[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES DIVISION, AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

A Mechanism for the N,N′-Dicyclohexylcarbodiimide-Caused Dehydration of Asparagine and Maleamic Acid Derivatives[1]

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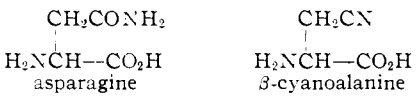
RECEIVED APRIL 4, 1964

The dehydration of N-butylmaleamic acid-1,1-O^{18} (**7b**) with dicyclohexylcarbodiimide results in the formation of N-butylmaleisoimide (**10**) and dicyclohexylurea (**6**), each possessing half of the O^{18} in the starting material. A similar dehydration of carbobenzoxy-L-asparagine (**1b**) produces carbobenzoxy-β-cyanoalanine (**2**) and dicyclohexylurea with approximately the same O^{18} distribution as observed in the maleamic series. These results are consistent with a mechanism in which the carboxamide oxygen is internally acylated by a carbodiimide-activated carboxyl group.

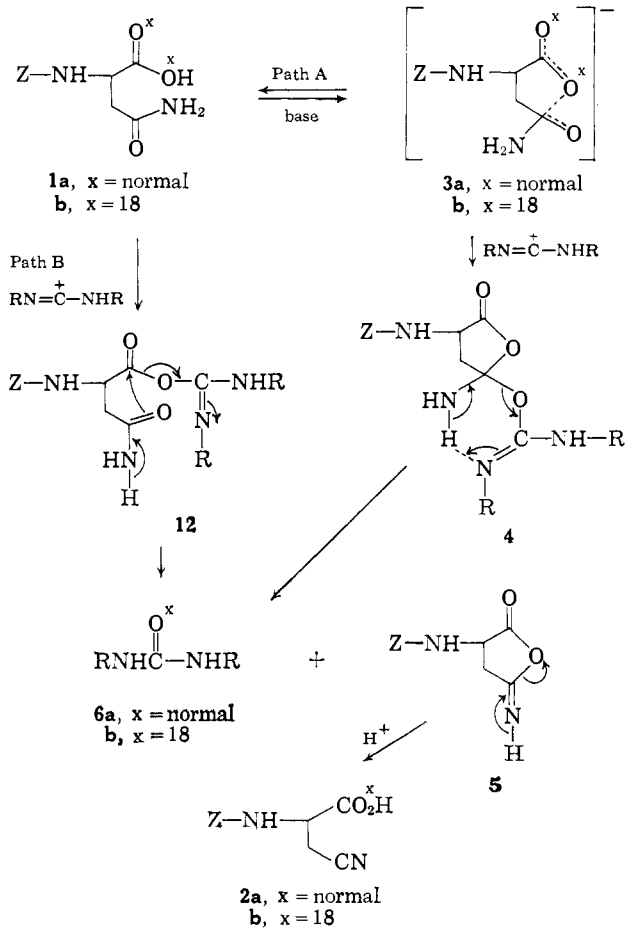
Introduction

The appearance of dehydration products during the syntheses of asparaginyl and, to a lesser extent, glutaminyl peptide bonds has been found by a number of workers.[2-5] Ressler[3] reduced an asparagine "anhydropeptide" with sodium in liquid ammonia, hydrolyzed the resulting peptide, and detected α,γ-diaminobuytric acid. She deduced that the anhydro product was probably a nitrile formed by dehydrating the asparagine amide group. Katsoyannis, *et al.*,[4] detected a nitrile peak in an infrared spectrum of one of their anhydro compounds. By the use of fractional precipitation techniques, they found nitrile formation when the coupling agent was tetraethyl pyrophosphite or N,N′-dicyclohexylcarbodiimide, but none when coupling was effected by the mixed anhydride procedure. We have re-examined the preparation of ethyl tosyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinate[6] using isobutyl chlorocarboxonate[7] and tetraethyl pyrophosphite.[8] By applying a new tool, thin layer chromatography, even the mixed anhydride products were shown to be contaminated with nitrile, though to a much lesser extent. Several other workers have described ω-nitrile formation.[9,10] A by-product, probably the nitrile, was found[11] in the preparation of *p*-nitrophenyl carbobenzoxy-L-asparaginate. It was noted that the ester, once formed, was stable to the action of N,N′-dicyclohexylcarbodiimide.



