ELECTROPHORETIC STUDY OF TRANSFERRIN AND PSEUDOCHOLINESTERASE ON VERVET MONKEYS (*Cercopithecus aethiops aethiops* L.)

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ELECTROPHORETIC STUDY OF TRANSFERRIN AND PSEUDOCHOLINESTERASE ON VERVET MONKEYS (*Cercopithecus aethiops aethiops*, L.1758)

A Thesis Submitted to the
School of Graduate Studies
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TABLE OF CONTENTS

CONTENT PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

ABSTRACT

Blood serum samples (n=34) were taken from a population of vervet monkeys inhabiting the terrain along the Awash River. Genetic variation on transferrin (Tf) and pseudocholinesterase (Psch) were studied using a non-denaturing vertical polyacrylamide gel electrophoresis system. No variation was detected on transferrin. Psch was found to be polymorphic with respect to mobility and activity level. Four phenotypes were distinguished based on this polymorphism. This included: single cathodal band with strong activity; single anodal band with strong activity; two bands of activity (one cathodal and the other anodal) and finally no band activity. These phenotypes could be explained based on a three allele model proposed from a previous study. These alleles are designated as $psch^1$ for the silent gene, $psch^2$ for the cathodal band and $psch^3$ for anodal band activities. The genotypes of each individual were constructed and the gene frequencies calculated. The gene frequency for $psch^1$, $psch^2$ and $psch^3$ were 0.0883 and 0.3088 and 0.6029 respectively. Test for goodness-of-fit for these alleles showed the population to be in a Hardy-Weinberg Equilibrium ($X^2_3=4.95$, $p > .05$) at present. However, when the results of this study were compared with a previous study (some 20 years ago) on the same area, the allelic frequencies have greatly changed. Possible factors responsible for the Tf monomorphism and Psch polymorphism are discussed. The Tf monomorphism may be due to the low migration rate; the antagonistic effect of the Psch third alle on rare Tf allele and the effect of sampling size. The shift in Psch allelic frequency polymorphism may be accounted for by environmental factors. The effect of the 1974 and 1985 drought periods is may have contributed to the dominance of the third allele. The Hitch-Hiking effect on the rapid shift of allele frequency and the effect of methods

employed are also discussed. Based on the allelic distribution, important sites for the conservation of vervet monkeys are recommended.

1. INTRODUCTION

Electrophoresis has become one of the most widely used molecular techniques for studying genetic variation. Several studies on quantifying genetic variation within natural populations have been undertaken for decades. This has consequently made it clear that genetic variation of proteins exists in the form of isoallelic variation. The premise is that the net charge difference during electrophoresis suggests an amino acid substitution or mutation (Thorpe and Sole-Cava, 1994).

Proteins are now generally considered as effective markers in various studies of genetics and evolution. Variant proteins are used by geneticists and taxonomists to estimate genetic polymorphism in natural and domestic populations; study differential gene expression during development and understand the mechanisms of the evolution of genes and proteins.

Therefore, the present work was formulated to see the role of evolutionary forces on selected proteins of vervet monkey natural populations. To fulfil this objective, two proteins were selected for analysis. These proteins were selected based on information from a previous study.

In 1973, vervet monkeys were trapped in the Awash National Park by The American Primate Biology Study Group from New York University. This sample included 7 social groups of vervets living along the Awash River. Blood samples were collected and starch gel electrophoresis was employed to study variation in serum and hemolysate

proteins. Of those proteins analyzed, two of them showed an interesting polymorphism. These were:

1. Transferrin having one common allele and one rare allele.
2. Serum cholinesterase having three activity level polymorphism; individuals with high, medium and nearly nil enzyme activity.

For transferrin analysis, 119 blood samples were taken from 7 social groups. These samples were run for 5 to 6 hours in starch gel at 35 v/cm. The gel was then stained with Amido Black. From that study, it was found that the rare allele of transferrin was restricted to E (0.88) and F (0.05) social groups only.

The pseudocholinesterase variation was determined from blood samples collected from 118 individual vervets. The samples were run in the same gel and running conditions as that of transferrin. The gel was then incubated with alpha naphthyl as substrate for band detection. It was found that this enzyme was polymorphic with respect to mobility and activity level.

Moreover, the total population size of the entire population was estimated (Table 1). The past population had a smaller number of individuals per social group. However, the sampling included almost all individuals from each social group.

Table 1. Estimated population size for the past sampled social groups (After Turdy, 1973).

Group	No. Sampled		Population
	transferrin	pseudocholinesterase	size
A	9	9	14
B	18	18	19
C	11	11	11
D	20	19	20
E	25	25	25
F	11	11	12
G	25	25	27
TOTAL	119	118	128

This study was, therefore, designed to see and illustrate the changes that may have occurred since the first study was undertaken 23 years ago. More specifically, questions that are to be answered are :

1. Is the rare allelic variant of transferrin present in the study population ?

2. Has the cholinesterase polymorphism still been maintained and if so have allele frequencies changed ?

2. LITERATURE REVIEW

2.1. TAXONOMY OF *CERCOPITHECUS*

Old world monkeys have their origin in Africa (Kingdon, 1971). Recently, they are distributed in both Eurasia and Africa. A total number of 94 species are distributed in Africa, Arabian Peninsula, Japan and East Indies (Delany and Happold, 1979). A small number of introduced species are found in Southern Spain (Nowak, 1991).

Monkeys of Africa are all included in family Cercopithecidae. This family is sub-divided into two sub-families, namely, Cercopithecinae and Colobinae. A total of 19 genera are included in the two sub-families. Of these, 9 belong to the former and the other 10 to the latter sub-family (Hill, 1966).

The genus *Cercopithecus* (Guenons) is included in the cercopithecinae sub-family. This genus contains 26 species, the largest genus in the entire family (Nowak, 1991). Its distribution is restricted to Africa South of the Sahara (Hill, 1966). Primate taxonomists find it difficult to assign specific ranks to the species of this genus. This problem has been mainly attributed to the wide geographical distribution of the genus and partly to the unreliability of coloration as a marker in primate taxonomy (Kingdon, 1990; Yalden *et al.*, 1977).

No consensus has been reached on this aspect but some taxonomists prefer the lumping of species. Particularly, the species *C. sabaeus* (green monkey), *C. aethiops* (grivet

monkey), *C. tantalus*, *C. cynosurus*, and *C. pygerythrus* (vervet) are lowered to sub-specific rank and included under *C. aethiops* species complex (Nowak, 1991). Following this trend, *C.a.sabaeus* represents the west African form; *C.a.aethiops* and *C.a.tantalus* the east African type. The last two, *C.a.cynosurus* and *C.a.pygerythrus*, represent more of the southern type. However, the exact geographical boundary and possible overlap is not clearly defined (Fedigan and Fedigan, 1988).

Yalden *et al.* (1977), on the other hand, treated *C. aethiops* as a superspecies and reported two species in Ethiopia alone -*C. aethiops* and *C. pygerythrus*. He also included several races but suggested that more information is needed on distribution of the species to arrive at a better taxonomy.

Therefore, it seems a matter of simplicity and convenience for taxonomists to follow a certain classification system.

In the present study, Kingdon's (1971) classification is used. He suggested *C.a.aethiops* and *C.a.tantalus* represent the more eastern and western regions of East Africa respectively. Hence, following his classification, *C.a.aethiops* will be represented in Ethiopia. *C.a.aethiops* is widely distributed in northern, central and southern parts of the country. It occupies different habitats including riverine, tropical deciduous or montane forest and relatively open *Acacia* savanna (Yalden *et al.*, 1977). In several parts of the country, it is known to intrude into human habitations and destroys many crop plants. Its altitudinal distribution in Ethiopia ranges from few hundred meters to 3000 meters above sea level (Hillman, 1993).

