Concerning the plasma protein and hematocrit values, table 10 also shows the wide variations which are characteristic. Labeo capensis has the highest mean hematocrit and plasma protein values and L. umbratus the lowest hematocrit and B. holubi the lowest plasma protein value. In the case of L. capensis, C. gariepinus and B. holubi, the male fish have higher mean values for both hematocrit and plasma protein concertrations and in L. umbratus the situation is reversed. (In the case of C. gariepinus many more females than males were studied.) These observations are discussed on p 42 and 45 .

4.2 Separation of one or more fractions

In the case of L. umbratus, L. capensis and B. holubi various fractions from the gel electrophoretogram were chosen and efforts made to isolate them. The next sections report these results.

4.2.1 Labeo umbratus separation

Precipitation with Na_2SO_4 according to the scheme of Hows (1923 a and b) yielded the effects shown in fig. 11 (supernatant fluid examined.) The fish used had an additional fraction no. 3a. (Due to the fact that additional fractions occur and also due to the variations found, blood from only one animal was used). Ten percent Na_2SO_4 caused the pronounced effects shown in fig. 11, removing fractions 4 and 8 (paper electrophoresis) and depressing others. Fourteen percent (14%), 18% and 22% Na_2SO_4 had very little additional effect; only depressing the peaks. Twenty-six percent Na_2SO_4 had the most pronounced effect and only peaks 14, 18

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and 20.and 21 remained (gel electrophoresis). At this stage it was possible to equate fractions 3 (plus 3a) and 7 (plus residue of 8) with fractions 13, 18 and 20 and 21. (Paper and gel electrophoresis respectively). According to the above results, it was decided to study peak 13 more closely.

Three (3) ml plasma were subsequently precipitated with 26% Na_2SO_4.

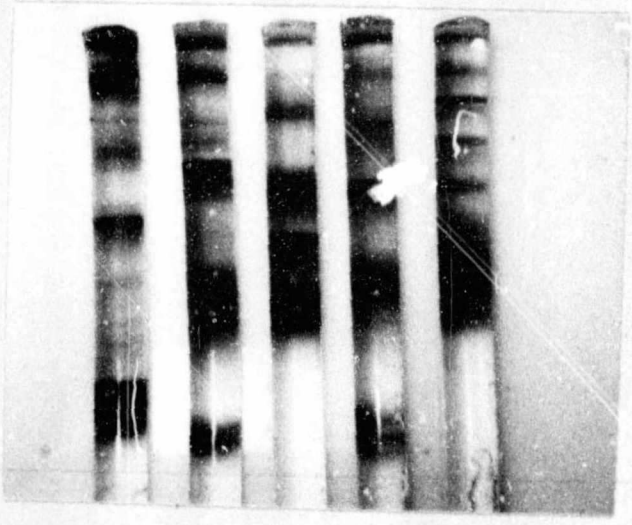

Plate 7 Comparative "standard" patterns obtained on gel electrophoresis. From left to right: Human, L. umbratus, C. gariepinus, L. capensis and B. holubi.

Control experiments showed that the 3 hour exposure in the waterbath had no influence on the normal electrophoretic pattern. After centrifugation, 2 ml supernatant were put on a Sephadex G-100 column. The elution pattern is shown in figure 12. Above the various peaks, the pattern obtained from fluid reapplied to polyacrylamide gels is shown in diagrammatic form. The two major fractions present, 13 and 18, were reasonably well separated but other small fractions were still present in both cases. The tubes containing the desired fractions were then pooled (gel 2 in figure 12) and dialyzed against several changes of distilled water and then freeze-dried (9,25 mg).

After re-running the dried fraction on polyacrylamide gels, the whole sample was subjected to iso-electric focusing in a pH gradient of 5 - 8. After 72 hours the effluent was read in the Perkin-Elmer spectrophotometer and the fractions visualized on gels. The result so obtained is shown in figure 13. Fraction 13 had an iso-electric point of pH 5,75 and after careful elution from the column it was found to be completely separated from the other fractions. Fraction 18 was found to have a rather low iso-electric point and due to the fact that it was found at the end of the pH gradient, the pH could not be measured accurately. It was found to be in the region of 4.2.

