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IDENTIFICATION OF SUSPECT SEA TURTLE MEAT SAMPLES AND DETERMINATION OF SPECIES, A LAW ENFORCEMENT PROBLEM

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U.S. DEPARTMENT OF COMMERCE
National Oceanic and Atmospheric Administration
National Marine Fisheries Service
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William G. Gordon, Assistant Administrator for Fisheries

INTRODUCTION

In 1973, the Congress of the United States passed the Endangered Species Act in order to provide for the conservation of endangered and threatened species (1). The Act, as amended in 1976, 1977 and 1978, clearly defined species of sea turtle which were endangered or threatened, and outlined unlawful actions subject to the jurisdiction of the United States. Law enforcement agents in several agencies were responsible for the execution of the provisions of the Act.

In order to enforce several of these provisions, including unlawful trade and possession of sea turtle, an effective means of identifying sea turtle meat by species was required. Since normal anatomical and morphological characteristics could not be used in the identification of meat samples, another method was demanded. Electrophoretic methods have been applied in the past to the successful speciation of numerous varieties of plants and populations of animals. In particular several closely related species of fishes as well as several crab species have been differentiated on the basis of sophisticated isoelectric focusing techniques (2-7).

Our investigations of both electrophoretic and isoelectric focusing techniques revealed the distinct advantages of the latter in differentiating turtle meat samples by species. Some of the advantages offered by the isoelectric focusing method were the relative speed of analysis, insensitivity to sample application technique, and high resolution with excellent reproducibility. The purpose of this paper is to explain the application of the isoelectric focusing technique to the resolution of extracted turtle meat proteins and hence to the identification of several species of threatened and endangered sea turtles.

EXPERIMENTAL PROCEDURE

CHEMICALS AND MATERIALS: Ready-made LKB PAG[1] plates: (pH ranges 3.5-9.5 and 5.5-8.5), acrylamide, methylene-N,N' bisacrylamide, ammonium persulfate, Coomassie Blue R-250 and a complete range of Ampholine solutions were purchased from LKB (Durham, NC). IsoGel agarose and Gelbond film were purchased from Marine Colloids (Rockland, ME). Research grade chemicals were purchased from Fisher Scientific (Atlanta, GA). Silane (3-methacryloxypropyltrimethoxysilane) was obtained from Alfa (Danvers, MA). Filter paper (S+S No.577) was obtained from Schleicher and Schuell (Keene, NH). Sorbitol and sulphosalicylic acid were obtained from Sigma (St. Louis, MO).

EQUIPMENT: Equipment used to produce the finished gels consisted of an LKB Multiphor basic unit, an LKB constant power supply, a Haake model circulating water bath, an LKB surface electrode, and an Ampholine electrofocusing kit for polyacrylamide gels.

TURTLE TISSUE: Authenticated (standard) samples of sea turtle species were not readily available due to their status as endangered or threatened species. Samples were obtained through the assistance of individuals associated with the NMFS Sea Turtle Recovery Team, through numerous law enforcement agents from NMFS, F&WS and Customs, and through several individuals associated with sea aquaria or reptile holdings in the United States. Muscle tissues were deemed acceptable if the sample was collected from a turtle suffering recent death and the carcass was maintained on ice or in a cold room until dissection. Most of the turtle deaths were the result of natural causes (disease, cold-induced torpor, old age) but some involved incidental catch in fishing gear, etc. Turtle carcasses recovered from beach wash-ups were considered unusable. Aquarium deaths, which were autopsied and subsequently frozen, were used. Species of turtles for which authenticated samples were available consisted of the following: Loggerhead (*Caretta caretta*), Atlantic green (*Chelonia mydas mydas*), Pacific green of Hawaiian origin (*Chelonia mydas*), Kemp's ridley (*Lepidochelys kempi*), olive ridley (*Lepidochelys olivacea*), hawksbill (*Eretmochelys imbricata*), and leatherback (*Dermochelys coriacea*) sea turtles, in addition to Central American river turtle (*Dermatemys mawi*) and southern yellowbelly (pond) turtle (*Pseudemys scripta scripta*). The sex of the turtle from which samples were collected was often unknown or not easily determinable and, therefore, not specified. All samples were collected from adult or subadult turtles unless otherwise stated.

DISC SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE): Solutions and conditions used during SDS-PAGE were those used by Weber and Osborn (8) and by Seaman and Nash (9).

ISOELECTRIC FOCUSING:

Silanization of glass gel supports: Glass gel supports were silanized by the method of Radola (10) in order to promote the adherence of polyacrylamide gels to the glass support throughout the staining process. This procedure entailed the complete submersion or spraying of thoroughly cleaned glass plates in 0.2% silane solution in ethanol/H_2O(1:1 V:V), followed by air drying for 15 minutes.

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[1] Reference to trade names does not imply endorsement by the NMFS.

Acrylamide gel preparation: Polyacrylamide gels were prepared by pouring 60 ml of gel solution into a glass-sandwich mold of 175 mm length X 90 mm width X 2 mm depth (rubber gasket). The gel solution was prepared according to the instructions in Tables IV and V of the LKB application note # 250(11). A typical 3.5 - 9.5 pH range polyacrylamide gel was prepared by the addition of 10 ml of 29.1% (w/v) acrylamide solution to 10 ml of 0.9% (w/v) bis-acrylamide solution, 7 ml of 87% (v/v) glycerol solution, 2 ml pH range 4-6 ampholyte solution, 0.2 ml pH range 5-7 ampholyte solution, 0.4 ml pH range 9-11 ampholyte solution, and 2.8 ml pH range 3.5-10 ampholyte solution. This mixture was brought to 60 ml with Milli-Q water and degassed for 10 minutes. The addition of 1% (w/v) ammonium persulfate was followed by gentle swirling and immediate pouring into the mold. The gelling process was complete in an hour. When gels thinner than 2 mm in thickness were prepared, proportionately less solution was mixed.

Agarose gel preparation: Agarose gels were prepared according to the method of Lundstrom(12) and by instructions available from Marine Colloids[2]. Two percent agarose was prepared in advance by dissolving 2 g Isogel in 100 ml of Milli-Q water at 90°C. The agarose was stored in 15 ml aliquots in screw-capped tubes at 4°C. Prior to pouring an agarose gel, a 15 ml aliquot was heated in a 90°C water bath, 13.5 ml of 20% sorbitol solution was added, and the solution was cooled to 80-90° C before adding the ampholyte solutions. To prepare a pH range 3.5-9.5 gel, 0.9 ml pH range 3.5-10, 0.3 ml pH range 2.5-4.0, and 0.3 ml pH range 5.0-8.0 ampholyte solutions were used. The final concentrations of the gel components were: 30 ml of 0.8% agarose solution containing 9.0% sorbitol and 5.0% ampholyte. The gel solution, cooled to 50-60°C, was poured into a mold preheated in a 50°C oven. The gel support was a layer of Gelbond film atop a 3 mm glass support plate. The sandwich was completed by clamping in place a 1 mm gasket on top of the gel bond and below a 1 mm top glass plate. The heated agarose gel solution was introduced into the sandwich via a preheated 50 ml syringe barrel fixed with a luer valve and 18 gauge needle. The agarose was allowed to gel at room temperature and was then "cured" in a humidity chamber at 4°C for at least 15 min.

Sea turtle sample preparation and application: Samples may be prepared and applied by a variety of means. The choice of methods for this research is described herein. Muscle protein of sea turtle was extracted by homogenization of the tissue with dilute phosphate buffer or Milli-Q water followed by centrifugation. In our experience, the resulting soluble protein extract did not differ in composition from that obtained by simple mincing and expression of tissue fluid. However, the latter method yielded a sample of higher protein concentration and permitted for rapid sample preparation. Extracted supernatants and expressed tissue fluids were applied to polyacrylamide gels by filter paper wicks, and to agarose gels by means of a LKB sample foil. The sample foil was a thin piece of plastic with rectangular slots cut through the plastic such that 1-2 ul of fluid may be contained in the cut area after the foil is laid on the gel surface. The filter paper wicks were rectangular and held 5-15 ul of tissue extract depending upon the size and thickness of the wick. The wicks were applied directly to the gel surface. In cases where limited sample was available (<100 mg), small slivers of tissue were placed directly on the gel with some success. However, direct tissue application usually produced streaking and skewing of the protein bands.

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[2] A brochure is available from FMC, Marine Colloids Division, Rockland, Maine, 04841.

Electrode solutions: For acrylamide gels, the electrode solutions were 1 M H_3PO_4 at the anode and 1 M NaOH at the cathode for pH range 3.5-9.5 gels or 2% ampholyte solution (pH range 4-6) at the anode and 1 M NaOH at the cathode for pH range 5.5-8.5 gels. The agarose gel electrode solutions were 0.1 M H_3PO_4 at the anode and 0.1 M NaOH at the cathode for pH range 3.5-9.5 gels.

Isoelectric focusing parameters: Procedures for isoelectric focusing were carried out in accordance with the instructions in LKB Application Note #250 (11). Laboratory prepared acrylamide gels were focused with the following parameters: power=25 W for 2 mm gels or 15 W for 1 mm gels, max. voltage=1.8 kV, and max. current = 200 mA. The prefocusing period was 30-40 min at reduced power followed by an additional 2.5-4.0 h of focusing. Sample wicks were placed on the gel following prefocusing and removed 30-45 min into the focusing period. Agarose gel parameters were: power= 25-30 W, max. voltage=1.5 kV, and max. current = 50 mA for 30-40 min. Improved sharpness in agarose gel patterns were noted when the gel was prefocused for 5 min at power range=15-20 W, max. voltage=1.5 kV and max. current=50 mA. In addition, focusing for the first 10 min with the sample application foil in place often aided in sample penetration and prevented surface run.

Determination of pH gradient: Near the end of the focusing period the pH gradient was determined for both agarose and acrylamide gels. This was accomplished by direct measurement with a flat surface electrode at 1 cm intervals at the focusing temperature of $4^{o}C$.

Fixing and staining procedures: Acrylamide gels were fixed and prepared for staining by the method described in LKB Application Note #250 (11). The fixing solution consisted of 5.5% sulphosalicylic acid in 11.5% in trichoroacetic acid. The staining and destaining solutions used were those described by Rhigetti and Drysdale (13). Each acrylamide gel was stained a minimum of 2 h in solution (I) and a minimum of 3-4 h with solution (II) prior to exhaustive destaining. On occasion only stain solution (II) was used. The gel could be preserved by soaking it in a glycerin solution. Stain solution (I) consisted of 0.05% Coomassie blue R-250 and 0.1% $CuSO_4$ in acetic acid:ethanol:water (10:65:25). Stain solution II consisted of 0.01% Coomassie blue R-250 and was otherwise, as in stain solution I. The destain solution consisted of acetic acid: ethanol:water (10:10:80), and the preservative solution was 10% glycerin in destain solution.

Fixing and staining of agarose gels involved an additional step which required dehydration of the gel (8). The agarose gel was fixed for 10 minutes immediately after focusing. The fixed gel was covered with a wet piece of filter paper and placed, filter paper-side down, on a stack of dry paper towels. A glass plate was placed over the gel backing and a 1000g weight was placed atop of this sandwich to assure even contact with the paper towels. After 15 minutes of dehydration over fresh paper towels, the gel dried to a "paper thin" layer. The filter paper was gently removed and wetted if necessary. The gel was dried with forced air or a heat lamp until it was clear and no longer tacky to the touch. The dried gel was rinsed with destain for 1-2 minutes and stained for 5-30 minutes with agitation. Destaining was accomplished by washing several times in destain solution and the gel was dried. The fixing solution consisted of 17.25g sulfosalicylic acid and 25g trichloroacetic acid in 150 ml methanol and 350 ml water. The stain solution consisted of 1.15g Coomassie blue R-250 in 1 liter destain solution which consisted of 500 ml 95% Ethanol and 160 ml acetic acid diluted to 2 liters with H_2O. Each solution was discarded after one use.

