D-[1-¹⁴C]Mannitol and [U-¹⁴C]sucrose as extracellular space markers for human spermatozoa and the uptake of 2-deoxyglucose

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Summary. [U-¹⁴C]Sucrose and D-[1-¹⁴C]mannitol were used to determine the tritiated water space of human spermatozoa to validate these compounds as markers for the extracellular space. Calculations based on 0.03 mM-[U-¹⁴C]sucrose gave a negative water space. The water space estimated with 0.03 mM-D-[1-¹⁴C]mannitol was unstable but a stable result was obtained with 0.3 mM-D-[1-¹⁴C]mannitol in incubations up to 2 h. The mean water space was $2.21 \pm 0.106 \mu\text{l}/10^8$ spermatozoa (mean $\pm$ s.e.m. for 6 batches of pooled semen). The water space was decreased or abolished by Triton X-100, cold shock, sonication or hypotonic treatment. The water space responded to changes in the osmolarity of the medium by increasing in dilute media. It is concluded that mannitol is an effective extracellular marker for human spermatozoa if concentrations ≥ 0.3 mM are used.

When the kinetics of the uptake of 2-deoxyglucose by the spermatozoa were studied by using mannitol as an extracellular marker, the transport was saturable and was inhibited by cytochalasin B. The K_m was 1.6 ± 0.33 mM and the $V_{\max}$ was 4.2 ± 0.52 nmol/ 10^8 spermatozoa/10 sec (mean $\pm$ s.e.m., $n = 4$).

Introduction

Experiments designed to study the transport of substances across membranes into cells or organelles frequently rely on rapid separation of the cells from the medium by centrifugation through a layer of silicone oil (see Wohlheuter, Marz, Graff & Plagemann, 1978) or by rapid filtration (Britten, Roberts & French, 1955). These techniques have been applied to the study of 2-deoxyglucose uptake by spermatozoa (Hiipakka & Hammerstedt, 1978a, b). Such separation procedures are efficient but the cells are inevitably contaminated with a small amount of the medium. The error introduced by this contamination is particularly significant with spermatozoa because of the very small intracellular water space, e.g. the water space of ram spermatozoa is 2–2.4 $\mu\text{l}/10^8$ cells (Hammerstedt, Keith, Hay, Deluca & Amann, 1979) compared with 150 $\mu\text{l}/10^8$ cells for mouse fibroblasts (see Wohlheuter *et al.*, 1978). The problem can be overcome to a large extent by including a substance which cannot penetrate the cells or bind to their surfaces in the incubation medium. The concentration of this 'marker' in the cell pellet allows the degree of contamination with medium to be determined and allowed for in subsequent calculations.

The present experiments were undertaken to determine whether [U-¹⁴C]sucrose or D-[1-¹⁴C]mannitol was a suitable extracellular marker for human spermatozoa and to measure the water space of these cells. Carboxyinulin, which is often used as an extracellular marker for other cells (see Wohlheuter *et al.*, 1978), was rejected because it binds to the surface of spermatozoa (Crabo *et al.*, 1975).

To illustrate the application of this technique the kinetic constants for the uptake of 2-deoxy-glucose by human spermatozoa were determined.

Materials and Methods

Chemicals

[U- ^{14}C]Sucrose, D-[1- ^{14}C]mannitol, $^3\text{H}_2\text{O}$ and 2-deoxy-D-[1- ^3H]glucose were obtained from Amersham International p.l.c., Amersham, Bucks HP7 9LL, U.K. Their purity was confirmed by thin-layer chromatography on 0.25 mm layers of silica gel G/UV254 in chloroform : methanol (2 : 1, v/v) or propan-1-ol : ethyl acetate : H_2O (75 : 10 : 15, by vol.). Silicone oils were supplied by Hopkins & Williams (P.O. Box 1, Romford, Essex RN1 1HA, U.K.) and other reagents were purchased from Fisons Scientific Apparatus (Loughborough LE11 0R9, U.K.) or Sigma Chemical Co. Ltd (Poole BH17 7NM, U.K.).

