DETERMINATION OF INOSOSES WITH AN ALKALINE SOLUTION OF COPPER(II) OXALATE–TARTRATE COMPLEX (THE SOMOGYI REAGENT) AND REACTION MECHANISMS INVOLVED*

ALEXANDER J. FATIADI

Division of Analytical Chemistry, Institute for Materials Research, National Bureau of Standards, Washington, D. C. 20234 (U. S. A.)

(Received March 3rd, 1970; accepted in revised form, November 2nd, 1970)

ABSTRACT

Four inososes have been analyzed with the Somogyi reagent** and empirical equations for their quantitative determination were derived. Results from spectrophotometric and electron-spin resonance studies of the mechanism of oxidation of inososes with the Somogyi reagent at 25 to 55° are in agreement with a one-electron transfer process; however, at 90 to 100°, extensive degradation of inososes by the Somogyi reagent occurs, doubtless caused by generation of transient radicals during the oxidation, as evidenced by results of a radical-scavenging experiment.

INTRODUCTION

Several analytical methods are available for the quantitative determination of pentahydroxycyclohexanones (inososes)†. In alkaline solution, one mole of an inosose usually reduces one mole of iodine (Wilstätter–Schudel titration), or one mole of dichlorophenolindophenol (the Tilmanns reagent)[1]. Polarography[2] in 100 mM phosphate buffer, with thallous chloride as the reference electrode, may also be used for estimation of inososes. Heyns and Paulsen[3] used cold Fehling reagent for the quantitative determination of a racemic mixture[4] of 2D-2,3,4,6/5-pentahydroxycyclohexanone (D-*epi*-inosose-2) (**1**) and 2L-2,4,5,6/3-pentahydroxycyclohexanone (L-*epi*-inosose-2) (**2**); here, one mole of the inosose produces one mole of cuprous oxide (Cu_2O); and it is assumed by the present author that this stoichiometry applies to all of the isomeric inososes.

It has now been found that the Somogyi reagent[5], widely used in the determination of reducing sugars, is an alternative for the quantitative determination of inososes. The reagent has been used successfully for determination of four inososes: the DL

*Part VII. Methods in Inositol Chemistry. For part VI, see *Carbohyd. Res.*, 12 (1970) 293.

**Electron-spin resonance (e.s.r.) studies of the structure of four reagents commonly used in the carbohydrate field, namely, the Somogyi reagent, the Fehling reagent, the Benedict reagent, and the Reeves reagent have been reported (see Ref. 25).

†The names of inositol derivatives used in this paper are based on the IUPAC–IUB Nomenclature for Cyclitols, *Eur. J. Biochem.*, 5 (1968) 1; names by an alternative system [H. G. Fletcher, Jr., L. Anderson, and H. A. Lardy, *J. Org. Chem.*, 12 (1951) 1238] are given in parentheses. The trivial name "inosose" for any pentahydroxycyclohexanone is used where convenient.

mixture[4] of **1** and **2**, the L enantiomorph[6] **2**, 2,4,6/3,5-pentahydroxycyclohexanone (*myo*-inososе-2)[4] (**3**), and 2D-2,3,5/4,6-pentahydroxycyclohexanone (D-*myo*-inosose-1)[7] (**4**).

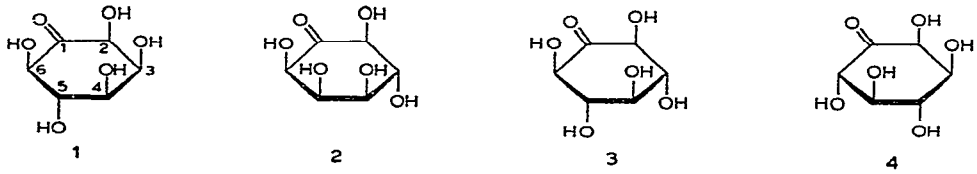


Fig. 1 shows a typical graph used in the quantitative determination of inosose (**1**+**2**); a straight line is obtained for titration, with a standard solution of sodium thiosulfate, of the iodine liberated (corresponding to the concentration of inosose). Table I shows the empirical equations used in the determination of inososes **1**–**4**, as derived from the graph shown in Fig. 1 and similar graphs for the other inososes.

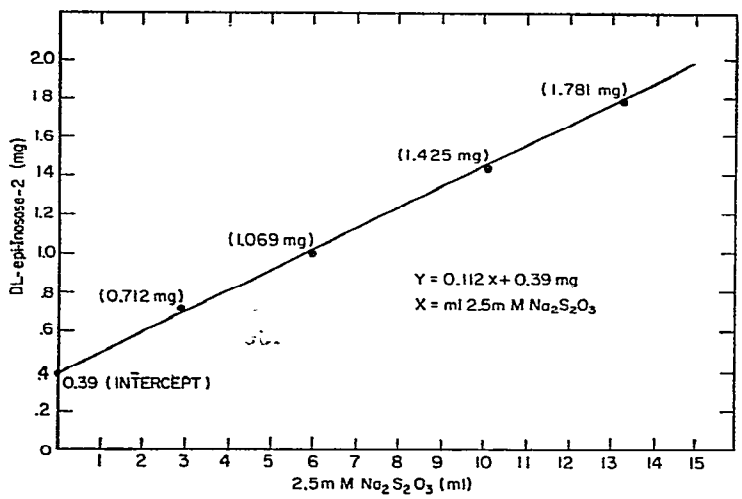


Fig. 1. A typical graph used in derivation of an empirical equation for the quantitative determination of inosose (**1**+**2**).

