NBS TECHNICAL NOTE

457

Organic Chemistry Section:

Summary of Activities July 1967 to June 1968

U.S. DEPARTMENT OF COMMERCE
National Bureau of Standards

NATIONAL BUREAU OF STANDARDS

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UNITED STATES DEPARTMENT OF COMMERCE
C. R. Smith, Secretary
NATIONAL BUREAU OF STANDARDS • A. V. Astin, Director

NBS TECHNICAL NOTE 457

ISSUED SEPTEMBER 1968

Organic Chemistry Section:

Summary of Activities July 1967 to June 1968

Edited by Robert Schaffer

Organic Chemistry Section
Analytical Chemistry Division
Institute for Materials Research

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FOREWORD

The Analytical Chemistry Division was established as a separate division at the National Bureau of Standards on September 1, 1963, and became part of the Institute for Materials Research in the February 1, 1964, reorganization. It consists at present of nine sections and about 100 technical personnel encompassing some 57 different analytical competences from activation analysis and atomic absorption to vacuum fusion and x-ray spectroscopy. These competences, and in turn the sections which they comprise, are charged with research at the forefront of analysis as well as awareness of the practical sample, be it standard reference material or service analysis. In addition it is their responsibility to inform others of their efforts.

Formal publication in scientific periodicals is a highly important output of our laboratories. In addition, however, it has been our experience that informal, annual summaries of progress describing efforts of the past year can be very valuable in disseminating information about our programs. A word is perhaps in order about the philosophy of these yearly progress reports. In any research program a large amount of information is obtained and techniques developed which never find their way into the literature. This includes the "negative results" which are so disappointing and unspectacular but which can often save others considerable work. Of importance also are the numerous small items which are often explored in a few days and which are not important enough to warrant publication--yet can be of great interest and use to specialists in a given area. Finally there are the experimental techniques and procedures, the designs and modifications of equipment, etc., which often must be covered in only a line or two of a journal article.

Thus our progress reports endeavor to present this information which we have struggled to obtain and which we feel might be of some help to others. Certain areas which it appears will not be treated fully in regular publications are considered in some detail here. Other results which are being written up for publication in the journal literature are covered in a much abbreviated form.

At the National Bureau of Standards publications such as these fit logically into the category of a Technical Note. In 1968 we plan to issue these summaries for all of our sections. The following is the fourth annual report on progress of the Organic Chemistry Section.

W. Wayne Meinke, Chief
Analytical Chemistry Division

PREFACE

This is the fourth in a series of NBS Technical Notes which are prepared annually to summarize the technical activities of the Organic Chemistry Section of the Analytical Chemistry Division. The present report, which covers the period from July 1, 1967 to June 30, 1968, describes much work that is still in progress and also provides accounts of studies that were completed during the year and are, or are about to be, published. In line with a uniform policy of the Division, the Section's programs have both a research and a sample aspect. About 60 percent of the total effort has been devoted to research and the remainder to Standard Materials.

For a great many years, because of their exceptional importance as natural products (to biochemical science and to industry) and as research materials, the chemistry of the carbohydrates has been a subject of central interest in the Section. The competences that have been developed in studying carbohydrate synthesis and reaction mechanisms, in developing methods of separation and purification, and in the characterization of molecular structure and conformation, are skills that are applied to all areas of organic chemistry where, within the mission of the National Bureau of Standards, the Section can uniquely serve the scientific needs of the Nation, e.g., with Standard Reference Materials for research and analysis, or conjointly with other research organizations, attack pressing national problems, e.g., air pollution, and clinical analysis.

Indeed, this year's report shows much of the Section's effort newly directed to the certification of organic Standard Reference Materials for use in clinical analysis, thereby helping clinical laboratories throughout the Nation to improve capabilities for performing their vital analytical tasks. In addition to continuing to provide needed clinical standards, a long-range research and development program is being

undertaken to investigate the clinically significant, organic compounds present in clinical samples, with the objective of developing better means for their characterization and determination.

Recent technological developments in starch hydrolysis have resulted in the industrial production of many new syrup types for which previously developed physical property relationships, currently in use in the industry and by consumers, are not entirely satisfactory. To remedy this, a Research Associate Program has been initiated, in cooperation with the Corn Industries Research Foundation, for restudying syrup properties by many of the most modern techniques. Dr. Daniel W. Vomhof and Mr. James Thomas are engaged in this program.

During the year, the staff of the Section was augmented with the appointment of Miss Barbara F. West, Dr. Robert F. Brady, Jr., and Dr. Bruce Coxon.

Robert Schaffer, Chief
Organic Chemistry Section

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JULY 1967 TO JUNE 1968
Edited by R. Schaffer

ABSTRACT

This report describes work in progress in the Organic Chemistry Section of the Analytical Chemistry Division of the NBS Institute for Materials Research. The following research areas are discussed:

Carbohydrate Synthesis, Structure, and Characterization: Novel Research Materials and Model Compounds. The reaction of sulfonic esters of D-mannitol with methoxide has been examined, and a new dianhydrohexitol has been isolated, and identified as 2,3:4,5-dianhydro-D-iditol. Methods for the esterification of tetrahydroxy-p-benzoquinone and benzenepentol, and the disproportionation of the former compound during acetylation have been studied. A novel method for acylation in 100% phosphoric acid has been developed. The usefulness of bromine for oxidizing cyclohexanehexols into inososes and diketoinositols was investigated. Acylations of certain inosose phenylhydrazones was proved to provide arylazocyclohexene derivatives, whereas previous workers had reported otherwise. NMR spectroscopy was used to determine the conformations of several bi- and tri-cyclic carbohydrate derivatives in solution, and the influence of substituents and of dipolar interactions on conformation was analyzed. The factors that determine the rates of reaction of the cyclic disulfones that are intermediates in the MacDonald–Fischer degradation of sugars were evaluated by pKa measurements and NMR analysis. Syntheses of "D-ribulose" and D-psicose have been carried out.

Clinical Chemistry. Extensive examinations of the properties of high-purity samples of creatinine, uric acid, and urea have been made, as a preliminary stage in the development of Standard Reference Materials for these compounds. The

application of phase solubility analysis and differential scanning calorimetry has been studied where appropriate for evaluations of purity. The properties and methods for determining the purity of SRM cholesterol were studied.

Properties of Polycyclic Air-Pollutants. The oxidation of a variety of polycyclic, aromatic compounds by periodic acid was studied and found to proceed by (1) a free-radical mechanism that gives rise to coupling products, or (2) a two-equivalent oxidation mechanism that leads to quinonoid derivatives, or both. On treatment with periodic acid, azulene, a non-benzenoid, aromatic compound, gave a free-radical-containing, polymeric material having ionic properties.

Analytical Chemistry of Corn Sugars and Related Products. As a preliminary but basic step in a program for developing relationships between the compositions and physical properties of corn syrups prepared by various processes, a variety of methods have been explored for assaying the water content in syrups.

In order to describe experimental procedures adequately, it has occasionally been necessary to identify commercial materials and equipment in this report. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

Key Words:

Carbohydrate structure and characterization, sulfonic esters of alditols, polyhydroxy cyclic compounds, acylation, n.m.r. spectroscopy, standard reference materials, 1,2:3,4-di-O-isopropylidene-D-erythro-pentulofuranose, D-psicose, clinical chemistry, creatinine, uric acid, urea, cholesterol, polycyclic air-pollutants, periodic acid, sucrose, dextrose, nicotinic acid, metallo-organic standard reference materials, analysis of corn syrups.

1. CARBOHYDRATE SYNTHESIS, STRUCTURE, AND CHARACTERIZATION: NOVEL RESEARCH MATERIALS AND MODEL COMPOUNDS

As one of the major categories of annually renewable, naturally occurring, organic materials, the carbohydrates are basic to many industries. Interest in the carbohydrates at NBS dates back to the early 1900's, when the Bureau first verified the calibration of polariscopes and other apparatus used by the Customs Bureau in levying duties on imported sugar. Shortly thereafter, sucrose was issued as a Standard Reference Material (SRM) for polariscopic measurements, and this was followed later by SRM dextrose used as a standard for reducing-sugar determinations. The active study of analytical and polarimetric methods continued for many years, during which time the Bureau became the "greatest repository of sugar technology in the country." The recovery of valuable chemicals from molasses and waste waters of sugar manufacture, the development of commercially feasible processes for the manufacture of sugars and derivatives, and the development of economically valuable products (gums, sugars, cellulose products) from farm waste-materials were additional activities pursued.

Research in the chemistry of rare sugars was stimulated when World War I cut off German sources of these and other organic compounds. The chemistry associated with the proximity and multiplicity of reactive substituent groups in each molecule, the multiple forms that each sugar may assume when dissolved, and the structural interrelationship of members of the carbohydrate family that make them ideally suited for use as model compounds for studying the fine details of organic reactions have been subjects of the Section's numerous publications.

With this extensive experience in carbohydrate chemistry, an intensive program in method development and synthesis of radioactive, position-labeled carbohydrates was undertaken in

the Section when carbon-14 and tritium became available and no effective sources existed that were able to provide the position-tagged sugars much needed for research in numerous, primarily biochemical, laboratories. Until commercial and other outside laboratories undertook to use the procedures developed in this Section, the Bureau served as the sole supplier of position-labeled sugars. The isotopic methods of analysis utilized in developing optimal syntheses of the labeled compounds were subsequently adapted and expanded for studying (a) complex reactions and their mechanisms, and (b) isotope effects; some aspects of these studies related closely to the biochemistry of the sugars.

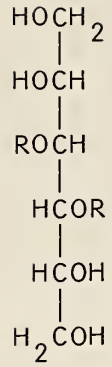
The carbohydrates have been and continue to be of immense scientific importance, and the development of the deepest possible understanding of their chemistry is a subject at the forefront of organic chemical research. The work described in this year's report concerns (i) novel methods for the introduction of functional groupings, whence they provide new materials, new derivatives, or better procedures for synthesizing those known previously, and (ii) some intense applications of nuclear magnetic resonance spectroscopy and other techniques for examining fundamental chemical properties of model compounds and for determining the structure of previously unknown compounds. As an outgrowth of research activities pursued earlier, four rare-carbohydrate SRM's were issued during the past year. In addition, D-*erythro*-pentulose ("D-ribulose") and D-psicose syntheses were carefully evaluated, and as a consequence, in the near future, stable derivatives of these two ketoses also will be made available as research standards, since there is no commercial source for either of these rare sugars.

A Reaction of Sulfonic Esters of Alditols with Methoxide

(R. S. Tipson and A. Cohen)

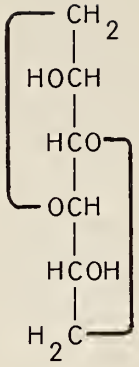
In Technical Note 427, we described the reaction of methanolic barium methoxide with the 3,4-dimethanesulfonate (1a) or 3,4-di-p-toluenesulfonate (1b) of D-mannitol to give a dianhydrohexitol. We showed that the structure of this dianhydrohexitol (A) was not that of the 1,4:3,6-dianhydride (2) or of the 1,4:2,3-dianhydride (3), but that it was one of the following: the 2,3:4,5-dianhydride (4), the 1,3:4,6-dianhydride (5), or the 2,3:4,6-dianhydride (6).

Compound A was found to have two free hydroxyl groups, because, on acetylation with acetic anhydride–pyridine (160 min at 25°C), it afforded a crystalline compound having an elementary composition agreeing with that calculated for a di-O-acetyl-dianhydrohexitol; yield 97%. A crystalline dibenzoate was also prepared; its properties differed from those expected for the 2,5-dibenzoate of 2 on the basis of those of the dibenzoate of the enantiomorph of 2 (a sample of which was kindly supplied by Dr. H. G. Fletcher, Jr., of the National Institutes of Health, Bethesda, Maryland 20014). The n.m.r. spectrum of compound A, measured in methyl sulfoxide-d_6, showed signals of the hydroxyl protons as a 2-proton, apparent triplet, and hence the two hydroxyl groups are both primary [1]. Hence, structures 5 and 6 are excluded from further consideration. This conclusion was confirmed by the observation that compound A, found to be stable in aqueous solution at 25°C, is unaffected by sodium metaperiodate in water during 24 hours at 25°C, showing that the two hydroxyl groups of compound A are not vicinal. As a further confirmation, the n.m.r. spectra of the diacetate and dibenzoate, measured in chloroform-d, showed a downfield shift of the signals of four protons. Consequently, the two anhydro rings of compound A must involve carbon atoms 2, 3, 4, and 5. Unless rearrangement occurred after the initial reaction, a



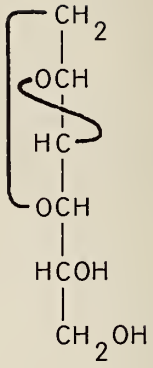
1a, R = Ms

1b, R = Ts



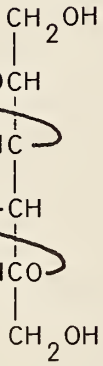
1,4:3,6-
DIANHYDRO-
D-IDITOL

2



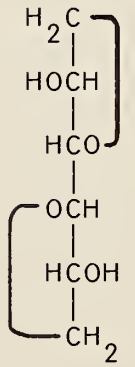
1,4:2,3-
DIANHYDRO-
D-IDITOL

3



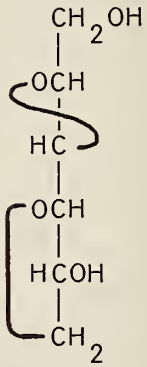
2,3:4,5-
DIANHYDRO-
D-IDITOL

4



1,3:4,6-
DIANHYDRO-
D-IDITOL

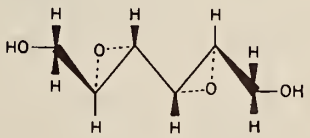
5



2,3:4,6-
DIANHYDRO-
D-IDITOL

6

2,5:3,4-dianhydride is ruled out because of the method of preparation, suggesting that compound A is the 2,3:4,5- or 2,4:3,5-dianhydride. The n.m.r. spectra of compound A and its 1,6-diacetate and 1,6-dibenzoate were consistent with either of these structures (and the respective esters thereof). The n.m.r. spectrum of compound A showed only four main groups of signals, and, for some compounds, this situation could be attributed to chemical-shift averaging caused by completely free rotation at the C-1—C-2, C-3—C-4, and C-5—C-6 bonds. However, inspection of molecular models of the 2,3:4,5- and 2,4:3,5-dianhydrides of D-iditol reveals that, with suitable orientation of the hydroxymethyl groups, each molecule can have a simple, two-fold axis of symmetry vertical to the C-3—C-4 bond. As compound A and its two diesters are optically active (and therefore lack a plane of symmetry), the optical asymmetry at C-2 differs from that at C-5, and that at C-3 differs from that at C-4. Consequently, it seems reasonable to attribute the observed chemical-shift equivalence of H-1 with H-6 or H-6', of H-1' with H-6' or H-6, of H-2 with H-5, and of H-3 with H-4, to the presence of the two-fold axis of symmetry. Such symmetry, which does not make the molecule optically inactive, is nevertheless sufficient to give chemical-shift equivalence of protons located similarly on each half of the chain of carbon atoms. Because the 2,4:3,5-dianhydride structure has two oxetan rings, seldom encountered in sugar chemistry, we have assigned the 2,3:4,5-



4

dianhydride structure (4) to compound A. We thank Mr. W. D. Dorko (National Bureau of Standards) and Mrs. Margaret Lake (Food and Drug Administration) for recording the n.m.r.

spectra, and Dr. B. Coxon of this Section for measuring and interpreting them.

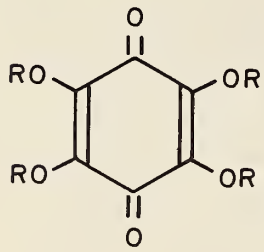
These results have been incorporated in an article which has been accepted for publication [2].

B. Polyhydroxy Cyclic Compounds

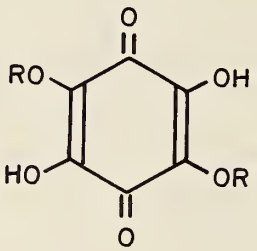
(A. J. Fatiadi)

1. Acylation of Tetrahydroxy-p-benzoquinone

The acetates of a simple enediol compound, the first oxidation product of benzenehexol (hexahydroxybenzene) 3a, namely, tetrahydroxy-p-benzoquinone (1a), were not described in recent reports by Verter and coworkers [3,4] on the preparation of other fatty acid esters of 1a. In order to fill this gap, we have prepared and characterized [5] the di- and tetra-acetates (compounds 2b and 1b, respectively) of 1a.



1



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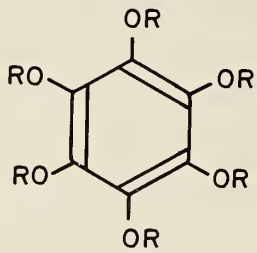

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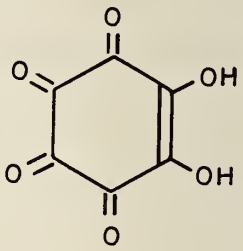


4

a. R = H
b. R = CH_3CO-
c. R = CH_3CH_2CO-
d. R = $CH_3CH_2CH_2CO$-
e. R = $(CH_3)_2CHCO$-

We attempted unsuccessfully to prepare diacetate 2b by the method [3] used by these authors for the preparation of the dipropionate 2c; however, a procedure employing simultaneous acetylation and hydrolysis ("hydrolytic acetylation") was successful in providing 2b in a yield of 85%. A mixture of tetrahydroxy-p-benzoquinone (1a) (3 g, 17.4 mmole) in acetone (50 ml) and acetic anhydride (20 ml) containing 20

drops of concentrated sulfuric acid was placed in a water bath at 50°C and stirred until the solid had dissolved (5 min). The rather dark solution was filtered through carbon, which was then washed with 10 ml of acetone. The filtrate and washing were combined, and concentrated to about 25 ml, and the solution was poured into a mixture of 50 ml of concentrated hydrochloric acid and 75 g of crushed ice. The mixture was stirred for 15 min. at room temperature, and the resulting product was filtered off, washed with ice-cold hydrochloric acid (6 M), and dried in a vacuum desiccator over potassium hydroxide; the yield of lustrous, gold-orange plates of diacetate (2b) was 2.5 g. Cooling of the filtrate (ice-bath) for 3-4 hr. gave an additional crop (1.3 g); total yield 3.8 g (85%), mp 246-248°C. The product was recrystallized quickly from warm, glacial acetic acid (prolonged heating causes decomposition) or from hot nitromethane, mp 250-251°C (with effervescence); lit. [6] mp 205°C.

By the same method, dipropionate 2c was prepared in a yield of over 60%; however, neither the dibutyrate 2d nor the diisobutyrate 2e was obtainable by this type of procedure.

a. Tetraacetate of Tetrahydroxy-p-benzoquinone

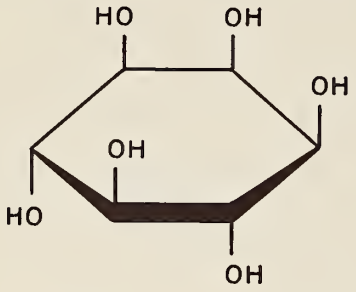
Refluxing of tetrahydroxy-p-benzoquinone (1a) with acetic anhydride for 3 minutes gave tetraacetate 1b in 75-80% yield; similarly, tetrapropionate (1c) was obtained in 70-75% yield; tetrabutyrate 1d (66-70% yield) and tetraisobutyrate 1e (78-81% yield) were also obtained.

b. Disproportionation Reactions of Tetrahydroxy-p-benzoquinone

In contrast, treatment of 1a with acetic anhydride and pyridine at 60-65°C gave benzenehexol hexaacetate 3b and rhodizonic acid (4) in nearly equal proportions (due presumably to a disproportionation). The other fatty acid esters of 3a have been prepared by an analogous procedure; compounds 3c, 3d, and 3e have been obtained in 48, 46, and 47% yields, respectively.

c. Esters of Benzenepentol

One-step aromatization of myo-inositol (5) (a cyclohexanehexol), following treatment with methyl sulfoxide–acetic anhydride in the presence of pyridine, has been reported previously [7]; the product, namely, pentaacetoxybenzene (6b) was isolated in about 50% yield.



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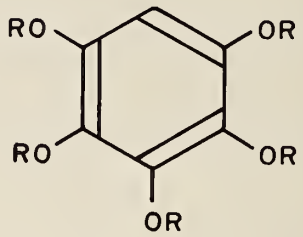


6

a. R = H
b. R = CH_3CO-
c. R = CH_3CH_2CO-
d. R = $CH_3CH_2CH_2CO$-
e. R = $(CH_3)_2CHCO$-

In order to test the general applicability of this procedure [7], compound 5 was separately treated with propionic, butyric, and isobutyric anhydrides [8]. As expected, compound 5 was converted into the corresponding fatty acid esters of benzenepentol (6a); namely, pentapropionate (6c) in 25-30% yield; pentabutyrate (6d) in 23-26% yield; and pentaisobutyrate (6e) in 21-23% yield. Hydrolysis of pentaacetoxybenzene (6b) with concentrated hydrochloric acid in methanol gave benzenepentol (6a) in 77-81% yield.

2. Acetic Anhydride–Phosphoric Acid as an Acetylating Agent

A satisfactory method was needed for acetylating the enolic compound, croconic acid. Attempts with all known acetylation procedures were incapable of providing an isolable acetate of that compound. However, when a reagent was used

that consisted of 100% syrupy phosphoric acid and acetic anhydride (mole ratio, about 1:1.7) the crystalline diacetate of croconic acid was obtained. The new reagent was found [9] to be effective for the acetylation of other difficultly and easily esterifiable hydroxylated compounds. These include carbohydrates, cyclitols, enols, phenols, sterically hindered secondary alcohols, and tertiary alcohols.

The active actylating species in the acetic anhydride–phosphoric acid reagent is believed to be monoacetic phosphoric anhydride. Use of 100% phosphoric acid in conjunction with other aliphatic acid anhydrides was found to be an effective means for the preparation of the propionate, butyrate, and isobutyrate of myo-inositol.

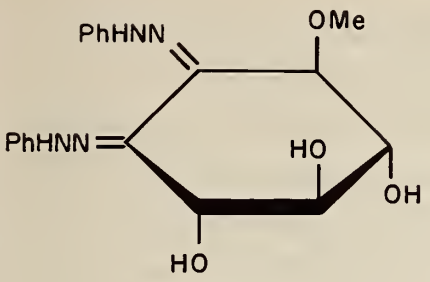
3. Bromine Oxidation of Inositols for Preparation of Inosose Phenylhydrazones and Phenylosazones

The methods usually employed for the preparation of the dicarbonyl derivatives of cyclohexanetetrols involve prolonged oxidation of inositols by Acetobacter suboxydans [10,11] or oxidation of inososes or their phenylhydrazones in the presence of phenylhydrazine and acetic acid [12]. The dicarbonyl derivatives are isolated as phenylosazones. The application of sodium hypobromite for oxidation of cyclitols has also been reported [13]; for example [14], myo-inositol was oxidized with bromine in the presence of a sodium carbonate buffer, and the mixture was then treated with phenylhydrazine to give DL-myo-inosose-1 phenylosazone (yield, about 7%). Similarly, phenylosazones have been obtained by oxidation of (+)-proto-quercitol [15] with aqueous bromine, and from oxidation of (-)-viburnitol with sodium hypobromite [16]; however, for the last two reactions, the yields were not stated.

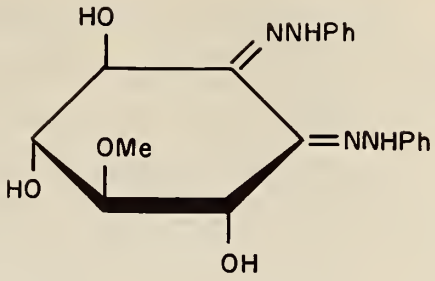
Except for a general statement by Posternak [13] that hypobromite oxidation of inositols provides unsatisfactory yields of inososes, there appears to be no literature report on the subject of the oxidation of inositols by bromine to

provide monoketoinositols. The bromine oxidation of cyclitols was studied in order to clarify whether this oxidation to inosose might be of special usefulness, in that the inososes obtained might be different from those resulting from the stereospecific catalytic and biological oxidations. The results were the subject of a paper [17] that has been accepted for publication.

It has been found that myo-inositol treated with bromine in a sodium acetate buffer, gave, on addition of phenylhydrazine, the usual phenylosazone, DL-myo-inosose-1 phenylosazone, in 22-30% yield. L-Inositol, on the other hand, gave D-myo-inosose-1 phenylhydrazone, a monoketone phenylhydrazone, (12% yield) as well as D-myo-inosose-1 phenylosazone, the expected diketone phenylosazone (28% yield). The corresponding enantiomorphs were obtained in 8 and 29% yields, respectively, from D-inositol. The oxidation reaction was also applied to pinitol and quebrachitol, the naturally occurring, monomethyl ethers of D- and L-inositol. The previously unknown diketone that results from quebrachitol (1-O-methyl-levo-inositol) was isolated as the phenylosazone 1 (29% yield), but no monoketone was isolated. From pinitol (5-O-methyl-dextro-inositol), an inosose derivative was not found either; although here too, a new diketone phenylosazone (2) was isolated (10-15% yield). Interestingly, compound 2 shows a rapid mutarotation in 1:1 (v/v) ethanol–p-dioxane. In the course of this study, a new phenylosazone 3 was prepared from DL-epi-inosose-2 phenylhydrazone; (+)-proto-quercitol (from acorns) was converted into its phenylosazone in 20% yield (a yield has not heretofore been reported), and myo-inositol was converted in low yield by bromine oxidation into the known compound DL-xylo-pentahydroxy-2-cyclohexen-1-one.



