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Further Investigations on Fish Tropomyosin and Fish Nucleotropomyosin

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The first method for preparing tropomyosin from muscles of several animals (rabbit, pig, horse and whiting) was described in 1948 by Bailey. Minced muscle is dehydrated with organic solvents and extracted with M-potassium chloride for 12 hr. Tropomyosin is isolated from the extract by precipitating at pH 4·3–4·5, redissolving in water at neutral pH, and fractionating with ammonium sulphate between 45 and 70 %. The amount of tropomyosin in rabbit muscle has been estimated by this method to be 0·47 g./100 g. fresh muscle weight.

Another method of preparation has been given recently by the author for carp muscle (Hamoir, 1951). The minced muscle is submitted to two successive extractions for short periods of time (10–20 min.) with potassium chloride-phosphate solutions: the first of pH 5·0 ($I = 0.25$), the second of pH 5·5 ($I = 0.6$) containing 0·3 % sodium adenosine-triphosphate. The first extract contains the muscle globulins soluble at low ionic strength and some tropomyosin; the second myosin, nucleotropomyosin and tropomyosin. The method of isolation of nucleotropomyosin and tropomyosin from these extracts does not differ essentially from Bailey's method. Nucleotropomyosin can easily be separated from tropomyosin: it coprecipitates with the myosin at low ionic strength and neutral pH while tropo-

myosin remains in solution. The total yield of tropomyosin and nucleotropomyosin isolated by this method from fish muscle does not amount to more than 0·1 g./100 g. fresh muscle weight.

The difference in yield by the two methods has led us to try to account for the discrepancy. Fish muscle was treated following the author's method and tropomyosin was extracted from the residue by Bailey's (1948) method. The total yield of tropomyosin was then comparable with that obtained by Bailey for rabbit muscle. New investigations have also been carried out on fish nucleotropomyosin. The content of ribonucleic acid and its splitting under various conditions have been examined more closely, and the ribonucleic acid has been more fully characterized by its ultraviolet absorption in N-perchloric acid and by quantitative estimation of the nitrogenous bases present.

METHODS

Preparation of fish tropomyosin and fish nucleotropomyosin

Carp muscle was used as starting material. Tropomyosin was prepared from the residue obtained after extracting twice by the author's method (Hamoir, 1951) and subsequently treating it according to Bailey's (1948) method. As some

simplifications have been introduced, a description of the preparation will be given here.

The residue was washed with 2 vol. of water (200 ml./100 g. fresh muscle) and 1 vol. of 1:1 ethanol-water. The dehydration was continued through two changes of ethanol (1 vol.) and two of ether (1 vol.). The pulp was immersed while still ether-damp in 7 vol. of M-KCl (700 ml./100 g. pulp) at pH 7 and was extracted with stirring for 12 hr. at room temperature. The insoluble residue was removed by centrifugation (10 min. at 10,000 rev./min.) and re-extracted twice for 2 hr. at neutral pH. Tropomyosin was precipitated from the combined extracts by first diluting with 9 vol. of water and acidifying to pH 4·6. The precipitate was washed twice with a dilute solution of acetate buffer (pH 4·6), and redissolved in 0·5M-KCl at neutral pH. The solution was then fractionated with neutral $(NH_4)_2SO_4$. The fraction isolated between 50 and 66% saturation corresponds to practically pure tropomyosin. The purity was assessed by electrophoresis (Dubuisson, Distèche & Debot, 1950) and by ultracentrifugation at 60,000 rev./min. (Spinco electric-driven ultracentrifuge). The isolation and purification of nucleotropomyosin were carried out as previously described (Hamoir, 1951).

The solutions of tropomyosin and nucleotropomyosin were usually dialysed for at least 1 week against several changes of NaCl-acetate buffer ($I = 0·4$ and pH 6·4) to remove any phosphate or ammonium ions present.

Analytical

Protein. Total nitrogen was estimated by the micro-Kjeldahl method and the concentrations of tropomyosin and nucleotropomyosin were calculated by assuming a N content of 16·7%, given by Bailey (1948) for tropomyosin. No appreciable error was introduced in this way in the evaluation of nucleotropomyosin, the nitrogen content of nucleic acid being close to that of tropomyosin (Smith & Markham, 1950).

Phosphorus. Total phosphorus was estimated by the perchloric acid method as modified by Allen (1940), using a photoelectric absorptiometer and the 608 Ilford filter.

Phospholipins. The ethanol- and ethanol-ether-soluble compounds of nucleotropomyosin were removed following the method of Ogur & Rosen (1950). The protein was precipitated with 5 vol. of ethanol and extracted with two changes of ethanol at room temperature. The residue was resuspended in a mixture of 3:1 ethanol-ether and boiled gently on a water bath for 5 min. The process was repeated and the extracts combined. After the removal of solvent, the amount of phospholipin was determined from the phosphorus content.

