THE DESOXYRIBONUCLEOPROTEIN OF SEA URCHIN SPERM

I. ISOLATION AND ANALYSIS*

by

MAURICE H. BERNSTEIN** AND DANIEL MAZIA

Department of Zoology, University of California, Berkeley 4, California (U.S.A.)

Some of the early work in KOSSEL's laboratory[1,2] had shown that nucleoproteins could be extracted from various tissues with distilled water. HAMMARSTEN[3] also made distilled water extracts of nucleoproteins. Unfortunately, these extractions were primarily made as preliminary steps in the preparation of basic proteins, by KOSSEL's group, and of DNA by HAMMARSTEN. More recently, FISCHER, BOETTGER AND LEHMANN-ECHTERNACHT[4], STERN *et al.*[5], and PETERMANN AND LAMB[6] have extracted nucleoproteins in dilute (0.001 *M*) solutions of arsenate or citrate, and have begun work on the nucleoproteins as such, rather than on their various components.

Reports to date have all involved an initial isolation of nuclei, and then the subsequent extraction of these nuclei. The direct extraction of a desoxyribonucleoprotein from sea urchin sperm is reported here.

MATERIALS AND METHODS

The sea urchin, *Strongylocentrotus purpuratus*, from northern California was used. The animals were cut open, the ripe testes were removed and cut into smaller pieces and allowed to shed in cold, filtered sea water. The shed sperm were filtered through a fine-grained non-woven cloth***. The sperm were washed twice in filtered sea water, and twice in 1/8 molar sodium citrate by centrifugation. The packed citrate-washed sperm were then suspended in cold distilled water. From this point all operations were performed in the cold. The addition of the water produced an immediate and very intense agglutination of the sperm, so that a cohesive, gelated sperm-mass was obtained. This sperm-mass was allowed to extract overnight at 2° C. The extract was then centrifuged, in the cold, at 7000 g, for twenty minutes. There was a clear-cut separation between the gelated sperm-mass and the supernatant solution, the latter was decanted and the sperm-mass resuspended in cold distilled water, and extracted again. As the sequence of extraction followed by centrifugation was repeated, the sperm-mass increased in volume as it took up more water of hydration. The process was continued until the material was completely hydrated and passed into solution, usually leaving a small residue of degraded material.

Frozen-dried material has been used in this work, as well as fresh. Subsequent extraction of the nucleoprotein of the dried material proceeded more rapidly than the fresh, but this was the only noticeable difference between the preparations.

The course of the extractions was followed by analyses of total protein, nucleic acid, and histone on the clear supernatant solutions decanted after centrifugation. The nucleic acid content of the extracts was determined from the specific absorption at 260 mμ in a (Beckman, Model DU) spectro-

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** Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council. Present address: Carnegie Institution of Washington, Cold Spring Harbor, New York.

*** NU-FAB, Essential Products, Inc., Los Angeles, California.

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photometer[7]. Equivalent nucleic acid values were obtained from a number of samples by the ultraviolet absorption method and by the DISCHE[8] diphenylamine reaction, indicating that essentially all of the nucleic acid of the sperm is DNA.

Total protein was measured by a quantitative biuret reaction[9]. Calibration of this method gave slightly higher values for egg albumin than for histone (0.080 and 0.065, respectively). The calibration with histone* has been used in the present study.

The modified MILLON'S reaction described by MIRSKY AND POLLISTER[10] was used for the determination of the histone content of the nucleoprotein extracts. The method was calibrated against histone*.

In the following, the term "histone" will be used to describe that (protein) fraction reacting in the MIRSKY AND POLLISTER *procedure. It will be seen (p. 605) that this fraction may include apparent degradation products of non-basic proteins as well as the basic histone protein.*

The non-histone protein (NHP) of the extracts was calculated from the difference between the total protein and histone.

RESULTS

With a small amount of sperm and a large volume of water, the complete extraction of the sperm can be achieved in a single step. This treatment will also obviate any residue of degraded material. To avoid handling such large volumes of water, and the resulting dilute solutions, the extraction has been achieved with a greater number of treatments with smaller volumes of water. In addition to the amount of sperm and the volume of water used, the time allowed for extraction is an important factor in determining the amount of material obtained. The time factor is particularly important during the early phases of the extraction process, the initial extractions requiring at least 12 hours for completion.

