Arginine Activates Glycolysis of Goat Epididymal Spermatozoa: An NMR Study

Anant B. Patel, Sudha Srivastava, Ratna S. Phadke, and Girjesh Govil
Tata Institute of Fundamental Research, Navynagar, Mumbai 400 005, India

ABSTRACT The present study explores the mechanism underlying the action of L-arginine on the metabolic activity of spermatozoa. Goat epididymal spermatozoa were incubated with different concentrations of L-arginine to determine its effect on the utilization of glucose, fructose, and pyruvate. NMR techniques have been applied to elucidate the effect of L-arginine, L-lysine, and L-ornithine on the glycolysis of epididymal goat spermatozoa. Whereas ^{31}P NMR has been used to estimate the change of pH in the presence of different concentrations of L-arginine, ^{13}C NMR has been used to estimate the substrate consumption and lactate production. At optimal concentration of L-arginine, the forward metabolic rates have been found to increase by two to three times over control experiments. Arginine is not consumed in these reactions, but acts as an activator. Longitudinal relaxation time (T_1) measurements indicate that the guanidino group of L-arginine plays an active role in binding to cells. The amino acid L-lysine is less effective, and L-ornithine is ineffective.

INTRODUCTION

It has been suggested that some of the amino acids detected in seminal plasma play an important role in spermatozoal metabolism and motility (Gassner and Hopwood, 1952). One of these is L-arginine, which is often referred to as a nonessential amino acid. It is indispensable for protein synthesis, and it plays a key role in modulating host defenses and cellular immunity (Eremin, 1997). During the active stages of growth, such as in infancy, the amount of L-arginine synthesized by the cells is not sufficient to meet the demand for this amino acid. L-Arginine takes part in sperm formation and has been found to be a basic component of the nucleoprotein of spermatozoa of various species (Adnan, 1970).

As early as 1944, Holt and Albanesi demonstrated a correlation between L-arginine deficiency and loss of normal functioning of testes. L-Arginine is known to increase spermatogenesis and acts as a source of energy for normal sperm motility in the form of arginine phosphoric acid. Schachter et al. (1973) have suggested that a deficiency in L-arginine due to poor diet, improper absorption, or abnormally active decomposition causes a subsequent derangement of metabolism in tissues undergoing frequent mitosis. Other reports (Bernard, 1967; Tanimura, 1967; Keller and Polakoski, 1975; Jungling and Bunge, 1976) describe a correlation between L-arginine deficiency and loss of spermatogenesis and a decrease in the motility of the sperm cells. Administration of L-arginine to oligospermic and asthenospermic patients results in an improvement in both the sperm count and motility without any side effects (Scibona et al., 1994; Aydin et al., 1995). However, no attempts have been made to investigate the mechanism underlying the action of L-arginine on the metabolic activity of spermatozoa.

In recent years, NMR has emerged as a powerful, nondestructive and noninvasive technique for the study of cell metabolism (Gupta, 1987; Gadian, 1995). In this paper, we report inferences drawn from NMR experiments conducted in real time on the action of L-arginine on viable spermatozoa under anaerobic conditions. In addition, the effect of related amino acids such as L-lysine and L-ornithine has been studied.

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Address reprint requests to Dr. Sudha Srivastava, National Facility for High Field NMR, Tata Institute of Fundamental Research, Homi Bhabha Road, Navynagar, Mumbai 400 005, India. Tel.: 2152971, Ext. 2271; Fax: 091-22-215-2110/2181; E-mail: sudha@tifrvax.tifr.res.in.

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MATERIALS AND METHODS

Chemicals

L-Arginine, L-lysine, L-ornithine, and 1-^{13}C isotope-labeled glucose were purchased from Sigma Chemical Co. (St. Louis, MO). Other isotope-labeled compounds were obtained from Isotech (Matheson, OH). Other chemicals used were of AR grade. Dulbecco's medium (pH 7.2) with 0.1% glucose (w/v) was used as a buffer and fuel for cellular metabolism, unless stated otherwise.

Cells

Spermatozoa recovered from random samples have been used to maintain the generality of the observations. The viability of the cells has been found to be normal up to at least 2.5 h after separation from the epididymis. Most experiments were therefore concluded within this period. Goat testes were procured from a slaughterhouse and used within 1 h of sacrifice. The epididymis was separated from the testes. The cells from the cauda region of the epididymis were collected by gentle mincing and tweezing in Dulbecco's buffer. Tissue pieces were removed by allowing the cell suspension to settle for 5 min. The cells were washed, made into a pellet by centrifugation, and then suspended in an appropriate quantity of buffer to attain the desired concentration of cells. The motility of the cells was checked from time to time with an optical microscope. Cells exhibiting more than 60% motility were used for all experiments. Testes from one animal were used for one set of experiments. Experiments were carried out at 298 K. Cells were counted with a cytometer, and cell concentration was maintained at 1×10^6 cells/ml in all experiments.

