3.5 *N* hydrochloric acid (10:3) as the developing solvent.[15] With this solvent the Cinchona alkaloids had the following R_f values: cinchonidine 0.52, cinchonine 0.56, quinine 0.58, quinidine 0.70. Radioactivity was detected on the paper at positions corresponding to these alkaloids. The crude alkaloid was dissolved in benzene and chromatographed on Woelm alumina (activity II–III). Elution was carried out with benzene followed by mixtures of benzene and chloroform. The fractions obtained by elution with a 1:1 mixture of benzene and chloroform contained quinine (detected by thin layer chromatography on basic silica gel). These fractions were combined (76 mg.) and crystallized twice from benzene, then from a mixture of benzene and petroleum ether. Finally the dried quinine was sublimed (160–165°, 0.001 mm.) affording 34.4 mg. of alkaloid, m.p. 174–175°. For degradation and further purification this quinine was diluted 25 times and crystallized from benzene. The diluted quinine (564 mg.) was dissolved in ethanol (10 ml.) and dilute sulfuric acid (4.25 ml. of a 2% solution). The solution was evaporated to dryness, water (25 ml.) added and boiled, adding two more drops of the 2% sulfuric acid. The hot solution was filtered and on cooling colorless needles of quinine sulfate ($B_2.H_2SO_4.2H_2O$) separated. After several crystallizations from hot water this material had a constant specific activity.

Oxidation of the Quinine sulfate-C^{14}.—Quinine sulfate (488 mg.) was dissolved in 10% sulfuric acid (10 ml.) and manganese dioxide (105 mg.) added. The mixture was raised to the boiling point and chromium trioxide (1.0 g.) dissolved in water (2 ml.) added during half an hour. The refluxing was continued for 3 hr. Hot water (90 ml.) and 15 *N* ammonia (20 ml.) were then added and after standing for 18 hr. on a steam-bath the mixture was filtered with the aid of Celite. The residue was extracted several times with hot dilute ammonia solution. The combined filtrates were evaporated to small bulk (15 ml.) and acidified with acetic acid yielding quininic acid (207 mg., 83%).

Decarboxylation of the Quininic Acid and Phenylation of the 6-Methoxyquinoline.—Dry quininic acid (205 mg.) was mixed with copper chromite catalyst[16] (205 mg.) and heated in a stream of carbon dioxide free nitrogen in a metal bath at 250–260° for 45 min. Carbon dioxide was liberated rapidly above 240° and was collected as barium carbonate by passing into 3% aqueous barium hydroxide solution. The metal bath temperature was then raised to 310° when 6-methoxyquinoline distilled as a pale yellow oil. This oil was washed out with toluene and dried over magnesium sulfate. The 6-methoxyquinoline dissolved in a 1:1 mixture of toluene and diethyl ether (5 ml.) was added at room temperature during 5 min. to a stirred solution of phenyllithium which had been prepared from lithium (22 mg.), bromobenzene (0.15 ml.) and ether (1 ml.). The yellow reaction mixture was then stirred at 80° for 5 hr. when a pale yellow solid separated. Finally the mixture was refluxed at 120° for 1 hr. After cooling, water was added and the organic layer extracted several times wih 2 *N* hydrochloric acid. The combined aqueous extracts were made basic with potassium hydroxide and extracted with chloroform. The dried chloroform on evaporation yielded a pale yellow solid which was sublimed *in vacuo* (120°, 0.001 mm.). Crystallization of the sublimate from aqueous ethanol yielded colorless plates of 6-methoxy-2-phenylquinoline (108 mg., 50%), m.p. 133–134°, not depressed on admixture with an authentic specimen.[10]

Anal. Calcd. for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.56; H, 5.81; N, 6.06.

Methylation and Oxidation of the 6-Methoxy-2-phenylquinoline.—6-Methoxy-2-phenylquinoline (51 mg.) was refluxed in methanol (20 ml.) with methyl iodide (3 ml.) in the presence of sodium carbonate (200 mg.) for 24 hr. Additional methyl iodide (1-ml. portions) were added 2, 8 and 16 hr. after the start of the reaction. The solvent was then removed and the residue dissolved in water (20 ml.) containing sodium carbonate (100 mg.). Potassium permanganate (3 g.) was added and the mixture refluxed for a few minutes and then allowed to cool slowly to room temperature with stirring. After 16 hr. the mixture was acidified with sulfuric acid and decolorized with sulfur dioxide. Extraction of the clear yellow solution with chloroform yielded benzoic acid which was purified by sublimation and crystallization from water (3.3 mg.); m.p. 121–122°, not depressed on admixture with an authentic specimen.

Activities of quinine and its degradation products are recorded in Table I, for the non-diluted material.

(15) D. J. Lussman, E. R. Kirch and G. L. Webster, *J. Am. Pharm. Assoc., Sci. Ed.*, **40**, 368 (1951).

(16) "Organic Syntheses," Coll. Vol. II, Ed. A. Blatt, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 142.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, N. Y.]

An Approach to the Specific Cleavage of Peptide Bonds. I. The Acyl Migration in Dipeptides Containing Hydroxyamino Acids in Anhydrous Hydrogen Fluoride

BY S. SAKAKIBARA,[1] K. H. SHIN AND G. P. HESS

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The N,O-acyl migration in dipeptides containing serine or threonine was studied in anhydrous hydrogen fluoride. In some instances this reaction was also studied in concentrated sulfuric acid. All experiments were followed by Van Slyke amino-nitrogen determinations, paper electrophoresis, and isolation, crystallization, and identification of the reaction products by classical means. The analytical procedures were standardized with synthetic N- and O-acyl peptides. The experiments presented indicate that the N,O-acyl migration in dipeptides proceeds in anhydrous HF in high yields (90%) without unspecific cleavage of amide bonds or the formation of significant amounts of side products. In contrast to this are the results obtained in sulfuric acid. In the case of glycyl-DL-serine, a maximum yield of 35% of the desired O-peptide was obtained and a great number of side products could be detected.

In view of the outstanding importance of obtaining amino acid sequences of proteins and the complex problems associated with the specific degradation of these molecules to small peptides amenable to structure determination, we have explored a method for the specific cleavage of peptide bonds using anhydrous hydrogen fluoride.

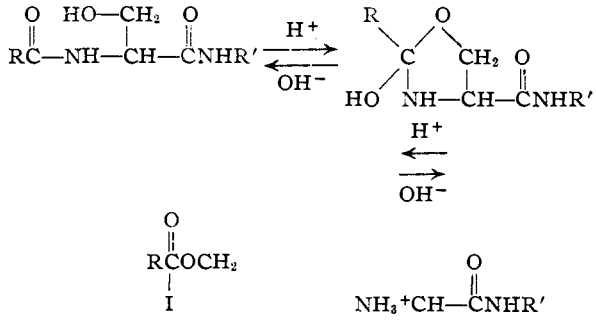
The reversible, *p*H dependent, N,O-acyl migration of β-amino alcohols was first investigated by Bergman[2a] in 1923; later studies[2b] suggested that a hydroxyoxazolidine (I) is the intermediate in this reaction.