TABLE I

DETERMINATION OF INOSOSES WITH THE SOMOGYI REAGENT

Inosose	*Number of determinations*[a]	*Concentration (mg/5 ml)*	*Equation*[b]	*S. D. based on % recovery (n = 12)*[c]
DL-*epi*-Inosose-2 (**1**+**2**)	16	0.35–1.78	Y = 0.112X+0.39	1.41
L-*epi*-Inosose-2 (**2**)	15	0.35–1.78	Y = 0.122X+0.39	1.62
myo-Inosose-2 (**3**)	16	0.35–1.78	Y = 0.100X+0.21	1.55
D-*myo*-Inosose-1 (**4**)	16	0.35–1.78	Y = 0.118X+0.38	1.66

[a]Heated for 30 min. [b]Y, mg of inosose in 5 ml; X, ml of 2.5 mM sodium thiosulfate. [c]S. D. is the percent standard deviation, and *n* is the number of determinations.

DISCUSSION

In analogy to reducing sugars, on treatment with the Somogyi reagent, inososes (**1**+**2**), **2**, **3**, and **4** produce non-stoichiometric quantities of cuprous oxide (Cu_2O), in contrast to the result of treatment of (**1**+**2**) with cold Fehling reagent[3]. Comparison of the copper-reducing power of the reducing sugars with those of the inososes, following treatment with the Somogyi reagent, indicated that D-fructose (not D-glucose) is structurally more related to inosose **3**; but inosose (**1**+**2**) behaves differently from **3**.

It was also observed that the copper-reducing power of inososes (**1**+**2**), **2**, **3**, and **4** is pH-dependent (sodium carbonate–sodium hydrogen carbonate ratio) and varies with the particular inosose.

It had previously been shown[5] that Somogyi oxidation of the reducing sugars has its highest efficiency at pH ~9.4. Isbell and co-workers[8] have demonstrated that the rate of enolization of the two isomeric inososes (**1**+**2**) and **3** in basic media is different, and is considerably higher for inososes than for sugars at the same pH. Consequently, a different copper-reducing power would be expected for each inosose, and this was actually observed. Differences in the copper-reducing power may also arise from the different conformations of the ligands[9] derived from several inososes on enolization. Thus, both the rate of enolization[8] and the conformation of the enol-ligands may affect the formation and the quantity of the Cu(II) formed prior to the oxidation.

Enolization of the inososes on treatment with the buffer solution (sodium carbonate–sodium hydrogen carbonate under the Somogyi conditions, pH ~9.4) was followed spectrophotometrically (u.v. and visible spectral regions). On treatment with the base, the original absorption band[3] for a mM solution of inosose (**1**+**2**) at 280 nm shifted to the 300–350-nm region, characteristic of an enolized inosose[3,10]. Acidification (dilute hydrochloric acid) of this solution, with brief warming (60 sec at 55°), followed by neutralization (sodium carbonate), gave a yellow solution having a strong absorption band at 305 nm typical of the anion of DL-*xylo*-pentahydroxy-2-cyclohexen-1-one[10]; however, the intensity of the band increased with time. Neutralization of the solution with 2M hydrochloric acid produced a shift of the maximum from the 305-nm to the 265-nm region; the latter band can be ascribed to the aromatic chromophore[11] of pentahydroxybenzene[10] (see Experimental).

Evidence for complex formation between an enolized inosose and the Somogyi reagent was obtained from a study of spectral changes in the visible region and from the changes in the shape of the e.s.r. spectra. Spectrophotometric titration of the Somogyi reagent (by use of the absorption band at 670–690 nm) with a mM solution of the inosose produced a gradual increase in absorbance without an appreciable shift in the maximum. This behavior reportedly[12,13] is characteristic for an association (complex formation) between a diol (in this case, an enediol) and the copper ion. Although the last few drops of the inosose solution caused turbidity in the cell (precipitation of Cu_2I_2), the ratio of copper to inosose was, nevertheless, close to 1:1

when the absorbance reached the maximum and the solution became somewhat turbid.

A typical e.s.r. spectrum of the Cu(II) complex in aqueous solution at room temperature shows a four-line, asymmetric pattern due to coupling of an unpaired electron of copper with the nuclear spin of the ^{63}Cu and ^{65}Cu nuclei ($I_{Cu} = 3/2$); ^{63}Cu and ^{65}Cu have similar nuclear moments[14]. The e.s.r. spectrum of the Somogyi reagent is shown in Fig. 2. In addition to a four-line, hyperfine structure, the spectrum shows a paramagnetic species absorbing at 3.245 kgauss (arrow, Fig. 2) that, presumably, arises from a superoxide ion $\dot{O}_2^-$.