In the development of this method for the extraction of nucleoprotein from sperm, it was soon observed that the extraction process followed a definite pattern; this is clearly shown in Table I. In the initial treatments with water, the hydration of the sperm-mass is the principal result, a preliminary or preparative procedure, which must precede the actual extraction of the nucleoprotein. Accordingly, Table I shows that only very small amounts of DNA are present in the first three extracts. On the other hand, substantial amounts of protein are found in these initial extracts. Extract IV represents an intermediate or transitional phase, here the relative proportion of nucleic acid becomes greater. These proportional relationships are most apparent in the ratios of Protein/DNA. In Extract V, the bulk of the nucleoprotein is actually extracted, and the Protein/DNA ratio, high in I, II, and III, reaches a minimum in V. VI is, then, the unextracted residue.

TABLE I

Extract	*DNA (mg)*	*Protein (mg)*	*Protein/DNA*
I	2.9	23.6	7.9
II	12.0	97.0	8.1
III	23.2	140.0	6.0
IV	45.3	129.3	2.9
V	143.0	365.0	2.5
VI	42.0	154.0	3.7

These data (Table I) would suggest that at least two principal entities are represented in these extracts. These entities may be identified as a protein not associated with

* Worthington Biochemical Corp., Freehold, N.J.

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nucleic acid, which is the predominant component in the early extracts, and a desoxyribonucleoprotein, the main portion of which appears in a single extract which constitutes the climax of the whole extraction process. In the example given in Table I, the "climax" is reached in extract V.

TABLE II

Extract	DNA		Histone		Non-Histone Protein		Total Protein/DNA
	mg	%	mg	%	mg	%	
I	76.0	19.9	65.0	16.9	242.0	63.2	4.04
II	103.0	25.1	87.8	21.3	220.0	53.6	2.98
III	168.3	27.3	145.9	23.7	302.1	49.0	2.67
IV	58.9	25.3	62.3	26.9	110.7	47.8	2.94
V	60.3	24.0	48.7	19.4	141.9	46.6	3.17
Total	466.5	24.6	409.7	21.6	1016.7	53.8	3.06

The pattern described above is also seen in the data of Table II, although it is not as clear, since they are taken from a preparation made with frozen-dried material, rather than with fresh, as was the case in Table I. It has been stated above that the lyophilized sperm are more readily extracted than the fresh material, and this point is demonstrated here. The preparation shown in Table I was made with fresh material, and culminated in the fifth extract. The extraction of lyophilized material, shown in Table II, reached a climax in the third extract, but was somewhat limited in that there was not sufficient water present to permit the "solation" of the total mass. The extraction of the nucleoprotein was completed in the fourth step, and the residual unextracted material is found in the fifth extract.

The preparation reported in Table II is presented in considerably greater detail, to permit further consideration of the changes in composition of the various extracts. In Table II, the extracts have been totaled, and the sum represents the composition of the original sperm. These totals have been verified by direct analysis of the whole sperm, and over a number of experiments the general agreement of the values has been within 5–10%. Compared with the total values of Table II, Extract I contains less-than-average amounts of DNA and of histone, and a greater amount of non-histone protein (NHP). Extract II has the same composition as the whole sperm, while III contains a major portion of the nucleoprotein and reflects this in containing above-average amounts of DNA and histone, but smaller amounts of non-histone protein. Extract IV is essentially similar to III, although somewhat richer in histone. V is the unextracted residue, consisting of degraded material which is no longer water-soluble, but reflects no significant change in composition. Here again, we may identify the nucleic acid-associated and the nucleic acid-free protein components of the sperm. The nucleic acid-free protein which forms the bulk of the early extracts, is apparently composed of non-histone protein. The "nuclear" fraction, the desoxyribonucleoprotein, contains DNA and histone, but, as we shall see, it also contains a considerable amount of non-histone protein.

Precipitation in dilute (0.14 *M*) NaCl has been described by GAJDUSEK (1950) as a suitable method for effecting the purification of water-extracted desoxyribonucleoproteins. In the present study the procedure has been carried out in the following manner:

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A measured volume of a suitable high-polymer DNA containing nucleoprotein extract is mixed with an equal volume of cold 0.28 *M* NaCl (final concentration 0.14 *M* NaCl). A fine precipitate forms immediately. However, if the container is allowed to stand in the cold, the precipitate becomes slightly flocculent and settles to the bottom. The supernatant solution can be decanted or siphoned off, and the precipitate re-dissolved by dilution with cold distilled water. The solution is now slightly cloudy, and is clarified by centrifuging for 30 minutes at 15,000 g. Analysis reveals that the sediment is primarily protein that has not been re-dissolved, and the bulk of the nucleoprotein is in the supernatant solution. This process is repeated several times, although a distinct limitation is imposed on the process by the instability of the material.