1



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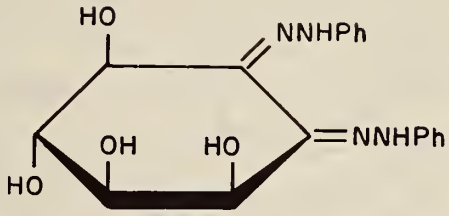


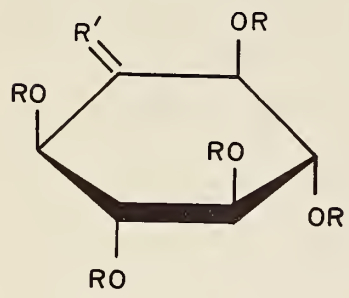
plus its enantiomer

3

4. Formation of Arylazocyclohexene Derivatives on Acylation of Certain Inosose Phenylhydrazones

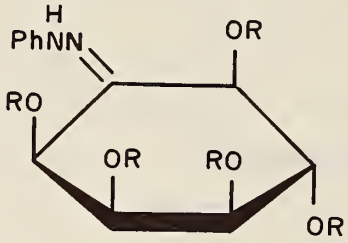
Recent communications by Wolfrom and coworkers [18] describing the preparation of arylazoethylene derivatives from aldose phenylhydrazones prompted our article [19] on related findings for inosose derivatives, in which were considered the products that result from acylation of myo-inosose-2 phenylhydrazone (1a) and DL-epi-inosose-2 phenylhydrazone (2a) in the presence of pyridine. Angyal and Matheson [20] had reported earlier that, on treating a solution of penta-O-acetyl-myo-inosose-2 (1d) in warm acetic acid with phenylhydrazine, they obtained a pale-yellow, crystalline compound (mp 112-113°C) to which they assigned the phenylhydrazone structure 1b. In our laboratory, it has been found that, under these conditions, compound 1d actually loses the elements of acetic acid, and yields the elimination product 6-phenylazo-5-cyclohexene-DL-ido-1,2,3,4-tetrol tetraacetate (3a) instead of the phenylhydrazone 1b.

The structure assigned to 3a was supported by its elemental and spectral analyses: u.v. and visible spectra λ_{max}^{MeOH} 222.5 (sh) (ε ~12,300); 228 (ε ~13,700); 233 (ε ~12,800); 302 (ε ~23,000), and 436 nm (ε ~400); i.r. spectrum $\nu_{max}^{Fluorolube}$ 1658 (C=C), 1584 (N=N), 1430 cm^{-1} (N=N); n.m.r. spectrum (p.p.m., $CDCl_3$), the olefinic proton at H-5 appears as a



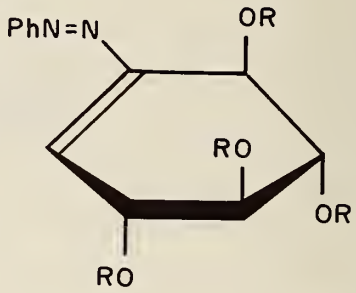
1

a. R = H, R' = NNHPh
b. R = Ac, R' = NNHPh
c. R = OCEt, R' = NNHPh
d. R = Ac, R' = O



2

a. R = H
b. R = Ac
c. R = OCEt



3

a. R = Ac
b. R = OCEt

quartet centered at 6.95, due to vicinal coupling with H-4 ($J_{4,5}$ ~2.5 Hz) and long-range coupling with H-1 ($J_{1,5}$ ~1.0 Hz).

Acylation of DL-epi-inosose-2 phenylhydrazone (2a) gave pentaacetate 2b (mp 134-135°C), and pentapropionate 2c (mp 112-113°C).

C. Determination of the Conformations of 1,2-O-Benzylidene-α-D-glucofuranose Derivatives by Nuclear Magnetic Resonance Spectroscopy

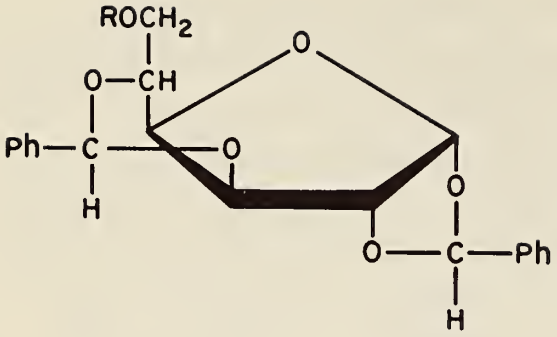
(B. Coxon)

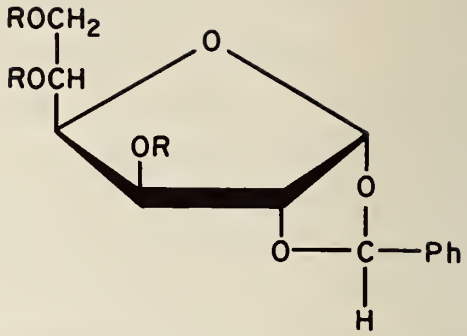
1. Introduction

The fundamental importance of the conformational analysis of carbohydrate molecules and, indeed, of organic molecules in general, has been reviewed in Technical Note 405 [21]. During the past ten years, nuclear magnetic resonance spectroscopy has provided a powerful experimental method for the determina-

tion of the conformations of organic molecules in solution, thereby eliminating much of the speculation that was apparent in earlier attempts at dealing with molecular topography. Additionally, this type of spectroscopy furnishes important new physical parameters, namely, chemical shifts, and nuclear spin–spin coupling-constants and their signs, which have proved to be extremely useful in the analysis and characterization of the structure of chemical compounds. Although the conformations are of considerable importance to an understanding of the kinetic and thermodynamic behavior of organic molecules in reaction, of even more profound implication is the characterization of the intramolecular and intermolecular interactions, both steric and dipolar, that govern the distribution of conformations and their relative stability. With greater knowledge of such interactions, it will be possible to predict favored conformations more accurately. This type of information is, for example, of basic importance in comparisons of the chemical reactivity of structurally related organic molecules.

The cylic acetals have always been valuable as intermediates in synthetic carbohydrate chemistry. The conformational influence of cyclic acetal substituents fused to furanose and pyranose rings is important, both from this point of view and as an aid to understanding the factors that determine the conformation that is adopted. In this study, the conformations of a number of bicyclic and tricyclic 1,2-O-benzylidene-α-D-glucofuranoses have been determined by proton magnetic resonance (p.m.r.) spectroscopy of their solutions at a radio-frequency of 100 MHz. The compounds [22] examined were 1,2:3,5-di-O-benzylidene-α-D-glucofuranose (1) and its 6-O-methyl, 6-O-acetyl, and 6-O-benzoyl derivatives (2, 3, and 4), and 1,2-O-benzylidene-α-D-glucofuranose (5) and its 3,5,6-tribenzoate (6). These derivatives were kindly supplied by Dr. H. G. Fletcher, Jr., of the National Institutes of Health.





1 R = H

2 R = Me

3 R = Ac

4 R = Bz

5 R = H

6 R = Bz

Compounds 5 and 6 afforded the opportunity for comparison of the conformational effect of the fusion of a 1,2-benzylidene group to a furanose ring with that of the fusion in a 1,2-O-isopropylidene-α-D-xylo-hexofuranose derivative [23]. As is well known, reactions of carbohydrates with aldehydes and ketones yield different types of ring systems for both the sugar moiety and the acetal moiety according to the particular catalysts and carbonyl compounds that are employed, and the conformational stability of the benzylidene acetals may be expected to have some bearing on this problem.

In order to assign a definite conformation to a carbohydrate ring system, it is desirable to measure as many as possible of the spin–spin coupling constants of the ring protons. Consequently, it is usually necessary that at least some of the signals of these protons be fully resolved. With molecules as complex as the benzylidene acetals mentioned, it is therefore necessary to study their proton magnetic resonance at a high magnetic-field strength (23,000 gauss or greater) in order to secure spectra that are amenable to a reasonably simple analysis. At this field strength, the chemical shifts of the nuclei may be sufficiently large to

ensure separation of several of the multiplets. Accordingly, the p.m.r. spectra of compounds 1-6 were recorded on a commercial, high-field, n.m.r. spectrometer at the National Physical Laboratory, Teddington, Middlesex, England, and the analysis and interpretation of the spectra were conducted at the National Bureau of Standards.

2. Chemical Shifts and Coupling Constants

In some of the p.m.r. spectra of 1-6, the proton multiplets overlapped one another in a complex manner. Hence, the spectral assignments were confirmed by frequency-swept, double-irradiation experiments, and by the chemical-shift differences (solvent shifts) between spectra measured in solutions in chloroform-d and in pyridine. Examples of the spectra (for compounds 2 and 6) are shown in figures 1 and 2, respectively. The spectra were, in most cases, analyzed by first-order methods, and the chemical-shift and coupling-constant parameters obtained in this way are listed in tables 1 and 2. The conformational significance of the proton–proton coupling-constants (J) was assessed by calculating approximate, vicinal proton–proton, dihedral angles (ϕ) from the theoretically derived equation of Karplus [24]

$$J = 4.22 - 0.5 \cos\phi + 4.5 \cos 2\phi,$$

which may be rewritten as

$$J = 9 \cos^2\phi - 0.5 \cos\phi - 0.28.$$

If this quadratic equation in cos ϕ is solved in the usual way, there is obtained

$$\phi = \cos^{-1} \frac{0.5 \pm [0.25 + 36(J + 0.28)]^{\frac{1}{2}}}{18}. \qquad (1)$$

This equation represents a convenient, explicit form for the calculation of approximate, theoretical, dihedral angles from vicinal-proton coupling-constants.

It often happens that a pair of vicinal protons in an organic molecule show an apparent zero coupling-constant, that is, the multiplets in the spectrum shown no resolvable split-

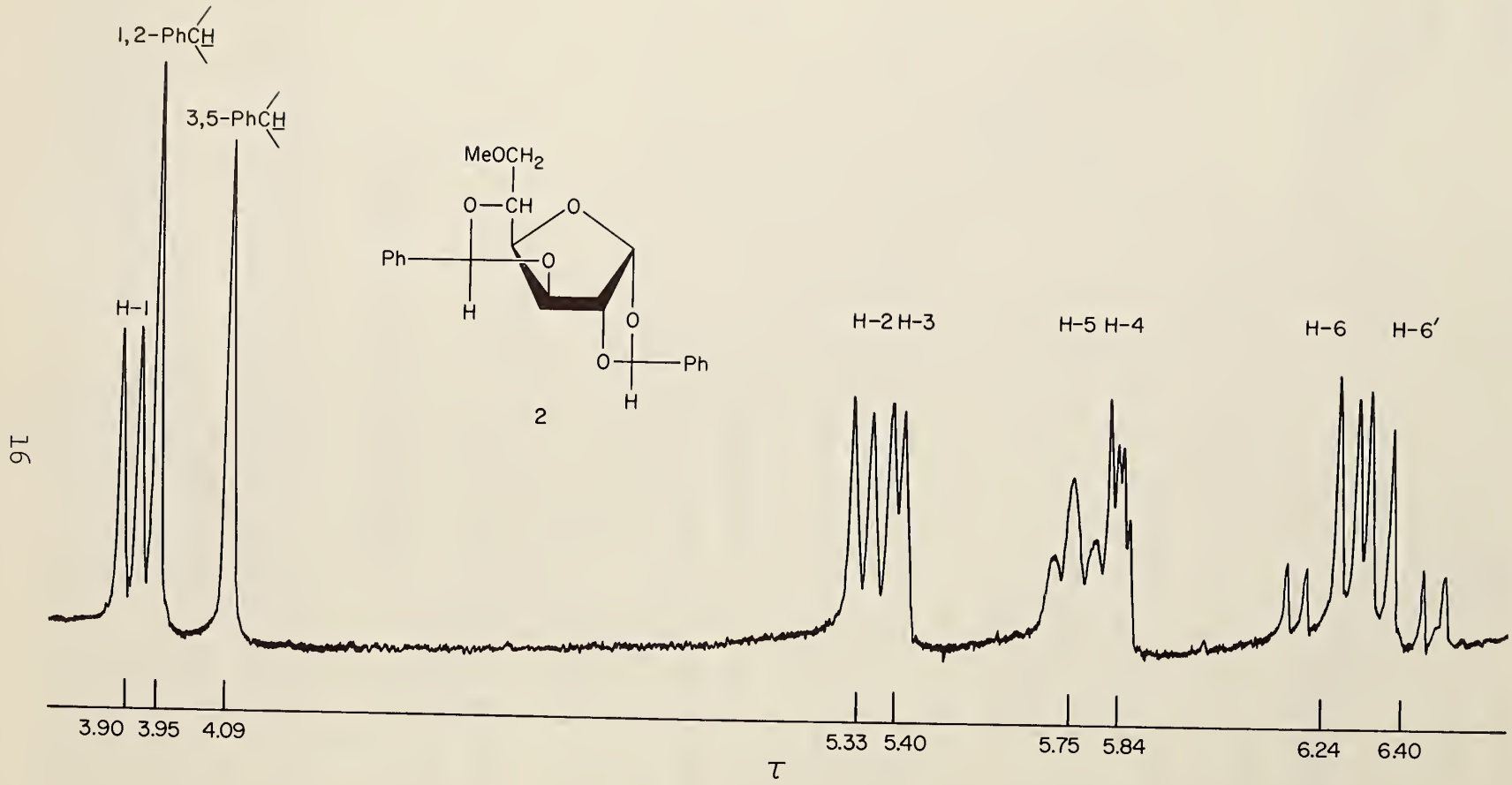


Figure 1. P.m.r. spectrum of 1,2:3,5-di-O-benzylidene-6-O-methyl-α-D-glucofuranose (2) in chloroform-d at 100 MHz. The signals of the methyl and phenyl groups are not shown.

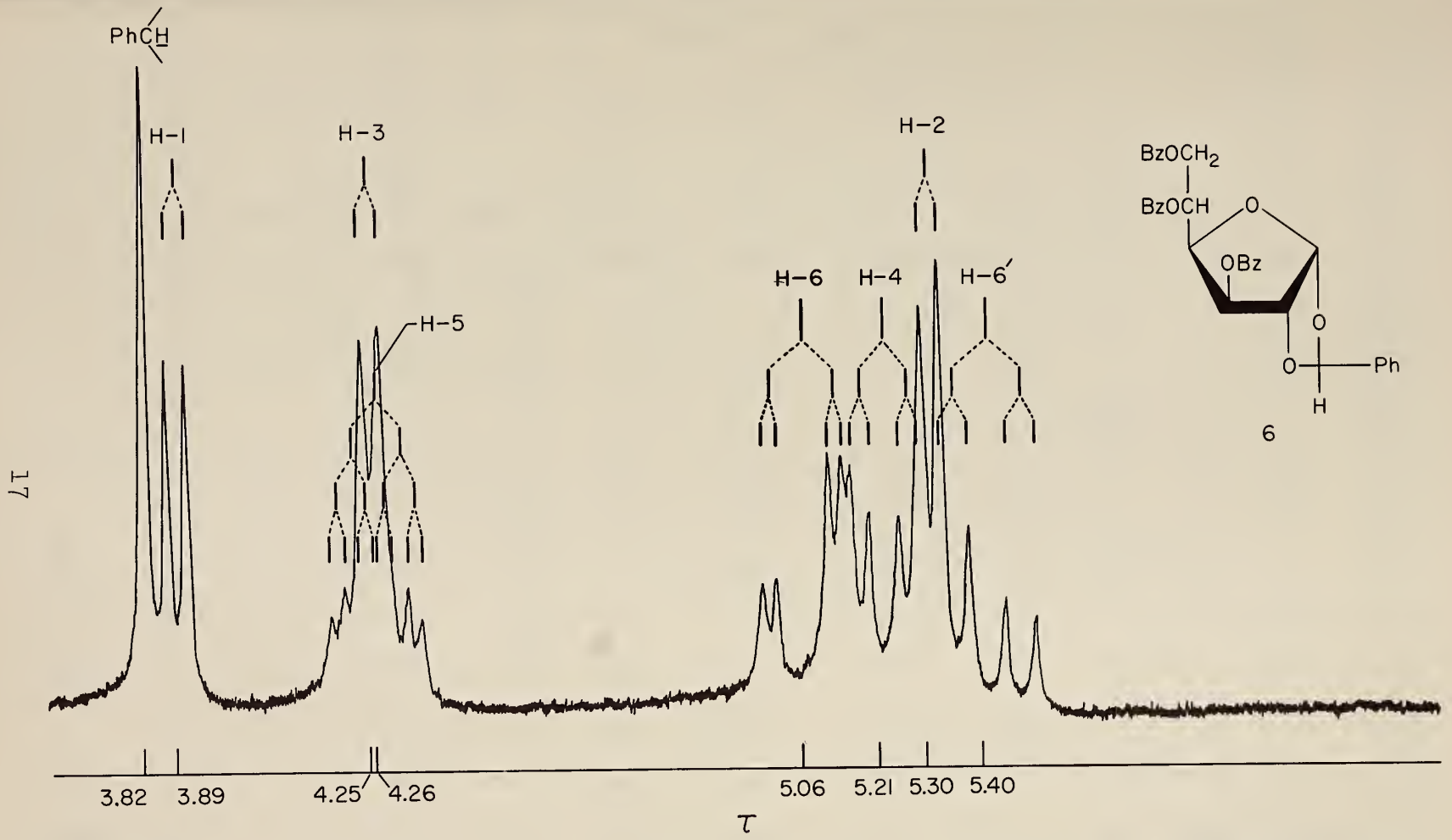


Figure 2. P.m.r. spectrum of 3,5,6-tri-O-benzoyl-1,2-O-benzylidene-α-D-glucofuranose (6) in chloroform-d at 100 MHz. The phenyl resonances are omitted.

Table 1. Chemical shifts (τ values) of 1,2-O-benzylidene-α-D-glucofuranose derivatives

Compound	Solvent[a]	H-1	H-2	H-3	H-4	H-5	H-6[b]	H-6'[b]	Ph	1,2-PhCH	3,5-PhCH	R-6[c]
1[d]	A	3.87d[e]	5.28d	5.45d	5.85[f]	5.81[f]	6.08q	6.14q	2.65m	3.96	4.19	8.15[g,h]
	B	3.65d	5.12d[f]	5.09d[f]	5.43[f]	5.44[f]	5.75q[f]	5.88q[f]	----[i]	3.71	3.77	3.52[g]
2[j]	A	3.90d	5.33d	5.40d	5.84q	5.75t[g]	6.24q	6.40q	2.70m	3.95	4.09	6.70
	B	3.68d	5.16d	5.22d	5.62q	5.53t[f,g]	6.24q[f]	6.38q[f]	----[i]	3.72	3.90	6.82
3[k]	A	3.89d	5.32d	5.50d	5.98q	5.64	5.47q[f]	5.84q	2.70m	3.97	4.28	7.99
	B	3.66d	5.13d	5.24d	5.68q[f]	5.38m	5.20q[f]	5.69q[f]	----[i]	3.73	3.99	8.09
4[m]	A	3.86d	5.27d	5.41d	5.84q	~5.48m[f]	5.24q[f]	5.52q[f]	2.68m	3.96	4.16	2.68m
									2.00m			2.00m
	B	3.61d	5.07d	5.13d	5.54q[f]	~5.16m[f]	4.92q	5.40q	----[i]	3.67	3.85	----[i]
									1.92m			1.92m
5[n]	B	3.67d	5.08d[f]	5.08d[f]	5.39m[f]	5.42m[f]	5.73m[f]	5.87q[f]	----[i]	3.75	----	3.58
6[p]	A	3.89d	5.30d	4.25d	5.21q[f]	4.26m[f]	5.06q	5.40q[f]	2.69m	3.82	----	2.69m
									2.13m			2.13m
	B	3.57d	5.00d	3.91d	4.82q[f]	3.91m[f]	4.81q[f]	5.21q	----[i]	3.47	----	----[i]

Table 2. First-order, proton coupling-constants (Hz) of 1,2-O-benzylidene-α-D-glucofuranose derivatives at 100 MHz

Compound	Solvent[a]	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$[b]	$J_{5,6'}$[b]	$J_{6,6'}$
1[c,d]	A	3.6	<0.4	2.3	----	5.2 (5.32)[q]	5.1 (5.005)[q]	11.4
	B	3.6	<0.4	----	----	∿4.9	∿5.7	∿11.5
2[j]	A	3.7	<0.4	2.5	1.5	3.7	4.3	10.3
	B	3.7	<0.4	2.4	1.2	4.3	4.8	10.3
3[k]	A	3.6	<0.4	2.4	1.35	6.1	3.5	9.7
	B	3.6	<0.4	2.4	1.2	∿7.7	3.1	9.9
4[m]	A	3.6	<0.4	2.4	1.5	8.1	4.3	13.1
	B	3.6	<0.4	2.2	1.2	7.4	4.0	10.7
5[n]	B	3.4	----	∿3.3	----	2.9	∿4.9	∿11.7
6[p]	A	3.6	<0.4	3.4	8.9	2.5	5.7	12.3
	B	3.6	<0.4	3.3	∿9.0	2.5	6.4	12.2

Footnotes to Tables 1 and 2

[a]Solvent: A is $CDCl_3$; B is C_5H_5N.

[b]H-6 is the methylene proton at lower field; H-6' is that at higher field.

[c]R-3, R-5, and R-6 refer to the O-substituents at C-3, C-5, and C-6, respectively.

[d]R-6 = H.

[e]Signal multiplicities are indicated by d(doublet), m(multiplet), q(quartet), or t(triplet).

[f]Incompletely resolved multiplet.

[g]Broad band.

[h]Assignment confirmed by deuterium exchange.

[i]Obscured by solvent signals.

[j]R-6 = Me.

[k]R-6 = Ac.

[m]R-6 = Bz.

[n]R-3 = R-5 = R-6 = H.

[p]R-3 = R-5 = R-6 = Bz.

[q]The figures in parentheses were obtained from an ABX analysis of the H-6 (A) and H-6' (B) signals, with H-5 as the X nucleus.

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ting, due to the mutual coupling of the protons. For most of the modern, high-resolution, n.m.r. spectrometers, the physical significance of this observation is that the coupling constant is less than 0.4 or 0.5 Hz, that is, less than the resolving power of the instrument. Although it has often been suggested in the literature that the non-observation of a vicinal coupling implies a proton dihedral angle of 90°, our re-examination of this situation, using equation 1, shows that this conclusion is only very approximately true. For a positive coupling in the range of 0 to 0.4 Hz, two dihedral angles of about 72-78° or 99-104° may be calculated, whereas, for a negative coupling in the range 0 to -0.2869 Hz, equation 1 implies that ϕ is about 78-88° or 88-99°. The value -0.2869 represents the minimum value of J that will allow a real solution for equation 1, corresponding to ϕ=88°. The latter two values are also obtained by equating to zero the derivative

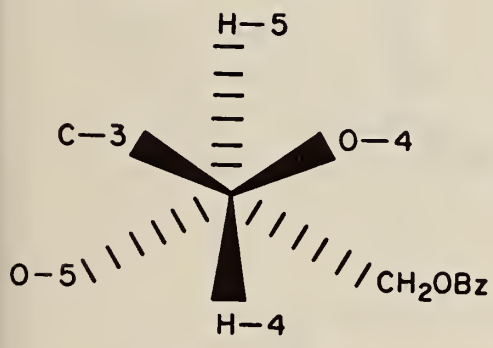
$$dJ/d\phi = 0.5 \sin\phi - 18 \sin\phi\cos\phi.$$

In compounds 1-4 and 6, the coupling constant ($J_{2,3}$) between the protons on C-2 and C-3 was not resolved, and, as its sign therefore cannot be determined, it can only be speculated from equation 1 that $\phi_{2,3}$ lies in the approximate range of 72-104°. These values represent the limits of the

dihedral angle for an "apparently zero" coupling constant and a finite reolution afforded by the spectrometer.

3. Conformations

In order that H-2 and H-3 may achieve a dihedral angle of 72-104°, it is necessary that the furanoid ring exist in one or more of those nonplanar conformations in which C-3 is above a plane containing C-1, C-2, and C-5 or the ring oxygen atom (O-4) or both (assuming that the ring is viewed in the usual orientation with O-4 at the back and C-1 on the right). Compound 6 displayed a large value (8.9-9.0 Hz) for $J_{4,5}$, indicating that the favored rotamer about the C-4–C-5 bond is 7, in which $\phi_{4,5}$ is 180°, and in which C-3 and C-6, and C-4 and C-5, are also in a true trans relationship. From consideration of all of the measured coupling constants of 6, its conformation was deduced to be that depicted in 8.



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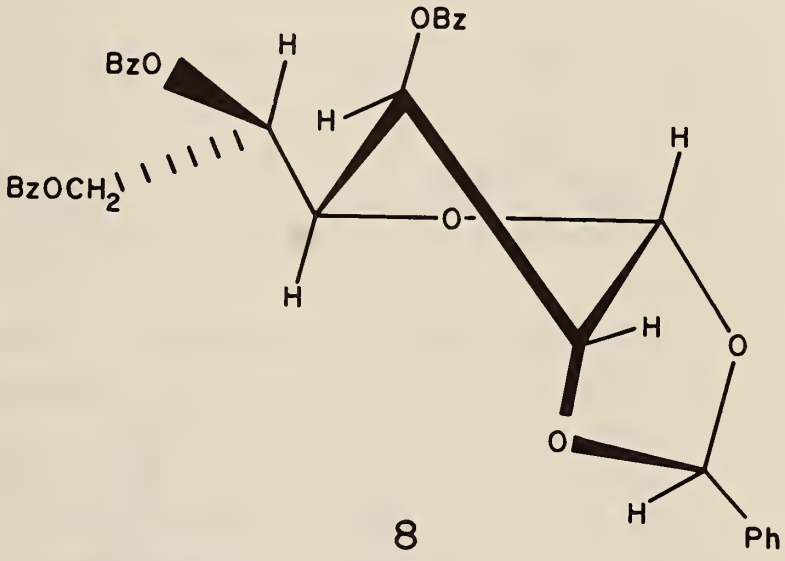
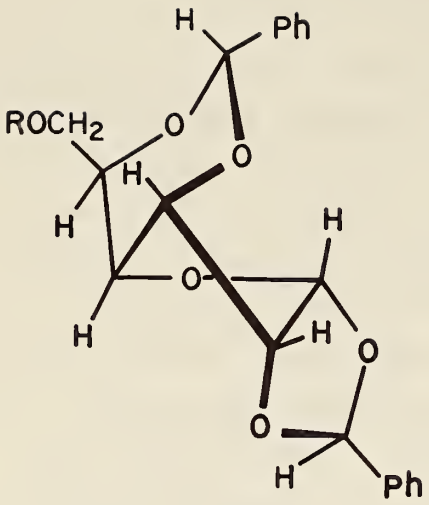


8

The coupling constants (see table 2) of the dibenzylidene acetals 1-4 indicated conformations intermediate between 9 and 10, and it was therefore inferred that fusion of a 3,5-benzylidene ring to the 1,2-O-benzylidene-α-D-glucofuranose system has the effect of distorting the symmetric, twist conformation of the furanoid ring (as in 8 and 9) towards the envelope conformation 10.



