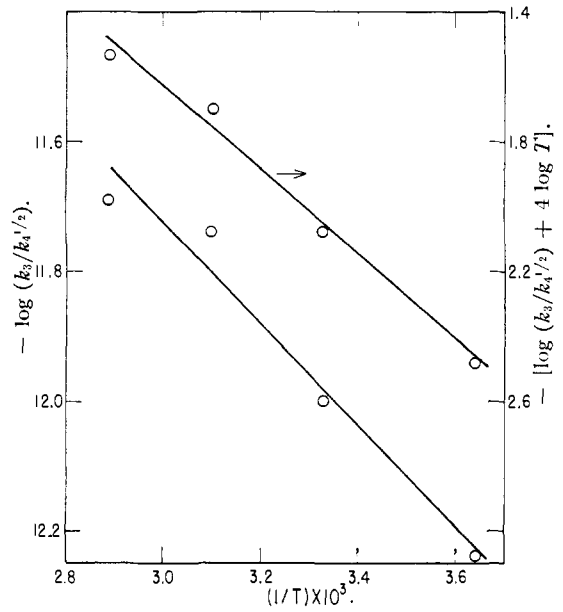


Fig. 4.—Arrhenius plot of rate constant ratios derived from slopes of Fig. 3; upper curve corrected for temperature dependence of diffusion.

An argument similar to the one we have already used suggests that 4 log $T$ should be subtracted from the values of log $k_5/k_2k_4^{1/2}$ to account for the temperature dependence of diffusion. Although such a treatment tends to correct the apparently anomalous value at 2°, the adjusted values do not lead to a linear Arrhenius plot unless the 2° value is neglected. The validity of this calculation is dubious for such a complex combination of rate constants. However, it may be observed that if the 2° result is not used, an activation energy, $E = E_2 + E_4/2 - E_5$, of about 9 kcal./mole is obtained. Since $E_2$ is about 13 kcal./mole in the gas phase[20] and $E_4$ may be taken as zero and $E_5$ is probably in the range of 0–4 kcal./mole, this value is reasonable.

**Effect of Allyl Alcohol.**—The effect of allyl alcohol, particularly at 27° where the highest concentrations were used, shows that the formation of gaseous products probably proceeds completely by a free radical mechanism. The virtual elimination of methane at an allyl alcohol concentration of 0.1 $M$ shows that simple hydrolysis of photoexcited acetone to yield acetic acid and methane, which had been assumed by early workers,[6] cannot account for methane formation.

An interesting result was the increase in quantum yield of methane and carbon monoxide at 2 and 27° for low alcohol concentrations. This does not occur at the higher temperatures. A possible explanation for this phenomenon is that methyl radicals in the solvent cage react with allyl alcohol much more rapidly than do acetyl radicals. This prevents recombination of acetyl and methyl to form acetone and allows acetyl radicals to escape the cage and actually obtain a higher concentration than in the absence of scavenger. This in turn favors the disproportionation reaction 5 which has relatively greater importance at low temperature because of the low activation energy usually associated with disproportionation reactions.

(20) J. C. Calvert and J. T. Gruver, THIS JOURNAL, **80**, 1313 (1958).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE UNIVERSITY, AMES, IOWA]

# The Starch–Iodine–Iodide Interaction. Part I. Spectrophotometric Investigations[1]

BY JOHN A. THOMA[2] AND DEXTER FRENCH

RECEIVED DECEMBER 10, 1959

Maltodextrins having six or more glucose units react with iodine–iodide solutions to form complexes which can be detected spectrophotometrically. Triiodide complexes exhibit absorption spectra essentially identical with the triiodide ion; hence triiodide complexes are not visually detectable. Dextrins with 9 or more glucose units give polyiodine complexes with enhancement of the absorption and shift to longer wave length, thus giving visible colors. Iodide ion was found to be necessary for the formation of blue starch–iodine complexes in aqueous solution. The apparent failure of amylose to form an iodide-free iodine complex suggests that amylose exists primarily as a random coil in aqueous solution.

## Introduction

The effect of chain length on the stoichiometry and absorption spectrum of the starch–iodine complex is known only semiquantitatively.[3,4] This deficiency has resulted principally from a lack of individual pure or adequately characterized starch saccharides, in the chain length range where small changes in chain length give large changes in (a) the tendency to form complexes and (b) the types of complexes formed.

Recently, maltodextrin saccharides up to $G_{18}$[5] have been isolated by column chromatography on cellulose columns.[6] These individual compounds afforded an unparalleled opportunity to examine definitively the effect of chain length on the starch–iodine complex.

