constant boiling (5.7 *N*) HCl for 24 hours. The amino acid content in the hydrolysates was determined by the method of Levy.[19] Since tryptophan is destroyed by acid hydrolysis, this amino acid was estimated from the ultraviolet absorption spectrum of the intact peptide according to the procedure of Goodwin and Morton.[31]

Paper Chromatography and Paper Electrophoresis.—Paper chromatography was carried out on Whatman No. 1 filter paper. The solvents used were *n*-butyl alcohol–acetic acid–water, 4:1:1 (BAW), and *sec*-butyl alcohol–10% NH_3, 85:15 (SBA). Zone electrophoresis on paper (Whatman 3 MM) was performed in a Spinco apparatus[32] for 8 hours at 200 volts with a collidine–acetic acid buffer of *p*H 7. Both paper electrophoresis and paper chromatography were conducted at room temperature. Color reactions were used to confirm the presence of histidine[33] and tryptophan.[34]

Enzymic Studies.—Crystalline α-chymotrypsin and trypsin were obtained from the Armour Laboratories; digestion of I and II with these enzymes was carried out at 25° for 24 hours in a solution of *p*H 9.0, with an enzyme–substrate ratio of $^1/_{100}$ (w./w.). A preparation of leucine aminopeptidase was kindly furnished by Drs. R. L. Hill and E. L. Smith; digestion was carried out at 25° for 8 hr. in a bicarbonate buffer of *p*H 8.8 containing 0.002 *M* $MgCl_2$, and with an enzyme–substrate ratio of $^1/_{100}$ (w./w.).

Assay of Melanocyte-stimulating Activity.[35]—The melanocyte-stimulating activity of the synthetic peptides was determined by the method described by Shizume, *et al.*,[21] with isolated skins of *Rana pipiens;* the unit of activity used is the same as that defined by these investigators. Bioassay was also carried out with hypophysectomized *Rana pipiens* (not more than 4 days after operation) as described by Hogben and Slome.[22]

Acknowledgment.—This work was initiated in the autumn of 1957 during a visit of C.H.L. to Basle, Switzerland. It is a pleasure to acknowledge the generous hospitality of Drs. A. Wettstein, R. Schwyzer and M. Brenner, and to thank these colleagues for many valuable discussions on general problems of peptide synthesis. This work was supported in part by grants from the National Institutes of Health of the U. S. Public Health Service (G 2907) and the Albert and Mary Lasker Foundation. E. S. wishes to thank the Conference Board of the Associated Research Councils for a Fulbright grant.

(31) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).
(32) F. G. Williams, Jr., E. G. Pickels and E. L. Durrum, *Science*, **121**, 829 (1955).
(33) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).
(34) I. Smith, *Nature*, **171**, 43 (1953).
(35) The authors wish to thank Barbara Solomon and C. W. Jordan, Jr., for their assistance in performing these assays.

BERKELEY 4, CALIF.

[CONTRIBUTION FROM THE FURMAN CHEMICAL LABORATORY, VANDERBILT UNIVERSITY, NASHVILLE, TENN.]

Resolution of N-Carbobenzoxy Amino Acids. Alanine, Phenylalanine and Tryptophan

BY LACY R. OVERBY[1] AND A. W. INGERSOLL

RECEIVED OCTOBER 5, 1959

Racemic N-carbobenzoxy derivatives of alanine, phenylalanine and tryptophan were resolved, respectively, with ephedrine, α-phenylethylamine and quinine. The resolution of N-carbobenzoxy-DL-alanine with inexpensive (−)-ephedrine provided what is regarded as the most convenient source of N-carbobenzoxy-D-alanine and of D-alanine. The reciprocal resolution of DL-ephedrine with N-carbobenzoxy-L-alanine easily provided (+)-ephedrine. These resolutions afford routes to all active forms of the acid and base. The resolutions of carbobenzoxyphenylalanine and tryptophan were less convenient and gave only moderate yields of pure isomers.

