the disulfide bonds by thioglycol. Evidently, urea is not a suitable solvent for investigations on changes of the optical rotation of proteins.

In proteins such as ovalbumin or pepsin, which are poor in disulfide bonds, the $[\alpha]_{ss}$ values are close to the molar rotation and therefore very high, *e.g.*, −3600° in ovalbumin. Since the error of our reading is approximately 2−3% of the observed angle, it amounts to ± 90° in ovalbumin and is of the same magnitude as the expected change in $[\alpha]_{ss}$. We are not able, therefore, to measure the difference in $[\alpha]$ between the oxidized and reduced form of ovalbumin or other proteins which are poor in dithio bonds. The contribution of the dithio bonds to the molecular rotation of these proteins is very small in comparison to the contribution by conformational factors.

Sulfitolysis was performed on several proteins in buffer, SDS or guanidine hydrochloride solutions. The changes in $[\alpha]_{ss}$ are given in Table III. Even though the initial values of $[\alpha]_{ss}$ varied considerably, the changes, Δ, were always positive and their magnitude similar to that observed in cystine and glutathione. The divergent results obtained with urea, shown in the bottom part of Table III, are analogous to those demonstrated in Table II and are attributed to the same cause. The increase in $[\alpha]_{ss}$ after reaction of a protein with sulfite plus oxidation (Table III, column C) is always lower than after reaction with sulfite only (column B). This is in agreement with the results shown in Table I, according to which the equivalent rotation of cystine (in buffer) on sulfitolysis changes by +42° (from −64 to −22°), however on oxidative sulfitolysis only by +22° (from −64 to −42°). The slight difference between the $[\alpha]_{ss}$ values of cystine in the Tables I and III is caused by the addition of sulfate in the control experiments recorded in Table III, column A.

TABLE III

CHANGES IN EQUIVALENT ROTATION AFTER REACTION WITH SULFITE[a]

Substance	A $[\alpha]_{ss}$	B RSSR → RSH + $RSSO_3H$ $[\alpha]_{ss}$	B Δ	C RSSR → $2RSSO_3H$ $[\alpha]_{ss}$	C Δ
Cystine (Bu)	− 67	− 22	+ 45	− 42	+ 25
Oxid. glutathione (Bu)	− 60	− 18	+ 42	− 29	+ 31
Lysozyme (SDS)	−141	− 95	+ 46	−105	+ 36
Bovine serum albumin (SDS)	−239	−203	+ 36	−215	+ 24
Bovine serum albumin (Gu)	−401	−365	+ 36	−377	+ 24
Chymotrypsin (SDS)	−478	−421	+ 57	−441	+ 37
In urea:					
Lysozyme	−132	−237	−105	−246	−114
Bovine serum albumin	−393	−409	− 16	−412	− 19
Insulin	−146	−154	− 8	−169	− 23

[a] Abbreviations: Bu = buffer, SDS = sodium dodecylsulfate, Gu = guanidine-HCl. The $[\alpha]$ values recorded in column A were measured in solutions containing sulfate instead of sulfite.

An exceptional behavior was observed in insulin which, after exposure to sulfite in urea or sodium dodecyl sulfate or to thioglycol in urea or guanidine hydrochloride, gave an increase in levorotation instead of the expected decrease. We attribute this exceptional behavior of insulin to the presence of two interchain bonds which cannot be cleaved by urea and prevent complete unfolding before their scission by thioglycol. It has been shown by Kay and Marsh[12] that conversion of the dimer of serum albumin into its monomer is accompanied by a decrease in levorotation. However, the dissociation of insulin into its two peptide chains after cleavage of the dithio bridges by thioglycol or sulfite may result in complete unfolding of the two peptide chains accompanied by an increase in levorotation. The increase in levorotation caused by this reaction seems to exceed the decrease in levorotation which accompanies the cleavage of the dithio bonds and is responsible for the abnormal behavior recorded in Table II (column C) and Table III.

In most of the experiments, the increase in equivalent rotation of the proteins was of the same order as that observed in cystine or glutathione, namely, +56 to +81° after reduction by thioglycol, +36 to + 57° after sulfitolysis and +24 to + 37° after

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(12) C. M. Kay and M. M. Marsh, *Biochim. Biophys. Acta*, **35**, 262 (1959).

oxidative sulfitolysis. The similarity of these values indicates that in proteins which are rich in dithio bonds, most of the change in $[\alpha]_D$ is caused by the cleavage of dithio bonds. Our observations give some information on the resistance of various proteins to denaturing agents and on the role of dithio bonds in maintaining the conformation of the native proteins. The stabilizing role of dithio bonds seems to be particularly strong in insulin where it is, most probably, responsible for the abnormal optical rotation of this protein after reduction or sulfitolysis.

