THE DESOXYRIBONUCLEOPROTEIN OF SEA URCHIN SPERM

II. PROPERTIES*

by

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The water-extraction of the desoxyribonucleoprotein of the sperm of the sea urchin, *Strongylocentrotus purpuratus*, has previously been described[1]. The extraction of desoxyribonucleoproteins in water was first described by LILIENFELD[2], and again by BANG[3]. These and subsequent workers were chiefly interested in the elementary analysis of the nucleoprotein or in one or more of its components, and relatively little work was done on the parent nucleoprotein. More recently, interest in this material has been reawakened by the work of STERN[4,5].

The extraction of a nucleoprotein in water is a slow and laborious task, requiring the handling of large volumes of liquid over an extended period of time. MIRSKY AND POLLISTER[6,7] have presented a much simpler procedure for the extraction of desoxyribonucleoproteins, based on their solubility in strong (1.0 molar) salt solutions. This method has found extensive application, and has been generally accepted as the method of choice for the study of nucleoproteins and their constituents.

In this paper, a few simple properties and characteristics of an undissociated desoxyribonucleoprotein, obtained by extraction in water, will be considered in some detail. Particular emphasis will be directed towards the elucidation of the physical state and the molecular and chemical configuration of the nucleoprotein. It will be shown that the characteristics of this undissociated desoxyribonucleoprotein are quite different from the properties exhibited by the nucleoproteins obtained by extraction in strong salt solutions.

MATERIALS AND METHODS

The isolation of the desoxyribonucleoprotein of the sperm of the sea urchin, *Strongylocentrotus purpuratus*, has already been described[1] and similar procedures have been followed in obtaining material for the present study. Further details concerning the various procedures and techniques utilized will be given in connection with the presentation of the results of the experiment.

* Supported by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

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RESULTS

Solubility in salt solutions

Available information on the solubility of nucleoproteins may be summarized in four salient points. LILIENFELD[2] showed that nucleoproteins are soluble in water, GAJDUSEK[8], that they are insoluble in dilute (0.14 *M*) NaCl. MIRSKY AND POLLISTER[7] demonstrated the solubility of nucleoproteins in strong (1.0 *M*) NaCl solutions, as well as the insolubility of this material when diluted to 0.14 *M* NaCl. STERN *et al.*[4] have shown that in strong salt solutions the DNA and protein (histone) are dissociated, and that the solution is actually a mixture of these two components. On dilution the nucleic acid and protein recombine to form a precipitate, which has been considered to be a nucleoprotein.

The solubility behaviour of desoxyribonucleoproteins in salt solutions represents one of the most characteristic properties of these compounds. To give a more complete picture, the solubility or the insolubility of the nucleoprotein has been determined over a range of concentrations. The specific absorption of the nucleic acid moiety (at 260 mμ) of the nucleoprotein has been utilized as the simplest and most sensitive measure of the amount of material remaining in solution. For these measurements, 1.0 ml aliquots of water-extracted nucleoprotein were mixed with varying amounts of 2.0 *M* NaCl, so that dilution to a constant volume of ten ml gave the desired salt concentration. To avoid complicating effects, the salt was always added last. The diluted samples were allowed to stand for a few minutes and then centrifuged to remove any precipitate that may have formed. The optical density of the clarified solutions was read at 260 mμ in a Beckman model DU spectrophotometer.

The results of a series of measurements of the solubility of the desoxyribonucleoprotein in NaCl solutions are shown in Fig. 1. The concentration (molarity) of NaCl has been plotted against the percent of the nucleoprotein that has been precipitated. The insolubility of the nucleoprotein rises sharply with increasing salt concentration, and is maximally precipitated at 0.14 *M* NaCl. As the salt concentration is raised further, there is a slow increase in solubility up to 0.7 *M* NaCl, and then very suddenly the material again becomes completely soluble.

