NBS TECHNICAL NOTE 507

Organic Chemistry Section:

Summary of Activities July 1968 to June 1969

U.S. DEPARTMENT OF COMMERCE
NATIONAL BUREAU OF STANDARDS

NATIONAL BUREAU OF STANDARDS

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UNITED STATES DEPARTMENT OF COMMERCE
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NATIONAL BUREAU OF STANDARDS • Lewis M. Branscomb, Director

TECHNICAL NOTE 507

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Organic Chemistry Section:

Summary of Activities July 1968 to June 1969

Edited by Robert Schaffer

Organic Chemistry Section
Analytical Chemistry Division
Institute for Materials Research
National Bureau of Standards
Washington, D.C. 20234

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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 require months to perfect and yet all too often must be covered in only a line or two of a journal article.

Thus our progress reports endeavor to present this infor-
tion which we have struggled to obtain and which we feel
ght be of some help to others. Certain areas which it
pears will not be treated fully in regular publications are
nsidered in some detail here. Other results which are
ing written up for publication in the journal literature
e covered in a much more abbreviated form.

At the National Bureau of Standards publications such as
ese fit logically into the category of a Technical Note.
1969 we plan to issue these summaries for all of our
ctions. The following is the fifth annual report on pro-
ess of the Organic Chemistry Section.

W. Wayne Meinke, Chief
Analytical Chemistry Division

PREFACE

This is the fifth annual progress report of the Organic Chemistry Section of the Analytical Chemistry Division, and it describes the activities of the Section from July 1, 1968 to June 30, 1969. Unlike the formal, journal publications that emanate from the Section from time to time, this report summarizes the activities of the entire Organic Chemistry Section during that period.

Following the established pattern within this Division, the programs of the Section have both a research and a sample aspect: about half of the total effort of the Section has been applied to research, and the remainder to certification of Standard Reference Materials (SRM's). The Section must concern itself with many subjects in organic chemistry in supporting the National Bureau of Standards' overall mission, which is to contribute, where possible, to the solution of nationwide problems through the enhancement of meaningful measurement. To that end, in the past year, much of the Section's effort was directed to meeting the needs for SRM's to serve clinical chemistry laboratories. This has entailed working with organic compounds that fall into a number of classical areas of organic chemistry. Indeed, in work planned, yet other areas will be included. Under the aegis of the National Institute of General Medical Services of the National Institutes of Health, the Section is about to undertake development of the competences that will be required in enabling the Bureau to certify enzyme and other protein SRM's.

To maintain overall unity in the Section's program, considerable attention is given to one major area of organic chemistry, the carbohydrates, developing therein the Section's competences in organic analysis, characterization, and synthesis. These competences are utilized to the maximum extent for the development of SRM's.

In this report, in order to describe procedures adequately, it has been necessary occasionally to identify commercial equipment. In no case does such identification imply NBS recommendation or endorsement, nor does it imply that the equipment identified is necessarily the best available for the purpose.

Robert Schaffer, Chief
Organic Chemistry Section

TABLE OF CONTENTS

TABLE OF CONTENTS (CONT'D)

TABLE OF CONTENTS (CONT'D)

LIST OF FIGURES

FIGURE NO.

LIST OF TABLES

ORGANIC CHEMISTRY SECTION: SUMMARY OF ACTIVITIES

JULY 1968 TO JUNE 1969

Edited by Robert Schaffer

ABSTRACT

This report by the Organic Chemistry Section of the National Bureau of Standards' Institute for Materials Research summarizes a year's scientific activities; consequently, it covers both work that was completed and some that is still in progress. It reports new work on carbohydrates and polyhydroxy cyclic compounds, and analytical studies leading to (a) the development of Standard Reference Materials for clinical chemistry, (b) characterizations of trace quantities of organic compounds, and (c) understanding the properties of starch hydrolyzates.

In research on carbohydrates, rules on selective hydrolysis of certain ketose acetals, a new crystalline derivative of potential use as an SRM for "D-ribulose," conformations of compounds having m- and p-dioxane rings, and a synthesis of ^{15}N-labeled "6-amino-D-glucose" are described.

Work on polyhydroxy cyclic compounds has shown that (a) phenylosotriazoles of diketoinositols favor a half-chair conformation, (b) mercuric acetate is a superior reagent for preparing these osotriazoles, (c) acetylation in 100% phosphoric acid gives an octaacetoxydibenzo-p-dioxin from 4,6/5-trihydroxy-1,2,3-cyclohexanetrione, whereas acetylation in pyridine had been shown to produce aromatized products, (d) periodic acid oxidizes phenylhydrazino to phenylazo derivatives, and (e) phenylformazans and bis(phenylhydrazones) form highly colored salts with strong acids.

In examining a variety of commercial samples of bilirubin in the course of developing an SRM for clinical analysis, data have been obtained by several techniques, including thin-layer chromatography and thermogravimetric analysis. Visible spectrophotometry gave data on the stability of bilirubin dissolved in a variety of solvents. Measurements were made by the diazo assay. A materials-balance analysis of bilirubin samples was developed.

Various chromatographic procedures, used to examine a supply of D-glucose as an SRM, are described. Measurements of optical rotation in water and methyl sulfoxide, and other analytical data, are also given. Some of the work pertaining to the creatinine, urea, calcium carbonate, uric acid, and cholesterol SRM's that have been issued are presented.

Finally, work on the detection and identification of trace quantities of a few organic compounds is summarized, and the description and statistical evaluations of several methods developed for the characterization of the properties of corn starch hydrolyzates are summarized.

Key Words:

6-Amino-6-deoxy-D-glucose-6-^{15}N, bilirubin analysis, calcium carbonate SRM, conformations by NMR, corn syrup analysis, creatinine SRM, D-glucose (clinical SRM), organic trace analysis, SRM's for clinical analysis, urea SRM, uric acid SRM.

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

The carbohydrates continue to be the Section's area of continued research specialization in organic chemistry. As an outgrowth of our previous work with isopropylidene acetals, a study of the selective hydrolysis of such substituents located at different positions on ketoses has enabled us to formulate some generalization on their selective cleavage. Advantage was taken of the high sensitivity to hydrolysis of 1,2-linked spiro acetals in obtaining crystalline 3,4-O-isopropylidene-"D-ribulose," which should prove most useful as a stable precursor that can be easily hydrolyzed further to give the rare sugar, which is known only as a syrupy material.

Proton magnetic resonance spectroscopy is an essential technique for laboratories doing fore-front research. In our work, this spectroscopy is providing understanding of the conformations of a variety of compounds, with much attention being given heterocyclic rings having two oxygen atoms, e.g., from simple compounds containing a p-dioxane ring to such complex substances as diastereoisomeric methoxymethylidene furanose derivatives with m-dioxane rings in their structures. Some newly synthesized precursors of ^{15}N-labeled 6-amino-6-deoxy-D-glucose have also been examined by p.m.r.

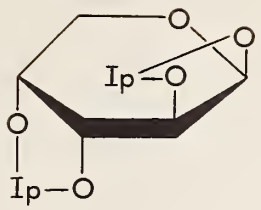
In the past few years, much effort has been devoted to the inositols and their oxidation products. This year's report reflects our continued interest in these substances; the emphasis here is the chemistry of the di- and tri-keto oxidation products and nitrogen-containing derivatives thereof.

A. Acid-catalyzed Hydrolysis of Isopropylidene Acetals of Some 2-Pentuloses and 2-Hexuloses

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

In Technical Note 457, we described the acid-catalyzed hydrolysis of some isopropylidene derivatives of ketoses. For this purpose, the use of such hydrolysts as hydrochloric acid or sulfuric acid leads to considerable decomposition of the free ketose liberated. This difficulty was overcome by use, as the hydrolyst, of oxalic acid [1]. This compound has the further advantages that (a) the anhydrous acid or its dihydrate may readily be weighed out for the preparation of an aqueous solution of any desired molarity, and (b) the hydrolysis may be rapidly arrested at any desired stage by addition of an excess of calcium carbonate, with subsequent removal of the resulting mixture of calcium oxalate and calcium carbonate by filtration. In an earlier study [2], it was found that 100 mM oxalic acid is suitable for hydrolyzing a variety of isopropylidene acetals of pentoses at 65 °C, and in determination of the rate of hydrolysis.

It has previously been shown [2] that, under these standard conditions of hydrolysis, the per-isopropylidenated aldopentoses [namely, 1,2:3,4-di-O-isopropylidene-β-L-arabinopyranose (1-e, the enantiomorph of 1), 1,2:3,5-di-O-isopropylidene-α-D-xylofuranose (2), 2,3-O-isopropylidene-α-D-xylofuranose (3), and 2,3-O-isopropylidene-D-ribofuranose (4)] are completely hydrolyzed to the corresponding free sugar within 2 hr at 65 °C, regardless of (a) the presence of a pyranoid (1) or a furanoid ring (2-4), or (b) the positions of attachment of the isopropylidene groups. However, as previously noted [2], hydrolysis of 2 to 1,2-O-isopropylidene-α-D-xylofuranose is complete in 40 min at 25 °C, but hydrolysis of 1-e to a monoisopropylidene acetal, at either 25 or 65 °C, could not be detected polarimetrically.



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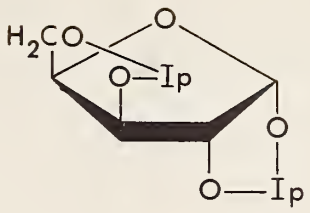

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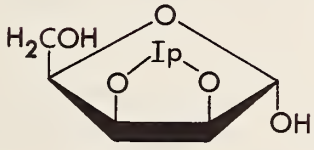

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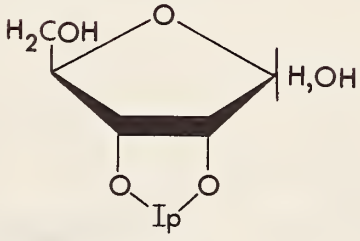
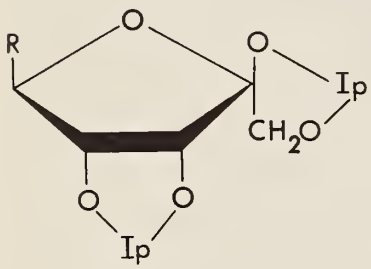


4

We have now studied the hydrolysis of 1; complete hydrolysis was effected in 1.5 hr at 65 °C, but the presence of any compound besides 1 and D-arabinose in the reaction mixture at any time could not be detected by thin-layer chromatography on Kieselguhr G (preheated for 1 hr at 110 °C) with 2:1 (v/v) ethyl acetate–pentane (detection with sulfuric acid spray, and charring). Hence, as regards acetals 1–4, the m-dioxane ring cis-fused at C-3,C-4 of 2 is much less stable than any of the cis-fused 1,3-dioxolane rings.

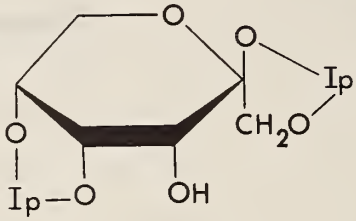
In contrast to these results for the acetals (1–4) of the aldopentoses, the sole 2-pentulose acetal for which a detailed study was made, namely, 2,3-O-isopropylidene-β-D-threo-pentulofuranose (9), required 8 hr for complete hydrolysis. We have therefore determined, by the technique

described earlier [2], the rate of hydrolysis of the diacetal of the other D-2-pentulose, namely, 1,2:3,4-di-O-isopropylidene-D-erythro-pentulofuranose (depicted as the β-D anomer 5a), and have compared it with the rates for some D-2-hexulose acetals, namely, 1,2:4,5-di-O-isopropylidene-β-D-ribo-hexulopyranose (6), 1,2:4,5-di-O-isopropylidene-β-D-erythro-2,3-hexodiulo-2,6-pyranose (7), 1,2:4,5-di-O-isopropylidene-β-D-arabino-hexulopyranose (8), and 2,3:4,5-di-O-isopropylidene-β-D-arabino-hexulopyranose (10). The β-D anomeric configuration of compounds 6 and 7 is



5a R = H

5b R = CH_2OH



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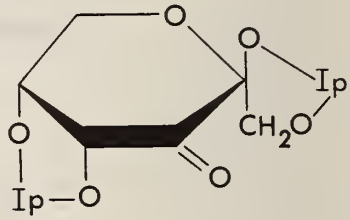

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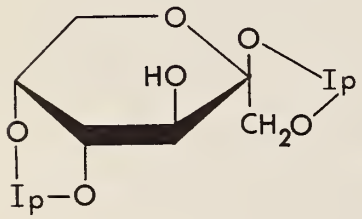

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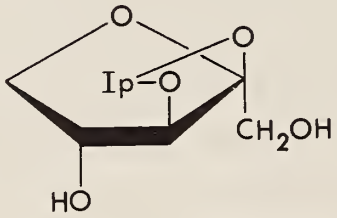

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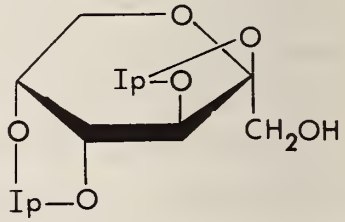


10

assigned because of their preparation [3,4] from compound 8, to which this configuration had been assigned by Ohle and

Just [5]. This anomeric configuration has now been proved by X-ray crystallographic study of a sample of compound 8 that we provided (R. D. Rosenstein and S. Takagi, University of Pittsburgh, personal communication, February 5th, 1969).

The results of hydrolysis of acetals 5a through 10, with 100 mM oxalic acid at 65 °C, are given in fig. 1; all values for $[\alpha]_D$ are calculated on the original weight of acetal. The final specific rotations, recalculated as the respective free sugar, are: for 5a, -16.6° (lit. [2] for enantiomorph, +16.6°); 6, +4.7° (lit. [6] +4.7°); 7, -43.8°; 8, -91.2° (lit. [7] -92.0°); 9, -33.2° (lit. [2] -33.2°); and 10, -90.3° (lit. [7] -92.0°).

From figure 1, it may be seen that these six compounds fall into three groups, namely, 5a (∿1.25 hr); 6, 7, and 8 (2–3 hr); and 9 and 10 (8 hr). Thus, of the four acetals (5a–8) having a spiro structure involving C-1 and C-2, the furanoid compound 5a is hydrolyzed about twice as fast as the pyranoid compounds (6, 7, and 8).

Presence of two spiro-fused, 5-membered rings in compound 5a evidently induces instability in the 1,2-isopropylidene group, because graded hydrolysis of 5a gives a monoacetal that we have isolated, and characterized as 3,4-O-isopropylidene-D-erythro-pentulofuranose, whereas graded hydrolysis of 6 (see ref. 4) and 8 (see ref. 8), in which there is less strain at C-2, causes removal of the 4,5-isopropylidene group. In contrast, the acetals 9 and 10 (that lack the spiro structure but have a hydroxymethyl group attached to C-2) are completely hydrolyzed at about one quarter of the rate found for compounds 6, 7, and 8, regardless of the presence of a furanoid (9) or a pyranoid ring (10). This behavior must be attributable to a stabilizing effect of the hydroxymethyl group at C-2 (compare 9 with 2, and 10 with 1). [Graded hydrolysis of 10 with acid causes [9] removal of the 4,5-isopropylidene group, to give a

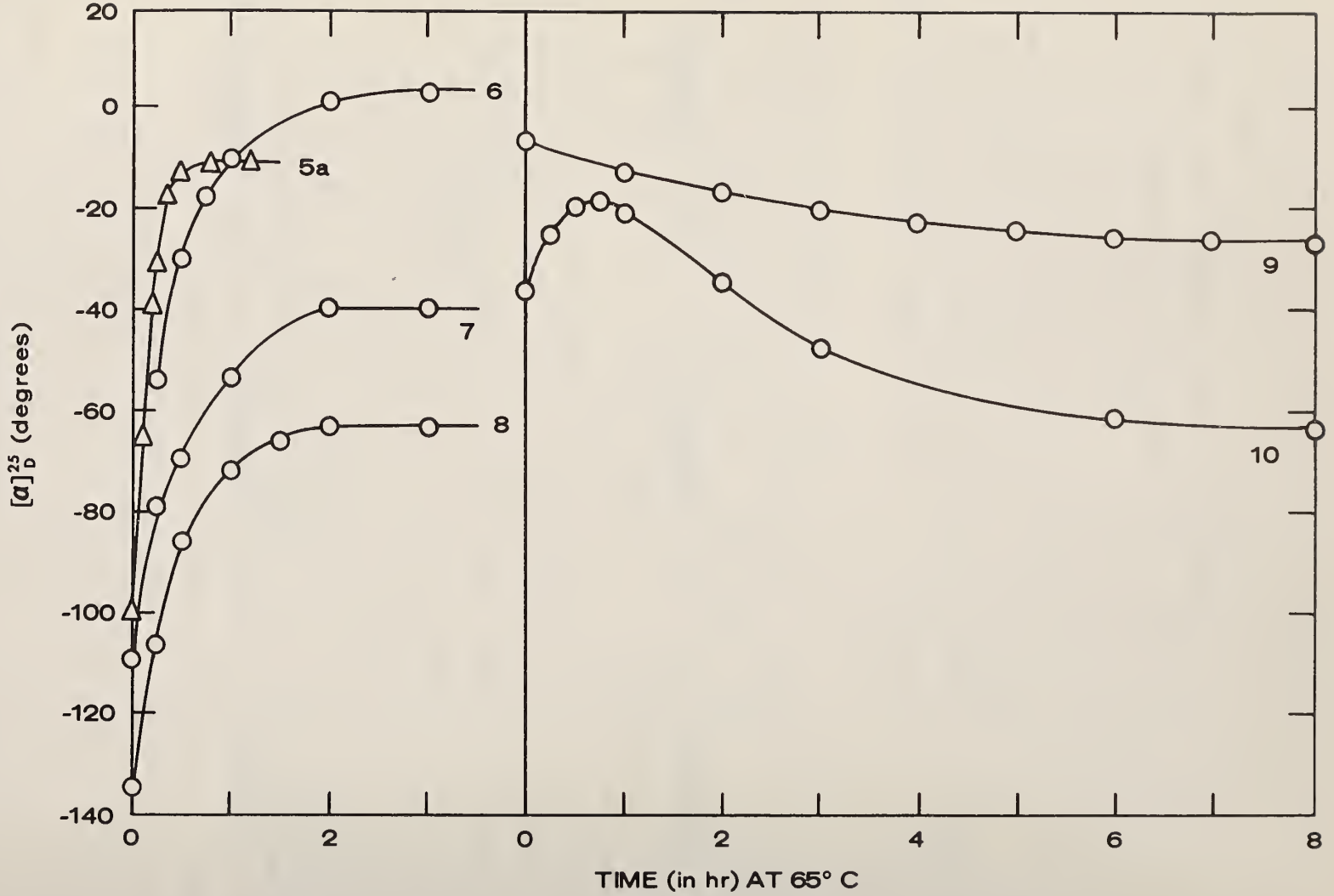
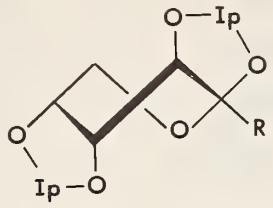


Figure 1. Rate of hydrolysis of isopropylidene acetals 5a through 10 in 100 mM oxalic acid (aqueous) at 65 °C.

pyranoid structure resembling the furanoid structure 9.]



1 R = H
10 R = CH_2OH

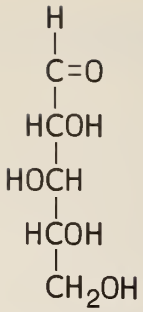
Determination of the rate of acid-catalyzed, complete hydrolysis to the free sugar, under the conditions described, therefore appears promising as a simple, rapid method for determining (a) the presence or absence of a spiro structure involving C-1 and C-2 in cyclic acetals of 2-ketoses (fast or slow hydrolysis, respectively), and (b) the presence of a furanoid ring if this spiro structure is present (very fast hydrolysis). As a check, hydrolysis of 1,2:3,4-di-O-isopropylidene-β-D-ribo-hexulofuranose (5b) was studied; it was rapid (∿50 min for completion).

This work has been published [10].

B. Improved Preparation of 1,2:3,4-Di-O-isopropylidene-D-erythro-pentulofuranose. New Derivatives of D-erythro-Pentulose

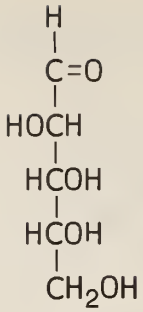
(R. S. Tipson and R. F. Brady, Jr.)

D-erythro-Pentulose (6) is a hitherto rare sugar that is an intermediate in many important biochemical processes. A crystalline derivative of D-erythro-pentulose was desired for issuance as a Standard Reference Material. Therefore, in order to prepare relatively large quantities of 6, we decided to (a) treat D-arabinose (2) with boiling pyridine [11], (b) remove the aldopentoses (1, 2, 3, and 4) from the



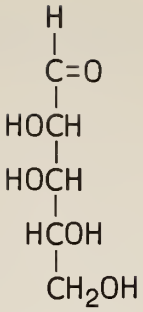
D-Xylose

1



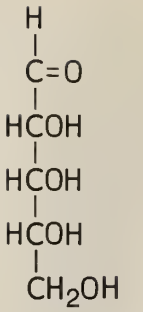
D-Arabinose

2



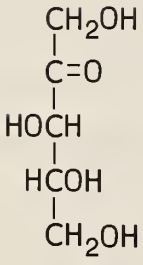
D-Lyxose

3



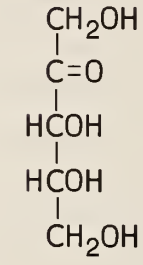
D-Ribose

4



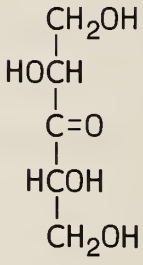
D-*threo*-Pentulose

5



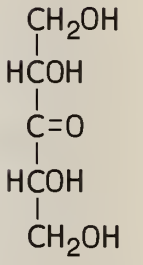
D-*erythro*-Pentulose

6



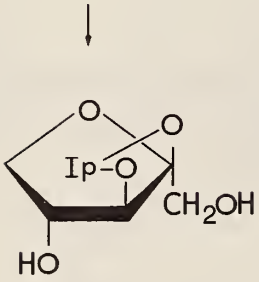
D-*threo*-3-Pentulose

7



erythro-3-Pentulose

8



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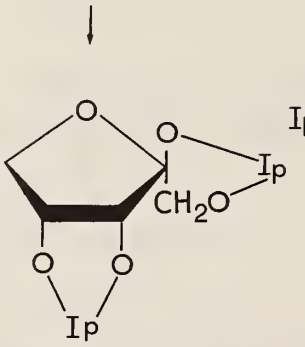

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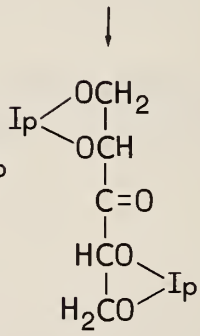

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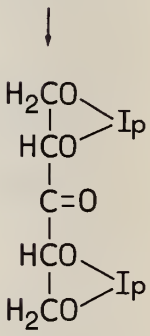

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initial reaction mixture (after removal of as much as possible of 2 by crystallization), and then (c) acetonate the mixture of pentuloses (5, 6, 7, and 8), and separate the mixture of one monoacetal (9) and three diacetals (10, 11, 12). It was found that pure 1,2:3,4-di-O-isopropylidene-D-erythro-pentulofuranose (10) is obtainable only by distillation, because the small content of diacetals 11 and 12 in the diacetal fraction is not removable by solvent partition.

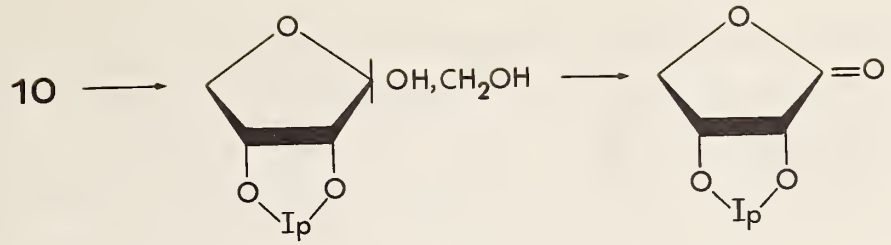
The diacetal 10 is completely hydrolyzed to D-erythro-pentulose (6) by 100 mM oxalic acid in 56 hr at 25 °C and in 72 min at 65 °C; in contrast, with 4:1 (v/v) acetic acid–water as the hydrolyst, treatment for 8 hr at 65 °C is required. In a polarimetric study of the rates of these hydrolyses, the optical rotation decreased steadily from the high value for 10 to the low (equibrium) value for 6, with no intermediate maximum or minimum; thus, polarimetric observation affords no evidence as to the intermediate formation or nonformation of a monoisopropylidene acetal of D-erythro-pentulose. Consequently, the hydrolysis was followed by t.l.c., and it was found that a monoacetal is, indeed, formed. The concentration of 10 decreases steadily, while that of 6 increases steadily; and the concentration of the monoacetal rapidly rises to a moderate value that is maintained until, near the end of the hydrolysis, the compound disappears. With this information, it then became possible to arrest the hydrolysis after the elapse of sufficient time to afford the optimal yield of monoacetal with minimal hydrolysis of the diacetal. The two acetals were then separated from free 6 by extraction with ether, and the monoacetal was separated from the diacetal (10) by extraction of the ether solution with water.

Purification of the monoacetal by distillation gave a syrup, $[\alpha]_D^{25}$ -63.0° (acetone), that was homogeneous by t.l.c. On slow crystallization from ether, one anomer, having $[\alpha]_D^{25}$

-5.4° (acetone), was isolated. Both the crystalline material and the syrup reduced Benedict solution rapidly, and both showed mutarotation in water, to give the same equilibrium rotation; consequently, one of the two free hydroxyl groups in the monoacetal is attached to C-2, the anomeric carbon atom. To determine the position of attachment of the other hydroxyl group, the monoacetal was oxidized with sodium metaperiodate; the crystalline product was identified as the known [12] 2,3-O-isopropylidene-D-erythrono-1,4-lactone (14), showing that the free hydroxyl groups in the monoacetal are attached to C-1 and C-2. Hence, the monoacetal is 3,4-O-isopropylidene-D-erythro-pentulofuranose (13), and the diacetal from which it was prepared is, as would be expected, 1,2:3,4-di-O-isopropylidene-D-erythro-pentulofuranose (10). Moreover, the properties of 14 were identical with those of authentic 14, showing that our sample of 10 was not contaminated by the corresponding diacetal of the enantiomorph of 6 (which might theoretically be formed in the isomerization with pyridine).

The optical rotation (in water) of the crystalline form of monoacetal 13 was more positive than that of the syrupy mixture of anomers, suggesting that the crystalline material is the α-D anomer. This assignment is supported by the results of p.m.r. spectroscopy; the low value for the chemical shift of the C-1 protons in the spectrum of a solution of the crystals suggests that C-1 may be trans to the 1,3-dioxolane ring fused at C-3,C-4. In addition, the appearance of the C-1 protons as a singlet fits the pattern shown by the anomeric triesters 17 and 18 (see later).

On treatment with acetic anhydride–pyridine, both the crystalline and the syrupy 13 were converted into a syrupy mixture, not yet crystallized, of two diacetates (15) that, by t.l.c., was found to contain the same two compounds, although in different proportions.



