*Biochem. J.* (1967) **105**, 1061
*Printed in Great Britain*



# Amino Acids in Ram Testicular Fluid and Semen and their Metabolism by Spermatozoa

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(*Received* 19 *May* 1967)

1. The testis of the ram secretes considerable amounts of amino acids (200 μmoles/day) into the fluid collected from the efferent ducts. The principal amino acid in this testicular fluid is glutamate, which is present in concentrations about eight times those in testicular lymph or in blood from the internal spermatic vein. 2. The concentration of glutamate in seminal plasma from the tail of the epididymis is about ten times that in testicular fluid, and, though glutamate is the major amino acid in ejaculated seminal plasma, its concentration is less than in epididymal plasma. 3. After the intravenous infusion of [U-$^{14}$C]glucose, labelled glutamate was found in the testicular fluid. Radioactivity was also detected in alanine, glycine, serine plus glutamine and aspartate. Alanine had the highest specific activity, about 50% of the specific activity of blood glucose. 4. When [U-$^{14}$C]glutamate was infused, the specific activity of glutamate in testicular fluid was only about 2% that in the blood plasma. 5. Testicular and ejaculated ram spermatozoa oxidized both [U-$^{14}$C]glutamate and [U-$^{14}$C]leucine to a small extent, but neither substrate altered the respiration from endogenous levels. 6. No radioactivity was detected in testicular spermatozoal protein after incubation with [U-$^{14}$C]glutamate or [U-$^{14}$C]leucine. Small amounts of radioactivity were detected in protein from ejaculated ram spermatozoa after incubation with [U-$^{14}$C]glutamate. 7. The carbon of [U-$^{14}$C]glucose was incorporated into amino acids by testicular spermatozoa; most of the radioactivity occurred in glutamate.

Glutamate is the principal free amino acid in the testis and semen of most mammalian species, where it occurs mainly in the seminal plasma rather than in the spermatozoa (see Mann, 1964, p. 97). Studies by Gassner & Hopwood (1952) and Hopwood & Gassner (1962) have demonstrated that the epididymis and testis are the most likely sources of amino acids in bovine seminal plasma and that the accessory glands produce only small quantities. Similar results were obtained by Lake & McIndoe (1959) and Ahluwalia & Graham (1966) for the origin and distribution of amino acids in cock semen. The present studies were designed to examine the amino acid composition of fluid collected directly from the testis of conscious rams (Voglmayr, Waites & Setchell, 1966) and to compare this with blood plasma, testicular lymph, epididymal plasma and seminal plasma from entire and vasectomized rams. An attempt was made to determine the origin of the amino acids in testicular secretion. The metabolism of glutamate and leucine by ejaculated and testicular ram spermatozoa and the formation of amino acids from glucose by testicular spermatozoa were also investigated.

## MATERIALS AND METHODS

*Animals.* Eight Merino rams, 3–5 years old and weighing 40–50 kg., were used. They were kept in a room at 21° with 12 hr. of light/day and were fed daily on 1000 g. of a mixture of equal parts of chaffed lucerne hay and oat grain.

*Collection of samples.* All experiments were done on conscious animals at least 24 hr. after surgery. Testicular secretion was collected from the efferent ducts by the technique described by Voglmayr *et al.* (1966) and Voglmayr, Scott, Setchell & Waites (1967). Testicular lymph was collected from a catheter inserted in a testicular lymphatic duct (Cowie, Lascelles & Wallace, 1964) and blood plasma from catheters in a femoral artery and the internal spermatic vein (Setchell & Waites, 1964). Epididymal semen was collected through a silicone rubber catheter (1 mm. external diam. 0·5 mm. internal diam.) in the vas deferens, and epididymal plasma was separated by centrifugation for 20 min. at 800 *g*. Seminal plasma was obtained by centrifuging ejaculated semen, which was collected by the procedure of Blackshaw (1954).

