The Electrophoretic Analysis of Protein Extracts from Striated Rabbit Muscle

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(Received 15 May 1946)

The electrophoresis method of Tiselius has been used only in a few instances for the study of muscle proteins. The purification of zymohexase has been followed by Bate Smith (1940), and Bailey (1942) has described experiments on myosin, which has been studied in more detail by Ziff & Moore (1944). Extracts of striated muscle from the rat and octopus have been examined electrophoretically by Mehl & Sexton (1942).

In an earlier paper (Dubuisson & Jacob, 1945*b*) the analysis of well-defined extracts from fatigued and resting frog striated muscle was described, but generally the characteristics of muscle proteins of mammals alone are relatively well established (Dubuisson, 1945). Further, electrophoretic examination reveals marked qualitative and quantitative differences between rabbit- and frog-muscle proteins. Consequently it was considered important to make a careful study of the muscle proteins of a mammal—the rabbit—with a view to defining accurately the characteristic properties of each of the main electrophoretic components.

METHODS

The electrophoresis apparatus used was that of Tiselius (1937), modified slightly (Dubuisson & Jacob, 1945*a*), the refractive index gradients being registered by the method of Longworth (1939).

The preparation of muscle extracts. Rabbits were killed, bled and skinned, and immersed in ice-water for about 15 min. This causes an intense vasoconstriction which expels the blood almost quantitatively from the muscle, since no haemoglobin or plasma protein can be detected electrophoretically in the muscle extract (Jacob, 1945). The muscles were then rapidly dissected and ground in a cooled Latapie mincer. The pulp was mixed with 0.8 vol. of cold solvent and extracted with continuous mechanical stirring for an hour at 0°. The tissue debris was spun down for 5 min. at 6000 r.p.m. and 0°, the supernatant fluid removed and dialyzed in collodion tubes (8 cm. long, 1 cm. diam.) against a large volume of buffer (2 l./30 ml. extract). During the dialysis, of at least 24 hr. duration, the outer fluid was twice renewed and the collodion tube and diffusate were kept slowly moving. The last diffusate was used as supernatant fluid for the electrophoresis.

The extraction and dialysis fluids have been varied as required. In the first instance the results obtained using the following solutions will be described: extraction fluid 0.048M- Na_2HPO_4 -0.006M- NaH_2PO_4 , pH 7.70, $\mu=0.15$;

dialysis fluid Na_2HPO_4 - NaH_2PO_4 ($\mu=0.1$)+0.05M- NaCl (total $\mu=0.15$), pH varied from 5.38 to 8.04. An ionic strength of $\mu=0.15$ allows relatively rapid electrophoresis without disturbing anomalies. For the extraction, pH 7.7 is optimal for all muscle proteins within the phosphate buffer range. Under these conditions the dialyzed extracts are deficient in the muscle proteins soluble only in phosphate buffers of higher ionic strength.

At all pH values a precipitate formed on dialysis which was removed both during and at the termination of dialysis by centrifuging. The quantity of precipitate varied considerably above pH 7.8, was least at pH 7.6, and became more abundant as the pH fell. The precipitates which formed above pH 7.1 were soluble in 0.5M-KCl, and these solutions showed double refraction of flow.

In a second series of experiments the ionic strengths of the extraction fluid and of the dialyzing medium were varied, and the composition is specified for each individual case. The collodion tubes were not always completely impermeable to protein, but the amount of protein which occasionally passed through the membrane was very small. Cellophan tubes were not available. No antiseptics were used, but the normal precautions against denaturation were taken. Even at 0° the extracts were stable for a very short time, the stability decreasing with pH. Accordingly, the electrophoresis experiments were made within 2-5 days after extraction. If a longer time was allowed to elapse, changes occurred in the diagrams. The descriptions here are confined to normal diagrams.

RESULTS

I. EXPERIMENTS AT CONSTANT IONIC STRENGTH

General description of diagrams

It is necessary first to summarize briefly the results already published (Jacob, 1945). At all pH values the electrophoretic diagrams show three groups of constituents (Figs. 1, 2). Group I, which accounts on the average for 72% of the refractive increment of the extracts, is the most important, and consists of those proteins with the highest isoelectric points falling in the range pH 6.0-6.75. Group II, representing 16.8% of the refractive increment, has isoelectric points in the region pH 5.0-5.5, while group III (5% to 8.6%) always has a considerable anodic mobility in phosphate buffers. The diagrams obtained between pH 7.4 and 8.0 (Fig. 1*a, b*) are the most complete. Group III separates first from the initial boundary after about 1 hr. electrophoresis at 4-5 V./cm., and consists of two peaks (*h* and *i*) which

Fig. 1a

Fig. 1b.

Fig. 1. Electrophoretic patterns (anodic) of extracts from perfused skeletal muscles of rabbit. Exp. 38: ionic strength, 0.15; pH 7.52; electrical field, 4.58 V./cm.

Durations of electrophoresis: (a) 11,100 sec. (the boundary I is still unique and too sharp to be recorded completely); (b) 34,080 sec. (the faster moving boundaries have left the photographic field). Arrows indicate the direction of migration from the position of the initial boundary quoted as 0. Convection means convection disturbances (see p. 89).

Fig. 2a

Fig. 2b.