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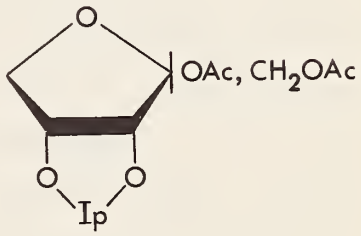

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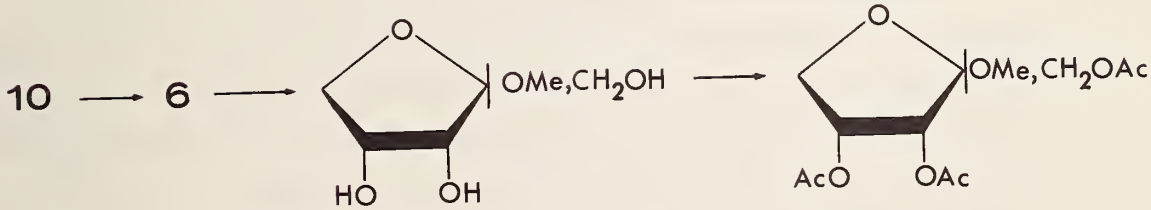

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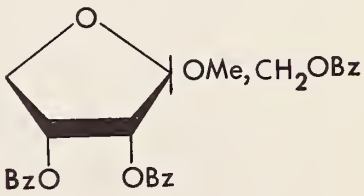

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Both the crystalline and the syrupy 13 were completely hydrolyzed to D-erythro-pentulose (6) by 4:1 (v/v) acetic acid–water in 1 hr at 65 °C. Acetylation or benzoylation of syrupy D-erythro-pentulose gave a syrupy mixture of esters, shown by t.l.c. to consist of three compounds, presumably the tetraesters of the acyclic form and of the two anomers of the furanose. Therefore, in order to obtain a less complex mixture of esters that might crystallize, a mixture of the anomers of methyl D-erythro-pentulofuranoside (16) was prepared by condensation of 6 with methanol containing 1% of hydrogen chloride; the resulting mixture was acetylated or benzoylated, yielding, in each instance, a mixture of two compounds (as shown by t.l.c.). Preparative t.l.c. of each mixture effected the isolation of each anomer; however, of the four compounds, 17, a and b; 18, a and b), only a methyl 1,3,4-tri-O-benzoyl-D-erythro-pentulofuranoside (probably the α-D anomer of 18) has thus far been obtained in crystalline form.

P.m.r. data for the D-erythro-pentulose derivatives show that the multiplicity of the signal due to the two protons on C-1 is quite definitely dependent on the configuration at the anomeric center (C-2). For the compounds presumed to be the α-D anomers, the protons on C-1 appear as a singlet, whereas, for those presumed to be the β-D anomers, they appear as two 1-proton doublets. It seems reasonable to suppose that this multiplicity is a consequence of the restricted rotation of the methylene group (C-1) when it is cis to the 3-acyloxy group. These assignments are supported by the positions of the signals caused by the anomeric methoxyl groups of the respective, acylated methyl glycosides; the signal appears at lower field for the presumed α-D anomers, in which the methoxyl group would be cis to the 3-acyloxy group. These tentative assignments agree, in each instance, with the relative, specific optical rotation of the

two anomers, and would therefore appear to permit unequivocal assignment of anomeric configuration to the anomers of compounds 17 and 18.

These results have been incorporated in an article which has been accepted for publication [13].

C. Conformational Analysis of Heterocyclic Compounds. The Favored Conformations of p-Dioxane-2,3-diol and its Diacetate

(R. F. Brady, Jr.)

1. Introduction

Relatively little is known about the favored conformations of saturated, six-membered, heterocyclic compounds. Often, such conformations have been considered to be the same as those for similarly substituted derivatives of cyclohexane, but the limited data available do not support this assumption.

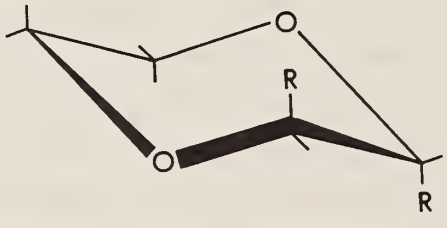
Saturated heterocyclic molecules having oxygen in the ring offer interesting variations on the cyclohexane series. A p-dioxane ring is smaller than a cyclohexane ring because the C-O bond (1.42 Å) is shorter [14] than the C-C bond (1.53 Å); this diminished ring-size may result in significant increase in steric and dipolar interactions between substituents [15] in some compounds. In addition, the polar nature of the C-O bond and of the unshared pairs of electrons on the oxygen atom may lead to dipolar interactions with polar ring-substituents. Furthermore, the C-O-C bond angle (112.5°) is slightly larger [14] than the C-C-C bond angle (111.5°), causing small differences in the related angle-bending force-constants and torsional potentials. Nevertheless, in p-dioxane, the free-energy barrier to chair inversion is lower than in cyclohexane, since distinct p.m.r. signals for axial and for equatorial protons are observed for cyclohexane [16] below -66.7 °C, but not for p-dioxane [17] even at -104 °C. The substitution of an oxygen atom for a methylene

group introduces the influence of the unshared pairs of electrons, which, apart from such consequences as van der Waals interactions [18] and London attractive forces [19], may result in the development of hydrogen bonding [20].

2. Results and Discussion

Crystalline p-dioxane-2,3-diol (1) is a bis-hemiacetal formed by the condensation of glyoxal with ethylene glycol. Only one isomer has ever been reported. The compound was first prepared by Head [21], and, by following his procedure, it has now been isolated in 37% yield.

The dipole moment of 1 in anhydrous p-dioxane (the dipole moment of which is zero [22]) is 2.18 D; this is comparable [23, 24] to the dipole moments of the trans-disubstituted compounds 2 and 3. The fact that these compounds exhibit appreciable dipole moments precludes a strict antiparallel orientation of the substituents [25], and indicates that the p-dioxane rings deviate from ideal chair-conformations due, in part, to 1,3-diaxial interactions between the substituents on C-2 (C-3) and the hydrogen atoms on C-6 (C-5). In 1, the two syn-axial interactions of a



1, R = OH

2, R = Cl

3, R = Br

4, R = OAc

hydroxyl group with a proton, in addition to the two interactions of the "axial" electron-pair on the ring-oxygen atom with an axial hydroxyl group, cause the molecule to assume a flattened-chair conformation. The observed dipole moment is too low for a cis disposition as, in this case, the two C-O bond dipoles (each 1.5 D [26]) would subtend a dihedral angle of 60° (or less) and give rise to an observed dipole moment of at least 2.6 D.

The 60-MHz p.m.r. spectrum of 1 in methyl sulfoxide-d_6 at 37 °C is shown in figure 2. The multiplet centered at 3.58δ is assigned to the methylene protons, and the multiplet centered at 4.27δ to the hydroxyl protons, and the methine protons appear as a multiplet centered at 6.30δ. The appearance of an AA'BB' pattern for the four protons on C-5 and C-6 indicates either that the molecule is held in a rigid conformation at room temperature, or that interconversion between conformations is rapid on the p.m.r. time-scale. To distinguish between these possibilities, the spectrum was measured at -7, 10, 66, 88, and 100 °C, and was found to be independent of temperature. (A 20% solution of 1 in methyl sulfoxide-d_6 solidifies below -7 °C. As an alternative solvent for 1 was not discovered, the p.m.r. spectrum could not be measured at lower temperatures.) In particular, no change was observed in the position or intensity of the lines in the methylene proton signal, and the multiplet width at 60 MHz was 58.0 ±0.1 Hz for each trial. This result indicates that 1 remains in a rigid conformation over the temperature range studied, and that there is no significant proportion of the Cee conformer present, even at 100 °C. The possibility of dynamic equilibrium involving a substantial proportion of a boat form at room temperature is also excluded, since the spectrum is unchanged at higher temperatures. This finding is noteworthy, considering that the boat form of p-dioxane is calculated [27] to have an enthalpy 9.2 kJ/mol greater

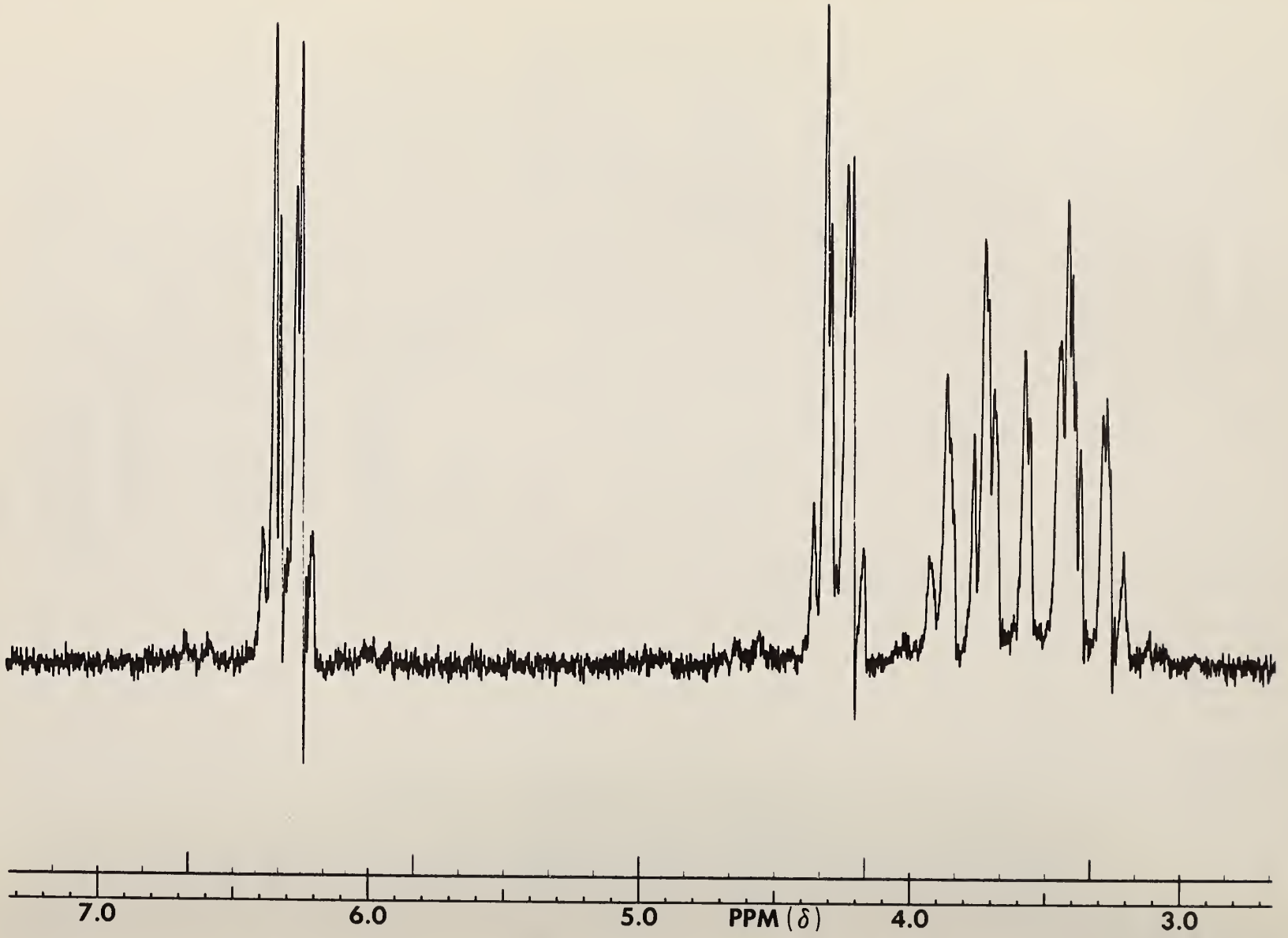


Figure 2. The p.m.r. spectrum of p-dioxane-2,3-diol at 60 MHz in methyl sulfoxide-d_6.

than that of the chair form, a difference sufficiently small to permit the existence of stable flexible forms for substituted derivatives.

The portion of the 60-MHz p.m.r. spectrum of 2,3-diacetoxy-p-dioxane (4) assigned to the protons on C-5 and C-6 is shown in figure 3 (upper trace). A computer-assisted analysis of the spectrum gave a set of coupling constants that generated the observed pattern (see figure 3). The pattern observed for the methylene protons at 100 MHz (see figure 4) was generated when the same coupling-constants and the chemical-shift difference at 100 MHz were used. The parameters obtained are given in table 1.

It may be seen from table 1 that the two vicinal coupling-constants for the trans protons have widely different magnitudes. The value of $J_{1,2}$, namely 12.24 Hz, is consistent with trans-diaxial protons, whereas that of $J_{3,4}$ (0.54 Hz) is consistent with trans-diequatorial protons. The value for the cis equatorial-axial protons is 3.39 Hz, consistent with this assignment.

The values found for the coupling constants are consistent with a flattened-chair conformation for 4. As for 1, such a conformation could be caused by the two 1,3-diaxial interactions of a proton with an acetoxyl group in the molecule. A similar conformation has been suggested [28] for trans-2,3-dichloro-p-dioxane (2); thus, it is noteworthy that an independent determination [29] of the coupling constants of 2 gave values that agree within ±0.2 Hz with those determined here for 4.

The p.m.r. spectrum of 4 was also recorded at -17, 10, 50, 74, 80, and 100 °C. Line positions and intensities remained constant over the entire range of temperature studied, and, in each trial, the width of the multiplet assigned to the methylene protons was 67.7 ±0.1 Hz at 60 MHz. These data are firm proof for the existence of only one conformation over the temperature range studied, as there is

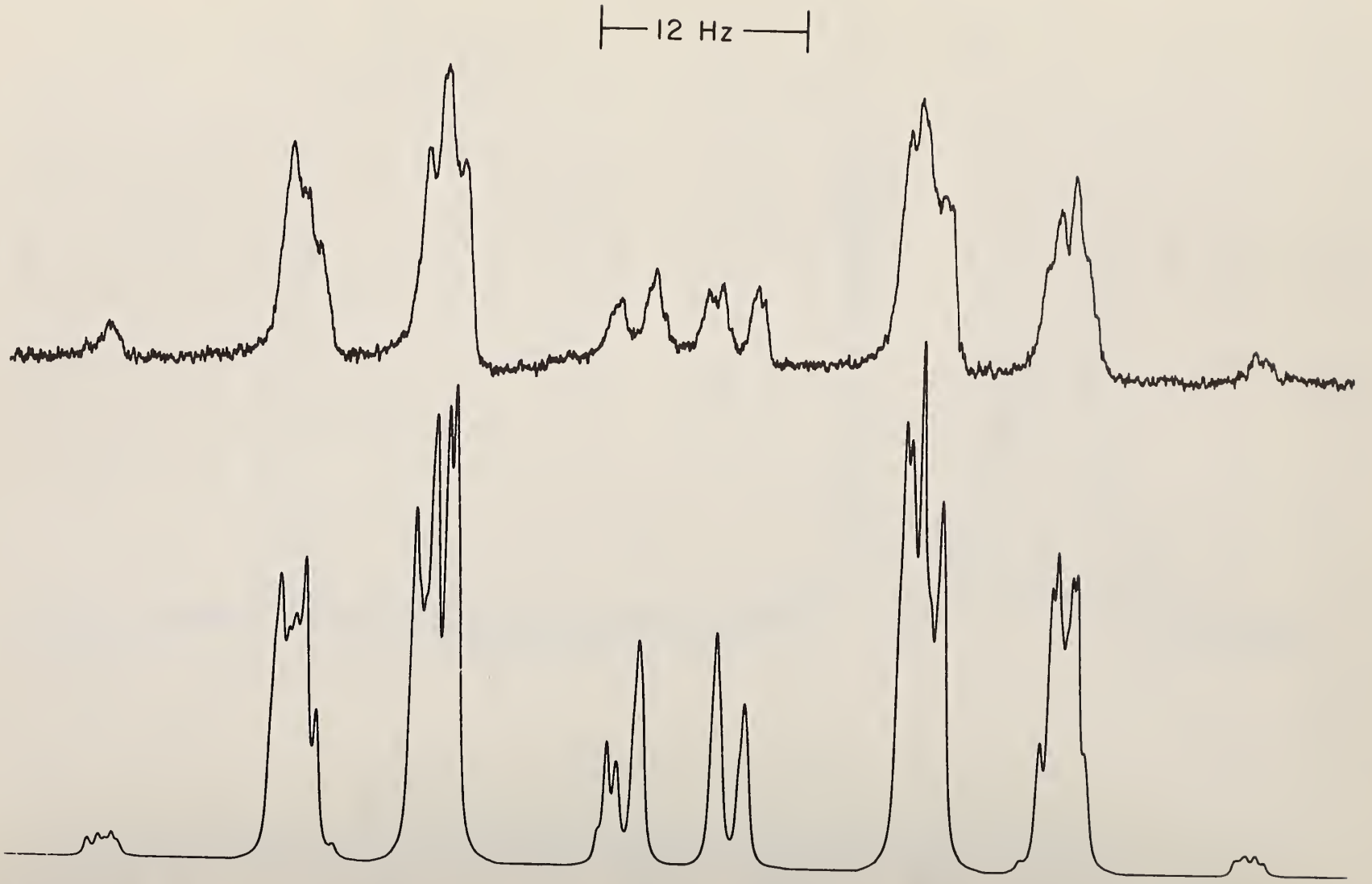


Figure 3. The observed (upper) and calculated (lower) p.m.r. spectra of the protons on C-5 and C-6 in 2,3-diacetoxy-p-dioxane at 60 MHz in acetone-d_6.

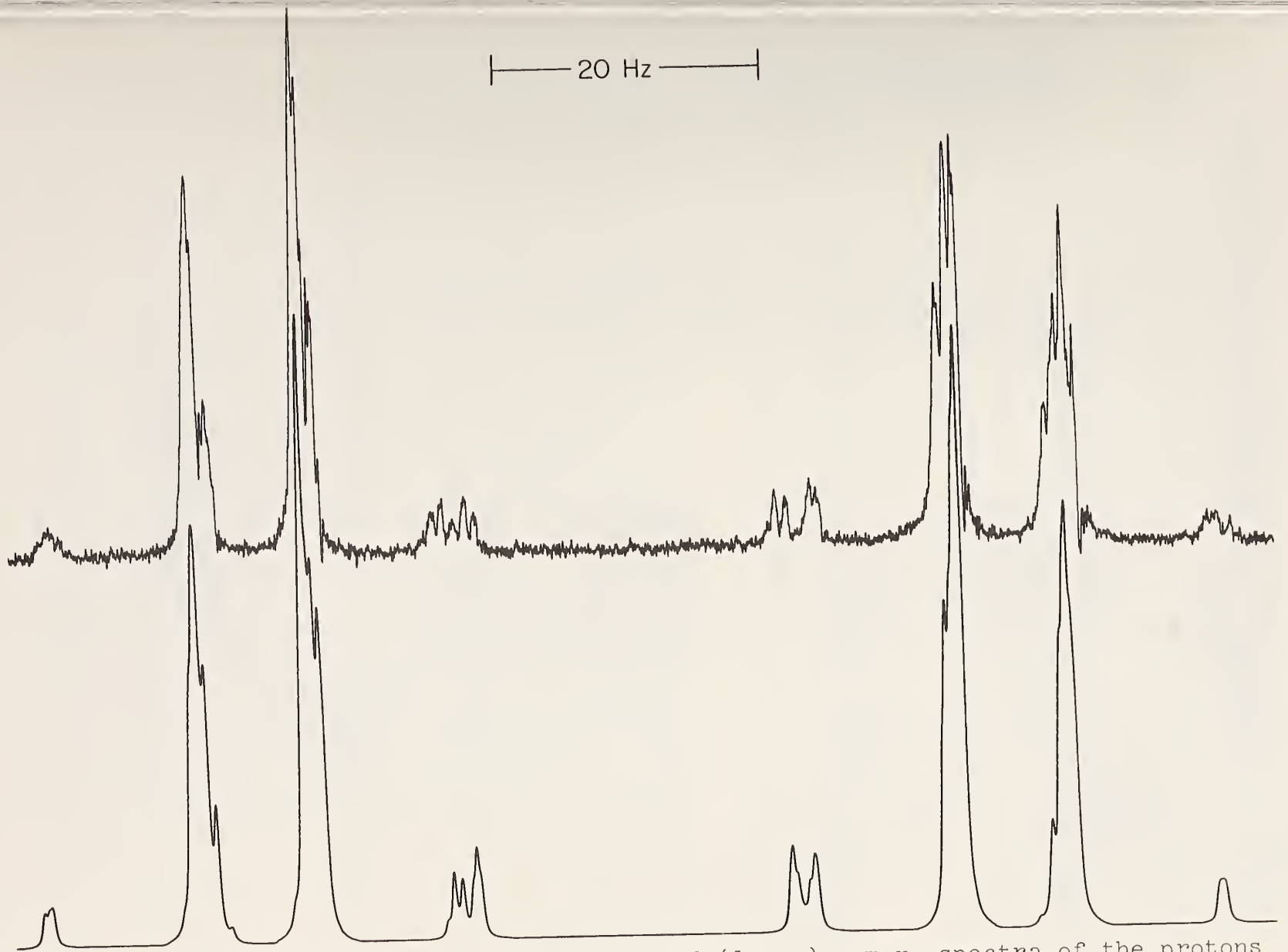
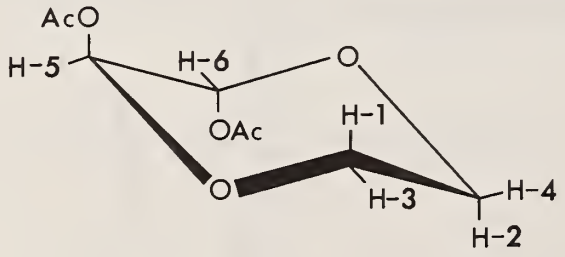


Figure 4. The observed (upper) and calculated (lower) p.m.r. spectra of the protons on C-5 and C-6 in 2,3-diacetoxy-p-dioxane at 100 MHz in acetone-d_6.

Table 1. Chemical shifts and proton–proton coupling-constants for 2,3-diacetoxy-p-dioxane (4a) in acetone-$\underline{d}_6$

Radio-frequency (MHz)	Chemical shifts[a] (Hz)	
60	$\nu_1 = \nu_2$	212.5
	$\nu_3 = \nu_4$	246.5
	$\nu_5 = \nu_6$	342.5
100	$\nu_1 = \nu_2$	354.3
	$\nu_3 = \nu_4$	411.0
	$\nu_5 = \nu_6$	570.9
	Coupling constants (Hz)	
	$\underline{J}_{1,2}$	12.24
	$\underline{J}_{1,3} = \underline{J}_{2,4}$	-12.31
	$\underline{J}_{1,4} = \underline{J}_{2,3}$	3.39
	$\underline{J}_{1,5} = \underline{J}_{2,6}$	-0.63
	$\underline{J}_{1,6} = \underline{J}_{2,5}$	0.04
	$\underline{J}_{3,4}$	0.54
	$\underline{J}_{3,5} = \underline{J}_{4,6}$	0.67
	$\underline{J}_{3,6} = \underline{J}_{4,5}$	0.00
	$\underline{J}_{5,6}$	-0.76

[a]Downfield from tetramethylsilane (ν 0.00 Hz) as the internal standard.



4a

no change in the p.m.r. spectrum at extreme temperatures. As for 1, the presence of a significant proportion of a boat form at room temperature is also excluded.

3. Conclusions

A flattened-chair conformation, with both substituents taking axial orientations, is favored for p-dioxane-2,3-diol and 2,3-diacetoxy-p-dioxane. The 1,3-diaxial interactions arising in each instance are lessened by the slight flattening of the p-dioxane ring.

D. Studies of ^{15}N-Labeled Amino Sugars. Synthesis and Spectroscopy of Derivatives of 6-Amino-6-deoxy-D-glucose-6-^{15}N

(B. Coxon)

1. Introduction

As part of a program for the synthesis, and spectral and conformational characterization, of isotopically labeled carbohydrate reference-materials of biomedical interest, a study of amino sugars labeled with ^{15}N (enrichment >99%) has been commenced. This work has been initiated by a synthesis of derivatives of 6-amino-6-deoxy-D-glucose-6-^{15}N, which, in its nonlabeled form, occurs as a component of the antibiotic Kanamycin [30].

2. Synthetic Methods

A suitable starting point for the synthesis was 1,2:3,5-di-O-isopropylidene-6-O-p-tolylsulfonyl-α-D-glucofuranose (1) [31]. Treatment of 1 with sodium iodide in 2-butanone gave an alternative starting-material, namely, 6-deoxy-6-iodo-1,2:3,5-di-O-isopropylidene-α-D-glucofuranose (2) [32]. Reaction of 1 or 2 with potassium phthalimide gave 6-deoxy-1,2:3,5-di-O-isopropylidene-6-phthalimido-α-D-

glucofuranose (3). After two recrystallizations, 3 had a purity of 99.5 mole %, as determined by differential scanning calorimetry [33].

Treatment of 3 with hydrazine gave 6-amino-6-deoxy-1,2:3,5-di-O-isopropylidene-α-D-glucofuranose (4), which was characterized as its known, crystalline salt with p-toluenesulfonic acid [34]. The free amine (4) was also converted into its N-acetyl derivative (5).

Reaction of 1 with potassium phthalimide-^{15}N afforded 3-^{15}N in 84% yield.

3. Magnetic Resonance Parameters

The magnetic resonance parameters of compounds 3–5 and their ^{15}N-labeled modifications have been studied initially by p.m.r. spectroscopy at 100 MHz. Spectra of the N-acetyl

Figure 5. A facility for computer-assisted analysis of n.m.r. spectra.

derivatives 5 and 5-^{15}N in pyridine-d_5 are shown in figure 6 (a and b, respectively). It may be seen that, even in the presence of a basic solvent, NH proton exchange is sufficiently slow to allow observation of the $J_{6,NH}$ and $J_{6',NH}$ couplings of 5, and additionally of $J_{^{15}N-H}$ in 5-^{15}N. A selection of proton–proton and proton–^{15}N coupling-constants is given in table 2. For 1,2:3,5-di-O-isopropylidene-α-D-glucofuranose derivatives (e.g., 1–5), $J_{4,5}$ is much larger than for 1,2:3,5-di-O-benzylidene-α-D-glucofuranose derivatives [35] or for a 1,2-O-isopropylidene-3,5-O-(methoxymethylidene) derivative [36], and it is apparent that the m-dioxane ring of 1–5 does not adopt predominantly the same conformation as in the latter derivatives. This aspect is being further investigated.

E. Synthesis, Equilibration, and Conformations of Diastereoisomeric 1,2-O-Isopropylidene-3,5-O-(methoxymethylidene)-6-O-p-tolylsulfonyl-α-D-glucofuranoses. Conformational Evidence from a Nuclear Overhauser Effect

(B. Coxon)

1. Introduction

A previous investigation of the conformations of a series of 1,2:3,5-di-O-benzylidene-α-D-glucofuranose derivatives by p.m.r. spectroscopy unexpectedly revealed that the m-dioxane ring in these molecules tends to assume that chair conformation having C-6 of the sugar axially attached [35]. Because the rationalization of this observation has been difficult, it appeared worth while to study further compounds of this type, in an effort to define the interactions that control their favored conformations.

Ribonucleoside derivatives in which the 2',3'-O-(alkoxymethylidene) substituent forms part of a five-membered

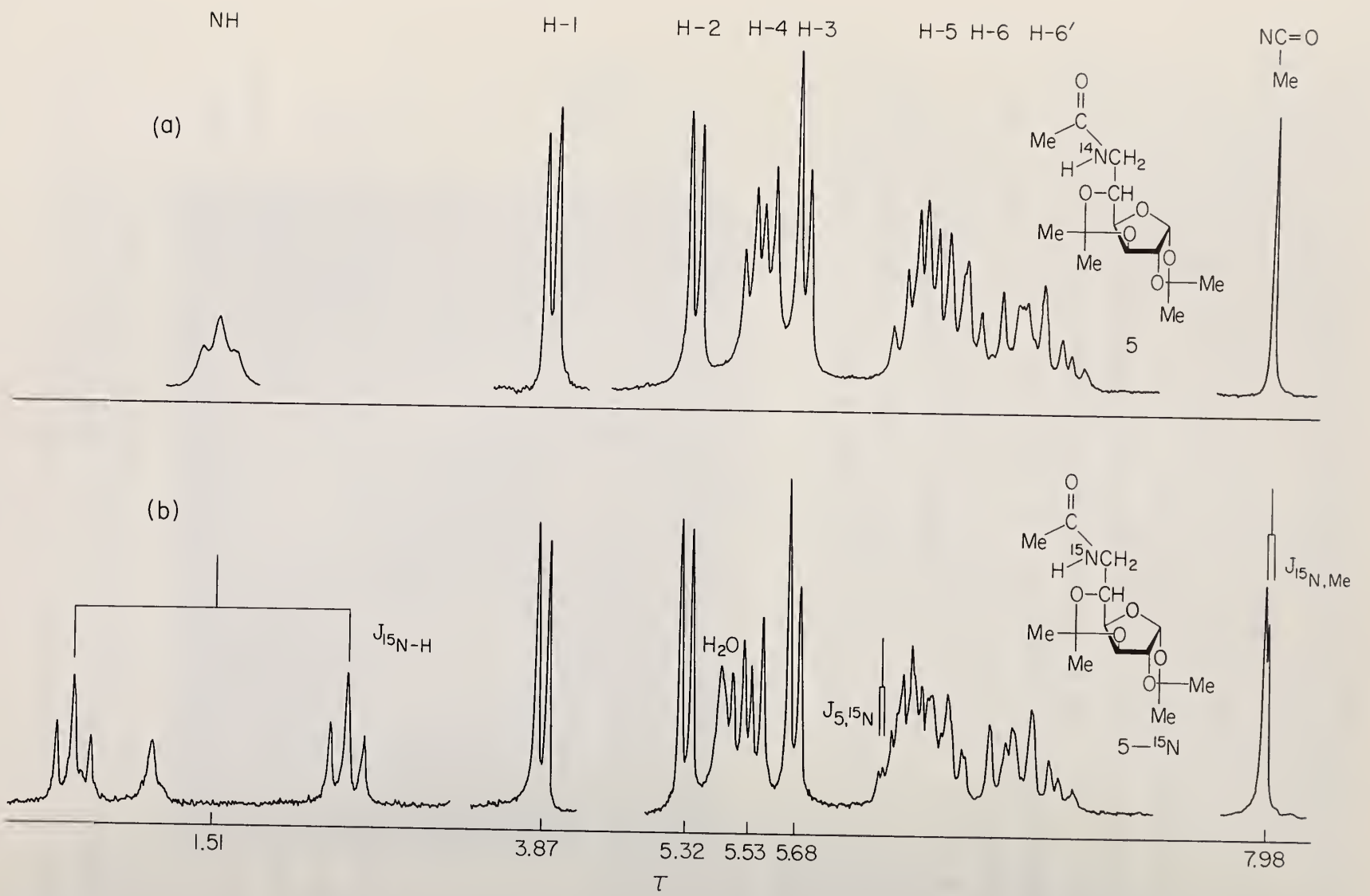


Figure 6. P.m.r. spectra of solutions in pyridine-d_5 at 100 MHz; the isopropylidene methyl resonances are omitted. (a) 6-Acetamido-6-deoxy-1,2:3,5-di-O-isopropylidene-α-D-glucofuranose (5), (b) 6-acetamido-6-deoxy-1,2:3,5-di-O-isopropylidene-α-D-glucofuranose-6-^{15}N (5-^{15}N).

