mito-oleo-linolein, and very small amounts of many other mixed glycerides. Trisaturated glycerides form little more than 2% of the whole fat.

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Fish Tropomyosin and Fish Nucleotropomyosin

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Tropomyosin has been prepared from muscles of several 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 pro-

portion of tropomyosin in the extract increases when the pH of the extraction fluid decreases and when the time of extraction is short. The tropomyosins are still very extractable when the duration of the extraction is 10 min. and the pH of the extract 5.5, but the amount of myosins going into solution under such conditions becomes much smaller. The proportion of the tropomyosins in the extracts can be further increased if the extraction is carried out with muscles kept for several months in the frozen state. Such treatment decreases the extractability of the myosins (Reay & Kuchel, 1936) and does not change the behaviour of the tropomyosins. Separation of nucleotropomyosin from tropomyosin in such extracts is easy. On dilution with water the former coprecipitates with the myosins while the latter remains in solution and precipitates only on acidifying the supernatant to pH 4.6. A final purification of the substances is carried out by ammonium sulphate fractionation. The details of the method are given below.

The preparation has been carried out throughout in the cold and all the separations have been done by centrifugation. Carp muscles cut with a freezing microtome into slices 40 μ . thick (Dubuisson, 1947) are extracted for 20 min. with 3 vol. of a KCl-phosphate solution (0.15 M-KCl and 0.1 M-KH₂PO₄ brought to pH 5). This extract is diluted with 3.5 vol. of cold water; the precipitate is discarded and the supernatant (I) kept.

The residue from this first extraction is re-extracted for 10 min. with 3 vol. of 0.5 M-phosphate solution of pH 5.5 containing 0.3% sodium adenosinetriphosphate. The

Fig. 1. Nucleotropomyosin
(magnification $\times 54$).

Fig. 2. Nucleotropomyosin
(magnification $\times 135$).

Fig. 3. Tropomyosin (magnification $\times 135$).

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 0.5M-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 0.5M-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 0.5M-KCl at neutral pH.

Both tropomyosins are isolated from these two solutions by $(\text{NH}_4)_2\text{SO}_4$ 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 $(\text{NH}_4)_2\text{SO}_4$ 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. $(\text{NH}_4)_2\text{SO}_4$ /l. and 0.01M-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 0.25M-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 Svedberg & Pedersen (1940), Appendix II.

The preparation of tropomyosin appears homogeneous by ultracentrifugation. It sediments as a single fairly symmetrical peak whatever the concentration (Fig. 4a), and the values of the sedimentation constants at different concentrations obtained from different preparations are in good agreement (Fig. 5 and Table 1). The sedimentation constant extrapolated to zero concentration is 2.85. The two values previously obtained for rabbit tropomyosin (Bailey *et al.* 1948) have been added to the diagram; they show excellent agreement with the present determinations.

Fig. 4. Sedimentation diagram of tropomyosin and nucleotropomyosin. Migration to the right. Tropomyosin (a): 0.89 %, 100 min. after full speed. Nucleotropomyosin (b): 0.67 %, 90 min. after reaching full speed.

The behaviour of the preparation of nucleotropomyosin is more complicated: it sediments as a main sharp peak which spreads much more slowly than tropomyosin and is always followed by a small component (Fig. 4b). The proportion of this component increases at low concentrations; it is not an impurity but a dissociation product of the main component, the sedimentation constant-concentration curve of which (Fig. 5) cannot therefore be extrapolated.

Several ultracentrifugations have been done to check the stability of nucleotropomyosin. Its sedimentation is unchanged in the presence of adenosinetriphosphate (ATP) or after keeping the concentrated solution at the pH of crystallization (5.4) for a few days. In acetate buffer of pH 3.5 nucleotropomyosin is slowly and irreversibly transformed in the cold as shown in Fig. 6: a new component sedimenting more slowly appears after 2 hr. (Fig. 6a) and becomes prominent after 20 hr. (Fig. 6b). Nucleotropomyosin as an entity disappears completely if the solution is kept a longer time at this pH. Sedimentation runs made with solutions of nucleotro-

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

Table 1. *Sedimentation data for tropomyosin and nucleotropomyosin at different concentrations in solutions*

Tropomyosin			Nucleotropomyosin		
(I=0.35, pH 7.1.)					
Sample no.	Protein concn. (g./100 ml.)	S_{20} corr. $\times 10^{13}$	Sample no.	Protein concn. (g./100 ml.)	S_{20} corr. $\times 10^{13}$
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.

