Fish Tropomyosin and Fish Nucleotropomyosin

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myosin has been prepared from muscles of eral animals (rabbit, pig, horse and whiting) by dehydrating the minced muscle with organic solvents and extracting with M-potassium chloride (Bailey, 1948). A detailed account of its isolation and properties has also been published (Bailey, 1948; Bailey, Gutfreund & Ogston, 1948; Astbury, Reed & Spark, 1948).

In the course of an extensive ultracentrifugal study on fish myosins, it has been found that two proteins showing the same solubility properties as tropomyosin can be isolated from fish muscles without previous treatment with organic solvents. Both tropomyosins have been obtained in crystalline form and their ultracentrifugal and electrophoretic behaviours have been investigated. One of these proteins is practically identical with Bailey's tropomyosin. Dr K. Bailey kindly suggested that the other, which had the larger sedimentation constant, might be an association of the first compound and nucleic acid. This was completely confirmed by phosphorus and ribose determinations and by the ultraviolet absorption. The name 'nucleotropomyosin' is proposed for this compound.

EXPERIMENTAL

Isolation

Extraction of fish muscles with salt solutions (mixtures of phosphate and potassium chloride) of varying pH between 5 and 7 shows that the proportion of tropomyosin in the extract incr the pH of the extraction fluid decreases an time of extraction is short. The tropom still very extractable when the duration traction is 10 min. and the pH of the extr the amount of myosins going into solution conditions becomes much smaller. The pr the tropomyosins in the extracts can be creased if the extraction is carried out wi kept for several months in the frozen st treatment decreases the extractability o sins (Reay & Kuchel, 1936) and does not behaviour of the tropomyosins. Sepa nucleotropomyosin from tropomyosin extracts is easy. On dilution with water coprecipitates with the myosins while the mains in solution and precipitates only on the supernatant to pH 4·6. A final purifica substances is carried out by ammonium fractionation. The details of the method below.

The preparation has been carried out throug cold and all the separations have been done by cer Carp muscles cut with a freezing microtome int thick (Dubuisson, 1947) are extracted for 20 min of a KCl-phosphate solution (0·15 M-KCl and 0· brought to pH 5). This extract is diluted with 3· water; the precipitate is discarded and the sup kept.

The residue from this first extraction is re-e 10 min. with 3 vol. of 0·5 M-phosphate solutio containing 0·3% sodium adenosinetriphosp

residue is discarded and the extract (II) is diluted with 7 vol. of water. A precipitate of myosins and nucleotropomyosin forms which is washed twice with water and redissolved in 0·5 M-KCl at neutral pH. The supernatant (II) and the supernatant (I) are mixed and brought to pH 4·6. The precipitate containing tropomyosin is washed twice with water and redissolved in 0·5 M-KCl at neutral pH.

Both solutions are now centrifuged for 30 min. at 14,000 rev. min. to remove some turbid material and are purified by a second precipitation by dilution with 8 vol. of water at neutral pH (nucleotropomyosin) or at pH 4·6 (tropomyosin). Both precipitates are washed twice with water and redissolved in 0·5 M-KCl at neutral pH.

Both tropomyosins are isolated from these two solutions by $(NH_4)_2SO_4$ fractionation at neutral pH: the major part of the total protein content of the solutions precipitates between 30 and 50 % saturation, whilst the tropomyosins precipitate between 50 and 66 % saturation. The precipitate can be redissolved very quickly by a slight dilution with water, giving a water-clear solution.

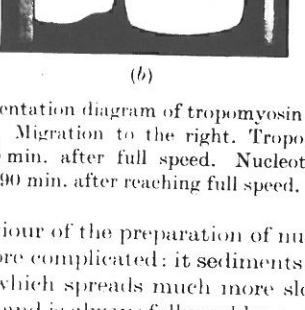
Although these methods of preparation are very reproducible, some variations are observed in the yields obtained, which are usually about 0·07 % of the wet weight for nucleotropomyosin and 0·03 % for tropomyosin. A better yield, amounting to 0·5 % of the fresh muscle weight, has been obtained after organic solvent treatment (Bailey, 1948). This discrepancy is not due to loss during the isolation. The quantity of tropomyosins and myosins extracted from fresh muscles under the conditions described amounts to 1 % of the wet weight and the tropomyosin content of the solution varies from 10 to 20 %. Fractionation by $(NH_4)_2SO_4$ is very effective: no precipitation of tropomyosin occurs before 40 % saturation while the major part of the myosins has already been removed. The denaturation of the myosins by organic solvent treatment probably increases the differences in extractability of the myosins and tropomyosins and allows a more complete removal of the tropomyosins.

