isomer of diacetyljervine (m.p. 173–175°, $[\alpha]_D$ −112°). The analytical sample was dried at 137° (0.1 mm.) for 3 hours.

Anal. Calcd. for $C_{31}H_{43}O_5N$ (509.7): C, 73.05; H, 8.50. Found: C, 73.22, 72.75; H, 8.21, 8.19.

The rotation and analytical findings were confirmed on a specimen obtained in a repetition of the experiment.

Acknowledgments—The authors are indebted to Mr. Joseph Alicino and his associates for the microanalyses, and to Dr. Nettie H. Coy and her colleagues, Mr. Carl Sabo and Mr. Charles Fairchild, for the ultraviolet and infrared measurements.

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[CONTRIBUTION FROM THE GEORGE HERBERT JONES LABORATORY, UNIVERSITY OF CHICAGO, AND THE ARGONNE NATIONAL LABORATORY]

The Synthesis of Poly-α-amino Acids in Anhydrous Hydrogen Fluoride[1]

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Solutions in anhydrous hydrogen fluoride of N-carboxyanhydrides of α-amino acids (NCA's) or of α-aminoacyl chloride hydrochlorides react to yield solutions of amino acid polymers. The reaction is complete within several hours at room temperature and has been made to yield polymers with average chain lengths of 25 to 30 units. Polymers have been prepared from the NCA's of DL- and L-leucine, L-alanine and DL-phenylalanine. Racemization occurs to the extent of about 40%; diketopiperazines are significant by-products. Sarcosine NCA yields chiefly N,N'-dimethyldiketopiperazine. It also has been possible to initiate polymerization in a sulfur dioxide solution or in the bulk NCA by addition of traces of hydrogen fluoride.

A recent report of the use of anhydrous hydrogen fluoride as a powerful solvent for proteins and polypeptides[2] has prompted an exploration into the possibility of its use as a solvent for the preparation of polypeptides. A growing peptide chain would not be likely to precipitate from hydrogen fluoride solution; thus one possible cause of chain termination would be eliminated, especially in the case of the simple bifunctional amino acids whose polymers are generally highly insoluble in other solvents. In the course of such a study, it has been found that N-carboxyanhydrides (NCA's) of amino acids, when dissolved in hydrogen fluoride, lose carbon dioxide and are converted to peptides.

N-Carboxyanhydrides of amino acids have been widely used for preparation of polymers and copolymers of amino acids.[3] Commonly, a solution of the anhydride is treated with an amine, alkoxide or hydroxide to initiate polymerization. Recently, polymerization has been induced by use of certain salts or tertiary amines in polar solvents. The mechanism of these polymerizations has been extensively investigated.[4a,b] A degree of control over chain length may be achieved by varying the concentration of the initiator used. On the other hand, the action of hydrogen chloride in ethanol on the N-carboxyanhydrides has been reported to lead to hydrochlorides of amino acid ethyl esters.[5] The present work indicates that L-leucine NCA may be converted to L-leucyl chloride hydrochloride by action of hydrogen chloride in toluene, although it may be recovered unchanged from anhydrous trifluoroacetic acid after several days storage at room temperature. In view of these facts, the polymerization which has been found to occur in the presence of hydrogen fluoride is perhaps unexpected.

The NCA's used in this study were those derived from L-alanine, DL- and L-leucine, DL-phenylalanine and sarcosine, although the bulk of the studies were carried out with leucine derivatives. These were placed in Fluorothene or nickel tubes attached to a suitable vacuum line. The reaction tubes were held at liquid nitrogen temperature as hydrogen fluoride was distilled in, then closed off from the line and brought to a suitable temperature. At room temperature or above there occurred appreciable gas evolution, which continued for about an hour. After removal of solvent by distillation under vacuum the infrared spectra of the products were measured without further manipulation. These spectra indicated that the products were peptide in nature, of the α-, folded, configuration.[6] In those cases in which thorough removal of solvent was effected, the loss of weight corresponded to one equivalent of carbon dioxide. The raw polymer was washed with water or water–dioxane to hydrolyze any remaining reactive functional groups and at the same time to separate the material into soluble and insoluble portions. In the case of the DL-leucine polymers the soluble product, about 15% of the total, proved to be chiefly 3,6-diisobutyl-2,5-diketopiperazine. Varying amounts of corresponding diketopiperazines were noted in the soluble fractions from other runs. In all cases the insoluble product was polypeptide and afforded only the corresponding amino acid on hydrolysis. No hydantoin-3-acetic acid derivatives were found among the reaction products nor were cyclic peptides other than diketopiperazines isolated. The optically active N-carboxyanhydrides used led to products which were as much as 40% racemized, although the amino acids themselves were unchanged by storage in hydrogen fluoride. Sarcosine N-carboxyanhydride, when treated with hydrogen fluoride, afforded a 60% yield of sarcosine anhydride, 1,4-dimethyldiketopiperazine, in contrast to a maximum of 20% diketopiperazine found

(1) Based on work performed under the auspices of the U. S. Atomic Energy Commission.

(2) J. J. Katz, *Nature*, **173**, 265 (1954).

(3) E. Katchalski, "Advances in Protein Chemistry," Vol. 6, Academic Press, Inc., New York, N. Y., 1951, p. 123.

(4) (a) D. G. H. Ballard and C. H. Bamford, *Proc. Roy. Soc.* (*London*), **A223**, 495 (1954); (b) D. G. H. Ballard and C. H. Bamford, Symposium on Peptide Chemistry, Special Publication No. 2, The Chemical Society, London, 1955, p. 25.

(5) E. Katchalski, ref. 3, p. 141.

(6) A. Elliott, *Proc. Roy. Soc.* (*London*), **A221**, 106 (1953).

in other polymerizates. It is known that polysarcosine is readily depolymerized to sarcosine anhydride on sublimation[7] and the present observation may be related to this fact.