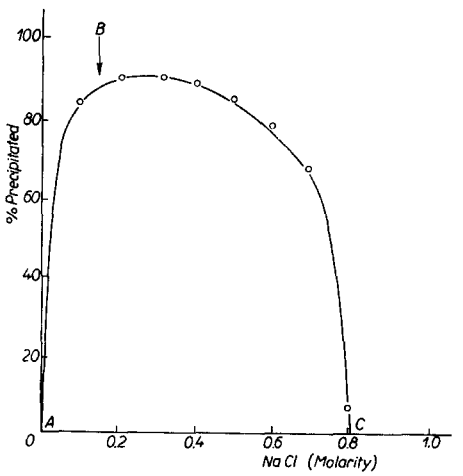

Fig. 1. The solubility of the water-soluble desoxyribonucleoprotein in varying concentrations of NaCl.

STERN and collaborators[4,5] have shown that nucleoproteins in strong (1.0 molar) salt solutions, are dissociated into their component parts, nucleic acid and protein (histone). These are the circumstances of the solubility of the nucleoprotein at point C of Fig. 1, and the increased solubility from points B to C is presumably a reflection of the progressive dissociation of the desoxyribonucleoprotein molecule. In the procedure of MIRSKY AND POLLISTER[7], a mixture of the dissociated nucleic acid and protein is obtained by extraction in molar NaCl. On dilution to 0.14 *M* NaCl, the nucleic acid and protein are recovered in the form of a fibrous precipitate of *re-combined* nucleoprotein.

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It is apparent that a complex product of cellular synthesis, such as a desoxyribonucleoprotein, will almost certainly not regain its original highly specific configuration once it has been dissociated by external influences. That this is actually the case is evidenced by the fact that this re-combined nucleoprotein is only very sparingly soluble in water, in contrast with the original water-soluble material.

The solubility properties of nucleoproteins described in the preceding paragraph, may be stated in terms of Fig. 1. Using a suitable source of material, by extraction at point A (distilled water), an undissociated desoxyribonucleoprotein is obtained. Extraction of similar material at point C (1.0 *M* NaCl) yields a mixture of dissociated desoxyribonucleic acid and protein. At point B (0.14 *M* NaCl), both the dissociated and the undissociated materials are precipitated. From point B, the undissociated desoxyribonucleoprotein can be returned to A, or carried all the way to point C. That is, the undissociated desoxyribonucleoprotein, precipitated in 0.14 *M* NaCl, is soluble in distilled water; and furthermore, it is soluble (and dissociated) in 1.0 *M* NaCl. On the other hand, the dissociated material precipitated at point B (0.14 *M* NaCl), can be returned to point C, but not to A. That is, the dissociated nucleoprotein extracted in 1.0 *M* NaCl can be precipitated in 0.14 *M* NaCl, but is no longer soluble in distilled water, only in strong salt solutions.

The cardinal feature of the preceding observations is that they illustrate the fact that a desoxyribonucleoprotein may exist in three distinctly different structural phases: the water-soluble undissociated desoxyribonucleoprotein; the dissociated mixture of desoxyribonucleic acid and protein, present in 1.0 *M* NaCl solutions; the re-combined nucleoprotein obtained by dilution of the preceding mixture to 0.14 *M* NaCl.

The dissociation of desoxyribonucleoproteins is not a specific property of strong NaCl solutions, but is, rather, a generalized property of strong salt solutions. Solubility studies similar to that described above for NaCl, have been made with several other salts, the results of these studies are shown in Fig. 2. In addition to NaCl, the solubility of desoxyribonucleoproteins has been studied in $(NH_4)_2SO_4$, $CaCl_2$, and NaH_2PO_4. Fig. 2 shows the typical pattern of the effects of salts on the sea urchin sperm nucleoprotein; in all cases, the water-soluble nucleoprotein is precipitated in 0.01–0.1 molar salt solutions. At higher concentrations, as the nucleoprotein is dissociated, it becomes soluble again. As seen in Fig. 2, the prin-

