D-MANNITOL, ERYTHRITOL AND GLYCEROL IN BOVINE SEMEN*

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Summary. An investigation into the distribution of freely occurring polyhydric alcohols in normal bovine semen has revealed the presence of three previously unreported constituents, namely D-mannitol (17 mg/100 ml), erythritol (6.9 mg/100 ml) and glycerol (*circa* 2 mg/100 ml). These three polyols, together with inositol and sorbitol were characterized by the preparation of suitable derivatives. Where applicable, the physical constants of the crystalline materials and of their derivatives were determined.

INTRODUCTION

Inositol, sorbitol (D-glucitol) and glycerylphosphorylcholine have already been identified in bovine seminal plasma (Hartree, 1957; King, Isherwood & Mann, 1958; Kahane & Levy, 1949; Dawson, Mann & White, 1956). The present study provides evidence for the occurrence of a small amount of glycerol (*circa* 2 mg/100 ml) as well as of two other polyols: D-mannitol and erythritol.

MATERIALS AND METHODS

Collection and processing of semen prior to analysis

Three composite samples of bovine semen (samples I, II and III) were obtained from two sources, namely the Cooperative Breeding Organizations of Minnesota and Wisconsin and from a group of experimental bulls located at the University farm. All semen ejaculates were collected by artificial vagina. Immediately after collection, all semen ejaculates were pooled. Samples I and II were then placed in a water bath at 100° C for 2 min. This treatment killed all the spermatozoa, and much of the heat-labile plasma protein was precipitated. After cooling, a volume of 95% ethanol equal to the total volume of the composite sample was added. This addition brought about further protein denaturation and precipitation. The semen was then placed in a cold room at 5° C until a sufficient volume had been accumulated. Sample I (23.4 ml) was used in the tentative qualitative analysis of the carbohydrate fraction. Sample II (111.7 ml) was used in the isolation and quantitative analysis of the polyols. Sample III (46.0 ml) was used in the quantitative determination of glycerol by a colori-

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metric technique. Sample IV (910 ml) was later obtained from the Milk Marketing Board, Thames Ditton, Surrey, England. Neither sample III nor sample IV was subjected to heat treatment, but immediately after collection and pooling of the ejaculates a volume of cold 95% ethanol equal to the original semen volume was added. Further deproteinization of sample III was effected by the addition of 5 vol. 95% ethanol. After centrifugation, the supernatant was decanted and the precipitate subjected to repeated washing by centrifugation with 50% methanol. The supernatant solutions were then pooled.

Isolation of carbohydrate fraction of seminal plasma before partition chromatography

Before chromatographic techniques could be employed to detect and isolate the free carbohydrates in bovine semen, inorganic ions and amino acids were removed by passing the material through ion exchange columns. Complete removal of proteins from samples I, II and IV was effected by transferring the material to dialysis bags (Visking Co., Chicago, Illinois) and dialysing the contents against frequent changes of de-ionized and distilled water at 5° C until such time as the dialysate no longer gave a positive Molisch test; after every change of water, the surface of the water was layered with toluene. The dialysed, protein-free semen extract was concentrated in a rotary evaporator *in vacuo*, and the concentrated extract shaken gently with half a volume of diethyl ether in a separatory funnel. This was repeated three times. De-ionization was effected by passing the protein and lipid-free material through columns containing Dowex 50 (H⁺ form) strong cation exchange resin (The Dow Chemical Co., Midland, Michigan) and then Duolite A 4 (OH⁻ form) weak anion exchange resin (Chemical Process Co., Redwood City, California). Frequently, the effluent collected from the anion exchange column was faintly alkaline due to 'bleeding' of the resin. In such a case it was neutralized on passing through a smaller column containing Amberlite IR120 (H⁺ form) strong cation exchange resin (Rohm and Hass Co., Philadelphia, Pennsylvania). Following de-ionization, the combined effluent and column washings were concentrated in a rotary evaporator *in vacuo* whereupon the pH of the carbohydrate fraction was checked with pH indicator paper as was also the presence or absence of amino acids by spraying a spot of the material on filter paper with ninhydrin reagent (Patton & Chism, 1951). When de-ionization had been completed, the fraction was concentrated further in the rotary evaporator and then transferred to a high vacuum pump. The fraction was then taken to dryness and weighed.

