tained by the procedure outlined by Frost and Pearson[20] for handling parallel first- and second-order reactions. The data for the two disulfides are in Table VIII. Where the k_1 value for a run is shown in parentheses it was obtained by the procedure referred to above. In the other cases the usual procedure could be employed. The limited solubility of dithiodipropionic acid unfortunately prevented extension of runs with that compound to higher disulfide concentrations.

(20) A. Frost and R. Pearson, "Kinetics and Mechanism," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1961, p. 165.

TABLE VIII

Disulfide	(H_2SO_4), M	(RSSR), M	$k_1 \times 10^4$, sec.$^{-1}$
PhSSPh[a]	0.60	0.10	(0.22)
		.20	0.83
		.30	1.7
$(HOOCCH_2CH_2S)_2$	0.60	.07	(0.13)
		.10	(0.25)

[a] The runs with phenyl disulfide were carried out by Mr. C. G. Venier.

[CONTRIBUTION FROM THE INSTITUTE FOR ATOMIC RESEARCH AND DEPARTMENT OF CHEMISTRY, IOWA STATE UNIVERSITY, AMES, IOWA]

The Mass Spectra of Dipeptides[1]

BY HARRY J. SVEC AND GREGOR A. JUNK

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The mass spectra of twenty-two dipeptides, several deuterated dipeptides, and two cyclodipeptides are reported. The interpretation of these spectra is consistent with work published previously on amino acids, peptides, and their more volatile derivatives. The observed dipeptide spectra are shown to be the summation spectra of the dipeptide and the cyclodipeptide formed by thermal cyclization in the mass spectrometer. Each spectrum, however, is unique so that identification and sequence determination is possible. Qualitative predictions of the mass spectra of pure cyclodipeptides and the methods used to detect the presence of a cyclodipeptide are given.

Valuable mass spectra of volatile derivatives of amino acids and peptides have been obtained by the use of externally heated sample inlet systems.[2,3] However, recent work has demonstrated the value of the crucible technique for the direct assay of solid samples of low vapor pressure without resorting to conversion of the samples to more volatile derivatives.[3-8] The mass spectra are obtained by placing the solids directly into the ionization chambers of a time-of-flight instrument[3,8] and a modified 60° sector instrument.[4] The accumulated mass spectra using this technique have been useful for the quantitative analysis of mixtures of amino acids,[5] for studies of the structures of peptides,[3] and for qualitative identification of dipeptides.[7] The study of the mass spectra of a series of amino acids[4,8] and deuterated amino acids has also been useful in furthering our understanding of ionization and unimolecular decomposition mechanisms.

The crucible technique has been extended to a series of representative isomeric dipeptides and two cyclodipeptides.[9] The observed spectra are correlated with the structures and the expected value[7] of the dipeptide mass spectra for qualitative identifications is confirmed.

(1) Work was performed in the Ames Laboratory of the U. S. Atomic Energy Commission.

(2) C.-O. Anderson, R. Ryhage, S. Ställberg-Stenhagen, and E. Stenhagen, *Arkiv Kemi*, **19**, 405 (1962).

(3) K. Biemann, "Mass Spectrometry," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, pp. 261–294 and references cited therein.

(4) G. A. Junk and H. J. Svec, *J. Am. Chem. Soc.*, **85**, 839 (1963).

(5) G. A. Junk and H. J. Svec, *Anal. Chim. Acta*, **28**, 164 (1963).

(6) G. A. Junk and H. J. Svec, *J. Org. Chem.*, **29**, 944 (1964).

(7) G. A. Junk and H. J. Svec, *Anal. Biochem.*, **6**, 199 (1963).

(8) K. Biemann and J. A. McCloskey, *J. Am. Chem. Soc.*, **84**, 3192 (1962).

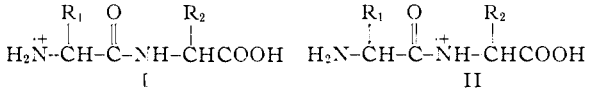
(9) Some cyclodipeptides are sold commercially as acid anhydrides, *i.e.*, glycine anhydride and alanine anhydride. In recent literature many additional designations such as 2,5-diketopiperazines, 2,5-piperazinediones, dioxopiperazines, and cyclic peptides are also used. The authors have standardized on the term cyclodipeptide because it is descriptive and unambiguous. Thus commercial glycine anhydride ($HNCH_2CONHCH_2CO$) is called cyclo(gly-gly) and the cyclic dipeptide from glycine and alanine ($HNCH(CH_3)CONHCH_2CO$) is called cyclo(gly-ala) etc.