Fig. 2. Modifications in the electrophoretic patterns related to pH changes at a constant ionic strength of 0.15. (a) Exp. 68 (anodic): pH 6.60; electrical field, 4.95 V./cm.; duration of electrophoresis, 13,800 sec. (b) Exp. 30: pH 6.30; electrical field, 4.79 V./cm.; duration of electrophoresis, 17,400 sec. (for this figure, the rate of motion of the schlieren diaphragm to that of the photographic plate is 1/14 instead of 1/20 as for the other patterns).

separate more completely from one another, the higher the pH. There may possibly be a peak faster than *h*, but owing to the small amount present and the wide spread of the peak, its estimation is outside the limits of accuracy of the method.

Group II separates after 2 hr. at 4–5 V./cm., and between it and group III is a broad band accounting for 2.6% of the refractive increment. The upper border of this band (*sp*) is almost parallel to the base-line. Group II consists of two bands, *j* the faster and smaller, *k* the slower and larger. Peak *j* occasionally resolves into two peaks, and *k* usually

the pH falls, whilst *k* and *j* resolve better than at higher pH values. Group III is apparently a single band.

The movement of the turbidity of the extracts can be followed by the more or less abrupt changes in blackening of the prints along the axis of migration. In general, part of the turbidity migrated with group III and at pH 8 and 7.6 entirely with *i*. Other changes in the blackening occurred at the peak of *sp* and group II, and in the cathode limb there was frequently a progressive increase in opacity from *i* to *k*.

Fig. 3. Mobility-pH curves for the chief constituents of rabbit skeletal muscle, at ionic strength 0.15

($\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4 + 0.05\text{M-NaCl}$).

■ = <i>n</i>	○ = <i>k</i> ₁	□ = <i>i</i>
× = <i>m</i>	△ = <i>k</i> ₂	+ = <i>h</i>
● = <i>l</i>	○ = <i>j</i>	

resolves into two peaks of approximately equal area of which the slower, *k*₁, migrates with the initial velocity of *k*.

Group I eventually resolves into several components of which *l*, the most electronegative, separates first, whilst *m* and *n* separate only after prolonged electrophoresis, the time required increasing with the pH. Between pH 7.4 and 6.5 (Fig. 2*a*) the three constituents of group I separate easily and completely, group II shows unaltered behaviour and the subdivision of group III becomes obscure. From pH 6.50 to 5.38 (Fig. 2*b*) constituents *l* and *m* of group I separate with greater difficulty as

The mobilities of the principal components plotted in Fig. 3 are more accurate and complete than those of a preliminary publication (Jacob, 1945). The main objections which can be raised against the determination of the mobilities of components in a complex protein mixture will be discussed later.

The results of the measurements showing the contribution of the various constituents to the total refractive increment are given in Tables 1 and 2. Table 1 demonstrates that over a limited pH range the total nitrogen content of extracts prepared by identical methods can vary by 20%, without

Table 1. Concentration measurements pH 7.10-7.8 ($\mu=0.15$)

Exp. no.	Rabbit no.	pH	Total N (mg./ml.)	Percentage amounts of constituents*							
				<i>n</i>	<i>m</i>	<i>l</i>	<i>k</i> ₁	<i>k</i> ₂	<i>j</i>	<i>sp</i>	<i>i</i> <i>h</i>
69	29	7.87	2.61†	47.5		25	11		4.5	—	—
67	29	7.85	4.29	48		22.5	8	6.5	3	2	10
23	8	7.71	5.00	50		21		13.5	5	2	4 4.5
35	12	7.70	?	45.5		27.5		11.5	3.5	—	—
21	8	7.68	4.97	48		22	8	6	4	3	3 6
1	1	7.60	4.12	48		25	9	3.5	3	2	3 6.5
38	14	7.53	3.24†	45.5		27		13.5	—	—	—
36	13	7.46	4.79	47		23		14	—	—	—
15	6	7.14	4.31	16.5	33.5	23		13	4	2	8
12	5	7.10	3.16	15	32	25		11.5	5	2.5	9
Arithmetic mean (<i>m</i>)‡				47.5		24		13.0	4	2.25	8.50
Standard deviation (S.D.)§				1.60		2.14		1.22	0.8	0.44	0.70
(S.D. × 100)/ <i>m</i>				3.4		8.9		9.3	20	19	8.2

* The values are practically independent of the δ and Svensson effects (p. 90).

† Corrected nitrogen value.

‡ Values accurate to 0.25.

§ Standard deviations given by $S.D. = \sqrt{\frac{\sum d^2}{n-1}}$.

Table 2. Variation of composition as a function of pH

Exp. no.	pH	Percentage amounts of constituents							
		<i>n</i>	<i>m</i>	<i>l</i>	<i>k</i> ₁	<i>k</i> ₂	<i>j</i>	<i>sp</i>	<i>i</i> <i>h</i>
6	8.02	38		27		12		7.5	3.5 6 6
Experiments from Table 1*	7.10-7.87	43.5-51.5		18.5-29.5	10-16	2-6	1.15-3.35	6.75-10.25	
	34	48		25	11.5	5.5	2	8	
3	6.75	15	33	25	10	3	4.5	2.5	7
68	6.60	15	30	29	9.5	3	4.5	2	7
17	6.53	15	29	27	14		5.5	2	7.5
18	6.40	17	29	25.5	8.5	4.5	6	2	7.5
37	6.30	18.5	54		8	5.5	4.5	3	6.5
83	6.13	19	55		12		5	3	6
82	6.04	17	55.5†		6	5.5	7	2.5	6.5
81	5.67	18.5	53.5		15		5.5	2	5.5