The carbobenzoxy derivatives of D- and L-amino acids are widely used for peptide synthesis because of their relatively simple preparation and the eventual easy removal of the carbobenzoxy group by reductive procedures or by the use of anhydrous acids. However, the requisite D-amino acids and many of the L-forms must now first be obtained through resolution of some other racemic derivative and subsequent hydrolysis to the free amino acid prior to conversion to the desired optically active carbobenzoxy derivative. A saving in time and material would be possible by preparation of the desired optically active derivatives by direct chemical resolution of carbobenzoxy-DL-amino acids. Because of the facile removal of the carbobenzoxy group, the method might also be of advantage as a source of free amino acids in instances where other resolutions are difficult or removal of acyl radicals from other types of derivatives is not satisfactory.

Several carbobenzoxy amino acids have been resolved through the asymmetric biosynthesis of the anilides.[2-6] Hunt and du Vigneaud[7] resolved the carbobenzoxy derivatives of β-amino-*n*-butyric acid and β-aminoisobutyric acid using (+)- and (−)-α-phenylethylamine.

In the present studies we sought to provide a broader test of the method with representative examples by preparing the pure D- and L-forms of carbobenzoxyalanine, carbobenzoxyphenylalanine and carbobenzoxytryptophan. Natural (−)-ephedrine forms a less soluble diastereoisomeric salt with N-carbobenzoxy-D-alanine, and this was obtained pure in high yields, providing an excellent source of D-alanine. Furthermore, DL-ephedrine was found to be easily resolved with active carbobenzoxyalanine. Therefore, starting with either N-carbobenzoxy-L-alanine or (−)-ephedrine and the corresponding racemic compounds, both active forms of each were easily produced by reciprocal resolutions. The process provides (+)-ephedrine, a rare and little studied compound.

N-Carbobenzoxy-DL-phenylalanine was resolved with α-phenylethylamine. Both active forms of

(1) Research Division, Abbott Laboratories, North Chicago, Ill.
(2) J. S. Fruton, G. W. Irving, Jr., and M. Bergmann, *J. Biol. Chem.*, **133**, 703 (1940).
(3) C. A. Dekker and J. S. Fruton, *ibid.*, **173**, 471 (1948).
(4) H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley and P. H. Lowy, *ibid.*, **176**, 1383 (1948); **184**, 529 (1950).
(5) H. T. Hanson and E. L. Smith, *ibid.*, **179**, 815 (1949).
(6) D. G. Doherty and E. A. Popenoe, Jr., *ibid.*, **189**, 447 (1951).
(7) M. Hunt and V. du Vigneaud, *ibid.*, **127**, 727 (1939).

the amine are available[8,9] and this allows the choice of producing either or both active forms of phenylalanine. Quinine gave a sparingly soluble salt with N-carbobenzoxy-D-tryptophan. Pure N-carbobenzoxy-L-tryptophan was not conveniently available from this resolution. The resolutions of phenylalanine and tryptophan required several crystallizations and gave only moderate yield of pure isomers. The methods are not as satisfactory as alternative procedures using acetyl derivatives.[10,11] In these cases resolutions of the carbobenzoxy derivatives would thus not be the method of choice for obtaining pure optically active amino acids.

Unsuccessful attempts were made to resolve N-carbobenzoxy-DL-alanine with fenchylamine, α-phenylethylamine and menthylamine. The following amines were unsuccessful in resolving N-carbobenzoxy-DL-tryptophan: α-phenylethylamine, ephedrine, brucine, cinchonine, fenchylamine and menthylamine. N-Carbobenzoxy-DL-phenylalanine was not resolved with fenchylamine.