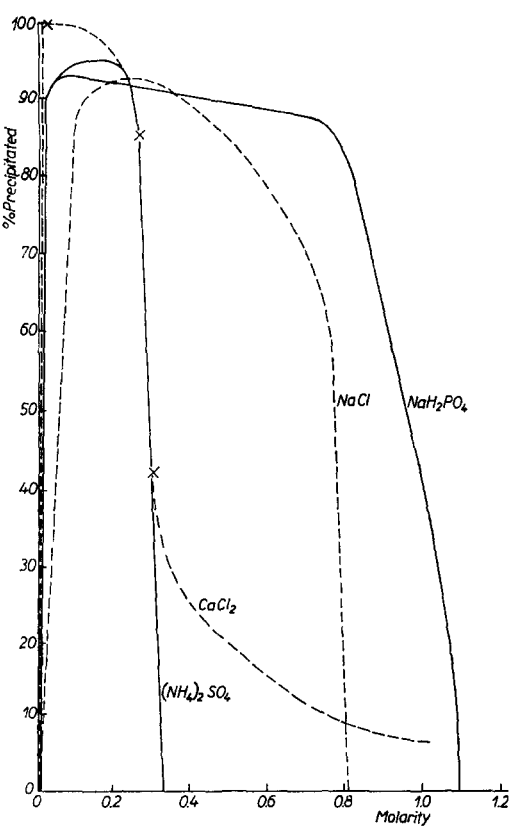

Fig. 2. The solubility of the water-soluble desoxyribonucleoprotein in solutions of $(NH_4)_2SO_4$, $CaCl_2$, NaCl, and NaH_2PO.

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cipal difference between the various salts is in the concentration at which the nucleoprotein is dissociated and, secondarily, soluble. Presumably, these values would be equivalent if the calculations were in terms of ionic strength instead of molarities. Unfortunately, it has not been possible to obtain sufficient data to permit the calculations of ionic strength on an empirical basis, and the theoretical calculation of ionic strength in strong salt solutions is not yet possible. Approximate calculations of ionic strength have shown, at least, that the spread of the dissociation values is reduced.

The dissociation of the nucleoprotein in the two uni-divalent salts, $CaCl_2$, and $(NH_4)_2SO_4$, occurs at the same concentration, about 0.3 molar (the coincidence of these curves is further evidence that the ionic strength of the salt solution is the critical factor in the dissociation of the nucleoprotein). However, it will be noted in Fig. 2 that the calcium ion does not permit the nucleoprotein to become completely soluble, even after it has been dissociated*. GILBERT, OVEREND AND WEBB[9] have shown that nucleohistones are precipitated by the heavy-metal cations, and that some of these precipitates, including the Ca-precipitate, are soluble in 2.0 molar NaCl. These authors did not investigate the effect of higher concentrations of the precipitants, but the fact of distinct cationic effects on the nucleoprotein seems to be well established.

Isoelectric point

The isoelectric point of the desoxyribonucleoprotein has been determined according to the method described by OSTER[10]. Duplicate samples of desoxyribonucleoprotein were set up to be titrated at the same time. Dilute HCl was added in small amounts, and each sample was stirred while the acid was being added. The titration of one sample was followed in a pH meter, the other sample was kept in Klett-Summerson photoelectric colorimeter (with a blue filter) and the turbidity of the sample was determined at each point. The maximal turbidity, attained at the isoelectric point gives a fairly sharp end-point to the titration. As shown in Fig. 3, the isoelectric point of the desoxyribonucleoprotein of sea urchin sperm is in the range of 2.3–2.7. This is well below the pH of any of the salt solutions that have been used in this study, and would preclude the possibility of isoelectric precipitations as a complicating factor in the solubility studies. Indeed, the presence of high concentrations of salt tends to lower the isoelectric point still further. Samples of desoxyribonucleoprotein titrated in the presence of 2.0 molar NaCl showed an isoelectric point in the region of pH 2.0–2.4.

