racemization of free tryptophan and of these derivatives will be described in detail elsewhere.[6]

Summary

The preparation, nitrogen content, melting point and optical rotation of seven derivatives of L-tryptophan are described. Five of these derivatives—N-phenoxyacetyl-L-tryptophan, *p*-phenylphenacyl-N-acetyl-L-tryptophan, *p*-phenylphenacyl-N-phenoxyacetyl-L-tryptophan, ethyl-N-acetyl-L-tryptophan and N-acetyl-L-tryptophan amide—have not been previously described.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF OKLAHOMA]

The Use of Amberlite Resin in a Separation of Xanthine and Guanine[1]

BY SAM C. SMITH AND SIMON H. WENDER

Hitchings[2] has described a method for the estimation of xanthine and guanine by making use of the color forming reaction of these purines with phenol reagent. In this method only 0.0007–0.0028 millimole of xanthine or guanine is required. These two purines can be separated from other interfering substances, but heretofore there has been no satisfactory method for separating such small amounts of xanthine and guanine from each other in a mixture of the two.

A description of a method for separating small amounts of xanthine from guanine from a mixture containing only these two purines by the use of amberlite resin and the analysis for each purine comprises the subject matter of the present article. Essentially, the method consists of the adsorption of xanthine and guanine from *p*H 1 hydrochloric acid by the sodium derivative of Amberlite IR-100 resin with subsequent elution of the xanthine by means of a suitable solution. (It is not known definitely that the phenomena reported are true examples of ion exchange. Hence, the terms "adsorption" and "elution" are used throughout this article.)

The amounts of xanthine and guanine are estimated by means of the reaction with the phenol reagent by the method of Hitchings, but modified for use with a Fisher Electrophotometer. The modified analysis enables one readily to obtain correct values on standards with the phenol reagent for the purine concentrations described in this article, although these concentrations of purines are even less than those of Hitchings. The amount of guanine is determined by difference between the amount of purine nitrogen in the original solution and the xanthine nitrogen eluted.

Experimental

Amberlite Resin.—The Amberlite IR-100-H cation exchanger used was manufactured by the Resinous Products and Chemical Company of Philadelphia. This resin, of analytical quality and in the form of the hydrogen derivative, was purchased from Fisher and Eimer and Amend Company of St. Louis. The resin was processed by a method based on the successful findings of Cleaver and others[3] in their studies of amino acids on exchange resins.

Amberlite IR-100 resin was ground in a ball mill and then sieved. The portion of resin which passed through a 60-mesh but not an 80-mesh screen was selected. The resin was then washed with several portions of distilled water to remove all small particles. The washing was continued till the supernatant liquid was practically clear. Next, 5% hydrochloric acid was added to the beaker containing the resin and allowed to stand, with intermittent stirring, for about twenty minutes. The acid was then decanted off, and the resin was washed with distilled water until the wash water gave no test for chloride ion with silver nitrate. A 5% solution of sodium carbonate was then added to the resin, and this was allowed to stand, with intermittent stirring, for about twenty minutes. The resin was again washed with distilled water until the wash water was neutral to litmus paper. The treated resin was then stored in a stoppered container in distilled water till ready for use.

The treated resin was introduced in a slurry into a well-cleaned 11 × 130 mm. chromatographic adsorption column to a height of 3 cm. Several small portions of distilled water were then put through the column until the effluate was absolutely clear. The level of liquid was at all times maintained above the top surface of the resin to prevent the formation of air pockets which would prevent the solution from contacting part of the resin.

Xanthine.—The xanthine used in this investigation was purchased from Schwarz Laboratories, and was further purified by the method of Levene and Bass.[4]

Guanine Hydrochloride.—Guanine hydrochloride purchased from Eastman was further purified according to the method of Hunter and Hlynka.[5]

Standard Purine Nitrogen Solution.—For constructing a calibration graph to be used in conjunction with the electrophotometer readings in determining the purine nitrogen content of solutions, a solution was made up containing 31.9 mg. of the previously purified guanine hydrochloride dihydrate in 100 cc. of 0.1 *N* hydrochloric acid. One cc. of this solution contained 0.1 mg. of nitrogen. In constructing the graph various different size samples of this solution were treated with phenol reagent, and the electrophotometer readings obtained, using filter number 650A, were plotted against mg. of purine nitrogen.

Eluting Solution.—For elution of xanthine from the adsorption column a solution of the following composition was used: Each 1000 cc. of solution contained 950 cc. of sodium hydroxide adjusted to *p*H 9, uncorrected, with a MacBeth *p*H meter, Model A, 25 cc. of sodium hydroxide–boric acid buffer, and 25 cc. of 0.1 *N* citric acid. The buffer was prepared by adding 21.3 cc. of 0.1 *N* sodium hydroxide to 50 cc. of 0.1 *M* boric acid and diluting to 100 cc.

(1) Taken from part of a thesis submitted by Sam C. Smith to the Graduate Faculty of the University of Oklahoma in partial fulfillment of the requirements for the degree of Master of Science. Present address: Department of Agricultural Biochemistry, University of Wisconsin, Madison, Wisconsin.