Although in strong acids this reaction has previously been investigated with only two model peptides,[3] both containing serine, a great number of

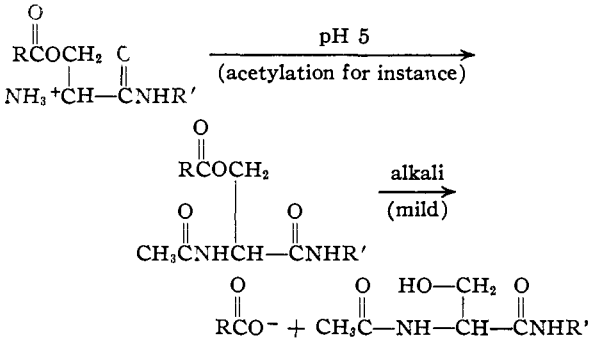
(1) Institute for Protein Research, Osaka University, Osaka, Japan.

(2) (a) M. Bergmann, E. Brand and F. Weinmann, *Hoppe-Seyler's Z. Physiol. Chem.*, **131**, 1 (1923); (b) A. P. Phillips and R. Baltzly, *J. Am. Chem. Soc.*, **69**, 200 (1947); W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).

(3) J. A. Moore, J. R. Dice, E. D. Nicolaides, R. D. Westland and E. L. Wittle, *J. Am. Chem. Soc.*, **76**, 2884 (1954); H. Hoermann, W. Grassman, E. Wuensch and H. Preller, *Ber.*, **89**, 933 (1956).



attempts have been made to induce acyl migrations in proteins by means of strong acids.[4] The acyl migration was then followed by attempts to block the newly formed amino groups at low *p*H and cleavage of the β-amino esters by mild alkaline[5] hydrolysis. The successful operation of these reactions leads to the specific cleavage of protein chains adjacent to seryl or threonyl residues.



Among the many conditions investigated for promoting acyl migration in proteins, the use of concentrated sulfuric acid appeared the most promising.[4-9] However, a great number of complications was encountered. The acyl migration occurred only in low yields, with unspecific hydrolysis of peptide bonds, sulfonation of some aromatic groups, and the formation of sulfate esters.[10] The modification of amide groups and of tryptophyl, phenylalanyl, tyrosyl, methionyl and cystinyl residues has been reported.[8,9]

Recent reports by Katz[11] suggested that anhydrous hydrogen fluoride is vastly superior to sulfuric acid as a solvent for proteins. In the instances investigated,[12] the biological activity of proteins was not altered irreversibly by anhydrous HF. Hydrogen fluoride (b.p. 19.5°) has the additional advantage over sulfuric acid in that it is easily removed from proteins dissolved in it. The fact that anhydrous HF is a very strong acid (the Hammett acidity function (H_0) is approximately -10)[13] suggested to us that the N,O-acyl migration might proceed in HF in good yields. This possibility has now been explored with the use of synthetic serine and threonine-containing peptides. For comparison purposes, some experiments were conducted using concentrated sulfuric acid as solvent.

Results and Discussion

The peptides or amino acid derivatives which were prepared for these studies are: glycyl-DL-serine, O-glycyl-DL-serine, acetyl-DL-serine, O-acetyl-DL-serine, glycyl-L-threonine and O-glycyl-L-threonine which has been synthesized for the first time.

In experiments using anhydrous hydrogen fluoride, about 0.1 g. of thoroughly dried peptide or amino acid derivative was placed in transparent Fluorothene (poly-(chlorotrifluoroethylene), Kel-F) reaction vessels which were attached to a monel vacuum line.[14] The details of the experimental procedure of the hydrogen fluoride and the sulfuric acid experiments are described in the Experimental section.

All experiments were followed by Van Slyke amino-nitrogen determinations and paper electrophoresis. Both analyses were performed on the starting material, the reaction mixture, and after treatment of the reaction mixture with bicarbonate to obtain the starting material. This procedure leads to the detection of hydrolysis products (amino-nitrogen value of reaction mixture after bicarbonate treatment minus amino-nitrogen value of starting material). However, because of the formation of side products, the Van Slyke amino-nitrogen determinations are reliable only in conjunction with other analytical techniques. Paper electrophoresis was used, and in some instances the experiments were further examined by a quantitative paper chromatography procedure, retention analysis, developed by Wieland.[15] The synthetic N- and O-acyl derivatives were used as standards for all procedures. The experiments in hydrogen fluoride were further substantiated by isolation and crystallization of the reaction products and their identification by classical means. Since hydrogen fluoride is a very weak acid in aqueous solutions, the compounds isolated from the HF reaction mixture are obtained either as the amines or the hydrochlorides, depending on the aqueous medium in which the products were dissolved.

The Van Slyke amino-nitrogen procedure was standardized with amino acids, the synthetic N- and O-acyl derivatives, and the O-acyl derivatives after treatment with bicarbonate. The data obtained can be found in Table I. Excellent agreement with the theoretical values were found in the case of amino acids, O-acetyl-serine and alanyl-serine. In the case of the glycyl dipeptides,

(4) For recent reviews see E. O. P. Thompson, in "Advances in Organic Chemistry," Interscience Publishers, Inc., New York, N. Y., 1960, p. 149; L. A. Cohen and B. Witkop, *Angew. Chem.*, **73**, 253 (1961); H. Zahn, *Chimia*, **15**, 378 (1961).

(5) D. F. Elliott, *Biochem. J.*, **50**, 542 (1952).

(6) P. Desnuelle and G. Bonjour, *Biochim. et Biophys. Acta*, **7**, 451 (1951).

(7) D. F. Elliott, in G. E. Wolstenholme and M. P. Cameron, eds., Ciba Symposium on "Chemical Structure of Proteins," Churchill, London, 1953, p. 129.

(8) L. Wiseblatt, L. Wilson and W. B. McConnell, *Can. J. Chem.*, **33**, 1295 (1955).

(9) L. K. Ramachandran and W. B. McConnell, *ibid.*, **33**, 1638 (1955).

(10) H. C. Reitz, R. E. Ferrel, H. Fraenkel-Conrat and H. S. Olcott, *J. Am. Chem. Soc.*, **68**, 1024 (1946).

(11) J. J. Katz, *Nature*, **178**, 265 (1954).

(12) J. J. Katz, *Arch. Biochem. Biophys.*, **51**, 293 (1954).

(13) H. H. Hyman, M. Kilpatrick and J. J. Katz, *J. Am. Chem. Soc.*, **79**, 3668 (1957).

(14) H. H. Hyman, Ph.D. Thesis, Illinois Inst. of Techn., 1960. A diagram of the line can be obtained from the Information Division, Argonne National Lab., Lamont, Ill., ANL negative #120-4450.

