EFFECTS OF ARECOLINE ON MAMMALIAN SOMATIC CHROMOSOME

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CONTENTS

	Page
CERTIFICATE	i
ACKNOWLEDGEMENTS	ii
INTRODUCTION	1
MATERIALS AND METHODS	13
RESULTS	20
DISCUSSION	39
CONCLUSION	51
BIBLIOGRAPHY	52

CERTIFICATE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted in part or full for any other degree or diploma of any University.

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INTRODUCTION

Cancer is a major problem in a number of countries. Though the incidence of this disease is very low among Indians its frequency is high in certain parts of the body. Thus oral and oropharyngeal cancers have a high incidence in the morbidity and mortality scene of cancer in India. Since the past 80 years a high frequency of oral and oropharyngeal cancer have been observed by all the hospitals treating cancer in India. In the cancer hospitals also the above trend is seen (Table-I).

In majority of these hospitals, a high proportion of cancer in males is located in the mouth and throat region and in females 50% to 70% of all cancer is in the cervix and breast together. This table suggests regional variations in the frequency ratio of certain cancer types. The data collected from various teaching hospitals in the country provides further evidence of regional variations in cancer types. Cancer of the base of tongue is common in Bombay and Gujarat. Hypopharynxlarynx cancer has a high frequency all over the country. The high frequency of lip cancer from Bihar, palate cancer from coastal districts of Andhra Pradesh, hypopharynx cancer in Assam are other features of this disease.

The high incidence of oral cancer in India and Far East is closely associated with the habits of betel chewing and tobacco smoking (Orr, 1933; Khanolkar, 1945; Sanghvi/etal., 1955; Shanta & Krishnamurty, 1959, 1963; Paymaster, 1962; Hirayama, 1966; Wahi, 1968; Jussawalla & Deshpande, 1971; Reddy et al., 1975 and Khanna et al., 1975).

Human oral carcinogenesis is evidently a complex interplay of many cofactors of intrinsic origin like genetic sensitivity of oral mucosa, oral hygiene and mucosa conditioning factors like diet, vitamin deficiency and extrinsic origin like chewing ingradients, edible oils to which the mucosa is exposed. Neoplastic transformation may be manifested by the appearance of leukoplakia, melanoplakia and submucous fibrosis in the buccal cavity (Paymaster, 1956; Padmavati & Reddy, 1960; Shanta & Krishnamurty, 1963; Martin & Koop, 1942).

Jussawalla & Deshpande (1971) at the Bombay Registry reported that the risk of developing cancer in the buccal mucosa was found to be 7.7 times higher in chewers than in nonchewers. Wahi (1976) based on the data collected by himself in Mainpur district of U.P. reported that the risk of cancer is 8 times higher among the daily chewers as compared to nonchewers. Again he established the fact that the earlier the age at which chewing was started the higher was the rate of oral cancer. For example, if the habit of

TABLE - I

Relative frequency of oral-pharyngeal and laryngeal cancer in six major cancer centres in India (1970-72).

(Source: Ind. J. of Cancer, Supp. Series I, 1973).

Cancer Centre	Oral-Pharyngeal-laryngeal cancer as percent of all cancer in		Total number of cancer - all sites	
	Males	Females	Males	Females
Tata Memorial Hospital, Bombay	51%	15%	14,772	9,406
M.P. Shah Cancer Hospital, Ahmedabad	58%	19%	5,706	2,433
Cancer Institute, Madras	51%	18%	2,419	3,101
M.N.J. Cancer Hospital and Radium Institute, Hyderabad	61%	21%	4,384%	7,327
Chittaranjan National Cancer Hospital, Calcutta	59%	16%	3,586	2,740
J.K. Institute of Radiology and Cancer Research, Kanpur	73%	17%	3,171	5,286

chewing started under the age of 14 years it was noted that the prevalence rate was 10 times higher in tobacco chewers than in nonchewers.

BETEL CHEWING

The chewing of betel quid is very common in South East Asia. The 'pan' quid is a combination of betel leaf (Piper betel), areca nut (Areca catechu) and slaked lime, tobacco, catechu (Acacia acacia catechu) and other aromatic spices like cloves, cardamum are often included as essential ingradients of the quid.

In recent years many attempts have been made to develop a theory of the origin of betel chewers cancer based on the chemical constituents of the quid. Extensive biochemical and other laboratory studies are going on, to assess the role of each ingradient that constitutes the total chew.

Aqueous extract of betel leaf by s.c. injections, however, failed to produce any tumors (Ranadive et al., 1976; Ranadive & Gothskar, 1978 and Bhide et al., 1979). It is interesting to note that betel quid produces less tumor than betel nut extract. It may be due to protective action of betel leaf because of the presence of chlorophyll (Ranadive et al., 1979).

The role of lime appears rather controversial, though some reports do suggest its correlation with the occurrence

of oral cancer. According to Tenneckoon and Bartleff (1969) lime might have an important action, but was used in such quantities that it is diluted by the saliva. Acanthosis of oral epithelium caused by lime was reported by Sirsat & Kandarkar (1968), Atkinson et al. (1964) postulated that lime might play a role of cocarcinogenic agent by virtue of tissue reaction it provokes. But Gothaskar et al. (1975) reported no neoplastic transformation of tissue treated with lime.

Tobacco as an ingradient of quid has been regarded as the promoter in changing the oral mucosa which eventuate in the development of oral cancer (Khanolkar, 1951; Singhvi, Rao and Khanolkar, 1955; Shanta and Krishnamurthy, 1963). Wahi (1968) reported an interesting relationship between the habit of tobacco chewing and the prevalence rate of oral cancer. He also reported that the risk for oral cancer among the chewers of Pattiwala tobacco was noted to be significantly lower than the risk of Mainpuri tobacco chewers though slightly higher than that of nonchewers. Extensive experimental works have therefore been carried out to test the carcinogenicity of a variety of tobacco extracts on various experimental models. Seven different fractions of sun-cured tobacco leaves extracted with various solvents-alcohol, benzene, chloroform, ether, water etc. were screened for

carcinogenic effect on mouse oral mucosa by continuous painting, over the entire life span of animals but no effect was observed in the oral mucosa (Mody & Ranadive, 1959). Acetone extract of Vadakkan tobacco when applied on skin with weekly painting of cocarcinogen, croton oil induced 26-28% epidermoid carcinoma thus confirming, for the first time, the presence of a weak carcinogenic principle in the total tobacco extract (Ranadive et al., 1965). In rodents, tobacco extract have completely failed to induce cancer in the oral mucosa, the target organ in human exposure (Mody & Ranadive, 1959; Gothoskar et al., 1975). This was considered as an incomplete carcinogen essentially requiring help of cocarcinogenic and/or syncarcinogenic co-factors for manifesting its carcinogenic effect. In fact previous workers like Muir & Kirk (1968) and Reddy & Anguli (1967) suggested the importance of other ingradients of betel quid besides tobacco in the induction of tumors in test animals. The preliminary work done by Ranadive et al. (1976) with the combination of betel nut extract with tobacco extract have indicated an enhanced effect with early cancer in the hamster cheek pouch, thus indicating the probability of enhancing effect of betel nut in carcinogenesis.

PHYSIOLOGICAL EFFECTS OF ARECA NUT

Areca nut is highly acidic and astringent to taste. It is neutralized by the addition of lime. Arecanut promotes

salivation (Eisen, 1946) mild exhilaration and to a certain extent of sleeplessness. It has some effect on the central nervous system.

ROLE OF ARECANUT AND ITS COMPONENTS IN CANCER

Dunham and Herrold (1962) and Woefel et al. (1941) reported that betel nut had no tumor inducing capacity. But later on in contrast to their experiment Suri et al. (1971) revealed that DMSO extract of betelnut is capable of inducing tumors in hamster cheek pouch at as high a rate as 38%. In 1976, Ranadive et al. though not quantitatively but qualitatively confirmed Suri's findings. Further subcutaneous injection of aqueous extract of betelnut yielded a high incidence (60%) of transplantable fibrosarcoma in swiss mice. Recently Ranadive et al. (1979) and Bhide et al. (1979) confirmed the tumor inducing ability of betelnut which produces 63% lesions in hamster cheek pouch and 58% of cancer in different organs of the body. Inhibition of DNA synthesis in lymphocytes and tumor cells in vitro by extract of betelnut is reported by Yang et al. (1979).

The polyphenolic fraction of betelnut which contains tannin produces 100% tumors consisting of 80% fibrosarcoma along with liver and lung tumors in swiss strain mice (Shivapurkar, 1978). About 50% of rats surviving tannic acid injections for 100 days produced liver tumor and process

could be enhanced to a greater degree of malignancy by adding acetylaminofluorene (Mozonyi & Korpassy, 1953). Kirby (1960) also reported the injection of tannin extract could produce tumor. Recently in 1979, Bhide et al. reported 17% tumor in liver and salivary gland. They also made it clear that betel nut extract produced a significant number of tumors of gastrointestinal tract where as feeding the polyphenol fraction failed to induce any tumor in the gastrointestinal tract. But s.c. injection produced 80% tumor. So it is assumed that the high frequency of tumor induction in the gastrointestinal tract by betelnut extract could be attributed to some constituent in betelnut other than tannin.

Alkaloids from different plants which are consumed either as food or local medicine are reported to be carcinogenic (Iwao Hirrono et al., 1977; Donald, 1972; Schoental, 1968) and mutagenic in different test system (Harumichi et al., 1979; Kolestsky, 1978; Hitoshi, 1980; Brink, 1965, 1969; Clark, 1959; Gladwin et al., 1977).

