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Carp Myogens of White and Red Muscles

GENERAL COMPOSITION AND ISOLATION OF LOW-MOLECULAR-WEIGHT COMPONENTS OF ABNORMAL AMINO ACID COMPOSITION

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1. The general composition of the carp myogens of white and red muscles was examined by electrophoresis and ultracentrifugation. 2. Eight and nine peaks were found in the electrophoretic analysis at pH 7·3 and I 0·075 of white and red muscle respectively. Lowering of the pH to 5 or 6 did not increase the number of peaks. The electrophoretic pattern of white-muscle myogen was remarkably different from that of red-muscle myogen, though ultracentrifugal analyses of the both types of myogen gave similar diagrams, in which about one-third of the total myogen sedimented slowly. 3. The pH–mobility curves of the myogen of white muscle indicated that the net charges of the components 2, 3 and 5 vary only slightly within the pH range 7·3–5·4, suggesting that their histidine content is very low. 4. The slow-sedimenting fraction of white-muscle myogen was isolated in fairly good yield by ammonium sulphate fractionation, by taking advantage of their high salting-out range, and the fraction was shown to be composed mainly of components 2, 3 and 5. 5. The same method of fractionation was applied to red-muscle myogen and the absence of the three components was confirmed. These results bring to light a new difference between the two types of fish muscle.

Various investigations have already been carried out on the myogens of carp white and red muscle. Their general composition has been examined by electrophoresis and ultracentrifugation (Hamoir, 1951, 1955*a*,*b*, 1962; Henrotte, 1960). Carp myoglobin (Hamoir, 1955*a*,*b*) and several other proteins have been crystallized (Henrotte, 1952, 1955, 1960; Hamoir, 1962). Glyceraldehyde phosphate dehydrogenase has been obtained in a high degree of purity (Ludovicy-Bungert, 1961) and aldolase partially purified (Shibata, 1958; Th. Grandjean, unpublished work). Extensive comparative work has been carried out by electrophoresis on extracts of fish muscle of low ionic strength, mainly by Connell (1953*a*,*b*) and Nikkilä & Linko (1955) (cf. also Dingle, Eagles & Neelin, 1955; Jebsen & Hamoir, 1958). The electrophoretic patterns were found to be characteristic of each species (Connell, 1953*b*) and to have in common a greater mean mobility than the one observed with similar extracts from higher vertebrates (Hamoir, 1955*a*,*b*, 1962).

Ultracentrifugal analyses have also been made on the myogens of carp (Hamoir, 1955*a*,*b*) and plaice (Jebsen & Hamoir, 1958) and on cod extracts (Connell, 1958). The same general pattern is obtained: fish myogen divides into three broad peaks of 1·3–1·5, 4·5–5 and 6·5–7·2s. The general occurrence of a slow-sedimenting fraction is remarkable. As it is also present in frog myogen but not in rabbit myogen (Deuticke, 1934), it appears to occur only in the myogens of cold-blooded vertebrates. Its amount evaluated from the ultracentrifugal patterns without taking into account the Johnston–Ogston effect is about 20% in plaice and cod, and 40% in carp. Several components of this fraction have been isolated from carp myogen (Henrotte, 1955, 1960; Hamoir, 1957, 1962); their molecular weights lie between 10000 and 15000 and their amino acid compositions are abnormal: they contain about 15% of phenylalanine, no tryptophan and usually no tyrosine. A protein having a similar composition also occurs in plaice myogen (Jebsen & Hamoir, 1958).

In the present work, we have tried to place these various results in their proper perspective. First, we have taken account of the wide differences that exist between white muscle and the red muscle located under the lateral line. The histological and physiological properties of these two kinds of muscle have been investigated extensively by Barets (1961), who showed that the former behaves as the quick-

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acting muscular fibres of the frog and the latter as the slow-acting fibres. According to Boddeke, Slyper & Van der Stelt (1959) the 'sprinters' (fishes such as pike and perch) should rely mainly on white muscle and the 'stayers' (such as salmon, trout and carp) on red muscle. On the other hand, the red muscle frequently contains about 1% of myoglobin and even sometimes about 3%, as in tuna (Matsuura & Hashimoto, 1959); it is also richer in vitamins (Braekkan, 1956; Higashi, 1962) and in lipids (Lovern, 1962). Preliminary experiments have shown that the electrophoretic patterns of the sarcoplasmic proteins of both kinds of muscle (Hamoir, 1955*a*,*b*), as well as the stability of the myofibrils towards salts (Hamoir, McKenzie & Smith, 1960), are different.

These two kinds of muscle have therefore been investigated separately. The myogens have been examined electrophoretically at an ionic strength of I 0·075 instead of 0·15 as before (Henrotte, 1960) and at various pH values to increase the resolution. The isolation of the slow-sedimenting fraction has been followed by ultracentrifugation and electrophoresis to correlate both patterns and to localize the proteins isolated previously from the whole myogens. The separation of the main components of the slow-sedimenting fraction and their properties is described below.

METHODS

Materials. Mirror carp (*Cyprinus carpio* L.) of between 1 and 2 kg. were killed by a blow on the head, decapitated, skinned and filleted without delay. The white muscle and the red muscle located under the lateral line were separated. To avoid mutual contamination, the intermediate region was removed. Any traces of red colour were eliminated from the white muscle and the 'cleaning' of the red muscle was extended until the white myosepta separating the red muscular fibres became very apparent.

Isolation of the myogens. Both kinds of muscle were minced with a domestic mincer at room temperature without delay. The further steps were carried out in the cold room at 2°. The muscle brei was mixed with cold water (0·8 ml./g.), gently stirred for 1 hr. and centrifuged for 15 min. at 25000*g*. A liquid layer of lipid material accumulated at the top of the extracts from red muscle; it was partially removed by filtration on cotton wool. The supernatants were dialysed in Visking cellulose tubing (18/32 in. or 23/32 in.) for 3 days against successive 2 l. batches of water with an efficient rocking system, the external solution being replaced five times. The globulins were centrifuged off at 25000*g* for 15 min., and the supernatants were filtered through cotton wool.

Analytical methods. After isolation, the myogen solutions were usually dialysed against a phosphate buffer, pH 7·3 and I 0·075 (15·4 mM-Na_2HPO_4–3·9 mM-NaH_2PO_4–25 mM-NaCl) and examined by electrophoresis and ultracentrifugation in this solvent. Other dialysis fluids of the same ionic strength and of various pH values used to determine pH–mobility curves were: (*a*) 7 mM-Na_2HPO_4–29 mM-NaH_2PO_4–25 mM-NaCl, pH 6·2; (*b*) 50 mN-NaOH–57 mN-acetic acid–25 mM-NaCl, pH 5·4; (*c*) 50 mN-NaOH–71·4 mN-acetic acid–25 mM-NaCl, pH 5·0. After dialysis, the protein content was determined by refractometry with sodium light at 25°, assuming a refractive index increment of 0·00182 for a 1% (w/v) solution (Hamoir, 1955*a*).

