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Glucose Requirement for Mouse Sperm Capacitation in vitro

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ABSTRACT

Requirements for the three energy substrates (glucose, lactate and pyruvate) which are present in Whitten's medium for fertilization of mouse ova *in vitro* by epididymal sperm were examined. Capacitation of sperm during a 3 h preincubation period occurred equally well in complete medium and in medium from which lactate and pyruvate had been omitted. The proportion of ova fertilized by sperm when lactate was omitted from the medium was not significantly different from that obtained with complete medium. Thus, glucose is the major source of energy for capacitation of epididymal mouse sperm, but the presence of cumulus cells or pyruvate is required to maintain ova viability during the incubation with sperm. Ova viability was not maintained by lactate in the absence of pyruvate. The substitution of fructose for glucose in Whitten's medium resulted in very low fertilization rates similar to those found when glucose was omitted from the medium. The rate at which CO_2 was produced from fructose by sperm during a 3 h incubation period was approximately one-half of the rate from glucose, suggesting that failure of fructose to support fertilization probably involves deficiencies in its transport or intracellular metabolism to provide adequate energy for sperm motility and capacitation. Rates of CO_2 production from carbon atoms (numbers 1 and 6) of glucose during incubation with sperm were not significantly different, suggesting that glucose is oxidized primarily by the glycolytic pathway during capacitation of sperm.

INTRODUCTION

Appropriate conditions for the preimplantation development of mouse zygotes in culture were not achieved until it was determined that the energy substrate requirements of the embryo change with advancing cleavages (Biggers et al., 1967). The incorporation of pyruvate into a modified Krebs-Ringer bicarbonate solution and the reduction of the oxygen concentration in the atmosphere to 5 percent prevented a block at the two-cell stage and allowed the one-cell mouse zygote to develop to the blastocyst stage (Whitten and Biggers, 1968; Whitten, 1971). Two-cell embryos can utilize lactate as an energy source for development (Brinster, 1965), whereas the 8-cell and later stage embryos metabolize glucose (Whitten, 1957). Thus, the successful preimplantation culture of mouse embryos was achieved when sodium pyruvate, sodium lactate and glucose were present in the modified Krebs-Ringer bicarbonate solution.

Fertilization of mouse ova *in vitro* with sperm collected from the uterus (Whittingham, 1968; Cross and Brinster, 1970) or the cauda epididymus (Toyoda et al., 1971; Miyamoto and Chang, 1972; Hoppe and Pitts, 1973) was achieved using a medium similar to that used for embryo culture. Thus, the examination of energy sources required by epididymal mouse sperm for their capacitation and for ova penetration in defined medium is now possible. Miyamoto and Chang (1973) observed that inclusion of lactate and pyruvate in a modified Krebs-Ringer bicarbonate solution containing serum albumin increased sperm motility and the number of sperm exhibiting the acrosome reaction. They inferred that these metabolic intermediates were required for sperm capacitation and fertilization of mouse ova *in vitro*. Studies described in the present report indicate that glucose is the major energy source utilized during the capacitation of cauda epididymal mouse sperm and that pyruvate is required for maintaining ova viability during incubation with sperm. Lactate is not required for sperm capacitation nor for maintaining ova viability during fertilization *in vitro*.

MATERIALS AND METHODS

In experiments designed to examine the energy sources required for sperm capacitation and fertilization *in vitro*, gametes from F_1 hybrid mice of the C57BL/10Wt X SJL/Wt cross or its reciprocal were