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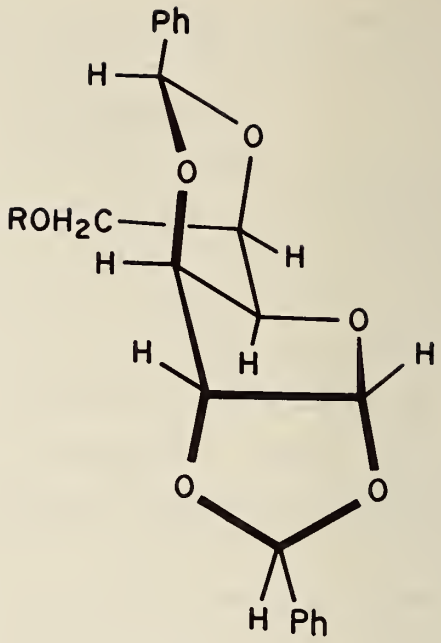
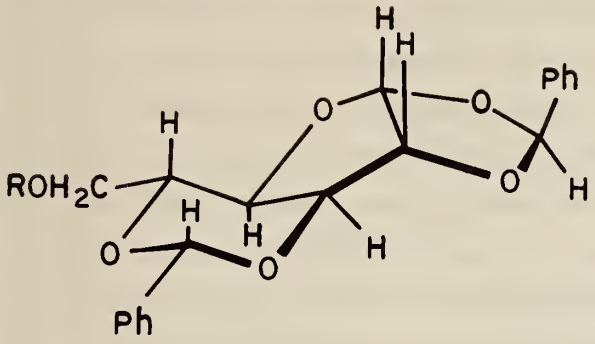


10

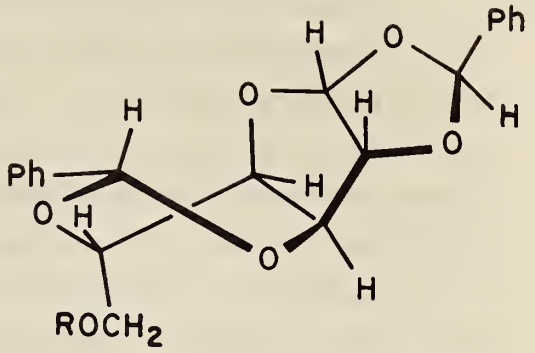
Because the m-dioxane ring in 1-4 is cis-fused to C-3 and C-4 of the furanoid ring, it can exist in either of two chair conformations, as in 9 (or 10) and 11. However, the small measured value of $J_{4,5}$ in 1-4 is inconsistent with the large dihedral angle of H-4 and H-5 in 11, and, hence, its contribution to any conformational equilibrium can only be small.

The skew form 12 provides a possible alternative to the chair forms 9 and 10 of the m-dioxane ring, as, in this conformation, H-4 and H-5 are also both equatorial and would be expected to display a small coupling-constant. This possibility was, however, discounted by analysis of the geometrical implications of the variation of $J_{3,4}$ on introduction of the 3,5-benzylidene group.

From the increased values of $J_{5,6}$ in the esters 3 and 4, it was deduced that solutions of these compounds contain a greater population of rotamer(s) about the C-5—C-6 bond, in which H-5 and H-6 are trans. As the H-6 and H-6' resonances cannot be individually assigned, this rotamer could be either

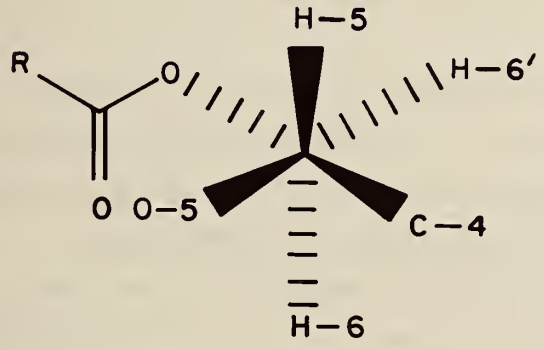


11



12

13 or 14, in which the acyloxy group at C-6 is trans to either C-5 or O-5, respectively. In the less-favored rotamer 15, in which $\phi_{5,6} = \phi_{5,6'}$, this acyloxy group is in gauche relationship with both C-4 and O-5.



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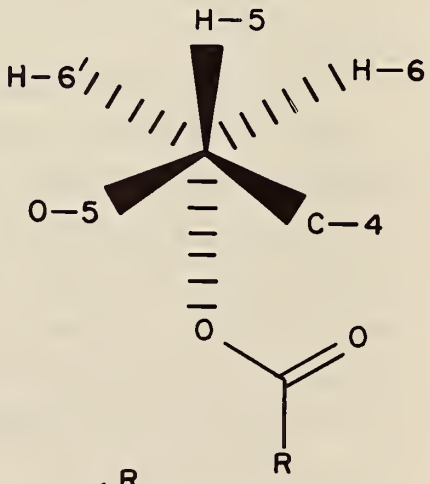
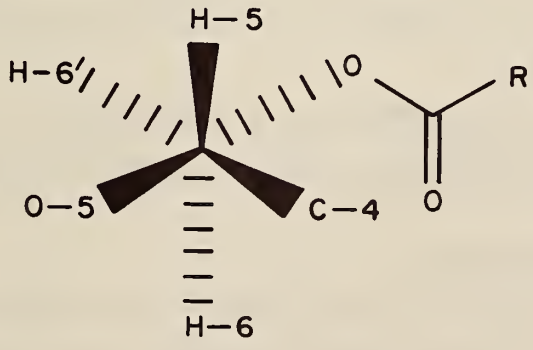


15



14

In view of the fact that hydroxymethyl, methoxymethyl, and acyloxymethyl substituents at C-5 of a pyranoid ring normally show a strong tendency to assume the equatorial orientation [25-29], it is surprising that the favored conformation (related to 9 and 10) of the dibenzylidene acetals contains C-6 in axial orientation of the m-dioxane ring. It is possible that dipolar interaction between the C-2—O-2 and C-3—O-3 bonds causes the O-2—O-3 dihedral angle to be maximized, as in conformations 8, 9, and 10 (170-180°). In the unfavored conformation 11, this angle is considerably smaller (85°). However, 11 also has some of the characteristics of an unstable, "H-inside" conformation [30], since H-2, H-5, and H-3,5 occupy endo positions with respect to the fused furanoid and m-dioxane rings. Conversely, 9 and 10 resemble a stable "O-inside" conformation [35], because O-3, O-4, and O-5 are endo with respect to the C-3—C-4 ring junction.

These results elucidate the observation that reactions of D-glucose with acetone (in the presence of zinc chloride, phosphoric acid, or other condensing agents) [31] do not give an appreciable yield of 1,2:3,5-di-O-isopropylidene-α-D-glucofuranose, because, if this compound exists in the same conformation (resembling 9 or 10) as the 1,2:3,5-di-benzylidene acetals, one of the methyl groups in its 3,5-isopropylidene moiety would be in syn-axial relationship with the $ROCH_2$ substituent on C-5.

D. Reactions, Conformations, and Acidity of Bis(ethylsulfonyl)-β-D-ribopyranosylmethane and Related Derivatives

(B. Coxon)

1. Introduction

One of the more useful methods for the conversion of an aldose (or a ketose) into its next lower homolog is the MacDonald—Fischer sulfone degradation [32]. This process involves the oxidation of a sugar dialkyl dithioacetal with hydrogen peroxide or a peroxy acid, to give a disulfone deri-

vative which is then degraded to the next lower sugar and bis(ethylsulfonyl)methane by treatment with a dilute base. The oxidation is complicated by the fact that three possible types of disulfone structure may be obtained. The initial product is, undoubtedly, an acyclic, saturated disulfone, formed merely by addition of four oxygen atoms to the diethyl dithioacetal. This type of product is, however, unstable and may suffer elimination of the elements of water from C-1 and C-2 to give an acyclic, unsaturated, disulfone derivative. Depending on the conditions of the reaction and the structure of the sugar, this alkene can then cyclize to give a third type of disulfone, which usually contains a pyranoid ring. Thus, cyclic disulfones are often the stable end-products of the oxidation reactions.

The rates of reaction of these various disulfones with dilute base differ widely, for, although the acyclic, saturated disulfones are degraded almost instantaneously to the next lower sugar, the unsaturated and cyclic disulfones, once formed, may require from two to fifteen days for complete reaction. Thus, a knowledge of the factors that influence the rate of degradation of the cyclic disulfones is important from the point of view of the preparative application of the reaction.

2. Disulfones Derived from D-Allose and D-Altrose

As part of a previous study of the general chemical and physical properties of cyclic disulfones, the oxidation by peroxypropionic acid of several diethyl dithioacetals having the D-allo and D-altro configurations was investigated [33]. Oxidation of 3-acetamido-3-deoxy-D-allose and -D-altrose diethyl dithioacetals gave (2-acetamido-2-deoxy-β-D-ribopyranosyl)bis(ethylsulfonyl)methane (1), and oxidation of the parent D-allose and D-altrose diethyl dithioacetals gave bis(ethylsulfonyl)-β-D-ribopyranosylmethane (2). The rate of reaction of 1 with dilute ammonium hydroxide to give 2-acetamido-2-deoxy-D-ribose was found to be much lower than

that of 2 to give D-ribose, and it became apparent that the rates of reaction of cyclic disulfones could be influenced by the inductive effect of the substituent at C-2. It had been discovered earlier [34] that certain other cyclic disulfones are weakly acidic, due to the electron-withdrawing character of the ethylsulfonyl groups and of the pyranoid ring [33]. The relationship of these acidic properties to the rates of degradation of the disulfones became obvious, and potentiometric titrations of the cyclic disulfones gave characteristic pKa values. Activity corrections were applied by using the Debye–Hückel theory as outlined next. The results are presented in table 3, with literature values for two related sulfones.

Table 3. pKa Values of substituted bis(alkylsulfonyl)methane derivatives

Compound	pKa	Reference
Bis(methylsulfonyl)methane	14.0	35
Bis(ethylsulfonyl)-α-D-lyxopyranosylmethane	10.1	
Bis(ethylsulfonyl)-β-D-ribopyranosylmethane (2)	9.9	
Bis(ethylsulfonyl)-(2-O-methyl-α-D-arabinopyranosyl)methane	9.6	
(2-Amino-2-deoxy-β-D-ribopyranosyl)-bis(ethylsulfonyl)methane hydrochloride	9.5	
	5.9	
(2-Acetamido-2-deoxy-β-D-ribopyranosyl)-bis(ethylsulfonyl)methane (1)	9.4	
Tris(ethylsulfonyl)methane	<1.0	36(a)

The titration curve for each of the cyclic carbohydrate disulfones listed in table 3 displayed a recognizable inflection at the neutralization point, whereas that for bis(ethylsulfonyl)methane did not. A pKa value for the latter compound

was not available in the literature, although that of its methyl homolog has been reported [35] to be 14. The pKa values of the disulfones were determined, from the half-neutralization point on the titration curve, by means of the equation [36(b)]

$$pK_a^T = pH + \log\frac{[HA]}{[A^-]} \pm \frac{0.505\sqrt{I}}{1 + 1.6\sqrt{I}},$$

where I is the ionic strength at half-neutralization of either the bis(ethylsulfonyl)pentopyranosylmethyl anion (positive activity term), or the pentopyranosylammonium cation (negative activity term). The carbohydrate disulfones showed pKa values in the range of 9.4 to 10.1, and the development of appreciable acidity in them is due to the introduction of the glycopyranosyl substituent, which is electron-attracting. The main influence must be the inductive effect of the ring-oxygen atom, as this electronegative atom is only three bonds removed from the ionizing hydrogen atom on C-1. It is apparent that the inductive effect of the substituent at C-2 also influences the pKa value of the disulfone, although to only a minor extent. There is, therefore, some correlation of these values with the rates of degradation of the disulfones. For example, compound 1, which is degraded very slowly, is the most acidic (pKa 9.4) of the disulfones examined. It must therefore be inferred that an electron-withdrawing substituent at C-2 tends to prevent recession of the electrons in the C-1—C-β bond, and, hence, the cleavage of this bond, thus inhibiting the degradation.

3. Deuterium Exchange Experiments

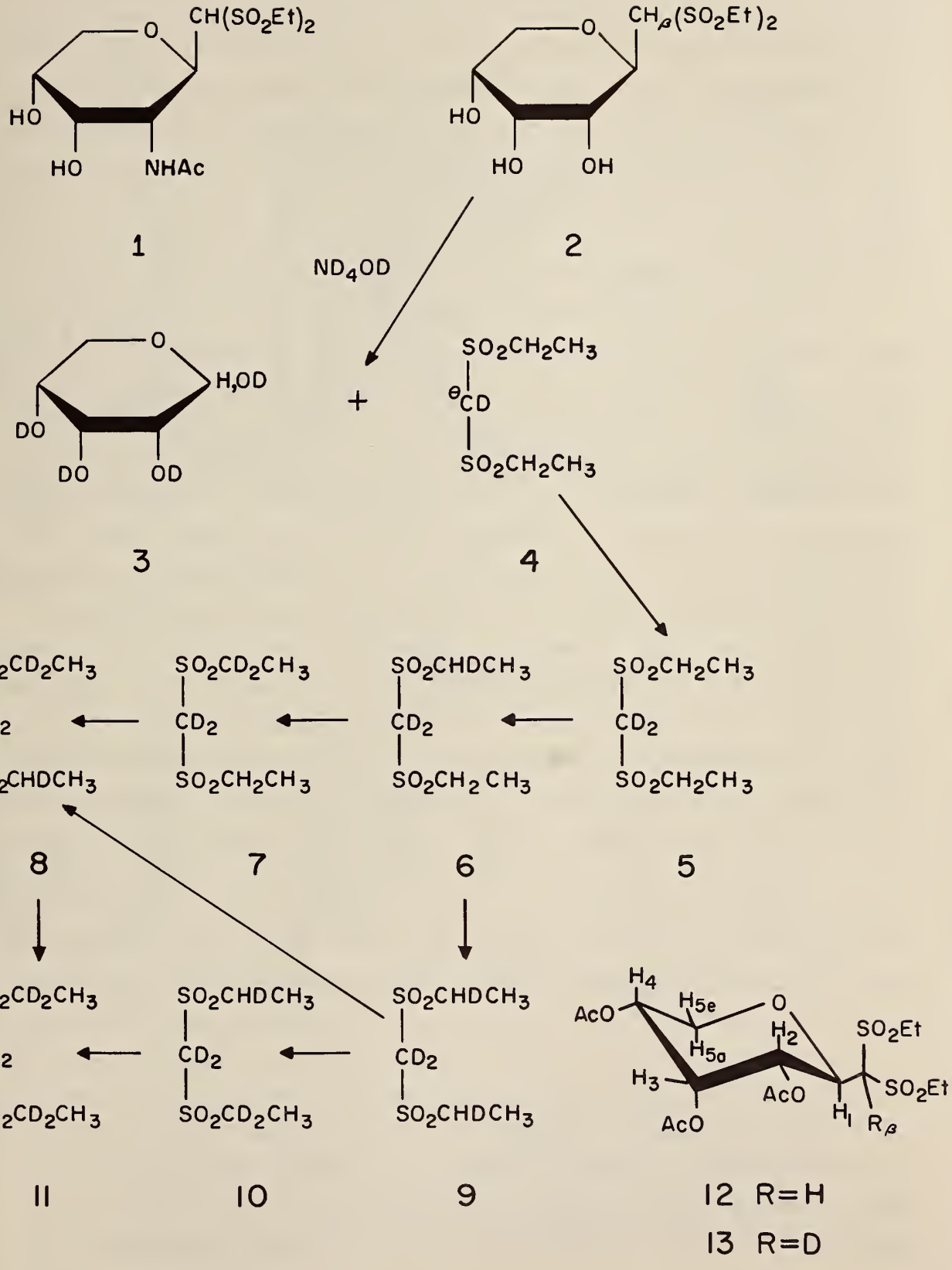
a. Bis(ethylsulfonyl)-β-D-ribopyranosylmethane (2)

Although the reaction of compound 2 with dilute ammonium hydroxide had previously been analyzed by polarimetry and paper chromatography [33], this reaction was examined further by means of proton magnetic resonance spectroscopy in order to gain information on the acidity of the disulfone and on the nature of the exchange processes involved in its reaction

with base. In ammonium hydroxide-$\underline{d}_5$ at pD 12 and 42°C, cleavage of $\underset{\sim}{2}$ was rapid. The complex series of changes observed in the p.m.r. spectrum of the solution is best interpreted in terms of the reaction sequence $\underset{\sim}{2} \rightarrow \underset{\sim}{3}$ + $\underset{\sim}{4} \rightarrow \underset{\sim\sim}{11}$ involving, initially, rapid duterium exchange of H-β in $\underset{\sim}{2}$, followed by a comparatively fast cleavage to deuterated $\underline{D}$-ribose ($\underset{\sim}{3}$) and bis(ethylsulfonyl)methane-$\underline{d}_2$ ($\underset{\sim}{5}$); and then a slower, but progressive, deuterium exchange of all α-protons of the ethyl groups, finally giving bis(ethylsulfonyl)methane-$\underline{d}_6$ ($\underset{\sim\sim}{11}$). The latter material was isolated, and characterized by its melting point and infrared and proton magnetic resonance spectra.

b. Bis(ethylsulfonyl)-(2,3,4-tri-O-acetyl-β-$\underline{D}$-ribopyranosyl)methane

In the previous work [33], evidence for the configuration and conformation of $\underset{\sim}{2}$ had been obtained from measurements of optical rotation and from periodate oxidation studies. Because cyclization of an intermediate 1,1-bis(ethylsulfonyl)-hex-1-ene-2,3,4,5,6-pentol can theoretically product two diastereoisomers, due to the introduction of new asymmetry at C-2, it was necessary, in each case, to prove the configuration at this carbon atom. Direct confirmatory evidence for the configuration and conformation of $\underset{\sim}{2}$ has now been obtained from the p.m.r. spectrum (see fig. 3a) of its triacetate ($\underset{\sim\sim}{12}$) measured at 60 MHz. Acetyl methyl signals at τ 7.82, 7.98, and 7.99 suggested the presence of one axial and two equatorial acetoxy substituents, respectively [37,38], and the spectral assignments shown in fig. 3a yielded first-order coupling-constants $\underline{J}_{1,\beta}$ 1.4, $\underline{J}_{1,2}$ 10.8, $\underline{J}_{2,3}$ 2.3, $\underline{J}_{3,4}$ 2.7, $\underline{J}_{4,5e}$ 5.8, $\underline{J}_{4,5a}$ 10.8, and $\underline{J}_{5e,5a}$ 11.4 Hz. These values compare favorably with the values $\underline{J}_{1,2}$ 10.1, $\underline{J}_{2,3}$ 2.8, $\underline{J}_{3,4}$ 2.6, $\underline{J}_{4,5e}$ 5.6, and $\underline{J}_{4,5a}$ 10.5 Hz reported previously [26] for acetamido-(2,3,4-tri-O-acetyl-β-$\underline{D}$-ribopyranosyl)-methane. The large values of $\underline{J}_{1,2}$ and $\underline{J}_{4,5}$ show that both H-1 and H-2, and H-4 and H-5a, exist mainly in diaxial relationship


CH(SO2Et)2
O
HO
HO
NHAc
1
CHβ(SO2Et)2
O
HO
HO
OH
2
ND4OD
+
H,OD
O
DO
DO
OD
3
SO2CH2CH3
θCD
SO2CH2CH3
4
SO2CD2CH3
CD2
SO2CHDCH3
8
SO2CD2CH3
CD2
SO2CH2CH3
7
SO2CHDCH3
CD2
SO2CH2CH3
6
SO2CH2CH3
CD2
SO2CH2CH3
5
SO2CD2CH3
CD2
SO2CD2CH3
11
SO2CHDCH3
CD2
SO2CD2CH3
10
SO2CHDCH3
CD2
SO2CHDCH3
9
H4
AcO
H5e
O
H2
H5a
SO2Et
H3
AcO
SO2Et
AcO
H1
Rβ
12 R=H
13 R=D

[37]. Since the triacetate of the α-D anomer of 2 would be expected to show a small value of $J_{1,2}$ in either of its chair conformations, this result demonstrates that the bis(ethylsulfonyl)methyl derivative has the β-D anomeric configuration, and that, in chloroform-d, it exists mainly in the conformation 12. The small value of $J_{1,\beta}$ indicates that the favored rotamer about the C-1—C-β bond is one or both of those in which H-1 and H-β are in gauche orientation [39], as depicted in 12.

In order to confirm the assignments of the H-1 and H-β multiplets in the spectrum (see fig. 3a) of the triacetate 12, the deuterium exchange of its weakly acidic H-β atom was investigated. Exchange did not occur readily under uncatalyzed conditions, although, with very vigorous mixing of the chloroform-d and deuterium oxide layers, H-α in the α-D-lyxo analog of 12 underwent exchange slowly (half-time 1 hr). In the presence of a small proportion of trimethylamine, exchange of H-β in 12 was rapid, as indicated by the disappearance of its doublet at τ 5.73 and the simplification of the H-1 signal to a doublet that no longer contained the small $J_{1,\beta}$ coupling (see fig. 3b). When isolation of the C-β-deuterated disulfone (13) was attempted, its deuterium was lost at a surprisingly high rate, owing, perhaps, to retention of some trimethylamine by salt formation with the disulfone. Although the spectrum (fig. 3a) of 12 shows the methyl protons of the ethyl groups as nonequivalent triplets, in the spectrum (see fig. 3b) of 13 measured in chloroform-d with one-third of a molecular equivalent of trimethylamine present, these protons are equivalent. That this difference is not due to a deuterium isotope-effect on the chemical shift of the ethyl groups was demonstrated by the fact that a sample of 13 prepared by equilibration of a chloroform-d solution of 12 with a deuterium oxide solution of sodium deuteroxide gave a spectrum in which the methyl protons were still nonequivalent. This result was confirmed by the observation that treatment of the chloroform-d solution of 12

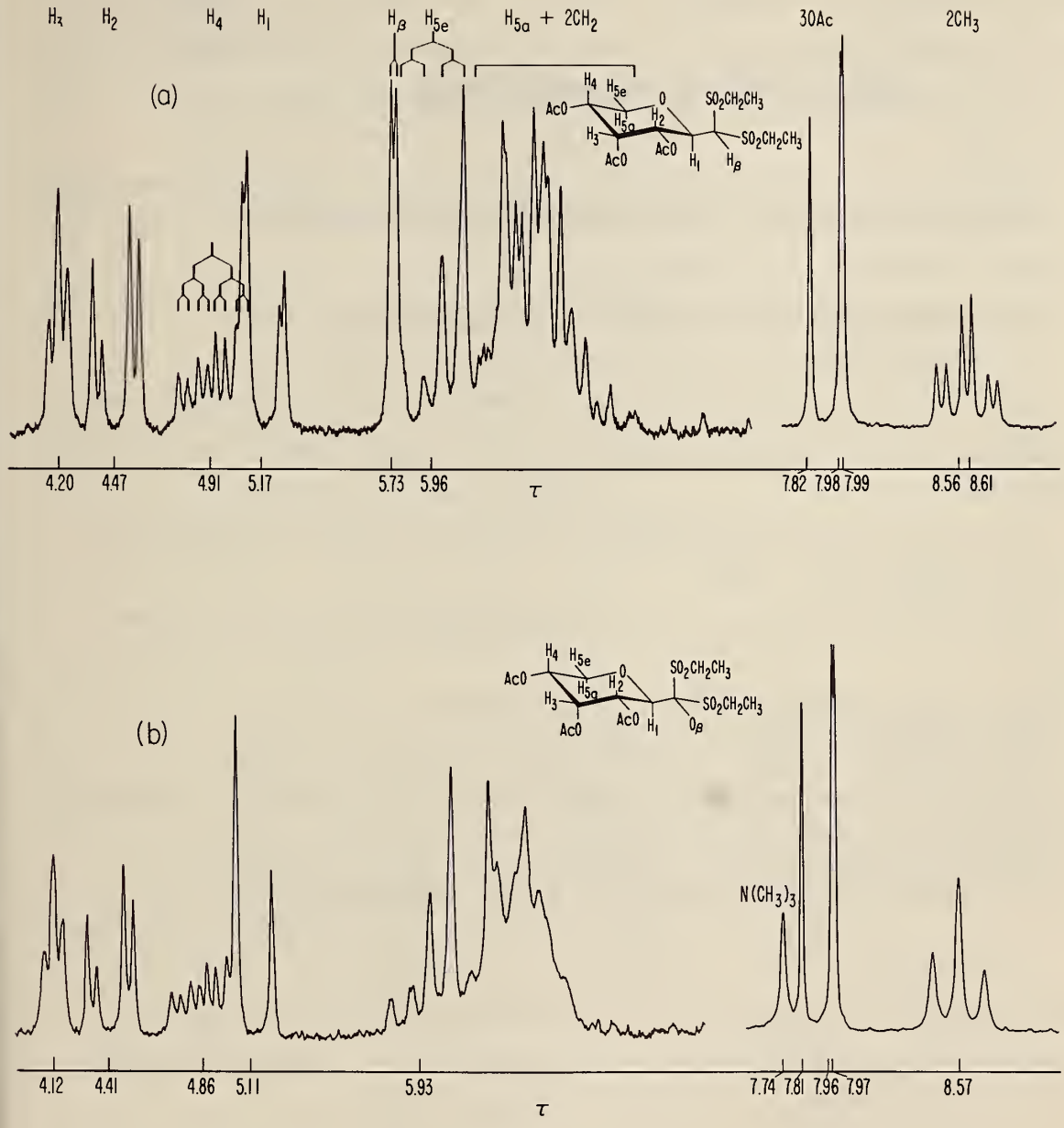


Figure 3. P.m.r. spectra in chloroform-d at 60MHz, with the methyl resonances at diminished amplitude: (a) bis(ethylsulfonyl)-(2,3,4-tri-O-acetyl-β-D-ribopyranosyl)methane (12), (b) bis(ethylsulfonyl)-(2,3,4-tri-O-acetyl-β-D-ribopyranosyl)methane-d (13) plus one-third of a molecular equivalent of trimethylamine.

with trimethylamine, alone, or in water, also caused the non-equivalence of the methyl groups to disappear, and, of course, produced chemical exchange of H-β. It therefore seems possible that the chemical-shifts effect in chloroform-d solution is due to some specific association between the nitrogen atom of trimethylamine and H-β or D-β in 12 and 13, respectively.