The solutions were examined in the electrophoretic cell described by Dubuisson, Distèche & Debot (1950), the recording of the patterns being made according to the Longsworth scanning method. Panchromatic films were used for the red-muscle extracts. The mobilities were calculated from the mean conductivity of the protein solution and the dialysis fluid at 1°, and the percentages of the peaks were evaluated with the perpendicular-extrapolation method of Tiselius & Kabat (1939). The standard deviations were given by the usual equation

$$\sqrt{\sum_{1}^{n} d^2/n-1}$$

where d corresponds to the difference with respect to the arithmetic mean, and n is the number of determinations. In the Figures, the upper part represents the ascending pattern and the lower part the descending one.

The ultracentrifugations were carried out in a Spinco model E ultracentrifuge at 59780 rev./min. and room temperature with a phase-plate recording system. The rates of sedimentation were corrected for water and 20°, assuming the partial specific volume given in Svedberg & Pedersen's (1940) Appendix II. The relative concentrations were calculated by the procedure of Tiselius & Kabat (1939), without taking into account the dilution occurring in the sector-shaped cell and the Johnston–Ogston effect (Johnston & Ogston, 1946; Harrington & Schachman, 1953).

Fractionation. The myogens were fractionated with ammonium sulphate (analytical reagent; E. Merck A.-G., Darmstadt, Germany) at 2° as follows. The clear water-

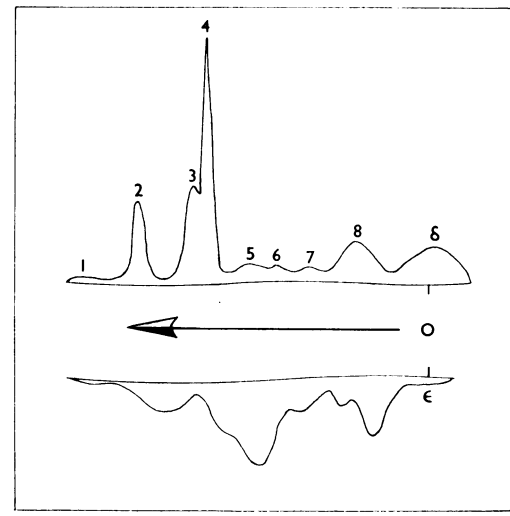

Fig. 1. Electrophoretic pattern of 1·55% (w/v) carp myogen (white muscle) in phosphate–NaCl buffer, pH 7·3 and I 0·075, after 322 min. at 5·84 v/cm.

dialysed solutions were brought to 77% saturation at neutral pH by the slow addition of 49·4g. of ammonium sulphate and 0·5ml. of N-NaOH/100ml. of solution (cf. the nomogram for 0° of Brenner-Holzach & Staehelin, 1953), with stirring. The stirring was continued for a further 10min. and the precipitate centrifuged for 20min. at 25000*g*. If necessary, the supernatant was filtered through cotton wool, brought to 100% saturation with solid ammonium sulphate and stirred for 30min. before centrifugation, when a second precipitate was isolated. The two precipitates were dissolved in water and dialysed against water. A slight precipitate that usually formed was removed by centrifugation. A subsequent dialysis against the usual buffer, pH7·3 and *I* 0·075, was made before examination.

When the fraction at 77–100% saturated ammonium sulphate was refractionated, it was freeze-dried after dialysis against water. The powder redissolved completely in water (11ml./g.). The solution obtained was then gently stirred and brought to 82% saturation at 2° by the addition of a neutral saturated solution of ammonium sulphate. The precipitate was immediately centrifuged off. The clear supernatant was saturated with solid ammonium sulphate and stirred for 30min. before centrifugation and collection of the precipitate.

RESULTS

Composition of the myogen of carp white muscle

Electrophoretic pattern at pH 7·3 *and I* 0·075. The ascending diagram (Fig. 1) shows eight components instead of the six observed at *I* 0·15 by Henrotte (1960). Their mobilities and proportions are given in Table 1. Component 1, which has not been described before, is observed regularly on the ascending and descending sides. The following peak, component 2, appears fairly homogeneous and separates well from the other components after a migration of about 5cm.; its percentage varies somewhat from one preparation to another. Components 3 and 4, which correspond together to about 50% of the total myogen, separate only partially from each other at the end of the run. In view of the poor resolution obtained, the amount of component 3 is not given in Table 1, though it was evaluated to vary from about 13% to a fairly low percentage. The proportion of component 4 is more constant. The two components 5 and 6 correspond to the asymmetrical ascending peak observed by Henrotte (1960) at pH 7·3 and *I* 0·15 following component 4. The amount of component 5 is usually larger (cf. Table 1) than that shown in Fig. 1. The separation of the two slow components 7 and 8 is also much improved on the ascending and on the descending sides under the present conditions.

pH–*mobility curves between* pH 7·3 *and* 5. As the amount of precipitate isolated after dialysis at pH 5·4 or 5·0 was negligible, the electrophoretic patterns obtained within the pH range 7·3–5 can be readily compared. The diagrams at pH 6·2 (Fig. 2) and pH 5·4 (Fig. 3) differ remarkably from each

Table 1. *Electrophoretic analysis of the myogen of carp white muscle in phosphate buffer*, pH 7·3 *and I* 0·075, *at a protein concentration of* 1·5% (*w/v*)

		Ascending								Descending							
Expt. no.	Peak ...	1	2	3	4	5	6	7	8	1	2	3	4	5+6	7	8
		(*a*) $10^5 \times$ Negative mobility (cm.^{2} v^{-1} sec.$^{-1}$)														
Mean of five determinations		5·8	4·74	3·86	3·53	2·88	2·41	1·80	1·14	5·7	4·40	3·5*	2·91	2·26	1·39	0·9
Standard deviation		0·25	0·14	0·14	0·14	0·03	0·03	0·03	0·11	0·22	0·14	—	0·04	0·05	0·13	0·09
		(*b*) Amount of component (% of total)														
383		2·1	16·1	52·9		5·6	3·0	2·6	17·6	3·2	19·3	45·4		11·3	5·9	14·9
387		2·9	8·8	43·2		13·0	6·2	5·2	25·2	3·9	9·8	36·7		20·4	6·9	17·9
434		—	14·5	51·4		11·0	7·2		15·0	—	17·2	43·1		20·3	19·5	
505		6·3	12·0	43·2		22·0			16·5	4·4	11·0	45·4		19·6		19·6
Mean		3·8	12·9	47·7		9·9	4·6	3·9	18·6	3·8	14·3	42·7		17·3	6·4	17·5
Standard deviation		2·2	3·2	5·2		3·8	—	—	4·5	0·6	5·3	4·1		5·2	—	—

* Approximate value.

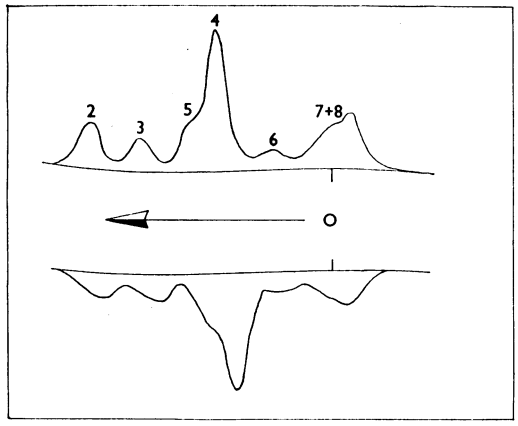

Fig. 2. Electrophoretic pattern of 1·55% (w/v) carp myogen (white muscle) in phosphate–NaCl buffer, pH 6·14 and I 0·075, after 322 min. at 5·5 v/cm.

