tions made here will lead to additional experimental investigation of this new type of isomerism. One of the most interesting and feasible should be the preparation of the trefoil, II.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, CAMBRIDGE 39, MASS.]

Mass Spectra of Organic Molecules. I. Ethyl Esters of Amino Acids[1]

BY K. BIEMANN, J. SEIBL AND F. GAPP

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The spectra of the ethyl esters of twenty-four amino acids have been determined and interpreted in terms of their structure. The results permit not only the identification of amino acids through their mass spectrum but also the determination of the structure of new compounds of this type. A detailed discussion of these mass spectra is given and serves as an illustration of some of the more important fragmentation reactions which complex organic molecules undergo on electron impact.

In the characterization or determination of the structure of amino acids, mobilities in chromatographic systems combined with color reactions and chemical degradation are still most commonly used. The spectroscopic methods, applied so successfully for such problems in other areas, are not generally utilized in this field. Two factors are mainly responsible for this situation. First, the variations among amino acids are not so much due to the presence of different functional groups as to their placement within the molecule, and often the only differences are size and structure of the side chain. Secondly, amino acids are primarily obtained by extraction of plant or animal tissues, in the metabolism of lower forms of life, or by degradation of proteins, which makes it difficult to obtain considerable amounts of the material in pure form. The increasing number of new amino acids found in plants and microörganisms makes it increasingly more imperative to search for additional means for their identification or for the determination of their structure.

Mass spectrometry seemed to us a method particularly suited to this field because it gives specific information about the arrangement of groups within a given molecule. In addition, the amount of sample required, a few tenths of a milligram, is well within the range which can be easily isolated from most sources.

There is one obstacle to the use of mass spectrometry for the characterization of amino acids: The sample has to be present in the vapor state in the ion-source of the instrument under conditions which do not lead to appreciable decomposition, if a spectrum representative of the original compound is to be obtained. Free amino acids, because of their zwitterion character, have a very low vapor pressure and frequently decompose if heated to the temperatures required for vaporization. It is therefore necessary to convert the free acids to more volatile derivatives which still retain all the structural features. Furthermore, it is important that the reaction employed is simple and can be done on a very small scale. To yield only one product the selected reaction should, if possible, be quantitative and, for general use, applicable to polyfunctional amino acids.

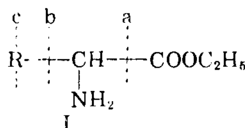
For this purpose, either the carboxyl group, the amino group or both have to be removed. A number of reactions come to mind: (a) esterification, reduction to an amino alcohol, decarboxylation, degradation to the next lower aldehyde or a Dakin–West reaction would remove the free carboxyl group, or (b) acylation or deamination with nitrous acid would dispose of the basic amino group. The first-mentioned reaction, esterification, was chosen for our purpose. It was expected to proceed smoothly and to apply to all carboxyl groups, not only to the one α to the amino group. Next, the alcohol moiety and the method of esterification had to be selected. Diazomethane is most frequently chosen for small-scale esterification of carboxyl groups. However, this reagent is unsuitable because it is known to methylate amino acids on nitrogen, which cannot be tolerated for our purpose: the amino acids would in part be converted to isomers of their next higher homologs and finally to non-volatile quaternary ammonium compounds. Furthermore, the methyl esters of α-amino acids dimerize most easily to diketopiperazines, a reaction which has to be avoided. As the best compromise between the tendency of dimerization, boiling points and molecular size, the ethyl esters were chosen as the most suitable derivatives and Fischer-esterification for the method of preparation. The latter leads first to the hydrochlorides from which the free ester has to be prepared. This is done on a large scale in the conventional way: extraction with a suitable solvent from an alkaline solution, followed by distillation. Almost all spectra discussed in this paper were obtained from such distilled samples. For work on a very small scale, particularly if quantitative results on mixtures are required, we prefer the conversion of the hydrochloride into the free ester in homogeneous solution (dichloromethane) with dry ammonia. The low boiling point of the solvent permits its removal before the sample is introduced into the mass spectrometer using the technique described in the Experimental part.

Discussion of the Mass Spectra

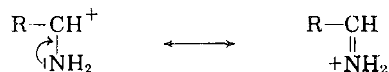
The peaks comprising the spectra of ethyl esters of amino acids are due to fragmentation of the molecule by preferred cleavage of those bonds

(1) A preliminary account of part of this investigation was presented at the 135th Meeting of The American Chemical Society, Boston, Mass., April, 1959, and was published in *Biochem. Biophys. Res. Comm.*, **1**, 307 (1959). This work was supported by a research grant (RG-5472) from the National Institutes of Health, Public Health Service.

which lead to energetically more favored, *i.e.*, best stabilized, positive ions. The characteristic peaks are due to breaking bond a, bond b or a bond of group R (formula I) particularly at highly substituted carbon atoms or at those bearing heteroatoms.



(a) **The "Amine-Fragment."**—Bond a in I is broken much more easily than in the ethyl esters of unsubstituted acids[2] because retention of the positive charge on the nitrogen-containing fragment results in a resonance-stabilized ion[3]

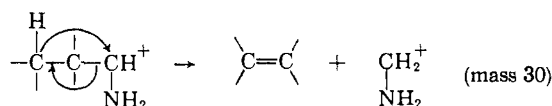


This bond (one removed from nitrogen) is cleaved preferentially in amines as was previously demonstrated on some simple alkyl amines.[4] Cleavage of bond b gives rise to a much smaller peak[5] (if R is alkyl) because in the resulting ion the positive charge is destabilized by the neighboring carbethoxy group.

In the spectra of α-amino esters devoid of additional functional groups the amine fragment is the most prominent one. Its mass is 29 + R, *i.e.*, 30, 44, 58, 72 and 86 for the ester of glycine, alanine, α-aminobutyric acid, valine and the leucines, respectively. Introduction of a heteroatom or aromatic system into the R-group increases the tendency for cleavage of other bonds, either in the original molecular ion or in the "amine-fragment," both factors leading to a lower abundance of the latter.

Such further decomposition of the amine-fragment arises from the elimination of neutral molecules like olefin, water, ammonia, hydrogen sulfide, mercaptans or ethanol. In almost all such cases the positive charge is retained on the nitrogen-containing fragment.

For aliphatic monoaminomonocarboxylic esters the elimination of an olefin from the amine fragment is the most common mode of further decomposition, giving rise to peaks of medium intensity. A peak at m/e[6] 30 in the spectra of most of these



(2) A. G. Sharkey, Jr., J. L. Shultz and R. A. Friedel, *Anal. Chem.* **31**, 87 (1959).

(3) In the following discussion of such fragments and their further decompositions the positive charge is shown, for reasons of simplicity, localized on the carbon atom, but it is understood that the actual situation is better represented by the resonance depicted above. Further decomposition of the amine fragment may proceed from either one of the two extreme resonance forms of this ion.

(4) J. Collin, *Bull. Soc. Roy. Sci. Liege*, **21**, 446 (1952).