Ribonucleic acid. The absence of ribonucleic compounds in the phospholipin fraction was checked by showing the absence of ribose. Ribose was determined by the method of Mejbaum (1939), adopting a 45 min. heating time for the development of the colour as proposed by Albaum & Umbreit (1947). The readings taken with a photoelectric absorptiometer and the 607 Ilford filter were referred to a calibration curve determined with D-ribose. The nucleotropomyosin precipitated by the organic solvents was dried *in vacuo* and used for the determination of the ultraviolet absorption of the nucleic acid and for the chromatographic analysis of the purine and pyrimidine bases. For the former, the residue was extracted with N-HClO₄ at room temperature for about 24 hr. according to Ogur & Rosen (1950) and the absorption of the extracted ribonucleic acid was measured with a Beckman universal spectrophotometer (model DU). The data were calculated as extinction/gram atom of phosphorus. For chromatographic analysis, the paper chromatographic method of Smith & Markham (1950) was slightly modified. Hydrolysis was carried out in N-HCl at 100° in a water bath for 20 min. instead of 1 hr. The extraction of the bases is complete under these conditions and the protein does not dissolve appreciably (Thomas, 1951). After elution of the spots of the chromatogram, the densities of the solutions were measured with the Beckman spectrophotometer. Concentrations were evaluated using the coefficients given by Smith & Markham (1950).

RESULTS

Tropomyosin content of fish muscle

Tropomyosin was estimated by Bailey's (1948) method on the residue left after dissolving out nucleotropomyosin and some tropomyosin following Hamoir's (1951) method. The extraction and isolation were carried out as quantitatively as possible. The yield of tropomyosin calculated from the nitrogen content of the solutions obtained amounted to 0·3% of the fresh muscle weight. As about 0·1% tropomyosin was removed before the treatment with organic solvents, the total content is approximately 0·4% of the fresh muscle weight.

This estimate is based on the supposition that the product is pure tropomyosin. As the protein was prepared by Bailey's method, the presence of significant amounts of nucleic acid seems unlikely. This was confirmed by determining the P content of two preparations, and the values of 0·05 and 0·13% found show that variations occur from one preparation to the other; however, the contamination with nucleic compounds is not very great. Electrophoresis and ultracentrifugation show that tropomyosin proper constitutes at least 95% of the total protein material. A small peak was found by electrophoresis migrating with a mobility of about 0·4 times that of tropomyosin. In the ultracentrifuge, this material sedimented with a rate 1·1 times that of tropomyosin. It is perhaps worth while to mention that this impurity seems not to be present in preparations in which the extraction of the dried residue was not exhaustive: a single extraction with M-potassium chloride or the extraction of a dried residue kept for a few weeks at room temperature gave lower yields of a tropomyosin which was pure by electrophoresis and ultracentrifugation.

In conclusion, the major part of tropomyosin of fish muscle does not go into solution by salt extractions of short duration at pH 5–6 and can easily be isolated from the residue after dehydration with organic solvents. Although the tropomyosin content of fish muscle has been assessed by the use of three types of extraction used in succession, the accuracy is sufficient to show that the amounts in carp and rabbit muscles are comparable.

The phosphorus of nucleotropomyosin

Since ribonucleoproteins are usually associated with phospholipins in the cytoplasm of animal cells, and since variations in the phosphorus content of nucleotropomyosin were observed, the nature of the phosphorus compounds of several preparations has been investigated. Nucleotropomyosin does not contain phospholipins. If the nucleoprotein is extracted with ethanol at room temperature or with boiling mixtures of ethanol and ethyl ether, no phosphorus or ribose is found in the extracts after evaporation of the organic solvents. The variations of the phosphorus content of several preparations are thus due to changes in the proportions of tropomyosin and nucleic acid. Table 1 shows that the content in nucleic acid can vary from 15 to 20 %.

Table 1. *Phosphorus content of nucleotropomyosin*

Preparation	P content (% of dry wt.)	Amount of nucleic acid* (%)	P (atom/mol. tropomyosin)†
A	1·20	14·3	24
B	1·26	15·0	25
C	1·72	20·5	37
D	1·48	17·6	31
E‡	1·47	17·5	30
Mean value	**1·43**	**16·98**	**29**

* Assuming a P content of 8·4 % of yeast nucleic acid (Smith & Markham, 1950).

† Assuming a molecular weight of 53,000 for tropomyosin (Tsao, Bailey & Adair, 1951).

‡ Taken from the previous paper (Hamoir, 1951).