The reported fraction 13 was dialyzed and freeze-dried. An extremely low yield was obtained - 0,7 mg. The absorption curve of this fraction is shown in fig. 14. The protein absorbed strongly at 275 and 220 nm and had a minimum value at 268 nm.

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The column used for initial separation (Sephadex G-100) was standardized with 2 mg alcohol dehydrogenase, 1,7 mg cytochrome C, 2 mg albumin fraction V (bovine), 2 mg Vit. B_{12} and blue dextran immediately after the plasma had eluted. Approximate average molecular weight of fraction 13 was then determined according to the method of Andrews (1965). Figure 15 shows the results obtained and it was found that fraction 13 had an approximate average molecular weight of 69,100. Fraction 18 apparently has a much higher average molecular weight (above 120,000) due to the fact that it eluted just after the front.

Finally, the remaining fraction 13 was dissolved in distilled water and a titration curve performed. At the same time a titration on bovine albumin (having the same concentration as that of fraction 13) was also performed. The results are shown in fig. 16. The curves obtained are very similar but were not very good, probably due to the low protein concentration.

A summary of the main results obtained on this fish, compared to that of the other two, is presented on p 39 .

4.2.2 Labeo capensis separation

Sodium sulphate precipitation yielded very similar effects to those obtained on L. umbratus (see fig. 17). The fish used, had a very small fraction 6 (paper electrophoresis) and both fractions 13 and 16 on gel electrophoresis. Ten percent Na_2SO_4 again caused the biggest effects, removing fraction 8 (paper electrophoresis) and smoothing the gelpattern in a way.

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Fourteen percent (14%), 18, 22% had little effect but 26% precipitated all the peaks except 5 and 9 (paper electrophoresis) and 13, 16, 18 and 20 - 22 (gel electrophoresis). It was now again possible to equate fractions 5 and 9 with fractions 13 and 16 and 18 and 20 to 22 (paper and gel electrophoresis) respectively. According to the above results it was thus decided to study fractions 13 and 16 more closely.

After precipitating 3 ml plasma (again no pooling) with 26% Na_2SO_4 and centrifugation, 2 ml supernatant were applied to a Sephadex G-100 column. The elution pattern is shown in fig. 18. The patterns for the two mudfish species were very similar and the separation achieved was again reasonably good. The tubes containing the desired fraction were then pooled (gel 2 fig. 18), dialyzed and freeze-dried.

After another analytical electrophoretic run the whole sample was subjected to iso-electric focusing in a pH gradient of 5 - 8 for 72 hours. The results obtained after reading the effluent spectrophotometrically and visualization on gels are shown in figure 19. Fraction 13 had an iso-electric point of 5,78, fraction 16 an iso-electric point of 5,41 and fraction 18 an iso-electric point of 4,3 (could again not be measured accurately - see p 34). Fractions 13 and 16 were not completely separated from one another. The tube containing the two partially separated fractions were pooled separately, dialyzed and freeze-dried. The yield at this stage was 9,11 mg and 6,74 mg respectively for fractions 13 and 16.

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The fractions were then subjected to preparative polyacrylamide electrophoresis in a 5% gel containing 0,1% S.D.S. (Tris-glycine buffer, pH 8,5). The pattern obtained is shown in figure 20 and it is immediately evident that the yield was very low and also that the separated fractions now migrated more than normally - 25,5 mm and 32,5 mm respectively compared to 20 and 28 mm previously.

Standardization of the column used for initial separation (Andrews), yielded identical results to those obtained for L. umbratus; approximate average molecular weight of 68,900 (see fig. 15) for fractions 13 and 16 and above 120,000 for fraction 18. The 2 fractions obtained by preparative gel electrophoresis were now pooled, concentrated with sucrose, dialyzed (repeatedly) and then applied to another Sephadex G-100 column. After standardization it was found that the approximate average molecular weight was 34,200. (The protein could just be detected in this case).

Prior to this determination, the absorption spectra of the two fractions were determined. These turned out to be identical to that of fraction 13 of L. umbratus (see fig. 14 for comparison).

At this stage virtually nothing of the proteins was left, and the study discontinued. See p 39 for summary and comparisons.

