PROTEIN POLYMORPHISM IN NORTHERN POPULATIONS OF FIELD MICE (*APODEMUS SYLVATICUS* L.)

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SUMMARY

An electrophoretic investigation was carried out on protein variation in different tissues of Apodemus sylvaticus. In addition to some ontogenetic studies, physicochemical methods were employed to characterise esterase enzymes and other proteins. The distribution of variant forms from thirteen localities in Iceland, Ireland, Scotland, Norway and Sweden were studied. Finally it was possible in some cases to acquire breeding data and clarify modes of inheritance.

Proteins:

Serum pre-albumins, albumin and ceruloplasmin are monomorphic, whilst post-albumin and transferrin may be polymorphic.

Red-cell proteins A and B are polymorphic.

Other tissue proteins were found to be uniform, except for a cathodally migrating protein fraction in striated muscle.

Esterases:

Most esterase fractions were found to be nonspecific carboxyl-esterases. Each tissue has its own esterase pattern, but also shares fractions with other tissues. The physicochemical tests, breeding, ontogenetic and population data make it feasable to propose a mode of inheritance for many esterase fractions.

Thus, the proposed loci Es-1, Es-2, Es-3, Es-4, Es-7 and Es-8 have each two codominant alleles. Es-6 has one dominant "silent" allele, and one recessive "producing" allele. These loci produce esterase in more than one tissue at the same time. There is a difference between all populations tested, the Iceland populations differing most from the rest.

It is suggested that chance "founder effects" is the chief factor for the uneven/

uneven distribution of the protein markers.

The physicochemical characters of proteins in Apodemus sylvaticus are similar to those established for other rodents.

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CHAPTER 1

INTRODUCTION

Introduction

Background to this investigation

The Long-tailed field mouse, Apodemus sylvaticus, occupies the deciduous woodland, Mediterranean and steppe zones of the western Palaearctic regions (Corbet, 1966). It extends furthest west to Ireland and east into Russia. Its northern limit is in Central Norway and Sweden and the southern limit is the South of France and northern Italy. It is one of the few mammalian species found in Iceland (Saemundsson, 1939). Field-mice exhibit very little variation over most of the area, except at the edges of the range particularly on the islands north and west of Britain (Barrett-Hamilton, 1900; Berry, 1969).

This variation in external characters (e.g. body size, skeletal characters and pelage colour) led to taxonomical sub-speciation and division into different races, mainly insular races (Matthews, 1952). These sub-species were thought to be relics of pre-glacial species, which had later evolved into the various races (Barrett-Hamilton and Hinton, 1910-1921 cited by Berry, 1969; Beirne, 1952). This "Classical" theory has been challenged by several authors (Delany, 1964; Delany and Healy, 1964; Berry et al. 1967; Berry, 1969, 1973).

Corbet (1961) proposed that the island races of small mammals are relatively recent introductions, through the agency of man. The differences observed in island populations compared to mainland ones, (mainly larger body size) was explained by influence of insular environmental factors (Foster, 1964).

Very little scientific literature exists on the Icelandic field-mouse. They are mentioned in the Sagas[1)] when their numbers increased and they were considered to be a plague. There is no mention of what type of mice they were/

1) 10-13th century

were, as the term mýs only means "mice". The present author being brought up and living in the Icelandic countryside has only seen Apodemus sylvaticus there, and the only place where house mice (Mus musculus) were caught was near Reykjavik.

The Icelandic field-mice are occupants of farmhouses, sheep sheds etc., but Icelanders call them húsa-mýs ("house mice") when found in houses and hagamys ("field mice") when found outdoors. The "plagues" come in hard winters, now and then; old farmers tell stories when mice got into the wool of the sheep when in their sheds and started eating them - the present author has not seen that happening. Travellers also mention mice, but usually in a folklore fashion Olafsen and Pouelsen ('Olafsson and Pàlson), 1772).

Lupton and Wykes (1938) studied mice in "Thrastaskogur" concentrating on external parasites of the mice. From those findings they suggested that the Icelandic field-mouse had more in common with Scandinavia than Britain. Degerbøl (1939) analysed external characters and suggests a new systematic name Apodemus sylvaticus grandiculus subsp. nov., mainly based on body size. He also suggests that it was introduced by the "first settlers' large transports of provenders and domestic animals".

It is generally accepted that the settlers came from Norway and Ireland ('Ola, 1973).

Berry (1969) made an extensive study of Apodemus sylvaticus at the North-west edge of its range using 20 non-metric variants of the skull as his method of analysis. From the data he estimated divergence between populations, arriving to the conclusion that Icelandic mice originated from Norway. He also suggests that Apodemus populations of the Scottish islands and Northern Ireland were brought from Norway by the Vikings.

Of the 60 Icelandic specimens that Berry used for his analysis, 40 were supplied/

supplied by the present author and blood and tissue samples of these were also used in this investigation. Berry (1973) emphasizes in a recent paper the effects of chance introduction on descendant populations i.e. the well known 'founder effects'.

Electrophoretic studies

The development of starch gel electrophoresis by Oliver Smithies (1955, 1959) provided a valuable tool to analyse the heterogeneity of proteins, thus making it possible to use biochemical markers that could not be observed before. Other authors modified the system by changing buffers e.g. Poulik (1957) and Ferguson and Wallace (1961). Combining histochemical methods with electrophoresis lead to the introduction of the zymogram by Hunter and Markert (1957) and the introduction of the term isozymes by Markert and Møller (1959). Several authors have applied this method to compare species (Lawrence et al., 1960; Paul and Fottrell, 1961; Coutinho et al., 1965; Holmes and Masters, 1968; Holmes and Massaro, 1969). Variations within species soon became evident and it was shown for many of these that molecular types are genetically controlled. Some of this biochemical genetic information will be reviewed in the discussion of the present results. General reviews in this field have been published by Lush (1966); Giblett (1969); Manwell and Baker (1970) and others. The present author has been unable to find but one publication on biochemical genetics of Apodemus (Engel et al., 1972) by others. To this should be added papers by Arnason, 1966; Pantelouris and Arnason, 1967 a and b; Arnason and Pantelouris, 1966.

Aims of the investigation

In the light of the above, the aims of the present investigation are:

(1)/

(1) To study the polymorphism of certain proteins and esterases in *Apodemus sylvaticus*.

(2) To compare protein and esterase patterns of various tissues in *Apodemus sylvaticus*.

(3) To characterize some of these proteins and esterases in particular by applying physicochemical methods.

(4) To test the genetic basis for the variations where possible. Facilities available for this task have been rather inadequate.

(5) To compare the populations under study, using biochemical markers.

(6) Relate the results to previous findings in other rodents, *Mus* in particular.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2 - MATERIALS AND METHODS.

I. ANIMALS AND SAMPLES

a) *Apodemus sylvaticus*.

The field-mice were obtained by trapping. "Longworth" small mammal traps were used in most cases, but in some cases the animals could be collected straight from the containers that farmers use to keep feeding stuffs. Where traps were used, they were either baited with Oxoid 41B animal diet or a mixture of compressed grain and fish meal in the form of small cubes. The bait seemed to serve the main purpose of keeping the animals from dying of starvation in the traps, rather than tempting them into the traps. In Iceland mice were mainly caught inside farm-houses, but in Norway, Scotland and Ireland in woods and fields.

After capture, the field-mice were kept in big rat-cages and fed "Oxoid 41B" animal diet. For the places of capture, see maps.

Collection of samples.

(1) Blood.

The animal was etherised and blood collected from the eye arteries, after removal of the eye-ball, into small heparinised tubes. The tubes were centrifuged to separate blood cells and sera. The sera were pipetted into clean tubes, which were sealed with parafilm and deep-frozen. The red cells were washed three times with isotonic saline and then deep-frozen. When used, the red cells were homogenised with aliquots of distilled water and then centrifuged./

centrifuged. The supernatant was used. As mentioned later, fresh-samples were used in some cases for comparison.

(2) Tissue samples.

The mouse corpses were deep-frozen immediately after th e collection of blood. They were thawn and dissected just before the collection of tissue samples. A small bit of tissue was cut up into smaller bits with a pair of scissors, put into a tube and then homogenised with an ultrasonic homogeniser (M.S.E.) in an aliquot of distilled water. The homogenates were centrifuged and the supernatants collected and used. It made no difference, whether the samples were centrifuged at 5.000 r.p.m. or 20.000 r.p.m. The tissues used were: liver, heart muscle, skeletal muscle (thigh-muscle). These were collected from all animals; kidney, testis, epididymis, fat, spleen and brain were collected from some animals for comparison of tissues. In some cases fresh samples were used to test the effects of freezing.

b) *Mus musculus*.

This ordinary mouse was only used for comparison with *A. sylvaticus*, and population studies were not carried out on this species. Both laboratory and feral mice were used. Trapping methods were the same as in the case of *A. sylvaticus*, but *M. musculus* is much more trap-shy than *A. sylvaticus*.

Collection and handling of samples.

The same methods were employed as described above, except the/

the laboratory strains used for comparison were usually killed one or two days prior to experiments.

II. ELECTROPHORESIS

The horizontal starch gel eledtrophoresis (Smithies, 1955) was used to separate proteins. The technical details of buffers, gels and stains are given below.

The apparatus was arranged as shown in figure 1. The vessels (A) and (B) contain the "vessel buffer" (borate). Each vessel has a partition (C), perforated at its lower half. This partition prevents the transfer of electrode reaction products into the gel. The electrodes are of platinum and are situated in the compartment further away from the starch-plate. The connection (D) between the starch gel plate and the vessel buffer is made with a double layer of lint covering about 2 cm. each side of the plate. The size of the perspex starch plates (E) was $17\frac{1}{2} \times 16\frac{1}{2}$ cm. depth .5 cm. Distance between the lint contacts was 10-12 cm. The voltage applied across the system is supplied by a power unit (F). At the end of the electrophoretic run, the starch plates were removed and sliced horizontally into two or more slices, which were then transferred to the staining trays. A thin layer of gel was removed from the upper surface where trailing of the proteins is worse. Where comparison of the two slices was wanted, "teeth" marks were made on the edge of the starch plate before the slicing to serve as points of reference.

(a)/

(a) Electrophoretic buffer systems:

The pH of the buffer plays a major part in the separation process in the starch gel. In contrast, on paper it is the ionic strength that matters most. The buffers used in this investigation wdre:

Vessel buffer pH8.6 - 8.7

Boric acid 18.6 gm.

Sodium hydroxide 2.0 gm.

are dissolved in H_2O and made up to 1 litre. The pH of the solution is checked and adjusted to pH 8.6-8.7 (usually some NaOH has to be added). It was found convenient to prepare a double strength stock solution and dilute it before use. The pH of the buffer in the vessels is checked before use and the content of the two vessels mixed. It was found, that the pH remains constant for several days, if the apparatus is used and kept in a cold room (2-4℃): Care is taken to fill the vessels to exactly the same level to avoid siphoning effects. The capacity of each vessel was 4 litres.

Alternative buffers for the starch-gels:

(1) Tris-citrate-borate, pH8.6

Tris (hydroxymethyl) aminomethane	8.0 gms.
Citric acid	1.5 gms.
Borate vessel buffer.	100 mls.

The above are dissolved in water made up to 1000 ml. This buffer proved suitable for the fast moving esterase fractions of/

of *Apodemus* serum, for soluble major proteins and for alkaline phosphatases. The stock solution was of fourfold concentration.

(2) Tris-citrate pH7.6

812.5 ml of 0.05M citric acid (10.51g/ 1)

187.5 ml of 0.76M Tris (92.07g/ 1)

These amounts are dissolved and mixed, the pH checked and, if necessary, adjusted to pH7.6. The buffer is diluted 1:10 before use for making starch plates. This system was found suitable for the separation of esterases, prealbumins, acid phosphatases, ceruloplasmins. It is of great importance that, with this buffer, background staining is much less of a problem, compared to buffer system (1). The same vessel buffer (borate pH8.7) was used with both types of gel buffers. Dilutions of the gel buffers were altered in some instances to effect specific separations, as will be indicated.

(3) Tris-citrate pH8.6

The same as (1), except no borate is added.

(b) Starch plates:

The commercially available "Connaught" hydrolysed starch was used. 10-14 gms of starch are used per 100 ml of gel buffer. The starch is dissolved in the buffer by swirling or stirring whilst the buffer is being heated slowly over a Bunsen flame, either in a sidearm "Pyrex" glass flask or a beaker. At a stage of the heating process, the starch granules rupture and the grey viscous/

viscous mass becomes transluscent. It is at this stage (around $74^{\circ}C$) that heating must be stopped. The flask is then connected to a vacuum pump. Under the negative pressure, the hot starch bubbles. When only large air bubbles form from the bottom of the container (this takes about a minute) pumping is discontinued. The extent of bubbling allowed must be standardised if reproducible results are to be acquired. This is very much a question of experience and practice. Now the starch is poured into a perspex tray (previously coated with a little paraffin) to fill it well above the edges. After about one minute a glass plate (also coated with paraffin) is laid on top. Positioning of the glass plate must be made carefully so as to prevent the trapping of air bubbles in the starch. The glass plate presses by its own weight down on tb the rims of the tray and thus provides to it an airtight seal. The plates are ready for use after 2-3 hrs, but were used mostly the following day without any ill effect. Sometimes a condensation of buffer is formed on the gel surfaces on standing. This is blotted off with filter paper before application of samples.

(c) Acrylamide plates. (Raymond and Wang modified)

"Cyanogum 41" (B.D.H.) was used. 7-7.5 gms. of "Cyanogum 41" were dissolved in 100 ml. of buffer (same as for starch). .1 ml. of TEMED (NNN'N' - tetramethyl - ethylene diamine) was added to each 100/

100 ml. of buffer. This was filtered and 1 ml. of 7% ammonium persulphate solution was added. Now the solution was swirled round in a side-arm flask, degassed and poured into the same moulds as used for the starch, covered with glass plates and allowed to set. The acrylamide plates were used only for few experiments, as most of the work had been carried out on starch.

(d) Application of samples and the electrophoretic run:

The samples (sera or tissue homogenates) are supported in the starch-gel by small pieces of Whatman filter paper Nos. 1 or 3, about .6 x .6 cm in size. The paper is soaked with the sample and is put into a vertical slit made in the starch-gel (acrylamide gel). After all samples have been applied, the slit is closed by pushing the starch portions towards each other, and a "compressor" piece of perspex is used to keep them in place. The gel is now sealed with a sheet of "Handiwrap". Alternatively, a glass plate is supported just above the gel surface, to minimize evaporation. In runs lasting for more than two hours it was found advantageous to use the Handiwrap rather than glass plates. The electrophoretic run was always done in the cold room at 2° - 4°C.

Voltage applied:

15-25 V/cm length of the plate

Tris-citrate gel pH7.6 and pH 8.0.

Esterases 20v/cm for 2-3 hrs

Phosphatases/

Phosphatases (acid)	15v/cm for 1-2 hrs
Prealbumins	20-25v/cm for 3 hrs

<u>Tris-citrate-borate gel pH8.6</u>:

Esterases	20v/cm for 4-6hrs
Proteins	20-25v/cm for 4-6hrs
Phosphatases (alkaline)	15-20v/cm for 1-2 hrs

The current applied varied, as the voltage was constant, from 2-4 mA/cm width of the plate

III. STAINING

(a) General protein stain

The starch gel slices were stained in the following solution for proteins :

(1) 8 gm nigrosine in

1000 ml Methanol

800 ml H_2O

200 ml Acetic Acid.

(2) Saturated solution of Amido Black 10B in the same methanol-water-acetic acid solution as above.

Equal amounts of (1) and (2) were then mixed and poured to cover the gel slices placed in a plastic tray. The staining was complete in 1-2 hrs, when the stain was poured off and 1-2% acetic acid in H_2O was poured on for cleaning the excess of stain off the gel. Up to 5 changes are necessary to give nearly colourless background. The last washing was usually done in methanol-water-acetic acid (50:50:10) as by doing so the background becomes nearly white. Where a transparent gel is preferable, the methanol wash is omitted. Another method is to transfer the starch gel slice to absolute alcohol for 12-24 hrs and then to glycerol for several days, which renders the gel transparent.

The acrylamide plates were stained in "weak" solution of Amido Black 10B in 10% acetic acid solution. The same acetic acid solution was used for destaining.

(b)/

(b) Lipoprotein stains

Three specific stains were used, Oil Red O, Sudan Black, Nile Blue Sulphate. For the first two stains, saturated solutions of the dye in methanol : acetic acid : water 60:10:30, were filtered and poured on to the gels. For the Oil Red O the following procedure was employed (Pert and Kutt, 1958):

The gel slices were kept in the dye solution for 48 hrs. with 3 changes of fresh stain. Washing in running tap water was then carried out until background stain was removed. Finally the gels were placed in a cleaning solution of methanol : acetic acid : water (60:10:30). Where Sudan Black was used the gels were kept in stain overnight and then transferred to similar washing solution. Several changes were needed.

For Nile Blue Sulphate the gels were immersed in 1% of the dye in 10% acetic acid for 10-20 minutes. Subsequent washing of the gels was done in continuously flowing tap water. Finally, the gels were placed in 1% acetic acid to bring up the lipoproteins.

(c) Glycoprotein stain

The method of Kapitany and Zebrowski (1973) was followed:

Immediately following electrophoresis the gels were fixed for

1/

1 hr in 12.5% TCA. The gels were then subjected to sample oxidation with 1% periodic acid for 2 hr and then washed for 2 hrs in 15% acetic acid. These steps were all carried out at room temperature.

The gels were then transferred to trays containing Schiff's Reagent (Lillie, 1951B) and stored in a refrigerator (in dark) for 2 hrs. The gels were destained in 7% acetic acid with frequent changes. In the last washes 15% acetic acid was used.

Destaining trays were covered to avoid light.

(d) Staining or labelling transferrins and other iron-binding proteins:

Two methods were employed:

(1) The Nitroso-R method (Mueller et al., 1962) was employed:

.5 gm Nitroso - salt

1.0 gm hydroxylamine hydrochloride

2.7 gm sodium acetate $.3H_2O$

1.5 ml glacial acetic acid

made up to 100 ml with distilled water.

Ferric ammonium sulphate was added to the serum prior to electrophoresis in an amount equivalent to approximately 5 μg Fe^{+++} per ml of serum. In other aliquots of the serum ferrous salt was added instead. The purpose of adding iron ions is the saturation of the transferrin molecules. The gels/

gels were soaked in the above solution for 15-120 minutes and then transferred to a solution of methanol : acetic acid : water (50:10:50). Iron-containing proteins stain green.

(2) Labelling with ^{59}Fe and autoradiography (Giblett et al. 1959)

Radioactive Iron ^{59}Fe is added to the sera to give a final concentration of 5 μc/ml serum. Electrophoresis is carried out as usual and the gel sliced in two halves. One slice is stained with a protein stain and the other covered with "Handiwrap" and an X-ray film placed on the gel and the position secured in the dark. The gels were kept in the darkroom for 1 week and then the X-ray films (Gevavert) were developed and later photographed.

(e) Staining for Ceruloplasmin

Two methods were used :

(1) Paraphenylene diamine hydrochloride staining. (Morell and Scheinberg, 1960). Approximately 0.227 gm of paraphenylene diamine hydrochloride is dissolved in 250 ml of .6M acetate buffer (pH5.7) immediately before use. This solution is poured on to the gel slices in a container which is then closed by a lid and incubated at 37°C. The ceruloplasmins show up as purple bands after one hour. Staining is due to oxidative darkening of paraphenylenediamine, catalysed/

catalysed by the ceruloplasmin. This same method can be used to show up amylases.

(2) O-dianisidine staining. (Owen & Smith 1961). One gm of o-dianisidine is dissolved in 50 ml of distilled water, by adding concentrated hydrochloric acid dropwise until the solution turns an amethyst colour. This is now diluted to 100 mls with distilled water. Incubation is carried out at 37°C in the following :

100 ml o-dianididine solution

100 ml .6M acetate buffer (pH 5.7)

300 ml ethanol (absolute)

The ceruloplasmins stain orange-brown.

(f) Peroxidase-staining.

The stain was prepared by dissolving 0.25 g o-dianisidine in a minimum of acetone or methanol. This was diluted to 100 ml with 0.1M acetate buffer pH4.5. Before use, 1.0 ml hydrogen peroxide solution (20 vol.) was added to the staining solution. The peroxidase bands stain brown.

