24493-40-1; 1,8-cineole, 470-82-6; (−)-camphor, 464-48-2; filifolone, 4613-37-0; isophorone, 78-59-1; α-fincholenic acid, 32082-53-4; α-fincholenic acid iodolacetone, 4627-35-4; (+)-chrysanthenone, 38301-80-3; (−)-chrysanthenone epoxide, 50763-19-4; (+)-verbenone, 18309-32-5.

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Mechanism of Cystine Racemization in Strong Acid

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Cystine (1) is the only naturally occurring amino acid which is racemized to a significant extent under the conditions commonly used for hydrolysis of proteins. A study of the mechanism of the racemization of cystine in refluxing 6 *N* HCl is reported. Various analog studies show that the racemization mechanism involves the formation of an acid enol which is stabilized by the inductive effect of a partially or fully charged β-heteroatom. In general, the requisite charge density may be induced through resonance as in the case of *S*-(2,4-dinitrophenyl)-L-cysteine or through protonation as in the case of 2,3-diaminopropionic acid, both of which racemize in acid at rates comparable to that of cystine. However, in the case of cystine, enolization does not occur in the intact molecule as a result of disulfide protonation. Rather, the intermediates in a concurrent, acid-catalyzed, disulfide interchange reaction are implicated as the species which undergo racemization.

All of the α-amino acids undergo some racemization during acid hydrolysis of protein, and the degree to which the various amino acids racemize has been quantitated by tritium incorporation experiments.[1] In the case of every naturally occurring amino acid but one, cystine (1), the rate of racemization is vanishingly slow under normal hydrolysis conditions. Cystine, on the other hand, is almost completely racemized after 120-hr exposure to refluxing 6 *N* HCl. When cystine, the first amino acid discovered, was initially isolated from kidney stones in 1810,[2] it was obtained in a relatively high state of optical purity, as the isolation did not involve extended heating in strong acid. It was not until 89 years after its discovery that cystine was isolated from horn hydrolysate[3] and shown to be the source of much of the sulfur known to be present in protein. In this very early work, the rotation of the cystine isolated was found to be dependent upon the duration of hydrolysis. In 1902,[4] the correct structure of cystine was elucidated and it was shown to be racemized completely by heating in hydrochloric acid. The existence of D, L, and meso forms was proposed in the inactive material, and all three forms were isolated in 1933.[5]

Somewhat surprisingly, in none of the above work or in other studies of this unique racemization[6] is there comment or speculation about the mechanism of the reaction. In the course of preparing a sample of DL-cystine for a separate study, our curiosity was aroused by the anomalous rate at which L-cystine racemized in acid, and we have examined this reaction with the objective of understanding its mechanism.

The decrease in rotation of a solution of cystine in refluxing 6 *N* HCl is first order in amino acid and has a half-life of about 20 hr. Unfortunately, dependence of the rate on acid concentration could not be fully investigated because of the rapid decomposition of cystine which occurs in more dilute, refluxing acid. Specifically, it was found that in refluxing 1 or 3 *N* HCl rather rapid decomposition occurred to give cysteine, alanine, glycine, and other products, production of which occurred rapidly enough to render racemization rate measurements meaningless. The extent of decomposition which occurred during racemization experiments was monitored by amino acid analysis and it was found that in 5 *N* or stronger HCl, decomposition occurred only very slowly.

The effect of the conjugate base of the mineral acid on racemization rate was investigated by comparing the results of experiments conducted in HCl, HBr, and H_2SO_4 at 110° (sealed tube) and constant H_0. Though these acids have widely variant anion nucleophilicities, the rate of racemization was essentially constant for all three. This ob-

servation may be interpreted to indicate that nucleophilic attack by the conjugate base is not involved in the rate-determining step of the racemization reaction.

In order to establish the effect of various structural parameters on the rate of racemization, a variety of analog amino acids were subjected to refluxing 6 *N* HCl. The tabulated results of these experiments appear in Table I, in which the amount of racemization is indicated as per cent decrease in optical activity over 20 hr, approximately the optical half-life of cystine under these conditions.