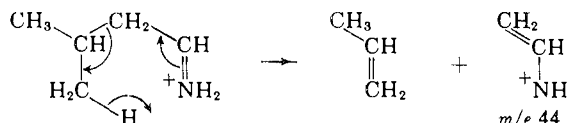
(5) While our work was in progress[1] C. O. Andersson reported in a brief note [*Acta Chem. Scand.*, **12**, 1353 (1958)] both these types of fragments in the methyl esters of amino acids.

(6) The mass spectrometer records the mass-to-charge ratio (m/e) of the particle rather than its mass. In this paper except where stated otherwise only single charged peaks are discussed, in which case m/e = mass.

compounds containing at least two carbon atoms in the side chain is formed by such an elimination. This mechanism is supported by the appearance of a metastable peak[7] at m/e 15.7 (calcd. 15.5) and at m/e 12.6 (calcd. 12.5) in the spectra of the esters of α-aminobutyric acid and norvaline, respectively.

The peak at m/e 30 is, therefore, an appreciable one in most amino esters, but absent in alanine and the aromatic amino esters because of the lack of the required γ-hydrogen atom. This reaction seems to be more favored in the unbranched isomers as illustrated by the abundance of m/e 30 in norleucine, leucine and isoleucine (45, 37 and 31%, respectively, of the abundance of the peak at m/e 86) and norvaline and valine (54 and 10% of the peak at m/e 72) (Figs. 13, 14, 15, 11 and 12). Serine ester also exhibits this peak, formed by migration of the hydroxyl-hydrogen and elimination of formaldehyde.

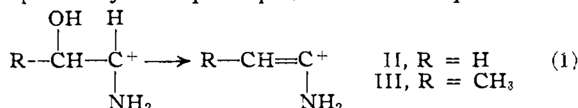
A different type of olefin elimination appears to operate in leucine ester (Fig. 14), which exhibits a rather intense peak at m/e 44 suggesting the mechanism (supported by a metastable peak at m/e 22.7, calcd. 22.5)



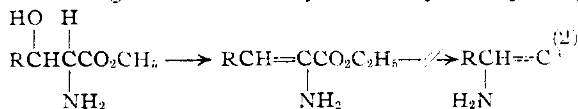
The high degree of substitution on Cγ- and the large number of available δ-hydrogens in leucine ester appears to aid the formation of this peak which is 50% as intense as the amine peak at m/e 86, but only about 10% in norleucine and isoleucine (Fig. 13 and 15), requiring a more complex rearrangement in the case of the latter.

Additional hydroxy, amino, carbethoxy, mercapto and thio ether groups give rise to elimination from the amine-fragment of water, ammonia, ethanol, hydrogen sulfide and mercaptans as illustrated by the spectra of the various polyfunctional amino acids:

Serine and threonine ester (Fig. 19 and 20) exhibit peaks at m/e 42 (II) and 56 (III), respectively. In principle, it would be possible that



the elimination of the elements of water takes place in the molecular ion, followed by cleavage of the Cα—C=O bond. The latter would now be attached to a double bond which, however, makes this fragmentation very unlikely. Hydroxy-



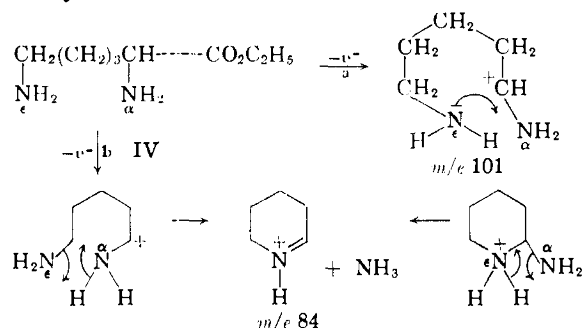
(7) A "metastable peak" is a broad peak of low intensity due to ions of mass m_1 decomposing after full acceleration to ions of mass m_2 in the vicinity of the exit slit of the ion source. The intensity maximum of this peak is found at mass m^* which is related to the two fragments as[8] $m^* = (m_2)^2/(m_1)$. The presence of such a peak is, therefore, an indication for the decomposition in one step of ion m_1 into ion m_2 plus a neutral particle of mass $(m_1 - m_2)$.

(8) J. A. Hipple and E. U. Condon, *Phys. Rev.*, **68**, 54 (1945).

proline also undergoes the elimination of H_2O from the amine fragment (mass 86) to give a peak at *m/e* 68.

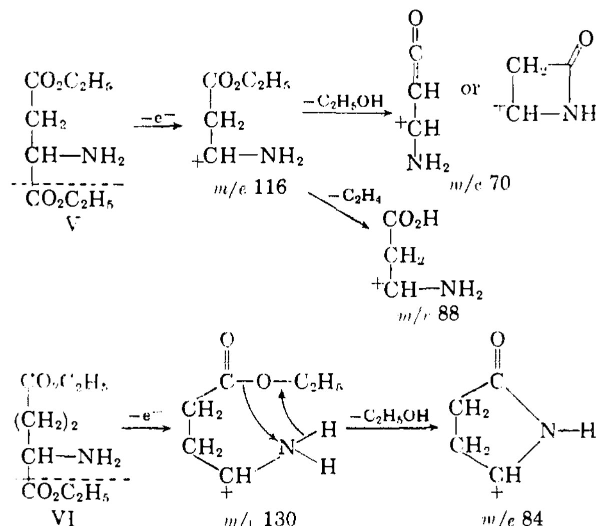
Additional support for the formation of fragments II and III *via* reaction 1 rather than 2 is provided by the appearance of metastable peaks at *m/e* 29.6 (calcd. 29.4) in the spectrum of serine ethyl ester and at *m/e* 42.6 (calcd. 42.4) for threonine ester.

The formation of a fragment of mass 84 from the one at mass 101 in the case of lysine ester (IV, Fig. 23) is of particular interest, because the former gives the most intense peak in the spectrum whereas the latter is rather small, although it is the amine fragment. This facile elimination of ammonia must therefore be assisted by the second amino group present. An attractive mechanism would be a cyclic one



Both path a and path b seem to be operative because the mass spectrum of the ethyl ester of lysopine (XIX), an N_α-substituted lysine derivative,[9] indicates that either one of the two amino groups may be eliminated. More detailed information will be obtained when the spectra of lysine esters, specifically labeled with N^{15} either in the α- or ϵ-position, are available.

A somewhat similar behavior is shown by the ethyl esters of aspartic acid (V, Fig. 1) and glutamic acid (VI, Fig. 22): The amine fragments are of mass 116 and 130, respectively, and both lose the elements of ethanol to give peaks at *m/e* 70 and

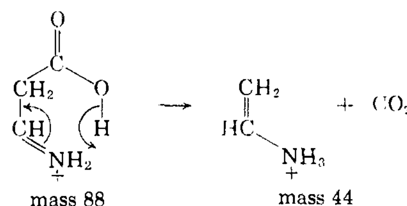


84. At least in the latter case we prefer a cyclic structure for the ions.

Although some of the fragments of mass 84 in the spectrum of glutamic ester arise *via* prior thermal cyclization of the ester to ethyl 2-pyridone-5-carboxylate in the inlet system of the mass spectrometer, most of the fragments come from the original ester. The thermal cyclization is rather slow and can be followed by plotting the intensity of *m/e* 130 *vs.* time.