Average molecular weights of the polypeptides were determined by end-group titration in dimethylformamide suspension,[8] using sodium methoxide to a thymol blue end-point. Molecular weights were calculated from the equivalent weights so obtained on the assumption that the products were polypeptides with carboxyl and amine hydrofluoride end groups, both of which are neutralized at the end-point. In a number of cases, titration was carried out with perchloric acid in dioxane with methyl violet as indicator.[8] Titers with this system were one-half those obtained using base and therefore corresponded to conversion of amine hydrofluoride to amine perchlorate. Several of the polymers were analyzed for amino nitrogen by a modification of the Van Slyke method[9] and the results were in agreement with the titration data. In one case, a dinitrophenyl derivative of the peptide was prepared and the chain length determined by comparison of the 1350 cm.$^{-1}$ nitro group vibration with the 1660 cm.$^{-1}$ amide absorption.[10] Results here also agreed with titration. None of the techniques used, however, ruled out the presence of large cyclic peptides.

A number of polymerization experiments were carried out to determine the effect of conditions on the product. When L-leucine N-carboxyanhydride was allowed to remain in hydrogen fluoride solution (1.5 g. in *ca.* 2 ml.) two hours at room temperature, the insoluble fraction of the product, obtained in 80% of the theoretical yield, had an average chain length of 19 units. The soluble product, exclusive of diketopiperazine, had an equivalent weight corresponding to a chain length of less than 4 units. When, in a similar experiment, the hydrogen fluoride was not removed until after one week at room temperature, the insoluble portion (average chain length 7 units) constituted only 20% of the theoretical yield. (A sample of polyleucine of average length 19 units was degraded to an average chain length of 11 units under the same conditions.) Although polymerization proceeded readily at room temperature, storage of a sample of the N-carboxyanhydride in hydrogen fluoride 24 hours at 0° yielded a product containing unreacted starting material.

Although provision was not made in this work for measuring carbon dioxide evolved, it early became obvious that sufficient pressure was not built up in the reaction vessel to account for the carbon dioxide eliminated from the N-carboxyanhydride. A polymerization experiment carried out under 8 atmospheres of carbon dioxide pressure led to a product of about one-half the average chain length obtained in a parallel run without added carbon dioxide. On the other hand, when the reaction was run at 57° with the reaction vessel open to a large volume, the insoluble product was obtained quantitatively and had an average chain length of 28 units. In this last case, the product precipitated from the hydrogen fluoride solution and the reaction was complete in a few minutes.

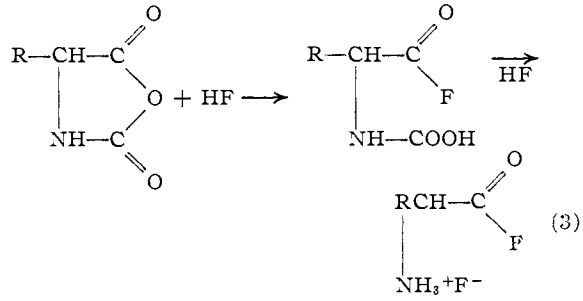
Polymerization of DL-leucine N-carboxyanhydride was shown to be possible in bulk, using only small amounts of hydrogen fluoride vapor (considerably less than equimolal), and also in sulfur dioxide solution, again using only catalytic amounts of the acid. Both methods resulted in high yields of insoluble polymer of average length 25 units.

It has generally been assumed, and substantiated by ebullioscopic and conductivity measurements,[11] that acyl halides and acid anhydrides are solvolyzed by anhydrous fluoride according to equations 1 and 2.

$$RCOCl + HF \longrightarrow RCOF + HCl \text{ (insoluble)} \quad (1)$$

$$RC(=O)-O-CR(=O) + HF \longrightarrow RCOF + RCOOH \quad (2)$$

Benzoyl fluoride has been so prepared from benzoyl chloride.[12] It would not be unreasonable to expect therefore that an N-carboxyanhydride would similarly react to yield an aminoacyl fluoride hydrofluoride (3). In one case, the concentration of acyl fluoride present in the raw product was sufficient to show moderate infrared absorption at 1855 cm.$^{-1}$ although in general hydrolysis by atmospheric moisture occurred before the infrared spectrum was measured. Polymerization of the aminoacyl fluoride is represented in eq. 4. Since amine hydrochlorides are converted to hydrofluorides and acyl chlorides to acyl fluorides by liquid hydrogen fluoride,[11] solution of an aminoacyl chloride hydrochloride in this solvent should lead to evolution of hydrogen chloride and the formation of polypeptide, if eq. 4 does describe the polymerization. L-Leucyl chloride hydrochloride is, in fact, converted to polypeptide on solution in anhydrous hydrogen fluoride. It may be mentioned here that succinic anhydride reacts with glycine ester hydrochloride when a mixture of the two solids is dissolved in hydrogen fluoride, although the yields of the corresponding succinamic acid were low (12%) under the conditions used. A more successful example was shown to be the reaction of *p*-nitrobenzoyl chloride with the same amine salt. These experiments seem to confirm



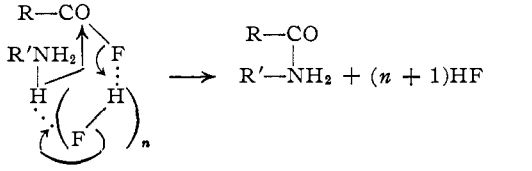
(3)

$$2RCHCOF \longrightarrow RCHCONHCH(R)COF + 2HF \quad (4)$$
$$\quad NH_3F \qquad\qquad NH_3F$$

(7) F. Wessely and F. Sigmund, *Z. physiol. Chem.*, **159**, 102 (1926).
(8) M. Sela and A. Berger, THIS JOURNAL, **77**, 1893 (1955).
(9) D. G. Doherty and C. L. Ogg, *Ind. Eng. Chem., Anal. Ed.*, **15**, 751 (1943).
(10) U. Scheidt and H. Restle, *Z. Naturforsch.*, **9b**, 182 (1954).
(11) For a review of reactions in solvent hydrogen fluoride, see G. Jander, "Die Chemie in wasseränlichen Losungsmitteln," Springer, Berlin, 1949.
(12) K. Fredenhagen, *Z. physik. Chem.*, **A164**, 176 (1933).