RESULTS

Disc-SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Sea Turtle Muscle Extracts: Separation of soluble sea turtle muscle protein by SDS-PAGE is presented in Figure 1 for later comparison with IEF separations. Although distinctions can be made between the various sea turtle species, there are but a few bands upon which this difference can be based. Columns 1-7 are gel patterns for suspect turtle meat samples co-electrophoresed along with authenticated sea turtle meat extracts (columns 8-16). Although tentative identity can be assigned, the decision is based on the location of approximately six bands at best. In looking at columns 8 through 16 obtained from authenticated sea turtle samples, those species closest in phylogeny have the most similar SDS-PAGE banding patterns; i.e. both ridleys (columns 10 and 11) and greens (columns 13 and 14) demonstrate this concept. Note that even though there appears to be several bands of identity for most of the sea turtle samples (area A for columns 8-11, 13, 14 and 16), the leatherback (column 9) appears to give a completely different banding pattern as does the river turtle (column 12).

Polyacrylamide Gel-Isoelectric Focusing (PAG-IEF) of Sea Turtle Muscle Extracts: Isoelectric focusing of soluble proteins extracted from sea turtle muscle samples (Figure 2), was performed in a thin-layer polyacrylamide gel to yield sharply delineated protein bands which correspond to the pI for a protein or group of proteins. In Figure 2, columns 1-4 and 12-15 correspond to authenticated turtle meat samples which were focused along with unknowns in columns 5-11. Although the authenticated samples focused in columns 12-15 suffer slightly from edge effects, surface smearing and interference at the sample wick placement site, the gel is otherwise one of the best examples of the resolving power of the isoelectric focusing technique as compared to SDS-PAGE (Figure 1). For example, 60 bands are easily discernible in column 5 and greater than 70 bands in column 10. In addition, Figure 2 may provide the best example of the similarity of the banding patterns for Atlantic (column 2) and Pacific (column 3) green turtle species, and yet still allow their differentiation; observe the banding pattern in the area encompassed by A.

Figures 3 through 6 provide additional examples of PAG-IEF of sea turtle meat extracts. Contained in Figure 3 are patterns for each of the six sea turtle species available to this study: Atlantic (AG) and Pacific (PG) green, olive (PR) and Kemp's (AR) ridley, hawksbill (HK), and loggerhead (LG). In addition, the Central American river turtle, (DM) and a Kemp's ridley juvenile (ARj) are included.

Figure 3 demonstrates several problems which may occur as a result of application of expressed tissue drip from thawed sea turtle muscle directly to the gel. "Tenting", as observed in columns 4, 5, 10, 11, 14, 15, 20 and 21 may be the result of excess salts in the sample. Streaking, as observed in 1, 3, 10, 14 and 20, arises when extracts applied to the gel do not penetrate the gel surface, but migrate on top of it. Column 21 demonstrates the result of changes in the pH gradient at the gel edge which are usually due to variations in gel thickness or to edge-running of the electrode wick solutions; curvature in the pattern results. The three olive ridley samples (PR) in the middle of the gel demonstrate the effect of sample state on density of individual bands within the pattern. These band density differences may reflect two conditions. First, the amount of total soluble protein is dependent upon the length of time the sample is held frozen and upon the condition of the meat when thawed. Second, each turtle

meat sample contains variable amounts of blood or interstitial fluid dependent upon the circumstances of death and the method of tissue storage. Thus samples 11 and 12 appear to contain more extractable protein than sample 13.

Figure 4 provides an excellent example of good resolution without many of the inherent problems associated with marine samples such as salt effects and protein precipitation. The apparent vertical lines are due to wrinkles in the cellulose cover sheet atop the gel. The heavy bands observed in the upper half of the gel are due to overloading of the sample; this condition enables the investigator to observe bands otherwise undetectable, due to the low quantity of protein comprising them and the limitations of the staining technique used. The region between pH 4.8 and 7.2 (designated B) is highly distinctive for each turtle sample. This region coupled with the less variable region between pH 7.2 and 9.5 (designated A) provides a good index for species identification of an unknown sample. Due to a lower sample load, Figure 5 allows the investigator the opportunity to examine the pH range 7.2-7.8 region (designated A) in detail. This is the region that is overloaded in Figure 4. Minor pattern differences become apparent throughout the gel that are not evident in the overloaded gel. Figure 6 is provided as an example of a gel with a moderate sample load. The river turtle (column 11), a freshwater species of significantly different morphological characteristics, yields a completely different banding pattern than those of the sea turtles. Also, although the overall pattern for sea turtle is similar for the five species, there are very specific pattern-keys to each of the sea turtle genera, Chelonia, Lepidochelys, Caretta and Eretmochelys in the pH range 5.2- 7.2. Several pattern differences can be seen between pH 5.5 and 5.8 (designated B) for Atlantic green (AG), Pacific green (PG) and olive ridley (PR) in columns 1,3, and 5. A key area for the differentiation between loggerhead (LG) in columns 16 and the other species is pH region between 6.0 and 7.2 (designated A). Figure 5 also demonstrates this reasonably well; banding-pattern differences are quite obvious in columns 18 and 20-23, in the pH region between 5.3 and 7.2 (designated B).

To further investigate the importance of the overall banding pattern in relation to recognition of a particular species, refer to Figure 7. This figure illustrates several important features of the PAG-IEF approach to speciation of meat samples. Foremost, is that the overall pattern for seven individual samples (columns 1-8) of the same species exhibit a highly replicative pattern. Columns 9-11 are duplicate applications of the same three samples applied in columns 1-3. It is apparent when comparing replicates in columns 1-3 and 9-11 that the method is highly reproducible and that minor differences in relative band densities and locations are "copied". In addition, an interesting sampling of yearlings (head-start Kemp's ridley which died in transit to the release site) in columns 4-8 was compared to three adult/subadult specimens in columns 1-3 (and duplicates 9-11). Aside from minor density variations in a few bands, the banding patterns for the yearlings were highly homologous. On the other hand, the adult/subadult banding-patterns showed some distinct banding density variations, particularly in the region of pH between 5.3 and 7.2 (designated A).

Agarose Gel Isoelectric Focusing (AG-IEF) of Sea Turtle Muscle Extracts: Agarose gels cast as broad range gels (pH range 3.5-9.5) for focusing of turtle meat samples are examined in Figures 8, 9 and 10. In Figure 8, authenticated sea turtle samples (columns 1, 2, 13-23) are compared against a "blind" selection (columns 3-12) of the same sample extracts. Figure 9 made use of the same extracts which were, focused

after one day of storage at $4^{o}C$; unknown samples are in columns 1-10 and authenticated samples are in columns 11-18. Storage for 24 hours did not cause substantial deterioration of the samples as evidenced by the clarity of patterns and density of bands. Figure 10 contains two authenticated samples for each turtle species, except river turtle and leatherback which are duplicates. Each of these gels demonstrate the sensitivity and resolution obtained with the AG-IEF method. The overall time involved in preparation, focusing, and staining is considerably less with the agarose method, and since the hazard of working with acrylamide is eliminated, the AG-IEF method is very attractive.

The resolution of a particular pH range can be improved by casting a gel with a narrower range of ampholytes. Figure 11 demonstrates the resolution of the proteins associated with the highly variable region of a pH range 3.5-9.5 gel utilizing a pH range 5.5-8.5 gel. Although the gel appears to have been cast with uneven thickness at the two ends, causing running, streaking and mishaped bands, the center of the gel serves to demonstrate the high resolving power of the technique. This narrower pH range provides such a high degree of resolving power that it is more appropriate for population studies, whereby samples of nearly identical patterns may be differentiated.

The constancy of overall pattern for two genera of sea turtle, Lepidochelys and Caretta are shown in Figures 12 and 13. Again, as observed earlier in the PAG-IEF of Kemp's ridley (Figure 7), an over-all characteristic pattern emerges for olive ridley (Figure 12). The samples are duplicate applications of ten individual extracts of olive ridley. While the greatest area of variability is observed in the pH 5.3-7.2 range in accord with the PAG-IEF pattern for Kemp's ridley close scrutiny reveals that this difference is in the density of the bands rather than representing a pattern difference. A potential area for further investigation is the pH region on either side of 5.6 where the pattern differences seem most significant. It is quite possible that a narrow range IEF gel alone or in combination with a specific enzyme stain could reveal individual differences.

Investigation of several loggerhead samples for degree of pattern homology reveals excellent replication of the protein banding pattern for seven of the loggerhead samples tested (Figure 13). An eighth loggerhead sample (columns 11 and 12) is the exception. This sample was obtained from a juvenile turtle with a carapace of 9 cm long and 7 cm wide. In addition to the presence of bands found in the other samples in the region between pH 6.7 and 7.5, there are additional bands present for the eighth loggerhead sample (columns 11 and 12 in Figure 13, A). Since these occur in the area of the gel where heme-containing proteins are usually observed prior to staining, it is possible that the extra bands represent "embryonic-type" blood proteins (fetal hemoglobin is an example) not observed in adult/subadult specimens. Minor differences in the other seven loggerhead samples were observed in the pH 5.7-6.5 range (Figure 13, B and C). Columns 3,4 and 5,6 appear to have several band density differences.

Utilization of IEF Banding Patterns of Muscle Protein Extracts to Identify Unknown Turtle Meat Samples: The observable differences in IEF protein banding patterns for each turtle genus can be utilized to identify unknown samples of suspected sea turtle meat. As a first step in the identification procedure of suspect turtle meat, the unknown sample(s) is run interspersed amongst standard samples of sea turtle (Figur 14). The unknown banding pattern (UK) is compared with those of the authenticated

samples for identity. The unknown is then run on a second gel alongside the authenticated sample(s) with which it is perceived to be most similar. As an example, the gel in Figure 15 is used to test for identity of unknowns perceived to be Atlantic green turtle during the initial testing phase. This gel confirms the species identification of the unknowns and demonstrates the constancy of IEF-patterns for the genus, Chelonia (Figure 15). Additional gels may be run to assist in identification. Changing the amount of sample applied to this gel may aid in the interpretation of a specific pH range. Figures 4-6 provide examples of changes in amount of protein applied to the gel. In addition, changes in the range of the pH gradient may be used to obtain increased resolution of a critical pH range, thereby assisting in the interpretation of that specific pH area (Figure 11).

Figure 16 illustrates the focusing of sea turtle samples received in preceding years and previously identified as olive ridley. Comparison of these samples is made with authenticated samples, one of which was received long after the original species identifications were performed (columns 3 and 7). Despite differences in total protein concentration (overall band densities), the patterns of the unknowns are highly consistent with the authenticated olive ridley patterns (columns 3 and 7).

DISCUSSION

Even a keen observer of taxonomic differences in species is often unable to distinguish closely related sub-species or populations by morphological characteristics which are, more often than not, indistinguishable. Yet these differences can be distinguished utilizing biochemical techniques such as electrophoresis coupled with specific enzyme stains or antibodies. These techniques rely on differences in specific proteins or protein groups. Protein formation is directed by genetic material, that is, DNA and RNA. Morphologic differences are a manifestation of the differences in the genetic material. Following this line of thought, it should be evident that each sea turtle species is a morphological expression of genetic differences and that these genetic differences can be observed in the direct expression of genetic material--protein. For the purpose of chemotaxonomy, protein differences can be exploited by the investigator using any of several biochemical approaches to reveal identity between authenticated samples and unknown samples. The species identification of marine animals from soluble protein extracts has been carried out by numerous investigators in the past (2-7, 12, 14). Starch gel, disc-polyacrylamide gel, and cellulose acetate strip electrophoresis have all been used with varying degrees of success. Each of these methods may produce variable protein patterns from analysis to analysis, introducing a source of error into the interpretation of the results. Isoelectric focusing, on the other hand, provides a technique which is highly reproducible due to the equilibrium nature of the focusing process. Provided that an uniform pH gradient is established, the protein pattern will be highly repetitive. The resolving power of isoelectric focusing can be at least 10 fold greater than SDS-PAGE, enabling the trained researcher to distinguish between closely related species. In theory, a "library gel" (12) may be used for comparisons of currently focused extracts against standard banding patterns for previously focused samples of authenticated species. In practice, a visual comparison is made more easily if the unknown and authenticated samples are placed in adjacent columns rather than residing on separate gels. A trained researcher can visually identify unknowns with a high degree of accuracy, as tested in double blind studies (3, 14).

