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Studies on Pituitary Lactogenic Hormone. XI. Reactions with Ketene[1]

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Some years ago in a preliminary note[2] we reported that when lactogenic hormone is in contact with a rapid flow of ketene for a short time its hormonal activity is destroyed. Though it was shown that the amino groups in the protein were completely acetylated no evidence was given to indicate that the tyrosine phenolic groups were not also involved in the reaction. In this paper we shall describe in more detail the reaction of ketene with the lactogenic hormone. The results appear to confirm the previous conclusion that the amino groups are essential for the biological action of this protein hormone.

Experimental

Lactogenic hormone preparations were prepared from sheep pituitaries according to the method of Li, *et al.*[3] The biological potency of the hormone, as assayed in pigeons,[3] is approximately 30 International Units (I. U.) per mg.

Ketene was generated in the apparatus previously described.[4] The acetylation experiments were carried out at 0° in two buffer solutions: 1 *m* acetate buffer of *p*H 4.0 and 0.75 *m* phosphate buffer of *p*H 7.0. The protein concentration was 10 mg. per cc. The *p*H of the solution after acetylation was determined with a glass electrode. The ketene treated hormone solutions were thoroughly dialyzed against distilled water and then lyophilized.

The amino nitrogen was determined by the manometric procedure of Van Slyke at 23°; the nitrous acid was allowed to react with the protein for twenty minutes. The extent of acetylation of the tyrosine groups in the hormone was estimated by the procedure of Herriott.[5]

Electrophoresis experiments were carried out in a Tiselius apparatus at 1.5°. The electrophoretic patterns were secured with the Longsworth scanning method.[6]

Results

The amino nitrogen content of five different samples of lactogenic hormone was found to be 0.74%. When ketene was allowed to react with the hormone in phosphate buffer for five minutes, 35% of the amino groups were covered while 75% of the tyrosine groups were acetylated. This is somewhat surprising, for it is generally believed[7] that amino groups are more easily attacked by ketene than the phenolic hydroxyls. If the reaction extended to fifty minutes, 20% of both amino and tyrosine groups are still uncovered. In one experiment we kept the flow of ketene at a slower rate; after three minutes treatment the amino groups were untouched while 20% of the phenolic groups[8] were acetylated. It is clear from these experiments that ketene reacts with the phenolic groups of tyrosine residues in the lactogenic hormone molecule more rapidly than with the amino groups.

In acetate buffer of *p*H 4.0, the extent of acetylation of both amino and phenolic groups after five minutes of treatment was practically identical. After longer treatment there appeared no significant difference in the rate of acetylation of these two groups. In comparison with the reaction in phosphate buffer the phenolic groups react slower with ketene in acetate solution while no distinction was observed in the acetylation of the amino groups.

In order to determine the homogeneity of the acetylated product we have subjected a preparation to electrophoretic examinations. Experiments were carried out in potassium chloride–hydrochloric acid solution of *p*H 2.2 and 0.10 ionic strength; results were recorded in Fig. 1. The acetylated hormone was quite homogeneous with respect to its electrochemical properties; when it mixed with the untreated hormone it migrated slower with a mobility of $+7.6 \times 10^{-5}$ sq. cm. per volt. sec. The untreated hormone possesses a mobility of $+8.1 \times 10^{-5}$ sq. cm. per volt sec.

The biological potencies of the acetylated hormone samples as assayed in pigeons are summarized in Table I together with the results of chemical analysis. The preparation with only 20% tyrosine groups acetylated, shows no loss of lactogenic potency. Another preparation, in which the tyrosine groups were covered to the extent of 75% and the amino groups to the extent of 35%, had decreased its potency from 30 I. U. per mg. to approximately 1.5 I. U. per mg. Moreover, preparation 02C, of which 70% of the tyrosine groups and 75% of the amino groups were acetylated, had a potency of less than 0.7 I. U. per mg. It would thus appear that the decrease in crop stimulating potency is due to the coverage of the amino groups. In order to sustain this conclusion we subjected the acetylated hormone

(1) Aided by grants from the Research Boards of the University of California and from the Rockefeller Foundation, New York, N. Y.

