from solution. The complexes with the toluene-*p*-sulphonyl derivatives are particularly insoluble. Where homogeneous reaction mixtures are possible, reduction of the peptide carbonyl becomes considerable even at low temperatures, thus presenting a serious drawback to the use of LiAlH_4 in the peptide and protein field.

A solution of AlH_3 in tetrahydrofuran in the presence of a little aluminium chloride has been found useful for the preparation of a number of β -hydroxyamides in good yield. Best results are obtained when the reducing solution is added to the ester gradually. In the preparation of β -hydroxyamides from the ethyl esters of glycylglycine and L-leucylglycylglycine using this reagent at a temperature of -40° losses due to amide reduction amounted to 5–10%.

LiBH_4 is the best of the three reducing agents described, in that most of the peptide derivatives are freely soluble in a tetrahydrofuran solution of the reagent and reduction of the ester group occurs at room temperature. Where suspensions are obtained it is possible to reflux the mixture with little risk of amide reduction.

SUMMARY

1. β -Hydroxyamides corresponding to a number of peptide esters and their toluene-*p*-sulphonyl derivatives have been prepared.

2. Quantitative reduction of the ester groups with lithium aluminium hydride (LiAlH_4) was impossible in many cases owing to the formation of insoluble aluminium complexes.

3. Both LiAlH_4 and aluminium hydride (AlH_3) reduce the peptide bond. Greater control of the reaction, however, is possible with the latter reagent.

4. Lithium borohydride (LiBH_4) is the most suitable reagent for reducing peptide esters. Homogeneous reaction mixtures are usually obtained and the minimum of side reactions take place.

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Systematic Degradation of Peptides from the Carboxyl End

By J. LEGGETT BAILEY*

Department of Biochemistry, University of Cambridge

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Chemical methods are available for operating on both the amino end and the carboxyl end of proteins and peptides. The amino end has been more amenable to the techniques of organic chemistry and the majority of degradative methods relate to this end group. Of the methods for the identification and systematic removal of amino acids at the carboxyl end of peptide chains, that of Schlack & Kumpf (1926) has received the most attention. This procedure which converts the *C*-terminal amino acid into a thiohydantoin has been re-investigated inde-

pendently by Waley & Watson (1951) and Tibbs (1951). Both groups of workers concluded that some modification in technique is necessary before the method can be used on proteins. The recently reported procedure of Kenner, Khorana & Stedman (1953) using diphenyl phosphoroisothiocyanatide brings about cyclization under very mild conditions, but it has yet to be applied to a protein. A new approach to the problem of degradation at the carboxyl end was made possible when Chibnall & Rees (1951) showed that the *C*-terminal ester groups in insulin methyl ester could be reduced quantitatively with lithium borohydride (LiBH_4) to produce *C*-terminal amino alcohols (I $\rightarrow$ II).

* Present address: The Rockefeller Institute for Medical Research, 66th Street and York Avenue, New York 21, U.S.A.

The selective removal of *C*-terminal amino alcohols from polypeptides reduced in this way appeared possible by a procedure involving two steps. Under certain conditions employing acids or acid chlorides, β -hydroxyamides (*N*-peptidylaminoalcohols (II)) may be converted into the corresponding β -amino esters (III). Reductive cleavage of the β -amino esters with LiBH_4 would liberate the *C*-terminal amino alcohols (IV).

migration in an aliphatic system made use of phosphorus pentachloride and thionyl chloride; one instance of the use of phosphorus oxychloride, however, is cited later by Bergmann & Brand (1923). The formation of oxazolines from β -hydroxyamides and the mechanism of ring-opening has been discussed by Attenburrow, Elliott & Penny (1948), Goldberg & Kelly (1948) and Fry (1950). A recent review of the subject with special

In acid media *N*, *O* migration of acyl groups (*V* $\rightarrow$ *VII*) is generally considered to occur via an intermediate hydroxyoxazolidine (*VI*). Reagents used for this purpose have been concentrated sulphuric and hydrochloric acids (Bettzieche, 1925) and ethanolic hydrogen chloride (Phillips & Baltzly, 1947). Experiments carried out in this laboratory using methanolic hydrogen chloride and boron trifluoride have been only partially successful and this type of reagent was abandoned.