## Experimental

**Materials.**—All inorganic reagents were commercial reagent grade and used without further purification.

(1) Journal Paper No. J-3746 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Proj. 1116. Supported in part by grants from the Corn Industries Research Foundation and General Foods, Inc.

(2) Brookhaven National Laboratory, Upton, Long Island, New York. Union Carbide and Carbon Fellow 1957–1958.

(3) M. A. Swanson, *J. Biol. Chem.*, **172**, 825 (1948).

(4) D. L. Mould, *Biochem. J.*, **58**, 593 (1954).

(5) $G_n$ designates a pure individual linear maltodextrin saccharide composed of $n$ D-glucose residues.

(6) J. A. Thoma, H. B. Wright and D. French, *Arch. Biochem. Biophys.*, **85**, 452 (1959).

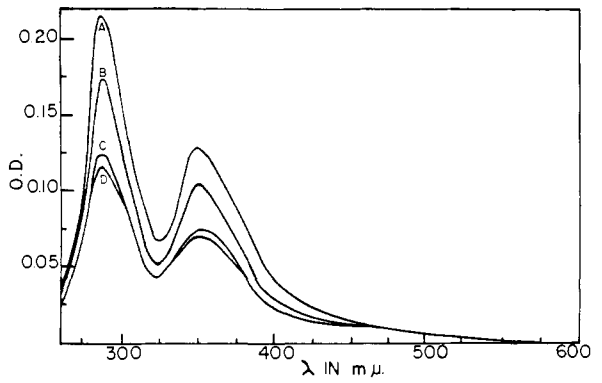


Fig. 1.—Ultraviolet and visible absorption of iodine complexes. Solutions contain $1.2 \times 10^{-4}$ $M$ $I_2$, $2.4 \times 10^{-4}$ $M$ KI and in addition 0.40% $G_{10}$ (curve A); 0.43% $G_8$ (curve B) and 0.54% $G_6$ (curve C). Curve D is a control containing only $I_2$ and KI. Light path was 2 mm. Blanks contained equivalent amounts of dextrin in $H_2O$.

Glycogen was purchased from the Nutritional Biochemicals Co. When the sample was added to $I_2$–KI solutions, a visible enhancement of the $I_2$ spectrum was just perceptible, an indication that the sample was highly branched.[7] Amylose was prepared by the procedure of Schoch[8] and three times recrystallized with 1-BuOH. The isolation by cellulose column chromatography of the maltodextrin saccharides which assayed between 87 and 92% carbohydrate with the anthrone reagent[9] has been described in another publication[6] by the authors. A mixture of $G_{18}$, $G_{19}$ and $G_{20}$ was obtained by continued elution of the cellulose column with two liters of solvent after the $G_{18}$ peak appeared.[6] The final solvent contained 42.5% $H_2O$, 25% EtOH and 32.5% 1-BuOH, by volume. These saccharides, while not completely resolved on the column, could just be resolved by paper chromatography at room temperature on 14″ sheets of Eaton and Dikeman #613 paper, when irrigated 6 times (by the ascending technique) with a solvent containing 28% $H_2O$, 37% EtOH and 35% $MeNO_2$, and then irrigated eight times with a solvent containing 33% $H_2O$, 37% EtOH and 30% $MeNO_2$. The saccharides above $G_{16}$ showed considerable streaking.

These large saccharides were made visible by spraying the papergrams with a solution containing 0.3% of $I_2$ and 0.15% KI in 90% methanol. This spray had the advantage that the developed papergram could be irrigated again with solvent if the saccharides had not been sufficiently resolved, because the $I_2$ moved with the solvent front. The hydrolysate from which the sugars were isolated, and previously identified $G_{16}$, served as reference materials.

**Spectra.**—Spectral measurements were made in capped quartz cells in a Beckman model DU spectrophotometer at room temperature.

**Methods.**—A nearly saturated stock $I_2$ solution was prepared by shaking $I_2$ crystals in water for a day at room temperature. The concentration of this solution was determined immediately before use by measuring the optical density of a 1:5 dilution at 460 mμ, using $\epsilon_{460} = 746$.[10] Other solutions were prepared by appropriate dilution of weighed amounts of reagents. $I_2$ solutions substantially free of $I^-$ were prepared by dissolving $I_2$ crystals in 0.2 $M$ aqueous $HIO_3$.