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used. Males used as sperm donors were 2 to 5 months of age, were housed individually and had not previously been mated. Virgin females, 2 to 6 months of age, were exposed to light from 0800 to 2200 h so that ovulation occurred at approximately 0600 h. Females were selected in proestrus in the afternoon (Champlin et al., 1973), killed 2 to 4 h after ovulation the following morning, and ampullae were removed and punctured to release ova into medium. The proportion of animals that ovulated under these conditions decreased from approximately 100 percent to about 20 percent while these investigations were being conducted. The mice would mate when paired with males during the evening, indicating that they were in estrus, and mating increased the incidence of ovulation. However, to facilitate collection of the numbers of ova needed, it became necessary to administer human chorionic gonadotropin (HCG). Females were selected in proestrus and 4 IU of HCG was injected IP. This treatment induced ovulation in nearly 100 percent of the mice and the number of ova collected from each animal was similar to the approximately 10 ova ovulated spontaneously in the F_1 hybrid (Hoppe, unpublished data). Induction of ovulation did not apparently affect the fertilizability of the ova. The reasons for the failure of the females to ovulate in the absence of treatment with HCG, even though they appeared to be exhibiting normal estrous cycling, is not known.

The procedures used for fertilization and preimplantation development *in vitro* of mouse ova have been described previously (Hoppe and Pitts, 1973). Since the publication of these procedures, I have obtained several bottles of paraffin oil (Saybolt, viscosity 125/135) which contains a substance(s) that is toxic to mouse gametes. The toxic factor was removed by washing the oil with 10 percent V/V of Whitten's complete medium for 4 to 5 days, followed by centrifugation at 2000g for 20 min. This treatment is analogous to equilibration of the paraffin oil with medium as reported earlier, except that the period of time for equilibration is increased.

The osmolarity of Whitten's (1971) medium was maintained at 290 mOSM, after either adding or removing energy sources, by adjusting the sodium chloride concentration. Crystalline bovine serum albumin (3 mg/ml), streptomycin sulfate, and penicillin were always present in the salt solution. Sperm suspensions were prepared by cutting the cauda epididymides into small sections in 1 ml of medium, usually without any energy substrates, and allowing the sperm to disperse into the medium. Ten microliters of the sperm suspension (approximately 2.5×10^5 sperm) was added to each watchglass containing ova. Cumulus cells were removed from ova, where applicable, by hyaluronidase (360 N.F.U./ml) and the ova washed three times with 1 ml aliquots of the respective media used in various experiments. Care was taken in all cases to prevent the contamination of either the media or the gametes with unwanted sources of energy.

Effect of Glucose, Pyruvate, Lactate and Cumulus Cells on Fertilization in vitro

Since Whitten's medium has been successfully used for the fertilization and preimplantation development *in vitro* of mouse ova, it can conveniently be employed to determine which of the three energy sources (glucose, pyruvate and lactate) that are included in the medium are required for fertilization. Experiments were designed to determine to what extent fertilization of ova occurred when complete medium was used, when all energy sources were omitted from the medium and when the only energy source was glucose (5.55 mM) or fructose (5.5 mM). The effect of fructose was examined since this sugar is present in high concentration in the male reproductive tract. Ova without cumulus cells were used in all experiments, except when the effect of cumulus cells was under examination. The ova were incubated for 6 to 7 h before they were placed in culture, and the number of cleaving zygotes was determined.

Initial experiments indicated that glucose was the major source of energy utilized during fertilization. However, either cumulus cells or pyruvate were needed to maintain ova viability since a number of ova incubated with sperm in medium containing glucose as the only energy source extruded the second polar body but failed to form pronuclei after 6 h of incubation. The possibility that pyruvate is also required for sperm capacitation was determined by examining the effect of preincubating the sperm in media with and without pyruvate upon their subsequent ability to fertilize ova. The experimental procedures for preincubating sperm were identical to those reported earlier (Hoppe and Whitten, 1974). The sperm were preincubated in Whitten's medium without lactate (calcium chloride was substituted for calcium lactate) for 3 h either with or without sodium pyruvate (0.23 mM), but in the presence of glucose, before adding ova free of cumulus cells to the dishes containing the sperm. At the end of the preincubation period pyruvate was added to dishes from which pyruvate had been omitted in order to maintain ova viability during fertilization. Ova free of cumulus cells were pipetted into dishes, removed after 1, 2, or 3 h of incubation, placed in culture and the number cleaving and developing to blastocysts was determined. Three experiments were conducted with a total of five observations per mean. The differences between means was determined using a t test (Steele and Torrie, 1960).