On the other hand, dehydration of the peptide hydroxyamides (*V*) to oxazolines (*VIII*) using phosphorus oxychloride and subsequent opening of the ring with dilute acid gave high yields of the β -amino ester salts (*VII*). The original studies of Bergmann, Brand & Dreyer (1921) of *N*, *O*-acyl

reference to *N*, *O* migration at the serine residues in proteins has been made by Elliott (1952). Many substituted 2-oxazolines (*VIII*) have been isolated by various workers as stable salts, which usually give the salts of the corresponding β -amino esters (*VII*) on treatment with water or dilute acid. In the presence of alkali the β -amino esters (*VII*) revert almost quantitatively to the original hydroxyamides (*V*).

β -Chloroethylamides (*X*) can arise by the action of Cl^- on the intermediate chlorophosphates (*IX*) or by ring cleavage of the oxazolines (*VIII*). This undesirable secondary reaction can be kept to a minimum by avoiding the more reactive acid chlorides such as thionyl chloride.

β -Hydroxamides can be prepared in high yield

from peptide esters by reduction with metal hydrides (Bailey, 1955). The present paper deals with the preparation of β -amino esters from peptide β -hydroxyamides, without isolation of the intermediate oxazoline derivatives, and subsequent reduction of these esters to liberate the terminal amino alcohols. In conjunction with the methods now available for the detection and estimation of amino alcohols on the micro scale (Grassmann, Hormann & Endres, 1953; Fromageot, Jutisz, Privat de Garilhe & Suquet, 1954; Chibnall & Rees, unpublished) the above principles might constitute a workable stepwise degradation of peptides from the carboxyl end.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected.

Aminoethanol (Ethanolamine). This was estimated by oxidation with periodic acid. The resulting formaldehyde (2 moles/mole of ethanolamine) was determined colorimetrically using chromotropic acid according to the modified micro method of Rees (unpublished).

O-Glycylaminoethanol dihydrochloride. The following synthesis was effected. β -Nitroethanol (1.5 ml.) was saturated with anhydrous HCl at -20° with rigorous exclusion of moisture. *N*-Carboxyglycine anhydride (100 mg.) was added to the solution and the mixture kept at -40° during 1 hr. when the temperature was allowed to rise slowly to room temperature. On dilution with ether crystals of *O-glycine β -nitroethanol ester hydrochloride* separated, m.p. 118–119°; yield 55 mg. (Found: N, 15.1. $C_4H_9O_4N_2Cl$ requires N, 15.2%). A further crop was obtained from the mother liquor on adding more ether. The nitro group was then reduced by means of iron and formic acid at room temperature. The mixture was filtered, concentrated *in vacuo* and the residue extracted with ethanol. A crystalline powder was obtained from the extract after addition of a few drops of ethanolic HCl and allowing to stand for some hr. The material was the dihydrochloride of *O-glycylaminoethanol* contaminated with a little glycine: no further purification was attempted. On titration of a sample with 0.0143 *N*-NaOH approximately 2 equivalents of alkali were consumed, and the quantitative conversion of the ester into *N-glycylaminoethanol* was confirmed by paper chromatography using the solvent system propanol–water–formic acid (80:19:1, by vol.). The R_F values for the hydroxyamide and the corresponding β -amino ester were 0.25 and 0.01 respectively.

Acyl migration via hydroxyoxazolidenes

Exploratory work was carried out using *N-glycylaminoethanol hydrochloride* on the 5 mg. scale, and progress was conveniently followed by paper chromatography. When the compound was treated with 3*N*-methanolic HCl at room temperature for 16 hr., no new ninhydrin-reactive material was detected. Heating for 2 hr. at 50° in a sealed tube, however, brought about the expected *N*, *O*-acyl shift and also alcoholysis of the *O-glycylaminoethanol* formed. The latter course was considerably suppressed when BF_3 -methanol was employed, although a higher temperature was necessary for the rearrangement to take place. An 80% rearrangement was possible under the following

conditions: (a) treatment with BF_3 -formic acid (0.2 ml.; b.p. 79–83°/15 mm.) for 2 hr. at 60° ; (b) treatment with BF_3 -acetic acid (0.2 ml.; b.p. 141°/760 mm.) for 3 hr. at 70° ; (c) treatment with $SnCl_4$ -formic acid (1:3, by vol.) for 4 hr. at 80° .