Application of the IEF technique to the species identification of sea turtles is an extension of this chemotaxonomic approach (2-7, 12, 14). The process adopted by us includes the extraction of water soluble proteins from authenticated and unknown meat samples, followed by co-focusing of the extracts on either PAG-IEF or AG-IEF. Once a tentative species identification is made, the unknown is then co-focused adjacent to this species and its closest morphologic relative, for example, olive and Kemp's ridley. In the final analysis several gels may be used for the comparison before a final species identification is concluded. Specific examples of this process have been presented in the results section (Figures 2-6, 11, 12, 14-16).

The results presented herein support the contention that isoelectric focusing is a suitable method to test suspect turtle meat for co-identity with authenticated sea turtle species. A high degree of identity of the unknown with one of the standards insures the researcher that the unknown sample is in fact a turtle in the genus of the authenticated sample. Identity along all regions of the banding pattern with but a few minor exceptions confirms species identity. This is illustrated in Figure 8 for olive versus Kemp's ridley, and Atlantic green versus Hawaiian green sea turtles. On the other hand, lack of identity over a major portion of the pattern assures the investigator that the unknown sample is not related to any of the genera of turtles used as standards (Chelonia, Lepidochelys, Caretta, Eretmochelys, Dermochelys and Dermatemys). Again, Figure 8 demonstrates the dramatic difference in IEF patterns between well separated genera of turtles; compare the pattern for D. mawi, a small river turtle with that for the sea turtles.

Although the number of standard sea turtle samples in our possession is limited, Figures 12-14 and 16 demonstrate the constancy of pattern derived from a specific species. Mackie (16) and Tsuyuki et al. (17) have demonstrated electrophoretic patterns for fish muscle extracts to be, generally, independent of age, sex, and physiological state. Because of limited sea turtle sample access, it has been impossible to explore the effect of sex, age and physiological state in a clear-cut manner. It is possible, though, to deduce from several of the available knowns, that hatchling and juvenile (10-15 cm length) turtles yield some additional features in their protein banding patterns (Figure 7 and 13) from those of adult/sub-adult turtles. These differences, though apparent, will not normally enter into the identification process of meat shipments arising from the slaughter of mature turtles for commercial profit, due to the lack of access to these turtles and to the fact that only a few grams of meat can be obtained from a turtle weighing less than 500 grams.

In conclusion, the problem of identification of meat samples suspected to be of sea turtle origin can be solved by the use of the isoelectric focusing technique. Both polyacrylamide gel isoelectric focusing (PAG-IEF) and agarose isolectric focusing may be used to identify the species of suspect sea turtle meat. While each method has individual advantages, both draw upon the unique resolving power of the method. Closely related species within a single genus can be readily distinguished by the trained researcher.

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Our deepest gratitude is extended to each and every individual who took the special effort and care to package and forward sea turtle tissue to us. Without the cooperation of the "turtle people" our efforts to develop this method would never have been possible. We would like to thank both Judith Krzynowek and Ronald Lundstrom for their encouragement, and excellent technical advice on isoelectric focusing of marine species. A special thank you goes to the law enforcement agents, whose zest for supporting the Endangered Species Act, spurred on our research when it seemed like an impossible feat.

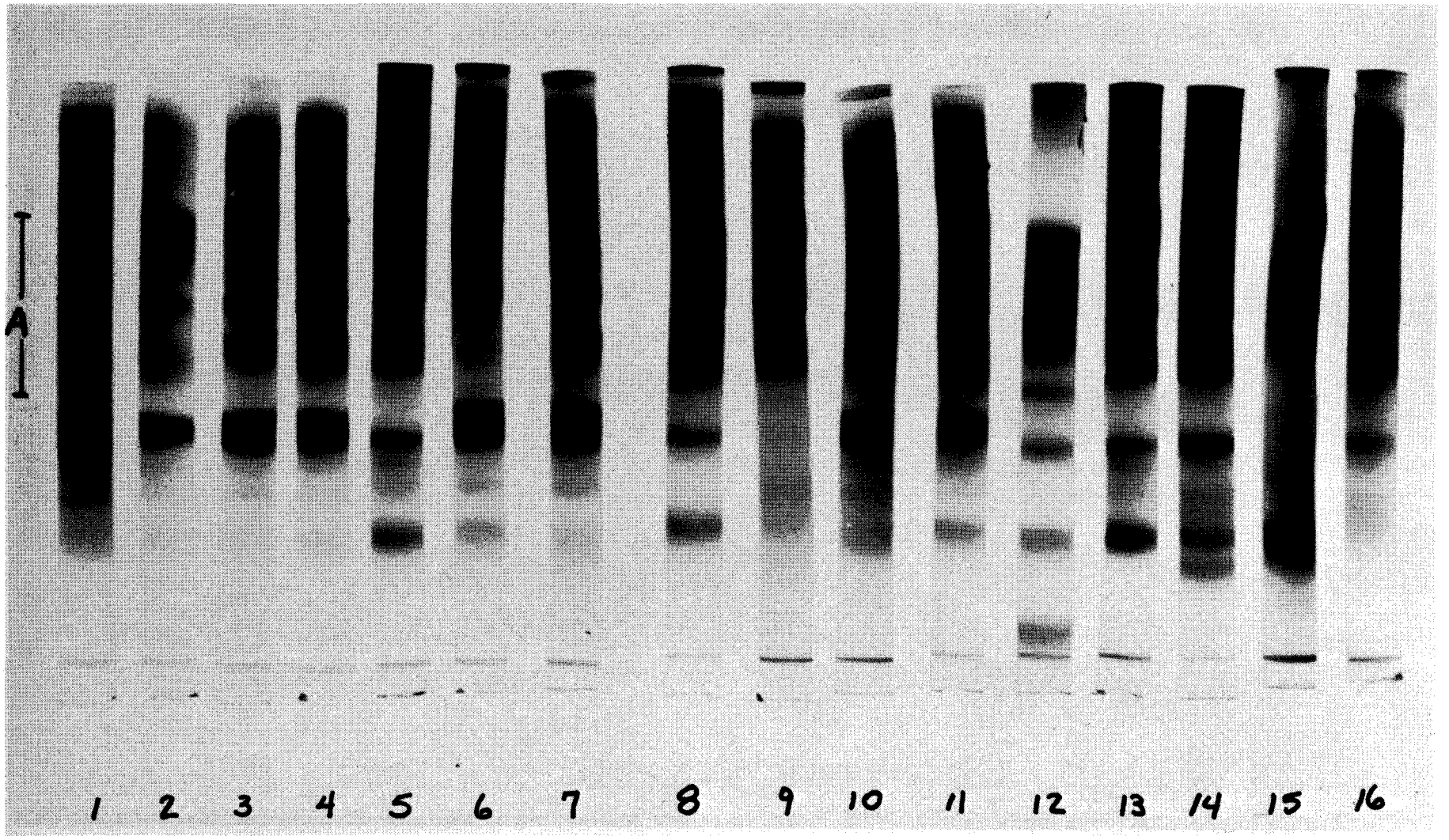

Figure 1. PAGE-disc gels of sea turtle muscle extracts. Gel columns 1-7 were unknown turtle meat samples; gel columns 8-16 were authenticated turtle meat samples: (8) loggerhead (LG), (9) hawksbill (HK), (10) olive ridley (PR), (11) Kemp's ridley (AR), (12) Dermatemys mawi (DM), (13) Atlantic green (AG), (14) Pacific green (PG), (15) leatherback (LB), (16) hawksbill (HK). The gels consisted of a running gel which was 5.0% in acrylamide and 0.8% bis-acrylamide, and a stacking gel which was 3.0% in acrylamide and 0.8% bis-acrylamide (8, 9).

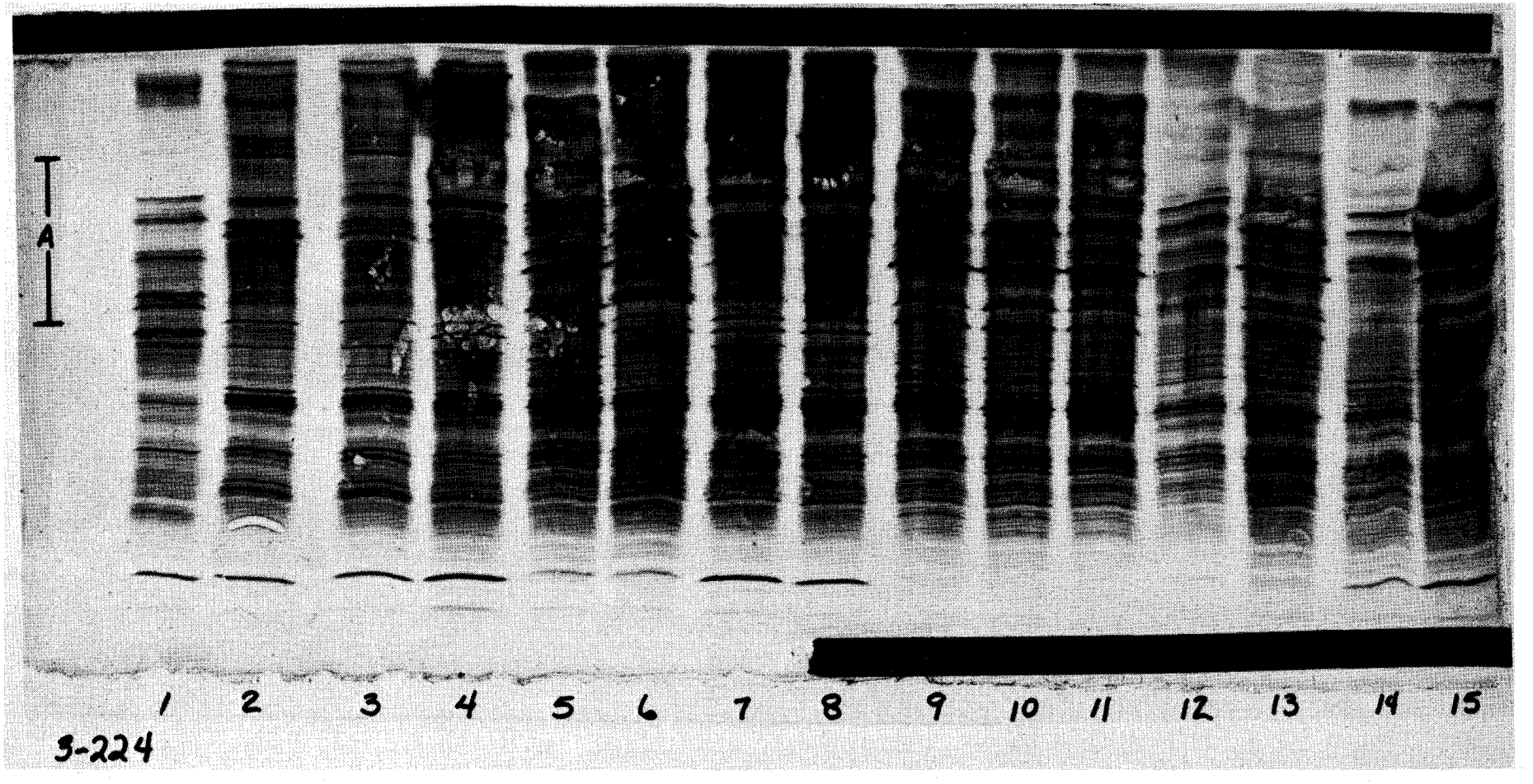

Figure 2. PAG-IEF of turtle muscle extracts on a pH gradient of 3.5-9.5 (laboratory prepared). Conditions were 1.8 kV max, 20 W and 200 mA max. The gel was focused for 1/2 hour followed by removal of wicks and focusing for two more hours. Samples 1-4 and 12-15 were standards and 5-11 unknowns: 1) D. mawi, (2) Pacific green, (3) Pacific green, (4) Atlantic green, (12) olive ridley, (13) olive ridley, (14) Kemp's ridley, (15) Kemp's ridley.