Zaoral and Rudinger,[12,13] making an asset of a liability, suggested using the nitrile as an amide "protecting" group. This was put into practice by Liberek.[14] On a preparative scale carbobenzoxy-L-asparagine (**1a**) has been dehydrated to carbobenzoxy-β-cyano-L-alanine (**2a**) using *p*-toluenesulfonyl chloride in pyridine,[12] or N,N′-dicyclohexylcarbodiimide.[15] Furthermore, methyl carbobenzoxy-L-asparagine could be dehydrated to methyl carbobenzoxy-β-cyano-L-alanine using *p*-toluenesulfonyl chloride and pyridine,[12] but surprisingly not by N,N′-dicyclohexylcarbodiimide.[16]

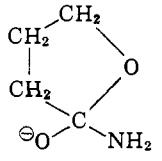
In an attempt to rationalize these results mechanistically, Liberek[16] suggested a mechanism (Path A) similar to that proposed earlier for the synthesis of N-substituted maleisoimides.[17] In Liberek's proposed



(1) Preliminary communication, R. Paul and A. S. Kende, *J. Am. Chem. Soc.*, **86**, 741 (1964).
(2) D. T. Gish, P. G. Katsoyannis, G. P. Hess, and R. J. Stedman, *ibid.*, **78**, 5954 (1956).
(3) C. Ressler, *ibid.*, **78**, 5956 (1956).
(4) P. G. Katsoyannis, D. T. Gish, G. P. Hess, and V. du Vigneaud, *ibid.*, **80**, 2558 (1958).
(5) Private communication from G. W. Anderson.
(6) J. Rudinger, J. Honzl, and M. Zaoral, *Collection Czech. Chem. Commun.*, **21**, 202 (1956).
(7) J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, **73**, 5553 (1951); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951).
(8) G. W. Anderson, J. Blodinger, and A. D. Welcher, *J. Am. Chem. Soc.*, **74**, 5309 (1952).
(9) R. O. Studer and V. du Vigneaud, *ibid.*, **82**, 1499 (1960).
(10) P. G. Katsoyannis, *J. Polymer Sci.*, **49**, 64 (1961).
(11) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
(12) M. Zaoral and J. Rudinger, *Collection Czech. Chem. Commun.*, **24**, 1993 (1959).
(13) J. Rudinger, *Collection Czech. Chem. Commun., Spec. Issue*, **24**, 103 (1959).
(14) B. Liberek, *Chem. Ind.* (London), 987 (1961).
(15) C. Ressler and H. Ratzkin, *J. Org. Chem.*, **26**, 3356 (1961).
(16) B. Liberek, *Bull. Acad. Polo. Sci. Ser. Sci. Chim.*, **10**, 227 (1962).
(17) R. J. Cotter, C. K. Sauers, and J. M. Whelan, *J. Org. Chem.*, **26**, 10 (1961).

mechanism carbobenzoxy-L-asparagine (**1a**) undergoes equilibrium formation of a five-membered ring, **3a**, in the presence of a base. This is attacked by protonated N,N′-dicyclohexylcarbodiimide[18] to give the intermediate **4**, which dehydrates *via* a six-membered ring to the substituted succinisoimide **5** and N,N′-dicyclohexylurea (**6a**). Proton loss carries the reaction irreversibly to the final nitrile **2a**.

The mechanism upon which path A was based was suggested by Cotter, Sauers, and Whelan[17] for N-substituted maleisoimide synthesis. As shown by path C this involves an N-substituted maleamic acid, **7a**, forming a five-membered ring, **8a**, under the influence of base. The latter reacts with protonated N,N′-dicyclohexylcarbodiimide to give **9**, which reacts further *via* a quasi-six-membered ring transition state to give the N-substituted maleisoimide **10a**. Such a path has considerable precedent.[19] However, if path C is correct, one might expect 4-hydroxybutyramide to undergo analogous dehydration under the influence of N,N′-dicyclohexylcarbodiimide to 4-hydroxybutyronitrile through an intermediate such as **11**. This dehydration could not be carried out in pyridine, with or without acid catalysis, nor could it be carried out using ethyl chlorocarbonate and triethylamine.