NMR

Experiments were carried out on a Bruker AMX 500 FT-NMR spectrometer. D_2O (10%) was used in the buffer for field-frequency locking. Sodium 3-trimethylsilyl[2,2,3,3-D]-propionate (TSP) has been used as a reference for ^1H and ^{13}C NMR chemical shifts. A 10-Hz exponential multiplication factor was applied before the Fourier transformation in both ^{31}P and ^{13}C NMR experiments.

The longitudinal relaxation time (T_1) for the ^{15}N nucleus was measured using the inversion recovery pulse sequence (D_1-π-VD-π/2-acq)$_n$, where D_1 is the relaxation delay, *VD* is the variable delay, and *n* is the number of transients accumulated (Freeman and Hill, 1969, 1970). T_1 was calculated from the inversion recovery signals with built-in Bruker software. For ^{13}C T_1 measurements, a gradient-based, sensitivity-enhanced, heteronuclear single quantum coherence pulse sequence incorporated with an inversion recovery sequence was used (Palmer et al., 1991).

Partition coefficients

The partitioning of L-arginine between the intra- and extracellular media was determined both by NMR and by a biochemical method. In NMR measurements, the required amount of washed packed cells (so as to maintain the cell count) was incubated for 1 h with a 10 mM solution of L-arginine labeled with ^{15}N at the guanidino group. The ^{15}N NMR spectrum of this sample was recorded. The sample was then centrifuged at 2000 rpm, and the pelleted cells were removed. The NMR spectrum of the supernatant solution thus obtained was also recorded. Care was taken to keep all of the conditions identical in the two experiments, including the NMR active volume in the sample tube. The difference between the integrated intensity of the spectrum of the cell suspension and that of the supernatant solution gives a measure of the intensity of the signal arising from L-arginine bound to the cells. Using this data, the fraction of arginine bound to the cells was estimated.

In the biochemical method, the ninhydrin test (Stewart and Young, 1969) was carried out to estimate the concentration of arginine present in the solution of arginine (10 mM) and in the supernatant obtained from cell suspension incubated with the arginine (10 mM), using the procedure outlined above. Optical density (OD) at 565 nm was monitored for this purpose. From the knowledge of the OD of free and supernatant solutions, the fraction of arginine bound to cells was calculated.

pH measurements

The ^{31}P NMR signal of inorganic phosphate (P_i) is a time average of signals due to various anionic forms of phosphoric acid, and therefore its chemical shift depends on the pH. At physiological pH, the main species are $H_2PO_4^-$ and HPO_4^-. The observed chemical shift of P_i signal therefore depends on the association constant (K_a) for the above reaction and the chemical shifts (R_1 and R_2) of the ^{31}P signal for the two anions. Because the chemical shifts may also depend on other environmental conditions, a correspondence between the chemical shift and pH has been established by generating a calibration curve. Dulbecco's buffer with ionic strength similar to that of the intracellular environment has been used, and the chemical shift positions of P_i signal at different pH have been determined. The observed chemical shifts have been fitted to the following modified form of the Henderson-Hasselbalch equation (Smith et al., 1985) with an iterative least-squares fitting program:

$$pH = pK_a + \log[(\Delta - R_1)/(R_2 - \Delta)] \quad (1)$$

where Δ is the absolute value of the observed chemical shift, and R_1, R_2, pK_a are used as unknown variables. The values of the above constants have been estimated as 0.06, 2.6, and 6.6, respectively, from the least-squares fit. The pH of the cell suspension can thus be calculated from the observed P_i chemical shift (Δ) values with Eq. 1.

Cellular metabolism

Three different isotope-labeled molecules, 1-^{13}C glucose, 1-^{13}C fructose, and 3-^{13}C pyruvate have been used as substrates. These molecules enter the glycolytic pathway at different stages. The requisite amount of L-arginine was added to the cell suspension, and incubation was allowed for half an hour for equilibration (Radany et al., 1981). The substrates were then added, and ^{13}C NMR spectra were recorded as a function of time. Thirty-two transients were accumulated in each case, which took ~1 min for each experiment. The mid-time for each experiment was taken as the corresponding experimental time. Intensity of the NMR signal was measured by peak integration (area under the curve) with built-in Bruker software. We feel that this is satisfactory for measuring the relative concentration of a particular molecule as a function of time. However, a more detailed procedure was adopted while measuring relative concentrations of different molecules because of the differences due to relaxation times that may affect the integrated peak intensities of different compounds, when short relaxation delays are used in the NMR experiments. The rate of the substrate consumption and lactate build-up were determined by acquiring data over a 2-h time period.

