A METHOD FOR THE DETERMINATION OF SUGAR ALCOHOLS AND THE FATE OF SOME OF THESE COMPOUNDS IN THE ANIMAL BODY

by

JANE AGNES MYERS

A Thesis

Presented to the Department of Biochemistry
and the Graduate Faculty of the University of Oregon
in partial fulfillment
of the requirements for the degree of
Master of Arts

May 1938

APPROVED:

[redacted]

Major Adviser

[redacted]

For the Graduate Committee

of the University of Oregon Medical School

TABLE OF CONTENTS

LIST OF TABLES

LIST OF CHARTS

A METHOD FOR THE DETERMINATION OF SUGAR ALCOHOLS AND THE FATE OF SOME OF THESE COMPOUNDS IN THE ANIMAL BODY

Introduction

Sorbitol has been brought to our attention the last year or two through the work of West and Burget of the University of Oregon Medical School. Previous to this time sorbitol was more or less a laboratory curiosity, expensive, hard to prepare, and of no particular interest. About 1935 sorbitol was put on the market at a reasonable price, making it available in large quantities.

Sorbitol is non-toxic and has enormous water binding capacity. It is rapidly excreted by the kidneys after intravenous injection and has 1.86 times the osmotic pressure of an equimolecular solution of sucrose as its molecule is about one half the size. Its solutions are less viscous and more easily injected than sucrose and are entirely stable to heat sterilization. Apparently sorbitol possesses many of the properties desirable of a physical diuretic (1).

There are several sugar alcohols in addition to sorbitol, some having the same size molecule, being isomeres, and others containing fewer carbon atoms. Theoretically, the smaller the molecule the greater will be its diuretic effect. However, other factors enter in. Some of the sugar alcohols having smaller molecules are not as soluble as sorbitol and therefore would not be as useful as diuretics.

General Properties of Sugar Alcohols. The sugar alcohols, or the polyhydric alcohols are solid crystalline compounds of sweet taste. Many occur as natural products.

They may be obtained by the reduction of the corresponding hydroxy aldehyde, hydroxy ketone, or hydroxy monobasic acid. Conversely, oxidation transforms them first into sugars and then into the corresponding acids.

$$\begin{matrix} CH_2OH \\ (CHOH)_4 \\ CH_2OH \end{matrix} \xleftarrow{red.} \begin{matrix} CHO \\ (CHOH)_4 \\ CH_2OH \end{matrix} \xrightarrow{oxid.} \begin{matrix} COOH \\ (CHOH)_4 \\ CH_2OH \end{matrix} \longrightarrow \begin{matrix} COOH \\ (CHOH)_4 \\ CH_2OH \end{matrix}$$

As a rule they cannot be volatilised without decomposition. Their derivatives are analagous to those of glycol and glycerol. Sugar alcohols are not fermented by yeast, and with the exception of dulcitol do not reduce alkaline copper solutions.

The following table points out some of the physical properties of the more common sugar alcohols.

Table I

Physical properties of Some of the Sugar Alcohols

Sugar alcohol	Sugar related to	Crystal form	Solubility gm/100cc.		M.P.	B.P.	Sweetness S ucrose-100	Formula
d-sorbitol	glucose	colorless rhombic	100	very sol. sol. in hot alcohol	165		54	CH_2OH HCOH HOCH HCOH HCOH CH_2OH
d-mannitol	mannose	fine needles or rhombic crystals	15	sol. cold H_2O; boiling alc. very sol. in absolute alc. insol. ether	166	295	57	CH_2OH HOCH HOCH HCOH HCOH CH_2OH
i-dulcitol	galactose	colorless prisms	6.2	sol. water v.s. alc. v.s. sol. ether	186	295	74	CH_2OH HCOH HOCH HOCH HCOH CH_2OH
dl-erythritol	erythrose	large quadratic crystals	? very sol.	s.s. in alc. insol. ether sol. water	126	331	238	CH_2OH HCOH HCOH CH_2OH

Occurrence. Sorbitol is found chiefly in the family Rosaceae. Bief (2) has found the following fruits to contain sorbitol: apples, pears, cherries, greengages, peaches, apricots, raisins, dried currants, sultanas, cherry plums, and dried dates. E. E. Strain (3) states that the best sources of sorbitol are fruits of the following species of plants: Pyrus, Photinea, Crataegus, Pyracantha, Cotoneaster, and Sorbus (Mountain Ash), all temperate zone plants. Sorbitol has also been found in grapes, grape wines, and red seaweed.

Mannitol has been found in the sap of the manna ash, in olives, sweet wines, larch, celery, sugar cane, brown seaweed, cassava, persimmons, Pelvetia canaliculata, Gardenia jasminoides, Laminaria digitata, Aspergillus fischeri, in twenty species of delphiniuş, in diseased wines, and in all samples of spiked leaf sandal. Busalt (4) has reported mannitol in aqueous extracts of asparagus, green French beans, cauliflower, savoy, carrots, and green peas. Imagaki (5) has found mannitol in twenty-seven varieties of mushrooms grown in Japan. Dox and Plaisance (6) state that mannitol occurs in all samples of corn, sunflower, and cane silage. Mannitol producing organisms are present in soil and milk.

Dulcitol has been found in the sap of the Madagascar ash, red seaweed, and in Melampyrum arvense.

Erythritol is found in Protococcus vulgaris and in many algae and lichens.

Other sugar alcohols are: pentaerythritol, inositol, adonitol, talitol, arabitol, and iditol, the last two of which only are found in nature.

substitutes for linseed oil, lubricants, polishing agents, or in the manufacture of varnishes etc.

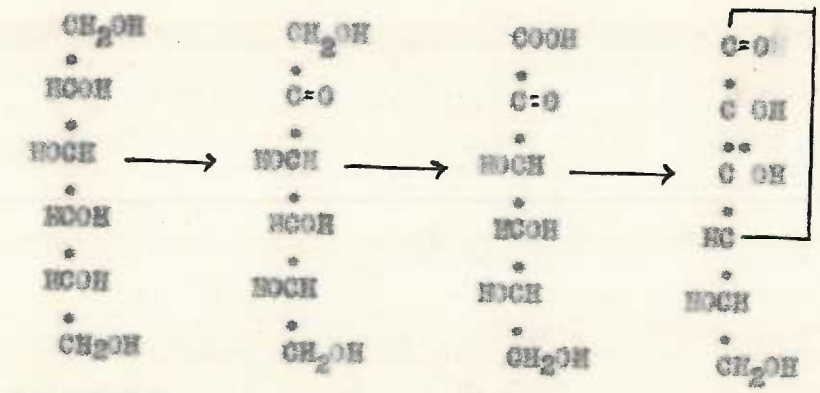
The dehydration products of sorbitol (11), or the products resulting from the action of chemical agents on these dehydration products, are used instead of glycerol, acetin, sugar, camphor, etc. in making printing colors, inks, cosmetics, soap, and varnishes.

"Cider sickness" is caused by a special organism, a bacillus, which attacks chiefly sweet ciders, leading to the reduction of the sugar to mannitol (12). Mannitol is a characteristic constituent of "diseased" wines (13).

Sorbitol is used in softening paper, especially parchment paper (14), in therapeutics, in organic synthesis, in manufacture of artificial resins, plastics, anti-freeze material, in the manufacture of explosives and cosmetics (15), as a water proofing and plasticising agent (16).

Pentaerythritol eleostearate and mannitol eleostearate are used in the preparation of rapidly drying paints and varnishes (17).

Sorbitol is used in the synthesis of vitamin C.



d-sorbitol — l-sorbose — 2 keto l-gulonic acid — l-ascorbic acid or vitamin C.

Chemical Reactions: To produce a lower from a higher polyhydric alcohol, as in the production of glycerol from sorbitol, the higher alcohol is catalytically hydrogenated at a high pressure and a high temperature in the presence of a weakly alkaline compound with the resultant splitting of the carbon to carbon bond.