*Infusions.* D-[U-$^{14}$C]Glucose (3 mc/m-mole) was diluted with sterile pyrogen-free 0·9% NaCl to give an activity of 1 μc/ml. A priming dose of 10 μc was given intravenously and then the dilute solution was infused by using a peristaltic pump (Technicon, Chauncey, N.Y., U.S.A.) for 5 hr. into a catheter in the recurrent tarsal vein at the rate of

0·15μc/min. L-[U-$^{14}$C]Glutamate (7·5mc/m-mole) was infused in like manner at 0·1μc/min. after a priming dose of 10μc. Collections of testicular secretion were made during and after the infusion and blood samples were taken from the internal spermatic vein after 1, 3 and 5hr. infusion.

*Analysis of amino acids.* Free amino acids were separated by precipitating the protein with 5 vol. of 1% (w/v) picric acid (Hamilton & Van Slyke, 1943) and treating the supernatant by the method of Stein & Moore (1954) before analysis on an amino acid analyser with a 130cm. × 0·63cm. column of Chromo-beads type A resin kept at 60° (Technicon). When radioactivity measurements were made on individual amino acids, the effluent from the column was passed through a stream-splitter. Two-fifths of the total was used for amino acid characterization and the remaining three-fifths was collected into tubes at a rate of 2·8ml./8min. for radioactivity assay. Each 2·8ml. of effluent was mixed with 10ml. of a mixture (1: 2, v/v) of Triton X-100 and toluene scintillation fluid containing 0·4% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene (Patterson & Greene, 1965) and counted in an automatic Tri-Carb scintillation spectrometer (Packard Instrument Co., La Grange, Ill., U.S.A.) with an efficiency of 40%.

*Incubation of testicular and ejaculated spermatozoa in* vitro. Spermatozoa for metabolic studies were prepared, counted and incubated by the procedures described by Voglmayr *et al.* (1967). The cells were incubated in Warburg flasks containing either [U-$^{14}$C]glutamate (2μc, 343μg./flask) or [U-$^{14}$C]leucine (2μc, 343μg./flask) in Krebs–Ringer diluent (Mann, 1945) buffered with 0·05M-tris, pH7·2. Oxygen uptake was measured by the direct Warburg technique (Umbreit, Burris & Stauffer, 1957). Radioactive $CO_2$ was collected by the method of Buhler (1962). To determine the incorporation of $^{14}$C-labelled amino acids into spermatozoal protein, the cells were separated from the media by centrifugation and protein extracted by the procedure of Bhargava, Bishop & Work (1959). The protein (1–2mg.) was dissolved by heating with 0·5ml. of N-NaOH. The solution was neutralized with 2N-HCl, mixed with 10ml. of the Triton X-100–toluene scintillation fluid described above and assayed for radioactivity.

*Radioactive substrates.* [U-$^{14}$C]Glucose, [U-$^{14}$C]glutamate and [U-$^{14}$C]leucine were obtained from The Radiochemical Centre, Amersham, Bucks. No impurities were detected in the glucose, when examined by paper chromatography with pyridine–propan-2-ol–acetic acid–water (8 : 8 : 1 : 4, by vol.) as the solvent system. [U-$^{14}$C]Glutamate and [U-$^{14}$C]-leucine were not contaminated with radioactivity in any other amino acid, when examined on the amino acid analyser by the procedures described above.

## RESULTS

*Amino acid concentrations.* The concentrations of amino acids in testicular lymph and blood plasma were very similar. The amino acid pattern of testicular fluid, on the other hand, was quite different from that of blood plasma or testicular lymph; the concentrations of many of the amino acids were less, but certain of the amino acids, particularly glutamate and aspartate, were present in much greater concentrations in testicular fluid than in blood plasma. In epididymal seminal

Table 1. *Amino acid concentrations in blood plasma and in the fluids secreted by the reproductive tract of the ram*

All values are expressed as μmoles/ml. Each set of values is the mean of the analyses of two (*) or three (†) samples collected usually on consecutive days. Each sample of seminal plasma was pooled from at least two rams before analysis.