* The relative compositions given are the maximum and minimum statistical values ($m \pm 2.5$ S.D.).† Only experiment corrected for δ effect.

alteration of the relative quantities of the various constituents. Variations of 20 % in the total concentration of extracts cannot be accounted for by such experimental variations as loss of protein through the collodion membranes, osmotic transfer of water during dialysis (Longworth, Curtis & Pembroke, 1945), or by accidental dilution during washings. They are more probably attributable to physiological variations in the amounts of extractable protein in different rabbits. It appears that they are related to differences in the imbibing power of pulped muscles. The error of the measurements of the relative concentrations is shown by the standard deviations given in the last column but one of Table 1. The method of extrapolation used in analyzing the curves (Tiselius & Kabat, 1939) and the uncertainty in the determination of the base-line position

(=0.1 mm.) account partly for the order of the standard deviation, but the rest must be due to physiological variation.

The standard deviation for *l* seems very high, but for this peak the perpendicular method of extrapolation is peculiarly unsuited, since (see below) between *l* and *m*, and *l* and *k*, are components obscured at $\mu=0.15$. The use of the analytical method described by Svedberg & Pedersen (1940) would not in this case have resulted in much improvement. However, various measurements on the diagram from one experiment have shown that this factor does not account for the great variability of *l*. It would appear that for this protein the physiological variations, or the losses through the membrane, are the greatest.

In Table 2 the relative concentrations of the components are shown as a function of pH. Owing to the variation in total nitrogen of the extracts it is

not possible to compare the absolute concentrations of the components. An approximate comparison is possible for extracts from the same rabbit dialyzed at various pH values. Direct measurements have indicated that the total refractive increment and total protein nitrogen decreases on either side of the range pH 7.4–7.7. That a maximum concentration is found in this region is in agreement with the minimal precipitation observed during dialysis. The relative decrease of $m+n$ at pH 8 is due to a decrease in solubility of one of these two proteins or of a complex formed by them. Similarly the relative reduction of group III at the more acid pH values indicates a loss of solubility, probably of a progressive nature, in this group. It is not possible to establish beyond doubt whether i alone finally disappears completely or whether a complex of i and h is partially precipitated, but the former is considered more probable, as will be shown in a further publication concerned with experiments at lower pH values.

Finally, the fairly constant relative values found for other proteins as the pH decreases suggests a lowering of their absolute concentrations. It is impossible to distinguish between loss of solubility and co-precipitation as a cause of this phenomenon.

II. EXPERIMENTS WITH VARYING IONIC STRENGTH

The classical muscle proteins myogen, globulin X, myosin and myoalbumin may be characterized by their differing solubilities at various ionic strengths. It seemed necessary to discover to what extent the electrophoretic diagrams are modified by variation of the ionic strength of the dialysis fluid or the extraction fluid or both together. However, such experiments reveal new problems rather than clarifying the original ones, on account of the complications of interaction between proteins such as co-precipitation and mutual effects on solubility. In this investigation no more than general information of a summary character is presented.

The mobilities have been found to vary with ionic strength, but the data obtained are insufficient to warrant publication here. The measurements of relative concentration are given in Table 3.

Decrease of ionic strength

Dialysis fluid. In order to prevent the appearance of disturbing anomalies, the lowest ionic strength used was $\mu = 0.10$, and this provides better conditions for electrophoretic separation. Thus, it is possible to resolve group I effectually (Fig. 4). After a 5 hr. electrophoresis at 7 V./cm., a peak s appears between k_1 and l in the anode limb, and after 7 hr. a further peak t separates between l and m . The total concentration of these extracts is about 80% of the corresponding extract at $\mu = 0.15$. At corresponding pH values the relative concentrations of the constituents remain unchanged, so that a slight proportional precipitation of all proteins occurs.

Extraction fluid. If the muscle pulp is extracted with distilled water, the ionic strength of the undialyzed extract is approximately $\mu = 0.10$, since for muscle juice $\mu = 0.21-0.26$ (Dubuisson, 1942). Rather than use large volumes of distilled water for the extraction, the ionic strength has been reduced by dialyzing the normal extract, containing about 4.5 mg. protein-N/ml. against a large volume of distilled water frequently renewed. After 4 hr. dialysis a copious precipitate was removed, a second of appreciable size after 16 hr., but after 24 hr. nothing further separated on centrifuging for 15 min. at 6000 r.p.m. The yellowish, slightly opalescent solution was dialyzed for 48 hr. against phosphate buffer pH 7.48, $\mu = 0.15$, and contained only 2.1 mg. protein-N/ml. The electrophoresis diagrams resemble those of the ordinary extracts except that i is absent and the presence of k_2 dubious (Table 3, Exp. 51). Therefore except for i and perhaps k_2 all the constituents of the normal extracts are at least partially mutually soluble in water. The disappearance of i and k_2 would account for the loss of a maximum of 0.45 mg. protein-N/ml., but since the total loss is 2.4 mg. N/ml. the other proteins must also have been partially precipitated but in varying proportions, h the least and k_1 perhaps not at all. These facts agree with the observations of Raffel, Pait & Terry (1940) and Svensson (1941) on the electrophoretic diagrams of the euglobulins and pseudoglobulins of blood serum.

