TABLE IV. Specificity in Inhibition of Proline-C^{14} Incorporation into Ribosomes by TZCA.

Amino acid	Specific activity No TZCA	Specific activity + 3.75 μmoles TZCA	% inhibition
L-lysine-C^{14} (specific activity, 220 μc/μmole)	1100	1248	.00
DL-tryptophan-2,3-H^3 (specific activity, 200 μc/μmole)	800	790	1.25
L-proline-C^{14} (specific activity, 180 μc/μmole)	2600	1250	52.0

similar to that of proline. The coding polynucleotide for this incorporation was a homopolymer of cytidine. t-RNA-TZCA-S^{35} was also isolated, and this presented further evidence that TZCA-S^{35} was incorporated into ribosomes following the formation of t-RNA-TZCA-S^{35}. Hence, these experiments show that TZCA may be used as a competitive inhibitor to the incorporation of proline into ribosomes in a cell-free system.

1. Meister, A., Stone, N., Manning, J. M., Adv. Chem. Ser., 1964, v44, 67.

2. Manner, G., Gould, B. S., Biochem. et Biophys. Acta, 1963, v72, 243.

3. Robertson, W., Van, B., Schwartz, B., J. Biol. Chem., 1953, v201, 689.

4. Njaa, L. R., J. Sci. Food Agr., 1961, v12, 757.

5. Johnson, A. B., Stricker, H. J., J. Biol. Chem., 1962, v237, 1876.

6. Debey, H. J., Mackenzi, I. B., Mackenzi, C. G., J. Nutrition, 1958, v66, 607.

7. Ratner, S., Clarke, H. T., J. Am. Chem. Soc., 1937, v59, 200.

8. Keller, E. B., Zamecnik, P. C., J. Biol. Chem., 1956, v221, 45.

9. Lowry, O. H., Rosebrough, H. J., Farr, A. L., Randall, R. J., ibid., 1955, v217, 111.

10. Siekevitz, P., ibid., 1952, v195, 549.

11. Zamecnik, P. C., Stephenson, M. L., Scott, I. F., Proc. Nat. Acad. Sci., 1960, v45, 811.

12. Nirenberg, M. W., Jones, O. W., Zeder, P., Clark, B. F. C., Sly, W. S., Pestka, S., Cold Spring Harbor Symposia, 1963, v28, 549.

13. Yarmolinsky, M. B., De La Haba, G. L., Proc. Nat. Acad. Sci., 1959, v45, 1721.

14. Kretsinger, R. H., Manner, G., Gould, B. S., Rich, A., Nature, 1964, v202, 438.

Received February 8, 1965. P.S.E.B.M., 1965, v119.

Characterization of the Basic Protein Associated With DNA in Mammalian Spermatozoa.* (30296)

DONALD M. HENRICKS AND DENNIS T. MAYER

Department of Agricultural Chemistry,† University of Missouri, Columbia

The basic proteins isolated from the nucleoprotein of salmon and sturgeon spermatozoa are protamines. A more complex and less basic protein, histone, is associated with DNA in somatic cell nuclei, such as calf thymus and chicken erythrocytes(1). The histones contain arginine and lysine in a ratio ranging from 0.5-1.5:1.0(2) and no cystine, except as a contaminant(3). Besides serving a structural purpose in the chromosome(4), histones have been hypothesized to serve as regulators of gene action(5). Evidence has been presented which indicates that DNA in these genes associated with histone is prevented from expressing itself(6).

In contrast to what is known about the basic proteins of other cells, there is a disturbing lack of knowledge about the basic protein associated with DNA in mammalian spermatozoa. This is due in some measure to the existence of a keratin-like protein in this cell which makes extraction of basic protein difficult. Green(7) presented evidence for the presence of a keratin membrane coating the head of the ram spermatozoon. Other work presented evidence for a keratin-like

* Supported in part by research grant G-3180 from Div. of Res. Grants, Nat. Inst. Health, U.S.P.H.S.

