Species	Fluid analysed	Mean Protein concentration %	Methods	Number of fractions	Remarks	Reference
Mudfish Barbel Tilapia	P P P	3,84-4,56 5,76 4,72	Polyacrylamide gel and Lowry	20 17 22	Marked differences Between S and P	Hattingh, 1972
Salmon	P	6,6	-	-	Season and spawning activity lowers the value	Robertson et al, 1961
Mackerel Carp	S	5,6 4,8	Paper electrophoresis	7	Season and culter influence value	Saito, 1957
Goldfish	P	3,6	Colorimetry	-	No sexual difference	Summerfelt et al, 1967
Carp	-	3,9	-	-	Starvation causes reduction	Sorvachov, 1957
Salmon	S	-	Paper electrophoresis	5	Increased temp. decreases A/G ratio. Photoperiod no effect	Meisner and Hickman, 1962
Rainbow Trout	P	-	Biuret. Paper electrophoresis	5	Method of capture and shock influences value of protein and fractions	Bouch and Ball, 1966

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3. MATERIALS AND METHODS

3.1 Animals

The fish used in this study were all healthy and sexually mature animals. They were either netted in various areas of the Vaal River or obtained from Provincial Fisheries in the same season (see tables 2, 4, 6 and 8). At the slightest sign of injury due to capture or transportation, the fish were discarded. In some cases blood was immediately drawn as the fish left the water, and in other cases the animals were transported to the laboratory in large oxygen supplied tanks and left for two weeks in aquaria in the laboratory at room temperature (see p41). The temperature difference between river and aquaria was minimal and all fish used were acclimatized to temperatures of 18 - 21°C. The fish which were kept in the aquaria were fed on trout pellets.

In tables 2, 4, 6 and 8 the sexual stage of the fish is indicated with a number after the sex. After blood was drawn the the abdomen was opened and the sex determined. If the fish was in a prespawning, spawning or postspawning condition this was indicated with the numerals 3, 4 or 5 respectively. In all other cases (without numerals) the animals were in a resting condition.

The weight of the fish was determined with a Mettler P 11 balance and the length measured was the forklength.

3.2 Plasma proteins

Prior to sampling of blood individual animals were anaesthetized in aqueous tricane methanesulphonate (MS 222 - 75 mg/L). Blood samples were then collected by cardiac puncture (Klontz and Smith, 1968), drawn up slowly in 5 ml syringes (containing a film of heparin - 5,000 units/ml). The fish were agitated as little as possible. In the case of the barbel it was found necessary to add a little more heparin then usual.

Plasma was obtained by centrifuging the blood in a Christ micro-centrifuge at 15,000 r.p.m. for 3 min (19Q,000 g). The fluid so obtained was immediately analysed.

3.2.1. Plasma protein content:

The plasma protein content was determined according to the method of Lowry et al (1951).

3.2.2 Electrophoresis

(a) Cellulose-acetate electrophoresis

For paper electrophoresis, a Beckman microzone chamber was employed using cellulose-acetate paper. This equipment was powered by a Beckman Duostat. Diethylbarbiturate buffer was used at a pH of 8,6, ionic strength 0,075. Plasma samples of 1 µ l were used. All runs were for 1 h at a constant voltage of 250 V and at a starting current of 3,5 mA and terminal current of 9,5 mA. The elec-

trophoresis papers were stained with Ponceau S and dried in the customary manner. They were scanned with a Beckman Densitometer R110 equiped with an integrator.

(b) Polyacrylamide gel electrophoresis

For the other method of electrophoresis, an Acrylophor electrophoretic chamber was employed using 5% polyacrylamide gels (see p 20) and applying 3 - 4 µ l of plasma. This chamber was powered by a Pleuger powerpack and the samples were run for 25 min. at 80 V and then for another 35 min. at 160 V in Tris-glycine buffer, pH 8,5. The gels were stained with amido black, destained in the customary manner in 5% acetic acid and scanned in the same Beckman Densitometer.

In both cases the electrophoretic fractions were assigned numbers in order of increasing mobility. Human blood was treated in the same way for comparison.

3.2.3 Separation and related aspects

In the case of Labeo umbratus, Labeo capensis and Barbus holubi one fraction (from the polyacrylamide electrophoretogram) was chosen and attempts were then made to obtain this fraction in pure form (see p 32).