Table 3. *Concentration measurements at different ionic strengths*

Exp. no.	Ionic strength of extraction liquid	Ionic strength of dialysis liquid	pH	Percentage amounts of constituents*					
				$n+m$	l	k_1+k_2	j	sp	$i+h$
Series									
Table 1	0.15	0.15	7.10–7.87	43.5–51.5	18.5–29.5	10–16	2–6	1.15–3.35	6.75–10.25
28	0.15	0.10	7.18	51.5	19.5	13	6	2.5	7.5
32	0.15	0.10	6.83	45	26.5	12.5	5	3	8
51	0.15	0.00	7.48	43.5	28	14? 0?	3.5	3	0 8
48	0.15	0.27	7.41	43	26	12	3.5	4	11.5
60	± 0.5	0.15	6.70	44.5	27.5	13.5	5	2	7.5

See note *, Table 1.

Increase of ionic strength

Dialysis fluid. It has been noted above that when an ordinary extract is dialyzed against phosphate buffer pH 7·7·6, $\mu = 0\cdot15$, a precipitate forms which is soluble in 0·5M-KCl, the solution showing double refraction of flow. This material remains in solution when the extract is dialyzed against phosphate buffer pH 7·41, $\mu = 0\cdot27$. The dialyzed solution is much more opalescent than normal, but no material deposits on centrifuging for 15 min. at 6000 r.p.m. Electrophoresis, which is slow on account of the high ionic strength, resolves group III into three components (Fig. 5) which represent 11·5 % of the total proteins (Table 3, Exp. 48). The opalescence migrates in the anode limb with the two slower components, in the cathode with the slowest of the three peaks.

Fig. 4. Complexity of group I. Anodic pattern of a prolonged electrophoresis at ionic strength 0·10. Exp. 32: pH 6·83; electrical field, 7·20 V./cm.; duration of electrophoresis, 26,110 sec. The faster moving boundaries have left the photographic field. The base-line is distorted owing to optical imperfection of the electrophoretic cell used in this particular experiment.

Extraction fluid. Extractions at ionic strengths about $\mu = 0\cdot5$, which are used for isolating the myosins of Edsall (Edsall, 1939, Table 3, Exp. 60) and of Banga & Szent-Györgyi (1942), after dialysis at pH 6·75 and $\mu = 0\cdot15$ give diagrams similar to those of normal extracts, both qualitatively and quantitatively. The analysis of the curves is difficult because of the low protein content, 1·542 mg. N/ml. for Exp. 60, and 0·882 mg. for Banga-Szent-Györgyi extracts. The physico-chemical and biological problems which these experiments present should be examined under more favourable conditions.

Extraction and dialysis fluids. Extraction fluid: 0·048M- Na_2HPO_4 , 0·006M- NaH_2PO_4 , 0·2M-NaCl, $\mu = 0\cdot35$, pH = 7·45. Dialysis fluid: 0·032M- Na_2HPO_4 , 0·004M- NaH_2PO_4 , 0·25M-NaCl, $\mu = 0\cdot35$, pH = 7·45.

These dialyzed extracts are very turbid and the electrophoresis diagram markedly altered (Fig. 6), but groups I, II and III are distinguishable. The component *sp* is present in much larger proportion

than at lower ionic strengths, for a comparable migration and height of group III, and completely masks *i*. Group II is atypical in appearance and is characterized by a very sharp peak in the position usually occupied by *j*. In the anode limb the turbidity coincides precisely with this peak, whilst in the cathode limb it is situated between *i* and *k*. Other less well-defined asymmetries occur and relative concentration measurements were impracticable.

Fig. 5. Emergence of a new constituent in group III in muscle extracted at an ionic strength of 0·15 but dialyzed at an ionic strength of 0·27. Exp. 48: anodic pattern; pH 7·41; electrical field, 4·12 V./cm.; duration of electrophoresis, 13,860 sec.

Increasing the ionic strengths of the extraction and dialysis fluids further involves electrophoresis runs of 24 hr. duration at least, which involves increased risks with respect to convection and diffusion effects. For the present a limit of ionic strength $\mu = 0\cdot35$ has been imposed.

Experiments at various pH values with acetate buffers as extractants have been made; owing to the buffering capacity of the pulp, these have up to the present merely been confirmatory of the results obtained on the effect of the pH of the dialysis fluid.

III. TECHNIQUE OF ANALYSIS OF THE DIAGRAMS

In order to define the physico-chemical limitations of the moving-boundary method, the effect of errors resulting from thermal convection, the configurational asymmetries between ascending and descending boundaries and the effect of various factors on mobility, have been experimentally examined. The main theoretical and experimental developments will be found in the papers of Tiselius (1930, 1937), Kekwick (1940), Longworth (1941, 1942), MacInnes & Longworth (1944), and Overbeek (1943), and only a brief discussion is necessary here.

peak preceding h , sp and δ , or ϵ . In the first of these, convections occur only in the cathode limb, irregularly at $\mu = 0.15$ and more frequently at higher ionic strengths (Fig. 6). After 2–3 hr. electrophoresis, sp is affected only on the cathode side; after 6–7 hr. δ becomes deformed (Figs. 1b, 4), and ϵ is never changed. All these convections disappear if the current is cut off for 1–1½ hr. It follows that the appearance of convection disturbances is not solely due to small density gradients, but a contributory condition is that the gradient should occur in a region of relatively high protein concentration, the viscosity of which opposes the dispersion of the convection by normal diffusion. Practically, these

Fig. 6a.

Fig. 6b.

