EXAMINATION OF BULL SEMEN AND OF THE BULL AND RABBIT TESTIS FOR THE PRESENCE OF CREATINE PHOSPHATE AND ARGININE PHOSPHATE

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There has been considerable controversy regarding the existence of phosphagens, such as creatine phosphate and arginine phosphate, in mammalian spermatozoa, though it is well documented that creatine phosphate forms a normal constituent of the testis (Treskin, 1872; Eggleton & Eggleton, 1929; Ennor & Rosenberg, 1952; White & Griffiths, 1958). Wajzer & Brochart (1947) were the first to claim that creatine phosphate and arginine phosphate were present in the 'sperme' of the boar. Later, Newton & Rothschild (1961) reported that bull semen contains labile phosphorus and attributed this, at least in part, to creatine phosphate, though White & Griffiths (1958), in a study involving both the spermatozoa and seminal plasma of a number of mammalian species (including the bull), were unable either to isolate phosphagens by a barium fractionation procedure or to demonstrate the appropriate guanidine kinases. On the other hand, there is good evidence for the occurrence of phosphagens in the spermatozoa of certain invertebrates. In two species of sea-urchins, Yanagisawa (1959) found creatine phosphate in the spermatozoa, and arginine phosphate in the eggs, and quite recently, Brooks, Lutwak-Mann, Mann & Martin (1971) demonstrated large amounts of arginine phosphate in octopus spermatozoa.

The present study was undertaken to obtain a more definite answer to the question of whether mammalian semen contains either creatine phosphate or arginine phosphate and, as an alternative approach to previous investigations, the experiments were designed to separate these phosphagens by chromatography on Dowex resin. Bull semen was collected by artificial vagina and extracted with an equal volume of 20% trichloroacetic acid (TCA) by methods based on those recently elaborated by Brooks (1970a) for the determination of adenine nucleotides. The semen was either added directly to ice-cold TCA in a centrifuge tube (Method I) or was first frozen in liquid N₂ and then ground with frozen TCA until the sample thawed (Method II). The testes of a mature bull and rabbit were sliced and frozen in liquid N₂ immediately after slaughter and were extracted by Method II. The extracts from semen and testes were centrifuged at 35,000 *g* for 5 min at –2° C. In order to minimize the breakdown of phosphagens, all following manipulations were carried out in a room main-

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tained at 2° C. The supernatant was quickly shaken with an equal volume of ether, six times, to remove TCA and neutralized with 1% NH_4OH . The material was then passed on to a 0.6 × 20-cm column of Dowex AG1X2 (200 to 400 mesh in the formate form) and the column was washed through with water until the effluent had a basal absorption at 254 nm. The column was eluted at 21.6 ml/hr (3.6-ml fractions) with a gradient of formic acid formed by 50 ml of water in the mixing chamber and 50 ml of 4 M-formic acid in the reservoir. In some instances, the neutralized extract was subjected to ultrafiltration in a pressure dialysis cell before it was applied to the column of Dowex 1 (Brooks, 1970b).

TABLE 1
RECOVERY OF CREATINE PHOSPHATE AND ARGININE
PHOSPHATE

<i>Material</i>	<i>Extraction method</i>	<i>% Recovery of phosphagen</i>
CP added directly to column		100
AP added directly to column		100
CP in 5 ml of water	I	80
10 ml seminal plasma + CP	I	80
6 ml semen + CP	I	84
6 ml semen + CP	II	80
4.5 ml semen + AP	I	86
5 ml semen + AP	II	82
Average recovery		82

CP = creatine phosphate, AP = arginine phosphate.

In the extraction and chromatographic procedures described in the text, 2.75 μmol of CP and 0.495 μmol of AP were added to the acid (20% TCA) immediately before the addition of semen.

In order to test for the presence of creatine phosphate and arginine phosphate, 0.5-ml samples taken from the column fractions were made 0.1 M with respect to HCl and heated at 65° C for 9 min in order to hydrolyse the phosphagens (Ennor & Rosenberg, 1952). The samples were then analysed for inorganic phosphate by the micromethod of Berenblum & Chain (1938) and for free guanidines by the method of Rosenberg, Ennor & Morrison (1956) in a final volume of 1 ml.

The sodium salt of arginine phosphate was prepared from the barium salt by shaking a solution with a little Dowex AG50WX8 (100 to 200 mesh in the Na^+ form). Good recoveries of both phosphagens were obtained when the standards were carried through the complete procedure (Table 1).

Under the chromatography conditions used, arginine phosphate was eluted before AMP, and creatine phosphate was eluted between AMP and inorganic phosphate. It was found that some material of high molecular weight, which was derived from seminal plasma, was not precipitated by TCA and was eluted at the position of arginine phosphate during chromatography. This material gave a positive reaction for arginine and interfered in the colorimetric determination

of inorganic phosphate by forming a precipitate on the addition of 2-methylpropan-1-ol; this interference was removed by submitting the extract to ultrafiltration before chromatography (Text-fig. 1).

Despite good recoveries of the added standards of creatine phosphate and arginine phosphate (Table 1), neither of these phosphagens could be detected in a series of twelve ejaculates (average volume of 7.2 ml and average sperm concentration of $1.1 \times 10^9/\text{ml}$). On the other hand, using the same procedure,

TEXT-FIG. 1. Chromatography of extracts from freshly ejaculated bull semen (average volume of 6.3 ml and average sperm concentration of $0.9 \times 10^9/\text{ml}$). - - - - -, Arginine; ———, labile phosphorus.

creatine phosphate was demonstrated without any difficulty in both bull and rabbit testes, which contained 102 and 206 nmol/g fresh weight, respectively. If spermatozoa did contain creatine phosphate or arginine phosphate, they should have been readily detected by the methods employed, which were sufficiently sensitive to detect as little as 10 nmol/ 10^9 spermatozoa. The possibility that bull semen may contain labile phosphorous compounds other than these two phosphagens still remains to be investigated.

Standard creatine phosphate (disodium salt) was obtained from the Boehringer Corp. and a sample of arginine phosphate (barium salt) was kindly donated by Professor H. Rosenberg and Dr J. F. Morrison (Australian National University).

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