(2) C. H. Li, M. E. Simpson and H. M. Evans, *Science*, **90**, 140 (1939).

(3) C. H. Li, M. E. Simpson and H. M. Evans, *J. Biol. Chem.*, **146**, 627 (1942).

(4) C. H. Li, *Science*, **90**, 143 (1939).

(5) R. M. Herriott, *J. Gen. Physiol.*, **19**, 283 (1935).

(6) L. G. Longsworth, THIS JOURNAL, **61**, 529 (1939).

(7) H. Staudinger, "Die Ketene," F. Enke, Stuttgart, 1912.

(8) It must be emphasized here that the acetylated hormone gave a full chromogenic value as expected on the basis of the tyrosine content after a *p*H 11.0 treatment for ten minutes. It is assumed throughout this paper that the fact that the tyrosine hydroxyl group is acetylated is based on the lowering of the Folin color as compared with that given by the same preparation after alkali treatment. Although virus proteins (ref. 9) after ketenization do not give full tyrosine color after short hydrolysis with alkali, the chromogenic values of tyrosine phenolic hydroxyl groups in the acetylated lactogenic hormone samples are easily recovered. As pointed out by the Referees, it will be more conclusive as to the mode of ketene action if the acetyl content of acetylated preparations can be determined. Due to the limitation of hormone supply, we were not able to perform acetyl analysis in the present investigation.

TABLE I

THE ACTION OF KETENE ON LACTOGENIC HORMONE

Preparation	Solvent	Rate of flow of ketene	Time of treatment, min.	pH Start	pH Final	Free NH_2 groups covered, %	Phenol groups covered, %	Assays in squabs: Total dose, mg.	Crop reaction[b]	Estimated I.U./mg.
88A	Phosphate buffer	Fast	5	7.0	6.0	35	75	2.0	+, +, +	1.5
								2.0[a]	+, +, +	
88B	Phosphate buffer	Fast	50	7.0	4.2	80	80	2.0	−, −, −	<1.5
								2.0[a]	−, −, −	
94	Phosphate buffer	Slow	3	7.0	6.5	0	20	0.5	2+, 2+, 2+	
								0.2	2+, +, +	30
								0.1	+, +, +	
02B	Acetate buffer	Fast	15	4.0	3.7	60	65	3.0	−, −, −	<1
								3.0[a]	−, +, −	
02C	Acetate buffer	Fast	180	4.0	3.2	75	70	4.0	−, −, −	<0.7
02D	Acetate buffer	Fast	5	4.0	3.9	35	35	4.0	2+, 2+, +	
								3.0	2+, 2+, +	
								2.0	+, +, +	2
								1.0	−, −, +	
								0.5	−, −, −	
								2.0[a]	+, +, +	
								1.0[a]	−, −, +	

[a] Before injection, the acetylated hormone had been treated at *p*H 11.0 for ten minutes. [b] The lactogenic activity was classified into one of four arbitrary groups according to the degree of proliferation in the crops, + being the minimal detectable response and the maximal reaction 4+.

to alkali treatment at *p*H 11.0 for ten minutes and then biologically assayed it after neutralization with hydrochloric acid. It is known that the oxygen-acetyl combination in tyrosine, hydrolyzes easily at *p*H 11[5,9]; in fact, the extent of coverage of the tyrosine groups is estimated in this way. If involvement of the tyrosine groups after acetylation is to any extent responsible for the loss of lactogenic activity and if the blockage of amino groups does not decrease the biological action of the hormone, one may expect some recovery of crop stimulating action after the *p*H 11 hydrolysis of the acetylated hormone. The results, as shown in Table I, indicate that there is no increase[10] of biological activity in acetylated hormone preparations after *p*H 11 treatment. It appears clear that the amino groups are essential for the biological activity of lactogenic hormone.

