Monograph on **Mannitol** 8/31/72

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MONOGRAPH

ON

MANNITOL

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MANNITOL

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MANNITOL

Summary

According to Touster and Shaw, metabolism of mannitol presumably begins in the liver, the first reaction probably being the oxidation of the polyol to D-fructose (29). Launiala reported that only a small part of orally administered mannitol was absorbed from the intestines in man (18), while Nasrallah and Iber found that man absorbs about 65% of ingested mannitol in the dose range from 50 to 100 g (20). Excretion of the absorbed mannitol was virtually complete after 48 hours (20).

Ellis and Krantz found that 25 g of ingested mannitol did not significantly influence either the level of blood sugar or the respiratory quotient of man (7). However, Nasrallah and Iber (20) and Field (10) reported that, in man, doses of 50 g and 100 g produced an increase in the level of blood sugar.

Wasserman and Comar observed an increase in intestinal absorption of calcium and strontium in the rat as a result of mannitol administration (31). Fournier et al. reported that mannitol increased the absorption, retention, and urinary excretion of calcium (11).

Launiala observed increased secretion of Na^+, Cl^-, and water when the small intestines of children were perfused with mannitol solution (18).

Segal and Smolarz-Dudarewicz reported that oral administration of 1 g of mannitol per kg of body weight significantly reduced the intra-ocular pressure of human subjects (23).

Whitaker et al. found that mannitol inhibits in vitro alpha-amylase activity (33).

Voegtlin et al. have found mannitol to be a weak antagonist of insulin (30).

Ellis and Krantz found that mannitol exhibited no toxicity for rats when fed to them as a 5% dietary component for 3 months. Monkeys also showed no effects from receiving 3 g of mannitol daily for 3 months. After ingesting 10 g of mannitol daily for 1 month, a human subject observed by Ellis and Krantz showed no untoward effects (7).

Ellis and Krantz on the basis of experiments with 12 volunteers reported that a single dose of 10-20 g of mannitol exerted a laxative effect on man (7).

A single case of sensitization was reported by Spaeth, et al., in which a woman given mannitol intravenously exhibited an anaphylactic reaction. However, over 1500 patients treated likewise with mannitol solutions showed no untoward reactions (25).

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MANNITOL

Chemical Information

I. Nomenclature

A. Common name
Mannitol

B. Chemical names
D-Mannitol
1,2,3,4,5,6-Hexanehexol

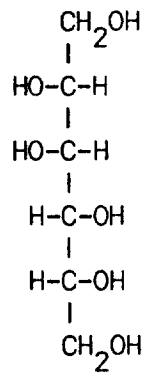
C. Trade names
Manicol
Mannidex
Diosmol
Osmitrol
Osmosal
Mannite

D. Chemical Abstracts Number -- 000069658

II. Empirical Formula

$C_6H_{14}O_6$

III. Structural Formula



IV. Molecular Weight

182.17

V. Specifications

A. Chemical

Meets A.C.S. Specifications

Specific Rotation	$+23.3^{\circ}$ - $+24.3^{\circ}$
Insoluble Matter	0.010 %
Loss on Drying at $105^{\circ}C$	0.050 %
Residue after Ignition	0.010 %
Acidity (as CH_3COOH)	0.005 %
Reducing Sugars	passes A.C.S. test
Heavy Metals (as Pb)	0.0005 %

B. Food grade

Mannitol	not less than 99.5%
Humidity (according to N.F. XII)	less than 0.5%
Reducing Sugars	less than 0.005%
Melting Point	$164-165^{\circ}C$
Heavy Metals	less than 10 ppm
Arsenic	none
Iron	none
Ash	less than 0.1%
Chlorides	less than 70 ppm
Sulfates	less than 100 ppm

C. Food codex

Assay	Not less than 98% and not more than the equivalent of 102% of $C_6H_{14}O_6$ after drying
Melting range	Between 165° and 168°
Specific rotation,	Between $+23.3^{\circ}$ and $+24.3^{\circ}$
Limits of Impurities	
Arsenic (as As)	Not more than 3 ppm (0.0003%)
Chloride	Not more than 70 ppm (0.007%)
Heavy metals (as Pb)	Not more than 10 ppm (0.001%)
Loss on drying	Not more than 0.3%
Reducing sugars	Passes test
Sulfate	Not more than 100 ppm (0.01%)

VI. Description

A. General characteristics

Mannitol appears as a white, crystalline powder, odorless, with a pleasant sweet taste. It is a compound endowed with great stability in the presence of light, humidity, metallic traces, heat.

B. Physical properties

Melting Point °C	166
Boiling Point °C 760 mm	Decomposes
Density--25°C	1.49
Viscosity cp. 25°C	Solid
Hygroscopicity	Low
Solvency (for oils)	Poor
Solubility in water @25°C	22%
Solubility in alcohol	Slight (1:83)
Solubility in ether	Insoluble
Solubility in pyridine	Soluble (1:2.3)
High Temperature Resistance	Stable

C. Stability

Stable in sealed containers.

VII. Analytical Methods

The Komarowsky reaction has been used to quantitatively determine the sugar alcohols. The sugar alcohols are heated in a reagent consisting of either p-hydroxybenzaldehyde or p-dimethylaminobenzaldehyde, thiourea, and concentrated sulfuric acid. The resulting colors are either orange, pink, or red, whose intensities follow the Beer-Lambert curve at their respective absorption maximums (range of 380-700 nm). The sugar alcohols in order of decreasing reactivity are: sorbitol; dulcitol; mannitol; erythritol; zylitol; arabitol; ribitol; glycerol; inositol. Carbohydrates and proteins interfere with this method and must be removed. Recovery from a complex milieu was 95-101% with a standard deviation of 1.06-1.26% (12).

A thin-layer chromatographic method has been developed that separates sorbitol, galactitol and mannitol from each other and from their corresponding hexoses. Solution mixtures are spotted on the plate and developed with 80% isopropanol. To detect the sugars, the plates are sprayed with 10% sulfuric acid in methanol and dried. The sugars appeared as deep charcoal spots and the polyols were light brown spots. Labeled sugars can subsequently be quantitated on a liquid scintillation counter. This technique has been used for sugars in urine samples (32).

Tibbling developed a method for quantitative determination of sugar alcohols such as sorbitol, mannitol and glycerol in serum based on the periodate oxidation method. The samples are read against a blank in a spectrophotometer at 570 nm. The interference of glucose and glycerol is negligible except where a high degree of specificity is required. The usable range is 3-500 mg/100 ml and the precision is 3 mg/100 ml. Recovery was 98-103% (27).

Britton developed a method for analyzing sugar alcohols in animal tissues and fluids. Samples are deproteinized with $ZnSo_4$ and $Ba(OH)_2$. When reducing sugars are not to be removed, the resin is bound with CO_2 and the sample washed down with water. Recovery of glucose, fructose, sorbitol and glycerol is quantitated. Glycerol, mannitol, sorbitol and

dulcitol remain chromatographically and electrophoretically identical. Paper chromatography is then carried out with these solvent systems: a) amyl alcohol-pyridine- water (4:3:2); b) butanol-acetic acid-water (4:1:5); c) phenol-water (5:2); d) butan-2-one-acidic acid-saturated aqueous boric acid solution (9:1:1). Nonreducing sugars in the presence of borate are detected with periodatebenzene while for reducing carbohydrates, aniline-diphenylamine and naphthoresorcinol are used.

Hexitols can be separated using paper electrophoresis in basic lead acetate. Electrophoresis is performed for 48 hours with a voltage gradient of 10 v/cm. To detect the spots, the paper is dipped in solutions of $AgNO_3$ in aqueous acetone and 40% HF-acetone. After allowing the paper to dry, it is dipped into an aqueous NaOH-ethanol solution. This method has been used for determining allitol, dulcitol, iditol, mannitol, talitol, glycerol, sorbitol, fructose, and inositol (1).

Mannitol can be extracted from food and blood and its trimethylsilane derivative analyzed by gas chromatography. A sample solution is treated to remove proteins, lipids and other impurities. The sample is dissolved in anhydrous pyridine, hexamethyldisilazane and trimethylchlorosilane. The trimethylsilane derivatives are purified and dissolved in n-hexane. This solution is injected in amounts of 0.1 to 1.0 microliters. These sugars can be analyzed: arabinose, rhamnose, xylose, fructose, mannose, galactose, glucose, mannitol, sorbitol, saccharose, and lactose. The program takes 45 to 50 min., and sorbitol recovery is 96-102% (6).

Mannitol and other polyols present in the urine can be detected using ion exchange chromatography. De-Acidite FF is used as a resin and is converted to the borate form using potassium tetraborate. The sample is dissolved in borate buffer, pH 9-10 and eluted with 0.18 and 0.36 M borate buffer adjusted to pH 9 with triethylamine. The eluant is analyzed by periodate oxidation. An aqueous chromotropic acid solution is added and the red color analyzed at 570 nm using a spectrophotometer. The polyols determined by this method are glycerol, threitol, erythritol, xylitol, arabitol, ribitol, glucitol, galactitol, mannitol, fucitol and rhamnitol. Recovery range is 99.0% ± 14.5% for mannitol (26).

VIII. Occurrence

Plants (15)

ash trees (bark), manna (Proust, 1806): 30-62% of the manna
- Fr. ornus (flowering ash)
- Fr. ornus (rotundifolia var.)
- Fr. excelsior (larger type ash tree)

Olive (trunk, branches, leaves, fruit)

Coffee and Cactus apuntia (fruit)

Aconite (root)

Cyclamen (tubers)

Syringa vulgaris (leaves)

Pinus abies and Abies excelsa (sap)

Seaweed (ex. Laminaria saccharina)

Fungi:	Lactarius pallidus:	up to 15% of dry substance
	Lactarius pyrogalus:	up to 15% of dry substance
	Agaricus integer:	up to 20% of dry substance

Beets

Figs and fig wine

Cider

Sourish wines and some wines from South Italy

Celery

Honey from Ethiopia, up to 3%

Date stones (as mannan)

Vegetable ivory (Hyphaea abissinica), as mannan

Phytelephas macrocarpa, as mannan

Gardenia jasminoides

Algae Sargassum natans (mannitol and mannitol-monoacetate)

Quercus glauca (about 4 g/kg)

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Biological Data

I. Acute Toxicity

Substance	Animal	Sex & no.	Route	Dosage (mg/kg)	Measurement	Ref.
Mannitol	Mouse	not given	P.O.	22,000	LD50	6
Mannitol	Mouse	not given	I.V.	16,800	LD50	6
Mannitol	Mouse	not given	I.P.	14,000-16,000	LD50	6
Mannitol	Rat	not given	P.O.	17,300		
Mannitol	Rat	not given	P.O.	13,000	MLD	2

Mice died with signs of central nervous system depression and gastrointestinal tract mucosal damage; rats died with predominately gastrointestinal tract signs (6).

A 65-year-old caucasian woman, admitted with chronic open-angle glaucoma, was given 500 ml of a 20% aqueous solution of mannitol in preparation for surgery. About 3 minutes after the mannitol was started, she began sneezing violently. Her nose became runny, her tongue swollen, and her breathing difficult. About 3 minutes later she was extremely anxious, dyspneic, cyanotic, and eventually unconscious. Four large wheals appeared on her chest. Air exchange was poor; wheezing was marked after mannitol was discontinued. Within 30 seconds respirations became more normal, and she regained consciousness. Cyanosis faded rapidly. Within 30 minutes the wheals disappeared. This case was the first reported even though over 1500 patients at this facility alone have been treated with mannitol solutions. Two months after the incident, the patient gave strongly positive reactions to mannitol in patch and scratch tests (25).

II. Short-Term Studies

Rat

Two groups of 20 white male rats, 20-28 days old and weighing about 40 g, were fed a balanced diet comprised of 40% carbohydrate. The control group's diet consisted of 40% dextrose; the test group's diet consisted of 35% dextrose and 5% crystalline mannitol. The study was conducted over a 3 month period. The study showed that dextrose was nutritionally superior to mannitol; however, no toxic effects attributable to mannitol were reported (7).

Monkey

Two rhesus monkeys used as controls were fed a stock diet and 3 test monkeys were fed 3 g of crystalline mannitol per day for 3 months. Blood sugar and urea-N were determined at regular intervals. At the conclusion of the experiment, animals were sacrificed and autopsies performed. Liver and kidney sections were prepared for micropathological examination and studies. The values of blood sugar and urea-N were comparable to

those of control animals, and test animals seemed to be unaffected pathologically by the mannitol (7).

Man

A study was conducted in which a normal individual ingested 10 g of crystalline mannitol daily for a month. Twenty-four hour urine samples were analyzed for sugar alcohol content. At the initiation and end of the study, blood studies were made and kidney function estimated. The RBC count was normal at all times. The renal excretion of phenolsulfonephthalein was indicative of normal kidney function. There was no effect shown on this individual by mannitol (7).

III. Long-Term Studies

None

IV. Special Studies

Laxative Effect

The laxative effect of crystalline mannitol was determined by Ellis and Krantz on the basis of 12 normal individuals tested. The laxative threshold was found to be 10-20 g of crystalline mannitol per man (7).

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Biochemical Aspects

I. Breakdown

No information from sources available.

II. Absorption - Distribution

In an experiment using isolated loops of the small intestine of the rat, Hoeber and Hoeber found that mannitol was completely unabsorbed (14).

However, Carr and Krantz reported that analysis of intestinal contents after administration of 4 ml of 15% mannitol solution by stomach tube indicated that the rats absorbed most of the administered dose (3).

When rats were given, orally, radioactive mannitol, 50% of the radioactivity was recovered in the expired CO_2 in 24 hours and 10% in the urine, thus indicating the possibility of at least 60% absorption. With I.P. injection Wick et al. found that mannitol was largely recovered in the urine with only 2-3% of its carbon in the expired CO_2 (34).

In vitro studies using hamster intestine showed that mannitol is not absorbed against a concentration gradient, indicating that it is absorbed passively (35).

In situ studies on the small intestines of dogs show that mannitol is absorbed slowly, absorption taking place faster in the duodenum than in the ileum (13).

In vivo studies on children in which the small intestine was perfused with mannitol solution resulted in only a small part of the mannitol being absorbed (18).

According to Nasrallah and Iber, who studied mannitol absorption in 10 human subjects, about 65% of ingested mannitol is absorbed in the dose range from 40 to 100 g. They also suggested that the mid or lower small intestine is the site of mannitol absorption. Subjects who had diarrhea excreted a higher percent in the stool (42.4% of the ingested dose) than those without diarrhea (25.4%) (20).

III. Metabolism and Excretion

According to Touster and Shaw, metabolism of mannitol presumably begins in the liver, the first reaction probably being the oxidation of the polyol to D-fructose (29).

Wick et al. found that when radioactive mannitol was given orally to rats, 50% of the mannitol carbon was expired as CO_2 within 24 hours and 10% was excreted in the urine (34).

Rats given 0.6 g of mannitol were killed after 6 hours. One-sixth of the mannitol was found in the bladder and excreted in the urine, while an equal amount was found unabsorbed in the intestines (3).

When 50 g of radioactive mannitol was ingested by human subjects, 17.5% of the dose was found in the urine and 31.5% in the feces within 48 hours. More than 90% of that excreted was recovered in the first 24 hr urine and feces collection, with little or no radioactivity recovered after 48 hours. When CO_2 was collected for 12 hours, 18.7% of the dose was recovered (20).

Carr et al. fasted rats for 24 hours and then fed them a diet of 33% mannitol and 67% cacao butter for 80 hours. They observed an increase in liver glycogen over the controls (1.23% vs. 0.14%). They also gave fasted rats 5 ml of a 25% solution of mannitol by stomach tube (35). No significant effect on the respiratory quotient of the rat was observed during the 2.5 hour period following administration (4).

Silberman and Lewis fasted young white rats for 24 hours and then fed them either 2 or 4 ml of a 15% solution of mannitol by stomach tube. After absorption periods of 2, 3, 4, and 6 hours, they found no significant increases in liver glycogen as compared with the values obtained for the control series (24).

Using the same procedures, Todd et al. (28) confirmed the results of Silberman and Lewis (24) and Carr et al. (4).

To confirm their previous results, Carr and Krantz again fed fasted rats a diet of 33% mannitol and 67% cacao butter for 72 to 80 hours. They again found an increase in liver glycogen over the controls (0.98% vs. 0.18%). In a further study on rats, they found that the administration of 4 ml of a 15% mannitol solution by stomach tube did not result in any increase in liver glycogen. However, they found that fasted rats given 5 ml of a 25% mannitol solution by stomach tube showed a slight, but significant, increase in respiratory quotient (3).

Carr et al. found that the administration by stomach tube of 4 g/kg of mannitol to fasted rabbits produced a slight but significant rise in the blood sugar level (4).

Ellis and Krantz gave rhesus monkeys 8 g/kg of mannitol by stomach tube and then, 3 hours later, tested for liver glycogen. They reported that mannitol under the conditions of the experiment, was incapable of producing significant glycogen deposition. In a further study they found that 25 g of orally ingested mannitol did not significantly influence either the level of blood sugar or the respiratory quotient of man (7).

Field reported that the administration of 100 g of mannitol to human subjects produced a rise in blood sugar of 10 mg/100 cc of blood, while the same amount of glucose resulted in a rise of 40 mg. The highest level of blood sugar was observed one hour after ingestion, dropping back to normal after another hour or two (10).

Nasrallah and Iber reported that C^{14} activity significantly above background was detected in blood following oral administration of 50 g of labeled mannitol in man. A peak in activity was reached after 5 or 6 hours, followed by a decline (20).

IV. Effects on Enzymes and Other Biochemical Parameters

Kameda et al. incubated hypertonic mannitol for 6 consecutive periods of 15 minutes each in intestinal loops of rats with intact blood supply. A decrease in glucose absorption was observed and histological damage to the surface epithelium of the intestinal mucosa was caused by osmotic removal of water from the cells. Isotonic mannitol did not affect absorption of glucose (17).

McHardy and Parsons reported that mannitol decreased, through osmotic restraint, absorption rates of water, sodium, and chloride in in situ rat intestine. In jejunal segments of rat intestines the net uptake of Na and I from isotonic fluids and the net entry into hypotonic fluid were both reduced when mannitol was added to the solutions (19).

In rats, Csaky and Ho found that, at low glucose concentrations, the replacement of glucose with mannitol inhibits intestinal glucose transport. They also found that mannitol increases the intracellular potassium concentration. They suggested that the effect on sugar transport was due to the intracellular increase of potassium (5).

Wasserman and Comar observed an increase in intestinal absorption of calcium and strontium in the rat as a result of mannitol administration and this was correlated to the prolonged residence of mannitol in the gut (31).

The supplementation of the diet of rats with 6% mannitol was found by Fournier et al. to result in increased calcium absorption and retention. They found that mannitol-fed rats retain almost twice the amount of ingested calcium as do the controls. They noted that along with increased calcium retention, mannitol greatly increases urinary calcium excretion (11).

Nasrallah et al. studied the effect of hypertonic mannitol solutions on fat absorption in in vivo studies of the jejunal loop in rats and showed that mannitol significantly inhibits fat absorption and impairs lipolysis (21).

Hindle and Code found, in in situ studies on the small intestines of dogs, that mannitol causes secretion of sodium and water as a result of its osmotic activity (13).

Launiala observed increased secretion of Na^+ and Cl^- when the small intestine (children) was perfused with mannitol solution. Hyperosmotic mannitol solutions also caused release of water from the intestinal walls. It was suggested that the osmotic effect of unabsorbed mannitol is the most important factor in the causation of the diarrhea of mannitol malabsorption (18).

Segal and Smolarz-Dudarewiez reported that oral administration of 1 g of mannitol per kg of body weight significantly reduced the intra-ocular pressure of human subjects. It was observed that the lowering of intra-ocular pressure was significantly greater for glaucoma sufferers than for healthy persons and that the lowering was much more pronounced for patients with higher initial pressures (23).

Ellis and Krantz reported that 10-20 g of ingested mannitol produces laxative action in man (7).

According to Nasrallah and Iber, oral doses of mannitol greater than 40 g generally produce diarrhea, as a result of osmotic activity (20).

Fell and Dingle (1966) investigated the effect of mannitol on embryonic (chick) cartilage and bone grown in organ culture. Histological examination showed that the cytoplasm of the connective tissue cells surrounding the cartilage was distended with small vacuoles. In the cartilage itself, this abnormal vacuolation appeared only near the articular surface and here the matrix was breaking down. According to the authors, the results suggest that the accumulation of undigested material in cells stimulates the release as well as the synthesis of lysosomal enzymes (8).

In a later study using limb-bone rudiments from 8-day embryonic chicks, Fell and Dingle (1969) reported that the addition of mannitol to the culture medium caused some thickening of the perichondrial tissue (9).

Whitaker et al. reported that mannitol inhibits in vitro alpha-amylase activity (31).

V. Drug Interaction

In experiments with rats, mannitol has been shown to be a weak antagonist of insulin (30).

Mannitol significantly reduced histidine absorption in rat jejunal loops in situ as a result of its osmotic activity (36).

VI. Consumer Exposure Information

In 1970 the total poundage of mannitol used in foods, not including baby food, was 112,200 (22).

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Some Non-Reducing Carbohydrates in Animal Tissues and Fluids

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The presence of sorbitol in ram seminal vesicles and sheep foetal blood was reported by Hers (1957*a*, *b*, 1960*a*, *b*), and evidence was also obtained that sorbitol is an intermediate in the conversion of glucose into fructose, the pathway being

$$\text{Glucose} + \text{NADPH} + \text{H}^+ \underset{\text{reductase}}{\overset{\text{aldose}}{\rightleftharpoons}} \text{sorbitol} + \text{NADP}^+$$

$$\text{Sorbitol} + \text{NAD}^+ \underset{\text{dehydrogenase}}{\overset{\text{iditol}}{\rightleftharpoons}} \text{fructose} + \text{NADH} + \text{H}^+$$

The present investigation confirms the presence of sorbitol, and a preliminary account has been given (Britton, 1958, 1960).

METHODS

Deproteinization and desalting. Samples were deproteinized with $ZnSO_4$ and $Ba(OH)_2$ (Somogyi, 1945), a 1:10 dilution being used, or with 5% trichloroacetic acid, which was extracted with ether before desalting. Desalting before chromatography was effected with Bio-Deminrolit (The Permutit Co. Ltd., London, W. 4). When it was not desired to remove the reducing sugars the resin was saturated with CO_2 (Woolf, 1953) and made into a column (vol. 2 ml., length 25 cm.). A portion (2–5 ml.) of the deproteinized solution was run slowly through the column, which was then washed with 3–4 ml. of water. Recoveries of glucose, fructose and sorbitol were quantitative. To remove reducing sugars fresh CO_2-free Bio-Deminrolit was used and 2–5 ml. of the deproteinized solution run slowly through the column in about 1 hr. The column was then washed with CO_2-free water to give a total volume of eluate of 20 ml. With this technique all but traces of reducing sugar (less than 0.5% of glucose and fructose appeared in the eluate when 100–1000 [illegible] the sugars was put through the column) were removed, and the recovery of glycerol and sorbitol was quantitative. Samples of glycerol, mannitol, sorbitol and dulcitol remained chromatographically and electrophoretically homogeneous after this treatment. All columns were well washed before use: this was especially necessary to remove formaldehydogenic material.

Chromatography. Paper chromatography was carried out with the solvent systems A, B, C and D of Table 1 on Whatman no. 1 paper. The solutions to be chromatographed were concentrated in a vacuum desiccator and volumes of 20–100 μl. were dried at the origin in a cold air draught. The amounts of extract applied were equivalent to between 1 and 7.5 ml. of the deproteinized filtrate. Non-reducing and reducing carbohydrates were localized by the method of Trevelyan, Procter & Harrison (1950) as modified by Smith (1960) for systems A, B and C. For system D, the method of Britton (1959*a*) was employed, 40% HF–acetone (1:40, v/v) proving suitable. Periodate–benzidine (Cifonelli & Smith, 1954) was also used to detect non-reducing carbohydrates in the presence of borate but it was much less sensitive and gave no indication of the amount of material on the spot. For reducing carbohydrates aniline–diphenylamine and naphtharesorcinol were used (Smith, 1960).

Elution from the paper after use of solvents A, B and C was effected by conventional techniques with water as the eluting agent. For system D a modified method was employed to remove the boric acid present. The area to be eluted (10–25 cm.^{2}) was cut out and dipped into a solution of 40% HF–acetone (1:20, v/v). The paper was allowed to dry at room temperature for about 1 hr. and was then eluted in the conventional way, contact with glass being avoided. To the eluate (1–2 ml.) was added an approximately equal volume of aq. 0.01% HF and the solution was run slowly through a column of Bio-Deminrolit saturated with CO_2 (vol. 10 ml., length 25 cm.). The column was further washed with 0.01% HF to give a total volume of eluate of 12 ml., the whole procedure taking about 1 hr. This removed boric acid but gave a quantitative recovery of glucose, fructose and sorbitol. The method appears to be generally applicable, but might not be suitable for highly acid-labile substances owing to the formation of fluoroboric acids on the paper.

Electrophoresis. To separate the hexitols paper electrophoresis was carried out in basic lead acetate (Frahn & Mills, 1959). A solution of basic lead acetate was prepared by shaking 58 g. of basic lead acetate (AnalaR; British Drug Houses Ltd.) with 1 l. of water and filtering. The electrophoresis paper (Whatman no. 1 paper, 57 cm. long) was dipped into the solution, well blotted and placed in a horizontal electrophoresis tank in which the paper hung freely, supported by transverse glass rods. After 30–60 min. had been allowed for capillary rise of solvent, spots containing 10–20 μg. of material were applied to the origin with a wire loop. Electrophoresis was carried on for 48 hr. at 3° with a voltage gradient of about 10v/cm. along the paper. The material was applied asymmetrically towards the negative pole; the position was important since movements of the solvent due to evaporation and endosmosis affect the relative separations obtained. Electrophoresis at room temperature gave a more rapid separation but the results were less reproducible and for rapid separation a totally enclosed strip as employed by Frahn & Mills (1959) is probably desirable. Detection of the sugar alcohols was effected by a modification of the method of Trevelyan *et al.* (1950). After the paper had dried at room temperature, it was dipped twice into a solution of $AgNO_3$ in aqueous acetone (prepared by adding 0·2 ml. of a saturated solution of $AgNO_3$ in water to 40 ml. of acetone and adding water drop by drop until the precipitate redissolved). The paper was then dipped into a solution of 40% HF–acetone (1:10, v/v) so that the paper was well saturated. After allowing the paper to dry for 15–20 min. at room temperature it was dipped into a solution of aqueous 50% NaOH–ethanol (1:10, v/v). The paper was hung in the dark or in subdued light for 1–2 hr. for the spots to develop and then photographed, since although fixation could be effected with ammonia or sodium thiosulphate these procedures led to a loss of density. If the impregnation with lead acetate had been heavy a second dip into the HF solution was sometimes required, but it was essential that most of the excess of HF should volatilize before the alkaline dip, although for maximum sensitivity it was necessary for a small amount of HF to be present. To accelerate the development of the spots the alkaline dip was repeated or heat applied. About 5 μg. of sorbitol was detected by the method.

For identification of glycerol, electrophoresis was carried out in 0·05 M sodium tetraborate on Whatman paper no. 1 at room temperature. Spots were made visible by the modified silver method of Britton (1959*a*).

Estimation of total hexitol. In foetal sheep blood the total hexitol present was estimated by the method of Britton (1959*b*), with Whatman paper no. 1 for chromatography.

Identification of sorbitol. Sorbitol dehydrogenase was prepared from sheep liver by the method of Todd (1954) except that the last acetone fractionation was omitted. To show reduction of NAD, 1·8 ml. of 0·2 M-glycine–NaOH buffer, pH 8·5, was placed in a cuvette, followed by 0·2 ml. of NAD solution (C. F. Boehringer und Soehne: NAD 75%, 25 mg./ml.) and 0·2 ml. of the unknown solution. A blank was set up at the same time. The extinctions were read at 340 mμ and 0·2 ml. of suitably diluted enzyme solution was added to both cuvettes. The change in E of the blank was used to correct the change in the unknown. To show the formation of fructose the total volume was kept to a minimum owing to the relatively high K_m of the enzyme: the volume of buffer was therefore brought to 0·5 ml. and 0·5 ml. of undiluted enzyme was added. About 100 μg. of the unknown dissolved in 0·2 ml. of water was taken and the mixture left overnight at room temperature. Under these conditions virtually complete conversion of sorbitol into fructose took place.

Acetylation of sorbitol was carried out with acetic anhydride and perchloric acid. A sample of the unknown (0·5–1 mg.) was evaporated to dryness in a small test tube and 0·5–1 ml. of a reagent prepared by mixing 0·1 ml. of 60% $HClO_4$ and 10 ml. of acetic anhydride was added. After about 20 min. $NaHCO_3$ solution and solid $NaHCO_3$ were added until the solution was neutral. The acetylated derivative was then extracted with $CHCl_3$ or ethylene dichloride. Crystallization was carried out by allowing some of the extract to dry on a glass slide. One drop of ethylene dichloride was added to dissolve the acetate and then a drop of redistilled amyl alcohol was added. By enclosing the slide in a chamber containing the solvents the rate of evaporation was slowed. Crystals were removed with a needle under a lower-power microsope and recrystallization was effected by the same technique. Alternatively the extract to be acetylated was dried on a glass slide and several drops of the acetylating reagent were added. The slide was placed in a chamber saturated with the vapour of the acetylating reagent and left for 20 min. The reagent was removed with a cold air draught and crystallization carried out as described above. By this technique the hexa-acetate from 100 μg. of sorbitol could be prepared.

Melting points were determined in a Kofler apparatus. X-ray crystallography on single crystals was carried out by Dr T. Lomer of the Physics Department, St Mary's Hospital Medical School.

Experiments with radioactive compounds. The compounds were obtained from The Radiochemical Centre, Amersham, Bucks. and used without further purification. Approximately 150 mg. of slices of seminal vesicle were incubated with 1 ml. of Krebs solution ('medium III', Krebs, 1950) in a Warburg apparatus at 37° for 2 hr. The reaction was stopped by adding 10% (w/v) trichloroacetic acid. In some experiments the supernatant was decanted and the slices were washed several times with Krebs solution. The supernatant and the tissue were then examined separately.

Rapid freezing. For observation of post-mortem changes the tissues were dropped into liquid N_2 and ground in a stainless-steel mortar and 1–2 g. of the powdered frozen material was triturated in 10 ml. of 10% trichloroacetic acid at 0° (LePage, 1948).

Specificity of methods. Glycerol and the hexitols lack specific colour reactions by which they may be detected on chromatograms. However, it could be shown that they did not react with aniline–diphenylamine or with naphtharesorcinol, whereas reaction occurred rapidly with periodate–benzidine and slowly with silver reagents. The failure of these substances to be removed by strongly basic resins also pointed to their non-reducing character. It is important to distinguish sorbitol from the other isomeric hexitols and their R_F values relative to sorbitol are listed in Table 1. Sorbitol in solvent systems A and B gave a complete separation from allitol and a partial separation from all the isomers with the exception of dulcitol. System D, on the other hand, gave a partial resolution from dulcitol.

A separation of the hexitols was possible by paper electrophoresis in basic lead acetate but relatively large quantities were required and these were not always available. In

typical electrophoresis the distances (cm.) of migration from the origin towards the negative pole were: talitol 11·8, mannitol 13·5, galactitol 16·4, sorbitol 17·8, iditol 21·6. The spots remained compact at the low temperature and these differences in migration were sufficient for a clear separation to be obtained.

The specificity of sorbitol dehydrogenase from sheep liver has been reported by Todd (1954) to be similar to that of rat liver (McCorkindale & Edson, 1954), except that a slight reaction (3% of the rate with sorbitol) took place with mannitol. Thus, among hexitols, the only substrates that react at a substantial rate are D-sorbitol and L-iditol, and these could be distinguished by chromatography of the products fructose and sorbose in solvent system C.

Glycerol is well separated from other lower sugar alcohols by electrophoresis in 0·05M-borate (Frahn & Mills, 1959).

Sources of material. The sheep foetal blood was obtained from the umbilical vessel shortly after exposure of the foetus by Caesarian section, the mother being anaesthetized with thiopentone or a spinal anaesthetic. For the surgical procedures the writer is indebted to Dr D. A. Nixon of the Physiology Department, St Mary's Hospital Medical School. The foetal rabbit, rat and guinea-pig blood was obtained by cardiac puncture under ether anaesthesia. Whale blood was collected 17–36 hr. *post mortem* from sperm whales caught in the Azores, by arrangement with the National Institute of Oceanography, Godalming, Surrey. The samples were deproteinized on collection with trichloroacetic acid. Foetal bloods of the llama, hippopotamus and rhinoceros were collected by Mr E. H. Tong and his staff at Whipsnade Zoological Park from the placentae at various times after delivery. The samples (2–30 ml.) were placed in bottles containing 100 mg. of a mixture of NaF and potassium oxalate (1:3, w/w) and refrigerated pending delivery to the Laboratory, where deproteinization was effected with $ZnSO_4$ and $Ba(OH)_2$.

Table 1. $R_{sorbitol}$ *values of hexitols in paper chromatography*

Solvent systems: A, amyl alcohol–pyridine–water (4:3:2, by vol.) (Kawerau, 1956); B, butanol–acetic acid–water (4:1:5, by vol.); C, phenol–water (5:2, w/v); D, butan-2-one–acetic acid–saturated aqueous boric acid solution (9:1:1, by vol.) (Rees & Reynolds, 1958).

Solvent system ...	A	B	C	D
Allitol	1·15	1·13	1·0	1·03
Dulcitol	1·03	1·02	0·97	0·81
Iditol	0·92	0·97	1·0	0·91
Mannitol	1·08	1·07	0·97	0·84
Talitol	1·10	1·09	1·02	0·96

RESULTS

Results of a chromatographic survey of some bloods and tissues are shown in Table 2. Traces of sorbitol and fructose were found in the foetal and maternal blood of the rabbit, guinea pig and rat and also in the maternal blood of the sheep. The quantities did not exceed 0·5 mg./100 ml. and the identification can only be considered tentative. With these species a third spot was also visible, on two-dimensional chromatograms in the position of mannose, the quantity involved being similar.

Further identification of sorbitol. Sufficient quantities of sorbitol-like material were available

Table 2. *Sorbitol, glycerol, fructose and inositol in some animal fluids and tissues*

Approximate concentrations were estimated from the density of the silver spot on the chromatograms, with the exception of the sorbitol content of sheep foetal blood which was estimated by the method of Britton (1959*b*).

	No. of specimens	Concentrations (mg./100 ml. or mg./100 g. of wet tissue)			
		Sorbitol	Glycerol	Fructose	Inositol
Foetal blood					
Sheep	6	7–22*	4	50–100†	10–31†
Llama (domestic breed)‡	1	15*	4	43†	10–20
Hippopotamus amphibius‡	1	15	4	60†	10–20
Rhinoceros unicornis‡	1	15	4	31†	10–20
Whale (sperm)§	4	10–20	4	30–100	10–20
Rabbit	3	Trace	1–2	Trace	3·2–13·9†
Guinea pig	3	Trace	1–2	Trace	2–10
Rat	3	Trace	1–2	Trace	2–10
Maternal blood					
Sheep	4	?Trace	1–2	Trace	0·6–2·7†
Rabbit	3	Trace	1–2	Trace	0·8–3·2†
Guinea pig	3	Trace	1–2	Trace	2–5
Rat	3	Trace	1–2	Trace	2–5
Ram seminal vesicle					
Post mortem	5	200	40	300	100
Frozen	3	10		300	100
Ram semen	4	10–20	0	247†	40

* Not homogeneous in solvent system D.
† Collected results of Huggett & Nixon (1961); Nixon (1954); Mann (1954).
‡ Blood from placenta after delivery.
§ Collected 17–36 hr. *post mortem*.

from the ram seminal vesicle and whale foetal blood to permit further identification. Thus it was shown that reaction occurred with sorbitol dehydrogenase (as judged by the reduction of NAD) at a rate compatible with the amount of material estimated chromatographically. Furthermore, the reaction was shown to go to virtual completion with the formation of a substance chromatographically indistinguishable from fructose. As a further verification the hexa-acetates were prepared and the single-crystal X-ray-diffraction patterns were indistinguishable from authentic sorbitol hexa-acetate. The melting points after recrystallization were 99°, not depressed on mixture with authentic sorbitol hexa-acetate.

Presence of other hexitols. When extracts of foetal sheep blood and foetal llama blood were chromatographed on system D a second spot could be seen running just behind the sorbitol position. This spot remained after the reducing sugars had been removed with Bio-Deminrolit and was still present when these extracts were chromatographed on system A and the hexitol area was eluted. It seemed probable therefore that other hexitols were present in addition to sorbitol and paper electrophoresis of the eluate on basic lead acetate showed three spots migrating in the positions of sorbitol, galactitol and mannitol. The concentrations of sorbitol and galactitol were similar, whereas the concentration of mannitol was rather smaller. Three independent samples of foetal sheep blood gave the same picture on electrophoresis and the chromatographic pattern of a further four samples was similar. One set of umbilical and arterial venous samples, which had been taken simultaneously, were chromatographed and there was no obvious difference between the arterial and venous samples. The specimen of foetal llama blood was a single one; the amniotic fluid, however, was similar in composition.

Further identification of glycerol. The glycerol fraction separated by chromatography was not adsorbed by CO_2-free Bio-Deminrolit. This and its reaction with the silver reagent on chromatograms and its partial volatility in steam indicated that it was probably a polyhydric alcohol. Electrophoresis in sodium borate buffer was carried out as a confirmatory measure except with the llama, hippopotamus and rhinoceros blood, where insufficient material was available.

Hexitol content of foetal sheep blood. Umbilical arterial and venous samples were taken from six foetal sheep and the total hexitol was estimated by the method of Britton (1959*b*). The results are shown in Table 3. The close correspondence of the arterial and venous concentrations suggests that there is no large production of hexitols by the placenta. The high values for sheep 5 are of interest, as this animal had been grossly anoxic during the operative procedure and had a high concentration of lactic acid. It was therefore thought that the sorbitol concentration might have been increased by a reversal of the sorbitol into fructose conversion which occurs in the foetal liver (Hers, 1957*a*, *b*; Andrews, Britton & Nixon 1959), due to a change in the $NAD^+/NADH+H^+$ ratio. However, chromatography of blood obtained from a number of foetuses that had been asphyxiated to foetal death showed no gross increase in sorbitol content, though with the technique used a small rise would not have been noticed.

Production of hexitol by the placenta. Blood obtained from the placenta after removal of the foetus contained larger quantities of hexitol, and subsequent studies on the perfused placenta (Andrews *et al.* 1959; H. G. Britton, A. St G. Huggett & D. A. Nixon, unpublished work) showed a production of hexitol. Chromatography of the samples obtained showed that the increase in concentration took place in the sorbitol fraction, although a small increase in the other isomers could not be excluded. Concomitant with the production of sorbitol an increase in the glycerol content was also noted.

Accumulation of sorbitol in the ram seminal vesicle post mortem. Since the conversion of sorbitol into fructose in the seminal vesicle is NAD-linked it was thought possible that the reaction might be reversed by anoxia. The seminal vesicles were removed from an anaesthetized animal and cut into portions. One piece was frozen immediately in liquid N_2, a second was frozen after 5 min. at room temperature, a third was frozen after 20 min. and the fourth was frozen after 2 hr. Equal quantities of the extracts were placed on a chromatogram and run with solvent system A. The results in Fig. 1 show that there is an increase in the sorbitol fraction on keeping at room temperature. The material in the post-mortem specimen appears to be largely, if not entirely, sorbitol. Unfortunately the sample that was frozen immediately was not examined in system D and the homogeneity of the sorbitol fraction is uncertain. To exclude the

Table 3. *Hexitol content of sheep foetal blood*

Sheep no.	Foetal age (days)	Concn. in umbilical blood (mg./100 ml.) Venous	Arterial
1	90	8·16	8·3
2	120	8·12	10·4
3	120	7·1	6·67
4	121	9·85	9·5
5	121	22·1	15·6
6	135	9·67	9·67

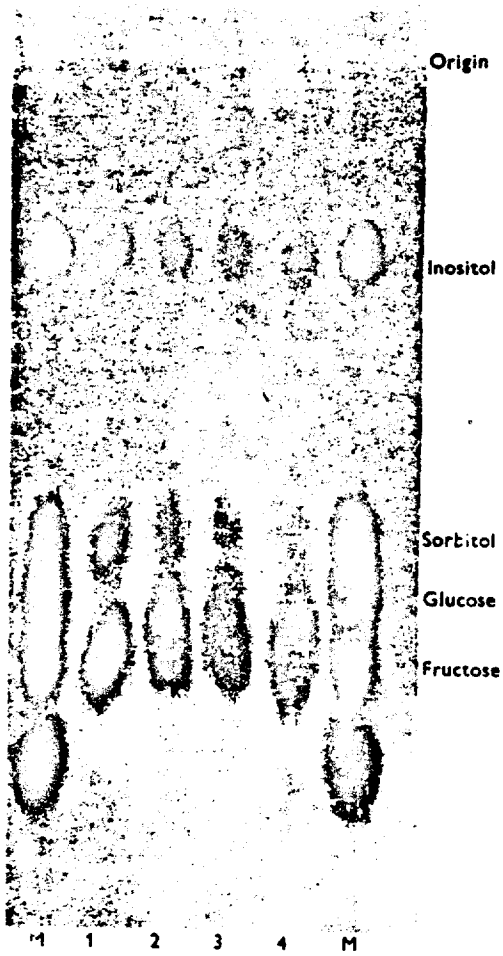

Fig. 1. Paper chromatogram showing post-mortem changes in sorbitol content of ram seminal vesicle. 1, Frozen in liquid N_2 immediately after removal; 2, frozen 5 min. after removal; 3, frozen 20 min. after removal; 4, frozen 2 hr. after removal. M, marker. Solvent system was amyl alcohol–pyridine–water (4:3:2, by vol.). Solvent ran off the paper.

possibility that the sorbitol was released from a readily hydrolysed ester some of the seminal vesicle was hydrolysed for 12 hr. at 100° in 6N-HCl. No increase in the sorbitol content was noted on subsequent chromatography.

Incubation of ram seminal vesicle with radioactive sugars. Slices of ram seminal vesicle were incubated with various radioactive sugars and the extract was chromatographed. The scans of chromatograms run with system D (Fig. 2) show that there is a conversion of glucose into sorbitol and fructose

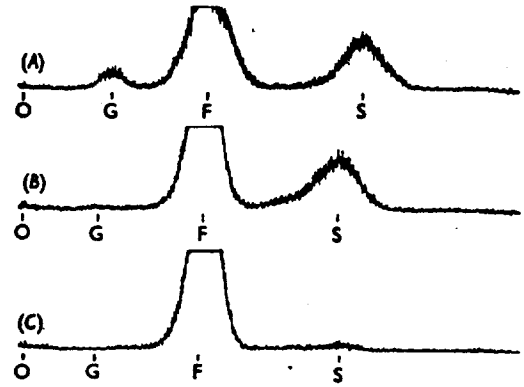

Fig. 2. Scans of radioactivity of paper chromatograms of extracts of seminal vesicle incubated with (*A*) [$^{14}C_6$]glucose, (*B*) [$^{14}C_6$]sorbitol and (*C*) [$^{14}C_6$]fructose. Chromatograms were run for 30 hr. in solvent system D (Table 1). Solvent ran off the paper. O, Origin of chromatogram; G, F and S, positions of glucose, fructose and sorbitol respectively as determined by parallel markers.

and of sorbitol into fructose but that there is no appreciable conversion of fructose into sorbitol. Experiments in which the supernatant and the tissue were examined separately gave qualitatively similar results. When the incubations were carried on anaerobically there was a decreased labelling of fructose when [^{14}C]glucose or [^{14}C]sorbitol was used but there was still no appreciable conversion of fructose into sorbitol.

DISCUSSION

These observations provide further evidence for the presence of sorbitol in sheep foetal blood (Hers, 1957*a*, *b*; 1960*a*), in ram semen (King, Isherwood & Mann, 1958; King & Mann, 1959) and in some other animal tissues and fluids. The association of this sorbitol with fructose might be expected since Hers (1957*b*; 1960*a*) found that fructose is formed from glucose by reduction to sorbitol and subsequent oxidation.

Galactitol and mannitol were only found in foetal sheep and llama blood. However, they might be present in other tissues since the methods of detection would not demonstrate small quantities of these isomers in the presence of sorbitol. The origin of the galactitol and mannitol is unknown but Hers (1957*b*; 1960*b*) showed that mannose and galactose are both reduced by ketose reductase. Galactose and mannose have not been observed in many chromatograms of sheep foetal blood but in this connexion the appearance of a faint spot corresponding to mannose in the maternal blood is perhaps relevant. It might also be that these

sugars are only transiently present because of dietary absorption, and the observation of Walker (1960) that the goat placenta (a species that also contains fructose in the foetal blood) is permeable to these sugars is of interest. The lens of the eye of the rat contains ketose reductase and when the animal is fed with galactose or mannose the corresponding sugar alcohol appears in the lens (van Heyningen, 1959), and this may also occur in the placenta.

Glycerol was present in all the tissues and fluids examined except ram semen. In this, however, large amounts of glycerylphosphorylcholine (Dawson, Mann & White, 1957) are present and evidence has been obtained of an esterase in the female genital tract hydrolysing this ester (White & Wallace, 1961). The concentration of inositol is high in fructose-containing fluids and tissues. The large rise in sorbitol content of the seminal vesicle *post mortem* (Table 2) was originally thought to be due to a reversal of the conversion of sorbitol into fructose by sorbitol dehydrogenase, since this enzyme is NAD-linked and anoxia might be expected to alter the NAD/NADH ratio. However, the failure to observe any appreciable labelling of sorbitol when seminal-vesicle slices were incubated with [^{14}C]fructose makes this unlikely. It therefore seems more likely that the sorbitol is derived from glucose and that under anoxic conditions there is a reduced conversion of sorbitol into fructose. The conversion of sorbitol into fructose must presumably be related to conditions under which the enzyme operates rather than to any cell-permeability phenomenon, since the supernatant and washed tissue slices gave similar chromatograms. However, King & Mann (1959) have shown that ram spermatozoa (which contain a sorbitol dehydrogenase) will reduce fructose to sorbitol under anaerobic conditions.

SUMMARY

1. Methods are described for the separation and identification of non-reducing carbohydrates.

2. Claims that sorbitol is present in various animal tissues and fluids have been confirmed and extended.

3. Galactitol and mannitol have been tentatively identified in foetal sheep and llama blood.

4. The concentration of sorbitol in the ram seminal vesicle increases rapidly after death. This appears to be due to reduction of glucose to sorbitol, rather than reduction of fructose to sorbitol.

5. The conversion of glucose and sorbitol into fructose by ram seminal vesicle has been confirmed.

6. Glycerol has been tentatively identified in all tissues and fluids examined except ram semen.

A sample of allitol was obtained as a gift from Professor T. Reichstein, University of Basle, and samples of iditol and of talitol were generously donated by Dr N. K. Richtmyer, Bethesda, Maryland, U.S.A. The work was aided by a M.R.C. grant to Professor A. St G. Huggett, F.R.S., Department of Physiology, St Mary's Hospital Medical School, London, W. 2, whom the author wishes to thank for his interest and encouragement.

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SUGAR ALCOHOLS

XX. THE FATE OF *d*-SORBITOL, STYRACITOL, AND *l*-SORBOSE IN THE ANIMAL BODY

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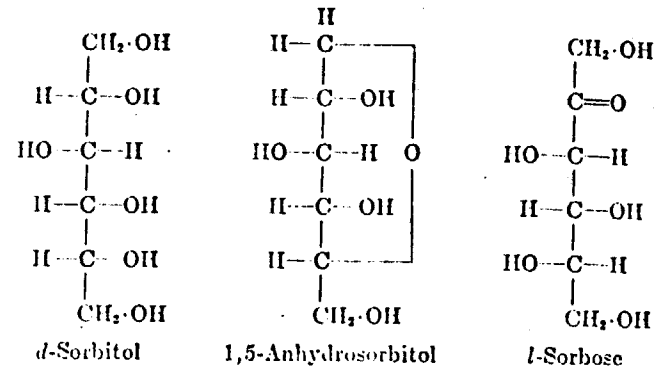
The sugar alcohols that have been previously studied generally serve as precursors of glycogen in rats and many are attacked by members of the colon *aerogenes* group of microorganisms with the production of acid and gas (1, 2). The anhydrides of these sugar alcohols are generally non-metabolizable by either microorganisms or the mammalian body. Recently we have observed that the anhydride, styracitol, was utilized by various members of the *Bacillus coli* group of organisms (3). To continue this study, the epimer of polygalitol, namely styracitol (1,5-anhydrosorbitol), was prepared in large quantities and experiments were conducted comparing the fate of this anhydride to sorbitol and the ketose, *l*-sorbose.

Approximately 100 gm. of styracitol were synthesized by the hydrogenation of tetraacetyloxyglucal and subsequent deacetylation of the reduction product according to the method of Zervas (4). The compound melted at 157° uncorrected; $[\alpha]_D^{24} = -18.2°$ (H_2O, $c = 1.5$); $[\alpha]_D^{20} = -53.4°$ (3.1 per cent H_3BO_3 in H_2O, $c = 3.3$). The *d*-sorbitol generously supplied by the Atlas Powder Company, Wilmington, was crystalline, non-reducing, with a melting point of 97°. The *l*-sorbose used in these studies was obtained through the kindness of Dr. P. A. Wells of the United States Department of Agriculture, m.p. 165°, $[\alpha]_D^{25} = -43.7°$ (H_2O, $c = 10$). The accompanying formulas show the relationship between the three compounds. Recent work by Freudenberg and Rogers (5) indicates that the structure of polygalitol is that which was assigned

formerly to styracitol, namely 1,5-anhydrosorbitol, and that styracitol may be 1,5-anhydromannitol.



Glycogen Storage in Livers of White Rats—Male rats weighing between 100 and 150 gm. were fasted 48 hours and placed in individual cages in a constant temperature room at 25°. The control rats were fed a basal diet of cacao butter and the experimental animals mixtures of cacao butter (67 per cent) and the experimental compound (33 per cent). The feeding period varied from 50 to 120 hours as indicated in Table I. The animals were anesthetized with sodium amytal, the livers removed, and the glycogen determined by the Good *et al.* (6) modification of Pflüger's method. The dextrose was determined by the Shaffer and Hartmann (7) method. The details of the foregoing procedure have been reported previously (1). The results are shown in Table I.

The results indicate that all three compounds are utilized by the fasting rat to form additional glycogen in the liver. In addition it was observed that long feeding periods with these compounds increased the total amount of glycogen in the livers of the rats and the animals did not lose weight as did the control animals. The excreted urine and the urine removed from the bladder at the time of killing did not yield detectable quantities of styracitol upon analysis. In previous experiments with the unmetabolized anhydrides invariably these compounds could be detected unchanged in the excreted urine.

Influence of Styracitol on Respiratory Quotient—Using the open circuit method of determining respiratory metabolism as reported previously, we determined the influence of styracitol upon the

fasting respiratory quotient of the white rat. As suggested by Chambers (8) the animals used in these studies were fasted 48 hours and lost approximately 12 per cent of their original body weight before the determination of the fasting R.Q. was made. The results as given in Table II indicate that the compound is not directly metabolized in the animal body as is dextrose which

TABLE I

Glycogen Storage in Livers of Rats after Feeding d-Sorbitol, Styracitol, and l-Sorbose

No. of Rats	Average food consumed		Feeding period	Liver glycogen		
				Maximal	Minimal	Mean
		gm.	hrs.	per cent	per cent	per cent
5	Cacao butter	8.5	72	0.17	0.03	0.13
10	Styracitol	1.6	50	0.80	0.20	0.40
4	Cacao butter	6.9	72	0.23	0.03	0.16
5	Styracitol	3.0	72	1.65	0.14	1.00
5	Cacao butter	7.8	69	0.25	0.04	0.11
6	Styracitol	2.5	69	0.77	0.15	0.35
2	"	3.5	93	2.36	1.77	2.06
5	Cacao butter	6.0	93	0.65	0.23	0.37
7	l-Sorbose	2.7	120	3.16	0.29	1.74
8	"	4.6	93	5.60	1.80	3.15
8	d-Sorbitol	3.8	89	2.36	0.21	1.16
5	"	4.2	93	1.96	0.55	1.37
5	Cacao butter	9.7	93	0.32	0.19	0.25

under the same experimental conditions gave an increase in the respiratory quotient from 0.716 to 0.800.

Styracitol and Blood Sugar Level Freudenberg and Felton, during a study of the capacity of various carbohydrate substances to elevate the blood sugar level of rabbits, reported that styracitol was physiologically inactive in two experiments (9). In the present study the capacity of styracitol to elevate the blood sugar level has been reinvestigated when this compound is administered by stomach tube. Since the influence of both sorbitol and sorbose

TABLE II

Respiratory Quotients during Fasting and after Styracitol

Rat No.	Weight	Styracitol, 40 per cent solution, by stomach tube	O_2 per 100 gm. per hr.	R.Q.	Length of determination
	gm.	gm.	gm.		hrs.
43	121	Fasting	0.147	0.723	2.5
		1.4	0.181	0.728	2.5
43	134	Fasting	0.150	0.719	3.5
		1.4	0.151	0.741	3.4
54	110	Fasting	0.182	0.735	3.5
		1.4	0.178	0.751	2.5
53	117	Fasting	0.189	0.732	2.7
		0.5	0.189	0.710	2.5
52	112	Fasting	0.155	0.743	2.9
		0.4	0.185	0.704	2.5
50	127	Fasting	0.166	0.719	2.5
		0.4	0.179	0.700	2.5
59	123	Fasting	0.184	0.725	2.7
		0.4	0.193	0.716	2.5
55	124	Fasting	0.193	0.721	3.2
		1.4	0.191	0.705	2.5
108	120	Fasting	0.190	0.732	2.6
		0.4	0.192	0.725	3.5
152	132	Fasting	0.175	0.739	2.5
		1.4	0.188	0.726	5.0
Mean		Fasting	0.173	0.728	
		Styracitol	0.182	0.721	

TABLE III

Influence of Styracitol on Blood Sugar Level of Rabbits

Rabbit No.	Weight	Styracitol	Blood sugar, mg. per 100 cc. blood				Samples
			Fasting	Maximal	Minimal	Mean	
	kg.	gm. per kg.					
1	0.67	2.0	108	109	86	99	Every ½ hr. for 4 hrs.
2	0.80	1.0	110	122	106	110	" ½ " " 4 "
3	0.85	4.0	99	112	95	104	" ½ " " 4 "
4	0.95	2.0	98	123	94	111	" ½ " " 4 "
5	1.36	1.0	100	112	86	98	" 10 min. " 1 hr.

upon the blood sugar level after ingestion has been reported, the compounds were not used. The results are shown in Table III.

Acute Toxicity—The value of the minimum lethal dose of mannitol *per os* previously reported was found to be greater than 1.3 gm. per 100 gm. of body weight for rats. Owing to the greater solubility of sorbitol much larger amounts could be administered. Doses of 1.3 gm. of sorbitol per 100 gm. of animal produced only mild depression and diarrhea in five animals. Quantities of 2.6 gm. per 100 gm. of body weight produced depression and death within 1 to 12 hours. Styracitol (two experiments) failed to produce any detectable effects in concentrations of 1.3 gm. per 100 gm. of rat. *l*-Sorbose in concentrations of 1.3 gm. per 100 gm. of rat (ten experiments) produced no toxic symptoms. It should be pointed out that these concentrations represent relatively massive amounts of the compound.

DISCUSSION

The data assembled indicate that the three chemically related compounds, *d*-sorbitol, styracitol, and *l*-sorbose give rise to additional glycogen storage in the livers of white rats. The effect was anticipated in the case of the ketose and the sugar alcohol, sorbitol, but the anhydride, styracitol, appears to be an exception to the general rule that anhydride formation among the sugar alcohols destroys glycogen-forming capacity. The sweet taste of this compound and its utilization by bacteria suggest utilization by the animal body. That styracitol is not directly metabolized is demonstrated by its inability to elevate the R.Q. and raise the blood sugar level of rabbits and indicates glycogen formation with subsequent utilization.

SUMMARY

1. *d*-Sorbitol, styracitol, and *l*-sorbose when fed in a basal fat diet for a period of days are stored as glycogen in the liver of the rat.

2. Styracitol does not raise the respiratory quotient or increase the O_2 consumption of the fasting rat when administered by stomach tube.

3. Styracitol does not elevate the fasting blood sugar level of the rabbit.

4. The results indicate that styracitol requires glycogen formation for utilization by the animal body.

5. These compounds are not toxic when administered by mouth to the white rat except in massive quantities.

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SUGAR ALCOHOLS

XII. THE FATE OF POLYGALITOL AND MANNITOL IN THE ANIMAL BODY

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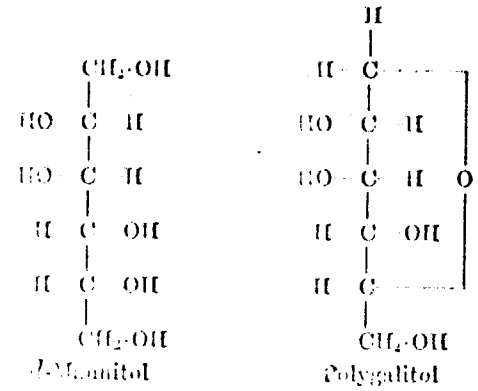
(Received for publication, March 21, 1938)

In a series of communications the authors have reported studies on the fate in the animal body and the decomposition by bacteria [illegible] of the sugar alcohols and their anhydrides. In general [illegible] of the sugar alcohols serve as precursors of glucose in rats [illegible] may give rise to acid and gas when attacked by members of [illegible] *aerogenes* (1, 2) group of microorganisms and the anhydrides of the alcohols behave as inert substances. In one series [illegible] experiments (3) extensive studies were carried out on the fate [illegible] mannitol and its 1, 4-anhydride, mannitan, in the animal body. [illegible] mannitol was found to give rise to additional liver glycogen in the [illegible] and mannitan behaved as an inert substance. Later Silberman and Lewis (4), using a different technique, reported that the [illegible] rat was incapable of using mannitol as a source of glycogen [illegible] fed this alcohol by stomach tube. Having prepared a [illegible] of polygalitol from *Polygala amara* and later having [illegible] in isolating the same compound from *Polygala senega* [illegible], the authors decided to investigate the fate in the body of the [illegible] anhydride and restudy the metabolism of mannitol.

Materials

The mannitol employed was of c.p. grade, generously supplied by the Atlas Powder Company, Wilmington, Delaware. The polygalitol was extracted from *Polygala amara*, by the method of [illegible], Sato, and Sato (6), kindly imported from Europe for this [illegible] by the Penick Company, New York. The compound [illegible] between 142-143° uncorrected; $[\alpha]_D^{16}$ = +17.8°. The

accompanying formulas show the relationship between the two compounds.



Recent work by Freudenberg and Rogers (7) indicates that the structure of polygalitol is that which was assigned formerly to sigonitol, namely 1,5-anhydrosorbitol, and *vice versa*.

Glycogen Storage with Mannitol and Polygalitol in Livers of White Rats. Male white rats weighing between 125 and 175 gm. were fasted for 24 hours and placed in individual cages. The control rats were fed a basal diet of cacao butter and the experimental animals mixtures of cacao butter (67 per cent) and the respective compound (33 per cent). The feeding was continued for from 72 to 80 hours. The animals were anesthetized with amytal, the livers extirpated, and the glycogen determined by the Good (8) modification of Pflüger's (9) method. The dextrose was determined by the Shaffer and Hartmann (10) method. The results are shown in Table I.

These results show that mannitol will and polygalitol will not store additional glycogen in the liver of the white rat under the foregoing experimental conditions. The average liver glycogen content of eighteen rats previously reported after mannitol feeding was 1.23 per cent.

The results reported by Silberman and Lewis were obtained by the administration of a 15 per cent mannitol solution by stomach tube. The experiment was repeated in this laboratory and the results are shown in Table II. These results clearly corroborate the findings of Silberman and Lewis and show that under these

...ditions the white rat is incapable of utilizing mannitol as a precursor of glycogen. Furthermore, analyses of the intestinal contents indicate the absorption of most of the mannitol and

TABLE I

Glycogen Storage in Livers of White Rats after Feeding Mannitol and Polygalitol

Rat No.	Weight	Cacao butter injected	Liver glycogen	Rat No.	Weight	Cacao butter and mannitol	Liver glycogen	Tissue glycogen
	gm.	*gm.*	*per cent*		*gm.*	*gm.*	*per cent*	*per cent*
1	101	4.0	0.12	15	124	10.0	1.52	
2	110	4.0	0.16	16	122	10.0	0.68	
3	106	5.1	0.19	17	129	9.0	0.53	
4	135	7.0	0.37	18	91	6.5	1.09	
5	121	3.5	0.10	19	119	10.0	0.70	
6	133	6.0	0.17	20	154	9.7	0.56	
				21	140	11.0	1.76	
Mean			0.18	22	131	12.1	1.58	
				23	134	13.4	1.11	
		Cacao butter and dextrose		24	130	11.2	0.38	
				Mean			0.98	
7	121	15.0	3.70					
8	135	13.0	2.34			Cacao butter and polygalitol		
9	125	14.5	2.97					
10	130	12.5	2.50					
11	124	19.2	2.36	25	131	6.3	0.16	0.06
12	128	18.8	3.35	26	130	3.6	0.21	0.05
13	136	19.8	3.71	27	125	6.3	0.16	0.06
14	122	15.5	4.19	28	143	8.1	Lost	0.04
				29	137	2.0	0.05	0.06
Mean			3.14	30	125	4.6	0.27	0.08
				31	129	4.6	0.42	0.09
				32	128	3.2	0.14	0.10
				33	125	2.0	0.12	0.10
				34	112	3.2	0.14	0.08
				Mean			0.18	0.08

...e amounts recovered from the urine were approximately one-...th of the total amount administered. Equal quantities of mannitol were administered to rats which were killed after 6 hours.

Approximately one sixth of the mannitol was found in the bladder and excreted in the urine, while an equal amount was found unabsorbed in the intestines. *In vitro*, mannitol passed through the rat's intestine in a typical Hédon's experiment (11). 75 per cent of the mannitol diffused through the intestinal wall within 5 hours.

Effect of Polygalitol and Mannitol on Respiratory Quotient—With certain minor modifications as adopted by the authors (12)

TABLE II

Glycogen Storage in Livers of Rats after Receiving Mannitol by Stomach Tube

Rat No.	Weight	Liver glycogen	Mannitol unabsorbed
Mannitol, 0.5 gm. per rat (1 cc. 15% solution)			
	gm.	*per cent*	*gm.*
1	125	0.03	0.031
2	137	0.09	0.041
3	118	0.16	0.058
4	150	0.06	0.052
5	167	0.05	0.035
6	140	0.05	0.063
7	156	0.09	0.054
8	179	0.07	0.035
Mean		0.08	
Controls given water			
9	146	0.07	
10	156	0.12	
11	150	0.05	
12	112	0.10	

0.782 gm. of mannitol was recovered from the combined excreted urine.

Haldane's (13) open circuit apparatus was employed. The other details of the experiments were set forth by the authors (3) previously. Results with mannitol over a longer time period indicate that under these experimental conditions mannitol does significantly raise the respiratory quotient of the white rat (Table III). Similar results with polygalitol are shown in Table IV.

Polygalitol and Insulin Shock In nineteen mice fasted for 12 hours and injected with insulin, polygalitol, in amounts equal to

that amount of glucose found effective, was incapable of relieving convulsions. Even massive doses of the anhydride failed to combat the symptoms of hypoglycemia. In this respect polygalitol resembles mannitol.

Polygalitol and Blood Sugar Level—It was shown previously that mannitol in doses of 4 gm. per kilo orally produced a mild hyperglycemia in rabbits. The results with polygalitol are shown in Table V. Control experiments in which an equal volume of water was administered produced corresponding changes in the blood sugar level. It is of interest to note that Freudenberg and Felton

TABLE III

Respiratory Quotients during Fasting and after Mannitol

Rat No.	Weight	O_2 per 100 gm. per hr.	25 per cent mannitol by stomach tube	R.Q.	Run in hrs.
	gm.	gm.			
671	126	0.178	Fasting	0.717	3.2
		0.171	5 cc.	0.778	3.1
672	148	0.164	Fasting	0.683	2.5
		0.159	5 cc.	0.740	3.6
673	131	0.176	Fasting	0.692	2.5
		0.185	5 cc.	0.734	4.0
674	123	0.154	Fasting	0.707	2.5
		0.135	5 cc.	0.751	4.3
675	130	0.178	5 "	0.730	7.6
676	123	0.186	5 "	0.727	7.6
Mean.....			Fasting	0.699	
			5 cc.	0.713	

(14) found the epimer of polygalitol, styracitol, ineffective in raising the blood sugar level of one rabbit.

Acute Toxicity—The value of the minimum lethal dose of mannitol previously reported (3) as 1.3 gm. per 100 gm. of body weight *per os* has been restudied. The acute toxic dose of mannitol is greater than 1.3 gm. per 100 gm. of rat, but owing to the limits of solubility the exact dose cannot be determined. Again severe depression of the animals receiving these amounts was observed. Polygalitol in doses of 1.3 gm. per 100 gm. of rat (two experiments) produced a violent diarrhœa within 4 hours and a marked diuresis.

TABLE IV

Respiratory Quotients during Fasting and after Polygalitol

Rat No.	Weight	O_2 per 100 gm. per hr.	40 per cent polygalitol by stomach tube	R.Q.	Run in hrs.
	gm.	gm.			
1	120	0.184	Fasting	0.733	2.5
		0.160	4 cc.	0.799	4.0
16	125	0.174	Fasting	0.726	2.5
		0.202	4 cc.	0.719	4.5
16	126	0.169	Fasting	0.728	2.8
		0.203	4 cc.	0.726	2.2
16	137	0.174	Fasting	0.725	2.9
		0.194	4 cc.	0.718	2.5
18	116	0.185	Fasting	0.739	2.5
		0.181	4 cc.	0.772	3.7
21	116	0.180	Fasting	0.748	2.8
		0.187	4 cc.	0.734	2.0
22	137	0.179	Fasting	0.740	2.1
		0.166	4 cc.	0.756	1.0
23	123	0.164	Fasting	0.738	3.6
		0.195	4 cc.	0.740	5.6
20	121	0.175	Fasting	0.728	2.7
		0.179	4 cc.	0.712	4.9
27	119	0.206	Fasting	0.713	2.5
		0.186	4 cc.	0.742	4.9
Mean.....		0.179	Fasting	0.732	
		0.185	4 cc.	0.745	

In this series $\sigma = 0.018$.

TABLE V

Influence of Polygalitol on Blood Sugar Level of Rabbits

Rabbit No.	Weight	Polygalitol	Blood sugar, mg. per 100 cc.						
			Fasting	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.
	kg.	gm.							
1	3.1	6.2	101		121	114	111	111	118
2	2.3	4.6	101	114	106	103	111	100	111
3	2.6	5.2	93	125	103	119	133		
4	2.3	9.2	118	125	112	125	123		
Mean.....			103	121	110	115	119	105	114

No respiratory or muscular depression was noted following administration of this compound. 24 hour samples of urine yielded nearly the total quantity of anhydride administered.

SUMMARY

1. The removal of a molecule of water from mannitol with the formation of its 1,5-anhydride, polygalitol, destroys the capacity of the former compound to be stored as glycogen in the liver of the white rat.

2. Mannitol when fed in a basal diet for a period of days is partially stored as glycogen in the rat's liver. Administration by stomach tube to fasting rats yields no significant deposition of glycogen within a 6 hour period, when the carbohydrate requirements of the animal may exceed glycogenesis.

3. With respect to insulin shock, mannitol and its 1,5-anhydride behave the same; *i.e.*, as inert compounds.

4. Polygalitol does not raise the respiratory quotient of the rat. In this series of experiments mannitol slightly but significantly increased the respiratory quotient.

5. The mild hyperglycemia observed in rabbits when mannitol is administered orally is not produced by polygalitol.

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THE FATE OF MANNITOL AND MANNITAN IN THE ANIMAL BODY

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A few years ago two of the authors (C. J. C. and J. C. K. (1)) became interested in the metabolism of the inulin obtained from burdock root. The hydrolysis of this polysaccharide, with the formation of levulose, directed their attention to the secondary alcohol, mannitol, and its first anhydride, mannitan.

The dried saccharin exudation, manna, obtained from *Fraxinus ornus* has been used for years in the medicine of folk-lore as a laxative. In 1883 Jaffe (2) observed that mannitol could be fed to dogs and recovered unchanged in large quantities from the urine. In rabbits the compound was partially decomposed. The resistance to oxidation in the body of mannitol is indeed interesting when contrasted with the behavior of sorbitol (3) which readily undergoes oxidation.

Sollmann (4) suggests the use of mannitol as a sweetening agent in the diabetic diet. In 1919 Field (5) studied the effect of the ingestion of 100 gm. of mannitol and certain other compounds on the blood sugar curves of normal colored males. The maximum rise with glucose was 40 mg. per 100 cc.; with mannitol the maximum rise was 10 mg. per 100 cc. The failure of mannitol to relieve insulin shock in white rats was observed by Voegtlin *et al.* (6). In 1929 Ariyama and Takahashi (7) studied the growth curves of white rats on an adequate diet with various carbohydrates. Mannitol was found to be far inferior to glycerol or ethyl alcohol. The bacteriological decomposition of mannitol has been investi-

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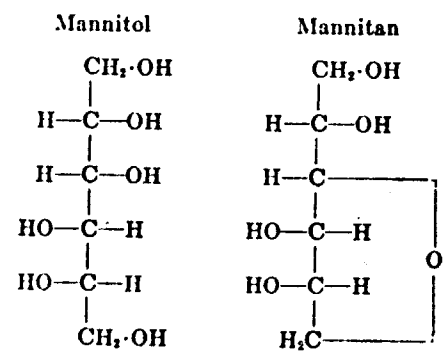
gated by many workers. This work is reviewed comprehensively by Eitel (8).

The authors found no account of the metabolism of mannitan in the literature. The purpose of this investigation is to study the fate of mannitol and its first anhydride, mannitan, in the animal body.

Materials Employed—The mannitol used in this investigation was supplied through the courtesy of Merck and Company, Inc., Rahway, New Jersey. The aqueous solution of the compound (1:10) is neutral to litmus and optically active. The mannitan was prepared from mannitol by a slight modification of the method of Vignon (9) in which the dehydration is accomplished by means of sulfuric acid. The compound was acetylated and the resulting ester distilled *in vacuo*. The fraction distilling between 200–210° at 10 mm. was saponified. The calculated percentage of CH_3COOH is 72.31, that found was 71.70. Through the chemical investigations of van Romburgh and van der Burg (10) who showed that α-vinyldihydrofurane could be obtained from mannitan tetraformate by reduction with hydrogen, the following structure has been assigned to mannitan and its relationship to mannitol may be observed.



Glycogen Storage with Mannitol and Mannitan in Livers of White Rats—White rats were fasted for a period of 24 hours and then placed in small individual cages. The control rats were given a weighed liberal supply of cacao-butter and allowed to continue on this diet of fat for a period of 80 hours. The experimental rats were fed mixtures of cacao-butter and mannitol and

mannitan respectively. The mixtures contained 33 per cent of mannitol and mannitan. The rats were then killed by exsanguination; the livers were immediately extirpated and the glycogen estimated by Pflüger's (11) method. The glucose formed by the hydrolysis of the glycogen was determined by the Shaffer and Hartmann (12) modification of the Folin-Wu method. In certain instances the rats were grouped and their livers analyzed. A few experiments were conducted with levulose for the purposes of comparison. The glycogen content of four livers was 2.89 per cent. The results of these experiments are shown in Table I.

A summary of Table I shows that the rats fed on a basal fat diet ingested an average of 10.9 gm. of fat; whereas, those fed the experimental diet with mannitol ingested an average of 9.3 gm. of food, 67 per cent of which was fat. Hence the amount of fat ingested by these rats was actually less than that ingested by the control rats; therefore, it is concluded that the additional glycogen storage is due to the presence of mannitol in the diet. On the other hand, mannitan does not give rise to increased glycogen storage, and in more than 70 per cent of the experiments the glycogen content of the liver was less than an amount capable of being detected by analytical procedure. By the method it is possible to detect 0.01 per cent of glycogen when a charge of 5 gm. of tissue is used. In an effort to establish the fate of these compounds in the body a series of experiments was conducted to measure the gaseous exchange of the white rat after the ingestion of each substance.

Effect of Mannitol and Mannitan on Respiratory Quotient—With certain minor modifications, Haldane's (14) open circuit apparatus was employed to measure the respiratory quotients.

The animals used in these experiments were fed a ration consisting of cod liver oil, wheat meal, corn-meal, lard, and meat scrapple. They were fasted for 48 hours prior to the experiment. No correction was made for the nitrogen content of the urine of the animals. This was deemed unnecessary for the purpose of this investigation as Cori and Cori (15) have shown that approximately 90 per cent of the metabolism in a fasting rat is fat oxidation.

The results of respiratory determinations on eight fasting rats for 2½ hour periods were 0.708, 0.703, 0.701, 0.709, 0.701, 0.690,

0.702, and 0.718 with a mean of 0.704. Four runs with glucose, according to the Cori technique, in which 4 cc. of a 60 per cent solution are administered by stomach tube, were made and the

TABLE I

Glycogen Storage with Mannitol and Mannitan in Livers of White Rats

Group No.	No. of rats	Food ingested: Type	Food ingested: Amount	Liver glycogen	Group No.	No. of rats	Food ingested: Type	Food ingested: Amount	Liver glycogen
			gm.	*per cent*				*gm.*	*per cent*
1	2	Cacao-butter	20.0	0.06	1	2	Cacao-butter and mannitan	30.0	
2	2		15.0	0.09	2	2		17.6	
3	2		10.0	0.32	3	2		20.0	
4	2		5.5	0.07	4	2		20.0	
5	3		32.0	0.18	5	2		15.6	
6	4		46.0	0.22	6	1		8.0	0.30
7	4		49.0	0.09	7	1		8.0	
8	4		56.0	0.03	8	1		6.5	
9	3		49.0	0.17	9	1		9.0	0.21
					10	1		7.5	
Average				0.14	11	1		8.0	0.23
					12	1		10.0	
1	2	Cacao-butter and mannitol	20.5	1.80	13	2		11.8	0.09
2	2		23.2	1.00	14	2		13.0	
3	2		23.5	1.60	15	2		16.5	
4	2		19.7	0.62	16	2		13.5	
5	2		22.4	1.60	17	2		13.5	
6	2		14.5	0.69	18	2		21.0	0.09
7	2		17.5	0.74	19	2		15.5	0.07
8	2		16.5	1.22	20*	1		6.5	
9	2		13.4	1.77	21*	1		7.5	
Average				1.23					

* Experiments on Groups 20 and 21 were conducted with mannitan prepared by the method of Berthelot (13) which consists essentially of dehydrating mannitol by prolonged boiling under a reflux condenser with several volumes of concentrated hydrochloric acid.

respiratory quotients were 0.888, 0.914, 0.838, and 0.883 with a mean of 0.881. The technique employed with glucose was used with mannitol. Owing to the insolubility of this compound, 5 cc. of a 25 per cent solution were administered. The results of these experiments are shown in Table II.

TABLE II

Respiratory Quotient during Fasting and after Mannitol or after Mannitan

	Weight of rat	O_2	R.Q.
	gm.	*gm.*	
Fasting	170	1.130	0.716
Mannitol	175	0.845	0.727
Fasting	160	0.790	0.744
Mannitol	165	0.782	0.705
Fasting	150	0.875	0.747
Mannitol	155	0.755	0.755
Fasting	205	1.019	0.728
Mannitol	210	0.823	0.713
Fasting	170	0.907	0.735
Mannitol	175	0.773	0.731
Mean, fasting			0.734
" mannitol			0.726
Fasting	182	0.834	0.720
Mannitan	187	0.677	0.770
Fasting	150	0.750	0.705
Mannitan	155	0.511	0.778
Fasting	147	0.783	0.720
Mannitan	152	0.613	0.740
Fasting	175	0.814	0.714
Mannitan	180	0.577	0.782
Fasting	180	0.792	0.743
Mannitan	185	0.564	0.826
Fasting	210	1.144	0.728
Mannitan	215	0.918	0.747
Fasting	123	0.601	0.675
Mannitan	128	0.570	0.765
Fasting	173	0.643	0.713
Mannitan	178	0.579	0.758
Fasting	185	0.638	0.695
Mannitan	190	0.739	0.763
Fasting	173	0.716	0.737
Mannitan	178	0.571	0.795
Fasting	160	0.675	0.725
Mannitan	165	0.683	0.716
Fasting	175	0.829	0.735
Mannitan*	180	1.266	0.758
Mean, fasting			0.718
" mannitan			0.766

* These values represent a 5 hour period after the administration of mannitan.

The results shown in Table II indicate that over a 2½ hour period the ingestion of mannitol does not significantly affect the respiratory quotient of a fasting white rat. The fasting quotients of these rats were somewhat higher than in the control series, indicating the advisability of the procedure herein employed of determining the fasting respiratory quotient on each animal before administering the substance under investigation. The difference between the two means of 0.008 (lower with mannitol) is considered insignificant by the authors and the experiment is interpreted to mean that under these conditions mannitol does not influence the respiratory quotient.

The next step in this investigation concerned the effect of mannitan on the respiratory quotient. The solubility of mannitan in water permitted the use of a 40 to 50 per cent solution. A volume of 4 cc. was administered. The results of these experiments also are shown in Table II.

Following this a series of respiratory quotients was determined on a group of fasting rats after the administration of mannitan as described previously. The fasting values were not determined prior to the administration of mannitan. These respiratory quotients were 0.782, 0.746, 0.750, 0.797, 0.729, and 0.774 with a mean of 0.763. The measurements of the respiratory quotients indicate that although the ingestion of mannitol does not raise the respiratory quotient, mannitan given under the same experimental conditions causes a slight but significant rise. Having observed in feeding experiments the depletion of liver glycogen in the feeding of mannitan, the attention of the authors was centered next on an explanation for the increased respiratory quotient after the ingestion of mannitan. It seemed to resolve itself into two possible explanations; *viz.*, the direct utilization of mannitan or the utilization of glycogen mobilized by its presence in the body.

To determine the answer to this question a series of tissue glycogen determinations was made on rats fasted and fed pure fat as previously described. A second series of experiments was conducted on rats fed fat plus mannitan under the conditions set forth in the liver glycogen feeding experiments. The tissue glycogen was determined by rapidly killing the rats by exsanguination and removing the liver and alimentary tract from the posterior end of the esophagus to the anus. The carcass was rapidly

weighed and cut into ten fragments by means of a cleaver. Each portion was transferred immediately to 100 cc. of a 60 per cent potassium hydroxide solution heated on a boiling water bath. The entire operation consumed not longer than 3 minutes. Hence, freezing of the tissues by means of solid carbon dioxide as suggested by Cori and Cori (15) seems to be unnecessary. After a period of 3 hours, the bones were removed by straining and the solution was made up to 250 cc. The glycogen was determined on a 50 cc. aliquot by the methods previously described.

The results with the control rats of this experiment show that the percentage of tissue glycogen was 0.109, 0.101, 0.133, 0.165, 0.131, 0.130, 0.172, and 0.212 with a mean of 0.144. With mannitan-fed rats, the following percentages of tissue glycogen were obtained: 0.148, 0.152, 0.133, 0.191, 0.138, 0.180, 0.133, 0.180, 0.194, and 0.205 with a mean of 0.164. These results indicate that when mannitan is fed according to the foregoing experimental procedure, the tissue glycogen of the rats shows no significant difference from the tissue glycogen content of rats fed cacao-butter alone.

During the respiratory quotient experiments, the injection by stomach tube of 2.5 gm. of mannitan seemed to exert a narcotic effect on certain of the animals, particularly those of relatively small body weight. This narcotic effect was observed on white rats injected intraperitoneally with mannitol solutions by Macht and Ting (16). Narcotic action was not observed during the course of the feeding experiments, although mannitol and mannitan each produced marked diarrhea.

In the belief that perhaps the mobilization of the tissue and liver glycogen was associated with the narcotic action of mannitan as manifested under the conditions of the respiratory experiments, the following procedure was employed.

Male rats of reasonably uniform weight were fasted for 48 hours. They were then given 2.5 gm. of mannitan by stomach tube by the technique previously described. The tissue glycogen was determined as set forth in the foregoing descriptions. The results of this experiment are shown in Table III.

These results present an entirely different picture from that obtained when mannitan is fed. When injected by stomach tube, there appears to be a diminution of tissue glycogen to approximately one-third the normal fasting value. The mobilization of the tissue glycogen by mannitan when given by stomach tube presents a possible explanation of its capacity to raise the respiratory quotient.

Acute Toxicity—The narcotic effect of mannitol and mannitan observed in these experiments prompted the determination of the acute toxicity of the two compounds. It seemed of special interest to observe, since the production of the anhydride grouping so radically changed the metabolism of the compound, whether or not a change in toxicity was concomitant with the change in its metabolism. The mannitol and mannitan were

TABLE III

Tissue and Liver Glycogen (Mannitan by Stomach Tube)

Weight of rat	Weight of tissue	Glycogen in tissues	
gm.	*gm.*	*gm.*	*per cent*
160	125	0.068	0.054
200	165	0.183	0.111
155	125	0.023	0.018
140	110	0.031	0.028
185	150	0.070	0.046
150	115	0.029	0.025
165	130	0.092	0.071
150	115	0.067	0.058
150	118	0.044	0.037
145	115	0.040	0.035
Mean			0.048

administered by stomach tube as previously described. The M.L.D. of mannitol by stomach tube for white rats (fifteen experiments) is approximately 1.3 gm. per 100 gm. of body weight. The M.L.D. of mannitan by stomach tube for white rats (nine experiments) is approximately the same as that for mannitol. The animals showed no evidence of convulsions prior to death. Death is apparently due to respiratory failure, as heart action continued after the cessation of respiration.

Fate of Mannitan in Body—To determine the presence or absence of mannitan in the urine of a group of rats fed mannitan and cacao-butter, the combined urine from eight rats (about 75 cc.)

was evaporated to dryness on a water bath, at a temperature not exceeding 75°. The residue, which was syrupy, was dried to a constant weight at 110° and mixed with washed sea sand. This mixture was extracted with absolute alcohol in a Soxhlet extractor. The alcohol was evaporated and the residue tested for mannitan. About 12 gm. of material were obtained, which coincided in physical properties and responded to the qualitative tests for mannitan. The residue gave a characteristic blue color when gently warmed with Froehde's reagent. It decolorized diluted acidified solutions of potassium permanganate in the cold. Neither glucose nor mannitol responds to this test. This experiment indicates that much of the mannitan ingested by the white rat appears unchanged in the urine.

Mannitan and Insulin Shock—The combatting of insulin shock with various carbohydrates and related compounds has been investigated by various workers. Noble and Macleod (17) found that levulose, galactose, and maltose provided temporary relief in insulin shock in rabbits and that glucose and mannose provided permanent relief of symptoms. Voegtlin *et al.* (6), as was stated previously, observed the failure of mannitol to relieve hypoglycemia. Accordingly the authors studied the influence of mannitan on insulin hypoglycemia. 5 units of insulin per 100 gm. of mouse were injected and at the beginning of the convulsive period 1.4 cc. of a 15 per cent mannitan solution per 100 gm. of mouse were injected intraperitoneally. When glucose was employed the same procedure was followed.

In thirteen experiments with mice, mannitan failed to relieve permanently hypoglycemia produced by insulin when administered in doses found to be effective when glucose was used.

Mannitol, Mannitan, and Blood Sugar Level—The influence of the ingestion of mannitol on the blood sugar level of humans was studied in a preliminary manner by Field (5). In the light of the foregoing findings in this study, it was thought to be of sufficient interest to study the influence of mannitol and its anhydride mannitan on the blood sugar level of rabbits. Fourteen rabbits weighing about 2 kilos were fasted for 48 hours. Their fasting blood sugar levels were determined by the Shaffer-Hartmann (12) method. 4 gm. per kilo respectively of glucose, mannitol, and mannitan were administered by stomach tube and at ½ hour

intervals subsequent determinations were made. A summary of the results obtained is shown in Table IV.

TABLE IV

Summary of Results. Glucose, Mannitol, and Mannitan

	Mg. per 100 cc. blood					
	Fasting	½ hr.	1 hr.	1½ hrs.	2 hrs.	3 hrs.
Glucose	97	179	255	246	243	197
Mannitol	105	159	155	154	119	143
Mannitan	101	111	103	95	106	111

DISCUSSION

In this investigation a series of twenty-six fasting respiratory quotients for white rats of various body weights has been reported. The mean of this series is 0.717. The probable error of a single determination of this series calculated by the formula, probable error $= \pm 0.6745 \sqrt{\Sigma(v^2)/(n-1)}$ is ±0.012. If one considers a significant difference to be of the order of magnitude of 3 probable error or ±0.036, it becomes evident that the mean 0.766 respiratory quotient obtained after mannitan ingestion represents a significant rise in the respiratory quotient.

It is therefore concluded from the gaseous exchange experiments that mannitan produces a slight but definite increase in the respiratory quotient. The question as to whether this is due to the catabolism of mannitan or the mobilization of tissue glycogen and its catabolism may be considered in the light of the following facts. The data assembled in Table III show that when mannitan is administered by stomach tube under the conditions of the experiment there was a marked diminution of tissue glycogen. The mean difference in tissue glycogen observed is 0.12 per cent. Setting the average weight of the rat tissue at 125 gm., 0.15 gm. of glucose becomes available for catabolism after the administration of mannitan. Theoretically the oxidation of mannitan alone would raise the respiratory quotient to 0.923. It would require the oxidation of relatively larger amounts of mannitan than glucose to raise the respiratory quotient to 0.766. In view of the facts that the injection of mannitan makes available the glucose for oxidation and that much of the mannitan can be recovered

unchanged in the urine, the authors are inclined to believe that the increase in repiratory quotient is due to the catabolism of glucose and not of mannitan.

An examination of the oxygen consumption of the fasting rats whose respiratory quotient was determined reveals that in twenty-six determinations an average of 0.174 gm. of oxygen was consumed per 100 gm. of rat each hour. An inspection of the oxygen consumption of rats after the injection of mannitan shows (average of nineteen) 0.139 gm. of oxygen consumed per 100 gm. of rat each hour or a diminution of 20 per cent. As the compound produces death by respiratory failure, the diminished oxygen consumption of the animals is due likely to depression of the respiratory center. An examination of the data in Table II shows that the injection of mannitol likewise produces a diminution in the oxygen consumption.

SUMMARY

1. The removal of 1 molecule of water from mannitol with the formation of its anhydride mannitan destroys the capacity of the former compound to be stored as glycogen in the liver of white rats.

2. Mannitol does not significantly affect the respiratory quotient of white rats. Mannitan produces a slight but definite rise in the respiratory quotient.

3. There is no significant difference in the toxicities of the compounds when injected by means of a stomach tube.

4. Mannitan is ineffective in relieving insulin shock.

5. Mannitan does not affect the fasting blood sugar level of rabbits when administered orally. Mannitol produces a slight but significant rise.