Crystallization. The undiluted salted-out precipitates are used for crystallization. This is carried out using the conditions previously described for tropomyosin (Bailey, 1948), i.e. by dialysing an approx. 1·5 % solution against a solution containing 16 g. $(NH_4)_2SO_4$ l. and 0·01 M-acetate buffer of pH 5·4. Nucleotropomyosin crystallizes in elongated prisms (Figs. 1, 2); tropomyosin in the quadrangular plates previously described by Bailey (Fig. 3). From a private communication of Dr Bailey it would appear that the crystalline form of nucleotropomyosin is not characteristic of this compound alone: similar crystals have already been obtained by him from fish (whiting) tropomyosin solutions prepared in the usual way.

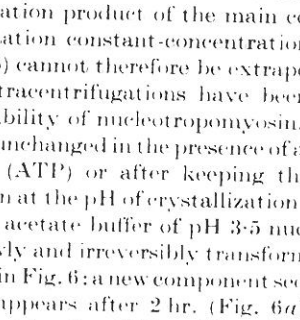
Ultracentrifugation

The solutions were examined in the Svedberg oil-turbine ultracentrifuge using the Philpot optical system (Philpot, 1938) at 60,000 rev. min. The tropomyosins were dissolved in a phosphate buffer of ionic strength 0·1 containing 0·25 M-sodium chloride. The pH of the solution was 7·1. The concentrations of the protein were determined refractometrically, assuming a specific refractive increment of 0·00180. The values of the sedimentation constants have been corrected to 20° and to a water basis, using the partial specific volume given by S Pedersen (1940), Appendix II.

The preparation of tropomyosin app geneous by ultracentrifugation. It sedi single fairly symmetrical peak whatev centration (Fig. 4*a*), and the values of the tion constants at different concentratio from different preparations are in good (Fig. 5 and Table 1). The sedimentatio extrapolated to zero concentration is 2·8 values previously obtained for rabbit tr (Bailey *et al.* 1948) have been added to th they show excellent agreement with t determinations.



(*a*)



(*b*)

Fig. 4. Sedimentation diagram of tropomyosin a tropomysin. Migration to the right. Tropon 0·89 %, 100 min. after full speed. Nucleotr (*b*): 0·67 %, 90 min. after reaching full speed.

The behaviour of the preparation of nuc myosin is more complicated: it sediments a sharp peak which spreads much more slo tropomyosin and is always followed by a sl ponent (Fig. 4*b*). The proportion of this co increases at low concentrations; it is not an but a dissociation product of the main co the sedimentation constant-concentration which (Fig. 5) cannot therefore be extrapol

Several ultracentrifugations have been check the stability of nucleotropomyosin. mentation is unchanged in the presence of ad triphosphate (ATP) or after keeping the trated solution at the pH of crystallization (few days. In acetate buffer of pH 3·5 nucl myosin is slowly and irreversibly transform cold as shown in Fig. 6; a new component sedi more slowly appears after 2 hr. (Fig. 6*a*) comes prominent after 20 hr. (Fig. 6*b*). tropomyosin as an entity disappears compl the solution is kept a longer time at this pH mentation runs made with solutions of nucle

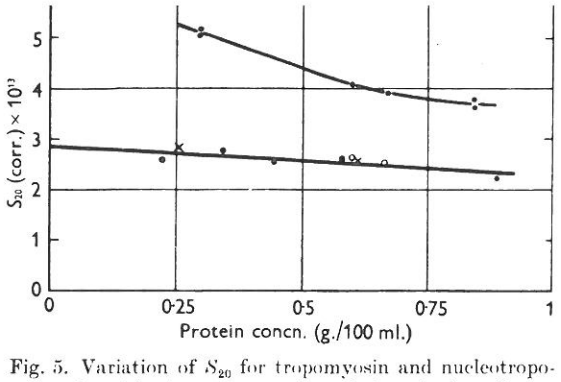


Fig. 5. Variation of S_{20} for tropomyosin and nucleotropomyosin with the concentration of the solution. Lower curve; tropomyosin; upper curve, nucleotropomyosin. ○, values taken from Bailey *et al.* (1948); ×, values obtained by transformation of nucleotropomyosin at low pH.

tropomyosin are transformed at low pH pomyosin.