Alkaline oxidation of reducing carbohydrates

The chromatographic resolution and detection of the polyols in bovine semen was greatly enhanced when part of the carbohydrate fraction of sample II, as prepared above but before de-ionization, was subjected to the procedure of alkaline oxidation (Smith & Unrau, unpublished data). Reducing sugars, e.g. fructose, are oxidized by this procedure to glyconic and saccharinic acids which are easily removed by passing through columns containing ion exchange resins.

Sample II (111.7 ml) yielded a fraction not de-ionized (after concentrating and drying) of 2.58 g. This was redissolved in water and made up to 25.0 ml.

An aliquot (12.0 ml) of this solution was transferred to a round-bottomed flask (Sample IIa = 53.6 ml) and after addition of 3 ml *N*-NaOH the mixture was heated for 4 hr in a boiling water bath while aerating from a capillary within the flask. The alkalinity of the reaction mixture was maintained at pH 10 or above by adding aliquots of the sodium hydroxide solution to neutralize the acids as they were formed. On completion of the reaction, the reaction mixture was passed successively through columns containing Amberlite IR120 (H^+ form) and Amberlite 401 (OH^- form) strong anion exchange resin. After the glyconic and saccharinic acids were removed the effluent contained only the non-reducing components.

A control experiment was subsequently carried out on 5.0 ml of an aqueous solution containing D-fructose (25.0 mg), sorbitol (1.25 mg), *m*-inositol (1.75 mg) and L- α -glycerylphosphorylcholine (17.5 mg) (crystalline free diester, Sigma London Chemical Co., Ltd), which was subjected to the complete carbohydrate isolation procedure including heating, addition of ethanol, extraction with ether and alkaline oxidation. After removal of acids, the material was chromatographed using solvents A and D. After spraying with ammoniacal silver nitrate and heating, both sorbitol and inositol as well as glycerol were clearly detected. Fructose and its degradation products had been completely removed. Neither mannitol nor erythritol could be detected. From the results of this experiment it was concluded that the isolation of glycerol could not be effected from a sample of semen which had undergone alkaline oxidation because of the possibility that its presence, and its concentration, might be artifacts arising from the degradation of glycerylphosphorylcholine. Hence it was considered necessary to characterize glycerol using an additional sample (sample IV, 910 ml) of bovine semen which was subjected to column partition chromatography after isolation of the carbohydrate fraction.

Quantitative determination of fructose in sample II (111.7 ml)

An aliquot of the unoxidized portion of the carbohydrate fraction obtained from sample II containing a minimum of 0.3 mg fructose was chromatographed on paper. The fructose band was located and excised as was a similar area from a blank chromatogram run concurrently. After elution with water, fructose was determined colorimetrically by the phenol-sulphuric acid method of Dubois, Gilles, Hamilton, Rebers & Smith (1951, 1956). The fructose content of the semen was calculated after reference to a previously prepared standard curve.

Paper partition chromatography

Throughout this study only descending chromatography was employed. Whatman No. 1 paper was used for qualitative analyses and for quantitative separations of mixtures not exceeding a total of 10 mg in weight. Recourse was had to Whatman No. 3MM paper for quantitative separations of mixtures up to a total of 75 mg. Four solvent systems were employed; A, Pyridine-ethyl acetate-water (1:2.5:3.5 v/v, upper phase), (Jermyn & Isherwood, 1949; McFarren, Brand & Rutkowski, 1951); B, *n*-Butanol-ethanol-water (3:2:1 v/v), (Whistler & Duffy, 1955); C, *n*-Butanol-ethanol-water (4:1:5 v/v) (Partridge, 1946); and D, Butan-2-one-glacial acetic acid-saturated aqueous boric acid

(9:1:1 v/v) (Rees & Reynolds, 1958). The detection of carbohydrates with the silver spray in the presence of borate was made possible by treatment with a solution of 40% hydrogen fluoride (1 to 4 vol.) and acetone (40 vol.) before spraying with ammoniacal silver nitrate (Britton, 1959). Three spray reagents were employed: (i) ammoniacal silver nitrate (Partridge, 1948; Hough, 1950), (ii) *p*-anisidine (Briggs, Garner, Montgomery & Smith, 1956; Christensen & Smith, 1957) and (iii) anthrone (Johanson, 1953). This last proved a useful specific reagent for the detection of ketosugars both on chromatograms and in spot tests.