The results of the purification described above are shown in Table III. The material used in this experiment was obtained by pooling four successive extracts, so that the series of repeated precipitations were begun with a relatively large amount of material. The extreme susceptibility to degradation, described above, can be seen in Table III. In the first precipitation, almost 50 % of the DNA and histone are recovered, but only 20 % of the non-histone protein is recovered. In the successive precipitations the recoveries of all components are much higher, but there is a gradual loss of water-soluble material, which includes all components of the initial extract and will ultimately result in complete loss.

TABLE III

	DNA		*Histone*		*Non-Histone Protein*		*Total Protein/DNA*
	mg	%	*mg*	%	*mg*	%	
Extract	350.8	27.0	319.9	25.4	598.1	47.6	2.61
Re-ppt. A	166.1	38.7	150.0	35.0	112.3	26.3	1.58
Re-ppt. B	118.4	35.7	127.8	38.6	85.4	25.8	1.80
Re-ppt. C	108.5	38.8	94.0	33.6	77.4	27.6	1.58
Re-ppt. D	87.3	36.2	88.5	36.7	65.1	27.0	1.76
Re-ppt. E	67.9	36.3	83.5	50.0	27.7	13.7	1.64
Re-ppt. F	43.9	31.5	77.5	55.7	17.9	12.8	2.17
Re-ppt. G	37.2	35.1	62.0	58.4	7.0	6.5	1.85
Re-ppt. H	23.8	33.9	46.3	66.1			1.94

The "purification" of the desoxyribonucleoprotein effected by precipitation in dilute salt solutions is seen in the changes in percentage composition shown in Table III. The original extract has a high (almost 50 %) non-histone protein (NHP) content, this is reduced by nearly half, to 27 %, in the first precipitation. The DNA and histone levels are essentially equivalent in the beginning, and remain so through the first four precipitations, although their levels rise slightly after the first precipitation, as a result of the decrease in NHP. The products recovered in the first four precipitations have the same composition, but it is clear that the amount of material precipitable is constantly diminishing. After the fourth precipitation (D) a consistent and progressive change in composition is seen. The percentage of histone rises, and at the same time the percentage of NHP falls, the gains and losses being nearly equivalent, while the nucleic acid remains constant. Further discussion of these observations will be deferred until later.

DISCUSSION

One of the objectives of the biochemical study of the chromosomes is the search for

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"units" of chemical organization between the highly complex chromosomes of microscopic dimensions, and the ultimate chemical components. The water-soluble nucleoprotein particles, described by the workers previously cited, and the nucleoprotein of the sea urchin sperm nucleus, now being studied in detail, merit examination from this standpoint. The present paper considers only the conditions of extraction and the stability of the desoxyribonucleoprotein described above. Data to be presented later consider its properties and morphology in more detail.

1. *Conditions of extraction*

The desoxyribonucleoprotein of the sea urchin sperm cannot be extracted efficiently by direct treatment with distilled water. In successive treatments with water, there is a progressive swelling, indicating that the material is undergoing hydration. However, the progress towards complete solution proceeds only at an extremely slow rate. The nucleoprotein particles become easily extractable after the sperm heads have been washed in solutions of sodium citrate or sodium phosphate, but sodium chloride does not affect them.

The mechanism of this effect of polyvalent anions has not been explained. The hydration of the nucleoprotein and the process whereby it is rendered soluble are clearly separable phenomena. In connection with the latter, several explanations present themselves. One, the pre-treatment acts on the surface of the sperm, opening it up for the exit of the large nucleoprotein particles. A second possibility is that the nucleoprotein particles in the compact chromosomes must be dissociated from each other as well as hydrated before they go into solution, and that the citrate or phosphate are effective here. In either case, it is probable that the action is one of sequestering Ca, Mg, or other heavy metal cations which may be involved in the structure of the sperm head.