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Effect of Mucosal Potassium on the Intestinal Glucose Transport* **

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Summary. The extracellular space (inulin or raffinose space) and the intracellular concentration of sodium and potassium were determined in the intestinal mucosal scrapings of rats following a perfusion of the intestine with isosmotic solutions of electrolytes or non-electrolytes. Following a perfusion with K_2SO_4 or mannitol, the intracellular concentration of potassium increased significantly. Perfusion with sorbitol or raffinose did not produce such change. Mannitol (but not sorbitol or raffinose) increased the rate of mediated diffusion but not that of active transport of glucose from the gut lumen. It is concluded that the increased glucose absorption is due to the higa intracellular concentration of potassium which causes an enhanced metabolic disappearance of the free intracellular glucose. In light of modern concepts regarding the intestinal absorption of glucose and of the findings of present and related studies, Verzár's theory concerning the role of intracellular metabolism (phosphorylation) in the absorption is reassessed.

Glucose and galactose are absorbed from the small intestine much faster than other monosaccharides. This "preferential absorption" phenomenon has been observed repeatedly since the turn of the century, and it has also been noted that it required the intact "vital" functioning of the epithelium as the process was inhibited by metabolic poisons, anoxia, or cooling [12]. Verzár suggested a theory in explanation of the preferential absorption [11]: Glucose and galactose undergo partial metabolic changes in the mucosal epithelium; this causes a decrease of the intracellular concentration of the sugar and results in a greater lumen-to-cell concentration gradient, which in turn increases the rate of influx of the sugar into the cell. Verzár and collaborators found that, following the feeding of glucose or galactose, concentration of the sugar-phosphate esters increased in the mucosa. It was therefore thought that the intermediary metabolic change responsible for the preferential absorption was phosphorylation [12].

Subsequent developments gradually modified the basic concepts concerning the intestinal absorption of sugars. The recognition that a carrier

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is involved in the transport process and that a sugar "pump" is responsible for the active, up-hill transport [13] indicated that the proposal of Verzár was not sufficient for an explanation of the preferential absorption of glucose and galactose: consequently, the question whether or not intracellular metabolic change could be involved at all in the complex process of intestinal sugar absorption needed a new reappraisal.

Recently in our laboratory the effect of high intraluminal potassium upon the intestinal absorption of glucose was studied in some detail [3]. The results of these studies suggested that potassium enhances the carrier-mediated disappearance of glucose from the lumen by increasing the rate of intracellular glucose metabolism, as manifested by a stepped-up turnover of mucosal glycogen and lactic acid production. It appears therefore that, under certain conditions, intracellular glucose metabolism may indeed play a role in the process of absorption. In continuation of the previous studies, the present paper describes experiments which indicate that the potassium effect is related to the intracellular concentration of the cation. Experiments will be presented showing that manipulations (other than the increase of the intraluminal concentration of potassium), which result in raising the intracellular potassium concentration, also stimulate the carrier-mediated diffusion of glucose from the intestinal lumen.

Materials and Methods

All reagents used in these experiments were of analytical grade. Glucose was determined by the glucose oxidase method [10], raffinose according to Roe [8], and sodium and potassium in a Baird-Atomic flame photometer with internal lithium standard. C^{14} in the dry tissue was determined after combustion [6] in the liquid scintillation spectrometer as described before [1].

Male Sprague-Dawley rats were used, starved for 16–24 hours before the experiments. The animals were anesthetized by a subcutaneous injection of 1.5 g/kg of urethan.

1. Determination of the extracellular space in the mucosa. The anesthetized rats were bilaterally nephrectomized. A solution of C^{14}-tagged inulin (10 mg/kg), or inert raffinose (1 g/kg), was injected intravenously. A loop of the upper small intestine was perfused *in situ* with an isosmotic solution, as indicated in Table, using Method I described before [2]. Glucose was present in the perfusate in a concentration of 50 mg% (2.1 mM). After a perfusion of 60 min, a blood sample was withdrawn from the abdominal aorta into a heparinized syringe, the animal was killed, and the perfused intestinal loop was rapidly removed and opened and the mucosa scraped off. The procedure from this point on was different, depending upon, whether C^{14} inulin or inert raffinose was used to estimate the extracellular space:

a) C^{14} inulin. The mucosal scraping was put into a small envelope of tared dryed filter paper. [6] It was then dryed at 50°C in a vacuum oven to obtain the total water content. The dryed tissue was combusted and the resulting $C^{14}O_2$ counted, as described above. The blood taken from the aorta was centrifuged, a sample of the plasma was deproteinized with equal volume of ice-cold methanol, and the C^{14} was measured in the filtrate.

b) Raffinose. In this case the mucosal scraping was divided. One part was dryed in a tared container at 50° C in vacuum to constant weight to obtain total water; the other part was boiled with 2 ml distilled water for 10 min, cooled, deproteinized according to SOMOGYI [9], filtered, and the raffinose determined in the filtrate. The plasma raffinose was determined the same way.

The apparent extracellular space was calculated by assuming a rapid and complete equilibration between the plasma and the extracellular space. The calculated inulin and raffinose spaces were in close agreement.

2. Determination of Na and K content of the mucosa. The animals were anesthetized and the intestine was perfused, as stated under Experiment I. After 1 hour the animals were killed, the intestines removed, and the mucosa scraped and treated as under 1b; but instead of raffinose the Na and K was determined in the filtrate which was obtained without deproteinization.

The intracellular concentration of the electrolytes in mucosa was calculated by the following formula

$$C_I = \frac{C_T V_T - C_M V_E}{V_I}$$

where C_I = intracellular concentration.
C_T = concentration in the total tissue water.
V_T = total tissue water.
C_M = concentration in the perfusate.
V_E = extracellular water.

In every instance it was assumed that the ionic composition of the extracellular space was identical with that of the perfusing fluid at the time of the removal of the intestine. The electrolyte composition of the perfusate was determined, therefore, at the end of each experiment.

Results

The combined results concerning the extracellular space and the electrolytes are reproduced in the Table.

Kinetics of glucose absorption in the presence of mannitol. The intestine was perfused *in situ*, as described above, with a solution containing varying concentrations of glucose and rendered isosmotic with mannitol. Fig. 1 illustrates the combined results of several experiments. As a control, the curve obtained with a mixture of glucose Na_2SO_4 is also illustrated. It is clear that, at low glucose concentrations, the replacement of glucose with mannitol inhibits the glucose transport. This finding is in accord with our previous results [4]. As the concentration of glucose in the perfusate is elevated, the rate of transport also increases until it exceeds that with sodium. The shape of the curve is very similar to that obtained previously in experiments when isosmotic K_2SO_4 was used instead of mannitol [3]. Since both procedures increase the intracellular potassium concentration, it is safe to assume that the effect on the sugar transport is due to the intracellular increase of potassium.

Similar experiments were conducted with raffinose and with sorbitol: neither caused an increase of the glucose transport above the level obtained with sodium. Also, as noted from the Table, none of them increased significantly the intracellular potassium of the mucosa.

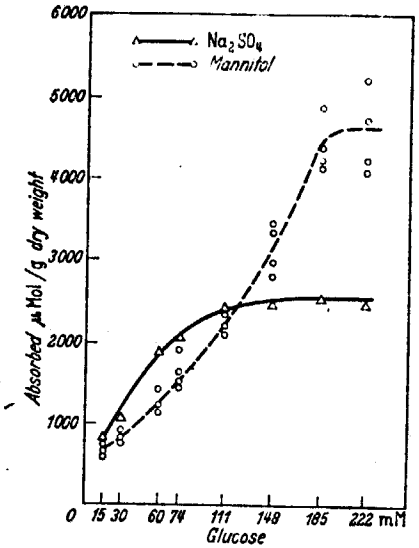

Fig. 1. Absorption of glucose from perfusates containing glucose and mannitol or Na_2SO_4 in mixtures made isosmotic in each instance. Ordinate: glucose disappearance from the lumen in 1 hr. Abscissa. Initial luminal concentration of glucose

Table. *Extracellular water (expressed as % of the total tissue water) and intracellular electrolytes (expressed as meq/L intracellular water) in the mucosal scrapings of the small intestine of rats perfused in situ for 1 hour with 50 mg % glucose dissolved in isosmotic solutions of various composition. The values represent mean with standard deviation. Numbers in bracket indicate the number of observations in each type of experiment*

Perfusate	Extracellular water		Intracellular electrolytes	
	Inulin	Raffinose	Na	K
1) Na_2SO_4	37 ± 3.6 (3)	30 ± 6.1 (3)	78 ± 6.7 (5)	164 ± 9.1 (5)
2) K_2SO_4	28 ± 4.3 (3)	24 ± 3.1 (3)	50 ± 10.4 (4)	194 ± 6.5 (5)
3) Li_2SO_4	29 ± 2.0 (3)	30 ± 3.2 (2)	58 ± 9.9 (5)	86 ± 12.4 (5)
4) Mannitol	30 ± 7.0 (3)	26 ± 4.0 (3)	43 ± 5.0 (6)	200 ± 16.5 (6)
5) Sorbitol	31 ± 2.8 (3)		65 ± 11.8 (3)	181 ± 7.0 (3)
6) Raffinose	29 ± 3.6 (3)		80 ± 14.0 (3)	163 ± 13.5 (4)

Discussion

That potassium ions may influence the intestinal transport of glucose was suggested repeatedly in the past [5, 7]. The actual absorption-stimulating effect of potassium was explained by RIKLIS and QUASTEL [7] as a direct stimulating effect upon the active transport system. However, CSÁKY and HO [3] found that the transport of non-metabolized but actively transported sugar, such as 3-0-methylglucose, was not affected by K, thus excluding the direct effect of high potassium upon the pump

or on the carrier. The latter authors also showed that, with high K in the lumen, the intestinal glycogen synthesis and lactic acid production increased. Thus, a logical explanation for the increased rate of absorption of glucose under the influence of K would be that the intracellular concentration of the free glucose decreases through metabolic conversion, creating a larger lumen-to-cell concentration gradient, which in turn facilitates the carrier-mediated diffusion.

The present experiments indicate that the potassium effect is most likely related to a high intracellular concentration of potassium. The latter could be readily achieved by perfusing the lumen of the gut with an isosmotic solution of K_2SO_4. Interestingly, however, similar increase of the intracellular potassium was obtained if the gut was perfused with isosmotic mannitol. The effect is related somehow to the mannitol molecule, as other non-penetrating electrolyte-free solutions, such as raffinose or sorbitol, did not produce it. This particular effect of mannitol was not further examined in the course of present study, it was noted only that high intraluminal mannitol concentration produced same changes in the kinetics of the intestinal glucose transport as did high intraluminal potassium. Both treatments caused an increase of the intracellular concentration of potassium; consequently, it is logical to assume that ultimately the latter is responsible for the effect upon the glucose transport.

The present experiments supplement the previous observations and help complete the understanding of the effect of potassium on the intestinal absorption of glucose. The latter, in turn, emphasizes a possible role of the intracellular glucose metabolism in the process of transfer.

The various factors which may be involved in the intestinal absorption of glucose could be summed up in the following. The structure of the brush border of the intestinal epithelium determines the first step of the absorption as an entry from the lumen into the cell. This process is mediated. The translocation proper can be caused either by a mediated diffusion or active transport, or a combination of both. As long as the carrier is unsaturated, Fick's law determines the rate of the mediated diffusion: *viz.*, the rate depends primarily upon the concentration difference of the sugar at the interfaces on both sides of the lumen-facing membrane. If the concentration difference is very small or nonexistent, the mediated diffusion ceases and the transport is caused by the pump. If glucose, after entering the cell, undergoes a metabolic change, the intracellular sugar concentration decreases, which in turn increases the gradient across the membrane causing an increased rate of diffusion. Thus the role of intracellular glucose metabolism, as proposed by VERZÁR, can be reassessed. Although it cannot serve as an explanation for the preferential absorption; nevertheless, such a process may play some role in case of sugars, which can be metabolized, by enhancing the rate of carrier-

mediated diffusion. If the initial concentration difference is nonexistent or negative, so that the transport proceeds via an active process, the increase of the intracellular potassium does not enhance the rate of sugar transport. This was corroborated in the present study as well as in the previous ones [3, 4].

Among the factors involved in the intestinal transport of glucose, the contribution by a circulation through the mucosa should not be neglected. Unfortunately, the quantitative role of this factor cannot be assessed at present, because of the lack of precise studies with regard to the intestinal microcirculation.

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QUANTITATIVE DETERMINATION OF MIXTURES OF MONOSACCHARIDES, DISACCHARIDES AND ALDITOLS AS THEIR TRIMETHYLSILYL ETHER DERIVATIVES BY GAS LIQUID CHROMATOGRAPHY

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SUMMARY

The gas chromatographic separation and determination of some important monosaccharides, disaccharides and alditols as their trimethylsilyl ether derivatives is described. This method is very rapid to determine these sugars in a single run in 45 to 50 min. The internal standard technique is used for quantitative determination.

An extraction method for sugars in biological materials and food is also described. The method is applied for the determination of glucose in whole blood.

INTRODUCTION

The simultaneous determination of sugars is important because some monosaccharides and disaccharides are usually present in food, and food for diabetics and dietary patients often contains sorbitol.

A considerable amount of work has been reported during the past few years on the gas chromatographic analysis of sugars, especially as their trimethylsilyl ether (TMSE) derivatives[1-8]. Sweeley *et al.*[1] gave a very detailed report on this subject.

EXPERIMENTAL

Reagents

The following sugars and alditols were obtained from E. Merck AG Darmstadt (Germany):

monosaccharides: aldopentoses: L(+)-arabinose & L(+)-xylose; aldohexoses: D-mannose, D(+)-galactose & D(+)-glucose; ketohexose: D(−)-fructose;

disaccharides: D(+)-saccharose & D(−)-lactose;

alditols: D(−)-mannitol and D(−)-sorbitol.

* Director: Prof. Dr. G. Verdonk.

The internal standard L(+)-rhamnose ($C_6H_{12}O_5$) and the sugar solvent pyridine were obtained from E. Merck; hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) from Carlo Erba (Italy).

Apparatus

The gas chromatograph, an F & M Model 402 with two columns in parallel equipped with a dual flame ionisation detector system (FID), was attached to a Honeywell recorder. A Hamilton 1-μl syringe was used for sample injection. The flash evaporator was from Büchi (Switzerland).

Columns

Two 120 cm × 0.25 cm i.d. U shaped glass columns packed with 3.8% UC W 98 (methyl vinyl substituted silicone gum) stationary phase on 80 to 100 mesh Gas Chrom Q (Applied Science Labs.) were employed. The packing was prepared by the filtration technique[9].

Chromatographic conditions

Carrier gas was nitrogen with a flow rate of 20 to 25 ml per min. Hydrogen and air flow rate were adjusted to obtain full base line compensation during the whole programme. The injection block temperature was about 50° higher than column oven temperature which started at 130° and was programmed with 3° per min till 270° was reached; 250° was detector temperature. The following electrometer settings were used: range, × 10; attenuator, between 16 and 64; samples of 0.1 to 1.0 μl were injected.

Sample preparation

Stock solutions of the different sugars were prepared containing 1 mg per ml water. From these solutions 5 ml amounts were pipetted into a vial and evaporated to dryness. Anhydrous pyridine (5 ml for 10 to 20 mg sugars), stored over KOH pellets, was added and the vial shaken vigorously to dissolve the sugars. (An excess of pyridine was used because of the difficulty to dissolve some sugars.) After dissolution HMDS (0.5 ml) was added, the vial stoppered and shaken vigorously for about 30 sec. Then TMCS (0.25 ml) was added and the stoppered vial was shaken again for at least 1 min. These manipulations were carried out at room temperature.

In principle, these solutions are ready to be injected. As the NH_4Cl precipitates it is recommended to extract the TMSE derivatives with a hexane after having evaporated the pyridine excess to dryness. The n hexane extract (5 ml) was filtered and the filtrate was evaporated under nitrogen. The TMSE was redissolved in 2 to 3 drops of n hexane. These samples were ready for injection.

Extraction of sugars

The following extraction method was [illegible] employed for all materials. The successive steps and the reagent amounts are related to blood sugar extraction. A known amount of total blood (3 g) was weighed in a vial and 3 ml of the 1 mg/ml rhamnose stock solution was added followed by 20 ml of absolute alcohol to make the proteins blood free. To this mixture was added 30 ml of a 50% EtOH solution to dissolve the sugars. This solution was warmed up in a water bath for 30 min

40° being shaken vigorously from time to time, filtered and the filtrate was washed with ether (2 × 30 ml) in a dropping funnel to get rid of the lipids. The water layer was evaporated to dryness and the TMSE derivatives prepared as described above.

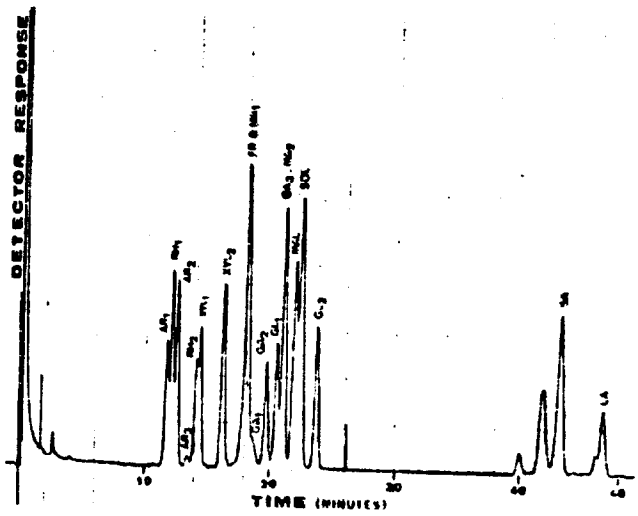


FIG. 1. Separation of eight sugars, two alditols, rhamnose [illegible] as TMSE derivatives on UC W-98 column. [illegible] indices; RH_1, RH_2, AR_1, [illegible] AR = arabinose, RH = rhamnose, [illegible] FR = fructose, MA = mannose, GA = galactose, GL = glucose, MAL = mannitol, SOL = sorbitol, SA = saccharose, LA = lactose.

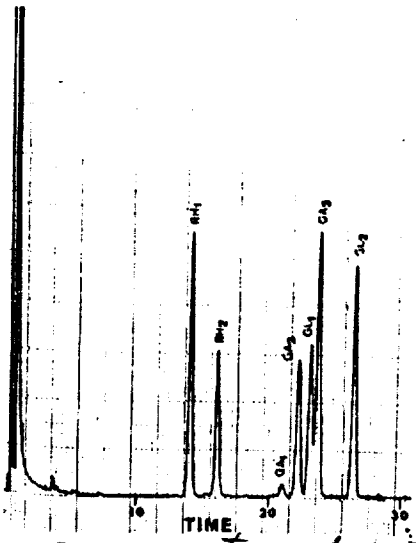


Fig 2. separation of the TMSE derivatives of glucose and galactose.

TABLE I

RELATIVE RETENTION TIMES FOR TMSE DERIVATIVES OF SUGARS RELATED TO THE FIRST RHAMNOSE PEAK

Component	*Retention times*		
	1	*2*	*3 (peak number)*
Arabinose	0.97	1.04	1.12
Rhamnose	1.00	1.17	
Xylose	1.10	1.35	
Fructose	1.50		
Mannose	1.50	1.73	
Galactose	1.54	1.62	1.73
Glucose	1.60	1.95	
Mannitol	1.81		
Sorbitol	1.85		
Saccharose	3.54		
Lactose	3.82		

RESULTS AND DISCUSSION

The object of these experiments is the simultaneous determination of monosaccharides, disaccharides and alditols in one gas-chromatographic run. The difficulty is to separate the anomeric forms of the sugars from each other. This can be done in 45 to 50 min by preparing TMSE derivatives followed by gas liquid chromatographic separation using a UC W-98 stationary phase with optimal conditions of nitrogen flow rate and temperature programme.

For aldoses are always two peaks observed, *i.e.* one peak for the α and one for the β anomer, and for some of them we have three (α, β and γ). A typical chromatogram is represented in Fig. 1, where a mixture of eleven compounds (all of them in the same concentration) was injected. The total time for determination was 48 min. In the presence of mannose there is an overlap of the first mannose peak (MA_1)

TABLE II

QUANTITATIVE ANALYSIS OF KNOWN SUGAR MIXTURES

Mixture	*% Recovery*							
	AR	*XYL*	*MA*	*GL*	*GA*	*FR*	*SOL*	*MAL*
RH AR	106							
RH XYL		105						
RH MA			106					
RH GL				101				
RH GA					97			
RH FR						75*		
RH SOL							96	
RH MAL								100
RH XYL MA		100	97					
		103	101					
RH GL GA				106	113			
				99	105			
RH GL FR				95		72*		
RH GL GA FR SOL				105	109	69*	102	

* The fructose peak area has to be multiplied by 1.4 to obtain the fructose concentration. The area of the peaks was determined by measuring the height and the width at ½ height.

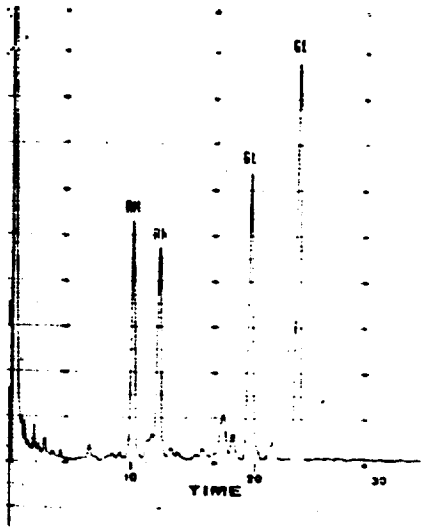
with the fructose peak (FR), and of the second mannose peak (MA) with the third galactose peak (GA_3).

Even the separation of the critical pair glucose and galactose can be obtained. This separation is shown in Fig. 2. The relative retention times related to the first rhamnose peak (RH_1) are given in Table I.

For some monosaccharides and alditols the quantitative aspect was examined. In case the peak areas of the same component are added, correction factors were found to be negligible except for fructose where the peak area has to be multiplied by 1.4. The values of a series of known sugar mixtures are given in Table II.

Blood sugar analysis

The method was applied to determine the glucose concentration in whole human blood. An adequate amount of rhamnose was added as standard before carrying out the sugar extraction. The TMSE were prepared, the mixture injected and the glucose concentration computed. A blood sugar chromatogram is shown in Fig. 3. The glucose concentration of some patients is given in Table III.



[illegible]

TABLE III

ANALYSIS OF GLUCOSE IN WHOLE BLOOD SAMPLES

Subjects	mg/100 ml
1. normal	70.0
2. normal	73.8
3. normal	78.3
4. diabetic	135.8
5. diabetic	200.0

ACKNOWLEDGEMENT

I wish to express my gratitude to Professor Dr. G. Verdonk for his interest in this work and his encouragement which greatly stimulated the conduct of the research.

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SUGAR ALCOHOLS

XXII. METABOLISM AND TOXICITY STUDIES WITH MANNITOL AND SORBITOL IN MAN AND ANIMALS*

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The fate of mannitol and sorbitol in the animal body has been investigated by a number of workers for many years. Nevertheless, the available data are quite confusing and the older literature relative to the metabolism of these compounds is controversial. This literature has been reviewed by Todd, Myers, and West (1) and by Blatherwick *et al.* (2).

Recently, however, experimental findings of workers in this field have been more consistent concerning certain phases of the metabolism of these sugar alcohols. Mannitol (1, 3, 4) and sorbitol (1, 5) definitely produce hepatic glycogen when fed to fasted rats over a period of days. Glycogen formation also occurs when sorbitol is given intraperitoneally to fasted rats (1, 6), but there is still some controversy concerning glycogen deposition following the oral administration of sorbitol (1, 2, 5). Mannitol does not deposit glycogen in the livers of fasted rats following administration either by stomach tube or intraperitoneally (1, 3).

Carr and Krantz (3) reported that the oral administration of mannitol only slightly increased the respiratory quotient of the rat and produced a mild hyperglycemia in rabbits. Todd *et al.* (1) found that sorbitol elevated the blood sugar in dogs after intravenous injection but mannitol did not. These authors state

* The expense of this investigation has been defrayed in part by a grant from the Atlas Powder Company, Wilmington, Delaware.

† The material contained in this paper is part of a thesis submitted by Fred W. Ellis to the Faculty of the Graduate School of the University of Maryland in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

further that after intravenous injection of sorbitol into dogs only 40 to 50 per cent of this compound was recovered in the urine. According to Waters (6), intravenous injection of sorbitol into fasting dogs produced only a mild hyperglycemia and markedly depressed the glucose tolerance curve of the normal and depancreatized animal. Smith, Finkelstein, and Smith (7) found that after the intravenous injection of mannitol and sorbitol into normal man 85 and 32 per cent, respectively, of these compounds appeared in the urine.

Since these later reports indicate considerable evidence of sorbitol utilization, this problem has been reinvestigated with particular reference to the possible use of sugar alcohols by man as substitute carbohydrates.

Materials—The compounds used in this study were crystalline mannitol and sorbitol and a commercial sorbitol syrup. This syrup is a non-crystallizing, aqueous solution of sorbitol having a polyhydroxylic compound content of about 83 per cent, and is manufactured by the electrolytic reduction of glucose. It is known under the trade name of "arlex."

EXPERIMENTAL

Nutritive Value in Rats—Groups of white male rats, 20 to 28 days old and weighing about 40 gm., were used in a feeding experiment over a period of 3 months. Twenty animals comprised each experimental group which received in the regular balanced diet 35 per cent dextrose and 5 per cent of one of the sugar alcohols. The diet fed to a control group of animals was composed of 40 per cent carbohydrate consisting entirely of dextrose.

The growth curves of these animals are shown in Fig. 1. These curves do not indicate any significant difference between the relative nutritional values of mannitol and sorbitol under the foregoing experimental conditions. However, dextrose appears to be superior and sorbitol syrup inferior to both mannitol and sorbitol under similar conditions. These results are in accord with those of Ariyama and Takahashi (8) who have previously shown that mannitol is inferior to dextrose in the diet of rats.

Glycogen Storage in Monkey—*Macacus rhesus* monkeys were fasted for 24 hours before receiving by stomach tube 8 gm. per kilo of body weight of the respective sugar alcohols. 3 hours later the

animals were anesthetized with sodium pentobarbital and two or three portions of liver were taken from different lobes of each animal for individual glycogen determinations. Small samples of liver were removed under aseptic conditions from two monkeys which recovered from the operation. All of the other animals were sacrificed for pathological examinations.

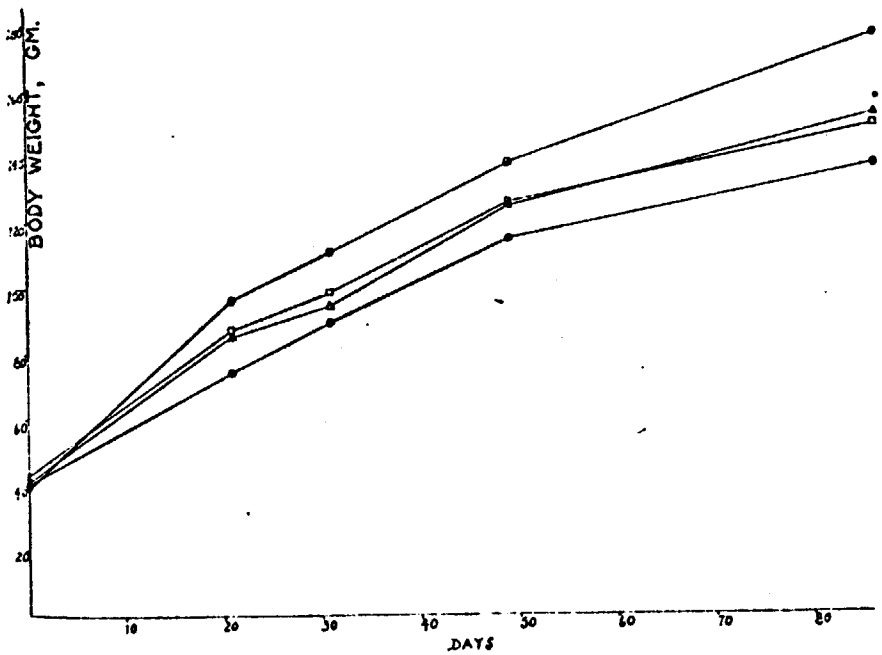

FIG. 1. Growth curves of rats receiving 5 per cent of mannitol and sorbitol in the diet. ○ represents glucose, △ sorbitol, □ mannitol, ◐ sorbitol syrup.

The glycogen was estimated by the procedure of Good, Kramer, and Somogyi (9) and the dextrose was determined according to the Shaffer-Hartmann method (10). The results are summarized in Table I. These results indicate that sorbitol significantly serves as a precursor of hepatic glycogen, mannitol questionably leads to glycogen deposition, and sorbitol syrup does not give rise to increased glycogen in the livers of rhesus monkeys under these conditions.

Toxicity in Monkeys—The chronic toxicity of these compounds was studied in rhesus monkeys by means of two feeding experiments, each of which lasted for 3 months. Nine experimental animals, divided into three equal groups, and two control animals were employed in each experiment. Each such group of animals

TABLE I

Glycogen Storage in Livers of Rhesus Monkeys after the Administration of Sugar Alcohols by Stomach Tube

24 hour fasting period.

Control, 30 cc. water			Sorbitol, 8 gm. per kilo		
Monkey No.	Weight	Liver glycogen, average	Monkey No.	Weight	Liver glycogen, average
	kg.	*per cent*		*kg.*	*per cent*
20	3.79	0.19	12	3.93	0.62
22	3.97	0.06	13	3.48	0.48
96	4.31	0.45	16	3.87	0.32
97	3.73	0.43	95	3.86	0.88
			98	4.18	0.66
			99	3.50	1.35
Mean.....		0.28			0.72
Mannitol, 8 gm. per kilo			Sorbitol syrup, 8 gm. per kilo		
17	3.55	0.62	11	3.51	0.25
89	3.56	0.47	14	3.12	0.22
90	3.98	0.48	15	3.78	0.17
100	3.52	0.49	91	3.88	0.51
101	3.72	0.27	92	4.10	0.45
102	3.38	0.87	94	3.25	0.46
Mean.....		0.53			0.34

was fed a different compound and each monkey received 3 gm. of the designated sugar alcohol in the daily diet.

The blood sugar and urea nitrogen were determined at regular intervals throughout the course of these feeding periods. The values obtained showed no significant variations from the normal values.

At the conclusion of each experiment the animals were sacrificed and routine autopsies were conducted. Sections of the livers and kidneys were prepared and examined by Dr. Henry Wollenweber,

pathologist in this department. Significant degenerative changes which might have been related to toxicity were evident in only one monkey, which was a member of the sorbitol group. In this animal inflammatory changes were noted in the kidney and the cytoplasm of the parenchymal liver cells showed areas of marked rarefaction. We interpret the liver changes as the result of kidney involvement rather than as the result of the feeding of sorbitol, since similar changes were not observed in any of the other animals receiving sorbitol.

Toxicity in Man—It has been shown previously (7) that the intravenous administration to normal man of as much as 80 gm. of either mannitol or sorbitol produced no acute toxic symptoms.

In this investigation the chronic toxicity of these compounds was studied in three normal individuals. Each person ingested 10 gm. of mannitol, sorbitol, and sorbitol syrup respectively each day for 1 month in three separate experiments. During each of these periods 24 hour urine samples were analyzed for sugar alcohol content by the method of Todd *et al.* (11). There was no evidence of significant excretion of any of these compounds in the urine.

At the beginning and end of each experiment blood studies were made and the kidney function was estimated. At no time did any appreciable changes occur in the red blood cell count. The renal excretion of phenolsulfonephthalein indicated no kidney damage.

Laxative Action in Man—The approximate laxative threshold of each compound was established in twelve normal individuals. The "threshold dose" was considered the minimum amount of each substance which produced very soft or watery stools. This action was produced in the majority of subjects after the oral administration of 10 to 20 gm. of mannitol, 20 to 30 gm. of sorbitol syrup, and about 50 gm. of crystalline sorbitol.

Respiratory Quotient and Blood Sugar Level in Man—The observations which comprise this part of the study were made in several normal individuals. Standard procedures employed in basal metabolism and carbohydrate tolerance curve determinations were followed in all experiments. The blood sugar concentration and the respiratory quotient were determined under basal conditions and at periods of ½ hour, 1 hour, and 2 hours after administration of the compound under investigation.

Expired air of 10 minute periods was collected in a Tissot tank and samples were taken for determination of respiratory quotient by the Haldane-Henderson technique. Capillary blood for sugar determinations was obtained from the finger tip after each respiration period and was analyzed by the Folin method (12).

Each basal subject ingested a definite quantity of dextrose and remained in an inclined position throughout the experiment. Under similar conditions on 3 subsequent days each person received an equal quantity of mannitol, sorbitol, or sorbitol syrup. In this routine manner it was possible to study the metabolic activity of each of these compounds in each individual.

TABLE II

Respiratory Quotient and Blood Sugar Level in Man after Dextrose, Sorbitol, and Mannitol

Substance	Amount administered	No. of experiments	Average values							
			Basal		½ hr.		1 hr.		2 hrs.	
			R.Q.	Blood sugar	R.Q.	Blood sugar	R.Q.	Blood sugar	R.Q.	Blood sugar
	gm.			*mg. per cent*		*mg. per cent*		*mg. per cent*		*mg. per cent*
Dextrose	50	2	0.81	82	0.79	123	0.87	115	0.92	109
Sorbitol	50	2	0.78	93	0.83	104	0.93	97	0.98	93
Dextrose	25	7	0.77	107	0.77	117	0.82	126	0.83	105
Sorbitol	25	7	0.77	104	0.81	110	0.80	105	0.83	103
" syrup	25	5	0.77	98	0.77	100	0.79	98	0.79	96
Mannitol	25	5	0.78	96	0.77	95	0.79	94	0.79	94

In most of these tests only 25 gm. of the respective compound were ingested, since larger amounts usually produced catharsis, which interferes to some extent with quantitative studies. In two subjects 50 gm. of sorbitol were used without severe intestinal disturbance.

The average values obtained in these experiments are shown in Table II. These results demonstrate the capacity of sorbitol to increase the respiratory quotient without appreciably elevating the blood sugar. Mannitol and sorbitol syrup did not significantly influence either the blood sugar or the respiratory quotient.

SUMMARY

1. In mice and rats the laxative action of mannitol and sorbitol limits nutritional studies when these compounds comprise a portion of the diet.

2. In the rhesus monkey, sorbitol is capable of storage as glycogen in the liver of the fasted animal. Under the same conditions mannitol is incapable of producing significant glycogen storage.

3. The feeding of 3 gm. per day of mannitol and sorbitol to rhesus monkeys over a period of 3 months produced no histopathological findings or toxicological indications which were attributed to these compounds.

4. In man, the daily ingestion of 10 gm. of mannitol and sorbitol, respectively, for 1 month produced no significant changes in the non-protein nitrogen, CO_2-combining power of the blood, or the red blood cell count. The phenolsulfonephthalein test indicated no kidney damage.

5. The approximate threshold of laxative action in man was determined.

6. In normal human subjects, sorbitol increased the respiratory quotient above the basal level. The effect was similar within 2 hours to that of an equal quantity of dextrose. The blood sugar curve remained practically normal after sorbitol. Mannitol and sorbitol syrup did not significantly influence either the blood sugar or respiratory quotient.

7. The laxative effect of mannitol and sorbitol syrup is greater than that of crystalline sorbitol.

The authors wish to thank Dr. C. Jelleff Carr and Dr. W. E. Evans, Jr., for their assistance in these studies.

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Extracellular Release of Lysosomal Enzymes in Response to Sucrose

By HONOR B. FELL and J. T. DINGLE. (*Strangeways Research Laboratory, Cambridge*)

The injection of sucrose into animals is known to damage the kidneys and liver, producing intense vacuolation of the cells (Janigan, Santamaria & Trump, 1960; Brewer & Heath, 1963). Wattiaux, Wattiaux-de Coninck, Rutgeerts & Tulkens (1965) have shown that in rat liver, sucrose also causes an increase in the size and fragility of the lysosomes.

In the present study, the effect of sucrose on embryonic (chick) cartilage and bone grown in organ culture has been investigated. Sucrose was readily taken up by the explants (38 μg. sucrose/μg. DNA phosphorus) but was not appreciably metabolized. It greatly increased both the release and total synthesis of lysosomal acid protease and acid phosphatase; for example when 7-day (cartilaginous) limb-bone rudiments were cultivated for 8 days in medium containing 0·08 M-sucrose, they released an acid proteolytic activity of 325 μg. tyrosine/hr./rudiment as compared with 46 μg. of tyrosine/hr./control rudiment without sucrose. The total enzyme synthesized (activity of rudiment + activity of medium) was 438 μg. tyrosine/hr./bone grown in the presence of sucrose, and that of the controls 118 μg. tyrosine/hr./rudiment. Histological examination showed that the cytoplasm of the connective tissue cells surrounding the cartilage was distended with small vacuoles which probably contained sucrose, though this has not yet been demonstrated; the cells appeared healthy and many mitoses were present. In the cartilage itself, this abnormal vacuolation appeared only near the articular surface and here the matrix was breaking down.

Sucrose produced similar cytological and biochemical changes in bone from 12–14 day embryos, but in such explants even the osteocytes were highly vacuolated and the well calcified matrix was undergoing a curious lysis.

When embryonic cartilage and bone are cultivated in medium containing complement-sufficient antiserum, the matrix breaks down, and the release of lysosomal enzymes is increased (Fell, Coombs & Dingle, unpublished). The addition of sucrose to medium containing a weak antiserum greatly enhanced both the dissolution of the intercellular material and the synthesis and release of acid proteolytic activity in explants of 7-day limb-bone rudiments; in these explants, unlike those with normal serum, cytoplasmic vacuolation extended far into the cartilage.

In preliminary experiments mannitol produced changes similar to those of sucrose, but glucose in the same concentration did not. The results suggest that the accumulation of undigested material in cells stimulates the release as well as the synthesis of lysosomal enzymes, a phenomenon that may prove important in pathology.

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ENDOCYTOSIS OF SUGARS IN EMBRYONIC SKELETAL TISSUES IN ORGAN CULTURE

I. GENERAL INTRODUCTION AND HISTOLOGICAL EFFECTS

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SUMMARY

The addition of sucrose to a chemically defined culture medium caused intense cytoplasmic vacuolation of the perichondrial, osteogenic and articular cartilage cells of limb-bone rudiments from 8½-day embryonic chicks grown in organ culture. The rest of the cartilage appeared unaffected by the sugar. The osteocytes were intensely vacuolated; the bone matrix was much less dense than that of the controls and had an abnormal fibrillar structure. When the explants were transferred to medium without sucrose, after 6 days vacuolation had almost disappeared.

The effect of the sucrose was dose-dependent; at a concentration of 0·32 M the sugar was highly toxic; at 0·16 M the explants survived and vacuolation of the chondrocytes extended further into the cartilage than at 0·08 M. A similar vacuolation of the cells in response to sucrose was seen in the isolated shafts of the limb-bones and in the mandibular rami from 11- to 13-day embryos; in the sucrose-treated explants osteogenesis was arrested and in places the bone showed osteolytic changes.

In the absence of glucose, 8½-day limb-bone rudiments failed to grow and rapidly degenerated in medium containing 0·08 M sucrose, indicating that sucrose was very little if at all metabolized.

Explants of 8½-day rudiments grown for 8 days in the presence of 0·08 M glucose showed no vacuolation; dextran had some effect, and both mannitol and sorbitol caused vacuolation.

GENERAL INTRODUCTION

The phenomenon of 'pinocytosis' – the ingestion of fluid – was first observed in metazoan cells by Lewis (1931) in studies on living cell cultures *in vitro*; he writes: 'Certain cells in our cultures, especially the macrophages, take in globules of fluid from the surrounding medium.' Lewis fully appreciated the physiological significance of this process: 'By pinocytosis the cells are able to take in substances which cannot diffuse into them or be taken in by ordinary phagocytosis of semi-solid particles. The importance of this phenomenon in cellular metabolism and in the economy of intracellular fluids, seems almost self-evident.' Although other workers confirmed Lewis's observations and recorded pinocytosis in other cell types, for many years the phenomenon aroused little interest, and its importance was not realized until the close relationship between pinocytosis and the lysosomal system was discovered (for review see Jacques, 1966). Nowadays phagocytosis and pinocytosis are usually included under the single term 'endocytosis' (de Duve, 1963) as they are

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[illegible] may similar phenomena. It used to be thought that some types of cell were incapable of endocytosis, but electron-microscopical evidence indicates that the process may take place at the submicroscopic level (micropinocytosis) and it probably occurs in most cells.

To render the four papers of the present series more intelligible to those who may not be familiar with the enormous literature on lysosomal physiology, it was decided to include in this Introduction the following brief account of what is known about the relationship between endocytosis and the lysosomal system (for recent reviews see Straus (1967), and de Duve & Wattiaux, (1966)).

When a cell is performing endocytosis, it puts forth groups of undulating membranes at the surface which infold globules of the surrounding fluid (pinocytosis) or solid particles adherent to the cell (phagocytosis); these folds become sealed off to form membrane-bound intracellular vesicles or phagosomes. At first the phagosome contains only ingested material, but it soon fuses with small, dense bodies, the primary lysosomes, which are laden with hydrolytic enzymes; these hydrolases are released into the phagosome and begin to digest its contents. As the digestive vacuole, or secondary lysosome as it is sometimes called, passes towards the interior of the cell, it rapidly loses fluid and becomes a solid residual body containing material in all stages of digestion. The cytoplasm may also include another type of digestive vacuole, namely autophagic vesicles in which some of the cell's own organelles (mitochondria, endoplasmic reticulum, etc.) are destroyed. An autophagic vacuole is formed by the development of a membrane around a small area of cytoplasm containing one or more organelles; like the phagosomes, the autophagic vacuoles fuse with primary lysosomes and thus acquire hydrolases which digest the imprisoned structures. Secondary lysosomes derived from both phagosomes and autophagic vacuoles may coalesce, thus complicating the cytological picture. Some of these points will be considered in more detail in the second paper of this series (by Glauert, Fell & Dingle).

Some years ago it was discovered that the injection of sucrose into animals damages the kidneys and liver, and causes vacuolation of the cells in these organs (Wilmer, 1944; Brewer & Heath, 1963). Recently Wattiaux, Wattiaux-de Connick, Rutgeerts & Tulkens (1964) examined the effect on the lysosomes of the liver, of sucrose injected into rats; they found that in the treated animals these organelles enlarged, were abnormally fragile but retained their enzymic activity. Sucrose was associated with the lysosomal fraction, and Wattiaux (1966) suggested that the dilatation of the lysosomes was due to the uptake of sucrose into these organelles, probably by pinocytosis. Since the lysosomes do not contain an invertase, the sugar could not be digested, and the hypertonicity thus produced would cause an inflow of water into the vesicles. It was interesting that the total activity of lysosomal hydrolases in the liver increased after the injection of sucrose, but the mechanism for this was not known (Wattiaux, 1966). The changes produced by the sugar were reversible.

Dingle and Fell became interested in the action of sucrose on embryonic skeletal rudiments in organ cultures, as the result of a chance observation. In connexion with another investigation (Fell, Dingle & Coombs, 1966), it was necessary to make control experiments on the effect of hypertonic medium on the synthesis and release

of lysosomal acid protease by skeletal explants. Hypertonicity was produced by the addition to the medium of either salts or sucrose; while the salts had no effect on the protease, the sugar caused a striking increase in both synthesis and release of the enzyme accompanied by intense vacuolation of the perichondrial, osteogenic and articular-cartilage cells. It was decided to investigate further this remarkable action of the sugar, as sucrose-treated cultures seemed to provide a promising model system in which to study certain physiological problems concerning the membrane systems of the cell.

Cytoplasmic vacuolation of cells cultivated in the presence of sucrose was first reported in 1935 by von Möllendorff. Recently, three studies have been made in this Laboratory on the effect of sucrose on cell cultures. Bernassi (1968) observed vacuolation and increased synthesis and release of lysosomal acid phosphatase in suspension cultures of fibroblasts of the *LS* strain; growth was only slightly inhibited. Nyberg & Dingle (personal communication) compared the cytological effects of sucrose and other sugars on Chinese hamster fibroblasts in monolayer cultures. Using time-lapse cinematography, Munro (1968) recorded the changes produced by sucrose in Chinese hamster fibroblasts in living cultures, and demonstrated that the vacuolation arose by pinocytosis followed by the abnormal persistence and accumulation of the pinocytotic vesicles in the cytoplasm.

The four studies of the present series were made on organ cultures of skeletal tissues from embryonic chicks; the first paper deals with the histological effects of the sugar, the second with the electron-microscopical changes, the third with the uptake and distribution of the sucrose in the explants as revealed by autoradiography, and the fourth with the biochemistry. A general discussion of all the results obtained is included in the fourth contribution.

HISTOLOGICAL EFFECTS

The following report concerns the histological changes produced by sucrose in embryonic (chick) cartilage and bone in organ culture. The recovery of the explants from the effect of sucrose, their response to different dose levels, the role of glucose in the action of sucrose and a comparison of the effects of sucrose with those of other polysaccharides, are described.

MATERIAL AND METHODS

Explants

The cartilaginous humerus, femur and tibia of 7- to 8½-day embryonic chicks, and the isolated shafts of the long bones and the mandibular rami from 11- to 13-day embryos were explanted.

Culture methods

The culture vessels were flat-bottomed dishes (internal diameter 3 cm, height 1 cm), each of which contained a shallow table of stainless steel mesh on which the explants

were laid; the height of the tables was such that 1·5 ml of medium just wetted the surface of the mesh. A pair of vessels was enclosed in a Petri dish (internal diameter 9 cm) on the floor of which were three circular layers of filter paper; two holes were cut in the paper to hold the culture dishes in place and to permit the explants to be examined under a dissecting microscope. The filter paper was moistened with a 0·9% solution of NaCl to maintain a moist atmosphere. After the cultures had been prepared, the Petri dishes were stacked on a special rack which was placed in a modified Fildes-Macintosh jar and gassed with a mixture of 5% CO_2 + 20% O_2 + 75% N_2; they were incubated at 38° C.

Medium. The rudiments were grown in a slightly modified version (BGJ5) (see Dingle, Fell & Coombs, 1967) of the chemically defined medium BGJ devised by Biggers, Gwatkin and Judah and described by Biggers, Gwatkin & Heyner (1961). Ascorbic acid was added at a concentration of 15 μg/100 ml of medium. In one experiment on 13-day bones the medium contained 15% heat-inactivated normal rabbit serum, but for the rest of the work the chemically defined medium alone was used. Normal BGJ5 contains 5 mg glucose/ml; for one series of experiments (see p. 96) the glucose was omitted. Sucrose (AR; B.D.H.), usually at a concentration of 0·28M, was added immediately before the culture medium was sterilized by filtration through a Millipore filter.

Every 2 days the used culture medium was removed, fresh medium was substituted and the explants were turned over with a pair of needles. The cultures were maintained for various periods of up to 8 days.

Histology

The explants were fixed in Zenker's solution containing 3–4% glacial acetic acid for about 45 min; the fixative was then replaced by Zenker's fluid without acetic acid in which the rudiments remained for 2–4 h, the time depending on the size of the explants, before being washed in several changes of tap water and dehydrated, cleared in cedar wood oil and embedded in the usual way.

Bones from 12- to 14-day embryos were hardened by passage through a graded series of ethanolic solutions to absolute alcohol, after which they were passed down the series to water and decalcified in formol nitric acid.

Sections were cut at 6 μ and stained with toluidine blue (30 min in 0·5% toluidine blue in 5% ethanol), Delafield's haematoxylin and chromotrop, azan, in celestine blue, Mayer's acid haemalum and van Gieson's stain, or by the periodic acid/Schiff (PAS) method, followed by Mayer's acid haemalum.

RESULTS

The effect of 0·28M sucrose on 7- to 9-day limb-bone rudiments

When first explanted, the rudiments (22 pairs; humerus, femur and tibia) consisted of cartilage already differentiated into small-celled epiphyses, broad zones of flattened proliferative cells, and a middle region of hypertrophic cartilage. The hypertrophic region was covered by a thin layer of early bone, sometimes associated with short

trabeculae, which had been deposited by the periosteal osteoblasts. Overlying the osteoblastic layer was a fairly thick, fibroblastic coat attached to which were a few tags of muscle.

When the living explants were examined after 2 days in culture, their surface had acquired a smooth, tidy appearance which persisted throughout the culture period. By the 4th day, the rudiments treated with sucrose were rather shorter than their paired controls and the perichondrial tissue was much thicker. These differences increased until the end of the experiment (8 days); at this stage the sucrose-treated explants were more flexible than their controls and less translucent when viewed by transmitted light. Both sets of rudiments had enlarged to several times their original size.

In histological sections of rudiments fixed at different stages of growth, the effect of the sugar was first distinguishable after $4\frac{1}{2}$ h incubation, when the cytoplasm of many of the perichondrial and some of the articular cells were seen to contain a large cluster of clear vacuoles; this was most obvious in tangential sections of the osteoblasts. After 2 days (Fig. 9), all the perichondrial and articular cells were honeycombed with vacuoles interspersed with PAS-positive granules of variable size which in many cells were very plentiful.

By the 8th day, the explants exposed to sucrose presented a remarkable histological picture. The closely packed perichondrial fibroblasts (Fig. 2) were distended by a mass of vacuoles so that their diameter was several times that of the corresponding cells in the controls (Fig. 1); many of the fibroblasts were undergoing normal mitosis. In preparations stained with azan (Fig. 2) each cell was seen to be enclosed by a thin fibrous sheath which appeared as a sharp blue line round the cytoplasm. The osteoblasts and osteocytes also were intensely vacuolated (Fig. 2), and the bone matrix was very abnormal. In the controls (Fig. 1), the bone matrix was dense and compact as in the normal embryo, but in the presence of the sucrose (Fig. 2) it was much less abundant and appeared as a mat of discrete fine fibres as if interfibrillar material were reduced or missing. Sometimes the periosteum became pulled away from the bone as the rudiment elongated, leaving a space bridged by osteoid strands and containing many free, vacuolated osteoblasts.

Unlike the bone, the cartilage (cf. Figs. 3, 4) showed no effect except at and immediately below the articular surface (cf. Figs. 5, 6). Only here did the chondrocytes undergo the intense vacuolation characteristic of the perichondrial cells, and only in this region was there loss of metachromasia from the matrix; elsewhere both cells and matrix were indistinguishable from those of the controls.

Recovery of 8- to 9-day limb-bones from exposure to 0·08 M sucrose

All the explants were grown for 2 days in the presence of sucrose; 12 were then fixed for histological study, and the remaining 18 transferred to control medium in which they were cultivated for periods ranging from 6 h to 6 days.

In the explants fixed immediately after 2 days' exposure to sucrose (Fig. 9), the perichondrial and articular cells showed the usual intense vacuolation which persisted almost unchanged for 2 days after transfer to control medium. At 4 days after transfer

the effect, though still conspicuous, was much reduced, and after 6 days (Fig. 10) the great majority of the cells appeared normal, though some of the deeper fibroblasts and osteoblasts remained highly vacuolated.

Dose response of 8-day rudiments to sucrose

When the living explants were examined after 2 days' cultivation, those grown in the highest concentration (0·32M) of sucrose were seen to be much smaller and more opaque than their paired controls grown without the sugar, and instead of forming a smooth tidy surface, as in the controls, the perichondrial tissue appeared rough and tattered as if it were disintegrating. The rudiments enlarged very little during subsequent cultivation, and by the 8th day were small, ragged and very limp. Histological study of 3 explants fixed and sectioned at this stage confirmed that this dose of sucrose was very toxic (Fig. 8); many of the perichondrial and cartilage cells were necrotic, the few chondrocytes that still appeared viable were very vacuolated and throughout the rudiment there was extensive though patchy loss of metachromasia from the matrix.

A dose of 0·16M sucrose was much less injurious. The total lengths of the rudiments increased to about two-thirds those of the paired controls, but the shafts were relatively shorter and the epiphyses larger than in the control explants. Examination of sections (9 pairs of explants) showed no necrosis, apart from a few isolated degenerate cells; mitosis was quite plentiful among the enormously vacuolated fibroblasts of the perichondrial tissue which formed an even thicker layer round the cartilage than in the rudiments treated with 0·08M sucrose. Some of the most superficial cells were rounded and were becoming detached from the surface, but showed no sign of degeneration. Vacuolation of the epiphysial chondrocytes (Fig. 7) and loss of metachromatic material from the matrix extended much further below the articular surfaces than in explants exposed to the lower dose of sugar (cf. Fig. 6). Elsewhere, the cartilage cells were more widely separated from one another than in their paired controls grown without sucrose, and the matrix had the curious 'bubbly' appearance described by Reynolds (1966) in similar limb-bone rudiments in which the cartilage had become abnormally hydrated during cultivation in a chemically defined medium without ascorbic acid. Very little bone and osteoid tissue was present in the rudiments treated with 0·16M sucrose; the newly formed matrix showed the abnormal fibrillar structure already described for the lower dose, and the osteoblasts and osteocytes were enormously distended with vacuoles. The osseous tissue presented a striking contrast to the thick layer of dense, trabecular bone that had been formed in the paired controls grown without sucrose.

Effect of 0·08M sucrose on 11- to 13-day limb-bones and mandibular rami

The explants (11 pairs) of *limb-bones* consisted of the shaft of trabecular bone from which the large cartilaginous ends had been cut off. Partly eroded hypertrophic cartilage occupied the distal regions of the marrow cavity, but in the middle segment the cartilage had been almost (11-day) or completely (12- to 13-day) replaced by

marrow. The bone was covered by a two-layered periosteum which was carefully preserved during dissection.

In the living cultures, the periosteum was seen to become much thicker in the sucrose-treated than in the control explants, but no other difference was detected. Sections of bones fixed after 6–8 days' cultivation in the presence of sucrose (cf. Figs. 13, 14), showed the usual intense vacuolation and consequent enlargement of the periosteal fibroblasts and osteoblasts; these changes were conspicuous also in the peripheral osteocytes, the endosteal osteoblasts and the reticulum cells of the marrow, but the osteocytes in the deeper, older bone either appeared unaffected or were necrotic. The haemopoietic tissue was largely degenerate in both sucrose-treated and control bones.

In preparations stained with azan, in the controls (Fig. 13) the outer coat of the periosteum was seen as a compact sheet of spindle-shaped fibroblasts and intercellular fibres; the underlying osteoblasts had deposited a thick, rather irregular, dense layer of bone, sometimes with cartilaginous areas, which was continuous with the system of fairly stout trabeculae surrounding the remains of the blood vessels and representing the rudiments of the Haversian canals (Fell, 1925). In the bones exposed to sucrose (Fig. 14), the outer coat of the periosteum contained abundant fibres, presumably collagenous, which in section formed a regular, blue-stained trellis-work between the oval, dilated fibroblasts some of which were in mitosis; the inner osteoblastic layer was similar, except that the fibrous intercellular lamellae were thinner and less regular. Unlike those of the controls, the osteoblasts of the sucrose-treated explants had ceased to form bone. The subperiosteal osseous layer stained less intensely than in the controls, and appeared to be undergoing an osteolytic disintegration (cf. Figs. 15, 16); no osteoclasts were encountered. The perivascular trabeculae were narrower than those of the controls but otherwise unchanged; the spaces between the trabeculae were often solidly packed with vacuolated cells.

The *mandibular* explants (4 pairs) consisted of paired rami from which the skin, muscle and Meckel's cartilage had been removed, and which thus consisted mainly of membrane bone and osteogenic tissue. After 2 days in culture, the living rami exposed to sucrose appeared more cellular than their controls, and by the 4th day the calcified bone was largely obscured by the greatly thickened periosteal tissue.

In section, the sucrose-treated explants (Fig. 12) were seen to consist largely of a mass of closely packed cells distended with vacuoles and dividing quite actively. The entire explant was rich in intercellular fibres, and the cells were separated from one another by regular fibrous lamellae. In the controls (Fig. 11), the cavities of the bone contained endothelial vessels, rather loosely arranged fibroblasts and osteoblasts, and in places a layer of endosteal osteoblasts adhering to the surface of the bone; in the sucrose-treated rami, however, many of the cavities were completely filled by a mass of vacuolated cells. In the younger bone, the osteocytes were intensely vacuolated and the matrix appeared to be disintegrating; although many osteoclasts were present in the controls, they were rare in the sucrose-treated explants. Osteogenesis, which was active in the controls, appeared to have ceased in the explants exposed to the sugar; there were, however, large areas of tissue in the latter showing

fine, discrete osteogenic fibres, similar to those described in the periosteal region of the 7- to 8-day rudiments treated with sucrose. It was not clear how much of this material was derived from the breakdown of pre-existing immature bone, and how much represented an abnormal attempt at osteogenesis. The most mature bone showed little change.

The glucose-dependence of the sucrose effect

When glucose was omitted from the medium, 8½-day limb-bone rudiments rapidly degenerated even in the presence of 0·08M sucrose.

In the living cultures, the explants in medium without glucose failed to enlarge; after 2 days those treated with sucrose were distinguishable from paired controls without either sucrose or glucose, only by their thickened perichondrium, and both sets were much smaller than rudiments with glucose and with or without sucrose. By the 8th day, the glucose-deficient explants showed no increase in size, whether sucrose was present or not, and had become very flabby, whereas the rudiments with glucose had enlarged to several times their original volume in the usual way.

Histological examination (9 pairs of explants) confirmed the observations on the living cultures. In this experiment all the explants were grown in medium containing 0·08M sucrose, but one of each pair was deprived of glucose. After 2–4 days (cf. Figs. 17, 18), both sets of rudiments showed thickening of the perichondrium due to vacuolation of the cells in response to sucrose. In the explants with glucose, however, the vacuolated perichondrial cells formed a compact tissue, while in those without glucose, the cells had rounded up and the perichondrium was beginning to disintegrate; there was also much necrosis in the absence of glucose and the metachromasia of the cartilage matrix had diminished. By the 8th day, the sucrose-treated, glucose-deficient explants were almost completely necrotic.

The effect of other sugars at a concentration of 0·08M on 8-day limb-bone rudiments

In the living cultures, explants treated with *glucose*, *mannose* or *maltose* for 8 days showed little effect, and there was no thickening of the perichondrium as in rudiments exposed to sucrose. *Mannitol*, however, caused some perichondrial thickening, and *melezitose* and *stachyose* were even more active in this respect than sucrose. The alcohol *sorbitol* caused an obvious and *dextran* a slight thickening of the perichondrial tissue as compared with untreated paired controls. All these agents, except glucose, mannose and maltose, increased the flexibility of the cartilage and all, except mannose, reduced growth in length to varying degrees.

The effects of some of these substances were examined in histological preparations. Sections (3 pairs of explants for each agent) showed no vacuolation of perichondrial cells in explants treated with hypertonic glucose, vacuolation closely resembling that produced by sucrose in rudiments exposed to mannitol and sorbitol, and less marked vacuolation after treatment with dextran.

DISCUSSION

Munro's observations on living hamster fibroblasts (Munro, 1968) showed clearly that in these cells, vacuolation induced by sucrose was due to the abnormal persistence of pinocytotic vesicles in the cytoplasm; it seems reasonable to assume that the vacuolation of the cells in the skeletal explants was produced by a similar mechanism. The vacuolation in the explants was reversible and gradually disappeared on withdrawal of the sucrose (cf. Figs. 9, 10). It was interesting that, as already noted in cell cultures by Nyberg & Dingle (personal communication), only compounds that are metabolized poorly or not at all (Paul, 1965) produced the effect in the organ cultures; that the skeletal explants cannot utilize sucrose to a significant degree, was suggested by the experiments in which 8-day limb-bone rudiments rapidly degenerated when explanted in chemically defined medium containing sucrose but no glucose.

In the 8-day limb-bones, vacuolation appeared throughout the fibroblastic and osteogenic tissue. Only the articular region of the cartilage was affected, however; here the cells were vacuolated and the matrix had lost most of its metachromasia. Elsewhere, both chondrocytes and intercellular material were histologically indistinguishable from those of the controls. This difference in response between the articular region and the deeper parts of the cartilage might have been due either to failure of the sucrose to penetrate into the interior of the explant, or to lack of pinocytotic activity of the inner chondrocytes. Experiments to be reported in the third paper of this series showed that sucrose permeated the entire explant but, unlike the articular and perichondrial cells, the deeper chondrocytes took up very little of the sugar. Thus the failure of all but the articular cartilage cells to respond to sucrose seems to be due to an inability to pinocytose the sugar. This problem will be considered further in the General Discussion in the fourth paper.

In this and subsequent papers of the series we are greatly indebted to Mr L. J. King for his skilful assistance with the organ culture, Miss B. Bunting and Mr P. Martin for assistance with the biochemical techniques and to Mrs E. Nyberg and Mrs K. S. Cusick for preparing the chemically defined medium. Mr M. Applin was responsible for the photographs. The work described in all four papers was supported by funds from the Medical Research Council and the Wellcome Trust to whom we express our grateful thanks.

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Fig. 1. Control. Part of the shaft of an 8½-day femur grown for 8 days in the chemically defined medium BGJ5. Note the compact two-layered periosteum (*f*, fibrous layer; *o*, osteoblasts) and layer of dense bone deposited on the cartilage. (Azan.)

Fig. 2. Sucrose treated. The opposite femur from the same embryo as the explant in Fig. 1 after 8 days in BGJ5 + 0·08 M sucrose. The periosteum is nearly four times as thick as in the paired control owing to the intense vacuolation of the cells; the osteocytes also are highly vacuolated and the bone matrix is much less dense. (Azan.)

Fig. 3. Control. Femur from an 8½-day embryo, grown in BGJ5 for 8 days (*d*, distal end). (Toluidine blue.)

Fig. 4. Sucrose treated. Opposite femur from the same chick as the explant in Fig. 3 cultivated for 8 days in BGJ5 + 0·08 M sucrose. The explant is rather shorter than its control and the layer of perichondrial tissue is thicker; the cartilage appears unaltered at this magnification. (Toluidine blue.)

Fig. 5. Control. The articular region at the distal end (*d*) of the rudiment shown in Fig. 3. (Toluidine blue.)

Fig. 6. Sucrose treated. The articular region at the distal end of the explant in Fig. 4. The chondrocytes at and for some distance below the articular surface are highly vacuolated and metachromasia has largely disappeared from the matrix; below this zone the cells and matrix appear normal. (Toluidine blue.)

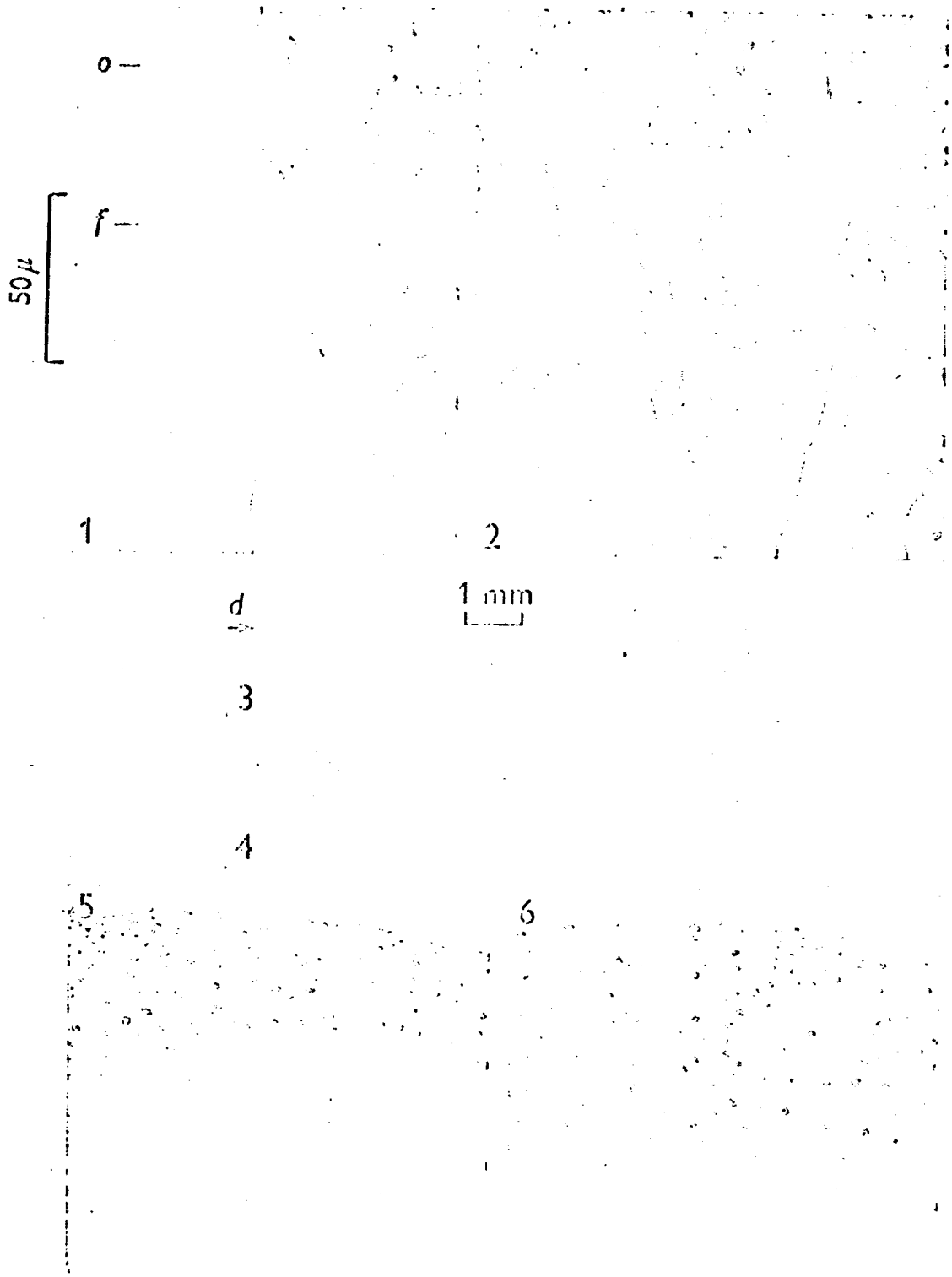
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Fig. 7. Sucrose treated. The articular region at the distal end of an 8½-day femur grown for 8 days in the presence of 0·16 M sucrose. Vacuolation of the chondrocytes is greater and extends further into the epiphysial cartilage than in explants treated with 0·08 M sucrose, cf. Fig. 6 (same magnification as Figs. 5, 6; toluidine blue).

Fig. 8. Sucrose treated. The distal end of an 8½-day femur from the same experiment as the explant shown in Fig. 7, after 8 days' cultivation in medium containing 0·32 M sucrose. At this concentration the sugar is very toxic and the epiphysial chondrocytes are pycnotic and dead (same magnification as Figs. 5–7; toluidine blue).

Fig. 9. Sucrose treated. Periosteum (*p*) and bone (*b*) of an 8½-day femur grown for 2 days in medium containing 0·08 M sucrose; note the intense vacuolation of periosteal fibroblasts and osteoblasts, and of the young osteocytes (PAS and Mayer's acid haemalum).

Fig. 10. Recovery. Periosteum and bone of an 8½-day femur from the same experiment as the rudiment shown in Fig. 9; this explant was exposed for 2 days to 0·08 M sucrose (cf. Fig. 9), then cultivated without sucrose for a further 6 days; cytoplasmic vacuolation has almost completely disappeared (PAS and Mayer's acid haemalum).

Fig. 11. Control. Mandibular ramus from a 14-day embryo grown in control medium for 8 days. Note periosteum (*p*) and young osteocytes (same magnification as Figs. 5–8; Delafield's haematoxylin and chromotrop).

Fig. 12. Sucrose treated. Fourteen-day mandibular ramus from the same experiment as the explant shown in Fig. 11, after 8 days' cultivation in the presence of 0·08 M sucrose; the cells of all types are intensely vacuolated (same magnification as Figs. 5–8, 11; Delafield's haematoxylin and chromotrop).

Fig. 13. Control. Part of the shaft of a tibia from a 13-day embryo cultivated for 8 days in BGJ5. Note the compact, two-layered periosteum in course of forming new bone. The Haversian space on the right is lined by osteoblasts but is otherwise empty except for a blood vessel (*bv*) at the top. (Azan.)

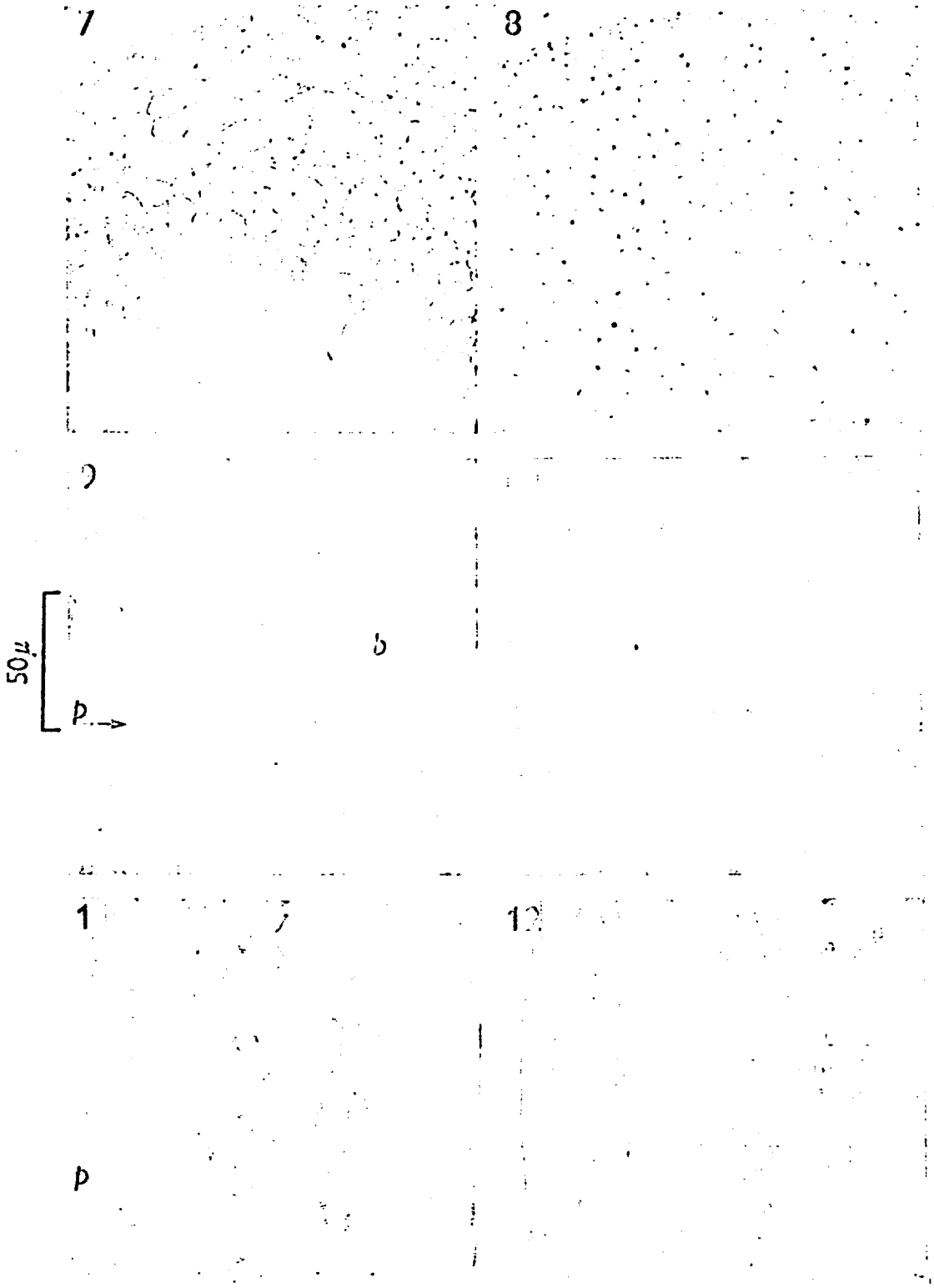
Fig. 14. Sucrose treated. Comparable region of the shaft of the opposite tibia from the same embryo as the explant in Fig. 13. The periosteum is greatly thickened, all the cells are much dilated and the two-layered structure has almost disappeared; note the regular fibrous lattice work between the cells. There is no new bone formation; the peripheral osteocytes are highly vacuolated and the Haversian space on the right is solidly packed with vacuolated cells. (Azan.)

Fig. 15. Control. Part of the shaft of a 13-day tibia cultivated for 8 days in BGJ5 + 15 % heat-inactivated rabbit serum, showing periosteum (*p*) and layer of bone. (Azan.)

Fig. 16. Sucrose treated. Comparable region of the opposite tibia from the same chick as the explant in Fig. 15, grown for 8 days in the same medium but with the addition of 0·08 M sucrose. The bone shows osteolytic changes; note the greatly enlarged lacunae and vacuolation of the periosteal cells (*p*) and osteocytes. (Azan.)

Fig. 17. Tibia from an 8-day embryo grown for 4 days in BGJ5 + 0·08 M sucrose + the normal amount (0·005 M) of glucose. The cartilage matrix is intensely metachromatic. (Toluidine blue.)

Fig. 18. Opposite tibia from the same chick as the explant in Fig. 11, cultivated for 4 days in BGJ5 + 0·08 M sucrose but without glucose. The rudiment has not grown and is largely necrotic. (Toluidine blue.)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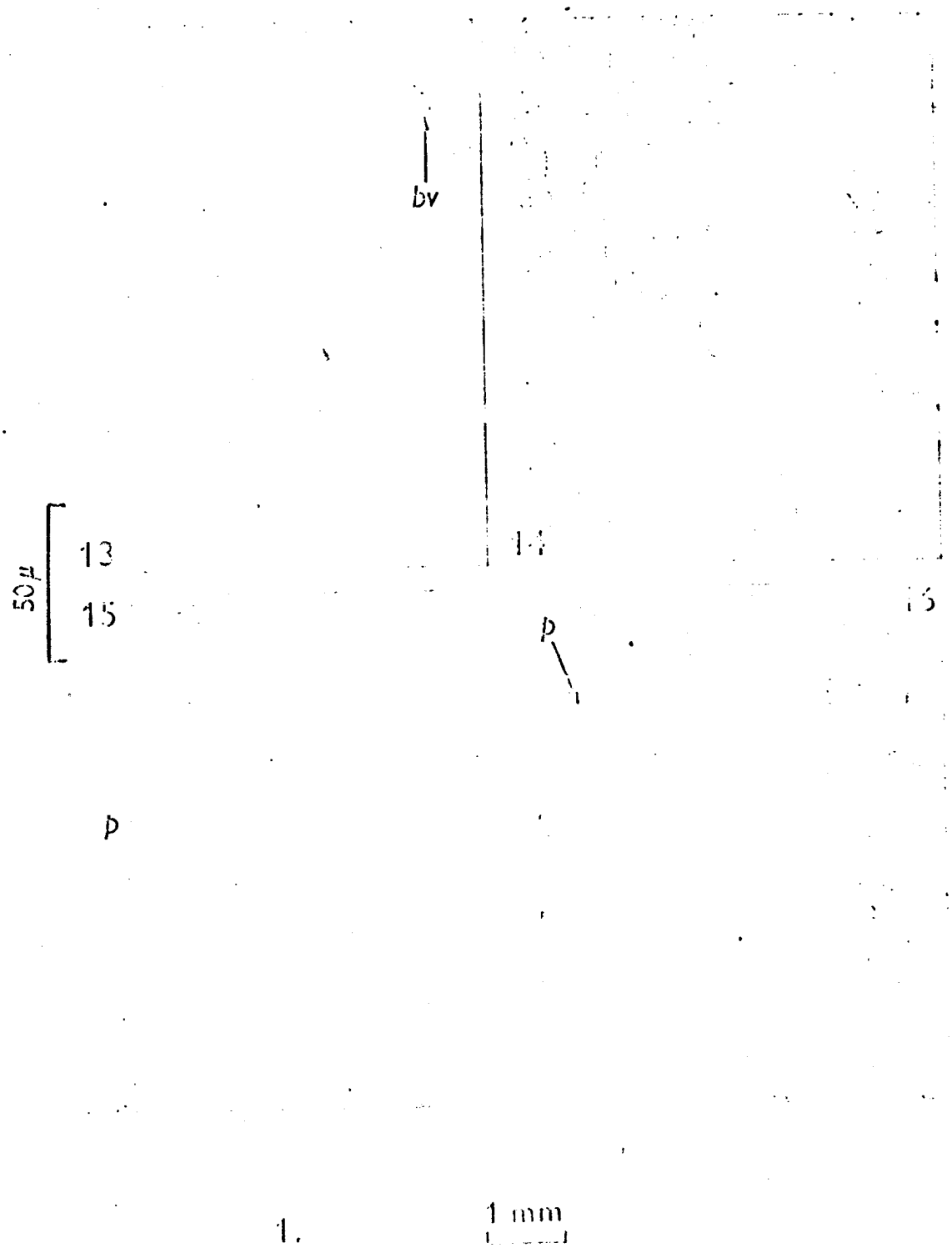

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SCIENTIFIC PROCEEDINGS

Abstracts of Communications.

One hundred second meeting.

New York Post Graduate Medical School, New York City, November 19, 1919. President Calkins in the chair.

17 (1477)

Blood sugar curves with glucose, lactose, maltose, mannite, and cane sugar.

By Cyrus W. Field.

These curves were obtained by feeding normal males colored, with 100 grams of the pure sugar, on a fasting stomach. The dose was as a rule given after the first sample of blood had been taken; this was as a rule at 8 A.M. The second, third and fourth samples were taken one, two and three hours after the ingestion of the sugar, which had been dissolved in a large glass of water.

The urines were tested for glucose up to three hours after the last sample of blood had been taken, and in none of the cases did a specimen ever show the slightest trace of a reducing substance with Benedict's qualitative solution.

The glucose curve was that with which all are familiar, that is rising to its highest point one hour after the ingestion, and then dropping to the normal at the end of the next hour or two.

Maltose gave the same curve as the glucose. Mannite gave the same time curve as the glucose, and maltose, but did not rise to the same height as the other two; the average of five cases gave an increase of only 10 milligrams per 100 c.c. of blood, while that for glucose was 40 milligrams, and for maltose 34 milligrams.

Cane sugar showed a curve that reached its height at the end of the second hour after its ingestion, and had dropped to normal at the third hour. Its average rise for 10 cases was 20 milligrams

per 100 c.c. of blood. Lactose shows only a very slight rise, 4 milligrams, and that at the end of the second hour, as was the case with the other disachrade, cane sugar. I have been unable to consult the literature and so will offer the figures for what they are worth.

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OSTEOGNETIC ACTION FROM HEXAHYDRIC ALCOHOL GLUCIDES AND FROM GLYCOLIC ACID. NEW PERSPECTIVE INTO GLUCIDE PHYSIOLOGY.

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The study of the influence of various glucides on calcium utilization in the rat recently led us to sketch a new physiological classification of those compounds. A first group holds the energy-glucides, namely glucose, saccharose, maltose and starch, of which the functional activities seem to be well understood. But these are substances hardly favoring utilization of calcium and skeletal development. On the other hand many terms of a series continuously growing as we perform our experiments have been shown to be very active with respect to ossification. To this second group belong the D and L xyloses, D and L arabinoses, galactose and lactose. These are glucides which, in order to differentiate them from the energy ones, and so as to stress their high osteogenetic value, we have called structural glucides (P. FOURNIER, 1954 c; P. FOURNIER ETAL, 1955).

The present work will discuss the manner of intervention of structural glucides in the bone metabolism.

By means of a series of experiments performed with young rats, this work sought to define the influence of administering various glucides and several of their acid or alkylated derivatives on the utilization of the calcium of the ration. We were guided by considerations of similarities in configuration or chemical affinity with glucides already studied, as regards the selection of the substances in our experiment. From another viewpoint, first keeping, then transposing the essentially structural role of some very common glucides in vegetal membranes, we studied their effects on the formation of animal support tissues.

Three experiments were performed successively, each comprising the study of a group of compounds that have one chemical property in common.

1. In the first experiment, we tested various glucides, that is, aldehyde function compounds: one pentose, the D-lyxose; two hexoses, fructose and mannose; one diholoside, the melibiose; one triholoside, the raffinose. Melibiose and raffinose are galactosides.

2. The second experiment concerns the study of the three hexahydric alcohols, sorbitol, mannitol and dulcitol, which resp. differ from glucose, mannose and galactose only by the substitution of a primary alcohol function for the corresponding hexose aldehyde function.

3. The testing of organic acids was the object of the third experiment.

Gluconic acid is derived from glucose by replacing the aldehyde function by an acid one. This experiment was required in view of the widespread use of calcium gluconate in bone therapy. Further the inferences from the first two experiments led us to experiment with another organic acid of very simple composition, namely glycolic acid.

The influence of each of those various compounds on calcium utilization was compared on one hand with a little active energy-glucide, starch, and, on the other, with a structural glucide of very high osteogenetic power, lactose.

TECHNIQUES

ANIMAL CARE

All experiments were performed with albino rats from 5 to 6 weeks old and weighing between 50 and 60 gm. The animals were homogeneously distributed in lots of 6.

In each of the three experiments, the rats from the control lot (1) are fed a starch-based diet of the following composition (%): wheat starch: 85.5; caseine: 4; peanut oil 8; dry yeast: 3; saline mixture: 3; titanium oxide: 0.5. For 100 parts, the saline mixture contains 45 of CO_3Ca; 26 of PO_4H_2K; 13.2 of ClK; 8.5 of ClNa; 3 of Mg hydrocarbonate; 2 Of $SO_4Mg\ 7OH_2$; 2 of ferric (ferrous?) oxalate; o.13 of FNa; 0.13 of $SO_4Cu\ 5OH_2$; 0.025 of crystallized potassium alum; 0.01 of IK; 0.005 of SO_4Mn.

The diets of the rats from the other lots differ from the one above only in that a given proportion of the compound investigated - glucide, alcohol or acid -, is substituted for an equivalent proportion of starch, with one exception, namely gluconic acid, where we did use 6% of calcium gluconate and thereby introduced 1½% of a saline mixture lacking calcium carbonate into this diet, so that the calcium content of the gluconic acid diet was the same as for the others.

Seven lots were made use of in the first experiment. The diets fed to the animals of lots others than the control held resp.: 3.5% of D lyxose (lot 2), 10% of mannose or fructose (lots 3 and 4), 7.6% of lactose or melibiose (lots 5 and 6), 11.2% of raffinose (lot 7). The ratios of the three galctosides -- lactose, melibiose and raffinose-- were so selected that the three diets contain the same proportion of 4% of galactose.

The second experiment was meant merely to study the influence of administering the three hexahydric alcohols on the calcium exchanges, but in fact deals with 8 lots. In addition to the control lot 1 (starch-based diet) and to lot 2 of which the lactose diet allows determining its sensitivity, with respect to the structural glucides , of the experimental rats, we made up 6 other animal lots all receiving diets containing 6% of one of the hexahyrdric alcohols or of one of the corresponding hexoses: sorbitol or glucose (lots 3 and 4), mannitol or mannose (lots 5 and 6), dulcitol or galactose (lots 7 and 8).

The third experiment comprises 4 lots. The first two as previously are the control lot which is fed on a starch-based diet and lot 2 corresponding to a diet of 10% lactose. The diet of rats in lot 3 contains 6% of calcium gluconate or 5.25% of gluconic acid. Glycolic acid is introduced into the animals rations of lot 4 in the amount of 1.5%.

All diets are prepared in the form of an unctuous paste containing one part of the powdered diet such as previously described and two parts of distilled water.

After three days of administration, the animals were individually placed in devices allowing to collect separately their urines and feces. Those devices have been previously described (FOURNIER & SUSBIELLE, 1952). The amounts of food consumed by each rat were carefully noted every day. The animals' weights were recorded twice a week.

ANALYTICAL TECHNIQUES

Though these experimental rats had been examined from various viewpoints, this report will only deal with the study of the calcium balance which, in order to be determined, requires calcium dosing in the feed, measurement in the urine and feces. The method we used for measuring all these is so conventional that we shall not describe it here, it having already appeared in a number of prior publications (FOURNIER,195C

FOURNIER & SUSBIELLE, 1952; FOURNIER ETAL, 1955).

RESULTS

There is one table listing the results of each experiment.

To the left and in the first column is listed the number of each lot. Under the caption of "diets" in the second is a listing of the tested substances and also of the proportions of those substances in the diets. The next four columns show the daily average values referring to calcium balance for each lot: the amounts of calcium ingested, absorbed, eliminated by means of urine and retained, the latter magnitude being the difference between the previous two. In last place are shown the efficiency coefficients, $Ca_{retained}/Ca_{ingested} \times 100$.

Lot	Diets	Ca (mg/day) ingested (a)	absorbed (b)	urinary (c)	retained (b-c)	efficiency 100 d/a
1	control (starch)	51.0	12.9	0.2	12.7	25
2	lyxose, 3.5%	43.0	23.4	0.6	22.8	53
3	mannose, 10%	47.4	29.1	1.6	27.5	58
4	fructose, 10%	48.1	13.4	0.3	13.1	27.2
5	lactose, 7.6%	48.0	22.8	0.7	22.1	46
6	melibiose, 7.6%	46.2	23.6	1.0	22.6	49
7	raffinose, 11.2%	46.8	23.7	1.2	22.5	48

TABLE I. Influence of various glucides on calcium utilization.

FIRST EXPERIMENT (GLUCIDES). These results are shown in table I.

Two of the glucides tested starch and fructose, show little efficacy with respect to utilization of calcium. On the other hand all the other glucides show effects so pronounced that their ingestion effectively doubles the calcium utilization. Lyxose and mannose from lots 2 and 3 are particularly active. The three galactosides, lactose, melibiose and raffinose also are effective and to the same extent (lots 5,6 and 7).

Whereas starch or fructose ingestion from lots 1 and 4 is accompanied only by a slight urinary calcium excretion, administration of the other five active glucides cause increases, sometimes appreciable ones, in this excretion. Thus for animals receiving mannose, the magnitude of urinary calcium excretion is eight times higher than among the controls. However this excretion is too low to appreciably affect calcium retention.

2nd EXPERIMENT (HEXAHYDRIC ALCOHOLS).

Table II shows the influence of the hexahydric alcohols on calcium exchanges and that of corresponding glucides. The results obtained by means of hexahydric alcohols are listed next to those from glucides of the same chemical configuration (lots 3 and 4,5 and 6,7,8).

Lot	Diets	Calcium (mg/day) ingested (a)	absorbed (b)	urinary (c)	retained (b-c)	efficiency 100 d/a
1	control (starch)	48.2	8.9	0.2	8.7	18
2	lactose, 12%	44.2	15.7	1.0	14.7	33
3	sorbitol, 6%	46.2	16.8	1.1	15.7	34
4	glucose, 6%	45.4	9.1	0.4	8.7	19.2
5	mannitol, 6%	43.2	16.7	1.2	15.5	35
6	mannose, 6%	45.8	16.7	1.6	15.1	33
7	dulcitol, 6%	48.3	17.1	1.0	16.1	33.3
8	galactose, 6%	45.0	9.3	0.3	9.0	20

TABLE II: Influence of various hexahydric alcohols and corresponding glucides on the calcium exchanges in young rats.

Sorbitol, mannitol and dulcitol, introduced in the same proportions in the animals' diets, improve calcium utilization in similar fashion. Their effects are appreciably the same as for lactose incorporated at twice the dosage (12% of lactose in lieu of 6% of hexahydric alcohol). In this experiment the remarkable mannose effect already previously observed is noted again.

Of the eight substances tested, only starch, glucose and -- in the minor proportion used by us -- galactose, evidence mediocre activity with respect to calcium utilization.

It should be noted that ingesting substances active with respect to calcium retention -- lactose, sorbitol, mannitol, dulcitol -- strongly increases urinary calcium excretion.

