Studies on the Mechanism of the Direct Acylation of Amino Acids and Related Compounds in Nonaqueous Solvents[1]

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Received September 3, 1964

The mechanism of direct acylation was investigated using L-leucine and dichloroacetyl chloride in anhydrous ethyl acetate as the model system. The kinetic data fit quite closely to the differential equation developed from theoretical considerations. Because of its bearing on the interpretation of the kinetic data, the solubility of L-leucine in anhydrous ethyl acetate was determined and was found to increase as the water content of the solvent decreased. The results permit a reasonable suggestion for the chemical structure of the intermediate formed in the course of the acylation process. The system is of interest as a heterogeneous reaction in which the rate of dissolution of the suspended, crystalline reactant is rate controlling.

The direct method of acylation[3-5] provides an easy method for the acylation of amino acids, peptides, and hydroxy acid esters. In contrast to the Schotten–Baumann reaction, direct acylation proceeds in anhydrous ethyl acetate without a base to accept the liberated hydrogen chloride. For preparative acylations, one suspends the amino acid in ethyl acetate, adds the acid chloride, and refluxes the mixture. The acylated amino acid, which is soluble in ethyl acetate, is separated from the unchanged amino acid by filtration. A crystalline product is usually obtained when the solvent is drawn off by a stream of air. Although the synthetic value of this reaction has been cataloged,[3-5] the reaction mechanism has not been investigated. Since amide bonds are formed (as well as ester), involving amino acids, interest is generated in what mechanistic analogies could be drawn to *in vivo* biochemical acylations. As a first step, a kinetic scheme for this reaction has been developed, using L-leucine and dichloroacetyl chloride for the initial model system. For kinetic purposes, it was profitable to determine the solubility of L-leucine in the reaction solvent. Qualitative observations were made on the effect of size and form of the suspended amino acid crystals on reaction rate. Additionally, other anhydrous solvents were tested as reaction media. The results allow the postulation of a reasonable structure for the responsible acylating intermediate.

Most important, the investigation is of interest as one of the rare instances allowing a quantitative kinetic study of a heterogeneous reaction between a crystalline and a dissolved organic reagent.

Experimental

Materials.—L-Leucine, General Biochemicals, Inc., was recrystallized from water and dried in a vacuum desiccator over phosphorus pentoxide. The amino acid flakes were pulverized to a fine powder using a Waring Blendor. The powder was forced through a 48-mesh (297 μ) sieve and caught on a 60-mesh (250 μ) sieve. The purity of the amino acid was confirmed by decomposition point range, optical rotation, and paper chromatography.

Dichloroacetyl chloride, Eastman reagent grade, was distilled under nitrogen before use (30 mm., 40–41°).

Ethyl acetate, J. T. Baker Chemical Co., anhydrous grade, was distilled at atmospheric pressure. The purity of the ethyl acetate was confirmed by boiling point range and refractive index. The initial product contained 0.02% water, as determined by the Karl Fischer method. By drying the above product over Molecular Sieves, the water content was reduced to 0.005%.

Kinetic Reaction Procedure.—Kinetic runs were carried out in the modified 250-ml. round-bottom Pyrex flask shown in Fig. 1. Capillary tube, A, was used to obtain reaction samples that were free of suspended amino acid particles. This capillary was also used to introduce a dry nitrogen gas atmosphere over the reaction mixture. The acid chloride was introduced by means of the outlet, B. Stirring was accomplished by a magnetic stirrer, C and D, encased in a polyethylene bag, E, which contained the indicating form of Drierite, F, to detect water leaks. Sampling was done with the aid of the Thomas–Seligson-type pipet shown in Fig. 2. Outlet A was connected to a source of vacuum, and outlet B to a 1:1 propanol–water diluent reservoir contained in a buret. The pipet stem, C, was fashioned from a 5-μl. pipet, though the total volume of the stem ranged from 0.081 ml. at 30° to 0.074 ml. at 65° (calibrated with mercury for the different temperatures of the runs). Tygon tubing, D, was used to connect the 10/30 $\overline{\S}$ male joint, E, to the pipet stem; the pipet stem was able to move through the joint.