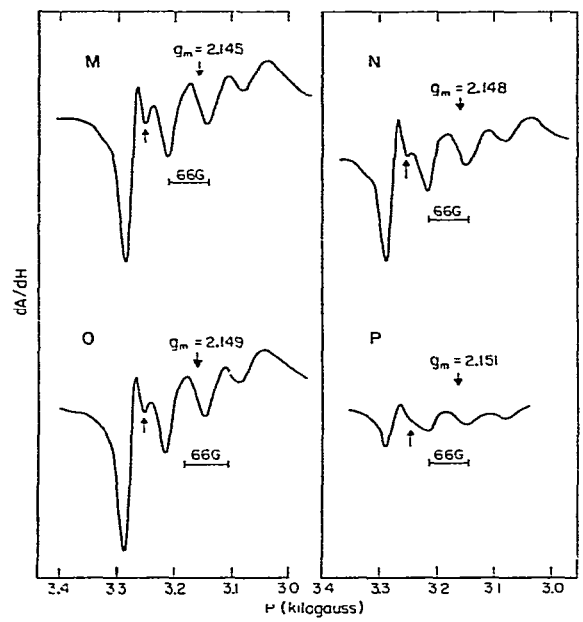


Fig. 2. E.s.r. spectral changes after titration of the Somogyi reagent (~0.4 ml) with mM inosose (1+2): spectrum M, the Somogyi reagent before titration (25°); spectrum O, after mixing with ~0.1 ml of mM inosose (25°); spectrum N, after mixing with ~0.2 ml (55°); and spectrum P, after mixing with ~0.4 ml (55°).

Similar titration of the Somogyi reagent in the e.s.r. cell with a mM solution of the inosose (1+2) resulted in the e.s.r. spectrum recorded (spectra M, N, O, P, Fig. 2). Addition of one drop of the inosose (1+2) solution to the Somogyi reagent (spectrum M, Fig. 2) at room temperature produced spectrum O (Fig. 2), in which the peak at 3.245 kgauss was decreased by 5 to 7%. The same solution was then mixed with another drop of the inosose solution, and the mixture was kept for 30 sec at 55°; the spectrum obtained (N) showed a decrease of this peak of about 80%. A further addition of inosose and warming (30 sec at 55°) produced a spectrum completely free from paramagnetic species absorbing at 3.245 kgauss. It is interesting that the mole ratio of inosose to copper was about 1:1 in the final solution when the peak at 3.245 kgauss had disappeared (the excess of Cu^{2+} concentration was deduced after

measurement of the visible spectra of both the original solution and the solution after mixing with inosose).

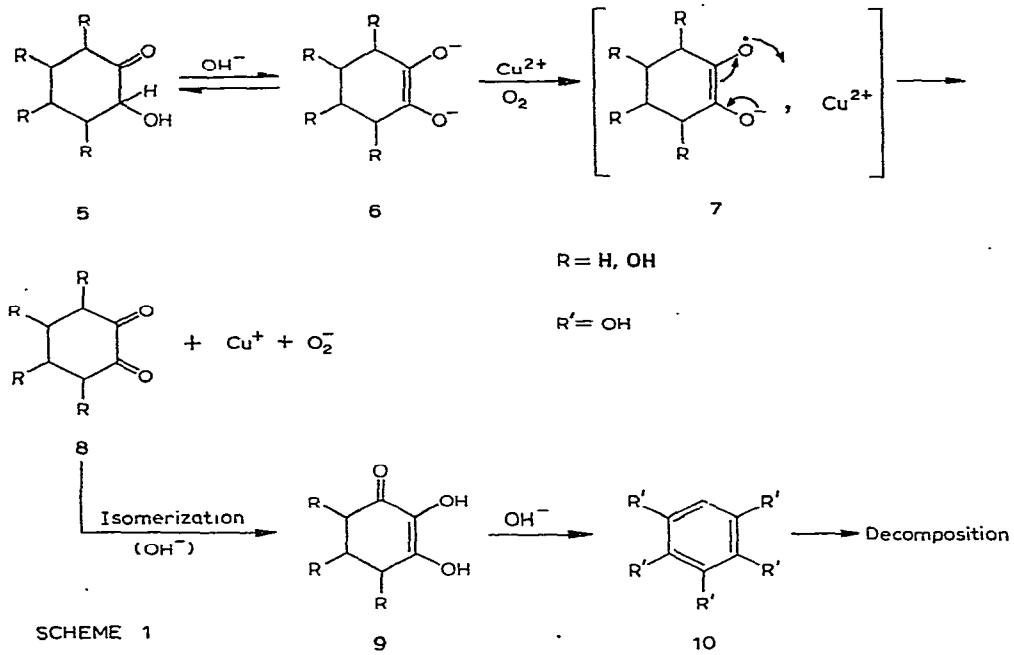
All of the observed changes (u.v., visible, and e.s.r. spectra) are indicative of several transformations prior to the oxidation (the electron-transfer step). The changes observed in the e.s.r. spectra (Fig. 2) can be explained as due to (*1*) simultaneous enolization and complex-formation between Cu(II) and an enolized inosose, and (*2*) an exchange of the bidentate ligands with approximately equal ligand fields, namely, a tartrate–oxalate ligand for a new, enolized, inosose ligand. The latter change is supported by (*a*) the observed change in shape of the e.s.r. spectra (from M to P, Fig. 2) and (*b*) almost no change in the g values for spectra M, N, O, and P. Alkaline solutions of Cu(II) complexes are known[15,16] to be one-electron (one-equivalent) oxidants. Kinetic studies have shown[15] that these oxidations are acid- or base-catalyzed, and that the alkaline oxidations are complicated by aldolization.

