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The Reduction of Esters of Simple Peptides by Metal Hydrides

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(Received 21 October 1954)

An investigation into the behaviour of simple peptide esters on reduction with metal hydrides has been carried out concurrently with the analytical work on proteins to be described later by Chibnall & Rees. Information was required concerning the conditions under which the reagents lithium borohydride (LiBH_4), lithium aluminium hydride (LiAlH_4), and aluminium hydride (AlH_3) reduced ester groups but failed to attack the peptide bond, and concerning methods for isolating pure reduction products for subsequent degradative studies. The following experimental work describes the preparation of β -hydroxyamides from (a) the esters of glycylglycine, L-leucylglycine and L-leucylglycylglycine and (b) the esters of the toluene-*p*-sulphonyl derivatives of glycylglycine, L-leucyl-L-phenylalanine and L-leucylglycylglycine. Fromageot & Jollès (1952) have reported yields of less than 50% for the diols produced from glutamyl- γ -glycine diethyl ester and glutathione diethyl ester by reduction with LiAlH_4 . The conditions for the total reduction of simple peptides to polyamines have been described by Karrer & Nicolaus (1952).

The main products and possible secondary products arising from the action of metal hydrides on a peptide ester may be summarized schematically as follows:

EXPERIMENTAL AND RESULTS

Materials

Tetrahydrofuran. This solvent (Imperial Chemical Industries Ltd., Billingham) was refluxed over K metal for 24 hr. and distilled, refluxed over LiAlH_4 and distilled, and stored in a dark bottle. The solvent rapidly peroxidizes and samples that have been kept longer than 2 weeks should be redistilled over LiAlH_4 before use.

Standard solutions of LiBH_4 and LiAlH_4 . Solutions between 1 and 2.5 M were prepared by refluxing the reagent (New Metals and Chemicals Ltd., London) (rough weight judged by bulk) with 30–40 ml. tetrahydrofuran for 2 hr., allowing to cool and centrifuging. The clear supernatant was poured off into a bottle from which the air had been displaced by dry N_2 . The reducing power of these solutions may be assessed by titration of the Li by standard acid or more accurately by the manometric method of Krynitsky, Johnson & Carhart (1948) depending on the volume of H_2 liberated on decomposition by acid. In the latter estimation a longer reaction time is necessary in the case of LiBH_4 . Decomposition of all boranes is assumed complete after about 30 min. The solutions can be kept for a few weeks but long storage brings about some change which is not understood.

Aluminium hydride. A 0.5 M solution of AlCl_3 was prepared by adding the resublimed material to tetrahydrofuran pre-cooled to -20° . On warming to room temperature the solid dissolves giving a practically clear solution. A solution

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of AlH_3 was made from a standard solution of LiAlH_4 and the 0.5M aluminium chloride solution according to the equation $3\text{LiAlH}_4 + \text{AlCl}_3 = 4\text{AlH}_3 + 3\text{LiCl}$ (Finholt, Bond & Schlesinger, 1947).

Peptide ester hydrochlorides. The methyl ester hydrochlorides of glycylglycine and L-leucylglycine were prepared by dissolution of the free peptides in saturated methanolic HCl and concentration of the solutions *in vacuo* at room temperature (cf. Wilson & Pacsu, 1942). L-Leucylglycylglycine ethyl ester hydrochloride was prepared by the method of Bailey (1950). N-Carboxy-L-leucine anhydride was coupled with glycylglycine ethyl ester dissolved in CHCl_3 and in the presence of methyldiethylamine at -60° . The bases were converted into hydrochlorides by means of ethanolic HCl and the tertiary base hydrochloride removed after addition of water by extraction with CHCl_3 . The aqueous layer was concentrated *in vacuo* and the residue crystallized from ethanol. L-Leucylglycylglycine ethyl ester hydrochloride crystallized in long prisms with one molecule of ethanol; yield 78%. (Found: N, 13.2. $\text{C}_{10}\text{H}_{18}\text{O}_5\text{N}_3\text{Cl}$ requires N, 13.5%.)