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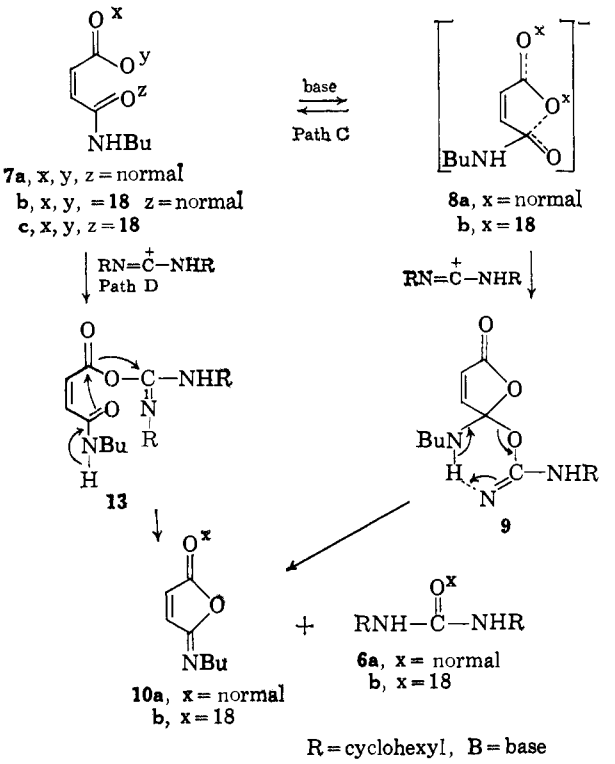
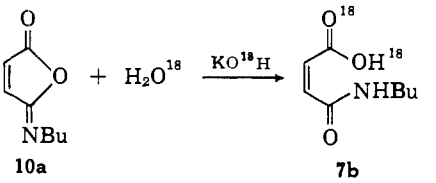
Both in the case of the maleamic acids and asparagine an alternative mechanism is available. In the asparagine series (path B) this utilizes as a first step[20] the addition of carbobenzoxy-L-asparagine (**1a**) across the carbodiimide linkage to give **12**. The amide oxygen then attacks the activated carbonyl splitting out N,N′-dicyclohexylurea (**6a**) to give an isoimide, **5**. Further reaction with base would give **2a**. Such a mechanism has been suggested by Stammer.[21] The nucleophilic properties of amide carbonyls have frequently been demonstrated in the literature. For example, it is considered to be the first step in azolactone formation from N-acylamino acid derivatives,[22a] and is also proposed for the mixed sulfonic–carboxylic anhydride dehydration of amides.[22b] The amide oxygen is a nucleophile in oxidative peptide cleavage.[22c]

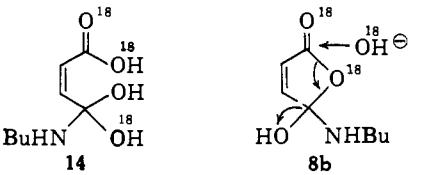
The same type of mechanism, path D, could be extended to N-butylmaleamic acid (**7a**); thus, the addition of protonated N,N′-dicyclohexylcarbodiimide would give **13**, which would decompose to form N-butylmaleisoimide (**10a**) plus the urea **6a**. In order to choose between the two types of mechanisms,[23] the reactions were followed by O^{18} labeling.



Maleamic Acid.—N-Butylmaleamic acid (**7a**) was examined first. It was labeled by carefully treating N-butylmaleisoimide with O^{18}-water containing potassium hydroxide-O^{18}. The possibility exists that the amide oxygen of **7b** might exchange with O^{18} under



these reaction conditions, perhaps by way of the intermediate **14**.[24] This could lead to totally labeled N-



butylmaleamic acid (**7c**). Another method of exchange would be hydroxyl-O^{18} attack on the carboxyl of **8b**, if **8b** exists. It is unlikely that any O^{18} was lost during the work-up of **7b**, since acid-catalyzed O^{18}-carboxyl exchange is known to be very slow.[25]

The accuracy in the agreement of the results with their predicted values (Tables I and II) argues against further exchange (see below).

Methyl N-butylmaleamate (**15**) was prepared from the acid **7b** using diazomethane. Several attempts to analyze **15** for O^{18} under our conditions failed. Unlabeled **15** was prepared by two other methods, the addition of sodium methoxide to N-butylmaleisoimide (**10a**) and the acid-catalyzed esterification of N-butyl-

(18) H. G. Khorana, *Chem. Rev.*, **53**, 145 (1953).

(19) See *Ann. Rept. Progr. Chem.* (The Chemical Society, London), **59**, 250 (1962), for a compilation of recent work in this area.

(20) H. G. Khorana, *Chem. Ind.* (London), 1087 (1955); G. Doleschall and K. Lempert, *Tetrahedron Letters*, 1195 (1963).

(21) C. H. Stammer, *J. Org. Chem.*, **26**, 2556 (1961).

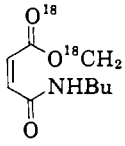
(22) (a) T. Wieland and H. Determann, *Angew. Chem. Intern. Ed. Engl.*, **2**, 368 (1963); (b) C. G. Overberger and E. Sarlo, *J. Am. Chem. Soc.*, **85**, 2446 (1963); (c) A. Patchornik, W. B. Lawson, and B. Witkop, *ibid.*, **80**, 4748 (1958). E. J. Corey and L. F. Haefele, *ibid.*, **81**, 2225 (1959); G. L. Schmir, L. A. Cohen, and B. Witkop, *ibid.*, **81**, 2228 (1959).

(23) In a similar study, D. V. Kashelikar, and C. Ressler, *ibid.*, **86**, 2467 (1964), report results which are in general agreement with ours.