## Results and Discussion

**Maltodextrins.**—In a preliminary experiment to determine the approximate chain length required for the formation of colored iodine complexes, 2% solutions of dextrins, $G_4$ through $G_{15}$, were spotted on filter paper and sprayed with the MeOH–$I_2$–KI reagent. The dextrins $G_9$ through $G_{12}$ stained yellow-brown to brown while the staining capacity of $G_8$ was questionable. From $G_{12}$ to $G_{15}$ the color changed from brown to reddish purple. This experiment suggested that under favorable conditions dextrins as small as $G_9$, and possibly $G_8$, could form iodine complexes with enhanced color.

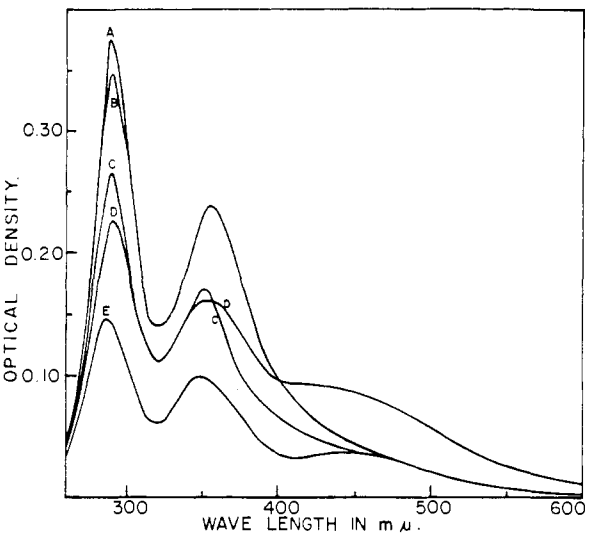


Fig. 2.—Ultraviolet and visible absorption of iodine complexes. Solutions contain $2.6 \times 10^{-4}$ $M$ $I_2$ and $1.2 \times 10^{-4}$ $M$ KI and in addition 0.96% $G_{11}$ (curve A); 0.80% $G_{12}$ (curve B); 0.87% $G_9$ (curve C); 0.32% mixture of $G_{18}$, $G_{19}$ and $G_{20}$ (curve D). Curve E is a control containing only $I_2$ and KI. Light path was 2 mm. Blanks contained equivalent amounts of dextrin in $H_2O$.

When the ultraviolet and visible absorption spectra of 0.4–0.5% solutions of $G_6$, $G_8$ and $G_{10}$ in the presence of $I_2$–KI were measured (Fig. 1), only an enhancement of the $I_3^-$ peaks at 290 and 355 mμ[12] was observed. This enhancement undoubtedly reflects the formation of dextrin $I_3^-$ complexes. This conclusion is supported by potentiometric titrations of the dextrins with $I_2$–KI to be reported in a subsequent paper.[11] Moreover, spectra of the α-$I_2$–KI system (α represents cyclohexaamylose) show absorption maxima at 290 and 353 mμ (Fig. 3, curves C and D) which have been demonstrated by Thoma and French to result from the formation of an $I_3^-$ complex.[12] The additional peak at 440 mμ observed when α was added to an $I_2$ solution (Fig. 3, curve D) was shown to result from the formation of an $I_2$ complex.[12] The $\alpha I_3^-$ peaks at 290 and 353 mμ could be repressed by adding $HClO_3$ to the system.[12]

In an effort to detect spectrophotometrically the visible iodine complexes with the larger dextrins, the dextrin concentrations were increased to approximately 1% and the ratio of $I_2$ to KI was changed from 1:2 to 2:1, a condition conducive to polyiodine formation. The dextrins $G_9$, $G_{11}$ and $G_{12}$ enhanced only the ultraviolet $I_3^-$ spectrum, but a visual enhancement was observed with the $G_{18}$, $G_{19}$, $G_{20}$ mixture in Fig. 2, curve D. Other spectra were not run at these low $I_2$–KI concentra-

(7) A. M. Liddle and D. J. Manners, *J. Chem. Soc.*, 3432 (1957).
(8) T. J. Schoch, *Advances in Carbohydrate Chem.*, **1**, 247 (1945).
(9) S. Siefter, S. Dayton, B. Novic and E. Muntwyler, *Arch. Biochem. Biophys.*, **25**, 191 (1950).
(10) A. D. Awtrey and R. E. Connick, THIS JOURNAL, **73**, 1842 (1951).
(11) J. A. Thoma and D. French, manuscript in preparation.
(12) J. A. Thoma and D. French, THIS JOURNAL, **80**, 6142 (1958).

tions because of the small quantities of material available.