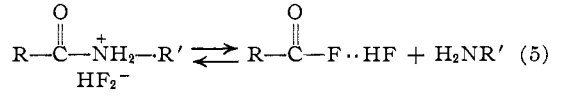
the hypothesis of eq. 4. Since the over-all reaction requires only two moles of hydrogen fluoride per completed chain, the observation that small amounts of hydrogen fluoride can induce polymerization, as in the bulk polymerization mentioned above, is in accord with the reaction scheme given.

The reaction of an amine with an activated carboxyl function such as an acyl halide proceeds by a nucleophilic attack of the amino group. If the amino group is protonated, no reaction is to be expected. Although the formation of polypeptides from aminoacyl chloride hydrochlorides has been reported,[13] such polymerizations are carried out in dimethylformamide solution on addition of base or in bulk by heating under vacuum at 180°. Both methods unblock the amino function by removal of hydrogen chloride. Hydrogen fluoride, although a weak acid in aqueous solution, is an extremely strong acid when anhydrous, the Hammett acidity function (H_0)[14] being approximately -10.[15] The concentration of free amino groups, even in the concentrated solution used, is therefore extremely low, much too low, approximate calculations indicate, to sustain a reaction which proceeds to completion in a matter of no more than hours. If it is taken as established that the intermediate in the polymerizations under discussion is the aminoacyl fluoride hydrofluoride, further explanation of the polymerization is necessary. It is suggested that the stability of the F—H· · ·F hydrogen bond assists acylation through formation of intermediates, involving several molecules of hydrogen fluoride, in which electron shifts occur as indicated. It is possible that similar intermediates occur in the

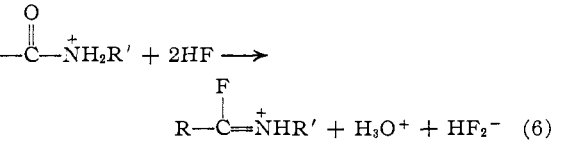


diazotization of amines in hydrogen fluoride.[16] The hydrogen fluoride catalyzed alkylation of toluene by *t*-butyl chloride has been reported to show kinetics with a high order of dependence on hydrogen fluoride pressure,[17] and there is possibility that a hydrogen-bonded intermediate occurs in this case also.

Were there no interfering reactions, the reactions of eqs. 3 and 4 would be expected to lead to extremely high molecular weight polymers, in contrast to the relatively short chains actually obtained. Since free amino acids can be recovered unchanged after prolonged storage in hydrogen fluoride, it may be expected that amino acid in the monomer or water in the solvent would act as chain-stopping reagents. Care was taken to ensure the dryness and purity of the N-carboxyanhydrides used, while the hydrogen fluoride used was shown to be 99.95% pure hydrogen fluoride. It is not believed that water is responsible for the chain lengths observed. The solvolysis represented in eq. 5 presents an alternative explanation. To explore this possibility, a mixture of excess sodium fluoride and N-carboxyanhydride was dissolved in hydrogen fluoride and left at room temperature for 24 hours. The product was found to be almost entirely water soluble even though the raw reaction product contained no unreacted monomer. Under normal reaction conditions, bifluoride ion may be present in the reacting solution as the result of protonation by the solvent of any of the species present. The chain-shortening effect of carbon dioxide may be of this nature.



It appears unlikely that the slow degradation of polymer on prolonged reaction times is due to the equilibrium proposed in eq. 5. More likely there is also occurring a dehydration reaction (6) which



produces water (and bifluoride ion) necessary for solvolysis of the peptide links or hydrolysis of acyl fluoride. The possibility of this reaction and the role of carbon dioxide in hydrogen fluoride solution are currently being investigated.

It was of interest to determine whether or not the polymerization of aminoacyl chloride hydrochlorides could be made to occur in other acidic solvents. Solution of L-leucyl chloride hydrochloride in trifluoroacetic acid resulted in formation of racemized leucine trifluoroacetate. Presumably hydrogen chloride and the volatile trifluoroacetic anhydride or trifluoroacetyl chloride, formed by anhydride interchange, escaped from the reaction mixture; N-trifluoroacetyl leucine was not present. In contrast, solution of the same acyl halide in glacial acetic acid led to formation of N-acetylleucine (racemized), in addition to uncharacterized products of peptide nature. Evidently, the acidity of the trifluoroacetic acid medium serves to prevent acylation of the leucine nitrogen by acyl chloride or anhydride groups present in the solution, while the lower protonating power of glacial acetic acid does not. Since anhydrous trifluoroacetic acid is of considerably lower acidity than anhydrous hydrogen fluoride,[18] these observations serve to indicate the unusual nature of the latter solvent.

Experimental[19]

Materials.—Hydrogen fluoride of purity greater than 99.6% was obtained in cylinders from the Harshaw Chemical Co. or the General Chemical Co. It was further purified on the vacuum line first by trap-to-trap distillation, and then by treatment with cobaltous fluoride, which serves to fluorinate residual water. By electrical conductivity measurements,

(13) M. Frankel, Y. Liwschitz and A. Zilkha, THIS JOURNAL, **76**, 2814 (1954).

(14) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 267.

(15) H. H. Hyman, M. Kilpatrick and J. J. Katz, unpublished.

(16) R. L. Ferm and C. A. VanderWerf, THIS JOURNAL, **72**, 4809 (1950).

(17) A. S. Gow and J. H. Simons, *ibid.*, **78**, 52 (1956).

(18) Unpublished work[15] indicates that H_0 for trifluoroacetic acid is about -3 while that for glacial acetic acid is reported as $+3.5$.[14]

(19) Infrared spectra were measured with a Perkin-Elmer Model 13 recording spectrophotometer using a sodium chloride prism. Samples were prepared as pressed potassium bromide disks.

hydrogen fluoride so prepared is of purity greater than 99.95%.

Sulfur dioxide was obtained in cylinders from the Matheson Co. and purified on the vacuum line by trap-to-trap distillation.

Apparatus.—Experiments with hydrogen fluoride were carried out on a metal vacuum line using transparent poly(chlorotrifluoroethylene) (Kel-F, Fluorothene) reaction tubes. These tubes were molded and are secured to the vacuum line by standard S.A.E. refrigeration flare fittings. The vacuum line is constructed of copper and nickel tubing and is fitted with Hoke–Phenix nickel diaphragm valves. A pressure of 10^{-5} mm. may be maintained by the use of a mechanical forepump and a mercury diffusion pump.