RESULTS AND DISCUSSION

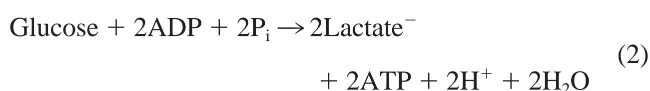
While interpreting experimental results, one should realize that because the sperms have been extracted from wild animals belonging to different age groups, the observations are qualitative indicators of the trend in the activation induced by L-arginine. The quantitative aspects of the effect of L-arginine depend on the quality and, in particular, on the initial motility of the sperm cells (Keller and Polakoski, 1975), and hence may vary from one set of experiments to another. This aspect is difficult to control in the experiments, and therefore the conclusions below are semiquantitative in nature.

^{31}P NMR

^{31}P NMR is particularly advantageous for in vivo studies, as it offers the possibility of monitoring energetics of viable cells. Fig. 1 *a* depicts the ^{31}P NMR spectrum of spermatozoa recovered from the cauda region of epididymis of sacrificed goat and suspended in Dulbecco's medium containing 0.10% (w/v) glucose. Assignments of the signals have been made by a spiking method and by comparing with the (Gadian, 1995; Elgarish, 1987) data reported in the literature (Navon et al., 1978; Kaplan et al., 1992). These are indicated in the figure caption.

Fig. 1 *b* shows the ^{31}P NMR spectrum of cells undergoing glycolysis, which have been incubated with 10 mM L-arginine. The effect of arginine on the rate of glycolysis is dramatic. The ATP signals build up with glycolysis, and P_i signal shifts up-field with a concomitant loss in its intensity. This may be explained by considering the phosphorylation of ADP with P_i in the presence of glucose by the following reaction:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i \rightarrow 2\text{Lactate}^- + 2\text{ATP} + 2\text{H}^+ + 2\text{H}_2\text{O} \quad (2)$$



