Some of the aspects of mass spectrometric structure analysis in organic chemistry may be summarized visually as shown by Figure 11, where the unknown

Fig. 11. The unknown mass spectrocopist.

mass spectroscopist is trying with apparent concentration and determination to reveal with the aid of his instrument some of the secrets of the shape and coherence of organic molecules.

Zusammenfassung. Massenspektrometrie ist die instrumentalanalytische Methode mit der grössten Anwendungsvielfalt im naturwissenschaftlichen Bereich. Auf dem Gebiet der organischen Chemie sind derzeit qualitative Strukturanalyse und die Analyse stabiler Isotopen die wichtigsten in einer ganzen Reihe von Applikationsmöglichkeiten. In strukturanalytischer Hinsicht handelt es sich im wesentlichen um eine chemische Reaktionsspektroskopie, die als Nebenprodukt der Ionenerzeugung anfällt. Man könnte diese Tatsache für synthetische oder präparative Zwecke ausnützen, wenn der Mengendurchsatz in einem Massenspektrometer nicht so klein wäre.

Der wissenschaftlichen Forschung sind in diesem Zusammenhang Aufgaben gestellt, welche die Entwicklung optimaler Geräte zum Ziel haben, eine wirkungsvolle Bearbeitung und Auswertung der riesigen Datenmengen, die besonders in Zusammenhang mit hochauflösender Massenspektrometrie und in direkter Kombination mit Gaschromatographie anfallen, sowie ein besseres Verständnis für die Vorgänge, die der massenspektrometrischen Strukturanalyse zugrunde liegen, um sichere Voraussagen zu ermöglichen.

In jüngster Zeit zeichnet sich eine Entwicklung ab, die sich das Massenspektrometer als vielversprechendes Instrument der chemischen Grundlagenforschung zunutze macht. Die Untersuchung des Zerfalls metastabiler Ionen scheint eine wirkungsvolle Methode zu werden, um Informationen über die Kinetik und Thermodynamik monomolekularer Abbaureaktionen zu erarbeiten.

SPECIALIA

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Comparative Acetoacetylation of Proteins

Among the acylation procedures for proteins, the acylation method with acetic anhydride[1], the trifluoroacetylation with ethylthioltrifluoroacetate[2] and the succinylation with succinic anhydride[3] have been described. Furthermore, other methods of amino group modification have been proposed, with reagents such as carbon disulphide[4], methylacetamidate or benzimidate[5,6].

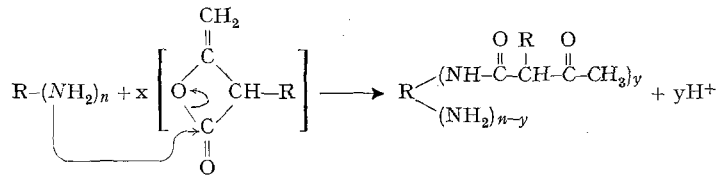
The acetoacetyl group, formerly proposed as a protective agent in peptide synthesis[7], seemed to combine the most advantageous characteristics of a reversible blocking and was employed in our laboratory for modification of proteins[8,9].

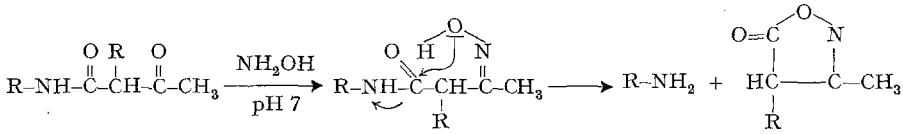
The acetoacetylation was carried out by using diketene. Hydroxylamine was chosen to remove the acetoacetyl group. Both the acetoacetylation and the deacetoacetylation step after hydroxylamine treatment are represented in the following general scheme:

This general reaction is dependent on the molar ratio of reagent x to protein $R-(NH_2)_n$, the number of the groups introduced may vary from a small number all the way up to $_n$, that is to complete coverage of amino groups of the protein. The reagent shows a strong preference for amino groups but reacts also with hydroxyl groups under suitable circumstances. An easy removal of the acetoacetyl function from residues other than amino groups has been shown to be possible after a carbonate-bicarbonate buffer treatment[8].