Fig. 6. Appearance of sp and group II in muscular extracts obtained and dialyzed at an ionic strength of 0.35; (a) anodic; (b) cathodic. Exp. 74: pH 7.45; electrical field, 1.75 V./cm.; duration of electrophoresis, 29,000 sec. Convection means convection disturbances (see p. 89).

Thermal convection

When the vertical gradient of density, the principal stabilizing factor in the moving boundary method, is very small, convection is unavoidable (Shedlovsky & Smadel, 1940; Longworth, 1942). Considering the present extracts, there are three regions in which low-density gradients may develop after varying periods of electrophoresis; the broad

disturbances are not of serious importance, and do not influence the mobility of adjacent components.

Asymmetry between ascending and descending boundaries

The main asymmetries are (Longworth & MacInnes, 1940): (a) the δ and ϵ effects, (b) the sharper form and faster migration of the ascending boundary.

(a) The δ and ϵ effects will only be considered at $\mu=0.15$ (Table 4). Above pH 7.1 these boundaries separate satisfactorily from the protein boundaries, and agreement between the percentages of the components in both limbs is obtained by excluding δ and ϵ (Table 4). The effect of ϵ is negligible and will not be further considered.

peaks, an almost isoelectric iso- δ peak and descending peaks. The ascending peaks, cathode I and anode *j*, *sp*, III, are decreased by the partial δ peak, and the iso- δ peak is increased by the sum of these effects which is greater in the cathode limb, since I accounts for 72 % of the total protein, than in the anode limb, where *j*, *sp*, and III together account for

Table 4. Comparison of constituents in anode and cathode limbs

Exp. no.	pH	mg. N/ml.	Group I		<i>k</i>		<i>n+m</i>		<i>l</i>	
			Anode	Cathode	Anode	Cathode	Anode	Cathode	Anode	Cathode
69	7.87	2.612	72.5	72.5	11	11	47.5	44	25	28.5
67	7.85	4.294	70.5	73	14.5	12	48	48	22.5	25
21	7.68	4.974	70	72.5	14	15	48	50	22	22.5
1	7.60	4.120	73	72	12.5	14	48	—	25	—
							<i>n</i>		<i>m</i>	
							Anode	Cathode	Anode	Cathode
15	7.14	4.318	73	73	13	12	16.5	—	33.5	—
12	7.10	3.160	72	73.5	11.5	11	15	13	32	30
3	6.75	3.320	73	72.5	13	11.5	15	19.5	33	21.5
60	6.70	1.542	72	74	13.5	11	14.5	15.5	30	17.5
68	6.60	3.472	74	73	12.5	11.5	15	15	30	29
									<i>m+l</i>	
									Anode	Cathode
37	6.30	3.092	72.5	73.5	13.5	14.5	18.5	18.5	54	55
83	6.13	?	74	73	12	12.5	19	18	55	54.5
82	6.04	?	74.5	66.5	11.5	16.5	17	15.5	57.5	51
81	5.67	?	72	63	15	24	18.5	16.5	53.5	46.5

In the pH range studied, the anodic and cathodic percentages of components *j*, *sp* and III are concordant within experimental error.

Below pH 7.14, the δ effect merges with the protein diagram, and to simplify the problem, consider first the diagrams in which group I is still represented by a single peak (Table 4, left-hand side). Close to pH 7.10, the δ boundary merges partially with the anode band of group I, so that a portion of it can be excluded. From pH 6.75 to 6.13 it is completely masked by group I (anodic), but the anode and cathode percentages of the constituents of this group are in agreement. Group I is therefore increased on the anode side by the effect, the magnitude of which is less than possible experimental error. As a first approximation this δ effect is caused by group II, *sp* and group III, that is, 28 % of the total protein of the extract, and is accordingly of little significance theoretically (Longworth, 1942), and neither is the error introduced in the estimates of II, *sp* and III.

From pH 6.04 to 5.67 an important δ effect interferes increasingly with the band *k*, migrating to the cathode, and the disagreement in the analyses is considerable.

The values obtained at pH 5.67 can be accounted for quantitatively if the anode and cathode diagrams are each considered as mixed, containing ascending

only 13 % of the total protein. The descending peaks are subject only to a negligible correction. At pH 6.04 the same argument can be applied taking *l* and *k* to be iso- δ boundaries.

Considering the asymmetries which exist within group I, no difficulties arise above pH 7.14 or below pH 6.13. Within the range pH 6.13–7.14 the situation is complex, and it seems necessary to consider the possible occurrence of protein interaction similar to that found for egg-white (Longworth, Cannan & MacInnes, 1940), and for ovalbumin-yeast nucleic acid mixtures (Longworth & MacInnes, 1942). It must be pointed out that at certain pH values the iso- δ boundaries can be different in the anode and cathode limbs because the conditions governing the migration of the δ boundary are not identical in the two limbs.

The Svensson effect (Svensson, 1943) revealed in the asymmetry of leading peaks has no measurable effect on the data obtained at $\mu=0.15$, since the percentage composition obtained at this ionic strength has been independent of the total protein concentration (Table 4, 69–60). Further, the composition does not change if the ionic strength is varied, provided that those proteins insoluble at

$\mu = 0.15$ have been precipitated previously during preliminary dialysis. There is no appreciable Svensson effect because the rapidly migrating components are present at low concentrations, and consequently modify very slightly the solution through which they pass and are not themselves affected by other gradients.

