amine (0.02 ml.). A d.c. current of 12 ma. at 16 v. was passed for 1 hr. After termination of the electroly ·s, the evolved gases were swept with nitrogen into a half-saturated barium hydroxide solution. The obtained barium carbonate was reprecipitated as described above to yield barium carbonate (31.6 mg., 78%). In a blank experiment 0.2 mg. of residue was obtained.

The solution in the electrolysis cell was dissolved in ether and washed with dilute hydrochloric acid, 1 *N* sodium hydroxide, and water. It was then dried and concentrated to yield a viscous residue XII (7.4 mg.). A thin layer chromatograph indicated the presence of several products, and the residue was counted as such.

[CONTRIBUTION FROM THE DIVISION OF PROTEIN CHEMISTRY, INSTITUTE FOR MUSCLE DISEASE, INC., NEW YORK, N. Y.]

An Oxygen-18 Study of the Dehydration of Asparagine Amide with N,N′-Dicyclohexylcarbodiimide and *p*-Toluenesulfonyl Chloride[1]

BY D. V. KASHELIKAR[2] AND CHARLOTTE RESSLER

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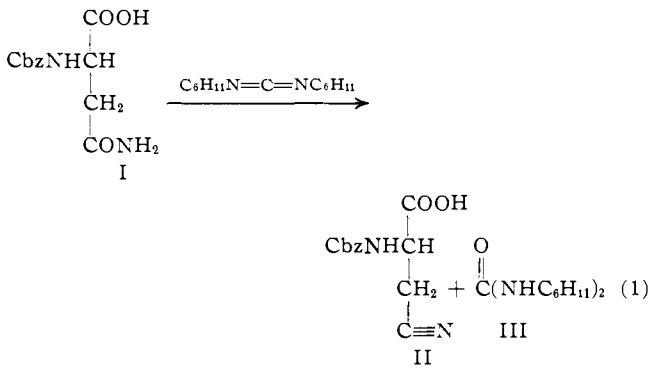
The mechanism of the dehydration of derivatives of certain amino acid amides to the corresponding amino acid nitriles with N,N′-dicyclohexylcarbodiimide in pyridine has been investigated by use of carbobenzoxy-asparagine labeled with oxygen-18 in the carboxyl group. Two suggestive routes have been considered, one of which involves formation of an activated addition intermediate between the carbodiimide and the asparagine carboxyl group, followed by intramolecular displacement of a dicyclohexylurea anion from the adduct by the *carboxamide* oxygen, succeeded by a base-catalyzed ring opening of the formed aminoisosuccinimide derivative. The second mechanism involves intramolecular interaction of the carboxylate anion with the *carboxamide* carbonyl and addition of the resulting anion at the *carboxamide* oxygen to the carbodiimide. Elimination of dicyclohexylurea from a cyclic transition state then gives rise to the common aminoisosuccinimide intermediate. The appreciable labeling of the formed N,N′-dicyclohexylurea supports the concept of an adduct between the carbodiimide and the asparagine carboxyl group. The results are consistent qualitatively with the first mentioned mechanism, although this interpretation is not considered to be unequivocal. To a smaller extent, the second mechanism, or a different one, may operate concurrently. The labeled carbobenzoxyasparagine was dehydrated also with *p*-toluenesulfonyl chloride in pyridine. The findings indicate that the reaction mechanisms with the two reagents may be similar. Syntheses of the L- and DL-isomers of carbobenzoxyasparagine labeled with oxygen-18 in the carboxyl group are presented.

Interest in the mechanism of the dehydration of asparagine amide encountered during the synthesis of certain asparagine peptides related to oxytocin[3] and vasopressin[4] has stemmed in part from the possibility of utilizing this reaction for analytical purposes in peptide chemistry, since the dehydrated asparagine residue can be easily converted to the readily identified 2,4-diaminobutyric acid.[3,5]

At the start of this study, it had been established that carbobenzoxy-L-asparagine (I) and carbobenzoxy-L-glutamine react in pyridine with the coupling agent N,N′-dicyclohexylcarbodiimide to afford in good yield N,N′-dicyclohexylurea and the ω-amino acid nitrile derivatives carbobenzoxy-β-cyano-L-alanine (II) and



carbobenzoxy-γ-cyano-α-L-aminobutyric acid.[5] Appropriate removal of the protecting groups resulted in reasonable syntheses of the free amino acid nitriles.[5,6]

Subsequent unpublished experiments showed, however, that, when the methyl ester of carbobenzoxy-L-asparagine or nicotinamide was treated in pyridine with N,N′-dicyclohexylcarbodiimide under similar conditions, dicyclohexylurea was not formed. Moreover, evidence had accumulated from syntheses of various asparagine and glutamine peptides that peptide couplings could be effected with N,N′-dicyclohexylcarbodiimide in satisfactory yield without apparent amide dehydration in instances in which the carboxyl group that was activated belonged to an amino acid residue other than asparagine or glutamine; *i.e.*, when the asparagine or glutamine residue was not in C-terminal position.[7] These observations suggested that the carboxyl group of the asparagine or glutamine residue might be participating, perhaps intramolecularly, in the dehydration of the carboxamide group of these residues.[8a] Evidence for intramolecular group participation has been available for a number of other reactions.[9,10]

(1) Aided by Muscular Dystrophy Associations of America and by U. S. Public Health Service Grant NB 04316.

(2) Visiting Research Fellow, 1961–1962.

(3) C. Ressler, *J. Am. Chem. Soc.*, **78**, 5956 (1956).

(4) D. T. Gish, P. G. Katsoyannis, G. P. Hess, and R. J. Stedman, *ibid.*, **78**, 5954 (1956); P. G. Katsoyannis, D. T. Gish, G. P. Hess, and V. du Vigneaud, *ibid.*, **80**, 2558 (1958).

(5) C. Ressler and H. Ratzkin, *J. Org. Chem.*, **26**, 3356 (1961).

(6) Removal of the carbobenzoxy group by hydrogenolysis in methanol with a palladium catalyst has since then been found somewhat more convenient than the procedure described, and it yields a γ-cyano-α-aminobutyric acid product that is easier to purify.

(7) An example is the synthesis of isoglutamine-oxytocin [C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **79**, 4511 (1957)].

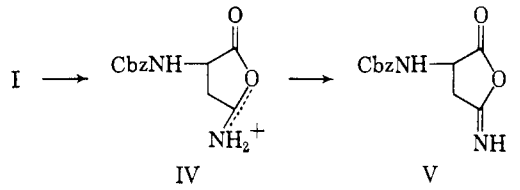
(8) (a) Since then, chiefly on the basis of similar observations that N,N′-dicyclohexylcarbodiimide fails to dehydrate asparagine ester or simple amino amide derivatives, this suggestion that the dehydration of the amide group of I is assisted by the neighboring carboxylate anion was made by B. Liberek [*Bull. Acad. Polon. Sci.*, **10**, 227 (1962)]. (b) That reaction 1 could proceed through a mechanism similar to that suggested by Cotter, Sauers, and Whelan[14] was also noted by this author.