Table 2. Coupling constants (Hz, first-order) of 6-amino-6-deoxy-1,2:3,5-di-O-isopropylidene-α-D-glucofuranose-6-^{15}N derivatives at 100 MHz

Derivative	Solvent	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$	$J_{6,NH}$	$J_{6',NH}$	^{15}N coupling constants[a]
4-d_2[b,c]	$CDCl_3$–D_2O[d]	3.7	<0.4	3.9	6.6	4.1	7.9	13.5	-----	------	---------
4-^{15}N	$CDCl_3$	3.6	<0.4	3.8	6.7	4.0	7.5	13.6	-----	------	$^3J_{5,^{15}N}$ 1.5 $^2J_{6,^{15}N}$ ∿0.5 $^2J_{6',^{15}N}$ ∿0.5
5-^{15}N	C_5D_5N	3.7	<0.4	3.7	6.4	∿4.4	∿8.3	∿14.4	5.7	5.7	$^3J_{5,^{15}N}$ 1.5 $^2J_{6',^{15}N}$ ∿0.5 $^1J_{^{15}N-H}$ 91.3 $^3J_{^{15}N,Me}$ 1.2
5-^{15}N-d[c]	C_5D_5N–D_2O (5:2 v/v)	3.7	<0.4	3.6	6.5	4.3	7.6	13.9	-----	------	$^3J_{5,^{15}N}$ 1.7 $^2J_{6,^{15}N}$ ∿0.7 $^2J_{6',^{15}N}$ ∿0.6 $^3J_{^{15}N,Me}$ 1.3

[a] Measurements from proton spectra. [b] ^{14}N Derivative. [c] N-Deuterated. [d] Overlayer.

ring are important intermediates in the preparation of ribo-oligonucleotides possessing the naturally occurring (3'→5') internucleotide linkage [37].

2. Synthetic Reactions

Acid-catalyzed reactions of 1,2-O-isopropylidene-6-O-p-tolylsulfonyl-α-D-glucofuranose (1) with trimethyl orthoformate in N,N-dimethylformamide gave syrups containing two major components in approximately equal proportions. P.m.r. spectra (similar, for example, to figure 7c) of the mixtures displayed two H-1 doublets, two H-2 doublets, and pairs of newly introduced methine (one-proton total) and methoxyl (three-proton total) singlets having similar chemical shifts. By chromatography on silicic acid, the mixtures were separated into crystalline (2) and syrupy (3) components that gave very similar p.m.r. spectra (see figures 7 and 8, respectively).

Treatment of either 2 or 3 with a mixture of trimethyl orthoformate, N,N-dimethylformamide, and sulfuric acid led, in each case, to a 6:5 mixture of 2 and 3, as indicated by integration of the methine and methoxyl singlets in the p.m.r. spectrum of each product mixture. Compounds 2 and 3 could also be equilibrated (2–3 min) by treatment of their solutions in chloroform-d with a trace of p-toluenesulfonic acid. The spectrum of an equilibrated solution is shown in figure 7c.

For further structural characterization of 2 and 3, their equilibrated mixture in p-toluenesulfonic acid–chloroform-d was treated with one molecular proportion of acetone. Studies of p.m.r. spectra of the mixture, and isolation from it of 1,2:3,5-di-O-isopropylidene-6-O-p-tolylsulfonyl-α-D-glucofuranose (4) demonstrated that methoxymethylidene substituents in both 2 and 3 were replaced by isopropylidene groups, with liberation of methyl formate.

The foregoing evidence indicates that 2 and 3 are

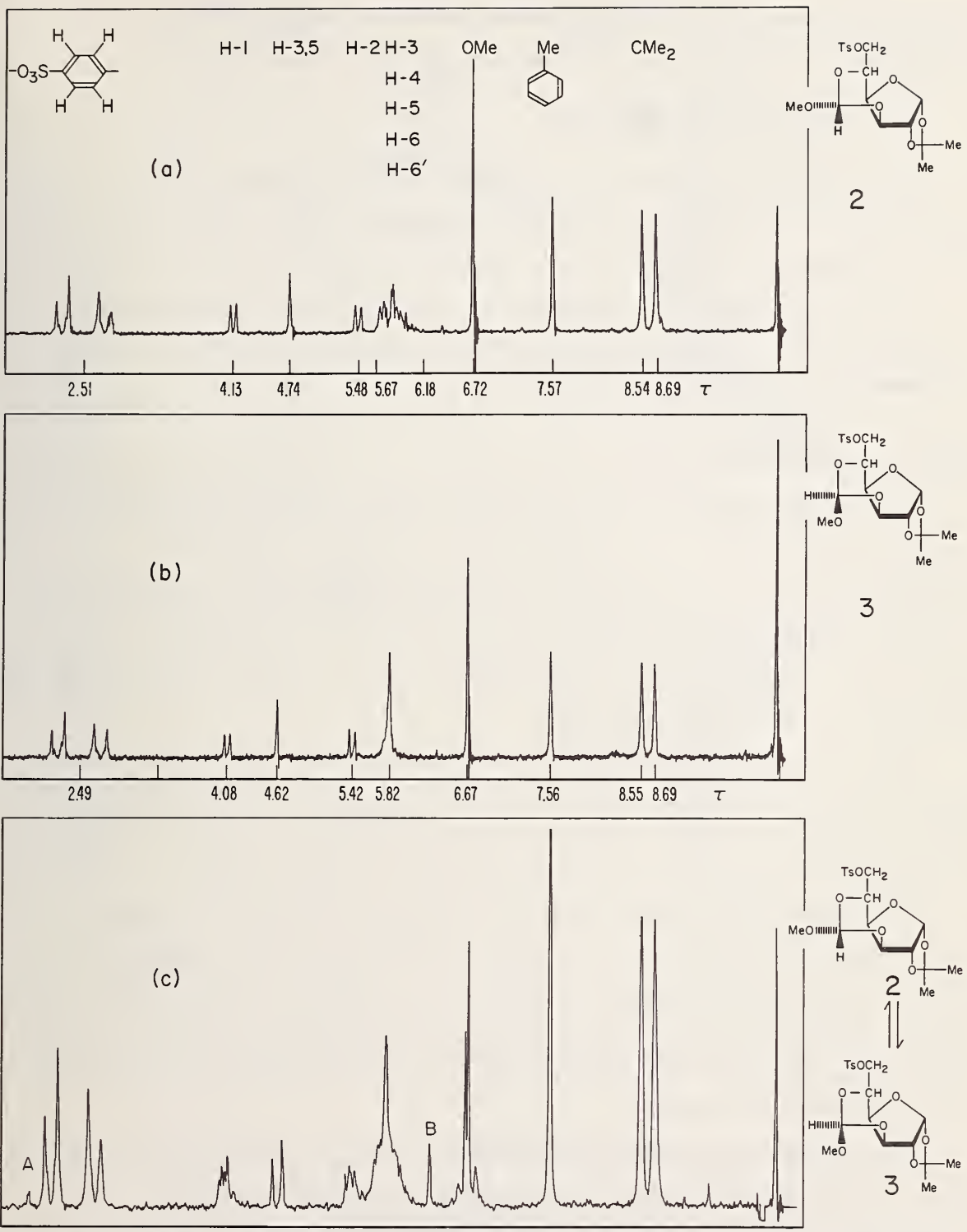


Figure 7. P.m.r. spectra in chloroform-d at 60 MHz: (a) 1,2-O-isopropylidene-3,5-O-[(endo-methoxy)methylidene]-6-O-p-tolylsulfonyl-α-D-glucofuranose (2), (b) 1,2-O-isopropylidene-3,5-O-[(exo-methoxy)methylidene]-6-O-p-tolylsulfonyl-α-D-glucofuranose (3), (c) 2 and 3 in equilibrium. Methyl formate signals appear at A and B.

diastereoisomeric 1,2-O-isopropylidene-3,5-O-(methoxy-methylidene)-6-O-p-tolylsulfonyl-α-D-glucofuranoses.

3. Reaction Mechanisms

The acid-catalyzed exchange of the 3,5-O-(methoxy-methylidene) substituent for an isopropylidene group may be rationalized in terms of the mechanism $\underset{\sim\sim}{2a} + \underset{\sim\sim}{3a} \rightarrow \underset{\sim}{6} \rightarrow \underset{\sim}{7} \rightarrow \underset{\sim}{4}$.

The most plausible mechanism for acid-catalyzed interconversion of the diastereoisomers $\underset{\sim}{2}$ and $\underset{\sim}{3}$ involves the intermediate, dioxo-carbonium ion $\underset{\sim}{5}$.

4. Conformations

To obtain reasonably accurate values of the vicinal coupling-constants, a 100-MHz spectrum (see figure 8a) of $\underset{\sim}{2}$ was subjected to an iterative computer-analysis. Initially, the proton group comprising H-1 to H-6' was analyzed as a seven-spin system. The theoretical spectrum from the iterative analysis is shown in fig. 8b.

By analogy with previous work [38], the near-equality of $\underline{J}_{1,2}$ and $\underline{J}_{3,4}$, and the small value of $\underline{J}_{2,3}$, for $\underset{\sim}{2}$ indicate that the shape of its furanoid ring is quite close to that of the $\underline{T}^3_2$ conformation, as in conformation $\underset{\sim\sim}{2a}$ (or $\underset{\sim\sim}{3a}$).

The relatively small value of $\underline{J}_{4,5}$, namely, 3.31 Hz, showed that H-4 and H-5 are disposed mainly in the gauche orientation, as in conformation $\underset{\sim\sim}{2a}$ (or $\underset{\sim\sim}{3a}$), in which the m-dioxane ring assumes that chair conformation having both of these protons equatorially attached but with C-6 of the sugar moiety axially attached.

The comparatively large values of $\underline{J}_{5,6}$ and $\underline{J}_{5,6'}$ (6.02 and 6.6 Hz, respectively) indicate large contributions from rotamers $\underset{\sim}{8}$ and $\underset{\sim}{9}$. From the coupling constants, it appears likely that $\underset{\sim}{3}$ has fundamentally the same type of conformation ($\underset{\sim\sim}{3a}$) as has $\underset{\sim}{2}$.

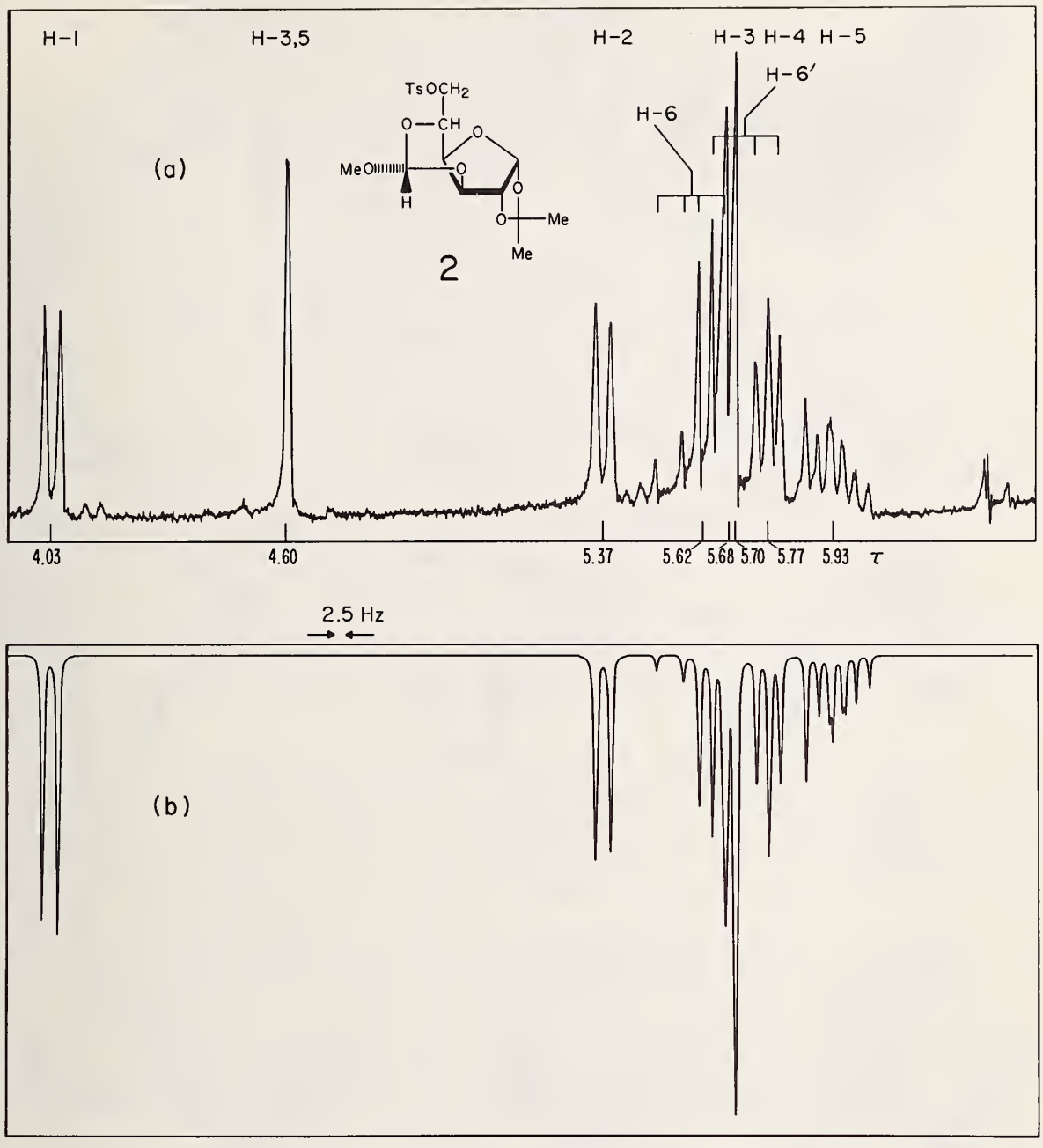
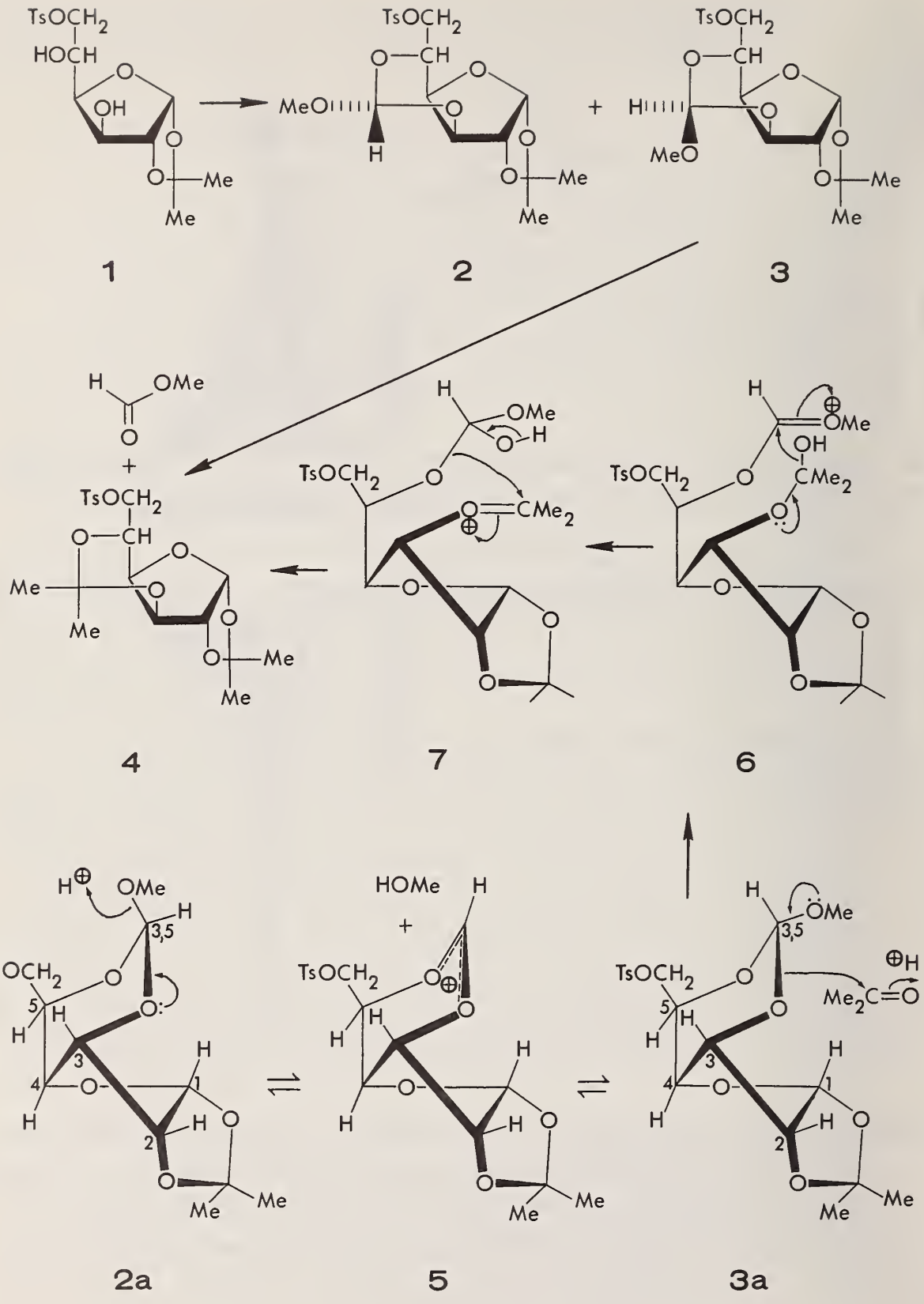


Figure 8. (a) Partial p.m.r. spectrum of 1,2-O-isopropylidene-3,5-O-[(endo-methoxy)methylidene]-6-O-p-tolylsulfonyl-α-D-glucofuranose (2) in 6:1 (v/v) acetone-d_6–pyridine-d_5 at 100 MHz. (b) Computed spectrum of same compound, from iterative analysis.


TsOCH2
HOCH
OH
Me
MeO
H
+
1
2
3
4
7
6
OMe
CMe2
2a
5
3a
HOMe
Me2C=O

5. Determination of Configuration

Evidence for the configuration of C-3,5 in 2 and 3 was obtained from three sources. It might be anticipated that the methoxyl substituent in 2 and 3 would show a doubled "anomeric effect" [39-45], due to the presence of two hetero oxygen-atoms in the attached m-dioxane ring.

Studies of pyranoid derivatives have shown that the anomeric effects of 1-substituents decrease on passing from a nonpolar to a polar solvent, due evidently to solvation of the intramolecular dipoles [46]. Accordingly, it was expected that, for the diastereoisomers 2 and 3, that diastereoisomer having its methoxyl group in the axial orientation on the m-dioxane ring would be destabilized with respect to its equatorial isomer on passing from equilibria in a nonpolar to those in a polar solvent. Studies of the equilibria of 2 and 3, in chloroform-d–N,N-dimethylformamide showed this to be the case.

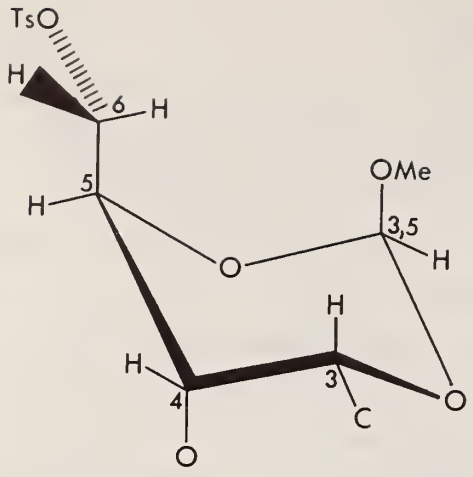
The configurations and conformations of 2 and 3 may, therefore, be represented as shown in 2a, and 3a, respectively. Crystalline 2 may therefore be described as 1,2-O-isopropylidene-3,5-O-[(endo-methoxy)methylidene]-6-O-p-tolylsulfonyl-α-D-glucofuranose, and syrupy 3 as its exo isomer.

The assignment of an equatorial position to H-3,5 of 2 (as in 2a) is also supported by the observation of its long-range coupling (0.42 Hz) with H-4, which is equatorially oriented with respect to the m-dioxane ring (see formula 8 or 9).

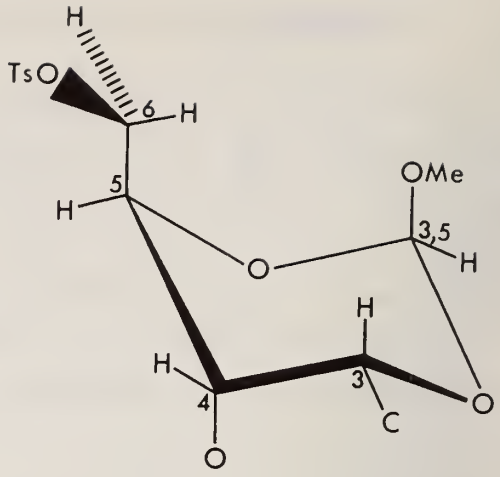
The chemical shifts of H-3,5 in 2 and 3 provided further evidence for the configuration of C-3,5 in these compounds.

6. Nonbonded Interactions

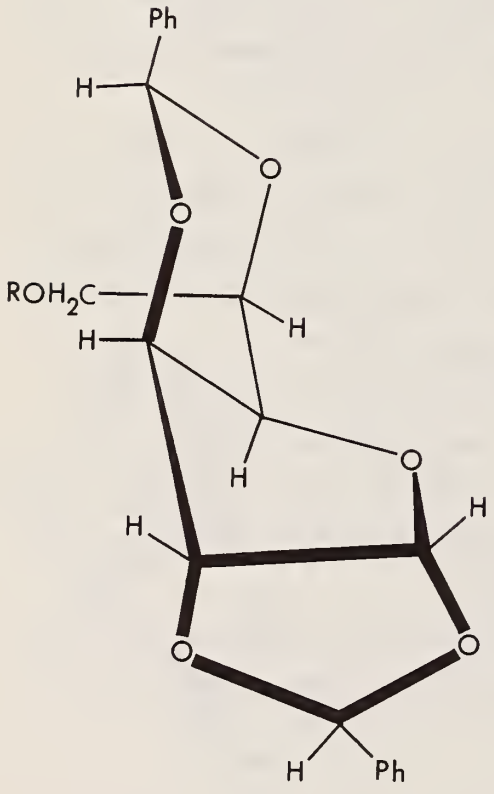
From the enthalpy change 3 → 2 in chloroform-d and N,N-dimethylformamide, it may be deduced that the stabilization


TsO
H
6
H
5
H
OMe
3,5
H
O
H
H
4
3
C
O
O

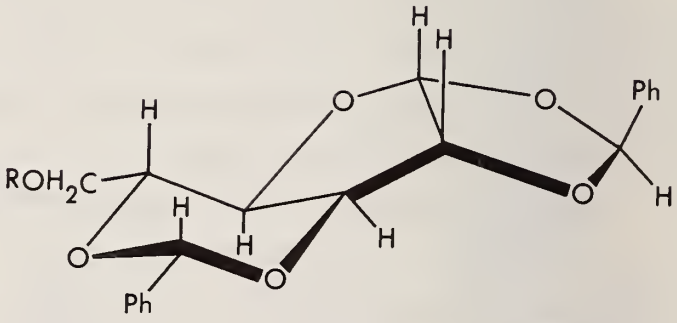
8


H
TsO
6
H
5
H
OMe
3,5
H
O
H
H
4
3
C
O
O

9


Ph
H
O
O
ROH2C
H
H
H
O
H
H
O
O
H
Ph

10


H
H
H
O
O
Ph
ROH2C
H
H
H
H
O
O
Ph
H

11

of 2 provided by the anomeric effect exceeds the destabilization from repulsive steric interaction.

The more important steric interactions in 2a and 3a and in conformations 10 and 11 of the related 1,2:3,5-di-O-benzylidene-α-D-glucofuranose derivatives have been calculated by using the Hill equation [47]: $E_v = \varepsilon\{-2.25[r/(r_1+r_2)]^{-6} + 8.28 \times 10^5 \exp[-r/0.0736(r_1+r_2)]\}$.

The results of these calculations suggest that the conformations favored by 1,2:3,5-di-O-alkylidene-α-D-glucofuranose derivatives can be explained in terms of conformational distortion of the m-dioxane ring and its attached substituents.

7. Intramolecular, Nuclear Overhauser Effect

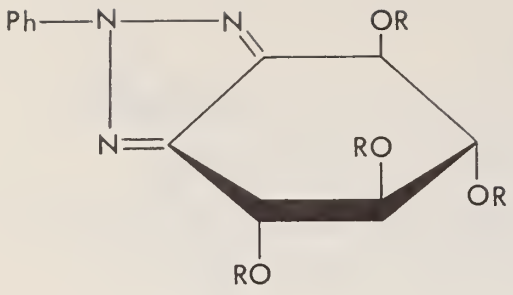
Experimental evidence for a favored orientation of the methoxyl group in 2 was obtained from an intramolecular, nuclear Overhauser experiment [48]. Saturation of the methoxyl protons of 2 resulted in a 25% enhancement of the integrated intensity of its methylidene proton resonance signal when compared with a similar experiment in which the irradiating frequency was applied off resonance.

