XIII. THE BASIC AND ACIDIC PROTEINS OF THE SPERM OF *ECHINUS ESCULENTUS*. DIRECT MEASUREMENTS OF THE OSMOTIC PRESSURE OF A PROTAMINE OR HISTONE.

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So far as we are aware no one has hitherto made a determination of the molecular weight in colloidal solution of any member of those interesting classes of proteins with marked basic properties known as the protamines and histones. These substances have been set down by workers upon them such as Miescher and Kossel as the simplest steps in natural protein synthesis which furnish a clue as to the constitution of the others, and it is rather remarkable that no attempts have been made to study their degree of complexity in solution. The present paper must only be regarded as a tentative experiment in this direction, to be made more complete when more material becomes available; for the chief difficulty lies in obtaining suitable material and working it up into a supply of pure protamine or histone. The substance which we have isolated from the ripe male gonads of *Echinus esculentus* appears to us to stand intermediately between a protamine and a histone, for it gives feebly the Millon's test as a reddish colouration on boiling, with no precipitate; and yet it possesses all the properties of a protamine. This substance dissolved as a sulphate in distilled water gave an osmotic pressure leading to a molecular weight in solution much less than that of an albumin or globulin, the figure being approximately 8780 when calculated to standard temperature and pressure.

It behaved as a true colloid in being perfectly indiffusible through parchment paper, and the osmotic pressure rose steadily to a maximum, and remained there at a constant level. Afterwards, the sulphate of the protein

was thrown out in apparently quite unaltered form by excess of alcohol, dried and weighed. The substance comes down quite sharply with excess of alcohol, as a white precipitate readily soluble again in distilled water. As this process was repeated several times before measurements were taken, it is quite certain that if there was any admixture it must have been that of very closely allied substances of this class.

The only other substance of this protein group from the echinodermata ever examined was a substance isolated from the sperm of *Arbacia* by Matthews [1897], and called by him *arbacin*. This substance is classed amongst the histones by Matthews chiefly because it gave a positive result with the Millon's test. The substance with which we were working, from a different species of echinus, in a first preparation gave a negative reply to the Millon's reagent, and in a second preparation gave a somewhat feeble red colouration but no precipitate, and in absence of more complete analysis we are consequently somewhat doubtful as to whether we ought to classify it as a protamine or histone, so for the present we prefer to leave the point open.

It may be of some interest to pause here to state that our original intention in isolating this substance at Port Erin in the Spring of 1911, just at the commencement of the active sexual period, was to observe whether protamines and histones acted as hormones to the earlier stages of cell-division in sexual reproduction.

The whole group of the protamines is intimately connected with the male reproductive organs, being found in these glands almost exclusively in fishes, and in greatest abundance just before the sperm cells are ripe for discharge. The histones are obtained from the ripe spermatozoa of fishes, but in addition extend up to the sperm cells of mammalia. Both protamines and histones are strongly basic bodies, showing a markedly alkaline reaction when in solution as free bases, and combining strongly with acids, such as sulphuric acid, in definite molecular proportions.

Now it was shown some years ago by Moore, Roaf, and Whitley [1905] that almost infinitesimal amounts of alkali added to sea-water increased the rapidity of cell-divisions in fertilized echinus eggs, and that a slightly greater amount of alkali hurried the process into marked abnormalities.

It was accordingly thought that small amounts of alkaline proteins, such as protamines or histones, present in the sperm, ought to have prominent functions in relationship to cell-division.

It was from this point of view that the protamine, or histone, from echinus sperm was prepared and its action tested (*a*) upon unfertilized ripe

eggs and (b) upon fertilized ripe eggs, alongside controls developed in sea-water only.

The results were uniformly negative: whether this may be due to our being unable to realize proper experimental conditions we do not know. It may well be, that protamine added as sulphate to sea-water is in some wise rendered inert and incapable of penetrating the ovum, while the same substance carried into the ovum as part of the sperm-nucleus may be most effectual. The occurrence of these peculiar proteins in the sexual organs would seem to indicate some relationship with a reproductive process, but we have quite failed to realize an experimental proof.