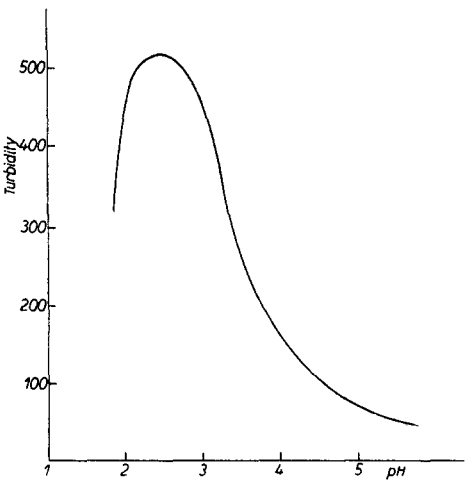

Fig. 3. Titration curve and isoelectric point of the desoxyribonucleoprotein of sea urchin sperm as determined by the turbidimetric method of OSTER[10].

Precipitation in alcohol

The distinctions that have previously been made with regard to the various con-

* The anions are considered to be innocuous, or, more probably, their interaction with the basic groups of the protein is responsible for the initial precipitation. In either case, there is no apparent specificity or selectivity among the anions.

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figurational states of the desoxyribonucleoprotein are also seen in the characteristics of the alcohol precipitation of the materials. One of the salient features of the MIRSKY AND POLLISTER preparation is the fact that when the nucleoprotein is extracted in molar saline, the precipitate obtained by dilution (with water) is very fibrous, and can be collected by winding around the end of a glass stirring rod. A similar precipitate is obtained if the molar saline extract is diluted with two volumes of 95% ethyl alcohol. It is clear that the fibrous nature of this precipitate is a property of the extremely asymmetric DNA molecule. If the water extracted (undissociated) nucleoprotein is precipitated with two volumes of 95% ethyl alcohol, a fluffy, white precipitate is obtained, which has no fibrous quality. If the same material is first made 1.0 molar in NaCl and then precipitated with alcohol, a fibrous precipitate is obtained. This simple procedure has been used routinely to test for the presence of high-polymer DNA.

Similar precipitation properties are observed if a solution of protamine is used as the precipitant, instead of alcohol.

These observations suggest that in the undissociated desoxyribonucleoprotein, the intrinsic asymmetry of the DNA molecule is masked or reduced by the combination of the DNA with protein, both histone and non-histone protein. When this complex conjugated nucleoprotein molecule is dissociated in strong salt solutions, the asymmetry of the nucleic acid component becomes manifest. It is also clear that, while the fibrous precipitate is composed of nucleic acid and protein, the structural configuration of this complex is not the same as that of the original undissociated desoxyribonucleoprotein.

Viscosity

Viscosity measurements have been made of the undissociated desoxyribonucleoprotein, and of the same material in 1.0 molar NaCl. These determinations were made in a constant temperature bath at 23° C, with an Ostwald viscometer. In all cases, a total volume of 5.0 ml was used in the viscometer. When dilutions were made, a 5.0 ml sample of the desoxyribonucleoprotein was used, with an equal volume of distilled water or 2.0 *M* NaCl. The results of one set of such measurements are shown in Table I. The original desoxyribonucleoprotein extract has a relative viscosity of 1.40. Dilution of the nucleoprotein extract with an equal volume of water should reduce the relative viscosity by one half, or, to a value of 1.20. A value of 1.19 is observed. Another sample similarly diluted with 2.0 molar NaCl instead of water, has a relative viscosity of 1.31. It is apparent that the relative viscosity of the nucleoprotein solution is increased in the presence of strong salt solutions. This increased asymmetry is attributable to the dissociation of the nucleic acid and protein components of the nucleoprotein preparation.

TABLE I

VISCOSITY OF THE UNDISSOCIATED DESOXYRIBONUCLEOPROTEIN, OF THE NUCLEOPROTEIN DILUTED WITH AN EQUAL VOLUME OF WATER, AND WITH AN EQUAL VOLUME OF 2.0 *M* NaCl

	Flow time (sec.)	*Relative viscosity*
DNP	134.2 ± 0.6	1.40
DNP + 1 Vol. H_2O	112.7 ± 0.2	1.19
DNP + 1 Vol. 2 m. NaCl	124.2 ± 0.3	1.31