Electrophoresis

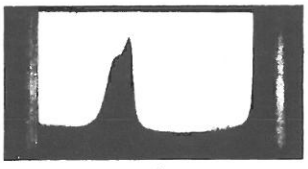
The experiments were performed with th electrophoresis apparatus slightly modi buisson & Jacob, 1945; Dubuisson, Di Debot, 1950). They lasted about 20 hr. a (1·65 V./cm.) and 7 hr. at $I = 0{\cdot}15$ (3·6 giving a migration of approx. 6 cm. in b No account was taken, in the calculati mobilities, of the differences in conductiv dialysis fluid and the protein solution, the the dialysis fluid being used for both limbs

Both preparations were homogeneous phoretically, showing a single component v only a slight admixture of impurities. No in mobility was observed between nuc

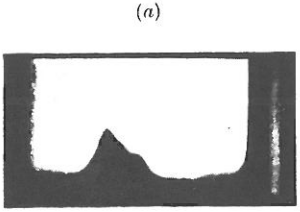
Table 1. *Sedimentation data for tropomyosin and nucleotropomyosin at different concentrations in* ($I = 0{\cdot}35$, pH 7·1.)

Tropomyosin			Nucleotropomyosin		
Sample no.	Protein concn. (g./100 ml.)	S_{20} corr. × 10^{13}	Sample no.	Protein concn. (g./100 ml.)	S_{20} corr. × 10^{13}
1	0·58	2·57	1	0·84	3·79
2	0·34	2·73	2	0·84	3·63
3	0·89	2·22		0·6	4·08
	0·44	2·52		0·3	5·11
	0·22	2·59	3	0·67	3·95
4*	0·25	2·82		0·3	5·14
5*	0·61	2·57			

* Obtained by transformation of nucleotropomyosin at pH 3·5.



(*a*)



(*b*)

Fig. 6. Sedimentation diagrams of nucleotropomyosin kept in acetate buffer pH 3·5 for 2 hr. (*a*, total concn. 1·1 %) and 20 hr. (*b*, total concn. 0·87 %); both 130 min. after reaching full speed. Migration to the right.

myosin kept 20 hr. or more in acetate buffer at pH 3·5 show that the peak corresponding to this new component has the same shape and rate of sedimentation as tropomyosin (Fig. 5 and Table 1). The

myosin and tropomyosin in several exp made on pure solutions and on mixtures of h pounds. The mobilities found are given in

Table 2. *Electrophoretic mobilities of tropo and nucleotropomyosin*

Conditions of electrophoresis	Mobilit (10^{-5} sq.cm./ Ascending value	D
NaCl-phosphate buffer of I 0·35 and pH 7·1 (I 0·1 for phosphate and 0·25 for NaCl)	−4·30	
NaCl-phosphate buffer of I = 0·15 and pH 7·4 (I = 0·1 for phosphate and 0·05 for NaCl)	−6·90	
NaCl-acetate buffer of I = 0·15 and pH 3·5 (I = 0·1 for acetate and 0·05 for NaCl)	+6·15*	

* Approximate value: one determination

The values obtained at $I = 0{\cdot}35$ and pH greatly from the mobilities of rabbit trop

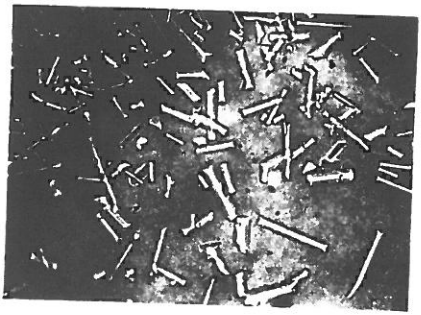


Fig. 1. Nucleotropomyosin (magnification × 54).