The oxidation of D-glucose and of acyloin by alkaline cupric complexes may proceed by transformation into an enol anion[15] which can again yield resonance-stabilized, semiquinone-type radicals (semidones); however, the stability of the semidone radical will depend[17] on the nature of the stabilizing groups on the adjacent two carbon atoms of the radical. Although these oxidations are zero-order with respect to Cu(II), they appear to be slower than the preceding enolizations[15]. As pointed out by Waters[15], the kinetics of these oxidations may depend on the stabilities of chelated cuprous and cupric complexes, and they are not yet fully understood. It is believed that radical-ions of semiquinone type are involved in the oxidation of L-ascorbic acid and of reducing sugars; in buffered solutions, however, such compounds are readily attacked by oxygen if a trace of copper or iron is present[15].

Similar oxidations are also observed in the aromatic series; for example, Cu(II) complex converts 1,4-benzenediol into *p*-benzosemiquinone[18], and 1,2-benzenediol into the less-stable *o*-benzosemiquinone[19].

It appears feasible at this stage to consider a radical type of mechanism in the oxidation of an inosose with the Somogyi reagent (boiling-water bath for 30 min). The possible steps in the oxidation are shown in Scheme 1; these may involve: (*a*) transformation of inosose **5** into enol-anion **6**; (*b*) reaction of a trace of oxygen to give the semidone radical intermediate **7**; and (*c*) transfer of an electron from a radical to a cupric ion, with formation of the diketo derivative **8**. In the presence of a base, the latter can isomerize through the enolic intermediate **9** to the aromatic compound **10**. The decomposition products can then arise either from intermediate **7** (because of the instability of the radical of this type) or from **10**. Both the Cu(II) complex and the radical intermediate **7** are expected to give, in the e.s.r. spectrum, some lines additional to that from the Cu(II) complex; however, this was not observed under the experimental conditions used. Indeed, the existence of transitory radicals, suggested as being **7** or its decomposition products, was indicated by the results of a radical-trapping experiment.

Applications of radical trapping to study of the short-lived free-radicals (without use of the flow-system technique[20]), and the use of effective radical scaven-



SCHEME 1

gers, such as certain nitroso compounds[21], was found useful in this study. Rapid heating (30 sec at 95°) of a mixture of the Somogyi reagent (0.3 ml) with inosose (1+2) (0.1 mmole) and 2-methyl-2-nitrosopropane (0.4 mmole, in *p*-dioxane or neat), quick cooling (ice-bath), and immediate measuring , gave an e.s.r. spectrum having a somewhat overlapped, weak triplet, with the coupling constant a^N 16.8 gauss, that can be ascribed to the nitroxide radical[20,22]. Hence, detection of the nitroxide radical derived from the radical scavenger indicates the presence of transient radicals in the oxidation mixture; and these are responsible for trapping of the 2-methyl-2-nitrosopropane.

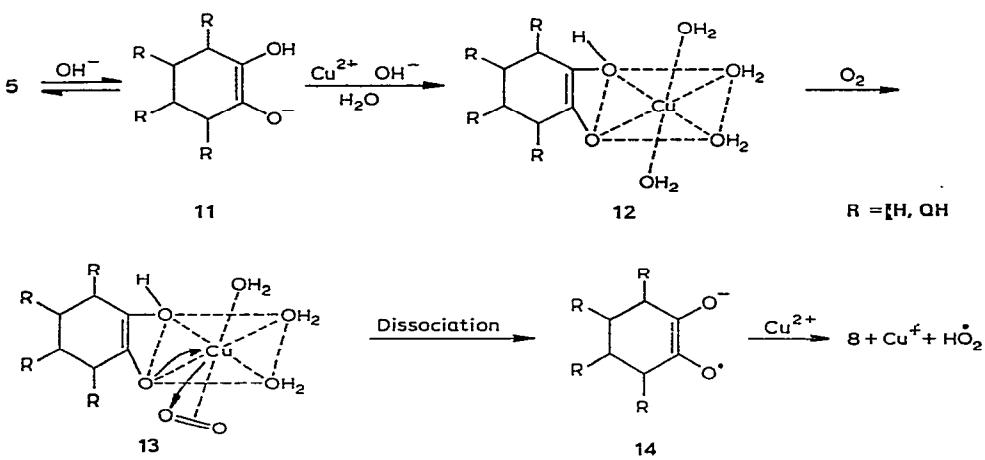
Although there was considerable interaction between the Somogyi reagent and 2-methyl-2-nitrosopropane*, the intensity of the nitrogen-triplet lines in the e.s.r. spectrum of a solution in the presence of inosose was much higher than for solutions from which inosose was absent or for which mixing of the components was performed at room temperature**.