(b) The ascending peaks are generally better separated and sharper than the descending except for h which is more sharp in the cathode than anode; but this may be a viscosity effect. In Table 5 are

of different bands during the course of a single experiment, or in comparing the results of different experiments.

In the first case the relevant errors which occur are: (1) in the calibration of each compartment of the cell; (2) in the determination of the position of the peak (this error can vary according to the peak considered, and is a function of duration and voltage) and (3) in the compression of the vaseline at the lower plate joint which causes a negligible increase of 'classical' asymmetry (see Table 6).

Table 5. Comparison of apparent mobilities of ascending and descending boundaries for three constituents

Exp. no.	pH	mg. N/ml.	$u \times 10^5$								
			h		A*	k_1			l		
			Asc.	Desc.		Asc.	Desc.		Asc.	Desc.	
6	8.02	3.90	-6.38	-6.26	A*	-2.31	-2.32	—	-1.48	-1.44	—
4	7.96	2.99	-6.30	-6.32	—	-2.23	-2.23	—	-1.45	-1.35	A
35	7.70	?	-5.72	-5.72	—	-2.30	-2.18	A	-1.53	-1.39	A
21	7.68	4.97	-5.92	-5.94	—	-2.29	-2.23	—	-1.48	-1.41	—
22	7.63	5.01	-5.86	-5.80	—	-2.16	-2.03	A	-1.43	-1.28	A
1	7.60	4.12	-5.70	-5.65	—	-2.18	-2.18	—	-1.35	-1.25	A
41	7.60	2.00	-5.87	-5.80	—	-2.25	-2.14	A	-1.41	-1.31	A
2	7.58	3.15	-5.58	-5.60	—	-2.09	-2.04	—	-1.26	-1.23	—
24	7.57	5.17	-5.80	-5.60	A	-2.24	-1.98	A	-1.48	-1.33	A
38	7.53	3.25	-5.57	-5.62	—	-2.13	-1.95	A	-1.43	-1.30	A
42	7.51	1.21	-5.83	-5.63	A	-2.05	-2.03	—	-1.34	-1.34	—
40	7.50	2.83	-5.74	-5.70	—	-2.25	-2.13	A	-1.43	-1.32	A
51	7.48	?	-5.64	-5.67	—	-2.10	-2.09	—	-1.35	-1.25	A
36	7.46	4.79	-5.43	-5.47	—	-2.04	-1.87	A	-1.37	-1.18	A
50	7.42	?	-5.75	-5.45	A	-2.12	-1.92	A	-1.43	-1.23	A
15	7.14	4.30	-4.90	-4.89	—	-1.78	-1.80	—	-0.95	-0.95	—
12	7.10	3.16	-4.87	-4.89	—	-1.78	-1.68	A	-0.93	-0.79	A
3	6.75	3.32	-4.50	-4.58	—	-1.49	-1.51	—	-0.75	-0.73	—
10	6.75	2.42	-4.93	-4.73	A	-1.64	-1.50	A	-0.87	-0.75	A
11	6.75	2.12	-4.65	-4.60	—	-1.47	-1.43	—	-0.68	-0.68	—
60	6.70	1.54	-4.62	-4.56	—	-1.41	-1.40	—	-0.77	-0.65	A
17	6.52	4.60	-4.38	-4.36	—	-1.36	-1.26	A	-0.70	-0.60	A
18	6.49	4.34	-4.44	-4.42	—	-1.34	-1.27	—	-0.70	-0.60	A
37†	6.30	3.09	-4.18	-4.20	—	-1.11	-1.10	—	-0.53	-0.51	—
83†	6.13	?	-4.08	-4.12	—	-0.85	-0.81	—	-0.05	-0.06	—
80†	5.92	?	-3.83	-3.83	—	-0.51	-0.47	—	+0.08	+0.08	—
81†	5.67	?	-3.55	-3.35	A	-0.25	-0.25	—	+0.50	+0.35	A
79†	5.62	?	-3.33	-3.26	—	-0.16	-0.12	—	+0.70	+0.55	A
78†	5.38	?	-2.71	-2.69	—	+0.25	+0.22	—	+1.13	+0.90	A

* A indicates that the asymmetry exceeds the experimental error.

† Experiments near the apparent i.e.p. of l (pH 6.00 ± 0.05). There is no asymmetry for l in this case. Thus, these experiments are not concerned in the calculation of the asymmetries of boundary l (Table 5).

‡ i.e.p. of k_1 pH 5.50 ± 0.05 . No asymmetry.

given the mobilities, both ascending and descending, of those constituents h , k_1 and l for which accurate measurements are possible. The interpretation of these data must be considered in relation to the mobilities and the errors in their measurement. The mobilities have been evaluated by the accepted methods and are apparent values, since the potential gradient has been taken as that in the initial protein solution and not the actual gradient occurring at each boundary. The errors occurring in these determinations do not have the same significance in comparing the ascending and descending mobilities

In comparing the results from different experiments, errors in conductivity determinations, pH and current measurement must also be considered. There are other systematic errors due to liquid displacement (Tiselius, 1930), and accidental errors which Longworth (1943) shows to be possible with even the best experimental arrangement. In this latter case an agreement of $\pm 0.10 \times 10^{-5}$ cm.² V.⁻¹ sec.⁻¹ is excellent and is not exceeded by most of our results (Fig. 3). Thus, in the former case, we have to consider an error of approximately $\pm 0.05 \times 10^{-5}$ cm.² V.⁻¹ sec.⁻¹, and owing to the relatively great

number of experiments it is likely that the asymmetry increases from h to k and from k to l (Tables 5 and 6). In every case it has been verified that this gradation cannot be due to accidental occurrences such as convection currents at sp on the cathode side, or to interaction between l , m and n . It seems to be correlated with the increase in the relative concentrations of the components h (5–6%), k_1 (8–10%), and l (22–28%). It follows that in the electrophoretic separation of a mixture of proteins, the velocity asymmetry is greatest for the component of highest relative concentration.

