certain of them, *e.g.*, bismuthyl ion, can be precipitated quantitatively by adding perfluoroacetic acid (at *p*H 0.5). In general, cations forming insoluble hydroxides can be precipitated in abnormal *p*H intervals by carrying out the precipitation in the presence of appropriate perfluoroacids. It is not yet known whether the perfluoroacid anion is combined in stoichiometric proportions in these precipitates.

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RECEIVED JULY 29, 1955

STUDIES ON ADRENOCORTICOTROPIN. XII. ACTION OF AMINOPEPTIDASE ON CORTICOTROPIN-A; EFFECT ON BIOLOGICAL ACTIVITY

Sir:

In previous communications from this laboratory, it has been shown that corticotropin-A is a straight chain polypeptide containing thirty-nine amino acid residues[1] with the N-terminal sequence: Ser.Tyr.Ser.Met.Glu.His.Phe.Arg.Try.... Our evidence for this sequence was based partly on chemical reactions on the intact molecule[2] and partly on a study of overlapping fragments produced by the action of endopeptidases on corticotropin-A.[3] In our hands, both of the chemical reagents used (dinitrofluorobenzene and phenyl isothiocyanate) and two of the endopeptidases (chymotrypsin and trypsin) attacked the molecule at several points simultaneously and therefore did not provide specific knowledge of the relationship between structure and physiological activity.[4] Pepsin, however, rapidly split three bonds near the C-terminus of corticotropin-A with no effect on activity and then slowly split the bond between glutamic acid in position 5 and histidine in position 6.[5] Serial application of the Sayers test indicated that the rupture of this bond was paralleled by a loss in physiological activity.

With the availability of highly purified aminopeptidase,[6] an opportunity was afforded of studying the effect of the serial removal of amino acids at the N-terminus of corticotropin-A. Five milligrams (approximately 1 micromole) of the purified hormone was incubated[7] with 2 units of a highly purified aminopeptidase preparation at 37° and aliquots were withdrawn at intervals over a 24-hour period. Quantitative amino acid determinations were made on portions of each aliquot by means of paper chromatography in the 2-butanol:ammonia system.[8] Figure 1 shows a plot of the amount of each amino acid released against the time of incubation. The situation is complicated somewhat by the presence of two serine residues in the sequence, but the results are in general agreement with the rates of hydrolysis for the individual amino acids as established by the University of Utah group with the corresponding amides.[9] The terminal amino acid, serine, is split very slowly (C_0 = 106) and therefore limits the rate of the more easily removed tyrosine (C_0 = 2,200). The second serine residue in position 3 again limits the more rapidly split methionine. Glutamic acid (position 5) apparently is also very slow and limits the next four amino acids, all of which have high rates. Glycine (position 10) is again very slow (C_0 = 18) and, due to the limited opportunity for reaction at this point, was not detected on our chromatograms.

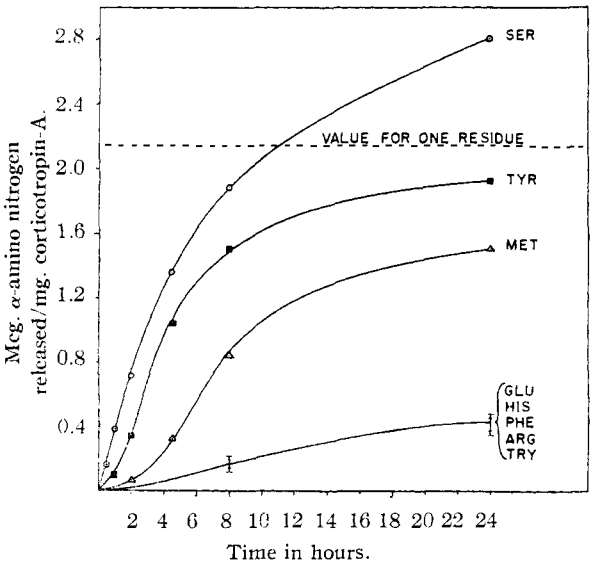

Fig. 1.—Release of amino acids from corticotropin-A by action of aminopeptidase. Since the corticotropin-A preparation contained salt, the value for one residue was an average value calculated from the amino acid composition after complete acid hydrolysis and taking into account the known number of residues of each amino acid.