L-Leucine (0.656 g., the quantity that would make a 0.1 *M* solution in 50 ml. of ethyl acetate if the amino acid were entirely dissolved) was suspended in 47 ml. of anhydrous ethyl acetate and allowed to reach saturation solubility. During this period the magnetic stirrer was allowed to reach a uniform rate and air was excluded from the reaction flask by forcing dry nitrogen through the capillary inlet, A, Fig. 1. A solution of dichloroacetyl chloride in 3 ml. of ethyl acetate was heated to reaction temperature and introduced into the stirred mixture through outlet B, Fig. 1. The clock was started at this point and the stopcock of outlet B was allowed to remain open as an escape vent for the nitrogen. A cold finger was placed in the portion of outlet B above the stopcock to reduce possible loss of solvent and acid chloride due to evaporation. At exactly 50 sec., the first sample was taken by withdrawing the nitrogen inlet tube in capillary A and attaching the sampling pipet to the capillary tube through the standard taper joint system. Under the demand of the vacuum, a sample of the reaction mixture was filtered through the glass wool filter of the capillary tube into the pipet filling the whole pipet cavity to the stopcock plug. The sampling-pipet assembly was now withdrawn from the capillary tube and the sample remaining in the capillary tube was forced back into the reaction mixture by reapplying the positive pressure of nitrogen. Meanwhile the contents of the sampling pipet stem were simultaneously discharged into test tubes and diluted by turning the stopcock so that the diluent would flow through the pipet. The reaction samples were diluted to a total volume of 1 ml. with propanol–water diluent from a buret connected to A, Fig. 2. This not only destroyed any acid chloride in the reaction sample, but diluted it tenfold. The test tubes were stoppered and set aside for the analytical procedure. The entire sampling process took approximately 10 sec. Samples were withdrawn every minute for the first 10 min. of the reaction.

Two aliquots, varying in size from 20 to 100 μl., of each reaction sample were placed into test tubes containing 2 ml. of 1 *N* KOH in 50% ethanol. The test tubes were capped and placed in boiling water. After 45 min., the test tubes were removed and

(1) Presented in part before the Division of Biological Chemistry at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept., 1963.

(2) Excerpted in part from the thesis submitted by C. B. Warren in partial fulfillment of the requirements for the degree of Master of Science.

(3) E. Ronwin, *J. Org. Chem.*, **18**, 127 (1953).

(4) E. Ronwin, *ibid.*, **18**, 1546 (1953).

(5) E. Ronwin, *ibid.*, **22**, 1180 (1957).

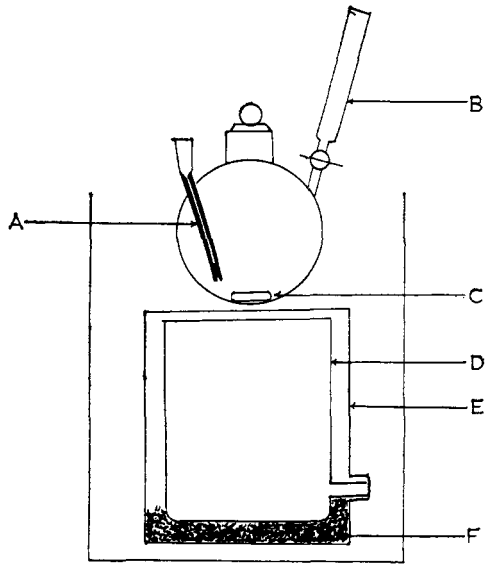


Fig. 1.—Reaction flask: A, capillary tube with glass-wool filter in tip and 10/30 ⅋ female joint on outer end; B, outlet with stopcock to permit entry of acid chloride solution and exit of nitrogen gas; C and D, magnetic stirrer; E, polyethylene bag; and F, Drierite.