(2) Hitchings, *J. Biol. Chem.*, **139**, 847 (1941)

(3) Cleaver, Hardy and Cassidy, THIS JOURNAL, **67**, 1343 (1945).

(4) Levene and Bass, "Nucleic Acids," The Chemical Catalog Co., Inc., New York, N. Y., 1931, p. 124.

(5) Hunter and Hlynka, *Biochem. J.*, **31**, 486 (1937).

Phenol Reagent.—The phenol reagent was prepared according to the method of Folin,[6] and to prevent precipitate formation, lithium sulfate was added as outlined by Folin and Ciocalteu.[7] Analysis for purine nitrogen with this reagent was carried out as follows: An aliquot of the solution was placed in a test-tube which was calibrated to contain 25.0 cc. To this were added 1.5 cc. of phenol reagent and 8 cc. of saturated sodium carbonate. The mixture was diluted to the mark, mixed, and placed in a beaker of water at a temperature of 40–50°. The tube was allowed to remain in the water for twenty minutes while it cooled slowly. At the end of this time the solution was analyzed with the electrophotometer and from the reading obtained the amount of purine nitrogen was determined, using the calibration curve.

Adsorption and Elution.—For studies on the adsorption and elution of xanthine, samples containing 0.1–0.225 mg. of purine nitrogen, such as numbers 1, 2 and 3 (Table I), were used. The xanthine was dissolved in *p*H 1 hydrochloric acid and was put through a column prepared as described above at the rate of about 2 cc. per minute. The column was rinsed with three 5-cc. portions of distilled water and the effluate analyzed with phenol reagent. The xanthine was practically completely adsorbed.

For elution of the adsorbed xanthine, from 1000 to 1350 cc. of eluting solution of the composition described above were put through the column at the rate of 3–4 cc. per minute, and by analysis of the eluate with phenol reagent it was found that practically quantitative elution was obtained (see Table I).

TABLE I

No.	Composition of solution: Xanthine nitrogen, mg.	Composition of solution: Guanine nitrogen, mg.	% of adsorption	Xanthine eluting soln., cc.	Nitrogen eluted, mg.	% Elution (or total recovery)
1	0.225	...	96	1250	0.234	104
2	.225	...	98.22	1250	.225	100
3	.225	...	96	1250	.221	98.22
'4	...	0.285	100	1000	.000	0.00
5	...	.470	100	800	.000	0.00
6	.112	.142	96.85	1000	.113	100.89[a]
7	.225	.285	98.82	1350	.224	99.56[a]
8	.225	.220	99.10	1250	.225	100.00[a]

[a] On basis of xanthine nitrogen.

In similar studies on guanine, samples containing 0.1–0.470 mg. of purine nitrogen, such as numbers 4 and 5 (see Table I), were used. The column was rinsed with three 5-cc. portions of distilled water and analysis of the effluate revealed complete adsorption (see Table I).

As can be seen from the table, the xanthine eluting solution, when used in amounts comparable to that required to elute practically all of the xanthine, eluted none of the guanine.

Mixtures of the two purines were then made up in *p*H 1 hydrochloric acid and put through columns in the same manner as were the solutions of a single purine. The eluting operation was then carried out with from 1000 to 1350 cc. of the eluting solution, and the eluate was checked with phenol reagent for total nitrogen content. As shown in Table I for samples 6, 7 and 8, values ranging from 99.56% to 100.89% recovery, as based on the amount of xanthine nitrogen in the mixture, were obtained.

In order to check the identity of the eluted material, ultraviolet absorption spectra were obtained on a xanthine eluate at *p*H 3, using a Beckman spectrophotometer with ultraviolet attachment. A maximum was obtained at 268 mμ. This maximum is characteristic for xanthine.[8] No trace of the characteristic maximum for guanine, 250 mμ, was obtained.[9] This confirmed the data obtained on samples of guanine alone, *i.e.*, no guanine is eluted by the xanthine eluting solution.

Numerous eluting solutions were tried for guanine, but none proved to be successful in removing all of the guanine. Evidence was also obtained that passage of the xanthine eluting solution through the column caused the guanine to be held more firmly by the resin.

Any purine nitrogen that was found in the original effluate from the adsorption process was attributed to xanthine, since it was previously found that the guanine was completely adsorbed.

Summary

1. A method has been described whereby small amounts of xanthine and guanine may be separated from each other in mixtures containing only these two purines.

2. The amounts of xanthine and guanine were determined by means of the color forming reaction with phenol reagent, modified for use with the Fisher Electrophotometer.

3. The identity of the separated material was checked by means of ultraviolet absorption spectra.

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(6) Folin, "Laboratory Manual of Biological Chemistry," D. Appleton-Century Co., New York, N. Y., 1934, p. 339.

(7) Folin and Ciocalteu, *J. Biol. Chem.*, **73**, 629 (1927).

(8) Loofbourow, Stimson and Hart, THIS JOURNAL, **65**, 148 (1943).

(9) Heyroth and Loofbourow, *ibid.*, **56**, 1728 (1934).