Spermatozoa

Ejaculates collected by masturbation were supplied by student volunteers who were free from urogenital infections. Spermatozoa were separated from the seminal plasma by centrifugation through a layer of Ficoll (Harrison, 1976) 1–4 h after ejaculation. The Ficoll solution was dialysed overnight against a large volume of phosphate-buffered saline (PBS) (Ford, Harrison & Waites, 1981) before use. Generally 5–10 ejaculates were pooled before centrifugation and the pellet of spermatozoa was suspended in PBS to give a concentration (mean $\pm$ s.e.m.) of $280 \pm 10 \times 10^6$ spermatozoa/ml ($n = 14$), for the water space experiments and of $270 \pm 20 \times 10^6$ spermatozoa/ml ($n = 13$) for the transport experiments. The concentration of spermatozoa was measured with a haemocytometer. All experiments were done at room temperature (about 20°C).

Separation of spermatozoa from the incubation medium

Spermatozoa were centrifuged on silicone oil gradients in 0.40 ml capacity plastic centrifuge tubes in an Eppendorf 'Zentrifuge 3200'. The bottom layer of the gradient was 50 μl Dow Corning 550 silicone fluid (relative density, 1.07). This served as a cushion to prevent the pellet entering the narrow tip of the tube whence it was difficult to extract. The upper layer was 100 μl of a mixture of silicone oils with a calculated relative density of 1.02. In early experiments this was Dow Corning 550 : Dow Corning 510/50 cs, 5 : 3 (v/v), mixed by heating; this mixture was clear when warm but quickly separated into an emulsion at room temperature. Later Dow Corning 550 : Dow Corning 200/1 cs, 4 : 1 (v/v), mixed by heating, was used and this mixture was stable indefinitely. In some experiments the volume of the upper oil layer was decreased to 75 μl and an aqueous layer consisting of 25 μl 7.5% (w/v) sucrose + 0.6% (v/v) Triton X-100 was put between the oil layers. This made it easier to isolate the pellet free of contamination from the upper aqueous phase (medium) and this system was used for all the experiments with 2-deoxyglucose.

Sperm suspension (0.2 ml) was placed on top of the oil layers and the tube was centrifuged at about 8000 g for 2 sec. The tube was frozen by standing it upright in liquid nitrogen and stored at -20°C . The sperm pellet was isolated by cutting out the region of the tube containing it. It was shaken out of the plastic ring and homogenized in 1.5 ml 5% (w/v) formic acid with an Ultra-Turrax blender. The denatured spermatozoa were removed by centrifugation and 1.0 ml of the supernatant was mixed with 10 ml scintillant (0.25% (w/v) 2,5-diphenyloxazole (PPO), 0.01% (w/v) 1,4-di-(5-phenyloxazolyl)-benzene (POPOP) in toluene : Triton X-100, 2 : 1 (v/v)) and counted using the $^3\text{H}/^{14}\text{C}$ settings on a Packard Tricarb Liquid Scintillation Spectrophotometer 3255. The activity of ^3H and ^{14}C in d.p.m. was calculated using polynomial equations fitted to curves obtained by plotting ^3H efficiency, ^{14}C efficiency and spillage of ^{14}C counts into the ^3H channel against the external channels ratio.

Calculations of the water space or of 2-deoxyglucose uptake

These were calculated from the formula:

Water space ($\mu\text{l}/10^8$ spermatozoa) or 2 deoxyglucose uptake ($\text{nmol}/10^8$ spermatozoa) =

$$\frac{T - (C \times R)}{n \times S}$$

where $T = {}^3\text{H}$ d.p.m. in the pellet; $C = {}^{14}\text{C}$ d.p.m. in the pellet; $R =$ ratio of ${}^3\text{H}$ d.p.m./ml to ${}^{14}\text{C}$ d.p.m./ml in the incubation medium; $S = {}^3\text{H}$ d.p.m./ μl water or ${}^3\text{H}$ d.p.m./nmol 2-deoxyglucose in the incubation medium; $n =$ number of spermatozoa in the pellet/ 10^8 .