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Electron microscopy

From the data presented above, one is led to the expectation that the undissociated desoxyribonucleoprotein will have a configuration dictated by the long, slender shape of the DNA molecule, the asymmetry of which has been reduced by the addition of protein in such a manner as to produce a thicker molecule of the same length as the DNA particle. Fig. 4 is an electron micrograph of an undissociated desoxyribonucleoprotein preparation, and it can be seen that the expected shape of the molecule is realized. In making this preparation, the nucleoprotein extract was sprayed on electron microscope grids that had been mounted on glass microscopic slides under a film of collodion. The sprayed grids were dried *in vacuo*, and shadowed with palladium. Under these conditions a marked tendency toward longitudinal aggregation is apparent. An individual particle is indicated in Fig. 4. This particle has a length of 4300 A.U., and all of the particles have a uniform thickness of 250–300 A.U., giving an axial ratio of 16:1. This is to be compared with the asymmetry factor of 35:1 that has been most frequently found in sedimentation studies on desoxyribonucleoproteins[4,5,11,12]. Further studies on the molecular configuration of the undissociated desoxyribonucleoprotein are in progress. This picture is presented now, primarily because it is, to the authors' knowledge, the first published picture of an extracted nucleoprotein particle that even approximates dimensions that could reasonably be expected to exist in a nucleoprotein molecule.

DISCUSSION

It is in many respects an unfortunate circumstance that nucleoproteins extracted in strong salt solutions are so easily recoverable in the form of a nucleoprotein precipitate. This has given rise to a widespread belief that the properties, and in particular the physical properties, of desoxyribonucleoproteins can advantageously be studied in strong salt solutions. From the data presented in this paper, it is clear that such is actually not the case, and that studies of the undissociated, water-soluble nucleoprotein must give due consideration to the medium in which the material is to be investigated.

With the information now available, we can make a few general remarks about the chemical and physical properties of the undissociated desoxyribonucleoprotein, and about the nature of the bonding between DNA and protein. The outstanding characteristic of the undissociated nucleoprotein is its extreme instability, with respect both to its structural integrity and to its water solubility. Once extracted the material must be kept cold, but even at 2° C the nucleoprotein degrades quite rapidly, degradation in this sense referring to the loss of high-polymer nucleic acid. The extent of this degradation was readily and almost routinely checked by the appearance of the alcohol precipitate of salt-dissociated material, and by the analyses reported previously[1]. This degradation or depolymerization was essentially complete in 48 hours, even when the material was kept in the cold. Apparently, the nucleic acid to protein linkage survives this degradation process, since the solubility properties shown in Figs. 1 and 2 are shown by degraded preparations that no longer contain any highly polymerized nucleic acid. This relative stability of the nucleic acid—protein linkage is surprising, since it is this same linkage that is broken by the dissociating action of strong salt solutions. This difference between the effects of aging degradation and the dissociation produced by strong salt solutions is illustrated diagrammatically in Fig. 5. In the diagram, it can