(15) Th. Wieland and L. Wirth, *Angew. Chem.*, **63**, 171 (1951).

the amino nitrogen values were higher than expected, in agreement with earlier reports.[16] It can also be seen from Table I that bicarbonate treatment of O-acetyl-DL-serine leads to complete conversion to the corresponding N-acyl derivative. Bicarbonate treatment of the O-glycyl-peptides gives values which are consistently too low. The time course of the O,N-acyl migration in sodium bicarbonate, as followed by Van Slyke amino-nitrogen determinations, can be seen in Fig. 1A.

TABLE I

VAN SLYKE AMINO-NITROGEN DETERMINATIONS OF AMINO ACID AND PEPTIDE STANDARDS

Compound	μMole used	Amino nitrogen found: Starting material μMole	%	After bicarbonate treatment[b] μMole	%
DL-Serine	11.1	11.0	99.2	...	...
L-Threonine	11.2	11.1	99.3	...	...
Glycine	17.6	17.7	100.6	...	...
Glycyl-DL-serine	12.4	14.3	115.0	...	...
O-Glycyl-DL-serine·HCl	503[a]	1005	99.8	564	97.5
Glycyl-L-threonine	12.7	14.9	117.0	...	...
O-Glycyl-L-threonine·HCl	452[a]	900	99.8	500	94.6
DL-Alanyl-DL-serine	12.8	12.6	98.5	...	...
O-Acetyl-DL-serine	13.3	13.3	100.0	0.0	100.0

[a] Refers to μmoles in 10-ml. aliquots taken for analysis contained between 10-20 μmoles of substance. [b] See text.

The amino-nitrogen values obtained with the standards were used to calculate the percentage acyl migration in hydrogen fluoride or concentrated sulfuric acid.

The N,O-Acyl Migration in Glycyl-DL-serine, DL-Alanyl-DL-serine and Glycyl-L-threonine in Hydrogen Fluoride.—A graphic representation of the time course of the conversion of glycyl-DL-serine to O-glycyl-DL-serine in hydrogen fluoride, as judged by Van Slyke amino-nitrogen determinations, appears in Fig. 1B. Equilibrium is reached fairly slowly and in 15 days 95% conversion to the O-peptide is obtained as judged by this method. The amino-nitrogen values obtained after treatment of the reaction products with bicarbonate (Fig. 1B) indicate a small amount of side products, possibly in equilibrium with the O-peptide. It can be seen (Fig. 1B) that the amino nitrogen value after bicarbonate treatment is initially lower than that of the starting material and then gradually approaches the theoretical value after a few days. This phenomenon, also observed with glycyl-L-threonine, is now under investigation. Nevertheless, the data indicate that the increase in amino-nitrogen content in hydrogen fluoride is due to the formation of the O-peptide and not due to the formation of hydrolysis products. Similar high conversion of the N-peptide to the O-peptide was obtained in the case of glycyl-L-threonine and DL-alanyl-DL-serine. The yields of O-peptide were 96 and 86%, respectively. The data are summarized in Table II. Since we were unable to prepare analytically pure, crystalline O-DL-alanyl-DL-serine HCl, the yields in this case were based on the theoretical amino-nitrogen value of the O-peptide.

(16) A. B. Kendrick and M. E. Hanke, *J. Biol. Chem.*, **132**, 739 (1940).

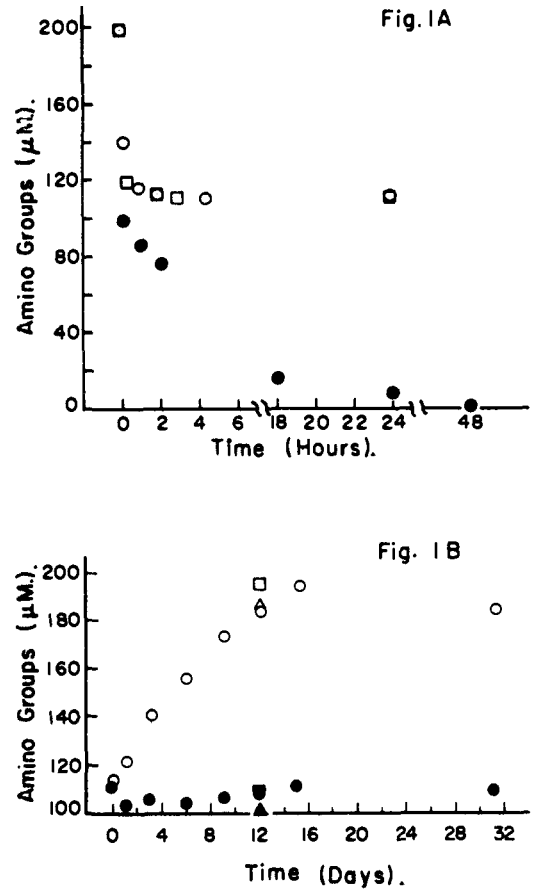


Fig. 1.—Time course of acyl migration in serine and threonine containing dipeptides and in O-acetyl-DL-serine: A, in 0.05 *N* sodium bicarbonate: O, O-glycyl-DL-serine; □, O-glycyl-L-threonine; ●, O-acetyl-DL-serine; B, in anhydrous hydrogen fluoride: O, glycyl-DL-serine; □, glycyl-L-threonine; △, DL-alanyl-DL-serine. The filled in symbols correspond to the amino-nitrogen values after bicarbonate treatment.

In Figs. 2 and 3 are presented the paper electrophoresis experiments with the synthetic peptides, the hydrogen fluoride reaction products, and the products obtained after bicarbonate treatment. These experiments substantiate the conclusions obtained from the amino-nitrogen determinations. The experiments reveal almost quantitative conversion of the N-peptides to the corresponding O-peptides in hydrogen fluoride. Only traces of side products could be detected in the reaction mixture. It should be noted in Figs. 2 and 3 that the synthetic peptides also contain traces of side products. The reason for this may be decomposition of the O-peptides during chromatography (see ref. 37). After bicarbonate treatment of the reaction products, only the N-peptide and traces of hydrolysis products were detectable by paper electrophoresis.