(g) Esterase staining

The method employed was based on the azo-dye coupling to naphthol (Nachlas and Seligman, 1949). A naphthyl ester is used as a substrate for the enzymes to act upon and hydrolyse the/

the ester into naphthol and acid. The azo-dye (Fast Garnet) then couples with the released naphthol and gives the stain precipitate. The following substrates were used:

Substrate	Solvent	Incubation buffer
1-naphthyl acetate	ethanol/acetone	phosphate or Tris-maleate
2-naphthyl acetate	- " -	- " -
1-naphthyl propionate	- " -	- " -
naphthol-AS-OL acetate	- " -	- " -
naphthol-AS-D acetate	- " -	- " -
6-bromo-2-carbo-naphthoxy - choline iodide	- " -	- " -
1-naphthyl phosphate sodium salt	water	Veronal-acetate

In all cases the coupler dye was Fast Garnet

The incubation buffers were prepared as follows :

Phosphate Buffer pH 6.0. Mix 289 ml of .5M KH_2PO_4 and 26 ml of .5M Na_2HPO_4 and make the mixture up to 1000 ml with distilled water. Before use, dilute 3:1 for gels of pH 7.6, and 1:1 for gels of pH 8.6 with distilled water.

Tris-Maleate Buffer pH 6.2 - 6.4

(a) 24.3 g Tris

19.6 g Maleic anhydride

in 1000 ml of distilled water.

(b)/

(b) 0.2M solution of NaOH. Mix 250 ml of (a) and 190 ml of (b) and make up to 1 with water. Adjust to pH 6.4.

Veronal-acetate Buffers.

(a) Veronal acetate stock solution :

Sodium acetate (anhydrous) 10.20 g.

Sodium diethylberbiturate 29.42 g.

Make up to 1000 ml with distilled water.

(b) 1 N HCl

Veronal-acetate-HCl pH 8.8:

100 ml of (a)

399 ml of distilled water

1 ml of (b)

Veronal-acetate-HCl pH 4.5:

100 ml of (a)

378 ml of distilled water

22 ml of (b)

Note on esterase staining :

The numerous esterase fractions give a staining reaction of unequal intensity. Some stain in a few seconds, but others take several hours. To be able to demonstrate all fractions, it is necessary to watch the plates carefully as/

as they stain and to photograph them intermittently.
This, incidentally, provides some indication of the relative reaction rates of individual fractions.

Note on phosphatases:

The procedure of Lawrence et al. (1960) was followed, except that Fast Garnet GBC (Gurr) was used, instead of Blue RR salt and Red 3GS. The incubation buffer was also different; Veronal-acetate buffer was found to be the best for phosphatase staining.

(h) Inhibitors

The following inhibitors were used in the present study:

Physostigmine sulphate (Eserine) 10^{-4} and	10^{-5}M
Neostigmine bromide	10^{-4}M
D.F.P. (di-isopropylfluorophosphate	10^{-4}M
P.C.M.B. (p-chloromercuribenzoic acid)	10^{-4}M
"Diamox" (acetazolamide)	10^{-4}M

In all the tests, the inhibitor was added, in required amount to the buffered Fast Garnet solution. The gel slices were incubated in this medium for 30 minutes and then the substrate 1-naphthyl acetate was added. Control gels were treated in the same way except that no inhibitor was added.
The usage of various substrates and inhibitors in order to classify esterases/

esterases is widely reported. It is appropriate to mention few authors in this field at this stage:

Koppayi and Karezmar (1950); Aldridge (1953); Markert and Hunter (1959); Augustinsern (1961); Hunter and Strachan (1961); Wright (1963) Oki et al. (1965); Tashian (1965); Arnason and Pantelouris (1966); Holmes and Masters (1966) and Holmes and Whitt (1970).

(i) Heat Treatment

Two methods were employed:

(1) Incubation of the starch plates at 56°C after electrophoresis (Holmes and Masters, 1967.

The gels were incubated at 56°C for different lengths of time after electrophoresis, then cooled down in the staining buffer prior to staining. Control gels were kept at room temperature for the same lengths of time.

(2) Incubation of samples at 56°C prior to electrophoresis.

The samples (homogenates, lysates and sera) were drawn into capillary tubes, these were then sealed in a bunsen flame. The capillaries were then put into a waterbath which was kept at 56°C. The incubation lasted for 5, 10 and 20 minutes for each of the three lots of capillaries respectively.

After the treatment the samples were run in starch gels along/

along with controls and stained in the usual fashion.

(j) Neurominidase Treatment

The samples were incubated with neuraminidase at $37^{o}C$ for three days prior to electrophoresis. Two control samples were run along with each treated with neuraminidase, one kept at $-20^{o}C$ the other kept at $37^{o}C$ for three days, but not treated with neuraminidase.

(k) Other treatments.

These will be described in Chapter 3, when the results are reported.

(l) Photographing and preserving of the stained starch-plates

Nigrosine-stained plates were photographed by reflected light (Philips photolita No. 1). The transparent and semi-transparent gels stained for esterases etc., were placed on a cold-light illuminator and photographed by transmitted light. In both cases the film used was Ilford Micro Neg Pan with an exposure time of 1 sec, f value 2.8 - 11.

Where necessary, gels were photographed at intervals during the staining reaction; thus obtaining a record of both strong and weak fractions.

Nigrosine-/

Nigrosine-stained plates were either stored in a container filled with the methanol-acetic acid-water washing solution or wrapped in handiwrap after a brief "dehydration" in absolute alcohol. Esterase-stained plates were found to keep best in 10%-20% methanol in the cold room. If too much alcohol is added, the stained bands dissolve and fuse. It is not advisable to add alcohol, until after the plates have been photographed.

CHAPTER 3

RESULTS

3. 1 BLOOD PROTEINS AND ENZYMES IN APODEMUS SYLVATICUS

A. The protein and enzyme patterns of red cell lysates.

a) The nigrosine-amidoblack stained pattern (Figs. 2,3,4, 5,6,47,51,60 and 61. Tables 1, 2 and 26)

When tris-citrate starch plates at pH 7.6 were used, the nigrosine-amidoblack stain revealed many fractions, most of which were faint and disappeared in samples that were kept frozen (at -20°C) for a long time (3-20 months, Fig. 5, I).

Fractions A, B, Hb and C are the strongest and remain unchanged at -20°C for at least 4 years. Fractions A and B are of special interest, as they suggest polymorphism. One or the other of them may be absent, but never are both absent from a sample (see Tables 1 and 2). Some effort was made to discover some properties of these proteins by the use of a series of substrate and inhibition tests. The idea that these fractions might be carbonic anhydrase, was tested by staining for esterases using a mixture of 1-naphthyl acetate and 2-naphthyl acetate as substrates. Carbonic anhydrase is selective for 2-naphthyl acetate (Tashian, 1965). Another approach was the use of "Diamox" as an inhibitor specific to carbonic anhydrase. The results of the tests were negative, and it was also found that no esterases/

esterases could be demonstrated that overlap with bands A and B (Fig. 3 I, II). When staining for peroxidases by the o-dianisidine method, no peroxidase fractions overlapped with A and B (Fig. 4). The Hb reacted strongly, as is to be expected. Neuraminidase treatment (Fig. 47) did not affect A and B. Fraction C does not show peroxidase activity.

Incubation of red cell lysates at 56°C for 10 minutes destroyed fractions A, B and C but not the Hb.

Fraction R (Fig. 6.I) is a non-haemoglobin fraction in *Mus musculus* (Biddle and Petras 1967).

(b) The esterase pattern (Figs. 2, 3, 10 and 11)

There are at least 11 erythrocytic esterase fractions. Polymorphism is suggested among fractions 1, 2, 3 and 4, but combinations are complex. Fractions 7, 8 and 9 show also polymorphism, but in a simple two allelic fashion where the alleged phenotypes are 7.8, 7.8.9 and 8.9. Unfortunately these could only be identified in fresh samples with any reliability. As present investigation had to be carried out in such a way that samples were collected during the winter months and electrophoretic examinations during the summer, very few of the samples were/

were fresh and therefore no extensive survey could be done on red cell esterases.

c) Haemoglobins (Figs. 2,6,7 and 11).

On tris-citrate borate starch gel pH 8.6. (Figs, 7 and 11) all *Apodemus sylvaticus* tested (60 in all) had two Hb-fractions, Hb1 and Hb2. The 60 samples were 20 Irish, 21 Scottish and 18 Icelandic and 1 Norwegian. They had to be used fresh, because if they are kept frozen Hb1 disappears (Fig. 11). It is worth mentioning that if one freezes the red cells of *Apodemus sylvaticus* - the haemoglobin is precipitated and can only be re-dissolved with difficulty in distilled water, and then only to a small extent.

At pH 7.6. there is only one Hb fraction in the electropherogram. This system on the other hand is the most suitable for esterases (as already shown) because it ensures that the Hb does not overlap with other fractions. This technique has been useful also in studies of red cell esterases of some other mammals, namely sheep and whales (own unpublished observations).

TABLE 1

Incidence of combinations of major protein fractions A and B of red cell lysates from *Apodemus sylvaticus*

Country	Area on Maps	Total no. of mice			A			AB			B		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	29	19	48	15	5	20	14	14	28			
	2	39	29	68	4	2	6	35	27	62			
	3	1		1				1		1			
	4	2	4	6	1	1	2	1	3	4			
	5	1	1	2				1	1	2			
IRELAND	6	10	10	20				10	10	20			
SCOTLAND	7	15	9	24				8	5	13	7	4	11
	7b	5	3	8	2	1	3	3	2	5			
NORWAY	9	8	12	20				8	8	16		4	4
	10	7	17	24					1	1	7	16	23

TABLE 2

Incidence of fractions A and B of red cell lysates in <u>Apodemus sylvaticus</u>

Country	Area on Maps	Total no. of mice			A			B		
		m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	29	19	48	29	19	48	14	14	28
	2	39	29	68	39	29	68	35	27	62
	3	1		1	1		1	1		1
	4	2	4	6	2	4	6	1	3	4
	5	1	1	2	1	1	2	1	1	2
IRELAND	6	10	10	20	10	10	20	10	10	20
SCOTLAND	7	15	9	24	8	5	13	15	9	24
	7b	5	3	8	5	3	8	3	2	5
NORWAY	9	8	12	20	8	8	16	8	12	20
	10	7	17	24		1	1	7	17	24

B. Protein and enzyme patterns of the serum.

A study of serum proteins and esterases of the mouse was carried out during the years 1964-1966. (Arnason, 1966; Pantelouris and Arnason, 1966, 1967 and 1967). These reports dealt with specificity tests on serum proteins of Irish samples of *Apodemus sylvaticus* and of certain laboratory strains of *Mus musculus*. The results of this previous research will not be repeated here.

a. The nigrosine-amido black stained pattern (Figs, 6,8,8A, 14, 47, 51, 60 and 61.

Many protein fractions were resolved (see above-mentioned references), but only the strongest and most stable ones are of interest in this survey.

1) Post-albumins.

Table 3 shows the frequencies of postalbumin fractions. The significance of these data will be analysed in later chapter. At this stage it is worth noting that the rare slower post-albumin was only found amongst the Glasgow and Oslo mice.

2) Transferrins.

In/

In contrast to the house mouse, the field-mouse does not show polymorphism in the transferrin-pattern to any extent. Nearly all samples were of the type Tf (14), 15, 16 (16 being the strongest). Fraction 17 was present in 3 Irish mice (out of 160) and in 1 Icelandic (out of 140). In no other specimen could this fraction be found. It can however be "produced" experimentally by incubation with neuraminidase (Fig. 47, S, k).

b) The esterase pattern (Figs. 9, 9A, 10,11,12,41,42,43, 45,55,58,59 and 62
Tables 4,5,6,7,8,9,

My earlier work (Arnason, 1966) dealt mainly with the serum esterases of both the field mouse as well as those of the house mouse. The present study deals with additional information based on improved resolution, new specificity tests, population studies and breeding tests, although limited.

1) Fast moving esterases (Figs, 9,9A,11,41,42,45,55,56 57,58 and 62)

These esterase fractions have been divided into groups on basis of tests presented in Chapter 3.4, 3.5 and 3.6./

3.6.

These are as follows:

Fraction 1. (Es-1)

This esterase fraction may occur as a strong or a medium fraction. In other cases it may be absent altogether (Figs. 9 and 62). Table 4 gives the results of populations survey. As shown there one slower variant of this fraction was found among the Scottish field mice. This fraction differs from most other fractions in its resistance to heat and neuraminidase treatment (Figs, 45 and 58).

Fraction 2 (Es-2)

This fraction may vary in intensity and therefore difficult to group into "strong" and "medium". It may be absent altogether. Table 5 gives population data. A fast variant of this fraction was observed among Scottish specimens. Fraction 2 has very low affinity for naphthol-AS-acetate, but is affected by neuraminidase and heat. Fraction 2b usually occurs as a faint fraction located just behind fraction 2 and is only observed after intense/

intense staining - this fraction is more apparent in old samples. Two sera from Scotland, (area 7) had strong fraction in the position of 2b, which has been labelled 2b' (Fig. 9A,II).

Fraction 3 (Es - 3)

This fraction may be present or absent, "strong" or "medium".

Table 6 has been composed by grouping this fraction arbitrily in groups: strong, medium and absent. It is noteworthy that this fraction stains initially red and then turns purple when mixture of 1-naphthyl and 2-naphthyl acetate is used as substrates and Fast Garnet as coupler dye.

Fractions 4,5,5b and 6 (Es-4)

Table 7 demonstrates polymorphism among these fractions. They all show similar substrate affinity and behave in the same way towards inhibitors (Arnason 1966 and Chapter 3.4.). These are the strongest serum fractions migrating just behind the albumin (Figs. 9 and 62)

Fraction 7 (Es - 5)

This/

This fraction occurs rarely as Table 9 indicates. It could not be studied to great extent because of how infrequently it was found.

2) <u>Slower moving esterases</u>.

<u>Zone X'</u> (Es-6)

Zone X' comprises fractions 8, 9 and 10 (Figs. 9A, 10, 12 and 62). These fractions are absent from all Icelandic sera, but present in all other samples tested. The intensity of fractions in this zone varies, probably depending on physiological condition. It is worth noting at this stage that the absence of zone X' in the Icelandic sera demonstrates similarity to the *Mus musculus* pattern (Fig. 12 b and m, also Arnason and Pantelouris, 1966)

Fractions 11 and 12 may vary in concentration. The former especially was always strong or moderately strong in mice from Jæren, but less so in other samples.

<u>Zone X</u> comprises many faint fractions, that may be altered by altering the concentration of the starch.

<u>Fractions 13,14,15 and 16</u> vary in concentration as seen in Fig. 12. This variation can be related to pregnancy. Fractions 15 and 16 increase in intensity during pregnancy and/

and are strongest on days 15-17 of pregnancy, when fraction P ("pregnancy fraction") is at its strongest; fractions 13 and 14 disappear at the same time, but return 2 days after parturition. These changes are comparable to the pregnancy changes in *Mus musculus* (Arnason, 1966). Fractions 17, 18 and 19 are faint and could therefore not be studied on old sera.

Fraction 20.

This esterase fraction is strong comparable in intensity to the fast moving fractions. In one animal from Skalakot (area 2b) this fraction was lacking along with fractions 15 and 16 (Fig. 9AI,d). Fraction 20 decreases in intensity on storage, whilst some slower fractions make their appearance. The first of these lies just in front of 21 with which it partly overlaps. Thus, fraction 20 behaves in a similar fashion to fraction C4 in human serum (Harris *et al.*, 1962, 1963a). Fractions 21 and 22 are faint, and sometimes absent.

These esterase-patterns show variation, some of which appears to be genetic whilst some is physiological.

C. Ceruloplasmins, (Figs. 13 and 14).

The oxidase activity of these proteins on p-phenylenediamine hydrochloride was used for their identification (Morell and Scheinberg, 1960).

After thorough investigation on samples from some 300 field mice (including a comparison of heparin-treated and untreated aliquots) no variation could be demonstrated. This casts doubt on a previous report of two phenotypes (Pantelouris and Arnason, 1967b). It would seem that the appearance of two 'phenotypes' was in fact caused by somewhat unequal heparinisation. For, if thoroughly heparinised, ceruloplasmin fraction 3 changes its mobility and takes up the same position as fraction 2 (Fig. 14). With a smaller amount of heparin both fractions 2 and 3 remain distinct. Fig. 13 shows the effect of incubation with the ions, Fe^{++}, Cu^{+} and Cu^{++}. The two latter ions have no effect, but Fe^{++} increases the mobility of fraction 3 to make it equal to that of fraction 2; an effect similar to that of heparinisation.

It can be concluded that the natural mobility of the ceruluplasmin leads to the appearance of band 3. In Fig./

Fig. 14, II the effect of heparin on the protein pattern as compared to ceruloplasmin pattern is shown. Heparinisation may affect also the pre - and post - albumin zones, in that it "decolourisеds" background staining at the site of fraction 1.

TABLE 3

Incidence of 'fast' and 'slow' postalbumins in Apodemus sylvaticus.

Country	Areas on maps	Total no. of mice	'Fast' Post Albumin	'Slow' Post Albumin
ICELAND	1	56	56	0
	2	72	67	0
	3	1	1	0
	4	6	5	0
	5	2	2	0
IRELAND	6	139	126	0
SCOTLAND	7	24	23	3
	7b	8	8	0
NORWAY	9	20	20	0
	10	22	20	3

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TABLE 4

Frequencies of phenotypic expressions of serum esterase fraction 1 in <u>Apodemus sylvaticus</u>

Country	Area on Maps	Total no. of mice			Fraction 1 Strong			Medium			Absent			Abnormal	
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f
ICELAND	1	23	27	50	5	8	13	14	13	27	4	6	10		
	2	41	30	71	12	11	23	21	13	34	8	6	14		
	2b	4	5	9	1	1	2	3	4	7					
	3	1		1	1		1								
	4	2	4	6				2	2	4		2	2		
	5	1	1	2				1	1	2					
IRELAND	6			11 6			22			65			29		
SCOTLAND	7	18	11	29	5	1	6	9	5	14	3	5	8	1 slow	
	7b	7	3	10							7	3	10		
NORWAY	8	22	20	42	5	2	7	11	8	19	6	10	16		
	9	8	12	20		2	2	4	6	10	4	4	8		
	10	9	15	24							9	15	24		

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TABLE 5

Incidence of serum esterase fraction 2 of *Apodemus sylvaticus*

Country	Area on Maps	Total no. of mice			Fraction 2 Present			Fraction 2 Absent			Fraction 2 Fast		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	23	27	50	23	27	50						
	2	41	30	71	41	30	71						
	2b	4	5	9	4	5	9						
	3	1		1	1		1						
	4	2	4	6	2	4	6						
	5	1	1	2	1	1	2						
IRELAND	6			115			115						
SCOTLAND	7	17	8	25	10	7	17	3		3	4	1	5
	7b	7	3	10	3	1	4	4	1	5		1	1
NORWAY	8	23	21	44	22	19	41	1	2	3			
	9	8	12	20	5	12	17	3		3			
	10	9	15	24	9	13	22		2	2			

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TABLE 6

Incidence of serum esterase fraction 3 in *Apodemus sylvaticus*.

Country	Area on Maps	Total no. of mice			Fraction 3								
					Strong			Medium			Absent		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	22	27	49	1		1	4	7	11	17	20	37
	2	41	30	71							41	30	71
	2b	4	5	9		1	1	1	1	2	3	3	6
	3	1		1							1		1
	4	2	4	6							2	4	6
	5	1	1	2		1	1				1		1
IRELAND	6			127			100			20			7
SCOTLAND	7	16	8	24	5	2	7	8	5	13	3	1	4
	7b	7	3	10	2	3	5	4		4	1		1
NORWAY	8	22	25	47	5	5	10	9	9	18	8	11	19
	9	8	12	20		4	4	8	8	16			
	10	9	15	24	2	6	8	6	7	13	1	2	3

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TABLE 7

Incidence of combinations of serum esterase fractions 4, 5, 5b and 6 in *Apodemus sylvaticus*

Country	Area on Maps	Total no. of mice			4			4-5			5			5 - 5b			5 - 6			5b			5b - 6			6		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	24	28	52							13	9	22				7	15	22							4	4	8
	2	41	29	70							3	8	11				37	17	54							1	4	5
	2b	4	5	9							2	1	3				2	3	5								1	1
	3	1		1													1		1									
	4	2	4	6							1		1				1	1	2								3	3
	5	1	1	2														1	1							1		1
IRELAND	6	102	46	148		2	2	4		4	41	23	64	2		2	35	14	49	1		1	1	1	2	18	6	24
SCOTLAND	7	15	10	25							7	3	10	1	2	3	5	3	8				1	2	3	1		1
	7b	7	3	10		1	1				5	2	7				2		2									
NORWAY	8	23	22	45							13	9	22		1	1	5	6	11	3	3	6	1	3	4	1		1
	9	8	12	20							8	8	16					4	4									
	10	8	14	22							2	4	6		3	3	2	4	6	1	1	2	1	2	3	2		2

TABLE 8

Incidence of serum esterase fractions 4, 5, 5b and 6 in <u>Apodemus sylvaticus</u>.

