4991

Cotton effect, which we have presented here, is primarily of theoretical significance. It may be helpful to those who are working on the development of detailed theories for optical rotatory dispersion, for it provides, on a relatively simple structural skeleton, three phenomena which a comprehensive theory would have to be able to explain.

(1) The fact that a Cotton effect should be associated with the aromatic absorption band of L-tyrosine, whereas none is found associated with the  $260 \text{-m}\mu$  absorption band of L-phenylalanine, is itself not an obvious result.

(2) Changes in the ionization states of the amino and carboxyl groups have no marked influence, and even ionization of the phenolic group leads only to an enhancement of the effect and a shift to higher wave length, in parallel with the enhancement and red shift of the absorption peak which accompanies the same process.

(3) On the other hand, chemical modification of the carboxyl and amino groups, which leaves the absorption

spectrum virtually unchanged, has a pronounced effect on the optical rotation. Acetylation of the amino group completely eliminates the Cotton effect. Esterification or amidation of the carboxyl group diminishes the magnitude of the effect by a factor of five.

An additional feature of our results is that they serve as a further reminder that procedures for analyzing the optical rotatory dispersion of proteins and polypeptides, so as to get information about the structure of the polypeptide backbone,<sup>7,11,12</sup> cannot be refined without limit. All such procedures require that the observed rotation be associated solely with the peptide carbonyl and imino absorption bands. Any contribution from side-chain chromophores will interfere, as has been pointed out before by Würz and Haurowitz<sup>13</sup> in connection with their studies of the effects of disulfide bonds, and by Fasman, *et al.*<sup>9</sup>

(11) E. Shechter and E. R. Blout, Proc. Natl. Acad. Sci. U. S., 51, 695, 794 (1964).

(12) E. Shechter, J. P. Carver, and E. R. Blout, *ibid.*, **51**, 1029 (1964).
(13) H. Würz and F. Haurowitz, J. Am. Chem. Soc., **83**, 280 (1961).

[Contribution from the Biochemistry Department, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania]

# Studies on Polypeptides. XXVIII. Elimination of the Methionine Residue as an Essential Functional Unit for *in Vivo* Adrenocorticotropic Activity<sup>1-4</sup>

## BY KLAUS HOFMANN, JOACHIM ROSENTHALER, ROBERT D. WELLS, AND HARUAKI YAJIMA

RECEIVED JULY 10, 1964

A synthesis is described of the eicosapeptide amide seryltyrosylseryl- $\alpha$ -amino-*n*-butyrylglutaminylhistidylphenylalanylarginyltryptophylglycyllysylprolylvalylglycyllysylarginylarginylprolylvaline amide (18 L). Experimental evidence is presented regarding the stereochemical homogeneity of the synthetic peptide. This compound, an analog of corticotropin<sub>1-20</sub> amide with the methionine replaced by  $\alpha$ -amino-*n*-butyric acid, possesses *in vivo* adrenal ascorbic acid depleting steroidogenic and hypoglycemic activity and is effective *in vitro* in bringing about melanocyte stimulation and lipid mobilization. The peptide amide raises the plasma corticoid level in man. Although the  $\alpha$ -amino-*n*-butyric acid analog is less active than its methionine congener, these findings eliminate the methionine residue of corticotropin as "functionally" essential for biological activity. The protected tridecapeptide N-acetylseryltyrosylseryl- $\alpha$ -amino-*n*-butyrylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N<sup>¢</sup>-formyllysylprolylvaline amide (12 L) was prepared and found to possess *in vitro* melanocyte-expanding activity.

#### Introduction

Delineation of the "functionally" essential amino acid residues<sup>5</sup> in a biologically active peptide is a prerequisite for understanding of its mode of action at the molecular level. Unequivocal identification of such amino acid residues represents a difficult problem with a peptide as complex as ACTH which contains 39 amino acid residues derived from 16 different amino acids. However, discovery that synthetic subfragments containing less than one-half the polypeptide chain are the carriers of the physiological activity of this hormone has somewhat simplified the task. In this study the adrenocorticotropically highly effective corticotropin<sub>1-20</sub> amide<sup>6</sup> (Chart I) was selected for exploration of the "functional" importance of the methionine residue.

Selection of methionine stems from the observation that oxidation of corticotropin with hydrogen peroxide largely destroys its adrenal stimulating activity. Exposure of oxidized material to such thiol reagents as thioglycolic acid or cysteine fully restores biological function.<sup>7</sup> Further inquiry into this phenomenon implicated the methionine sulfur as the site where reversible oxidation-reduction takes place.<sup>8</sup> On the basis of this evidence one may conclude that the methionine sulfur is involved in the physiological function of ACTH. Claims to the effect that Raney nickel desulfurized corticotropin also exhibits the characteristic

<sup>(1)</sup> The authors wish to express their appreciation to the U. S. Public Health Service and the National Science Foundation for generous support of this investigation.

<sup>(2)</sup> The peptides and peptide derivatives mentioned in this communication (with exception of glycine) are of the L-configuration. In the interest of space conservation the customary L-designation for individual amino acid residues has been omitted.

<sup>(3)</sup> A preliminary communication (paper XXVII in this series) of some of the material presented in this paper has appeared: K. Hofmann, R. D. Wells, H. Yajima, and J. Rosenthaler, J. Am. Chem. Soc., 85, 1546 (1963).

<sup>(4)</sup> Some of the results have been presented at the Laurentian Hormone Conference, Sept. 5, 1961; see *Rec. Progr. Hormone Res.*, **18**, 41 (1962).

<sup>(5)</sup> See K. Hofmann, Brookhaven Symp. Biol., 13, 184 (1960), for definition of this term.

<sup>(6) (</sup>a) K. Hofmann, T. Liu, H. Yajima, N. Yanaihara, C. Yanaihara, and
J. L. Humes, J. Am. Chem. Soc., 84, 1054 (1962); (b) K. Hofmann, H.
Yajima, T. Liu, N. Yanaihara, C. Yanaihara, and J. L. Humes, *ibid.*, 84, 4481 (1962).

<sup>(7) (</sup>a) M. L. Dedman, T. H. Farmer, and C. J. O. R. Morris, *Biochem. J.*,
59, xii (1955); (b) H. B. F. Dixon, *Biochim. Biophys. Acta*, 18, 599 (1955);
(c) H. B. F. Dixon and M. P. Stack-Dunne, *Biochem. J.*, 61, 483 (1955);
(d) T. H. Farmer and C. J. O. R. Morris, *Nature*, 178, 1465 (1956).

<sup>(8)</sup> M. L. Dedman, T. H. Farmer, and C. J. O. R. Morris, *Biochem. J.*, 78, 348 (1961).



oxidation-reduction behavior of the genuine hormone could not be substantiated.<sup>9</sup> In order to test the essential nature of the methionine sulfur for activity we synthesized and biologically evaluated an analog of corticotropin<sub>1-20</sub> amide in which the methionine residue is replaced by a residue of  $\alpha$ -amino-*n*-butyric acid. The observation that this peptide amide exhibits a significant level of adrenocortical activity in the rat and in man unequivocally eliminates the methionine sulfur as a "functionally" essential unit of the ACTH molecule.

The synthesis of an analog (XIV) of the melanocyteexpanding hormone  $\alpha$ -MSH in which methionine is replaced by  $\alpha$ -amino-*n*-butyric acid, which contains glutamine in *lieu* of glutamic acid and whose lysine  $\epsilon$ amino group is formylated, is also described. The compound exhibits melanocyte-expanding activity.

The over-all scheme which was employed in the present study (see Chart II) was developed in our laboratory and was used in the preparation of  $corticotropin_{1-10}$ ,<sup>10</sup>  $corticotropin_{1-13}$ ,<sup>10</sup>  $corticotropin_{1-16}$ ,<sup>11</sup>  $corticotropin_{1-20}$ amide,<sup>6</sup> and corticotropin<sub>1-23</sub>.<sup>12</sup> In these syntheses we employed the acetyl group for protection of the N-terminal amino group and the formyl group for masking of the  $\epsilon$ -amino groups of lysine. In the present study these groups were replaced by the more readily removable tbutoxycarbonyl group first described by Carpino13 and introduced into the peptide field by McKay and Albertson<sup>14</sup> and by Anderson and McGregor.<sup>15</sup> Carpino showed as early as 1957<sup>13</sup> that this group can be removed smoothly from certain amino components by short exposure to trifluoroacetic acid. Several years later this technique was applied to peptide synthesis by Schwyzer, et al.<sup>16</sup> In the present study trifluoroacetic acid was used in the final deblocking step. Experiments reported in this paper were repeated several times with essentially identical results.

Synthesis of Protected Decapeptide XVII.—Seryl- $\alpha$ amino-*n*-butyrylglutamine (VII) was synthesized from glutamine (II) by a stepwise procedure outlined in Chart

(9) M. L. Dedman, T. H. Farmer, and C. J. O. R. Morris, *ibid.*, **66**, 166 (1957).

- (11) K. Hofmann, N. Yanaihara, S. Lande, and H. Yajima, *ibid.*, **84**, 4470 (1962).
- (12) (a) K. Hofmann, H. Yajima, N. Yanaihara, T. Liu, and S. Lande, *ibid.*, 83, 487 (1961); (b) K. Hofmann, H. Yajima, T. Liu, and N. Yanaihara, *ibid.*, 84, 4475 (1962).
- (13) L. A. Carpino, ibid., 79, 98 (1957).
- (14) F. C. McKay and N. F. Albertson, *ibid.*, 79, 4686 (1957).
- (15) G. W. Anderson and A. C. McGregor, *ibid.*, 79, 6180 (1957)
- (16) R. Schwyzer, W. Rittel, H. Kappeler, and B. Iselin, Angew. Chem., **72**, 915 (1960).