(24) M. L. Bender, R. D. Ginger, and K. C. Kemp, *ibid.*, **76**, 3350 (1954); M. L. Bender and R. D. Ginger, *ibid.*, **77**, 348 (1955).

(25) J. G. Burr, "Tracer Applications for the Study of Organic Reactions," Interscience Publishers, Inc., New York, N. Y., 1957, p. 188.

maleamic acid (**7a**), in an attempt to pin down the cause of our difficulties. It was found in each case there was contamination by N-butylmaleimide. However, even after careful purification of the ester **15** to remove the imide, an interpretable spectra could not be obtained. Probably too high a temperature was used in the mass spectrometer.



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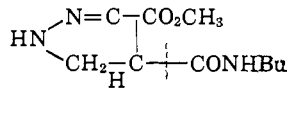

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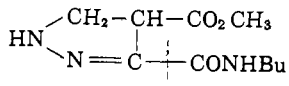

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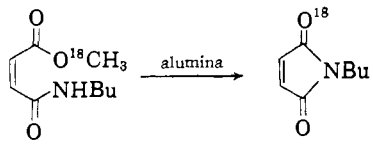
An excess of diazomethane with **7b** gave methyl 4-(or 3)-(N-butylcarbamoyl)-2-pyrazoline-3-(or 4)-carboxylate (**16** or **17**).[26] This conveniently cleaved *in* the mass spectrometer to give a peak of mass 127 (as shown by the dotted line) permitting a determination of the amount of O^{18} labeling in the acid group of **7b**.

The ester **15** on treatment with alumina lost the elements of methanol to give N-butylmaleimide (**18**) which contained slightly less than half of the O^{18}. Thus the amide portion of **7b** cannot contain a significant amount of O^{18}, since **18** should then contain more than half of the O^{18}. It is difficult to conceive of the loss of methanol to give **18** involving the amide oxygen of **15**.



15 **18**

The labeled maleamic acid **7b** was dehydrated to N-butylmaleisoimide (**10b**) using N,N′-dicyclohexylcarbodiimide. The N,N′-dicyclohexylureas **6b** and **10b** were examined for O^{18} by mass spectrometric analysis. The results, summarized on Table I, are in agreement with path D and exclude path C.

TABLE I

N-BUTYLMALEAMIC ACID-1,1-O^{18} (**7b**) DEHYDRATION[a]

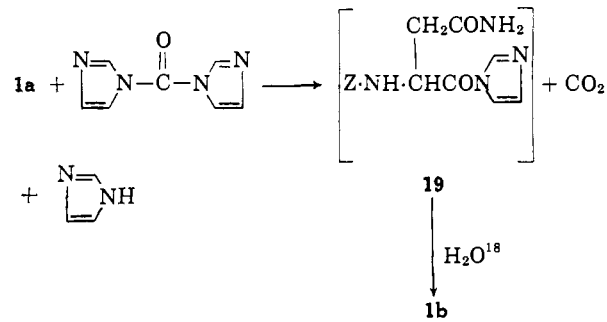
Compound	% of molecules containing one excess O^{18} Predicted by path C	Predicted by path D	Found (±0.3)
Methyl 4-(or 3)-(N-butylcarbamoyl)-2-pyrazoline-3-(or 4)-carboxylate (16,17)[b,c]			10.7
N-Butylmaleimide (18)[d]			4.7[e]
N,N′-Dicyclohexylurea (6)[f]	0	5.4	5.7
N-Butylmaleisoimide (10)[g]	10.7	5.4	6.0

[a] The O^{18} assays were made by comparing the highest significant (mass) peak to the peak at +2 mass units. In each case unlabeled samples were used as standards. The peaks used are cited for each compound. [b] Prepared using 11% H_2O^{18}. [c] 127 − methyl 2-pyrazoline-3-(or 4)-carboxylate ion. [d] Molecule ion peak. [e] Theory − one-half of 16 (17) or 5.4%. [f] 125 − phenyl isocyanate ion. [g] 110 and 111 − loss of C_3H_7 and C_3H_6.

(26) While no attempt was made to chose between structure **16** and **17**, it seems more likely that **16** is correct. From a comparison of σ-constants of an amide and an ester group (J. Hine, "Physical Organic Chemistry," 2nd Ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 90) a negative charge from the polarized double bond of the amic acid would be more stabilized α to the ester group.

(27) (a) H. A. Staab, *Ann.*, **609**, 75 (1957); (b) G. W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80**, 4423 (1958); (c) R. Paul and G. W. Anderson, *ibid.*, **82**, 4596 (1960).