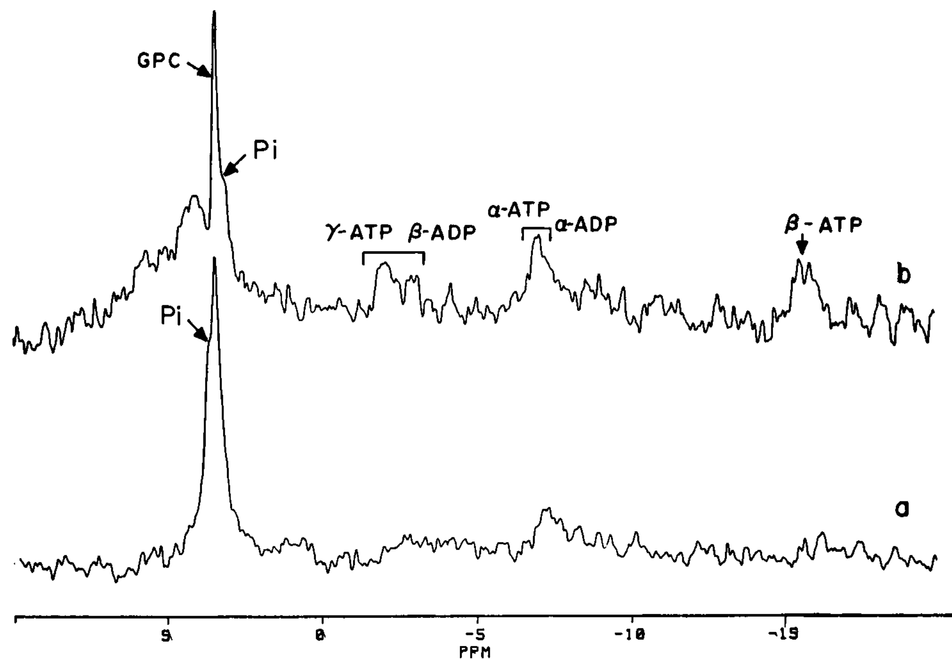


FIGURE 1 ^{31}P NMR spectrum (202.5 MHz) of spermatozoa obtained from the cauda region of epididymis of sacrificed goat. (*a*) Cells undergoing glycolysis in Dulbecco's medium (initial pH 7.2), containing 10% (w/v) glucose but no arginine. (*b*) Cells undergoing glycolysis after incubation with L-arginine (10 mM) for 30 min. Spectral parameters used are 2-s relaxation delay, 10-μs pulse width corresponding to 60° flip angle and 10-kHz spectral width. Chemical shifts have been reported with respect to a phosphocreatine signal. The resonance signal at -2.30 ppm arises from the γ-P-ATP (with a small contribution from β-P-ADP). Peaks from α-P-ATP and the β-P-ATP can be observed at -6.78 ppm and -15.44 ppm, respectively. A signal at 3.0 ppm corresponds to inorganic phosphate (P_i). The P_i resonance is a composite signal with contributions from intra- and extracellular inorganic phosphate, which are not significantly different at normal pH and therefore overlap. A strong signal slightly up-field to P_i corresponds to glycerophosphoryl choline (GPC).

A progressive up-field shift of the P_i signal can be ascribed to the acidification of the extra- and intracellular environments due to the formation of lactate after glucose metabolism (Parrish et al., 1989). The enhancement of the ATP signals is indicative of higher rates of phosphorylation in the presence of arginine. The above reaction is therefore catalyzed by the presence of small concentrations of L-arginine.

Attempts have been made to use changes in pH as a parameter to estimate the influence of L-arginine on the metabolic activity of the spermatozoa. For this purpose, changes in the chemical shift of the P_i signal have been used to estimate the change in pH as a function of time, during glycolysis of cells incubated with different amounts of arginine (Fig. 2). It is observed that for a set of identical conditions (i.e., number of cells/ml, temperature, viability, motility, pH, etc.), the relative rate of change in pH increases up to ~20 mM L-arginine, and thereafter it declines (Fig. 2). It is worth noting that large concentrations of L-arginine lead to deactivation of glycolysis.

The addition of L-arginine increases the pH of the cell suspension (30 mM arginine changes the pH of the cell suspension to 7.8). To check if there is a direct influence of pH on metabolic activity, a control experiment has been done by suspending the cells in buffers of different pH in the absence of L-arginine. It has been found (data not shown) that glycolysis is independent of pH over the range of 6.2–8.2 under normal conditions. Similar observations on effect of pH have been reported earlier (Babcock et al., 1983). We can therefore conclude that at concentrations below 30 mM, L-arginine increases the metabolic activity of spermatozoa, but above these concentrations it deactivates the metabolism.

^{13}C NMR

The rate of glycolysis and the metabolic pathway of sperm cells have been studied by ^{13}C NMR. Three specifically labeled substrates (glucose, fructose, and pyruvate) have been added to cell suspensions to monitor the metabolism of such molecules, starting with different steps of the glycolytic pathway. It is known that in the first few steps of glucose metabolism, there are secondary pathways before one reaches fructose. Similarly, at the level of pyruvate metabolism, the metabolism can lead to the formation of lactate and alcohol under anaerobic conditions and to the formation of coenzyme A as a gateway to the Krebs cycle. The use of these three substrates therefore allows one to monitor arginine-induced metabolic conversions at various points in the glycolytic pathway.

Fig. 3 shows the relevant portion of a ^{13}C NMR spectrum of spermatozoa incubated with 1-^{13}C glucose. Assignments of ^{13}C resonances arising from glucose and its metabolites have been made using standard procedures (Canioni et al.,

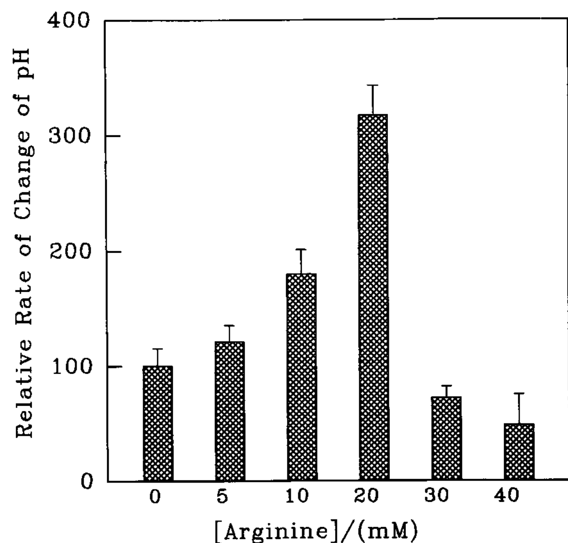


FIGURE 2 Relative rate of pH change of cell suspension with varying concentrations of L-arginine. pH has been estimated using NMR spectrum as discussed in the text. The rate of change of pH as a function of time during the first 60 min of glycolysis by the cells incubated with arginine has been compared with the corresponding rate of cells in the absence of arginine (control). Data are expressed with respect to the control, taking it as 100. The increase in the rate of metabolism in the presence of small concentrations of L-arginine can be clearly seen.

