DISCUSSION

As the most of the cells show a chromosome number ranging from 66-79, they may be regarded as the stemline cells. Again the cells show a peak of chromosome numbers at 73 which may be taken as the modal number. However, the percentage of cells showing this number is only 22, whereas another 28% of cells have chromosome number 72 or 74. As such the chromosome numbers ranging from 72-74 should be regarded as the nodal number for this cell-line. The average chromosome numbers of this cell-line is found to be 74.7 which is slightly higher than the modal number. This is possibly due to a number of polyploid cells which were counted at random basis. As the normal mouse cells have 40 chromosomes, this cell-line should be regarded as a hypotetraploid one. However, as the chromosomes are very small and as they cannot be identified easily it was not possible to identify which of the chromosome groups are present in less number than is expected in a tetraploid.

The microchromosomes were not included in determination of the modal number. The microchromosomes are generally regarded as the accessory chromosome (cf. 173,178) although in many species as in birds, microchromosomes are always present in normal cells and their presence is a constant feature of the normal karyogramme. The minutes resemble the fragments or even like non-chromosomal materials. However, the chromatic nature of them is established by the Feulgen reaction, characteristic

fluorescence with acridine orange and also of being labelled after ^{3}H-thymidine incorporation. That they were not fragments was evident from anaphase studies as in no case they were found lagging. It seemed that they have a very regular distribution through anaphasic movement. In almost 80% of the cells they were regularly present and their number varied from 1-4 although most of the cells have 2 to 3 minute chromosomes. This pattern of distribution is a characteristic of the accessory chromosome. In tumour cell-lines too, all the chromosomes are not responsible for the genetic make-up of a particular cell-line (242). It was shown by Ghosh and Ghosh (242) in HeLa cells that different chromosome types may be represented in karyogrammes of modal cells showing identical chromosome number, but leading to chromosomal polymorphism. As expected that tetraploid or hypertetraploid cell-lines have a number of extra chromosome or parts thereof which are in excess and are not essential for the genetic integrity of that cell-line. As such, when we consider for a particular chromosome it is essential that the chromosome must be present in all the cells, at least in one dosage for example, the metacentric chromosome which has arisen due to fusion of two telocentric chromosomes in this particular cell-line is present only in 60% of cells. It is quite possible that in the rest 40% of cells, although this chromosome is missing, the two original telocentric chromosomes are present. This may also be true for other chromosomes, but as they cannot be identified

properly, this cannot be demonstrated. As such, absence of the microchromosomes in about 20% of the cells does not necessarily indicate that they are accessory in nature. However, these chromosomes were thought to be new in origin. This cell-line maintained in a different strain (strain A) was cytologically described by Ghosh (243) earlier. The microchromosomes were absent in this cell population. As such it may be considered that these chromosomes are new in origin and are present in access to the modal number. There is a possibility, therefore, that they may be accessory in nature as these minute accessories are present often in different malignant cell-lines (234, 235, 236). Small, chromosome-like structures have long been reported in human neoplasms (188, 244). Such chromosomes were also described from Rous sarcoma by Mark (189) which he called as double minutes. Levan et al. (191) reported the occurrence of minute chromatin bodies or double minutes in SEWA ascites tumour. Later on, in 1977 the same group of scientists (192) found that besides double minutes which were acentric, this tumour contained another type of minute chromosome, which they called microchromosomes. The microchromosomes could be seen in 12-60% of the cells depending on the type of the tumour and their number varied from 0 to 3. In contrast to the double minutes, they were centric. The presence of a small centric chromosome was also reported in mouse DBA lymphoma cell-line (245). However, this chromosome was found to be present in

almost all the cells. The nature of the microchromosomes in this MS 180 cell-line was, therefore, studied through banding analysis and other procedures which allow us to detect the nature of the chromatin material.

Although the main target was to evaluate the nature of the chromatin material of these microchromosomes, but some of the chromosomes revealed interesting banding pattern and as such was studied along with. The nature of these chromosomes will be discussed at first.

Banding analysis of the chromosomes

C-bands induced through alkali or heat treatment produced identical results. As all the mouse chromosomes are acrocentric in nature, the C-bands appear at one end of the chromosomes except in the metacentric one. Some of the chromosomes however showed C-bands on both ends of the chromosome; one of them is a long one which is present in almost all the cells (Figs. 16, 17,18,19). The regularity of the other chromosome could not be ascertained as they are not distinguishable. Ghosh (243) presumed that presence of C-bands at both ends of the chromosome might either indicate they are dicentric or more likely that the centromere is situated at one end and the NOR is situated at other end of the chromosome. Although the Ag-NOR staining revealed that some of the telomeres are associated with the nucleolus formation, but whether they were centromeric end

or the other end could not be clearly ascertained, as the chromosomes were much swollen. The N-banding procedure, on the other hand, did not produce persistent results. Various numbers of chromosomes showed N-bands in different plates (Fig. 27). In most cases, the C-banded regions of some of the chromosomes revealed N-bands too. By this procedure too, it was not possible to identify the nature of the seemingly dicentric chromosome.