II. Benzyloxycarbonyl- $\alpha$ -amino-*n*-butyric acid (I), originally described by Waley,<sup>17</sup> was recently employed by du Vigneaud, et al.<sup>18</sup> in the synthesis of an analog of oxytocin. In the Experimental section we describe a procedure for synthesis of this compound which appears to afford superior yields. The optical rotation of our material agrees satisfactorily with the values reported by du Vigneaud but differs significantly from that given by Waley.<sup>17</sup> Preparation of t-butoxycarbonylseryltyrosylseryl- $\alpha$ -amino-*n*-butyrylglutamine (XV) was approached in two ways. In the first the tripeptide VII was acylated with benzyloxycarbonylseryltyrosine azide  $(IX)^{19}$  to give VIII which was decarbobenzoxylated by hydrogenolysis to the pentapeptide X. This was converted into the N-t-butoxycarbonyl derivative XV by treatment with t-butyl azidoformate. In the second route, preferable from an over-all yield standpoint, the tripeptide VII was acylated with t-butoxycarbonylservityrosine azide (XVI). However, purification of the final product prepared in this manner proved difficult. The protected pentapeptide (XV) was transformed into the azide (XVIII) which was coupled with histidylphenylalanylarginyltryptophylglycine (XIX)<sup>20</sup> to give the protected decapeptide XVII. Following purification by countercurrent distribution the material was obtained in the form of the monoacetate trihydrate by lyophilization. The protected decapeptide formed a single spot on paper chromatography in the Partridge system and its acid hydrolysate contained the constituent amino acids in the ratios predicted by theory.

Synthesis of the Protected Decapeptide Amide XXVI. —Hydrogenation of benzyloxycarbonylprolylvalylglycine methyl ester in presence of a palladium catalyst but in absence of acid afforded the free crystalline tripeptide ester XX which was coupled with N<sup> $\alpha$ </sup>-benzyloxycarbonyl-N<sup> $\epsilon$ </sup>-t-butoxycarbonyllysine (XXI)<sup>21</sup> to give N<sup> $\alpha$ </sup>-benzyloxycarbonyl-N<sup> $\epsilon$ </sup>-t-butoxycarbonyllysylprolylvalylglycine methyl ester (XXII) which was converted into XXIII by saponification. Methyl N<sup> $\alpha$ </sup>benzyloxycarbonyl-N<sup> $\epsilon$ </sup>-t-butoxycarbonyllysyltoxycarbonyllysinate was prepared via the mixed anhydride procedure as described previously for the corre-

- (18) V. du Vigneaud, G. S. Denning, Jr., S. Drabarek, and W. Y. Chan.,
  J. Biol. Chem., 239, 472 (1964).
  (19) K. Hofmann, T. A. Thompson, H. Yajima, E. T. Schwartz, and H.
- (19) K. Hofmann, I. A. Inompson, H. Yajima, E. I. Schwaltz, and H. Inouye, J. Am. Chem. Soc., 82, 3715 (1960).
- (20) (a) K. Hofmann, M. E. Woolner, G. Spühler, and E. T. Schwartz, *ibid.*, **80**, 1486 (1958); (b) K. Hofmann and S. Lande, *ibid.*, **83**, 2286 (1961).
  (21) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44**, 159 (1961).

<sup>(10)</sup> K. Hofmann and H. Yajima, J. Am. Chem. Soc., 83, 2289 (1961).

<sup>(17)</sup> S. G. Waley, Biochem. J., 68, 189 (1958).



sponding formyl derivative.<sup>22</sup> The physical properties of this compound agreed well with those recorded<sup>21</sup> for the same compound prepared by the use of dicyclohexylcarbodiimide.<sup>23</sup> Hydrogenation of the benzyloxycarbonyl dipeptide methyl ester gave the partially protected dipeptide ester XXIV which was coupled with XXIII to give the protected hexapeptide methyl ester XXV. During synthesis of benzyloxycarbonylnitroarginylprolylvaline amide, an intermediate in the preparation of arginylarginylprolylvaline amide triacetate tetrahydrate (XXVIII), according to our published procedure,6b formation of a by-product was occasionally observed which decreased the yield of the desired compound. This by-product, identified as benzyloxycarbonylnitroarginine amide, appears to arise from unreacted mixed anhydride during extraction of the reaction mixture with ammonium hydroxide. Substitution of sodium bicarbonate for ammonium hydroxide in this extraction step eliminates this difficulty.

The protected hexapeptide methyl ester XXV via the hydrazide was converted into the azide XXVII which was treated with the tetrapeptide amide XXVIII to give crude XXIX. Without isolation XXIX was hydrogenated in aqueous methanol containing acetic acid to give XXVI. The crude partially protected decapeptide amide was purified by chromatography on carboxymethylcellulose (CMC) using pH 6.8 ammonium acetate solutions of increasing ionic strength for elution. The homogeneous compound was obtained by lyophilization in the form of its triacetate hexahydrate.

Synthesis of Eicosapeptide Amide XXXI.-The protected decapeptide derivatives XVII and XXVI in the form of the hydrochlorides were coupled in dimethylformamide (DMF) solution by the use of N,N'-carbonyldiimidazole.<sup>24</sup> The homogeneous protected eicosapeptide amide XXX was isolated from the reaction products by chromatography on CMC. This material was deblocked by short exposure to trifluoroacetic acid. Trifluoroacetate ions were exchanged for acetate ions with Amberlite IRA-400 in the acetate cycle and the resulting crude acetate salt of XXXI was subjected to chromatography on CMC. The final product gave a single ninhydrin-, Pauly-, Sakaguchi-, and Ehrlichpositive spot on paper chromatography. The peptide formed a sharp single band when subjected to disk electrophoresis on polyacrylamide gel<sup>25</sup> at pH 4.6 (Fig. 1). Identification of peptides on the gel involves dyeing with such materials as Buffalo Black NBR. Although this dye appears to have good affinity for most proteins we find that it fails to stain certain peptides. Whereas arginylarginylprolylvaline amide and peptide XXXI accept the stain, such peptides as S-peptide,<sup>26</sup> phenylalanylglutamylarginylglutaminylhistidylmethionine,<sup>27</sup> and lysylglutamylthreonylalanylalanylalanyllysylphenylalanylglutamylarginylglutaminylhistidylmethionine<sup>27</sup> cannot be visualized. Thus, formation of a sharp band on gel electrophoresis does not constitute unequivocal proof for homogeneity. The acid hydrol-

(23) J. C. Sheehan and G. P. Hess, *ibid.*, 77, 1067 (1955).

(26) F. M. Richards, Proc. Natl. Acad. Sci. U. S., 44, 162 (1958)

ysate of peptide XXXI contained the constituent amino acids in the theoretically expected ratios. The eicosapeptide amide was digestible by leucine aminopeptidase (LAP), but as has been observed previously with similar peptides<sup>6b,12b,28</sup> recovery of amino acids from the N-terminal was higher than that from the Cterminal. The LAP digest contained glutamic acid and not glutamine. Based on this observation we concluded<sup>3</sup> that exposure to trifluoroacetic acid brings about deamidation of glutamine. An extensive study of the behavior of glutamine-containing peptides related to the amino acid sequence of S-peptide<sup>29</sup> failed to substantiate this previous conclusion. The present finding that Pronase digests of peptide XXXI contain glutamine and not glutamic acid proves that the peptide was not deamidated during deblocking. Deamidation of glutamine must have occurred during the LAP procedure.

Synthesis of  $\alpha$ -MSH Analog XIV.—For preparation of XIV the pentapeptide X was N-acetylated,<sup>19</sup> the Nacetyl derivative XI was converted via the methyl ester and hydrazide into the azide XII which was in turn reacted with the partially protected octapeptide amide XIII.<sup>22</sup> The reaction product XIV was purified by chromatography on CMC. The tridecapeptide amide XIV was obtained as a fluffy amorphous powder in the form of its diacetate heptahydrate. The compound formed a single ninhydrin-negative, Pauly- and Sakaguchi-positive spot on paper chromatography in the Partridge system. The amino acid ratios in an acid hydrolysate of the peptide derivative were those expected by theory.

### Discussion

Oxidation of the pigmentation hormone  $\beta$ -MSH<sup>30</sup> with hydrogen peroxide brings about a marked lowering but not a complete loss of biological function. We observed that exposure of N-acetylseryltyrosylserylmethionylglutaminylhistidylphenylalanylarginyltryptophylglycyl-N<sup>e</sup>-formyllysylprolylvaline amide to hydrogen peroxide decreases in vitro melanocyte-expanding activity from approximately  $10^{10}$  to  $10^7$  MSH units/g.<sup>4</sup> Studies with model peptides related to the sequence of this peptide demonstrated that the only recognizable change which occurred during the hydrogen peroxide treatment was conversion of the methionine residue to its sulfoxide.<sup>4</sup> It could thus be expected that the  $\alpha$ -MSH derivative XIV should exhibit the ability to expand melanocytes. Its activity (2  $\times$  10<sup>8</sup> MSH units/ g.) is of the same order of magnitude as that of ACTH but is only approximately 1% that of natural  $\alpha$ -MSH.<sup>31</sup> This result eliminates the methionine sulfur as an essential element for melanophoretic activity, a finding which is in complete agreement with the observation that a series of peptides structurally related to  $\alpha$ -MSH but not containing methionine have melanocyte-expanding activity.<sup>32</sup> In view of the previously demonstrated dependence of adrenocorticotropic activity of ACTH upon the presence of the intact thioether sulfur it was surprising to find that peptide XXXI in which methionine is replaced by  $\alpha$ -amino-*n*-butyric acid exhibits a significant level of biological potency. Assays of vari-

- (30) H. B. F. Dixon, Biochim. Biophys. Acta., 19, 392 (1956).
- (31) T. H. Lee and A. B. Lerner, J. Biol. Chem., 221, 943 (1956)
- (32) See K. Hofmann, Ann. Rev. Biochem., 31, 213 (1962), for a compilation of peptides with MSH activity.