The water space represents the volume in the pellet accessible to ${}^3\text{H}_2\text{O}$ but not to $[\text{U-}^{14}\text{C}]\text{sucrose}$ or to $\text{D-[1-}^{14}\text{C]mannitol}$. If the ${}^{14}\text{C}$ markers are unable to penetrate the plasma membrane it will equal the intracellular water space, but if they cross the membrane or bind to it it will be decreased and may assume a negative value.

Water space as a function of time

Spermatozoa were mixed with ${}^3\text{H}_2\text{O}$ (10 $\mu\text{Ci}/\text{ml}$) and either 0.03 mM- $[\text{U-}^{14}\text{C}]\text{sucrose}$ or 0.03 mM- or 0.3 mM- $\text{D-[1-}^{14}\text{C]mannitol}$ (1 $\mu\text{Ci}/\text{ml}$). The mixture was agitated frequently and samples were removed at various times up to 120 min. When 0.3 mM-mannitol was used the sperm suspension was divided so that the larger portion was about twice the volume of the smaller. Both portions were kept in similar conditions but $\text{D-[1-}^{14}\text{C]mannitol}$ and ${}^3\text{H}_2\text{O}$ were added to the small portion 90 min after they had been added to the large one.

The effect of the concentration of spermatozoa on the water space

Portions of sperm suspension were mixed with ${}^3\text{H}_2\text{O}$ (10 $\mu\text{Ci}/\text{ml}$), 0.3 mM- $\text{D-[1-}^{14}\text{C]mannitol}$ (1 $\mu\text{Ci}/\text{ml}$) and PBS buffer to yield cell concentrations in the ratios 3:2:1. The most concentrated suspension contained about 220×10^6 spermatozoa/ml. After standing for 5 min, 0.20 ml samples were removed from the suspensions and centrifuged (8000 g) on silicone oil gradients.

Effect of various treatments on the water space

Control spermatozoa were held in PBS buffer. The other treatments were: cooling on ice for 5 min and addition of 0, 0.0008, 0.008 or 0.08% (v/v) Triton X-100 to the medium and standing on ice for 5 min, centrifugation (600 g_{av} , 10 min, room temperature) and resuspension in ice-cold 10 mM-potassium phosphate pH 7.4 for 5 min, sonication (6 $\times$ 10 sec bursts, 100 W, 6–8 μm peak to peak with 20 sec between each burst). After the treatment period ${}^3\text{H}_2\text{O}$ (10 $\mu\text{Ci}/\text{ml}$) and 0.3 mM- $\text{D-[1-}^{14}\text{C]mannitol}$ (1 $\mu\text{Ci}/\text{ml}$) were added to the suspensions which were stood for 5 min at room temperature before 0.20 ml samples were removed and centrifuged.

The effect of the osmolarity of the medium on the water space

The pooled semen was divided into aliquots which were centrifuged in different tubes and the sperm pellets were resuspended in 20 mM-Hepes pH 7.4 containing 50, 80, 110, 140, 170 or 200 mM-NaCl. Tritiated water (10 $\mu\text{Ci}/\text{ml}$) and 0.3 mM- $\text{D-[1-}^{14}\text{C]mannitol}$ (1 $\mu\text{Ci}/\text{ml}$) were added to the sperm suspensions which were stood for 5 min before 0.20 ml samples were removed.