2.2. PROPERTIES OF TRANSFERRIN

2.2.1. Chemical Properties of Transferrin

Serum transferrin is one of the most important members of iron-binding proteins. Such iron binding property was first observed in egg white (Charlwood, 1963). The chicken's protein is commonly known as conalbumin or more properly ovatransferrin. This discovery led to the search for a homologous substance in humans. The human serum contains transferrin which is commonly described as siderophilin or ß1-metal binding protein. Other parts of human body also contain a functionally similar protein in the milk, lactoferrin. These studies and others consequently led to the recognition of transferrin in all vertebrates (Kamboh and Ferrell, 1987).

Transferrin is a three dimensional globular bilobed molecule. Each lobe has a single site for binding iron. This provides the characteristic property of transferrin to bind two ferric ions per mole (Beckman and Beckman, 1987). The iron binding process occurs only in the presence of bicarbonate or carbonate ligands (Karin and Mintz, 1981).

Chemical studies on vertebrate transferrin showed its molecular weight to be in the range of 68,000 to 90, 000 daltons (MacGillivray *et al.*, 1982). Transferrin is a glycoprotein and contains about 6% carbohydrate residue in humans (Beckman and Beckman, 1987). Two identical chains of carbohydrates are attached to the asparagen residues of the protein (Yang, 1984). These chains are composed of 6 mannose, 6 galactose, 8 N-acetylglucosamine and 4 sialic acid residues.

Moreover, aminoacid sequence studies suggested the existence of two structural domains in all transferrins (Park *et al.*, 1985). These domains correspond to the two iron binding bilobed structures of transferrin. The two regions show a higher (50%) internal sequence homology within NH_2 and COOH terminals (Young, 1984). Crystallographic analysis on human transferrin supported the presence of two structural domains corresponding to the sequence homology regions (Weitkamp, 1985). These regions are found in amino acid residues at positions 1-336 and 337-679 of human transferrin. From such studies, it became clear that transferrin is a monomeric protein made up of two homologous proteins. Such a sequence homology was hypothesized to be a product of intra-genic duplication during the course of evolution (Yamato *et al.*, 1983). The ancestral transferrin is believed to have half the molecular weight of the present form with a single Fe binding site (Chudoba and Jablonska, 1978; Rozhkov and Rozhkova, 1984).

This hypothesis is now supported at the gene level. Characterization of two overlapping gene clones of transferrins from liver DNA library of humans suggested the occurrence of two genes in a single segment of DNA (Beckman and Beckman, 1987). They are composed of equal number of exons interrupted by variable sizes of introns. However, no single vertebrate species has been found in which the single lobed evolutionary precursor persists.

The biological role of the duplicated structures within a given transferrin remained to be a matter of speculation. Some suggested the evolutionary role of duplication to be for reducing glomerular filtration (Young, 1984). The doubling of the structure might

have conferred an evolutionary advantage by avoiding exclusion of transferrin from serum in the glomeruli of the kidney (Yamato *et al.*, 1983).

2.2.2. Genetic Variants of Transferrin

Electrophoretic analysis of the serum protein of vertebrates reveals ß-globulin variation. Transferrin electrophoretic variants exist in wide array of species. Transferrin variants are widely distributed in fish, amphibians, reptiles, birds and mammals (Charlwood, 1963; Yamato *et al.*, 1983).

In humans, there are 18 electrophoretically detectable genetic variants (Kamboh and Ferrell, 1987). These variants were also screened for their variability by amino acid sequencing. Transferrins such as TfD, TfDchi, and TfB2 are a result of single amino-acid substitutions from the most common variant -TfC (Ekblom *et al.*,1983; Philips and Azari, 1971). TfD is a result of Asp-Gly mutation at position 277. TfDchi is due to His-Arg transition at position 300 and TfB2 due to Gly-Glu conversion. These variants are products of A to G, A to G and G to A mutations respectively in the second nucleotide of the three codons (Young, 1984).

Several workers indicated that these variants lie within the homologous sequence. But within the homologous sequence there are even more conserved regions. These regions within the two domains have a very high percentage of internal nucleotide homology (Beckman and Beckman, 1987; Delbruck and Henkel, 1979). These highly conserved parts were represented as A, B, and C blocks. Codons for tyrosine and histidine

residues are predominantely found in these extremely conserved regions (MacGillivray *et al.*, 1982). Tyrosine and histidine are known to be involved in iron binding (Fletcher and Huens, 1968). This may account for little divergence seen in these regions since they are critical for the survival of species. Hence, the regions elucidate the evolutionary constraint for divergence of transferrin. They also indicate that selection has been operating more strongly on some exons of transferrin than others (Karin and Mintz, 1981).

2.2.3. Biological Role of Transferrin

Transferrin is the principal plasma protein for the transport of iron. Its concentration correlates well with the total iron-binding capacity of serum (Tietz, 1987). It transports iron from absorption (e.g. intestine) and storage sites (e.g. liver spleen and bone marrow) to sites of iron utilization. Iron is utilized by all proliferating cells (Jamieson, 1973). The foetal cells also depend on transferrin for iron transport (Graham, 1975).

The most important function of transferrin, however, is its involvement in red blood cell synthesis (Windle *et al.*, 1963). It also affects cell division not only by limiting iron supply but also by acting as cell growth stimulant (Yang, 1984). It has received wider attention in cancer studies because of its involvement in cell proliferation.

This function of transferrin will be fulfilled after passing through various stages. The first stage involves binding of transferrin-Fe complex to specific transferrin receptors (Graham, 1975). Most of these receptors are found in placental cells, reticulocytes,

mammary tumour cells , B and T lymphoblastoid cells and chorioncarcinoma cells. Endocytosis of the iron complexed protein takes it to the lysozyme. Fusion of the protein with lysozyme is followed by liberation of the metal. The low pH of lysosome is known to contribute to the liberation of iron free transferrin back to the cytoplasm (Philips and Azari, 1971).

Transferrin is synthesized in the liver and to a small extent in the reticuloendothelial system, testes and ovaries (Tietz, 1987). The exact mechanism of its biosynthesis has been a point of discussion and debate for some time. Some workers suggested that both halves of the protein are synthesized from the same segment of DNA (Park *et al.*, 1985). These two fragments undergo fusion into a single polypeptide chain of double molecular weight. However, an alternative explanation was suggested. This second hypothesis was proposed by assuming a partial gene duplication and synthesis of a single polypeptide chain without any fusion process. Hence, this latter idea seems plausible in view of the evidence for duplication of transferrin genes during evolution. Similar situations exist where the light chains of antibodies (globulins) as well as non-heme iron carrying proteins such as ferrodoxins and rubredoxins are products of duplicated genes (Beckman and Beckman, 1987).

2.3. PROPERTIES OF PSEUDOCHOLINESTERASE

2.3.1. Chemical Nature of Pseudocholinesterase

Esterases are a group of hydrolytic enzymes that are known to hydrolyse a wide range of esters. Cholinesterases belong to this group as they are able to hydrolyse cholinesters. They are distinguished from other esterases by their sensitivity to eserine inhibition (Ord and Thompson, 1950).

