XXXI. THE PROTEINS OF ELECTRICAL TISSUE

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As a corollary to the more general study of muscle proteins [Bailey, 1937, 2] an investigation of the proteins of electrical tissue is of especial interest, for the electrical organs of some elasmobranch fish in the genus *Raia* and *Torpedo* arise from the same embryonic rudiments as those of muscle, and earlier studies by Ewart have shown that the development of the electrical layer takes place at the expense of the fibrils. In the primitive organ of *R. radiata*, for instance, the metamorphosis to electrical tissue is only partially complete, the muscle fibrils developing into club-shaped cells bearing a richly innervated cup-like electrical layer and exhibiting the characteristic striations of skeletal muscle [Ewart, 1888]; in *R. circularis* and *R. fullonica* the electric cups are retained, while in *R. batis* they have developed into discs, and in all these three latter species the striations are no longer observable [Ewart, 1892]. It is in *R. clavata* and *Torpedo*, however, that the change is most marked, for here the electrical organs consist of cones or columns of cells separated by septa identical with those between the myotomes of muscle and each cell carries a nerve plate (i.e. the electrical layer), an alveolar layer and a mucilaginous material of high refractive index.

The metamorphosis of muscle rudiments to richly innervated discs suggests that the organ is essentially a modified muscle in which the contractile mechanism, generally considered to be the protein myosin, has disappeared, and that the reactions supplying electrical energy in the one case and contractile energy in the other are comparable. This supposition is supported by experiments of Baldwin & Needham [1937] and of Baldwin [1938] who find that the cyclical reactions of the glycolytic system, which are intimately associated with the liberation of energy in muscle working anaerobically, are catalysed by enzymes also common to *R. clavata* and *Torpedo*, the two species which they studied. To supplement this hypothesis, an investigation of the protein components and their comparison with those of skeletal muscle have been undertaken, and an attempt has been made to show whether the striations in the organ of *R. radiata* are coincidental with the presence of myosin, whether other globulins are also present and whether these latter have disappeared from the organs of *R. clavata* and *Torpedo* where metamorphosis from muscle rudiments to electrical tissue is most complete. The tissues have thus been compared with muscle with particular reference to their N partition, the physico-chemical properties and cataphoretic isoelectric point of the fractionated proteins, and in the case of *Torpedo* protein chemical analyses are presented.

Previous chemical studies are relatively sparse, and almost solely devoted to species of *Torpedo*. An investigation undertaken by J. Davy [1832] at the request of Sir Humphry Davy records for the tissue the low total solid content of 7%, whilst Matteucci [1838] gives figures of 9.2 and 10.4%; the latter found in the organ lactic acid, fatty substances, protein, traces of "gelatin", sodium

and potassium. Frerichs & Städeler [1858] discovered urea and Schultze [1859] reported creatinine, lactic acid, calcium phosphate, sodium chloride, sulphate and a mucous substance precipitated by acetic acid. The tissue insoluble in water he identified as fibrous tissue. Weyl [1881; 1883; 1887] added to this list nuclein, xanthine, hypoxanthine, cholesterol and made a detailed analysis of the ash. He prepared the mucin from the alcohol- and ether-extracted organ by treatment with dilute NaOH and subsequent precipitation with dilute acetic acid. After purification the product gave the following analysis: C, 52.5; H, 7.2; N, 13.2; S, 1.03%. Baglioni [1906] carried out a proximate analysis of the tissue, recording a glycogen content of 0.09% of the fresh weight. Later investigations have centred upon the phosphagen content [Kisch, 1930; Baldwin, 1933] and on the role of adenylic compounds [Baldwin & Needham, 1937; Baldwin, 1938].

