The R_f values of the nucleosides obtained were: adenosine 0.20; guanosine 0.42; cytidine 0.61; uridine 0.62. The spots of the products together with appropriate blanks were cut out and eluted and the amounts and ratios of the component nucleosides of the fragments were determined spectrophotometrically. These results are recorded in parentheses in Tables II and III.

Acknowledgment.—The authors are indebted to Dr. P. Kaesberg and Dr. L. E. Bockstahler for their help in preparing the bromegrass mosaic virus.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY MEDICAL CENTER, DURHAM, NORTH CAROLINA]

Optical Rotatory Dispersion of L-Tyrosine[1]

BY T. M. HOOKER, JR., AND CHARLES TANFORD

RECEIVED JULY 20, 1964

Aqueous solutions of L-tyrosine display a prominent Cotton effect which is associated with the aromatic absorption band near 280 mμ. The effect is enhanced and shifted to higher wave length when the phenolic group is ionized, but is not greatly affected by changes in the ionization states of the amino or carboxyl groups. On the other hand, the Cotton effect is considerably reduced in amides and esters of tyrosine, and is absent altogether in N-acetyl derivatives. No corresponding Cotton effect is associated with the 260-mμ absorption band of L-phenylalanine.

The most prominent feature of the optical rotatory dispersion of the α-amino acids[2-4] is a Cotton effect which shows its first extremum in the region from 220 to 230 mμ, and which has been associated with the carboxyl group absorption band near 210 mμ. Schellman[2,3] has predicted, however, that the aromatic amino acids should have an additional Cotton effect, associated with the aromatic chromophore, and the purpose of this paper is to show that such a Cotton effect indeed appears prominently in the optical rotatory dispersion of aqueous solutions of L-tyrosine. On the other hand, it has not been observed with L-phenylalanine, and it is either not present at all, or considerably less prominent, in a number of derivatives of L-tyrosine.

Experimental

L-Tyrosine was an A-grade sample from the California Corporation for Biochemical Research. The same sample has been used for solubility studies,[5] and proved free from impurities by the criterion that its apparent solubility remained unchanged when the amount of solid phase added exceeded the saturation amount more than tenfold. L-Phenylalanine was obtained from Mann Research Laboratories. It also has been used for solubility studies,[5] and found free from impurities. The tyrosine derivatives which were used were purchased from Mann Research Laboratories or from Cyclo Chemical Corporation. The purest available grades were obtained. These derivatives were used only for preliminary survey experiments.

Optical rotations were measured with a Cary Model 60 spectropolarimeter, using an Osram 450 watt xenon lamp as light source. Measurements were made at 25°, most of them using cells with a 1-cm. light path. In the data reported for tyrosine the amino acid concentration was between 1.5 and 1.6 × 10^{-3} M. The phenylalanine data represent a composite of two curves, using concentrations of 5.4 × 10^{-5} M and 6.8 × 10^{-3} M, respectively. All results are reported in terms of molecular rotations

$$[\phi] = \frac{3}{n^2 + 2} \frac{M_0}{100} [\alpha]$$

where n is the solvent refractive index, M_0 the molecular weight, and $[\alpha]$ the specific rotation at any wave length. The refractive index values for pure water[6] were used, since the small amounts of acid or base, present in most of the solutions to adjust the pH, should have no appreciable effect on the refractive index.

Since solutions with high absorbance may sometimes lead to artifactual optical rotation data,[7] great care was taken to make certain of the validity of our results. The peak absorbance of our tyrosine solutions (1.6 = 10^{-3} M, 1-cm. light path) was about 2.2, which is well within the permissable range for the Cary spectropolarimeter. Solutions of lower concentration were studied as controls, and data were also obtained for 1.6 × 10^{-3} M solutions in cells with a 0.1-cm. light path. Such data were somewhat less precise, because the observable rotation diminishes with the absorbance, but agreed with all other data within the limits of error.

In one experiment a curve was obtained for a solution of 1.06 × 10^{-3} M tyrosine (peak absorbance 1.5), and a second curve was obtained with two cells in the light path, one containing the same solution, and the other containing an equal concentration of *dl*-tyrosine. The peak absorbance was now 3.0, which is near the limit which the instrument can handle. Nevertheless, no significant change in the optical rotatory dispersion curve was observed.