The experiments were carried out by introducing a given amount of N-carboxy anhydride (or other reagent) into the reaction tube, manipulations being carried out in a good drybox. The reaction tube was then affixed to the line and evacuated. The desired amount of hydrogen fluoride was then introduced into the reaction tube by distillation with liquid nitrogen as the refrigerant. The tube was then closed off from the line and allowed to warm to the desired temperature and remain for a predetermined length of time. On reopening the tube to the line no pressure surge was noted. The product was recovered by distilling the hydrogen fluoride away and freed as much as possible from hydrogen fluoride by long continued pumping in vacuum.

Polymerization of L-Alanine N-Carboxyanhydride.—L-Alanine N-carboxyanhydride, prepared by treating a dioxane suspension of L-alanine with phosgene, was twice crystallized from ether–petroleum ether and had m.p. 90–92°.[20] The anhydride (1.44 g., 0.0125 mole) was dissolved in *ca.* 2 ml. of anhydrous hydrogen fluoride. On warming to room temperature (25°) the clear colorless solution began to evolve gas. No attempts were made in this or subsequent experiments to collect or measure this gas. Gas evolution ceased within two hours and the solution, now considerably more viscous, allowed to remain at 25° for 18 hours before removal of hydrogen fluoride by distillation. Firmly bound traces of solvent were removed by pumping at 10^{-5} mm. for several days. The resulting fluffy, friable white glass weighed 0.95 g. (calculated for conversion to peptide of infinite chain length, 0.89 g.). This product did not appear to be soluble in water, 4 *N* sodium hydroxide, dioxane, 6 *N* hydrochloric acid or dimethylformamide, although it swelled somewhat in the last four solvents, especially when warmed. Its infrared spectrum showed bands at 1660, 1630 (partially resolved) and 1525 cm.$^{-1}$ consistent with a mixture of the α- and β-forms of poly-L-alanine.[6] The crude reaction product as obtained above (2.60 g.) was ground to a fine powder and suspended in 50 ml. of boiling water. The mixture was allowed to cool, the insoluble material was removed by centrifugation and washed with another 50 ml. of boiling water. The residue was washed with 100 ml. of 50% ethanol and dried to a white powder at 50° and 0.2 mm., weight 0.79 g. The combined washings were concentrated under reduced pressure to a volume of 50 ml. and lyophilized to yield a white powder, weight 1.85 g.

The insoluble product was titrated as a suspension in freshly distilled dimethylformamide using sodium methoxide in methanol–benzene and thymol blue as an indicator.[8] The equivalent weight was thus determined to be 500 (*n* 13.5). A Van Slyke amino nitrogen determination was carried out according to the method of Doherty and Ogg[9]; calculated for *n* 15: amino N, 1.27; found: amino N, 1.39, 1.18. The infrared absorption spectrum showed bands at 1630 and 1535 cm.$^{-1}$.

The extracted (soluble) product was further investigated as follows. A 200-mg. portion dissolved in water was passed through a column of Dowex-50 cation-exchange resin in the hydrogen form. Elution with water yielded 68 mg. of L-alanine anhydride. Paper chromatography of this fraction, before recrystallization (butanol–water–acetic acid, developed by the chloroimide method of Rydon and Smith[21]) showed it to be apparently homogeneous. However, the infrared spectrum of the raw fraction possessed slight absorption at 1535 cm.$^{-1}$ in addition to the C=O absorption at 1680 cm.$^{-1}$, indicating the presence of a small amount of peptides other than the diketopiperazine. One recrystallization from a small amount of ethanol afforded material melting at 291° dec. (reported m.p. 297°[22]). Further recrystallizations did not appreciably raise the melting point. No further fractions were eluted with water; elution with 1 *N* hydrochloric acid yielded 90 mg. of amorphous material which did not give the picric acid test for diketopiperazines[23] but did show a positive biuret test and possessed amide (1635, 1530 cm.$^{-1}$) and carboxyl (1725 cm.$^{-1}$) absorption in the infrared. Titration of this fraction indicated an equivalent weight of 220 (*n* 5.4). A 2,4-dinitrophenyl derivative was prepared[24] and carefully washed with benzene to remove traces of dinitrofluorobenzene and the corresponding phenol. Comparison of NO_2 absorption at 1340 cm.$^{-1}$ with the CONH absorption at 1645 cm.$^{-1}$ led to an estimated chain length of 5.3 residues.[10]

Hydrolysis of a small portion of the initial raw product was carried out in a 1:1 mixture of concentrated hydrochloric and glacial acetic acids. After 4 hours at 105° a clear solution was obtained. Only after heating for a total of 48 hours was a chromatographically homogeneous sample of alanine obtained, however. Since similar treatment of L-alanine resulted in some racemization, the hydrolyzed sample could not be used for determining the degree of racemization in the polymerization process. It was shown, however, that L-alanine recovered from solution of anhydrous hydrogen fluoride retained full optical activity, as did L-alanine prepared by hydrolysis of its N-carboxyanhydride.

Polymerization of DL-Leucine N-Carboxyanhydride. A. In Hydrogen Fluoride.—The anhydride used was prepared by the phosgene method[20] and had m.p. 47–48° (reported 48–50°[25]) after recrystallization from ether–petroleum ether; it (1.54 g., 0.0098 mole) was dissolved in about 2 ml. of hydrogen fluoride and the clear solution allowed to remain at 25° for 24 hours. Gas was evolved during the first hour. Excess solvent was distilled off and the residue stored one week at 25° and 10^{-5} mm. The measured over-all weight loss was 0.51 g. (calcd. for conversion to peptide of infinite length 0.43 g.). The raw product showed infrared absorption at 1660 and 1540 cm.$^{-1}$ but no higher frequency carbonyl absorption, indicating it to be polypeptide in nature. It was triturated with 6 ml. of pure dioxane; the mixture was diluted to 25 ml. with water and filtered. The residue was washed with water (25 ml.) and dried under vacuum to yield 0.83 g. of material, 75% on above basis. The combined filtrates were lyophilized to a white powder, 0.18 g. (16%).

