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Studies with Corticotropin. III. Determination of the Structure of β-Corticotropin[1] and its Active Degradation Products

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The complete amino acid sequence of pure β-corticotropin has been derived primarily from its trypsin and chymotrypsin digestion products which were quantitatively separated and sequentially degraded. Pepsin digestion and partial acid hydrolysis were used to determine the sequences of two portions of the peptide chain. No evidence has been adduced to prove that fewer than 24 amino acid residues are sufficient for corticotropin activity.

The study of the pepsin digestion of β-corticotropin reported in the preceding paper[2] indicated that a peptide containing 28 amino acids was the smallest biologically active product. Mild acid degradation of β-corticotropin (see ref. 2 and below) produced active products which are apparently slightly smaller (24 and 25 amino acid residues). More extensive enzymatic degradation of the hormone was then carried out, in order to elucidate its complete structure and to determine whether smaller fragments had any degree of corticotropin activity. This work has enabled us to propose[3] a structure for the major component of hog corticotropin. This study is now reported in detail. None of these smaller fragments was found to be active, and therefore, practical synthesis in competition with the natural product seems unlikely.

For determination of the structure of the "essential" portion of the hormone, β-corticotropin was selected rather than the smaller active pepsin product P4, since preparation of the latter involved considerable time and loss of material.[2] The presence in β of the additional 11-amino acid C-terminal portion was not expected to complicate this work since its structure was known.[2]

Determination of structure by partial acid hydrolysis, sequential chemical degradation of the intact molecule and specific cleavages was considered. Specific cleavage by trypsin and chymotrypsin appeared most promising because of the presence of seven basic and six aromatic amino acid residues in β-corticotropin. These enzymes would be expected to cleave the peptide chain at different points and to preserve special structural features which might be destroyed by partial acid hydrolysis. Specific degradation to a small number of peptides was necessary since only a small quantity of pure hormone was available. Partial acid hydrolysis appeared less promising, since the very large number of products from random cleavage of a 39-amino acid peptide would present a formidable fractionation problem and would require a prohibitively large sample of hormone. In addition, the virtual impossibility of determining the theoretical yields of products would eliminate this highly desirable aspect of any such degradation. Of all the available sequence methods, only the Edman procedure[4] appeared to have the necessary general applicability for total sequence determination. However, its possibilities were found to be limited by low yields of end group, random cleavage during the acidic cyclization step and low solubility of polyphenylthiocarbamyl derivatives.

Possible disadvantages of the enzymatic method are the greater amount of sequential degradation made necessary by the larger fragments, as compared to partial acid hydrolysis, and the chance of rearrangements catalyzed by the enzymes. The sequence work was feasible with the newer methods. Transpeptidation, of the type reported by Waley and his associates,[5] was a more serious objection. Minimal exposure to the enzymes and dilute substrate solutions were used to favor the desired process. In addition, the approach to the structure outlined below would eliminate from consideration as part of the hormone structure any minor products arising from transpeptidation. The requirement that the products from two enzymes of different specificity give a single consistent structure for the hormone would further reduce the possibility of arriving at an erroneous structure as a result of transpeptidation.

The determination of the structure of β-corticotropin involved the following steps. The two sets of fragments produced by trypsin and chymotrypsin were each quantitatively separated by countercurrent distribution and the purity of the separated fragments was examined in several ways. Quantitative amino acid analyses and peptide sequence studies were then carried out on each major product. Using these data, the yields as percentages of theory were calculated for the products. Finally, a single consistent structure was derived, based on the high yield fragments, which accounted for the major portion of the weight of the original hormone. The enzymatic cleavages observed constitute additional knowledge of enzyme specificity on a complex substrate.

The first enzymatic degradation of β-corticotropin was carried out with trypsin, which rapidly inactivated the hormone. Preliminary paper chromatographic studies of digests prepared using various times and amounts of enzyme indicated that degradation to 6–8 peptides could be accomplished by 4-hour digestion at an enzyme:substrate ratio of

(1) Studies with Corticotropin. I. R. G. Shepherd, *et al.*, THIS JOURNAL, **78**, 5051 (1956).

(2) Studies with Corticotropin, II, P. H. Bell, *et al.*, *ibid.*, **78**, 5059 (1956).

(3) (a) P. H. Bell, *et al.*, *ibid.*, **76**, 5565 (1954); (b) K. S. Howard, R. G. Shepherd, E. A. Eigner, D. S. Davies and P. H. Bell, *ibid.*, **77**, 3419 (1955).

(4) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(5) S. G. Waley and J. Watson, *Nature*, **167**, 361 (1951); *Biochem. J.*, **57**, 529 (1954); K. G. Blau and S. G. Waley, *Biochem. J.*, **57**, 538 (1954).

1:100. Cellulose column partition chromatography of the digest, using the system 1-butanol *vs.* 20% acetic acid, was unsatisfactory because of low recoveries of the separated components. On the other hand, countercurrent distribution in this solvent system permitted quantitative recovery. A preliminary 200-transfer distribution showed gross fractionation into two peaks, with the slower one beginning to separate into at least three materials. Although this solvent system was not optimal for the separation of the components of the slow peak, it was selected for extended countercurrent distribution since it was volatile and thus involved no possible solute losses during isolation. In Fig. 1 is shown such a distribution of the tryptic digest of

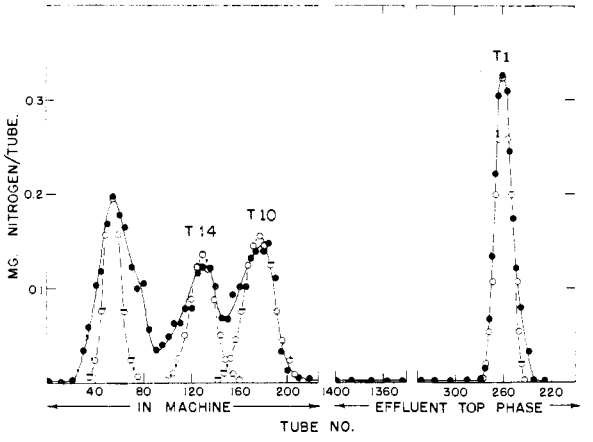

Fig. 1.—Countercurrent distribution of trypsin-digested β-corticotropin in 1-BuOH *vs.* 20% AcOH after 1400 transfers (200 mg. charged in 7 tubes of 220 tube machine): ●—●, concentrations in effluent top phase and in contents of machine; O——O, theoretical curve.

200 mg. of β-corticotropin after 1400 transfers,[6] the top phase passing out of the apparatus at tube 220. The volatility of the solvent was especially advantageous, since direct paper chromatography of lyophilized samples enabled more critical evaluation of the progress of the distribution than did nitrogen analysis and was necessary for analysis of the very dilute solutions present after several thousand transfers. Paper chromatographic analysis of the effluent and the contents of the machine at 1400, 4900 and 8200 transfers was used to determine the location of each fragment and the value of continuing the distribution. Completion of the separation of the minor basic peptides was accomplished by paper chromatography of fractions of the final contents of the distribution machine. The weight, yield and calculated distribution coefficient[7] of each product from the tryptic digest are shown in Table I. Total weight recovery was 93%.

Chymotrypsin digestion, which also led to rapid inactivation of β-corticotropin, was shown by preliminary studies to produce about six peptides after 24 hours at an enzyme:substrate ratio of 1:100. A 179-mg. sample of digest prepared using these conditions was subjected to countercurrent distribution as above. Paper chromatography was used for analysis at 2810, 4850, 5550 and 9500 transfers and for preparation of pure samples of C2 and C16. The recovered peptides, their weights, yields and calculated distribution coefficients are also shown in Table I. In this case the total weight recovery was only 73% since several minor peptides, not examined as to composition, are not included in the totals. The subsequent studies of the peptides of Table I which revealed their structures made it possible to express their recovered weights in terms of the theoretical yield. These major products are not numbered consecutively because the other arbitrarily numbered sections of the countercurrent distribution were ultimately shown to contain insignificant amounts of material. In Table I and Fig. 2 the fragments produced by trypsin and chymotrypsin are designated by the letters T and C, respectively.

Any trypsin and chymotrypsin degradation product listed in Table I was considered suitable for structural study only when it had satisfied the following criteria of purity: (1) was separated from its nearest neighbor by countercurrent distribution, a method of high resolving power; (2) contained a single component on paper chromatography in 5:4:1 1-butanol:water:acetic acid; (3) gave integral mole ratios upon quantitative amino acid analysis[2] by paper chromatography; and (4) had a single C-terminal and N-terminal group.