Most of the n.m.r. spectra described herein were recorded on a Varian Associates Model A-56/60 spectrometer at Southern Illinois University, Carbondale, Illinois.

E. Rare Carbohydrate Standard Reference Materials

1. Standards Issued During the Past Year

As a result of the work described in our Technical Note 427 [40], the following standards have been issued during the past year:

(i) 1,2-O-isopropylidene-β-L-idofuranose as Standard Reference Material 1591;

(ii) 2,3-O-isopropylidene-β-D-threo-pentulose as SRM 1592;

(iii) levo-inositol as SRM 1593; and

(iv) quebrachitol as SRM 1594.

Reproductions of the individual Certificates of Analysis are given on the following pages.

2. 2,3-O-Isopropylidene-β-D-threo-pentulofuranose

(R. S. Tipson)

In Technical Note 427 [40], the preparation of 2,3-O-isopropylidene-β-D-threo-pentulofuranose (13) for use as a standard reference material was described. Further improvements in its preparation have now been devised.

In 1936, Levene and Tipson [41] reported that the syrupy mixture of sugars obtained by boiling a solution of D-xylose (1) in dry pyridine for 4.5 hours, followed by removal of the pyridine and as much as possible of unchanged 1, could be resolved to a considerable extent by acetonation followed by fractional distillation of the resulting mixture of mono- and di-isopropylidene acetals. They thus isolated four acetals,

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Alexander B. Trowbridge,
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 1591

1,2-*O*-Isopropylidene-β-L-idofuranose

This Standard Reference Material is certified regarding its identity and has the following properties:

Melting Point	114° – 115°C
Specific Rotation $[\alpha]_D^{20}$	–27.8° (c 2; water)

Neither of the enantiomorphs of idose has been reported as a crystalline material, and the syrupy sugar is unstable, isomerizing to a ketose or losing water to form an anhydro-sugar. Reference samples of this sugar are obtained by hydrolysis of this Standard Reference Material with acetic acid. Thus, 2 mg of the Standard Reference Material dissolved in 0.5 ml of 70-percent acetic acid and warmed at 70°C for 2.5 hr provide L–idose free of the starting material and containing only a trace of impurity, possibly 1,6–anhydro–L–idopyranose.

Washington, D. C. 20234
January 18, 1968

W. Wayne Meinke, Chief
Office of Standard Reference Materials

U. S. Department of Commerce
Alexander B. Trowbridge,
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 1592

2,3-*O*-Isopropylidene-β-D-*threo*-pentulose

This Standard Reference Material is certified regarding identity and has the following properties:

Melting Point	70°–71°C
Specific Rotation $[\alpha]_D^{22}$	+1.6° (c 4; acetone)

This compound is also known by the synonyms monoacetone–D–lyxoketose, monoacetone–D–lyxulose, monoacetone–D–xyloketose, and monoacetone–D–xylulose.

To obtain the free pentulose (the crystalline form of this sugar has never been reported) dissolve 2 mg of the Standard Reference Material in 1 ml of water, add 1.25 mg of oxalic acid dihydrate and heat the solution to 65°C for eight hours. Cool, add finely powdered calcium carbonate, with stirring, until the solution is neutral to congo red. Filter and wash the calcium salts with water. Combine the filtrate and washings and evaporate to dryness at 30°C at 25 mm Hg. Dissolve the syrup in absolute ethanol, filter, and evaporate the filtrate to dryness.

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A. V. Astin, Director

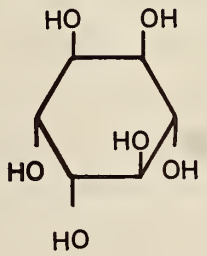
Certificate of Analysis

Standard Reference Material 1593

levo-Inositol

This Standard Reference Material is certified regarding its identity and has the following properties and spatial structure:

Melting Point	243° – 244°C
Specific Rotation $[\alpha]_D^{22}$	−64.9° (c 2; water)



levo–Inositol

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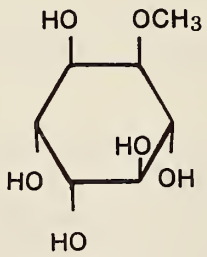
Standard Reference Material 1594

Quebrachitol

This Standard Reference Material is certified regarding its identity and has the following properties and spatial structure:

Melting Point ---------- 191° – 193°C

Specific Rotation $[\alpha]_D^{20}$ ---------- −80.5° (c 2.37; water)



Quebrachitol

Washington, D. C. 20234
January 18, 1968

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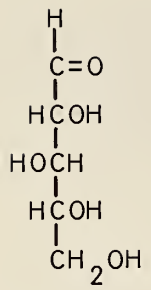
Office of Standard Reference Materials

namely, crystalline 1,2:3,5-di-O-isopropylidene-α-D-xylofuranose (5) derived from unchanged 1, syrupy 2,3-O-isopropylidene-α-D-lyxofuranose (6) (the crystallization of which, from this reaction, was reported in Technical Note 427), crystalline 2,3-O-isopropylidene-β-D-threo-pentulofuranose (13), and a syrupy mixture (A) of unidentified di-O-isopropylidenepentoses (free from 5) having n_D^{25} 1.4414 and $[\alpha]_D^{25}$ -47.8° (acetone).

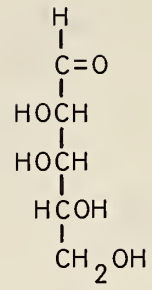
On complete hydrolysis with 0.1 M oxalic acid, mixture A gave a mixture of pentoses having $[\alpha]_D$ -5.7° (calculated as pentose; in water). These properties suggested that A consisted mainly of 1,2:3,4-di-O-isopropylidene-D-erythro-pentulofuranose (14), the enantiomorph of which had been shown by Levene and Tipson [41] to have n_D^{25} 1.4422 and $[\alpha]_D^{28}$ +105.5°(acetone), and to give, on hydrolysis, L-erythro-pentose having $[\alpha]_D^{27}$ +16.6° (water). A new sample of mixture A has now been prepared. It was hydrolyzed with 0.1 M oxalic acid at 25°C for 40 minutes, and the acid was then neutralized by addition, with stirring, of an excess of calcium carbonate. The suspension was filtered, and the filtrate was extracted with chloroform. The extract was dried (anhydrous sodium sulfate) and evaporated to dryness, affording a product that was identified as 1,2:3,4-di-O-isopropylidene-D-erythro-pentulofuranose (14). The aqueous layer was evaporated to dryness, giving a syrup that was treated with absolute ethanol; a trace of insoluble calcium salts was removed by filtration. The filtrate was evaporated to dryness, and the resulting syrup was treated with anhydrous ether (20 volumes). Part of the syrup, consisting of free sugars, remained undissolved; the ether solution was evaporated to dryness, giving a syrup consisting of monoisopropylidene acetals. These products are undergoing further study.

By use of modern techniques (paper and column chromatography), Fedoroňko and Linek [42] have recently re-examined the mixture of pentoses obtained by the action (8 hours) of boiling pyridine on D-xylose. They found that, in addition



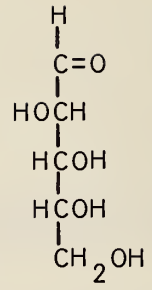
D-Xylose

1



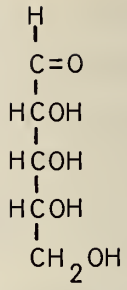
D-Lyxose

2



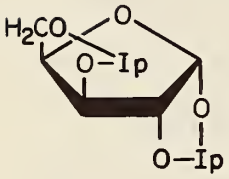
D-Arabinose

3



D-Ribose

4



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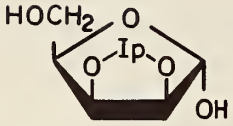

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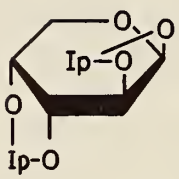

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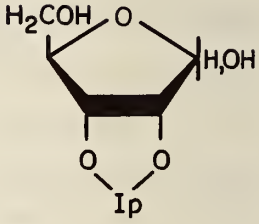

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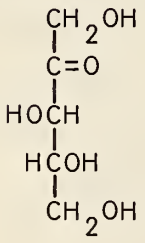
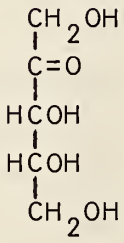


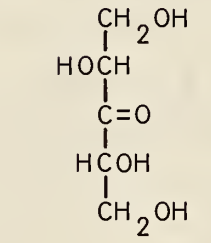
D-threo-Pentulose

9



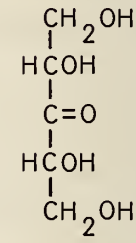
D-erythro-Pentulose

10



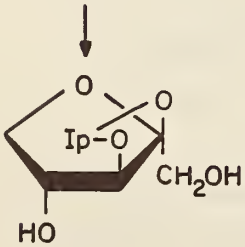
D-threo-3-Pentulose

11



erythro-3-Pentulose

12



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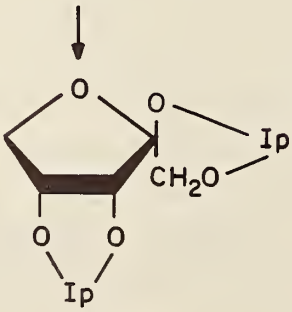

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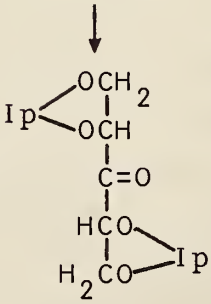

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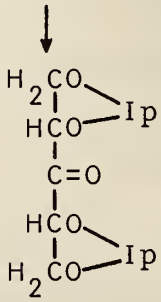

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to the pentoses already mentioned (1, 2, 9, and 10), it contains D-arabinose (3), D-ribose (4), D-threo-3-pentulose (11), and erythro-3-pentulose (12), all in unstated proportions. In restudying the reaction with a view to increasing the yield of acetal 13, it was decided to remove the aldopentoses (1, 2, 3, and 4) from the initial reaction mixture (after removal of as much as possible of 1 by crystallization), and then to acetonate the mixture of ketopentoses (9, 10, 11, and 12). Such removal of an aldose from a solution of an aldose and a ketose was achieved in 1950 by Gabrielson and Samuelson [43] by use of Amberlite IRA-400 (HSO_3^-) ion-exchange resin, and the method has since been improved [44,45].

D-Xylose (1) was epimerized on a larger scale (150 g per batch) than hitherto, and, after removal of some 1 by crystallization, the mixture of pentoses was passed through a column of Amberlite IRA-400 (HSO_3^-) which had been prepared as described by Adachi and Sugawara [46] (see also, Ref. [47]), and the mixture of ketopentoses was isolated from the effluent and treated as described by Zinner and Rehpenning [47]. The mixture of compounds 9, 10, 11, and 12 was acetonated, giving a mixture of 13, 14, 15, and 16 as a syrup that contained a preponderance of 13, because, without distillation of the syrup, the monoacetal 13 crystallized from it, affording an almost solid mass of crystals. The monoacetal 13 was readily separated from the diacetals (14, 15, and 16) by dissolving the mixture of acetals in water and extracting with ether; 13 remains in the aqueous layer, and the mixture of the three diacetals is obtained in the ether layer.

3. 1,2:3,4-Di-O-isopropylidene-D-erythro-pentulofuranose (R. S. Tipson and R. F. Brady, Jr.)

D-erythro-Pentulose (10) is a rare sugar that is an intermediate in the oxidative pathway of metabolism of D-glucose in animals and yeast, and it is an early product of photosynthesis in plants. It is therefore of considerable interest to biochemists.

The sugar was synthesized in 1934 [48] by pyridine-catalyzed epimerization of D-arabinose (3) and isolated as the crystalline (o-nitrophenyl)hydrazone [49] in 1935. In the following year, Levene and Tipson [41], working with the enantiomorph (3e), improved the preparative value of the reaction by acetonating the mixture of pentoses that is formed, and separating the resulting mixture of acetals by fractional distillation. In this way, they isolated L-erythro-pentulose (10e) as its crystalline 1,2:3,4-diisopropylidene acetal (14e). L-Ribose (4e) was also isolated, as its 2,3-isopropylidene acetal (8e).

Federoňko and Linek [42] have recently shown that treatment of D-arabinose (3) with boiling pyridine for 4 hours gives a mixture of the same pentoses as was obtained from D-xylose (1). Obviously, however, the proportions of the pentoses in the mixture will be different, starting with 1 or 3, unless the reaction is permitted to proceed to equilibrium in both instances.

In order to make D-erythro-pentulose (10) available as a standard reference material, it was decided to apply Levene and Tipson's procedure to D-arabinose (3), to remove the aldopentoses (1, 2, 3, and 4) from the initial reaction mixture (after removal of as much as possible of 3 by crystallization), and then to acetonate the mixture of ketopentoses (9, 10, 11, and 12). Zinner and Rehpenning [47] have achieved the separation of the aldopentoses from the ketopentoses obtained from D-arabinose (3) by use of a column of Wofatit SBW (HSO_3^-) ion-exchange resin. Dowex-1 (HSO_3^-) was used by Lindberg and Slessor [45] for similar separation of the pentoses from L-arabinose (3e).

D-Arabinose (3) was epimerized on a large scale (150 g per batch), and, after removal of some 3 by crystallization, the mixture of pentoses was passed through a column of Amberlite IRA-400 (HSO_3^-) ion-exchange resin, prepared as described

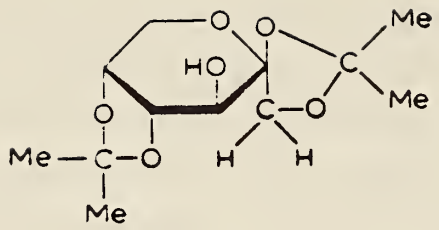
by Adachi and Sugawara [46] (see also, Zinner and Rehpenning [47]). The mixture of ketopentoses (9, 10, 11, and 12) that was isolated from the effluent was acetonated, giving a mixture of the acetals 13, 14, 15, and 16. The monoacetal 13 was then readily separated from the diacetals (14, 15, and 16) by dissolving the mixture of acetals in water and extracting with ether. (a) The monoacetal remained in the aqueous layer, and evaporation of this solution gave a crystalline compound that was identified as 2,3-O-isopropylidene-β-D-threo-pentulofuranose (13). (b) The ether layer was evaporated to dryness, and the resulting mixture of 14, 15, and 16 was fractionally distilled under high vacuum, affording crystalline 1,2:3,4-di-O-isopropylidene-D-erythro-pentulofuranose (14). Compounds 15 and 16 are under further study.

Because of the low melting point (+5 °C) of compound 14, a more suitable derivative is being sought for use as a standard reference material. A sample of the diacetal 14 was therefore hydrolyzed with 0.1 M oxalic acid, to give free D-erythro-pentulose (10), and attempts were made to crystallize the free sugar, and to prepare a crystalline derivative having a melting point above 25°C. Attempts to prepare a crystalline cyclohexylidene acetal by condensation with cyclohexanone, or of a crystalline peracetate or perbenzoate have, thus far, been unsuccessful.

4. Studies of D-Psicose Derivatives

(R. S. Tipson and B. F. West)

D-Psicose (D-ribo-hexulose) (4) is a rare sugar that occurs in Itea plants and in the antibiotic psicofuranin. In 1967, McDonald [50] described a new synthesis of the sugar, from D-fructose, that involved the following steps. 1,2:4,5-Di-O-isopropylidene-D-fructopyranose (1) was oxidized with methyl sulfoxide—acetic anhydride to 1,2:4,5-di-O-isopropylidene-D-erythro-2,3-hexodiulose (2); it was stated that a small proportion of the 3-acetate (5) of compound 1



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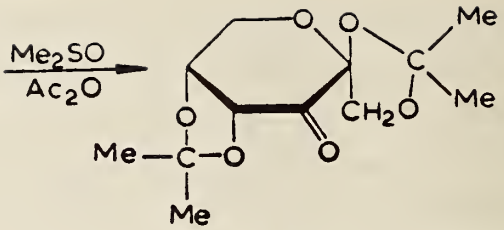

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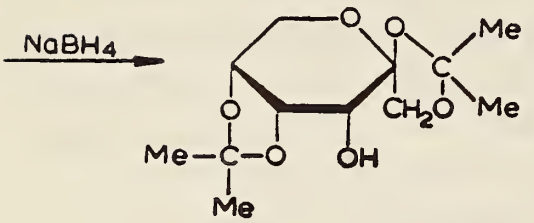

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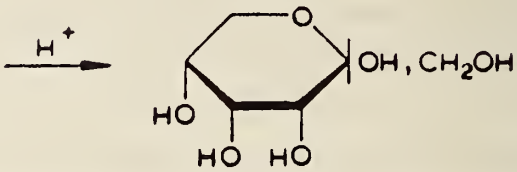

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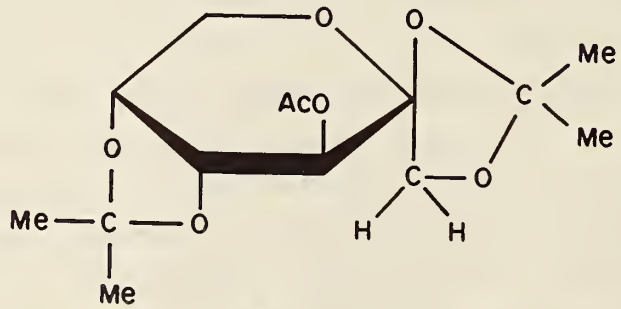
was also formed, but that, on evaporation of the reaction



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mixture, followed by crystallization of 2, compound 5 remained in the mother liquor. Reduction of this diulose acetal (2) with sodium borohydride in aqueous methanol gave, by stereospecific reaction, 1,2:4,5-di-O-isopropylidene-D-ribo-hexulopyranose (3) almost exclusively, together with a trace of the corresponding D-fructose acetal (1), presumably removed on recrystallization of compound 3. Acid hydrolysis of compound 3 then gave D-psicose (4).

Because this synthesis provides a direct means for producing D-psicose, which heretofore had been hard to obtain, it was decided to repeat this procedure to produce a supply of the crystalline di-O-isopropylidene-D-psicose available for issuance as a reference compound under the Bureau's Standard Reference Materials Program. McDonald's article was published in September, 1967, and, in December, 1967, a more detailed article describing similar work was published independently [51]. Many of the conclusions described in the latter article had already been arrived at by us.

In our study, McDonald's procedure was first repeated in accordance with her instructions. Compound 1 was oxidized with a solution of acetic anhydride (1 molar proportion) in methyl sulfoxide (70 molar proportions), and crude compound 2 was isolated in a yield of 50% of the theoretical, as compared with the 70% yield of crude product reported by McDonald [50]. Because of this result, a series of experiments was performed in order to determine what molar proportion of acetic anhydride would optimize the yield of diulose acetal 2 from 1. [In the course of this work, the reaction was followed by thin-layer chromatography on Silica Gel G, and we found that 1:3 (v/v) ethyl acetate–pentane is preferable to the 3:1 (v/v) ethyl acetate–petroleum ether used by McDonald as the developing solvent.] The results are given in table 4, from which it would

Table 4. Comparison of yield of diulose acetal (2) obtained with various molar proportions of acetic anhydride and methyl sulfoxide, per molar proportion of acetal 1

Molar proportion to 1		Yield of compound 2	
Acetic anhydride	Methyl sulfoxide	Crude (%)	Recrystallized (%)
1	70	50	
2	42		54
16	42		50
21	42	61	52

appear that a molar ratio (of acetic anhydride to 1) of 2:1 gives a slightly higher yield of compound 2 than a ratio of 1:1, but that no improvement in yield is obtained by a further increase in the ratio. In this connection, we made a comparison of the molar proportions that had been given in the literature for other such oxidations; these are listed in table 5, together with information from articles published subsequent to that of McDonald [50]. From table 5, it may be seen that, for different compounds to be oxidized, the ratio of acetic anhydride to the alcohol has lain in the range of 23.1:1 to 1.08:1, and for the ratio of methyl sulfoxide to the alcohol, in the range of 564:1 to 14.5:1. It would therefore appear that, in this reaction, the proportions of reactants employed, over the range studied, are not critical.

Table 5. Molar proportions of acetic anhydride and methyl sulfoxide, per molar proportion of the alcohol, employed in oxidations at 25-30°C

Molar proportion		Reaction time (hours)	References
Acetic anhydride	Methyl sulfoxide		
21.5	42.6	18-24	[52]
21.5	42.6	24	[53]
21.5	564.0	78-96	[54]
21.5	42.5	24	[55]
1.08	70.5	48	[50]
6.5	28.3	36	[56]
23.1	45.8	24	[51]
7.4	14.5	48	[57]

According to Albright and Goldman [58], the reacting species is formed by reaction of acetic anhydride with methyl sulfoxide:

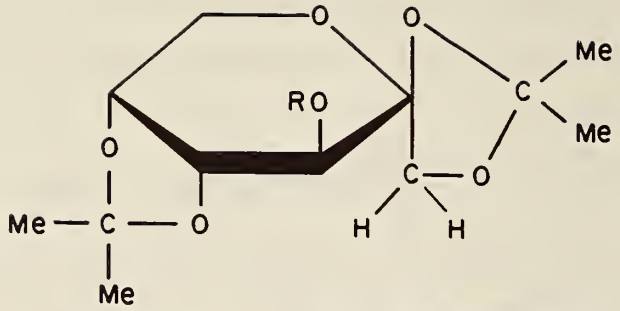
$$Ac_2O + Me_2SO \rightarrow [Ac\text{-}O\overset{+}{S}Me_2]O\bar{A}c$$

and this acetoxysulfonium acetate then reacts with the alcohol to give, via the intermediate alkoxysulfonium salt, the ketone and methyl sulfide.

$$\overset{|}{\underset{|}{C}}(H)OH + [Ac\text{-}O\overset{+}{S}Me_2]OA\bar{c} \rightarrow \left[\overset{|}{\underset{|}{C}}(H)O\overset{+}{S}Me_2\right] OA\bar{c} \rightarrow \overset{|}{\underset{|}{C}}{=}O + Me_2S$$

This reaction sequence would indicate that one molar proportion of acetic anhydride and one molar proportion of methyl sulfoxide, per molar proportion of compound 1, should be sufficient to effect complete oxidation of 1 to 2. However, the intermediate acetoxysulfonium ion must be present in sufficient concentration for nucleophilic attack by compound 1 to occur. Consequently, only sufficient methyl sulfoxide need be employed to provide the one molar proportion required for the reaction together with a sufficient excess to act as the solvent. Possibly, the solvent effect could be provided by dry benzene or dry ether, thus eliminating the rather tedious removal of excess of methyl sulfoxide at the end of the oxidation.

In all of these experiments, we found that, accompanying compound 2, there was a large proportion (25% or more) of a different, crystalline compound (A) that, on the basis of thin-layer chromatography [Silica Gel G, with 3:1 (v/v) ethyl acetate–petroleum ether; sulfuric acid spray for detection], had been regarded by McDonald [50] as being the 3-acetate (5) of compound 1. However, we found that the by-product A, which had mp 81-82°C and $[\alpha]_D^{24}$ +28° (c 1.25, chloroform), showed no carbonyl (or hydroxyl) absorption in its infrared spectrum, and therefore could not be the acetate 5. To verify this conclusion, we prepared an authentic specimen of acetate 5 by the method of Fischer and Noth [59], and found this compound to have mp 74-76°C, $[\alpha]_D^{24}$ -171.3° (c 1.0, ethanol); lit. [59] mp 76-77°C, $[\alpha]_D$ -176.3° (c 0.083, ethanol); its infrared spectrum, which shows bands at 1755 (C=O) and 1225 cm^{-1} (C-O), is entirely different from that of compound A. A mixture of compound A



6 (R = $MeSCH_2$-)

with compound 5 had mp 63°C. By-product A was identified as the 3-(methylthio)-methyl ether (6) of compound 1, and this conclusion has also been reached in an article (published after completion of this aspect of our work) by Tatchell and co-workers [51].

The (methylthio)methyl ether produced in the reaction may be formed [58] by either pathway (1) or (2).

$$Ac\text{-}O\overset{+}{S}Me_2 \rightarrow AcO^{-}CH_2=\overset{+}{S}Me \qquad (1a)$$

$$\overset{|}{C}(H)OH + AcO^{-}CH_2=\overset{+}{S}Me \rightarrow \overset{|}{C}(H)OCH_2\text{-}SMe \qquad (1b)$$

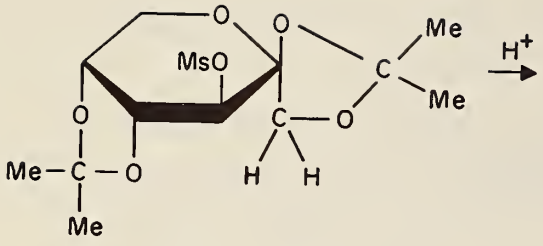
$$\overset{|}{C}(H)O\overset{+}{S}Me_2 \rightarrow \overset{|}{C}(H)O\overset{+}{S}\begin{matrix}Me\\ \diagup\\ \diagdown\\ \bar{C}H_2\end{matrix} \rightarrow \overset{|}{C}(H)\bar{O}\overset{+}{S}\begin{matrix}Me\\ \diagup\\ =\\ CH_2\end{matrix} \rightarrow \overset{|}{C}(H)OCH_2\text{-}SMe \qquad (2)$$

To effect purification of the diulose acetal (2), McDonald reported using methanol as the recrystallization solvent. McDonald [50] found mp 101-102°C; $[\alpha]_D^{25}$ -100.4° (c 2.41, acetone). Petroleum ether (bp 30-60°C) or chloroform–hexane has been found to be satisfactory for the recrystallization. After three recrystallizations, the yield was 41.5%; mp 99-102°C; $[\alpha]_D^{25}$ -97.5° (c 2.40, acetone) $[\alpha]_D^{24}$ -126.4° (c 1.91, chloroform); homogeneous by t.l.c. Tatchell et al. [51] obtained a 53% yield of purified 2; mp 101.5-102.5°C; $[\alpha]_D^{20}$ -126.4° (c 1, chloroform). Hence, our results are in close agreement with those of Tatchell

and co-workers [51]. We have repeated their method of isolation of compound 2, and find it satisfactory.