4.2.3 Barbus holubi separation

The fish used in this case came from the Hartebeespoort Dam

Provincial Fisheries Department and were obtained in the early spring. On paper electrophoresis, the patterns were identical to those reported earlier. Gel electrophoresis, however, showed a difference in the area of fraction 12. In the patterns reported earlier (see p 28) only one prominent band 12 was always present. In the case of the fish now used, 3 very closely associated bands were present. This difference is shown in fig. 21 where it can be seen that fraction 12 is now a diffuse band and not a prominent peak. The rest of the patterns were identical to those reported earlier and possible reasons for this variation are discussed on p 45 . (In the case of the mudfish, fish caught at the same locality and season were used for all studies.)

Fig. 21 also shows the results obtained by Na_2SO_4 precipitation of the plasma proteins. These are more complicated than in the case of the previous fish species. Ten percent (10%) Na_2SO_4 precipitated fractions 1, 2 and 3 and flattened peaks 5 and 7 in the case of the paper electrophoretogram. Fourteen percent (14%), 18% and 22% Na_2SO_4 flattened these peaks more and 26% precipitated everything except peaks 6 and 8. In the case of gel electrophoresis the tendency to flatten is also noticed and with 26% Na_2SO_4 it is found that only fractions 8, 12 (3 bands) and 17 are left.

The fish used were relatively small, although sexually mature (350 g) and it was therefore decided to pool the plasma from 4 fish. The plasma from each fish was first run on gels and these proved to be identical. Four (4) ml of plasma were then

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precipitated with 26% Na_2SO_4 and applied to a larger Sephadex G-100 column (93 cm) than used previously. The elution pattern obtained is shown in figure 22 and it can be seen that the separation is not much better than in the case of the other two fish using shorter columns. During the elution, it was found that the tubes containing fraction 8 absorbed strongly at 405 nm. This important point is discussed on page 41 .

The tubes containing fraction 12 in partially purified form were pooled, the sample dialyzed and freeze-dried (yield - 10,5 mg). The sample was then again subjected to iso-electric focusing in a pH gradient of 5 - 8. The elution pattern and gel patterns so obtained are shown in figure 23. Fraction 12 (3 bands) was found to have an iso-electric point of 5,81 and fraction 17 an iso-electric point of 3,8 (not measured accurately).

Approximate average molecular weight determination according the Andrew's method showed that fraction 12 (3 bands) had a value of 55,500, and fraction 17 a value of 89,100. The fractions obtained from the iso-electric column were then again placed on 5% polyacrylamide gels containing 0,1% S.D.S. after dialysis and freeze-drying. No differenceswere observed when compared to the distances moved on normal gels.

4.2.4 SUMMARY

Table 12 summarizes the relevant results on the 3 fish species studied. Precipitation with 26% Na_2SO_4 gave very similar re-

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sults except in the case of B. holubi where an additional fraction, no. 8, was not precipitated. The fractions studied were very similar in distance moved in the gels for the two mudfish species and the average molecular weights were again very similar. An interesting point here is fraction 16 of L. capensis which had an iso-electric point of 5,41. Barbus holubi proteins had characteristics different from those of the mudfish and S.D.S. had no effect here. In the case of L. capensis a profound effect was seen which indicates that dimer formation is possible and probable in these proteins. The absorption spectra for the three fish proteins were identical.

From the present results, it seems that L. capensis and L. umbratus are very similar in both electrophoretic pattern and in the characteristics of some of their fractions. C. gariepinus and B. holubi each have their own patterns and in the case of B. holubi, fraction characteristics. At this stage it is also possible to see that equating of a fish pattern of the species studied with that of the human and applying the nomenclature of the latter, would not be a very profitable technique (see p 25).

5. DISCUSSION

The work of Bouch and Ball (1966) conclusively shows that the method of capture has an important effect on the plasma protein pattern, components and concentration. Inter alia, they showed that the plasma protein concentration starts decreasing within

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60 minutes after capture and this downward trend is maintained for at least 300 min. (termination of the experiment). Hattingh (1973) also showed that in at least the mudfish and yellowfish, plasma hemoglobin concentration is high after capture and this high level is maintained for at least two weeks after capture. This is probably due to hemolysis (shock-reaction?). It is therefore of vital importance in this kind of work to state the method of capture and to use only animals that have in no way been injured. The fish so obtained should then be kept for at least two weeks in aquaria in the laboratory to overcome the emotional stress and other influences of capture.