Finally, the reaction products were identified by classical means. Crystalline O-glycyl-DL-serine was isolated from a 15-day hydrogen fluoride re-

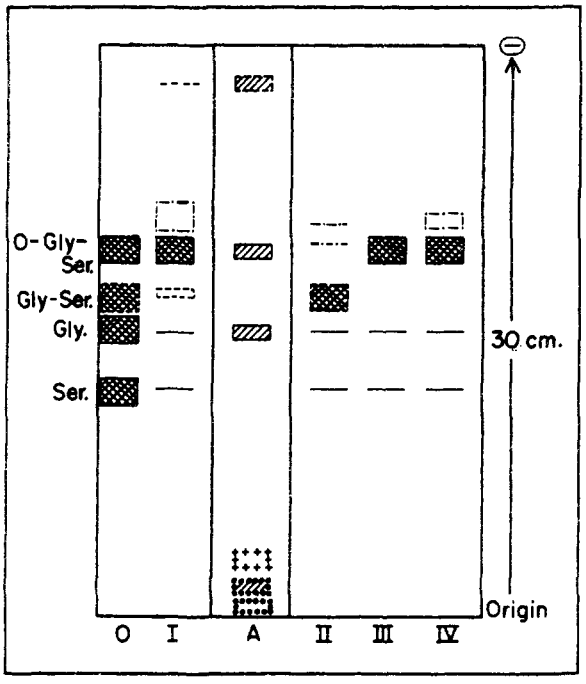


Fig. 2.—N,O-Acyl migration in glycyl-DL-serine; paper electrophoresis experiments: O, mixture of synthetic compounds; I, 12-day hydrogen fluoride reaction mixture; II, 12-day hydrogen fluoride reaction mixture after bicarbonate treatment (see text); III, synthetic O-glycyl-DL-serine·HCl; IV, crystalline O-glycyl-DL-serine·HCl isolated from 15-day hydrogen fluoride reaction mixture; A, 6-day concentrated sulfuric acid (see text) reaction mixture. Paper electrophoresis experiments were run in *N* acetic acid, 15 volts cm.$^{-1}$, at 0° for 3 hours. About 2 μmoles, calculated on the basis of starting material, was used in each experiment. Shading and size of spots denotes relative concentrations; lines denote traces of material. Lines surrounding spots denote color: ninhydrin purple ———; ninhydrin yellow with a change to purple after several hours - - - -; ninhydrin yellow with a change to purple after one day - · - · -; ninhydrin yellow ++++; ninhydrin yellow-brown · · · ·.

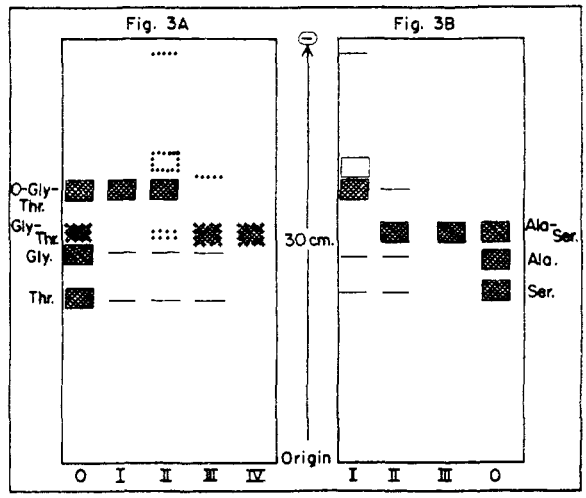


Fig. 3.—N,O-Acyl migration in glycyl-L-threonine and DL-alanyl-DL-serine; paper electrophoresis experiments: A, Experiments with glycyl-L-threonine: O, mixture of synthetic compounds; I, synthetic O-glycyl-L-threonine·HCl; II, 12-day hydrogen fluoride reaction mixture; III, 12-day hydrogen fluoride reaction mixture after treatment with bicarbonate; IV, crystalline glycyl-L-threonine isolated from 15-day hydrogen fluoride reaction mixture after treatment with triethylamine (see text); B, experiments with DL-alanyl-DL-serine: O, mixture of synthetic compounds; I, 12-day hydrogen fluoride reaction mixture; II, 12-day hydrogen fluoride reaction mixture after treatment with bicarbonate; III, crystalline DL-alanyl-DL-serine isolated from 15-day hydrogen fluoride reaction mixture after treatment with triethylamine (see text). Experiments were run in *N* acetic acid, 15 volts cm.$^{-1}$, at 0° for 3 hours. About 2 μmoles, calculated on the basis of starting material, was used in each experiment. Shading and size of spots denotes relative concentrations; lines denote traces of material. Lines surrounding spots denote color: ninhydrin purple, ———; ninhydrin yellow with a change to purple after several hours,. . . .; ninhydrin yellow with a change to purple after one day, ++++.

action mixture in 70% yield as the monohydrochloride and identified by classical procedures. After glycyl-L-threonine or DL-alanyl-DL-serine was allowed to stand in hydrogen fluoride for 15 days, the reaction products were treated with base and the dipeptides were obtained in crystalline form and identified. The results of the paper electrophoresis experiments with these crystalline compounds appear in Figs. 2 and 3. The specific rotation of glycyl-L-threonine ($[\alpha]^{23}D$ −16.3) and glycyl-L-threonine obtained by the bicarbonate-induced conversion of synthetic O-glycyl-L-threonine ($[\alpha]^{21}D$ −15.9°) are nearly the same. Similarly, treatment of the 12-day hydrogen fluoride reaction products with bicarbonate and crystallization of glycyl-L-threonine ($[\alpha]^{23}D$ −15.8°) indicated only small changes in specific rotation. Therefore, hydrogen fluoride, as other strong acids, does not racemize the β-carbon.[2,5] The infrared spectra of the allo and threo compound are quite different.[17] Threonine has an absorption band at 870 cm.$^{-1}$, while allothreonine has an absorption peak at 835 cm.$^{-1}$. Therefore, intactness of configuration of the β-carbon[2,5] could be further substantiated by comparison of the infrared spectra of synthetic glycyl-L-threonine with the compound isolated after hydrogen fluoride and bicarbonate treatment. The measurements of $[\alpha]D$ also indicate that significant racemization of the α-carbon is not obtained in hydrogen fluoride.

N,O-Acyl Migration in Glycyl-DL-serine in Concentrated Sulfuric Acid.—For comparison purposes the behavior of glycyl-DL-serine in concentrated sulfuric acid was studied in some detail. The results of these experiments appear in Table III and Fig. 2, experiment A. If one interprets the amino-nitrogen values only in terms of hydrolysis products (amino-nitrogen value of reaction products after bicarbonate treatment minus amino-nitrogen value of starting material), starting material and O-peptide, the yield of O-glycyl-DL-serine is at best 36% (Table III). The paper electrophoresis experiments (Fig. 2, experiment A) indicated the presence of a great number of side products. Some of these are due to the conversion

(17) R. J. Koegel, J. P. Greenstein, M. Winitz, S. M. Birnbaum and R. A. McCallum, *J. Am. Chem. Soc.*, **77**, 5708 (1955).

TABLE II

N,O-ACYL MIGRATION IN HYDROGEN FLUORIDE

Compound (μmoles in reacn. mixt.)	HF, ml.	Days of reacn.	Amino nitrogen, μmoles: Reaction mixture	O-Derivative, %[a]	Reacn. mixt. after bicarbonate treatment (see text)	N-Derivative, %[b]
Glycyl-DL-serine (617)	5	1	750	8	635	94
		3	875	32	640	94
		6	955	47	635	94
		9	1080	71	650	96
		12	1135	82	670	99
		15	1210	96	680	100
		31	1130	81	670	99
Glycyl-L-threonine (568)	5	12	1110	96	630	100
DL-Alanyl-DL-serine (568)	5	12	1040	86	570	101
N-Acetyl-DL-serine (680)	5	6[c]	10	1.5	...	...
		25	29	4.2 (5)[d]	...	...
	5[e]	1	50	7.2	4	99.4
		3	47	7.0 (6.3)[d]	8	98.8
	5[f]	3	54	8.0	..	..

