considered that the permeability characteristics merely reflect the chemical structure of the membrane and are not in themselves of importance as mechanisms regulating metabolism.

SUMMARY

1. The water and solute content of rat-liver mitochondria have been measured during swelling or shrinking of the particles.

2. When mitochondria swell in 0.25M-sucrose solution there is an increase in the fraction of mitochondrial water penetrated by sucrose. The shrinkage due to transfer of mitochondria from 0.25M-sucrose to solutions of a higher osmoticity is mainly due to the loss of water from the sucrose-free space.

3. In mitochondria partially permeated by sucrose it is necessary to assume for osmotic equilibrium that the endogenous potassium is solely within a compartment separate from the sucrose.

4. When magnesium or manganese cause a shrinkage of mitochondria there is a loss of potassium that is never greater than that expected from the volume change of the sucrose-free water.

5. When mitochondria have swollen to a state when they contain more than 4 l. of water/kg. of solids they become completely permeable to sucrose and in further swelling the volume changes are due to an uptake of the suspending medium. Below 4 l. of water/kg. of solids the mitochondria are able partially to exclude some components of the medium.

6. Calcium and magnesium have antagonistic effects on mitochondria. Calcium increases the uptake of sodium and increases the loss of potassium, whereas magnesium decreases both these processes.

7. The problems of mitochondrial permeability are discussed.

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Selective Determination of Sugars Manifesting Enediol Isomerism by means of Reaction with Tetrazolium

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2:3:5-Triphenyltetrazolium chloride in aqueous alkaline solution (Wallenfels, 1950) has been used as a selective spray reagent on paper for the detection of sugars manifesting 1:2-enediol isomerism (Bell & Dedonder, 1954; Feingold, Avigad & Hestrin, 1956; cf. also Barker, Bourne, Grant & Stacey, 1956; Haq & Whelan, 1956; Côté, 1959; O'Donnell & Percival, 1959). It has been noted by Bacon (1959) that the reaction of 2:5-diphenyl-3-(4-styrylphenyl)tetrazolium chloride yields a

deeper colour than does the reaction with triphenyltetrazolium chloride. In the present paper a selective quantitative assay of sugars, manifesting enediol isomerism, is based on reaction with these tetrazolium reagents and is used in particular for the selective determination of oligofructosides bearing a substituent on C-6 (compounds of the type of 6-R-fructose) in the presence of those bearing a substituent on C-1 (compounds of the type of 1-R-fructose).

METHOD

Reagents. 2:3:5-Triphenyltetrazolium chloride (TTC) was an aqueous 1% (w/v) solution. This keeps for about one week at room temperature in the dark. TTC (Schwarz Inc., New York) gave a deeper colour on interaction with sugars than a preparation supplied by National Biochemicals Co., Cleveland, Ohio, U.S.A., and was used throughout the present investigation. 2:5-Diphenyl-3-(4-styrylphenyl)tetrazolium chloride (DSTC) (May and Baker, Ltd., Dagenham) was an aqueous 0.2% solution. This keeps at room temperature in the dark for at least one month.

Other reagents were: sodium hydroxide (0.5N); borate (1 vol. of 0.2N-NaOH was mixed with 1 vol. of 0.2N-sodium tetraborate); acidic methanol [10% (w/v) acetic acid in methanol]; acidic propanol [10% (w/v) acetic acid in propan-2-ol].

Procedure. Aqueous sugar solution is deproteinized if necessary by addition of 4% (w/v) trichloroacetic acid. The cleared solution is adjusted with N-alkali to pH 7.0. If a zinc-barium mixture is used for this purpose, excess of barium must be avoided.

To a test tube containing 0.7 ml. of neutralized sugar solution in water are added, with mixing, 0.1 ml. of tetrazolium reagent (TTC or DSTC) and then 0.2 ml. of NaOH solution (final pH of mixture, 12.4). Control tubes were set up with known amounts of aldose or ketose, and the mixtures incubated in a water bath at 37°. After a time specified below, the reaction was terminated and the pigment formed completely dissolved by adding 2.0 ml. of acidic solvent (acidic methanol with TTC; acidic propanol with DSTC). The colour is stable in the acidified solution at room temperature for at least 2 hr. The extinction was measured at 485 m μ with TTC and at 510 m μ with DSTC. Measurements were carried out in the Beckman DU spectrophotometer in cells of 1 cm. light-path. The final volume of reaction mixture was 3.0 ml.