The comparison of the C-bands and G-bands reveal that trypsin induces some more bands, mostly interstitial, in addition to C-bands to appear in these chromosomes (Figs. 23 and 24). However, the bands at the centromeric regions are much darker than the interstitial bands.It is particularly true for cells treated with longer period of trypsin where the chromosomes swell to a considerable degree and finally only the deep-stained centromeres remain readily recognizable (Figs. 25 and 26).

DNase I produces bands which are intermediate between C-bands and G-bands (Figs. 21 and 22). In an earlier report (246) we reported the production of C-bands through short DNase I treatment. The chromosomes showed deep bands at their centromeric regions. However, the lighter bands in the intercalary regions of chromosomes are produced regularly. This intercalary bands are very faint and as such were overlooked for convenience. In 1978, Rattner et al. (247) showed that

DNase I selectively degrades mouse metaphase chromosomes leaving the centromeric heterochromatin. However, in our preparation, a longer treatment with DNase I resulted in the total disappearance of the banded nature of the chromosome. Earlier in 1977, Schweizer (74) used DNase I to produce R-bands. If chromomycin A_3 stained chromosomes were treated with DNase I and then stained with Giemsa, the chromomycin A_3 fluorescent positive bands stained darkly. This is most likely due to the protection against DNase I by chromomycin A_3 bound to the GC-rich DNA. From our experiment, it seems that centromeric heterochromatin or even intercalary heterochromatin are less sensitive to DNase I than the rest of the chromosomes. As proteolytic enzymes also produce such type of bands and moreover even C-bands are produced with longer treatment with these enzymes, it was thought that DNA at the centromeric region and intercalary bands may have a stronger association with protein and as such are less sensitive to this enzyme. To see, how far the action of this enzyme is on the DNA of the chromatin material, particularly in the very short period of treatment leading to the production of the bands. The total amount of DNA degraded in this period was calculated. It was found that even with this very short period of treatment about 18% of DNA was extracted from the chromosomes. In control preparations, which are incubated in distilled water containing $MgSO_4$ only, we found an extraction of about 9% of DNA in 2 hours. Perhaps, intra-

cellular DNase become active in such case. 2 hours of DNase digestion resulted in the extraction of only 23% of DNA. As the concentration of DNase was a very meagre, it may be possible that the enzyme was used up within a short period and there was no more action of the enzyme. However, the experiments indicate that the action of the enzyme is very quick and even within a short period of treatment leading to the production of bands, a good amount of DNA becomes extracted. This may account for the appearance of the bands particularly in the centromeric regions of chromosomes.

DNase banding of the chromosomes again produce identical results. The metacentric one shows bands at the median region, and the 'alleged dicentric' one at both ends of the chromosome.

The Q-banding procedure did not produce any bands. It is almost established that the chromosome regions enriched with AT bases show bright fluorescence with quinacrine (6-9). Earlier it was observed by Rowley and Bodmer (19) that mouse centromeres did not show Q-bands although it is well known that mouse centromeres are rich in AT. These authors tried to explain this phenomenon by considering that quinacrine binds with guanidine as supposed originally (1) and as such mouse centromeres should not show Q-bands, as they are poor in GC content. However, as it became established that AT-rich DNA enhances Q-fluorescence, it was puzzling why mouse centromeres should not

show Q-bands. Weisblum (20) could establish that while AT-rich DNA segments enhances Q-fluorescence GC-rich sequences on the other hand, quenches it. If an AT-rich chromosome segment is interspersed with GC sequences, no enhancement of fluorescence can be observed. He (20) claimed that although mouse centric DNA is enriched in AT, these base pairs are not present in long sequences but are interspersed with GC pairs. As such the AT-GC base pair ratio is critical for the Q-fluorescence. However, the fluorescence of Hoechst 33258 is not quenched by GC sequences (248). This drug also binds with AT-rich DNA. As such this drug also shows Q-banding pattern in general. Chaudhuri and Ghosh (249) were able to demonstrate that Hoechst 33258 can show positive Q-bands in mouse chromosomes at the centromeric regions. The chromosomes in this cell line also exhibited bright Q-bands at the centromeric regions. The bi-armed chromosome again showed bands at the centric region. A small chromosome exhibited bands at both ends of the chromosome (Fig. 39) whereas the alleged dicentric one showed band at one end only (Fig. 41).

