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Enzymatic Determination of Sorbitol in Animal Tissues.* (24493)

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Chemical methods for determination of sorbitol and related polyols depend upon degradation of these substances by ferricyanide(1, 2) or periodate(3,4). These procedures suffer from the disadvantage of extreme lack of specificity, since a wide variety of sugars other than acyclic polyols react with these reagents. Although interference by substances such as glucose can be minimized by pretreatment with fermenting yeast(3) or by examining the reactions under carefully controlled conditions (4), application of these methods to determination of polyols in tissue extracts is not altogether satisfactory. Purification of a soluble liver polyol dehydrogenase(5) which catalyzes the reversible oxidation of certain 5, 6 and 7 carbon polyols to ketoses(6,7) made possible the present method for enzymatic estimation of sorbitol. As little as 1 μg of sorbitol can be measured by rapid and simple spectrophotometric procedures, not subject to interference by glucose or other naturally occurring sugars.

Methods and materials. Spectrophotometric measurements were made with Beckman model D.U. spectrophotometer using cells of 1 cm light path. Most sugars and polyols used were obtained from Pfanstiehl Laboratories, Waukegan, Ill., and were of the highest grade of purity. L-Iditol(8) was donated by Dr. Jean Sice. DPN and TPN were more than 80% pure when assayed enzymatically (9,10). DPNH was prepared by either chemical(11) or enzymatic(12) reduction of DPN. Ketoses were estimated by the methods of Dische and Borenfreund(13) or Roe (14). Protein was determined spectrophotometrically(15). Molar extinction coefficient of pure DPNH at 340 μ was assumed to be 6220.

Results. Purification of soluble polyol dehydrogenase of liver. Assay. Activity of the enzyme at each stage of purification was determined in a reaction mixture containing 0.033 M sodium pyrophosphate buffer pH 9.1, 0.0006 M DPN and 0.033 M sorbitol. Total volume was 1.5 cc. The reaction was initiated by addition of enzyme in total volume of 0.1 cc or less. Absorbency at 340 μ was measured at 30 second intervals at 25°. Reaction velocities were determined from zero order portions of plots of change in absorbency *vs.* time. One unit of activity was defined as equal

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to change in absorbency of 0.001/minute. Specific activities were defined as units/mg protein. Enzyme activity was proportional to amount of protein added over a wide range of concentration.

Initial extract. This was based on the procedure of Blakley(6). Either fresh or frozen rat liver was chopped with scissors and washed many times with cold tap water. After washing with deionized water the tissue was homogenized in Waring blendor with 4 volumes of 0.01 M sodium phosphate buffer pH 7.8 at 2°. The homogenate was centrifuged at 3.000 x g for 20 minutes and the precipitate discarded. In some experiments the pH of initial extract was adjusted to 4.7 by addition of 10 N HCl at 2°, and the precipitate which formed was discarded. The supernatant fluid was adjusted to pH 7.4 with 5 N NaOH and a further precipitate removed. The supernatant fluid resulting from either type of treatment was then treated with chloroform (0.25 vol) and ethanol (0.25 vol) according to Blakley(6). The aqueous extracts resulting from this treatment were dialyzed against deionized water at 2° until free of organic solvents. Any insoluble material remaining after dialysis was removed.

Ammonium sulfate fractionation. Solid ammonium sulfate was added slowly at 2° until the saturation was 0.4. The mixture was stirred one hour and the precipitate removed. Ammonium sulfate was added to the supernatant fluid until saturation had been raised to 0.7. Incomplete recoveries of enzyme were obtained if the saturation with ammonium sulfate was raised to only 0.6 as stated elsewhere(5). The precipitate was harvested and dialyzed against deionized water until free of ammonium sulfate. Insoluble material remaining after dialysis was removed.

Ethanol fractionation. The solution was cooled to 0° and ethanol (−25°) was added dropwise until a final concentration of 20% was reached. After addition of all of the ethanol, the mixture was stirred for 10 minutes and centrifuged. In a similar manner the precipitates resulting from addition of ethanol to final concentrations of 30, 40 and 50% were collected. The ethanol fractions were suspended in and dialyzed against deionized water at 2°; any insoluble material remaining after dialysis was removed. The material possessing the highest specific activity for sorbitol oxidation was in the 40-50% ethanol fraction. However, this was only the case if the ethanol fractionation was carried out as described above. If ethanol was added in one step to a final concentration of 40%, a great deal of polyol dehydrogenase coprecipitated with a large bulk of inert protein.