A minor path of decomposition of the amine fragment of mass 116 in aspartic ester is the loss of ethylene from the carbethoxy group to give a peak at *m/e* 88, a process analogous to the one which the ester-fragment undergoes (see below). The corresponding peak for glutamic ester coincides with the peak at *m/e* 102 of another origin.

The formation of the fragment of mass 44 in aspartic ester (Fig. 1) is similar to the one discussed for leucine involving, in this case, the elimination of carbon dioxide rather than propylene

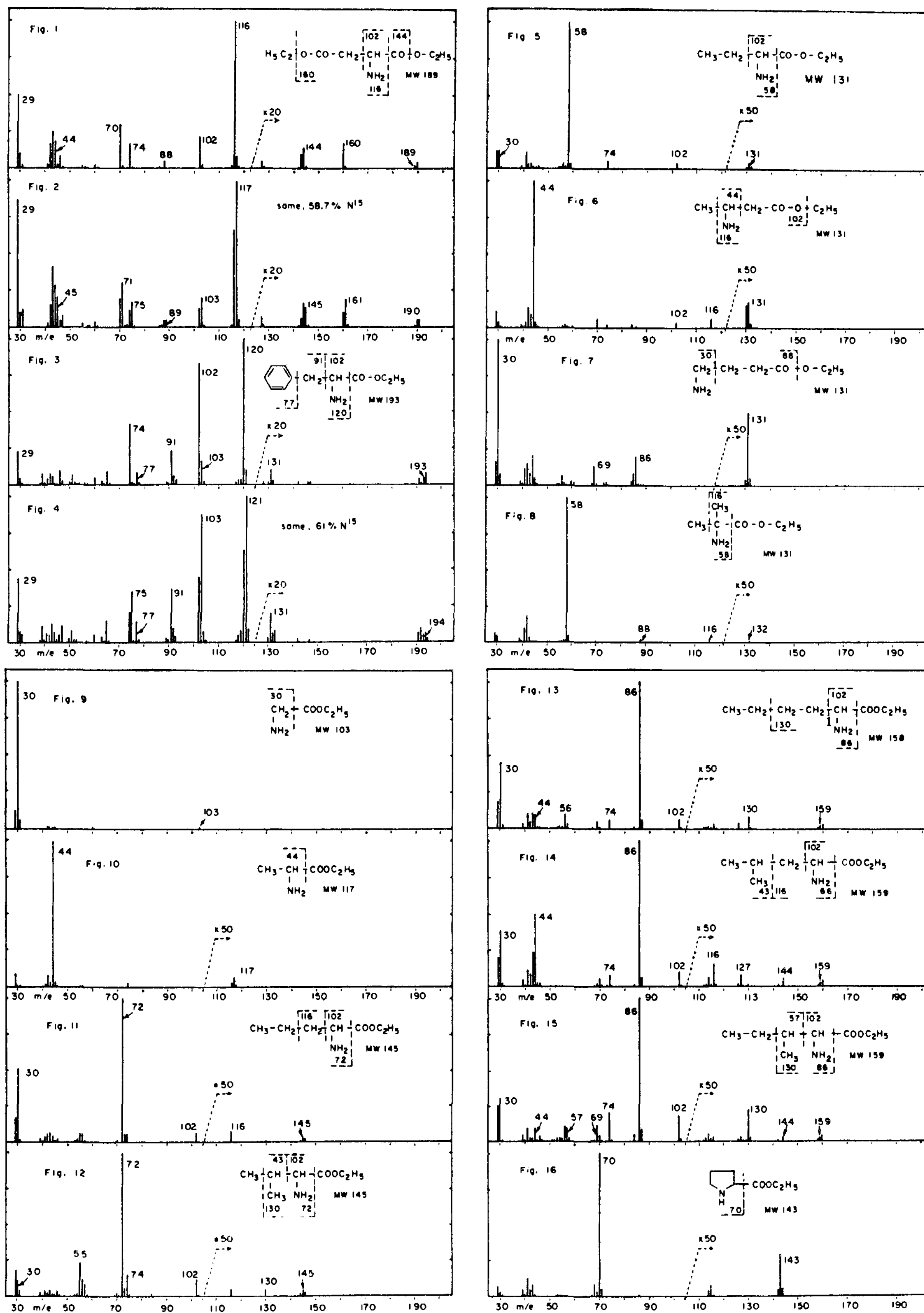


The spectrum of the ethyl ester of labeled aspartic acid (58.7% excess N^{15}) is compared with the spectrum of the unlabeled one in Figs. 1 and 2. All peaks containing the nitrogen atom appear as doublets in the approximate ratio 4:6 (the variations are due to the contributions of C^{13}, changing with the number of carbon atoms in the fragment). One of the two peaks (N^{14} containing) remains at the mass of the corresponding unlabeled fragment; the second which contains N^{15} is one mass unit higher. The composition assigned to the peaks in the spectrum of aspartic ester in the present discussion are in agreement with these two spectra: Peaks at *m/e* 70, 74, 88, 102 and 116 all contain nitrogen and are therefore shifted one mass unit in the proportions of the N^{15} content of the sample. Fragments of mass 27 and 29, arising from the ethyl group are not influenced. Similarly, Figs. 3 and 4 compare the spectra of labeled and unlabeled phenylalanine ester (61.0% excess N^{15}). These spectra also confirm the presence of nitrogen in the fragments of mass 74, 102 and 120 and its absence in *m/e* 29, 77 and 91, for example. The spectra of a considerable number of N^{15}-labeled amino esters have been obtained in this Laboratory.[10] While space does not permit their reproduction here, they corroborate all the assignments made in this paper concerning the spectra of the ethyl esters of glycine, alanine, valine, leucine, isoleucine, glutamic acid, serine and α,ϵ-labeled lysine.