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Kinetics of the Reaction of Ammonia and Nitric Oxide in the Region of Spontaneous Ignition[1]

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RECEIVED JULY 25, 1960

Rates of the NO–NH_3 reaction were measured within the temperature range of 850 to 1050° using a flow technique and were adequately represented by the expression

$$\frac{d(H_2O)}{dt} = \frac{3.2 \times 10^{10} \exp(-58400/RT)(NH_3)^{1/2}(NO)}{1 - k_2(NH_3)^{1/2}(NO)}$$

Hydrogen, but no N_2O, was found in the reactor effluent in addition to the major products, N_2 and H_2O. Spontaneous ignition was observed above 960° and the boundaries of inflammable reactant compositions were used to obtain k_2. The temperature dependence of the iguition limits gave an activation energy of 30.0 kcal. for k_2.

Investigations of high temperature reaction systems involving NO are of importance for interpretation of combustion phenomena. Nitric oxide supported flames, including the NO–NH_3 flame, have received considerable attention recently.[2-4] The spontaneous ignition temperature of stoichiometric mixtures of NO and NH_3 has been reported.[5] Kinetic studies of the NO–NH_3 reaction at lower temperatures have resulted in a number of disparities.[6-8] There is a lack of agreement on the kinetic order of the reaction, the activation energy and even the reaction products.

The present work is an attempt to assist in the elucidation of an apparently complex reaction by procuring kinetic data in the temperature region of spontaneous ignition.

Experimental

Materials.—Anhydrous ammonia from two sources was used. Shell Chemical Co. refrigeration grade NH_3 was condensed by means of liquid air and the non-condensable gas was pumped off. A repetition of this procedure gave a product which, upon chromatographic examination, showed only a trace of N_2. A majority of the rate measurements was made with Matheson Co. anhydrous ammonia, specified as having 99.9% minimum purity. The same purification procedure was employed at first, but later it was established that use of NH_3 taken directly from the cylinder had no effect on the measured rates.

Nitric oxide from Matheson Co., specified as having 99.0% minimum purity, was purified in the same manner as was the NH_3. With this procedure it was not possible to eliminate traces of nitrous oxide, an impurity whose possible presence in the first of the two cylinders used was indicated by infrared analysis. The content of the second cylinder was analyzed by the supplier and was alleged to contain no N_2O, 0.275% N_2 and 0.14% NO_2. Ascarite was used to remove NO_2 from the NO and to dry the helium, H_2 and N_2O which were obtained from commercial sources. Matheson Co. "prepurified" N_2 and helium were used as diluent alternately without detectable differences in the measured reaction rates.

Methods.—A flow technique was employed for measurement of both reaction rates and explosion limits. Use was made of the same quartz reaction vessels, mixing chambers, furnace, temperature controls and measuring devices which have been described in previous reports.[9]

Temperatures at the center of the reaction vessel were controlled to ± 1°. Measurement of the temperature profile along the axis of the furnace demonstrated that the temperature gradient between entrance and exit of the reactor was approximately 1° for the smaller vessel and approximately 3° for the larger vessel. The total pressure of the reactant mixture was approximately 80 cm. for all of the rate measurements. A total flow rate of either 100 or 1000 ml./min. was maintained for various reactant compositions by addition of diluent gas.

An improved design of the capillary tube flowmeters and use of Hoke metering valves facilitated control of the gas flow rates and extended the ranges of the meters to permit measurement of flow rates from 5 to 1000 ml./min. White paraffin oil was the fluid used in the flowmeters, which were calibrated individually for specific gases.

Reaction rates were determined by absorption of water vapor in Ascarite. The presence of NH_3 in the effluent necessitated the selection of Ascarite, rather than the more common desiccants. Gravimetric measurement of the amount of H_2O collected during an appropriate time interval permitted the determination of the steam content of the reactor effluent with a precision of ± 2%.

A gas chromatograph[10] was used to ascertain qualitatively the purity of the reactants and to identify the reaction products.

Results

In addition to water the presence of N_2 and H_2 in the reactor effluent was established by gas chromatography. It was also demonstrated that in the absence of NO, but under the same reactor conditions, considerable decomposition of NH_3 occurred. No unequivocal evidence of N_2O was

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