| | Blood plasma | | | | Testicular lymph | Testicular fluid | | Epididymal plasma | | Seminal plasma from vasectomized rams* |
|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid | Arterial Ram 1* | Venous Ram 1* | Arterial Ram 4* | Venous Ram 4* | Ram 1† | Ram 1† | Ram 2* | Ram 3* | Seminal plasma* | |
| Aspartate | 0·01 | 0·03 | 0·05 | 0·04 | 0·02 | 0·31 | 0·33 | 0·42 | 0·51 | 0·05 |
| Threonine, Glutamine, Asparagine, Serine | 0·22 | 0·24 | 0·22 | 0·22 | 0·28 | 0·50 | 0·44 | 9·14 | 3·08 | 0·20 |
| Glutamate | 0·16 | 0·15 | 0·31 | 0·37 | 0·16 | 1·82 | 1·70 | 18·00 | 5·16 | 0·08 |
| Glycine | 0·81 | 0·86 | 0·77 | 0·82 | 0·53 | 1·96 | 1·05 | 1·10 | 0·85 | 1·09 |
| Alanine | 0·16 | 0·18 | 0·24 | 0·24 | 0·14 | 0·39 | 0·39 | 1·43 | 0·93 | 0·27 |
| Valine | 0·11 | 0·11 | 0·14 | 0·14 | 0·17 | 0·02 | 0·04 | 0·59 | 0·36 | 0·12 |
| Isoleucine | 0·05 | 0·05 | 0·06 | 0·05 | 0·07 | 0·01 | 0·01 | 0·36 | 0·19 | 0·06 |
| Leucine | 0·09 | 0·08 | 0·12 | 0·10 | 0·11 | 0·02 | 0·02 | 0·95 | 0·73 | 0·18 |
| Tyrosine | 0·02 | 0·02 | 0·04 | 0·04 | 0·03 | 0·02 | 0·02 | Trace | Trace | 0·05 |
| Phenylalanine | 0·05 | 0·03 | 0·03 | 0·03 | 0·05 | 0·01 | 0·01 | Trace | Trace | 0·04 |
| Lysine | 0·10 | 0·10 | 0·15 | 0·15 | 0·12 | 0·06 | 0·03 | 0·65 | 0·92 | 0·55 |
| Histidine | 0·07 | 0·06 | 0·07 | 0·07 | 0·09 | 0·06 | 0·07 | 0·13 | 0·42 | 0·12 |
| Arginine | 0·09 | 0·09 | 0·11 | 0·11 | 0·08 | 0·02 | 0·01 | 0·26 | 0·53 | 0·04 |

Table 2. *Specific activities* (μC/g. *of* C) *during and after an intravenous infusion* (0–5 *hr.*) *of* [U-$^{14}$C]*glucose*

| Time of sample (hr.) | Blood plasma | | Time of collection (hr.) | Testicular fluid | | | | |
|---|---|---|---|---|---|---|---|---|
| | Glucose | Glutamate | | Glutamate | Aspartate | Glutamine + asparagine + serine (as glutamine) | Glycine | Alanine |
| 3 | 12·5 | 0·63 | 3–5 | 1·8 | 2·1 | 2·0 | 0·3 | 2·9 |
| 5 | 10·4 | 0·66 | 5–8 | 3·1 | 3·7 | 2·3 | 1·3 | 6·4 |
| | | | 8–23 | 1·5 | 1·3 | 1·1 | 0·3 | 0·9 |
| | | | 23–32 | 0·1 | 0·3 | 0 | 0·3 | 0·5 |

Table 3. *Metabolism of* [U-$^{14}$C]*glutamic acid and* [U-$^{14}$C]*leucine by testicular and ejaculated ram spermatozoa*

Substrate oxidized is calculated from the $^{14}CO_2$ collected during incubation. Values are given as mean ± S.E.M. from three rams. Washed testicular and ejaculated spermatozoa ($10^8$ cells/flask) were incubated for 3 hr. at 37° in Krebs–Ringer diluent containing [U-$^{14}$C]glutamate or [U-$^{14}$C]leucine (2 μC, 343 μg./flask).