The explanation of the spectra which will be discussed here is consistent with the interpretation of the mass spectra of amino acids.[4,8] However, the correct interpretation depends upon the behavior of dipeptides when heated to sublimation temperatures. Experimental evidence of thermal cyclization is cited and the observed spectra are shown to be the sum of the mass spectra of the dipeptide and the corresponding cyclodipeptide.

Results and Discussion

Qualitative Identification.—Partial mass spectra of six dipeptides have been reported previously.[7] These spectra and the partial mass spectra of sixteen additional dipeptides are recorded in Table I. The amine fragment peak from the amino acid which is in the N-terminal position is given a value of 100 and all the other peaks are made relative to this peak. Uniqueness of the spectra is apparent from inspection of the table. The value of the spectra for the unambiguous identification of dipeptides is thus confirmed. Since this variety of dipeptides gives characteristic spectra without exception, it seems safe to conclude that most dipeptides can be characterized from their mass spectra.

Molecular Ionization.—The ionization of both dipeptides and cyclodipeptides must be considered before the observed mass spectra can be satisfactorily explained. It has been shown that the energetically favorable process in the ionization of an amino acid[4] is removal of one of the unbonded electrons from the nitrogen atom. A dipeptide has two such nitrogen atoms and hence the two most probable molecule ions are I and II. Molecule ion I is more stable than II



and hence its existence is more probable. The observed fragmentations of the dipeptides reflect the greater stability of I.

TABLE I

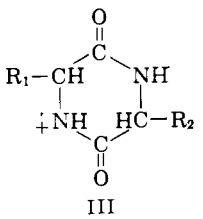
RELATIVE FRAGMENTATION PATTERNS OF DIPEPTIDES AT THE MASSES UNIQUE FOR THE CONSTITUENT AMINO ACIDS

m/e	Gly-ala	Ala-gly	Gly-val	Val-gly	Gly-leu	Leu-gly[c]	Gly-pro	Pro-gly	Gly-ser	Ser-gly	Pro-met
30	100	29	100	17	100	67	100	13	100	577	7
44	39	100	7	7	13	61	12	14	88	392	19
58[a]	1	1	1	3	1	...	1	1	4	21	2
60	...	...	...	...	...	3	...	1	7	100	2
61[b]	...	...	...	...	...	3	...	...	...	4	19
70	1	1	7	2	2	7	90	100	3	23	100
72	...	...	9	100	...	2	...	1	1	8	2
86	2	2	1	1	6	100	...	1	3	23	2
120	...	...	...	...	...	...	...	...	...	...	1
P	1.0	0.3	1.8	0.2	1.2	0.3	0.6	1.8	1.0	...	0.8

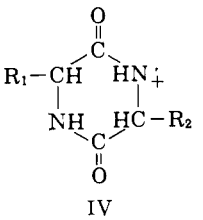
m/e	Gly-phe	Phe-gly	Gly-met	Ala-leu	Leu-ala[c]	Ala-pro	Pro-ala	Val-leu	Leu-val[c]	Ala-val	Val-ala
30	100	42	100	3	65	5	3	47	45	1	5
44	60	17	119	100	75	100	24	45	49	64	14
58[a]	3	4	14	...	4	5	...	8	2	2	2
60	1	1	5	...	...	2	...	...	...	..	...
61[b]	...	...	150	...	...	...	...	...	...	..	...
70	12	3	7	2	6	237	100	17	7	..	2
72	7	1	2	...	...	2	2	100	30	2	100
86	3	1	17	3	100	3	1	5	100	..	...
120	6	100	...	...	...	...	...	...	...	..	...
P	2.9	0.6	4.8	0.04	0.005	0.4	...	0.006	0.3	0.6	0.2

[a] This *m/e* is included in the table to show the low level of interference to be expected if a peptide containing α-aminobutyric acid is analyzed. [b] Not from an amine type fragment but a sensitive peak for the presence of methionine in the dipeptide. [c] The large peaks at 44 and 30 are caused by subsequent fragmentation of the *m/e* 86 fragment.

The most probable molecule ions from cyclodipeptides are III and IV. The stabilities of molecule-ions III and IV should be nearly equal because of the symmetry of the molecule. The observed fragmentation patterns confirm this conclusion.