2-Deoxyglucose uptake

PBS (0.1 ml) containing 0 to 10 mM-2-deoxy-D-[${}^3\text{H}$]glucose and 1 mM- $\text{D-[1-}^{14}\text{C]mannitol}$ was placed on top of the silicone oil gradients and 0.1 ml sperm suspension was added and mixed by sucking in and out of the 'Eppendorf' pipette tip (from which the narrowest constriction at the end of the tip had been cut off). Care was taken not to disturb the gradient and the mixing process could

be completed in 2 sec. The tube was transferred to the microcentrifuge which was started 10 sec after the spermatozoa had been added, except in determination of the time course when a range of times was allowed; 6 sec was the shortest time which could be studied reliably. The tubes were frozen in liquid nitrogen immediately after centrifugation and the pellets were processed in the same way as for the water space measurements. The specific activity of the 2-deoxy-D-[1-³H]glucose was adjusted according to its molarity, e.g. 0.1 mM solutions contained about 50 Ci/mol and 10 mM solutions about 0.5 Ci/mol. This was necessary to economise in the use of the radioactive sugar whilst ensuring that an adequate number of counts were present in the pellet at all 2-deoxyglucose concentrations. The activity of D-[1-¹⁴C]mannitol was adjusted so that the ratio ³H d.p.m./ml to ¹⁴C d.p.m./ml was about 10.

In some experiments 0.4 mM-cytochalasin B, 2 mM-phloridzin or 20 mM-D-glucose was mixed with 2-deoxyglucose solutions before the addition of the spermatozoa. In other experiments the spermatozoa were preincubated with 1.0 mM-cytochalasin B for 5 min before they were added to the 2-deoxyglucose solution.

The kinetic constants were calculated using an iterative non-linear regression programme (Duggleby, 1981). The points were weighted according to their standard deviations.

Results and Discussion

The silicone oil separation procedure was quite efficient and only 3–5 µl of the extracellular medium were carried down with the sperm pellet. However, this was equivalent to 10–15 µl/10⁸ spermatozoa or roughly 6 times the intracellular water space. Greater contamination occurred in some cases but this resulted in obviously discrepant results which were excluded from further analysis.

When human spermatozoa were incubated with ³H₂O and 0.03 mM-[U-¹⁴C]sucrose the apparent water space was negative and declined further during the first hour of the incubation (Text-fig. 1). Therefore sucrose must be bound to the surface of the spermatozoa or be transported into the intracellular compartment, and low concentrations of sucrose are not suitable as extracellular markers for human spermatozoa.

The water space measured with 0.03 mM-D-[1-¹⁴C]mannitol was about 2 µl/10⁸ spermatozoa after short periods of incubation but declined to about 0.3 µl/10⁸ spermatozoa after 1 h (Text-fig. 1). When the concentration of D-[1-¹⁴C]mannitol was increased to 0.3 mM the measured tritiated water space was stable for 2 h and the water space was the same whether the 'space markers' were present throughout the incubation or were added after 90 min (Text-fig. 1).

A plot of the total water space against sperm concentration yielded a straight line (Text-fig. 2). However, the line did not pass through the origin and this could result in a substantial overestimate of the water space with concentrations of < 100 × 10⁶ spermatozoa/ml. In the concentration range used for the experiments the maximum error from this source was < 4%. The tritiated water space was 2.21 ± 0.106 µl/10⁸ spermatozoa (mean ± s.e.m. (6 batches of pooled semen with at least 4 observations per batch)). This compares with 2.0–2.4 µl/10⁸ spermatozoa measured by electron spin resonance in ram spermatozoa (Hammerstedt *et al.*, 1979) and about 1.5 µl/10⁸ spermatozoa measured by gravimetric and morphometric methods in bull spermatozoa (Drevius, 1972). The overall dimensions of ram, bull and human spermatozoa are quite similar (Mann, 1964). Calculations from morphometric measurements give the total volume of 10⁸ human spermatozoa as 1.7 µl (see Mann & Lutwak-Mann, 1981) or 2.6 µl (Glander & Dettmer, 1978). Therefore, the results of our measurements are similar to those obtained by other procedures although they seem a little higher than the morphometric measurements would suggest in view of the very sparse cytoplasm in the mature sperm cell.