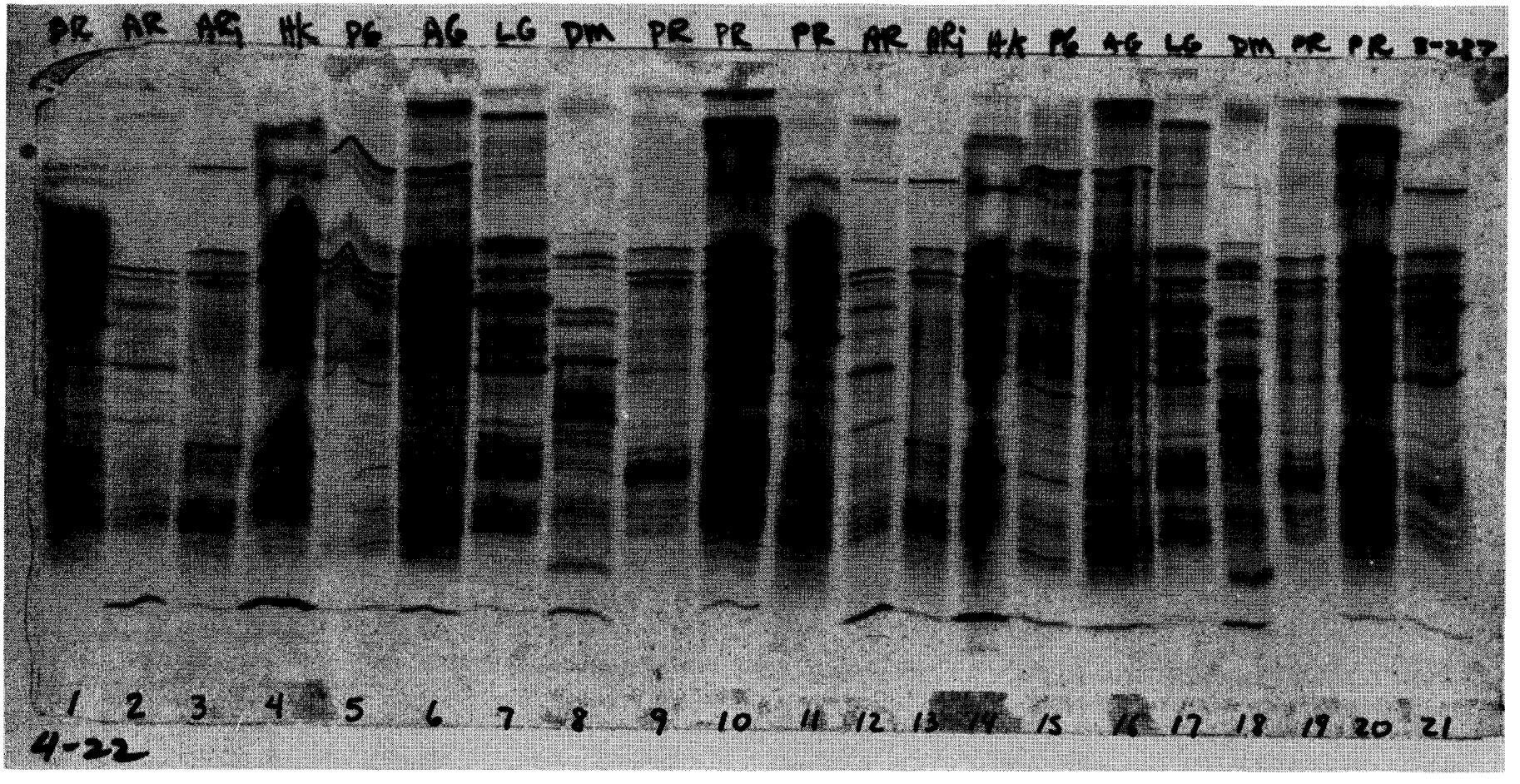

Figure 3. PAG-IEF of turtle thawed muscle drip on a pH gradient of 3.5-9.5 (LKB PAG-plate). Conditions used for focusing were 1.8 kV max, 25 W, and 200 mA max. The gel was focused for 35 minutes with sample wicks in place, and two hours following removal of the wicks. Columns 1-10 and 21 utilized full wicks and columns 11-20 used 3/4 wicks. Authenticated samples were as labeled: (PR) olive ridley, (AR) Kemp's ridley, (ARj) Kemp's ridley juvenile, (HK) hawksbill, (PG) Pacific green, (AG) Atlantic green, (LG) loggerhead, (DM) D. mawi. An unknown sample was focused in column 21.

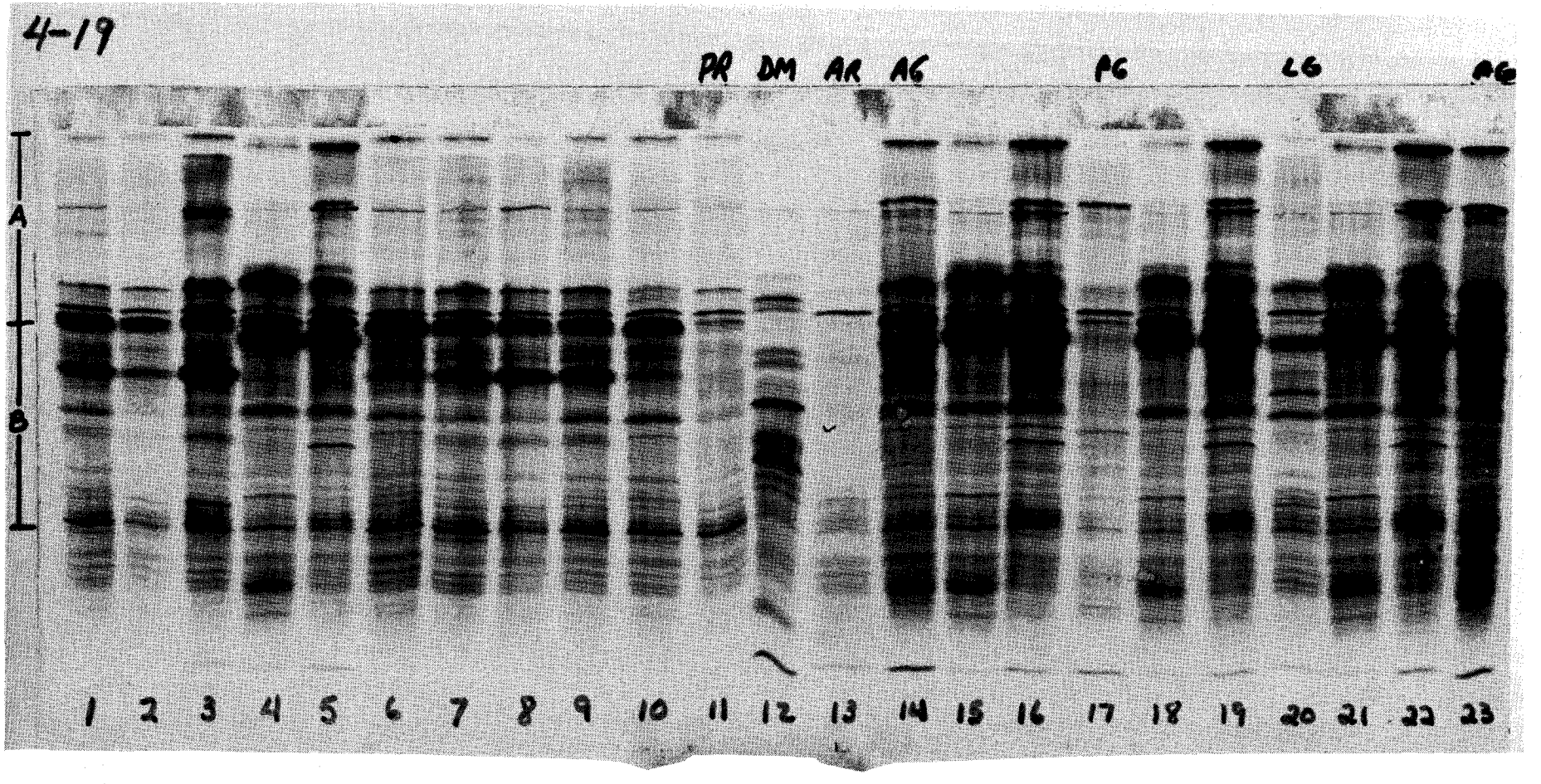

Figure 4. PAG-IEF of turtle muscle extracts on a pH gradient of 3.5-9.5 (LKB PAG-PLATE). Conditions used for focusing were 1.8 kV max, 25 W and 200 mA max. The gel was prefocused for 35 min and focused for 2 hours after sample placement. Standards were focused in columns 11-14, 17, 20 and 23 as labeled. Unknowns were focused in columns 1-10, 15, 16, 18, 19, 21 and 22.