Experimental[12]

Synthesis of N-Carbobenzoxy Derivatives.—The compounds were prepared by a generally applicable procedure. The amino acid was exactly neutralized with sodium hydroxide in 5 to 10 parts of water. The solution was cooled to 5° in an ice-bath and stirred mechanically. From dropping funnels one equivalent each of sodium hydroxide solution and benzylchloroformate in toluene[13] were added at rates to maintain a slightly basic solution and a temperature of 5–10°. The mixture was washed twice with ether and the carbobenzoxy derivative precipitated in the cold by a slight excess of hydrochloric acid. The crude product was crystallized from appropriate solvents.

a. **N-Carbobenzoxy-DL-alanine.**—The yield of crude material was almost 100%, m.p. 90–95°. It was recrystallized from benzene, giving a 93% yield in two crops, m.p. 113.5°, reported[14] m.p. 114–115°.

b. **N-Carbobenzoxy-L-alanine** was prepared from natural L-alanine. Upon acidifying the aqueous solution of the sodium salt an opalescent mixture was obtained. Fine needles formed overnight at 4°, yield 86%, m.p. 84–85°, $[\alpha]^{25}D$ −13.59° (*c* 4, glacial acetic acid) unchanged after recrystallization from benzene. Bergmann and Zervas[14] reported m.p. 84°, $[\alpha]^{25}D$ −14.3° (glacial acetic acid).

c. **N-Carbobenzoxy-DL-phenylalanine.**—The crude product was oily but soon solidified. After drying 24 hours in vacuum it could be powdered in a mortar; yield 96%, m.p. 102–103°. The yield was 90% after recrystallization from benzene, m.p. 102–103°, in agreement with literature values.[14]

d. **N-Carbobenzoxy-DL-tryptophan.**—After addition of all of the reagents the sodium salt of the carbobenzoxy derivative separated from the alkaline solution as a viscous mass. Acidification gave an oil which soon solidified and was treated as described above; yield 97%, m.p. 165° (not sharp). Recrystallizations were in general unsatisfactory, but it was crystallized conveniently from ethyl acetate; over-all yields 85–87%, m.p. 168–169°, literature[15] value 169–170°.

Resolution of N-Carbobenzoxy-DL-alanine. (a) Separation of Diastereoisomeric Salts with (−)-Ephedrine.—N-Carbobenzoxy-DL-alanine (11.7 g.) and (−)-ephedrine (8.7 g.) were dissolved in 75 cc. of warm ethyl acetate. Cooling at room temperature gave 9.4 g. of the less soluble diastereoisomeric salt of N-carbobenzoxy-D-alanine, $[\alpha]^{25}D$ −23.88° (*c* 2, methanol). This was recrystallized from 275 cc. of ethyl acetate, giving 8.3 g., $[\alpha]^{25}D$ −23.92° (*c* 2, methanol). Thus the first crystallization yielded (92%) a diastereoisomeric salt with maximum rotation. No more crystalline material was obtained from the filtrates containing the more soluble salt.

b. **N-Carbobenzoxy-D-alanine** was obtained from the less soluble salt after acid decomposition by a generally applicable procedure: The salt (7.0 g.) was dissolved in 100 cc. of water and neutralized with a slight excess of dilute hydrochloric acid. Crystallization at 4° yielded 3.7 g. of N-carbobenzoxy-D-alanine, m.p. 84–85°, $[\alpha]^{25}D$ +13.65° (*c* 4, glacial acetic acid). The melting point and rotation were not changed by recrystallization from benzene. The constants agree closely with those of the L-form.

The aqueous acid filtrate containing ephedrine was combined with other similar material for recovery of either the free base or the hydrochloride. The free base was extracted into ethyl acetate after neutralizing the solution with sodium hydroxide. Alternately, the acid solution was evaporated to dryness under reduced pressure and the residue washed with acetone to remove traces of carbobenzoxy derivatives. The ephedrine hydrochloride remained as a fine powder. The products could be reused without further purification.