3rd EXPERIMENT: ORGANIC ACIDS

Table III lists the results obtained from experiments with gluconic and glycolic acids (lots 3 and 4). The results should be compared with the control rats feeding on a starch diet (lot 1) and those fed a structural glucide, lactose, lot 2.

Lot	Diets	Calcium (mg/day) ingested (a)	absorbed (b)	urinary (c)	retained (b-c)	efficiency (100 d/a)
1	controls (starch)	41.0	11.0	0.2	10.8	26
2	lactose, 10%	38.0	18.7	0.6	17.1	45
3	gluconic acid, 5.25%	41.0	18.8	0.8	18.0	44
4	glycolic acid, 1.5%	39.1	17.9	0.60	17.3	44

TABLE II: Influence of gluconic and glycolic acids on calcium utilization.

As in the other two experiments, we notice that starch, an energy glucide, is little active with respect calcium utilization. But lactose is very effective. The influence of gluconic and glycolic acids on absorption, retention, utilization and elimination in the urine are appreciably the same as for lactose.

DISCUSSION

From the whole of the results obtained, it is noted that all the compounds tested with respect to calcium utilization were effective. This increased calcium retention signifies that the animal being administered those active compounds will immediately develop a more intense ossification. The exact relationships between calcium utilization and skeletal building will be soon discussed by one of us (Y. DUPUIS). Further, in view of the fact that during nursing there is no demineralization of the female rat for practical purposes would lead us to assume that conversely, during growth, calcium fixation will produce bone structuring; FOURNIER & SUSBIELLE, 1953; FOURNIER, 1954 a.

We shall discuss separately the results from each of the three experiments, showing the relation of facts and hypotheses that led us to understand first the mode of operation of structural glucides on ossification, then to infer from this disparate set of structural substances the simple and natural compound by means of which all these substances are likely to evidence their common activity.

1. RELATIONSHIP BETWEEN STRUCTURAL GLUCIDES AND OSSIFICATION.

Of the five new glucides tested in the first experiment, four were shown very active with respect to calcium utilization. Only one, fructose, practically is without effect on the magnitude of calcium exchanges with respect to starch and therefore must be classified as an energy glucide. The poor osteogenetic capability of fructose caused no surprise since we had already observed that saccharose is

no more active than glucose and starch (FOURNIER ETAL, 1955; FOURNIER, 1954 c).

Thus four more glucides will be added to the group of numerous structural ones. We are therefore tempted to classify as a structural glucide any that is not considered being an energy glucide. We shall examine separately the cases for lyxose, mannose, and for the two galactosides, melibiose and raffinose.

a) LYXOSE. The remarkable lyxose activity with respect to ossification must be compared to that of four other pentoses previously studied. All the pentoses studied so far therefore are of the active kind, but this is not the case for the hexoses, of which certain are the energy kind, while others are structural. Further, pentose activity seems independent of optical properties, since for arabinose as well as for xylose, the two inverse optics L and D were shown active.

b) MANNOSE. This hexose must be included among the glucides of the structural kind which, improving calcium utilization in rats, favors ossification. This property certainly must be compared with the fact that in the natural state, mannose in found virtually only in the mannanes, that is, in its structural function. We were led to establish a connection of a similar kind between the animal and vegetable kingdoms with respect to the osteogenetic actions from galactose, xylose and arabinose which are widespread in the vegetal membranes in their polymerized galactane form, also of xylane and arabane forms.

May one state from the osteogenetic action of mannose that the utilization mode of this glucide in no way is connected with those of the energy glucides? Let it be observed that mannose like glucose and fructose may be fermented by means of beer yeast.

The few researches undertaken with respect to the biological mannose properties compare these to glucose's. At first sight, the results of those researches are very discordant. COOK & LORBER (1952) injected mannose or glucose with radioactive first carbon into fasting young rats, and found that those two "oses" are of similar glycogenoforming efficacy, so that these authors concluded there was direct conversion from mannose into glucose in the organism, without breaking

the hexosic chain. BAILEY & ROE (1944) showed that when mannose is parenterally administered to the rabbit, there is increase of glycogen in the liver but that on the other hand mannose ingestion is little effective. The latter fact confirms the observations of DEUEL ETAL (1938) who, working with rats, observed that ingested mannose only to a very slight degree has the capacity of forming hepatic glycogen.

Ingested mannose does not seem to follow the glucose metabolic path since in contrast to the latter, it is not glycogen-forming but rather it is very active with respect to ossification; glucose is not.

Mannose's capacity to supply glycogen depending on parenteral or digestive administration allows the assumption that the liver plays an essential role in the utilization of mannose in a manner favorable to ossification.

c) GALACTOSIDES

Three galactosides in this experiment, namely lactose, melibiose and raffinose were administered in such doses to the animals that there always was the same proportion of galactose. Similar activities were obtained.

Differences with respect to the respective galactose and lactose activities, -- in particular the fact the animals only made use of 50% of the galactose received -- had led us to report the very superior effectiveness of lactose in the presence of beta molecular galactose form (P. FOURNIER ETAL, 1955; FOURNIER, 1954 a). Now, galactose in melibiose and raffinose is in alpha form and those glucides are just as active as lactose. To the contrary of what we had thought previously, the galactoside efficacy is thus independent of the isomeric galactose form they contain.

How is one to understand that these galactosides all are much more active than equivalent quantities of galactose, unless one assumes that they are being used without therefor their constituting galactose being freed?

Such interpretation, which actually is the object of special work, may be based on 2 observations of different orders: on one hand, the search for digestive enzymes capable of hydrolyzing the galactosides having proved negative, ANAGNOSTOPOULOS & PETEK (1953) raised the question how and at which level those glucides might be absorbed; on the other hand, RUTTER ETAL (1953) observed that lactose administered to the chick per os results in lactose but not galactose being present

in the animals' blood. For galactose ingestion, this glucide and also lactose is found in blood.

d) Hypothesis with respect to structural glucide action. Considering these ten structural glucides, namely five pentoses, two hexoses, two diholosides and one triholoside, let us attempt comprehending how they may manifest their common action toward ossification.

Our first care was to write the formulas that were developed and to compare them to each other. They are such that no matter how manipulated, there is no coincidence.

In the face of this architectural heterogeneity, we must admit that the mechanism exerted by all these compounds with respect to the ossification process must be a simple one. Otherwise one would have to assume transpositions, radical inversions of different and complex kinds according to the case involved.

Examination of the glucide configuration revealing very little, we considered bone composition, more particularly that of osseine, because, so far as we were concerned, bone condition above all depends on the synthesis of this protide; see FOURNIER ETAL,1953; FOURNIER, 1954 a

Osseine contains an enormous proportion of glycocoll, about 25%, with respect to the other body protides, seral or muscular.

Could the structural glucides be the glycocoll forerunners?

Immediately there are arguments in favor of such a hypothesis, and they may be divided into four classes:

a) glycocoll is the simplest aminated acid, and this is a noteworthy fact if one assumes that the osteogenetic power of the seemingly so disparate group of structural glucides may only be explained by a simple mechanism.

b) of all the aminated acids, glycocoll is the only one lacking rotational power. Now for the two cases where we researched the influence of the two D and L forms from the same glucide on the calcium utilization, those forms were shown active. Thus the stereochemical properties of the glucides, which are decisive from the point of view of energy utilization , seem insignificant when their plastic qualities are considered.

c) Since the structural glucides are fare more significant than the energy glucides with respect to ossification activity, then it must be that the utilization process of the two glucide groups is different.

Thus we must seek from the start a metabolism other than that which makes two tri-oses from glucose. The osteogenetic activity of the five pentoses we researched allows us to assume that all structural glucides free one common molecule with two carbon atoms, a molecule that would generate the glycocoll, which is the only dicarbonated amino acid.

d) Despite the considerable research into the origins of glycocoll, the matter remains obscure. It is known however that the animal intoxicated by benzoic acid may synthetize appreciable quantities, but it is not clear from which compounds glycocoll is derived. We shall show below how we were led to consider that the precise origin of the aminated acid of the simplest form could not be found because it must be capable of forming in the course of the utilization of the most common metabolites. IN this respect one should observe that animals not receiving structural glucides, that is, the control rats of each experiment for instance whose diet contains a very large proportion of starch, do fix a significant quantity of calcium of their rations. They do structure their skeleton but less easily than those animals in whose diet there is a slight proportion of structural glucides.

2. OSTEOGENETIC POWER OF THE HEXAHYDRIC ALCOHOLS DERIVED FROM THE "OSES".

Sorbitol, mannitol and dulcitol all are active with respect to ossification.

In view of the lack of bibliographical data conerning the physiological aptitudes of dulcitol, we shall restrict ourselves to observing that this compound has an osteogenetic power equivalent to that of the two other hexahydric alcohols studied, but that it is much more pronounced than galactose's, a hexose which chemically corresponds to it. We have on earlier occasion reported the peculiarity of galactose action, since it must be administered in strong doses before its effect on calcium utilization will be revealed (FOURNIER, 1954 b).

Which has been so far the physiological significance of mannitol and sorbitol? All experimenters liken the activities of those compounds to that of glucose. This comparison occurs so as to be particularly favorable to glucose, particularly as concerns the power of glycogen-

formation. Under such conditions, mannitol is incapable of providing hepatic glycogen, whether in the monkey rhesus (ELLIS & KRANTZ, 1941) or in the rat (TODD ETAL, 1939). When under the influence of mannitol, glycogen will deposit in the liver only is mannitol administration is accompanied by that of cacao butter (TODD ETAL, 1939).

There has been more discussion with respect to glycogen generation. The same authors considering mannitol to be completely ineffective state that sorbitol is glycogen-forming. One must however note the unexplained differences regarding the experiments performed with the rhesus monkey, where one crystallized sorbitol solution was shown to be active while a sorbitol syrup was not (ELLIS & KRNATZ,1941). For rat having fasted for 40 hours, glycogen will deposit in the liver when sorbitol is administered by stomach tube or by ip. injection. But the effect from this hexahydric alcohol is much more pronounced ip. than per os. Further, simultaneous administration of cacao butter much increases the sorbitol glycogen-forming power (TODD ETAL, 1939).

On the other hand, other workers show that sorbitol lacks the physiological properties of glucose. ROCHE & RAYBAUD (1933) observed no hepatic glycogen formation following sorbitol administration to a fasting guinea-pig, nor any respiratory quotient increase in man, nor any improvement in the condition of the insulinized rabbit. BLATHERWICK ETAL (1940) considered as being non-detectable any capacity of sorbitol to create glycogen after a fasting time of 24 hours, though after a longer fasting time, there is tendency to increase.

In summary, it seems that under normal physiological conditions, sorbitol and mannitol do not have the physiological properties of glucose. Only in exceptional circumstances will sorbitol display such: either when administered parenterally, so that the direct and definitely decisive action of the liver is excluded, or when under the influence of Phlorhizine; see ROCHE & RAYBAUD, 1933; or after a prolonged fast, see BLATHERWICK, 1941, the effect being a considerable drop in an organism's lipidic reserves and its dipping into its proper lipidic reserves. The latter condition allows a better understanding of the glycogen-forming power of mannitol and sorbitol that are administered with certain lipids.

Because of their slight capacity for replacing glucose, the efficacy of mannitol and sorbitol with respect to ossification, which is quite superior to glucose's, deserves being stressed.

We previously noted that mannose displays osteogenetic power. The similar mannitol activity, when mannitol and sorbitol are chemically close, is no surprise. But the same order of chemical kinship between sorbitol and glucose does not cause the same biological common efficacy because, with respect to clacium utilization, sorbitol is closer to mannitol and mannose than to glucose.

Thus the structural properties of a large group of glucides extend to some of their non-glucide derivatives and even to derivatives of the energy glucides. This observation entails two important consequences regarding the evolution of our experimentation:

a) since the hexahydric alcohols derived from the "oses" are active, it became probable that the acids from the oxidation of the aldehyde functions of the oses are equally effective with respect to ossification. These acids form the group of gluconic acids of which the model is gluconic acid;

b) utilization of those structural compounds which increase incessantly as we keep on experimenting, must correspond to a very general metabolism. Considering the metabolism of the energy glucides, it was reasonable that we led our research in the direction of the action of those organic acids on calcium utilization.

Because being very widespread in certain vegetal membrane constituants, we had wished first to research the activities of galacturonic or glycuronic acids with respect to ossification. In view of our conceptions of structural metabolism of the bone tissue, the latter acid held particular interest. It is a normal constituent of the organism that seems to arise from glucose by means of an oxidation process that is different from the classical tricarboxylic cycle. It may well be that glycuronic acid is metabolically related to glycocoll, these two compounds concurrently being capable of combining with benzoic acid in the intoxicated animal.

Because it was impossible to secure those uronic acids, we were forced to skip this experimental phase and to search at once for the common compound, forerunner of glycocoll, that could cause all these structural compounds.

First, from which end of the glucidic chain may the release of the dicarbonated molecule be effected? Very likely from the end of the molecule primary alcohol, since modifications in the aldhyde end cause no changes in activity; also, the two L and D forms of the same glucide are active.

Next we had to conceive the nature of this dicarbonated molecule. We shall state why we thought this molecule had to be glycolic acid.

3. GLUCONIC AND GLYCOLIC ACID EFFECTS ON CALCIUM UTILIZATION.

The reasons for the experiments with those two acids are so different that the discussion of the results obtained from those acids will take place separately.

a) GLUCONIC ACID. The remarkable activity of gluconic acid with respect to ossification only confirms the known properties. It is known that calcium gluconate is classically one of the "remineralizers" most commonly used. The sole advance we are contributing is a matter of interpretation. The definite osteogenetic properties of calcium gluconate are much less due to its calcium part than to its gluconic share. As other structural compounds, gluconic acid very likely intervenes in the synthesis of the protein web of the bone,a synthesis that conditions the fixation of mineral matter.

b) GLYCOLIC ACID. It may seem paradoxical that this aminated acid should not have been immediately examined with respect to calcium utilization when the plastic role of a large number of compounds was performed by the intermediary of glycocoll. Recently we verified that ingested glycocoll is without osteogenetic properties, which is hardly surprising since the liver transforms glycocoll into glycogen.

In order to validate our hypothesis, we had to determine which compound could simultaneously arise from the structural glucides and from their derivatives and cause glycocoll.

F. Chao etal (1953) confirmed that glycolic acid $CH_2OH-COOH$ is a fore-runner of glycocoll, whereas the acetic and lactic acids do not cause glycocoll and pyruvic acid only indirectly. The same workers report certain hypotheses according to which glycolic acid forms in the course of pentose utilization, -- an origin that agrees well with our own conceptions of the metabolic actions of the structural glucides.

We chose the ratio of glycolic acid in our experiment in conformity with our hypothesis of structural glucides being the fore-runners of glycocoll and intervening in the bone metabolism by the intermediary of a dicarbonated molecule. Thus a diet containing 1.5% of glycolic acid should produce appreciably the same physiological effect as the diet containing 10% of lactose. Experiment has shown that under such conditions, glycolic acid and lactose improve to the same degree the calcium utilization.

These results seem to confirm indeed that glycolic acid is the fore-runner of glycocoll and that the structural compounds cause glycolic acid. The latter point is significant if one considers that if glycolic acid is assumed being the precursor of glycocoll, little is gained unless one may state the origin of the glycolic acid.

It is important to stress the importance of glycocoll origin, an aminated acid which has been revealed as being a genuine rotating plate with respect to metabolism by the use of radioactive isotopes. Rats not requiring glycocoll specifically, indicates this aminated acid occurs in the course of energy-glucide utilization. When there is weight equilibrium in the animal, the amount of bone formation as measured from calcium utilization indeed is proportional to the ingested quantity of an essentially amylaceous diet (RANDOIN ETAL, 1955). Opposing such a conception is the fact of acetic and lactic yielding no glycocoll and pyruvic acid only indirectly (CHAO ETAL, 1953).

Such facts are only contradictory if the glucides are utilized only in one manner, namely energetically. This classical notion is not wholly satisfactory because certain compounds cannot be fitted into the energy cycle, such as glycuronic acid, even though close to glucose. But especially we were led to individualize a large number of glucides and derivatives according to their plastic properties, this group

being so complete already that energy metabolism seems to us a fundamental utilization mode though rather peculiar. For a given Glucide we must assume there are two ways of utilization, one energetically that makes two trioses from one hexose, and the other structurally by-passing that phase and which more or less at once supplies a two-carbon molecule. It will be observed in favor of the theory of such bi-metabolism that the structural glucide effect with respect ossification seems to of the same kind as for that of energy glucides, though much more intense.

The favorable effect from glycolic acid on ossification allows thinking that this acid, fore-runner of glycocoll, is this dicarbonated molecule. Indubitably there is large physiological significance in a body of which the existence in animal tissues (BERNARD & GREBUS,1951) and in the excreta (NORDMANN ETAL, 1954) so far remained an enigma. It was learned recently that glycolic acid is the simplest compound appearing during the first moments of photo-synthesis and its unexpected presence has remained unexplained in the sequence of the energy-glucides gensis (SHOU ETAL,1950). Might it be the first term of a metabolic structure series?

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Quantitative Determination of Sugar Alcohols by the Komarowsky Reaction

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SUMMARY

When heated in a reagent consisting of a cyclic aldehyde, thiourea, and concentrated sulfuric acid, the sugar alcohols, including glycerol and inositol, give orange, pink, or red colors whose intensities follow Beer's law. The reagent is stable for 6 weeks or more in an amber bottle under refrigeration. Of the aldehydes tested, *p*-hydroxybenzaldehyde and *p*-dimethylaminobenzaldehyde were the most sensitive. Generally, the reactivity of the sugar alcohols was as follows: sorbitol > dulcitol > mannitol > erythritol > xylitol > arabitol > ribitol > glycerol > inositol. Carbohydrates and other interferences were removed by acid hydrolysis followed by degradation with hot alkali and treatment with ion-exchange resins. Hydrolysis, degradation, and removal of degradation products can also be done in a single operation with a mixture of ion-exchange resins. Important factors for reproducible quantitative results are the concentration of acid, thiourea, and aldehyde in the reagent, the time and temperature of heating, and the particular aldehyde used. Recovery from complex milieu was good (95–101.8%).

INTRODUCTION

Usually, the sugar alcohols are determined by subjecting them to oxidation with periodate in alkaline solution to produce formaldehyde, which is then measured by reaction with chromotropic acid to give a red or wine color with an absorption maximum at 570 mμ (Snell *et al.*, 1961). Because of their increasing importance in the food and pharmaceutical industries (Merck and Co., 1960; Genest and Chapman, 1962) and their physiological and metabolic roles in animals and higher and lower plants (Carr and Krantz, 1945; Hajny 1964a,b; Anderson *et al.*, 1961, 1962; Touster and Shaw, 1962), simple, rapid, and easily executed tests for this class of compounds are needed. It was recently shown that the anthrone reagent could be used for the colorimetric determination of sugar alcohols (Graham, 1963). However, both this and the chromotropic acid reagent are rather unstable in sulfuric acid and must be prepared fresh each day.

The Komarowsky reaction has been used for the determination of aliphatic alcohols (Coles and Tournay, 1942). Although it is thought that the reaction is not given by polyalcohols, 2-ethyl-1,3-hexanediol was determined by the use of *p*-dimethylaminobenzaldehyde in strong sulfuric acid (Bowman *et al.*, 1959), and Komarowsky-type spray reagent has been used for the detection of polyols (Godin, 1954). Preliminary investigations showed that, with proper control of reaction time, reaction temperature, and the concentrations of sulfuric acid and of the aldehyde used, the sugar alcohols can be determined quantitatively by this reaction. The addition of 2.5–5% thiourea to the acid-aldehyde mixture provides a reagent which is stable for as long as 6 weeks if kept in an amber bottle and refrigerated.

Carbohydrates constitute the most common interference in the determination of sugar alcohols. Adcock (1954) removed interferences through degradation in sodium carbonate and treatment with ion-exchange resins. White (1958) suggested preliminary hydrolysis of nonreducing carbohydrates such as sucrose to facilitate their degradation and removal. Anderson *et al.* (1961) proposed a similar method using ion-exchange resins only. In the procedure presented here, carbohydrates were removed by preliminary heating in hydrochloric acid followed by treatment with hot dilute sodium hydroxide and, finally, filtration over a bed of ion-exchange resins.

EXPERIMENTAL

Materials. The sugar alcohols (Nutritional Biochemicals Corp., Cleveland, Ohio) and aldehydes (Eastman Chemicals, Rochester, N.Y.) and the concentrations used are shown in Table 2. Sulfuric acid was reagent grade, 95–98%, sp. gr.

1.8407–1.8437. Thiourea (Baker Chemical Co.), was reagent grade, m.p. 171.5–172.5°C. The concentrations used are shown in Table 1. Ligroin, b.p. 66–75°C, highest purity (Fisher Scientific Co.), was used.

Aldehyde-thiourea-sulfuric acid reagents. Thiourea was placed in a 100-ml volumetric flask, and 80 ml of concentrated sulfuric acid was added. After the thiourea was dissolved (with heating, if necessary), the desired amount of the particular aldehyde was added and the mixture was heated for 10 min at 80°C. After cooling to room temperature (29±1°C), the volume was made up to 100 ml with concentrated sulfuric acid and the mixture transferred to an amber bottle and stored in the refrigerator when not being used.

Equipment. The equipment used was a Beckman DU spectrophotometer, borosilicate ground-glass-stoppered test tubes, 250-ml ground-glass-stoppered volumetric flasks, and ion-exchange columns. The columns were made from glass tubings, 15-mm inner diameter and 60 cm long. They were packed to a height of 10 cm with a 1:1 mixture of AG50 W-X8 resin (H form, 100–200-mesh) and AG1 X8 resin (OH form, 100–200-mesh) obtained from Bio-Rad laboratories, Richmond, California. The resin was held in place by a plug of glass wool.

General procedure. One ml containing 25–1000μM of the sugar alcohol in water was placed in borosilicate ground-glass-stoppered test tubes. The water was removed by drying for 8 hr at 105°C. After cooling, 10 ml of the reagent was added to each test tube and the tubes were heated for 60 min in a water bath (99±1°C). The tubes were cooled for 5 min in an ice-water bath and allowed to stand at room temperature (29±1°C) for 30 min, after which the color developed was measured against a reagent blank at the wave length of maximum absorption.

The wave length of maximum absorption for each sugar alcohol-reagent mixture was determined by treating the mixtures as described above. The absorbance of the color developed was measured over the wave-length range of 380–700 mμ against distilled water. A plot of absorbance as a function of the wave length established the wave length of maximum absorption. The results for *p*-hydroxybenzaldehyde are shown in Fig. 1.

Factors influencing the reaction. Preliminary investigations showed that the time and temperature of heating, the concentration of acid, and

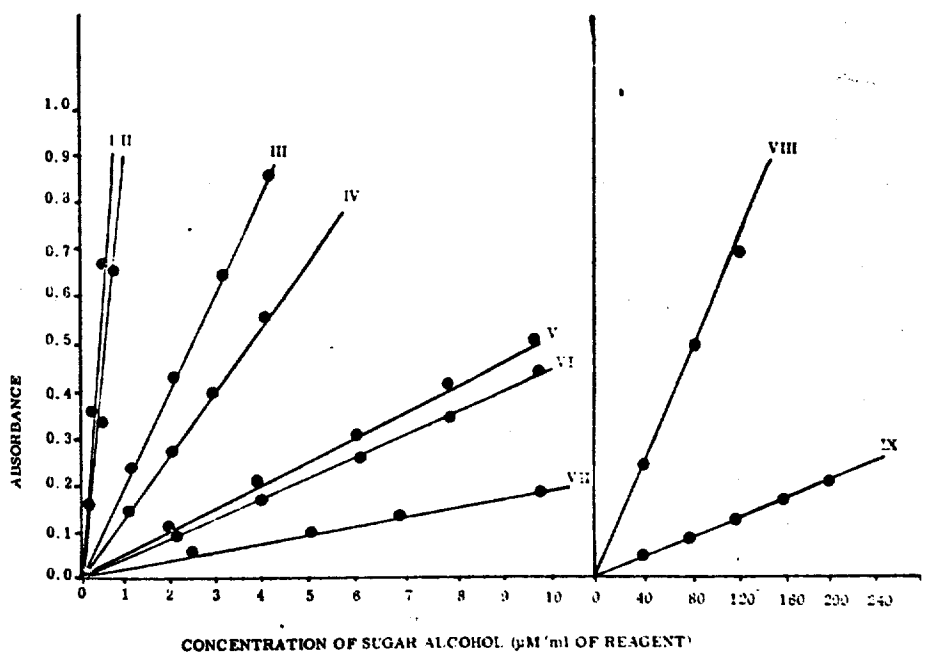

Fig. 1. Absorption spectrum of sugar alcohols in *p*-hydroxybenzaldehyde-H_2SO_4-thiourea reagent.

I = Dulcitol	IV = Ribitol	VII = Xylitol
II = Mannitol	V = Glycerol	VIII = Inositol
III = Sorbitol	VI = Erythritol	IX = Reagent blank

other factors were critical for maximum color development. Therefore, these and other variables were investigated under the conditions described in the general procedure. The results are shown in Table 1.

Comparative sensitivity of the various aldehyde reagents. To assess the sensitivity of the various aldehydes used, a fixed amount of each sugar alcohol was treated with each of the aldehyde reagents according to the general procedure. The results are shown in Table 2.

Quantitative response of the sugar alcohols. After the limits of the variables were established, the quantitative response of the various sugar alcohols was tested. For this, increasing quantities of each sugar alcohol were treated according to the general procedure, and the colors developed were measured at the wave length of maximum absorption against the appropriate reagent blank. The results, using *p*-hydroxybenzaldehyde, are shown in Fig. 2.

Removal of carbohydrates. Since sugars will interfere, these were removed by a modified version of the procedure outlined by Anderson *et al.* (1961). An aqueous mixture of 10 ml (500μM) of sorbitol and 10 ml (500μM) of sucrose was placed in a 250-ml Erlenmeyer flask, and 20 ml of 1N hydrochloric acid was added. The mixture was heated for 30 min in a boiling-water bath. Then 2 ml of 10N NaOH was added and heating was continued for another 20 min. Three of the cationic resin and 3 g of the anionic resin were added and the mixture was shaken and allowed to stand for 10 min at room temperature (29 ± 1°C) and finally filtered over the ion exchange column. The column was washed with 200 ml of double-distilled water, the filtrate was collected in a 250-ml volumetric flask and made up to volume with double-distilled water, and one ml was used for assay according to the general procedure. Mixtures contained only sorbitol and only sucrose were treated similarly. The unmodified method of Anderson *et al.* (1961) was also tried for the removal of interferences.

Recovery of sorbitol from complex mixtures. Sorbitol (500μM) in 5 ml of distilled water was added to separate 100-ml glass-stoppered centrifuge bottles containing the materials shown in Table 3. The mixtures were heated for 30 min at 100°C and cooled, and 50 ml of ligroin was added. The mixtures were shaken for 30 min and centrifuged for 10 min at 2500 rpm and the ether layer was removed by careful siphoning. The ether extraction procedure was repeated, and the combined ether layers were washed twice with 5-ml portions of distilled water. The aqueous layers were removed and added to the residues. The residues were heated over a steam bath to remove residual ether, and after cooling, 50 ml of absolute methanol was added. The mixture was heated for 15 min on a steam bath, with a small funnel placed in the mouth of the bottle to minimize evaporation, and filtered while still hot into a 250-ml volumetric flask. The centrifuge bottles were washed twice with 10-ml portions of hot 90% methanol which was poured over the residue on the filter paper. The filtrate was evaporated almost to dryness on a steam bath.

Table 1. Influence of variables on color development in the interaction of sugar alcohols in the Komarowsky reaction.

Variable	Range investigated	Limits for reproducibility		Value or condition selected	
		Max	Min	Rgt A[a]	Rgt B[b]
Temperature of heating (°C)	60-100	100	95	100	100
Time of heating at 100°C (min)	15-120	60	60	60	60
Normality of H_2SO_4 in reagent	18-36	36	36	36	36
Amount of aldehyde in reagent (mg/100 ml of reagent)	25-400	200	200	200	250
Amount of thiourea in the reagent (% w/v)	0-10	5.0	2.5	5.0	2.5
Stability of color developed (hr)	0-240	4.0	0.5	0.5	0.5
Stability of reagent itself (days)	0-70+	56	42	42	42
Diluent for color developed	Water and several organic solvents tried			Concentrated H_2SO_4	

[a] Reagent A, *p*-Dimethylaminobenzaldehyde + thiourea in conc. H_2SO_4.
[b] Reagent B, *p*-Hydroxybenzaldehyde + thiourea in conc. H_2SO_4.
For aldehyde [illegible], 6, 7, 8, and 9 (legend in Table 2), 2.5% thiourea was used and a level of 1% for aldehyde No. 4.

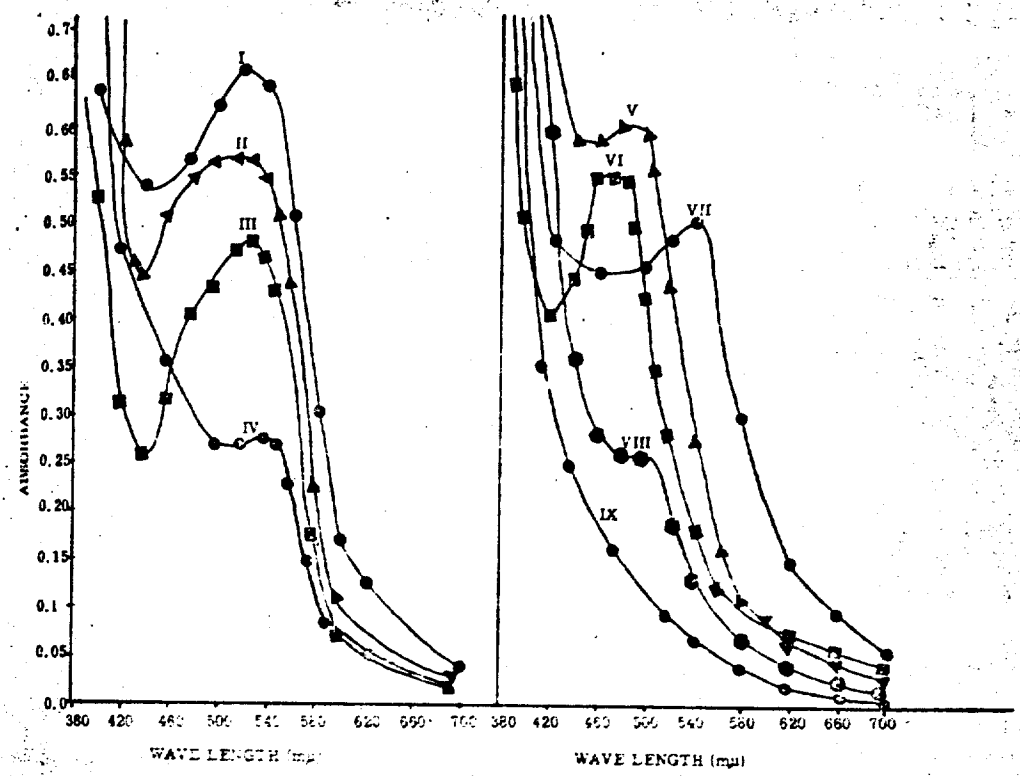

Fig. 2. Quantitative response of the sugar alcohols when treated with the *p*-hydroxybenzaldehyde-H_2SO_4-thiourea reagent.

I = Sorbitol	IV = Erythritol	VII = Ribitol
II = Dulcitol	V = Xylitol	VIII = Glycerol
III = Mannitol	VI = Arabitol	IX = Inositol

the residue was treated for the removal of interferences as outlined previously, and then 1-ml portions of the filtrate were assayed for sorbitol according to the general procedure. The results are shown in Table 3. The above procedure served well for materials which are low in proteins.

Deproteinization of milk and high-protein foods. Milk and other materials high in protein were deproteinized by adjusting the pH of the medium to 3.5 with glacial acetic acid. The pH was followed with a Beckman Zeromatic pH meter. Any precipitate formed was concentrated by centrifugation for 15 min at 1500 rpm and the supernatant was poured over Whatman No. 2 filter paper. The filtrate was collected, made up to 50 ml with distilled water, and 10 ml of freshly prepared 50% (w/v) trichloroacetic acid was added. The mixture was heated at 70°C for 10–15 min, cooled, filtered and treated for the removal of carbohydrates as described in the general procedure. Then, 1-ml portions of the filtrate were assayed for sorbitol according to the general procedure.

Instead of trichloroacetic acid, 2 g of sodium hexametaphosphate may be added and the mixture allowed to stand for 30 min at room temperature (29 ± 1°C). The remainder of the procedure remains the same.

Reproducibility. Ten separate determinations were done on solutions of sorbitol made up in distilled water, and 5 determinations on sorbitol recovered from complex milieu. The standard deviations were calculated from the data obtained.

RESULTS AND DISCUSSION

Of the aldehydes used, *p*-dimethylaminobenzaldehyde and *p*-hydroxybenzaldehyde were the most sensitive. The latter is preferable since, generally, the colors produced with the sugar alcohols have maximum absorption at higher wave lengths. *p*-Chloro-

Table 2. Relative sensitivity of various aldehydes in the Komarowsky reaction of sugar alcohols.

Amt. of aldehyde used (mg/100 ml)	Sugar alcohol (μM/ml of reagent).[a] Sorbitol 0.5	Dulcitol 0.5	Mannitol 1.0	Erythritol 1.0	D-arabitol 10	Xylitol 10	Glycerol 40	Inositol 40
200	0.66 (450)	0.55 (450)	0.72 (450)	0.52 (480)	0.60 (490)	0.58 (540)	0.14 (490)	0.22 (490)
250	0.59 (520)	0.56 (520)	0.86 (520)	0.56 (460)	0.45 (540)	0.50 (540)	0.24 (490)	0.05 (490)
200	0.56 (530)	0.47 (530)	0.57 (530)	0.13 (540)	0.10 (540)	0.31 (540)	0.14 (540)	0.17 (540)
50	0.24 (510)	0.24 (510)	0.29 (500)	0.215 (510)	0.29 (500)	0.48 (500)	0.45 (510)	0.07 (510)
(ml/100 ml) of reagent[1]								
0.01	0.03 (480)	0.05 (480)	0.12 (480)	0.52 (460)	0.01 (460)	0.38 (460)	0.00 (470)	0.06 (480)
0.05	0.36 (480)	0.25 (480)	0.65 (480)	0.33 (460)	0.06 (460)	0.09 (460)	0.00 (480)	0.20 (480)
0.01	0.00 (480)	0.00 (480)	0.00 (480)	0.30 (460)	0.00 (460)	0.0 (460)	0.00 (480)	0.16 (480)
0.02	0.09 (480)	0.06 (480)	0.18 (480)	0.40 (460)	0.26 (460)	0.05 (460)	0.00 (480)	0.00 (480)
0.01	0.05 (480)	0.05 (480)	0.12 (480)	0.78 (460)	0.03 (460)	0.40 (460)	0.00 (480)	0.14 (480)

[a] Numbers in parentheses represent wave length of maximum absorption of the sugar alcohol-aldehyde reagent mixture.
[b] Aldehydes: 1. *p*-Dimethylaminobenzaldehyde; 2. *p*-Hydroxybenzaldehyde; 3. *p*-Chlorobenzaldehyde; 4. Vanillin; 5. Furfural; 6. Benzaldehyde; 7. Cinnamaldehyde; 8. Salicylaldehyde; 9. *p*-Anisaldehyde.

benzaldehyde and vanillin gave colors with absorption maxima at 500–510 mμ, but the sensitivity was always much lower than with *p*-dimethylaminobenzaldehyde or *p*-hydroxybenzaldehyde. The intensities were always much less with the liquid aldehydes than with the solid ones. Since the solid aldehydes can be more readily purified (by recrystallization) and weighed out than the liquid aldehydes, these are to be preferred, with *p*-hydroxybenzaldehyde and *p*-dimethylaminobenzaldehyde, respectively, the first and second choices.

The reactivity of the sugar alcohols can be listed as sorbitol > dulcitol > mannitol > erythritol > xylitol > arabitol > ribitol > glycerol > inositol.

Since color development decreased considerably as the acid concentration decreased, concentrated sulfuric acid was used throughout the experiment. Inadequate drying of the sample will lead to low results, and alcohol and other solvents must be absent.

A heating time of 1 hr or more is needed for maximum color development. Although heating for 90 min gave slightly higher absorbance, the color was somewhat less stable than when heating was done for 60 min at 100 C, the selected condition.

For consistent results, the temperature must be maintained at 99 ± 1 C.

If the concentration of the aldehyde is too low, maximum color development will not occur, while if it is too high the reagent will be too highly colored, and, as a consequence, high reagent blanks will be obtained.

As the concentration of thiourea was increased, the stability of the reagent also increased. A concentration of 5% (w/v) was chosen since little added stability was achieved beyond this level and higher levels caused too intense yellowing and decreased sensitivity of the reagent. The thiourea serves as an antioxidant to prevent or retard color development in the aldehyde-H_2HO_4 mixture.

Aging of the colors affects the absorbance. Generally, at the shorter heating times (15–30 min) the absorbance increased considerably with aging. Since the minimum change in absorbance with aging occurred after heating for 45–75 min, a heating time of 60

min at 100°C was selected. Under these conditions, no change in absorbance occurred after aging for 60 min at room temperature (29±1°C), and after 6 hr the change was only 0.05 absorbance unit.

Recoveries from complex milieu were good (Table 3). With materials which are low in lipids, for example, some leaf extracts, the preliminary ether extraction can be omitted. The removal of proteins in blood will be more complete if $ZnSO_4$-$Ba(OH)_2$ (Somogyi, 1945) is used. With milk and materials high in protein the acetic acid-trichloroacetic acetic or acetic acid-sodium hexametaphosphate procedures are preferable. Complete deproteinization is essential since tryptophane will give a positive reaction.

The procedure of Anderson *et al.* (1961) for removal of interferences is quite suitable and is somewhat less destructive to the sugar alcohol than the direct acid-alkali treatment. However, the latter procedure is less time consuming. In both cases the recovery of pure sorbitol must be ascertained since, if destruction occurs, erroneous results will be obtained. The completeness of the removal of carbohydrates can be readily checked by heating a 1-ml portion of the filtrate for 5 min at 100°C with the anthrone-thiourea reagent (Graham, 1946). If a bluish-green color appears, destruction is incomplete. In this case, another sample should be treated carefully according to the procedure outlined until a negative anthrone test is obtained. Alternately, 50 ml of the same sample may be treated with 0.5 ml of 2N NaOH and heated for 6–10 min in a boiling-water bath, treated with the mixed resin, filtered over the ion-exchange column, the filtrate collected, made up to volume with distilled water and an aliquot assayed as described under the general procedure. Materials like tryptophane, ascorbic acid, sorbic acid, lactic acid, piperine, etc., are either destroyed or removed during the preliminary treatment to rid the solution of interferences. However, ethylene glycol, propylene glycol, and the polyoxyethylene and polyoxypropylene esters and glycols are not removed. When these are present, they can be separated by chromatographic procedures (Moore *et al.* 1960; Genest and Chapman, 1962) prior to elution and colorimetric determination of the sugar alcohol. The proposed method does not distinguish between individual sugar alcohols and, if the material being examined contains more than one sugar alcohol, chromatographic separation prior to elution and colorimetric determination would also be nec-

Table 3. Recovery of sorbitol from complex mixtures.[a]

Sorbitol added (μM)	Other material present	Method for removal of interferences: Sorbitol recovered (percentage) Method 1[b]	Method 2[c]
0.0	Carrageenan (200 mg)	0.0	0.0
0.0	Starch (200 mg)	0.01	0.00
0.0	Sodium carboxymethylcellulose (200 mg)	0.01	0.01
0.0	Gum acacia (200 mg)	0.00	0.01
0.0	Pectin (200 mg)	0.02	0.0
0.0	Sucrose (500 μM)	0.0	0.1
500		98.0	100.6
0.0	Spinach leaf extract	0.0	0.05
500	Spinach leaf extract	97.5	99.9
0	Milk (3 g)	0.1	0.12
500	Milk (3 g)	101.1	101.8
0	Biscuits (2 g)	0.05	0.00
500	Biscuits (2 g)	95.0	99.2

[a] Average of 3 different determinations.
[b] Method 1, mixture heated in hydrochloric acid followed by hot alkali and ion-exchange treatment.
[c] Method 2, as per Anderson *et al.* (1961).

essary. Possible interference from seaweeds and other products which contain sugar-alcohol moieties (Touster and Shaw, 1962; Lohmar and Goepp, 1949), when these products themselves are not being analyzed, should not be ignored.

The mechanism of the Komarowsky reaction is still not fully known. Dehydration of the compound by strong sulfuric acid to produce olefinic-type intermediates which condense with the aldehyde to give characteristic colors, is thought to occur (Nogare and Mitchell, 1953). With alcohols, a mechanism involving carbonium ion formation and aldol condensation to form colored products has been proposed (Duke, 1947). The following sequence of reactions was postulated:

$$R_2CHOH + H^+ \longrightarrow R_2HC^+ + H_2O \qquad [1]$$

$$R_2HC^+ + RCHO \longrightarrow R-\overset{H}{\underset{+}{C}}-\underset{H}{OCR_2} \qquad [2]$$

$$\left[R-\overset{H}{C}-O-CR_2 \atop \quad \diagdown H \diagup\right] \longrightarrow RCH_2 + R_2$$

$$C=O \qquad [3]$$

$R_2C = O + RCHO \longrightarrow$ colored aldol condensation products. In acid solution, the condensation product resonates, resulting in color formation. The elimination or inhibition of color formation by added water, observed here, indicates that dehydration is involved. As in the reaction with the anthrone reagent (Graham, 1963), chain length seems to be an important factor in color production since the hexitols (sorbitol, dulcitol, and mannitol) were the most reactive, followed by the pentitols (xylitol, arabitol, and ribitol). The intermediate reactivity of erythritol indicates that configuration may also be important. Since glycerol was rather inactive, this probably reflects the opinion of Duke (1947) that, with primary alcohols, the equilibrium concentration of primary carbonium ion is extremely small, and since the equilibrium concentration of secondary carbonium ions is relatively higher than that of primary carbonium ions, oxidation of secondary alcohols goes on more readily than that of primary alcohols. Additionally, aldol condensation products involving ketones (from secondary alcohols) are more stable to polymerization than are the aldehyde products (from primary alcohols). This supports the finding that, generally, the greater the number of secondary alcohol groups possessed by the sugar alcohol, the greater the reactivity, as with the hexitols. The refractory nature of the cyclohexane ring probably accounts for the rather low activity of inositol.

The procedure has the distinct advantage of employing highly stable reagents, and it allows for much flexibility since one of several aldehydes may be used. Although the sugar alcohols can be determined by the anthrone reagent (Graham, 1963) and the chromotropic acid method (Snell *et al.*, 1961), both of these reagents are highly unstable in concentrated sulfuric acid. This imposes a severe limitation on routine analyses where many samples must be handled.

The method has good precision and reproducibility. For *p*-hydroxybenzaldehyde, the aldehyde of choice, the standard deviation was found to be 0.98% for pure sorbitol and 1.06–1.26% for the assay of sorbitol added to complex mixtures.

A linear response was obtained when the absorbance of the color produced was plotted against the concentration of the sugar alcohol present. For *p*-hydroxybenzaldehyde sorbitol, this relationship can be expressed by the equation: $X = 0.8203\ Y - 0.0034$

where X = concentration (μM) of sorbitol per ml of reagent

Y = absorbance at 540 mμ

$$\text{Percent sugar alcohol in sample} = \frac{X\ (\text{mol. weight} \times 10^{-3})\ (\text{dilution factor})}{\text{sample weight (mg)}} \times 100$$

Similar equations can be derived for any other sugar alcohol-aldehyde mixture.

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Some differences between duodenal and ileal sorption[1]

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HINDLE, WILLIAM, AND CHARLES F. CODE. *Some differences between duodenal and ileal sorption.* Am. J. Physiol. 203(2): 215–220. 1962.—Previous studies have shown that large exchanges of water and sodium occur in both directions across the mucosa of the duodenum between the contents and the blood as if to develop and maintain equilibrium with the plasma. In contrast, in the ileum the exchanges are directed to absorption of the contents rather than equilibration. These findings indicate differences in function of the mucosa of the two sites. To illuminate these, isosmotic and hypertonic solutions of a slowly absorbed substance (mannitol) were placed in duodenal and ileal segments of the small bowels of dogs. The changes in the composition of these solutions and the bidirectional fluxes of sodium and water have been determined. Although mannitol was absorbed by the two sites at equal rates and the osmolality of the contents of both regions changed in a parallel fashion, the composition of the contents in the two sites underwent quite different alterations. In the duodenum the volume was unchanged if the solution was isosmotic and increased if it was initially hypertonic, while in the ileum it decreased in both instances. Sodium always entered the duodenal contents in large quantities, while little appeared in the ileal contents.

PREVIOUS INVESTIGATIONS have indicated a difference between absorption in the upper and lower portions of the small bowel (1–4). The duodenum has been studied infrequently, and its function has seldom been contrasted with that of more distal segments. Results reported in 1960 indicated that the mucosa of the duodenum equilibrates its contents with blood by maintaining nearly equal, large movements of both water and sodium into and out of the duodenum, and that the mucosa of the ileum is more rigidly fixed to serve the function of absorption of water and sodium (5). We undertook to test this hypothesis by contrasting the changes in identical solutions placed in the duodenum and ileum. For this purpose solutions of mannitol, which is slowly absorbed, were used.

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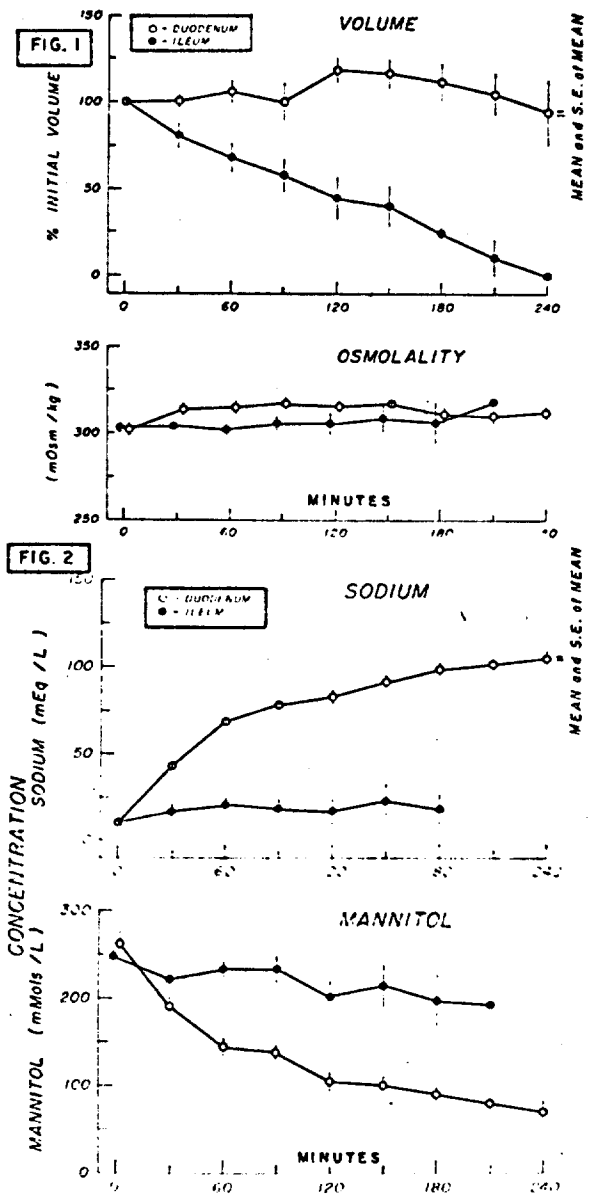
METHODS

Tests were performed on healthy, fasting mongrel dogs anesthetized with pentobarbital sodium. The anesthetic used, the surgical procedures, the intestinal cannulas, and the general plan of the experiments were identical with those previously reported (5, 6). Cannulas composed of spools and connecting tubing were introduced through longitudinal incisions in the antimesenteric border of the bowel. The duodenum was entered through its second portion; after the ligament of Treitz was cut, the spools were tied in place with the proximal one always just distal to the entrance of the pancreatic ducts and the distal spool close to the duodenojejunal junction. The mean distance of the proximal spool from the pylorus was 13 cm. The ileal segments were isolated in a similar fashion. The mean distance of the distal spool from the ileocecal valve was 16 cm. After completion of each test the loops were removed, opened along their mesenteric borders, pinned on a cork board, and their dimensions measured. The mean serosal area of the duodenal loops was 89 sq cm, and that of the ileal loops was 102 sq cm.

The test solutions placed in the loops contained 5.46 or 10.92 g mannitol/100 ml distilled water. These concentrations are equivalent to 300 and 600 mOsm/kg water, respectively. In tests on five animals deuterium (D_2O) and radiosodium (Na^{22}) were added to these solutions to give an initial concentration of D_2O 2.5% and of Na^{22} 5 mc/100 ml.

Soon after preparation, the loops were rinsed with a 0.154 M solution of NaCl at 37 C until the returning liquid was clear. After as much of the washing fluid as possible had been removed, the loops were left for at least 30 min. The temperature of the animals was maintained between 36 and 38 C.

In each test identical solutions were placed in the duodenal and ileal loops, and in the majority the observations on the two loops were almost simultaneous. Initially, about 30 ml of the test solution was placed in each loop and mixed by repeated withdrawal and readmission. Any air mixed with the solution was expelled. The volume was then measured by withdrawing as much of the contents as possible into a



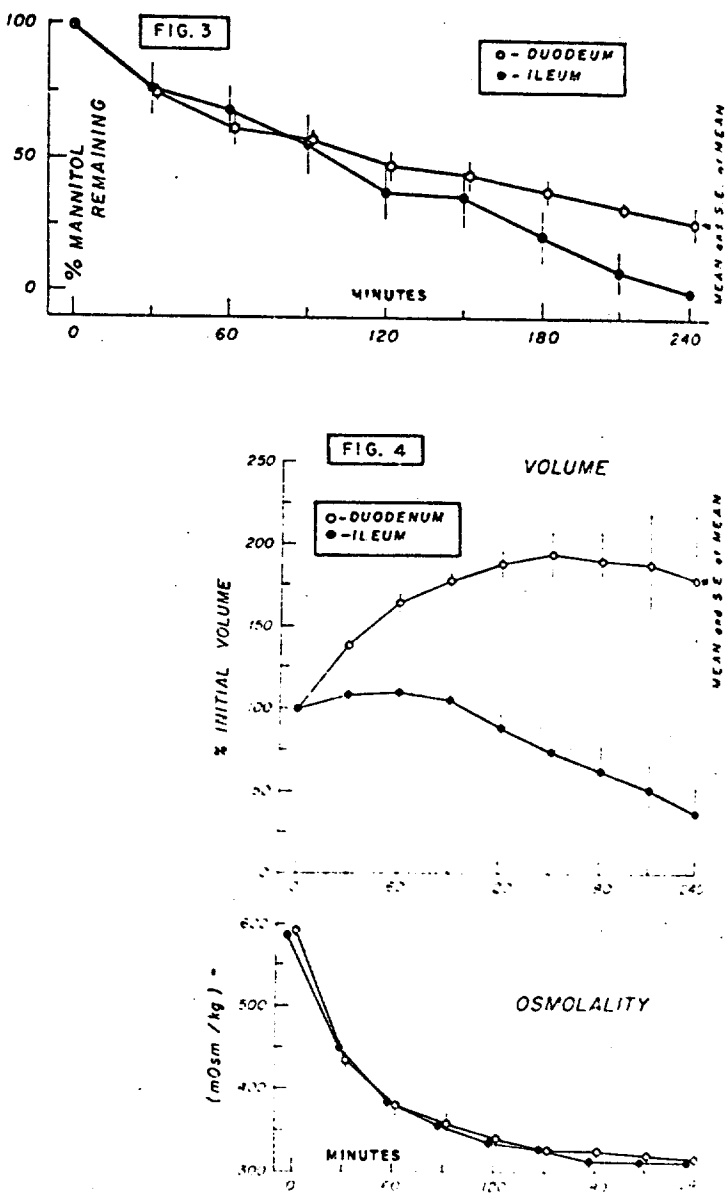


FIG. 1. Changes in volume and osmolality of isosmotic solution of mannitol in duodenal and ileal loops.

FIG. 2. Changes in concentration of sodium and mannitol when isosmotic solutions of mannitol were placed in segments of duodenum and ileum.

FIG. 3. Percentage of initial mannitol remaining in separate segments of duodenum and ileum when filled at 0 time with an isosmotic solution of mannitol.

FIG. 4. Changes in volume and osmolality of hypertonic mannitol solution in duodenal and ileal loops.

calibrated syringe; samples were removed for analysis. These procedures required approximately 1 min to complete. The volume and the composition determined by analysis of the contents at this time were designated as the initial volume and composition of the test solution, rather than the volume and composition of the solution which had actually been placed in the intestine. At regular intervals thereafter, usually every 30 min (but in two tests, every 20 min), the volume of the contents was measured, and samples were taken. When the test had been completed, the loops were washed out with saline solution (0.154 M NaCl) and left for at least 30 min before the procedure was repeated with another test solution.

The volumes measured during the tests were later corrected for the volume of the samples removed and to represent changes per 100 sq cm of serosal surface of loop. To these corrected volumes was added an estimate of the volume remaining in the loop and cannula, which could not be withdrawn by suction and which was determined by isotope dilution.

Immediately after removal of the samples, duplicate determinations of the osmolality were made with a Fiske osmometer. The concentrations of mannitol, Na^{23}, Na^{22}, and D_2O were determined after appropriate dilution of the sample. Mannitol was estimated by the method of Corcoran and Page (7); Na^{23} by use of flame photometer; Na^{22} in a sodium-iodide (thallium-activated)

well type of scintillation counter, and deuterium in a mass spectrometer, after reduction of the water over hot zinc.

Number of tests and expression of results. Eleven dogs were used in the first group of tests and five in the second group. No isotopes were used in the first tests, the object being to determine changes in the volume and composition of the solution left in the loops for a prolonged period (usually 4 hr). In the second group of tests D_2O and Na^{22} were added to the solutions of mannitol to determine the bidirectional movements of water and sodium. In these, estimates of osmolality were not attempted because the D_2O complicated the determinations.

In tests on 13 of the 16 animals simultaneous observations were made on duodenal and ileal loops. The results from these were treated as paired data. Statistical analysis was usually unnecessary, since the differences were consistent and large. The most convenient expression of the changes in volume was found to be as a percentage of the initial volume. Likewise, the most satisfactory expression of the changes in the quantity of mannitol in the contents was as a percentage of the amount of mannitol initially contained in the loops. The terms "insorption," "exsorption," "absorption," and "enterosorption" are used according to definitions recently recommended (8). The rates of insorption and exsorption of sodium and water were calculated according to the exponential method which has been previously described (5).

RESULTS OF TESTS WITHOUT ISOTOPES

Isosmotic Solution of Mannitol (300 mOsm/kg Water)

Volume. In the duodenum the volume did not change significantly during a period of 4 hr. By contrast, in the ileum the volume of fluid decreased progressively until none remained at the end of 4 hr (Fig. 1).

Osmolality. In the duodenum the osmolality increased slightly from an initial mean value of 302 mOsm/kg of water to a mean maximum of 317 mOsm/kg at 90 min and remained at approximately this level for the remainder of the test. The osmolality of the solution in the ileum did not alter significantly during the 4-hr period of the experiment (Fig. 1).

Sodium. Before its introduction into the gut the test solution contained no sodium. During the 1st hr of the test the concentration of sodium in the solution in the duodenum rapidly increased to 70 mEq/liter (Fig. 2) amounting at this time to a net accumulation of 2.5 mEq sodium in the contents. Subsequently the rate of accumulation was more gradual, and the quantity of sodium in the loop at 4 hr was approximately 3.5 mEq; this was present at a concentration of 105 mEq/liter. In sharp contrast, the maximal concentration of sodium reached in the ileum after 1 hr was only 20 mEq/liter. The total quantity of sodium in the loop at this time was 0.5 mEq (Fig. 2). During the remainder of the test no significant change in the concentration of sodium occurred and the quantity in the loop gradually decreased.

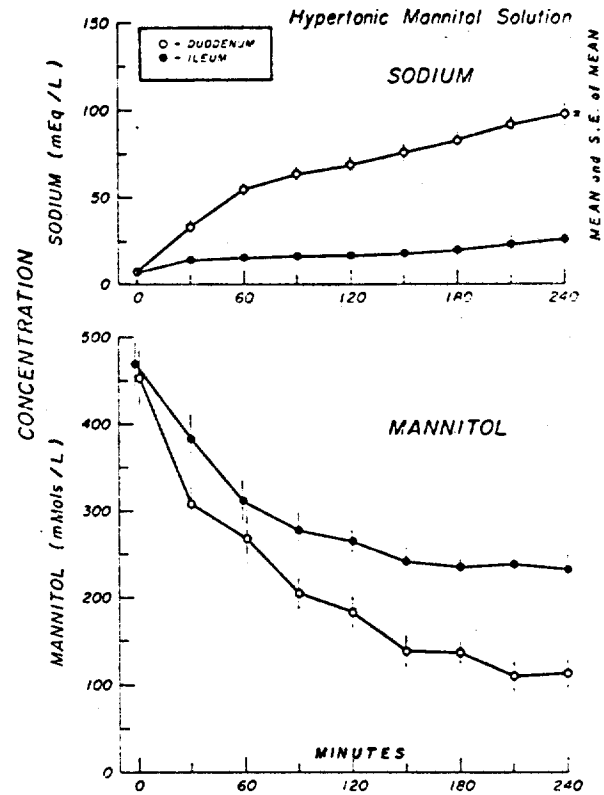


FIG. 5. Changes in concentration of sodium and mannitol when hypertonic solutions of mannitol were placed in segments of duodenum and ileum.

Mannitol. During the first 90 min of the test, mannitol disappeared from both segments of the bowel slowly and at almost identical rates (Fig. 3). No significant difference was found between the percentages remaining in the two loops until 3.5 hr after the beginning of the test. Although the disappearance rates of mannitol were similar in the duodenum and the ileum, the changes in concentration were different. In the duodenum, the mean initial concentration of 262.5 mmoles/liter rapidly decreased in 2 hr to 105.2 mmoles/liter, and then more gradually, to reach 70.3 mmoles/liter after 4 hr. In contrast, the decrease in the concentration of mannitol in the ileum was linear and much less: the mean initial concentration of 247.0 mmoles/liter decreasing to only 192.1 mmoles/liter even after 3.5 hr (Fig. 2).

Hypertonic Solution of Mannitol (600 mOsm/kg Water)

Volume. A rapid increase occurred in the volume of the contents of the duodenum. This reached about 200% in 2–2.5 hr and then remained constant. In contrast, the volume in the ileum reached a maximum of 111.1% at 60 min, and then decreased progressively in each subsequent 30-min period (Fig. 4). At 4 hr only 41% of the initial volume remained in the ileum as compared with 186% in the duodenum.

Osmolality. Both loops of gut rapidly reduced the hypertonic solution almost to isotonicity at identical rates, a value of 320 mOsm/kg being reached in 3 hr,

Sodium. As with the isosmotic solution, sodium entered the duodenal contents quickly and in large quantity, while little appeared in the ileum (Figs. 2 and 5). The quantity of sodium which accumulated in the duodenum increased progressively and had reached 5.8 mEq at the end of 4 hr. In the ileum the maximal quantity of sodium was reached after 1 hr and was approximately 0.5 mEq; thereafter the quantity present gradually decreased. The rates at which the concentrations increased in the contents were actually slower with the hypertonic than with the isosmotic solution, but the final concentrations attained were the same (Figs. 2 and 5).

Mannitol. The amount of mannitol in the duodenum changed little for a period of 1 hr and then decreased gradually during the next 3 hr. At the end of the test 57% of the original amount remained in the loop. The amount of mannitol in the ileum decreased steadily throughout the 4-hr period; at 4 hr only 17% of the original quantity remained (Fig. 6).

The concentration of mannitol decreased in both the duodenum and the ileum, but in the duodenum this decrease was more rapid than in the ileum and continued throughout the study. In the ileum, however, the concentration reached a level of approximately 240 mmoles/liter after 2.5 hr and then remained at this value for the remaining 1.5 hr (Fig. 5).

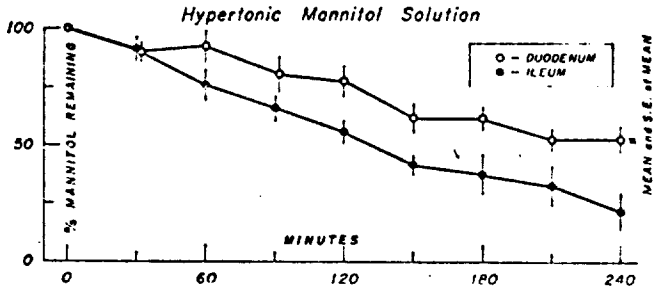


FIG. 6. Percentage of initial mannitol remaining in separate segments of duodenum and ileum when filled at 0 time with a hypertonic solution of mannitol.

RESULTS OF TESTS WITH ISOTOPES

The changes in volume, concentration of mannitol, percentage of the initial dose remaining, concentration of sodium and quantity of sodium accumulating in the loops were essentially the same in the group receiving isotopes as in the group that received no isotopes.

Bidirectional movements of sodium and water with isosmotic mannitol solution. In the five duodenal loops studied with isotopes, 1800 to 2100 microequivalents (μEq) of sodium were exsorbed in each of three consecutive 30-min periods (Fig. 7). During these three periods the quantity of sodium insorbed increased progressively from 413 μEq in the first period to 1181 μEq in the third period. The result of these opposing movements of sodium was a pronounced accumulation of sodium in the duodenal

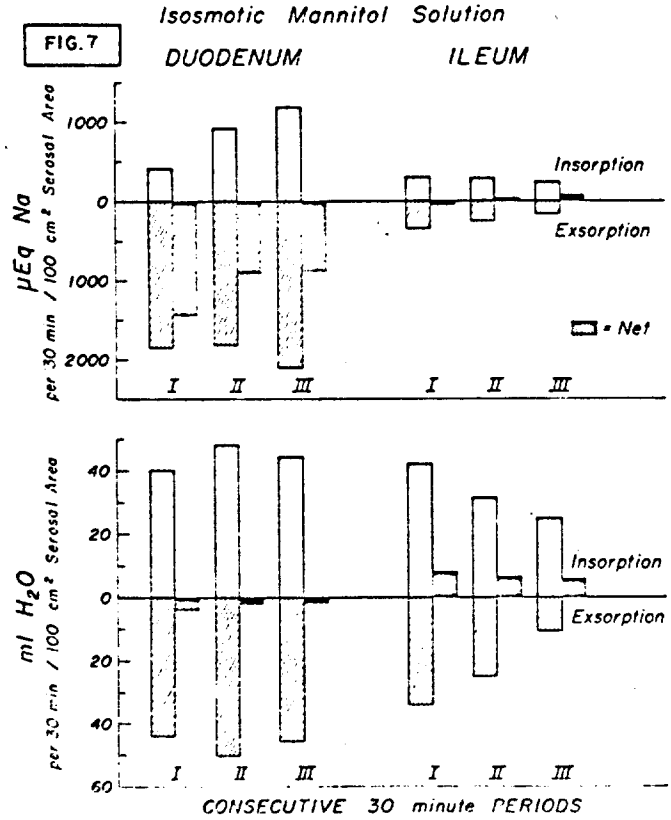


FIG. 7. Bidirectional movements of sodium and water when isosmotic solutions of mannitol were placed in isolated segments of duodenum and ileum.

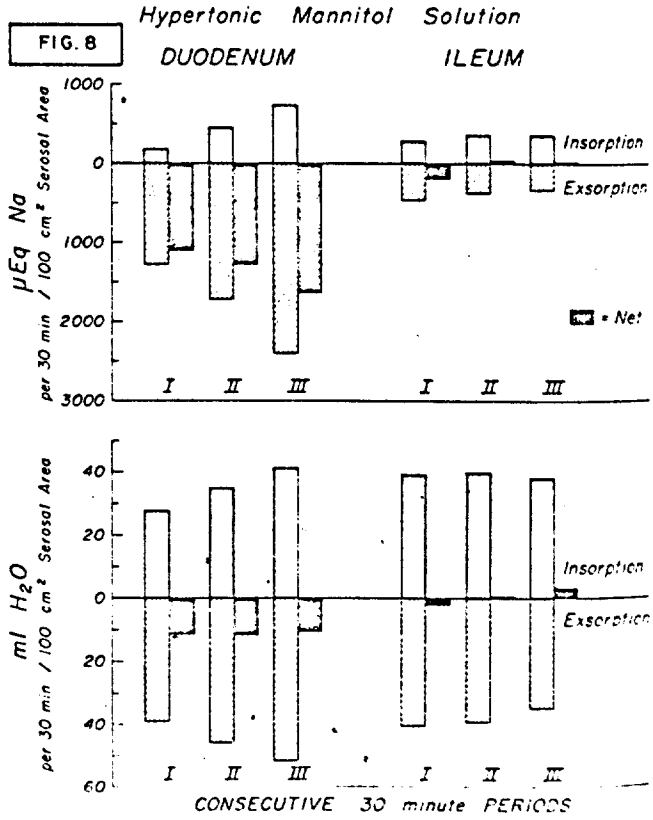


FIG. 8. Bidirectional movements of sodium and water when hypertonic solutions of mannitol were placed in isolated segments of duodenum and ileum.

contents, the rate of this accumulation gradually decreasing during the 90 min. In contrast to these large movements of sodium in the duodenum, the rate of exsorption of sodium into the ileum was 350 μEq in the first 30 min, less than a fifth that in the duodenum, and became progressively smaller in subsequent periods. Insorption of sodium was approximately 300 μEq in each of the 30-min periods and remained relatively constant. The net change resulting from these movements was an initial small enterosorption of sodium followed by an equally small absorption of sodium (Fig. 7).

The volumes of water insorbed and exsorbed by the duodenum were large (40–46 ml/30 min/100 sq cm), and as exsorption was slightly larger than insorption, a small volume of water was added to the contents in each 30-min period. In the ileum insorption was always larger than exsorption, resulting in the absorption of water and a gradual decrease in the volume of the contents (Fig. 7).

Bidirectional movements of sodium and water with hypertonic solution of mannitol. Large quantities of sodium again moved in both directions across the mucosa of the duodenum. Exsorption was slightly less than 1300 μEq in the first 30-min period but had increased to 2400 μEq by the third period. Insorption also increased progressively from 200 to 750 μEq in consecutive 30-min periods, but was much less than exsorption, so that a large quantity of sodium was enterosorbed. Again, the quantities of sodium moving across the ileal mucosa were smaller than those traversing the duodenal mucosa. Insorption was constant at approximately 300 μEq/30 min throughout the 90-min period, but exsorption showed a small but progressive decline from 450 to 325 μEq from the first to the third period. These movements produced a small net accumulation of sodium in the ileum in the first 30-min period, followed by little change in the quantity of sodium in the contents during the second and third periods (Fig. 8).

In the duodenum the volume of water exsorbed during the first 30-min period was smaller than when the isosmotic mannitol solution was used (Figs. 7 and 8). However, this quantity progressively increased until 52 ml were exsorbed in the third 30-min period, and this was considerably more (by 10 ml) than the largest quantity exsorbed with the isosmotic solution. The volume of water insorbed also increased progressively from 28 to 41 ml/30 min/100 sq cm (Fig. 8). In each period insorption was considerably less than exsorption; hence a large increase occurred in the duodenal contents (Fig. 8).

In the ileum the volumes of water insorbed and exsorbed were approximately equal (40 ml/30 min/100 sq cm) and constant during the first two periods. In the third period insorption was greater than exsorption so that some decrease in volume occurred (Fig. 8). The volumes involved were not particularly different from those moved when the isosmotic solution was placed in the ileum.

COMMENT

In general our results are similar to those obtained by Nobécourt and Vitry, who carried out absorption studies on isolated segments of rabbit small bowel in 1904 (1). Using distilled water and saline solutions of different strengths, they found marked differences between the behavior of duodenal and ileal loops. No change in volume occurred when water was placed in the duodenum, but the volume increased in proportion to the concentration of the solution when saline solutions were used. On the other hand, the ileum absorbed both water and weak saline solutions, although the volume of those solutions which had initially been more hypertonic increased. The quantity of chloride increased in the duodenum with all except the strongest saline solutions, but decreased in the ileum with all the saline solutions tested. No doubt Nobécourt and Vitry determined the chloride rather than the sodium content because of the difficulty of making estimations of sodium at that time, but it is likely that the changes in sodium were approximately parallel to those of chloride.

Using solutions of mannitol, we too have found that the volume of the duodenal contents shows little tendency to change when the solution is isosmotic, whereas the volume of ileal contents decreases. With hypertonic solutions, on the other hand, volume increases markedly in the duodenum and decreases or is only moderately increased in the ileum.

In our studies mannitol was absorbed at a slow but appreciable rate from both the duodenal and ileal segments. This rate is probably more rapid in the intact

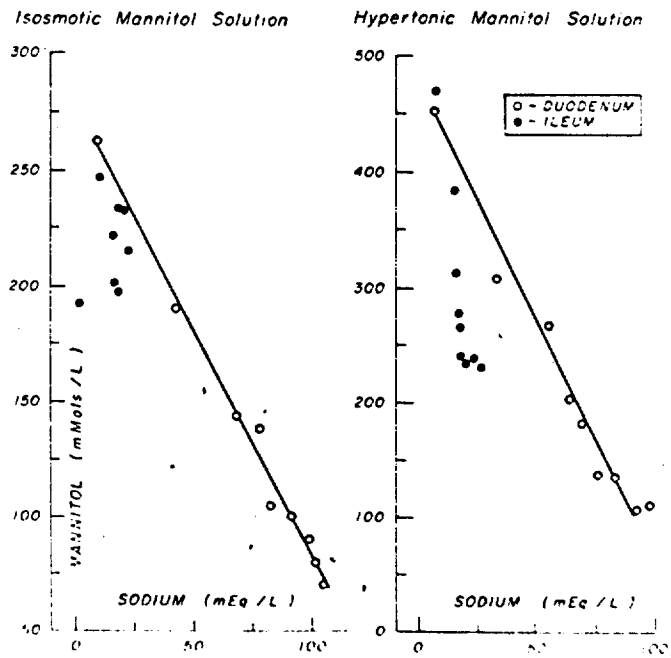


FIG. 9. Relationship between concentrations of sodium and mannitol in duodenum and ileum with isosmotic and hypertonic mannitol solutions.

bowel of dogs than that in the isolated segments of ileum of rats studied by Curran and Solomon (9, 10), for they considered that the quantity of mannitol absorbed under the conditions of their study was negligible.

By providing a slowly absorbed material, mannitol served to illuminate the differences between the sorptive mechanisms of the duodenum and ileum. In the presence of mannitol the movements of sodium and water were strikingly different in the two sites. These differences occurred despite the fact that mannitol disappeared from the two sites at almost the same rate and even though the osmolality of the hypertonic solution was reduced at the same rate in the two sites. Sodium entered the duodenum in quantities sufficient to raise its concentration in the contents to nearly that of the plasma, and water entered in amounts adequate to maintain the volume of the isosmotic solution and to increase that of the hypertonic solution. Across the ileal membrane, on the other hand, the exsorption of sodium was small and barely exceeded insorption, leading to the accumulation of only a tiny quantity in the contents. Water was absorbed from the ileal contents almost from the start of each test, so that no significant increase in volume occurred even with the hypertonic solution. A linear relationship existed between the changing concentrations of mannitol and sodium in the duodenal solution which did not occur in the ileal solution (Fig. 9).

These studies indicate that when mannitol is present in the duodenal contents, the mucosa tends to bring the concentrations of sodium and mannitol toward those of the plasma, while water is brought into the contents in sufficient quantities to maintain the volume of an isotonic solution and to dilute a hypertonic solution. In contrast, the mucosa of the ileum does not perform these functions. It quickly and effectively sets about its function of absorption, moving the mannitol into the blood without the associated movements of sodium and water which occur in the duodenum.

The "equilibrating action" of the duodenum may aid the absorption of some substances in the distal portion of the small bowel. For example, Curran (10) has shown in rat ileum that absorption of water does not occur in the absence of solute movement. Sodium added to the contents in the duodenum may facilitate the absorption of water lower in the small bowel. The presence of sodium has been shown by others to be essential for the insorption of glucose and possibly for the insorption of some amino acids from the small bowel (11–14). The addition of large quantities of sodium to the contents in the duodenum may aid the absorption of these substances in the lower portions of the small bowel.

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EXPERIMENTS ON THE ABSORPTION OF ORGANIC SOLUTES IN THE SMALL INTESTINE OF RATS

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For many years, it has been generally accepted that intestinal absorption of dissolved substances is more than a mere diffusion. This belief has been established mainly on the basis of the statement of Nagano ('02) in Roehmann's Laboratory, that hexoses like glucose, galactose, fructose, mannose, though very nearly related to each other in regard to their chemical and physico-chemical properties, are very strikingly different in their rates of absorption, the more physiological sugars showing the stronger absorption. Another old statement, likewise at variance with diffusion being assumed as the ruling factor of absorption, is the following: Katzenellenbogen ('06) (see also Höber, '26) in the laboratory of one of us, has pointed out that Cl ions, in presence of organic compounds of low diffusion rate, like mannitol, move from the intestinal content into the body against the concentration gradient. Clearer evidence of the same effect has been presented by Goldschmidt and Dayton ('19) applying, instead of mannitol or another non-electrolyte, the slowly diffusing Na_2SO_4, and more recently by Burns and Visscher ('34) and Ingraham and Visscher ('36) emphasizing again the influence on the Cl transport of ions with lower mobility, like SO_4, phosphate, citrate in comparison with those of higher mobility, NO_3, SCN. Now, considering that the mechanism of entrance and transport of dissolved substances in the living beings is one of the central problems of general physi-

[1] A preliminary report has been published in the Proceedings of the Society for Experimental Biology and Medicine, 1936, vol. 34, pp. 486–488.

ology, it seems to be of great value to obtain more knowledge of the factors involved in the higher rate of penetration of some solutes in comparison with others. In this respect, it is conceivable that the finding of Magee and Reid ('31) that phosphate augments the intestinal absorption of glucose, but not of xylose, has stimulated the interest in the problems of absorption remarkably, although the interpretation of this effect is still ambiguous. All the more, it seems profitable to extend the fairly small experimental basis, providing support for the idea that diffusion is an essential factor of absorption and, by doing so, to trace a sharper line between merely physico-chemical and—what may be called—physiological factors, where processes in the interior of the cells are involved. This plan appeared to be promising also for the reason that knowledge concerning the physico-chemical properties of organic membranes has largely increased since the time when physical chemistry was considered to be more extensively serviceable in this field of physiology.

To arrive at such a distinction between physico-chemical and physiological factors, we decided to study absorption 1) of some more unphysiological, but indifferent compounds, i.e., substances which are chemically fairly inactive in the body, not poisonous, not metabolized, and 2) of some more physiological substances, turning our interest chiefly to the amino acids, the behavior of which has been investigated only occasionally.

METHODS

The experiments have been carried out under nembutal anaesthesia with rats of 150 to 200 gm. body weight, starved for 24 hours. Five hundredths cubic centimeter per 100 gm. body weight were injected subcutaneously. The greatest part of the small intestine was isolated by ligatures. Small glass canulae were fixed on both ends of the loop. The loop was carefully washed with warm Ringer's solution and emptied by soft pressing. In each experimennt, the loop was refilled several times with equal volumes (4 cc.) of different solutions, each of these being equimolar to the others. After identical intervals, the loop was emptied by rinsing with Ringer and pressing again softly. In this way, four to six different solutions could be administered in one experiment. The total

residues of solute contained in the loop were analytically determined. We have resigned to measure the volumes emptied as well as the final osmotic pressure. Though apparently rather rough, de facto the method turned out to give generally satisfactory results.

The fluid gained in each period of an experiment was precipitated with tungstic acid. Insofar as amino acids or acid amides were concerned, the material contained in the loop was determined by the Kjeldahl method applying for titration n/20 sulphuric acid. Sugars were titrated following Benedict's prescription, taking into a small test tube not more than 1 to 3 cc. of the copper reagent. For N-free substances we applied Bang's method using n/10 chromic acid and n/10 thiosulphate. Blank determinations have to be made not with distilled water, but with NaCl solution containing the same percentage of Cl as the solutions collected from the intestinal loop.

The results calculated from the analyses are expressed either as per cent absorbed of the total amount introduced into the loop or as absolute amount absorbed, given in millimols.

THE ABSORPTION OF UNPHYSIOLOGICAL SUBSTANCES

We have investigated the behavior of a number of polyhydric alcohols and of aliphatic acid amides. Provided these substances would penetrate the intestinal wall as they would pass an artificial diffusion membrane, we could expect an influence chiefly 1) of the molecular volume (MV)[2] in case the structure of the membrane would be sieve-like, resembling that of an inert filter, and 2) an influence of partition coefficients, if the character of the membrane would be that of a lipoid-like solvent allowing the solute present to be distributed between both phases.[3] As a matter of fact, such behavior is fairly generally accepted to characterize the surface membrane of living cells. However, concerning total living organs, like the intestinal wall, our knowledge is at present rather poor in this respect, although, obviously, it is desirable to be well informed for each organ, how far substances are enabled to pass as they would through an inert diaphragm and how far they have to suffer some kind of intermediate alterations

[2] The MV values recorded in this paper are those calculated from Kopp's formula.

[3] The partition coefficients concerned are in general those of ether: water and of olive oil: water taken from the paper of Collander and Bärlund ('33).

on their pathway. To some extent this question has been attacked by Amberson and Höber ('32) on the cat's salivary gland and by Schmengler and Höber ('33) on the kidney tubules of the frog. In both cases the isolated and artificially perfused glands, tested with indifferent lipoid-insoluble non-electrolytes, appeared to be permeable in the direction blood vessel-lumen like a filter only to such molecules, the volume of which does not exceed a certain limiting value. In addition, the experiments on the salivary gland furnished evidence that the permeating power of distinctly lipoid-soluble compounds surpasses that of lipoid-insoluble ones, even though the former

TABLE 1

Absorption of polyhydric alcohols. Solutions: 0.1 molar in one-third Ringer. Each period of absorption: 25 minutes

	SUBSTANCE	MV	PART. COEFF. ETHER: WATER	PART. COEFF. OLIVE OIL: WATER	PER CENT ABSORPTION
Ia	Mannitol	189.2	0.00001	.....	0
b	Glycerol	87.8	0.00066	0.00007	88.6
c	Erythritol	130.2	0.00011	0.00003	41.4
d	Mannitol	189.2	0.00001	.....	0
IIa	Mannitol	189.2	0.00001	.....	0
b	Erythritol	130.2	0.00011	0.00003	23.6
c	Glycerol	87.8	0.00066	0.00007	69.8
d	Glycerol	87.8	0.00066	0.00007	75.7
e	Erythritol	130.2	0.00011	0.00003	30.2

ones have the larger volume (i.e., butyramide in comparison with acetamide). Now, we have carried on studies upon the question of passive permeability of total organs taking as another subject the intestine which provides, for this purpose, especially suitable conditions.

1. *The absorption of polyhydric alcohols*

The rates of absorption of the following substances have been compared: ethylene glycol, glycerol, erythritol, mannitol. Table 1 shows the outcome of two experiments with the same substances to give an idea as to the consistency of the result under our experimental conditions.

The result is obvious: the absorption rates of the polyhydric alcohols rise with decreasing MV. In both experiments the absorption rate of mannitol is 0.

2. *The absorption of aliphatic acid amides*

The same rule is valid with acid amides. This has been proved by comparison of acetamide, lactamide, urea, malonamide and succinamide, all of them being fairly lipoid insoluble. Table 2 gives an example.

Now, having suggested that the evidence of such a conspicuous connection between absorption rate and MV, as demonstrated in these experiments, supports the concept that absorption is like the diffusion across a porous membrane,

TABLE 2

Absorption of acid amides. Solutions: 0.06 molar in two-thirds Ringer. Each period of absorption: 25 minutes

	SUBSTANCE	MV	PART. COEFF. ETHER : WATER	PART. COEFF. OLIVE OIL : WATER	PER CENT ABSORPTION
a	Succinamide	126.4	.....	.....	3.4
b	Lactamide	98.5	0.0018	0.00058	42.5
c	Acetamide	68.7	0.0025	0.00083	78.2
d	Lactamide	98.5	0.0018	0.00058	36.7
e	Succinamide	126.4	.....	.....	6.3
f	Acetamide	68.7	0.0025	0.00083	68.4

the results obtained still need some further comments. Obviously, the differences of absorption rates of the chemicals applied in our experiments are far greater than the differences in the rates of free diffusion, as determined usually with membranes provided with wide pores, f.i., with membranes of Jena sintered glass, diffusion-coefficient ratios as large as 1 : 2 being considered very large. However, we know mainly from investigations of Michaelis and of Collander that with sieve-like membranes, where the pore diameter approaches more or less the diameter of the molecules of the solutes, the diffusion rates differ to a much greater extent from each other (Höber, '36). We conclude, therefore, that the intestinal membrane resembles such a molecular sieve.

This conclusion will be supported later by another observation (p. 410).

Another point needy of discussion is the following: it might be concluded from tables 1 and 2 that the differences of absorption rates are dependent as well upon the MV as upon the lipoid solubilities, the smaller volumes being correlated with the higher partition coefficients. As a matter of fact, it seems hardly possible to decide at present whether minute partition coefficients like those indicated in tables 1 and 2, though comparatively different from each other, have any significance. For instance, supposing that the plasma membrane of the cells is like a monomolecular film of lipoid, such a film would behave like a porous structure with its intermolecular spaces involving porosity and, due to the existence of thermal agitation, involving the alternative formation of wide and narrow pores. In this regard, such a film would resemble a rigid molecular sieve, like a collodion membrane, the various pore diameters fluctuating around an average value. However, the same film could resemble a solvent layer, provided the lipoid molecules would show a distinct affinity toward solute molecules approaching them from a bordering solution. Following the action of such an adhesion force, 'lipoid-soluble' molecules with volumes not surpassing a limiting value would be allowed to pass some intermolecular spaces, where 'lipoid-insoluble' ones of the same size would not be allowed, solely the axial, not the total column of solvent in such a space being available for their passage. Consequently, the strength of those adhesion forces would be the main factor determining whether such a lipoid film would disclose the properties of a sieve or those of a layer of oil.

3. *Comparison between polyhydric alcohols and acid amides*

From the foregoing, it could be expected that indifferent 'lipoid-insoluble' non-electrolytes of equal MV will be absorbed with the same speed. However, they fail to do so. We have found that, under comparable conditions, the amides are more slowly absorbed than the polyhydric alcohols and that

an amide with two —$CONH_2$-groups moves more slowly across the intestinal wall than one with one such group. This is shown by table 3.

Actually, the differences in the absorption rates are even larger in favor of the alcohols than it appears from this table,

TABLE 3

Absorption of polyhydric alcohols and of acid amides compared

SUBSTANCES COMPARED		PER CENT ABSORPTION			
		alc.	am.	alc.	am.
Erythritol (130.2) (0.00011)	Malonamide (104.4) (0.00030)	18.2	12.6	23.2	5.8
Glycerol (87.8) (0.00066)	Lactamide (98.5) (0.0018)	56.6	41.8	57.4	44.7
Erythritol (130.2) (0.00011)	Succinamide (126.4)	51.5	12.2	36.3	16.4
Ethylenglycol (65.5) (0.0068)	Urea (59.2) (0.00047)	71.3	34.6	76.4	39.4
Erythritol (130.2) (0.00011)	Malonamide (104.4) (0.00030)	89.2	25.9	87.1	12.8
		mono-am.	di-am.	mono-am.	di-am.
Acetamide (68.7) (0.0025)	Urea (59.2) (0.00047)	83.7	49.9	80.2	48.1
Lactamide (98.5) (0.0018)	Malonamide (104.4) (0.0003)	69	27	66	

Numbers in parentheses mean MV and partition coefficients ether: water. Numbers headed by alc. and am. (di-am., mono-am.) mean per cent absorption of alcohols and amides alternatively attempted.

since the amounts of alcohols have been determined by the Bang method, and its titration values mostly appear to be fallacious to some small extent by the reducing effect of some additional amounts of organic substances leaving the intestinal tissue during the experiment.

What is the reason for this remarkable difference in the absorption rates? It will be shown later (p. 412) that the preferential absorption of the polyhydric alcohols is by no means indicative of a preferential absorption as brought about by some special cellular activity, further that such a preferential absorption is by no means conflicting with the view that the absorption of the alcohols is a diffusion process as well as that of the amides. On the other side, it seems impossible at present to give a definite explanation of this behavior. But, at least, this phenomenon resembles a number of other observations well known from studies on permeability of singular cells. Jacobs ('31) was the first to turn the attention to the striking fact that the red cells of mammals and reptiles are more permeable to urea than to ethylene glycol, whereas for those of fishes and birds the inverse relation holds true. Wilbrandt ('31) generalized these statements, as, studying permeability to a number of non-electrolytes with various plant species, substances containing in their molecule acid amide groups turned out to enter the cells either more rapidly or more slowly than those containing alcoholic OH-groups, according to either one or another species being concerned. This generalization has been proved to be justified by many other observations (Höber and Ørskov, '33; Collander and Bärlund, '33; Höfler, '32, '34; Hofmeister, '35; Wahry, '36). The following interpretation has been proposed by one of us (Höber, '30, '31). The cell surfaces differ from each other by the composition of their building material. Among others, their lipoids, due to a more or less acidic character, could display a greater or smaller affinity to amides. As a matter of fact, such a behavior can be imitated by a mixture of oil and oleic acid which is a better solvent for acid amides than is neutral oil (Watzadse, '29; Collander and Bärlund, '33). In a corresponding manner, a lack of affinity to amides could be granted by a more basic character. But it seems possible also to find lipoid solvents exhibiting special affinities to polyhydric alcohols (Fleischmann, '28). Finally, instead of some real dissolving power, the structure of the cell surface might

exhibit particular adsorption affinities toward different substances (Höber, '36).

4. *The absorption of lipoid-soluble compounds*

Lipoid solubility, in the following experiments, means a more definite affinity to a lipoid-like solvent than in the previously described experiments with polyhydric alcohols and with acid amides. The results of three experiments appear in table 4.

TABLE 4

Influence of lipoid solubility on the absorption of acid amides

	SUBSTANCE	MV	PART. COEFF. OIL: WATER	PER CENT ABSORPTION	REMARKS
Ia	Lactamide	98.5	0.00058	65	Solution: 0.06 mol. in one-half Ringer. Each period 25 minutes
b	Valeramide	134.7	0.023	81	
c	Lactamide	98.5	0.00058	69	
d	Malonamide	104.4	0.00008	27	
e	Valeramide	134.7	0.023	89	
f	Lactamide	98.5	0.00058	66	
IIa	Acetamide	68.7	0.00083	81.3	Solution: 0.18 mol. in one third Ringer. Each period 10 minutes
b	Butyramide	112.7	0.0095	84	
c	Acetamide	68.7	0.00083	76.3	
d	Butyramide	112.7	0.0095	80	
IIIa	Malonamide	104.4	0.00008	13.3	Solution: 0.1 mol. in one third Ringer. Each period 25 minutes
b	Succinimide	102.9	0.0049	84.3	
c	Malonamide	104.4	0.00008	13.2	
d	Succinimide	102.9	0.0049	79.3	

The first experiment in table 4 indicates the influence of lipoid solubility insofar as valeramide, with its partition coefficient (oil: water) being about forty times greater than that of lactamide, is more rapidly absorbed than the latter one, though its MV is one-third greater. In the second experiment acetamide and butyramide have been compared, the MV of the second surpassing by far that of the first. However, the absorption rate of butyramide is as great as that of acetamide or a little greater, apparently on account of its higher lipoid solubility. The third experiment displays an enormous difference in the absorption rates of malonamide and succinimide, the MV values being very near to each other, but

the oil: water coefficient being 60 times higher with succinimide, than with malonamide. This difference may partially be due to the two acid amide groups contained in the malonamide molecule.

