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.

(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).

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.

[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).

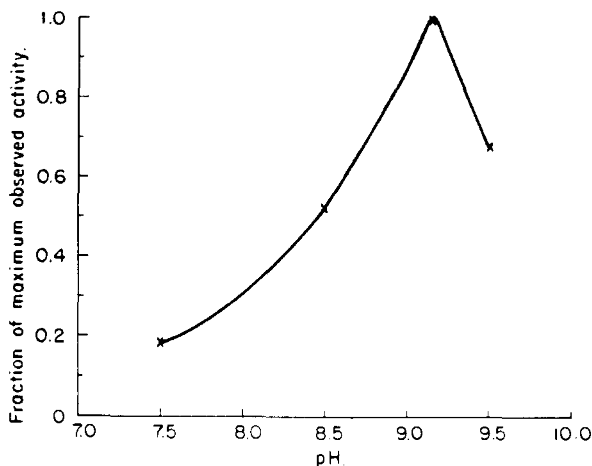

Fig. 1.—Effect of *p*H on dialysis of enzymatic activity through 18/32 Visking membranes. Trypsin I, 5 mg./ml. was dissolved in 0.1 *M* sodium phosphate buffer, *p*H 7.5, or in 0.05 *M* sodium borate buffers, *p*H 8.5, 9.1 or 9.5. The enzyme solutions were allowed to autolyze at 25° inside 18/32 Visking cellophane membranes. The enzymatic activity was assayed continuously for 20 hr. in the dialysate using the dye–fibrin substrate.

clease[2,3] and papain[4] have offered convincing evidence that at least in case of some enzymes a major reduction in size of the molecule is possible without a substantial loss in catalytic activity. It would be desirable, therefore, to investigate a great number of enzymes in the hope of obtaining a small molecule without a substantial loss of its catalytic properties. A simple method is needed which permits a rapid evaluation of the degradation experiments and which permits judgment as to whether a more complete investigation of the problem is worthwhile. The elegant dialysis method of Craig, *et al.*,[5,6] appears to provide such a procedure, since it has been demonstrated that the rates of escape of molecules through 18/32 and 20/32 cellophane membranes are correlated with the size and shape of the molecules and are quite reproducible under standardized conditions.

The application of this method to degradation studies, carried out with several different trypsin preparations, is described in this paper. A preliminary report of some of these experiments has appeared earlier.[7,8] Our previous conclusion that the total structure of trypsin is not required for catalytic activity has since been confirmed by Viswanatha, *et al.*[9]

Experimental

Materials.—Trypsin was purchased from the Worthington Biochemical Corporation, Freehold, New Jersey, as the $MgSO_4$ salt. Before use, the enzyme preparations were dialyzed at 4° against 0.001 *N* HCl until salt free and then lyophilized. The experiments reported in this paper were performed with once crystallized trypsin (Trypsin I), lots T526 and T582 used interchangeably and twice crystallized trypsin (Trypsin II) lot Tr571. Trypsin I has a low specific activity toward α-N-toluene-sulfonyl-L-arginine methyl ester (TAMe), k_0 = 1.1 m*M*. TAMe/min./mg. trypsin N. Trypsin II represents a highly purified trypsin preparation, with the same specific activity, k_0 = 1.9 m*M* TAMe/min./mg. trypsin N, as highly purified trypsin preparations used in other laboratories.[10,11]

Denaturation of trypsin II was carried out by heating a solution of 5 mg./ml. enzyme in 0.05 *M* sodium borate, *p*H 9.1, in a water-bath at 85–90° just long enough to start precipitation. The material remained finely dispersed. After the suspension had cooled to room temperature it was diluted with an equal volume of buffer containing 5 mg./ml. untreated trypsin II.

Analytical Methods.—Esterase activity was determined by the direct electrometric titration method of Schwert, *et al.*,[10] using 0.01 *M* TAMe as substrate in 15 ml. of 0.015 *M* phosphate buffer, *p*H 7.9 at 25°. Enzymatic activities are expressed as zero-order velocity constants (k_0) which were calculated from the initial slopes of the alkali uptake curves.