[a] (μMoles of amino-nitrogen observed − μmoles amino-nitrogen of N-acyl compound) × 100/μmoles of amino-nitrogen of O-acyl compound − μmoles of amino-nitrogen of N-acyl compound). The amino-nitrogen values used for the N-acyl and O-acyl compounds were the experimentally determined values listed in Table I. The amino-nitrogen value of O-alanyl-serine was assumed to be twice the experimentally determined value of the N-peptide. [b] The amino-nitrogen value obtained after treating the synthetic O-acyl compound with bicarbonate was taken as 100% (see Table I). O-Alanyl-serine was assumed to be quantitatively converted to the N-peptide. The experimentally determined amino nitrogen value of alanyl-serine (see Table I) was taken as 100%. [c] Before the end of the experiment the reaction mixture was heated to 50° for 6 hours. [d] This value was obtained by retention analysis (see text). [e] Commercial HF without drying over cobaltous fluoride was used. [f] The reaction mixture contained 0.5 ml. of H_2O.

TABLE III

N,O-ACYL MIGRATION IN SULFURIC ACID

Compound (μmoles in reacn. mixt.)	H_2SO_4, ml.	Days of reacn.	Amino-nitrogen, μmoles: Reacn. mixture	O-Derivative, %[c]	Reacn. mixt. after bicarbonate treatment (see text)	N-Derivative %[d]
Glycyl-DL-serine (617)	2[a]	3	840	25	690	101
		6	920	40	720	106
		12	920	40	710	104
N-Acetyl-DL-serine (680)	2[a]	3	5.9	0.9	..	..
	2[b]	3	21	3.0	..	..

[a] Concentrated sulfuric acid (see text). [b] Commercial 96% sulfuric acid. [c] See footnote *a* in Table II. [d] See footnote *b* in Table II.

of the serine hydroxyl group to the sulfate ester.[18] Treatment of serine with sulfuric acid for only two hours under the conditions used in this experiment leads to the isolation of the crystalline O-serine sulfate ester in over 70% yield. It may therefore be noted in Fig. 2A, that while one can detect free glycine, free serine or the starting material are not discernible.

N,O-Acyl Migration in Acetyl-DL-serine in Concentrated Sulfuric Acid and in Hydrogen Fluoride.—Acetyl-serine has often been used as a model compound for studies of the N,O-acyl migration. However, the reaction has never been followed quantitatively. As can be seen from Tables II and III, the choice of this compound appears unfortunate. The maximum yield of O-acetyl-serine in HF is 8%, and in sulfuric acid 3%. The amino nitrogen value of the reaction products in hydrogen fluoride were further checked by a quantitative paper chromatography procedure.[18] Both methods give essentially the same data. The low yields of O-acetyl-DL-serine in sulfuric acid are not too surprising in view of two serious side reactions, the rapid conversion of N-acetyl-serine to the sulfate ester[18] and the ester interchange between O-acetyl-serine and sulfuric acid. When O-acetyl-DL-serine is placed in sulfuric acid under the conditions used in the experiments, the sulfate ester of serine can be isolated in crystalline form in 60% yield after two days of reaction. The low yield of O-acetyl-serine in hydrogen fluoride was unexpected in view of the results obtained with the dipeptides. The electronegativity of the free amino group of the dipeptides cannot be the only important difference, as far as N,O-acyl migration is concerned, between the dipeptides and acetyl-serine. Experiments with chloracetyl-serine in hydrogen fluoride indicated a conversion to the O-acyl compound of less than 10%. A 70% N,O-acyl shift in chloracetyl-serine has been reported in ether–HCl.[19] This yield is probably due to the insolubility of O-chloracetyl-serine in

(18) H. C. Reitz, R. E. Ferrel, H. Fraenkel-Conrat and H. S. Olcott, *J. Am. Chem. Soc.*, **68**, 1024 (1946).

(19) I. Levi, J. W. R. Weed, G. Laflamme and A. E. Koller, *Can. J. Chem.*, **39**, 2491 (1961).

ether-HCl. Preliminary experiments suggest that the low yield of O-acetyl-serine in hydrogen fluoride is due to the formation of serine esters. The high reactivity of the serine hydroxyl group in hydrogen fluoride is demonstrated by the observation that equal molar mixtures of acetic acid and serine in hydrogen fluoride lead to the formation of O-acetyl-serine in 73% yield in two days. Similarly, the reaction of acetic anhydride with DL-serine in anhydrous hydrogen fluoride leads to the isolation of crystalline O-acetyl-DL-serine in 95% yield. It is noteworthy that in this reaction, crystallization from water–ethanol yields the free amine and not the hydrofluoride as one might expect. The behavior of acetyl-serine in hydrogen fluoride is under investigation.

The amino-nitrogen data, the paper electrophoresis experiments and the crystallization of the products after HF treatment leave little doubt that with the peptides used, N,O-acyl migration proceeds in anhydrous HF in high yield and without unspecific cleavage of amide bonds or significant formation of side products. Another advantage of hydrogen fluoride is that it is a weak acid in aqueous solution and peptides are easily converted to their free amines. In contrast to this are the experiments with glycyl-DL-serine in concentrated sulfuric acid. While the amino-nitrogen determination indicated a maximum yield of 36% of the desired O-peptide, the paper electrophoresis experiments indicated that a great number of side products is formed.

The N,O-acyl migration has been studied with a number of model compounds, and steric and inductive effects affect the acyl migration.[20] The influence which steric or inductive effects in peptides have on the N,O-acyl migration is not known. These questions must be investigated with model peptides before assessing the use of anhydrous hydrogen fluoride as an agent for inducing acyl migration in proteins. Experiments along these directions are in progress.

Experimental[21]

Reagents.—Hydrogen fluoride of 99.5% purity was obtained from the Matheson Chemical Co. It was further purified on a vacuum line by trap-to-trap distillation and then by drying over cobaltous fluoride at room temperature and further distillation. Hydrogen fluoride so prepared is of purity greater than 99.95%.[22]

The 96% sulfuric acid used was Baker analyzed reagent; "100%" sulfuric acid (referred to as concentrated sulfuric acid) was prepared by mixing 95% sulfuric acid with appropriate amounts (as determined from the specific gravity) of 30% fuming sulfuric acid (Baker analyzed reagent). The glacial acetic acid was Baker analyzed reagent. Sodium nitrite, potassium iodide, potassium permanganate, sodium hydroxide and anhydrous ether were all Mallinckrodt analytical reagent grade.

