THE COMBINATION OF PROTAMINE WITH DESOXYRIBONUCLEIC ACID

by

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MIESCHER[1] in 1897 found that an insoluble precipitate was formed on mixing aqueous solutions of protamine sulphate and the sodium salt of desoxyribonucleic acid (DNA). The nucleohistones and nucleoprotamines occurring naturally in living cells are, however, soluble in water—this property was utilised by BANG[2] and HAMMARSTEN[3] in their isolation—though not in physiological saline[4]. The nucleoprotein isolated from the sperm heads of fish consists largely[1] if not entirely[5] of protamine and DNA. This water-soluble nucleoprotamine, though insoluble in dilute solutions of electrolyte (*e.g.* 0.2 *M* sodium chloride), dissolves with dissociation in strong salt solutions from which the DNA and protamine can be separated by dialysis or precipitation[6]. On mixing aqueous solutions of the DNA and protamine thus obtained an insoluble fibrous precipitate is formed having the same composition as the original soluble nucleoprotamine. An attempt is made in this paper to determine the differences in structure between the original and reconstituted complex.

EXPERIMENTAL

Preparation of nucleoprotamine from herring sperm was carried out by the method of HAMMARSTEN[3] except that the complex was precipitated with 0.14 *M* NaCl instead of $CaCl_2$. The product was completely water-soluble though it dissolved only slowly and was found to contain 65% DNA and 33% protamine. In agreement with FELIX *et al.*[5] the whole of the complex was accounted for within experimental error as DNA and protamine. DNA of high molecular weight[7], with which the subsequent work was done, was kindly provided by Dr J. A. V. BUTLER. The protamine sulphate, salmine, was obtained from British Drug Houses Ltd.

Combination of DNA with protamine was determined by mixing equal volumes of aqueous solutions containing known strengths of the two materials. The precipitate formed was centrifuged off and its composition was calculated from the concentration of DNA and protamine in the supernatant liquid, which was analysed spectrophotometrically. The concentration of protamine was determined by the Sakaguchi reaction, the colour being estimated at 520 mμ[8] and calibrated with protamine. DNA did not interfere in this estimation. The DNA was estimated by determining the absorption at 260 mμ; a correction was applied for the absorption due to protamine.

The viscosity of DNA and nucleoprotamine were determined in a horizontal capillary viscometer as described by ALEXANDER AND HITCH[9].

RESULTS

Table I shows that the combination of DNA and protamine is non-stoichiometric and that complexes of widely varying composition are precipitated on mixing solutions

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of different concentrations. In the concentration range examined in these experiments DNA and protamine could not exist together in neutral solution. The minimum concentration of protamine in a complex with DNA was found to be approximately 30%, and, when insufficient protamine was present to form such a complex, part of the DNA remained in solution and all the protamine was precipitated. With more than one part of protamine per two parts of DNA some of the protamine remains in solution but all the DNA is precipitated. The quantity of protamine in the complex in excess of 30% is determined by the concentration of protamine remaining in solution. The precipitate once formed can still combine with protamine if more of this is added to the solution in equilibrium with the complex. Under all these conditions the complex is precipitated in the form of white fibres which are soluble in strong salt solutions (*e.g.* 2 *M* sodium chloride) from which the protamine can be completely removed by dialysis through "cellophane".

TABLE I

REACTION OF DNA AND PROTAMINE IN AQUEOUS SOLUTION

Original concentration %		*Final concentration %*		*Composition of precipitate*
DNA	*Protamine*	*DNA*	*Protamine*	*g protamine/g DNA*
0.2	0.4	0.0	0.20	1.01
0.2	0.3	0.0	0.13	0.83
0.2	0.2	0.0	0.065	0.66
0.2	0.15	0.0	0.027	0.62
0.2	0.10	0.001	0.0	0.50
0.2	0.05	0.11	0.0	0.51
0.1	0.1	0.0	0.034	0.68
0.05	0.15	0.001	0.112	0.78

Staining tests in neutral solutions show that complexes containing approximately one third of protamine stain readily with basic dyes and not at all with acid dyes. Complexes containing more protamine stain preferentially with acid dyes though they will combine with basic dyes which on combination displace some of the protamine. This staining behaviour is in agreement with the generally accepted concept that the combination of DNA and protamine is the result of electrostatic interaction between the arginine groups of protamine and the phosphate anion in the DNA. An exactly stoichiometric complex would consist of 63% DNA (containing 9% of phosphorus) and 37% of salmine (containing 85% of arginine[10]). Protamine-rich complexes have excess basic groups available for combining with acid dyes (*i.e.* sodium salts of organic acids), whereas in complexes with excess DNA phosphate groups are available for combining with basic dyes (*i.e.* chlorides or sulphates of organic bases).