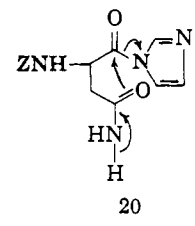
Asparagine.—The study of asparagine derivatives was more difficult because they were not volatile in our mass spectrometer and the O^{18} assays could only be done indirectly. Carbobenzoxy-L-asparagine (**1a**) was treated with N,N′-carbonyldiimidazole[27] to give an acyl imidazole **19** (unisolated). Reaction with labeled water gave **1b**. The formation of **19** is probably quantitative. However, the reaction of acyl imidazoles



with water under neutral conditions is slow[27c]; thus the conversion to **1b** was probably incomplete. During the work-up procedure, unlabeled water and acid were added. This would cause any remaining **19** to go to unlabeled carbobenzoxy-L-asparagine (**1a**). As a net result of this process, **1b** contained 8.8% O^{18}. Quantitative reactions for both steps should have given 11%, since 11% O^{18} water was used. The reaction was carried out at −15° where the formation of nitrile *2* was kept below 5%. The relative sizes of the spots obtained on thin layer chromatography was used as a crude quantitative measurement of **2**. This point is important since the assay procedure for **1b** is based on the same reaction.

Two recrystallizations gave **1b** chromatographically pure. To determine the extent of labeling, **1b** was treated with N,N′-carbonyldiimidazole and the carbon dioxide formed in this reaction captured. The carbon dioxide should contain one-half of the O^{18} in **1b**. Since further side reaction of **19** to give a nitrile, **2**, would proceed by **20** (path B) without the release of any more carbon dioxide, this side reaction would not interfere with the assay.

It may be argued, however, that the nitrile present could originate from some carbobenzoxy-L-asparagine (**1**) existing as **3b**. This would react with N,N′-carbonyldiimidazole through an intermediate such as **21**, similarly to path A. If this happened, the carbon dioxide evolved would be unlabeled. Thus if **1b** were actually 11% labeled and the assay gave 8.8%, a minu-



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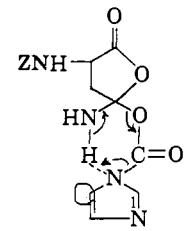

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(27) (a) H. A. Staab, *Ann.*, **609**, 75 (1957); (b) G. W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80**, 4423 (1958); (c) R. Paul and G. W. Anderson, *ibid.*, **82**, 4596 (1960).

mum of 20% nitrile, **2**, should be detected. Since only a very small amount of nitrile was found, the interference of **21** in the assay procedure may be discounted. A more likely source of nitrile is **20**.

The labeled carbobenzoxy-L-asparagine (**1b**) was dehydrated using N,N'-dicyclohexylcarbodiimide by the method of Ressler and Ratzkin.[15] The N,N'-dicyclohexylurea (**6b**) had approximately one-half of the label of **1b**. The nitrile **2b** could not be freed of a small amount of **1b**. An assay of **2b** was carried out in a manner similar to **1b**. The nitrile was treated with N,N'-carbonyldiimidazole and the carbon dioxide evolved was captured. It contained one-half of the O^{18} of **2b** which in turn contained one-half the O^{18} of **1b**. Thus path A proved to be incorrect for the asparagine derivatives (see Table II).

TABLE II

CARBOBENZOXY-L-ASPARAGINE-1,1-O^{18} (**1b**) DEHYDRATION[a]

Compound	% of molecules containing one excess O^{18} Predicted by path A	Predicted by path B	Found (±0.3)
Carbon dioxide from carbobenzoxy-L-asparagine (**1b**)[b,c]			4.4
N,N'-Dicyclohexylurea (**6**)[d]	0	4.4	3.6
Carbon dioxide from carbobenzoxy-β-cyano alanine(**2**)[c]	4.4	2.2	2.7[e]

[a] Footnote *a*, Table I; molecule ion peak. [b] The preparation of **1b** was carried out using 11% H_2O^{18}. [c] From the reaction of the acid with N,N'-carbonyldiimidazole. [d] Footnote *d*, Table I. [e] The carbobenzoxy-β-cyanoalanine was contaminated with some **1b** which could not be removed.

Roderick and Bhatia[28] studied the dehydration of amic acids. Using trifluoroacetic anhydride as the dehydrating agent, they dehydrated N-substituted maleamic acids to isoimides. With saturated amic acids, symmetrical anhydrides were usually formed. One exception was N-substituted succinamic acids. These underwent acyl exchange giving succinic anhydride plus a trifluoroacetamide. The authors mentioned that several α-substituents might force the molecule into a favorable conformation for cyclization. Apparently in the asparagine case, carbobenzoxyamino- is enough. A corollary to path B, suggested by the work of Roderick and Bhatia,[28] might be the formation of symmetrical carbobenzoxy-L-asparagine anhydride from **12** and **1a**. This would then go on to the isoimide **5**. The present work does not permit one to distinguish between formation of **5** directly from **12** or through an intermediate symmetrical anhydride.