*The interaction of the Somogyi reagent with 2-methyl-2-nitrosopropane caused substantial decrease in intensity of the Cu(II) complex, four-line, e.s.r. spectrum, and little or no nitroxide radical was observed; also, there was a considerable interaction between the nitroxide radical formed and iodide ions present in the reagent. However, trapping of the radical can be successful if a small excess of the nitroso reagent is used and the experiment is performed quickly. Somewhat less effective as the radical scavengers in this experiment were 2-nitrosopropane, *N*-nitrosodimethylamine, and 5-nitrososalicylic acid (the last two reagents are soluble in water).

**Experiments conducted at room temperature gave e.s.r. spectra that showed very weak or no nitrogen-triplet lines due to nitroxide radical.

Supposedly operating in the oxidation of the inosose by the Somogyi reagent is an alternative reaction-mechanism that may involve a ligand exchange; an electron transfer is favored from the results of a study involving enolization, association, and ligand exchange. In a mixture containing the oxidizing metal ions (Cu^{2+} ions, the Somogyi reagent) and the oxidizable ligand (RCHO, R–C(=O)–CH(OH)–R, or an enolized inosose), progressive increase in absorbance at 670–690 nm was observed. This change can be ascribed to the formation of a complex in which an electron can be removed from a ligand (an enolized inosose) and transferred to the metal ion, so that the metal is reduced (the formation of cuprous iodide was observed). In an analogous case, the charge-transfer band observed was ascribed[23] to the transfer of an electron from a ligand to the metal ion.

The steps in this mechanistic approach (Scheme 2) may involve (*a*) enolization of inosose **5** to enol-anion **11**, (*b*) formation of an association complex **12**, followed



11 12 13 14

SCHEME 2

by a ligand exchange, (*c*) formation of the oxygen–Cu^{2+} adduct **13**, and (*d*) transfer of an electron from an anion ligand *via* Cu^{2+} ion to oxygen, followed by dissociation of the adduct **13** to give the semiquinone (semidone) radical **14** (identical with *7*); the latter is then oxidized by Cu^{2+} to the diketo derivative **8**. This mechanism is similar to those proposed by Taqui Khan and Martell[24] for metal [Cu(II) and Fe(III)] catalyzed oxidation of L-ascorbic acid by molecular oxygen; it also suggests possible existence of an oxygen–copper adduct as a reaction intermediate. An analogous oxygen–copper adduct has recently been observed[25] in e.s.r. studies of similar systems.

EXPERIMENTAL

General methods. — N.m.r. spectra were recorded with a Varian A-60 spectrometer, with tetramethylsilane (τ, 10.00) as the internal standard. U.v. and visible

spectra were recorded with a Beckman DK-2 or Cary 14 spectrophotometer. E.s.r. spectra were recorded with a Varian Model 4500 EPR spectrometer* with 100-kHz field-modulation and detection, operating at ~9.2 and ~9.5 GHz. The klystron frequency was measured with a transfer oscillator and frequency counter. The magnetic field was measured by a proton gaussmeter monitored by the same frequency counter. All spectra were recorded in duplicate or triplicate, and the results of the measurements were averaged. Replicate readings for the frequency-counter meter agreed within ±3%. The solutions were examined in a Varian Model V-4548 aqueous-solution sample-cell at various temperatures (25 to 60°). In the e.s.r. spectra of liquids (at 25°), g_m is defined as the middle point of the hyperfine lines of the spectrum. A measured volume of the inosose solution was introduced directly into a cell by means of a microsyringe equipped with a long needle to facilitate mixing. The inosose solutions were prepared according to the published procedures indicated in the text.

Somogyi reagent. — This reagent was prepared according to the standard method[5]: copper(II) sulfate (26mM, 0.65%), potassium–sodium tartrate (Rochelle salt) (60mM, 1.2%), sodium carbonate (200mM, 2.0%), sodium hydrogen carbonate (300mM, 2.5%), potassium iodate (11.5mM, 1.0%), potassium iodide (0.08%), and potassium oxalate (100mM, 1.8%).

Other reagents and materials. — Sodium thiosulfate solution (2.5 mM, freshly prepared by dilution of 100mM $Na_2S_2O_3$); ~2.5M sulfuric acid; 1% starch solution. Pipets (1, 2, 3, 4, 5, and 10 ml); buret (25 ml); test tubes (250 × 25 mm); glass bulbs; water bath, ice bath, magnetic mixer.

