

HEMOGLOBINS AND SERUM PROTEINS IN FOUR NORTH ATLANTIC SEALS, STUDIED BY ELECTROPHORESIS

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INTRODUCTION

An investigation of polymorphic characteristics to be applied in population studies of harp seals, *Pagophilus groenlandicus* (Erxleben), and hooded seals, *Cystophora cristata* (Erxleben), was started in 1962. Erythrocyte antigens were tried, but as the seals are caught in the pack-ice of northern waters, it proved difficult to get enough fresh material for these investigations. Although several freezing media were tried, the blood cells ruptured from freezing and thawing, and this complicated the analyses. Attention therefore was shifted to electrophoretic studies of hemoglobins and serum proteins.

The purpose of the present paper is to give a description of hemoglobins and serum proteins of harp seals, hooded seals, ringed seals, *Pusa hispida* (Schreber), and bearded seals, *Erignathus barbatus* (Erxleben), as these proteins appear after starch-/agar-gel electrophoresis. The first step towards identification of populations is to reveal individual differences within species. Therefore most attention has been paid to this problem. The results presented here will form the basis for further investigations.

MATERIAL AND METHODS

An account of the collected material is given in Table 1.

Table 1. Hemoglobins and sera from four North Atlantic seals collected in the years 1963—1964.

	Year	Harp seal		Ringed seal		Bearded seal		Hooded seal	
		Hemo-globins	Sera	Hemo-globins	Sera	Hemo-globins	Sera	Hemo-globins	Sera
Newfoundland area	1963	8	—	—	—	—	—	5	—
» »	1964	200	208	—	—	—	—	155	201
Jan Mayen area ..	1963	166	132	2	1	—	—	166	130
White Sea	1963	105	105	—	—	—	—	—	—
Denmark Strait ..	1964	—	—	—	—	—	—	83	83
Barents Sea	1963	130	103	15	14	46	40	3	2
» »	1964	—	16	—	—	—	—	—	—
Total		69	554	17	15	46	40	412	416

Part of the material of harp and hooded seals has been taken from pups. Blood from mothers and their pups has been sampled to get direct observations on heredity. Except this, all specimens have been sampled at random.

Sampling has been carried out as described for harp seals (NÆVDAL 1966).

Most of the specimens were collected by observers from the Institute of Marine Research on Norwegian commercial sealing vessels in the years 1963—64. The sample from the Denmark Strait was collected on the Danish sealing vessel «Ejnar Mikkelsen», and the sample from White Sea was collected by Soviet scientists.

Because of unfavourable weather and primitive equipment it has proved difficult completely to avoid lysis of erythrocytes and obtain sera free from hemoglobins. Much attention therefore has been given to the problem of analysis of sera containing some hemoglobins.

The electrophoretic technique described by SICK (1961) and MØLLER (1966) with starch-/agar-gel medium and at pH 9.0 was adopted for analysis of all specimens. The electrophoresis was run for 75 minutes to discover also the weaker components on the electrophoretograms. Electrophoresis in «difcoagar» at pH 6.3, as described for the harp seal transferrins (NÆVDAL 1966) has to some extent been carried out for serum proteins.

Proteins were stained with Amido Black 10B (Merck), and the benzidine staining process was used to stain hemoglobin/haptoglobin complexes.

An autoradiographic technique (GIBLETT, HICKMAN, AND SMITHIES 1959) used to identify harp seal transferrins (NÆVDAL 1966), has also been applied to hooded seal sera.

RESULTS

HEMOGLOBINS

The electrophoretogram of the harp seal hemoglobins is described elsewhere (NÆVDAL 1966). All specimens from the three other species have given exactly the same pattern as the normal hemoglobins of the harp seal: a major fraction (hem A) moving towards the anode, and a minor fraction (hem A₂) moving towards the cathode at pH 9.0.

Three specimens with deviating hemoglobins, supposed to be genetically controlled, were found among the harp seals. In the other species no deviating specimens could be detected, but here it should be stressed that the number of specimens is much higher for the harp seal than for any of the other species.

It has been possible to compare the electrophoretograms from fresh and frozen hemoglobins of hooded seals because hemoglobin specimens have been available from three living pups kept at the Institute, but no differences were found. Neither were any difference found between hemoglobins from pups and hemoglobins from older animals.

