# 2. MATERIALS AND METHODS

## 2.1 Materials

2.1.1 The racial groups belonging to different ethnic population of North-Bengal.

The four principal groups belonging to different ethnic population of North Bengal were investigated cytogenetically for chromosomal variations within and between the groups. These groups consist of Bengalis, the Plain Tribes, the Nepalis and the Hill Tribes. On the basis of Linguistics, Ecology and Origin, the groups investigated may be distinguished as follows.

2.1.2 Basis of classification of different groups

Linguistics : Linguistically the four groups may be divided into two main classes, Bengali speaking and the Nepali speaking. The Bengalis and the Plain Tribes generally speak Bengali, whereas the Nepalis and the Hill Tribes invariably speak Nepali.

Ecology : Ecologically, the Bengali speaking groups considered in the present study are permanent residents of the plains of North Bengal whereas the Nepali speaking are natives of the Darjeeling District, dwelling at altitudes of 2500 ft (MSL) to 8500 ft (MSL).

Origin : Racially, the four groups studied here are the descendents of the Mongolo-Dravadians, Dravadians, aryans and the Mongoloids. The details of the racial origin with characteristic features of the different groups are seperately dealt with in the following text.

2.1.3 Characteristic features of different groups and sub-groups.

2.1.3.1 Bengalis : The Bengalis of North Bengal are descendants of Mongolo-Dravedians, with a blend of Indo-Aryan blood in the higher groups. They are marked by their relatively long heads (dolichocephalic), a straight finely cut nose, a long symmetrically narrow face, a well developed forehead, regular features and a high facial angle. The complexion is normally light transparent brown or "Wheat Coloured". It is one of the most distinctive type in India and its members can be recognised at a glance.

This group has been divided into (a) Brahmins, (b) Kshatriyas and Vaishnus, and (c) the Sudras on the basis of their social customs, religious beliefs and occupation. For exact identification of the group to which the individuals belonged different titles of the subjects were used as the criteria (Risley 1891). However, in Bengalis, a few titles like Roy, Sarker, Pal, Nandi, Neogi and Chowdhury were encountered any one of which might represent any one of the three classes. In such cases each individual was interviewed personally at the time of blood collection and the class which he/she represented was ascertained and placed accordingly.

(a) Brahmins are those who teach and practice religion and those who wear the sacred thread around their necks. The following titles were placed in this group - Lahiri, Misra, Gangulí, Bakshi, Bhattacharya, Chatterjee, Mukherjee and Chakravarty (Plate 1).

(b) Kshatriyas and Vaishnus are the military Chieftans and landlords, the writers and the traders. The titles of Mitra, Pal, Neogi, Chowdhury, Nandi, Roy, Ghosh, Guha, Ghatak, Sarkar, Basu and Sen were placed in this group (Plate 2).

(c) Sudras are the occupational class who by profession are barbers, washermen, shoemakers, smiths, weavers and those who deal in oils, salt and leather works. The titles encountered were Saha, Seal, Halder, Karmakar, Dhasker,and Mandol (Plate 3).

2.1.3.2 Plain Tribes : The Plain Tribes are the oldest of the Indian races having their origin in the Dravadians. The most characteristic representative of this group studied here is the Santhal followed by the Mundas and Oraons. In a typical specimen the stature is short or below average; complexion very dark, hair plentiful with a tendency to curl, eyes dark, head long, nose very broad, sometimes depressed at the root, but not so as to make the face appear flat, large mouth provided with thick and sometimes protruding lips.

(a) Santhals have been placed under Proto-Australoid or Pre-Dravadian stock. The traditional occupation of this group is hunting, fishing and cultivation. However, in North Bengal, majority of the Santhals are attached to the tea plantations as labourers, and some are in agriculture. Titles of Murmu, Soren, Sankh, Kora and Hansda were encountered (Plate 4).

(b) The name Munda is of Sanskrit origin meaning headman of a village. It is a large Dravadian tribe of Chota Nagpur, closely akin to the Santhals (Roy 1912). At present, they are mostly absorbed in tea plantations. They have title of Munda (Plate 5).

(c) The traditions of the Oraons people point to the Deccan as their original home (Roy 1915), and they are closely associated with the Mundas. They have titles of Ekka, Lakra (Plate 6).

(d) The Rajbansis also known as "Koch" and in the present study placed in the Plain Tribes, are the original inhabitants of Cooch Behar and its neighbouring area (Risley 1891). According to Risley (1891) and Dalton (1872), the Rajbansis have originated from Dravadian stock with suspected admixture of Mongolian blood. They have flat square faces, eyes black and oblique, hair black and straight, in some curling, nose flat and short; cheek bones prominent, beard and whisker rather deficient, colour of the skin in most cases is black. They are mainly agriculturists and they have titles of Kalburman, Burman, Roy, Koch and Mech (Plate 7).

