kin's nomenclature, based on X-ray diffraction patterns, seems the most promising available to correlate properly the polymorphic forms of a number of different triglycerides and to associate them with corresponding forms of other long chain compounds.

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Summary

The "even" members of the homologous series of triglycerides, trilaurin through tristearin, have been studied by X-ray diffraction and thermal methods and found to exhibit monotropic trimorphism. This is in line with the conclusions of previous observers, notably Malkin.

Manifestation of different crystal structures by a given triglyceride was clearly established by the classical work of Malkin, *et al.*, as the underlying cause of the multiple melting of triglycerides. The names of polymorphic forms were based by Malkin upon X-ray diffraction patterns in a manner to relate logically the triglyceride forms to corresponding forms of other long chain compounds. It resulted that the forms for tristearin, *etc.*, were called (by Malkin) gamma (glassy), alpha, and beta in order of increasing melting point. Unfortunately, according to the present study, there appears to have been a faulty association of X-ray diffraction pattern with melting point in the work of Malkin. This association was correct in the case of the highest melting beta form. However, the lowest melting form (called gamma, glassy or vitreous by Malkin) actually exhibits Malkin's alpha pattern and in accordance with Malkin's original intention is therefore named the *alpha form.* The intermediate form (called alpha by Malkin) actually exhibits Malkin's beta prime pattern (not reported by him for tristearin, etc., but for mixed glycerides). It is therefore named the *beta prime form.* The gamma name and pattern and the concept of the glassy state of triglycerides should be eliminated.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE OHIO STATE UNIVERSITY]

Separation of Sugar Acetates by Chromatography[1]

BY W. H. MCNEELY,[2] W. W. BINKLEY[3] AND M. L. WOLFROM

Application of chromatographic methods to the separation of sugars has been made by Hayashi,[4] who reported the separation of D-glucose and sucrose on a charcoal column. Reich[5] employed the colored *p*-phenylazobenzoates of the sugars and this general method has been extended successfully by Coleman and associates[6] to a very considerable number of typical separations. Employing the procedure of fractional elution (liquid or flowing chromatogram), Talley, Reynolds and Evans[7] and Jones[8] have applied chromatographic methods to the separation of sugar derivatives. Bell[9] has utilized the partition (chloroform–water–silica gel) chromatographic procedure of Martin and Synge[10] in the establishment of a new O-tetramethyl-D-glucopyranose end-group assay for polysaccharides. The Molisch reagent was used by Bell to locate the zones on the developed, extruded and oven-dried column.

Although the above-mentioned chromatographic procedures represent a considerable advance over methods previously available for the separation of sugar mixtures, they have disadvantages. We wish to report herein the successful application of the chromatographic brush method established by Zechmeister and collaborators[11] to the separation of the well-characterized sugar acetates. This method consists in extruding the developed, colorless chromatogram and making the zones visible by brushing a line along the length of the column with a brush dipped in a reagent capable of forming a colored reaction product with the adsorbed substance. Such a procedure avoids the previous necessity of either working with colored sugar derivatives or resorting to empirical chromatography. A search for a suit-

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(2) Hoffmann-La Roche Post-doctoral Fellow of The Ohio State University Research Foundation.

(3) Sugar Research Foundation Post-doctoral Fellow of The Ohio State University Research Foundation.

(4) F. Hayashi, *J. Biochem.* (Japan), **16**, 1 (1932); *C. A.*, **27**, 8 (1933).

(5) W. S. Reich, *Compt. rend.*, **208**, 589, 748 (1939); *Biochem. J.*, **33**, 1000 (1939).

(6) G. H. Coleman, A. G. Farnham and A. Miller, THIS JOURNAL, **64**, 1501 (1942); G. H. Coleman and C. M. McCloskey, *ibid.*, **65**, 1588 (1943); *cf.* J. K. Mertzweiller, D. M. Carney and F. F. Farley, *ibid.*, 2367.

(7) E. E. Talley, D. D. Reynolds and W. L. Evans, *ibid.*, **65**, 573 (1943).