Acridine orange also reveal Q-banding pattern although it is based on different principle. It is claimed that the emission property of acridine orange changes when the dye binds with single-stranded or double-stranded nucleic acids (35). As such for the production of Q-band through acridine orange one has to denaturate the DNA in the chromosome. Although it is claimed that acridine molecules stack on single-stranded DNA

which should fluoresce red. In our preparations, the chromosome arms were found to show red to yellowish green fluoresce whereas the centromeric regions fluoresce green. It is not often possible to distinguish them in the photographs. Moreover, acridine orange cannot tell us the nature of chromatin as precisely as quinacrine or Hoechst 33258 can. However, all the regions producing C-bands are found to produce acridine orange bands too.

Metacentric chromosome

It is present in almost 60% of the cells and this chromosome can be taken as a marker as it is the largest chromosome and has a centromere in the nearly median region. However, in many cells, this chromosome appeared as sub-metacentric. As mouse chromosomes are all telocentric, this chromosome should arise due to fusion of two acrocentric chromosomes. This is evident from the C-banding pattern as in many cases, two C-bands at the centromeric region were found side by side (Figs. 16 and 18). However, in some cases, one large C-band could be observed (Fig. 25). In most cells, both arms are almost equal in size. This is possible if two homologous chromosomes fuse at their centromeres. However, G-banding pattern indicates clearly that the two arms are not homologous as one of the arm has an extra band. It has been already established by Ghosh (243) that this bi-armed chromosome has arisen due to Robertsonian translocation. This has been further supported by Chakraborti and Chakraborti (250).

C-bands is associated with the formation of the nucleolus whereas Q-banding procedure did not reveal any band. But Hoechst 33258 banding indicates the presence of only one brightly-stained band at one end of this chromosome as Hoechst-33258 stains the AT-rich DNA associated with the centromere more brilliantly than the rest of the chromosome. It may be inferred that the alleged 'dicentric' chromosome has actually one centromere (Fig. 41). It is possible that at the other end it has got a heterochromatic region, but this heterochromatin is not associated with the centromere or kinetochore formation. Heterochromatins are in general sticky in nature (254,255) and as such attracts nearby heterochromatic regions. This leads to the stickiness of the chromatids, which retains the chromatids side by side for a longer period than usual.

A small dicentric chromosome

C-band procedure reveals that some chromosomes often show two C-bands on either end of the chromosome. Apart from the alleged 'dicentric' one, one or two small chromosomes were found to have two C-positive heterochromatic regions on both ends. However, it is not possible to distinguish these chromosomes as they are small in size & have no other special feature. As such it is not possible to ascertain their regular presence in the cell-line. In these cases too, one of the C-band regions may not represent the centromere or kinetochore at all. However, Hoechst-33258 banding showed the presence of one such chromosome

with two centromeres (Fig. 39). The occurrence of dicentrics have been reported in many plant and animals by following treatment with different mutagens,but instability in subsequent cell-division leads to their quick elimination within a few cell-generations. In malignant cell-lines chromosomal rearrangements are often found. It is possible that some dicentrics may arise due to this rearrangements. However, they are maintained only for few generations in the cell population. This small dicentric chromosome may represent such a case.

Banding analysis of the microchromosomes

All the C-banding procedures reveal that the microchromosomes are devoid of C-bands. As these chromosomes are very small, it was not expected that microchromosomes would regularly show band but rather a deep or light staining after the banding procedures. Both alkali and heat denaturation processes produced identical results. The microchromosomes are lightly stained with Giemsa. Identical results were also obtained with DMase I banding. They are lightly stained indicating the absence of centromeres. However, with G-banding procedure, the microchromosomes showed the intensity of the stain as produced in the intercalary bands. In G-banded chromosomes three different regions could be obtained, one deeply-stained centromeric bands, medium stained intercalary bands and non-band light-stained regions (Figs. 23 and 24). The microchromosomes showed the intensity as the intercalary bands. As these intercalary bands represent tighter

chromatin condensation in the heterochromatic regions (55), the microchromosomes could be considered as heterochromatic in nature, but are different from the centromeric heterochromatin.