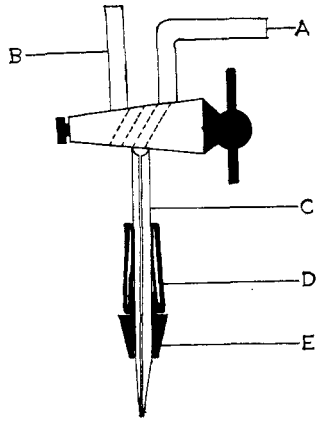


Fig. 2.—Sampling pipet: A, outlet connecting to vacuum source; B, outlet to buret containing 50% propanol–water diluent; C, pipet stem; D, tygon tubing; and E, 10/30 ⅋ male joint.

the solution was neutralized with 1 *N* HCl.[6] The concentration of free amino acid in the hydrolyzed samples was determined by the ninhydrin reaction.[7]

Solubility Determinations.—L-Leucine (0.656 g.) was suspended in anhydrous ethyl acetate at various temperatures and stirred 1 hr. which assured saturation. The mixture was then poured through a sintered-glass filter which was preheated to the desired temperature by pouring through warm solvent. The clear solution was than taken to less than 1-ml. volume with the aid of a stream of air and 3 ml. of 1 *N* HCl was added to the residue (this increased the aqueous solubility of the L-leucine without affecting its quantitative determination). Then 3 ml. of 1 *N* KOH solution was added and aliquots of the resulting solution were analyzed for the quantity of L-leucine present by the ninhydrin procedure.

Results and Discussion

Solubility of L-Leucine in Ethyl Acetate.—As a result of its bearing on the interpretation of the kinetic data, as will be indicated below, the determination of the solubility of the amino acid in ethyl acetate was of value. The results are presented in Fig. 3, for the temperature range between 35 and 65° and for solution in ethyl acetate containing 0.02%, as well as 0.005% water. It is obvious that the lower the water content of the solvent, the greater the solubility of the amino acid. This is interesting in view of the fact that L-leucine is soluble in water to the extent of 2.3 g./100 g. of water at 0° to 3.8 g./100 g. of water at 75°, which is some 10,000 times greater than its solubility in the most anhydrous ethyl acetate available to us.[8]

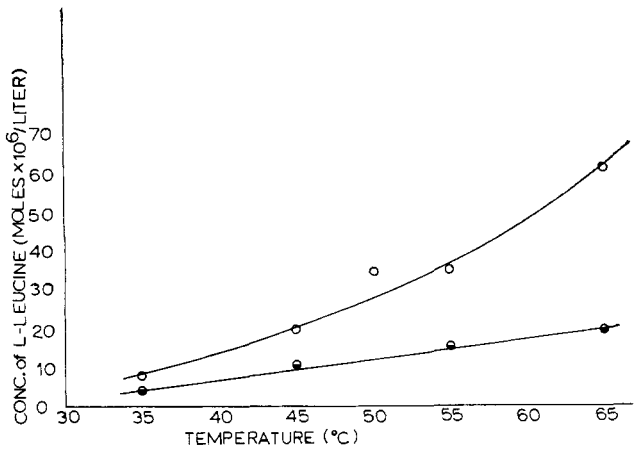


Fig. 3.—Solubility of L-leucine in ethyl acetate as a function of temperature: ◒, ethyl acetate containing 0.02% water; and O, ethyl acetate containing 0.005% water.

While the time to attain saturation varied with temperature, saturation occurred within 30 min. at all temperatures in the range from 35 to 65°. As it is important from the kinetic viewpoint, as well as from the question of reproducibility, the amino acid was always brought to saturation by allowing a 45- to 60-min. equilibration period with the solvent at the reaction temperature prior to the addition of the acid chloride.

Kinetic Studies.—Consider the system where S = suspended L-leucine (a surface area); $[L_s]$ = dissolved L-leucine; $[A]$ = acid chloride; $[p]$ = products. These relations and rate expressions can be derived.

$$S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [L_s] + [A] \xrightarrow{k_3} [p]$$

The reaction is commenced with the amino acid at its saturation solubility from which one of two cases holds: either the amino acid remains at saturation in the solvent (case I; that is the rate is governed by a homogeneous reaction), or the rate of solution of the amino acid becomes rate controlling (case II; that is the rate of dissolution of the solid reagent is insufficient to maintain the equilibrium concentration of the amino acid in solution).