Cysteine (**2**) and *S*-methylcysteine (**3**) do not undergo appreciable racemization under the standard conditions. These amino acids differ from cystine to the extent that they do not incorporate either a disulfide linkage or a second intramolecular α-amino acid group. Hence, their lack of reactivity implicates one of these two structural parameters in the cystine molecule.

If the inclusion of a companion intramolecular α-amino acid function is the key to the reactivity of cystine, replacement of one of the disulfide sulfur atoms by a methylene group should have no effect on racemization rate and should result in a structure that would racemize significantly more rapidly than normal amino acids. In fact, such a compound, cystathionine (**4**), was prepared. Neither cystathionine or the analogous lanthionine (**5**) racemize appreciably faster than alanine. In addition, the ω-carboxy amino acids aspartic (**9**) and glutamic (**10**) do not racemize nor does the ϵ-amino acid, lysine (**11**). These results clearly indicate that inclusion of an ω-amino or carboxyl group, or both, is not sufficient to activate the molecule toward the acid-catalyzed racemization which cystine undergoes.

The results above implicate the disulfide linkage as the activating structural parameter. Hence, a number of structural analogs of cystine, which include the disulfide linkage, were prepared for testing. Homocystine (**6**), in which the disulfide linkage is insulated from the chiral carbons by two methylene groups, does not racemize to a significant extent. Penicillamine disulfide (**7**), which can be considered a β-tetramethyl analog of cystine, does not racemize.

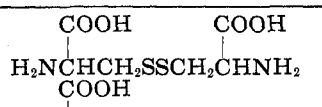
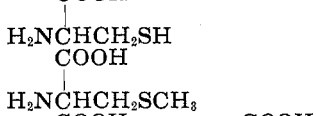
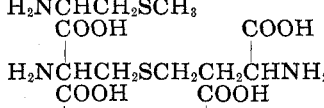
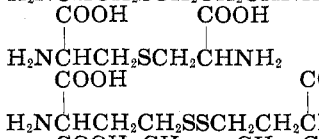
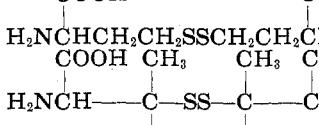
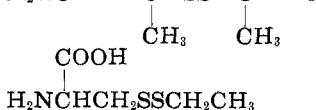
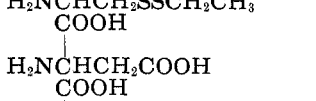
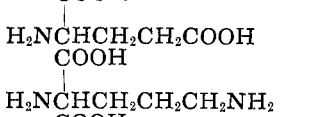
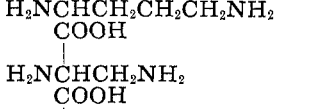
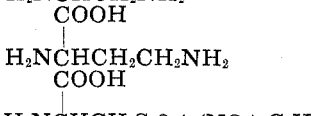
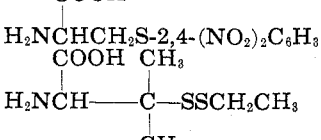
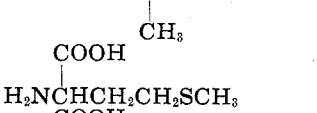
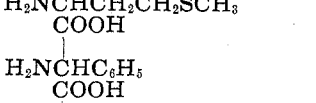
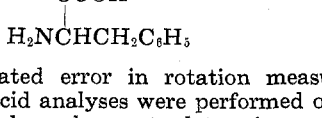
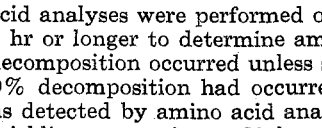
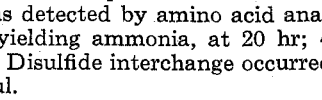
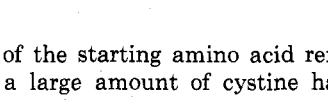
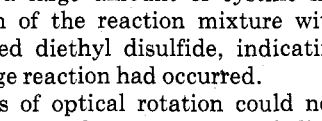
The inability of penicillamine disulfide to racemize under conditions which result in rapid racemization of cystine could be the result of steric inhibition of the mechanism or could result from absence of the β hydrogens present in cystine. If, for example, enamine formation was the source of the racemization, replacement of the β hydrogens by methyl groups would prevent the occurrence of reaction.

