Carbohydrate Research, 47 (1976) 345–353

Note

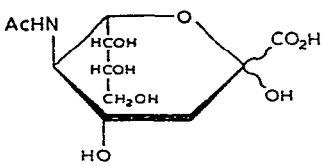
Reactivity of the sialic acid derivative 5-acetamido-3,5-dideoxy-L-*arabino*-heptulosonic acid in the resorcinol and thiobarbituric acid assays*

BARRY P. PETERS AND NATHAN N. ARONSON, JR.

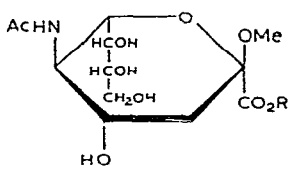
Althouse Laboratory, Department of Biochemistry, The Pennsylvania State University, University Park, Pennsylvania 16802 (U. S. A.)

(Received September 11th, 1975; accepted for publication in revised form, December 15th, 1975)

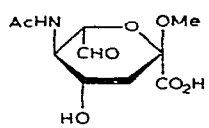
The preparation of a seven-carbon analogue of *N*-acetylneuraminic acid (**1**), 5-acetamido-3,5-dideoxy-L-*arabino*-2-heptulopyranosonic acid (**6**), has been described[1–3], and this derivative has been used[2,4–13] as a model compound to study the metabolic functions of **1**. Preliminary studies in our laboratory indicated that **6** gives results radically different from those of **1** in the thiobarbituric acid assay of Warren[14], the Svennerholm resorcinol assay[15], and the direct and indirect Ehrlich's colorimetric estimations for *N*-acetylneuraminic acid[16,17], thus making this analogue of sialic acid difficult to determine quantitatively. During our study, the difficulty of determining quantitatively **6** has also been noted by others[2,3,8]. Because of its potential usage for determining the biological significance of the parent sialic acid, the reactivity of **6** in the common colorimetric procedures used for the determination



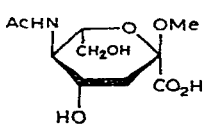
1



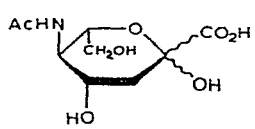
2 R = Me
3 R = H



4



5



6

*This work was supported by grant AM-15465 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, United State Public Health Service, and by a grant from the Brown–Hazen Fund of the Research Corporation.

of sialic acids was evaluated and is explained on the basis of the mechanisms of the characteristic color formation. A structure (**4**) having an aldehyde group at C-7 is proposed for the intermediate forming the chromophore in the Svennerholm resorcinol assay.

Blix *et al.*[18] have described the time course of the periodate oxidation of *N*-acetylneuraminic acid (**1**). Study of such an oxidation reaction by the resorcinol assay indicated that coincident with the evolution of the first mol of formic acid, which arises from C-8, the intensity of the resulting resorcinol chromogen increased 2.9 fold. However, further oxidation resulting in the evolution of an additional 0.55 mol of formic acid per mol of **1** caused a 42% decrease of the maximum value. The seven-carbon oxidation product having a carbonyl group at C-7 is, therefore, more reactive in the resorcinol reaction than **1**, whereas the six-carbon oxidation product is not chromogenic. A similar result was observed by Jourdian *et al.*[19]. These changes in the extinction value of the resorcinol chromophore were accompanied by changes in the visible spectrum. Unoxidized sialic acid shows a maximum absorption at 585 nm. During oxidation, the spectrum of the chromophores (Fig. 1) showed both an increase in extinction value and a shift of the maximum absorption to a second peak at 630 nm. Further oxidation resulted in a decrease of the extinction value and appearance of a third peak at 540–550 nm.