Hypotonic lysis, sonication or 0.08% Triton X-100 decreased the measured water space to zero. The negative values were probably produced by mannitol binding to intracellular components

Text-fig. 1. The tritiated water space in human spermatozoa measured with 0.03 mM-[U-¹⁴C]sucrose (□) or 0.3 (△) mM-[1-¹⁴C]mannitol as the extracellular marker during incubation for 2 h at room temperature (~20°C). △, Marker present throughout; ▲, marker added after 90 min. Values are mean ± s.e.m. (*n* = 3-5).

(Table 1). Cold shock reduced the water space to about 50% of the control value and low concentrations of Triton X-100 had no further effect. This could reflect a selective effect on different cell compartments or variation in the sensitivity of different spermatozoa. Even centrifugation followed by resuspension in PBS buffer caused a significant decrease in water space, suggesting that the sperm plasma membrane is quite vulnerable to mechanical damage (Table 1).

Spermatozoa swell in hypotonic media (Drevius, 1972) and this produced easily detectable changes in their water space (Text-fig. 3). The water space in the spermatozoa roughly doubled when the calculated osmolality of the medium was decreased from 430 to 130 mosmol/l.

The data presented here demonstrate that mannitol is an effective extracellular marker for human spermatozoa when concentrations ≥ 0.3 mM are used. Lower concentrations did not yield reliable results. This discrepancy is probably caused by the presence in the sperm suspension of a small number of binding sites with a high affinity for mannitol. These sites may be in cells with leaky membranes since lysis of the spermatozoa resulted in the binding of mannitol (Table 1). The

Text-fig. 2. The effect of dilution of the sperm suspension on the total water space (mean $\pm$ s.e.m., $n = 6$). Data from 2 experiments were normalized as a percentage of the space in the undiluted sample (241 or 220×10^6 spermatozoa/ml) and pooled.

Table 1. The effect of various treatments on the tritiated water space of washed human spermatozoa with $0.3 \text{ mM-D-[1-}^{14}\text{C]mannitol}$ as extracellular marker

Treatment	No. of measurements	Tritiated water space ($\mu\text{l}/10^8$ spermatozoa)
Controls	11	$2.29 \pm 0.07^*$
0°C for 5 min	6	1.21 ± 0.21
0.0008% Triton X-100	3	1.25 ± 0.16
0.008% Triton X-100	3	1.30 ± 0.14
0.08% Triton X-100	4	-0.86 ± 0.10
Resuspension in $10 \text{ mM-potassium phosphate pH } 7.4$ at 0°C	4	-1.73 ± 0.78
Sonication (6×10 sec bursts at 100 W , $6\text{--}8 \mu\text{m}$ peak to peak, 20 sec between bursts)	3	0.20 ± 0.04
Centrifugation ($800 g_{av}$, 10 min, 20°C)	7	1.65 ± 0.86

Values are mean $\pm$ s.e.m.

* Significantly different from each treatment value, $P < 0.001$ (t test).

fractional change in mannitol concentration produced by such binding would clearly be greater when the concentration was low. The increase in error with time (Text-fig. 1) could represent the degeneration of increasing numbers of spermatozoa or the time needed to penetrate partial permeability barriers. Sucrose was not a good extracellular marker although it is quite likely that its performance would be better if higher concentrations were used.