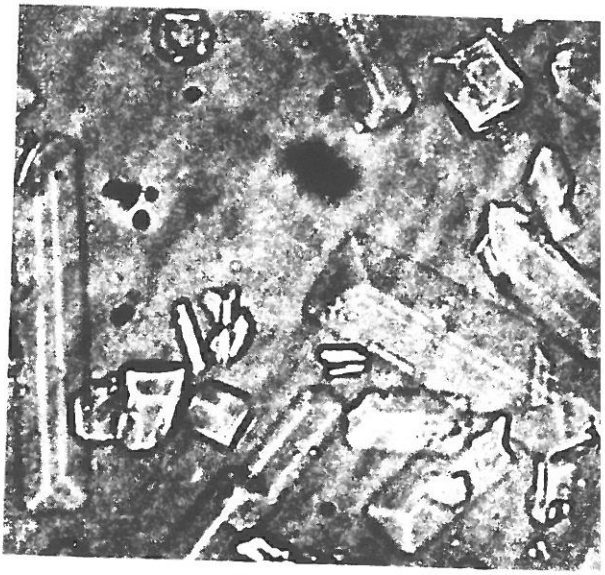


Fig. 2. Nucleotropomyosin (magnification × 135).

Fig. 3. Tropomyosin (magnification × 135).

under the same conditions (ascending, 5·6; descending, 4·9: Dubuisson, 1950). Further investigations will be necessary to determine the cause of this difference.

Nucleic acid content

Liberation of the nucleic acid was performed by heating the protein solution in a boiling-water bath in 5 % (w/v) trichloroacetic acid for 45 min. The protein was removed by filtration and the ribose content of the filtrate was determined according to Mejbaum (1939), using a 45 min. heating time as proposed by Albaum & Umbreit (1947). Measurements were made with a photoelectric absorptiometer using the 607 Ilford filter with a maximum transmission at 600 mμ. Readings were referred to a calibration curve obtained by measuring the absorption of different amounts of D-ribose under the same conditions. As both deoxyribose and ribose react with the orcinol reagent of Mejbaum (1939), the diphenylamine reaction specific for deoxyribose was performed on the trichloroacetic filtrate under the conditions described by Dische (1930). The total phosphorus contents of nucleotropomyosin and tropomyosin were determined by the perchloric acid method as modified by Allen (1940), using the same photoelectric absorptiometer as for ribose and the 608 Ilford filter.

The ultraviolet spectrum of both proteins dissolved in the phosphate-sodium chloride buffer of pH 7·1 and $I = 0{\cdot}35$ was measured against the solvent with the Beckman universal spectrophotometer (model D.U.). (These solutions had already been used in the ultracentrifugal experiments.) Readings were taken at 3 mμ. intervals between 220 and 350 mμ. The concentrations of the solutions, expressed in g./100 ml., were determined by micro-Kjeldahl assuming a nitrogen content of 16·6 % (Bailey, 1948) and were used for the calculation of the extinction coefficient $E^{1\%}_{1\,cm.}$.

Both preparations were precipitated by an equal volume of 10 % (w/v) trichloroacetic acid and kept in a boiling-water bath for different intervals to determine the time required for complete extraction (Table 3). (A slight liberation of nucleic acid occurs even after precipitation at room temperature.) Approximately constant values were obtained after about 20 min. for tropomyosin and 30 min. for nucleotropomyosin. A heating period of [illegible] therefore chosen for the determinations.

Table 3. *Rate of nucleic acid liberation at* 10 *in* 5 % *(w/v) trichloroacetic acid*

Duration of heating (min.)	Ribose liberated (mg./g. prote	
	Tropomyosin	Nucleotropor
0	4·4	5·35
10	10·2	32·1
18	10·4	—
21	—	34·6
30	12·7	44·0
45	11·0	44·0
60	10·0	—

The ribose and phosphorus contents foun given in Table 4. In view of the very differen bration curves given by ribose and deoxyribos the orcinol reagent (Schneider, 1945) and the agreement observed between the ribose and phorus determinations, the presence of deoxy is very unlikely. Tests for deoxyribose carried both preparations of nucleotropomyosin fai give any evidence of the presence of this suga

Samples of the first preparation were us measurements of the ultraviolet absorption (

