Thiobarbituric acid reacting material in Erigeron annuus pers

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Abstract Erigeron was hydrolyzed in acid directly, and the acid hydrolysate was fractionated by ion-exchange chromatography (Amberlite IR-120 and Dowex 1×2) and by gel filtration using Sephadex G-15. The gel filtrated TBA positive fraction was submitted to analysis for comparison with sialic acid from ovomucin. Sialic acid was not detected in Erigeron.

The structure of TBA positive fraction can be formulated as $HOCH_2(CHOH)_5CH_2COCOOH$ according to molecular weight determination, identification of chromogen in TBA test, IR absorption, NMR spectra, elementary analysis, periodate oxidation, analysis of functional groups, some coler reaction and so on. This compound showed rather similar properties to sialic acid in chromatographic and colorimetric method of analysis.

Introduction

Since standard procedures of TBA assay for determination of sialic acid were established by L. Warren[1] and by D. Aminoff[2], respectively, their methods have been extensively used when sialic acid was studied. Thus, there was a tendency among researchers dealing with sialic acid in some connection with TBA to produce pink color with maximum absorption at 549 nm belong to sialic acid. In fact, G. F. Springer[3], F. C. Mayer et al.,[4] Onodera et al.,[5] D. L. Correll[6] and M. Kanamori et al.[7] indicated occurrence of sialic acid in plant kingdom mainly on experimental basis of TBA reaction. But as studies made progress, it has been recognized that it is not only sialic acid that reacts with TBA in pink color. That is why F. C. Mayer and his coworkers[4] had to concluded that it was not yet determined whether the compound occured as acetyl, diacetyl, glycolyl or methyl derivatives. The occurrence of sialic acid in a glycopeptide isolated from chlorella celles[4] has been demonstrated by paper chromatography and color reaction. However, recent surveys and reviews of plant glycoproteins and glycolipids either do not mention sialic acid as a compound or suggest that it does not occur in these compounds in plant[8-14].

As to sialic acid in animal, however, it was well purified, often obtained in a crystal form and, therefore, identified with various other methods including a number of color reactions, X-ray diffraction, elementary analysis, paper chromatography, UV absorption, IR absorption, oxidation and reduction reaction, and determination of functional groups etc.. As far as sialic

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acid of animal origin is concerned, the metabolic reactions involved in the synthesis of of sialic acid has been elucidated, in spite of much remaining room for discussion on its biological function.

This paper describes a substance in Erigeron which was found to be remarkably possitive to TBA reaction. The substance was expected to be a sialic acid at first. In fact, it had been very difficult to show the difference in property between the substance in Erigeron and sialic acid at the beginning of isolation and purification processes. Marked differences were caught, however, when organic chemical procedures, such as IR and NMR, were used for more detailed analysis of their structures. We will discuss the way how the substance in Erigeron was found to be 2-keto-3-deoxy nonate through the explanation of these differences from sialic acid.

Methods and Materials

Sialic acid-like substance isolated from Erigeron in the way described below was analyzed in comparison with N-acetyl neuraminic acid from ovomucin. All the reagents used in this experiments were of c. p. grade.

Isolation of TBA reacting material from Erigeron

Erigeron of 500g dry weight was repeatedly hydrolysed in 10 volumes of 0.1 N sulfuric acid for one hour at 80°C. The residue was filtered off and pH of the filtrate was adjusted to 7 with barium hydroxide solution, followed by centrifugation to remove the precipitate. The supernatant liquid was passed through Amberlite IR-120 H^+ form (5×35cm). After appropriate washing with water, the combined effluent was poured on Dowex 1×2 $HCOO^-$ form (5×35cm). Sialic acid-like substance was eluted with 0.3 N formic acid and TBA possitive fractions were collected for lyophilization. For further purification, the lyophilized sample was rechromatographied on Amberlite and Dowex ion exchange resins. The concentrated eluate which was highly TBA possitive was applied on Sephadex G-15. The gel filtrated TBA possitive fractions were once dried and dissolved in minimum amount of water. Ten volumes of methanol was added, followed by the add addition of 30 volumes of ethyl ether. The precipitate was filtered off and the filtrate was dried by evaporating the organic solvents. Slightly brownish powder thus obtained was submitted to analysis for comparison with sialic acid from ovomucin.