(9) For some of these see J. Hine, "Physical Organic Chemistry," 2nd Ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 141.

(10) In the present series of compounds there is suggestive evidence that the carboxyl group of β-cyano-L-alanine can catalyze the hydrolysis of the cyano group. This amino acid is completely hydrolyzed within 1 hr. at 100° in 0.1% solution in 0.35 *N* H_2SO_4 to a mixture of asparagine and aspartic acid. In contrast, β-aminopropionitrile, as determined by paper electrophoresis, yields no detectable β-alanine and appears quite stable, as had been observed for the latter for even a longer heating period (J. T. Garbutt and F. M. Strong, Abstracts, 128th National Meeting of the American Chemical Society, Minneapolis, Minn., 1955, p. 26A.

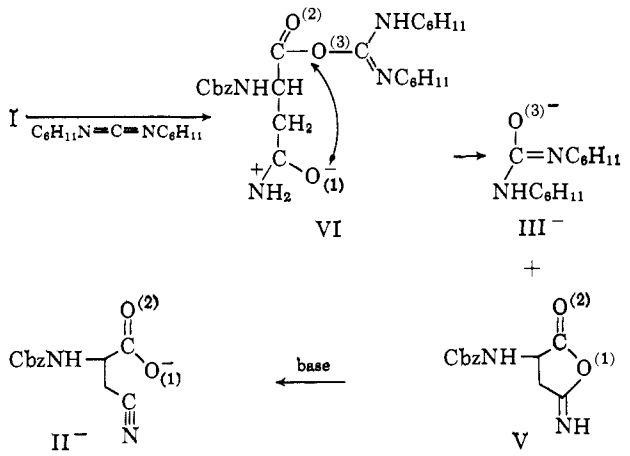
However, possible participation in the dehydration of a carboxamide by a neighboring carboxyl group through mediation of a reagent that appears capable of a 1:2 addition reaction has not been studied in detail before and is the principal subject of the present paper.[11]

Stammer, who had also observed the formation of II from I, noted the possibility that isoimide intermediates, such as IV and V, might be formed through interaction of the carboxamide group of I with the activated carboxyl group.[12] For condensation reac-



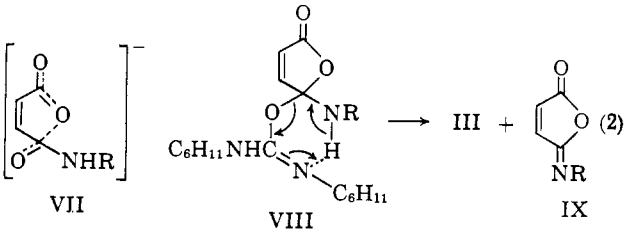
IV V

tions which employ N,N′-dicyclohexylcarbodiimide, such as couplings of carboxylic acid and amino components in the synthesis of peptides, it had been proposed that the first step might be the formation of an adduct between the carboxylic acid and the carbodiimide.[13] Thus, a route for the dehydration of carbobenzoxyasparagine to II was conceivable in which an early step could be the formation of intermediate VI as the product of addition of I to N,N′-dicyclohexylcarbodiimide. Intramolecular displacement of a dicyclohexylurea anion (III^-) by the carboxamide oxygen in VI would then yield the isoimide V, which could be converted with base to the β-cyanoalanine derivative II^-.



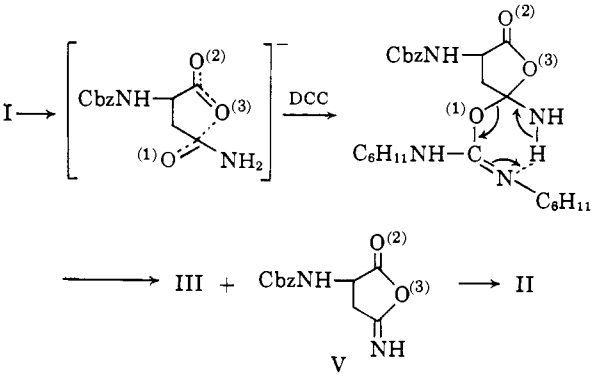
Mechanism A-1

For their somewhat similar synthesis of N-substituted isomaleimides by the dehydration of N-substituted maleamic acids with N,N′-dicyclohexylcarbodiimide, Cotter, Sauers, and Whelan, however, suggested an intramolecular mechanism which involves the formation of a ring-closed species such as VII through donation of a proton to the base or to the carbodiimide.[14] Subsequent reaction with the dehydrating agent was invoked to give a quasi-6-membered-ring transition state (VIII) which would then form the N-substituted isoimide IX (eq. 2). A mechanism similar to the latter,



VII VIII IX

represented as mechanism B, would, like mechanism A-1, be consistent with the supposed intramolecular nature of the dehydration of carbobenzoxyasparagine with N,N′-dicyclohexylcarbodiimide.[8b] Both mecha-



Mechanism B

nisms lead through the common isoimide intermediate V to the β-cyanoalanine product II. Use of oxygen-18 might allow one to distinguish between mechanisms A-1 and B. If II is formed from I through mechanism A-1, the *amide* oxygen of the carbobenzoxyasparagine would appear in the carboxyl group of the β-cyanoalanine derivative, and the oxygen of the dicyclohexylurea would be derived from the *carboxyl* oxygen of I, whereas in mechanism B, the oxygen atoms of the carboxyl group of carbobenzoxy-β-cyanoalanine are both derived from the carboxyl group of I, and the oxygen atom of the dicyclohexylurea would come from the *amide* oxygen of I. A study of reaction 1 was therefore undertaken with the use of carbobenzoxy-L-asparagine that was labeled with oxygen-18 in the carboxyl group. For preparation of the latter, carbobenzoxy-β-cyano-L-alanine methyl ester (XIa), obtained by treating carbobenzoxy-β-cyano-L-alanine with diazomethane, served as starting material. Isotope was introduced by hydrolyzing ester XIa back to carbobenzoxy-β-cyano-L-alanine (IIa) in an organic medium containing water enriched with oxygen-18 and an equivalent of base. Water containing a high concentration of isotope was used for the hydrolysis in view of the dilution during the oxygen isotope analysis[15] of the introduced isotope

(11) An independent investigation of this subject which also employed an oxygen tracer technique has recently been reported (R. Paul and A. S. Kende, *J. Am. Chem. Soc.*, **86**, 741 (1964)). Different routes were used for the synthesis of the labeled asparagine derivative and for the preparation of samples for mass spectrometric analysis. In agreement with the present study, their results indicate that mechanism A, as represented herein, can be an important pathway for reaction 1.