Cholinesterases have a high molecular weight ranging between 2-12 x 10^6. Despite their high molecular weight, however, their substrates are in the small molecular weight range. This has raised the possibility that the enzymes may contain a number of active sites. These active sites are in turn composed of two sub-sites. The anionic site is for the attachment of cationic nitrogen atom of the substrate and the second site for hydrolysing the ester moiety (Cucuiano, 1988).

The presence of these sites accounts for strong affinity of cholinesterases for positively charged substrates. Several inhibitors of cholinesterase, e.g. physostigmine were found to be effective in their ionized state only (Goedde and Agarwal, 1978).

There are two important types of cholinesterases in the vertebrate body, namely, acetylcholine (E.C.3.1.1.7) and pseudocholinesterase (E.C.3.1.1.8). Acetylcholinesterase, also known as 'true' cholinesterase or acetylcholine hydrolase,

differs from pseudocholinesterase ('non specific', acyl-choline acyl hydrolase) in many respects.

Acetylcholinesterase is distributed in the grey matter of the central nervous system, in sympathetic ganglia, motor end plates and red blood cells (Augustinsson, 1958). Pseudocholinesterase is restricted to the liver, intestinal mucosa, pancreas, white matter of central nervous system and plasma (Cucuiano, 1988).

Acetylcholinesterase does not metabolize butyrate substrates and is inhibited by high concentration of its natural substrate -acetyl choline. Although pseudocholinesterase is active on acetyl choline, neither its natural substrate nor its function are known (Leibel, 1987). It is not affected by high concentrations of acetyl choline. The affinity of acetylcholinesterase to acetyl choline is 30 times greater than that of pseudocholinesterase (Sklar and Sonn, 1988). This could be due to the presence of more number of ionized sites in the former. Two anionic sites are present in acetylcholinesterase while pseudocholinesterase has only one (Leibel, 1987).

Nevertheless, both enzymes show some similar chemical characteristics. They can metabolize acetyl choline and other non-choline esters. They are also sensitive to eserine inhibition.

$$CH_3COOCH_2CH_2N^+(CH_3)_3 + H_2O \rightarrow CH_3COOH + HOCH_2CH_2N^+(CH_3)_3$$

Acetyl choline Choline

The mechanism of the hydrolytic process is not fully understood. But, the proposed mechanism is thought to involve a combination of the carboxyl group of cholinesters with a G-H group on the enzyme surface. The G group is an imidazole and this complex decomposes to free choline and the acyl-enzyme (Sklar and Sonn, 1988). The acyl enzyme then undergoes hydrolysis to restore the free enzyme.

2.3.2. Cholinesterase Isoenzymes

The occurrence of multiple cholinesterases were first reported on rats (Ord and Thompson, 1950). Such electrophoretically distinct serum esterases are now commonly found in primates and other vertebrates.

Serum esterases became widely known after the use of suxamethonium. Suxamethonium is a drug used to relax muscle during surgery (Weitkamp, 1985). Chemically, it resembles acetylcholine. In normal patients undergoing surgery this chemical is hydrolysed rapidly by pseudocholinesterase.

$$(CH_3)_3N^+CH_2.CH_2O.CO.CH_2.CH_2.COOCH_2.CH_2.CH_2.N^+(CH_3)_3$$

Suxamethonium (Succinyl dicholine)

However, in some, suxamethonium sensitive patients, this drug is not hydrolysed and as a result they undergo a prolonged apnoea after completion of the surgery. This effect was found to be due to a genetically determined less active variant of normal pseudocholinesterase. Family studies showed this to be true. Homozygous people for

the usual (normal) gene never experience apnoae (Cucuiano, 1988). People with homozygous atypical (unusual) gene have lower activity and this includes the suxamethonium sensitive individuals. They suffer from the side effects of the drug. An intermediate activity is found in people heterozygous for the usual and atypical variants (Yang, 1984).

Such studies revealed that pseudocholinesterase is a product of four alleles at single locus, the Eu, Ea, Ef and Es. The EuEu genotype produces normal phenotypes and other genotype combinations produce weak activity (Young, 1984). No activity is found in EsEs individuals and they are classified as homozygous for the silent gene. Such effects are seen when either the enzyme is absent or when a non-functional (defective) enzyme is produced (Roed, 1987).

Mutations on the active centres of this enzyme are responsible for the absence of activity. They result in an increased Km (Michaelis constant) diminishing the affinity of the enzyme for substrates and competitive inhibitors such as dibucaine and fluoride (Teitz, 1987). A higher degree of inhibition by these inhibitors indicates normal enzyme and vice versa.

3. MATERIALS AND METHODS

3.1. SITE SELECTION, SAMPLE SOURCE AND PREPARATION

Vervet monkeys were trapped from the Awash National Park (Gotu or Campsite 1) in June, 1995 (Figure 1). This site was selected for its easy access to blood sampling. The study was originally designed to collect blood samples from as many social groups of vervets as possible from the Awash National Park. However, the sampling site in the present study was confined to Campsite 1 where the G group had previously been sampled. Sampling on other sites was difficult mainly due to the encroachment of the nomadic people into the Awash National Park as a result of shortage of rain during the sampling period. Subsequent sampling attempts failed to materialise as there was no sufficient fund for covering later expenses. So, the study was limited to only one social group near the campsite. This group provided samples from which the objectives of the present study could still be satisfied. The results of this study group were also compared with data from the previous study in the same area. This was incorporated into the present study as it was considered to provide some additional data for extrapolating the results.

A total of 38 monkeys were trapped out of which 34 of them were taken for blood analysis. The trapping material was made of thin wires. The wire was meshed into 3 by 4 cm rectangular sizes. The trap was accompanied by a v-shaped metal bar. The monkeys were then attracted by corns or pieces of food spread on the ground within the trap. The monkeys will be trapped when the rope connected to the metal bar is pulled

down by a person concealed from the trap. The metal will keep the monkey inside by restricting its movement within the trap.

Individuals were then tranquillized by injecting Ketamin (Ketaset ™ HCl). The drug was given intramuscularly. The sites of injection were mostly on the femoral muscles. Blood samples were taken from the inguinal veins just after tranquillization. The blood was drawn into heparinized vacutainer tubes. These samples were centrifuged at 3000 rpm for 20 minutes in the Park. The serum part was isolated and transferred into cryogenic vials. The vials were stored and transported to the laboratory in Addis Ababa in liquid nitrogen. During this tranquillization stage other parameters determined were: sex, age and weight of individuals. Age determination was based on dental eruption and formula. These data were taken as it was believed that they will give some valuable information in future studies.

3.2. PREPARATION OF STOCK SOLUTIONS

3.2.1. Polyacrylamide Solution

Polyacrylamide stock solution was prepared by combining 87.6 g of acrylamide with 2.4 g of N N'-Methylene-bis-acrylamide. Distilled water was then added to make a final volume of 300 ml. This solution gives a 30% T and 2.67% C acrylamide: bis-acrylamide ratio.