One may conclude that once a peptide bond has been formed on the α-carboxy of asparagine, subsequent peptide-forming reactions will not lead to dehydration of the asparagine amide bond.

Experimental

All melting points were taken on a Fisher–Johns block and are corrected. Thin layer chromatography (t.l.c.) was carried out on silica gel plates. System 1 was chloroform–methanol, 2:1; system 2 was 5% methanol in chloroform. The chromatograms were developed using chlorine.[29] The mass spectrographic analyses were carried out in a Consolidated Electrodynamics Corp. 21-103C, with the inlet at 200°, at 70 e.v. and 95 μa.

N-Butylmaleisoimide[17] (**10a**).—In a modification of the method of Cotter, *et al.*,[17] a solution of 10.3 g. (0.505 mole) of N,N'-dicyclohexylcarbodiimide[30] in 50 ml. of methylene chloride was added dropwise, with stirring, over a 1-hr. period to 8.6 g. (0.050 mole) of N-butylmaleamic acid[31,32] in 50 ml. of chloroform. The urea started to precipitate shortly after the addition had commenced. After standing overnight, the N,N'-dicyclohexylurea was filtered off (11.0 g., theory 11.1 g.). The filtrate was concentrated under vacuum. Distillation of the residue gave 7.10 g. (93%) of isoimide, b.p. 51–55° (1 mm.), $n^{26}D$ 1.4884; lit.[29] 52%, b.p. 80–83° (3 mm.), $n^{25}D$ 1.4890; infrared spectrum (carbon tetrachloride) no NH or OH, 5.49, 5.54, 5.90 μ.

Anal. Calcd. for $C_8H_{11}NO_2$: C, 62.72; H, 7.24; N, 9.14. Found: C, 62.69; H, 7.21; N, 9.07.

N-Butylmaleisoimide-O^{18} (**10b**).—The above reaction was repeated on a 6.5 mmolar scale using N-butylmaleamic acid-1,1-O^{18}. The N,N'-dicyclohexylurea-O^{18} obtained as a by-product was purified by washing with boiling chloroform to give m.p. 229–232°. The O^{18} content of the urea and of the isoimide were determined in a mass spectrograph.

N-Butylmaleamic Acid-1,1-O^{18} (**7b**).—Addition of a solution of 3.06 g. (0.020 mole) of N-butylmaleisoimide (**10a**) in 10 ml. of dry tetrahydrofuran to a solution of 2.80 g. (0.025 mole) of potassium *t*-butoxide[33] in 10 ml. of 11% O^{18}-water[34] gave a two-phase system. After 1 min. of rapid stirring, a homogeneous solution was obtained. As slight warming was noted, the solution was cooled in an ice bath. The pH was 8. After 15 min., the tetrahydrofuran was removed under aspirator vacuum. The residue was cooled to 0° and acidified with 6 *N* hydrochloric acid. The thick mixture was diluted with water, and the crystalline product collected. It was washed with water and air-dried overnight, giving 2.82 g. (82%), m.p. 79.5–81.5°. The maleamic acid was recrystalized from 21 ml. of 48% methanol–water. The product was collected, washed with 20 ml. of ice-cold 50% methanol–water, and air-dried to give 2.03 g. (59%) of product, m.p. 82–83.5°. The recrystallization should have removed any labile deuterium picked up from the O^{18} water.

Methyl N-Butylmaleamate[32] (**15**). **Method A.**—Undistilled diazomethane[35] (*ca.* 2 mmoles), in an ethereal solution, was added to 352 mg. (2.05 mmoles) of N-butylmaleamic acid in 2 ml. of methanol and 15 ml. of ether at 0°. Immediately after the addition, 1 ml. of acetic acid was added. The solution was washed with three 10-ml. portions of aqueous sodium bicarbonate and 10 ml. of water. The washes were extracted with 25 ml. of ether. The combined organic layers were dried over anhydrous sodium sulfate and concentrated to 237 mg. (64%) of an oil. The oil was distilled using a micro sublimation apparatus as a molecular still. The distillate was dissolved in carbon tetrachloride. Insoluble recovered nitrosomethylurea (7 mg., used as the diazomethane source) was filtered off. The filtrate was redistilled giving 122 mg. of ester (33%); t.l.c.-2 indicated 2 slight impurities, R_f 0.01, 0.40, and the ester 0.57; $n^{28}D$ 1.4761; infrared peaks 3.00, 3.37, 5.78, 6.1 (broad), 6.48, 6.96, and 7.15 μ. The mass spectrometric analysis confirmed the structure. Unfortunately, every attempt at repeating the mass spectrogram on a labeled sample prepared from **7b** in the same manner failed under the conditions used.

Anal. Calcd. for $C_9H_{15}NO_3$: C, 58.36; H, 8.16; N, 7.56. Found: C, 58.62; H, 8.29; N, 7.69.