Table 6. *The asymmetries of the boundaries h , k_1 and l*

Boundary	h	k_1	l
Frequency of asymmetry*	0.21	0.42	0.73
Mean deviation†	+0.045	+0.071	+0.10
Mean deviation as % of mobility‡	+1%	+4%	+8%

* Frequency of asymmetry is the ratio of the number of cases of asymmetry (A, Table 5) to the total number of experiments.

† The difference between ascending mobility and descending mobility is taken as positive when ascending > descending (classical asymmetry).

‡ Mean deviation as percentage of mobility of descending boundary, near pH 7.6.

The factor of individual concentration seems to be important. For h there is practically no asymmetry, and even if the errors in all experiments were such as to increase the classical asymmetry to a maximum ($2 \times 0.05 \times 10^{-5}$ cm.² V.⁻¹ sec.⁻¹) the mean deviation as percentage of mobility should be +3%, and in only five out of twenty-nine experiments has this value been exceeded. The component h therefore migrates nearly ideally without change in its concentration throughout the liquid column, which is remarkable, since above pH 6.75 it migrates in a more varied medium than any of the other components.

In practice the medium modifies during the course of electrophoresis (Longworth & MacInnes, 1940), and during the first hour the mobilities of the ascending and descending boundaries of h may differ by c. 10% but become equal after 2–3 hr. This may be a consequence of the regulation function of Kohlrausch (1897), studied in relation to salt ions by MacInnes & Longworth (1932).

It may be concluded, that in the electrophoresis of a protein mixture at favourable ionic strength ($\mu = 0.15$) self-regulation occurs most markedly for the constituents of lowest concentration. This regulation which tends to equalize the apparent mobilities in the ascending and descending limbs introduces qualifications to the conclusions of Longworth & MacInnes (1940). These conclusions

are, however, a satisfactory approximation, and since we have not observed a negative asymmetry in conformity with the predictions of these authors, their view has been adopted and the values have all been taken from the descending limb in constructing the curves of Fig. 3.

Miscellaneous factors

The mobility measurements have proved to be substantially independent of a number of factors which theoretically might possibly have had an effect. This is particularly true of the total protein concentration (Table 7) which would be expected to

Table 7. *Independence of mobility with respect to total concentration of extract*

Exp. no.	mg. N/ml.	pH	Descending mobility		
			h	k_1	l
42	1.214	7.51	5.65	2.05	1.35
40	2.832	7.50	5.70	2.15	1.30
22	5.016	7.63	5.80	2.05	1.30

influence the viscosity of the medium through which the boundaries migrate, particularly h in the cathode limb, and so alter the mobility. These observations disagree with those of Longworth & MacInnes (1940) on ovalbumin, but agree with the findings of Stenhagen & Teorell (1939) on thymonucleic acid. The author agrees with the latter's distinction between real viscosity which enters into the electrophoretic equation, and apparent viscosity called plasticity which depends on protein concentration and does not affect the mobility.

The magnitude of the potential gradient has no measurable influence (Table 8) which is contrary to

Table 8. *Independence of mobility with respect to potential gradient and length of dialysis*

Exp. no.	V./cm.	Length of dialysis (hr.)	mg. N/ml.	pH	Descending mobility		
					h	k_1	l
23	2.86	88	5.002	7.70	5.85	2.15	—
21	4.22	40	4.974	7.68	5.95	2.25	1.40
22	6.76	64	5.016	7.63	5.80	2.05	1.30

the findings of Stenhagen & Teorell (1939) who obtained a 3% increase in mobility for unit increase in potential gradient. Exp. 22 should have furnished a measurable effect

$$\left(5.85 + \frac{3 \times 4}{100} \times 5.85 = 6.55 \right)$$

according to the above results.

The temperature increase in a potential gradient of 5 V./cm., determined with thermocouples is

0.2–0.3°, which would have a negligible effect on the viscosity and the conductivity of the solution. Duration of dialysis has no effect provided it is sufficient but not so prolonged as to cause denaturation of certain of the proteins. Thus muscle proteins do not behave like haemocyanins (Tiselius & Horsfall, 1939). Reversible electrodes of the Cu/CuSO₄ or Ag/AgCl type may be used indiscriminately.

In conclusion, the mobility curves given characterize proteins in mixtures where one component may influence another, and are likely to be useful in the identification of constituents ultimately obtained pure.

DISCUSSION

In extracts of striated rabbit muscle freed from blood, there exist above pH 7.10 and at ionic strengths 0.10–0.15 at least eleven electrophoretically distinguishable components. It is difficult to establish the exact relationship between these components and the classical muscle proteins, but there are some points worth indicating. At an ionic strength of 0.15, the isoelectric points (I.E.P.) of the group I proteins are n 6.75, m 6.20, l 6.00, and for t and s close to pH 6.00. The soluble portion of group I seems to correspond with the myogen of Weber (1934) (I.E.P. 6.3) which has been known for some time to be a mixture of proteins (Finn, 1932; Baranowski, 1941). This identification is supported by the findings of Bate Smith (1940). It is not possible to place zymohexase precisely.