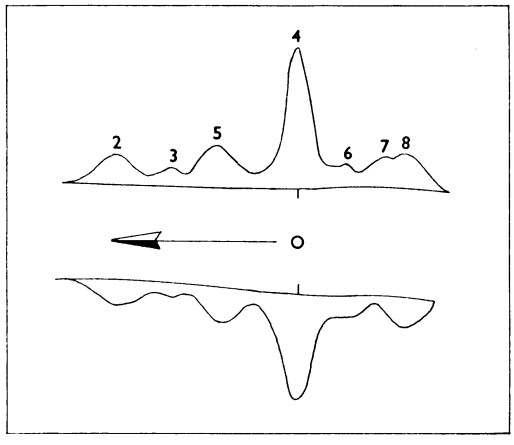

Fig. 3. Electrophoretic pattern of 1·5% (w/v) carp myogen (white muscle) in acetate-NaCl buffer, pH 5·43 and I 0·075, after 420 min. at 3·3 v/cm.

other and from the one obtained at pH 7·3 (Fig. 1). These changes are in agreement with previous experiments suggesting that components 3 and 5 should move more quickly than the main component 4 at lower pH, the isoelectric points being respectively 4·2 and 4·55 for components 3 and 5 (Hamoir, 1957) and 5·55 for component 4 (Henrotte, 1960). Peak 5 effectively migrates in front of peak 4 at pH 6·2 and separates very well at pH 5·4. Peak 1 is usually not visible at pH 6·2 or 5·4 (it could be observed in front of component 2 on the ascending side only in one experiment at pH 6·2). Components 7 and 8 are approximately isoelectric at about pH 6

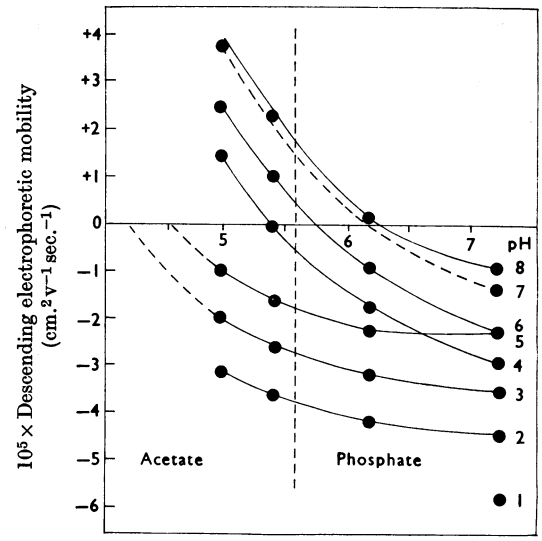

Fig. 4. Descending mobilities of the components of carp myogen (white muscle) in phosphate–NaCl or acetate–NaCl buffer, I 0·075, as a function of pH.

and do not separate from the δ- and ε-effects; the behaviour of component 7 under these conditions cannot therefore be accurately defined. The lowering of pH does not increase the number of components but improves some separations. The net charges of the components 2, 3 and 5 vary only slightly within the pH range 7·3–5·4 (Fig. 4). The curves of components 3 and 5 are extrapolated by broken lines to the isoelectric points 4·2 and 4·55 determined previously for the crystalline component isolated by Henrotte (1955) and a slow-moving impurity of this preparation (Hamoir, 1957). The isoelectric point of 5·38 corresponding to component 4 is somewhat lower than the value of 5·55 previously determined by microcataphoresis for a crystalline component moving at the rate of this peak (Henrotte, 1960). However, as the linear variation of the mobility then assumed by Henrotte (1960) between pH 4·5 and 6·5 is unlikely to be correct, our present value appears more exact. Component 7 does not separate well from component 8 at pH values below 7·3; its mobility has been tentatively represented by the broken line in Fig. 4. The isoelectric points of the components 6, 7 and 8 are respectively about 5·75, 6·2 and 6·3.

These results were compared with similar curves corresponding to muscle extracts of low ionic strength of rabbit (Jacob, 1947, 1948) and of post-rigor cod (Dingle *et al.* 1955). The only component of rabbit extracts whose isoelectric point is in the range found for components 2, 3 and 5 is component h; it corresponds probably to serum albumin (cf.

Goffart-Louis, 1959; Moreau-Collinet & Hamoir, 1961; Hartshorne & Perry, 1962), which has an isoelectric point of 4·7 in acetate buffer, I 0·15 (Hughes, 1954). The main component 4 is also absent in rabbit extracts; its isoelectric point, 5·4, is lower than those of the main components l, m and n of rabbit extracts, which are respectively 6·0, 6·2 and 6·75 (Jacob, 1947), and its mobility at pH 7·3 is about 2·2 times that of component l (Henrotte, 1960; Moreau-Collinet & Hamoir, 1961; Hamoir, 1962). The slow components 6, 7 and 8 are apparently the only ones corresponding to the main components of rabbit extracts. The comparison of the mobilities of glyceraldehyde phosphate dehydrogenase and aldolase isolated from rabbit and carp muscles confirms this analogy. Rabbit aldolase and glyceraldehyde phosphate dehydrogenase migrate with the l and m + n peaks respectively (Distèche, 1953; Cori, Slein & Cori, 1948), whereas the carp enzymes are found in the region of peaks 7 and 8 (Ludovicy-Bungert, 1961; Th. Grandjean, unpublished work).

The electrophoretic analysis of cod muscle extracts (Dingle *et al.* 1955) suggests also the presence of two groups of proteins, a first one consisting of the components 2, 3 and 4 described by these authors, with very low isoelectric points and with mobilities decreasing slowly in the pH range 6–7·4 (especially component 4), and a second one with isoelectric points between about 4·8 and 6. An improved separation of these two groups was observed at pH 5, as with the carp extracts. Though further experiments are necessary to confirm the existence of these two groups of proteins in cod muscle, the nearly constant mobility of component 4 also observed in this fish between pH 7·5 and 6 strongly suggests that it belongs to the group of proteins corresponding to components 2, 3 and 5 of the carp extracts.