SERUM PROTEINS

General

In the combined starch- and agar-gel medium at pH 9.0, all serum proteins except the γ -globulins moved towards the anode. The γ -globulins were found as a more or less strong diffuse zone on the cathodic side of the point of application. Between the point of application and the albumins, which had the highest electrophoretic mobility, the other proteins were found as more or less outstanding bands on the electrophoretogram. Among these fractions, representing the α - and β -globulins, each species presented its own characteristic pattern, and the number of fractions and their distance from the point of application varied between species. Variations were also found within species, especially the presence or absence of fractions, but also in relative strength of fractions. In particular these variations were very complicated for the hooded seal.

Harp seal

A generalized pattern from routine electrophoresis in starch-/agar-gel at pH 9.0, never found in any single animal, is shown in Fig. 1 a. In addition to the γ -globulins and albumins a total of eleven fractions of different strength were seen. Fig. 1 b, c, and d show outlines of the electrophoretograms of three specimens differing in some of the proteins.

The components labelled as I, II, and III represent the proteins demonstrated to be transferrins (Tf A, Tf B, and Tf C respectively). One or two of these fractions were found in each animal, permitting the establishment of six phenotypes (NÆVDAL 1966). Three of these are shown in Fig. 1 b, c, and d (AA, AC, and BB).

Component IV usually is very faint, and often not seen at all (Fig. 1 b and c). This fraction may cause some difficulties for the determination of transferrin groups because it is located close to the Tf C band. However, the IV-band is much weaker than any of the transferrins, so the problem is not serious.

The protein labelled V occurs in all specimens. It is a relatively strong fraction usually giving a well defined band in the gel.

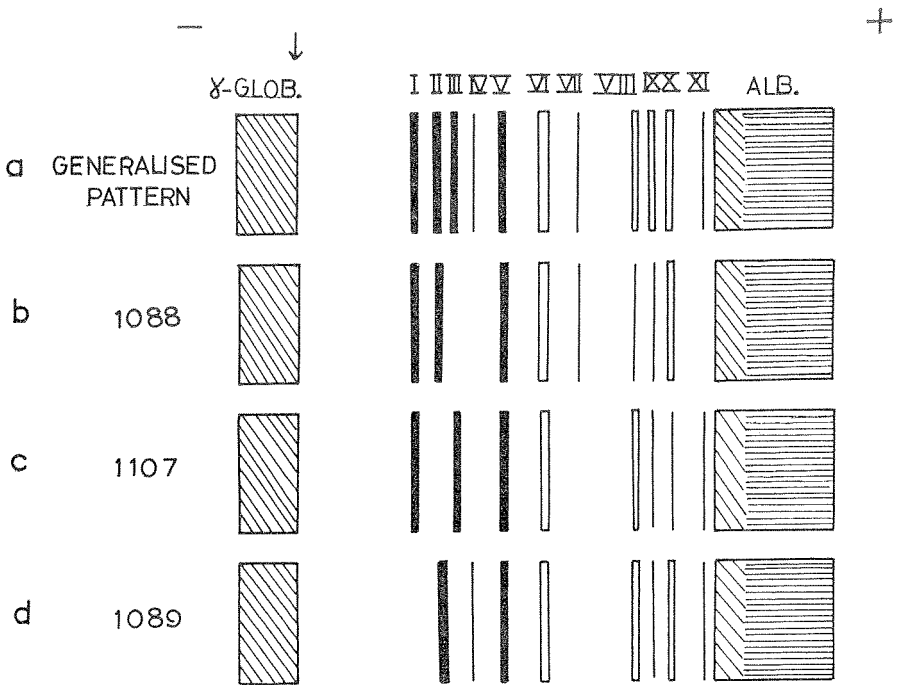


Fig. 1. Outlines of harp seal serum protein patterns by starch-agar-gel electrophoresis at pH 9.0. Solid bars : sharp and strong bands. Open bars : sharp, but weak bands. Single lines : faint bands. Horizontal shading : strong diffuse zones. Cross hatched : weak diffuse zones. Arrow indicates point of application.

The VI-band represents the haptoglobins. When excess hemoglobins are added to the sera before electrophoresis, this band stains with benzidin. No clear differences were found staining hemoglobinfree sera with Amido Black before and after addition of hemoglobins, but the VI-band was somewhat stronger in the last case. Some times a slightly higher electrophoretic mobility was indicated for the hemoglobin/haptoglobin complexes than for the free haptoglobins. But in contrast to the results for hooded seals (p. 46), this difference was very small.

