depressed on admixture with the authentic derivative of octanoic acid. (Found: C, 56.2; H, 6.1; Br, 23.4%. Calc. for $C_{10}H_{21}O_2Br$: C, 56.3; H, 6.3; Br, 23.5%.)

SUMMARY

1. Serratamic acid, m.p. 138° (decomp.), is produced by several strains of organisms of the *Serratia* group.

2. Serratamic acid is readily hydrolysed by both mineral acids and alkalis. Acid hydrolysis yields L-serine and an acid, $C_{10}H_{20}O_3$, tentatively identified as a hydroxydecanoic acid, which is oxidized to octanoic acid or one of its isomers.

3. The linkage between the amino acid and hydroxy acid appears to be of the peptide type.

4. Serratamic acid does not account for the antibiotic activity of strains of *S. marcescens* producing this acid.

5. The growth of organisms of the *Serratia* group to produce serratamic acid affords a convenient source of L-serine.

REFERENCES

- Bergey, D. H. (1948). *Manual of Determinative Bacteriology*, 6th ed. London: Baillière, Tindall and Cox.
- Breed, R. S. & Breed, M. E. (1926). *J. Bact.* **11**, 76.
- Daft, F. S. & Coghill, R. D. (1931). *J. biol. Chem.* **90**, 341.
- Fischer, E. & Jacobs, W. A. (1907a). *Ber. dtsh. chem. Ges.* **40**, 1058.
- Fischer, E. & Jacobs, W. A. (1907b). *Ber. dtsh. chem. Ges.* **40**, 3718.
- Fuller, A. T. & Horton, J. M. (1950). *J. gen. Microbiol.* **4**, 417.
- Jarvis, F. G. & Johnson, M. J. (1949). *J. Amer. chem. Soc.* **71**, 4124.
- McChesney, E. W. & Swann, W. K. jun. (1937). *J. Amer. chem. Soc.* **59**, 1116.
- Nicolet, B. H. & Shinn, L. A. (1941). *J. biol. Chem.* **139**, 687.
- Stokes, J. L. & Gunness, M. (1945). *J. biol. Chem.* **157**, 65.
- Van Slyke, D., Hiller, A. & MacFadyen, A. (1941). *J. biol. Chem.* **141**, 681.
- Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.
- Woodbine, M., Kolasiewicz, A. & Seaman, A. (1954). *Nature, Lond.*, **174**, 406.
- Wrede, F. & Rothaas, A. (1934). *Hoppe-Seyl. Z.* **226**, 95.

Paper-electrophoretic Analysis of Protein Extracted at Low Ionic Strength from Fish Skeletal Muscle

BY O. E. NIKKILÄ AND R. R. LINKO

Department of Biochemistry, University of Turku, Finland

(Received 16 December 1954)

The muscle proteins of fish are considered to be very similar in their general properties to corresponding mammalian proteins. Reay (1935), Reay & Kuchel (1936), Subba Rao (1948) and Dyer, French & Snow (1950) applied the fractionation method of Smith (1934, 1937) in studies of the muscle proteins of several species of marine fish and found protein fractions similar to those present in rabbit muscle. Roth (1947) reported that protein fractions of some freshwater fish resemble in their general properties those in rabbit and frog muscles. Smith's fractionation scheme is, however, out of date and some of the results obtained by the method are incorrect. On the basis of the most reliable values of the above workers, fish muscle seems to differ most characteristically from mammalian muscle only in its very low content of connective tissue and perhaps also in the albumin content.

Using the free electrophoretic method, Hamoir (1949, 1951) has compared carp muscle proteins with those from rabbit muscle and found both similarities and differences between them. The electrophoretic patterns of codling muscle at low ionic strength obtained by Connell (1953a) using the Philpot-Svensson system show the same general characteristics as those of rabbit or frog muscle.

Later Connell (1953b) found considerable differences in minor details of the patterns obtained in free electrophoresis of muscle extracts from several species of fish, but concluded that the components of all the patterns may be resolved into the same mobility groups as those of mammalian muscle extracts described by Jacob (1947).

The present study has been undertaken to determine the electrophoretic properties of fish muscle-protein extracts at low ionic strength by using both paper and free electrophoretic methods. The diagrams for extracts obtained under identical conditions from several species of fish are compared. An extract of pike muscle has been fractionated by ethanol precipitation, and the behaviour of the successive fractions in paper electrophoresis is discussed.