General procedure[5]. — A solution (5 ml) containing 0.25 to 2.0 mg of inosose was added to the Somogyi reagent (5 ml) in a test tube containing a magnetic microbar. It was mixed by gentle shaking, covered with a glass bulb, and kept for 30 min in a boiling-water bath. The suspension was rapidly cooled (ice–water) to 35–40°. Sulfuric acid (2.5M, 1 ml) was added with stirring, being cautious that all of the cuprous oxide (Cu_2O) dissolved promptly. After about 2 min, the liberated iodine was titrated with 2.5mM sodium thiosulfate (starch indicator; duplicate). A blank titration on 5 ml of the reagent was made after it had been diluted with an equal volume of water, heated, and cooled.

The difference between the blank titer and that in a determination is equivalent to the copper reduced, and is thus related to the weight of inosose present.

By plotting the concentration (mg of inosose per ml) against the volume of 2.5mM sodium thiosulfate used (which is equivalent to the copper reduced), a graph is obtained [as shown, for the inosose (**1**+**2**), in Fig. 1] from which an empirical equation for the particular inosose can be calculated in the usual way[5].

Examination of the reaction path. Spectrophotometric evidence for formation of a complex. — A sample of the Somogyi reagent (4.5 ml, 26mM in copper, λ_{max} 685 nm,

*Mention of commercial instruments does not imply recommendation or endorsement by the National Bureau of Standards.

A_{685} ~0.70*) was titrated spectrophotometrically with 50mM aqueous inosose (**1**+**2**) at room temperature. Addition of the first drops (0.1-ml increments) of inosose solution produced the expected slight decrease in absorbance consistent with dilution and with enolization of the inosose in the (basic) Somogyi solution. However, further addition of the inosose caused a rapid rise in absorbance without a shift in the position of the maximum (at 685–688 nm), and this can be attributed[12,13] to the formation of a complex. Titration was continued until a white or tan solid (Cu_2I_2) was about to be formed (cloudiness in the cell). About 2.1 ml of the inosose solution was used; at this point, the mole ratio of copper to inosose was approaching 1:1; however, determination of the ratio of enolized inosose to copper was obscured by the precipitated solid.

Spectrophotometric evidence for enolization and aromatization of inosose in the presence of the Somogyi reagent. — A dilute Somogyi reagent (about 0.1mM in copper) was titrated with inosose solution (**1**+**2**) (about mM) and examined spectrophotometrically in the region of 250–330 nm. On mixing with the Somogyi reagent, the original peak characteristic of an inosose (λ_{max} 280 nm) shifted to 306 nm, and this behavior is characteristic of the enolization of an inosose**. Acidification of this solution (with dilute hydrochloric acid) produced a new peak (at 266 nm) that is characteristic of an aromatic absorption.

Analogous results were observed when a solution of the inosose was treated with a 100mM sodium carbonate solution until the solution reached pH 9.4 (the Somogyi reagent).

Isolation of pentaacetoxybenzene. — A solution (0.5mM) of inososes (**1**+**2**, 40 mg) in water (110 ml) was mixed with the Somogyi reagent (110 ml) in a 250-ml flask, and stirred for 30 min at 55–60°. The cuprous oxide that formed was filtered off on a layer of Celite, and the filtrate was acidified with acetic acid containing some sodium sulfite (to remove any free iodine), and evaporated to dryness at 60°. The product was acetylated by stirring overnight at room temperature with 4:1 acetic anhydride–pyridine (40 ml). The suspension was poured into ice–water, and the mixture was extracted with four 30-ml portions of 1:1 (v/v) chloroform–dichloromethane. Concentration and recrystallization from 95% ethanol gave colorless crystals of pentaacetoxybenzene (5 mg, 13%) m.p. 165° (lit.[10] m.p. 166–168°); λ_{max}^{MeOH} 266 nm (ε ~550); n.m.r. data (chloroform-*d*; tetramethylsilane as internal standard, τ 10.00): τ 2.80, 2.70 (aromatic proton, two lines) and methyl protons of acetyl groups at τ 7.75 and 7.72 in the ratio of 1:15, which agrees with the structure proposed. On acidification (hydrochloric acid) of the original filtrate, concentration, and treatment with phenylhydrazine, a mixture of bis(phenylhydrazones) resulted.

*The spectrum of the freshly prepared Somogyi reagent showed a maximum at about 670 nm (absorbance 0.7); however, on aging of the solution, the maximum slowly shifted to 690–705 nm with an increase in absorbance to 1.2–1.5; thus, dilution was necessary, so that the latter measurements could be made.

**The presence of the enolized inosose was evident from analysis of the e.s.r. spectrum of the Cu(II)-chelate mixture, prepared on treatment of the acidified (acetic acid) solution with an aqueous solution of cupric sulfate.

In another experiment, the original mixture was kept for 30 min at 55°; acidification (2M hydrochloric acid), followed by concentration, gave a solid which, on acetylation with phosphoric–acetic anhydride reagent[26], gave a small proportion of white crystals (m.p. 165°) identical with an authentic sample of pentaacetoxybenzene[20]. However, when acetylation was performed with pyridine–acetic anhydride, the crude mixture, in addition to pentaacetoxybenzene, contained a trace of 1,2,3,5-tetraacetoxybenzene [t.l.c. with 1:4 (v/v) acetic acid–benzene] which may have arisen from aromatization of the original inosose[27].