End-group titration of the insoluble product with sodium methoxide showed an equivalent weight of 890 (*n* 15.3); while titration with perchloric acid in dioxane using methyl violet indicator indicated an equivalent weight of 1870 (*n* 16). Van Slyke amino nitrogen determinations were also carried out: calcd. for *n* 15.5: amino N, 0.77; found: 0.68, 0.87. The infrared spectrum of this material showed amide bands at 1660 and 1540 cm.$^{-1}$ and NH absorption at 3280 and 3060 cm.$^{-1}$.

The soluble (extracted) material, judged by its infrared spectrum which lacked absorption at 1550 cm.$^{-1}$ was largely leucine anhydride. Several recrystallizations from ethanol–water yielded DL-leucine anhydride, m.p. 269–270°, with carbonyl absorption at 1678 cm.$^{-1}$.

B. **Bulk Polymerization.**—The N-carboxyanhydride (1.57 g., 0.010 mole) was treated with a small, unmeasured amount of hydrogen fluoride gas. On storage at 25° for two days, the originally crystalline starting material swelled to a fluffy glass. Traces of hydrogen fluoride were removed under high vacuum. The resulting product, after transfer from the reaction tube, weighed 1.00 g. Its infrared spectrum indicated it to be chiefly polypeptide, but a small amount of unreacted NCA absorbing at 1780 and 1852 cm.$^{-1}$ was present. The work-up followed the procedure outlined above; on the same basis, the insoluble material was obtained in 75% yield (0.86 g.). End-group titrations using sodium methoxide indicated an equivalent weight of 1300 (*n* 23); Van Slyke determinations yielded: amino N, 0.37, 0.52, 0.45; calcd. for *n* 28: N, 0.43. Calcd. for *n* 25: Dumas N, 12.21; found, 11.90.

C. **Polymerization in Sulfur Dioxide.**—An estimated 1.0 g. of the anhydride was dissolved in 3 ml. of sulfur dioxide and the clear solution allowed to remain at 25° for 48 hr. No

(20) A. C. Farthing, *J. Chem. Soc.*, 3213 (1950).

(21) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

(22) E. Fischer, *Ber.*, **39**, 453 (1906).

(23) E. Abderhalden and E. Komm, *Z. physiol. Chem.*, **139**, 181 (1924).

(24) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(25) H. Leuchs and W. Geiger, *Ber.*, **41**, 1721 (1908).

gas was evolved. Several ml. of hydrogen fluoride gas was then allowed to enter the reaction tube. Slow gas evolution was noted within 24 hr., and after storage 4 days more at 25° a precipitate had formed. The solvent was removed, leaving a white powder, again containing traces of starting material, which was worked up as before. The insoluble product, which accounted for 80% of the raw product, had an equivalent weight of 1600 (*n* 27.7).

Polymerization of L-Leucine N-Carboxyanhydride. A. Effect of Storage in Hydrogen Fluoride.—The anhydride used, prepared by action of phosgene on a dioxane suspension of the amino acid,[20] was recrystallized twice from ether–hexane, m.p. 77–78°. In all cases it was dissolved in 2 ml. of hydrogen fluoride per 1.5 g. to give a clear solution which evolved gas on reaching room temperature (25°). Gas evolution ceased after 1 hr. A series of runs was made in which the solution was allowed to remain at room temperature for varying lengths of time before removal of solvent. Pumping was continued for 24 hr. to obtain a sensibly dry product.

The raw polypeptides had amide absorption at 1660 and 1540 cm.$^{-1}$ and no bands corresponding to unreacted starting material (L-leucine NCA shows three bands at 1855, 1810 and 1755 cm.$^{-1}$) although the product from run C had a band of medium intensity at 1855 cm.$^{-1}$ and a distinct shoulder at 1725 cm.$^{-1}$. These last two absorptions probably correspond to acyl fluoride and carboxyl produced by its hydrolysis, respectively. The products were worked up as in the case of the DL-leucine polymers and molecular weights were determined by end-group titration. The results are described in Table I. All runs were made with the same batch of NCA.

Small amounts (21 mg.) of material with infrared spectrum identical with DL-leucine anhydride (carbonyl absorption at 1670 cm.$^{-1}$) were isolated by crystallization from 50% ethanol of 80 mg. of the soluble product from run A. By passing the soluble product from run B through Dowex-50 in the hydrogen form the same substance was also isolated to the extent of 10% of that fraction. The melting points of these products could not be raised by recrystallization from ethanol–water above 270–271°, the reported melting

TABLE I

EFFECT OF STORAGE IN HF ON THE POLYMERIZATION OF LEUCINE NCA

Run	*t*[a]	Insoluble Yield,[b] %	Insoluble Equiv. wt.[c]	*n*	Soluble Yield,[b] %	Soluble Equiv. wt.[c,d]
A	2 hr.	70	1090	18.6	10	520
B	24 hr.	68	780	13.2	19	..
C	7 days	16	420	7.0	62	220

[a] Total time at 25° before removal of solvent begun. [b] Based on total conversion to peptide of infinite chain length. [c] Titrations using 0.097 *N* sodium methoxide in methanol–benzene and a suspension of *ca.* 50 mg. of peptide in 1 ml. of freshly distilled dimethylformamide which showed a negligible blank. A green color stable to shaking 10 min. at 50° in the absence of air was taken as the thymol blue endpoint. Results could be duplicated to ±5%. [d] Of soluble fraction prior removal of diketopiperazine.

point of inactive leucine anhydride.[26] All of the soluble products gave positive biuret and picric acid tests, indicating the presence of peptide and diketopiperazine. They also possessed carboxyl absorption (1725 cm.$^{-1}$) in the infrared, in addition to peptide bands.

B. **Racemization.**—A portion of the soluble product from run C above was dissolved in 6 *N* hydrochloric acid and heated on a steam-cone for 40 hr. The solution was then concentrated under reduced pressure and the solid product dried over sodium hydroxide. Paper chromatography revealed only one component, leucine, in the hydrolysate. This sample of leucine had $[\alpha]^{20}D$ 9.0°. L-Leucine itself, similarly treated, had $[\alpha]^{20}D$ 13.3°.