5. *Absorption as limited by a certain molecular size*

The two experiments reported in table 1 seem to prove that, during an absorption period of 25 minutes, mannitol failed entirely to be absorbed. The question arises whether this behavior would be indicative of the existence of a limiting cross section of the pores of a sieve-like membrane. If so, we could expect 1) that extending the absorption period over more than 25 minutes would have no differential effect on

TABLE 5

Experiments concerning the limiting size of MV. Solutions: 0.1 mol. in one-third Ringer

MANNITOL C_6 189.2		MANNITOL C_6 189.2		SORBOSE C_6 183.2		INOSITOL C_6 178.8		ADONITOL C_5 160.0		ERYTHRITOL C_4 130.2	
After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.	After min.	P.c. unabs.
10	104	20	100	15	92	15	105.5	15	97.7	15	94.7
20	108	40	100	30	92	30	103	25	82.9	30	71.2
30	113.7	60	101.8	45	89	47	103	35	80.0	45	60.3
40	113.5	110	97.7	60	79	60	107.6	45	75.1	60	43.3

the absorption rate, and 2) that other substances resembling mannitol in their physico-chemical properties likewise would fail to be absorbed. Experiments where conditions have been varied in these directions are comprised in table 5.

In these and in a number of other experiments the results have been secured by Bang titration of the intestinal contents. This is the reason why, with mannitol and inositol, the absorption rates are partially negative, in other words, some substances reacting with chromic acid have escaped from the gut wall into the content of the loop (p. 407). On the other hand, the experiments with sorbose, adonitol and erythritol show a distinct increase of absorption with time, although presumably partially veiled by a corresponding exit of material

from the gut wall. As a matter of fact, we may be permitted to interpret the results of this series of experiments as indicating a certain limit of molecular size, the size of mannitol (C_6) and inositol (C_6) being above, that of adonitol (C_5) and erythritol (C_4) being below this limit. This concept being accepted, sorbose could be expected to be unable to pass the intestinal wall like mannitol or inositol, unless the hexoses would display a preferential absorption different from diffusion. In this case sorbose would display a lesser degree of preferential absorption than for instance glucose or galactose.

However, we may have to raise the question whether the behavior of mannitol and of inositol can be looked upon only as caused by a threshold porosity of the intestinal membrane, or whether it might be due to low lipoid solubility. We have already faced this problem without arriving at a definite answer (p. 408). The partition coefficients ether: water are <0.00001 for mannitol and 0.00011 for erythritol, respectively. Interpreting the greater absorption rate of erythritol as inconsistent with its MV, even such a low partition coefficient 0.00011 would have to be considered as an effective lipoid-solubility (Marklund, '36). But, taking into account a number of other observations, it seems to be more reasonable to adhere to the concept of a sieve-like architecture. For instance, comparing the permeating power of the polyhydric alcohols and sugars from glycerol (C_3) to raffinose (C_{18}) in regard to various cells and tissues, one fails to meet the distinct difference in the rate of penetration always lying between C_5 and C_6 as in the intestine. For the sulfur alga Beggiatoa (Ruhland and Hoffmann, '25; Schönfelder, '30) and for the marine alga Chaetomorpha (Hoffmann, '37) the border line is drawn between C_{12} (disaccharide) and C_{18} (trisaccharide), for the placenta of mammals (Schlossmann, '32) between disaccharide C_{12} and hexose C_6, whereas the fresh water plant Chara, like the gut wall, is enabled to distinguish between hexose and pentose (Collander and Bärlund, '33). Furthermore, mentioning still larger molecules, the glomerular membrane of

the kidney has been found to be freely permeable to inulin (MW = 5000) (Richards et al., '34; Shannon, '34), and to permit even ovalbumin (MW = 36,000) to pass, but not serum globulin (MW = 4 × 36,000) (Bayliss, Kerridge and Russell, '33). In all these cases, it seems to be appropriate to account for the smallest permeability by only a small number of comparatively wide pores being accessible to the largest molecules.

6. *Absorption at different concentrations*

So far, the absorption of unphysiological substances seems to be like a diffusion across an inert membrane. This concept is in agreement with further experiments, where the

TABLE 6

Absorption of urea, erythritol and malonamide at different concentrations

UREA			ERYTHRITOL			MALONAMIDE		
After 15 minutes			After 25 minutes			After 35 minutes		
Molar	P.c. abs.	Mmol abs.	Molar	P.c. abs.	Mmol. abs.	Molar	P.c. abs.	Mmol. abs.
0.06	20.9	0.050	0.06	54.1	0.130	0.066	16.9	0.045
0.09	19.0	0.068	0.09	65.0	0.235	0.132	16.8	0.089
0.12	17.1	0.082	0.12	62.2	0.297	0.198	16.5	0.131
0.18	20.0	0.144	0.18	54.4	0.393	0.264	18.4	0.194
0.24	17.8	0.170	0.24	55.5	0.535			

influence of concentration upon absorption rates has been studied (table 6). Under those conditions, considering that the solution in touch with the outside of the membrane, the circulating blood plasma, has a fairly constant composition and that, particularly, the concentration of the substance submitted to the absorption is there equal to 0, we have to expect from Fick's law of diffusion that, with equal amounts of solution and with equal lengths of time, the percentage of absorption will be approximately constant. For singular cells, evidence of a corresponding behavior is chiefly available from the more recent experiments by Jacobs ('33, '34, '36) and by Collander and Bärlund ('33). With the intestine the theoretical requirements have been satisfied at first by experiments of

Verzár ('35), showing that the absolute amount absorbed of the 'unphysiological' sugars, mannose, sorbose, xylose rises more or less in proportion to the concentration applied, in other words, that the relative amounts absorbed are practically constant. The same relation, within a certain range of deviations, holds true in our experiments with compounds of smaller molecular size.

It is of particular interest to learn from these experiments that polyhydric alcohols as well as acid amides are equally absorbed according to Fick's law. This means, that the preferential absorption of the polyhydric alcohols in comparison with the acid amides is by no means a sign of cellular activity, as taken into consideration before (p. 408).

THE ABSORPTION OF AMINO ACIDS

The experiments so far referred to have provided evidence that, after applying several criteria, the influence upon the rate of absorption of molecular size, of lipoid solubility, of time, of concentration, of characteristic features of the molecular structure, we may be allowed to conclude that absorption of a number of unphysiological chemically fairly inert organic non-electrolytes is essentially like a diffusion process. Now, we shall apply some of these criteria to see, whether substances of great physiological importance, the amino acids, behave otherwise. At first we have studied the influence of MV.

1. The absorption of amino acids compared with that of other substances with regard to their molecular volume

In studying this connection, we have to take into account that the amino acids present a particular behavior in their diffusion rates. It is conspicuous already from old observations of Overton ('02) concerning the osmotic properties of frog muscles that amino acids like glycin or alanin enter, if at all, with very great slowness, although one could expect them to exceed, f.i., erythritol—which enters—because of their smaller molecular size. Still more evident is their inertia in penetrating the cell surface in the experiments of Ruhland and

Hoffmann ('25) and of Schönfelder ('30) on Beggiatoa, which is remarkable for its fairly incomparable behavior as a molecular sieve, and where the permeation rates of glycin, alanin, asparagin, leucin have been found to be abnormally low. The reason for this slowness is not of a physiological nature, since, according to Schmengler ('33), the slowness becomes evident also in diffusion experiments with collodion membranes, when the amino acids are compared with other substances of corresponding MV. Obviously, for several reasons, this slowness

TABLE 7

Amino acids compared with acid amides, polyhydric alcohols and sugar. Indicating, whether the absorption from equimolar solutions of amino acids is faster or slower

AMINO ACID	MV_{calc}	MV_{eff}	ABSORPTION RATIO	SUBSTANCE COMPARED WITH AMINO ACID	MV
Glycin	78	~110	<	Acetamide	69
Glycin			>	Lactamide	98
Glycin			>	Malonamide	104
Glycin			>	Succinamide	126
dl, α-alanin	98.5	~150	=	Glycerol	87.8
dl, α-alanin			>	Succinamide	126
dl, α-alanin			>	Erythritol.	130
dl, α-aminobutyric acid	120	....	>	Malonamide	104
dl, α-aminobutyric acid			>	Succinamide	126
dl, asparagin	130	~170	>	Malonamide	104
dl, asparagin			>	Succinamide	126
dl, asparagin			>	Erythritol	130
dl, asparagin			>	Xylose	153
dl, α-aminovaleric acid	142	....	>	Erythritol	130
dl, α-leucin	164	~190	=	Malonamide	104

is based upon the ampholyte character of the amino acids, according to which in aqueous solution they are practically entirely present as zwitterions, i.e., as strong dipoles enlarging their MV, between others, by the formation of a shell of water molecules. Consequently, a porous membrane such as the intestinal wall could be expected to be passed also comparatively slowly. But the contrary is true. This will be learned from table 7, which comprises briefly our main results, and from table 8, which shows more in detail the general

course of these experiments. The columns headed in the tables by MV show the values calculated from Kopp's formula and the approximate effective values as derived from the diffusion experiments of Schmengler.

It follows from the fourth column in table 7 that, in nearly all experiments reported, the absorption of amino acids exceeds that of the substance correlated. Only, comparing glycin with acetamide, the latter turned out to be faster, obviously, because in this case the effective molecular size of

TABLE 8

Three experiments concerning amino acids as compared with acid amide, polyhydric alcohol and sugar

	SUBSTANCES	MV		PER CENT ABSORBED		REMARKS
Ia	Malonamide, xylose	104	153	11.3	13.1	Solutions: 0.09 mol. in
b	Asparagin, xylose	~170	153	65.7	10.5	one half Ringer. Each
c	Malonamide, xylose	104	153	11.1	6.9	period 20 minutes
d	Asparagin, xylose	~170	153	72.7	18.7	
IIa	Malonamide	104		11.3		Solutions: 0.05 mol. in
b	Aminobutyric acid	(120)		66.2		two thirds Ringer.
c	Malonamide	104		9.1		Each period 25
d	Aminobutyric acid	(120)		66.1		minutes
IIIa	Erythritol	130		7		Solutions: 0.1 mol. in
b	Aminovaleric acid	(142)		28.4		one-third Ringer.
c	Erythritol	130		12.6		Each period 25
d	Aminovaleric acid	(142)		29.5		minutes

glycin is overbalancing. For the same reason, alanin is assumed not to surpass glycerol in speed, leucin not malonamide. For the rest it appears that, even with the MV of the amino acid rather largely exceeding that of the compared substance, like in the experiments with aminobutyric acid and malonamide, with aminovaleric acid and erythritol and particularly with asparagin and malonamide, the amino acid is the faster one.

In table 8 the numbers in brackets mean the MV_{calc} of aminobutyric and aminovaleric acid, the MV_{eff} not being available. In the first experiment, mixtures of two substances have been introduced into the intestinal loop, both components being

analyzed one beside the other. The far greater absorption of the amino acid is evident in this as well as in the other two experiments.

2. *Absorption at different concentrations*

Considering the results so far described, the question arises whether the preferential absorption of the amino acids resembles that of the polyhydric alcohols in respect to the acid amides (p. 407), in other words, whether the diffusion of the amino acids is merely speeded up by some adsorption affinities exerted by the pore walls of the sieve-like structure of the surface membrane, so that the concentration gradient inside

TABLE 9

Absorption of glycin, alanin, asparagin and valin at different concentrations

GLYCIN			ALANIN			ASPARAGIN			VALIN		
	After 20 min.			After 20 min.			After 20 min.			After 20 min.	
Mol.	P.c. abs.	Mmol. abs.	Mol.	P.c. abs.	Mmol. abs.	Mol.	P.c. abs.	Mmol. abs.	Mol.	P.c. abs.	Mmol. abs.
0.071	71.0	0.201	0.055	48.5	0.107	0.026	64.3	0.067	0.056	62.0	0.139
0.152	55.2	0.313	0.110	45.9	0.202	0.052	53.4	0.112	0.084	37.0	0.124
0.212	52.3	0.445	0.165	40.7	0.268	0.078	41.8	0.132	0.115	27.8	0.128
0.283	45.5	0.515	0.220	26.8	0.235	0.104	28.4	0.118	0.167	15.0	0.100
									0.223	10.6	0.094

the pores would be raised. In order to answer this question, we have studied the influence of varying concentrations upon the rate of absorption, in the same manner as done with other non-electrolytes. Provided that absorption would be like a diffusion, the percentage absorption would turn out to be constant, the absolute absorption to be increasing. Some of our experimental results are recorded in table 9.

Evidently, the behavior of amino acids is different from that of the other non-electrolytes as described above. Whereas, according to table 6, the percentage of absorption of urea, of erythritol and of malonamide turned out to be fairly constant, whatever concentration was introduced for absorption, the percentage absorption of amino acids falls off with rising

molarity. This is just what has been observed in earlier experiments of Auchinachie, Macleod and Magee ('30), of Donhoffer ('35) and chiefly of Verzár ('35) with the 'physiological' sugars, particularly with glucose and galactose, in contrast to the 'unphysiological' sugars, like mannose, xylose, arabinose. Apparently it means that, beside the concentration gradient, there is still another factor involved as a driving force in the absorption of special solutes through the intestinal wall. This factor displays its effect most vigorously with low molarities, where the concentration gradient is relatively ineffective. Therefore, the percentage absorption is highest at those low concentrations. Whenever diffusion would fail to work, the special factor alone would be responsible, in other words, the absolute amount absorbed would appear to be independent from the concentration present. This conclusion seems to be justified by the data in table 9 especially in regard to the amino acid with the highest MV, valin, whereas, with glycin, allowing a larger diffusion because of its low MV, an increase of concentration is more or less paralleled by an increase in the absolute amount absorbed. However, the experiments carried out so far may not be numerous enough to permit a definite conclusion.

How to explain this special behavior of some sugars and that of the amino acids? Since Wilbrandt and Laszt ('33) have published their well-known observations upon the influence of iodoacetate on the absorption of hexoses and pentoses, there has been much discussion of the possibility, that, by an intermediary phosphorylation and dephosphorylation of the sugars, variable in their rates from one sugar to the other, the absorption of some of these might be speeded up. The main objection to this concept is resting on the evidence, that, wherever cellular transport is associated with cellular metabolism, iodoacetate seems to work as an inhibitor. Regarding our own experiences, this follows from observations concerning the absorption of Cl ion and the secretion of phenol red by the tubular wall of the frog kidney, both moving from a lower to a higher level of concentration (Ferrari and

Höber, '33), further from observations concerning the inhibitory effect of iodoacetate on the accumulation and secretion of dyestuffs in the liver (see also Koll-Schröder, '34). Consequently, phosphorylation cannot be taken as mediating primarily the higher rate of absorption of sugars. More specific appears to be the influence of another poison, phlorizin, which displays a strong inhibitory effect on the absorption of the 'physiological' sugars not only in the kidney, but also in the intestine, whereas the absorption of amino acids in these organs remains unaffected by phlorizin (Lundsgaard, '33; Wertheimer, '33). In this case as well as in others, where we are dealing with specific transporting cellular properties, obviously useful for the total organism—as f.i., in the frog kidney the secretory transport of metabolic end products, like urea, uric acid, thiocyanate, the absorbing transport of Cl, glucose, aminoacids (Laug and Höber, '36)—we have to demand a special metabolic reaction as initiating the specific transport, which might be detected by a systematic investigation of the local influence of a great variety of poisons upon the isolated transporting structure. We are continuing our studies in this direction.

SUMMARY

1. In experiments on isolated loops of the small intestine of rats, the absorption of a number of organic substances has been followed. The absorption rates of these substances have been compared by filling alternatively into the loop equal amounts of the equimolar solutions for equal time periods. Further the influence of varying concentrations upon the rate of absorption has been investigated.

2. In the series of polyhydric alcohols (ethylenglycol—mannitol), the absorption rate falls off with rising molecular volume. The absorption rate of mannitol is approximately equal zero. Varying the concentration of a substance shows the percentage of absorption to be rather constant. It is concluded, that absorption of these substances is like a diffusion through a sieve-like membrane, the maximal pore diameter of

which controls the limiting molecular volume allowing permeation.

3. The absorption of aliphatic acid amides is analogous to that of the polyhydric alcohols except the fact that the amides are absorbed more slowly than the alcohols of corresponding molecular volume. A similar behavior has been encountered in studies upon the permeability of single cells of animals and plants. The differences are assumed to be based on special affinities between the pore walls of the membranes and the solutes.

4. Substances of distinct lipoid solubility are absorbed at a higher rate than lipoid insoluble substances of very low lipoid solubility.

5. Amino acids are absorbed at a higher rate than could be expected from their calculated molecular volume and even at a higher rate considering the volume of the zwitterions of the ampholytes being enlarged by a shell of water dipoles. Varying the concentration, it appears that the percentage of absorption is not constant, but falls off with rising concentration. Evidently, these substances of physiological importance resemble the 'physiological' sugars (glucose, galactose). Their absorption is not like a diffusion, but is a process complicated by the presence of an accelerating factor, the effect of which becomes more visible with lower than with higher concentrations.

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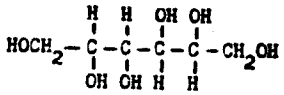
MANNITOL

Synonyms D-Mannitol; Mannite

Chemical name 1,2,3,4,5,6-Hexanehexol

Empirical formula $C_6H_{14}O_6$

Structural formula



Molecular weight 182.17

Definition Mannitol consists of D-mannitol and contains, after drying, not less than 98 per cent. and not more than the equivalent of 102 per cent. of $C_6H_{14}O_6$.

Description Mannitol is a white crystalline solid which is odourless and has a sweet taste.

Uses Sweetening agent, humectant, sequestrant.

Biological Data

Biochemical aspects

D-mannitol occurs widely in nature in a variety of plants, algae, fungi and certain bacteria. L-mannitol does not occur naturally. Traces of mannitol have been identified occasionally in human urine (Pitkänen & Pitkänen, 1964).

D-mannitol has a slow rate of absorption from the intestinal tract and exerts laxative properties. The laxative threshold for man was found to lie between 10 and 20 g of D-mannitol per single dose (Ellis & Krantz, 1941). D-mannitol fed to dogs was excreted in large quantities in the urine (Jaffe, 1883). When ^{14}C-D-mannitol was given at a rate of 240 mg/rat orally to non-fasted rats, about 50 per cent. of the radioactivity was recovered in the expired $^{14}CO_2$ (Wick et al., 1954). In similar experiments, also using ^{14}C-D-mannitol at a rate of 500 mg/kg body-weight, fasted rats oxidized 40 per cent. of the dose to $^{14}CO_2$, non-fasted rats 68 per cent.; 9.74 per cent. was stored in the carcass; 1.28 per cent. in the liver and 6.32 per cent. was excreted in the urine (Gongwer, 1963). Feeding D-mannitol to rats and dogs led to a small but significant increase of liver glycogen (Carr et al., 1933; Todd et al., 1939; Silberman & Lewis, 1933; Carr & Krantz, 1938).

When ^{14}C-D-mannitol was administered to rats by i.p. injection, 77-97 per cent. of the dose was excreted in the urine within 24 hours, and only 2-3 per cent. of the mannitol carbon was oxidized to $^{14}CO_2$. Additional experiments using injection directly into the portal vein showed that mannitol could be oxidized by the liver only (Wick et al., 1954).

I.v. administered D-mannitol was completely cleared by the kidneys of 2 dogs at rates identical to inulin and creatinine (Smith et al., 1940). Mannitol did not elevate the blood sugar level of dogs upon i.v. injection at a rate of 22.5 g/dog (Todd et al., 1939).

In man, i.v. administration of D-mannitol is practiced for induction of diuresis in oliguria or for forced diuresis in poisoning cases or to measure the extracellular fluid compartment. There is an extensive literature available on these aspects (Milne, 1965; Widdowson et al., 1964). Following the i.v. injection of 10 g D-mannitol into man, 81 per cent. of the dose was excreted unchanged in the urine and up to 80 g produced no toxic effect (Smith et al., 1940). Administration of 25 g of D-mannitol on 3 subsequent days to man did not significantly influence either the blood sugar level or the respiratory quotient (Ellis & Krantz, 1941). When 100 g D-mannitol was fed to man, the maximum rise in blood sugar level was 10 mg per cent. (Field, 1919).

Acute toxicity

Animal	Route	LD_{50} (mg/kg body-weight)	References
Mouse	oral	22 000	Gongwer, 1960
	i.v.	16 800	Robb, 1964
	i.p.	14 000-16 000	Beck et al., 1936
Rat	oral	17 300	Gongwer, 1960

Mice diet with signs of central nervous system depression and gastrointestinal tract mucosal damage; rats died with predominantly gastrointestinal tract signs (Gongwer, 1960; Gongwer, 1961).

Short-term studies

Rat. Groups of 20 male rats were fed 35 per cent. sucrose plus 5 per cent. D-mannitol or 40 per cent. sucrose (control group) over a period of 3 months. The growth curves showed that D-mannitol was nutritionally inferior to sucrose (Ellis & Krantz, 1941). These results are in accord with earlier findings that mannitol is inferior to sucrose, as judged by weight gain of rats (Ariyama et al., 1929).

Monkey. Three rhesus monkeys were each fed 3 g of D-mannitol daily, for 3 months. Two animals were employed as controls. No toxic signs nor pathological changes were observed (Ellis & Krantz, 1941).

Man. The i.v. injection of 10 g of D-mannitol daily over a period of 1 month produced no significant changes in non-protein nitrogen, or CO_2-combining power of blood, red cell count or renal function (Ellis & Krantz, 1941).

Long-term studies

No data are available.

Comments

The poor absorption shown in the metabolic studies and a long clinical experience in man support the safety of D-mannitol.

Evaluation

The many studies that have been carried out in man provide a basis for evaluation.

Estimate of acceptable daily intake for man

	mg/kg body-weight
Unconditional acceptance	0-50
Conditional acceptance	50-150

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Functional and histological injury to intestinal mucosa produced by hypertonicity

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KAMEDA, H., T. ABEI, S. NASRALLAH, AND F. L. IBER. *Functional and histological injury to intestinal mucosa produced by hypertonicity.* Am. J. Physiol. 214(5): 1090–1095. 1968.—Solutions of glucose, mannitol, urea, and polyethylene glycol (PEG) in isotonic NaCl were placed in 8-cm long jejunal or ileal loops of rats with intact blood supply. The incubated solution was removed after 15 min and replaced with fresh material for six consecutive periods. Isotonic NaCl containing 14 milliosmols/liter glucose showed uniform absorption of glucose, water, and Na over the 90 min of study. At the end of six study periods, the mucosa was intact to light microscopy. When 550 milliosmols/liter of mannitol, urea, or PEG were added to the above-mentioned solution it resulted in impairment of glucose absorption with a net increase in its luminal content and a histological injury to the surface epithelium of the intestinal mucosa. Similar results were found in both jejunum and ileum. These studies demonstrate an injury both to the glucose-absorbing mechanism and to the blood-luminal barrier to glucose diffusion.

hypertonic solutions; mannitol; glucose absorption; intestinal injury; intestinal absorption

HYPERTONIC SOLUTIONS of mannitol placed in the human intestinal tract result in the movement of fluid from the body into the intestinal lumen (6). The oral administration of hypertonic mannitol produces a profuse diarrhea in humans. The diarrheal fluid is isotonic and contains approximately 40 mEq/liter of sodium and 20 mEq/liter of potassium (7). During the course of investigating the origin of this fluid and electrolytes in the rat, there was evidence of impairment of glucose absorption and damage to the intestinal mucosa. The following experiments were designed to define better this disordered glucose transport and histological injury.

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METHODS

Two hundred male Wistar rats, weighing 300–500 g were used in these experiments. After anesthesia with ether, segments of intestine were prepared for in vivo study by the method of Cocco and co-workers (2). Segments (8–10 cm long) of proximal jejunum, distal ileum and colon just distal to the cecum were tested. Utmost care was taken to keep an intact blood supply to the segments as judged by the pink color and pulsating arcades. The test solutions (2 ml) were placed in the intestinal segment for 15 min and then recovered by flushing the loop gently with air. The procedure was repeated for six consecutive 15-min studies in each loop using fresh solutions each time. Animals were sacrificed at various time intervals to take biopsies from the experimental loops. The biopsy tissues were fixed in Hartman's solution and studied under the dissecting microscope. Eight to ten sections were mounted on each slide and at least four slides were prepared from each biopsy tissue. The sections were stained with hematoxylin and eosin. Table 1 indicates the composition of the various test solutions.

Each solution was tested in the ileal loops in 3–10 rats. Six consecutive studies were performed in each animal. Most solutions were also tested in six or more additional animals to study jejunum and colon segments. The results recorded represent the average of all test periods in at least three animals. Histological study was carried out on 21 animals.

The volume of material recovered from each study period was measured in a small graduated cylinder. Glucose, sodium, and potassium were determined on the recovered solutions. When mannitol was used, its concentration was also determined in the fluid.

Glucose was determined by the reducing technique of Hoffman (10) modified for the AutoAnalyzer. All glucose samples were treated with buffered D-glucose oxidase and reanalyzed. The glucose level is recorded as the difference. Sodium and potassium values were determined by flame photometry using internal lithium standardization. Mannitol concentrations were deter-

mined by the method of Corcoran and Page (3) and osmotic activity by freezing-point depression.

RESULTS

Absorption of glucose added to isotonic saline is illustrated for each of the three levels of the intestine in Fig. 1*A*. The number of the consecutive perfusion is shown

TABLE 1. *Composition of test solutions (mM)*

Solution	NaCl	Glucose	Mannitol	PEG	$MgSO_4$	Urea	Osmolarity*
A	154						270
B	154	14					280
C	77		140				270
D	77	14	140				285
E	154		550				880
F	154	14	550				890
G	154	14				550	850
H	154	14		550			2,000–4,000†
I	154	14			275		600
J	154	14	140				410
K	154	14	220				510
L	154	14	280				600

* Expressed as milliosmols/liter and determined by freezing-point depression. † Calculated value is 300.

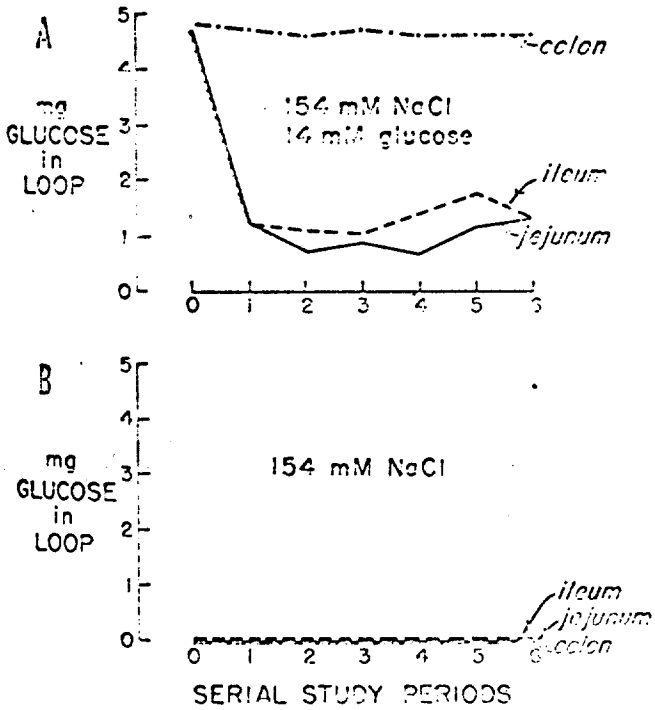

FIG. 1. *A*: glucose absorption from isotonic solution at three different levels of the intestine. At zero is indicated the amount of glucose put in the loop. The numbers along the *ordinate* indicate the amount of glucose recovered from the loops at the end of the study periods specified along the *abscissa*. Note uniform absorption of glucose from the ileum and jejunum throughout the 6 study periods. There is no significant difference in glucose recovered from the loops in *periods 1* and *6*. There was uniform absorption of water also averaging 12% in jejunum and ileum and 8% in the colon. *B*: shows that no glucose was recovered from any of the intestinal segments when isotonic saline alone was used.

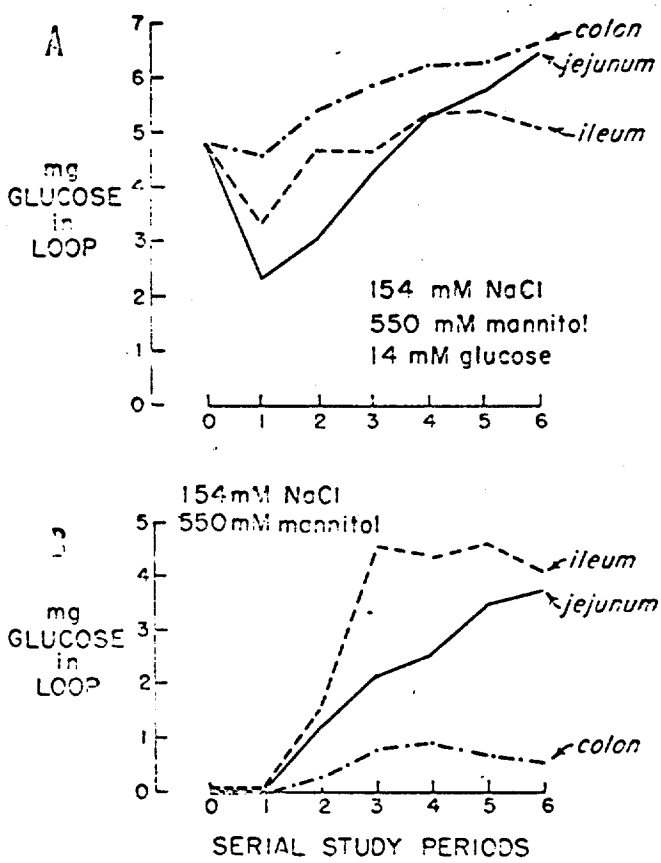

FIG. 2. *A*: observe initial absorption of glucose from hypertonic mannitol and progressive increase in amount of glucose recovered with each study period. Comparison of glucose absorption during first and last 2 study periods showed a statistically significant difference (t = 13.8 at P of 0.01). The volume also increased significantly in each loop compared to isotonic solution (t = 29.5 at P of 0.01). Average increase above the 2 ml placed in each loop at beginning of the study was 1.1 ml for jejunal loops, 0.65 ml for ileal loops, and 0.35 ml for loops of the colon. Volume of fluid entering the loops and sodium concentration was not significantly different in 1st or 6th test periods, despite the marked change in glucose. *B*: with omission of glucose from the test solution, glucose influx into the loop was observed at all levels of the intestine, again increasing with serial study periods. Glucose recovered is statistically significant for 2nd and subsequent periods for jejunum and ileum and for 3rd and subsequent periods for the colon.

along the abscissa. The values on the ordinate indicate the amount of glucose recovered from the loop at the end of each incubation. At zero is indicated the amount of glucose placed in each loop at the beginning of each of the six study periods. Glucose was absorbed from the ileum and jejunum, but not from the colon. No significant change in the sodium concentration was observed at any level of the intestine when isotonic solutions were tested. Water was absorbed from all three levels (mean 18% in jejunum and ileum, and 8% in colon). No glucose was recovered from the loops when isotonic saline alone was tested (Fig. 1*B*). The glucose and water absorption were uniform over the six periods of perfusion.

The results, when 10% mannitol in isotonic saline and glucose was tested, (*solution F*, 890 milliosmols/liter) are illustrated in Fig. 2. There was an appreciable increase in

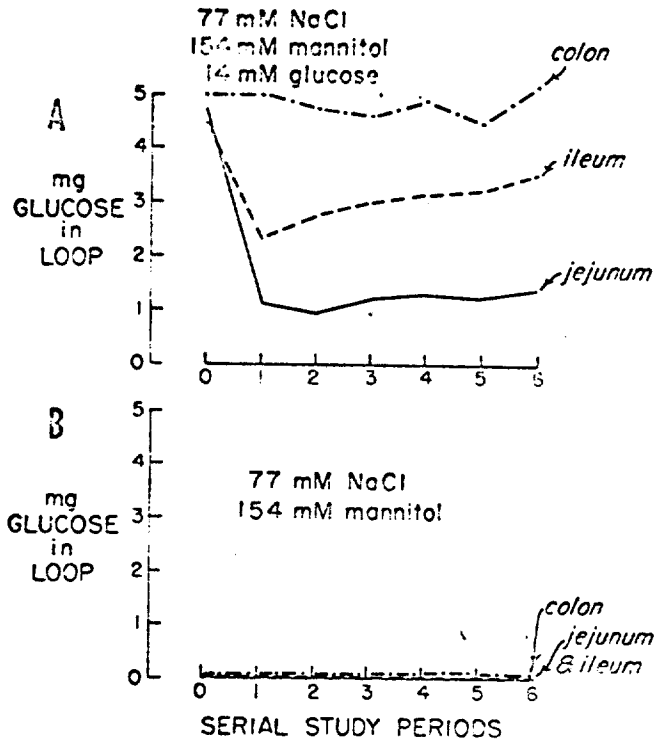

FIG. 3. *A*: glucose absorption proceeded normally for isotonic solutions containing mannitol. *B*: no influx of glucose observed.

the volume of the fluid recovered from all three levels of intestine, but more in the jejunum than in the ileum and colon. The volume increase after 10% mannitol in isotonic saline and glucose was significantly greater than the volume recovered after isotonic solutions (t = 29.5 at P of 0.01). The sodium concentration diminished in all in proportion to the increased influx of water. Glucose absorption occurred in the first study period (Fig. 2*A*). The glucose concentration (not illustrated) was the same as that observed in the control experiments with *solution A*. On subsequent study periods, progressively increasing amounts of glucose were recovered from the loop. After the third study period there was net movement of glucose into the loops at all levels of the intestine. Comparison of glucose absorption during the first and last two study periods showed a statistically significant difference (t = 13.8 at P of 0.01). Figure 2*B* indicates the results when glucose was omitted from the hypertonic solution (*solution G*). After the third study period, glucose appeared in the segments taken from all levels of the gut. This change became progressively more severe.

Figure 3*A* illustrates that glucose absorption from an isotonic solution of half saline and half mannitol with glucose (*solution D*) is similar to absorption from saline and glucose. Glucose is absorbed by ileal and jejunal segments but not by colon (Fig. 3*A*). When no glucose was present (*solution G*), none was recovered from the various loops (Fig. 3*B*).

Figure 4 illustrates the effect of exposing the intestine to hypertonic solutions for the first four study periods, then to an isotonic solution. It is observed that the changes produced during incubation with the hypertonic solution (*solution F*) persist for at least 45 min afterwards, even in the presence of an isotonic solution. Thus, in Fig. 4, glucose absorption in the ileum and jejunum has ceased by the third period and did not return after an isotonic solution (*solution B*) was used. In Fig. 5, glucose appeared in the loop after the second study period with *solution E*, and continued to appear even though *solution A* was placed in the loop. Colon loops were not studied in these experiments. Figure 6 demonstrates the effect of hypertonic solutions made by replacing mannitol with equimolar amounts of urea, polyethylene glycol (PEG, mol wt 1,000) or magnesium sulfate ($MgSO_4$). The effect of these hypertonic solutions on glucose absorption are similar to those with hypertonic mannitol (Figs. 2 and 6). Note in Table 1 that PEG alters the freezing point far more than its concentration would predict and this may account for the increased effect. The effects of varying the concentrations of mannitol in saline on glucose absorption from the ileum are illustrated in Fig. 7. It is observed that solutions containing 410 or more milliosmols/liter interfere with glucose absorption, leading to appearance of glucose in the loops.

Histological Examination. Samples of jejunum were examined after 90 min of study for all solutions except *C* and *D*. Biopsies of ileum were examined after *solutions B, F, G, H, I, J, K*, and *L*. Very few biopsies of colon were

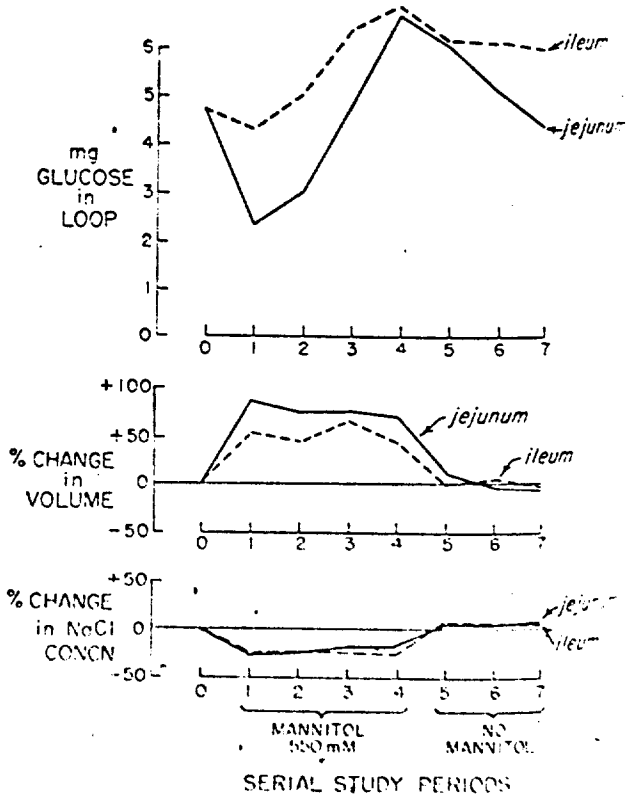

FIG. 4. Hypertonic mannitol (*solution F*) was used for 4 study periods and then substituted by isotonic solution (*solution B*). Glucose absorption did not return to normal (as in Fig. 3), indicating that hypertonic mannitol produced persistent damage.

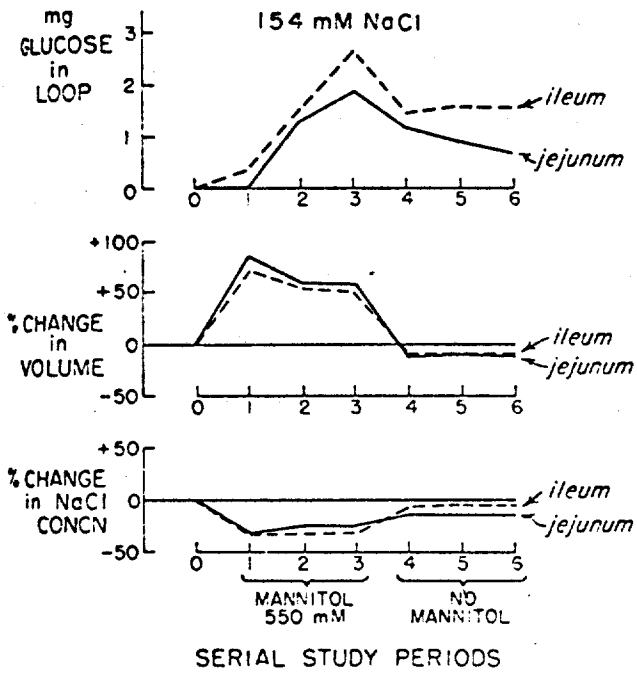

FIG. 5. After 3 study periods with glucose-free hypertonic mannitol and substitution of it with isotonic saline, glucose could still be recovered from the loops, again indicating persistence of damage produced by hypertonic mannitol.

made for study. The changes in the ileum and jejunum were similar for any given solution. Examination of the control biopsies under the dissecting microscope revealed no gross abnormality. Ninety minutes after 10% mannitol in isotonic saline (with or without glucose) the surface epithelium appeared flat. Microscopic examination of the intestinal mucosa indicated lymphatic dilation in all specimens. In the controls (*solution B*), the surface epithelium was uniformly intact after six serial study periods (Fig. 8). Severe injury to the surface epithelium occurred after 90 min of study with 10% mannitol in isotonic saline (with or without glucose, *solutions E* and *F*) (Fig. 9) and the injury was clearly discernable at 30 min (Fig. 10). In all instances, there was an excellent correlation between the finding of surface epithelial injury by light microscopy and impairment of absorption of glucose (Table 2).

DISCUSSION

Hindle and Code (9), in a study of hypertonic mannitol in isolated loops of dog small intestine, observed osmotic equilibration only after 3-4 hr. The 15-min study periods employed here were not sufficiently long to permit osmotic equilibration to occur when hypertonic solutions were tested. Maximum fluid movement occurred in the jejunum and decreased the osmolarity from approximately 880 milliosmols/liter to 560 milliosmols/liter. Thus, the surface epithelium was constantly bathed in a hypertonic solution during the period of these studies.

Glucose absorption from small intestinal segments was rapid when isotonic solutions were studied as has been shown by previous investigators (1, 5, 13). When hypertonic solutions were employed, glucose absorption occurred during the first study period but during the subsequent study periods, progressively increasing amounts of glucose were recovered from the loops usually exceeding the amount put in the segment, indicating net movement of glucose from the blood into the loop. Histological sections taken at various time intervals following the study with hypertonic solutions, indicated definite damage to the surface epithelium, most marked at the tips of the villi. The damage increased in severity with prolongation of the exposure to hypertonic solution (Table 2). Even when glucose-free hypertonic solutions were placed in the loops, glucose could be recovered from them as the epithelial injury developed.

Glucose absorption and the blood-to-lumen barrier to glucose passage seem to occur at two different sites. Glucose absorption was shown by Kinter and Wilson to be carried on by the mature intestinal epithelial cells located at the tips of the villi (11). The resistance to the blood-to-lumen passage of glucose is probably dependent upon the integrity of the entire mucosa. A break in the mucosa at any point might be expected to permit passage of glucose from blood to lumen without interfering with

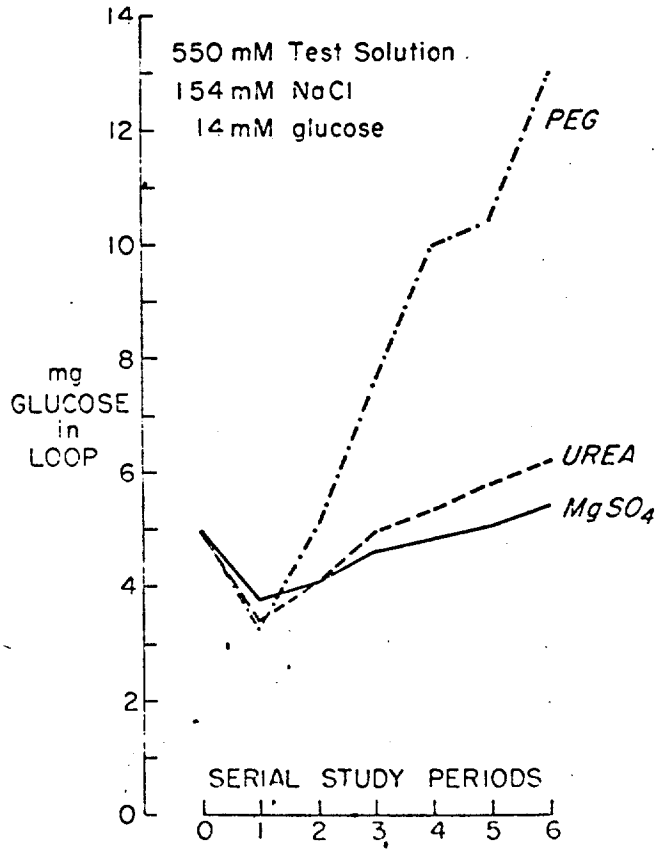

FIG. 6. Glucose recovered from successive incubations with hypertonic solutions of urea, $MgSO_4$, and polyethylene glycol (mol wt 1,000). Ileal loops in three animals were studied with each agent. In all, the amount of glucose recovered during the 6th period is statistically significantly greater than the amount recovered in the 1st period.

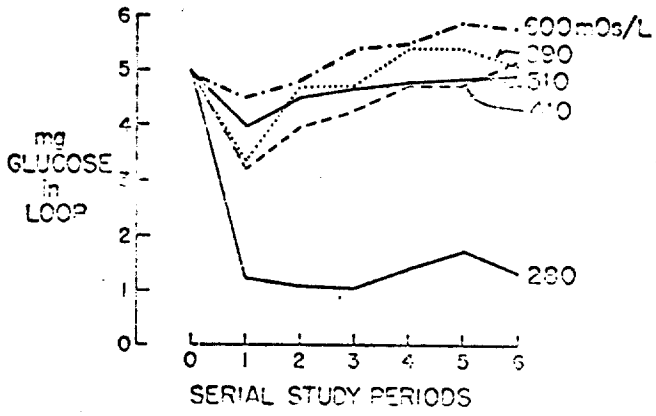

FIG. 7. Isotonic saline containing 14 mM glucose, and mannitol added to bring total osmolarity to the designated figure. Bottom line, marked 290, represents experiments with the control *solution B*. Three animals were studied with ileal loops for each solution. In all studies employing total osmolarity of 710 and higher, the 6th incubation produced significantly more glucose than lower levels.

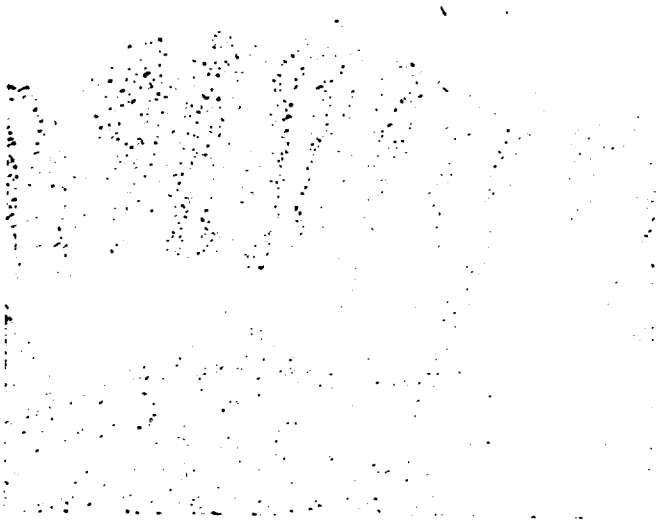

FIG. 8. Intestinal mucosa after 6 study periods with isotonic solution (*solution B*). There was dilation of lymphatic spaces but note that the epithelial cells are intact (104 ×).

glucose absorption. On the other hand, injury without destruction to the more mature epithelial cells might impair absorption without altering the blood-to-luminal barrier.

Marner et al. (12) studied the effect of 50% glucose solution on segments of pig intestine. Although water and sodium values were similar throughout a series of six 15-min incubations as in these experiments, marked histological changes were present after 1 hr. The changes were described as swollen and edematous villi and exfoliated epithelium.

Helb et al. (8) investigated the effects of hypertonic glucose (50% solution) on the absorption of chloride against an osmotic gradient in Thiry-Vella loops of dog ileum. They found an inhibition of the chloride transport after instillation of hypertonic glucose persisting for about 90 min. No histological examination was made.

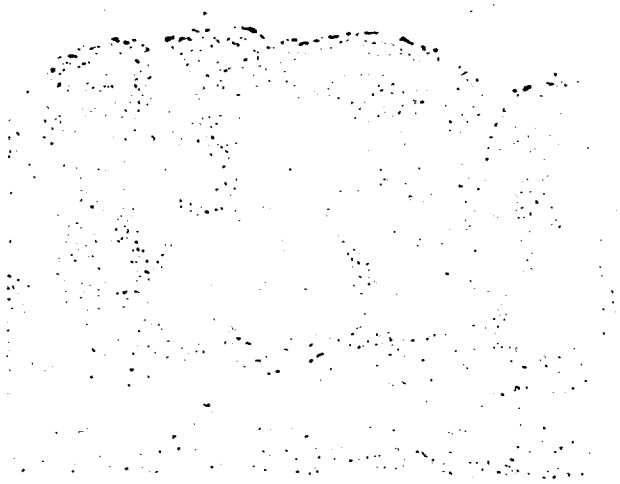

FIG. 9. Intestinal mucosa after 6 study periods with hypertonic mannitol (*solution F*). Note the celiaclike lesion (104 ×).

TABLE 2. *Comparison of impairment of glucose absorption with histological injury after various times of incubation with solution F*

Time Interval, min	Impairment of Glucose Absorption	Surface Necrosis
15	0–1+	1+
30	1+	2+
45	2+	2+
60	3+	3+
90	3+	4+

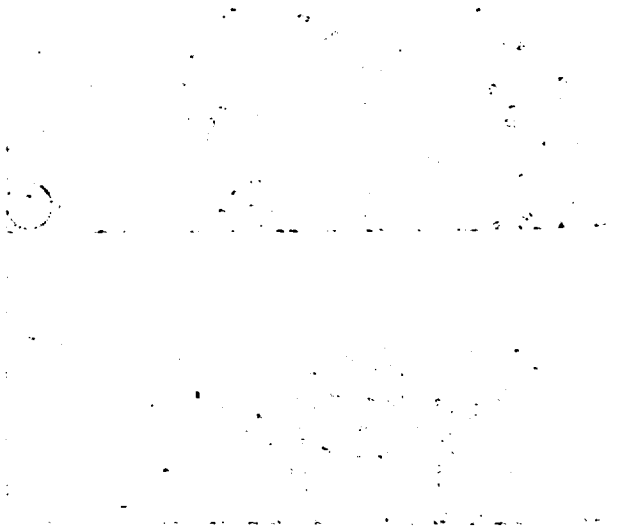

FIG. 10. High power (430 ×) view of the epithelial cells of the tips of the villi after the 2nd study period with isotonic solution and hypertonic mannitol, *solution B*. Note the normal appearance of the cells in *solution A*, in comparison to the deep stain taken by the epithelial cells in *solution B*, plus the loss of the cytoplasm of the cells—indicating that the early injury starts at the tip of the villi where the old cells are located.

Hypertonic mannitol (mol wt 182), urea (mol wt 60), $MgSO_4$ and PEG (mol wt 1,000) produced similar effects on glucose absorption and epithelial histology. Urea probably penetrates the epithelial cell, mannitol may penetrate the cell, but PEG almost certainly does not do so, suggesting that the osmotic injury produced by mannitol and PEG is due to drawing water out of the cell, or out of the cell membrane while the injury produced by urea is secondary to water entering the cell and denaturing the intracellular contents.

The histological sections (as in Fig. 10) of the early lesion showed striking localization of the injury to the cells located at the tips of the villi. These cells are exposed to the most severe osmotic insult, for cells nearer the crypt would quite possibly be exposed to more dilution of the hypertonic solution. Furthermore, the cells at the tip are the oldest on the villus and are near the end of their life span and are, therefore, possibly more susceptible to the osmotic injury. The localization of the injury to these cells apparently leads to a greater impairment of glucose absorption than to water or electrolyte movement. The blood-luminal barrier to glucose passage is so destroyed due to destruction in the epithelium. On the other hand, water and sodium absorption is not progressively impaired with the various solutions as one continues the study, suggesting that the injury, though marked histologically, does not affect the transport of these materials. We, like Hindle and Code (3), find no deviation in water and electrolyte transport with time. Thus, in Figs. 5 and 6, when the hypertonic solution was replaced by an isotonic one, water and sodium absorption was observed, even though glucose absorption had stopped.

The origin of the increased glucose in the lumen following exposure to glucose-free solutions must be considered. The final concentration of glucose in the lumen was occasionally 250–275 mg/100 ml. The additional glucose recovered presumably came from the blood or tissues and represented glucose, not the phosphate esters which are not determined by our method. The blood sugar was measured in representative animals and found to vary from 120 to 200 mg/100 ml. In these animals, although the total luminal content of glucose increased, the luminal glucose concentration was less than the plasma glucose concentration. It is presumed that the same relationship occurred between the final luminal and blood glucose concentration in the rest of the experiments, thus it would appear that passive diffusion in the direction of the concentrated gradient is the basis for finding net increase of glucose in the lumen.

We were not able to demonstrate a change in the absorption of glucose when an isotonic solution containing mannitol was employed (*solution D*), indicating that the impairment of glucose absorption is not due to mannitol per se. Other hypertonic solutions (PEG, urea, $MgSO_4$) behaved like hypertonic mannitol, suggesting that the disordered transport of glucose and the histological injury are due to the hypertonicity of the solution. An electron microscopic injury to renal tubular cells was demonstrated by Discala et al. (4) during osmotic diuresis with mannitol. The intestinal injury we observed by light microscopy was more severe, probably because of the higher osmolarity of the fluid bathing the intestinal epithelium and the greater fragility of the cells at the tips of the villi.

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The Effect of Unabsorbed Sucrose and Mannitol on the Small Intestinal Flow Rate and Mean Transit Time

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Abstract: LAUNIALA, K. The Effect of Unabsorbed Sucrose and Mannitol on the Small Intestinal Flow Rate and Mean Transit Time. *Scand. J. Gastroenterol.* 3, 665-671, 1968.

The effect of different concentrations of unabsorbable solute on the movement of water and electrolytes and on the mean transit time was studied in children by perfusing a 50 cm segment of proximal small intestine. Sucrose was the unabsorbable solute used in a child with congenital sucrose-isomaltose malabsorption and mannitol in three normal children.

In both cases a steady state was approached in which the intestinal fluid was isotonic to the extracellular fluid and contained about 95 mEq/l of Na^+ and about 95 mmoles/l unabsorbable solute and glucose. Concentrations of the test substance exceeding 100 mmoles/l thus caused net secretion of water and electrolytes. This resulted in an accelerated flow in the distal part of the test segment. The flow rate was further increased by shortening the mean transit time, which was proportional to the content of unabsorbable solute.

The findings suggest that in disaccharide malabsorption the increase in intraintestinal volume leads to accelerated transit through the small intestine, in proportion to the quantity of disaccharides ingested.

Key-words: Diarrhoea; disaccharidases; electrolytes; intestine, small; intestinal absorption; movement disorders

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There is evidence to suggest that the osmotic effect of unabsorbed disaccharides is the most important factor in the causation of the diarrhoea of disaccharide malabsorption. In patients with a disaccharidase deficiency the unabsorbed disaccharide will cause considerable movement of water and electrolytes into the small intestinal lumen (Kern & Struthers 1966, Launiala 1968). The only observation of an accelerated transit through the small intestine associated with this increase of intraintestinal volume is a radiological one (Laws & Neale 1966), and the relationship between the amount of unabsorbable disaccharide (or other unabsorbable solute) and the mean intestinal transit time has not previously been analysed.

The object of this investigation is to clarify the effect of different concentrations of unabsorbable solute on the movement of water and electrolytes in the small intestine and on the mean transit time. Sucrose was used as an unabsorbable solute in a child with CSIM* and mannitol in children with normal intestines.

MATERIAL AND METHODS

A 2½-year-old girl with CSIM, (for clinical data see Launiala & Visakorpi 1968), and three mentally retarded children, called 'normal children' in this paper, aged 8 months, 1½ years, and 4 years, who showed no evidence of gastrointestinal disease and had normal findings in a d-xylose and a butterfat loading (Hadorn, Zoppi, Launiala, Shmerling & Prader 1966) test were studied.

Transnasal intubation of the small intestine was performed with bilumen tubing in which the prox-

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* Following abbreviations are used:
CSIM congenital sucrose-isomaltose malabsorption
PEG polyethylene glycol

imal opening was 50 cm from the distal opening. The tubing and the intubation procedure have been described elsewhere (Launiala 1968). The position of the tube was checked by fluoroscopy injecting liquid contrast medium (60% Urografin[R], Schering A.G., Berlin) through the channels. The perfusions were done when the proximal opening was in the duodenum 2-3 cm from the pylorus in the CSIM patient, and in the proximal jejunum in the others. This position was rechecked after each perfusion and no movement was observed on any occasion. No reflux of contrast medium into the stomach was observed in the CSIM patient. After a fasting of at least 8 hours, a continuous infusion of a test solution was started through the proximal opening at a constant rate (3.00 ± 0.07 ml/min), using a roller-type pump. The solution contained 45 mEq/l of sodium, 5 mEq/l of potassium, 50 mEq/l of chloride, 5 g/l of PEG (polyethylene glycol 4000) as unabsorbable marker (e.g. Fordtran 1966) and 0,50, 100 or 150 mmoles/l of sucrose or mannitol for the CSIM patient and for the others, respectively, with glucose added to the total non-electrolyte solute concentration of 200 mmoles/l. The patients had two perfusions with different solutions done on the same day. To obtain a steady state the infusion was continued for at least 60 min before a 0.3 ml bolus of 5% Bromsulphalein[R] (BSP) (Hynson, Westcott and Dunning, Inc., Baltimore, USA) was injected via the proximal opening and timed collection of samples of the effluent (later called 'samples') by slight suction through the distal opening at intervals of 2-3 minutes was begun. A second bolus of BSP was injected 30 to 60 minutes later, once the BSPwas no more visible on alkalinization of the samples. BSP is known to be unabsorbed in the gut (Fordtran, Levitan, Bikerman, Burrow & Ingelfinger 1961, Maddrey, Serebro, Marcus & Iber 1967). The perfusion was stopped after the last sample was obtained.

The dye dilution curve described by Dillard, Lastman & Fordtran (1965) was constructed from the BSP concentration of the samples measured according to O'Brien & Ibbott (1962). Following the peak the dye concentration decreased exponentially as [illegible] by Dillard et al. (1965). The mean transit time was estimated by dividing the area of the dye dilution curve by a vertical line in two halves using planimetry (Ziljlstra & Mook 1962). In 13 double determinations of the mean transit time the values deviated from the mean by an average of 7.3 per cent.

For other analyses samples from one perfusion were pooled. For determination of PEG, the sugars, and mannitol, a $Zn(OH)_2$ precipitation was done adding to one part of the sample four parts of water, two parts of 0.3 N NaOH, and two parts of 5% $ZnSO_4$ solution. The PEG was determined by my modification of the method of Hyden (1955) as follows. To 2 ml of diluted supernatant fluid 0.4 ml of 20% $BaCl_2$ solution was added to precipitate the remaining SO_4 ions. After centrifugation at 10,000 × g for 20 minutes, one part of the supernatant fluid was mixed with one part of trichloracetic acid-$BaCl_2$ reagent of Hyden (1955), and after the mixture had been standing for 45 minutes the turbidity was measured with a spectrophotometer at 450 nm. Standards treated identically to the samples were used as reference. The recovery of varying concentrations of PEG, added to intestinal fluid, was 96-106%, mean 102% (26 exps.), and the SD in 608 double determinations was 2.2%.

Mannitol was analysed by the method of Corcoran & Page (1947), glucose by the Tris-glucose-oxidase method (Dahlqvist 1961), sucrose as glucose after complete hydrolysis with invertase in 0.1 M acetate buffer at pH 4.6 (Bergmeyer & Klotzsch 1962), sodium and potassium with a flame photometer (Eppendorf), chloride by coulometric titration (FIL Chloride Meter), and osmolality with a freezing point osmometer (Fiske Osmometer) after centrifugation of the samples.

The equations used in the calculations were as follows.

$$F = \frac{I \cdot PEG_0}{PEG_x}, \quad K = \frac{C_x \cdot PEG_0}{C_0 \cdot PEG_x} - 1$$

$$M_w = F - I, \quad M_e = K \cdot I \cdot C_0$$

where F is the flow rate at the level of the distal opening, I the infusion rate, PEG_x the concentra-

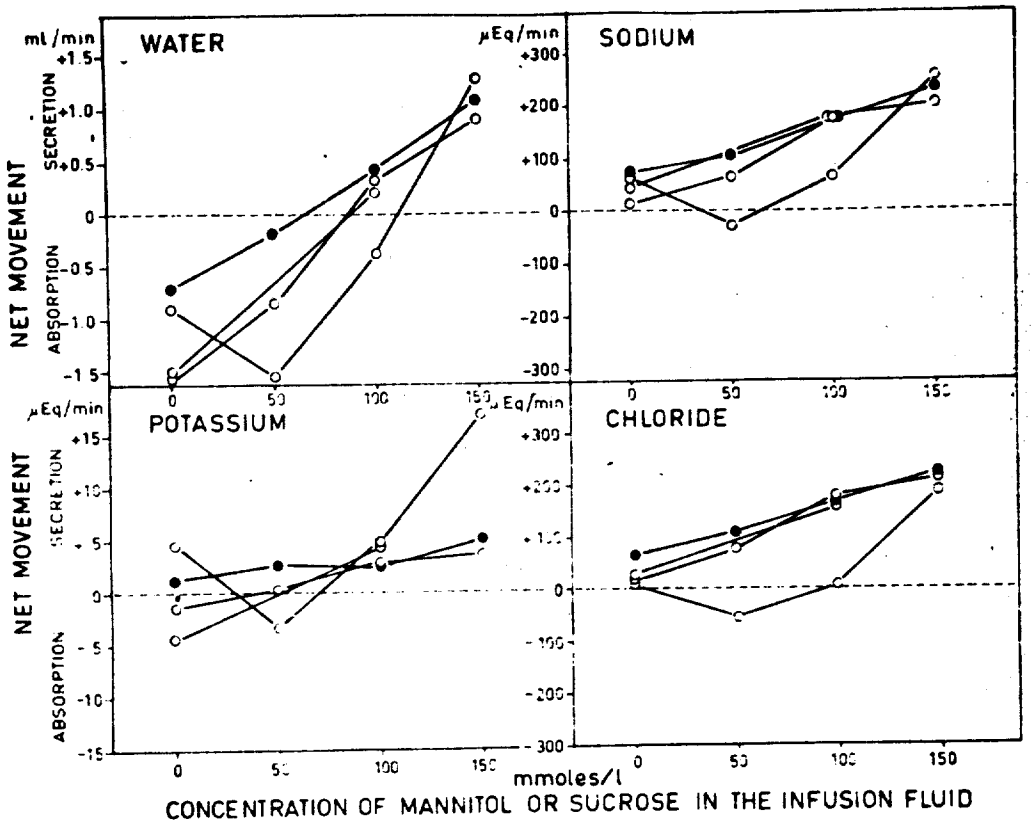

Fig. 1. Net movement of water, Na^+, K^+ and Cl^- in a 50 cm segment of proximal small intestine in 'normal' children (open symbols) and in a child with congenital sucrose-isomaltose malabsorption (black symbols) when the segment was perfused with solutions containing different concentrations of glucose and of mannitol in 'normal' children or sucrose in a child with congenital sucrose-isomaltose malabsorption.

tion of PEG, C_x the concentration of a solute in the sample, PEG_0 the concentration of PEG, and C_0 the concentration of the solute in the perfusion fluid; K is the net movement index, indicating absorption and secretion, M_W is the content of water, and M_e that of electrolytes absorbed from ( ) or secreted into ( ) the test segment.

RESULTS

Most of the glucose was absorbed in the 50 cm test segment of the proximal small intestine, whereas only a little of the mannitol and hardly any sucrose was absorbed (Table I). The presence of mannitol in the perfusion solution in the normal children had an effect on the movement of electrolytes and water identical to that of sucrose in the child with CSIM. There was net secretion of Na^+ and Cl^- in most perfusions even in the absence of unabsorbable solute, and this secretion was enhanced with increasing concentrations of sucrose or mannitol as substituted for glucose in the perfusion solution (Fig. 1). The $[Na^+]$ in the effluent samples varied from 69 to 119 mEq/l (Table II). Thus the net movement of ions was such as to bring the $[Na^+]$ in the luminal fluid to around 95 mEq/l. It thus seemed that the jejunal mucosa was unable to maintain a $[Na^+]$ gradient higher than about 40-45 mEq/l from blood plasma to lumen. The $[K^+]$ of the perfusion solution was close to that of plasma and [illegible] net K^+-

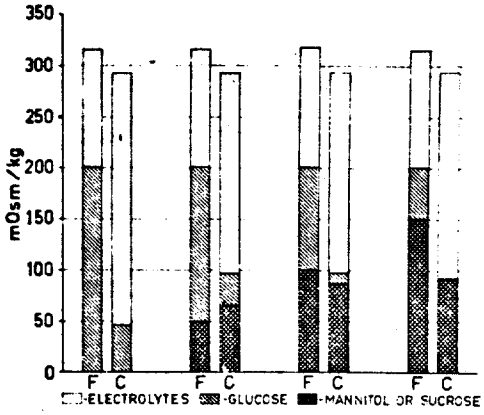

Fig. 2. The proportion of the solutes from the total osmolality of perfusion fluids (F) and samples (C) collected at the end of the 50 cm segment of proximal small intestine perfused with test fluids containing different concentrations of glucose and of mannitol ('normal' children) or sucrose (a child with congenital sucrose-isomaltose malabsorption).

Table I. *Absorption of glucose and of mannitol or sucrose from the test solutions in a 50 cm proximal small intestinal segment of 'normal' children and one child with congenital sucrose-isomaltose malabsorption (CSIM)*

	Concentration of mannitol or sucrose and glucose in the perfusion solutions mmoles/l			
Mannitol or sucrose ...	0	50	100	150
Glucose	200	150	100	50
Glucose* (all subjects)	81**	85	88	97
	(58-97)	(67-99)	(69-100)	(94-100)
	n = 7	n = 4	n = 6	n = 6
Mannitol* (normal children)	–	16	9	18
		(12-21)	(0-23)	(6-23)
		n = 3	n = 4	n = 4
Sucrose* (child with CSIM)	–	8	3	10
			(1-5)	(9-10)
		n = 1	n = 2	n = 2

*absorption in per cent of the amount of the substance in test solution
**mean and range, n = number of tests performed

Table II. *Concentration* of electrolytes in the perfusates*

	Concentration of mannitol or sucrose in the perfusion solutions mmoles/l			
	0	50	100	150
Na, mEq/l	107	87	93	95
	(95-119)	(69-105)	(78-104)	(91-98)
K, mEq/l	8.2	6.8	6.0	5.5
	(6.9-9.5)	(6.2-7.7)	(4.9-7.8)	(4.4-7.5)
Cl, mEq/l	101	84	86	90
	(78-116)	(60-103)	(61-96)	(79-98)

*mean and range

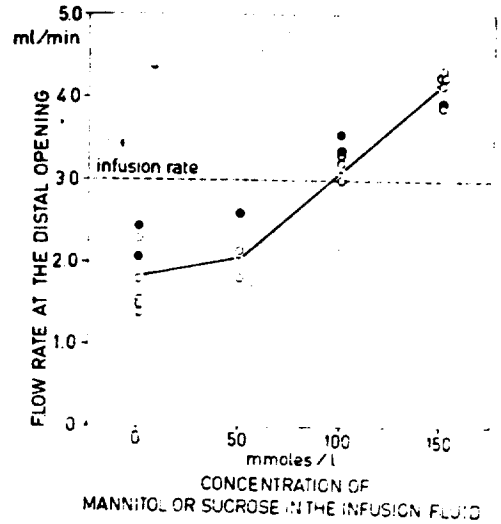

Fig. 3. Flow rates at the end of the 50 cm segment of proximal small intestine when test fluids containing different concentrations of mannitol ('normal' children, open symbols) or sucrose (a child with congenital sucrose-isomaltose malabsorption, black symbols) were infused through the segment.

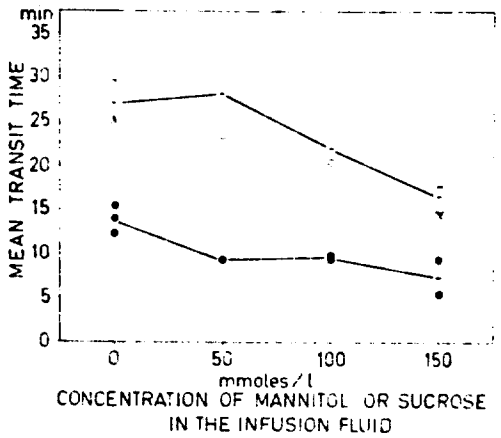

Fig. 4. The mean transit times of the intestinal contents in the 50 cm segment of proximal small intestine when test fluids containing different concentrations of mannitol ('normal' children, open symbols) or sucrose (a child with congenital sucrose-isomaltose malabsorption, black symbols) were infused through the segment.

movement occurred. The [K^+] in samples varied from 4.4 to 9.5 mEq l, mean 6.6 mEq l (Table II). The mucosa thus seemed not to build up any significant [K^+] gradient.

The intestinal fluid always remained isoosmotic to plasma (Fig. 2), i.e. water readily followed the net total solute movement. When sucrose or alternatively mannitol was present in the test fluids, the concentration of this plus glucose varied in the samples from 57.2 to 133.4 mmoles l, mean 94.3 mmoles l (Fig. 2). The steady state situation approached was thus one in which the unabsorbable solute retained a volume of fluid in the lumen threefold the volume of its isotonic solution, two-thirds of the solute being electrolytes.

In perfusion with solutions containing 100 mmoles l or 150 mmoles l of sucrose or mannitol, there was net secretion of water and thus actual increase in the fluid volume proportional to this concentration of unabsorbable solute. The flow rate, i.e. the volume of fluid passing a cross-section per unit time, was at the distal end of the test loop in on the average 1.86 ml min when no unabsorbable solute was present, and 4.14 ml min when the perfusion solution contained 150 mmoles l of unabsorbable solute (Fig. 3).

In Fig. 4 the mean transit times are plotted against the concentration of unabsorbable solute in the perfusion solution. The mean transit time decreased with increasing unabsorbable solute concentration. In the jejunal loop of the normal children it was on the average 28 minutes at zero concentration and 16.5 at 150 mmoles l. The corresponding figures in the loop of the CSIM patient, which included duodenum, were 14 and 7 minutes.

DISCUSSION

Most estimates of the transit time through the small intestine have generally been obtained by a variety of indirect methods (Farrar & Bernstein 1958, Mattson, Perman & Lagerlot 1960, Cornell 1961, Laws & Neale 1966, Park 1967, Rossack Stedeford & [illegible] Dillard 1965 have introduced dye dilution curve method circulatory physiology (eg. Dow 1956 Zierler

1958) into determination of the mean transit time of a segment of small intestine. Although flow conditions in the small intestine are not, even when a solution is being infused at a constant rate, nearly as stable as in the blood circulation, measurement of the mean transit time by means of the dye dilution curve is quite reproducible. The individual values obtained in six consecutive measurements in one and the same person by Dillard et al. (1965) deviated from their mean by an average of 6.6 per cent. In my present 13 double determinations, likewise, the values deviated from the mean by an average of 7.3 per cent.

The presence of mannitol in normal small intestine had an effect identical to that of sucrose in sucrase-deficient small intestine. The fact that the mean transit times through the test segment were shorter in the CSIM patient than in the 'normal' children is probably due to duodenum being part of the test segment of the CSIM patient; normal passage of duodenum is a few seconds (Connell 1961).

When unabsorbable solute was present in the jejunal lumen, the steady state was approached in which the luminal fluid was isotonic to the plasma and [Na^+] about 95 mEq/l, the concentration of the unabsorbable solute thus being about 95 mmoles/l. This is in fair agreement with the observation of Dillard et al. (1965) of inhibition of net water movement in the small intestine by the presence of 70 mmoles/l of mannitol in perfusion solution. I have previously reported concentrations of 60-90 mmoles/l of unabsorbable disaccharide in the proximal jejunum following a test meal containing 150 mmoles/l of the disaccharide in children with congenital disaccharide malabsorption. In addition to the unabsorbability of the disaccharide or mannitol, the physiological basis of this phenomenon lies in the free permeability to water and the relatively poor capability of concentrative Na^+-transport of the jejunal mucosa, which is also reflected in inability to maintain a [Na^+] more than ca. 100 mEq/l in the luminal fluid.

Although accurate calculations are not possible, it seems that the flow rate increased mainly through acceleration of transit and not through widening of the cross-section of the lumen. For example, an increase in the flow rate to 2.2-fold was accompanied by shortening of the mean transit time by 40% (Figs. 3 and 4).

My present results confirm the previous observations that in the diarrhoea of disaccharide malabsorption an increased volume of fluid is retained in the lumen of the small intestine by the osmotic effect of disaccharide (Kern & Struthers 1966, Launiala 1968). This osmotic effect is potentiated by what probably is poor capability of concentrative Na^+-transport of the small intestine so that actually volume is retained which is about three times that of the isotonic solution of the disaccharide. The increase in fluid volume accelerates the transit through the small intestine, this acceleration being proportional to the ingested amount of the unabsorbable disaccharide.

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THE ABSORPTION OF WATER AND SALT FROM THE SMALL INTESTINE OF THE RAT.

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We have previously reported the results of experiments on the absorption of inorganic phosphate from the small intestine of the rat under controlled conditions [McHardy and Parsons, 1956]. During these experiments the net absorption rates of water and some electrolytes were also measured and these findings are described in this paper.

Methods

The experimental technique and the methods of determining the phosphate concentration, pH and tonicity of the fluid circulating in the intestinal segment have been described previously [McHardy and Parsons, 1956].

Analyses.—Sodium and potassium were estimated with a direct-reading flame photometer [Parsons and Cox, unpublished] and chloride by the method of Sendroy [1937]. Glucose was estimated by the method of Hulme and Narain [1931], and urea by the Kjeldahl procedure on protein-free filtrates of the circulating fluid.

At the end of each experiment the circulated segment was removed from the animal and its length and wet weight were measured; after six hours drying in an oven at 105°–110° C. the dry weight was measured and the water content found by subtraction. At the end of certain experiments blood was taken from the heart with anaerobic precautions and the plasma obtained was used for electrolyte determinations.

Calculation and Expression of Results.—In calculating the absorption rate of water, phosphate was used as a "volume indicator" as described for sugars by Fullerton and Parsons [1956]. the initial and final volume of circulating fluid being determined from the initial and final phosphate content and concentration. The absorption rates of the various solutes were calculated from the initial and final concentrations and the initial and final volumes of circulating fluid, due allowance being made for the quantities and volumes removed in sampling. The net absorption rates were expressed as microlitres of water, or as microequivalents or micromoles of solute absorbed per milligram dry weight of intestine per hour (μl., μEq, or μM/mg./hr.). Mean values were derived from

experiments on at least six animals, including at least twelve one-hour absorption periods.

The detailed composition of the fluids used for circulation is given elsewhere [McHardy and Parsons, 1956].

Results

Effects of Hydrogen-ion Concentration.—In the first sets of experiments (Series I), fluids having mean initial pH values of 4·4, 5·6, 6·7, 7·2 or 7·9 were circulated through jejunal segments. Solutions at pH 7·2 and 7·9 were also circulated through ileal segments. All the solutions contained 160 mEq/l. Na and were buffered with 50 mM/l. phosphate. The experimental results are set out in fig. 1 and Table I.

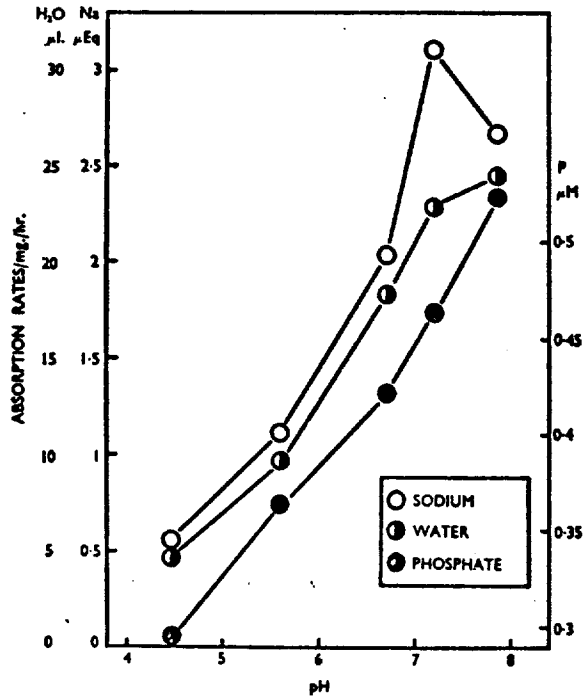

Fig. 1.—Showing the mean absorption rates of water and sodium found when solutions of differing initial pH were circulated in jejunal segments. For comparison the absorption rates of phosphate [McHardy and Parsons, 1956] are also shown.

It can readily be seen that the more alkaline the fluid circulated the greater was the net absorption rate of water, which increased fivefold in jejunal segments from 4·6 µl./mg./hr. at mean initial pH 4·4 to 24·4 µl./mg./hr. at pH 7·9. In ileal segments the rate increased slightly from 18·6 µl./mg./hr. at pH 7·2 to 20·3 µl./mg./hr. at pH 7·9.

TABLE I.—THE MEAN NET ABSORPTION RATES OF WATER, Na and Cl FOUND WHEN SOLUTIONS OF DIFFERING pH, ALL CONTAINING 160 mEq/L. Na AND BUFFERED WITH 50 mM/L. PHOSPHATE, WERE CIRCULATED. NOMINAL pH IS pH CALCULATED FROM THE PHOSPHATE CONTENT, MAKING NO ALLOWANCE FOR EFFECTS OF GASSING WITH CO_2. THE TONICITY OF 160 mM NaCl SOLUTION IS 1 UNIT.

Fluid No.	No. of periods	Tonicity, units	mean pH			Absorption rates, per mg. dry wt. per hour			Nominal conc. of Cl : mEq/l.
			Nominal	Initial	Final	H_2O : μl.	Na : μEq	Cl : μEq	
A. *Jejunal segments*									
11	12	0·99	4·5	4·4	5·6	4·6 ± 1·2	0·56 ± 0·16	0·38 ± 0·14	110
12	13	0·96	5·8	5·6	5·9	9·6 ± 0·6	1·21 ± 0·15	0·78 ± 0·09	105
13	12	0·91	6·9	6·7	6·6	18·2 ± 1·0	2·03 ± 0·16	0·72 ± 0·11	80
14	18	0·81	*ca.* 8·6	7·2	7·0	22·9 ± 1·2†	3·11 ± 0·19	0·48 ± 0·09	60
15*	12	0·81	8·0	7·9	7·1	24·4 ± 1·8	2·67 ± 0·32	0·56 ± 0·13	62·5
B. *Ileal segments*									
14	13	0·81	*ca.* 8·6	7·2	7·2	18·6 ± 1·4	2·21 ± 0·26	1·13 ± 0·14	60
15*	12	0·81	8·0	7·8	7·5	20·3 ± 1·6	2·02 ± 0·25	1·16 ± 0·13	62·5

* Gassed with air only. † Mean of 30 periods.

Over the pH range 4·4 to 7·2 a similar marked increase in the net absorption rate of sodium, from 0·56 μEq/mg./hr. at pH 4·4 to 3·11 μEq/mg./hr. at pH 7·2, was found in jejunal segments. In contrast to the behaviour of water absorption and also of phosphate absorption [McHardy and Parsons, 1956] no further increase in sodium absorption was found between pH 7·2 and 7·9; in both jejunal and ileal segments the rate declined slightly.

The absorption rate of chloride was nearly twice as great at pH 6·7 as at pH 4·4, though the concentration of Cl was 30 mEq/l. less at the higher pH level; but as there was a different concentration of Cl in each of the five fluids, it is impracticable to comment on the absorption rates of this ion in terms of the hydrogen-ion concentration alone.

Effects of Tonicity and Sodium Concentration.—In a further series of experiments in which the effect of reducing the sodium concentration was studied, the circulating fluids were buffered at a nominal pH of 6·9 with 25 mM/l. phosphate; the absorption rates observed were compared with those found from a solution containing 160 mEq/l. Na, 120 mEq/l. Cl, and having tonicity 0·92 units (the reference solution). The results of the experiments are set out in Table II.

In jejunal segments, when a solution having half the tonicity of the reference solution and containing 80 mEq/l. Na and 40 mEq/l. Cl was circulated, there were considerable changes in the absorptive pattern. The absorption rate of water increased by an amount not statistically significant, but the absorption of sodium and chloride was replaced by a marked net entry of both substances into the intestinal lumen. In ileal segments, when the solution of half-tonicity and salt concentration was circulated water absorption increased ($p < 0·05$), there was no net movement of sodium but absorption of chloride occurred, although at about a quarter of the rate found from the reference solution.

Effects of Non-electrolytes.—It will also be seen from Table II that when solutions containing 80 mEq/l. Na and 40 mEq/l. Cl but with the tonicity restored to 0·94 units by the addition of 148 mM/l. mannitol, were circulated through jejunal segments, the mean absorption of water was zero (0·2 ± 0·9 μl./mg./hr.), the entry of sodium into the intestinal contents practically ceased and the Cl entry was greatly reduced. In ileal segments the water absorption ceased, sodium absorption began to occur, but the chloride absorption rate was the same as from the half-isotonic solution.

In further experiments with jejunal segments, additions of 37 mM/l. of glucose, urea or mannitol, chosen as substances absorbed at different rates, were made to the solution. The results of these experiments are shown in Table III, together with the absorption rates of glucose and urea. The rate of absorption of mannitol was not measured.

When the solution containing 37 mM/l. mannitol was circulated, the absorption rate of water compared with that from the reference solution

TABLE II.—THE MEAN NET ABSORPTION RATES OF WATER, Na AND Cl FOUND WHEN FLUIDS OF DIFFERING TONICITY AND SALT CONCENTRATION, ALL CONTAINING 25 mM/L. PHOSPHATE BUFFER AT NOMINAL pH 6·9, WERE CIRCULATED. A NEGATIVE VALUE OF ABSORPTION INDICATES NET APPEARANCE OF THE SUBSTANCE IN THE INTESTINAL LUMEN.

Fluid No.	No. of periods	Description of fluid. Name	Concentrations of Na (mEq/l.)	Concentrations of Cl (mEq/l.)	Tonicity, units	Absorption rates per mg. dry wt. per hour: H_2O : μl.	Na : μEq	Cl : μEq
A. *Jejunal segments*								
31	12	Reference	160	120	0·92	18·1 ± 1·8	1·91 ± 0·20	1·25 ± 0·21
32	16	Half-tonicity	80	40	0·46	23·4 ± 1·8	−0·34 ± 0·14	−0·84 ± 0·10
33	12	Tonicity restored with mannitol	80	40	0·94	0·2 ± 0·9	−0·08 ± 0·10	−0·48 ± 0·07
B. *Ileal segments*								
31	12	Reference	160	120	0·92	18·8 ± 2·0	2·74 ± 0·10	2·50 ± 0·11
32	12	Half-tonicity	80	40	0·46	27·5 ± 2·6	0·17 ± 0·16	0·60 ± 0·18
33	12	Tonicity restored with mannitol	80	40	0·94	1·7 ± 1·8	0·35 ± 0·14	0·63 ± 0·17

was greatly reduced (from 18·1 to 2·3 μl./mg./hr.) and that of Cl virtually abolished. The sodium absorption rate was also considerably reduced below the reference level.

The absorption rates from the solution containing glucose did not differ significantly from those found with the reference solution, though the sodium absorption rate was slightly increased. With the urea-containing solution the water and chloride absorption rates were reduced to less than half the reference level, but the Na absorption was almost identical with that from the reference solution though less than that

TABLE III.—THE MEAN NET ABSORPTION RATES FROM JEJUNAL SEGMENTS OF WATER, SODIUM AND CHLORIDE FOUND WHEN ADDITIONS OF 37 mM/L. GLUCOSE, UREA OR MANNITOL WERE MADE TO THE REFERENCE SOLUTION CONTAINING 160 mEq L. Na, 120 mEq L. Cl AND 25 mM/L. PHOSPHATE AT NOMINAL pH 6·9. EACH VALUE THE MEAN OF OBSERVATIONS ON 12 EXPERIMENTAL PERIODS. THE TONICITY OF FLUID 31 WAS 0·92 UNITS, AND OF THE OTHER FLUIDS 1·04 UNITS.

Fluid No.	Additions	Absorption rates, per mg. dry wt. per hour			
		H_2O : μl.	Na : μEq	Cl : μEq	Glucose or urea : μM
31	None	18·1 ± 1·8	1·91 ± 0·20	1·25 ± 0·21	..
34	37 mM/l. glucose	16·7 ± 0·8	2·46 ± 0·19	1·40 ± 0·12	2·22 ± 0·09
35	37 mM/l. urea	8·6 ± 1·2	1·92 ± 0·16	0·54 ± 0·18	0·69 ± 0·19
36	37 mM/l. mannitol	2·3 ± 1·0	1·19 ± 0·27	0·07 ± 0·14	..

from the glucose-containing solution. The absorption rate of urea itself was about one-third of the rate of glucose absorption found with an identical concentration of glucose.

Comparison of Jejunum and Ileum.—Jejunal and ileal absorption rates could be compared in five sets of experiments, and the results of these comparisons are set out in Table IV.

It can be seen that under most circumstances there was not any tendency for greater absorption of water from either region. With sodium the important finding was a greater tendency towards entry into the jejunum when the concentration in the circulating fluid was halved to 80 mEq/l., the difference between the two regions being significant ($p < 0·05$) under these conditions.

In contrast to this was the finding of consistently greater chloride absorption rates in the ileum, under all conditions studied. There was, for example, marked absorption from solutions containing about 40 mEq/l. Cl, whereas net entry of Cl into the jejunum took place.

Plasma Electrolyte Concentrations.—The concentrations of sodium and chloride were measured in the plasma at the end of many of the experiments, and it was thus possible to correlate these values with the concentrations in the intestinal lumen and the absorption rate of each electrolyte. The results of these comparisons are set out in Table V.

TABLE IV.—THE MEAN DIFFERENCES (JEJUNAL-ILEAL) IN THE NET ABSORPTION RATES OF WATER, Na and Cl BETWEEN SEGMENTS CIRCULATED WITH FLUIDS OF THE SAME COMPOSITION. STATISTICALLY SIGNIFICANT DIFFERENCES ARE MARKED WITH AN ASTERISK *. NEGATIVE DIFFERENCES INDICATE A GREATER ILEAL ABSORPTION RATE. FOR FLUID 14 THE WATER ABSORPTION DATA ARE BASED ON 30 PERIODS IN JEJUNAL SEGMENTS, MAKING A TOTAL OF 43 PERIODS.

Fluid No.	No. of periods			Composition of fluid			Mean differences		
	Jejunal	Ileal	Total	Tonicity, units	Conc. of Na, mEq/l.	Conc. of Cl, mEq/l.	H_2O, μl./mg./hr.	Na, μEq/mg./hr.	Cl, μEq/mg./hr.
14	18	13	31	0·81	160	60	4·3 ± 2·1*	0·90 ± 0·31*	− 0·66 ± 0·16*
15	12	12	24	0·81	160	62·5	4·1 ± 2·4	0·65 ± 0·40	− 0·60 ± 0·18*
31	12	12	24	0·92	160	120	− 0·8 ± 2·6	− 0·75 ± 0·37	− 1·25 ± 0·41*
32	16	12	28	0·46	80	40	− 4·0 ± 3·1	− 0·52 ± 0·21*	− 1·44 ± 0·14*
33	12	12	24	0·94	80	40	− 1·4 ± 2·0	− 0·42 ± 0·17*	− 1·11 ± 0·18*

TABLE V.—THE VALUES OF THE PLASMA Cl AND Na CONCENTRATIONS AT THE END OF TWO HOURS' CIRCULATION COMPARED WITH THE MEAN CONCENTRATION IN THE CIRCULATING FLUID. THE MEAN NET ABSORPTION RATES OF EACH ION ARE SHOWN, MARKED WITH AN ASTERISK IF SIGNIFICANT ABSORPTION HAS TAKEN PLACE FROM A SOLUTION WITH CONCENTRATION LESS THAN THAT IN THE PLASMA.