Literature is poor on the alkaloid part of betelnut. Arecoline is one of the important alkaloid of areca nut. Its content is about 0.8% in some riped nuts (Goswami & Ahmed, 1956).

PROPERTIES OF ARECOLINE

1, 2, 5, 6-Tetrahydro-1-methyl-3-pyridine carboxylic acid methyl ester; methyl 1, 2, 5, 6-tetrahydro-1-methyl nicotinate; methyl 1-methyl - $\Delta^{3'4}$ -tetrahydro-3-pyridine carboxylate; methyl N-methyl tetra hydronicotinate.

$C_8H_{13}NO_2$; mol. wt. 155.9 (C 61.91%, H 8.44%, N 9.03%, O 20.62%).

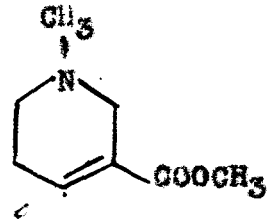


Fig:1: STRUCTURE OF ARECOLINE

It is extracted from the seeds of the betelnut palm- Areca catechu L., Palmaceae (Catechu).

PHYSICAL PROPERTIES

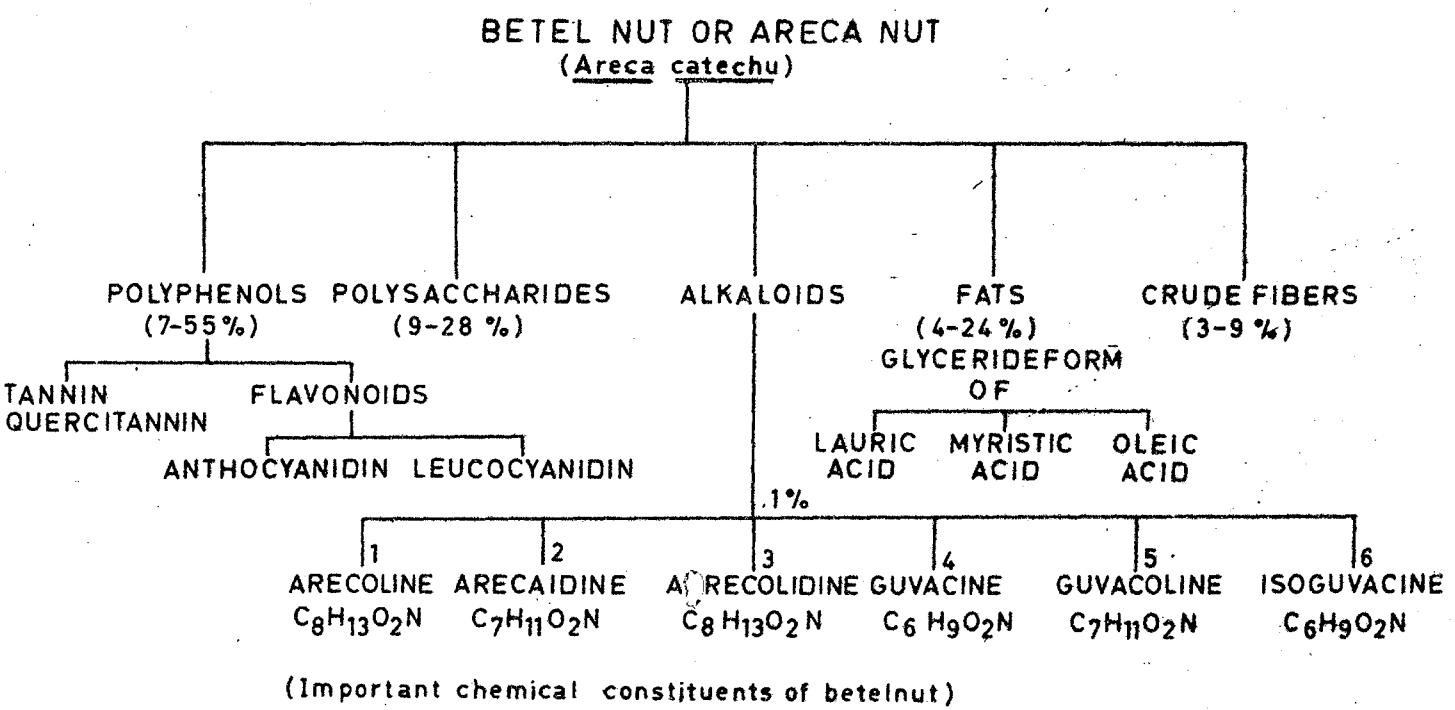
Arecoline, a colourless substance with a strongly alkaline reaction is volatile in steam and forms well defined salts. Boiling point is 209° C. It is miscible with water, alcohol and ether. It is soluble in chloroform. Hydrolysis of arecoline with hydrochloric acid under pressure or boiling hydroiodic acid or hot alkalies, eliminates a methyl group and produces crystalline arecaidine. It is also found in amorphous form (synthesized) which is soluble in water.

PHYSIOLOGICAL PROPERTIES

Arecoline stimulates the brain. Arecoline administered to rats showed various effect on central nervous system i.e. increased motor activity (Hertz, 1962), decreased conditioned avoidance response (Hertz, 1962; Hertz & Yacoub, 1964; Pfeiffer & Jenny, 1957), activation of electroencephalogram (Riech et al., 1962). Arecoline causes cataleptogenic effects (Green, 1979). It has been shown that arecoline has a very short duration of action and its effects are maximal at this time. Semenov (1978) reported that the Cholinomimetic arecoline led to a rapid initial increase in ^{45}Ca uptake by rat brain synaptosomes. The increase in ^{45}Ca uptake through the action of arecoline is connected with the activation of Na channels. It may exert a deleterious effect on the dental enamel (Riker, 1958). It is used as veterinery medicine for taenoids.

As the literature is very poor on the biological action of alkaloids of arecanut the present study has been taken up to see the Chromosome breaking ability of arecoline a major alkaloid of arecanut. Certain plant alkaloids are both mutagenic as well as carcinogenic (vide supra). Synthetic arecoline induced lung tumors in 20% animals (ICMR Bulletin, 1978). Recently the cell transformation ability, an assay that is good predictor of animal carcinogenicity, of arecoline was reported by Ashby et al. (1979).

Mutation has long been suggested to be the mechanism of carcinogenesis. The hypothesis that cancer is induced by alteration of the primary structure of DNA seems to be well supported by the correlation of the carcinogenic and mutagenic effects of many compounds. There is a high correlation between carcinogenicity and mutagenicity: 90% of carcinogens are mutagenic (McCann, 1975). So it is worthwhile to see the mutagenic effects of arecoline on the mammalian chromosome and this could shed light on the possible carcinogenic effect of betelnut alkaloids. Hence this study has been taken up.



(Important chemical constituents of betelnut)

FIG. 2

MATERIALS AND METHODS

A. ANIMALS:-

Swiss albino mice were used in these experiments. Young adult animals of both the sexes (10-12 week old) were obtained from All India Institute of Medical Sciences Experimental Animal Facility, New Delhi. The animals were kept in aluminium cageswith rice husk and maintained in air conditioned room. They were given Hindustan Lever mouse feed and fresh water ad libitum.

B. ARECOLINE INJECTION:-

Arecoline was obtained from Sigma Chemical Company, U.S.A. Solution was prepared by dissolving the chemical in physiological saline at the concentration of 5 mg/ml. Fresh solution was used every time. The solution was prepared in an amber coloured bottle to protect it from light.

C. EXPERIMENTAL DESIGN:-

Table-II shows the experimental design. The doses of arecoline administered were 2 mg, 1 mg, 0.5 mg and 0.25 mg. The time of exposure were 10, 20 and 30 days for each dose. Six animals of both the sexes were taken for each set of experiment. The animals were injected with arecoline stock solution daily by intra peritoneal route (i.p.) at the dose

TABLE - II

Particulars of the Experimental Design

(A)

Experiment	No. of animals used & sex	Concentration of dose injected	Days of Exposure	Remarks
Expt-I	3♂ +3♀	1mg + 1mg	10 days	-
Expt-II	3♂ + 3♀	1mg	10 days	-
Expt-III	3♂ + 3♀	.5mg	10 days	-
Expt-IV	3♂ + 3♀	.25mg	10 days	-
Expt-V	3♂ + 3♀	Control	10 days	-

(B)

Experiment	No. of animals used & sex	Concentration of dose injected	Days of Exposure	Remarks
Expt-I	3♂ + 3♀	1 mg + 1mg	20 days	-
Expt-II	3♂ + 3♀	1 mg	20 days	-
Expt-III	3♂ + 3♀	.5 mg	20 days	-
Expt-IV	3♂ + 3♀	.25 mg	20 days	-
Expt-V	3♂ + 3♀	Control	20 days	-

(C)

Experiment	No. of animals used & sex	Concentration of dose injected	Days of Exposure	Remarks
Expt-I	3♂ + 3♀	1mg + 1mg	30 days	-
Expt-II	3♂ + 3♀	1mg	30 days	-
Expt-III	3♂ + 3♀	.5mg	30 days	-
Expt-IV	3♂ + 3♀	.25mg	30 days	-
Expt-V	3♂ + 3♀	Control	30 days	-

levels of 2 mg, 1 mg, 0.5 mg and 0.25 mg. Only in case of 2 mg dose level, two injections (1 mg each) were given with a gap of 6 hrs in order to avoid animal mortality.