2.1.3.3 Nepalis : The Nepalis of North Bengal mainly residing in the hills of Darjeeling District, North Bengal may be classified as (a) Brahmins, Chettris (b) Newars (c) Kiranti, Tamangs, Mogars, Gurungs and (d) the Sudras.

(a) Brahmins are those who preach and practice religion and those who wear the sacred thread around their necks. Chettris are the warrior class. This group is the descendant of the Aryan stock. Titles of Sharma, Poudel, Adhikari and Chettri were encountered (Plate 8).

(b) Newars are the trading class of Nepal. They are believed to be an admixture of Aryo-Mongolian blood (Histe 1930). The titles encountered were Pradhans and Shrestha (Plate 9).

(c) Kiranti, Magars, Gurungs and Tamangs are the typical representatives of the Mongoloid stock. They have broad heads, complexion fair/dark with a yellowish tinge, hair on face scanty; stature small or below average, nose fine to broad, face characteristically flat and eyelids oblique. The Gurungs and the Tamangs screened had titles of Gurung and Mokten whereas in Kiranti titles of Rai, Limboo, Subba and in the Magars titles of Thapa and Rana were encountered (Plates 10, 11,12).

(d) The Sudras are the occupational class known as Kami (Black Smiths), Darjee or Damai (Tailor) and Sarki (Cobbler) in Nepali. Their original home land is Nepal, now well settled in Darjeeling. They are of Aryan origin. Their titles are Sarki, Darjee, Viswakarma, Nasaily and Baraily (Plate 13).

2.1.3.4 Hill Tribes : The Hill Tribes studied in the present investigation inhabit the high altitude belts of Darjeeling District. The sub-groups considered here are the (a) Lepchas (b) the Sherpas and the (c) Bhutias. All these sub-groups have their origin from the Mongolian stock. According to their linguistic position the Hill Tribes belong to the Tibeto-Himalayan group of Tibeto-Chinese family, but now they speak mostly in Nepali (Das et al.1966).

(a) Lepchas are the earlier inhabitants of Sikkim who dwelt there before the first Tibetians arrived from Eastern Tibet and in their masterful way took over control of the country. Gradually they migrated and now they are mainly concentrated in the District of Darjeeling. They are typical Mongoloids with short stature and fair complexion. They have titles of Lepcha (Plate 14).

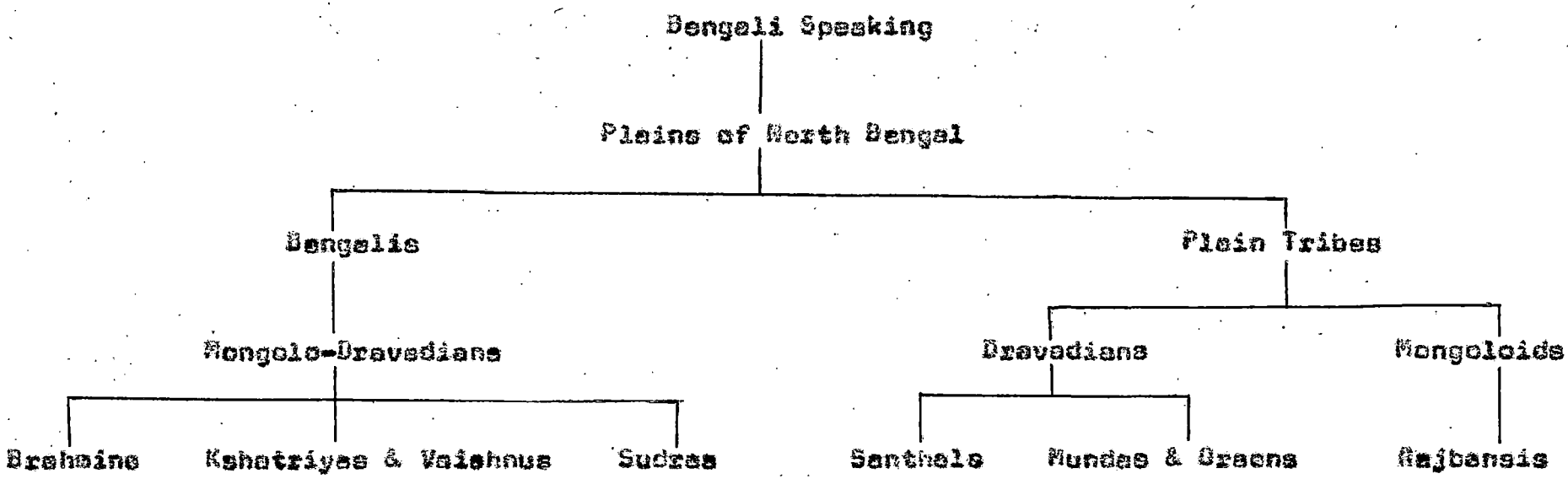
(b) Sherpas are typical mongoloids and are the mountain race. Sherpas are the porter class and are very hardy. They can be seen in large numbers in Darjeeling District. They use the title of Sherpa (Plate 15).