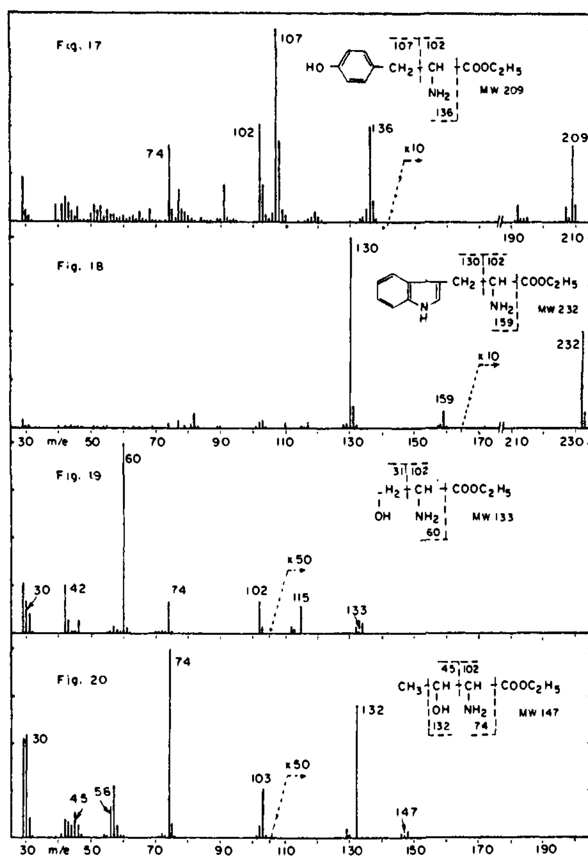
The elimination of H_2S from the amine fragment (mass 76) of cysteine ethyl ester (Fig. 24) is analogous to the formation of the same fragment (II) from serine ester. Similarly, methionine

(9) K. Biemann, C. Lloret, J. Asselineau, E. Lederer and J. Polonsky, *Biochim. et Biophys. Acta*, **40**, 369 (1960).

(10) We are indebted to Dr. G. G. J. Deffner for the determination of these spectra.



Mass spectra of ethyl esters of amino acids in the region of mass 29-235: All peaks of $\geq 0.5\%$ of the intensity of the largest peak are shown, and of correspondingly lower intensity in the enlarged portions of the spectra (on the right of the slanted



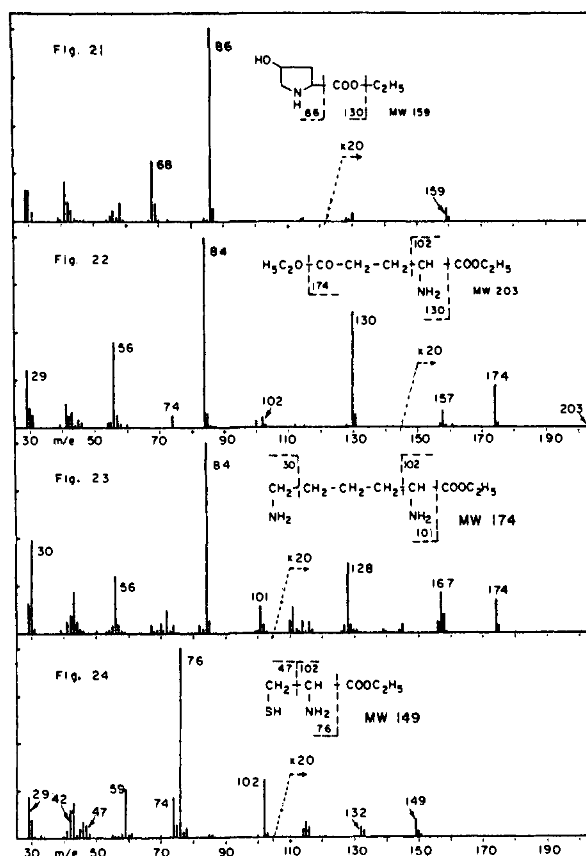
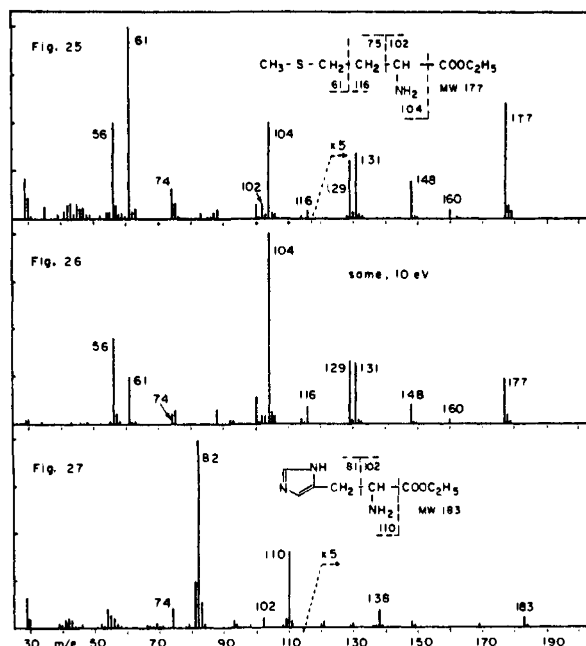
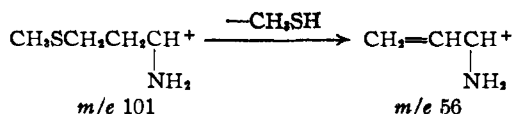


Fig. 1, aspartic acid; Fig. 2, aspartic acid-N[15]; Fig. 3, phenylalanine; Fig. 4, phenylalanine-N[15]; Fig. 5, α-aminobutyric acid; Fig. 6, β-aminobutyric acid; Fig. 7, γ-aminobutyric acid; Fig. 8, α-aminoisobutyric acid; Fig. 9, glycine; Fig. 10, alanine; Fig. 11, norvaline; Fig. 12, valine; Fig. 13, norleucine; Fig. 14, leucine; Fig. 15, isoleucine; Fig. 16, proline; Fig. 17, tyrosine; Fig. 18, tryptophan; Fig. 19, serine; Fig. 20, threonine; Fig. 21, hydroxyproline; Fig. 22, glutamic acid; Fig. 23, lysine; Fig. 24, cysteine; Fig. 25, methionine; Fig. 26, same at 10 ev.; Fig. 27, histidine.



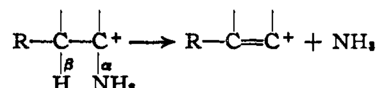
arrows, indicating the factor by which the actual intensity had been increased for the purpose of reproduction).

ester (Fig. 25) gives a rather intense peak at m/e 56. While the fragment of mass 56 may con-



ceivably arise by prior elimination of CH_3SH from the molecular ion followed by loss of the carbethoxy group, a metastable peak at m/e 30.3 (calculated 30.2) supports the course of reaction depicted above.

Finally, the elimination of NH_3 from the amine fragment should be mentioned. It leads to olefin-type particles



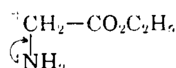
Such peaks are generally of low intensity unless C_β is highly substituted or bears an aryl group. In valine, isoleucine and phenylalanine ester (Fig.

12, 15 and 3) the peaks at m/e 55, 69 and 103[11] are 22, 11 and 11% of the intensity of the amine peaks.

Proline ester (Fig. 16) is somewhat exceptional since it not only has no further functional groups but also is cyclic, both factors contributing to the increased stability of the amine fragment which can decompose further only by cleavage of two bonds. The intensity of peak m/e 70 is therefore unusually high and the few other peaks in the spectrum are quite low.