Measurement of the intracellular water space allows an estimate of the intracellular concentration of metabolites to be made. The results must be treated with caution because of potential sources of error which include intracellular compartmentation and binding of metabolites to cell

Text-fig. 3. Two experiments to illustrate the effect of the osmolarity of the medium (calculated assuming activity = concentration) on the water space of human spermatozoa. Values are mean $\pm$ s.e.m. (Exp. 1 (●), $n = 6-8$; Exp. 2 (▲), $n = 3-4$).

protein. However, they provide a starting point to relate in-vitro enzymology to the living cell. For example, human spermatozoa contain about 12 nmol ATP/10⁸ spermatozoa (Ford & Harrison, 1981) which corresponds to 12/2.21 nmol/μl or 5.4 mM-ATP. This is greatly in excess of the K_m of (sea urchin) dynein ATPase, i.e. 26–110 μM-ATP depending on incubation conditions (Gibbons & Frank, 1972), and would be sufficient to inhibit completely the activity of human sperm phosphofructokinase in the absence of activators such as AMP (Peterson & Freund, 1970).

The uptake of 2-deoxyglucose from a 0.1 mM-solution was linear with time for at least 40 sec (Text-fig. 4a) but became non-linear after about 10 sec with 1.5 mM-2-deoxyglucose (Text-fig. 4b). In some experiments, including those illustrated, an extrapolation of the graph to zero time crossed the ordinate above the origin. The intercept corresponded to the apparent uptake of 2-deoxyglucose in the presence of high concentrations of inhibitors of 2-deoxyglucose transport (Text-fig. 4a). Therefore the discrepancy was not due to a very rapid initial phase of facilitated diffusion but is likely to be caused by the rapid binding of 2-deoxyglucose or an impurity to material in the sperm suspension. To circumvent this problem, control incubations with cytochalasin B-treated spermatozoa were included in all subsequent experiments. The transport system for 2-deoxyglucose was saturable and demonstrated Michaelis–Menten kinetics (Text-fig. 5). It was effectively blocked in spermatozoa exposed to 1.0 mM-cytochalasin B although the motility of these cells was the same as that of controls when examined under the light microscope. The kinetic constants were: K_m 1.6 $\pm$ 0.33 mM; V_{max} 4.2 $\pm$ 0.52 nmol/10⁸ spermatozoa/10 sec. In comparison to values from other species converted to the same units (ram, K_m 0.16, V_{max} 3.9; bull, K_m 0.13, V_{max} 7.2; rat K_m 0.2, V_{max} 13; Hiipakka & Hammerstedt, 1978a, b; W. C. L. Ford & D. W. Hamilton, unpublished results) the maximum rate of 2-deoxyglucose accumulation by human spermatozoa is similar but the affinity of the transport system for 2-deoxyglucose is unusually low. Our values are supported by the observation that the rate of accumulation of [U-¹⁴C]glucose by human spermatozoa was dramatically increased when its concentration was raised from 1.0 to 10.0 mM (Peterson & Freund, 1969). Hexokinase from bull spermatozoa is not inhibited by cytochalasin B (Hiipakka & Hammerstedt, 1978a) and the K_m of hexokinase from human sperm for glucose is 0.1 mM. Thus it is

Text-fig. 4. The time course of the uptake of 2-deoxyglucose from (a) 0.1 mM and (b) 2.5 mM solutions and the effect of inhibitors: (○) control, (●) 0.2 mM-cytochalasin B, (△) 1 mM-phloridzin, (▲) 10 mM-D-glucose. Values are mean $\pm$ s.e.m. for 5 (a) or 4 (b) observations.

Text-fig. 5. The effect of 2-deoxyglucose concentration on the rate of uptake (○) and its inhibition in spermatozoa treated with 1 mM-cytochalasin B (●). Values are mean $\pm$ s.e.m. for $n = 4$.

unlikely that phosphorylation of 2-deoxyglucose rather than its transport is the rate limiting step in our experiments.

The $V_{\max}$ for 2-deoxyglucose transport corresponds to about $1.5 \mu\text{mol}/10^8$ spermatozoa/h. This is greater than the glycolytic rate, about 0.25 (Ford & Harrison, 1981) or about $0.5 \mu\text{mol glucose}/10^8$ spermatozoa/h (Peterson & Freund, 1969) even before the effect of temperature is taken into account. Therefore glucose transport will only limit the rate of glycolysis when the glucose concentration is low.

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