Clinical Uses: Mannitol hexanitrate (18) and erythritol tetranitrate (19) have been found to be of value in lowering blood pressure.

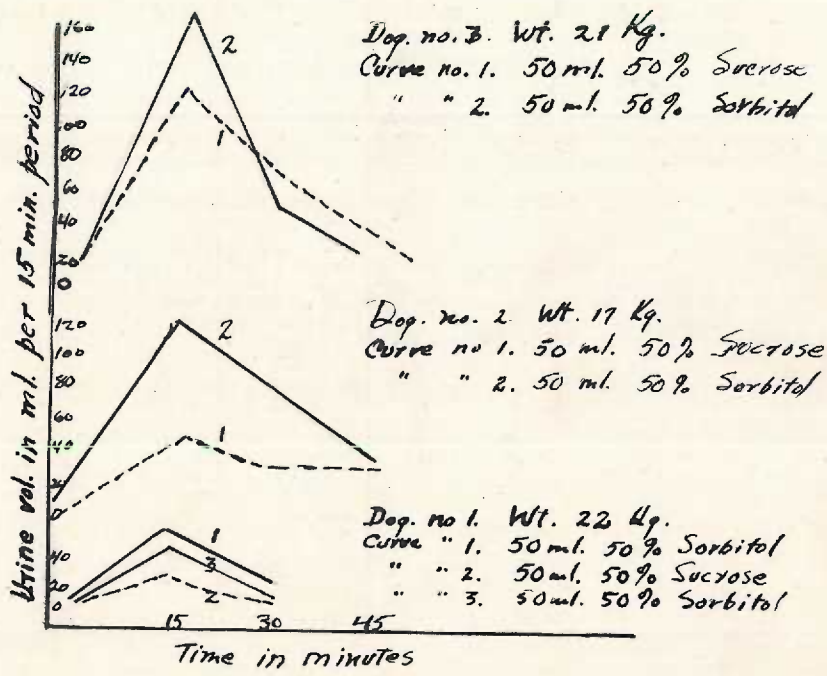
Leake and coworkers (20) have found that by the repeated administration of erythrol tetranitrate they could develop a tolerance to the headache producing action of the drug and to the changes which it produced in the blood pressure and pulse rate.

For some time sorbitol has been used for the relief of intracranial pressure both in brain injuries and post traumatic headaches, advanced arterial hypertension, and to cause diuresis. Strohm (21) recommends the use of sorbitol in certain cases of anuria.

West and Burget (22) compared the diuretic effect of sorbitol and sucrose. They used a 50 per cent solution of each and injected 50 ml. as the experimental dose. In one dog sorbitol was injected first, followed by sucrose, and then a second injection of sorbitol was given. The time interval between injections was long enough to allow the urine out-put to return to normal. Sorbitol had a more powerful diuretic action than sucrose even after the dehydration produced by the previous injection of sucrose. In two more dogs they gave sucrose first and then followed it by sorbitol. In one case Ringer's solution was given to compensate for the fluid lost. Sorbitol was more efficient as a diuretic than sucrose in each instance.

Chart I

Comparison of the Diuretic Effects of Sorbitol and Sucrose



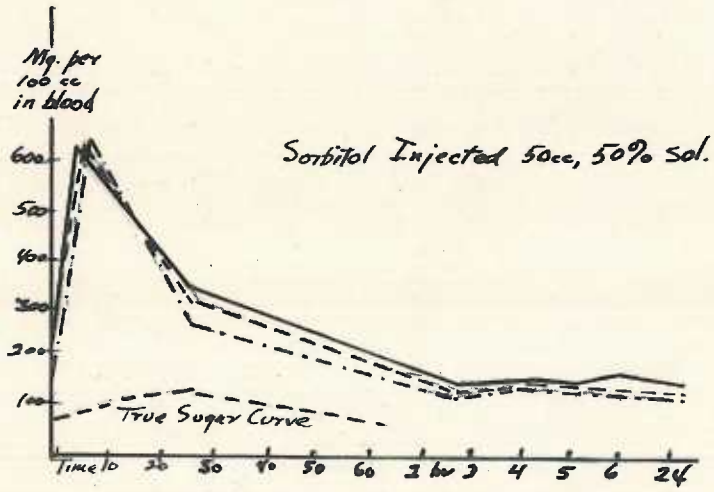
Next they compared the action of mannitol with sorbitol (private communication), using 15 per cent solutions and injecting 150 cc. Mannitol was injected first, followed by sorbitol one hour later. Sorbitol gave a considerably larger diuretic effect, but when this was followed by a second injection of mannitol after another hour, the mannitol was just as effective as the sorbitol. It may have been that the glomeruli had not opened with the first mannitol injection, thus accounting for the lower urine output.

In clarified blood the reduction to the sorbitol reagent was not entirely accounted for by the glucose present indicating that non-sugar reducing substances remained in the filtrate.

Calculated as sorbitol the total reduction amounted to about 120 mg. per cent in dog blood. Upon intravenous injection of 50 ml. of a 50 per cent sorbitol solution into a dog, the sorbitol content of the blood increased to 500-600 mg. per cent in 5 to 10 minutes. It fell to half or less in 30 minutes, and in two hours was practically back to the basal level. The true sugar curve also exhibited a sharp increase, reaching 150 mg. per cent in 30 minutes. After intravenous injection with 50 ml. of 50 per cent sorbitol solution, the volume of urine output per ml. was determined. The output of urine was increased about 500 per cent in 15 minutes, and over a period of 48 hours some 40 per cent of the sorbitol was recovered in the urine.

Chart II

Sorbitol Content of the Blood after Intravenous Injection of Sorbitol.



Since this demonstration of the diuretic effect of sorbitol, it has seemed of value to study methods for its determination and its fate in the animal body.

Part I A Method for the Determination of Sugar Alcohols

The determination of sorbitol in wines has for many years presented considerable difficulty. The usual methods were gravimetric, in which sorbitol was precipitated as hexaacetyl sorbitol (23), dibenzol sorbitol (24), hexabenzoate (25), a ferric barium complex (26), or as the chlorobenzaldehyde (27). These methods are time consuming and present technical difficulties.

The effect of different compounds upon the optical properties of alcohols has been used as the basis for quantitative polarimetric determinations. Methods have been described using the effect of sodium metaborate (28), alkaline arsenous oxide (29), and acidic ammonium molybdate (30).

Sorbitol has been determined electrolytically (31). In an alkaline solution containing potassium ferricyanide, sorbitol forms a ferric-sorbitol complex which ionises and sets up a measurable potential from which may be calculated the amount of sorbitol present.

The reducing power of the polyalcohols has been the basis for quantitative iodometric determinations. Fleury and Marque (31) determined mannitol, inositol, and dulcitol by the oxidizing power of an alkaline solution of potassium mercuric iodide. The excess of iodine was titrated with sodium thiosulfate. Nagyar (33) oxidized polyhydric alcohols quantitatively in strong alkaline solution with sodium hypobromite under the influence of filtered light rays, and the excess of sodium hypobromite was back titrated iodometrically. Malaprade (34) determined erythritol by the action of periodic acid

and alkaline periodates on the polyalcohol. Potassium iodide was added and the liberated iodine was titrated with thiosulfate. Periodic acid is expensive and the reaction takes from 2 to 3 hours to go to completion. Rappaport and Riefer (35) also described a method for the use of periodates in the determination of polyalcohols.

Basis of Method Used in our Experimental Work.