TABLE I

PEPTIDES FROM TRYPSIN AND CHYMOTRYPSIN DIGESTIONS OF β-CORTICOTROPIN

Peptide	Calcd. K^a	Weight recovd.,[b] mg.	% of theory[c]
	Trypsin (200 mg.)		
T1	5.0	73[d]	95[d]
T10	.13	38	90
T14	.09	34	90
T15		(*ca.* 2)	
T16, T18		*ca.* 9	*ca.* 30
T17	.04	22	70
T19		(*ca.* 1)	
	Total	186 mg.	93%
	Chymotrypsin (179 mg.)		
C2	0.8	10	100
C4	.4	10	100
C5	.3	11	90
C7	.2	24	100
C10	.05	60	70
C16		16	70
	Total	131 mg.	73%

[a] Corrected to org./aq. vol. ratio of 1.0. [b] Corrected for amount used for analysis (*ca.* 3%). Some peptides, such as T1, were weighed as anhydrous "free bases" and others, such as T17, as hydrated acetate salts. [c] Calculated from the relation of the amount of peptide found by quantitative amino acid analysis of the hydrolysate to one mole of anhydrous peptide "free base" possible from β-corticotropin. [d] Corrected for T2 formed.

(6) The nomenclature used for the degradation products in the preliminary reports (*cf.* ref. 3) of this work has been simplified here. Thus the designation for the βTr(1) peptide becomes T1 in this paper, βTr(10) becomes T10, etc. In a similar fashion, the chymotrypsin peptide nomenclature has been simplified, changing βCh(4) to C4, etc.

(7) L. C. Craig and D. Craig, "Techniques of Organic Chemistry," Interscience Publishers, Inc., New York, N. Y., 1950, p. 289.

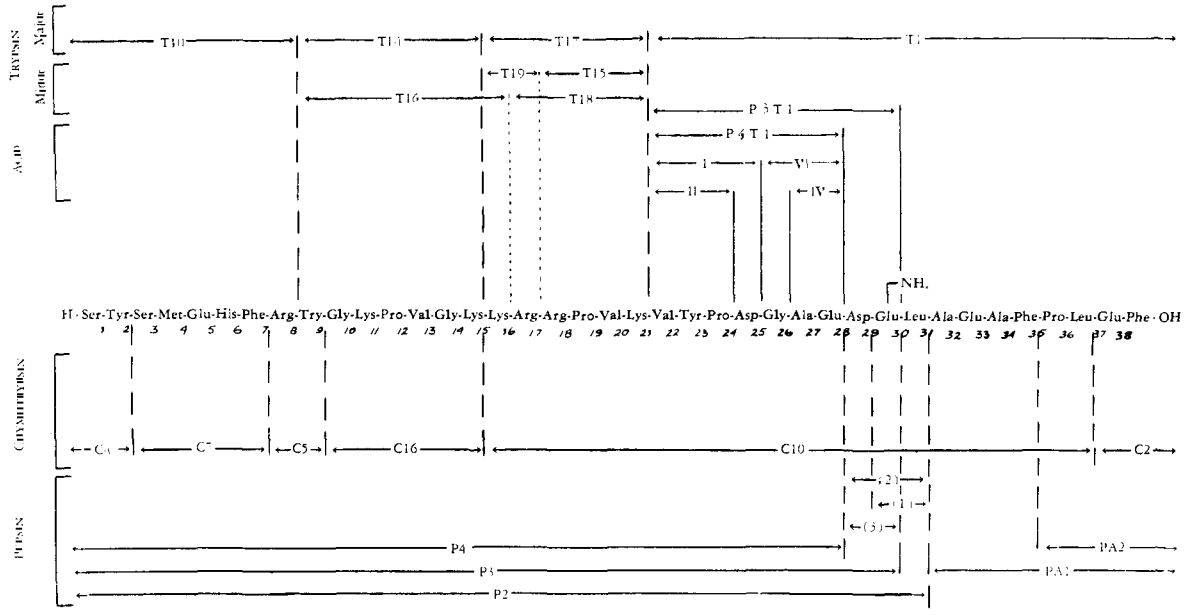

Fig. 2.—Structure and degradation of β-corticotropin. The degradative agents are shown at the left; the fragments produced by each agent are grouped in the horizontal area enclosed by the brackets. The dotted lines designate minor trypsin cleavages. The small peptides under pepsin arise from two separate digestions.[2]

For N-terminal sequence work the Edman phenyl isothiocyanate method[4] was adapted to use on a micro scale. Carboxypeptidase[8a] degradation appeared to have more general applicability than any of the chemical C-terminal sequence methods,[8b] and a procedure permitting direct quantitative amino acid analysis by paper chromatography was employed. Whenever possible, the Edman and carboxypeptidase methods were applied to each peptide until the derived sequences overlapped.

Using the sequences found in the peptides from the various cleavages, it was possible to arrange the fragments so as to form a unique sequence for β-corticotropin. This process involved the following additional considerations: (1) the N- and C-terminal data in the starting material; (2) the locations of methionine, histidine and tryptophan, which occur only once in the molecule; (3) the relation of the quantitative amino acid analysis and of the sequences of the fragments to each other; (4) the relation of the quantitative amino acid analysis of the proposed structure to that of the starting material; and (5) the yield of the fragments.

In the following discussion, the structure (Fig. 2) will be derived by establishing which fragments arise from the N-terminus of the hormone and then adding sequences to the C-terminus of the partial structure. β-Corticotropin has serine N-terminal from previous work[1] using three different methods. Tyrosine and methionine were shown to be in the second and fourth positions, respectively, by sequential degradation of P2 and P3 which have the same N-terminus[2] as β. Since T10 contains the only methionine and both serines present in the hormone and is the only tryptic product containing these amino acids, it must be the N-terminal fragment. It has the required N-terminal serine and has tyrosine and methionine in the second and fourth positions. The corresponding N-terminal fragment in the chymotrypsin digest must also have serine N-terminal and contain tyrosine. C4 is the only such peptide containing serine and tyrosine and having the same sequence as the N-terminal portion of T10 and of β. C7 must be a portion of the T10 sequence, since it contains the remaining serine and the only methionine and histidine residue (as does T10) and is the only chymotryptic peptide containing the latter two amino acids. It must adjoin C4, since its N-terminal sequence constitutes the third and fourth amino acids of T10 and of β and since it comprises the remainder of the amino acids of T10 except for the C-terminal arginine. The sequences of T10 (complete except for the positions of histidine and glutamic acid) and of C4 and C7 (both complete) establish the sequence of the first to eighth amino acids.

Since the C-terminus of T10 is . . . His–Phe–Arg·OH and of C7 is . . . His–Phe·OH, an arginine N-terminal peptide from the chymotrypsin digest would provide the basis for connecting a trypsin fragment to the C-terminus of T10. C5, H·Arg–Try·OH, is the only arginine N-terminal peptide in the chymotrypsin digest, and hence it must adjoin C7. The tryptic cleavage to give T10 with arginine C-terminal will simultaneously give a tryptophan N-terminal product such as T14. The latter must be the adjacent portion since it contains the only tryptophan residue present in the hormone. These cleavages of the proposed sequence . . . $Phe_{\overline{7}}Arg_{\overline{8}}Try_{\overline{9}}$ . . . at bond 8 by trypsin and at bonds 7 and 9 by chymotrypsin are consistent with the specificity of these enzymes.[8a] The completely determined sequence in T14 then established the structure through the 15th amino acid.

(8) (a) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 126 (1950); E. L. Smith, *Adv. in Enzymology*, **12**, 226 (1951). (b) For a discussion of other methods considered see Experimental.

C16 is the chymotryptic fragment which represents the T14 sequence except for the tryptophan.

Trypsin produces only three major cleavages of β-corticotropin yielding just four major products (T10, T14, T17 and T1). T1 contains the same C-terminal sequence[1] as β-corticotropin and contains the leucine, aspartic acid and alanine among others which are removed by pepsin degradation.[2] Therefore, T1 must be the C-terminal fragment and T17 should be the fragment between T14 and T1. Positive evidence for the linkage of T14 to T17 is furnished by the minor cleavage products T16 and T18. They represent the same portion of the chain as T14 and T17 since they contain the single tryptophan, all the lysine and the remaining arginine, valine, glycine and proline. T16 and T14 must have a common origin since there is only one tryptophan residue in the molecule. Therefore, T16 and T18 arise from an alternate cleavage which was found to occur to a small extent. T16 was shown to be merely T14 with an additional lysine at the C-terminus, hence the lysyl–lysine bond thus established must involve T17 since it contains all the rest of the lysine. The amino acids in the 16th–21st positions were determined from the complete sequence of T17. T18, on the basis of its composition and its C-terminal valyl–lysine sequence (as in T17), represents the other product from the cleavage producing T16. Another trypsin cleavage occurs in still smaller amount at bond 17 between two arginines to form T19 and T15, which represent the N- and C-terminal portions, respectively, of T17.

The analysis of the chymotrypsin fragment C10 corresponds to the sum of T17 plus T1 less the glutamyl–phenylalanine C-terminus (C2) of the latter. The finding that leucine is C-terminal in C10, and that its N-terminal sequence is the same as the T17 sequence means that it comprises the T17 plus T1 portion of the peptide chain. This fact is an additional reason for linking T14 to T17. C10 also provides positive evidence for connecting T1 to T17 through the lysyl–valyl bond (no. 21), since the N-terminal valine of T1 and the C-terminal lysine of T17 are internal in C10. The fragments T10, T14, T17 and T1, which comprise over 90% of the trypsin digest of β-corticotropin, account quantitatively for its entire amino acid composition. The fragments C4, C7, C5, C16, C10 and C2 likewise account for the entire amino acid content of the hormone.