Within wide ranges of sugar concentration and reaction time (see Experimental), the reduction of tetrazolium progresses linearly with time at a rate which varies directly as the sugar concentration. The following are suitable reaction times: 60 min. for aldose and 12 min. for ketose in the range 0.1–1.5 μ moles of sugar with TTC; 75 min. for aldose and 15 min. for ketose in the range 0.02–0.25 μ mole of sugar with DSTC.

The sensitivity of the test is increased eightfold if the reaction time is doubled and the DSTC concentration increased fourfold.

EXPERIMENTAL

At temperatures used in different procedures of sugar assay on the basis of reaction with tetrazolium (Fischer & Dörfel, 1954; Mattson & Jensen, 1950; Fairbridge, Willis & Booth, 1951) both inulobiose (1-R-fructose) and levanbiose (6-R-fructose) reduced TTC readily. At a relatively lower temperature (37°), however, a TTC reagent described by Cheronis (1956) manifested selective reactivity to 6-R-fructose and fructose as compared with 1-R-fructose, and to aldose as compared with 2-deoxyaldose (see below, 'Influence of

carbohydrate structure'). Factors affecting the selective reactivity of this reagent were therefore examined more fully.

Influence of pH. The velocity of reaction was markedly increased with increase in pH, as has been previously noted by Cheronis (1956). At pH 11.4 (0.1 ml. of 0.2N-sodium hydroxide, 0.1 ml. of 0.2N-sodium tetraborate, 0.1 ml. of TTC solution and 0.7 ml. of sugar solution), for instance, the rate of reduction of TTC by glucose was not more than one-fiftieth of that at pH 12.4 (standard system). Tetrose (erythrose), trioses (glyceraldehyde, dihydroxyacetone) and glycolaldehyde, however, reduced TTC rapidly even at pH 11.4 [$E_{1\text{ cm.}}^{485\text{ m}\mu}/\mu\text{mole}$ (in 3.0 ml. of reaction mixture)/10 min., 0.3, as compared with < 0.02 for glucose].

Choice of oxidant. Rate of production of pigment with DSTC was about five times that with TTC, both with ketose and aldose. Acidic methanol, a good solvent of TTC-formazan (Fischer & Dörfel, 1954), failed to dissolve the DSTC product readily. DSTC-formazan proved, however, to be readily soluble in acidic propanol.

Influence of time and concentrations of reactants. The course of the reduction was linear in time over a major part of the reaction. The initial reaction rate varied directly with the sugar amount over a wide range (Fig. 1) and affords a ready means of measuring sugar concentration (Fig. 2). As may be seen in Fig. 2, the limit yield in contrast with the rate of formation of formazan was constant in the designated range of carbohydrate amount (0.25–0.50 μ mole). With smaller amounts of carbohydrate (< 0.2 μ mole), the limit yield of formazan varied positively with the carbohydrate amount, but in a non-linear manner. Increase in the concentration of oxidant gave some increase in initial reaction rate and permitted enhancement of the sensitivity of the reaction without loss of selectivity (Fig. 3). The tested increase in oxidant concentration failed, however, to establish a condition in which the limit yield of formazan became a linear function of the sugar amount.

Influence of temperature. The reaction rates of glucose and fructose with TTC were about 1.3 times as much at 40° as at 37°. At 97° rates of the reaction were extremely high and difficult to measure. At this elevated temperature, moreover, selectivity as between levanbiose and inulobiose and as between glucose and 2-deoxyglucose was almost completely abolished.

Influence of carbohydrate structure. In the aldose series, changes at C-2 which impede 1:2-enediol isomerism resulted in marked depression of the reaction rate. The 2-deoxyaldoses tested, for example, were all almost non-reactive. A variety of alterations (e.g. epimerization, substitution of radicals in hydroxyl at C-4 and C-6, elongation or

Fig. 1. Influence of concentration and type of reductant on initial rate of reaction. Standard reaction conditions were used, with DSTC as oxidant and with fructose (○), glucose (Δ) and levanbiose (●) as reductant. With TTC, as with DSTC, reaction rate was a linear function of the carbohydrate concentration, rates of inulobiose and levanbiose with TTC being as 1:20.