F. Polyhydroxy Cyclic Compounds

(A. J. Fatiadi)

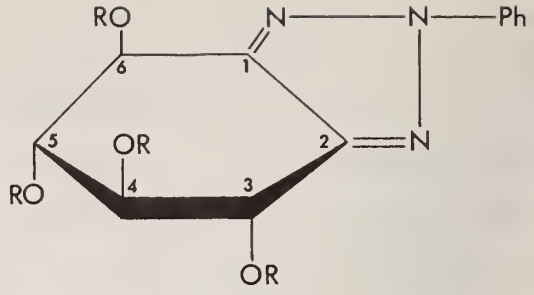
1. Evidence for a Half-chair Conformation of Certain Osotriazoles from Inositols

As part of a continuing interest in the chemistry of inositol derivatives, D- (1a), L- (2a), and DL- (1a–2a) 1,2-diketo-myo-inositol phenylosotriazoles [49] have been prepared in 42–45 percent yield from the corresponding inosose phenylosazones by use of a new reagent for this reaction, namely, mercuric acetate; the yields are higher



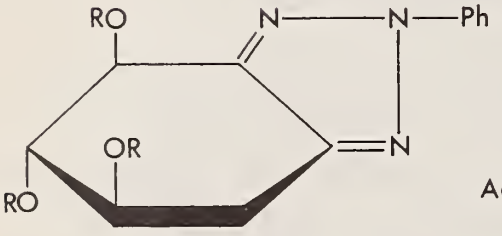
1

D



2

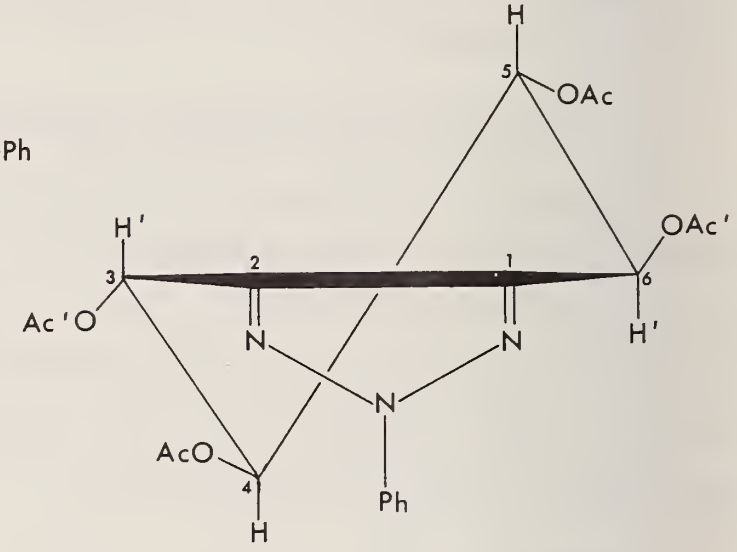
L



3

a, R = H

b, R = Ac



4

than those obtained with the cupric sulfate reagent originally used in the preparation of the DL-osotriazole (la-2a) by Anderson and Aronson [50].

The p.m.r. spectra of the diketoinositol osotriazoles la (mp 234-235 °C), 2a (mp 233-234 °C), and la-2a (mp 277-278 °C), and their tetraacetates lb (mp 146-147 °C), 2b (mp 145-146 °C), and lb-2b (mp 193-194 °C), hitherto not analyzed, revealed some interesting features, including: (1) the presence of a simple, two-fold axis of symmetry (for example, for the optically active compounds la and 2a), and (2) their ring-

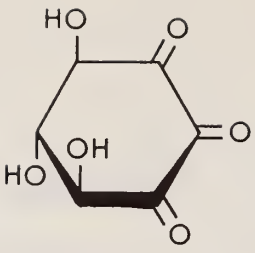
proton p.m.r. spectra are symmetrical about a midpoint, making them a new example of the important, four-nucleus, AA'BB' system. However, as expected, the p.m.r. spectra of the osotriazoles from D-proto-diketoquercitol (3a), m.p. 195-196 °C, (or its tetraacetate 3b) and from D-diketopinitol or D-diketoquebrachitol do not show a symmetrical pattern.

From inspection of Dreiding models, it appeared that, in solution, the favored conformation for the osotriazole tetraacetate 1b-2b would be the half-chair conformation (H_4^5) depicted in 4. The coupling constant ($J_{BB'}$) of 7.5 Hz deduced for H-4 and H-5 indicates a trans-diaxial orientation; the H_4^5 conformation would also have H-5 and H-6 axial, and this fits the value calculated (J_{AB} 6.5 Hz). The small coupling-constants between H-3 and H-6 ($J_{AA'}$, -1.25 Hz) and between H-4 and H-6 ($J_{AB'}$, 0.5 Hz) indicate the influence of the neighboring, planar, osotriazole ring, and this gives additional support for the half-chair conformation deduced for 1b-2b.

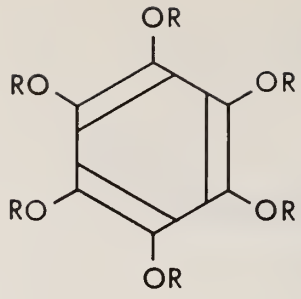
The analysis of the p.m.r. spectrum of tetraacetate 1b-2b (and others) has been verified by means of the calculated spectrum (UEA/NMR2 Program). This work has been published [51].

2. Novel Aromatization of a Trihydroxycyclohexanetrione (Triketoinositol) to a Dibenzo-p-dioxin Derivative

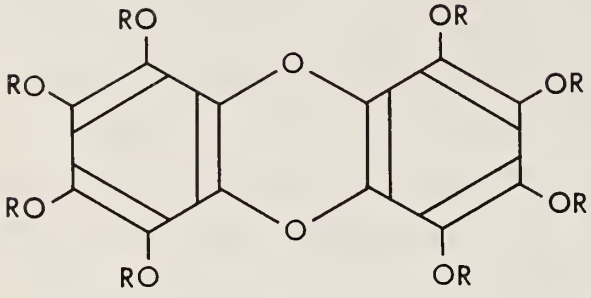
It had been shown [52] that, on acetylation in the presence of pyridine, 4,6/5-trihydroxy-1,2,3-cyclohexanetrione is aromatized to benzenehexol hexaacetate in high yield. It has now been found that acetylation of 1 in the presence of an acidic catalyst (100% phosphoric acid) gives a new adduct, the fully hydroxylated dibenzo-p-dioxin 3a, isolated as its octaacetate 3b.



1



2 a, R = H
b, R = Ac



3 a, R = H
b, R = Ac

A mixture of 1 monohydrate [52] (1 g; 5.17 mmoles) with acetic anhydride–phosphoric acid reagent (60 ml; 12 ml of 100% syrupy H_3PO_4 and 48 ml of acetic anhydride, homogeneously mixed [53]) was stirred at 85 °C until a solution was obtained (60 min). The somewhat brownish solution was decomposed with ice-water to give 3b, yield 1.3 g (77.5%). The crude product was recrystallized from warm acetic acid, and a sample was dried at 110 °C/0.1 torr for 2 hr; mp 300-302 °C (298-300 °C, slow heating); $\lambda_{max}^{(MeOH)}$ 232 (ε ∿47.5),* 278 (ε ∿4.5), and 295 (sh) nm (ε ∿3.0). The formation of 3a can be envisaged as an acid-catalyzed nucleophilic attack of 2a on 1.

*Throughout this Technical Note, the symbol ε represents $\varepsilon_{m\underline{M}}$ (not $\varepsilon_{\underline{M}}$).

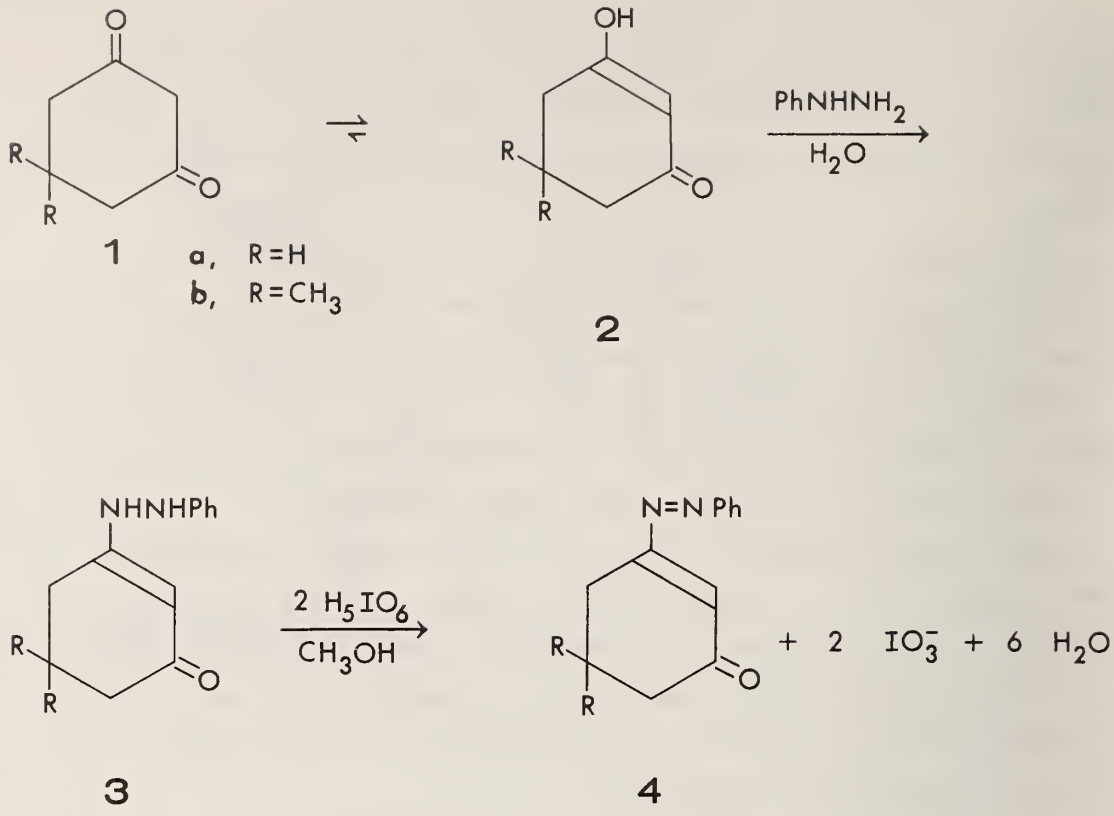
The structure of 3b was confirmed by i.r., n.m.r., and high-resolution mass-spectroscopy; ν_{max}^{KBr} 1785(vs) (C=O), 1650(m) (C=C, aromatic), 1615(w), 1480(vs) (phenyl ring), 1210(vs), 1180(vs) (acetate), and 1062(vs) cm^{-1} (=C-O, aryl ether).

The p.m.r. spectrum of 3b (~12% solution in methyl sulfoxide-d_6, with tetramethylsilane as the internal standard) showed only two narrowly spaced singlets of equal intensity (separation 5 Hz), at τ 7.69 and τ 7.65, arising from two groups of methyl protons of the acetyl groups on O-1, O-4, O-5, and O-8, and on O-2, O-3, O-6, and O-7.

The mass spectrum (70 eV) of 3b showed a molecular-ion peak (parent ion) at m/e 648.00; calcd. for $C_{28}H_{24}O_{18}$, 648.492. The spectrum also showed seven ion-peaks (moderate to strong intensities) due to the consecutive loss of ketene (CH_2=C=O) group from the acetate groups at m/e 606, 564, 522, 480, 438, 396, 354, and 312.

3. Conversion of Certain Cyclic Phenylhydrazino Derivatives into Phenylazo Compounds with Periodic Acid

In connection with studies [54] of certain cyclic, vinyl phenylazo compounds as possible intermediates in the formation of bis- or tris-(phenylhydrazones), there was a need to obtain pure samples of 3-oxo-1-phenylazo-1-cyclohexene (3a) and 5,5-dimethyl-3-oxo-1-phenylazo-1-cyclohexene (3b). One possible way to obtain these compounds is to convert the corresponding enols of 1,3-cyclohexanediones [for example, the enol of 5,5-dimethyl-1,3-cyclohexanedione (1b)] into the phenylhydrazino derivative, such as 2b; the oxidation of the latter would give the needed azo compound 3b.



It has been found that treatment of a solution of 3a or 3b (one mole) in methanol (or glacial acetic acid) with an aqueous solution of periodic acid (two moles) at room temperature produces the corresponding azo compounds 4a and 4b in 90% yield.

It is believed that clear-cut conversion of the vinyl phenylhydrazino compounds 3a and 3b into the corresponding azo compounds 4a and 4b with periodic acid may indicate the general character of this reagent for deprotonation of this type of compound.

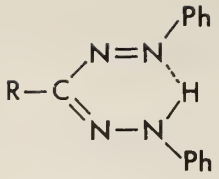
4. Phenylhydrazono–phenylazo Tautomerism. Reactions of Phenylformazans and Certain Bis(phenylhydrazones) with Strong Acids

(A. J. Fatiadi and H. S. Isbell)

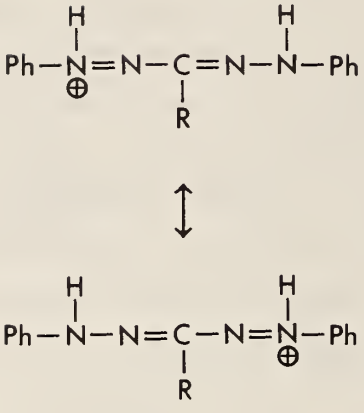
The behavior of diphenylformazans, 2-oxo-1,3-bis-(phenylhydrazones), 1,2-bis(phenylazo)ethylene, and the phenylhydrazone of 4-oxo-1-phenyl-5-phenylazo-3-pyridazinecarboxaldehyde on protonation has been examined spectrophotometrically. These compounds form purple, blue, or green protonated cations. The nature of the spectral changes suggests that the highly colored cations have resonance-stabilized structures. Phenylosazones and bis(phenylhydrazono) compounds that cannot form resonance-stabilized cations on protonation do not usually give the blue-color reaction. Structures have been proposed for the cations derived from diphenylformazans and certain 2-oxo-1,3-bis(phenylhydrazones).

Treatment of the red, enolic tautomer of 2-oxo-1,3-bis-(phenylhydrazono)cyclohexane with perchloric acid in acetic acid yielded a new, stable, dark-blue, crystalline salt. Dissolution of this salt in acetic acid, followed by addition of ice–water gave a yellow hydrate, previously described, of the keto tautomer. Dissolution of the hydrate in ethanol, and warming, regenerated the red enolic tautomer.

E.s.r. measurements of colored solutions of 2-oxo-1,3-bis(phenylhydrazones) or diphenylformazans did not show the presence of radical species, thus indicating the ionic character of the products formed on protonation. The neutral molecule, for example, D-mannose diphenylformazan, has a chelate resonance-structure 1 in which the imino hydrogen atom spans the phenylhydrazono group and the phenylazo group. Apparently, strong acids rupture this chelate, and the resulting, resonance-stabilized cation may have a structure encompassing the resonance forms depicted in 1a.



1



1a

Where $R = -(CHOH)_4 CH_2OH$

The electron system for the cation of diphenylformazan la encompasses 17 atoms: only two of the resonance forms are illustrated. Delocalization of the positive charges over the resonance system in la may account for the bathochromic shift found in the spectrum of 1 on protonation.

These results have been incorporated in an article which has been accepted for publication [55].

2. CLINICAL CHEMISTRY

Those familiar with the activities of the Organic Chemistry Section know of its long-time connection with sugar technology and carbohydrate research, and may have had occasion to call on us for samples of the rare carbohydrates that were prepared here in the past, or for ^{14}C- and ^{3}H-labeled carbohydrates that for a long while were only available through our work. Recently, new supplies of a few rare carbohydrate derivatives were being made available as Standard Reference Materials; but the Section is no longer serving as a source for labeled carbohydrates, since these materials are now widely available from commercial laboratories utilizing our published procedures.

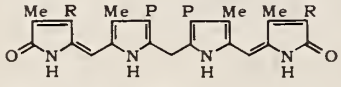
A few years ago, the Section devoted considerable effort to developing oil-soluble compounds of 24 different metals for the purpose of providing standards for the spectrographic measurement of these metals in lubricating oils, a type of analysis that very effectively measures engine wear. Such analyses are now in widespread use in the air, rail, and trucking industries.

Most recently, we have begun to develop SRM's for clinical analysis. Cholesterol was the first of these to be issued, and some of the present report is concerned with an evaluation of its shelf-life. Creatinine, urea, and uric acid, as well as calcium carbonate, have been issued during this past year. Much of the present report is devoted to work on bilirubin and on a standard for clinical glucose determinations.

A. Bilirubin

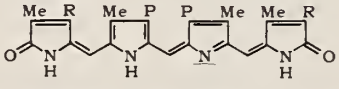
(R. S. Tipson, R. F. Brady, Jr., A. J. Fatiadi, and B. F. West)

Of the bile pigments, bilirubin (1) is the constituent of major interest in clinical chemistry. These pigments include biliverdine (2), mesobilirubin (3), and other compounds having four pyrrole rings linked at their alpha positions by carbon atoms. Many of these pigments are interconvertible; for example, 1 (red-orange) may be oxidized to 2 (green), or reduced to 3 (orange-yellow). Thus, a chloroform solution of 1 exposed to air and light changes color rapidly, demonstrating the ease of interconversion. The various bile pigments may usually be distinguished by their absorption spectra in the visible range.

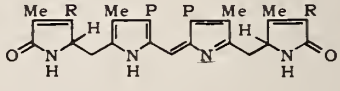


1, Bilirubin, R = $-CH=CH_2$

3, Mesobilirubin, R = $-CH_2-CH_3$



2, Biliverdine, R = $-CH=CH_2$



4, Urobilin, R = $-CH_2CH_3$

where P = $-CH_2CH_2CO_2H$

Bilirubin is derived primarily from the hemoglobin of mature red-blood cells, and secondarily from heme or porphyrin-like compounds formed during the synthesis of heme. As erythrocytes are destroyed in the reticulo-endothelial system, the hemoglobin is degraded, and the porphyrin ring of heme is converted into biliverdine, which is then reduced to bilirubin. The bilirubin is complexed by albumen in the blood, and is thus transported to the liver. There it is conjugated with two D-glucosyluronic acid groups by reaction thereof with the two carboxyl groups of bilirubin; this conjugation is necessary for its secretion into the bile. From the bile, the conjugated bilirubin passes into the intestinal tract; it is reduced to urobilin (4) (brown) by bacteria in the large intestine, and is eliminated. Di-(D-glucosyluronic acid) bilirubinate may pass into the urine; severe cases of bilirubinuria result in visible discoloration of the urine.

Because bilirubin analyses have major medical significance, particularly in the detection of such diseases as hepatitis or jaundice, and because of the difficulties encountered in these analyses, it is important that a well characterized bilirubin reference standard be available for use in clinical laboratories for standardizations and for comparing bilirubin methodologies.

1. Volatile Matter in Bilirubin Samples

(B. F. West)

Commercial samples of bilirubin were dried under vacuum to determine the content of volatile matter. A preliminary drying experiment was performed on a single lot of bilirubin. Two samples of ∿400 mg were accurately weighed (on a micro-balance) into each of two tared, dry vials. The samples were heated in an Abderhalden dryer at 58 °C (refluxing acetone)/ ∿0.3 torr for one hour, and then allowed to cool under vacuum.

The samples were weighed, and redried at 65 °C (refluxing methanol), and then at 111 °C (refluxing toluene). At each temperature, there was some loss in weight. The total loss in weight was 0.066%.

A second drying experiment was performed with three different samples of commercial bilirubin. The heating was conducted for 1.5 hours at 111 °C (refluxing toluene)/∿0.3 torr. The results are given in table 3.

Table 3. Loss in weight of samples of commercial bilirubin at 111 °C/∿0.3 torr

Manufacturer's lot number	Loss in weight, %
7941-I	0.065
39148	0.14
5310	0.38

2. Ash Content of Bilirubin Samples

The ash was determined on 100-mg samples of various commercial bilirubins. The results, obtained by R. A. Paulson and W. P. Schmidt, are shown in table 4.

Table 4. Ash content of samples of commercial bilirubin

Manufacturer's lot number	Ash, %
FP	0.07
7970-I	0.11
39148	0.13
39142	0.14
7941-H	0.16
7970-H	0.16
5310	0.23

3. Spectrochemical Analysis of Ash from Bilirubin Samples

The proportions of metallic elements present in the ash of commercial bilirubin from three sources were determined, and tentative limits of detection for common elements not detected in these samples were established. Ashing and testing techniques were developed for 25- and 100-mg samples of bilirubin. Limits of detection of an element in the range of 0.1 to 1 ppm of bilirubin were established for 22 common elements by using synthetic gallium oxide as the reference standard; the results of the analyses are shown in table 5. The analyses were performed by Martha M. Darr, Spectrochemical Analysis Section, NBS. The results given in table 5 show that the principal metallic constituents in the ash from the three bilirubin samples were Al, Ca, Fe, Mg, Na, Si, and Zn; but that there were distinct differences in the concentration levels of the different elements among the samples. For example, sample 1 (Lot 7941-I) shows the highest single metallic impurity, namely, sodium (500 ppm), which is probably a residue from the method used in isolating the product. On the other hand, sample 2 (Lot 39148) shows an appreciable content of iron (200 ppm) which may indicate the presence of a heme-like impurity in the product. The contents of Al, Ca, Mg, Si, and Zn in these commercial bilirubins differ widely; especially, the presence of Si (50 ppm) in sample 1, as compared with traces of it in the other two samples.

4. Solubility of Bilirubin

(A. J. Fatiadi and R. F. Brady, Jr.)

The solubility of bilirubin in various solvents is (in decreasing order) 1-methyl-2-pyrrolidinone, 2-pyrrolidinone, methyl sulfoxide, N,N-dimethylformamide, chlorobenzene, chloroform, acetone, glacial acetic acid, ethyl acetate, benzene, methanol, and acetonitrile.

Table 5. Proportions (ppm, by weight) or elements in ash from samples of commercial bilirubin

Element	Sample[a] 1	2	3	E[b]	Element	Sample[a] 1	2	3	E[b]
Ag	0.4	<0.2	<0.2	<0.1	Na	500[d]	50[d]	100[d]	
Al	20[c]	1	1	<0.1					
Au	-	-	-	-	Ni	-	0.4	0.2	0.2
B	-	-	-	0.5					
Be	-?	-?	-?	0.1	Pb	-	0.2	0.4	0.2
Bi	-	-	-	0.5	Pd	-	-	-	-
Ca	50[c]	10[c]	5	0.2	Pt	-	-	-	-
Cd	-?	-?	-?	1.0					
Co	-	-	-	0.5					
Cr	-?	2	2	1.0	Rh	-	-	-	-
					Ru	-	-	-	-
Cu	0.5	5	0.5	<0.1	Sb	-	-	-	0.1
					Si	50[c]	5	5	0.1
Fe	50[c]	200[c]	50[c]	0.2	Sn	-	-	0.2	0.2
Ga	-	-	-						
Ge	-	-	-	0.5					
Hf	-	-	-						
					Ti	0.1	0.2	0.4	0.1
In	-	-	-	0.1					
Ir	-	-	-						
La	-	-	-		V	-	-	-	0.2
Mg	20[c]	5	10[c]	<0.1					
Mn	1	-	-	0.5	Zn	5	10[c]	2	1.0
Mo	-	0.1	0.2	<0.1	Zr	-	-	-	

[a] Bilirubin sample 1, lot 7941-I; 2, lot 39148; 3, lot 5310.

[b] Estimated limits of detection as metal (ppm) in bilirubin.

[c] Concentration estimated (above highest standard).

[d] Visual estimate, without a specific standard on this plate.

Solubility data for bilirubin were desired for possible use in phase-solubility analysis. Therefore, 500-mg quantities of bilirubin were equilibrated by shaking for 7 days in a bath at 13.0 ±0.1 °C with 500 ml of different solvents. Undissolved material in each mixture was removed by filtration (Millipore), and an aliquot of the filtrate was evaporated to dryness. The results are given in table 6.

Table 6. Approximate solubilities of bilirubin[a] at 13.0 °C

Solvent	Solubility, mg/ml of solvent
Methanol	0.14
Dichloromethane	0.47
Acetone	0.72
Chloroform	1.33

[a] Lot number 39148.

5. Spectra of Bilirubin Samples in the Visible Range

(B. F. West)

Bilirubin has a characteristic spectrum in the visible range. For solutions in chloroform, the maximum is at 453 nm, with a millimolar extinction coefficient (ε) reported as lying in the range of 55.0 to 60.8. Purified samples of bilirubin give [56] a value of ε that converges on ∿60.8. Generally, it has been supposed that samples of bilirubin that have a significantly lower ε value are less pure, although it has been noted [56] that such absorption curves alone are inadequate as a measure of the purity of bilirubin.

A spectrophotometric survey of some samples of commercial bilirubin was made as follows. A sample of ∿3 mg (accurately weighed on a microbalance) was quantitatively transferred to a 500-ml volumetric flask containing ∿200 ml of chloroform

at 20 °C, the suspension was vigorously shaken until the bilirubin had dissolved according to visual inspection, and the solution was placed in a water bath at 20 °C. It was then made to 500 ml with chloroform at 20 °C. (All solutions were made under dim incandescent light.) The final solution was mixed thoroughly, and spectral measurements were immediately made by use of a 1-cm cell in a Cary 14 spectrometer (with use of the automatic, slit-control setting), against a chloroform blank. For these measurements, the cell compartment was not thermostated; the temperature therein was ∿22 °C. The values of ε for commercial samples of bilirubin are given in table 7.

Table 7. Millimolar extinction coefficients of samples of commercial bilirubin

Manufacturer's lot number	ε
45054	56.3
39148	57.3
29191	57.6
5310	59.6
7941-J	59.9
FP[a]	59.9

[a] Cell thermostated at 25 °C.

6. Stability of Bilirubin Samples in Various Solvents

(A. J. Fatiadi)

The following is the general procedure for stability studies on bilirubin samples. A sample of a commercial bilirubin (3.126 mg) was quickly dissolved in warm chloroform (450 ml, 3 min of stirring, water bath at 60 °C), quickly cooled to about 20 °C, and diluted to 500 ml. This stock solution showed an absorption maximum at 453 nm, with an

absorbance of 0.640. The stock solution was kept at 3–5 °C in the dark (refrigerator). Periodically, a portion was withdrawn and quickly warmed to room temperature, and its absorbance determined. This value was compared with that obtained immediately after dissolution of the bilirubin. On the assumption that the only material present that showed absorption at 453 nm was bilirubin, the percentage loss of bilirubin with time was calculated; the results are given in table 8.

7. Effect of Antioxidants on Stability of Bilirubin Solutions

(A. J. Fatiadi)

A sample of bilirubin (lot number 7941-I; 3.58 mg) was dissolved in methyl sulfoxide (10 ml), and an aliquot (1 ml) was diluted to 50 ml; A_{454} 0.743. This solution, kept at room temperature (in the dark) for 48 hours, showed a decrease in absorbance of 9%. However, addition of a sample of L-ascorbic acid (15 mg) to a similar solution lessened the decomposition of bilirubin (after 48 hr) to 2.5%, showing the stabilizing effect of L-ascorbic acid.

Addition of 2,5-di-(tert-amyl)hydroquinone (5 mg) failed to diminish the decomposition (loss, about 8% after 48 hr).

8. Synthesis of Mesobilirubin IXa

(B. F. West)

In order to be able to identify one of the impurities that might be present in commercial samples of bilirubin, attempts were made to synthesize one of the most probable impurities [57], namely, mesobilirubin. This compound differs from bilirubin in having two ethyl groups in place of the two vinyl groups of bilirubin.

Table 8. Stability of samples of commercial bilirubin in solution (10 μM), as shown by decrease in A_{453}

Bilirubin lot number	Solvent	Time (hours)	Decrease in concentration, %
39142	chloroform	144	5
		528	19
		1152	23
7970-I	chloroform	24	1
		144	5.8
		336	24.5
		552	26.3
783889	chloroform	141	9.4
		312	17.3
		528	23
		1152	25
68-B-1300	chloroform	140	19
		336	29
		552	31
		1176	34
	N,N-dimethyl-formamide	144	16
		360	20
		624	30
		984	38
7970-I	N,N-dimethyl-formamide	42	10
		190	19
		406	32
		1032	57
7970-I	30 mM sodium hydroxide	6	7
		30	46

Mesobilirubin is a yellow solid having, in chloroform, λ_{max} 434 nm, with a millimolar extinction coefficient [58] of 54.6. Following the procedure of Gray, Kulczycka, and Nicholson [58], a solution of bilirubin in 100 m$\underline{M}$ sodium hydroxide was hydrogenated for 3 hours at room temperature and atmospheric pressure in the presence of palladium-on-carbon. However, three times the theoretical volume of hydrogen was consumed. Consequently, the following modification was used. A sample of bilirubin (0.2 g) was dissolved in $\underline{M}$ sodium hydroxide (15 ml) in a 250-ml flask provided with a magnetic stirrer and covered with aluminum foil to exclude light. In a second flask, similarly equipped, were placed 0.1 g of palladium-on-carbon and 10 ml of $\underline{M}$ sodium hydroxide. The necks of the two flasks were connected with a T-tube which led also to a gas-measuring reservoir, to a vacuum, and to a source of hydrogen. With stirring of the contents of both flasks, the assembly was alternately evacuated and filled with hydrogen six times, and finally left filled with hydrogen. Then, the flask containing the catalyst was inverted, so that the catalyst was added to the sodium bilirubinate solution. When the theoretical amount of hydrogen had been absorbed ($\sim$1 hr), the reaction was stopped, and the mixture was filtered.