III



IV

Instrumental difficulties encountered during the course of the work prevented obtaining even approximate estimations of the appearance potentials of the parent and principal fragment ions. Without this corroborative evidence, ionization of dipeptides is assumed to be similar to that which has been shown to occur with the free amino acids.[4] The observed fragment ion currents for each sample are consistent with this assumption of ionization localized at one of the nitrogen positions.

Cyclization Evidence.—Critical inspection of the complete mass spectra of the dipeptides reveals strong evidence that the observed spectra are not due to pure components. In summary this evidence is: (1) simple fragmentation processes cannot be formulated which explain the large peaks due to the amine fragment $R_2CH{=}\overset{+}{N}H_2$; (2) some of the dipeptides have large peaks at m/e = 18 and at the m/e of the parent minus water; (3) peaks of significant intensity are observed at certain common masses from isomeric dipeptides and these peaks cannot be produced by simple fragmentation. Examples of such peaks are the 70, 85, and 100 mass fragments from gly-ala and ala-gly; the 85, 99, 127, and 142 masses from gly-leu and leu-gly; the 99, 113, 126, 141, and 156 masses from ala-leu and leu-ala; and the 112, 126, 127, 141, 154, 169, and 184 masses from val-leu and leu-val. The ion currents at these masses can best be attributed to fragmentation of the cyclodipeptides. The amine fragments $R_2CH{=}\overset{+}{N}H_2$ can also be formed from the cyclodipeptides as will be shown below.

First confirmation of the suspicion that the mass spectra were the summation spectra of dipeptides and cyclodipeptides was the observation of slight discoloration of some of the samples when the crucibles were removed from the ion chamber. Gross and Grodsky[10] have reported the extent of cyclodipeptide formation for five dipeptides which were sublimed at 170–215°. They found some cyclodipeptide in each of the sublimates and as much as 7% in the gly-leu sublimate. Since the dipeptides must be heated to 120–160° to achieve a source pressure of $\sim 10^{-6}$ torr, some cyclization probably occurs in the ion chamber of the mass spectrometer prior to ionization.

Further evidence of cyclization was obtained from the mass spectra of several dipeptides which had been deuterated at the active sites. The $(R_1CH{=}\overset{+}{N}HD)/(R_1CH{=}\overset{+}{N}D_2)$ ratios from these deuterated samples are recorded in the second column of Table II. The ratios were calculated from the peaks which were observed after the spectra had stabilized. The change of the ratios with the time is illustrated in Fig. 1 which shows the typical variation observed for val-gly-d_4. Twenty minutes after the filament of the mass spectrometer was turned on the ratio was 1.4. This value decreased rapidly for 1.5 hr. and then more slowly for an additional 6 hr. after which the value was constant at 0.45.[11]

(10) D. Gross and G. Grodsky, *J. Am. Chem. Soc.*, **77**, 1678 (1955).

(11) The observed change in the ratio was not due to hydrogen–deuterium exchanges on the walls of the ion chamber. Extended conditioning of the walls with D_2 and D_2O both before and during the assay did not affect the time required for the ratio to stabilize or the value of the ratio once it ceased to change.

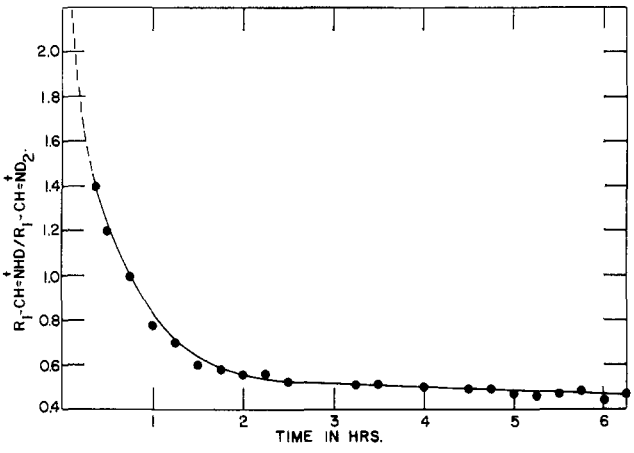


Fig. 1.—Variation in $R_1CH{=}\overset{+}{N}HD/R_1CH{=}\overset{+}{N}D_2$ from val-gly-d_4.