Elucidation of the sequence of T1 by the two sequence methods employed posed a special problem. The N-terminal sequence H·Val–Tyr–Pro . . . was clearly demonstrated by the Edman method. However, at each of the fourth, fifth and sixth stages of degradation, several amino acid "end groups" were obtained as a result of random cleavage. Carboxypeptidase degraded the C-terminus . . . Pro–Leu–Glu–Phe·OH only until it reached the other proline present in T1. The 11 amino acid C-terminal sequence (29th–39th positions) was derived[2] from quantitative amino acid analysis and sequence data on P2, P3 and on the eight small peptides (five peptides (1, 2, 3, PA1, PA2) are shown in Fig. 2) isolated from pepsin digests of β and of P2.

The sequence in the four remaining positions (25th–28th) was determined primarily from data obtained by partial acid hydrolysis. The pepsin product[2] P4, consisting of amino acids 1–28, has this sequence at its C-terminus. However, this substance was rather unreactive to carboxypeptidase yielding only a small amount of glutamic acid. By analogy with the results above, it was expected that trypsin would split off from the carboxyl end of P4 a heptapeptide, which would be the simplest peptide containing this still-to-be-determined sequence. From a tryptic digest of P4, this heptapeptide (P4T1, K 0.39) was separated by a 486-transfer countercurrent distribution in 1-butanol *vs.* 20% acetic acid, analysis of which is shown in Fig. 3. This material was judged to be pure[9] by comparison with the theoretical curve (Fig. 3), by paper chromatography and by quantitative amino acid analysis. The latter and the subsequent determination of the N-terminal sequence demonstrated that the material in this peak was the desired heptapeptide.

Attempted determination of the structure of P4T1 by the Edman reaction clearly gave H·Val–Tyr–Pro . . . and, in the subsequent stages, decomposition as found with T1. P4T1 gave no reaction with carboxypeptidase, but its phenylthiocarbamyl derivative liberated traces of alanine and glutamic acid. A new approach seemed necessary and the acid lability observed during the Edman reaction made partial acid hydrolysis promising. Deriving the structure of P4T1 from its fragments was facilitated by the fact that each amino acid occurred only once and by the partially known sequence. Paper chromatography of a partial acid hydrolysate separated the major components I, II, IV and VI (Fig. 2). They were considered to be pure on the basis of re-chromatography, quantitative amino acid analysis and sequential degradation. The complete sequences of all four were determined by the Edman method. These results formed a unique over-all sequence for the 22nd–28th positions since the N-terminal sequence of the starting material was known. Additional evidence for the sequence shown is the C-terminal glutamic indicated by a modified Dakin–West reaction on P4T1.

The results of all these degradations provide an adequate basis for proposing a single consistent sequence (Fig. 2) for β-corticotropin. The question of alternate linkage has been considered in the case of each polyfunctional amino acid. Linkage through the ε-amino groups is ruled out by the reaction[1] of all the lysine in β with dinitrofluorobenzene, as well as by the Edman degradation results on the lysine-containing fragments. Alternate linkage of the chain through the γ-carboxyl of glutamic acid was also ruled out by the Edman degradation results in the case of bonds 5, 30, 33 and 38. Application of a modified Dakin–West reaction to P4T1 indicated that bond 25 was formed by

(9) When, in an early experiment, a mixture of P4 and P3 such as is present in an unfractionated vigorous pepsin digest of β-corticotropin was used for trypsin digestion, this peak was a partly resolved doublet of P4T1 and P3T1 after 500 transfers. P3T1 is the closely related nonapeptide resulting from similar cleavage of P3 by trypsin. Since the composition of these two peptides is qualitatively the same, and the properties are so similar, the fact that both are present might easily be overlooked.

the α-carboxyl group of the aspartic acid. This reaction gave equivocal results with P3T1 and leaves the status of bonds 28 and 29 uncertain. The cleavage of bond 28 by pepsin suggests an α-linkage.

An incomplete structure has been proposed[10a] for hog Corticotropin-A, which appears to be merely the alkali-deamidated form of β-corticotropin. The proposed structure is consistent with this possibility except for a difference in the 25th–28th amino acid sequence, which involves only the transposition of aspartic acid from the 25th position to the 28th position. White and Landman's[10a] sequence of . . . Gly–Ala–Glu–Asp . . . is based on the results of Edman degradation. In our hands this region of the peptide chain was so unstable once the . . . Pro–Asp . . . bond was split by the Edman degradation that a valid assignment of a sequence seemed impossible. Thus their sequence . . . Gly–Ala–Glu–Asp . . . could result from decomposition and actually be the 26th–29th amino acid sequence in T1 or in a P3T1 contaminant[9] of P4T1. The analysis and sequence of peptide I, valyltyrosylprolylaspartic acid, showed that this fragment was pure and clearly placed aspartic acid in the 25th position. Therefore, the . . . Gly–Ala–Glu . . . sequence, which is common to White and Landman's structure and ours, would have to be in positions 26–28. Further evidence for placing it in this location is the C-terminal glutamic acid in P4T1.

Subsequent publication[11] of a proposed structure for sheep corticotropin presents an interesting comparison. Its over-all composition is reported to be the same as hog β-corticotropin except for a single amino acid unit (leucine replaced by serine) and one or more additional unsubstituted amide groups. It is noteworthy that the species difference in composition occurs in the "non-essential" C-terminal sequence: . . . Ala–Ser . . . at the 31st–32nd positions as compared to . . . Leu–Ala . . . in Fig. 2. The portion of the molecule which appears[2] to be essential for corticotropin activity (amino acids 1–24) has been assigned exactly the same structure as reported[3a] from these laboratories for hog corticotropin. A sequence for the adjacent "unstable" 25th–28th amino acid region has been proposed which differs from the two discussed above but involves the same four amino acids for these four positions. Edman degradation results on their "trypsin peptide no. 2,3" are subject to the reservation mentioned above. On the basis of the Edman degradation results on T1 we had earlier[3a] tentatively also proposed this sequence, but the results of partial acid hydrolysis of P4T1 compelled us to discard it. Their assignment of sequence is based primarily on the isolation of peptide WOE-4, H·Ala(Gly,Glu,Asp)·OH. A peptide of this composition can arise from the 26th–29th units in the β-corticotropin structure but would not have alanine N-terminal as reported. It is to be hoped that further work will either clarify the nature of this portion of the hormones or demonstrate a peculiar structure[12a] which is responsible for the various results.

(10) (a) W. F. White and W. A. Landman, THIS JOURNAL, **77**, 1711 (1955); (b) W. F. White, *ibid.*, **77**, 4691 (1955).

(11) C. H. Li, I. I. Geschwind, R. D. Cole, I. D. Raacke, J. I. Harris and J. S. Dixon, *Nature*, **176**, 687 (1955).

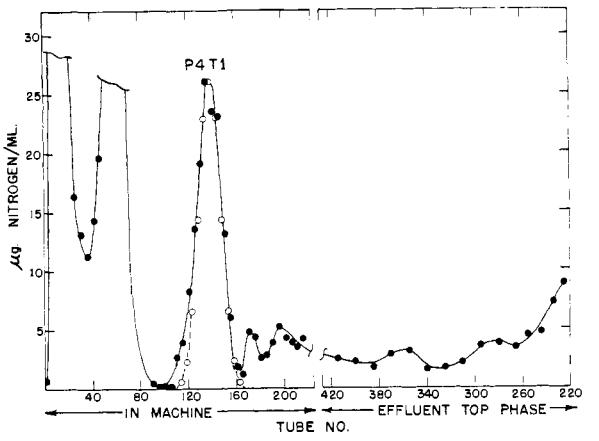

Fig. 3.—Countercurrent distribution of trypsin-digested P4 in 1-BuOH *vs.* 20% AcOH after 486 transfers (81 mg. charged in 3 tubes of 220 tube machine): ●——●, concentrations in effluent top phase and in contents of machine; O——O, theoretical curve.

Certain information about the detailed structure necessary for corticotropin activity can be deduced from various degradations of the hormone. Pepsin digestion to P2, P3 and P4 removes 8, 9 and 11 amino acids, respectively, without changing the corticotropin potency. The failure of acid degradation of β-corticotropin (using conditions more drastic than those required to split completely bonds 24 and 25 in P4T1) to destroy corticotropin activity suggests that an additional four amino acids, in positions 25–28, are also not essential for activity.[12b] All the other fragments shown in Fig. 2 were inactive at a very high dosage (40 μg./100 g. rat). Inactivation of β-corticotropin by alkali is associated with cleavage of the first, second and third bonds (Fig. 2) and inactivation of Corticotropin-A by pepsin is accompanied by cleavage of the fifth.[10a] Inactivation of Corticotropin-A by aminopeptidase has been reported by White[10b] to involve cleavage of bonds 1 and 2. Thus, extensive degradation at the carboxyl end of the hormone can be carried out without loss of activity, while all reactions involving the amino end have caused inactivation. The foregoing observations indicate that any active peptides smaller than those reported to date must retain the amino terminal sequence intact and be larger than the inactive octapeptide, T10.