Column partition chromatography of sample IV (910 ml)

The methods of column chromatography employed were first described by Hough, Jones & Wadman (1948, 1949). A tightly packed column of powdered cellulose (Whatman 'Chromedia' CC31), contained in a glass fractionating column (85 × 5 cm), was used. The column was enclosed in a water jacket maintained at 30°C and was irrigated with solvent B.

Approximately one half (*circa* 2.72 g) of the carbohydrate fraction (*circa* 5.37 g) obtained from sample IV, after dissolving in water to give a thin syrup, was introduced on to the column with a small transfer pipette and allowed to soak into the cellulose. A solvent reservoir was then placed in position. The eluate was collected in aliquots of *circa* 10 ml by means of an automatic fractionating device (L.K.B. 'Radi Rac'). Small drops of eluate from every fifth tube were then placed in chronological order and chromatographed on paper. Appropriate aliquots of the eluate were then selected and combined. The solvent was removed by distillation in a rotary evaporator *in vacuo*. After tentative identification, the polyols isolated from samples IIa and IV were crystallized and/or their derivatives prepared as described below.

Identification of m-inositol, D-mannitol, sorbitol and erythritol from sample IIa (53.6 ml)

The areas of the paper chromatogram corresponding to the various polyols were excised and eluted with water. The solutions were filtered through wads of cotton. The filtrates were heated with small quantities of activated charcoal ('Darco', Eastman Kodak Company) and were then filtered, while still hot, through wads of tightly packed cotton and the filtrates evaporated *in vacuo* to dryness. In order to identify these fractions attempts were made to induce crystal formation by the addition of a few drops of aqueous methanol. In all cases these were successful and recrystallizations of the products were effected from either aqueous methanol or aqueous ethanol.

The crystalline products obtained from the inositol and sorbitol fractions were acetylated by the sodium acetate-acetic anhydride method of Bates & Associates (1942). Recrystallizations of the acetylated derivatives were effected from hot methanol. (A melting-point determination carried out on the crystalline product obtained from the 'sorbitol' fraction revealed that this was mannitol which had been separated by fractional crystallization. Attempts made to induce crystallization of the mother liquor failed. Both fractions were acetylated.)

The crystalline product obtained from the erythritol fraction was converted

into the tetra-*p*-nitrobenzoate derivative prepared by dissolving the crystals in a small quantity (1.0 ml) of dry pyridine, adding *p*-nitrobenzoyl chloride (20% molar excess) and heating at 80° C for 1 hr. Excess aroyl halide reagent was destroyed by the addition of a few drops of water to the cooled reaction mixture which was poured into a cold saturated solution of sodium bicarbonate and left overnight. The product was extracted with chloroform and anhydrous magnesium sulphate added. The solution was filtered and evaporated to dryness. The tetra-*p*-nitrobenzoate of erythritol was crystallized and recrystallized from a mixture of 1 part chloroform and 2 parts ethanol.

Identification of glycerol separated by column chromatography of sample IV (910 ml)

The product obtained after treatment with charcoal and decolorization of the fraction of the eluate corresponding to glycerol was a clear viscous syrup. The tri-*p*-nitrobenzoate derivative of glycerol was prepared as described previously. The product was crystallized and recrystallized from the chloroform ethanol mixture.

Quantitative determination of glycerol separated by paper chromatography of sample III (46.0 ml)

The concentration of free glycerol in this sample was determined colorimetrically by the method of periodate oxidation and the reaction of the formaldehyde liberated with chromotropic acid (Lambert & Neish, 1950). The combined supernatants were evaporated to dryness *in vacuo* and the residue (934 mg) redissolved in water and made up to 5.0 ml in a volumetric flask. An aliquot of this (378 μ l) was applied by micropipette to a chromatogram of Whatman No. 3MM paper. This was developed in solvent B and the area corresponding to glycerol excised and eluted with water (25.0 ml). A blank chromatogram was run concurrently and an area equal to that of the first was excised and treated in a similar manner. The eluates were filtered and 20.0 ml withdrawn from each and evaporated to dryness in round-bottomed flasks. These were taken up in minimal volumes of water and transferred to two 5.0 ml volumetric flasks. The periodate oxidation of the contents was carried out in the manner prescribed, using 1/10 the quantities of reagents.

The amount of glycerol present in the sample was calculated by reference to a previously prepared standard curve.

