Volumetric Determination of Amino Acid and Peptide Hydrazides

Peptide azides are often used in the preparation of polypeptides[1,2]. For successful coupling by the azide method the purity of the peptide hydrazide, the precursor of the azide[3], should be determined. A simple and rapid method for quantitative determination of peptide hydrazides is therefore of importance.

Acid hydrazides are amphoteric[4]. They are also reducing agents[4]. Their determination may therefore be based on acid-base titration or on titration with oxidizing reagents. Isonicotinic acid hydrazide, for example, has been determined both potentiometrically and volumetrically by various titrants based on the above properties of this class of compounds[5–10].

Peptide hydrazides have been determined by a gas volumetric method[11]. This determination was based on the oxidation of the peptide hydrazide by iodine and measurement of the nitrogen evolved. BRENNER and HOFER[12] have titrated glycine and phenylalanine peptide hydrazides directly with sodium hypobromite (NaOBr) using methyl red indicator. While this work was in progress GREVEN[13] reported the use of perchloric acid in the titrimetric determination of a peptide hydrazide.

The purpose of this communication is to report the investigation of several volumetric methods for the determination of peptide hydrazides. Known methods of volumetric and potentiometric acid hydrazide determination were tested for their general applicability as volumetric methods.

Experimental. The peptide hydrazides were prepared by the hydrazinolysis of the corresponding methyl or ethyl ester. Elemental analysis and thin-layer chromatography of the hydrazide showed the material to be pure. Tert-butyloxycarbonylhydrazide (*t*-Boc-hydrazide) was purchased from EGA-Chemie (KG), West Germany and tested without further purification. All other reagents used were analytical grade.

The titrations were performed on 5–15 mg of material. 0.1 *N* perchloric acid ($HClO_4$) in glacial acetic acid (CH_3COOH) was prepared and standardized as described elsewhere[14]. The compound to be determined was dissolved in 1 ml glacial acetic acid and titrated with the above reagent. The end-point was determined by a violet to green colour change of the methyl violet indicator.

An aqueous solution of 0.1 *N* sodium hypobromite (NaOBr) was prepared by dissolving an equivalent amount of bromine in 0.1 *N* sodium hydroxide. This solution was then standardized against sodium thiosulphate[15]. The peptide hydrazide was dissolved in 1 ml glacial acetic acid to which was added 1 ml 2 *N* sulphuric acid and titrated with the hypobromite solution. The end-point was observed by a colour change of red to colourless of the methyl red indicator[12].

An aqueous solution of 0.1 *N* potassium permanganate ($KMnO_4$) was prepared and standardized[15]. 1 ml of 2 *N* sulphuric acid was added to the peptide hydrazide dissolved in dimethylformamide. This solution was titrated with the permanganate solution. The end-point, a change in colour from colourless to pink, is stable for 10 sec and is detectable without the aid of an indicator.

The determination of peptide hydrazides was also tested with aqueous solutions[15] of ceric sulphate ($Ce(SO_4)_2$), potassium dichromate ($K_2Cr_2O_7$), chloramine-T, and potassium ferricyanide ($K_3Fe(CN)_6$). Non-aqueous determinations using perchloric acid in dioxane[16], potassium *t*-butoxide[17], and bromine (Br_2) in glacial acetic acid were also tested for their applicability.

[1] E. SCHRODER and K. LUBKE, *The Peptides* (Academic Press, New York 1965), Vol. 1, p. 79.
[2] R. G. DENKEWALTER, D. F. VEBER, F. W. HOLLY and R. HIRSCHMAN, J. Am. chem. Soc. *91*, 502 (1969).
[3] T. CURTIUS, Chem. Ber. *35*, 3226 (1902).
[4] S. SIGGIA, *Quantitative Organic Analysis via Functional Groups*, 3rd edn. (John Wiley and Sons Inc., New York 1963), p. 543.
[5] J. F. ALICINO, J. Am. pharm. Soc., Sci. Ed. *41*, 401 (1952).
[6] A. ANASTASI, E. MECARELLI and L. NOVACIC, Mikrochemie *40*, 53 (1952).
[7] J. VULTERIN and J. ZYKA, Chemické Listy *48*, 839 (1954).
[8] VON A. BERKA and J. ZYKA, Pharmazie *13*, 81 (1958).
[9] K. W. MERZ and M. SCHIRM, Naturwissenschaften *39*, 570 (1) (1952).
[10] B. SINGH, S. S. SAHOTA and M. P. GUPTA, J. Ind. chem. Soc. *38*, 189 (1961), and references cited therein.
[11] H. MEDZIHRADSZKY-SCHWEIGER, Acta chim. hung. *34*, 213 (1962).
[12] M. BRENNER and W. HOFER, Helv. chim. Acta *44*, 1794 (1961).
[13] H. M. GREVEN, 10th European Peptide Symposium, Abano, Italy (1969).
[14] J. S. FRITZ, Analyt. Chem. *22*, 1028 (1950).
[15] A. I. VOGEL, *Textbook of Quantitative Organic Analysis*, 2nd edn. (Longmans, Green and Co. Ltd., London 1958).
[16] J. S. FRITZ, Analyt. Chem. *22*, 578 (1950).
[17] S. EHRLICH-ROGOZINSKY, A. PATCHORNIK, personal communication.