Properties of purified enzyme. The purified preparations exhibited specific activities for sorbitol oxidation ranging from 1600 to 2500 units/mg protein. At a final concentration of 0.033 M, L-iditol, ribitol and xylitol were oxidized at 96, 83 and 56% of the rate of sorbitol. TPN was reduced by sorbitol at less than 2% of the rate with DPN. The enzyme was stable for some months when stored at −20°, but repeated freezing and thawing was deleterious. In the standard assay system with sorbitol omitted, and containing 100 units of enzyme, DPN was not reduced by glucose (0.05 M), glycerol (0.02 M) n-propanol (0.02 M), m-inositol (0.01 M) and ethanol (0.02 M). At pH 7.4 in phosphate buffer, DPNH was oxidized by D-fructose (0.03 M) and L-sorbose (0.03 M) was oxidized at 86% the rate of fructose. DPNH was not oxidized upon the addition of DL-glyceraldehyde, dihydroxyacetone, monohydroxyacetone or potassium pyruvate (0.01 M).

Equilibria. Others(6,7) have shown that the soluble polyol dehydrogenase of mammalian liver catalyzes, *inter alia,* the following reactions:

Sorbitol + DPN$^+$	$\rightleftarrows$ D-Fructose + DPNH + H$^+$		(1)
L-Iditol + "	$\rightleftarrows$ L-Sorbose +	*Idem*	(2)
Ribitol + "	$\rightleftarrows$ D-Ribulose +	"	(3)
Xylitol + "	$\rightleftarrows$ L-Xylulose +	"	(4)

The equilibrium constant $K = [\text{Ketose}][\text{DPNH}]/[\text{Polyol}][\text{DPNH}]$ for reaction (1) was found by Blakely(6) to be 0.24 at pH 8.0 and 20°. The equilibrium of other pyridine nucleotide linked alcohol dehydrogenases is displaced to the right with rise in pH, since a proton is liberated when the coenzyme is reduced(9,17). The equilibrium for sorbitol oxidation by the purified enzyme was determined at various pH values. The vessels contained 200 units of enzyme/1 cc of reaction

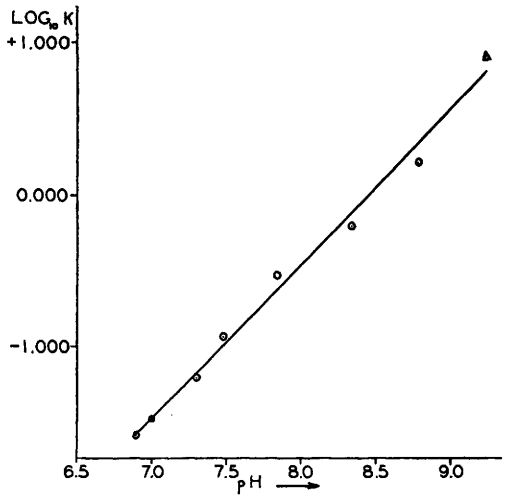

FIG. 1. Variation of equilibrium constant for transformation of sorbitol to fructose with pH. ○, Tris buffer; ●, phosphate buffer; △, pyrosphosphate buffer.

mixture, and initial concentration of DPN was 0.00016 M. At each pH, mixtures of sorbitol and D-fructose were added at approximately 100 to 300 times the molar concentration of DPN. Equilibrium was considered to be established when no further change in absorbency at 340 mμ occurred within 30 minutes. Three different ratios of sorbitol to fructose were employed for determination of K at each pH value. A plot of $\log_{10}K$ against pH yielded a straight line of unit slope (Fig. 1). The thermodynamic equilibrium constant $K_{II} = K \times [H^+]$ was estimated graphically to be 3.35×10^{-9} for reaction (1) at 25°. This is in good agreement with the value of K at pH 8.0 reported by Blakley(6) and with a previously reported value(5) of 2.09×10^{-9} obtained from determinations made at a smaller number of pH values. The value of K for reaction (3) was calculated from amount of DPNH formed in the presence of limiting concentrations of ribitol and excess DPN, on the assumption that 1 mole of D-ribulose was formed/mole of DPN oxidized. In sodium pyrosphosphate buffer of pH 9.0 an average value of $K = 0.107$ was found. The value of K for the xylitol ↔ D-xylulose reaction (equation 4) was not determined. However, it was observed that at pH 9, in the presence of 0.0005 M DPN, addition of 0.05 μmole of xylitol/1 cc of reaction mixture led to formation of 89% of the theoretical amount of DPNH, which would be expected if oxidation of xylitol had gone to completion. It is clear that there is a large difference between equilibrium constants for reactions 3 and 4.