This series of papers[1,2] has presented the preparation and determination of the structure of pure β-corticotropin. This substance is considered to be pure in spite of the presence of another hormonal activity, intermedin. The significance of the latter has been carefully investigated, and we have concluded that the pure peptide hormone possesses both kinds of biological activity. A similar exam-

(12) (a) The occurrence of a small amount of an isomer of P4T1 in its partial acid hydrolysate may indicate such a peculiarity in structure which may also be responsible for the resistance to carboxypeptidase digestion. (b) A similar "active unit" (radiation molecular weight 2400 ± 800) is indicated by the deuteron bombardment *vs.* biological activity studies of R. G. Child, A. W. Moyer, H. R. Cox and E. Pollard, *Arch. Biochem. Biophys.*, **61**, 291 (1956).

ple of the presence of two biological activities in a pure hormone is provided by arginine–vasopressin. The synthetic peptide, as well as the pure natural peptide, retains 15% avian vasodepressor (oxytocic) activity.[13] Although the intermedin activity of the various pure corticotropin substances is of a "low" order, we do not feel it can be disregarded. The constancy of the intermedin-to-corticotropin activity ratio for preparations with very widely different histories and properties strongly suggests that the minimum structural requirements for the type 2 intermedin[14a] activity are to be found within the amino acid sequence of the β-corticotropin structure. The main corticotropin fraction (which has had the δ_1-intermedin fraction removed) of oxycellulose–ACTH has been separated[1] into α_1, α_2, α_3, β, γ_1, γ_2 and γ_3 corticotropin components, each of which retained the intermedin activity. The pepsin-digest products P2, P3 and P4 from β-corticotropin were isolated as three distinct countercurrent distribution peaks and again each retained the intermedin activity.[2] Deamidation of β produced a more acidic substance,[1] in contrast to the more basic pepsin degradation products, and again the intermedin accompanied the product into a new ion exchange column peak.

Alkali-boiled β, on distribution for 100 transfers in 1-butanol *vs.* 0.5% trichloroacetic acid, gave two widely separated peaks (K *ca.* 15 and 0.02), both of which had low corticotropin activity (at *ca.* 10 μg./100 g. of rat) and high intermedin activity (at *ca.* 0.1 μg./100 g. of frog). Thus the new peak (K 0.02) contained corticotropin activity with a different distribution coefficient from the starting material. These results suggest that the β-corticotropin structure elicits both hormone responses[14b] and that alkali treatment markedly alters the quantitative relation of the two responses.

Both intermedin and corticotropin activities are rapidly destroyed by mild trypsin and chymotrypsin digestion. No intermedin activity was present in any of the degradation fragments (Fig. 2) of trypsin and chymotrypsin digests when tested at a very high dosage.

Experimental

Trypsin Digestion as Used on β-Corticotropin and Its Pepsin Product, P4 (Enzyme:Substrate Ratio, 1:100).—An aqueous solution of β-corticotropin (0.5 mg./ml.) was treated with $^1/_{200}$ volume of trypsin (Worthington Biochemical Sales, "2X crystallized, salt-free") solution (1.0 mg./ml.), and with sufficient dilute ammonia to adjust the final *p*H to 7.8–8.0. β-Corticotropin is not entirely soluble at this *p*H, but the slight turbidity of the solution disappeared within 2–3 minutes after enzyme addition. The stoppered solution was held at 25° for 4 hours and then lyophilized.

Chymotrypsin Digestion of β-Corticotropin (Enzyme: Substrate Ratio, 1:100).—An aqueous solution of β-corticotropin (0.5 mg./ml.) was treated with $^1/_{200}$ volume of chymotrypsin (Worthington Biochemical Sales, "crystalline, salt-free") solution (1.0 mg./ml.), and with sufficient dilute ammonia to adjust the final *p*H to 7.8–8.0. The stoppered solution was held at 25° for 24 hours and then lyophilized.

Partial Acid Hydrolysis of P4T1.—The peptide solution (0.6 mg./ml.) in 0.1 N HCl was sealed (*ca.* 1.5 ml./tube) in 0.7 cm. i.d. bomb tubes and placed in an oven at 110°. The liquid in such a tube was shown to reach oven temperature within 15–20 minutes. Preliminary experiments indicated that all the P4T1 had disappeared by 2 hours and that, at 2–4 hours, there was a maximum of small peptide material present. In 24 hours there appeared to be complete conversion to peptide II (valyltyrosylproline) and amino acids. The main sample (6 mg.) was heated in 7 tubes for a total of 2 hours. After heating, the tubes were cooled in ice, opened and pooled. Evaporation was carried out on a steam-bath under a stream of nitrogen. The sample was dissolved in water and re-evaporated, the total drying process requiring a maximum additional heat exposure of 45 minutes.

The hydrolysis mixture was separated by chromatography as a band on a paper sheet, using 5:4:1 1-BuOH:H_2O:HOAc. Strips cut from each side were sprayed with ninhydrin and used to guide the cutting of the chromatogram into "horizontal" strips containing the separated peptides, which were then eluted with 0.1 N HCl. Re-chromatography in the same solvent system was used to confirm the presence of a single component and to prepare samples of higher purity. Chromatography in at least one other solvent system was used to check the homogeneity before quantitative analysis and sequence determinations.

Countercurrent Distribution. (A). Trypsin Digest.—The 200 mg. of trypsin-digested β-corticotropin was charged in the micro Craig machine[2] in 7 tubes (*ca.* 7.7 ml. total) as a saturated solution in equilibrated bottom phase from 1-BuOH:20% HOAc. After about 20 transfers an emulsion formed and the settling time had to be increased to 20 minutes. The settling time was decreased gradually to 4 minutes at n = 600 and to 0.5 minute at n = 2000. The shaking time was 3 minutes throughout. Initially the top phase volume was 1.2 ml., but at n = 1000 it was increased to 1.6 ml. in order to increase the effective distribution coefficient of the remaining material having low K-values. The effluent top phases were collected three in a tube up to n = 800 and ten in a tube for the remainder. The analysis of the distribution is outlined above and the results at n = 1400 are given in Fig. 1. The deviation of the fast peak (Fig. 1) from the theoretical curve was shown by paper chromatography to be due to the presence of a small amount of T2, which is apparently an ester of the main component, T1. The top phase was allowed to pass out of the machine until 5000 transfers had been carried out. At this point the top phase was recycled in the machine for a total of 8207 transfers. The important products were present in the following effluent pools: T2, 232–244; T1, 245–280; T10, 1600–2200; T14, 2500–4100; T15, 4600–4900; at n = 8207, T19 was present in tubes 1–60 in the machine, T16 and T18 in tubes 190–220 and 0–30, and T17 in tubes 140–210. As can be seen from these data, a low coefficient (*e.g.*, K 0.1) material forms a very much wider peak in the effluent top phases than in the machine because of the small fraction moving out with each transfer. Pure samples of T16, T18 and T19 were prepared by paper chromatography.[2]

The important products are not numbered consecutively because the areas between recognized peaks which were pooled and numbered arbitrarily were found to contain no significant fragments. The distribution fractions were pooled on the basis of nitrogen and paper chromatographic analysis. Recognition of the peaks of most of the fragments was not possible on the basis of nitrogen analysis because of the low concentrations and proximity of the peaks. Paper chromatography using several specific tests constituted a very critical method which permitted very precise cutting of even overlapping peaks (*e.g.*, C4, C5 and C7). The various peptides were recognized by their R_f values and by differences in their color with ninhydrin (heating at 105° for 20 minutes for peptides) and in their reactions to specific tests. Effluent cuts at selected intervals were lyophilized and spotted on a paper sheet for chromatography in 5:4:1 1-BuOH:H_2O:HOAc. The developed chromatogram sheet

(13) V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, THIS JOURNAL, **76**, 4751 (1954).

(14) (a) Two types of intermedin activity were found in corticotropin preparations and were clearly separated (ref. 1) by countercurrent distribution: (1) high activity which is not increased by alkali treatment (δ_1-fraction is of this type), and (2) low activity which is potentiated by alkali treatment. The α-, β- and γ-corticotropins and the active degradation products P2, P3 and P4 fall in this latter class. Hot dilute alkali (0.1 M NaOH, 100°, 20 min.) rapidly destroys the corticotropin activity with a simultaneous 30- to 100-fold increase in the intermedin activity of type 2 material. After alkali treatment both types of intermedin have a more prolonged action than untreated material. (b) H. B. F. Dixon [*Biochim. Biophys. Acta*, **19**, 393 (1956)], using a different approach, reached the same conclusion.

was cut into strips containing one sample and each strip was then cut into longitudinal sections. These sections were subjected to the Durrum test, the Sakaguchi test for arginine, the dimethylaminobenzaldehyde test for tryptophan, the *p*-chlorobenzenediazonium coupling test for histidine and/or tyrosine, and the platinic iodide test for methionine (*vide infra*).