In order to ascertain whether the racemization of cystine involved the β protons, L-cystine was refluxed in 6 *N* DCl–D_2O. After 70 hr (3+ half-lives), nmr analysis of the isolated cystine showed that over 80% exchange had occurred at the α position but no measurable exchange (less than 3%) had occurred at the β position. Clearly, racemization does not involve an enamine-like intermediate or any other structure which requires rehybridization of the β carbon. It would appear, therefore, that penicillamine disulfide does not racemize either because of some steric inhibition or because of the inductive effect of the β-methyl groups.

In order to gain further insight into the question of steric inhibition to racemization in penicillamine disulfide, *S*-(ethylthio)-L-cysteine (**8**) was subjected to the reaction conditions. This unsymmetrical disulfide incorporates the requisite disulfide linkage and retains the unsubstituted β carbons. When **8** was refluxed in 6 *N* HCl, a loss of rotation occurred at a rate similar to that of cystine. However, amino acid analysis of the reaction mixture showed that only 20% of the starting amino acid remained after 55 hr and that a large amount of cystine had been produced. Extraction of the reaction mixture with methylene chloride yielded diethyl disulfide, indicating that a disulfide interchange reaction had occurred.

The loss of optical rotation could not be attributed to racemization of the unsymmetrical disulfide, however, as we found that the interchange reaction is rapid compared to loss of rotation. In fact, within 1 hr, an equilibrium

Table I
Amino Acid Racemization Rates

No.	Compd	% loss optical rotation at 20 hr[a]
1		50[b]
2		0
3		0[b]
4		5[b,c]
5		5[b,d]
6		5[b]
7		0[b]
8		*b, f*
9		5[b]
10		5[b]
11		0
12		86[b]
13		5
14		38[b,e]
15		*b, f*
16		5
17		17
18		0

[a] Estimated error in rotation measurements is ±5%. [b] Amino acid analyses were performed on the refluxed solution at 20 hr or longer to determine amount of decomposition; no decomposition occurred unless specified. [c] Approximately 40% decomposition had occurred at 72 hr. [d] Meso isomer was detected by amino acid analysis. [e] 11% decomposition, yielding ammonia, at 20 hr; 45% loss of optical activity. [f] Disulfide interchange occurred; rotation data not meaningful.

mixture of disulfides is produced. By chance, the molecular rotation of 8 (234°) is almost exactly half that of cystine (466°) in 6 *N* HCl. Therefore, conversion of two molecules of 8 into one molecule of cystine by disulfide interchange would not in itself cause a change in the magnitude of the measured rotation if no racemization occurred during the interval required for interchange. This reaction might be considered to have simply produced a solution of cystine which then racemized at its normal rate.

The experiment described above led to attempts to determine whether there is a mechanistic relationship between the disulfide interchange reaction and racemization. The mechanism of disulfide interchange in acid is not clearly understood. Initially, it was proposed that the reaction proceeded *via* a sulfenium ion mediated chain reaction.[7] More recent work[8] suggests that this mecha-

$$\text{initiation}\quad RSSR + H^+ \longrightarrow RSH + RS^+ \qquad (1)$$

$$\text{interchange}\quad RS^+ + R'SSR' \longrightarrow RSSR' + R'S^+ \qquad (2)$$

nism is incorrect, as some evidence indicates that the rate-determining step involves nucleophilic displacement by acid anion on a sulfur activated by a prior electrophilic attack. In addition, homolytic cleavage and a resulting

$$X^- + RS\overset{+}{S}(H)R \longrightarrow RSX + RSH \qquad (3)$$

radical mechanism are known to occur under some conditions.[9] None of these mechanisms are consistent with all of the data; perhaps all are operative.

In any case, cystine and most other disulfides are certainly undergoing some interchange reaction in acid solution. Demonstration of this interchange in a symmetrical disulfide such as cystine would require an isotopic labeling experiment, which has not been carried out. It has been shown, however, that *N,N'*-bis(2,4-dinitrophenyl)-cystine interchanges with cystine itself[10] and we have demonstrated the rapid interchange of cystine with diethyl disulfide. There is little doubt that interchange occurs upon heating cystine in acid. The question of a mechanistic relationship between this interchange reaction and racemization led to a new series of experiments.