The changing ratio can be explained if cyclization occurs as the ion chamber heats to the temperature necessary for sufficient volatilization of the dipeptide. As the temperature increases, the higher volatility[12]

TABLE II
EXPERIMENTAL EVIDENCE FOR CYCLIZATION OF DIPEPTIDES

Sample	$R_1CH{=}\overset{+}{N}HD$[a] / $R_1CH{=}\overset{+}{N}D_2$	Σ Cyclodipep.[b]	C-terminal[c] or $R_2CH{=}\overset{+}{N}H_2$
Ala-val	...	4	4
Leu-ala	0.5	8	6
Ala-leu	...	8	3
Val-gly	0.8	12	5
Val-ala	...	13	12
Gly-leu	1.0	14	7
Ala-gly	1.3	15	28
Pro-ala	...	15	23
Leu-gly	...	15	7
Gly-ala	1.8	16	34
Gly-phe	...	19	6
Leu-val	...	19	30
Gly-val	2.3	21	9
Phe-gly	...	21	37
Pro-gly	...	22	9
Gly-pro	...	32	90
Ala-pro	...	44	237
Val-leu	5.9	48	5

[a] From deuterated dipeptide data. [b] Per cent of total ionization. [c] Relative to a value of 100 for the N-terminal amine fragment ($R_1CH{=}\overset{+}{N}H_2$).

of the cyclodipeptide causes these molecules to predominate in the first vapor which leaves the solid sample in quantity sufficient to produce measurable ion currents. As the temperature increases further, dipeptide molecules begin to vaporize in measurable quantity. This simple picture of the complex equilibria of thermal cyclization and volatilization explains the observed variation in the relative vapor concentrations of cyclodipeptide and dipeptide. Thus the changing ratio of mono- to dideuterated amine fragments as a function of time is clarified if the origin of the monodeuterated amine fragment can be proved to be from the cyclodipeptide. This proof is given in the ensuing discussion of the fragmentation of cyclodipeptides.

Fragmentation Processes of Cyclodipeptides.—Two pure symmetrical cyclodipeptides, cyclo(gly-gly) and cyclo(ala-ala), were studied. Their mass spectra are given in Fig. 2. The amine fragment peaks at m/e = 30 from cyclo(gly-gly) and m/e = 44 from cyclo(ala-ala) are the largest peaks in the spectra. One hydrogen atom must rearrange during the fragmentation according to 1 and/or 2.

(12) It takes less than 1 hr. to collect 25 mg. of cyclo(gly-gly) and cyclo(ala-ala) at a sublimation temp. of 140°. Less than 5 mg. of the dipeptides gly-ala and ala-gly sublime after 1 hr. at 170°.

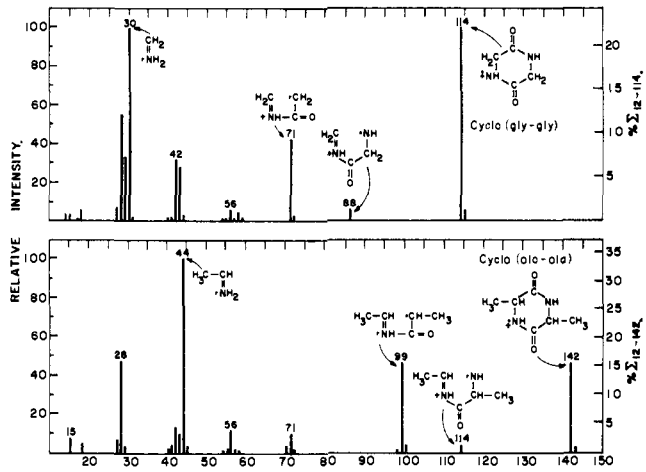
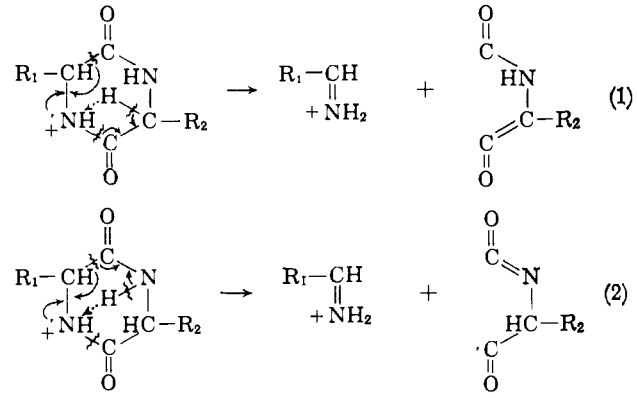


Fig. 2.—Mass spectra of cyclo(gly-gly) and cyclo(ala-ala).