Splitting of nucleotropomyosin at pH 3·5

It has been shown that nucleotropomyosin kept in the cold in an acetate buffer of $I = 0·15$ and pH 3·5 (acetate, 0·1 M and sodium chloride, 0·05 M) transforms slowly: a precipitate forms which is easily removed by centrifugation, and ultracentrifugation of the supernatant showed a progressive disappearance of nucleotropomyosin and the formation of tropomyosin (Hamoir, 1951). It was sought to discover whether this splitting is due to a degradation of the nucleic acid or corresponds to a separation of the nucleic acid and the protein. Dilute solutions of nucleotropomyosin kept in the cold were dialysed overnight in cellophan bags against water and afterwards against a small volume of the acetate buffer of pH 3·5 for 2 or 3 days in order to obtain a complete disappearance of nucleotropomyosin in the supernatant. No nucleic compounds were found in the diffusate: tests for ribose, phosphorus and nitrogenous bases (ultraviolet absorption) were negative. By centrifuging of the contents of the bag, a supernatant was obtained which contained tropomyosin and only small amounts of phosphorus (Table 2). The nucleic acid thus appeared to be bound to the insoluble fraction. This was washed twice with acetate buffer of pH 3·5, resuspended in a solution of $I = 0·4$ and pH 6·4 (acetate buffer, 0·1 M, and sodium chloride, 0·3 M) and dialysed for a few days against the latter buffer; a partial dissolution was observed. The phosphorus and the nitrogen contents of the redissolved fraction are given in Table 2, together with values determined on the fraction soluble at pH 3·5.

These results show that when nucleotropomyosin is kept for 2 or 3 days at pH 3·5, it splits into tropomyosin and an insoluble fraction containing practically all the nucleic acid, and from which nucleotropomyosin of a nucleic acid content of about 40 % can be prepared. This is insoluble at low pH and differs, therefore, in solubility from tropomyosin itself. When the duration of the acid treatment of the original nucleotropomyosin amounted to 2 or 3 hr., the ultracentrifuge has revealed components sedimenting at a rate intermediate between those of tropomyosin and of nucleotropomyosin (Hamoir, 1951). It can therefore be concluded that 'artificial' nucleotropomyosins can be obtained of a nucleic acid content lower than 15 % or higher than 20 %, and that it seems probable that the proportions of the two components can be altered continuously.

Ultraviolet absorption of the nucleic acid

The ultraviolet spectrum of the nucleic acid in N-perchloric acid is given in Fig. 1. The curve obtained conforms with the previous values reported on nucleotropomyosin (Hamoir, 1951), taking into account the fact that the extraction with perchloric acid slightly affects the absorption (Ogur & Rosen, 1950). A small shift of the maximum from 258 to 261 mμ. occurs while a trough corresponding to 231 mμ. is now accurately defined. The noticeable absorption at about 280 mμ. already observed with nucleotropomyosin is due to the high content of the nucleic acid in cytosine and guanine (see below). The ϵ_P, i.e. the extinction of a solution

Table 2. *Composition of two fractions resulting from the splitting of nucleotropomyosin at pH 3·5*

Preparation	Fraction considered	P content (% of dry wt.)	Amount of nucleic acid* (%)	P (atom/mol. tropomyosin)*
A	Supernatant	0·38	4·5	7
	Precipitate redissolved	3·28	39·0	92
B	Supernatant	0·19	2·3	3
	Precipitate redissolved	3·3	39·3	93

* Calculated as in Table 1.

containing one gram atom of phosphorus/l. measured in a cell 1 cm. thick, has been calculated at 261 mμ. for several preparations: values of 10,420, 10,900 and 10,460 were obtained, giving a mean of 10,590. Only slightly different (10,800) is the value found by Ogur & Rosen (1950) for yeast ribonucleic acid after perchloric acid treatment.
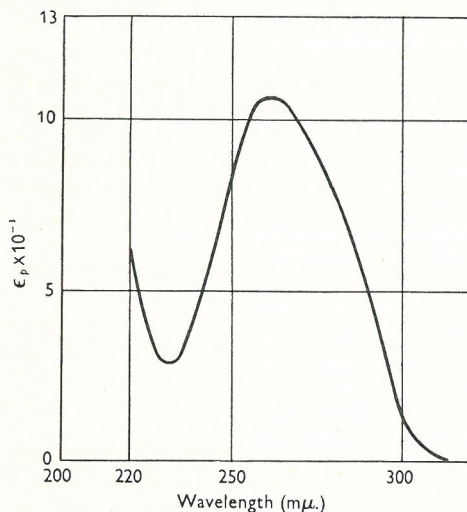

Fig. 1. Ultraviolet absorption spectrum of the ribonucleic acid of fish nucleotropomyosin in N-perchloric acid. Ordinate: extinction of a solution containing one atom P/l. in a cell 1 cm. thick. Abscissa: wavelength (mμ.).