Initially salt precipitation with sodium sulfate was carried out for 3 hours at 37°C. The salt concentrations used were 11%, 14%, 18%, 22% and 26%. This method is according to the

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scheme of Howe (1923 a and b) and the salt was added to 0,5 cc plasma to give the required concentration. The various mixtures were then subjected to electrophoresis to see the effect. Control experiments were performed to see the effect of this high temperature on the pattern of normal blood and it was found that the patterns before and after the three hour exposure were identical.

Precipitation with 26% sodium sulphate was found to be the most effective (see p 32) and 3 cc. plasma from all three fish were subsequently precipitated with this concentration. After 3 hours the suspension was spun down in a Sorvall refrigerated centrifuge at 30,000 g for 10 min. and the supernatant liquor was subjected to one or more of the following separation procedures:

(a) Preparative electrophoresis (Shandon), on 5% polyacrylamide containing 0,1% SDS. Tris-glycine buffer, pH 8,5, was used both for electrophoresis and for elution (separation achieved at 350 V and 30mA). Elution was carried out at $0^{o}C$ at 12-15 ml/h and was recorded with a LKB Uvicord recorder at 254 nm. Fractions of 2ml each were collected with a refrigerated Ultrorac fraction collector. Absorbance of all fractions obtained were also read in a Perkin-Elmer spectrophotometer at 260 and 280 nm. 25 µ L of the fractions obtained were run on 5% polyacrylamide-gel electrophoresis for analytical purposes.

(b) Chromatography on Sephadex G-100 equilibrated with Tris-HCl buffer, pH 7,5 and 0,05 M. Column sizes were 2,5 x 37,5 cm; 1,5 x 65 cm and 2,5 x 93 cm respectively for the

mudfish, yellowfish and barbel. Elution and recording were carried out as in (a) above. The same columns were used for approximate average molecular weight determinations according to the method of Andrews (1965) using analytically pure albumin (Fraction V), cytochrome C, vitamin B_{12}, alchohol dehydrogenase and blue dextran to standardize the columns (see p 35).

(c) Isoelectric focusing carried out in a LKB 8010 electrofocusing column using a linear 0-50% sucrose density gradient and LKB ampholine of pH range 5 - 8 at a concentration of 1%. The column was maintained at $0^{o}C$ and electrofocusing was carried out for 72 hours at a potential of 700 V.

After completion of electrofocusing, 2,0 ml fractions were again collected and read. pH was measured with a Radiometer model 25 pH meter to an accuracy of 0,02pH units.

After separation and visualization of the fractions, the aliquots containing the desired protein were pooled and dialyzed against several changes of distilled water at $0^{o}C$. The sample so obtained was freeze-dried in a Virtis freeze-drier and weighed on a Mettler 5 decimal place balance.

Absorption spectra of the proteins were obtained on a Perkin-Elmer spectrophotometer and the titration curve of a Labeo umbratus protein was performed on a Radiometer titrigraph and autoburette ABU 13, using 0,01 N NaOH and HCl.

Minor experimental details will be described in the various sections.

3.2.4 Chemicals.

All chemicals used were analytically pure (A.R.) and most were obtained from Merck. The MS 222 used was obtained from Sandoz. The ampholine used for iso-electric focusing was obtained from LKB and the albumin, cytochrome C, Vitamin B_{12}, alcohol dehydrugenase and blue dextran used for standardization of the columns, from Seravac.

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4. RESULTS

4.1 Cellulose-acetate and polyacrylamide gel electrophoresis

Preliminary experiments were performed on both methods to determine the exact times necessary for proper separation (within the limits of the technique) and it was found that 1 hr was adequate in the case of paper electrophoresis at 250 V. In the case of gel electrophoresis, 25 min. at 80 V and 35 min. at 160 V sufficed. In the latter case it was also found necessary to see the effect of the concentration of acrylamide on the separation and experiments were performed with 3%, 5%, 7½%, 10%, 12½% and 15% gels. Typical results so obtained are shown in plates 1 and 2. The 3% gels presented a diffuse picture in the case of some bands and it was found that the 5% gel showed a well defined pattern with good resolution. All subsequent studies were therefore performed on 5% gels.