The VII-band was not seen in all the slides (Fig. 1 c and d), and it usually was very weak. This may represent a case of real difference between specimens, but the absence could also be caused by low protein concentration in the sera.

The three proteins VIII, IX, and X seemed to be closely related. The relative strength of these bands vary to a great extent between individuals, but they are always seen at least as faint bands, and it is very difficult to separate the specimens into groups according to relative strength of these proteins.

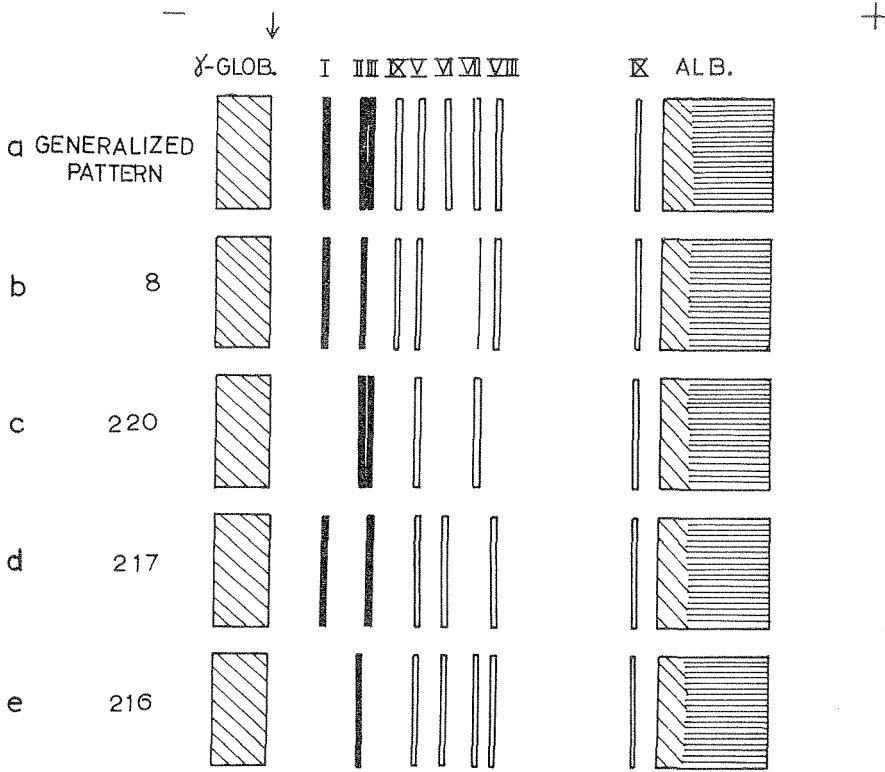


Fig. 2. Outlines of ringed seal serum protein patterns by starch-/agar-gel electrophoresis at pH 9.0. Legend: Fig. 1.

Band XI was seen as a very weak but well defined band on some of the slides. It is located near the albumins, and may be screened by these stronger zones. Therefore it cannot be decided whether the presence or absence of this component represent real difference between individuals.

Ringed seal

A generalized pattern of the starch-/agar-gel electrophoretograms of the ringed seal is shown in Fig. 2 a. The serum of one single animal never showed all fractions. Four individual patterns are given in Fig. 2 b, c, d, and e.

The fractions I, II, and III are very similar to the transferrins of the harp seal, and one or two of these fractions were found in each individual. The same fractions were found in «difcoagar» at pH 6.3, but it was