2. *Stability*

The nucleoprotein particles described in this paper are remarkably unstable. In addition to a progressive depolymerization of DNA, the instability expresses itself in two ways. First, the preparation degrades rapidly, merely on standing in the cold or on repeated precipitations with 0.14 *M* NaCl. The degradation products are soluble in the NaCl solution, so that ultimately none of the material is recoverable by precipitaton. This characteristic is apparent during the first precipitations, although the composition of the precipitate recovered does not change (Table III). Second, there is an apparent alteration in the composition of the precipitated nucleoprotein, which becomes striking after several precipitations. In Fig. 1, this alteration is seen to be an apparent increase in the ratio of "histone" to total protein. This ratio increases rapidly, while the ratio of total protein to DNA does not rise appreciably (Table III). It would appear that the instability of the nucleoprotein is further expressed as a conversion of "non-histone" protein to "histone". This observation motivated a closer study of the significance of the histone method.

In this study, the protein of the sea urchin sperm has been arbitrarily separated into two categories. These have been identified simply as histone and non-histone protein, the histone has been determined directly, while the NHP has been calculated from the difference between total (biuret) protein and histone. The identification of the histone has been based on the solubility of histones in 1.88 *M* H_2SO_4 that is 0.34 *M* in $HgSO_4$, according to the method described by MIRSKY AND RIS[11]. Solubility in this reagent is,

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however, not a specific property of histone, but rather a general characteristic of low-grade proteins and protein fragments. The identification of a histone fraction by this method is, then, not strictly accurate, and may include more than just histones. This inexactness will be more pronounced in any situation in which the presence of degraded proteins or protein fragments is at all likely. This point is illustrated by the data of Table IV, determinations have been made of the "biuret" protein and of the "histone"

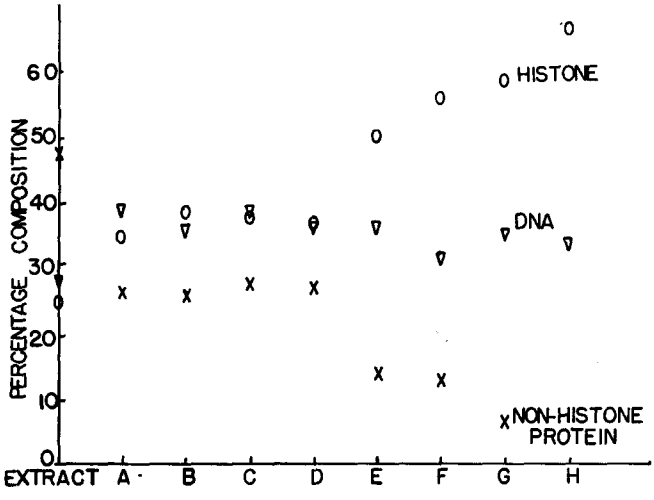

Fig. 1. Changes in the composition of the water-soluble desoxyribonucleoprotein of sea urchin sperm during repeated precipitation in 0.14 *M* NaCl.

protein in crystalline bovine serum albumin in water, and of the same material after incubation with crystalline pepsin at pH 1.6 for one hour. The "biuret" protein gives the same values in both cases, but the "histone" protein, negligible before digestion, in the digested sample is even higher than the "biuret" protein. (In terms of material required, the "histone" method is four times as sensitive as the biuret.) Similarly, proteose-peptone, also shown in Table IV, is apparently 80 % "histone".

TABLE IV

	Biuret Protein (Mg per ml)	*"Histone" Protein (Mg per ml)*
Crystalline Bovine Serum Albumin	2.36 ± 0.05	0.077 ± 0.012
Crystalline Bovine Serum Albumin + Crystalline Pepsin (0.09 mg per ml) 1 hour, pH 1.6	2.30 ± 0.02	3.53 ± 0.015
Proteose-Peptone	4.43	3.69

It is evident that this test is not specific for histones, but may include other partially degraded proteins. We may conclude, therefore, that the protein fraction of the nucleoprotein particle must be undergoing degradation on standing or during repeated precipitations in the cold, the degradation products reacting as "histone" and

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remaining associated with those particles that are still precipitable in 0.14 *M* NaCl.

These various considerations would indicate that the composition of the "pure" desoxyribonucleoprotein of the sea urchin sperm is most clearly shown in the material recovered in the first precipitations. The composition of this material is in good agreement with the findings of MIRSKY AND RIS[11] on the composition of isolated chromosomes. On the other hand, the composition of the original extract coincides with the values for isolated nuclei and sperm reported by STEDMAN[12]. These observations merely re-state the obvious fact that the composition of whole nuclei will not be the same as the composition of one specific portion of the nucleus, the chromosomal substance.