Ultracentrifugal pattern. The few ultracentrifugations of carp white-muscle myogen described so far (Hamoir, 1955*a*; Henrotte, 1960) suggest fairly wide variations in the percentages of the three peaks observed. Some complementary experiments have therefore been made at pH 7·3 and I 0·075 to determine more exactly the ultracentrifugal composition of this material (Fig. 5 and Table 2). The rate of sedimentation of the median peak is fairly constant, but those of the slow and the fast peaks are more variable from sample to sample. The fraction sedimenting more quickly is obviously heterogeneous; the slow one appears more homogeneous, but the variation in migration velocity observed suggests that it also corresponds to a mixture of components of slightly different rates of sedimentation. The slow-sedimenting peak amounts usually to about 30%, and not to 40–50% as suggested by previous results (Hamoir, 1955*a*; Henrotte, 1960). However, this value remains higher than those (20–24%)

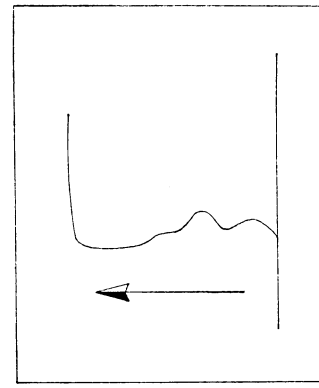

Fig. 5. Ultracentrifugal pattern of 1% (w/v) carp myogen (white muscle) in phosphate–NaCl buffer, pH 7·3 and I 0·075, after 60 min. at 59 780 rev./min., angle 60°.

Table 2. *Ultracentrifugal analysis of the myogen of carp white muscle in dilute phosphate–sodium chloride buffer*, pH 7·3

Expt. no.	Concn. (%, w/v)	$S_{20,w}$ (s) Slow	Median	Fast	Amount (% of total) Slow	Median	Fast
Taken from Hamoir (1955*a*): I 0·15	1·07	1·70	4·85	6·86*	53·5*†	38·8*†	7·8*†
Taken from Hamoir (1955*a*): I 0·15	1·1	1·48	4·75	7·12*	37·7†	47·0†	15·4†
Carp a: I 0·075	1·0	2·2*	4·9	6·7	29·8	47·0	23·2
Carp 17: I 0·075	1·0	1·5	5·2	7·0	32·4	51·5	16·2
Carp 19: I 0·075	1·0	1·1*	4·9	5·7*	30·8	49·9	19·4
Carp 22: I 0·075	1·0	1·6	5·5	7·6*	35·6	47·4	17·1
Mean		1·6	5·0	6·9	32·2	49·0	19·0
Standard deviation		0·1	0·3	—	2·5	2·1	3·1

* Approximate or abnormal value not used for calculation of the mean.

† Recalculated value differing somewhat from that published previously and not used for calculation of the mean.

found for codling extracts of low ionic strength (Connell, 1958) and for plaice myogen (Jebsen & Hamoir, 1958).

It has been shown previously that the slow and the median peaks contain components 3 and 4 respectively (Henrotte, 1954, 1955, 1960), whereas carp glyceraldehyde phosphate dehydrogenase is part of the fast fraction (Ludovicy-Bungert, 1961). The localization of other electrophoretic peaks in the ultracentrifugal pattern is examined below after the description of the isolation of the slow-sedimenting fraction by ammonium sulphate fractionation.

Composition of the myogen of carp red muscle

Electrophoretic pattern at pH 7·3 *and I* 0·075. As small fast components vanish fairly soon during the electrophoresis, the patterns corresponding to two electrophoresis times are reproduced in Fig. 6. The amount of the components migrating in front of the main peak, or component 4, is lower here than in previous ones (Hamoir, 1955*a*,*b*), which correspond to preparations somewhat contaminated with white muscle. In Table 3, the components having apparently the same mobilities as in white muscle at pH 7·3 are represented tentatively by the same numbers, and the new symbols 1a and 1b are used for peaks not present in white-muscle diagrams. The very small component, 1a, migrating very quickly, is indeed sometimes visible on the ascending side at the beginning of the run. The second one, 1b, is more constantly present in red-muscle myogen. On the descending side, a single fast component is observed regularly with a mobility suggesting that it corresponds to both ascending components 1a and 1b. The two following heterogeneous peaks migrate with mobilities that are fairly close to those of components 1 and 2 of white muscle. A separation of component 3 from the main peak 4 has never been observed in red-muscle extracts; in view of the slight asymmetry of this peak, however, its presence cannot be excluded. Component 5 is not present.

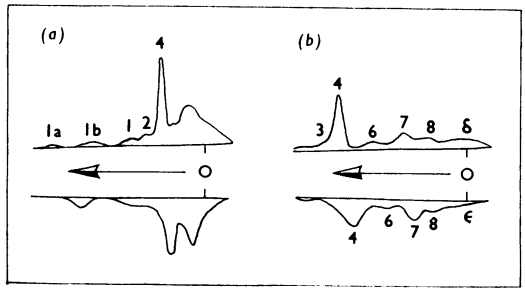

Fig. 6. Electrophoretic patterns of 1·5% (w/v) carp myogen (red muscle) in phosphate–NaCl buffer, pH 7·3 and *I* 0·075, after 174 min. (*a*) and 501 min. (*b*) at 4·74 v/cm.

Table 3. *Electrophoretic analysis of the myogen of carp red muscle in phosphate or acetate buffer, I* 0·075, *at a protein concentration of* 1·5% (*w/v*)

		Ascending								Descending							
Expt. no.	Peak ...	1a	1b	1	2	3	4	6 or M	7	8	1a	1b	1	2	3	4	6 or M	7	8
								(*a*) $10^5 \times$ Negative mobility (cm.^{2} v^{-1} sec.$^{-1}$)									
pH 7·3 (mean of six determinations)		11·3	9·28	5·81	4·5	—	3·45	2·43	1·63	1·03	9·29		5·40	4·21	—	2·94	2·10	1·39	0·89
Standard deviation		—	0·56	0·30	0·28	—	0·07	0·12	0·10	0·02	0·44		0·27	0·22	—	0·10	0·12	0·09	0·10
pH 5·4 (mean of three determinations)		—	—	3·82	2·31	1·19	0·27	+0·27	+1·62				3·80	2·29	—	0·22	+0·22	+1·81	
								(*b*) Amount of component observed at pH 7·3 (% of total)									
Electrophoresis no. 21		—	—	9·2		32·4	14·7	43·8			—		9·2		38·3	14·0	38·5		
Electrophoresis no. 17		1·0	4·0	5·6	4·3	40·0	11·4	19·5	14·2		7·7		7·5	5·7	39·7	10·6	17·8	11·4	
Electrophoresis no. 48		—	1·9	4·7	4·8	33·2	11·9	19·0	24·5		4·5		7·4	5·5	36·4	10·0	18·0	18·3	
Electrophoresis no. 54		1·7	2·1	5·8	5·1	37·7	11·5	22·0	14·1		4·1		4·7	4·8	37·0	11·9	24·5	12·9	
Mean		1·35	2·7	5·4	4·7	35·8	12·4	20·2	17·6		5·4		6·5	5·3	37·8	11·6	20·1	14·2	
Standard deviation		—	1·2	0·6	0·4	3·6	1·6	1·6	6·0		2·0		1·6	0·5	1·5	1·8	3·8	3·6	

Table 4. *Ultracentrifugal analysis of the myogen of carp red muscle in phosphate buffer*, pH 7·3 *and* I 0·075

Expt. no.	Concn. (%, w/v)	$S_{20,w}$ (s) Slow	Median	Fast	Amount (% of total) Slow	Median	Fast
Carp a	1·0	1·7*	5·0*	6·9*	26·2	53·4	20·4
Carp 17	0·5	2·1	5·1	7·9	32·0	50·7	17·4
Carp 18	0·5	2·4	5·3	7·2	38·1	46·2	15·8
Carp 19	0·5	1·0	4·9	7·1	32·1	52·5	15·4
Carp 21 to 24	0·5	1·2	5·0	7·6*	44·3	42·8	12·9
Mean		1·7	5·1	7·4	34·5	49·1	16·4
Standard deviation		0·7	0·2	0·4	6·9	4·5	2·8

* Approximate value not used for calculation of the mean.