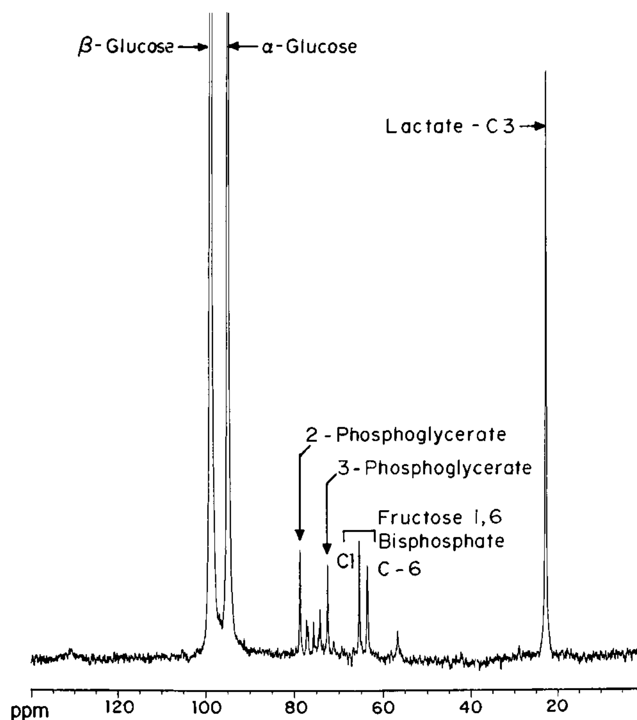


FIGURE 3 ^{13}C NMR spectrum (125.76 MHz) of spermatozoa obtained from the cauda region of goat epididymis. Spermatozoa were incubated with 1-^{13}C-labeled glucose, and the spectrum was recorded after 2 h of glucose addition. Spectral parameters are 25-kHz spectral width, 60° flip angle, 2-s relaxation delay, and 1024 transients with power-gated broadband proton decoupling. Signals: α and β anomers of 1-^{13}C glucose, 3-^{13}C lactate, and intermediates of the glycolytic pathway such as 2-phosphoglycerate, 3-phosphoglycerate, and fructose-1,6-bisphosphate. There are some additional signals that could not be assigned.

1983; Santos et al., 1985). The two intense signals at 94.7 ppm and 98.4 ppm correspond to α and β anomers of D-glucose, respectively. The other strong signal centered at 22.9 ppm has been assigned to 3-^{13}C (methyl group) of lactate. Several other signals in the region 60–80 ppm are assigned to glycolytic intermediates and have been marked accordingly.

The intensities of signals arising from the substrate and 3-^{13}C lactate have been monitored as a function of time with the different substrates glucose, fructose, and pyruvate. The time evolution of glucose and lactate signal intensities for cells incubated with different concentrations of L-arginine is shown in Fig. 4, *A* and *B*, respectively. The profile shows that initially there is a large substrate consumption, which tends to slow at longer times. A similar behavior, i.e., an increase in signal intensity, is observed for lactate generation. The slower glucose consumption at longer times is partly due to the decrease in glucose concentration and partly to the decrease in the motility of cells under anaerobic conditions.

The data have been fitted to a first-order rate equation to determine the rate constants for substrate consumption and lactate generation. The results of such an analysis are shown in Fig. 5. One observes that the rates of glucose consumption and lactate generation increase monotonically with an increase in the L-arginine concentration until 30 mM and drops thereafter. The maximum increase in the glycolytic rate is found to be two to three times of that of the control.

The percentage increase in the rate of substrate consumption and that for lactate generation studied for three different substrates (glucose, fructose, and pyruvate) in the presence of 10 mM L-arginine are given in Table 1. The rates are in the order glucose > fructose > pyruvate. The data again shows a two- to threefold increase in the rate of substrate consumption and lactate production due to the presence of L-arginine. Thus one can infer that stimulating action of L-arginine induces a higher glycolytic rate in spermatozoa at all three levels of the pathway.

Anaerobically incubated bull and ram spermatozoa that maintain high motility utilize the substrate at a higher average rate. A positive correlation between the rate of substrate consumption and the degree of motility has been drawn (Peterson and Freund, 1976). Therefore, both ^{31}P and ^{13}C NMR results, which show enhancement in the substrate consumption, lead to the conclusion that L-arginine enhances sperm motility.