Proteolytic activity was measured by the azocasein[12] or the dye–fibrin method.[13] The dye–fibrin method has been standardized for a trypsin concentration of 0.4 to 2.0 μg. per assay solution. This method allowed a continuous determination of enzyme activity in the dialysate since the inactive dialyzable products do not interfere with the assay.

Protein concentration was estimated by means of either ultraviolet absorption,[14] total nitrogen determination[15] or the Copper–Folin method of Lowry, *et al.*[16] All methods were standardized against each other.

Free amino groups were measured by the photometric ninhydrin method of Moore and Stein.[17]

Diffusion Studies.—A modification of the method of Craig and co-workers[5,6] was used to obtain a large standard surface which results in reproducible dialysis rates. Both 18/32 and 20/32 Visking cellophane membranes purchased from the Visking Corporation, Chicago, Ill., were employed. The volumes of solution used were 4 ml. inside the membrane and 5 ml. outside.

Results

The effect of *p*H on the appearance of enzyme molecules which can diffuse through 18/32 Visking membranes is illustrated in Fig. 1. In experiments with trypsin I autolysates, the enzymatic activity in the dialysates was greater at *p*H 9.1 than at any other *p*H tested. At this *p*H the enzyme molecules diffusing through this membrane in 20 hr. corresponded to 20 μg. of trypsin. When experiments with trypsin II preparations were performed under identical conditions and at the same time, enzymatic activity was not detected in the dialysates.

The effect of initial enzyme concentration in the autolysis solution, on the appearance of enzyme molecules which can diffuse through 18/32 Visking membranes, is illustrated in Table I. In experiments with trypsin I autolysates, the enzymatic activity in the dialysates was two times greater when the initial enzyme concentration was 5 mg./ml. than when the initial concentration was either 2 or 10 mg./ml. The maximum observed activity in the dialysates corresponded to 40 μg. of trypsin.

(2) C. B. Anfinsen, W. F. Harrington, Aa. Hvidt, K. Linderstrom-Lang, M. Ottesen and J. Schellman, *Biochim. et Biophys. Acta*, **17**, 141 (1955).

(3) W. I. Rogers and G. Kalnitsky, *ibid.*, **23**, 525 (1957).

(4) R. L. Hill and E. L. Smith, *ibid.*, **19**, 376 (1956).

(5) L. C. Craig and T. P. King, This Journal, **77**, 6620 (1955).

(6) L. C. Craig, T. P. King and A. Stracher, *ibid.*, **79**, 3729 (1957).

(7) E. Wainfan and G. P. Hess, Abstracts, Am. Chem. Soc., 132nd Meeting, New York, Sept. 1957, p. 83c.

(8) G. P. Hess and E. Wainfan, This Journal, **80**, 501 (1958).

(9) T. Viswanatha, R. C. Wong and I. E. Liener, *Biochim. et Biophys. Acta*, **29**, 174 (1958).

(10) G. W. Schwert, H. Neurath, S. Kaufman and J. E. Snoke, *J. Biol. Chem.*, **172**, 221 (1948).

(11) J. I. Harris, *Nature*, **177**, 471 (1956).

(12) J. Charney and R. M. Tomarelli, *J. Biol. Chem.*, **171**, 501 (1947).

(13) G. P. Hess, E. I. Ciaccio and W. L. Nelson, *Federation Proc.*, **16**, 195 (1957).

(14) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).

(15) M. J. Johnson, *J. Biol. Chem.*, **137**, 575 (1941).

(16) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. L. Randall, *ibid.*, **193**, 265 (1951).

(17) S. Moore and W. H. Stein, *ibid.*, **176**, 367 (1948).