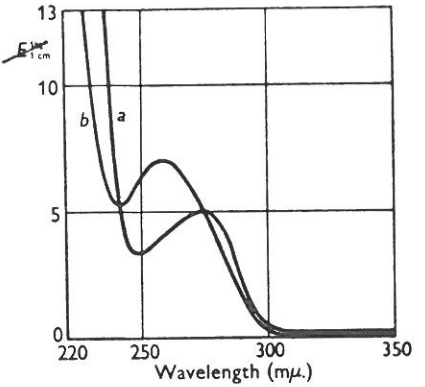


Fig. 7. Ultraviolet absorption spectra of tropo (curve *a*) and nucleotropomyosin (curve *b*) in a pho NaCl buffer of pH 7·1 and $I = 0{\cdot}35$. Ordinate: E tropomyosin and $0{\cdot}25\,E^{1\%}_{1\,cm.}$ for nucleotropomyo

Nucleotropomyosin shows the well known acid maximum at about 260 mμ., and tropo a maximum at 274 mμ. due to its content

Table 4. *Ribose and phosphorus contents of tropomyosin and nucleotropomyosin*

		Ribose (%)	P (%)	Ribose/P (mol./atom)	Pentos acid c (%
Preparation I	Tropomyosin	1·05	—	—	2·
	Nucleotropomyosin	4·38	—	—	9·
Preparation II	Tropomyosin	1·22	0·20	1·25	2·
	Nucleotropomyosin	6·75	1·47	0·99	15·

Calculated from the ribose content.

matic amino-acids. The two spectra are very similar to those obtained by Markham, Matthews & Smith (1948) on a plant virus and its specific protein.

In conclusion, tropomyosin prepared by the present method is less pure than Bailey's tropomyosin. It contains a few per cent of pentosenucleic acid which could probably be very easily removed by keeping it for a certain time at low pH. Nucleotropomyosin has a much higher content of pentosenucleic acid, which shows some variation from one preparation to the other. The presence of pentosenucleic acid in muscle is well known from the work of Davidson & Waymouth (1944) on sheep and fowl muscles and of Schneider & Klug (1946) on rat muscles, but no analytical data seem to be available on fish. It can be assumed from the yield of 0·05–0·1% of the fresh weight found by the previous authors for pentosenucleic acid that the amount in fish muscle is high enough to explain the present analytical results.

DISCUSSION

Comparison of the data obtained allows some conclusions to be drawn on the influence of the nucleic acid content on the macromolecular behaviour of tropomyosin. The presence of nucleic acid does not affect the electrophoretic mobility, as has already been observed for mixtures of proteins and nucleic acid (Greenstein, 1944) and for nucleoproteins (Markham *et al.* 1948). A content of nucleic acid of less than 3% does not influence the ultracentrifugal behaviour of tropomyosin. A higher content (about 10%) greatly changes the shape and dimensions of the molecule: a new component, named nucleotropomyosin, appears with a different rate of sedimentation which seems also uninfluenced by a variation of a few per cent in the nucleic acid content. Solutions showing an intermediate behaviour between tropomyosin and nucleotropomyosin in the ultracentrifuge were only very rarely observed.

Owing to the presence of ATP during extraction, the question arises whether nucleotropomyosin is not formed by some reaction with ATP. As it could be isolated from extracts made without addition of ATP to the extractant, and as preliminary experiments have shown that the content of ATP of fish muscles kept in a frozen state is extremely
the fresh weight), this hypothesis s

Nucleotropomyosin appears as
tuent of muscle extracts made at
ionic strength to bring the myosins
dilution of the extract with water i
with the globulins. The phosphorus
myosins and actomyosins previousl
hot organic solvents (Dubuisson, 19
is probably due to the presence of
already suggested by Bailey (194
Deutsch, Knappeis & Munch-Peters

The present results confirm the
myosin is a natural component of the
work will be necessary to know
myosin arises from the association
with pentosenucleic acid during ex
exists in fish muscle. As tropomyosi
ably located in the fibril (Bailey, 194
nucleic acid is a component of the
not unreasonable to think that b
could be associated *in situ*.

SUMMARY

1. Two proteins showing the solub
of tropomyosin have been isolated fr
by salt extraction.

2. Although the two compounds ar
able by electrophoresis, they crystall
forms and behave very differently in
fuge. These differences have been r
different nucleic acid contents.

3. It is concluded that one compoun
identical with Bailey's tropomyosin
one is a combination of the same c
pentosenucleic acid.

4. An especially mild method of
nucleoprotein is described and the
both compounds in muscle discussed.

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Director of the Lister Institute, for the
available by him, and Dr K. Bailey for a
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Belgian Government.

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