C. Variation of Conditions.—The results of these experiments are given in Table II. *Dilution:* The anhydride (1.32 g., 0.0084 mole) was dissolved in 10 ml. of hydrogen fluoride. At 25° little gas evolution was noted. After 24 hr. at 25° solvent was removed and the product worked up as usual. *Thionyl chloride:* Thionyl chloride (0.5 ml.) and hydrogen fluoride (3 ml.) were mixed and allowed to remain at 25° for 2 days before distilling the solution onto 1.77 g. (0.011 mole) of anhydride. Little gas evolution was noted. After 24 hr. at 25° the product was worked up as usual. The raw product was shown to be free of sulfur by sodium fusion analysis. *Sodium fluoride:* A mixture of 1.0 g. of sodium fluoride (anhydrous) and 1.73 g. (0.011 mole) of the anhydride was treated with 3 ml. of hydrogen fluoride. Not all of the sodium fluoride dissolved. Extremely vigorous evolution of gas occurred on warming to 25° and the reaction tube was cooled slightly to moderate the reaction until gas evolution ceased (*ca.* 15 min.). A gelatinous precipitate was then observed below the clear supernatant. After 24 hr. at 25° the mixture was worked up in the usual manner, except that the insoluble product was washed thoroughly with an extra 25 ml. of water to remove the last of the inorganic salt. The filtrate and washes were lyophilized and the residue extracted with 60 ml. of absolute ethanol to yield 430 mg. of material reported in Table II as soluble product. *Lowered temperature:* A solution of the anhydride (0.80 g., 0.0051 mole) in 1.5 ml. of hydrogen fluoride was held at 0–5° for 20 hr. Hydrogen fluoride was then distilled off at 0°. Little gas evolution was observed. The infrared spectrum of the raw product, a viscous oil, indicated the presence of unreacted NCA which was, of course, hydrolyzed in the work-up. *Carbon dioxide:* A nickel reaction tube was used. Hydrogen fluoride (1.5 ml.) was distilled onto 1.1 g. of the NCA and the liquid nitrogen was replaced by a Dry Ice–acetone cooling bath before the tube was charged to 120 lb. pressure with carbon dioxide (measured at room temperature). After the tube was closed off from the line, it was allowed to warm to room temperature and stored for 24 hr. before being worked up in the usual way. The product was a very viscous oil almost completely soluble in 6 ml. of dioxane, but precipitated on addition of water. A sample of L-leucine was treated in the same fashion and recovered unchanged. *Elevated temperature:* The reaction was carried out in the usual manner, using 1.3 g. of NCA and 4 ml. of hydrogen fluoride, except that the reaction tube was placed in a 57° bath as soon as the solid had dissolved in the thawed solvent. Within a few minutes, a granular precipitate had formed; in no other experiment using hydrogen fluoride alone was a precipitate noted. After 12 min. the reaction mixture appeared to have solidified, and solvent was removed rapidly, leaving a white powder with no detectable odor of hydrogen fluoride. During the period at 57° the tube was open to a manifold of

TABLE II[a]

EFFECT OF VARYING CONDITIONS ON THE POLYMERIZATION OF LEUCINE NCA

Run	Insoluble Yield, %	Insoluble Equiv. wt.	*n*	Soluble Yield, %	Soluble Equiv. wt.	*n*
B, Table I	68	780	13.2	19	..	..
Dilution	21	650	11.0	68	220	3.5
Thionyl chloride	71	980	17.0	7	..	..
Sodium fluoride	6	480	8.0	..	180	2.8
Low temperature	22	400	6.7	69	200	3.2
Carbon dioxide	57	440	7.4	32	205	3.3
Elevated temp.	100	1610	27.8	0	..	..

[a] Same footnotes apply as for Table I. Where equivalent weights for soluble fractions are not given, fraction is chiefly diketopiperazine. All soluble products gave positive picric acid tests, however.

ca. 400-ml. capacity; a pressure increase of 170 mm. was observed, corresponding to one-third of the theoretical yield of carbon dioxide. The infrared spectrum of the raw product indicated presence only of peptide.

Degradation of Polyleucine by Hydrogen Fluoride.—A sample of polyleucine of average degree of polymerization 19 was dissolved in hydrogen fluoride and stored 8 days at 25°. Solvent was then distilled off and the residue pumped dry. The infrared spectrum of the product was identical with that of the initial peptide, with amide absorption at 1660 and 1535 cm.$^{-1}$ and no observable acyl fluoride or carboxyl absorption. Neither the product or starting material gave positive ninhydrin tests. End-group titration of thoroughly dried samples indicated a molecular weight of 1300 (*n* 11.2).

(26) E. Fischer, *Ber.*, **34**, 448 (1901).

Polymerization of DL-Phenylalanine N-Carboxyanhydride.—DL-Phenylalanine NCA was prepared by treatment of a dioxane suspension of the amino acid with phosgene[20] and had m.p. 127.5–128.5° (reported 128–129°). A solution of 1.37 g. of this anhydride (0.0072 mole) in about 2 ml. of hydrogen fluoride evolved gas at 25° and was allowed to remain at that temperature 24 hr. before being worked up exactly as were the leucine polymers.

The raw polymer and the insoluble product had similar infrared absorption spectra, the important bands of which included N–H absorption at 3280 and 3060 cm.$^{-1}$, amide absorption at 1650 and 1535 cm.$^{-1}$ (phenyl absorption interferes with this latter) and monosubstituted phenyl at 700 and 750 cm.$^{-1}$. End group titration of the insoluble product with sodium methoxide indicated an equivalent weight of 1100 (n 14.7) and a Van Slyke amino nitrogen determination showed amino N, 0.65; calcd. for n 15: 0.62. A small amount of this product was hydrolyzed by heating at 100° for 48 hr. in a 1:1 mixture of acetic acid and concentrated hydrochloric acid. Paper chromatography of the hydrolysate using butanol–acetic acid–water gave only one ninhydrin-active spot, identical with phenylalanine.