The behaviour of the red colour of the extracts is somewhat variable from one experiment to another. The change of light-absorption occurs in the region of peak 6 or 7, or both, according to the experiment. As the surface of peak 6 appears to vary proportionally to the absorption, we have assumed that it corresponds to myoglobin. However, a fraction of the myoglobin is apparently sometimes denatured by the exhaustive dialysis of the extract against water, giving rise to a second change in absorption in the region of the following electrophoretic peak (cf. Rumen, 1959; Edmundson & Hirs, 1962). This peak and the slowest one move fairly close to one another, with mobilities comparable with those of components 7 and 8 of white muscle. Comparison of the percentages of the various peaks also reveals large differences between white and red muscle: in red muscle the amounts of components 2, 3 + 4 and 5 are much lower, whereas those of components 6 and 7 are much larger (cf. Table 4). The slow component 8 is apparently the only one that remains unchanged in both kinds of extracts.

Electrophoretic pattern at pH 5·4 *and* I 0·075. The myogen of red muscle remains entirely in solution after dialysis against acetate buffer, pH 5·4 and I 0·075. The peaks 1a and 1b shown in Fig. 6(*a*) have not been observed at this pH (Figs. 7*a* and 7*b*). Components 1 and 2 appear to be very similar to those seen at pH 7·3 (cf. Fig. 6*a*), but their mobilities at pH 5·4 correspond respectively to those of components 2 and 3 of white muscle. As peak 1 of white muscle is not visible at pH 5·4, a similar decrease of mobility may occur in the present case, it being not apparent in white muscle because of the presence of the large peak 2. Components 2 and 3 of red muscle move much more slowly than peaks 2 and 3 of white muscle at pH 5·4 (cf. Table 2), and do not therefore correspond to the same material. The reverse appears to be true for the main peak 4. Carp myoglobin does not separate from this peak at pH 5·4; its isoelectric point lies at about pH 5·3 in acetate buffer. A broad peak moving backwards is seen on the ascending side instead of the two poorly separated components of Fig. 3; the decreased resolution observed for components 7 and 8 of white muscle at pH 5·4 is thus still more pronounced here. But the mobilities agree fairly well, suggesting that these components do not differ notably in both kinds of muscle.

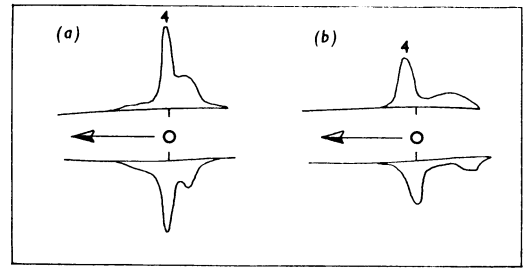

Fig. 7. Electrophoretic patterns of 1·5% (w/v) carp myogen (red muscle) in acetate–NaCl buffer, pH 5·43 and I 0·075, after 223 min. (*a*) and 505 min. (*b*) at 3·48 v/cm.

Ultracentrifugal analysis. As the high absorption due to the presence of myoglobin obscures the pattern, the analyses have been made usually at the total concentration of 0·5%. The diagrams obtained do not differ significantly from that of Fig. 5 corresponding to the white muscle; the ultracentrifugal composition (Table 4) also seems very similar (cf. Table 3), though the electrophoretic patterns of both types of myogen are very different. The presence of myoglobin in the slow peak does not increase the percentage of this fraction.

Isolation of the slow-sedimenting fraction of white muscle by ammonium sulphate fractionation and identification of some electrophoretic and ultracentrifugal peaks

It has been shown previously (Henrotte, 1955; Hamoir, 1957; Jebsen & Hamoir, 1958) that some components of this slow-sedimenting fraction are precipitated at neutral pH at between 90 and

100% saturation of ammonium sulphate. The method of fractionation based on this high salting-out range is described in the Methods section.

Fractions isolated with 0–77% *and* 77–100% *saturated ammonium sulphate.* The ultracentrifugation shows that the area of the slow-sedimenting peak amounts to 20·9 and 73·3% in the fractions isolated with 0–77% and 77–100% saturated ammonium sulphate respectively (Figs. 8*a* and 8*b*). The electrophoretic patterns of the same preparations (Figs. 9*a* and 9*b*) reveal an increased concentration of peak 4 in the 0–77% saturated ammonium sulphate fraction and of components 1, 2, 3, 5 and 8 in the 77–100% saturated ammonium sulphate fraction. These results suggest that components 2 and 5 are sedimenting slowly but that they are already partially precipitated at 77% saturation with ammonium sulphate. They amount together in the electrophoretic pattern (Fig. 9*a*) to 17·9% (ascending limb). The slow-sedimenting peak (Fig. 8*a*) corresponds to 20·9% of the total area. The agreement appears satisfactory in view of the positive error in the estimation of the slow-sedimenting material due to the Johnston–Ogston effect. As the 0–77% saturated ammonium sulphate fraction amounts to about 70% of the myogen, the slow fraction lost in this step corresponds to about $17{\cdot}9 \times 0{\cdot}7 = 12{\cdot}5\%$ of the myogen.

Refractionation of the 77–100% *saturated ammonium sulphate fraction with* 0–82% *and* 82–100% *saturated ammonium sulphate.* The first fraction (Fig. 10*a*) corresponds to about 19% of the myogen;

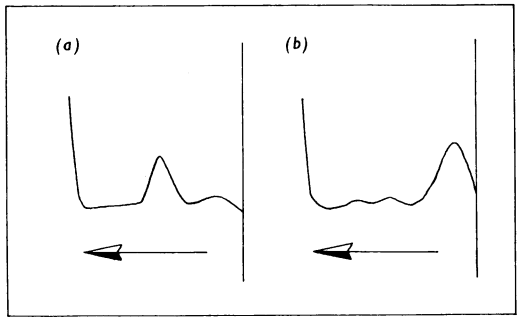

Fig. 8. Ultracentrifugal patterns of the two fractions isolated by ammonium sulphate fractionation of carp myogen (white muscle) in phosphate–NaCl buffer, pH 7·3 and I 0·075, at a protein concentration of 1% (w/v) after 64 min. at 59780 rev./min., angle 45°. (*a*) Fraction isolated with 0–77% saturated ammonium sulphate; (*b*) fraction isolated with 77–100% saturated ammonium sulphate.