The fluorescence banding pattern reveal again that the microchromosomes do not have centromeric heterochromatin. They did not show enhanced fluorescence either through acridine orange banding or through Hoechst-33258 staining which indicate that these chromatin are not rich in AT-base pairs. On the other hand, anaphase studies indicate that these chromosomes are regularly distributed during mitosis. As such they cannot be considered as acentric fragments. The regular anaphasic distribution of these chromosomes indicate that they should have functional centromeres. As such to be sure the replication pattern of these chromosomes was studied. It is known that the centromeric heterochromatin is late-replicating (256). If the microchromosomes had centromeres, they would also show late-replication pattern. The metaphase plates with labelled chromosome which are obtained first after radioactive incorporation, i.e., after 5 hours of incorporation of radioactive precursor showed silver-grains mainly on the centromeric regions of all the chromosome (Figs. 30 and 31). In no case, grains could be observed on microchromosomes. On the other hand, the labelled metaphase plates which are obtained with longer period of treatments showed grains all over most of the chromosomes. The micro-

chromosomes in these preparations also reveal labelling (Figs. 32 and 33) indicating that they replicate not at the fag end of the S-phase but rather at an earlier period.

The only alternative is to consider that the microchromosomes are associated with nucleolus formation and may be distributed regularly with its attachment to the nuclear remnants. However, Ag-NOR staining clearly reveals that these chromosomes are not actively associated with the nucleolus formation.

From the banding analysis it can be said that the microchromosomes are devoid of centromere, but they are heterochromatic in nature. The microchromosomes are neither associated with the nucleous formation. Most of the former studies of the microchromosomes in different cell-lines indicate the presence of centromere in the microchromosome as the microchromosome in bladder carcinoma (257), DBA lymphoma cell line (245) or the large double minutes in SEWA mouse ascites tumour (258). The same authors reported the presence of other type of microchromosomes without functional centromeres in this SEWA cell-line too. It has been claimed that the double minutes are attached to or enclosed by the nucleolar matter persisting around the chromosome ends and at anaphase they are transported to the poles together with the nucleolar matter through hitchhiking. The work of Balaban-Malenbaum and Gilbert (259) in human neuroblastoma cell-line has demonstrated that homogeneously staining

regions (or HSR) and C-bandless double minutes may be related. It has been proposed that these dms arise through the breakdown of HSR. Both HSR and dms have been functionally related to the production of increased amounts of specific gene products (260). It is tempting to correlate the microchromosomes with these acentric double minutes or the C-bandless minutes (CM) of Levan and Levan (258). It is possible that these microchromosomes represent non-centromeric telomeric ends of some chromosome and as such show intercalary heterochromatic bands after G-banding procedure.

In many of our preparations, the metacentric chromosome appeared to be sub-metacentric (Fig. 10), the telomeric end of the smaller arm missing very often. This end shows G-bands. It is quite possible that in these cells this end is present as microchromosome. Some other chromosomes may also be involved in their production. However, how they are distributed regularly in anaphase poses a problem. There is no evidence to claim that they are transported like the double minutes by attaching themselves to other chromosome ends. Alternatively it is also possible that these microchromosomes or the minutes are not present in the normal cell complement, but some of the chromatid ends perhaps being more labile leads to the production of these fragments during preparational stress, as artifacts. It is quite possible that in this abnormal neoplastic cell-line some chromosomes have labile telomeric ends which lead to the regular production of the minutes during treatment with colchicine or hypotonic solu-

tion; but actually they are not present as minutes in the normal cells.

However, alternatively, the absence of C-bands do not tell clearly the absence of kinetochores too (253) as all the C-bands do not represent kinetochores. In many species of plants and animals C-bands appear at non-kinetochoric regions. It is possible that the microchromosomes have functional kinetochores, but they do not show C-bands neither they are AT-rich. This is particularly evident in endoduplicated cells (Fig. 42a) where the two sister chromosomes remain attached by their centromeres as the kinetochores fail to separate in the previous mitosis. The occurrence of endoreduplication is a very common phenomenon in this cell line (251) and in other mouse ascites cell line (261). In the metaphase plate showing endoreplication two microchromosomes are often found side by side. This is only possible if the microchromosomes have kinetochores which failed to divide in their previous mitosis. However, the two chromatids in the microchromosomes can never be obtained. It is likely that the two chromatids of these small microchromosomes which consist fully of heterochromatin remain closely together due to heterochromatic attraction and due to the smallness of their size, the sister chromatids cannot be detected before anaphase movement. From the present observations, it cannot be definitely said whether the microchromosomes are acentric fragments or preparational artifacts or chromosomes without centromere but

with functional kinetochore. However, their regular distribution and presence in the stemline cells and their behaviour in endoreplicated cells add more weightage to the later possibility.