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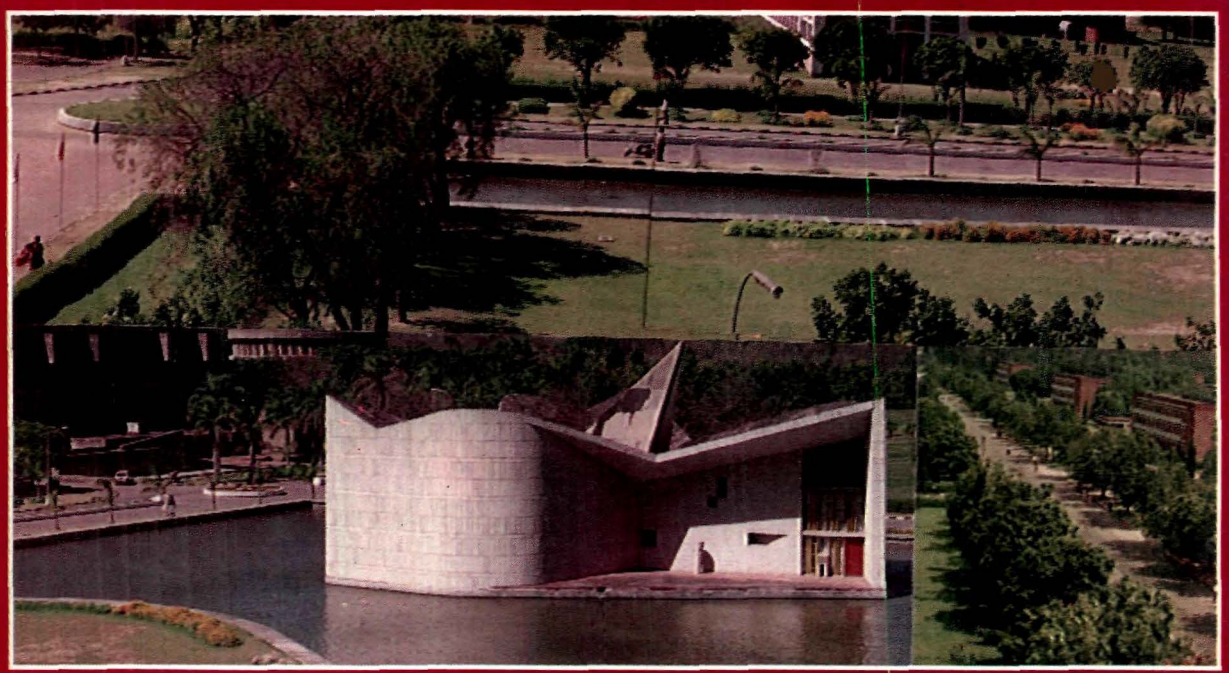

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MOLECULAR BASIS OF METAL CARCINOGENESIS : A REVIEW

H.M. Dani and Parminder Kaur

Department of Biochemistry, Panjab University, Chandigarh -160 014.

Carcinogenic Potentials of Metals for Humans

Metals are ubiquitous in the environment to which human beings are constantly being exposed. Roles played by metals in mankind's development are of immense significance. Industrial and domestic uses of metals have introduced their substantial amounts into the atmosphere and surrounding environment (Zelikoff and Cohen, 1997). General population is also exposed to a number of metals, but at lower levels than industrial population which has been most extensively exposed (Hayes, 1997).

Amongst the list of metallic elements, about 40 are considered as metals of common use out of which compounds of 30 have been known to produce toxicity (Winder *et al.*1997). Metals are probably some of the oldest toxicants known to humans. In human diet, both essential and toxic metals are present simultaneously. Some metals are essential for human nutrition while others enter as contaminants from foodstuffs. All living organisms are found to contain molecules having metals as common moieties which are involved in a wide variety of biological processes (Rojas *et al.* 1999).

Absorption, deposition and excretion of metals are largely dependent on physical factors such as solubility, ionization, particle size and chemical nature. All metals in their ionic forms can accumulate and reach toxic levels in our bodies. This is true even for the most important metal 'iron'. Metals in their ionic forms can act as catalysts and create an avalanche of free radicals. One metal can catalyze thousands of molecules that generate free radicals for the formation of newer free radicals. Finally, cells can be destroyed and therefore no amount of ionic metals in our bodies can be regarded as safe.