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

(13) H. G. Khorana, *Chem. Ind.* (London), 1087 (1955).

(14) R. J. Cotter, C. K. Sauers, and J. M. Whelan, *J. Org. Chem.*, **26**, 10 (1961).

(15) Oxygen-18 analyses were carried out by Analytica Corporation, New York, N. Y., by mass spectrometric analysis of carbon dioxide obtained on combustion of the carbobenzoxy-β-cyanoalanine, carbobenzoxyasparagine, and N,N′-dicyclohexylurea compounds. Samples of 5 to 10 mg. of these were combusted directly with $HgCl_2$ in the presence of 15 mg. of $Hg(CN)_2$ at 475–525° for 5 hr. in evacuated, sealed capillary tubes according to the procedure described, with some modifications [D. Rittenberg and L. Ponticorvo, *Internal. J. Appl. Radiation and Isotopes*, **1**, 208 (1956–1957)]. Atom % excess O^{18} was derived from ha he ion intensity of mass 46 relative to those of mass 44 and mass 46. Correction for the normal abundance of $CO^{16}O^{18}$ of 0.395 was made. Possible error was estimated as within 3%. The calculated values for atom % excess O^{18} per oxygen in carboxyl group

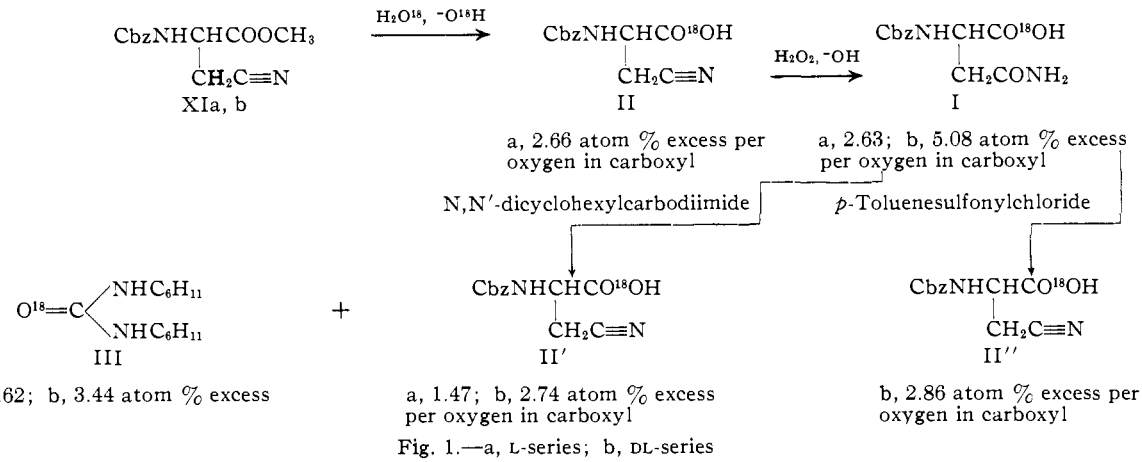


Fig. 1.—a, L-series; b, DL-series

by one nonisotopic *carboxyl* oxygen atom and by the two nonisotopic *carbobenzoxy* oxygen atoms already present in XI. Analysis[15] showed that the isotope in carbobenzoxy-β-cyano-L-alanine-*carboxyl*-O^{18} (IIa) formed by hydrolysis in the presence of dioxane corresponded to the introduction of one atom of labeled oxygen. Compound IIa was converted to carbobenzoxy-L-asparagine-*carboxyl*-O^{18} (Ia) with hydrogen peroxide in alkaline solution by a modification of the procedure suggested by Liberek[16] for converting protected β-cyanoalanyl peptide tertiary butyl esters to the corresponding asparaginyl peptide esters. Although the carboxyl group of IIa had not been protected, Ia was obtained in fair yield. Isotopic analysis of the latter showed a 1.27-fold dilution in proceeding from IIa to Ia, which indicated that one unlabeled oxygen atom had been introduced in converting the labeled carbobenzoxy-β-cyanoalanine to the corresponding labeled carbobenzoxy asparagine. Thus, unless an unforseen compensating oxygen exchange reaction has occurred during the hydration of the cyano group, carbobenzoxy-L-asparagine prepared in this way contains oxygen-18 in the carboxyl group but not in the *amide* or *carbobenzoxy* oxygen atoms. The carbobenzoxy-L-asparagine-*carboxyl*-O^{18} (Ia) was allowed to react with 1.05 equivalents of N,N'-dicyclohexylcarbodiimide in pyridine. The carbobenzoxy-β-cyano-L-alanine and N,N'-dicyclohexylurea thus formed were isolated and purified by recrystallization, and each was analyzed for oxygen isotope content. The distribution of isotope found in the two products is given in Fig. 1 along with the scheme used for preparing the starting carbobenzoxy-L-asparagine-*carboxyl*-O^{18} (Ia).

The starting carbobenzoxy-L-asparagine (Ia) had a concentration of 2.63 atom % excess O^{18} per oxygen in the carboxyl group. For mechanism B it would be expected that the dicyclohexylurea (IIIa) obtained from the latter would have no excess oxygen-18 and that the carboxyl group of the carbobenzoxy-β-cyano-L-alanine (IIa) would have the same concentration of O^{18} as that of Ia, *i.e.*, 2.63 atom % excess O^{18} per oxygen in the carboxyl group. For mechanism A-1, IIIa would have 2.63 atom % excess O^{18} and II'a would have in its carboxyl group half the isotope present in the carboxyl group of Ia, *i.e.*, 1.31 atom % excess O^{18} per oxygen in the carboxyl group. The formed dicyclohexylurea had 1.62 atom % excess O^{18}, which would suggest that reaction 1 may proceed through mechanism A-1 to the extent of 1.62/2.63 × 100, or 62%, with the remaining 38% of reaction 1 proceeding possibly through mechanism B. For 62% of the amide dehydration to proceed through mechanism A-1 and 38% through mechanism B, the oxygen isotope concentration in the β-cyanoalanine derivative II'a would be expected to be 1.81 atom % excess O^{18} per oxygen in the carboxyl group. However, the value for II'a was 1.47 atom % excess O^{18} per oxygen in the carboxyl group, representing 56% of the isotope concentration in the carboxyl group of the starting Ia. Interpreted independently, the latter figure could suggest 88% mechanism A-1 and 12% mechanism B. Thus, the isotope found in the β-cyanoalanine product would favor mechanism A-1 to a greater extent than the amount of isotope in the dicyclohexylurea would indicate. Since these two products were not isolated in quantitative yield, the formation of some labeled by-product has not been excluded. A precise estimate of the degree of mechanism B on the basis of the isotope content of III may therefore not be warranted, inasmuch as the difference between the oxygen isotope concentration of the carboxyl group of I and of the dicyclohexylurea is a quantitative measure of the occurrence of mechanism B only when the isotope of I appears in no product other than II and III. However, when it was assumed that the latter were the only products, the finding that II'a was lower in isotope concentration than expected for the degrees of mechanisms A-1 and B suggested by the concentration of isotope found in the dicyclohexylurea IIIa raised the question whether some loss of isotope had occurred through solvent–*carboxyl*-oxygen exchange when II'a was allowed to stand in the final aqueous acidified reaction mixture from which it was subsequently isolated. It also seemed possible that some racemization may have occurred.[17] Reaction 1 was