Thiobarbituric acid assay

Sialic acid-like substance was determined at various steps of isolation with Beckman spectrophotometer or multipurpose spectrophotometer after color development of Aminoff's TBA reaction.[2] In relation to the structural interpretation of sialic acid-like substance, mechanism involved in this reaction will be discussed later in this paper. It seems, however, to be worthy of mentioning here that the chromogen of this two step reaction is not formed until periodate oxidation of sample substance is completed before the addition of TBA.

Paper chromatography

Using sialic acid as reference, one dimension paper chromatography was performed at 20°C

on Toyo filter paper No. 2 with following solvents ; n-butanol : acetic acid : water=4: 1 : 5, and n-butanol : pyridine : water=6 : 4 : 3. After 17 hr development, spot were located by either TBA assay reagents of alkaline silber nitrate.

Silica gel thin layer chromatography

In addition to the reagents used in the paper chromatography, heated sulfuric acid was used for spot detection to check the homogeneity of samples. Other conditions were the same as those of paper chromatography.

High voltage paper electrophoresis

Electrophoresis was performed on Toyo filter paper No. 51A (3×30cm) at pH 3.6 in pyridine-acetate buffer for 20 min with a current condition of 10 mA/cm. Migrated components were located with the same method as in the case of paper chromatography.

Other color reactions

Direct Ehrlich reaction and Orcinol reaction were performed according to Werner and Odin[15], and Svennerholm[16]. These are typical one step reaction sensitive to sialic acid. The former gives a purple color and the latter a dark purple color.

Paper electrophoresis

A current of 1 mA/cm was applied in 5 hr electrophoresis on Toyo filter paper No. 51A with pH condition of 4.0 in acetate buffer (μ=0.05). Migrated components were detected by TBA assay reagents.

Qualitative analysis of functional groups of sialic acid-like substance

Carboxyl group was detected in then ext way. After the addition of thionylchloride, sample was heated to dryness and added by alcohol saturated with hydroxylamine hydrochloride before it was neutralized with alcohol solution of sodium hydroxide and heated. Carboxyl acids colored dark purple when 1% ferric chloride was added in the acidic condition. Keto group was detected by the formation of yellow precipitate with 2, 4-dinitrophenylhydrazine. Amine group was checked with ninhydrin and p-dimethylaminobenzaldehyde. The first amine group was detected by the characteristic odor formed in the carboxyl-amine reaction.

Infrared analysis

Infrared spectra were obtained from KBr disks with a Shimazu IR-27C.

Nuclear magnetic resonance

5-30 mg of samples were submitted to NMR analysis which was recorded at 60 MHz in D_2O solution with a Varian DA-60IL spectrometer. Tetramethylsilane was used as an internal reference.

Color reactions specific to 2-Keto-3-deoxy sugar acids

Semicarbazide and o-phenylenediamine reactions were employed in detecting 2-keto-3-deoxy

acids quantitatively. In addition, modified TBA assay was tried to confirm whether or not color developed at lower temperature than 100°C in the normal reaction.

Periodate oxidation

Periodate oxidation was carried out in the similar codition to that previously reported by Blix[17]. Reaction temperature was changed to 50°C in order to avoid over-oxidation. Formation of the chromogen for TBA assay was traced as oxidation reaction proceeded up to 60 min after oxidation started. It was calculated how many moles of periodate should be consumed by one mole sample for the complete formation of the chromogen.

Elementary analysis

Hydrogen, carbon and nitrogen were determined with usual method.

Results and Discussion

The sample once chromatographied roughly in larger volume fraction (50 ml) with Dowex 1×2 was again applied to a column chromatography in a closer condition as shown in Fig. 1. It is apparent that three fractions which are all possitive to TBA reaction were separated and that the last one (Fraction 3) is a quantitatively main fraction. In fact, it showed very clear absorption curve in TBA reaction which is ideally similar to that of authentic sialic acid, negligibly low at 450 nm and sharply high at 549 nm with a small shoulder around 510 nm.