Although lactate is the chief product of sperm glycolysis, it does not account for all of the sugar utilized anaerobically (Graves et al., 1966; Nevo et al., 1970; O'Shea and Voglmayr, 1970; Dacheux et al., 1979a,b; Voglmayr and White, 1971). The reasons may be the ability of spermatozoa to convert glucose into other metabolites such as phospholipid, inositol, etc. We have tried to correlate the rate of glucose consumption and that of lactate generation by giving longer

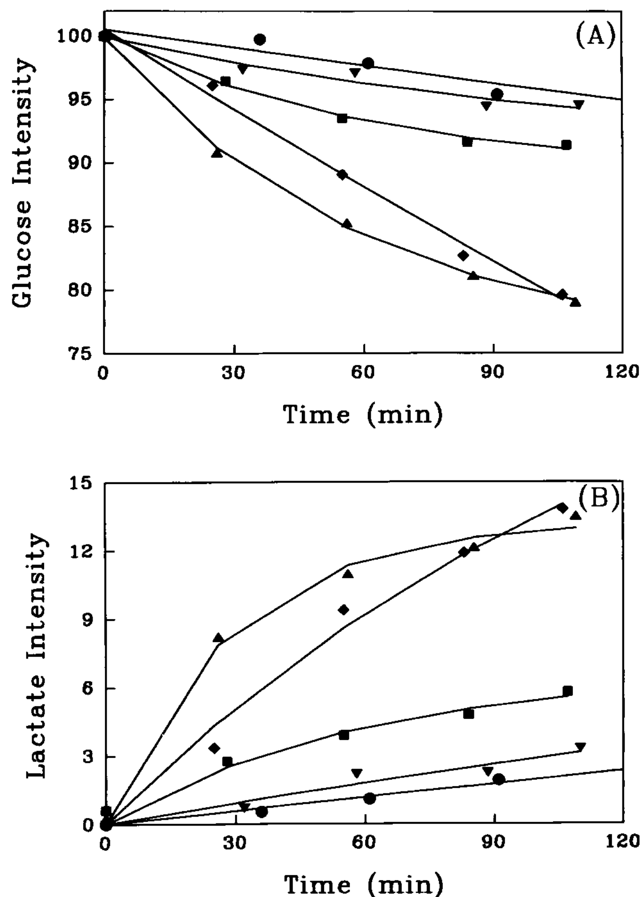


FIGURE 4 Variation in ^{13}C signal intensity with time. (*A*) Decrease in glucose concentration ($\alpha + \beta$). (*B*) Increase in lactate concentration. Cells were incubated for 30 min with different amounts of L-arginine. ●, 0 mM; ▼, 4 mM; ■, 10 mM; ▲, 30 mM; ◆, 40 mM. Glucose and lactate signals of subsequent spectra were integrated and are expressed with respect to the glucose signal of the first spectrum, which is assigned as 100.

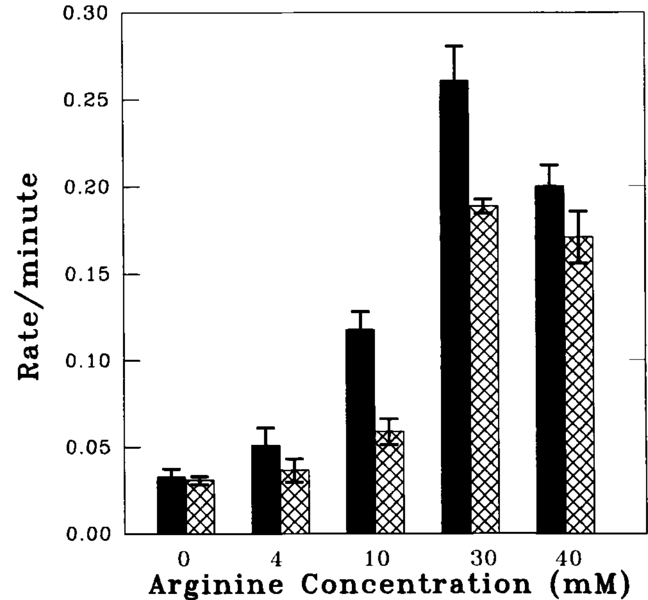


FIGURE 5 Rate of glucose consumption and that of lactate production for different concentrations of L-arginine. The data were fitted to a first-order rate equation (decaying) to determine the rate constant for substrate consumption, whereas for lactate generation a rising exponential equation was used for the calculation of the rate constant. ■, Glucose consumption rate. ▩, Lactate production rate.

relaxation delays so that the amounts of the two compounds can be estimated more quantitatively. Indeed, the amount of lactate generated was found to be smaller than the amount of glucose consumed. On the other hand, when pyruvate was used as the substrate, almost 100% of the label was found to go to the lactate signal. Unfortunately, difficulties in obtaining precise quantitative information from ^{13}C signal intensities does not allow us to comment on this problem more precisely.