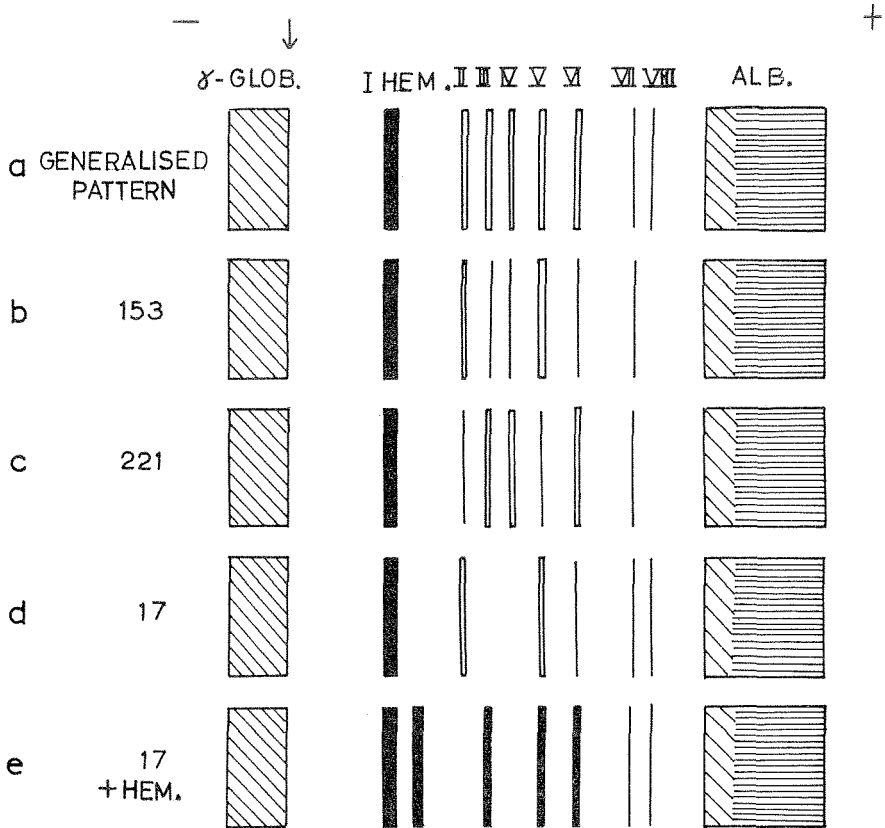


Fig. 3. Outlines of bearded seal serum protein patterns by starch/agar-gel electrophoresis at pH 9.0. Legend: Fig. 1.

difficult to distinguish between the bands II and III with both techniques. Band I alone was found in «difcoagar» (two specimens), but these sera contained too much hemoglobin to be analysed at pH 9.0.

Individual differences were also seen for bands IV, V, and VI, but because they were weak, it was difficult to separate the specimens into groups according to these bands.

No clear-cut qualitative difference was found after adding hemoglobins, but the VIII-band stained with benzidine and became stronger when excess hemoglobin was added to the sera, and therefore represents the haptoglobins. Band VII, when it occurred, seemed to become somewhat weaker in these cases.

The weak IX-band was seen in all specimens, but often it was partly screened by the albumins.