When the reaction was performed under the Somogyi conditions (boiling-water bath for 30 min), none (or only traces) of the pentaacetoxybenzene or an enol form of a diketoinositol could be recovered; however, the crude mixture contained, in addition to oxalic acid and erythraric acid, a small proportion of galactaric acid, identified as the bis(benzimidazole) by t.l.c. with 1:1:3 (v/v) benzene–acetic acid–methanol; the R_F values were comparable to those of authentic samples[28,29].

Isolation of DL-xylo-*2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol.* — A solution (0.5mM) of inosose (**1**+**2**, 40 mg) in water (110 ml) was mixed with the Somogyi reagent (110 ml) and kept for 45 min at room temperature. The precipitated solid (Cu_2I_2) was filtered off (layer of paper pulp); the filtrate was made neutral with acetic acid (25 ml) containing sodium hydrogen sulfate (250 mg), phenylhydrazine (10 g) was added, and the mixture was stirred for 30 min at room temperature. The crude bis(phenylhydrazone) was separated, and dried for 24 h at room temperature. The pink to red mixture was then extracted with acetone (25 ml), and the red extract was rapidly filtered. The light-colored solid was primarily the bis(phenylhydrazide) of oxalic acid (m.p. 176°, crystallization from acetic acid). The acetone extract was placed on a column (2 × 35 cm) of Florisil, and eluted with 1:2 (v/v) methanol–acetic acid. A red band was collected, concentrated, and recrystallized from methanol (5 ml) to give dark-red, lustrous crystals (18 mg), m.p. 183–185°; the product was identical with an authentic sample of DL-*xylo*-2-oxo-1,3-bis(phenylhydrazono)cyclohexane-4,5,6-triol[10,30].

Rechromatography of a forerun of the red band (m.p. 183–185°), gave, as the major component, a small proportion of an orange-red solid. The R_F value of this compound was close to that of the bis(phenylhydrazone) obtained on condensation of phenylhydrazine with the "reductone", described by von Euler and Glaser[31], prepared by treatment of inosose (**1**+**2**) with ~700mM sodium hydroxide.

Good results in isolation of the bis(phenylhydrazone) (m.p. 183–185°) were obtained by the following procedure: after reaction of the inosose with the Somogyi reagent at room temperature and filtration, the solution was carefully acidified with acetic acid (to about 15–20%) and treated with an excess of phenylhydrazine. The bis(phenylhydrazide) of oxalic acid was immediately precipitated; it was quickly filtered off, and the clear, red filtrate was diluted with water (to about 4–5% in acetic acid) and kept for several hours or overnight at room temperature. The crystalline bis(phenylhydrazone) was purified by recrystallization (methanol) or column chromatography.

E.s.r. study of the oxidation of inosose (**1**+**2**) *with the Somogyi reagent.* — An aliquot (0.4 ml) of Somogyi reagent (26mM) was pipetted into a cell, and the e.s.r. spectrum was recorded at 25 ±3° (see Fig. 2, spectrum M) and after mixing with ~0.1 ml of mM inosose (25°) (spectrum O). The solution was next mixed twice, consecutively, with one drop (~0.1 ml) of mM aqueous solution of inosose (**1**+**2**), and warmed for 30 sec at 55 ±3°, and spectra N and P were obtained. An interpretation of the changes observed is presented in the Discussion.

Examples. — (*a*) *Analysis of crude inosose* (**1**+**2**). The crude inosose (**1**+**2**, 36 mg, 0.2 mmole) was dissolved in water (100 ml); an aliquot (5 ml) was added to the Somogyi reagent (5 ml); the mixture was heated for 30 min at 95°, and processed as already described. There was consumed (blank) 9.57 ml of 2.5mM sodium thiosulfate. $Y = (0.112 \times 9.57) + 0.39 = 1.45$ mg of reducing product in 5 ml of solution, or 29 mg in 100 ml, corresponding to 80% of reducing compound in the crude inosose.

(*b*) *Analysis of crude* 3D-*3,5/4,6-tetrahydroxy-1,2-cyclohexanedione bis(phenylhydrazone)* [L-*inosose-1 phenylosazone*][32]. A sample of crude osazone (50 mg) was treated with Amberlite IR-120 (H^+, 5 ml) in water (5 ml) to hydrolyze any 2D-2,3,5/4,6-pentahydroxycyclohexanone phenylhydrazone [L-inosose-1 phenylhydrazone] according to the published procedure[33]. The filtrate (about 10 ml) was treated with the Somogyi reagent (5 ml), and processed as already described. There was consumed (blank) 15.2 ml of 2.5mM sodium thiosulfate. $Y = (0.118 \times 15.2) + 0.38 = 2.17$ mg of inosose or 3 mg of inosose phenylhydrazone; thus, the crude osazone contained 6% of L-inosose-1 phenylhydrazone.

ACKNOWLEDGMENT

The author thanks N. Adams (NBS) for the e.s.r. measurements.