Since it seemed that the disulfide linkage and/or its associated interchange intermediates might be in some way the source of the species prone to racemization, we attempted to uncouple the interchange and the racemization. That is, a set of experiments was designed to determine whether racemization could occur without interchange and vice versa.

Since the existence of a free radical interchange mechanism has not been eliminated, we attempted to inhibit a presumed homolytic interchange in order to study the effect of interchange inhibition on racemization rate. When 10 mol % of hydroquinone was added to *S*-(ethylthio)-L-cysteine (8) and the resulting solution was refluxed in 6 *N* HCl in the absence of air and light, the interchange rate was the same as that observed for a sample refluxed open to the air, in the light, and without the radical scavenger. Though this type of experiment is not completely conclusive, it must be interpreted to mean that either the interchange is not primarily free radical in mechanism or that our inhibition conditions were inadequate.

It has been noted in an earlier experiment that penicillamine disulfide does not racemize at a rate even near that of cystine. Yet penicillamine disulfide (7) differs from cystine only by inclusion of the β-methyl groups. Since β hydrogens are not necessary for the racemization is indicated by the isotope-exchange experiment, it was postulated that the methyl groups might, in some way, prevent the interchange reaction and thereby the racemization. We found that penicillamine disulfide (7) does, in fact undergo disulfide interchange but at a rate much slower than that of cystine. This was demonstrated by refluxing each of these disulfides in 6 *N* HCl to which a quantity of diethyl disulfide had been added. At intervals, the reaction mixtures were analyzed (by amino acid analysis) for the production of the corresponding unsymmetrical disulfides, the concentration of which, as a function of time, was a measure of interchange rate. It was found that cystine undergoes interchange with diethyl disulfide about ten times as fast as penicillamine disulfide does.

We have shown then, that the exchange rate and the racemization rate have a certain correlation. That is, penicillamine disulfide racemizes more slowly than cystine, and it also exchanges more slowly than cystine. This observation does not prove a direct relationship between the two reactions; however, a common intermediate may be involved in both racemization and interchange, with the activation energy for the interchange being lower than that for racemization.

A crossover experiment was conducted to determine whether the presence of the intermediates in the interchange reaction (*e.g.*, sulfenium ions) could induce racemization *via* intermolecular reaction. This experiment consisted of refluxing mixtures of racemic cystine with L-alanine, with L-cysteine, and with *S*-methyl-L-cysteine. In none of these three cases was there any evidence of an increased racemization rate. Therefore, the interchange intermediates are not capable of promoting racemization *via* an intermolecular reaction.

Both of the postulated ionic mechanisms for disulfide interchange in acid, though different, involve an intermediate in which the cysteinyl residue bears a full or partial positive charge on the sulfur atom. Thus, it is possible that the electron-withdrawing inductive effect of a charged sulfur atom at the β position might be sufficient to increase the acidity of the α proton enough to lead to racemization. The likelihood of such a proposal is buoyed by the fact that the proton on the chiral carbon is already exposed to the inductive effect of protonated, geminal amino and carboxyl groups. Thus, the combination of strong electron-withdrawing groups (NH_3^+, S^+, COOH)[11] and the potential for stabilization of the conjugate base by acid enol formation may well be responsible for the rapid racemization of cystine. However, since one would not expect the disulfide to be more basic than the thio ether,[12] and since neither *S*-methyl-L-cysteine, the corresponding thioether, or cysteine, the corresponding mercaptan, racemize under conditions which racemize cystine, simple protonation of the β sulfur cannot be the source of the charged intermediate. This intermediate must then arise as a result of the interchange reaction, a path uniquely available to disulfides.

The hypothesis above would predict that 2,3-diaminopropionic acid (12) should racemize as rapidly as cystine, if not faster. Consistent with the hypothesis, 2,3-diaminopropionic acid was found to racemize at a dramatic rate. In addition, the insulating effect of a single methylene group, as in 2,4-diaminobutyric acid (13), renders the molecule inert to racemization. This observation is consistent with the similar observations on cystine and homocystine. It is also consistent with the observation that phenylglycine (17) racemizes while phenylalanine (18) does not. In the case of 17, the acid enol is stabilized by phenyl conjugation, resulting in rapid racemization. In 18, insulation by the methylene group is sufficient to confer optical stability.