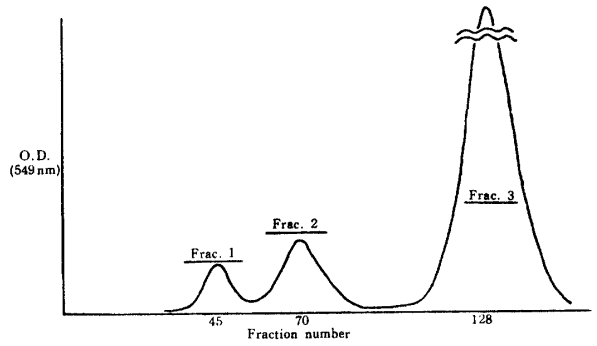

Fig. 1. Chromatography of Thiobarbituric Acid (TBA) Reacting Materials of Erigeron on a Column (2.5×25cm) of Dowex 1×2 (formate from)
Elution was performed with 0.3N formic acid at the rate of 0.5 ml per minute after the column was washed with water until no sugar was detected by Molish test. Fraction volume was 5 ml. Fractions were collected every 10 minutes and 0.1 ml alipuot was used for TBA assay

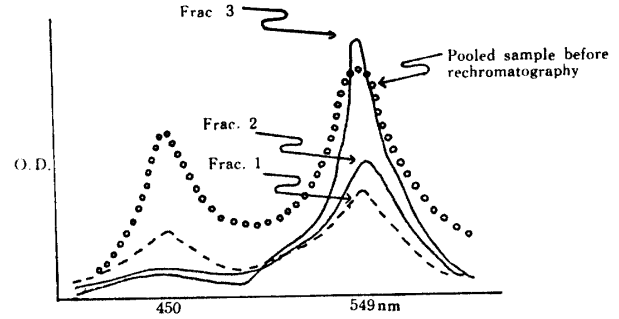

Fig. 2. Absorption Spectra of Various TBA Reacting Materials of Erigeron in TBA reaction

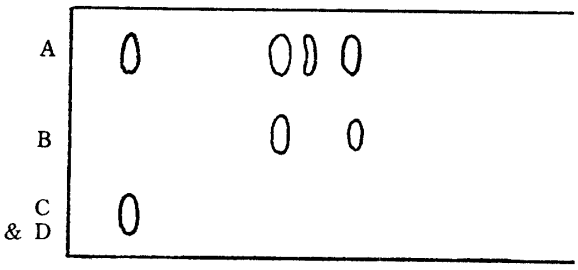

Fig. 3. Paper Chromatography of TBA Reacting Materials Isolated from Erigeron on Toyo Filter Paper No 2 with Following Conditions.
Solvent ; n-butanol : acetic acid : water=4 : 1 : 5
Running time ; 16 hr
Temperature ; 20°C
Spot detection ; TBA and alkaline silver nitrate
Samples were ; A—pooled sample before rechromatography
B—fraction 2 on rechromatography
C—fraction 3 on rechromatography
D—sialic acid

Figure 2 shows how much the peak at 450 nm is due to some unkonwn contamination which were, in relatively large amount, contained in the sample before rechromatography. In addition, fraction 1 seemed to be an unexpected fraction for the reason that the TBA absorption spectrum was more or less similar to that of contaminated sample. Therefore, it was put aside from the present experiment. The rest of the samples, that is, sample before rechromatography, fraction 2, and fraction 3 were com-pared with authentic sialic acid with paper chromatography. The result showed that the rechromatography on Dowex 1×2 was quite an effective step for the purification of the contaminated sample which at least contained four components as shown in Fig. 3. Three of four components were well separated by Dowex treatment and again here it was proved that fraction 3 is paper chromatographically indistinguishable from sialic acid. Fraction 2, on the other hand, which had rather good absorption sepctrum in TBA reaction, was found here unworthy of further analysis owing to its heterogeneity. A problem now encountered is that it is necessary to check whether or not the last remained fraction 3 was