Apparatus.—Experiments with hydrogen fluoride were carried out on a monel vacuum line fitted with Hoke nickel diaphragm valves.[14] The reaction vessels (1.5 × 15 cm.) were transparent Fluorothene tubes attached to the vacuum line by standard S.A.E. refrigeration flare fittings. The line was maintained at a pressure of 0.1 mm. by the use of a mechanical pump.

Reaction in Hydrogen Fluoride.—Thoroughly dried peptide or amino acid derivative 0.1 g. was placed into a Fluorothene reaction vessel which was then attached to a vacuum line. Five ml. of hydrogen fluoride was distilled into the reaction vessels which were held at liquid nitrogen temperature. The reaction mixture was then allowed to come to room temperature. After various time intervals the hydrogen fluoride was removed under reduced pressure. The reaction products were then dried *in vacuo* overnight over both phosphorus pentoxide and potassium hydroxide. The reaction mixture was then dissolved in 1% acetic acid and quantitatively transferred, as checked by Kjeldahl nitrogen determinations, to 10-ml. volumetric flasks. Aliquots from the 10-ml. volumetric flasks were used in the analytical procedures.

Reaction in Sulfuric Acid.—Thoroughly dried peptide or amino acid derivative 0.1 g. was placed in a round-bottom flask; 2 ml. of sulfuric acid was then slowly added to the reaction vessel at −20° with stirring. The reaction mixture was then allowed to come slowly to room temperature and allowed to stand for various time intervals under protection from moisture. After various time intervals the material was removed from the sulfuric acid solution by precipitation with 200 ml. of anhydrous ether at 0°. The precipitate was removed by centrifugation, repeatedly washed with cold ether, and dried overnight *in vacuo* over phosphorus pentoxide. The material was then dissolved in 1% acetic acid and quantitatively transferred, as checked by Kjeldahl nitrogen determinations, to 10-ml. volumetric flasks. Aliquots from the 10-ml. volumetric flasks were used in the analytical procedures.

Reaction in Bicarbonate.—Two-ml. aliquots from the 10-ml. volumetric flasks (see above) were transferred to 50-ml. volumetric flasks; 0.2 g. of sodium bicarbonate was added and the flasks were made up to volume. The solutions were kept at room temperature. At various time intervals 5-ml. aliquots were removed and used for Van Slyke amino-nitrogen determinations. Experiments with synthetic O-acetyl-DL-serine, O-glycyl-DL-serine and O-glycyl-L-threonine indicated almost quantitative conversions of the O-acetyl derivatives to the corresponding N-peptides, in the bicarbonate solutions, using the Van Slyke amino-nitrogen procedure (Fig. 1A, Table I) and paper electrophoresis (Figs. 2 and 3) as criteria.

Van Slyke Amino-nitrogen Determination.—The procedure of Van Slyke[23] as modified by Kendrick and Hanke[16] was used. All determinations were run in triplicate. The data obtained with the synthetic compounds which were used as standards appear in Table I.

Kjeldahl Nitrogen Determination.—The micro-Kjeldahl method of Johnson[24] was used.

Paper Electrophoresis.—Immediately after transferring the products from the HF reaction vessels to 10-ml. volumetric flasks, 30 μl. (≈ 2 μmoles) was applied to a strip of Whatman #3 MM filter paper. The experiments were run in *N* acetic acid, 15 volts cm.$^{-1}$, at 0° for 3 hours.

Quantitative Paper Chromatography.—The experiments with N-acetyl-DL-serine in hydrogen fluoride were followed by a quantitative paper chromatography procedure, retention analysis, developed by Wieland.[15] The reaction products from the 10-ml. volumetric flasks were first separated by paper chromatography, using 1-butanol–acetic acid–water, 4:1:1 (v./v.) as solvent. The area corresponding to O-acetyl-DL-serine was then subjected to retention analysis using the following modification of the developing solution used by Wieland[15]: 1% cupric acetate solution, 160 ml. of acetone, 80 ml. of isopropyl alcohol, 3 drops of glacial acetic acid. The method was standardized with synthetic O-acetyl-DL-serine samples which were subjected side by side with 10-μl. aliquots from the 10-ml. volumetric flasks to both paper chromatography and retention analysis. With each unknown, 10 μl. of the following standards was run: 0.017 *M*, 0.034 *M*, 0.051 *M*, 0.068 *M*, 0.51 *M*, O-acetyl-DL-serine. With synthetic O-acetyl-DL-serine one obtained ±5% of the theoretical value.

(20) C. A. Grob and C. Wagner, *Helv. Chim. Acta*, **38**, 1699 (1955).

(21) All melting points were taken on the hot-stage and are uncorrected. Specific rotations were calculated on the basis of the anhydrous compound. Microanalyses were performed by the Scandinavian Microanalytical Laboratory, Herlev, Denmark. Water analysis and the analysis of glycyl-L-threonine was performed by Miss K. Koike, Institute for Protein Research, Osaka University, Osaka, Japan.

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N-Acetyl-DL-serine was prepared according to the procedure of Narita,[25] m.p. 130–132° (reported[25] m.p. 129–132°).

O-Acetyl-DL-serine·HCl was prepared in 95% yield according to the procedure of Sheehan, Goodman and Hess[26] for the preparation of O-acetyl-L-serine, d. 155–157°.

Anal. Calcd. for $C_5H_{10}O_4NCl$: C, 32.71; H, 5.49; N, 7.63. Found: C, 32.87; H, 5.62; N, 7.62.

The compound was converted to the free base, d. 144° (reported[27] d. 144°).

DL-Alanyl-DL-serine, d. 210–217°, was obtained from Mann Research Laboratory, 136 Liberty St., New York, N. Y. Before using this dipeptide in the experiments it was dried *in vacuo* over phosphorus pentoxide at 100° for 24 hours; d. 210–217°. Paper electrophoresis experiments (Fig 3B) revealed a single spot corresponding to DL-alanyl-DL-serine.

Carbobenzoxy-glycyl-DL-serine Benzyl Ester.—To a solution of DL-serine benzyl ester hydrochloride[28] (29 g., 0.125 mole) and triethylamine (17.5 ml., 0.125 mole) in freshly distilled chloroform there was added 41.3 g. (0.125 mole) of carbobenzoxy-glycine *p*-nitrophenyl ester[29] and the solution was allowed to stand overnight at room temperature. The solvent was then replaced by ethyl acetate, and the solution was washed in succession with *N* ammonia, water, *N* hydrochloric acid and water. The ethyl acetate was dried over anhydrous sodium sulfate and concentrated under reduced pressure to an oil which crystallized on addition of absolute methanol. The yield of fine needles was 30.1 g. (74.7%), m.p. 142.5–143.5°, (reported[30] m.p. 141–142°).