The fish used in this study were all obtained by netting and blood was either obtained immediately on capture or after a delay of at least 2 weeks. The plasma protein patterns so obtained were identical. The variations observed in the various fish (see p28) were present in blood obtained by either method and it can be assumed that the methods of capture and transportation give reliable results although a certain amount of experimental error is definitely still present.

The method of taking blood also merits some discussion. Wedemeyer (1970) has shown that MS-222 is a powerful stressing agent for fish and many of the effects of this substance have not been clearly elucidated up to date. The doses used in this study fall into the range suggested (Klontz and Smith, 1968) but more work is necessary to be able to state definitely whether MS-222 has or has not an effect on the plasma proteins. The fish were found to be sedated enough for cardiac puncture by the dosage used but it must be borne in mind that a certain amount of agi-

tation is always present in netting fish and this effect cannot be eliminated.

Cardiac puncture is again a recognized technique for obtaining blood in fish (Klontz and Smith, 1968) and it has been stressed that small syringes should be used due to the fact that excessive negative pressure causes hemolysis. The plasma used in this study was always inspected for hemolysis and if present, the sample was rejected. A certain amount of hemoglobin is however always present (see p 43).

The anticoagulant used in this study (heparin) was found to be very effective and the syringes were only coated with this fluid. In the case of the barbel, however, this technique was found not to be very effective and a small amount of heparin was added to the blood (this was later corrected for). It is a well known fact that fish have a very rapidly activated blood clotting mechanism (Wolf, 1959) and this is again apparently related to the degree of agitation (Klontz, 1967).

From the foregoing, it is evident that a fair amount of inherent experimental error is still present in the techniques for obtaining fish blood and also that the influences of anaesthetics, anticoagulants, agitation, etc. have not yet been clearly elucidated. These factors influenced the results obtained to a unknown extent.

Variations in the hematocrit value seem to be a characteristic of

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fishes. Young (1949) for instance, found that repeated hematocrits on individual fish (opaleye) during several months showed that the values always become lowered when a fish lost appetite or become supposedly diseased. Sano (1960) found an influence of gonad development on the hematocrit of both sexes of rainbow trout. The values reported in tables 2, 4, 6 and 8 also show a large variation and this could not be correlated with weight, length or reproductive activity but the male fish had higher mean values than the females except in the case of L. umbratus. Seeing that within one species all the animals were caught in the same season, the latter can therefore also not influence the hematocrit. Diet could be a possible factor seeing that it is not known to what extent the trout pellets given were appreciated. It is also known that the hematocrit correlates well with the hemoglobin concentration in these fish (Hattingh, 1973) but not with the red blood cell count (unknown for C. gariepinus). More work is therefore needed to elucidate these problems fully. At present it can only be said that in L. capensis, B. holubi and C. gariepinus the males usually have higher hematocrit values than the females, but the effects, if any of age season, activity and captivity are unknown in these fish. Due to the large individual variations the differences between males and females are of doubtful value.

The variations observed in the plasma protein concentrations also seem to be a fish characteristic. In the case of L. capensis and C. gariepinus the values were again higher in the males than in the females; in the case of L. umbratus, the females had higher values and in case of B. holubi no differences were ob-

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served. Due to the very large individual variations, the differences observed were not statistically significant and these differences are therefore again of rather doubtful value. Various factors could influence the plasma protein concentration. In the first place, the diet during captivity is of importance. The work of Summerfelt et al (1967) shows clearly that this factor influences the concentration and components. As has been said before, it is not sure to what extent the trout pellets were eaten, and some fish might not have been fully nourished. In the second place age might influence the concentration (Sano, 1960) as well as disease (Fujiya, 1961). As far as could be seen, only healthy fish were used in this study but this factor is of obvious importance due to the fact that minor injuries due to capture and transportation could have induced mild infections. In the third place, the stage of sexual activity is known to influence the concentrations (Thomas and McCrimmon, 1964). No correlations could be found here but this might have been due to the relatively small numbers of fish investigated. If a hundred or more of each species were used, this influence might have come to light. A fourth factor is pollution. Even in South Africa, an important factor in designing an experiment, is to obtain an "unpolluted fish". No work has been done on the influence of pollution on the fish plasma proteins (as far as is known to the author) and it is also not known to what extent the water in which the fish were caught (various localities) was polluted. The water was certainly not clean and this factor would in all probability influence the concentrations.