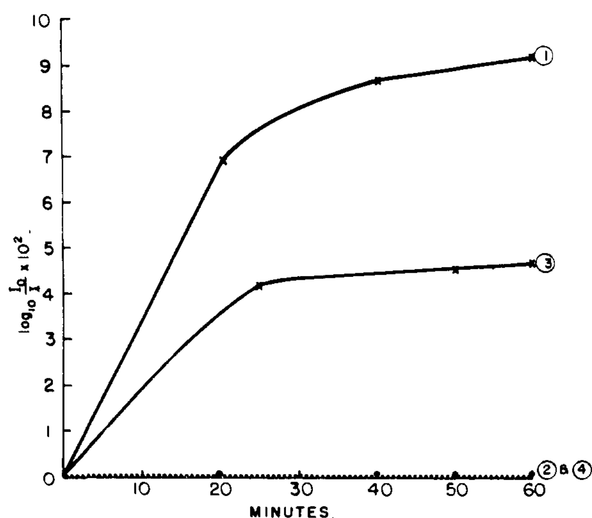

Fig. 2.—Dialysis of enzyme activity of various trypsin autolysates through 18/32 Visking cellophane membranes. The following solutions in 0.05 M sodium borate, pH 9.1 were used. 5 mg./ml. trypsin I, curve 1; 5 mg./ml. trypsin II, curve 2; 2.5 mg./ml. trypsin II, curve 2; 2.5 mg./ml. trypsin II plus 2.5 mg./ml. heat denatured trypsin II, curve 3; 2.5 mg./ml. trypsin II plus 2.5 mg./ml. casein, curve 4. The solutions were allowed to stand at 25° for 15 minutes and were subsequently transferred to 18/32 Visking cellophane bags. The dialysis was carried out at 37° and the enzymatic activity in the dialysate was assayed continuously for 60 minutes, using the dye–fibrin substrate.

When experiments with trypsin II preparations were performed under identical conditions and at the same time, no enzymatic activity was detected in the dialysates.

TABLE I

INFLUENCE OF INITIAL TRYPSIN CONCENTRATION ON DIALYSIS OF ENZYMATIC ACTIVITY THROUGH 18/32 VISKING MEMBRANES

Trypsin I solutions, containing 2, 5 or 10 mg. of enzyme per ml., were allowed to autolyze at 25° for 15 minutes in 0.05 M sodium borate buffer, pH 9.1, and were subsequently transferred to 18/32 Visking cellophane bags. The dialysis was carried out at 37° and the enzymatic activity in the dialysate was assayed continuously for 45 minutes using the dye–fibrin substrate.

Mg. enzyme per ml.	Fraction of max. obsd. activity
2	0.5
5	1.0
10	0.4

The effect of adding inactive protein to the autolysis reaction mixture, on the appearance of enzyme molecules which can diffuse through 18/32 Visking membranes, is illustrated in Fig. 2. Curves 1 and 2 represent control experiments. Dialysis of trypsin I autolysates (curve 1) again resulted in the appearance of enzyme activity in the dialysates during the 60 minute experiment. The enzyme activity in the dialysates corresponded to 25 μg. of trypsin. Curve 2 shows the results of experiments in which 5 mg./ml. or 2.5 mg./ml. of trypsin II was allowed to autolyze. In both cases, enzymatic activity could not be detected in the dialysates. When identical dialysis experiments were