For case I, it is self-evident that

$$dp/dt = v = k_3[L_s][A] \quad (1)$$

(6) It was found empirically that hydrolysis was complete within 30 min., the extra 15 min. being employed as a precautionary measure. L-Leucine was not destroyed by the hydrolytic step.

(7) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 376 (1948).

(8) The seeming incredibility of this result stimulated a reinvestigation by Ronwin and Horn. The unpublished results of experiments in which the water content of the ethyl acetate ranged from 0.005 to 0.1% show an unmistakably gradual, *decreasing* solubility of the amino acid in these mixtures as the water content increases. At 65°, the drop in solubility of L-leucine in going from ethyl acetate containing 0.005% water to ethyl acetate containing 0.05% water is approximately one-half.

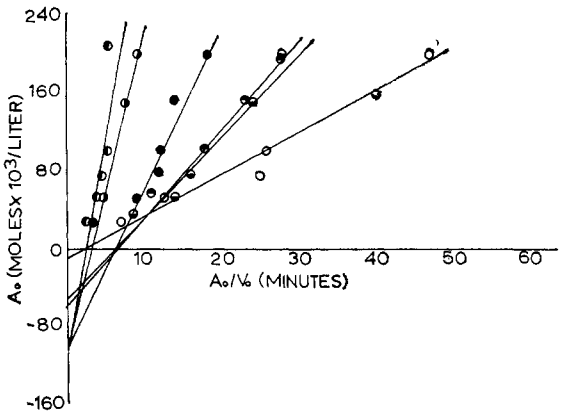


Fig. 4.—Plot of the initial concentration of dichloroacetyl chloride *vs.* the initial concentration of dichloroacetyl chloride/initial rate of the reaction run in ethyl acetate containing 0.02% water and saturated with L-leucine: ○, 35°; ◒, 40°; ◓, 45°; ●, 50°; ◑, 55°; and ◐, 65°.

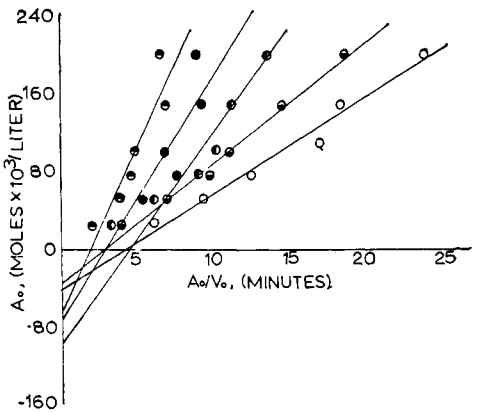


Fig. 5.—Plot of the initial concentration of dichloroacetyl chloride *vs.* the initial concentration of dichloroacetyl chloride/initial rate of the reaction run in ethyl acetate containing 0.005% water and saturated with L-leucine: ○, 40°; ◒, 45°; ◐, 50°; ●, 55°; and ◓, 65°.

For case II, assuming a steady state for $[L_s]$

$$d[L_s]/dt = 0 = k_4S - (k_5S + k_1[A])[L_s] \quad (2)$$

Solving for $[L_s]$ and substituting in eq. 1 yields[9a]

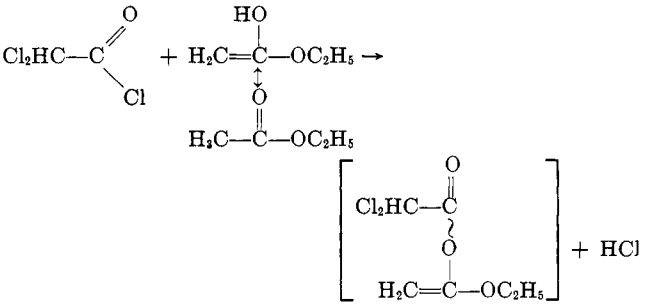
$$[A] = k_4S[A]/v - \frac{k_5S}{k_1} \quad (3)$$

If eq. 3 holds, then the plot of the initial acid chloride concentration, $[A]$ *vs.* the initial acid chloride concentration divided by the initial velocity of the reaction, $[A]/v$, will be a straight line with a negative intercept equal to k_5S/k_1 and a positive slope equal to k_4S. Figures 4 and 5 are plots of $[A]$ *vs.* $[A]/v$ at temperatures ranging from 35 to 65° and water concentrations of 0.02 and 0.005%. The fit of the empirical results to the theoretical equation is satisfactory. All the points fall within the interval allowed by experimental error. The correlation coefficient ranges from 0.90 to 0.95.