Experimental

Nitrogen partition of the tissues. The methods evolved by Smith [1934; 1937] and by Reay & Kuchel [1936] for the partition of N between the proteins of skeletal muscle were adopted with minor variations. The total coagulable N (T.C.N.) was determined by mixing a weighed aliquot of minced tissue suspended in water with an equal volume of 10% trichloroacetic acid. After centrifuging, the protein was washed 4–5 times with 5% trichloroacetic acid until free from the non-coagulable nitrogenous substances, which, occurring mainly as urea, are present in large amounts in elasmobranch tissue. The N of the washed protein was determined by the Kjeldahl method. Another aliquot of tissue was exhaustively extracted at 0° by grinding with silver sand in presence of 7% LiCl; the extract was separated by centrifuging, the grinding repeated at least 10 times, and the combined extracts were adjusted with salt solution to a volume containing 0.02–0.04 g. N/100 ml., and the protein in aliquots of this solution was determined by precipitating with trichloroacetic acid as before. Other portions of the tissue in certain instances were exhaustively extracted with $N/20$ NaOH and $N/50$ HCl.

In the case of skeletal muscle, the proteins dissolving in the LiCl solution represent the major portion of the intracellular globulins and albumins, viz. myosin, globulin X and myogen, but the residual intracellular fraction which, according to Smith [1937], consists mainly of denatured globulin X, dissolves, together with the salt-soluble intracellular fraction, in dilute acid or alkali; the protein undissolved by these latter is the muscle stroma. Myosin is estimated by dilution of the LiCl extract with 20 vol. of ice-cold water and the gelatinous precipitate which settles out overnight at 0.07–0.08 M LiCl is centrifuged off, washed with water and digested in the Kjeldahl flask. The non-coagulable N has been estimated in the LiCl and other extracts by subtracting the protein-N from the total N of the extract.

In Table I a detailed comparison between the N partition of the skeletal muscle and that of electrical tissue is made for three separate specimens of *Torpedo* treated immediately after death. For the muscle, 81–83% of the T.C.N. is soluble in LiCl and of this 65–71% occurs as myosin; of the salt-insoluble residue (17–19% of the T.C.N.) a further 5–10% is included in the acid or alkali extracts. The stroma fraction of 7–12% is considerably higher than the 3–5% reported by Reay & Kuchel [1936] for haddock muscle but there is as yet no systematic evidence that this fraction is constant in different muscles. The other figures presented here are in good agreement with the analyses of these authors.

Table I. *Distribution of N in g./100 g. wet tissue*

	Muscle			Electrical tissue						
	<i>T. mar-</i> <i>morata</i> (1)	<i>T. mar-</i> <i>morata</i> (2)	<i>T. ocel-</i> <i>lata</i> (3)	<i>T. mar-</i> <i>morata</i> (1)	<i>T. mar-</i> <i>morata</i> (2)	<i>T. ocel-</i> <i>lata</i> (3)	<i>R. clavata</i> *		<i>R. radiata</i> *	
Dry weight	23.2	23.8	26.2	10.8	10.6	11.8	10.4	10.0	12.0	12.8
Non-coagulable N	1.66	1.74	1.72	1.75	1.66	1.68	1.06	1.07	0.47	0.51
Total coagulable N	2.345	2.534	2.828	0.39	0.36	0.44	0.818	0.80	1.51	1.52
Coagulable N:										
In LiCl extract	1.89	2.11	2.34	0.25	0.22	0.314	0.333	0.30	0.65	0.67
In N/20 NaOH extract	2.09	—	—	0.27	0.227	0.34	—	—	—	—
In N/50 HCl extract	—	2.35	2.48	0.175	0.146	0.20	—	—	—	—
Myosin	1.67	1.65	1.90	Nil	Nil	Nil	Nil	Nil	0.15	0.09

* T.C.N. of *R. clavata* muscle 2.63; *R. radiata* muscle 2.41. Dry weight of *R. clavata* muscle 23.4%; *R. radiata* muscle 19.5%.

Fig. 1.