c. **N-Carbobenzoxy-L-alanine** along with some of the racemic compound was obtained from the more soluble salts in the original ethyl acetate mother liquors. Evaporation of the ethyl acetate, followed by decomposition of the salts with hydrochloric acid as above, gave the partially resolved material, m.p. 83–84°, $[\alpha]^{25}D$ −10.4° (*c* 4, glacial acetic acid). Systematic recrystallization from benzene indicated that the DL-form was less soluble than the active form. The impure N-carbobenzoxy-L-alanine therefore could not be purified by fractional crystallization from benzene. However it was easily obtained in pure form through the diastereoisomeric salts of (+)-ephedrine, made available by resolution of DL-ephedrine with N-carbobenzoxy-L-alanine, as shown below.

d. **D-Alanine** was obtained by hydrogenolysis of the corresponding carbobenzoxy derivative by a generally applicable procedure. Five grams of N-carbobenzoxy-D-alanine was dissolved in 50 cc. of methanol containing 2 cc. of concentrated hydrochloric acid. About 0.2 g. of palladium black[16] was added. The mixture was stirred vigorously and hydrogen passed in at atmospheric pressure. Carbon dioxide was detected almost immediately in the exit gases. After 45 minutes the catalyst was removed by filtration, and the methanol solution evaporated to dryness under reduced pressure. The residue was dissolved in 95% ethanol and the free amino acid precipitated with a slight excess of ammonium hydroxide, and then recrystallized from aqueous ethanol. The recrystallized D-alanine had $[\alpha]^{25}D$ −14.23° (*c* 2, 6 *N* HCl).[17] Dunn and Rockland[18] reported $[\alpha]^{25}D$ +14.47° (*c* 10, 5.97 *N* HCl) for L-alanine.

Resolution of DL-Ephedrine.—N-Carbobenzoxy-L-alanine (11.1 g.), prepared by synthesis from L-alanine, and 8.2 g. of DL-ephedrine[19] were dissolved in 100 cc. of ethyl acetate by warming. Crystallization at room temperature gave 8.7 g., $[\alpha]^{25}D$ +21.04° (*c* 2, methanol). This was the essentially pure salt of (+)-ephedrine. It was recrystallized from 175 cc. of ethyl acetate and gave 7.2 g. (76% based on the racemic form taken), $[\alpha]^{25}D$ +23.32°, unchanged by further recrystallizations. The salt was decomposed with acid as described above, giving pure (+)-ephedrine hydrochloride, $[\alpha]^{25}D$ +34.59° (*c* 4, water), and N-carbobenzoxy-L-alanine; $[\alpha]^{25}D$ of −32.5 to −38.6° have been recorded for (−)-

(8) A. W. Ingersoll, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 506.

(9) H. D. DeWitt and A. W. Ingersoll, THIS JOURNAL **73**, 5782 (1951).

(10) L. R. Overby and A. W. Ingersoll, *ibid.*, **73**, 3363 (1951).

(11) L. R. Overby, *J. Org. Chem.*, **23**, 1393 (1958).

(12) Melting points are uncorrected and were determined in capillary tubes.

(13) H. E. Carter, R. L. Frank and H. W. Johnston, *Org. Syntheses*, **23**, 13 (1943).

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

(15) E. L. Smith, *J. Biol. Chem.*, **175**, 39 (1948).

(16) R. Willstätter and E. Waldschmidt-Leitz, *Ber.*, **54B**, 113 (1921).

(17) When one lot of commercial (−)-ephedrine was used for the resolution slightly impure D-alanine was obtained. Evidently this ephedrine contained some base that formed a sparingly soluble salt with the L- or DL-acid. This was not separated from the D-acid salt by recrystallization. The values reported here were for D-alanine obtained with (−)-ephedrine purified by recrystallization of the hydrochloride.

(18) M. S. Dunn and L. B. Rockland, *Advances in Protein Chem.*, **3**, 296 (1947).

(19) Merck and Co., Inc., Rahway, N. J.

ephedrine hydrochloride.[20] Most values for the pure material are near −34°.