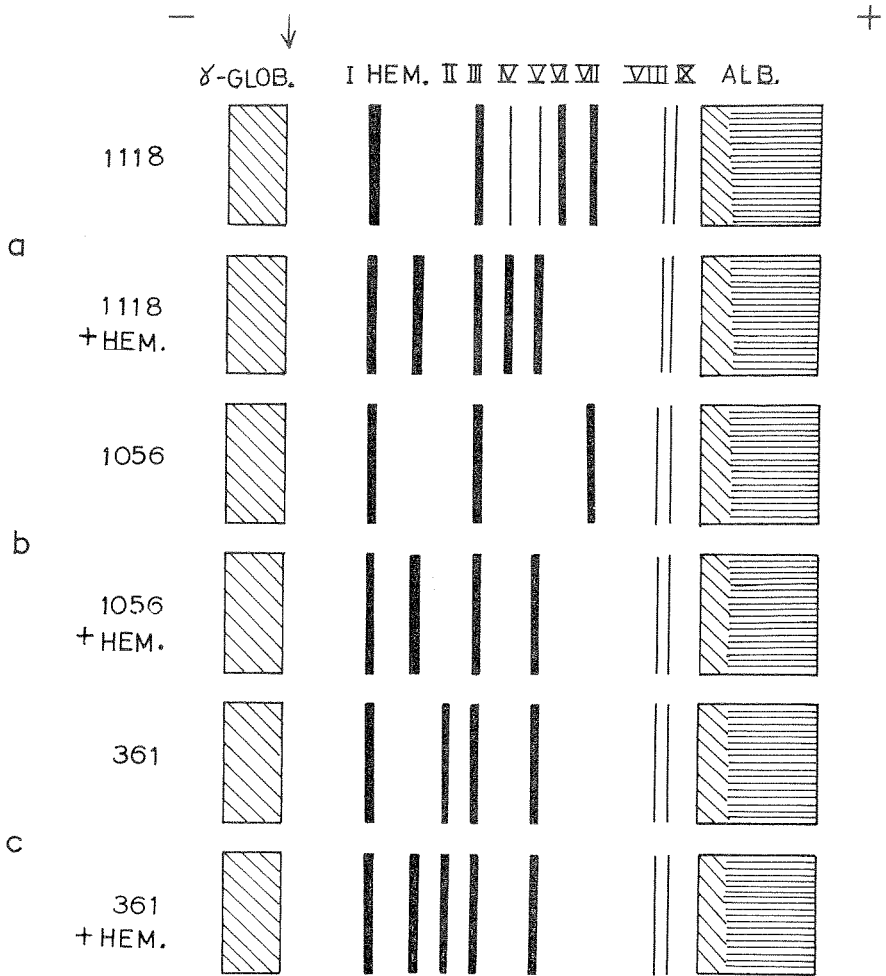


Fig. 5. The effect of adding hemoglobins to the sera of hooded seal, analyzed by starch-agar-gel electrophoresis. Band IV and V stained with benzidine. Legend: Fig. 1.

weaker bands on the electrophoretograms. Fig. 3 b, c, and d show three individual electrophoretograms from sera free from hemoglobins.

Band I was the the strongest and often had a diffused appearance which indicates that it may be composed of two proteins.

Band no. II, III, IV, V, and VI appeared with varying strengths, and although it seemed clear that some individual differences existed in these proteins (Fig. 3 b, c, and d), well-defined results could not be obtained. After addition of hemoglobin to sera, some of the electrophoretograms changed pattern (from d to e in Fig. 3) and the fractions

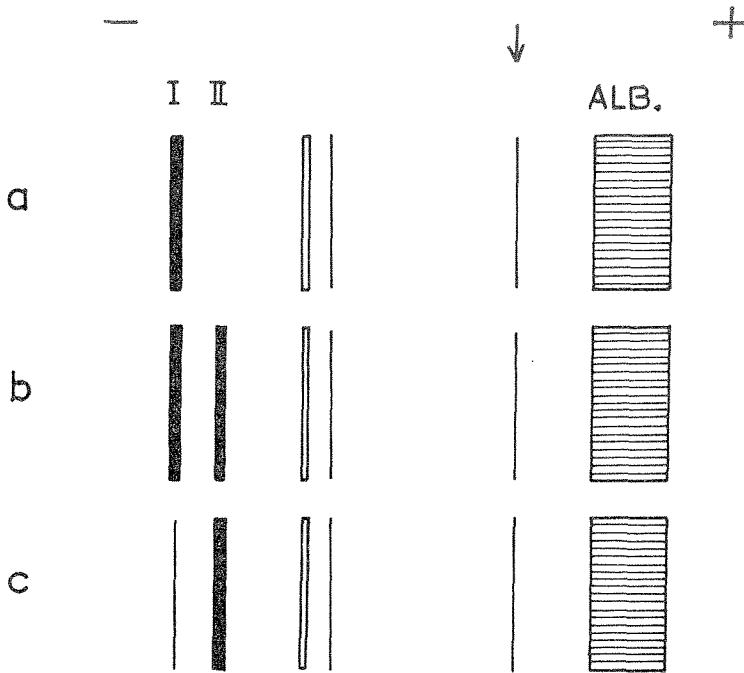


Fig. 6. Haptoglobin types of hooded seal sera revealed by agar-gel electrophoresis at pH 6.3. Only band I stained with benzidine. Legend : Fig. 1.

III, V, and VI stained with benzidine. In these cases the fractions therefore represent haptoglobins. Other sera, however, with similar electrophoretograms did not change at all after addition of hemoglobins.

The fractions VII and VIII were not seen on all slides, and often VII occurred alone. These bands were very faint, and it proved impossible to obtain clear-cut results from all specimens.

Hooded seal

The most complicated patterns were found in electrophoretograms of sera from this species, and several individual differences were discovered. A generalized pattern from electrophoresis in starch-/agar-gel is shown in Fig. 4 a, and six individual patterns of sera which appeared to be free from hemoglobins are shown in Fig. 4 b - g.

Band I, which was demonstrated by autoradiography to represent transferrins, was found in all specimens. In some cases, however, this band was diffuse, indicating a compound of two proteins with similar electrophoretic mobility.

Individual variations were found in band II and III. In some sera both bands appeared with equal strength, in others one of them was lacking. More often, however, the bands appeared with very different strength.

It has been possible to classify the specimens in three groups (phenotypes), II-II, II-III, and III-III respectively, according to the occurrence of these bands. Specimens with only band II or with band II and traces of band III were classified as II-II, when both bands occurred with the same or nearly the same strength, the specimens were ascribed to group II-III; and specimens with only band III, or with band III and traces of band II, were classified as III-III.