<sup>(22)</sup> K. Hofmann, E. Stutz, G. Spühler, H. Yajima, and E. T. Schwartz, J. Am. Chem. Soc., 82, 3727 (1960).

<sup>(24)</sup> R. Paul and G. W. Anderson, ibid., 82, 4596 (1960).

<sup>(25)</sup> R. A. Reisfeld, U. J. Lewis, and D. E. Williams, Nature, 195, 281 (1962).

<sup>(27)</sup> K. Hofmann, F. Finn, W. Haas, M. J. Smithers, Y. Wolman, and N. Yanaihara, J. Am. Chem. Soc., 85, 833 (1963).

<sup>(28)</sup> W. F. White, *ibid.*, **77**, 4691 (1955).
(29) K. Hofmann, *et al.*, J. Am. Chem. Soc., in press

ous batches of peptide XXXI by the adrenal ascorbic acid depletion method ranged from  $31.2 \pm 4.2$  to 48.4 $\pm$  10.4 IU/mg. On subcutaneous administration potencies ranging from  $21.6 \pm 3.3$  to  $32.4 \pm 3.0$  IU/ mg.<sup>33</sup> were recorded. The melanocyte-expanding activity was  $1.6 \times 10^7$  MSH units/g.<sup>34</sup> Lebovitz<sup>35</sup> evaluated the steroidogenic activity of the aminobutyric analog XXXI in the rat against corticotropin  $A_1$  and found the compound to possess 74% the activity of this highly purified hormone on a weight basis. The in vitro adipokinetic and in vivo hypoglycemic effects of the aminobutyric acid analog were of a comparable order of magnitude. The plasma corticoid elevating activity in man (intravenous administration) of peptide XXXI corresponds to 30 IU/mg. Under oxidative conditions<sup>7</sup> which bring about practically complete deactivation of corticotropin the  $\alpha$ -amino-*n*-butyric acid analog showed insignificant diminution in potency. However, we have occasionally observed inactivation of XXXI under more drastic conditions of oxidation.<sup>36</sup> It is of considerable importance to note that  $corticotropin_{1-20}$  amide (Chart I), when assayed with corticotropin  $A_1$  as the standard, possesses 183% the in vivo steroidogenic activity of that hormone on a weight basis. Since the molecular weight (2443) of corticotropin<sub>1-20</sub> amide is approximately one-half that of corticotropin  $A_1$  (4567) it would appear that on a molar basis this synthetic corticotropin fragment is practically as active as the standard. In similar assays the adipokinetic and hypoglycemic activity of corticotropin<sub>1-20</sub> amide were of the same order of magnitude, *i.e.*, 150-200% that of corticotropin A1.35

The observation that different chemical modifications of the methionine residue in the functionally important section of the corticotropin molecule, *i.e.*, conversion of the sulfur to the sulfoxide or sulfone or replacement of the S-methyl moiety by a hydrogen atom, affect the biological properties of this molecule in a strikingly different manner is significant. This finding shows clearly that the oxidation-reduction behavior of a physiologically active peptide containing methionine may not provide valid information regarding the "functionally" essential nature of the methionine residue.

In this connection it is of interest to note that based on its oxidation-reduction behavior methionine has also been implicated as "functionally" essential for the biological activity of parathyroid hormone.<sup>37–39</sup>

Our findings suggest caution in accepting this conclusion.

(38) A. H. Tashjian, Jr., D. A. Ontjes, and P. L. Munson, Federation Proc., 22, 420 (1963).

(39) (a) A. H. Tashjian, Jr., and P. L. Munson, J. Chronic Diseases, 16, 269 (1963); (b) A. H. Tashjian, Jr., D. A. Ontjes, and P. L. Munson, Biochemistry, 3, 1175 (1964).



Fig. 1.—Disk electropherogram of peptide XXXI on polyacrylamide gel.

#### Experimental<sup>40</sup>

Benzyloxycarbonyl- $\alpha$ -amino-*n*-butyric Acid (I).—Benzyloxycarbonyl chloride (13.7 ml.) was added with stirring to an ice-cold solution of  $\alpha$ -amino-*n*-butyric acid [Calbiochem. Corp., Lot No. 610523;  $[\alpha]^{26}D + 20.6^{\circ}$  (c 4.0, 6 N HCl);  $R_{f}^{1}$  0.38;  $R_{f}^{2}$  0.11; containing minor ninhydrin-positive impurities] (10.0 g.) dissolved in 1 N sodium hydroxide (110 ml.). Stirring was continued for 2 hr. at ice-bath temperature when the solution was extracted with three portions of ether. The aqueous phase was acidified to Congo red with 2 N hydrochloric acid, the product was extracted into ether, and the extract was washed with three 50-ml. portions of 1 N hydrochloric acid and with several portions of water. Evaporation of the dried ether solution (Na<sub>2</sub>-SO<sub>4</sub>) gave an oil which crystallized on standing under petroleum ether (b.p. 30-60°); yield 21.3 g. (80%), m.p. 77-78°; [α]<sup>29</sup>D 10.3° (c 0.78, absolute ethanol); lit.<sup>18</sup>  $[\alpha]^{19.5}$ D -10.5° in absolute ethanol.

Anal. Calcd. for  $C_{12}H_{15}O_4N$ : C, 60.7; H, 6.4; N, 5.9. Found: C, 61.1; H, 6.5; N, 6.0.

Benzyloxycarbonyl- $\alpha$ -amino-*n*-butyrylglutamine (III).—A mixed anhydride, prepared in the usual manner from benzyloxycarbonyl- $\alpha$ -amino-*n*-butyric acid (4.74 g.) in freezing dry dioxane (20 ml.) with tri-n-butylamine (4.76 ml.) and ethyl chloroformate (1.92 ml.), was added slowly with stirring to a chilled solution of glutamine (II, 2.92 g.) and triethylamine (2.78 ml.) in water (20 ml.). The mixture was stirred in an ice bath for 1.5 hr. when the bulk of the dioxane was removed in vacuo at a bath temperature of 40–50°. The residue was acidified to Congo red with 1 N hydrochloric acid and the organic material was extracted with three 100-ml. portions of ethyl acetate. The organic layers were reextracted with three 50-ml. portions of 1 N hydrochloric acid followed by several portions of water and were dried over sodium sulfate. Evaporation of the ethyl acetate gave a gelatinous product which was collected, dried, and recrystallized from a mixture of methanol and ether; yield 3.47 g. (47%), m.p. 185-187°;  $[\alpha]^{29}D + 4.22^{\circ} (c \ 0.31, DMF); [\alpha]^{29}D - 14.5^{\circ} (c \ 0.51, 95\%)$ ethanol);  $[\alpha]^{27}D - 20.6^{\circ} (c 0.71, 3\% \text{ NH}_4\text{OH})$ ; ninhydrin negative. Anal. Calcd. for C<sub>17</sub>H<sub>23</sub>O<sub>6</sub>N<sub>3</sub>: C, 55.9; H, 6.3; N, 11.5.

Found: C, 55.8; H, 6.4; N, 11.6.

 $\alpha$ -Amino-*n*-butyrylglutamine (IV).—The above benzyloxycarbonyl derivative (8.07 g.) in methanol (230 ml.) containing glacial acetic acid (0.7 ml.) was hydrogenated over a palladium catalyst in the usual manner. Peptide which precipitated in the course of the reaction was redissolved by addition of small portions of water. The catalyst was removed by filtration, the filtrate evaporated to dryness *in vacuo*, and the residue dissolved in 1% ammonium hydroxide. The solution was filtered, the pH adjusted to about 6 by addition of glacial acetic acid, and ethanol (200 ml.) was added to precipitate the peptide which was collected and dried; yield 4.68 g. (92%), m.p. 233–234° dec.;  $[\alpha]^{29}p + 24.7°$  (c 0.39, water);  $[\alpha]^{26}p + 19.4°$  (c 0.62, 10% NH<sub>4</sub>-OH); single ninhydrin-positive spot with  $R_{\rm f}^1$  0.30.

Anal. Caled. for  $C_9H_{17}O_4N_3$ : C, 46.7; H, 7.4; N, 18.2. Found: C, 46.9; H, 7.5; N, 18.1.

<sup>(33)</sup> Ascorbic acid depleting activity was determined in 24-hr. hypophysectomized rats according to the method of "U. S. Pharmacopeia," Vol. XV, against the U.S.P. reference standard. We are much indebted to Dr. Joseph D. Fisher of Armour Pharmaceutical Company, Kankakee, Ill., for the biological assays.

<sup>(34)</sup> We wish to express our thanks to Dr. A. B. Lerner of the Department of Medicine, Yale University School of Medicine, for the MSH assays which were performed according to the method of K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).

 $<sup>(35)\,</sup>$  We thank Dr. Harold E. Lebovitz and the late Frank Engel for these results which will be published in detail elsewhere.

<sup>(36)</sup> H. A. Boright, F. L. Engel, H. E. Lebovitz, J. L. Kostyo, and J. E. White, Jr., *Biochem. J.*, **83**, 95 (1962).

<sup>(37)</sup> H. Rasmussen and L. C. Craig, Rec. Progr. Hormone Res., 18, 269 (1962).