The filtrate was made neutral with a slight excess of $\underline{M}$ acetic acid (30 ml), and a precipitate formed immediately. This tan-colored precipitate was filtered off; it rapidly darkened to a green-black cake. The dry solid weighed 0.2 g.

A portion of this material was treated with warm ethanol; it left a yellow, insoluble material which was filtered off and dried. The yellow product, in chloroform, showed λ_{max} 433 nm with a millimolar extinction coefficient of 54.2, properties that correspond approximately to those of mesobilirubin IXa.

This product was compared with bilirubin by thin-layer chromatography (on polyamide) with 9:1:2 methanol–10% ammonia–water, and found to be slightly impure. In addition to the major, light-yellow component (mesobilirubin), there appeared to be, in the tailing of the mesobilirubin spot, a slightly darker yellow color having an R_F slightly higher than that of bilirubin. This impure, synthetic mesobilirubin IXa was used in the t.l.c. examination of samples of bilirubin.

9. Synthesis of Biliverdine

(A. J. Fatiadi)

a. Oxidation of Bilirubin with Ferric Chloride

A reference sample of biliverdine was prepared by oxidation of bilirubin with $FeCl_3$ according to a published procedure [59]. Starting from 150 mg of bilirubin, there was obtained 34 mg (22.6%) of biliverdine. The product had λ_{max}^{MeOH} 238(shoulder; sh) (ε ∿6.0); 277 (ε ∿22.0); 315(sh) (ε ∿28.0); 374 (ε ∿39.8), and 650 nm (ε 12.1); lit. [60] λ_{max}^{MeOH} 375-380 (ε ∿40.5) and 640-650 nm (ε 12.9).

b. Oxidation of Bilirubin with Acidified Methyl Sulfoxide

A sample of bilirubin (200 mg) in a test tube was treated with methyl sulfoxide (10 ml) and concentrated hydrochloric acid (0.3 ml); the suspension was placed in a water bath at 90-95 °C and stirred for 60 min. The dark-green solution was then made less acidic with aqueous sodium acetate (0.2 ml, 2 M), and adsorbed on Whatman filter paper (20 x 20 cm); this was dried in a hood for 24 hr. The paper was then cut into small strips and extracted with warm 1:1 acetone–methanol (2 x 100 ml); evaporation of the extracts gave a dark-green solid (120 mg); it had λ_{max}^{MeOH} 375-382 and 665-670 nm. T.l.c.

on polyamide with 2:1 (v/v) ethyl acetate–methanol revealed the presence of some impurity that did not move from the origin, indicating the possible presence of a biliverdine salt or a complex with methyl sulfoxide. The product was further purified by adsorption on magnesium sulfate (30 g) followed by elution with methanol, concentration, and extraction with ether (3 x 100 ml). Recrystallization from methanol, by concentration and cooling, gave shiny green crystals of biliverdine; yield 42 mg (21%); the u.v. and visible spectra of this material were close to those of that prepared by oxidation with ferric chloride.

10. Thin-layer Chromatography of Bilirubin

(B. F. West)

Thin-layer chromatography of bilirubin was performed in an attempt to detect and possibly identify impurities in bilirubin samples. Thin-layer chromatography of bilirubin has been studied by various workers [61–64], usually for bilirubin as a component of urine or blood samples. There are reports of satisfactory identification of bilirubin, biliverdine, mesobilirubin, and D-glucosyluronic acid bilirubinate fractions in such biological samples. However, we are unaware of any thin-layer chromatographic studies to determine traces of impurities in purified, free bilirubin.

In order to detect and identify small proportions of impurity in commercial samples of bilirubin, relatively large volumes of a solution of bilirubin in chloroform were spotted on thin-layer plates of silica gel, microcrystalline cellulose, and polyamide, respectively. Under spot-overload conditions, bilirubin does not behave satisfactorily on thin-layer plates. Depending on the solvent system and the adsorbent, either (a) no significant migration of bilirubin is observed, or (b) the bilirubin component migrates almost with the solvent front, with considerable "tailing."

Table 9. Mobilities of bilirubin, biliverdine, and mesobilirubin in t.l.c.[a]

Solvent system	Silica gel		Cellulose		Polyamide	
	BR	BV	BR	BV	BR	mesoBR
Chloroform	0	0	1	1	0.79	0.9
Methanol	0	0.81				
4:10:1 2-Propanone–propionic acid–water	0	0.7	1			
7:4:3:3 1-Butanol–acetone–propionic acid–water	0	0.83	1			
2-Propanone	0	0.74				
16:8:3 Benzene–ethyl acetate–ethanol	0	0.5	1	1		
90:25:4 Benzene-p-dioxane–acetic acid	0.83		1			
2:4:1:1 Butylacetate–methanol–NH_3–water	0.88		1			
25:4:3 95% Ethanol–25% NH_3-water	0.88		0			
10:1 Chloroform–methyl sulfoxide	1					
100:1 Chloroform–methyl sulfoxide	0					
1:1 Chloroform–methyl sulfoxide	0		1			
Methyl sulfoxide	0.38		1			

Table 9. (Continued)

4.7:3 Chloroform–methanol	1	1		
49:1 Chloroform–methanol	1	1		
4:1 Chloroform–methanol	1	1		
1:4 Chloroform–methanol	0.78	1		
10:1 Methanol–methyl sulfoxide	0			
1:1 Methanol–methyl sulfoxide	1			
9:1:2 Methanol–10% NH_3–water			0.59	0.66
3:1 Methanol–water			0	0 (BV 0)
1:1 Chloroform–methanol			0.49	0.7
3:1 Chloroform–methanol			0.85	0.9
1:1 Chloroform–acetone			0.32	0.4 (BV 0)
3:1 Chloroform–acetone			0.93	0 (BV 0)

[a] BR = bilirubin; BV = biliverdine; mesoBR = mesobilirubin.

For the commercial samples studied, with a variety of solvent systems and adsorbents, no detectable impurities could be separated. Commercial biliverdine and synthetic mesobilirubin, assumed to be most probable as impurities in bilirubin samples, were spotted on the same plates, for comparison. None of the commercial samples of bilirubin gave spots that corresponded to these compounds. Under the conditions studied, bilirubin could be located and identified by its own color.

The solvent systems and adsorbents employed to determine the mobilities of bilirubin and biliverdine are given in table 9.

In every system in which bilirubin or mesobilirubin migrated, the resulting spot streaked considerably, so that distinct spots were not obtained. The compounds and their location were evident from the colors of the spots; bilirubin, golden yellow; biliverdine, dark green; and mesobilirubin, light yellow. The most satisfactory solvent system and adsorbent for distinguishing between the structurally very similar bilirubin, mesobilirubin, and biliverdine was 9:2:1 methanol—10% ammonia—water on polyamide. The spots not only showed their characteristic colors, but fluoresced under ultraviolet light (254 nm), after the solvents had been thoroughly removed by drying the developed plate. (The polyamide layer did not contain a fluorescent indicator.) When commercial samples of bilirubin were chromatographed with this solvent system and adsorbent, neither mesobilirubin nor biliverdine was detected.

The conclusion drawn from the thin-layer chromatographic examination of the commercial preparations of bilirubin is that, if impurities are present, they are in proportions that afford amounts below the detection limits of the technique.

11. Mass Spectra of Bilirubin Samples

(R. F. Brady, Jr.)

The mass spectrum of several samples of bilirubin was determined on an LKB Model 9000 mass spectrometer, made available through the courtesy of Dr. Henry M. Fales, National Institutes of Health. Some of the more prominent ions in the spectra, and their relative abundances, are listed in table 10. Samples from three different sources gave the same spectra.

Table 10. Mass-spectral data for bilirubin[a]

m/e	Relative abundance
211	96.4
227	98.8
241	68.6
286	100.0
300	83.8
584	7.9
585	5.2
586	1.8
587	0.6
588	0.3

[a] Lot numbers 7322, 8162, and 39148.

The molecular ion for bilirubin is seen at m/e 584, and is accompanied by the normal cluster of bilirubin isotopic peaks at higher mass-to-charge ratios. Other prominent ions in the spectrum are formed when bilirubin splits at the central methylene group, giving ions of masses 300 and 286. Evidence for biliverdine (m/e 582) or mesobilirubin (m/e 588) and expected, associated, isotopic ions was not found.

12. Thermogravimetric Examination of Bilirubin Samples

(A. J. Fatiadi)

Several different samples of commercial bilirubin (∿5–6 mg) were analyzed for thermal stability in (a) dry air, and (b) dry nitrogen with a Dupont TGA apparatus (see fig. 9). The rate of heating was 2 °C/min up to 150 °C, 5 °C/min up to 250 °C, and 10 °C/min up to 500 °C. The results of these experiments are summarized in table 11. The thermogravimetric curves, shown in fig. 10, illustrate the fact that bilirubin, whether heated in air or under nitrogen, is surprisingly stable; that is, sample weights remain almost constant until a rapid decomposition is initiated. Curve 5 shows a thermogram typical of those

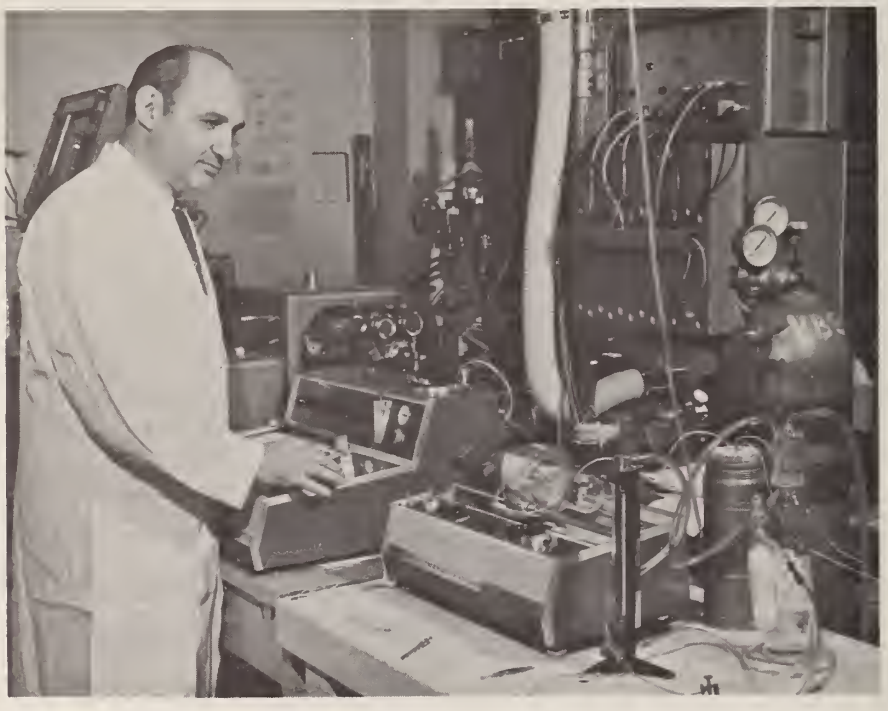


Figure 9. Thermogravimetric equipment used for the analysis of bilirubin.

Table 11. Decomposition of various commercial bilirubins in dry air, as shown by thermogravimetric analysis

Bilirubin		Weight, mg	Beginning of rapid decomposition, °C	Beginning of recoil, °C	End of recoil, °C	Recoil temperature difference, °C	Sample weight at start of recoil, %
Sample number	Lot number						
1	39148	4.80	227	290	238	52	76.2
2	5310	4.68	250	350	270	80	75.2
3	7941-I	4.52	260	290	285	5	81.9
4	FP	5.79	277	346	292	54	72.5

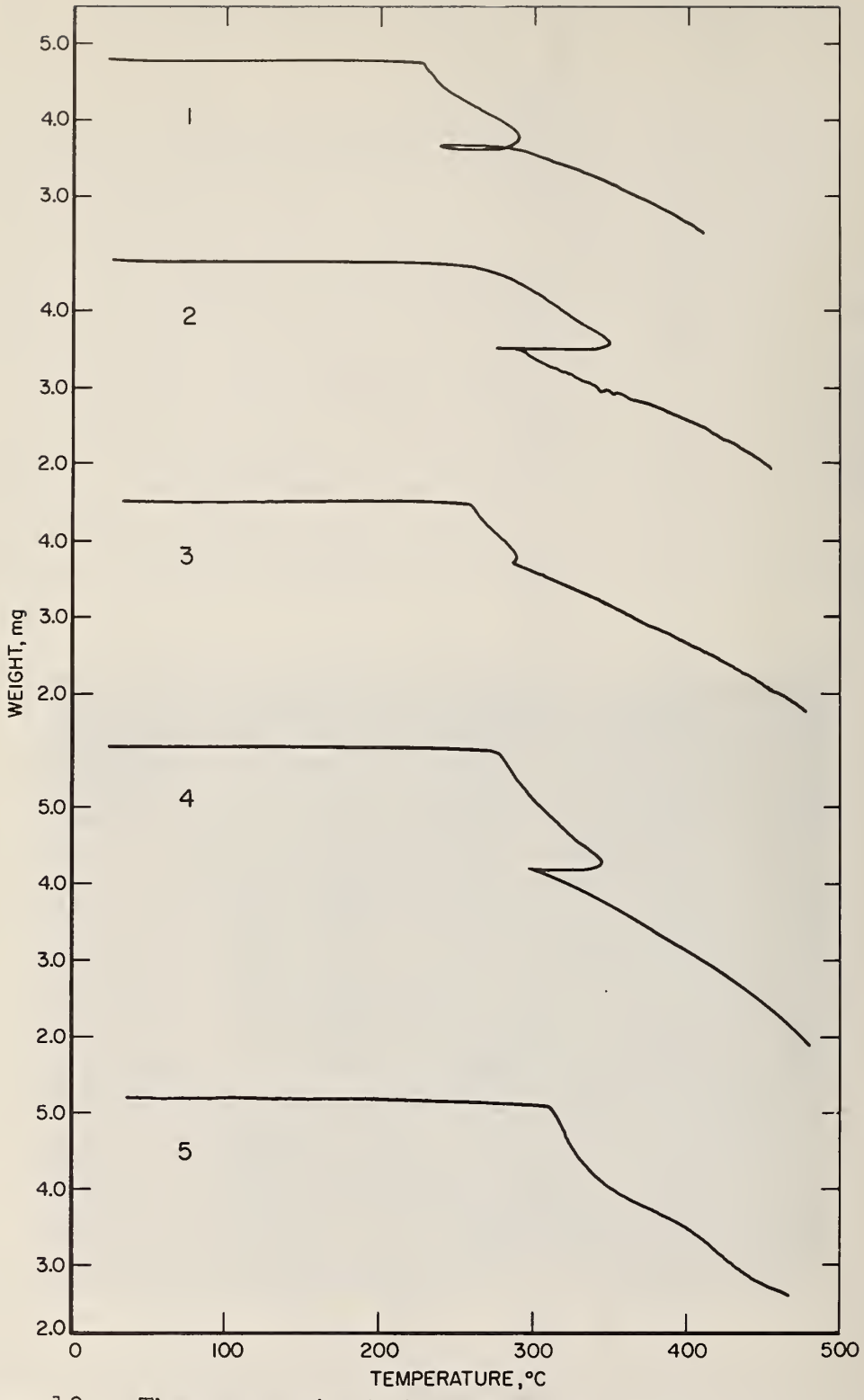


Figure 10. Thermogravimetric curves for various samples of commercial bilirubin (1, lot 39148: 2, lot 5310; 3, lot 7941-I; 4, lot FP; and 5, lot 39148 under nitrogen).

obtained with bilirubin samples heated under nitrogen; it shows that there is no evidence of the sudden drop in sample temperature ("recoil") that occurs with samples heated in air (see curves 1–4), and, furthermore, the temperature at which rapid decomposition begins is much higher under nitrogen than in air. The initial decomposition temperatures of the different bilirubin samples under nitrogen ranged from ∿300 to ∿315 °C, with most values clustering near the higher one. On the other hand, as shown in curves 1–4, initial decomposition temperatures in air are quite different from sample to sample, ranging from 227–277 °C. Thus, decomposition in air appears to provide a means for semi-quantitatively discerning the purities of the samples, assuming that the purity determines the decomposition temperature.

The drop in sample temperature observed in the thermograms of bilirubin samples heated in air is unusual. This type of thermogravimetric curve has been reported for sodium oxalate, and has been associated with an overheating generated within the sample, which (for the sodium oxalate) was due to the evolution of carbon dioxide. With the bilirubin samples, an acrid gas is evolved, but it has not yet been identified. That this overheating of the sample does not occur with bilirubin under nitrogen suggests involvement of an oxidative reaction. The thermogravimetric curves were recorded by J. T. Sterling of the Analytical Coordination Chemistry Section, N.B.S.

13. Tests for Bicarbonate-extractable Materials

(R. F. Brady, Jr., and B. F. West)

a. Purpose of the Test

Bilirubin is produced commercially from gallstones or from bile. In general, the preparation consists in saponifying the starting material with alkali (to remove the D-glucosyluronic groups), acidifying, and extracting the bile

pigments into a suitable organic solvent. The bile pigments obtained contain the weakly acidic bilirubin, contaminated by a neutral fraction and a more strongly acidic fraction. The bilirubin is separated by careful conversion into a bilirubinate salt, followed by careful neutralization and crystallization, but this process does not completely remove the impurities.

b. Qualitative Determination

The commercial bilirubins surveyed in this laboratory contained various proportions of pigments more acidic than bilirubin. A measure of the relative proportion of impurities in commercial samples was obtained by a simple test suggested by Mr. Arthur G. Holstein of Pfanstiehl Laboratories, Inc., Waukegan, Illinois. About 10 mg of bilirubin is weighed accurately, transferred to a separatory funnel, and dissolved, with shaking, in 50 ml of chloroform (unless otherwise indicated in table 12). The solution is extracted with 10 ml of 5% aqueous sodium bicarbonate, and the layers are separated. The absorbance at 420 nm of the bicarbonate extract is measured in a 1-cm cell, and the absorbance values are normalized by dividing ten times the absorbance by the sample weight. In our work, for added information, a second extraction was performed 30 min after the first, a third extraction was done one hour after the second, and normalized absorbances were obtained in the same way. The results are given in table 12.

c. Refinements in the Qualitative Procedure

The simple, extractables test showed promise of being made into a quantitative method; to do so, the following refinements were introduced.

i. In view of the sensitivity of bilirubin solutions to decomposition, the use of heat to dissolve samples in chloroform was avoided; however, it was found that samples dissolved incompletely, even after prolonged (15 min)

Table 12. Bicarbonate-extractable material in samples of commercial bilirubin

Manufacturer's lot number	Solvent	Normalized absorbance ($\underline{A}_{420}$) of the bicarbonate layer		
		First extraction	Second extraction	Third extraction
7970-I [a]	$CHCl_3$	0.21	0.07	0.09
	$CHCl_3$	0.26	0.11	0.12
	$CHCl_3$ [b]	0.23	0.08	0.26 [c]
	$\underline{o}$-$C_6H_4Cl_2$	0.18	0.08	0.11
	CH_2Cl_2	0.28	0.11	0.14
7941-I	$CHCl_3$	0.22	0.11	0.11
7941-H	$CHCl_3$	0.26	0.05	0.02
5310	$CHCl_3$	1.00	0.18	0.10
39148A	$CHCl_3$	1.66	0.47	0.24

[a] Dried for 8 hr at 60°/0.1 torr.

[b] Alcohol-free.

[c] The chloroform solution was exposed to light between the 2nd and 3rd extractions.

shaking by hand with chloroform at room temperature. Filtration showed residues that were approximately 3% of the sample weight. Instead of hand shaking, the mixture was agitated in an ultrasonic bath for 2 min, and it was then found that the residues amounted to only about 0.01%.

ii. Solution preparation and extractions were performed only under dim incandescent light; fluorescent light was specifically excluded.

iii. Solutions were filtered through tared membranes (0.5-mm Millipore). The membranes did not change in weight after the passage of pure chloroform.

iv. In early experiments, extractions were performed in separatory funnels having greased stopcocks, and films were found to form at the interface of the layers.

This may have been due both to the grease and to fine particles that had passed through the paper initially used for filtration. Teflon stopcocks and stoppers were used subsequently, and, with the use of better filtration (Millipore), the formation of films was no longer encountered.

v. The bicarbonate extracts were neutralized as rapidly as possible, to minimize the duration of exposure of the extracted material to the alkalinity (and, hence, decomposition). If the extracts contained significant proportions of impurity (~5% of the sample weight), precipitation occurred at this stage.

vi. The filtered, neutralized, combined, aqueous extract was back-extracted with chloroform. Some bilirubin-like material (λ_{max} ~450 nm) was obtained. Its visible spectrum corresponded in shape with that of bilirubin, but the difference in wavelength maximum cannot yet be explained.

vii. For measurements of absorbance, solutions were made to volume in calibrated flasks at 20 °C, and spectra were recorded in cells thermostated at 25 °C.

viii. In the calculation of materials balances, allowance was made for the aliquots taken for the various measurements. The amounts of material extracted were obtained by drying the extracts and weighing the residues.

d. Quantitative Analysis by the Bicarbonate Extraction–Materials Balance Method

The procedure, employing ~0.1 g of a bilirubin sample, consists in dissolving it in about 200 ml of chloroform, filtering, washing and weighing any insoluble residue, and, with due precaution for temperature control and careful aliquoting from a 250-ml volume, starts with the absorbance measurements of the dissolved sample. The chloroform solution is then successively extracted with 2 portions of aqueous

sodium bicarbonate and 2 portions of water, and these extracts are neutralized before further processing. After dilution of the extracted chloroform layer to 500 ml (to eliminate any turbidity due to water), large aliquots are diluted for absorbance measurements, and other (larger) aliquots are evaporated to dryness to obtain the bilirubin concentration.

The neutralized, combined, aqueous extracts are back-extracted with portions of chloroform until the chloroform extracts show no absorbance at ∿450 nm. Each chloroform extract is washed twice with water, the aqueous layers being recombined with the chloroform-extracted, neutralized, aqueous layer before the latter is subsequently re-extracted with additional portions of chloroform. The absorbances and the solids content of these chloroform extracts are measured as before. As a check for complete extraction of the impurities, the initial chloroform solution that had been successively extracted is again extracted in the same way, and the aqueous extracts are similarly processed.

Table 13 summarizes the results obtained with four commercial samples.

Table 13. Materials-balance analysis of the purity of samples of commercial bilirubin

Lot number	Number of determinations	Sample recovery, %	Minimum purity, %
39148A	3	99.63	90.4
7322	3	99.76	93.4
8162	3	99.79	97.5
FP[a]	2	99.83	99.7

[a] To dissolve this sample, it was necessary to heat the mixture for 2 min in a bath at 80 °C; then the mixture was ultrasonically shaken for 2 min.

14. The Diazo Reaction for the Assay of Bilirubin

(B. F. West)

Bilirubin is routinely assayed in bile and blood by application of a diazo coupling reaction with p-diazobenzenesulfonic acid (p-sulfobenzenediazonium hydroxide). Depending on the pH, a blue or red diazo dye is obtained. The concentration of this dye, which can be measured spectrophotometrically, gives a quantitative measure of the bilirubin present.

Because of the sensitivity of test results to experimental conditions, many modifications of the test have been introduced. In biological samples, the test conditions are designed to distinguish between free bilirubin and bilirubin that is esterified with D-glucosyluronic acid groups. In commercial preparations, however, only free bilirubin is present. In all cases, for the diazo reaction mixture to develop full color, an accelerator, either an alcohol (ethanol or methanol) or caffeine, is added.

As previously noted, the spectral absorption curve of the azo dye is affected by the pH of the solution. One general method, that of Malloy and Evelyn, uses acidic conditions; a blue color develops, and the maximum absorption is obtained at pH 1.5, but the intensity falls off rapidly, even at slightly lower pH. The other general method, that of Jendrassik and Grof, uses a buffer solution, containing sodium acetate and sodium benzoate, that affords only slightly acidic conditions and gives rise to a red color.

The extinction coefficients observed for "azo-bilirubin" in chloroform–ethanol, or aqueous caffeine–acetate mixtures, are different from those observed for reactions performed in serum and plasma. Clinical workers find that more-consistent results are obtained with the biological fluids as the solvent for the reaction.

In the present investigation, a modified Malloy–Evelyn method [65] was employed. Certain additional modifications of this procedure were introduced, to improve the precision and accuracy of the results. The experiments were performed in dim incandescent light. The volumes of bilirubin solution and coupling reagent employed were increased, so that more-accurately measured aliquots could be taken. Furthermore, the acidity of the sulfanilic acid solution was increased by doubling the amount of the hydrochloric acid added. The amount of bilirubin treated was also doubled, to increase the absorbance measured. Finally, instead of dissolving the bilirubin sample in a sodium carbonate solution, in which it is very susceptible to degradation, the bilirubin sample was dissolved in methyl sulfoxide.

The millimolar extinction coefficient for the diazo dye is calculated from the absorbance of the diazo dye and the weight of bilirubin used in the reaction. It has been reported that, for bilirubin of $\varepsilon \sim 60.0$, the ε of the diazo dye is ~ 64.0. The values of ε for the various "azo-bilirubin" preparations obtained with some commercial bilirubins by using the modifications described are shown in table 14. A solution of each commercial sample was analyzed three times, and the $\underline{A}_{545}$ values are the averages. Standard deviations are reported for the $\underline{A}_{545}$ values.

Table 14. Millimolar extinction coefficient of "azobilirubin" from samples of commercial bilirubin

Bilirubin (manufacturer's lot number)	"Azobilirubin"		Standard deviation
	$\underline{A}_{545}$	ε	
39148	0.446	63.2	0.006
7941-I	0.461	65.0	0.010
7322	0.470	66.5	0.018

15. Examination of Bilirubin Samples by X-Ray Diffraction

CuKα radiation was used to obtain X-ray powder diffraction patterns for the commercial samples of bilirubin. These measurements were made on samples in the form in which they were supplied. As the stability of bilirubin to the radiation was of concern, repeated scans were made on one sample; but no difference was evident between the pattern of reflections initially obtained and that of the fourth repeat. Also, the possibility was considered that moderately elevated heating of a sample might, at some stage in its isolation, have affected its diffraction pattern, but the pattern, obtained with a portion of the same sample after heating it for 2 hr at 65 °C/0.3 torr, was essentially unchanged.

On comparing the patterns of bilirubin samples from different commercial sources, only one was found to be markedly different, displaying fewer and different reflections; the other patterns more or less resembled each other, although there were noticeable differences: some showed sharper reflections; others had displaced, or apparently absent, reflections. The sharper peaks indicate a more regular order in the crystalline packing. However, without certain knowledge of the processing conditions through which each preparation had passed, no conclusions have been reached regarding the differences in these diffraction patterns. This work was performed by H. E. Swanson.

16. Oxidation of Biliverdine

(A. J. Fatiadi)

a. Action of Periodic Acid on Biliverdine in Methyl Alcohol

A solution (18.7 μM) of synthetic biliverdine in methyl alcohol was prepared; it had A_{374} 0.75, ε ∿40.0 (peak A) and

A_{650} 0.22, ε ~11.8 (peak B). On addition of 0.2 ml of 100 mM aqueous periodic acid to 50 ml of this solution, peak A disappeared within 30 sec. Peak B shifted to give a new peak (C) at 695 nm that reached maximum intensity within 30 sec, and then decreased continuously, and disappeared in 60 min. A new peak (D) appeared at 580 nm that decreased continuously, and a new peak (E) at 482 nm that increased continuously.

In the u.v. region, a strong peak at 277 nm disappeared, and a new peak at 225 nm appeared.

b. Action of Iodic Acid on Biliverdine in Methyl Alcohol

The same behavior was observed when a solution (22.1 μM) of synthetic biliverdine in methanol was treated with iodic acid (aq. HIO_3, 100 mM). However, the disappearance of peak A was much slower; it required about 45 min to decrease to 5% of its original height. In the u.v. region, the peak at 277 nm disappeared, but that at 238 nm shifted only slightly, to 236 nm.