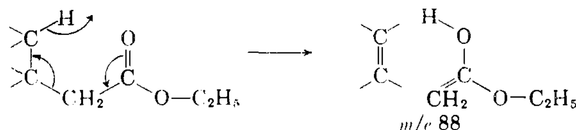
Presentation of mass spectra in the form of the ratios of each peak to the most intense one in the spectrum is common practice and also followed in the figures of this paper. It does not clearly indicate the sometimes considerable difference in intensity among various spectra. For this reason we present in Table I the relative equimolar intensities of the highest peaks in the spectra of a number of amino esters (m/e 116 in aspartic ester = 100).[12]

(b) **The "Ester Fragment."**—If the C_α–C_β-bond (b in I) is broken in an α-amino ethyl ester, a fragment is formed in which the positive charge is again stabilized by the attached nitrogen atom



This fragment of mass 102, is, therefore, characteristic of all ethyl esters of primary α-amino acids. Obviously, it is absent in the spectra of proline and hydroxy-proline ester, and negligible in glycine and alanine ester, because hydrogen or methyl is less easily lost than larger groups. A high degree of branching or an aryl group on the β-carbon, as in valine, isoleucine, phenylalanine and tyrosine further facilitates the cleavage of the β–γ bond and gives rise to higher peaks at m/e 102 (14, 17, 83 and 100%, respectively, of the intensity of the amine peak of the corresponding ester).

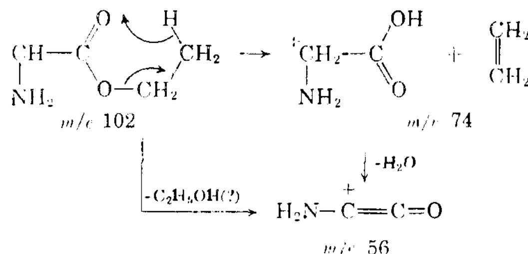
Two facts about this fragment deserve further comment: first, its relatively low abundance, compared with the amine peak in which similarly a carbon–carbon bond next to nitrogen is broken; secondly, unsubstituted ethyl esters never give the corresponding fragment of mass 87 by simple cleavage of the C_α–C_β bond but instead a fragment of mass 88, which arises *via* a cyclic rearrangement[13]



This rearrangement appears to be a favorable mode of decomposition of such esters because it allows the rupture of the C_α–C_β bond without production of an ion $^+CH_2CO_2C_2H_5$ in which the positive charge is particularly destabilized by the neighboring, partly positive, carboxyl-carbon. A nitrogen atom at C_α adds adequate stabilization to such an ion to make the simple cleavage of the C_α–C_β bond possible without rearranging a hydrogen atom. On the other hand, over-all stabilization is still not as good as in the absence of the carbonyl group, and the intensity of the fragment of mass 102 is therefore lower than one would expect otherwise.

The rearrangement mentioned above is operative in the ethyl esters of β-hydroxy-α-amino acids, like serine and threonine, due to the greater[13] mobility of hydrogens attached to oxygen over those attached to carbon and leads to a fragment of mass 103 (Figs. 19 and 20).

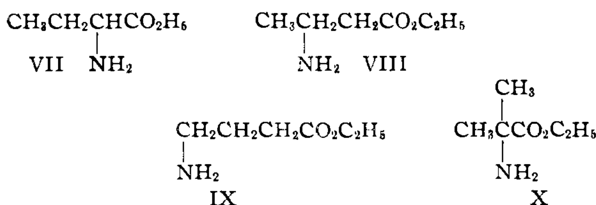
Further decomposition of the ester-fragment leads to peaks at m/e 74 and m/e 56



The first step manifests itself in many spectra by a metastable peak at m/e 53.9 (calcd. 53.8), whereas the formation of the peak at m/e 56 may occur in the two different ways shown above.

Clearly, the ester peak can be used to deduce the substituents, if any, on C_α on N_α, leading to displacement of this peak to correspondingly higher masses.

A discussion of the spectra of esters derived from the isomeric aminobutyric acids will illustrate the information one can derive from a consideration of the amine peak and ester peak:



Only ethyl α-aminobutyrate (VII) and ethyl α-aminoisobutyrate (X) (Figs. 5 and 8) are primary α-amino esters and exhibit a strong peak at m/e 58, clearly the amine fragment. VII gives the peaks at m/e 102 and 74 indicating a normal, unsubstituted α-amino ethyl ester, whereas these peaks are missing in X but appear instead 14 mass units higher at m/e 88 and 116. The methyl group at C_α is responsible for this shift. In principle, it could also be attached to the nitrogen as in N-methylalanine. The high intensity of the amine peak at m/e 58, coupled with the almost negligible intensity of the molecular ion (compared with the other isomers) indicates that a bond at a highly substituted carbon atom is broken in the formation of this ion.

The spectra of the isomeric β- and γ-aminobutyric esters VIII and IX (Figs. 6 and 7) give the expected

(11) Corrected for the C^{13} isotope peak of m/e 102.

(12) These values were obtained from mixtures of known composition, using aspartic acid as internal standard. We are indebted to Dr. W. Vetter for these determinations.

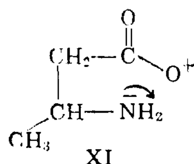
(13) F. W. McLafferty, *Anal. Chem.*, **31**, 82 (1959).

TABLE I

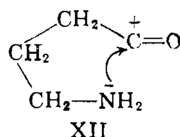
COMPARISON OF THE EQUIMOLAR INTENSITIES OF REFERENCE PEAKS IN VARIOUS AMINO ESTERS

Ethyl ester of	Mass of peak	Rel. intensities
Glycine	30	130
Alanine	44	136
α-Aminobutyric acid	58	128
Valine	72	117
Leucine	86	117
Isoleucine	86	127
Proline	70	157
Phenylalanine	120	102
Tyrosine	107	35
Tryptophan	130	130
Serine	60	83
Threonine	74	75
Hydroxyproline	86	96
Aspartic acid	116	*100*
Glutamic acid	84	105
Methionine	61	93

peaks at m/e 30 and 44. The ester fragment appears at m/e 116 in VIII, but the corresponding peak at m/e 130 in IX is exceedingly small, because loss of hydrogen is very unfavorable if there are other modes of fragmentation available to the molecular ion. Interestingly there is a peak of m/e 102 in VIII.[14] We attribute this peak to the fragment XI arising through loss of the ethyl group in the alcohol moiety because the same fragment (mass 102) is also formed from the corresponding butyl ester. This mode of fragmentation, not found in α-amino esters, seems to be aided by the possible stabilization of the charge on oxygen by the amino group.

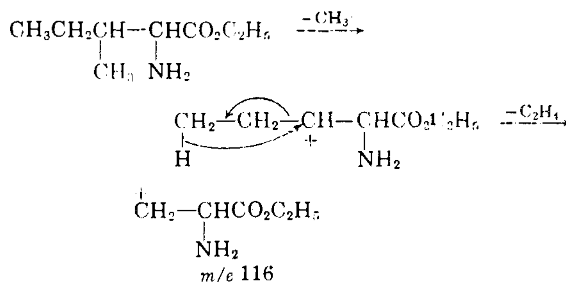


A somewhat similar process gives rise to the peak m/e 86 in the γ-isomer: it is due to loss of the ethoxy group, and also this fragment XII, not formed to a comparable extent from the other isomers, appears to receive additional stabilization from the γ-amino group.