The relative occurrence of processes 1 and 2 were determined from the mass spectra of cyclo(gly-gly) and cyclo(ala-ala) deuterated at the imido positions. These data indicate that in the case of cyclo(gly-gly) the hydrogen from the nitrogen position migrates 25% of the time and in cyclo(ala-ala) the migrating hydrogen comes from the nitrogen position only 20% of the time. Hence the amine fragments from symmetrical cyclodipeptides are formed primarily by process 1. It is expected that these results also apply to unsymmetrical cyclodipeptides such as those formed in the mass spectrometer. Thus the monodeuterated amine fragment $R_1CH{=}\overset{+}{N}HD$, is formed in high yield from the deuterated cyclodipeptide by process 1.

Other important fragmentations occur with cyclodipeptides. Two of those which were established from a comparison of the deuterated spectra with the undeuterated spectra are given here. (a) Elimination of CO

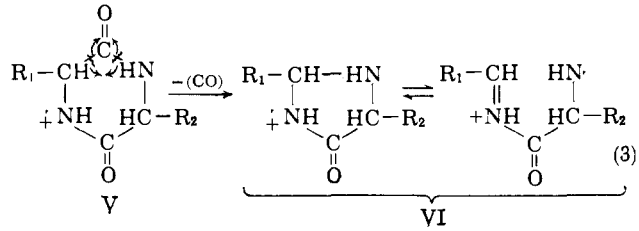


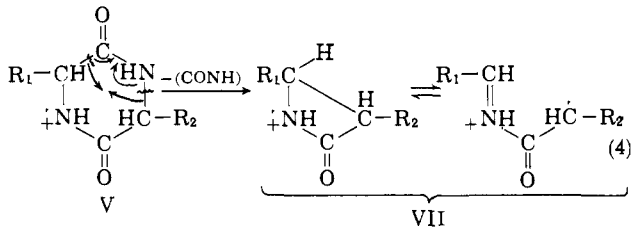
TABLE III

SELECTED PEAKS IN THE MASS SPECTRA OF DIPEPTIDES

	% Total ionization[a]								
	Gly-ala	Ala-gly	Gly-val	Val-gly	Gly-leu	Leu-gly	Gly-pro[c]	Pro-gly[c]	Gly-phe
A	21.7	26.9	28.3	35.9	32.2	15.2	6.8	15.8	6.8
A′	..	..	..	0.1	..	..	...	..	..
B	..	..	..	0.7	..	0.3[b]	...		..
C + F	..	..	..	..	..	..	...	..	..
D	..	..	0.1	..	0.1	..	0.4	0.3	0.4
E	..	..	0.1	..	1.8[b]	..		..	0.2[b]
	Phe-gly	Ala-leu	Leu-ala	Ala-pro[c]	Pro-ala[c]	Val-leu	Leu-val	Ala-val	Val-ala
A	8.3	51.8	13.8	6.4	28.1	6.6	9.4	63.7	42.3
A′	0.1	0.1	..	...	..	...	..	..	..
B	2.2[b]	..	..[d]	...		...[e]	..	..	..[d]
C + F	..	..	..	...	..	...	..	..	..
D	0.1	0.1	..	...	0.1	...	..	..	0.4
E	..	0.4[d]	..		..	...	0.4[e]	0.2	..

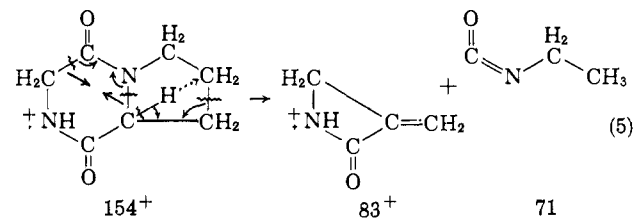
[a] The dipeptides which contain serine and methionine are not included since their mass spectra are more complicated because other hetero-atoms are present. [b] Includes rearrangement peak at m/e = 132. [c] The rupture of the β bond within the cyclic structure of proline is not considered. [d] Includes rearrangement peak at m/e = 146. [e] Includes rearrangement peak at m/e = 188.