Intraperitoneal injections of saline were given to the control animals.

D. MITOTIC ARREST AND SACRIFICE:-

An i.p. injection of colchicine (Sigma Chemical Co., U.S.A.) was given to inhibit mitoses on animals on the respective days of sacrifice. The colchicine was prepared in physiological saline at the concentration of 4 mg/ml and injected at a dose of 1 ml/100 g of body weight.

The animals were sacrificed by cervical dislocation 2 hr after injection of the colchicine and 10/20/30 days after chemical treatment or saline water injection for control series.

E. BONE MARROW CELL RECOVERY:-

Skin and muscle tissue were removed from both femora and humeri immediately after sacrifice. The bones were punctured at both the ends by needle. The bone marrow was flushed out through a 24-gauge needle containing physiological saline into a 15 ml centrifuge tube. This process was done twice. Then material were mixed vigorously by an 18 gauge

needle to assure dissociation of the cells. Then a final volume of 7 ml was attained by adding physiological saline.

F. INCUBATION AND HARVEST:-

The cell-saline mixture was centrifuged in a centrifuge (ZANETZKI-K70) using swinging out rotor at 1000 rpm for 10 minutes and the supernatant fraction was discarded. The material was agitated properly after centrifugation. A seven millilitre hypotonic solution of 0.075 M KCl was added to the residual cell pellet and the material was mixed thoroughly with a disposable pasteur pipette. All the fat mass was discarded by using the pipette. The cells were incubated in a water bath maintained at 37°C for 25 minutes and then recentrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and material was agitated vigorously. Freezer-chilled Carnoy's fixative (methanol: acetic acid, 3:1) was added to give a total volume of 5 ml/ tube. The cells were resuspended and then refrigerated at 4°C for 2 hrs. The cells were again centrifuged for 10 minutes at 1000 rpm and the supernatant was discarded. Fresh Carnoy's solution was added to give a volume of 5 ml/ tube and the cells were resuspended; then the tubes were refrigerated for 24 hr at 4°C before slide preparation was started.

G. SLIDE PREPARATION:-

Greese free cleared slides were dipped into chromic acid overnight and washed thoroughly in tap water. These were again dipped in soap water for half-an-hour and washed properly in tap water andkept for 2 hrs. in running water. The slides were rinsed in distilled water and fresh distilled water was added and kept on freezer before cell spreading over it.

The fixed cells were centrifuged for 10 minutes at 1000 rpm, the supernatant was discarded; and 0.8-1.0 ml fresh Carnoy's solution was added to each tube depending upon the quantity of the cells. The cells were resuspended and then dropped from two feet high on slides that had been chilled in ice bucket after taking out from freezer. The fixative content was wiped out from the under surface of slide and kept for 2 to 3 minutes on a hot plate maintained at 60°C. Slides were taken out and placed in a slide box for about 24 hrs.

H. STAINING AND MOUNTING OF SLIDES:-

Slides were stained by 10% Geimsa prepared by phosphate buffer (p^H . 6.8) solution for 20 to 30 minutes. These slides were then rinsed in the same buffer and dried. Dehydration was done with double washes of acetone, acetone : Xylol (1:1) and pure xylol and mounted by DPX mount. 100 metaphase spreads per animal were studied.

I. CRITERIA FOR THE STUDY OF CHROMOSOMAL ABNORMALITIES:-

In order to keep the number of errors as small as possible it is necessary to limit one self to a small number of distinctly identifiable aberration types.

The following chromatid aberrations were taken into consideration:

Chromatid break:-

A chromatid break is only diagnonised if a distal fragment is dislocated.

Chromosome break:-

If two chromatids are dislocated completely.

Translocation chromosome:-

The translocation chromosomes can only be recognized:

a) if they are considerably larger or smaller than normal chromosome;

b) if the structure of the acrocentric chromosome has been altered to metacentric, dicentric or polycentric chromosome.

Ring chromosome:-

Formation of ring chromosome is easily identifiable having two fragments or one joined fragment with one ring.

Inversion:-

Only pericentric inversion are recognisable. These lead to metacentric chromosome whose length remains unchanged. The occurrence of a metacentric chromosome of approximately normal size makes a distinction between a translocation and a pericentric inversion impossible.

Deletions:-

Deletions and deficiencies are not recognisable. To avoid bias in the results, every well spread and complete metaphase was analysed. The chromosomes are required to be easily countable, or, on cells with multiple aberration the single elements have to be well distinguished. Almost all metaphases fulfilled these requirements.

RESULTS

TREATED SERIES:

1. Toxicity:- Arecoline is highly neurotoxic. After i.p. injection of it, violent shivering, salivation, inhibition of spontaneous activity were noticed. After 30 minutes of injection the animals came back to normal condition.

2. Mortality:- Lower doses like 0.25 mg, 0.5 mg and 1.0 mg did not kill the animals. But a single dose of 2 mg was fatal to many animals, hence this dose was fractionated into two injections with a gap of six hours.

3. Mitotic Index:-

Mitotic indices were not investigated though they could have served as an indicator of the effect of the arecoline treatment. On account of the arrangement of the experiment, in which the stimulation of some marrow was variable, these values could not be compared. But in general the mitotic index decreased considerably when the days of exposure increased.

4. Morphological Studies on Chromosome Aberrations:-

Different types of chromosomal abnormalities such as chromatid break, chromosome break, ring chromosomes, cells with multiple breaks and pulvurization of chromosomal compliments were observed. The data of chromosomal analysis are presented in Table-III, IV & V. It has been found that no

TABLE - III. Showing Different types of aberrations at 10 days of exposure to arecoline.

S.L.	DOSE	No. of animals treated	No. of Cells Studied	Type of aberrations						Mean of number of damaged cells	Total No. of damaged cells
				Chromatid break	Chromosome break	Ring	Multiple break	Pulverization	Ploidy		
1	2 mg	6	600	70	10	2	6	4	0	15.33± 0.76 S.E.	92
2	1 mg	6	600	57	9	1	1	1	0	11.5± 1.3 S.E.	69
3	0.5 mg	6	600	18	1	0	0	0	0	3.16± 1.06 S.E.	19
4	0.25 mg	6	600	13	0	0	0	0	0	2.16± 0.30 S.E.	13
5	Control	6	600	5	0	0	0	0	0	.833± 0.28 S.E.	5

TABLE - IV Showing different type of aberrations at 20 days of exposure to arecoline.

S.L.	DOSE	No. of animals treated	No. of Cells studied	Type of Aberrations						Mean of number of damaged cell	Total No. of damaged cells
				Chromatid break	Chromosome break	Ring	Multiple break	Pulverization	Ploidy		
1	2 mg	6	600	60	6	0	4	5	0	12.5± 1.61 S.E.	75
2	1 mg	6	600	15	4	1	3	0	0	3.83± 0.65 S.E.	23
3	0.5 mg	6	600	17	0	0	0	1	0	3 ± 0.63 S.E.	18
4	0.25 mg	6	600	10	0	0	1	0	0	1.83± 0.40 S.E.	11
5	Control	6	600	6	0	0	0	0	0	1 ± 0.25 S.E.	6

TABLE - V Showing different type of aberrations at 30 days of exposure to arecoline.

S.L.	DOSE	No. of animals treated	No. of Cells studied	Type of Aberrations						Mean of number of damaged cells	Total No. of damaged cells
				Chromatid break	Chromo-some break	Ring	Multi-ple break	Pulvu-rization	Ploidy		
1	2 mg	2*	200	16	2	0	1	1	0	10±1 S.E.	20
2	1 mg	6	600	20	1	0	0	0	1	3.66± 0.76 S.E.	22
3	.5 mg	6	600	16	1	0	1	0	0	3 ± 0.36 S.E.	18
4	.25 mg	6	600	6	0	0	1	0	0	1.16± 0.30 S.E.	7
5	Control	6	600	6	0	0	0	0	0	1 ± 0.25 S.E.	6

* 4 animals died towards the end of follow up period.

sex dependent differences in the frequencies of chromosomal aberration exists. The aberrations like gap is not taken into account because of its controversy though several cases of gaps were observed (Fig. 8).

(a) Chromatid break:-

Chromatid breaks are observed at almost all dose levels of arecoline treatment:

i) 2 mg dose level: In 10 days of exposure 10 cases of chromatid breaks were observed which is highest in number among different doses and days of exposure. At 20 days of exposure 60 cases of chromatid breaks were observed. At 30 days of exposure the frequency of chromatid breaks are less. Only 16 cases of chromatid breaks are seen.

ii) 1 mg dose level: The frequency of chromatid breaks is less than the breaks observed in the highest dose. In 10 days of exposure 57 cases, in 20 days 15 cases and in 30 days 20 cases of chromatid breaks are seen.

iii) 0.5 mg dose level: The frequency of chromatid breaks are much less than that seen in the above dose. In 10 days of exposure 18 cases, in 20 days of exposure 17 cases and 30 days 13 cases of chromatid breaks are seen.

iv) 0.25 mg dose level: Here the lowest number of chromatid breaks are seen. In 10, 20 and 30 days of arecoline exposure the chromatid breaks are 13, 10 and 6 respectively.