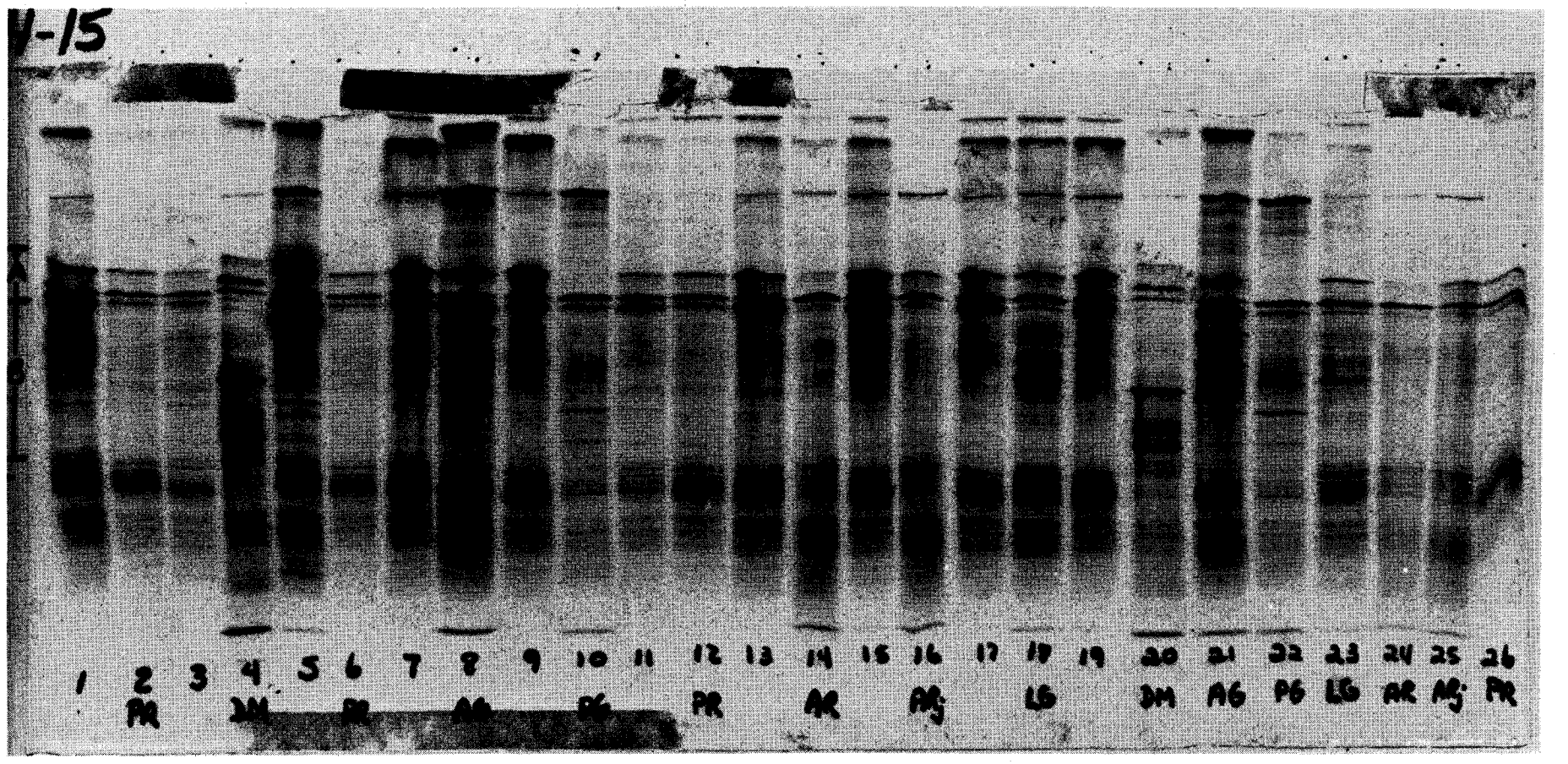

Figure 5. PAG-IEF of turtle muscle extracts on a pH gradient of 3.5-9.5 (LKB PAG-plate). Conditions used for focusing were 1.8 kV max, 25 W and 200 mA max. The gel was focused for a total of 2.5 hours. Standards were focused in even numbered columns through 20 and columns 21-26 as labeled. Unknowns were focused in odd numbered columns through 19.

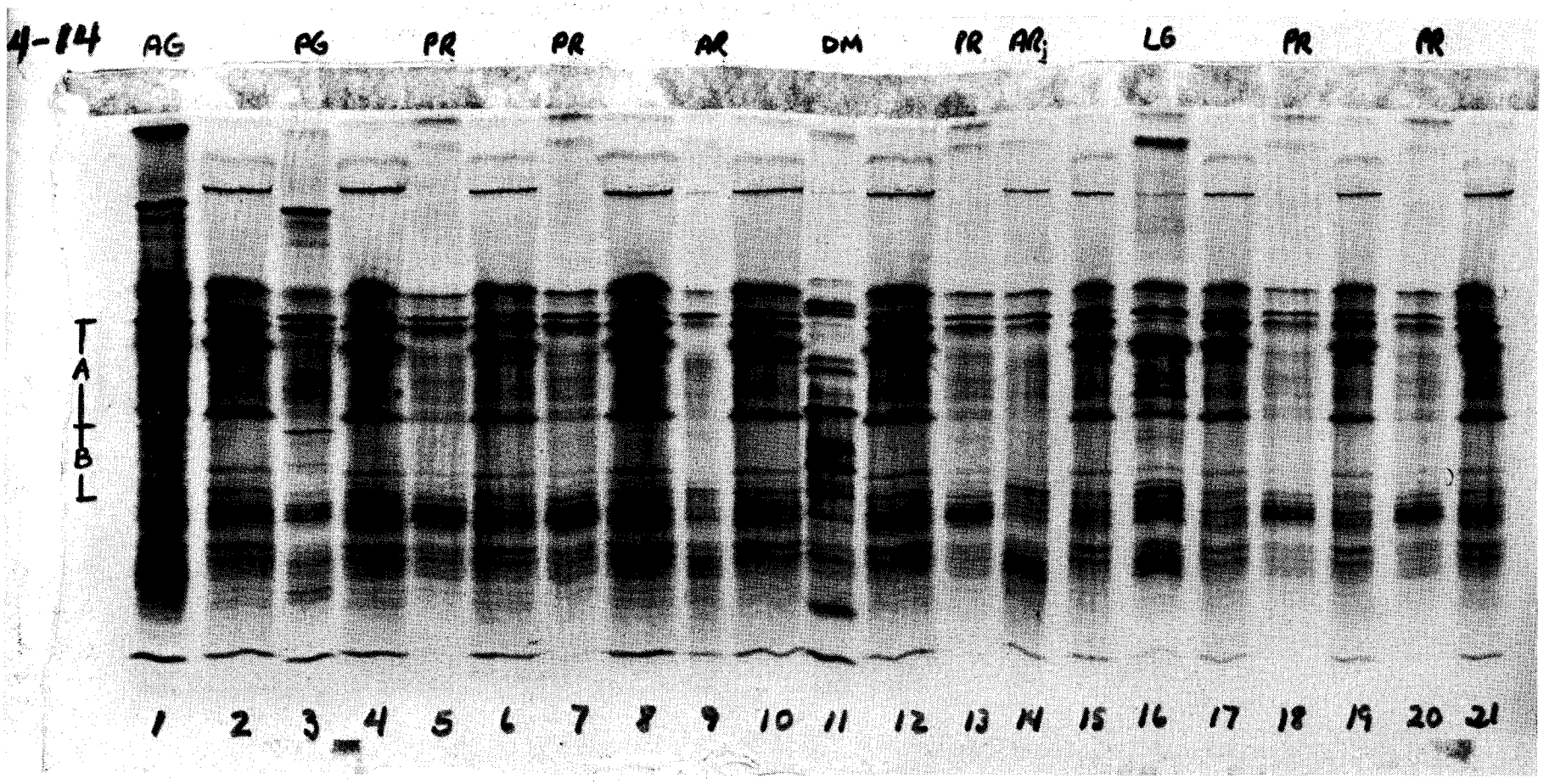

Figure 6. PAG-IEF of turtle muscle extracts on a pH gradient 3.5-9.5 (LKB PAG-plate). Conditions used for focusing were 1.8 kV max, 22 W and 200 mA max. The gel was focused for a total of 2 hr 10 min. Standards were focused in columns 1, 3, 5, 7, 9, 11, 13, 14, 16, 18 and 20 as labeled. Unknowns were focused in columns 2, 4, 6, 8, 10, 12, 15, 17, 19 and 21.

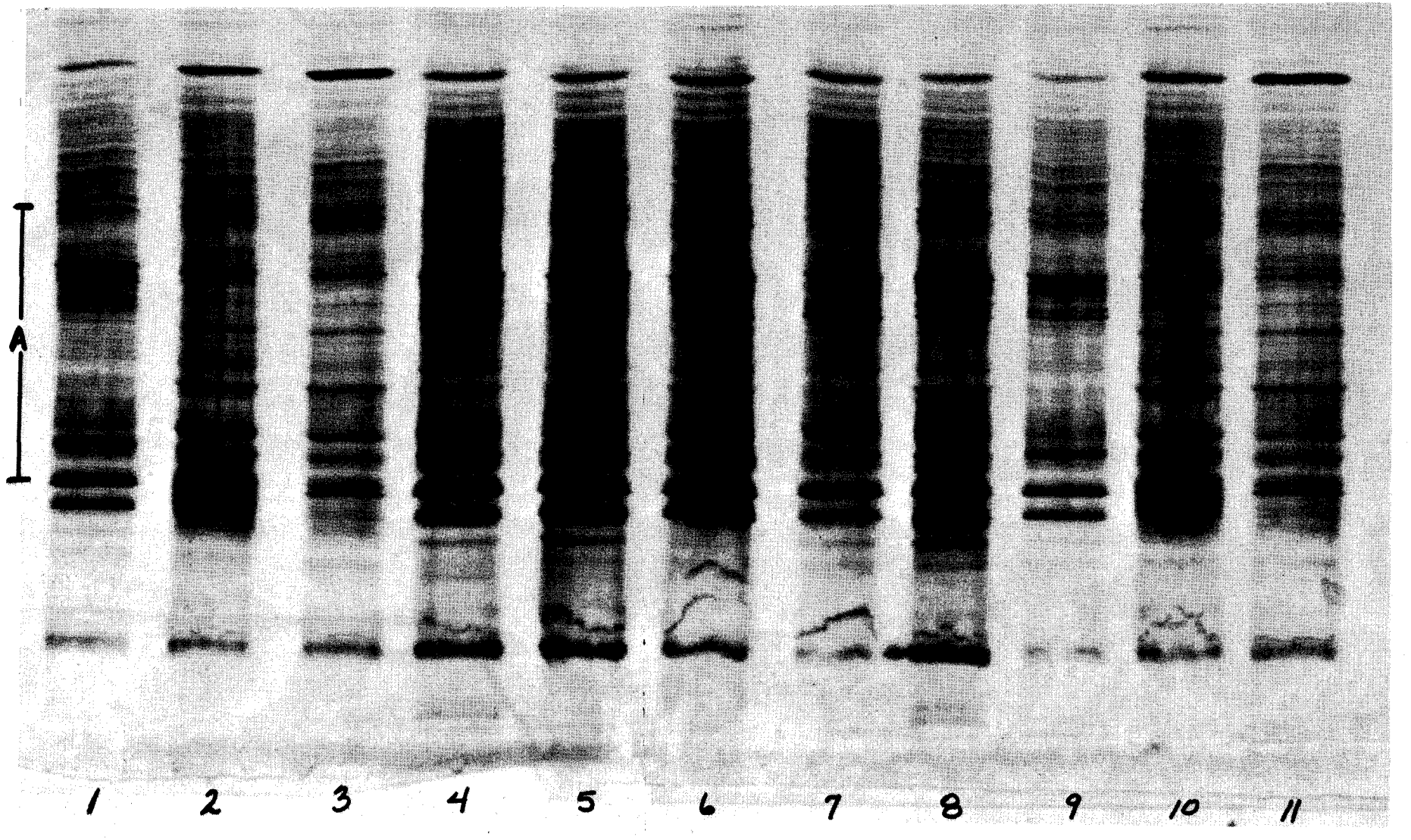

Figure 7. PAG-IEF of Kemp's ridley muscle extracts on a pH gradient of 3.5-9.5 (1.5 mm laboratory prepared gel). Conditions used for focusing were 1.8 kV max, 20 W and 200 mA max. The gel was focused for a total of 2 hr 50 min. Columns 1-3 and 9-11 were Kemp's ridley (AR) mature and columns 4-8 were Kemp's ridley juveniles (ARj), approximately one year old.