(8) J. K. N. Jones, *J. Chem. Soc.*, 333 (1944).

(9) D. J. Bell, *ibid.*, 473 (1944).

(10) A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35**, 1358 (1941); A. H. Gordon, A. J. P. Martin and R. L. M. Synge, *ibid.*, **37**, 79, 86, 92 (1943).

(11) L. Zechmeister, L. de Cholnoky and (Mlle.) E. Ujhelyi, *Bull. soc. chim. biol.*, **18**, 1885 (1936); L. Zechmeister and O. Frehden, *ibid.*, **22**, 458 (1940); L. Zechmeister and W. H. McNeely, THIS JOURNAL, **64**, 1919 (1942); L. Zechmeister, W. H. McNeely and G. Sólyom, *ibid.*, 1922.

able brush reagent, capable of locating the zones of the invisible chromatogram, led to the selection of aqueous alkaline permanganate. This reagent was suitable for non-reducing as well as for reducing sugars, and even for sugar alcohols. The brush reagent streak on the extruded column reacted with the invisible zones at rates characteristic of the particular sugar acetate constituting the zone (Table I). Many of the common adsorbents were found to be suitable in some degree but "Magnesol," a synthetic hydrated magnesium acid silicate ($2MgO \cdot 5SiO_2$), was chosen as the most versatile.

Table I

Comparative Rates of Distinct Zone Formation for Sugar and Sugar Alcohol Acetates on "Magnesol" with Alkaline Permanganate as Brush Reagent

Substance	Time, sec.
keto-D-Fructose pentaacetate	10
β-D-Glucopyranose pentaacetate	30
β-Maltose octaacetate	60
Sucrose octaacetate	120–180
Raffinose hendecaacetate	120–180
(*levo*)-Sorbitol hexaacetate	180–240
L-Rhamnitol pentaacetate	240–300

Exploratory experiments demonstrated that the sugar acetates fell into general groups determined by small changes in the nature of the developing agent. A ratio of 500 parts by volume of benzene to 1 part of ethanol moved the fully acetylated monosaccharides tested, except *keto*-D-fructose pentaacetate, down the column while the acetates of the di- and trisaccharides were held near the top. A 100 to 1 ratio readily washed the monosaccharide acetates off the column while moving the disaccharide acetates to the bottom with a moderate speed and leaving the trisaccharide acetates near the top of the column. Application of these results led to the separation of the acetates of the following two component sugar mixtures (1:1): β-D-glucose from *keto*-D-fructose, β-maltose, sucrose, and raffinose; β-maltose from sucrose, and raffinose; raffinose from sucrose. The reagent was also suitable for the detection of the fully acetylated sugar alcohols, and the separation of (*levo*)-sorbitol hexaacetate from L-rhamnitol pentaacetate was effected readily. The results of this investigation are tabulated in Table II. The recovery of each component of the mixtures was nearly quantitative. From the properties of the recovered fractions recorded in Table II, it can be seen that the purity of each component was excellent without recrystallization. In the examples shown in Table II, the upper and lower zones of the chromatograms were separated by relatively large interzones, enabling each component to be recovered in a high state of purity. Preliminary experiments indicated that the differences in adsorption affinity between a number of pairs of substances such as α- and β-D-glucose pentaacetate were smaller and that rechromatographing probably would be necessary for their separation.

The separation of the sugar acetates by application of the chromatographic brush method shown in the present work offers a number of advantages for the isolation, separation and identification of sugars over the methods previously available. The empirical liquid chromatogram or fractional elution method is tedious and is very difficult to apply to the problem of isolating all of the components of a complex, naturally occurring, mixture of sugars. While the use of colored sugar deriva-

Table II

Chromatographic Separation of Sugar Acetate and Sugar Alcohol Acetate Pairs on "Magnesol" (50 g. "Magnesol"–"Celite" Mixture 5:1)