The reduction of purified compound 2 (free from 1 and 6) was effected with sodium borohydride (9.5 molar proportions) in water (0.155 g per ml); this solution was quickly added, with stirring, to an ice-cold solution (0.035 g per ml) of the diulose acetal (2) in 1:3 (v/v) water—methanol. After 30 minutes, t.l.c. with 1:3 (v/v) ethyl acetate—pentane indicated that the reaction was complete, and that only one product appeared to be present. The solution was evaporated to dryness under diminished pressure, the residue was dissolved in water, and the solution was extracted repeatedly with chloroform or ether. The extract was dried, and then evaporated to a clear syrup which crystallized on trituration with petroleum ether (bp 30-60°C). The crude product was, in fact, a mixture of 1,2:4,5-di-O-isopropylidene-D-psicopyranose (3) with compound 1; it was recrystallized from petroleum ether (bp 30-60°C); yield 87.5%, mp 64.5-67°C; $[\alpha]_D$ -111.0° (c 2.12, acetone). McDonald [50] reported a yield of 60%, and mp 60-61°C, but did not report the specific rotation. Tatchell and co-workers [51] obtained an 87% yield of 3 after three recrystallizations from petroleum ether; mp 64-65°C; $[\alpha]_D^{20}$ -126.8° (c 1, chloroform); homogeneous by gas—liquid chromatography of its trimethylsilyl ether.

We have also studied the method of Jones and Nicholson [60] for the preparation of D-psicose from D-fructose. This method involves the methanesulfonylation of compound 1 to give 1,2:4,5-di-O-isopropylidene-3-O-(methysulfonyl)-D-fructopyranose (7). Compound 7 was then hydrolyzed to the monoacetal (8), and this was hydrolyzed further, with 0.1 M sulfuric acid, to give 3-O-(methylsulfonyl)-D-fructose (depicted as the pyranose form 9). Compound 9 was treated with 0.3 M sodium hydroxide solution, causing simultaneous deesterification and epimerization at C-3 to give D-psicose (4).



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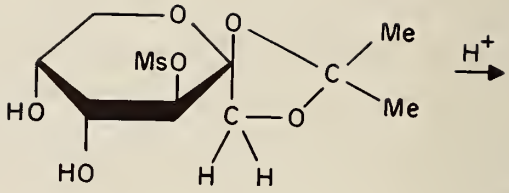

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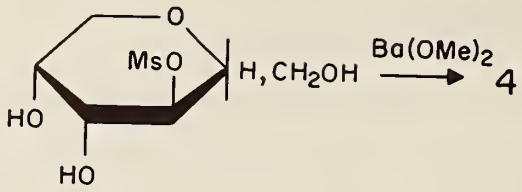

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As we have found that de-esterification—epimerization does not occur with the 1,2-isopropylidene acetal (8) of 9, the reaction presumably involves formation of 2,3-anhydro-D-psicose as an intermediate.

Because sulfuric acid is generally regarded as unsuitable for the hydrolysis of ketose derivatives, we examined the hydrolysis of 7 with 0.1 M oxalic acid at 65°C. During the course of the hydrolysis, crystalline 8 separated out; after 2 hours, the suspension was cooled and filtered. The yield of 8 was 75.5% of the theoretical, and compound 9 was present in the filtrate [as shown by t.l.c. with 10:1 (v/v) chloroform—methanol]. In a repetition of the experiment, except that the solution was boiled under reflux, all of the crystals had dissolved after 1.5 hrs; and, after neutralization with calcium carbonate and filtration, compound 9 was isolated from the filtrate. Also, 4:1 (v/v) acetic acid—water, in which the monoacetal 8 is readily soluble, proved to be a satisfactory hydrolyst. This work is being continued.

2. CLINICAL CHEMISTRY

Laboratories providing clinical analysis are called on to perform ever greater numbers of determinations; an annual growth in output of 20 percent or more is not uncommon. To contend with this problem, automated methods and other fast methods of analysis are coming into widespread use. Despite the earnest efforts of clinical chemists and pathologists to achieve high standards of precision and accuracy under these burgeoning workloads, surveys taken throughout the Nation clearly demonstrate that actual attainment is far from ideal. As a consequence, a vigorous program for upgrading performance in these essential laboratories is being undertaken under the aegis of professional societies, industry, and governmental organizations. To facilitate this program, the National Bureau of Standards' Office of Standard Reference Materials is underwriting the development of a series of organic and inorganic Standard Reference Materials certified for use in clinical chemical analysis. Each standard material, certified as to its purity, will enable medical laboratories to calibrate their instruments, to test their procedures, and to relate their analytical results to a common base; hence, their widespread use should prove fundamental for instituting the needed improvement.

Only a small proportion of clinical laboratories perform analyses for anywhere near the gamut of medically significant constituents, and although standards for virtually all of these analyses are needed, the Section is addressing itself initially to standards for bilirubin, cholesterol, creatinine, D-glucose, urea, and uric acid, because analyses for these six constituents preponderate over all others and are conducted by virtually all clinical laboratories.

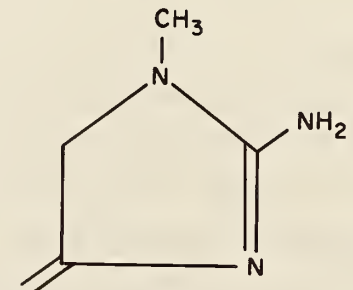
Cholesterol was the first of the standards to be undertaken, and, during the year, a provisional standard cholesterol was issued. Because the shelf-life of this standard is of

great concern, periodic re-examinations have to be carried out; those performed to date are described in this report, along with additional techniques for the determination of its purity. Substantial progress with creatinine, uric acid, and urea has been made; descriptions of the preliminary examinations of samples from several suppliers are included in this report, although work with the standards has begun since the time these reports were assembled. Our first studies on bilirubin have also begun. Work with this compound, which is well known to be exceedingly unstable, is expected to require much effort and time before a standard bilirubin can be issued. Descriptions of these early experiments are being deferred until a more comprehensive report can be made. Work toward the issuance of a clinical standard for D-glucose is expected to commence shortly. Finally, a D-fructose standard for clinical analysis has also been requested and begun. This work also is too incomplete for presentation now.

A. Creatinine

(R. S. Tipson and R. F. Brady, Jr.)

As an extension of the efforts of this Section to provide standard reference materials for clinical use, the amino acid derivative creatinine (1) is being examined. The criteria



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established by this research will be used to certify a supply of creatinine for issue as a Standard Reference Material.

Samples of creatinine of the best grade available were obtained from eight commercial sources. Each was subjected to intensive scrutiny by a variety of chromatographic, spectrophotometric, and other methods. In addition, samples of sarcosine (2), cyanamide (3), and creatine (4) were subjected to the same kind of examination. These compounds are the most likely contaminants of creatinine (1), since it is produced commercially either by the condensation of 2 with 3, or by the cyclization of 4 with hydrochloric acid.

$$CH_3NHCH_2COOH + H_2NC{\equiv}N \rightarrow 1 + H_2O$$

2 3

$$CH_3N(C(=NH)NH_2)CH_2COOH \xrightarrow{HCl} 1 + H_2O$$

4

1. Some Physical Properties of Creatinine

Creatinine is a colorless, crystalline compound, which melts with decomposition at about 300°C and is optically inactive. It is soluble in water (1 g in 11 ml at 25°C), slightly soluble in methanol or ethanol, and almost insoluble in ether, chloroform, or acetone [61]. The pH of a saturated aqueous solution (0.805 M) at 25°C is 7.8.

2. Preparation of Purified Creatinine for Comparison

Creatinine of the best commercial grade was recrystallized by dissolution in the minimal volume of distilled water, followed by precipitation with acetone [62]. It is incompletely dried at 25°C/0.1 torr over phosphorus pentaoxide in 24 hours. An elevated temperature (110°C for 24 hours) leads to complete drying.

Anal. Calc. for $C_4H_7N_3O$: C, 42.5; H, 6.2; N, 37.2. Found: C, 42.5; H, 6.0; N, 37.2; Cl, <0.02; ash, 0.02%.

3. Loss of Weight on Drying

For each commercial sample, the loss in weight on drying was less than 0.5% after 24 hours at 25°C/0.1 torr. All samples were so dried prior to quantitative study.

4. Chromatography of Creatinine

The samples of creatinine were studied by thin-layer chromatography (t.l.c.) and by paper chromatography.

a. Thin-layer Chromatography

Plates were prepared by coating glass microscope slides (75 x 25 mm) with a slurry of Kieselguhr G in distilled water; after being dried, the plates were activated by heating at 110°C for 1 hour, and were stored in a desiccator prior to use. Samples were spotted as saturated solutions in methanol, and developed in 4-oz, screw-capped jars lined with Whatman No. 1 chromatography paper. Four solvent systems were employed, namely, (a) 7:1:2 (v/v) butanol–acetic acid–water [63]; (b) 460:1:39 (v/v) butanone–diethylamine–water [64]; (c) 19:1 (v/v) methanol–water [65]; and (d) 4:1:1 (v/v) butanol–ethanol–water [66]. The R_F values of the various compounds in these systems are listed in table 6.

Table 6. R_F values in thin-layer chromatography

Solvent	Creatinine	Sarcosine	Creatine	Cyanamide
a	0.28	0.14	0.19	0.84
b	0.08	0.05	0.04	0.86
c	0.61	0.34	0.32	0.98
d	0.31	0.10	0.13	0.75

After the plates had been air-dried, the spots were made visible in one of five ways.

(1) The plates were heated on a hot plate until the material charred. Well-defined, brown spots on a clear, white background are produced in this way. Only cyanamide was not made visible by this technique.

The following indicator sprays were used as alternative methods of detecting the compounds.

(2) 5:1 (v/v) Methanol—concentrated sulfuric acid, followed by charring as above. This technique invariably produced black spots on a dark-gray background, and was inferior to method 1. Creatine and cyanamide were not detectable with this spray.

(3) Aqueous ferric chloride solution (2%) [64]. This reagent produced yellow spots on a tan background; definition of the spots was improved considerably when the plates were charred after spraying as in (1) above. This spray did not detect creatine.

(4) Chloranilic acid (0.1%) in amyl acetate [66]. This spray produced orange spots on a pale-blue background. As in other methods, the spots could be made darker by charring. The sensitivity of this reagent makes it very suitable for creatinine, but it does not make cyanamide visible.

(5) Fresh sodium picrate solution, prepared by mixing 5 volumes of saturated, aqueous picric acid solution with 1 volume of 10% aqueous sodium hydroxide solution [62]. This spray produces dark-red spots on a yellow background, and gives a positive reaction with creatinine, sarcosine, creatine, and cyanamide. The spots may be made darker by charring. This spray was considered to be the most satisfactory means of detecting the spots.

b. Paper Chromatography [62]

Ascending paper-chromatograms of creatinine were obtained on strips of Whatman No. 1 chromatography paper (40 x 4 cm) by use of the following developers: (a) 18:2:5 (v/v) butanol—acetic acid—water, R_F 0.38; and (b) 5:1 (v/v) phenol—water containing 15 mg of 8-quinolinol per 100 ml, and run in the presence of 2 M ammonia, R_F 0.93. A spray of fresh sodium picrate solution [method (5), t.l.c.] was used for detecting the spots.

c. Gas–liquid Chromatography

A sample of creatinine was trimethylsilylated (by D. H. Freeman, D. Enagonio, and C. B. Romain), and the product was examined by gas–liquid chromatography (g.l.c.). A solution of creatinine (50 mg) in 4 ml of pyridine containing 0.5 ml of hexamethyldisilazane and 4 drops of chlorotrimethylsilane was refluxed for 1.5 hours. The solution was cooled to room temperature, and 1 µl was chromatographed on a Hewlett–Packard Model 5750 vapor-phase chromatograph equipped with a stainless-steel column (0.32 x 183 cm) of 10% silicone gum-rubber (U.C.W.-98) on 80–100-mesh Chromosorb W. The column temperature was programmed from 100-300°C at 30°C/min. Helium was used as the carrier gas at a flow rate of 40 ml/min, and a flame-ionization detector was used. The chromatogram showed two main peaks, a symmetrical doublet with a mean retention time of 4.4 minutes. This is compatible with the formation of isomeric trimethylsilylated derivatives of creatinine. G.l.c. studies are being continued in order to obtain unequivocal data.

d. Results

With the exception of the gas–liquid chromatographic data, which are still under investigation, each sample of creatinine appeared to be homogeneous by each of these chromatographic methods.

5. Spectrophotometric Methods

a. Ultraviolet (u.v.) Spectrophotometry

Approximately 1 g of creatinine was accurately weighed (to 10 µg) and dissolved in water in a 1-liter volumetric flask. The u.v. spectrum of each sample in a 100-fold dilution of the original solution was recorded with a Cary Model 14 u.v. spectrophotometer. The u.v. spectra were measured, against water as the blank, from 300 to 210 nm at a scanning speed of 20 nm/min. Creatinine shows one absorption peak, at $\lambda_{max}^{H_2O}$ 234 nm (log ε 3.853) [67].

Ultraviolet spectrophotometry showed for creatine $\lambda_{max}^{H_2O}$ 190 nm (log ε 4.09), sarcosine 265 (2.32), and cyanamide 214 (2.65). The u.v. spectrum of only one of the creatinine samples showed contamination by sarcosine. No other contaminants were revealed in any of the other commercial samples by the u.v. studies.

b. Infrared (i.r.) Spectroscopy

Infrared spectra were recorded with a Perkin-Elmer Model 257 grating infrared spectrophotometer. Creatinine in a potassium bromide pellet gives a well-defined i.r. spectrum having major bands at 3260 (NH_2 asymmetric stretch), 3040 (NH_2 symmetric stretch), 2810 (CH symmetric stretch), 1688 (C=O stretch), 1665 (C=N stretch), 1588 (NH_2 asymmetric bend), 1498 (NH_2 symmetric bend), 1418 (CH_3 symmetric bend), 1330, 1242, 1210, and 1202 (doublet), 1118, 1040 (all C-C or C-N stretches), 842, 812, and 680 cm^{-1} (all NH_2 rocking). The absence of impurities in commercial samples may be inferred from the absence of bands at 1052, 908, and 905 cm^{-1} for sarcosine, and at 2260 cm^{-1} for cyanamide (the strongest bands, only, are listed for impurities); however, the absence of bands at these positions cannot be interpreted quantitatively. All of the commercial samples of creatinine showed a small band at 2260 cm^{-1}. Since cyanamide was not detected in their u.v. spectra, this peak may be attributed to a trace of cyanide ion in the creatinine. This impurity was removed by recrystallization from 4 M ammonia by addition of acetone.

As far as we are aware, the i.r. spectrum of creatinine has not been published, although it was formerly available on a punch-card, now out of print [68]. Consequently, the i.r. spectrum for the range of 2.5 to 16 μm is reproduced in Fig. 4 (see page 63).

6. Stability of Creatinine

Creatinine appears to be stable indefinitely at room temperature when stored as an anhydrous powder [62]. There

is, however, an equivocal report in the literature stating that, in solution in aqueous hydrochloric acid, or aqueous acetic acid, creatinine undergoes fairly rapid hydrolysis, at 10-50°C, to give an equilibrium mixture with creatine [69]. Therefore, in order to evaluate the stability of creatinine under routine conditions in the clinical chemistry laboratory, several typical solutions of creatinine were prepared and stored at room temperature. The u.v. spectra of the solutions were measured after the elapse of various intervals of time.

Solution 1. Creatinine (1.38863 g) was dissolved in water, and the solution was made to 1 liter with water. A 50.00-ml aliquot of this solution was diluted to 1 liter with distilled water in a second, 1-liter volumetric flask; then, a 50.00-ml aliquot of the resulting solution was diluted further to 250 ml.

Solution 2. Creatinine (1.51159 g) was dissolved in sufficient 0.1 M hydrochloric acid to make 1 liter of solution ("Stock Standard" [70]). For the u.v. measurements, a solution was prepared by diluting 10.00 ml to 1 liter with distilled water immediately before use.

Solution 3. A 1.00-ml aliquot of solution 2 was diluted to 100.00 ml with distilled water. The u.v. spectrum of this solution was measured without dilution ("Working Standard" [70]).

Solution 4. Creatinine (0.17477 g) was weighed out, transferred to a 1-liter volumetric flask, and dissolved in some distilled water. One equivalent (14.17 ml) of 0.109 M hydrochloric acid was added, and the solution was made to the mark with distilled water. For each u.v. measurement, 10.00 ml of this solution was diluted to 100.00 ml with distilled water, immediately before use.

Solution 5. A saturated solution of creatinine in methanol was prepared by weighing excess material (ca. 1 g) into a 125-ml Erlenmeyer flask, adding 80 ml of methanol,

heating the solution at 45°C under a reflux condenser for 3 hours, and allowing it to stand at room temperature. The solution was separated from the residue by filtration, and its u.v. spectrum was obtained (against methanol as the blank).

During the first 4-week period of examination, none of these solutions showed any dimunution in ε_{max}. This investigation is continuing.

7. Quantitative Determinations of Creatinine

a. Potentiometric Titration

The tertiary amine function in creatinine can be titrated with 0.1 M hydrochloric acid. The end-point is, however, very gradual and cannot be determined with standard indicators. The titration curve, determined potentiometrically, also shows an indefinite end-point; however, the first derivative of this curve, $\Delta pH/\Delta V_{H}+$, shows a sharp maximum corresponding to the end-point. (Calculation of the curve of the first derivative is facilitated if the titrant is added in small aliquots having constant volume.) Results of a triplicate analysis of one sample of creatinine by this technique are given in table 7.

Table 7. Analysis of creatinine by titration

Experiment	Mole titrated	Mole found	Error
1	0.89197	0.89489	+0.327%
2	0.99628	1.00171	+0.545%
3	0.83620	0.83821	+0.240%
		Average error	+0.371%

b. Differential Scanning Calorimetry [71-73]

The Perkin-Elmer DSC-1B Differential Scanning Calorimeter was employed as another possible means of measurement of the purity of the creatinine samples. Not unexpectedly, however, the amount of energy required for heating the sample to its decomposition temperature differed for each trial, and also for

each set of operating parameters, This variability was attributed to the decomposition of creatinine at its melting point. Hence, differential scanning calorimetry is not suitable for analysis of creatinine.

c. Phase Solubility Analysis [74]

The solubility of a material in a certain solvent is, like its melting point or index of refraction, a fundamental physical property of the material. This property is exploited in phase solubility analysis, which was employed in an attempt to obtain quantitative data on the purity of creatinine. This technique measures the amount of material dissolved when increasing amounts of the material are equilibrated in a constant volume of solvent. In saturated solutions of the material being tested, the increasing amounts of the mixture that are dissolved with increasing sample-weight are assumed to be due to the increasing amount of dissolved impurity. Thus, the amount of impurity in the sample can be determined. The solubility of creatinine in methanol, absolute ethanol, 95% ethanol, ethyl acetate, and pyridine was measured, in order to determine the most suitable solvent for the phase solubility analysis. Methanol, in which creatinine has been found soluble to the extent of 8.1 mg/g, was chosen. Sealed tubes containing weighed, progressively larger weights of creatinine and uniform weights of methanol were allowed to equilibrate in a water bath at 25.1±0.1°C. The weights of creatinine were so chosen that at least one tube would contain an undersaturated solution. After equilibration, aliquots of each solution were separated from any undissolved creatinine, evaporated to dryness, and weighed. A graph of system composition (mg of sample per g of solvent) versus solution composition (mg of solute per g of solvent), known as the phase solubility diagram, was drawn from the data. The content of solids throughout the range of saturated solutions studied was found to increase slightly. The sample of creatinine investigated was found to be about 99.0% pure by this technique. This method is general-

ly considered accurate to ±0.1%.

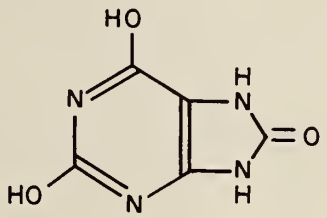
8. Creatinine as a Standard Reference Material

A large quantity of that commercial sample found to be of the highest purity is on order. This material will be examined by the useful techniques described, for issuance as a Standard Reference Material.

B. Uric Acid

(R. S. Tipson and B. F. West)

Because clinical chemists require standard samples of uric acid (1), the development of such a standard was under-



1

taken. As a preliminary, the properties of eight different commercial specimens were examined.

1. Some Physical Properties of Uric Acid

Uric acid is a colorless, crystalline compound which decomposes at about 400°C and is optically inactive. It is slightly soluble in water (ca. 1 g in 15,000 ml at 25°C), soluble in aqueous solutions of alkali hydroxides or carbonates, sodium acetate, or sodium phosphate, and almost insoluble in alcohol or ether [75]. Commercial uric acid is obtained from snake excrement or guano, by dissolution in base, followed by precipitation with acid.

As a check of solubility of samples under the conditions used in clinical chemistry, a solution containing 1 mg of uric acid per ml of solution was prepared as follows. Lithium carbonate (0.50 g) was dissolved, with stirring, in 150 ml of water at 60°C in a beaker. Uric acid (ca. 1 g) was care-

fully weighed and transferred to a 300-ml Erlenmeyer flask having a funnel in the neck. The hot solution of lithium carbonate was then added to the flask, care being taken to wash all traces of the uric acid in to the flask, and the suspension was shaken until all of the uric acid had dissolved. The solution was then cooled to room temperature, transferred to a 1-liter, volumetric flask, and rinsed in with water (to a volume of *ca.* 500 ml). Formaldehyde solution (37%; 20 ml) was added, and the mixture was shaken. Glacial acetic acid (3 ml) was added next, and the solution was shaken until evolution of carbon dioxide had ceased. The solution was then diluted to 1 liter with water, the flask was stoppered and shaken, and the solution was preserved in a refrigerator.

2. *Preparation of Purified Uric Acid for Comparison*

Purification of a commercial sample of uric acid was accomplished as follows [75]. Lithium carbonate (1.1 g) was dissolved in 75 ml of distilled water at 90°C, and to the solution was added *ca.* 2.5 g of uric acid. The suspension was stirred at 90°C until the uric acid had dissolved, and to the resulting solution were added activated carbon (Darco G-60) and Celite. The suspension was vigorously stirred, and then filtered while hot. The filtrate was cooled to room temperature, and glacial acetic acid (2.5-, 2.0-, and 4.0-ml portions, at 15-minute intervals) was slowly added dropwise, with stirring, until no more crystallization of uric acid occurred. The crystals were filtered off with suction, washed with distilled water, and briefly air-dried. Three recrystallization performed in this way afforded a sample of crystalline uric acid whose u.v. and i.r. spectra were found to be practically identical with those of the starting material (before recrystallization). The compound was dried for 24 hours at 25°C/0.1 torr.

Anal. Calc. for $C_5H_4N_4O_3$: C, 35.7; H, 2.4; N, 33.3. Found: C, 35.7; H, 2.5; N, 33.3; ash, 0.02%.

3. Loss of Weight on Drying

In order to avoid possible decomposition of the uric acid by heating, each specimen was dried to constant weight at 25°C/0.1 torr; for each commercial sample, the loss in weight was less than 0.13% after 24 hours at 25°C/0.1 torr. All samples were so dried prior to quantitative study. Oven-drying at 110°C for 18 hours causes a somewhat greater loss in weight.

4. Chromatography of Uric Acid

The samples of uric acid were studied by thin-layer chromatography and by paper chromatography.

a. Thin-layer Chromatography

Thin-layer chromatography was performed on plates (7.5 x 2.5 cm) of Silica Gel G, activated at 100°C, with the following solvent systems: (a) 1:1 (v/v) isorpropy alcohol–water; (b) water saturated with butanol; (c) butanol saturated with water; (d) 2:23 (v/v) butanol–water; and (e) 10:6:5:3 (v/v) ethyl acetate–methanol–water–heptane. The spots were made visible with iodine vapor, after the plates had been air-dried.

In each of these solvent systems, each of the commercial samples of uric acid showed the presence of only one component. The R_F values were, for solvent a, 0.53; b, 0.92; c, 0.92; d, 0.80; and e; 0.0.

b. Paper Chromatography [76]

Paper chromatography was performed by the ascending technique on strips of Whatman No. 1 chromatography paper, with the following solvent system: 9:3:2:2:4 (v/v) butanol–acetone–aqueous ammonia (5%)–acetic acid–water. An aqueous solution of each sample was spotted onto a strip. The spot was made visible under u.v. light (254 nm). Each of the commercial samples showed the presence of only one component.

c. Gas–liquid Chromatography [77]

Gas–liquid chromatography was performed by D. H. Freeman, D. Enagonio, and C. B. Romain, on a trimethylsilylated sample of uric acid. The trimethylsilyl derivative was prepared by

treating the uric acid with hexamethyldisilazane, with chlorotrimethylsilane as a catalyst, in pyridine under reflux for 1.5 hours. The analysis was performed by injection of a sample (1 μl) of this liquid. A Hewlett–Packard gas chromatograph Model 5750 was used with a stainless-steel column (0.32 x 183 cm), and the column support was Chromosorb W (80–100 mesh) with a liquid phase of 10% (w/w) silicone gum-rubber (U.C.W.-98). The column temperature was programmed from 100-300°C at 30°C/min. Helium was used as the carrier gas at a flow rate of 40 ml/min. A chart speed of 0.5 in/min was maintained, and detection was by flame ionization. A single, major peak was noted; this was well formed, with a retention time of 6.7 minutes. Two very minor peaks, having relative retention times of 0.97 and 1.08 minutes, were also observed; whether they represent minute traces of impurity in the uric acid, or artifacts formed during trimethylsilylation has not yet been decided. Now that the usefulness of this examination has been established, it will be applied routinely for study of samples of uric acid.