(c) Bhutias are also mongoloids. The people of Sikkim were known as Dan-jong-pa i.e. people of Dan-jong, the Tibetian name for Sikkim. Europeans and Indians styled them Bhutias. The term is applied to all the Tibetian race being derived from 'Bhot', the Indian name for Tibet. They are mainly agriculturist traders and graziers. A large number of Bhutias are now well settled in high altitude areas of Darjeeling District. They have titles of Bhutia (Plate 16).

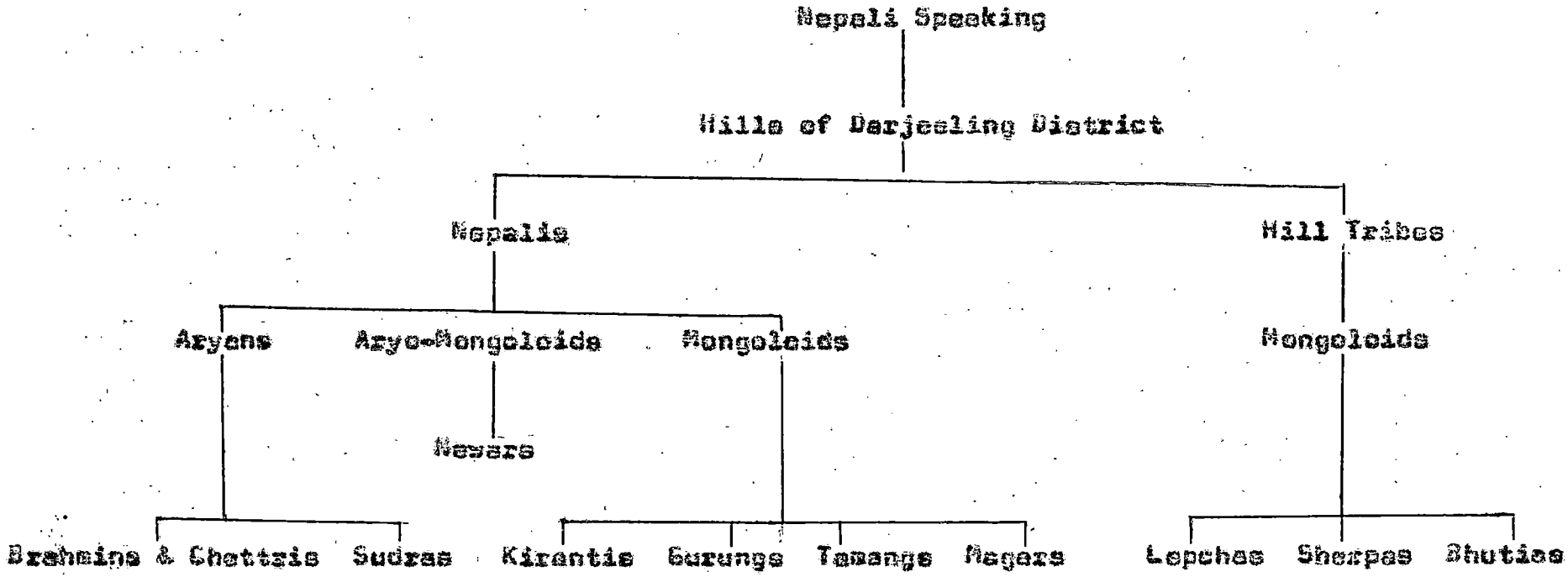
2.1.4 A summary of principal groups and sub-groups