17. Oxidation of Bilirubin

(A. J. Fatiadi and H. J. Elseroad)

A study was made of the action of a variety of oxidants on commerical samples of bilirubin, dissolved in various solvents, with the object of devising a new way of assaying samples of bilirubin for purity.

a. With Iodic Acid (HIO_3) in Water

In each experiment, 0.2 ml of the oxidizing solution (10 mM) was added to 50 ml of the bilirubin solution.

i. In Methyl Sulfoxide

A solution (11.3 μM) of bilirubin showed ε_{454} ~60.3 (peak A), and a shoulder at 420 nm (peak B). The oxidant was added, and, within 2 minutes, peaks A and B had disappeared;

new peaks appeared at 374 nm (peak C), 415-430 nm (peak D), and 670 nm (peak G). Peak C increased in intensity for 20 min, then decreased, and disappeared in ∿175 min. The intensity of peak G decreased continuously; it disappeared in ∿170 min. Peak D increased gradually in intensity for ∿165 min and then decreased slightly.

ii. In Methanol

A solution (12.4 μM) of bilirubin showed ε_{447} ∿60.0 (peak A) and a very slight shoulder at ∿418 nm. When the oxidant was added, peak A disappeared immediately, and new peaks appeared at 373 nm (peak C), 488 nm (peak E), 590 nm (peak F), and 685 nm (peak G). The intensities of peaks C, F, and G decreased continuously, reaching about 10% of the original intensity in 85 min. The intensity of peak E increased by about 45% in ∿60 min, and then decreased.

The new peak E (at 488 nm) was not observed when other solvents were used.

iii. In N,N-Dimethylformamide

A solution (16.0 μM) of bilirubin showed ε_{456} ∿59.5 (peak A). When the oxidant was added, peak A disappeared immediately, and new peaks appeared at 377 nm (peak C), 650 nm (peak G), and, after ∿10 min, at 547 nm (peak F). The intensities of peaks C and G gradually decreased to ∿5% of the original intensity in 45 min; that of peak F increased for 10 min and then decreased. This behavior differs from that encountered on oxidation of the same solution with periodic acid (see page 73).

iv. In 1-Methyl-2-pyrrolidinone

A solution (13.3 μM) of bilirubin showed ε_{453} ∿60.6 (peak A). When the oxidant was added, peak A disappeared immediately, and new peaks appeared at 383 nm (peak C), 420 nm (peak D), and 660 nm (peak G). The intensities of these peaks decreased to 20% of the original values in

45 min. This behavior differs from that shown on oxidation of the same solution with periodic acid (see below).

v. In 2-Pyrrolidinone

A solution (13.9 μM) of bilirubin showed ε_{454} ∿60.3 (peak A). On addition of the oxidant, this peak disappeared, and new peaks at 378 nm (peak C) and 694 nm (peak G) were formed. The intensity of peak C decreased, and the peak shifted to a lower wavelength; that of peak G also decreased with time.

b. With Periodic Acid (H_5IO_6) in Water

In each experiment, 0.2 ml of the oxidant (10 mM) was added to 50 ml of the bilirubin solution.

i. In Methyl Sulfoxide

A solution (13.3 μM) of bilirubin showed ε_{453} ∿59.9 (peak A) and a shoulder at 419 nm (peak B). On addition of the oxidant, peaks A and B disappeared within 2 min (see fig. 11). A new peak appeared at 380 nm (peak C), a shoulder at 425 nm (peak D), and a peak at 695 nm (peak E). The intensity of peak C decreased 36% in 3 hr and 84% in 64 hr, while the maximum slowly shifted to 372 nm. Peak E correspondingly decreased in intensity, without a shift in the maximum, and the band had disappeared completely after 64 hr. The intensity of peak D decreased relatively slowly; the decrease after 64 hr was ∿65%, with no shift in the position of the maximum. In table 16, for purposes of intercomparison, the new peak E is labeled as peak G.

ii. In Methanol

A solution (6.0 μM) of bilirubin showed ε_{448} ∿59.9 (peak A) and a slight shoulder at 417 nm (peak B). On addition of the oxidant, peak A vanished immediately, and new peaks appeared at 485 nm (peak E), 580 nm (peak F), and 690 nm (peak G). The intensity of peak E increased ∿90% in

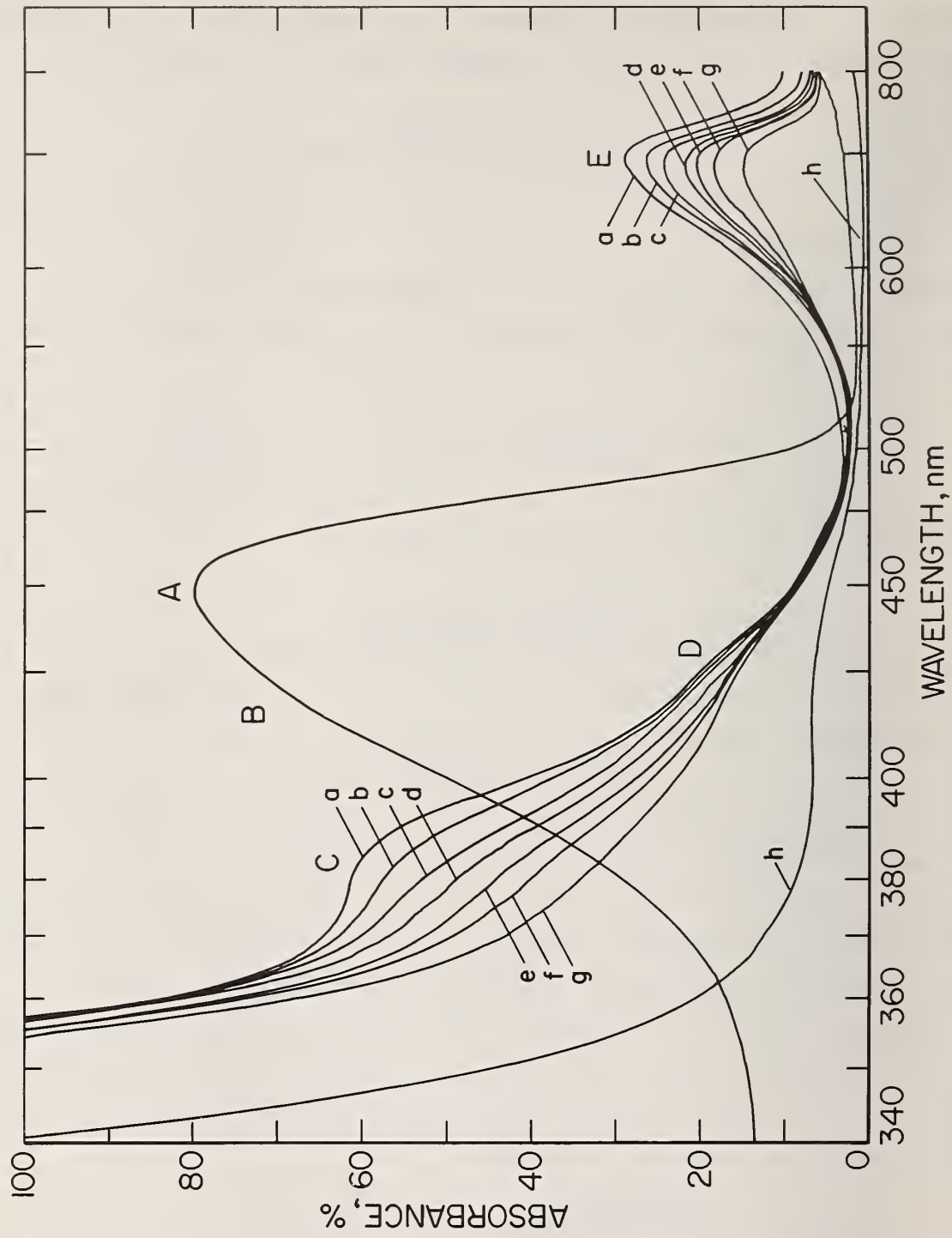


Figure 11. Oxidation of bilirubin (in methyl sulfoxide) with periodic acid at 25 °C. A and B, peaks for bilirubin; C, D, and E, new peaks. Times of recording: (a) 2 min, (b) 30 min, (c) 60 min, (d) 90 min, (e) 120 min, (f) 150 min, (g) 180 min, and (h) 64 hr.

110 min and ∿130% in 145 min; that of peak F increased by ∿9% in 25 min and then decreased; that of peak G decreased ∿95% in 145 min.

A peak at 373 nm, produced with iodic acid as oxidant, was not formed. The intensity of peak E (485 nm) increased rapidly, whereas, with iodic acid as the oxidant, it increased more slowly.

iii. In N,N-Dimethylformamide

A solution (12.8 μM) of bilirubin showed ε_{456} ∿59.8 (peak A), but no shoulder at 420 nm. On addition of the oxidant, peak A disappeared in <2 min, and new peaks appeared at 377, 600, and 668 nm; these diminished in intensity with time (∿35% in 70 min). The consumption of periodic acid (moles per mole), determined by the arsenite procedure, was ∿2.18 (30 min) and ∿2.48 (45 min).

A peak at 600 nm was produced; this was not observed when other solvents were used.

iv. In 1-Methyl-2-pyrrolidinone

A solution (14.1 μM) of bilirubin showed ε_{455} ∿59.9 (peak A). On addition of the oxidant, peak A changed to a shoulder (peak D) of A_{440} 0.22; this disappeared within 20 min. New peaks appeared at 386 nm (peak C), 662 nm (peak G), and (strong) <350 nm. The intensity of peak C increased for 20 min and then decreased; that of peak G decreased continuously (∿30% in 60 min).

v. In 2-Pyrrolidinone

A solution (13.9 μM) of bilirubin showed ε_{454} ∿60.3 (peak A). On addition of the oxidant, this peak disappeared, and new peaks appeared at 385 nm (peak C), 660 nm (shoulder, G), and 695 nm (peak X). With time, the intensity of peak C increased, and those of G and X decreased.

vi. In p-Dioxane

A solution (13.9 μM) of bilirubin showed ε_{449} ∿59.5 (peak A). On addition of the oxidant, peak A disappeared, and new peaks appeared at 386 nm (peak C), 610 nm (peak F), and 710 nm (peak X). Peak C decreased in intensity with time. After 5 min, peaks F and X became a peak at 575 nm that decreased by 30% in 30 min.

vii. In Acetonitrile

A solution (6.98 μM) of bilirubin showed ε_{449} ∿60.1 (peak A). On addition of the oxidant, peak A disappeared, and new peaks appeared at 388 nm (peak C), 582 nm (peak F), and 700 nm (peak X). With elapse of time, the intensity of peaks C and F increased, and that of peak X decreased.

viii. In Bis(2-methoxyethyl) Ether

A solution (9.68 μM) of bilirubin showed ε_{446} ∿58.0 (peak A). On addition of the oxidant, the intensity of peak A decreased by ∿20% and it shifted to 452 nm; weak peaks appeared at 387 and 642 nm that decreased in intensity with time.

c. With Sodium Periodate ($NaIO_4$) in Water

i. In Aqueous Sodium Hydroxide (30 mM)

A solution (17.8 μM) of bilirubin showed ε_{425} ∿39.5. Addition of 0.2 ml of the oxidant to 50 ml of this solution caused this peak to decrease by 85% in 80 min, with a shift to 400-410 nm. Weak peaks appeared at 550 and 640 nm. The rate of oxidation was much lower than with periodic acid in an organic solvent. About the same behavior was observed on oxidation of the same solution with sodium iodate.

d. With m-Chloroperoxybenzoic Acid

i. In 1-Methyl-2-pyrrolidinone

A solution (12.9 μM) of bilirubin showed ε_{454} ∿60.4 (peak A). On addition of 0.2 ml of the oxidant (100 mM,

in 1-methyl-2-pyrrolidinone) to 50 ml of the bilirubin solution, the intensity of peak A gradually decreased and shifted to lower wavelength, as shown in table 15. A weak peak at 576 nm was also formed.

Table 15. Change in λ_{max}, and in its intensity, on oxidation of bilirubin with m-chloroperoxybenzoic acid

Elapsed time	λ_{max} (nm)	Total decrease in intensity, %
30 sec	420	38
5 min	415	58
10 min	410	63
25 min	405	70

e. With Ferric Chloride–Hydrogen Peroxide

i. In Methyl Sulfoxide

A solution (10.9 μM) of bilirubin showed ε_{452} ~60.3 (peak A) and a shoulder of ε_{419} ~49.8. The oxidant consisted of 10 mmoles of ferric chloride and 0.2 ml of 50% hydrogen peroxide in 50 ml of methyl sulfoxide. On addition of 0.2 ml of this solution to 50 ml of the bilirubin solution, peak A disappeared, and new peaks appeared at 343 nm (peak X) and 680 nm (peak G). Peak X decreased in intensity by 15% in 10 min, and peak G by 3% in 15 min.

The action of this oxidant differs from those of the other oxidants used.

f. Summary

These oxidation studies indicate that, in the presence of iodic or periodic acid, bilirubin is initially oxidized to biliverdine, which is then further oxidized to a red product (λ_{max} 482-485 nm) as shown by the fact of its formation from biliverdine. Much of the pertinent data on the effect of various oxidants on bilirubin is summarized in table 16.

Table 16. Effect of various oxidants on bilirubin, as revealed by spectrophotometry in the visible region

Oxidant	Lot no.	Solvent[a]	Initial solution of bilirubin: Concentration (μM)	Peak A: λ_{max}	Peak A: A	Peak B: λ_{max}	Peak B: A
Iodic acid	39142	Me_2SO	11.9	453	0.72	420(sh)	0.59
	39142	MeOH	12.4	447	0.75	418(sh)	0.62
	7941-I	DMF	16.0	456	0.95		
	7941-I	1-Me-2P	13.3	453	0.81		
	39142	2-P	13.9	454	0.84		
Periodic acid	39142	Me_2SO	13.3	453	0.80	419(sh)	0.66
	39142	MeOH	6.0	448	0.36	417(sh)	0.32
	7941-I	DMF	12.8	456	0.77		
	39142	1-Me-2P	14.1	455	0.85		
	39142	2-P	13.9	454	0.84		
	7941-I	p-D	13.9	449	0.83		
	7941-I	MeCN	6.98	449	0.42		
	39142	$(MeOEt)_2O$	9.68	446	0.57		
Sodium periodate	39142	(30 mM) H_2O-NaOH	17.8	425	0.70		
m-Chloroperoxybenzoic acid	39142	1-Me-2P	12.9	454	0.78		
$FeCl_3$–H_2O_2	7941-I	Me_2SO	10.9	452	0.66	419(sh)	0.54

Table 16 (Continued)

After addition of oxidant

Oxidant	Peak C		Peak D		Peak E		Peak F		Peak G	
	λ_{max}	A	λ_{max}	A	λ_{max}	A	λ_{max}	A	λ_{max}	A
Iodic acid	374	0.84	415-30	0.26 (170 min)					680	0.27
	373	0.34			488	0.34 (55 min)	590	0.19	685	0.18
	377	0.40					547	0.16 (10 min)	650	0.18
	383	0.44	420	0.18 (45 min)					660	0.19
	378	0.94							694	0.17
Periodic acid	380 →372 ---	0.61	425(sh)	0.21					695	0.29
					485	0.51 (85 min)	580	0.17	690	0.18
	377	0.41					600	0.21	668	0.22
	386	0.35	440(sh)	0.22					662	0.11[b]
	385	0.77	465(sh)	0.15					660	0.25[c]
		0.98 (45 min)								
	386	0.19					610 →575	0.22 0.20 (5 min)		d
	388	0.85 (15 min)					582	0.33		e
	387	0.21	452	0.18					642	0.10

Table 16 (Continued)

After addition of oxidant

Oxidant	Peak C		Peak D		Peak E		Peak F		Peak G	
	λ_{max}	A	λ_{max}	A	λ_{max}	A	λ_{max}	A	λ_{max}	A
Sodium periodate			420	0.48 (1 min)						
			→410	0.18 (40 min)			550	0.05	640	0.04
m-Chloroperoxybenzoic acid			420	0.49 (1 min)						
			→405	0.24 (25 min)			576	0.03		
$FeCl_3$–H_2O_2									680	0.27[f]

[a] Me_2SO, methyl sulfoxide; DMF, N,N-dimethylformamide; 1-Me-2P, 1-methyl-2-pyrrolidinone; 2-P, 2-pyrrolidinone; p-D, p-dioxane; MeCN, acetonitrile; and $(MeOEt)_2O$, bis(2-methoxyethyl) ether. [b] Peak at <350 nm (u.v. region). [c] Peak at 695 nm (A 0.351). [d] Peak at 710 nm (A 0.16). [e] Peak at 700 nm (A 0.27). [f] Peak at 343 nm (A 0.80).

B. D-Glucose (Clinical)

(B. Coxon)

A supply of microcrystalline, "anhydrous" α-D-glucose was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Illinois, for evaluation as a standard reference material. It had been packed in polyethylene bags contained in five cardboard drums. Samples were removed from the top (T) and the bottom material (B) in each drum. The samples of material are designated hereafter as samples 1T and 1B, 2B, 3B, 4B, and 5B.

1. Paper Chromatography of D-Glucose

a. On Whatman Filter Paper No. 1

Solutions of samples 1B, 2B, 3B, 4B, and 5B (100 mg of each) in water (1 ml for each) were prepared. The solutions were stored in a refrigerator when not in use. Aliquots (5 µl and 10 µl) of each solution were applied to Whatman No. 1 filter paper, and paper chromatography was performed by the descending method with 6:4:3 (v/v) butyl alcohol–pyridine–water.

Chromatograms were sprayed with the following reagents:

i. Sodium metaperiodate solution (20 mM), followed after 2 min by ammoniacal silver nitrate solution (5%).

ii. Sodium metaperiodate solution (20 mM), followed after 2 min by 2% p-anisidine hydrochloride in butyl alcohol–ethanol–water.

iii. Ammoniacal silver nitrate solution (5%).

With sprays (i) and (ii), D-glucose was detected as a colorless spot on a colored background. Spray (iii) afforded the most evident detection of the D-glucose, but, on storage in the dark for 2 weeks or more, chromatograms that had been

sprayed with spray (iii) developed, at the point of origin, small dark spots with a lighter corona. However, these were not attributable to impurities in the sugar, as such spots could also be produced by spotting distilled water on the paper.

As some typical, "pure," commercial samples of D-glucose have been found [66] to contain the rare disaccharide sophorose (2-O-β-D-glucopyranosyl-D-glucose) [67], further chromatograms were prepared on which aliquots (1 µl and 5 µl) of a 1% solution of this disaccharide were applied, together with solutions of D-glucose samples 1B–5B (5-µl and 10-µl aliquots of 10% solutions). With sprays (i) and (iii), only one spot was obtained from each D-glucose sample. On the other hand, the sophorose (10 µg) from the 1-µl aliquot of its solution was at the lower limit of detection with sprays (i) and (iii), and it may therefore be inferred that, if sophorose is present, its proportion in samples 1B–5B is below 1%.

b. On Seed-test Paper

Portions (50, 100, 150, 200, 250, and 300 mg) of sample 2B of D-glucose were applied to seed-test paper (45 x 60 cm) by spotting aliquots (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 ml) of a solution of the sample (25 g) in water (total volume of solution, 100 ml). Portions of distilled water (0.5 and 1 ml) were also applied, as a control. The paper chromatogram was developed by the descending method in 6:4:3 (v/v) butyl alcohol–pyridine–water in a large, chromatographic tank, and, after it had been dried in the air, it was sprayed with reagent (i). Only a large, single spot, elongated for larger quantities, was observed for each quantity of D-glucose taken. There was no evidence for the presence of sophorose.

Hence, paper chromatography revealed no evidence of impurities in the D-glucose.

2. Thin-layer Chromatography of D-Glucose

T.l.c. was conducted on layers (20 x 20 cm) of non-activated Silica Gel (Merck). Solutions were prepared from each of samples 1B–5B [(a) 1% (in methanol), and (b) 10% (in water)], and aliquots (1 µl) of each solution were applied to the layers. After development with 5:1 (v/v) methanol-chloroform, the plates were sprayed with either (i) ammoniacal silver nitrate solution (5%), or (ii) 5% sulfuric acid, and were then heated at 115 °C for 0.25 hr. Reagent (i) was found to be the more sensitive as a detecting spray.

Solutions (a) gave a single, dark apot having R_F 0.62. However, solutions (b) showed, also, a circular spot at the point of origin. This phenomenon was investigated further by t.l.c. of larger aliquots (5, and 10 µl) of solutions (a), together with aliquots (1 µl) of solutions (b).

If the chromatogram was developed immediately after spotting, separated spots at the origin were obtained only from solutions (b). On the other hand, if the spotted plates were stored at room temperature for 1.25 hr before development, separated spots at the origin were observed for all solutions tested.

The foregoing observations indicate that the spots at the origin are artifacts caused by chemical reactions (possibly, oxidation or polymerization) in the layer, prior to development. These reactions obviously occur more readily when the D-glucose samples are applied in aqueous solution than when they are applied in methanolic solution. This experience emphasizes that the thin-layer chromatograms should be developed as soon as possible after spotting. In some cases, this requirement may preclude the application of many different sample spots to a single plate.

T.l.c. showed no evidence of impurities in the samples of D-glucose.

3. High-pressure, Liquid Chromatography

An aqueous solution of D-glucose, sample 1T (concentration, 120 mg/100 ml) was analyzed by means of the Oak Ridge National Laboratory high-pressure, chromatography system (by Dr. Donald Young at the National Institutes of Health). By this system, the sample was detected, at 485 nm, as a single peak; and, at the level of detection of the system, the sample was found to be free of any impurities.

4. Optical Rotation of D-Glucose

Measurements were made at 20 °C, on solutions of samples 1B, 3B, 4B, and 5B in basified water, or in methyl sulfoxide, or both, by using Rudolph and Perkin–Elmer Model 141 polarimeters. Water thermostated at 20 °C was circulated through the jackets of the sample cells (1 dm, and 2 dm).

For measurements on mutarotated D-glucose in aqueous solution, samples (∿5 g, accurately weighed) of D-glucose were made up to a volume of 25.0 ml at 20 °C, with the inclusion of concentrated ammonium hydroxide (1 drop). Measurements on non-mutarotated D-glucose were made by dissolving samples (∿2.5 g) in dry methyl sulfoxide (total volume, 25.0 ml) at 20 °C. The measured rotation was constant during a period of 45 min.

For all calculations, the sample weights in air were corrected to weights *in vacuo* by application of the factor $1 + (k/1000) = 1.00064$, where, for D-glucose, $k = 0.64$.

Representative results, indicative of the similarity of the readings given by the two instruments, are given in table 17 for sample 5B.

It has previously been postulated [68] that D-glucose, D-xylose, and D-mannose each exist as a single anomeric form in methyl sulfoxide, because of complex-formation involving *cis*-hydroxyl groups in axial-equatorial relationship; *e.g.*,

in α-D-glucopyranose, the hydroxyl groups on C-1 and C-2. The large, positive, optical rotation of the solutions in methyl sulfoxide indicated that the samples consisted primarily of α-D-glucose.

Table 17. Values for the specific rotation of a solution of D-glucose in methyl sulfoxide

Polarimeter	Concentration [a] in solution	Time after dissolution (min)	$[\alpha]_D^{20}$, degrees
Rudolph	10.05	20-30	+112.65
Perkin–Elmer automatic	10.05	14	+112.55

[a] In g per 100 ml of solution.

Measurements made on solutions of Sample 4B in ammoniacal distilled water (c 20.065) by using a Perkin–Elmer polarimeter are given in table 18.

Table 18. Specific rotation, at various wavelengths, of an equilibrated solution of D-glucose in water

Wavelength (λ), nm	$[\alpha]_\lambda^{20}$, degrees, [a] calc. from wt. in air	$[\alpha]_\lambda^{20}$, degrees, [b] calc. from wt. in vacuo
589 (Na-D)	+53.22	+53.19
578	55.46	55.43
546	62.76	62.72
436	104.53	104.46
365	159.17	159.07

[a] It has been demonstrated [69] that, below pH 10.5, the equilibrium specific rotation of D-glucose is constant with respect to pH.

[b] Expected values of $[\alpha]_D^{20}$ +53.04° and $[\alpha]_{546}^{20}$ +62.89° were calculated from the empirical equations [70]

$$[\alpha]_D^{20} = +52.50 + 0.0227c + 0.00022c^2$$

and $$[\alpha]_{546}^{20} = 62.032 + 0.04257c,$$

where c is the concentration (g in vacuo/100 ml of solution).

The accuracy of the automatic polarimeter was checked by measurement of several standard quartz plates. Since there was no mechanism for thermostating the quartz plates (and aluminum supports) while they were in the analyzing cavity of the polarimeter, they were pre-equilibrated for 1–2 hr at 20 °C (NBS calibrated thermometer). The plate and support were then rapidly transferred to the cavity, and a measurement was made immediately. Because the analyzer of the Model 141 polarimeter requires relatively long periods (3–4 min) to rotate to large angular values, the polarimeter was normally preset to an angle near to the expected value. With this technique, it was possible to obtain a measurement within ∿5 sec after the plate had been inserted into the cavity. The results are given in table 19.

Table 19. Effect of gradual heating of quartz plates in a polarimeter

Quartz control plate No.	Nominal α_{546} (degrees)	λ(nm)	Time (sec)	α (degrees)[a]
1724-NBS-1965	+38.927	546	3	+38.874
			60	+38.877
			120	+38.880
			180	+38.883
1727-NBS-1965(B)	-13.691	546	3	-13.673
			60	-13.675
			120	-13.677
			180	-13.678

[a] As determined on a Perkin–Elmer Model 141 polarimeter.

The slowly changing values of the angular rotation represent the effect of the warming of the plate.

If the ratio of the nominal rotation of the quartz plates to the rotation measured on the Model 141 instrument may be used as a correction factor for the specific rotation of D-glucose measured at 546 nm with sample 4B, the value $[\alpha]_{546}^{20}$ +62.81° is obtained; this compares well with the anticipated empirical value of +62.89°.

5. Proton Magnetic Resonance Spectroscopy of D-Glucose

The proton magnetic resonance spectrum of a solution of sample 3B (50 mg) in methyl sulfoxide-d_6 (0.5 ml) was recorded on a Varian Associates Model A-60 n.m.r. spectrometer at 60 MHz, with tetramethylsilane as the internal reference standard. The spectrum showed a sharp doublet at τ 3.71, (α-1-OH, $J_{1,OH}$ 4.6 Hz), a triplet at τ 5.0 (H-1), a doublet at τ 5.1 (4-OH, $J_{4,OH}$ 5.1 Hz), a doublet at τ 5.29 (3-OH, $J_{3,OH}$ 4.4 Hz), and a doublet at τ 5.48 (2-OH, $J_{2,OH}$ ~5.2 Hz) which was assumed to overlap with a triplet at τ 5.54 (6-OH). Other complex bands appeared at τ 6–7 (H_2C-6, and ring protons other than H-1) together with a sharp singlet at τ 6.41 (water).

Further p.m.r. spectra of the solution were recorded after it had been stored at room temperature for three weeks. The spectrum had by then developed an additional doublet at τ 3.38 (β-1-OH, $J_{1,OH}$ 6.5 Hz) whose chemical shift and splitting agree with those of the β-1-OH of β-D-glucose [68]. It is obvious, therefore, that mutarotation of α-D-glucose does occur (slowly) in methyl sulfoxide; and, hence, it is questionable whether 1-OH and 2-OH of this sugar form a stable, bidentate complex with a molecule of methyl sulfoxide, as had been suggested by Rao and Foster [69]. The assignments are based on those published by Casu et al. [71]. Previous assignments of H-1 by these [72] and other [69] authors have been shown to be incorrect [73].

In this work, hydroxyl assignments were confirmed by deuterium exchange. When the partially mutarotated solution was diluted with deuterium oxide (0.2 ml), all of the multiplets in the region of τ 3.3–5.8 that had been assigned to hydroxyl groups disappeared, leaving only a doublet ($J_{1,2}$ 3.1 Hz) at τ 4.92, due to H-1 of the α-D anomer, and some ill-defined peaks at slightly higher field.

In conclusion, n.m.r. spectroscopy confirmed that the commercial sample of D-glucose is mainly the α-D anomer, and it did not afford any evidence of organic impurities.

6. Drying of D-Glucose

Aluminum pans were constructed from foil, and were pre-dried overnight at 70 °C/1 torr. D-Glucose samples 4B and 5B (~95 g of each, accurately weighed) were placed in the pans, and were dried at 70 °C/0.6–2.4 torr for a total of 114 hr. The pans of sample were cooled in a desiccator over phosphorus pentaoxide and weighed periodically. Constant sample-weight was reached after 50 hr; each sample had then lost 0.012% of its weight.