In the case of *N*-L-leucylglycylaminoethanol (5 mg.) best results were obtained by warming the compound with BF_3 -formic acid (0.1 ml.) for 1 hr. at 60° . Although the main spot on the chromatogram corresponded to the β -amino ester (R_F , 0.28; solvent system, propanol–water–formic acid), the presence of other faint ninhydrin spots indicated secondary products. Treatment of the β -hydroxyamide with 97.5% H_2SO_4 at 21° for 3 days as described by Elliott (1952) gave no β -amino ester.

Acyl migration via intermediate oxazolines

N-Glycylaminoethanol and $SOCl_2$ at room temperature yielded glycyl- β -chloroethylamide (R_F , 0.45) and unchanged hydroxyamide. Interaction of a solution of L-leucylglycylaminoethanol in nitromethane with $SOCl_2$ at -10° or $POCl_3$ at room temperature gave unchanged hydroxyamide, the β -amino ester and β -chloroethylamide (R_F , 0.44). When the reactions were carried out in dimethylformamide, the β -chloroethylamides were the main products.

When the *N*-toluene-*p*-sulphonyl derivatives of the hydroxyamides were used, excellent yields of corresponding β -amino ester hydrochlorides were obtained.

*N-Toluene-*p*-sulphonyl-O-glycylaminoethanol hydrochloride.* *N*-Toluene-*p*-sulphonylglycylaminoethanol (44 mg.) was dissolved in acetonitrile (0.3 ml.) containing $SOCl_2$ (0.05 ml.; 4 mol. prop.) at -10° . At the end of 2 hr. the excess $SOCl_2$ was removed *in vacuo* at -10° and the residue decomposed with water. Concentration of the solution afforded *N-toluene-*p*-sulphonyl-O-glycylaminoethanol hydrochloride* as rectangular prisms in 85% yield. Recrystallized from ethanol–ether the compound had m.p. 177.5–178°. (Found: C, 42.7; H, 5.5, N, 9.3. $C_{11}H_{11}O_4N_2S$ requires C, 42.8; H, 5.5; N, 9.1%). Rearrangement by means of selenium oxychloride (0.05 ml.) gave a similar yield of the β -amino ester hydrochloride. In this case after decomposition with water Se was removed by precipitation with H_2S .

*N-Toluene-*p*-sulphonyl-O-leucylphenylalaninol hydrochloride.* *N*-Toluene-*p*-sulphonylleucylphenylalaninol (45 mg.) was dissolved in acetonitrile (0.1 ml.) and $POCl_3$ (0.1 ml.) and left at room temperature for 3 hr. On removal of the solvents *in vacuo* a crystalline mass was obtained. This was assumed to be the intermediate oxazoline hydrochloride which on addition of water decomposed to give *N-toluene-*p*-sulphonyl-O-leucylphenylalaninol hydrochloride*; yield 44 mg., 90%. This compound which was insoluble in water, was recrystallized from ethanol giving needles, m.p. 197.5°. (Found: C, 58.0; H, 6.9; N, 6.1. $C_{22}H_{21}O_4N_2S$ requires C, 58.1; H, 6.8; N, 6.2%).

*N-Toluene-*p*-sulphonyl-O-leucylglycylaminoethanol hydrochloride.* *N*-Toluene-*p*-sulphonylleucylglycylaminoethanol (114 mg.) was suspended in acetonitrile (0.2 ml.) and $POCl_3$ (0.2 ml.) added. The clear solution was left at room temperature for 3 hr., the solvent removed *in vacuo* and the residue decomposed with water. After 2 hr. the solution was decanted from a small amount of insoluble material, neutralized with $BaCO_3$, centrifuged to remove barium phosphate and concentrated to dryness at room temperature. The residue was extracted with hot ethanol and the extract concentrated to a small volume. After addition of ethyl acetate

and allowing to stand overnight fine needles of the β -amino ester hydrochloride were deposited; yield 107 mg., 87%, m.p. 166–167°. (Found C, 47.1; H, 7.0. $C_{17}H_{25}O_4N_2S$ requires C, 48.4; H, 6.6%.) Treatment of *N*-toluene-*p*-sulphonylleucylglycylaminoethanol with $SOCl_2$ in acetonitrile at 0° gave an 85% yield of the corresponding β -chloroethylamide. Recrystallized from ethanol–ether (needles) this compound had m.p. 159.5–160°. (Found C, 50.8; H, 6.5. $C_{17}H_{25}O_4N_2S$ requires C, 50.6; H, 6.4%.)

