THE CHEMISTRY OF TEA LEAVES.

II. THE ISOLATION OF GUANINE NUCLEOTIDE AND CYTOSINE NUCLEOTIDE.

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(Received for publication, January 14, 1927.)

INTRODUCTION.

In an earlier paper, the preparation of adenine nucleotide from tea leaves was reported (1). It was stated there that neither guanine nucleotide, guanine, nor cytosine nucleotide had been found. That is not surprising since the methods used earlier were such that in that particular case they may have easily precluded the evidence for the presence of these substances. Modifications in the procedure have made possible the isolation of both guanine nucleotide and cytosine nucleotide.

DISCUSSION.

In the general procedure for the preparation of the nucleotides, the substance from which they are to be prepared is usually hydrolyzed in an alkaline medium. The hydrolysate is made slightly acid with acetic acid and the nucleotides precipitated as the lead salt by means of a neutral lead acetate solution. The lead salt is centrifuged or filtered, washed, decomposed with hydrogen sulfide, filtered, and the excess hydrogen sulfide removed by aeration. The filtrate is then evaporated to a syrup at a low temperature in a vacuum and the resulting syrup hardened with alcohol. The hardened material usually consisting solely of mixed nucleotides is then dissolved in the smallest possible amount of hot water (ordinarily 2 to 3 times its weight). The solution is then made faintly alkaline with concentrated ammonia. On cooling this becomes an opaque gel consisting of the ammonium salts of the nucleotides.
The ammonium salt of guanine nucleotide can be completely removed by addition of 1 to 1\(\frac{1}{2}\) volumes of absolute alcohol. The guanine nucleotide is then prepared from the precipitate. The other nucleotides are prepared from the filtrate by fractional crystallization of their brucine salts from 35 per cent alcohol.

In the preparation of the nucleotides from tea leaves the extraction is made with dilute alkali to insure the conversion of any nucleic acid, which may be present, to the nucleotides. After straining through cloth the extract is acidified and filtered. The filtrate on treatment with neutral lead acetate gives a heavy brown precipitate which is centrifuged, washed, decomposed with hydrogen sulfide, and filtered. The filtrate is aerated, evaporated to a small volume in a vacuum at a low temperature, and hardened with absolute alcohol. This hardened material, instead of being nearly 100 per cent nucleotides as is usually the case, consists of about 10 to 20 per cent nucleotides and 80 to 90 per cent impurities. It will not dissolve in 2 to 3 times its weight of warm water but sometimes requires 15 to 20 times its weight. It was at this point that the error was made in the previous work. When an equal volume of alcohol is added a heavy black precipitate separates which consists largely of impurities. Unfortunately, it does not contain enough guanine nucleotide to enable one to detect guanine by the usual process of acid hydrolysis, precipitation with ammonia, decolorization with animal charcoal in acid solution, and final precipitation from a colorless solution with ammonia. The precipitate obtained does not contain a trace of guanine as shown by a negative murexide test. The crude adenine fraction (the filtrate above) was also tested but a negative test for guanine was obtained, no doubt due to loss in purification. Some guanine nucleotide however was undoubtedly present in the adenine fraction since the analyses of the brucine salt did not appreciably change on recrystallization from 35 per cent alcohol, a result which has recently been observed when guanine nucleotide was known to be present.

Since adenine and guanine almost invariably occur together, it seemed unlikely that it should not be so in this case. A slight modification of the above procedure changed the findings almost entirely. Instead of adding 1 or 1\(\frac{1}{2}\) volumes of alcohol to a solution of the crude nucleotide in about 15 parts of water, \(\frac{1}{2}\) to \(\frac{3}{4}\)
of the volume is added and the impurities, consisting chiefly of phosphates, are precipitated leaving principally nucleotides in solution. In this way about 75 per cent of the impurities are removed and then the manipulations of the nucleotides are about the same as with the mixed nucleotides obtained from yeast nucleic acid.

The crystalline guanine nucleotide obtained in this investigation was prepared by the method of Buell and Perkins of this laboratory (2). It has the characteristic crystalline structure, the same number of molecules of water of crystallization, and the same chemical composition as the crystalline guanine nucleotide, prepared from yeast nucleic acid.

The cytosine nucleotide has also the characteristic crystalline structure and same chemical composition as that obtained from yeast nucleic acid and from the pancreas.

The occurrence of the above two nucleotides in tea leaves along with the one reported earlier would seem to indicate that they are present in the form of nucleic acid. In view of the fact that nucleic acid is such an indefinite substance and that it is impossible or extremely difficult to prepare, no attempt was made to isolate it. But since the nucleotides are relatively easy to isolate and since their properties are well defined, methods were used whereby any combined nucleotides (nucleic acid) would be hydrolyzed before any attempt was made to isolate them from tea leaves. For this purpose the extraction was made with 2.5 per cent sodium hydroxide.