| | Oxygen uptake (μl./$10^8$ cells/3 hr.) | | Substrate oxidized (μg./$10^8$ cells/3 hr.) | |
|---|---|---|---|---|
| | Testicular | Ejaculated | Testicular | Ejaculated |
| No substrate | 27 ± 1·68 | 22 ± 3·03 | | |
| Glutamate | 25 ± 3·48 | 22 ± 0·81 | 2·36 ± 0·54 | 3·42 ± 0·31 |
| Leucine | 25 ± 1·31 | 25 ± 4·22 | 0·26 ± 0·04 | 0·59 ± 0·22 |
| Glucose (from Voglmayr *et al.* 1967) | 32 | 70 | 81 | 337 |

plasma, glutamate and most of the other amino acids except glycine were present in even higher concentrations than in testicular fluid. The amino acid concentrations were lower in seminal plasma than in epididymal plasma, and in the seminal plasma from vasectomized rams the concentrations of amino acids were very similar to those in blood plasma (Table 1).

*Distribution of radioactivity in amino acids in testicular fluid after infusion of* [U-$^{14}$C]*glucose and* [U-$^{14}$C]*glutamate*. After the infusion of [U-$^{14}$C]-glucose, radioactivity was detected in alanine, glycine, glutamate, serine plus asparagine plus glutamine and aspartate isolated from testicular fluid. The radioactivity was mainly in glutamate, although alanine reached the highest specific activity, about 50% of that of the blood glucose. The specific activity of glycine was appreciably less than the others. The specific activity of all the amino acids labelled reached a maximum during the 3 hr. collection after the infusion was stopped (Table 2). The specific activity of the blood plasma glutamate was much less than that of the glutamate in the testicular fluid. The concentrations of amino acids in the testicular fluid remained reasonably constant during the experiment.

After infusion of [U-$^{14}$C]glutamate for 5 hr., the specific activity of glutamate in testicular fluid was 0·13 μC/g. of C, whereas that of the glutamate in the blood plasma was 6·4 μC/g. of C. Radioactivity was also detected in aspartate and possibly in glutamine in testicular fluid.

*Metabolism of* [U-$^{14}$C]*glutamate and* [U-$^{14}$C]-*leucine by ram spermatozoa*. Neither glutamate nor leucine significantly increased the respiration of testicular or ejaculated spermatozoa above endogenous levels when incubated in Krebs–Ringer diluent (Table 3). Further, no significant change in oxygen uptake was observed when testicular spermatozoa were incubated in phosphate buffer, pH 7·2 (White, 1953), containing various concentrations of glutamic acid (in the range 0–3000 μg./ml. of medium). Glucose (5 mM) stimulated the oxygen uptake of testicular spermatozoa incubated in phosphate buffer, pH 7·2 (controls: 24 μl./$10^8$ cells/3 hr.; glucose: 52 μl./$10^8$ cells/3 hr.), and the addition of different amounts of glutamate (0–3000 μg./ml. of medium) did not affect this. Both glutamate and leucine were oxidized by spermatozoa, but the quantity was considerably less than the amount of glucose oxidized under similar conditions. The amount of glutamate oxidized was about ten times that of leucine (Table 3).

Negligible amounts of radioactivity were detected in protein extracted from testicular spermatozoa after 3 hr. incubation with [U-$^{14}$C]leucine or

Table 4. *Pattern of amino acid formation from* [U-$^{14}$C]*glucose by testicular spermatozoa*

Values are given as disintegrations/min./$10^8$ cells. Washed testicular spermatozoa ($10^8$ cells/flask) were incubated in either phosphate free Krebs–Ringer diluent (2·5$\mu$c, 505$\mu$g. of glucose/flask) for freshly collected cells, or in phosphate buffer, pH 7·2 (1·25$\mu$c, 508$\mu$g. of glucose/flask), for stored spermatozoa.

| Amino acid | Testicular spermatozoa | Testicular spermatozoa (after 6 days storage at 1°) |
|---|---|---|
| Aspartic | 1620 | 2190 |
| Glutamic | 43200 | 11070 |
| Glycine | 1260 | 200 |
| Alanine | 5770 | 640 |

[U-$^{14}$C]glutamate. However, radioactivity was present in small amounts in the protein of ejaculated spermatozoa (185 disintegrations/min./mg. of protein) after incubation with [$^{14}$C]glutamate.