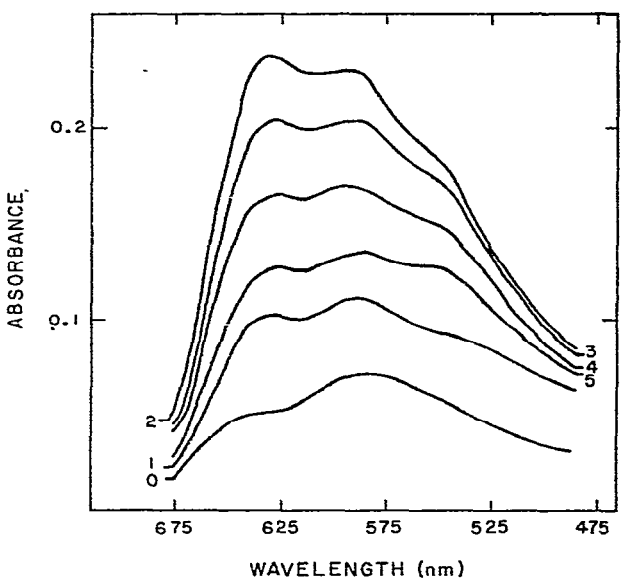


Fig. 1. Spectral changes in the resorcinol chromophore during the periodate oxidation of **1**. Time of periodate oxidation (0), 0 min; (1), 0.4 min; (2), 6.2 min; (3), 37 min; (4), 120 min; and (5), 240 min.

Periodate oxidation of the methyl β-glycoside of sialic acid[20] (**3**) gave one mol of formic acid and the C-7 aldehyde (**4**) with a change of the spectrum of the

resorcinol chromophore that appeared to be identical to that occurring during the first 10 min of the oxidation of free sialic acid (Fig. 1). In contrast to the observation made during the sialic acid oxidation, the color obtained with the resorcinol reagent did not change after further periodate oxidation of **3**, as already reported by Jourdian *et al.*[19]. This confirms the role played by the intermediate **4** in the resorcinol assay. Sodium borohydride reduction of **4** gave **5** showing a decrease by 33% of the absorbance at 585 nm of the resorcinol chromophore (see Fig. 2). The explanation that the decrease in absorbance is due to the reduction of the aldehyde group at C-7 was verified by the borohydride treatment of **3**, which caused no change in the color obtained with resorcinol.

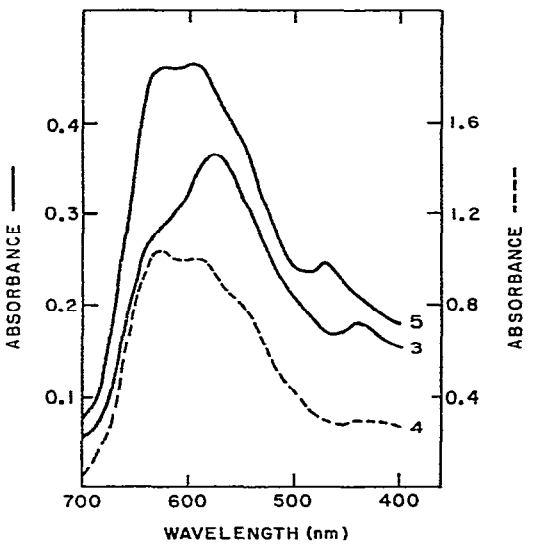


Fig. 2. Visible absorption spectra of resorcinol chromophore arising from **3**, **4**, and **5**. The resorcinol assay was performed on each of these three derivatives (approximately 0.15 μmol).

The rate of appearance of resorcinol color at time points during the 100°-heating step for this assay was determined for **3**, **4**, and **5** (see Fig. 3). Both **3** and **5** exhibited lag periods with inflection points at 5–10 min. The presence of an aldehyde group at C-7 (**4**), however, eliminated this lag period, and a rapid color development reaching 3 times the absorbance of either of the other two derivatives having an alcohol group at C-7 was observed. The enhancement of the rate and extent of color formation by the aldehyde group suggests that the lag periods observed are required for the formation of such an aldehyde group in the case of **3** and **5**, and this is the rate-limiting step in the color development. The spectra given in Fig. 2 are those occurring at 20 min during this experiment.

After periodate treatment of **6** at ambient temperature for 20 min, equivalent to the normal oxidation step in the Warren assay, only 34% of the color was observed.