5. Spectrophotometric Methods

a. Ultraviolet Spectrophotometry

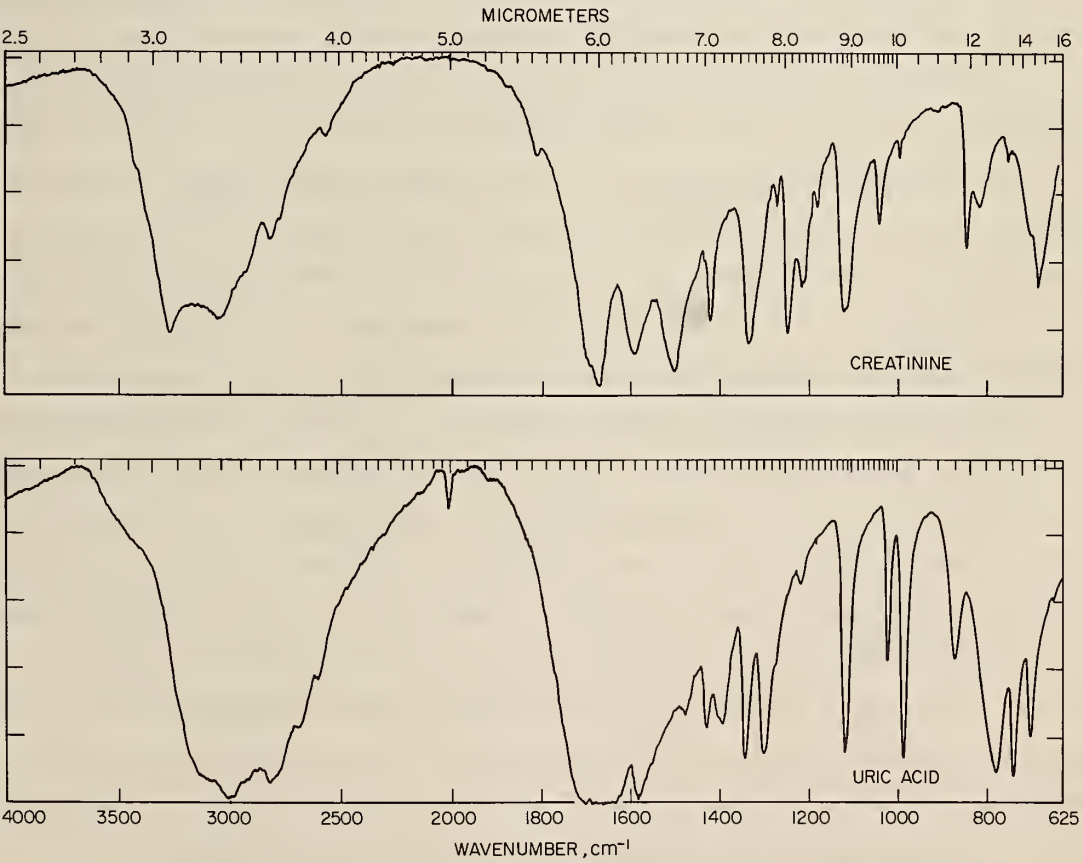
For each sample, the uric acid (0.0167 g; 94.4 mmoles) was dissolved in 2 ml of saturated lithium carbonate solution (1.52 g of lithium carbonate in 100 ml of water) at 25°C, and the solution was diluted to 100 ml with water. For recording the ultraviolet spectrum, an aliquot (1.00 ml) of this solution was diluted to 25.00 ml with distilled water. The u.v. spectrum was recorded with a Cary Model 14 spectrophotometer, against a solution of lithium carbonate as the blank, from 330 to 210 nm at a scanning speed of 20 nm/min. The purified sample of uric acid showed $\lambda_{max}^{pH\ 7.5}$ 292 nm (log ε 4.080). Commercial samples showed $\lambda_{max}^{pH\ 7.5}$ 292 nm (log ε 4.060 to 4.089).

Because the values of the extinction coefficient are recorded in the literature [75] for stated values of pH, the ultraviolet spectra were also recorded for solutions of the

les of uric acid in glycine buffer solution of pH 9.45. results for the purified sample were $\lambda_{max}^{pH\ 9.45}$ 292 nm ; ε 4.086); lit. [75] $\lambda_{max}^{pH\ 9.4}$ 292 nm (log ε 4.097). A ercial sample had $\lambda_{max}^{pH\ 9.45}$ 292 nm (log ε 4.087).

b. Infrared Spectroscopy

The infrared absorption spectrum of each sample of uric l, in a potassium bromide pellet, was recorded with a tin-Elmer Model 257 grating infrared spectrophotometer. ough the i.r. spectrum of uric acid for the range of 15 23 μm has been published [78], that for the range of 2 to m seems only to have been made available on a punchcard, out of print [79]. Consequently, the i.r. spectrum for range of 2 to 15 μm is reproduced in Fig. 4. For each



ure 4. Infrared absorption spectra of creatinine and uric acid, each in a potassium bromide pellet.

of the commercial samples, the i.r. spectrum was identical with that shown in Fig. 4. The principal bands are at 3021 (NH, OH, CH), 1675 (C=O), 1342 (NH), and 1299 cm^{-1} (NH).

6. Stability of Uric Acid

It has been reported [75] that lithium carbonate solutions of uric acid at pH 7.5 are unstable at room temperature. In order to gain information in this regard, stock solutions of uric acid were prepared according to published procedures [80], and the solutions were stored under a variety of conditions, and then examined after the elapse of various periods of time by means of their u.v. spectra.

Stock solutions of uric acid were prepared by dissolving 100 mg (accurately weighed) of uric acid and 60 mg of lithium carbonate in ca. 50 ml of water at 25°C, and diluting to exactly 100.00 ml with distilled water. Samples of stock solution in glass-stoppered Erlenmeyer flasks were kept (a) in the dark at 25°C, at 5°C (refrigerator), and at -7°C (freezer), and (b) exposed to sunlight, or to laboratory light, at room temperature (25°C). Measurements were made with 10.00-ml aliquots of each solution, diluted to 1 liter with distilled water. After 1 week, the solution exposed to sunlight showed almost complete decomposition; it was devoid of a peak at λ292 nm. Those solutions kept at 25°C (light and dark) for 1 week showed considerable diminution of ε_{292}, but those solutions stored in a refrigerator or in a freezer for 10 days remained unchanged. The decomposition might be due to microbiological action; however, this possiblity was not explored.

Consequently, it is imperative that clinical chemists who use stock solutions of uric acid should minimize the length of time that the solutions are handled at room temperature.

7. Uric Acid as a Standard Reference Material

On the basis of the foregoing tests, the most suitable supplier of uric acid was selected, and a large batch of it was ordered. This material will be subjected to many of the

same tests, before being issued as a Standard Reference Material.

C. Urea

(B. Coxon)

In order to assess the quality of urea available from commercial sources and to ascertain the suitability of any of them as a standard material, the purity of four samples of urea (samples 1, 2, 3, and 4) was examined by means of various analytical techniques. Solutions for chromatography were prepared by dissolving a portion (100 mg) of each of the urea samples in methanol (1.0 ml). These solutions were stored in a refrigerator when not in use.

1. Thin-layer Chromatography

Thin-layer chromatography (t.l.c.) was carried out by the ascending method on glass plates, either 20 x 20 cm or 20 x 5 cm, which were coated with an aqueous slurry of Silica Gel G (Merck). The plates were air-dried, but were not further activated. The solvent mixtures (i) 9:1 (v/v) chloroform–methanol, (ii) 1:1 (v/v) dichloromethane–methanol, and (iii) 1:2 (v/v) dichloromethane–methanol were tested as developers. In solvent (i), the urea barely moved from the point where it had been deposited, as indicated by a dark spot seen under ultraviolet irradiation. Solvent (iii), in which the urea had R_F 0.6, was selected as the most suitable solvent for general testing. In order to obtain an impure sample of urea for purposes of comparison, a portion of urea, sample 2, was heated at 180°C for five hours at atmospheric pressure (sample 5). Ammonia was evolved, and the material then gave a strong positive test for biuret (see p. 66).

As with samples 1-4, a 10% w/v solution of 5 was prepared. The samples were tested for the presence of biuret and cyanuric acid by chromatography against commercial samples of these compounds, which are known to be products of the pyrolysis of urea [81]. Biuret and cyanuric acid were spotted as saturated

solutions in 20% aqueous methanol. The following indicating reagents were tested.

a. 5% Sulfuric Acid

The sprayed plates were heated at 150°C. Samples 1-4 each showed as a single, colorless spot with a dark-brown corona on a light-brown background. Sample 5 showed the same spot, and also a darker, slightly faster-moving spot having the same R_F value as the spot which showed for biuret. However, the appearance of the latter spot was different, in that its front was dark and crescent-shaped. Cyanuric acid was not detected by this reagent.

b. 1% Ninhydrin in Ethanol

This reagent was found to be insensitive for urea. A faint, purple corona was produced on prolonged heating of the plate at 120°C, but this disappeared on further heating.

c. 0.2% Ninhydrin in 19:1 (v/v) Acetone–Pyridine Followed by 1% Ferric Chloride in Methanol

This combination of sprays has been recommended as the reagent of choice for the detection of mixtures of urea, hydroxyurea, O-carbamoylhydroxylamine, and (2-hydroxyethyl)urea [82]. Samples 1-3 each showed as a single, colorless spot on a yellow background; the contrast was not good, however.

d. Biuret Reagent

This was prepared by mixing a 3% solution of copper sulfate (12.5 ml) with 10% sodium hydroxide solution (500 ml) [83]. When sample 4 was mixed with biuret reagent in a test tube, a strong positive test for biuret (red coloration) was obtained. However, when t.l.c. of sample 4 on silica gel was carried out, nothing could be detected with the biuret reagent when used as a spray, either before or after heating the developed plate at 150°C for 10 min. In an effort to intensify the color, higher concentrations of reagents were used. Although sample 4 responded as a faint purple coloration when its undeveloped spot on filter paper was sprayed with saturated copper sulfate solution followed by 10% sodium hydroxide, no coloration was

observed when it was spotted on layers of silica gel. Similar results were obtained when a spray consisting of a saturated solution of copper sulfate in concentrated ammonium hydroxide was used. It appears, therefore, that the color intensity of the biuret reaction is insufficient for this reagent to be a useful indicating spray for t.l.c. on silica gel.

e. Iodine Vapor

The developed plates were kept in a closed tank containing iodine crystals. Samples 1-5 and the biuret sample showed faint, dark streaks stretching about half way along the plate from the starting point. The significance of these streaks is obscure. Samples 1-5 each showed an intense spot corresponding to urea. However, a component at this position also appeared to be present in the commercial biuret as a major impurity. The most intense spot in the chromatogram of the biuret moved slightly faster than urea, but was preceded by an additional, minor component having the same mobility as an impurity observed in the sample of cyanuric acid. Near to the starting line, the biuret chromatogram also showed a crescent-shaped spot, and the cyanuric acid showed a dark ring. Urea samples 3, 4, and 5 showed three minor components having mobilities greater than that of urea. The least mobile of these components had the same mobility as the major component of the biuret sample. The fastest-moving component travelled almost with the solvent front, and was also present in samples 1 and 2, although it was least intense in sample 2. The remaining minor component (in 3, 4, and 5) had the same mobility as cyanuric acid. Thus, it appeared that urea samples 3, 4, and 5 contained biuret and cyanuric acid as impurities, whereas the biuret sample contained an appreciable proportion of urea and some cyanuric acid. Under ultraviolet light, the iodine-detected urea showed as a dark spot.

f. 1% p-(Dimethylamino)cinnamaldehyde (DMCA) in 1:1 (v/v) 95% Ethanol–6 N Hydrochloric Acid [84]

Immediately on spraying with this reagent, urea and biuret showed as bright-red spots. Heating the sprayed plates at 70°C intensified the color, and produced white centers in the urea spots. For urea sample 2, a pale-red spot was detected at the starting line. However, this "component" was proved to be an artifact by first allowing the developing solvent to travel only one-third of the length of the plate (at which point the urea component could be seen as a white spot at about one quarter of the distance along the plate). The plate was then removed from the tank, dried, and redeveloped so that the solvent ran the full length of the plate. On spraying with DMCA, the main urea component immediately appeared as a homogeneous, red spot, and on heating at 70°C, a faint, pale-red spot appeared at the origin, plus another having an apparent R_F 0.25 at the location of the white spot that was observed following the first development. The major urea spot was not white-centered and had an apparent R_F 0.75. Biuret was not detected in sample 2 by use of spray (f).

g. 2% p-(Dimethylamino)benzaldehyde (DMB) in 4:1 (v/v) 95% Ethanol–6 N Hydrochloric Acid [84]

After being sprayed, the plates were heated at 70°C, whereupon urea and biuret appeared as bright-yellow spots. Urea sample 2 displayed a pale-yellow, artifactual spot at the starting point. However, with spray (g), biuret was not detected in this sample.

2. Paper Chromatography

Paper chromatography was performed on Whatman No. 1 filter paper by the descending technique, with 6:4:3 (v/v) butanol–pyridine–water as the developing solvent. In this system, urea had R_F 0.24, and biuret had R_F 0.37. These compounds were detected by means of sprays (f) (DMCA) and (g) (DMB) described above. DMCA was found to be a sensitive reagent for urea, producing a bright-red spot on a colorless

background immediately after spraying. On storage, this spot became pale-yellow on a dark-red background. Biuret also gave a bright-red spot with this reagent.

With the DMB reagent, urea and biuret were detected on paper as bright-yellow spots on a colorless background. On standing for several weeks, the urea spots became green, whereas the biuret spots faded to the color of the background. By means of these sprays, biuret was detected in urea samples 4 and 5, and urea was detected in the commercial sample of biuret.

3. Spectrophotometric Examination of Biuret–Copper Complex

To a portion (5.0 g) of each urea sample was added biuret reagent (5.0 ml), prepared as described above. The mixture was warmed briefly to dissolve the urea, and the solution was diluted to 10.0 ml with water. A blank solution was prepared by dilution of the biuret reagent (5.0 ml) to 10.0 ml with water. Spectra of the urea solutions in 2-cm cells were recorded with a Cary Model 14 spectrophotometer over a wavelength range of 700-300 nm, with the reagent solution as a blank in the reference beam. Each spectrum was recorded 15 min after dissolution of the urea. The following results were obtained.

Sample	λ_{max} (nm)	Absorbance
1	505	0.194
2	570	0.026
3	510	0.152
4	490	0.303
Biuret[a]	520	0.031

[a] 0.000611 g

From these data, urea sample 2 was estimated to contain 0.01% of biuret, and the other samples, proportionately larger concentrations.

4. Determination of the Purity of Urea by Differential Scanning Calorimetry (DSC)

These determinations were made with a Perkin-Elmer Differential Scanning Calorimeter Model DSC-1B [71] (see figure 5). The computational method used was that of Plato and Glasgow [73]. The DSC instrument measures the differential thermal energy required to maintain both the sample and the reference channels at the same temperature during the analysis. If the sample undergoes an endothermic transition (e.g., melting) as its temperature is increased at a constant rate, the instrument is programmed to replenish the energy absorbed by the sample by an increased energy input to the sample, so as to ensure that it is isothermal with the reference. Since the instrument recorder plots the rate of heat input with respect to time, (dq/dt) against time (t), the area under the curve obtained gives the heat of the transition directly. For samples having a definite melting point, the purity is measured by analysis of the degree of premelting observed in the plot of dq/dt against t. To compensate for the thermal lag in the sample pan of the system, it is necessary to perform a separate scan of a reference material of high purity; this is usually indium metal containing 99.9999% of indium. This reference material is also used to calibrate the relative planimeter measurements employed for analysis of the areas under the curves. The calibration depends on an independent measurement of the heat of fusion of indium. The value used in this work was 780 cal/mole.

For calculation of the purity of the sample material, the area of premelting on the curve is divided into fractions that are individually measured with a planimeter. A plot of the reciprocal of the fraction melted ($1/F$) against the absolute temperature of the sample (T) is made. According to the theoretically derived equation $T = T_o - [T_o - (T_m/F)]$, where T_o is the true melting-point of the pure sample and T_m the observed melting point, this plot should be a straight line.

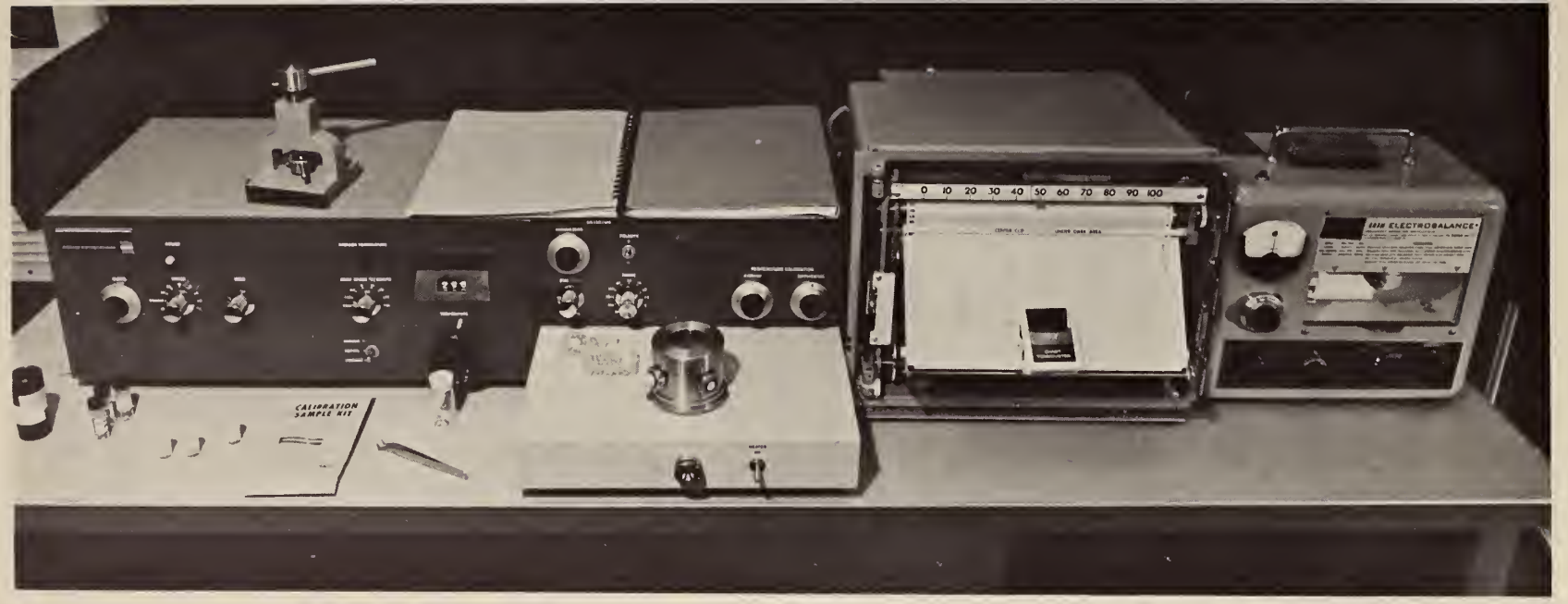


Figure 5. Differential scanning calorimeter having a sample-holder assembly and multi-range, bidirectional, linear temperature programmer with eight speeds.

However, in practice, for materials which are not of extremely high purity, it is not certain at what temperature premelting commences, and hence it is uncertain where the true base-line of the experimental scan should b located. As a consequence, a curved plot of $1/F$ against T is usually obtained, and it is necessary to apply systematic corrections to the measured areas until the plot is linearized. The true melting-point of the sample (T_o) may then be obtained from the intercept on the graph, and the melting-point depression ($T_o - T_m = \Delta T$) is given by the slope of the straight line. The mole percentage of total impurity is given by the function $100\Delta H_f \cdot \Delta T/RT_o^2$, where ΔH_f is the heat of fusion of the sample, and R is the gas constant. To evaluate this function, a measurement of ΔH_f is made. As the area under the curve has been calibrated by reference to the standard heat of fusion of indium, the heat of fusion of the sample is obtained from the total corrected area under the sample curve.

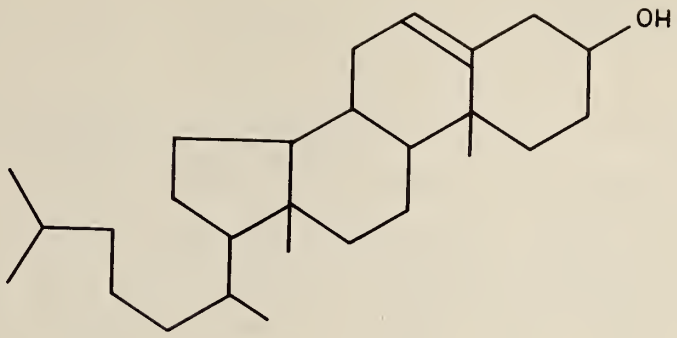
The purity of sample 2 was analyzed by this method. For such a determination, an approximately 2-mg sample of finely pulverized urea, sealed in the customary type of aluminum container, was heated rapidly to about 5°C below its melting point and then, while recording the transition, at a rate of 0.625°C/min to beyond melting. Repetition of the measurement using once-melted specimens produced inferior plots. Therefore, only initial melting patterns were analyzed. The results obtained on three different specimens of urea sample 2 are as follows.

Determin.	C	ΔT	ΔH_f (Cal/mol)	ΔH_f (J/mol)	Mole % purity
1	8.0	0.106	3,590	858	99.88
2	8.5	0.102	3,600	860	99.89
3	8.0	0.108	3,650	872	99.88

C is the percentage correction applied to the measured total area of the DSC curve, in order to linearize the plot of $1/F$ versus T.

D. Cholesterol

(A. Cohen)



The certification of a supply of cholesterol of high purity as a Standard Reference Material for use in clinical chemical analysis was undertaken at the request of the College of American Pathologists and the American Association of Clinical Chemists. The cholesterol standard was made available for issuance in December, 1967, and is the first in the series of organic chemical standards for use in clinical chemistry to be certified by the National Bureau of Standards.

1. Source of the Material

Cholesterol, isolated from brain and spinal cord of sheep, was supplied by Distillation Products Industries, Rochester, New York, where the isolated natural product was purified according to the method of Fieser [85]. This process consists of preparing crystalline cholesterol dibromide, and then regenerating the cholesterol by debromination, and crystallizing from methanol. The product was dried at room temperature under vacuum. Our recrystallizations of portions of this material did not significantly affect any of the physical properties; consequently, no further processing of the stock supply of cholesterol was carried out in our laboratory, although, for some tests, specially treated samples were prepared (as described in the relevant sections).

2. Some Physical Properties of Cholesterol

a. Melting Point

Fieser [86] has shown that the temperature at which cholesterol melts depends on the conditions employed during the measurement: in a soft-glass, capillary tube open at one end to air, melting occurs at a temperature significantly lower than for a sample in a capillary tube that is evacuated and then sealed from air. The standard cholesterol behaved in this way. Melting-point determinations at heating rates of approximately 0.5°C/min on several samples exposed to air in Pyrex capillary tubes, and repeated redeterminations (4 times) after melts had been allowed to cool and crystallize, showed a regular decrease in the temperature at which the last, minute crystals melted. With cholesterol samples sealed in capillaries after they had been evacuated to less than 1 torr, the melting of the last, minute crystals occurred at 148.7°C (corrected), and remelting of the last crystals of resolidified melts occurred at 148.7°C through several such cycles.

The melting point was also obtained by a modification of the technique described by Enagonio, Pearson, and Saylor [87], in which temperatures were measured by use of a platinum resistance thermometer calibrated in accordance with the International Practical Temperature Scale (1948). The melting point of a macro sample of the cholesterol, measured at a pressure of less than 1 torr (to exclude air) was 148.8±0.1°C. (Work performed by C. Stanley and T. W. Mears, Office of Standard Reference Materials, N. B. S.)

b. Optical Rotation

The specific optical rotation of highly purified cholesterol was reported by Fieser [86] to be -39.9° (c 1.73, chloroform); that of another sample as -39.6° (c 1.49). Our cholesterol showed $[\alpha]_D^{20}$ -40.0° (c 2.241, chloroform).

c. Elementary Analysis

Calc. for $C_{27}H_{46}O$: C, 83.87; H, 11.99. Found: C, 83.86±0.08; H, 11.98±0.09. (Determinations were performed by W. P. Schmidt and R. A. Paulson of the Analytical Chemistry Division.)

3. Spectroscopic Properties

a. Infrared Spectrum

The infrared spectrum of the cholesterol corresponded to that recorded in the Atlas of Steroid Spectra [88], which was obtained for a KBr pellet. In a mineral-oil mull, the spectrum shows bands at ν_{max}^{Nujol} 3380, 1670, 1330, 1270, 1253, 1243 (sh), 1236, 1219, 1190, 1160, 1140 (sh), 1130, 1109, 1084, 1058, 1024, 1008, 986, 960, 952, 938, 927, 885, 840, 800, 737, 723, and 700 cm^{-1}, and corresponds closely to the spectrum for the KBr pellet.

b. Ultraviolet Spectrum

A 0.92-percent solution of the cholesterol in dichloromethane was examined with a Cary Spectrophotometer, Model 14. The slit-width of the instrument reaches a maximum at 230 nm with this solvent. The spectrum of the solution showed a shoulder (ε 13.3) at 232.5 nm; otherwise, there was no marked, selective absorption, and the solution was essentially transparent from 270 to 400 nm. In absolute ethanol (0.49-percent solution), the spectrum showed no significant absorption from 230 to 400 nm.

The u.v. spectrum was also recorded for a sample of the cholesterol that had been twice sublimed. (The sublimation was conducted under a pressure of 0.03 torr with the sublimation apparatus immersed in a bath at 143°C.) The u.v. spectrum of an almost saturated solution of the sublimed cholesterol in dichloromethane showed peaks at 247, 283.5, 292.8, 309.5, and 322.5 nm, and, possibly, an additional one near 345 nm. A comparable solution of the standard showed no significant absorption at these positions. Appearance of these peaks in the spectrum of the sublimate indicated that new impurities

had been formed during sublimation.

c. Proton Magnetic Resonance Spectrum

A solution of cholesterol (0.077 g) in chloroform-d (0.5 ml) was examined by proton magnetic resonance spectroscopy at 100 MHz. The spectra were measured under frequency-swept conditions, with tetramethylsilane (τ = 10.00) as the internal reference standard and also to provide an internal, lock signal. The following signals were observed: (1) a complex multiplet (actually a sharp doublet, with additional, weak, outlying lines) centered at τ 4.65, assigned to the vinyl proton; (2) a complex multiplet at τ 6.49, assigned to the proton in the α-position to the hydroxyl group; (3) a complex envelope of peaks in the τ 7.5-9.3 region, assigned to the remaining methine and methylene protons; (4) a sharp singlet at τ 8.25 [this singlet at τ 8.25, superimposed on the envelope (τ 7.5-9.3), was assigned to the hydroxyl proton, because, on equilibration of the solution with deuterium oxide (0.4 ml), this singlet disappeared]; (5) also superimposed on the methylene envelope were five intense, sharp lines at τ 9.00, 9.05, 9.11, 9.18, and 9.32. The singlets at τ 9.00 and 9.32 were assigned to the C-19 and C-18 angular methyl groups, respectively. The three remaining lines were interpreted to be two overlapping doublets with spacings of $\sim$6 Hz, centered at τ 9.09 and 9.15. From intensity considerations, these doublets were assigned as the C-21 methyl group, and the C-26 and C-27 pair of methyl groups, respectively, with each methyl group coupled to a methine proton in the α-position.