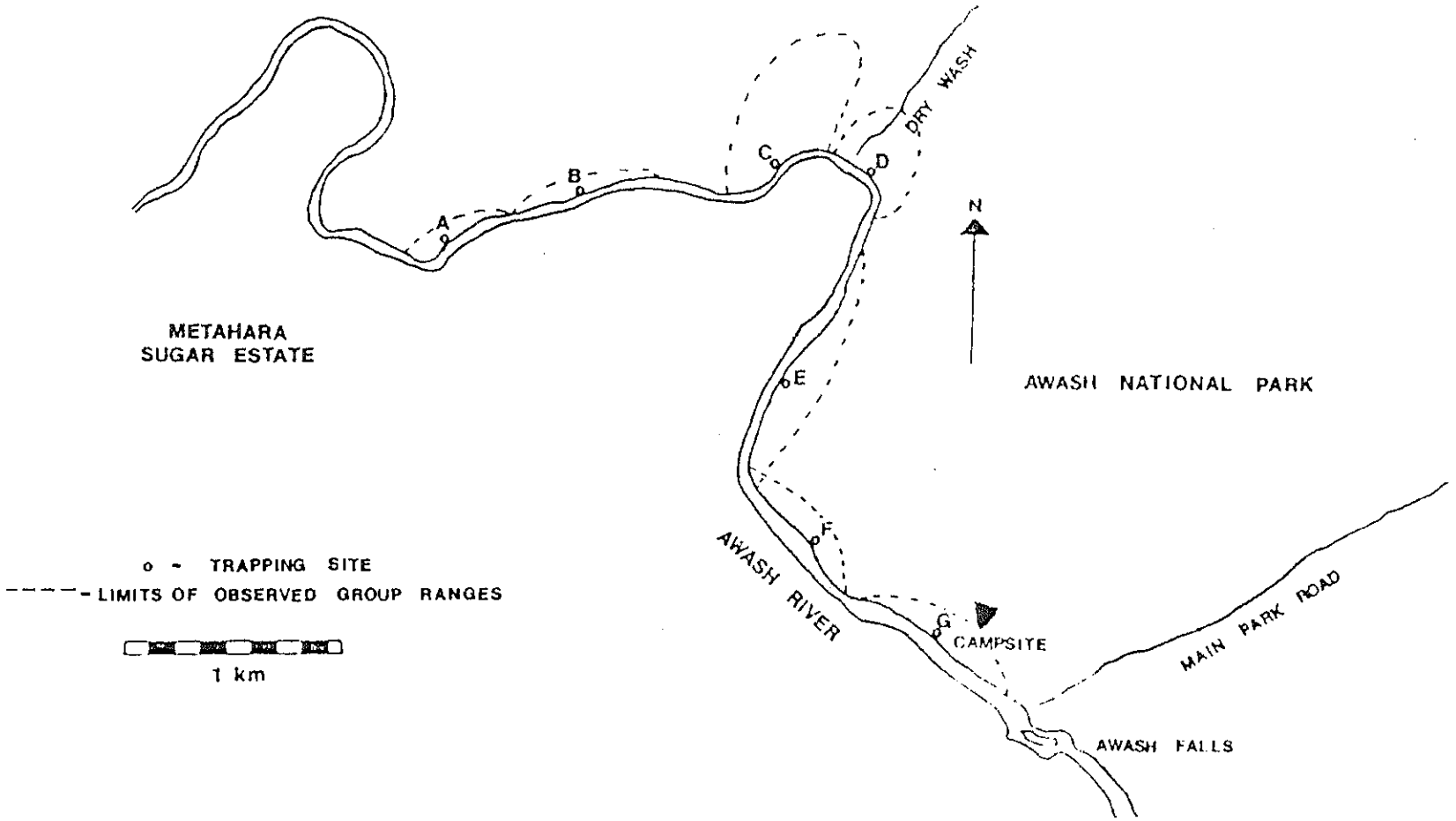

Fig. 1. Location of study site of the previous and present study groups (After Turdy, 1977). Arrow indicates the present study site. Letters stand for previous study social groups.

Where:

$$\%T = \frac{\text{gms acrylamide + bis-acrylmide}}{\text{Total volume}} \times 100$$

$$\%C = \frac{\text{gms Bis-acrylamide}}{\text{gms acrylamide + bis-acrylamide}} \times 100$$

3.3. BUFFER PREPARATION

The electrophoretic buffer system used was that of Jolly and Allen (1965) .

3.3.1. Stacking Buffer

A tris (hydroxymethyl) amino methane-HCl stacking buffer was used. 19.85 g of sucrose (0.58M) and 0.73 g of tris (0.06M) were dissolved in 1000 ml of distilled water. The buffer system was adjusted with HCl to pH 7.3.

3.3.2. Separating Buffer

The separating buffer used was Tris-HCl at pH 7.2.

This was prepared by dissolving 13.3 g of tris to 1000 ml of distilled water which produces a 0.11M of tris.

3.3.3. Electrode Buffer

The electrode buffer was made up of Tris- glycine. This was prepared by combining 0.96 g (0.008M) of Tris with 2.85 g of glycine (0.038M) and dissolving it in 1000 ml of distilled water. This solution has a pH of 8.5.

3.3.4. Loading Buffer

Loading buffer was prepared by mixing bromophenol with a defined amount of stacking buffer. This was done by mixing 1 ml of stacking buffer to .001 μg of bromophenol.

3.4. GEL PREPARATION

Various gel concentrations of stacking and separating gels were used for transferrin and pseudocholinesterase. A 3% stacking and a 7.5% separating gel concentrations were used for transferrin. The same stacking gel concentration but an 8% separating gel was used for pseudocholinesterase. These gel concentrations were achieved following the gel formula indicated in Tables 2 and 3.

3.5. RUNNING CONDITIONS

3.5.1. Electrophoresis Apparatus

The electrophoretic apparatus for power supply used was a 1000/500 Bio-rad model. This power supply provides both auto and manual control systems for presetting the desired amount of current and voltage for the gel. Vertical slab gel apparatus was used for running gels. The slab can be used to simultaneously run up to four gels.

Table 2. Gel formula for preparing resolving gel.

CONTENT	7.5%	8%
SEPARATING BUFFER	3.75 ml	3.75 ml
POLYACRYLAMIDE	3.75 ml	4.0 ml
DISTILLED WATER	7.5 ml	7.25 ml
10% APS[1]	100 μl	100 μl
TEMED[2]	10 μl	10 μl
TOTAL	15 ml*	15 ml

* The contents were doubled for big gels.

[1] Ammonium persulphate $(NH_4)_2S_2O_8$

[2] N,N,N',N'-tetramethylethylenediamine $(C_6H_{16}N_2)$

3.5.2. Sample Loading

Samples were thawed in cold ice or refrigerator until liquid suspension was obtained. A 5 μl of undiluted serum sample was taken using Hamilton syringe. This was mixed with an equal amount of protein loading buffer. The Hamilton syringe was thoroughly washed with running buffer after each loading.

Table 3. Gel formula for preparing stacking gel.

CONTENTS	3% GEL
STACKING BUFFER	2.0 ml
POLYACRYLAMIDE	.8 ml
DISTILLED WATER	5.2 ml
10% APS[1]	100 μl
TEMED[2]	10 μl
TOTAL	8 ml*

* The contents were doubled for big gels.

[1] Ammonium persulphate $(NH_4)_2S_2O_8$

[2] N,N,N',N'-tetramethylethylenediamine $(C_6H_{16}N_2)$

Human serum was loaded on a separate well along with monkey serum samples to allow easy comparison of bands. The gels were kept in cold room (5°C) during electrophoresis. The samples were subjected to electrophoresis at 400 volts in a constant current of 18 ma/gel for stacking and 21 ma/gel for separating gel. This running condition lasted for 2.5 hours in which case the dye (bromophenol) has migrated near the end of the gel. This running condition was employed for both transferrin and pseudocholinesterase. But, a longer running time (3 hours) was used for pseudocholinesterase in which case the indicator dye diffuses out of the gel.