The stoichiometry of the reaction is of obvious interest to an understanding of the acylation mechanism. In the acylation of *m*-nitroaniline in benzene by benzoyl chloride, Hinshelwood[9b] noted that one-half of the amine served as acceptor of the liberated hydrogen chloride. On the other hand, Ronwin[3-5] noted preparative yields, based on the amino acid, of over 50% (occasionally quantitative) in several cases, although the greater number of reactions resulted in less than 50% yields. For the specific case at hand, Ronwin reported a yield of 28% dichloroacetyl-L-leucine.[4] Using 0.1-mole quantities of each of the reactants in 50 ml. of anhydrous ethyl acetate at 50°, under an atmosphere of dry nitrogen gas, a quantitative yield of dichloroacetyl-L-leucine was obtained. The difference in yield is simply explained in that Ronwin's original reaction conditions, adopted empirically for preparative purposes only and without study of the effect of conditions, are at some variance with the more ideal reaction conditions used in this study. The result establishes the reaction stoichiometry as 1:1 amino acid–acid chloride, which supports the rate equation developed for the reaction and dictates that any proposed mechanism for the reaction must not require any of the amino acid to serve *solely* as acid acceptor. The result is of additional interest since the suspended amino acid is almost immediately, upon inclusion of the acid chloride into the reaction medium, converted to the hydrochloride salt; nevertheless, it goes on to react so that virtually none of it is limited to the function of acid acceptor. It is also obvious that by choosing proper reaction conditions, preparative yields can be pushed to quantitative levels.

Proposed Reaction Intermediate.—When the amino acid, suspended in ethyl acetate, is quickly treated with acid chloride and immediately filtered off, the filtered material is no longer free amino acid but identical with the hydrochloride salt. Since the water content of the ethyl acetate, even at 0.02%, is insufficient to create total hydrolysis of the acid chloride quantities used, the origin of the hydrogen chloride demands explanation. Additionally, when reaction is attempted between the hydrogen chloride salt of the amino acid and the free acid corresponding to the acid chloride, no product is obtained. Further, the determination of the molecular weight of the acid chloride in ethyl acetate by the boiling-point elevation procedure resulted, both in the case of dichloroacetyl chloride as well as for benzoyl chloride, in values which are almost one-half their actual molecular weights (Table I); yet, free benzoic acid in ethyl acetate yields the correct molecular weight by the same procedure.[10] Thus, the acid chlorides act in ethyl acetate as though they yield two particles.

The above experimental results indicate that (1) the acid chloride must be the initial and sole source of the hydrogen chloride produced, and (2) the acid chloride reaction to yield the hydrogen chloride must leave a



HCl + amino acid → amino acid hydrochloride

(9) (a) For the reader who may wonder, the symbols k_2 and k_3 were deliberately not used. (b) C. N. Hinshelwood, *J. Chem. Soc.*, 1353 (1936).

(10) International Critical Tables, Vol. III, McGraw-Hill Book Co. Inc., New York, N. Y., 1928, p. 340.

TABLE I

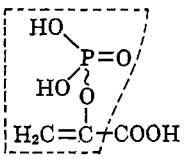
THE APPARENT MOLECULAR WEIGHT OF DICHLOROACETYL CHLORIDE AND BENZOYL CHLORIDE IN ETHYL ACETATE DETERMINED BY BOILING POINT ELEVATION

Acid chloride	Molality[a]	B.p. elevation, °C.[b] Calcd.	B.p. elevation, °C.[b] Obsd.	Mol. wt. Obsd.	Mol. wt. Actual	Mol. wt. ratio, obsd./actual
Dichloroacetyl chloride	0.436	0.59	1.02	89.4	147.4	0.59
Benzoyl chloride	0.617	0.85	1.64	72.4	140.5	0.52