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Due to the fact that the fish from the various species were all caught in the same season and locality (except see pp 37 and 65), these factors could not have had any influence on the variations in concentration. These factors do influence the concentration (Mairs and Sinderman, 1960; Saito, 1957) and it would be interesting to repeat some of this work on similar fish obtained from other localities and then to see whether the individual variations now occur at a higher or lower mean value. A seasonal variation would probably be associated with a sexual variation as well greater numbers of fish would then have to be used.

It is also known that temperature has an effect on the concentration (Meisner and Hickman, 1962) as well as the stage of the life cycle (Sano, 1960). The temperature in the waters where the fish were caught ranged between 13 - 16°C and the fish were all mature. These two factors could therefore not have had an effect in this case.

From the foregoing it is evident that except for diet, pollution, age and sex which might have influenced the concentrations, the plasma proteins of all four species investigated varies considerably and this could not be correlated with body weight and length. In general it can be concluded that this individual variation is a characteristic of fishes and this agrees with the work of Tsuyuki and Roberts (1965), Mulcahy (1970) and others (see table 1).

Concerning the paper electrophoretic patterns obtained on the four species it is clear from fig. 9 that each species has a characteristic pattern distinguishable from the others. The two mudfish species have very similar patterns but differ sufficiently for

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identification (number of fractions and relative mobilities). Certain similarities are also present in the patterns of L. capensis, L. umbratus and C. gariepinus but the pattern of B. holubi is distinct. The paper electrophoretogram can therefore be used as a means of identification of these four species but is not sensitive enough for routine analysis of plasma within one species (see later). As has also been said on p 24 the application of the human nomenclature to fish blood is not advisable although certain similarities exist (see p 28).

The variations observed in the percentage protein of the various fractions of the paper electrophoretogram, is interesting. The same factors which influence the plasma protein concentration (see above) persumably influence the pattern obtained on electrophoresis. It is therefore extraordanary that no correlation could be found between the patterns and sex, sexual activity and body weight and length. As in the case of the protein concentration, pollution, age, diet and disease probably influence the protein concentration of the various fractions, but was not elucidated in this study (see above for references).

The additional fractions observed in the patterns of C. gariepinus and L. umbratus were probably due to slight experimental differences during different experiments, and also during the same run (different areas of the paper). In the case of L. umbratus, 30% of the animals studied showed these fractions (see table 4) and in this case some other influence (experimental or environmental) is pro[illegible] work.

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For routine analysis of the plasma protein,pattern polyacrylamide gel electrophoresis was found to be superior to paper electrophoresis (Thompson, 1967). The comparative summary in fig. 10 shows that from 19 to 22 components were obtained by the method (compare with the 8 to 10 components obtained with paper electrophoresis). The similarity in the patterns are obvious from fig. 10 but each pattern is specific enough for each species to be used as a means of identification. Even in the case of the two mudfish species investigated the patterns are in a way distinct (number of fractions and mobilities).

The factors discussed earlier that could influence the percentage protein of the various fractions and the total plasma protein concentration (see p 43) would presumably show up better in this method of separation. Especially in the case of B. holubi where 3 bands were observed in "band 12" in fish caught at the Provincial fisheries and only one band in fish caught at Villiers, season and locality could have had an influence. It should also be borne in mind that Saito (1957) showed that the patterns from wild and hatchery reared fish differ. Here again factors such as diet, sexual activity, pollution and age might have had a pronounced effect but were unfortunately not controlled in this study. The relative numbers of fish used, were also small (Meirs and Sindenman, 1960). No sexual differences in the patterns were observed and this corresponds to the work of Summerfelt et al (1967) but differs from that of Thomas and McCrimmon (1964) and Saito (1960). It is possible that this variation is only found in certain species of fish. Due to the fact that the nets used were fairly

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selective concerning length and thus also size, only fish of relatively similar dimensions were caught and this eliminated sexually immature fish. The age variations were however not influenced.