Country	Area on Maps	Total no. of mice			4			5			5b			6		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	24	28	52				20	24	44				11	19	30
	2	41	29	70				40	25	65				38	21	59
	2b	4	5	9				4	4	8				2	4	6
	3	1		1				1		1				1		1
	4	2	4	6				2	1	3				1	4	5
	5	1	1	2					1	1				1	1	2
IRELAND	6	102	46	148	6		6	82	37	119	4	1	5	54	21	75
SCOTLAND	7	15	10	25				13	8	21	2	4	6	7	5	12
	7b	7	3	10		1	1	7	2	9				1		1
NORWAY	8	23	22	45				18	16	34	4	7	11	7	9	16
	9	8	12	20				8	12	20					4	4
	10	8	14	22				4	11	15	2	6	8	5	6	11

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TABLE 9

Incidence of serum esterase fraction 7 in Apodemus sylvaticus

Country	Area on Maps	Total no. of mice			Fraction 7 Present			Fraction 7 Absent		
		m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	24	28	52	3	2	5	21	26	47
	2	41	29	70	1	1	2	40	28	68
	2b	4	5	9				4	5	9
	3	1		1				1		1
	4	2	4	6				2	4	6
	5	1		1				1		1
IRELAND	6	102	46	148	5	2	7	97	44	147
SCOTLAND	7	15	10	25		1	1	15	9	24
	7b	7	3	10				7	3	10
NORWAY	8	23	22	45				23	22	45
	9	8	12	20				8	12	20
	10	8	14	22				8	14	22

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3.2 PROTEIN AND ESTERASE PATTERNS OF STRIATED - AND HEART MUSCLE HOMOGENATES OF APODEMUS SYLVATICUS

A. Nigrosine stained pattern.

a) Protein pattern of skeletal muscle homogenates of Apodemus sylvaticus

(Figs. 15,16,17,47,48,49,50,51,60, 68 and 73. Tables 10, 11 and 12).

Electropherograms of skeletal muscle homogenates comprise at least 19 protein fractions, when amido-black-nigrosine is used as the stain (Fig. 15). These have been grouped arbitrily into four zones A-D to simplify demonstration and discussion.

Zone A. fractions 1-5:

Fractions 1, 2 and 3 may be separated to a different degree depending on the percentage of starch in the gel and the electrophoretic conditions.

Fraction 4 has always the same migration rate as the serum albumin and may indeed be an albumin contamination of the muscle homogenates.

Fraction 5 similarily seems to correspond to the serum post-albumin/

albumin (Fig. 50).

No polymorphism was observed in zone A.

Zone B, fractions 6-12

Fraction 6 is the strongest fraction in the skeletal muscle homogenate. All other fractions in this zone are faint and no polymorphism was observed.

Fractions 8 and 9 correspond to serum transferrins (Fig. 15)

and

Fraction 11 overlaps a peroxidase-active fraction (Fig. 50)

Zone C, fractions 13-17:

The appearance of this zone depends on the duration of the electrophoretic run and how fresh the starch plate is (Figs. 15 and 50). In a short run the fractions pile up near the origin and may be mixed up with fractions of zone D.

Fraction 13 is the strongest in this zone. The others are usually faint and do not keep well on prolonged storage. Heat and neuraminidase treatments destroy all fractions in this zone (Figs. 47,48,60 and 61) . Fraction 13 stains faintly with P.A.S. (Fig. 51)

When either Tris-citrate borate pH 8.6. (Fig. 17,I) or, even better, Tris-citrate pH 8.6. (Fig. 68) were used fraction

C'/

C' is demonstrated. This fraction is present in some Icelandic samples and one Swedish one.

Table 12 shows incidence of fraction C'.

Zone D, fractions 18 and 19

Tables 10 and 11 show the incidence of these fractions. When fraction 19 occurs and fraction 18 is absent, fraction 19 is much "heavier" than when both 18 and 19 occur together (Fig. 15). In some cases they may be absent (Fig. 15 f and h) and in one Scottish specimen a different, rare type was observed (Fig. 16,e). Fractions in zone D migrate cathodally at pH 7.6.

b) The protein pattern of cardiac muscle homogenates of *Apodemus sylvaticus*

(Figs. 15,16,47,48,49,50,51,60,61 and (73)

The electropherograms of heart muscle homogenates are arbitarily divided into 5 zones comprising at least 19 fractions.

Zone A, fractions 1-5

Fractions 1,3,4 and 5 of the cardiac muscle correspond to the same numbers of the skeletal muscle, but fraction 2 of the heart is faster moving than fraction 2 of the skeletal muscle (Fig. 16). Fraction 3 is destroyed by neuraminidase.

Zone A_2, fractions 6 - 8

Fractions 6 and 7 are usually faint, but fraction 8 is the strongest in/

in this zone. The skeletal muscle homogenates have no corresponding fractions to these.

Zone B, fractions 9-14

In this zone fraction 12 is the strongest. It is destroyed by neuraminidase and stains with P.A.S. Fraction 11 corresponds to 9 in skeletal muscle - both overlap the strongest Tf band (16).

Fraction 14 overlaps a peroxidase-active fraction (Fig. 50). More peroxidase active bands move anodally at pH 8.6. than pH 7.6. (Figs. 49 and 50) - these have no corresponding protein fractions.

Zone C, fractions 15 and 16.

Fraction 15 is strong and overlaps fraction 13 of the skeletal muscle, it is affected by neuraminidase and heat (Figs. 47 and 60). Fraction 16 is very likely a haemoglobin contamination in the heart homogenates.

Zone D, fractions 17-19

These cathodally migrating proteins are much fainter than the cathodally migrating fractions of the skeletal muscle.

Fraction 17 overlaps fraction 19 of the skeletal muscle, but fractions 18 and 19 do not have any corresponding ones. There is a polymorphism in this zone, but this could not be studied, because/

because fractions were too faint.

B. The esterase pattern of skeletal and cardiac muscle homogenates.

(Figs. 18,19,20,21,22,23,39,40,41,42,43,44,45,46,52,53,54, 55,56,57,58,59,63,64 and 65 Tables 4,5,6,7,13,19,20 and 25)

In contrast to the protein patterns of these two tissues which differ widely, the esterase pattern of these two are more or less identical, except for few fractions in zone C. Polymorphisms are observed in zones A and C.

Zone A, fractions 1-8

Most fractions in this zone correspond to the fast moving esterases of the serum and can only be resolved using Tris-citrate borate pH 8.6. (Tables 4,5,6,7 and 25. Figs. 19,21 and 52)
Skeletal - cardiac muscle esterase fractions 2 and 4 do not have their equivalent in the serum, it is however possible that in the serum, fraction 2 (cardiac) is overlapped by the strong serum fraction 1.

Fraction 4 may be absent, weak or strong, but because of variation in strength it was too difficult to tabulate. Differences between populations were apparent and are reported in Chapter 3.6. Specificity tests of above mentioned fractions are tabulated in Chapter 3.4

Zone/

Zone B.

This zone does not show any esterase activity after normal staining time, but prolonged incubation produces very faint esterase smear in stored samples in this area, Fresh samples may reveal faint fractions in zone B on prolonged staining, but as explained previously only few fresh samples could be obtained.

Zone C, fractions 12-27

Four main types are observed here - each comprising many fractions, only the strongest ones being numbered. The relative positions of the fractions may also change, depending on the electrophoretic system used (Figs. 18,63,64 and 65). On this basis I have chosen to describe and discuss these in terms of zones. Population data are shown in Table 13 and specificity properties in Tables 19 and 20. It is worth stressing that the same animal has always the same type of C-zone(the number of fractions may vary) in all tissues sampled. The four above mentioned types in order of decreased migration rate of the strongest fractions of the zone are:

C_I : This is the fastest type. The strongest fractions are 16, 19 and 20' at pH 7.6. (Fig. 63). At Tris-citrate/

citrate pH 8.6. this zone is completely separated from C_{II}. The Swedish samples had only Type C_I.

C_{II} : The main fraction is 20 and the slowest is 21 at pH 7.6. This is the only type found in the Scottish specimens.

C_{III} : The characterstic fractions are 23 the strongest and 24 the slowest at pH 7.6 (Fig. 18). This type was only found in Norway.

C_{IV} : The slowest type with characteristic fractions 26 and 27. Found only in Iceland and Ireland.

In all four types there may occur minor differences, regarding number of bands or relative positions. It is not known whether these intra-type variations are genetical, physiological or due to handling of samples. Storage at -20°C did not seem to alter the type; samples retain their C-type even after storage for four years. These esterases are therefore stable when kept frozen.

Faster moving fractions of zone C are more pronounced in skeletal than cardiac muscle homogenates (Fig. 23). Results of breeding tests will be reported in Chapter 3.5.

TABLE 10

Incidence of combinations of cathodally migrating muscle proteins in *Apodemus sylvaticus*.

Country	Area on maps	Total no. of mice			Fractions Absent			18-19			19		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	13	15	28	2	1	3	3	3	6	8	11	19
	2	21	16	37	6	2	8	3	5	8	12	9	21
	3	1		1							1		1
	4	2	4	6							2	4	6
	5	1	1	2							1	1	2
IRELAND	6	10	10	20							10	10	20
SCOTLAND	7	14	9	23							14	9	23
	7b	7	3	10							7	3	10
NORWAY	8	25	23	48							25	23	48
	9	8	12	20				2	2	4	6	10	16
	10	7	17	24				1	5	6	6	12	18

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TABLE 11

Incidence of muscle protein fractions 18 and 19 in Apodemus sylvaticus.

Country	Area on Maps	Total no. of mice			Fractions 18			Fractions 19		
		m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	13	15	28	3	3	6	11	14	25
	2	21	16	37	3	5	8	15	14	29
	3	1		1				1		1
	4	2	4	6				2	4	6
	5	1	1	2				1	1	2
IRELAND	6	10	10	20				10	10	20
SCOTLAND	7	14	9	23				14	9	23
	7b	7	3	10				7	3	10
NORWAY	8	25	23	48				25	23	48
	9	8	12	20	2	2	4	8	12	20
	10	7	17	24	1	5	6	7	17	24

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TABLE 12

Incidence of c^1 protein in thigh muscle homogenates of <u>Apodemus sylvaticus</u>.

Country	Area on Maps	Total no.			Fraction c^1 Present			Fraction c^1 Absent		
		m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	17	17	34	9	8	17	8	9	17
	2	29	28	57	15	16	31	14	12	26
IRELAND	6	10	10	20				10	10	20
SCOTLAND	7	12	7	19				12	7	19
	7b	7	3	10				7	3	10
NORWAY	8	25	23	48				25	23	48
	9	8	12	20				8	12	20
	10	5	8	13				5	8	13

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TABLE 13

Incidence of different phenotypes of heart and striated muscle esterases in zone C of *Apodemus sylvaticus*.

Country	Area on Maps	Total no. of mice			Zone C: C II			Zone C: C III			Zone C: C IV		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	17	25	42	8	9	17				9	16	25
	2	33	25	58	27	13	40				6	12	18
	3	1		1							1		1
	4	2	4	6		2	2				2	2	4
	5	1		1	1		1						
IRELAND	6	10	10	20	10	8	18					2	2
SCOTLAND	7	15	9	24	15	9	24						
	7b	7	3	10	7	3	10						
NORWAY	8	24	24	48	17	20	37	7	4	11			
	9	8	12	20	4	4	8	4	8	12			
	10	7	18	25		3	3	7	15	22			

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3.3 ESTERASE AND PROTEIN PATTERNS OF LIVER HOMOGENATES OF APODEMUS SYLVATICUS.

A. Esterase pattern of liver homogenates

a) Description of zones.

(Figs. 24, 25,26,27,28,29,30,31,32,33,35,36,38,39,40,41,42,43,45, 45,46,53,54,55,56,57,58,59,66,69,72, and 74. Tables 13,14,15 16,17,21 and 25)

Liver esterase zymograms exhibit the strongest as well as the greatest number of fractions found in any tissue. This presents us with the problem of separation as well as staining; long electrophoretic runs are necessary as well as photographing of plates at intervals during staining procedure. The liver esterase zymogram comprises at least up to 37 fractions arbitrarily divided into four zones A-D. Polymorphism occurs in all 4 zones (Tables 13,14,15,16, and 17. Figs. 25, 26 and 28)

Zone A, fractions 1-10

This zone is divided into fast and slow region:

Fast region: fractions 1-7 (Tables 14, 5 and 15). These fractions are very faint, therefore a prolonged staining time is required (Fig. 26), this in turn may cause diffusion of enzyme bands so resolution is decreased. Control runs were necessary on many occasions.

Fraction 1 is the fastest esterase fraction of any time in *Apodemus sylvaticus*, and rarely occurs on its own, but along with fraction 2, which overlaps serum esterase fraction 1 (Tables 14 and 25, Figs. 26 and 27).

Fraction 3, which is always faint and fraction 4 overlap serum esterase fraction 2 and demonstrate the same polymorphism (Table 5). The comparative situation of fractions 1 and 2 is more complicated as described in Chapter 3,4.

Incidence of fractions 5,6 and 7 is given in Table 15; as shown there fraction 6 occurs in all samples, but fractions 5 and 7 may be present or absent. All these fractions have average intensity, but tend to fuse on prolonged staining. The faster fractions in this region (1,2,3 and 4) have low affinity for napthol-AS- acetate, but the slower (5,6 and 7) break down this substrate.

Slow region (E_s- 4), fractions 8-10

Fractions 8, 8b and 9 correspond to serum esterase fractions 5, 5b and 6 respectively and react in the same way, but are less intense (Figs. 26,42,46,55, Tables 7,8,18,21 and 25). Compare Figs. 11 and 33 for serum and liver fractions of the same animals.

Fraction 10 is nearly always faint.

Zone B, (E_s- 7b) fractions 11-15

Polymorphism is observed here (Figs. 26,27,32 and 66 Tables 16 and 17) The separation in this zone depends on the length of the electrophoretic run. All fractions, except 11 are strong and react to all substrates but 6-bromo-2-carbonaphthoxy choline iodide; they are heat sensitive (Fig. 58) and fractions 14 and 15 at least (Fig. 46) affected by neuraminidase (see next Chapter 3.4)

Zone B (E_s-^{7a}) - the "Icelandic" Type

All Icelandic specimens tested (about 200) showed very little reactivity in this zone in contrast to most other specimens (Fig. 24).

The/

The "near silence" in this zone is a "marker" for Icelandic field mice. Only two animals outside Iceland exhibited the "emptiness" in zone B – these were Irish specimens (Fig. 29). Liver homogenates of Mus musculus stained for esterases lack activity in this zone (Fig. 32 e,l; Figs. 33,69,71 and 74).

Prolonged staining brings up fractions in above mentioned zone in the Icelandic samples. The strongest fraction usually overlaps fraction 13, but the position may vary somewhat in some zymograms. The other fractions are fainter, but some may overlap fractions of the "usual" B-zone type. Because of how weak the Icelandic B-zone is a tabulation of its fractions was impracticable. This "near silence" of the Icelandic mice is shown in chapter 3.5 to be genetical (Fig. 66)

Zone C (E_S- 8), fractions 16-37

(Figs. 24,25,26,27, and 66, Tables 13 and 21)

As a whole this is the part of the zymogram with most intense esterase activity. This stems from numerous and close fractions in this zone, which are only separated with difficulty. Sometimes the fractions may cluster to form dense bands. Four main typed occur (Figs. 24,25 and 66)

C_I, a zone reaching from fraction 16 to 25

C_{II}, reaching from fraction 16 to fraction 33 (31-33 are very faint)

C_{III}, reaching from fraction 16 to fraction 35 (35 is very faint)

C_{IV}, reaching from fraction 16 to fraction 37

The zone C has affinity for all substrates tested but 6-bromo-2-carbonaphthoxy choline iodide. Neuraminidase treatment decreases the intensity of the fractions and slows down the mobility (Fig. 46). Heat destroys this zone, except the "strongest" fractions of each type (Figs. 58 and 59)

Zone D.

Three fractions are foond here (Fig. 28), migrating cathodally in Tris-citrate gel at pH 7.6. These can only be demonstrating after 12 hours staining, and only in samples having zone IV.

b) Foetal liver esterases of Apodemus Sylvaticus

(Figs. 30,31 and 32)

Six foetuses were obtained from a pregnant female from Norway (Jaeren). The age of the foetuses was estimated at 15 days according to earlier experience (Arnason, 1966). The livers of these were pooled two in each lot thus giving three samples. The next age group that was obtainable were 12 days old young.

These sporadic samples give only a limited idea of the ontogenetic changes of the liver esterase fractions. The 12 days old young showed the adult pattern - probably not quite so strong. The foetal pattern will be described with reference to the adult pattern.

Zone A.

Fast region.

Only fractions 5, 6 and 7 were observed here and these were the strongest fractions in the foetal liver not much weaker than the corresponding adult ones (Fig. 30).

Zone B.

Fractions 13,14 and 15 did stain here but far less intensely than in adult samples. Fractions 11 and 12 are missing in the foetal homogenates (Fig. 30).

Zone C.

No activity was observed here in the foetal samples, except for faint reaction of fractions 16 and 17 (Fig. 32).
It is a striking observation to compare the emptiness of this zone on the foetal zymogram to the reactiveness on the adult zymogram (Fig. 32). The difficulty in rearing and catching of field mice prohibited further study into this field.

B. <u>Protein pattern of liver homogenates.</u>

(Figs. 35,47,48,49,50,60,61 and 73)

An attempt was made to study the protein fractions of liver homogenates, but this is next to impossible because of the large number and faintness of the protein fractions (Fig. 35). On storage these faint fractions are turned into continuous smear. Only fresh samples could be used and these were scarcely available as explained earlier. Despite all this a few points are worth noting.

The neuraminidase treatment seems to destroy most of the liver protein fractions (Fig. 47), thus implying that they are sialo proteins (Svensmark, 1961). Incubating the liver homogenates at 56°C for 10 minutes clears away the protein smear and the pattern that is left is more or less the cardiac protein pattern (Figs. 60 and 61). The strongest protein band of the liver overlaps the serum albumin and very likely is serum albumin contamination of the liver.

TABLE 14

Incidence of combinations of liver esterase fractions 1 and 2 in *Apodemus sylvaticus*.

Country	Area on Maps	Total no. of mice			Fractions 1 and 2											
					Absent			1			1 - 2			2		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	22	22	44		2	2	1	1	2	21	18	39		1	1
	2	10	13	23		1	1				10	12	22			
	4	2	4	6		1	1				2	3	5			
IRELAND	6	10	10	20	4	5	9				6	5	11			
SCOTLAND	7	17	8	25	5	3	8				12	5	17			
	7b	7	3	10	7	3	10									
NORWAY	9	8	12	20	4	8	12				4	4	8			
	10	7	17	24	3	7	10				4	10	14			

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TABLE 15

Incidence of liver esterase fractions 5, 6 and 7 in *Apodemus sylvaticus*.

Country	Area on Maps	Total no. of mice			Fractions 5, 6 and 7: 5 - (6)			Fractions 5, 6 and 7: (6) - 7		
		m	f	m+f	m	f	m+f	m	f	m+f
ICELAND	1	21	23	44				21	23	44
	2	11	12	23				11	12	23
	4	2	4	6				2	4	6
IRELAND	6	10	10	20	4	3	7	6	7	13
SCOTLAND	7	16	9	25	14	7	21	2	1	3
	7b	7	3	10	6	2	8	1	1	2
NORWAY	9	8	12	20	8	8	16		4	4
	10	7	17	24	4	9	13	3	8	11

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TABLE 16

Incidence of combinations of liveresterase fractions 11, 12, 13, 14 and 15 in <u>Apodemus sylvaticus</u>

Country	Area on Maps	Total no. of mice			11 (12)			11,12,13,14			11,12,13,14,15			11,13			11,13,14			11,13,14,15			(13)*			13,14		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
IRELAND	6	10	10	20				2	1	3	6	7	13				1	1	2				1	1	2			
SCOTLAND	7	18	7	25	1		1				2	1	3	3		3	9	4	13	3	2	5						
	7b	7	3	10										1		1	4	2	6		1	1				1		1
NORWAY	9	8	12	20				8	8	16								4	4									
	10	8	17	25					1	1	4	4	8	1		1	3	4	7		5	5					3	3

*) All Icelandic mice are of this type, lacking zone B or expressing faint esterase fractions in zone B

TABLE 17

Incidence of liver esterase fractions 11, 12, 13, 14 and 15 in *Apodemus sylvaticus*.