Substance	Wt., mg.	Developing agent Vol. ratio, benzene–ethanol	Vol., cc.	Relative zone location	Recovered materials: M. p., °C. Found	M. p., °C. Accepted value	$[\alpha]_D$ (20–25°, $CHCl_3$, $c < 5$) Found	$[\alpha]_D$ (20–25°, $CHCl_3$, $c < 5$) Accepted value	Wt., mg.	Yield %
β-D-Glucose pentaacetate	124.0	200:1	450	Lower	129–130	132	+ 4.0	+ 3.8	118.5	95.5
β-Maltose octaacetate	130.0			Upper	159–160	160	+62	+ 63	124.7	95.7
β-D-Glucose pentaacetate	125.1	200:1	350	Lower	130–131	132	+ 4.1	+ 3.8	115.8	92.4
Sucrose octaacetate	125.6			Upper	86–88	89	+59	+ 60	124.9	99.4
β-D-Glucose pentaacetate	126.5	200:1	400	Lower	129–130	132	+ 4.1	+ 3.8	123.5	97.6
Raffinose hendecaacetate	123.9			Upper	99–100	100–101	+99	+101	122.5	98.8
β-D-Glucose pentaacetate	125.2	500:1	500	Lower	130–131	132	+ 3.9	+ 3.8	115.0	91.7
keto-D-Fructose pentaacetate	127.7			Upper	64–66	70	+32	+ 35	113.8	89.2
β-Maltose octaacetate	125.2	300:2.25	300	Lower	158–159	160	+62	+ 63	116.2	92.8
Sucrose octaacetate	126.8			Upper	86–88	89	+61	+ 60	119.4	94.3
β-Maltose octaacetate	124.7	100:1	300	Lower	157–158	160	+63	+ 63	122.0	97.9
Raffinose hendecaacetate	125.1			Upper	97–98	100–101	+99	+100	122.9	98.0
Sucrose octaacetate	125.1	100:1	375	Lower	86–88	89	+61	+ 60	123.4	98.6
Raffinose hendecaacetate	125.7			Upper	99–100	100–101	+98	+100	122.2	97.4
L-Rhamnitol pentaacetate	121.7	500:1	350	Lower	Sirup	Sirup	−31	− 30[a]	103.8	85.3
(*levo*)-Sorbitol hexaacetate	126.1			Upper	97–98	99	+ 9.4	+ 10	125.9	99.7

[a] Unpublished results of this Laboratory.

tives allows the zones to be visible without column extrusion, the *p*-phenylazobenzoates are not as well characterized as the acetates and are more difficult to prepare. Since the acetate group is relatively small, inherent differences in the carbohydrate structure are not so masked as when a larger substituent is employed. Thus, the sugar residue constitutes 44.9% of glucose penta-acetate but only 14.3% of glucose penta-*p*-phenylazobenzoate.

Experimental

The adsorbent employed was "Magnesol" (Westvaco Chlorine Products Co., South Charleston, West Virginia), a synthetic hydrated magnesium acid silicate ($2MgO \cdot 5SiO_2$).[12] It is necessary to examine the extrusive and adsorptive properties of each new lot of this material. "Celite" (No. 535, Johns-Manville Co., New York, N. Y.) was used as a filter aid. A mixture of 5 parts (by wt.) of "Magnesol" and 1 part of "Celite" was extracted at room temperature with a large volume of acetone and air-dried at room temperature. An amount of 50 g. of this mixture was packed in a chromatographic tube (35 mm. in diameter, 230 mm. in length) with the aid of suction. After the top of the column had been moistened with 10 cc. of benzene, there was added 5 cc. of a benzene solution containing *ca.* 250 mg. of a 1:1 mixture of the two sugar acetates to be separated. The amount and composition of the benzene (thiophene-free)–ethanol (absolute) solution then employed for developing each column is shown in Table II. The column was extruded and immediately brushed with a freshly prepared 1% solution of potassium permanganate in 2.5 *N* sodium hydroxide. The zones were detected by the appearance in the brush streak of yellow to orange bands separated by green interzones of adequate width. The brush mark exhibited a series of color changes from an initial green, through a yellow and orange, to a final light brown. The green interzones were caused by the relatively slower reaction of the solvent on the column. Eventually, the brush streak assumed a uniform, light brown color.