Component h , perhaps totally water-soluble, may be myoalbumin, and represents 7–8 % of the water-soluble proteins. At pH 5.38 it still has a high anodic mobility in agreement with Bate Smith (1937). For myoalbumin at pH 7 and $\mu = 0.1$ Bate Smith (1940) gives a mobility which can correspond only with h . The other components (k_1 , k_2 , j , sp and i) may represent myosin and globulin X, whose isoelectric points are near pH 5.00 (Weber, 1934). It may be objected that at ionic strength $\mu = 0.15$, the myosins of Edsall and of Banga & Szent-Györgyi are not sufficiently soluble to appear in the present diagrams. However, Bate Smith (1937) has demonstrated that it is difficult to precipitate the myosin of Edsall from solutions containing phosphate, and further it is probable that in addition to the effect of ions the presence of other proteins affects the solubility. Of k_1 little is known except that its isoelectric point is 5.50. The components k_2 , j , sp and i are accompanied at $\mu = 0.15$ by a turbidity the distribution of which is difficult to explain. Of these four proteins, i is at present the best characterized by its insolubility in distilled water, and complete loss of solubility below pH 6.0. They are important since it is in this region of the diagram that changes occur when the protein responsible for birefringence

is kept in solution, or those proteins more soluble at $\mu = 0.35$ than at $\mu = 0.15$ are investigated. These last considerations suggest that electrophoretically there is not one but several myosins, if myosin is defined as a globulin much more soluble at $\mu = 0.27$ –0.35 than at $\mu = 0.15$. This disagrees with the results of Bailey (1942) but agrees with those of Ziff & Moore (1944).

It is possible that the electrophoretic method may introduce important corrections to the existing classification of muscle proteins, but further experiments are necessary to establish this. In a previous publication (Jacob, 1945), the main differences between the muscle proteins of the rabbit and frog have been described. In a private communication, J. W. Mehl has indicated that extracts of rat muscle are similar electrophoretically to those of rabbit.

SUMMARY

1. Saline extracts of striated rabbit muscle have been examined in the Tiselius-Longworth electrophoresis apparatus.

2. In the extract obtained with phosphate of pH 7.70 (0.048M-Na₂HPO₄–0.06M-NaH₂PO₄, $\mu = 0.15$) and dialyzed against phosphate buffer containing 0.05M-NaCl, total $\mu = 0.15$, there are nine components divisible into the following three groups: group I consisting of three proteins, n , m and l ; group II, three proteins, k_1 , k_2 and j ; group III, which at pH 7.4 and above contains two proteins h and i , and below pH 7.4 is apparently homogeneous; between groups II and III is found a heterogeneous component sp .

3. The mobility curves of the first eight components have been established at ionic strength $\mu = 0.15$ over the pH range 5.38–8.04. From these the following isoelectric points have been interpolated: n , 6.75 ± 0.05 ; m , 6.20 ± 0.05 ; l , 6.00 ± 0.05 ; and k_1 , 5.50 ± 0.05 .

4. Some of the constituents show marked variations in solubility over the pH range studied.

5. At an ionic strength $\mu = 0.1$, two further proteins resolve in group I.

6. When the extracts are dialyzed against distilled water the protein i is apparently completely precipitated.

7. Extraction or dialysis at higher ionic strengths ($\mu = 0.27$ –0.35) shows that the proteins with higher solubilities in this range migrate close to k_1 , j , sp and i .

8. Various aspects of technique apposite to the study of the protein mixtures have been examined experimentally. These include: the effects of thermal convection; the δ boundary and Svensson effect and their significance in the measurement of relative concentration; asymmetries in the ascending and descending mobilities of three of the consti-

tuents of the mixtures; and the influence of extract concentration, potential gradient, and duration of dialysis on the mobility.

9. The relation between the observed electrophoretic components and the classical muscle proteins has been examined.

The author (Chargé de Recherches du F.N.R.S.) wishes to express his gratitude to Prof. Dubuissou for his hospitality, encouragement and advice, and to Mrs Bruylant for technical aid. He is greatly indebted to Dr R. A. Kekwick for translating the text. The work was made possible by grants from the Fondation Nationale pour les Recherches Scientifique de la Belgique.

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Studies in Vitamin A

1. A CHROMATOGRAPHIC METHOD FOR SEPARATING FREE AND ESTERIFIED VITAMIN A

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(Received 16 August 1946)

Partition between immiscible solvents, usually light petroleum and aqueous methanol, has long been in use to separate carotenoid hydrocarbons from hydroxyl-containing 'xanthophylls'. Gillam & Senior (1936) found that in the absence of much cholesterol, vitamin A alcohol could be removed from light petroleum by seven extractions with 90 % aqueous methanol. Reed, Wise & Frundt (1945) effected a partial separation of free and esterified

vitamin A by extracting light petroleum solutions with 95 % aqueous methanol; 98 % of the esterified and 27 % of the free vitamin remained epiphasic and simple equations permitted the proportions of combined and free vitamin to be calculated. As in the earlier work of Gillam & Senior (1936), the presence of cholesterol adversely affected the separation. This interference is a serious drawback to the use of partition methods in that biological