EXPERIMENTAL.

Extraction of Tea Leaves.—The procedure was the same as that described in the earlier paper and need not be repeated here.

Preparation of Crude Nucleotides.—The filtrate obtained in the procedure referred to above was slowly treated with a 25 per cent solution of neutral lead acetate until no further precipitate formed on addition of more lead acetate to a portion of the filtered liquid. About 20 cc. of lead acetate were added in excess of the above requirement and the solution was usually allowed to stand overnight. The precipitate was centrifuged from the pale yellow liquid and washed three times by grinding with cold distilled water in the centrifuge cups. The washed precipitate was suspended in
hot water and decomposed with hydrogen sulfide. The dark brown filtrate from the lead sulfide was freed from hydrogen sulfide by aeration. It was then concentrated under reduced pressure at 40–50°, to a small volume and finally in a vacuum desiccator to a thin syrup. This syrup was hardened by pouring it into 10 times its volume of absolute alcohol. The finely divided light brown precipitate, which formed, was filtered and washed twice with 100 cc. of absolute alcohol at each washing. When dry the precipitate varied in weight from 10 to 15 gm. A total of 24 pounds of tea leaves was carried through the procedure up to this point, in lots of 2 pounds each, and yielded a total of 135 gm. of crude nucleotides. The crude nucleotides were dissolved in 10 to 12 times their weight of boiling water, let stand overnight, and filtered from a small amount of insoluble material. The solution was then made faintly alkaline with ammonia and a liter of absolute alcohol was added. The dark gelatinous precipitate was centrifuged from the dark brown alcohol solution and washed once with 40 per cent alcohol. No guanine could be found in the precipitate. The combined mother liquor and washings were evaporated to dryness by means of an electric fan at room temperature. The residue was dissolved in 10 times its weight of boiling water and made distinctly acid with a very small amount of acetic acid. Neutral lead acetate was added until no further precipitate was formed when tested as above. The hot solution was cooled by allowing it to stand overnight. The lead precipitate was centrifuged from the supernatant fluid and washed three times. The washed precipitate was suspended in hot water and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was aerated to remove the excess hydrogen sulfide and concentrated to a thin syrup by the usual process. The syrup was hardened with absolute alcohol and dried in a vacuum desiccator.

Separation of Nucleotides into Two Fractions.—The hardened material obtained above (20 gm.) was dissolved in 4 times its weight of warm water and made faintly alkaline with concentrated ammonia. When cool, 120 cc. of absolute alcohol were added to this solution and a heavy precipitate was obtained. It was centrifuged and washed once with 60 per cent alcohol. The precipitate constitutes the guanine fraction and the filtrate and washings constitute the adenine fraction.
Preparation of Guanine Nucleotide.—The guanine cake obtained above was dissolved in the smallest possible amount of hot water, cooled, treated with an equal volume of absolute alcohol, and centrifuged at high speed. This process was repeated three times. The final precipitate was dissolved in about 20 times its weight of hot water, made faintly acid with acetic acid, treated with a slight excess of lead acetate solution, cooled, and centrifuged. The precipitate was washed twice, suspended in hot water, and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was aerated and precipitated again with lead acetate solution. The precipitated lead salt was washed three times, suspended in hot water, and decomposed with hydrogen sulfide. The filtrate from the lead sulfide was aerated and this time the nucleotide was precipitated as the silver salt by means of silver nitrate solution. The silver salt was centrifuged and washed two or three times with distilled water, suspended in hot water, and the silver precipitated as the sulfide by means of hydrogen sulfide. Colloidal silver sulfide may be obtained, and if so, it is almost impossible to obtain a clear filtrate. However the colloidal nature may be changed by addition of 1 or 2 drops of 10 per cent hydrochloric acid, a procedure which does not interfere with the crystallization of the guanine nucleotide. The water-clear filtrate from the silver sulfide was aerated to remove the excess hydrogen sulfide. It was concentrated in a vacuum below 40° to a small volume and then allowed to evaporate further by exposure, in an open dish, until the guanine nucleotide crystallized out. The crystals had the characteristic whetstone shape as those obtained by Buell and Perkins (2) from yeast nucleic acid.

I. Micro-Pregl for P.
   5.246 mg. gave 28.06 mg. ammonium phosphomolybdate.
II. Micro-Dumas-Pregl for N.
   7.474 mg. gave 1.126 cc. N at 20° and 755 mm.