Metals are an important emerging class of carcinogens. However, the molecualr basis of metal carcinogenesis is completely shrouded in mystery because several hypotheses (Kasprzak, 1995) like DNA base modification and cross linking, DNA strand breaks, oxidative attack on DNA by active oxygen and other free radicals, enhancement of lipid peroxidation, inhibition of DNA repair, alterations in the structures of transcription factors and DNA methylation to silence genetical activity of genes for normal cell functioning

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have been propounded from time to time but none has been put on a firm footing. Extensive epidemiological evidence exists for three metals namely nickel, chromium and cadmium as human carcinogens. Some others, that are suspected to have carcinogenic potentials in man are lead, beryllium, arsenic, antimony and cobalt; but limited experimental and epidemiological data exist for these metals (Waalkes *et al.* 1992; Hayes, 1997).

Nickel Carcinogenicity

Nickel is a toxic, mutagenic and carcinogenic metal of significant occupational and environmental concern. It is a constituent of over 3000 metal alloys and is used for a huge range of purposes such as in the automobile industry, electronics, coinage (some coins may have 99.8% nickel), stainless steel (contains about 10% nickel), nickel-cadmium batteries, many household products, medical appliances and in the cheap jewellery (IPCS, 1991; Savolainen, 1996).

Nickel is eighth element of first transition series in the Periodic Table. Its electronic configuration is $4s^2 3d^8$ with atomic number 28. Various oxidation states exhibited by nickel are +2, +3 and +4. The existence of the transition elements in different oxidation states means that their atoms can lose different number of electrons.

Biologically nickel is considered to be a new trace element with tissue concentration in ng/g amounts. Although there is substantial evidence for nickel as an essential element for animals, a deficiency state in humans has not been clearly defined. The exposure and toxicity to nickel compounds is based on the human activities associated with them. Nickel carbonyl is a volatile liquid at room temperature and is responsible for almost all cases of acute nickel toxicity (Barceloux, 1999).

NiIII/NiII redox couple facilitates free-radical reactions producing reactive oxygen species, which may represent one of the molecular mechanisms for genotoxicity and carcinogenicity of nickel compounds. Soluble nickel compounds may cause DNA damage by producing hydroxyl radicals through Fenton/Haber Weiss reaction. All constituents of DNA including deoxyribose, pyrimidines and purines may be attacked by highly reactive $OH^{\bullet}$ radicals producing oxidised purines and pyrimidines that constitute promutagenic lesions. $OH^{\bullet}$ attack upon nucleoprotein can also produce DNA - protein crosslinks. The damage produced by $OH^{\bullet}$ radicals cannot be protected by adding $OH^{\bullet}$ scavangers because of 'site-specific' generation of $OH^{\bullet}$ radicals by reaction of DNA-bound nickel and endogenous H_2O_2 in cells (vonSonntag, 1987; Oleinick *et al.*,1987;

Stoewe and Prutz, 1987; Dizdaroglu *et al.*,1991; Arouma *et al.*,1991; Klein *et al.*,1991; Higinbotham *et al.*, 1992; Huang *et al.*,1994).

Exposure of human epithelial cells to nickel has been reported to alter p53 gene structure which is considered to act as a tumour supressor(Baker *et al.*, 1990; (Baker *et al.*, 1990; Diller *et al.*,1990; Mercer *et al.*, 1990; Maehle *et al.*, 1992). In codon 238, T-C transition mutation was revealed in abnormal p53 expression exposed to nickel. This point mutation in the tumour suppressor gene induces immortalization and thereby accelerates the process of multistage carcinogenesis (Maehle *et al.*, 1992). The finding that nickel promotes microsatellite mutations in human cancer cell lines raises the possibility that genetic instability may be a mechanism involved in nickel carcinogenesis (Zinolddinyl *et al.*, 2000).

Nickel ions may act as a weak substitute for Mg^{2+} during DNA replication by some polymerases such as *E. coli* DNA pol. L and human DNA pol. α, hence inhibiting the DNA polymerase activity. This altered processivity and fidelity of DNA polymerase activity may contribute significantly to nickel induced mutagenicity and genotoxicity (Snow *et al.* 1993).

Nickel compounds possessing similar elemental composition but varying in physicochemical properties can produce different biological effects. Carcinogenic, water insoluble nickel compounds (crystalline nickel sulphides and oxides) slowly dissolve in body fluids and readily enter cells by phagocytosis, releasing nickel ions that may interact with chromatin. A unique feature of nickel carcinogenesis is the damage produced by nickel in nickel responsive genomic regions. Due to their abundance, histones are likely targets for nickel ions among nuclear macromolecules (Broday *et al.* 2000; Bal *et al.* 2000). At nontoxic doses, nickel decreased the levels of histone H_4 acetylation *in vivo* in both yeast and mammalian cells, affecting only lysine 12 in mammalian cells and all of the four lysine residues in yeast. In yeast, lysine 12 and 16 were more greatly affected than lysine 5 and 8. A histidine Ni^{2+} anchoring site is present at position 18 from the NH_2 terminal tail of H_4 (Broday *et al.*, 2000).