Mode of action of arginine

Attempts have been made to trace the metabolites of L-arginine, by employing ^{1}H NMR. The ^{1}H NMR spectrum of cells incorporated with arginine does not show any signals other than those arising from native L-arginine after 2 h (Fig. 6). The arginine signals do show a downfield shift on incorporation into the cells, but do not show a decrease in intensities during glycolysis. This indicates that L-arginine itself is not metabolized, but only acts as an activator. Additional confirmation of this finding has been obtained by performing the above experiment with ^{15}N-labeled L-arginine at the guanidino group and monitoring the ^{15}N NMR spectrum with time. It was observed that the ^{15}N NMR signal remains unchanged, and no additional signal arising from metabolites of L-arginine could be observed. This further indicates that L-arginine itself is not metabolized. However, it has been observed that the L-arginine signal is broadened considerably after incubation with the cells. This indicates that L-arginine binds to the cells.

To gain more insight into the mode of binding, the ^{15}N longitudinal relaxation time (T_1) has been measured using ^{15}N-labeled L-arginine at the guanidino group. To calculate the T_1 of the bound fraction of L-arginine from observed T_1 data, the following equation has been used:

$$1/(T_1)_{obs} = n_{aq}/(T_1)_{aq} + n_b/(T_1)_b \qquad (3)$$

where n_b and n_{aq} are the cell-bound and free fractions of L-arginine, and $(T_1)_b$ and $(T_1)_{aq}$ are the corresponding spin lattice relaxation times. From the knowledge of $(T_1)_{obs}$ and $(T_1)_{aq}$, an estimate of $(T_1)_b$ can be obtained.

TABLE 1 Substrate consumption and lactate generation by spermatozoa in the presence of 10 mM arginine

Substrate	Percentage increase in substrate consumption rate*	Percentage increase in lactate production rate*
Glucose	170	230
Fructose	100	130
Pyruvate	30	70

*Expressed with respect to that in the absence of L-arginine.

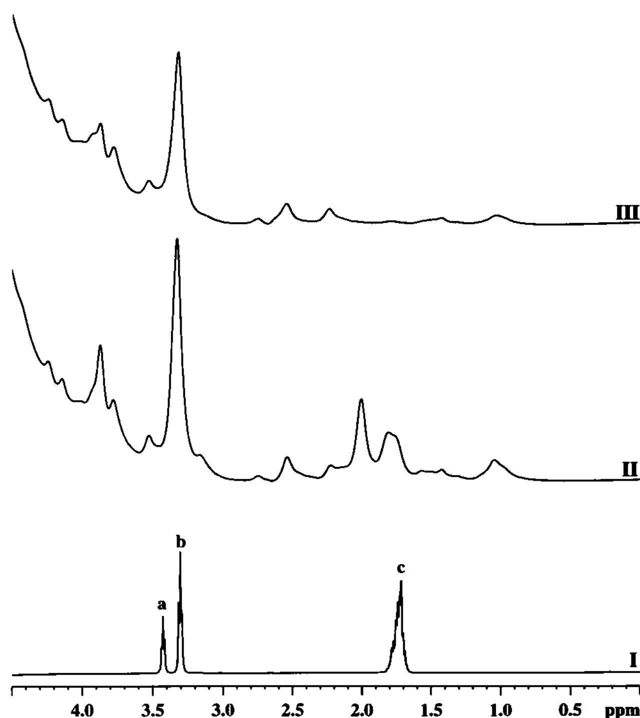


FIGURE 6 ^{1}H NMR spectra (500 MHz). (*I*) Arginine (10 mM) in buffer. (*II*) After 120 min of incubation of arginine with the cell. (*III*) Cell suspension in buffer. Assignments of arginine signals are as (*a*) CαH, (*b*) CδH, and (*c*) CβH and CγH protons. The strong signal at 3.3 ppm is due to GPC present in the cell.

Arginine is highly hydrophilic in nature. The partition fraction of arginine in the cell, calculated using methodology discussed earlier, gives rise to a partition fraction of ~10%. Using the above equation, $(T_1)_b$ was then calculated to be 2.87 s, as compared to $(T_1)_{aq}$, which is 5.25 s for free amino acid. This change in the ^{15}N relaxation time indicates that the guanidino group of L-arginine binds to cells, thereby giving rise to higher correlation times and lower T_1 values.