<sup>(40)</sup> See ref. 6b for analytical procedures and general experimental methods. The chlorine test was carried out as described by J. Barrollier, Naturwiss., **48**, 554 (1961).  $R_{\rm f}^1$  refers to the Partridge system [S. M. Partridge, Biochem. J., **42**, 238 (1948)];  $R_{\rm f}^2$  refers to the 2-butanol-ammonia system [J. F. Roland, Jr., and A. M. Gross, Anal. Chem., **26**, 502 (1954)];  $R_{\rm f}^2$  refers to the pyridine system [S. G. Waley and J. Watson, Biochem. J., **55**, 328 (1953)]. With the latter system  $R_{\rm f}$  values are expressed as multiples of the distance traveled by a histidine marker. The following abbreviations are used: But =  $\alpha$ -amino-n-butyric acid; *t*-Boc = *t*-butoxycarbonyl; Z = benzyloxycarbonyl; Ac = acetyl; F = formyl; Gluta = glutamine; DMF = dimethylformamide.

Methyl t-Butoxycarbonylseryltyrosinate.-t-Butoxycarbonylserine hydrazide<sup>41</sup> (2.2 g.) was dissolved in 1 N hydrochloric acid (20 ml.) containing sodium chloride (2 g.) at a temperature of  $-10^{\circ}$  and a precooled  $(-10^{\circ}) 2 N$  solution of sodium nitrite (6) ml.) was added slowly with cooling. The precipitated oily azide was extracted with three 30-ml. portions of precooled  $(-15^{\circ})$  ethyl acetate. The organic layers were washed with ice-cold 1 N sodium bicarbonate and water and dried at  $0^{\circ}$  over sodium sulfate. The solution was concentrated to a volume of approximately 30 ml. in vacuo at room temperature<sup>42</sup> and an icecold solution of methyl tyrosinate (prepared from 2.5 g. of the hydrochloride) in tetrahydrofuran (20 ml.) was added to the concentrate. The mixture was kept at 5° for 24 hr., the solvents were removed in vacuo, and the residue was dissolved in ethyl acetate. The solution was washed with ice-cold 2 N aqueous citric acid solution, saturated sodium bicarbonate, and water and dried over sodium sulfate. The solvent was removed and the residue crystallized on addition of ether; yield 2.2 g. (53%), m.p. 117-119°;  $[\alpha]^{28}D = -13.3^{\circ}$  (c 0.97, methanol);  $R_{t}^{1}$  0.94, single ninhydrin-negative, Pauly-positive spot.

Anal. Calcd. for  $C_{18}H_{26}O_7N_2$ : C, 56.5; H, 6.9; N, 7.3. Found: C, 56.6; H, 7.1; N, 7.6.

t-Butoxycarbonylseryltyrosine Hydrazide Monohydrate.-Hydrazine hydrate (1.6 ml.) was added to a solution of methyl tbutoxycarbonylseryltyrosinate (1.4 g.) in methanol (15 ml.) and the mixture was kept at room temperature for 14 hr. The solid hydrazide which had precipitated was collected and dried over sulfuric acid in vacuo. For purification the material was recrystallized from water; yield 1.2 g. (82%), m.p. 199–200° dec.;  $[\alpha]^{28}$ D -18.6° (c 1.20, 50% v./v. aqueous acetic acid).

Anal. Calcd. for C17H26O6N4 H2O: C, 51.0; H, 7.0; N, 14.0; O, 28.0. Found: C, 51.1; H, 7.2; N, 14.1; O, 28.0.

Benzyloxycarbonylseryl- $\alpha$ -amino-n-butyrylglutamine  $(\mathbf{VI})$ .-An ethereal solution (approximately 100 ml.) containing benzyloxycarbonylserine azide (V, prepared from 5.06 g. of the hydrazide)48 was shaken for 48 hr. at a temperature of 1-5° with a solution of  $\alpha$ -amino-*n*-butyrylglutamine (IV, 2.31 g.) in water (100 ml.) and triethylamine (1.39 ml.). The aqueous phase was then separated from the ether layer and was extracted with ethyl acetate. The aqueous layer was evaporated to dryness in vacuo, the residue was dissolved in 75 ml. of 1-butanol (equilibrated with 5% acetic acid), and the solution was extracted, in countercurrent fashion, with six 10-ml. portions of 5% aqueous acetic acid. Evaporation of the butanol phases gave a solid residue (2.60 g.)which was recrystallized from 50% aqueous methanol; yield 2.12 g. (47%), m.p. 186–187° dec;  $[\alpha]^{29}D = -36.2°$  (c 0.27, 3% NH<sub>4</sub>OH);  $R_{f}^{1}$  0.77; chlorine positive, ninhydrin negative. Anal. Calcd. for C<sub>20</sub>H<sub>28</sub>O<sub>8</sub>N<sub>4</sub>: C, 53.1; H, 6.2; N, 12.4.

Found: C, 52.8; H, 6.2; N, 12.4.

Seryl-a-amino-n-butyrylglutamine (VII).-The benzyloxycarbonyl derivative (VI, 3.74 g.) was hydrogenated over a palladium catalyst in 80% aqueous methanol (100 ml.) containing 3 ml. of 10% acetic acid. Water was added to dissolve precipitated peptide, the catalyst was removed by filtration, the filtrate was evaporated to a small volume in vacuo, and the pH was adjusted to approximately 6.0 by addition of ammonium hydroxide. Addition of ethanol (approximately five times the volume of the aqueous phase) precipitated the peptide. The suspension was kept in a refrigerator overnight when the peptide was collected and recrystallized from aqueous ethanol; yield 2.22 g. (84%), m.p. 245–246° dec.;  $[\alpha]^{27}D = -29.2^{\circ}$  (c 0.27, water);  $R_{\rm f}^1 = 0.20$ ; amino acid ratios in acid hydrolysate, ser0.98glu0.97but1.05; amino acid ratios in LAP digest, ser + gluta<sub>1.56</sub> but<sub>1.00</sub> (100%).

Anal. Calcd. for  $C_{12}H_{22}O_6N_4$ : C, 45.3; H, 7.0; N, 17.6. Found: C, 45.4; H, 7.4; N, 17.3.

Benzyloxycarbonylservltyrosylservl- $\alpha$ -amino-n-butyrylglutamine Hydrate (VIII).-Benzyloxycarbonylseryltyrosine azide19 (IX, prepared from 1.3 g. of the hydrazide) was added to an icecold solution of seryl- $\alpha$ -amino-*n*-butyrylglutamine (VII, 0.50 g.) in water (3 ml.), triethylamine (0.22 ml.), and DMF (10 ml.), and the mixture was kept at 3° for 3 days. The solvents were removed in vacuo, the residue was dissolved in 3% ammonium hydroxide (slight warming required), and the solution was extracted with three 50-ml. portions of ethyl acetate. The organic

layers were discarded and the aqueous phase was acidified, evaporated to dryness in vacuo, and the residue dissolved in 1-butanol (equilibrated with 5% acetic acid). The butanol solution was then extracted with six 20-ml. portions of 5% acetic acid and evaporated to dryness in vacuo. The residue was crystallized from 80% aqueous ethanol; yield 0.45 g. (40%), m.p. 190–194°;  $[\alpha]^{28}$ D -20.3° (c 0.58, glacial acetic acid); ninhydrin-negative, tyrosine-positive spot;  $R_{\rm f}$  0.74; amino acid, ratios in acid hydrolysate, ser2.00tyr0.70but1.01glu0.97.

Anal. Calcd. for  $C_{32}H_{42}O_{12}N_6$  H<sub>2</sub>O: C, 53.3; H, 6.2; N, 11.7; O, 28.9. Found: C, 53.1; H, 6.7; N, 11.7; O, 28.9.

Seryltyrosylseryl- $\alpha$ -amino-*n*-butyrylglutamine Trihydrate (X). The above benzyloxycarbonyl derivative (VIII, 1.10 g.) was hydrogenated in the usual manner over palladium in 50% v./v. aqueous methanol (60 ml.) containing 2 ml. of glacial acetic acid. Water (30 ml.) was added at the conclusion of the hydrogenation to dissolve precipitated peptide and the catalyst was removed by filtration. The filtrate was evaporated to dryness in vacuo, the residue dissolved in water (30 ml.), and the peptide precipitated from the filtered solution by addition of absolute ethanol (approximately 90 ml.). The product was collected and dried in vacuo over phosphorus pentoxide; yield 0.81 g. (85%), m.p. 241-242° dec.;  $[\alpha]^{28}D = -27.3°$  (c 0.21, 2 N hydrochloric acid);  $R_{\rm f}$  0.27, single ninhydrin- and tyrosine-positive spot; amino acid ratios in acid hydrolysate, ser1.98tyr0.95but1.10glu0.95; amino acid ratios in LAP digest, ser<sub>1.94</sub>tyr<sub>1.02</sub>but<sub>1.04</sub>gluta<sub>1.02</sub>glu<sub>0.14</sub>

Anal. Calcd. for  $C_{24}H_{36}O_{10}N_6\cdot 3H_2O$ : C, 46.3; H, 6.8; N, 13.5; O, 33.4. Found: C, 46.1; H, 6.9; N, 13.7; O, 33.4.