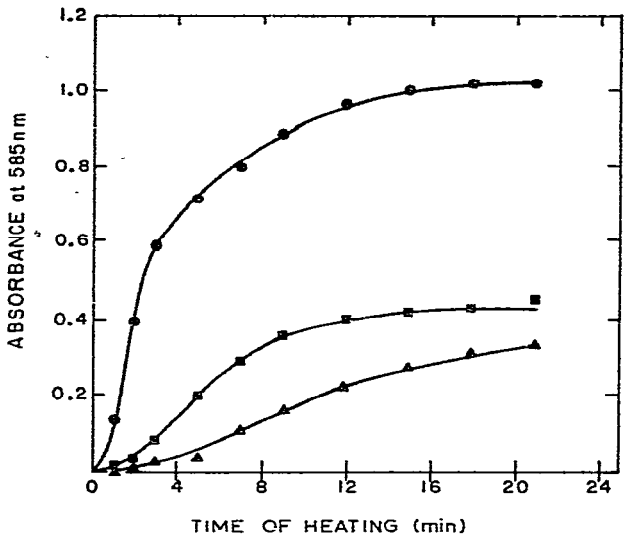


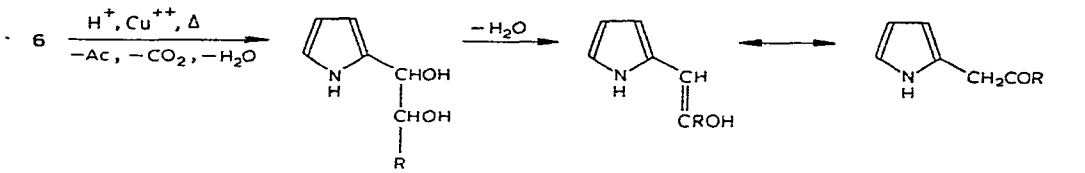
Fig. 3. Effect of heating step in the Svennerholm resorcinol method on the development of color arising from 3 (▲), 4 (●), and 5 (■).

As reported by Van Lenten and Ashwell[2], and also by Liao *et al.*[8], the absorbance values of **1** and **6** in the Warren assay are approximately identical when the oxidation of **1** is performed at ambient temperature and that of **6** at 37°, each for 20 min. Complete periodate oxidation of **6** required 5 h at ambient temperature, whereas sialic acid under the same conditions is completely oxidized within 2 h. Since complete periodate oxidation of sialic acid, required for the thiobarbituric acid chromogen formation, can only occur when the pyranose ring is opened, an explanation for the resistance of **6** to oxidation is the possible formation of an internal lactone that would stabilize the pyranose ring. Compound **6** may form two lactones[21]. In **1**, the bulky, exocyclic chain, which should remain equatorial, does not allow the proper pyranose ring conformation ($^5C_2$) for lactone formation. Under the strong acid conditions of the oxidation, such lactone formation would be favored. Other evidence for lactone formation from **6** is the presence, on thin-layer cellulose chromatograms irrigated in 7:3 propanol–water, of two minor, fast-moving, resorcinol-positive spots in addition to the major, slow-moving spot. This observation was also made by Klenk *et al.*[22]. The fast-moving spots may correspond to lactone forms. Secondly, **6** does give a slightly positive response in the hydroxamic acid test, which is also a characteristic of lactones.

The mechanism of the resorcinol assay for sialic acid has not been determined. The periodate–resorcinol modification of the assay provides some insight[19]. Borohydride reduction of the carbonyl group of sialic acid destroyed the color-forming capacity. Periodate oxidation (without borohydride reduction) caused a 2.5–5 fold increase in molar response of free and glycosidically-bound sialic acid. Prolonged oxidation caused a decrease in the absorbance of **1**, whereas glycosidically bound **1**

was stable under these conditions. Neuberger and Ratcliffe[23] noted that (methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosid)onic acid yielded a color with a low absorbance in the periodate–resorcinol reaction, and subsequent saponification of the *O*-acetyl groups increased the color production to that given by **1**. On these bases, it has been suggested that the C-7 aldehyde may be the major substance involved in the condensation with resorcinol. Our results clearly substantiate this proposal for an aldehyde intermediate in the resorcinol assay.

When heated in strong acid solution (4.8M hydrochloric acid in this assay), sialic acid derivatives underwent a number of complex transformations resulting in the formation of heterocyclic derivatives[16]. The glycosidic linkage was hydrolyzed, the carboxyl group was lost quantitatively as carbon dioxide, and *N*-deacetylation occurred[18]. Free neuraminic acids are known to exist in aqueous solution primarily as the internal Schiff base[24] formed by condensation of the amino group at C-5 with the C-2 carbonyl group. Acid-catalyzed dehydration of this form of neuraminic acid produced a tetrahydroxybutylpyrrole (**7**) in the case of **1** and a dihydroxyethylpyrrole (**8**) in the case of **6**. Formation of a pyrrole chromogen, in the resorcinol assay, was consistent with the elevated absorbance (120% compared to that of **1**) of the *N*-deacetylated derivatives (unpublished result), the primary amino groups of which need not be liberated by the acid hydrolysis prior to cyclization.