As none of these methods seemed applicable to a routine analysis of sugar alcohols, work was begun on a new method which could be used for the rapid estimation of small amounts of such compounds in biological materials. It was thought in the beginning that it would be advisable to adopt, if possible, the principles of one of the accepted sugar methods. With this in mind the first attempts were made following the principles of the Hagedorn-Jensen glucose method. To a strongly alkaline solution of potassium ferricyanide, sorbitol was added and the mixture heated. It was apparent from the color change that reduction of the ferricyanide had occurred. It was felt that such a reaction could be made the basis of a method for the estimation of small amounts of sorbitol. The following reactions represent the principles involved in the method described in this paper.

An alkaline solution of potassium ferricyanide is the oxidizing agent. Upon heating in the presence of sorbitol the ferricyanide radical is reduced to ferrocyanide with concomittant oxidation of sorbitol. The oxidation is terminated by cooling. The ferrocyanide produced is precipitated as insoluble zinc ferrocyanide by the addition of

an excess of zinc sulfate. Upon the addition of acid and potassium iodide the remaining, or unused, ferricyanide liberates iodine, which can be determined by titration with sodium thiosulfate. Thus the amount of sorbitol originally present is easily calculated.

$C_6H_{14}O_6 + Fe^{+++}$(ferricyanide) $\longrightarrow$ oxidation prod. $+ Fe^{++}$(ferrocyanide)

$Fe^{++} + ZnSO_4 \longrightarrow$ insoluble zinc ferrocyanide

$Fe^{+++} + KI \xrightarrow{HAc} I_2 + Fe^{++}$

$I_2 + Na_2S_2O_3 \longrightarrow 2NI + Na_2S_4O_6$

Development of the Method

Method first used:

Procedure: Sugar tubes were charged with 5 ml. of a solution containing close to 0.5 mg. of sorbitol. Five ml. of ferricyanide reagent were added, the tubes covered with glass bulbs and heated in a boiling water bath for 45 minutes. The tubes were cooled and to each was added 2.5 ml. of zinc sulfate reagent, followed by 5 ml. of glacial acetic acid. The titrations were carried out with 0.005 N sodium thiosulfate and starch indicator was added near the end point. Water blanks were also run.

Reagents:

1. 7.5 gm. $K_3Fe(CN)_6$ dissolved to one liter in 2.5 N NaOH
2. 15 per cent $ZnSO_4$ in water solution. 5 per cent KI added just before using.
3. Glacial acetic acid.
4. 0.005 N $Na_2S_2O_3$.

Calculations: The titration values of known amounts of sorbitol are subtracted from the value of the water blank. The number of mg. present in the tubes divided by the ml. of titration difference gave a factor which was used in the calculation of unknown samples of sorbitol.

Application of the method:

Recoveries of sorbitol from blood and urine using the mercury sulfate-barium carbonate precipitation procedure (described later) were tried with poor and varying results. The values for blood were consistently poorer than for urine. The presence of glucose in blood was suspected of interfering, and the recovery of sorbitol in the presence of glucose in pure solutions, with and without precipitation by mercury sulfate was attempted with varying results and poor titration checks. It was impossible to obtain satisfactory titration checks in triplicate samples. It was decided that the method should be improved before further application.

Colorimetric Determination of Sorbitol:

A colorimetric method for the determination of sorbitol based on the Folin-Wu method for the determination of glucose was tried. The sorbitol was oxidized by heating for thirty minutes with a ferricyanide reagent in alkaline solution (0.1 per cent $K_3Fe(CN)_6$ per liter 1 N NaOH). After cooling, ferric sulphate was added and the solution acidified with phosphoric acid. Ferri-ferrocyanide (Prussian blue) was formed, the intensity of which was matched against prepared standards in the colorimeter.

A gum ghatti solution was added to keep the color in suspension.

This method was unsuccessful as varying amounts of green made it impossible to match with a blue standard even when using a prepared yellow filter.

A Mercury Method for the Determination of Sorbitol:

A solution of mercuric acetate, potassium iodide, and potassium iodate in 5N. NaOH was employed as the oxidizing agent. After heating sorbitol with this reagent, the solution was cooled and acidified with concentrated hydrochloric acid and the liberated iodine titrated against potassium thiosulfate. The titration difference given by this method was from one to two ml. per mg. of sorbitol. With such small differences, minute quantities of sorbitol could not be determined accurately.

It seemed advisable to return to the first method used and to try to improve it by varying the different factors entering into the reactions.

Variation of Factors:

Ferricyanide Reagent: It was necessary to consider the possibility of the formation of ferric hydroxide in the reagent. It is known that the ferricyanide radical decomposes in the presence of strong alkali according to the following equations.

$$K_3\,Fe(CN)_6 \xrightarrow{H_2O} 3K^+ + Fe(CN)_6^{\equiv}$$

$$Fe(CN)_6^{\equiv} + NaOH \longrightarrow Fe^{+++} + 6\,CN^-$$

$$Fe^{+++} + NaOH \longrightarrow Fe(OH)_3$$

$$Fe^{+++} + HI \longrightarrow I_2 + Fe^{++}$$

That this reaction occurred in our reagent was demonstrated by filtering the reagent through a sintered glass filter and collecting $Fe(OH)_3$ on the filter. This presence of $Fe(OH)_3$ would make for a non-homogeneous solution, and as varying quantities of $Fe(OH)_3$ present during the oxidation would lead to varying titration values, it was evident that some change in procedure was necessary.

The first attempt was made by adding different salts to the stock oxidizing reagent of 7 gm. of potassium ferricyanide per liter of 2 N NaOH. As potassium fluoride and potassium pyrophosphate form unionizable ferric salts, they were separately added in an attempt to minimize the formation of ferric hydroxide. Neither of these salts accomplished this purpose. As will be seen later this difficulty was overcome through a different approach.

It was noticed that a newly prepared reagent gave better results than one several days old. Perhaps the formation of ferric hydroxide could be eliminated by preparing a water solution of ferricyanide and combining it with alkali just before using. As this gave no better results it was decided to hunt for some other source of error.

Sodium chloride and sodium sulfate were added in turn with the hope of minimizing the solubility of air in the reagent, but brought no improvement. Rochelle salts and potassium cyanide were both tried in hopes of introducing a stabilizing factor, but did not help.

Because of the greater solubility of potassium fluoride and potassium pyrophosphate in KOH than in NaOH, reagents were prepared using these potassium salts in KOH, the latter being used in place of

NaOH in the oxidizing solution. Compared to the corresponding reagents prepared with NaOH and sodium salts, no improvement was observed.

It was felt that perhaps oxidation from the air was occurring during the heating, so different means of preventing this were tried. Instead of the customary glass bulbs covering the sugar tubes, rubber stoppers with capillary glass tubing were used. Overlaying the solution with a thin coat of paraffin or mineral oil was also tried. These procedures raised the titration values of the blanks and of the unknown sorbitol solutions, but the results were not improved. The use of the customary glass bulbs was continued.

A variation in alkalinity was tried in hopes that a less alkaline solution would lessen the ionization of ferricyanide and the formation of ferric hydroxide. The same reagent was prepared in 1, 1.5, and 2 N. NaOH but the titrations showed no better checks than when a more alkaline oxidizing reagent was used. The use of 2 N. alkali was continued as it gave a greater titration difference per unit of sorbitol present and therefore a more accurate method.

Not only the amount of acid but also the type of acid was thought to be a factor in the ionization of the precipitate zinc ferrocyanide. Phosphoric, citric, and acetic acids were added in different concentrations after cooling the tubes. It was found that mineral acids could not be used as they allowed too much ionization of zinc ferrocyanide. Acetic acid was found to be more satisfactory than the others tried and was therefore used in the remainder of the work.

Zinc Reagent. Next the constituents of the zinc reagent were varied. In order to prevent the formation of sulfuric acid upon hydrolysis of zinc sulfate, other salts of this metal were used. Of those tried the acetate was found to be most satisfactory.

Zinc acetate and potassium iodide were added separately, varying the time interval between the additions. The tubes were allowed to stand different lengths of time before titrating. No improvement in titration values resulted from these varying procedures.