N-Acetylservltyrosylservl- $\alpha$ -amino-n-butyrylglutamine Dihydrate (XI).—Acetic anhydride (0.4 ml.) was added in three portions over a period of 45 min. to an ice-cold solution of servityrosylseryl- $\alpha$ -amino-*n*-butyrylglutamine (X, 0.77 g.) in water (20 ml.) and sodium bicarbonate (0.70 g.) and the mixture was stirred for 90 min. following the addition. The solution was acidified with glacial acetic acid and was evaporated to dryness in vacuo. The residue was dissolved in ice-cold 1 N sodium hydroxide (7 ml.) and the solution was kept at ice-bath temperature for 40 min. after which time it was acidified to Congo red with 1 N hydrochloric acid. The precipitate was collected and recrystallized from 50% aqueous ethanol; yield 0.57 g. (71%), m.p. 206–209°;  $[\alpha]^{28}$ D - 17.2° (c 0.487, DMF);  $R_{i}^{1}$  0.57, single tyrosine-positive, ninhydrin-negative spot; amino acid ratios in acid hydrolysate, ser1.98glu1.00tyr0.88but1.12.

Anal. Calcd. for  $C_{26}H_{38}O_{11}N_6 \cdot 2H_2O$ : C, 48.3; H, 6.5; N, 13.0; O, 32.2. Found: C, 48.5; H, 6.7; N, 13.1; O, 32.1.

N-Acetylseryltyrosylseryl-a-amino-n-butyrylglutamine Hydrazide Hydrate.-An ether solution of diazomethane was added to a solution of the N-acetylpentapeptide XI (1.04 g.) in DMF (20 ml.) and methanol (10 ml.) until the yellow color remained, and the mixture was kept at ice-bath temperature for 5 min. The excess of diazomethane was destroyed by addition of a drop of glacial acetic acid, the solution was evaporated to dryness in vacuo, and the residue was dissolved in boiling methanol (100 ml.). Hydrazine hydrate (1 ml.) was added to the hot solution and the mixture was kept at room temperature for 24 hr. when approximately one-half the volume of methanol was evaporated. The resulting gelatinous hydrazide was collected and dried over sulfuric acid in vacuo; yield 0.99 g. (96%), m.p. 208-210°; a sample for analysis was recrystallized from aqueous methanol; m.p. 218-220°.

Anal. Calcd. for  $C_{26}H_{40}O_{10}N_8 \cdot H_2O$ : C, 48.6; H, 6.6; N, 17.4; O, 27.4. Found: C, 48.4; H, 6.6; N 17.4; O, 27.1.

 $N-Acetylseryltyrosylseryl-\alpha-amino-n-butyrylglutaminylhistidyl$ phenylalanylarginyltryptophylglycyl-N -formyllysylprolylvaline Amide Diacetate Heptahydrate (XIV).-N-Acetylseryltyrosylseryl- $\alpha$ -amino-*n*-butyrylglutamine azide (XII) prepared from 62 mg. of the hydrazide in the manner previously described was added to an ice-cold solution of histidylphenylalanylarginyl tryptophylglycyl-Ne-formyllysylprolylvaline amide22 (XIII, 59 mg.) in dimethylformamide (0.4 ml.) containing 0.07 ml. of triethylamine, the solution was kept at 0° for 48 hr., and water (70 ml.) was added. This solution was added to a CMC column  $(1.5 \times 15 \text{ cm.})$  which was successively eluted with the following pH 6.8 ammonium acetate buffers: 0.01 M (250 ml.); 0.02 M (150 ml.); 0.025 M (1000 ml.), and finally 0.03 M (500 ml.). Individual fractions approximately 10 ml. each were collected and the desired material located by its absorbancy at 280 m $\mu$ . The 0.025 M eluates which contained the desired material were pooled, evaporated to a small volume in vacuo, and finally lyo-

<sup>(41)</sup> B. Iselin and R. Schwyzer, Helv. Chim. Acta., 44, 169 (1961)

<sup>(42)</sup> Prolonged exposure of this azide to temperatures above  $20^{\circ}$  brings about conversion to 4-(t-butoxycarbonylamino)oxazolidone-2; m.p. 188-190°; [ $\alpha$ ]<sup>28</sup>D -109.2° (c 1.21, methanol).

<sup>(43)</sup> J. S. Fruton, J. Biol. Chem., 146, 463 (1942).

philized to constant weight giving a fluffy colorless powder; yield 63 mg. (70%);  $[\alpha]^{29}D - 51.1^{\circ}$  (c 0.23, 10% acetic acid);  $R_{l}^{1}$  0.42; ninhydrin negative, Pauly positive; amino acid ratios in acid hydrolysate, ser<sub>2.00</sub>tyr<sub>0.89</sub>but<sub>1.00</sub>glu<sub>0.97</sub>his<sub>1.08</sub>phe<sub>1.13</sub>arg<sub>1.00</sub>gly<sub>1.13</sub>lys<sub>1.05</sub>pro<sub>1.08</sub>val<sub>1.13</sub>; *in vitro* MSH activity, 1.5 to  $2.6 \times 10^{8}$  MSH units/g. in various assays.

Anal. Caled. for  $C_{77}H_{108}O_{19}N_{22} \cdot 2CH_4COOH \cdot 7H_2O$ : C, 51.5; H, 6.8; N, 16.3; O, 25.4. Found: C, 51.4; H, 7.1; N, 16.1; O, 25.6.

N-t-Butoxycarbonylseryltyrosylseryl- $\alpha$ -amino-n-butyrylglutamine Dihydrate (XV). A. From the Pentapeptide X with t-Butyl Azidoformate.--- A solution of t-butyl azidoformate (0.10 ml.) in dioxane (3 ml.) was added at room temperature to a solution of seryltyrosylseryl- $\alpha$ -amino-n-butyrylglutamine (X, 0.31 g.) in 80% (v./v.) aqueous dioxane (1.6 ml.) containing triethylamine (0.16 ml.) and the mixture was warmed at 40° for 24 hr. The solvents were removed in vacuo, the residue was dissolved in 1-butanol (previously equilibrated with 10% acetic acid), and the solution was washed with six 40-ml. portions of 10% acetic acid. The butanol layer was concentrated to a small volume in vacuo, ether (approximately three times the volume) was added, and the suspension was kept at 5° for 2 to 3 hr. when the precipitate was collected and washed with ether. The colorless solid was dried in vacuo over P2O5 and KOH pellets; yield 0.24 g. (69%), m.p. 146–148° dec.;  $[\alpha]^{28}D = -30.8^{\circ}$  (c 0.26, methanol);  $R_{f}$  0.73, single Pauly-positive, ninhydrin-negative spot.

Anal. Calcd. for  $C_{29}H_{44}O_{12}N_6 \cdot 2H_2O$ : C, 49.4; H, 6.9; N, 11.9; O, 31.8. Found: C, 49.6; H, 6.9; N, 12.0; O, 31.8.

B. From N-t-Butoxycarbonylseryltyrosine Azide (XVI) and Seryl- $\alpha$ -amino-n-butyrylglutamine (VII).—Sodium nitrite (0.59 g.) was added to an ice-cold solution of N-t-butoxycarbonvlservltyrosine hydrazide (3.25 g.) in 1 N hydrochloric acid (17 ml.)and water (10 ml.) and the mixture was kept at ice-bath temperature for 5 min. Ice-cold saturated sodium bicarbonate was added until the solution turned "Alkacid" test paper light green (approximately pH 8), and the precipitate was collected by filtration, washed with ice-water, and dried at 5° in vacuo over  $P_2O_5$ . This azide (XVI) was added to a solution of seryl- $\alpha$ amino-n-butyrylglutamine (VII, 2.31 g.), dissolved in a mixture of water (6 ml.), triethylamine (1.0 ml.), and DMF (6 ml.), and the mixture was kept with stirring at 5° for 36 hr. Approximately 60 ml. of 2.5% ammonium hydroxide was added to the suspension and the mixture was extracted with four 40-ml. portions of ethyl acetate. The ethyl acetate extracts were washed with two 20-ml. portions of 5% ammonium hydroxide and the combined aqueous layers were adjusted to pH 7 with acetic acid and evaporated to a small volume in vacuo. The resulting gelatinous mass was dissolved in 1-butanol and the solution was washed with six 50-ml. portions of 10% acetic acid. The combined organic layers were then evaporated to a small volume in vacuo and the product was precipitated by addition of four volumes of ether. The suspension was kept at  $5^{\circ}$  for 2 hr. when the precipitate was collected, washed with ether, and dried in vacuo at room temperature over P2O5 and KOH pellets; yield 2.07 g. (40%), m.p. 146–150° dec.;  $[\alpha]^{28}D - 30.8^{\circ}$  (c 0.26, methanol);  $R_{f}^{1}$  0.77, single chlorine-positive, ninhydrin-negative spot. Calcd. for C29H44O12N8.2H2O: C, 49.4; H, 6.9; N, Anal.

11.9; O, 31.8. Found: C, 49.5; H, 7.0; N, 11.7; O, 31.6.

N-t-Butoxycarbonylseryltyrosylseryl- $\alpha$ -amino-n-butyrylglutamine Hydrazide Hydrate.-Ethereal diazomethane was added to solution of t-butoxycarbonylseryltyrosylseryl-a-amino-nа butyrylglutamine dihydrate (0.92 g.) in methanol (150 ml.) until the yellow color remained and the mixture was kept at icebath temperature for 10 min. The excess of diazomethane was destroyed by addition of 1 drop of glacial acetic acid and the solvents were removed in vacuo. The oily residue was washed with ether and dissolved in methanol (35 ml.), the solution was filtered, and hydrazine hydrate (0.88 ml.) was added. The mixture was kept at room temperature for 24 hr., then kept at 5° for 2 hr., and the precipitate was collected and washed with ice-cold methanol, then with ether, and dried over sulfuric acid; yield  $0.76 \text{ g.} (83\%), \text{m.p. } 200-202^\circ; R_f^1 0.73, \text{ single tyrosine-positive,}$ ninhydrin-negative spot. A sample for analysis was recrystallized from 30% (v./v.) aqueous methanol.