The enhancement of both extinction and rate of color development afforded by a carbonyl group at C-7 (Fig. 3) suggests the participation of this group in color formation. A carbonyl group was introduced into **7** or **8** by dehydration to give **9** and **10**, respectively, which equilibrated to **11** and **12**, respectively. In the longer polyhydroxy side chain of **7**, only 1 out of 6 dehydrations would result in a carbonyl group at the appropriate carbon (assuming equal statistical weight for each possible dehydration), as opposed to 1 out of 2 in the chromogen arising from **6**. This would explain the higher extinction of **6** in the resorcinol assay and the rapid color formation by **4**.

Electrophilic aromatic substitution of activated aromatic rings by carbonyl groups in acid solution have been widely studied[25], and we suggest that such an attack on resorcinol by the carbonyl group resulting at C-7 leads to the final resorcinol chromogen. The proof of the structure of these proposed pyrrole intermediates (**11, 12**) requires further evidence, such as chemical synthesis of the derivatives and

positive evidence of their reactivity with the resorcinol reagent to yield a product having the proper chromophore.

Aliquots of standardized solutions (see below) of the derivatives of sialic acid were subjected to colorimetric analysis in the Warren assay for free sialic acid, and in the Svennerholm resorcinol assay for bound sialic acid. The extinction of the chromophore from each derivative in each of the assays was determined and its visible absorption spectrum recorded. Results are listed in Table I as molar absorbance at the specified wavelength.

TABLE I

MOLAR EXTINCTIONS OF SIALIC ACID DERIVATIVES IN COLORIMETRIC ASSAYS

Compounds	*Thiobarbituric acid method*	*Resorcinol method*	
	(λ_{max} *549 nm*)	(λ_{max} *585 nm*)	(λ_{max} *630 nm*)
1	63,000 (59,000, ref. 14)	8,400 (8,500, ref. 15)	7,050 (6,500, ref. 1)
2	0	7,500	5,980
3	0	6,900	5,890
4	0	21,300	23,000 (27,900, ref. 19) (29,000, ref. 23)[a]
5	0	13,500 (11,400, ref. 1)	15,100 (13,600, ref. 1)
6	21,000[b] 56,500[c]	16,500 (7,200, ref. 2)	17,900

[a]See also refs. 1, 8, and 31. [b]Periodate oxidation at 22°. [c]Periodate oxidation at 37°.

Solutions of **4** and **5** could not be determined by formaldehyde evolution. Their respective extinctions in the Svennerholm assay were calculated on the basis of the color given with resorcinol by **3** and its subsequent 3-fold increase in extinction upon oxidation to **4**, and of the 33% decrease in the extinction of **4** that occurs upon subsequent reduction of the aldehyde group. These results indicated that all derivatives of sialic acid (free or bound) do not have the same resorcinol extinction. The presence of a methyl glycoside reduced the color yield by 15% (**1** or **6**), whereas the presence of an *N*-deacetylated amino group enhanced the color by 20%. Spectra of all nine-carbon compounds were identical, and spectra of all seven-carbon compounds were identical, except **4**, the spectrum of which differed from both (see Fig. 2). The spectra of **5** and **6** did exhibit minor variations from analysis to analysis, as shown by the relative sizes of the peaks at 585 and 630 nm.

EXPERIMENTAL

Materials. — *N*-Acetylneuraminic acid (**1**) was a product of Koch–Light Laboratories (Colnbrook SL3 OBZ, England). Sodium (meta)periodate (analytical grade reagent) and cadmium iodide were purchased from Fisher Chemical Co. All ion-exchange resins were Analytical Grade and purchased from Bio-Rad Labs (Rockville Centre, N.Y. 11570).

Preparation of **2**–**5**. — These compounds were prepared essentially by the method of Yu and Ledeen[21]: Methyl (methyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosid)onate (**2**) was prepared by boiling at reflux crystalline **1** in anhydrous methanol containing dry AG 50X8 (H^+, 200–400 mesh) ion-exchange resin; (methyl 5-acetamido-3,5-dideoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosid)-onic acid (**3**) from **2** by mild saponification[21,26]; (methyl 5-acetamido-7-aldehydo-3,5-dideoxy-α-L-*arabino*-2-heptulopyranosid)onic acid (**4**) by periodate oxidation of **3**; and (methyl 5-acetamido-3,5-dideoxy-α-L-*arabino*-2-heptulopyranosid)onic acid (**5**) by sodium borohydride reduction of **4**.

