AB spectrum averaging to an A_2 spectrum,[8] then the free-energy barrier separating the boat and chair forms is calculated to be about 11.7 kcal/mol, with an estimated maximum error of ±0.5 kcal/mol.[7,9]

The diketone IV-d_4 also showed a temperature-dependent spectrum for the benzylic protons. At −110° at 60 MHz an apparent AB quartet ($\Delta\nu_{AB}$ = 24.4 Hz, J_{AB} = 12.6 Hz) was observed for the benzylic protons, and the coalescence temperature was −92°. Although the proportions of chair and boat forms were not determined, it is likely that both forms occur as with I. With the assumption previously mentioned for I, the free-energy barrier separating the conformations of the diketone is calculated to be about 8.8 kcal/mol. The lower barrier in the diketone compared to the hydrocarbon is understandable since the transition states should have expanded bond angles compared to the ground states, and the diketone has an sp^2-hybridized carbon atom on each bridge.

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(8) R. J. Kurland, M. B. Rubin, and W. B. Wise, *J. Chem. Phys.*, **40**, 2426 (1964).

(9) Because of the difference in populations and hence in the free energies of the boat and chair forms, the barrier for the chair–boat conversion is actually 0.25 kcal/mol less than for the boat–chair conversion.

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A Racemization Test in Peptide Synthesis[1]

Sir:

Several methods of measuring the extent of racemization in peptide synthesis have been reported in the literature.[2] We have been attempting to find a convenient racemization test with the application of an amino acid analyzer though Bodanszky and Conklin already reported the use of the analyzer in the system involving the coupling of acetyl-L-isoleucine and glycine ethyl ester.[3] We introduce here a simple and accurate procedure to detect the degree of racemization and report the results of the influence of several coupling reagents on the extent of racemization during peptide synthesis.

Our proposed sequence is shown in Scheme I.

Scheme I

$$\text{Z-Gly-L-A-OH} + \text{H-L-B-OBzl} \xrightarrow[\text{of A residue)}]{\text{(with partial racemization}} \text{Z-Gly-A-B-OBzl (L,L isomer plus D,L isomer)} \xrightarrow{H_2\text{-Pd}} \text{Gly-A-B (L,L isomer plus D,L isomer)}$$

(1) Presented at the 1st American Peptide Symposium, New Haven, Conn., Aug 14, 1968.

(2) For reviews, see: M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, New York, N. Y., 1966, p 137; E. Schröder and K. Lübke, "The Peptides," Vol. I, Academic Press, New York, N. Y., 1965, p 319.

(3) M. Bodanszky and L. E. Conklin, *Chem. Commun.*, 773 (1967).

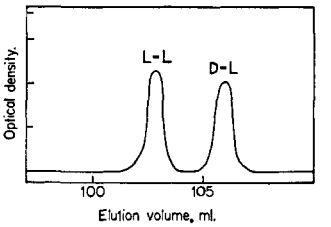

Figure 1. Chromatogram of Gly-Ala-Leu using an amino acid analyzer.

The crude benzyloxycarbonyl (Z) tripeptide benzyl ester (OBzl) is subjected to hydrogenolysis, and the hydrogenated material is submitted to the analyzer.

We first tried to discover a good system of glycyl tripeptide diastereomers for separation by the analyzer. We synthesized the pure L,L and D,L isomers of Gly-Lys-Glu, Gly-Lys-Asp, Gly-Orn-Glu, Gly-Orn-Asp, Gly-Glu-Lys, and Gly-Asp-Lys, with the surmise that a diastereomeric mixture of the polyfunctional neutral tripeptides might be efficiently separated under appropriate conditions. We observed, however, that all mixtures gave incomplete separation.[4] Therefore, we selected rather simple systems preparing several Gly-Ala-B tripeptides in which B could be Ala, Val, Leu, Pro, and Ser residues. We observed that a diastereomeric mixture of Gly-Ala-Val or Gly-Ala-Leu was separated completely with a Hitachi amino acid analyzer (Model KLA-3B) with spherical Dowex 50 resin in a 0.9 × 50 cm column under the conditions: flow rate 60 ml/hr, jacket temperature 55°, and 0.2 *M* standard citrate buffer at pH 4.25 as solvent. Gly-Ala-Leu (I) was our preferred system for the racemization test because it is not overlapped by either Leu or Gly-Ala; Leu was eluted at 58 ml of effluent volume, Gly-Ala at 73 ml, L,L-I at 129 ml, and D,L-I at 159 ml.

We prepared pure I (L,L and D,L) by the azide method. The azide in ethyl acetate derived from Z-Gly-L(or D)-Ala-$NHNH_2$[5] (mp 133°) was added to H-L-Leu-OBzl *p*-toluenesulfonate[6] (II) (mp 156°) in dimethylformamide (DMF) and triethylamine (TEA). The pure Z-Gly-Ala-Leu-OBzl (III) (L,L, 70%, mp 102°; D,L, 64%, mp 125°) obtained was hydrogenated to yield crystalline I (L,L with $0.25H_2O$, 92%, $[\alpha]^{25}D$ −84.5° (H_2O); D,L with $0.5H_2O$, 88%, $[\alpha]^{25}D$ +29.6° (H_2O)). Figure 1 shows the pattern of a mixture of 0.6 μmol each of I L,L and D,L by the analyzer. The limit of detection of D,L in L,L was studied with a synthetic mixture of both isomers. When a mixture of I (L,L) (6 μmol) and I (D,L) (0.06 μmol) was analyzed, a distinct peak of D,L isomer was observed. Even at 1000 parts of L,L (6 μmol) and 1 part of D,L, a small peak of D,L isomer could still be recognized. It will be evident that our method is more sensitive in detecting the slight occurrence of racemization than the Anderson test[2] which has been used widely nowadays. We applied this method in the detection of racemization in the azide procedure. Crude III (L,L) was directly hydrogenated, and a part of the filtrate was submitted to the analyzer. The material showed only single peak by I (L,L), using

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(5) M. Bergmann and L. Zervas, *J. Biol. Chem.*, **113**, 341 (1936).

