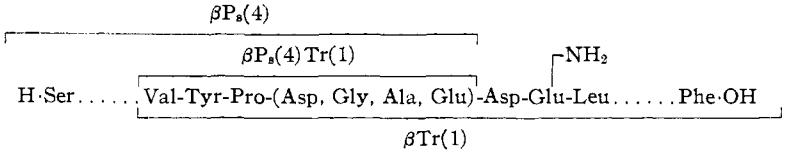
STRUCTURE OF β-CORTICOTROPIN: FINAL SEQUENCE STUDIES

Sir:

An earlier communication from these laboratories[1] reported the methods of purification and essentially all of the structure of β-corticotropin. We now wish to present additional results which are sufficient to complete the entire sequence.

The previous work established the sequence of the first twenty-four amino acids from the amino end as well as the first eleven amino acids from the carboxyl end. The simultaneous failure of the two sequence methods being used left an unknown section of four amino acids as shown in the following diagram.

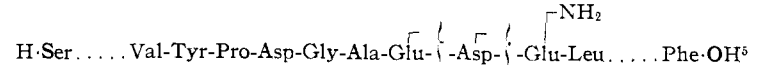


The trypsin fragment βTr(1) from β-corticotropin was too acid-labile in this unknown region to permit sequence determination by the Edman[2] method. Each of the first three Edman degradations gave only one amino acid per step: valine, tyrosine and proline, in that order. However, the fourth stage of the Edman study gave a predominance of alanine along with significant amounts of glutamic and aspartic acids, glycine, phenylalanine and leucine, which clearly indicates extensive side-reactions. The fifth degradation gave the same amino acids in nearly equimolar amounts.

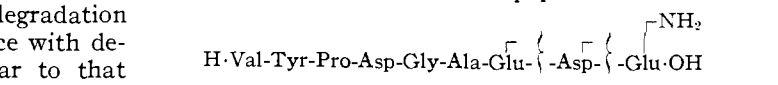
Although pepsin exposes the unknown sequence by removing the eleven amino acids from the carboxyl end, the product βPs(4) is unreactive to carboxypeptidase. It is clear from the diagram that the simplest fragment containing the uncertain portion would be the heptapeptide cleaved from the carboxyl end of βPs(4)[1] by trypsin. This βPs(4)Tr(1) was separated from the trypsin digest mixture by a 500-transfer countercurrent distribution in 20% acetic acid *vs.* *n*-butanol (obs. $K = 0.39$). Its amino acid analysis (1.1 Ala, 1.0 Asp, 1.0 Gly, 1.0 Glu, 0.9 Pro, 0.7 Tyr, 1.1 Val) showed it to be the expected heptapeptide. Edman degradation clearly gave a Val-Tyr-Pro . . . sequence with decomposition in the fourth stage similar to that found in βTr(1).[3] Carboxypeptidase failed to digest even this simpler peptide. Work on model peptides suggests that this is due to the proximity of aspartic and glutamic acids rather than to absence of an α-carboxyl terminus.

The acid lability and unimolar ratio of amino acids made partial acid hydrolysis promising for study of the structure of βPs(4)Tr(1). Hydrolyses using 12*N* HCl at 25° and 0.1*N* HCl at 105° for varying times were followed by paper chromatography. The former conditions were slower and less specific than the latter. Treatment with 0.1*N* HCl for 2 hours completely destroyed the parent peptide and gave a maximum amount of intermediate peptides with a minimum of conversion to free amino acids. Four peptides were isolated from such an acid digest of βPs(4)Tr(1) using a 5:4:1 *n*-butanol:water:acetic acid paper chromatographic system. Amino acid analysis of the hydrolysates of these peptides along with their Edman degradations established that their structures were: H·Val-Tyr-Pro·OH, H·Val-Tyr-Pro-Asp·OH, H·Ala-Glu·OH, and H·Gly-Ala-Glu·OH. Since valine was N-terminal in the starting material, these fragments are sufficient to establish that the sequence in βPs(4)Tr(1) is H·Val-Tyr-Pro-Asp-⟨-Gly-Ala-Glu·OH. The C-terminal glutamic acid was confirmed by the Dakin reaction for free α-carboxyls.[4] The aspartic acid failed to undergo this reaction under conditions causing reaction of C-terminal asparagine in a model peptide. Therefore, we conclude that the aspartic acid is α-linked to the glycine. This, then, establishes the sequence and the nature of the links in the entire structure of βPs(4), the smallest active pepsin-digestion product having clinical activity equal to the native hormone.