MATERIALS

The following ten species of fish caught on the west coast of Finland were studied in the experiments: Baltic herring (*Clupea harengus membras* (L.)); whitefish (*Coregonus lavaretus* (L.)); roach (*Rutilus rutilus* (L.)); ide (*Idus idus* (L.)); bream (*Abramis brama* (L.)); eel (*Anguilla anguilla* (L.)); pike (*Esox lucius* (L.)); flounder (*Platichthys flesus* (L.)); perch (*Perca fluviatilis* (L.)); pike-perch (*Lucioperca lucioperca* (L.)).

Most of the fish were obtained alive and none had been dead for more than a few hours. The sex and the weights of the individuals were recorded. None of them were observed to be abnormal in size or appearance. The fish were filleted and skinned and the musculature from both sides used in the extractions.

METHODS

The fillets were passed through a meat mincer and then ground with fine sand. The muscle pulp was mixed with an equal volume of phosphate buffer (0.0156M- Na_2HPO_4 + 0.0035M- NaH_2PO_4 ; pH 7.55; ionic strength 0.05) and extracted by gentle mechanical stirring for 2–2.5 hr. The extracts were cleared of the insoluble pulp by centrifuging at 2250 g for 30 min. In the case of free electrophoresis, the extracts were dialysed in cellophan bags against the above-mentioned buffer for about 48 hr. with frequent changes of outer liquid, and the bag contents centrifuged. The pH values and the protein contents of the extracts and dialysed solutions were determined immediately before the electrophoretic analysis. The operations mentioned were carried out near 0°, with the exception of paper-electrophoretic analysis which was conducted at room temperature.

The free electrophoresis was carried out at 4.4° using the Philpot-Svensson modification of the conventional Tiselius technique.

The filter-paper electrophoretic analysis were conducted in an apparatus of type LKB 3276 (LKB-Produkter Fabriks AB, Stockholm). In general, the run in the phosphate buffer with $I=0.05$ and pH 7.55 gave a good resolution of the proteins. In some cases, treatment of the filter paper with serum albumin according to the method described by Mariani (1952) resulted in an even better resolution. The most suitable paper for the electrophoretic run in a vapour-saturated cassette was found to be the Carl Schleicher & Schüll no. 2043 B (120 g./m.²) filter paper. As a rule four strips measuring 40 × 425 mm. or one sheet measuring 180 × 425 mm. can be placed in the cassette. In the first case four runs and in the second case seven runs may be carried out at the same time. The sample solution was applied to the filter paper at right angles to the direction of the migration along a line 30 mm. long and, when using the sheet, along a line 10 mm. long. A sample of solution containing about 4% (w/v) of protein suitable for the strips was found to be approx. 14 $\mu\text{l.}$; for the sheets 6 $\mu\text{l.}$ of a solution containing 0.75% (w/v) protein. For the comparison of the results for different samples it is important that the sample quantities are as nearly as possible equal. The point of application of the sample was chosen so that the most rapidly and the most slowly migrating protein components were situated at an equal distance from the middle point of the strip after the completed run. In all experiments 296 v, giving a current of 5–7 mA, and a running time of 17 hr. were used.

A shift of a protein in the direction of the cathode observed in filter-paper electrophoresis is caused by electroendosmosis. This flow must be taken into consideration particularly when determining the isoelectric points of proteins. Some substance without significant charge which would not have a mobility of its own in an electric field but would move with an electro-osmotic liquid may give an index of the degree of this flow. Kunkel & Tiselius (1952) have shown dextran to be a suitable substance to use

as indicator. The pretreatment of filter paper with serum albumin for the purpose of weakening the binding of protein to the paper was shown to result in a weaker electroendosmosis at the same time.

Following the treatment described by Kunkel & Tiselius (1952), the filter papers used in the electrophoresis were dried for 30 min. at 105° and stained with bromophenol blue dissolved in ethanol sat. with HgCl_2 , and subsequently washed with 0.5% acetic acid. For the detailed estimation of the proteins, segments 1–4 mm. in length were cut from the paper strips. These pieces were eluted for 30 min. with 3–6 ml. of 0.01N- NaOH and the optical density of the eluate measured at 575 m μ . with a Unicam spectrophotometer.