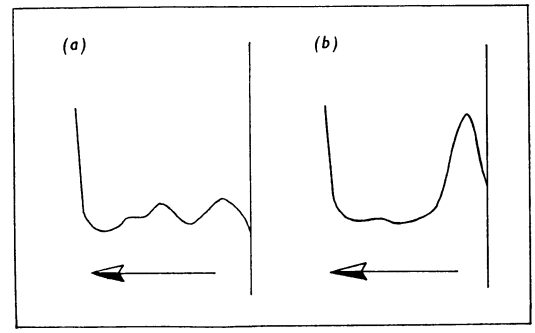

Fig. 10. Ultracentrifugal patterns of the two fractions isolated by refractionation of the 77–100% saturated ammonium sulphate fraction of Figs. 8(*b*) and 9(*b*) in phosphate–NaCl buffer, pH 7·3 and I 0·075, at a protein concentration of 1% (w/v), after 74 min. at 59780 rev./min., angle 45°. (*a*) Fraction isolated with 0–82% saturated ammonium sulphate; (*b*) fraction isolated with 82–100% saturated ammonium sulphate.

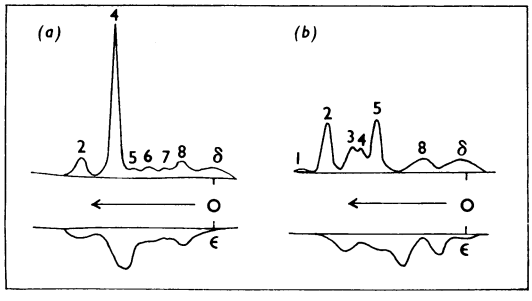

Fig. 9. Electrophoretic patterns of the two fractions isolated by ammonium sulphate fractionation of carp myogen (white muscle) in phosphate–NaCl buffer, pH 7·3 and I 0·075, at a protein concentration of 1·5% (w/v). (*a*) Fraction isolated with 0–77% saturated ammonium sulphate after 295 min. at 5·8 v/cm.; (*b*) fraction isolated with 77–100% saturated ammonium sulphate after 280 min. at 6·3 v/cm.

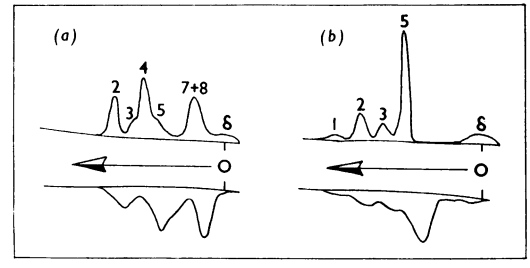

Fig. 11. Electrophoretic patterns of the two fractions isolated by refractionation of the 77–100% saturated ammonium sulphate fraction of Figs. 8 (*b*) and 9 (*b*) in phosphate–NaCl buffer, pH 7·3 and I 0·075, at a protein concentration of 1·5% (w/v). (*a*) Fraction isolated with 0–82% saturated ammonium sulphate after 300 min. at 5·7 v/cm.; (*b*) fraction isolated with 82–100% saturated ammonium sulphate after 258 min. at 5·4 v/cm.

the components sedimenting at 4·5 and 7s amount to 56·1% of the total area in Fig. 10(*a*) but to a lower value, 47·2%, in another similar experiment. Electrophoresis of this mixture (Fig. 11*a*) reveals the presence of the main components 2, 4 and 7 + 8, of a small amount of component 5 and of traces of component 3; peaks 4 and 7 + 8 are particularly large and amount together to 60·6% of the total mixture. When the two fast ultracentrifugal peaks amounted to 47·2%, peaks 4 and 7 + 8 of the corresponding electrophoretic pattern also amounted together to a lower value, 45·6%. The 82–100% saturated ammonium sulphate fraction (Fig. 10*b*) contains only 3·5% of an impurity moving at about 7s. The electrophoretic composition of the same preparation (Fig. 11*b*) is, however, fairly heterogeneous: it contains peaks 1, 2, 3, 5 and a very small amount of peak 7 or 8 corresponding to 2–3%. The higher value of 6 ± 2% for peak 1 suggests that it is a slow-sedimenting component, and the ultracentrifugal impurity at about 7s corresponds to the traces of peaks 7 or 8. The 82–100% saturated ammonium sulphate fraction represents about 12% of the myogen, or 40% of the slow-sedimenting peak if this peak corresponds to 30% of the myogen.

Correlation between the electrophoretic and ultracentrifugal analyses

Component 1 is not present in the fractions isolated with 0–77% and 0–82% saturated ammonium sulphate. It corresponds to a very soluble protein. Its amount, in the fraction isolated with 82–100% saturated ammonium sulphate, is 6 ± 2% as estimated from electrophoresis (Fig. 11*b*). On the other hand, the amount of the slow electrophoretic impurity in the same pattern is only 2–3%. This last value is nearer to that found for the fast ultracentrifugal impurity. These results suggest, as mentioned above, that component 1 sediments slowly. This last correlation, however, remains doubtful in view of the experimental error: the low-molecular-weight myokinase, present in the 82–100% saturated ammonium sulphate fraction (G. Siebert, unpublished work), should appear in the electrophoretic pattern in the region of peaks 7 or 8 if its mobility is similar to the one of rabbit myokinase (Noda & Kuby, 1957). The rate of sedimentation of component 1 therefore remains doubtful; this point is re-examined by Pechère & Focant (1965). Components 2, 3 and 5, present in large concentrations in the 82–100% saturated ammonium sulphate fraction, appear as the main components of the slow-sedimenting fraction. They differ, however, in salting-out range. Component 2 is partially precipitated by 77% saturated ammonium sulphate and is present in increased concentration in the 0–82% saturated ammonium sulphate fraction; it is the least soluble component of this group of proteins. Component 3 is absent in the fraction isolated with 0–77% saturated ammonium sulphate; the small amount present in the fraction isolated with 0–82% saturated ammonium sulphate does not separate well from peak 4; it is less soluble than component 1 but more soluble than component 2. The solubility of component 5 appears to be slightly lower than that of component 3: it occurs in small amount in the 0–77% saturated ammonium sulphate fraction and is always visible in the 0–82% saturated ammonium sulphate fraction. The slow-sedimenting peak of the myogen appears therefore to be made up of components 2, 3, 5 and perhaps 1. Peaks 2, 3 and 5 together represent (Table 1) 35·8% of the total myogen if component 3 amounts to 13% (maximal value). In view of the over-estimation of the slow-sedimenting peak due to the Johnston–Ogston effect and of the variable content of component 3, the agreement with the ultracentrifugal mean value of 32·2% (Table 3) appears satisfactory.

The median ultracentrifugal peak contains a protein, which has been crystallized, migrating in the region of peak 4 (Henrotte, 1952, 1960). In view of the homogeneity of this electrophoretic gradient (cf. Fig. 1) and of the parallelism observed between the amount of peak 4 and that of the median ultracentrifugal peak in various fractions (fractions isolated with 0–77% and 0–82% saturated ammonium sulphate), the whole peak 4 is probably sedimenting with the rate of Henrotte's (1952, 1960) crystalline protein. But, as the percentage of the median ultracentrifugal peak is still higher, about 10% of this fraction appears to be made of components migrating more slowly than peak 5 by electrophoresis.