Preparation of **6**. — To **5** (150 μmol) in water (30 ml) was added 3M sulfuric acid (0.3 ml), and the mixture was heated at 80° for 90 min, and then cooled in an ice-bath and the pH adjusted to 7 with M sodium hydroxide. The hydrolyzate was filtered to remove the humin, evaporated to 3 ml, and desalted by chromatography on a column of Sephadex G-10 (1.5 × 110 cm; flow rate, 6 ml/h). The resorcinol-positive fractions were pooled, evaporated to 1 ml, and rechromatographed on Sephadex G-10 to remove the last traces of sodium sulfate. Compound **6** was characterized by preparing **5**, m.p. 152° (dec. with browning), and the quinoxaline derivative according to Kuhn and Gauhe[1], m.p. 208–210°.

Anal. Calc. for $C_{10}H_{17}NO_7$: C, 45.62; H, 6.51; N, 5.32. Found: C, 45.67; H, 6.54; N, 5.22.

Anal. Calc. for $C_{15}H_{19}N_3O_5$: C, 56.07; H, 5.96; N, 13.07. Found: C, 55.92; H, 6.04; N, 12.92.

Measurement of the time course of periodate oxidations. — Unless otherwise stated, all periodate oxidations were conducted at sialic acid concentrations between 1–1.5 μmol/ml and a periodate concentration of 10 μmol/ml at 0°. Periodate consumption was determined by the method of Avigad[27], formaldehyde evolution by the chromotropic acid procedure[28] scaled down to a 2-ml final volume, formic acid by a novel application of Lambert's linear starch–cadmium iodide reagent[29], and in some instances sialic acid by the methods of Warren[14] or Svennerholm[15]. These determinations were performed simultaneously on the same oxidation mixture. Periodate was used as a primary standard, and the periodate oxidation of standard amounts of erythritol was used to generate standard solutions of formaldehyde and formic acid. Erythritol was not used as a primary standard, but aliquots of a stock solution were standardized, employing the u.v. absorption of periodate at 310 nm to measure the periodate consumption[30].

Quantitative determination of derivative solutions. — Solutions of each of the

derivatives of sialic acid, except **4** and **5**, were determined quantitatively by the amount of formaldehyde evolved after periodate oxidation of each derivative. Sodium periodate (10 µl, 50 µmol/ml) was added to an estimated 1 µmol/ml solution (50 µl). After 3 h for **1**, **2**, and **3**, and after 8 h for **6**, the reaction was terminated by addition of an arsenite solution (100 µl) and formaldehyde was determined. Concentration of each of the derivatives was calculated on the assumption that 1 mol of formaldehyde is liberated per mol of sugar.

Analytical techniques. — Free sialic acid was determined by the thiobarbituric acid assay of Warren[14] or by the indirect Ehrlich's reaction[17]. Glycosidically-bound sialic acid was determined by the resorcinol assay of Svennerholm[15]: The resorcinol reagent (0.5 ml) was added to a sample (0.5 ml) containing 0–0.3 µmol of sialic acid. The mixture was heated for 15 min at 100°, the tubes were cooled in cold water, and butanol (2.0 ml) was added to each tube. After thorough mixing, the absorbance at 585 nm was immediately measured. All spectra were measured with a Varian Model 635 UV-Vis Recording Spectrophotometer.

Spectral changes in the resorcinol chromophore during the periodate oxidation of **1**. — Visible spectra were taken of the resorcinol products at various time intervals during the oxidation of **1** (Fig. 1). Compound **1** (0.95 µmol/ml) was oxidized at 0°. To measure resorcinol-positive material, 0.5-ml aliquots were added to 1,2-ethanediol (100 µl, 10% v/v) to terminate the oxidation. Each sample was then layered on a column (1 ml) of Dowex 1 (X-8, Cl^-, 200–400 mesh) ion-exchange resin, and eluted first with water (4 ml), followed by 0.12M sodium chloride (9 ml). The resorcinol reaction was performed on this salt eluate (0.5 ml).