Country	Area on Maps	Total no. of mice			Fractions 11			12			13			14			15		
		m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
IRELAND	6	10	10	20	9	9	18	8	8	16	10	10	20	9	9	18	6	7	13
SCOTLAND	7	18	7	25	18	7	25	2	1	3	17	7	24	14	7	21	5	3	8
	7b	6	3	9	5	3	8				6	3	9	5	3	8		1	1
NORWAY	9	8	12	20	8	12	20	8	8	16	8	12	20	8	12	20			
	10	8	17	25	8	14	22	4	5	9	8	17	25	7	17	24	4	9	13

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3.4 SPECIFICITY TESTS AND COMPARISON OF VARIOUS TISSUE HOMOGENATES OF APODEMUS SYLVATICUS

A. Specificity tests

a) Characterization of the protein fractions.

(Figs. 2,3,4,7,8A,13,14,47,48,49,50,51,60 and 61)

In previous sections of this chapter the results of various tests have been described. This section will summarize these findings. The tests used included: Glycoprotein staining (P.A.S.), lipoprotein staining, peroxidase staining, esterase staining, iron labelling and staining, paraphenylene diamine hydrochloride staining, effects of some metal ions, neuraminidase test and heat incubation.

1) P.A.S. staining revealed several glycoprotein fractions in the serum (Fig. 51). This was to be expected and agrees with earlier findings (Arnason, 1966). Fraction 16 of the serum is the strongest - this is the main transferrin fraction. Apart from transferrin contamination the only fraction in other tissues tested to stain with this method was 13 of the skeletal muscle.

2) Lipoprotein staining only revealed faint smear in the middle of the zymogram in the case of the serum. All tissues showed staining at the point of application.

3) Peroxidase staining is demonstrated in Figs. 4, 49 and 50. In addition to staining haemoglobins following fractions showed reaction:

Red cell lysates: fraction 1 and 2 (Fig. 4)

Cardiac muscle: fraction 14, (Fig. 50)

Skeletal muscle: fraction 11 (Fig. 50)

It is worth noting that at pH 8.6., the heart muscle homogenates have strong peroxidase activity nearly 2/3rds of the whole zymogram from the origin, some activity is also observed in the liver (Fig. 49). At pH 7.6. this migrates cathodally, indicating that this could be Hb-contamination.

4) Iron-binding protein was observed overlapping serum fraction 16 (Fig. 8A) when autoradiography was applied and ^{59}Fe used as marker. The nitroso-R method gave similar results, though less specific.

5) Ceruloplasmins are described in Chapter 3.1,C. They are situated between serum protein fractions 15 and 16 at pH 7.6., when not heparinized - (Fig. 14).

6) Neuraminidase treatment had effect on various protein fractions as shown in Figs. 47 and 48.

Red cell lysates:	no effect
Serum:	most pronounced is the effect on Tf fractions, but other fractions are also affected (Arnason, 1966)
Skeletal muscle:	All fractions except 1, 6 and 19 seem affected (Fig. 47)
Heart muscle:	Fractions 3,4,5,12,13,14,15,16 and 18 are either destroyed or the mobility is changed by this method.
Liver:	All fractions more or less destroyed.

7) Heat incubation of homogenates at 56°C for 5-10 minutes resulted in disappearance of certain fractions:

Red cell lysates: Fractions A,B and C are destroyed

Serum: The only observable affect is the disappearance of Pre-albumin fractions (Fig. 60).

Skeletal muscle: Fractions 9-17 are all destroyed. Fraction 6 and 19 are not affected.

Heart muscle: Only fractions 3 and 15 are destroyed.

Liver: The protein smear disappears and what is left is similar to the heart pattern.

Kidney: Same as the liver pattern, except fraction overlapping heart fraction 2 is missing.

Testis: This tissue has its characteristic protein pattern, counting at least 14 fractions. The ones destroyed by heat are: 2,3,5,7, 10 and 11.

b) Phosphatases (Fig. 38)

Only a limited work was done on phosphatases in the present survey, but the following points can be made: The acid phosphatase activity was especially strong in B and C Zones of spleen and testis homogenates and in B zone of epididymis. Liver, kidney, heart, and skeletal muscle had some activity in zone C. Serum phosphatases were discussed in previous work (Arnason, 1966).

c) Characterization of esterase fractions.

(Figs. 39, 40,41,42,43,44,45,46,53,54,55,56,57,58 and 59; Tables 18, 19,20,21,22,23 and 24)

The methods employed here include the use of different substrates and inhibitors, incubation with neurominidase and heat sensitivity tests.

Substrates/

Substrates and inhibitors are listed in Chapter 2, III. The results of tests are given in Tables 18-24. Some explanatory notes are added here:

Substrates:

In addition to 1-naphthyl acetate listed in the tables, 2-naphthyl acetate and 1-naphthyl propionate were used (Figs. 53,54 and 55). All these substrates gave more or less the same results, but 2-naphthyl acetate showed slower reaction. Exception to this is fraction 3 in skeletal and heart muscle, and fractions (4), 5,6 and 7 in the liver. When a mixture of 1-naphthyl acetate and 2-naphthyl acetate were used, fractions 2, 2b' and 3 of the serum and fractions 3,4 and 5 of skeletal and heart muscle stained red in the early phases of the staining. Other fractions were purple i.e. the former have greater affinity for 2-naphthyl acetate than 1-naphthyl acetate.

Fraction 12 of the testis and brain show greater affinity for 1-naphthyl propionate than for other naphthyl compounds.

Naphthol-AS-acetate is broken down by many fractions, but usually at a slower rate than naphthyl compounds. Of particular interest is that the fastest fractions do not hydrolyse naphthol -AS- acetate. These are serum fractions 1,2,2b and fractions 1,2,3 and 4 of skeletal, heart muscle, brain and testis as well as liver and kidney fractions 1,2,3 and 4. Fractions 5,6 and 7 of liver and kidney react strongly with this substrate in contrast to fraction 4 of heart, muscle, brain and testis.

Zone X' of serum does not break this substrate down (Fig. 54), but fractions 9 and 10 of brain and testis do.

The fractions that react most strongly with naphthol-AS-acetate are fractions in zone B, particularly fraction 13 of liver and kidney and fraction 12 of testis and brain. Faster moving fractions of zone C show also/

also high affinity in this respect (Figs. 42,54 and 55)

6-bromo-2-carbonaphthoxy choline iodide is broken down by some serum fractions (Table 18), particularly fractions 3-6, 16 and 20. In other tissues fractions corresponding to serum fractions 3-6 break this substrate down to some extent, but far less efficiently than the serum does. The fact that most fractions react with many substrates underline the difficulty in grouping esterases on the bases of substrate reactions alone.

Inhibitors:

Three groups of esterase inhibitors were used (Holmes and Masters, 1966)

1. Cholinesterase inhibiting:

   physostigmine sulphate (10^{-4} and 10^{-5} M) and neostigmine bromide (10^{-4}M)

2. Carboxyl - (Ali-) esterase and choline esterase inhibiting:

   D.F.P. (di-isopropyl fluorophosphate 10^{-4}M)

3. Arylesterase inhibiting:

   P.C.M.B. (p-chloromercuribenzoate 10^{-3}M). The effect of these inhibitors is tabulated in Tables 18-24.

   Physostigmine and neostigmine were only shown to inhibit some serum fractions, partially: The fastest moving ones (1-2b) and middle fractions (15-16) and fraction 20. Zone X' is inhibited to some extent by neostigmine, but not physostigmine. The inhibitory effects on other tissues are only noticed in fractions 12 and 13 in heart and muscle homogenates and the fastest fractions of the liver.

   D.F.P. inhibits nearly all fractions, except the strongest serum fractions (3-6) and fraction 13 and faster fractions of zone C in the liver.

   P.C.M.B. failed to have any effect, probably partly due to the difficulty in dissolving this compound in the media necessary for the procedure.

Heat treatment.

When starch plates were incubated in a waterbath for 20 minutes at 56°C after electrophoresis no effect was observed (Martin and Petras, 1971). If on the other hand the samples were incubated at 56°C for different lengths of time prior to electrophoresis drastic changes were observed (Pelzer, 1965) (Figs. 58 and 59 and Tables 18-24). The following points are worth stressing:

Liver: All fractions are nearly completely destroyed after 5 minutes. incubation except 8,9 and 23, 24, and 25 which are partially destroyed. Kidney: Fractions 5, 6 and 7 are not destroyed after 10 minutes incubation in contrast with the same fractions in the liver. All fractions in zone C are only partially inhibited after 10 minutes.

Serum: Fraction 1 is the only band that is not affected after 10 minutes incubation. Fraction 20 is split in two after 5 minutes incubation i.e. a new faster moving fraction is formed (Fig. 58). All other fractions are destroyed in 5 minutes. Heart, skeletal muscle and testis: Fraction 1 is resistant to this treatment. Fractions 3 and 4 disappear in 5 minutes. Fractions 5, 6 and 7 are only partially destroyed in 5 minutes and have not disappeared after 10 in contrast to fractions 3, 5 and 6 of the serum. In zone C the fractions are only partially inactivated after 10 minutes comparible to fraction 25 of the liver.

All esterase fractions of all tissues were destroyed after 20 minutes incubation at 56°C.

Neuraminidase treatment:

The effect of this enzyme on the serum of Apoderus has already been described (Arnason, 1966). It is found that all fractions, except 1 are affected (Figs. 43 and 45). Fraction 2 takes up the position of 2b (2b') after this treatment. The other serum fractions are slowed down or disappear. All zones of the liver homogenate are affected, especially zone A, where the fractions nearly disappear. In zone B the slower fractions are obliterated and in zone C the faster moving fractions decrease in intensity and the slower ones increase their intensity and probably take up a new position (Fig. 46).

In heart and skeletal muscle homogenates the fractions in zone A show comparable changes as the corresponding fractions of the serum. Zone C is changed in such a way as to reduce intensity of fractions and even make them disappear (Fig. 45, M, h).

B. Comparison of esterase fractions of various tissues.

Table 25 gives the relative positions and intensity of esterase fractions in various tissues. The specificity tests give some idea which of these have similar function or make up and the population studies clarify further which of the overlapping fractions in different tissues could be the same fractions. This will be discussed in Chapter 4. It is stressed here that every animal had the same type of zone C for all the homogenised tissues tested i.e. an animal with a fast C-zone in one tissue has fast C-zone in all tissues even if number of fractions between tissues may vary e.g. liver and heart. For a demonstration of this compare Figs. 63,64,65,66 and 67, and also Figs. 18 and 33

Fig. 18,a is from the same animal as Fig. 33,b

Fig. 18,b ... ... Fig. 33,c

Fig. 18, c and d ... ... Fig. 33, d and e

Fig. 18,e ... ... Fig. 33,f

Fig. 18,f ... ... Fig. 33,g

Fig. 18,g ... ... Fig. 33,h

Fig. 18, h, i and j ... ... Fig. 33, i,j and k.

The above-mentioned figures and indeed most of the figures underline common factor for zone C in all tissues.

TABLE 18

Serum esterases of *Apodemus sylvaticus*. Tests with various substrates and inhibitors.

(Substrates: +++: strong ++: medium +: weak ± : trace)
(Inhibitors: +: no inhibition ±: partial inhibition -: inhibition)

SUBSTRATES					INHIBITORS			HEAT	
6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate	Zone	Fraction	neo-stig-mine	physo-stig-mine	D.F.P.	Incubated at 56°C 5 min.	10 min.
		++		1	±	±	-	+	+
+		++		2	±	±	-	-	-
+		++		2b	±	±	-	-	-
+	++	+++	A	3	+	+	±	-	-
?	++	+++		4	+	+	±	-	-
+	++	+++		5	+	+	±	-	-
+	++	+++		5b	+	+	±	-	-
+	++	+++		6	+	+	±	-	-
+	?	+		7	?	±	?	-	-
		+		8)	±	+	-	-	-
		++	B	9) X'	±	+	-	-	-
		++		10)	±	+	-	-	-
±	(±)	+		11	+	+	-	-	-
±	(±)	+		12	+	+	-	-	-
		+		X	+	+	-	-	-
		+		13	+	±	-	-	-
+	(±)	+		14	+	±	-	-	-
+	±	++		15	±	±	-	-	-
+	+	++		P_1	±	±	-	-	-
++	+	++		16	±	±	-	-	-
		+		17	+	±	-	-	-
±		+	C	18	+	-	-	-	-
		+		19			-	-	-
++	++	+++		20	±	±	±	±	±
		+		21					
		+		22					
		¥		23					

TABLE 19

Esterases of skeletal muscle homogenates of *Apodemus sylvaticus*. Tests with various substrates and inhibitors.

(Substrates, +++: strong ++: medium. +: weak. ±: Trace)

(Inhibitors +: no inhibition. ±: partial inhibition. −: inhibition)

SUBSTRATES			Zone	Fraction	INHIBITORS			HEAT	
6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate			neo-stigmine	physo-stigmine	D.F.P.	Incubated at 56°C 5 min	10 min
		±		1	+	+	−	+	+
		±		2	+	+	−	−	−
		+		3	+	+	−	−	−
		±	A	4	+	+	−	−	−
±	+	++		5	+	+	−	+	±
±	+	++		6	+	+	−	±	±
±	+	++		6b	+	+	−	±	±
±	+	++		7	+	+	−	±	±
		+		8	+	+	−	−	−
		+	B	12	±	±	−	−	−
		+		13	±	±	−	−	−
		±		15	+	+	−	±	−
		+		16	+	+	−	+	−
		+		17	+	+	−	+	±
	±	++		18	+	+	−	+	±
		+		19	+	+	−	+	±
	+	++	C	20	+	+	(±)	+	+
		+		21	+	+	−	±	±
		+		22	+	+	−		
	+	++		23	+	+	−		

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TABLE 19 (Contd.)

6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate	Zone	Fraction	neostigmine	physostigmine	D.F.P.	Incubated at 56°C 5 min	10 min
		+		24	+	+	-	no data	no data
		+		25	+	+	-		
	+	++		26	+	+	-		
		+		27	+	+	-		

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TABLE 20

Esterases of <u>cardiac muscle homogenates</u> of <u>*Apodemus sylvaticus*</u>. Tests with various substrates and inhibitors.

(Substrates +++: strong ++: medium +: weak ±: trace)
(Inhibitors +: no inhibition ± : partial inhibition −: inhibition)

SUBSTRATES					INHIBITORS			HEAT	
6-bromo-2-carbo-naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate	Zone	Fraction	neo-stig-mine	physo-stig-mine	D.F.P.	Incubated at 56°C 5 min	10 min
		+		1	+	+	−	+	+
		±		2	+	+	−	−	−
		+		3	+	+	−	−	−
		±		4	+	+	−	−	−
±	+	++	A	5	+	+	−	±	±
±	+	++		6	+	+	−	±	±
±	+	++		6b	+	+	−	±	±
±	+	++		7	+	+	−	±	±
		+		8	+	+	−	−	−
		+	B	12				−	−
		+		13				−	−
		±		15	+	+	−	+	−
		±		16	+	+	−	+	±
		±		17	+	+	−	+	±
	+	++		18	+	+	−	+	±
		+		19	+	+	−	+	±
±	+	+++	C	20	+	+	(±)	+	+
		+		21	+	+	−	+	±
		+		22	+	+	−		
	+	++		23	+	+	−	no data	no data
		+		24	+	+	−		
		+		25	+	+	−		
	+	+++		26	+	+	−		
		+		27	+	+	−		

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TABLE 21

Esterases of <u>liver homogenates</u> of *Apodemus sylvaticus*. Tests with various substrates and inhibitors.
(Substrates, +++: strong ++: medium +: weak ±: trace)
(Inhibitors, +: no inhibition ±: partial inhibition -: inhibition)

SUBSTRATES			Zone	Fraction	INHIBITORS			HEAT	
6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate			neo stig-mine	physo-stig-mine	D.F.P.	Incubated at 56°C 5 mins.	10 mins.
		+		1	±	±	-	±	-
		+		2	±	±	-	±	-
		±		3	±	±	-	-	-
		+		4	±	±	-	-	-
	+	+	A	5	+	+	-	-	-
	+	+		6	+	+	-	-	-
	+	+		7	+	+	-	-	-
±	+	++		8	+	+	-	±	±
?	+	++		8b				±	-
±	+	++		9	+	+	-	±	-
	±	±		10	+	+	-	-	-
	++	+++		11	+	+	-	-	-
	++	+++	B	12	+	+	-	-	-
	+++	+++		13	+	+	(±)	-	-
	+	+++		14	+	+	-	-	-
	+	+++		15	+	+	-	-	-
	++	++		16	+	+	-	-	-
	++	+		17	+	+	-	-	-
	±	±		18	+	+	-	-	-
	±	±	C	19	+	+	-	-	-
	±	±		20	+	+	-	-	-
	±	+		21	+	+	-	-	-

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TABLE 21 (Contd.)

6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate	Zone	Fraction	neo stig-mine	physo stig-mine	D.F.P.	Incubated at 56°C 5 mins	10 mins
	±	+		22	+	+	-	-	-
	+	+		23	+	+	-	-	-
	+	+	C	24	+	+	-	-	-
	++	+++		25	+	+	(±)	±	±
	±	+		26	+	+	-	-	-
	+	++		27	+	+	-	-	-
	+	+		28	+	+	-	-	-
	++	+++		29	+	+	(±)		
	±	+++		30	+	+	(±)		
	±	+		31	+	+	-		
	+	+		32	+	+	-		
	+	+		33	+	+	-		
	+	+		34	+	+	-		
	+	++		35	+	+	(±)	no data	no data
	±	+		36	+	+	-		
	±	±		37	+	+	-		

TABLE 22

Esterases of <u>kidney homogenates</u> of <u>Apodemus sylvaticus</u>. Tests with various substrates and inhibitors.
(Substrates: +++: Strong ++: medium +: weak ±: trace)
(Inhibitors: +: no inhibition ±: partial inhibition −: inhibition)

SUBSTRATES					INHIBITORS			HEAT	
6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate	Zone	Fraction	neo stigmine	physostigmine	D.F.P.	Incubated at 56°C 5 min	10 min
		±		1	+	+	−	+	+
		+		2	+	+	−	+	+
		±		3	+	+	−	−	−
		++		4	+	+	−	−	−
	+	++	A	5	+	+	−	+	+
	+	++		6	+	+	−	+	+
	+	++		7	+	+	−	−	−
±	+	++		8	+	+	−	±	±
?	+	++		8b	+	?	?	?	?
±	+	++		9	+	+	−	±	−
		±		10	+	+	−	−	−
		±		11	+	+	−	−	−
		±	B	12	+	+	−	−	−
	+	+		13	+	+	(±)	−	−
	±	±		14	+	+	−	−	−
	±	±		15	+	+	−	−	−
	±	+	C	16	+	+	−	±	±
	±	+		17	+	+	−	+	±
	±	+		18	+	+	−	+	±

TABLE 22 (Contd.)

6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate	Zone	Fraction	neo stigmine	physostigmine	D.F.P.	Incubated at 56°C 5 min	10 min
	±	+	C	19	+	+	-	+	±
	±	+		20	+	+	-	+	±
	±	+		21	+	+	-	+	±
	±	+		22	+	+	-	+	±
	±	+		23	+	+	-	+	±
	+	+		24	+	+	-	+	±
	++	++		25	+	+	(±)	+	±
	±	±		26	+	+	-		
	±	+		27	+	+	-		
	±	±		28	+	+	-		
	±	++		29	+	+	(±)		
	+	++		30	+	+	-		
		±		31	+	+	-		
		±		32	+	+	-		
		±		33	+	+	-		
		±		34	+	+	-	no data	no data
		+		35	+	+	-		
		±		36	+	+	-		
		±		37	+	+	-		

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TABLE 23

Esterases of brain homogenates of *Apodemus sylvaticus*. Tests with various substrates and inhibitors.

(Substrates. +++: strong. ++: medium +: weak ±: trace)
(Inhibitors. +: no inhibition ±: partial inhibition - inhibition)

SUBSTRATES			Zone	Fraction	INHIBITORS			HEAT	
6-bromo-2-carbo-naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate			neo-stig-mine	physo-stig-mine	D.F.P.	Incubated at 56°C 5 min.	10 min.
		+		1	+	+	-	+	+
		±		2	+	+	-	-	-
		±		3	+	+	-	-	-
		+		4	+	+	-	-	-
	±	+		5	+	+	-	±	±
	+	++	A	6	+	+	-	±	±
	±	++		6b	+	+	-	±	±
±	+	++		7	+	+	-	±	±
		±		8	+	+	-	-	-
		±	B	9	+	+	-	-	-
		++		10	+	+	-	-	-
	+	±		12	+	+	(±)	-	-
		+		18	+	+	-	-	-
	±	++	C	20	+	+	(±)	±	±

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TABLE 24

Esterases of testis homogenates of <u>Apodemus sylvaticus</u>. Tests with various substrates and inhibitors.
(Substrates +++: strong ++: medium +: weak ±: trace)
(Inhibitors +: no inhibition ±: partial inhibition -: inhibition)

SUBSTRATES					INHIBITORS			HEAT	
6-bromo-2-carbo naphthoxy choline iodide	naphthol -AS- acetate	1-naphthyl acetate	Zone	Fraction	neostigmine	physostigmine	D.F.P.	Incubated at 56°C 5 min	10 min
		+		1	+	+	-	+	+
		±		2	+	+	-	-	-
		+		3	+	+	-	-	-
		±		4	+	+	-	-	-
	±	++		5	+	+	-	+	±
	+	++	A	6	+	+	-	+	±
	+	++		6b	+	+	-	+	+
±	+	++		7	+	+	-	+	±
		+		8	+	+	-	-	-
	±	+	B	9	+	+	-	-	-
	±	+		10	+	+	-	-	-
	++	±		12	+	+	(±)	-	-
	±	+		18	+	+	-	±	-
	+	++		20	+	+	(±)	+	±
	±	+		22	+	+	-		
		+		24	+	+	-		
		+		26	+	+	-		
								no data	no data

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Table 25

A comparison of relative position and intensity of esterase fractions of various tissue homogenates from

<u>*Apodemus sylvaticus*</u>.

