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141. Masashi Tomoda: Colorimetric Determination of Pentoses. III.[1] Determination with Pyrogallol Reagent.

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Colorimetric determination of pentoses with polyhydric phenol reagent, with a mixture of hydrochloric acid and acetic acid as a solvent, was examined and it had been found that the pyrogallol reagent effects good coloration and that it could be used for the determination of pentoses.[1] However, accuracy of determination with this reagent was found to be low by comparison of the absorbance of the colored solution using five samples containing 20 μg./ml. of D-xylose, giving standard deviation of 0.0216, average value of absorbance of 0.383, and coefficient of variation of 5.7%. It was found from experiments that the addition of a trace of iron salt to the reagent eliminated scattering of observed values and that the pyrogallol reagent could be used for determination of pentoses.

It is already known that the presence of an iron salt in the orcinol-hydrochloric acid reagent for determination of pentoses gave a more marked coloration and many methods have been devised.[2~4] In the case of the pyrogallol reagent, increase of coloration by the addition of iron salt is small and there is almost no change in the degree of coloration or precision by variation of the concentration of iron salt in the range of 0.1 to 5×10^{-3} moles. There is also no difference according to the use of ferric chloride and ferric ammonium sulfate as the iron salt. The use of cobalt or nickel chloride in place of iron salt fails to show any effect like the iron salt.

Absorbance in the wave length of maximum absorption was measured with five samples containing 20 μg./ml. of xylose using the new pyrogallol reagent and the values obtained were 0.398 for average value of absorbance, 0.0025 for standard deviation, and 0.6% for coefficient of variation. Absorption curves obtained by this method with D-xylose, D-fructose, L-rhamnose, D-glucose, N-acetylneuraminic acid, D-glucuronic acid, and sodium deoxyribonucleate in aqueous solution are shown in Fig. 1. Both pentoses and hexuronic acids have absorption maximum at 500 mμ but the coloration of uronic acids is low. Other carbohydrates also undergo slight coloration and D-fructose showed a fairly strong coloration, with absorption maximum at 475 mμ. Substances other than pentoses and uronic acids have the same absorbance at 500 and 430 mμ, and the effect of their presence can be eliminated by observing the difference of absorbancies at 500 and 430 mμ, even if such substances are present with pentose.

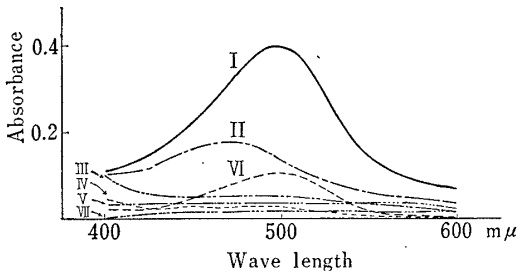

Fig. 1. Absorption Curves for the Colours produced by Pyrogallol-Fe^{3+} Reagent

I : xylose (sample = 20 μg./ml.)
II : fructose
III : rhamnose
IV : glucose
V : sialic acid
VI : glucurone
VII : DNA

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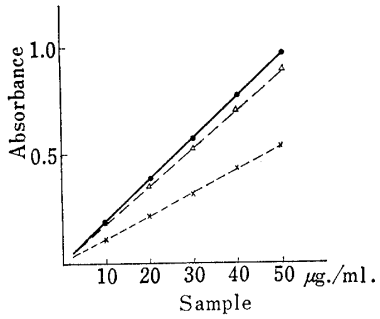
1) Part II : Yakugaku Zasshi, **82**, 1447 (1962).
2) G. L. Miller, R. H. Golder, E. E. Miller : Anal. Chem., **23**, 903 (1951).
3) W. R. Fernell, H. K. King : Analyst, **78**, 80 (1953).
4) Z. Dische : Methods of Biochem. Anal.' **2**, 349 (1955).

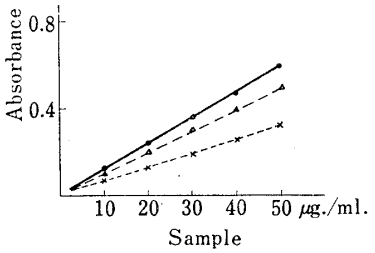
Measurement of absorbance of D-xylose, D-ribose, and L-arabinose at 500 mμ and calculation of difference in their absorbancies at 500 and 430 mμ showed that the coloration is linearly proportional to concentration in the range of 10~50 μg./ml. (Figs. 2 and 3). Determination of sodium ribonucleate by this method was also found to be possible within a concentration of 250 μg./ml., as shown in Fig. 4. It is clear from the experimental results reported in Part I of this series[5] that the determination of pentosans is possible under the present conditions. The degree of coloration of D-ribose is 90% and that of L-arabinose is 54% of that of D-xylose at 500 mμ.

It is now clear that the determination of pentoses, pentosans, and D-ribose in nucleic acid can be carried out by the present method. D-Glucosamine and serum albumin do not undergo this coloration and their presence, as well as that of sodium chloride, does not interfere in this determination.