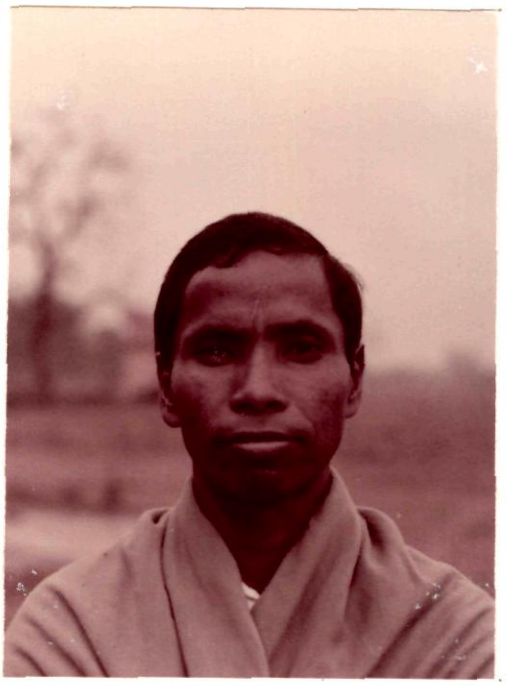
A summary of the principal groups belonging to different ethnic populations of North Bengal and their sub-divisions based on Linguistics, Ecology and origin is presented in the following charts 1 and 2.