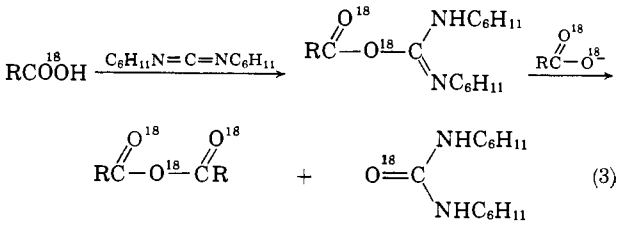
of the carbobenzoxy-β-cyanoalanine compounds were obtained by multiplying the experimentally derived values for atom % excess O^{18} by the factor 4/2, stemming from the dilution of the 2 *carboxyl* oxygen atoms by the 2 nonisotopic *carbobenzoxy* oxygen atoms which occurs during the combustion. For the corresponding calculated values per oxygen in carboxyl group of the carbobenzoxyasparagine compounds, the experimentally derived values were multiplied by the factor 5/2 in view of the dilution of the 2 *carboxyl* oxygen atoms by the 2 nonisotopic *carbobenzoxy* oxygen atoms and 1 nonisotopic *amide* oxygen atom.

(16) B. Liberek, *Chem. Ind.* (London), 987 (1961).

17) After this work was well underway, β-cyano-L-alanine became available from a natural source.[25] It was noted that even the best samples of car(bobenzoxy-β-cyano-L-alanine prepared by the carbodiimide method, which had satisfactory elemental analysis, had a melting point some degrees lower than this derivative prepared from the natural material. Other reported melting points for synthetic carbobenzoxy-β-cyano-L-alanine have covered even a considerably wider range (see preparation of 1a). Moreover all compounds of the present L-series, derived from carbobenzoxy-β-cyano-L-alanine prepared in this way, melted 0.5–3° lower than reference compounds

therefore repeated using as starting material the DL-isomer of carbobenzoxyasparagine-*carboxyl*-O^{18} (Ib). The latter was prepared through a route similar to that used to obtain the L-compound. Product II'b was isolated directly from the acidified reaction mixture by extraction with ethyl acetate. The results obtained on dehydrating the DL-compound Ib were very similar to those with the L-compound. The dicyclohexylurea (IIIb) was appreciably labeled, to an extent which could suggest 68% participation through mechanism A-1. The carbobenzoxy-β-cyano-DL-alanine dehydration product II'b had in its carboxyl group 2.74/5.08, or 54%, of the isotope concentration of the carboxyl group of the starting Ib, which, interpreted independently, could indicate a pathway which involved 92% mechanism A and 8% mechanism B. In both experiments, the appreciable labeling of the oxygen of the N,N'-dicyclohexylurea, and, to an even greater degree, the amount of isotope in the β-cyanoalanine product, suggest that mechanism A-1 could represent a significant path for reaction 1. However, the possibility that this reaction can proceed to some extent by a path such as mechanism B has not been excluded.[17] Inasmuch as examination of both products leads to some quantitative difference in the derived conclusions, the coexistence, to a smaller degree, along with mechanism A-1, of a pathway other than mechanism B has also not been excluded.

Some qualifications are necessary regarding the interpretation of labeling of dicyclohexylurea oxygen as evidence for mechanism A-1. It is recalled that one of the major reactions that simple carboxylic acids tend to undergo with N,N'-dicyclohexylcarbodiimide is to form the symmetrical anhydride of the carboxylic acid along with N,N'-dicyclohexylurea.[18,19] If the latter reaction proceeds through the route which has been suggested for it,[18] then it might not be unexpected to obtain some dicyclohexylurea labeled with oxygen-18 from any carboxylic acid labeled with oxygen-18 (eq. 3). The



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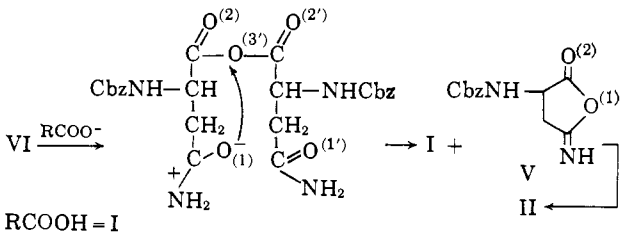
appearance of isotopic dicyclohexylurea, *per se*, thus would not necessarily have to indicate an intramolecular mechanism and its appearance in reaction 1 is therefore considered evidence which is consistent with but not proof for intramolecular mechanism A-1. For example, if some symmetrical anhydride of I is formed, another portion of I could be dehydrated by the latter anhydride intermolecularly. Such a route would similarly lead to labeled dicyclohexylurea and to a β-cyanoalanine product with a lower isotope concentration in its carboxyl group than in the starting material I. This, however, seems a less attractive possibility than an intramolecular pathway. Moreover, the fact that N-substituted isomaleimides can be prepared by dehydrating N-substituted maleamic acids with dicyclohexylcarbodiimide[14] suggests, by analogy, an intramolecular mechanism for the dehydration of I. It may be noted that if a symmetrical anhydride intermediate is actually formed from I, probably by the reaction of VI with a second mole of I, it could decompose intramolecularly and afford the same isoimide intermediate V and product II according to the path indicated as mechanism A-2. The latter path would be



Mechanism A-2

indistinguishable from mechanism A-1 in the present isotope experiment. A more definitive assessment of the possible importance of either mechanism A-1 or A-2 compared to mechanism B might be obtained from a study with carbobenzoxyasparagine labeled with isotope in the *amide* oxygen. Interpretation of the appearance of isotope in the dicyclohexylurea would not then require consideration of a conceivably unrelated formation of anhydride indicated in eq. 3. In addition, in mechanism A, but not in B, isotope derived from the amide position and having migrated intramolecularly in full to the carboxyl group would still be present in the dehydration β-cyanoalanine product II. In the current experiments the presence of isotope in the dicyclohexylurea oxygen does, nevertheless, provide some direct evidence relevant to mechanism A-1, in that it indicates that an intermediate may be formed of the nature of VI, *i.e.*, an adduct between the carbodiimide and the carboxyl group of I. This would then also tend to give support to the suggestion