The only positive result obtained by treatment of the fertilized eggs was that an extract of the crude sperm powder, after treatment with alcohol as described below, added in small amount, such as 1 in 4000, to sea-water inhibited cell-division and induced irregularities. Sperm nuclein appeared to possess a like action, but the protamine sulphate was quite inert.

The substance was separated from the male gonads by the well-known process of Kossel. About sixty well-grown echini were captured upon the breakwater at low tide on April 14th, 1911. The gonads of each individual were examined microscopically for sex identification and a mass of about 700 g. of spermatid gonads was obtained. This material was broken up finely and taken out in a large volume of water acidulated with acetic acid which causes the sperm cells to agglutinate and separate off. This was collected, extracted once with cold alcohol, and then exhausted of all fats and lecithides by four extractions with hot alcohol, and one with ether. The ether took out practically nothing on account of the previous exhaustive treatment with hot alcohol. The dried residue from all this extraction, of 700 g. of moist gland, amounted to only 21.68 g. This dried material remaining to the end, contains the protamines or histones and the nucleins or nucleic acids in combination together as nucleoproteins. To obtain the basic proteins (protamines or histones) it is extracted, still following Kossel's instructions, with five times its volume of half per cent. sulphuric acid, shaking vigorously at each extraction for about 15 minutes, followed by separation through fine silk gauze and pressing out. About three extractions were found to exhaust all protamine or histone from the material. After subsequent complete filtration through filter paper, the sulphuric acid extracts were precipitated with three volumes of absolute alcohol, yielding a nearly pure white precipitate which after standing for 24 hours was decanted and filtered by a Buchner filter. The precipitate dissolved readily in distilled water, and was purified by dissolving and reprecipitating with alcohol four times. It was

then dried after washing thoroughly with ether and weighed. The weight of the precipitate from 700 g. of moist gland, or 21.68 g. of dried tissue residue after alcoholic and ethereal extraction, was 1.95 g.

The residue from the extraction of the tissue with dilute sulphuric acid is exceedingly rich in nucleins or nucleic acid. It was freed from acid and dried by extraction with alcohol and ether, and then left to extract over night in the cold with one per cent. solution of caustic soda. When treated with the dilute alkali it swells up into a jelly which is almost solid in consistency and cannot be filtered in this condition, but it aggregates somewhat on adding three times its volume of absolute alcohol. It was left over night in this condition, but next morning was still unfilterable through paper. It was cleared of grosser debris by filtration through the finest silk bolting so giving a colloidal suspension free from large particles. When this alkaline alcoholic suspension was made just acid by addition of normal hydrochloric acid, a white flocculent precipitate was obtained which settled rapidly. This filtered quite readily leaving a very fine almost white precipitate on the paper. This precipitate was washed with distilled water over night, and until the washings were practically neutral next morning. It contained no protamine or histone as was shown by extracting it with dilute sulphuric acid and none was obtained from it at any stage, showing that the 1.95 g. mentioned above represented practically the total amount in the glands. The above precipitate caused by the acid in the alkaline extract, representing nuclein or nucleic acids, was weighed and found to be 7.45 g. A second extraction of the tissue residue separated by the silk filtration, yielded by the same method of alkaline extraction, followed by alcohol and acid, about 1.12 g. of a similar nucleic product, so that the total amount of acidic material was $7.45 + 1.12 = 8.57$ g. in combination with 1.95 g. of basic protein. The final residue after extraction with both acid and alkali was a substance which swelled up into a thick jelly in dilute alkali but did not dissolve in it, and shrank up when treated with dilute acid and was obviously an insoluble fibrinous form of protein. The weight after treating with alcohol, drying and weighing was 9.10 g. This material, calculating from the above weights, and the total weight of dried tissue extracted, is seen to form roughly half of the organic residue remaining after extraction with dilute acetic acid followed by alcohol and ether.

A second extraction of a different batch of male gonads made afterwards in Liverpool gave similar results.