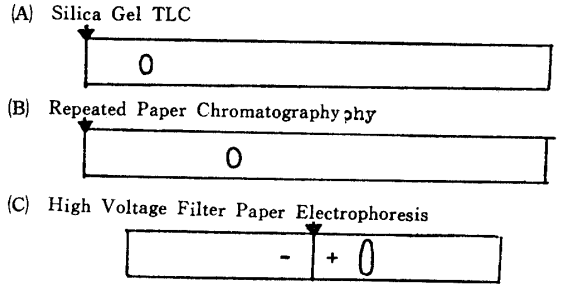

Fig. 4. Chromatographic and Electrophoretic Homogeneity of Fraction 3
(A) Solvent system was ; n-butanol : acetic aoid : water=4 : 1 : 5. Spot was made visible by spraying sulfuric acid and heating the whole plate.
(B) Repeated paper chromatography in the same solvent system as in the silica gel TLC. The second time chromatography was carried out after drying the paper of the first time one. Spot was made visible by TBA.
(C) High voltage paper electrophoresis was performed in the condition of 60 V/cm, 10 mA/cm, 20 min. and pH 3.6. Migrated band was located by silver nitrate.

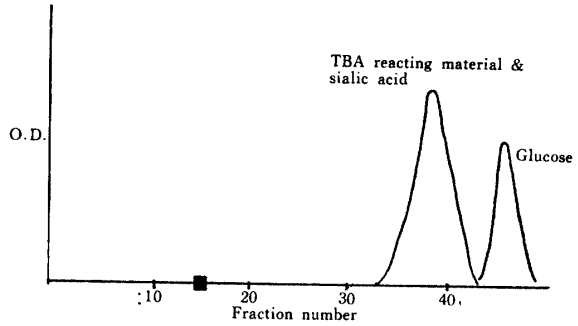

Fig. 5. Chromatogrphy of TBA reacting Material of Erigeron on a Column (1×55cm) of Sephadex G-15
An alipuot of 5 ml was applied to the column. Elution was performed with deionized water at a rate of 0.5 ml per minute. Fraction volume was 2.7 ml. 0.1 ml aliquot was used for TBA assay.

pure, or to check its homogeneity in another word. For this purpose, four methods were used; silica gel thin layer chromatography, repeated paper chromatography, high voltage paper electrophoresis and gel filtration on Sephadex. Reagents employed for spot detection were sulfuric acid, TBA, and alkaline silver nitrate, although othe reagents, for instance, iodine and ammonium metavanadate, might have been used. In none of these cases, however, there was any evidence for necessity to purify the sample further. Additionally, within the limit of the method employed, gel filtration pattern brings about an idea that molecular weight of the sample is about 300, well larger than that of glucose and the same as or close to that of sialic acid.

So far, the sample 3 isolated in considerably high purity showed several properties which made us expect that it might be sialic acid. In fact, a few papers[3-14] reporting the existence of sialic acid in plants is based on not many experimental evidence. For example, F. C. Mayer et al[4]. made their conclusion concerning the occurrence of sialic acid in plant seed from results of paper chromatography and some color reactions including TBA reaction. It is sure that TBA reaction had been a key reaction to identify sialic acid unti several years ago. However, as the mechanism involved in the TBA reaction has been made clearer, although not completely understood yet, several other compounds have been found positive to TBA reaction. Such compounds are 2-keto-3-deoxy-sugar acid, deoxyribose, 2-aminopyrimidine, and shikimic acid etc. In spite of the absence of decisive evidence for the occurrence of sialic acid in plants, sialic acid from animal and microorganism sources has been extensively studied with additional methods such as X-ray diffraction, IR spectrum, enzyme assay for synthesis and decomposition, and elementary analysis etc. So it was considered that it was too early to call the sample substance from Erigeron sialic acid and, instead, that it was proper to call it sialic acid-like or TBA reacting material.

The first experimental fact that made it clear that the sample is different from sialic acid of ovomucin was the result of paper electrophoresis. Figure 6 shows distinct difference in mobility to the cathod, where the sample moved a little bit further than sialic acid. At this point, however, our expectation that the sample sustance may be sialic had not gone because sialic acid is a general nomenclature for all the delivatives of neuraminic acid. Sialic acid of

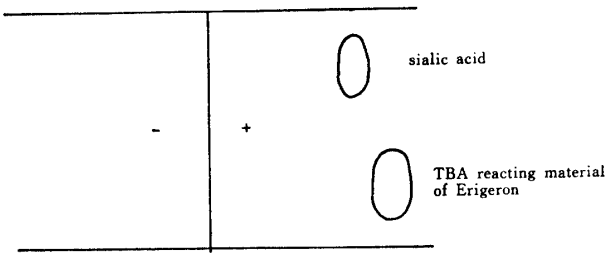

Fig. 6. Paper Electrophoresis
Conditions of five hour electrophoresis were 1mA/cm, pH 4.0 in acetate buffer (μ=0.05). Spots were located by TBA.