Highly carcinogenic insoluble nickel compounds induce an increase in chromatin condensation. As a result, cancer related genes that are actively expressed in euchromatin, may become condensed into heterochromatin and are stabilized by subsequent DNA methylation. Actively expressed genes have less cytosine methylation in their promoters whereas hypermethylation of cytosine in promoters is a characteristic of inactive genes. Thus nickel induced increases in heterochromatin condensation and hypermethylation of DNA may cause inherited inactivation of critical tumor suppressor or

senescence genes. Loss of cellular senescence and the acquisition of cellular immortality is an extremely important step in nickel-induced human carcinogenesis. (Costa *et al.*, 1994; Costa, 1995; Lee *et al.*, 1995; Costa, 1996; Broday *et al.*, 2000).

Nickel has been shown to inhibit the nucleotide excision repair pathway induced by UV irradiation. Ni(II) was found to interfere with DNA-protein interactions involved in the damage produced after UV irradiation by reducing the specific binding of a protein to UV damaged DNA. Repair of base modifications like 7,8-dihydro-8-oxoguanine and DNA strand breaks induced by visible light are also completely inhibited by non-cytotoxic concentrations of nickel ions (Hartwig *et al.*, 1996).

Epidemiological evidence suggests that certain exposures to metals may increase the risk of cancer in the progeny. This effect may be associated with promutagenic damage to sperm DNA. The latter is packed with protamines which might sequester carcinogenic metals and moderate the damage. Human protamine P_2 has an amino acid motif at its N-terminus that can serve as a heavy metal trap especially for nickel (II) (Liang *et al.*, 1999).

It has been postulated that metal toxicity may, at least in part, be due to autoimmunity, since an autoimmune disorder exists for all the major target organs affected by heavy metals (Zelikoff and Cohen, 1997). Activation of vascular endothelium is a key event in the initial phase of an inflammatory reaction *e.g.* to contact allergens. Effects of contact sensitizer, $NiCl_2$, on endothelial expression of intercellular adhesion molecule-1, vascular cell adhesion molecule and endothelial leukocyte adhesion molecule-1 was studied by Goebeler *et al.* (1993). Nickel chloride was found to upregulate these adhesion molecules on human umbilical vein endothelial cells in a dose and time-dependent manner. Goutet *et al.* (2000) investigated the *in vivo* effect of nickel sulphate on the pulmonary non-specific immune defences. It was found that natural killer cell activity and alpha-tumor necrosis factor (TNF-alpha) secretion are sensitive targets for instilled nickel sulphate in Wistar rats.

Differential expression of several genes was observed in human lung A_{549} cells exposed to nickel. Ni_3S_2 and $NiCl_2$ were found to induce a gene Cap^{43}, which expressed a 3.0 kb mRNA encoding a protein of 43,000 dalton. This induction of Cap^{43} gene is nickel specific in several rat organs after oral exposure and also in tested rodent and human cell lines *in vitro*. In colon cancer also, Cap^{43} is expressed after nickel exposure. Elevation of free intracellular Ca^{2+} caused by nickel exposure is a primary signal for Cap^{43} induction, which may play an important role in cancer pathogenesis

(Zhou *et al.*, 1998). Induction of hypoxia like condition in nickel-trasnformed rodent cells resulting in increased activity of hypoxia-inducible factor (HIF-1) responsive promoters may increase HIF-1-dependent transcription possibly leading to nickel induced carcinogenesis. Induction of Cap^{43} is dependent upon HIF-1, because only HIF-1 proficient cells induced Cap^{43} when exposed to either hypoxia or nickel (Salnikow *et al.*, 1999; Salnikow *et al.*, 2000; Salnikow and Costa, 2000). Recent experiments in our laboratory have shown that nickel carcinogenicity may be due to its specific binding to RNA and not DNA (unpublished work), both *in vivo* and *in vitro*.

Chromium

Chromium is an important industrial metal, an environmental pollutant and is one of the best-documented human and animal carcinogens (Kim and Yukow, 1996). Occupational and environmental exposure to chromium compounds has been widespread. Occupational exposure of chromium to humans occurs mostly through extraction, production of ferrochrome (an iron-containing alloy with about 60% chromium), electroplating, welding, tanning, glassware-cleaning solutions, wood preservatives and in the manufacture of pigments, safety matches and magnetic tapes (Bonde and Christensen, 1991; Barceloux. 1999).

Chromium is fourth element of first transition series and its electronic configuration is $4s^1 3d^5$ with atomic number 24. The metal exists in all oxidation states from –2 to +6, but stable states of chromium found in nature are reduced Cr(III) and Cr(VI) or chromate (Snow, 1991; Singh *et al.*, 1998; Barceloux, 1999).