(6) N. Izumiya and S. Makisumi, *J. Chem. Soc. Japan*, 78, 662, 1768 (1957).

Table I. Extent of Racemization during Peptide Bond Formation[a]

No.	Coupling reagent	Additional component	Solvent	Tertiary amine	Reaction time, hr	Reaction temp, °C	Yield of tripeptide I, %	Extent of racemization
1[b]	Isobutyl chloroformate		THF	TEA	15	25	87	9.5
2[b]			THF	NMM	15	25	91	2.4
3[c]		HOSu	THF	TEA	2	25	55[d]	1.1
4[c]		HOSu	THF	NMM	2	25	63[d]	0.2
5	DCC		THF	TEA	48	0	73	22
6			THF	NMM	48	0	77	21
7[e]		HOSu	THF	TEA	48	0	98	0.0
8[c]	NEPIS		CH_3CN	TEA	24	25	95	1.8
9			CH_3CN	NMM	24	25	72	1.7
10[c]	EEDQ		THF	TEA	7	25	97	0.2
11			THF	NMM	7	25	91	0.2

[a] All components (II, IV, coupling reagent, HOSu, and tertiary amine) in the coupling were of equivalent weight. NMM, N-methylmorpholine. [b] The procedure following that in the literature[8] was noted in detail in this text. [c] The procedure was similar to that described in the literatures: MA,[10] NEPIS,[14] and EEDQ.[15] [d] The lower yields compared with that using the MA method without HOSu may be due to the extraction operation with isopropyl ether[10] of the reaction mixture of Z-Gly-L-Ala-OH, isobutyl chloroformate, and HOSu. [e] The procedure reported[11] was slightly modified; the components were of equivalent weight and the temperature was 0° during the entire reaction.

a load of up to 6 μmol. The result agreed with the fact that the azide procedure has been considered to be safe to avoid racemization.[7]

We have now employed this procedure to examine the influence of racemization of several coupling reagents. A typical procedure was as follows. The reaction conditions (temperatures and reaction times) were similar, as described in the literature.[8] To Z-Gly-L-Ala-OH[9] (IV; 1 mmol) (mp 133°) was added isobutyl chloroformate (1 mmol). After the solution was left standing at −15° for 12 min, II (1 mmol) in THF (5 ml) and TEA (1 mmol) was added, and the mixture was left at 25° for 15 hr. After evaporation, ethyl acetate was added to the residue. It was washed with dilute HCl and then $NaHCO_3$ solution, dried with Na_2SO_4, and evaporated; yield of crude solid (V), 474 mg. Part (47.4 mg) of V was hydrogenated in 90% acetic acid, and the filtrate was evaporated. The residue was dissolved in 0.2 *M* citrate buffer at pH 4.25 (10 ml), and part (0.7 ml) of the solution was submitted to the analyzer; the yield of I (L,L plus D,L) from IV was calculated as 87%, and the extent of racemization,[3] which is defined as $\{100[\text{I (D,L)}]\}/\{[\text{I (L,L)}] + [\text{I (D,L)}]\}$, was calculated as 9.5.

The experiments are summarized in Table I. The results shown confirm the observation of Anderson, *et al.*,[8] on the role of tertiary bases in the racemization on the mixed anhydride (MA) method. The results also indicate that the addition of N-hydroxysuccinimide (HOSu) on the MA[10] and dicyclohexylcarbodiimide (DCC) method[11,12] decreased the racemization remarkably, and the use of N-ethyl-5-phenylisoxazolium-3′-sulfonate (NEPIS)[13,14] and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[15] caused only slight racemization. An easy and straightforward procedure such as the racemization test described in this paper is useful to detect possible racemization when a new coupling method will be developed in future.

(7) Recently, two papers have reported the occurrence of racemization even in the case of the azide procedure: G. W. Anderson, J. E. Zimmermann, and F. M. Callahan, *J. Am. Chem. Soc.*, 88, 1338 (1966); H. Determann in "Peptides," H. C. Beyerman, A. Van de Linde, and W. Maassen van den Brink, Ed., North-Holland Publishing Co., Amsterdam, 1967, p 73.

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Chemical Information from Forbidden Hyperfine Lines in Electron Paramagnetic Resonance. Copper Complexes

Sir:

The allowed transitions ($\Delta M_S = \pm 1$, $\Delta M_I = 0$) in electron paramagnetic resonance yield useful information on spin density distribution, and this has been much used in chemical studies of bonding. In contrast, although the intensities and positions of the so-called forbidden transitions (particularly those where $\Delta M_S = \pm 1$, but $\Delta M_I = 0$) usually carry information about the over-all charge distribution (namely, the nuclear quadrupole coupling constants and thus the electric field gradient at the magnetic nucleus), these transitions have not been much exploited by chemists.

The forbidden hyperfine lines are made allowed and are shifted and split by a combination of nuclear Zeeman interaction and nuclear electric quadrupole coupling with the molecular electric field gradient.[1] In general, the nuclear gyromagnetic ratio is well known, and an analysis of the forbidden lines gives the nuclear quadrupole coupling constant as well as some information as to the relative signs of the parameters in the spin Hamiltonian.[1] Lyons and Kedzie[2] recently demonstrated for the case of Mn^{2+} in an axial site that the complete diagonalization of the spin Hamiltonian is

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