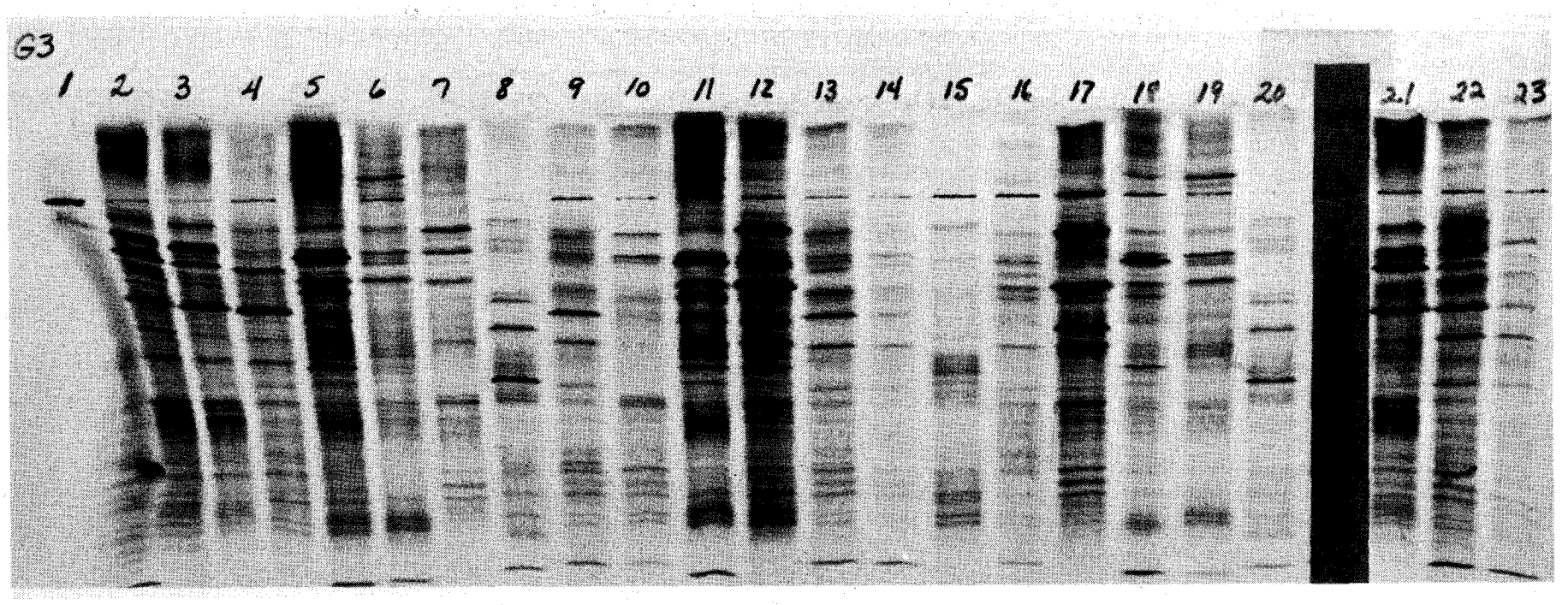

Figure 8. AG-IEF of authenticated turtle muscle extracts on a pH gradient of 3.5-9.5. Conditions used for focusing were 1.5 kV max, 25 W and 50 mA max. The gel was prefocused for 5 min at 15W and focused for 30 min. Columns 1, 2 and 13-23 were standard samples. Columns 3-12 contained a random "blind" selection of the standards and were considered as unknowns. The authenticated samples were: 1, 2, 21(PR); 13, 14, 22, 23(AR); 15(LB); 16(HK); 17(LG); 18(AG); 19(PG); 20(DM).

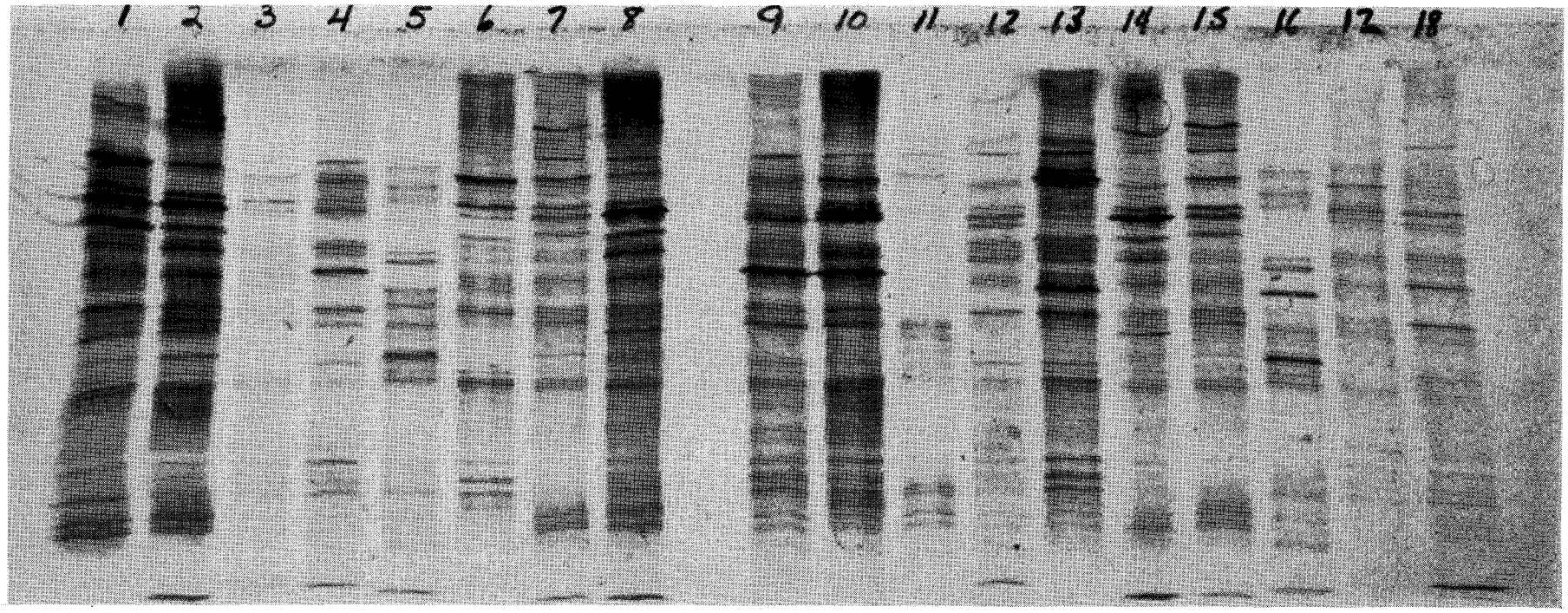

Figure 9. AG-IEF of turtle meat samples on a pH gradient of 3.5-9.5. Conditions used for focusing were 1.5 kV max, 25 W and 50 mA max. The gel was focused for 30 min. Authenticated turtle meat samples were in columns 11-18 as labeled, while columns 1-10 contained a "blind" sampling of the authenticated samples.

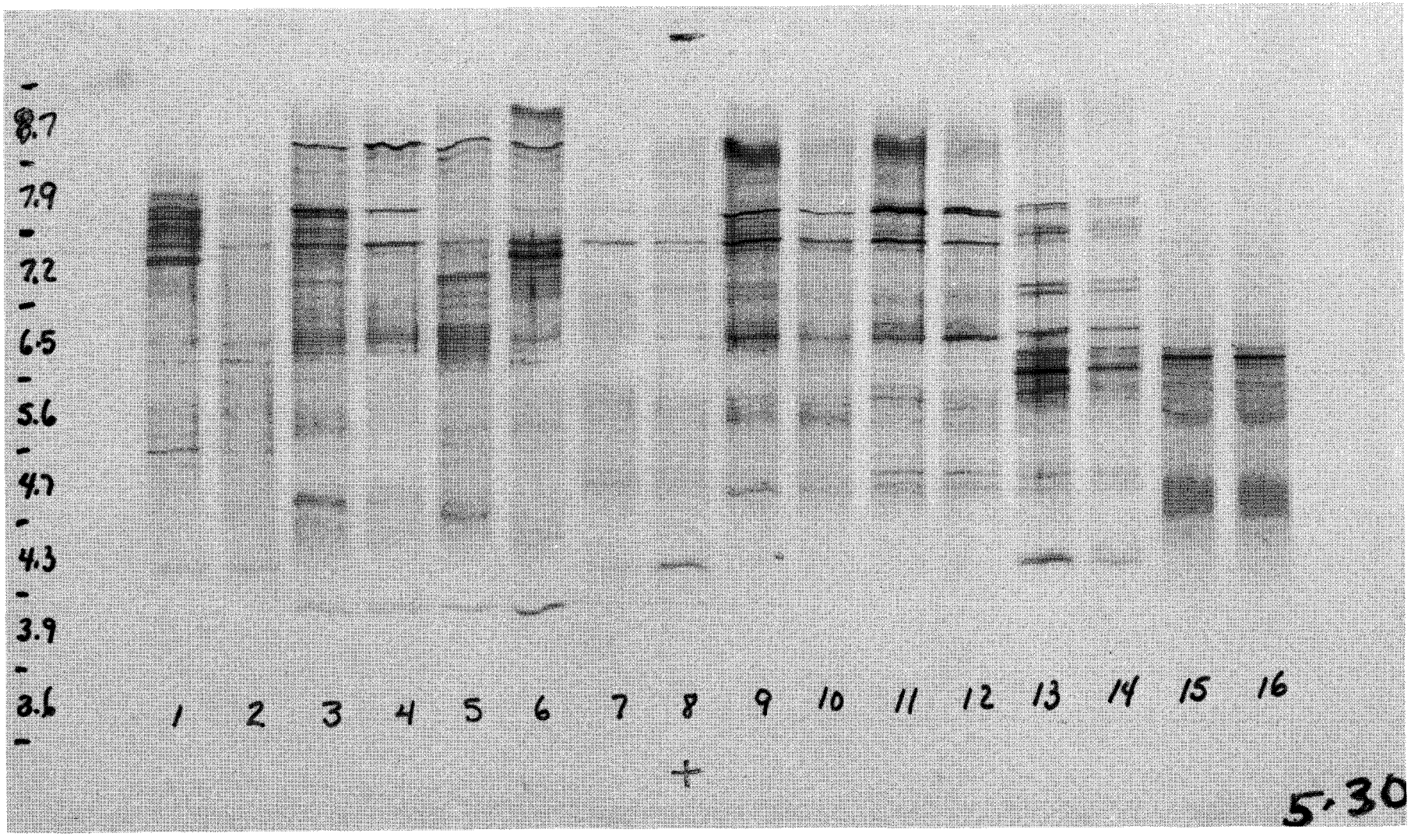

Figure 10. AG-IEF of turtle meat samples on a pH gradient of 3.5-9.0. Conditions used for focusing were 1.5 kV max, 30 W and 50 mA max. The gel was focused for 25 min. The samples were: columns 1 and 2(HK); 3 and 4(PG); 5 and 6(AG); 7 and 8(AR); 9 and 10(PR); 11 and 12(LG); 13 and 14(DM); 15 and 16(LB). All columns were unique individual samples, except those for DM and LB which were duplicates of a single sample.