The bands IV, V, VI, and VII seemed to be closely connected with haptoglobins. The VI and VII-bands, when occurring, disappeared after addition of hemoglobins to the sera before electrophoresis. Band V stained strongly and band IV weakly with benzidine. In Fig. 5 some electrophoretograms of sera before and after addition of hemoglobins are shown.

In sera which apparently were free from hemoglobins, band VI and band VII occurred alone or together. In some sera both bands were lacking. After addition of hemoglobins, these bands disappeared and band V became visible, either alone or together with band IV (Fig. 5 a and b). In specimens without band VI or VII no qualitative changes in the electrophoretograms were detected after addition of hemoglobins, but band V became somewhat stronger (Fig. 5 c).

Using «difcoagar» at pH 6.3, clear individual differences were found in the haptoglobins. The pattern shown in Fig. 6 a was found for sera free from hemoglobins, and all specimens contained the component labelled II. After addition of hemoglobins, however, the specimens could be separated into three groups. In group one no changes occurred, and no bands stained with benzidine. All sera from pups belonged to this group, but the characteristics were also found among older animals. In the second group (Fig. 6 b) a new component (labelled I) occurred while band II persisted although with a somewhat reduced strength. Only band I stained with benzidine. The third group is represented in Fig. 6 c. Here band II disappeared, except for a faint trace, while band I was very strong and stained with benzidine.

It has not been possible to compare these results with the haptoglobin types at pH 9.0.

The faint bands VIII and IX (Figs. 4 and 5) were not found in all specimens. Sometimes only IX occurred, and sometimes both were missing. These bands are very faint, however, and they are located near

the stronger albumins, so the suggested individual differences may be apparent only.

In sera from pups («bluebacks») some of the proteins were found as they occurred in sera from older animals, although often weaker. However, the bands IV - VII were diffuse and very weak in blueback-sera.

DISCUSSION

Except three specimens from harp seals, all analysed hemoglobins have given the same result, and accordingly all the four seal-species seem to be monomorphic in the composition of hemoglobins as far as it can be revealed by this type of electrophoresis. However, «abnormal» hemoglobins like these found in harp seals may also exist in the other three species. Too few specimens have been analysed yet to decide this. The deviating harp seal hemoglobins occur at such low frequencies that even if they are genetically controlled, it is not at all certain that they represent a real case of polymorphism. They might for instance be caused by new mutations which are too unfavourable to be established at high frequencies in the populations.

The transferrin groups of harp seals have been found to be controlled by three autosomal, co-dominant alleles (NÆVDAL 1966). A similar mode of inheritance is indicated by the I, II, III-fractions of the ringed seal sera, although the specimens from this species are too few to allow statistical tests of the distribution of phenotypes and its accordance with Hardy-Weinberg's law. However, six phenotypes should be expected and five have been found. The expected type III-III is missing, but band III is rare in the available specimens and the expectance of an individual homozygous for the gene supposed to control it, therefore is very low when the number of specimens is as small as in this case.

Autoradiography has been used only for sera from harp and hooded seals. In both cases the transferrins were found in the fraction or group of fractions with the lowest mobility towards the anode. It is probable that this is also the case for the other two species, and accordingly it is possible that fraction I of the bearded seal sera and fractions I, II, and III of the ringed seal sera are transferrins, being monomorphic in bearded seals and polymorphic in ringed seals.

Interpretation of the observed individual differences in fractions next to the transferrins in harp, ringed and bearded seals is difficult. For the harp seal very small intraspecific differences have been found in these fractions (band IV), and for the other two species the bands are too weak for classification.

For the hooded seal, however, it is possible to separate the specimens into groups according to the presence or absence of the bands II and III

(although weak bands may cause some difficulties in classification). The frequencies of these proteins or the genes supposed to control them, therefore may be used to investigate the population problem for this species. The provisional results show that the distribution of phenotypes seems to be in good accordance with Hardy-Weinberg's law, provided that these proteins are controlled by two allelomorphic genes.

BLUMBERG, ALLISON and GARRY (1960) found individual differences in haptoglobins of the fur seal, *Callorhinus ursinus*, and these variations seemed to be under genetic control, although no proof of this could be given. Individual differences in the haptoglobins are very clear in hooded seal, and also indicated in the bearded seal. No interpretation of the bearded seal haptoglobins can be given, because some specimens had three components of hemoglobin/haptoglobin complexes, while others seemed to have none.