The amounts of zinc acetate and potassium iodide were varied. From 1 ml. to 6 ml. of a 10 per cent solution of each of these salts were added to different tubes in an attempt to determine their optimum concentration for the reactions. Without a large excess of zinc and potassium iodide, the end point of the titration was very poor. Upon standing before titrating, triplicate samples showed a progressive increase in titration value and further amounts of iodine appeared a few minutes after completion of the titration, indicating that the reaction had not gone to completion. When 2 ml. were used the end point was very sharp and no additional iodine was liberated after the titration.

The order of the addition of zinc acetate, potassium iodide, and acid was varied. The addition of acid before zinc resulted in the formation of Prussian blue. However, by mixing equal portions of a zinc acetate-potassium iodide solution and glacial acetic acid just before use, and adding these three components at once, not

only were the number of steps in the procedure lessened, but the reaction went to completion in a satisfactory manner.

It was found that when a sorbitol solution stood in the presence of the alkaline ferricyanide reagent, slight oxidation took place in the cold. It was realised that the time involved in filling the tubes allowed different reaction times in different tubes before heating. This oxidation was not avoided at 0 C. To eliminate this error the tubes were filled one at a time and immediately placed in the water bath. The titration checks were much improved, but still the formation of ferric hydroxide in the reagent produced an error. To eliminate this the ferricyanide and the alkali were added separately. A ferricyanide reagent in water solution which does not oxidise sorbitol was added to all the tubes containing known amounts of sorbitol, and the alkali then added to each tube separately immediately before placing in the water bath. For the first time the titration values checked, and continued to check from day to day.

The Method as Finally Worked Out:

The following procedure was used for the determination of sorbitol.

Reagents:

1. 1.0 per cent potassium ferricyanide in water.
2. 5 per cent sodium sulfate in 3.33 N sodium hydroxide
3. A solution of 12 per cent potassium iodide and 15 per cent zinc acetate.

4. Glacial acetic acid

5. 0.005 N sodium thiosulfate

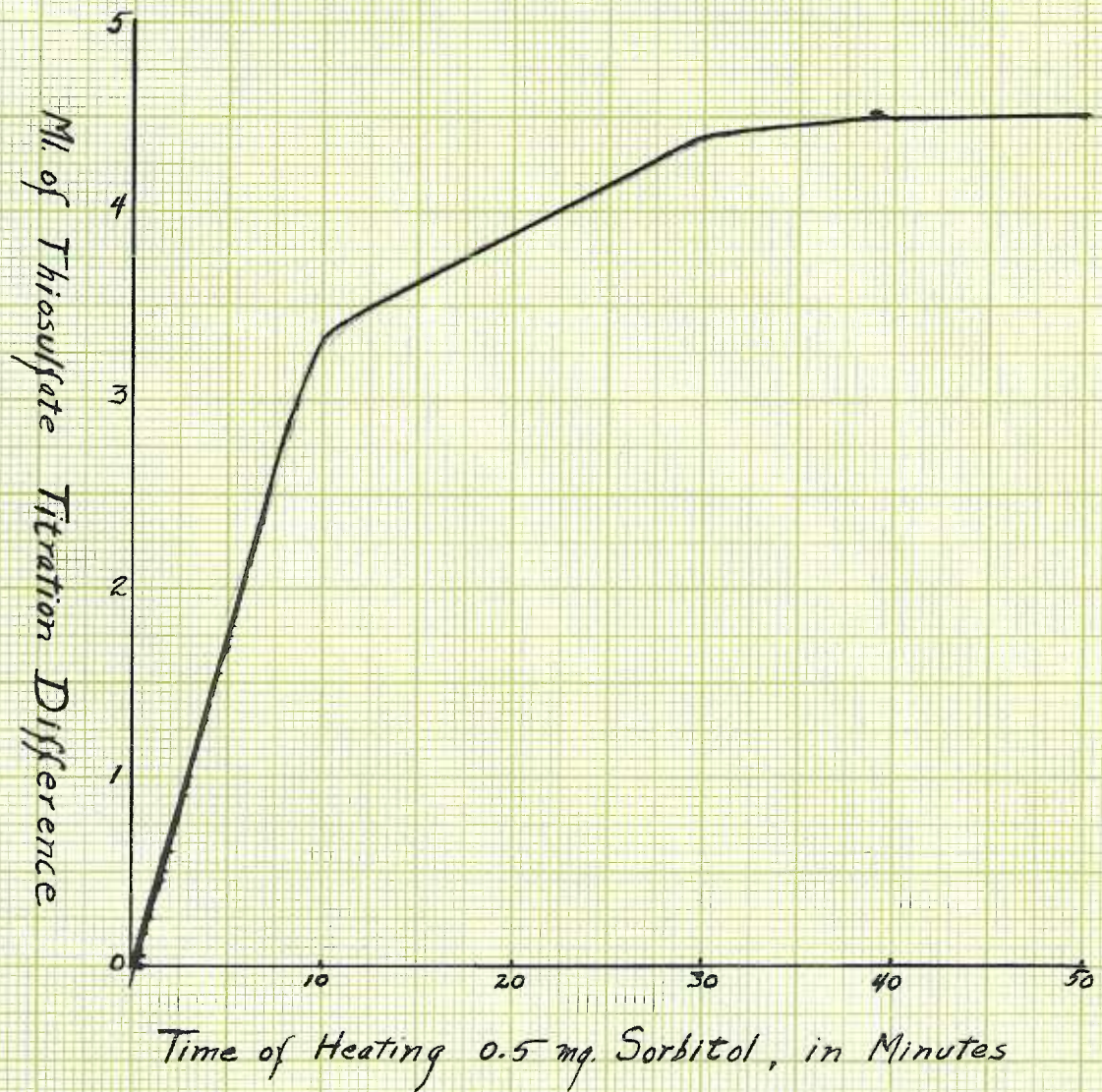
Procedure: Triplicate pyrex sugar tubes were charged with 5 ml. of a solution containing from 0.1 to 1.0 mg. sorbitol. Three 5 ml. water blanks were run simultaneously. To each sugar tube were added 3 ml. of reagent one. 5 ml. of reagent two were quickly blown in, charging three tubes in rapid order. Each of the three tubes was covered with a glass bulb, shaken to provide thorough mixing of the solution, and immediately placed in a boiling water bath. The time required to fill three tubes with reagent two and to place them in the water bath should not exceed one minute. Each set of three tubes was heated exactly thirty minutes, then plunged in a cold water bath. Reagents three and four were mixed in equal proportions. 5 ml. of this solution were blown in to each tube from a fast flowing pipette. Each tube was shaken to insure mixing. The liberated iodine was titrated with 0.005 N. sodium thiosulfate using a few drops of starch indicator near the end point.

When sorbitol was heated with the oxidizing agent the major portion of the reaction took place in the first several minutes. After this the speed of the reaction slowed down considerably. When time was plotted as abscissa and titration difference as ordinate the curve rose sharply and then gradually straightened out. In order to determine the shortest time of heating commensurate with the accuracy desired, 0.5 mg. samples of sorbitol were heated with the oxidizing reagent for varying lengths of time.

Chart III shows that at 30 minutes the curve had reached a plateau, indicating that the oxidation was proceeding slowly. This 30 minute period was chosen as the standard heating time in the succeeding work.

Chart III

Ml. of Titration Difference per Ten Minute Periods of Heating 0.5 mg. Sorbitol



Calculations: With the reagents and conditions above, 0.5 mg. of sorbitol gave a titration difference of 4.36 ml. of thiosulfate. Each ml. of titration difference then represented $\frac{1}{4.36}$ of 0.5 or 0.115 mg. of sorbitol. Factors for amounts of sorbitol from 0.1 to 1.0 mg. were thus determined and used in the calculation of unknown amounts of sorbitol. The ml. of titration difference were plotted against the mg. of sorbitol present. After determination of the number of ml. of titration difference, the number of mg. present in an unknown sample of sorbitol could be read directly from this curve.