4.1.1 *L. capensis* electrophoresis

The fish used were all caught in the Vaal River (Barrage) except for 2 which were obtained from a locality down river (Villiers). On paper electrophoresis, the plasma separated into 10 components. Table 2 summarizes all the data obtained on 22 fish and an immediate observation is the big variations in both hematocrit and plasma protein concentration. A constant pattern was obtained on electrophoresis which is shown on figure 1. Variations in the percentage protein were observed in various fractions, especially in fractions 5, 7 and 9. These variations could not be correlated with length,

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weight, sex or hematocrit (see table 2). Compared to the pattern of the human (fig. 1), it can be seen that different mobilities are involved and care should therefore be applied in naming the fractions of L. capensis after the human nomenclature. It might indeed be argued that the patterns are very similar and that the fish one is not as expanded as that of the human. Fractions 2 and 3 would then represent gamma globulins and fibrinogen; fractions 4 and 5 the $beta_2$ globulins; fraction 6 the $beta_1$ globulins; fractions 7 and 8 the $alpha_1$ and $alpha_2$ globulins and fractions 9 and 10 the albumins. In later sections (p 28) this argument shall be shown to be incorrect.

From table 2 it is clear that the major fractions present are 5, 6, 8 and 9 and they represent a mean of 7,94, 6,94, 4,33 and 4,59 mg/ml protein respectively.

In the case of polyacrylamide electrophoresis, a more complicated picture was obtained. Plate 3 shows the typical patterns obtained with that of the human on the left. This is again a fairly constant pattern but variations are evident in the middle third of the pattern in the percentage protein of various fractions. This variation is illustrated more clearly in fig. 2 and table 3. The "normal" or "standard" pattern is the full pattern in fig. 2 and the prominent peaks are 1, 3, 6, 9, 13, 18 and 20 - 22. The variations are found in peaks 13 - 16. In a small percentage of fish two prominent peaks, 13 and 14 are found and in isolated cases an extra peak 16 is

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observed. In other cases, no peak 13 is present, but a peak 14. The differences between prominent peak 13 or 14 and also between 13 and 14 (if both are present) from different fish, might be due to small differences in experimental technique (variation in pH) but on the other hand, these might indeed represent different fractions. The various peaks were numbered according to their relative mobilities and not according to the distance moved and it would seem that these are indeed different patterns in different fish within one species (different protein concentrations). These variations could not be correlated with length, weight, sex nor hematocrit and was also not reflected in the paper electrophoretogram.

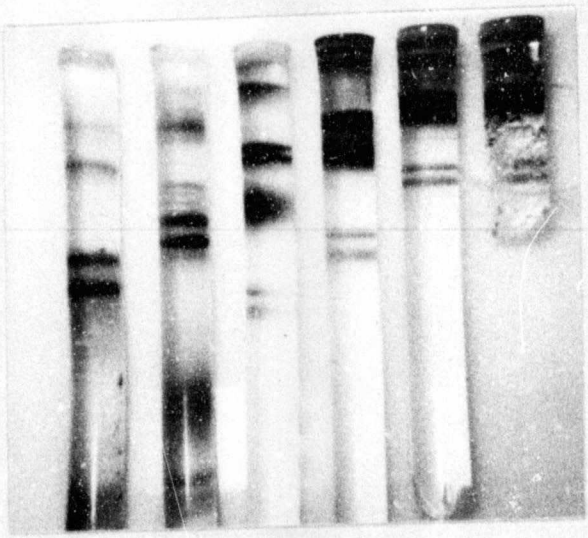

Plate 1 Polyacrylamide gel electrophoresis of L. capensis plasma. From left to right the gels are 3%, 5%, 7½%, 10%, 12½%, and 15%. The best resolution was obtained on the 5% gel.

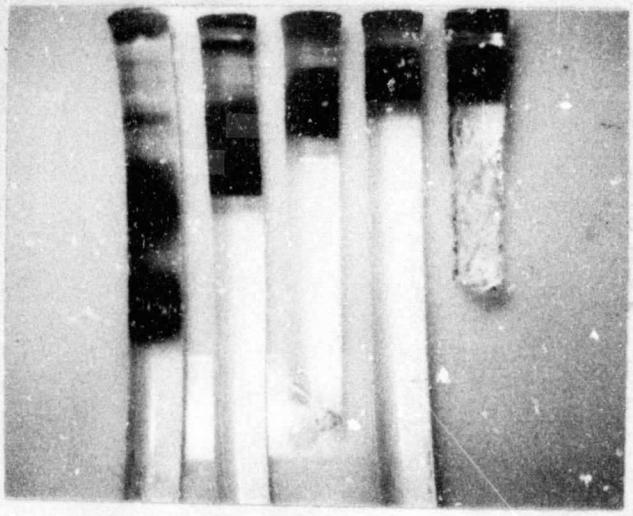

Plate 2 Polyacrylamide gel electrophoresis of C. gariepinus plasma. From left to right the gels are 5%, 7½%, 10%, 12½% and 15%. The best resolutions were again obtained on the 5% gel.