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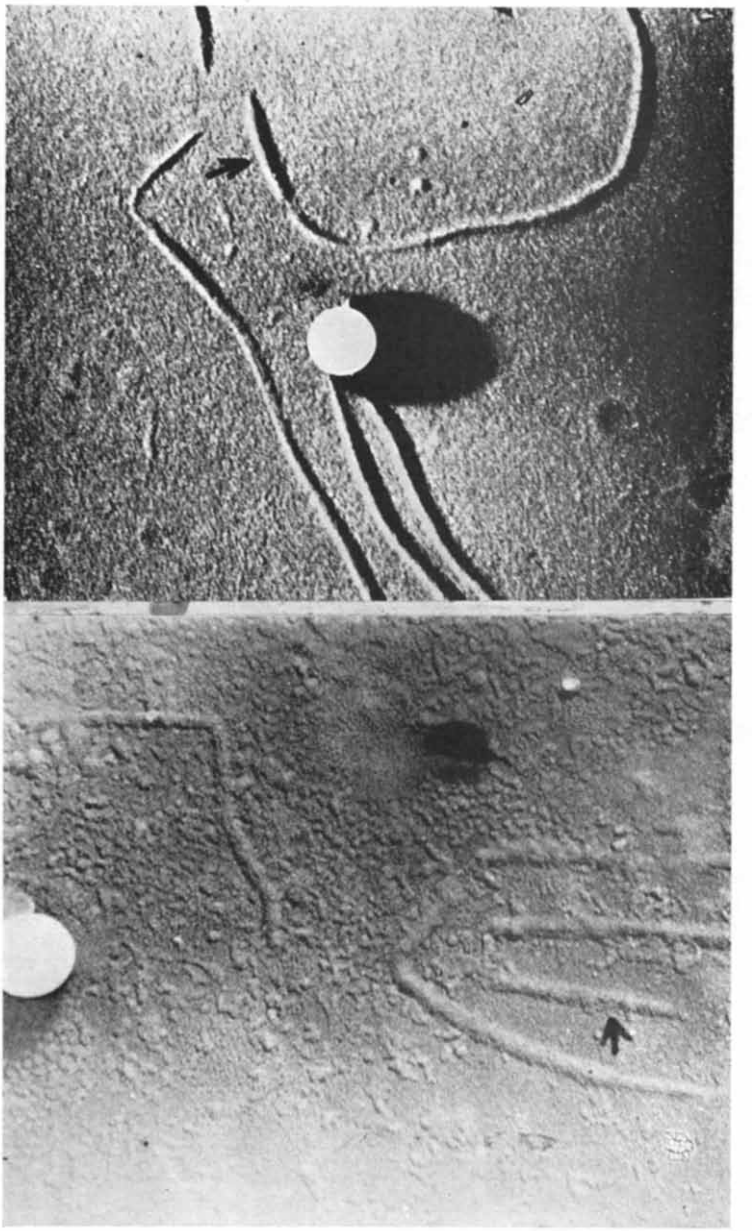

Fig. 4. Electron micrographs of the desoxyribonucleoprotein particles. Preparations were sprayed, and shadowed with palladium. The white spheres are polystyrene particles, having a diameter of 0.26 microns. Individual particles are indicated by arrows. The tendency toward longitudinal aggregation is apparent.

be seen that the degradation consequent to aging is a splitting *across* the nucleoprotein molecule, parallel to the planes of the "stack of discs" made by the individual nucleotides of the DNA. The product of this degradation is apparently a low-molecular-weight fragment, containing nucleic acid and protein in essentially the same combinations as found in the original particles[1].

In contrast to this, the effect of strong salt solutions is to split the molecule down its long axis, separating nucleic acid and protein components. The nucleic acid thus liberated is free to express its intrinsic asymmetry, accounting for the viscosity of the salt-dissociated nucleoprotein solutions, and for the fibrous nature of the alcohol precipitate.

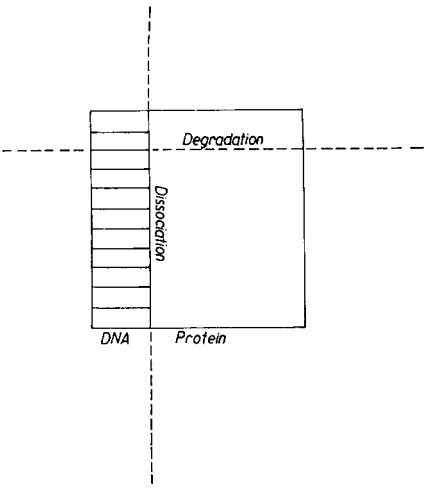

Fig. 5. Schematic representation of the desoxyribonucleoprotein particle, to show the difference between the degradation due to aging, and the dissociation effected by strong salt solutions.

The phenomenon of aging degradation described above is probably not attributable to desoxyribonuclease activity. The sperm have been initially treated with sodium citrate, a potent though not universal inhibitor of desoxyribonuclease at concentrations well below those used here. Furthermore, all attempts, to date, to show desoxyribonuclease activity in sperm have been unsuccessful.