In the same manner, factors and curves were obtained for mannitol and glucose. Graph IV shows the amount of oxidation, expressed as ml. of titration difference, obtained with amounts of sugar alcohols varying from 0.1 to 1.0 mg.

Chart IV

Relation of Concentration to Oxidation

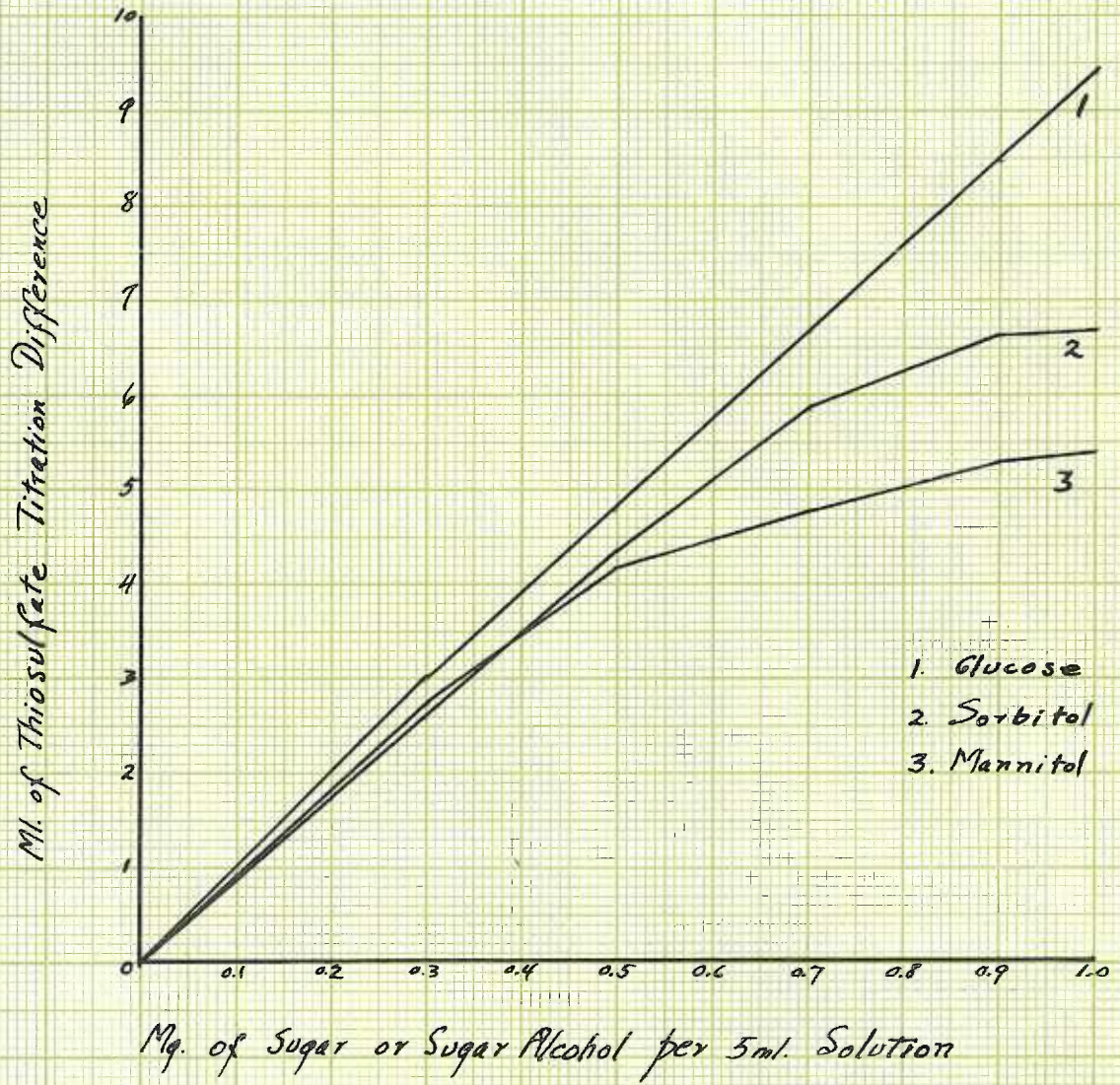


Table II gives the factors determined as above for sorbitol, mannitol, and glucose. These factors may be used for calculating unknown amounts of these sugar alcohols.

Table II

Factors of Sugar Alcohols for Varying Concentration

Sugar or Sugar Alcohol	No. of mg. present					
	0.1	0.3	0.5	0.7	0.9	1.0
Sorbitol	.109	.114	.116	.120	.136	.150
Mannitol	.107	.110	.120	.141	.170	
Glucose	.103	.103	.104	.105	.106	.107

Application of the Method

Recovery from Pure Solutions: Of the various methods of clarifying blood or urine for sugar determinations, that of West, Scharles, and Peterson (36) proved satisfactory for our work. By this method the precipitation is accomplished with a 23 per cent solution of mercury sulfate in 2 N. Sulfuric acid. The excess is neutralized with solid barium carbonate and the remaining traces of mercury removed with zinc dust. By this procedure not all of the non-sugar reducing substances were removed from blood or urine although a rather constant value was found for various specimens. The following data indicate that the addition of this reagent did not interfere with the recovery of sorbitol and mannitol from pure solutions of these compounds.

Procedure: To a solution containing a known amount of sorbitol or mannitol, 5 ml. of $HgSO_4$ reagent were added and the mixture neutralised with 8 gm. of solid $BaCO_3$. The suspension was shaken to rid it of CO_2. If the suspension was not neutral to blue litmus, more $BaCO_3$ was added until neutrality was reached. The mixture was filtered and 1 gm. of zinc dust was added to the filtrate and the solution again filtered through No. 2 Whatman paper. Determinations were run on 5 ml. aliquots of this filtrate. Table III shows the recoveries obtained after this treatment.

Table III

Recovery of Sorbitol and of Mannitol from Pure Solution following Treatment with $HgSO_4$ Reagent

Sugar Alcohol	Mg. used	Total volume in ml.	Mg. recovered	Per cent Recovered
Sorbitol	3.0	35	3.18	106
Sorbitol	5.5	60	5.28	96
Sorbitol	5.5	60	5.39	98 — 100 Average
Mannitol	3.0	35	3.3	110
Mannitol	5.0	50	4.9	98
Mannitol	5.0	100	5.4	108 — 106 Average

It is seen from the above that excellent recoveries were possible from pure solutions of the sugar alcohols. Attempts to recover sorbitol and mannitol from urine were carried out as follows.

Recovery from Urine: Known amounts of sorbitol or of mannitol were added to urine and after treatment with $HgSO_4$ reagent the sugar alcohol was determined in the clarified filtrate.

Procedure: In each of two Erlenmeyer flasks were placed 10 ml. of urine. To one 75 ml. of water were added and to the other 75 ml. of a solution containing 30, 40, or 50 mg. of sorbitol. 15 ml. of the $HgSO_4$ reagent were added to each and the procedure followed as outlined above. The final filtrates were diluted 1:10 or as required, depending on the original concentration, and the sorbitol determined. Recoveries of mannitol were also made following the same technique. Table IV indicates that the recovery of these two compounds from urine is high enough to make the method valuable for physiological work.

Table IV

Recovery of Added Sorbitol and Mannitol from Urine after Precipitation with $HgSO_4$ Reagent

Sorbitol			Mannitol		
Mg. added to 10 ml. urine	mg. recovered	per cent recovery	Mg. added to 10 ml. urine	mg. recovered	per cent recovery
50	4.5	90	50	4.75	95
50	4.7	94	50	4.55	91
50	4.8	96	50	4.5	90
50	4.25	85	50	4.60	92
50	4.6	92			
		91 average			92 average
40	3.4	85	40	3.76	94
40	3.78	94	40	4.15	104
40	3.54	86	40	3.68	92
40	3.68	92			
		89 average			96 average
30	2.79	93			

Although the recoveries were not exceptionally good they were considered satisfactory for biological work. It can be stated that in general only larger variations in concentration of the sugar alcohols in urine would be of significance and that the method is satisfactory under these conditions.