*Formation of amino acids from* [U-$^{14}$C]*glucose by testicular spermatozoa.* Radioactivity was observed in several amino acids after incubating freshly collected testicular spermatozoa for 3hr. in phosphate-free Krebs–Ringer diluent containing [U-$^{14}$C]glucose. Most of the $^{14}$C radioactivity was present in the glutamate fraction (80% of the total amino acid counts) with much less radioactivity being present in aspartate, alanine and glycine (Table 4). A similar distribution of radioactivity was observed in the amino acids formed from incubating testicular spermatozoa (which had been stored for 6 days at 1°; see Voglmayr *et al.* 1967) in phosphate buffer, pH 7·2 (White, 1953), containing [U-$^{14}$C]glucose for 3hr. at 37° (Table 4).

## DISCUSSION

It is clear from these results that there are major differences in the free amino acid composition of the fluid inside and outside the seminiferous tubules. Further, it seems that the testis synthesizes from glucose most of the amino acids present in high concentrations in testicular fluid with the possible exception of glycine. The testis is similar in this regard to the mammary gland (Black & Kleiber, 1961) and brain (Lindsay & Bachelard, 1966). Testicular spermatozoa retain similar synthetic activity (Table 4) but lose it during their passage through the epididymis (Voglmayr *et al.* 1967). It would also appear that the seminiferous tubules are not readily permeable to glutamate, thus probably facilitating the maintenance of the concentration difference between testicular fluid on the one hand and testicular lymph and blood plasma on the other. The even higher concentration of glutamate found in epididymal seminal plasma is probably due to selective resorption of fluid in the head of the epididymis (Crabo, 1965) and the selectivity of the resorption is emphasized by the similar concentrations of glycine found in testicular fluid and epididymal plasma. The highest concentrations of glutamate reached are comparable with those found in avian ejaculated seminal plasma (Lake & McIndoe, 1959), with which mammalian epididymal seminal plasma is analogous because there are no accessory reproductive organs in birds (see Mann, 1964, p. 59).

From the volume of testicular fluid produced (Voglmayr *et al.* 1967), and the amino acid concentrations, it can be calculated that the testis secretes enough amino acids to account for most of that found in the semen. Rams can produce up to 20 ejaculates a day each of about 0·4ml. (Mattner & Braden, 1967), but under these conditions the concentrations of amino acids in the semen would probably be less than those reported above (Kirton, Hafs & Hunter, 1964). Because there is much more cellular debris in ejaculated semen (Voglmayr *et al.* 1967), the failure of suspensions of testicular spermatozoa to incorporate either glutamate or leucine into spermatozoal protein supports the suggestion of Martin & Brachet (1959) that the results of Bhargava *et al.* (1959) and Abraham & Bhargava (1963) were due to contamination of the ejaculated spermatozoa with other cells.

The role of glutamate in testicular secretion, epididymal semen and ejaculated semen is obscure. It does not seem to constitute a significant energy source, nor does it affect the respiration of testicular spermatozoa; some amino acids have been shown to be beneficial to spermatozoa during storage (see Mann, 1964, p. 166) and glutamate may be important during the period of spermatozoal maturation in the epididymis. However, the importance of these amino acids may lie within the testis, rather than in the semen. Aspartate, glycine and glutamine are involved in the synthesis of purine and pyrimidine bases (see Buchanan, 1960; Crosbie, 1960) and the high concentrations established in the testicular fluid may be a reflection of the creation of especially favourable conditions for nucleic acid synthesis within the seminiferous tubules.

T.W.S. gratefully acknowledges the receipt of a Queen Elizabeth II Fellowship during this work.

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