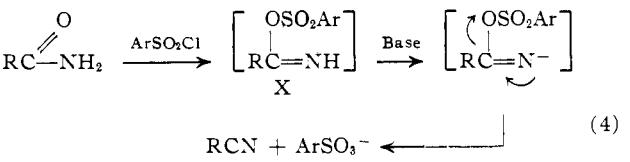
even after extensive recrystallization. It seemed possible that some racemization had occurred in the original preparation of II from which XIa had been derived and in the subsequent conversion of Ia to II'a, and it was also possible in the hydrolysis of XIa to IIa. This possibility had been noted independently for the hydrolysis reaction [B. Liberek, *Bull. Acad. Polon. Sci.*, **10**, 407 (1962)]. In addition, samples of recrystallized synthetic β-cyano-L-alanine prepared by the carbodiimide dehydration generally have been found to contain 0.5 to 1% of asparagine. The latter has been attributed to the presence of carbobenzoxyasparagine as an impurity in the carbobenzoxy-β-cyanoalanine, probably residual starting material that was difficult to remove completely from the latter by repeated crystallization and which did not significantly affect the elemental analyses. In the present study, examination of the oxygen-18 labeled carbobenzoxy-β-cyanoalanine starting material IIa and IIb, and the labeled carbobenzoxy-β-cyanoalanine products II'b and II''b, after removal of the carbobenzoxy group by hydrogenolysis, showed in these compounds, in addition to the expected products, the presence of 1 to 6% asparagine. Corrections of the isotope contents calculated for the carboxyl group of the β-cyanoalanine compounds and of Ia and Ib for these have not been made. However, it is noted that incomplete removal from the dehydration products II'a, II'b, or II''b of starting carbobenzoxyasparagine Ia or Ib would be reflected in a high isotope content of the carbobenzoxy-β-cyanoalanine product when reaction 1 proceeds according to mechanism A to the extent of 68 to 100% with the remainder according to mechanism B. The presence of 4% of Ib in II'b would then lead to a conclusion for mechanism A that is low by less than 3%. If carbobenzoxyasparagine has been formed as a by-product during the hydrolysis of XI and has not been quantitatively removed, part of the isotope of I would be contained in the *amide* oxygen. For 3.9% of impurity of this type in Ia, a conclusion based on the isotope of the dicyclohexylurea would be about 4% low for mechanism A when only the latter is involved, and it would not be affected appreciably when the dehydration proceeds 62% according to mechanism A with the remainder according to mechanism B. The effect of 1.2% impurity in Ib would not be appreciable. In the DL-series with dicyclohexylcarbodiimide, the extent of mechanism A based on the isotope of the β-cyanoalanine compound should therefore be about 3% higher than indicated.

(18) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958).

(19) I. Muramatsu and A. Hagitani, *Nippon Kagaku Zasshi*, **80**, 1497 (1959); *Chem. Abstr.*, **55**, 6394 (1961).

that an adduct of this type, an O-acylisourea, can exist as an intermediate in the formation of acid anhydrides and in peptide coupling reactions with the carbodiimide reagent.[13,18]

Certain other dehydrating agents, which include several arylsulfonyl halides, are capable, even under rather mild conditions, of converting a variety of primary carboxamides to nitriles without requiring participation of a neighboring carboxyl group.[20] For example, *p*-toluenesulfonyl chloride,[21] in contrast to N,N′-dicyclohexylcarbodiimide, can dehydrate the methyl ester of carbobenzoxy-L-asparagine in pyridine to the methyl ester of carbobenzoxy-β-cyano-L-alanine. Some indirect support has been given for sulfonation of *amide* oxygen in preference to *amide* nitrogen as an intermediate step in the dehydration of a primary carboxamide with benzenesulfonyl chloride represented by eq. 4.[20] In place of intermediate X a cyclic transi-



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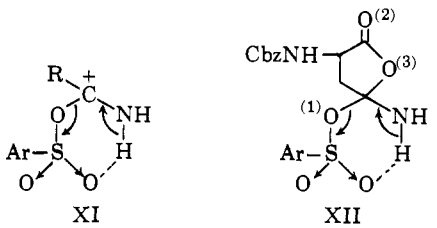
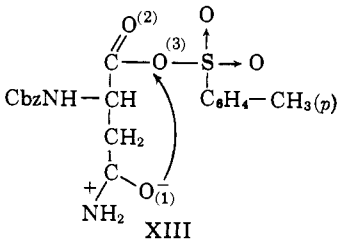
tion state of the nature of XI may also be considered. Intramolecular assistance by a carboxylate ion could then make XII a likely intermediate in the dehydration of an amino acid amide derivative such as I. A pathway which involves the latter intermediate is analogous





to mechanism B considered for the carbodiimide dehydration, and it has been suggested for the dehydration of I with an arylsulfonyl halide (*cf.* ref. 8). It was therefore of interest to attempt to determine the course of the dehydration of I with a reagent of the latter type and to compare it with the findings with dicyclohexylcarbodiimide. Another portion of the carbobenzoxy-DL-asparagine-*carboxyl*-O^{18} (Ib) was therefore treated with *p*-toluenesulfonyl chloride in pyridine. The procedure used by Zaoral and Rudinger[21] with the non-isotopic L-isomer was essentially followed with modifications in the amount of *p*-toluenesulfonyl chloride and in reaction temperature. Since only the β-cyano-DL-alanine derivative was isolated, conclusions concerning the route of the dehydration are based on the oxygen isotope content of the latter. Within this limitation, the dehydration with *p*-toluenesulfonyl chloride appears to resemble closely the carbodiimide dehydration, since the product II″b had in its carboxyl group 56% of the isotope concentration in the carboxyl group of the starting asparagine derivative Ib, a result very similar to that obtained in the dehydration with the carbodiimide. Thus the carboxyl group appears to participate also in the dehydration of the carboxamide group of I with *p*-toluenesulfonyl chloride. An addition compound between the carboxyl group of I and the *p*-toluenesulfonyl group, such as XIII, which is analogous to VI, becomes a likely intermediate, and a route involving intramolecular displacement in XIII of a *p*-toluenesulfonate anion by the *carboxamide* oxygen in analogy to mechanism A-1, or one analogous to mechanism A-2, becomes a major possibility as a pathway for the dehydration of I with *p*-toluenesulfonyl chloride. The presence of a carboxyl group that is apparently appropriately situated therefore seems to have effected a change in the mechanism of amide dehydration with the latter reagent. In the case of dicyclohexylcarbodiimide, it has made possible an amide dehydration reaction that would not otherwise occur with this reagent, probably chiefly through a primary reaction of the carboxyl group with the carbodiimide.[22]

Experimental[23]