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

bigger and more asymmetrical particles of nucleotropomyosin are transformed at low pH into tropomyosin.

Electrophoresis

The experiments were performed with the Tiselius electrophoresis apparatus slightly modified (Dubuisson & Jacob, 1945; Dubuisson, Distèche & Debot, 1950). They lasted about 20 hr. at $I = 0.35$ (1.65 V./cm.) and 7 hr. at $I = 0.15$ (3.65 V./cm.), giving a migration of approx. 6 cm. in both cases. No account was taken, in the calculation of the mobilities, of the differences in conductivity of the dialysis fluid and the protein solution, the value for the dialysis fluid being used for both limbs.

Both preparations were homogeneous electrophoretically, showing a single component with no, or only a slight, admixture of impurities. No difference in mobility was observed between nucleotro-

myosin and tropomyosin in several experiments made on pure solutions and on mixtures of both compounds. The mobilities found are given in Table 2.

Table 2. *Electrophoretic mobilities of tropomyosin and nucleotropomyosin*

Conditions of electrophoresis	Mobility (10^{-5} sq.cm./V./sec.)	
	Ascending value	Descending value
NaCl-phosphate buffer of $I = 0.35$ and pH 7.1 ($I = 0.1$ for phosphate and 0.25 for NaCl)	-4.30	-3.90
NaCl-phosphate buffer of $I = 0.15$ and pH 7.4 ($I = 0.1$ for phosphate and 0.05 for NaCl)	-6.90	-6.20
NaCl-acetate buffer of $I = 0.15$ and pH 3.5 ($I = 0.1$ for acetate and 0.05 for NaCl)	+6.15*	+5.76*

* Approximate value: one determination only.

The values obtained at $I = 0.35$ and pH 7.1 differ greatly from the mobilities of rabbit tropomyosin

under the same conditions (ascending, 5·6; descending, 4·9: Dubuissou, 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 m μ . 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 $I = 0\cdot35$ 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 m μ . intervals between 220 and 350 m μ . 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 $E_{1\text{cm}}^{1\%}$.

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 45 min. was therefore chosen for the determinations.

Table 3. Rate of nucleic acid liberation at 100° in 5% (w/v) trichloroacetic acid

Duration of heating (min.)	Ribose liberated (mg./g. protein)	
	Tropomyosin	Nucleotropomyosin
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 found are given in Table 4. In view of the very different calibration curves given by ribose and deoxyribose with the orcinol reagent (Schneider, 1945) and the good agreement observed between the ribose and phosphorus determinations, the presence of deoxyribose is very unlikely. Tests for deoxyribose carried out in both preparations of nucleotropomyosin failed to give any evidence of the presence of this sugar.

Samples of the first preparation were used for measurements of the ultraviolet absorption (Fig. 7).

Fig. 7. Ultraviolet absorption spectra of tropomyosin (curve *a*) and nucleotropomyosin (curve *b*) in a phosphate-NaCl buffer of pH 7·1 and $I = 0\cdot35$. Ordinate: $E_{1\text{cm}}^{1\%}$ for tropomyosin and $0\cdot25 E_{1\text{cm}}^{1\%}$ for nucleotropomyosin.

Nucleotropomyosin shows the well known nucleic acid maximum at about 260 m μ ., and tropomyosin a maximum at 274 m μ . due to its content of aro-

Table 4. Ribose and phosphorus contents of tropomyosin and nucleotropomyosin

		Ribose (%)	P (%)	Ribose/P (mol./atom)	Pentosenucleic acid content* (%)
Preparation I	Tropomyosin	1·05	—	—	2·36
	Nucleotropomyosin	4·38	—	—	9·9
Preparation II	Tropomyosin	1·22	0·20	1·25	2·75
	Nucleotropomyosin	6·75	1·47	0·99	15·2

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. Nucleotropyosin 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 nucleotropyosin, 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 nucleotropyosin in the ultracentrifuge were only very rarely observed.

Owing to the presence of ATP during extraction, the question arises whether nucleotropyosin 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 low (≤ 0.01 % of the fresh weight), this hypothesis seems unlikely.

Nucleotropyosin appears as a normal constituent of muscle extracts made at sufficiently high ionic strength to bring the myosins into solution. On dilution of the extract with water it is precipitated with the globulins. The phosphorus content of many myosins and actomyosins previously extracted with hot organic solvents (Dubuisson, 1941; Bailey, 1942) is probably due to the presence of nucleic acid, as already suggested by Bailey (1946) and Buchtal, Deutsch, Knappeis & Munch-Petersen (1949).