Recovery from Blood: The first attempts to recover added sorbitol from blood gave very low recoveries. As glucose is oxidized very rapidly by the ferricyanide reagent it was apparent that its presence in blood was introducing a complicating factor. During the heating process the glucose undoubtedly reduced a portion of the ferricyanide and consequently there remained less to react with the sorbitol.

This assumption was born out by the data obtained when recoveries of sorbitol from glucose solutions were determined. The results indicated that under these conditions the reduction per unit of sorbitol present was less than when sorbitol alone was employed. By determining the factor for sorbitol in the presence of varying concentrations of glucose and employing the revised factor in calculating the recoveries from blood, far better results were found. In other words, fairly good recoveries had been made from blood, but the calculations were incorrect and thus indicated much poorer recoveries.

Procedure: To each of two flasks were added 5 ml. of whole blood. To one were added, for example, 115 ml. of water and 5 ml. of the $ZnSO_4$ reagent. To the other were added a solution containing 5 mg. of sorbitol and water sufficient to make 115 ml.

After the addition of 5 ml. of $HgSO_4$ reagent both flasks were treated as previously described. These volumes were varied and it was found that a total of from 100 or 125 ml. was convenient when 5 ml. of blood were used. The addition of 5 ml. of sorbitol in this volume allows an amount which together with the blood glucose yields in a 5 ml. aliquot a total reduction well within the limits of the method.

The following table (Table V) indicates the percentage recovery of sorbitol added to dog blood. Included also are data on the recovery of sorbitol from glucose solutions. The amounts of sorbitol and glucose were so arranged that the 5 ml. aliquot used for analysis contained quantities of these two compounds similar to that contained in the blood filtrates prepared from blood and added sorbitol.

Table V
Recovery of Added Sorbitol from Blood and from Glucose Solutions

Blood			Glucose		
mg. added to 5cc blood	mg recovered	percentage recovered	mg. sorbitol added	mg. recovered	percentage recovered
5	4.35	87	5	5.1	102
5	4.75	95	5	4.7	94
5	4.6	92	5	4.6	92
5	5.0	100	5	4.8	96
5	4.7	94	5	2.25	105
5	4.6	92	5	5.1	102
5	4.8	96	5	4.8	96
5	5.3	106	5	5.4	108
5	4.1	81			99 average
5	4.1	81			
		92.4 average			

It is evident that the recoveries were in some instances quite low (81 to 96 per cent). These recoveries are not all that might be desired, but at the same time suffice for the type of work for which the method is employed.

Part II The Fate of Sugar Alcohols in the Animal Body

The dried saccharin exudate, manna, obtained from Fraxinus ornus has been used for years in the medicine of folk lore as a laxative. In 1893 Jaffe observed that mannitol could be fed to dogs and recovered unchanged in the urine. However, he found that rabbits utilized the compound well. The resistance of mannitol to oxidation in the body is interesting when contrasted with the behavior of sorbitol which readily undergoes oxidation (37).

In 1919 Field (28) studied the effect on blood sugar one, two, and three hours after the ingestion of 100 gm. of mannitol and other sugars by normal colored males. The maximum rise with glucose was 40 mg. per cent and with mannitol 10 mg. per cent. Urines were tested for glucose for three hours after the last sample of blood was taken, and in no case did the specimen show a trace of reducing substance with Benedict's qualitative solution.

In 1925 Voegtlin (39) observed the failure of mannitol to relieve insulin shock in white rats.

The same year Uglow (40) pointed out that dulcitol had a retarding effect upon the enzymes pepsin, pancreatic lipase, and diastase in the intestinal tract. He pointed out that weak alkalis and acids decomposed dulcitol, forming aminophenols, which changed oxyhemoglobin to methemoglobin. The same decomposition products arise in the animal body and may be detected in the urine. The poisonous activity of dulcitol upon the blood was due to these decomposition products. A dose of 0.1 gm. per kg. body

weight caused sickness. He concluded that its use as a substitute for sugar should be avoided.

In 1929 Kaufman (41) recommended sorbitol for use in the diet of the diabetic. He stated that it was sweet, easily absorbed, could be oxidised by the diabetic organism, hence spared protein, led to glycogen formation, and did not elevate blood sugar.

In 1929 Reinwein (42) also recommended the use of sorbitol in the treatment of diabetes. He noted that following the administration of sorbitol there was observed no rise in blood sugar and no decrease in carbohydrate tolerance. Dyspeptic symptoms followed doses of 75 gm. but were absent if smaller doses were given. A rise in respiratory quotient and disappearance of hypoglucemic symptoms indicated the utilization of the compound by the organism.

Donhoffer (43) stated in 1930 that sorbitol produced an increase in blood sugar in normal metabolism and in diabetes. However, a month or two later he published another article (44). He had fed fasting subjects 50 gm. of sorbitol in 300 ml. tea. In the blood of the normal subject following administration of sorbitol, approximately equal gains were observed in both glucose and sorbitol. In the blood of the diabetic, however, the amount of glucose increased enormously, reaching a maximum in 105 minutes, but the sorbitol was no greater than in the normal subject. Since sorbitol is easily synthesized into glycogen the sorbitol hyperglucemia seemed to result from the same causes as hyperglucemia from the ingestion of glucose and other carbohydrates.

Carr and Krantz and coworkers (45) at the University of Maryland Medical School have worked on the fate of dulcitol and mannitol in the animal body. They worked on white rats after fasting them 24 hours. The controls were given a liberal supply of cacao butter and allowed to continue on this diet for 80 hours. The experimental rats were fed mixtures of 33 per cent mannitol and dulcitol and killed by exsanguination. Glycogen was determined by Pflüger's method, and glucose by Shaffer-Hartman. Liver glycogen from mannitol was increased about 000 per cent, and from dulcitol 300 percent, while the tissue glycogen decreased 40 per cent with dulcitol.

According to these authors, neither dulcitol nor mannitol affected the respiratory quotient. Mannitol, when injected intraperitoneally produced diarrhea in rats. 1.3 gm. of mannitol per 100 gm. rat given by stomach tube was toxic, killing them by respiratory paralysis. Dulcitol exhibited no toxic symptoms. With rabbits, mannitol produced a slight but significant rise in blood sugar, but dulcitol produces no rise.

There seems to be a difference of opinion as to whether or not sorbitol forms liver glycogen. Ehrlich (46), cited by Carr and Krantz, has established the fact that in the dog sorbitol raised liver glycogen comparable to the rise after the ingestion of glucose, 97 per cent was utilized and 0.5 to 3 per cent was eliminated in the urine. Hyperglucemia was less than after the ingestion of glucose.

In direct contradiction to this, Rayhaud and Roche (47) at the Hospital of Marseille, working with guinea pigs, found in every case

proof of the non-transformation of sorbitol into glycogen. They administered 2.5 gm. of sorbitol intra-peritoneally and fed controls caterpillars.

Roche and Bayhand have also experimented on rabbits and found that sorbitol was not transformed into glycogen in fasting rabbits. They were looking into this problem in hopes of being able to use sorbitol in the diabetic diet, but concluded from this and other work that they could not recommend its use. They also stated that it had no effect on insulin hypoglucemia.

Other Frenchmen, Bertrand, Nadais, and Labbe (48), investigating sorbitol as a possibility for the diabetic diet, stated that sorbitol was poorly absorbed and not better tolerated than glucose, and that it caused gastric disturbances.