SERUM	R.B.C.	MUSCLE	HEART	BRAIN	TESTIS	LIVER	KIDNEY
						1+(+)	
1++		1++	1++	1+	1+	2+(+)	2+
		2+	2(+)	2(+)	2(+)	3+	3+
2++	1+(+)	3++	3++	3++	3++	4+(+)	4++
						5++	5++(+)
2b[1]+(+)		4+(+)	4++	4++	4+(+)	6++	6++(+)
						7++	7++(+)
3+++	2+	5+(+)	5++	5+	5+		
4++							
5+++	3+(+)	6+(+)	6++	6+(+)	6+(+)	8+(+)	8+(+)
5b+++		6b+(+)	6b++	6b+(+)	6b+(+)	8b+(+)	8b+(+)
6+++	4+	7+(+)	7++	7+(+)	7+(+)	9+(+)	9+(+)
7+++		8+	8+	8+	8+	10+	10+
				9+	9+		
8+				10++	10++	11++	11(+)
9++	5+(+)						
10++	6+(+)					12++(+)	12(+)

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Table 25 (contd.)

SERUM	R.B.C.	MUSCLE	HEART	BRAIN	TESTIS	LIVER	KIDNEY
11+							
12+	7+			12++	12++	13+++	13(+)
X+	8+(+)					14+++	14(+)
13+	9+					15+++	15(+)
14+						16++(+)	16+
						17+	17+
15++		12+(+)	12+(+)			18+	18+
		13+(+)	13+(+)			19+	19+
P1++		15+	15+			20+	20+
16++	10+	16+	16+			21+	21+
		17+	17+			22+	22+
17+		18++	18++(+)	18+(+)	18+(+)	23++	23+(+)
18+		19++	19++	19+(+)	19+(+)	24++	24+(+)
19+	11+	20+++	20+++	20++(+)	20+++	25+++	25++
						26+	26+
20+++		21+	21+	21		27++	27++
						28+	28+
21+		22++	22++		22++	29++(+)	29+(+)
						30++(+)	30+(+)

Table 25 (Contd.)

SERUM	R.B.C.	MUSCLE	HEART	BRAIN	TESTIS	LIVER	KIDNEY
22+		23++	23++			31+	31+
						32+	32+
23+		24++	24++		24++	33++	33+(+)
		25+	25+	No Data		34++	34+(+)
		26++(+)	26++(+)		26++	35++(+)	35+(+)
						36++	36(+)
		27+(+)	27+(+)			37+(+)	37(+)

3.5 BREEDING TESTS

Only three matings were successful. Two of these were carried out at the University of Lund, Lund, Sweden.

The first mating was between a feral male from Iceland and female from Sweden; the second mating was between male and female from Sweden that came from an inbred stock, these were sibs. The third mating was carried out in Belfast, but only serum was tested from this mating. From the fourth mating, only the offspring were obtainable for testing: No back crosses could be carried out.

The following mating numbers will be used in this Chapter:

Mating 1 : male (Iceland) X female (Sweden)

Mating 2 : male (Sweden) X female (Sweden)

Mating 3 : male (Ireland) X female (Ireland)

Mating 4 : male (Iceland) X female (Iceland)

(parents not typed)

a) Inheritance of protein fractions

(Figs. 5,8,14,15 and 68, Tables 26 and 27)

Albumins, post-albumins and transferrins (the usual type, fraction 16) were present in all parents and offsprings. The skeletal muscle protein fraction 19 was present and 18 absent in parents and offsprings. Table 26 gives segregation of red cell lysate proteins A and B. Table 27 gives segregation of C^1 protein of skeletal muscle homogenates. The fainter protein fractions would not be investigated in the present work. The ceruloplasmins were monomorphic.

b) Inheritance of esterase fractions

(Figs. 62,63,64,65,66 and 67, Tables 28,29,30,31,32,33,34 and 35)
The results are tabulated in most cases and the nomenclature used is that recommended/

recommended by IUPAC-IUB Commission on Biochemical Nomenclature (1971). The fractions have been divided into eight groups (Es-1 -Es-8) based on specificity tests. The intensity of fractions was estimated visually from series of photographs taken at different intervals as staining proceeded and with different photographic exposures. Fractions were scored for intensity as strong, medium or absent where applicable. The postulated mode of inheritance is presented in the following Tables and Figures:

Table 28; Fig. 62:

Serum esterase fraction 1 (Es-1)

Table 29; Fig. 62:

Serum esterase fraction 2 (Es-2)

Table 30; Fig. 62:

Serum esterase fraction 3 (Es-3)

Table 31; Figs. 9 and 62:

Serum esterase fractions 5 and 6 (Es-4)

Table 32; Figs. 12 and 62:

Serum esterase zone X^1 (Es-6). It is worth noting here that the proposed allele for absence (the silent allele $Es\text{-}6^a$) seems dominant.

Table 33; Fig. 66:

Liver esterase zone B (Es-7). This is a good example of quantitative biochemical genetics, where the heterozygote is intermediate as regards activity compared to the parents. In mating number 2 (parents from Sweden) some variations occur in fraction 15 of this zone. Two out of 14 offsprings lacked this/

this fraction, but both parents possessed it. It is not clear whether this is genetical (and then both parents must be heterozygous for fraction 15) or physiological. Both individuals lacking fraction 15 were females.

Tables 34 and 35; Figs. 63,64,65,66 and 67:

Esterase zone C (Es-8). This zone is the varied one in appearance but is present in all tissue homogenates (see Chapter 3.4). Each tissue has its characteristic expression regarding number of fractions, but the migration rate of the zone as a whole is comparable for all tissues tested for any one animal (see previous section). It is however helpful to give some account of each tissue separately:

a) Skeletal muscle esterases of zone C are demonstrated in Figs. 63 and 65, at pH 7.6 (Tris-citrate) and pH 8.6 (Tris-citrate) respectively Both electrophoretic systems show that the offspring have a hybrid pattern as compared to the parents. The multiple fractions of the C_I - C_{II} zone at pH 8.6. should be noticed, although it is difficult to show them up on a photograph.

b) Cardiac muscle esterases of zone C are demonstrated in Figs. 63,II; 64,I and 64,II at pH 7.6 (Tris-citrate), pH 8.6 (Tris-citrate borate) and pH 8.6 (Tris-citrate) respectively. At pH 7.6 the fraction 20' of the mother is not produced in the offspring (Fig. 63,II) as is the case for skeletal muscle so that the hybrid offsprings have their father's type. Similarly in Tris-citrate borate at pH 8.6 the offsprings show the father's type, but in Tris-citrate pH 8.6 (Fig. 64,II) the offsprings have multiple fractions in zone C, whereas the father/

father has three fused fractions here.

The relative position of serum fraction 20 should be noted.

c) Liver esterases of zone C can be seen in Fig. 66. The father (c) and the mother (d) have multiple fractions, arranged mainly in two clusters in each. The "clusters" (see dots on photograph) or "main fractions" in the father and mother do not overlap; These are designated C_{II} and C_{I} respectively. The offsprings did all show a multiple fraction in this zone, covering the area of both C_{I} and C_{II} i.e. a hybrid zone is formed. A detailed analysis of zone C in the liver esterase zymogram can only be made by watching the stain reaction, as bands fuse readily.

d) Kidney esterases of zone C are demonstrated in Fig. 67,I. The system is the same as in Fig. 66. The argument for the liver esterases applies for the kidney esterases.

e) Brain esterases of zone C can be observed in Fig. 67,II. The system is Tris-citrate pH 7.6, the same as in Fig. 63. The same phenomenon is seen here as in other tissues i.e. the main fraction of the mother's homogenate is faster than that of the father - C_{I} and C_{II}. The offsprings have fused intermediate zone. It is pointed out here again that the brain homogenates have fewer fractions than any other tissue tested.

Table 26

Segregation of protein fractions A and B of red cell lysates of <u>Apodemus sylvaticus</u>.

Mating number	Phenotype of parents				Phemetype of offspring					
					A			B		
	m		f		m	f	m+f	m	f	m+f
1	A	X	A	Obs.	8	7	15			
				Exp.			15			
2	B	X	B	Obs.				8	6	14
				Exp.						14
4				Obs.	2	3	5	2	3	5
				Exp.			5			5

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Table 27

Postulated mode of inheritance of C[1] protein fraction of skeletal muscle homogenates in

<u>Apodemus sylvaticus</u>.

Mating number	Parents: Phenotype	Parents: Proposed genotype	Offspring: Phenotype	C' present C' +			C' absent C' -			Offspring: Proposed genotype	
	m x f	m x f		m	f	m+f	m	f	m+f	C/c	c/c
1	c'- x c'+	c/c x C/c	Obs.	4	2	6	4	5	9	6	9
			Exp.			7.5			7.5	7.5	7.5
										X^2 = 0.6000	
2	c'- x c'-	c/c x c/c	Obs.				8	6	14		14
			Exp.						14		14

C allele for presence of fraction c'

c allele for absende (recessive) of fraction c'

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Table 28.

Postulated mode of inheritance of serum esterase fraction 1 (Es - 1) in *Apodemus sylvaticus*.

Mating number	Parents: Phenotype m	f	Parents: Proposed genotype m	f	Number of offspring	Proposed genotype Es-1^{b}/Es-1^{b} m	f	m+f	Es-1^{a}/Es-1^{b} m	f	m+f	Es-1^{a}/Es-1^{a} m	f	m+f	X^2
1	strong X	absent	Es-1^{b}/Es-1^{b} X	Es-1^{a}/Es-1^{a}	Obs.				8	7	15				
					Exp.						15				-
2	strong X	medium	Es-1b/Es-1^{b} X	Es-1^{a}/Es-1^{b}	Obs.	4	4	8	4	2	6				
					Exp.			7			7				0.2858
3	strong x	medium	Es-1^{b}/Es-1^{b} X	Es-1^{a}/Es-1^{b}	Obs.	2	1	3	1	1	2				
					Exp.			2.5			2.5				0.2000
4						1	1	2	1	2	3				

Es -1^{a} codominant allele for absence of fraction 1

Es -1^{b} codominant allele for presence of fraction 1

Table 29.

Postulated mode of inheritance of serum esterase fraction 2 (Es-2) in *Apodemus sylvaticus*.

Mating number	Phenotype (m × f)	Proposed genotype (m × f)		$Es\text{-}2^b/Es\text{-}2^b$ m	f	m+f	$Es\text{-}2^a/Es\text{-}2^b$ m	f	m+f	$Es\text{-}2^a/Es\text{-}2^a$ m	f	m+f	X^2
1	medium x strong	$Es\text{-}2^a/Es\text{-}2^b$ x $Es\text{-}2^b/Es\text{-}2^b$	Obs.	2	3	5	6	4	10				1.666
			Exp.			7.5			7.5				
2	strong x strong	$Es\text{-}2^b/Es\text{-}2^b$ x $Es\text{-}2^b/Es\text{-}2^b$	Obs.	8	6	14							-
			Exp.			14							
3	strong x strong	$Es\text{-}2^b/Es\text{-}2^b$ X $Es\text{-}2^b/Es\text{-}2^b$	Obs.	3	2	5							-
			Exp.			5							
4							2	3	5				

$Es\text{-}2^a$ codominant allele for absence of fraction 2

$Es\text{-}2^b$ codominant allele for presence of fraction 2

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Table 30.

Postulated mode of inheritance of serum esterase fraction 3 (Es-3) in *Apodemus sylvaticus*.

Parents			Number of offspring										
Mating number	Phenotype	Proposed genotype		Proposed genotype									X^2
				$Es\text{-}3^b/Es\text{-}3^b$			$Es\text{-}3^a/Es\text{-}3^b$			$Es\text{-}3^a/Es\text{-}3^a$			
	m f	m f		m	f	m+f	m	f	m+f	m	f	m+f	
1	absent X strong	$Es\text{-}3^a/Es\text{-}3^a$ X $Es\text{-}3^b/Es\text{-}3^b$	Obs.				8	7	15				
			Exp.						15				-
2	strong X strong	$Es\text{-}3^b/Es\text{-}3^b$ X $Es\text{-}3^b/Es\text{-}3^b$	Obs.	8	6	14							
			Exp.			14							-
3	strong X strong	$Es\text{-}3^b/Es\text{-}3^b$ X $Es\text{-}3^b/Es\text{-}3^b$	Obs.	2	3	5							
			Exp.			5							-
4							1	1	2	1	2	3	

$Es\text{-}3^a$ codominant allele for absence of fraction 3

$Es\text{-}3^b$ codominant allele for presence of fraction 3.

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Table 31.

Postulated mode of inheritance of serum esterase fractions 5 and 6 (Es-4) in <u>Apodemus sylvaticus</u>.

Parents					Number of offspring										
Mating number	Phenotype		Proposed genotype			Proposed genotype									X^2
						$Es\text{-}4^b/Es\text{-}4^b$			$Es\text{-}4^b/Es\text{-}4^d$			$Es\text{-}4^d/Es\text{-}4^d$			
	m	f	m	f		m	f	m+f	m	f	m+f	m	f	m+f	
1	5-6 x	6	$Es\text{-}4^b/Es\text{-}4^d$ X $Es\text{-}4^d/Es\text{-}4^d$		Obs.				7	1	8	1	6	7	
					Exp.						7.5			7.5	0.0666
2	6 X	6	$Es\text{-}4^d/Es\text{-}4^d$ X $Es\text{-}4^d/Es\text{-}4^d$		Obs.							8	6	14	
					Exp.									14	-
3	5 X	5-6	$Es\text{-}4^b/Es\text{-}4^b$ X $Es\text{-}4^b/Es\text{-}4^d$		Obs.		1	1	3	1	4				
					Exp.			2.5			2.5				1.8000
4						1	1	2	1	2	3				

$Es\text{-}4^b$ codominant allele for presence of fraction 5

$Es\text{-}4^d$ codominant allele for presence of fraction 6.

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Table 32.

Postulated mode of inheritance for serum esterase zone X^1 (Es-6) in <u>Apodemus sylvaticus</u>.

Parents			Number of offspring										
Mating number	Phenotype	Proposed genotype		Proposed genotype									X^2
				$Es\text{-}6^b/Es\text{-}6^b$			$Es\text{-}6^a/Es\text{-}6^b$			$Es\text{-}6^a/Es\text{-}6^a$			
	m f	m f		m	f	m+f	m	f	m+f	m	f	m+f	
1	absentXpresent	$Es\text{-}6^a/Es\text{-}6^a X Es\text{-}6^b/Es\text{-}6^b$	Obs.				8	7	15				
			Exp.						15				-
2	presentXpresent	$Es\text{-}6^b/Es\text{-}6^b X Es\text{-}6^b/Es\text{-}6^b$	Obs.	8	6	14							
			Exp.			14							-
3	presentXpresent	$Es\text{-}6^b/Es\text{-}6^b X Es\text{-}6^b/Es\text{-}6^b$	Obs.	3	2	5							
			Exp.			5							
4										2	3	5	

$Es\text{-}6^a$ dominant? allele for absence of zone X^1

$Es\text{-}6^b$ recessive? allele for presence of zone X^1

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Table 33.

Postulated mode of inheritance of esterase zone B (Es-7) of liver homogenates in _Apodemus sylvaticus_.

Mating number	Phenotype m × f	Proposed genotype m × f		Es-7^b/Es-7^b m	f	m+f	Es-7^a/Es-7^b m	f	m+f	Es-7^a/Es-7^a m	f	m+f	X^2
	Parents		**Number of offspring (Proposed genotype)**										
1	"near silent"Xstrong	$Es\text{-}7^a/Es\text{-}7^a$ X $Es\text{-}7^b$X$Es\text{-}7^b$	Obs.				8	7	15				
			Exp.						15				-
2	strongXstrong	$Es\text{-}7^b/Es\text{-}7^b$ X $Es\text{-}7^b/Es\text{-}7^b$	Obs.	8	6	14							
			Exp.			14							-
4										2	3	5	

$Es\text{-}7^a$ codominant allele for "near silence" of zone B

$Es\text{-}7^b$ codominant allele for presence of zone B

Table 34

Inheritance of esterase zone C (Es-8) in *Apodemus sylvaticus*.

Mating number	Parents: Phenotype			Number of offspring: Phenotype C_I			C_{II}			$C_I - C_{II}$			C_{IV}		
	m		f	m	f	m+f	m	f	m+f	m	f	m+f	m	f	m+f
1	C_{II}	X	C_I							8	7	15			
2	C_I	X	C_I	8	6	14									
4				1	1	2							1	2	3

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Table 35

Postulated mode of inheritance of esterase zones C_I (Es-8a) and C_{II} (Es-8b) of *Apodemus sylvaticus*.

Mating number	Phenotype m	Phenotype f	Proposed genotype m × f		$Es\text{-}8^a/Es\text{-}8^a$ m	$Es\text{-}8^a/Es\text{-}8^a$ f	$Es\text{-}8^a/Es\text{-}8^a$ m+f	$Es\text{-}8^a/Es\text{-}8^b$ m	$Es\text{-}8^a/Es\text{-}8^b$ f	$Es\text{-}8^a/Es\text{-}8^b$ m+f	$Es\text{-}8^b/Es\text{-}8^b$ m	$Es\text{-}8^b/Es\text{-}8^b$ f	$Es\text{-}8^b/Es\text{-}8^b$ m+f
1	C_{II} X	C_I	$Es\text{-}8^b/Es\text{-}8^b$ X $Es\text{-}8^a/Es\text{-}8^a$	Obs.				8	7	15			
				Exp.						15			
2	C_I X	C_I	$Es\text{-}8^a/Es\text{-}8^a$ X $Es\text{-}8^a/Es\text{-}8^a$	Obs.	8	6	14						
				Exp.			14						

Parents: Mating number, Phenotype, Proposed genotype. Number of offspring: Proposed genotype.

$Es\text{-}8^a$ codominant allele for presence of C_I

$Es\text{-}8^b$ codominant allele for presence of C_{II}

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3.6 COMPARISON OF VARIOUS PROTEIN SYSTEMS OF NORTHERN POPULATIONS OF APODEMUS SYLVATICUS.

A. The environment of the mice.

The field mice used in this study came from the following localities (Maps 1 and 2):

Iceland:

1. Laugarvatn area:

These mice came from farmhouses near Laugarvatn, mainly barns and sheep-sheds, as the mice were mainly caught during the wintertime and spring. Only four mice were caught outside in a field, just outside a barn.

2. Stora-Mörk area:

This farm is about 100 km from Laugarvatn, from which it is separated by many big glacial rivers. Bridges were not built over all of them until 1930, but the first bridge was built before 1900.
Again, all the mice were caught in barns and sheds. About half of the total number of the Stora-Mörk mice came from one sheep-shed. They were not found to differ from the rest statistically and were therefore pooled along with the others.

2b. Skalakot area.

Only nine mice were caught at Skalakot, a farm about 25 km east of Stora Mörk in a sheep shed.
The rest of the localities in Iceland are far apart and separated by rivers and glaciers. As mentioned before the longtailed fieldmouse (*Apodemus sylvaticus*) in Iceland lives in the habitats which are elsewhere occupied by *Mus musculus*.

Ireland:

6. Belfast area:

The mice in this study were caught in the Saintfield Estate, just outside Belfast./

Belfast. Usually traps were placed at edges of woods, 10 paces apart. No animal was caught inside a house.

Scotland:

7. Glasgow area:

This area is a woodland estate - called Garscube Estate, very similar to Saintfield and the traps were again placed at the edge of the wood.

7b. Cumbernauld area:

The same type of environment as the previous two places, about 20-30 km from Garscube.

Norway.