Anal. Caled. for  $C_{29}H_{46}O_{11}N_8 \cdot H_2O$ : C, 49.7; H, 6.9; N, 16.0; O, 27.4. Found: C, 49.6; H, 7.1; N, 15.7; O, 27.3.

N-t-Butoxycarbonylseryltyrosylseryl- $\alpha$ -amino-*n*-butyrylglutaminylhistidylphenylalanylarginyltryptophylglycine Monoacetate Trihydrate (XVII).—This entire operation was performed at ice-

salt bath temperatures  $(-3 \text{ to } -10^\circ)$  in a cold room  $(5^\circ)$  and all solutions used were refrigerated prior to their use. t-Butoxycarbonylseryltyrosylseryl- $\alpha$ -amino-n-butyrylglutamine hydrazide monohydrate (0.205 g.) was dissolved in 90% (v./v.) aqueous DMF (2 ml.) with warming, the solution was cooled at -3 to  $-10^{\circ}$ , and 1 N hydrochloric acid (0.6 ml.) was added followed by 20.7 mg. of sodium nitrite dissolved in water (0.5 ml.). This solution containing the azide (XVIII) was stirred vigorously for 4 min. and enough DMF containing 10% of triethylamine was added to turn "Alkacid" test paper green (approximately pH 7.5-8.0). A solution of histidylphenylalanylarginyltryptophylglycine monoacetate dihydrate (XIX)<sup>20a,b</sup> (0.11 g.) in DMF (1.2 ml.) containing 0.3 ml. of 10% triethylamine in DMF was added and the mixture was kept at 5° for 24 hr. At this point a second portion of azide XVIII (prepared from 0.068 g. of the hydrazide as described above), was added and the mixture stored for another 24 hr. at 5°. Paper chromatography of the reaction mixture showed the presence of three Pauly-positive spots with  $R_{\rm f}^1$  values of 0.24, 0.53, and 0.73, respectively, with the fastest moving component producing a yellow spot. Water (400 ml.) was added to the reaction mixture, the solution was filtered, and the filtrate was applied to a CMC column ( $3 \times 15$ cm.) (at room temperature) which was then eluted successively with the following pH 6.8 ammonium acetate buffers: 0.01 M (250 ml.); 0.02 M (950 ml.); 0.03 M (150 ml.); and 0.05 M(450 ml.). Individual fractions, 13 ml. each, were collected at a flow rate of 4 ml. per min. and the absorbancy of individual tubes was determined at 275 m $\mu$ . The 0.02 M eluates (tubes 89-127) containing the desired decapeptide were pooled, the bulk of the water was removed in vacuo, and the concentrate was lyophilized to constant weight; yield 153 mg. (74%). Chromatographic analysis of this material in the Partridge system showed the presence of two components with  $R_{\rm f}^1$  values of 0.50 (major) and 0.38 (minor). For further purification a sample of this material (178 mg.) was dissolved in 10% acetic acid equilibrated with 1butanol and this solution was added to the first four tubes of a 200-plate countercurrent machine and the material was distributed between 1-butanol and 10% acetic acid (200 transfers). Spectrophotometric evaluation at 280 mµ revealed the presence of three components located respectively in tubes 37-55 (minor), 109-135 (major), and 154-181 (minor). The contents of the tubes containing the major peak was pooled, the bulk of the solvent was removed in vacuo at 40°, and the concentrate lyophilized to constant weight; yield 151 mg.;  $[\alpha]^{27}D - 27.2^{\circ}$  (c 0.24, 50% acetic acid);  $R_{\rm f}^1$  0.53, ninhydrin-negative, Paulyand Sakaguchi-positive spot; amino acid ratios in acid hydrolysate, ser2.09tyr1.00but1.09glu0.94his1.00phe0.96arg1.00gly0.96.

Anal. Calcd. for  $C_{53}H_{55}O_{17}N_{17}$ ·CH<sub>3</sub>COOH·3H<sub>2</sub>O: C, 53.3; H, 6.5; N, 16.2; O, 24.0. Found: C, 53.2; H, 6.7; N, 16.0; O, 24.1.

**Hydrochloride**.—The acetate salt (0.93 g.) was dissolved in water (5 ml.), the solution was cooled at 0°, and 0.5 N hydrochloric acid (0.24 ml.) was added. The mixture was kept at 0° for 3 min. when it was frozen and lyophilized. The resulting colorless fluffy powder was ninhydrin negative; yield essentially quantitative.

Methyl Prolylvalylglycinate (XX).—Methyl benzyloxycarbonylprolylvalylglycinate<sup>22</sup> (5.68 g.) was hydrogenated in methanol over a palladium catalyst until evolution of carbon dioxide ceased. The catalyst was then removed by filtration and the filtrate was evaporated to dryness *in vacuo* to give a colorless solid which was recrystallized from a mixture of ethanol and ether; yield 3.5 g. (91%), m.p.  $142-143^\circ$ ;  $[\alpha]^{31}$ D  $-74.3^\circ$  (*c* 1.39, methanol);  $R_{f^1}$ 0.58, ninhydrin positive.

Anal. Caled. for  $C_{13}H_{23}O_4N_3\colon$  C, 54.7; H, 8.1; N, 14.7. Found: C, 54.9; H, 8.3; N, 14.7.

Methyl N- $\alpha$ Benzyloxycarbonyl-N<sup>e</sup>-t-butoxycarbonyllysylprolylvalylglycinate (XXII).—A mixed anhydride prepared in the usual manner from N $\alpha$ -benzyloxycarbonyl-N<sup>e</sup>-t-butoxycarbonyllysine<sup>21</sup> (XXI, 1.97 g.) in freezing dioxane (25 ml.) with tri-*n*-butylamine (1.24 ml.) and ethyl chloroformate (0.48 ml.) was added with stirring to a chilled solution of methyl prolylvalylglycinate (1.34 g.) in tetrahydrofuran (20 ml.) and the mixture was stirred at 0° for 30 min. and at room temperature for 1 hr. The solvents were evaporated *in vacuo*, the residue was dissolved in cooled (4°) ethyl acetate, and the solution was washed successively with icecold 2 N aqueous citric acid, sodium bicarbonate, and water, and was dried over sodium sulfate. Evaporation of the solvent gave a solid residue which was recrystallized from a mixture of ethyl acetate and petroleum ether; yield 2.4 g. (79%), m.p. 94-97°;  $[\alpha]^{3i}D - 78.7^{\circ}$  (c 1.18, methanol); amino acid ratios in acid hydrolysate,  $lys_{0.93}pro_{0.95}val_{1.06}gly_{1.06}$ .

Anal. Calcd. for  $C_{32}H_{49}O_{9}N_{5}$ : C, 59.3; H, 7.6; N, 10.8. Found: C, 59.3; H, 7.9; N, 10.9.

Nª-Benzyloxycarbonyl-N\*-t-butoxycarbonyllysylprolylvalylglycine (XXIII).-Normal sodium hydroxide (2.5 ml.) was added to a solution of XXII (1.29 g.) in methanol (15 ml.) and water (20 ml.) and the mixture was kept at room temperature for 2 hr. The pH was then adjusted to approximately 7 by addition of solid citric acid and the bulk of the solvents was removed in The residue was dissolved in 0.5 N ammonium hydroxide vacuo. (75 ml.) and the solution was washed with three 75-ml. portions of ethyl acetate which were re-washed with two 25-ml. portions of 0.5 N ammonium hydroxide solution. The combined aqueous layers were cooled to -5 to 0°, the pH was adjusted to about 3 by addition of solid citric acid, and the solution was extracted with three 150-ml. portions of ethyl acetate which were washed successively with two 30-ml. portions of ice-cold 1 N citric acid and two 30-ml. portions of saturated sodium chloride solution. Evaporation in vacuo of the sodium sulfate dried organic layers gave an oil which was dissolved in ethyl acetate, and petroleum ether (b.p. 30-60°) was added until the solution became cloudy. On standing needles appeared; yield 1.20 g. (95%); m.p. 104-108°; lit.<sup>44</sup> m.p. ca. 72–95°;  $[\alpha]^{31}D - 77.1^{\circ}$  (c 1.58, methanol); single chlorine-positive, ninhydrin-negative spot,  $R_{\rm f}^1$  0.91. A sample was recrystallized from a mixture of ethyl acetate and petroleum ether for analysis; m.p. 115-117°

Anal. Calcd. for  $C_{31}H_{47}O_9N_5$ : C, 58.8; H, 7.5; N, 11.0; O, 22.7. Found: C, 58.7; H, 7.6; N, 10.9; O, 22.9.

Methyl N<sup>a</sup>-Benzyloxycarbonyl-N<sup>e</sup>-t-butoxycarbonyllysyl-N<sup>e</sup>-tbutoxycarbonyllysinate.---A mixed anhydride, prepared in the usual manner from N<sup>a</sup>-benzyloxycarbonyl-N<sup>e</sup>-t-butoxycarbonyllysine (XXI, 2.31 g.) in freezing dioxane (20 ml.) with tri-n-butylamine (1.45 ml.) and ethyl chloroformate (0.57 ml.), was added with stirring to a cold (5°) solution of methyl N<sup>e</sup>-t-butoxycarbonyllysinate hydrochloride<sup>21</sup> (1.8 g.) and triethylamine (0.85 ml.) in tetrahydrofuran (15 ml.) and the mixture was stirred for 30 min. in an ice bath and for 1 hr. at room temperature. The solvents were evaporated in vacuo, the oily residue was dissolved in ethyl acetate, the solution was washed with ice-cold 2 N citric acid, with sodium bicarbonate solution, and with water, and dried over sodium sulfate. Evaporation of the ethyl acetate gave an oil which crystallized when kept under petroleum ether (b.p. 30-60°). The material was recrystallized from a mixture of ethyl acetate and petroleum ether; yield 2.76 g. (73%), m.p.  $70-74^{\circ}$ ;  $[\alpha]^{31}$ D -11.2° (c 1.95, methanol);  $[\alpha]^{28}$ D -6.0° (c 1.96, acetone); lit.<sup>21</sup> m.p. 78-84°;  $[\alpha]^{30}$ D -5.3  $\pm$  1° (c 2.0, acetone).