Cr(VI) is the toxicologically active form because it is isostructural with phosphate and sulphate and is readily taken up by the gastrointestinal tract and penetrates to many tissues and organs by an anion transport system. Most of the intracellular Cr(VI) form is metabolically reduced via reactive intermediates to stable trivalent form, Cr(III). Cr(III), which is the most abundant form of chromium, is unable to enter the cells and hence is considerably less toxic. However intracellular Cr(III) and reactive intermediates formed such as Cr(V) can react slowly and are capable to form co-ordinate covalent interactions with biological ligands such as DNA and proteins and hence can be mutagenic and genotoxic (Miller *et al.*,1991; Snow, 1991; Snow, 1994; Costa, 1997; Singh *et al.*, 1998; Barcceloux, 1999).

Exposure to soluble hexavalent chromium from drinking water and surrounding environment resulted in high accumulation of Cr(III) in all tissues and organs. Increased

risk of Hodgkins disease, leukemia, lymphomas and cancers of bone, prostate, stomach, genital organs, kidneys and bladder caused by hexavalent Cr reflects the ability of Cr(VI) to penetrate all tissues in the body (Costa, 1997).

Trivalent chromium interacts with DNA through phosphate backbone without exhibiting any base specificity. Cr (III) mediates DNA protein crosslinks by binding with reactive amino acids and linking these to the phosphate backbone of DNA. Amino acids tyrosine, cysteine, methionine and threonine were found to exhibit more activity than any other amino acid in being complexed to DNA by Cr(III) *in vitro* (Miller *et al.*, 1991; Lin *et al,*. 1992. Salnikow *et al.*, 1992). *In vitro* actin was found to be the major protein (20%) of the protein component in chromate-induced DNA-protein crosslinks. DNA-protein crosslinks were also demonstrated in circulating leukocytes and liver and kidney of rats exposed to $K_2Cr_2O_4$ (Lei *et al.*, 1995). DNA-protein corsslinking has been proposed as a biomarker of chromate exposure and genotoxic damage which quantitatively shows exposure to chromate (Kuykendall *et al.*, 1996).

Chromium has been shown to facilitate the formation of hydroxyl radical from hydrogen peroxide (Shi and Dalal, 1990; Shi *et al.*, 1993). Intracellular metabolism of chromium (VI) may lead to oxidative stress which may account for the ability of Cr(VI) compounds to induce their genotoxicity, mutagenicity and carcinogenicity. Incubation of Cr(VI) with ascorbate generates Cr(V), Cr(IV) and ascorbate-derived free radicals. Intermediates produced in the reaction of chromium(VI) with dehydroascorbate cause single strand breaks in plasmid DNA. Cr(V) can form complex with reductant moieties such as glutathione *etc.* These Cr(V) species and ascorbate-derived free radicals have been suggested to play an important role in chromate-induced carcinogenesis by producing DNA single strand breaks, interacting with chromatin and oxidizing purine and pyrimidine nucleotides in DNA (Faux *et al.*, 1992; Shi *et.al.*, 1994; Shi and Dalal, 1994; daCruzFresco and Kortenship, 1994; Hamilton *et al.*,1998; Leonard *et al.*, 2000). Lung cancer and respiratory tract toxicity have been associated with occupational exposure to hexavalent chromium compounds such as lead chromate. The formation of lead inclusion bodies in normal human lung cells exposed to lead chromate indicates that ionic lead is released from the particles and thus might contribute to cell toxicity. Internalization and dissolution of lead chromate particles and the interaction of ionic chromium and lead with DNA may be components of the lead chromate carcinogenesis (Singh *et al.*, 1999).

Reactive intermediates produced during the process of chromium reduction may also interact with DNA to form lesions capable of obstructing DNA replication (Bridgewater *et*

al., 1994). DNA replication is inhibited by high Cr(III) concentration while micromolar concentration of Cr(III) can substitute for Mg^{2+} that may weakly activate the Klenow fragment of *E. coli* DNA pol. 1 and hence enhance the nucleotide incorporation. This leads to the alteration in the enzyme kinetics as a 4-fold increase in enzyme processivity and a 2-fold decrease in fidelity due to bypass of DNA polymerase from DNA lesions. Thus low levels of DNA-bound Cr(III) ions may contribute to increased rate of spontaneous mutagenesis and carcinogenesis during DNA replication, both *in vitro* and *in vivo* (Snow and Xu, 1991; Snow, 1994).

Apoptosis as the mode of cell death induced by chromium may also be considered as a component of chromium induced multistage carcinogenesis (Blankenship *et al.*, 1994). Transcription factors AP-1, NF-Kappa B, Spl and YB-1 may regulate transcription of many inducible genes. Nuclear binding levels of these transcription factors to their cis-acting elements might be altered significantly by chromium exposure, which may play a vital role in metal induced carcinogenesis due to altered gene expression. Activation of transcription factors and altered binding to DNA may be due to the Cr-mediated free radical reactions or through interactions with critical protein sulfhydryls (Ye *et al.*, 1995; Shumila *et al.*, 1998; Kaltreider *et al.*, 1999).