In comparison, it is seen that the dry weight of *Torpedo* electrical tissue is $< \frac{1}{2}$ and the T.C.N. only $\frac{1}{6} - \frac{1}{7}$ that of muscle and of this total protein, 61–77% is soluble in salt, 63–77% in N/20 NaOH and 40–45% in N/50 HCl. The comparatively large deviation in the amount of extractable coagulable N between individual organs of different specimens has been confirmed many times and may be explained by an observation of Ewart that the discs of connective tissue increase in diameter with age and decrease in thickness, whilst the amount of “gelatinous insulating material”—shown later to be a mucin—is considerably augmented; the proportion of intracellular N to stroma-N might therefore depend upon the age of the specimen. The figures reveal, however, that whereas in individual specimens, salt or alkali are equally effective in extracting the intracellular protein, the coagulable N extracted by acid is far lower, and in this respect the system differs fundamentally from muscle. For detection of myosin, it is not possible to utilize the sol obtained by exhaustive extraction of the tissue since the protein concentration is too low. More concentrated extracts obtained by extracting 1 part of tissue with 1 part of 14% LiCl have never yielded a precipitate. After 20-fold dilution with water, the faint opalescence which appears is due most probably to a small amount of apparent

globulin which precipitates when the LiCl sol is dialysed to a molarity of 0.03. This precipitate, which is small in comparison with the protein remaining in solution, is not a characteristic tissue globulin since it does not coagulate on heating.

The dry weight of the electrical tissue of *R. clavata* is about $\frac{1}{2}$, and the T.C.N. approximately $\frac{1}{3}$ that of the corresponding muscle; only 37–41 % of the total protein is soluble in LiCl, and salt extracts containing 0.5 g. N/100 ml. showed only a faint turbidity when diluted with 20 vol. of water. Although myosin thus appears to be absent, a heavy globulin precipitate was obtained on dialysis to a salt molarity of 0.002. The protein remaining in solution did not precipitate when the salt concentration was further reduced. The amounts of globulin obtained from various LiCl extracts bear a constant ratio to the amount of coagulable N in the extract (Table II).

Table II

	(a)	(b)	(c)
Coagulable N extracted by LiCl as % of T.C.N. (A)	31.4	35.4	35.4
Coagulable N in globulin fraction as % of (A)	26.4	27.8	26.3

Mean coagulable N of globulin fraction = 26.8 % of the salt-soluble protein or 9 % of the T.C.N.

The comparatively large amount of salt-insoluble protein in this tissue is to be expected from the known existence of relatively dense septa of connective tissue separating the electric discs.

Experiments with the electrical tissue of *R. radiata* were greatly handicapped by the small size of the organ, 100 dissections yielding about 6.2 g. of wet tissue. The dry weight of the organ is somewhat higher than that of previous tissues and the T.C.N. now reaches $\frac{2}{3}$ the value of *R. radiata* muscle. About 44 % of the T.C.N. is extractable by LiCl, and on dilution to a salt molarity of 0.07 a myosin-like precipitate amounting to 6–10 % of the T.C.N. separates. This precipitate, after centrifuging, was largely denatured and did not give the strong elastic film characteristic of myosin proper; moreover, the myosin prepared from *R. radiata* muscle did not denature under comparable conditions. Dialysis of the LiCl extract to lower salt concentrations (0.004 *M*) yielded an abundant globulin precipitate which was heat-coagulable. The total globulin precipitated on complete dialysis, determined in two separate extracts, amounted to 21 % of the T.C.N. and the amount of globulin salted out by saturation with NaCl amounted to a comparable figure of 19 %.

Thus of all the electrical tissues examined, that of *R. radiata* most closely resembles the protein system obtaining in muscle not only in the amount of T.C.N. but also in the comparatively large proportion of globulin, some of which precipitates at a salt molarity usually adopted for separation of myosin, but which has not been identified as myosin proper. When the results are represented diagrammatically (Fig. 1) the comparison between muscle and electrical tissue is seen to advantage. It may also be noted from this diagram that the non-coagulable nitrogenous constituents are highest where the T.C.N. is least, and in *Torpedo* amount to 1.7 % of the wet weight of the electrical organ.