(c) **Fragmentation of the Molecular Ion at the R- Group.**—In Sections a and b we have discussed fragments formed by cleavage of one of the two C–C bonds to which the α-amino group is attached. The majority of the ions in the mass spectra of α-amino esters are formed in this way, including those obtained by further decomposition of the primary fragment. If these bonds remain intact, and others present in the molecule are cleaved instead, a third group of fragments is formed. These are particularly useful if one wants to obtain detailed information about the structure or to distinguish among isomers. The abundance of such peaks will naturally depend on the ease of fragmentation of those bonds *vs.* the C–C bonds α- to the amino group and is particularly aided by the presence of highly substituted carbon atoms or of heteroatoms in the side chain of the amino ester. Proline ester (Fig. 16), lacking any such groupings, is a perfect example, giving only very few fragments of low intensity besides the very strong amine peak. In methionine ester (Fig. 25), on the other hand, we find many peaks of considerable intensity among which the amine peak is far from the highest, although one may be tempted to look at it as isosteric to norleucine ester. The presence of a sulfur atom instead of CH_2 completely changes the fragmentation of the molecule.

Branching in the side chain favors fragmentation at that point, and the isomeric leucines illustrate this influence. Whereas the peaks at m/e 43 and 57 are of low intensity in norleucine ester (Fig. 13), the former is more intense and much higher than the latter in leucine ester (Fig. 14), while the opposite holds for isoleucine ester (Fig. 15). These peaks are due to fragmentation at C_γ and C_β with retention of the positive charge on the C_3H_7 and C_4H_9 fragment, respectively. On the other hand, if an alkyl group is lost from one of the highly substituted carbon atoms and the charge is retained on the remaining part of the molecule, peaks for $M-15$,[15] $M-29$, $M-43$ and $M-57$ (masses 144, 130, 116 and 102, respectively) are obtained. The last one is the ester peak which we have already discussed. The others, $M-15$ and $M-43$, are more abundant in leucine ester whereas in isoleucine m/e 130 ($M-29$) is the highest peak of this group. The small one at m/e 116 in Fig. 15 corresponds to the loss of three carbon atoms which cannot be derived by simple cleavage of a C–C bond but from a two-step process which is less probable and leads therefore only to a small peak. Norleucine ester behaves similarly to n-hydrocarbons in this respect: It does not lose methyl, but ethyl and propyl. Figures 13, 14 and 15 show that these peaks are of very low intensity (below 1% of the intensity of the amine peak). They occur, however, in a mass region otherwise free of peaks and are, therefore, easily detected because of the mass spectrometer's wide range of sensitivity. The small peak at m/e 127 found in all three isomers is at-



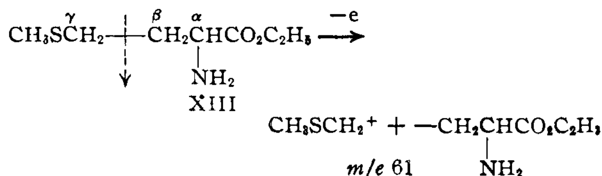
(14) The intensity of this peak is much too high to be due to the presence of ethyl α-aminobutyrate. The sample of VIII used for the spectrum was gas chromatographically pure.

(15) Denotes a fragment of mass corresponding to the molecular weight M minus 15 mass units.

tributed to the elimination of ammonia from the molecule and then loss of methyl.

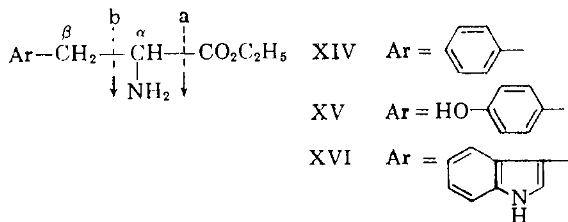
The presence of functional groups in the side chain of the amino acid gives rise to more intense peaks, either by their elimination in the form of water, ammonia, ethanol, mercaptans or hydrogen sulfide in a manner already discussed for the amine fragment or by direct cleavage of a carbon–carbon bond. Examples of the first case are peaks in lysine ester (Fig. 23) at *m/e* 167 and 128 (*M* minus NH_3, *M* minus C_2H_5OH) and *m/e* 131 ($M-46$) and 129 ($M-48$) in methionine ester (Fig. 25) due to loss of ethanol or methyl mercaptan.

More significant are peaks due to direct cleavage of a bond at a carbon atom to which a hetero-atom is attached. If this functional group is at the β-carbon atom as in serine, threonine or cysteine, the C_α–C_β bond is cleaved with particular ease giving rise to an enhanced ester peak at *m/e* 102 (*m/e* 103 in threonine, see above) and to an appreciable one at the mass of the remaining part of the molecule (mass 31 in serine, 45 in threonine and 47 in cysteine ester). A functional group at the γ-carbon atom, as in methionine ester (XIII), similarly leads to cleavage of the C_β–C_γ bond. In this case the positive charge is retained almost exclusively on the sulfur-containing fragment,



where it is stabilized by the sulfur atom; its retention at the β-carbon would lead to a primary carbonium ion lacking any additional stabilization.

The ethyl esters of phenylalanine (XIV), tyrosine (XV) and tryptophan (XVI) provide us with interesting examples in which the substituent at the β-carbon atom is able to accommodate the positive charge so well that the peaks corresponding to rupture of the C_α–C_β bond are rather intense, particularly the one containing the β-carbon. The increase in π-electron density in the aromatic



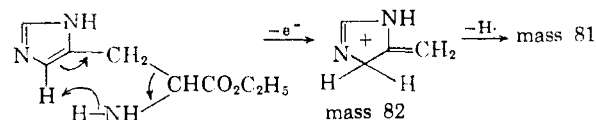
nuclei in the series phenylalanine, tyrosine and tryptophan is expressed in the increased intensity of the peak due to the $(Ar–CH_2)^+$ fragment. The probability of formation of this ion *vs.* the amine peak (cleavage at a) and the ester peak (cleavage at b but retention of the positive charge at C_α) increases remarkably in this series (Table II; arabic numbers are relative intensities within each spectrum).