3.6. STAINING

The gels for transferrin were stained with comassie blue. The comassie blue stain contained the following composition:

Comassie blue	2.5 g
95% ethanol	800 ml
Acetic acid	200 ml
Distilled water	1000 ml

The gel for transferrin detection was kept for 5 hours in this solution.

3.7. DESTAINING

The gels were destained for several days (5 to 7) until a relatively transparent gel was obtained. The destainer solution was made up of the following recipe:

Methanol	250 ml
Glacial acetic acid	500 ml
Distilled H_2O	4250 ml

3.8. BAND IDENTIFICATION

Transferrin bands were detected visually and corroborated densitometrically on the basis of their position and intensity (Figure 2).

Pseudocholinesterase bands were detected by incubating the gel at ambient temperature in the following reaction mixture:

50 ml of 0.2M Tris-HCl at pH 7.0

3 ml of alpha naphthyl acetate solution as substrate and

0.05 g of Fast Red TR salt.

The above reaction mixture was taken from the following stock solutions. The substrate solution was prepared from 1 g of alpha naphthyl acetate dissolved first in 50 ml of acetone. Distilled water was then added when all the substrate was fully dissolved to make a final volume of 100 ml. The defined concentration of 0.2M of tris was made by adding 2.422 g of tris and the solution was adjusted with HCl to pH 7.0. The gels were incubated in this solution for 30 minutes.

Vervet pseudocholinesterase bands were then identified after incubation with substrate. This was mainly done by comparing it with the human serum. This enzyme is a highly intense band which lies within the same electrophoretic mobility zone as the vervet's.

This allowed the differentiation of pseudocholinesterase bands from other non-specific activities.

3.9. CALCULATION OF GENETIC PARAMETERS

3.9.1. Number of Genotypes

The number of genotypes is given as: $n^2 + n / 2$

where n is the number of alleles.

3.9.2. Total Heterozygosity

Total heterozygosity is given as:

Ht = 1 - total homozygosity

The total homozygosity is calculated as:

Homozygosity = $q_1^2 + q_2^2 + ... q_n^2$

where n is for the n^{th} allele

3.9.3. Fixation Index

Wright's (1978) F statistics was used.

$$F = 1 - \frac{\text{observed heterozygosity}}{\text{expected heterozygosity}}$$

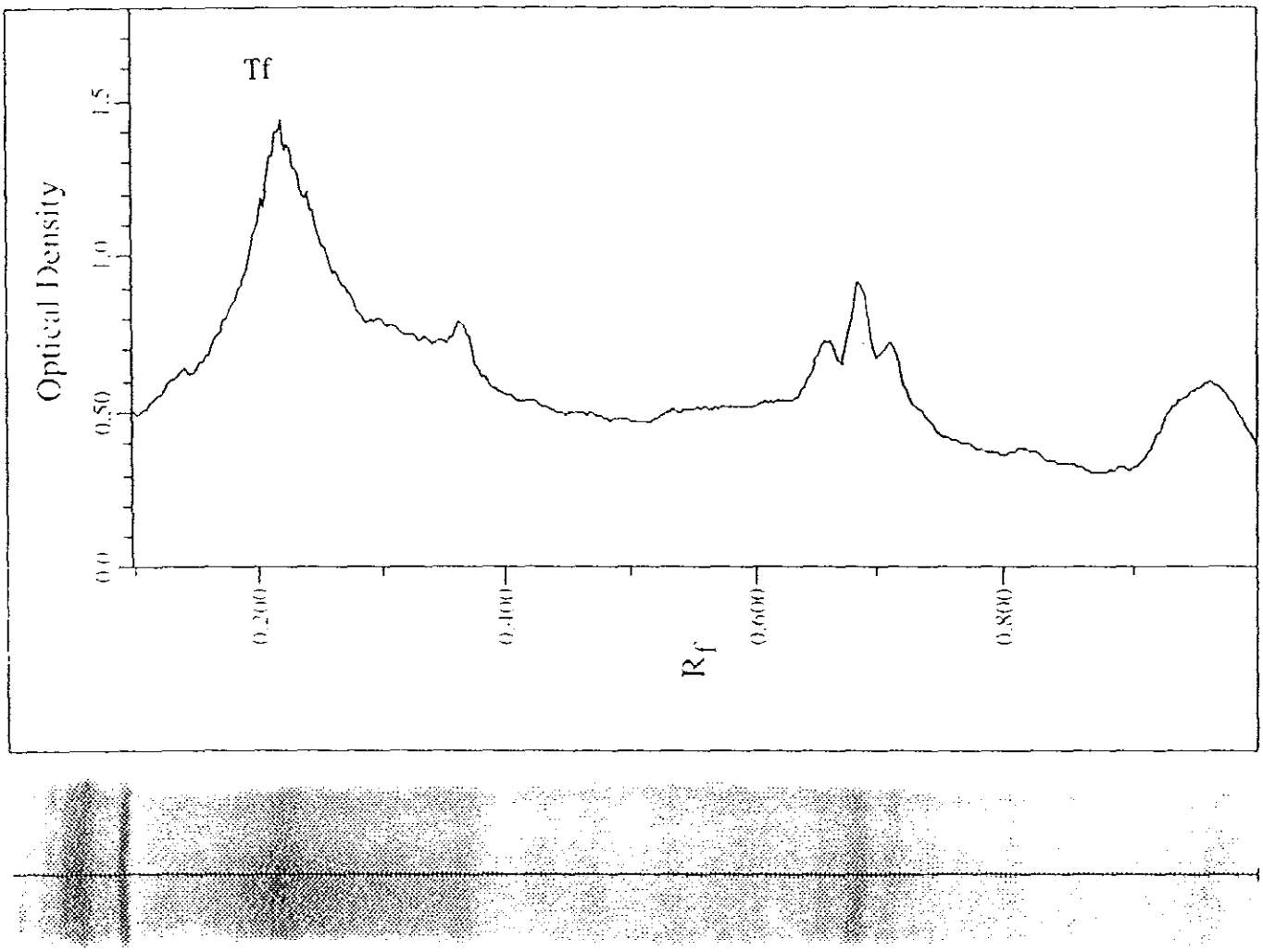

Fig. 2. Densitometric scanning of transferrin. High peak near the origin indicates location of transferrin. Tf is for transferrin and Rf indicates migration distance from origin.

3.9.4. Genetic Identity (I) and Genetic Distance (D)

Nei's (1972) measure of genetic distance was used.

$$I = \frac{\Sigma aibi}{(\Sigma ai^2\ \Sigma bi^2)^{1/2}}$$

where:

ai = frequency of allele i in population a

bi = frequency of allele i in population b

$$D = - \ln I$$

Where:

I is the genetic identity.

4. RESULTS

4.1. TRANSFERRIN

Only the common transferrin phenotype was found in all individuals (n=34). Other variants of transferrin were not found in this study population (Figure 3).

4.2. PSEUDOCHOLINESTERASE

In this sample, four phenotypes of pseudocholinesterase and different levels of activity were found. This included a single cathodal band with strong activity; a single anodal band with strong activity; a two-band activity and finally no band activity (Figure 4).