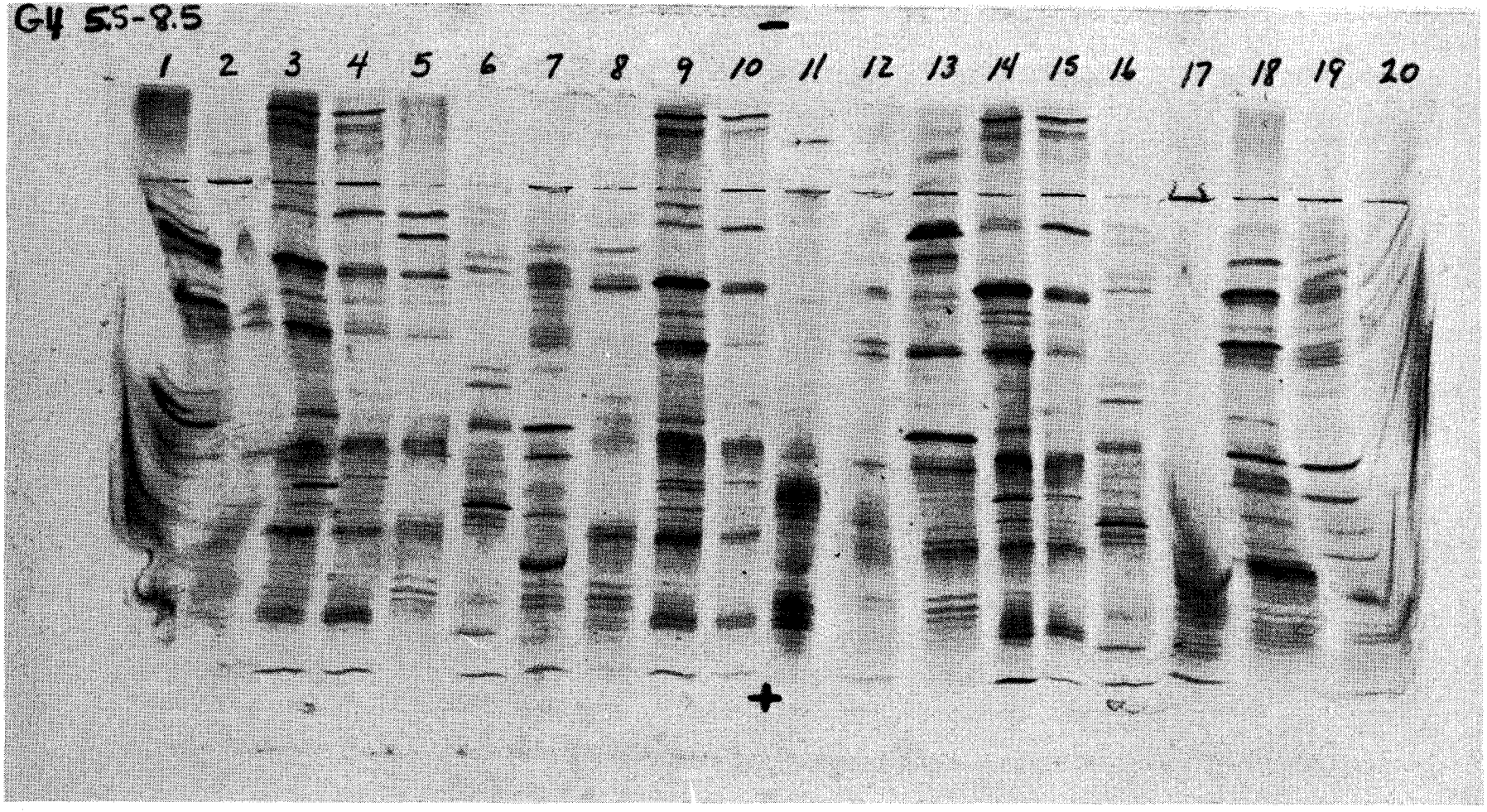

Figure 11. AG-IEF of turtle meat samples on a pH gradient of 5.5-8.5. Conditions used for focusing were 1.5 kV max, 25 W and 50 mA max. The gel was focused for 30 min. The samples were: 1, 2, 13, 14(AR); 3, 4, 20(PR); 5, 15(DM); 6, 17(PG): 7, 12, 18(AG); 8, 16(LG); 9, 19(HK); 10, 11(LB).

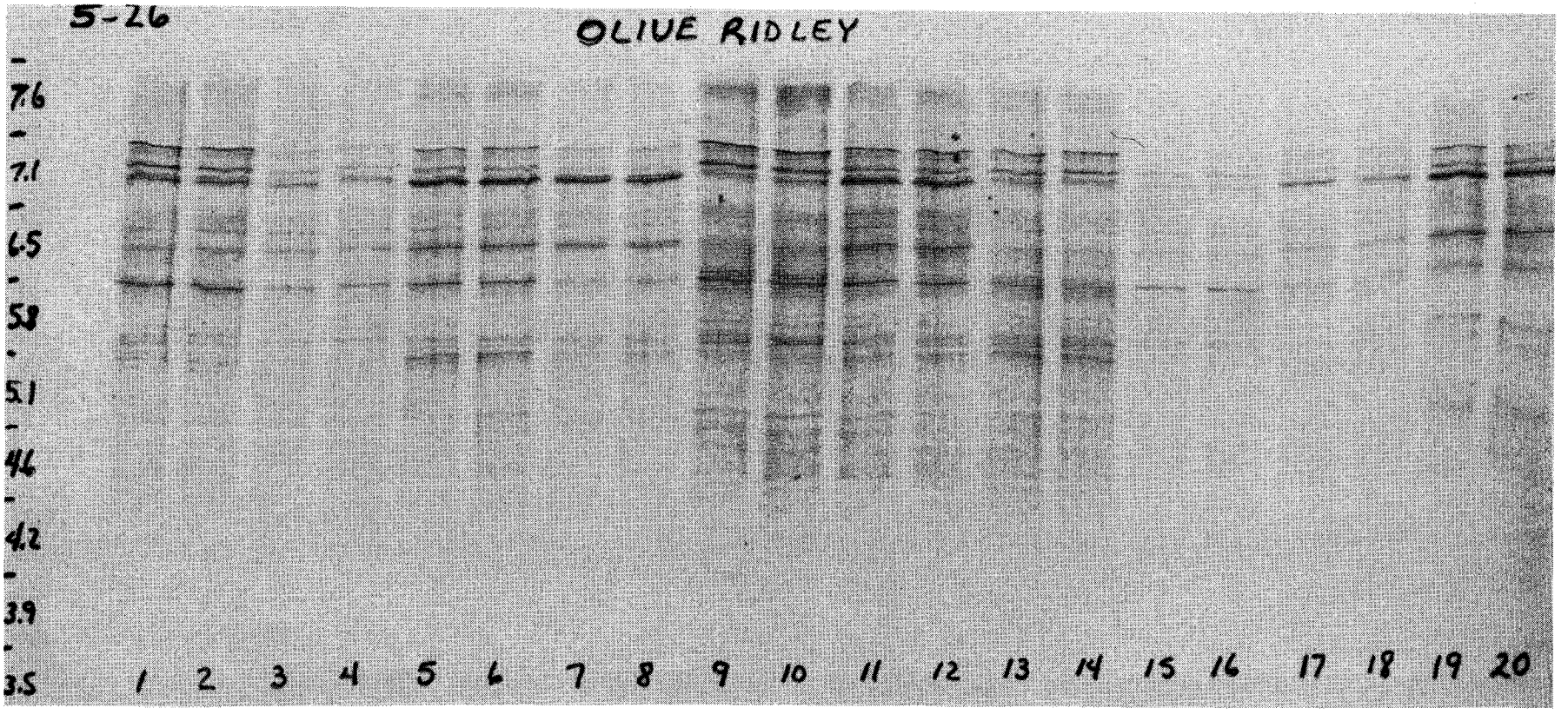

Figure 12. AG-IEF of olive ridley muscle extracts on a pH gradient of 3.5-9.0. Conditions used for focusing were 1.5 kV max, 30 W and 50 mA max. The gel was focused for 35 min. Each individual sample was run in duplicate, side by side; i.e., 1 and 2 are duplicates as are 3 and 4, etc.

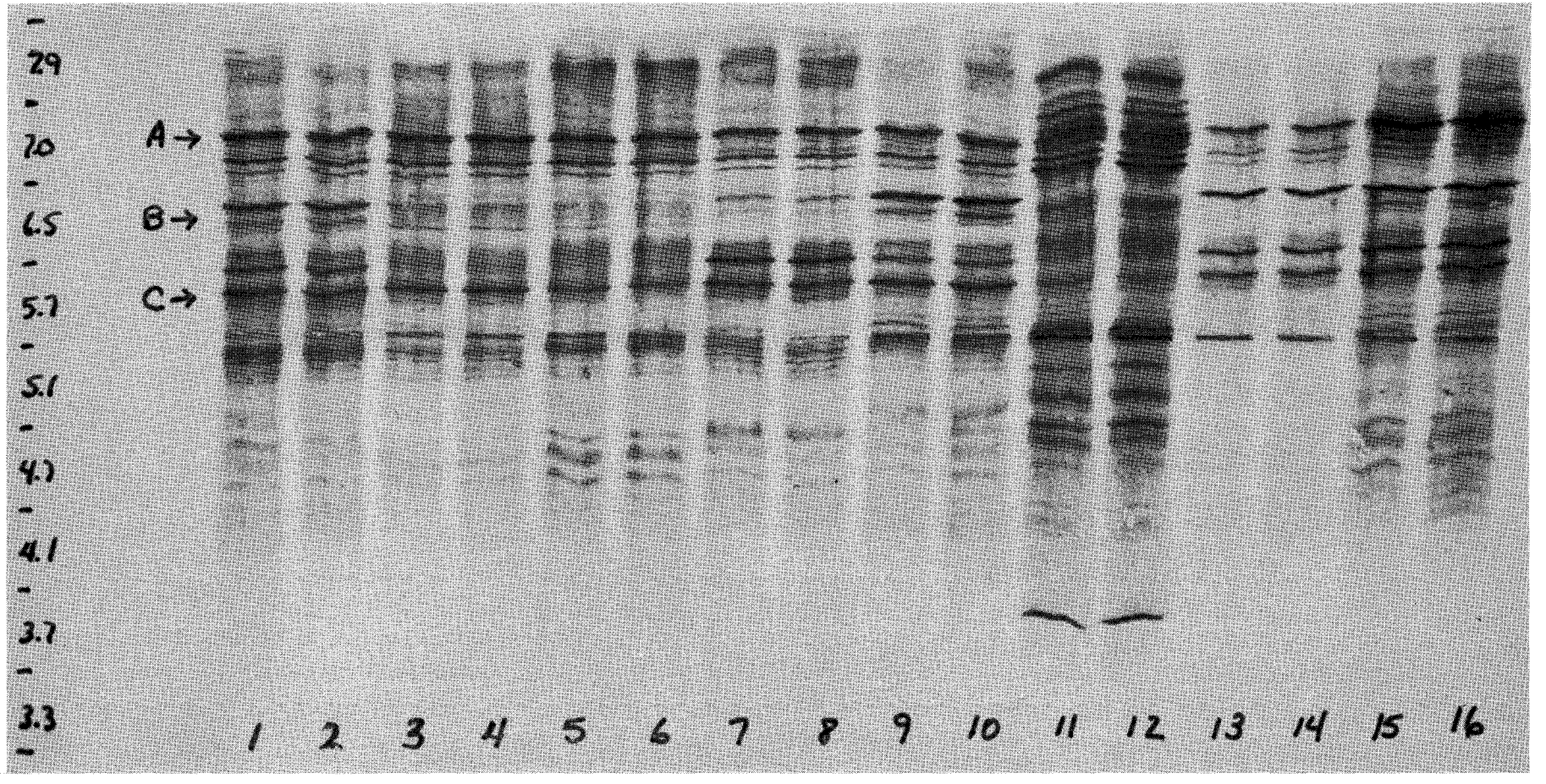

Figure 13. AG-IEF of loggerhead muscle extracts on a pH gradient of 3.5-8.0. Conditions used for focusing were 1.5 kV max, 30 W, and 40 mA max. The gel was focused for 40 min with the sample foil in place during the entire focusing period. The sample size was 2.5 ul per lane and each sample was run in duplicate, side by side.