and (b) elimination of CONH



In the cases of cyclo(gly-gly) and cyclo(ala-ala) the masses of the charged fragments produced according to eq. 3 are 86 and 114 and according to eq. 4 are 71 and 99. With other cyclodipeptides the masses of these fragments change with the masses of R_1 and R_2. Thus characteristic peaks are observed at masses 85 and 100 from cyclo(gly-ala) and cyclo(ala-gly); at 113 and 128 from cyclo(gly-val) and cyclo(val-gly); at 127 and 142 from cyclo(gly-leu), cyclo(leu-gly), cyclo(ala-val), and cyclo(val-ala); at 141 and 156 from cyclo(ala-leu) and cyclo(leu-ala); and at 169 and 184 from cyclo(val-leu) and cyclo(leu-val). Peaks are also observed at lower m/e values due to the further fragmentation of VI and VII. The process which produces the lower mass fragments depends on the ease of formation of the fragments (determined primarily by the structure) and the relative stabilities of the charged and neutral products of the decomposition.

Peaks which are characteristic of some cyclodipeptides shift two mass units lower when proline is present. For example, cyclo(gly-val) and cyclo(val-gly) have a significant peak at m/e = 85 and none at m/e = 83, while cyclo(gly-pro) and cyclo(pro-gly) have a significant peak at m/e = 83 and none at m/e = 85. The exact fragmentation mechanism has not been unequivocally established but the fragment at m/e = 83 probably forms according to eq. 5,



The same bond ruptures can occur in cyclo(ala-pro) and cyclo(pro-ala). However, the mass of the charged fragments shifts from 83 to 97. This mass shift was observed with both cyclo(ala-pro) and cyclo(pro-ala).

The peaks due to elimination of CO and CONH from the charged cyclodipeptide and the peaks due to the further decomposition of these fragments were summed and the sums are given in column 3 of Table II. These values give an estimate of the relative amount of contamination due to cyclodipeptide in the vapor above heated dipeptides. The stabilized $(RCH{=}\overset{+}{N}HD)/(RCH{=}\overset{+}{N}D_2)$ ratios can also be used to estimate this contamination. Good correlation exists between the two sets of data.

Fragmentation Processes of Dipeptides.—The two most probable molecule ions and the possible fragments which can be formed by single β-bond rupture of a dipeptide are shown in Fig. 3. The contribution of each

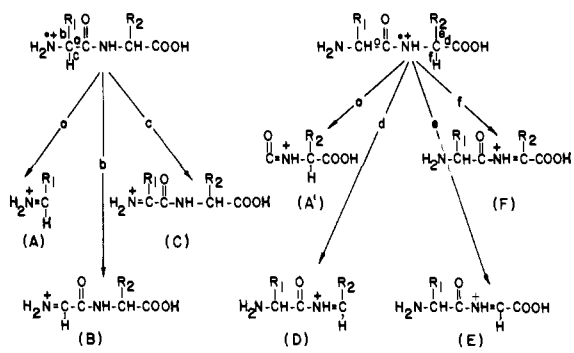


Fig. 3.—β-Bond ruptures of dipeptides.

of these fragments to the total ion current is recorded in Table III. These data indicate the strong tendency for preferred ionization at the N-terminal acid. The only significant peaks are due to the amine fragment

(A) which is formed by bond rupture β to the charged amino nitrogen.

Conclusions

Three types of complex, low vapor pressure organic compounds (amino acids,[4,8] dipeptides,[7] and cyclodipeptides) have been studied. A mutually consistent explanation of the ionization and fragmentation processes can be developed because of the similarity of the functional substituents. Ionization occurs at the nitrogen atom resulting in virtual charge localization. The main fragmentation is then due to bond ruptures β to the charged nitrogen. Consequently all the fragments which cause large peaks in the mass spectra contain nitrogen if other hetero atoms are not present. The remarkable predictability and similarity of the mass spectra of these three types of compounds confirm this interpretation.

The value of the mass spectra of dipeptides for qualitative identification and structural work needs little additional comment. The results show that the spectra are unique for each sample. The significance of these characteristic spectra in relation to the present and future use of the mass spectrometer for structure determinations of both amino acids and peptides is evident.

The cyclization of the dipeptides during the vaporization does not detract significantly from the usefulness of the mass spectra. Accurate conclusions concerning the structure and identity of the samples are possible even when most of the observed ion currents are from the cyclic product. However, knowledge that cyclization has occurred is imperative for an accurate and complete explanation of the spectra.