Cadmium and Arsenic

Cadmium is an important inorganic toxicant in the environment and was classified as human carcinogen in 1993 (Waalkes, 2000). Human prostate and lung cancers have been associated with cadmium exposure in some epidemiological studies. Inhalation of cadmium oxide is a major route to human exposure (Waalkes and Rehm, 1994; Mekenna *et al.*, 1997; Martin *et al.*, 2001). Cadmium inhalation in rats results in pulmonary adenocarcinomas, supporting a role in human lung cancer. Prostate tumors and preneoplastic proliferative lesions can be induced in rats after oral ingestion and injection of cadmium. Cadmium has an androgen like activity in epithelial cells of prostate and could enhance the androgen response when given in its combination. This androgen like activity of cadmium implies its role as a carcinogen in the prostate cancer (Waalkes, 2000., Ye *et al.*, 2000; Achanzar *et al.*, 2001).

Apoptosis is triggered by oxidants and it plays a key role in eliminating preneoplastic cells. Cadmium could act to select for apoptotic defective cells *in vivo* thus increasing the tumor formation. Suppressed oxidant induced apoptosis by cadmium could leave preneoplastic and neoplastic cells alive and this can eventually lead to cancer development (Achanzar *et al.*, 2000; Eneman *et al.* 2000).

Arsenic may potentiate cadmium nephrotoxicity during the long term combined exposures to cadmium and arsenic (Liu *et al.,* 2000). Tobacco smoke is also a route of cadmium exposure to humans, where it acts in synergy with other carcinogens present in the tobacco smoke. N_7 guanines in major groove of DNA are potential target sites for cadmium binding in this process (Prakash *et al.,* 1998). The association between multiple cadmium exposures and enhanced metastatic potential of the ensuing tumors may have important implications in chronic exposures to cadmium or in cases of co-exposure of cadmium with organic carcinogens, as in tobacco smoking (Waalkes *et al.,* 2000).

In addition, carcinogenesis has been demonstrated by arsenic exposure. Increased risk of skin, urinary bladder, hepatocellular and respiratory tract cancers are associated with environmental and occupational exposure to arsenic. It promotes the tumor formation by modulating the signal pathways responsible for cell growth (Simeonova and Luster, 2000; Lu *et al.,* 2001). Arsenite induces translocation of protein kinase C (PKC) epsilon, PKC delta, and PKC alpha from cytosol to membranes (Chen *et al.*, 2000).

Arsenic in rodent cells, has been shown to produce aberrant gene expression and activation of the proto-oncogene c-myc. *In vitro*, arsenic, cadmium, mercury and zinc alter the expression of several model inducible genes by inhibiting the binding of transcription factors to specific DNA regions, resulting in altered gene expresssion. This altered expression of genes and oncogenes may play a significant role in metal induced carcinogenesis (Shimizu *et al.,* 1998; Shumilla *et al.,* 1998). Arsenite reduces p53 levels while concomitantly increasing the p53 regulatory protein mdm2 levels in a dose- and time-dependent manner. Disruption of the p53-mdm2 loop regulating cell cycle arrest can act as a model for arsenic-related skin carcinogenesis and it may be important in tumors with elevated mdm2 levels (Hamadeh *et al.,* 1999).

Arsenic, a human carcinogen, is reduced and methylated mainly in liver cells generating a number of intermediate reactive moieties which could lead to the formation of DNA-protein crosslinks (Ramirez *et al.,* 2000). Exogenous methylated arsenic species and endogenous ascorbic acid can cause the release of iron from ferritin, the iron-dependent formation of reactive oxygen species, and DNA damage. This reactive oxygen species pathway could be a mechanism of action of arsenic carcinogenesis in man (Ahmed *et al.*, 2000). Increased production and accumulation of 72kDa stress protein (Hsp72) by oral administration of dimethylarsenic acid (DMA) to mice may occur specifically in target organs for arsenic carcinogenesis (Kato *et al.*,2000). DMA has a promoting effect on skin tumorigenesis in K6/ODC transgenic mice (Morikawa *et al.* 2000). Repeated arsenate

exposure was associated with proliferative and preneoplastic lesions of the uterus, testes, and liver. Estrogen treatment has been associated with proliferative lesions and tumors of the uterus, female liver, and testes supporting a hypothesis that arsenate might somehow act through an estrogenic mode of action (Waalkes *et al.*, 2000).