The water solubility of the undissociated nucleoprotein is also maintained, apparently, in a very sensitive situation. Precipitation in dilute NaCl is the only treatment that has been found that can be successfully used on this material and permit its recovery in a water-soluble form, and there are limitations here as well. When precipitated in dilute salt, the material can be recovered by mild centrifugation. However, if the precipitate is packed too tightly by the centrifugation, it may no longer be soluble in water. Alcohol precipitation renders the nucleoprotein insoluble in water. Lyophilization was attempted, but was found to be impracticable, since freezing the aqueous nucleoprotein extract makes it precipitate on thawing, and again the material is no longer soluble in water. On the other hand, whole sperm, the source material, can be lyophilized and the undissociated desoxyribonucleoprotein extracted.

These observations suggest that the water solubility of the undissociated nucleoprotein is a function of a property (possibly electrostatic charge) which is easily and irreversibly upset, but which, while maintained intact, permits the molecule to become sufficiently hydrated to be soluble in water.

In the preceding paper[1], it has been shown that precipitation of the undissociated desoxyribonucleoprotein in 0.14 *M* NaCl followed by re-solution in water purifies in the sense that it removes a portion of the non-histone protein. Presumably, the protein that is removed is not attached or is more loosely attached to the nucleic acid moiety. The fact that not all of the NHP is removed implies that this non-histone fraction is attached to the nucleic acid, or is at least an integral part of the nucleoprotein molecule. MIRSKY[14] in a recent publication has suggested that there is an attachment between the non-histone protein fraction of chromosomes and the nucleic acid.

It has also been found in the present study that this nucleic acid-protein linkage

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is resistant to surface denaturation. An aqueous nucleoprotein extract was shaken with chloroform, according to the deproteinizing procedure of SEVAG *et al.*[15]. Protein was removed by this procedure, but in essentially the same sense as described above for the purification by precipitation in dilute saline. That is, all of the nucleic acid was recovered, as was all of the histone, but only a small fraction of the non-histone protein remained attached to the nucleoprotein. This phenomenon has not been thoroughly investigated, but would suggest itself as a possible means of effecting the rapid removal of "extraneous" protein associated with the nucleoprotein.

We may, then, formulate a picture of the undissociated desoxyribonucleoprotein in the following terms: the long axis of the molecule (the backbone) is formed by the desoxyribonucleic acid component. Protein, both histone and a smaller amount of non-histone protein, is attached to the nucleic acid in such a manner as to reduce the asymmetry of the molecule from the 300:1 ratio of the DNA to the 35:1 of the nucleoprotein; this reduction in asymmetry can be effected by an approximately six-fold increase in the diameter of the molecule. These proteins are, furthermore, linked to the nucleic acid in such a manner that they are readily split off by the action of concentrated salt solutions, yet the linkage is resistant to the degradation of the nucleic acid and to surface denaturation. This whole complex exists in such a state (of electrostatic charge) that it can be hydrated sufficiently to make it soluble in distilled water, but this water solubility is lost if the molecule is dissociated by concentrated salt solutions, or if it is precipitated by alcohol, or if a water solution of the material is frozen.

These then, are the properties of an undissociated desoxyribonucleoprotein. They are not shared by other configurational states of similar materials. The uniqueness of the undissociated nucleoprotein cannot be over-emphasized, particularly since this material presents distinct advantages for the speculative interpretation of chromosomal behaviour on a molecular basis.

SUMMARY

Some of the physical and chemical properties of the undissociated desoxyribonucleoprotein of the sperm of the sea urchin, *Strongylocentrotus purpuratus*, have been investigated. The desoxyribonucleoprotein is soluble in distilled water, precipitated by physiological saline, and is in general insoluble in salt solutions in the range of 0.01 to 0.1 molar.

The undissociated desoxyribonucleoprotein is soluble in and dissociated by strong salt solutions. The range of this effect is from 0.34 molar for $(NH_4)_2SO_4$ to 1.1 molar for NaH_2PO_4. Ionic strength is believed to be the common denominator of this phenomenon.