Glycyl-DL-serine.—Carbobenzoxy-glycyl-DL-serine benzyl ester (9.9 g., 0.03 mole) was dissolved in 200 ml. of warm dioxane. Two grams of 10% palladium-on-charcoal was added to the solution and hydrogen bubbled through the solution for 3 hours with stirring. Then, 20 ml. of H_2O was added and hydrogenation was continued for 1 more hour. The catalyst was removed by filtration, washed with water, and the filtrate and washing were concentrated under reduced pressure to about 10 ml. On addition of absolute ethanol, 5 g. (92.6%) of the crystalline product was obtained as the monohydrate, d. 197–205° (reported[3,31] d. 196–198°). Before using this dipeptide in the experiments it was dried *in vacuo* over phosphorus pentoxide at 100° for 16 hours to remove the water of crystallization; d. 197–205°. Paper electrophoresis revealed a single substance corresponding to glycyl-DL-serine (Fig. 2).

Anal. Calcd. for $C_5H_{10}O_4N_2$: C, 37.03; H, 6.22; N, 17.28. Found: C, 36.86; H, 6.39; N, 17.22.

O-Glycyl-DL-serine thydrochloride was prepared according to a previously published procedure[3]; d. 168–170° (reported[3] d. 168–170°). Paper electrophoresis experiments with this compound revealed, in addition to the desired dipeptide, traces of glycine and serine (Fig. 2).

Anal. Calcd. for $C_5H_{11}O_4N_2Cl$: C, 30.23; H, 5.58; N, 14.11. Found: C, 30.30; H, 5.86; N, 13.70.

Carbobenzoxy-glycyl-L-threonine Ethyl Ester.—To a solution, maintained at 0°, of 5.5 g. (0.03 mole) of L-threonine ethyl ester hydrochloride[32] (prepared by Fischer esterification) and 4.2 ml. (0.03 mole) of triethylamine in 50 ml. of freshly purified chloroform there was added slowly 8.3 g. (0.025 mole) of carbobenzoxy-glycine *p*-nitrophenol ester[29] with vigorous shaking. After the solution was allowed to stand overnight at room temperature, the solution was filtered, and washed in succession with *N* ammonia, water, *N* hydrochloric acid and water. After drying the solution over anhydrous magnesium sulfate, the solvent was removed under reduced pressure, and the light yellow sirup crystallized on standing in a refrigerator. Recrystallization from ethyl acetate–petroleum ether (b.p. 60–70°) yielded 6.7 g. (79%) of colorless fine needles, m.p. 80–82°. A small sample was recrystallized from the same solvent for analysis; m.p. 82–83.5°, $[\alpha]^{25}D$ −1.9° (*c* 5.0, 95% ethanol).

Anal. Calcd. for $C_{16}H_{22}O_6N_2$: C, 56.79; H, 6.55; N, 8.28. Found: C, 56.96; H, 6.68; N, 8.24.

Glycyl-L-threonine.—Carbobenzoxy-glycyl-L-threonine ethyl ester (6.8 g., 0.02 mole) was dissolved in 50 ml. of dioxane, and 20 ml. of *N* sodium hydroxide (0.02 mole) was slowly added during a 15-minute period with stirring. After standing at room temperature for 10 more minutes the reaction mixture was adjusted to *p*H 2.5 with 6 *N* hydrochloric acid. The dioxane was removed under reduced pressure and the product was extracted into ethyl acetate. The ethyl acetate solution was dried over anhydrous sodium sulfate and evaporated *in vacuo* to give 6 g. of a sirup which could not be crystallized. The sirup was dissolved in a mixture of 50 ml. of methanol, 30 ml. of water and 1.5 g. of 10% palladium-on charcoal was added. Hydrogen was then bubbled through the solution for 3 hours with stirring. The catalyst was removed by filtration and the solution was concentrated under reduced pressure to about 10 ml. On addition of a small amount of ethanol, 3.8 g. (89%) of the desired dihydrate was obtained, which was air-dried at room temperature; d. 227–228°, $[\alpha]^{23}D$ −16.3° (*c* 1.68, H_2O); reported[33] $[\alpha]^{25}D$ −16.2° (*c* 2.0, H_2O).

Anal. Calcd. for $C_6H_{12}O_4N_2 \cdot 2H_2O$: C, 33.96; H, 7.60; N, 13.20; H_2O, 16.98. Found: C, 34.31; H, 7.71; N, 13.34; H_2O, 17.29.

Before using this dipeptide in experiments it was dried *in vacuo* over phosphorus pentoxide at 110° for 24 hours to remove water of crystallization; d. 227–228°.

Anal. Calcd. for $C_6H_{12}O_4N_2$: C, 40.90; H, 6.87; N, 15.90. Found: C, 40.71; H, 7.05; N, 15.52.

O-Glycyl-L-threonine Monohydrochloride.—The peptide was synthesized using the mixed anhydride method of Vaughan and Osato.[34] Carbobenzoxy-glycine[35] (5.2 g., 0.025 mole) and triethylamine (3.5 ml., 0.025 mole) were dissolved in 100 ml. of methylene chloride. The solution was cooled to −10° and isobutyloxycarbonyl chloride (3.42 g., 0.025 mole) was added dropwise to the solution with stirring. After 20 minutes, a 30-ml. solution of methylene chloride containing 6.3 g. (0.025 mole) of carbobenzoxy-L-threonine[36] and 3.5 ml. (0.025 mole) of triethylamine was added and the reaction mixture was allowed to stand overnight at 4°. The solution was then washed with three 50-ml. portions of a 1% sodium bicarbonate solution, dried over anhydrous sodium sulfate, and concentrated to a sirup under reduced pressure. The sirup was dissolved in ethyl acetate, and the product was extracted into water. The aqueous solution was acidified with 6 *N* hydrochloric acid, the product was extracted into ethyl acetate, the ethyl acetate solution was dried over anhydrous sodium sulfate and concentrated to a sirup *in vacuo*, (7.7 g. (70%), 0.018 mole). The sirup was dissolved in a solvent containing 20 ml. of ethanol and 17 ml. of *N* hydrochloric acid, and then 2 g. of 10% palladium-on-charcoal was added and hydrogen was bubbled through the solution for 3 hours with stirring. After removing the catalyst, concentrating the solution under reduced pressure and flushing with dry toluene, the solutes were dissolved in 50 ml. of methanol with careful heating. The solution was filtered and ethanol was added (100–150 ml.) until the solution became turbid. The product was precipitated carefully by adding small amounts of ether. The material was filtered, washed with ethanol–ether, and dried over phosphorus pentoxide *in vacuo* at room temperature: first crop, 1.5 g. (38%), d. 138–140°, $[\alpha]^{23}D$ +0.4° (*c* 2.26, H_2O); second crop, 0.3 g. (7.5%), d. 135–140°.

Anal. Calcd. for $C_6H_{13}O_4N_2Cl \cdot 0.5H_2O$: C, 32.51; H, 6.37; N, 12.64. Found: C, 32.81; H, 6.73; N, 12.21.

Paper electrophoresis revealed a strong ninhydrin-positive (purple) band and traces of glycine and threonine. Treatment of the O-peptide with bicarbonate followed by electrophoresis revealed as the main component, a substance corresponding to glycyl-L-threonine (Fig. 2).