Histidine ethyl ester appears to be least aromatic in this respect. The amine peak (*m/e* 110) is much higher than the ester peak at *m/e* 102. The imidazolyl-methyl fragment of mass 81 appears to be considerable, but a metastable peak at *m/e* 80 indicates that at least part of it is formed from mass 82, the most intense peak in the spectrum. We would therefore suggest the fragmentation scheme indicated in Fig. 27 and in addition a rearrangement for the formation of the fragment of *m/e* 82

Table II

Relative Abundance of Selected Peaks in the Spectra of Aromatic Amino Esters

	XIV	XV	XVI
Amine peak	100	51	8.5
Ester peak	83	52	4
$(ArCH_2)^+$	23	100	100
Mol. wt.	1.3	4	5.2



(d) **The Molecular Weight.**—The presence in the molecule of a number of bonds easily broken decreases the number of molecular ions able to survive for about 10^{-4} sec., the time required to be fully accelerated. For this reason, the peak at the molecular weight of the amino ester is always very small (0.05% to 0.2% of the highest peak in the spectrum) unless there is present in the molecule a grouping which can tolerate the loss of an electron particularly well. Such is the case in the aromatic amino acids (see Table II). The thio-ether group of methionine also adds to the stability of the molecular ion, the intensity of which is 12% of the highest peak (*m/e* 61). This value is somewhat exaggerated because of the relatively low intensity of *m/e* 61 in XIII. It is more realistic to compare the intensities of peaks in different spectra if expressed in percentage of total ion yield (the sum of all peaks) instead of the highest peak. This value is 2.32% for tryptophan ethyl ester, 3.4% in methionine ethyl ester and in leucine ethyl ester, as an example of the major group of amino esters, 0.025%.

The presence of sulfur in methionine ester (XIII, Fig. 25) is indicated by the peak at *m/e* 179 due to the molecular species containing S^{34} (natural abundance about 4.2%). Its intensity is 5.2% of the peak at *m/e* 177 due to the combined additional contributions of the heavy isotopes of carbon, nitrogen, oxygen. Consideration of these isotope peaks frequently makes it possible to obtain information about the presence or absence of certain heteroatoms and also aids in the interpretation of the spectrum. It can be seen from Fig. 25 that fragments of *m/e* 61 and 104 contain sulfur because they are accompanied by peaks of the required intensity at *m/e* 63 and 106. The absence of an appreciable peak at *m/e* 58 does not allow sulfur in the fragment of *m/e* 56, in agreement with the structure assigned to these fragments earlier in the discussion.

The low intensity of the molecular weight peak sometimes makes it difficult to identify or distinguish it from other small peaks due to impurities.

The peak one mass unit above the molecular weight can be used to recognize the latter.[16] This so-called "$M + 1$ peak" arises through hydrogen abstraction from another molecule by the molecular ion during a collision process (RH may or may

$$X^+ + RH \longrightarrow XH^+ + R\cdot$$

not be equal to X which denotes the molecule under consideration). Formed in a second-order reaction, the abundance of XH^+ ions is not directly proportional to the concentration of X^+ in the ion source like all other ions, but to the product $[X^+][RH]$. The ratio of the intensity of the XH^+-ion *vs.* all other ions varies, therefore, if the sample pressure or the conditions in the ion source are changed.[17] Once the $M + 1$ peak has been positively identified, subtraction of one mass unit gives the molecular weight of the amino ester. It follows from the preceding that the latter must be equal to the mass of the amine-peak plus 73 mass units ($CO_2C_2H_5$) for all α-amino ethyl esters. thus providing an additional check on the interpretation of the spectrum.

The spectra discussed have all been obtained using an electron beam of about 70 ev. energy. Frequently it is advantageous to scan the spectrum in addition, employing energies of only about 10 ev. Such spectra, while of lower intensity, are greatly simplified. Fragments not due to the rupture of one of the two bonds at C_α (a or b in I) are considerably suppressed or even absent, and the amine and ester peaks are still more significant. Particularly the identification of the former is facilitated in cases where it could be doubtful. The fragment of mass 44 in leucine ethyl ester is 50% as intense as the peak at *m/e* 86 at 70 ev. while it is only about 8% at 10 ev. Mass 61 is the largest peak in methionine ester, while the amine peak at *m/e* 104 is only 50.4% as high. Using electrons of lower energy, the situation is reversed and *m/e* 61 is only 30% of the peak at *m/e* 104 (Fig. 26). Ions formed by further decomposition of the amine fragment require more energy for their formation than the latter and are, therefore, suppressed. Similarly, the processes discussed under (c) appear to require more energy than the formation of the amine fragment and cannot compete favorably. The relative intensities of the M and $M + 1$ peaks also increase.

Conclusion

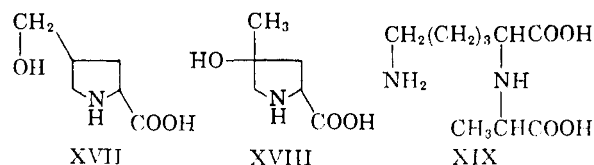
Most of the significant peaks in the mass spectra of esters of amino acids can be correlated with their structure. Because of the interplay of various functional groups in these molecules, the spectra represent an interesting summary of the fragmentation processes taking place in the ion source of the mass spectrometer.

As a result of the present study, the applications of mass spectrometry in the field of amino acid chemistry are numerous:

It provides a method for the rigorous identification of an amino acid merely by brief interpretation of the spectrum of its ethyl ester followed by comparison with the spectrum of an authentic sample. The presence of other amino acids as impurities may be detected simultaneously. Individual components in a mixture of amino acids can be identified without prior separation, because the spectrum of the mixture of esters is the algebraic sum of the spectra of the individual components. In this case, it is of advantage to obtain the spectrum using electrons of about 10 ev. energy. As indicated earlier, the spectra are simplified, and amine peaks, the most useful for the recognition of most of the amino esters, are more pronounced. On the other hand, for the differentiation of isomers, like leucine, isoleucine and also hydroxylproline, the more discriminating 70-ev. spectrum is additionally required. It is used to measure the peaks in which these compounds differ most.

Based on the knowledge gained in the course of the present investigation concerning the mass spectra of amino esters, we have devised methods for the quantitative determination of amino acid mixtures[18] and for the determination of N^{15} in the individual components of such mixtures without prior separation.[19]

The ultimate purpose of our investigation of the mass spectra of amino acid derivatives was, however, to provide the basis for the use of mass spectrometry in the determination of the structure of unknown amino acids. That this has been accomplished already has been demonstrated in two instances. The mass spectrum of the proline derivative obtained from apple peels or twigs permitted[20] a decision in favor of XVII[21] and against the possible alternative XVIII.[22] As another example, the structure of lysopine (XIX) was derived[9] entirely from the mass spectrum of its ethyl ester.



For the determination of the structure of an amino acid it will be advantageous in some cases to use the spectra of other derivatives in addition to the ethyl esters. As was pointed out during the discussion of the spectra presented in this paper. comparison of ethyl esters and butyl esters aid in the identification of fragments containing the alcohol moiety while N-alkylation (*e.g.*, dimethylation with $CH_2O/H_2/Pd$) marks the amino group.[23]

In the interpretation of a mass spectrum one has to keep in mind the possibility of a compound undergoing thermal reaction in the inlet system of the mass spectrometer. If such is the case, the

(16) F. W. McLafferty. *Anal. Chem.*, **29**, 1782 (1957).