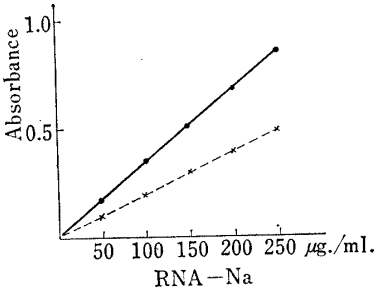
○——○ xylose △——△ ribose
×------× arabinose

Fig. 2. Optical Density at 500 mμ of Various Amounts of Pentoses



○——○ xylose △——△ ribose
×------× arabinose

Fig. 3. Difference between the Opitical Density at 500 mμ and at 430 mμ of Various Amounts of Pentoses



•——• E (500 mμ)
×------× ΔE (500~430 mμ)

Fig. 4. Optical Density of Various Amount of RNA-Na

Coefficient of variation by the known representative methods for colorimetric determination of pentoses is 0.7% for the orcinol method[2] and 1.2% for the phloroglucinol method.[6] The present pyrogallol method is better in accuracy and has eliminated the effect of the presence of substances other than pentose and hexuronic acid.

Experimental

The New Pyrogallol Reagent——A solution was prepared by dissolving pyrogallol in conc. HCl-AcOH (2:1, v/v) mixture to the concentration of 0.6% and 99 ml. of this solution was mixed with 1 ml. of 0.01*M* $FeCl_3$ solution. This reagent is almost colorless and should be prepared just before the use.

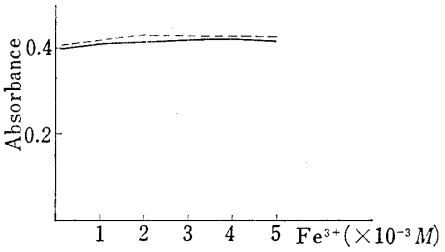
Measurement of Absorbance and Absorption Curve——The sample solution (1 ml.) was added to each of a series of test tubes (25×200 mm.) with a glass stopper. The reagent (4 ml.) was added and the content of the tube was well mixed. At the same time, a blank (1 ml. of water plus 4 ml. of the reagent) was set up, heated for 20 min. in a boiling water bath, and cooled to room temperature immediately in cold water. The absorbance was measured by Hitachi spectrophotometer type EPU-2 and absorption curve was measured by Hitachi automatic recording spectrophotometer type EPS-2.

Effect of the Addition of Iron Salt——Relationship between the absorbance at 500 mμ and the concentration of Fe^{3+} ion in the reagent with a sample of D-xylose (20 μg./ml.) is shown in Fig. 5.

Effect of Heating Time——Relationship between the absorbance at 500 mμ and heating time in a boiling water bath with the sample of D-xylose, D-glucose (20 μg./ml.), and sodium ribonucleate (100 μg./ml.) is shown in Fig. 6. The maximum coloration was obtained when xylose sample was heated for 20 min. In the case of ribonucleic acid, the coloration almost the maximum for 20 min.'s heating and, in the case of D-glucose, the coloration became higher with increase of heating time.

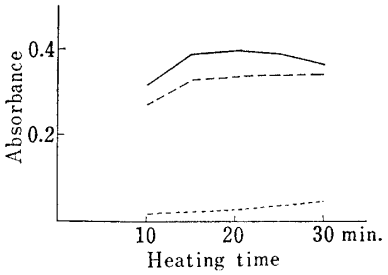
5) M. Tomoda, S. Kamiya, M. Kimura : Yakugaku Zasshi, 82, 126 (1962).
6) H. von Euler, L. Hahn : Svensk Kem. Tidskr, 58, 251 (1946) (C. A., 41, 2108 (1947)).

Stability of the Color Developed——Relationship between the absorbance at 500 mμ and time at room temperature after heating with the sample of D-xylose (20 μg./ml.) is shown in Fig. 7. The color was stable within 30 min. and decreased gradually thereafter.



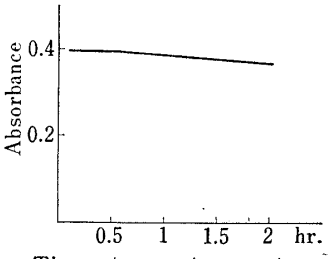
---- $NH_4Fe(SO_4)_2 \cdot 12H_2O$
—— $FeCl_3$
sample : xylose 20 μg./ml.

Fig. 5. Relationship between the Optical Density at 500 mμ and Ferric Ion Concentration



—— xylose 20 μg./ml.
---- RNA·Na 100 μg./ml.
------ glucose 20 μg./ml.

Fig. 6. Relationship between the Optical Density at 500 mμ and Heating Time



sample :
xylose 20 μg./ml.

Fig. 7. Stability of Colour Development

The writer is indebted to Prof. Tamio Yamakawa, Institute for Infectious Diseases, University of Tokyo, for his kindness in providing the sample of N-acetylneuraminic acid. He is grateful to Miss Masako Kimura for her technical assistance in the experiment.

Summary

A method using pyrogallol-ferric chloride-hydrochloric acid-acetic acid reagent was described for the colorimetric determination of pentose, pentosan, or ribonucleic acid. Coloration by this method was 90% in D-ribose and 54% in L-arabinose of that of D-xylose. Pentoses can be determined up to 10～50 μg./ml. Hexuronic acid showed an absorption curve similar to that of pentoses but its coloration was much lower than that of pentoses. It is possible to eliminate the influence of other carbohydrates by estimating the difference in absorbances between 500 and 430 mμ. The presence of D-glucosamine, serum albumin, and sodium chloride does not interfere in this coloration.

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