Method B.—When N-butylmaleisoimide (3.44 g., 0.0225 mole) was added to 10 ml. of methanol containing a few drops of 1 *N* sodium methoxide a violent reaction (splattering) occurred and an instantaneous deep red color developed. Even though the catalytic amount of base may have sufficed for the completion of the reaction, 22.5 ml. of 1 *N* sodium methoxide in methanol was added. The solution was neutralized with glacial acetic acid, most of the color disappearing. It was then concentrated under vacuum. The residue was dissolved in ether and washed with 1 *N* hydrochloric acid, aqueous sodium bicarbonate, and water. The organic layer was dried over anhydrous sodium sulfate. It was concentrated under an air stream. The residual oil was distilled to give 1.80 g. of a colorless oil, b.p. 86–92° (0.25 mm.), $n^{25}D$ 1.4594, 43% yield; infrared spectrum: 2.90, 5.60 (sh), 5.85, 5.95 (sh), 6.52 μ. The 5.60 μ shoulder indicated the product was probably contaminated with some N-butylmaleimide; t.l.c.-2, R_f 0.62 (blue), 0.70 (white).

(28) W. R. Roderick and P. L. Bhatia, *J. Org. Chem.*, **28**, 2018 (1963).
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(33) MSA Research Corp., Callery, Pa.
(34) Weizmann Institute, Rehovoth, Israel.
(35) F. Arndt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 165.

Anal. Calcd. for $C_9H_{15}NO_3$: C, 58.36; H, 8.16; N, 7.56. Found: C, 57.89; H, 8.33; N, 7.20.

Method C.—Heat was applied to a solution of 17.1 g. (0.10 mole) of N-butylmaleamic acid and 1.58 g. of benzenesulfonic acid (Eastman) in 40 ml. of methanol and 400 ml of benzene. After 1 hr. of reflux the condenser was removed and most of the solvent was boiled off. More methanol (200 ml.) and benzene (200 ml.) were added and the process was repeated. After a third repetition, the residue was dissolved in ether and washed with two portions of saturated aqueous sodium bicarbonate, then once with water. The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was distilled giving 3.0 g. of N-butylmaleimide, b.p. 50–51° (0.02 mm.), $n^{25}D$ 1.4725, infrared spectrum identical with a known sample, t.l.c.-2 R_f 0.72 (white); 1.7 g. of mixture, b.p. 51–110° (0.02 mm), $n^{25}D$ 1.4745; and 7.8 g. of methyl N-butylmaleate (42%), b.p. 109–113° (0.02 mm.), $n^{25}D$ 1.4752, t.l.c.-2 R_f 0.57.

Methyl 4-(or 3)-(N-Butylcarbamoyl)-2-pyrazoline-3-(or 4)-carboxylate (16 or 17).—A large excess of undistilled diazomethane (*ca.* 5 mmoles)[35] in ether was added to 101 mg. (0.59 mmole) of N-butylmaleamic acid in 0.5 ml. of methanol and 2 ml. of ether. The yellow solution was permitted to stand for 15 min. A precipitate started to form. The color of the solution was discharged with acetic acid. The precipitate was collected and dried to give 110 mg. (82%) of product, m.p. 163–165°. This was recrystallized from 8 ml. of ethyl acetate to give 82 mg. (61%), m.p. 164.5–166°, of pyrazoline, t.l.c.-2 R_f 0.25. The infrared peaks (chloroform), in μ, 2.93 (sh) 3.04 (m), 5.90 (s), 6.08 (s), 6.40 (sh), 6.45–6.55 (s), 6.88 (s), 7.09 (s), 7.31 (m), 7.57 (m), 8.90 (s), and ultraviolet λ_{max}^{EtOH} 294 (ϵ 8970) showed similarity to those reported for methyl 3-pyrazolinecarboxylate,[36] infrared peaks (chloroform), in μ, 2.87 (m), 5.82 (s), 6.37 (m), 6.90 (s), 7.09 (s), 7.31 (m), 7.57 (m), 8.85 (s), ultraviolet λ_{max}^{EtOH} 292 mμ (ϵ 10,200). The mass spectrometric analysis was in good agreement with the structure, as was the proton magnetic resonance spectrum. The same reaction was carried out with labeled acid **7b**.

Anal. Calcd. for $C_{10}H_{17}N_3O_3$: C, 52.85; H, 7.54; N, 18.49. Found: C, 52.45; H, 7.40; N, 18.45.