The soluble product was examined without success for the presence of Friedel-Crafts acylation products.[27]

Sarcosine N-Carboxyanhydride in Hydrogen Fluoride.—Sarcosine N-carboxyanhydride was prepared by the phosgene method and recrystallized from chloroform–petroleum ether, m.p. 104–105° (reported 105° [28]). Its infrared spectrum showed carbonyl absorption at 1852 and 1768 cm.$^{-1}$. This material (0.46 g., 0.004 mole) was dissolved in 2 ml. of hydrogen fluoride to give a yellow solution which slowly evolved gas at 25°. After 20 hr. the solvent was removed to yield a tan, water-soluble solid which showed, in the infrared, amide absorption at 1660 cm.$^{-1}$ and complete absence of absorption corresponding to starting material; this spectrum was identical to that of sarcosine anhydride. A portion of the raw product (180 mg.) was extracted with dioxane, and the dioxane concentrated under reduced pressure to yield, in two crops, 110 mg. of fine needles, m.p. 136–152°. Recrystallization from ethanol–ether yielded sarcosine anhydride, m.p. 145–146° (reported 149–150°[29]), undepressed on mixing with a known sample. The dioxane-insoluble residue (53 mg.) was not investigated; its spectrum exhibited carboxyl and amide absorption.

Leucyl Chloride Hydrochloride in Hydrogen Fluoride.—L-Leucine (5 g.) was suspended in 100 ml. of carbon tetrachloride and 9 g. of phosphorus pentachloride was added to the mixture. The mixture was shaken at 20° for 24 hr. At the end of this period the mixture was filtered rapidly under dry nitrogen and the residue washed with 100 ml. of carbon tetrachloride followed by two 100-ml. portions of anhydrous ether. The product was dried in vacuum and stored over phosphorus pentoxide. The infrared spectrum of the white micro-crystalline material so obtained showed strong absorption at 1780 cm.$^{-1}$ due to the acyl chloride carbonyl and only a weak bond at 1740 cm.$^{-1}$ due to unreacted carboxyl. No amide absorption was noted.

The acid chloride so prepared (2.2 g.) was dissolved in slightly more than 3 ml. of hydrogen fluoride. Vigorous evolution of gas ensued. After 48 hr. at 25° the product was worked up in the usual manner. The raw product exhibited infrared absorption at 1720 (carboxyl), 1660 and 1530 cm.$^{-1}$ (amide). The insoluble fraction (400 mg., 30%) had equiv. wt., 675 (n 11.5) and infrared absorption identical with other polyleucine samples. The soluble portion (480 mg., 35%) had equiv. wt., 220 (n 3.6).

Reaction of Succinic Anhydride with Glycine Ethyl Ester in Hydrogen Fluoride.—Glycine ethyl ester hydrochloride (1.40 g., 0.01 mole) and freshly sublimed succinic anhydride (1.04 g., 0.01 mole) were treated for about 5 days with 3 ml. of hydrogen fluoride. All solid dissolved in the acid and evolution of gas was noted. On removal of the hydrogen fluoride, the residue was triturated thoroughly with 20 ml. of 0.5 N hydrochloric acid and the resulting mixture extracted with three 30-ml. portions of methylene chloride. The extract was concentrated under reduced pressure to an oil which rapidly crystallized as rosettes (240 mg.) melting at 95–96°. Recrystallization from methylene chloride–petroleum ether afforded fine needles, m.p. 99–100°, of N-carbethoxymethylsuccinamic acid. *Anal.* Calcd. for $C_8H_{13}O_5N$: C, 47.29; H, 6.45; N, 6.89. Found: C, 47.50; H, 6.63; N, 6.60.

A similar experiment was performed using glycine ester hydrochloride and p-nitrobenzoyl chloride. The infrared spectrum of the raw product contained bands for acyl fluoride (1825 cm.$^{-1}$), ester (1745 cm.$^{-1}$) and amide (1645 and 1540 cm.$^{-1}$). By a work-up similar to the above, it was possible to isolate p-nitrohippuric ester in good yield.

Effect of Hydrogen Fluoride on Various Compounds.—The following compounds were recovered in good yield (directly or after one recrystallization) after storage for at least 24 hr. in hydrogen fluoride at 25°: succinimide, L-alanine (unracemized), γ-ethyl glutamate, N-carboethoxyglycine, diketopiperazine, caprolactam, cyclohexanone oxime, hydantoin and 2,4-dinitrochlorobenzene. Glycine ethyl ester hydrochloride was converted to the hydrofluoride without formation of peptides, although the hydrofluoride was not characterized fully. The product of hydrogen fluoride treatment of p-nitrobenzoyl chloride was shown to be free of chlorine. Methionine was degraded; hydrogen sulfide and methyl mercaptan were among the products.

L-Leucyl Chloride Hydrochloride from the N-Carboxyanhydride.—L-Leucine NCA (1.2 g.) was dissolved in 70 ml. of dry toluene and the solution saturated with anhydrous hydrogen chloride. After 24 hr. at 20° some precipitate was present. Anhydrous ether (100 ml.) was added to force out a heavy white precipitate which was collected by rapid filtration. The white microcrystalline product (1.1 g.) was dried in vacuum and stored over phosphorus pentoxide. It did not have a definite melting point but decomposed above 270°. A sample was dissolved in distilled water and neutralized with sodium hydroxide. Titration was carried out for chloride by the Mohr method. Calcd. for $C_6H_{13}NOCl_2$: Cl, 38.1. Found: Cl, 36.8.

An 0.6-g. portion of the product was dissolved in absolute ethanol and the solution allowed to remain for 1 hr. Diluting with ether precipitated 0.41 g. of L-leucine ethyl ester hydrochloride, m.p. 133.5–134.0°.[30]

L-Leucine N-Carboxyanhydride with Trifluoroacetic Acid.—The anhydride (1.20 g.) was dissolved by shaking in 3 ml. of anhydrous trifluoroacetic acid and the solution was allowed to remain at room temperature for 6 days. No gas evolution was noted. Solvent was removed by lyophilization and the product shown to be identical with starting material, although its melting point (71–73°) was low. No amide absorption was detected in its infrared spectrum, which was identical in all respects with that of L-leucine N-carboxyanhydride.