N-Toluene-p-sulphonyl-L-leucylglycylglycine ethyl ester. Toluene-p-sulphonyl-L-leucine (1 g.; m.p. 124°) prepared according to McChesney & Swann (1937) was warmed with SOCl_2 on the steam bath for 1 hr. and the excess reagent removed *in vacuo* at room temperature. The crystalline acid chloride was dissolved in CHCl_3 (10 ml.) and added slowly with cooling to glycylglycine ethyl ester (from 1.4 g. hydrochloride) in the same solvent. The mixture was allowed to stand overnight, filtered from glycylglycine ethyl ester hydrochloride and the filtrate concentrated *in vacuo*. The residue was taken up in benzene and an equal volume of light petroleum (b.p. $40-60^\circ$) added. After a few hours clusters of needles appeared. Two further crystallizations from benzene gave toluene-p-sulphonyl-L-leucylglycylglycine ethyl ester; yield 0.84 g., m.p. $151.5-152^\circ$. (Found: C, 54.2; H, 6.9; N, 9.6. $\text{C}_{19}\text{H}_{29}\text{O}_6\text{N}_3\text{S}$ requires C, 53.6; H, 6.8; N, 9.8%.) Prepared similarly, toluene-p-sulphonylglycylglycine ethyl

ester crystallized in needles from benzene m.p. $88-88.5^\circ$ (Found: C, 50.1; H, 5.7. $\text{C}_{13}\text{H}_{18}\text{O}_5\text{N}_2\text{S}$ requires C, 49.7; H, 5.7%) and toluene-p-sulphonyl-L-leucyl-L-phenylalanine methyl ester in fine needles from benzene-light petroleum, m.p. $84-85^\circ$ (Found: C, 61.8; H, 6.9. $\text{C}_{23}\text{H}_{30}\text{O}_5\text{N}_2\text{S}$ requires C, 61.9; H, 6.7%).

Reduction of peptide esters with LiBH_4

Preliminary experiments were conducted using the conditions employed by Chibnall & Rees (to be published). Insoluble complexes were obtained leading to incomplete reduction of the ester groups. When the volume of solvent was decreased so that the solution became about M with respect to the LiBH_4 the peptide esters dissolved and the solutions remained clear. Reduction of the ester groups then exceeded 90%.

An example of the procedure finally adopted is as follows. L-Leucylglycylglycine ethyl ester hydrochloride (160 mg.) was dried over P_2O_5 and NaOH and then *in vacuo* at 70° for 3 hr. The crystals were transferred to a small flask which had been flushed with dry N_2 . Anhydrous tetrahydrofuran (0.4 ml.) and 2.3M- LiBH_4 solution (0.47 ml.; 2 mol. prop.) were added and the clear solution left for 17 hr. at room temperature. The mixture was then cooled to -10° , decomposed with N-HCl (1.1 ml.), left for a few hr., and neutralized to bromothymol blue with N-LiOH. The solution was concentrated to dryness, the residue dissolved in water (1 ml.) and applied to a short Dowex-50 (X8, 200 mesh) column (6.5 $\times$ 1.5 cm.). On some occasions at this stage slight disturbance of the column occurred due to evolution of hydrogen from the slow hydrolysis of small amounts of higher boron hydrides. On elution with 0.1N- NH_3 the β -hydroxyamide formed by the reduction of the peptide ester travelled down the column as a visible band adjacent to the neutralization front. The fraction containing the β -hydroxyamide (20-30 ml.) was concentrated *in vacuo* over P_2O_5 , the crystalline residue dissolved in ethanol and treated with the calculated amount of ethanolic HCl to give

Table 1. β -Hydroxyamides prepared by reduction of peptide esters with LiBH_4

R_F values in propanol-formic acid-water (80:1:19, by vol.) computed proportionally from an arbitrary standard for ethanolamine of $R_F = 0.35$.