Fluid No.	No. of animals	Chloride: Circulating fluid Nominal mEq/l.	Chloride: Circulating fluid Mean mEq/l.	Chloride: Plasma conc. mEq/l.	Chloride: Absorption rate, mEq/segment/2 hrs.	Sodium: Circulating fluid Nominal mEq/l.	Sodium: Circulating fluid Mean mEq/l.	Sodium: Plasma conc. mEq/l.	Sodium: Absorption rate, mEq/segment/2 hrs.
A. *Jejunal segments*									
Control	8	..	..	104 ± 1	..	..	..	152 ± 2	..
32	8	40	46	84 ± 1	−0·44 ± 0·07	80	82	128 ± 2	−0·19 ± 0·10
33	6	40	41	89 ± 1	−0·38 ± 0·06	80	73	140 ± 3	−0·06 ± 0·08
34	5	120	119	101 ± 1	0·82 ± 0·16	160	154	149 ± 4	1·47 ± 0·27
B. *Ileal segments*									
32	6	40	43	89 ± 1	0·37 ± 0·06*	80	82	135 ± 3	0·16 ± 0·08
33	6	40	37	98 ± 1	0·37 ± 0·12*	80	72	145 ± 3	0·20 ± 0·09*
31	6	120	118	104 ± 2	1·37 ± 0·09	160	155	151 ± 1	1·59 ± 0·07

Control determinations of plasma Na and Cl, made on animals which were anæsthetized but not circulated, are also shown in the table.

It was of interest to record the conditions under which significant absorption of Cl or Na took place when the concentration in the lumen was less than that in the plasma, the so-called "absorption against a gradient". It will be seen from the table that this occurred from ileal segments, with Cl when fluids containing nominally 40 mEq/l. Cl (Nos. 32 and 33) were circulated, and with Na when fluid containing nominally 80 mEq/l. Na and 148 mM/l. mannitol was circulated. In the experiments with fluids containing around 60 mEq/l. Cl (Nos. 14 and 15, Table I) no plasma chloride determinations were made, but there was considerable absorption from both jejunum and ileum, the lumen concentration being between 70–80 mEq/l. This is lower than the lowest plasma concentrations found under unfavourable circumstances when chloride was entering the lumen, so there must be a strong presumption that Cl absorption against a gradient was taking place, not only in the ileum but also in the jejunum.

Hydration of the Intestinal Wall.—In a set of control experiments with animals which had been anæsthetized but not circulated, the water content of intestinal segments was found to be linearly related to the dry weight. The parameters of this relation are given in Table VI,

TABLE VI.—THE PARAMETERS OF THE LINEAR RELATIONS BETWEEN THE WATER CONTENT AND THE DRY WEIGHT, AND BETWEEN THE WATER CONTENT AND WATER ABSORBED.

	Jejunum	Ileum
A. Control Segments		
Water content and dry weight		
Number of segments	10	9
Mean dry weight: mg./cm.	9·4 ± 0·4	9·4 ± 0·3
Mean water content: mg./cm.	28·8 ± 1·0	28·7 ± 1·0
Slope of regression line of closest fit: mg. water/mg. dry weight	1·96 ± 0·44	2·51 ± 0·72
Intercept of line: mg. water/cm.	10·39 ± 4·16	5·44 ± 6·61
Result of F test for linearity	F = 20·15 (1, 8) $p < 0·01$	F = 12·3 (1, 7) $p < 0·01$
B. Absorbing Segments		
Water content and water absorbed		
Number of segments	48	25
Slope of line of closest fit: mg. increase in water content/ml. absorbed in 2 hours	12·0 ± 1·7	14·8 ± 1·8
Intercept of line: mg. water content/mg. dry weight	2·97 ± 0·04	3·10 ± 0·06
Result of F test for linearity	F = 48·2 (1, 46) $p < 0·001$	F = 64·1 (1, 23) $p < 0·001$

and it will be seen that the water content and dry weight were the same in the jejunum and ileum and that the dry weight accounted for about 25 per cent of the total weight.

In segments which had been circulated there was a well-marked linear relation in both jejunum and ileum between the water content of the segment at the end of two hours' circulation and the volume of water absorbed during that time. This relation is set out in Table VI and illustrated in fig. 2. It will be seen that in both the jejunum and

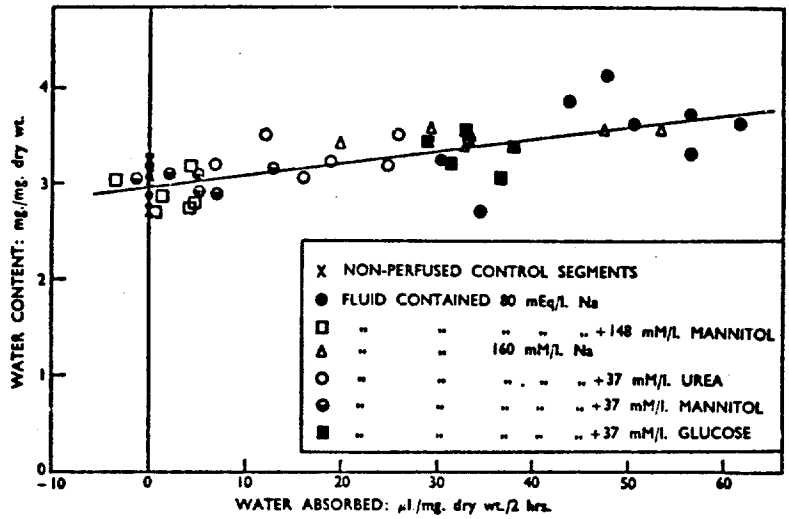

FIG. 2.—Showing the linear relation between water content of jejunal segments and the volume of water absorbed by or entering the segments during two consecutive absorption periods. The regression line of closest fit is shown.

the ileum about 1·5 per cent of the volume absorbed appeared in the intestinal wall as an increase in water content at the end of two hours. It is also apparent that the water content was highest after circulation with the half-isotonic fluid (No. 32); and it will be recalled that there was considerable absorption of water from this fluid but that Na entered the jejunum and was not absorbed in the ileum. The water content of the whole intestinal wall would thus seem to be related to the net water absorption rather than to the net absorption of Na.

If it is assumed that the control segments absorbed little water, it will be seen that the vertical scatter of the control points on the graph is comparable to that of the circulated segments.

Discussion

As we have pointed out previously [McHardy and Parsons, 1956], it is impossible to prepare a series of solutions differing only in a single

variable, if a buffer such as phosphate is used. The various absorption rates should therefore be thought of in terms of the solution circulated, rather than of any one variable.

Effects of Hydrogen-ion Concentration.—The marked increases in the absorption rates of water and sodium with increasing alkalinity are immediately apparent. If the results are considered in terms of tonicity also, it is noticeable that large increases in absorption rate coincided with large differences in pH and small differences in tonicity (*e.g.* between solutions 11 and 12, pH difference 1·2 units; tonicity difference 0·01 tonicity units). It would seem probable that the absorption rates were more closely influenced by the pH of the circulating fluid than by the tonicity.

There was no evidence, in the range studied, for an optimum pH for water absorption. This is similar to the findings for phosphate absorption. The absence of any increase in Na absorption between pH 7·2 and 7·9 suggests that an optimum pH may lie in this range, but in the absence of results with solutions at still higher pH values it is impossible to state this categorically. Ponz and Larralde [1950] have reported an optimum for glucose absorption by rat jejunum at pH 7·0.

The effect of the hydrogen-ion concentration on chloride absorption is obscured by the differences in chloride concentration between the various solutions, but the finding that the absorption rate was nearly twice as great at pH 6·7 as at pH 4·4, though the concentration was about 30 mEq/l. less at the higher pH, suggests that an effect was present.

The magnitude of the pH effect is striking. Parsons [1956] has suggested that part of the sodium absorption in the intestine may take place through a sodium-hydrogen ionic exchange, and it might be surmised that such a process would be inhibited by increasing the H^+ concentration. A comparable finding in the case of sodium is that of Schoffeniels [1955], who has shown clearly that both the influx and net transport of Na ions across frog skin are dependent upon the pH of the bathing solution on either side, both the net transport and the inward flux being reduced in the presence of increasing H-ion concentrations. The effect of decreasing pH on water movement in the present experiments could be explained as a secondary consequence of the reduced salt movement if, as is discussed below, net water absorption occurs as a result of a primary salt absorption.

Effects of Non-electrolytes: Water Absorption.—Water absorption from solutions containing different non-electrolytes varied greatly. The presence of mannitol at a concentration of 148 mM/l. almost completely inhibited water absorption from isotonic solutions in both jejunum and ileum, and at a concentration of 37 mM/l. in the presence of 160 mEq/l. Na there was again almost negligible water absorption from the jejunum. These effects and the differing water absorption rates from solutions of

the same tonicity and ionic composition containing glucose, urea or mannitol can be explained as osmotic effects.

The important factor is the osmotic pressure of substances which cannot penetrate the mucosal cells, as opposed to the tonicity of the solution as a whole. The less readily a substance is absorbed, the more effective is its osmotic pressure in opposing water absorption. The water absorption rates in Table III can be seen to be roughly in proportion to the absorption of glucose and urea and to that of mannitol if we assume that its absorption rate is very small [Höber and Höber, 1937].

This phenomenon has been described in several animal tissues. Jacobs and Stewart [1947], noting the effect of sucrose in preventing the colloid-osmotic hæmolysis of erythrocytes made abnormally permeable to cations, remark that "A substance of this sort may be effective in such low concentrations as at first sight to suggest that it must have some non-osmotic protective effect on the cell-surface". The phenomenon has been observed in kidney slices by Deyrup [1953] and in isolated rat intestine and butanol-treated leucocytes by Wilson [1954]. Both these authors point out that the tissues swell in saline solutions and shrink in solutions of non-penetrating non-electrolytes alone.

The theory can be correlated with our observations in which the degree of hydration of the intestinal wall was found to be closely related to the water absorption rate, which in its turn is influenced by osmotic restraint. It is worth noting that the increase in water content *in vivo* was only 1·5 per cent of the water absorbed. This may be partly due to the efficient lymphatic drainage of the intestine, which has been shown to increase threefold in rats when isotonic saline was present in the intestine [Borgström and Laurell, 1953]. Nevertheless, the fact that the water content of the intestinal wall increased means that water was being absorbed more rapidly than it could be removed by the circulatory system and the lymphatics.

Effects of Non-electrolytes: Salt Absorption.—The other striking finding, which can also be seen from the data in Table III, was that in the presence of 37 mM/l. mannitol the absorption of Cl, and also that of Na, was greatly reduced from solutions in the jejunum containing concentrations of these ions greater than those in the plasma. In order to offer an interpretation of this effect we must assume one of two possible modes of net absorption. In the first, net water absorption might be consequent upon osmotic differences set up by solute absorption. In the second, solute absorption would follow a primary water absorption process, such as entrainment of solutes in a water stream. If a mechanism of the first type is assumed, the restraint on water absorption by 37 mM/l. mannitol might be sufficient to permit the establishment of local concentration gradients (for example at the lumen border of the cells) due to primary solute uptake. Net solute transport would then

be reduced either because the solute absorption processes in the jejunum have a limited capacity for doing work against a gradient, or because of an increased "leak" down the gradient into the lumen. In comparing the jejunum and ileum, we present evidence that the ability of the jejunum to perform net absorption against a concentration gradient is limited.

It has already been seen that net Cl absorption occurred "against a gradient" from solutions in the jejunum containing initially about 60 mEq/l. Cl, and in which the concentration did not rise above 80 mEq/l. (Fluids 14 and 15, Table I). But these fluids were alkaline (pH 7·2 and 7·9), which may increase the capacity of the systems responsible for moving solutes or reduce the leak, and contained no non-electrolytes which might restrain water movement. It may thus be imagined that in this instance the uptake of Cl was accompanied by sufficient water to maintain in the steady state a reasonable gradient across the critical boundary. The high Na absorption rate along a favourable concentration gradient in these experiments might further assist Cl uptake by favouring water movement from the intestinal lumen.

In experiments with fluids containing 80 mEq/l. Na and 40 mEq/l. Cl, the effect of mannitol appeared to be to reduce net entry of Na and Cl and, in the ileum, even to favour net Na uptake. In the absence of measurements of the unidirectional rates of movement to and from the intestinal contents, it is not possible to say whether this effect is due to increased activity of the process leading to salt uptake or not.

Comparison of Jejunum and Ileum.—The experiments provide further details of the differing absorptive patterns of the upper and lower small intestine. The variation in activity towards glucose has been expressed in the form of gradients by Fisher and Parsons [1950], but as no exact measurements of the position of the segments were made, the present results cannot be expressed in this form.

In contrast to the jejunum, the ileum proved markedly superior in the absorption of Cl under all conditions studied, and especially in moving Cl ions against a concentration gradient (Table II, B), a phenomenon which is well known for dog intestine [Visscher *et al.*, 1944]. From hypotonic solutions in the ileum there was no net entry of salt, and when the tonicity was restored with mannitol appreciable sodium absorption occurred (Table II, B).

Little difference in water and sodium absorption can be found at the two sites. The large entry of sodium into hypotonic jejunal contents bears out Visscher's dictum that "The jejunum is obviously unfitted for the absorption of sodium against a concentration gradient . . . and the ileum is able under optimum conditions to accomplish positive net movement" [Visscher *et al.*, 1944]. This statement was based on the results of experiments with ^{24}Na in chronic segments in dogs, which

showed that the permeability to Na of the intestinal wall from blood to gut decreased from jejunum to colon. A similar state of affairs seems to exist in the rat.

Relation between Water and Solute Absorption.—In certain experiments it was possible to estimate approximately the rate of absorption

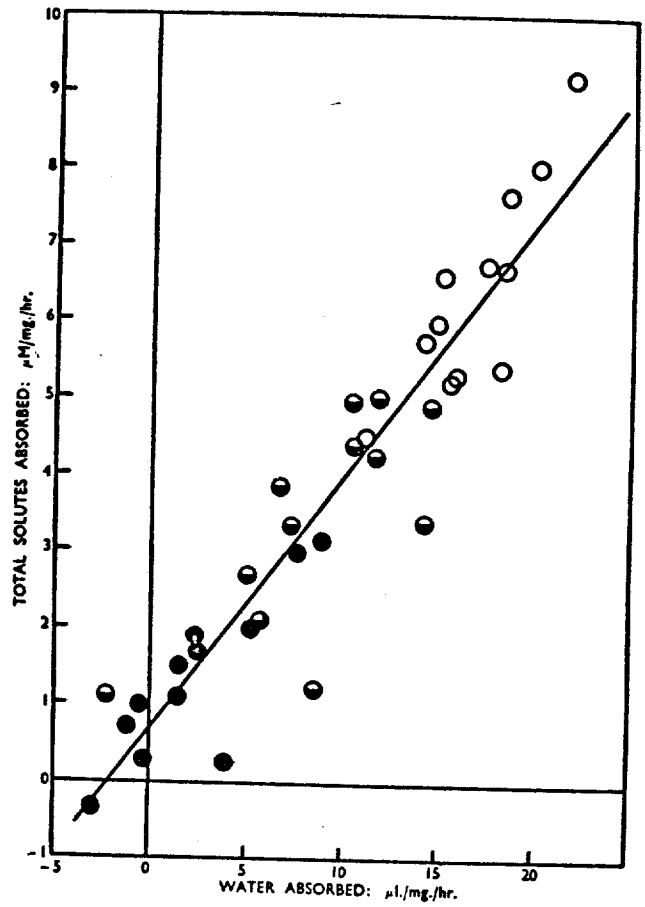

FIG. 3.—Showing the linear relation between the total solutes absorbed and the volume of water absorbed by jejunal segments. Circulated fluid, reference solution with addition of 37 mM/l. glucose (○), urea (●), or mannitol (◒). The calculated regression line is also added.

or appearance of all the important solutes in the circulating fluid. Assuming that only sodium, chloride, phosphate, potassium and bicarbonate, with glucose and urea when present, were involved in the exchanges across the intestinal wall and that mannitol was not absorbed, the algebraic sum of the absorption rates could be taken and an estimate obtained of the net gain or loss of solutes to the animal. This was expressed in μOsM/mg./hr., assuming unit ionic activities.

A statistically significant ($p < 0{\cdot}01$) linear relation was found between the total absorption of solutes and the absorption rate of water in each set of experiments. An example of this is shown in fig. 3, in which the absorption rates found in the jejunum with fluids containing 37 mM/l. of added glucose, urea or mannitol are plotted together. It can be seen that the points lie around a straight line passing close to the origin, the position along the line depending on the non-electrolyte added. The slope of the line of closest fit in this relation was 331 ± 20 mOsM/l. and represents the apparent concentration at which the solutes were absorbed. It agrees well with the nominal concentration of solutes in the lumen (342 mOsM/l.). This observation means that the fluid leaving the intestine was approximately isotonic with that in the lumen. We have already described two possible modes of net absorption, in which either solute or water absorption was the primary process. Only if the secondary process lagged appreciably behind the primary process in either instance could the net fluid leaving the intestine have a tonicity different from that in the lumen. It is thus impossible from the data to distinguish the two modes of absorption. Either might occur, with the secondary absorption rapidly following the primary process.

Further interpretation must require that the measurements of net absorption be resolved into components representing movement in each direction across the intestinal mucosa.

Summary

1. The net absorption rates of water and sodium, measured *in vivo* in the jejunum and ileum of the rat, were greater from alkaline than from acid solutions, over the pH range 4·4–7·2.

2. Chloride was absorbed more rapidly from the ileum than from the jejunum under all conditions studied.

3. Chloride was absorbed by the ileum from solutions containing between 20–50 mEq/l. less than plasma. Sodium was absorbed by the ileum from isotonic solutions containing between 45–75 mEq/l. less than plasma, but no absorption occurred from hypotonic solutions.

4. The decreasing absorption rates of water from similar solutions containing glucose, urea or mannitol are accounted for in terms of relative osmotic restraint on absorption.

5. The water content of the intestinal wall was found to be linearly related to the dry weight of the segment and to the amount of water absorbed by the segment. About 1·5 per cent of this amount remained in the intestinal wall after two hours circulation.

6. The net uptake of Na and Cl from isotonic fluids and the net entry into hypotonic fluids in jejunal segments were both reduced when mannitol was added to the solutions.

7. With solutions containing 160 mEq/l. sodium in the jejunum, the fluid absorbed was approximately isotonic with that in the lumen.

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Mannitol Absorption and Metabolism in Man

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Abstract: Mannitol-^{14}C was administered orally in doses of 25 to 100 g to five subjects who had cirrhosis of the liver and to five subjects who had normal liver function. Mannitol was also assayed by a chemical method and measurement of radioactivity in urine and stool. A mean of 17.5% of the mannitol was recovered in the urine. The total amount recovered in the stool and urine averaged 50% in 48 hours; an average which indicated appreciable loss by metabolism. As much as 17% of the orally administered radioactivity was estimated to be in the form of expired $^{14}CO_2$ in 12 hours. After mannitol-^{14}C was administered intravenously, almost all was recovered in the urine and stool. As the dose of orally ingested mannitol was increased, more frequent loose stools occurred. When diarrhea occurred, a higher percentage of the ingested dose was found in the stool. These results indicate that at least one-sixth of orally ingested mannitol is absorbed and about one-third is metabolized.

KEY INDEXING TERMS:

Mannitol absorption — *Mannitol metabolism* — *Oxidation of mannitol to CO_2*
Osmotic diarrhea — *Oxidation of hexosels*

Studies of intestinal physiology in man and animals have assumed that mannitol is a nonabsorbable molecule which remains intact in the intestine.[1,2] Yet there have been contradictory reports, particularly in animal studies, regarding the absorption and metabolism of mannitol. In 1883 Jaffe demonstrated the absorption by feeding dogs mannitol and recovering large quantities in the urine.[3] Other investigators subsequently have demonstrated absorption of mannitol in animals.[4-7] On the other hand, there are reports in the literature that deny absorption in animals[8-10] and humans,[2] although metabolism of mannitol has been most extensively studied in animals.[2-5,11,12] This paper will demonstrate that mannitol is absorbed and metabolized in man.

Methods

Patients and Materials

Sixteen human volunteers (age 20 to 66) were hospitalized in a clinical research unit for the study. Seven had cirrhosis of the liver and one had Wilson's disease. Eight had no clinical or

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laboratory evidence of hepatic dysfunction or malabsorption; their primary diagnoses were alcoholism (two subjects), resolving pneumonia, essential hypertension, healed duodenal ulcer, and inactive tuberculosis (two subjects). One subject was normal.

Chemically pure unlabeled d-mannitol and uniformly labeled ^{14}C-d-mannitol were provided by Atlas Chemical Industries, Inc. The ^{14}C-d-mannitol was purified by column chromatography using the middle migrating 20% for these studies. This material migrated as a single spot in water-isopropanol (1:4) chromatography, and the mean recovery of radioactivity from elution of this spot was 99.0%. The specific activity of mannitol was 8 μc/mmole.

Human Studies

Ten subjects were utilized in experiments with oral administration. If mannitol was recovered in the urine, then absorption was considered to have taken place. If the total amount of mannitol recovered in the urine and stool over several days was less than the amount ingested, then the discrepancy was interpreted as indicating that the mannitol had been metabolized.

Six additional subjects were studied to show directly that the mannitol had been metabolized by the appearance of radioactive CO_2 in the expired air after oral or intravenous administration of mannitol-U-^{14}C. The production of radioactive CO_2 after oral administration was compared with the production after intravenous administration to determine the relative importance of the intestine to portal vein route of absorption.

After an overnight fast, ten patients ingested mannitol trace labeled with 10 μc of mannitol-U-^{14}C as a 5% solution over a 30 minute period. Four subjects had total urine and stool collections for 96 hours; analysis revealed that little or no radioactivity was recovered in the stool or urine after 48 hours (mean 0.6%, maximum 2.2% of ingested dose). Therefore, all subsequent studies consisted of two 24 hour collections of stool and urine. During the study all ten patients were confined to a metabolic ward to assure completeness of collections; creatinine determinations on all urine collections indicated that the collections were complete. Eight of the ten subjects were given both a small and a large dose of mannitol in sequential studies to determine if absorption is dose related. Doses greater than 40 g generally produce diarrhea, which may lessen absorption.[13]

After the oral administration studies were completed, six additional subjects were studied to determine the specific activity of expired CO_2 after they received mannitol. Three subjects were given 10 g of mannitol labeled with 10 μc of mannitol-U-^{14}C intravenously as a 10% solution. Three other subjects were given 50 g of trace labeled mannitol orally. All six subjects had samples of the expired CO_2 collected in 1 mmole of Hyamine, according to the method of Kaihara and Wagner.[14] Collection of 1 mmole of CO_2 was made immediately before the administration of mannitol and at 30 or 60 minute intervals afterwards for as long as 12 hours. Specific activity values for expired CO_2 were converted to total radioactivity excreted by assuming a CO_2 production of 600 mmoles CO_2/hour.[15] Each group of three subjects consisted of two with normal liver function and one with cirrhosis. The two normal subjects who were taking mannitol orally had hourly samples of blood taken for measurement of radioactivity.

Chemical and Radioactive Methods

The quantity of mannitol was determined in all urine, stool, and standard solutions by the method of Corcoran and Page.[16] In this reaction, formaldehyde is generated from mannitol and subsequently is used to develop a spe-

cific color. Nonmannitol formaldehyde generation is significant in urine, blood and particularly stool. The nonmannitol formaldehyde generation in urine and blood is fairly constant in the same patient from day to day, permitting the use of urine or blood not containing mannitol for a blank correction.

In the stool, the value for nonmannitol color producing material may vary over a fivefold range from day to day. This blank value may represent as much as 5 g of mannitol/sample and assumes greatest statistical importance in those samples with the least amount of true mannitol. A stool blank for purposes of this study was determined for each patient by collecting a 24 hour stool and diluting it to the same degree as the mannitol containing stools.

The recovery of mannitol by the chemical method from water and from a single urine is 100% with mean replicate variability of 2%. The recovery from a homogenate of stool is 94% with mean replicate variability of 5%.

Mannitol was crystalized from the urine and stool of six subejcts by the following method. A measured amount of stool homogenate, 30 to 50 g in an equal amount of water or urine, 30 to 50 ml, was filtered through a thin layer of activated charcoal in a Buchner funnel. The filtrate was evaporated to near dryness under vacuum in a rotary evaporator. An aliquot was taken from the concentrated specimen and crystallized by the addition of 100% ethanol at 4°C. The recovered crystals were dissolved in four volumes of warm water and recrystallized by the addition of ethanol at 4°C. The recovered crystals were identified as mannitol from their shape and from their chromatographic behavior on paper in water-isopropanol (1:4) solvent.

The radioactivity on test solutions, urine and serum, was measured by the following method: 160 μL samples were pipetted into a 20 ml glass counting vial containing 1 ml of 1 mmole Hyamine Hydroxide. Seventeen milliliters of scintillation solution (500 ml of toluene, 500 ml of dioxane, 300 ml of methanol, 104 g of naphthalene, 6.5 g of 2.5 diphenloxadole and 130 mg of 1,4 bis[5-phenyl-2-oxazolyl]benzene) were then added. The mixture was cooled and counted in a Packard Tri-Carb Liquid Scintillation Counter.

All stool samples were thoroughly homogenized to a semiliquid by adding a measured volume of water when needed. Stool radioactivity was determined by the combustion technique of Kelly and associates,[17] as modified by Abei and Iber.[18] Recovery of added ^{14}C by this technique averages 95%. The counting vials used to collect expired CO_2 were treated with 17 ml of scintillation solution and then counted.

In Vitro Studies

To determine the quantitative importance of bacterial breakdown, a 1% solution of mannitol trace labeled with mannitol-U-^{14}C in agar broth solution was inoculated with pure cultures of *Staphylococcus aureus, Streptococcus fecalis,* or fresh whole stool from four different patients. Each incubation mixture was placed in a flask sealed with a stopper to which a small cup containing Hyamine was attached. Quadruplicate samples and distilled water controls were placed in a shaker for 24 hours at 37°C. At the end of this time, marked turbidity had occurred, indicating bacterial growth. The culture medium was acidified to pH 2 with HCl and shaken for 30 minutes to assure evolution of CO_2. The Hyamine cup was placed in a counting vial to measure the ^{14}C activity.

Results

The recovery of mannitol in urine after oral ingestion was significant in all subjects. Whether the recovery was determined by radioactive or chemical methods, the data, as presented in Table 1, are quite similar; 17.5 and

TABLE 1

Fecal and urinary recovery of orally ingested mannitol in 48 Hours

Subject	Diagnosis	Dose (g)	% Total Recovery		% Urinary Recovery		% Stool Recovery	
			Isotope	Chemical	Isotope	Chemical	Isotope	Chemical
1	Resolving pneumonia	42.0	86.6	58.5	15.6	24.6	65.0	73.8
		100.4*	72.1	72.2	14.6	8.2	57.5	64.0
2	Wilson's disease	48.0	32.2	32.3	17.4	16.7	14.8	15.5
		98.0	22.7	17.3	13.2	8.5	9.5	8.7
3	Cirrhosis	55.0*	70.8	79.5	22.3	19.4	48.5	60.5
		72.6*	46.5	46.2	14.3	15.8	26.2	30.4
4	Normal	42.2*	59.9	34.4	23.5	27.3	36.4	7.1
5	Alcoholism	84.6*	75.4	62.5	4.2	2.3	71.2	60.2
6	Alcoholism	46.0*	50.5	63.2	27.0	28.2	23.5	35.0
		68.0*	69.3	160.0	28.2	36.6	41.3	67.8
7	Cirrhosis	42.0	24.6	26.4	13.5	11.9	8.1	14.5
		68.0	29.2	38.8	8.7	7.1	20.5	31.7
8	Essential hypertension	28.0					16.2	38.0
		76.0*					31.1	38.1
9	Cirrhosis	32.0	56.0	156.6	18.1	23.2	37.9	70.8
		60.0	38.0	69.1	7.1	13.3	31.1	55.8
10	Cirrhosis	28.0	23.8	44.1	21.3	39.5	2.5	4.6
		84.0*	52.6	25.2	25.9	16.3	26.7	8.9
Average ±SD			50.0 ±20.0	57.0 ±27.0	17.5 ±7.0	19.1 ±11.0	31.5 ±19.0	38.1 ±24.0

* Patient developed diarrhea.

able blank values on stool when no mannitol is present. Studies on jejunal and ileal aspirates free from mannitol also indicate variable amounts of color in the Corcoran and Page method for mannitol. From these data we conclude that results on stool, utilizing the Corcoran and Page chemical method, are less accurate than the radioactivity measurements and will discuss our data largely from the radioactivity determinations.

A substantial amount of mannitol was recovered in urine after oral ingestion (mean 17.5%) indicating appreciable absorption. Such absorption has previously been reported in animals[6,7] but has not been observed during perfusion experiments of the jejunum or ileum in man.[2] We have no direct data on the site of absorption; the graph depicting the rise in blood radioactivity with time (Figure 1) shows a rise over the first two hours followed by a plateau of two to four hours. Such a slow rise and prolonged elevation suggest that the mid or lower small intestine is the site of absorption. Fordtran et al. did not observe such absorption in perfusion experiments.[2] In their experiments, they utilized the Corcoran and Page method for mannitol analysis to determine disappearance from the fluid in short study periods. It is possible than an increase in nonmannitol chromogenic material replaced small amounts of mannitol that were absorbed, but this is speculation.

The overall recovery of mannitol in 48 hours was 50% in stool and urine. The large amounts excreted in the first 24 hours and the progressively smaller amounts on subsequent days make it very unlikely that additional intact mannitol remained in the body. The other half of the mannitol seems to have been converted into smaller molecules that were not excreted either in the urine or stool. Metabolism of mannitol to carbon dioxide by either intestinal bacteria or the experimental subject seemed a reasonable means to explain this loss. The specific activity of expired CO_2 increased for eight hours after oral ingestion (Figure 2), indi-

FIGURE 1. THE PLASMA RADIOACTIVITY LEVEL AFTER ORAL ADMINISTRATION OF TRACE LABELED MANNITOL IN TWO SUBJECTS. Note that [illegible] to five hours

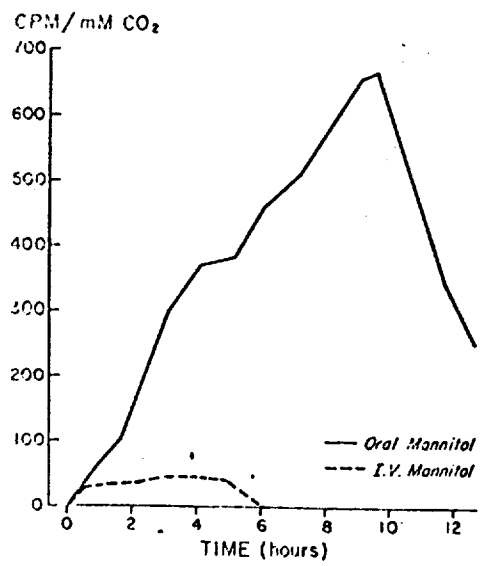

FIGURE 2. THE SPECIFIC ACTIVITY OF THE EXPIRED CO_2 AFTER INTRAVENOUS AND ORAL ADMINISTRATION OF MANNITOL. Note that the peak value of CO_2 was not reached until nine to 10 hours after oral ingestion

cating significant production of radioactive CO_2. Total CO_2 production was not measured, but assuming a production of 600 mmoles/hour for resting man, a value of 18.7% of the administered dose was recovered as radioactive CO_2/12 hours. Even if the estimate of CO_2 production was in error, large amounts of $^{14}CO_2$ have been demonstrated to follow mannitol ^{14}C ingestion.

In contrast, very little $^{14}CO_2$ is produced after intravenous administration (Figure 2), with 2.5% in the six hours the maximum estimate. We are not able to fully explain this difference; however, three factors seem pertinent. The amount of mannitol oxidized by the body organs (possibly liver) may be proportional to both the concentration reaching that organ and the duration of exposure. The dose of mannitol given intravenously (10 g) was less than that given orally (50 g), and the intravenous dose presumably persisted for a shorter period of time because it is excreted at the full glomerular filtration rate by the kidney.[19] In contrast, the oral dose rises and falls more slowly (Figure 1). A second explanation is found in the experiments of Wick et al., in a study of mannitol oxidation in rats and its recovery as CO_2.[6] They found 2 to 3% of the mannitol as CO_2 after intravenous administration, 50% as CO_2 after oral administration, and 70% after intrasplenic administration (directly into the portal venous system). They postulated direct oxidation by the liver increased following the exposure of the liver to the higher concentration. A third possibility is that intestinal organisms split the mannitol and allow for its subsequent absorption and further metabolism.

Our data on incubation of mannitol in culture medium with stool or specific organisms suggest only that the breakdown of mannitol to CO_2 at concentrations present in the bowel in our study subjects was limited. It is known that bacteria, such as *Staphylococcus aureus* and *Streptococcus fecalis*,[20,21] are capable of oxidizing mannitol. Under the conditions of our in vitro experiments, the production of CO_2 by bacteria was too little to account for the events in man.

Experiments in rats led Wick et al. to conclude that mannitol is oxidized by the liver.[6] One might suppose that patients with liver disease would have impaired oxidation compared to subjects with normal liver function. Analysis of our data indicates no significant difference between the fraction of the ingested dose that was metabolized (i.e., the ingested dose minus the portion recovered in urine and stool) in the seven patients with liver disease and the five patients with normal liver function. The two patients who metabolized mannitol least (hence had higher portion of the dose recovered) did not have known liver disease. Our subjects with cirrhosis were stable and all had serum bilirubin levels of less than 3.0 mg/100 ml.

Diarrhea produced by oral administration of mannitol depends on the dose of mannitol and the amount of that dose remaining unabsorbed in the stool. Patients who had diarrhea excreted a higher percent of the dose in the stool (42.4% of the ingested dose) than those without diarrhea (25.4%). The fraction of the dose recovered in the stool in those patients with diarrhea (42.4%) was lower than that found in our previous studies (62.0% by chemical method).[22] However, the dose used in our previous studies was 200 g, at least twice the dose used in these studies. In the present study there is a relationship between the dose of mannitol ingested and the amount of mannitol appearing in the stool; even though the fraction of the dose in the stool does not increase. Since mannitol produces diarrhea by its osmotic activity, it follows that higher dosages will produce more diarrheal fluid.

From these studies it can be estimated that approximately 65% of the

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FAT AND GLUCOSE MALABSORPTION AFTER HYPERTONIC MANNITOL

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Hypertonic solutions impair glucose absorption and produce histological injury to the intestinal mucosa of rats (1). The reversibility of these changes was not determined, and no previous efforts were made to evaluate the effects of hypertonic solutions on other parameters of intestinal absorption. The purpose of this paper is to study the effect of hypertonic mannitol solutions on intestinal absorption of fat and to determine the reversibility of the functional and histological injury produced by hypertonicity.

MATERIALS AND METHODS

Preparation of Intestinal Loops:

Twenty-one male Wistar rats, weighing 350-450 gm were fasted for 24 hours prior to the study. Under light ether anesthesia the abdomen was opened through a mid-ventral incision. The duodenum and the jejunum were identified. A small incision was made in the first part of the duodenum about 2 cm proximal to the common bile duct. The flanged end of a polyethylene tube (PE 200) was introduced through this incision and secured in place with a ligature. Another small incision was made in the proximal ileum and a similarly flanged polyethylene tube was ligated in place. The blood supply as well as the biliary and pancreatic drainage were carefully preserved. The abdomen was closed with silk sutures, anesthesia was discontinued, and the animals were restrained (2).

Fat absorption studies:

In 12 experiments the intestinal segment was perfused with a solution of 10% mannitol in 0.5% saline (600-950 mOsm/L) which was delivered through a Harvard pump at the rate of 1 ml/min for 60 minutes. In 9 control experiments the intestine was perfused with isotonic saline at the same rate and for the same period of time. At the end of the perfusion period 2 ml of labeled fat emulsion were slowly instilled into the intestinal segment over a five minute period. The test solution was prepared as follows: 1 ml of a triglyceride emulsion, Lipostrate CB (Ediol, A grade-Calbiochem), and 2μc of ^{14}C-triolein (New England Nuclear Corp.) in 100μl of benzene were added to 19 ml of distilled water. The benzene was evaporated by blowing air over the solution. The mixture was thoroughly homogenized in a Virtis 23 homogenizer at high speed. Samples (10μl) were transformed from the stock solution to counting vials before and after

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19.1%, respectively. In the majority of the patients receiving two doses the recovery of radioactivity in the urine was about the same fraction of the total ingested dose at each of the two dose levels. Overall, a little less than one-fifth of the mannitol appeared in the urine; indicating significant absorption occurred.

The recovery of mannitol in the stool proved more variable from patient to patient. By the radioactivity measurements, 31.5% of the ingested dose was found in the stool in all patients and 49.9% in those with marked diarrhea. Those with diarrhea had significantly more mannitol in the stool than those without diarrhea ($p < 0.05$). The recovery of mannitol in the stool determined by the chemical test was much higher than the mannitol determined by radioactivity (mean 130%, $p < 0.05$).

The total amount of mannitol recovered in urine and stool over 48 hours was usually much less than the dose administered with an average recovery of 30% of the ingested dose by radioactivity measurements and 57% by chemical measurements. The excretion of radioactivity in the urine and stool was rapid. In the four patients studied for four days, a mean of 94% of the four day total was excreted in the first day (minimum 91%) and less than 1% by the third day. No radioactivity was detected after the third day. In the 18 studies reported in Table 1, more than 90% of the radioactivity was recovered in the first 24 hour stool and urine collection.

Specific Activity

The specific activity of mannitol recrystallized from the urine and stool of six subjects is shown in Table 2. Specific activity is expressed as a percent of the specific activity of the administered mannitol. The average specific activity of the mannitol crystals from the urine was 96.4% and from the stool 108.2%. Neither value ($p < 0.2$ and $p < 0.35$, respectively) is significantly different from the material administered. On the other hand, when the

TABLE 2

*Comparison of specific activity of crystals and whole specimen**

Subject	Dose (g)	Sp. Act. Urine: Crystals (%)	Sp. Act. Urine: Whole Urine (%)	Sp. Act. Stool: Crystals (%)	Sp. Act. Stool: Whole Stool (%)
1	19.0	67.0	63	103.0	87
	199.4	122.0	128	111.0	95
2	48.0	87.5	104	115.0	93
	98.0	124.0	154	115.0	109
3	39.0	106.0	108	102.0	80
	72.0	86.0	90	No Cryst.	84
4	44.2	100.0	81	104.0	67
5	34.0	92.5	121	91.5	121
6	46.0	86.0	94		
	67.0	90.0	85	99.0	61
Mean		96.4	102.8	108.2	88.5
± SD		14.8	± 26.3	± 20.4	± 19.3
p Value Compared to Standard		$p < 0.25$	$p < 0.40$	$p < 0.15$	$p < 0.05$

* Values as Percent of Standard

specific activity is calculated on either the whole urine or stool specimens, values of 102% in the urine and 88.5% in the stool were obtained. The values for whole urine and for recrystallized mannitol from urine are not significantly different ($p < 0.10$), but the values of the stool are statistically different ($p < 0.05$).

Blood Radioactivity

In two subjects, ^{14}C activity significantly above background was detected in blood following oral administration of labeled mannitol. A peak seemed to be reached after five to six hours followed by a decline (Figure 1).

Expired CO_2

After orally administered mannitol, the cumulative radioactivity in the expired CO_2 in six hours was 2.5 and 4.5% of the ingested dose, and the specimen taken at six hours indicated that the excretion was still rising (Figure 2). In one subject, CO_2 was collected over 12 hours and 18.7% of administered dose was recovered as CO_2. However, with intravenous administration only 1.0, 1.1, and 2.5% of the administered dose were recovered in six hours, and the levels were falling.

Bacterial Breakdown of Mannitol to CO_2

Incubation of mannitol-U-^{14}C with *Staphylococcus aureus* or *Streptococcus fecalis* led to recovery of only 1% of the dose as $^{14}CO_2$. Incubation with whole fresh stool led to recovery of $^{14}CO_2$ at the level of background readings; there was no significant production in our experiments in 24 hours.

Discussion

Two independent methods were employed for the detection of mannitol, a chemical one and a radioactive one. A variety of naturally occurring substances give rise to formaldehyde and the subsequent color in the Corcoran and Page mannitol method,[16] and these substances provide particular problems in measuring mannitol recovery in serum, urine, and stool. On the other hand, the presence of radioactivity in the stool and urine demonstrates only the presence of the carbon skeleton of the mannitol and does not indicate what chemical form it is in. We would anticipate from our study of the methods that the radioactivity measurements would be more reliable and specific.

To further evaluate this specificity, mannitol crystals were obtained and purified from the urine in ten specimens and from the stool in eight specimens (Table 2). The specific activity of these crystals was not significantly different from that of the ingested mannitol, indicating that they are unaltered, ingested mannitol. The specific activity was calculated for the whole urine and was the same as that for the ingested material. This similarity indicates that in urine there was no significant quantity of radioactivity that was not mannitol and that the chemical mannitol measured by the Corcoran and Page method is not contaminated with nonmannitol color. In marked contrast, the specific activity of whole stool was significantly lower than the specific activity of the ingested material. This finding suggests that there were increased amounts of formaldehydegenic material in the stool that was not adequately corrected by the blank method. If the data in Table 1 are calculated so that the stool recovery by the chemical methods is expressed as a percentage of the radioactivity recovery, then the mean value is 130.7%.

We believe that the marked variability between the data derived from the radioactive method and that derived from the chemical one results from the marked variability of chemical determinations of mannitol in the stool. This variability in turn stems from the vari-

ingested mannitol is absorbed in the dose range from 40 to 100 g. Doses above 40 g are more likely to produce diarrhea than smaller doses. Of the absorbed mannitol, about one-third is excreted intact in the urine and the remainder is oxidized, presumably by the liver to CO_2.

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drawing out the 2 ml test solution to check the homogeneity and stability of the emulsion. The fat emulsion was stable for at least one hour since counts of aliquots at various time intervals agreed within 5%. Two hours after the labeled emulsion was placed in the intestine the common bile duct was ligated and the whole intestinal loop was removed (3). Its contents were measured into a graduated cylinder and then the intestine was washed with 10 ml of chloroform:methanol (2:1) solution.

Labeled lipids were extracted by the method of Folch (4). To each ml of solution recovered from the intestinal segment, 20 ml of chloroform:methanol (2:1) solution were added. After adequate shaking, the extraction mixtures were allowed to stand for 30 minutes and were then filtered. Four ml of water were added for each 20 ml of starting chloroform:methanol solution. A separatory funnel was used to collect the chloroform layer. After its volume was measured, 5-10 ml aliquots were pipetted into counting vials and evaporated to dryness under a stream of air.

In 6 control and 7 mannitol experiments the remainder of the chloroform phase was used to determine the distribution of radioactivity among various lipid subclasses. The chloroform phase was dried under nitrogen and the lipids were dissolved in 0.2 ml of benzene. Aliquots (10-20 µl) were chromatographed on silica gel G thin layer plates. The solvent system consisted of hexane:diethylether:glacial acetic acid (70:20:1) and good separation was achieved after 45 minutes. A standard mixture of fatty acids, tri-, di-, and monoglycerides (Applied Science Laboratories, Incorporated) was run on each plate. Lipid classes were located by spraying with Rhodamine 6G and identified by comparison of their movement with that of the standards. Areas containing labeled fatty acids and labeled glycerides were scraped from the plate into counting vials.

The dried chloroform:methanol extracts were dissolved in 17 ml of scintillation fluid (500 ml of toluene, 500 ml of dioxane, 300 ml of methanol, 104 gm of naphthalene, 6.5 gm of 2,5 diphenyloxazole and 130 mg of 1,4 bis-(5-phenyl-2-oxazolyl) benzene. Silicic acid scraped from plates was suspended in 10 ml of scintillation fluid. Radioactivity was assayed in a Packard Tri-Carb Scintillation Spectrometer.

Reversibility of the functional and histological injury:

Twenty male Wistar rats were studied. Functional reversibility of the lesion was studied by following glucose absorption. A preliminary effort to follow reversibility of fat absorption was discontinued because of the higher operative mortality.

The experimental procedure was similar to that used for fat studies except that an 8-10 cm segment of the mid jejunum was cannulated with polyethylene tubes (5). In 14 experiments the segment was perfused for 1 hour with 10% mannitol in isotonic saline at the rate of 1 ml/min; 6 control intestinal segments were perfused with isotonic saline at the same rate and for the same time. A 2 ml test solution, containing 5 mg glucose in 0.9% saline, was placed in the jejunal loop for 30 minutes and then recovered by gently flushing the loop with air and collecting the solution in a 5 ml graduated cylinder. At the end of the experiment a small biopsy was taken from each loop and placed in buffered formalin in all but four of the experimental rats. In the latter group, the continuity of the intestine was reestablished by anastomosing the loop to the small intestine. [illegible]

[illegible]ls were placed on clear fluids for 48 hours and then fed a regular diet. Glucose absorption in the [illegible] mannitol treated loop was tested in three rats 7 days and in one rat 30 days after surgery. Biopsies were taken at the end of the experiment and the animals were sacrificed. Glucose concentrations were determined by the reducing technique of Hoffman modified for the autoanalyzer (6). Ten to fifteen serial histological sections were made on each biopsy and stained with hematoxylin and eosin.

Fat and glucose absorption were calculated from the difference between the administered dose and the quantities recovered.

RESULTS

In these animals whose intestine was perfused with normal saline and then injected with 2 ml of a triglyceride lipid, 0.5-2.0 ml of clear fluid was recovered from the intestinal segment. In contrast, 6-12 ml of turbid fluid was recovered from those animals first perfused with 10% mannitol in normal saline. After perfusion with saline, absorption averaged 82% of the administered lipid, compared with only 48% absorption after mannitol perfusion (Fig 1). Fat absorption after saline perfusion was significantly different from that after mannitol perfusion, with $P < 0.025$.

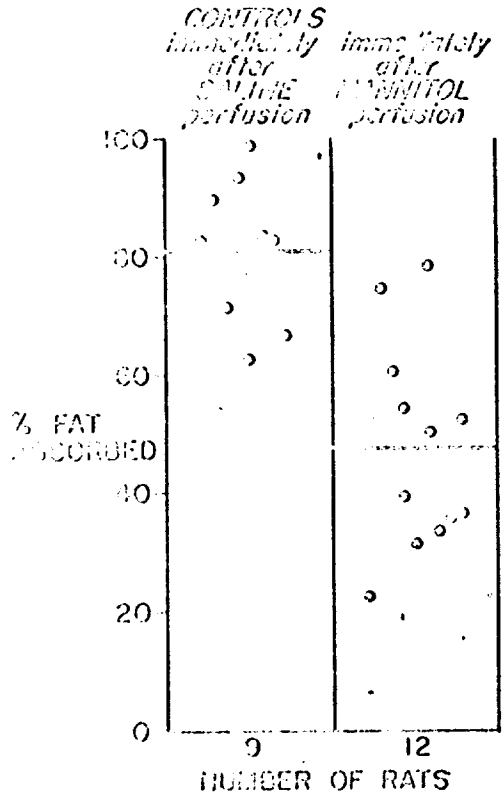


Fig 1. Comparison of the [illegible] of fat absorption after mannitol perfusion and in control rats perfused with saline. Each dot represents a single experiment.

Analysis of the individual fat components recovered from the intestinal lumen revealed striking differences between the saline and mannitol-perfused animals. Table I shows that more than 1/3 of the labeled triolein was recovered as di- and triglycerides after mannitol perfusion compared to a recovery of only 8% in higher glycerides in the control rats. There was also a significantly greater amount of unabsorbed fatty acid and monoglyceride in the mannitol-treated rats.

TABLE I

Distribution of Unabsorbed Labeled Lipids Among Lipid Subclasses*

	Saline Perfusion**	Mannitol Perfusion+	P
Fatty acids	6.0 ± 3.4	15.6 ± 5.4	< .0025
Monoglycerides	3.2 ± 2.1	10.2 ± 4.2	< .005
Fatty acids + monoglycerides	9.2 ± 5.3	25.3 ± 9.3	< .0025
Diglycerides	2.6 ± 1.3	12.4 ± 5.1	< .0025
Triglycerides	5.4 ± 3.1	23.7 ± 8.6	< .0005
Diglycerides + triglycerides	8.0 ± 4.1	36.1 ± 11.4	< .0005

*All values are expressed as the percentage of the administered labeled triolein recovered in each lipid fraction after the absorption period.

**Values are averages from 6 experiments ± S.D.

+Values are averages from 7 experiments.

Glucose absorption proceeded normally after isotonic saline perfusion, averaging 89% of the administered dose. While after perfusion with hypertonic mannitol there was an average absorption of only 32% (Fig 2). When mannitol-treated rats were allowed to recover for seven days, there was full return of intestinal absorptive capacity; an average of 85% of the administered glucose was absorbed (Fig 2).

Figure 3 shows a representative biopsy taken after treatment with hypertonic mannitol. The villi are short and blunt with loss of polarity of the epithelial cells and necrosis at the tips of the villi. Figure 4 is a representative biopsy taken seven days after treatment with hypertonic mannitol. The villi are tall and fingerlike and the epithelial cell architecture again appears normal.

DISCUSSION

Earlier studies indicated that the instillation of hypertonic solutions into rat small intestine interfered with glucose absorption and produced marked epithelial injury, particularly to the crest of the villus as opposed to the crypt area (1). Similar treatment with isotonic solutions did not produce cellular injury or interfere with glucose absorption.

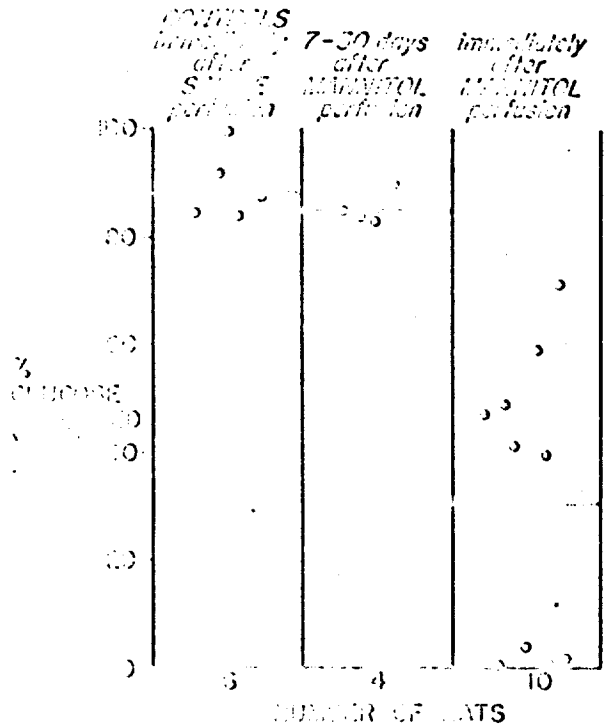


Fig. 2. Comparison of the extent of glucose absorption in control experiments immediately after hypertonic mannitol perfusion and 7-30 days after perfusion with mannitol. The numbers at the bottom represent the number of rats studied.

Our present studies demonstrate that malabsorption of fat also followed instillation of hypertonic mannitol solutions. After perfusion with isotonic saline, 82% of the administered fat was absorbed from the jejunal loop. On the other hand, only 48% of the label was absorbed when administered after perfusion with hypertonic mannitol solutions. The epithelial cell damage, which was again evident on histological examination (Fig 3), could not be solely responsible for the defect in fat absorption since there was clear evidence of impaired intraluminal hydrolysis following mannitol perfusion. Thus, more than 1/3 of the labeled triolein was recovered from the intestinal loop as di- and triglycerides after mannitol perfusion as compared to 8% after saline perfusion. In addition, in the mannitol treated rats there was a significantly greater amount of unabsorbed fatty acids and monoglycerides. These results imply that mannitol perfusion interferes with both the breakdown of higher glycerides and the subsequent absorption of monoglycerides and fatty acids by the epithelial cell.

Several possible mechanisms can be suggested for the fat malabsorption following hypertonic mannitol perfusion. Intestinal epithelial injury by mannitol-treatment is probably responsible for the increased volume of residual fluid in the jejunal loop, noted both in previous studies of glucose absorption (1) and in the present experiments. This increased volume of intraluminal fluid may impair lipolysis because of the resultant dilution

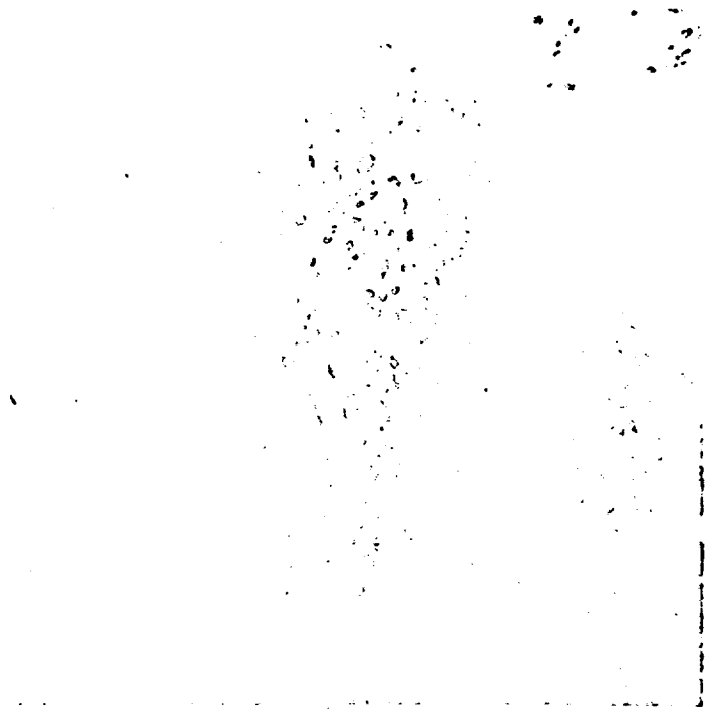
Fig 3. Jejunal biopsy immediately after mannitol perfusion. Villi are blunt with damage to the epithelial cells at the tips of the villi and infiltration with inflammatory cells (340 X)

of pancreatic lipase and bile salt concentration or possibly by altering the pH of the intestinal contents. No measurements were made of the pH of the luminal fluids.

Factors other than dilution may have decreased the luminal concentration of lipase or bile salts. Epithelial cell damage may reduce the release of pancreozymin in response to a fat load and consequently lower the amount of pancreatic lipase released into the intestinal lumen. Moreover, the intraluminal content of lipase and bile salts may be depleted excessively during the mannitol perfusion. Finally, injury to the ileal mucosa may impair bile salt reabsorption with a resultant decrease in the pool of bile salts available for release into the intestinal lumen.

Not even the diminished absorption of fatty acids and monoglycerides can be attributed with assurance to a defect in the cellular phase of fat absorption, since diminished luminal bile salt concentration may interfere with micelle formation. Thus, although cellular damage is ultimately responsible for the fat malabsorption, it seems possible that most or all of the changes are in the intraluminal phase of fat digestion.

The reversibility of the anatomic and functional lesion was studied utilizing glucose as the test material. When mannitol treated rats were allowed to recover for seven days, there was full return of the intestinal absorptive capacity: an average of 85% of the administered glucose was absorbed as opposed to only 32% immediately after mannitol



1. Jejunal biopsy seven days after mannitol perfusion. Note the return to normal in the length configuration of the villi (310 X).

treatment. The recovery in the intestinal absorption of glucose was accompanied by a return to normal in histological appearance. The reduction in glucose absorption can be attributed solely to the cellular injury, but impaired intraluminal lipolysis plays an important role in the more complex process of fat absorption. The reversibility of fat malabsorption could not be studied in the same manner because the longer period of incubation needed for fat studies greatly reduced the postoperative survival rate of these rats.

SUMMARY

The effect of hypertonic mannitol solutions on fat absorption was studied in the jejunal loops of 21 rats in vivo. An average of 82% of the administered neutral fat was absorbed in the control experiments as opposed to only 48% in the mannitol-treated animals. Much of the administered triolein was recovered as di- and triglycerides after mannitol perfusion, indicating an impairment in lipolysis. A histological injury to the intestinal mucosa was demonstrable after mannitol perfusion. The histological appearance of the mucosa and the intestinal absorptive capacity for glucose returned to normal in less than seven days.

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RESEARCH INTO THE EFFECTS FROM SEVERAL POLYALCOHOLS AS PERORALLY ADMINISTERED OCULAR-PRESSURE REDUCING MEDICINES.

P Segal & J Smolarz-Dudarewicz (2 figures & 3 tables)

The influence of osmotic means on the intra-ocular pressure has long been known. Mannitol and urea intra-venously applied are the best known of this group of medecines within the recent past. BELLOWS etal in 1938 made use of iv. sorbitol and achieved encouraging results; nevertheless this medecine did not prove itself well. With respect tu urea, there were empirical observations (BERING & AVMAN) that the pressure changes of the cerebro-spinal liquid are a function of the gradient of the osmotic pressure between serum and cerebro-spinal liquid. So long as the osmotic pressure of the serum is higher than th osmotic pressure of the cerebro-spinal liquid, the intra-cranial pressure will drop. The moment there is osmotic equilibrium, intra-cranial pressure lowering will cease. It is known there is great similarity between cerebró-spinal and intra-ocular liquids. Though not every detail of the functioning of osmotic medicines with respect to intra-ocular pressure has been precisely investigated, one may assume they cause water migration from the inside of the eye into the vascular milieu and that they are one of the main factors in intra-ocular pressure lowering. It is important in terms of clinical consideration that an osmotically effective medecine be made of small molecules, that it be non-toxic and do not penetrate into the eye, so that a high gradient between blood and eye environment be maintained.

Numerous communications show that both Mannitol and urea, when iv. injected, will produce a very strong osmotic effect and may be successfully used in treating glaucoma (KLOETTI ETAL, SMITH & DRANCE, GALIN ETAL, WEISS ETAL, ADAMS ETAL, HILL ETAL, PAUFIQUE ETAL, ESPILDOZA ETAL, POLLIOT & REGNAULT). Unfortunately urea often causes fairly dangerous complications and may not be applied to persons suffering from kidney insufficiencies, or liver insufficiencies. While Mannitol is not toxic in practice (WISE), iv. infusions often are inconvenient in many aspects. In view of the danger of circulation overloading, caution must be exerciced when using Mannitol with persons suffering from heart-kidney insufficiencies (GOLDBERG).

Three years ago a team of Italian workers (CANTORE ETAL, VIRNO ETAL) published good results obtained from perorally administered glycerol for glaucoma treatment. They observed that this drug, in a manner similar to iv. administered Mannitol and urea, reduces brain edema and lowers intra-ocular pressure. Their work aroused great interest. Reports from further workers (CASEY & TREVOR-ROPER, THOMAS ETAL, CHARAN ETAL, AVASTI & SRIVASTAVA) confirmed the effectiveness of glycerol, and above all for acute glaucoma. Besides anti-glaucoma applications, this drug also was used for operational preparations in cataract patients (JAFFE & LIGHT, TREVOR-ROPER ETAL).

We began working with glycerol in 1963, after a study of CANTORE's results. Initial results were published in 1964 and discussed at the meetings in Rostock and Cracov. In order to better evaluate the drug, a compendium of many clinical researches was put together. At the same time the applicability of other polyalcohols was investigated, namely of Mannitol and sorbitol as anti-glaucoma drugs from peroral administration.

PROPERTIES OF THE DRUGS USED

Glycerol is a three-valent alcohol very soluble in water and of sweet taste. Its molecular weight is 92 and it is readily absorbed by the intestine. It is metabolized to a large extent in the body. It oxidizes in carbon dioxide and water and generates 4.32 kg cal/gm. Glycerol causes an effect similar to urea's by yielding 75 gm 996 mOs per dose (urea yields 50 gm 833 mOs per dose).

Glycerol occurs in animal tissues, in which it is an ingredient of fats and lipoids. FERBER & RABINOVICH showed the human body's complete tolerance by feeding 60 patients with glycerol. When administered to one group of students for 50 days in doses of 1-3 gm/kg of body weight, glycerol caused no somatic symptoms. There was no toxic symptom in dogs treated for one year with a daily dose of 9 gm/kg of body weight (JOHNSON ETAL). SLOVITER in 1958 administered iv. glycerol to humans in the form of 5% glucose solutions and observed no somatic symptoms. A 30% glycerol solution when applied iv. may cause transient hemoglobinuria (CANTORE ETAL).

Glycerol will only slightly increase the blood sugar content; it does not cause significant electrolyte interferences.

Mannitol is a 6-valent alcohol of molecular weight 182 and of sweet taste, 20% soluble in water. It occurs in nature in the fruit of the ash tree. According to many workers (JAFFE, PAUFIQUE, SMITH), it

will only be slightly metabolized in the body and according to others (HILL, ADAMS, POLLIOT, SEEGER), it is subject to no metabolism. In tests with rats, CARR showed that Mannitol and sorbitol may form glycogen sources for the liver. Mannitol does not penetrate cell membranes and remains in the extra-cellular spaces; it will be rapidly removed by the malpighian bodies of the kidneys and will not be reabsorbed in the little canals, so that a pronounced osmotic effect is obtained. It will temporarily increase the plasma volume for iv. injections; the plasma volume will be maximum at the time of infusion end and rapidly returns to normal after the drug has been removed from the kidneys. When perorally administered, Mannitol acts as a laxative. It will slightly affect blood sugar (BRIGGS). KJELDSEN used Mannitol in cases of intra-ocular bleedings. Mannitol is used in internal medecine for measuring the duration of filtration of the malpighian bodies in the treatment of acute ascites and kidney damages (LAMPE).

Like other alcohols having more than 3 OH groups, sorbitol is akin to the monosaccharide group. It is a 6-valent alcohol of molecular weight 182, soluble in water and of pleasant taste; it occurs in nature in mountain ash and cranberry berries; it is subject to partial metabolism in the body. Following reabsorption in the small intestine, it will be changed into fructose by dehydrogenases, then it will be built up to glucose and inserted into glycogen metabolism (MAURER). Sorbitol is synthetically made by means of glucose reduction. Though it contains the same amount of calories as sugar, it raises the blood sugar level to a much lesser extent and may therefore be used by diabetics (ROETH, BELLOWS). It also acts cholecysto-kinetically and gall-evacuating, and, furthering intestinal peristalsis, it is used by radiologists when examining the digestive tracts (REINHARDT). It acts as a purgative when applied perorally. No damaging effects have been observed in the body (FIJALKOVSKI & ZAJGNER), but it may cause heart overloading by increasing the rate of blood circulation (BELLOWS ETAL). It diffuses only little into the front chamber of the eye.

Glycerol and sorbitol were administered in a 50% water solution; Mannitol was administered in a 50% water mixture.

OUR OWN OBSERVATIONS

1. INFLUENCE ON INTRA-OCULAR PRESSURE

A. GLYCEROL

We administered perorally glycerol in the amounts of 0.25-1.5 gm/kg of body weight (mostly 1.0 gm/kg, i.e., to 112 persons) to 125 persons. Only 8 received doses of 0.25 gm/kg, and only a minimal hypotensive effect being observable, this group will be omitted from further discussion. We evaluated the findings from 157 persons, of whom 65 were healthy (126 eyes) and 92 were patients (173 eyes) with diverse forms of glaucoma. In most, glycerol caused a lowering of intra-ocular pressure, occurring most of the time within 30-45 minutes from drug administration. Maximum hypotensive effect was recorded after 1-2 hours. It was then determined that intra-ocular pressure returned to the initial stage after 4-5 hours. In a few cases a temporary increase in pressure was observed, exceeding by a few mm Hg the initial values.

The size and duration of intra-ocular lowering of pressure depends on several factors, namely: whether the drug is being administered to healty of ailing persons; from the dose, and from the initial values of the pressure. Also certain individual deviations were observed for many persons with similar clinical profiles. Two points were important from the practical viewpoint:

first, glycerol lowered the intra-ocular pressure in such eyes where neither Miotika nor Diamox had any effect;

second, we were capable in many cases to prolong the hypotensive glycerol effect by administering drugs to the ailing which previously had been ineffective.

For most of the ailing, we restricted ourselves to a one-time application of glycerol The drug was administered more than once to 200 patients (twice to 14, three times to 5 and four times to 4) in intervals of several hours or the next day. Among those patients who received the drug repeatedly, either the same effect as after the first administration was observed or else the pressure lowering upon the next administration was larger. In such cases the graph is in the form of descending steps. Fig. 1 shows typical curves of intra-ocular pressure following glycerol application.

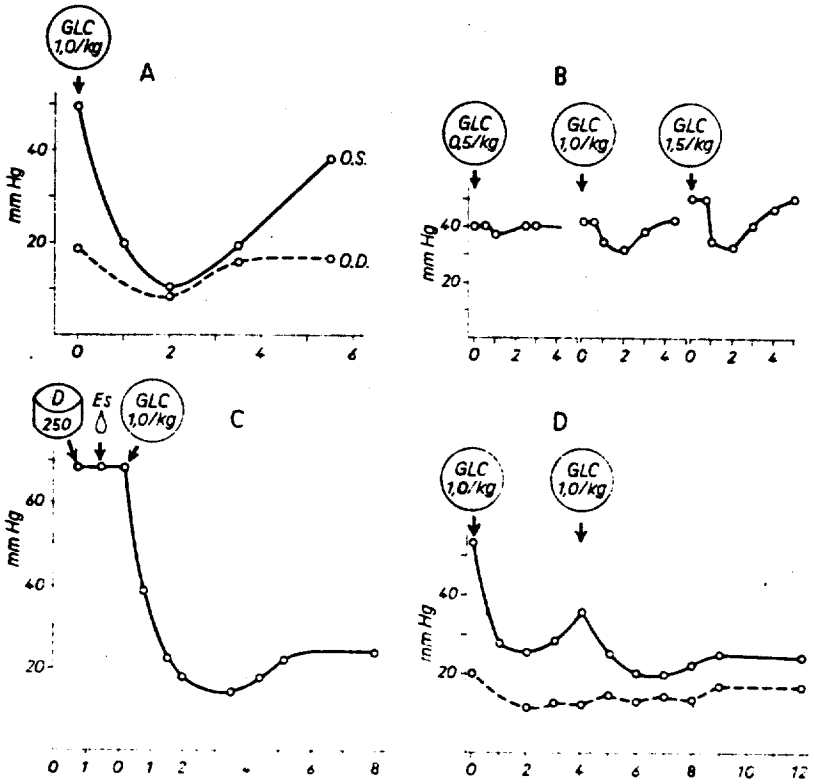

Fig.1.A. Characteristic influence of glycerol administrations in doses of 1 gm/kg in cases of acute glaucoma. There is clear lowering of pressure lasting 3 hours. In the second eye (glaucoma simplex), there is a lowering of equal length though not as clear. B. Characteristic change in behavior of intra-ocular pressure in the case of glaucoma absolutum following administration of glycerol doses of 0.5, 1.0 and 1.5 gm/kg of body weight. C. A characteristic example of a clear hypotensive glycerol effect in the case of an acute glaucoma attack in the course of iridocyclitis that had been treated with large doses of corticosteroids and where neither Diamox nor Eserin lowered the pressure. A renewed dose of Diamox after 5 hours extended the hypotensive effect of glycerol. D. Influence of a second glycerol administration for glaucoma acutum, administered 4 hours after first dose, that is, at the time of the beginning renewed hypertonia. The second dose caused an even more pronounced lowering of pressure. Effect of "descending steps".

Table 1 shows collected data of the effects from various glycerol doses for healthy and ailing individuals. These data show that the hypotensive glycerol effect is much more pronounced among the glaucoma patient than among the healthy persons. After using similar drugs, the averages of intra-ocular pressure lowering among the glaucoma sick were 3.2-3.4 times larger than among the healthy persons. Statistical analysis of the corresponding groups shows that these differences are

significant. At the same time one may notice that the increase in doses from 0.5 to 1.0 gm/kg causes a lowering of pressure among the healthy persons that is 2.2 times larger (4.8:2.1). When doses are changed from 0.5 to 1.5 gm/kg, the lowering is 2.5 times as large (5.3:2.1). Corresponding comparisons of findings among the glaucoma sick yielded almost the same figures: 2.1 for increasing the doses from 0.5 to 1.0 gm/kg (14.2:6.8) and 2.8 when the doses were raised from 0.5 to 1.5 gm/kg ([illegible]). Statistical analysis of the data showed that the di[illegible] [illegible]en the doses of 0.5 and 1.0 gm/kg and bet[illegible] 1.5 gm/kg are significant both for the healty [illegible] the glaucoma sufferers; but the differences between [illegible] of 1.0 and 1.5 gm/kg are not significant.

e 'kg	healthy persons number of eyes	average lowering of pressure mm Hg	glaucoma sufferers number of eyes	average lowering c pressure mm Hg
0.[illegible]	7	2.1	27	6.8
1 [illegible]	101	4.8	117	14.2
[illegible]	18	5.3	29	18.8

LE 1: Dependence of hypotensive glycerol effect on dosage (tabl shows the number of eyes).

Another question had to be answered: To what extent is the glycerol pressure-lowering effect a function of the initial intra-ocular pressure? Table 2 shows the corresponding data in summary form. For better comparison, data are shown only for persons given 1 mg/kg of glycerol, since these were the majority. As shown by the data regarding healthy eyes, the mean magnitudes for pressure lowering increase from 3.0 mm Hg for eyes with the lowest initial pressure (less than 12 mm Hg) to 6.3 mm Hg in eyes with initial pressures exceeding 17 mm Hg. In general however the average lowering of pressure in healthy eyes is modest (less than 5 mm Hg) and clearly less than in glaucoma-stricken eyes (average pressure lowering: 14.2 mm Hg).

There is a clear difference in the group of glaucomatous eyes between the reaction in such eyes with closed angle where a very large lowering of pressure occurs (22.8 mm Hg on the average) and in such eyes with open angles (mean lowering of 9.2 mmHg). It seems however that

B. MANNITOL

Mannitol was applied to 31 persons orally in doses of 1 gm/kg (7 healthy ones and 24 glaucoma sufferers). As for glycerol treatment, it was observed here that the hypotensive effect of mannitol is less in healthy eyes than in glaucomatous ones (2.6 mm Hg and 7.1 mm Hg resp.). The largest lowering in pressure took place for closed angle glaucoma cases, 9.1 mm Hg on the average; it was less pronounced in open angle glaucoma cases, being 6.1 mm Hg on the average. The pressure lowering also was more pronounced for eyes with higher initial pressure. The values for the other groups being small, statistical analysis was undertaken only foropen angle eyes; it showed the differences were significant.

C. SORBITOL

Sorbitol was administered to 31 persons (4 healthy ones and 27 glaucoma sufferers); 28 persons received doses of 1.0 gm/kg and 3 persons 1½ gm/kg. For the sake of better overview, only the results for the 1.0 gm/kg were listed. Findings for 1½ gm/kg showed no appreciable differences.

As for glycerol and mannitol treatments, it was observed here too that the lowering of intra-ocular pressure was appreciably less for healthy persons than for glaucoma sufferers, namely 2.3 and 7.1 mm Hg resp. on the average; among the latter, it was much more pronounced than for patients with higher initial pressures.

2. TONOGRAPHIC EXAMINATIONS

Among some of the subjects, for a total of 92 eyes, tonography was carried out by means of a standard SCHIOETZ tonometer with a 5.5 g weight (and with a 7.5 g weight for 3 persons) and for a duration of 4 minutes. The "C" coefficient of outflow rate was set according to the MOSES tables of 1958, sclero-rigidity having been previously determined by means of comparative tonography, the corresponding values having been read off the FRIEDENWALD tables (1955). These tests were performed twice for each patient, prior to administration of the drug, C_1, and at the peak of its effectiveness, that is, 1½-2 hours after administration, C_2. Graphs were plotted for 34 eyes after glycerol administration, for 35 eyes after mannitol administration, and for 23 eyes after sorbitol administration.

the kind of angle in the front chamber is not as critical as the level of the initial pressure as regards the magnitude of pressure-lowering, which ordinarily is significantly higher in glaucomatous eyes with closed angles than with open ones. For patients with the latter, the magnitudes of pressure lowering were the higher, the larger the initial values (for instance, eyes with 45 mm Hg on the average experienced a lowering of 18 mm Hg, and eyes with 55-70 mm Hg experienced a lowering of 28 mm Hg)). W_e observed a similar dependence among the glaucoma sufferers with open angles.

TABLE 2. Dependence of hypotensive glycerol, mannitol and sorbitol effects (1 gm/kg) on the initial value of the intra-ocular pressure and on clinical glaucoma kind.

A. healthy persons

Initial pressure mm Hg	GLYCEROL no of eyes	GLYCEROL mean pressure lowering	MANNITOL no of eyes	MANNITOL mean lowering of pressure	SORBITOL no of eyes	SORBITOL mean pressure lowering
6-12	17	3.0	1	0.0	2	1.0
12-17	60	4.6	6	2.5	1	2.0
17-22	24	6.3	7	3.0	3	3.6
TOTAL	101	4.8	14	2.6	6	2.3

B. Glaucoma sufferers

Kind of glaucoma	initial pressure mm Hg	GLYCEROL no. of eyes	GLYCEROL mean press. lower.	MANNITOL no. of eyes	MANNITOL mean press. lower.	SORBITOL no. of eyes	SORBITOL mean press. lowering
closed angle	25-45	8	18.0	10	8.3	11	8.3
	45-55	11	22.0	-	-	3	12.3
	55-70	10	28.0	1	18.0	3	11.0
	total	29	22.8	11	9.1	17	9.5
open angle	25	39	7.0	18	4.7	9	3.3
	25-37	32	11.6	15	9.2	17	6.8
	total	71	9.2	33	6.6	26	5.6
secondary		17	20.4	2	5.0	1	6.0
TOTAL		117	14.2	46	7.1	44	7.1

The majority of both healthy and glaucomatous eyes showed no changes in outflow prior or after drug application that could be considered significant. This finding applies as well to glycerol for 21 out of 35 cases as to mannitol for 20 of 35 cases and for sorbitol for 18 of 23 cases.

3. LABORATORY TESTS

In order to determine any changes occurring after glycerol administrations, a series of biochemical investigations was resorted to. The behavior of the sodium and potassium levels and the alkali supply in the blood serum, also the alcohol level and the sugar curve were carefully monitored and a hemocrit was carried out. The recordings were two-fold: immediately prior to oral glycerol administration of 1 gm/kg and 1½ hours afterward at the time of the drug' maximum effect. The alcohol level in 30 examinations and the sugar curve of 21 examinations in no case showed appreciable changes. There were no appreciable differences as regards the sodium and potassium levels or the alkali supply for 36 persons. Among a third of those examined, certain differences were observed which were neither large nor bilateral. Their statistical evaluation showed them to be insignificant. The hematocrit for 24 examinations in most cases, namely 14, remained unchanged or varied only within empirical limits. Among 5 of the remaining 10 persons there was an increase, and for 5 there was a decrease of the red hematocrit corpuscles. Examinations of sodium and potassium levels in the 25 persons administered mannitol and 15 persons administered sorbitol showed similar results; similar considerations apply to the alkali supply in the blood serum.

4. SIDE EFFECTS

As shown by our observations, all of the drugs tested by us do not cause more serious complications. However fairly unpleasant side effects were observed among an appreciable part of our subjects. The frequency of the side effects depended on the drug dosage administered. Table 3 lists the individual symptoms. Each patient was exhaustively questioned about his complaints. Many side effects could thus be determined which would have gone unnoticed if no more than the spontaneous complaints on the part of the subjects had been elicited.

TABLE 3. Side effects from glycerol, mannitol and sorbitol (%)

Symptoms	glycerol 157 persons	mannitol 31 persons	sorbitol 31 persons
headaches	27.0	13.0	16.0
nausea	20.0	32.0	22.0
thirst, mouth dryness	8.5	0.0	10.0
vomiting	4.0	3.0	0.0
arterial fluctuations	3.0	6.0	6.0
diuresis	2.5	0.0	10.0
sleepiness	2.5	0.0	6.0
feeling of neck stiffness	1.0	0.0	0.0
dizziness	0.5	3.0	0.0
chills	0.5	0.0	0.0
diarrhea	0.5	90.0	52.0
numbness	0.0	0.0	3.0
total number of persons with side effects	50.0	93.0	93.0

The following points deserve emphasis:

a) in parallel with larger dosages, there were appreciably more side effects in the group treated with glycerol. They occurred in a third (35%) of the persons treated with 0.5 g/kg, in half of those treated with 1.0 g/kg (48%), and in about two thirds (68%) of those treated with 1.5 g/kg.

b) The sum of the side effects caused by glycerol is significant. For half of those tested, there occurred more or less serious complaints. The percentage of side effects following mannitol and sorbitol was even higher, -- 9/10 of those tested.

c) complaints due to the osmotic effect from the drug on the central nervous system (headaches, sleepiness, neck numbness, dizziness) as caused by glycerol were predominant -- they occurred for about 50 of the 157 subjects. Stomach disorders occupy second place: nausea and vomiting (38 times) and dehydration symptoms: thirst, mouth dryness, diuresis (20 times). These symptoms most of the time lasted 2-3 hours and essentially overlapped the duration of the hypotensive effect of the drugs.

Intestinal disorders were predominant for those persons treated with mannitol and sorbitol; mostly it was diarrhea (in half the cases following sorbitol and in 9/10 of the cases following mannitol absorption). Stomach complaints were as frequent as for the use of

glycerol, but central nervous system complaints were far fewer percentage-wise. Diarrhea following mannitol and sorbitol ingestion mostly occurred within 5-7 hours. For many it lasted up to 18 hours and was quite bothersome. These complaints are the reason the number of healthy subjects was limited.

DISCUSSION

As noted from the listed data, the drugs we applied orally act hypotensively upon the intra-ocular pressure. Their effect is larger in glaucoma sufferers than in healthy persons. It is the more pronounced the larger the initial pressure.

By comparing the effects from those medecines, it was determined that glycerol's is the most pronounced. The compilation of the data from the 3 medecines in 1 gm/kg doses shows there is appreciable difference in their effectiveness with regard to healthy and glaucomatous eyes. Significant differences exist between glycerol and the other two drugs, but not between mannitol and sorbitol.

For some of the ailing, an investigation was undertaken concerning the effects from different medecines. Fig. 2 shows several typical curves.

If the tonometric curves following administration of glycerol, sorbitol and mannitol are compared, it will be observed that the lowering of intra-ocular pressure following glycerol is larger but of shorter duration (2-3 hours) than is the case for mannitol and sorbitol.

We consider it to be very significant that for the drugs that were tested, the hypotensive effect will be the more pronounced the higher the pressure at the time of drug administration. Statistically treated results from many groups encompassing a large number of observations prove that the correlation coefficients between the initial pressure and the magnitude of the induced lowering of pressure are particularly high and thus also prove the significance of this phenomenon.

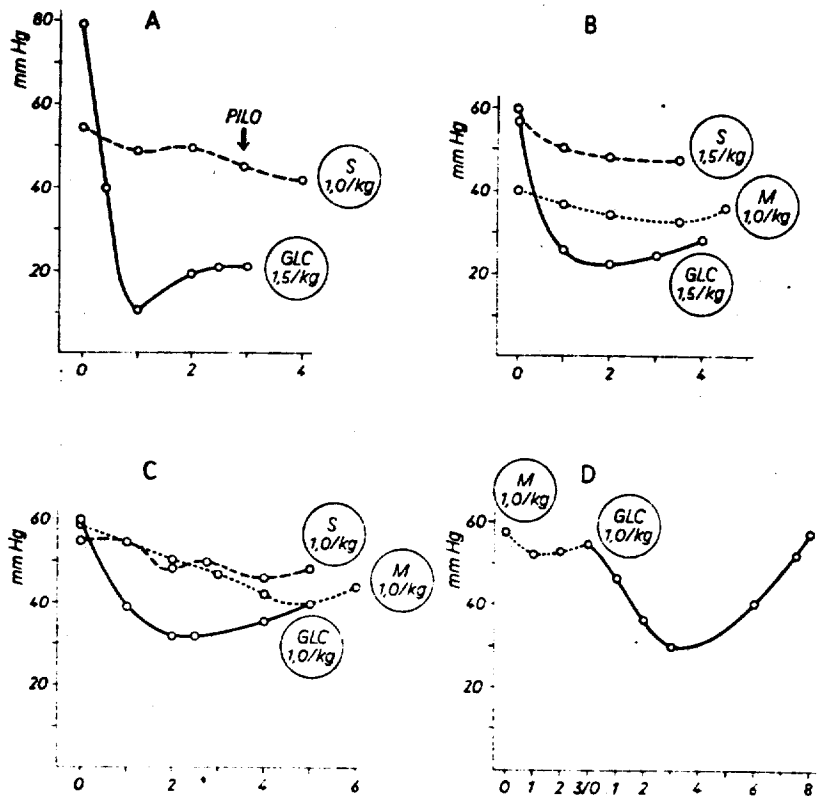

Fig. 2. - comparison of efficacies following administrations of glycerol, mannitol and sorbitol, for the same patients. A. - Case of acute glaucoma attack; there is clear lowering of pressure following glycerol absorption; two days later, there is a much lesser effect from sorbitol. -B. Glaucoma secundarium in the course of an iridocyclitis: very weak sorbitol effect; 10 days later, glycerol adminstered at the same high initial pressure caused a much more pronounced lowering of pressure. 3 weeks later, for a low initial pressure, mannitol caused a slight lowering of pressure. -C. Glaucoma acutum responding slightly to sorbitol and mannitol. Glycerol causes a somewhat more pronounced effect (curves were executed in 2-3 day time intervals). -D. Acute glaucoma attack; there is moderate response after mannitol ingestion and a clear lowering of pressure after glycerol's.

As shown from the experiments, it is for acute glaucoma and for chronic glaucoma with high pressure that the hypotensive effect usually is the most pronounced.

Even though the hypotensive effect of the drugs we used is of short duration, those medecines may be of considerable use when intra-ocular pressure may not be lowered in other ways. They may be of use prior to operations of intra-bulbar and anti-glaucoma kinds. In contrast to other workers who recommend the use of glycerol prior to cataract operations, we are of the opinion that such use is fairly

ticklish
in view of the frequent nausea and vomiting. Such complications are most intensive among fasting patients. Though side effects do often complicate the efficacy of those three medecines, there are no serious disorders, as was already mentioned. For patients, the most bothersome effect is diarrhea from mannitol and sorbitol.

We consider glycerol the most valuable of the three drugs because of its larger hypotensive effect and because of its fewer and shorter side-effects. Further advantages are its low cost, its easy accessibility and the convenient manner of administration. Glycerol's hypotensive effect is due largely to its rapid gastro-intestinal reabsorption. As a consequence there is increase in the osmotic blood pressure which causes electrolyte migration from the tissue into the bed of the circulatory blood, so that eye dehydration ensues. Conceivably the hypotensive effect observed after glycerol administration is caused by a much faster reabsorption than is the case for mannitol and sorbitol. Different opinions prevail with respect to mannitol's gastro-intestinal reabsorption. According to our observations, however, mannitol is at least partly reabsorbed in in the digestive tract and does exert an osmotic effect. But one cannot rule out that the hypotensive effect from mannitol and sorbitol is to some extent the consequence of the diarrhea dehydration of the body. Such hypothesis would have been confirmed by the longer-term pressure lowering following mannitol and sorbitol, just as glycerol. As expected, the tonographic tests for most of the persons examined showed no appreciable differences in the behavior of the outflow conditions whether after glycerol or after mannitol and sorbitol administrations.

Our own laboratory analyses showed no differences in alcohol levels or in the behavior of the sugar curves.

The sodium and potassium levels and also the blood serum alkali supply were normal. These results however do not contradict the concept of an osmostic effect of those drugs, since in the course of electrolyte migration from the tissue into the blood bed and the elimination of those drugs from the kidneys, their continuous level compensation occurs because of homeostasis in the serum.

Besides the above mentioned data, empirical research into potassium exchange in the erythrocytes of the human blood (BOBINSKI ETAL) was carried out by means of radioactive potassium K_{42}. We found that glycerol exerts no hyperosmotic action on the red blood corpuscles whether in vitro or in vivo and that therefore the potassium exchange rate will be slowed down in the blood corpuscles. Though results from such a model experiment do not suffice to prove the mechanism of the glycerol effect on the eye, one may assume that this medecine will act in similar inhibiting fashion on the epithelium of the corpus ciliare and on the production of chamber water.

Summary: The authors investigated the hypotensive effect of perorally administered glycerol, mannitol and sorbitol in a 50% solution.

The hypotensive effect is most pronounced after administration of glycerol. The decrease of intraocular tension lasts for 3—5 hours.

Tonographic studies did not reveal any significant changes in the outflow facility of the aqueous humour; laboratory examinations did not yield any evident disturbances in the mineral and carbohydrate metabolysis.

Side-reactions were observed, most frequently headaches, nausea, vomiting and diarrhea, but there were no serious complications.

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Glycogen Formation after Oral Administration of Mannitol to White Rats.

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Glycogen formation is stated to occur after the oral administration of mannose to animals, but the conversion of mannitol, the hexahydric alcohol which may be obtained by reduction of the aldehyde group of mannose, to glucose or glycogen has not been definitely proven. It has been maintained that sorbitol,[1] the hexahydric alcohol derived from glucose, may alleviate the hypoglycemic symptoms in rabbits which have received insulin and when administered to diabetic patients may increase the respiratory quotient. Rosenfeld[2] was unable to observe any significant increases in liver glycogen after feeding mannitol to dogs. Pflüger[3] in a critical evaluation of the data of Külz concluded that the increased glycogen deposition obtained by this investigator was within the limits of experimental error.

[1] Reinwein, H., *Deut. Arch. klin. Med.*, 1929, **164**, 61.

[2] Rosenfeld, G., *Centr. inner Med.*, 1900, **21**, 177.

[3] Pflüger, E. F. W., *Das Glykogen und seine Beziehungen zur Zuckerkrankheit*, Bonn, 1905, 2nd Ed., 215.

We have studied the glycogen content of the liver after the oral administration of *d*-mannitol by the method of Cori. Young white rats were fasted 24 hours and fed either 2 or 4 cc. of a 15% solution of mannitol by stomach tube. The amount of mannitol fed was determined by passing the mannitol solution through the stomach tube into an evaporating dish, which was then placed in an oven and dried to constant weight. After absorption periods of 2, 3, 4, and 6 hours, the rats were killed and the glycogen content of the liver was determined as described previously.[4]

TABLE I.

Glycogen content of liver of white rats after oral administration of Mannitol.

Wt. after fast gm.	Absorption period hr.	Mannitol fed mg.	Liver Glycogen %
143	2	214	0.07
160	2½	214	0.05
137	3	214	0.08
129	3	214	0.12
130	4	250	0.16
135	4	250	0.08
110	6	505	0.19
114	6	505	0.05
115	6	505	0.07
120	6	505	0.08
123	6	505	0.06
132	6	505	0.09
136	6	505	0.07
145	6	505	0.07
Average value for 9 control animals			0.05 (range 0.02-0.06).

The results are presented in Table I. The glycogen values for the livers of a control series of 9 rats which received 2 cc. of water after 24 hour fasting periods varied from 0.02 to 0.06% with an average of 0.05%. Inspection of the data of the table fails to reveal any significant increases in liver glycogen after oral administration of mannitol as compared with the values obtained for the control series.

After these experiments were completed, Carr and his co-workers[5] reported a similar study of the glycogen content of the liver of white rats, previously fasted and then fed a mixture of cacao-butter and mannitol (33%) over a period of 80 hours. The glycogen content

[4] Catron, L. F., and Lewis, H. B., *J. Biol. Chem.*, 1929, **81**, 553.

[5] Carr, C. J., Musser, R., Schmidt, J. E., and Krantz, J. C., Jr., *J. Biol. Chem.*, 1933, **102**, 721.

of the liver of the rats fed mannitol and cacao-butter ranged from 0.62 to 1.80% with an average value of 1.23%, as compared with the average glycogen content of the livers of control rats fed cacao-butter alone of 0.14%.