Such phenotypes can be produced if the activity is controlled by three alleles at a single loci. Hence, the alleles are denoted as: $Psch^1$ for the silent allele producing no activity, $Psch^2$ for the cathodal band and $Psch^3$ for the anodal band activity. This model will predict the existence of the following six genotypes and their respective phenotypes:

$Psch^1Psch^1$ -silent gene producing no activity

$Psch^2Psch^2$ -cathodal strong band

$Psch^3Psch^3$ -anodal strong band

$Psch^1Psch^2$ -cathodal weak band

$Psch^1Psch^3$ -anodal weak band

$Psch^2Psch^3$ -Two bands (one cathodal and one anodal)

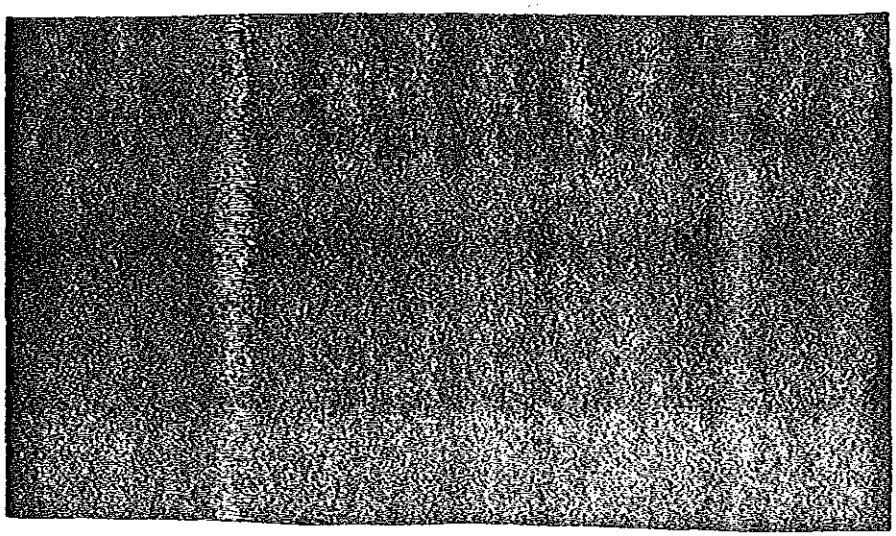

Fig. 4. Activity and mobility polymorphism in pseudocholinesterase of vervet monkeys. Numbers and signs are as defined in Figure 3. Arrow indicates position of pseudocholinesterase (see appendix 2 to interpret bands).

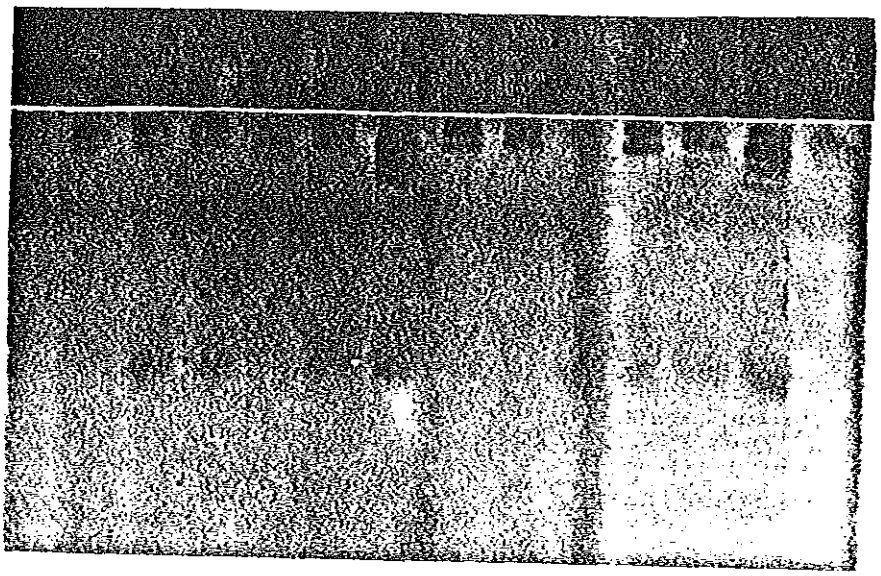

Fig. 4. continued

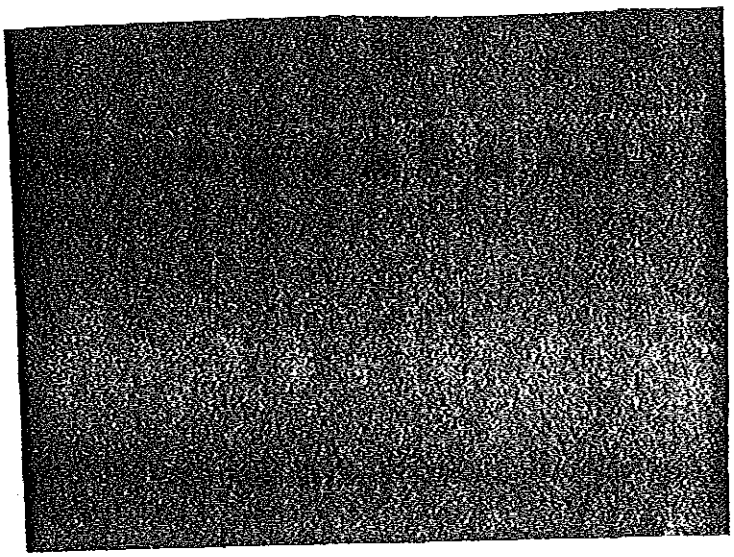

Fig. 4. continued

The frequency of each allele and its distribution were calculated based on the above hypothesis (Table 4). The X^2 test for goodness-of-fit showed that the population is in Hardy-Weinberg-Equilibrium ($X^2_3 = 4.904$, $p > .05$).

4.2.1. Number of Genotypes

The expected genotypes were found to be six assuming three alleles of pseudocholinesterase. In this study, only four phenotypes of pseudocholinesterase were found.

4.2.2. Fixation Index

The deviations between the observed and expected heterozygosity were calculated on the basis of allelic frequency of pseudocholinesterase (Table 5).

4.2.3. Genetic Identity and Genetic Distance

The genetic identity and genetic distance were calculated based on the data on pseudocholinesterase locus (Table 6).

Table 4. Phenotype distribution and allele frequency of pseudocholinesterase among sampled vervets.

	Phenotype*							
	1-1	1-2	1-3	2-2	2-3	3-3	Total	Allele Frequency
No. of individuals	3	0	0	5	11	15	34	-
No. of Pch^1 alleles	6	0	0	0	0	0	6	.0883
No. of $Psch^2$ alleles	0	0	0	10	11	0	21	.3088
No. of $Psch^3$ alleles	0	0	0	0	11	30	41	.6029
No. of alleles	6	0	0	10	22	30	68	-

* Psch was omitted and only the numbers are shown representing the phenotype composition.

Table 5. Heterozygosity and F values among the present and past populations.

Social Group	Observed Heterozygosity	Expected Heterozygosity	F-Values
A	.67	.54	-0.22
B	**.39**	**.56**	**0.32**
C	.27	.35	0.23
D	.63	.64	0.02
E	.60	.45	-0.21
F	.36	.39	0.08
G	.44	.55	0.17
P*	.41	.51	0.19

P* stands for the present population and other letters are as indicated in Figure 1.

Table 6. Computed I (above diagonal) and D values (below diagonal) for all social groups.