Ethanol fractionation of pike muscle extract. The fractionation of pike muscle proteins extracted with the phosphate buffer was carried out with ethanol at low temperatures. The addition of the ethanol, which had been precooled to –40°, took place through capillary tubing. The temperature of the extract, which was 0.5° at the beginning of the fractionation, was gradually decreased as the ethanol concentration increased. After the ethanol content of the extract had risen to at least 20% (v/v), the temperature was maintained at or below –5°. After the addition of the desired amount of ethanol, the solution was allowed to stand, usually overnight in a cold room, after which the precipitate was removed by centrifuging. The precipitate was cooled to –20° and dried *in vacuo*. During the evacuation the temperature of the precipitate decreases considerably, but the ethanol prevented it from freezing, so that freeze-drying in the ordinary sense of the word did not take place. The precipitate dried completely in 1 hr. The precipitate was then dissolved in the phosphate buffer. The proteins separated in this way did not show either a loss in solubility or a change of their electrophoretic behaviour.

RESULTS

The paper-electrophoretic diagrams of the protein extracted from different species of fish under the same conditions are reproduced in Fig. 1. The more obvious components, which were also visually detected on the strips, have been numbered arbitrarily from 1 to 8 in the order of decreasing mobility. Several measurements were performed on the different samples from fishes of each species. The reproducibility of the tests was satisfactory. In the case of bream and pike, the electrophoresis was carried out using filter paper pretreated with serum albumin. The division of the diagrams into components is somewhat arbitrary and probably deviates from the 'true' picture in some cases. It is emphasized that similar figures in different diagrams do not necessarily refer to the same components. Thus, for example, it is quite possible that the extract of roach muscle contains two fast-moving components, although the diagram does not show any peak between the main components and the fastest component as do all the other diagrams obtained in the present investigation. In general, the diagrams show that considerable differences exist between the electrophoretic

properties of muscle proteins extracted from different species of fish with a phosphate solution of low ionic strength.

The electrophoretic patterns of pike muscle extract obtained in free solution of ionic strength 0.05 and pH 7.55 are shown in Fig. 2. The similarity between the ascending and descending limbs is apparent. The free electrophoretic patterns and the paper-electrophoretic diagrams generally run parallel, and exhibit differences only in some details. The resolution of proteins on filter paper has been appreciably greater than that in free solution, and consequently the details in the paper-electrophoretic diagrams are more distinct.

Table 1 summarizes the distribution and the mobilities of the protein components present in

pike muscle in free electrophoresis. The mobilities in Table 1 represent averages of the values measured for the ascending and the descending limbs. These values agree satisfactorily with the results of Connell (1953b). The values given in Table 1 relate to Fig. 2.

The fractionation of pike muscle extract is outlined in Fig. 3. The protein-nitrogen distribution among various fractions of pike muscle extracts is given as percentage of the nitrogen content of the total precipitated protein in Table 2.

The separated fractions were examined electrophoretically at various pH values. The proteins of different fractions were run in parallel on the same sheet of filter paper. The electrophoretic migration of pike muscle proteins after ethanol fractionation

Fig. 1. Paper-electrophoretic diagrams of extracts of fish skeletal muscle in sodium phosphate buffer, at $I=0.05$, pH 7.55. The dextran line represents the starting-point after allowing for electroendosmosis and the solid line the point of protein application.

is shown in Fig. 4. The fractions were numbered I–VI in the order of precipitation by ethanol.

The protein fraction I did not move at all in electrophoresis at various pH values in the range 4.25–7.55. It was found to give typical colour reactions of carbohydrates. Thus the fraction may consist of glycoprotein either alone or together with a complex composed of protein and buffer or mixed denatured proteins. Fraction II contains two components which may be separated better from each other in a low pH range. At low pH, however, the amount of protein remaining at the starting line increased considerably, which may be

due to denaturation of the protein. The components are probably identical with peaks 5 and 6 in Figs. 1 and 2. The respective isoelectric point values of the electrophoretic components obtained were 5.8 and 6.2. Fraction III appeared to be electrophoretically homogeneous at pH 7.55 but at pH 5.95 it showed two components. These components may correspond to the peaks 3 and 4 in Figs. 1 and 2. The greater part of fraction III consisted of component 4; the isoelectric point of the main component was found to be 5.4. Fraction IV was

Finely minced and ground pike muscle extracted for 2.5 hr. with 1.5 vol. of sodium phosphate buffer ($I=0.05$, pH 7.55) at 0° and centrifuged at 2250 *g* for 30 min.

Fig. 2. Electrophoretic patterns of extract from skeletal muscle of pike from analysis conducted in free solution. Upper part, descending limb; lower part, ascending limb. sodium phosphate buffer, $I=0.05$, pH 7.55; electrical field, 12.7 v/cm.; duration of electrophoresis, 3900 sec.