Almost 86.4% of the aberrations observed are of the chromatid break type and rest are other types of chromosomal aberrations. Random breaks are observed on the chromatid either at proximal, middle or distal end of the chromatid. The frequency of single break per cell is more (Fig. 11) than double breaks per cell (Fig. 8). Highest number of breaks are obtained in the highest dose (2 mg) on 10 days of exposure. The frequency of breaks decreases when the exposure time increases and the dose of chemical decreases.

(b) Chromosome Breaks:-

Chromosome breaks are observed at almost all dose levels of arecoline but not at all the intervals of observation. The frequency of chromosome breaks is less than chromatid breaks. The results are as follows:

i) 2 mg dose level: At 10 days of exposure 10 cases of chromosome breaks are noted which is the highest among different groups. In 10 and 30 days of exposure only 6 and 2 cases of chromosome breaks are seen respectively.

ii) 1 mg dose level: The frequency of chromosome breaks is slightly less than that seen at the highest dose level. At 10 days of exposure 9 cases of chromosome breaks are seen which is highest among different groups as compared to other intervals at this dose level. In 20 and 30 days of exposure of chemicals only 4 and single case of chromosome breaks are seen respectively.

iii) 0.5 mg dose level: Here only 2 cases of chromosome breaks are noticed. At 10 and 30 days of exposure single chromosome break in each case is observed. However, at 20 days not a single chromosome break is observed.

iv) 0.25 mg dose level: Chromosome break frequency is very less at this dose level. Only at 30 days of exposure one chromosome break has been seen. At 10 and 20 days not a single case of break could be observed.

Thus only 9.51% of chromosome breaks are observed. There is no regional specificity of break. It is either at proximal, middle or distal part of the chromosome. In one cell both chromosome and chromatid breaks are observed (Fig. 7). But in no case more than one chromosome break per cell is seen. In most of the cases the breaks are identical and at a corresponding site. But only in one case both the chromatids are broken at different position.

(c) Cells with Multiple Breaks:-

The incidence of multiple breaks is less than that of the chromatid and chromosome breaks.

i) 2 mg dose level: Highest number of cells having multiple breaks are seen only at this dose level. At 10 days of exposure 6 cases of multiple breaks are seen. At 20 and 30 days of exposure 4 and one case of multiple breaks have been seen respectively.

ii) 1 mg dose level: At this dose level the frequency of multiple breaks is less than that seen at the above dose level. It includes only 4 cases of multiple breaks. One is in 10 days of exposure and other three are in 20 days of exposure. In 30 days of exposure no case of multiple break cell has been observed.

iii) 0.5 mg dose level: Only single case of multiple break cell is seen at 30 days of exposure in this dose. At 10 and 20 days not a single case of multiple break cell has been observed.

iv) 0.25 mg dose level: Here also only single cell has been observed having multiple break at 20 days of exposure. At 10 and 30 days of exposure no cell having multiple break was seen.

Thus 4.61% of cells having multiple breaks have been seen. It includes number of chromatid as well as chromosome breaks (Fig. 10). Highest number of multiple breaks are seen in the highest dose level at 10 days of exposure and the frequency decreases as the dose of chemical decreases.

(d) Ring Type of Chromosomes:-

Ring type chromosome structures are also observed after arecoline treatment although the frequency is very less:

i) 2 mg dose level: Only 2 cases of ring formation are observed in 10 days of chemical exposure. But in 20 and 30 days not a single case of ring is observed.

ii) 1 mg dose level: One in each case of ring structure has been observed at 10 and 20 days of chemical exposure. At 30 days not a single ring is seen.

iii) 0.5 mg dose level: No case of ring structure is observed at any interval of observation.

iv) 0.25 mg dose level: Here also not a single ring chromosome is seen at any interval.

In total 4 cases of ring structure are seen and its percentage is only 1.03. Single cell having one ring and one fragment are also seen (Fig. 9). In no case cell having more than one ring has been observed. Ring chromosomes are

observed only at highest dose. Below 1 mg dose level not a single ring is observed.

(e) Pulvurized Chromosomes:-

The frequency of cells with pulvurized chromosomes is higher than that of the ring chromosomes and slightly less than that of the multiple breaks:

i) 2 mg dose level: Highest number of cells with pulvurized chromosomes are observed at this dose level. At 10, 20, and 30 days of exposure 4, 5 and 1 cases of cells with pulvurized chromosomes are seen respectively.

ii) 1 mg dose level: Single cell with pulvurized chromosome is seen at 10 days. At 20 and 30 days no case of pulvurized chromosomes is seen.

iii) 0.5 mg dose level: Here also single cell having pulvurized chromosomal compliments is observed at 20 days of exposure. No cell with pulvurized chromosomes is seen at 10 and 30 days of exposure.

iv) 0.25 mg dose level: Not a single cell having pulvurized chromosomal compliments is seen at this dose level at different intervals of observation.

3.10% of cells with pulvurized chromosome are seen. In some cases, except one or two chromosomes (Fig. 12) rest

of the chromosomes are fragmented, while as in others all the chromosomes are fragmented. The frequency of cells with pulvurized chromosome increases as the dose of the chemical increases.

(f) Single case of tetraploid cell is observed in 1 mg dose level at 30 days of exposure.

Besides the above effects in several cases C-mitotic effect and corrosiveness of chromosomes are observed.

5. Variation of aberrent metaphase cells in bone marrow after i.p. injection of arecoline:-

Aberrant metaphase cells in the bone marrow at 10, 20 and 30 days after chronic i.p. injection of 2 mg, 1 mg, 0.5 mg and 0.25 mg of arecoline are studied. The aberrant metaphase cells began to increase at 10 days after chronic injection of arecoline and gradually decrease as the days of treatment increases which is well depicted by the Fig. 3 .

6. Dose-response relationship:-

The effect of arecoline on the chromosomes of metaphase cells shows very good dose-response relationship. The percentage of chromosomal abnormalities increases as the dose of arecoline increases. The dose response relationship is evident from the Figs. 4,5&6.

Fig. No. 3. Variation of affected metaphase cells (%) in the bone marrow after i.p. injection of arecoline at the concentration of 2 mg, 1 mg, 0.5 mg and 0.25 mg contained in saline. Here control is taken as 100 percent.

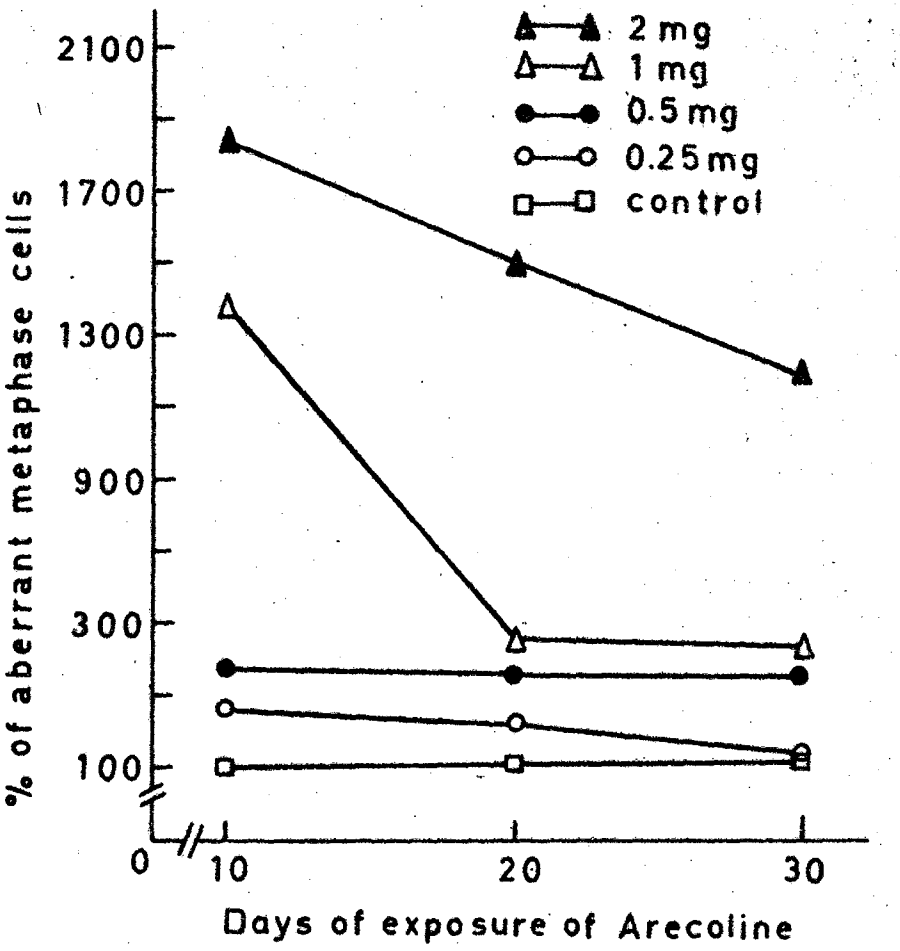

2 mg
1 mg
0.5 mg
0.25 mg
control
2100
1700
1300
900
300
100
0
% of aberrant metaphase cells
10
20
30
Days of exposure of Arecoline

Fig. No. 4. Percentage of aberrant cells at 10 days after administration of 2 mg, 1 mg, 0.5 mg and 0.25 mg of arecoline taking control as 100 percent.