In attempting to relate the release of amino acids to physiological activity, the 4.5-hour fraction was selected for assay. At this point a total of approximately two-thirds of a residue of serine had been released. Since the rate for tyrosine is about twenty times that for serine, the value for tyrosine can be taken as a measure of the amount of the first serine unit released, and the difference between the serine and the tyrosine values as the amount of the second serine unit released. Thus, at 4.5 hours more than one-half of both the first serine and the tyrosine had been removed. Assay of the 4.5

(1) W. F. White and W. A. Landmann, THIS JOURNAL, **77**, 1711 (1955).

(2) W. A. Landmann, M. P. Drake and W. F. White, *ibid.*, **75**, 4370 (1953).

(3) W. F. White and W. A. Landmann, *ibid.*, **77**, 771 (1955).

(4) Throughout this paper the terms "physiological activity" or simply "activity" refer to depletion of adrenal ascorbic acid, as measured by the subcutaneous method, U. S. Pharmacopeia, Vol. XV, p. 176.

(5) W. F. White, THIS JOURNAL, **74**, 4194 (1954).

(6) The preparation used in this work was kindly furnished by Dr. Emil L. Smith of The University of Utah College of Medicine. The method of preparation is described in *J. Biol. Chem.*, **212**, 255 (1955).

(7) The reaction was carried out in 0.005 *M* ammonium veronal at *p*H 8.5 and containing 0.0025 *M* magnesium chloride. The aminopeptidase preparation had been treated with diisopropylfluorophosphate at the time of preparation. A small amount of toluene was added to the mixture before incubation.

(8) For details of the quantitative chromatographic estimation of amino acids, *cf.* J. F. Roland, Jr., and A. M. Gross, *Anal. Chem.*, **26**, 502 (1954).

(9) E. L. Smith and D. H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955).

hour fraction[10] showed that 64% of the physiological activity had been destroyed. Since only 15% of the methionine (position 4) and, therefore, also of the second serine (position 3) had been removed at 4.5 hours, neither of these units could have been involved in the loss of activity. Thus one, or both, of the first two amino acids in the sequence appear to be necessary for physiological activity.[11]

Acknowledgment.—The author wishes to acknowledge the technical assistance of Mr. A. M. Gross.

(10) As control, a sample of corticotropin-A was incubated for 4.5 hours in the buffer, but without aminopeptidase. No loss in activity was detected. The corticotropin-A sample used in this work had an activity of 110 units per milligram of peptide.

(11) An interesting sidelight on the aminopeptidase reaction was the effect of the hydrolysis on the solubility of corticotropin-A. At the concentration used in the experiment (5 mg./ml.), corticotropin-A is not completely soluble at *p*H 8.5. However, as the aminopeptidase reaction proceeded, the cloudy appearance of the reaction mixture became less pronounced and by 4.5 hours had cleared completely. Thus the first two amino acid units also appear to be associated with the solubility of corticotropin-A.

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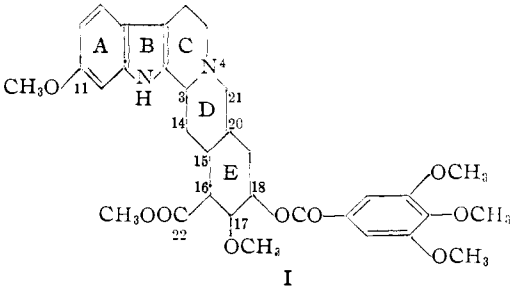
RECEIVED JULY 22, 1955

THE STEREOCHEMICAL FORMULATION OF RESERPINE

Sir:

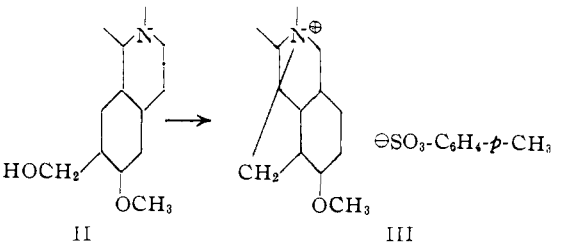
As an adjunct to studies taken up in the derivation of the gross structures for reserpine (I)[1,2] and deserpidine (I with the C-11 methoxyl replaced by hydrogen),[3] there was obtained in-