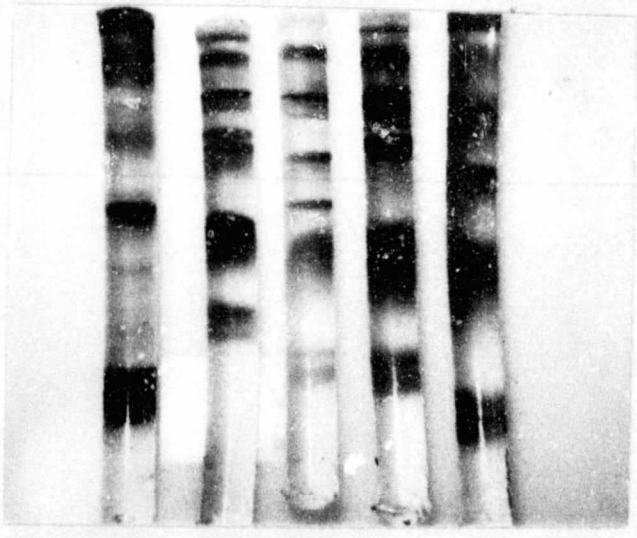

Plate 3 Polyacrylamide gel electrophoresis of L. capensis plasma compared to that of the human (left). Major variations are found in the middle third of the pattern.

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Comparison to that of the human again shows that very few similarities are present. Fraction 18 of L. capensis always contained the largest amount of protein (table 3) and this could therefore represent an albumin if the pattern of the human is considered (human albumin contains the most amount of protein - Putnam, 1965).

Finally mention should be made of the fact that some fish contained additional small fractions not present in the "standard" electrophoretogram. These fractions are shown in table 3 and it can be seen that they represent a small amount of protein. This could again be due to experimental technique (see p 48 for a full discussion of this) or could also be definite additional fractions.

4.1.2 L. umbratus electrophoresis

The fish used were again all obtained from the Vaal River. Paper electrophoresis yielded 8 fractions and table 4 summerizes all the data obtained from 16 fish. A big variation in hematocrit and plasma protein concentration is again evident. A constant pattern was obtained on paper electrophoresis and is shown in fig. 3. In 30% of the animals, fractions 2 and 3 separated into two and this is depicted as fractions 2a and 3a in table 4, (see next section). The major fractions present were fractions 3, 4 and 7. No correlation between variations in percentage protein and the additional fractions on the one hand and length, weight, sex or hematocrit on the other could be obtained.

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In comparison to the human pattern the same arguments concerning nomenclature can be applied as in the previous section. This will however not be done due to arguments outlined on p 51 of the discussion. Comparison of the mudfish patterns and those of the other two fish species is made on p 28 .

On polyacrylamide electrophoresis 21 fractions were obtained Plate 4 and fig. 4 show these results. A fairly constant pattern was obtained. The "standard" pattern is again the full pattern in fig. 4 and the prominent peaks are 4, 7, 9, 13, 18 and 20 (talbe 5). Less variation is found here than in the case of L. capensis but peaks 13 to 15 are again variable. In some cases two peaks are observed, a 13 and a 14 or a 13 and a 15. Peak 13, however, is present in all cases. In some cases the double peak pattern is reflected in the paper electropherogram with the additional fractions 2a and 3a but this is not so in all cases. It is therefore not possible to refer to any peak in the paper electropherogram as being represented by a particular peak in the gel electropherogram or vice versa. The variations observed could again not be correlated with any variable in table 4.

There is little correlation between the pattern of L. umbratus and that of the human except that peak 18 might again be regarded as a albumin due to the fact that it contains the largest amount of protein. In the case of L. umbratus only one additional fraction not present in the "standard" electrophoretogram was observed and that was a peak 10a in the case of fish 14. (table 5).