From these data, it appeared that at low $I_2$–KI concentrations (*ca.* $10^{-3}$ or below) a chain length of about 18 glucose units was required for the production of visual iodine complexes. Considerably different conclusions were arrived at by Swanson[3] who examined iodine-staining properties of amylose hydrolysates.

The $G_{16}$-iodine complex (Fig. 3, curve B) exhibited an enhancement in the visible region of the spectrum. Although this enhancement was detectable spectrophotometrically in the more concentrated $I_2$–KI solutions, it was almost completely masked to the eye by the intense $I_3^-$ absorption.

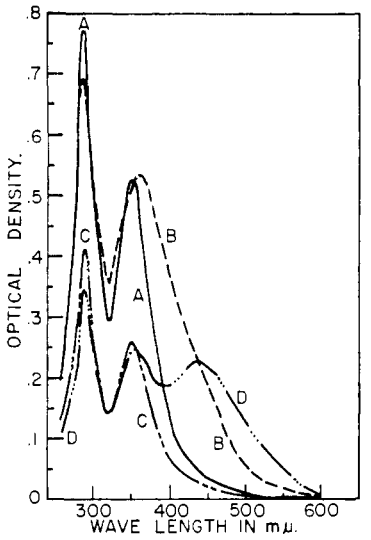


Fig. 3.—Ultraviolet and visible absorption of iodine complexes. Solutions contain $4.4 \times 10^{-3}$ *M* $I_2$, 0.25 *M* KI (curve A); $4.4 \times 10^{-3}$ *M* $I_2$, 0.25 *M* KI, 7.0 mg. $G_{16}$ per ml. (curve B); $1.1 \times 10^{-5}$ *M* $I_2$, $2.5 \times 10^{-5}$ *M* KI, $1.2 \times 10^{-3}$ *M* α (curve C); $2.6 \times 10^{-4}$ *M* $I_2$, $4.8 \times 10^{-3}$ *M* α (curve D). Light path was 0.5 mm. for curves A and B and 10 mm. for curves C and D, $H_2O$ was the blank.

Although this small visible enhancement is probably caused by a diiodine complex ($I_6^=$, demonstrated potentiometrically in a forthcoming paper[11]) present in small amounts, the visible maximum which appears to be in the region of 400 to 440 mμ is certainly different from the visible maximum at 510–520 mμ for complexes which also contain two moles of iodine per mole of dextrin observed by Mould. The most plausible explanation of these observations is that two diiodine complexes are formed, one associated with one $I^-$ at low $I^-$ concentrations and one associated with two $I^-$'s at high $I^-$ concentration. The uptake of $I^-$ as well as $I_2$ undoubtedly will be modified considerably by the chain length.

**Amylose and Glycogen.**—Numerous investigators have implicated $I^-$ in the formation of the starch–iodine complex. Nevertheless, the absolute dependence of the formation of colored complexes on $I^-$ has not been demonstrated, and some authors[13,14] have proposed that $I^-$ is not mandatory for the formation of a colored complex.

When formation of $I^-$ by the hydrolysis of $I_2$ was repressed by the addition of $HIO_3$ to an amylose –$I_2$ solution, the resulting spectrum was essentially identical with that of $I_2$ in $HIO_3$ (Fig. 4, curve D).[15]

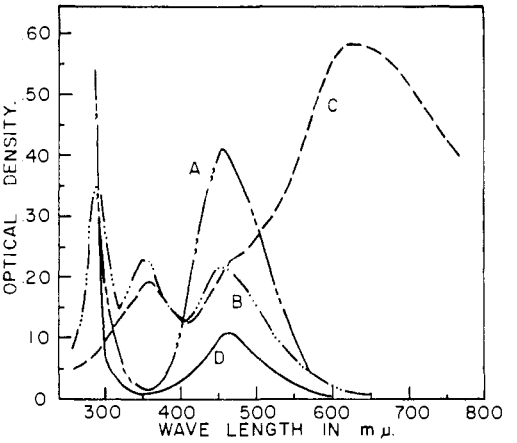


Fig. 4.—Ultraviolet and visible absorption of amylose and glycogen-$I_2$–$HIO_3$ systems. Solutions contain 0.16% glycogen, $5.2 \times 10^{-4}$ *M* $I_2$, 0.2 *M* $HIO_3$ (curve A); 0.16% glycogen, $2.6 \times 10^{-4}$ *M* $I_2$ (curve B); 0.08% amylose, $5 \times 10^{-5}$ *M* $I_2$ (curve C); 0.08% amylose, $1.3 \times 10^{-4}$ *M* $I_2$, 0.2 *M* $HIO_3$ (curve D). Light path was 10 mm. Blanks contained equivalent amounts of glycogen and amylose in $H_2O$.