General properties of electrical tissue proteins. The quantitative results indicate that all types of electric tissue contain a water-soluble protein; in *Torpedo* the whole of the salt-soluble protein is also water-soluble and in *R. clavata* and *R. radiata* a water-soluble fraction with properties similar to those of *Torpedo* protein is obtained after removal of globulins by complete dialysis. The dialysis liquors have a pH of approximately 7, and at this reaction the water-soluble protein is not coagulated by heat, is not immediately precipitated by $\frac{1}{2}$ saturated

(NH_4) $_2\text{SO}_4$, saturated NaCl or by addition of 1–2 vol. of alcohol. The globulins on the other hand precipitate under these conditions and are heat-coagulable. The water-soluble fraction is precipitated at pH 4.5–5 and in this range it can be denatured by heating; at lower pH values it passes into solution and on neutralizing it again precipitates at the point of minimum solubility and re-dissolves at pH 7.5. In marked contrast to these properties the water-soluble fraction (myogen) of mammalian muscle, investigated by Smith [1937], and of *R. radiata* muscle, investigated by the author, is heat-coagulable, and although it denatures on acidification in absence of salt it does not precipitate until the solution is neutralized. The properties of the water-soluble proteins of electrical tissue are not unlike those of some mucins and nucleoproteins and the preparation of the solid protein from *Torpedo* was undertaken to characterize it more completely.

Preparation of Torpedo protein. The minced tissue was extracted with an equal volume of water or of 14% LiCl, stirred mechanically for 1 hr. and centrifuged. The extraction was once more repeated and the combined extracts were dialysed at 1° against distilled water. After centrifuging away a small amount of precipitate, the liquid was filtered through paper pulp, acidified to pH 4.5 and the supernatant was decanted from the protein precipitate which was now dissolved at pH 7.5, centrifuged, and precipitated as before. It was dried thoroughly in alcohol and ether, and a chance evaporation of these liquors revealed the presence of lipid which is discussed in a later section.

Two main samples of protein were analysed (Table III), *A* being prepared from a LiCl extract and *B* from a water extract.

Table III

Figures given are in percentages of total (ash free) dry wt.

	<i>A</i>	<i>B</i>
Ash	0.6	1.2
N	14.4	14.5
Amide-N	1.21	1.23
P	0.27	0.18
Carbohydrate	~ 6.5	~ 6
Glucosamine	3.5	3.8
Total S	1.38	1.33
SO $_4$ -sulphur split off on hydrolysis by HCl	0.47	0.49
Cystine-S (differential oxidation)	0.40	0.39
Methionine-S (by difference)	0.51	0.45
Tyrosine	3.9	—
Tryptophan	1.32	—

The methods adopted in this analysis are described elsewhere: amide-N, total S, tyrosine and tryptophan [Bailey, 1937, 1]; P [Fiske & Subbarow, 1925]; cystine-S by differential oxidation [Lugg, 1938]; carbohydrate, the method of Tillmans & Philippi [1929] adapted by Pirie [1936]; glucosamine, the Zuckerkandl & Messiner-Klebermass method [1931] modified by Hewitt [1938].

The presence of carbohydrate, glucosamine, and of ethereal sulphate split off after hydrolysis with 5*N* HCl at 100° for 12 hr., confirms the inference, derived from physico-chemical tests, that the protein belongs to the class of mucins. The P appears also to be an integral part of the protein; it is not split off by dilute *N*/70 HCl in the cold, and only 26% is freed by digestion with *N* NaOH at 100° for 5 min. A P-rich residue was obtained in the following way: 1.58 g. of protein (P, 0.3%) were digested with 10 ml. of 0.5% pepsin solution in 30 ml. *N*/70 HCl at 27° for 2 days. The undigested sediment after washing

and drying amounted to 23% of the original protein and gave on analysis: ash, 1.0; N, 13.6; P, 0.8; S, 1.6%. Whether this residue is a nuclein cannot categorically be affirmed since attempts to isolate nucleic acid from the amount of material available have failed.