Payne, Lawrence, and McCance (49) have also observed that there was a slight rise in blood sugar after giving sorbitol, but much less than with glucose. They remarked that this was not understood, but concluded that sorbitol might be used for a sweetening agent for diabetics as it does not enter directly into carbohydrate metabolism. They found that sorbitol had no effect in relieving insulin hypoglucemia and failed to increase liver glycogen in starved rats. However, intense hyperaemia and dilatation were produced in the small intestine. They felt that sorbitol probably was not absorbed and acted as a foreign sugar. This tendency toward intestinal irritation limits doses to 50 gm. a day.

Silbermann and Lewis have investigated glycogen formation from mannitol (50). Young white rats were fasted 24 hours and fed 2 or 4 ml. of 15 per cent solution of mannitol by stomach tube. After absorption periods of 2, 3, 4, and 6 hours the rats were killed and the liver glycogen determined. The data failed to reveal any significant increase over the control series. They concluded that mannitol did not serve as a readily available source of glycogen.

As the literature presented such conflicting statements as to the utilization of sugar alcohols by the animal body, work was begun to determine the formation of liver glycogen in rats from various sugar alcohols.

Procedure: White rats, after 48 hours of starvation, were given a sugar alcohol by stomach tube, intra-peritoneally or subcutaneously. After a suitable length of time to allow formation of liver glycogen, the rats were killed by decapitation, the livers immediately removed, ground in a meat grinder, and approximately 1 gm. placed in 2 ml. of 30 per cent KOH in a 50 ml. conical pyrex centrifuge tube. The time required to kill the rat and place the liver in alkali did not exceed 1½ minutes. The tubes were weighed before and after the addition of the liver. The tubes were heated about ten minutes in a boiling water bath, or until the mixture became homogenous. 1.1 volumes of 95 per cent ethyl alcohol were added to each tube to precipitate the glycogen. They were then heated to boiling to flocculate the glycogen and then cooled and centrifuged. The supernatant liquid was poured off and the tubes heated in a

boiling water bath to drive off the remaining alcohol. 5 ml. of 0.6 N. HCl were added to each tube and the tubes heated for two hours in a boiling water bath to hydrolyse the glycogen to glucose. The amount of glucose present was determined by the Shaffer-Hartmann method. As 1.06 gm. of glucose are equivalent to 1 gm. of glycogen, the amount of glycogen present was easily calculated. The following tables show the results of these determinations.

Table VI

Glycogen Storage in the Liver of Starved White Rats following the Administration of Sorbitol

No. of rats	Hours starved	Method of administration	Time in hours of administration and quantity used.	Killed: hours after zero hour	Av. per cent liver glycogen
5	48	Stomach tube	1cc 50% 0 1cc 50% 4.5	8	1.060
3	48		none	**	0.148
4	48	Stomach tube	1cc 50% 0 1cc 50% 4	7	0.530
3	48		none	**	0.091
5	48	stomach tube	2cc 25% 0 2cc 25% 2	5	0.480
5	48		none	**	0.570
5	48	stomach tube	2cc 20% 0 2cc 20% 1 2cc 20% 2 2cc 20% 3 2cc 20% 4	5	0.49
5	48		None	**	0.39
2	40	subcutaneous	1cc 25% 0 1cc 25% 1	5	2.15
2	40		none	**	0.44
3	48	subcutaneous	2cc 12.5% 0 2cc 12.5% 1	2	1.53
3	48		none	**	0.02
4	48	subcutaneous	2cc 12.5% 0 2cc 12.5% 0	2	2.28
4	48		none	**	0.40
5	48	intra-peritoneal	4cc 12.5% 0 4cc 12.5% 2	6	2.53
5	48		none	**	0.57
5	48	intra-peritoneal	4cc 12.5% 0 4cc 12.5% 2	6	1.720
5	48		none	**	0.264

*Rats starved 24 hrs., fed 2 hrd., starved 48 hrs.
** Controls killed immediately after experimentals.

Table VII

Glycogen Storage in the Liver of Starved White Rats following the Administration of Mannitol

No. of rats	Hours starved	Method of administration	Time of administration and quantity used Time in hrs.	Killed: hours after zero hour	Av. per cent liver glycogen
4	48	stomach tube	1.5cc 20% 0 1.5cc 20% 2 1.5cc 20% 4	5.5	0.25
4	48		none	**	0.43
5	48	stomach tube	2cc 20% 0 2cc 20% 1 2cc 20% 2 2cc 20% 3 2cc 20% 4	5	0.26
5	48		none	**	0.28
5	48	stomach tube	2cc 20% 0 2cc 20% 2	4	0.33
5	48		none	**	0.33
5	48	intra-peritoneal	4cc 12.5% 0 4cc 12.5% 2	5	0.27
5	48		none	**	0.33
4	48	intra-peritoneal	4cc 10% 0	4	0.24
4	48		none	**	0.43
5	48*	intra-peritoneal	4cc 12.5% 0 2	5	0.214
5	48*		none	**	0.264

*Rats starved 24 hrs., fed 2 hrs., starved 48 hrs.
** Controls killed immediately after experimentals.

Table VIII

Glycogen Storage in the Liver of Starved White Rats following the Administration of Erythritol

No. of rats	Hours starved	Method of administration	Time in hours of administration and quantity used	Killed: hours after zero hour	Av. per cent liver glycogen
5	48	stomach tube	1.5cc 25% 0 1.5cc 25% 2 1.5cc 25% 4	6	0.15
5	48		none	**	0.11
4	48	stomach tube	1.5cc 25% 0 1.5 cc 25% 2	4	0.420
2	48		none	**	0.298
4	48	stomach tube	1cc 25% 0 1cc 25% 2	5	0.451
4	48		none	**	0.03
3	46	sub-cutaneous	2cc 12.5% 0 2cc 12.5% 1	2	0.610
4	46		none	**	0.247
4	46	sub-cutaneous	2cc 12.5% 0 2cc 12.5% 1	2	0.620
3	46		none	**	0.131
3	46	intra-peritoneal	4cc 12.5% 0	2	0.61
3	46		none	**	0.13
4	48	Intra-peritoneal	4cc 7.5% 0	2	0.109
2	48		none	**	0.298
5	48	Intra-peritoneal	4cc 12.5% 0 4cc 12.5% 2	5	0.249
5	48		none	**	0.264

*Rats starved 24 hrs., fed 2 hrs., starved 48 hrs.
** Controls killed immediately after experimentals.

It will be seen from the above tables that the administration of sorbitol to starved rats led to the storage of liver glycogen. Mannitol, on the other hand, allowed so much storage. The data are inconclusive with regard to erythritol. To substantiate these findings sorbitol and mannitol were given intravenously to dogs to determine if they could be converted to glucose in the blood, as such a transformation is a pre-requisite to the formation of liver glycogen.

Formation of Blood Sugar in Dogs.

The procedure was as follows: 150 ml. of a 15 per cent mannitol solution were injected intravenously in a dog. Blood samples were taken before and after the injection, clarified by the mercury sulfate precipitation procedure, and a glucose determination made on the filtrate. Two days later the procedure was repeated on the same dog, injecting 150 ml. of a 15 per cent sorbitol solution.

The true sugar content of the blood increased after the intravenous injection of sorbitol, reaching a peak of 118 mg. per cent in 40 minutes. The sugar content of the blood failed to show any increase after the injection of mannitol. Chart V shows the true sugar content of the blood after the injection of each.

Chart V

True Sugar Content of the Blood After Intravenous Injection of Sorbitol and Mannitol into Dogs

Curve 1 Sorbitol

" 2 Mannitol

Discussion

Part I

In the estimation of sorbitol by the method outlined heretofore, several points may well be emphasized. It has been our experience that the ferricyanide solution undergoes some change on standing for a few weeks. After this time poor results are obtained with its use and we have found it necessary to prepare new reagents from time to time. When the quantity of sugar alcohol contained in the 5 ml. aliquot used for analysis approaches .7 to 1.0 mg. triplicate checks are generally rather poor indicating that this amount exceeds the limit of the method. Although this constitutes a drawback to the method it is easily overcome by proper dilution.