(B) **Chymotrypsin Digest.**—The *delayed* appearance of an emulsion was also observed here and the emulsification was much worse than in the trypsin case. It was necessary to dilute the solution and charge the 179-mg. sample in 24 tubes (*ca.* 27 ml.). The distribution and analysis were carried out as above. The major products were found in the following effluent pools: C2 along with some C3, 400–500; C4, 600–800; C5, 800–1000; C7, 1000–1300; C10, 3300–5000; at $n = 9518$ (total transfers) three minor components were present in the machine in addition to C16 in tubes 116–210 and C13 in tubes 5–115. Pure C2 was separated by paper chromatography from a small amount of peptide contaminant, C3. This C3 material corresponded to the lysine-through-leucine region (Fig. 2, amino acid units 21 through 37) on the basis of its quantitative amino acid analysis.

The distribution samples were recovered by lyophilization of the pooled aqueous phases after all the material had been extracted from the butanol by water or 1 *N* acetic acid. Petroleum ether was added to the solutions of components of higher *K* in order to facilitate extraction.

One other distribution system, 1-BuOH *vs.* 5% trichloroacetic acid, was tried for separation of the basic fragments in the tryptic digest of β-corticotropin. T10 (K 5.5) was easily separated from a peak (K *ca.* 0.9) comprising the six T14–19 peptides. This system was set aside because of the possibility of incomplete recoveries, but this and related systems warrant further study.

Stepwise Degradation of Peptides from the Amino End.—On the basis of preliminary studies, the Edman phenyl isothiocyanate method[4] was selected. Application of the method in its original form to β-corticotropin and to basic peptides was impossible due to insolubility of the poly-phenylthiocarbamyl derivatives in the cyclization solvent, nitromethane. However, the solubility in glacial acetic acid at 80° was found to be sufficient for micro work. An extraction procedure was designed for use on peptide samples of about 1 mg. Numerous other modifications of the Edman method subsequently published[15] have not been investigated here. Regeneration of the amino acid from the phenylthiohydantoin was decided to be the only practical way to obtain simultaneous identification and quantitative determination of the end group on a microgram scale.

The procedure for corticotropin peptides was as follows: the peptide ($2.0–50 \times 10^{-7}$ mole, *ca.* 200 μ g.–5 mg.) was dissolved in 2.0 ml. of 50% aqueous pyridine at 40° in the reaction vessel, and 2×10^{-4} mole of phenyl isothiocyanate (Eastman Kodak Co.) was added; 0.1 *N* NaOH was used to bring the *p*H to 8.75 (glass electrode) and to maintain it while stirring at 40° for 45 minutes. The reaction mixture was extracted with C.P. benzene, once with 2 volumes, and three times with 1 volume. The benzene phase, containing only pyridine and reagent, was routinely discarded. The aqueous phase was adjusted at 25° to *ca.* *p*H 4 (congo red paper) with 0.5 *N* HCl and immediately extracted four times with 1 volume of ethyl acetate to separate the peptide derivative from the salt formed. This extraction was omitted when the peptide contained arginine or histidine, since the basicity of the phenylthiocarbamyl (PTC) derivatives of such peptides does not permit extraction at this *p*H. This extract was evaporated to dryness at 25° in a stream of nitrogen and the residue was dissolved in 0.5 ml. of glacial acetic acid. The tube was plugged with glass wool and the solution was saturated with hydrogen chloride gas. It was then heated for 15 minutes at 75–80° (water-bath), stirring with a slow stream of HCl. The solution was evaporated immediately *in vacuo* over solid sodium hydroxide.

The residue was extracted with ethyl acetate to remove the cleaved phenylthiohydantoin (PTH) from the residual peptide. This separation was carried out as a four-tube countercurrent extraction, with the residue dissolved in water in the first tube, 0.05 *N* HCl in the second and water in the remaining two. Three successive 0.5-ml. portions of ethyl acetate were equilibrated in turn with each of the 0.5-ml. aqueous phases. The organic phases were then combined, as were the aqueous phases. If the peptide contained histidine, the aqueous phase was adjusted to *ca.* *p*H 8 (alkacid paper) with 0.1 *N* NaOH and the four-tube countercurrent extraction with ethyl acetate was repeated using *p*H 8 $NaHCO_3$ as aqueous phase. This extraction removed histidine PTH from the residual peptide. The aqueous solution of the residual peptide was lyophilized before further application of the reaction.

The ethyl acetate extract(s) containing the PTH of the N-terminal amino acid was evaporated to dryness in a nitrogen stream at 25°. A solution of the residue in 0.2 ml. of carbonate-free $Ba(OH)_2$ (0.25 *N*) was sealed in a bomb tube (diam. 0.7 cm.) and heated in an air oven at 150° for 2 hours to regenerate the amino acid. This dilution was required for complete recovery of aspartic and glutamic acids which were otherwise adsorbed by the $BaCO_3$. A stream of CO_2 was bubbled through the solution on the steam-bath and, after centrifugation, the $BaCO_3$ precipitate was washed twice with 0.5 ml. of hot water. One drop of concd. HCl was added to the combined supernate and washings (to eliminate the loss of glutamic acid by pyrrolidone formation observed at neutrality) and the aqueous solution was evaporated twice under nitrogen (steam-bath). The regenerated N-terminal amino acid was identified and quantitatively determined by paper chromatography[2] alongside suitable standards.

Edman End-group Results.—Proof that the end of a peptide chain had been reached was obtained by demonstrating that a single amino acid[16] was liberated when the presumed final peptide bond had been cleaved. This information established that a peptide had the minimum length possible on the basis of its quantitative amino acid analysis.

Serine and arginine are not obtained as end groups in the Edman procedure above. Serine PTH is so unstable to the acid cyclization conditions (characteristic red product) and to alkali that no serine was ultimately obtained. Failure to obtain an end group, coupled with the red color at cyclization, was sufficient to identify the position of the serine. The basic arginine PTH cannot be extracted from the residual peptide and a blank end group was interpreted as arginine if no serine was present. Formation of the extractable guanidino-PTC derivative at *p*H 13 was not used for arginine peptides since the reaction was far from complete (20–40%).

The end group from synthetic tryptophyllysylglycine, and from free tryptophan, consisted of a 5–10% yield of **tryptophan** accompanied by two break-down products, R_f 0.58 and 0.25, which contained arylamine[17] as well as α-NH_2 groups. The extensive decomposition and by-product formation was shown to occur during the acid cyclization and not during alkaline hydrolysis. With the tryptophan-containing peptide, T14, milder cyclization–cleavage conditions (glacial HOAc, $^1/_{20}$ saturated with HCl, at 40° for 30 minutes) gave an 11% yield of tryptophan and neither of the decomposition products. General application of the arylamine test would prove helpful in unequivocal end-group identification and should eliminate the possibility of mistaking these products for amino acids.

Identification and quantitative measurement of **lysine** in the alkaline hydrolysates was made difficult by the effect of residual salt in the lysine region of the chromatogram. Confirmatory identification of lysine by hydrolysis of part of the unknown PTH in 48% HBr (several artifact spots also formed) was used.

Quantitative measurement of the liberated amino acid was desired as an indication of the homogeneity of the peptide and as a basis for designation of the predominant amino acid as the end group. Traces of other constituent amino acids are often visible in this procedure and generally increase in amount as succeeding stages are carried out. The cause of the poor yields of the N-terminal amino acid generally obtained was investigated. Synthetic phenylthiohydantoins were found to be quantitatively converted to amino acids by the alkaline hydrolysis, and standard amino acids were quantitatively recovered after exposure to "acid cyclization" and alkaline hydrolysis. However, consider-

(15) *Cf.* refs. in H. Fraenkel-Conrat and J. I. Harris, THIS JOURNAL, **76**, 6058 (1954).

(16) C-terminal lysine was identified as ε-PTC-lysine which had R_f 0.65 in 5:4:1 1-BuOH:H_2O:HOAc.

(17) R. G. Shepherd, *Proc. Am. Phar. Manuf. Assoc.*, p. 30, Feb. 1949.

able decomposition (50–70%) resulted from exposure of the phenylthiohydantoins and phenylthiocarbamyl derivatives to the acid-cyclization. This decomposition was assessed on the basis of the amount of amino acid found after alkaline hydrolysis. Modifications of the above conditions employing aqueous acid, less HCl, lower temperature or less time gave varying degrees of peptide bond cleavage in PTC-valylalanine. Under the mildest conditions (gl. HOAc, $^1/_{20}$, saturated with HCl, at 40° for 30 minutes) giving 80–100% cleavage, alanine PTH was found to be stable (82–92%). When these conditions were applied to various peptides, the yield of end group was about 30%, indicating that a critical phase of the decomposition occurs during the cleavage and/or cyclization. Many of the peptides were less soluble under these milder conditions. Low yields of end group and instability of the PTH derivative has been observed elsewhere[15,18] using different acidic conditions. The yield of end group generally decreased as the stepwise degradation proceeded. However, in spite of these various difficulties, interpretation of the results was possible since the terminal amino acid was usually present in the end-group hydrolysate in 5- to 10-fold the amount of any others.