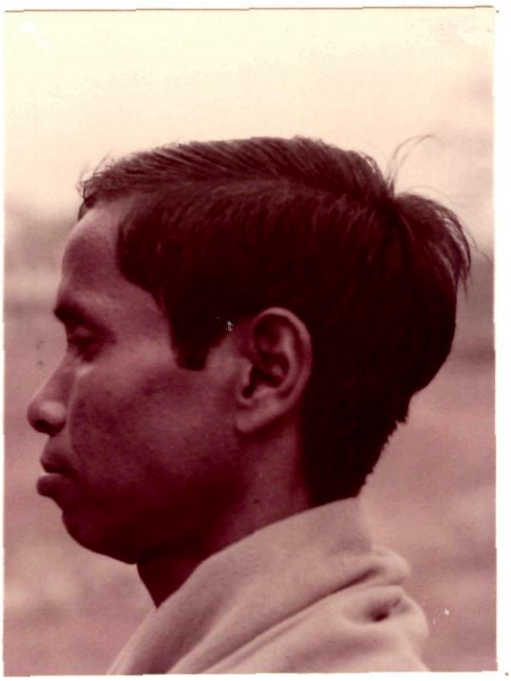
CHART 1

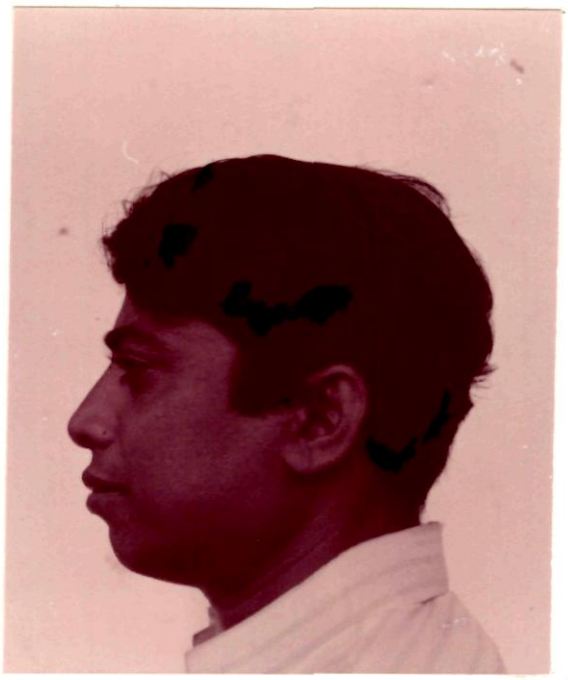


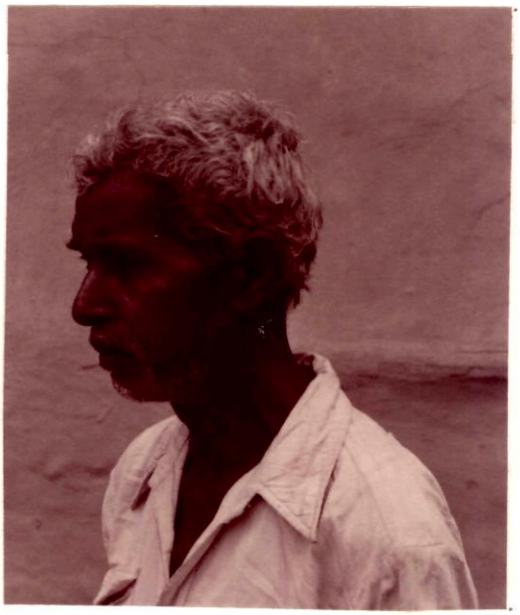
## CHART 2

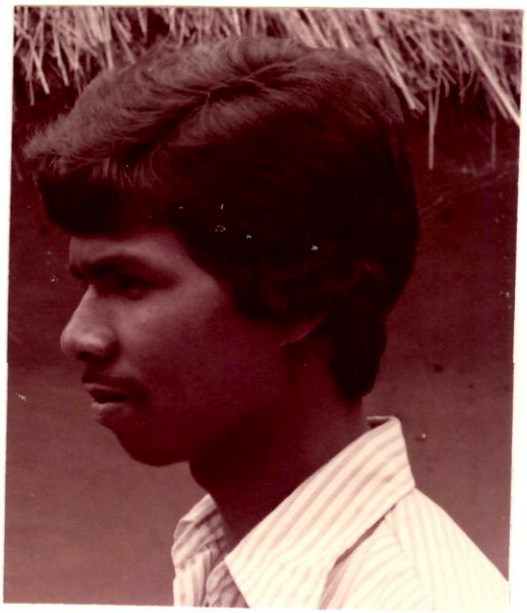

Nepali Speaking
Hills of Darjeeling District
Nepalis
Hill Tribes
Aryans
Aryo-Mongoloids
Mongoloids
Mongoloids
Newars
Brahmins & Chettris
Sudras
Kirantis
Gurungs
Tamangs
Magars
Lepchas
Sherpas
Bhuties