L-Leucyl Chloride Hydrochloride with Trifluoroacetic Acid.—A solution of 1.88 g. of L-leucyl chloride hydrochloride in 3 ml. of trifluoroacetic acid was stored at room temperature. Slow evolution of gas was noted over the first 45 min. and at the end of this period the contents of the flask had become a mass of fine needles. These needles dissolved and remained in solution on the addition of 5 ml. more solvent. After 24 hr. solvent was removed by lyophilization. Paper chromatograms revealed leucine as the only ninhydrin active constituent. The product gave a negative biuret test. The infrared spectrum did not reveal the presence of peptide or trifluoroacetamido groups. A solution of the product in ethanol, treated with pyridine, yielded a precipitate of highly racemized leucine $[\alpha]^{20}D$ 4°. Titration with sodium methoxide in methanol–benzene of a dimethylformamide suspension of the product indicated an equivalent weight of 107; calcd. for leucine trifluoroacetate 123.

L-Leucyl Chloride Hydrochloride with Acetic Acid.—Warmed to 50°, 1.60 g. of the acid chloride dissolved in 10 ml. of glacial acetic acid and slowly evolved gas. After 5 hr. at 50° the solution was concentrated under vacuum to yield a colorless glass. Trituration with 3 ml. of water yielded 400 mg. of a crystalline product, m.p. 151–153° which on recrystallization from water, yielded acetyl-DL-leucine, m.p. 160–162°.[31] The mother liquors on concentration yielded a gum which gave a positive biuret test but could not be crystallized. This product exhibited infrared absorption at 1720 (carboxyl), 1665 and 1550 cm.$^{-1}$ (amide).

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(29) F. Mylius, *Ber.*, **17**, 287 (1884).
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(31) E. Fischer, *ibid.*, **34**, 449 (1901).

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CHICAGO, ILLINOIS

COMMUNICATIONS TO THE EDITOR

EVIDENCE FOR THE EXCRETION OF FORMIMINOGLUTAMIC ACID FOLLOWING FOLIC ACID ANTAGONIST THERAPY IN ACUTE LEUKEMIA

Sir:

Rats on a diet deficient in folic acid (FA) excrete a heat- and alkali-labile derivative of glutamic acid[1] presumably identical with N-formiminoglutamic acid (I) (α-formamidinoglutaric acid).[2] It was suggested recently that with a suitable extract of mammalian liver the formimino group of (I) may be transferred to tetrahydro-FA.[3] Recent publications[4,5] give evidence that the degradation, of formiminoglycine is also FA dependent. Preliminary microbiological and chromatographic evidence shows that a compound so far indistinguishable from synthetic (I) is excreted in the urine of children with acute leukemia during FA antagonist therapy.

The glutamic acid excretion patterns of various individuals are in Table I. The microbiological response to the unheated urine samples should be a measure of the glutamine and free glutamic acid present in the urine, but not of (I) which is microbiologically inactive. In contrast, the microbiological response to heated urine samples should be a measure of any glutamic acid originally present plus glutamic acid arising from (I) during heating. The glutamine present in unheated urine was destroyed by the heating process and did not contribute to the microbiological activity of the heated samples. Glutamic acid activity in urine from normal individuals could be detected only when the samples were unheated and paper chromatography indicated that the activity was due to glutamine. In contrast, urine specimens from two children with acute leukemia being treated with N - [4 - {N - (2,4 - diamino - 6 - pteridyl) - methyl]-N-methylamino}-benzoyl]-glutamic acid (Methotrexate) contained glutamic acid activity when the samples were either heated or unheated. Moreover, when the antagonist was discontinued, a marked drop in the glutamic acid content of the heated urine samples was observed. These observations could be explained by assuming that (I) was being excreted and that its presence may be associated with the degree of FA deficiency induced by the antagonist. An alternate explanation would be that these patients excreted glutamic acid rather than glutamine. However, evidence was found favoring the first of these two explanations as follows.

Synthetic (I) was separated by paper chromatography from glutamic acid (Strip A and B, Fig. 1). One hundred ml. of urine (representing 16% of the 24-hour total from Case 1, during Methotrexate therapy) was treated with charcoal (see footnote, Table I), filtered, and a mercury precipitation performed as described elsewhere[6] for the isolation of (I). The precipitate was decomposed with hydrogen sulfide, filtered and the filtrate was lyophilized and reconstituted with 10 ml. water; 80% of the original glutamic acid activity in the heated microbiological assay was recovered. The

TABLE I

EXCRETION OF GLUTAMIC ACID ACTIVITY IN NORMAL INDIVIDUALS AND CHILDREN WITH ACUTE LEUKEMIA TREATED WITH 4-AMINO-10-METHYLPTEROYLGLUTAMIC ACID (METHOTREXATE)

Description of individual studied	Glutamic acid excreted,[a] mg./day: Urine unheated[b]	Urine heated[b]
Normal individuals		
12 yr. old boy	59	None detected
6 yr. old boy	60	None detected
Adult female	91	None detected
Acute Leukemia[c]		
Case 1, 12 yr. old boy, on Methotrexate 2.5 mg. per day, 30 days	38	45
Case 1, off Methotrexate 10 days	55	20
Case 2, 14 yr. old girl, on Methotrexate 5 mg. per day, 14 days	35	39
Case 2, off Methotrexate 3 days	12.5	3.5

[a] Determined with *Lactobacillus arabinosus* 17-5 by the method of Henderson and Snell[7]; sensitivity about 5γ glutamic acid per ml. [b] Unheated test samples were sterilized by Seitz filtration and added aseptically to sterile cooled media. Heated samples were autoclaved for 15 minutes at 15 lb. pressure at *p*H 7, then added aseptically to sterile media. [c] Urine from these cases was adjusted to *p*H 2–3 and stirred with 2% charcoal (Darco G-60) for 30 minutes, filtered, and the filtrate brought to *p*H 7 for the microbiological assay. Such a procedure effectively removed Methotrexate present in urine; control experiments showed such urine contained no growth inhibiting substances.

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