These results complete the structure of β-corticotropin, βPs(3)[1] and βPs(2),[1] except for the possible β linkage of the other aspartic acid and the possible γ linkage of *one* of the glutamic acids as illustrated



An attempt to resolve this uncertainty by means of the Dakin reaction on the nonapeptide



gave equivocal results. The cleavage of the aspartyl–glutamine bond by carboxypeptidase and of the glutamyl–aspartic bond by pepsin suggests α-peptide linkages.

(1) Paul H. Bell, *et al.*, THIS JOURNAL, **76**, 5565 (1954).

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

(3) White and Landman, in a publication [THIS JOURNAL, **77**, 1711 (1955)] which appeared after this manuscript was prepared, proposed a sequence based on the Edman method for this region of corticotropin-A. This sequence was almost the same as the erroneous one based on our Edman results on a fragment from β-corticotropin.[1] Our subsequent partial acid degradation conclusively placed aspartic acid and showed that the H·Ala··Glu·OH, also obtained by the Armour workers, came from the -COOH end of βPs(4)Tr(1). White, *et al.*, used a qualitative Edman analysis, but in our work we have found that valid conclusions cannot be drawn even from quantitative Edman data because of concomitant decomposition in this region. This difficulty of interpretation is illustrated by the following Edman results on βPs(4)Tr(1): 4th stage—Ala, Glu, Gly and/or Asp in ratios of 5:1.5:1; 5th stage—Ala, Glu, Gly, 1:1:1; 6th stage—Ala, Glu, Gly and/or Asp, 4:1:2.

(4) G. H. Cleland and C. Niemann, THIS JOURNAL, **71**, 841 (1949).

(5) The amide-free corticotropin-A of White and Landman (ref. 3) could have been derived from an amide-containing precursor, since we observed in our earlier work that alkaline conditions similar to theirs caused decomposition of the glutamine amide without loss of activity. Recently Li, Geschwind, Dixon, Levy and Harris [*J. Biol. Chem.*, **213**, 171 (1955)] reported a similar observation on sheep corticotropin. The identity of the peptide chains of β-corticotropin and of corticotropin-A is to be expected if the latter is simply a deamidation product of this mild alkaline degradation. In addition, the homogeneity of corticotropin-A in terms of our α-, β- and γ-corticotropins[1] needs to be established before comparisons are made. Corticotropin-A comprises approximately ¾ of the activity of oxycellulose ACTH, which we have shown contains eight *active* components, the most abundant of which (β-corticotropin) amounts to only 33%.

Lack of time and material does not permit us to obtain additional evidence regarding these alternate linkages. Logical approaches would be by the use of the Edman method on the aspartyl-glutaminyl-leucine peptide[1] and by partial acid degradation of the nonapeptide mentioned above. Details of this and the earlier work will be reported shortly.

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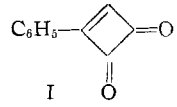
PHENYLCYCLOBUTADIENOQUINONE

Sir:

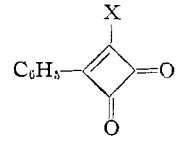
No authentic cylobutadiene derivatives appear to have yet been prepared except for biphenylene[1a] and related substances[1b,2,3] where the four-membered rings are part of fused-ring systems.[4] The failure to detect cyclobutadiene or its non-fused ring derivatives among the products of appropriate synthetic reactions can be ascribed either to excessive ring strain or unfavorable electronic configurations.[3,5] Strong evidence that ring strain is not the most important factor is now provided by a synthesis of the quite stable phenylcyclobutadienoquinone (phenylcyclobutendione, I) which to a first approximation is expected to have the same degree of ring strain as phenylcyclobutadiene.



I

Phenylacetylene with trifluorochloroethylene at 120° for 24 hours gave 1,1,2-trifluoro-2-chloro-3-phenylcyclobutene[6]; b.p. 52–53° (0.4 mm.), $n^{25}D$ 1.5117. *Anal.* Calcd. for $C_{10}H_6F_3Cl$: C, 54.92; H, 2.77. Found: C, 54.83; H, 2.89. Hydrolysis[6] of the adduct with 92% sulfuric acid at 100° afforded bright-yellow crystalline I in 75% yield; m.p. 152–153° (dec.) after recrystallization from acetone. *Anal.* Calcd. for $C_{10}H_6O_2$: C, 75.94; H, 3.82; mol. wt., 158. Found: C, 76.00; H, 3.85; mol. wt. (Rast), 151. I had λ_{max} = 286 mμ, ϵ 2.3 × 10^5 and strong infrared absorption at 5.6 μ. A chloroform solution of I with 30% hydrogen peroxide yielded phenylmaleic anhydride, m.p. 119–120°, which did not depress the m.p. of an authentic sample.[7] Reduction of I with amalgamated zinc and hydrochloric acid yielded phenylcyclobutane which was identified by comparison of its infrared spectrum with material previously prepared.[6] I is much more stable to water and oxygen than *o*-benzoquinone. It is apparently not reduced by agents such as hydrogen over platinum to the corresponding hydroquinone.