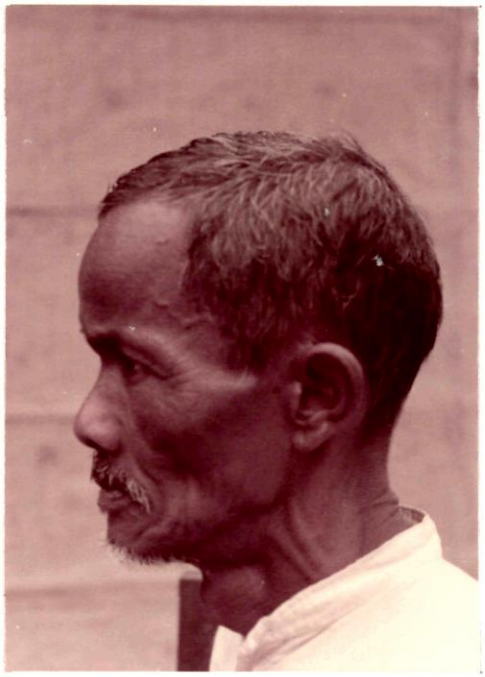


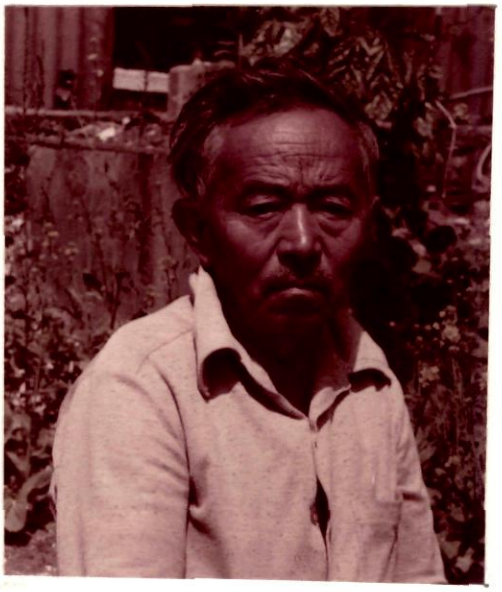


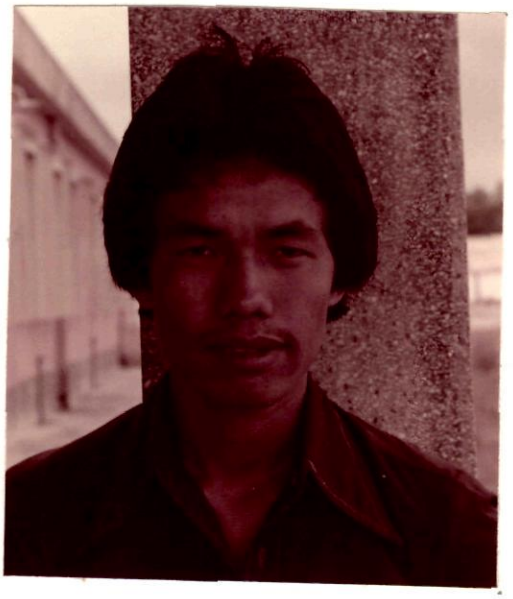


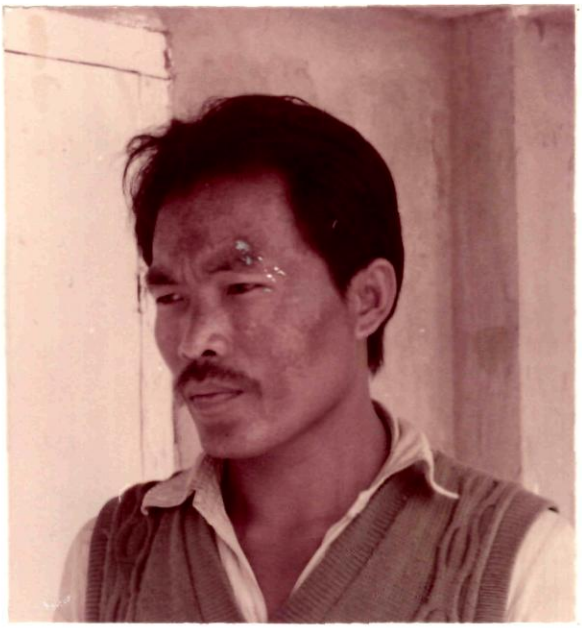


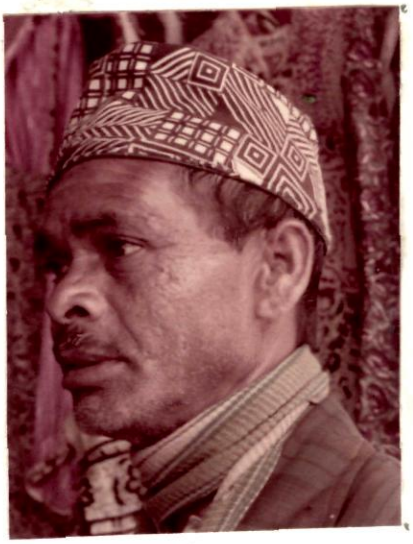


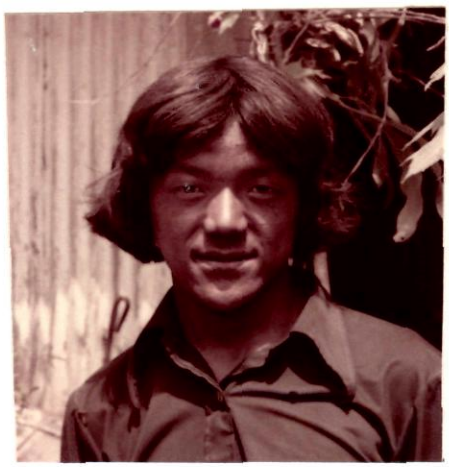


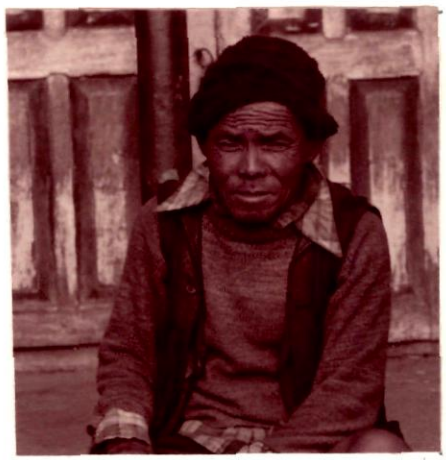


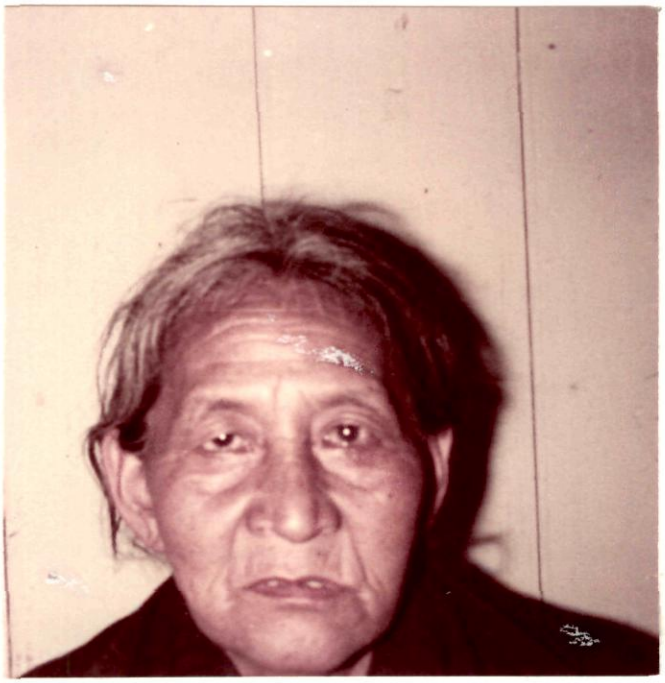












## 2.2 Methods

### PERIPHERAL BLOOD LEUKOCYTE CULTURE

### 2.2.1 Collection of samples

Human peripheral venous blood was drawn from the brachial vein of normal subjects belonging to different ethnic population of North Bengal, from the different health centres, blood banks and hospitals. However, most of the samples were obtained from the North Bengal Medical College and Siliguri blood banks, Kurseong Sub-Divisional Hospital, Darjeeling Sadar Hospital, and Health centres of Dowhill, Bagdogra, Sukna, Naxalbari, Bengdubi, Phansheria and from the tea estates of North Bengal.