β -Hydroxyamide	Yield (%)	M.p. (uncorr.) Solvent for recryst. and cryst. form	R_F	Analysis	
				Found (%)	Required (%)
Glycylaminoethanol hydrochloride	90	126-5° (90% ethanol, rectangular prisms)	0.25	C, 31.3; H, 7.3; N, 18.0	($\text{C}_4\text{H}_{11}\text{O}_2\text{N}_2\text{Cl}$) C, 31.1; H, 7.1; N, 18.1
L-Leucylaminoethanol hydrochloride	90	159.5-160° (ethanol-ether, flat prisms)	0.61	C, 45.3; H, 9.0	($\text{C}_8\text{H}_{19}\text{O}_3\text{N}_2\text{Cl}$) C, 45.6; H, 9.0
L-Leucylglycylaminoethanol hydrochloride	83	161-162° (ethanol-ether, long prisms)	0.58	C, 44.9; H, 8.3	($\text{C}_{10}\text{H}_{22}\text{O}_3\text{N}_2\text{Cl}$) C, 44.9; H, 8.2
Toluene-p-sulphonylglycylaminoethanol	87	126-126.5° (water, prisms)	—	C, 48.9; H, 5.9; N, 10.2	($\text{C}_{11}\text{H}_{16}\text{O}_4\text{N}_2\text{S}$) C, 48.6; H, 5.9; N, 10.3
Toluene-p-sulphonyl-L-leucylphenylalaninol	85	165.5-166° (acetonitrile, long prisms)	—	C, 62.8; H, 7.1; N, 6.6	($\text{C}_{22}\text{H}_{30}\text{O}_4\text{N}_2\text{S}$) C, 63.1; H, 7.1; N, 6.7
Toluene-p-sulphonyl-L-leucylglycylaminoethanol	94	176° (methanol-ethyl acetate, large rhombohedra)	—	C, 54.0; H, 7.4; N, 10.7	($\text{C}_{17}\text{H}_{27}\text{O}_5\text{N}_3\text{S}$) C, 53.0; H, 7.0; N, 10.9

the hydrochloride. The latter crystallized in long prisms on addition of ether to the solution; m.p. 161–162°; yield 115 mg. Data on the β -hydroxyamides prepared in this way are given in Table 1.

The procedure for the reduction of *N*-toluene-*p*-sulphonyl derivatives was as follows: 1.75M-LiBH₄ solution (0.34 ml.; 2.25 mol. prop.) was added to *N*-toluene-*p*-sulphonyl-L-leucylglycylglycine ethyl ester (100 mg.) in anhydrous tetrahydrofuran (0.7 ml.) and the clear solution left at room temperature overnight. The mixture was cooled, diluted with 50% ethanol (4 ml.) and decomposed with dry Dowex-50 (200 mg.) to give a solution of about pH 8. After allowing to stand at this pH for a few hr. further resin was added to lower the pH to about 5 and the mixture kept at room temperature for a further 24 hr. The resin was filtered off, the filtrate concentrated to dryness *in vacuo* and the boric acid removed by evaporating several times with anhydrous methanol-benzene (2:1). Addition of ethyl acetate to the residue gave the crystalline β -hydroxyamide; yield 820 mg., 94%. Recrystallized by dissolving in the minimum volume of methanol and adding ethyl acetate the compound has m.p. 176°.

sumably diamines) resulting from reduction of the peptide carbonyl.