Determination of sorbitol. From the value of K_{II} for reaction (1) it may be calculated that at pH 9 in a final volume of 2 cc, containing initially 1 μmole of DPN, the conversion of 0.2 μmole of sorbitol to D-fructose is more than 98% complete. Thus it would be expected that addition of sorbitol in quantities of 0.2 μmole or less would result in formation of stoichiometric amounts of DPNH. This was found to be the case. In the presence of 0.1 μmole of added sorbitol, further addition of 0.1 μmole of D-fructose did not alter detectably the final change in absorbency, as would be expected from the value of K_{II}. Addition of D-fructose in molar concentrations 100 times that of sorbitol reduced the final change in absorbency to about one-half of the theoretical. The following procedure was adopted to determine sorbitol in tissue extracts. The tissue was deproteinized by 1 of 2 methods. The first of these involved homogenization of tissue with 9 volumes of 5% TCA. The homogenate was centrifuged, the residue washed with TCA, and the combined acid soluble extract used. The second method of deproteinization was effected by homogenizing with 9 volumes of 66% ethanol. The homogenate was centrifuged and the precipitate washed with 66% ethanol. The combined ethanol extracts were stripped of ethanol under reduced pressure, and the aqueous residue extracted 3 times with chloroform. Traces of chloroform were removed from the aqueous extract by aeration. The deproteinized extracts were then de-ionized by passage over columns of the anion-cation strong base, strong acid ion exchange resin Amberlite MB-1 (Rohm and Haas) until free of ionic material as judged by conductivity determinations. The de-ionized extracts were concentrated under reduced pressure. At this stage, total polyol present was determined, using a suitable aliquot in final volume of 2 cc containing 100 μmole of sodium pyrosphosphate buffer of pH 9.1, 1 μmole DPN and sufficient amounts of purified liver enzyme to allow the

system to come into equilibrium within 20 minutes. An aliquot of de-ionized extract was also subjected to cysteine-carbazole reaction of Dische and Borenfreund(13) to determine level of ketoses present. Aliquots of de-ionized extracts were also chromatographed on Whatman number 42 filter paper in an ascending system at 25° using 85% ethanol as solvent. Standards of sorbitol (100 μg) and ribitol (100 μg) were run alongside these samples. The following values for $R_{sorbitol}$ (movement of substance / movement of sorbitol) were observed in this system: L-iditol, 0.94; D-mannitol, 1.00; ribitol, 1.20; glycerol, 1.42; m-inositol, 0.23; D-glucose, 0.98 and D-fructose, 0.98. Location of the carbohydrates on the chromatograms was determined by spraying with ammoniacal silver nitrate. In each determination, 2 chromatograms were run side by side. One of these was sprayed to determine the position of carbohydrates present, while the other was cut into strips 1 cm in length. Each strip was eluted with a small volume (3 cc or less) of water, and a suitable aliquot of the eluate was used for determination of polyol present by the enzymatic method.

Recoveries of greater than 92% were found if a solution of pure sorbitol was treated with TCA, and subjected to de-ionization and paper chromatographic procedures here described. Sorbitol could not be detected in either rat liver or in heparinized rat blood (adult males weighing between 350 and 400 g), under conditions where 2 mg of sorbitol / 100 g of tissue could have been detected. It may be added that after de-ionization of the deproteinized extracts of these tissues, amount of ketose present was insignificant, and incapable of leading to errors in determination of sorbitol due to alteration of the equilibrium of reaction(1) by D-fructose. Sorbitol added to deproteinized, heparinized rat blood was also recovered to the same extent.