The isoelectric point of the undissociated desoxyribonucleoprotein is in the region of pH 2.3–2.7

Precipitation of the undissociated nucleoprotein particle in alcohol gives a light fluffy precipitate, indicating a very marked reduction in asymmetry as compared with the fibrous precipitate obtained by alcohol precipitation of the nucleoprotein from a strong salt solution. The increased viscosity of the nucleoprotein in strong salt solution provides further proof that the undissociated desoxyribonucleoprotein is dissociated into nucleic acid and protein components by the action of strong salt solutions.

Electron microscopy has shown the undissociated desoxyribonucleoprotein particle to be approximately 4300 A.U. in length and 250–300 A.U. in width.

RÉSUMÉ

Certaines des propriétés physiques et chimiques du désoxyribonucléoprotéide non dissocié des spermatozoïdes de l'oursin *Strongylocentrotus purpuratus* ont été étudiées. Ce désoxyribonucléoprotéide est soluble dans l'eau distillée, précipité en milieu physiologique salin et, de manière générale, il est insoluble dans les solutions salines allant de 0.01 à 0.1 molaire.

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Le désoxyribonucléoprotéide est soluble et il se dissocie dans les solutions salines fortes. Cet effet se produit entre les concentrations 0.34 molaires pour $(NH_4)_2SO_4$ et 1.1 molaire pour NaH_2PO_4. Nous pensons que la force ionique est le dénominateur commun de ce phénomène.

Le point iso-électrique du désoxyribonucléoprotéide non dissocié se situe dans la région allant de pH 2.3 à 2.7.

La précipitation par l'alcool des particules du désoxyribonucléoprotéide non dissocié fournit un léger précipité duveteux ce qui indique une réduction marquée de l'asymétrie par rapport au précipité fibreux qu'on obtient par précipitation à l'alcool du nucléoprotéide en solution saline concentrée. L'accroissement de la viscosité du nucléoprotéide en solution saline concentrée constitue une preuve supplémentaire de ce que les solutions salines fortes dissocient le désoxyribonucléoprotéide non dissocié en ses constituants nucléique et protéique.

La microscopie électronique a montré que les particules du désoxyribonucléoprotéide non dissocié ont approximativement 4300 A de long et 250–300 A de large.

ZUSAMMENFASSUNG

Einige der physikalischen und chemischen Eigenschaften des undissoziierten Desoxyribonukleoproteins aus dem Sperma des Seeigels, *Strongylocentrotus purpuratus*, wurden untersucht. Das Desoxyribonukleoprotein ist in destilliertem Wasser löslich, wird durch physiologische Kochsalzlösung ausgefällt und ist im allgemeinen in Salzlösungen in einem Bereich von 0.01 bis 0.1 *M* unlöslich.

Das undissozierte Desoxyribonukleoprotein ist unter Dissoziation in starken Salzlösungen löslich. Dieser Effekt wird in einem Bereich von 0.34 *M* bei $(NH_4)_2SO_4$ bis zu 1.1 *M* bei NaH_2PO_4 beobachtet. Es wird angenommen, dass dieses Phänomen hauptsächlich durch die Ionenstärke verursacht wird.

Der isoelektrische Punkt des undissozierten Nukleoproteins liegt im pH-Gebiet von 2.3–2.7.

Die Ausfällung der undissozierten Nukleoproteinteilchen mit alkohol gibt einen leichten, flaumigen Niederschlag und zeigt so eine sehr bemerkenswerte Verminderung der Asymmetrie an, verglichen mit dem durch Alkoholausfällung aus starken Salzlösungen erhaltenen faserigen Niederschlag des Nukleoproteins. Die erhöhte Viskosität des Nukleoproteins in starken Salzlösungen liefert einen weiteren Beweis dafür, dass das undissoziierte Desoxyribonukleoprotein in Nukleinsäure und Proteinkomponenten durch Einwirkung der starken Salzlösung dissoziiert ist.

Aufnahmen mit dem Elektronenmikroskop zeigten, dass die undissoziierten Desoxyribonukleoproteinteilchen ungefähr 4300 A lang und 250–300 A breit sind.

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Received October 4th, 1952