Chromatographic analysis of the nitrogenous bases

A better characterization of the ribonucleic acid of nucleotropomyosin has been gained by the quantitative analysis of the purine and pyrimidine bases present. Four paper chromatograms were run with four different preparations, giving in each case four well separated spots; these were eluted and each compound characterized by its ultraviolet spectrum. The spots correspond to guanine, adenine, cytidylic and uridylic acids. The relative concentrations found are given in Table 3, the adenine concentration being taken as unity. The proportions of the different bases differ notably from the values obtained for yeast nucleic acid (Smith & Markham, 1950), and such high proportions of guanine and cytosine have already been found in the ribonucleic acids extracted from pig pancreas (Vischer & Chargaff, 1948) and from ox pancreas (Kerr,

Seraidarian & Wargon, 1949) and seem to be characteristic of animal ribonucleic acids. As deoxyribonucleic acid is only slightly hydrolysed in these conditions and does not move on the chromatogram (Thomas, 1950), the lack of absorbing material at the starting point affords an independent confirmation of the absence of deoxyribose in nucleotropomyosin.

DISCUSSION

A more accurate representation of nucleotropomyosin emerges from the present results. The compound is not a molecular entity but an association of ribonucleic acid and tropomyosin in varying proportions. Some variations occur from one preparation to another and the amount of nucleic acid can be greatly increased by keeping nucleotropomyosin at pH 3·5, when a part dissociates leaving a fraction richer in nucleic acid.

Nucleotropomyosins of a nucleic acid content of 15–20 % have the same solubility properties as tropomyosin: they are salted out by ammonium sulphate in the same range of concentration; they precipitate at low ionic strength at the same pH and crystallize under identical conditions. When, however, the nucleic acid content reaches about 40 %, a difference in solubility is observed at low pH.

Nucleotropomyosin probably pre-exists in muscle and seems not to be formed during extraction. Ribonucleic acids occur always as ribonucleoproteins in the cells (Szafarz, 1951), and there is no reason at present to suppose a splitting of the original ribonucleoprotein and a subsequent combination of tropomyosin and nucleic acid.

To determine the tropomyosin content of fish muscle, three extractants have been used successively. Three tropomyosin fractions were thus obtained which showed surprising variations in composition: the first and the third contained only small amounts of phosphorus while the second was mainly nucleotropomyosin. If nucleotropomyosin pre-exists in muscle, and if no specific variations have to be considered between fish and mammals, the very low phosphorus content of tropomyosin found by Bailey (1948) could be attributed to a splitting of the nucleoprotein in the fibre in the course of dehydration with organic solvents. The most likely although very hypothetical explanation of the previous results seems, therefore, to be that tropomyosin may exist in muscle partly dissolved in

Table 3. *Mole ratio of bases in the nucleic acid of nucleotropomyosin*

	Preparation 1	Preparation 2	Preparation 3	Preparation 4	Mean value
Adenine	1	1	1	1	1
Guanine	2·0	2·1	2·0	2·3	2·1
Cytosine	1·8	1·9	1·8	1·9	1·85
Uracil	1·0	1·15	(0·6)*	—	1·1

* Minimal value due to a too high blank; not used for the calculation of the mean.

the muscle juice in minute quantities (first fraction) and as nucleotropomyosin very partially extracted in the conditions used (second fraction) and whose major part is split into tropomyosin and nucleic acid by dehydration with organic solvents (third fraction). The content in ribonucleic acid of the fibre of skeletal muscle which amounts in the case of the rat to 0·06–0·12 % (Schneider & Klug, 1946) agrees with this hypothesis. The question arises whether the strong basophily of the anisotropic bands of striated muscle (Dempsey, Wislocki & Singer, 1946; Clavert, Mandel & Jacob, 1949) could not be due to the location of nucleotropomyosin at that level in the cell, but more experiments are obviously needed to determine how and where tropomyosin occurs in the muscle fibre.

SUMMARY

1. Fish muscle contains approximately 0·4 % of tropomyosin and does not differ noticeably, therefore, from rabbit muscle in this respect.

2. Nucleotropomyosin does not contain phospholipins; its content of nucleic acid varies between 15 and 20 % and can be increased to 39 % by a partial splitting at pH 3·5.

3. The ultraviolet spectrum and the extinction coefficient of the ribonucleic acid of nucleotropomyosin in perchloric acid do not differ much from the same values of yeast nucleic acid.

4. The ribonucleic acid of nucleotropomyosin contains roughly twice as much guanine or cytosine as adenine or uracil, a composition which appears to be typical of animal ribonucleic acids.

5. It is suggested that, *in situ*, nearly all the muscle tropomyosin could be associated with ribonucleic acid and be located in the anisotropic bands.

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