	P	A	B	C	D	E	F	G
P	.0000	.4780	.4796	.2515	.6905	.4276	.4689	.4306
A	.7381	.0000	1.000	.9015	.9617	.9788	.8823	.9978
B	.7348	.0000	.0000	.9338	.9617	.9600	.8398	.9978
C	1.380	.1037	.0685	.0000	.8200	.8386	.6004	.9309
D	.3703	.0390	.0390	.1985	.0000	.9381	.8526	.9439
E	.8496	.0214	.0408	.1760	.0639	.0000	.9454	.9777
F	.7574	.1252	.1746	.5101	.1595	.0561	.0000	.8501
G	.8426	.0022	.0022	.0716	.0577	.0226	.1624	.0000

5. DISCUSSION

Genetic variation at the molecular level has been found in the majority of organisms studied so far. This has raised the question of how this variation is maintained in natural populations. Two theories have been put forward to explain this observation. One school proposes that the majority of allelic variation is maintained by random genetic drift as proposed by Kimura (Roed, 1987). The other school as proposed by Lewontin, asserts that balancing selection is responsible for maintaining the allelic polymorphism (Roed, 1987). However, the issue is still unresolved.

Genetically determined transferrin polymorphism has been widely seen in many vertebrate species. This has aroused the possibility that selection may be operating to maintain this polymorphism. It was hypothesized that some form of balanced selection is working on the transferrin locus. In mice and man, some allelic variants of transferrin are associated with reproductive fitness (Beckmann and Beckmann, 1987). TfC2 is associated with spontaneous abortion and premature birth in humans (Yang, 1984). In cows and ruminants, some alleles were also known to influence production of milk, fattening and fertility (Rozkhov and Rozkhova, 1984). Roed (1987) found high correlation between some transferrin alleles and body weight in reindeer. Body weight is an important determinant of fitness in reindeer. Female's body weight influences the calf's birth weight and its future survival and males are affected during fighting for reproductive access. Selective forces have also been implicated in view of the physiological importance of transferrin. It has bacteriostatic effect and thus deprives iron which is essential for the growth of pathogens. It is conceivable that transferrin

allelic variants could be positively selected if they result in high affinity for iron (Kamboh and Ferrell, 1987). This is because allelic variants with higher affinity for iron will efficiently deplete iron essential for the growth of pathogenic organisms. Besides, segregation distortion of transferrin alleles was observed in man and deer mice (Beckmann and Beckmann, 1987). It was found that only some alleles of paternal transferrin could penetrate the egg. Hence, these studies seem to indicate the non random distribution of alleles and their differential contribution to survival. Such situation will of course lead to maintenance of polymorphism.

This study shows that the transferrin variant is absent (0%) in the present population. This is the lowest percentage reported in vervet monkeys. Barnicot and Hewlett-Emmett (1971) reported the variant transferrin in 1 out of 25 animals. Moreover, in the 1973 study in the Awash National Park vervets, the proportion of the variant type was the highest (5.9%) when the entire population was considered (Turdy, 1977). However, this proportion was distributed in only two study sites -E and F (Figure 1). The other groups didn't show the transferrin variant. The present population was sampled from or near the G site of 1973 study populations. The G group had no transferrin polymorphism but was very adjacent to E and F.

If one considers the behaviour of vervet monkeys, it becomes clear that migration has a vital role in gene flow. Vervets have a social behaviour that facilitates gene exchange among neighbouring troops. Migration is only possible through transfer of young males and females remain in their own group (Fedigan and Fedigan, 1988). Such exchange occurs more among social groups who have a previous history of exchange. Thus

migration becomes readily possible between familiar social groups. Data on the distribution of transferrin variant on sex and age groups are not available from the previous study. Assuming its distribution to be uniform with age and sex, the possibility exists that the variant can be represented in the present population. The effect of migration, however, may not be apparent in view of the highly restricted distribution of the protein in the Awash National Park. A longer period is, of course, needed for rare variant alleles to spread and be represented in other social groups. Through time, the variant could spread to all social groups. Maturity in vervets takes about 3 years and it can be seen that for the last 23 years about 7 generations of juveniles of vervet monkeys have passed. This must have given enough chance for the spread of the rare type from E and/or F to the G group. The G group is where the present sample was taken (see Fig. 1). If the migration rate was high, it could have been detected in the present population.

The migration rate per annum is unknown and it is difficult to assess the contributions of these generations to gene flow. The absence of the rare type could rather be due to the low rate of migration rather than due to a complete absence of migration. Migration does occur among these social groups as can be seen from Table 5. The calculated F values for population B (F= 0.32) is the highest being followed by C (F= 0.23). The present study population has an F value of 0.19 and is followed by G (F= 0.17). The F values indicate that the present population has a low migration rate (high inbreeding) when compared to other populations (A,D,E,F). Particularly, the observed heterozygosity is high for populations A, D and E. As a result, there is a high migration or introgression rate in these populations. Taking into account these

observations, it appears that individuals migrating from E or F are at a selective disadvantage. The juvenile migrants containing the rare type will not be having a chance to produce offsprings representing their genes. Hence, the gene pool will not be composed of the variant transferrin. A plausible explanation for such a selective disadvantage will be an epistatic gene interaction between the transferrin and pseudocholinesterase loci. The restricted distribution of the rare type in E and F gives a clue. The third allele of pseudocholineseterase and rare transferrin allele seem to have some kind of antagonistic interaction. In populations E and F where the variant transferrin is found, the third allele of pseudocholinesterase is absent. Whereas in the present population, where the third allele is dominant, the rare type is not represented. Moreover, E, F and C have the least number of genotypes (3); A,B,G and the present population have a medium number of genotypes (4) and only population D shows all phenotypes (6). The smaller number of genotypes is a result of the absence of the third allele.

Therefore, it appears that individuals having both protein variants are negatively selected. The presence of the third allele may affect the three dimensional conformation of the rare transferrin variant. Since the structure of proteins is critical for proper functioning, such interaction will result in abnormal proteins. Thus, both factors may be responsible for lack of the rare variant in the present population.

However, the effect of sampling size on the present observation is also important to consider. The sampled population (n=38) is slightly greater than 50% of the entire

population (n=70) and hence there was a possibility to obtain the variant phenotype if a bigger sample size was taken.

The situation regarding the pseudocholinesterase polymorphism is more complicated as its physiological importance is unknown. As a result, there is no literature to account for this polymorphism in terms of its selective value. Even though the population is in Hardy-Weinberg Equilibrium for the three alleles, the frequency has changed greatly since the previous study (Table 7). It can be seen that the situation for gene frequency has shown a significant shift. The average frequency for allele 3 (anodal) was the lowest (.059) in the previous population but it has the highest frequency (.6029) in the present population. Although, alleles 1 and 2 are found in both the past and present population, their frequency was higher in the former.

Such a rapid shift of allelic frequency with short period (1973-1995) in nature is hard to explain. It may be that the gel medium used in the present study has a bearing on band interpretation. Although both gels are quite remarkable for their high resolution of serum proteins, polyacrylamide gel has an additional advantage. Its pore size can be manipulated as 'desired' in such a way that it matches the three dimensional globular size of a given protein. During the initial stage of the experiment for optimization of gel concentration a critical problem was faced . Very few activities were obtained when 5% acrylamide gels were used. However, when the gel concentration was increased from 7% to 8% more bands appeared on the gel on the same individuals. The bands on lower concentration of gel were highly diffused and difficult to classify into various activities. Therefore, the sieving effect of acrylamide gels contributed to concentrate the

bands. By the same analogy, the starch gel used in the previous study may have influenced and there by contributed to the low proportion of 3-3 genotypes and increased the value of silent genes and genotypes. This remains to be investigated since to what extent this effect is due to the difference in physical properties is unknown.