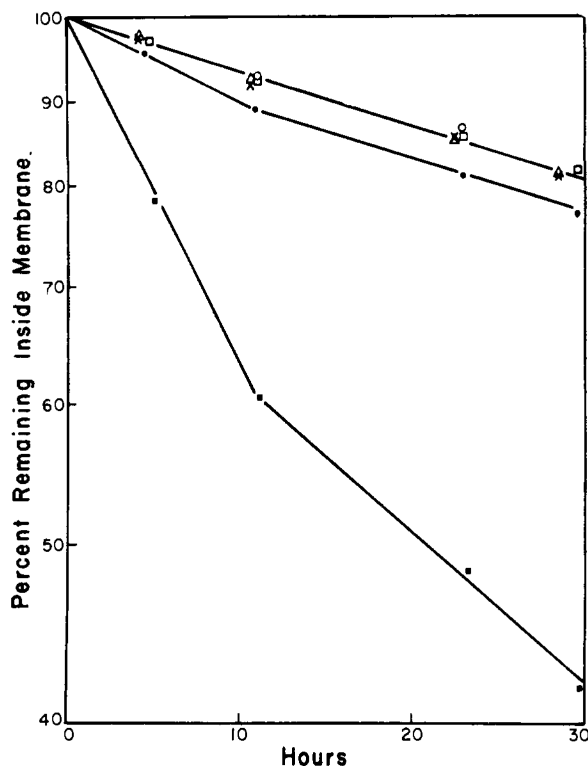

Fig. 3.—Dialysis of enzyme activity and protein nitrogen through 20/32 Visking membranes. O, trypsin I (enzyme activity); □, trypsin I (protein nitrogen); △, trypsin II (enzyme activity); ●, autolysate trypsin I (enzyme activity); ■, autolysate trypsin I (protein nitrogen); X, autolysate trypsin II (enzyme activity). Trypsin I and trypsin II solutions, 5 mg./ml., were allowed to autolyze for 100 minutes in 0.05 M sodium borate buffer, pH 9.1, 25°. The pH of the autolysates was then adjusted to pH 3.0 at 0° and the dialysis experiments were carried out at 25°. Final concentration of solution: 0.03 M sodium borate, 0.01 M acetic acid, 0.05 M calcium chloride. The dialysis experiments with trypsin I and trypsin II were carried out in a solution of identical composition, pH 3.0, 25°. Enzymatic activity was assayed using azo-casein as substrate.

performed with solutions containing 2.5 mg./ml. of trypsin II and 2.5 mg./ml. of heat denatured trypsin II (curve 3), enzymatic activity was found in the dialysates. If 2.5 mg./ml. of casein was substituted for denatured trypsin II (curve 4), dialysis of enzyme molecules could not be detected.

The escape curves of enzyme molecules through 20/32 Visking membranes, which are known to be permeable to trypsin,[6] are plotted in Fig. 3. The escape rates of trypsin I and trypsin II preparations were identical as judged by both determination of protein nitrogen and quantitative enzyme assay. The escape curve for enzyme molecules present in trypsin I autolysates gave a distinct break after about 10% of the enzymatic activity had dialyzed. The molecules associated with the second part of the escape curve dialyzed with the same rate as trypsin I and trypsin II and had a 50% escape time of approximately 90 hr. Subtracting this escape rate from the first part of the curve, one obtains a 50% escape time of about 10 hr. for the fast

moving component. The enzymatic activity of the component having a 50% escape time of 10 hr. was equivalent to 200 μg. of trypsin and represented 2% of the total activity in the autolysate. Parallel dialysis experiments of trypsin I autolysates through 18/32 Visking membranes at 0° revealed that an equivalent of 10 μg. of trypsin, 5% of the minor component of the trypsin I autolysate, dialyzed through these membranes in 12 hr.

Dialysis experiments with trypsin II autolysates through Visking 20/32 membranes at 25°, run simultaneously with the experiments described for trypsin I autolysates, revealed only a single component with an escape rate identical to the one found for trypsin I and trypsin II. Similarly, when trypsin II autolysates were allowed to dialyze through 18/32 Visking membranes at 0°, in experiments run side by side with those described for trypsin I autolysates, the dialysates were found to be free of enzymatic activity even after 24 hr.

Re-dialysis, through Visking 20/32 membranes, of the first 10% of material escaping through these membranes at 25° was not possible because of instability of this fraction. In dialysis experiments at 25°, the minor component represented 2% of the total activity, while in identical experiments at 0° it represented 6% of the total activity. Further evidence that this rapidly dialyzing component was less stable than trypsin was seen in experiments run at 37°. When autolysates of trypsin I were dialyzed through 18/32 membranes and assayed continuously at 37° with dye–fibrin as shown, for example, in Fig. 2, activity in the dialysates was seen to fall sharply and virtually disappeared in 1 hr. Trypsin, assayed under the same conditions of temperature, *p*H, buffer and substrate, still had over 60% of its initial activity present after the same time interval.

It can be seen in Fig. 3 that the escape curve for protein nitrogen in trypsin I autolysates gave a distinct break at about the same time that a break was observed in the escape curve for the enzyme molecules. After 10 hr. when the rate of diffusion of enzyme had returned to that of trypsin, the protein nitrogen was still dialyzing at a considerably faster rate. The number of free amino groups inside the membrane, 20 per 23,000 molecular weight, remained higher than that for the unautolyzed enzyme. Control experiments with trypsin I and trypsin II gave a value of 9 free amino groups per 23,000 molecular weight.