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Glycyl-L-threonine from O-Glycyl-L-threonine.—To a solution of 0.5 g. (2.3 millimoles) of O-glycyl-L-threonine in 5 ml. of H_2O there was added dropwise 0.35 ml. (2.5 millimoles) of triethylamine. After 1 hour at room temperature the excess triethylamine was removed *in vacuo* and 100 ml. of absolute ethanol was added to the reaction mixture. A total of 0.34 g. (71%) of crystalline product was obtained, d. 227–228°, $[\alpha]^{21}D$ −15.9° (*c* 1.49, H_2O). The infrared spectra of this material and of synthetic glycyl-L-threonine were indistinguishable.

The Rearrangement of Glycyl-DL-serine to O-Glycyl-DL-serine in Hydrogen Fluoride.—One gram (6.17 millimoles) of glycyl-DL-serine was dissolved in 10 ml. of anhydrous hydrogen fluoride and allowed to stand at room temperature for 15 days. The hydrogen fluoride was then removed under reduced pressure and the reaction products were dried both over phosphorus pentoxide and potassium hydroxide *in vacuo*. The solids were dissolved in 6 ml. of *N* hydrochloric acid. The solution was treated with a few mg. of Darco. After filtration, about 50 ml. of absolute ethanol was added to the solution until it became turbid and the solution was treated again with a few mg. of Darco and filtered. The solution was then concentrated to about 3 ml. under reduced pressure, and about 200 ml. of absolute ethanol was added to afford 0.65 g. (53%) of the hydrochloride as colorless fine crystals, d. 159–161°. Another crop, 0.2 g., had a d. of 140–145°. The combined yield was 69%. The infrared spectrum of the main crop and synthetic O-glycyl-DL-serine monohydrochloride were indistinguishable. Paper electrophoresis experiments with both fractions indicated the presence of O-glycyl-DL-serine as the main product and a trace of an impurity (Fig. 2).

O-Glycyl-DL-serine. HCl (0.6 g., 3 millimoles) was dissolved in water and the *p*H of the solution was adjusted to about 7.0 by careful addition of triethylamine. After 1 hour the excess triethylamine was distilled off under reduced pressure. Addition of ethanol afforded 0.47 g. (86%) of the crystalline glycyl-DL-serine, d. 185–190°. Recrystallization from water–ethanol afforded 0.39 g. (71%) of crystals, d. 193–194° (reported[8,11] d., 196–198°). The infrared spectra of this material and of the starting material, glycyl-DL-serine, were indistinguishable.

The Rearrangement of Glycyl-L-threonine to O-Glycyl-L-threonine in Hydrogen Fluoride.—Glycyl-L-threonine (0.28 g., 1.6 millimoles) was dissolved in 5 ml. of anhydrous hydrogen fluoride and allowed to stand at room temperature for 15 days. The hydrogen fluoride was then removed under reduced pressure and the reaction products were dried both over phosphorus pentoxide and potassium hydroxide *in vacuo*. The solids were dissolved in about 2 ml. of water, a few mg. of Darco were added and the solution was filtered. Van Slyke amino-nitrogen analysis and paper electrophoresis experiments indicated that the material consisted essentially of O-glycyl-L-threonine.

The *p*H of the solution was adjusted to about 7.0 with triethylamine and the mixture was then allowed to stand at room temperature for 1 hour. The excess triethylamine was distilled off under reduced pressure. Addition of 50 ml. of ethanol to 2 ml. of the aqueous solution afforded 230 mg. (68%) of crystalline glycyl-L-threonine, d. 227–228°, $[\alpha]^{20}D$ −15.6° (*c* 2.0, H_2O). Recrystallization of a sample from water–ethanol yielded 207 mg. (61%), d. 227–280°, $[\alpha]^{22}D$ −15.8° (*c* 2.0, H_2O).

The Rearrangement of DL-Alanyl-DL-serine to O-DL-Alanyl-DL-serine in Hydrogen Fluoride.—DL-Alanyl-DL-serine (0.78 g., 4.4 millimoles), was dissolved in 5 ml. of anhydrous hydrogen fluoride and allowed to stand at room temperature for 15 days. The hydrogen fluoride was then removed under reduced pressure and the reaction products were dried both over phosphorus pentoxide and potassium hydroxide *in vacuo*. The solids were dissolved in 3.9 ml. of *N* HCl (3.9 millimoles). The solution was treated with a few mg. of Darco and filtered. Slow addition of absolute ethanol yielded 4 crystalline fractions. The combined yield was 0.62 g. (63%), d. 135–150°. The infrared spectrum revealed a characteristic ester carbonyl band at 1740 $cm.^{-1}$. Paper electrophoresis experiments indicated a main, ninhydrin positive component with a mobility similar to synthetic O-glycyl-DL-serine.

This material (0.34 g., 1.6 millimoles) was dissolved in about 2 ml. of water. The *p*H of the solution was adjusted to about 7.0 with triethylamine and the mixture was then allowed to stand at room temperature for 1 hour. The excess triethylamine was distilled off under reduced pressure. Absolute ethanol was added to about 2 ml. of the aqueous solution until turbid and the solution was allowed to stand overnight in a refrigerator to yield 0.16 g. (52%) of crystalline DL-alanyl-DL-serine, d. 192–200°. Paper electrophoresis revealed a single spot with a mobility identical to the starting material, DL-alanyl-DL-serine.

Conversion of O-Acetyl-DL-Serine Hydrochloride to the Sulfate Ester of DL-Serine.—O-Acetyl-DL-serine hydrochloride (1 g., 5.4 millimoles) was dissolved in 5 ml. of 96% concentrated sulfuric acid in an ice-bath. After 2 days the products were precipitated with 200 ml. of anhydrous ether at 0°. The precipitate was removed by centrifugation repeatedly washed with cold ether, and dried *in vacuo* overnight over phosphorus pentoxide. The product was crystallized from ethanol–acetone–ether to yield 0.6 g. (60%) of the crystalline sulfate ester of serine, d. 199–203° (reported[18] d. 203–205°). The infrared spectrum of this material was indistinguishable from the infrared spectrum of the sulfate ester of serine synthesized by an independent procedure.[18]

Reaction of DL-Serine with Acetic Anhydride in Hydrogen Fluoride.—To 1.05 g. (0.01 mole) of DL-serine and 2.5 ml. (0.027 mole) of freshly distilled acetic anhydride there was added 5 ml. of anhydrous hydrogen fluoride. After 23 hours at room temperature the hydrogen fluoride was removed under reduced pressure and the products were dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. The solids were taken up in about 2 ml. of H_2O. Paper electrophoresis indicated that the product was O-acetyl-serine with a trace of serine. Addition of ethanol to the aqueous solution yielded 1.4 g. (95%) of crystalline O-acetyl-DL-serine, d. 144° (reported[27] d. 144°).

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