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It should be mentioned that numerical correlation of fractions of the different fish is not possible due to the fact that different relative mobilities are involved. The patterns for L. umbratus and L. capensis are very similar but fractions 10 have relative mobilities of 65 and 67% respectively. Other small differences exist and shall be pointed out on p 28 .

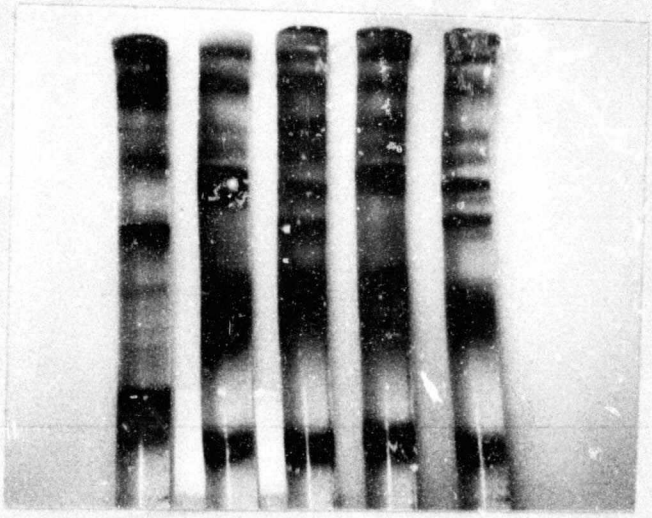

Plate 4 Polyacrylamide gel electrophoresis of L. umbratus plasma compared to that of the human on the left. Major variations are again found in the middle third of the pattern.

4.2.3 Clarias gariepinus electrophoresis

The fish used in this case were obtained in the early spring from the Lowveld Provincial Fisheries Department. Paper electrophoresis yielded 8 components with only one case of an additional fraction - fish 3 which had a fraction 5a (see table 6). As with the previous animals, wide variations in the plasma protein concentrations and hematocrits are characteristic (table 6). The constant pattern obtained is shown in fig. 5. The major bands present were 3, 4, 5, 6 and 8 and the biggest amount of variation was also observed here. As with the previous fish, no correlation could be obtained with this and the other variables. The comparison with the human is indeed striking here, except for the gamma globulins and fibrinogen. Fraction 8 also contains the greatest amount of protein and this is then a significant point when compared with the other fish species (see p 28).

The results obtained with polyacrylamide gel electrophoresis is shown in table 7, plate 5 and figure 6. The variation in the percentage protein of existing fractions is more pronounced in the case of this fish than in any other species investigated. The variation is mostly in fractions 14 to 19 as shown in fig. 6. In the standard pattern the prominent fractions are 3, 6, 9, 10, 16, 17 and 20 and 21.

Fraction 21 again contains the greatest amount of protein and similarities between the barbel and human plasma are evident. No additional small fractions were observed as in the case of

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the other fish and the last fraction (22) migrated less than human albumin. (In the mudfish this was not the case.)

4.1.4 Barbus holubi electrophoresis

All fish used were obtained from the Vaal River in the winter. Nine components were found on paper electrophoresis. Table 8 and fig. 7 shows the results obtained. As with the previous fish, wide variations in plasma protein concentration and haemotocrit are evident. The 9 fractions obtained were more constant than in the other fish species and no. 6 fraction was the only one to show a wide variation. The pattern is difficult to equate with that of the human and the prominent peaks are 5, 6, 7 and 8 with the latter two having the greatest percentage protein.

Polyacrylamide electrophoresis yielded a rather standard pattern with only one band, no. 15, which showed the variation also found earlier. Table 9, plate 6 and fig. 8 show the results obtained. The prominent peaks are 3, 6, 8, 12 and 17. Fraction 17 contained the greatest percentage protein and this feature is therefore constant in all species examined. Only one additional fraction was observed, a fraction 7a in fish 11 (table 9). Migration of the last fraction in this case was also less than in the human.

4.1.5 SUMMARY

At this stage a summary of the main results so far reported would be appropriate. Table 10 summarizes the results on the

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paper electrophoresis of the 4 fish species investigated. Care should be taken in equating the fractions of L. umbratus with that of the other fish due to the fact that fractions 2a and 2b found in 30% of the fish investigated, have been incorporated into the table (see p 69). Figure 9 compares the electrophoretic patterns and it can be seen that the two mudfish species have very similar patterns, with that of L. umbratus expanded more than that of L. capensis. The barbel and yellowfish have characteristic patterns of their own which are rather different from that of the mudfish.