C-Terminal Sequence Determination by Carboxypeptidase Digestion.—The carboxypeptidase digestion procedure[19] for the degradation of peptides from the carboxyl end was modified to permit direct quantitative paper chromatography of lyophilized aliquots. To this end, a volatile ammonium acetate–ammonium bicarbonate buffer was used to dissolve the enzyme, simultaneously effecting increased enzyme solubility and maintenance at the optimum *p*H for enzymatic digestion. An antibiotic mixture of penicillin, streptomycin and octyl alcohol was added to the digest to prevent bacterial consumption of the liberated amino acids (glutamic acid is especially susceptible to such consumption), and did not contribute to the amino acid blank. Carboxypeptidase, even when recrystallized several times, still contained enough enzyme impurities to split partially the trypsin- and chymotrypsin-labile bonds in β-corticotropin, P2, P3 and P4.[2] Treatment of the enzyme with diisopropyl fluorophosphate (DFP)[20] reduced this effect, but the DFP-treated enzyme was considerably less soluble, hence it was not used on many of the simpler peptides. The "enzyme blank" was satisfactorily reduced by multiple washing of the enzyme with distilled water. The rate of liberation of amino acids depends on the nature of the C-terminal amino acid and the penultimate amino acid as well.[8a]

Procedure.—The peptide substrate was dissolved in water or *p*H 7.8 buffer (6:1 mixture of 1.0 *M* solutions of ammonium acetate and ammonium bicarbonate) at 1.0 mg./ml. concentration. The washed enzyme was dissolved in the buffer at 0.4 mg./ml. and an equal volume was added to the substrate, along with a $^1/_{20}$ volume of the antibiotic mixture and 1–2 microdrops of octyl alcohol. The *p*H was found to be 7.7–7.9. The final solution contained, per ml., 0.5 mg. of substrate, 0.2 mg. of enzyme, 10 μg. of streptomycin, 6 μg. of penicillin, was saturated with octyl alcohol, and was 0.25 *M* in ammonium salts at *p*H 7.8.

Aliquots were removed at various time intervals (2–72 hours), lyophilized and re-lyophilized from 0.1 *N* HOAc. Quantitative paper chromatography[2] revealed the identity and amounts of liberated amino acids. It is generally advisable to elute and hydrolyze the spots to ensure that new peptides formed by digestion of the substrate are not interpreted as amino acids (*cf.* peptide T14 below).

Dakin–West Reaction[21] as Identification of the C-Terminus.—A note[22] by Turner and Schmerzler on such use of this reaction was the basis of our initial work, but we were unable to get satisfactory results with their conditions. The Dakin reaction has been used to prepare various α-acylamino methyl ketones from amino acids by treatment with acetic anhydride and pyridine at 60–150° for 0.5–5 hours. For end-group determination, the use of a high temperature (150°)[22] to ensure the most complete reaction leads to considerable decomposition of the peptide and of the reagents themselves. In line with the observation[23] that stirring increases the yield of the reaction, we found that stirring with a stream of nitrogen gave satisfactory reaction at a lower temperature (80°). An atmosphere of nitrogen, either under these conditions or in a sealed tube, decreased the amount of decomposition. The amino ketone present in the acid hydrolysate is ninhydrin-positive, and we found it advisable to oxidize it to the corresponding pyrazine in ammoniacal solution prior to chromatography.

The conditions giving the least decomposition of peptide (30–50%) and of reagents, with at least 70% reaction were: $2.0\text{–}40 \times 10^{-7}$ mole (*ca.* 200 μg.-4.0 mg.) of peptide heated at 80° for 6.5 hours in 0.3 ml. of C.P. acetic anhydride and 0.2 ml. of distilled dry pyridine. The mixture was continuously stirred with a stream of nitrogen and the resulting evaporation was counteracted by additions of 3:2 Ac_2O: pyridine to constant volume. After the reaction, 0.2 ml. of water was added and the sample was evaporated on a steam-bath under nitrogen. The residue was re-evaporated twice from water and then hydrolyzed for 21 hours in 0.6 ml. of 6 *N* HCl. The hydrolysate was evaporated and re-evaporated twice from water prior to the addition of 1.0 ml. of 0.5 *N* NH_4OH and 20 minutes aeration. After evaporation, appropriate amounts were spotted for paper chromatographic analysis.

Carbodiimide Method[24a] for C-Terminal Stepwise Degradation.—The peptide (10^{-3} mole) was dissolved in the minimal amount (1–10 ml.) of absolute alcohol and di-*p*-tolylcarbodiimide, in 10% excess, was added. The mixture was allowed to stand for 24 hours at 25° in a closed vessel, after which it was made up to 100 ml. with alcohol. After the addition of 100 ml. of 0.02 *N* NaOH, the mixture was warmed 10–15 minutes (steam-bath), cooled, filtered and the solid washed with alcoholic alkali and water. The residual acylpeptide remains in the alkaline solution, while the precipitate contains the tolylureidoaminoacyltoluidide of the C-terminus. Amino acid analysis of the precipitate and of the solution were carried out to determine how cleanly the end group was split off.

The results on various synthetic peptides showed that the size of the penultimate side chain controlled the extent of the desired reaction. If the penultimate R group were H, as in carbobenzoxyglycyl-L-leucine,[24b] carbobenzoxyglycyl-DL-phenylalanine[24c] and in phthaloylglycyl-DL-alanine,[24d] the C-terminal amino acid came off cleanly. If, however, the penultimate R group was large, as in carbobenzoxy-DL-valyl-DL-alanine[24c] and carbobenzoxy-L-leucylglycine,[24e] no end group cleavage could be demonstrated. When reaction did occur, the original peptide was regenerated. In view of this blocking of the reaction by certain side-chains present in β-corticotropin, the method was set aside.

Schlack–Kumpf Method[25] for C-Terminal Stepwise Degradation.—This procedure was adapted to a micro scale on small model peptides, but application to β-corticotropin gave disappointingly low yields and multiple[26] end groups. The limitations of applicability of the method have been reported.[27]

Akabori Method[28] for Identification of the C-Terminus.—The method was satisfactorily adapted to small peptides but application to β-corticotropin gave low yields and multiple[26] end groups along with a peptide containing lysine, proline and valine. Such a tripeptide could result from the cleavage of bonds 10 and 13, or bonds 18 and 21 (Fig. 2), apparently by the water of hydration.[1] The amount of this material (R_f 0.90 in 1-BuOH *vs.* concd. NH_4OH, 3:2 vol. ratio) increased with reaction time (5 as compared to

(18) H. Fraenkel-Conrat and B. Singer, THIS JOURNAL, **76**, 180 (1954).

(19) K. Hoffman and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940); A. A. Plentl and I. H. Page, *J. Exp. Med.*, **79**, 205 (1944).

(20) E. W. Davie and H. Neurath, THIS JOURNAL, **74**, 6305 (1952).

(21) H. D. Dakin and R. West, *J. Biol. Chem.*, **78**, 91, 745 (1928); G. H. Cleland and C. Niemann, THIS JOURNAL, **71**, 841 (1949).

(22) R. A. Turner and G. Schmerzler, *ibid.*, **76**, 949 (1954).

(23) R. H. Wiley and O. H. Borum, *ibid.*, **70**, 2005 (1948).

(24) (a) H. G. Khorana, *J. Chem. Soc.*, 2081 (1952); (b) S. Goldschmidt and M. Wick, *Ann.*, **575**, 217 (1952); (c) J. R. Vaughan and R. L. Osato, THIS JOURNAL, **74**, 676 (1952); (d) G. W. Anderson, J. Blodinger, R. W. Young and A. D. Welcher, *ibid.*, **74**, 5304 (1952); O. H. Emerson, U. S. Patent 2,498,665; (e) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, 111, 238 (1935).

(25) P. Schlack and W. Kumpf, *Z. physiol. Chem.*, **154**, 125 (1926); S. G. Waley and J. Watson, *J. Chem. Soc.*, 2394 (1951).

(26) The additional end groups result from random splitting of peptide bonds.

(27) J. M. Swan, *Australian J. Sci. Research*, **A5**, 711, 721, 728 (1952); V. H. Baptist and H. B. Bull, THIS JOURNAL, **75**, 1727 (1953).

(28) S. Akabori, K. Ohno and K. Narita, *Bull. Chem. Soc. Japan*, **25**, 214 (1952).

18 hours). A good evaluation of the Akabori method has been published by Locker.[29]

Qualitative Tests for Amino Acids.—A number of specific amino acid tests were routinely used in the separation and isolation of the peptides for preliminary characterization and also for the detection of peptides in microgram concentrations during countercurrent distributions. The tests were carried out on paper, either on a developed chromatogram or on a small sample which had been spotted on paper. Semiquantitative results could be achieved by comparison with serially diluted standards, charged in identical fashion.