8. Bergen area:

Similar type of environment as in Scotland and Ireland and the traps were laid in similar way, except in this instance "backbrakers" were used instead of "live" traps. The nights of capture were all frosty nights ce. 5-8°C frost. Of course blood could not be collected from these animals, so that the "fast" esterase zone was typed from heart and skeletal muscle samples.

9. Jaeren:

In this area the "live" traps were placed as before at the edge of woodland near "Revtangen" Bird Observatory.

10. Oslo area:

Same trapping method as before in a bushy woodland near Hvalstad, just outside Oslo.

Sweden:

11. Lund.

These were an inbred stock of Apodemus sylvaticus originally caught in woods near Lund in South-Sweden. The Lund mice were only used in breeding tests as they were inbred (see 3.5).

B. The distribution of protein markers.

Tables 1-17 give the incidence of certain enzymes and other proteins in the above mentioned populations. Sections 3.4 and 3.5 of Chapter 3 subdivide the proteins population markers. It is however clear that more knowledge of the genetics of these proteins is necessary for the full use for this purpose.

The results of the comparison of protein marker frequencies are given in Tables 36-44.

a) Nigrosine-amidoblack stained protein markers.

Table 36 gives the percentage frequencies of various polymorphic protein fractions in the populations under study. Only the main differences will be underlined in this section and discussed in Chapter 4.

1) Red cell proteins A and B.

These proteins, as will be argued later, are most likely controlled by two different loci. Fraction A seems to be fixed in all Icelandic populations as fraction B seems to be fixed in the Norwegian populations. The incidence of A in the Oslo area is as low as 4%.

2) Serum proteins

Slow post albumin fractions were only found amongst mice from Scotland (7) and Norway (10) in about the same frequency, but was absent from other localities.

Fast post albumin was present in all individuals tested from areas 1, 7b and 9, and present in 91% - 96% of individuals tested from localities 2,6,7 and 10. This means there is no significant difference between countries.

Slow transferrin (serum fraction 17)

was only found in Iceland (2) and Ireland (6) at a frequency of 1-2%

3)/

3) Skeletal muscle proteins.

Fraction 18 was found in Iceland (1,2) and Norway (9,10) in the frequency of about 20-25%.

Fraction C' only occurred in Iceland (1,2) in about half of the samples, but in no other country could it be observed.

b) Markers with esterase activity

Tables 37-40 compare the observed phenotypes of four proposed loci (Es-1 - Es-4) with those expected as a result of Hardy - Weinberg equilibrium. The implication of the result will be discussed in Chapter 4.

Serum esterase fraction 7 was found in Iceland (1,2), Ireland (6) and Scotland (7), but in a low frequency (4- 10%). It was not found in Norway.

Table 42 states frequencies of fractions in zone A and B of liver-zymograms.

Zone A.

Fraction 5 is absent from the Icelandic populations, but present in all others, the frequency ranging from 35% in Ireland (6), and 54% in Oslow (10) to 80% - 84% in Scotland (7,7b) and Jaeren (9) in Norway.

Zone B. Es-7.

In addition to "near-silence" in all Icelandic populations ($Es\text{-}7^a/Es\text{-}7^a$) and "strong" in other populations ($Es\text{-}7^b/Es\text{-}7^b$) there is variation in the pattern where fractions may be missing. The most outstanding differences are in the frequencies of fractions 12 and 15. Fraction 12 is least frequent in Scotland (7, 7b) and occurs in about 36% of the mice from Oslo (10) and 80% in Ireland (6) and Jaeren (9).

Fraction 15 is absent in Jaeren (9) and low in Scotland (7,7b). Just over half/

half the Oslo (10) specimens have it and two thirds of the Irish (6).
Table 43 gives frequencies of various types of zone C. It is here that the most outstanding differences between countries occur.

C_I was found in all animals from Sweden and probably in one animal from Ireland (Fig. 37,H,b) in a hybrid form.

C_{II} is observed in all countries and is the only type found in Scotland.

C_{III} occurred only in Norwegian populations, the frequencies ranging from 23% - 88%

C_{IV} was found in Iceland and Ireland.

Table 44 summarises allele frequencies at six proposed esterase loci. Some of the populations seem to have one allele fixed e.g. at loci Es-1, Es-2, Es-3, Es-6 and Es-7. For discussion see Chapter 4.

Table 36.

Comparison of frequencies of various protein fractions from different tissues in northern populations of *Apodemus sylvaticus*.

Country	Locality	Red Cells		Serum			Skeletal muscle	
		Fraction A	Fraction B	Slow Post-alb	Fast Post-alb.	Slow Tf (17)	Fraction 18	Fraction C1
ICELAND	1	1.00	0.58	0.00	1.00	0.00	0.21	0.50
	2	1.00	0.91	0.00	0.93	0.01	0.22	0.54
	4	1.00	0.67	0.00	0.83	0.00	0.00	0.00
IRELAND	6	1.00	1.00	0.00	0.91	0.02	0.00	0.00
SCOTLAND	7	0.54	1.00	0.13	0.96	0.00	0.00	0.00
	7b	1.00	0.63	0.00	1.00	0.00	0.00	0.00
NORWAY	8						0.00	0.00
	9	0.80	1.00	0.00	1.00	0.00	0.20	0.00
	10.	0.04	1.00	0.14	0.91	0.00	0.25	0.00

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Table 37

A comparison of the numbers of observed phenotypes of Es-1 (fraction 1) with those expected as a result of a Hardy-Weinberg equilibrium.

			Proposed genotype							
		Total	$Es\text{-}1^b/Es\text{-}1^b$		$Es\text{-}1^a/Es\text{-}1^b$		$Es\text{-}1^a/Es\text{-}1^a$		X^2	P
Country	Locality	no.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.		
ICELAND	1	50	13	14.54	27	24.91	10	11.05	0.4379	> .50
	2	71	23	22.27	34	34.99	14	13.76	0.0561	> .90
	2b	9	2	3.35	7	4.28	0	1.37	2.9621	> .05
	4	6	0	0.65	4	2.61	2	2.61	2.1252	> .10
IRELAND	6	116	22	25.62	65	57.80	29	32.58	1.8017	> .10
SCOTLAND	7	28	6	5.92	14	13.91	8	8.16	0.0047	> .90
	7b	10					10			
NORWAY	8	42	7	6.39	19	19.98	16	15.63	0.1433	> .70
	9	20	2	2.45	10	9.10	8	8.45	0.1957	> .60
	10	24					24			

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Table 38.

Observed phenotypes of Es-2 (fraction 2)

Country	Locality	Total no.	Present: $Es\text{-}2^{b}/Es\text{-}2^{b}$ or $Es\text{-}2^{a}/Es\text{-}2^{b}$ Obs.	Absent: $Es\text{-}2^{a}/Es\text{-}2^{a}$ Obs.
Iceland	1	50	50	
	2	71	71	
	2b	9	9	
	4	6	6	
Ireland	6	115	115	
Scotland	7	25	22	3
	7b	10	5	5
Norway	8	44	41	3
	9	20	17	3
	10	24	22	2

Table 39

A comparison of the numbers of observed phenotypes of Es-3 (fraction 3) with those expected as a result of Hardy-Weinberg equilibrium

Country	Locality	Total No.	Proposed genotype: Es-3b/Es-3b Obs.	Es-3b/Es-3b Exp.	Es-3a/Es-3b Obs.	Es-3a/Es-3b Exp.	Es-3a/Es-3a Obs.	Es-3a/Es-3a Exp.	x^2	P
ICELAND	1	49	1	0.71	11	11.08	37	37.08	0.1192	> .70
	2	71					71		-	
	2b	9	1	0.44	2	3.09	6	5.48	1.1679	> .25
	4	6					6		-	
IRELAND	6	127	100	96.13	20	28.73	7	2.15	7.3207	< .005
SCOTLAND	7	24	7	7.50	13	11.80	4	4.70	0.2596	> .60
	7b	10	5	4.90	4	4.20	1	0.90	0.0226	> .95
NORWAY	8	47	10	7.50	18	22.7	19	16.90	2.0673	> .10
	9	20	4	7.2	16	9.6	0	3.2	8.8888	< .001
	10	24	8	8.60	13	11.50	3	3.80	0.4060	> .50

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Table 40

A comparison of the numbers of observed phenotypes of Es-4 (fractions 5 and 6) with those expected as a result of a Hardy-Weinberg equilibrium.

Country	Locality	Total No.	Proposed genotype: $Es\text{-}4^b/Es\text{-}4^b$ Obs.	Exp.	$Es\text{-}4^b/Es\text{-}4^d$ Obs.	Exp.	$Es\text{-}4^d/Es\text{-}4^d$ Obs.	Exp.	x^2	P
ICELAND	1	52	22	20.6	22	24.2	8	7.2	0.4649	> .40
	2	70	11	20.4	54	34.8	5	14.8	21.4135	< .001
	2b	9	3	3.20	5	4.30	1	1.5	0.2932	> .60
	4	6	1	0.65	2	2.65	3	2.69	0.3803	> .05
IRELAND	6	145	70	61.26	49	65.97	26	17.76	9.4356	< .001
SCOTLAND	7	25	13	11.56	8	10.98	4	2.56	1.7495	> .10
	7b	9	7	6.65	2	2.17	0	0.18	0.0489	> .90
NORWAY	8	39	23	20.78	11	15.37	5	2.84	3.7532	> .05
	9	20	16	16.20	4	3.60	0	0.20	0.2469	> .50
	10	20	9	7.20	6	9.60	5	3.20	2.8125	> .05

Table 41

Frequencies of serum esterase fraction 7 in field mice (Apodemus sylvaticus) from Iceland, Ireland, Scotland and Norway

Country	Locality	Total no.	Frequency of fraction 7
IRELAND	1	52	0.10
	2	70	0.03
	2b	9	0.00
	4	6	0.00
IRELAND	6	148	0.08
SCOTLAND	7	25	0.04
	7b	10	0.00
NORWAY	8	45	0.00
	9	20	0.00
	10	22	0.00

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Table 42

Frequencies of various esterase fractions in liver esterase zones A and B in field mice (<u>Apodemus sylvaticus</u>) from Iceland, Ireland, Scotland and Norway.

Country	Locality	Total No.	Zone A		Zone B*				
			5	7	11	12	13	14	15
ICELAND	1	44	0.00	1.00					
	2	23	0.00	1.00					
	4	6	0.00	1.00					
IRELAND	6	20	0.35	0.65	0.90	0.80	1.00	0.90	0.65
SCOTLAND	7	25	0.84	0.16	1.00	0.12	0.96	0.84	0.32
	7b	9	0.80	0.20	0.89	0.00	1.00	0.89	0.11
NORWAY	9	20	0.80	0.20	1.00	0.80	1.00	1.00	0.00
	10	24	0.54	0.46	0.88	0.36	1.00	0.96	0.52

*Fractions in zone B in Icelandic mice were too faint to be read with certainty.

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Table 43

Frequencies of various types of esterase zone C (Es-8) in field mice (<u>Apodemus sylvaticus</u>) from Iceland, Ireland, Scotland and Norway.

Country	Locality	Total No.	Zone C		
			CII	CIII	CIV
ICELAND	1	42	0.40		0.60
	2	58	0.70		0.30
	4	6	0.33		0.67
IRELAND	6	20	0.90		0.10
SCOTLAND	7	24	1.00		
	7b	10	1.00		
NORWAY	8	48	0.77	0.23	
	9	20	0.40	0.60	
	10	25	0.12	0.88	

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Table 44

Allele frequencies at five proposed esterase loci in field mice (Apodemus sylvaticus) from Iceland, Ireland, Scotland and Norway.

		Proposed protein locus									
		Es-1		Es-3		Es-4		Es-6		Es-7	
Country	Locality	$Es\text{-}1^a$	$Es\text{-}1^b$	$Es\text{-}3^a$	$Es\text{-}3^b$	$Es\text{-}4^b$	$Es\text{-}4^d$	$Es\text{-}6^a$	$Es\text{-}6^b$	$Es\text{-}7^a$	$Es\text{-}7^b$
ICELAND	1	0.47	0.53	0.87	0.13	0.63	0.37	1.00	0.00	1.00	0.00
	2	0.44	0.56	1.00	0.00	0.54	0.46	1.00	0.00	1.00	0.00
	2b	0.39	0.61	0.78	0.22	0.60	0.40	1.00	0.00	1.00	0.00
	4	0.66	0.33	1.00	0.00	0.33	0.67	1.00	0.00	1.00	0.00
IRELAND	6	0.53	0.47	0.13	0.87	0.65	0.35	0.00	1.00	0.10	0.90
SCOTLAND	7	0.54	0.46	0.44	0.56	0.68	0.32	0.00	1.00	0.00	1.00
	7b	1.00	0.00	0.30	0.70	0.86	0.14	0.00	1.00	0.00	1.00
NORWAY	8	0.61	0.39	0.60	0.40	0.73	0.27			0.00	1.00
	9	0.65	0.35	0.60	0.40	0.90	0.10	0.00	1.00	0.00	1.00
	10	1.00	0.00	0.40	0.60	0.60	0.40	0.00	1.00	0.00	1.00

3.7 AN ELECTROPHORETIC COMPARISON OF APODEMUS SYLVATICUS AND MUS MUSCULUS.

In my earlier work (Arnason, 1966) I have made an extensive comparison of serum proteins and esterases of these two species. The results will not be dealt with here in any detail, but will be mentioned where relevant. The purpose of the present comparison is to review data published subsequently on *Mus musculus*, as only very limited study has been made of *Apodemus sylvaticus*.

A. Nigrosine - amidoblack stained proteins of various tissues. (Figs. 6,8,11,35,49,50,68 and 73).

a) Serum.

The albumin of *Apodemus sylvaticus* has a little slower mobility than the albumin of *Mus musculus*. The latter species has no fraction corresponding to the post albumin of the former (Fig. 8). The "normal" or faster transferrin variant of *Apodemus sylvaticus* (fraction 16) comes in between the main fractions of Trf-ab (Cohen, 1960; Shreffler, 1960, Cohen and Shreffler, 1961).

b) Red cell lysates.

Haemoglobins of *Apodemus sylvaticus* (Hb 1 and Hb 2, Figs. 7 and 11) have slower migration rate than those of the house mouse (Gluecksohn - Waelsch *et al*, 1957; Russell and Gerald, 1958; Popp and St. Amand, 1960; Petras, 1967, Petras and Martin, 1969)

Non-haemoglobin proteins of *Apodemus sylvaticus* A, B and C have no over-lapping protein fractions in haemolysates of *Mus musculus* (Fig. 6,I). The *Mus musculus* has strong protein fraction, just in front of the Hb (in pH 7.6). This is most likely the non-haemoglobin protein described by Riddle and Petras (1967).

c) Other tissue homogenates.

1) Skeletal muscle proteins.

The strongest protein fractions of the skeletal muscle in Apodemus sylvaticus, fractions 6 and 13 have overlapping strong fractions in Mus musculus (Figs. 50, 68 and 73). The same applies to fraction C' in Apodemus sylvaticus which has a corresponding fraction in Mus musculus. (Fig. 68). On the whole the patterns of both species are very similar.

2) Cardiac muscle proteins.

The patterns of both species are again very similar, except for pre-albumin fractions differ in mobility, Mus musculus lacks the post-albumin of Apodemus sylvaticus and Apodemus sylvaticus lacks a fraction immediately in front of its fraction 8 (Fig. 50).

3) Liver proteins

These are faint in both species and can only be demonstrated in fresh material (Fig. 35). The two species differ markedly in the faint fractions. The strongest fraction (albumin) of Mus musculus is a little faster than that of Apodemus sylvaticus. No differences in migration rate of liver-albumin was found in this study (Wilcox, 1972, 1973) nor indeed of the serum albumin (Petras, 1972).

B. Esterase patterns of various tissues.
(Figs. 9A, 10,11,12,18,32,33,34,56,69,70,71,72 and 74).

The esterases of *Mus musculus* have been thoroughly investigated in contrast with *Apodemus sylvaticus*. (See Chapter 1 and 4).
In the following pages I shall try to compare the electrophoretic position of the various esterases of the two species.

Serum.

The Es-1 (IV) of *Mus musculus* the "albumin esterase" (Popp and Popp, 1962; Pelzer, 1965; Ruddle and Roderick, 1965) has a position similar to 2b of *Apodemus sylvaticus*, the Es-1b overlaps 2b' of the serum or 4 of heart in *Apodemus sylvaticus*.

Es-2 (V) of *Mus musculus* is faster than Es-1 (Petras, 1963, Popp, 1967) and is situated just behind serum esterase fraction 1 (Es-1) of *Apodemus sylvaticus* (Fig. 11, e and 33, e)

Es-5 of *Mus musculus* (Petras and Biddle, 1967) overlaps serum esterase fraction 16 of *Apodemus sylvaticus*, but no genetical variation could be found in the latter. Zone X' has no corresponding fractions in *Mus musculus* (Arnason and Pantelouris, 1966).

b) Red cell lysates

Only limited study into erythrocytic esterases of *Apodemus sylvaticus* could be carried out in present work as explained earlier.

Es-3 (Pelzer, 1965; Popp, 1966; Martin and Petras, 1971) of *Mus musculus* probably correspond to fractions 7, 8 and 9 of *Apodemus sylvaticus* (Fig. 2).

c) Skeletal and cardiac muscle homogenates.

In comparing tissue esterases of the two species I shall use the nomen - clature of zones used by Ruddle and Harrington (1967), who divided the mouse esterase zymogram into five zones I (the slowest) - V (the fastest). Zone IV of the house mouse overlaps zone A in the field mouse and zone II of/

of the former overlaps zone C in the latter, more exactly type C_{III} (Figs. 18, 70 and 72). Note the improved separation of fractions in Zone II at pH 7.6.

d) Liver and kidney homogenates.

Zone V (Es-2) overlap or is just behind fraction 1 (Es-1) of *Apodemus sylvaticus* and zone IV (which includes Es-1) corresponds to the slower fractions of zone A in *Apodemus sylvaticus* (Es-2, Es-3 and Es-4). The B zone has no overlapping zone in *Mus musculus* no matter which electrophoretic system was used (Figs. 33, 69 and 74). Zone III of *Mus musculus* overlaps the faster fractions of zone C in *Apoddmus sylvaticus* (Figs. 32,33,69,71 72 and 74). When tris-citrate (no borate) pH 8.6 was used a further separation of the C -zone is accomplished thus giving better comparison (Fig. 74).

Zone II overlaps the slower fractions of zone C. The Es-6 variants described by Petras and Sinclair (1969) are probably shown in Fig. 33, d,e. This type of phenomenon also occurs in *Apodemus sylvaticus* (see Chapter 4).

e) Brain and testis homogenates

As in *Apodemus sylvaticus* the brain zymogram is faint in *Mus musculus*. Zone IV is similar in both tissues and so is zone A. Zone II of the testis is comparable with zone II of skeletal or heart muscle as is the case with zone C of *Apodemus sylvaticus* for the same tissues.

Zone II of the brain in *Mus musculus* has fewer bands than the testis (Fig. 72) and so does zone C in *Apodemus sylvaticus* for the same tissues.

CHAPTER 4

DISCUSSION

4.1. GROUPING OF BIOCHEMICAL MARKERS.

A. Interpretation of electropherograms

An electropherogram with all its fractions or bands is comparable to a large number of letters that can be arranged into sensible words and sentences if the know-how is there. To start with all the letters look alike, but by applying various physical and chemical methods their true identity may be revealed. The analogy is particularly applicable to the present work on *Apodemus sylvaticus*. Apart from my earlier work (Arnason, 1966; Pantelouris and Arnason, 1966; Arnason and Pantelouris, 1966; Pantelouris and Arnason, 1967 a, b) I have only been able to find one publication dealing with biochemical polymorphism in this species. This report by Engel *et al.* (1972) deals with polymorphism of LDH B^+ and LDH B^- in erythrocytes of *Apodemus sylvaticus*. The polymorphism and mode of inheritance was found to be similar to that established in the case of *Mus musculus*.

Having no comparable work on *Apodemus* to refer to or consult, makes the task more difficult, particularly concerning classification or identification of the fractions.

B. Classification of general proteins.

Standard methods for protein identification were used: general and specific stains; radioactive labelling; effect of cations, heparin, neuraminidase and heat treatment etc. (see Chapter 2). As the protein pattern seemed to be "conservative" compared to the esterases the classification of proteins used by other authors could be applied to the *Apodemus* pattern. The pattern obtained in the field mouse is very similar to other rodent species indeed , particularly *Mus musculus*. The exception to this is the red cell protein pattern, where *Apodemus sylvaticus*/

sylvaticus has 3(2) non-haemoglobin proteins, but *Mus musculus* 2(1) proteins of comparable type (Riddle & Petras, 1967). Although the proteins studied seemed to be stable and therefore handling did not present a problem, there are other factors that can influence the pattern apart from genetics:

a) *Ontogenesis* (Hartshone and Perry, 1962; Pantelouris and Hale, 1962; Pantelouris and Arnason, 1966, 1967; Gitlin and Boesman, 1967);

b) *Pregnancy* (Cons and Glass, 1963, Afonso and de Alvarez, 1964)

c) *Sex and Hormonal dependent* factors (Reuter and Kennes, 1966; Pantelouris and Arnason, 1967);

d) *temperature* (Grau and Lee, 1963);

e) *pathological* (Hughes, 1960);

g) *metal ions* (Aoki and Hori, 1964) and

f) *post-mortem changes* (Scopes and Lawrie, 1963).