[a] Solvent, ethyl acetate containing 0.005% water. For molecular weight of dichloroacetyl chloride: weight of ethyl acetate was 67.759 g.; weight of dichloroacetyl chloride was 4.360 g. For molecular weight of benzoyl chloride: weight of ethyl acetate was 71.134 g.; weight of benzoyl chloride was 6.170 g. [b] Cottrell boiling point apparatus employing a Beckman thermometer. All boiling points were determined in a nitrogen atmosphere.

form which retains acylating activity and which is not the free acid. These considerations lead to the suggestion that the hydrogen chloride is generated in the preceding manner which also gives rise to a reasonable structure that can be expected to be actively acylating and, additionally, accounts for the molecular weight observations.

The intermediate in brackets would essentially be a "high energy" anhydride type. Although there is no scientific reason why a precedent for the above compound type is necessary in order to validate the suggestion, some degree of precedence is found in the consideration of the structure of phosphoenol pyruvate,



wherein the difference with the proposed intermediate shown above is simply that one-half of the "anhydride" is phosphate in phosphoenol pyruvate and carboxylate in the proposed intermediate.

Upon reaction with amino acid (or amino acid hydrochloride), the intermediate would yield the acylated amino acid and free the hydrogen chloride as well as a molecule of solvent.

Other Experiments.—It was observed that the acylation of L-leucine by dichloroacetyl chloride did not proceed in the following solvents: triethyl orthoformate, methyl formate, N,N-dimethylformamide. The reaction does proceed in *n*-butyl acetate, *n*-propyl acetate, and methyl acetate. In each case, the product (dichloroacetyl-L-leucine) was identified by its m.p. 119–122°.[4] These results tend to support the acylating intermediate structure proposed above. It is of interest that the product from the methyl acetate reaction is difficult to crystallize, though success is eventually achieved; whereas, the product from the other acetates crystallizes fairly readily.

Although a quantitative study has yet to be undertaken, definite evidence was obtained that both particle size and form (flake or powder) of the amino acid affect the rate of reaction. This led to the use of a uniformly purified and treated batch of L-leucine throughout this study.

Acknowledgment.—The authors are indebted to Dr. Kenneth F. O'Driscoll of this department for his development of the rate equation and for his helpful discussions throughout this study.

The Preparation and Some Reactions of N′-Fluorodiimide N-Oxides,[1] R—N(O)=NF

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Received January 13, 1964

A variety of N-substituted N′-fluorodiimide N-oxides, R–N(O)=NF, have been prepared by the reaction of nitroso compounds with either tetrafluorohydrazine or pyridine–difluoramine mixtures. Some reactions of those novel azoxy compounds with nucleophilic reagents were investigated and a new synthesis of unsymmetrical azoxy compounds was discovered. Reduction yielded anilines or hydrazines.

The preparation and thorough characterization of N-trifluoromethyl-N′-fluorodiimide N-oxide (I, R = CF_3) was reported recently.[2] This N-fluoroazoxy compound was obtained from the ultraviolet or thermally activated reaction of trifluoronitrosomethane and tetrafluorohydrazine. Two convenient methods for the preparation of alkyl- and aryl-N′-fluorodiimide N-oxides from nitroso compounds in solution are reported here.

(1) This research was supported by the Advanced Research Projects Agency under Army Ordnance Contract No. DA-01-021 ORD-11909.

(2) J. W. Frazer, B. E. Holder, and E. F. Worden, *J. Inorg. Nucl. Chem.*, **24**, 45 (1962).

In inert solvents such as chlorobenzene, carbon tetrachloride, or methylene chloride, C-nitroso monomers absorb tetrafluorohydrazine (N_2F_4) at subatmospheric pressure and are converted to the corresponding N′-fluorodiimide N-oxide (Table I). The reaction proceeded readily at 0–20° if the blue-green color of the nitroso monomer were visible in the solution. Only with the last three nitroso dimers listed in Table I was heating necessary; at 60–80° in chlorobenzene solution sufficient monomer was present to cause these reactions to proceed at a reasonable rate. N′-Fluorodiimide N-oxides also were produced when di-