Rate of color formation from **3**, **4**, *and* **5** *during the heating step of the resorcinol assay.* — Three sets of tubes containing either **3**, **4**, or **5** (0.15 µmol/tube), respectively, were assayed by the resorcinol procedure[15] (Fig. 3). At time intervals during the 100°-heating step, tubes were removed from the heating bath and immediately cooled in ice to stop the reaction. The remainder of the resorcinol assay was completed in the normal manner for each sample.

REFERENCES

1 R. Kuhn and A. Gauhe, *Chem. Ber.*, 98 (1965) 395–413.
2 L. Van Lenten and G. Ashwell, *J. Biol. Chem.*, 246 (1971) 1889–1894.
3 R. L. McLean, M. Suttajit, J. Beidler, and R. J. Winzler, *J. Biol. Chem.*, 246 (1971) 803–809.
4 M. Suttajit, L. E. Reichert, and R. J. Winzler, *J. Biol. Chem.*, 246 (1971) 3405–3408.
5 M. Suttajit and R. J. Winzler, *J. Biol. Chem.*, 246 (1971) 3398–3404.
6 O. O. Blumenfeld, P. M. Gallop, and T.-H. Liao, *Biochem. Biophys. Res. Commun.*, 48 (1972) 242–251.
7 M. Suttajit, C. Urban, and R. L. McLean, *J. Biol. Chem.*, 246 (1971) 810–814.
8 T.-H. Liao, P. M. Gallop, and O. O. Blumenfeld, *J. Biol. Chem.*, 248 (1973) 8247–8253.
9 J. L. Vaitukaitis, J. Hammond, G. T. Ross, J. Hickman, and G. Ashwell, *J. Clin. Endocrinol. Metab.*, 32 (1971) 290–293.
10 J. L. Vaitukaitis, R. Sherins, G. T. Ross, J. Hickman, and G. Ashwell, *Endocrinology*, 89 (1971) 1356–1360.
11 H. B. Bosmann, *Biochim. Biophys. Acta*, 279 (1972) 456–474.
12 B. B. Kirschbaum and H. B. Bosmann, *Nephron*, 11 (1973) 26–39.

13 R. J. BERNACK AND H. B. BOSMANN, *Eur. J. Biochem.*, 34 (1973) 425–433.
14 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971–1975.
15 L. SVENNERHOLM, *Biochim. Biophys. Acta*, 24 (1957) 604–611.
16 I. BERGGARD AND L. ODIN, *Ark. Kemi*, 12 (1958) 581–595.
17 D. AMINOFF, *Biochem. J.*, 81 (1961) 384–392.
18 G. BLIX, E. LINDBERG, L. ODIN, AND I. WERNER, *Acta Soc. Med. Ups.*, 61 (1956) 1–25.
19 G. W. JOURDIAN, L. DEAN, AND S. ROSEMAN, *J. Biol. Chem.*, 246 (1971) 430–435.
20 E. KLENK, H. FAILLARD, F. WEYGAND, AND H. H. SCHÖNE, *Hoppe–Seylers Z. Physiol. Chem.*, 304 (1956) 35–52.
21 R. K. YU AND R. LEDEEN, *J. Biol. Chem.*, 244 (1969) 1306–1313.
22 E. KLENK, L. HOF, AND L. GEORGIAS, *Hoppe–Seylers Z. Physiol. Chem.*, 348 (1967) 149–166.
23 A. NEUBERGER AND W. A. RATCLIFFE, *Biochem. J.*, 133 (1973) 623–628.
24 R. SCHAUER, *Angew. Chem.*, 12 (1973) 127–138.
25 J. E. HOFMANN AND A. SCHRIESHEIM, in G. A. OLAH (Ed.), *Friedel–Crafts and Related Reactions*. Vol. II, Wiley, New York, 1964, pp. 597–640.
26 J. D. KARKAS AND E. CHARGAFF, *J. Biol. Chem.*, 239 (1964) 949–956.
27 G. AVIGAD, *Carbohydr. Res.*, 11 (1969) 119–123.
28 D. A. MACFADYEN, *J. Biol. Chem.*, 158 (1945) 107–133.
29 J. L. LAMBERT, *Anal. Chem.*, 23 (1951) 1247–1251.
30 G. V. MARINETTI AND G. ROUSER, *J. Am. Chem. Soc.*, 77 (1955) 5345–5349.
31 R. G. SPIRO, *J. Biol. Chem.*, 239 (1964) 567–573.