Results

Figure 1 shows typical data for L-tyrosine at three pH values, and also gives for comparison the optical rotatory dispersion curve for L-phenylalanine, which has no Cotton effect above 225 mμ. The figure shows that the peak and trough of the effect lie at 283 and 260 mμ, respectively, at neutral pH. The midpoint thus lies at 272 mμ, close to the absorption peak which occurs in neutral solution at 275 mμ. At high pH, the peak and trough shift to 304 and 274 mμ, respectively, and the midpoint at 289 mμ now lies closer to the alkaline absorption peak of tyrosine, which occurs at 294 mμ.

The point midway between the trough and peak of the curves of Fig. 1 should not be regarded as the exact center of the Cotton effect because Cotton effects which occur at lower wave lengths evidently contribute to the rotation in the 280-mμ region. To obtain a precise quantitative measure of the 280-mμ Cotton effect would require that the contribution of these other Cotton effects could be estimated accurately and subtracted from the data. If this could be done, then the peak and trough rotations would become equal, but opposite in sign, and the midpoint would correspond to the absorption wave length of the transition responsible for the effect. The required correction cannot be estimated with the data presently available. It is evident, however, that the rotation due to lower wave length Cotton

(1) This work was supported by research grants from the National Science Foundation and from the National Institutes of Health, U. S. Public Health Service.

(2) J. A. Schellman in C. Djerassi, "Optical Rotatory Dispersion," McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p. 210.

(3) J. Strem, Y. S. R. Krishna-Prasad, and J. A. Schellman, *Tetrahedron*, **13**, 176 (1961).

(4) J. P. Jennings and W. Klyne, *Biochem. J.*, **86**, 12P (1963).

(5) Y. Nozaki and C. Tanford, *J. Biol. Chem.*, **238**, 4074 (1963).

(6) "Landolt-Bornstein Physikalisch Chemische Tabellen," Julius Springer, Berlin, 1931.

(7) P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 402 (1961).

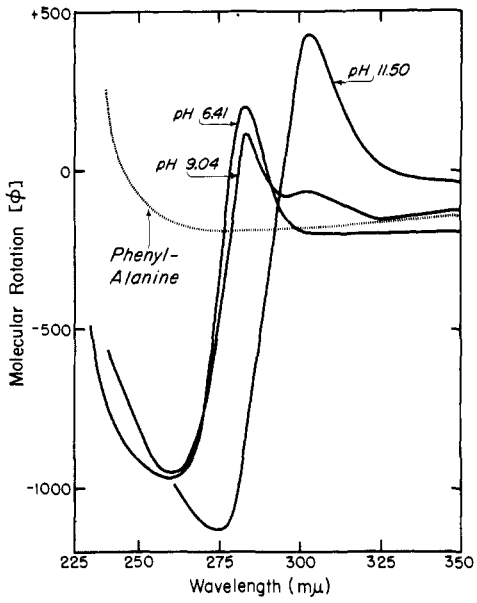

Fig. 1.—Optical rotatory dispersion for L-tyrosine at three pH values, in water at 25°. Comparable data for L-phenylalanine are also shown. The results are essentially independent of amino acid concentration.

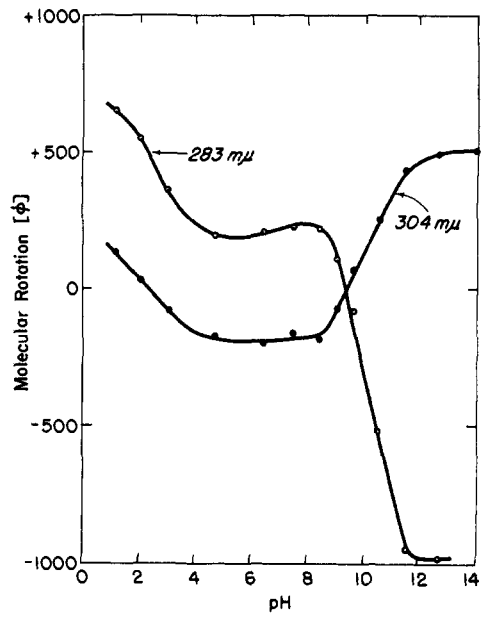

Fig. 2.—Molecular rotation of L-tyrosine at the peak wave lengths as a function of pH. The large changes between pH 8 and 12 coincide with the dissociation of the phenolic hydroxyl group.

effects must be negative in the region of interest here. This implies that the first derivative of the rotation with respect to wave length should be positive and the second derivative negative. Subtraction of a rotation with these characteristics would increase the midpoint wave length above the apparent midpoints estimated in the preceding paragraph. and would diminish the difference between peak and trough rotations somewhat. (It is perhaps reasonable to obtain a value for the peak height by extrapolation of the background rotation from higher wave lengths. This leads to a molecular rotation at the peak of about 500°.)