Table 1. Average distribution and mobilities of components of pike muscle extract in sodium phosphate buffer ($I=0.05$, pH 7.55)

	Component				
	1	2	3	4+5	6+7
Fraction of total protein (%)	4.1	13.2	4.0	48.3	20.3
Mobility (cm. ² sec. ⁻¹ v ⁻¹ × 10 ⁻⁵)	10.9	7.8	5.4	3.5	1.9

Fig. 3. Fractionation scheme employed for pike muscle proteins.

Table 2. Nitrogen distribution in pike muscle-protein fractions after ethanol fractionation (Fig. 3)

Fraction	Precipitated protein N as % of total N	Appearance of the dissolved fraction
I	31.9	Turbid, viscous
II	42.8	Slightly turbid, reddish
III	18.5	Clear
IV	4.0	Clear
V	2.3	Clear
VI	0.5	Clear

electrophoretically heterogeneous and contained traces of fraction III together with a part of the following fraction. Fraction V was found to contain only one component in the electrophoresis at both high and low pH values. This component may correspond to the peak 2 in Figs. 1 and 2. The isoelectric point of the protein present in fraction V was 4.8. Fraction VI contained the protein giving peak 1 in Figs. 1 and 2; in addition it contained some of the material of fraction V. The isoelectric point of the new component was 4.4.

Fig. 4. Migration of protein components of different fractions of pike muscle extract in filter-paper electrophoresis at $I=0.05$, pH 7.55. Electrical field, 6.3 v/cm.; duration of run, 17 hr. The extracts had been previously fractionated by ethanol at low temperature. Fractions I-VI were numbered in the order of precipitation by ethanol.

The quantitative estimation of the protein components separated by filter-paper electrophoresis was not carried out because the different proteins have somewhat different dye-binding capacities, which makes their colorimetric determination inaccurate. For the purpose of the present investigation, the determination of the number of the protein components found in fish muscle extracts and their electrophoretic behaviour were more important than their quantitative distribution on filter paper.

DISCUSSION

In spite of the inexpensive apparatus and simple procedure, filter-paper electrophoresis as employed for muscle-protein studies offers advantages in some respects over the classical methods in free solution. Thus, the paper method permits the use of smaller samples of lower concentration and the analysis of several samples at the same time. In addition, in paper electrophoresis it is possible to avoid the inaccuracies associated with the sensitive optical methods employed in free

electrophoresis when the solution is poorly translucent.

The paper-electrophoretic diagrams of muscle extracts from several species of fish have the same general features but show some characteristic differences in details (Fig. 1). There is an over-all similarity in that the number of the electrophoretic peaks varies in a very small range from 6 to 8. Nine diagrams out of ten show two rapidly migrating components in comparatively low concentrations. The absence of one of them in the diagram for the roach muscle extract may be due to a lower sensitivity of the method used or to a very low concentration of the component. The components next in order of migration form quantitatively the largest group. This group of proteins generally consists of three members. The slowest components form a somewhat more heterogeneous group than the two preceding ones. This group probably consists of three or four components, which are in some cases superimposed in some degree. In contrast to these similarities, the diagrams given in Fig. 1 show that some marked differences exist between the muscle extracts from different species of fish. The number and the relative quantities of the components vary characteristically from species to species. The form of diagram of each species has been found to be constant enough to permit identification of the fish by means of electrophoretic analysis of the muscle extract obtained under the same conditions.

Parallel analysis of the proteins present in pike muscle extract conducted in free solution and on filter paper revealed very similar electrophoretic properties. On filter paper, however, the resolution was better than in free electrophoresis. In contrast, the distribution and mobilities of components can perhaps be more easily measured in the latter case.

The combination of ethanol fractionation at low temperatures with electrophoretic analysis proved to be a very suitable method for the study of proteins. Using this method, an extract of pike muscle was divided into six fractions which contained all the components found in electrophoresis before fractionation. By running each of the fractions separately in parallel on filter paper, it was possible to compare the electrophoretic properties of the components of different fractions. The positions of components could readily be distinguished on the filter paper. In some cases fractions homogeneous according to the electrophoretic analysis at one pH value proved to be heterogeneous at another pH. However, paper electrophoresis as at present conducted is probably not sensitive enough to reveal all the components which according to some enzymological investigations may be present in very low concentration in muscle extracts of low ionic strength.

SUMMARY

1. Muscle extracts of ten different species of fish have been examined by the paper-electrophoretic method in sodium phosphate buffer at $I=0.05$ and pH 7.55.