I

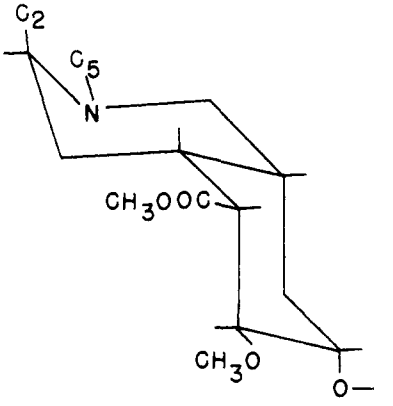
formation requiring for reserpine a *cis* nature for the substituents at C-16 and C-18[1] and also a *cis* juncture for the D and E rings.[4,5] We have now secured compelling evidence for a *cis* relationship of the hydrogens at C-16 and C-20, which, in turn, taken together with certain previously recorded observations, provides for the complete stereochemical formulation of this complex base.

Reserpinol (II) (Calcd.: C, 71.32; H, 8.16. Found: C, 70.96; H, 8.23), m.p. 254–255.5°, obtained by lithium aluminum hydride reduction of methyl reserpate tosylate (V),[1] was converted, on



II III

treatment with *p*-tosyl chloride in pyridine under normal conditions, to a high-melting (330–333°, dec.) solid which we consider to be the quaternary salt III (Calcd.: C, 66.39; H, 6.90. Found: C, 66.18; H, 6.88), by reason of the following characteristics: (i) infrared absorption at 8.2–8.6, 8.91, 9.62 and 9.86 μ, indicative of the *p*-tosyloxy anion,[6] (ii) ultraviolet absorption maxima at 222 mμ (log ϵ 4.70), 269 mμ (log ϵ 3.80) and 294 mμ (log ϵ 3.93), nearly identical with those of reserpinol hydrogen tosylate and therefore signifying an unmodified indole ring, and (iii) in 66% dimethylformamide, no titratable groups between *p*H 3 and 13, and recoverable after treatment, in solution, with alkali.[7] It is apparent that the salt III, which possesses a bridged bicyclic system defined by atoms 4 and 15 through 22, can result from N-4 attack on C-22 of the unisolated II-tosylate only if C-21 and the carbomethoxyl group originally present in reserpine are attached in a *cis* fashion to ring E. The above finding, coupled with the equatorial nature of the carbomethoxyl group as indicated by the stability of methyl reserpate to sodium methoxide in boiling methanol,[1] is incorporated in the expression IV for reserpine.



IV

Further, the demonstration that reserpine possesses the less stable configuration at C-3[3] implies that C-2 is joined through the *axial* bond of C-3, thereby placing the C-3 hydrogen equatorial and *trans* to the hydrogen at C-15 and C-20. Finally, the stereochemical course of elimination to methyl anhydroreserpate (VII) and the concurrent internal

(1) L. Dorfman, A. Furlenmeier, C. F. Huebner, R. Lucas, H. B. MacPhillamy, J. M. Muller, E. Schlittler, R. Schwyzer and A. F. St. André, *Helv. Chim. Acta*, **37**, 59 (1954).

(2) N. Neuss, H. E. Boaz and J. W. Forbes, THIS JOURNAL, **76**, 2463 (1954).

(3) H. B. MacPhillamy, L. Dorfman, C. F. Huebner, E. Schlittler and A. F. St. André, *ibid.*, **77**, 1071 (1955).

(4) P. A. Diassi, F. L. Weisenborn, C. M. Dylion and O. Wintersteiner, *ibid.*, **77**, 2028 (1955).

(5) E. E. van Tamelen, P. D. Hance, K. V. Siehrasse and P. E. Aldrich, *ibid.*, **77**, 3930 (1955); see also reference (3).

(6) F. L. Weisenborn and D. Burn, *ibid.*, **75**, 259 (1953).

(7) Formation of a higher molecular weight salt from II through intermolecular alkylation seems unlikely since yohimbyl alcohol monotosylate (R. C. Elderfield and A. P. Gray, *J. Org. Chem.*, **16**, 506 (1951))—wherein internal quarternization is sterically impossible—is recovered after being refluxed in benzene solution for two hours, and since methyl reserpate is not alkylated by isobutyl tosylate under similar conditions.