SUMMARY

A procedure has been described for extracting the desoxyribonucleoprotein of the sperm of the sea urchin, *Strongylocentrotus purpuratus*, using distilled water as the extraction medium. The procedure requires treatment of the sperm with sodium citrate, or phosphate, prior to extraction with water; the effect of this pre-treatment is not known.

The extracted desoxyribonucleoprotein can be purified by precipitation in 0.14 *M* NaCl. The result of this purification is the removal of approximately half the non-histone protein present in the extract. The purified desoxyribonucleoprotein contains 35–37% DNA, an equal amount of histone, and 25–27% non-histone protein. The analytical method for "histone" is shown to be non-specific. The significance of the analyses reported here is discussed.

RÉSUMÉ

Un procédé pour l'extraction d'un désoxyribonucléoprotéide à partir de spermatozoïdes de l'oursin *Strongylocentrotus purpuratus* est décrit; dans ce procédé, l'extraction se fait au moyen d'eau distillée après traitement préalable par du citrate ou du phosphate de sodium; l'effet de ce traitement préalable n'est pas connu.

Le désoxyribonucléoprotéide ainsi isolé peut être purifié par précipitation dans NaCl 0.14 *M*. Cette purification résulte dans l'élimination d'environ la moitié des protéines autres que les histones qui étaient présentes dans l'extrait. Le désoxyribonucléoprotéide purifié contient 35–37% d'ADN, une quantité égale d'histone et 25–27% de protéines distinctes des histones. Il est montré que la méthode analytique pour le dosage de l'"histone" n'est pas spécifique. La signification des analyses décrites dans le présent travail est discutée.

ZUSAMMENFASSUNG

Es wird ein Verfahren zur Extraktion des Desoxyribonukleoproteins aus dem Sperma des Seeigels, *Strongylocentrotus purpuratus*, beschrieben, das destilliertes Wasser als Extraktionsmittel verwendet. Das Verfahren erfordert eine der Extraktion mit Wasser vorhergehende Behandlung des Spermas mit Natriumcitrat oder -phosphat. Die Wirkung dieser Vorbehandlung ist nicht bekannt.

Das extrahierte Desoxyribonukleoprotein kann durch Ausfällen mit 0.14 *M* NaCl gereinigt werden. Das Resultat dieser Reinigung ist die Entfernung von ungefähr der Hälfte des im Extrakt anwesenden Nicht-Histon-Proteins. Das gereinigte Desoxyribonukleoprotein enthält 35–37% DNS, einen gleich grossen Anteil Histon und 25–27% Nicht-Histon-Protein. Es wurde gezeigt, dass die analytische Methode für "Histon" nicht spezifisch ist. Die Bedeutung der hier berichteten Analysen wird besprochen.

REFERENCES

1 H. LILIENFELD, *Z. Phys. Chem.*, 18 (1893) 473.
2 W. HUISKAMP, *Z. Phys. Chem.*, 32 (1902) 145.
3 E. HAMMARSTEN, *Biochem. Z.*, 144 (1924) 383.
4 F. G. FISCHER, I. BOETTGER AND H. LEHMANN-ECHTERNACHT, *Z. Phys. Chem.*, 271 (1941) 246.
5 K. G. STERN, G. GOLDSTEIN, J. WAGMAN AND J. SCHRYVER, *Federation Proc.*, 6 (1947) 296.
6 M. L. PETERMANN AND C. M. LAMB, *J. Biol. Chem.*, 176 (1948) 685.
7 B. THORELL, *Acta Med. Scand.*, Suppl. 200 (1947) 1.
8 Z. DISCHE, *Mikrochem.*, 8 (1930) 4.
9 H. W. ROBINSON AND C. G. HOGDEN, *J. Biol. Chem.*, 135 (1940) 707, 727.
10 A. E. MIRSKY AND A. W. POLLISTER, *J. Gen. Physiol.*, 30 (1946) 117.
11 A. E. MIRSKY AND H. RIS, *J. Gen. Physiol.*, 34 (1951) 475.
12 E. STEDMAN, *Phil. Trans. Roy. Soc., B*, 235 (1951) 565.

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