Other Metals

Damage to DNA produced by transition metals is considered mainly due to generation of free radicals via Fenton reaction. Zinc finger proteins control the transcription of a number of genes by acting as transcription factors. Some metals such as cobalt, cadmium, copper and iron have an ability to substitute zinc in zinc finger proteins resulting in the production of free radicals damage which DNA in close proximity (Sarkar, 1995, Conte *et al.*, 1996; Buchko *et al.*,2000).

In mammals, iron is an essential moiety for the functions of many enzymes and is also responsible for oxygen transport by haemoglobin. 'Free' iron is considered quite cytotoxic to produce carcigogenic and mutagenic effects. Production of reactive oxygen species and oxidative stress by 'Free' or 'Catalytic' form of iron leads to lipid peroxidation, DNA oxidation, protein damage and activation of a variety of reducing protective mechanisms via signal transduction. These effects are thought to play an important role in iron-induced carcinogenesis (Toyokoni, 1996). Iron overload predisposes experimental animals as well as humans to an increased risk of intestinal, colon, hepatic, pulmonary and mammary carcinogenesis (Rezazadeh and Athar, 1997).

Metals have been shown to increase the production of reactive oxygen species (ROS) and/or to inhibit antioxidant activities, directly or indirectly leading to oxidative injuries. Those metals that directly increase the production of reactive oxygen species include iron, copper, chromium, vanadium and manganese. Iron (Alleman *et al.*,1985; Halliwell and Gutteridge, 1986) and copper (Chan *et al.*,1982) are important cofactors in the Fenton reaction to produce reactive oxygen species (ROS). Production of reactive oxygen may play a vital role in lead induced cytotoxicity and it may act as tumour promoter in human fibroblast cells (Iiwua and Yang, 1998). Perpetual ROS generation by metals can cause specific molecular changes resulting in the activation or inactivation of transcription factors that may alter gene expression leading to cell proliferation, differentiation and carcinogenesis. ROS signalling is critical for the responses of cytokines, growth factors and activation or inactivation of transcription factors that promote carcinogenesis (Ding *et al.*, 2000).

Conclusions

Metal carcinogenesis is increasing as an occupational biohazard, particularly due to the synergistic effects of metals even with low doses of organic carcinogens. Understanding the molecular basis of metal carcinogenesis is still in its infancy, as both genetic and epigenetic effects of various carcinogenic metals have been reported. In the light of known facts at present about the molecular basis of tumorigenicity caused by the metals, it may not be out of place to comment that metal carcinogenesis might not ultimately be explained by a uniform molecular mechanism for all the carcinogenic metals.

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CYTOTOXIC EFFECTS OF MONOCROTOPHOS (MCP) - AN ORGANOPHOSPHATE ON LUNG OF RATS-A LIGHT MICROSCOPIC STUDY

P. K. Mittal and Sangeeta

Department of Zoology, Panjab University, Chandigarh –160 014

Abstract

MCP, an organophosphorus insecticide caused necrotic changes in alveolar epithelium, bronchiolar epithelium, emphysematous lesions and haemolysis in lungs following oral administration to female albino rats at the rate of 0.28mg/100gms of body weight i.e. $1/5^{th}$ of ld_{50}, for 15 days and 30 days. This leads to increased surface tension in lungs due to decreased secretion of surfactant and hypoxia. The damage is time dependent being more in 30 days treatment than 15 days treatment. The recovery group (30 days treatment of rats with MCP followed by 15 days recovery) showed only partial recovery from the toxic effects of MCP.

INTRODUCTION

Monocrotophos (3-hydroxy-N-methyl-cis-crotonamide dimethyl phosphate),an organophosphorus insecticide has both systemic and contact actions and has been used against a wide range of insects including mites, boll worms, sucking insects, leaf eating beetles and other larvae on a variety of crops[18]. Its residual effect in/on different important vegetable crops in/on soil has been studied[9]. Lung is an important organ which helps in exchange of gases between blood and air in the alveoli. The contraction and dilation of smooth muscles in the airways is controlled by autonomic nervous system which involves cholinergic nerve fibres[6]. The organophosphorus pesticides have been reported to inhibit the action of enzyme acetylcholinesterase at neuronal junctions[14]. These xenobiotics reach lungs along with blood after absorption from small intestine and thus interfere with synaptic chemistry. This would hamper the normal contraction and dilation of smooth muscles of the airways which may lead to asphyxia. As is reported by Holmstedt, the immediate cause of death is asphyxia in almost all cases related to toxicity of organophosphates[7]. A few workers[2–5,8,12,13,15] have reported effects of some pesticides on the lungs of vertebrates. To the best of knowledge of present workers, a little work is available on MCP toxicity on lungs. Therefore, the light microscopic study of lungs has been undertaken to see the histopathological changes after MCP treatment in rats.