Anal. Calcd. for  $C_{31}H_{50}O_9N_4$ : C, 59.8; H, 8.1; N, 9.0. Found: C, 59.6; H, 8.1; N, 9.0.

Methyl N<sup>a</sup>-Benzyloxycarbonyl-N<sup>e</sup>-t-butoxycarbonyllysylprolyl $valylglycyl-N^{\epsilon}-t-butoxycarbonyllylsyl-N^{\epsilon}-t-butoxycarbonyllysinate$ (XXV).-N,N'-Carbonyldiimidazole<sup>24</sup> (0.47 g.) was added to an ice-cold solution of Na-benzyloxycarbonyl-Ne-t-butoxycarbonyllysylprolylvalylglycine (XXIII, 1.64 g.) in dimethylformamide (15 ml.) and the solution was stirred with ice cooling for 1.5 hr. A solution of methyl Ne-t-butoxycarbonyllysyl-Ne-t-butoxycarbonyllysinate (XXIV, prepared from 1.76 g. of the benzyloxycarbonyl derivative<sup>21</sup>) in DMF (15 ml.) was then added and the mixture was kept at room temperature for 4 hr. The solvent was removed in vacuo and the residue was dissolved in ice-cold ethyl acetate (150 ml.) and washed with three portions of icecold 2 N citric acid, three portions of saturated sodium bicarbonate, and finally with water. The sodium sulfate dried organic layer was evaporated to a small volume in vacuo and the product was precipitated by addition of ether; yield 2.50 g. (80%), m.p. 117-118°;  $[\alpha]^{29}D - 48.1^{\circ}$  (c 0.78, methanol);  $R_{\rm f}^1$  0.93, chlorine positive, ninhydrin negative; amino acid ratios in acid hydrolysate, lys<sub>2.9</sub>pro<sub>1.6</sub>val<sub>1.0</sub>gly<sub>1.0</sub>; lit.<sup>44</sup> m.p. 119-126°; [α]<sup>25</sup>D  $-50.8^{\circ} \pm 0.5^{\circ}$  (c 1.89, methanol).

Anal. Calcd. for  $C_{54}H_{89}O_{15}N_{9}$ : C, 58.7; H, 8.1; N, 11.4. Found: C, 58.7; H, 8.3; N, 11.2.

N°-Benzyloxycarbonyl-N<sup> $\epsilon$ </sup>-t-butoxycarbonyllysylprolylvalylglycyl-N<sup> $\epsilon$ </sup>-t-butoxycarbonyllysyl-N<sup> $\epsilon$ </sup>-t-butoxycarbonyllysine Hydrazide.—Hydrazine hydrate (95%, 0.15 ml.) was added to a solution of the above methyl ester XXV (552 mg.) in methanol (5 ml.) and the solution was kept at room temperature for 24 hr. Ether (10 ml.) was then added and the mixture placed in a refrigerator to complete precipitation. The resulting gelatinous hydrazide was collected, washed with ether, and dried over sulfuric acid *in vacuo*; yield 400 mg. (76%), m.p.  $142-145^{\circ}$ ;  $[\alpha]^{29}D - 54.8^{\circ}$  (c 0.86, methanol).

Anal. Caled. for  $C_{53}H_{39}O_{14}N_{11}$ : C, 57.6; H, 8.1; N, 13.9. Found: C, 57.9; H, 8.3; N, 13.9.

N<sup>e</sup>-t-Butoxycarbonyllysylprolylvalylglycyl-N<sup>e</sup>-t-butoxycarbonyllysyl-Ne-t-butoxycarbonyllysylarginylarginylprolylvaline Amide Triacetate Hexahydrate (XXVI).-A solution of Na-benzyloxycarbonyl-Ne-t-butoxycarbonyllysylprolylvalylglycyl-Ne-t-butoxycarbonyllysyl-N<sup>e</sup>-t-butoxycarbonyllysine hydrazide (1.49 g.) in DMF (12 ml.) and water (5.5 ml.) was cooled at -5 to  $-10^{\circ}$  and ice-cold 0.5 N hydrochloric acid (5.4 ml.) followed by ice-cold 1 N sodium nitrite (1.35 ml.) was added. After 5 min. the pH was adjusted to approximately 7 by addition of 5% (v./v.) triethylamine in DMF, and ice-water was added. The precipitated azide XXVII was collected, washed with ice-water, and dissolved in DMF (12 ml.). This solution was added to a solu $tion \, of \, arginy larginy lproly lvaline \, amide \, triacetate \, tetrahydrate^{6b.\,46}$ (XXVIII, 476 mg.) in DMF (12 ml.) and 5% (v./v.) triethylamine in DMF (2.13 ml.). The mixture was kept at 5° for 24 hr., the solvents were removed in vacuo, and the residue was dissolved in a mixture of 1-butanol and 1% acetic acid. The butanol layer was extracted with six 15-ml. portions of 1% acetic acid and then evaporated to dryness in vacuo. The residue, dissolved in 90% aqueous methanol (50 ml.) containing 1% of glacial acetic acid, was hydrogenated over a palladium catalyst in the usual manner. The catalyst was removed by filtration and the filtrate was evaporated to a small volume and finally lyophilized. The crude material (1.72 g.) dissolved in water (100 ml.) was applied to a CMC column (3  $\times$  19 cm.) which was eluted successively with the following pH 6.8 ammonium acetate buffers: 0.01 M (300 ml.); 0.025 M (500 ml.); 0.050 M (500 ml.); 0.075 M (200 ml.); and finally 0.10 M (800 ml.). Individual fractions of 10 ml. each were collected and spot tests with Sakaguchi reagent served to locate the desired material in the 0.10 M eluates (tubes 167-217). The contents of these tubes were pooled, concentrated to a small volume in vacuo, and finally lyophilized to constant weight, giving colorless fluffy material; yield 816 mg. (76%);  $[\alpha]^{27}D - 69.8^{\circ}$  (c 0.15, 10% acetic acid); single ninhydrin- and Sakaguchi-positive spot;  $R_{f}^{1} 0.68$ ;  $R_{f}^{2} 0.95$ ; amino acid ratios in acid hydrolysate, lys3.06pro1.94val2.02gly1.00arg1.98-

Anal. Calcd. for  $C_{67}H_{122}O_{16}N_{20} \cdot 3CH_{3}COOH \cdot 6H_{2}O$ : C, 50.1; H, 8.2; N, 16.0; O, 25.6. Found: C, 50.0; H, 8.6; N, 16.2; O, 25.6.

t-Butoxycarbonylseryltyrosylseryl- $\alpha$ -amino-n-butyrylgluta $minyl histidyl phenylalanylarginyl tryptophylglycyl-N^{\epsilon}-t-butoxy car$  $bonyllysylprolylvalylglycyl-N^{\epsilon}-t-butoxycarbonyllysyl-N^{\epsilon}-t-butoxy$ carbonyllysylarginylarginylprolylvaline Amide Acetate Hydrate (XXX).—N,N'-Carbonyldiimidazole<sup>24</sup> (10.5 mg.) was added to a solution of carefully dried t-butoxycarbonylseryl $tyrosylservl-\alpha$ -amino-*n*-butyrylglutaminylhistidylphenylalanylarginyltryptophylglycine hydrochloride (XVII, prepared from 93 mg. of the monoacetate trihydrate) in DMF (1 ml.) at 0°. The solution was stirred in an ice bath for 65 min. and an ice-cold so-butoxycarbonyllysyl-N\*-t-butoxy-carbonyllysylarginylarginylprolylvaline amide hydrochloride (XXVI, prepared from 88 mg. of the triacetate hexahydrate) in DMF (0.8 ml.) containing 10% (v./v.) triethylamine in DMF (0.13 ml.) was added. The mixture was stirred for 1 hr. at 0°, then kept at room temperature for 48 hr. Ethyl acetate (approximately 300 ml.) was added, the suspension centrifuged, and the precipitate washed with additional ethyl acetate and recentrifuged. The material was freed of solvent in vacuo, dissolved in water (45 ml.) and DMF (4 ml.), and the solution was applied to a CMC column  $(3 \times 8 \text{ cm.})$  which was eluted successively with the following pH 6.8 ammonium acetate buffers: 0.075 M (300 ml.); 0.10 M (300 ml.); and 0.15 M (450 ml.). Individual fractions, 6 ml. each, were collected at a flow rate of 2 ml, per min. and absorbancy at  $280 \text{ m}\mu$  served to locate the desired product in the 0.15 M eluates (tubes 100-150) which were pooled, the bulk of the solvent was removed in vacuo, and the residue was lyophilized, giving a fluffy colorless powder; yield 63.1 mg.(38%);

<sup>(44)</sup> R. Schwyzer, W. Rittel, and A. Costopanagiotis, *Helv. Chim. Acta*, **45**, 2473 (1962).

<sup>(45)</sup> Substitution of saturated sodium bicarbonate for the previously employed 3% ammonium hydroxide for washing of benzyloxycarbonylnitroarginylprolylvaline amide employed in the preparation of arginylarginylprolylvaline amide (XXVIII) was found to be advantageous.