The major variations observed in the polyacrylamide electrophoretic patterns as shown in fig. 2, 4, 6 and 8 could also have been due to experimental differences and not purely due to the factors discussed in the previous paragraph. Plate 3 shows the gel patterns of L. capensis plasma proteins and it can be seen that similar fractions do not exactly correspond (this is also observed in some of the other gels). This was due to shrinkage of some of the gels after prolonged storage (they were only photographed after a delay of some weeks). This variation was never observed in fresh gels and seeing that they were immediately scanned after destaining, this cannot explain the major variations. Another factor to be borne in mind is that the major variations were also found in blood from different animals during the same experiment (8 samples run at the same time). The same applies to the additional bands observed in some fish as tabulated in tables 3, 5, 7 and 9. It therefore seems that these variations are real in some cases; probably a polymorphism (Bernet and Tsuyuki, 1967) or due to an uncontrolled or unknown environmental factor (Roberson et al, 1961; Saito, 1957; Shell, 1961 and Thomas and McCrimmon, 1964).

From the foregoing it can again be concluded that gel electrophoresis is a valuable tool in both the separation of plasma proteins and identification of closely related fish species (L. capensis and L. umbratus). Each of the species investigated has a characteristic pattern but variations do occur within a framework.

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Compared to some of the published patterns of fish plasma or serum proteins (fig. 24) it can also be seen that the four species investigated have characteristic patterns generally in line with reported results.

The scheme introduced by Howe in 1923 for the precipitation of plasma proteins formed the basis of one of the systems of nomenclature applied to these proteins. (Pennel, 1960). By using different salts, various proteins were precipitated and according to a system using Na_2SO_4, the proteins still in solution after precipitation with 22% Na_2SO_4 were classified as albumins. It is not possible to obtain highly purified products with this method and it should only be used as the first step prior to chromatographic separation (Putnam, 1965). From figs. 11, 17 and 21 it is clear that the different salt concentrations used did diminish some of the fractions in all three fish studied, but only a few of these were entirely removed. A further point is that 26% Na_2SO_4 was found to be more effective than 22% Na_2SO_4 in the case of these fish. (In the case of ox blood done as a control, the same applied - various fractions were completely precipitated, others were diminished and after 22% Na_2SO_4 precipitation, it was not only the albumin band that remained). According to the classical scheme then the fractions remaining in plasma after the 22% precipitation are albumins. This would then not agree with another nomenclature, that of Tiselius, in which the albumins have the highest electrophoretic mobility in a restricted p/H. (Putnam, 1965). It can be seen from the mentioned figures that this is not the case and this nomenclature is therefore not applicable.

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It could be argued that the columns used in the case of L. capensis and L. umbratus for separation of the proteins after precipitation, were too short. The separations achieved were reasonable and in the case of B. holubi where a much longer column was used, the same degree of separation was achieved. If columns of 100 cm in length with Sephadex G-200 were used, better separation would possibly have resulted. After a further separation step, either preparative gel electrophoresis or iso-electric focusing or both, the fractions obtained were completely separated from each other in the case of L. umbratus (fig. 13) and L. capensis (fig. 20) but not in the case of B. holubi (fig. 23 - fraction 12). This latter protein could not be separated into 3 individual components by the methods used. Mahler and Cordes (1968) point out that several criteria are necessary before a protein is pure: crystallinity, solubility curves, ultracentrifuge studies, electrophoretic studies, etc. The criterium used in this study was only the presence of one band on gel electrophoresis and it can therefore not be said that any of the fractions isolated were definitely pure.

The method of approximate average molecular weight determination according to Andrews is known to have a experimental error of approximately ten percent (Andrews, 1965). It is also known that the sugar content of the proteins influences the results obtained. Anthrone determinations done on samples of the small amount of protein available after separation were negative. It can therefore be concluded that the results summarized in table 12 concerning molecular weight are correct within ten percent.

The range reported for the molecular weight of albumin in the literature is from 65,000 (rat) to 70,000 (equine)(Putnam, 1965). The

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