† Missouri Agri. Exp. Station, Journal Series No. 2878. Approved by Director.

protein in bovine spermatozoa(8). Berry and Mayer(9) bombarded bovine spermatozoa with fine glass beads (0.17-0.18 mm) prior to extraction of a basic protein which precipitated from acid solution upon addition of NH_4OH. These properties indicated a histone-like protein had been isolated. Bril-Petersen(10) used performic acid to dissolve the keratin barrier in bovine spermatozoa. This treatment left an insoluble fraction having a high arginine content. The freeze-dried supernatant fluid contained material part of which was soluble in HCl. Both the insoluble residue and the HCl-soluble fractions were high in arginine and cysteic acid content.

The intention in the present research was to determine whether a basic protein existed in porcine spermatozoa, and if so, whether it was a protamine, histone, or an entirely different protein.

Methods. Semen was collected from healthy boars which were used for breeding studies at the Swine Reproduction Research Laboratory, University of Missouri. The spermatozoa were washed free of seminal fluid and submitted to 2 different fractionation procedures in an effort to isolate a basic protein. The first procedure involved a bombardment of spermatozoa with fine glass beads in 0.85% NaCl in a Servall omnimixer at full speed. This was followed by extraction for 24 hours with 0.85% saline. The supernatant was lyophilized and the resulting material was extracted with 0.1 N HCl. This supernatant did not give a precipitate upon addition of NH_4 OH. Similar treatment of bull spermatozoa had yielded an acid-soluble, NH_4OH-insoluble fraction(8). It was lyophilized in order to determine if any material (F1) had been extracted by the HCl. About 4% of the original sperm cells had been extracted by the acid. This fraction was submitted to an amino acid analysis.

The second fractionation procedure involved the treatment of porcine spermatozoa with performic acid. Before attempting to disrupt the keratin component, the spermatozoa were treated with 0.3 N HCl to remove any acid-soluble material (F2). If not done, this material would contaminate a fraction (F4a) obtained later in the procedure. The residue was treated with performic acid for 18 hours at 4°C. The mixture was centrifuged to give a residue (F3) and a supernatant which was lyophilized. The material obtained upon lyophilization was extracted 3 times with 0.1 N HCl and centrifuged. The material which was not soluble was labeled F5. The supernatant was filtered through a Sephadex (G-25) column which separated a protein (F4a) from DNA degradation products.

The fractions were defatted by extracting with 1:1 $CHCl_3$-methanol for 2 hours at 56°C and submitted to complete amino acid analysis. For analysis, a sample was hydrolyzed in a specially designed, evacuated, sealed glass tube for 24 hours at 110°C and analyzed by ion exchange chromatography using a Beckman amino acid analyzer. For comparative purposes a sample of whole calf thymus histone was also analyzed. The fractions were also analyzed for DNA content by the Webb and Levy method(11) and for protein content by a modified biuret method (12).

Results. In Table I is shown a complete amino acid analysis of the only fraction (F1) obtained from the first procedure. This fraction was quite acidic in composition. Therefore the presence of a basic protein is not likely.

Also presented in Table I, are the amino acid compositions of the 4 fractions obtained with the second procedure. In the second procedure the initial extraction with 0.3 N HCl gave a material (F2), which was approximately 9.6% of the cell weight. It was a neutral protein. After performic acid treatment, 3 fractions were obtained: F3, a quite resistant residue which was basic in amino acid content, F4a, and acid-soluble protein which was neutral. All 3 fractions contained half-cystine in its oxidized form, cysteic acid, with the highest concentration present in the acid-soluble protein (F4a). The composition of the whole calf thymus histone is given in Table I also. In this protein the arginine to lysine ratio is 1:1 and only a trace of half-cystine is present.

The acid-soluble protein (F4a) cited above was separated from 2 other sub-fractions

TABLE I. Amino Acid Analyses of Protein Fractions from Porcine Spermatozoa.

Amino acids are expressed as g/100 g of protein. No corrections were made for hydrolytic losses of amino acids.