RESULTS

Preliminary qualitative chromatographic analyses of bovine semen had indicated the presence of several discrete spots, three of which corresponded with *m*-inositol, sorbitol and D-fructose. These have all been isolated previously by other workers (Hartree, 1957; King *et al.*, 1958; Mann, 1954, 1946a, b, c).

Further analyses on paper performed on sample I revealed the presence of two additional components which moved faster than fructose but were present in very much lower concentrations as assessed visually by the area and intensity of their spots on spraying with ammoniacal silver nitrate. These components gave no reaction with *p*-anisidine, from which it was concluded that they were

non-reducing and therefore probably polyols. These two unknown components corresponded to erythritol and glycerol.

Tentative identification of the various carbohydrate components in bovine semen was made by running aliquots of the carbohydrate fraction of sample I against authentic standard solutions in three different solvent systems A, B and C. Since D-fructose is the principal reducing sugar of bovine semen and since

TABLE 1

CHROMATOGRAPHIC MOBILITIES OF VARIOUS CARBOHYDRATE COMPONENTS OF A COMPOSITE SAMPLE OF BOVINE SEMEN (SAMPLE I = 23.4 ml) AND OF STANDARD SOLUTIONS IN THREE DIFFERENT SOLVENT SYSTEMS (A, B AND C), IN RELATION TO THE MOBILITY OF FRUCTOSE ($R_{Fr} = 1$)

Material chromatographed		Solvent systems		
		A	B	C
Component (C) - Standard (S)		C-S	C-S	C-S
1	- Inositol	0.21-0.21	0.23-0.23	0.13-0.13
2	- Sorbitol	0.74-0.74	0.82-0.82	0.73-0.73
3	- Mannitol*	0.77-0.77	0.87-0.87	0.75-0.75
4	- Fructose	1.00-1.00	1.00-1.00	1.00-1.00
5	- Erythritol	1.35-1.35	1.53-1.53	1.75-1.75
6	- Glycerol	1.74-1.74	1.98-1.98	2.53-2.53

* The chromatographic mobility values of mannitol are included here for reference. This polyol was not detected in the preliminary qualitative analyses and its presence was not revealed until it crystallized spontaneously from the 'sorbitol' fraction of sample II. Closer scrutiny of the spot corresponding to sorbitol revealed that it was slightly elongated in the direction of solvent flow and was therefore probably heterogeneous.

TABLE 2

CHROMATOGRAPHIC MOBILITIES OF DEGRADATION PRODUCTS FOLLOWING ALKALINE OXIDATION OF AN AQUEOUS SOLUTION CONTAINING *m*-INOSITOL, SORBITOL, D-FRUCTOSE AND L- α -GLYCERYLPHOSPHORYLCHOLINE, AND STANDARD SOLUTIONS IN TWO DIFFERENT SOLVENT SYSTEMS (A AND D), IN RELATION TO THE MOBILITY OF FRUCTOSE ($R_{Fr} = 1$)

Material chromatographed		Solvent systems	
		A	D
Product (P) - Standard (S)		P-S	P-S
1	- Inositol	0.19-0.19	0.09-0.09
n.d.	- Mannitol	n.d.-0.69	n.d.-1.47
2	- Sorbitol	0.71-0.71	1.68-1.68
n.d.	- Fructose	n.d.-1.00	n.d.-1.00
n.d.	- Erythritol	n.d.-1.41	n.d.-2.85
3	- Glycerol	1.95-1.95	3.95-3.95

n.d., Non-detectable.

it appeared to move chromatographically at about half the rate of the fastest component, it was decided that in comparing the mobilities of the various components in the different solvent systems D-fructose would serve as a convenient reference ($R_{Fr} = 1.0$). The results of these studies are presented in

Table 1. From the chromatographic evidence it was concluded that bovine semen contained the polyols erythritol and glycerol as well as inositol and sorbitol. The presence of D-mannitol as a constituent of normal bovine semen was not revealed in the initial chromatographic analyses.