The β -amino ester hydrochlorides rearranged back to the original β -hydroxyamide on neutralization with alkali. The course of *N*, *O*-acyl migration could be conveniently followed on the small scale by determination of amino nitrogen in the Van Slyke–Neill apparatus (reaction time 11 min.).

Step-wise degradation of N-toluene-p-sulphonyl-L-leucylglycylglycine ethyl ester

The conversion into *N*-toluene-*p*-sulphonyl-L-leucylglycylaminoethanol (94%) has been described by Bailey (1955) and subsequent rearrangement to the β -amino ester (87%) is described above.

Reductive cleavage of the β -amino ester. *N*-Toluene-*p*-sulphonyl-*O*-leucylglycylaminoethanol hydrochloride (79 mg.) was moistened with tetrahydrofuran (0.2 ml.) and reduced at room temperature with 3*M*-LiBH₄ solution (0.22 ml.) during 16.5 hr. The reaction mixture was cooled, decomposed with dilute HCl and a vacuum applied to remove the tetrahydrofuran. The oily precipitate crystallized after 30 min. and was the new β -hydroxyamide, *N*-toluene-*p*-sulphonyl-L-leucylaminoethanol; 45 mg., 82%. After recrystallization from ethyl acetate–ether the compound (needles) had m.p. 151.5°. (Found: C, 55.0; H, 7.6; N, 8.5. $C_{15}H_{21}O_4N_2S$ requires C, 54.9; H, 7.3; N, 8.5%.) The aqueous filtrate remaining after filtration of the β -hydroxyamide was made up to 100 ml. and a sample taken for oxidation with periodic acid. The aminoethanol found corresponded to an 85% reduction of the β -amino ester.

Rearrangement of *N*-toluene-*p*-sulphonyl-L-leucylaminoethanol. The β -hydroxyamide (35 mg.) was dissolved in acetonitrile (0.1 ml.), $POCl_3$ (0.1 ml.) added and the mixture left for 3 hr. at room temperature. After removal of the solvents *in vacuo* and decomposition of the residue with water the new β -amino ester hydrochloride was isolated by neutralizing the solution with $BaCO_3$ as described above. *N*-Toluene-*p*-sulphonyl-*O*-leucylaminoethanol hydrochloride was recrystallized from acetonitrile (needles) and had m.p. 181–182.5; yield, 33 mg., 86%. (Found: C, 48.9; H, 7.1; N, 7.8. $C_{15}H_{21}O_4N_2S$ requires C, 49.4; H, 6.8; N, 7.7%.)

Reductive cleavage of the β -amino ester. Reduction of *N*-toluene-*p*-sulphonyl-*O*-leucylaminoethanol hydrochloride with LiBH₄ solution in the usual way liberated ethanolamine in 91% yield. Reduction with AlH_3 as described by Bailey (1955) gave a 90% recovery of ethanolamine. For the purpose of a systematic degradation methods for separation of the main products from undesirable secondary products are necessary and have been tentatively explored. A synthetic mixture of the β -amino ester, β -hydroxyamide and β -chloroethylamide corresponding to any particular peptide could be resolved very simply by electrodialysis in a three-compartment cell. Using 0.2*N* acetic acid in all compartments 98% of the β -amino ester migrated from the centre to the cathode compartment; the neutral compounds

for the main part remained in the centre compartment, a small amount migrating to the anode compartment. High voltage zone electrophoresis on thick filter paper was also found useful for following the course of acyl migration. The β -amino esters which migrated towards the cathode could be detected by the ninhydrin reaction whereas the traces of neutral compounds responded to the chlorination technique of Rydon & Smith (1952).

SUMMARY

1. Warm methanolic hydrogen chloride and boron trifluoride in formic acid bring about *N*, *O*-acyl migration in *N*-glycylaminoethanol and *N*-L-leucylglycylaminoethanol in good yield. Alcoholysis of the β -amino esters is considerable with the former reagent; a number of secondary products not identified are produced with the latter reagent.

2. *N*, *O*-Acyl migration in β -hydroxyamides derived from *N*-toluene-*p*-sulphonyl peptide esters has been achieved in 85–90% yield using phosphorus oxychloride at room temperature. The β -amino ester hydrochlorides have been isolated in crystalline form.

3. Reductive cleavage of the β -amino esters with lithium borohydride liberates the terminal amino alcohol in 85–90% yield. The residual peptide at this stage is simultaneously converted into a new β -hydroxyamide capable of rearrangement with phosphorus oxychloride.

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