A more concentrated solution of cholesterol (0.152 g) in chloroform-d (0.5 ml) was also examined. In the spectrum of this solution, the sharp singlet due to the hydroxyl proton occurred at lower field (τ 7.87), thus providing further evidence for its assignment. A different assignment has been given by Roberts and Casino [89], in which a doublet at τ $\sim$7.7, forming part of the methylene envelope, was assigned

to the hydroxyl proton. Since, in our work, the doublet was unchanged by equilibration of the solution with deuterium oxide, and because the hydroxyl proton could not be expected to resist deuterium exchange, it must be concluded that their assignment is incorrect. No definite signals corresponding to impurities were detected. (Spectra were recorded and interpretations made by Dr. B. Coxon of this Section.)

d. Mass Spectrum

Mass spectroscopy of the cholesterol in an Associated Electrical Industries Mass Spectrometer, Model MS-9, by use of a direct, introductory probe and heating at 150°C, revealed the presence of two very small peaks of greater mass to charge ratio than that for the cholesterol ion peak. The small peaks indicated masses of 14 and 28 mass units greater than that of the cholesterol peak; higher attenuation showed a third impurity that was 44 mass units greater than that of the cholesterol. (Measurements by H. Fales of the National Institutes of Health, Bethesda, Maryland 20014.)

4. Chromatographic Methods

a. Gas–liquid Chromatography

Solutions of the cholesterol in ethyl acetate were injected into a Glowall Corporation Chromolab 320 Gas Chromatograph fitted with a column of Gas-Chrom Q treated with 3-percent OV-17 as the stationary phase and heated at 240°C. Peaks having the following retention times relative to that of cholesterol, and the area of each peak relative to that of cholesterol, are shown in table 8.

Table 8. Gas-chromatographic behavior of the standard cholesterol

Relative retention time	Area relative to cholesterol (percent)
0.5	0.1
1.2	0.3
1.6	0.1
1.8	0.1

b. Thin-layer Chromatography

i. Survey of Chromatographic Conditions

Cholesterol dissolved in chloroform (4 mg/ml) was spotted in 8-, 16-, and 24-µg quantities on t.l.c. plates (coated with Silica Gel G, preactivated at 110°C for 1 hr). Evaporation of the chloroform at the applied spots was conducted under a jet of nitrogen gas. Chromatography was performed with a variety of developing solvents, and, after being dried with nitrogen, the plates were sprayed with 1:5 (v/v) sulfuric acid–methanol and charred on a hot plate. The conditions surveyed, and the resulting observations, are given in table 9.

ii. T.l.c. of a Degraded Cholesterol

A sample of the standard cholesterol in a tube exposed to air was heated in an oil bath at 165°C for 1.5 hr, and then dissolved in chloroform and examined by t.l.c. The conditions and results of the chromatography are shown in table 10.

iii. Artifacts in the T.l.c. of Cholesterol

Cholesterol (0.091 g) as a solution in chloroform was applied as a streak to an activated, 20 x 20 cm plate, coated 1-mm thick with Silica Gel G, the chloroform being evaporated from the streak with nitrogen. The plate was developed for 10.5 cm by use of 1:1 (v/v) benzene–chloroform, and was dried under nitrogen; the cycle was repeated 3 times. After final drying of the plate, a thin coating of silica gel was transferred from the plate by gently pressing to it a 10-inch strip of masking tape in the direction of the development and removing the tape. Spraying the transferred material with the sulfuric acid–methanol reagent, and oven-heating at 115°C, revealed the position of the cholesterol. This zone, between R_F 0.20 and 0.29, was scraped from the t.l.c. plate and extracted with chloroform; an 88-percent recovery of the cholesterol was obtained. The recovered cholesterol was crystallized from chloroform plus methanol, and collected by filtration under nitrogen. The crystallized product (24 µg) was applied

Table 9. T.l.c. data on SRM cholesterol

Developing solvent	R_F	Conditions for visibilization	Identification
Acetone	0.78	visible, uv[a]	cholesterol
	0.00	uv (faint)	artifact[b]
Acetone–benzene [1:1 (v/v)]	0.72	visible, uv	cholesterol
	0.00	uv (faint)	artifact[b]
Benzene	0.05	visible, uv	cholesterol
Benzene–chloroform [1:1 (v/v)] (3 developments)	0.26	visible, uv	cholesterol
	0.20	uv (faint)	artifact[b]
	0.09	uv (faint)	artifact[b]
	0.00	uv (faint)	artifact[b]
Benzene–ethyl acetate [1:1 (v/v)]	0.55	visible, uv	chloroform
	0.00	uv (faint)	artifact[b]
Ethyl acetate	0.67	visible, uv	cholesterol
	0.00	uv (faint)	artifact[b]
Heptane	0.00	visible, uv	cholesterol
Chloroform (1 development)	0.16	visible, uv	cholesterol
(3 developments)	0.66	uv (faint)	unknown
	0.53	visible, uv	cholesterol
	0.42	uv (faint)	artifact[b]
	0.28	uv (faint)	unknown
	0.22	uv (faint)	artifact[b]
	0.08	uv (faint)	unknown
	0.00	uv (faint)	artifact[b]

[a]Visible when illuminated with 366-nm light.

[b]Evidence given on previous page.

Table 10. T.l.c. of a degraded sample of SRM cholesterol

Developing solvent	R_F	Conditions for visibilization	Identification
Chloroform (1 development)	0.10 tailing from	visible	cholesterol
	0.10	visible	unknown
	0.00	visible	unknown (blue)
Chloroform (3 developments)	0.64	uv[a] (faint)	unknown (blue)
	0.54	uv[a] (faint)	unknown (pink)
	0.44	visible, uv	cholesterol
	0.35	uv[a] (faint)	
	0.13	visible	unknown (brown)
	0.06	visible	unknown (blue)
	0.00	uv[a] (faint)	

[a]Visible under 366-nm light.

as a spot to another plate, along with a separate spot for an equal quantity of unchromatographed standard cholesterol and a separate spot for an equal volume of the chloroform used as the solvent for applying the two samples. The plate was developed three times, as in the initial experiment. Spraying and charring showed a corresponding response with both of the cholesterol samples, and no response from the spotted solvent. The two cholesterols showed visible charring at R_F 0.29 and, under 366-nm light, showed spots at R_F 0.29, 0.18, 0.08, and 0.00. Hence, the spots at R_F 0.18, 0.08, and 0.00 were artifacts. Another plate spotted with the same materials (chromatographically purified cholesterol, standard cholesterol, and solvent only), developed three times, sprayed with antimony trichloride in chloroform, and heated for 10 min at 117°C, gave the same pattern of response.

With 160 μg of cholesterol, spotted on an activated 250-μm thick, 20 x 20 cm plate of Silica Gel G, and developed with chloroform and dried, the new position of the cholesterol was evident by its ability to reflect ordinary light. On repeating the cycle of developing and drying twice more, each new position of the cholesterol could be observed. After spraying with methanolic sulfuric acid and charring, examination under a 366-nm lamp revealed trace responses at all three of the previous positions of the cholesterol, i.e., one at the origin and the two positions where reflections were noted after the first and second developments. It is clear that the process of evaporation of the chloroform from contact with the cholesterol and the silica gel was giving rise to the artifacts.

iv. Impurities in the SRM Cholesterol by T.l.c.

Chloroform development for 10 cm of a 250-μm thick, activated, Silica Gel G-coated plate, spotted with 160 μg of the standard cholesterol, drying with gaseous nitrogen, spraying with the methanolic sulfuric acid reagent, and charring, revealed a faintly visible spot at R_F 0.28 in addition to that due to the cholesterol (R_F 0.18). Under 366-nm light, the former had a faint pink color. This ultraviolet illumination also revealed overlapping white spots at the origin (artifact) and slightly above the origin (possible impurity). Another t.l.c. plate, 20 x 20 cm, spotted with 160 μg of cholesterol, developed in chloroform, and dried (but not sprayed), was then turned at a 90° angle and re-developed in chloroform. After spraying and charring, the white spot ahead of the origin was no longer evident under 366-nm light, but the visible charred spot (R_F 0.28 in the original direction) was clearly distinguishable.

Smith and coworkers [90] have employed 1:1 (v/v) ethyl acetate–heptane as developing solvent for examining the composition of cholesterol that had undergone oxidative degrada-

tion during many years of storage. They demonstrated the presence of components such as the 7α-, 7β-, and 25-hydroxy derivatives of cholesterol, cholest-5-en-7-one, 5α-cholestane-3β,5,6β-triol, and cholesta-3,5-dien-7-one. The SRM cholesterol (50 μg), applied as a spot on a 250-μm, 20 x 20 cm, activated Silica Gel H plate, plus separate spots for 0.1-, 0.2-, and 0.3-μg quantities of the cholesterol were chromatographed with the ethyl acetate—heptane mixture. After spraying with methanolic sulfuric acid and heating for 15 min at 115°C, the spot for the 50 μg of cholesterol was visible at R_F 0.48. That for the 0.3 μg was barely visible (R_F 0.52), but a response for any lesser amounts was questionable. Under the 366-nm lamp, the 50 μg of cholesterol was found to have components at R_F 0.37 (weak), 0.26 (medium), 0.19 (weak), and 0.0 (strong); there was also faint streaking from R_F 0.26 to the origin. Under the ultraviolet lamp, spots from the 0.1-, 0.2-, and 0.3-μg quantities were all visible at R_F 0.48. The intensity of response was noticeably greatest at 0.3 μg; that from 0.1 μg was distinct. The response from the 0.2 μg of cholesterol was significantly greater than that from the component at R_F 0.26 in the chromatogram of the 50 μg of cholesterol. Also, the response from the 0.1 μg was greater than that at R_F 0.37 or at R_F 0.19. Assuming the spot at R_F 0.0 to be an artifact, the purity of the standard thus appeared to be at least 99%.

c. Paper Chromatography

Smith [91] has reported some useful techniques for the paper chromatography of cholesterol. Whatman No. 3 MM paper was used for ascending chromatography with o-chlorobenzene for development, and Whatman No. 1 paper impregnated with 2-phenoxyethanol (by dipping the paper into a 10-percent solution of the compound in acetone), with heptane for development; antimony trichloride in nitrobenzene, or a sulfuric acid—acetic anhydride—acetic acid reagent, was used for visibilization.

In our studies using the ascending technique, 8, 16, 24, and 32 μg of standard cholesterol were chromatographed along with a spot containing 120 μg of degraded cholesterol (preheated in air at 160° for 1.5 hr). On spraying with antimony trichloride, and heating in an oven at 110°C, the 24-μg and larger spots were detected. The 16-μg spot was barely detectable. Cholesterol appeared as a violet spot at R_F 0.89 in this system. The degraded cholesterol showed, in addition, a blue spot at R_F 0.41. Ultraviolet light (366 nm) brought out no additional impurities. The spray containing sulfuric acid detected only cholesterol under ordinary illumination; under 366-nm light, the degraded cholesterol showed a white region tailing to the origin from R_F 0.07.

In the ascending technique, 30-, 60-, 90-, and 120-μg quantities of the standard cholesterol were examined. Only the antimony trichloride reagent was used for detection, and only the degraded cholesterol gave evidence of an impurity, as a blue spot at $R_{cholesterol}$ 0.24.

5. Selenium Dioxide Test for Lathosterol

Fieser [86] has developed a very sensitive color test for 5α-cholest-7-en-3β-ol (lathosterol) that utilizes selenium dioxide. The test was tried according to the conditions specified. Cholesterol (1.5 mg) was dissolved in 0.5 ml of benzene, and treated at room temperature with 1.0 ml of the selenium dioxide reagent (prepared by dissolving 1.1 g of selenium dioxide in 2 ml of hot water and diluting to 100 ml with glacial acetic acid). The mixture remained colorless for over one hour. With 50 mg of the cholesterol and the same quantity of the reagent, the mixture remained colorless for over 0.5 hr. Hence, the test for lathosterol was considered negative.

6. Liebermann–Burchard Analysis

Quantitative estimations of cholesterol, based on the color produced in its reaction with the Liebermann–Burchard reagent, are frequently performed in clinical laboratories.

The reagent is a solution of sulfuric acid in acetic anhydride and acetic acid. The uniformity of response by the standard cholesterol was studied.

The clinical procedure adopted for use with the standard was the method of Ness, Pastweke, and Peacock [92]. A 0.1-percent (wt/vol) solution of the standard cholesterol in glacial acetic acid was prepared. The Liebermann–Burchard reagent was prepared from 300 ml of acetic anhydride, 150 ml of glacial acetic acid, 50 ml of sulfuric acid, and 10 g of sodium sulfate. The densities of the two solutions were determined in order to eliminate inaccuracies due to drainage from the pipettes used in their delivery; the volumes could then be determined by weighing each delivered aliquot. Approximately 2.9 ml of the cholesterol solution and 9.9 ml of the reagent were quickly weighed and combined, mixed on a vortex stirrer, transferred to a water bath at 25±0.1°C, and absorbancies were measured at 535 nm in a Cary Spectrophotometer, Model 14, after exactly 40 min reaction. For calculation of molar absorbancy, the volume of each reaction mixture was assumed to equal the sum of the calculated volume of each reactant combined. The molar absorption for 6 separate determinations was 590±1 liters/mole cm. Repetition of the analysis on another date gave a molar absorbancy of 591±3 liters/mole cm. (The uncertainties represent the standard deviation of the mean.)

7. Issuance of the Cholesterol as a Provisional Standard

The properties of the cholesterol that had been studied by November, 1967, showed the material to be at a high state of purity; none of the measurements were in conflict with this evaluation. Of the techniques employed with the cholesterol, g.l.c. was considered most satisfactory for providing a quantitative measure of its purity. Assuming that the areas of all peaks were proportional to their concentrations and that the minor peaks represent the impurities in the choles-

terol sampled, g.l.c. gave the purity as 99.4±0.3 percent, estimated at the 95% confidence level.

In light of the reported instability of cholesterol during long-term storage, periodic study of this cholesterol was planned for a 2-year period in order to determine its shelf-life as stored at NBS, and as it might be kept in a clinical laboratory. This interval would afford additional opportunity for evaluations of purity to be performed. Meanwhile, to fill the need of clinical chemists for a standard, the cholesterol was issued as a provisional standard reference material. (The material issued is packaged, under nitrogen, in brown-glass, sealed vials that are wrapped in opaque outer containers.)

8. Post-issuance Studies of the Standard Cholesterol

At several-month intervals since its issuance as a standard, samples of the standard that had been stored under various conditions were re-examined by some of the techniques used initially. Occasion was also taken to apply some additional methods for assaying its purity. Only some of the findings, obtained about 6 months after the date of issuance of the standard, are discussed.

a. Thin-layer Chromatography

Samples of the standard (a) kept packaged under nitrogen, (b) unpackaged, stored in a freezer, and (c) unpackaged, exposed to air at room temperature, were examined in 50-μg quantities by t.l.c. on a Silica Gel G plate with the ethyl acetate–heptane developing mixture used earlier. Reference quantities (0.1, 0.2, and 0.3 μg) of the packaged standard were also spotted on the plate. After spraying with methanolic sulfuric acid and heating at 115°C for 15 min, the chromatogram showed visible charring of the 0.3- and 50-μg quantities of cholesterol. Under 366-nm light, all the fractional-μg quantities spotted were evident, as well as additional spots behind the 50-μg spots of cholesterol. The latter spots appeared in the same pattern (and with the same intensities relative to each other and to the fractional-μg spots) as had been observed in

U. S. Department of Commerce
Alexander B. Trowbridge,
Secretary
National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 911
Cholesterol

This Standard Reference Material is certified as a chemical standard to aid manufacturers in meeting the specifications for cholesterol for use in clinical analysis.

This cholesterol was purified according to the method of L. F. Fieser [J. Am. Chem. Soc. 75, 5421 (1953)], which consists of preparing cholesterol dibromide, regenerating the cholesterol by reacting the dibromide with zinc dust, and crystallizing. The purity is 99.4 ± 0.3 percent.

Purity was determined by gas chromatography, thin-layer chromatography, and mass, infrared, and nuclear magnetic resonance spectroscopy, Gas-chromatographic examination, using Gas–Chrom Q treated with 3 percent OV–17 as the stationary phase at 240°C, showed impurity peaks at the following retention times relative to cholesterol:

Relative Retention Time	Area Relative to Cholesterol Peak (Percent)
0.5	0.4
1.2	0.3
1.6	0.1
1.8	0.1

Thin-layer chromatography on silica gel developed with chloroform, sprayed with sulfuric acid in methanol, and heated to 110° revealed two trace contaminants under ultraviolet irradiation. The greater contaminant appeared at the head of the cholesterol; the lesser, just beyond the origin. By two-dimensional chromatography, the greater contaminant was found as a separate spot. No quantitative values were assigned to these spots.

Response to the Liebermann–Burchard test was very uniform. A molar absorbance of 590 ± 1 at 535 nm was obtained by using 2.9 ml of 0.1 percent (wt/vol) cholesterol solution in glacial acetic acid and 9.96 ml of reagent prepared from 300 ml of acetic anhydride, 150 ml of acetic acid, 50 ml of sulfuric acid, and 10 g of sodium sulfate. The volumes in each analysis were calculated from the weights of each solution delivered and the densities. Measurements of absorbance, with the reaction performed at 25°C, were obtained 40 minutes after mixing. Molar absorbances higher than 590 could be obtained with mixtures containing less acetic acid, but the objective was to obtain high reproducibility and not to maximize the molar absorbance [Ness, Pastewka, and Peacock, Clin. Chim. Acta 10, 229 (1964)]. All measures of precision used in this certificate are estimated at the 95 percent confidence level.

WASHINGTON, D. C. 20234
October 20, 1967

W. Wayne Meinke, Chief
Office of Standard Reference Materials

(over)

The melting point of this material is 148.8 ± 0.1°C at a pressure of less than 1 mm of Hg. This value was obtained by using a modification of the technique described by Enagonio, Pearson, and Saylor [Temperature, Its Measurement and Control in Science and Industry, Vol. III, Part 1, pp. 219–230. The Reinhold Publishing Co., New York, N.Y. (1962)]. Temperatures were measured by use of a platinum resistance thermometer calibrated in accordance with the International Practical Temperature Scale (1948).

The capillary melting point is 148.7°C (corrected). The melting point is that temperature at which the last trace of crystalline cholesterol melted. The cholesterol was under a pressure of less than 1 mm of mercury, and was heated at the rate of 0.5°C per min. Solidification of the melt followed by remelting gives this same value repeatedly.

The specific rotation, $[\alpha]^{20}D$, is −40° (c 2.241, $CHCl_3$). Microchemical analysis found 83.86 ± 0.08 wt percent carbon and 11.98 ± 0.09 wt percent hydrogen. The theoretical percentages based on $C_{27}H_{46}O$ are 83.87 wt percent carbon and 11.99 wt percent hydrogen.

Mass spectroscopy revealed the presence of contaminants having masses 14 and 28 units greater than that of the cholesterol parent peak. Infrared and nuclear magnetic resonance spectroscopy did not reveal the presence of any significant impurities. The selenium dioxide test for lathosterol was negative [Fieser, J. Am. Chem. Soc. 75, 4395 (1953)].

Preliminary indications of storage life were measured by storing a sample under vacuum at 80°C for one month. No changes were noted in melting point or infrared spectrum. The purity of the cholesterol will be followed over a 2-year period. If these additional measurements indicate a significant departure from the purity as stated, users of this material will be notified.

The Standard Reference Cholesterol is packaged in 0.5–g quantities sealed under nitrogen in screw–cap vials.

Analyses were conducted by A. Cohen, D. H. Freeman, R. T. Leslie, R. A. Paulson, C. B. Romaine, R. Schaffer, and C. L. Stanley of the NBS Institute for Materials Research, and H. F. Fales of the National Institutes of Health.

the original examination of the standard cholesterol. Thus, t.l.c. provided no evidence of instability of the standard, even after exposure to air at room temperature.

b. Liebermann–Burchard Analysis

Analyses by this method were performed as previously described, and the results obtained are shown in table 11. The

Table 11. Liebermann–Burchard results with the standard cholesterol, six months after issuance

Cholesterol sample number	Storage conditions	Molar absorbance at 535 nm (liters/mole cm)
1	packaged, and stored at 25°C	595±2
2	unpackaged, and stored in freezer	597±2
3	unpackaged, but open to air at 25°C	598±2

tabulated molar absorbancies (±1 standard deviation, based on determinations with 6 aliquots per sample) are about 1 percent higher than the molar absorbance determined initially. However, a variation of this magnitude may be attributable to instrument performance and therefore cannot be considered to signify an alteration in the composition of the standard. Similarly, the differences in the tabulated values cannot in themselves be considered as showing decomposition of cholesterol that was exposed to air.

c. Differential Scanning Calorimetry

Representative samples of SRM cholesterol were examined by differential scanning calorimetry as an additional method for ascertaining purity. (Analysis by Dr. R. F. Brady, Jr.) These measurements were performed with specimens of cholesterol under a nitrogen atmosphere. A weighed quantity of cholesterol in an open aluminum dish was placed on the calori-

meter sample-pan, over which was subsequently placed a loose-fitting, hollow, hemispherical aluminum dome (ca. 1.0 cm in diameter). The reference pan, covered similarly, held an empty aluminum dish. The calorimeter cover was then locked in place, and the assembly was successively evacuated to about 30 torr with a vacuum pump, and filled to atmospheric pressure with dry nitrogen. The evacuation and filling processes were repeated six times. With the sample under one atmosphere of nitrogen, the calorimeter temperature was raised from 298 to 414°K at a rate of 80°CC/min, and then from 414 to 423°K at 0.625°C/min (see also the discussion on p. 70).

Endotherms recorded under the foregoing conditions showed little premelting and high peak amplitudes, evidences of high purity; these characteristics are in marked contrast to those of endotherms obtained when the melting transition was studied with cholesterol exposed to air. For example, using cholesterol specimens sealed in all-aluminum containers (∿0.2 cm^3 in volume) without provision for exclusion of air, the resulting endotherms exhibited wide ranges of premelting and small peak amplitudes, which indicated impurity at the 1.1 mole percent level. Presumably, the higher level of impurity calculated from these endotherms is attributable to the oxygen sealed within the sample container. (The beneficial effect of evacuating and sealing cholesterol in capillary tubes in determinations of its melting point has been discussed previously, see p. 74).

Some results obtained by differential scanning calorimetry with the standard cholesterol, six months after issuance, are shown in table 12, in which melting transitions were recorded for cholesterol samples under a nitrogen atmosphere.

Purity determinations by the DSC method are regarded as reliable to 0.1 mole percent; hence, the packaged standard appears to be more nearly at the upper limit of the 99.4±0.3 percent purity at which it was provisionally certified. The

Table 12. DSC results with the standard cholesterol, six months after issuance

Cholesterol sample number	Storage conditions	Purity (moles percent)
1	packaged, and stored at 25°C	99.84
2	unpackaged, and stored in freezer	99.77
3	unpackaged, but open to air at 25°C	99.73

difference in purity between the packaged and the exposed sample (0.11 mole percent) is essentially within this range of reliability, and therefore the difference does not provide unequivocal evidence of decomposition; however, the trend in these purity values and the trend in molar absorbancies by the Liebermann–Burchard analysis suggest that evidence of slight decomposition is indeed being detected in the cholesterol exposed to air at room temperature.

d. Phase Solubility Analysis

The packaged, standard cholesterol was also examined by phase solubility analysis, another technique that had not been used with the cholesterol during the initial study. (This work was performed by Dr. R. F. Brady, Jr.; see also p. 58.) Weighed quantities of packaged standard were allowed to equilibrate in sealed glass ampoules with equal weights of heptane (Eastman, highest purity) for 6 days at 25±0.1°C. A phase solubility diagram was constructed from the original weights mixed and the analyzed compositions of the equilibrated solutions. The purity derived from the phase solubility diagram amounted to 99.96 percent, a value that agrees very well with the 99.84 mole percent that was found by differential scanning calorimetry for the same material.

3. PROPERTIES OF POLYCYCLIC AIR-POLLUTANTS

Among the organic compounds that are common components of polluted air, having entered the atmosphere as a result of various incomplete, fuel-combustion processes, are a number of polycyclic, aromatic compounds, some of which are carcinogenic. With their continual introduction into the atmosphere, they may pose a considerable health hazard, and an understanding of the fate of these substances is a matter of concern to the Nation.

Laboratory-simulated, natural conditions of exposure were employed in determining that pyrene, as a representative of the group, is susceptible to degradation. It was found that many of the same products that resulted from this exposure could be obtained by treating pyrene with periodic acid, a reagent which had not been used previously as an oxidant of polycyclic, aromatic molecules, and the mechanism of the periodate-induced reaction of pyrene was studied. The applicability of the reagent for obtaining reference degradation compounds from other polycyclic, aromatic pollutants was also investigated.

A. Periodic Acid, A Novel Oxidant of Polycyclic Aromatic Hydrocarbons

(A. J. Fatiadi)

In this laboratory, it has been shown [93] that treatment of a solution of pyrene (1) (1 mole) in glacial acetic acid with an aqueous solution of periodic acid (1 mole) at 50°C produces 1,1'-bipyrene (2) in a yield of over 70%, the first report of a simple, one-step preparation of 2.

Evidence was obtained later [94] showing that the dimerization of 1 to 2 proceeds through a radical intermediate: the e.s.r. spectrum of this radical shown in Fig. 6 (spectrum A).