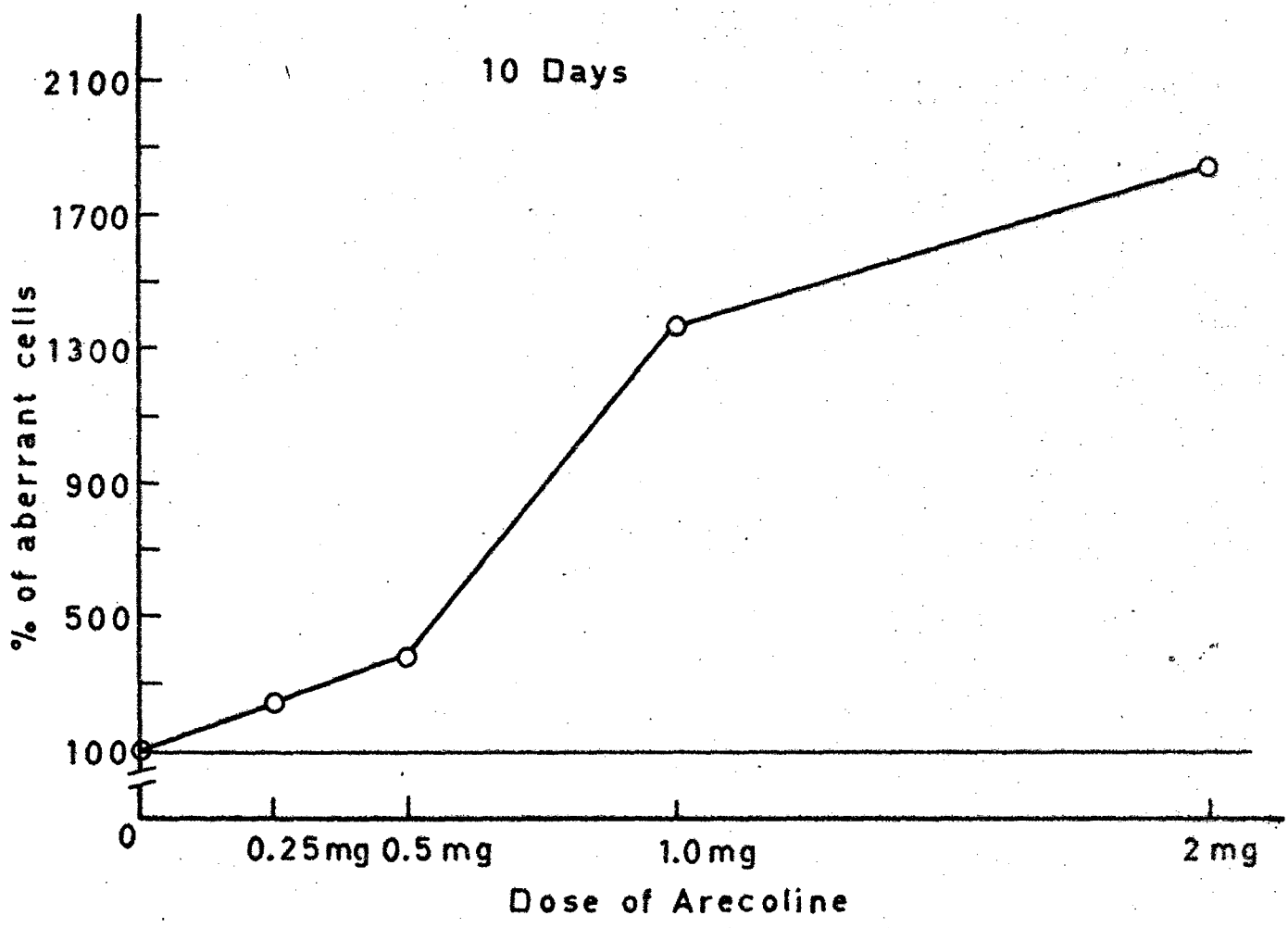

10 Days
2100
1700
1300
900
500
100
0
% of aberrant cells
0.25mg
0.5 mg
1.0mg
2 mg
Dose of Arecoline

Fig. No. 5 . Percentage of aberrant cells at 20 days after administration of 2 mg, 1 mg, 0.5 mg and 0.25 mg of arecoline taking control as 100 percent.

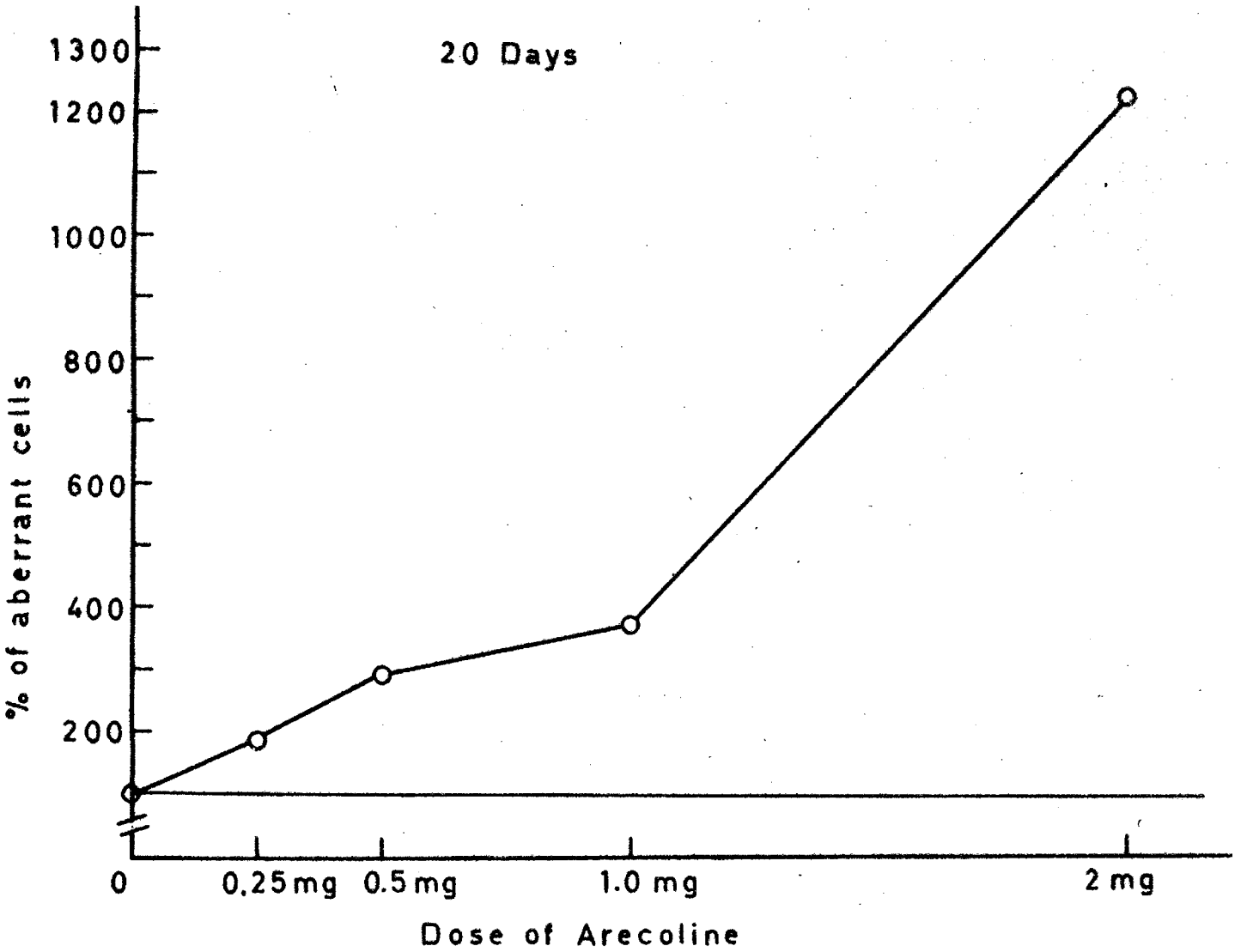
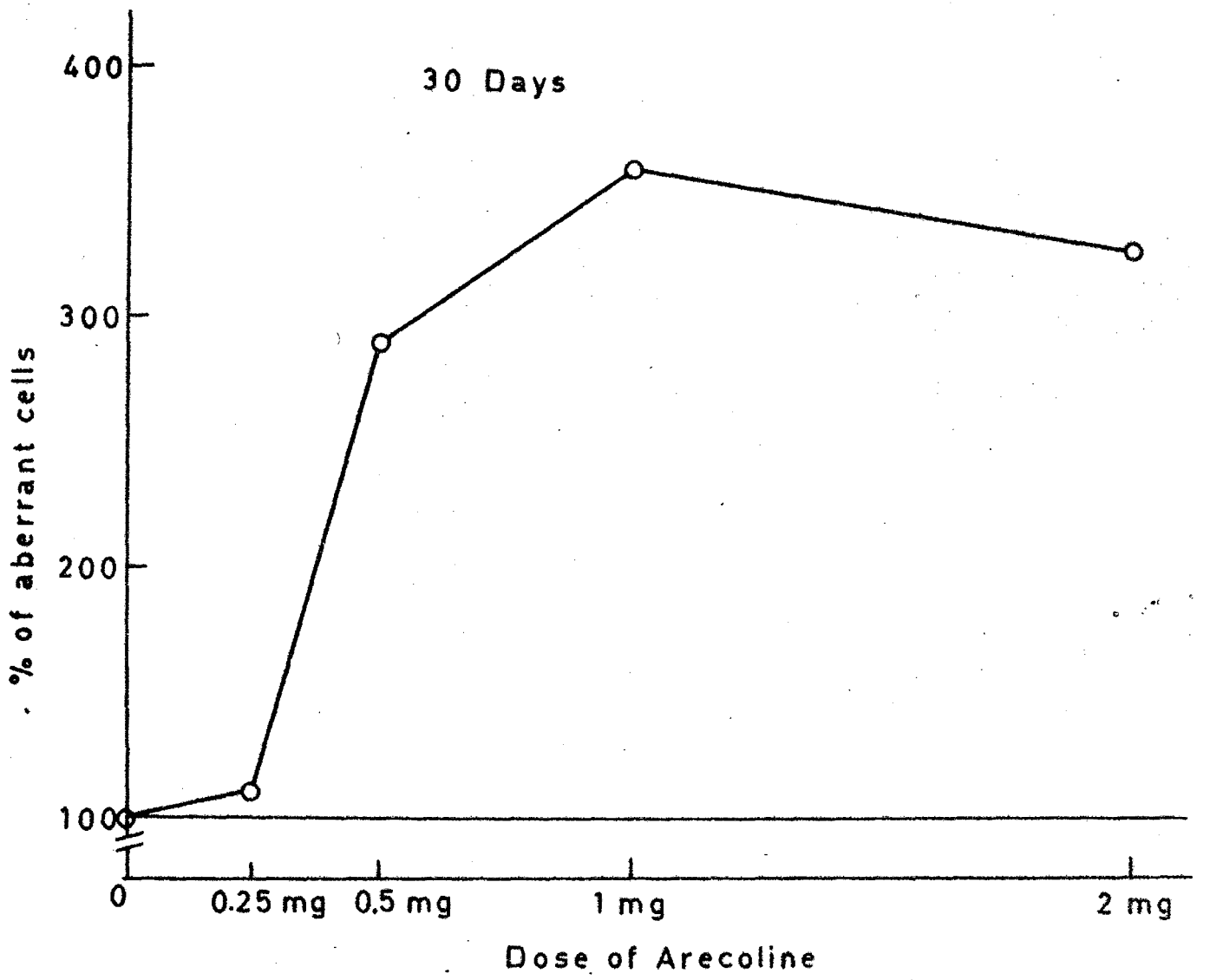