ovomucin has been already elucidated to be N-acetyl neuraminic acid. Consequently, the assured fact is that the sample substance is not N-acetyl neuraminic acid, but may be one of the other delivatives of neuraminic acid. At least, there were some substantial experimental bases on which it could be confirmed that its frame structure of carbon atomes in the molecule is the same as or quite similar to that of sialic acid. First of all, infrared spectrum supported our idea about molecular structure. The spectrum of the sample is nearly completely consistent with that of N-acetyl neuraminic acid (Na salt) of ovomucin. An absorption at 1680 cm^{-1} is certainly due to carbonyl group. Differences were found at ·1720, 1570 and 1320 cm^{-1}. The absence of carboxyl absorption at 1720 cm^{-1} in N-acetyl neuraminic acid is explainable when it is considered that the absorption due to carboxylic acid moves to 1600 cm^{-1} in it salt form. A weak absorption at 1320 cm^{-1} cna't be well ascribed to any functional group at present, but that at 1570 cm^{-1} is certainly ascribable to -NHR group. Accordingly, only difference between the sample from Erigeron and sialic acid rom ovomucin that is understood from IR spectra is the absence of -NHR group in the sample. R of sialic acid from ovomucin is acetyl which has very sharp single peak at 2.07 ppm on NMR spectrum as seen in Fig. 8. It should not be overlooked that doubel peaks due to $-CH_2-CO-$ protons overlapped with that of acetyl protons. Methyne protons cause peaks around 4 ppm. Ratio of 2 ppm protons and 4 ppm protons was calculated from the spectrum by comparison of the integrated area of each peak, which made 5 : 7, perfectly consitent with sialic acid. On the other hand, spectrum of the sample shows no peak ascribable to acetyl protons, but it has two double peak due to methylene

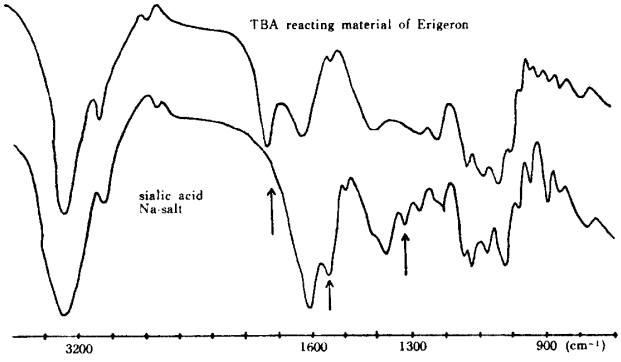

Fig. 7. Infrared Spectra
After mixing samples of 1-2 mg with KBr of 250mg, they were placed in a dessicator overnight and pressed into pellet.

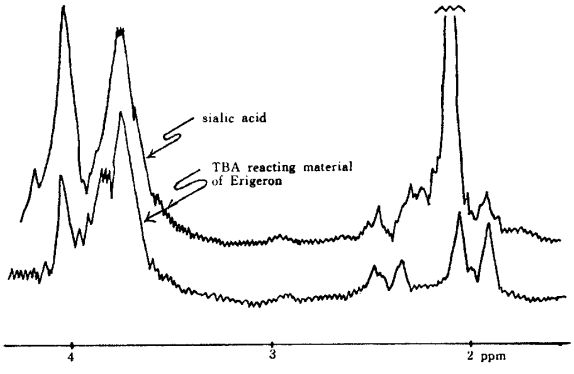

Fig. 8. Nuclear Magnetic Resonance Spectra
30mg of samples were analyzed in D_2O solvent with tetramethlsilane as an internal reference.