50 mg of lysozyme (Fluka) or ribonuclease (Fluka) were dissolved in 4 ml of distilled water and diketene (T. Schuchard GmbH & Co.) was added in a suitable

molar ratio to the protein. The pH was kept at a constant value of 8.5 by addition of standard alkali. The acetoacetylated protein was purified by gel-filtration and the extent of the acetoacetylation reaction was estimated: (a) by the increase of an absorbance peak at 278 nm due to the enolization of the carbonyl function; (b) by a colorimetric reaction with 3% $FeCl_3$ in aqueous solution





quantitated spectrophotometrically at 540 nm; (c) by the reaction with ninhydrin which gave the extent of the amino group protection; (d) by the determination of terminal amino group with dinitrofluorobenzene or 2 Cl-3,5-dinitropyridine[10] followed by amino acid analysis.

An easy removal of the acetoacetyl from residues other than amino groups was obtained by treatment with 0.2 *M* carbonate-bicarbonate buffer pH 9.5, for 11 h at 25°C. In order to remove the acetoacetyl function from the amino groups, 20 mg of the modified protein were dissolved in 4 ml of aqueous hydroxylamine at pH 7 and 25°C for 8 h.

The chromatographic behaviour of the deacetoacetylated protein was identical with that of the native protein on CG-50 Amberlite[8] and its electrophoretic mobility was also identical with the native, whereas the electrophoretic mobility of the acetoacetylated protein showed an evident change in the overall charge of the protein molecule.

The Table summarizes the results obtained in the reversible acetoacetylation of lysozyme compared with the results already obtained with ribonuclease. In both cases the treatment with hydroxylamine yielded proteins with the same enzymic activity as the original one. The 2 proteins we have chosen show, however, a different susceptibility towards acetoacetylation, which is related to the different conformation they may have in solution.

The acetoacetylated proteins are much more resistant towards tryptic hydrolysis, because acetoacetylation protects the lysyl bonds and only the arginyl bonds are cleaved after tryptic treatment[9]. Besides, the velocity of the tryptic hydolytic reaction is slowed down as any other enzymatic attack, due to the diminished solubility of the acetoacetylated substrate.

From our studies, acetoacetylation appears to be a specific type of acylation reaction with a reagent easy to handle experimentally. Besides diketene provides a nucleus for the attacking of various functional groups and thus makes it possible to introduce such groups into proteins and other macromolecules under mild simple conditions. The acetoacetyl group also provides a method for the introduction of a heavy atom into a protein after enolization of its carbonyl function. Such a method can be successfully exploited in X-ray structural work.

Acetoacetylation increases the overall negative charge of a protein, thus increasing the electrostatic repulsion of the macromolecule which can expand. This may cause dissociation in proteins composed of subunits, as in the case of succinylation[3].

To summarize, we can conclude that the acetoacetylation may be considered as a general method for protein modification and can also provide an insight into the conformational susceptibility.

Riassunto. È stata studiata l'acetoacetilazione reversibile dei gruppi aminici presenti nella ribonucleasi A pancreatica di bue e nel lisozima del bianco di uovo di pollo. I risultati sono stati confrontati e correlati con la diversa conformazione e stabilità delle due proteine.

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Acetoacetylation of lysozyme and ribonuclease

Protein	Reagent	Mole reagent added/mole protein	Activity (%)	Activity after NH_2OH treatment
Lysozyme	Diketene	10	60	100
		30	15	100
		50	10	98
		70	5	95
		100	2	100
Ribonuclease	Diketene	10	100	100
		30	90	98
		50	60	100
		70	25	96
		100	0	100

[1] R. K. Brown, J. Dutieux, R. Delaney, E. Leikhim and B. J. Clark, Ann. N.Y. Acad. Sci. *81*, 524 (1959).
[2] R. F. Goldberger and C. B. Anfinsen, Biochemistry *1*, 401 (1962).
[3] A. F. S. A. Habbeeb, H. G. Cassidy and S. J. Singer, Biochim. biophys. Acta *29*, 587 (1958).
[4] T. C. Merigan, W. J. Dreyer and A. Berger, Biochim. biophys. Acta *62*, 122 (1962).
[5] M. J. Hunter and M. L. Ludwig, J. Am. chem. Soc. *84*, 3491 (1962).
[6] M. L. Ludwig and R. J. Byrne, J. Am. chem. Soc. *84*, 4160 (1962).
[7] F. D'Angeli, F. Filira and E. Scoffone, Tetrahedron Letters *10*, 605 (1965).
[8] A. Marzotto, P. Pajetta and E. Scoffone, Biochem. biophys. Res. Commun. *26*, 517 (1967).
[9] A. Marzotto, P. Pajetta, L. Galzigna and E. Scoffone, Biochim. biophys. Acta *154*, 450 (1968).
[10] A. Signor and L. Biondi, Ricerca scient. *4*, 165 (1964).