A possible effect of sodium lactate on sperm capacitation was examined by removing ova at various times after mixing with sperm to observe any differences in the proportion of ova fertilized in Whitten's medium with or without sodium lactate but with pyruvate and glucose. Ova free of cumulus cells were pipetted into dishes with or without lactate and 10 ul of sperm suspension prepared in medium without lactate were added to each dish. The dishes were then removed after 1, 2 or 3 h or incubation and the ova placed in culture where the number of cleaving ova and the number developing to blastocysts were observed. Five experiments were carried out with a total of 10 observations, except for 3 h where only nine observations were made.

Carbon Dioxide Production from Glucose and Fructose

Carbon dioxide production from glucose [^{14}C (U)] or fructose [^{14}C (U)] (New England Nuclear) by

epididymal sperm was assayed by incubating the sperm in 10 ml Erlenmeyer flasks containing 0.5 ml of Whitten's medium without pyruvate or lactate but with 1 mg/ml of either glucose or fructose and 2 ml of paraffin oil. The specific activity of the glucose and fructose containing medium was 0.33 and 0.36 mc/mM, respectively. Twenty microliters of cauda epididymal sperm suspension, prepared in Whitten's medium without any energy source were pipetted into each flask. Flasks without sperm were prepared to serve as background controls. The flasks were sealed with rubber septum through which a plastic well was suspended above the paraffin oil and were gassed with 5 percent CO_2, 5 percent O_2, and 90 percent N_2. Incubations were carried out for 1, 2 and 3 h at 37°C on a gyrotory shaker (60 rpm), after which 0.1 ml of 4 N H_2SO_4 was added to the medium and 0.3 ml of hydroxide or hyamine 10-X (Packard) was injected into the plastic well. Flasks were then gently shaken at room temperature (approximately 22°C) for 90 min in order to collect the $^{14}CO_2$ into the hydroxide of hyamine. In initial experiments, it was found that 90 min was required to collect approximately 95 percent of the $^{14}CO_2$ released from $NaHCO_3$ (^{14}C) under the conditions of these experiments. The plastic wells were then carefully removed from the flasks and placed in scintillation vials containing 10 ml of scintillation fluid. The scintillation fluid contained 5 gm of 2,5-diphenyloxazole (PPO, Packard) and 0.2 gm of 1,4-bis-2 (4-methyl-5-phenyloxazolyl) benzene (Popop, Packard) per liter of toluene (Fisher)-Triton X-100 (Packard), 2:1. $^{14}CO_2$ produced from glucose and fructose by sperm was counted in a Nuclear Chicago Unilux scintillation counter allowing sufficient time to count a minimum of 2000 counts per sample. The sperm concentration in the flasks was determined with a hemocytometer. Three experiments were conducted with a total of six observations per time period. Data were expressed as dpm/10^6 sperm, the differences between means were determined by analysis of variance (Steele and Torrie, 1960).

Production of $^{14}CO_2$ by sperm from glucose carbon atoms labeled either in the 1 or 6 position was determined by procedures that were similar to those described for measuring $^{14}CO_2$ production from glucose and fructose. Glucose [1-^{14}C] or [6-^{14}C] was added to the Whitten's medium containing 1 mg/ml glucose with a specific activity of 0.3 mc/mM.

RESULTS

Effect of Glucose, Fructose, Pyruvate, Lactate and Cumulus Cells on Fertilization in vitro

Table 1 summarizes the results of a number of experiments in which fertilization was carried out in media containing various sources of energy. The use of Whitten's medium without energy substrates resulted in the fertilization of 16 percent of the cumulus cell free ova. Sperm exhibited very sluggish motility in the absence of all energy sources. The addition of glucose maintained normal sperm motility, but only 20 percent of the ova were fertilized, as indicated by the proportion of the ova that cleaved. A more important observation was that a significant number of the ova had extruded a second polar body after 6 h of incubation with sperm, but there was a failure in pronuclei development. Ova transferred into Whitten's medium for culture usually did not cleave but fermented, suggesting that the ova had been irreparably damaged during 6 h incubation in medium containing glucose as the energy source. When the medium contained glucose alone and the ova were surrounded by cumulus cells, normal fertilization rates were obtained (94 percent). The beneficial effect of the cumulus cells may be due to their ability to produce pyruvate from glucose for the mainte-

TABLE 1. Fertilization of mouse ova by epididymal sperm in medium containing various energy substrates.