When production of $I^-$ was not repressed, the characteristic blue starch–iodine complex resulted (Fig. 4, curve C). This experiment, in addition to demonstrating the requirement of $I^-$ for the formation of the starch–iodine complex in aqueous solution, suggested that aqueous, uncomplexed amylose is not predominantly in a helical configuration. If it were helical, one would expect a shift in the $I_2$ spectrum upon addition of amylose similar to the shift observed when the cyclic α-dextrin was added to $I_2$–$HIO_3$.[12] By studying the physical properties of amylose in a number of solvents, Everett and Foster[16] have demonstrated that amylose exists as a random coil. Holló and Szejtli,[17] on the other hand, investigating the viscosity of amylose–iodine solutions as a function of bound iodine, concluded that native amylose exists predominantly in a helical configuration.

In an attempt to explain the high binding capacities of amylopectin and glycogen at high iodine concentrations, it has been proposed that molecular $I_2$ and even $I_4$ may be bound.[18,19] *A priori* this conclusion can be challenged because the high iodine binding is only achieved in iodide-rich systems where the iodine exists primarily as triiodide

(13) E. O. Forster, unpublished Ph.D. dissertation, Columbia University, 1951. Forster did not add enough $HIO_3$ to prevent the formation of $I^-$ by the hydrolysis of $I_2$.

(14) R. E. Rundle and D. French, THIS JOURNAL, **65**, 1707 (1943).

(15) When the $I_2$–$HIO_3$ reagent was added to amylose solutions, a small amount of rose-colored complex formed which slowly disappeared. To prevent formation of the colored complex, the amylose solutions were made 0.2 *M* in $HIO_3$ prior to mixing.

(16) W. W. Everett and J. F. Foster, THIS JOURNAL, **81**, 3464 (1959).

(17) J. Holló and J. Szejtli, *Periodica Polytech.*, **2**, 25 (1958); *C. A.*, **52**, 18231 (1958).

(18) F. J. Hybart, unpublished Ph.D. dissertation, University of Birmingham, 1952.

(19) R. S. Higginbotham, *Shirley Institute Memoirs*, **23**, 171 (1949).

ion. In systems containing amylopectin or glycogen, iodide would compete with polysaccharide for the available "molecular" iodine ($I_2$ or $I_4$). Although amylopectin or glycogen do not bind significant amounts of iodine from iodide-free solutions, it is conceivable that the changes in configuration of the polysaccharide resulting from triiodide complex formation may condition the polysaccharide for adsorption of molecular iodine. Based on experience with the Schardinger dextrins,[12] it was our expectation that the absorption of $I_2$ by starch should be accompanied by a measurable shift of the $I_2$ spectrum. Manners[7] has reported that a number of highly branched glycogens had visible absorption maxima ranging from 420 to 470 m$\mu$.

In the absence of $HIO_3$, the spectrum of the glycogen-$I_2$ solution (Fig. 4, curve B) was similar to that of the $\alpha I_2$ solution (Fig. 3, curve D). Although there is a significant amount of glycogen $I_3^-$ complex formed, the small shift in the $I_2$ spectrum (3 to 5 m$\mu$) was not significant. This visible maximum when corrected for absorption by glycogen $I_3^-$ (assuming $\epsilon_{I_3^-}$ equal to that of glycogen $I_3^-$) produced a peak identical to that produced by $I_2$ within experimental error ($\epsilon_{max}$ $I_2$ = 460 m$\mu$) and suggested that $I_2$ was not bound by glycogen, at least not as a helical complex. When $HIO_3$ was added to a glycogen–$I_2$ solution (Fig. 4, curve A), no spectral shift of $I_2$ was encountered. Therefore, spectral shifts reported to accompany the addition of glycogen to $I_2$ solutions may most likely be attributed to the formation of glycogen $I_3^-$ or polyiodide complexes.