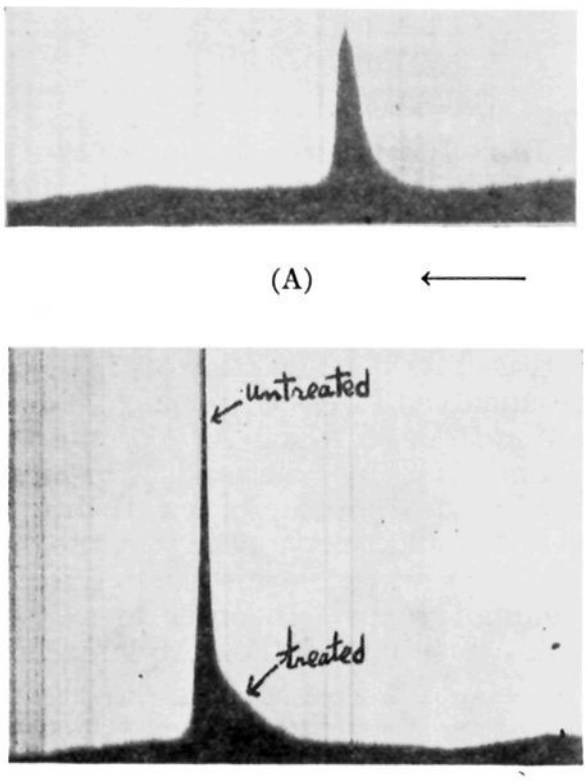

Fig. 1.—Electrophoresis of acetylated lactogenic hormone; *p*H 2.2 potassium chloride–hydrochloric acid solution of 0.10 ionic strength at 1.5°. Potential gradient, about 2.5 volts per cm. All ascending boundaries. A. Preparation 02D after electrophoresis for 300 minutes. B. A mixture of 02D and untreated hormone after electrophoresis for 400 minutes.

Discussion

The fact that studies of the behavior of one protein toward a specific reagent cannot be utilized to infer a corresponding behavior on the part of another protein has been pointed out by other investigators.[11,12] In ketene experiments with pepsin[13] and insulin[14] it was noted that amino groups could be blocked without modification of the tyrosine residues in the molecule. The present work, however, shows that both the phenolic hydroxyl and amino groups in lactogenic hormone are attacked at almost the same rate by ketene and that the amino groups cannot be acetylated with-

(9) G. L. Miller and W. M. Stanley, *J. Biol. Chem.*, **141**, 905 (1941).

(10) It should be remembered that the untreated lactogenic hormone is not inactivated in *p*H 11 for ten minutes.

(11) A. White, *Ann. N. Y. Acad. Sci.*, **43**, 361 (1943).

(12) B. F. Chow, *ibid.*, **43**, 316 (1943).

(13) R. M. Herriott and J. H. Northrop, *J. Gen. Physiol.*, **18**, 35 (1934).

(14) K. G. Stern and A. White, *J. Biol. Chem.*, **122**, 371 (1938).

out involving the tyrosine groups in the molecule.

The effect of *p*H on the extent of acetylation with ketene also reveals differences in the chemical characteristics of different proteins. Herriott[5,13] found that the acetylation of the tyrosine phenolic group in pepsin is slower at *p*H 4.0–4.5 than at *p*H 5.0–6.0. In tobacco mosaic virus[9] and serum albumium[15] no effect of *p*H was observed in acetylating the tyrosine phenolic group. The results herein reported resemble the findings of Herriott.

The employment of other reagents, such as phenyl isocyanate[16] and nitrous acid[17] has already demonstrated the essentiality of the amino groups for the biological effects of lactogenic hormone. The results of the present investigation confirm this conclusion. So far there are no indications of the existence of a prosthetic group in lactogenic hormone; the published data rather suggest that the structural make-up of the whole molecule is necessary for its physiological action. All modifications of the molecular structure thus far studied[18,19,20] tend to destroy the specific function of the hormone.

(15) C. H. Li and A. Kalman, unpublished results.

(16) A. C. Bottomley and S. J. Folley, *Nature*, **145**, 304 (1940).

(17) C. H. Li, W. R. Lyons, M. E. Simpson and H. M. Evans, *Science*, **90**, 376 (1939).