N-Butylmaleimide[32] **(18).**—A 9 cm. × 1 cm. column of alumina, Woelm activity grade 1 (6 g.), was prepared and a solution of 93 mg. (0.5 mmole) of methyl N-butylmaleamate-1,1-O^{18} (**15**) in carbon tetrachloride placed on it. The column was eluted, slowly with carbon tetrachloride, benzene, methylene chloride, and ether in that order. The elution was followed by t.l.c.-2. Those fractions giving a white spot at R_f 0.72 were combined and distilled in a microsublimation apparatus at 70° (9 mm.), lit.[32] 97° (8 mm.). The infrared spectrum (liquid film) gave peaks at 5.63 (sh) and 5.85 (s) μ indicative of a maleimide[17]; no N–H or secondary amide peaks were detected. The spectrum was similar to that of an available sample of N-methylmaleimide.[32] Proton magnetic resonance and mass spectrometric analysis further confirmed the structure. The same reaction had previously been carried out on an unlabeled sample.

(36) J. A. Moore, *J. Org. Chem.*, **20**, 1609 (1955).

Anal. Calcd. for $C_8H_{11}NO_2$: C, 62.53; H, 7.38; N, 9.21. Found: C, 62.72; H, 7.24; N, 9.14.

Carbobenzoxy-L-asparagine-α-carboxyl-O^{18} (**1b**).—A solution of 5.12 g. (19.3 mmoles) of carbobenzoxy-L-asparagine, Mann, in 20 ml. of dry dimethylformamide was cooled to −15° and 3.79 g. (22.0 mmoles, 94% purity) of N,N'-carbonyldiimidazole[27] added. The temperature was maintained at −20 to −15°. A solution formed during the first 30 min. After 2–3 hr., 4.7 ml. of 11% O^{18} water[34] was added. The solution was stored at −20° for 1 hr. and at 0° overnight. The solvent was removed under high vacuum at 30°. The solid residue was dissolved in 150 ml. of water, quickly cooled to 0°, and acidified with 6 *N* hydrochloric acid. After 15 min. at 0° the precipitate was collected, washed with water, and dried in a steam cabinet to give 5.5 g. (slightly damp) of product, m.p. 161–163°. This was recrystallized twice from methanol to give 2.33 g. of O^{18}-labeled carbobenzoxy-L-asparagine (46%), m.p. 165.5–168°, $[\alpha]^{25}D$ +5.2 ± 3.2° (*c* 1.5, acetic acid), both identical with the starting material.[37] Product recovered from the mother liquors was not combined with the main material; t.l.c.-1 showed that a small (estimated at less than 5%) amount of carbobenzoxy-β-cyanoalanine formed during the reaction was removed in the recrystallization.

A 0.815-g. (3.06 mmoles) sample of the labeled carbobenzoxy-L-asparagine (**1b**) was dissolved in 5 ml. of dimethylformamide and cooled to −15°. A cold solution of 0.82 g. (4.56 mmoles, 91% purity) of N,N'-carbonyldiimidazole in 5 ml. of dimethylformamide was added. The carbon dioxide evolved was trapped in a two-stoppered bulb and sent for O^{18} analysis.

Carbobenzoxy-β-cyano-L-alanine[15] **(2b).**—The reaction was carried out as described by Ressler and Ratzkin,[15] using carbobenzoxy-L-asparagine-α-carboxy-O^{18} (**1b**) and N,N'-dicyclohexylcarbodiimide. The carbobenzoxy-β-cyano-L-alanine-α-carboxyl-O^{18} (**2b**), obtained in 62% yield, m.p. 130–131°, $[\alpha]^{25}D$ −46 ± 4.7° (*c* 1, dimethylformamide), lit.[12,15] m.p. 131–132°,[38] $[\alpha]^{25}D$ −45.2° (*c* 1, dimethylformamide), was shown by t.l.c.-1 to be contaminated with a little carbobenzoxyasparagine. All attempts at removing this failed. The N,N'-dicyclohexylurea obtained as a by-product was assayed for O^{18}.

A 0.323-g. (1.30 mmoles) sample of **2b** in 3 ml. of dimethylformamide was cooled to −15° and treated with a cold solution of 0.356 g. (2.00 mmoles, 91% purity) of N,N'-carbonyldiimidazole. The carbon dioxide evolved was trapped in a two-stoppered bulb and assayed for O^{18} by mass spectrometric analysis.

Acknowledgment.—The authors wish to thank Mr. L. Brancone and staff for analyses, Mr. W. Fulmor and staff for rotations and spectra, and especially Mr. A. H. Struck and staff, Stamford, for mass spectrometric analyses and interpretations. We wish also to thank Dr. G. W. Anderson for his interest in and encouragement with this problem.

(37) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932), give m.p. 165°, $[\alpha]^{18}$ +7.6° (*c* 1.7, acetic acid).

(38) Dehydration with *p*-toluenesulfonyl chloride has been shown by two groups[12,15] to give a higher melting point, 133–134°.