REFERENCES

1 T. POSTERNAK, *The Cyclitols*, Holden–Day, San Fransicso, Calif., 1965, p. 156.
2 D. H. COUGH AND W. PIGMAN, *Anal. Chem.*, 24 (1952) 1364.
3 K. HEYNS AND H. PAULSEN, *Chem. Ber.*, 86 (1953) 833.
4 T. POSTERNAK, *Biochem. Prep.*, 2 (1952) 57.
5 M. SOMOGYI, *J. Biol. Chem.*, 70 (1926) 599; P. A. SCHAFFER AND M. SOMOGYI, *J. Biol. Chem.*, 100 (1933) 695; F. J. BATES AND ASSOCIATES, *Polarimetry, Saccharimetry and the Sugars*, Circular, C440, National Bureau of Standards, 1942, p. 196; E. J. MCDONALD AND B. Y. FOLEY, *J. Ass. Offic. Agr. Chem.*, 43 (1960) 645.
6 T. POSTERNAK, *Helv. Chim. Acta*, 29 (1946) 1991.
7 G. POST AND L. ANDERSON, *J. Amer. Chem. Soc.*, 84 (1962) 471.
8 H. S. ISBELL, H. L. FRUSH, C. W. R. WADE, AND C. E. HUNTER, *Carbohyd. Res.*, 9 (1969) 163.
9 A. M. SARGESON, in R. L. CARLIN (Ed.), *Transition Metal Chemistry*, Marcel Dekker, New York, 1966, Vol. 6, p. 303; M. M. JONES, *Mechanisms of Inorganic Reactions, Advan. Chem. Ser.*, 48. (1965) 153.
10 A. J. FATIADI AND H. S. ISBELL, *J. Res. Nat. Bur. Stand.*, 68A (1964) 287.
11 A. J. SCOTT, *Ultraviolet Spectra of Natural Products*, Macmillan, New York, 1964, p. 91.
12 E. J. MCDONALD, *J. Org. Chem.*, 25 (1960) 111.
13 F. A. COTTON AND G. WILKINSON, *Advanced Inorganic Chemistry*, Interscience, 2nd ed., 1966 pp. 157, 675.

14 W. B. LEWIS AND L. O. MORGAN, in R. L. CARLIN (Ed.), *Transition Metal Chemistry*, 1968, Vol. 4, p. 82.
15 W. A. WATERS, *Mechanisms of Oxidation of Organic Compounds*, Wiley, New York, 1964, p. 87.
16 R. STEWART, *Oxidation Mechanisms*, Benjamin, New York, 1964, p. 159.
17 G. A. RUSSELL, in E. T. KAISER AND L. KEVAN (Eds.), *Radical Ions*, Interscience, New York, 1968, p. 87; *Science*, 161 (1968) 423.
18 M. BERSOHN AND J. C. BAIRD, *An Introduction to Electron Paramagnetic Resonance*, Benjamin, New York, 1966, p. 134.
19 P. B. AYSCOUGH, *Electron Spin Resonance in Chemistry*, Methuen., London, 1967, p. 356.
20 S. TERABE AND R. KONAKA, *J. Amer. Chem. Soc.*, 91 (1969) 5655.
21 C. LAGERCRANTZ AND S. FORSHULT, *Nature*, 218 (1968) 1247; A. MACKOR, T. A. J. W. WAJER, AND T. J. DE BOER, *Tetrahedron*, 24 (1968) 1623.
22 A. L. BUCHACHENKO, *Stable Radicals*, Consultants Bureau, New York, 1965, p. 126.
23 L. E. ORGEL, *An Introduction to Transition-Metal Chemistry Ligand-Field Theory*, Wiley, New York, 1960, p. 100.
24 M. M. TAQUI KHAN AND A. E. MARTELL, *J. Amer. Chem. Soc.*, 89 (1967) 4176; 89 (1967) 7104.
25 A. J. FATIADI, *J. Res. Nat. Bur. Stand.*, 74A (1970) 723.
26 A. J. FATIADI, *Carbohyd. Res.*, 6 (1968) 273.
27 T. POSTERNAK, *Helv. Chim. Acta*, 19 (1936) 1333; 24 (1941) 1045.
28 D. A. ROSENFELD, J. W. PRATT, N. K. RICHTMYER, AND C. S. HUDSON, *J. Amer. Chem. Soc.*, 73 (1951) 5907.
29 R. LOHMAR, R. J. DIMLER, S. MOORE, AND K. P. LINK, *J. Biol. Chem.*, 143 (1942) 551; J. STANĚK AND V. WOLLRAB, *Monatsh. Chem.*, 91 (1960) 1064.
30 A. J. FATIADI, *Carbohyd. Res.*, 9 (1969) 177.
31 H. VON EULER AND A. GLASER, *Ark. Kemi*, 8 (1955) 61.
32 A. J. FATIADI, *Carbohyd. Res.*, 8 (1968) 135.
33 A. J. FATIADI, *Carbohyd. Res.*, 1 (1966) 489.