Discussion

The dialysis experiments with the 18/32 membranes provide a rapid means for studying the effectiveness of various autolysis conditions. The optimum conditions for the appearance of enzyme molecules which can diffuse through 18/32 Visking membranes were found to be autolysis of 5 mg./ml. of trypsin I at *p*H 9.1 and 25°. The simultaneous experiments in which trypsin II was autolyzed under identical conditions and which consistently yielded dialysates which did not contain measurable enzymatic activity, provided good internal controls. These experiments served to eliminate the effects of *p*H, temperature and buffer on the permeability of the membranes to trypsin, as possible causes for the appearance of enzyme in the dialysates.

The amount of enzyme found in the dialysates under optimal conditions corresponded to 20–40 μg. of trypsin, an amount 50–100 times higher than the minimum concentration which can be quantitatively determined by the dye–fibrin assay method.[13] This low concentration is partly due to the instability of the enzyme in the dialysates at 37°. The inactivation of the dialyzable enzyme was seen to proceed much faster than that of trypsin under the same conditions.

It is significant that the enzyme concentration in the dialysate was two and one-half times greater when the initial enzyme concentration in the autolysis reaction was 5 mg./ml. than when it was 10 mg./ml. Since the rate of diffusion of trypsin through 20/32 Visking membranes is independent of enzyme concentration in the range studied, increasing amounts of trypsin inside the 18/32 membranes should produce increasing amounts of enzyme in the dialysate, if the enzyme activity in the 18/32 dialysate represented native trypsin. This was not found to be the case.

The autolysis of mixtures of native and denatured trypsin II also resulted in the appearance of enzyme activity in the dialysates. This suggests that denatured trypsin competes successfully as substrate with the enzyme molecules which can dialyze through the 18/32 Visking membranes and that the presence of this competitive substrate is required to allow detectable amounts of dialyzable enzymatic activity to accumulate. These experiments and the following considerations suggest that trypsin I consists of a mixture of native and denatured protein.

The specific activity of trypsin I preparations is low. Dialysis experiments with trypsin I autolysates result in enzymatic activity in the dialysates, while dialysis experiments with trypsin II autolysates result in enzymatic activity in the dialysates only when denatured trypsin II is initially added to the reaction. Trypsin I activity, trypsin I protein nitrogen and trypsin II have the same escape rates through 20/32 Visking membranes. While the contamination of trypsin I by another enzyme is not excluded, its contribution to the enzyme activity in the dialysate can only be considered as an additional effect.

The experiments with the 18/32 Visking membranes, which are permeable to enzyme molecules present in trypsin I autolysates, but which are not permeable to enzyme molecules in trypsin II autolysates, include an unknown time factor. Since the effects of *p*H, buffer composition and temperature on the permeability of the 18/32 Visking membranes are not known, and trypsin itself is not very stable under the conditions of the experiment, it appeared possible that very minor alterations in structure of the enzyme present in the autolysates could produce the observed effect.

Semi-quantitative information about the escape rates and concentration of the enzyme present in the autolysates, however, can be obtained by the use of membranes permeable to trypsin.[6] The 50% escape time of trypsin I and trypsin II through

20/32 Visking membranes under the conditions used is the same, about 90 hours. At 25°, 2% of the enzyme present in trypsin I autolysates has a 50% escape time of about 10 hr. or about nine times faster than that for trypsin I or II under these conditions. The observation that the concentration of the enzyme with the faster escape rate was about three times higher when dialysis was carried out at 0° indicates that this molecule is considerably less stable at 25° than trypsin. In control experiments, trypsin was found to be stable for 30 hr. at 25° under the same experimental conditions.

It is significant that trypsin I autolysates, identical with those which reveal two enzymatically active components in dialysis experiments through 20/32 Visking membranes at 25°, contain an enzyme that can diffuse through 18/32 Visking membranes at 0°. Trypsin II autolysates which reveal only a single component in dialysis experiments with 20/32 Visking membranes at 25°, with an escape rate identical to that of trypsin, do not contain an enzyme that can dialyze through 18/32 membranes at 0°.