A small amount of the protamine (or histone) sulphate was used at Port Erin for the investigation of effects upon cell-division, the remainder was

afterwards made up into a solution in distilled water containing approximately two per cent. of protamine sulphate. This was introduced against distilled water in the type of osmometer already described in previous papers [Moore and Roaf, 1907]. The dialysing membrane was parchment paper and complete equilibrium was established in three days' time as shown by the figures given below. At the end negative results were obtained on testing for protamine on the water-side. The protamine solution on the solution side of the membrane was quite sweet at the end of the experiment, and the protamine was precipitable from solution by alcohol as at the beginning. The solution had of course become somewhat diluted by expansion, and a determination of the amount of protamine sulphate by precipitation by excess of alcohol, filtering on to a Gooch crucible, drying by washing with alcohol and ether and weighing gave 1.65 per cent. of protamine sulphate. This gave a steady osmotic pressure of 34 millimetres of mercury at 15°, which works out at 19.3 millimetres for a one per cent. solution at 0°, and leads to a molecular weight in solution of the protamine solution of 8780.

The following is the protocol of the experiment:

	Date	Time	Manometric reading	Temperature
1911.	Nov. 21st	12 noon	388	15°
	„ 22nd	9.30 a.m.	391	13.5°
	„ 23rd	9.45 a.m.	396	14.0°
	„ „	5.30 p.m.	397	16.5°
	„ 24th	10.0 a.m.	401	15.0°
	„ „	6.0 p.m.	403	16.0°
	„ 25th	9.45 a.m.	406	14.5°
	„ „	5.45 p.m.	406	15.5°
	„ 26th	10.0 a.m.	406	14.5°
	„ „	5.30 p.m.	406	15.5°
	„ 27th	9.30 a.m.	406	15.0°
	Opened.	Zero Pressure	389	

Constant

Osmotic Pressure = $2(406 - 389) = 34$ millimetres of mercury. Percentage of protamine sulphate = 1.65.

As has clearly been shown by Moore and Roaf, and confirmed by Lillie and others, the molecular weight of colloids in solution varies with the solvent, amounts of neutral salts, and acidity or alkalinity. Such reagents affect the colloidal aggregation. Still it is of interest to compare the complexity of solution aggregation of this basic protein, with averages for other proteins, and colloids.

The following results have been obtained by various observers:

Colloid	Osmotic pressure for one per cent. of Colloid	Molecular weight in solution
Gelatin (Moore and Roaf) ...	9.2 mm.	18,500
Egg albumin (Moore and Parker) ...	13.1	13,000
Serum (" ") ...	2.8	60,800
Alkalized Serum (" ") ...	17.0	10,000
Sodium Oleate (" ") ...	10.4	16,300
" Palmitate (" ") ...	10.0	17,000
" Stearate (" ") ...	9.4	18,000
Caseinogen (Moore, Roaf and Webster) ...	129.0	1,400
Haemoglobin (Hüfner and Gausser) ...	—	15,000 to 16,000
" (Reid) ...	—	65,000
" (Roaf) under varying con- ditions from ...	—	5,000 to 70,000
" but under more natural con- ditions and in neutral solution about ...	—	14,000 to 18,000
Histone and Protamine (present communi- cation) ...	19.3	8,780

The solution complexity accordingly is less than that of the coagulable proteins, but is considerably higher than that of the acidic protein caseinogen, which is by far the lowest of all proteins hitherto examined. The alkalized serum and the histone lie nearly at the same level.

It is interesting to compare the molecular weight in solution as given directly by osmotic pressure with the molecular weights assigned from analysis by Kossel and his co-workers, and with the molecular weights of the amino-acids (hexone bases) which the protamines yield on hydrolysis. The molecular weights given by Kossel for salmine and sturine lie between 800 and 900, so that even after allowance is made for the inorganic acid in the sulphate there must be eight or more such large molecular groups in the solution aggregate to yield 8,000 to 9,000 as found. The mean molecular weight of the amino-acids forming this class of protein may be taken as 150, and, making allowance for the sulphate in combination, this leads to the conclusion that there are about 40 molecules of such amino-acids united to form the complex, or colloidal solution aggregate, of the protamine, or histone, in solution.

At present, similar measurements of the molecular complexity in solution of nucleoproteins and nucleic acids are being carried out.

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