20 Days
1300
1200
1000
800
600
400
200
% of aberrant cells
0
0.25mg
0.5mg
1.0 mg
2 mg
Dose of Arecoline

Fig. No. 6. Percentage of aberrant cells at 30 days after administration of 2 mg, 1 mg, 0.5 mg and 0.25 mg of arecoline taking control as 100 percent.

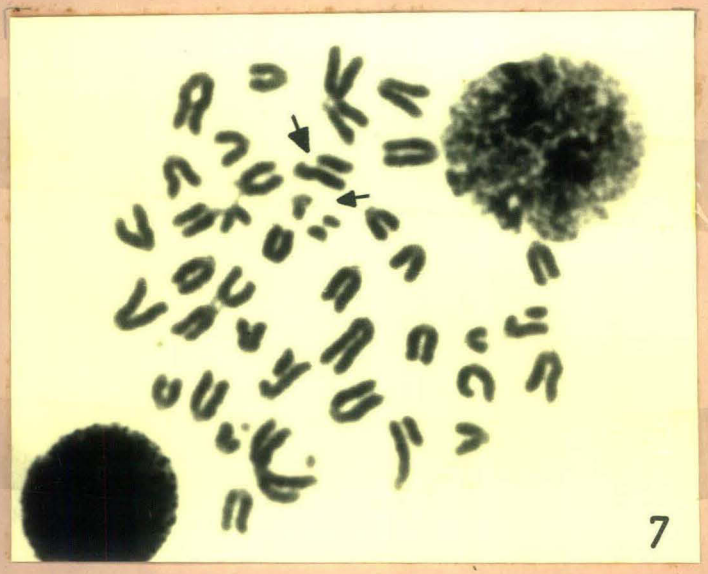

30 Days
400
300
200
100
% of aberrant cells
0
0.25 mg
0.5 mg
1 mg
2 mg
Dose of Arecoline

CONTROL SERIES:

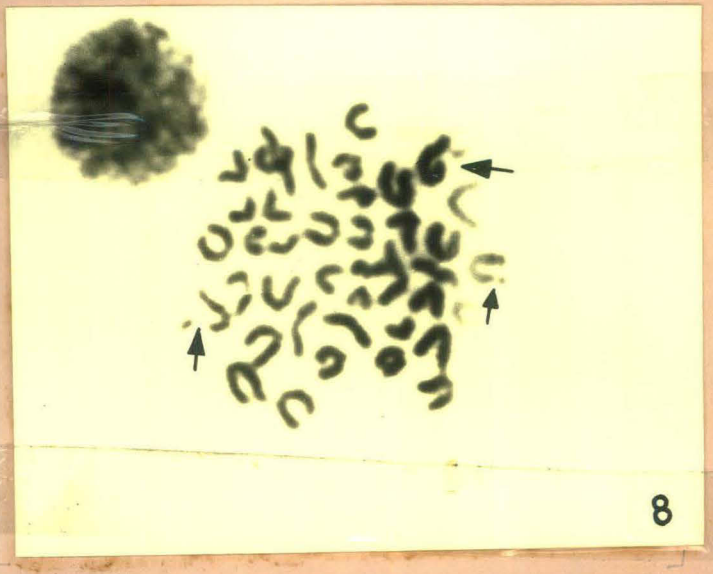
In the control series of experiment very few cases of abnormalities are seen. The aberrations are confined to only chromatid break types. At 10 days of saline treatment only 5 cases of chromatid breaks are seen. At 20 days and 30 days 6 cases of chromatid breaks in each set are observed. In all 0.944% of abnormalities are observed in the control set of experiments which is very less in comparison to the treated series.

Fig. 7. Chromosome & chromatid breaks.(x 3750)

Fig. 8. Two chromatid breaks and one gap.(x 3750)



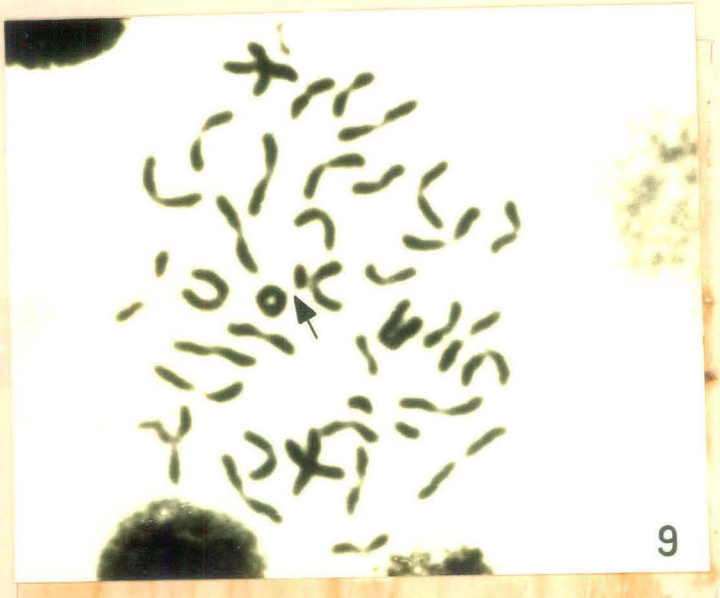
7



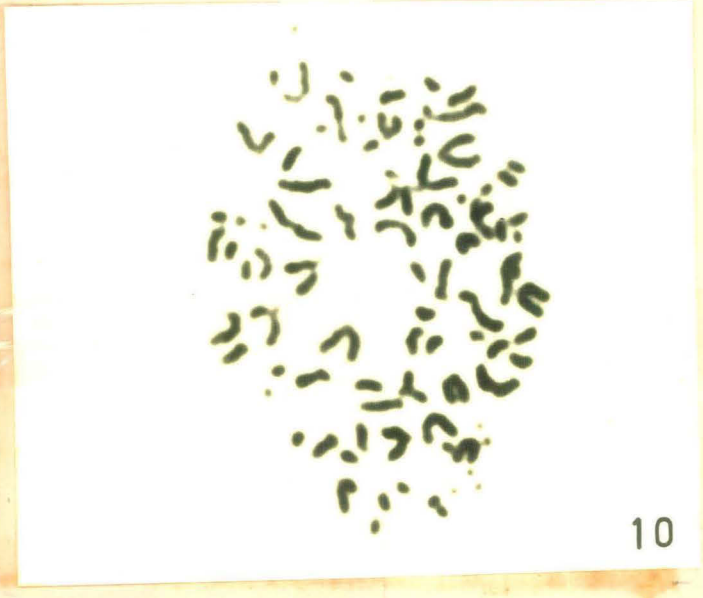
8

Fig. 9. Ring structure & fragment.(X 3750)

Fig. 10. Multiple breaks of chromosomal compliments.(X 3750)