and methyne protons, ration of which is probable 2 : 7. And its whole pattern looks very similar to that of N-acetyl neuraminic acid except the absence of 2.07 ppm peak. Accrodingly, it was clearly proved that there is no N-acetyl group in the sample the basic structure of which has almost all the same carbon scheleton as sialic acid. Thus, it was assumed according to the experimental results mentioned above that the sample was a 2-keto-3-deoxy-sugar acid which is generally formulated as $HOCH_2(CHOH)_nCH_2COCOOH$. In fact, there have been a few papers reporting 2-keto-3-deoxy hexonic acid, 2-keto-3-deoxy heptonic acid and 2-keto-3-deoxy octonate of biological origin as highly TBA possitive materials. The first process in the structural analysis was to know the structural situation of the first four carbons in the molecule. It is in general accepted that formyl pyruvic acid is the chromogen in TBA color reaction. The chromogen is formed from the first four carbon atoms of sialic acid molecule in the first step of periodate oxidation before TBA is added to develope pink color. Figure 9 shows that both the sample and sialic acid, when oxidized by periodic acid, formed the same chromogen for TBA test. One of the two spots on the chromatogram should be formyl pyruvic acid and the other is probably a partially oxidized portion. Therefore, the first four carbons were determined to in the same form as sialic acid; $-HCOH-CH_2-CO-COOH$. As the previous discussion indicated, IR and NMR spectra also supported the existence of -COOH, -CO, and $-CH_2$ respectively in the molecule of the sample substance. Additional evidence for the presence of -COOH and -CO groups was obtained by frequently used and handful spot test; hydroxyl amine and 2, 4 DNP tests. Semicarbazide and phenylene diamine

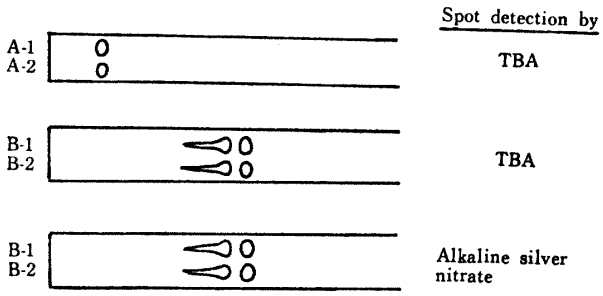

Fig. 9. Thin Layer Chromatography of Chromogen in TBA reaction
After complete formation of chromogen by periodate oxidation performed in the same condition as in TBA reaction, reaction mixture was subjected to cellulose thin layer chromatography using solvent system of n-butanol : acetic acid : water=4 : 1 : 5.
Samples were A-1 : sialic acid before periodate treatment
A-2 : TBA reacting material before periodate treatment
B-1 : sialic acid after periodate treatment
B-2 : TBA reacting material after periodate treatment

Table. 1. Spot Tests of Functional Groups

Spot test	Functional group to detect	Result
Hydroxyl amine	COO^-	+
2 , 4 DNP	CO	+
Ninhydrin	NH_2	−
p-DMAB	NH_2	−
Carbylamine	NH	−

Table. 2. Qualitative Tests on TBA reacting Material of Erigeron and Sialic acid

Color Reaction	Sample	Sialic acid
a. Bial	−	+
b. Ehrlich	−	+
c. Ehrlich (modified)	+	+
d. Alkaline silver nitrate	+	+
e. Ninhydrin	−	−
f. Semicarbazide	+	+
g. Phenylene diamine	+	+
h. TB A (modified)	+	−

a. Reaction with orcinol in HCl containing ferrous ammonium sulfate
b. Reaction with p-dimethylbenzaldehyde after alkali treatment
c. Acetylacetone was mixed before reaction b
d. Alkaline silver nitrate was sprayed onto a paper where sample was spoted, and whole paper was heated
e. Usual ninhydrin test
f. O. D. was measured at 250nm according to Weissbach et al.
g. Reaction was performed on a paper according to Wei ssbach et al.
h. Reaction temperature with TBA was 20°C

reactions indicated the sample substance is a keto acid. In the case of sialic acid, the fifth carbon is in the form of HCNHR (N-acyl). The presence of this form in the sample, however, was denied by IR analysis etc. The presence of $HCNH_2$ (amine) is inconceivable, either, in view that the sample was not adsorbed by cation exchange resin and that IR spectrum showed no amine absorption at 1600 cm^{-1}. An established fact is that elementary analysis found no nitrogen in the sample. With the combination of NMR data which indicated that number of hydrogen atoms in a molecule is 9, the rest of the structure was assumed to be $-(CHOH)_4 CH_2OH$. Thus, the whole structure can be formu lated as $HOCH_2(CHOH)_5CH_2COOH$. If this is a real structure of the TBA reacting material isolated from Erigeron, 5 moles of periodate are to be consumed by one mole of sample material to leave one mole formyl pyruvic acid, one mole formaldehyde and four moles formic acid. Figure 10 indicates one mole sample