Amino acids	F1	F2	F3	F4a	F5	Histone*
Lys	5.0	6.6	6.3	4.9	6.4	14.0
His	2.6	2.9	2.8	2.6	2.3	2.3
Arg	5.6	7.1	18.4	31.8	11.3	13.1
Asp	9.2	9.2	7.7	5.0	8.4	5.0
Glu	14.6	10.5	9.1	7.1	10.3	10.9
Thr	5.6	5.8	3.8	3.1	4.1	5.4
Ser	5.6	5.6	5.0	4.8	5.1	4.6
Pro	6.1	6.2	4.6	5.3	5.0	4.3
Gly	4.9	5.0	3.1	2.2	3.5	4.1
Ala	4.7	3.8	3.8	3.6	4.1	8.8
Val	5.7	5.6	4.4	3.9	4.5	5.6
Isol	3.9	5.7	4.2	2.6	4.0	4.6
Leu	7.7	7.4	7.2	4.1	7.2	8.2
Tyr	4.0	6.3	.2	.2	.2	3.1
Phe	5.2	5.4	4.1	1.7	4.2	2.7
Half-cys	3.1	2.7	7.1†	11.2†	6.3†	.4
Met	2.8	1.2	2.0†	1.6†	2.1†	1.5
Tot. basic A.A.	13.2	16.6	27.5	39.3	20.0	29.4
Tot. acid A.A.	23.8	19.7	16.8	12.1	18.7	15.9

* Histone was extracted from calf thymus and supplied by California Biochem. Corp.

† These are values for cysteic acid and methionine sulfone, respectively.

when the acid solution (HCl) was filtered through Sephadex gel. The first fraction contained all of the measurable protein and some DNA. The last 2 fractions contained no protein, and were high in DNA content presumably DNA degradation products. Table II presents the results of a determination of the DNA and protein content of the fractions obtained from performic acid treatment, and the amount of the total protein located in each fraction. Over half of the protein was located in the residue, while the rest was split almost equally between the 2 fractions solubilized by the performic acid. The use of gel filtration resulted in a substantial purification of the protein in fraction F4. The residue contained 15.8% DNA, while an aliquot removed from the sample of spermatozoa after 18 hours treatment with performic acid contained 11.8% DNA.

Discussion. None of the fractions obtained from the two procedures in this study could be classified as a classical histone or protamine. An attempt to disrupt the keratin component physically (procedure 1) gave a fraction containing an acidic protein. The initial extraction of boar spermatozoa with 0.3 N HCl prior to performic acid treatment gave a protein which was neutral in its amino acid content. This could be material outside the keratin moiety including material from the tail of the spermatozoon.

The drastic treatment of spermatozoa with a peroxide, performic acid, oxidized all cystine and cysteine present to cysteic acid and all methionine present to its sulfone. This treatment split the cell into 3 fractions. Treatment of bovine spermatozoa with performic acid by Bril-Petersen *et al*(10) gave qualitatively the same 3 fractions. However, there were quantitative differences. In porcine spermatozoa, a lesser proportion of the protein was present in the residue (F3). It contained some lysine and histidine, and about one-half as much arginine as the residue fraction in bovine spermatozoa. The residue (F3) in bovine spermatozoa contained neither lysine nor histidine. The acid-soluble protein (F4a) in porcine spermatozoa assumes a greater importance than in bovine spermatozoa. It is present in a greater quantity and contains the highest content of arginine and half-cystine, when compared to the other 2 fractions (F3 and F5). This could be due in part to a greater solubilization of a protein in the cell high in these 2 amino

TABLE II. Composition of Fractions Contained from Performic Acid Fractionation of Porcine Spermatozoa.*

Fraction	% DNA	% Protein	% Recovery†	% of total protein/fraction
F3 (residue)	15.8	74.8	70.2	51.8
F4 (acid-soluble)	4.7	80.0	92.8	24.7
F5 (acid-insoluble)	5.9	70.8	76.7	23.5

* Data are based on 2 samples of spermatozoa submitted to fractionation procedure.