With all this in mind the protein pattern will be discussed in Chapter 4.2.A.

C. *Esterases*

The main effort in the present investigation was concentrated on enzymes that hydrolyse 1-naphthyl acetate. The first step in differentiating between various groups was made by Richter and Croft (1942), when observing that all enzymes classified as cholinesterases were completely inhibited by 10^{-5}M eserine. They introduced the term "*aliesterases*" for those esterases resistant to eserine but hydrolysing aliphatic esters. Aldridge (1953 a and b) described esterases A and B; the A-type hydrolysed diethyl-p-nistrophenyl phosphate (E600) while the B-type was inhibited by E600. The third type was described by Bergmann *et al.* (1957)and called C esterase; it was inhibited by heavy metal ions and activated by certain organic mercurials./

mercurials. Another approach to the problem of esterase classification can be made by using substrates differing in the alcoholic part of the ester. The name arylesterase was given to those hydrolysing aromatic esters (Augustinsson, 1959). Augustinsson (1961) clarified the terms used for esterase classification:

1) Arylesterases, A esterases, aromatic esterases: Preference for aromatic substrates, resistant to organophosphorus compounds as well as eserine, activated by Ca^{2+}, but inhibited by sulfhydryl reagents; proposed active site: ... Cys....

2) Aliesterases, B esterases, simple esterases; Hydrolyse aliphatic as well as aromatic esters, resistant to eserine and sulfhydryl reagents but inhibited by organophosphorous compounds; proposed active site: ... Ser....

3) Choline esterases, nonspecific choline esterases, pseudocholine esterases: Preference for choline esters, but can hydrolyse aliphatic and aromatic esters, inhibited by eserine and organophosphorus compounds, but not by sulfhydryl reagents; proposed active site ... Ser ....

On the basis of phylogenetic data, Augustinson (1959 and 1967) proposed an evolution model for aryl-, ali - and cholinesterases from an ancestral proprionyl carboxylesterase possessing some cholinesterase characteristic at the same time. This common "ancestry" of the esterase types could probably explain some of the multiplicity found by the different authors. When Hunter and Markert (1957) applied histochemical techniques to stain for esterases after electrophoresis, using Smithies' method (1955), the multiplicity of these enzymes was even more apparent than before. The authors proposed the term "<u>zymogram</u> to refer to strips in which location of enzymes is demonstrated by histochemical methods". The term "<u>isozyme</u>" for/

for multiple enzymes with same characters was proposed by Markert and Møller (1959) working on LDH.

Holmes and Masters (1967a and b; 1968; 1969) made an extensive study of esterases of various tissues from several mammalian species, taking into account the ontogenetic changes. They grouped the enzymes into four main groups: carboxylesterases, aryl-esterases, acetylesterases and cholinesterases. Then on basis of physicochemical and developmental parameters they demonstrated that these classes are all heterogeneous: In the cavian tissues they found 10 forms of carboxylesterases, 4 arylesterase forms, 5 forms of cholinesterases and 5 forms of acetyl esterases. In rat tissues: 15 carboxylesterases, 5 arylesterases, 10 cholinesterases and 3 acetylesterases.

Recently Choudhury (1972) has challenged the subgrouping of esterases into acetyl-, aryl- or carboxyl esterases on the grounds of substrate hydrolysis or organophosphate sensitivity. He suggests that all non-specific esterases belong to one enzyme system. His conclusions are based on investigation of nonspecific esterases in a large number of organs from rat. "In starch gel electropherograms the capacity of ester-hydrolysis was seen to decline predictably with successively longer chain carbon substrates. An increasing susceptibility to organophosphate inhibition was observed with progressive lengthening of the acyl chain in the substrate molecules. Attempts to hybridize an organophosphate-sensitive esterase with a resistant type yielded a few additional esterase species." He then proposes a new model where the esterase species are built from subunits, which may be shared with the next species hence overlap in specificities. This view will be discussed further at a later stage.

D./

D. Non-genetic variation

Proteins and esterases may exhibit modification of the electrophoretic pattern due to reasons other than genetic.

Ontogenesis (Bernsohn et al., 1963; Manwell, 1966; Maynard, 1966; Pantelouris and Arnason, 1966; Stave, 1967: Holmes and Masters 1967 a and b: Holmes and Whitt, 1970); pregnancy (Cons and Glass, 1963; Pantelouris and Arnason, 1966); hormonal effect (Shaw and Koen, 1963 ; Leeuwin, 1965 and 1966; Allen and Moore, 1966; Arnason, 1966; Angeletti and Angeletti, 1967; Ambrus and Black, 1968); physiological (Meir et al., 1964; Lewis and Hunter, 1966); cold climate (Baker et al., 1962); irradiation (Hunter et al., 1968); action of proteases (Saeed et al., 1971; Arnason and Bjarnason, 1972); neuraminidase or sialidase Svensmark, 1961; Pantelouris and Arnason, 1967b) and effect of heat incubation (Pelzer, 1965; Holmes and Masters, 1966; 1967 a and b).
All the above mentioned factors as well as electrophoretic conditions (Arnason 1966) have to be considered when applying esterases as biochemical markers.

4.2 GROUPING OF BIOCHEMICAL MARKERS IN PRESENT STUDY

A. Proteins of various tissues of *Apodemus sylvaticus*

a) Serum proteins

This investigation was not focussed on these, but some explanations are necessary as no references to serum proteins of *Apodemus sylvaticus* could be found except in my previous work (Arnason, 1966).

1) Pre-albumins.

At least 3 prealbumins can be demonstrated in *Apodemus sylvaticus* on starch gel at pH 7.6 (Arnason, 1966). This is comparable to results with other rodents (Reuter and Kennes, 1966; Claxton *et al*. 1974; Biggers and Dawson, 1971). Neither sex differences were observed (Reuter and Kennes 1966) nor any variations that could be shown to have genetic basis (Kristjansson, 1963; Gahne, 1966; Biggers and Dawson, 1971; Claxton *et al*; 1974.) The existence of such types of variation cannot however be excluded.

2) Albumin

No variations in electrophoretic mobility was found in *Apodemus sylvaticus*. Reports exist on albumin polymorphism in *Mus musculus* (Petras, 1972); *Peromyscus* various species (Welzer *et. al*; 1965; Brown and Welser 1968; Biggers and Dawson, 1971); sheep (Tucker, 1968); horse (Gahne 1966) and man (Weitkamp *et. al*. 1969). The reason for not finding differences in the albumin means that variants are either rare or the electrophoretic systems used are not suitable to resolve these variants.

3) Post-albumins.

The "fast" post-albumin is found in the majority of field mice, but the "slow"

"slow" variant is rare. Whether the "slow" variant is "genuine" or conformational variant of the "fast" post-albumin could not be decided. It was only found in Scotland and Norway (Tables 3 and 36). The post-albumin is neither affected by heat (Fig. 61) nor neuraminidase (Arnason, 1966). No corresponding protein was found in Mus musculus, but post-albumin is reported in Peromyseus polionotus (Biggers and Dawson, 1971).

4) Transferrins.

Serum fractions 14, 15 and 16 are shown to be iron-binding (Nitroso-R method; ^{59}Fe-labelling) (Fig. 8.A and Arnason, 1966). They are slowed down by neuraminidase (Fig. 47) (Blumberg and Warren 1961; Poulik 1961; Chen Shi-Han and Sutton, 1967). Fraction 16 stains intensely with P.A.S. (Fig. 51) (Kapitany and Zebrowski, 1973). These fractions are therefore considered to be transferrins. A rare variant, where fraction 17 is the most intense (same as the pattern produced by neuraminidase) was found in Ireland and Iceland (Table 36). Many rodent species have transferrin polymorphism on genetic bases; Mus (Shreffler, 1960; Cohen, 1960; Cohen and Shreffer, 1961); Rattus rattus Moriwaki et al, 1969); Microtus (Maurer, 1967) and Peromyscus (Rasmussen and Koehn, 1966).

As reported by Pantelouris and Arnason (1967) the slower Trf-variant (17) was found in three individuals, one of these was a female that later gave birth to litter of three, one of which carried the "slow" variant. On basis of this and comparing with results obtained by the other authors cited, it is suggested that the transferrins of Apodemus sylvaticus are determined genetically by two co-dominant alleles: Trf^a giving the faster type (main fraction 16) and Trf^b giving the slower type (main fraction 17).

5)/

5) Ceruloplasmins.

As described in Chapter 3.1,C, the previous report of two phenotypes is doubtful (Pantelouris and Arnason, 1967b). An extensive survey of some 400 individuals did not show any polymorphism. The "fast variant" was produced by heparinisation (Templeton, 1969). (Fig. 14). This effect is also produced by Fe^{++} (Fig. 13) but not Cu^{+} nor Eu^{++} (Baker 1967). Aoki and Hori (1964) demonstrated similar phenomenon where "a zone of particular component disappears abruptly at a certain concentration of cation, while a new zone appears".

The human ceruloplasmin is thought to consist of two pairs of non-identical polypeptide chains, long and short; it is affected by neuraminidase, and is probably allelically polymorphic (Poulik, 1968).

b) Red cell proteins.

1) Haemoglobins.

Two Hb - fractions were observed in all fresh haemolysates tested when Tris-citrate borate pH 8.6 was used (Chapter 3.1,A,c). Only a limited number could be investigated, 60 samples from four countries. Freezing precipitates the Hbs and they are hard to dissolve again and Hb1 is missing after freezing (Figs. 7 and 11). The red cell lysates of *Mus musculus* did not behave in this way. Haemoglobin variants are found in rodents f. ex-*Mus* (Russell and Gerald, 1958; Petras and Martin, 1969) and *Peromyscus* (Rasmussen *et al.* 1968). It is therefore most likely that *Apodemus* might also reveal some polymorphism if extensive surveys could be carried out. Time and facilities did not allow the present author to investigate this point further.

2) Non-haemoglobin proteins.

Several non-haemoglobin proteins are described in Chapter 3.1.A,a. The strongest ones are A, B and C. A and B are polymorphic and probably governed/

governed by two independent loci, as will be discussed later in this chapter. Several tests were carried out in order to identify these proteins. These included:

Testing for carbonic anhydrases by using an esterase staining method with mixture of 1-naphthyl acetate and 2-naphthyl acetate as substrates (Tashian and Shaw, 1962; Tashian, 1965; Tashian _et al._, 1968). This failed to give any corresponding esterase fractions to A, B and C, so if they are carbonic anhydrases they differ from human (see above) and Cetacean (own observations, unpublished) carbonic anhydrases in this respect. No esterase fraction stained red (2-naphthyl acetate) and no inhibition effect was noticed with "Diamox" (acetazol-amide) (Figs. 2 and 3). No peroxidase activity could be demonstrated in these fractions, so they are not haemoglobins (Fig. 4). Neuraminidase treatment and P.A.S. staining did not affect these fractions, so they are not glycoproteins (Fig. 47). Incubation at 56°C for 10 minutes destroyed all of them (Fig. 60). Biddle and Petras (1967) have reported non-haemoglobin erythrocytic protein in _Mus musculus_. Two variants were found, Pro-1a (C57BL/10) and Pro-1b (C3H) that are probably governed by two codominant alleles at an autosomal locus. Fraction B in Fig. 6,I.e,d is probably Pro-1b. Fractions A and B are possibly equivalent to Pro-1 of _Mus musculus_, although electrophoretic mobility differs. Non-haemoglobin proteins have been reported in man (Hart _et al._ 1964).

c) _Skeletal and heart muscle proteins_

The skeletal and heart muscle proteins give fairly uniform pattern in all individuals. The number of fractions and general distribution is comparable with other authors' results (Hughes, 1959, 1960, 1961: Giles/

Giles, 1962; Hartshorne and Perry, 1962; Denuce, 1962).
The identification of fractions is difficult, but skeletal muscle fraction 13 and heart fraction 15 are probably myoglobins, as they are overlapping bands found strong in both tissues (Boyer et. al, 1963). The peroxidase activity overlapping strong nigrosine bands is very weak, but fraction 6 at pH 8.6 could come under this classification (Fig. 49 I, M,a and II, M,a). The faster albumin fraction in Fig. 49 could be grouped as "myoalbumin" and the slower as serum albumin (Hughes, 1960). Variations were found in skeletal muscle fractions C' (Tables 12 and 36) and 18 (Tables 11 and 36). Certain evidence suggests allelic control of C' (Table 27). On the other hand there is no evidence for this being the case with fraction 18. It is conceivable that the presence of fraction 18 or indeed absence of both 18 and 19 (Fig. 15) reflects some kind of conformational polymorphism. Changes in sarcoplasmic proteins due to external factors have been reported (Hughes, 1960; Grau and Lee, 1963; Scopes and Lawrie, 1963; Barron et al., 1965). It is possible that pathological conditions (then only found in Iceland and Norway) may well be the reason for fraction 18 (Barron et al., 1965). Ultrasonic homogenisation could possibly cause this breakdown of fraction 19 to give faint 19 and 18 and finally no fraction at all (Fig. 15), but fraction 19 is shown by experiment to resist thermal denaturization to certain extent (Fig. 60). Despite the doubt whether this polymorphism is allelic or conformational it is tabulated in Tables 10, 11 and 36.

d) Protein of other tissues.

Liver, kidney and testis all had fractions corresponding by mobility to serum albumin and transferrins, and probably representing contamination by serum. No individual variation similar to those described by Wilcox (1972) for such 'liver albumins' of presumed serum origin, were observed.

e) Conclusions.

Pre-albumin, albumin and ceruloplasmin of serum were all of one type. Same applied to haemoglobin and most tissue proteins. Serum post-albumins, transferrins and red cell proteins A and B are all polymorphic.

B. Esterases of various tissues of *Apodemus sylvaticus*.

In this section I shall discuss the fractions of each zone in terms of proposed esterase loci where applicable. The allocation of fraction to each "locus" is based on physicochemical tests (Holmes and Masters, 1966; 1967 a and b), breeding and population data. All the esterases in question probably belong to non-specific carboxyl esterases, except for few serum esterases. This is in good agreement with the findings of other workers in different animal species (Womack, 1972; Choudhury, 1972). Most fractions are found in more than one tissue, and some probably in all.

a) Red cell esterases

Erythrocyte esterases could only be studied on a limited scale as they disappear on storage. The fast-moving esterases present a complex pattern (Fig. 2) which might conceivably be complicated by serum contamination (Pelzer, 1965) or reflect a genuine red cell esterase polymorphism (Randerson, 1965; Tashian, 1962; 1965; Schiff and Stormont 1970). No carbonic anhydrase could be detected using mixture of 1-naphthyl and 2-naphthyl acetate (Tashian, 1969). Fractions 7, 8 and 9 are extremely labile and could be homologous to Es-3 of *Mus musculus* (Martin and Petras, 1971). The effect of heparin on these esterases are not ruled out (Templeton, 1969; Chapman *et. al.*, 1974).

b) Zone A

Es-1: Serum fraction 1; fraction 1 and (2) of cardiac - and skeletal muscle, brain and testis and fractions (1) and 2 of liver and kidney have same electrophoretic mobility and respond in the same way to physico-chemical tests (Tables 18-25). This is the only fraction in this zone that/

that is resistant to heat and neuraminidase treatment (Figs. 45 and 58). The fast fraction 1 of liver and the slow fraction 2 of heart etc. are most likely tissue-specific forms of Es-1 (Schiff and Stormont, 1970; Womack, 1973) or conformational isozymes (Kaplan, 1968). One animal had a slower version of serum fraction 1 (Table 4) which could either be an allelic variant or a conformational variant.

Es-2: serum fraction 2; fraction 3 of skeletal - and heart muscle, testis and brain; fraction 3 and 4 of liver and kidney. This esterase has some substrate and inhibitor specificity as Es-1, except it stained red at first when mixture of 1-naphthyl and 2-naphthyl acetate was used, as did 2b' and 3 of serum. Es-2 is heat and neuraminidase sensitive as are Es-3 and Es-4. Es-1 and Es-2 do not break down naphthol- -AS-acetate as Es-3, Es-4 and Es-5. Fraction 2b' (Fig. 9A) is probably a true allelic variant of 2. In samples from Scotland a slightly faster variant was observed (Table 5) in 6 samples out of 35. Whether this phenomenon is based on genetical grounds or conformational changes is not certain. Es-2 of Apodemus sylvaticus is probably analogous to Es-2 of Mus musculus (Petras, 1963) and Es-1 of the rat (Augustinsson and Henricson, 1966; Womack, 1973).

Es-3: serum fraction 3; fraction 5 of skeletal - and heart muscle, testis and brain; very faint if any expression in liver and kidney, but electrophoretic mobility similar to liver fraction 7. Es-2 and Es-3 are present in foetus and newborn, as Es-4 is absent and develops later (Arnason 1966). On the other hand Es-3 and Es-4 react both strongly with naphthol -AS- acetate (Tables 18-24). This fraction overlaps the albumin and is in many ways analogous to Es-1 in the house mouse (Popp and Popp, 1962) and Es-2 in the rat (Womack, 1972a).

Es-4/

Es-4: fractions 5 and 6 of serum; fractions 6 and 7 of heart, skeletal muscle, testis and brain; fractions 8 and 9 of liver and kidney. On grounds of physioochemical tests, ontogenesis, breeding tests and population data these are most likely the genuine product of a two allele loci. Serum fractions 4 and 5b and overlapping fractions in other tissues are possibly either break-down products of the others e.g. by losing sialic acid molecule or hormonally induced fractions (Gasser et. al, 1973). Es-4 in Apodemus sylvaticus has many things in common with albumin esterases in Rattus norvegicus (Gasser et. al., 1973; Womack, 1973).

Es-5: Serum fraction 7. As mentioned earlier this fraction is rate, and data do not permit any discussion.
Fraction 4 of heart-, skeletal muscle, testis and brain is variably expressed in some instances, probably of physiological nature.
Fractions 5, 6 and 7 of liver and kidney have same elctrophoretic position as heart fraction 4, but different physiochemical behaviour.

6) Zone B.

Es-6: Zone X' of serum. This is a silent zone in Icelandic mice, but present in all mice from other countries. Breeding data suggest a dominant allele for the silent form (Fig. 62; Table 32). These esterases are true aliesterases as they neither hydrolyse naphthol -AS- acetate nor 6-bromo-2-carbonaphthoxy choline iodide.

Es-7: fractions 11-15 of liver and kidney. This locus expresses itself only faintly in the kidney. The breeding studies suggest quantitative mode of inheritance, involving a "near silent" allele producing fractions with low activity and a "usual" allele producing normal activity (Liddell

et/

et al., 1962; Augustinsson and Olsson, 1961; Seneonoff and Robertson, 1968; Arnason *et al*. in press).

The "near silent" allele was found in all Icelandic and 2 Irish samples. It might seem likely that Es-6 and Es-7 was the same locus, judging from distribution alone, but they have different substrate specificity as Es-7 hydrolysis naphthol -AS- acetate. Brain and testis fractions 9 and 10 belong to zone B, but differ from fractions of Es-6 and Es-7 in physicochemical as well as genetical terms as they are present in all samples including samples from Iceland.

d) Zone C:

This zone expresses itself variably in various tissues. Heart-, skeletal muscle and testis are very similar, which is in agreement with results obtained by Holmes and Masters (1967b) studying rat tissues. Liver and kidney are similar, but have many more fractions than heart etc., again in agreement with the rat..

The brain has only few fractions here again as in the rat (Holmes and Masters, 1967b). The absence of this zone in foetal liver of *Apodemus sylvaticus* (Figs. 30 and 31) agrees with findings in cavian and rat foetal liver (Holmes and Masters, 1967a and b). Tissue diversity in zone C of *Apodemus sylvaticus* is remarkably like that in zone II of *Mus musculus* as demonstrated by Ruddle and Harrington (1967).