Treatment	No. observed	No. ova	No. fertilized	Percent (range)
None	7	76	12	16 (0-42)
Glucose	4	54	11	20 (8-31)
Glucose + cumulus cells	3	31	29	94 (82-100)
Glucose + pyruvate + lactate (Whitten's medium)	7	82	72	88 (64-100)
Fructose	4	53	4	8 (0-15)
Fructose + cumulus cells	3	25	0	0
Fructose + pyruvate + lactate	7	82	20	24 (0-56)

Fertilization was observed when gametes were incubated in Whitten's medium containing 1 mg/ml (5.5 mM) of either glucose or fructose in combination with ova surrounded by cumulus cells or in medium additionally supplemented with pyruvate (0.23 mM) and lactate (23.29 mM). Incubations were carried out in watchglasses containing 0.5 ml of the respective medium and 2 ml of paraffin oil at 37°C with shaking (60 rpm) in an atmosphere of 5 percent CO_2, 5 percent O_2, and 90 percent N_2. After 6 to 7 h of incubation, ova from each watchglass (approx. 10) were placed in culture to observe the number cleaving.

nance of ova integrity (Biggers et al., 1967; Donahue and Stern, 1968). Fructose substituted for glucose did not support fertilization of ova. Sperm motility was very sluggish in the presence of fructose after 6 h of incubation, and the addition of a small amount of medium containing glucose to these dishes resulted in an instantaneous increase in sperm motility.

Fertilization with sperm preincubated either with or without pyruvate for 3 h before being added to cumulus cell-free ova is shown in Table 2. Although a slightly higher percentage (average of 13 percent) of the ova were fertilized after 1, 2 and 3 h of incubation with sperm that had been preincubated in the presence of pyruvate, the differences were not significantly different ($P>0.01$). No significant differences were observed in the development of the zygotes to blastocysts. The proportion of ova fertilized at the various hourly intervals are similar to the results of a previous study using preincubated sperm (Hoppe and Whitten, 1974). Also, comparison of the fertilization results in Table 2 with those in Table 3 indicates that sperm were capacitated during the preincubation period.

Fertilization of ova was not affected by deleting lactate from the medium, nor was there any significant difference in the percentage of zygotes developing to blastocysts (Table 3(. These results indicate that capacitation of sperm and fertilization of ova can be accomplished in the absence of lactate.

TABLE 2. The effect of preincubating epididymal mouse sperm in Whitten's medium with or without pyruvate on their subsequent ability to fertilize cumulus cell-free ova.

	Without pyruvate				With pyruvate			
Time (h)	No. ova	No. fertilized	Percent (range)	Percent of zygotes developing to blastocysts	No. ova	No. fertilized	Percent (range)	Percent of zygotes developing to blastocysts
1	58	36	62 (38-91)	80	56	43	77 (45-100)	81
2	58	42	72 (43-100)	71	58	47	81 (71-100)	76
3	60	47	78 (50-100)	89	58	54	93 (78-100)	85

Ten microliters of a cauda epididymal sperm suspension prepared in Whitten's medium without lactate and pyruvate was pipetted into watchglasses containing 0.5 ml of medium without lactate and either with or without pyruvate (0.23 mM) and 2 ml of paraffin oil. After 3 h incubation of sperm under standard conditions approximately ten ova free of cumulus cells were pipetted into each watchglass and sodium pyruvate (0.23 mM) was added to those watchglasses containing sperm that had been preincubated without pyruvate. Ova were removed at hourly intervals and placed in culture tubes to observe the number cleaving and developing to blastocysts. Three experiments with a total of 5 observations per treatment were performed.