† % Recovery pertains to grams of total amino acids per 100 g protein recovered from column during A.A. analysis.

acids than was possible in the case of bovine spermatozoa.

This acid-soluble fraction (F4a) could be the functional corollary of the histone of somatic cell nuclei. Like the histones it is quite basic and is acid soluble. Unlike the histones, it has a ratio of arginine to lysine of 5:1, and it has a high content of half-cystine. It is very possible that a number of proteins are present in this fraction, some of them typical histones, others proteins containing a high content of arginine and cystine. In addition, accompanying the F4a fraction is an acid-insoluble protein (F5) which is present in as great a quantity as the acid-soluble protein, and is quite different in composition. It is interesting to note that the degraded DNA did not accompany this fraction. Of note also in regard to the DNA is the fact that the major portion of the DNA in the cell was not solubilized by the performic acid. Somehow, it was protected by the insoluble residue (F3).

This study indicates that in porcine spermatozoa the use of the term "keratin membrane" is inadequate to describe the basic keratin-like protein present. Much of the protein of this cell appears to be a unique keratin, unique because of its high content of the basic amino acids, especially arginine. Much of the protein solubilized by performic acid could be the more soluble components of this keratin. Physiologically, a spermatozoon in which the DNA is embedded in an insoluble basic keratin-like protein would be well adapted to perform its function, that of transporting its genetic material to the ovum. Hence, one could postulate the involvement of a keratinase in the fertilization process. The bonding of DNA to the keratin could be performed *via* a salt linkage between guanidino groups of arginine and phosphoric acid groups of DNA. Thus this basic keratin-like protein in a mammalian germ cell could be the counterpart of the histones in somatic cells as has been suggested previously(10). This protein may replace and fulfill the function of a histone in mammalian spermatozoa.

Summary. None of the fractions obtained from porcine spermatozoa resembled the classical histones or protamines in amino acid content when two different isolation procedures were utilized. A physical attack on the keratin by glass beads appeared to give inadequate penetration to release the other proteins of this cell. A chemical attack with performic acid appeared quite adequate. Much of the protein in this cell is a basic keratin. The properties of such a protein would allow it to protect the DNA during its journey to the ovum.

The authors wish to express their gratitude to Dr. Benedict Campbell, University of Missouri, Biochemistry Department, for material aid necessary for this study, and to Dr. Bril-Petersen for advice with regard to treatment of spermatozoa with performic acid.

1. Mann, T., Biochemistry of Semen and of the Male Reproductive Tract, John Wiley & Sons, New York, 1963, p153.

2. Murray, K., in The Nucleohistones (Ed., Bonner, J., Ts'O, P.) Holden-Day, Inc., San Francisco, 1964, p15.

3. Phillips, D. M. P., Prog. Biophys. Biophys. Chem., 1962, v22, 211.

4. Zubay, G., in The Nucleohistones (Ed., Bonner, J. Ts'O, P.) Holden-Day, Inc., San Francisco, 1964, p95.

5. Stedman, E., Stedman, E., Nature, 1950, v166, 780.

6. Mirsky, A. E., Osawa, S., in The Cell, (Ed., Brachet, J., Mirsky, A. E.) Academic Press Inc., New York, 1961, p677.

7. Green, W. W., Anat. Rec., 1940, v76, 467.

8. Berry, R. E., Mayer, D. T., Exp. Cell Research, 1959, v18, 398.

9. ———, ibid., 1960, v20, 116.

10. Bril-Petersen, E., Westenbrinik, H. G. K., Biochim., Biophys. Acta, 1963, v76, 152.

11. Webb, J. M., Levy, H. B., J. Biol. Chem., 1955, v213, 107.

12. Goa, J., Scand. J. Clin. Lab. Invest., 1953, v5, 218.

Received February 10, 1965. P.S.E.B.M., 1965, v119.