Table II shows that less precipitation takes place in more dilute solutions; when the ratio of DNA to protamine is kept constant progressively more of the two components remain in solution until with 0.006% DNA no insoluble complex is formed. A white powder, freely soluble in water, is obtained if these dilute solutions are evaporated under vacuum or precipitated with salt. This material will be referred to as the soluble complex and resembles in many respects the naturally occurring nucleoprotamine obtained by water extraction.

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TABLE II

REACTION OF DNA AND PROTAMINE IN VERY DILUTE SOLUTIONS

Original concentration %	*% of DNA precipitated with the following ratios of protamine to DNA*		
	0.80	*0.66*	*0.33*
0.3	100	100	55
0.15	100	91	44
0.06	78	76	18
0.03	36	58	7.3
0.015	10.0	0.0	11.2
0.006	0.0	0.0	0.0

A soluble complex containing 38% protamine was obtained by mixing 0.0024% DNA with 0.0015% protamine and evaporating under vacuum. The solubility and viscosity of the powder thus obtained was compared with that of the naturally occurring nucleoprotamine and both materials showed exactly the same behaviour in the following tests. Both materials were precipitated from aqueous solution on making the solution 0.14 *M* with respect to sodium chloride but dissolved in concentrated salt solutions (*e.g.* 2 *M* sodium chloride). The protamine could be removed by dialysis from solutions in strong salt but not from solutions in 0.01 *M* sodium chloride. From this it can be concluded that both the soluble synthetic complex and the nucleoprotamine dissociate in salt but are undissociated in water or very dilute salt solutions.

The viscosity of both materials in water was very much less than that of a solution containing the same quantity of DNA only (see Table III). In 2 *M* sodium chloride however the viscosity of the complex was almost the same as that of DNA. Similar results were obtained by STERN[11], who found that the viscosity of a water-soluble nucleohistone was greater in 2 *M* sodium chloride than in water. The water-soluble complex is not very stable and on standing after more than a few days becomes progressively less soluble in water.

TABLE III

THE VISCOSITY AT A SHEAR RATE OF 50 sec^{-1} OF NUCLEOPROTAMINE

Solution	*Relative viscosity*
0.1% DNA in water	9.3
Nucleoprotamine solution containing equivalent of 0.1% DNA in water	1.8
As above in 2 *N* KCl	3.4

DISCUSSION

These results can be interpreted in the following way. In the naturally occurring material a number of protamine molecules lie end-to-end exactly parallel to the nucleic acid molecule (*cf.* ASTBURY[12]), but since the complex contains only 33% protamine not all the phosphate groups are taken up in combination with basic groups and the molecule has a negative charge which renders it soluble. On the addition of small quantities of electrolyte (*e.g.* 0.14 *M* sodium chloride) the width of the electric double layer sur-

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rounding the ionised phosphate groups is decreased sufficiently to enable the nucleoprotamine molecules to approach close enough for interaction by secondary valency forces. There are many possibilities for the formation of hydrogen bonds and the large size of the molecule promotes Van der Waals' attraction, so that interaction leading to aggregation and precipitation is to be expected once these forces can come into play. ALEXANDER AND STACEY[13] showed that the aggregation of dyes by the addition of salt occurred by an essentially similar mechanism to the one proposed here. Concentrated salt solutions on the other hand dissociate the complex by breaking the electrostatic link between the basic groups of the protamine and the phosphate anions of DNA (*cf.* GREENSTEIN[14]). The rupture of comparable links in protein fibres by concentrated salt solutions was observed by ALEXANDER[15].