Es-8: It is not clear whether all fractions in zone C are governed by one locus, but the limited breeding data suggest that majority of them are. Petras and Sinclair (1969) found that fractions in zone II of *Mus musculus* are governed by two alleles at one antosomal locus, with the allele for absence of the multiple-band phenotype being dominant. It is quite/

quite probable that the faster fractions in zone C are governed by another locus - this is supported by the fact that faster fraction separate out of zone C under certain electrophoretic conditions (Figs. 64 and 74). Under these conditions faster fractions of zone C become more like zone II of _Mus musculus_ (Ruddle and Harrington, 1967). Womack (1973) demonstrates mode of inheritance at Es-4 locus in the rat, which seems similar to the behaviour of Es-8 of _Apodemus sylvaticus_ (see 4.3). The argument that the same locus is responsible in heart, muscle, testis, brain liver and kidney for respective zone C is supported by similar finding by Womack (1973). The tissue environment could be responsible for the ultimate phenotype of the gene product.

e) _The mouse with the missing serum esterase 20_.

Fig. 9A shows serum zymogram of _Apodemus sylvaticus_. Sample d lacks fractions 20, 16 and 15. These fractions show all cholinesterase activity. They have similar electrophoretic behaviour as human pseudocholinesterase fractions C4, C3 and C2 respectively. Both sets of fractions are sialoproteins (Svensmark, 1961; Arnason, 1966) and heat labile. The question arises whether the locus responsible for the "silence" in _Apodemus sylvaticus_ is homologous with Es_1 locus in man responsible for the Es_1^s gene (Liddell _et_. _al_., 1962; Goedde and Altland, 1968; Arnason _et_. _al_. in press). The author has also observed that sheep, house mouse, rat, cow, horse and whales (_Balaenoptera physalus_; _Balalnoptera borealis_ and _Physeter catodon_) all have a serum esterase fraction corresponding to C4 of human and fraction 20 of _Apodemus sylvaticus_. An equivalent fraction is also found in gulls (Genus Larus) (Palsdottir/

(Palsdottir and Arnason, unpublished).

f) Notes on physicochemical tests.

In general most of the esterases already discussed are probably non specific carboxylesterases (Choudhury, 1972): Exceptions from this are the eserine sensitive serum esterases. All esterase zones are affected in some way by neuraminidase so they are likely to be glycoproteins. Heat inhibition was not observed when the gels were incubated after electrophoresis but widespread inhibition occurs when samples were incubated prior to electrophoresis (Fig. 58). It seems that the gel "matrix" protects the protein molecule in some way. The difference in heat sensitivity in homologous fractions of various tissues (e.g. liver and kidney) could be attributed to different ionic environment in each tissue.

g) Conclusions.

Esterases are found in all tissues in multiple forms. Each tissue has its own pattern, but shares the majority of its fractions with some other tissues. The esterases described overlap in substrate specificity and sensitivity to inhibitors - and most can be classified as non-specific carboxylesterases. Physicochemical tests, breeding and population data indicate at least 8 loci governing the esterase pattern. These loci express themselves in most tissues, but the intensity of esterase fraction may vary from tissue to tissue.

Zone C has different number of fractions in different tissues, but is still governed by the same locus, Es-8 in all of them.

4.3 Interpretation of the breeding tests and proposed loci.

Chapter 3.5 and Tables 26-35 give the results of the limited number of breeding tests or rather family survey, that could be carried out.

I shall deal here with a few major points which can be confidently derived from these rather limited data.

A. Red cell proteins A and B.

The breeding data (Table 26) show that where parents are both A (or B) the offspring also are of the parental type. On the other hand the population data (Tables, 1, 2 and 36) suggest as likely two autosomal loci for the A and B proteins. Locus A, autosomal with two alleles, a dominant allele 'producing' protein A and a recessive allele 'not producing' protein A.

Locus B, autosomal with two alleles, a dominant allele 'producing' B and a recessive allele non protein producing.

This hypothesis agrees with all data obtained. One locus - two allele inheritance could not explain why all Icelandic samples have A, and all Irish and Norwegian all B phenotype.

B. The muscle protein C'.

A one autosomal locus - two allele hypothesis is presented in Table 27. The admittedly limited data fit in this hypothesis well.

C. Inheritance of esterases, Es-1 - Es-8

Tables 28-35 give the postulated mode of inheritance. All results agree statistically/

statistically with one locus - two allele mode of inheritance for each of the proposed loci:

Es-1; Es-2; Es-3; Es-4; Es-6; Es-7 and Es-8.

$Es\text{-}6^a$ reflects probably a dominant and $Es\text{-}6^b$ a recessive allele. All the other pairs of alleles are codominant.

The occurrence of a number of "non-esterase producing" or "null" alleles brings up the question whether it is compatible with life to lack so many enzymes?

Several conexplanations may be conceivable:

(1) The "silent" allele produces an "inactive" enzyme. This enzyme may be physiologically functional but inactive on the histochemical substrates used.

(2) The "silent" allele produces no enzyme. A deletion of genetic material or regulatory mutant may be involved.

(3) The "silent" allele produces an esterase inhibitor. The results from pooled samples does not suppport this hypothesis.

(4) The "silent" might not be silent at all, but "hidden" by overlapping fraction governed by another locus.

(5) In Mus musculus it has been shown that Es-1; Es-2; Es-5; Es-6; and Es-7 are linked and situated on chromozome 8; Es-3 on chromosome 11 and Es-8 on chromosome 7 (Popp, 1965; Popp, 1967; Petras and Biddle, 1967, Petras and Sinclair, 1969; Ruddle et al., 1969; Nichols and Ruddle, 1973; Chapman et. al. 1974) and in Rattus norvegicus Es-1; Es-2; Es-3 and Es-4 are all linked (Womack, 1973). Augustinsson (1967) has proposed that multiple forms of esterases occurred by gene duplication. This hypothesis could explain that high number of genuine "silent" genes is tolerated/

tolerated as the products of the next linked locus can take the job over.

The similarity between *Mus*, *Rattus* and *Apodemus* has been demonstrated throughout this discussion. It is therefore possible to suggest that most esterase loci of *Apodemus sylvaticus* are linked and have originated by duplication.

D. Conclusions

Red cell protein A is governed by one autosomal locus with two alleles: the "protein producing" allele is dominant and the recessive is silent. The red cell protein B is inherited in the same way and has its own locus. The C' muscle protein also has present and silent alleles. Most esterase fractions are amounted to one autosomal locus with two codominant alleles. The proposed loci Es-1, Es-2, Es-3 and Es-5 control one fraction each. Es-4 governs serum esterase fractions 5 and 6 and "equivalent" fractions in other tissues. Es-6 governs zone X' in serum, the Es-6^a allele (silent) being dominant. Es-7 has a quantitative effect on zone B of liver, the Es-7^a being "near-silent" Es-8 expresses itself differently in different tissues, but each allele affects many fractions.

4.4 Implications of population data using biochemical markers.

A. Previous work.

In recent years several authors have used biochemical markers in population studies of e.g. *Mus*, *Microtus* and *Peromyscus*. No such data exist on *Apodemus*, except the data already presented by myself (Arnason, 1966).

Petras/

Petras (1967) studied commensal populations of Mus musculus in Michigan over a four year period. He examined four loci controlling biochemical variants, two of which were polymorphic (Es-2 and Hbb). Numerical deficiency of heterozygotes of both Es-2 and Hbb loci was noted. This was explained by the "existence of small random breeding units with limited migration between them, and the frequent occurences of numerical bottlenecks".

There were no significant changes from year to year.

In a later examination of Mus musculus from Ontario Petras et. al. (1969) found six polymorphic loci (Hbb, Es-2, Es-3, Es-5, Ldr-1 and A) and three monomorphic loci (Es-1, Pro-1 and Trf-1). This study and data from other investigators suggest broad geographic distribution of these polymorphisms. The conclusion was that polymorphic loci are probably as frequent in populations of Mus as in Drosophila (Lewontin and Hubby, 1966) and man (Harris, 1966; Lewontin, 1967; Harris et al. 1968). Similar results were obtained by Selander et al. (1969), and Selander (1970), working on Mus musculus, using 36 proteins governed by 41 genetic loci. Roderick et al. (1971) compared biochemical polymorphisms in feral and inbred mice (Mus musculus) with comparable results.

Seasonal variations as in frequency of biochemical phenotypes has been reported for Mus musculus (Berry and Murphy, 1970; Berry, 1970) and changes of protein markers in fluctuating populations of Microtus (Tamarin and Krebs, 1969; Myers and Krebs, 1971). Semeonoff and Robertson (1967) report both density-dependent and seasonal fluctuations involving the E_1 locus in Microtus agrestis.

Evidence for 'founder effects' on frequencies of biochemical markers has been published for Mus musculus (Hunt and Selander, 1973) and Peromyscus polionotus (Biggers and Dawson, 1971).

B. Interpretation of present data.

Chapter 3.6 describes some of the results presented in Tables 1-17 and 36-44. The number of samples are admittedly low, but comparable to the numbers used in many previous publications. In this brief discussion I shall only try to interpret each table first and then discuss results as a whole.

a) Table 36:

All Icelandic and Irish field mouse populations have a fixed allele for red cell protein A, and so does the Cumbernauld (7b) population. Protein B seems to be fixed in Norway, Ireland (only 20 samples) and the Garscube (7) population in Scotland. In this sense Iceland and Norway populations differ. The cause of this cannot be determined.
The Garscube (7) and Oslo (10) populations are the only ones to have slow post-albumin, but in low frequency. No significant differences can be observed in incidence of the fast-post albumin.
The slow Trf variant is found in Ireland and Iceland, but the frequency is too low to be decisive. If the frequency of muscle protein 18 has a genetical basis, (see 4.2.A) there is a marked similarity between Icelandic and Norwegian populations. Muscle protein C' was found only in Iceland and in the Swedish laboratory stock.

Tables 37-40:

Table 37 compares numbers of observed phenotypes of Es-1 with those expected/

expected as a result of Hardy-Weinberg equilibrium. All populations show statistical agreement with the hypothesis at 5% level and 1 d.f., except the Cumbernauld (7b) and Oslo (10) population that were fixed for the silent allele, probably due to founder effect.

No selection was found here. Table 38 deals with Es-2 in the same way. The outstanding point here is that all Icelandic and Irish populations are fixed for allele Es-2^b, as the others are polymorphic. This favours closer relation between Icelandic and Irish mice, than Icelandic and Norwegian mice.

Table 38 compares observed phenotypes of Es-3 with those expected as a result of Hardy-Weinberg equilibrium.

The pooled data suggest a significant difference, probably indicating selection.

The deficiency of heterozygotes in the Ireland population could be explained by the fact that it probably consisted of small random breeding units.

Petras (1967) claims 'this should be expected in populations of all small mammals and most bisexual species, since they are generally composed of individuals whose parents have limited dispersal.'

The reason for excess heterozygotes in the Oslo population is not known.

Table 40 shows that there is no selection for certain phenotypes, judging from the pooled data, which are within statistical limits.

Two populations show a significant difference from the Hardy-Weinberg equilibrium. The Stora-Mörk (2) population has great excess of heterozygotes. It is not obvious what causes this, but all these mice came from sheep sheds. No difference in frequency was observed between different/

different sheep sheds. Selection by environmental factors artificial fertilizers, fish-meal cubes etc., might be responsible. The Irish population shows deficiency in heterozygotes which can again be explained by small breeding units.

Table 41

Fraction 7 is only found in Iceland, Ireland and Scotland, but not Norway, again hinting closer relationship between Iceland and Ireland-Scotland.

Table 42 demonstrates that fraction 7 of the liver is fix ed in the Icelandic mouse population, but polymorphic in the others. Fractions in zone B (Es-7) are faint in all Icelandic populations.

Table 43 demonstrates the most outstanding difference between the populations. The absence of C_{IV} from Norwegian populations and presence of C_{III} raises the question whether these two forms are in fact one and the same but assuming different electrophoretic mobility under different conditions. No experimental manipulation succeeded in converting one form into the other, so they have to be considered 'genuinely' different.

Table 44 summarises allele frequencies at six proposed esterase loci. Outstanding features are that all Icelandic populations have fixed alleles at loci Es-2, Es-6 and Es-7 Ireland, Scotland and Norway have fixed alleles at Es-6, but a different one from Iceland. Scotland and Norway have fixed alleles at Es-7, but Ireland is polymorphic. In Iceland there is a significant difference between Laugarvatn (1) and Store-Mork (2) at Es-3, the latter location has the silent allele only/

only, probably a true 'founder effect'. All what has been observed here is in good agreement with the findings of other authors in Mus e.g. Selander et. al. (1969) and Hunt and Selander (1973). These authors found many of the alleles in Mus were fixed in one locality, but not another. There were also differences in sub-species of Mus musculus, where one sub-species had a fixed allele but not the other and vice versa.

The Icelandic and the Irish field mouse populations show similarities in the frequency of: Red cell protein A, slow Trf, Es-2, and, to a lesser extent, Es-7 and Es-8.

The Icelandic and the Norwegian field mouse populations show similarities in the frequencies of: muscle protein 18, Es-3.

The "silent allele" for zone B is found in Iceland and also in Ireland.

The frequencies in general hint stronger Irish, than Norwegian origin for the Icelandic field mice, but results like these have to be interpreted cautiously as to tracing back the origin of populations. It is however feasible to suggest that field mice came to Iceland both from Ireland and Norway, on the basis of historical (see Introduction) evidence as well as from these data here described.

This may seem to be in disagreement with previous findings (Berry, 1969), but need not be, as different systems are used as markers.

The frequency of polymorphic loci out of 17 loci tested (see Tables 36-44) in these populations varied from 23.5% to 53%. This range is similar to observations in other animals (Harris, 1966; Lewontin, 1967; Lewontin and Hubby, 1967; Petras et al. 1969)

The striking difference between the populations in this study is best explained/

explained by founder effects, due to chance introduction or populations descending from few survivors, say after a severe winter.

C. Conclusions.

Most populations investigated differed significantly from one another in the frequencies of protein markers. Fixed alleles are common. These differences are attributed mainly to "founder effects".

Modes of inheritance are postulated which fit the population data. Certain characteristics specific to Iceland have been found e.g. fixed alleles Es-6^{a} and Es-7^{a}. About 40% of the protein loci in this investigation were polymorphic.

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APPENDIX

List of publications by Alfred Arnason:

1. Ontogenesis of serum esterases in *Mus musculus*

   J. Embryol. exp. Morph. 1966 Vol. 16 55-64.

2. Serum esterases of *Apodemus sylvaticus* and *Mus musculus*

   Comp. Biochem. Physiol. 1966 Vol. 19 53-61

3. Serum proteins of *Apodemus sylvaticus* and *Mus musculus*

   Comp. Biochem. Physiol., 1967 Vol. 21 533-539.

4. Further observations on mouse serum esterases.

   Comp. Biochem. Physiol., 1967 Vol. 20, 647-652.

5. Um sameindir nokkurra eggjakvituefra (protein-gerdir) hja rjupum.

   Natturufraedingurinn, 1970. Vol. 40, 171-196.

6. Genetic variation in the eel. II Transferrins, haemoglobins and esterases in the eastern North Atlantic. Possible interpretations of phenotypic frequency differences

   Genet. Res., Camb. 1970 vol. 16, 277-284.

7. Genetic variation in the eel. III. Comparisons of Rhode Island and Icelandic populations. Implications for the Atlantic eel problem

   Marine Biology, 1971 Vol. 9(3), 242-249.

8. Post-mortem changes of human serum esterases. An electrophoretic study.

   Acta path. microbiol. 1972. Vol. 80A, 841-846.

9. Öndunarlömun af vöðvaslappandi lyfi vegna arfgengs skorts a serumcholinesterasa.

   Læknablaðið 60 arg., 1.-2. Tbl., Jan.-Febr. 1974

ADDENDUM TO THE DISCUSSION ON RED CELL PROTEINS A AND B

The brief discussion of these two electrophoretic fractions in p. 136 requires some amplification:

Table 45 gives the observed numbers of combination AA, AB and BB and the numbers expected on a hypothesis entertained in the first instance: of two codominant alleles. It is apparent that for most locations the data significantly disagree ($P < .001$) with the hypothesis. Only two (7B and 10) out of ten samples appear to agree with the hypothesis, but one of these samples comprises 8 individuals only and the phenotypic distributions in the other (1'AB', 23'BB') is very unlikely to occur under a Hardy-Weinberg equilibrium. It appears therefore that the one locus - two allele hypothesis finds no support in the data. In addition, if the above hypothesis was accepted, there would be necessary to assume further selection against B/B individuals in Iceland, as they are not found there; and similar selection against A/A individuals in Norway and Scotland. This would be hard to explain.

An alternative and more likely hypothesis is presented in Table 46. Two autosomal loci A and B are proposed to be involved, each consisting of two alleles, one dominant and one recessive.

The breeding data of Table 26 could then be explained in the following way:

Mating 1, proposed genotypes of parents: A/A, b/b x A/A, b/b.

Mating 2, proposed genotypes of parents: a/a, B/B x a/a, B/B.

Mating 4, proposed genotypes of parents: A/A, B/B x A/A, B/B

A/a, B/B x A/A, B/b

etc.

It is apparent from Table 46 that heterozygotes cannot be detected, as the "silent" is hidden in the heterozygous phenotypic group. The term "fixed allele" used on p. 140 might not be applicable in all cases. The same is true for $Es\text{-}2^{b}$, described on p. 141.

Table 45.

A comparison of the numbers of observed phenotypes of Red cell proteins A and B with those expected as a result of Hardy-Weinberg Equilibrium.

Country	Locality	Total no.	Proposed genotype A/A		A/B		B/B		x^2	P
			Obs	Exp	Obs	Exp	Obs	Exp		
Iceland	1	48	20	24.20	28	19.77	0	4.03	8.1846	< .001
	2	68	6	19.83	62	33.78	0	14.39	47.6105	< .001
Ireland	6	20	0	5.00	20	10	0	5.00	20.000	< .001
Scotland	7	24	0	1.75	13	9.46	11	12.79	3.3233	> .05
	7b	8	3	3.8	5	3.4	0	0.8	1.7214	> .10
Norway	9	20	0	3.2	16	9.60	4	7.20	8.8888	< .001
	10	24	0	0.01	1	0.94	23	23.05	0.0144	> .90

One locus, two autosomal allele hypothesis

A codominant allele for presence of fraction A

B codominant allele for presence of fraction B

Table 46.

Observed phenotypes of Red cell fractions A and B, based on two loci, four allele hypothesis.

Country	Locality	Total no.	Locus A		Locus B	
			Fraction A present	Fraction A absent	Fraction B present	Fraction B absent
			A/A and A/a	a/a	B/B and B/b	b/b
Iceland	1	48	48	0	28	20
	2	68	68	0	62	6
	4	6	6	0	4	2
Ireland	6	20	20	0	20	0
Scotland	7	24	13	11	24	0
	7b	8	8	0	5	3
Norway	9	20	16	4	20	0
	10	24	1	23	24	0

Locus A

A dominant allele for presence of fraction A

a recessive allele for absence of fraction A

Locus B

B dominant allele for presence of fraction B

b recessive allele for absence of fraction B

ADDENDUM TO THE DISCUSSION ON ESTERASE FRACTIONS Es-6, Es-7 and Es-8

The following is an amplification of the points made in p. 138 (re Es-6), p. 132-133 (re Es-7) and p. 133-134 and Table 34 (re Es-8):

Es-6

All Icelandic mice were silent at this locus, but mice from other countries expressed esterase fractions in zone X' (Es-6) (see p. 132, 138). Table 32 gives the limited breeding results available. Mating 1, between male from Iceland, which was 'silent' and female from Sweden, which had esterase fractions in zone X' resulted in 15 offsprings. All these lacked esterase fractions in zone X' (Es-6). This is the only evidence at present for the suggestion of a "dominant silent allele".

The "silence" at locus Es-6 might not be "true silence" at all (see p. 137). It is possible that Point (1): the "silent" allele produces an apparently "inactive" enzyme, which might be functional fails to react with the substrates used in the tests. Point (4): The "silent" might again not be true silent, but "hidden" by overlapping fraction governed by another locus.

Es-7

Referring to p. 57, Table 16, p. 64, Table 42, p. 112 and p. 132-133 it is obvious that the "near silent" allele(s) found in the Icelandic samples is (are) only one (few) of many alleles involved in the inheritance of fractions in zone B of liveresterases. The involvement of other loci cannot be excluded. This might well apply to mating 2 in Table 33, see also P. 88-89.

Es-8

Referring to p. 50-51 it may be concluded that the pattern in zone C may be a complex one. This is discussed further on p. 118 and p. 133-134. Table 34 gives limited data on inheritance of some of this complex pattern.

Matings 1 and 2 give patterns which can be explained by two codominant alleles at one locus (see Table 35). Mating 4, where there is no information on the parents resulted in 2 offsprings of type CI and 3 offsprings of type CIV (Table 34).

These results hint similar inheritance as described by Petras and Sinclair (1969) for zone II of Mus (p. 133), where one allele was dominant for absence of the multiple-band phenotype. Similarly either zone CI or CIV might be governed by a dominant allele for its presence. This could explain the results of mating 4.