The fast-sedimenting fraction contains glyceraldehyde phosphate dehydrogenase (Ludovicy-Bungert, 1961), which migrates with peak 7 or 8; the similarity of carp and rabbit aldolase (Th. Grandjean, unpublished work) suggests that this carp enzyme is also sedimenting at this rate. The increases of peak 8 in the fractions isolated with 77–100% and 0–82% saturated ammonium sulphate are paralleled by the enlarged fast ultracentrifugal peaks of these fractions. In the fraction isolated with 0–82% saturated ammonium sulphate, the slow electrophoretic peak corresponds to 18·5 ± 1%, whereas the fast ultracentrifugal one amounts to 14·8%. The major part of the slow electrophoretic peak 8 thus corresponds to the heterogeneous fast ultracentrifugal fraction.

Composition of the slow-sedimenting fraction of red-muscle myogen

With red muscle, the electrophoretic analysis reveals that the peaks 2, 3, 5 and perhaps 1 that form

the slow-sedimenting fraction of white muscle are absent or at most present to only a very small extent. The lack of the slow-sedimenting components of white muscle is compensated in the ultracentrifugal pattern of red muscle by the presence of myoglobin. To check this point, the method of fractionation used for the isolation of the slow-sedimenting fraction of white muscle was applied to red-muscle myogen. However, as the presence of lipids in red-muscle myogen sometimes prevents the separation in saturated ammonium sulphate, the first precipitate obtained at 100% saturation was spun down in a Spinco model L ultracentrifuge at 80000*g* for 1hr.

Fractions isolated with 0–77% *and* 77–100% *saturated ammonium sulphate.* The 0–77% saturated ammonium sulphate fraction corresponds to about 90% of the myogen instead of 70% in white-muscle myogen. This higher amount is due to the lower content of the components corresponding to peaks 2, 3 and 5 and to the fact that myoglobin is precipitated notably in this fraction, which is more coloured than the 77–100% saturated ammonium sulphate fraction. The salting-out range 75–95% saturated ammonium sulphate found for this protein by Hamoir (1955*a*,*b*) was due to the much lower protein concentration of these previous experiments. The ultracentrifugal pattern of the 0–77% saturated ammonium sulphate fraction does not differ significantly from the original one; in the electrophoretic pattern of one experiment, peak 4 increased slightly from 34·6 to 39%, whereas peak 8 decreased from 12·4 to 8·2% and the percentages of the other peaks, particularly of 6 and 7, at which region the myoglobin migrates, remained unchanged. Wider differences were, however, observed in the 77–100% saturated ammonium sulphate fraction (Figs. 12*a* and 12*b*). Some heterogeneous material sediments between the slow and the median peaks; in one case this fraction and the slow peak amounted to 60% of the total area, the median peak corresponding to only 17% and the fast one to 23%. These changes are in agreement with the electrophoretic diagram of Fig. 12(*b*), in which peak 8 corresponds to 30% of the total, peak 4 to only 14·3% and peaks 1 and 7 to 9 and 33% respectively. In another fractionation, the myoglobin was visibly more completely precipitated by 77% saturated ammonium sulphate; in the patterns of the 77–100% saturated ammonium sulphate fraction, the slow-sedimenting peak amounted to only 30% and the electrophoretic peak 7 to 15%; on the other hand, the proportion of the fast-sedimenting peak increased to 47% and the corresponding slow electrophoretic peak 8 to 45%. Although 90% of the myogen had been removed at this step, the slow-sedimenting fraction was thus still very impure. The only component the concentration of which had increased considerably was component 1, the peak of which was larger and more homogeneous than in the corresponding fraction of white muscle (cf. Fig. 9*b*).

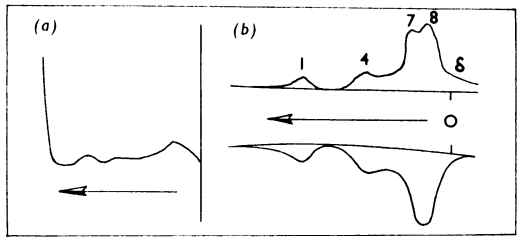

Fig. 12. (*a*) Ultracentrifugal pattern of the 77–100% saturated ammonium sulphate fraction of carp myogen (red muscle) in phosphate–NaCl buffer, pH 7·3 and *I* 0·075, at a protein concentration of 0·5% after 76min. at 59780rev./min., angle 45°; (*b*) electrophoretic pattern of the same fraction at a protein concentration of 1·5% (w/v) after 337min. at 3·62v/cm.

Refractionation of the 77–100% *saturated ammonium sulphate fraction with* 0–82% *and* 82–100% *saturated ammonium sulphate.* Three-quarters of the 77–100% saturated ammonium sulphate fraction is precipitated in the 0–82% saturated ammonium sulphate fraction; this precipitate amounts to 8–9% of the total myogen, whereas the 82–100% saturated ammonium sulphate fraction amounts to less than 3%. Although the amount precipitated up to 82% saturation with ammonium sulphate is so large, its ultracentrifugal pattern differs somewhat from that (Fig. 12*a*) relating to the 0–77% saturated ammonium sulphate fraction: the percentage of the slow ultracentrifugal peak and of the adjacent heterogeneous material decreases to 51%, whereas those of the median and fast peaks increase to 22·5 and 26·5% respectively. However, the electrophoretic diagram remains practically unchanged, except for peak 1, which decreases from 9% to about 5·5%. The amount of the 82–100% saturated ammonium sulphate fraction was too small to be examined electrophoretically. In the ultracentrifugal pattern (Fig. 13) three fractions are still present: a fast one, amounting to about one-third of the total area and sedimenting with a corrected rate of 7·5s; a heterogeneous median one (separating badly from the slow peak) which was evaluated approximately to 13%; and the slow one, with a rate of 1·7s, which accounts for about 54%. Although from the electrophoretic analysis of the 0–82% saturated ammonium sulphate fraction it can be deduced that the concentration of the peak 1 is much increased in the 82–100% saturated ammonium sulphate fraction, the persistence of fast components in the ultracentrifugal pattern of the 82–100% saturated ammonium sulphate fraction does not allow proof

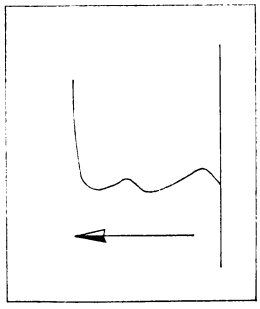

Fig. 13. Ultracentrifugal pattern of the 82–100% saturated ammonium sulphate fraction isolated after refractionation of the 77–100% saturated ammonium sulphate fraction of Fig. 12 in phosphate–NaCl buffer, pH 7·3 and I 0·075, at a protein concentration of 0·5% (w/v) after 60 min. at 59780 rev./min., angle 45°.

that peak 1 is a slow-sedimenting protein. The slow peak of the 82–100% saturated ammonium sulphate fraction represents only 1·3% of the myogen of red muscle instead of 12% in white muscle. The red muscle thus does not contain components 2, 3 and 5 of white muscle but appears to contain the small component 1.