It is impossible to compare the results of this series with our own experiments since the treatment of the animals was quite different in the 2 series. Under the experimental conditions used by us, it is evident that mannitol did not serve as a ready available source of glycogen. A similar lack of ready utilization of mannitol is shown by the reported failure of mannitol to relieve insulin shock in white rats.[6]

Anaphylactic Reaction to Mannitol

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Mannitol administered intravenously produced a severe reaction resembling anaphylaxis in a healthy 65-year-old woman with glaucoma and a positive allergic history, who had never knowingly received the medication before. Skin testing suggested an immediate-type hypersensitivity of the patient to mannitol. Since mannitol is an important component of most fungi it is possible that the initial sensitizing episode occurred 40 years previously when the patient received "allergy shots."

MANNITOL, traditionally used to measure glomerular filtration, has found favor for a variety of other purposes and has gained an enviable record of safety.[1-8] This report describes a serious allergic reaction to intravenously administered mannitol.

Report of a Case

A 65 year-old white housewife was admitted to the Wills Eye Hospital with chronic open-angle glaucoma, first diagnosed two years before. Her intraocular pressure had been adequately controlled by acetzolamide sodium and pilocarpine hydrochloride until shortly prior to admission, when surgical intervention was thought necessary because of a sudden increase of pressure.

The patient had bronchial asthma from early childhood until the birth of her only child when she was 40 years old. Thirty seven years prior to admission she was hospitalized for ten days because of an allergic reaction to "hay fever shots." She developed marked urticaria three or four times following administration of penicillin. Procaine hydrochloride, used during a dental procedure, caused severe swelling of the entire face and neck. Since she was allergic to standard brands of pilocarpine eye drops, she used a specially compounded solution containing no buffering agent or preservative. Her history and review of systems were otherwise negative.

At the time of admission to the hospital intraocular pressure in the left eye was 40 mm Hg; otherwise the patient's physical examination was entirely within normal limits. She was in no distress, and appeared to be in excellent health.

The morning of admission she received acetazolamide sodium, 250 mg orally following a light breakfast. Pilocarpine eye drops were instilled at 8AM and again at noon. Lunch was omitted. No other medications of any kind were administered until late afternoon, at which time an intravenous infusion of mannitol, 20%, in 500 ml of water was started, in preparation for surgery. Other preoperative medications were not given at that time and had not been given previously. About three minutes after the mannitol was started, the patient began sneezing violently, her nose became runny, her tongue swollen, and breathing difficult. About three minutes later, she was extremely anxious, dyspneic, cyanotic, and lost consciousness. Four large wheals were noted on the chest. Air exchange was poor; wheezing was marked. Mannitol was discontinued and 0.4 cc epinephrine hydrochloride 0.1% given subcutaneously. Within 30 seconds respirations became more normal and the patient regained consciousness. Cyanosis faded rapidly. Within 30 minutes the wheals had disappeared.

Glycerol, 60 cc, administered orally, resulted in a fall of intraocular pressure to 9 mm Hg. The following day under general anesthesia a peripheral iridectomy with thermal sclerotomy was performed without complication. Postoperative recovery was entirely satisfactory.

Comment

Mannitol, an alcohol derived from mannose, has been used in the prophylaxis and treatment of oliguria,[2-4] as an osmotic diu-

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retic,[5,6] and to lower intracranial pressure.[7] It has also become a valuable aid in the treatment of acute glaucoma, possessing certain advantages over urea, glycerol, and parasympathomimetic agents.[8] Extensive use in several institutions has supported its safety.[2,4,8] More than 1,500 patients in the Wills Eye Hospital have received the medication without a serious untoward reaction. An allergic reaction has not been reported.

Since an anaphylactic reaction to a simple carbohydrate is unusual it is essential to be sure that there could be no other cause. Both the patient and those caring for her state that except for pilocarpine ophthalmic drops no medication of any kind was administered at any time. The mannitol used was a standard, commercially available product containing only water, mannitol, and sodium hydroxide; nothing had been added to this. The solution was administered from a previously unopened bottle by means of a standard, commercially available infusion set. Other bottles from the same batch did not produce similar reactions in other patients.

Two months after the initial reaction, skin testing with mannitol produced a positive reaction. Immediately after a light scratch was made on the arm where a drop of mannitol, 25%, had been placed, a large flare (25 × 30 mm) developed, and a few seconds later a 5 × 10 mm wheal. The patient commented that the area felt as if it were burning; no systemic symptoms occurred. The reaction was transient, fading in 30 minutes. Three control scratch tests done at the same time were all negative, producing neither flare, wheal, nor burning sensation. Furthermore, similar testing with mannitol of three control subjects produced no reaction. The mannitol used in skin testing was manufactured by a different pharmaceutical company from that used in preparation for surgery.

It is not known how the subject of this report became sensitized to mannitol. It had never before been used as part of her glaucoma treatment, and she took no medication of which it was a component. However mannitol occurs widely in nature, particularly in fungi where it exceeds glucose quantity and may even replace it entirely. Forty years prior to the present episode an attempt had been made to desensitize the patient by means of "allergy shots." These injections contained fungal products the may have provided the initial sensitizing episode.

The mannitol used in skin testing was provided by Harvey Laboratories.

Generic and Trade Names of Drugs

Acetazolamide sodium—*Diamox Parenteral.*
Mannitol—*Osmitrol.*
Pilocarpine hydrochloride—*Isopto-Carpine, Pilocar, Pilocel, Pilovise, Palocarp.*
Procaine hydrochloride—*Novocain, Kirkane, Gericaine H-3, Lactocaine, Naucaine, Necaine, Scurocaine.*
Epinephrine hydrochloride—*Adrin, Vaponefrin.*

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ION EXCHANGE CHROMATOGRAPHY OF POLYOLS

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Polyols have been detected in many biological fluids and tissues[1]. A systematic study of polyols has been hindered by lack of suitable methods of separation, although chromatographic methods have been devised for particular problems such as the determination of glycerol[2] and propylene glycol[3] in tobacco.

The most widely used method is based on the work of KHYM AND ZILL[4] who separated polyols as their borate complexes on strongly basic anion exchange resins in the borate form. The disadvantage of this procedure lies in the use of dilute borate buffers for elution with the consequent production of broad solute peaks.

Analysis of polyols is also hampered by lack of a specific analytical method; assays based on the measurement of periodate uptake are relatively insensitive and sugars interfere to a large extent.

In the present work these disadvantages have been overcome by elution from ion exchange resins with concentrated borate buffers and by estimating the formaldehyde produced by periodate oxidation of polyols.

These techniques have been applied to the analysis of urinary polyols.

MATERIALS AND METHODS

Chemicals

The author is indebted to Dr. C. E. STICKINGS of Imperial College, London, for a sample of L-threitol.

Glycerol was used without further purification. Other commercially available polyols were purified by recrystallisation from absolute or aqueous ethanol. After repeated recrystallisations some polyols were found to be impure when analysed by the column procedure described here. For recovery experiments it was found convenient to prepare these polyols in solution by reducing the corresponding Analar grade aldose sugar with sodium borohydride by the method of ABDEL-AKHER, HAMILTON AND SMITH[5]. The disappearance of reducing sugar was followed to completion by measuring reducing power by the method of PARK AND JOHNSON[6]. Suitably diluted samples of these solutions were used without further treatment.

Analar grade sodium metaperiodate and chromotropic acid "for formaldehyde analysis" were used. Bisulphite solutions were prepared from B.D.H. ampoules. D-Mannitol was obtained from the Radiochemical Centre, Amersham.

Column chromatographic procedure

De-Acidite FF (3-5 cross-linked [illegible] 200 mesh: Permutit Co. Ltd.) was

exhaustively washed with water and the fines removed by decantation. The resin was regenerated in large glass columns by passing 5 column volumes of 2 *N* sodium hydroxide. After washing excess alkali from the column with water, the resin was converted into the borate form by passing 5 column volumes of 0.5 *M* potassium tetraborate. Excess borate was washed from the resin with distilled water; the resin was then extruded and stored moist.

The analytical columns were jacketed and were 60 cm in length and 0.8 cm internal diameter. The resin was thoroughly degassed at 40–50° on a vacuum pump and then poured as a thick slurry (0.5 vol. water) to give a column 50–55 cm in length. Water at 35° circulated through the column jacket as the resin was poured and throughout the chromatographic procedure. The sample, dissolved in 1–2 ml of 0.1 *M* borate pH 9–10, was applied when excess liquid had just drained to the top of the resin bed. In the same way 1–2 ml washings were applied. Eluting buffer was then immediately pumped onto the column *via* a small de-aeration tube[7] placed in a circulating water bath at 35°. The pump was adjusted to give a column flow rate of 25 ml per h and 5 ml fractions were collected using an Isco volumeter (Shandon Scientific Supplies). Eluting buffers were 0.18 and 0.36 *M* boric acid adjusted to pH 9 with triethylamine. The column was eluted with 0.18 *M* buffer up to fraction 80 when 0.36 *M* buffer was introduced. Conductivity measurements showed that the 0.36 *M* buffer emerged from the column at fraction 84–90.

Analysis of column eluates

Following periodate oxidation formaldehyde was estimated by a modification of the methods described by WEST AND RAVAPORT[8] and by BARTLETT[9].

The reactions were carried out in Pyrex test tubes. To 2 ml samples were added 0.5 ml 0.01 *M* sodium metaperiodate in *N* sulphuric acid. After mixing, the solutions were allowed to stand for at least 10 min at room temperature. Next 0.2 ml 10% (w/v) sodium bisulphite was added with immediate mixing followed by 0.2 ml 2% (w/v) aqueous chromotropic acid solution. Finally 3 ml conc. sulphuric acid was added from a burette; the solutions were mixed well using a vortex mixer and the tubes were then placed in a boiling water bath for 1 h. After some 10 min in the bath the tubes were briefly removed and the contents again well mixed with the vortex mixer before the tubes were returned to the bath; incomplete mixing at this stage resulted in the formation of a precipitate which interfered in the spectrophotometer readings. The timing for both the periodate oxidation and the colour development stage in the water bath was not found to be critical. 10–30 min sufficed for the oxidation and 45–60 min for the colour development. Reagents were freshly prepared and added by automatic pipette or syringe.

In the presence of borate, full development of the red colour was inhibited by an amount proportional to the amount of borate present. For a constant level of borate however, the colour intensity was found to be directly proportional to the amount of polyol oxidised. The polyols examined in the present procedure were eluted by 0.18 *M* or 0.36 *M* borate solutions; it was found convenient to dilute 1 ml samples of column fractions to 2 ml with either 0.18 *M* buffer or water so that all samples were 0.18 *M* with respect to borate before addition of periodate. The resulting colours were then related to a standard curve prepared using pure erythritol dissolved in 0.18 *M* buffer. Colours were read at 570 mμ in a Unicam SP 500 spectrophotometer;

in the reaction mixture described 0.1 μmole polyol (yielding 2 moles formaldehyde per mole) gives an extinction of 0.5 with a path length of 1 cm.

Of the common sugars which were tested in this assay only fructose interfered to any extent giving an extinction of 0.1 per 0.1 μmole.

Radioactivity measurements

Radioactivity was counted in a Packard Tri Carb scintillation counter. 0.2 ml samples of column fractions were diluted with 2.8 ml absolute alcohol and 7 ml of scintillation fluid. The scintillation fluid contained 5 g 2,5-diphenyloxazole and 0.3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in 1 l of toluene.

Paper chromatography

Those fractions which on analysis were found to contain a solute peak were combined. Triethylamine was removed with Zeo-Carb 225 (Permutit Co. Ltd.) and the boric acid was distilled off as methyl borate by repeatedly evaporating to dryness in the presence of methanol[4]. The resulting residues were dissolved in 0.1 ml water and spotted on to sheets of Whatman No. 3 MM paper. The papers were irrigated for 18 h by descending flow with the solvent, ethyl acetate–pyridine–water saturated with boric acid (12:5:4 by vol)[10]. Chromatograms were allowed to dry in air; polyols (and sugars) were revealed using the anisidine–periodate dip method of BEAN AND PORTER[11].

Preparation of urine samples for analysis

Urines were collected over a 24 h period and kept at 4° with a few drops of toluene added as preservative. Samples were processed within 24 h of the last collection. 10 ml portions were added to 15 ml of moist Bio-Deminrolit which had been converted to the HCO_3^- form by passing an excess of CO_2 through a suspension of the resin in water. The urine–resin slurry was magnetically stirred for 30 min after which the resin was filtered off and washed well. The filtrate and washings were freeze-dried and kept in a deep freeze. For analysis the residues were dissolved in borate buffer and applied to the column as described.

RESULTS

Under the conditions described a mixture containing nine polyols is almost completely resolved into nine discrete solute peaks (Fig. 1a). The effect of temperature on the separation was particularly marked; at 25° the affinity of the polyol–borate complexes for the resin is lowered so that many of the polyol peaks overlap, whilst at 45° polyols are so strongly bound to the resin that the time required for elution is impractical. At 35° only mannitol and galactitol overlap; when the less commonly occurring polyols, rhamnitol and fucitol are included in the mixture they overlap arabitol and glucitol respectively (Table I).

Recoveries of individual polyols after the separation of various mixtures are shown in Table I, which also indicates the fraction numbers at the peak positions. These positions varied by up to ten fractions from the values quoted but the order of elution of the polyols remained constant.

Absorption of hexitol by strongly basic ion exchange resins was reported by ROSI[illegible] *et al.*[12] [illegible] the procedure for desalting urine described earlier involved the [illegible] effect of this procedure on the recoveries of

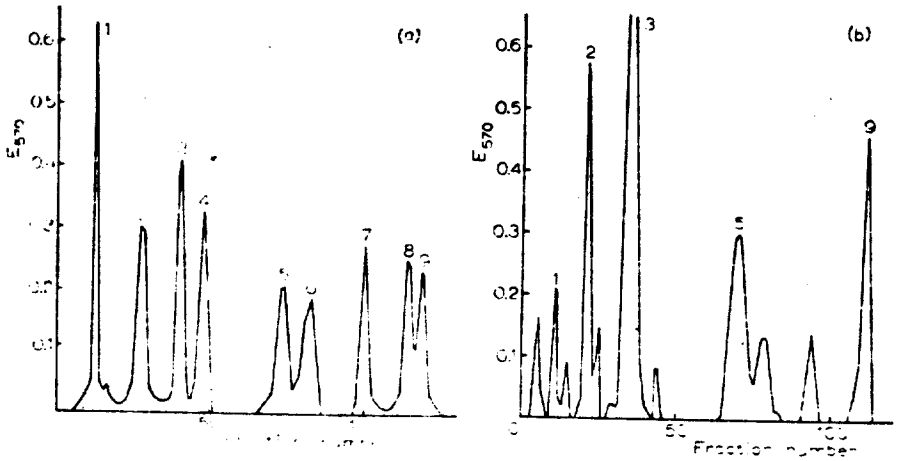

Fig. 1. Elution curves of polyols in a synthetic mixture (a) and from 10 ml of a normal urine (b); conditions as described in the text. Peaks are identified as follows: 1 = glycerol; 2 = threitol; 3 = erythritol; 4 = xylitol; 5 = arabitol; 6 = ribitol; 7 = glucitol; 8 = galactitol; 9 = mannitol.

micromole amounts of polyols was therefore examined. The results presented in Table II indicate that appreciable losses of all the common polyols resulted from the use of the desalting procedure.

The method has been applied to the analysis of urines from apparently healthy individuals; a typical elution pattern is shown in Fig. 1b. Five polyols were consistently found in the urines which were examined: glycerol, threitol, erythritol, arabitol, and mannitol. With the exception of glycerol the polyols were identified from their elution positions and by paper chromatography; mannitol was also identified by co-chromatography with ^{14}C-mannitol. The method described earlier[1] for removing boric acid from column fractions resulted in the complete loss of glycerol which was therefore identified solely by its elution position.

The 24 h excretion values of the five polyols which were found are presented in Table III.

TABLE I

RECOVERIES AND PEAK FRACTION NUMBERS OF POLYOLS

Recoveries are expressed as the average percentage recovery ± standard deviation. The number of determinations is given in brackets. The peak fraction number is the average of at least 5 determinations.

Polyol	*Recovery*	*Peak fraction number*
Glycerol	108 ± 5.4 (6)	12
Threitol	100 ± 3.0 (7)	20
Erythritol	92.2 ± 4.7 (7)	30
Xylitol	94.0 ± 11.2 (8)	40
Arabitol	89.2 ± 4.2 (8)	51
Ribitol	84.4 ± 5.0 (10)	59
Glucitol	89.5 ± 14.1 (13)	101
Galactitol	92.0 ± 7.3 (5)	114
Mannitol	99.0 ± 14.5 (5)	117
Fucitol	95.6 ± 7.0 (6)	99
Rhamnitol	89.5 ± 4.8 (6)	97

TABLE II

RECOVERIES OF POLYOLS AFTER DESALTING BY THE PROCEDURE DESCRIBED IN THE TEXT

Recoveries are presented as percentages of untreated samples. The values for three separate experiments are shown with the mean value in brackets.

Polyol	*Recovery*
Glycerol	70, 68, 60 (66)
Threitol	62, 60, 55 (59)
Erythritol	60, 49, 53 (54)
Xylitol	53, 64, 60 (59)
Arabitol	72, 60, 68 (67)
Ribitol	70, 77, 73 (73)
Glucitol	38, 48, 50 (45)
Galactitol	45, 43, 36 (41)
Mannitol	44, 36, 40 (40)

TABLE III

EXCRETION OF GLYCEROL, THREITOL, ERYTHRITOL, ARABITOL AND MANNITOL IN μMOLES PER 24 HOURS

The figures for each polyol were calculated from elution curves obtained by the methods described in the text and were corrected for losses incurred in the desalting procedure.

Sample	*Sex*	*Age*	*Glycerol*	*Threitol*	*Erythritol*	*Arabitol*	*Mannitol*
1	F	19	33	164	724	252	307
2	F	17	108	495	715	243	540
3	F	20	94	156	690	315	285
4	F	20	45	122	600	313	1390
5	M	30	55	250	990	400	150
6	M	23	68	198	1060	323	120
7	M	16	124	156	695	425	385
8	M	33	73	203	610	485	190
9	M	37	42	78	445	122	122

DISCUSSION

Recently gas chromatographic methods have been described (see for example SWEELEY *et al.*[13]), for the analysis of sugars and polyols which offer distinct advantages in terms of speed and sensitivity over the methods described here. However, ion exchange methods are useful in the analysis of unknown mixtures as the analytical procedures used often serve to partly characterise solute peaks which can also be collected for identification by other methods. WELLS *et al.*[14] described a method for the analysis of sugars and polyols in serum and urine by gas chromatography of the corresponding trimethylsilyl ethers; mannitol was detected as a constituent of normal urine.

The presence of mannitol, erythritol[15], arabitol[16] and threitol in normal urine may be signific[illegible] [illegible]FINDALE AND EDSON[17] found that rat liver polyol de[illegible] [illegible]ritol, erythritol or arabitol; the human urinary polyo[illegible] [illegible]s of carbohydrate metabolism.

The occurrence of threitol is noteworthy as this polyol has previously only been detected in bacterial[18], and plant material[19], although BATT *et al.*[20] showed that in rats ^{14}C-L-erythrulose is converted in part to threitol. The guinea pig liver enzyme reported by HOLLMAN AND TOUSTER[21], which converts D-xylulose to xylitol will also convert L-threitol to L-erythrulose. In humans threitol metabolism may possibly be linked to that of L-erythrulose but little is known about the metabolism of this sugar.

The fact that only small amounts of polyols are normally found in urine suggests that rapid screening methods for urinary polyols may be valuable in clinical diagnosis since it is known that large amounts of arabitol are excreted by pentosuric individuals[16]. Similarly galactitol has been isolated in quantity from the urines of patients with galactosemia[22] and from the urine of an individual with a possible generalised deficiency of the enzyme galactokinase[23].

SUMMARY

A method is described for the separation of 0.5–1.0 micromole amounts of some common polyols by ion exchange chromatography. Application of the method to the analysis of urinary polyols has confirmed the presence of arabitol, erythritol and mannitol in normal human urine; threitol has also been detected. The 24 h excretion values of some polyols are presented.

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A Routine Method for Micro Determination of Mannitol in Serum

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Abstract: TIBBLING, G. A Routine Method for Microdetermination of Mannitol in Serum. *Scand. J. clin. Lab. Invest.* **22**, 7-10, 1968. A simplified method for the determination of mannitol in serum is described. The method is based on oxidation of mannitol with periodic acid followed by colorimetric determination of the formaldehyde formed by the use of chromotropic acid. The interference of glucose and glycerol present in serum is generally negligible, except where a very high degree of specificity is required. The small sample volume (50 µl) required for analysis renders the method suitable for capillary blood sampling.

Key-words: Serum; mannitol; spectrophotometry

During the past years the increasing use of hypertonic mannitol infusion in treatment of patients with renal failure (5, 10, 11) and cerebral edema (2) has created a need for estimating the serum level of mannitol, particularly where repeated infusions are made.

The most convenient methods for determination of mannitol are based on the oxidation of mannitol by periodate, yielding two moles of formaldehyde and four moles of formic acid per mole of mannitol. Formed formaldehyde is then determined spectrophotometrically by using a colorimetric reagent. With the procedure recommended by Corcoran & Page (4) the range for determination of mannitol in serum is small (10-60 mg per 100 ml of serum), which makes the method laborious when unknown samples with a wide range of mannitol concentrations have to be determined. Furthermore, the excess of periodate and iodate present in sample after oxidation of mannitol is removed by addition of stannous chloride, which has to be freshly prepared and standardized daily. Sodium arsenite is more convenient for this purpose, since it is stable in solution (7). As colorimetric reagent Bailey (1) used a modification of the phenyl hydrazine-ferricyanide procedure of Kersey, Maddocks & Johnson (6). With this reagent, zinc and barium ions have to be removed from the supernatant, after deproteinizing to eliminate interference with the ferricyanide reagent, which is not necessary when using chromotropic acid reagent.

The aim of this study was to develop a routine method for determination of mannitol in serum, based on the principles mentioned above.

METHODS

Reagents

Zinc sulphate, 0.087 M. *Barium hydroxide*, 0.083 M. *Postassium periodate*, 0.02 M in 1 N sulfuric acid. *Sodium arsenite*, 0.2 M.

Chromotropic acid reagent

300 ml of concentrated sulfuric acid is mixed with 150 ml of distilled water. 1 g of 4,5-dihydroxy-2,7-naphtalene disulphonic acid, disodium salt (Eastman-Kodak), is dissolved in 100 ml of distilled water, filtered off if not clear, and added to the diluted sulphuric acid. The solution should be prepared at least two days before use, as a fresh solution may result in poor color development. The reagent is stored in a dark, air tight bottle at 4° C. P

can then be used for at least two months. *Standard:* 400 mg mannitol (Merck) per 100 ml of distilled water.

Collection of sample and deproteinization

Serum (50 μl) from venous or capillary blood is transferred to a small disposable tube containing 1 ml of 0.087 M $ZnSO_4$. Alternatively, 50 μl of capillary blood may be collected directly into a tube containing this solution. In this case, however, it is necessary to estimate the hematocrit for calculating the mannitol concentration in serum from the concentration obtained in whole blood. For complete deproteinization, 1 ml of 0.083 M $Ba(OH)_2$ is added to each tube followed by centrifugation. It should be observed that the pH of the supernatant must not exceed 7.0 for complete deproteinization. When samples have to be stored, it is recommended that serum or supernatants should be kept at 4° or below freezing to avoid liberation of glycerol due to *in vitro* lipolysis of triglycerides.

Procedure

From the clear protein-free supernatant, 0.3 ml is transferred to a glass stoppered test-tube followed by addition of 0.1 ml of 0.02 M potassium-periodate in sulphuric acid. Ten minutes later 0.1 ml of sodium arsenite is added. After another five minutes or more, 3 ml of chromotropic acid reagent is transferred to each tube. The samples are heated on a boiling water bath for 30 minutes. After boiling for about one minute the glass-stoppers are placed loosely on the tubes. At the end of the boiling period the samples are cooled to room temperature and read against blank in a spectrophotometer at 570 nm.

EXPERIMENTS AND DISCUSSION

Periodic acid oxidation

The oxidation of mannitol by the present method was studied at two different concentrations (200 and 400 mg per 100 ml of water) and with an oxidation time varying up to twenty minutes (Fig. 1). Since optimal oxidation was already obtained within ten minutes, this time was chosen for the method. It is, however, not advisable to prolong the oxidation due to increasing interference from glucose.

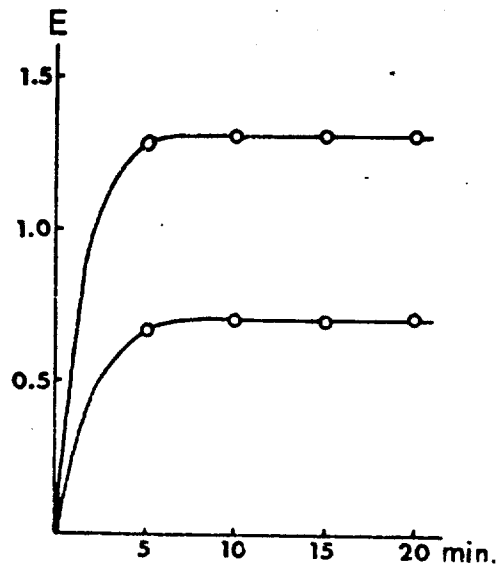

Fig. 1. Oxidation rates of mannitol at 200 and 400 mg per 100 ml of water. A sample volume of 50 μl was analysed according to the whole procedure. Oxidation was arrested at the respective time by addition of sodium arsenite.

The oxidation was arrested by addition of a sufficient molar excess of arsenite to reduce remaining periodate and iodate to iodide, which does not interfere with the chromotropic acid reagent (7).

Blank and standard curve

The absorbance of the blank varied between 0.03-0.06, increasing with the age of the chromotropic acid reagent over several months.

The standard curve passed through the origin and was linear at least to an absorbance of 1.8 corresponding to a concentration of mannitol in serum of 500 mg per 100 ml. The molar absorptivity for mannitol and glycerol

as well as sorbitol determined according to the present method was found to be $3.1 \cdot 10^{-7}$. These results are equivalent to the molar absorptivity calculated from the results given by Carlson (3) in his method for determination of triglycerides using the same principle for determination of liberated glycerol.

Reliability of the method

From the results of 51 pairs of duplicate analyses, the precision of the method was S.D. = 3 mg of mannitol per 100 ml of serum, tested within a range of 20-470 mg per 100 ml.

The accuracy of the method was estimated from the results of recovery experiments, which are presented in Table I. The Table shows that the accuracy was satisfactory. In five samples of whole blood containing mannitol, the concentration was determined both on whole blood and serum. When the mannitol concentrations obtained in whole blood were converted into concentrations in serum by means of the respective hematocrit values found (range 23-42 per cent), the results were in accordance with those obtained in serum (mean 100 per cent, range 98-103 per cent).

The sensitivity of the method was judged from the results of precision. From these calculations, the S.D. was found to be 1.5 mg of mannitol per 100 ml of serum, and the 95 per cent level of confidence will thus be 3 mg per 100 ml. Therefore, mannitol concentrations below 3 mg per 100 ml of serum had to be considered indistinguishable from zero.

Table I. *Recovery of mannitol*

Number of samples	Mannitol* added mg/100 ml	Recovery per cent mean SD (range)
8	76	100 2.5 (97 - 103)
2	173	97 : 102
12	302	99 : 1.5 (97 101)

* Mannitol added refers to the concentration after dilution in serum.

Table I. *Oxidation rate of glucose in the present method*

Glucose mg per 100 ml of water	Absorbance at 570 nm 5 min.	10 min.	15 min.	20 min.
100	0.002	0.010	0.013	0.024
200	0.015	0.021	0.025	0.044
400	0.029	0.044	0.061	0.084

A sample volume of 50 μl was analysed according to the whole procedure.

Oxidation time with periodate. Reaction was arrested by addition of excess sodium arsenite as described in procedure followed by colorimetrical determination of the formaldehyde formed.

The specificity of the method was studied with regard to interference from glucose and glycerol. In Table II results are presented for three different concentrations of glucose tested with a varying time of periodic acid oxidation. It is obvious that there is a proportional increase in the absorbance both with increasing concentration of glucose and time of oxidation. The mean absorbance for each 100 mg of glucose per 100 ml of water and per min. of oxidation was found to be 0.001, which corresponds to a mannitol concentration of 0.28 mg per 100 ml of water. With the oxidation time chosen for the method (10 min.) the interference of glucose on the determination of mannitol in serum will therefore be about 3 mg of mannitol per 100 ml of serum for each 100 mg of glucose present per 100 ml of serum.

As the molar absorptivity for glycerol was equal to that of mannitol, the interference from glycerol present in serum was calculated to be one mg of mannitol per 100 ml of serum for 0.055 mM glycerol. The mean fasting level of glycerol from healthy subjects and patients with non-metabolic disorders (n = 75) has been found to be 0.065 ± 0.021 (S.D.) mM in whole blood (9), which is about 11 per cent lower than the corresponding value of

serum (8). The ordinary interference from glycerol present in serum from subjects with non-metabolic disorders will thus be less than two mg of mannitol per 100 ml of serum, when in vitro lipolysis of triglycerides can be avoided.

The absorbance obtained from 44 serum specimens containing no mannitol and collected from subjects with non-metabolic disorders, showed a range of 3.5 to 11.2 mg per 100 ml and a median concentration of 5.6 mg of mannitol per 100 ml of serum. In 20 of these specimens the absorbance was measured from unoxidized supernatant fluid, which was performed by reversing the order of addition of periodate and arsenite. When these results were taken into consideration the remaining absorbances of the oxidized serum specimens showed a normal distribution corresponding to a mean value of 4.0 ± 0.9 (S.D.) mg of mannitol per 100 ml of serum (range 2.3-5.0 mg per 100 ml). These results agree well with the errors calculated for glucose and glycerol, which are about 4 mg of mannitol per 100 ml of serum. For simplicity an approximate correction can be done by reducing the mannitol concentration obtained in serum by about 6 mg per 100 ml. For a high degree of specificity, as in the investigation of patients with metabolic disorders, the factors discussed above which interfere with the method must be taken into consideration.

When zinc sulphate and sodium hydroxide was used for protein precipitation the serum blank values varied greatly corresponding to mannitol concentrations of 3-30 mg per 100 ml of serum. An increase of the median value of about three times that obtained by the method was found, which makes this a less useful precipitant for this technique.

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Conclusion

The advantage of the present method is the small sample volume required for analysis and the simplicity of the procedure employed. Furthermore, sorbitol and also glycerol were determined in the same way as mannitol. It is therefore concluded that homologous alcohols to mannitol can be estimated by the method in equimolar concentrations with those given for mannitol.

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ON THE METABOLISM OF SORBITOL AND MANNITOL*

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Following the demonstration (1) that sorbitol acts as an efficient diuretic in dogs, we have interested ourselves in a study of the metabolism in experimental animals of this sugar alcohol and one of its isomers, mannitol. A review of available literature concerning the metabolism of these compounds reveals considerable difference of opinion as to utilization, glycogen formation, etc., in animals. Variations in species response and in techniques employed may in part explain the discrepancies reported. The value of sorbitol in the diabetic diet is similarly controversial.

As early as 1883 Jaffe (2) demonstrated that after feeding mannitol to dogs large quantities can be recovered unchanged from the urine. Rosenfeld (3) observed no significant increase in liver glycogen in dogs after feeding mannitol. Following the administration of 100 gm. each of several carbohydrates dissolved in a glass of water to fasting colored males, Field (4) reported an average increase in blood sugar of 10 mg. per cent when mannitol was given compared to a 40 mg. per cent increase after the ingestion of glucose. Ariyama and Takahashi (5) reported mannitol to be far inferior to starch as a carbohydrate source for rats. It was also noted by Lafon (6) that in mice mannitol is utilized to a very slight extent or not at all and is toxic in sufficient quantity. However, it was reported that sorbitol is not toxic up to 30 per cent of the ration and that it seems to be completely metabolized. On the other hand Lecoq (7) observed that both mannitol and sorbitol are completely utilized by pigeons when they comprise 35 per cent, and the only carbohydrate source, of a lipid-rich ration. 66 per

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cent of either in the diet resulted in death of the animals. Voegtlin, Dunn, and Thompson (8) studied a number of carbohydrates and listed mannitol as slightly or doubtfully protective to rats previously given normal lethal doses of insulin.

Only two papers have come to our attention regarding the possibility of glycogen deposition in fasted rats following the administration of mannitol. Carr, Musser, Schmidt, and Krantz (9) fasted rats for 24 hours and then fed cacao-butter containing 33 per cent mannitol. After 80 hours on this diet these animals showed large increases in liver glycogen compared to controls fed cacao-butter only. Silberman and Lewis (10) administered 2 to 4 ml. of 15 per cent mannitol by stomach tube to rats fasted 24 hours and after absorption periods of 2, 3, 4, and 6 hours found no increase in liver glycogen over controls which had received like amounts of water. These authors stated that their results cannot be compared with those of Carr *et al.* because of the great dissimilarity in technique employed. We are unaware of any work substantiating or refuting these results.

Embden and Griesbach (11) reported that sorbitol solution perfused through livers from dogs previously phlorhizinized resulted in the formation of a mixture of fructose and glucose. However, lactic acid was formed when liver tissue from dogs fasted but not phlorhizinized was employed. Koike (12) found increased blood sugar and blood lactic acid following the intraperitoneal administration of sorbitol to rabbits, but Roche and Raybaud (13) state that this alcohol is not transformed into liver glycogen by the fasting rabbit and that it is without effect on insulin hypoglycemia in these animals. Payne, Lawrence, and McCance (14) reported the failure of ingested sorbitol to increase the liver glycogen content of fasted rats. Thannhauser and Meyer (15) fasted dogs and then fed lean meat and glucose or lean meat and sorbitol for 3 days. The liver glycogen content of both groups showed about equal values and they stated that sorbitol is built into liver glycogen as readily as is glucose.

A number of authors (14, 16-18) are of the opinion that sorbitol has a place in the diabetic diet, while others (19-23) feel that it is to be avoided in such a diet.

Because of the controversial nature of the data and interpretations relative to the metabolism of sorbitol and mannitol in the

animal body, the writers have undertaken experiments in an attempt to clarify the situation.

Recovery from Urine of Sorbitol Given Intravenously—Normal female dogs were catheterized and then given 50 ml. of 50 per cent sorbitol intravenously over a period of 5 minutes. At varying intervals for 24 hours urine was collected by catheterization, or from the metabolism cages if voided, and kept in an ice box under toluene for sorbitol estimations. (Methods for the determination of sorbitol and other sugar alcohols are given in the preceding paper.) It is seen from the data in Table I that approximately 40 to 50 per cent of the injected sorbitol was recovered from the urine. Practically no excretion of sorbitol occurred after the first 24 hours. The remaining 50 or 60 per cent was apparently metabolized, as will be shown in a later discussion. Four such experiments were carried out on each of two dogs; representative data are given in Table I.

The figures in Table I are subject to some error and are to be seen as a close approximation only. With the method of determination employed dog urine exhibits considerable reduction (5 to 10 mg. per ml.) and consequently sorbitol determinations on urine in these experiments actually give total reduction which is not all attributable to the sorbitol present. Total reduction was determined on 24 hour urine samples from two dogs for 7 consecutive days. A range of 0.4 to 2.6 gm. reduction per day was found with an average of 1.4 gm. daily (average of both animals). Considering this amount of reduction from sources other than sorbitol during the above experiments, an average error in sorbitol recovery of about 12 per cent is introduced. The figures in the third column of Table I are thus high. The average per cent recovery in Table I is 44.7 and after the above correction is applied this figure becomes 39 per cent, which may be closer to the true value.

Clearance of Sorbitol from Blood Following Its Intravenous Injection—Normal dogs were given 50 ml. of 50 per cent sorbitol intravenously over a period of 5 minutes. Blood samples were withdrawn just previous to and at varying intervals following the injection. The samples were oxalated and subjected to sorbitol estimation at once. In a number of instances this work was carried out concurrently with the urine studies cited above. In Fig. 1 representative curves are presented for three dogs. Sor-

bitol was rapidly cleared from the blood following the high values attained a few minutes after the end of the injection. In all instances a peak of 400 to 700 mg. per cent was reached a few minutes after the injection and generally a value rather near the basal figure was attained within 2 or 3 hours. However, there is a

TABLE I

Recovery of Sorbitol from Dog Urine after Intravenous Injection of 50 Ml. of 50 Per Cent Sorbitol Solution

Dog No.	Recovered from urine in 24 hrs.	Recovery
	gm.	*per cent*
1	10.13	40.5
	12.14	48.5
3	9.53	38.1
	12.95	51.8

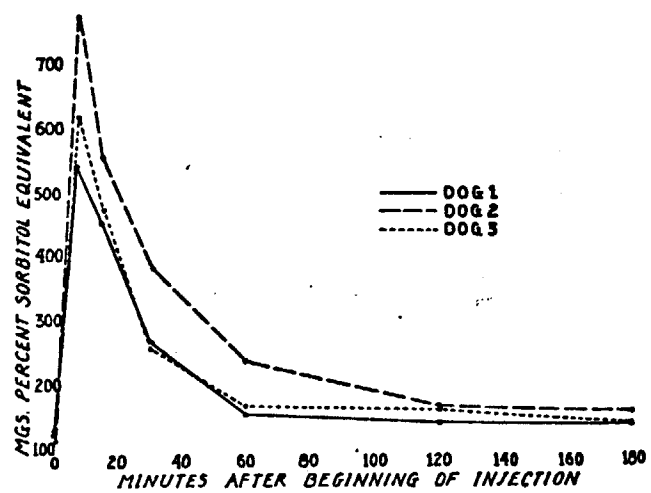

FIG. 1. Blood sorbitol curves following intravenous injection

distinct tendency for the curve, although flattened at 2 or 3 hours, to remain above the basal figure for several hours. This phenomenon has not yet been investigated. In eight such experiments on these three dogs the curves all fall within the limits of curves plotted in Fig. 1.

It is to be remembered that these curves are not true values for

sorbitol but represent total reduction including glucose, sorbitol, and undoubtedly other non-sugar reducing substances. It is felt that the validity of the data is little affected by this circumstance, as basal values were always determined. This figure was found to be quite constant; in eleven experiments in which basal blood reduction was determined with our method a range of from 118 to 146 mg. per cent was found, while in seven of these instances values between 130 and 140 mg. per cent were noted.

Blood Sugar Level Following Injection of Sorbitol and Mannitol—The reports in the literature on the conversion of sorbitol to glucose in experimental animals are controversial. Under certain conditions mannitol may lead to the formation of glucose, although little work has been reported on this point. Quantitative data and information regarding the speed of such reactions are lacking.

Normal dogs were given 50 ml. of 50 per cent sorbitol solution intravenously over a 5 minute period. Blood sugar was estimated on aliquots of the blood samples drawn for sorbitol studies, as described above. Clarification of the blood was accomplished by the method described by Steiner, Urban, and West (24) and the sugar was estimated by the Shaffer-Somogyi technique (25) with their Reagent 50. Mannitol and sorbitol do not reduce this reagent.

Fig. 2 indicates the nature of the true sugar curves. It will be seen that a peak of approximately double the basal value is reached about 30 minutes after the injection and that in 2 or 3 hours the basal figure is approached.

As mannitol is much less soluble (15 parts per 100 at 18°) than sorbitol, it was necessary to use relatively dilute solutions in order to compare the two compounds. In these experiments a dog was anesthetized with nembutal and given 150 ml. of 15 per cent mannitol intravenously over a period of 5 minutes. Blood samples were taken as previously and sugar estimations made at once. 3 days later the same dog was employed similarly except that 150 ml. of 15 per cent sorbitol were administered. The curves in Fig. 3 indicate that under these conditions sorbitol leads to an increase in blood sugar, while mannitol does not. The drop in the sugar curve after mannitol may be a result of blood dilution.

Formation of Glycogen from Sorbitol and from Mannitol in Fasted Rats—As the literature offers some confusion regarding the

possibility of conversion of sorbitol and mannitol into glycogen in experimental animals, we have administered these compounds to rats with the hope of clarifying the situation with regard to this species at least. Adult white rats, weighing from 150 to 250

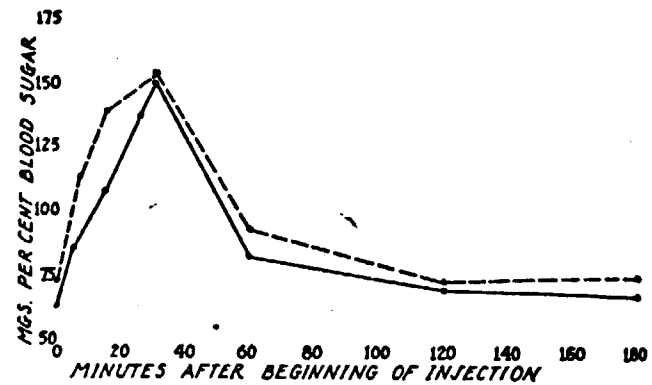

FIG. 2. Blood sugar curves for Dog 3 following 50 ml. of 50 per cent sorbitol given intravenously.

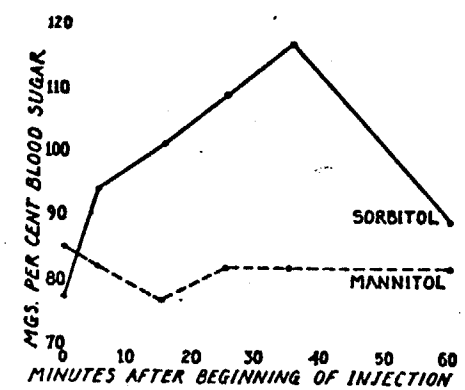

FIG. 3. Blood sugar curves for Dog 4 after the intravenous administration of 150 ml. of 15 per cent sorbitol or mannitol.

gm., from our stock colony were fasted for 40 hours and then given a solution of sorbitol or mannitol by stomach tube or by intraperitoneal injection. After various absorption periods the animals were decapitated, the livers removed and ground in a small meat grinder, and a sample immediately placed in a weighed tube

of alkali. With a little practice this entire operation can be completed in about 1 minute. The glycogen was determined by the Good-Kramer-Somogyi method (26). A number of the animals in each experiment received no supplement and acted as a control group.

TABLE II

Glycogen Storage in Livers of Fasted Rats (40 Hours) Following Administration of Sugar Alcohols

No. of rats	Substance	Method of administration	Total given	Dose volume	Solution	Interval between doses	Time killed after first dose	Per cent liver glycogen average
			ml.	*ml.*	*per cent*	*hrs.*	*hrs.*	
3	Sorbitol	Stomach tube	2	1	50	4	8	1.06
3	None		0				8	0.14
5	Sorbitol	Intraperitoneally	8	4	12.5	2	6	1.73
5	None		0				6	0.26
5	Mannitol	Stomach tube	4	2	20	2	5	0.33
5	None		0				5	0.33
5	Mannitol	Intraperitoneally	8	4	12.5	2	5	0.27
5	None		0				5	0.33

TABLE III

Glycogen Deposition in Livers of Fasted Rats (24 Hours) Subsequently Fed for 72 Hours

Eight animals were used in each instance.

Food consumption	Fed	Average weight loss	Average glycogen
gm.		*gm.*	*per cent*
13.4	Cacao-butter only	37	0.27
11.3	" + 33% mannitol	35	0.97
12.4	" + 33% sorbitol	43	0.79

Table II presents data from several experiments. It is apparent that sorbitol is readily converted into liver glycogen, while mannitol is not handled in the same manner under these experimental conditions.

Glycogen Storage in Previously Fasted Rats after Long Periods of Mannitol Feeding—Our results are in accord with those of Silberman and Lewis (10) that mannitol *per os* does not lead to glycogen deposition in fasted rats when only a few hours are allowed for absorption. However, the situation is apparently different when such animals are allowed to feed over long periods on mannitol and fat (Carr *et al.* (9)). We have further substantiated the findings of these authors using their technique, although our results show considerably less glycogen deposition.

Rats were fasted 24 hours and then fed cacao-butter containing 33 per cent mannitol or 33 per cent sorbitol *ad libitum* for a period of 72 hours. Controls were given cacao-butter only. At the end of this period the animals were sacrificed and their liver glycogen estimated, as previously described. The data in Table III indicate that the ingestion of either of these sugar alcohols leads to the deposition of liver glycogen under these conditions.

DISCUSSION

It was recently pointed out by Mirski *et al.* (27) that, when rats previously fed high protein diets were fasted, glyconeogenesis was highly intensified. This protein effect was also evident in animals which had been fasted 3 days, for, as explained by these workers, the rat organism uses up its store of carbohydrate during the first 24 hours of fasting and after that is living on its stock of protein and fat. We have noticed that on several occasions when our animals were fasted more than 48 hours liver glycogen values were found which were considerably above the usual 0.1 to 0.3 per cent for fasting rats. This we feel is explainable on the basis of the protein effect described by the above authors. Low figures for liver glycogen can be obtained regularly by fasting rats 40 hours.

As far as we are aware this paper presents the first data indicating that in the dog the intravenous injection of sorbitol results in an elevation of the blood sugar. We are also unaware of other attempts to study quantitatively the recovery of sorbitol from the urine or its clearance from the blood after its administration to dogs.

The writers wish to express their appreciation to Dr. H. C. Speel of the Atlas Powder Company for kindly supplying sorbitol.

and mannitol for this work. They are indebted to Dr. Hance Haney for assistance in the dog experiments.

SUMMARY

1. The intravenous injection of sorbitol leads to an increased blood sugar in dogs. Mannitol does not exhibit this action.

2. Shortly after the injection of 50 ml. of 50 per cent sorbitol into dogs, blood clearance curves for this compound show a peak and a rapid decrease within the 1st hour. At 2 hours values close to the basal figure are reached but are maintained slightly elevated for several hours.

3. Under the same conditions about 40 to 50 per cent of the sorbitol can be recovered from the urine in 24 hours. After this time the excretion is negligible. The remainder of the sorbitol is apparently utilized.

4. When sorbitol is administered to fasted rats by stomach tube or by intraperitoneal injection, a deposition of liver glycogen occurs within 8 hours. Mannitol similarly administered does not lead to such a deposition.

5. When fasted rats are fed over a 72 hour period on mixtures of either sorbitol and cacao-butter or mannitol and cacao-butter, glycogen is deposited in their livers.

6. It is evident that sorbitol is much more readily converted into glucose and glycogen in the animal body than is mannitol.

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PHYSIOLOGICAL REVIEWS

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Biochemistry of the Acyclic Polyols[1,2]

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POLYOLS, or polyhydric alcohols, are, strictly speaking, simply organic chemicals containing more than two hydroxyl groups. However, common usage has generally limited the term to reduction products of sugars and their closely related derivatives. The simplest acyclic polyol is therefore glycerol, the reduction product of either glyceraldehyde or dihydroxyacetone. Glycerol and inositol have been recognized as substances of major physiological importance in organisms ranging from bacteria to mammals. Other polyols have interested investigators primarily from the viewpoint of toxicology, chemotherapy, the biochemical potentialities of microorganisms, or the composition of plants. In recent years, there has been a considerable expansion in knowledge of polyol metabolism in microorganisms, and, in addition, evidence has accumulated indicating that polyols are normal intermediates in the metabolism of mammals.

It is the purpose of this paper to review the biochemistry of polyols other than glycerol and inositol, except where discussion of these two substances is especially relevant to a point under consideration. The wide occurrence of polyols in nature, particularly in lower forms of life, points to their great physiological importance. Table 1 summarizes the natural occurrence of both free and combined forms of the acyclic polyols. References to the more important and more recent findings are given. For detailed listings of the many plant species that contain polyols, other reviews may be consulted (27, 55, 215). Table 1 omits the listing of the numerous microorganisms capable of excreting polyols into culture media when they are given appropriate sugars as nutrients.

[1] Previously unpublished work mentioned in this paper was done at Vanderbilt University under a research grant from the National Science Foundation.

[2] Abbreviations: DPN, DPNH, TPN, and TPNH: oxidized and reduced forms of di- and triphosphopyridine nucleotide, respectively; GA, D-glucuronic acid; GA-1-P, D-glucuronic acid 1-phosphate; Xu, xylulose; Pi, inorganic phosphate; UMP, uridine monophosphate; ADP and ATP, adenosine di- and triphosphate, respectively; UDPG, UDPGal, and UDPGA; in order, uridine diphosphate glucose, galactose, and glucuronic acid.

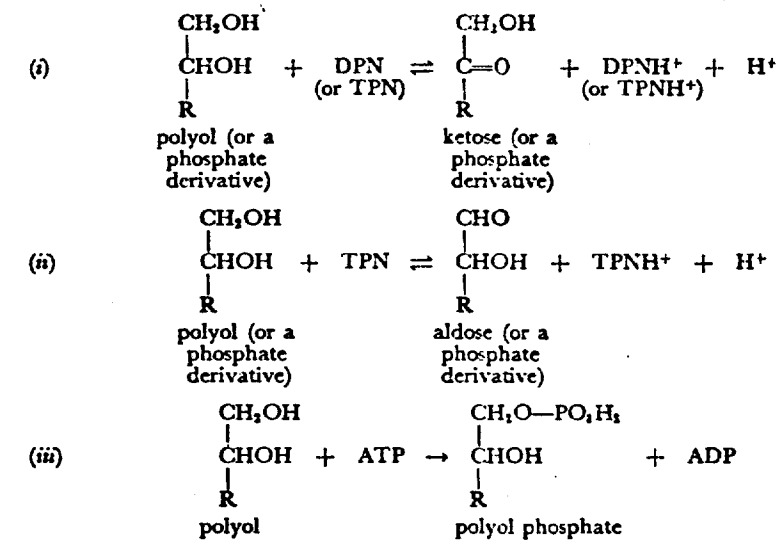
TABLE I. NATURAL OCCURRENCE OF THE ACYCLIC POLYOLS

POLYOL	SOURCE	REF.
Erythritol	Algae	55, 215
	Grasses	55, 215
	Lichens	55, 212, 215
	Fungi	54, 142, 157, 234, 240
	Residue from fermented molasses	43
	Urine of normal humans	301
D-Arabitol	Lichens	14, 213, 236
	Fungi	55
	Uredospores of wheat-germ rust	247
	Residue from fermented molasses	43
L-Arabitol	Urine of pentosuric humans	300
DL-Arabitol	Urine of normal humans	300
Ribitol	In riboflavin and its derivatives throughout Nature	
	Bacteria (in cell walls and in cytidine diphosphate ribitol of gram-positive bacteria, and in pneumococcal polysaccharide)	161, 195
	Plants	55, 215
	Honeydews of wax scale insects (Ceroplastes)*	152
Xylitol	Rat lens (after D-xylose feeding)	167, 168
Sorbitol	Algae (seaweed)	55, 215
	Fungi (yeast)	108
	Higher plants of many types, especially fruit trees	55, 215
	Silkworm (diapause egg of *Bombyx* silkworm)	94
	Animals (fetal blood, seminal vesicles and plasma, the lens of the alloxan-diabetic or D-xylose-fed rat)	59, 60, 164, 166–168, 200, 201, 204
D-Mannitol	Bacteria (*L. arabinosus*)	21
	Algae (e.g. all brown seaweeds)	215
	Grasses	156
	Lichens	55
	Fungi	55
	Higher plants of many types	55, 215
L-Iditol	Berry of mountain ash (*Sorbus aucuparia*)	39
Dulcitol	Algae (seaweed)	55
	Fungi (yeast)	55, 215
	Higher plants	55, 215
	Honeydew of *Aphis euonymi* Fabr.*	229
Perseitol	Avocado	55, 215
	Algae	215
Volemitol	Lichens	55, 215
	Fungi (mushroom)	55, 215
	Plants (primrose)	55, 215
β-Sedoheptitol	Plant (*Sedum spectabile*)	85
D-Erythro-D-galacto-octitol	Avocado	85

* It has been suggested that the polyol may be of dietary origin (152).

It is to be expected that many polyols have escaped detection during chemical analysis of tissues, since methods for their detection are few and sensitive procedures are comparatively recent in development. Lacking the carbonyl group present in sugars, the polyols do not respond to either common reducing tests or to the color tests so useful in carbohydrate chemistry. Analysis for polyols generally takes advantage of their reactivity with periodic acid or other reagents reactive with the 1,2-diol grouping. The periodic acid procedure, in conjunction with paper chromatographic techniques (19, 135, 149, 300), offers a sensitive method which, however, requires confirmation by other procedures able to rule out sugars or amino alcohols.

Since metabolites often do not accumulate in amounts sufficient for detection, even more convincing evidence that polyols are important in metabolism is the wide occurrence of enzymes that have polyols as substrates. The reactions catalyzed by these enzymes are of three types:



As expected, polyol phosphates are susceptible to the action of phosphatases. Other enzymatic transformations of polyols will probably be discovered as work proceeds on the biosynthesis of complex polyol-containing substances.

The naming of enzymes catalyzing reactions of types (*i*) and (*ii*) has long been a problem. Designations as "dehydrogenases" and as "reductases" are unfortunately now concurrently in use for some enzymes. It is hoped that the recommendations on nomenclature to be made in August, 1961 by the Commission on Enzymes of the International Union of Biochemistry will serve to reduce the number of names of enzymes acting on polyols, without sacrificing specificity. Another difficulty involves the designation of substrate specificity. Suggested names have sometimes indicated: (*a*) the essential chemical groups in the substrates, (*b*) the most typical substrate, (*c*) the physiological substrate, or (*d*) the substrate with greatest affinity for the enzyme. Most of these bases for nomenclature require extensive testing with a wide variety of possible substrates. Finally, there is the question of multiplicity of enzymes in preparations available. The

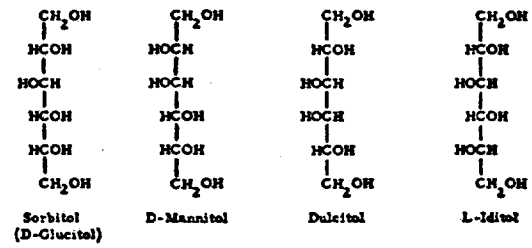


FIG. 1. Formulas of some hexitols.

specificity of an enzyme can be assigned with certainty only when it has been obtained in pure, crystalline form. Only one polyol dehydrogenase has been prepared in this state (275).

It should also be emphasized that the nomenclature of the polyols themselves is often confusing. The systematic method of naming polyols containing no more than six carbons is based on the name of the theoretical aldose precursor. There are, however, common names based on a major natural source of the polyol. Also, the prefix meso is occasionally added to indicate that a substance is devoid of optical activity (e.g. it is usually used with erythritol, but not with galactitol). A few examples follow (trivial or common names of the polyol are given in parentheses):

D-glucose + 2H → D-glucitol (sorbitol; D-sorbitol is incorrect (215))
L-glucose + 2H → L-glucitol
D- or L-galactose + 2H → galactitol (dulcitol)
D- or L-ribose + 2H → ribitol (adonitol)
D-arabinose + 2H → D-arabinitol (D-arabitol)
D- or L-erythrose + 2H → erythritol (meso-erythritol)

The naming of longer polyols is more complicated; two recent reviews on polyol chemistry may be consulted (55, 215). When isotopically labeled polyols are employed, proper designation of the labeled position is of course necessary. Sorbitol labeled on C_1 or C_6 with tritium may be named D-glucitol-1-t or L-gulitol-1-t, respectively (139).

Metabolic considerations at times lead to names which are not in strict conformance with nomenclature rules. A polyol esterified with phosphate on a primary hydroxyl should logically be designated as a 1-phosphate. However, since L-ribitol 1-phosphate is probably derived from D-ribose 5-phosphate, the polyol phosphate is referred to as D-ribitol 5-phosphate (18). Such deviations are useful, provided that the basis for the common name is made clear.

A few general comments about the physiological importance of polyols may be useful before individual substances and transformations are discussed. As with other classes of biochemicals, a number of different functions can be discerned or inferred: (*a*) structural, as in the ribitol phosphate polymers in cell walls of bacteria; (*b*) interconversions of sugars, as in the transformation of D-glucose into D-fructose via sorbitol in male accessory organs; (*c*) energy storage, as is likely in plants containing large amounts of mannitol polymers; and (*d*) coenzyme regulation, as in bacteria that excrete large amounts of polyols formed by reduction of sugars in culture media. These and other suspected functions will be discussed in greater detail later. Finally, the ease with which microorganisms adapt to growth on un-

common sugars and to polyols facilitates the development of systems useful in the study of adaptive enzyme formation.

I. Metabolism of Polyols in Mammals

Hexitols

Sorbitol (D-glucitol). Among the hexitols (Fig. 1), sorbitol is probably the most widely distributed in nature. It is the only reduction product of D-glucose and one of the two products from D-fructose, and its sweetness and hygroscopicity have made it useful in food manufacturing. It is therefore not surprising that of all the polyols sorbitol has been the most extensively studied in mammalian systems. Although there had been previous studies of its utilization (255), investigation of this substance was greatly stimulated by the work of Embden and Griesbach (125), who, in 1914, showed that perfusion of sorbitol through the liver of fasted or phloridzinized dogs yields a mixture of glucose and fructose, the former presumably being derived from the latter. This hypothesis was supported by the subsequent demonstration that the administration of sorbitol to an individual with fructosuria is followed by a large increase in the excretion of the ketose (7). These findings, together with the suggestion that sorbitol might be useful in the diet of patients with diabetes mellitus (293), and the finding that sorbitol exerts an antiketogenic effect on liver slices from starved rats (116), led to numerous studies on the metabolism and therapeutic value of this hexitol (81, 123). Although many of these studies initially appeared to yield conflicting results, there is now clear evidence that administered sorbitol is readily utilized and that it is a normal metabolic intermediate.

The utilization of administered sorbitol has been demonstrated in the rat (52, 78, 162, 193, 285, 315), mouse (205), rabbit (203), pigeon (209), dog (293, 296, 317), monkey (122), and man (293). Little sorbitol is excreted if moderate amounts are administered (203, 205); larger doses lead to urinary sorbitol (296). Most studies have shown that orally administered sorbitol can increase liver glycogen, although there are reports to the contrary (52, 241, 315). In an individual with essential fructosuria there was found an increase in blood fructose but not glucose (272). Transient fructosemia has been reported to accompany the intravenous administration of sorbitol to rabbits (261). Studies on the metabolism of sorbitol have been complicated by the fact that it is slowly absorbed from the gastro-

Table 2. Distribution of labeling in muscle and liver glycogen after intracardiac injection of glucose-1-C^{14}, fructose-1-C^{14}, and sorbitol-1-C^{14} into rats (162)

Carbon atom*	Percentage of total activity in glucose isolated 1 hour after injection of					
	Glucose-1-C^{14}		Fructose-1-C^{14}		Sorbitol-1-C^{14}	
	Liver glycogen	Muscle glycogen	Liver glycogen	Muscle glycogen	Liver glycogen	Muscle glycogen
1	86	95	54	65	60	57
6	4	0.5	30	25	32	33

* C_2, C_3, C_4, and C_5 were also determined. They contained relatively little radioactivity.

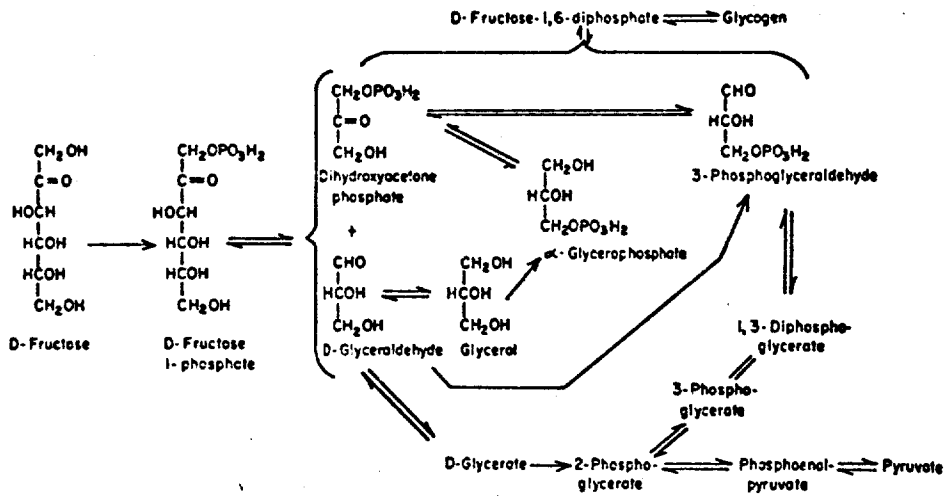


FIG. 2. Metabolism of D-fructose in liver.

intestinal tract (226, 239). As will be discussed later, this property of sorbitol has interesting nutritional consequences.

Isotopically labeled sorbitol has been used to substantiate the evidence that sorbitol is metabolized via fructose. C^{14}-sorbitol leads to labeled CO_2, urinary glucose, and liver and muscle glycogen in the rat (285, 317). Hers (162), by comparing the distribution of C^{14} in the carbon chains of glycogen following the administration of sorbitol-1-C^{14}, fructose-1-C^{14}, and glucose-1-C^{14}, obtained clear evidence that the pathways of sorbitol and fructose to glycogen are very similar, and, unlike that of glucose, involve cleavage of the carbon chain (Table 2). These results are in full agreement with enzymatic studies on the utilization of fructose in liver, where fructose-1-phosphate is the first metabolic product. This is split into two trioses before hexose intermediates of the Embden-Meyerhof glycolytic pathway are formed (Fig. 2).

Following the demonstration by Breusch (57, 58) that liver brei can metabolize sorbitol, Blakley (51) showed that the polyol is converted directly into D-fructose in a DPN-linked reaction. This transformation will be discussed in detail in the section dealing with enzymatic aspects. The distribution of sorbitol dehydrogenase activity in human tissues has been determined (144). The enzyme is not detectable in normal human serum (329), but it may be markedly elevated in the sera of patients with acute viral hepatitis (144, 329) or with liver metastases from malignant disease (329). It is one of the many enzymes that increase rapidly (in several hours) in the sera of rats treated with carbon tetrachloride (329). The diagnostic value of serum sorbitol dehydrogenase levels is undoubtedly the basis for the commercial availability of "SDH" assay kits.

In accord with the observation that appreciable levels of sorbitol dehydrogenase are present only in the liver, kidney, and male accessory organs, peripheral tissues are unable to metabolize sorbitol. Wick and Drury (318) showed that C^{14}-sorbitol injected into an eviscerated, nephrectomized rabbit does not give rise to $C^{14}O_2$. Moreover, since the polyol has a volume of distribution corresponding to the extra-

cellular compartment, whether or not insulin is given, sorbitol does not enter peripheral cells. However, although many other polyols and sugars are unable to replace glucose in promoting the growth of tissue cultures, sorbitol has been found to yield limited growth of a wide variety of animal and human tissues, both normal and neoplastic (25, 115, 231).

The value of sorbitol in diabetes has been widely debated. It should first be mentioned that, in the normal rat, injected sorbitol and glucose are fairly similar in the extent to which they are oxidized to CO_2 and converted to glycogen (285, 317). In the alloxan-diabetic rat, both sorbitol and glucose injected intraperitoneally are strongly glucogenic and are poor precursors of CO_2 (285), but ingested sorbitol is well metabolized (320). Even large amounts of C^{14}-sorbitol administered orally to humans are efficiently metabolized to $C^{14}O_2$, both in normal and in severely diabetic subjects (75% and 65% conversion, respectively) (1). However, in diabetic subjects, a single oral dose of 35 g of sorbitol causes a much smaller increase in blood sugar levels than does glucose, the blood sorbitol levels remaining negligible (1). The slow absorption of sorbitol from the gastrointestinal tract allows the polyol to serve as an energy source without causing a marked elevation in blood sugar levels (196, 270). On the basis of the similar antiketogenic activity of sorbitol and fructose in rats and the convenient preparation and administration of sorbitol, Todd (295) has suggested that the hexitol be considered as a possible adjunct to insulin therapy in the treatment of human diabetic ketosis. Daughaday (105) recently stated that "sorbitol would appear to have promise in providing slowly absorbed carbohydrate between meals and at night for individuals prone to hypoglycemic attacks." The hexitol does not have any effect on the insulin requirement of diabetics (270).

Large amounts (50 g) of sorbitol may have a laxative effect (317). This property of the hexitol has led to its use as an adjunct to therapy with ion-exchange resins, the hexitol preventing impaction of the resin and promoting fluid and electrolyte losses through the bowel (258). Moreover, it was suggested that sorbitol alone may promote dehydration in overhydrated patients. Intravenously administered sorbitol has been reported to be a diuretic (210).

It is interesting that dietary sorbitol is able to lower or eliminate requirements for B vitamins in the diets of animals. This phenomenon, first reported by Morgan and Yudkin in 1957 (232), has been demonstrated in the rat (86, 87, 153, 175), mouse (233), and man (316). The slow absorption of sorbitol permits it to influence the intestinal flora, leading to an increase in microorganisms which synthesize the B vitamins. Lactose, a sugar which is absorbed fairly slowly, also has a moderate vitamin-sparing action (153).

Sorbitol enhances the absorption of vitamin B_{12} in rats (150) and in normal human subjects (96). Unfortunately, the polyol seems to be ineffective in per-

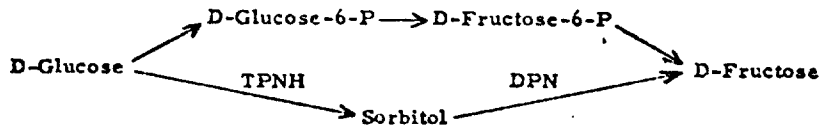


FIG. 3. Alternative pathways for the conversion of D-glucose to D-fructose.

TABLE 3. SORBITOL AND FRUCTOSE CONTENT OF FRESH SEMINAL PLASMA (201)*

SPECIES	NO. SAMPLES ANALYZED	SORBITOL (MG/100 ML)	FRUCTOSE (MG/100 ML)
Ram	5	26–120 (12)	150–600 (12)
Rabbit	1	80 (2)	40–150 (4)
Bull	4	10–134 (4)	120–540 (14)
Boar	4	6–18 (4)	20–40 (4)
Stallion	3	20–60 (3)	1 (4)
Dog	2	1 (3)	1 (5)
Cock	3	10–15 (14)	1 (14)
Man	1	10 (3)	154 (3)

* Figures in parentheses indicate number of individuals in pooled samples.

nicious anemia patients (121). It is of interest that, in pregnant rats, sorbitol also promotes the transport of vitamin B_{12} from maternal tissues to the fetus (252). The mechanism of this effect on vitamin transport has not been elucidated.

In the last few years sorbitol has been recognized as a normal metabolite in mammals, namely, as a precursor of fructose. In 1954 Williams-Ashman and Banks (323) found that the seminal vesicle, dorsal prostate, and coagulating gland of the rat contain an enzyme similar to "sorbitol dehydrogenase" of liver (51, 57, 58, 218) and suggested that this enzyme might be involved in the formation of D-fructose, the characteristic sugar of seminal plasma (220, 221). It had previously been proposed by Mann and Lutwak-Mann (222) that seminal fructose arises from the successive conversion of glucose into glucose 6-phosphate and fructose 6-phosphate, the latter finally being hydrolyzed by a phosphatase (Fig. 3). Hers (163, 164, 166) proposed an alternative mechanism in which glucose is reduced to sorbitol, followed by dehydrogenation of the polyol to form fructose (Fig. 3). He has offered the following evidence for this mechanism: (*1*) Slices of sheep seminal vesicles convert glucose-1-C^{14} into sorbitol and fructose (the label still being at C_1), and sorbitol only into fructose, while fructose is not transformed into either sorbitol or glucose. (*2*) The same tissue contains an enzyme, "aldose reductase," which catalyzes the reduction of glucose to sorbitol in the presence of TPNH. (*3*) Inhibitors of aldose reductase prevent the conversion of glucose to sorbitol and fructose, and inhibitors of sorbitol dehydrogenase block the conversion of sorbitol into fructose. (*4*) Tissues usually have TPNH/TPN and DPN/DPNH ratios greater than 1 (146); these ratios would explain the observed irreversibility of the glucose-fructose conversion in intact seminal vesicle tissue. (*5*) The fresh tissue contains polyol material, a large portion of which appears to be sorbitol. This mechanism for the production of seminal fructose has received general support, although the site of one or the other of the reactions probably still requires further study. Sorbitol has also been found in extracts of the coagulating gland of the rat (59).

Sorbitol has been identified as a constituent of the seminal plasma of many species, including man (200, 201). Table 3 shows that there is a rough correlation between the sorbitol and fructose concentrations of seminal plasma. Ram spermatozoa oxidize sorbitol to fructose in the presence of DPN, and sorbitol dehydrogenase has been isolated from this tissue (200, 201). The spermatozoa are able to convert both fructose and glucose into sorbitol.

The high concentration of fructose in the fetal blood of ungulates led Hers (164, 166) to explore the possibility that it, too, is formed from glucose via sorbitol. The glucose-reducing aldose reductase was found in the placentas of the sheep and cow but not of the human or rabbit, and it was undetectable in other tissues, except, of course, those concerned with the production of seminal fructose. Sorbitol dehydrogenase was found to be very active in fetal liver of sheep, the placenta showing much lower and variable activity. Fetal blood of the sheep has been reported to contain sorbitol-like material (59, 60, 164, 166, 204), and improved separation methods have led Britton (60) to suggest that mannitol and dulcitol are present with the sorbitol in the hexitol fraction. He has also suggested that the sorbitol of ram seminal vesicle and of fetal whole blood may be formed post mortem (59).

Hers believes that almost all fetal fructose is formed in the liver from sorbitol produced by the placenta. However, Huggett et al. (185), using a perfusion technique, have shown that the isolated sheep placenta can produce fructose from glucose. This transformation would require a higher activity of sorbitol dehydrogenase than was found by Hers in this tissue.

The concentration of fructose in the fetal blood of primates is small, but, as in ungulates, it is higher than that of maternal blood. Slices and "homogenates" of human placenta are able to produce fructose in vitro (154). The homogenates produce fructose when incubated with fructose 6-phosphate, glucose 6-phosphate, or sorbitol plus DPN. Since human placenta contains sorbitol dehydrogenase but neither aldose reductase nor appreciable amounts of sorbitol, the sorbitol mechanism cannot be the basis for the fructose production. Enzymatic studies led Hagerman et al. (154) to the conclusion that the ketose originated from fructose 6-phosphate as a result of the action of a nonspecific alkaline phosphatase.

A TPN-linked aldose reductase has also been extracted from rat lens by van Heyningen (167, 168) after she had observed that the administration of large quantities of aldoses leads to the accumulation of polyols almost exclusively in this tissue. Sorbitol (4.0 mg/g) was detectable in the lens of alloxan-diabetic rats and in much smaller amounts (0.4 mg/g) in rats given cataractogenic doses of D-xylose, but only traces were present in glucose-fed animals. Rat lens also contains a DPN-linked polyol dehydrogenase capable of converting several polyols to ketoses, including sorbitol to fructose. The role of these enzymes in the lens is unknown.

TABLE 4. EFFECTS OF POLYOLS ON ACETOACETATE FORMATION IN LIVER SLICES FROM STARVED RATS (160)

SUBSTRATE ADDED	O_2 UPTAKE*	ACETOACETATE FORMED*	RELATIVE ACETOACETATE PRODUCTION (ENDOGENOUS = 100)
None	19.9	4.36	100
Sorbitol	18.2	2.56	59
Xylitol	18.8	2.64	61
Ribitol	16.2	3.36	77
Mannitol	15.4	4.20	96
Dulcitol	14.1	4.00	92
Fructose	17.5	3.04	70

* Results expressed as μmole/100 mg wet tissue.

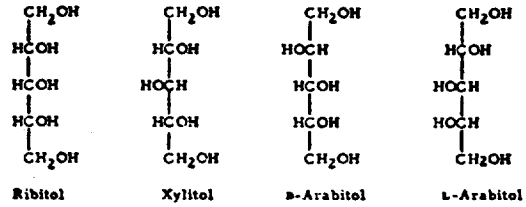


FIG. 4. Formulas of the pentitols.

The antiketogenic action of sorbitol on liver slices remains unexplained, but there is now evidence that polyols having this activity are substrates of L-iditol dehydrogenase (sorbitol dehydrogenase) (160). In Table 4 are shown the effects of polyols on acetoacetate formation in liver slices of starved rats. The most active substances, sorbitol, xylitol, ribitol, and, interestingly, fructose as well, are all known to be substrates of the dehydrogenase, whereas dulcitol and mannitol are not. Moreover, when the polyol concentration is lowered, the influence of sorbitol is diminished more than that of xylitol (160). This finding is consistent with the postulation that the dehydrogenase plays a role in the phenomenon (perhaps by generating DPNH), as xylitol has a greater affinity for the dehydrogenase than does sorbitol.

D-Mannitol. D-Mannitol is widely distributed in the plant kingdom, being, for example, the principal component of the solidified sap (manna) of a variety of ash. Jaffe (192), in 1883, showed that the hexitol is largely recoverable from the urine of dogs after oral administration. For many years, conflicting reports appeared as to whether mannitol can serve as a glucose or glycogen precursor or give other evidence of its utilization (80, 82, 122, 131, 271, 278, 296, 313). These studies indicated that orally administered, but not parenterally administered, mannitol is partially utilized (81). Studies with C^{14}-mannitol have substantiated this conclusion, oral and intravenous doses yielding $C^{14}O_2$ to the extent of 50% and 2 to 3%, respectively (319). The metabolism of mannitol presumably begins in the liver, the first reaction probably being the oxidation of the polyol to D-fructose.

Ishihara et al. (189) have reported that the formation of glycogen following mannitol feeding is increased if cholic acid is given simultaneously.

Mannitol has been tentatively identified as a constituent of the blood of the fetal sheep (60).

Of the many cell cultures grown in the presence of mannitol in place of glucose (25, 115, 231), only with a strain of mouse lymphoblasts has the hexitol shown any growth promoting ability (25).

The fact that mannitol is nontoxic and is poorly utilized when given parenterally has led to two uses in medicine and physiology. Since it is filtered by the renal glomeruli and is neither reabsorbed nor excreted by the tubules, mannitol can be used to measure glomerular filtration rate (111, 225, 278). It has also been widely used to determine extracellular fluid volume (99, 120).

Since only 10 to 20 g of mannitol can be ingested by humans without having a laxative effect, its use in foods and pharmaceutical products has been limited. However, it is now being used as a sweetening agent in food for diabetics. The poor absorption of mannitol is the basis for its ability, when used as a long-term

slow infusion, to produce a good osmotic diuresis in patients, who lose more water than salt (230).

Other hexitols. These have not been extensively studied, nor have they been found to have any physiological importance in mammals. Orally administered dulcitol has been reported to be a precursor of liver glycogen in the rat (79). However, it was a poorer precursor than mannitol, and no effect on blood glucose was detectable. It is not unlikely that the low utilization is a consequence of poor absorption. The simultaneous administration of cholic acid is reported to be required for the production of liver glycogen from an oral dose of dulcitol (189), but the conversion was, nevertheless, very small.

Cataractogenic doses of D-galactose lead to marked accumulation of dulcitol in the lens of the weanling rat, the TPN-linked aldose reductase undoubtedly being responsible for the conversion to the hexitol (167). The heart and leg muscles also contained dulcitol in small amounts. The relationship of the production of dulcitol to the cataract often accompanying galactosemia in humans is unknown.

The presence of dulcitol in fetal sheep blood has been provisionally reported by Britton (60).

For studies on anhydrides of hexitols, the reader may consult an earlier review (81).

Pentitols

Until very recently, not only were the pentitols (Fig. 4) not assigned any physiological importance, they had not even been studied biologically (81). Compared to the hexitols, the pentitols are rather limited in natural occurrence. During the last few years, however, xylitol has been found to be a metabolic intermediate in mammalian liver (176, 177, 219, 299, 309), arabitol has been isolated from

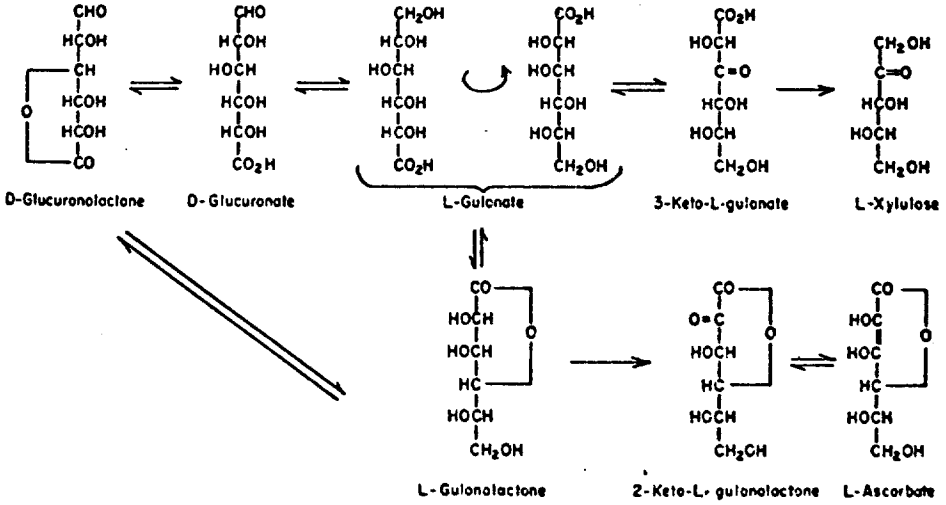


FIG. 5. Transformation of D-glucuronic acid and L-gulonic acid and their lactones into L-ascorbic acid and L-xylulose. The direct reduction of glucuronolactone to gulonolactone has support from only one laboratory (223). The physiological significance of the reaction has not been established.

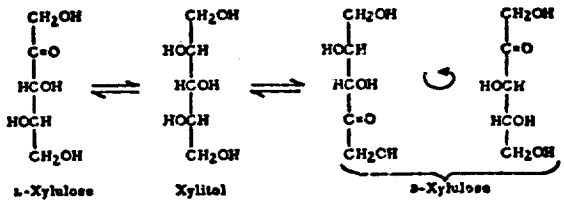


FIG. 6. Enzymatic interconversion of L-xylulose, xylitol, and D-xylulose.

human urine (300), and a ribitol derivative has been found to be a constituent of bacterial cell walls (195). Moreover, induction of cataract by xylose administration has been shown to cause accumulation of xylitol in the lens but not in other tissues (167, 168). These findings will be discussed here, except for the work on bacterial cell walls, which will be reviewed later in this paper.

Xylitol. The discovery that xylitol functions in mammalian metabolism stemmed from investigations on L-xylulose, the characteristic urinary sugar in essential pentosuria. The work of Enklewitz and Lasker (126) on pentosuric individuals suggested that the pentulose is derived from D-glucuronolactone. Touster et al. (305) obtained similar evidence in normal subjects and suggested mechanisms for the transformation. With isotopically labeled compounds, it was then shown that C_6 of D-glucuronolactone is lost in the conversion, C_1 becoming C_5 of L-xylulose (307) (Fig. 5). The fact that glucuronic acid is also the precursor of L-ascorbic acid in the rat (182, 188) led to enzymatic work in several laboratories which demonstrated that L-gulonic acid is an intermediate both in this transformation and in the formation of L-xylulose (67, 72, 74, 151, 158, 190, 197). The existence of lactonases in tissues led to difficulties in assessing the relative importance of lactone and salt forms of the sugar acids (119, 325, 334, 335). However, it is now clear that oxidation of L-gulonate leads to L-xylulose (16, 64, 66, 73, 274), whereas oxidation of L-gulonolactone is required for L-ascorbate production (63, 65, 74, 197, 223) (Fig. 5).

Since both laboratory findings and reasoning by analogy with other inborn errors of metabolism suggested that essential pentosuria involves the accumulation and excretion of a metabolite which is readily disposed of in normal, but not in pentosuric, individuals, studies were undertaken to find an enzymatic system which could metabolize L-xylulose. It was found that guinea-pig liver slices, homogenates, and mitochondria were equally capable of causing the disappearance of the pentulose when incubated in appropriate media (304, 309). Suspected products such as sugar phosphates were not formed; rather, a nonreducing, periodate-reactive substance accumulated as the L-xylulose concentration decreased. The polyol product was isolated and characterized as xylitol, one of the two possible reduction products of L-xylulose (309).

The solubilization of the mitochondrial enzyme system by Hollmann and Touster (176, 177) opened the way for the elucidation of the role of xylitol in liver. With the soluble preparation it was shown that the interconversion of L-xylulose and xylitol is catalyzed by a TPN-linked enzyme of high substrate specificity. There was also found in the extract a second xylitol dehydrogenase, of low substrate specificity, this enzyme utilizing DPN and converting xylitol to D-xylulose rather than to L-xylulose. The two enzymes furnished an enzymatic

mechanism for the conversion of L-xylulose to D-xylulose (Fig. 6). The DPN-linked enzyme closely resembles the nonmitochondrial L-iditol dehydrogenase (sorbitol dehydrogenase) first studied by Blakley (51, 174).

The interconversion of xylitol and D-xylulose at once raised the possibility that D-glucuronate, L-gulonate, and L-xylulose are metabolized via the pentose phosphate pathway, in which D-xylulose 5-phosphate is a key intermediate. The formulation of this pathway by Hollmann and Touster (176, 177) required support from experiments in intact animals and also the demonstration of the existence of a D-xylulokinase in liver. This enzyme was reported by Hickman and Ashwell (172) to occur in calf liver, thus completing the enzymatic basis of the pathway. These and other investigations led to the formulation (72, 219) of the cyclic pathway for glucose metabolism shown in Fig. 7. The designations, "glucuronate-xylulose cycle" (298, 311) and "glucuronate-ascorbate pathway," are useful in differentiating the two metabolic pathways which share a common initial sequence of reactions. Other suggested designations, some referring to more limited aspects of the cycle, are the glucuronic acid pathway (68), the uronic acid pathway (180),

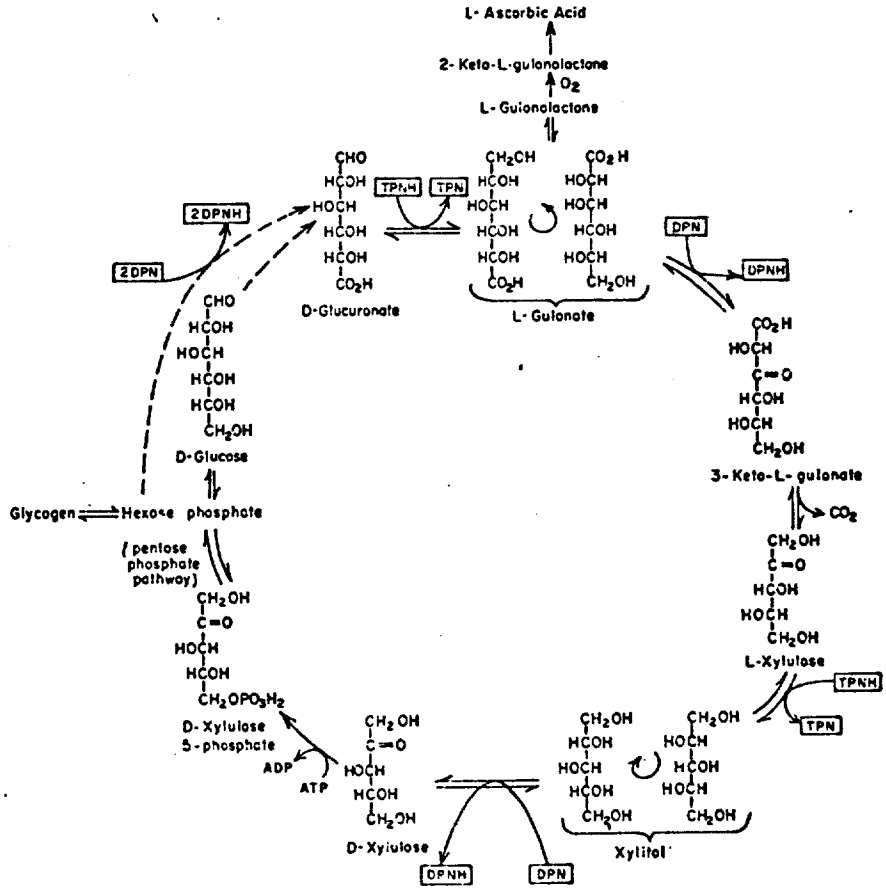


FIG. 7. Glucuronate-xylulose cycle. *Dashed arrows*, leading to glucuronate, indicate uncertainty regarding intermediates (see Fig. 9 and text).

TABLE 5. INCORPORATION OF C^{14} INTO LIVER GLYCOGEN AFTER ADMINISTRATION OF D-ARABITOL-1-C^{14}, L-ARABITOL-1-C^{14}, D-RIBITOL-1-C^{14}, AND D-XYLITOL-1-C^{14} (219, 219a)

SUBSTANCE INJECTED	EXPERIMENTAL ANIMAL	% ADMINISTERED C^{14} IN GLYCOGEN	RELATIVE % C^{14} IN GLUCOSE CARBON ATOM NO.					
			1	2	3	4	5	6
D-Arabitol-1-C^{14} (1.5 μc, 10 mg)	Guinea pig ♀ (595 g)	0.21						
D-Arabitol-1-C^{14} (1.5 μc, 10 mg)	Rat ♂ (295 g)	0.35						
L-Arabitol-1-C^{14} (4.5 μc, 15 mg)	Guinea pig ♀ (775 g)	3.2	37.3					23.8
L-Arabitol-1-C^{14} (4.4 μc, 17.5 mg)	Guinea pig ♀ (544 g)	2.9	38.7	22.9	4.1	1.8	13.0	19.8
L-Arabitol-1-C^{14} (5.2 μc, 10 mg)	Rat ♂ (350 g)	1.0	38.2					24.3
L-Arabitol-1-C^{14} (4.0 μc, 15.9 mg)	Rat ♂ (370 g)	0.9	32.2	25.4	6.6	1.4	14.3	20.5
D-Ribitol-1-C^{14} (3.0 μc, 15 mg)	Guinea pig ♀ (800 g)	17.2	63.6	9.0	24.1	1.1		1.0
D-Ribitol-1-C^{14} (3.0 μc, 15 mg)	Rat ♀ (254 g)	22.3	66.1	10.1	16.5	4.1		4.5
D-Xylitol-1-C^{14} (6.70 μc, 10 mg)	Guinea pig ♀ (765 g)	23.8	67.1	0.4	25.6	2.0		2.1
D-Xylitol-1-C^{14} (6.70 μc, 10 mg)	Rat ♂ (474 g)	11.8	67.1	0.6	25.1	2.2		3.3
D-Ribose-1-C^{14} (4.30 μc, 10.4 mg)	Rat ♀ (356 g)	7.1	69.5	2.4	28.5	2.9		1.4

the C_6 oxidation pathway (171), and the uridine monophosphate shunt (291). The existence of the cycle has been substantiated by tracer experiments utilizing suitably labeled D-glucuronolactone (70), L-gulonolactone (118), and xylitol (219). Table 5 shows that D-xylitol-1-C^{14} labels C_1 and C_3 of liver glycogen as predicted for a 5-carbon substance entering the pentose phosphate pathway without prior chain cleavage (179).