Similar proteins have been described in the literature; eel-slime, investigated by Müller & Reinbach [1914], has similar physico-chemical properties and gives a comparable analysis (N, 14.0–14.4; S, 1.16–1.29; P, 0.33–0.49%) and in its natural state is associated with lipid. The “nucleoalbumin” isolated by Hammarsten [1885] from snail liver possesses similar properties, giving on analysis, N, 14.3; S, 1.06; P, 0.42%; when digested with pepsin a nuclein containing 2.1% P was obtained. In *Torpedo* extracts it was at first suspected that the P might arise from the abundant nuclei of the electrical layer, the isolated protein thus consisting of a mixture of mucin and nucleoprotein. Since, however, the P occurs in Müller & Reinbach’s protein, which is an external skin secretion, it is unlikely that such admixture is possible, and although the P reacts as nucleic acid-P, its exact assignation to a definite prosthetic group must await further research.

Isoelectric points of electrical tissue proteins. These were determined by the cataphoretic method of Dummett & Bowden [1933] using 0.01 *M* phthalate buffers. Dried and undried preparations of proteins were ground with a little water and lightly centrifuged; aliquots of the particle suspension in the supernatant were added to a buffer series and the approximate velocity of migration was plotted against the *pH* value of the solution, measured after completion of the experiment by the hydrogen electrode. The values are given in Table IV.

Table IV

	Isoelectric point
1. <i>Torpedo</i> mucin, dried preparation, fat-free	3.15
2. <i>Torpedo</i> mucin + fat, undried	3.1
3. <i>R. clavata</i> mucin, dried preparation, fat-free	3.5
4. <i>R. clavata</i> mucin + fat, undried	3.6
5. <i>R. clavata</i> globulin, dried, fat-free	4.1
6. <i>R. clavata</i> globulin + fat, undried	4.1
7. <i>R. radiata</i> mucin + fat, undried	3.55
8. <i>R. radiata</i> mucin + fat, undried	3.6
9. <i>R. radiata</i> globulin, dried, fat-free	4.1
10. <i>R. radiata</i> myosin (from muscle)	4.6

Note on the preparations listed in Table IV. The globulin fractions 5 and 6 were obtained by dialysis of the tissue extracts and were washed thoroughly with water; their purification was not undertaken as they were partly denatured; globulin 9 was obtained by saturation of the LiCl extracts with NaCl. The mucins 3 and 4 were prepared after removal of globulins by dialysis, the residual liquors being filtered through paper pulp and acidified; the precipitated protein was then purified by redissolving at *pH* 7.5 and reprecipitating twice. The mucin of *R. radiata* was more difficult to prepare, owing to contamination of the dialysate with colloidal globulin; preparation 7 was obtained by saturating the dialysate of the LiCl extract with NaCl when a small amount of globulin separated and, after dialysis of the residual liquor, the protein was precipitated by acidification; preparation 8 was obtained by saturating the LiCl extract with NaCl and, after removal of the globulin by centrifuging and of NaCl by dialysis, the residual liquor on boiling gave a slight coagulum, which was filtered off and the mucin precipitated by acidification. The myosin (10) was prepared by the Edsall [1930] method and twice precipitated from salt solution.

Within 0.1–0.2 pH unit from the designated isoelectric point, differences in the direction of migration could sometimes be observed only in the case of the globulin fractions, indicating, as indeed is most likely, some degree of non-homogeneity. On the other hand, the isoelectric point of the mucin fractions was very sharp, and the low values obtained confirms their assignation to this group of proteins. The globulin fractions in bulk have an isoelectric point lower than that of *R. radiata* myosin, which in turn is lower than the reported values for that of mammalian muscle. The isoelectric point of the protein-lipoid complex does not differ greatly from that of the alcohol-extracted protein.