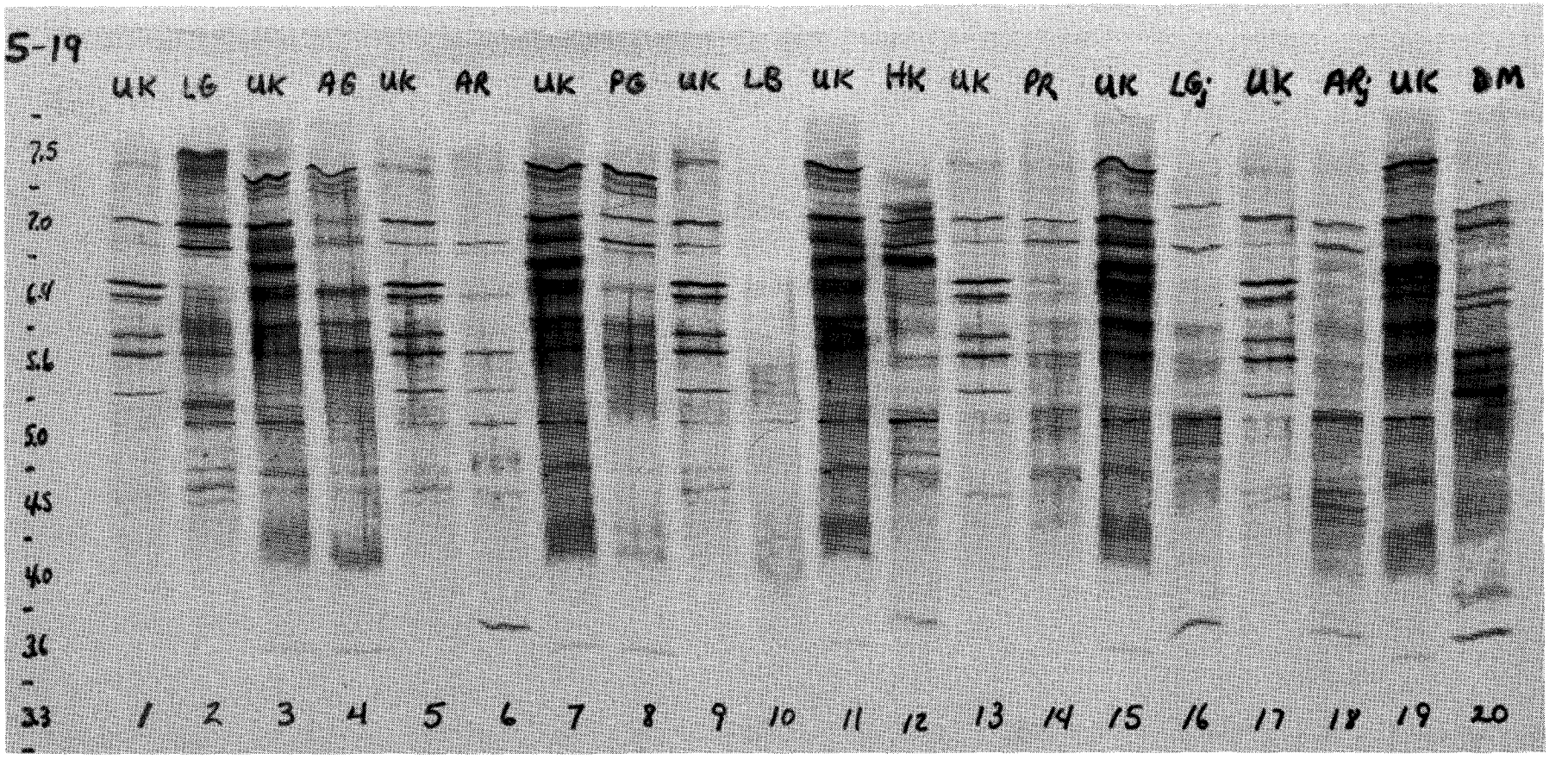

Figure 14. AG-IEF of turtle meat samples on a pH gradient of 3.3-8.0. Conditions used for focusing were 1.5 kV max, 30 W and 50 mA max. The gel was focused for 35 min. The sample size was 3 ul. The samples were as labeled.

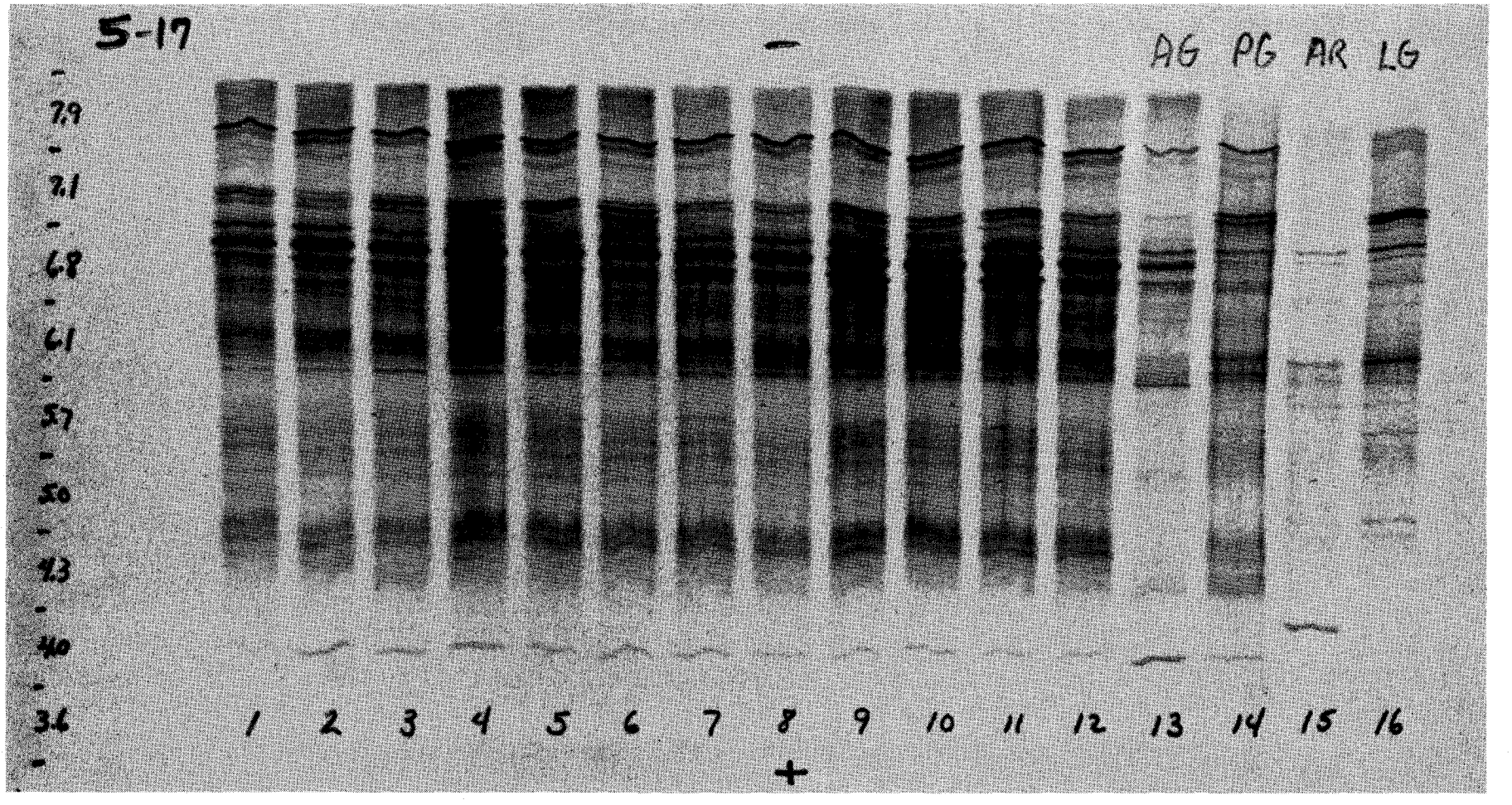

Figure 15. AG-IEF of turtle meat samples previously identified as Atlantic green. Conditions used for focusing were 1.5 kV max, 30 W, and 50 mA max. The gel was focused for 30 min. Authenticated Atlantic green was in column 13 with other authenticated samples in columns 14-16 as labeled.

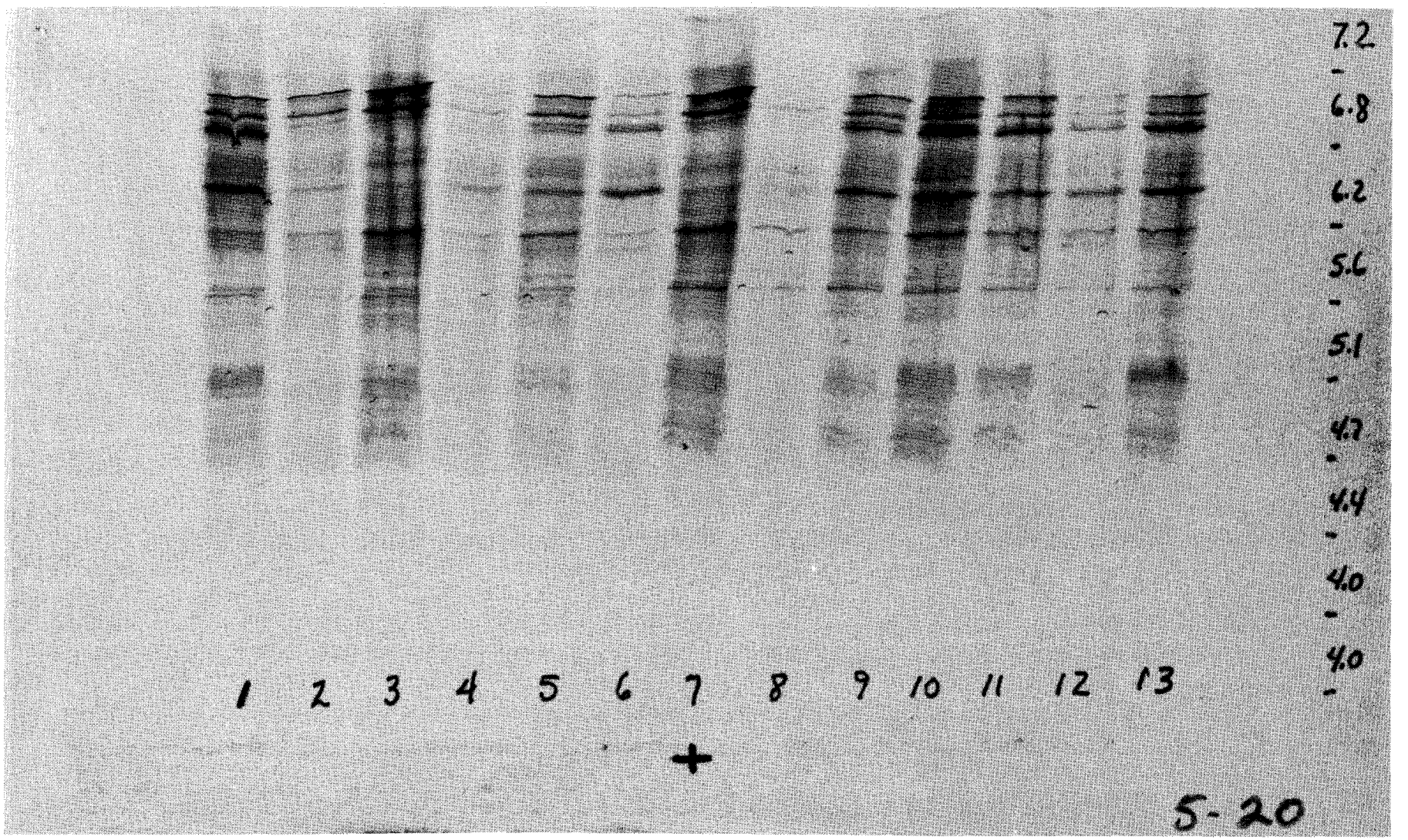

Figure 16. AG-IEF of turtle meat samples, previously identified as olive ridley, on a pH gradient of 4.0-7.5. Conditions used for focusing were 1.4 kV max, 25 W and 50 mA max. The gel was focused for one hour. Columns 3 and 7 contained an authenticated olive ridley sample.