The formation of the insoluble precipitate on mixing relatively concentrated solutions of DNA and protamine result from the failure of the two macromolecules to align themselves, so that one protamine molecule is associated not with one DNA molecule only but links a number of these together. This behaviour is to be expected when two solutions are mixed in which the molecules are not oriented. The mixture precipitates due to electrostatic cross-linking and the three-dimensional network is insoluble although charged groups are present. Non-stoichiometric combination would be expected in such a system.

According to this interpretation it is to be expected that when the DNA and protamine are mixed in exceedingly dilute solution no precipitation will occur since the molecules are too far apart for the electrostatic cross-linking to take place and each protamine molecule combines with only one DNA molecule.

The soluble complex so formed would be very similar to the natural nucleoprotamine and this in agreement with the results reported in this paper. HAMMARSTEN[3] prepared a soluble complex between DNA and protamine by mixing these in 0.1 *N* sodium hydroxide and subsequently neutralising the solution. No cross-linking can take place in the alkaline solution when the basic groups of the protamine are unionised and can align themselves by secondary forces parallel to the DNA, and on neutralisation firm combination via electrostatic links takes place. This method of preparing a soluble complex is not very satisfactory since irreversible changes take place on making DNA alkaline[16].

The fall in viscosity of DNA on combination with protamine may either be due to a decrease in asymmetry or a decrease in molecular interaction which contributes to the viscosity especially at low rates of shear[9,17]. Experiments are now being undertaken to determine this point by studying the shape of the soluble complex by light scattering.

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SUMMARY

Water-insoluble fibrous complexes of deoxyribonucleic acid (DNA) and protamine of widely varying composition were obtained by mixing aqueous neutral solutions of the two materials. Depending on the relative proportions of DNA and protamine the complexes stained preferentially with acid or basic dyes. If the DNA and protamine were mixed in very dilute aqueous solution no precipitation took place: a powder soluble in water but insoluble in dilute salt solution was left in drying. This material resembles the undissociated nucleoprotein obtained by extracting cells with water, and the protamine is associated with the DNA in aqueous solutions (*i.e.* cannot be removed by dialysis) but is dissociated in concentrated salt solutions.

Structures are proposed for the water-soluble and insoluble complexes which account for their physical properties.

RÉSUMÉ

Des complexes d'acide désoxyribonucléique et de protamine ont été obtenus en mélangeant des solutions aqueuses neutres des deux substances; ces complexes sont fibreux et insolubles dans l'eau. Selon les proportions d'acide désoxyribonucléique et de protamine qu'ils renferment, les complexes fixent de préférence les colorants acides ou basiques. Aucun précipité ne se forme lorsqu'on mélange des solutions très diluées d'acide désoxyribonucléique et de protamine; la solution, évaporée à sec, laisse une poudre soluble dans l'eau mais insoluble dans une solution saline diluée. Ce produit ressemble à la nucléoprotéine non dissociée qu'on obtient en extrayant les cellules par l'eau et la protamine y est associée à l'acide désoxyribonucléique en solution aqueuse (la dialyse ne l'élimine pas); dans les solutions salines concentrées, au contraire, elle est dissociée.

Des structures sont proposées qui rendent compte des propriétés physiques des complexes solubles et insolubles dans l'eau.

ZUSAMMENFASSUNG

Wasserunlösliche, faserige Komplexe von Desoxyribonukleinsäure (DNS) und Protamin von sehr wechselnder Zusammensetzung wurden beim Mischen der wässrigen neutralen Lösungen beider Substanzen erhalten. Je nach den entsprechenden Anteilen von DNS und Protamin liessen sich die Komplexe vorzugsweise mit sauren oder basischen Farbstoffen färben. Werden DNS und Protamin in sehr verdünnten wässrigen Lösungen gemischt, so tritt keine Ausfällung ein: Beim Eintrocknen blieb ein in Wasser lösliches, aber in verdünnten Salzlösungen unlösliches Pulver zurück. Es ähnelt dem undissoziierten Nukleoprotein, das bei der Extraktion von Zellen mit Wasser erhalten wird; sein Protamin ist an die DNS in wässrigen Lösungen gebunden (d.h. es kann nicht durch Dialyse entfernt werden), ist aber in konzentrierten Salzlösungen dissoziiert.

Es werden Strukturen für die wasserlöslichen und -unlöslichen Komplexe vorgeschlagen, die ihre physikalischen Eigenschaften erklären.

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