The lipid material associated with electrical tissue proteins. All globulin and mucin fractions prepared from salt or water extracts of electrical tissue contain considerable amounts of lipid which can be separated by extraction with hot alcohol and ether. After evaporation of the extracts, the residue was freed from traces of protein by re-extracting with chloroform. The amount of lipid associated with various preparations of protein was investigated only in the case of *Torpedo* mucin, and this was not constant; e.g. the lipid contents as % of six lipid-protein preparations were 15.2, 14.0, 22.4, 23.0, 23.0, 14.9 % respectively.

The combined lipid fractions from several preparations of *Torpedo* mucin were combined, re-extracted with chloroform, evaporated and dried at 100° to constant weight. The material contained 1.92 % P and 1.4 % N. The free cholesterol was estimated in the usual way by precipitation with digitonin, and the cholesterol as ester, after saponification with *N* sodium ethoxide and removal of unsaponifiable material. The free fatty acids were next extracted and weighed and the contents of phosphatides and glycerides present in the original lipid fractions were then calculated on the assumption that the P was due exclusively to lecithin, and the fatty acids of both to oleic acid. The following figures were thus obtained:

Phosphatide	...	...	...	...	...	50.6 %
Free cholesterol	...	...	...	...	...	19.4 %
Unsaponifiable matter other than cholesterol	...	...	...	...	...	10.0 %
Glycerides	...	...	...	...	...	7.1 %
Total	...	...	...	...	...	87.1 %

The amount of cholesterol ester was insignificant.

It is of immediate chemical interest to consider the function of lipid in its association with mucin, since this combination, found also in eel-slime, is probably of general occurrence in the fish mucins. It appears to act as a dispersing agent for the protein since its removal by alcohol extraction leads to complete insolubility of the protein above and below its isoelectric point, and although the denaturing action might be ascribed to the use of organic solvents, it is significant that the original protein complex is not affected by the stronger denaturing actions of heat and acid. In its native state the complex has the solubility characteristics of the mucoids, but this term has been avoided because it has not been possible to assess how much the lipid fraction contributes to the solubility of the protein *per se*. Although the mucin-lipoid complex is stable in neutral solution, those of the globulins lose their solubility in salt after dialysis, and the globulin-LiCl extracts denature slowly on standing. These phenomena are probably due to the denaturing action of phosphatide acting in the manner described by Hardy & Gardiner [1910] for the plasma proteins and resulting, in the view of Chick [1914] in the production of euglobulin from pseudoglobulin.

SUMMARY

1. Since the electrical and muscular tissues of elasmobranchs arise from the same embryonic rudiments, a comparative study of the protein systems of the electrical tissue of *Torpedo*, *Raia clavata* and *R. radiata* has been made to ascertain whether such systems are comparable with those of muscle.

2. The N-partition of *Torpedo* muscle and of the three types of electrical tissue has been determined. Quantitatively, electrical tissue is characterized by a low total solid and low protein content; the amount of total coagulable N is highest in *R. radiata*, decreasing progressively in *R. clavata* and *Torpedo*, and the proportion of this N existing as globulin decreases in the same way. In *Torpedo* the extractable protein is almost entirely a mucin and this is common to the other electrical tissues examined.

3. The cataphoretic isoelectric points of the globulin fractions and the mucins have been determined.

4. All protein fractions are associated with comparatively large amounts of lipid, which was isolated from *Torpedo* mucin and analysed. It consists mainly of phosphatide, free cholesterol and glyceride.

5. A partial analysis of *Torpedo* mucin is presented. It is shown to contain ethereal sulphate, carbohydrate and glucosamine, and non-labile P, which is obtained in a P-rich residue after digestion with pepsin. Attempts to isolate nucleic acid have failed.

6. The results support the view that electrical tissue, whilst retaining the enzymes associated with the glycolytic system of skeletal muscle, differs mainly in the absence of myosin, which is no longer necessary for contraction. The protein system is most dissimilar from muscle in those tissues which, from the histological standpoint, are farthest removed from muscle (e.g. *Torpedo*) and most similar in incompletely metamorphosed tissue (e.g. *R. radiata*).

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