Dried samples of D-glucose were found to be hygroscopic.

7. Ash Content of D-Glucose

The ash content of the D-glucose was determined by W. P. Schmidt by heating samples (20 g) to 750 °C. For samples 3B, 4B, and 5B, ash contents of 0.004, 0.002, and 0.002%, respectively, were found.

8. Undissolved Residue in D-Glucose

The residue of samples 3B, 4B, and 5B (10 g of each) that remained undissolved by water was determined by W. P. Schmidt to be 0.002, 0.006, and 0.001%, respectively.

9. Nitrogen Content of D-Glucose

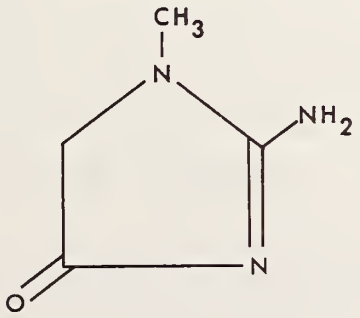
Sample 2B (1 g) was analyzed by W. P. Schmidt for nitrogen by the Kjeldahl method. Since the limit of detection by this method is 0.001%, and because no nitrogen was detected, it may be stated that the nitrogen content of sample 2B is less than 0.001%.

Spectrographic analysis of samples 3B and 5B is under way. When the results of this analysis are known, analyses for individual trace elements will be performed.

C. Creatinine

(R. F. Brady, Jr.)

Preliminary work on the analysis of creatinine (1) for issuance as a clinical Standard Reference Material has been described in Technical Note 457.



1

Refinements have been made in the phase-solubility analysis of 1. In early work, samples of creatinine, dried for 24 hr at 25 °C/0.1 torr over phosphorus pentaoxide and then analyzed by phase-solubility analysis (in absolute methanol), had been found to be 99.0% pure; but this value seemed unreasonable in the light of information that had been gathered by other techniques. It was postulated that this low value might be due to incomplete drying of the sample; the presence of water in the sample would cause the solubility of creatinine in the methanol to increase, and this increase

in the amount of 1 dissolved (with increased amounts of 1 equilibrated) would have been interpreted as being impurity. To overcome this uncertainty, the phase-solubility analysis has now been performed with 19:1 (v/v) methanol–water and with 19:1 (v/v) ethanol–water as the solvents. By this means, any contribution of water in the sample to the solubility was made negligible in relation to that of water in the solvent. In this way, the purity found was 99.8%, and repetition of the analysis of a thoroughly dried sample of 1 with absolute methanol as the solvent confirmed this value.

Creatinine was issued as Standard Reference Material 914. A reproduction of the Certificate of Analysis is shown on the following pages.

D. Urea

(B. Coxon)

On the basis of a previous investigation of the properties and purity of four commercial samples of urea [75], a sample of a commercial urea was selected as being the most suitable product for issuance as a Standard Reference Material. Bulk material was supplied in the form of well defined, colorless crystals packed in polyethylene bags contained in cardboard drums. Portions from each container were analyzed, and are designated hereafter as box or sample 1,2,3, or 4.

1. Thin-layer Chromatography of Urea

Solutions were prepared as follows:

(a) A sample (101 mg) from each container of urea was dissolved in methanol (1.0 ml) and 10 µl of each solution was applied to the chromatographic plates.

(b) A suspension of biuret (101 mg) in methanol (1.0 ml) was boiled briefly, and aliquots (5 µl) of the supernatant solution were used.

U. S. Department of Commerce
Maurice H. Stans
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 914

CREATININE

This Standard Reference Material is certified as a chemical of known purity for use in the calibration and standardization of procedures employed in clinical analysis.

Purity	99.8	percent
Volatile matter	0.03	percent
Chloride	0.07	percent
Ash	0.003	percent
Insoluble matter	0.001	percent

The value of the purity has an estimated inaccuracy of 0.1 percent.

The creatinine used for this Standard Reference Material was obtained from the Pfanstiehl Laboratories, Inc., of Waukegan, Illinois. Analyses were performed by D. A. Becker, R. F. Brady, Jr., M. M. Darr, T. E. Gills, R. A. Paulson, W. P. Schmidt, and R. S. Tipson of the Analytical Chemistry Division.

The overall direction and coordination of technical measurements leading to the certification were under the chairmanship of R. Schaffer.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by T. W. Mears.

Washington, D. C. 20234
September 24, 1968

W. Wayne Meinke, Chief
Office of Standard Reference Materials

(over)

The homogeneity of the creatinine was determined by paper, thin–layer, and gas–liquid chromatography.

Volatile matter was determined by measurement of loss of weight of the creatinine after heating for 24 hours at 110 °C.

Phase–solubility analysis [1] of an oven–dried sample of the Standard Reference Material, with absolute methanol as the solvent, indicated the purity to be 99.82 weight percent. Phase–solubility analysis of the undried creatinine with 95 percent methanol, and 95 percent ethanol, indicated purities of 99.81 and 99.76 weight percent, respectively. Potentiometric titration of the Standard Reference Material required 99.82 percent of the theoretical amount of hydrochloric acid.

The certified value for chloride was obtained by titration with mercuric nitrate of a solution of the products resulting from an oxygen–flask combustion of the Standard Reference Material.

The ultraviolet absorption spectrum of a solution of the creatinine in water showed an extinction coefficient (ϵ_{max}) of 7140 ± 30 at 234 nm. The measure of uncertainty is the standard deviation of a single measurement, and should not be considered to be a certified measure of inaccuracy for the extinction coefficient. Infrared absorption and nuclear magnetic resonance spectra provided no evidence of impurities.

An emission spectrometric analysis for metallic constituents in the ash from this Standard Reference Material showed the following present as major constituents: silicon, aluminum, sodium, titanium, iron, and magnesium. Neutron activation analysis of the bulk Standard Reference Material indicated the presence of the following approximate concentrations of elements: chlorine, 330 ppm; copper, 0.2 ppm; manganese, 0.07 ppm; sodium, 30 ppm.

[1] Mader, W. J., 'Phase–Solubility Analysis" in Organic Analysis, Vol II, Interscience Publishers, Inc., New York, 1954, p. 253.

(c) A suspension of cyanuric acid in water was boiled briefly, and aliquots (5 µl) of the supernatant solution were spotted on chromatograms.

The solutions described in (a), (b), and (c) were separately applied to 20-cm glass plates coated with non-activated layers of Silica Gel G (Merck). The layers were developed by the ascending method with 2:1 v/v methanol–dichloromethane. Developed components were detected with 1% p-(dimethylamino)cinnamaldehyde reagent [76] (DMCA), or iodine vapor.

With DMCA, urea was revealed as a white-centered red spot (R_F 0.5). Biuret (red spot, R_F 0.65) was detected in the commercial sample of biuret, and in the impure sample of urea, but not in boxes 1–4 of urea. Cyanuric acid gave a dark-purple fluorescence under ultraviolet light, but was not detectable in the pure samples of urea. Similar results were obtained with iodine vapor; with it, urea and biuret were detected as brown spots.

Plates that were sprayed with 50% sulfuric acid and then heated at 150 °C did not show any significant spots, either under room lighting or under ultraviolet light, thus indicating that, if other organic impurities were present in the urea, their proportion was at a very low level.

2. Paper Chromatography of Urea

Aliquots (10 µl for urea, 20 µl for biuret) of the solutions prepared for t.l.c. were applied to Whatman No. 1 filter paper, and the chromatogram was then developed by the descending method with 6:4:3 (v/v) butanol–pyridine–water. The separated components (urea, R_F 0.38; and biuret, R_F 0.51) were detected with

(i) DMCA. No heating was necessary for visibilization of the spots, but heating at 70 °C for 15 min intensified their red color.

(ii) 5% Silver Nitrate Solution. The chromatogram was stored in the dark for 10 days, after which time, urea was detected as a brown spot.

Urea samples 1–4 were homogeneous, according to the results of paper chromatography.

3. Infrared Spectroscopy of Urea

Urea (sample 1) was pressed into a potassium chloride disc at 12 tons/in^2, and the i.r. spectrum was recorded on a Perkin–Elmer Model 257 grating spectrometer. The resulting spectrum showed ν_{max} 3450s, 3350s, 3265m, 3225m (shoulder), 1680s, 1626s, 1601s, 1460s, 1155m, 787w cm^{-1}, and was identical with NBS Standard Spectrum No. 486, which had been obtained for recrystallized urea (Eastman).

4. Proton Magnetic Resonance Spectroscopy of Urea

Urea samples 1 and 3 (210 mg of each) were each dissolved in a portion of deuterium oxide (0.4 ml) and, after 30–70 min, the n.m.r. spectra of the solutions were recorded on a Varian Associates A-60 spectrometer at 60 MHz, with 1:1 (v/v) tetramethylsilane–carbon tetrachloride as the external reference. Proton exchange did not occur readily under these conditions, as shown by the observation of a broad singlet at τ' 4.07 (NH_2). One other resonance was observed, a sharp singlet at τ' 5.2 (HOD). No peaks for impurity were detected.

5. Ultraviolet Spectrophotometry of Urea

Urea samples 1–4 (5.0 g of each) were each dissolved in a portion of water (10.0 ml), and the u.v. spectra of the aqueous solutions (2-cm cells) were recorded with a Cary Model 14 spectrophotometer, with water in the reference cell, over the wavelength range of 220–750 nm. The absorbances observed are shown in table 20.

A weak absorption shoulder was observed at 280 nm.

Table 20. Absorbances at various wavelengths in the u.v. spectra of aqueous solutions of various samples of urea

Box	Absorbance at			
	$\lambda_{<220}$	λ_{280}	λ_{560}	λ_{565}
1	very strong	0.096	0.019	---
2	very strong	0.129	0.029	---
3	very strong	0.088	---	0.015
4	very strong	0.098	---	0.015

6. Determination of Biuret

The reagent for biuret estimations was prepared by mixing a 3% aqueous cupric sulfate solution (12.5 ml) with 10% sodium hydroxide (500 ml). Aliquots (5.0 ml) of the resulting solution were added to urea samples 1–4 (5 g of each, accurately weighed), and the crystals were dissolved by gentle warming of the suspensions. The solutions were diluted to 10.0 ml, and their absorption spectra in the visible region were recorded with a Cary Model 14 spectrophotometer, with diluted alkaline cupric sulfate reagent in the reference cell. (The baseline was set initially by recording reagent versus reagent.) The absorbances observed at the absorption maximum of the biuret–copper complex (560-565 nm) were corrected by subtraction of the corresponding absorbances of the urea samples in water alone (see part 5, above).

The quantities of biuret present were determined by reference to absorbances measured on three solutions of a commercial biuret in the diluted copper reagent. The results obtained from the standard solutions (a) and the urea samples (b) are given in table 21.

Table 21. Absorbances of solutions of (a) biuret and (b) various samples of urea in alkaline cupric sulfate solution

(a) Wt. of biuret (mg)	λ_{max} (nm)	Absorbance	Absorbance per mg of biuret	ε
1.012	550	0.012	0.012	6.1
4.248	525	0.054	0.013	6.6
8.450	505	0.107	0.013	6.5
(b) Urea sample	Absorbance[a]	Corrected absorbance[a]	% of biuret	
1	0.059	0.040	<0.06[b]	
2	0.066	0.037	<0.06	
3	0.057	0.042	<0.07	
4	0.061	0.046	<0.07	

[a] At 560-565 nm. [b] Less than, because chromatography indicated that the commercial sample of biuret contained some urea.

7. pH of Urea in Aqueous Solution

The pH values of solutions of urea samples 1–4 (2.5 g) in carbon dioxide-free, distilled water (25.0 ml, total volume) were measured with a Corning, expanded-scale, pH meter and were found to be 7.06, 7.06, 6.97, and 7.30, respectively.

8. Drying of Urea

a. At Room Temperature over Phosphorus Pentaoxide

The urea samples were pulverized in a mortar, and each sample (∿11 g, accurately weighed) was dried over phosphorus pentaoxide at room temperature/0.01 torr, with a solid carbon dioxide–dichloromethane trap, for a total of 206 hr. The samples were weighed periodically during this time. The weight of each sample decreased progressively, at an approximately constant rate. After 206 hr, the mean total loss in weight ranged between 2.84 and 3.08%, a mean rate of 0.0144% per hr.

b. At Room Temperature (No Desiccant)

Urea (32.5 g, from box 2) was powdered, weighed, and then dried at room temperature/0.01 torr for 48 hr with two liquid nitrogen traps.

The sample was then dried for a further 304 hr, but with two solid carbon dioxide—dichloromethane traps. The results shown in table 22 were obtained.

Table 22. Loss in weight of urea at 25 °C/0.01 torr after various time-intervals

Drying time (hr)	$\underline{w}$ (% per hr)[a]	Total wt-loss (%)
24	0.00031	0.0075
48	0.00026	0.0137
118	0.00020	0.0278
190	0.00013	0.0369
326	0.00012	0.0539
352	0.00026	0.0605

[a]Mean % loss in weight per hr since previous weighing.

After a total of 352 hr, air was admitted to the traps through a calcium chloride tube. The condensate was a colorless, turbid liquid (1.00 g) which turned anhydrous cupric sulfate blue, and gave a faint red spot when spotted on filter paper, sprayed with the reagent for urea (DMCA), and heated.

Karl Fischer determinations (by J. H. Thomas) on two specimens of the dried material showed that it still contained water (0.073 and 0.086%).

c. At 115 °C

Urea sample 2 (∿10 g, accurately weighed) was dried at 115 °C in an oven at atmospheric pressure. Periodic weighing gave the results shown in table 23.

Table 23. Loss in weight of urea at 115 °C/760 torr after various time-intervals

Drying time (hr)	$\underline{w}^a$ (%)	Total wt.-loss (%)
1	0.089	0.089
2	0.064	0.153
3	0.050	0.203
4	0.048	0.251
122	27.577	27.828

[a] Weight loss (%) since previous weighing.

The excessive loss in weight, and the fact that the dried material was no longer soluble in water, indicated that extensive decomposition had taken place.

9. Change in Weight on Exposure to the Atmosphere

Urea sample 2 (15 g) was exposed to the atmosphere over a 15-day period. After 1 day, the sample weight had increased by 0.0033%, but, after a further 14 days, the weight of the sample was 0.006% less than the initial weight.

It was concluded that urea shows little tendency to gain or lose moisture when exposed to air in the laboratory.

Confirmation was obtained from Karl Fischer determinations (by D. W. Vomhof and J. H. Thomas) on the exposed material, which showed a moisture content (0.17%) that was comparable with that (0.19%) of freshly opened samples.

10. Determination of the Purity of Urea by Differential Scanning Calorimetry

The experimental method used was that of Plato and Glasgow [77, 78].

The area of the recording chart was calibrated by observation of the melting-point transition of ultrapure

indium (3.0545 mg). The calibration factor was 0.02340 mcal/unit area, the area being measured by a planimeter.

The results obtained (sample weights 0.7695-0.8705 mg) are shown in table 24. The results indicated a high degree of homogeneity in material from different containers.

Table 24. Purity of various samples of urea, as determined by differential scanning calorimetry

Urea, box no.	ΔT (degrees)	ΔH_f (kcal/mol)	ΔH_f (kJ/mol)	Purity, mole %
1	0.133	3.76	15.7	99.85
2	0.169	3.83	16.0	99.80
3	0.147	3.82	16.0	99.83
4	0.153	3.74	15.6	99.82

11. Determination of the Purity of Urea by Phase-solubility Analysis

(R. F. Brady, Jr.)

The solubility of a pure compound in a pure solvent is a fundamental physical property of the compound. This property is exploited in phase-solubility analysis, which has been employed in an attempt to obtain quantitative data on the purity of urea. This technique measures the amount of material dissolved when increasing amounts of a substance are equilibrated with 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 proportion of impurity in the sample can be determined. Isopropyl alcohol, in which urea has been found to be soluble to the extent of 29.0 mg/g at 25.0 °C, was chosen. Sealed tubes containing weighed, progressively larger weights of urea and uniform weights of

isopropyl alcohol were allowed to equilibrate in a water bath at 25.0 ±0.1 °C. The weights of urea were so chosen that at least one tube contained an unsaturated solution. After equilibration, aliquots of each solution were separated from any undissolved urea, and evaporated to dryness, and the residues were 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. This diagram is shown in fig. 12. The content of solute throughout the range of saturated solutions studied was found to increase slightly. The sample of urea investigated was found to be 99.82 weight-percent pure, in agreement with the value of 99.82 mole-percent purity obtained by differential scanning calorimetry. Phase-solubility analysis is generally to be considered accurate to within ±0.1%.

Urea was issued as Standard Reference Material 912. A reproduction of the Certificate of Analysis is shown on the following pages.

E. Calcium Carbonate

Calcium carbonate for use in clinical laboratories was issued as Standard Reference Material 915. A reproduction of the Certificate of Analysis is shown on the following pages. Dr. Robert Schaffer coordinated the work leading to its certification.

F. Uric Acid

As a result of the work described in Technical Note 457, uric acid was issued as Standard Reference Material 913. A reproduction of the Certificate of Analysis is shown on the following pages.

Figure 12. Phase-solubility diagram of urea in isopropyl alcohol at 25 °C.

U. S. Department of Commerce
Maurice H. Stans
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 912

UREA

This Standard Reference Material is certified as a chemical of known purity for use in the calibration and standardization of procedures employed in clinical analysis.

Purity	99.7	percent
Moisture	0.18	percent
Biuret	0.07	percent
Ash	0.002	percent
Insoluble matter	0.002	percent

The value of the purity has an estimated inaccuracy of 0.1 percent.

The urea used for this Standard Reference Material was obtained from the Mann Research Laboratories of New York, N.Y. Analyses were performed by D. A. Becker, R. F. Brady, Jr., B. Coxon, M. M. Darr, T. E. Gills, R. A. Paulson, W. P. Schmidt, J. Thomas, and D. W. Vomhof of the Analytical Chemistry Division.

The overall direction and coordination of technical measurements leading to the certification were under the chairmanship of R. Schaffer.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by T. W. Mears.

Washington, D. C. 20234
September 24, 1968

W. Wayne Meinke, Chief
Office of Standard Reference Materials

A satisfactory method for drying this Standard Reference Material was not found. Either incomplete drying, or excessive (and progressive) weight loss occurred, depending on the degree and intensity of heating and evacuation. However, it was found that this material showed little tendency to gain or lose weight (± 0.003 percent) when exposed to laboratory air for a two–week period. Hence, the analyses were performed on this Standard Reference Urea without attempting to lower its moisture content. It is recommended that, after the withdrawal of portions of the Standard Reference Material, the container should be tightly closed. Thus protected, it may be assumed that the moisture content of the Standard Reference Material will remain unchanged. The reported moisture content was determined by the Karl Fischer titration method.

The apparent purity of this Standard Reference Material was determined by two methods. Differential scanning calorimetry [1], performed with a Perkin–Elmer model DSC–1B* instrument on the urea heated through its melting transition, indicated an apparent purity of 99.82 ± 0.003 mole percent. Phase–solubility analysis [2] of the urea in isopropyl alcohol indicated an apparent purity of 99.82 weight percent. These values are designated as "apparent purity", because neither method accounted for the moisture content of the urea. Biuret was estimated spectrophotometrically by use of 50 percent (wt/vol) solutions of urea in aqueous alkaline cupric sulfate measured at its absorption maximum near 560 nm. Measured in a 2–cm cell, a 50 percent (wt/vol) solution of urea in water showed a very strong absorption below 220 nm, a weak absorption (0.10±0.03) at 280 nm, and a very weak absorption (0.02 ± 0.01) at 560 nm.

Neither paper nor thin–layer chromatography indicate any evidence of biuret or cyanuric acid. Neither infrared absorption nor nuclear magnetic resonance spectroscopy reveal any unexpected peaks and, hence, they give no evidence of impurity.

The melting range of this Standard Reference Material was 131.5 to 132.4 °C when measured in an open capillary tube heated at 0.5 °C/min.

A 10 percent (wt/vol) solution of this Standard Reference Material in water free from carbon dioxide showed a *p*H of 7.0 ± 0.1 at 20 ° C.

An emission spectrometric analysis for metallic constituents in the ash from this Standard Reference Material showed the following present as major constituents: aluminum, silicon, iron, sodium, nickel, calcium, magnesium, and manganese.

Neutron activation analysis of the bulk Standard Refcrence Material indicated the presence of the following approximate concentrations of elements: aluminum, 0.9 ppm; chlorine, less than 1 ppm; copper, 0.27 ppm; gold, 0.0001 ppm; manganese, 0.008 ppm; sodium, 9 ppm; zinc, 0.24 ppm.

[1] Plato, C. and Glasgow, A. R., Jr., Abstracts of Papers, 154th American Chemical Society Meeting, September 1967, p. 44A; Submitted to Anal. Chem. September 1968.

[2] Mader, W. J., "Phase Solubility in Organic Analysis" in Organic Analysis, Vol II, Interscience Publishers, New York, 1954, p. 253.

*Note – The use of proprietary designations in this certificate are for information only, and should not be construed as an endorsement of the product by either the Departmcnt of Commerce or the National Bureau of Standards.

U. S. Department of Commerce
Maurice H. Stans
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 915

Calcium Carbonate

This Standard Reference Material is certified for use in the calibration and standardization of procedures employed for the determination of calcium in clinical analysis. The sample consists of highly purified calcium carbonate, and chemical assay as well as analysis for specific impurities indicate that the material may be considered to be essentially pure.

Purity	99.9+ percent
Water	0.01 ± 0.005 percent

Replicate samples taken from a randomly selected region of the undried material were assayed by a coulometric acidimetric procedure. The results from nine independent determinations, based on expression of the assay as calcium carbonate, indicate a purity of 99.99+ percent with a standard deviation of 0.003 percent. Samples equilibrated at a relative humidity of 90 percent and assayed by this coulometric procedure showed a maximum moisture adsorption of 0.02 percent as compared to samples that were dried for 6 hours at 210 °C. The moisture content, similarly measured, on samples equilibrated at 75 percent relative humidity, was found to be 0.01 percent. The water content was determined by the Karl Fischer method.

The calcium carbonate used for this Standard Reference Material was obtained from the J. T. Baker Chemical Company, of Phillipsburg, New Jersey. Analyses were performed by C. E. Champion, E. R. Deardorff, G. Marinenko, O. Menis, T. C. Rains, T. A. Rush, W. P. Schmidt, B. F. Scribner, V. C. Stewart, J. K. Taylor, and D. W. Vomhof.

The overall direction and coordination of technical measurements leading to the certification were under the chairmanship of R. Schaffer.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by J. L. Hague.

Washington, D. C. 20234
March 4, 1969

W. Wayne Meinke, Chief
Office of Standard Reference Materials

Supplemental Information

The material was examined for compliance with the specifications for reagent grade calcium carbonate as given in *Reagent Chemicals*, 4th edition, published by The American Chemical Society. The material was found to meet or exceed the minimum requirements in every respect. Examination by thermal gravimetric analysis indicated the loss of a minute proportion of weight below 175 °C (volatile matter) and the composition was stable above this temperature until a temperature of 625 °C, above which decomposition (evolution of CO_2) set in.

A semi-quantitative survey for trace contaminants by emission spectroscopy indicated the presence of less than 0.001 percent of copper, iron, magnesium, manganese and silicon in the material. By atomic absorbtion, magnesium was evaluated at 1.0, sodium at 0.4, and strontium 2.1 parts per million (ppm) ; potassium was less than 0.4, lithium less than 0.05, and barium much less than 10 ppm. Neutron activation analysis indicated copper 0.9, manganese 0.6 and sodium 0.5 ppm. Copper was determined at 1 ppm by spectrophotometry.

U. S. Department of Commerce
Maurice H. Stans
Secretary

National Bureau of Standards
A. V. Astin, Director

Certificate of Analysis

Standard Reference Material 913

URIC ACID

This Standard Reference Material is certified as a chemical of known purity for use in the calibration and standardization of procedures employed in clinical analysis.

Purity	99.7 percent
Volatile matter	0.14 percent
Ash	0.057 percent

The value of the purity has an estimated inaccuracy of 0.1 percent.

The ash was found to be composed principally of salts of sodium and potassium. Aluminum, calcium, iron, phosphorus, and silicon were found in proportions estimated to be between 1 and 10 percent of the ash. Cobalt, copper, manganese, nickel, and zinc were each present to an extent not exceeding 1 percent of the ash.

The uric acid used for this Standard Reference Material was obtained from the Pfanstiehl Laboratories, Inc., of Waukegan, Illinois. Analyses were performed by D. P. Enagonio, R. A. Paulson, W. P. Schmidt, V. C. Stewart, R. S. Tipson, and B. F. West of the Analytical Chemistry Division.

The overall direction and coordination of technical measurements leading to the certification were under the chairmanship of R. Schaffer.

The technical and support aspects concerning the preparation, certification, and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by T. W. Mears.

Washington, D. C. 20234
September 24, 1968

W. Wayne Meinke, Chief
Office of Standard Reference Materials

The uric acid used in this Standard Reference Material was found to be homogeneous by paper and thin–layer chromatography with several solvent systems. This homogeneity was further verified by gas–liquid chromatography of the trimethylsilylated material.

Volatile material was determined by heating the sample overnight at 110 °C.

An emission spectrometric analysis for metallic constituents in the ash from this Standard Reference Material showed the following present as major constituents: sodium, potassium, aluminum, calcium, iron, phosphorus, and silicon. Neutron activation analysis of the bulk Standard Reference Material indicated the presence of the following approximate concentrations of elements: sodium, 170 ppm; copper, 2.4 ppm, manganese, 0.028 ppm.

The ultraviolet absorption spectra of the material exhibited the following absorption maxima:

In lithium carbonate solution, *p*H = 7.6.

at 292 nm, ϵ_{max} = 12,560 ± 10

at 236 nm, ϵ_{max} = 10,000 ± 80

In a glycine-buffered solution, *p*H = 9.6

at 292 nm, ϵ_{max} = 12,650 ± 50

at 234 nm, ϵ_{max} = 9,890 ± 50

The measures of uncertainty given are the standard deviation of a single measurement. They should not be considered to be a certified measure of inaccuracy for the extinction coefficients.

A 100–g sample of uric acid was extracted successively with 500 cm^3 each of water, absolute ethanol, benzene, and water. The ultraviolet absorption spectrum of each of these extracts showed no absorption bands other than those of uric acid. Only uric acid was detected by thin–layer chromatography of these same extracts.

G. Cholesterol. Post-issuance Studies

In Technical Note 457, we reported analyses performed on the cholesterol SRM some six months after issuance. Since then, further studies have been carried out at two additional half-year intervals. The objective of this continuing study is to evaluate the shelf-life of the standards, for it is known that cholesterol may undergo decomposition, and this imperils the standard.

1. Thin-layer Chromatography of Cholesterol Samples

(A. Cohen)

Chromatography was performed on activated Silica Gel H plates with 1:1 (v/v) ethyl acetate–heptane, as described in Technical Note 457. At one year after issuance and at one-half year later still, the bulk supply of standard material (that had been stored in a freezer) showed no significant evidence of greater impurity than had been detected in the material when originally tested or at six months after issuance. SRM packaged samples, which are bottled under nitrogen, behaved much like the freezer-stored supply. However, samples that had been kept at room temperature in amber bottles loosely covered to ensure exposure tc air showed a significant growth of impurities that we estimated as amounting roughly to 1% at one year of exposure and to greater than this after six months more.

2. Liebermann–Burchard Analysis of Cholesterol Samples

(A. Cohen)

New measurements made by our modification (see Technical Note 457) of this method showed no significant difference between the packaged, the freezer-stored, or the air-exposed cholesterol at one year after issuance; for these samples, molar absorbances (liters/mole cm) at 535 nm were

596 ±2, 597 ±1, and 597 ±3 (averages of 5 or 6 aliquots per sample ± 1 standard deviation), respectively. These values are almost identical with those obtained on similarly stored samples at six months after issuance. Thus, these results do not distinguish between the better and poorer cholesterol.