This result is supported by measurements of ^{13}C relaxation times (T_1) for arginine and ornithine (which lacks a guanidino group and does not activate glycolysis), in free solution and when incorporated into cell suspensions. Results indicate a large change in arginine ^{13}C T_1 values from free solution to that in cell suspension (Table 2). The change progressively increases from the α-carbon to the δ-carbon of the side chain. This indicates the involvement of the guanidino group end of the amino acid in the binding. However, the observed change in ornithine ^{13}C T_1 is very small. This small change could be due to the effect of cell inhomogeneity in the sample.

The above observation is in agreement with the report from Radany et al. (1981), in which competitive experiments with L-arginine analogs and amino acids show the active role of the guanidino group in L-arginine transport across the sperm cell. This indicates that L-arginine plays an important role in enhancing membrane permeability and thereby increased substrate permeation/utilization and concomitant lactate production.

To understand the role of the chain length and the functional group of L-arginine, experiments have been performed in the presence of amino acids related in structure to L-arginine, such as L-lysine and L-ornithine. The specificity of L-arginine action is strongly suggested by the fact that L-ornithine, which lacks a guanidino group and has a similar chain length, is unable to induce significant stimulations (Fig. 7). L-Lysine, which lacks a guanidino group but has a side chain length longer than that of L-arginine, induces glycolytic activity, which, although less, is comparable to that of L-arginine. It can thus be inferred that the chain length of the amino acid as well as the terminal guanidino group play crucial roles in inducing stimulating action. These results support earlier reports in which the special function of L-arginine has been established by comparing the efficacy of other amino acids in the treatment of spermatogenetic derangements. It is worth noting that whereas L-lysine caused considerable improvement, all other amino acids were totally ineffective (Keller and Polakoski, 1975).

CONCLUSION

It is thus concluded that the presence of relatively small concentrations of L-arginine not only enhances metabolism and spermatogenesis, but also the synthesis of adenosine triphosphate, an energy-rich compound essential for sperm motility. The side chain and guanidino group play essential roles. The results indicate that sperm cells require L-arginine for their motility, which shows the possibility of its potential clinical usefulness when human semen with subnormal motility is utilized for artificial insemination.

The NMR spectra were recorded at the National Facility for High Resolution NMR, located at TIFR, Mumbai, and supported by the Department

TABLE 2 Longitudinal relaxation time (T_1) of arginine and ornithine (in free solution and in cell suspension)

	^{13}C longitudinal relaxation time of different carbons (ms)					
	Arginine			Ornithine		
	Solution	With cells	% decrease	Solution	With cells	% decrease
αC	1818 ± 72	1658 ± 254	9	1904 ± 96	1737 ± 205	9
βC	752 ± 65	500 ± 92	34	744 ± 131	605 ± 106	19
γC	752 ± 65	462 ± 101	39	—	—	—
δC	790 ± 92	374 ± 51	53	908 ± 95	845 ± 206	7

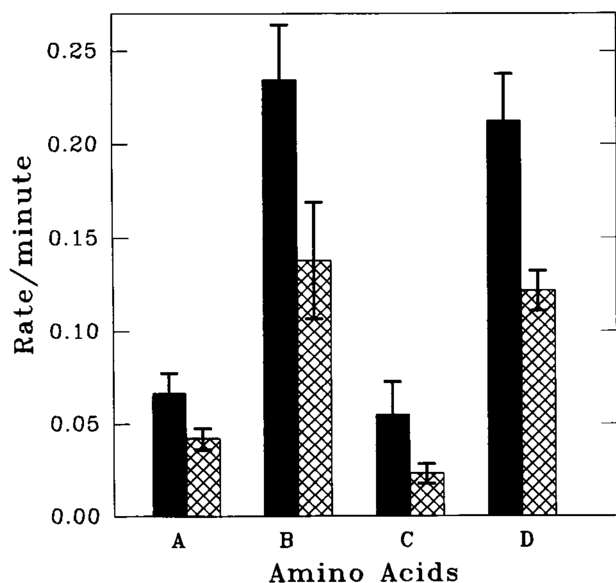


FIGURE 7 Effect of amino acids on the rate of glucose consumption and lactate production. The spermatozoa were incubated with 10 mM of amino acids for 30 min. (*A*) Control. (*B*) L-Arginine. (*C*) L-Ornithine. (*D*) L-Lysine. ■, Rate of glucose consumption. ▩, Lactate generation.

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