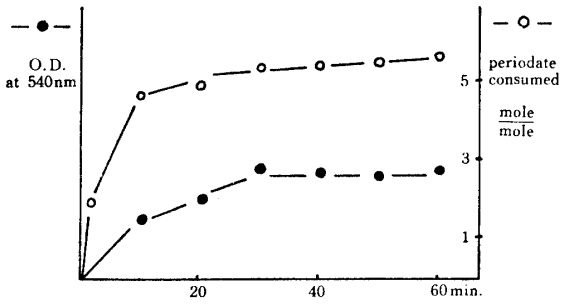

Fig. 10. Formation of Chromogen for TBA test by Periodate Oxidation
At the given intervals, alipuots were taken from a reaction mixture of 1/3×10 mole sample and 1/2×10 mole metaperiodate in an acetate buffer of pH 4.0 for the measurement of periodate consumption by iodine titration and for the measurement of formaldehyde formation by chromotropic acid test. TBA test was performed at the same intervals.

needed 5 moles periodic acid for the full formation of the chromgoen of TBA test which took about 15 to 20 minutes at 5°C.

Though there is not little argument on the reliability of such a structural conclusion made from periodate oxiation, the sample from Erigeron is tentatively described in the form of $HOCH_2(CHOH)_5CH_2COCOOH$ according to molecular weight determination, identification of chromogen in TBA test, IR analysis, NMR spectra, and some color reactions.

There have been not few investigations on 2-keto-3-deoxy sugar acids. It is well known that phosphorylated gluconate is metabolically connected with glucose. A. Weissbach and J. Hurwitz proved that 2-keto-3-deoxy heptonate has some relation with pyruvate and erythrose as indicated in the metablic process of shikimic acid. W. F. Vincent and J. A. Cameron reported that 2-keto-3-deoxy octonate seemed to be associated with bacterial cell wall. Although no study was made as to the metabolic passway of the nonate isolated by us, it would be reasonable to assume that the nonate consists of two components, pyruvate and hexose. Taking it into consideration that the heptonate above mentioned is synthesized from pyruvate and four carbon sugar, it seems natural that the nonate is a product of aldol condensation of the two components.

It is well known that sialic acid exists in animals and in some microorganisms as a constituent of mucoproteins, mucopolysacchrides, and mucolipids. The definite biological function of sialic acid has not been dissoved yet in the face of an important discovery that certain mucoproteins are endowed with biological activities which are lost upon treatment with an influenza virus in accompany with the release of sialic acid.

In october, 1963, We first reported the presence of sialic acid in plant tissues at the Annual Meeting of the Biochemical Society of Japan. Also We isolated N-acetyl-neuraminic acid from Japanese honeyworst. This result was reported at the Annual Meeting of the Agricultural Chemical Society of Japan, April 1, 1965. But I could not recognized clearly whether this material was real N-acetylneuraminic acid or not.

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要旨 ：ヒメジオンの直接酸加水分解物を，イオン交換（アンバーライト IR-120 とダウエックス 1×2）クロマトグラフィーと，セファデックス G-15 を用いるゲルロ過で分画した。得られたチオバルビツール酸反応陽性画分は，オボムチンのシアル酸と比較するため，各種の分析に供された。その結果，ヒメジオンの酸加水分解物からは，シアル酸が検出されなかった。

得られたチオバルビツール酸反応陽性物質の構造は，分子量の測定，TBA テストによる発色団の同定，赤外吸収，NMR スペクトル，元素分析，過ヨード酸酸化，官能基の検索および各種の呈色反応などの結果から，$HOCH_2(CHOH)_5CH_2COCOOH$ と決定された。この化合物は，クロマトグラフ的にも呈色反応的にも，シアル酸に非常によく類似した性質を有していた。