Five to eight ml of blood drawn with a sterilised glass syringe was collected in a sterile heparinised universal container and transported to the laboratory in ambice-flask. Heparin was obtained from Biological Evans (Bombay), and the concentration used per culture bottle was 0.1 ml of 5000 i.u./ml.

All chromosomal observations were made on cells from cultured peripheral blood using a modification of the whole blood micromethod of Hungerford (1965).

2.2.2 Preparation of culture media

Medium 199 (Wellcome, U.K.) was used as the culture media. Ten grams of the dry media was dissolved in 950 ml of sterile, triple distilled water and 50 ml of 4.4% of sodium bicarbonate was added to make the total volume 1000 ml. The colour of the media was light red having an approximate pH 7 - 7.2. The media was supplemented with antibiotics Penincillin sodium 100 i.u./ml (Alembic, India) and Streptomycin sulphate 0.1 mg/ml (Sarabhai, India). The media was filtered through a millipore filter and collected in tightly stoppered conical flasks and stored at $4^{\circ}C$.

2.2.3 Collection of human AB serum

290 to 300 ml of AB blood was collected in a sterile blood bottle, swirling the bottle in one direction as the blood dripped from the vein of the doner. The bottle was swirled continuously for another ten minutes after collection, so that the fibrinogen formed a clump. The blood was carried to the laboratory in anice flask and kept at room temperature until all the R.B.C. and fibrinogen clot settled down and the serum formed a seperate upper layer. The serum was pipetted to sterile universal containers in the culture chamber and centrifuged at 3000 r.p.m. for 20 min to separate completely the serum from the R.B.C. and fibrinogen. The serum was pipetted into screw cap tubes and placed in a beaker of water at 55°C for 45 minutes. The heat inactivated serum was stored at -20°C.

Phytohaemagglutinin 15 (PHA) (Wellcome, U.K.) was reconstituted with 5 ml sterile, distilled water and used to stimulate the cells.

2.2.4 Culture setting

5 ml of media was pipetted in a universal culture bottle and supplemented with 1 ml of AB serum. 0.1 ml of reconstituted PHA was added to each culture bottle followed by 0.3 ml of the blood sample. Carbon-dioxide was introduced by blowing through a pipette and the container was tightly closed.

2.2.4.1 Incubation : The cultures were incubated in a vertical position at 37.5°C for 72 h to obtain a rich mitotic index. The cultures were rotated at least twice a day with care to maintain the temperature at 37.5°C. The ph of the cultures were kept slightly acidic throughout incubation period.

2.2.4.2 Harvesting : 0.3 ml of 0.04% of Colcemid (CIBA) giving a final concentration of 12 µg/ml was added per culture and reincubated for 2½ h to 3 h before termination of incubation to arrest the cells at early metaphase and metaphase stages. The cultures were centrifused lightly (1000 - 1200 r.p.m.) for 5 min and the supernatant removed. 10 ml of pre-warmed 0.56% of KCl solution was added as hypotonic solution and reincubated for 20 min at 37.9°C. The suspension was centrifused for 5 min and the supernatant removed. The deposits were agitated and freshly prepared glacial acetic acid and absolute methanol (1:3 v/v) was gradually added and kept at room temperature for at least 30 minutes. The suspension was centrifused, supernatant removed, and the residue resuspended in the fresh fixative. The process was repeated two to three times.

2.2.5 Preparation of slides

Scrupulously clean slides were labeled and kept in chilled distilled water. Two to three drops of the cell suspension was dropped from a height of 6 inches on the wet slides kept at an angle of 45° and quickly run through a flame to hasten drying.

2.2.5.1 Staining : The slides were stained with 5% buffered Giemsa Stain for 5 min and rinsed in distilled water. The slides were dried and mounted in DPX .