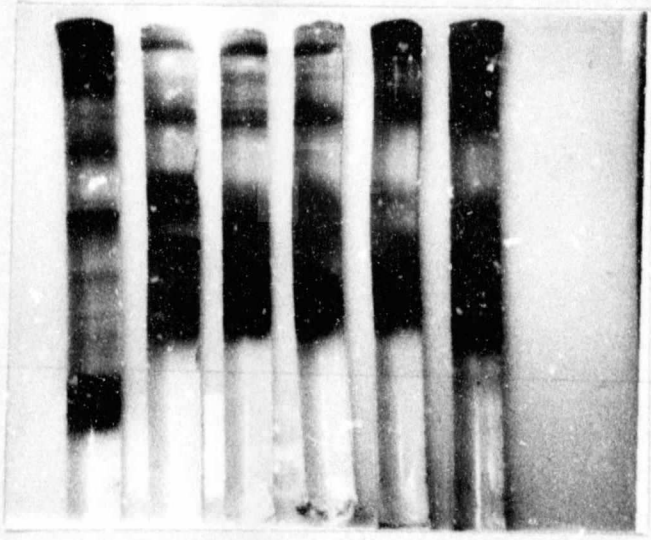

Plate 5 Polyacrylamide electrophoresis of C. gariepinus plasma compared to that of the human on the left. Major variations are found in the middle third of the pattern.

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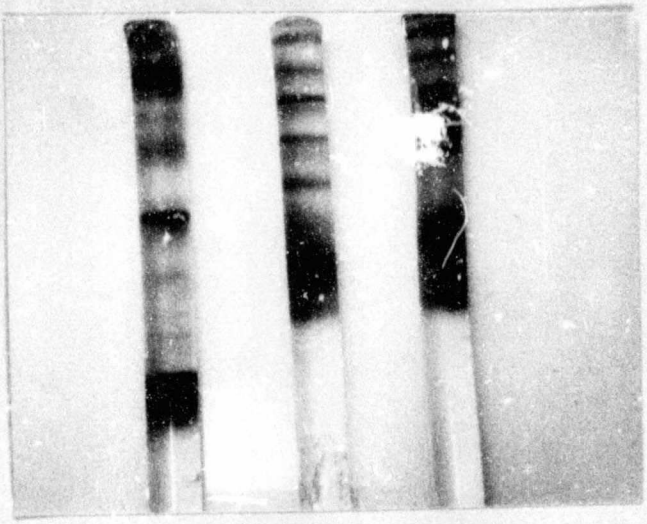

Plate 6 Polyacrylamide electrophoresis of B. holubi plasma compared to that of the human on the left. In the middle third of the pattern the variation is evident.

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Fraction 8 of the barbel is characteristic in that it contains the greatest percentage protein (fraction with greatest relative mobility). The yellowfish fractions migrated less than that of the other fish. In the case of L. capensis and L. umbratus, fraction 5 and fraction 3 respectively contain the greatest percentage protein and in the yellowfish fraction 6. The characteristic wide variations in hematocrit and plasma protein concentration are shown in table 10 and it is evident that L. capensis contains the highest and B. holubi the lowest plasma protein concentrations.

Table 11, plate 7 and figure 10 summarize the results obtained on gel electrophoresis. Only "standard" patterns are presented. As was the case with paper electrophoresis, the patterns of L. umbratus and L. capensis are here again very similar. The variations observed are also similar (see figures 2 and 4). In all four cases (figure 10) a relatively immobile fraction (1) is found and the last prominent peak in all four cases contains the greatest percentage protein (L. umbratus and L. capensis, no. 18; C. gariepinus no. 21 and B. holubi no. 17). B. holubi and C. gariepinus last fractions migrated less than that of the other two species. Closer inspection of fig. 10 will show that the prominent peaks in all four cases are very similar in distance moved except peak 16 and 17 in the case of C. gariepinus. The similarity in the curves are more striking here than in the case of paper electrophoresis.

Finally, it is mentioned again that no correlation between the variations observed in the electrophoretic patterns and the other tabulated variables was found.

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