It is of interest to note that Wootton and Hess used the dialysis method of Craig and co-workers for the analysis of acetyltrypsin autolysates and concluded that, under their experimental conditions, an active degradation product of the enzyme does not accumulate in detectable amounts,[18] an observation which has recently been confirmed.[19] When the dialysis experiments of acetyltrypsin autolysates[18] and trypsin autolysates were followed by a determination of enzymatic activity, protein nitrogen, and free amino groups of the dialysates and the material remaining inside 20/32 Visking membranes, the results could be explained on the basis that acetyltrypsin is autolyzed by a cleavage of all susceptible amide bonds of one molecule at a time, while trypsin is autolyzed by an essentially random cleavage of amide bonds of all molecules present. Therefore, trypsin autolysis proceeds under conditions that would allow the accumulation of an enzyme molecule of altered structure, while the autolysis of acetyltrypsin does not.

(18) J. F. Wootton and G. P. Hess, *Biochim. et Biophys. Acta*, **29**, 435 (1958).

(19) I. E. Liener, *ibid.*, **30**, 252 (1958).

Craig and co-workers have determined the escape rates of a number of different peptides and proteins through 18/32 and 20/32 Visking membranes and concluded that, as in free diffusion, the molecular weight and shape of the molecules are the major factors which influence the escape rates of these molecules through membranes.[6,20] The data presented are therefore consistent with a difference in size and/or shape between trypsin and the fast moving enzymatic component in autolysates of trypsin I and in autolysates of mixtures of trypsin II and denatured trypsin II. The data also suggest that this fast moving enzymatically active component is considerably less stable than trypsin. This observation assigns a definite function to the amino acid residues of trypsin which are not directly involved in the active site of the enzyme, namely that of stabilizing the active site. Some modifications of this part of the molecule appear to be permissible.

Acknowledgments.—These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Cornell University, NR 108-417. The work was also supported in part by U. S. Public Health Service Grant 4842.

(20) L. C. Craig, W. Konigsberg, A. Stracher and T. P. King, in "Symposium on Protein Structure," A. Neuberger, Ed., John Wiley and Sons, Inc., New York, N. Y., 1958, pp. 104–115.

ITHACA, NEW YORK

COMMUNICATIONS TO THE EDITOR

MANY-MEMBERED CARBON RINGS. XXI. RESOLUTION OF [10]PARACYCLOPHANE-12-CARBOXYLIC ACID

Sir:

We wish to report evidence for restricted rotation about carbon–carbon single bonds in the carbocycle [10]paracyclophane. The *o*-carboxylic acid derivative of this hydrocarbon, [10]paracyclophane-12-carboxylic acid (I), has been resolved into pure optical antipodes through its cinchonidine salts. Isolation of enantiomorphs of the acid I thus comprises another cogent example of molecular dissymmetry arising from restricted rotation about single bonds.[1] In this particular instance the position of the *p*-decamethylene bridge must be restricted to regions above or below the faces of the benzene nucleus.

Enantiomorphs of
[10]Paracyclophane-12-carboxylic Acid (I)
(*p*-Decamethylenebenzene-*o*-carboxylic Acid)

The acid I, free of the isomeric [10-*m*]cyclophane-12-carboxylic acid,[2] was synthesized from authentic [10]paracyclophane[3] (n^{27}D 1.5332) *via* a

(1) (a) Lüttringhaus and H. Gralheer, *Ann.*, **557**, 108, 112 (1947); A. Lüttringhaus and G. Eyring, *Angew. Chem.*, **69**, 139 (1957); *Ann.*, **604**, 111 (1957).
(b) D. J. Cram and N. L. Allinger, THIS JOURNAL, **77**, 6289 (1958); D. J. Cram, R. J. Wechter and R. W. Kierstead, *ibid.*, **80**, 3126 (1955).

(2) A. T. Blomquist and F. Jaffe, *ibid.*, **80**, 3405 (1958).

(3) R. E. Stahl, Thesis, Cornell University, Ithaca, N. Y., 1954; D. J. Cram and H. U. Daeniker, THIS JOURNAL, **76**, 2743 (1954).