Similarly, no difficulties were encountered in estimation of sorbitol added to rat urine. It must be emphasized that the de-ionization procedure employed precluded interference by ionic substrates which would form DPNH by action of DPN-linked dehydrogenases which might be present in the purified liver enzyme used. Failure of glucose, glycerol, ethanol and inositol to reduce DPN in the presence of liver ketose reductase also excluded interference by these substances. Paper chromatography of de-ionized extracts of rat liver showed the presence of material which reacted with ammoniacal silver nitrate and migrated to the same positions as glycerol, glucose and m-inositol.

In 2 separate experiments it was found that the coagulating gland of sexually mature rats contained an hexitol in amounts equivalent to 25 and 37 μmoles / 100 g of fresh tissue, and which migrated on paper to the same position of sorbitol. Work from this laboratory showed that fructose-secreting accessory sexual tissues of the rat and guinea pig catalyzed the transformation of sorbitol to fructose readily(5,18). The finding of an hexitol resembling sorbitol in the coagulating gland of the rat, which secretes large amounts of fructose, suggests that sorbitol may be an intermediate in biosynthesis of fructose by this tissue. This contention is in full accord with studies of Hers(19,20) on seminal vesicle of the sheep. The latter tissue, which also secretes fructose, contained sorbitol in its secretion, and also enzymes which reduce glucose to sorbitol and oxidize sorbitol to fructose.

Summary. An enzymatic method for determination of sorbitol is described. The method is based upon reduction of DPN by sorbitol catalyzed by a purified preparation of soluble ketose reductase of rat liver. No polyol which reacts with this enzyme could be detected in blood or liver of the rat, whereas considerable amounts of an hexitol resembling sorbitol were found in the coagulating gland.

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Chemical Studies of Mucopolysaccharides of Rat Blood Platelets. (24494)

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Isolation of a sulfated mucopolysaccharide from blood platelets of rats has been reported (1). Subsequent investigation on larger quantities of extracts of rat platelets (200 to 500 μg) revealed another mucopolysaccharide-rich fraction that is not sulfated. This report describes the separation and further chemical characterization of these two fractions by paper electrophoresis, hydrolysis, and paper chromatography.

Materials and methods. Male Sprague-Dawley rats weighing 272-477 g were used as platelet donors. Platelets labeled *in vivo* with $Na_2S^{35}O_4$ and harvested by the procedure described(1) were lyophilized or frozen for storage. Mucopolysaccharides (MPS) were extracted from platelets by alkali treatment(1) or by trypsin digestion. The trypsin digestion medium was made by adding 0.1 ml of a 0.1% trypsin† solution to 0.9 ml of M/10 $NaHCO_3$ buffer (pH 8.3, M/1000 with respect to $CaCl_2$). One milliliter of this mixture was added for each 13.3 x 10^9 platelets and reacted for 1 week at 34°-38°C. The undissolved material was removed by centrifugation at 2190 x g for about 30 minutes. The supernatant was neutralized with 1 N HCl, diluted to 10 ml with water, and repeatedly treated with chloroform-amyl alcohol reagent (2) to remove protein. The resulting supernatant was dialyzed and lyophilized(1). In addition to the method of electrophoresis used before [essentially that of Rienits(3)], we also used as the electrolyte a 35% solution of propanol in M/15 phosphate buffer (pH 6.4) a solvent system similar to that used by Kerby(4) for chromatographic separation of heparin and chondroitin sulfate(CSA). The extract was electrophoresed on strips of Whatman #2 filter paper (3.0 x 30.5 cm) for 5 hours at 10.8 V/cm. The two MPS-rich fractions were located on paper strips by staining with Alcian blue. Each MPS-rich fraction was eluted from unstained paper electrophoresis strips run by the Rienits procedure, dialyzed, lyophilized, and hydrolyzed by modification of the method of Glegg and Eidinger (5). Two hundred to 500 μg (estimated by comparing staining density of small samples on paper strips with that of known amounts of CSA) of each fraction and 12 mg of Dowex 50 cation exchange resin‡ in the hydrogen form and 0.1 ml of water were heated at 100°C for 33 or 40 hours in a sealed tube with a volume of about 3 ml. In this procedure hexosamines released during hydrolysis adsorb to the resin, and sugars and uronic acids

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† The trypsin was a crystallized, lyophilized, salt-free preparation (TL 550) obtained from Worthington Biochemical Sales Co.

‡ Dowex-50 resin of analytical grade, 50 to 100 mesh, obtained from Bio-Rad Laboratories.