Fig. 2. Course of formazan production as a function of time. Standard reaction mixture with DSTC as oxidant. Fructose (μmole): 0.5 (Δ); 0.25 (▲). Glucose (μmole): 0.5 (○); 0.25 (●).

restricted abbreviation of carbon chain) effected in aldose at carbon sites other than C-2 failed to depress the reaction rate. An exception in this respect occurred, however, in two methylpentoses (L-rhamnose, L-fucose) but not in a third (D-thevetose). Substitution of $-\text{CH}_2\cdot\text{OH}$ for $-\text{H}$ at C-1 in aldose, to yield ketose, resulted in a marked enhancement of reaction rate. Fructose reacted about five times as rapidly as glucose (cf. Fig. 1). Abbreviation of the length of the carbon chain to afford triose (D-glyceraldehyde) likewise gave a marked enhancement of the reaction rate. The aldoses tested and their reactivities are listed in Table 1.

In the ketose series, substitution of glycosyl in the OH group at C-1 gave a marked depression in reaction rate. For example, equimolar amounts of inulobiose, inulotriose and higher members of this series reacted with TTC about one-twentieth as rapidly as levanbiose and about one-tenth as rapidly as fructose. This may be related to the suppressive effect of substitution at C-1 on 1:2-enediol isomerism. However, normal ketose reactivity characterized acetylmethylcarbinol. Substitution at C-6 in fructose with resulting restriction in the size of the oxygen ring gave a significant increase in the reaction rate observed both with TTC and DSTC (cf. levanbiose with fructose, Fig. 1). The size of the substituent on C-6 did not alter the rate of reaction appreciably, since equal

Fig. 3. Influence of oxidant concentration on initial rate of reaction. Standard reaction conditions were used, but at varying concentrations of DSTC with 0.25 μmole of glucose. In the tested range, the reaction was followed throughout its linear course.

Table 1. *Reaction of aldoses and related substances with triphenyltetrazolium chloride*

A standard reaction mixture (3.0 ml.) was used, with 60 min. incubation time. Sugars are of D-configuration except where otherwise specified.

<i>Reactive compounds</i> ($E_{1.0\text{ cm.}}^{485\text{ m}\mu}/\mu\text{mole}$, 0.4 ± 0.1)		
Monosaccharides	Unsubstituted sugars	Glycolaldehyde,* glyceraldehyde,* erythrose* Ribose,† ribose 6-phosphate, xylose,† arabinose,† L-rhamnose,‡ L-fucose‡ Glucose,† galactose,† mannose,† glucose 6-phosphate D-glycero-L-galactoHeptose, D-threo-L-galacto-octose
		Glucuronate,† galacturonate,† glucurono-δ-lactone
	Uronic acids	
	Amino sugars	Glucosamine,† N-acetylglucosamine‡
	O-Methyl-substituted	6-Deoxy-3-O-methylglucose (thevetose), 3-O-methylglucose
Disaccharides	3-Glycosylglycose	Nigerose, laminaribiose
	4-Glycosylglycose	Maltose,† cellobiose,† lactose
	6-Glycosylglycose	Isomaltose, gentiobiose, melibiose,† 6-fructosylglucose
<i>Relatively inert compounds</i> ($E_{1.0\text{ cm.}}^{485\text{ m}\mu}/\mu\text{mole}$, ≤ 0.01)		
Monosaccharides	2-Deoxyaldoses	2-Deoxyribose,§ 2-deoxyglucose,§ 2-deoxyallose, 2-deoxygalactose,§ 2:6-dideoxyallose (digitoxose)
	2-O-Methylaldoses	2-O-Methylglucose, 2:3-di-O-methylglucose, 2:3-di-O-methylxylose
Disaccharides	2-Glycosylglycose	Sophorose, 2-fructosylglycose, kojibiose, 2-β-xylosyl-L-arabinose
	1-Glycosylglycose	Sucrose, trehalose
Other compounds	—	Methyl α-glucoside, α-glucose 1-phosphate, formaldehyde, acetaldehyde, glyoxalate, pyruvate, oxaloacetate, oxoglutarate

* Reaction conducted at pH 11.4.

† With DSTC this sugar gave $E_{1.0\text{ cm.}}^{510\text{ m}\mu}/\mu\text{mole}$, 2.0 ± 0.4 .

‡ Compound showed relatively low activity ($E_{1.0\text{ cm.}}^{485\text{ m}\mu}/\mu\text{mole}$, $0.04-0.08$).

§ With DSTC this sugar gave $E_{1.0\text{ cm.}}^{510\text{ m}\mu}/\mu\text{mole}$, ≤ 0.01 .

Table 2. *Reaction of ketoses with triphenyltetrazolium chloride*

A standard reaction mixture (3.0 ml.) was used, with 12 min. incubation time. Sugars are of D-configuration except where otherwise specified.