(18) C. H. Li, W. R. Lyons and H. M. Evans, *J. Biol. Chem.*, **139**, 43 (1941).

(19) C. H. Li, *ibid.*, **155**, 45 (1944).

(20) H. Fraenkel-Conrat, M. E. Simpson and H. M. Evans, *ibid.*, **142**, 107 (1942).

It is of interest to note that pepsin,[13] insulin,[14] human chorionic gonadotrophin,[21] tobacco mosaic virus[9] and β-amylase[22] are not diminished in their biological potencies by acetylation of their amino groups with ketene, whereas the potencies of diphtheria toxin[23] pituitary gonadotrophins,[21] pregnant mare serum gonadotrophin,[24] alkali phosphatase[25] and lactogenic hormone (as shown in this paper) depend upon the free amino groups in the molecule.

Summary

The reaction between ketene and lactogenic hormone has been studied in *p*H 4.0 and 7.0 buffer solutions at 0°. When acetylated lactogenic hormone preparations were assayed in pigeons, the crop-sac stimulating action was always diminished, except in one case where 20% of tyrosine phenolic hydroxyls were covered and the amino group was untouched. It was, therefore, concluded that the amino groups are essential for the biological activity of the hormone.

(21) C. H. Li, M. E. Simpson and H. M. Evans, *ibid.*, **131**, 259 (1939).

(22) C. E. Weill and M. L. Caldwell, THIS JOURNAL, **67**, 212 (1945).

(23) A. M. Pappenheimer, Jr., *J. Biol. Chem.*, **125**, 201 (1938).

(24) C. H. Li, H. M. Evans and D. H. Wonder, *J. Gen. Physiol*, **23**, 733 (1940).

(25) B. S. Gould, *J. Biol. Chem.*, **156**, 365 (1945).

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Effect of Molecular Weight and Method of Deësterification on the Gelling Behavior of Pectin[2]

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Introduction

The objective of the research described in this paper and the preceding ones[3,4,5] has been to find an explanation for the marked difference in gelling behavior between pectinic acids deësterified by acid and by enzyme catalysis.

The gelling properties of pectin, as of any high polymer, depend upon molecular weight and type of molecular surface. The latter can be readily ascertained from the degree of esterification[3] and acid behavior,[4] but the determination of molecular weight presents difficulties because of the complex colloidal behavior of pectin in water solution.

To avoid these complications, Schneider and co-workers[6,7,8] nitrated pectin and measured molecular weights in acetone solution, a procedure analogous to that commonly used in cellulose chemistry. They also made parallel measurements on pectin acetates from the same samples and obtained viscometric molecular weights agreeing with those of the nitrates. We therefore conclude that the viscometric molecular weight of nitrated pectin in acetone solution is a property of the size of the molecule and not of its surface. This is in contrast to the behavior of pectin in water, where the surface of the molecule is one of the controlling factors in determining viscosity.[9]

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Portions of this paper were included in "Factors Influencing the Behavior of Pectinate Gels," by C. H. Hills, H. H. Mottern, G. C. Nutting and R. Speiser, presented at the meeting of the Division of Agricultural and Food Chemistry, American Chemical Society, held in New York, N. Y., September 13, 1944.

(3) C. H. Hills and R. Speiser, *Science*, in press.

(4) R. Speiser, C. H. Hills and C. R. Eddy, *J. Phys. Chem.*, **49**, 328 (1945).

(5) R. Speiser, C. R. Eddy and C. H. Hills, *ibid.*, in press.

(6) F. A. Henglein and G. Schneider, *Ber.*, **69B**, 309 (1936).

(7) G. Schneider and M. Ziervogel, *ibid.*, **69B**, 2530 (1936).

(8) G. Schneider and U. Fritschi, *ibid.*, **69B**, 2537 (1936).

(9) G. L. Baker and M. W. Goodwin, *Delaware Agr. Expt. Sta. Bull.*, 234 (1941); H. S. Owens, H. Lotzkar, R. C. Merrill and M. Peterson, THIS JOURNAL, **66**, 1178 (1944). Also see reference included in footnote 2 and Fig. 11 of ref. 5.