Better success attended experiments using AlH₃. The following procedures afforded the best yields of the β -hydroxyamides using this reagent. The ester to be reduced (10–100 mg.) was weighed in a 5 ml. reaction vessel and dried *in vacuo* for 2–3 hr. at 60°. Sufficient 0.5M AlCl₃ solution (0.2–3.0 ml.) to dissolve the ester was introduced into the reaction vessel which was fitted with a mercury-sealed stirrer, and the air displaced by dry N₂. By means of a micrometer and syringe the calculated volume of freshly prepared AlH₃ solution (1.5–2.0 equivalents) was drawn into a micro-burette tube which was then immediately fitted into a side-arm of the reaction vessel. The latter was cooled to –40° (solid CO₂-butanol), the stirrer set in motion and the reducing agent delivered gradually during a period of 1 hr. The temperature of the bath was allowed to rise gradually to –10° during 8–12 hr. The reaction mixture was decomposed with ethanolic HCl and after removal of the Al as Al(OH)₃ the isolation procedure was the same as that described above. The results of a few experiments selected for comparison purposes are shown in Table 2.

Table 2. Reduction of peptide esters with LiAlH₄ and AlH₃

Ester	Reducing agent	Conditions	Yield of β -hydroxyamide isolated (%)	Other products
Glycylglycine ethyl ester	LiAlH ₄	Suspension in tetrahydrofuran at –50°; 6 hr. reaction	None	Aminoethanol, glycine, glycylglycine, strongly basic product
	LiAlH ₄	Solution in anhydrous pyridine at –50°; 3 hr. reaction	40	Aminoethanol, glycine, glycylglycine, strongly basic product
	AlH ₃	4 hr. at room temp.	48	Strongly basic product
	AlH ₃	4 hr. at –40°	70	Traces of strongly basic product
L-Leucylglycylglycine ethyl ester	LiAlH ₄	Suspension in <i>N</i> -methylmorpholine-tetrahydrofuran; 2 hr. at room temp.	47	Leucylglycylglycine, strongly basic product
	AlH ₃	4 hr. at –40°	74	Traces of strongly basic product

Reduction of peptide esters with AlH₃ and LiAlH₄

Preliminary experiments with glycine ethyl ester hydrochloride showed that reaction with a solution of LiAlH₄ in tetrahydrofuran was very slow at –65°. On allowing the temperature to rise gradually the reaction became vigorous at about –10°. In the case of peptide esters, although 90% reduction of the ester groups was realized, the yields of β -hydroxyamides isolated never exceeded 50%. When the reaction mixtures were passed through a short column of the strong-base resin, Dowex-2, evidence of strongly basic secondary products was obtained. These materials reacted with periodic acid but were almost completely resistant to acid hydrolysis; they could be eluted from Dowex-50 by means of N-NaOH solution. When run on paper chromatograms in propanol-formic acid they appeared as elongated spots behind each β -hydroxyamide; in phenol-water they ran to the solvent front as do most basic substances. The low yields of β -hydroxyamides isolated were attributed mainly to the production of these basic substances (pre-

It was of interest to assess the amount of reduction of a simple amide such as *N*-acetyl aminoethanol using the optimum conditions described above for AlH₃. Whereas *N*-acetyl aminoethanol reacts very slowly with periodic acid, *N*-ethyl aminoethanol arising from reduction of the acyl group gives the usual two molecules of formaldehyde. On reduction of the amide with AlH₃ (2 equivalents) at –40° to –10° during 17 hr. only 4.4% of the theoretically possible *N*-ethyl aminoethanol was produced. An even lower yield of 1.5% *N*-ethyl aminoethanol was obtained after reduction of the amide with LiBH₄ for 3 hr. in tetrahydrofuran under reflux conditions.

DISCUSSION

Reduction of peptide esters to the corresponding β -hydroxyamide with LiAlH₄ proceeds rapidly at room temperature. Reaction is considerably moderated at low temperature but it is difficult to bring about quantitative reduction of the ester group if insoluble aluminium complexes separate

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.

I wish to express my thanks to Dr A. C. Chibnall, F.R.S., for his advice and interest in this work which was carried out during tenure of an Imperial Chemical Industries Research Fellowship.

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Systematic Degradation of Peptides from the Carboxyl End

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(Received 21 October 1954)

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).

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