2.2.5.2 Photomicrography : The slide was scanned and suitable metaphase spreads were photographed at 1250 X with a Carl Zeiss Fluoval Microscope attached with a 35 mm camera. The film used was KB 14, 20 ASA/ KB 17, 40 ASA. The developing medium used was Kodak D 76 for film and D 163 for prints. Fixing was done in Kodak's Acid fixing salt with hardener. Printing was done on Agfa Gevaert single weight extra glossy, hard/normal papers.

2.2.5.5 Karyotype preparation : Well spread metaphase plates with 46 chromosomes were selected and photographed. From the print each chromosome was cut seperately and then arranged into pairs of 22 autosomes and 2 sex chromosomes (Denver 1960, Paris Conference 1972, 1975). The arranged chromosome were pasted neatly on a white board and the chromosome number and group written. The symbols p and q were used to designate the short and long arms of each chromosome respectively, inv for inversion and h for secondary constriction.

2.2.6 Chromosome banding

A number of different techniques have been developed for producing characteristic pattern of light and dark bands on the arms of the chromosomes. One of these techniques, first described by Arrighi & Hsu (1971), brings out a pronounced band in the centromere region of certain chromosomes notably chromosome 1, 9 and 16 and is known as C-bands. Usually they are located on the long arms but in about 3% of cases it may be wholly or partly on the short arms giving different band on chromosome shapes.

The technique, first used with mouse chromosome (Pardue & Gall 1970) involves an alkali treatment of the cells *in situ* and subsequent incubation in a salt solution. This procedure is believed to denature the DNA and then allow partial annealing of the DNA strands during incubation (Craig Holmes & Shaw 1971). When the slides are then treated with Giemsa stain, a darkly stained area is produced at the centromere of every chromosome. In addition very prominent blocks of heterochromatin are stained in the secondary constriction regions of chromosomes 1, 9 and 16. The Y chromosome is unique in that it has a large heterochromatin block that covers the distal half of the long arms.

The methods of Sumner (1972) and Arrighi & Hsu (1971) have been followed with slight modifications to obtain C-bands.

2.2.6.1 C-banding (Sumner 1972) : Seven to eight day old slides were used to obtain C-bands. Slides were treated with 0.2 N HCl for 1 h and thoroughly rinsed in three changes of distilled water. Slides were dried and then treated with 4% Barium Hydroxide solution at 50°C for 9-12 min and immediately rinsed in several changes of distilled water. Slides were dried and incubated in 2 X SSC (0.3 M sodium chloride & 0.03 M tri-sodium citrate) at 65°C for 60-90 min and again rinsed in water. Slides were dried and stained with 5% Giemsa (Gurr) for 30 min and rinsed in distilled water. The slides were mounted in DPX and scanned.

The method of Arrighi & Hsu (1971) was also employed to obtain C-bands. Slides were treated with 0.033 N Sodium Hydroxide pH 12 for 30-120 sec at 20°C and rinsed in distilled water. The slides were incubated in 2 X SSC for 24 h at 65°C followed by 5% Giemsa staining for 15 minutes. After mounting the slides in DPX they were observed and photographed.

2.2.6.2 C-band location : Scoring was done by direct microscopic visual examination. C-band variations in chromosomes 1, 9 and 16 were recorded with respect to size, shape and position of the centromere. The size and shape of the C-bands on chromosomes 1, 9 and 16 were recorded on a minimum of 5 well spread C-banded plates. The size of the centromeric heterochromatin was scored as very large (VL), large (L), medium or normal (M) and small (S).

2.2.6.3 C-band Screening : Normal C-bands on chromosomes 1, 9 and 16 are usually located on the long arms proximal to the centromeres. In some cases, the whole or part of the C-band is located on the short arm proximal to the centromere. In the present investigation, a variant was scored when at least one third of the C-band was located on the short arm as partial inversion and a total inversion if all the C-band material was on the short arm. A small C-band was frequently observed on the short arm, but this was not scored as a variant for two reasons : (a). its presence on the short arm was not consistent from cell to cell in an individual and (b).when its classification was not clear, suggesting that the C-band was probably the result of technical rather than biological variation.

The relatively non-variable 16 p was chosen as a standard reference (medium category) for comparison of cell to cell and individual to individual variations in length, as suggested by Patil and Lubs'77. Chromosome 16 is easily recognizable by the C-band technique alone. To evaluate the reliability of scoring in case of variants, photomicrographs were taken and from these prints chromosome pairs 1, 9 and 16 were selected for analysis. They were evaluated by visually comparing the size and position of C-band pattern of each pair with the pasted karyotype with 'Normal' banding pattern. If the C-band was larger than the standard karyotype the classification was (+) or +/N for the heteromorphic pair or +/+ for the homomorphic pair. Similarly, if the C-band was shorter the classification was (-). The C-band variations (+/-) were further classified as VL, L or S.