Carbon Dioxide Production from Glucose and Fructose

The rate of CO_2 production by epididymal sperm from glucose was approximately twice that from fructose during 3 h of incubation (Table 4). Thus, it is possible that fructose failed to support fertilization because it was not metabolized rapidly enough to provide energy required for maintenance of motility and sperm capacitation. Rates of CO_2 production by sperm from glucose labeled in either the 1-^{14}C or 6-^{14}C position were not significantly different over a 3 h incubation period (Table 4). Apparently sperm oxidize glucose predominantly by the glycolytic pathway.

DISCUSSION

Of the three energy substrates present in Whitten's medium, glucose is the only one that is required for sperm capacitation and fertiliza-

TABLE 3. Fertilization of cumulus cell-free ova by epididymal mouse sperm in Whitten's medium with and without lactate.

Time (h)	No. observed per treatment	Without lactate				With lactate			
		No. ova	No. fertilized	Percent (range)	Percent of zygotes developing to blastocysts	No. ova	No. fertilized	Percent (range)	Percent of zygotes developing to blastocysts
1	10	100	10	10 (0-27)	80	97	6	6 (0-22)	83
2	10	100	32	32 (0-67)	78	101	19	20 (10-56)	95
3	9	90	42	47 (20-70)	78	91	40	44 (20-89)	92

Gametes were incubated under standard conditions in watchglasses containing 0.5 ml of medium with or without lactate (23.29 mM) and 2 ml of paraffin oil. Ten microliters of a cauda epididymal sperm suspension prepared in Whitten's medium without lactate was pipetted into each watchglass containing approximately ten cumulus cell-free ova. At three hourly intervals after adding sperm, ova from each watchglass were placed in culture tubes and the number cleaving and developing to blastocysts were observed. Five experiments were performed.

tion of mouse ova *in vitro*. However, either cumulus cells which metabolize glucose to pyruvate (Biggers et al., 1967; Donahue and Stern, 1968) or pyruvate are required to maintain ova viability during fertilization. Recently it has been reported that pyruvate was required to maintain the viability of rat gametes during fertilization *in vitro* (Tsunoda and Chang, 1975). My observations that pronuclei failed to develop during fertilization of ova in the absence of pyruvate is in agreement with the findings of Whittingham (1969). In the latter study, zygotes cultured in the absence of pyruvate failed to complete the process of syngamy. Evidence that pyruvate is required during fertilization by the ova and not by the sperm is indicated by the fact that sperm motility was maintained and capacitation occurred during 3 h of preincubation in a pyruvate-free medium. However, it is conceivable that sperm might need a brief exposure to pyruvate before ova penetration since normal fertilization was only observed in the presence of pyruvate. Similarly, lactate is not required for sperm capacitation or for maintenance of ova viability because the proportion of ova fertilized 1, 2 and 3 h after mixing with sperm in medium with and without lactate was not significantly different. It has been well documented that lactate is not metabolized by the zygote until after the first cleavage (Brinster, 1965).

These results appear to contradict the observations of Miyamoto and Chang (1973), who reported that a medium containing pyruvate, lactate, glucose and serum albumin was required to achieve optimal sperm capacitation and fertilization of mouse ova with surrounding cumulus cells. A recent report has shown that pyruvate and lactate are required to achieve the acrosome reaction in guinea pig sperm and that the presence of glucose or other glycolyzable sugars have an inhibitory effect on the acrosome reaction (Rogers and Yanagimachi, 1975). Although it is possible that the discrepancy may be a consequence of mouse strain or species differences in the energy sources required by sperm, a more plausible explanation has to do with the fact that the conditions used to determine the energy sources required for sperm capacitation in the previous reports referred to above were more aerobic (20 percent O_2 atmosphere) than in the present studies (5 percent O_2 atmosphere). Under relatively anaerobic conditions energy for capacitation

TABLE 4. Production of CO_2 from glucose [^{14}C(U), 1-^{14}C, and 6-^{14}C] and fructose [^{14}C(U)] by epididymal mouse sperm.