MATERIALS AND METHODS

Adult female albino rats of Wistar strain, weighing 100-150 gms, were obtained from animal house, Panjab University, Chandigarh. The rats were divided into four groups: T_1, T_2, R and C, each group having 3-5 animals. These were provided with rat chow

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and water *ad libitum*. Each group was administered MCP (technical grade) at the rate of 0.28mg/100 gms of body weight (i.e. 1/5th of LD_{50} value) of MCP for different time periods daily through gavage. T_1 group rats were administered MCP for 15 days. T_2 for 30 days and R group animals were given MCP for 30 days and then these were allowed to recover for 15 days on normal diet. C group was run simultaneously under the same conditions on normal diet.

After dosing for a given period, the overnight fasted rats of each group were killed by cervical dislocation. The chest was then opened and the lungs were taken out in 0.9% physiological saline. These were cleared off extraneous material in saline and then were cut into small pieces followed by fixation in Bouin's fluid and formal calcium (FCa) for 24 h. The Bouin-fixed tissue was then dehydrated in various ascending grades of alcohol, cleared in benzene and was finally embedded in paraffin wax at 60°C. 7μ thick sections were cut on the microtome. FCa fixed tissues were post chromated in dichromate calcium, embedded in gelatin and 10μ thick sections were cut on cold microtome.

For histopathological studies, Delafield's haematoxylin eosin (H/E) staining was carried out[1]. For histochemical studies, mercuric bromphenol blue staining was done to locate the sites of proteins. Feulgen staining for DNA, periodic acid Schiff (PAS) staining for carbohydrates, acid haematein for phospholipids and Sudan black B (SBB) for general lipids was done[10].

RESULTS

In all the four groups stained with H/E, the respiratory bronchioles and alveoli were observed along with blood vessels.

Bronchioles : The bronchioles are the branches of fourth or fifth order of primary bronchi. Histologically, these bronchioles in C group showed an epithelium which was thrown into wavy longitudinal folds. Two major cell types, more of them ciliated than non-ciliated were present. The ciliated cells extend from lumen to basement membrane with nuclei projecting towards the lumen. The non-ciliated cells showed spherical nuclei under oil immersion. On the outside of the basement membrane was seen lamina that completely encircled the wall (Figs. 1,5).

In T_1 group, the epithelial wall of bronchioles showed disruption at places indicating damage to epithelial cells. The lamina outside the basement membrane surrounding epithelium was also not continuous. A mass of necrotic epithelial cells of alveolar walls showed concentration in between bronchiole and blood vessel which may be forming oedema (Figs. 2,6).

In T_2 group, there was further disintegration of the tissue indicating severe damage (Figs. 3,7).

In R group, not much recovery was seen. Nuclei of bronchiolar epithelial cells showed pycnosis. Basement membrane underlying epithelium was not continuous. Broncho-constriction could also be seen (Figs. 4,8).

Pulmonary Alveoli : Alveolar epithelium in C group showed two types of cells namely squamous alveolar cells (Type I cells) and Great alveolar cells (Type II cells). The connective tissue beneath the epithelium which served as the air blood barrier is thinnest for proper exchange of gases. Type II alveolar cells were roughly cuboidal in shape and had vesicular and relatively large nuclei (Figs. 1,5).

In T_1 group, the epithelial wall surrounding the alveolar spaces showed damage. Some of the alveoli showed widening due to coalescence of adjacent alveoli forming emphysematous lesions (Figs. 2,6).

In T_2 group, the damage was even more. The alveolar walls were found to be broken at places and alveoli appeared to be irregular. Necrotic cells showed accumulation at places (arrow) (Figs. 3,7).

R group, showed very little recovery as some of the alveolar spaces showed widening and some were like that of C group (Fig.4,8).

Histochemically, sites of DNA showed decreased staining but increase in number of cells in all the treated groups showing pulmonary damage. Carbohydrates also showed a decrease in all the treated groups. Sites of proteins showed a decrease in T_1 group but an increase in T_2 group indicating stress of pesticide. Phospholipids and lipid granules in various cells of bronchiole and alveoli showed a decrease in T_1, T_2 and R groups as compared to C group, however, the decrease was less in T_2 group and R group (Figs. 5-8).

DISCUSSION

During present study it was observed that alveolar spaces are lined by two types of cells: Type I squamous alveolar cells and Type II great alveolar cells. Type I cells help in gaseous exchange[17] and damage to these as observed in T_1, T_2 and R groups may impair the proper gaseous exchange resulting in asphyxia. Type II (great alveolar cells) are secretory in nature[6,17]. They produce the surfactant which is a surface active material that helps in reducing surface tension at respiratory surface. The surfactant is a complex mixture of phospholipids (dipalmitoyl lecithin), proteins and ions. After treatment with MCP, damage to Type II cells had been observed in T_1, T_2 and R groups which may result in less production of surfactant and increase the surface

tension. Histochemical observations also revealed decrease in phospholipids and proteins in treated groups. Some earlier workers[12,15] have reported degenerative changes in the lungs after treatment with mephosfolan and VX chemical respectively.