Procedures.—In all dehydration reactions with N,N′-dicyclohexylcarbodiimide and tosyl chloride considerable care was taken to obtain an anhydrous condition, since low melting products of II have been obtained in reaction 1 with pyridine which had not been recently dried. Pyridine of analytical grade was dried over BaO and distilled. It was stored in a dry cabinet. Recrystallizations of the β-cyanoalanine compounds were carried out with dried, distilled solvents in dried glassware. Hydrogenolyses for analytical purposes were carried out by bubbling a stream of H_2 gas through a solution of 6 ml. of methanol containing 2 mg. of the carbobenzoxy compound in the presence of 6 mg. of palladium black. Evolution of CO_2 usually ceased within 5 to 7 min., and the reaction was then continued for 10 min. The mixture was filtered, the catalyst was washed with a small volume of hot water, and the combined solutions were concentrated under reduced pressure to dryness. Chromatographic ninhydrin analyses were carried out on the residues with the Beckman Model 120 automatic amino acid analyzer. The system pH 3.25 at 30° on the 150-cm. column was used for neutral and acidic amino acids,[24] and a modified system, pH 4.26 at 50° on the 50-cm. column, was used for basic substances. Carbobenzoxy-β-cyanoalanine (II) under these conditions is normally expected to yield 40 to 50% of β-cyanoalanine, 20 to 40% 2,4-diaminobutyric acid, ammonia, a total of 5 to 11% of two unidentified basic substances which are eluted at about 165 and 175 ml., and approximately 1% each of two unidentified neutral or acidic substances which are eluted at about 142 and 177 ml. The unidentified substances are formed under similar conditions, sometimes in somewhat smaller amount, also from recrystallized β-cyano-L-alanine which is chromatographically free of these components. They are therefore considered to be by-

(20) C. R. Stephens, E. J. Bianco, and F. J. Pilgrim, *J. Am. Chem. Soc.*, **77**, 1701 (1955).

(21) M. Zaoral and J. Rudinger, *Collection Czech. Chem. Commun.*, **24**, 1933 (1959).

(22) An extension of these studies has led to a convenient micro procedure for the identification of asparagine and glutamine residues in peptides (C. Ressler and D. V. Kashelikar, manuscript in preparation).

(23) Melting points were taken in capillaries and are corrected. The melting points of most compounds in this series are highly sensitive to the rate of heating and the temperature at which they are placed in the bath. Compounds were placed in a bath about 10° below the m.p. and were heated at a rate of about 2° per min. Samples which were being compared were melted simultaneously. Elemental analyses were performed by Micro-Tech Laboratories, Skokie, Ill., or by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. The H_2O^{18} containing 10.1 atom % was purchased from Isomet Corporation, Palisades Park, N. J., and the H_2O^{18} containing 1.62 atom % was obtained from Yeda Research and Development Co., Ltd., at the Weizmann Institute of Science, Rehovoth, Israel.

(24) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

products formed during the hydrogenolysis. Concentrations of unidentified substances were calculated with the constant for leucine. Recoveries in terms of total products present after hydrogenolysis were close to the expected values.

Carbobenzoxy-β-cyano-L-alanine Methyl Ester (XIa).—Five batches of carbobenzoxy-β-cyano-L-alanine[5] (1.25 g. each), dissolved in a minimum volume of methanol, were treated with a slight excess of diazomethane in 20 ml. of ether. Hexane was added to incipient crystallization and, after standing at 5° overnight, a total of 5.3 g. of needles was collected, m.p. 91.5–92.5°. A second crop of 0.2 g., m.p. 89.5–90.5°, was obtained. Recrystallization from 1,2-dichloroethane–hexane yielded 4.65 g. (70.4%) of XIa which melted at 92.5–93.5°, reported[21] m.p. 93–94°.

Carbobenzoxy-β-cyano-L-alanine-*carboxyl*-O^{18} (IIa). (a) Hydrolysis in Dioxane.—A solution of 1.4 g. of XIa in 2.8 ml. of purified dioxane and 1.5 ml. of H_2O^{18} (1.62 atom % excess) was stirred magnetically in a closed 5-ml. ground joint test tube, and 0.95 ml. of a solution of 0.239 g. of NaOH in 1.0 ml. of the H_2O^{18} was added dropwise over a 40-min. period. During the addition pH was maintained between 8 and 9. After 15 min. the solution was brought to pH 7 with 20 μl. of concentrated HCl. The solution was frozen, and it was first lyophilized for 2 hr. and then evaporated to dryness under vacuum with a rotary evaporator. The residue was dissolved in 3 ml. of water, and the solution was cooled and acidified to pH 2 with a few drops of 6 *N* HCl. After 30 min. in the cold, the white, crystalline product was filtered off and washed several times with cold water and then dried under vacuum over P_2O_5; wt. 1.25 g., m.p. 118–120°. Two recrystallizations from ethyl acetate–hexane yielded 1.03 g. (77.8%) which melted at 132–133.5°; reported m.p. 133–134°,[21] 131–132°,[5] 136°,[25] 126–128°.[8,12] Atom % excess O^{18} of this sample of IIa was 0.33. The expected value for introduction of 1 atom of labeled oxygen is 0.39 atom % excess O^{18} based on the concentration of 1.62 atom % excess for the H_2O^{18} with the dilution by the nonisotopic NaOH.

IIa. (b) Hydrolysis in Acetone.—Hydrolysis similar to that described under a was carried out in which 1.12 g. of XIa, dissolved in 2.5 ml. of acetone and 1 ml. of H_2O^{18}, was treated with a solution of 0.18 g. of NaOH dissolved in 1 ml. of H_2O^{18}, in portions, over a 20-min. period. The H_2O^{18} was a sample recovered from a similar reaction. It originally had 10.1 atom % O^{18}; its concentration was not redetermined. The white solid obtained on acidification to pH 2 weighed 833 mg. and melted at 124–125°. Four recrystallizations from 8-ml. portions of 1,2-dichloroethane left 432 mg. (41%) which melted at 131.5–133°. Hydrogenolysis followed by amino acid analysis showed in addition to the expected products 3.9% asparagine. Atom % excess O^{18} of IIa was 1.33; calculated 2.66 atom % excess O^{18} per oxygen in carboxyl group.