(12) U. S. Patent 2,076,545 (1937); *C. A.*, **31**, 4023 (1937); U. S. Patents 2,163,525, 2,163,527, 2,163,528 (1939); *C. A.*, **33**, 8049 (1939).

The average rates of appearance of the zones are shown in Table I. The zones were cut out and the brush marks removed with a scalpel. Elution of each zone was effected with 125 cc. of acetone and the acetone was removed by evaporation at room temperature. The properties and yields of the residues so obtained are shown in Table II. Adequate purity was present without any further purification.

In a typical separation, the chromatogram was developed in ninety minutes. The brush reagent located a β-maltose octaacetate zone (35 mm. broad) 25 mm. below the top of the column and beneath this, separated by a 60-mm. interzone, the β-D-glucose pentaacetate zone (22 mm. broad). Different lots of "Magnesol" may lead to variations in these data.

Preliminary experiments indicated that the differences in adsorption affinity between the following pairs would be such that rechromatographing would probably be required for their separation: α- and β-D-glucose pentaacetate; α-D-arabinose tetraacetate and α-D-glucose pentaacetate; α-D-arabinose tetraacetate and *keto*-D-fructose pentaacetate; D-mannitol hexaacetate and (*levo*)-sorbitol hexaacetate; β-D-glucose pentaacetate and (*levo*)-sorbitol hexaacetate.

Summary

The chromatographic brush method with aqueous alkaline permanganate as brush reagent and "Magnesol" as adsorbent has been employed successfully in the separation of a selected number of two component (equal portions) mixtures of fully acetylated sugars and sugar alcohols. The following separations were effected: β-D-glucose pentaacetate (I) from β-maltose octaacetate (II); I from sucrose octaacetate; I from *keto*-D-fructose pentaacetate; I from raffinose hendecaacetate; II from sucrose octaacetate; II from raffinose hendecaacetate; raffinose hendecaacetate from sucrose octaacetate; (*levo*)-sorbitol hexaacetate from L-rhamnitol pentaacetate. The components were recovered in nearly quantitative yield and in excellent purity.

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[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY,[1] PHILADELPHIA, PENNSYLVANIA]

On the Presence of a Proteolytic Enzyme in Casein

BY ROBERT C. WARNER AND EDITH POLIS

The presence of a proteolytic enzyme in milk was first reported by Babcock and Russell.[2] They studied the decomposition of skim milk in the presence of antiseptics and the formation of soluble nitrogenous products during the ripening of cheese and attributed the observed proteolysis to an enzyme, "galactase." Others[3,4] have studied the same problem, and the general results of Babcock and Russell have been confirmed. No successful preparations of concentrates of the enzyme have been made.

We have found that almost all of the proteolytic activity in milk is precipitated along with the casein when milk is acidified. The activity is extremely difficult to separate from casein and is absent from the usual casein preparations only if they have been exposed for long periods to alcohol. It is thus present in both commercial casein and most purified laboratory preparations.

The fact that casein may undergo proteolysis in solution with the formation of products soluble at *p*H 4.6 has been noted by Robertson[5] and Walters.[6] However, they believed that their

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) Babcock and Russell, *Ann. Rep., Wisconsin Agr. Exp. Sta.*, **14**, 161 (1897); Babcock, Russell and Vivian, *ibid.*, **15**, 77, 93 (1898).

(3) Van Slyke, Harding and Hart, N. Y. Agr. Exp. Sta. Bulletin 103, 215 (1901).

(4) Thatcher and Dahlberg, *J. Agr. Research*, **11**, 437 (1917).

(5) Robertson, *J. Biol. Chem.*, **2**, 317 (1906–1907).

(6) Walters, *ibid.*, **11**, 267 (1912); **12**, 43 (1912).