Experiment	Fructose [^{14}C(U)]	Glucose		
		[^{14}C(U)]	[1-^{14}C]	[6-^{14}C]
1	1710 ± 239[a]	2894 ± 158[a]	660 ± 71[a]	434 ± 68[a]
2	1974 ± 324[a]	4719 ± 489[b]	1178 ± 129[ab]	957 ± 138[a]
3	3377 ± 775[ab]	6969 ± 664	1963 ± 291[b]	1923 ± 361[b]

Mean dpm/10^6 sperm ± S.E. with same superscripts are not significantly different ($P>0.05$) for glucose [^{14}C(U)] versus fructose [^{14}C(U)] and [1-^{14}C] versus [6-^{14}C] glucose. Twenty microliters (approx. 5×10^5 sperm) of a cauda epididymal sperm suspension prepared in Whitten's medium without any energy course was pipetted into 10 ml Erlenmeyer flasks containing medium with either 1 mg/ml (5.55 mM) of glucose [^{14}C(U), 0.33 μc/μM, 1-^{14}C and 6-^{14}C, 0.30 μc/μM] or fructose [^{14}C(U), 0.36 μc/μM] and 2 ml of paraffin oil. Flasks were incubated at 37°C with shaking (60 rpm) in an atmosphere of 5 percent CO_2, 5 percent O_2, and 90 percent N_2. After the specified incubation period, 0.1 ml of 4 N H_2SO_4 was added to the medium and the CO_2 was collected in 0.3 ml of hydroxide of hyamine 10-X during 90 min of incubating the flask at room temperature. The radioactivity in the hydroxide of hyamine was counted. Three experiments with a total of 6 observations were performed except for 5 observations at 2 h incubation with uniformly labeled glucose.

and fertilization may be generated largely by glycolysis, whereas under more aerobic condition oxidation of pyruvate and lactate may have been facilitated and anaerobic glycolysis inhibited (Pasteur effect). An atmosphere containing 5 percent oxygen is approximately optimal for the preimplantation development of mouse embryos in culture (Whitten, 1971; Haidri et al., 1971). However, information is not available as to whether or not similar anaerobic conditions exist in the mouse oviduct.

The rate of CO_2 production from glucose by epididymal sperm was approximately twice that produced from fructose. Oxidation of glucose was linear during the 3 h incubation period and no irregularity in the rate of glucose oxidation could be correlated with the occurrence of sperm capacitation. Sperm incubated in the presence of fructose were agglutinated and exhibited sluggish motility resembling the appearance of sperm freshly collected from the epididymis. This motility pattern continued over several hours of incubation. However, the introduction of a small amount of medium containing glucose into the center of a watchglass in which the sperm were incubated with fructose resulted in an instantaneous dispersion of the sperm and an increase in motility that was propagated peripherally as the medium diffused in the watchglass. A possible explanation for the failure of fructose to provide sufficient energy for the maintenance of sperm motility and capacitation involves a deficiency either in the transport or intracellular metabolism of fructose as compared to glucose.

Rates of CO_2 production by mouse sperm from carbon atoms number 1 and 6 of glucose were not significantly different and are in agreement with results obtained with epididymal sperm from bulls and rams (Wu et al., 1959). Freshly ejaculated rabbit sperm also oxidizes the C-1 and C-6 of glucose at similar rates, but preferentially oxidizes the C-1 atom after capacitation *in utero*, indicating an increase in the activity of the pentose shunt (Hamner and Williams, 1963; Mounib and Chang, 1964; Murdock and White, 1967). No such shift in the oxidation of C-1 and C-6 of glucose was observed during capacitation *in vitro* of mouse sperm, indicating that glycolysis is probably the major pathway of glucose utilization during capacitation.

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