The haptoglobin types of the hooded seal, although complicated, are of greater interest because it seems to be possible to use also these characteristics in population studies of the species. There are, however, some problems. The haptoglobin variations at pH 9.0 are best seen when analysed as free haptoglobins (Fig. 5, band VI and VII), but when none of these bands occur, it is often impossible to decide whether they are really lacking or whether traces of hemoglobins bind the haptoglobins as hemoglobin/haptoglobin complexes.

Haptoglobin-bands are very weak in sera from pups, and the hemoglobin-binding capacity seems to be nearly absent in such sera. Therefore mother/pup combinations can not be used to solve the question of heredity for these proteins, and sera from pups can not be used for determination of haptoglobin types. This is unfortunate because pups account for the greater part of the catches and consequently they are the easiest to sample. However, the study of haptoglobins will be continued with sera from adult animals.

BLUMBERG et al. (1960) described some individual differences in postalbumins of fur seals, and SMITHIES (1959) has described polymorphism in the postalbumins of man. The variations of the bands VIII-XI for harp seals, VII and VIII for bearded seals, and IX and X for hooded seals may correspond to this, but the bands are very weak.

Some of the individual variations found by starch-/agar-gel electrophoresis at pH 9.0 can also be demonstrated in «difcoagar»-gel at pH 6.3. At this pH the hemoglobins are precipitated in the gel near the application point, and do not disturb the electrophoretograms of serum proteins. During sampling it has proved difficult to avoid all lysis of cells, but with the present methods also sera containing some hemoglobin may be analysed.

The methods are comparatively rapid, permitting analysis of large samples within short periods of time, and this of course is a factor of some importance in population studies.

One disadvantage of the methods is that small amounts of sera are analysed, and some of the individual differences therefore appear only in the presence or absence of very weak fractions, making it impossible to classify all specimens from these characteristics.

Other methods might reveal additional individual differences in hemoglobins as well as in serum proteins, and it would be worth while to try technical modifications. The results obtained with the present methods, however, encourage further investigations, and will be applied in future studies of seal populations.

SUMMARY

Hemoglobins and serum proteins of four species of pinnipeds have been analysed by starch-/agar-gel electrophoresis at pH 9.0, the serum proteins also by agar-gel electrophoresis at pH 6.3.

The original purpose of the work was to discover polymorph characteristics to be used in population studies of the two most valuable species: the harp seal and the hooded seal. During sampling also some specimens from ringed and bearded seals have been collected, and descriptions of the obtained electrophore'ograms are given. The greatest attention has been paid to intraspecific variations detectable by these relatively simple electrophoretic methods.

For all species, several individual differences have been found. Particularly the transferrin variations in harp and ringed seals (probable transferrins) and haptoglobin variations in the hooded seal are conspicuous.

Individual differences in other serum proteins have been found for all species, but they are most obvious in the hooded seal. Although no definite proof can yet be offered (except for the transferrins of the harp seal), it is probable that most of the variations are genetically controlled.

The transferrins of harp seals, proteins labelled II and III of hooded seals, and perhaps also the haptoglobins of the hooded seal seem to be suitable characteristics for studies of population problems.

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REFERENCES

- BLUMBERG, B. S., ALLISON, A. G., and GARRY, B. 1960. The haptoglobins, hemoglobins and serum proteins of the Alaskan fur seal, ground squirrel and marmot. *J. cell. Com. Physiol.*, 55 : 61—71.
- GIBLETT, E. R., HICKMAN, C. G. and SMITHIES, O. 1959. Serum transferrins. *Nature, Lond.*, 183 : 1589—1590.
- MØLLER, D. 1966. Serum transferrins in cod. *FiskDir. Skr. Ser. HavUnders.*, 14 : 51—61.
- NÆVDAL, G. 1966. Protein polymorphism used for identification of harp seal populations. *Årbok Univ. Bergen*, 1965(9) : 1—20.
- SICK, K. 1961. Haemoglobin polymorphism in whiting and cod. *Coun. Meet. Int. Coun. Explor. Sea*, 1961 (18) : 1—8. [Mimeo.]
- SMITHIES, O. 1959. An improved procedure for starch-gel electroporesis. Further variations in the serum proteins of normal individuals. *Biochem. J.*, 71 : 585—587.

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