The results of the control experiment, involving the alkaline oxidation of an aqueous solution of the components already known to occur in bovine semen, and in concentrations approximating those which have been reported in the literature are presented in Table 2. From the experimental evidence afforded

TABLE 3

QUANTITIES AND CONCENTRATIONS OF POLYOLS ISOLATED FROM A COMPOSITE SAMPLE (SAMPLE IIA = 53.6 ml) TOGETHER WITH THE PHYSICAL CONSTANTS OF THE CRYSTALLINE PRODUCTS AND OF THEIR DERIVATIVES

<i>Polyol</i>	<i>Quantity (mg)</i>	<i>Concentration (mg/100 ml)</i>	<i>m.p. and m.m.p. (°C)</i>	<i>Derivatives</i>	<i>m.p. and m.m.p. (°C)</i>	$[\alpha]_D^{20}$
<i>m</i> -Inositol	12.7	23.7	225	Hexa-acetate	218 to 219	Inactive
D-Mannitol	9.1	17.0	165 to 166	Hexa-acetate	126	+24.6° (concn. 0.7 in CHCl ₃)
Sorbitol	9.0	16.8	Not crystallized	Hexa-acetate	98 to 99	+6.7° (concn. 0.9 in CHCl ₃)
Erythritol	3.7	6.9	119 to 120	Tetra- <i>p</i> -nitrobenzoate	239	Inactive
Glycerol*	5.4	ca. 2.0	Syrup	Tri- <i>p</i> -nitrobenzoate	195 to 196	Inactive
Glycerol†	—	1.71				

A fructose determination carried out by the method of Dubois *et al.* (1951, 1956) on an unoxidized aliquot of the carbohydrate fraction of sample II gave 519 mg/100 ml.

* Obtained from column partition chromatography of sample IV (910 ml).

† Determined colorimetrically by the method of Lambert & Neish (1950) on sample III.

by this control experiment it was concluded that the characterization of glycerol could not be effected in a sample of semen which had been subjected to alkaline oxidation (see 'Materials and Methods').

Table 3 summarizes the results obtained and the physical constants observed when the polyols, previously only tentatively identified, were characterized in sample IIA. The results reported in this table were confirmed when, in addition to glycerol, the other polyols identified were isolated and characterized in sample IV (910 ml) with results comparable to those reported below.

DISCUSSION

The isolation of inositol and sorbitol as constituents of normal bovine semen serves to confirm the findings of other workers in this field (Hartree, 1957; King *et al.*, 1958). However, the isolation of D-mannitol, erythritol and free glycerol have been hitherto unreported in the literature, although the presence of 'bound' glycerol in the form of glycerylphosphorylcholine has been acknowledged. The experimental finding that both D-mannitol and sorbitol are present

in bovine semen in approximately equal concentrations does suggest that both D-mannitol and sorbitol may arise simultaneously from the reduction of D-fructose.

The isolation of erythritol as a constituent of normal bovine semen is of great interest. At the time of its isolation no mammalian tissue or fluid had been reported as containing this tetritol, although its presence has been reported in certain algae, lichens and grasses (Bamberger & Landsiedl, 1900; Tischer, 1936; Hesse, 1861; Lindberg, 1955). In a report on the metabolism of the sugar alcohols and their derivatives, Carr & Krantz (1945) expressed the opinion that erythritol and its two optically active isomers D- and L-threitol would be found to be refractory to metabolism by the animal body. This hypothesis was supported by Batt, Dickens & Williamson (1960), who, during the course of a comprehensive study on tissue utilization of tetroses and tetritols, found that [1— ^{14}C]erythritol was not appreciably converted into $^{14}\text{CO}_2$ or ^{14}C -glucose when incubated with slices of rat-liver tissue.

Among the first reports on the occurrence of erythritol in mammalian tissues is that of Touster, Hecht & Todd (1960) who isolated erythritol in crystalline form from the urine of two fasting men and established its identity by chromatography, melting point and X-ray diffraction pattern; and Williams, Pearce & Smith (1961) who isolated erythritol from bovine foetal tissue and prepared a crystalline derivative. This foetal product was found to be concentrated in the chorion, the allantoic fluid and the placental tissue. It was also found to stimulate the growth of the *Brucella abortus* organism.

Since this investigation was completed both erythritol and glycerol have been isolated from the semen of the domestic cock (Ahluwalia & Graham, 1963). These observations as well as the absence of mannitol in fowl semen have been confirmed by Clark (unpublished data).

In the absence of experimental evidence it is largely a matter of conjecture as to what the site and mode of biosynthesis of these polyols may be, but their chemical nature might suggest that the hexose monophosphate oxidation shunt reactions may play a more important role in the metabolism of the genital tract accessory glands and their secretions than has hitherto been realized.

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