We have obtained data at lower wave lengths than shown in Fig. 1 which indicate that tyrosine may have a Cotton effect associated with the aromatic absorption peak near 230 mμ. However, optical rotation measurements in the 230-mμ region are also strongly influenced by the 210-mμ Cotton effect ascribable to the carboxyl group. We feel that an interpretation of the data obtained with tyrosine below 250 mμ must await the results of a systematic study of the ultraviolet optical rotation of amino acids which lack the side chain chromophore, so that evaluation of the contribution from the carboxyl group Cotton effect may be made.

Figure 2 shows a more detailed examination of the effect of pH on the optical rotation. The rotation at the two peaks seen in Fig. 1 is plotted *vs.* pH, and it is seen that two sigmoid curves are obtained between pH 8 and 12, for the decrease in rotation at 283 mμ and the increase at 304 mμ. These curves presumably reflect the disappearance of the un-ionized phenolic group and the appearance of the ionized form, respectively. Both curves are essentially superimposable on the spectrophotometric titration curve of the phenolic group of tyrosine[8] and give the same pK of 10.15.

Figure 2 also shows a rise in molecular rotation below pH 3, where protonation of the carboxyl group occurs. This rise, however, represents an increase in the background rotation in the 280–300 mμ region, which is to be ascribed to changes in the low wave length Cotton effect associated with the carboxyl group. The Cotton effect associated with the arommatic absorption band appears not to be influenced appreciably by the state of ionization of the carboxyl group. Figure 2 shows that it is also uninfluenced by dissociation of the α-amino group of tyrosine, which occurs predominantly near pH 8.

In addition to the experiments shown in Fig. 1 and 2, we have carried out preliminary survey experiments on several derivatives of tyrosine. In view of the apparent lack of interdependence between the observed Cotton effect and the state of ionization of the carboxyl and amino groups, it is surprising to find that chemical modification of the carboxyl and amino groups completely or nearly abolishes the effect. Plain dispersion curves were obtained down to below 250 mμ for N-acetyl-L-tyrosine, N-acetyl-L-tyrosine ethyl ester, and for the hydantoin of L-tyrosine, all measured in methanol. Cotton effects near 275 mμ were observed for L-tyrosinamide and L-tyrosine ethyl ester (both in methanol), but their magnitudes are quite small, the peak height in the molecular rotation being about 100° compared to the peak height of about 500° in Fig. 1.

Discussion

The Cotton effect reported for L-tyrosine in this paper has been predicted on the basis of analysis of data at higher wave lengths by Schellman.[2,3] It has been observed experimentally by Fasman, *et al.*,[9] as an incidental part of a study of poly-L-tyrosine. Data presented in an earlier report by Billardon,[10] claiming to show Cotton effects in the aromatic absorption region both for L-tyrosine and L-phenylalanine, differ markedly from the findings here reported.

We feel that the more detailed examination of this

(8) C. Tanford and G. L. Roberts, Jr., *J. Am. Chem. Soc.*, **74**, 2509 (1952).

(9) G. D. Fasman, E. Bodenheimer, and C. Lindblow, *Biochemistry*, in press. We are grateful to Dr. Fasman for providing us with a copy of this paper in advance of publication.

(10) M. Billardon, *Compt. rend.*, **251**, 535, 1759 (1960).

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

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

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

(3) On the other hand, chemical modification of the carboxyl and amino groups, which leaves the absorption spectrum virtually unchanged, has a pronounced effect on the optical rotation. Acetylation of the amino group completely eliminates the Cotton effect. Esterification or amidation of the carboxyl group diminishes the magnitude of the effect by a factor of five.

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

(11) E. Shechter and E. R. Blout, *Proc. Natl. Acad. Sci. U. S.*, **51**, 695, 794 (1964).
(12) E. Shechter, J. P. Carver, and E. R. Blout, *ibid.*, **51**, 1029 (1964).
(13) H. Würz and F. Haurowitz, *J. Am. Chem. Soc.*, **83**, 280 (1961).

[CONTRIBUTION FROM THE BIOCHEMISTRY DEPARTMENT, UNIVERSITY OF PITTSBURGH SCHOOL OF MEDICINE, PITTSBURGH, PENNSYLVANIA]

Studies on Polypeptides. XXVIII. Elimination of the Methionine Residue as an Essential Functional Unit for *in Vivo* Adrenocorticotropic Activity[1-4]

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

RECEIVED JULY 10, 1964

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

Introduction

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

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

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

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

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

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

(5) See K. Hofmann, *Brookhaven Symp. Biol.*, **13**, 184 (1960), for definition of this term.

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

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

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