The present results confirm the view that tropomyosin is a natural component of the muscle. Further work will be necessary to know if nucleotropyosin arises from the association of tropomyosin with pentosenucleic acid during extraction or pre-exists in fish muscle. As tropomyosin is quite probably located in the fibril (Bailey, 1948) and pentosenucleic acid is a component of the cytoplasm, it is not unreasonable to think that both compounds could be associated *in situ*.

SUMMARY

1. Two proteins showing the solubility properties of tropomyosin have been isolated from fish muscle by salt extraction.
2. Although the two compounds are indistinguishable by electrophoresis, they crystallize in different forms and behave very differently in the ultracentrifuge. These differences have been related to their different nucleic acid contents.
3. It is concluded that one compound is practically identical with Bailey's tropomyosin and the other one is a combination of the same compound with pentosenucleic acid.
4. An especially mild method of splitting this nucleoprotein is described and the occurrence of both compounds in muscle discussed.

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The Effect of Starvation on the Pseudo-Cholinesterase Activity of the Liver and Serum of Rats

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In man the serum cholinesterase has been found to be low in many diseases affecting the cells of the liver and to rise again to normal levels with recovery, and the measurement of the activity in the serum has been suggested as a test of liver function (McArdle, 1940; Faber, 1943; Kaswin, 1945). McCance, Widdowson & Hutchinson (1948) showed that the serum possessed subnormal activity during undernutrition, and that normal activity returned with recovery. Waterlow (1950) took liver biopsies and simultaneous samples of serum from infants during and after a period of malnutrition, and found that the enzyme activities increased in both tissues as nutrition improved.

Some have assumed that the pseudo-cholinesterase found in the serum has been made by the liver and discharged into the circulation, but the experiments undertaken by others to prove this on animals have not been conclusive. This has partly been due to a tendency for each species and sex to respond to the same stimulus in a different way, but the difficulties have been enhanced by the variable amounts of 'true' cholinesterase found in the serum and organs of the domestic and laboratory mammals. Thus chloroform and carbon tetrachloride poisoning raised the cholinesterase in the serum of dogs (Steensholt & Venndt, 1945; Brauer & Root, 1947), but ligaturing the pancreatic duct, phosphorus poisoning, or undernutrition made no permanent impression on the serum activity (Hutchinson, 1951). Brauer & Root (1947) found that the cholinesterase activity of the liver in dogs was depressed after prolonged carbon tetrachloride poisoning. In rats, carbon tetrachloride lowered the serum cholinesterase in females but not significantly in males, and it reduced the activity in the livers of both sexes (Ellis, Sanders & Bodansky, 1947; Brauer & Root, 1946). Hutchinson (1951) observed that feeding half

rations for 3 months halved the weight of adult male rats, but did not lower the activity of the serum cholinesterase. On the other hand, Mendel, Hawkins & Nishikawara (1948) determined the serum cholinesterase of males before and after a 12-day fast and found that the serum activity fell by 50%. Milch (1950) also found a fall in activity after feeding a protein-free diet for 6 weeks. No similar experiments have been carried out on female rats, although other liver enzymes have been followed during starvation in both sexes (Miller, 1948).

No significant sex difference has been found in the serum cholinesterase activity of dogs (Brauer & Root, 1947), or of human beings except during pregnancy (Levine & Hoyt, 1949). In rats, however, the females have much more cholinesterase in their livers and sera than the males (Birkhäuser & Zeller, 1940; Sawyer & Everett, 1947). Both tissues contain true and pseudo-cholinesterase, but it is the latter which is responsible for the sexual variation (Mundell, 1944; Sawyer & Everett, 1946).

In the present study, the cholinesterase activity of the liver and the serum of male and female rats was followed during a 6-day period of starvation. At the same time, other changes in the composition of the liver cell during starvation were investigated by one of us (M.F.H.) and these will be reported in a later paper.

METHODS

Animals. Thirty-five male and thirty-five virgin female black and white hooded rats of the Lister strain, 3-4 months old, were used. Until the fast, they were fed on the stock diet consisting of bread, milk, whole wheat, wheat germ, yeast, fish meal, oatflakes and bran. The rats were weighed twice a week for 3 weeks before the fast to ensure that they were growing at a normal rate. The males and females were then divided into five comparable groups of seven rats, according to their mean weight for the 3 days before the fast,