Essential pentosuria is a harmless, rare, recessive genetic abnormality found thus far only in Jews and Arabs (170, 206, 207, 298). The evidence that the individual with the metabolic error lacks the L-xylulose-xylitol enzyme is quite convincing (298), although there has not yet been an opportunity to assay this enzyme in a sample of pentosuric liver. The failure of pentosurics to convert L-xylulose to xylitol is indicated by (*a*) the increase in blood xylulose of pentosuric, but not of normal, individuals after administration of D-glucuronolactone (56, 137) and (*b*) the inability of D-glucuronolactone-U-C^{14} to label urinary ribose in the pentosuric (169). In the latter test, the urinary ribose of a normal subject contained the isotope. The accumulated L-xylulose in pentosuric individuals probably is reduced in part to L-arabitol by the relatively nonspecific DPN-linked polyol dehydrogenase (Fig. 8). The urinary L-arabitol is in fact labeled when a pentosuric is given D-glucuronolactone-1-C^{13} (300).

Glucuronolactone loading tests have been employed to detect heterozygous

carriers of the pentosuric gene as they develop blood xylulose levels intermediate between these of normal and pentosuric subjects (137). It has been observed that parents of pentosuric individuals excrete more L-xylulose normally than do subjects from families in which the metabolic error is absent (O. Touster and R. W. Hester, unpublished observation). For a more extensive discussion of the pentosuric defect, other reviews may be consulted (170, 298, 299). However, in relation to the wider significance of glucuronate metabolism, several additional matters should be discussed here.

The role of the cycle in animals lacking gulonolactone oxidase and therefore requiring a dietary source of vitamin C remains to be elucidated. There is indirect evidence that little carbohydrate is actually metabolized through the cycle (171). It is conceivable that it serves in the production of a sugar whose biosynthesis is still unknown or that the large number of oxidation-reduction reactions in the cycle help to regulate tissue coenzyme levels. As shown in Fig. 7, TPNH is always used in reductive steps, whereas DPN is used in dehydrogenations. It is noteworthy that tissue concentrations of TPNH and DPN in liver are higher than those of TPN and DPNH, respectively. It may be recalled that TPNH and DPN are also required for the two-step conversion of glucose to fructose through sorbitol. The antiketogenic effect of xylitol is discussed above, at the end of the section on sorbitol.

A very intriguing matter is the remarkable stimulatory effect on the pathway shown by a wide variety of substances, including many drugs and carcinogenic hydrocarbons. Two drugs, aminopyrine and antipyrine, enhance the excretion of L-xylulose in pentosuric subjects (126) and of L-ascorbic acid in rats (216). Similarly, menthol increases urinary L-xylulose in the human (126) and L-ascorbic acid in the rat (S. Hollmann and O. Touster, unpublished observations), although borneol effects L-xylulose excretion (126) but not L-ascorbic acid excretion (71). The carcinogenic hydrocarbons induce a very long period of elevated ascorbic acid excretion, one 10-mg injection of 3-methylcholanthrene causing a rat to excrete for weeks many times the normal amount of the vitamin (100). Burns and his colleagues (71, 127) have clearly demonstrated that the enhancement of ascorbic acid excretion by barbital and Chloretone is due to increased rate of synthesis of the vitamin and its precursors, D-glucuronate and L-gulonate, and that stimulatory agents are relatively ineffective in hypophysectomized rats (71).

Further evidence that protein synthesis is involved in the mechanism of action of these agents is the finding that ethionine blocks the action of barbital and

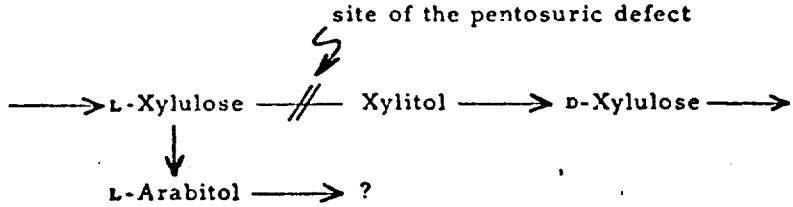


FIG. 8. Site of the pentosuric defect and interrelationship of the urinary pentitols.

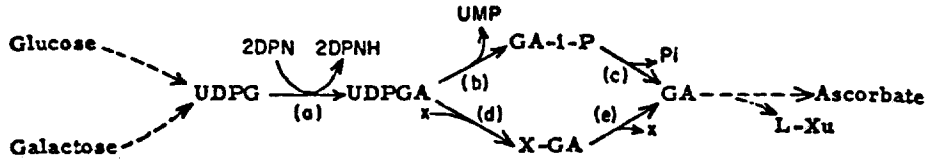


FIG. 9. Alternative pathways for the biosynthesis of glucuronate and ascorbate. (*a*) Uridine diphosphate glucose dehydrogenase; (*b*) "UDPGA pyrophosphatase"; (*c*) a phosphatase; (*d*) glucuronyl transferase; (*e*) β-glucuronidase?

methylcholanthrene on ascorbic acid excretion (302). Finally, von der Decken and Hultin (107) have shown that the liver microsomes of methylcholanthrene-treated rats have increased RNA-protein ratios and enhanced ability to incorporate L-leucine-C^{14}.

There is now evidence that all the enhancing agents do not act through a common mechanism. Chloretone and barbital, but not the carcinogenic hydrocarbons or aminopyrine, increase the levels of UDPG dehydrogenase (302a, 303). This enzyme produces UDPGA, which is undoubtedly the precursor of glucuronate (Fig. 9). The mechanism of action of the hydrocarbons may involve their reported elevation of glucuronyl transferase (UDP-transglucuronylase, glucuronosyl transferase) of liver (187). Aminopyrine, as well as the hydrocarbons, increase the concentration of this enzyme in rat liver without elevating UDPG dehydrogenase (303a). Conney et al. (101a) have reported that UDPG dehydrogenase from Chloretone-treated rats is higher than that of normal rats and more stable as well.

Support for the concept that all enhancing agents do not act through the same mechanism is provided by the finding that methylcholanthrene, unlike barbital and Chloretone, increases ascorbate production without enhancing glucuronate synthesis (106). Doubts about the importance of the UDPGA pyrophosphatase pathway (145) (Fig. 9) for the synthesis of D-glucuronate have been expressed in two recent publications (243, 337), and evidence in favor of an alternate route involving glucuronyl transferase was reported (243). Moreover, the inhibition in vitro of UDPGA pyrophosphatase by barbiturates (302a) and the relative ineffectiveness of the enhancing agents in animals deficient in the transferase (303a) point to a greater role of the transferase route than the pyrophosphatase route in the production of ascorbate, at least under the influence of the enhancing agents. The wider significance of glucuronyl transferase is indicated by the pathological conditions resulting from genetically induced deficiencies of the enzyme (10, 17, 77, 114, 259) and by the ability of sex hormones to influence its level in liver (187).

It should be emphasized that elucidation of the mode of action of the enhancing agents is of importance not only in regard to the mechanism and function of the glucuronate pathway but also in connection with pharmacological problems and metabolic adaptation. Since many of the agents that affect the pathway also increase levels of several microsomal enzyme systems involved in drug metabolism (69, 101), questions of drug detoxication and tolerance are involved (69).

Many problems obviously remain in relation to glucuronate metabolism. For example, all substances that stimulate glucuronate formation would be expected to

increase the production of both ascorbic acid and L-xylulose. This seems not to be so from a comparison of the effects of menthol and borneol on ascorbic acid excretion by the rat and L-xylulose excretion by pentosuric individuals. Preliminary studies have failed to show proportional effects of enhancing agents on L-ascorbic acid and L-xylulose excretion in rats (R. W. Hester, S. Shumaker, and O. Touster, unpublished observations).

Two alternative pathways from UDPGA to glucuronate have already been mentioned. A third possible source of glucuronate is inositol. Charalampous (84) found a kidney enzyme that catalyzes the conversion of inositol into D-glucuronate. Experiments carried out in vivo with isotopically labeled inositol have yielded urinary glucose or liver glycogen with labeling patterns expected from degradation of the cyclitol via the glucuronate-xylulose pathway (4, 244, 245). However, a study of inositol metabolism in liver homogenates suggests the existence of more than one degradative pathway (253). Labeled inositol gives rise in the intact rat to labeled D-glucuronate and L-gulonate but not to L-ascorbate, presumably because the L-gulonate formed in the kidney does not penetrate the liver cells which synthesize the vitamin (75). In further support of the uridine nucleotide route is the fact that galactose is an even better precursor of ascorbate than glucose (127), the galactose presumably being converted to UDPG via UDPGal. The physiological significance of the inositol metabolizing enzyme is still unknown.

D-Xylose-induced cataract in weanling rats is accompanied by the accumulation of xylitol in the lens, to the extent of 4 mg/g, as well as smaller amounts of sorbitol. Both polyols are found in the lens of the xylose-fed adult rat, which is not susceptible to xylose cataract, as well as in the lens of weanling rats allowed to recover from cataract. The formation of xylitol from D-xylose is undoubtedly under the influence of the TPN-linked aldose reductase in the lens (167, 168). Also present in the lens is a DPN-linked polyol dehydrogenase capable of interconverting xylitol and D-xylulose.

$$\text{D-xylose} \xrightleftharpoons{\text{TPNH}} \text{xylitol} \xrightleftharpoons{\text{DPN}} \text{D-xylulose}$$

The enzymatic reduction of D-xylose has not been demonstrated in liver. Nevertheless, it has been suggested that the failure of this organ to accumulate xylitol after xylose administration could in part be due to the further metabolism of the pentitol via D-xylulose and subsequent reactions of the glucuronate-xylulose cycle (168). It is interesting that, in intact calf lens in vitro, conversion of D-xylose to D-xylonic acid is a more prominent reaction than reduction of the aldose to xylitol (168).

Arabitol (*D and L*). There is only one report of the presence of L-arabitol in nature, namely, in human urine. Touster and Harwell (300), examining pentosuric urine for the possible occurrence of xylitol, found L-arabitol instead. Isolation experiments on two different urine samples indicated that their subject excreted several hundred milligrams of the pentitol daily. As mentioned in the preceding section, it is considered likely that the L-arabitol originates from the large amount of L-xylulose present in pentosuric individuals. When the urine of fasted, normal individuals was examined, a smaller amount of a pentitol was found. This was

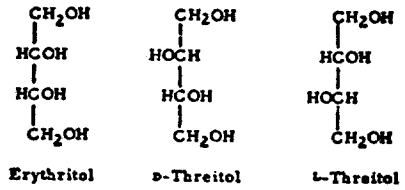


FIG. 10. Formulas of the tetritols.

identified as DL-arabitol. Its origin is unknown, but it is possible that the L-arabitol is derived from metabolic transformations and the D-isomer derived from the diet. This is a speculation based only on the fact that D-arabitol has been isolated from several plants (Table 1.).

The utilization of D-arabitol-1-C^{14} and L-arabitol-1-C^{14} in the rat has been studied (219a, 306, Table 5). The D-isomer led to extremely low labeling of liver glycogen, but the L-isomer caused appreciable incorporation of the isotope, a result conforming to the hypothesis that this pentitol is a metabolite. However, the labeling pattern in the carbon chains of the glycogen was complex and suggested extensive chain cleavage of the L-arabitol before conversion to a hexose derivative.

Ribitol. Except for its presence in riboflavin, this pentitol has not been found in animals. However, D-ribitol-1-C^{14} is an extremely efficient precursor of liver glycogen in the rat (219a, 306, Table 5). The labeling of the hexose chains predominantly in C_1 and C_3 indicates that most of the ribitol enters the pentose phosphate pathway without prior chain cleavage. The DPN-linked polyol dehydrogenase of the liver presumably converts the ribitol to D-ribulose, which may then be phosphorylated by ATP in the presence of a ribulokinase (306).

Tetritols

Erythritol. This tetritol (Fig. 10) has been isolated in crystalline form from the fasting urine of male human beings (301) and from bovine fetal tissues (322a), where it appears to serve as a growth factor for *Brucella abortus*, an organism that grows preferentially in fetal tissues. Erythritol could not be isolated from bovine maternal tissues. Erythritol-1-C^{14} is not appreciably converted to $C^{14}O_2$ or C^{14}-glucose in rat liver slices (32), and earlier attempts to find evidence that orally administered tetritol is metabolized in rats gave negative results (33, 227). The recent discovery of erythritol in animal tissues and fluids should encourage further work on its metabolism and physiological significance.

D-Erythritol 1-phosphate and D-erythrulose 1-phosphate are interconverted by α-glycerophosphate dehydrogenase derived from rabbit muscle, the tetrulose derivative reacting at 10% the rate of dihydroxyacetone phosphate (97).

L-Threitol. Only this threitol enantiomorph has been studied in mammals. Interest in it derives from the fact that it is reversibly converted to L-erythrulose by the DPN-linked polyol dehydrogenase of guinea-pig liver (31, 173, 177), and L-erythrulose can serve as a substrate of transketolase, an enzyme of the pentose phosphate pathway. L-Threitol preparations labeled with C^{14} in terminal carbons have been shown to yield $C^{14}O_2$ and C^{14}-glucose slowly in rat liver slices, L-erythrulose perhaps being the first intermediate in the metabolism of the polyol (32).

The remaining steps are even more uncertain, as no clear picture evolved from examination of the C^{14} distribution in the glucose derived from L-erythrulose-C^{14}.

II. Mammalian Enzymes Acting on Polyols

DPN-Linked Polyol Dehydrogenases

The lack of purity of these enzymes often permits only tentative conclusions regarding their specificity and distribution. Comparison of the preparations will be largely postponed until the enzymes from individual tissues have been discussed briefly.

Enzyme derived from the soluble fraction of liver ("L-iditol dehydrogenase"). Breusch (57, 58), using the Thunberg technique, first demonstrated in vitro the sorbitol dehydrogenase activity of brei prepared from the liver of starved cats. Blakley (17) made an extensive study of this enzymatic activity in a variety of species, organs, and cellular fractions. His work, together with the subsequent study of McCorkindale and Edson (218), led to definite conclusions regarding the substrate specificity and coenzyme dependence of the preparations obtained from rat liver. On the assumption that all substrates were dehydrogenated by the same enzyme, the specificity study led to the designation of the enzyme as "L-iditol dehydrogenase," since this polyol possesses at both ends of the molecule the cis C_2 and C_4 hydroxyl groups required for activity. The transformations involving L-iditol and sorbitol are shown in Fig. 11. The reversibility of these reactions and their linkage to DPN were demonstrated. The enzyme was found in the soluble fraction of liver

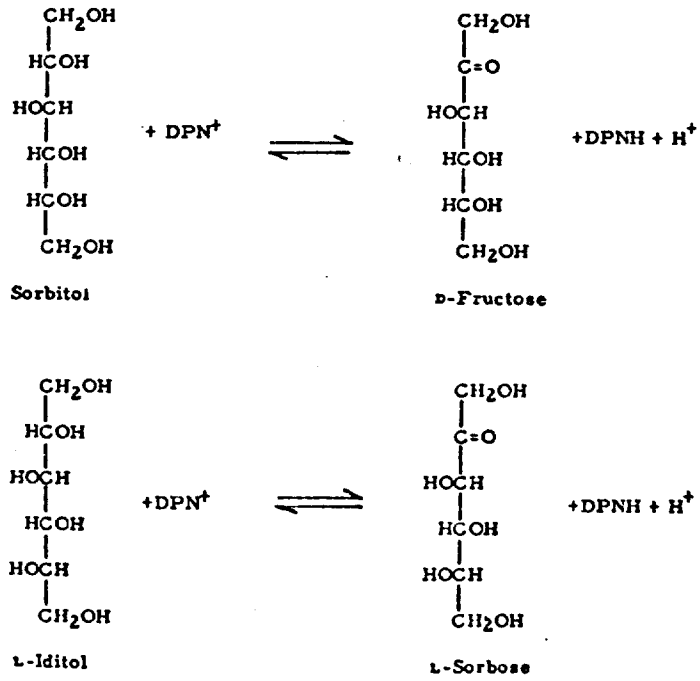


Fig. 11. Two reactions catalyzed by L-iditol dehydrogenase.

TABLE 6. RELATIVE ACTIVITIES OF POLYOL SUBSTRATES OF DPN-LINKED POLYOL DEHYDROGENASES FROM DIFFERENT TISSUES

POLYOL (PRODUCT)	ENZYME AND ASSAY CONDITIONS (pH AND POLYOL CONCENTRATION)			
	GUINEA-PIG LIVER MITOCHONDRIA ENZ. pH 8.1;0.006 M[a] (174)	SHEEP LIVER CRYST. ENZ. pH 8.1;0.006 M[a] (276)	GUINEA-PIG SEMINAL VESICLE ENZ. pH 8.9; 0.013 M (324)	RAM SPERMATOZOA ENZ. pH 8.6;0.033 M (201)
Sorbitol (D-fructose)	110	75	196	127
L-Iditol (L-sorbose)	115	51	198	114
Xylitol (D-xylulose)	100	100	100	100
Ribitol (D-ribulose)	48	13	149	46
D-Glycero-D-glucoheptitol (D-altroheptulose)	58	28		
Allitol (D-allulose)	29			
L-Threitol (L-erythrulose)	26			
D-Mannitol (D-fructose)[b]	0[c]	Oxidized[d]	10[e]	22
L-Arabitol (L-ribulose)[b]	Oxidized[f]	Oxidized[g]		0
Dulcitol (?)	0[c]	Oxidized[d]		
L-Glycero-D-glucoheptitol[h] (perseulose)	0[c]			

[a] This is the optimal concentration for xylitol with the mitochondrial enzyme (174).

[b] The ketose given is the presumed product.

[c] The lack of reactivity was found in tests with a less purified preparation of the mitochondrial enzyme (177).

[d] Weak activity was found. Conditions were different from those shown in the Table (276).

[e] The concentration was 0.033 M instead of 0.013 M used with the other substrates.

[f] Under other conditions, L-arabitol reacts weakly with the guinea-pig liver mitochondria enzyme and with the nonparticulate enzyme of rat liver (O. Touster, M. Escobedo, G. Montesi, and S. Hollmann, unpublished observations).

[g] Under other conditions, L-arabitol reacted at 23% of the sorbitol rate (276).

[h] This heptitol is listed because, although it was not tested with any of the four preparations shown, it had previously been found to be a substrate for the nonparticulate enzyme of rat liver (218).

homogenates, and partial purification was attained. A rather wide distribution of the enzyme was found among the animals whose livers were assayed, and tests on various organs of the rat indicated that the enzyme is limited to the liver and kidney. However, as will be noted below, similar activity is indeed present in certain other tissues of this species. Purification studies in several laboratories (178, 294, 323) have now culminated in the crystallization of the enzyme by Smith (275). This accomplishment permits resolution of questions regarding the possible multiplicity of the nonparticulate DPN-linked polyol dehydrogenases of mammalian liver.

Enzyme derived from guinea-pig liver mitochondria ("*DPN-xylitol* (D-*xylulose*) *dehydrogenase*"). This enzyme was discovered during studies on the metabolism of L-xylulose (176, 177). The xylitol produced from the pentulose is reoxidized to D-xylulose by a DPN-linked enzyme in the mitochondria. Hollmann (174) later studied the specificity and other properties of this dehydrogenase and proposed the name "D-erythro-1,2,4-polyalcohol dehydrogenase." Although the substrate

specificity was quite similar to that of L-iditol dehydrogenase of the nonparticulate portion of liver, the difference in cellular location and small differences in specificity led to the conclusion that the two enzymes are not identical (174).

Enzyme derived from male accessory organs and spermatozoa ("ketose reductase"). Williams-Ashman and his co-workers (323, 324) discovered and partially purified a DPN-linked dehydrogenase from male accessory organs of the guinea pig and rat and obtained evidence from specificity and inhibition studies that one enzyme is responsible for the oxidation of both hexitols and pentitols. Moreover, inhibition and rate studies suggested that this enzyme is identical with the dehydrogenase isolated from the soluble fraction of liver. A very similar enzyme was later discovered by King and Mann (200, 201) in the spermatozoa of many species and was prepared in a soluble form. The substrates for this enzyme are the same as those of the liver enzyme, sorbitol and L-iditol being the most active.

Enzyme derived from lens ("sorbitol dehydrogenase"). Dialyzed extracts of rat lens, in the presence of DPN, interconvert xylitol and xylulose (167, 168). By analogy with the action of DPN-linked polyol dehydrogenases of other tissues, the xylulose was assumed to be the D-isomer. The extracts also oxidize sorbitol to D-fructose, but no further information is available.

Comparison of the enzymes. The evidence now indicates that the DPN-linked polyol dehydrogenases of animal tissues are very similar to each other in properties. However, since small differences among them have been reported, and since only the sheep liver enzyme has been crystallized (275), the individual names should be retained for the time being. The similarity between the mitochondrial and nonparticulate enzyme of liver led Hollmann (174) to test whether the latter is derived from the former during homogenization. His evidence suggested that DPN-linked polyol dehydrogenase activity does indeed occur in both cell fractions. Therefore, Hickman and Ashwell's convenient preparation of the enzyme from acetone powder (173, 308) leaves open the question as to how much of the enzyme prepared in this manner is derived from each of the two fractions. It is clear from the data in Table 4 that partially purified mitochondrial enzyme of guinea-pig liver and crystalline sheep liver enzyme, when tested under identical conditions (but in different laboratories), show different relative reaction rates for the various polyols.

The constancy of the relative reactivity of various polyols during purification of the enzyme from guinea-pig liver mitochondria (174), from rat liver soluble fraction (323), and from sheep liver (through crystallization of this enzyme) (276), indicates that a single enzyme in each tissue is acting upon all the polyols. The

TABLE 7. INFLUENCE OF pH AND SUBSTRATE CONCENTRATION ON RELATIVE RATES OF POLYOL OXIDATION BY SEMINAL VESICLE ENZYME (324)

SUBSTRATE	CONCENTRATION (M)	RELATIVE RATE OF OXIDATION* pH 8.9	pH 7.5
Sorbitol	0.013	100	100
L-Iditol	0.013	101	97
Ribitol	0.013	76	38
Xylitol	0.013	51	68
Xylitol	0.003	81	73

* Rate of oxidation with sorbitol assigned 100 at a given pH.

TABLE 8. THERMODYNAMIC EQUILIBRIUM CONSTANTS FOR THE MITOCHONDRIAL DPN-LINKED POLYOL DEHYDROGENASE AND MICHAELIS CONSTANTS FOR THE POLYOL SUBSTRATES (174)

POLYOL	K_m (M)	E
Sorbitol	3.38×10^{-2}	4.19×10^{-10}
L-Iditol	4.18×10^{-2}	4.23×10^{-10}
Xylitol	4.96×10^{-4}	4.18×10^{-11}
Ribitol	1.78×10^{-2}	1.75×10^{-11}
D-Glycero-D-glucoheptitol	1.47×10^{-2}	5.54×10^{-11}
Allitol	3.70×10^{-2}	1.44×10^{-11}

relative rates of oxidation of the polyol substrates vary not only with the source of enzyme, but also with the pH. Table 6 lists all substrates of this group of enzymes and compares polyol oxidation rates by different preparations. The assays were all performed in alkaline media, but conditions varied as noted in the Table. Xylitol is the only polyol showing substrate inhibition (174, 324). Moreover, xylitol has been shown to have the highest affinity for the mitochondrial enzyme (174). Therefore, at very low concentrations, the relative rates of polyol oxidation become more favorable towards this pentitol. The data of Williams-Ashman et al. (324) are given in Table 7 to illustrate the influence of pH and of substrate concentration (xylitol) on the rate of dehydrogenation of polyols. The most complete data on K_m values and equilibrium constants are available for the mitochondrial enzyme (Table 8).

The assignment of the name "DPN-xylitol (L-xylulose) dehydrogenase" to the mitochondrial enzyme reflects the origin of its discovery and its high affinity for xylitol [$K_m = 5.96 \times 10^{-4}$ M (174)] and for L-xylulose [$K_m = 6.6 \times 10^{-4}$ M (173)]. The designation "D-erythro-1,2,4-polyalcohol dehydrogenase" (174) is almost equivalent to the name "L-iditol dehydrogenase" suggested by McCorkindale and Edson (218) on the basis of extensive substrate specificity studies on the soluble rat liver enzyme, but it does emphasize the lack of essentiality of the hydroxyl group on C_3. The nomenclature is complicated further by the use of the names "sorbitol dehydrogenase" and "ketose reductase" for preparations from various sources. The minimum structural requirement for substrate activity is shown in Fig. 12.

The liver dehydrogenases are inhibited by sulfhydryl-binding agents, such as *p*-chloromercuribenzoate (173, 276) and iodoacetate (174, 177, 308) and by metal chelators, e.g., 8-hydroxyquinoline (174), *o*-phenanthroline (276), and Versene (164, 165, 276; contrary result in 173). DPNH protects the seminal vesicle enzyme against Versene (164). The importance of metal ions is further suggested by the fact that the inhibitory action of 2.5×10^{-3} M cysteine on a liver preparation is completely reversed by 2.5×10^{-4} M $ZnSO_4$ (174). On the other hand, the enzyme derived from the seminal vesicles of the rat, when tested at pH 9.0 in pyrophosphate buffer, is completely inhibited by 5×10^{-4} M $AgNO_3$ or 5×10^{-4} M $HgCl_2$, but not by 9×10^{-4} M $CuSO_4$ (324). Moreover, 2×10^{-4} M 2,3-mercaptopropanol is strongly inhibitory, but 4×10^{-2} M cysteine is not (324). These results serve to emphasize the great importance of assay conditions, or the likelihood that significant structural differences exist in the various DPN-linked polyol dehydrogenases in spite of their very similar substrate specificity.

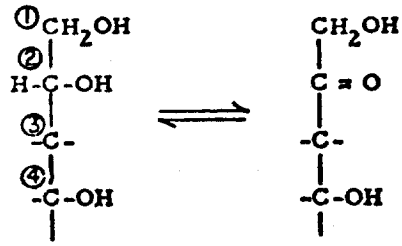


FIG. 12. Structural requirement of substrates of DPN-linked polyol dehydrogenase of liver. The substituents on C_4 may be H and H or H and OH. The other substituents on C_4 may be H and H or H and the rest of a polyol.

The mechanism of the dehydrogenation has not been elucidated in detail, but the three-point attachment of substrate to enzyme first suggested by Edson (117) is in harmony with the structural requirement for a primary alcohol possessing a cis-2,4-dihydroxy configuration. The relative unimportance of a hydroxyl on C_3 is shown by the reactivity of 3-deoxyribitol (3-deoxyxylitol) (174). In fact, in the pentitol series, the substituent on C_3 may affect the reaction rate by steric hindrance, as the rate decreases in the order: 3-deoxyribitol (H on C_3), xylitol (trans OH on C_3), ribitol (cis OH on C_3) (174). Also, xylitol has a much greater affinity for the enzyme than does ribitol (Table 11). As expected from this reasoning, sorbitol has greater affinity than does allitol (Table 8). However, these are only suggestive observations on a limited number of substrates.

Weak substrate activity has been reported for D-mannitol (200, 201, 276, 324) and L-arabitol (276, 308, 323). The reactivity of the latter substance may be undetectable (324) if a relatively high substrate concentration is not employed (H. G. Williams-Ashman, private communication; G. Montesi and O. Touster, unpublished work). The responses of L-arabitol and D-mannitol are not in harmony with the postulated cis-2,4-dihydroxy requirement. Perhaps, under suitable conditions, a significant portion of the molecules of these polyols exist, or can be forced into, a proper configuration for the three-point attachment. The reactivity of L-arabitol is much more sensitive to replacement of DPN by DPN analogs than is that of other substrates (G. Montesi and O. Touster, unpublished observations).

The reactivity of ketoses has been occasionally compared. With the mitochondrial enzyme, ketopentoses were found to react more rapidly than ketohexoses (177). However, with enzyme prepared from liver acetone powder, D-ribulose has been reported to be inactive under conditions very favorable for the reduction of D-xylulose and L-erythrulose (173). Since all other mammalian DPN-linked polyol dehydrogenases act on D-ribulose this discrepancy is a puzzling one. In another laboratory, similar preparations of the enzyme from acetone powder showed the expected activity towards this pentulose (308). It has been stated that the D-ribulose response is dependent on variations in the calcium phosphate gel used for the purification of the enzyme (G. Ashwell, private communication).

TPN-Linked Polyol Dehydrogenase From Guinea-Pig Liver Mitochondria

Touster et al. (309) found that this mitochondrial enzyme completely accounted for the ability of guinea-pig liver slices to utilize L-xylulose, the product being xylitol. Hamster, rat, and monkey liver, and guinea-pig kidney, presumably contain the enzyme, as indicated by the moderate rate of L-xylulose disappearance

The distribution of the enzyme (164, 165), which is present in the placentas and seminal vesicles of the sheep and in the placenta of the cow but absent from human and rabbit placenta and from liver, kidney, and brain, suggests that the enzyme occurs only in fructose-forming tissue. However, as indicated on page 189, the human placenta can form fructose from glucose in spite of the absence of the reductase (185). The broad specificity of the reductase is shown in Table 9. There is no evidence that a multiplicity of enzymes is responsible for the activity of the substrates shown. The enzyme is specific for TPN, which has a protective action against heat denaturation at 55 C (165).

Aldose reductase has been demonstrated in dialyzed extracts of rat lens (167, 168). The identity of the various aldose reductases has not been shown, nor have the enzymes been purified greatly.

Hers (165) has also reported that a different TPN-linked enzyme, "aldehyde reductase," occurs in liver, this enzyme being inactive towards aldohexoses and aldopentoses but utilizing D-glucosone, D-glucuronolactone, DL-glyceraldehyde, benzaldehyde, and alphatic aldehydes. The substrate specificity of this enzyme suggests that it may be TPN-L-gulonate dehydrogenase (223a), which converts D-glucuronate into L-gulonate for the production of L-ascorbic acid and L-xylulose.

III. Insects

A few studies on insect nutrition have included polyols among the carbohydrates tested (310). Sorbitol and D-mannitol were able to prolong life when fed to honeybee larvae, *Caliphora erythrocephala*, *Drosophila melanogaster*, *Tribolium confusum* and silkworms (*Bombyx mori*); dulcitol, ribitol and erythritol were also used by some of the insects (Table 10). Sorbitol, mannitol and dulcitol promoted glycogen synthesis in the starved mosquito (*Aedes aegypti*) but were not as effective as glucose (322). Blood trehalose and fat-body glycogen were formed readily from sorbitol and mannitol (but not from dulcitol or erythritol) in previously starved silkworms (181).

Chino (94, 95) has found sorbitol (identified by paper chromatography) in diapause eggs of the *Bombyx* silkworm. At the onset of diapause the glycogen content of eggs decreases abruptly and gradually reaches a minimum level about 30 days after oviposition. The loss of glycogen is accounted for quantitatively by the formation of sorbitol and glycerol. During diapause, metabolism is much reduced and

Table 10. Utilization of polyols by insects

Insect	Polyol tested: Sorbitol	D-mannitol	Dulcitol	Ribitol	Erythritol
Honeybee larva* (314)	+	+	+		
*Caliphora erythrocephala** (134)	+	+	−		−
*Drosophila melanogaster** (159)	+	+	−	+	+
Tribolium confusum larva* (34)	+	+			
Aedes aegypti† (322)	+	+	+		
Bombyx mori larva‡ (181, 191)	+	+	−		−

* Tested for prolonging survival.
† Tested for glycogen synthesis.
‡ Tested for prolonging survival, glycogen synthesis, and trehalose synthesis.

TABLE 9. SUBSTRATES OF ALDOSE REDUCTASE (165)

SUBSTRATE	RELATIVE VELOCITY	K_m APPROX. (M)
D-Glyceraldehyde	110	5×10^{-4}
L-Arabinose	56	1.5×10^{-1} (3×10^{-1})
D-Ribose	55	2×10^{-1}
D-Xylose	56	1.5×10^{-1}
L-Xylose	56	3×10^{-2}
D-Galactose	43	3×10^{-1}
D-Glucose	51	2×10^{-2}
D-Mannose	60	5×10^{-2}
D-Glucosone	100	4×10^{-4}
D-Glucosamine	30	5×10^{-2}
D-Glucuronolactone*	70	3×10^{-4}
L-Fucose	46	2×10^{-2}
D-Rhamnose	38	7×10^{-1}
D-Glycero-L-alloheptose	19	3×10^{-2}
D-Glycero-D-galactoheptose	30	10^{-2}
D-Glycero-L-galactoheptose	33	5×10^{-2}
D-Glycero-D-guloheptose	26	2×10^{-2}
D-Glycero-L-mannoheptose	17	3×10^{-2}

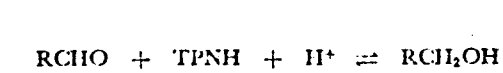
* Glucuronic acid reacted almost as well as the lactone.

catalyzed by homogenates of these tissues (O. Touster, S. Hollmann, and B. M. Silbert, unpublished observations). Treatment of the insoluble portion of osmotically ruptured mitochondria with butanol liberates the dehydrogenase in soluble form, thus permitting spectrophotometric studies of its properties (176, 177). The use of liver acetone powder has simplified the preparation and purification of the enzyme (173, 308).

The dehydrogenase is active only with TPN as coenzyme, and L-xylulose and xylitol are the most active substrates (177). D-Threitol and D-erythrulose have recently been shown to serve as substrates (31), and L-ribulose is very weakly active (173). This enzyme, which was named TPN-xylitol (L-xylulose) dehydrogenase before the D-threitol reactivity was discovered, therefore appears to be among the polyol dehydrogenases with the most rigorous substrate requirements. The equilibrium constant for the L-xylulose-xylitol interconversion is 2.97×10^{-11} (174). For L-xylulose, the K_m is reported to be 2.9×10^{-4} M (173), for xylitol, 2.54×10^{-2} M (174). Different enzyme preparations and different assay conditions were used to determine these two constants. The importance of the sulfhydryl group of the enzyme is indicated by inhibition by *p*-chloromercuribenzoate (173) and iodoacetate (174, 177, 308). Metal dependence is suggested by inhibition with 8-hydroxyquinoline (174) and by cysteine, the latter being reversed by zinc sulfate (174).

TPN-Linked Aldose Reductase

Hers (163) first demonstrated the existence of this enzyme in studies on the origin of seminal fructose from glucose. The reaction catalyzed by the enzyme is as follows:



$$RCHO + TPNH + H^+ \rightleftharpoons RCH_2OH + TPN^+$$

the cytochrome system becomes inactive. There is also a decrease in fat content, greater than can be accounted for by oxygen consumption, without an accumulation of the intermediates of fatty acid catabolism. Chino has suggested that intermediates derived from glycogen are reduced to sorbitol and glycerol by reactions with TPNH or DPNH so that the coenzymes may be regenerated in the oxidized form. When diapause is terminated sorbitol and glycerol disappear to form hexose. Whereas the conversion of glycogen to sorbitol and glycerol takes place under anaerobic conditions, the reverse process requires a supply of oxygen.

Faulkner (128, 129) has observed TPN-linked dehydrogenase activity in silkworm hemolymph capable of reducing glucose-6-phosphate to sorbitol phosphate and glyceraldehyde to glycerol. Similar activity has been extracted from larval fat body and gut, pupal and adult forms of *Bombyx mori*. The reactions may well be involved in the formation of sorbitol and glycerol in diapause eggs. Substrate specificity studies on the hemolymph dehydrogenase showed no activity towards D-fructose, L-sorbose, D-glucose or sorbitol, nor to a mixture of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Among the various sugars that were reduced are D-ribose 5-phosphate (to ribitol phosphate), D-erythrose (to erythritol), and galactose 6-phosphate. Faulkner (128) has suggested that some of the unidentified phosphorus-containing compounds isolated from silkworm hemolymph may be polyol phosphates.

Dulcitol has been isolated from the honeydew of *Aphis Euonymi* Fabr. (229) and ribitol from that of Ceroplastes species (152). Since the plants on which these insects were feeding contained dulcitol and ribitol, respectively, it is likely that the polyols were of food origin and not metabolic products of the insects (152).

IV. Plants

The metabolism of polyols in plants has received little attention compared with that given to their occurrence and distribution [see reviews by Barker (27) and Bourne (55)].

The high concentration of mannitol (248), dulcitol (208) or perseitol (194) in certain plant exudates has suggested (55) that polyols may have a role in protecting injured tissue.

Mannitol formation is associated with photosynthetic activity in the brown algae. The mannitol content of fronds shows a wide seasonal variation, reaching a maximum about midsummer. In young fronds, as photosynthesis proceeds the mannitol content rises from 5% to as much as 36% of the dry weight of *Laminaria cloustonii* (46, 47). The concentration of mannitol within the frond is highest in the newly formed tissues (49), and the mannitol content is greatest at depths where the light intensity is optimal for photosynthesis (48). Using $C^{14}O_2$, Bidwell (40) has found that the main product of photosynthesis is mannitol in the brown algae (compared with sucrose in the green algae and galactosylglycerol in the red algae). Under favorable conditions 65% of the total C^{14} assimilated by frond sections of *Fucus vesiculosus* was in mannitol, which accounted for 95% of the alcohol-soluble compounds (41); most of the remaining C^{14} was found in the alcohol-insoluble alginic acid (42). Studies on the incorporation of $C^{14}O_2$ into carbohydrates of

detached plum leaves (3) revealed a rapid synthesis of sorbitol following photosynthesis and also a conversion of carbohydrates to sorbitol in the dark. A high proportion of the CO_2 assimilated was incorporated into sorbitol and the turnover of sorbitol was comparable with that of starch.

In contrast to the seaweeds, the mannitol content of olive trees increases during the winter (238). The leaves of *Gardenia jasminoides* contain no mannitol in summer but accumulate the polyol during winter; the mannitol content reaches a maximum just after the coldest month. Leaves detached from the plant in summer synthesize and accumulate mannitol when placed in a cold environment (7–9 C); light has no apparent effect on the process (15). There was no apparent correlation between the polyol content of woody plants and their frost hardiness (256).

Reserves of polyols, built up during periods of anabolic activity, can be used subsequently for the supply of energy or conversion to other substances. Plums (112, 113) and pears (198) ripening on the tree accumulate sorbitol as well as sucrose and reducing sugars. Fruit detached from the tree and stored lose sorbitol rapidly but maintain their sugar content until the sorbitol concentration drops to a low level.

Plant tissues have been found to make selective use of exogenous polyols supplied to them. According to Steele (283), sorbitol (but not D-mannitol or dulcitol) is converted to starch in detached leaves of *Rosaceae*; the leaves of *Adonis vernalis* and of *Oleaceae* use dulcitol and D-mannitol, respectively. Sorbitol was converted to cellulose in wheat shoots after splitting of the carbon chain; D-mannitol and D-arabitol were not used (2, 62).

It has generally been supposed that plant polyols originate from sugars by reduction and that reoxidation to sugars is the first step in their metabolism. The simultaneous occurrence of certain pairs of polyols and ketose sugars (e.g., perseitol and D-mannoheptulose in avocado, sorbitol and D-fructose in the *Rosaceae*, and D-mannitol and D-fructose in the brown algae) has stimulated suggestions that enzymes for their interconversion may occur widely in plants (117, 184). Such enzymes have been isolated from animal tissues and microorganisms, and DPN-linked dehydrogenase activity towards sorbitol and D-mannitol has been observed in extracts of *Sorbus aucuparia* berries (117). Other enzymes acting on aldoses or phosphorylated polyols and sugars could equally well be involved in plant polyol metabolism.

V. Microorganisms

Occurrence

In contrast to the widespread occurrence of polyols in plants there are few reports of acyclic polyols occurring as normal constituents of microorganisms (Table 1). Free dulcitol has been isolated from *Torulopsis utilis* (*Candida utilis*) cells grown on wood-sugar wort (132). A mannoside of erythritol, free or esterified to fatty acids, occurs in numerous species of the corn smut *Ustilago* (54, 157). Sorbitol and arabitol, as well as glycerol, are lipid constituents of the yeast *Rhodotorula graminis* (108). Riboflavin is a derivative of ribitol, which can be released as the free polyol by the hydrolytic enzyme, riboflavin hydrolase, extractable from

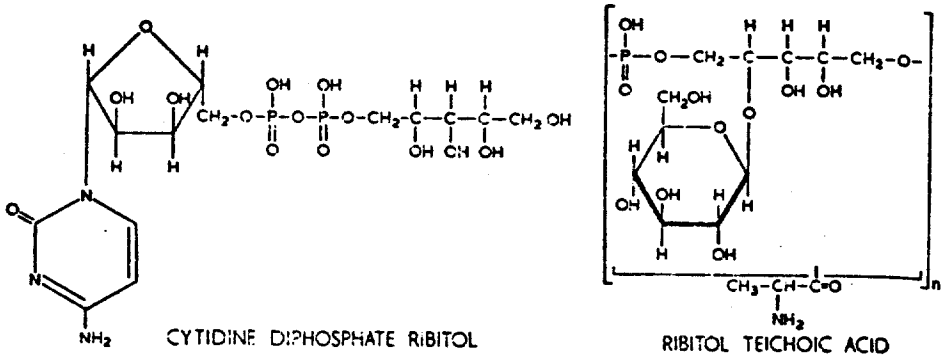


FIG. 13. Structure of cytidine diphosphate ribitol and structure of repeating unit of ribitol teichoic acid from *Bacillus subtilis*.

Pseudomonas riboflavina (336). The biosynthesis of the vitamin has recently been reviewed (61). Ribitol is a major constituent of the cell walls of many gram-positive bacteria (12, 13, 186).

Baddiley and his collaborators found in *Lactobacillus arabinosus* two new cytidine nucleotide derivatives (24), which were identified as cytidine diphosphate ribitol and cytidine diphosphate glycerol (Fig. 13) (21). Subsequent examination of *L. arabinosus* led to a further discovery, that the cells contained appreciable quantities of ribitol phosphate and of glycerol phosphate in combined forms (22). The ribitol-containing compound was located in the cell wall of *L. arabinosus* and similar compounds were isolated from *Staphylococcus aureus* and *Bacillus subtilis*. These compounds, which are known as ribitol teichoic acids, are composed of ribitol phosphate residues joined in phosphodiester linkage. Ribitol teichoic acid obtained from *B. subtilis* has one molecule of D-glucose attached to each ribitol residue by a β-glucosidic bond and a molecule of D-alanine esterified to a hydroxyl group of the glucosylribitol residues (Fig. 13). The compound from *L. arabinosus* is similar in structure but some ribitol residues have two glucosyl substituents while others have none. Ribitol teichoic acid from *S. aureus* contains N-acetylglucosamine in place of glucose (11, 12, 20). Analogous glycerophosphate polymers (glycerol teichoic acids), combined with D-alanine but without sugar residues, occur in the cell walls of some bacteria. Ribitol and glycerol teichoic acids have been found, either separately or together, in the cell walls of a large number of lactobacilli, staphylococci and other bacteria (13, 23, 186, 257). Glycerol teichoic acids, or very similar compounds, have been obtained from whole cells of a great many bacteria, even when no such polymer was present in the cell wall (13, 23, 217, 228). A recent publication reviews the structural aspects of teichoic acids in more detail (195).

There is a correlation between the occurrence and location of teichoic acids in lactobacilli and their serological behavior (23) (cf 217). Ribitol phosphate is one component of the polymer chain of an antigenic polysaccharide (SVI) from pneumococcus (161).

Nothing is known of the enzymic pathways for teichoic acid syntheses, but

cytidine diphosphate ribitol and cytidine diphosphate glycerol are considered to be likely intermediates in forming the polymer chains. Traces of cytidine diphosphate glycerol and/or cytidine diphosphate ribitol have been detected in *Lactobacillus* sp. other than *L. arabinosus*, in *B. subtilis* and in *S. aureus* (98). Treatment with gentian violet causes *S. aureus* Copenhagen to accumulate relatively large quantities of cytidine diphosphate ribitol (290). The enzymic synthesis of cytidine diphosphate ribitol from cytidine triphosphate and D-ribitol 5-phosphate, also of cytidine diphosphate glycerol from cytidine triphosphate and α-glycerophosphate, has been shown to take place in extracts of *L. arabinosus*, *S. Aureus* and other microorganisms according to the following reactions (264, 265):

cytidine triphosphate + D-ribitol 5-phosphate ⇌ cytidine diphosphate ribitol + inorganic pyrophosphate

cytidine triphosphate + L-α-glycerophosphate ⇌ cytidine diphosphate glycerol + inorganic pyrophosphate.

While the origin of ribitol phosphate is not known, by analogy with enzymic reactions to be described below, it could possibly be formed by the reduction of D-ribulose 5-phosphate or D-ribose 5-phosphate, or, alternatively, in two stages, by the reduction of ribulose or ribose to ribitol followed by phosphorylation of ribitol to D-ribitol 5-phosphate. It is of interest to note that D-mannitol 1-phosphate has been isolated from ethanolic extracts of *L. arabinosus* (21) and that an enzyme capable of interconverting D-fructose 6-phosphate and D-mannitol 1-phosphate is active in lactobacilli (327).

Formation of Polyols as the End Products of Carbohydrate Dissimilation

Mannitol may be formed during bacterial spoilage of wine, and many species of bacteria isolated from fermenting mixtures of plant origin possess the ability to reduce D-fructose to D-mannitol. Mannitol is not formed, however, from glucose and other aldoses (143, 288). Studies by Peterson and Fred (136, 242) on some of these organisms showed that, during the early stages of fermentation, up to half of the fructose consumed by *Lactobacillus pentoaceticus* was reduced to mannitol. If the fermentation was prolonged, mannitol was used up slowly and converted to the normal end products of carbohydrate fermentation (136, 242). Eltz and Vandemark (124) found that extracts of *L. brevis* (*L. pentoaceticus* sp.) catalyzed the oxidation of DPNH by D-fructose, but not by D-fructose 6-phosphate, and the reduction of DPN by D-mannitol but not by sorbitol. Somewhat similar mannitol dehydrogenase activity has been described by Barker (28) in extracts of a cider-spoilage organism that forms mannitol and removes fructose under anaerobic conditions in cider. Resting cells and freeze-dried cells of this organism (which resembles *L. brevis*) were able to oxidize several polyols besides mannitol and reduce a number of ketose sugars to polyols. It may be noted that Wolff and Kaplan (327) examined extracts of *L. brevis* but detected no mannitol phosphate dehydrogenase activity.

Many species of molds form D-mannitol from glucose, mannose and other sugars but not from fructose. The yield of mannitol may amount to 50% under conditions of restricted aeration. Under aerobic conditions mannitol and other

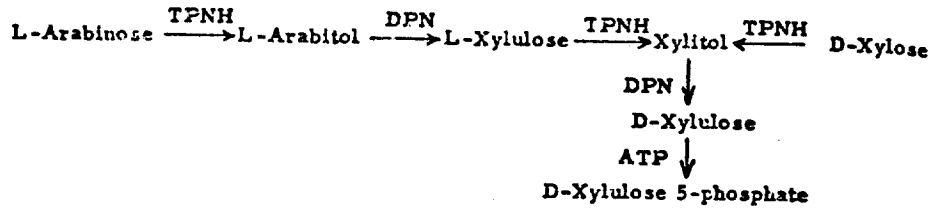


FIG. 14. Proposed metabolic pathway for D-xylose and L-arabinose in *Penicillium chrysogenum* (91).

polyols can support growth (44, 45, 102, 133, 147). While it is probable that fructose is the immediate precursor of mannitol in the mannite bacteria a different pathway is present in molds. In extracts of *Piricularia oryzae* and some *Aspergillus* species Yamada et al. (333) have found a D-mannitol 1-phosphate dehydrogenase that reduces D-fructose 6-phosphate with DPNH to D-mannitol 1-phosphate. In addition the extracts contained a specific phosphatase that releases mannitol from D-mannitol 1-phosphate.

It would seem that mannitol may be formed under anaerobic conditions in order to regenerate DPN from DPNH. However, some organisms form polyols aerobically. *Leuconostoc mesenteroides* converts fructose to mannitol in about 33% yield during growth under both aerobic and anaerobic conditions (30, 50). A number of osmophilic yeasts studied by Spencer and his collaborators (281, 282) are able to ferment up to 60% of the glucose supplied in the medium to one or more of the polyols: glycerol, erythritol, D-arabitol and mannitol. Increasing oxygen tension in the medium increased the yield of polyols while the formation of ethanol was decreased. Increasing the concentration of inorganic phosphate had the opposite effect, implying that polyols were formed as a result of the aerobic dissimilation of glucose.

The mechanism of polyol formation from glucose has been studied in *Torulopsis magnoliae* (279, 280) and of erythritol formation in *Ustilago maydis* (148) with the use of isotopically labeled sugars.

Polyols as Intermediates in Carbohydrate Metabolism

It was pointed out by Edson (117) that two polyol dehydrogenases could form a system for the conversion of one sugar isomer to another. Examples of such a mechanism have been described in a previous section of this review: in the glucuronate-xylulose cycle and in the conversion of glucose to fetal and seminal fructose. In each case a reducing sugar is reduced to a polyol by the action of a TPNH-dependent enzyme and the polyol is oxidized to a second sugar by a DPN-linked enzyme.

For pentose metabolism in the filamentous fungi and yeasts Chiang and Knight (91) have proposed a pathway that involves the formation of pentitol intermediates. According to the scheme D-xylose is converted, via xylitol, to D-xylulose, and L-arabinose is also converted to D-xylulose, but via L-arabitol, L-xylulose and xylitol. The individual reactions summarized in Fig. 14 have been shown to take place in enzyme preparations from *Penicillium chrysogenum*. Extracts of cells grown either on

D-xylose (88, 93) or on L-arabinose (90, 92) as sole carbon source contained a TPNH-dependent aldopentose reductase that catalyzed the reduction of D-xylose to xylitol and L-arabinose to L-arabitol. The reductase, an inducible enzyme, has been purified twentyfold from extracts of xylose-grown cells and its properties studied. Attempts to demonstrate an oxidation of polyol to aldose were unsuccessful and the equilibrium is considered to lie far on the side of aldopentose reduction (88). Veiga, Bacila, and Horecker (312), studying a similar enzyme from *Candida albicans*, were able to demonstrate the reversibility of the reaction. Extracts also contained DPN-linked pentitol dehydrogenase activities for the oxidation of xylitol to D-xylulose (88) and of L-arabitol. The product formed from L-arabitol was a mixture of L-xylulose and L-ribulose in the ratio of three to one (90, 92). Substrate competition studies have indicated that the oxidations of L-arabitol to L-xylulose and of xylitol to D-xylulose were catalyzed by one enzyme while a second dehydrogenase was responsible for the oxidation of L-arabitol to L-ribulose (91).

Aldopentose reductase and pentitol dehydrogenase, together capable of converting D-xylose, via xylitol, to D-xylulose, have been detected in extracts of several *Penicillium* and *Aspergillus* species, a number of other molds and two yeasts [*Rhodotorula glutinis* and *Torulopsis utilis* (*Candida utilis*)] (89). Veiga et al. (312) have concluded that the same reactions occur in *C. albicans* and have studied the enzymes involved.

Another induced enzyme found in extracts of L-arabinose-grown *P. chrysogenum* is capable of reducing L-xylulose with TPNH to xylitol. Since the extracts

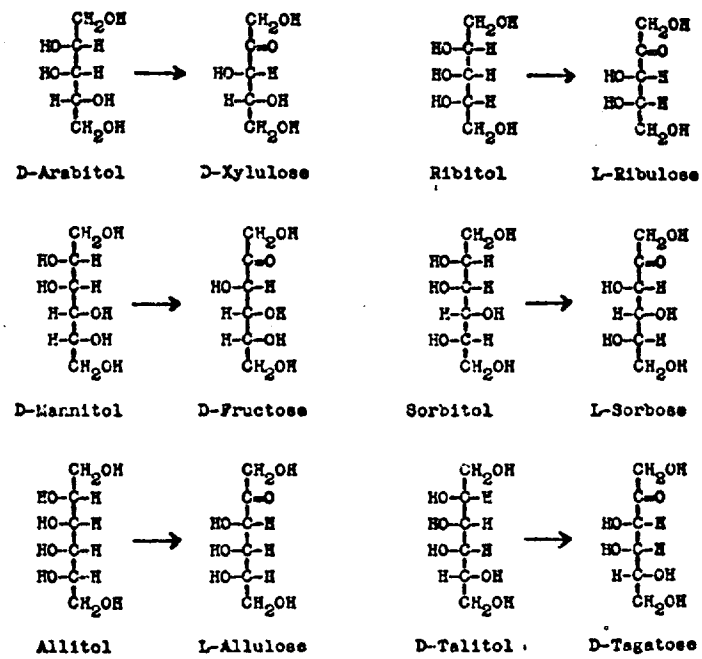


FIG. 15. Pentitols and hexitols oxidized by whole cells of *Acetobacter suboxydans* and their respective products.

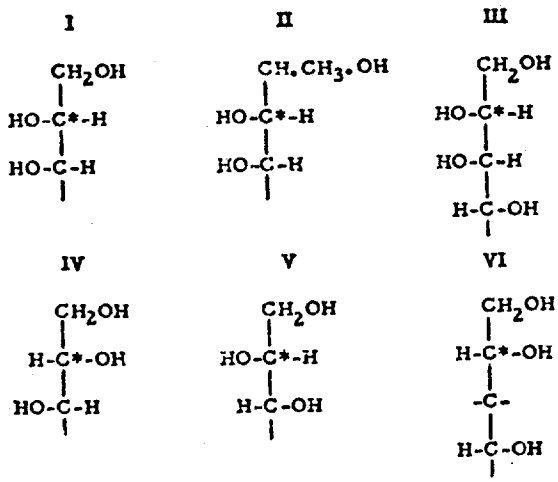


FIG. 16. Substrate configurations required by polyol-oxidizing enzymes with group specificity. *I* and *II*, D-mannitol dehydrogenase; *III*, TPN-linked D-mannitol dehydrogenase; *IV*, galactitol dehydrogenase; *V*, D-iditol dehydrogenase; *VI*, L-iditol dehydrogenase.

were unable to phosphorylate L-xylulose (or L-ribulose) but contained an active D-xylulokinase, Chiang and Knight (91) suggested that L-xylulose formed from L-arabinose, via L-arabitol, would be converted to D-xylulose, via xylitol.

The pathway of pentose dissimilation in bacteria involves epimerases and kinases that have not been found in *P. chrysogenum*, and the polyol-intermediate pathway available to molds and yeasts has not been detected in several bacteria that have been examined (6, 89).

Polyol intermediates have been invoked to explain unexpected labeling patterns in carbohydrate metabolism (267, 292). In order to explain the labeling of mycelial glucosan in *Aspergillus niger* grown on C^{14}-labeled D-ribose, Sumere and Shu (292) suggested that some D-ribose-1-C^{14} was transformed, via D-ribulose and D-arabitol, to D-xylulose-5-C^{14}. It is interesting to note that aldopentose reductase from *P. chrysogenum* (88, 92) and *C. albicans* (312) reduces D-ribose and that this enzyme has been found in *A. niger* (89). Ribitol is oxidized in extracts of *P. chrysogenum* (91) and so it is possible that ribose metabolism in *A. niger* may proceed by successive conversions to ribitol, D-ribulose, D-arabitol and D-xylulose.

Utilization of Polyols by Microorganisms

A very large number of microorganisms can use polyols as sources of carbon and energy. Since different families and species vary in their abilities to metabolize individual polyols, growth and fermentation tests employing polyols have been devised to aid in identification and classification. References in the literature to polyol utilization are too numerous to be considered individually; this review will be restricted to studies that illuminate in some degree the enzyme reactions involved.

Acetobacter species. The metabolism of polyols in *Acetobacter* has been studied more extensively than in any other bacteria. Several reviews have been written on the subject (35, 36, 141, 249) and the present article will describe the early work briefly. At the turn of the century, Bertrand discovered that D-sorbose, which he

had isolated from the fermented juice of sorbus berries, was not a constituent of the berries. Instead, they contained sorbitol, which, during the extraction procedure, had been converted to sorbose through bacterial action. The organism responsible was identified later as *Acetobacter xylinum*. Bertrand extended his studies to the metabolism of other polyols and observed that their oxidation to ketoses by *A. xylinum* was favored by a cis-relationship of two secondary hydroxyl groups adjacent to a primary alcohol group (37, 38). Similar selective oxidation of polyols was shown by other species of *Acetobacter*, among which *A. suboxydans* showed superior cultural characteristics and oxidative behavior (202). Bertrand's studies on *A. xylinum* were repeated on *A. suboxydans* and extended to include the oxidation of other polyols (140, 155, 250, 284, 297). Figure 15 shows the pentitols and hexitols oxidized by *A. suboxydans* and the products formed in each case. Hudson and his collaborators (155) pointed out that the hydroxyl group of the secondary alcohol group involved in the oxidation and the cis-vicinal secondary hydroxyl groups have a D-configuration with respect to the primary alcohol group (configuration *I*, Fig. 16). The Bertrand-Hudson rule states that only polyols possessing this configuration are oxidized to sugars by *A. suboxydans*. The rule has been proved for tetritols and heptitols as well as for pentitols and hexitols (246, 286, 287).

A. suboxydans is a useful tool in the laboratory for preparing rare ketose sugars from more readily obtainable polyols, and for identifying polyols. Ketoses may be obtained in almost quantitative yield under certain conditions, which have been employed in large-scale industrial preparations of L-sorbose (141).

Work on the oxidation of omega-deoxy-polyols (53), including the identification of L-fuco-4-ketose as the product from L-fucitol (254), extended the Bertrand-Hudson rule to cover those polyols that were oxidized by *A. suboxydans* but did not conform to configuration *I* (Fig. 16). The specific structure in this case is illustrated by configuration *II* (Fig. 16).

It was suggested by Edson (117) that the oxidation of polyols by *A. suboxydans* could be explained by the presence of an enzyme having a group substrate specificity defined by the Bertrand-Hudson rule. Arcus and Edson (8, 9) examined cell-free extracts of *A. suboxydans* and found polyol-oxidizing activity associated with insoluble cytochrome-containing particles. The particle preparation oxidized D-mannitol to D-fructose, sorbitol to L-sorbose, and other polyols possessing the configuration defined by the Bertrand-Hudson rule. It is possible that the particles were derived from the protoplast membrane (109). Unlike other polyol dehydrogenases the particulate enzyme is most active about pH 5 to 6, which is optimal for cell growth, and does not require the addition of DPN or TPN for activity. The particles took up oxygen in the presence of polyol substrates but could also couple polyol oxidation to the reduction of ferricyanide or dyes such as phenol blue.

Other workers have confirmed the Bertrand-Hudson specificity of *A. suboxydans* particulate preparations (109, 130, 289, 321). Similar enzymic activity associated with particles has been found in *A. melanogenum* (289), but particles from a number of other species of *Acetobacter* are more restricted in their activity towards polyols (109, 289).

cens[3] isolated by means of dulcitol enrichment was able to oxidize dulcitol as well as mannitol and sorbitol (262, 263). At least three DPN-linked polyol dehydrogenases, oxidizing a wide variety of polyhydric alcohols, could be induced under suitable conditions. A labile D-mannitol dehydrogenase was induced by growth on D-mannitol or sorbitol; the properties of this enzyme were not studied. Sorbitol and dulcitol (galactitol) induced the formation of galactitol dehydrogenase, considered to be specific for the oxidation of polyols possessing configuration *IV* (Fig. 16), e.g., dulcitol to D-tagatose, sorbitol to D-fructose, L-iditol to L-sorbose, L-talitol, L-arabitol and xylitol. Dulcitol in the growth medium also induced the formation of dehydrogenase activity catalyzing the oxidation of D-iditol to D-sorbose, L-glucitol to L-fructose, D-talitol to D-allulose, and xylitol to L-xylulose. It was considered that one enzyme, D-iditol dehydrogenase, was responsible for these oxidations and that it had a substrate specificity enantiomorphic to that of galactitol dehydrogenase (configuration *V*, Fig. 16). Although D-iditol dehydrogenase is induced by dulcitol, dulcitol is apparently not oxidized (to L-tagatose) by the enzyme.

Aerobacter aerogenes. Studies on the metabolism of pentitols in *Aerobacter aerogenes* strain PRL-R3 by Wood and his collaborators (5, 6, 331, 332) have shown that the first step in dissimilation is the oxidation of polyol to ketopentose catalyzed by induced DPN-linked dehydrogenases, each specific for only one pentitol. Ribitol is oxidized to D-ribulose by ribitol dehydrogenase, which has been purified from *A. aerogenes* ATCC 9621 by Fromm (138, 237). The enzyme does not oxidize sorbitol, dulcitol, xylitol (138), or D- or L-arabitol (331). Ribitol dehydrogenase is induced not only by ribitol but by a number of reducing sugars and carbohydrates which are not substrates: D-arabinose (but not D-ribose or D-xylose), xylitol, and D- and L-arabitol (331).

D-Arabitol and ribitol induce the formation of D-arabitol dehydrogenase in *A. aerogenes* PRL-R3 (331, 332). Preparations of the enzyme (purified 18-fold) oxidized D-arabitol to D-xylulose but did not attack the other pentitols. In the reverse reaction D-xylulose was reduced with DPNH but D-fructose, L-sorbose and the other ketopentoses did not react. A D-arabitol dehydrogenase with different properties has been obtained by Lin (211) from *A. aerogenes* ATCC 1033. This enzyme oxidized D-mannitol to D-fructose at half the rate of D-arabitol oxidation. Sorbitol was oxidized at a slow rate (7% of that with D-arabitol). The substrate specificity of the enzyme resembles that of the TPN-linked mannitol dehydrogenase from *A. suboxydans* (configuration *III*, Fig. 16). TPN would not replace DPN as coenzyme. The enzyme was induced by D-arabitol but not by ribitol, D-fructose or D-mannitol; in fact, mannitol represses the induction of the enzyme by D-arabitol.

A. aerogenes PRL-R3 grown on L-arabitol forms a DPN-linked dehydrogenase oxidizing L-arabitol to L-xylulose, and extracts of a mutant strain that can grow on xylitol are able to oxidize xylitol also to L-xylulose (5). Extracts of erythritol-grown cells contain erythritol dehydrogenase activity (191a).

While pentitols are oxidized to ketopentoses before phosphorylation and

[3] This organism has only recently been identified as *Pseudomonas fluorescens* by Dr. M. E. Rhodes, Department of Microbiology, University of Reading, England (1961, unpublished).

The particulate enzyme preparation from *A. suboxydans* can account for all the polyol oxidative properties of the whole cells. But when Arcus and Edson (8, 9) examined extracts of the bacterium they also found soluble DPN-linked dehydrogenase activity towards D-mannitol, sorbitol, xylitol and a number of other polyols. Whereas the particulate preparation oxidized sorbitol to L-sorbose the DPN-linked oxidation of sorbitol formed D-fructose and took place more rapidly at an alkaline pH; D-fructose was also the product of DPN-dependent D-mannitol oxidation. To account for this, and for the fact that there is no configuration common to all the polyols oxidized, Arcus and Edson (9) considered that more than one DPN-linked polyol dehydrogenase was present in *A. suboxydans* extracts. The enzyme oxidizing mannitol was named DPN-linked D-mannitol dehydrogenase to distinguish it from the Bertrand-Hudson enzyme.

Bygrave and Shaw (76) have confirmed the presence of DPN-linked D-mannitol dehydrogenase in extracts of *A. suboxydans* and separated it from the DPN-linked enzyme that oxidized sorbitol. On the other hand, Cummins, King, and Cheldelin (103, 104) failed to detect the mannitol enzyme in crude extracts but purified the DPN-linked sorbitol dehydrogenase some fourteenfold. In addition, they discovered a TPN-dependent oxidation of D-mannitol to D-fructose and sorbitol to L-sorbose. The TPN-linked dehydrogenase has been purified partially (76) and separated from the DPN-linked sorbitol dehydrogenase. Since the TPN-linked enzyme also oxidizes D-arabitol (to D-xylulose) but not other pentitols and hexitols, it appears to be specific for the oxidation of polyols with configuration *III* (Fig. 16). TPN-Dependent mannitol dehydrogenase activity has been found in extracts of *A. aceti* (110), *A. melanogenum* (289), and *Gluconobacter liquefaciens* (289). Both DPN- and TPN-linked dehydrogenase activity towards mannitol and TPN-linked activity to sorbitol have been found in *A. mesoxydans* (289).

Thus *A. suboxydans* contains at least four enzymes oxidizing polyols. Only the activity of the particulate enzyme is apparent in growing cultures or in resting cell suspensions incubated at an acid pH. No explanations have been offered for the functions of the other polyol dehydrogenases in the living cell. The activity of the DPN-linked sorbitol dehydrogenase is apparent in resting cell suspensions incubated at pH 7.5 since sorbitol is oxidized to D-fructose (as well as L-sorbose) under these conditions (9).

Mycobacterium species. Extracts of *Mycobacterium avium* were found to reduce DPN in the presence of D-mannitol (but not in the presence of sorbitol, dulcitol or erythritol) and to oxidize DPNH in the presence of D-fructose (199). An earlier publication mentions DPN-linked dehydrogenase activity towards D-mannitol and sorbitol in extracts of *M. phlei* (117).

Pseudomonas fluorescens. Sebek and Randles (260) studied the oxidation of mannitol and sorbitol by adaptive enzymes in *Pseudomonas fluorescens.* Cells grown on D-mannitol or sorbitol were able to oxidize D-mannitol to D-fructose and sorbitol to L-sorbose. There was evidence to suggest that growth on sorbitol also induced another enzyme oxidizing sorbitol to D-fructose.

The ability to grow on sorbitol and mannitol is a common phenomenon in the *Pseudomonadaceae* but growth on dulcitol is rare (251). One strain of *Ps. fluores-*

conversion to common metabolic intermediates, it appears that hexitol metabolism in *A. aerogenes* proceeds by way of phosphorylation to a hexitol phosphate and subsequent oxidation to 2-ketohexose 6-phosphate (138, 214, 331).

Yeasts. The utilization of polyols, mannitol and sorbitol in particular, by yeasts is widespread and has been studied as an aid to taxonomy (29). D-Mannitol oxidation with DPN has been observed in extracts of brewer's yeast (235), *Candida utilis* (9, 83) and *Saccharomyces cerevisiae* (328). Arcus and Edson (9) found DPN-linked dehydrogenase activity towards sorbitol, xylitol, L-rhamnitol and D-arabitol as well as to mannitol in extracts of *C. utilis*. The oxidation product of mannitol was D-fructose, and sorbitol was converted to a mixture of D-fructose and L-sorbose, suggesting that more than one polyol dehydrogenase was present in the extract. However, a brief communication from Horecker's laboratory (83) indicates that one enzyme is responsible for the oxidation of both mannitol and sorbitol to fructose and of xylitol to D-xylulose. The reduction of D-xylose to xylitol by yeasts and the subsequent oxidation of xylitol to D-xylulose (89, 312) has been described in a previous section of this article. A small amount of dehydrogenase activity to D-mannitol 1-phosphate was found to accompany a greater activity towards D-mannitol in *S. cerevisiae* (328).

Metabolism of polyol phosphates. The oxidation of polyols through their phosphorylated derivatives was discovered independently in two laboratories. Marmur and Hotchkiss (183, 224) studied the oxidation of mannitol in extracts of mannitol-grown *Diplococcus pneumoniae*. They were unable to detect the oxidation of mannitol but found that extracts catalyzed the reduction of DPN with mannitol phosphate and the oxidation of DPNH with D-fructose 6-phosphate. Wolff and Kaplan (326, 327) observed the reduction of D-fructose 6-phosphate with DPNH in extracts of *Escherichia coli*. The product was identified as D-mannitol 1-phosphate and this phosphate was oxidized with DPN to D-fructose 6-phosphate. The enzyme responsible for these reactions has been named D-mannitol 1-phosphate dehydrogenase and has been purified some thirtyfold from crude extracts. It appears to be highly specific for DPN, mannitol 1-phosphate and fructose 6-phosphate, since it is without action on TPN or a wide variety of other sugar phosphates. Mannitol phosphate dehydrogenase is present in glucose-grown cells of *E. coli* but the level of enzyme activity is increased greatly in extracts of cells grown on mannitol or sorbitol (328). A survey of other organisms revealed the presence of mannitol phosphate dehydrogenase in *A. aerogenes*, *L. casei*, *L. plantarum*, *Leuconostoc mesenteroides*, and *Saccharomyces cerevisiae*, but no enzyme activity was reported in extracts of *Clostridium kluyveri*, *Lactobacillus brevis*, *Pseudomonas fluorescens*, or *Neurospora crassa* (327). The enzyme has been found in *Piricularia oryzae*, *Aspergillus oryzae*, and *A. niger* by Yamada et al. (333) together with a specific mannitol phosphatase.

Further studies on *E. coli* (328) indicate that enzymes are induced by sorbitol and dulcitol for the oxidation of sorbitol 6-phosphate (D-glucitol 6-phosphate) and dulcitol phosphate (D-galactitol 6-phosphate), respectively. Extracts from sorbitol-grown cells (but not mannitol or dulcitol-grown cells) oxidized sorbitol 6-phosphate with DPN, probably to D-fructose 6-phosphate; extracts from dulcitol-

(but not mannitol- or sorbitol-) grown cells reduced D-tagatose 6-phosphate with DPNH. Extracts of *L. mesenteroides* contained sorbitol 6-phosphate dehydrogenase activity. Sorbitol 6-phosphate dehydrogenase has been partially purified from *L. casei* by Shockley and Pride (268) and the enzyme appears to be specific for sorbitol phosphate and DPN. Earlier studies on some unidentified species of lactobacilli (269, 277) had shown that induced enzymes were formed for the dissimilation of mannitol, sorbitol, and dulcitol and had indicated that the oxidations of nonphosphorylated polyols to ketoses were involved. It would now appear more likely that crude cell-free extracts of the organism were able to phosphorylate polyols prior to oxidation (cf 268). Sorbitol phosphate and dulcitol phosphate dehydrogenases have been found in *A. aerogenes* (214).

The first step in the dissimilation of polyols by *Acetobacter* and *Pseudomonas* and of pentitols by *A. aerogenes* appears to be the direct oxidation of polyol to ketose. In contrast, this reaction does not seem to be important for hexitols in the coli-aerogenes and lactic acid bacteria where, presumably, hexitols are phosphorylated prior to oxidation. Some indirect evidence has been reported for the phosphorylation of mannitol and sorbitol in *E. coli* (328). Marmur and Hotchkiss (224) were unable to detect mannitol phosphorylation in extracts of mannitol-adapted *Diplococcus pneumoniae*. The phosphorylation of mannitol, sorbitol, and erythritol by ATP in dried cells of *Propionibacterium pentosaceum* has been demonstrated by Barker and Lipmann (26) and D-erythritol 4-phosphate was shown to be the product of erythritol phosphorylation in extracts of the same organism (266). Because the phosphorylation reactions took place slowly these authors doubted their metabolic significance. Since the propionibacteria ferment a wide range of polyols (330), it would be interesting to know whether oxidation takes place prior to or after phosphorylation. Highly purified L-ribulokinase from *A. aerogenes* can phosphorylate ribitol and L-arabitol as well as D- and L-ribulose (273). The enzyme is induced by growth on L-arabinose and its normal metabolic role is to phosphorylate L-ribulose formed from L-arabinose. The enzyme is not induced by growth on ribitol, and ribitol phosphate is probably not an intermediate in ribitol dissimilation. The phosphorylation of ribitol and L-arabitol by L-ribulokinase may be fortuitous, or it might be concerned with the formation of some structural component (e.g. teichoic acid) (331).

VI. General Considerations on Polyol Dehydrogenases

Before there had been any extensive studies on enzymes involved in polyol metabolism, a simple hypothesis was advanced by Edson (117, 218) to explain the occurrence of polyols in plants and their utilization in animals and microorganisms. The hypothesis assumed that a general reaction, polyol + $DPN^+ \rightleftharpoons$ ketose + DPNH + H^+, was catalyzed by three types of stereospecific polyol dehydrogenases: D-mannitol dehydrogenase, with substrate specificity defined by the Bertrand-Hudson rule, oxidizing D-mannitol to D-fructose and sorbitol to L-sorbose (configuration *I*, Fig. 16); L-iditol dehydrogenase, requiring substrates with configuration *VI* (Fig. 16), oxidizing sorbitol to D-fructose but not mannitol or dulcitol; a galactitol dehydrogenase specific for polyols possessing

TABLE 11. ENZYMES RELATED TO D-MANNITOL DEHYDROGENASE

Enzyme	Configuration Required for Substrate	Pentitols and Hexitols Oxidized
D-Mannitol dehydrogenase in particles from *Acetobacter suboxydans* (9)	CH_2OH HO-C*-H HO-C-H	D-arabitol, ribitol, allitol, D-mannitol, sorbitol, D-talitol
TPN-linked D-mannitol dehydrogenase from *A. suboxydans* (104, 76)	CH_2OH HO-C*-H HO-C-H H-C-OH	D-arabitol, D-mannitol, sorbitol
D-Arabitol dehydrogenase of Lin (211) from *Aerobacter aerogenes*	CH_2OH HO-C*-H HO-C-H H-C-OH H-C†-OH	D-arabitol, D-mannitol (sorbitol, slowly)
D-Arabitol dehydrogenase of Wood (331, 332) from *A. aerogenes*	CH_2OH HO-C*-H HO-C-H H-C-OH CH_2OH	[illegible]bitol
D-Mannitol dehydrogenase from ropy-cider organism (28)	CH_2OH HO-C*-H HO-C-H -C- H-C-OH	D-mannitol, D-talitol

* Site of oxidation. † Configuration preferred.

configuration *IV* or *V* (Fig. 16), oxidizing dulcitol (9). The presence of one or more of these enzymes in various tissues could permit the oxidation of each polyol known to be utilized by microorganisms and animals and the synthesis of each polyol known to occur in plants.

This hypothesis has proved to be too simple. During the last 6 years a large number of polyol-oxidizing enzymes have been detected in many different tissues. The reactions catalyzed by these enzymes include oxidations and reductions involving polyols, aldoses, and ketoses, both in free and phosphorylated forms. These enzymes vary from having group specificities similar to the types proposed by Edson to being specific for one polyol substrate only. Since substrate specificity studies on the enzymes have seldom been complete, it would be premature to attempt to formulate rules which could cover these more recent findings. However, it is possible to relate a number of enzymes to the D-mannitol dehydrogenase type (Table 11): the TPN-linked mannitol dehydrogenase of *A. suboxydans*, the D-arabitol dehydrogenases obtained from *A. aerogenes*, and the mannitol dehydroge-

TABLE 12. ENZYME CATALYZED INTERCONVERSIONS OF POLYOLS* AND SUGARS

POLYOL	SUGAR	COENZYME AND SOURCE OF ENZYME
Polyol-ketose		
D-Arabitol	D-Xylulose	Nil,† *A. suboxydans* (9, 130); DPN, *A. aerogenes* (211, 331, 332); TPN, *A. suboxydans* (76)
L-Arabitol	L-Ribulose	DPN, *P. chrysogenum* (92)
L-Arabitol	L-Xylulose	DPN, *P. chrysogenum* (92), *A. aerogenes* (5)
Ribitol	D-Ribulose	DPN, mammals (150, 174, 177, 201, 218, 324), *A. aerogenes* (138, 140, 331, 332)
Ribitol	L-Ribulose	Nil,† *A. suboxydans* (9)
Xylitol	D-Xylulose	DPN, mammals† (150, 168, 174, 177, 201, 218, 324), *P. chrysogenum* (88), *C. albicans* (312)
Xylitol	L-Xylulose	DPN, *Ps. fluorescens* (263), *A. aerogenes* (5); TPN, guinea-pig liver mitochondria (174, 177), *P. chrysogenum* (91)
Allitol	D-Allulose	DPN, liver (177, 218)
Allitol	L-Allulose	Nil,† *A. suboxydans* (9)
Dulcitol	D-Tagatose	DPN, *Ps. fluorescens* (263)
Sorbitol	D-Fructose	DPN, mammals (51, 150, 168, 174, 177, 201, 218, 324), *Ps. fluorescens* (263), *C. utilis* (9), *A. suboxydans* (9, 76, 103, 104, 130)
Sorbitol	L-Sorbose	Nil,† *A. suboxydans* (9, 130, 321); DPN, *C. utilis* (9); TPN, *A. suboxydans* (76, 104)
L-Glucitol	L-Fructose	DPN, *Ps. fluorescens* (263)
D-Iditol	D-Sorbose	DPN, *Ps. fluorescens* (263)
L-Iditol	L-Sorbose	DPN, mammals (51, 150, 174, 177, 218, 324), *Ps. fluorescens* (263)
D-Mannitol	D-Fructose	Nil,† *A. suboxydans* (9, 130, 321); DPN, *A. suboxydans* (9, 76), *L. brevis* (28, 124), *A. aerogenes* (211), *C. utilis* (9), brewer's yeast (235); TPN, *A. suboxydans* (76, 103, 104), *A. aceti* (110)
D-Talitol	D-Allulose	DPN, *Ps. fluorescens* (263)
D-Talitol	D-Tagatose	Nil,† *A. suboxydans* (9, 297)
Polyol-aldose		
L-Arabitol	L-Arabinose	TPN, *P. chrysogenum* (92), *C. albicans* (312)
Xylitol	D-Xylose	TPN, mammals‡ (165, 168), *P. chrysogenum* (88), *C. albicans* (312)
Sorbitol	D-Glucose	TPN, mammals‡ (165, 168, 201)
Polyol phosphate-ketose phosphate		
D-Galactitol 6-phosphate	D-Tagatose 6-phosphate	DPN, *E. coli* (328)
Sorbitol 6-phosphate	D-Fructose 6-phosphate	DPN, *E. coli* (328), *L. casei* (268)
D-Mannitol 6-phosphate	D-Fructose 6-phosphate	DPN, *Diplococcus pneumoniae* (224), *E. coli* (327), *Piricularia oryzae* (333)
Polyol phosphate-aldose phosphate		
D-Ribitol 5-phosphate	D-Ribose 5-phosphate	TPN, silkworm (128)
D-Sorbitol 6-phosphate	D-Glucose 6-phosphate	TPN, silkworm (128)

* The list is limited to fully hydroxylated acyclic pentitols and hexitols.

† Particulate enzyme from *A. suboxydans* that is active without the addition of cofactors.

‡ Compare Table 12 for other substrates of mammalian aldose reductase.

nase activity of a *Lactobacillus* sp. These enzymes are more highly specific in their substrate requirements than the cytochrome-linked D-mannitol dehydrogenase from *A. suboxydans*, but the only polyols oxidized are those that are among the substrates for the Bertrand-Hudson enzyme.

In considering the mechanism of polyol dehydrogenase action it has been suggested that polyol substrates are bound to the enzyme surface by hydrogen bonds whose number and spatial arrangement determine the enzyme specificity (117). The form of the sugar product could be open chain, pyranose or furanose. Barker et al. (28) have pointed out that, in addition, it is necessary to consider the various boat and chair conformations. Where the relative rates of ketose reduction by polyol dehydrogenases have been measured, it is noticeable that ketopentoses are reduced to polyols much more rapidly than are ketohexoses. This would indicate that ketopentoses in solution can take up the reactive form required by the enzyme more readily than ketohexoses. Since the former cannot form a pyranose ring and the latter are predominately pyranose, it would appear that some form other than the pyranose is required by these enzymes. On the other hand, Barker et al. (28) concluded that the polyol dehydrogenase of a *Lactobacillus* sp. prefers the 1-C pyranose form; unfortunately, their published studies were not performed on a cell-free enzyme preparation.

Many microbial polyol dehydrogenases are inducible enzymes; either they are found in cell-free extracts only when the organism has been grown in the presence of a specific inducer or the enzyme concentration can be increased greatly by an inducer (263, 312, 327, 331). Other polyol dehydrogenases are constitutive enzymes and seem not to be affected by substances that might act as inducing agents (109). The phenomena of "inducible" and "constitutive" enzymes, with reference to polyol dehydrogenases, has been discussed by Wolff and Kaplan (328). The induction of a polyol dehydrogenase has generally been observed with the substrate as inducer. However, there are examples of enzyme induction by polyols and sugars that are not substrates (263, 331), and one case has been reported of a substrate for a group specific enzyme that not only fails to induce the enzyme but represses its induction by another substrate (211).

Many reports of polyol oxidations, even those that have been effected in vitro by enzyme preparations, do not state the nature of the oxidation product. Table 12 lists well-documented polyol-sugar interconversions and the enzymes catalyzing the reactions.

In conclusion, it should be emphasized that, when it seems apparent that the utilization or formation of a polyol is not serving specifically in the biosynthesis or breakdown of cellular elements, it is possible that the usefulness of the transformation lies in the transfer of hydrogen on coenzymes. For example, reversible sequential reactions involving DPN and TPN allow these reactions to serve a "transhydrogenase" function. Recent work has made it increasingly evident that various metabolic pathways can be regulated by the availability of coenzymes in the appropriate state of oxidation or reduction. Future studies of the physiological importance of the polyols may be more fruitful if this concept plays a significant role in the formulation of experiments.

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THE ANTAGONISTIC ACTION OF CERTAIN SUGARS, AMINO ACIDS AND ALCOHOLS ON INSULIN INTOXICATION[1]

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The object of this paper is to record some observations which, we believe, may assist in the elucidation of the still incompletely understood mechanism of the physiological action of insulin. The work is based on the well-known discovery of Banting, Best, Collip, Macleod and Noble (1) who found that the characteristic symptoms supervening in rabbits after the administration of an excessive dose of insulin can be rapidly relieved by the injection of glucose. The question naturally arises as to whether carbohydrates other than glucose, amino acids, alcohols and, generally, substances which may be converted within the body into glucose, have an antagonistic effect similar to that of glucose. This idea occurred also to Noble and Macleod (7) who put it to an experimental test. Their method is briefly the following:

After taking blood samples of normal rabbits the animals receive an injection of insulin in such amounts as to produce convulsions. As soon as the convulsions supervene, the substance to be tested for its antagonistic effect is injected subcutaneously and at intervals other blood samples are taken, these being analyzed for sugar. The disappearance of the symptoms is taken as a criterion of the activity of the substances tested. Noble and Macleod found that "the only sugar which can definitely antidote the symptoms that accompany the hypoglycemia due to insulin is glucose." Mannose was found almost as efficient as glucose. Levulose, galactose and maltose cause only a slight improvement in the symptoms, in spite of a marked increase in blood sugar. The rise in blood sugar in this case is evidently not due to glucose, but must be attributed to the presence of these reducing sugars. Arabinose, xylose, sucrose, lactose, sodium lactate and glycerol are inactive. From this it is evident that only two aldohexoses (glucose and mannose) are effective antagonists of insulin and that blood sugar estimations are only of limited value in the study of this kind of antagonism.

[1] In this paper the term "insulin intoxication" is used to denote the pathological condition produced by the administration of toxic doses of insulin to normal animals.

It is obvious that the method employed by Noble and Macleod is only adapted for the investigation of the antagonistic action of substances which are very easily and rapidly converted into glucose by hydrolysis, tautomeric change or other means. For this reason a method of broader scope would decidedly enhance the usefulness of insulin in the study of metabolism. We have utilized for this purpose a method which we suggested recently for the biological standardization of insulin (10).

METHOD. Healthy, non-pregnant albino rats are kept on a standard diet for several weeks previous to the experiment. About 18 hours before the experiment the animals are placed in a ventilated incubator room (28° to 30°C.) and starved. The minimum lethal dose of insulin (defined as the lowest dose which yields a mortality of 90 to 100 per cent in a set of ten animals) is then determined by injecting the drug subcutaneously in graded doses, i.e., 1 cc., 2 cc., 4 cc., etc., per kilogram bodyweight. Having thus established the M.L.D. of insulin, we can proceed to test the antagonistic action of various substances. This is done by injecting several sets of 10 rats each, under the above mentioned conditions, with the M.L.D. of insulin, this being *immediately* followed by the administration, by means of a stomach tube, of graded doses of the substances to be tested. It was found that 8 grams of glucose per kilogram are required to counteract the fatal action of a M.L.D. of insulin. The following protocol illustrates such an experiment:

NUMBER OF RATS	INSULIN	GLUCOSE PER KILO	MORTALITY
	M.L.D.	*grams*	*per cent*
10 (controls)	1	None	100
10	1	5	20
10	1	6	10
10	1	8	0
10	1	10	0

In the same manner there was tested a considerable variety of other substances. It is of course always necessary to establish the toxicity of these substances before their antagonistic action against insulin is tested. Our method is limited to substances which have no considerable inherent toxicity, but fails for instance with acetic aldehyd, which was found to be quite toxic in the amount required for our purpose.

The method has an accuracy of about 20 to 50 per cent or, in other words, it is possible to establish the protective dose of a given substance against insulin within 20 to 50 per cent. If the test be repeated several times it is quite feasible to reduce the error to 20 per cent. Some of the active substances were also tested as to their effect on the removal of the symptoms of insulin intoxication, thus making use of the method of Noble and Macleod.

In some cases blood sugar estimations were made on animals which had received a M.L.D. of insulin together with a protective dose of an active substance, in order to determine whether the blood sugar values remained normal. For this purpose the blood from several decapitated rats killed from 1 to 24 hours after injection was analyzed separately for blood sugar. Each figure in tables 1 and 2 therefore indicates the blood sugar of an individual rat.

Discussion of results. It has been pointed out that the method used by us yields information as to whether a certain substance given in proper amounts prevents the fatal action of a minimum lethal dose of insulin. We know from the work of the Toronto School that *glucose* may be considered as a physiologic antidote of insulin and our work with standard-bred rats has shown that about 8 grams of glucose per kilo administered by mouth at the time of the subcutaneous injection of a M.L.D. of insulin protected the animals from death. A larger dose of glucose is needed to completely prevent the hypoglycemia.

It will be seen from the data of table 1 that two other hexoses besides glucose, i.e., *galactose* and *fructose*, offer very good protection. These observations are somewhat at variance from a quantitative point of view with the *curative action* of these sugars as observed in rabbits by Noble and Macleod (7) and quite recently in mice by Herring, Irvine and Macleod (4). These investigators arrive at the conclusion that fructose and galactose are less efficient in alleviating the symptoms.

The two disaccharides tested, i.e., *maltose*, *lactose*, *sucrose* and *trehalose*, also exert a powerful preventive action, about equal to that of glucose.

Inulin yielded negative results, as would be expected. Hydrolysis of inulin yields fructose. The negative results obtained with this polysaccharide therefore indicate lack of digestion, and this view was confirmed by the *necropsy*, which showed the presence of large amounts of the undigested carbohydrate in the intestines.

Of the two alcohols included in this work, *glycerol* showed a remarkable effectiveness, whereas *mannitol* was only slightly active (table 2).

The antagonistic effect of glycerol on the hypoglycemia following insulin is illustrated by the data in table 1. It will be noted that the 12 control animals showed the usual marked drop in blood sugar from an average value of 0.113 per cent before injection to 0.01 per cent after injection, whereas the rats having received 6 grams of glycerol per kilogram had a considerably higher blood sugar, and very mild symptoms. A larger amount of glycerol undoubtedly have prevented the hypoglycemia.

In view of this very great *preventive* action of glycerol it was decided to carry out a few experiments on the *curative* action of the substance on the removal of already established symptoms. Observing the usual technic a minimum lethal dose of insulin was given to six rats. (Twelve con-

trols similarly injected all died within four hours.) The six animals received intraperitoneally from 1 to 6 grams of glycerol (20 per cent solution) per kilogram bodyweight. In every case there was noted a marked improvement in the severity of the symptoms, the convulsions or coma

TABLE 1

Sugars

		Dose per kilo	Mortality	Number of animals	Mortality of controls receiving only insulin*
		per cent	*per cent*		*per cent*
Pentoses	l-Arabinose	7	60	10	100
	Xylose	7	70	10	80
Hexoses	Glucose	6	30	10	100
		8	0	10	100
		10	0	10	100
	Galactose	7	20	10	80-100
		8	20	10	80-100
		10	0	10	80-100
	Fructose	3	45	20	80
		4	25	20	80
		5	17	30	80
		6	0	10	80
Disaccharides	Maltose	3	30	10	90
		4	20	10	90
		5	0	10	90
	Lactose	3	20	20	80
		4	20	20	80
		5	3	30	80
	Sucrose	3	37	20	80-90
		4	15	20	80-90
		5	10	20	80-90
		6	0	20	80-90
	Trehalose	6	0	10	80
Polysaccharide	Inulin	5	100	10	90
		6	90	10	90

* It will be noted that some of the [illegible] yielded a slightly lower mortality than the definition of the M.L.D. [illegible] Under these circumstances the protective dose of the [illegible] may be slightly lower than would have been the case had a full M.L.D. of [illegible] given.

disappearing within ten minutes. Relapse occurred in two animals, but four out of the six survived.