The more soluble diastereoisomeric salts gave the pure alanine derivative and partially resolved (−)-ephedrine. The recovery of N-carbobenzoxy-L-alanine was almost quantitative, and this could be successively used to resolve more ephedrine. The (+)-ephedrine so obtained could be used for resolving carbobenzoxyalanine, giving more N-carbobenzoxy-L-alanine. Eventually large amounts could be built up.

Resolution of N-Carbobenzoxy-DL-phenylalanine. a. Separation of the Diastereoisomeric Salts with (−)-α-Phenylethylamine.—N-Carbobenzoxy-DL-phenylalanine (30 g.) and (−)-α-phenylethylamine (12.2 g.) were dissolved in 100 cc. of warm benzene. Crystallization at room temperature yielded 21 g., $[\alpha]^{25}D$ +17.75° (*c* 6.5, 95% ethanol). The theoretical amount of each diastereoisomeric salt was 21 g., but the salt did not consist solely of one form. Recrystallization from 95% ethanol gave 10.8 g., $[\alpha]^{25}D$ +27.50°, unchanged by further recrystallization. Systematic fractionation of the ethanol mother liquor gave an additional 2.2 g. of the pure salt of the L-acid, $[\alpha]^{25}D$ +27.91° (*c* 6.5, ethanol). Total recovery was 62% based on the amount taken in the racemic mixture.

b. N-Carbobenzoxy-L-phenylalanine and (−)-phenylethylamine were recovered from the less soluble salt by alkaline decomposition. The salt was dissolved in 25 cc. of warm 95% ethanol and poured into 25 cc. of water containing an exact equivalent of sodium hydroxide. The amine was extracted with benzene and the extracts dried with solid sodium hydroxide and distilled to recover the benzene and the amine. The aqueous ethanol layer containing the sodium salt of the carbobenzoxy derivative was evaporated to remove most of the ethanol, and the volume made to 50 cc. with water. A slight excess of hydrochloric acid was added with ice cooling. N-Carbobenzoxy-L-phenylalanine separated as an oil which solidified; yield 9.2 g. (100% based on the salt taken). It was recrystallized from freshly distilled xylene after filtering off traces of sodium chloride. The first crop of crystals was 7.5 g., m.p. 86–87°, $[\alpha]^{25}D$ nearly zero in acetone or methanol, +4.98° (*c* 4, glacial acetic acid), and −5.80° (*c* 4, *N* NaOH). These values were unchanged by recrystallizations from xylene. The recovery from xylene mother liquors was almost quantitative, but prolonged heating to remove solvent gave colored material. Bergmann and associates[21] prepared N-carbobenzoxy-L-phenylalanine from natural phenylalanine and reported $[\alpha]^{25}D$ +4.9° (glacial acetic acid) and m.p. 126–128°. Our recrystallized product showing m.p. 86–87° gave optically pure phenylalanine upon removal of the carbobenzoxy group.

c. N-Carbobenzoxy-D-phenylalanine mixed with some of the racemic compound and the (−)-amine were recovered from the more soluble salts present in the original benzene and ethanol liquors as described above. The partially resolved carbobenzoxy derivative (30 g.) and (+)-α-phenylethylamine (12.2 g.) were combined in 100 cc. of warm 95% ethanol. The estimated amounts of diastereoisomeric salts of the L-acid and D-acid were 29 and 13 g., respectively. Crystallization in three crops gave a total of 23 g. of solids and a sirupy residue. Recrystallization from 50 cc. of 95% ethanol gave 20.3 g., $[\alpha]^{25}D$ −27.63° (*c* 6.5, 95% ethanol). The salt was decomposed with alkali as above, giving N-carbobenzoxy-D-phenylalanine. After recrystallizing from xylene it had m.p. 86–87°, $[\alpha]^{25}D$ −4.90° (*c* 4, glacial acetic acid). The more soluble salts were decomposed and reserved for reworking with other similar material.