Carbobenzoxy-L-asparagine-*carboxyl*-O^{18} (Ia).—To 380 mg. of IIa (1.33 atom % excess O^{18}) in a 5-ml. test tube cooled in an ice bath was added cautiously with magnetic stirring 0.8 ml. of 2 *N* NaOH solution followed by 1.5 ml. of 30% H_2O_2 (Merck Superoxol). After 3 hr. at room temperature the solution was cooled and acidified to pH 2 with 0.28 ml. of 6 *N* HCl. The white solid was collected by filtration, washed with cold water, and dried under vacuum; wt. 340 mg., m.p. 152–157°. Three recrystallizations from water left 142 mg. (35%) which melted at 160–161.5°, or 2° below a reference sample.[23,26] Hydrogenolysis followed by amino acid analysis showed in addition to the expected amount of asparagine 3.3% aspartic acid. Atom % excess O^{18} of Ia was 1.05; calculated 2.63 atom % excess O^{18} per oxygen in carboxyl group. The expected value for introduction of 1 atom of unlabeled oxygen into IIa is 2.66 atom % excess O^{18} per oxygen in carboxyl group.

Dehydration of Carbobenzoxy-L-asparagine-*carboxyl*-O^{18} (Ia) with N,N′-Dicyclohexylcarbodiimide (DCC).—A solution of 66 mg. of Ia (1.05 atom % excess O^{18}) in 0.3 ml. of pyridine in a small, dry, stoppered test tube was treated with a solution of 55 mg. of DCC in 0.2 ml. of pyridine at 16–18°, as described for the reaction on a larger scale.[5] The N,N′-dicyclohexylurea, 45 mg., obtained by filtration of the reaction mixture, was combined with the solid which remained undissolved after water had been added to the residue left after removal of the pyridine. This was recrystallized twice from methanol; **IIIa**, wt. 30 mg., m.p. 231.5–232.5°, reported[27] m.p. 227–228°, 1.62 atom % excess O^{18}, was obtained. The carbobenzoxy-β-cyano-L-alanine, precipitated from the acidified solution, and a second crop, obtained on lyophilization of the mother liquor to a small volume, were collected by filtration and were combined; wt. 43 mg., crude yield 70%, m.p. 128–129°. After having been thoroughly dried, this material was recrystallized twice from ethyl acetate–hexane; II′a, wt. 18 mg., m.p. 132–133.5°, 0.737 atom % excess O^{18}, calculated 1.47 atom % excess O^{18} per oxygen in carboxyl group, was obtained.

In separate experiments an attempt was made to evaluate the stability of DCC under the conditions used to isolate the dicyclohexylurea. Solutions of 55 mg. of DCC in pyridine were concentrated under vacuum either to dryness or to 0.5 ml., and 2-ml. volumes of water were added. Dicyclohexylcarbodiimide was recovered unchanged in both cases as a solid which melted at 34° and was soluble in cold methanol. There was no evidence of formation of the high melting, less soluble dicyclohexylurea. When 210 mg. of nonisotopic I in pyridine was treated with 171 mg. of DCC and the reaction mixture was cooled prior to collection of the dicyclohexylurea, the solid (3 mg.) obtained on addition of water to the residue left after removal of the pyridine amounted to only 1.7% of the total dicyclohexylurea obtained. A similar reaction mixture from which the bulk of the dicyclohexylurea had been separated by filtration without prior cooling yielded solid in a quantity which represented 4.6% of the total dicyclohexylurea isolated. Thus, in the dehydration reaction of labeled I, it is unlikely that a significant amount, if any, of nonisotopic dicyclohexylurea is formed when the pyridine solution, which may contain some unreacted DCC, is concentrated and the residue is diluted with unlabeled water.

Carbobenzoxy-β-cyano-DL-alanine.—A modification of the procedure described for dehydrating carbobenzoxy-L-asparagine with *p*-toluenesulfonyl chloride[21] was used. In our hands the latter procedure, which employed excess reagent, led with the L-isomer to dark insoluble tarry products. Trial experiments with purified tosyl chloride and anhydrous pyridine showed that a small (5%) excess of the chloride is sufficient and yields a satisfactory product but that with larger proportions unsatisfactory products and insoluble by-products are obtained.

Carbobenzoxy-DL-asparagine was prepared from commercial DL-asparagine as described for the L-isomer.[5] It was purified by two recrystallizations from methanol. It melted at 163.5–164°, approximately 2° higher than the L-isomer under the indicated conditions.[23] To a solution of 4.76 g. of carbobenzoxy-DL-asparagine in 25 ml. of dry distilled pyridine in a 100-ml. round bottom flask was added with magnetic stirring a solution of 3.6 g. of purified tosyl chloride in 5 ml. of pyridine in portions over 35 min. The temperature was maintained at 30–35° during the addition by cooling the reaction flask in a water bath. When addition was complete, the clear, almost colorless, solution was heated for 40 min. in an oil bath maintained at 50°. The solution was then allowed to cool to room temperature and was concentrated under high vacuum with a water bath at 30°. The viscous residue was dissolved in 20 ml. of water, and the solution was cooled to 0–5°. It was then adjusted to pH 1–2 by the slow addition of cold 6 *N* HCl. Cold water was added to the clear solution which brought about precipitation of a white solid. After 1 hr. in the cold the solid was collected and pressed dry on the filter. The solid was resuspended 3 times in cold water and was washed on the filter with cold water until the washings were chloride free. It was then dried under vacuum over P_2O_5; wt. 3.86 g. (87%), m.p. 106–108.5° dec. Recrystallization from ethyl acetate–petroleum ether with removal of a small first crop gave 81% recovery of product as mica-like plates which melted a little more sharply at 106.5–107.5° dec. The sample was recrystallized for analysis with no change in melting point.

Anal. Calcd. for $C_{12}H_{12}N_2O_4$: C, 58.1; H, 4.87; N, 11.3. Found: C, 57.9; H, 4.90; N, 11.2.

Samples recrystallized from dichloromethane have also given satisfactory analyses. Frequently the material crystallizes from this solvent in part as needles. The latter form is obtained more consistently when the compound is allowed to crystallize slowly from a solution cooled to 5°, m.p. 112.5–113.5° dec. After 2 recrystallizations of the latter from ethyl acetate–petroleum ether, with seeding with the lower melting material, it is reconverted to plates which melt at 106.5–107.5° dec.

Carbobenzoxy-β-cyano-DL-alanine Methyl Ester (XIb).—

(25) C. Ressler, *J. Biol. Chem.*, **237**, 733 (1962).

(26) M. Bergmann and L. Zervas, *Chem. Ber.*, **65**, 1192 (1932).

(27) A. F. McKay, W. R. R. Park, and S. J. Viron, *J. Am. Chem. Soc.*, **72**, 3659 (1950).

Carbobenzoxy-β-cyano-DL-alanine (7.5 g.) was converted to the ester with diazomethane as described for the L-isomer. The product separated from the reaction mixture as long needles after addition of petroleum ether and cooling; wt. 5.5 g. (69%), m.p. 82–83°. The ester was recrystallized from methanol, ether, and petroleum ether (12:20:80 parts per g.). The sample prepared for analysis was obtained from starting acid of analytical purity. It was dried under high vacuum at room temperature for 7 hr. and melted at 82–83°.