TABLE 2

Alcohols, acids and amino acids

	Number per kilo	Number of animals	Mortality	Mortality of controls receiving insulin alone
	grams		*per cent*	*per cent*
Mannitol	3	10	70	100
	1	10	30	100
	5	10	80	100
Glycerol	4	10	80	88
	5	10	0	88
	6	10	0	88
Lactic acid (sodium salt)	2	10	100	90
	4	10	90	90
Pyruvic acid (sodium salt)	10.6	9	100	80
Glycin	6	10	100	80
	7	10	80	80
d-Alanin	6	10	20	70
	7	10	0	70
Glutamic acid	2	20	95	100
	4	20	95	100

* Toxicity not determined on account of insufficient supply of substance.

TABLE 3

Insulin preceded by olive oil 4 to 6 hours

Insulin per kilo	Number of animals	Mortality	Mortality of controls receiving only insulin	Interval between olive oil and insulin injection
units		*per cent*	*per cent*	*hours*
4.5	10	60	100	6
9	10	70	100	6
13.5	30	60	100	6
18	30	57	100	4–6
27	30	56	100	4–6
36	30	30	95	4

Whether glycerol is a direct antidote for insulin or whether it must be first converted into glucose cannot be decided on the basis of this evidence.

The [illegible] possibility would presuppose a remarkable speed in transformation of glycerol into glucose.

Noble and Macleod failed to obtain any [illegible] of the symptoms in rabbits as a result of subcutaneous injections of undiluted glycerol. The reason for the discrepancy between our observations and those of these workers may be due to differences in the mode of administration or

TABLE 4

Influence of glycerol on the [illegible] following insulin

INSULIN	GLYCEROL PER KILO	NUMBER OF ANIMALS	BLOOD SUGAR AFTER INJECTION				
			1 hour	2 hours	3 hours	4 hours	24 hours
	grams		per cent	per cent	per cent	per cent	per cent
1 M.L.D.	6	*12	0.113	0.068	0.083	0.095	0.115
			[illegible]	0.065	0.068	0.085	0.115
							0.113
							0.120
1 M.L.D. (controls)	None	12	[illegible]	0.047	0.049		

* The other 9 animals died of insulin intoxication within 4 hours after injection.

TABLE 5

Influence of alanin on the [illegible] following insulin

INSULIN	ALANIN PER KILO	NUMBER OF ANIMALS	BLOOD SUGAR AFTER INJECTION				
			1 hour	2 hours	3 hours	4 hours	24 hours
	grams		per cent	per cent	per cent	per cent	per cent
1 M.L.D.	7	12	[illegible]	0.068	0.066	0.057	0.170
			[illegible]	0.061	0.050	0.061	0.161
			[illegible]	0.065			
Average			0.073	0.065	0.058	0.059	0.165
1 M.L.D. (controls)	None	12	[illegible]	0.054			

* The remaining 10 animals died of [illegible] intoxication within 3 hours after injection.

perhaps to a difference in the animal [illegible]. In all events our observations agree with the results of [illegible] showed that glycerol can give rise to glucose and they [illegible] with some recent observations of Thomas (9) indicating that [illegible] has a marked antiketogenic action in diabetes. Our results are [illegible] with the findings of

[2] Recently we were able to [illegible] action of glycerol (2 to 6 gm. per kilo) administered [illegible] per cent solution.

[illegible] and [illegible] (o) who showed that carbohydrate is formed from [illegible], when the respiratory quotient is below 0.8.

[illegible] and [illegible] are supposed to be readily convertible into [illegible] in the mammalian body. These two acids were therefore tested in the form of the [illegible] salts as to their activity against insulin, with negative results. Noble and Macleod had previously found lactic acid [illegible] as a curative agent. The negative results with lactic acid are [illegible] and indicate that, at least under the conditions of our experiments, the transformation of lactic acid into glucose does not take place at sufficient speed to prevent death from insulin. This absence of [illegible] is not due to lack of absorption of the substance from the gastro-intestinal tract, as shown by the fact that this tract was found to be practically empty at necropsy.

[illegible] *glycin*, *dialanin* and *glutaminic acid*—showed [illegible] is [illegible] in preventing insulin death, being about [illegible] as glucose.

Glycin [illegible] which may be due to a slow rate of absorption from the intestines. The latter, at necropsy, were found to be [illegible] with a large amount of fluid, presumably containing the unabsorbed [illegible]. Glutaminic acid also was inactive. The influence of alanin [illegible] with insulin on the hypoglycemia following the latter [illegible] by the data in table 5. It will be noted that alanin decreases the hypoglycemic effect considerably and there is no doubt that a larger dose of the amino acid would prevent altogether the occurrence of hypoglycemia.

The results with these three amino acids are of interest in connection with the question of their conversion in the body into glucose. It is well known that [illegible] have shown that alanin fed to a phloridzinized dog [illegible] elimination of glucose in the urine, the [illegible] per cent of the theoretical amount, [illegible]. These results were confirmed by Dakin and Dudley [illegible] with glycin, glutaminic acid and some [illegible] indicated conversion into glucose. [illegible] in the determination as to whether [illegible] with the substance to be tested has [illegible] have their weak points, [illegible] the belief that a direct conversion [illegible] place but rather that it [illegible] between the amino acid or peptide [illegible] with the result that other amino acids [illegible] free." In view of this [illegible] it is certainly gratifying [illegible] concerned, seems to be con-

firmed by our experiments on the antagonistic action of alanin against insulin. We fully realize that the latter evidence is also of an indirect nature and we do not want to fail to mention that the antagonism between alanin and insulin may perhaps be due to the possibility that alanin by being metabolized has a sparing action on glucose. However, this interpretation probably is not correct in view of the fact that the amount of alanin necessary to antagonize a M.L.D. of insulin is practically the same as in the case of glucose.

Inasmuch as glycerol proved itself to be such a powerful physiological antagonist against insulin it was of interest to test out the effectiveness of *olive oil,* which contains about 10 per cent of glycerol in combination with fatty acids. The oil was given by stomach tube 4 to 6 hours before the injection of insulin, in order to allow a sufficient time for digestion. The data of table 3 clearly indicate that olive oil has a moderate protective action and the results would have been even more pronounced had it been possible to give an amount of oil equivalent to 6 grams of glycerol (60 grams of olive oil).

It should be pointed out that the antagonistic action of all the active substances showed itself not only in the prevention of death but also in the milder character of the symptoms, in fact it was noted that with the minimum protective dose of glycerol and alanin for instance, the symptoms were either absent or consisted only of depression.

The principal conclusion to be drawn from our experiments is that various carbohydrates, glycerol and at least one amino acid are physiological antagonists of insulin. This hormone therefore is not only strictly related to carbohydrate metabolism, but has relations to protein and fat metabolism. Whether these latter relations are direct or indirect cannot be determined on the basis of the present evidence. In all events it is advisable to regard the function of insulin in metabolism from a broad viewpoint.

SUMMARY

1. Various sugars are physiological antagonists of insulin: glucose, galactose, fructose, maltose, lactose, sucrose and trehalose. l-Arabinose and xylose have only a questionable activity. Inulin is inactive, due probably to lack of digestion.

2. Mannitol has a slight antagonistic action. Glycerol is very effective, both as a preventive of insulin death and as a curative agent. Glycerol decreases the hypoglycemia following insulin.

3. Lactic acid and pyruvic acid are inactive.

4. Glycin and glutaminic acid are inactive, whereas d-alanin is effective in about the same amount as glucose. Alanin decreases the hypoglycemia following insulin.

5. Olive oil, in the doses used, has a moderate activity, which is probably due to its glycerol component.

6. The physiological mechanism of insulin not only involves carbohydrate metabolism, but also that of the proteins and fats.

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Carbohydrates and Gastrointestinal Absorption of Radiostrontium and Radiocalcium in the Rat.* (24922)

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Ability of lactose to increase gastrointestinal absorption and retention of calcium has been known for many years and has been adequately confirmed under various conditions and in different species. The effects of lactose on calcium metabolism and other physiological functions have been reviewed recently by Duncan(1) and Atkinson *et al.*(2). Although the action of lactose has been widely studied, little attention, with one exception, has been given to the influence of other sugars and sugar derivatives on calcium. Fournier and his colleagues(3-5) determined the net retention of calcium in rats as influenced by ingestion of sugars by use of conventional calcium balance technic. Certain sugars, in addition to lactose, were capable of enhancing calcium retention in the growing and lactating rat; *e.g.*, xylose, mannose, raffinose, mannitol and sorbitol. It was not feasible with calcium balance method to determine extent

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to which carbohydrates increased gastrointestinal absorption or acted by direct effect on skeletal growth. The work presented here, based on tracer procedures, distinguishes between skeletal and absorptive effects. The technic was used to estimate effect of various sugars and sugar derivatives on gastrointestinal absorption of radiocalcium and radiostrontium in a way similar to that previously described for study of the influence of various amino acids on intestinal absorption(6).

Materials and methods. Male rats of Wistar strain weighing about 90-110 g were fasted 24 hours with only drinking water available. At this time, the rats were lightly anaesthetized with ether and given, by gavage, 1 ml of test solution containing 20 μc Ca^{45}, 2 μc Sr^{85}, 0.42 millimole of test substance and 0.04 millimole of calcium chloride. The animals were killed at 24 hours after dosing and the femurs removed for radioassay. Calcium 45 was determined by oxalate-precipitate method described by Comar(7) and Sr^{85} was measured by counting excised whole femur in a well-type scintillation detector. It has previously been shown that the appearance of Ca^{45} and Sr^{85} in bone 24 hours after oral administration is a measure of gastrointestinal absorption(6) and, more specifically, that lactose administered under similar conditions does not directly influence movement of alkaline earths from blood to bone(8). In the latter study, the ratio of femur Sr^{85} to absorbed Sr^{85} as directly measured was not affected by presence of lactose. Radioassay data are expressed in terms of "% of administered dose" or as "% of ingested dose."

To study further this lack of lactose effect on skeletal metabolism, a balance-type technic was employed in which young growing male rats on Rockland stock diet were placed on diets that contained either 30% sucrose or 30% lactose. When rats were placed on experimental diets, the drinking water was replaced with distilled water containing 0.02 μc Sr^{85}/liter; the rats were continued on this regime for 7 days. By use of graduated drinking tubes, the volume and therefore the amount of activity ingested was determined. Radiostrontium or radiocalcium ingested in this manner has been shown to equilibrate with, and be metabolized similarly to, dietary calcium in the young growing rat(9). The feces and urine were collected separately over the 7-day period and at this time the rats were killed and the contents of the digestive tract was removed and combined with the pooled feces. The pooled feces plus digestive tract contents, pooled urine samples and the left femur from each rat were radioassayed for Sr^{85}.

Carbohydrates were obtained from Nutritional Biochemicals Corp., and used without further purification. All sugars employed were the natural forms.

Results. The data on comparative effect of a number of sugars and sugar derivatives on radiostrontium absorption and, in certain instances, on radiocalcium absorption are summarized in Table I. This compilation represents 3 separate experiments in which femur Sr^{85} values for control groups were standardized to a value of 1.3% for comparative purposes. However, results from each experiment were treated by analysis of variance method to determine any statistical significance of effects of test substances. First, there was parallel behavior between Ca^{45} and Sr^{85} in regard to effects on absorption, *e.g.*, xylose and lactose increased absorption of both alkaline earths whereas glucose and galactose had no effect on either ion. This parallelism holds in this laboratory for numerous substances, including some 20 amino acids and Vit. D(6,8,10).

Although radiostrontium responds qualitatively in the same way as does radiocalcium, there are certainly quantitative differences in over-all metabolism of these alkaline earths, a subject that has been studied intensively (11). Differences in the present study are shown by Sr^{85}/Ca^{45} ratios listed in Table I; here the Sr^{85}/Ca^{45} ratio of the ingested solution is taken as unity. The Sr^{85} Ca^{45} ratio of 0.61 for the control group indicates that proportionally more Ca^{45} than Sr^{85} was transported from gut to bone. There was a definite increase in the Sr^{85}/Ca^{45} ratios of xylose and lactose-treated groups; this elevation of the Sr^{85}/Ca^{45} ratio by substances that en-

TABLE I. Influence of Carbohydrates on Gastrointestinal Absorption of Sr^{85} and Ca^{45} in the Rat.

Test substance	Femur conc. of radionuclide* (% dose/femur) Sr^{85}	Ca^{45}	Sr^{85}/Ca^{45}	Change in femur Sr^{85} content (%)	Effectiveness in enhancing Sr^{85} absorption (present study)	Effectiveness in enhancing Ca. retention (after Fournier *et al.*) †
Control	1.3 ± .1	1.9 ± .1	.61			
D-Glucose	1.1 ± .1	2.1 ± .2	.50	− 15	ineffective	ineffective
Sucrose	1.4 ± .1			+ 8	"	"
D-Galactose	1.5 ± .1	2.5 ± .2	.61	+ 15	"	slightly effective
D-Fructose	1.6 ± .2			+ 23	"	
Cellobiose	2.1 ± .2			+ 62	effective	
L-Sorbose	2.5 ± .1			+ 92	"	effective
D-Ribose	2.5 ± .2			"	"	
D-Xylose	2.6 ± .4	3.6 ± .3	.71	+100	"	effective
Lactose	2.6 ± .4	3.2 ± .2	.76	"	"	"
Raffinose	2.6 ± .2			"	"	"
Melibiose	2.6 ± .3			"	"	"
D-Glucosamine	2.7 ± .2			+108	"	
D-Mannitol	2.8 ± .2			+115	"	effective
D-Sorbitol	2.8 ± .3			"	"	"

* Values represent mean ± stand. error of mean; 6 rats/group; mean body wt about 90-110 g.

† See text for references.

hance alkaline earth absorption had been noted previously(6). The reason for this change in ratio is unknown but may be due to mass effects or the effect of these substances on processes of discrimination.

The relative effectiveness of test substances on Sr^{85} absorption may be readily noted in column 5 of Table I. The Sr^{85} contents in femurs of glucose, galactose, sucrose and fructose-treated groups were not significantly different from those of control group. All other sugars or their derivatives greatly enhanced Sr^{85} absorption, with cellobiose being least effective. Also in Table I, the data of Fournier *et al.* (*loc cit*) as obtained from the balance technic are summarized for comparison. There is excellent agreement between the 2 studies.

In Table II, results of the balance study with Sr^{85} are presented. The lactose-supplemented group absorbed and retained more of the ingested Sr^{85} than did the sucrose-supplemented group when measured directly or by bone content; these differences were significant at $p<0.01$. An important conclusion from this study is that the enhanced retention of Sr^{85} was accounted for by increased gastrointestinal absorption of Sr^{85}. This is shown by the similarity of "absorbed-retained" ratios for the 2 groups. Thus, there was no evidence that lactose had a direct effect on skeletal metabolism in these studies.

Discussion. From our study, it seems apparent that there was no general pattern of specificity in enhancement of Ca^{45} and Sr^{85} absorption by carbohydrates. Also there did not appear to be any correlation between effectiveness in promoting alkaline earth ab-

TABLE II. Comparison of Sucrose- and Lactose-Supplemented Diets on the Absorption and Retention of Radiostrontium.*

Diet	Fecal excretion of Sr^{85} (% ingested dose) †	Urinary excretion of Sr^{85} (% ingested dose) †	% absorption of Sr^{85} (by difference)	% retention of Sr^{85} (by difference)	Femur content of Sr^{85} (% ingested dose) †
Sucrose	78 ± 3	2.4 ± .3	22	20	.82 ± .09
Lactose	68 ± 2	4.4 ± .8	32	28	1.18 ± .04

* Basal diet contained casein 20%, starch 33%, vegetable oil 10%, brewer's yeast 2%, salt mixture U.S.P. #2 4%, vit. supplement (Nutritional Biochemical) 1%; sucrose and lactose added at 30%; 5 rats/group with mean initial body wt of 193 g.

† Values represent % of total ingested Sr^{85} that was excreted in feces and urine over the 7 day experimental period; data given as mean ± stand. error of mean.

sorption and any usual physical or chemical properties of the carbohydrate, which included pentoses, hexoses, disaccharides, trisaccharides, hexitols and hexosamines. This was in contrast to the specificity of the influence of individual amino acids on Ca^{45} and Sr^{85} absorption where L-lysine and L-arginine were more effective than any of 20 amino acids tested(6).

An interesting relationship may exist, however, between the physiological behavior of carbohydrates and their ability to enhance Sr^{85} absorption and calcium retention. Glucose and galactose are absorbed rapidly from the digestive tract and essentially by active metabolic processes; rate of absorption of glucose and galactose is decreased by presence of monoiodoacetic acid, phlorizin, an abnormal pH, and takes place at a constant rate, independent of concentration(12). Fructose absorption also occurs rapidly but takes place by 2 processes: simple diffusion and an active process that depends on transformation of intestinal fructose to glucose(12). The pentoses, sorbose and glucosamine are absorbed by passive diffusion as reported by Verzár(12) and Wilson and Vincent(13). Among disaccharides, lactose is absorbed more slowly than sucrose(14), the difference probably being due to the less rapid hydrolysis of lactose by intestinal enzymes. Cellobiose is absorbed much less rapidly by the rat than is glucose but can be utilized for glycogen synthesis(15). Information on comparative rates of absorption of sorbitol, mannitol, raffinose and melibiose or their comparative rates of enzymatic hydrolysis was not found. Thus, from information available, it appears that effectiveness in enhancing Sr^{85} absorption and calcium metabolism may be positively correlated with 2 physiological characteristics: (a) prolonged residence time of the effective carbohydrate in the gastrointestinal tract or (b) absorption by a passive mechanism. Although the exact meaning of this apparent relationship is not known, it is indicated from these studies that the site of action of effective carbohydrates is the gastrointestinal tract. This is in contrast to the theory of Fournier *et al.* (*loc cit*) that lactose, xylose, and other stimulatory sugars operate by directly influencing skeletal ossification.

Summary. 1. The influence of 14 carbohydrates on gastrointestinal absorption of radiostrontium and radiocalcium was studied in the rat. 2. Cellobiose, sorbose, ribose, xylose, lactose, raffinose, melibiose, glucosamine, mannitol and sorbitol significantly enhanced alkaline earth absorption whereas glucose, galactose, fructose and sucrose had little or no effect. 3. In a balance-type study with Sr^{85}, enhanced retention due to dietary lactose as compared with dietary sucrose could be accounted for by increased intestinal absorption rather than by direct effect on skeletal ossification. 4. Effectiveness in ability to enhance calcium and strontium absorption appeared to be positively correlated with a prolonged residence time of the carbohydrate in the gut or absorption of carbohydrate by a passive mechanism.

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Thin-Layer Chromatographic Separation of Hexitols and Corresponding Hexoses

The hexitols, sorbitol and galactitol, have become of increasing biological importance, the former because of its accumulation in nervous tissue of diabetic animals (1) and the latter because of its presence in the tissues and urine of patients with congenital galactosemia (2) and in the tissues of animals fed a high galactose diet (3). Galactitol has been considered the etiological factor in galactose cataract formation (4).

Metabolic studies with galactitol have necessitated a convenient method of separation of this polyol from galactose as well as from other common hexitols. Paper chromatographic procedures have failed to achieve the desired results (5). Gas chromatography has resolved this difficulty but requires laborious procedures and expensive equipment (6). Recently Nemec *et al.* (7) employed silica gel G on glass plates to separate sorbitol, galactitol, and mannitol from their related aldoses but were unable to separate these hexitols from each other. This communication describes the use of borate impregnated silica gel G on thin layer to effect the separation of these polyols from their corresponding hexoses and from each other.

Materials and Methods. 0.02 *M* borate buffer, pH 7.85, was made up fresh as a 3% solution of 0.02 *M* sodium borate, pH 9.2, in 0.02 *M* boric acid, pH 6.6; 120 ml of the resulting solution was added to 60 gm silica gel G, according to Stahl (Brinkmann Instruments). The mixture was shaken manually for 90 sec and spread onto 20 × 20 cm glass plates to a thickness of 250 μ using the Desaga spreader. After drying overnight the plates were placed into a 126° C oven for 60 min, then stored at room temperature in a wooden box with desiccant. Before using, the plates were reactivated for 30 min in the 126° oven.

Unlabeled sugars were obtained from Phanstiehl Laboratories, Inc.

Galactitol-1-^{14}C 2.26 μc/mg, was provided by the National Bureau of Standards and galactose-1-^{14}C obtained from New England Nuclear was diluted with unlabeled galactose to 2.26 μc/mg.

With a 2 μl pipet, 2 μl of each of the unlabeled 30 mM solutions was spotted 3.5 cm above the bottom of the plate. The plates were run in a glass tank. The solvent, 80% isopropanol in water, was run at room

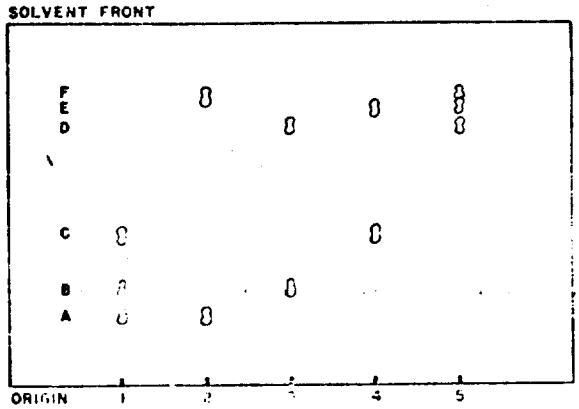

FIG. 1. Representation of chromatogram in which three hexoses had been run with their respective sugar alcohols on borate-impregnated silica gel G. Applications were made as follows: (1) galactitol, sorbitol, mannitol; (2) sorbitol, glucose; (3) galactitol, galactose; (4) mannitol, mannose; (5) galactose, glucose, mannose. Resulting R_f values were as follows: (A) sorbitol, 0.192; (B) galactitol, 0.269; (C) mannitol, 0.415; (D) galactose, 0.711; (E) mannose, 0.750; (F) glucose, 0.788.

temperature to a predetermined solvent front 13 cm above the origin (approximately 4 hr). After the plates were removed and dried at room temperature, the procedure was repeated twice using the same solvent. To detect the sugars, the plates were sprayed with a 10% solution of sulfuric acid in methanol and dried at 126° for 40 min. All of the sugars appeared as deep charcoal spots after 15 min and the polyols were noted after 30 min as light brown spots.

When labeled galactose and galactitol were used 2 μl of 30 mM galactitol or 1 μl of 100 mM galactose was applied and after development of the plate the area above the origin was divided into 1 cm portions, 3 cm in width. The 1 cm portions were scraped from the plates into small glass counting vials; 10 cc of liquid scintillator, made as 5% solution of Liquifluor (New England Nuclear Co.) in toluene was added to each counting vial. Samples were counted in the Packard model 3365 liquid scintillation counter.

Results and Discussion. Figure 1 is a representation of a chromatogram

in which each of the three hexoses was run with its respective sugar alcohol. Also, the three sugars were run as a mixture, as were the three polyols. A series of thin-layer experiments indicated that the separation of the hexoses and polyols was reproducible and the separation and relative positions of the three polyols were also constant in these studies.

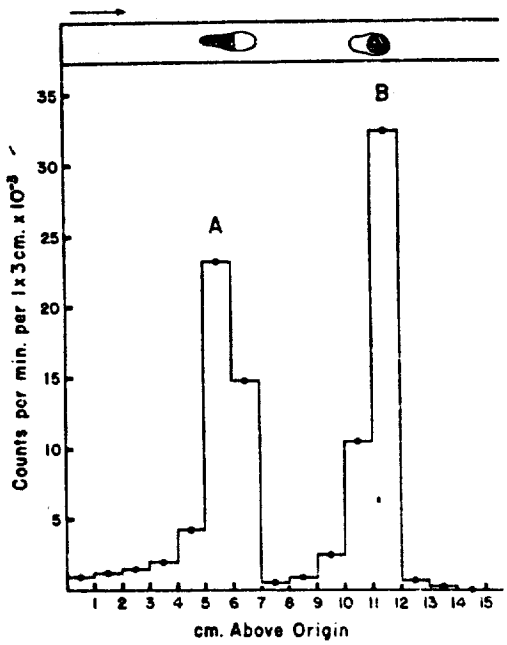

FIG. 2. Sketch of chromatograph analyzed by spraying as well as scraping. 2 μl of 30 m*M* galactitol (2.26 μc/mg) and 1 μl of 100 m*M* galactose (2.26 μc/mg) were spotted together as a mixture, in duplicate at the origin. The bar graph represents the radioactivity recovered in the 1 × 3 cm sections scraped; circles indicate the spots visualized after spraying. Identity of peaks are: (A) galactitol; (B) galactose.

Application of this method has also been made to the separation of pentoses from their corresponding pentitols. Resulting R_f values were as follows: D-arabitol, 0.469; D-arabinose, 0.692; L-arabitol, 0.476; L-arabinose, 0.684; ribitol, 0.561; ribose, 0.500; xylitol (two resulting spots), 0.453 and 0.253; and D-xylose, 0.669. Although it was possible to separate D- and L-arabinose and D-xylose from their respective polyols, it was only possible to separate ribitol from the other three pentitols. The method, however, can be employed to separate the pentitols and the hexitols. (See R_f values in Figure 1).

Figure 2 shows the results of a chromatograph of a mixture of galactose-1-^{14}C and galactitol-1-C^{14}, applied as adjacent spots, where, after

running, one of the chromatographs was analyzed by spraying and the other by counting. The sketch indicates that the sugar and the polyol are clearly separated by this system and that the radioactivity peak for both migrates to the same position on the plate as the visual spot.

Borate-impregnated silica gel G has proved to be a simple means for the detection of galactitol in the urines of patients given labeled galactitol and in various animal tissues which had been incubated with labeled galactitol in metabolic experiments (8), thus eliminating the need for complicated analysis by gas chromatography.

ACKNOWLEDGMENTS

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MODIFICATION OF ENZYMIC ACTIVITY
I. EFFECT OF ORGANIC ADDITIVES ON α-AMYLASE ACTIVITY

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SUMMARY

Alcohols inhibit α-amylase activity in the decreasing order of ethanol, isopropanol, propylene glycol, glycerol, ethylene glycol and methanol. Inhibition is directly proportional to the molar concentration of these alcohols and there is a linear relationship between the effect of the alcohols on the dielectric constant of water and their effect on α-amylase activity. Urea, glycine and saccharin have no effect on α-amylase activity at low concentrations but are inhibitory at high concentrations. Anionic and non-ionic surfactants, ranging in hydrophilic-lipophilic balance from 2 to 18, and methyl celluloses have very little effect on α-amylase activity. Inhibition by carbohydrates decreases in the order disaccharides, sugar alcohols and monosaccharides. Maltose is the most inhibitory of the carbohydrates tested.

INTRODUCTION

A great deal of work has been done on reagents which, when present in very low concentrations, inhibit enzyme-catalyzed reactions; however, very little information is available on the effect of large concentrations of relatively innocuous reagents such as alcohols, carbohydrates, surfactants, thickeners, etc.

Although there have been no comprehensive studies of the effect of these organic compounds on enzyme catalyzed reactions, there are many isolated reports. Glycerol has been shown to be a stabilizer as well as an inhibitor of enzymic activity[1-4]. Alcohols such as methanol, ethanol, propanol and isopropanol inhibit some enzymes while they activate others[5-10]. 8 *M* urea denatures many enzymes, some reversibly while others retain their activity[11,12]. Mono- and disaccharides in moderate concentration, *i.e.*, 0.5 *M*, were reported to have marked inhibitory effects on the activity of pancreatic proteinase[1]; however, sucrose, fructose and glucose have an enhancing effect on chymotrypsin activity[13]. Sucrose may tend to increase or decrease the rate of denaturation of proteins[14]. Amino acids are reported as being both activators and inhibitors of enzyme reactions[1,15]. Other substances which may affect the stability

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of proteins or the activity of enzymes are guanidine hydrochloride[12], soaps and detergents[15, 18], heparin[17, 18], and certain organic acids[19].

The present work is concerned with the effect of relatively large concentrations of alcohols, urea, glycine, saccharin, surfactants, methyl celluloses and carbohydrates on the hydrolysis of starch by hog-pancreas α-amylase.

MATERIALS AND METHODS

Crystalline hog-pancreas α-amylase from Nutritional Biochemical Corporation was used in these studies. The substrate was reagent-grade soluble starch purchased from Mann Research Laboratories, Inc. The Tergitols were gifts from the Union Carbide Co.; the non-ionic surfactants were gifts from the Atlas Powder Co.; and the methyl celluloses were gifts from the Dow Chemical Co. All other compounds used were chemically pure and reagent grade.

The concentration of the enzyme was determined spectrophotometrically using the value of $E_{1\,cm}^{1\%}$ at 280 mμ = 26 (ref. 20) and a molecular weight of 45000 for calculating the molar concentration[21]. The activity of the enzyme was 11500 moles maltose produced per minute per mole enzyme by the modified method of SCHALES AND SCHALES[22] and under the conditions described below.

The modified method of SCHALES AND SCHALES[22] was used to determine the effect of the alcohols, urea, glycine and saccharin on the activity of α-amylase on starch. The reaction mixture, at pH 7.3, contained 0.05 M Tris–HCl, 0.50% soluble starch, $1.84 \cdot 10^{-9}$ M crystalline hog-pancreas α-amylase and suitable amounts of the substances being tested. Enzyme was added to initiate reaction. At appropriate time intervals (0–28 min), samples were removed from the reaction vessel, maintained at 25.0 ± 0.1°, and pipetted into the alkaline ferricyanide solution to stop the reaction and for determination of the amount of reducing groups produced. The assay was standardized against maltose. Reducing-group liberation was linear with time under these conditions.

The iodine assay method of SCHWIMMER[23] was used to determine the effect of saccharin, the carbohydrates, the surfactants and the methyl celluloses on the activity of α-amylase. The reaction mixture, at pH 7.3, contained 0.05% soluble starch, 0.05 M Tris–HCl buffer, $2.14 \cdot 10^{-9}$ M crystalline hog-pancreas α-amylase and suitable amounts of the substances being tested. Enzyme was added to initiate reaction. The iodine reagent was 0.0035 M with respect to iodine and 0.25 M with respect to potassium iodide. At various time intervals (0–28 min), a 0.50-ml aliquot of the reaction mixture, maintained at 25.0 ± 0.1°, was removed and added to 2.0 ml of the iodine reagent, the volume adjusted to 5.0 ml with water and the absorption read at 500 mμ in a DU spectrophotometer. Reaction was linear with time under these conditions and independent of substrate concentration.

RESULTS AND DISCUSSION

Effect of alcohols

Fig. 1 shows that the amount of inhibition of the activity of α-amylase on starch by the alcohols tested is directly proportional to the molar concentrations of these compounds. The alcohols inhibit in the decreasing order of ethanol (0–3.3 M),

isopropanol, propylene glycol, glycerol, ethylene glycol and methanol. It was found that the slope of the line for ethanol inhibition changed in the region of 3–4 M. The reason for this is not clear. If it were due to a precipitation of the substrate or enzyme or a denaturation of the enzyme the curve should bend downward rather than upward. It is surprising that some of these reagents did not produce a sudden loss in activity due to enzyme precipitation or denaturation. Apparently the enzyme did not precipitate because it was too dilute ($1.84 \cdot 10^{-9}$ M) and also because it was

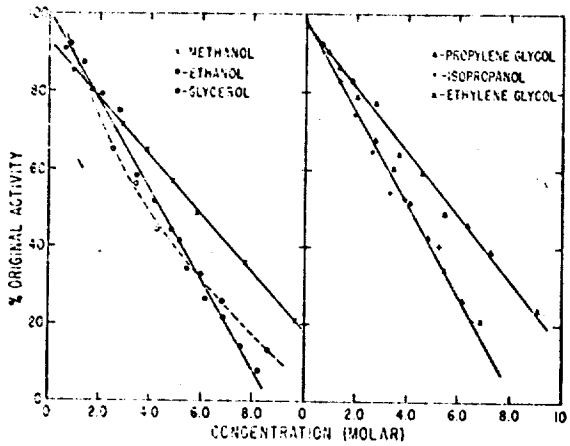

Fig. 1. Effect of alcohols on the activity of α-amylase.

stabilized by the starch. Both ethanol and isopropanol did produce some turbidity in the reaction mixtures at the higher concentrations but this did not appear to change the nature of the reaction as the slopes were constant at these high concentrations.

The rate constants at 1.0 M of the compounds tested have been calculated. In Fig. 2 the logarithm of the ratio of rate constant k at 1.0 M alcohol additive to rate constant k_0 in the absence of alcohol additive *vs.* the reciprocal of dielectric constant, D, is plotted. From the data for the limited number of compounds studied it would appear that there is a linear relationship between the effect of the alcohol on the dielectric constant of water and its effect on the α-amylase–starch system. Again, ethanol appears to have a peculiar effect. Values reported in Fig. 2 for ethanol at 4.0–6.7 M are extrapolated from slope of line (per cent inhibition *vs.* concentration) in this region.

The effect of ethanol, methanol, glycerol, isopropanol, and ethylene glycol on the dielectric constant of water at 25° was calculated from the data of ÅKERLÖF[21]. The effect of propylene glycol on the dielectric constant of water was estimated from its dielectric constant of 29.46, reported by TIMMERMANS *et al.*[25] and its similarity to ethylene glycol.

HAMMOND AND GUTFREUND[6] correlated the effect of isopropanol on ficin activity with its effect on the dielectric constant of water. They found that v_{max} decreased as the dielectric constant decreased while K_m was increased. In a study of the effect

of methanol and isopropanol on the papain-catalyzed hydrolysis of α-benzoyl-L-argininamide STOCKELL AND SMITH[5] reported that both alcohols increased K_m; however, k_o remained essentially constant at 38° at pH 5.2 or 6.9, up to 50% methanol, but decreased in the presence of 30% isopropanol. They concluded that there is apparently no simple consistent effect of dielectric strength on either K_m or k_o under the experimental conditions used. Observations on the effect of dielectric constant on the activity of trypsin and α-chymotrypsin by CASTAÑEDA-AGULLÓ AND DEL CASTILLO[7] indicated that at pH 7.5 trypsin is positively charged and α-chymotrypsin is negatively charged[8]. Further study on the combined effects of dielectric

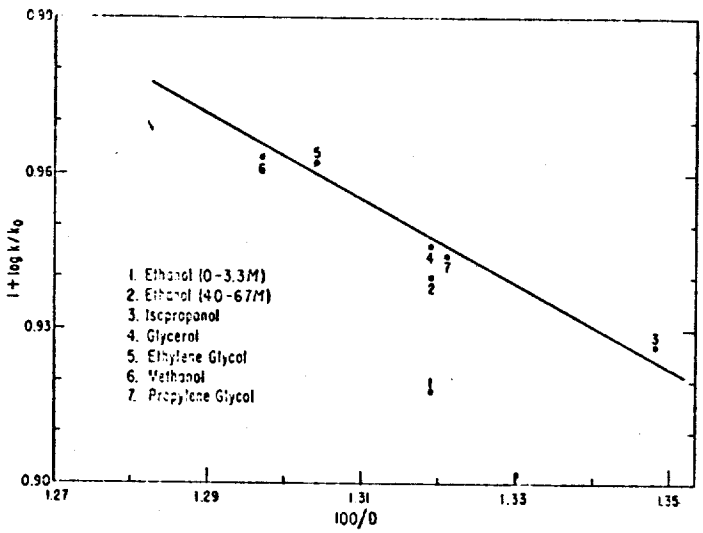

Fig. 2. Relationship between the effect of alcohols on the dielectric constant of water and the activity of α-amylase.

constant and pH indicated that the charge on α-chymotrypsin is reversed around pH 6.6 (ref. 26).

The following equation has been calculated by AMIS[27] for the reaction of ions and dipolar molecules:

$$\ln k_D' = \ln k_{\infty}' + \frac{ZC\mu}{DkTr^2} \qquad (1)$$

Where k_D' and k_{∞} are the rate constants at dielectric strength D and D_{∞}, Z is the valence of the ion, C is the electronic charge, μ is the dipole moment of the molecule, D is the dielectric constant, k is the Boltzmann constant, T is the absolute temperature and r is the reacting distance between the two molecules. This equation predicts that if D increases the rate should decrease for a positive ion and *vice versa* for a negative ion. If, on the other hand, D should decrease the opposite effect would be true. From Fig. 2 it is seen that as D increases the reaction rate for α-amylase on starch increases. According to the AMIS equation the active form of α-amylase at pH 7.3 would appear to be the negative ion. The same would appear to be true of ficin at pH 6.3 (ref. 6) and α-chymotrypsin at pH 7.8 (ref. 8). On the other hand, the active form of trypsin

appears to be the positive ion[7]. This may explain why alcohols increase the activity of trypsin on many synthetic substrates and decrease the activity of α-amylase on starch, and ficin and α-chymotrypsin on synthetic substrates.

Effect of urea, glycine and saccharin

If the hypothesis of a correlation between the effect of a substance on the dielectric constant of water and the activity of α-amylase is valid, then substances which increase the dielectric constant of water should increase the activity of α-amylase. Urea, glycine and saccharin (up to at least 0.021 *M*) are reported to increase the dielectric constant of water[28,29]. As shown by the data in Table I these compounds do not significantly increase the activity of α-amylase. There is inhibition of activity at the higher concentrations as would be expected. While these compounds do not increase the activity of α-amylase as expected, they do not refute the hypothesis advanced.

TABLE I

EFFECT OF UREA, GLYCINE AND SACCHARIN ON THE α-AMYLASE–STARCH SYSTEM

Urea		Glycine		Saccharin	
M	Activity*	*M*	Activity*	*M*	Activity*
0.5	101.0 ± 1.3	0.067	102.0	0.004	102.0
1.0	101.7 ± 3.9	0.13	103.9 ± 2.7	0.010	95.0
1.5	101.6 ± 5.4	0.20	97.8 ± 0.9	0.021	100.1
2.0	101.2 ± 4.8	0.27	94.3 ± 2.5	0.042	101.6
2.5	95.1 ± 4.1	0.33	75.8 ± 4.1	0.084	100.5
3.0	90.1 ± 3.9	0.40	70	0.142**	102.8
3.5	77.9 ± 3.2	0.53	72	0.284	97.4
4.0	72.2 ± 5.8			0.426	81.4
5.0	39.9 ± 3.6			0.568	68.0
6.0	21.1 ± 4.4			0.710	68.0

* Expressed as percentage of the activity in the absence of additive.

** Activities from 0.142 through 0.710 *M* saccharin determined by iodine assay method of SCHWIMMER, the others were determined by alkaline ferricyanide method of SCHALES AND SCHALES.

Effect of surfactants and methyl celluloses

The results of the effect of a large number of surfactants on the activity of α-amylase are presented in Table II. These were tested at the relatively high concentrations of 1% for screening purposes. Both anionic and non-ionic surfactants, ranging in hydrophilic–lipophilic balance from 2 to 18 have very little effect upon the activity. Tween 80 was tested over a wide concentration range. There is an increase in inhibition as a function of concentration, but this is rather small, being only 20% at 1.2% Tween 80. It is surprising to find such a small effect of these surfactants on activity. One might expect that the surfactant in aqueous solution would orient itself at the surface of the enzyme and thus cause interference with the catalysis.

Perhaps, at these concentrations, the major portion of the surfactant goes into micellular formation.

In the category of anionic surfactants, some of the monocarboxylic acids were tested. The effects of the potassium salts of acetic, butyric, propionic and octanoic acids at 0.5 M on activity are shown in Table II. Only the higher fatty acids give appreciable inhibition. Octanoate was examined in greater detail (Table II). It is

TABLE II

EFFECT OF SURFACTANTS AND METHYL CELLULOSES ON THE α-AMYLASE–STARCH SYSTEM

*A. Anionic Compounds**

Chemical name (trade name)	*Concn.*	*Original activity (%)*
Sodium 2-ethyl hexyl sulfate (Tergitol-08)	1% (v/v)	96.0 ± 0.0
Sodium tetradecyl sulfate (Tergitol-4)	1% (v/v)	92.6 ± 3.7
Sodium di(2-ethyl hexyl)phosphate (Tergitol p-28)	1% (v/v)	95.5 ± 6.7
Sodium heptadecyl sulfate (Tergitol-7)	1% (v/v)	96.0
Potassium acetate	0.5 M	76
Potassium butyrate	0.5 M	74
Potassium propionate	0.5 M	85
Potassium octanoate	$5 \cdot 10^{-5}$ M	72
	$2 \cdot 10^{-4}$ M	75
	$1 \cdot 10^{-3}$ M	76.5
	$5 \cdot 10^{-3}$ M	101
	$2 \cdot 10^{-2}$ M	91.5
	$1 \cdot 10^{-1}$ M	83
	$5 \cdot 10^{-1}$ M	49
Potasium laurate	$5 \cdot 10^{-6}$ M	100
	$2 \cdot 10^{-5}$ M	100
	$1 \cdot 10^{-4}$ M	85.5
	$1 \cdot 10^{-2}$ M	55

*B. Non-ionic compounds***

Chemical name (trade name)	*HLB****	*Original activity (%)§*
Polyoxyethylene lauryl ether (Brij)	9.5	98.7 ± 5.4
Polyoxyethylene oxypropylene stearate (G-2162)	15.7	85.5 ± 4.0
(G-2800)		84.6
(G-3608)		92.0 ± 1.3
Polyoxyethylene stearate (Myrj-45)	11.1	81.4 ± 0.7
Polyoxyethylene stearate (Myrj-52)	16.9	94.6 ± 4.7
Polyoxyethylene stearate (Myrj-53)	17.9	80.0 ± 2.0
Glyceryl mannitan laurate (NNO)	7.1	80.0 ± 4.0
Sorbitan monolaurate (Span-20)	8.6	90.0 ± 13.0
Sorbitan trioleate (Span-85)	1.8	91.4 ± 17.3
Polyoxyethylene sorbitan monopalmitate (Tween-40)	15.6	84.0 ± 4.0
Polyoxyethylene sorbitan monopalmitate (Tween-60)	14.9	87.5 ± 8.0
Polyoxyethylene sorbitan monostearate (Tween-61)	9.6	70.0 ± 8.0
Polyoxyethylene sorbitan tristearate (Tween-65)	10.5	85.5 ± 5.4
Polyoxyethylene sorbitan monooleate (Tween-80)	15.0	90.0 ± 1.3 (0.040)§§
		80.0 ± 6.0 (0.120)
		84.0 ± 1.3 (0.200)
		81.4 ± 2.7 (0.600)
		81.4 ± 5.3 (1.00)
		80.7 ± 0.7 (1.20)

As pointed out in the section on the effect of alcohols there appears to be a positive correlation between their effect on the dielectric constant of water and their effect on the activity of α-amylase. It would be nice if such a correlation could be extended to explain the effect of carbohydrates on α-amylase activity.

With the exception of glucose and sucrose, very little information is available on the effect of carbohydrates on the dielectric constant of water. MALMBURG AND MARVOTT[33] have determined the dielectric constants of aqueous solutions of glucose and sucrose and ÅKERLÖF[21] has reported the dielectric constants of aqueous *d*-

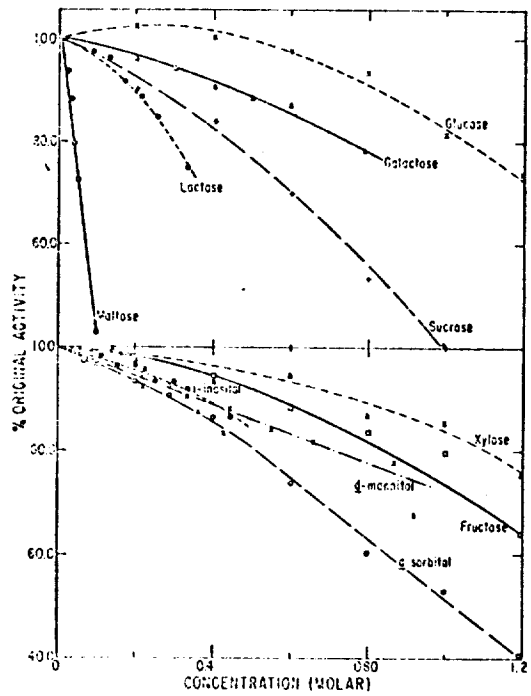

Fig. 3. Effect of carbohydrates on the activity of α-amylase.

mannitol. Other workers have reported that the dielectric constants of aqueous solutions of starch, maltose and glucose are identical[31].

The dielectric constants of aqueous solutions of glucose, sucrose and *d*-mannitol are not identical (for 10 wt.%: glucose, 76.14; sucrose, 76.19; and *d*-mannitol, 77.12) but the differences among them are not great enough to explain the differences in the inhibitory properties of the three compounds. Their effect on the dielectric constant of water decreases in the order glucose, sucrose and *d*-mannitol, yet their inhibitory abilities decrease in the order sucrose, *d*-mannitol and glucose. FARBER AND WYNNE[1] reported the inhibitory effects of glucose, galactose, fructose, sucrose, maltose and lactose on pancreatic proteinase to be largely independent of the configurations of the sugars and to be related to a disturbance of the effective water concentration of the digestion mixture. They found all these sugars to be equally effective in inhibiting pancreatic proteinase activity.

It does not appear that the effect of the carbohydrates on the dielectric constant of water can explain the differences in the inhibitory properties of these compounds on α-amylase activity. Despite SCHWIMMER's finding that maltose and limit dextrins were non-competitive inhibitors of malt α-amylase[32] it appears from the data in Fig. 3 that the structures of the compounds are important factors in their effect on α-amylase activity. Maltose is several times more inhibitory than is sucrose or lactose. The calculated I_{50} values for these compounds are: maltose, 0.08 M; lactose (extrapolated), 0.53 M; and sucrose, 0.86 M. If structure were one of the important factors determining inhibition, maltose would be expected to be the best inhibitor; it differs only in size from the substrate. The lactose molecule differs from maltose in two important aspects: (a) the linkage of the glucose moiety to the galactose is of the β-configuration while the glucose moiety is linked to the other glucose by an α-linkage in maltose and (b) the position of the hydroxyl group on C-4 on one of the moieties. It is suggested that the first one of these is the more important; however, the position of the hydroxyl group on C-4 has some influence as shown by the difference between glucose and galactose. Sucrose differs in several important aspects from maltose: (a) one glucose is replaced by fructose, (b) the linkage is $1 \rightarrow 2$ between glucose and fructose, (c) the fructose moiety exists as the furanose ring rather than the pyranose ring as does the glucose moiety and (d) because of (b), sucrose is a non-reducing sugar. On the basis of the data on the sugar alcohols, *d*-sorbitol and *d*-mannitol, it is suggested that (d) does not play an important function in explaining the difference between the inhibitory ability of sucrose and maltose. It should be noted that sucrose is approx. 4 times more inhibitory than ethanol, the most inhibitory of the other alcohols tested.

The inhibitory ability of the hexose monosaccharides decreases in the order galactose, fructose and glucose. These compounds are much less inhibitory than are the disaccharides. It is interesting to note that the position of the hydroxyl group on C-4 makes a difference in the degree of inhibition (compare glucose and galactose). Fructose is more inhibitory than is glucose. It is suggested that this difference is due to the primary alcohol group (C-1) in fructose rather than the keto group being more inhibitory than the aldehyde group. This is partially inferred from the data on the sugar alcohols. Fructose exists predominantly in a furanose rather than a pyranose ring.

It does not appear that increasing the carbon chain by one CHOH group increases the degree of inhibition (compare xylose and glucose).

The sugar alcohols are more inhibitory than the corresponding parent sugars. *d*-Sorbitol is more inhibitory than *d*-mannitol and *i*-inositol which are about equally inhibitory. It is interesting to note that the replacement of the aldehyde group of glucose by a primary alcohol group has greatly increased the inhibitory properties. The I_{50} value for *d*-sorbitol is 1.03 M, for glucose it is approx. 1.8 M.

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The Oxidation of Mannitol.* (20826)

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The value of mannitol, an isomer of sorbitol, as an adjunct to the diabetic diet has recently been reviewed by Olmsted(1). It was concluded that mannitol could not be of much importance as an adjunct to the diabetic diet, since 80% or more of the mannitol administered is excreted in the urine. These conclusions were apparently based on the data obtained by IV administration(2-6). Mannitol has also been known to form significant amounts of liver glycogen after oral administration(2,7), and under these conditions it is unlikely that mannitol can be excreted in the amounts indicated above. However, no posi-

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TABLE I. Recovery of C^{14} after Administration of R.A. Mannitol.

	Hours of CO_2 collection	Experiment No. 1	2	3	4	5	6	7
		I.P. injection		Oral feeding				Spleen injected
% administered radioactivity recovered in expired CO_2 (accumulative)	3	.4	0	3.5	.2	.5	.3	.5
	6	.8	.5	21	4	5	4	70
	9	1.1	1.7	35	11	13	12	71
	12	1.3	2.1	38	29	25	23	71
	18	1.6	2.4	46	42	51	47	71
	24	2.1	3.2	48	46	63	58	71
Urine		97	77	13	11	8	8	5
Stool						12	12	1
Total recovery		99	80	61	57	83	78	78
mg mannitol/admin.		12	180	2	240	240	240	2
Animal wt. g		183	150	183	155	190	190	190

tive conclusions regarding the metabolic utilization of a substance can be obtained from liver glycogen data; for this, labeled material must be used. The differences in regard to the utilization of the sugar alcohol may be due to the technics employed in the administration. In order to clarify the overall metabolic fate of mannitol, we have studied its rate of oxidation and excretion after oral and IP administration. Mannitol uniformly labeled with C^{14} was employed.

Methods. Preparation of radioactive mannitol. Mannitol C^{14} was prepared by the catalytic reduction of C^{14} uniformly labeled glucose with Raney nickel in alkaline solution. The procedure is similar to that used for the preparation of sorbitol(8). The mannitol was purified by a series of crystallizations from water and finally from aqueous ethyl alcohol. The product was identified as mannitol by its M.P. (162-163°C) and mixed M.P. (163-164°C) with an authentic mannitol sample (164°C). In addition, the specific activity of a mixture of labeled mannitol and carrier mannitol did not change on repeated crystallization. *Animal treatment.* Non-fasted male albino rats were used. The mannitol was given intraperitoneally, orally, and in one experiment directly into the portal system by way of the spleen. For the latter experiment the spleen was exteriorized the day before the experiment. The methods used for collecting the expired CO_2 and for measurement of radioactivity are the same as that used for sorbitol(8).

Results and discussion. The data given in Table I show that mannitol, when given parenterally, is excreted to a large extent in the urine, which is in agreement with numerous previous reports. The high urinary excretion after parenteral injection is balanced by a low rate of oxidation of about 2-3%. On the other hand, oral feeding of the sugar alcohol leads to oxidation values of about 50% of the injected radioactivity. These divergent results regarding mannitol utilization, obtained by the two methods of administration, indicate either that the mannitol is oxidized, initially at least, only by the liver or that mannitol is acted on by the intestinal flora before absorption from the intestinal lumen. In the latter case the metabolic utilization of fed mannitol is due to the absorption of conversion products of bacterial action. In order to differentiate between these two possibilities, mannitol was injected directly into the portal system by way of the spleen. In this experiment (Exp. 7), 71% of the mannitol was oxidized, indicating that the liver is the most likely site of mannitol oxidation and that bacterial action in the intestinal tract does not play a measurable role in the oxidation. One must assume from these results that mannitol cannot be oxidized by tissues other than the liver and also that the kidneys have a low renal threshold for the sugar alcohol. In parenteral injection a large fraction of the material is subjected largely to the excretory action of the kidneys before it is made available to the liver, and it is rapidly excreted, due to the low renal threshold, before it can be oxidized. In oral feed-

ing practically all of the material must pass first through the liver before it is put into the general circulation: hence the oxidation values of 48-63% are obtained. It is of interest to note that Dominguez, Corcoran and Page(4) in evaluating the use of mannitol in renal function concluded from their studies that the utilization of mannitol in humans, although small after IV injection, is presumably metabolic.

Although metabolites are routinely given parenterally in many experimental studies in order to by-pass the intestine, it is obvious from the results reported here that data obtained from parenteral injection should be examined with comparable data obtained after oral administration for a true evaluation of the metabolic pathway.

Summary. Radioactive mannitol has been administered to rats with the following results: 1. When given orally, about 50% of the mannitol carbon is recovered in the expired CO_2 in 12 hours. 2. With intraperitoneal injection, the mannitol is largely recovered in urine with only 2-3% of the mannitol carbon recovered in the expired CO_2. 3. By injection directly into the portal system by way of the spleen, oxidation comparable to or greater than that obtained by oral administration is obtained. It is concluded from these results that mannitol can be oxidized by the body if it is administered by a route that makes it first available to the liver, as in oral feedings.

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THE SPECIFICITY OF SUGAR TRANSPORT BY HAMSTER INTESTINE*

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Active transport of sugars by the small intestine has been convincingly demonstrated by the finding that transport takes place against a concentration gradient. This has been shown to occur, *in vivo*, with the compounds, D-glucose[1], and 3-O-methyl-D-glucose[2], and, *in vitro*, by means of recently developed techniques, with D-glucose[3], 3-O-methyl-D-glucose[4], D-galactose[5], and with 1-deoxy-D-glucose and 6-deoxy-D-glucose[6]. Certain other compounds, notably D-mannose and D-fructose, have been found not to be actively transported by intestinal mucosa, *in vitro*[4]. Our interest in extending this work is to define more completely the limits of specificity of the transport process. If all the sugars are transported by the same process, a knowledge of these limits would indicate which functional groups of the sugar molecule may enter into reaction during active transport.

MATERIALS AND METHODS

The technique for use of sac of hamster intestine as described by WILSON AND WISEMAN[7] were followed. Incubation was for [illegible] at 37° in bicarbonate saline under an atmosphere of 95% O_2 and 5% CO_2. Analysis of the various compounds was accomplished by the method described in the references cited: reducing sugars, 2-deoxy-D-glucose[9], glucosamine[10], 6-deoxyhexoses[11], polyhydric alcohols and the [illegible] compounds[12]. The 1-deoxy compounds (also known as 1,5-sorbitan and 1,5-mannitan) were obtained from Dr. N. K. RICHTMYER, 6-deoxy-D-glucose from Dr. E. HARDEGGER, 6-deoxy-D-galactose from Dr. D. TURK, 7-deoxy-D-glucoheptose from Dr. J. SOWDEN, and D-anose from Dr. H. S. ISBELL. The remaining compounds were obtained commercially.

EXPERIMENTAL

The concentration gradients developed by the intestinal sac preparations with four different sugars are shown in Fig. 1. Glucose and 7-deoxyglucoheptose were transported against large and probably comparable gradients. The gradient for glucose cannot be estimated in this particular experiment owing to the small amount of sugar remaining on the mucosal side. The gradient for 7-deoxyglucoheptose is more than fifty-fold. It is noteworthy that 7-deoxyglucoheptose is not metabolized by intestinal mucosa. For comparison with these sugars, an example is given of a sugar that is less well transported, 6-deoxy-D-galactose, and of one not transported, [illegible]. In the experiment with D-glucosamine there was observed a fall in the

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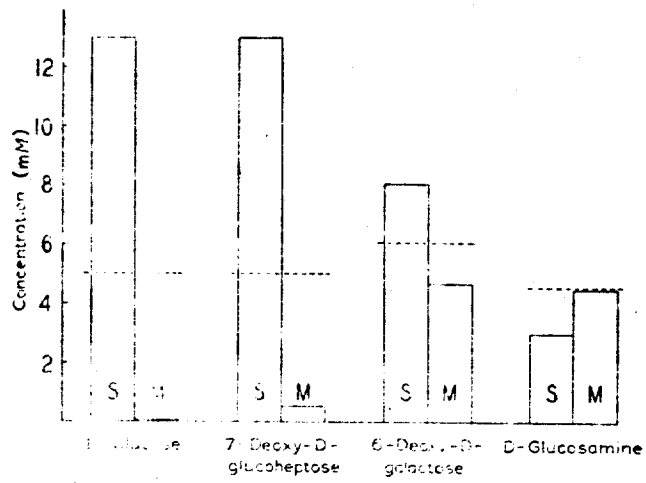

Fig. [illegible] Concentration gradient developed across the wall of everted sacs of hamster intestine. Conditions as given in the text. The initial concentration of sugar on each side was the same, as indicated by the dotted line. S = serosal side, M = mucosal side.

serosal concentration. This [illegible] be accounted for by the intake of fluid (there was an increase in serosal volume from 1.0 to 1.2 ml) and by the diffusion of sugar into the tissue where it is not assayed.

Table I lists the sugars and related compounds which have been found to be transported across the wall of the hamster intestine against a concentration gradient. The rate measurements [illegible] in the table were made at a single concentration for each sugar and comparison of rates should be made with regard for the possibility that the rates are not maximal. The effect of concentration on the rate of transport has been evaluated for only [illegible] sugars. Fisher and Parsons found an apparent Michaelis constant of [illegible] M for glucose[illegible] and of $3.5 \cdot 10^{-2}$ M for galactose[5] transport by rat intestine [illegible]. Apparent Michaelis constants of $4 \cdot 10^{-4}$ M and

TABLE I

SUGARS [illegible] TRANSPORTED AGAINST A CONCENTRATION GRADIENT BY HAMSTER INTESTINE

[illegible]	[illegible]	Initial concentration of compound on [illegible]
	[illegible]	moles/l
D-glucose	12.[illegible]	0.005
3-methyl-D-glucose	7	0.007
6-deoxy-D-glucose	2.0	0.004
7-deoxy-D-glucoheptose*	4.5	0.005
D-glucoheptulose*	0.7	0.005
D-galactose	12.3	0.005
6-deoxy-D-galactose*	1.2	0.006
D-allose*	1.0	0.005
1-deoxy-D-glucose	1.3	0.001

* Substance not previously tested.

[illegible] p. 32.

[illegible] M have been found for the transport by hamster intestine of 6-deoxy-D-[illegible] and 1-deoxy-D-glucose respectively, using a new technique[11].

Table II lists those compounds tested with the everted sac method which were not transported against a concentration gradient.

TABLE II

GALACTOSE-RELATED COMPOUNDS NOT TRANSPORTED AGAINST A CONCENTRATION GRADIENT BY HAMSTER INTESTINE

[illegible] sugars	D-[illegible]mannoheptulose*
[illegible] sugars	D-[illegible]ketose, [illegible]-ribose, D-mannose, D-glucosamine*, N-acetyl-D-glucosamine*
	6-deoxy-D-glucose*, L-galactose, 6-deoxy-L-mannose*, 6-deoxy-L-galactose
[illegible] sugars	D-xylose, D-ribose, L-arabinose, D-arabinose*
[illegible]	[illegible]-deoxy-D-[illegible]mannose, L-sorbitan, mannitol*, sorbitol*, glycerol*

* [illegible] not previously tested.

In all fifteen sugars and related compounds not previously studied have been tested for active transport by the everted sac technique. The new information on specificity so obtained which was not implicit in former studies is: (1) that ketoses can be transported as demonstrated by the transport of D-glucoheptulose, and (2) that certain groups substituted at carbon atoms 1 or 6 of glucose do not prevent active transport.

A generalization with respect to the specificity of the transport process may be made. Those compounds were transported which possess a D-pyranose structure, a methyl or substituted methyl group at carbon five of this structure and a hydroxyl group in the glucose configuration at carbon two.

SUMMARY

[illegible] specificity [illegible] against a concentration gradient *in vitro* has been tested with [illegible] sugars and related [illegible]. Those compounds were transported which possess a D-[illegible] structure, a methyl or substituted methyl group at carbon five of this structure, and a [illegible] hydroxyl group in the glucose configuration at carbon two.

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Competition for transport between methyldopa and other amino acids in rat gut loops[1]

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YOUNG, J. A., AND K. D. G. EDWARDS. *Competition for transport between methyldopa and other amino acids in rat gut loops.* Am. J. Physiol. 210(5): 1130–1136. 1966.—Transport of L- and D-methyldopa and their inhibition of natural amino acid transport was studied in situ, with rat jejunal loops. L-Methyldopa was absorbed very slowly although significantly more than the D isomer. In a latin-square experiment, the effects of L- and D-methyldopa and mannitol (as a poorly absorbed control) on histidine transport were compared. Mannitol significantly reduced histidine absorption, indicating sensitivity of histidine transport to adverse osmotic gradients. D-Methyldopa reduced histidine absorption but not significantly more than mannitol. L-Methyldopa reduced histidine absorption significantly more than mannitol and D-methyldopa. It was thought that L-methyldopa inhibited amino acid transport mechanism(s) having specificity for L configurations. Mannitol and L- and D-methyldopa were all without effect on glucose absorption, suggesting that methyldopa had no nonspecific effects on gut metabolism. Further studies demonstrated inhibition of transport of glutamic acid, glycine, lysine, taurine, and phenylalanine, but not methionine or proline by L-methyldopa, suggesting that it had a more general inhibitory action on amino acid transport than competitive inhibition of one transport mechanism. Incidental evidence suggested that the amino compound, taurine, was absorbed by a process other than simple diffusion.

intestinal amino acid transport; inhibition of intestinal amino acid transport; methyldopa inhibition of intestinal amino acid transport; methyldopa transport by intestine; intestinal transport of methyldopa; taurine absorption by intestine

IN A PREVIOUS INVESTIGATION it was shown that the synthetic L-alpha amino acid, methyldopa,[2] was reabsorbed from the renal tubule by a mechanism for which it had a very low affinity, probably located in the proximal convolution. In addition, the drug was found to be capable of increasing the clearance of histidine over a wide range of plasma histidine concentrations (1–10 μmoles/ml). Stop-flow analysis demonstrated that methyldopa inhibited the transport mechanism for histidine which was located in the proximal tubule (43). Similarly, intestinal absorption of L-methyldopa, although only slight, was greater than for the D isomer,[2] suggesting that the intestinal transport of methyldopa involved more than a mechanism of simple diffusion (40).

Intestinal and renal amino acid transport systems have previously been reported to resemble one another very closely. Thus, in humans suffering from the genetically determined diseases of renal amino acid transport, cystinuria and Hartnup disease, it has been found that similar disorders exist also in the intestinal amino acid transport systems (18, 19). Studies in experimental animals have also shown that intestinal amino acid transport resembles that seen in the kidneys in showing specificity for L forms of amino acids (3, 7, 13, 14, 22) and in possessing separate transport systems for various groups of amino acids. Thus, separate transport systems have been described for the di-amino acids (3, 7, 9), for the neutral amino acids (3, 7, 9, 13, 23), and for the imino acids, and possibly glycine (10, 20, 21).

L-Methyldopa is given orally to humans for the treatment of hypertension in doses of up to 4 g/day. Because this was observed to be associated with a mild fall in serum albumin concentrations (6), and in view of the known action of methyldopa on renal amino acid transport (43), it was thought possible that the drug might also inhibit absorption of amino acids from the intestine. We decided to investigate the intestinal handling of L- and D-methyldopa in the rat and observe any effects that the drug might have on the intestinal transport of L-histidine and other naturally occurring amino acids.

The results obtained, using ligated loops of upper jejunum in situ, showed that methyldopa had a similar action on small intestine and kidney, partially inhibiting the transport of L-histidine. However, on screening a variety of amino acids in the intestinal preparation, the

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[2] Throughout this paper the terms methyldopa and L-methyldopa will be used synonomously to mean: L-alpha-methyl-3,4-dihydroxy-phenylalanine; the term D-methyldopa will be used to refer to the D, or unnatural, isomer of methyldopa.

data obtained suggested that methyldopa inhibited the absorption, not only of the amino acids sharing the neutral transport system, but also other amino acids. Thus absorption of glutamic acid and lysine also appeared to be affected by methyldopa. A preliminary report of part of this work has already been published (42).

MATERIALS AND METHODS

Male albino or hooded rats (200–250 g in wt), anesthetized with ether and im sodium pentobarbital (20 mg/kg), were submitted to tracheostomy. A laparotomy was then performed and a 5-cm segment of upper jejunum (and duodenum below the entrance of the common bile duct) was ligated and cannulated with polyethylene tubing at both ends. The intestinal loop was then returned to the abdomen and the laparotomy closed with the cannulas protruding.

Test solutions. Solutions for introduction into the intestinal loops were prepared from a solution containing 0.1% (w/v) phenolsulphonphthalein (PSP) dissolved in a peritoneal dialysis solution (Dianeal, Baxter-DHA, Australia) consisting essentially of an isotonic Ringer-lactate solution to which 15 g/liter glucose had been added. The osmolarity of this fluid was 375 milliosmols/liter.

For the first series of experiments with histidine three test solutions were prepared, *solution H* (containing 10 mmoles/liter histidine), *solution L* (10 mmoles/liter histidine and 100 mmoles/liter L-methyldopa), and *solution D* (10 mmoles/liter histidine and 100 mmoles/liter D-methyldopa). As the results from this series of experiments indicated that there might have been a nonspecific inhibitory action attributable to osmotic effects of L- and D-methyldopa, in addition to any specific effect on histidine transport, it was decided to perform further series of experiments with osmotic controls. For this purpose a fourth test solution, *solution M* (containing 10 mmoles/liter histidine and 100 mmoles/liter mannitol), was prepared.

In subsequent experiments with other naturally occurring amino acids (alanine, lysine, glycine, taurine, glutamic acid, phenylalanine, methionine, and proline) only two test solutions were prepared, *solutions M* and *L*.

In the experiments where L- and D-methyldopa absorption was studied a solution similar to *M* was prepared, except that 10 mmoles/liter of either L- or D-methyldopa was substituted for the naturally occurring amino acid.

Experimental procedure. The loop was carefully rinsed with the Dianeal-PSP solution and then with the appropriate test solution. After emptying the loop with air, 1 ml of the test solution was introduced into the loop with a calibrated tuberculin syringe, and the cannulas were then clamped. Exactly 15 min later the clamps were removed and the loop contents expelled with air into bottles containing 0.05 ml glacial acetic acid as a preservative. These solutions were stored frozen until required.

In the histidine experiments, three series, each of six animals, were studied. The first series received *solutions H, L,* and *D*; the second series, *H, L,* and *M*; and the third *H, D,* and *M*. The three test solutions were given to each rat in a different sequence, following a double latin-square design, thereby permitting elimination of any effects which may have been attributable to the sequence in which the treatment solutions were given. Thus, in the analysis of variance of the data, the total sums of squares were partitioned into variations due to differences between animals, to differences between test solutions, and to differences between the time periods in which the test solutions were given.

In subsequent investigations with amino acids other than histidine only two test solutions were used, *M* and *L*. In other respects the procedure followed was the same as for the histidine series.

Chemical methods. PSP was measured by adding 0.05 ml of the solution to 5 ml of a boric-acid/borate buffer, pH 10, and reading the optical density of the resulting purple-colored solution in a Beckman DU spectrophotometer at wavelength 560 mμ. Solutions containing methyldopa tended to darken slightly with time following the addition of the alkaline buffer; the error arising from this was of the order of 2% but it was avoided altogether by reading the optical densities of such solutions immediately after the addition of the test solution to the buffer.

Glucose in the intestinal loop samples containing methyldopa could not be measured by the ordinary, nonspecific, ferricyanide reduction method (11) or by a glucose oxidase method (12), since methyldopa was found to reduce ferricyanide and to take up the peroxide liberated by glucose oxidase. It was found adequate to separate glucose from methyldopa by ascending, one-dimensional paper chromatography using a technique of double development in butanol/acetic acid/water (40). Suitably placed glucose markers on each chromatogram were stained with a silver nitrate reagent (28). The regions of the chromatograms corresponding in position to the stained glucose markers were cut out and the glucose eluted into 2 ml water for the determination of the glucose concentration by the ferricyanide reduction method (11) adapted for use in a Technicon Auto-Analyzer.

Amino acids in intestinal loop solutions were usually separated by one-dimensional, ascending paper chromatography on 8-inch sheets of Whatman no. 4 filter paper developed twice in butanol/acetic acid/water (40, 43, 44). As alanine and methionine did not separate completely from methyldopa, nor phenylalanine from PSP, they were separated; instead, by high-voltage paper electrophoresis (44) in a buffer of pH 1.85. All amino acids except proline were stained on paper with ninhydrin and quantitated spectrophotometrically. In the working range (0.025–0.100 μmole amino acid on the chromatogram) the coefficient of variation was

as low as 2% for alanine and as high as 10% for methyldopa; histidine at 5.6% was intermediate (44). Recoveries performed with these synthetically prepared solutions always averaged 100%.

Proline and methionine were determined by use of the radioisotope-dilution technique. Traces of C^{14}-labeled amino acids introduced into the test solutions were counted in a Packard Tri-Carb liquid-scintillation spectrometer by a paper-strip technique described in a previous paper (43). Methionine concentrations determined in this way showed excellent agreement with ninhydrin colorimetric determinations done on the same solutions. Likewise proline, determined by the isatin color reaction (28), gave similar results.

Calculation of percent absorption of glucose and amino acids from intestinal loops. To estimate the percentage absorption of glucose and amino acids from loop samples the concentration of these substances in each sample was measured and then corrected for any water movement across the intestinal mucosa by reference to the change in the concentration of PSP according to the following formula:

$$\text{percent absorption of substance } X = 100 - 100\left[\frac{C_x \cdot I_{PSP}}{I_x \cdot C_{PSP}}\right]$$

where I_x and C_x are the initial and final concentrations, respectively, of substance X, and I_{PSP} and C_{PSP} are the initial and final concentrations, respectively, of PSP. A similar formula was used by Schanker et al. (26).

Initial concentrations were determined on aliquots of surplus test solution not utilized in each experiment. Following introduction into the gut loop this test fluid would be expected to be diluted by any residual fluid not previously expelled from the loop with air. The true initial amino acid concentration of the test fluid might therefore be different from that measured in the solution in vitro. The difference would, however, be unlikely to be very great since the gut loop was routinely rinsed out with 5 ml of whatever test solution was about to be introduced for the 15-min study period, just prior to the commencement of each experiment. In any case the residual volume of the rinsing fluid was always small.

Statistics. Analysis of variance, and partitioning of sums of squares by the method of orthogonal comparisons, were carried out according to methods described by Snedecor (30).

Chemicals. All naturally occurring amino acids were purchased in pure form from L. Light & Co., Colnbrook, England; methyldopa and its D isomer were the generous gifts of Mr. I. McD. Cooke, Merck, Sharp & Dohme (Australia) Pty. Ltd., and Drs. K. C. Mezey and R. Pingeon, Merck, Sharp & Dohme (International) and Merck, Sharp & Dohme Research Laboratories, Rahway, New Jersey; mannitol was purchased from May & Baker, Dagenham, England; phenolsulphonphthalein from British Drug Houses, Pty. Ltd.; Dianeal from Baxter-DHA, Australia; and C^{14}-labeled amino acids from The Radiochemical Centre, Amersham, Bucks, England.

RESULTS

PSP recovery. Six rats were each given two test solutions, L and M, for time periods of 15 min. After expulsion of the test solutions the loop was washed out with 50 ml Dianeal and the washings combined with the recovered test solution. This was then made up to 100 ml with pH 10 buffer and the PSP concentration determined spectrophotometrically; the total PSP recovered was then calculated.

The mean recovery value, expressed as a percentage of the amount of PSP originally present in the test solutions, from all 12 observations in the 6 animals was 99.4% ± 3.9% SD. Analysis of variance of the data showed that the PSP recoveries from the two test solutions were not significantly different; furthermore, there was no significant difference in the recoveries from different animals nor was there a significant difference between the recoveries from solutions which were given first to each animal and those given second.

Water shifts into the four test solutions. Figure 1A illustrates the change in PSP concentration observed in each test solution following the period of 15-min of exposure to the action of the intestinal mucosa. The change in concentration has been expressed as a percentage of the initial concentration, and can be taken as indicating the percent water gain (or loss) by the solution in the loop. The mean value for each group of experiments has been indicated on the figure.

From the analysis of variance of the data it was found that the difference between water gained by *solution H* and the other three test solutions was highly significant ($P < 0.001$); the difference between *solutions L* and *M* was probably significant ($P < 0.05$); and the difference between *solutions D* and *M* was not significant ($P > 0.05$). The difference between water gained by the solutions in the three successive time periods was also not significant ($P > 0.25$).

Glucose absorption from the four test solutions. Figure 1B illustrates the percent absorption of glucose, expressed as a percentage of the amount of glucose initially present, from the four test solutions. The data were derived from the same series of experiments as for Fig. 1A. Mean values have been indicated on the figure. Analysis of variance of the data failed to demonstrate any significant difference between the glucose absorption percentages from the four test solutions ($P > 0.25$). The glucose absorption from the solutions given in the three successive time periods also did not differ significantly ($P > 0.25$).

Histidine absorption from the four test solutions. Figure 2 illustrates the percent absorption of histidine from the four test solutions. The data were derived from the same series of experiments as for Figs. 1, *A* and *B*. The mean values for the percent absorption of histidine from each test solution have been included in the figure legend.

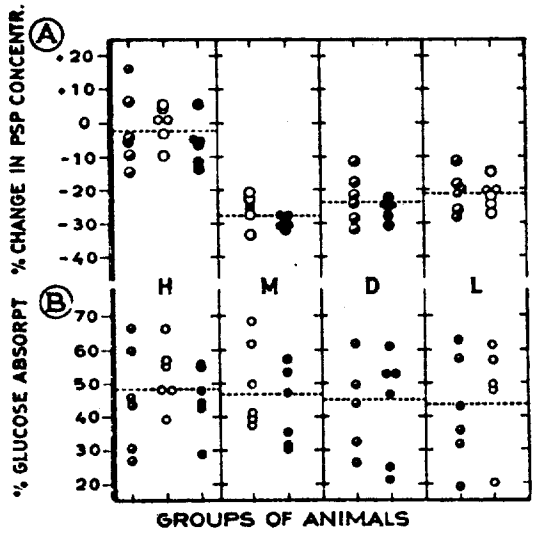

FIG. 1. *A:* Percent change in PSP concentration, an indication of transmucosal water shifts, in the four test solutions. *Solution H* contained histidine dissolved in Dianeal-PSP; *solutions L* and *D* contained, in addition to histidine, L- and D-methyldopa, respectively. *Solution M* contained histidine and mannitol. The symbols ⊖, O, and ● have been used to distinguish between the first, second, and third series of six animals studied. Horizontal broken lines indicate the mean values obtained from all animals given a particular test solution. *B:* Percent absorption of glucose from the four test solutions from the same experiments as are depicted in Fig. 1*A*. All four test solutions contained glucose, 15 g/liter. Symbols and horizontal broken lines are explained in legend to Fig. 1*A*.

From the analysis of variance (see Fig. 2) it was found that the absorption of histidine from *solution L* was significantly less than from the other three solutions ($P < 0.05$); similarly, the absorption of histidine from *solution H* was significantly greater than from the other three solutions ($P < 0.05$). Only the difference between histidine absorption from *solutions M* and *D* was not significant ($P > 0.25$). In all three series of experiments no significant difference in the histidine absorption in the three time periods was observed ($P > 0.25$). The *F* values and their significance levels derived from the analysis of variance and a partition of the treatment sums of squares by use of orthogonal comparisons have been indicated in Fig. 2.

Absorption of other amino acids from solutions M and L. The inhibitory effect of L-methyldopa on the absorption of a large range of naturally occurring amino acids is indicated in Table 1. Nine series of animals were studied. In each series histidine in *solutions M* and *L* was replaced by an equimolar amount of either phenylalanine, proline, methionine, alanine, glycine, lysine, taurine, or glutamic acid. In the case of phenylalanine, absorption was found to be complete from both test solutions after 15 min so the experiment was repeated with time periods of only 5 min duration. The mean percent absorption of each amino acid from *solutions M* and *L* is listed in Table 1. Also indicated in the table are the *F* values (and the probability levels) for the significance of the difference between the absorption of each amino acid from *solutions M* and *L*, derived from the analysis of variance. The difference was found to be significant at the 5% point or better in the case of alanine, glycine, lysine, taurine, glutamic acid, and phenylalanine (studied with 5-min time periods). In the case of proline and methionine the difference was regarded as not significant since the probability levels were only 10%.

In Table 1 the percentages of each amino acid absorbed by the intestinal loops from *solution M* have been ranked in descending order so as to indicate the relative rates of transport of each amino acid in a given period of time. Viewed in this way, the data suggested that the amino acids could be divided into a number of subgroups on the basis of their relative transport rates: *1*) Phenylalanine (100% absorption in 15 min); *2*) alanine, methionine, and proline (85% absorbed in 15 min); *3*) lysine, histidine, and glycine (50-60% absorption in 15 min); *4*) glutamic acid and taurine (21% absorption in 15 min); *5*) L-methyldopa (4.5% absorption in 15 min); *6*) D-methyldopa (no appreciable absorption in 15 min).

Absorption of L- and D-methyldopa. A series of experiments was performed in which rats were given two test solutions each, one containing L-methyldopa and the other, D-methyldopa. The mean absorption percentage for L-methyldopa after 15 min was 4.5%; there was no appreciable absorption of D-methyldopa. The difference between the absorption percentages for L- and D-methyldopa was found to be significant at the 5% point.

DISCUSSION

Water movements. Many workers have shown that the organic acid, PSP (phenol red), is not absorbed appreciably across the intestinal mucosa (26, 29, 31), although a small amount of absorption occurred when the dye was in contact with the intestinal mucosa for 3 hr (15). A number of workers have used it as an indicator of water movements in studies on the intestinal absorption of various solutes (24, 25, 27). The complete PSP recoveries obtained in the present experiments amply confirm the suitability of the dye for this purpose.

The large volume of water gained by *solutions M, L,* and *D,* in comparison with the amount gained by *solution H,* was presumably due to the osmotic effects of the poorly absorbed compounds, mannitol and L- and D-methyldopa. Thus, mannitol has been shown to be absorbed very slowly from the intestine by a passive process (37) and studies presented in the present paper demonstrate that L- and D-methyldopa also are very slowly and incompletely absorbed. *Solution H,* although hyperosmotic to plasma due to its content of glucose and histidine, was considerably less hypertonic than *solutions M, L,* and *D.* Since more than half of the glucose and histidine in *solution H* was absorbed during the 15-min study period it is not surprising that the mean net water gain by this solution was considerably less than

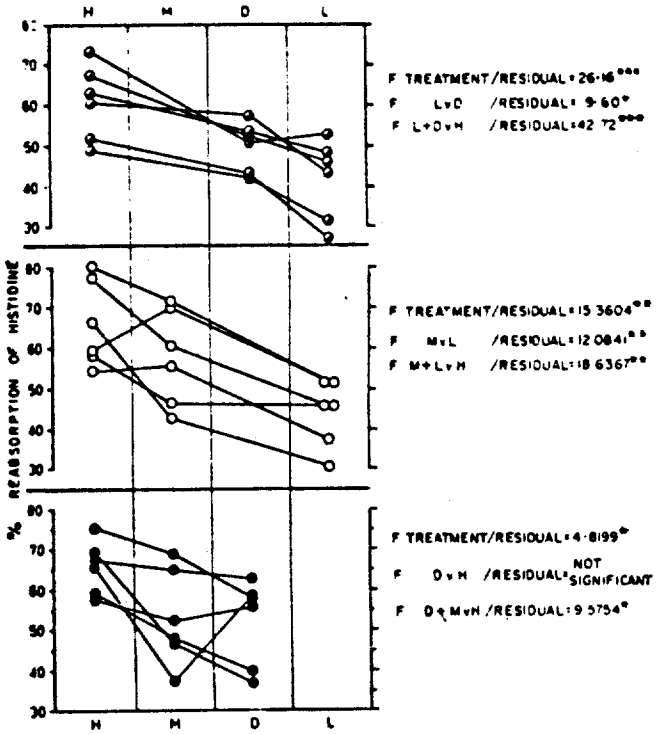

FIG. 2. Percent absorption of histidine from the four test solutions from the same experiments as are depicted in Fig. 1, *A* and *B*. All four test solutions contained 10 mmoles/liter histidine. Unlike the data presented in Fig. 1, however, the data from the three series of experiments have been plotted separately, enabling the three observations made in each animal to be connected up with straight lines. The symbols have the same meanings as in Fig. 1, *A* and *B*. The mean percent absorption from all animals given a particular test solution were: *solution H*, 64.4%; *M*, 55.5%; *D*, 50.9%; and *L*, 42.3%. The results of the analysis of variance, including a partitioning of each treatment sum of squares by means of orthogonal comparisons, have been indicated beside the data from each series of animals. * = Probably significant ($P < 0.05$); ** = significant ($P < 0.01$); *** = highly significant ($P < 0.001$).

by the solutions containing poorly absorbed compounds such as mannitol, in addition to glucose and histidine.

Glucose absorption. Verzar (32) was the first to demonstrate that glucose was actively absorbed by the intestinal mucosa. The present observations agree well with his data, suggesting that our loop preparations were functioning "normally."

The demonstration that L- and D-methyldopa and mannitol had no significant effect on glucose transport suggested that these drugs had no deleterious effects on general cell metabolism and that any effects which they had on amino acid transport were confined to a stage in the transport process not shared with the hexose transport process. As the glucose and amino acid absorption from the test solutions did not vary significantly in the different time periods the function of the loop preparations was regarded as stable for the duration of the experimental period.

Effect of L- and D-methyldopa and mannitol on absorption of histidine and other amino acids. The present studies demonstrated that histidine was rapidly absorbed from the intestinal loop. Histidine absorption has been studied in the rat and hamster intestine by a great many workers and found to be absorbed by an active transport mechanism available to many neutral amino acids (1–3, 8, 17, 39).

The fall in histidine absorption from 64.4 to 55.5% following the addition of the poorly absorbed mannitol to the test solution was thought to indicate an inability of the transport mechanism to operate efficiently against a large osmotic gradient. Such an osmotic sensitivity has also been observed in studies on the transport of D-methionine and alpha-amino-isobutyric acid by isolated, everted, intestinal loops of the rat (4, 14). Consequently, when assessing the action of L- and D-methyldopa on histidine transport by the intestine, it was found necessary to use *solution M*, containing mannitol, as a control in order to allow for the osmotic effect of L- or D-methyldopa on histidine transport.

The observation that histidine absorption from *solution L* (containing L-methyldopa) was significantly less than from *solution M* (containing mannitol) demonstrated that L-methyldopa had a definite inhibitory action on histidine absorption over and above any osmotic effect. Competition among neutral amino acids, including histidine, for absorption from the intestine of the rat and other animals has been very fully documented (3, 7, 13, 17, 22, 23, 38). In addition, although it has been shown that substitution of a methyl group for the alpha-hydrogen of an amino acid resulted in a diminished rate of intestinal transport, alpha-methyl amino acids were still found to be transported weakly by the intestine and to have an inhibitory action on the transport of other amino acids (17). It seemed possible that the inhibitory action of alpha-methyldopa on the intestinal transport of histidine was of a similar nature. However, it was not possible to state with certainty that the inhibition was competitive in nature, since Michaelis-Menten studies have not yet been performed.

The inhibitory effects of L-methyldopa on the transport of a large number of naturally occurring amino acids, including neutral, basic, and acidic amino acids, as well as the amino compound, taurine, was surprising since it is widely believed that the amino acid transport systems for basic and neutral amino acids are discrete (36). In order to assess the significance of this observation of a wide spectrum of activity for methyldopa, it would be necessary to compare its efficacy as an inhibitor with the efficacy of other amino acids under identical conditions. The wide spectrum of activity of methyldopa might then prove to be due to overlapping of intestinal amino acid transport systems. In this context it is interesting to note that Christensen (5) has described an amino acid transport system in Ehrlich ascites cells common to several neutral amino acids and also the basic amino acid, lysine; similarly, L-methionine has been shown to have an inhibitory action on the intestinal transport of L-lysine (36). Extensive overlapping of basic, neutral, and acidic amino acid transport systems has been observed in the dog kidney (33–35).

TABLE 1. *Percent absorption of various amino acids from solutions M and L by rat small intestinal loops*

L-Amino Acid	Amino Acid Absorbed, %		Signif. of Diff. Betwe L and M	
	Soln M	Soln L	F	P
Phenylalanine*	100.0	100.0		
Phenylalanine* (5 min)	47.2	43.4	12.19	<0.05
Alanine†	86.5	81.3	6.62	<0.05
Methionine†	85.0	81.1	4.27	$0.05 < P < 0.10$
Proline	84.5	80.0	4.33	$0.05 < P < 0.10$
Lysine	59.0	40.6	36.70	<0.001
Histidine	55.5	42.3	12.08	<0.01
Glycine	50.7	31.9	24.97	<0.01
Glutamic acid	22.2	3.8	87.17	<0.001
Taurine	20.9	16.0	12.20	<0.05
Methyldopa‡	4.5			

* Phenylalanine absorption was 100% for both solutions when placed in the intestinal loop for 15 min; the experiment was repeated with 5-min time periods. † Six animals only in these series. ‡ There was no appreciable absorption of D-methyldopa and the difference between the absorption of D- and L-methyldopa was significant at the 5% point.

Rather than an inhibitory effect on amino acid uptake at the mucosal surface of the intestinal cells there still remains the alternative possibility that methyldopa acted on amino acid absorption by interfering with amino acid transport within the cell or at the serosal surface. Transport mechanisms at these sites may be less specific (20).

Intestinal transport of L- and D-methyldopa and naturally occurring amino acids. Earlier studies (40) from this laboratory suggested that L-methyldopa was more readily absorbed from the intestine than its D isomer. These observations were confirmed more directly by the present studies. A number of experiments, performed by several different groups of workers (2, 3, 8, 17), have shown that D-amino acids were less well absorbed than L forms, although there is ample evidence to suggest that the unnatural isomers have some affinity for the normal amino acid transport mechanisms (3, 7, 10, 22). Although the present studies did not demonstrate any absorption of D-methyldopa in 15 min it still seemed possible that some absorption would have been demonstrable had the length of the time period been extended since studies with oral feeding of D-methyldopa in the rat have shown that a small amount of the D isomer was excreted in the urine following a large oral dose (40).

Since L-methyldopa was absorbed faster than the D isomer, and, in addition, could interfere with the absorption of naturally occurring amino acids, it seemed most probable that methyldopa was transported, albeit slowly, by the intestinal amino acid transport mechanism(s) normally available to naturally occurring amino acids. If this be so then methyldopa is one of the few drugs absorbed from the intestine by a mechanism other than simple diffusion (16).

With the exception of taurine, the intestinal transport of all the naturally occurring amino acids used in the present investigation has been studied by many other workers (see ref. 36 for a detailed review of the subject). The present studies confirm the generally held view that different naturally occurring amino acids are transported at different rates, with glycine and glutamic acid among the slowly transported and phenylalanine and methionine among the rapidly transported ones. In this paper the transport of another amino compound, structurally similar to the amino acids, taurine, has been studied. It was found that taurine was very slowly transported by the intestine (at about the same rate as glutamic acid) but, as it was transported at four times the rate of L-methyldopa and its transport could be inhibited by methyldopa, it seemed necessary to postulate that it was absorbed by some mechanism more specific than simple diffusion. Studies which we have performed in the rat kidney indicate that there may also be a specific transport mechanism available to taurine in the proximal tubule (41).

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