At any rate, in the absence of direct evidence the present result must be viewed from another aspect. Chudoba and Jablonson (1978) showed a rapid reversal of transferrin allele frequency under artificial selection pressure in their follow up study from 1960-1974.

Under natural conditions, such rapid changes in allele frequency are not reported. Therefore, a more plausible explanation would be that direct selection pressure on the pseudocholinesterase locus may not be responsible. This would imply that the allelic frequency shift may be correlated with alleles affecting the fitness of individuals in the population. Since the importance of pseudocholinesterase has never been demonstrated, it is more likely that this shift is a result of selection at a linked loci controlling other fitness related characters. Thus, the Hitch-Hiking effect may have contributed to this situation. The breeding system of vervets is also important to consider in this regard. During stress (either from habitat decline, resource limit or high predation), vervets use a different strategy. They split into smaller group sizes and forage independently. Such splitting strategy is a stochastic process. Females non-randomly split into their matrilineal lines. As a result certain degree of kinship breeding will prevail and result in a shift of allelic frequency. The Kenyan vervet monkey shows no polymorphism of pseudocholinesterase and they are known to have faced splitting (Dracopoli *et al.*,

1983). It is possible that the present population may be founded by such splitted groups. It is also probable that migration of individuals from other groups may result in increasing the frequency of allele 3. But it is not known whether this population has undergone such splitting in the past 20 years. The population is located along the Awash river and the Park has been well gazetted for a long time. So the decline in food resource and human intervention in that population is unlikely. However, it should be remembered that the 1974 and 1985 drought periods may have affected the population demography in relation to other species competing for a similar ecological demand. But, the exact effects of these periods are hardly possible to quantify.

Therefore, it appears that the shift in allelic frequency can not be attributed to a single evolutionary force.

Table 7. Distribution of pseudocholinesterase phenotypes among social groups of vervet monkeys

Group	No. of Animals	Phenotypes						Allele Frequency			Hardy Weinberg X^2 Values
		1-1	1-2	1-3	2-2	2-3	3-3	1	2	3	
A	9	2	5	0	1	1	0	0.500	0.444	0.056	1.887
B	18	6	4	3	5	0	0	0.527	0.388	0.083	6.275
C	11	7	3	0	1	0	0	0.772	0.227	0.000	0.564
D	19	3	6	3	3	3	1	0.394	0.394	0.210	0.474
E	25	4	15	0	6	0	0	0.460	0.540	0.000	1.077
F	11	1	4	0	6	0	0	0.272	0.727	0.000	0.082
G	25	9	9	0	5	2	0	0.540	0.420	0.040	3.715
P	34	3	0	0	5	11	15	0.0883	0.3088	0.6029	4.09

6. CONCLUSION

The present study has shown that transferrin (Tf) is monomorphic while the pseudocholinesterase (Psch) locus is polymorphic. Tf monomorphism in the present population may be due to the low migration rate from social groups containing the rare phenotype. The migration rate has been found to be low as indicated from the high F-value of the present study group. Added to this effect is the possible interaction between the Tf and Psch loci. It is interesting to see that both the past and present social groups show a similar pattern with respect to allelic distribution. Whenever the third allele of Psch is present or dominant, the rare variant of Tf appears to be absent. This may imply that there is an antagonistic interaction between the two allele which disfavour their mixing as a result of migration. So such individuals that do enhance such situation will be negatively selected. This will further the elimination of individuals containing the rare type. However, the effect of optimum sampling size may have also contributed to such observation. In the previous study, although the population size was small, the majority of the individuals were sampled which may have increased the chance for detecting the rare phenotypes. There is a possibility that the other 50% unsampled individuals from the present study may contain the variant.

Regarding the Psch locus, this study has shown that the polymorphism is still maintained and are in Hardy-Weinberg Equilibrium. However, this study has also shown that the allelic frequency in natural populations are not static in time. The frequency of alleles has greatly changed and shifted towards the third allele. This rapid shift in allelic composition may be a result of group fission as result of the two drought

periods (in 1974 and 1985) which had affected Ethiopia. Hence, the present social group may simply be a result of splited groups. Such splitting behaviour in vervets is a stochastic process which results in a non-random distribution of alleles. So, it appears that the present social group may not be a direct descendent of the G social group. The other explanation may be the Hitch-Hiking effect. The third allele of Psch may be linked to a selectively advantageous allele. This will favour its dominant frequency distribution in the preset social group. Nevertheless, the results of this study ought to cautiously be interpreted as the methods employed for the present and previous studies are different. Such methodological difference may have influenced band interpretation.

7. RECOMMENDATION

Based on the allelic distribution of the present study, the following are recommended:

1. The present study shows that sites for conservation of vervet monkeys are A, D or E since they contain the highest observed heterozygosity (.67, .63, .60 respectively). The higher the heterozygosity the higher the magnitude of gene diversity. Site C should be included as a conservation site as it has the highest D value (D= 1.30). It is the furthest in its genetic composition and its gene pool must be included in future management plans.

2. The entire population be screened to see the variant transferrin in other groups and also to study the polymorphism of pseudocholinesterase.

3. It is also important to use other methods of determining pseudocholinesterase activity such as dibucaine number rather than subjectively evaluate activities visually.

4. Inhibition tests be performed to characterize the pseudocholinesterase variation since the substrate used is non specifically metabolized by other esterases.

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9. APPENDIX

Appendix 1. Age, sex and weight of vervet monkeys trapped at Camp site 1.

Vervet code no.	Sex	Weight in Kg	Age
1	M	2.1	Sub-adult
2	M	3.1	Adult
3	M	3.5	"
4	M	1.15	Young
5	F	1.4	"
6	M	1.75	Sub-adult
7	F	1.2	Young
8	M	2.5	Sub-adult
9	M	2.8	"
10	F	1.45	"
11	M	4.5	Adult
12	M	2.0	Sub-adult
13	F	3.2	Adult
14	M	-	Infant
15	F	1.8	Sub-adult
16	M	1.4	Young
17	M	1.5	"
18	M	1.4	"
19	M	4.0	Adult
20	M	4.4	"
21	M	3.55	"
22	F	2.5	"
23	M	-	Infant
24	F	2.5	Adult
25	M	-	Infant
26	F	2.65	Adult
27	F	1.0	Young
28	F	1.7	Sub-adult
29	F	1.65	"
30	F	2.9	Adult
31	M	-	Infant
32	M	4.35	Adult
33	F	1.3	Young
34	M	2.6	Sub-adult
35	M	4.9	Adult
36	F	1.45	Sub-adult
37	F	2.9	Adult
38	F	3.1	"

- Indicates infant monkeys whose weight and blood sample could not be taken.

Appendix 2. Formulation of the genotypes of individual vervets for pseudocholinesterase activity polymorphism.

Code #	01	02	03	04	05	06	07	08
Genotype	2-3	3-3	2-2	2-2	2-2	2-3	3-3	2-3

Code #	9	10	11	12	13	15	16
Genotype	3-3	3-3	2-3	1-1	2-2	2-3	2-3

Code #	17	18	19	20	21	22
Genotype	3-3	3-3	3-3	2-3	3-3	2-2

Code #	24	26	27	28	29	30
Genotype	2-3	3-3	3-3	1-1	2-3	2-3

Code #	32	33	34	35	36	37	38
Genotype	2-3	3-3	1-1	3-3	3-3	3-3	2-3

Note: The symbol Psch was omitted for convenience.

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