The extent to which the sulfur must carry charge is demonstrated in the observation that *S*-(2,4-dinitro-

phenyl)-L-cysteine (14) racemizes at a rate only slightly slower than that of cystine. Since cysteine itself does not racemize, it is difficult to postulate a mechanism that does not depend upon the influence of the charged sulfur. It would seem that the rate may be dependent upon the degree of charge induction and that the actual cystine intermediate must have a degree of charge somewhere between that induced by the 2,4-dinitrophenyl group and the full charge on the protonated nitrogen of 2,3-diaminopropionic acid.

In summary, it is proposed that in refluxing 6 *N* HCl, cystine is undergoing fairly rapid disulfide interchange and that some intermediate in this exchange reaction carries at least a partial positive charge on sulfur. The combined inductive effect of geminal carboxyl and protonated amino groups together with the added boost from this charged sulfur renders the α proton sufficiently acidic to allow acid enol formation, resulting in racemization. The racemization reaction is far slower than the interchange reaction but factors which increase or decrease the interchange rate likewise affect the racemization rate.

Experimental Section

Melting points are uncorrected. Amino acid analyses were performed on a Beckman Model 120C amino acid analyzer. Optical rotations were obtained on a Bendix ETL-NPL automatic polarimeter, Type 143A, using an all-glass 2-cm cell. Elemental analyses were performed by the Analytical Laboratory, University of California, Berkeley.

Materials. L-Methionine, L-cystine, D-penicillamine, L-cysteine, L-aspartic acid, L-glutamic acid, L-2,4-diaminobutyric acid, L-lysine, and L-phenylglycine were obtained from commercial sources and used as supplied. The following amino acids were synthesized by standard literature procedures.

D-Penicillamine disulfide (7) was prepared by O_2 oxidation of D-penicillamine,[13] mp 199–201° (lit.[14] mp 204–205°).

S-Methyl-L-cysteine (3) had mp 240° (lit.[15] mp 247–248°), $[\alpha]^{20}D$ − 10° (*c* 1, 1 *N* HCl) [lit.[13] $[\alpha]^{20}D$ − 9.6° (*c* 1, 1 *N* HCl)].

L-Lanthionine (5) was prepared from serine and cysteine, mp 297–299° (lit.[16] mp 293–295°), $[\alpha]^{20}D$ + 6° (*c* 1, 1 *N* NaOH) [lit.[16] $[\alpha]^{20}D$ + 6° (*c* 1, 1 *N* NaOH)].

L-Cystathionine (4) had $[\alpha]^{20}D$ − 21.5° (*c* 1, 1 *N* HCl) [lit.[17] $[\alpha]^{20}D$ − 23.5° (*c* 1, 1 *N* HCl)].

S-(2,4-Dinitrophenyl)-L-cysteine hydrochloride (14) was prepared from cysteine and 2,4-dinitrofluorobenzene at pH 4.2.[18] Further purification was necessary by cation-exchange chromatography (Bio-Rad AG50W-X8, H^+ form, 200–400 mesh), mp 180–181° (lit.[18] mp 182–184°), $[\alpha]^{20}D$ + 82° (*c* 1, 6 *N* HCl).

L-Homocysteine had mp 255–265° (lit.[19] mp 281–284°), $[\alpha]^{20}D$ + 69° (*c* 1, 1 *N* HCl) [lit.[19] $[\alpha]^{20}D$ + 79° (*c* 1, 1 *N* HCl)].

L-2,3-Diaminopropionic acid (12) was purified by cation-exchange chromatography (Bio-Rad AG50W-X8, H^+ form, 200–400 mesh), $[\alpha]^{20}D$ + 22.5° (*c* 1, 1 *N* HCl) [lit.[20] $[\alpha]^{20}D$ + 25° (*c* 1, 1 *N* HCl)].