The determination of peptide hydrazides

Hydrazide[a] No.		Mol. wt.	$HClO_4/CH_3COOH$		NaOBr		$KMnO_4$	
			Mol. wt. found	% error	Mol. wt. found	% error	Mol. wt. found	% error
1	*t*-Boc	132	127	3.8	126[b]	4.5	123	6.8
2	Z-Gly	223	220	1.3	214	4.0	217	2.8
3	Z-Ser	253	249	1.6	255	0.8	240	5.1
4	Z-Im-Bz-His	377	370[c]	1.9	360	4.5	371	1.6
5	α, ε-di-Z-Lys	412	422	2.4	422	2.4	420	1.9
6	Z-Gly-Gly	280	274	2.1	270	3.6	273	2.5
7	Z-Ser-Ser	339	337	0.6	328	3.2	328	3.2
8	Z-Phe-Phe	460	461	0.2	445	3.3	425	7.6
9	Z-Phe-Gly	370	359	3.0	352	4.9	364	1.6
10	Z-Ala-Leu	350	329	6.0	333	5.0	329	6.0
11	Z-Ala-Ala-Ala	379	375	1.1	340[d]	10.3	381	0.5

[a] L-Amino acids were used throughout. The abbreviations are: *t*-Boc, tertiary-butyloxycarbonyl; Z, carbobenzoxy; Im-Bz, N-benzyl-imidazole; Gly, glycine; Ser, serine; His, histidine; Lys, lysine; Phe, phenylalanine; Ala, alanine; and Leu, leucine. [b] This titration was performed without sulphuric acid. [c] This mol. wt. determination is based on 2 equivalents acid to 1 equivalent hydrazide, since the imidazole ring is also titrated by $HClO_4$[18]. [d] This determination was performed on 2 mg of material.

Results and discussion. The experimental error inherent in the determinations was 3%. This error arises from the deviations possible in the weighings of the sample and the droplet size.

The mol. wt. of a peptide hydrazide determined by perchloric acid titration was calculated from the following stoichiometry:

$$RCONHNH_2 + HClO_4 \rightarrow RCONHNH_3^+ ClO_4^-$$

The titration using the perchloric acid in glacial acetic acid and methyl violet indicator had sharp end-points while the same acid in dioxane using thymol blue as indicator gave poor end-points. The former titrant gave results which on the average were within the experimental error (Table).

The stoichiometry for the titration of peptide hydrazides with NaOBr is[12]

$$RCONHNH_2 + 2HOBr \rightarrow RCOOH + N_2 + 2HBr + H_2O.$$

The end-point with methyl red indicator is sharp and the deviation of the molecular weight found from the true mol. wt. is on the average 3.6% (Table).

The oxidation of acid hydrazides by $KMnO_4$ involves a 3.5 electron change per hydrazide group[9].

$$4RCONHNH_2 + 3.5O_2 \rightarrow RCON = NOCR + 2RCOOH + 3N_2 + 5H_2O.$$

The titrant does not need an indicator and the mol. wt. determinations had the same accuracy as the sodium hypobromite determinations (Table).

Base titration of the peptide hydrazides with potassium *t*-butoxide[17] (0.05 *N*) using any of the following indicators gave obscure end-points: *o*-nitroaniline, tropaerolin 00 or brilliant cresol blue. The best end-point was obtained with *o*-nitroaniline, the error being 10–15%.