(1) **Diazonium coupling test**[30] for **histidine and tyrosine** (sensitivity 3 μg.): using diazotized *p*-bromoaniline the spots are red for histidine and brown for tyrosine.

(2) **Dimethylaminobenzaldehyde test**[31] for **tryptophan** (sensitivity 0.4 μg.): the purple spots turn blue on exposure to HNO_2 fumes, which serves as a confirmatory test. Arylamines give yellow or orange spots.

(3) **Durrum dye test**[32a] for **certain peptides**: our reagent was 2.8 g. of $HgCl_2$ and 3.6 g. of brom phenol blue in 360 ml. of methanol. The sensitivity was *ca.* 1×10^{-7} mole of peptide. The test was usually carried out on chromatograms which had been reacted with ninhydrin.

We found the test helpful throughout this work for distinguishing peptides from one another and from amino acids, generally. The following materials gave a positive test: T10, T14, T15, T16, T17, T18, T19, C5, C7, C10, P2, P3, P4 and β; these gave a negative test: T1, T2, C4, P4T1, I, II, IV, VI and PA1. A recent note by Geschwind and Li[32b] reported that this test was specific for histidine and its peptides. However, it is clear from the series of peptides above that this is not the case. Although the size of the peptide may modify the response, size alone does not determine the response. Thus, a large peptide such as T1 (18 residues) is negative, while small peptides such as C5, T19 and C7 (2, 2 and 5 residues, respectively) are positive. The test appears to depend on the presence of sufficient basic, heterocyclic or sulfur-containing amino acids to form a mercuric ion–dye complex which is insoluble in the final washing in tap water.

(4) **Iodoplatinic Acid Test**[33a] for **Methionine** (sensitivity *ca.* 10 μg).

(5) **Isatin test**[34a] for **proline**: for N-terminal proline peptides, the following procedure is more sensitive than the acetic acid method[34b]: the strip is sprayed with 0.2% isatin in water-saturated 1-BuOH containing 0.5% chloroacetic acid and heated at 105° for 20–25 minutes; sensitivity: 2×10^{-5} mole of peptide; 1×10^{-6} mole of proline.

(6) **Nessler periodate test**[35] for **N-terminal serine** sensitivity: 1×10^{-7} mole of NH_3 (*ca.* 1 μg. of serine).

(7) **Ninhydrin Test.**[2]

(8) **Sakaguchi Test**[36] for **Arginine** (sensitivity: 2 μg.).

Analyses and Structures of β-Corticotropin Peptides.—A standard format is used in describing the results for each peptide with the deduced structure (designated by the Brand convention[37]) given at the beginning. Of the qualitative tests which were applied, only those are listed which were positive. The R_f given is that obtained in 1-BuOH:H_2O:HOAc (5:4:1), with the ninhydrin color in parentheses. The calculated[7] K is the distribution coefficient in *n*-BuOH:20% HOAc. All amino acid quantities are as moles/mole of peptide. For the Edman reaction the moles of amino acid liberated per mole of peptide are listed for each successive stage, with the stage number in parentheses. Where more than one amino acid was detected, the one designated as the end group is italicized. The moles of amino acid liberated per mole of peptide by carboxypeptidase are given at various time intervals.

Trypsin Peptides

T1 H.Val–Tyr–Pro(Asp,Gly,Ala,Glu,Asp,Glu–NH_2Leu, Ala,Glu,Ala,Phe,Pro)Leu–Glu–Phe·OH

R_f: 0.72(purple) Calcd. *K:* 5 Qual. tests: Tyr

Analysis (7 detns.): 3.3 Ala; 2.3 Asp; 4.1 Glu; 1.1 Gly; 2.2 Leu; 1.9 Phe; 1.6 Pro; 0.8 Tyr; 1.0 Val; 0.8 NH_3

Edman: (1) 0.30 Val; (2) 0.21 Tyr; (3) *0.30 Pro*, 0.04 Ala, 0.03 Phe; (4) 0.06 Ala, 0.04 Leu, 0.03 Glu, 0.03 Phe, 0.02 Asp, 0.02 Gly; (5) 0.06 Ala, 0.03 Phe, 0.02 Glu, 0.02 Gly, 0.02 Pro

Carboxypeptidase: (12 hr.) 0.77 Phe; 0.38 Glu; 0.23 Leu
(120 hr.) 1.00 Phe; + Glu[38]; 1.00 Leu

T2 Ester (?) of T1

R_f: 0.84 (purple) Calcd. *K:* 8

Analysis (3 detns.): 3.1 Ala; 2.3 Asp; 3.7 Glu; 1.3 Gly; 1.9 Leu; 1.8 Phe; 1.7 Pro; 0.9 Tyr; 0.9 Val

Carboxypeptidase: (12 hr.) no reacn. except a small amount corresponding to the T1 contaminant

T10 H·Ser–Tyr–Ser–Met(Glu,His)Phe–Arg·OH

R_f: 0.36 (brown) Calcd. *K:* 0.13 Qual. tests: Arg, His, Met, Tyr

Analysis (6 detns.): 1.2 Arg; 1.1 Glu; 1.0 His; 0.9 Met; 1.0 Phe; 1.9 Ser; 0.9 Tyr

Edman: (1) Blank (= Ser); (2) 0.10 Tyr; (3) Blank (= Ser); (4) 0.003 Met

Carboxypeptidase: (12 hr.) 0.99 Arg; 0.65 Phe; + His; + Glu

T14 H·Try–Gly–Lys–Pro–Val–Gly–Lys·OH

R_f: 0.20 (purple) Calcd. *K:* 0.09 Qual. tests: Try

Analysis (7 detns.): 1.9 Gly; 1.9 Lys; 1.0 Pro; 1.1 Try (Folin analysis); 1.0 Val

Edman: 1st detn.: (mild cyclization conditions) (1) 0.11 Try; (2) 0.06 Gly; (3) 0.01 Lys. 2nd detn.: (usual conditions) (1) 0.07 Try + acid decomposition products; (2) *0.07 Gly*, 0.01 Lys; (3) *0.03 Lys*, 0.01 Pro, 0.01 Gly; (4) 0.09 Pro; (5) 0.02 Val; (6) *0.01 Gly*, 0.003 Lys; (Residue) 0.03 Lys

Carboxypeptidase: (12) hr.) 0.60 Lys; 0.11 Gly
(44 hr.) 0.97 Lys; 0.80 Gly; tr. Val
(68 hr.) 0.99 Lys; 0.80 Gly; + Val
(116 hr.) 1.00 Lys; 1.00 Gly; + Val

Three peptides isolated from the carboxypeptidase digest were hydrolyzed and had the following amino acid compositions: (Try,Gly,Lys,Pro), R_f 0.36; (Try,Gly,Lys,Pro,Val), R_f 0.45; (Try,Gly,Lys,Pro,Val,Gly), R_f 0.61.

T15 H·(Arg,Pro)Val–Lys·OH

R_f: 0.08 (red-purple) Calcd. *K:* *ca.* 0.05 Qual. tests: Arg

Analysis (2 detns.): 1.1 Arg; 1.0 Lys; 1.0 Pro; 0.9 Val

Carboxypeptidase: (48 hr.) 0.90 Lys; + Val

T16 H·(Try,Gly,Lys,Pro)Val–Gly–Lys–Lys·OH

R_f: 0.13 (red-purple) Calcd. *K:* *ca.* 0.05 Qual. tests: Try

Analysis (2 detns.): 2.3 Gly; 3.1 Lys; 0.9 Pro; 1.0 Try (Folin analysis); 1.0 Val

Carboxypeptidase: (20 hr.) 1.45 Lys; 0.45 Gly
(50 hr.) 2.03 Lys; 0.74 Gly; 0.03 Val
(2nd expt. at 68 hr.) 1.90 Lys; 0.84 Gly
(2nd expt. at 92 hr.) 2.14 Lys; 1.05 Gly; 0.07 Val

T17 H·Lys–Arg–Arg–Pro–Val–Lys·OH

R_f: 0.02 (blue) Calcd. *K:* 0.04 Qual. tests: Arg

Analysis (6 detns.): 2.2 Arg; 2.1 Lys; 1.0 Pro; 0.8 Val

Edman: (1) 0.06 Lys; (2) Blank (= Arg); (3) Blank (= Arg); (4) 0.13 Pro; (5) 0.03 Val; (Residue) 0.08 Lys

Carboxypeptidase: (50 hr.) 1.05 Lys; + Val

T18 H·(Arg,Arg,Pro)Val–Lys·OH

R_f: 0.10 (red-purple) Calcd. *K:* *ca.* 0.05

Analysis (2 detns.): 2.2 Arg; 1.1 Lys; 1.0 Pro; 0.9 Val

Carboxypeptidase: (48 hr.) 0.88 Lys; + Val

T19 H·(Lys,Arg)·OH

R_f: 0.05 (blue) Calcd. *K:* *ca.* 0.05

Analysis (2 detns.): 1.0 Arg; 1.1 Lys

P3T1 H·(Val,Tyr,Pro,Asp,Gly,Ala,Glu,Asp,Glu–NH_2)·OH

R_f: 0.36 (purple) Calcd. *K:* 0.39

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(38) Decreased by bacterial consumption before antibiotic procedure above was used.