DISCUSSION

The electrophoretic analysis of the myogens of carp white and red muscle separates eight and nine peaks respectively at pH 7·3 and I 0·075, instead of six and five as observed previously at pH 7·1 and I 0·15 (Hamoir, 1955*a*,*b*; Henrotte, 1960). Lowering of the pH to 5 or 6 does not increase the number of peaks, though this effect does occur with muscle extracts of post-rigor cod (Dingle *et al.* 1955). The presence of the electrophoretic components described above therefore appears to be fairly constant under the conditions of our experiments. However, in view of the large influence of the buffer used on such electrophoretic patterns (Moreau-Collinet & Hamoir, 1961), a further splitting of the somewhat heterogeneous slow-moving peaks might be possible under other ionic conditions.

The general patterns for white and red muscles show a high mean electrophoretic mobility that differentiates the fish muscle extracts of low ionic strength from those of warm-blooded vertebrates (Hamoir, 1951, 1955*a*,*b*, 1962; Connell, 1953*a*,*b*). The difference is much more pronounced with white muscle. This myogen, indeed, contains proteins of high mobility at neutral pH (components 2, 3 and 5) that separate well from the other components at pH 5 or 6 and are apparently absent from red muscle. Component 4 is the only main component of the red-muscle extracts contributing to their higher mean mobility.

The pH–mobility curves (Fig. 4) show that the components 2, 3 and 5 of white muscle have isoelectric points at or below 4·5 and only a small buffering action in the pH range 6–7·3, suggesting a low content in histidine. This result is in agreement with data on the amino acid compositions of the components 3 (Henrotte, 1955, 1960; Hamoir, 1962) and 5 (Hamoir, 1962) (see also Konosu, Hamoir & Pechère, 1965). The similar electrophoretic behaviour of components 2, 3 and 5 and the comparison of the total percentage of these electrophoretic peaks with that of the slow ultracentrifugal peak suggest that they form the slow-sedimenting fraction of the white-muscle myogen. The general occurrence of this slow-sedimenting fraction in fish muscle extracts (Jebsen & Hamoir, 1958; Connell, 1958), the presence of a fast electrophoretic peak of similar amino acid composition in plaice (Jebsen & Hamoir, 1958) and the occurrence in cod muscle extracts of low ionic strength of a major component with a mobility that barely varies between pH 7·4 and 6 suggest that we are dealing with a group of proteins characteristic of fish white muscle.

These components with a low rate of sedimentation are isolated in fairly good yield by ammonium sulphate fractionation on account of their high salting-out range. The nearly pure ultracentrifugal preparation obtained from white-muscle myogen contains large proportions of components 2, 3 and 5, which are the major components of this group of proteins. The same method of fractionation, when applied to red-muscle myogen, gives strikingly different results, confirming the absence there of components 2, 3 and 5. It shows, however, that component 1 is also present in small amount in this tissue, and is perhaps part of the slow-sedimenting peak in view of its high solubility. These results bring to light a new difference between white and red muscles of fish: the proteins of low molecular weight and abnormal amino acid compositions that represent about 30% of the albumins of carp white muscle are practically or completely absent (with the possible exception of the small component 1) in red muscle, which contains instead a comparable amount of myoglobin.

The study of mammalian and avian red muscle has shown that the development of the cytochrome system increases with the myoglobin content (Lawrie, 1950, 1953*a*,*b*). Fish red muscle is in fact characterized by a higher content of respiratory enzymes (Umemura, 1951*a*,*b*) and of succinate dehydrogenase (Fukuda, 1958) than white muscle, and by a much increased ability to oxidize fatty acids (Bilinski, 1963). The high value suggested by our results for myoglobin in carp red muscle indicates that the cytochrome system must be much

more developed in this muscle than was supposed hitherto. The comparison of the mode of life of various species of fish and that of the development of white and red muscle (Boddeke *et al.* 1959), as well as that of the physiological behaviour of both kinds of muscle (Barets, 1961), suggest that the white muscle is particularly suited to store and produce 'energy-rich' phosphate anaerobically. However, the fraction isolated from white muscle by 82–100% saturation with ammonium sulphate, when tested for various enzymic activities, gave negative or nearly negative results for lactate dehydrogenase, α-glycerophosphate dehydrogenase, pyruvate kinase, enolase, phosphoglycerate kinase, glyceraldehyde phosphate dehydrogenase, aldolase, malate dehydrogenase and cathepsin. Myokinase was the only enzyme the activity of which was not negligible (G. Siebert, unpublished work; Pechère & Focant, 1965). The biological function of these small molecules thus remains obscure.

Peak 4 is present in both kinds of muscle at about the same concentration. Its homogeneity suggests that it corresponds entirely to Henrotte's (1952, 1960) crystalline component with a molecular weight of 67000. It is the main component of the median ultracentrifugal peak. At pH 7·3 and I 0·075 it moves 2·2 times as fast as the fastest main component 1 of rabbit myogen (Moreau-Collinet & Hamoir, 1961). Its mobility decreases normally at lower pH, in agreement with its content of histidine (Henrotte, 1960). Its isoelectric point is 5·4, whereas that of rabbit component 1 was evaluated to be about 6·5 under our experimental conditions (Moreau-Collinet & Hamoir, 1961). A similar component has been observed in cod myogen (peak 5) (Connell, 1953*a*; Dingle *et al.* 1955) and in plaice myogen (peak 4) (Jebsen & Hamoir, 1958; Henrotte, 1960). This fraction appears to be typical of fish myogen, but its biological function has not yet been determined.

The electrophoretic mobilities of peaks 7 and 8 do not differ from those of the main components 1 and m+n respectively of rabbit myogen. They contain the carp's glyceraldehyde phosphate dehydrogenase (Ludovicy-Bungert, 1961) and aldolase (Th. Grandjean, unpublished work), which migrate in the U-tube in a manner similar to that of the corresponding rabbit enzymes. On the other hand, the major part of the slow electrophoretic peak 8 sediments at 7–8 s, as do several glycolytic enzymes that represent an important fraction of the myogen. It seems therefore that the slow components 7 and 8 correspond, as in rabbit muscle, to the main glycolytic enzymes.

The subdivision of fish myogen into three groups of protein suggested by previous results raises again the question of the possible occurrence in myogen of proteins devoid of enzymic activity. It is generally assumed that the entire myogen consists of enzymes (Mommaerts, 1950; Bailey, 1954; Perry, 1960). According to Czok & Bücher (1960), however, the 15 muscle enzymes crystallized so far correspond respectively to only 66 and 44% respectively of the myogen of the rabbit white and red muscles. Thus, in spite of the high specialization of the muscle tissue, no enzymic activity can yet be assigned to a notable portion of rabbit myogens. The study of fish muscle shows that in such a calculation account should be taken of the notable lipid metabolism of the red muscle, and that a large inactive fraction probably occurs in the white muscle of the cold-blooded vertebrates at least. Clearer evidence in support of this opinion is given by Konosu *et al.* (1965) and Pechère & Focant (1965).

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