<i>Reactive compounds</i> ($E_{1.0\text{ cm.}}^{485\text{ m}\mu}/\mu\text{mole}$, 0.4 ± 0.1)		
Monosaccharides	Unsubstituted sugars	Dihydroxyacetone*
		Xylulose
		Fructose,† tagatose,† L-sorbose,† fructose 6-phosphate
		Glucoseheptulose, sedoheptulose†
	Oxogluconate	2-Oxogluconate, 5-oxogluconate
	Other compounds	Acetylmethylcarbinol
Di- and oligo-saccharides	—	Turanose
		Maltulose
		Isomaltulose, levanbiose, oligolevans (n , 3–8)
<i>Relatively inert compounds</i> ($E_{1.0\text{ cm.}}^{485\text{ m}\mu}/\mu\text{mole}$, ≤ 0.05)		
Compounds of type 1-R-fructose	—	1-Glucosylfructose, inulobiose,† oligoinulins (n , 3–8),† fructose 1:6-diphosphate, A-series of oligolevans (Zelikson & Hestrin, 1961)‡
Other compounds	—	Acetylacetone
* Reaction conducted at pH 11.4.		
† With DSTC this sugar gave $E_{1.0\text{ cm.}}^{510\text{ m}\mu}/\mu\text{mole}$, 2.0 ± 0.4 .		‡ $E_{1.0\text{ cm.}}^{510\text{ m}\mu}/\mu\text{mole}$, 0.045.

* Reaction conducted at pH 11.4.

† With DSTC this sugar gave $E_{1.0\text{ cm.}}^{510\text{ m}\mu}/\mu\text{mole}$, 2.0 ± 0.4 .

‡ $E_{1.0\text{ cm.}}^{510\text{ m}\mu}/\mu\text{mole}$, 0.045.

yields of colour per mole were found with levanbiose, levantriose and higher members of this series. A variety of alterations effected at C-3, C-4 or C-5 (e.g. introduction of substituents, epimerization, elongation or restricted abbreviation of carbon chain) did not markedly affect the reaction rate. A compound (2-oxogluconate) in which the keto group is vicinal both to a secondary carbinol and to a carboxyl group reacted with about the same rapidity as fructose itself. Abbreviation of the carbon chain to afford triose (dihydroxyacetone) gave a marked enhancement in the rate of reaction. The classes of ketoses tested and their reactivities are listed in Table 2.

Interfering substances. Several alcohols (methanol, ethanol, propan-2-ol) markedly augmented the rate at which formazan was formed from TTC by the action of a variety of ketoses. The selectivity of the assay as between levanbiose and inulobiose was almost completely abolished by small amounts of such solvents, e.g. by 0.2 ml. of methanol in 3.0 ml. of standard reaction mixture. When TTC was used as a spray on paper, the selectivity of the reagent as between compounds of the type of 1-R- and 6-R-fructose was likewise almost abolished in the presence of several common alcohols (butan-1-ol, propan-1-ol, propan-2-ol, ethanol, methanol).

DISCUSSION

The method presented above permits the selective determination of 6-R-fructose or free fructose in the presence of an equal amount of 1-R-fructose and that of aldose in the presence of an at least thirtyfold quantity of 2-deoxyaldose within limits of error less than 5%. With DSTC as oxidant, under conditions of prolonged reaction and elevated concentration of oxidant, as little as 5 μ m-moles of sugar can be determined by means of this technique.

Although the proposed method is simple, rapid and highly selective, its reliability as an analytical procedure in application to biological mixtures suffers from the circumstance that the measurement of sugar concentration is based on rate of formation rather than on limit yield of formazan.

SUMMARY

1. Tetrazolium derivatives in aqueous solution at pH 12.4 are readily reduced at 37° by sugars

manifesting enediol isomerism, but not by sugars in which such isomerism is impeded by presence of a substituent in a carbinol group vicinal to the carbonyl group.

2. A method is presented for the determination of 6-R-fructose and fructose in the presence of 1-R-fructose, and of aldose in the presence of 2-deoxyaldose by means of reaction with tetrazolium derivatives. Conditions are defined in which the rate of the reduction is a linear function of the sugar concentration over a wide concentration range.

3. The use of 2:5-diphenyl-3-(4-styrylphenyl)-tetrazolium in this reaction permits smaller amounts of sugar to be determined than was possible with the use of 2:3:5-triphenyltetrazolium.

4. The selectivity of reaction as between 6-R- and 1-R-fructose is abolished if certain alcohols at high concentration are present in the reaction mixture during formazan production.

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