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[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS, MINNESOTA]

# Reactivity of Sulfhydryl and Disulfide in Proteins. V. Reversal of Denaturation of Bovine Serum Albumin in 4 *M* Guanidine Hydrochloride or 8 *M* Urea and of Splitting of Disulfide Groups in 4 *M* GHCl

BY I. M. KOLTHOFF, ADA ANASTASI[1] AND B. H. TAN

RECEIVED JANUARY 25, 1960

The protein obtained after denaturation of bovine serum albumin (BSA) in 4 *M* guanidine hydrochloride or 8 *M* urea at *p*H 5 at 25° and appropriate dilution has the same viscosity, specific optical rotation and reactive disulfide (zero) as native protein, provided the dilutions are made within one hour of standing of the denaturation mixtures. This reversibility is instantaneous upon dilution of the denaturation mixture in 4 *M* GHCl but is attained only after 15 minutes upon dilution of a denaturation mixture in urea. After longer times of standing exchange reactions between sulfhydryl and disulfide make the denaturation irreversible with regard to the above properties. In 4 *M* GHCl at *p*H 5 the reaction between BSA and sulfite

$$P\left\langle\left(\begin{matrix}S\\S\end{matrix}\right)_m + n\ SO_3^= \rightleftharpoons P\left\langle\left(\begin{matrix}S^-\\SSO_3^-\end{matrix}\right)_n\right\langle\left(\begin{matrix}S\\S\end{matrix}\right)_{m-n}$$

was allowed to proceed to the right and then from right to left by removal of sulfite (or bisulfite). The reversal was found quantitative, all disulfide groups were reformed and by extrapolation the intrinsic viscosity was found the same as that of untreated BSA in 4 *M* GHCl, provided the protein concentration was less than 0.25%. At higher concentrations crosslinking reactions occur during the reversal.

Part of the work described in this paper deals with the reversal to the native state of bovine serum albumin (BSA) after denaturation in 4 *M* guanidine hydrochloride (GHCl) or 8 *M* urea and dilution to such a concentration that the GHCl or urea has no measurable denaturing effect. In addition to viscosity and optical rotation the reactivity of disulfide groups with sulfite served as indicators for the degree of reversal from the denatured to the native state. In previous papers[2,3] it has been shown that after denaturation of BSA in 4 *M* GHCl or 8 *M* urea and appropriate dilution the sulfhydryl group is not oxidized by oxygen or ferricyanide while it is oxidized readily in the denatured state. Since sulfhydryl is not oxidized in the native state, the denaturation can be reverted as far as this property is concerned.

A more extensive study has been made of the reversal of the splitting of disulfide groups with sulfite in BSA at *p*H 5 in 4 *M* GHCl solution. For low molecular weight disulfides (RSSR) the reaction $RSSR + SO_3^= \rightleftarrows RSSO_3^- + RS^-$ is reversible.[4,5] The breaking of all 17 or part of the disulfide bonds in denatured BSA with sulfite is accompanied by a very marked structural change as evidenced by a large increase of the intrinsic viscosity.[6,7] If the reaction could be forced back

$$P\left\langle\left(\begin{matrix}S\\S\end{matrix}\right)_m + n\ SO_3^= \rightleftharpoons P\left\langle\left(\begin{matrix}S^-\\SSO_3^-\end{matrix}\right)_n\right\langle\left(\begin{matrix}S\\S\end{matrix}\right)_{m-n} \quad (1)$$

quantitatively from right to left, it is hardly to be expected that the S–S bonds can be reformed in the original position. In the present study disulfide bonds were broken at *p*H 5 and the reaction reversed quantitatively by removing sulfite (for convenience sulfite refers to all charge forms of sulfurous acid), using a procedure described in the experimental part. The intrinsic viscosity and the optical rotation of the reverted protein were com-

(1) On leave from S. A. Farmitalia, Milano, Italy.

(2) I. M. Kolthoff, Ada Anastasi, W. Stricks, B. H. Tan and G. S. Deshmukh, THIS JOURNAL, **79**, 5102 (1957).

(3) I. M. Kolthoff and Ada Anastasi, *ibid.*, **80**, 4248 (1958).

(4) W. Stricks and I. M. Kolthoff, *ibid.*, **73**, 4569 (1951).

(5) C. Cecil and J. R. McPhee, *Biochem. J.*, **59**, 234 (1955); **60**, 496 (1955); McPhee, *ibid.*, **64**, 22 (1956).

(6) I. M. Kolthoff, Ada Anastasi and B. H. Tan, THIS JOURNAL, **80**, 3235 (1958).

(7) I. M. Kolthoff, Ada Anastasi and B. H. Tan, *ibid.*, **81**, 2047 (1959).