9



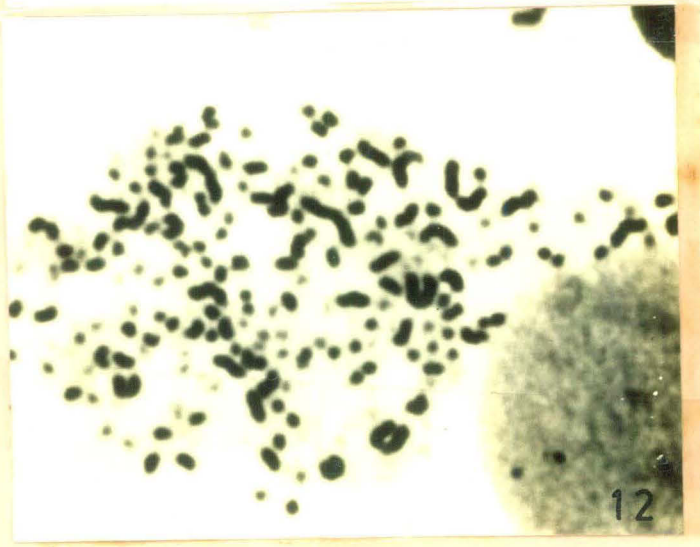
10

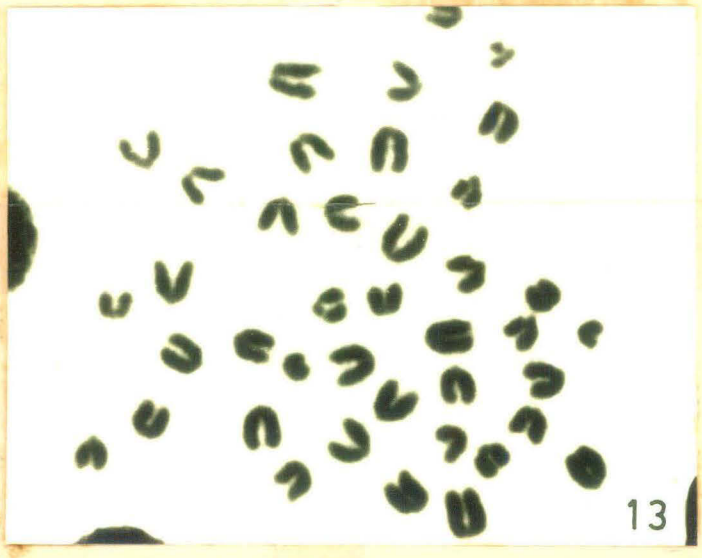
Fig. 11. Single chromatid break.(x 3750)

Fig. 12. Pulvurization of chromosomal compliments.(x3750)

Fig. 13. Normal metaphase plate (2n = 40)(x 3750)


11


12


13

DISCUSSION

Present study clearly demonstrates that administration of arecoline to mice would elicit several varieties of chromosomal aberrations in the bone-marrow cells. Control animals receiving only the vehicle (physiological saline) did not show any appreciable degree of aberrations in the bone marrow cell chromosomes.

Earlier also some evidences have been reported that some plant alkaloids do have similar type of chromosome breaking ability in different test materials including bone marrow. Alkaloid heliotrine has the mutagenic activity in *Drosophila* (Clark, 1959; Brink, 1965, 1969) and chromosomal aberrations in the root tip cells of *Allium cepa* (Avanzi, 1963) and in rat lung cells in culture (Umeda et al., 1977; Hitoshi et al., 1980). Gladwin (1977) observed significant number of aberrations in the bone marrow preparation after treatment with higher doses of ergot derivatives. Other pyrrolizidine alkaloids like lasiocarpine, petasitenine, senkirkine, clivorine, LX-201, fukinotoxin, ligulanidine and monocrotaline have been tested to be mutagenic in the *Salmonella*/mammalian microsome test and modified *Salmonella* microsome test (Green et al., 1975; Koletsky, 1978; Hiromichi et al., 1979; Williams, 1980). Hitoshi et al. (1980) have reported the chromosomal aberration and mutation in cultured mammalian cells exposed

to pyrrolizidine alkaloids, heliotrine, petasitenine, lasiocarpine and senkirkine. The Vinca rosea alkaloid, Vinblastine also induces various chromosomal aberrations - thin unreplicated chromosomes, chromosomal segments and chromosome pulvurization of various extents (Palyi, 1969). After treatment of mice in vivo, vinblastine and vincristine gave a positive response in the micronucleus test (Heddle et al., 1977 and Maier et al., 1976). Rapora (1978) reported a significant increase in sister chromatid exchange on exposure to higher dose of vincristine.

Gebhart et al. (1969) observed gaps and breaks and few exchanges induced by vincristine. However, the effect on chromosomes in bone marrow of a hamster treated with vincristine remained negative even at high dose (Muller et al., 1978).

Present investigation documents a number of chromosomal aberrations induced by different doses of arecoline in mouse bone marrow cells. These structural lesions include chromatid breaks, chromosome break, cells with multiple breaks of chromosome, ring shaped chromosome and cells with pulvurized chromosome compliments. Mostly the aberrations are chromatid break type (86.41%). Besides the chromatid breaks, chromosome breaks are also observed indicating the effect of chemical both at G_1 and G_2 phases of cell cycle. Appearance of

pulvurized chromosomes indicates the drastic effect of arecoline on the genetic material.

The dose-response relationship at 10 and 20 days intervals is nearly linear. At 30 days interval it is linear up to 1.0 mg dose level but beyond this dose there is decline in the response. Well, it has to be admitted here that at 2 mg dose level at this interval only two animals (the rest died toward the end of study due to excessive loss of body weight) were available for screening the bone marrow and this may not be sufficient to conclusively demonstrate that at higher doses there is decline in aberrations.

Another interesting thing is that longer the duration of treatment for any given dose of arecoline, lesser is the damage to the chromosome. This may be due to the following reason:

Bone marrow is a cell renewal system comprising progenitor and multiplier compartments. The toxic and cytogenetic effects of arecoline in different cell populations of bone marrow could be different. It has already been demonstrated that chronic administration of arecoline to mice would lead to decline in the number of cells in the functional compartment (blood) of this renewal system (Shahabuddin, 1978). If the cell-damaging and/or chromosome-

damaging action of arecoline for any given dose varies with segment of the cell renewal assembly line of bone marrow, we could deduce that the observed decline of chromosomal aberrations at later intervals might be due to depletion and/or non-production of cells population which are highly sensitive to arecoline. This explanation, of course, needs emphorical demonstration.

Another possibility, although seems to be very remote, is that chronic administration of arecoline augments the efficiency of repair mechaniams involved in normalization of chromosomal structures after exposure to toxic substances. This also needs experimental verification.

The highest number of aberrations are reported at the highest dose (13.35%) which is significantly more than that in the control series (.94%). But it is less than the chromosomal aberrations manifested by the treatment of highly alkylating agents like cyclophosphamide, MMS, and EMS. So it is assumed that though arecoline has the chromosome breaking capacity yet it is very weak mutagenic agent like other alkaloids (vide supra).

Arecoline interferes with the biosynthesis of macromolecules. It decreases the incorporation of ^{3}H-thymidine in muscle and kidney tissue which may finally inhibit DNA synthesis. However, DNA synthesis is increased in liver

and lung tissue (Shivapurkar, 1979). Sedgley (1968) reported that the alkaloid vinblastine decreased the rate of synthesis of DNA but did not inhibit the capacity for the cells to synthesize new DNA. Fraysainet et al. (1969) have reported that DNA synthesis is inhibited by inhibiting the incorporation of ^{3}H-thymidine by the injection of lasiocarpine. Lasiocarpine strongly inhibits polymerase activity.

It has been reported that the net RNA levels were decreased in arecoline treated mice. This decrease is probably due to a very significant increase in RNase activity (Shivpurkar et al., 1979). The uptake of ^{3}H-uridine into soluble RNA of Ehrlich ascites cells recovered from mice treated with vinblastine depressed by about 80% compared with the control value. Since the specific activity both uridine and cytidine is reduced, it appears that the alkaloid inhibits the synthesis of the whole chain of soluble RNA (Creasey and Morkin, 1964). Following the administration of lasiocarpine, prominent nuclear abnormalities occur accompanied by a decrease in hepatic RNA and protein content (Svoboda et al., 1966; Reddy et al., 1968). Arecoline also decreases the protein contents of the tissue (Shivpurkar, 1978).

All the above interference may be the manifestation of the breaking of the DNA strand by arecoline.

Arecoline is a monofunctional alkylating agent as it loses only one of its methyl group during metabolism (Boyland, 1969) and by virtue of its addition reaction across the reactive Δ^3 - ethylenic bond (Nery, 1971). This methyl group may alkylate the DNA strand. It has been reported that alkaloids can bind with nucleic acids, but the bond disappear at high ionic strength indicating that they are weak electrostatic bonds (Semmel, 1971). Mattocks (1969) has reported the alkylating capacity of pyrrolizidine alkaloids such as retrosine, monocrotaline, lasiocarpine and heliotrine. McLean (1970) also postulated the theoretical mechanism of alkylation and chromosome breaking effect of pyrrolizidine (Senecio) alkaloids. The breaking of the chromosomes were due to the cross linkage of DNA strand by bifunctional group of the alkaloids. Although some monofunctional agents are excellent chromosome breaker (Smith et al., 1955; Swanson, C.P. et al., 1959).

Boyland and Nery (1969) reported that in aqueous media there was no evidence of reaction between arecoline and the bases of the nucleic acids; again they had mentioned that such reactions might occur <u>in vivo</u> because of the presence of microsomal enzymes. Fortunately, in 1971, Nery had reported the binding of arecoline with nucleic acids and protein.

Possible Mechanism of Alkylation and Chromosome Break

Biological alkylating agents are chemicals that transfers alkyl groups to biologically important macromolecules and accepts one hydrogen atom under physiological condition. It has been investigated in many biological systems (Review-Ross, 1962; Lawley, 1966; Loveless, 1966; Singer, 1975).