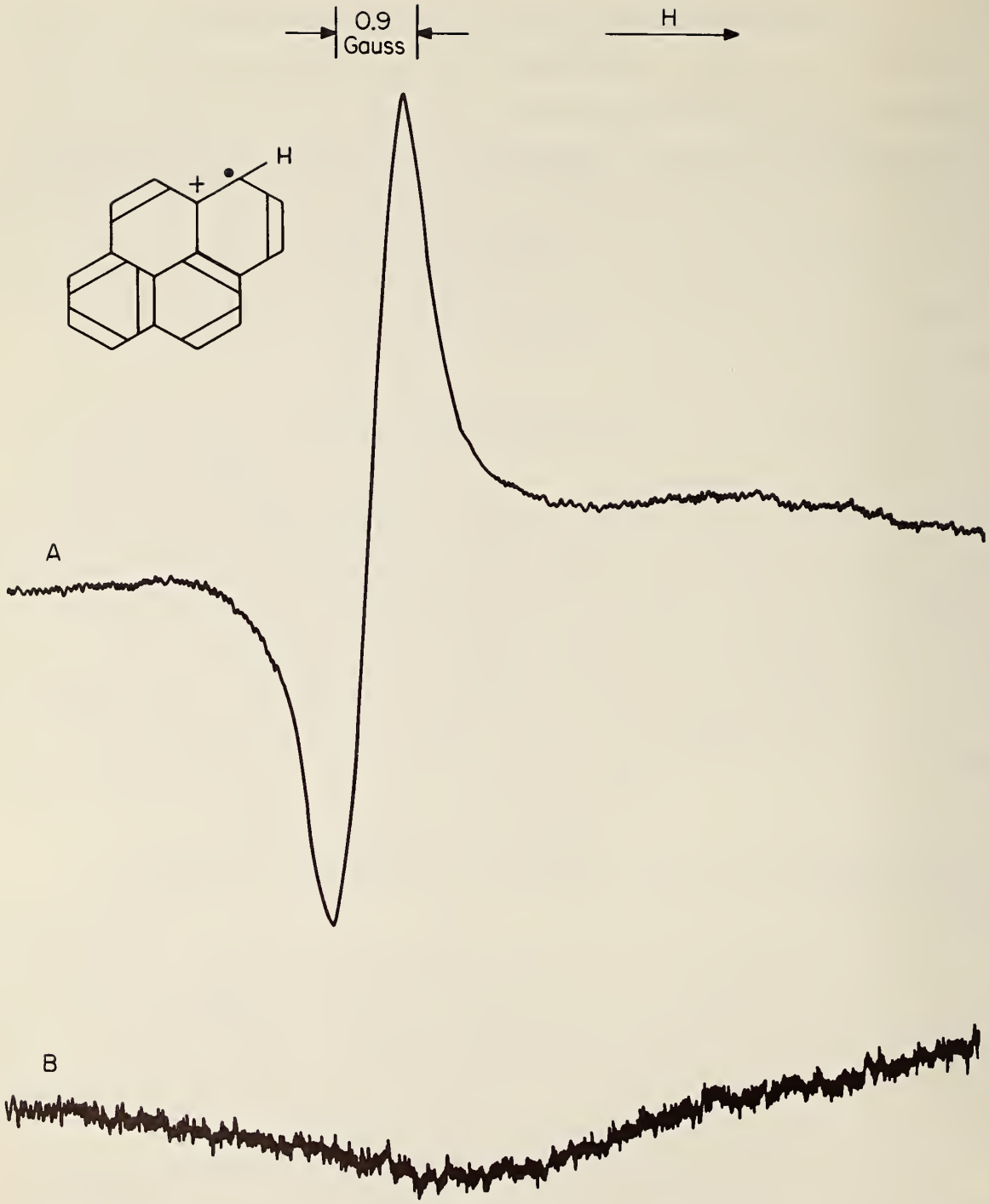
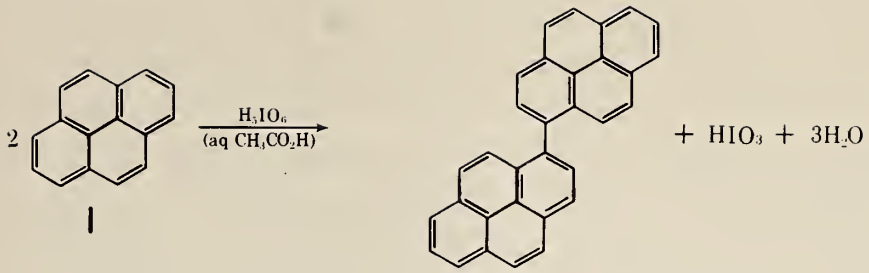


Figure 6. (A) The electron-spin resonance spectrum of pyrene radical prepared by treatment of pyrene (in acetic acid) with periodic acid, H_5IO_6 (in water) at room temperature, g = 2.0041. (B) Anthracene (in N,N-dimethylformamide) on treatment with periodic acid in water. Sample B seen under comparable conditions. No radicals were observed.



1

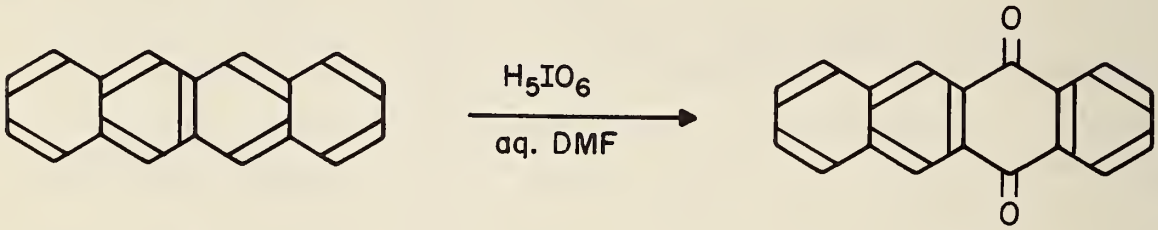
2

As has previously been shown [95], on exposure to air, warmth, and ultraviolet irradiation, pyrene (1) adsorbed on soil is converted into compound 2, as well as 1,6- and 1,8-pyrenediones plus other products. This finding was part of a study of the fate of this hydrocarbon, which occurs commonly as a constituent of polluted air. Some of these oxygenated pyrene derivatives were also found among the products of periodic acid-oxidized pyrene [93]. It was of interest to elucidate the effect of periodic acid on other polycyclic, aromatic hydrocarbons, and to determine whether other polycyclic, aromatic quinones might be obtained, as well as dimeric products. For determinations of the fate of these other aromatic pollutants, such derivatives were needed.

It was found [94,96] that, of a number of polycyclic aromatic hydrocarbons studied, many are oxidized by periodic acid in aprotic solvents containing a small proportion of water. Treatment of a solution of fluorene in glacial acetic acid with aqueous periodic acid yielded a coupling product, 1,2-bis(2,2'-biphenylylene)ethylene, in about 10% yield. Here again, the dimerization proceeds through a radical intermediate whose spectrum was recorded by e.s.r. [96]. In other hydrocarbon oxidations, however, only quinones were obtained. In contrast to the two reactions in which dimerizations were found to occur, the oxidations of these other aromatic hydrocarbons by periodic acid to quinones afforded no evidence for the presence of radical intermediates. E.s.r. monitoring of

the reaction of periodic acid with anthracene, either in aqueous acetic acid or aqueous N,N-dimethylformamide (in the range of 0 to 100°C), indicated that there is no radical participation in the oxidation (spectrum B, Fig. 6). Hence, for these oxidations, a second mechanism must be involved.

Usually, the latter group of polycyclic, aromatic hydrocarbons is oxidized to quinones in yields that range from 50 to 80%; for example, treatment of a solution of naphthacene (3) in N,N-dimethylformamide with aqueous periodic acid at 120°C for 5 minutes gave 5,12-naphthacenequinone (4) in an 80-85% yield.



3 4

By use of an approximately 1:4 molar ratio of hydrocarbon to periodic acid, the following were oxidized: anthracene in N,N-dimethylformamide, at 95°C for 5 minutes, and then 70°C for 30 minutes, to anthraquinone (91-95%); naphthalene in glacial acetic acid, at 110°C for 5 minutes and then 70°C for 30 minutes, to 1,4-naphthoquinone (70-76%); and phenanthrene in p-dioxane, at 95°C for 5 minutes and 30°C for 30 minutes, to phenanthrenequinone (50%). In the same way, benz[a]anthracene (in N,N-dimethylformamide) was oxidized to benz[a]anthracene-7,12-dione (78-82%); pentacene (in N,N-dimethylformamide) to 6,13-pentacenequinone (81-87%), and acenaphthene (in acetic acid) to acenaphthenequinone (65-70%). By use of a ca. 1:2 molar ratio of hydrocarbon to periodic acid, anthrone (in acetic acid) was oxidized to the quinone (94%). When sodium metaperiodate was used instead of periodic acid, none of these reactions were observed. Little or no reaction was observed on attempted periodic acid oxidation of biphenyl, chrysene,

coronene, perylene, picene, p-terphenyl, and triphenylene.

The mechanism for the oxidation of naphthalene (7) to 1,4-naphthoquinone (9) apparently proceeds by a two-equivalent oxidation. This mechanism involves electrophilic attack by periodic acid, as shown in 7a, with formation of intermediate 7b. Collapse of 7b by loss of iodic acid, and addition of water to the cationic center of 7b, affords the 1,4-dihydro-1,4-diol (7c), which is then rapidly oxidized by periodic acid to dione 9. The last step in this sequence of reactions is supported by the observation that 1,4-naphthalenediol (7d) is oxidized with periodic acid to dione 9 in a yield of 80%. E.s.r. monitoring of this reaction revealed no radicals, and showed a spectrum similar to spectrum B (Fig. 6).

The application of periodic acid is being extended to a study of the oxidation of nonbenzenoid, aromatic hydrocarbons. Room-temperature treatment of azulene [97] with periodic acid results in the deposition of a free-radical solid (Fig. 7, spectrum A) which is extremely stable to air and heat. A sample purified by precipitation (three times) from aqueous N,N-dimethylformamide was used in all analyses and measurements. The sample (dried at 150°C/0.05 torr) is a black, glass-like, amorphous (X-ray evidence) powder which is strongly paramagnetic, giving an e.s.r. spectrum identical with that of the crude material. Microanalysis indicated that the repeating unit of the product consists of three azulene-like moieties, and one iodine and four oxygen atoms. Magnetic susceptibility measurements indicated that the number of radicals is about 20% of the number of repeating units. Conductivity measurements in N,N-dimethylformamide suggest that the product is a weak electrolyte. The viscosity of a solution of the product (in N,N-dimethylformamide) showed an intrinsic viscosity of about 0.53 $dl \cdot g^{-1}$, which indicates that the degree of polymerization of the product may be of the order of 20—100 repeating units.

7
H_5IO_6
7a
7b
H_2O
$+ H_2O$
H_2O
7c
$+ HIO_3 + H_2O$
H_5IO_6
7d
H_5IO_6
9

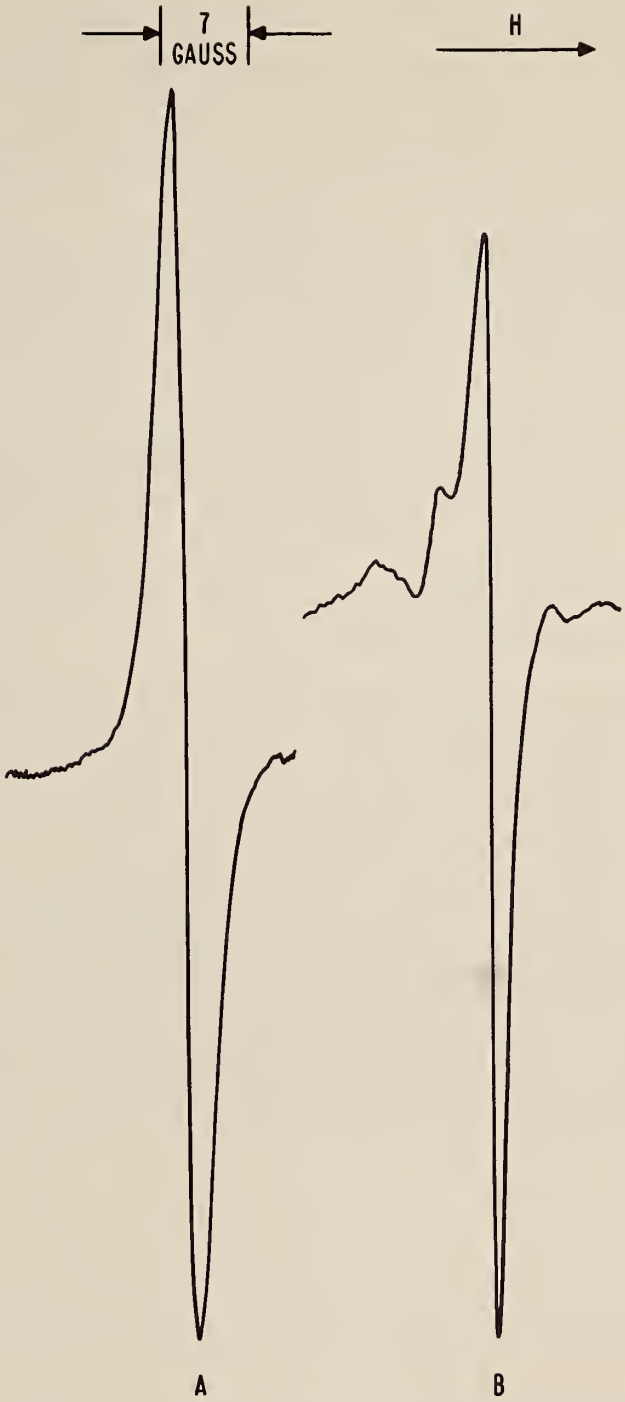


Figure 7. The e.s.r. spectrum (A) of the solid radical prepared by treatment of azulene (in acetic acid) with periodic acid, H_5IO_6, (in water) at room temperature, g = 2.0034±0.0002; the e.s.r. spectrum (B) of the solid radical obtained on treatment of azulene (in acetic acid) with conc. nitric acid at room temperature; g = 2.0087±0.001.

The product is not soluble in aqueous base and is little affected on fusion with potassium hydroxide. Its u.v. spectrum shows $\lambda_{max}^{HCONMe_2}$ at 292, 370 (sh), and 590 (sh) nm, which are spectral shifts of 90-100 nm as compared to the peaks of azulene [$\lambda_{max}^{HCONMe_2}$ 326, 351, 548, 579, 620, and 682 nm]. I.r. maxima (Nujol) were observed at 1700, and at 1590 and 1570 cm^{-1} (aromatic C=C stretchings, observed also for azulene); but the spectrum shows no significant absorption between 1400 and 625 cm^{-1} (fingerprint region).

An attempt was made to sublime a sample of the product (0.25 g) at 395°C/0.05 torr for 1 hour; the unsublimed residue (0.22 g, 88%) (Found: C, 84.0; H, 2.9; I, 9.9%) was still strongly paramagnetic and gave the same symmetrical e.s.r. spectrum (spectrum A, Fig. 7).

The stability of this radical property is shown by the fact that further heating at 485°C for 15 minutes (evolution of iodine) left a residue (Found: C, 93.5; H, 3.1; I, 2.9%) that was still paramagnetic; however, the intensity of the e.s.r. spectrum of the residue (little change in amount) had diminished considerably. Similar high stability of the radical was observed when heating was conducted in air or in oxygen-enriched air. Considering all information presented above, a polymeric, charge-transfer complex involving iodine appears to be a possible structure of this radical.

4. OTHER STANDARD REFERENCE MATERIALS PROCESSED THROUGH THE ORGANIC CHEMISTRY SECTION

A. Sucrose, Standard Reference Material 17a; Reissuance of the Standard

A specially selected supply of sucrose, provided by the California and Hawaiian Sugar Refining Corporation, was subjected to the physical testing procedures that had been employed in determining the suitability of previous samples of sucrose for certification as the standard. The optical rotatory properties, the moisture and ash content, and the reducing properties of the sucrose were found to be acceptable.

B. Dextrose, Standard Reference Material 41a; Reissuance of the Standard

Highly refined dextrose, obtained from Pfanstiehl Laboratories, Inc., was tested for the conformity of its physical properties with those of the dextrose standard previously issued. The new dextrose supply was determined to have the properties required for it to serve as the standard.

C. Nicotinic Acid, Standard Reference Material 148

Nicotinic acid, a standard to be used for the microdetermination of carbon, hydrogen, and nitrogen, is a newly issued standard. The analyses used in its certification were performed within other Sections of the Division of Analytical Chemistry, but the Organic Chemistry Section provided overall supervision. The nicotinic acid, as a new standard, is currently issued with a provisional certification.

D. Reissuance of Metallo-organic Standard Reference Materials

New supplies of lead, magnesium, and zinc cyclohexanebutyrates and silver 2-ethylhexanoate for use in the spectroscopic determination of these metals in petroleum products were studied, and found suitable as Standard Reference Materials. The lead salt, obtained from Sadtler, Research

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Alexander B. Trowbridge,
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 17a

Sucrose

Moisture	less than 0.01%
Ash	less than 0.001%
Reducing substances, estimated as invert sugar [1]	less than 0.02%

Each 100 ml of a normal sucrose solution contains 26.000 g of dried substance, weighed with brass weights in air (760 mm pressure, 20 °C, 50 percent relative humidity). At 20 °C, this solution in a 200-mm polariscope tube reads 100 °S (International Sugar Degrees). The illumination is white light filtered through a 15-mm layer of a 6-percent solution of potassium dichromate. The International Sugar Scale was defined and adopted by the International Commission for Uniform Methods of Sugar Analysis at the Eighth Session, Amsterdam, 1932 [2], [3].

The rotation in circular degrees of the normal sucrose solution observed in a 200-mm polariscope tube, for wavelength 5461 Å is 40.763° and for wavelength 5892.5 Å is 34.617°.

The specific rotations of sucrose for the normal solution are:

$$[\alpha]^{20\ °C}_{5461\ Å} = 78.342° \qquad 26 \text{ g per } 100 \text{ ml}$$

$$[\alpha]^{20\ °C}_{5892.5\ Å} = 66.529° \qquad 26 \text{ g per } 100 \text{ ml}$$

[1] F. J. Bates and R. F. Jackson, Bull. B. S. 13, 67 (1916).

[2] Proceedings of the Eighth Session, International Commission for Uniform Methods of Sugar Analysis, Intern. Sugar J. 35, 17 (1933).

[3] F. J. Bates and Associates, NBS Circular C440, pp 79, 775 (1942).

The sucrose was supplied by the California and Hawaiian Sugar Refining Corporation of Crockett, California. Chemical and physical testing to determine its suitability as a standard reference material were performed by B. S. Carpenter, B. Coxon, R. A. Paulson, R. Schaffer, and B. F. West of the Analytical Chemistry Division.

Washington, D. C. 20234
December 26, 1967

W. Wayne Meinke, Chief
Office of Standard Reference Materials

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Alexander B. Trowbridge,
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 41a

Dextrose

Ash	less than 0.01%
Moisture	less than 0.02%

The specific rotation of dextrose at equilibrium is represented by the formula

$$[\alpha]^{20\,^{\circ}C}_{5461\,\text{Å}} = 62.032^{\circ} + 0.04257^{\circ}\,c,$$

where c is grams of anhydrous dextrose weighed in vacuo and contained in 100 ml of solution. This formula is valid for values of c between 6 g and 32 g. [Bull. B. S. 13, 633 (1916) S293; NBS Circular C440, p. 83 (1942)].

$$[\alpha]^{20\,^{\circ}C}_{D} = 52.7 \qquad c = 4 \text{ g per } 100 \text{ ml.}$$

Dextrose absorbs surface moisture which can be removed by drying in vacuum at 60 to 70 °C.

The dextrose was obtained from the Pfanstiehl Laboratories, Inc. of Chicago, Illinois. Chemical and physical testing to determine its suitability for a standard reference material were performed by B. S. Carpenter, B. Coxon, R. A. Paulson, R. Schaffer, and B. F. West of the Analytical Chemistry Division.

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December 26, 1967

W. Wayne Meinke, Chief,
Office of Standard Reference Materials.

Laboratories, Inc., was issued as Standard Reference Material 1059b; the magnesium salt, suppled by Distillation Products Industries, was issued as SRM 1061b. The zinc salt, from Sadtler Research Laboratories, was issued as SRM 1073b, and the silver salt, prepared by Distillation Products Industries, was issued as SRM 1077a. Analyses used in these certifications were performed in other Sections of this Division.

5. ANALYTICAL CHEMISTRY OF CORN SYRUPS AND RELATED PRODUCTS

(D. W. Vomhof and J. H. Thomas)

A Research Associate Program, in co-operation with the Corn Industries Research Foundation, is being conducted to study some physical properties of corn syrups and their interrelationships. Of immediate concern to the industry is the relationship between refractive index, density, and dry-substance content as functions of moisture content and average molecular weight.

Corn syrups are made either by acid hydrolysis of corn starch or by partial hydrolysis with acid followed by further hydrolysis of the resultant oligomers of large molecular weight by use of any of a variety of enzymes. The syrups have a broad distribution, both with respect to molecular size and to percentage of D-glucose and malto-oligosaccharides; and different syrup types are characterized by their particular ranges of saccharide distribution. The majority of the physical properties of syrups, e.g., viscosity and volume of mixing, are dependent on their moisture content, as well as on their saccharide distributions.

Before reliable data for the evaluation of physical properties can be obtained, the moisture content of the syrups being studied must be known accurately. The present standard industrial method for directly determining the moisture content of corn syrups employs the vacuum-oven, filter-aid technique [98]. However, this method has the disadvantage of requiring almost 24 hours for obtaining a moisture determination. A more serious disadvantage inherent in the method is the variable extent of saccharide degradation that occurs at the oven temperature (100°C) during the time period required for the volatilization and removal of the water. The extent of this degradation seems to be related to the proportion of dextrose or maltose in the sample. The error may become significant when this method is used with syrups

having high contents of dextrose and maltose.

Alternatives to this method for the direct determination of the moisture content of syrups and related products are therefore being evaluated, prior to our undertaking studies of other physical properties. Requirements for a suitable, direct method of moisture analysis are accuracy, precision, freedom from interference (either by the saccharides present or the ash content), freedom from degradation or chemical action resulting in the production of volatile substances or the consumption of moisture, and, finally, reasonable rapidity.

A. The Near-Infrared Method

For obtaining moisture content, one direct method which appears to satisfy these requirements is a near-infrared spectrophotometric method specific for water. The method being investigated for its applicability to syrups is essentially that reported by Rader [99]; it depends on measurement of the absorption of energy at 1.9 μm by water molecules. The energy absorbed is directly proportional to the quantity of water present. Hydroxyl groups of saccharide molecules do not interfere.

In this method, calibration is achieved by determining the absorbance of solutions containing known concentrations of water in a suitable organic solvent. The solvents that we have employed are N,N-dimethylformamide or methyl sulfoxide. Solutions of the syrups are prepared in solvent from the same lot as that used for calibration, care being taken to insure their complete dissolution. The net absorbance is obtained by subtracting the baseline absorbance at 1.83 μm from the peak absorbance (at about 1.93 μm). The concentration of water in the sample solution is determined from the calibration curve, and the weight-percentage of water in the original syrup is calculated from the equation

$$F = (W/S) \times 10^2$$

where F = the weight-percentage of water in the syrup, W = the weight-percentage of water in the sample solution, and S = the weight-percentage of syrup in the sample solution.

The repeatability of the method is within ±0.03 weight-percent of moisture in the original syrup.

B. Thermogravimetric Analysis

Thermogravimetric analysis (TGA) is another method undergoing investigation. In this method, the loss in weight of a sample is determined as a function of increasing temperature. In its application to moisture analysis, a plateau results when the moisture in a sample has been volatilized, and before thermal decomposition has begun.

The percentage of water in a material is calculated by dividing the final weight (at the plateau) by the initial weight of the sample and multiplying the quotient by 100.

The difficulties in transferring controlled quantities of a thick syrup to the sample holder of the TGA apparatus are formidable; hence, a commercial syrup cannot be measured directly. When diluted with water, the syrup can be handled readily; however, a small loss, caused by evaporation of water from the sample during assembly of the apparatus, introduces relatively large error in the analysis.

TGA is applicable to solidified and spray-dried starch hydrolyzates, because sample loading presents fewer problems with these more readily fragmented "syrups." Since the usual, industrial, quality-control methods, such as those using refractive index or specific gravity, cannot be readily run with these types of products, TGA may prove of particular usefulness.

C. Other Methods

For the determination of moisture, methods that are in preliminary stages of evaluation for applicability to syrups are Karl Fischer titrimetric procedures and infrared spectrophotometry utilizing the 6.05-μm absorption band of water.

6. PERSONNEL AND ACTIVITIES

A. Personnel Listing

R. Schaffer, Section Chief

R. S. Tipson

B. Coxon

R. F. Brady, Jr.

A. J. Fatiadi

A. Cohen

B. F. West

D. W. Vomhof, Research Associate

J. H. Thomas, Research Assistant

B. Publications

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C. Manuscripts in Process of Publication

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A. J. Fatiadi. (Manuscript accepted for publication in J. Res. NBS)

Reaction of Some Sulfonic Esters of D-Mannitol with Methoxide; Synthesis of 2,3:4,5-Dianhydro-D-iditol.
R. S. Tipson and A. Cohen. (Manuscript accepted for publication in Carbohydrate Research)

P.M.R. Evidence for the Conformations of 1,2-O-Benzylidene-α-D-glucofuranose Derivatives.
B. Coxon. (Manuscript accepted for publication in Carbohydrate Research)

Acylation of Tetrahydroxy-p-benzoquinone.
A. J. Fatiadi. (Manuscript accepted for Journal of Chemical and Engineering Data)

Bromine Oxidation of Inositols for Preparation of Inosose Phenylhydrazones and Phenylosazones.
A. J. Fatiadi. (Manuscript accepted for publication in Carbohydrate Research)

Isotopic Methods in Carbohydrate Chemistry.
R. Schaffer. (Manuscript accepted for "The Carbohydrates," edited by W. Pigman and D. Horton, in press)

Infrared Spectroscopy of Carbohydrates.
R. S. Tipson and F. S. Parker. (Manuscript accepted for "The Carbohydrates," edited by W. Pigman and D. Horton, in press)

Esters of Benzenepentol (Pentahydroxybenzene).
A. J. Fatiadi. (Manuscript completed for Journal of Chemical and Engineering Data)

7. ACKNOWLEDGMENTS

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