The reaction of $K_3Fe(CN)_6$ with acid hydrazides is[8]

$$RCONHNH_2 + 4K_3Fe(CN)_6 + 4NaOH \rightarrow 3K_4Fe(CN)_6 + Na_4Fe(CN)_6 + N_2 + 3H_2O.$$

When the peptide hydrazide is dissolved in DMF and excess NaOH is added, the direct titration with $K_3Fe(CN)_6$ produces no colour change at the equivalence point. However, if excess $K_3Fe(CN)_6$ is present in the above peptide solution, the excess NaOH may be titrated with the aid of phenolphthalein indicator (red to yellow) and the mol. wt. may be determined for the peptide hydrazide. Such determinations gave only 10–15% accuracy. 0.2 *N* $K_3Fe(CN)_6$ in 0.1 *N* NaOH using phenolphthalein as indicator was tested as a direct titrimetric method. In this case the colour change (yellow to red) occurs before the equivalence point but slowly reverts to the initial colour. The reappearance of the initial colour indicates that peptide hydrazides are slowly oxidized by $K_3Fe(CN)_6$. Therefore this oxidant is a poor volumetric titrant for this class of compounds.

Similar difficulties were encountered with the oxidants $Ce(SO_4)_2$, $K_2Cr_2O_7$ and chloramine-T.

Determinations of the peptide hydrazide made with Br_2 in glacial acetic acid involved a colour change at the end-point of colourless to faint-yellow in the absence of an indicator. Since there was so little difference between the initial and the final colour, the mol. wt. of the hydrazides could not be determined with any confidence.

In conclusion it may be stated that peptide hydrazides can be determined by simple titrimetric methods. Of the titrants tested, perchloric acid in glacial acetic acid with methyl violet as indicator appears to be the best as its stoichiometry is one-to-one, the end-point is sharp and stable and it gives the most accurate mol. wt. determinations. If the peptide hydrazide has an acid titratable group (e.g. compound 4, Table) the equivalence point changes accordingly. If, however, one wishes to perform aqueous titrations, then an oxidizing titrant would be of choice. Of those oxidants tested $KMnO_4$ or NaOBr with methyl red indicator appear to be the best. The disadvantage for general use of oxidizing titrants for determination of peptide hydrazides is the existence of oxidizable amino acids. Thus it is advantageous to have available both an acidimetric and an oxidimetric method.

Résumé. Nous avons déterminé par titrage volumétrique une série d'hydrazides peptidiques et testé les titrages acides, basiques et oxidants. Un tableau condense les résultats obtenus avec les trois meilleurs réactifs de titrage (acide perchlorique dans l'acide acétique glacial, l'hypobromite de sodium et le permanganate de potassium) qui sont tous utilisables à cet effet.

M. FRIDKIN and H. J. GOREN[19]

Department of Biophysics,
The Weizmann Institute of Science,
Rehovot (Israel), 29 December 1969.

[18] A. PATCHORNIK and S. SHALTIEL, Bull. Res. Coun. Israel *11A*, 224 (1962).

[19] Acknowledgment. H. J. GOREN is grateful for the Post-doctoral Fellowship awarded him by the National Research Council of Canada, which enabled him to carry out this research.

Culture de cellules embryonnaires d'Urodèles sur un support-gel d'acrylamide

Les travaux de RAPPAPORT et al.[1] ont montré que pour qu'une cellule adhère, s'étale et se différencie sur une surface, celle-ci doit présenter certaines caractéristiques physico-chimiques, notamment: 1. une charge électrique suffisante par unité de surface pour permettre l'attachement d'un type cellulaire donné; 2. un mécanisme de protection de ces «sites» contre l'excrétion des protons par la cellule après attachement. Selon ces auteurs, ces paramètres doivent s'adapter aux caractéristiques de la cellule, sa taille, son métabolisme, ainsi que la densité de charge de sa membrane cytoplasmique.

D'autres auteurs se sont également intéressés au problème de «l'attachement» de cellules à un support. En particulier CURTIS[2] a développé plusieurs théories de «l'attachement» des cellules au verre. Il suppose que cet attachement résulterait d'un équilibre entre les forces adhésives de van der Waals et les forces électrostatiques de répulsion dues aux charges de surface.

Nous nous sommes inspirés des travaux d'HOFFMAN et al.[3] pour étudier l'action de certaines substances biologiques, séparées par électrophorèse sur gel, sur la différenciation cellulaire. Nous avons, dans ce travail préliminaire, recherché un support-gel permettant l'étalement et la différenciation de cellules embryonnaires d'Urodèles, selon une technique de culture déjà décrite[4,5].

Matériel. Les cellules utilisées pour effectuer ce travail sont des cellules embryonnaires de *Pleurodeles waltlii* et d'*Ambystoma mexicanum* prélevées sur la plaque et les