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<th>Found, P</th>
<th>Found, N</th>
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<tr>
<td>P</td>
<td>7.77</td>
<td>7.74</td>
<td>7.43</td>
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<tr>
<td>N</td>
<td>17.54</td>
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Preparation of Guanine and Guanine Chloride.—A sample of 182.8 mg. of the above nucleotide was hydrolyzed for 1 hour in a
test-tube immersed in a steam bath. The pale yellow solution was cooled slightly and carefully neutralized with ammonia. Further addition of ammonia was made to make a 2 per cent solution and the mixture was allowed to stand 24 hours at room temperature. The guanine was filtered on a micro asbestos filter which had been previously dried in a desiccator to constant weight. The guanine was dried similarly. Yield, 65.8 mg.

The guanine obtained above was dissolved in 15 cc. of hot 5 per cent hydrochloric acid and filtered. On cooling the characteristic macroscopic needles of guanine chloride crystallized out. They were recrystallized once from dilute hydrochloric acid and analyzed.

I. Water of crystallization.
73.8 mg. samples lost 11.9 mg. in a desiccator over sulfuric acid.

II. Micro-Dumas-Pregl for N.
3.63 mg. gave 1.009 cc. N at 28° and 755 mm.

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<td>C₉H₇N₄O₂·HCl·2H₂O</td>
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<td>I. Water of crystallization</td>
<td></td>
<td>10.10  16.10</td>
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<tr>
<td>N</td>
<td></td>
<td>31.32  31.24</td>
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<tr>
<td>II. Micro-Dumas-Pregl for N</td>
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<td>10.12  31.24</td>
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Preparation of Cytosine Nucleotide.—The adenine fraction of the alcoholic filtrate obtained above consisted chiefly of the ammonium salts of adenine nucleotide and cytosine nucleotide. This was evaporated to dryness by means of a current of air, dissolved in 3 times its weight of water, and treated with an equal volume of absolute alcohol to remove last traces of guanine nucleotide. If all the guanine was not removed this process was repeated. The precipitate which formed was filtered off and the alcoholic filtrate was treated with an equal volume of hot water, made faintly acid with acetic acid, and the nucleotides precipitated by means of lead acetate. The free nucleotides were prepared from the lead salt in the usual way and hardened with absolute alcohol. They were then dissolved in a small amount of hot water and neutralized with a hot solution of brucine in alcohol. The brucine salts were filtered off and recrystallized nine times from 35 per cent alcohol. The results of analysis after each fractionation are as follows:
Crystallization No. & Analyses, N & Calculated for: \\
& per cent & Cytosine nucleotide, N & Uracil nucleotide, N & per cent \\
1 & 8.27 & 7.92 & 6.79 \\
2 & 7.97 & & \\
3 & 8.16 & & \\
4 & 7.84 & & \\
5 & 7.83 & & \\
6 & 7.51(?) & & \\
7 & 7.82 & & \\
8 & 7.85 & & \\
9 & 7.81 & & \\

The final precipitate and the residues obtained by evaporation of the last four filtrates were combined, suspended in hot water, and an excess of ammonia was carefully added. The brucine was allowed to crystallize out overnight, filtered off, and the filtrate extracted three times with a small amount of chloroform. The solution was made faintly acid with acetic acid and lead acetate was added. The precipitated lead salt was decomposed in the usual way for obtaining the free nucleotides and instead of concentration in a vacuum the solution was allowed to evaporate spontaneously at room temperature. The characteristic crystals of cytosine nucleotide were formed.

I. Micro-Dumas-Pregl for N.
   4.884 mg. gave 0.555 cc. N at 22° and 756 mm.

II. Micro-Pregl for C and H.
   4.241 mg. gave 1.74 mg. water.

III. 4.241 " " 5.16 " CO₂.

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<th>Calculated for cytosine nucleotide</th>
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<td>N</td>
<td>13.90</td>
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<td>H</td>
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<td>C</td>
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The author wishes to express his sincere appreciation to Dr. O. Wintersteiner, Rockefeller Foundation Fellow, from Professor Pregl's laboratory at Graz, who has done several of the micro analyses reported in this paper while testing out his apparatus here. He also supervised the others.
SUMMARY.

Guanine nucleotide and cytosine nucleotide were prepared from dried tea leaves. Both nucleotides possessed the characteristic crystalline structure and properties of those obtained from yeast nucleic acid. It seems very probable that the nucleic acid, which is characterized by the presence of a pentose, is a naturally occurring product in tea leaves.

BIBLIOGRAPHY.