Many sites of DNA are very prone to being attacked by alkylating agents. The numerous sites of reactions in nucleic acids for monofunctional alkylating agents are now known to include the following: in adenine residues, N-1, N-3 and N-7; in guanine, N-3, N-7 and O-6; in cytosine, N-1, N-3 and O-2 and in thymine N-3 and O-4.

Arecoline being a monofunctional alkylating agent may react on the above mentioned site. The major site of reaction for many alkylating agent is the N-7 position of guanine and some times accounts for upto 90% of the total alkylatinn.

Secondary effect of the alkylation (methylation) of guanine, is the loosening of the bond between the base (guanine) and the sugar phosphate chain leading to the removal of purine base (guanine) forming apurinic gaps. This depurination may lead to the breakage of the chromosome.

But at this stage it is very difficult to say actually in which base it is reacting and in this area much work is needed.

Probable Mechanism of Carcinogenesis:-

Day by day data on the role of alkaloids on carcinogenesis are accumulating although there is no clear cut picture of the alkaloids causing cancer. The evidence has been reviewed by Barnes and Schoental (1958), Schoental (1968), Bull et al. (1968) and Styles et al. (1960). Campbell (1956) reported the incidence of primary liver tumor in the fowl injected with Ragwort alkaloid senecionine. Harris and Chen (1970) again reported the liver tumors, intrahepatic metastasis, pulmonary metastasis and hepatic angiosarcomas with multiple nodules after prolonged administration of dried Senecio longilobus in the diet of rats. The role of epoxides in carcinogenecity by pyrrolizidine alkaloids were discussed by Schoental (1970). Svoboda & Reddy (1972) observed 61% of hepatocellular carcinoma and 33% of squamous cell carcinoma of skin of rat treated with Lasiocarpine. They also reported that the tumors could be transplanted successfully through five generations. Schoental (1975) reported the pancreatic islet cell and other tumors in rats given heliotrine a monoester pyrrolizidine alkaloid and nicotinamide. He also

observed a single case of adenoma of the pancreatic islet cells only by the injection of heliotrine. Liver tumors were also reported by the administration petasitenine and senkirkine in rat (Hirono et al., 1977; Hirono et al., 1979).

Arecoline produced 20% lung tumors in the mouse (ICMR Bull. 1978). In 1979, Ashby et al. proved the carcinogenic ability of arecoline by cell transformation method. But the exact mechanism of tumor is not yet known. The probability of carcinogenesis may be attributed to the following reasons.

Methylation of DNA and later effect of chromosome breakage may be a probable cause of carcinogenesis. There have been numerous attempts to correlate overall methylation of DNA with the incidence of tumors in various tissues (Schoental, 1967; Swan and Magee, 1968; Den Engelse, 1969-70). Number of alkylating agent reacts with the electrophilic sites of DNA, only some of which will cause mutations leading to cancer.

Secondly effects of chemicals on the main molecular biosynthesis play an important role in carcinogenesis. Many of carcinogens inhibit the DNA synthesis and secondary effect like decrease of RNA synthesis and protein content.

Fig. No. 14

I. Arecoline

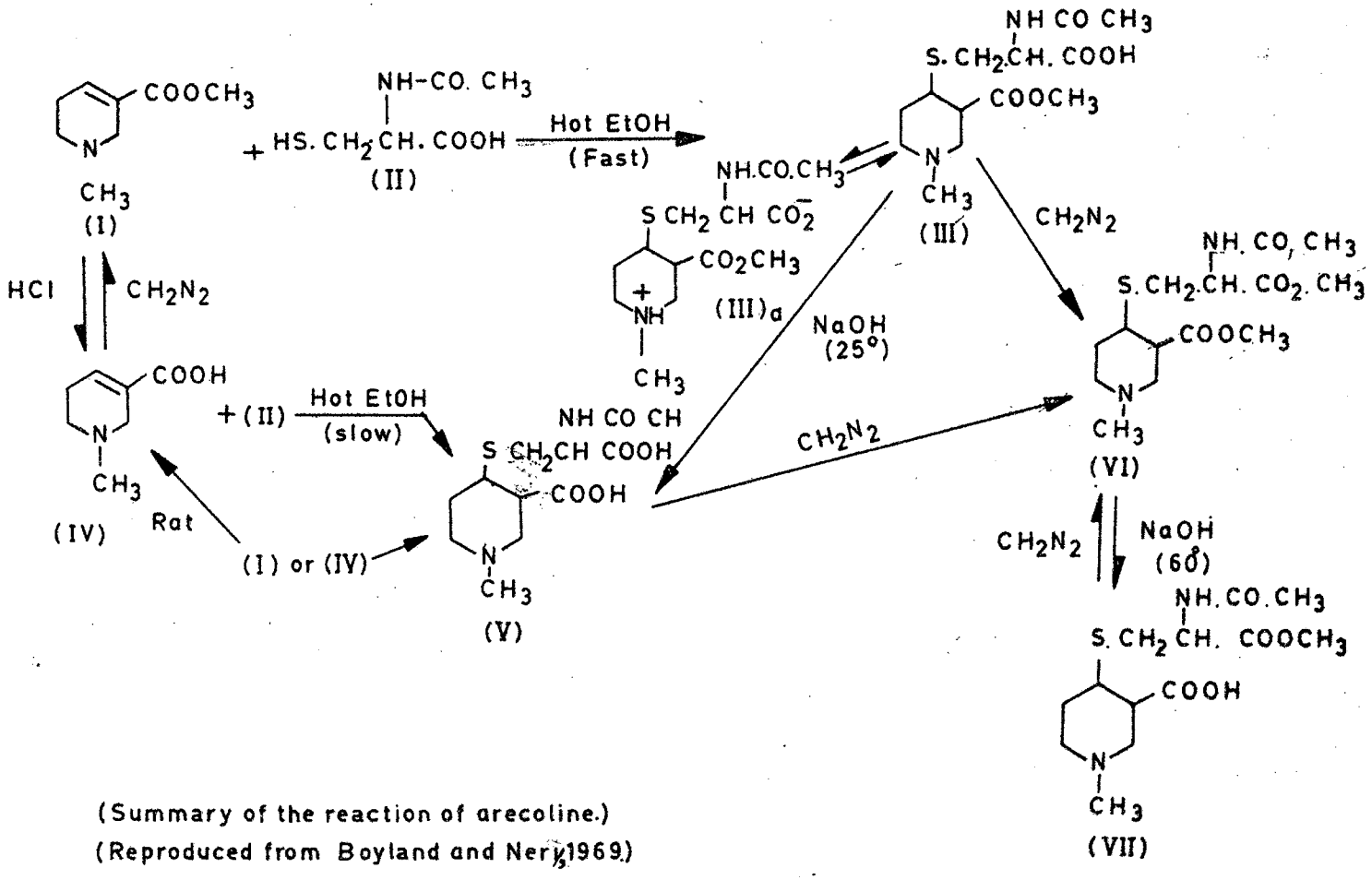
II. Cysteine

III. N-Acetyl-S(3-methoxycarbonyl-1-methylpiperid-4-yl)-L-cysteine

IV. Arecaidine

V. N-Acetyl-S-(3_Carboxy-1-methyl piperid-4-yl)-L-cysteine

VI. N-Acetyl-S-(3-methoxycarbonyl-1-methyl piperid-4-yl)-L-cysteine methylester



(Summary of the reaction of arecoline.)
(Reproduced from Boyland and Nery, 1969.)

It may be due to strand separation or DNA polymerase activity. As mentioned earlier, arecoline inhibits DNA synthesis, and decreases RNA synthesis and protein content.

Several carcinogens possess an activated carbon double bond which is known to react with biologically occurring nucleophiles. Such type of reaction are detected by the isolation of cysteine or methionine adducts (Chasseaud, 1979) and can be taken as an indicator of the established carcinogenecity of the compound. Arecoline is converted into cysteine adduct amongst other metabolites (Nery, 1971; Boyland and Nery, 1969) (Fig.14). Similarly, cysteine adduct has been isolated from rats injected with carcinogen, vinyl chloride (Green and Hathway, 1975). But all the compounds that react with sulphur compounds and forming adducts are not carcinogens (Harrington, 1967).

Lastly, arecoline is metabolised by 1-oxide formation (Nery, 1971). The effect of N-oxidation of tertiary amine drugs is often to increase the biological action of drugs, i.e. the carcinogenic action of aminoquinoline (Arad and Nakayama, 1952) and 4-nitroquinoline (Kawazoe, Tachibana, Aoki and Takahara, 1967). So it may be one of the evidences of carcinogenesis.

Besides the above evidences on mutagenic and carcinogenic properties of arecoline the exact mechanism

is not known. Arecoline as an alkylating agent can be fully confirmed by further mutagenic and carcinogenic experiments in different test materials. Much work has to be done to know the exact position of reaction with the nucleotides of DNA.

CONCLUSION

Arecoline, the alkaloid of betelnut at the given dose levels in the present experiments on mice does not evoke mortality except at 2 mg dose level. Arecoline treatment decreases the motitic index of the bone marrow cells of mice. Arecoline causes the chromosomal aberrations in the bone marrow cells of mice. The aberrations are chromatid break, chromosome break, cells with multiple breaks, ring structure of chromosome and cells with pulvurized chromosomal compliments. The frequency of chromatid break is more. So arecoline is a weak mutagenic chemical.

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* Original is not seen.