(17) We prefer the latter method because it is simpler and less time-consuming: The spectrum is run under normal conditions with the controls on the ion-source set to the "focused" position. The scan of the higher mass range is repeated after switching to "non-focused." If the ionizing current is simultaneously increased to counter-balance the decrease in intensity due to this change, all "normal" peaks will be unaltered while the $M + 1$ peak is considerably higher than before.

(18) K. Biemann and W. Vetter, *Biochem. Biophys. Res. Comm.*, **2**, 93 (1960).

(19) K. Biemann and G. G. J. Deffner, *ibid.*, **4**, 283 (1961).

(20) K. Biemann, G. G. J. Deffner and F. C. Steward, *Nature*, **191**, 380 (1961).

(21) G. Urbach, *ibid.*, **175**, 170 (1955).

(22) A. C. Hulme and F. C. Steward, *ibid.*, **175**, 171 (1955).

(23) K. Biemann and W. Vetter, unpublished.

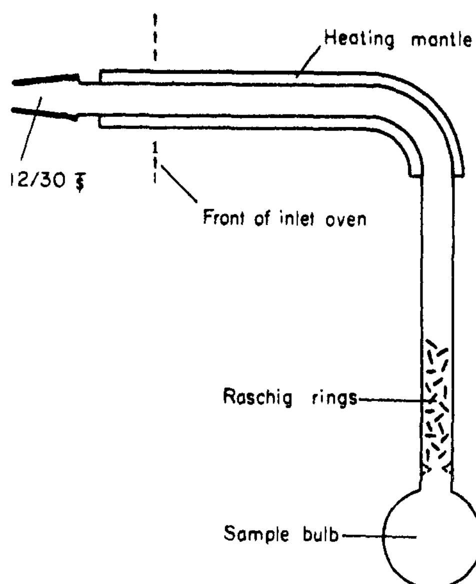
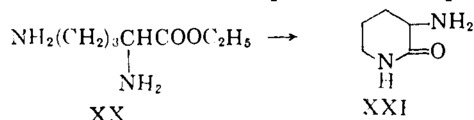


Fig. 28.—Sample introduction tube.

spectrum of the reaction product is obtained instead of that of the original molecule. In contrast to the esters of lysine (IV) and γ-aminobutyric acid (IX) the ethyl ester of ornithine (XX), for example, is stable only in the form of the hydrochloride while the free base lactamizes particularly fast on distillation.[24] The spectrum of the product,



3-amino-piperidone-2 (XXI), does not of course bear any resemblance to the one expected for an α-amino ester. Such knowledge about the chemical reactivity of certain molecules sometimes permits the elimination of a structure which otherwise would have to be considered on the basis of the mass spectrum alone. One of the arguments[7] for the placing of the primary amino group in lysopine as shown in structure XIX was based on the fact that the product of esterification exhibited all the characteristics of an α-amino ester, containing an additional carbethoxy group. This indicated that none of the two amino and two carboxyl groups were in a spatial relationship similar to the one in ornithine because no facile cyclization of lysopine ester took place.

Experimental

Ethyl Esters of Amino Acids.—The amino acids (Mann Res. Labs., MA-grade) were converted to the ester hydrochlorides by Fischer-esterification (2 hr. reflux with excess ethanol, saturated with dry hydrogen chloride), with the exception of glycine, L-tyrosine, L-tryptophan, L-glutamic acid and L-cysteine, the ethyl esters of which were commercially available (Mann).

(24) E. Fischer and G. Zemplén, *Ber.*, **42**, 4878 (1909).

The free ethyl esters of most of the amino acids discussed in this paper have been prepared and characterized repeatedly before, and only the conditions of their preparation from the hydrochlorides merit to be summarized:

The ethyl esters of glycine, L-alanine, D, L-α,β and γ-aminobutyric acid, α-aminoisobutyric acid, D,L-norvaline, L-valine, D,L-norleucine, L-leucine, D,L-isoleucine, L-proline, L-phenylalanine, L-methionine, L-aspartic acid and L-glutamic acid were obtained by repeated extraction with ether of an aqueous solution of the hydrochloride which was made alkaline with potassium carbonate (except with sodium hydroxide and saturation with potassium carbonate in the case of glycine ester). The ethereal solutions were dried, the solvent removed, and the remaining ester distilled under reduced pressure.

The polyfunctional amino esters are more difficult to extract, and the more polar ones have to be distilled quickly and under low pressure to prevent polymerization. The conditions of extraction and boiling points are summarized in Table III.

TABLE III

Ethyl ester of	Extraction	B.p. °C.	Mm.
D,L-Serine	a	80–90[d]	0.1
D,L-Threonine	a	95–100[d]	1.0
L-Hydroxyproline	b	111–112	0.4
L-Tyrosine	a	108–109[e]	
L-Tryptophan	a	170–180[d]	0.08
L-Lysine	c	100–110[d]	0.15
L-Cysteine	a	90–100[d]	0.2
L-Histidine	c	185–190[d,f]	0.08

[a] Concd. aqueous solution of the hydrochloride saturated with $NaHCO_3$ and repeatedly extracted with ethyl acetate. [b] Suspension of hydrochloride in ether saturated with dry ammonia, NH_4Cl filtered off. [c] Two equiv. of EtONa added to ethanolic soln. of hydrochloride, NaCl filtered off. [d] Temperature of oil-bath. [e] Melting point. [f] Immersed in oil-bath preheated to 180°.

The ethyl ester of N^{15}-L-aspartic acid was prepared in the same way as the unlabeled esters, but using only about 10 mg. of the free acid.

Mass Spectra.—The spectra were determined with a CEC 21-103C mass spectrometer, equipped with a heated inlet system operated at 140°. Ionizing current 10 μamp., electron energy 70 ev. (unless stated otherwise), sample size 1–5 μmoles. All the samples had been distilled or redistilled just prior to the determination of their spectra.

Preparation of Ethyl Esters of Amino Acids on a Small Scale and Their Introduction into the Mass Spectrometer.—If only small amounts of material are available, the following technique is used: The amino acid (0.5–5.0 micromoles) is refluxed with 2 ml. of ethanolic hydrochloric acid (10%) for 2 hr. and the solvent evaporated over potassium hydroxide pellets in a vacuum desiccator. The residue is dissolved (or suspended) in 2 ml. of dichloromethane and dry ammonia is passed briefly through the solution. The suspension of ammonium chloride formed is filtered through a cotton plug into a distilling tube (Fig. 28) and most of the solvent distilled off by immersion of the bulb in water of 60°. The standard tapered joint (12/30) of the tube is then connected with the port on the heated inlet system of the mass spectrometer. Remaining solvent is removed with the aid of the pumping system of the instrument, while the bulb is cooled to −50°. On closing the valves leading to the vacuum pump, current is applied to the heating jacket, an auxiliary electrical heater is placed over the vertical part of the tube, and the amino ester vaporizes into the mass spectrometer. The spectrum of the ethyl ester of N^{15}-labeled phenylalanine (Fig. 4) was obtained in this manner.