Anal. Calcd. for $C_{13}H_{14}N_2O_4$: C, 59.5; H, 5.38; N, 10.7. Found: C, 59.6; H, 5.22; N, 10.5.

Carbobenzoxy-β-cyano-DL-alanine-*carboxyl*-O^{18} (IIb).—Sodium, 360 mg., was added in portions to 8 ml. of methanol. The solution was then concentrated under vacuum to dryness, and the residue was dissolved in 1 ml. of H_2O^{18} with 10.1 atom %.[23] This solution was then added in small portions with magnetic stirring to a solution of 2.62 g. of XIb dissolved in a mixture of 0.98 ml. of H_2O^{18} with 10.1 atom % and 5.25 ml. of dioxane. The latter had been freshly refluxed and distilled over sodium. The hydrolysis was carried out and the product was isolated as described for the L-compound; wt. 2.24 g., m.p. 109.5–110.5° dec., with sintering at 107°. The material was recrystallized twice to constant m.p. by dissolving it in 130 ml. of warm dichloromethane. The mixture was filtered and the solution was concentrated under vacuum to 40 ml. and then cooled; wt. 1.5 g. (60.5%), m.p. 112.5–113.5° dec. The material was dried to constant weight under high vacuum and showed satisfactory elemental analyses for C and H. For reference, a small sample of the latter was recrystallized from ethyl acetate–petroleum ether as plates which melted at 107–108.5° dec. Hydrogenolysis followed by amino acid analysis showed in addition to the expected products 1.2% asparagine.

Carbobenzoxy-DL-asparagine-*carboxyl*-O^{18} (Ib).—To a cooled (ice bath) solution of 1.36 g. of carbobenzoxy-β-cyano-DL-alanine-*carboxyl*-O^{18} (IIb) in 3.1 ml. of 2 *N* NaOH solution was added dropwise with stirring 5.5 ml. of 30% H_2O_2. The temperature tended to rise as O_2 was evolved. After 3 hr. at room temperature the solution was cooled and acidified with 6 *N* HCl. The product was isolated as described for the L-compound except that it was kept 25 min. in the cold before filtration, after which it was resuspended in cold water to help free it from adherent chloride. It was dried under vacuum over P_2O_5; wt. 1.12 g., m.p. 162–164.5°. The product was dissolved in 20 ml. of hot methanol, and the solution was filtered and concentrated until crystallization started; wt. 850 mg., m.p. 163.5–164.5°. A second similar crystallization yielded 518 mg. (36%), m.p. 164–164.5°. A second crop was obtained from the combined mother liquors; wt. 410 mg., m.p. 161–163°, which, after 2 recrystallizations, weighed 265 mg. (total yield 54%) and melted at 163.5–164.5°. The material showed satisfactory elemental analyses for C, H, and N. Hydrogenolysis followed by amino acid analysis indicated a quantitative amount of asparagine; atom % excess O^{18} of Ib was 2.03, calculated 5.08 atom % excess O^{18} per oxygen in carboxyl group. The expected value for introduction of 1 atom of labeled oxygen and 1 atom of unlabeled oxygen into XIb is 2.02 atom % O^{18}, based on the concentration of 10.1 atom % for the H_2O^{18}.

Dehydration of Carbobenzoxy-DL-asparagine-*carboxyl*-O^{18} (Ib) with *p*-Toluenesulfonyl Chloride.—To a solution of 200 mg. of carbobenzoxy-DL-asparagine-*carboxyl*-O^{18} (Ib) in 1 ml. of dry pyridine was added a solution of 152 mg. of recrystallized tosyl chloride in 0.25 ml. of pyridine, in portions, with magnetic stirring over 10 min. The temperature was maintained at 26–30° during the addition. The reaction mixture was treated and was worked up as described for the nonisotopic compound with the exception that the white solid product obtained upon acidification was removed by four extractions with 10-ml. portions of ethyl acetate. The combined extracts were washed twice with 10-ml. portions of cold water and were dried over $MgSO_4$ and concentrated under vacuum to dryness. The viscous residue was dissolved in 2 ml. of dry dichloromethane, and petroleum ether (b.p. 30–60°) was added to incipient crystallization. After an overnight period at 5° an additional 5-ml. portion of petroleum ether was added. The crystalline carbobenzoxy-β-cyanoalanine was collected on the filter; wt. 149 mg., m.p. 105–106° dec. The product, 133 mg., was then treated with 1 ml. of ether, and a small amount of insoluble material was removed by filtration. The residue left on evaporation of the solvent was recrystallized twice from dichloromethane with seeding to give II''b, wt. 49 mg., m.p. 106–108° dec. Hydrogenolysis followed by amino acid analysis showed in addition to the expected products 5.8% asparagine. Atom % excess O^{18} of II''b was 1.43, calculated 2.86 atom % excess O^{18} per oxygen in carboxyl group.

Dehydration of Carbobenzoxy-DL-asparagine-*carboxyl*-O^{18} (Ib) with N,N'-Dicyclohexylcarbodiimide.—To a solution of 200 mg. of Ib in 1 ml. of dry pyridine was added a solution of 163 mg. (5% excess) of DCC in 0.5 ml. of pyridine at 16–18° in portions over 10 min., as described.[5] After 3 hr. at this temperature the mixture was cooled to 5°, and the dicyclohexylurea was collected by filtration and washed with 1 ml. of pyridine; wt. 168 mg. This material was recrystallized twice by dissolving it in 5 ml. of hot methanol and concentrating the solution to incipient crystallization; IIIb, wt. 143 mg., m.p. 232–233°, atom % excess O^{18} 3.44.

The filtrate was concentrated under high vacuum to 0.5 ml. and was then diluted with 2–3 ml. of cold water. After 1 hr. in the cold, the mixture was filtered, and the filtrate was cooled and acidified to pH 1 with 6 *N* HCl. The partly crystalline product was extracted four times with 10-ml. portions of ethyl acetate, and the extract was dried over $MgSO_4$ and concentrated under vacuum to dryness. The residue was dissolved in 2 ml. of dichloromethane, and an equal volume of petroleum ether was added. After several hours in the cold the white carbobenzoxy-β-cyano-DL-alanine was filtered off and dried under high vacuum; wt. 170 mg., m.p. 98.5–105° dec. The product, 129 mg., was dissolved in about 1 ml. of ether, and some insoluble material was removed by filtration. The residue left after evaporation of the ether was crystallized twice from dichloromethane with seeding to give II'b, wt. 45 mg., m.p. 106–108° dec. Hydrogenolysis followed by amino acid analysis showed in addition to the expected products 4.1% asparagine and 0.6% unidentified basic material which was eluted at 135 ml. Atom % excess O^{18} of II'b was 1.37, calculated 2.74 atom % excess O^{18} per oxygen in carboxyl group.

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