 $[\alpha]^{26}$ D – 54.7° (c 0.17, 10% acetic acid); single Pauly-, Sakaguchi-, and Ehrlich-positive, ninhydrin-negative spot on paper chromatography,  $R_{f^1}$  0.61, and on paper electrophoresis in pyridinium acetate pH 3.5; amino acid ratios in acid hydrolysate, ser<sub>2.17</sub> tyr<sub>0.96</sub>but<sub>1.13</sub>glu<sub>1.00</sub>his<sub>1.04</sub>phe<sub>1.09</sub>arg<sub>2.01</sub>gly<sub>1.91</sub>lys<sub>2.80</sub>pro<sub>2.00</sub>val<sub>1.91</sub>, MSH activity *in vitro* 2.2 × 10<sup>6</sup> MSH units/g.

 $Seryltyrosylseryl-\alpha$ -amino-*n*-butyrylglutaminylhistidylphenyla lany larginy ltryptophylglycyllysylprolylvalylglycyllysyllysylarginylarginylprolylvaline Amide Acetate Hydrate (XXXI).-The protected eicosapeptide amide XXX (45 mg.) was dissolved in anhydrous trifluoroacetic acid (0.6 ml.) and the solution was kept at room temperature for 20 min. Water (4 ml.) was added and the solution was immediately frozen and lyophilized. The residue was dissolved in water (15 ml.). Amberlite IRA-400 (acetate cycle) (approximately 3 ml. settled in water) was added, and the suspension was stirred for 50 min. at room temperature. The mixture was filtered, the resin was washed with several portions of water, and the filtrate and washings were concentrated to a small volume in vacuo and then lyophilized, giving a colorless fluffy powder; yield 41.8 mg. This material was dissolved in water (30 ml.) and the solution was applied to a CMC column  $(1.5 \times 20 \text{ cm.})$  which was eluted successively with the following ammonium acetate buffers: pH 6.8, 0.075 M (30 ml.); pH 6.8, 0.25 M (40 ml.); pH 8.5, 0.25 M (40 ml.); pH 9.0, 0.25 M (100 ml.); pH 9.5, 0.025 M (100 ml.); and pH 10.0, 0.25 M (100 ml.). Individual fractions, 5 ml. each, were collected at a flow rate of approximately 2 ml. per min. Absorbancy at 280  $m\mu$  located the desired material in the pH 9.5, 0.25 M eluates (tubes 71-109) which were pooled, the bulk of the solvent was removed in vacuo, and the concentrate was lyophilized to constant weight, giving a colorless fluffy powder; yield 25.6 mg.,  $[\alpha]^{28}$ D  $-61.7^{\circ}$  (c 0.09, 10% acetic acid); single ninhydrin-, Pauly-, Sakaguchi-, and Ehrlich-positive spot with  $R_{\rm f}$ <sup>3</sup> 0.7 imessingle band on disk electrophoresis on polyacrylamide His: gel25 at pH 4.6; amino acid ratios in acid hydrolysate, ser1.97tyr<sub>0.91</sub>but<sub>0.94</sub> glu<sub>1.02</sub> his<sub>1.02</sub>phe<sub>0.98</sub>arg<sub>2.91</sub>gly<sub>1.89</sub>lys<sub>8.31</sub> pro<sub>2.16</sub>val<sub>1.89</sub>; acid ratios in LAP digest, ser2.1tyr1.00but0.0>glu0.74his0.57phe0.65arg0.78 $try_{0.48}gly_{0.52}lys + orn_{1.04}pro_{0.70}val_{0.57}$ ; 0.26 µmole of peptide liberated 0.16 µmole of glutamine on digestion with Pronase, glutamic acid was not detectable in the digest on paper chromatograms or on the amino acid analyzer; the peptide did not liberate amino acids on treatment with carboxypeptidase A; MSH activity<sup>34</sup>  $1.6 \times 10^7$  MSH units/g.; adrenal ascorbic acid depleting activity i.v.  $31.2 \pm 4.2$  to  $48.4 \pm 10.4$  IU/mg.

**Pronase Experiments.**—Pronase (Calbiochem. Lot. No. 502117, 45000 P.U.K./g.) (0.1 mg.) in 0.025 M ammonium acetate buffer, pH 7.4 (0.1 ml.), was incubated for 24 hr. at 40° with a solution of peptide XXXI (1.5–2.5 mg.) in 0.025 M ammonium acetate buffer, pH 7.4 (0.1 ml.). Digestion was stopped by evaporating the solution to dryness *in vacuo* over P<sub>2</sub>O<sub>5</sub> and KOH. The residue was dissolved in 0.2 N sodium citrate buffer, pH 2.2, for amino acid analysis.

Acknowledgment.—The skillful technical assistance of Mrs. Chizuko Yanaihara, Mrs. Maria Günther, Mrs. Jemele Hudson, Miss Priscilla Holland, Miss Judy Montibeller, and Mr. John Humes is gratefully acknowledged.

[CONTRIBUTION OF THE DEPARTMENT OF CHEMISTRY, BRANDEIS UNIVERSITY, WALTHAM, MASSACHUSETTS]

# Kinetics of Hydrolysis of Dicarboxylic Esters and Their $\alpha$ -Acetamido Derivatives by $\alpha$ -Chymotrypsin<sup>1</sup>

By SAUL G. COHEN AND JOHN CROSSLEY

RECEIVED JULY 2, 1964

The diethyl esters of malonic, succinic, glutaric,  $\alpha$ -acetamidomalonic, N-acetylaspartic, and N-acetylglutamic acids are hydrolyzed to the monoesters by  $\alpha$ -chymotrypsin, the latter three stereospecifically to the  $\alpha$ - acids. The kinetic parameters  $K_m$  and  $k_3$  have been determined for each ester. Values of  $k_3/K_m$  for the six compounds are, respectively, 1, 7.5, 1.7, 23, 950, and 64, the succinate hydrolyzing most rapidly of the unsubstituted compounds, the aspartate most rapidly of the  $\alpha$ -acetamido compounds. The second carbethoxyl group associates at the  $\beta$ -aryl site of the enzyme, most effectively, leading to lowest  $K_m$ , when  $\beta$  relative to the hydrolyzing group. Its effectiveness is compared with other  $\beta$ -substituents. The  $\alpha$ -acetamido group leads to increased reactivity by increasing  $k_3$ .

## Introduction

Esters of glutaric acid and of  $\beta$ -substituted glutaric acids,<sup>2</sup> RO<sub>2</sub>CCH<sub>2</sub>CHXCH<sub>2</sub>CO<sub>2</sub>R, X = H, HO, CH<sub>3</sub>-CONH, and CH<sub>3</sub>CO<sub>2</sub>, are hydrolyzed by  $\alpha$ -chymotrypsin,<sup>2</sup> the  $\beta$ -hydroxy<sup>3</sup> and the  $\beta$ -acetamido<sup>4</sup> compounds stereospecifically, and the  $\beta$ -acetoxy compound more rapidly but nonstereospecifically.<sup>2,5</sup> The unsubstituted glutarate itself was hydrolyzed more readily than the  $\beta$ -hydroxy and the  $\beta$ -acetamido compounds. The corresponding derivatives of butyric acid are inert or may be hydrolyzed exceedingly slowly and apparently without stereospecificity.<sup>3,6</sup> The second carbethoxyl group in the glutarates, like the  $\beta$ -aryl group in "natural" substrates of this enzyme, contributes to reactivity and stereospecificity in these reactions. Dicarboxylic esters may provide a group of relatively watersoluble substrates in which a significant structure-reactivity relation may be studied in an enzymatic reaction, and it seemed of interest to examine a series of unsubstituted and  $\alpha$ -acetamido-substituted diesters. Diethyl N-acetylaspartate, the analog in this set of ethyl Nacetyl- $\beta$ -phenylalaninate, was examined first and found to be hydrolyzed by  $\alpha$ -chymotrypsin with high effectiveness and stereospecificity.<sup>7</sup> We are now reporting on the kinetics of the enzymatic hydrolysis of the diethyl esters of malonic, succinic, glutaric, adipic,  $\alpha$ -acetamidomalonic, and N-acetylglutamic acids. Diethyl  $\alpha$ -acetamidomalonate had been reported previously to be hydrolyzed stereospecifically by  $\alpha$ -chymotrypsin.<sup>8</sup>

## Results

Preparative experiments were carried out first to establish whether all the compounds would, in fact, be hydrolyzed by  $\alpha$ -chymotrypsin and to allow characterization of the products. Diethyl malonate, 0.6 g., was hydrolyzed at pH 7.2 by 0.1 g. of the enzyme, 73%

<sup>(1)</sup> We are pleased to acknowledge generous support of this work by the Division of Research Grants, National Institutes of Health, Grant No. GM 04584.

<sup>(2)</sup> S. G. Cohen and J. Crossley, J. Am. Chem. Soc., 86, 1217 (1964).

<sup>(3)</sup> S. G. Cohen and E. Khedouri, ibid., 83, 4228 (1961).

<sup>(4)</sup> S. G. Cohen and E. Khedouri, *ibid.*, 83, 1093 (1961).

<sup>(5)</sup> S. G. Cohen, J. Crossley, E. Khedouri, R. Zand, and L. H. Klee, *ibid.*, **85**, 1685 (1963).

<sup>(6)</sup> S. G. Cohen, Y. Sprinzak, and E. Khedouri, ibid., 83, 4225 (1961).

<sup>(7)</sup> S. G. Cohen, J. Crossley, and E. Khedouri, *Biochemistry*, 2, 820 (1963).

<sup>(8) (</sup>a) S. G. Cohen and L. Altschul, *Nature*, **183**, 1678 (1959); (b) S. G. Cohen and L. H. Klee, *J. Am. Chem. Soc.*, **82**, 6038 (1960).