d. L-Phenylalanine and D-phenylalanine were prepared from the corresponding crude carbobenzoxy derivatives by hydrogenolysis as described above for alanine; $[\alpha]^{25}D$ −35.16 and +34.94° (*c* 1.7, water). The accepted value for L-phenylalanine is −35.1°.[18]

Resolution of N-Carbobenzoxy-DL-tryptophan. a. Separation of the Diastereoisomeric Salts with Quinine.—The racemic carbobenzoxy derivative (7.6 g.) and 7.6 g. of quinine were heated under reflux for 30 minutes with 100 cc. of acetone, an amount insufficient to dissolve all the salts. After cooling to room temperature the solid was filtered and air-dried; yield 12.2 g., $[\alpha]^{25}D$ −91.02° (*c* 2, methanol). The acetone liquor yielded no more solid. The crystals were twice recrystallized from isopropyl alcohol giving 4.9 g., $[\alpha]^{25}D$ −102.5° (*c* 2, methanol). This was the maximum rotation and represented a recovery of 65% of the less soluble diastereoisomer.

b. N-Carbobenzoxy-D-tryptophan was recovered from the less soluble salt by alkaline decomposition as described above for the salts of carbobenzoxyphenylalanine. The free quinine was removed by filtration and purified by precipitating with alkali from a decolorized solution of the hydrochloride. The aqueous alkaline filtrate from the decomposition was extracted once with chloroform to remove traces of quinine and then acidified with acetic acid. N-Carbobenzoxy-D-tryptophan precipitated as a gelatinous mass. It was filtered by suction and most of the associated water was removed by pressing the filter cake. The dry weight was 3.1 g. (theoretical 3.3 g.), m.p. 136–137°, $[\alpha]^{25}D$ +15.55° (*c* 5, 1 equiv. NaOH). Hanson and Smith[5] reported m.p. 124–126° and $[\alpha]^{29}D$ +15.4°.

c. D-Tryptophan, obtained from the carbobenzoxy derivative by the above-described hydrogenolysis, had $[\alpha]^{25}D$ +31.19° (*c* 1, water). The literature[18] value for L-tryptophan is −32.15°.

d. Impure N-carbobenzoxy-L-tryptophan was obtained from the more soluble quinine salts. It had $[\alpha]^{25}D$ −10.11° (*c* 5, 1 equiv. NaOH). No attempt was made to purify the N-carbobenzoxy-L-tryptophan contained in this material.

(20) T. A. Henry, "The Plant Alkaloids," Fourth Edition, The Blakiston Co., Philadelphia, Pa., 1949, p. 636.

(21) M. Bergmann, L. Zervas, H. Rinke and H. Schleich, *Z. physiol. Chem.*, **224**, 33 (1934).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND NUTRITION, CORNELL UNIVERSITY]

Enzymatically Active Products of Trypsin Autolysis

BY ELSIE WAINFAN AND GEORGE P. HESS

RECEIVED JUNE 1, 1959

The autolysis of trypsin under appropriate conditions has been found to result in enzyme molecules which dialyze through 18/32 Visking cellophane membranes and whose dialysis rate through the larger pore 20/32 membranes is more rapid than that of trypsin. The accumulation of the faster diffusing molecules in detectable amounts was found to be dependent on *p*H, trypsin concentration and initial proportion of native and denatured trypsin present in the reaction mixture. Under standardized conditions, the 50% escape time of trypsin through 20/32 membranes was found to be 90 hr., and that of the faster dialyzing enzyme approximately 10 hr. The latter enzyme is less stable at 25° than trypsin, as indicated by its presence to the extent of about 5% in dialysis experiments carried out at 0° and only 2% in identical experiments at 25°.

Introduction

The large size and complex structure of enzymes provide formidable obstacles to the understanding of the relationship between structure and enzymatic function. Recent work with pepsin,[1] ribonu-

(1) G. E. Perlmann, *Nature*, **173**, 406 (1954).