2. The recorded diagrams are similar in their main features, but differ in details characteristically from species to species. Thus it is possible to identify the fish by means of an electrophoretic analysis of the muscle extract obtained under prescribed conditions.

3. Pike muscle extract was analysed electrophoretically both on filter paper and in free solution. The diagrams obtained by both methods were found to run generally parallel.

4. Fractionation by precipitation with ethanol was applied to effect separation of the protein components extracted from pike muscle at low ionic strength. The proportions and the electrophoretic properties of protein components of different fractions were examined. The nature of the components of diagrams obtained before and after fractionation has been discussed.

The authors express their thanks to Professor E. Uroma, chief of the State Serum Institute, for permission to use the Tiselius electrophoretic apparatus and to Mr A. Louhivuori, Ph.M., for valuable assistance in conducting the electrophoretic analysis in free solution.

REFERENCES

- Connell, J. J. (1953*a*). *Biochem. J.* **54**, 119.
 Connell, J. J. (1953*b*). *Biochem. J.* **55**, 378.
 Dyer, W. J., French, H. V. & Snow, J. M. (1950). *J. Fish. Res. Bd Can.* **7**, 585.
 Hamoir, G. (1949). *Bull. Soc. Chim. biol., Paris*, **31**, 118.
 Hamoir, G. (1951). *Symp. biochem. Soc.* **6**, 8.
 Jacob, J. J. C. (1947). *Biochem. J.* **41**, 83.
 Kunkel, H. G. & Tiselius, A. (1952). *J. gen. Physiol.* **35**, 89.
 Mariani, A. (1952). *R.C. Ist. sup. Sanit.* **15**, 337.
 Reay, G. A. (1935). *Rep. Fd Invest. Bd, Lond.*, no. 65.
 Reay, G. A. & Kuchel, C. C. (1936). *Rep. Fd Invest. Bd, Lond.*, no. 93.
 Roth, E. (1947). *Biochem. Z.* **318**, 74.
 Smith, E. C. B. (1934). *J. Soc. chem. Ind., Lond.*, **53**, 351.
 Smith, E. C. B. (1937). *Proc. Roy. Soc. B*, **124**, 136.
 Subba Rao, G. N. (1948). Physico-chemical investigations on the muscle proteins of some commercial species of fish caught in N.E. Scottish waters. Ph.D. Thesis, University of Aberdeen.

The Amino Acids and other Ampholytes of Urine

3. UNIDENTIFIED SUBSTANCES EXCRETED IN NORMAL HUMAN URINE*

By R. G. WESTALL

Medical Unit, University College Hospital Medical School, London, W.C. 1

(Received 16 December 1954)

The application of the displacement method of chromatography on ion-exchange resins described in Part 1 of this series of papers (Westall, 1952*b*) has led to the isolation from urine of a number of previously unknown amino acids or ampholytes: β -aminoisobutyric acid (Crumpler, Dent, Harris & Westall, 1951), porphobilinogen (Westall, 1952*a*) and felinine (Westall, 1953). In the course of these studies certain other ninhydrin-reacting substances, many of which were unstable to acid hydrolysis, were observed but the amounts of these compounds were usually too small to permit further investigation. The existence of unknown substances in human urine which liberate free amino acids on acid treatment has been recognized for a long time (Henriques & Sørensen, 1909) and many other workers, notably more recently Boulanger, Biserte & Courtot (1952) and Carsten (1952), have added fresh evidence for the occurrence of such substances in urine. The elution diagram for normal urine (Stein, 1953) also showed a number of small peaks representing unknown ninhydrin-reacting substances, which disappeared after acid hydrolysis, in

addition to the larger peaks representing the commonly occurring amino acids. In this method, which uses only 2–5 ml. of urine, the analysis could not be expected to show substances excreted in amounts of less than 5 mg. per day, especially if they were peptides giving a relatively low colour yield with the ninhydrin reagent.

The work to be described now is an attempt to investigate further the ninhydrin-positive unidentified compounds in a sample of 100 l. of urine from a normal adult male subject. As a first method of isolation and fractionation of the ampholytes, ion-exchange chromatography has been employed using the displacement technique with its advantages of high capacity. This was followed by two-way paper chromatographic analysis of all the fractions obtained, with other methods of analysis as required.

EXPERIMENTAL

Urine (100 l.) was collected from one healthy, adult male subject over a period of 3 months. Only full 24 hr. specimens were taken and breaks were made over weekends owing to storage difficulties. The full collection represented the total output of 80 days. Each specimen was stored

* Part 2 of this series: Westall (1953).