Samples which contain proline exhibit the strongest tendency to cyclize as was evident from the intense peak due to the amine fragment $R_2CH{=}\overset{+}{N}H_2$ characteristic of the C-terminal acid and the summation of the peaks characteristic of the cyclodipeptide. This observation is consistent with the already known cyclization of dipeptide esters containing proline[13] and of free dipeptides in acidic and hot water solutions.[14]

A final conclusion, supported by data given in Table II, is the apparent selective ionization and fragmentation of cyclodipeptides. Three observations are listed as evidence for cyclization. However, the relative number of $R_2CH{=}\overset{+}{N}H_2$ fragments from the C-terminal acid is not always consistent with the ratio and the summation of the peaks from the cyclodipeptide. An extreme example of this is the data from val-leu and leu-val. Both the ratio and the summed cyclodipeptide data suggest that val-leu forms much more cyclodipeptide than leu-val. However, comparison of the peaks due to $R_2CH{=}\overset{+}{N}H_2$ fragments suggests that leu-val has the greater amount of cyclodipeptide. This discrepancy is completely explained if selective ionization and subsequent fragmentation occurs at the valine nitrogen position. It is obvious that other cyclodipeptides exhibit this same effect though probably to a lesser degree. Unfortunately, accurate predictions of the extent of this selectivity are not possible due to the absence of data on samples of pure mixed cyclodipeptides.

(13) E. Snith and M. Bergmann, *J. Biol. Chem.*, **153**, 627 (1944).
(14) E. Abderhalden and E. Komm, *Z. physiol. Chem.*, **139**, 147 (1924).

Experimental

Material.—All the chemicals were commercial preparations. The gly-ala, ala-gly, gly-pro, gly-val, gly-leu, and gly-met were grade C dipeptides obtained from Calbiochem. The gly-ser, ser-gly, and ala-val were research grade from Mann Research Laboratories. The leu-gly, leu-ala, leu-val, cyclo(gly-gly), and cyclo(ala-ala)[9] were the best grade available from Nutritional Biochemicals Corp. All the other dipeptides were obtained from Schwarz Bioresearch Inc., Yeda preparations.

Sampling Procedure.—Dipeptide crystals were packed into a small clean borosilicate glass crucible and degassed for 1–2 hr. at 80° in a vacuum oven. The crucible, containing the sample, was then inserted directly into the ion chamber of the mass spectrometer. The two cyclodipeptides were treated in a similar manner except the degassing conditions were at 60° for 1 hr.

Vacuum Sublimation.—The procedure followed was that described by Gross and Grodsky.[10] Six of the dipeptides and the two cyclodipeptides were vacuum sublimed. Mass spectra of these sublimed samples were compared to the mass spectra of the samples which were degassed in the vacuum oven. No significant difference in the stabilized mass spectra were observed.

Mass Spectra.—The mass spectra were established using a modified General Electric analytical mass spectrometer which has already been described.[4] Heat radiation from the filament of the electron gun was sufficient to heat the ion chamber to the desired temperature which was adjusted by rotating the electron collimating magnet. This rotation changes the trajectory of the electron beam so that a greater or lesser fraction of the total electrons falls on the trap electrode depending on the direction of rotation. Since the emission regulator maintains a constant trap current, the effect of the rotation of the magnet is to increase or decrease the power to the filament of the electron gun. The range of temperature employed was 120 to 160° for dipeptides and 80 to 120° for cyclodipeptides. The electron energy was held constant at 70 v. The ion accelerating voltage was 2000 v. and magnetic scanning was employed.

Deuterations.—The samples were deuterated at the labile positions by dissolving them in a 100:1 excess of 99+% D_2O. The deuteration time and temperature were arbitrarily set at 15 min. and 20° for convenience. The extent of exchange was not enhanced when longer times and temperatures as high as 80° were employed. The solid samples were recovered from the D_2O solutions by lyophilization.

Infrared Spectra.—Infrared spectra of the samples before and after mass analysis were used to confirm the appearance of cyclodipeptide contamination. Broadening of the bands characteristic of the pure dipeptides and the appearance of a few minor bands at some frequencies were observed. These changes in the spectra were attributed to the presence of a small amount of cyclodipeptide formed during the sampling procedure required for the mass analysis.

The infrared spectra were also used to confirm exchange at both nitrogen positions when dipeptides are dissolved in D_2O and recovered by lyophilization. Shifts in the frequencies due to nitrogen–hydrogen stretching were observed in all the samples studied.

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