In the earlier work a heating period of 45 minutes was employed. A glance at Chart III indicates that although after 30 minutes of heating the oxidation is still proceeding somewhat rapidly it was felt that by using care in the timing of this heating period the shorter interval could be used successfully especially in view of the advantage gained in time.

As previously stated the zinc Acetate solution must be mixed with the acid immediately preceeding its use. On standing any length of time iodine is liberated and after this has occurred it cannot be employed in the method. It has also been found more satisfactory to prepare small amounts of the Zinc acetate solution, enough say, to last only several days. After this solution has stood a week or two iodine is liberated more rapidly upon the addition of acid than in one freshly prepared.

The end point is very sharp and easily seen after a little experience. The change is from the starch-iodine color to a milky white.

Recoveries of added sorbitol or of mannitol from urine are generally 90 per cent or over. From blood as low as 81 per cent recovery was obtained on several occasions. However, these recoveries suffice for the type of work for which the method is employed.

Part II

Sorbitol Mannitol

In view of the similarity of these two molecules it would be expected that the animal organism would handle them in somewhat the same fashion. Contrary to this expectation it was found that the administration of mannitol to starved rats did not lead to the storage of liver glycogen while the administration of sorbitol led to a copious storage of this substance in the liver. This held true whether the sorbitol was administered per os, subcutaneously or intraperitoneally.

The concentration of the solution used and the time for absorption are important considerations. Enough work has not been done to state the optimum for either of these variables. By our technique the largest storages of sorbitol were found after subcutaneous or intraperitoneal injection. That subcutaneously injected sorbitol leads to the formation of liver glycogen indicates that a passage through the intestinal wall is not a pre-requisite for the oxidation of sorbitol to glucose in the animal organism.

In no case did the administration of mannitol lead to liver glycogen formation. It cannot be stated definitely that this compound is unable to act as a precursor of glycogen but under our experimental conditions it did not. By the administration of more doses and by allowing either shorter or longer absorption times it might be shown that mannitol may undergo such a transformation.

The data regarding true blood sugar formation following the administration of sorbitol and of mannitol to dogs substantiate the above findings, i.e., blood sugar increased following intravenous sorbitol and remained unchanged following the injection of mannitol under like conditions.

Conclusions

(1) A method is described for the estimation of sugar alcohols. By this method .1 to .7 mg. may be determined. The accuracy of the method makes it applicable to physiological work.

(2) The method has been applied successfully to sorbitol and mannitol. Other work than herein described indicates that the method is also applicable to dulcitol, erythritol, inositol, and pentaerythritol.

(3) Studies have been made on the possibility of glycogenesis in starved white rats after the administration of sorbitol and mannitol. It has been demonstrated that the former under our working conditions led to the formation of liver glycogen while the latter did not. The data regarding erythritol in this connection were not conclusive. As further evidence for the ability of the animal organism to form glucose from sorbitol and not from mannitol, these compounds were administered intravenously to dogs and the true blood sugar determined after the administration. The data indicated that the former led to glucose formation and that the latter did not.

BIBLIOGRAPHY

(1) West, R.S., and Burget G.E., Proc. Soc. Exp. Biol. Med., 35, 105 (1936).

(2) Reif, O., L. Untersuch Lebensmen, 68, 179-86 (1934).

(3) Strain, H.H., J.A.C.S., 59, 2264 (1937).

(4) Busalt, E., Biedermanns Lentr., 47, (1918).

(5) Imagaki, S., J. Pharm. Soc. Jap., 54, 726 (1934).

(6) Dox, A.W., and Plaisance, G.P., Science, 46, 192-3 (1917).

(7) Farbenind, I.G., Br., 301, 655, Dec. 19, 1927.

(8) Bergeim, Frank, U.S. 1, 751, 439, March 18, 1929

(9) Stettbacher, A., Z. ges Schiess Sprengstoffw, 25, 461 (1930).

(10) Farbenind, I.G., Fr. 703, 792. Oct. 7, 1930.

(11) Ibid. Fr. 705, 428. Dec. 7, 1927

(12) Tutin, F., Biochem. J. 19, 416 (1925).

(13) Neihaus, C., Farming South America 6, 445 (1932).

(14) Meyer, K.H., Muller, J., and Hoffman, U. U.S. 1,731, 679. Oct. 15, 1930.

(15) Frylender, N.N., Rev. Prod. Chim. 40, 129 (1937).

(16) Matagrin, A., Papeterie 58, 1077 (1936).

(17) Shen, T., and Kuo, C., Chio Tung Univ. Research Inst. S.40.

(18) Herrman, R., Leake, C., Loevenhart, A., and Muehlberger, C., J. Pharmacol., 27, Proc. 259 (1926).

(19) Vetlesen, H.J., Norsk. Mag. Laegev., 22, 120 (1934).

(20) Leake, C., Loevenhart, A., and Muehlberger, C., J. Pharmacol. 41, 103-19 (1931).

(21) Strohm, J.C., Western J. Surgery 200, April, 1930.

(22) West, R.S., and Burget, C.E., Proc. Soc. Exp. Biol. Med., 25, 103-7 (1926).

(23) Werder, J., Mitt. Lebensmm Hyg. 21, 121-3 (1930).

(24) Ibid., 20, 14-5 (1929).

(25) Kruszewski, P., Przemyst Chem. 18, 144-6 (1934).

(26) Traube, Wilhelm, Ger. 559, 228. April 23, 1935.

(27) Vogt, E., Z. Untersuch Lebensm. 69, 567-91 (1935).

(28) Boeskin, J., Rec. Trav. Chim., 54, 863 (1935).

(29) Badreau, J., J. Pharm. Chim., 224, 12 (1921).

(30) Frerejacque, M., Compt. Rendu, 200, 1410 (1935).

(31) Komarek, K., Czechoslavakian Coll., 7, 404 (1935).

(32) Marque, J., and Fleury, P., J. Pharm. Chim. (8), 10, 241 (1929).

(33) Magyar, K.S., Tanszag Ertesitoje, 9, 125-47 (1937).

(34) Malaprade, L., Bull. Soc. Chim., (5), 1, 833 (1934).

(35) Riefer, R., Ch. Ab., 31, 77-86 (1937).

(36) West, R.S., Scharles, F., and Peterson, V., J. B. C., 82, 137 (1929).

(37) Carr, C.J., Musser, R., Schmidt, J.R., and Krantz, J.C., J. B. C. 102, 721 (1933).

(38) Field, C.W., Proc. Soc. Exp. Biol. Med., 17, 29 (1919).

(39) Voegtlin, C., Am. J. Physiol., 75, 574 (1925).

(40) Uglow, W.A., Arch. Hyg., 95, 89 (1925).

(41) Kaufman, E., Klin. Wschschr., 8, 66 (1929).

((42) Heinwein, E., Dent. Arch. Klin. Med. 164, 61 (1929).

(43) Donhoffer, S., Dent. Arch. Klin. Med. 167, 252 (1930).

(44) Ibid., 169, 242 (1930).

(45) Carr, C.J., and Krantz, J.C., J. B. C., 107, 371-6 (1934).

(46) Carr, C.J., and Krantz, J.C., J.P.E., 102, 721 (1933).

(47) Raybaud, A., and Roche, A., La Presse Medicale 42, 172 (1934).

(48) Bertrand, E., and Labbe, M., Bull. Acad. Med., 112, 8 (1934).

(49) Payne, W., Lawrence, K., and McCance, R., Lancet 2, 1257 (1933).

(50) Silbermann, A., and Lewis, E., Proc. Soc. Exp. Biol. Med., 31, 253 (1933).