I can be converted to a variety of stable substitution products (II–VII) which have some chemical properties analogous to those of substituted benzo- and naphthoquinones.



II, X = Cl
III, X = Br
IV, X = I
V, X = –OCH_3
VI, X = –NH_2
VII, X = –OH

I reacted with chlorine or bromine in acetic acid to yield the yellow halogen derivatives II and III, respectively. II had m.p. 114–115°. *Anal.* Calcd. for $C_{10}H_5O_2Cl$: C, 62.36; H, 2.62. Found: C, 62.20; H, 2.54. III had m.p. 128–129°. *Anal.* Calcd. for $C_{10}H_5O_2Br$: C, 50.66; H, 2.13. Found: C, 50.64; H, 2.13. Reduction of III with amalgamated zinc and hydrochloric acid gave phenylcyclobutane. III reacted rapidly with alcoholic silver nitrate and, with sodium iodide in acetone, yielded IV, m.p. 163–165° (dec.). *Anal.* Calcd. for $C_{10}H_5O_2I$: C, 42.26; H, 1.77. Found: C, 42.38; H, 1.87. Methanolysis of III afforded pale yellow V, m.p. 151–152° (*Anal.* Calcd. for $C_{11}H_8O_3$: C, 70.20; H, 4.28. Found: C, 69.51; H, 4.16) while ammonia in dry benzene gave the colorless essentially neutral amino derivative (VI), m.p. 279–281° (dec.). *Anal.* Calcd. for $C_{10}H_7O_2N$: C, 69.36; H, 4.07; N, 8.09. Found: C, 69.37; H, 4.04; N, 8.08. With dilute acetic acid, III was hydrolyzed to colorless VII, m.p. 205–208° (dec.). VII is soluble in water, is an exceptional strong acid for a C, H, O compound ($pK_A \sim 1$) and gives a magenta color with ferric chloride solution. *Anal.* Calcd. for $C_{10}H_6O_3$: C, 68.96; H, 3.48; neut. equiv., 176. Found: C, 68.87; H, 3.61; neut. equiv., 174. VII is converted to V with diazomethane.

The stable existence of I–VII suggests the possibility that quinone-type derivatives of unknown cyclic polyolefins such as pentalene and heptalene might be more stable than the parent hydrocarbons.

Investigation of the chemical and physical properties of I–VII is being continued.

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A NEW REAGENT FOR RESOLUTION BY COMPLEX FORMATION; THE RESOLUTION OF PHENANTHRO-[3,4-*c*]PHENANTHRENE[1]

Sir:

We wish to report the synthesis of 2-(2,4,5,7-tetranitro-9-fluorenylideneaminoöxy)-propionic acid, III, a useful reagent for the optical resolution of certain aromatic compounds which do not have the functional groups usually needed to effect resolution by conventional reagents.

The dextro and levo forms of III were made by condensing 2,4,5,7-tetranitrofluorenone, I, with

(1) (a) W. C. Lothrop, THIS JOURNAL, **63**, 1187 (1941); (b) **64**, 1698 (1942).

(2) R. F. Curtis and G. Viswanath, *Chem. and Ind.*, 1174 (1954).

(3) M. P. Cava and J. F. Stucker, *ibid.*, 446 (1955).

(4) An excellent survey of attempts to prepare non-fused ring cyclobutadiene derivatives was recently presented by E. R. Buchman, Abst. of A.C.S. meeting, Sept. 13, 1954, p. 9-O.

(5) For references and discussion see J. D. Roberts, A. Streitwieser, Jr., and Clare M. Regan, THIS JOURNAL, **74**, 4579 (1952).

(6) *Cf.* J. D. Roberts, G. B. Kline and H. E. Simmons, Jr., *ibid.*, **75**, 4765 (1953).

(7) L. E. Miller, H. B. Staley and D. J. Mann, *ibid.*, **71**, 374 (1949).

(1) A part of this material was presented before the Division of Organic Chemistry at the 127th meeting of the American Chemical Society, Cincinnati, Ohio, March, 1955.