S-(Ethylthio)-L-cysteine (8) was prepared by reaction of cysteine with diethyl sulfenyl thiocarbonate.[21] Cysteine hydrochloride monohydrate (8.73 g, 50 mmol) was added to 100 ml of ethanol–water (2:1). To this solution was added diethyl sulfenyl thiocarbonate (8.4 g, 50 mmol) dissolved in 50 ml of ethanol and the reaction mixture was stirred vigorously under nitrogen at room temperature after 3 drops of triethylamine was added. Stirring was continued for 5 hr followed by evaporation of the solvent and crystallization of the residue from aqueous ethanol, yield 5 g, mp 190–193°, $[\alpha]^{20}D$ + 129° (*c* 1.5, 6 *N* HCl).

Anal. Calcd for $C_5H_{11}NO_2S_2$: C, 33.1; H, 6.1; N, 7.7; S, 35.4. Found: C, 33.2; H, 6.1; N, 7.7; S, 35.2.

Ethyl penicillamine disulfide (15) was prepared essentially as 8, in methanol at 50° for 7 hr, from D-penicillamine hydrochloride (5.07 g, 27 mmol) and diethyl sulfenyl thiocarbonate (4.53 g, 27 mmol) and was crystallized from ethanol–ether, mp 148–151°, $[\alpha]^{20}D$ + 111° (*c* 1, 1 *N* HCl).

Anal. Calcd for $C_7H_{16}NO_2S_2Cl$ (0.6 mole fraction as HCl salt): C, 36.7; H, 6.8; N, 6.1. Found: C, 36.9; H, 7.1; N, 5.9.

DCl–D_2O was prepared essentially as described,[22] except that both SO_2 traps were at Dry Ice temperature and the DCl–D_2O was boiled to remove residual SO_2. Nmr of the DCl–D_2O solution showed it to be as isotopically pure as the D_2O used in the hydrolysis, 99.8%.

Table II
Rates of Disulfide Interchange with $CH_3CH_2SSCH_2CH_3$

Time, min	% of cystine (1) interchanged	% of penicillamine disulfide (7) interchanged
0	0	0
5	10	1
10	24	4
20	39	9
77	42	27
192	42	51

Racemization Rate Studies. In a typical experiment, 100 ml of 6 *N* HCl in a round-bottom flask equipped with reflux condenser was brought to reflux (110°) in a thermostated (120°) oil bath. The flask was removed from the bath and cooled to about 100°, and about 1.0 g of the amino acid was added; immediately the flask was returned to the oil bath, whereupon refluxing ensued within a few minutes. All of the amino acid dissolved within the first few minutes and, as soon as solution was effected, a 5-ml aliquot was removed and cooled to room temperature in an ice bath, and the initial rotation measured. Subsequent aliquots were obtained and their rotations were measured in a similar manner.

Rates of Disulfide Interchange. The relative rates of disulfide interchange between cystine (1) and penicillamine disulfide (7) and diethyl disulfide were measured in the following manner. Disulfide 1 (0.25 g) or 7 (0.31 g) was placed in 25 ml of 6 *N* HCl and the solution was brought to reflux (110°) in a thermostated (120°) oil bath. At time t = 0, pure diethyl disulfide (1.1 g) was injected by syringe into the refluxing solution and the flask was shaken. Some of the diethyl disulfide dissolved, but the solution remained heterogeneous. An aliquot of the aqueous solution was removed at the appropriate time, cooled to about 0°, diluted (1:20 in pH 2.2 buffer), and immediately placed on the column of the amino acid analyzer. The results of these disulfide interchange studies are given in Table II.

Registry No.—**1**, 56-89-3; **2**, 52-90-4; **3**, 1187-84-4; **4**, 6899-07-6; **5**, 922-55-4; **6**, 462-10-2; **7**, 20902-45-8; **8**, 17885-24-4; **9**, 56-84-8; **10**, 56-86-0; **11**, 56-87-1; **12**, 4033-39-0; **13**, 1758-80-1; **14**, 23815-63-6; **15**, 38261-80-2; **16**, 63-68-3; **17**, 2935-35-5; **18**, 63-91-2; diethyl sulfenyl thiocarbonate, 30453-25-9; L-cysteine hydrochloride, 52-89-1; D-penicillamine hydrochloride, 2219-30-9; diethyl disulfide, 110-81-6.

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