3. Phase-solubility Analysis (PSA) and Differential Scanning Calorimetry (DSC)

(R. F. Brady, Jr., and D. A. Niner)

PSA and DSC were applied to cholesterol samples as described in Technical Note 457, where it was reported that, at six months after issuance, PSA indicated 99.96 weight-percent purity, and DSC, 99.84 mole-percent purity for the freezer-stored cholesterol. After the elapse of an additional year, the purity values for this material were found to be 99.6 weight-percent and 99.5 mole-percent, and samples of packaged SRM cholesterol showed purities of 99.6 weight-percent and 99.5 mole-percent by DSA and DSC, respectively. At the time of the most recent reevaluations by DSC, new measurements of the purity of SRM urea were obtained, and these values found were almost exactly those obtained earlier. Thus the changes in the weight- and mole-percent purities of the cholesterol appear to be real. Since the SRM cholesterol was originally certified as 99.4 ±0.3 percent, and our recent work still shows a purity on the high side of this range, the SRM continues to meet the specifications stated. Further evaluations will continue.

3. DETECTION AND IDENTIFICATION OF TRACES OF ORGANIC COMPOUNDS

In much of the Section's work, particularly that leading to the certification of SRM's, traces of impurities are encountered, and the identification of these trace substances is usually desired. As a guide for identification, there may be, in the literature, information on impurities that have previously been identified in work on the major component, or use may be made of knowledge on the origin (natural source) or the method of synthesis of the compound that is the major constituent. Occasionally, grossly impure commercial preparations can provide a source for the impurities.

T.l.c. with several different adsorbents and developing solvents is frequently employed for establishing the identity of a known reference compound with the impurity; however, such evidence alone is seldom entirely satisfactory. Additional characterization is needed, e.g., mass spectra, ultraviolet and infrared spectrophotometry, etc. Since an infrared spectrum is almost always unique for a compound, characterization by infrared spectroscopy in combination with t.l.c. usually provides firm proof of identity; our work began with a comparisom of these two techniques.

To ascertain the relationship between the quantities of compounds that could be detected by t.l.c. versus those needed for full characterization of them by infrared spectroscopy, micro-sampling techniques for infrared spectroscopy were studied along with the limits of detection for the same compounds in t.l.c. The approach was to utilize compounds for which each of the techniques would be quite sensitive. Some polynitro compounds were employed, because (a) nitro groups have high absorptivity in the infrared region, and (b) there are sensitive methods for detecting such compounds on thin-layer chromatograms.

A. Thin-layer Chromatography

(A. Cohen)

For determining detection-limits by t.l.c., aliquots of a solution of 2,4,6-trinitrotoluene in acetone (5 g/l) were transferred, as separate spots, to silica gel plates containing a 254-nm activated, fluorescent indicator (Silica Gel GF) such that the quantities of compound in individual spots ranged from 5 to 40 ng. After developing with 9:1 (v/v) benzene–pentane and drying under nitrogen, the chromatogram, examined with 254-nm light, showed no evidence of the nitro compound in spots containing less than 40 ng; but, at 40 ng, there was some suggestion of detection. On spraying the chromatogram with ethylenediamine in methyl sulfoxide [79], there was questionable detection at 15 ng, and certain detection of the spots with 20-, 30-, and 40-ng quantities of the compound, by a faint pink coloration.

With hexahydro-1,3,5-trinitro-s-triazine, taken as an example of a nonaromatic trinitro compound, the quantities studied ranged from 30 to 150 ng. Benzene–acetone (4:1 v/v) was used for development of the plates (Silica Gel GF). On examination under 254-nm light, only spots containing 120 ng or more were detected definitely; there was considerable uncertainty for amounts at the 60- and 90-ng level. For making the spots visible under ordinary light, the plate was treated as follows: it was first sprayed with methanolic potassium hydroxide [80] and warmed at 115 °C for 5 min, and then sprayed with a Benzidine Blue reagent [81]. Quantities of the compound, 60 ng or more, were visible as weak blue or purplish spots after this treatment.

These examples showed that, with visual observation, fluorescence quenching provided no advantage over colorimetric tests for detection of the two polynitro compounds. Nevertheless, fluorescence quenching has the advantage of revealing the presence of the compounds without altering them chemically,

and thus it affords the option of isolating the chromatographically separated compound and utilizing it for additional characterization.

B. Infrared Spectroscopy

(A. Cohen)

Methods previously reported for the preparation of microsamples for infrared spectroscopy employed (a) freeze-drying of the sample plus potassium bromide, (b) grinding of the sample with potassium bromide in a volatile solvent, or (c) adding the compound, in a volatile solvent, to a preground specimen of potassium bromide [82]. In our work, a weighed quantity of preground potassium bromide (5–7 mg) and an aliquot of a solution of the compound in chloroform or acetone were combined in a small test-tube, and mixed gently with a glass rod. Evaporation of the solvent was speeded with a gentle stream of nitrogen. The dry mixture was transferred to a 100-mm, ultramicro die-assembly (Perkin–Elmer) for pressing into a disc, after initial evacuation of the assembly at about 2 torr. The discs produced weighed 4 to 5 mg. The sample (in the potassium bromide disc) was mounted in a 4X beam condenser, and examined with Perkin–Elmer infrared spectrometers (Models 257 and 621) without ordinary-scale expansion. Fully characteristic spectra were obtained when a disc contained 4 to 5 μg of a compound. Below this amount, the spectra did not show the less-intense bands, and therefore were less characteristic of the compound. With 0.5 μg of compound in the disc, only the most intense bands in the spectra of the polynitro compounds were barely discernible above the noise level. With use of ordinate-scale expansion, improvement by a factor of 2 or 3 could be reached. Nevertheless, the quantities of the compounds needed for providing characteristic infrared spectra were found to be many times greater than those necessary for useful examination by t.l.c. This subject is undergoing further study.

4. ANALYTICAL CHEMISTRY OF CORN SYRUPS AND RELATED PRODUCTS

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

This Research Associate Program, supported by the Corn Industries Research Foundation and devoted to the study of corn syrup hydrolyzates, has completed its initial phase, the development of assured methods for the evaluation of the moisture content of these hydrolyzates. The methods are described in this report. The additional physical properties that are to be evaluated are briefly sketched.

A. Direct Determination of Moisture

Prior to undertaking studies of other physical properties, alternatives to the vacuum-oven method for the direct determination of the moisture content of starch hydrolyzates were evaluated. Requirements for a suitable direct method of moisture analysis are: a high degree of accuracy, a low level of imprecision, freedom from interference (either by the saccharides or the ash present), freedom from degradation or chemical action resulting in the production of volatile substances or the consumption of moisture, and, finally, reasonable rapidity. Three methods have been developed which satisfy these criteria.

1. Near-infrared Spectrophotometry

One direct method found suitable is a near-infrared, spectrophotometric method specific for water. The determination depends on measurement of the absorption of energy at 1.9 μm by water molecules. The amount of energy absorbed is directly proportional to the quantity of water present. Hydroxyl groups of saccharide molecules do not interfere.

Calibration is achieved by determining the absorbance of solutions containing known concentrations of water in a suitable solvent and the results are plotted graphically.

The best solvent for this purpose appears to be methyl sulfoxide (Me_2SO). Solutions of the syrups are prepared in solvent from the same lot as that used for calibration, care being taken to ensure complete dissolution of the syrup. The net absorbance, measured with a millimeter rule, is obtained by subtracting the base 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

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

where $\underline{F}$ = the weight-percentage of water in the original syrup, $\underline{W}$ = the weight-percentage of water in the sample solution, and $\underline{S}$ = the weight-percentage of syrup in the sample solution.

The near-infrared absorption spectra of water, sucrose, and a corn syrup in Me_2SO are shown in figure 13. The hydroxyl groups of the saccharide are the major contributors to the absorbance in the region from 2.0 to 2.1 μm. The minimum at 2.0 μm is dependent on both the content of saccharide and of the water in the solution.

The increase in absorbance is linear over the range from 0.0 to 1.3 weight-percent of moisture in the sample solution. The standard deviation of the method, based on 24 degrees of freedom, is 0.034 weight-percent of water for the moisture content of the original syrup. The detection limit [83] (having a 95% chance of being detected) is 0.0071 weight-percent of water in the sample solution. The time required for the analysis of a syrup, including the preparation of the solution and the calculations, is about 90 minutes.

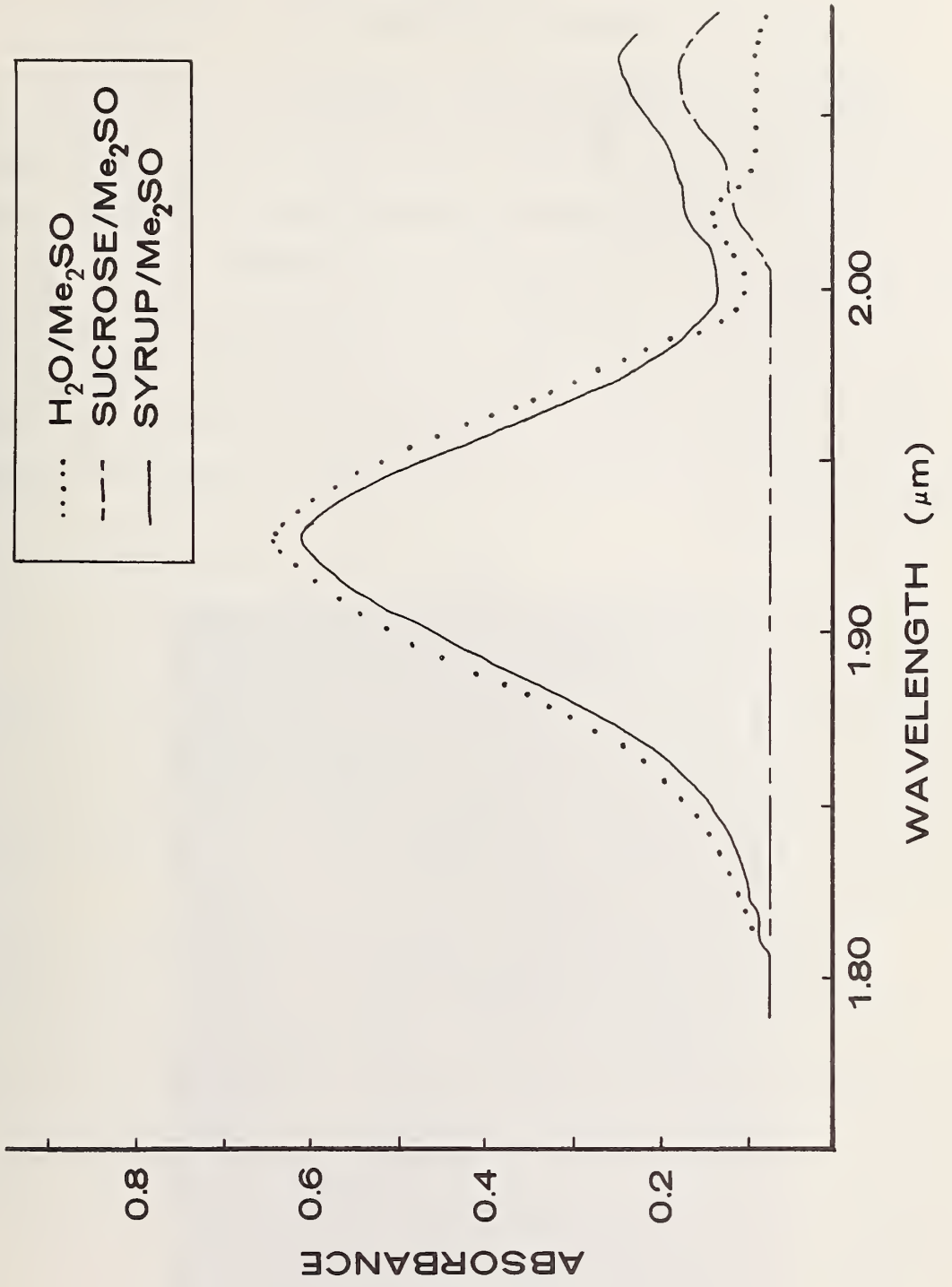


Figure 13. Near-infrared absorption spectra of water, sucrose, and a corn syrup (each dissolved in methyl sulfoxide).

2. Infrared Spectrophotometry

Another spectrophotometric method, not acceptable for our work because of inaccuracy and imprecision, is suitable for many applications where a knowledge of moisture content is required. In this method, calibration and sample solutions are prepared as in the near-infrared method. Me_2SO is the solvent of choice. The determination depends on the measurement of the energy absorbed by the water molecules when the sample is exposed to infrared radiation at 1640 cm^{-1}. The net amount of energy absorbed is proportional to the quantity of water present, with no interference from the hydroxyl groups of the saccharide.

The transmittance at 1786 cm^{-1} is taken as the base level in determining the net absorbance. In this method, unless an infrared spectrophotometer is used that can operate in the absorbance mode, the values recorded are in percent transmittance; these values must be converted into absorbance units prior to preparing the working calibration plot. As a consequence, the method suffers in terms both of accuracy and precision. The calculations are handled in the same way as for the near-infrared method.

The change in absorbance with increasing concentration of water is linear over the range from 0 to 1.4 weight-percent of water in the sample solution. The standard deviation of the method, based on 21 degrees of freedom, is 0.135 weight-percent of water in the original syrup (about 20% of water). The detection limit [83] (at the 95% level) is 0.016 weight-percent of water in the sample solution. The total time required for an analysis is about 90 minutes.

3. Gas–solid Chromatography

A third direct method for determination of moisture, comparable in terms of accuracy and precision to the near-infrared and vacuum-oven methods, utilizes gas–solid chromatography. In this procedure, the components (of a solution) having different partition coefficients are separated as they pass through a column. In the present work, the solid support was Porapak Q (Waters Associates), 80–100 mesh, in a copper column (0.25 in O.D. x 5 ft). The carrier gas was helium. The column was operated at 220 °C (isothermal), and the separated components were detected with a thermal conductivity detector operated at a temperature of 220 °C. The sample size was 1 µl.

Figure 14. Gas-chromatography equipment for the determination of water in starch hydrolyzates.

Calibration standards and sample solutions are prepared on a weight basis, with Me_2SO as the solvent. The solvent serves as an internal standard in this method. The ratio of the water peak-height to the solvent peak-height (measured with a millimeter rule) is plotted against the concentration of water in the solution. The water present in the original syrup is calculated by using the equation discussed for the near-infrared method (see p. 112).

To improve the accuracy and precision of the results, three aliquots of a solution are injected at 1.5-minute intervals, and the resulting peak-height ratios are averaged. This averaged ratio is used in conjunction with the calibration curve. Fig. 15 shows a chromatogram of three injections of a syrup solution.

Over the concentration range of 0.6 to 1.8 weight-percent of water, the peak-height ratio changes linearly with increasing concentration of water in a solution. The standard deviation of the method, based on 12 degrees of freedom, is 0.052 weight-percent of water in the original syrup (about 20% of water). The time required for an analysis is comparable to that required for the near-infrared method.

4. The Karl Fischer Titration

For the determination of water, another direct method that has been widely used is the Karl Fischer titrimetric method [84]. In this method, a known quantity of sample is placed in the reaction vessel containing Karl Fischer (KF) reagent. The water in the sample reacts with the KF reagent and thus changes the conductivity of the solution. Karl Fischer reagent is then added to the system until the endpoint, as determined by the conductivity shown by a milliammeter, has been re-established.

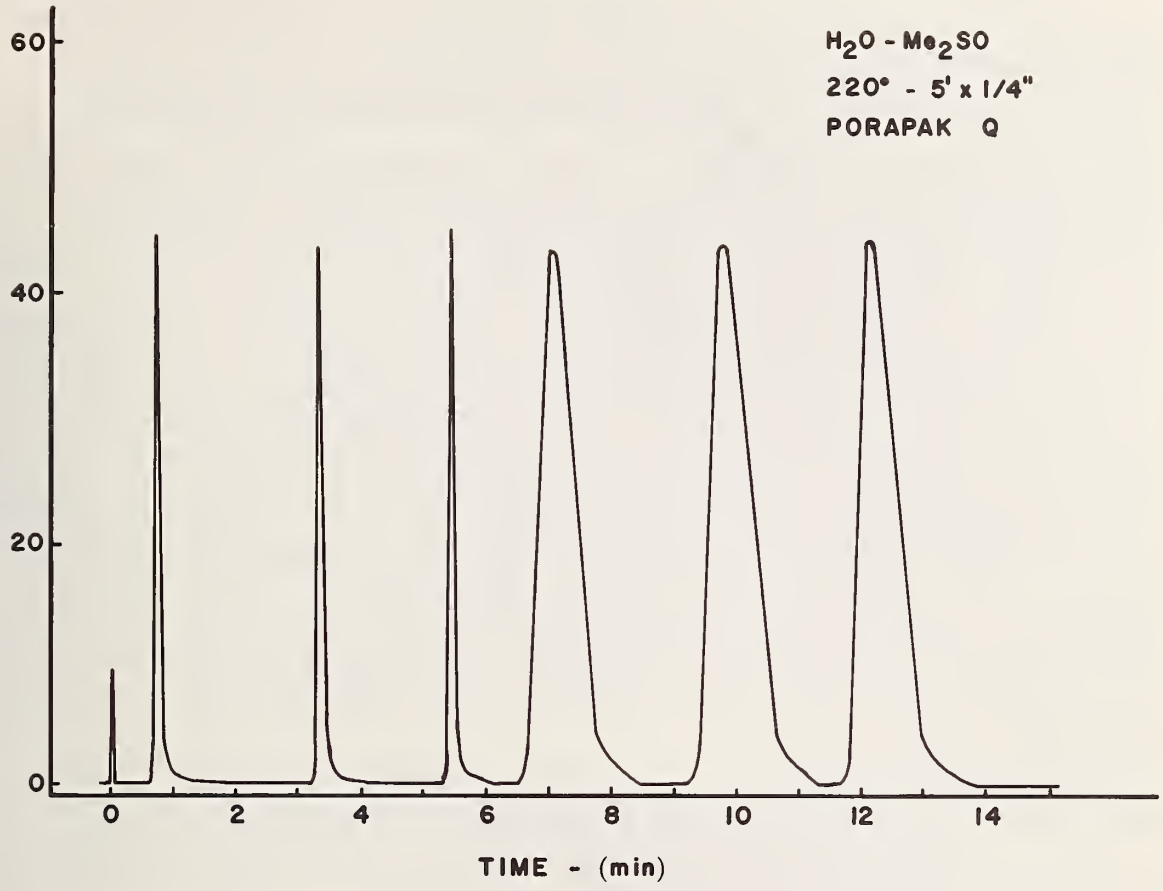


Figure 15. Chromatogram of three injections of a syrup solution.

This method is not directly adaptable for use with undiluted syrups because of their viscosity and their low solubility in pyridine. Furthermore, when a syrup is diluted with a suitable solvent to lower the viscosity, other problems arise The most serious problems are that, with syrups, the moisture content determined by the Karl Fischer method is usually found to be lower than that determined by other methods, often by as much as 0.3 weight-percent, and the precision of the method is very poor. These problems do not arise with granular materials that do not require dilution.

We have recently observed that, when a commercial diluent (Fisher Scientific SO-K-5) is added to the reaction vessel prior to the analysis, both the accuracy and the precision of the results are acceptable. Thus, a syrup having a Dextrose Equivalence value of 29 was diluted with water, resulting in three concentrations. These solutions were analyzed, in duplicate, for moisture content by the Karl Fischer method. In one case, the commercial diluent was added to the reaction vessel; in the second, N,N-dimethylformamide—methanol (DMF—MeOH) was added, and the moisture content was also determined by the near-infrared method, in duplicate. Estimates of precision, based on these results, are shown in table 25. In this case, the accuracy was comparable for all methods. Additional experiments are planned, to find the reasons for the imprecision when other solvents are substituted for the commercial diluent.

Table 25. Composition and estimates of precision of two Karl Fischer procedures, in comparison with the near-infrared method, for water

	SO-K-5	DMF	Near-infrared
Average, dry solids value (wt-%)	80.61	80.59	80.664
Range of values (wt-%)	0.44	1.06	0.374
Standard deviation	0.16	0.37	0.143
Degrees of freedom	6	6	6

B. Physical Properties of Hydrolyzates

1. Characterization of Hydrolyzates

The Dextrose Equivalent (DE) is the criterion employed by the corn wet-milling industry to indicate the extent of hydrolysis of starch in the manufacture of corn syrups and sugars. It is defined as the percentage of reducing sugars, calculated as dextrose and expressed on a dry-substance basis. Reducing sugars are determined by the method of Lane and Eynon, which uses an alkaline copper tartrate reagent. Although this approach is suitable for starch hydrolyzates, it is unsatisfactory when heat-labile sugars (such as D-fructose) or nonreducing sugars (such as sucrose) are present. In these cases, an alternative means of characterizing the hydrolyzate is desirable.

One method that appears suitable is the determination of the number-average molecular weight ($\overline{M}_n$). This determination can be made rapidly and economically by using a commercially available osmometer to measure the freezing-point depression of a dilute aqueous solution of the syrup. The $\overline{M}_n$ is calculated by using the equation

$$\overline{M}_n = K_f 10^3 W_2 / \Delta T_f W_1$$

where K_f is the molar depression constant (1.86 for water), W_2 is the weight of solute, W_1 is the weight of solvent, and ΔT_f is the freezing-point depression (°C). Solutions containing up to 5 percent of solids can be used. Ash contents up to 0.6 percent, calculated on an original dry-solids basis, do not interfere at these dilutions.

The $\overline{M}_n$ values obtained by measurements of freezing-point depression agreed quite well with those calculated from saccharide distribution data and from an empirical formula that includes the DE value and the percent of dextrose in the syrup.

When this approach is used, D-fructose behaves as if it were dextrose, and sucrose behaves as maltose. The time required to obtain a measure of hydrolysis by this method is less than that required to obtain a DE value. Furthermore, manipulative technique is not an important source of error, as it can be in the titration technique.

2. Other Physical Properties

Other physical properties of hydrolyzates and their solutions that are currently being investigated are: refractive indices, optical rotations, densities, and volume expansion properties. These are being studied as functions of moisture content, ash content, saccharide distribution, and temperature.

5. 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

H. J. Elseroad, Summer Employee (1968)

D. Niner, Guest Worker (1969)

B. Publications

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C. Manuscripts in Process of Publication

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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).

Synthesis of the Two D-2-Pentuloses. New Derivatives of D-erythro-Pentulose.
R. S. Tipson and R. F. Brady, Jr. (Manuscript accepted for publication in Carbohydrate Research)

Phenylhydrazono–phenylazo Tautomerism. III. Reactions of Phenylformazans and Certain Bis(phenylhydrazones) with Strong Acids.
H. S. Isbell and A. J. Fatiadi. (Manuscript accepted for publication in Carbohydrate Research)

A Comparison of the Spectrophotometric and Gas-chromatographic Methods with the Vacuum-oven Procedure for the Determination of Moisture in Starch Hydrolyzates.
D. W. Vomhof and J. H. Thomas. (Manuscript accepted for publication in Ann. Proc. Am. Soc. Brewing Chemists)

Studies of ^{15}N-Labeled Amino Sugars. Synthesis and Spectroscopy of Derivatives of 6-Amino-6-deoxy-D-glucose-6-^{15}N.
B. Coxon. (Manuscript accepted for publication in Carbohydrate Research)

The Synthesis, Equilibration, and Conformations of Diastereoisomeric 1,2-O-Isopropylidene-3,5-O-(methoxymethylidene)-6-O-p-tolylsulfonyl-α-D-glucofuranoses. Conformational Evidence from a Nuclear Overhauser Effect.
B. Coxon. (Manuscript submitted for publication in Carbohydrate Research)

Novel Aromatization of a Trihydroxycyclohexanetrione (Triketoinositol) to a Dibenzo-p-dioxin Derivative.
A. J. Fatiadi. (Manuscript submitted for publication in Carbohydrate Research)

Process for Producing Inositol Hexasulfate (a Patent).
A. J. Fatiadi. (Submitted to NBS patent office on March 3, 1969)

Conformational Analysis of Heterocyclic Compounds. The Favored Conformations of p-Dioxane-2,3-diol and its Diacetate.
R. F. Brady, Jr. (Manuscript submitted for publication in Journal of Organic Chemistry)

Determination of Moisture in Starch Hydrolyzates by Near-infrared and Infrared Spectrophotometry.
D. W. Vomhof and J. H. Thomas. (Manuscript submitted for publication in Analytical Chemistry)

Determination of Moisture in Syrups by Gas–Solid Chromatography.
D. W. Vomhof and J. H. Thomas. (Manuscript completed for publication in Analytical Chemistry)

Conversion of Certain Cyclic, Phenylhydrazono Derivatives into Phenylazo Compounds with Periodic Acid.
A. J. Fatiadi. (Manuscript completed for publication in Journal of Organic Chemistry)

D. NBS Reports

NBS 9972 - Report on Determination of Water in Starch Hydrolyzates by Near-infrared Spectrophotometry.
D. W. Vomhof, J. H. Thomas. 1/15/69

NBS 9974 - Report on Determination of Water in Starch Hydrolyzates by Infrared Spectrophotometry.
D. W. Vomhof, J. H. Thomas. 1/16/69

NBS 9999 - Report on Direct Determination of Water in Starch Hydrolyzates by Use of Gas-Solid Chromatography.
D. W. Vomhof, J. H. Thomas. 2/26/69.

E. Lectures

9/7/68 - Methods for the Determination of Moisture in Syrups and Related Materials. Starch Round Table, Pennsylvania State University. D. W. Vomhof.

9/10/68 - A Comparison of Methods for the Direct Determination of Water in Starch Hydrolyzates. Carbohydrate Division, 156th American Chemical Society Meeting, Atlantic City, New Jersey. D. W. Vomhof, J. H. Thomas.

9/10/68 - Studies of the Conformations of 1,2-O-Benzylidene-α-D-glucofuranose Derivatives by P.m.r. Spectroscopy. Carbohydrate Division, 156th American Chemical Society Meeting, Atlantic City, New Jersey. B. Coxon.

9/16/68 to 11/4/68 - Lectures on "Instrumental Methods of Qualitative Organic Analysis," Graduate School, NBS. R. F. Brady, Jr. Titles: (1) Introduction to Mass Spectrometry; (2) Interpretation of Mass Spectra; (3) Introduction to Nuclear Magnetic Resonance Spectrometry; (4) First-order Spin-patterns in N.M.R.; (5) Complex Spin-spin Splitting in N.M.R.; (6) Structural Determination by N.M.R.;

(7) Infrared Spectrophotometry in Organic Analysis; (8) Elucidation of Molecular Structure by Complementary Spectrometric Techniques.

10/11/68 - Determination of Carbohydrate Conformations by N.m.r. Spectroscopy. U. S. Army Natick Laboratories, Natick, Massachusetts. B. Coxon.

12/4/68 - O. R. Rodig, R. F. Brady, Jr., and R. J. Sysko, "The Biosynthesis of Citrinin," Twentieth Southeastern Regional Meeting, American Chemical Society, Tallahassee, Fla.

2/14/69 - Synthesis of the Epimeric D-2-Pentuloses. New Derivatives of D-erythro-Pentulose. Fourth Middle Atlantic Regional Meeting, American Chemical Society, Washington, D. C., R. S. Tipson, R. F. Brady, Jr.

4/14/69 - Direct Determination of Water in Starch Hydrolyzates By Use of Gas-Solid Chromatography. Carbohydrate Division, 157th American Chemical Society Meeting, Minneapolis, Minnesota. D. W. Vomhof, J. H. Thomas.

4/14/69 - The Influence of Saccharide Composition on the Refractive Index of Syrups. Carbohydrate Division, 157th American Chemical Society Meeting, Minneapolis, Minnesota. D. W. Vomhof.

4/14/69 - Synthesis and Equilibration of Diastereoisomeric 1,2-O-Isopropylidene-3,5-O-(methoxymethylidene)-6-O-p-tolylsulfonyl-α-D-glucofuranoses. 157th American Chemical Society Meeting, Minneapolis, Minnesota. B. Coxon.

5/2/69 - Changes in Nitrogen Fractions Associated With Fruit Abscission in Cotton. Washington Area Section, American Society of Plant Physiologists, Beltsville, Maryland. D. W. Vomhof.

5/6/69 - A Comparison of Spectrophotometric and Gas-Chromatographic Methods with the Vacuum-Oven Procedure for the Determination of Moisture in Starch Hydrolyzates. American Society of Brewing Chemists, Baltimore, Maryland. D. W. Vomhof, J. H. Thomas.

6. ACKNOWLEDGMENTS

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Sustained maximum growth in a free market economy, without inflation, under conditions of full employment and equal opportunity

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- **Office of Foreign Commercial Services**
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JULY 1969

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