Analysis (3 detns.): 1.2 Ala; 1.8 Asp; 1.7 Glu; 1.0 Gly; 1.0 Pro; + Tyr[39]; 0.8 Val

Peptide P4T1 and its Partial Acid Hydrolysis Products

P4T1 H·Val–Tyr–Pro(Asp,Gly,Ala)Glu·OH

R_f: 0.46 (purple) Calcd. *K:* 0.39

Analysis (3 detns.): 1.1 Ala; 1.0 Asp; 1.0 Glu; 1.0 Gly; 0.9 Pro; 0.6 Tyr[39]; 1.0 Val

Edman: (1) 0.35 Val; (2) 0.19 Tyr; (3) 0.17 Pro; (4) 0.07 Ala, 0.02 Glu, 0.01 Asp and/or Gly; (5) 0.03 Ala, 0.02 Glu, 0.03 Gly; (6) 0.04 Ala, 0.01 Glu, 0.02 Asp and/or Gly

Carboxypeptidase: (136 hr.) No reacn.; traces of alanine and glutamic acid were liberated from the phenylthiocarbamyl deriv.

Dakin reaction: Glu C-terminal, Asp α-linked. (analysis after reacn. and hydrol.: 0.9 Ala; 1.1 Asp; 0.3 Glu; 1.3 Gly; ++ Pro (not measured); 1.0 Tyr; 1.0 Val)

I H·Val–Tyr-Pro-Asp·OH

R_f: 0.59 (purple)

Analysis (1 detn.): 1.0 Asp; 0.8 Pro; 0.6 Tyr[39]; 1.3 Val

Edman: (1) 0.55 Val; (2) 0.30 Tyr; (3) 0.90 Pro; (Residue) 0.80 Asp

II H·(Val,Tyr,Pro)·OH

R_f: 0.70 (purple)

Analysis (1 detn.): 1.1 Pro; 0.9 Tyr; 1.0 Val

IV H·Ala–Glu·OH

R_f: 0.31 (purple) identical with synthetic H·Ala–Glu·OH

Analysis (1 detn.): 1.0 Ala; 1.0 Glu

Edman: (1) 0.26 Ala; (Residue) 0.65 Glu

VI H·Gly–Ala–Glu·OH

R_f: 0.25 (yellow) identical with synthetic H·Gly–Ala–Glu·OH[39a]

Analysis (1 detn.): 0.8 Ala; 0.9 Glu; 1.3 Gly

Edman: (1) *0.23 Gly*, 0.05 Ala; (2) 0.27 Ala; (Residue) *0.17 Glu*, 0.24 "Ala." This persistence of "alanine" in the residue may represent carry-over from stage 2 or unreacted alanylglutamic acid.

An additional peptide, R_f 0.54, was also isolated from the hydrolysis mixture. This peptide had an analysis identical with the starting material (1.1 Asp; 1.0 Ala; 1.0 Glu; 1.1 Gly; 1.0 Pro; 0.7 Tyr[39]; 1.3 Val), but chromatography beside intact P4T1 indicated that they were not identical. This "isomer" may be the result of some rearrangement within the acid portion of the peptide; its structure remains obscure.

Chymotrypsin Peptides

C2 H·(Glu,Phe)·OH

R_f: 0.66 (purple) Calcd. *K:* 0.8

Analysis (2 detns.): 1.0 Glu, 1.0 Phe

C4 H·Ser–Tyr·OH

R_f: 0.39 (brown) Calcd. *K:* 0.4 Qual. tests: Tyr, N-terminal Ser

Analysis (4 detns.): 1.1 Ser; 0.9 Tyr (1.1 Tyr, Folin analysis)

Edman: (Residue after stage 1) 0.83 Tyr

C5 H·Arg–Tyr·OH

R_f: 0.39 (purple) Calcd. *K:* 0.3 Qual. tests: Arg, Try

Analysis (2 detns.): 1.0 Arg; 1.1 Try (Folin analysis)

Edman: (residue after stage 1) 0.71 Try

C7 H·Ser–Met–Glu–His–Phe·OH

R_f: 0.39 (brown) Calcd. *K:* 0.2 Qual. tests: Met, His, N-terminal Ser

Analysis (5 detns.): 1.0 Glu; 1.1 His; 0.9 Met; 0.9 Phe; 1.1 Ser

Edman: (1) Blank (= Ser); (2) 0.14 Met; (3) *0.09 Glu*, 0.009 Met; (4) ++ His; (Residue) ++ Phe

Carboxypeptidase: (3 hr.) 0.39 Phe
(20 hr.) 0.67 Phe; 0.10 His
(44 hr.) 0.70 Phe; 0.35 His; 0.04 Glu
(70 hr.) 0.86 Phe; 0.64 His; 0.12 Glu; 0.04 Met; + Ser

C10 H·Lys–Arg–Arg–Pro–Val(Lys,Val,Tyr,Pro,Asp, Gly,Ala,Glu,Asp,Glu,Leu,Ala,Glu,Ala,Phe, Pro)Leu·OH

R_f: 0.11 (purple) Calcd. *K:* *ca.* 0.5 Qual. tests: Tyr

Analysis (2 detns.): 3.3 Ala; 2.1 Arg; 2.4 Asp; 3.4 Glu; 1.0 Gly; 1.6 Leu; 2.0 Lys; 0.9 Phe; 3.4 Pro; 0.6 Tyr[39]; 2.1 Val

Edman: (1) 0.08 Lys; (2) Blank (= Arg); (3) Blank (= Arg); (4) 0.07 Pro; (5) *0.06 Val*, 0.03 Pro, 0.008 Ala; (6), (7) and (8) traces of several amino acids

Carboxypeptidase: (20 hr.) 0.53 Leu

C16 H·(Gly,Lys,Pro,Val,Gly,Lys)·OH

R_f: 0.02 (brown) Calcd. *K:* *ca.* 0.01

Analysis (2 detns.): 1.7 Gly; 1.8 Lys; 1.4 Pro; 0.9 Val

Intermedin Activity.—Assays for the melanophore-expanding activity of ACTH fractions were routinely carried out using normal *Rana pipiens* frogs, light-adapted for at least 24 hours. Copper sulfate (1:40,000) was a successful prophylactic against "red leg" infection.[40] The drug was injected into the dorsal lymph sac and the Hogben melanophore index[41] was determined after one hour (maximum response time; duration of detectable response *ca.* 4 hours). Comparison of hypophysectomized and normal frogs indicated that the speed, degree and duration of reaction was somewhat greater in the hypophysectomized animals, but relative potencies were the same. Use of *Xenopus laevis* as the test animal gave results similar to those with *Rana pipiens*, and the less manageable *Xenopus* offered no advantage. Control tests with *Rana pipiens* demonstrated that excitement, pain, 4% sodium chloride, 6% acetic acid or 0.5% trichloroacetic acid caused no melanophore expansion. Adrenalin temporarily and rapidly reversed the melanophore expansion of both normal and hypophysectomized animals. In the case of the long-acting alkali-boiled fractions, administration of adrenalin caused a rapid contraction followed by a re-expansion of melanophores.

The minimum effective dose (m.e.d.) of β, acid-boiled β (0.1 *N* HCl for 6 hours) and desamido-β[1] was 3–5 γ/100 g. frog. After boiling with alkali (0.1 *N* NaOH for 20–30 minutes), the m.e.d. of β was 0.1 γ/100 g. of frog. The m.e.d. of δ_1-intermedin[1] was 0.1–0.2 γ/100 g. of frog both before and after boiling with alkali. This difference in potency produced by alkali can be used to distinguish δ_1-intermedin (type 1) from β-intermedin (type 2) activity.[14a] Both types gave a more prolonged response (24 hours, or longer at 10–20 times the m.e.d.).

The trypsin and chymotrypsin degradation products shown in Fig. 2 and Table I were inactive at 400 γ/100 g. of frog.

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(39) It was repeatedly observed that tyrosine was recovered in low yield from P3T1, P4T1 and certain of the acid hydrolysis products of the latter. A brown ninhydrin-positive material (R_f 0.55) was always formed. Hydrolysis in a nitrogen atmosphere reduced this decomposition, but did not eliminate it. Tyrosine in other peptides from β, and in certain synthetic peptides studied, did not exhibit this phenomenon. Indeed, the "catalyzing factor" must involve aspartic acid, because tyrosine can be recovered normally from H·Val–Tyr–Pro·OH (II), but not from H·Val–Tyr–Pro-Asp·OH (I).

(39a) Unpublished work of J. R. Vaughan, Jr., using carbobenzoxyglycyl-L-alanine and the mixed anhydride method [THIS JOURNAL, **74**, 676 (1952)].

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