Furthermore, damage to bronchiolar epithelium and also non-ciliated (Clara) cells has been observed during present study. Although the functional significance of these cells is subject to speculation, evidence suggests that these are secretory in nature and may produce a component of surfactant[11]. Therefore, the damage to Clara cells may affect the synthesis of products like phospholipids and proteins which are important constituents of surfactant.[2,3,5,16]

MCP treatment also caused emphysematous lesions in T_1 and T_2 groups lungs, characterized by destruction of alveolar walls and consequently marked enlargement of alveolar air spaces and loss of pulmonary capillaries which may impair gaseous exchange leading to hypoxia.

Necrotic cells showed concentration in between blood vessel and bronchioles in T_1 and T_2 groups which may develop an oedema. Increase in number of cells was confirmed after Feulgen staining for DNA. Oedema has also been reported in lungs of sheep after administration of a VX another organophosphate[15].

Therefore, it can be concluded that MCP treatment causes pulmonary damage and the necrotic effects of this pesticide cannot be reversed after 15 days of the treatment.

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ABBREVIATIONS USED IN THE FIGURES

AS	Alveolar Space
BD	Bronchiolar Duct
BR	Bronchiole
BV	Blood Vessel
EC	Epithelial Cells
M	Macrophage
MC	Muscular Coat
TI	Type I Squamous Alveolar Cell
TII	Type II Great Alveolar Cell

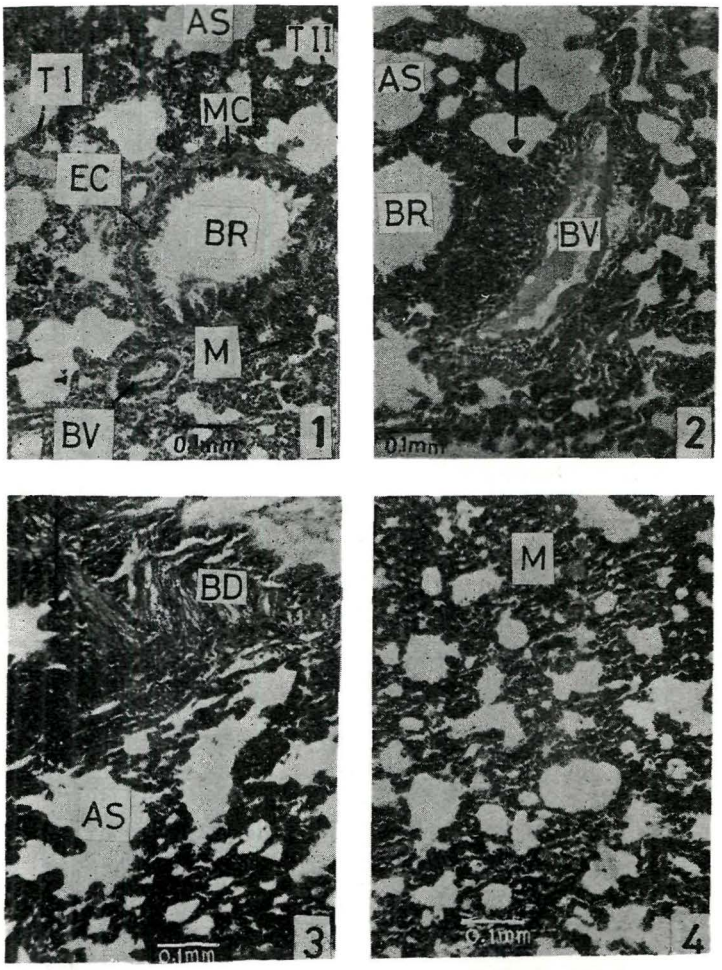

Fig.1. T.S. Lung of rat showing the nuclei and cytoplasmic granules in various cells of respiratory bronchiole and alveoli in C group. Bouin, H/E, X125.

Fig.2. T.S. Lung of rat showing necrotic changes in respiratory bronchiole and alveoli in Ti group. A mass of nuclei of damaged cells show concentration in between bronchiole and blood vessel (arrow). Bouin, H/E, X125.

Fig.3. T.S. Lung of rat showing severely damaged alveolar walls in T_2 group. Arrow shows the mass of nuclei of damaged cells near bronchiolar duct. Bouin, H/E, X125.

Fig.4. T.S. Lung of rat in R group showing little recovery in this part of lung. Bouin, H/E, X125.

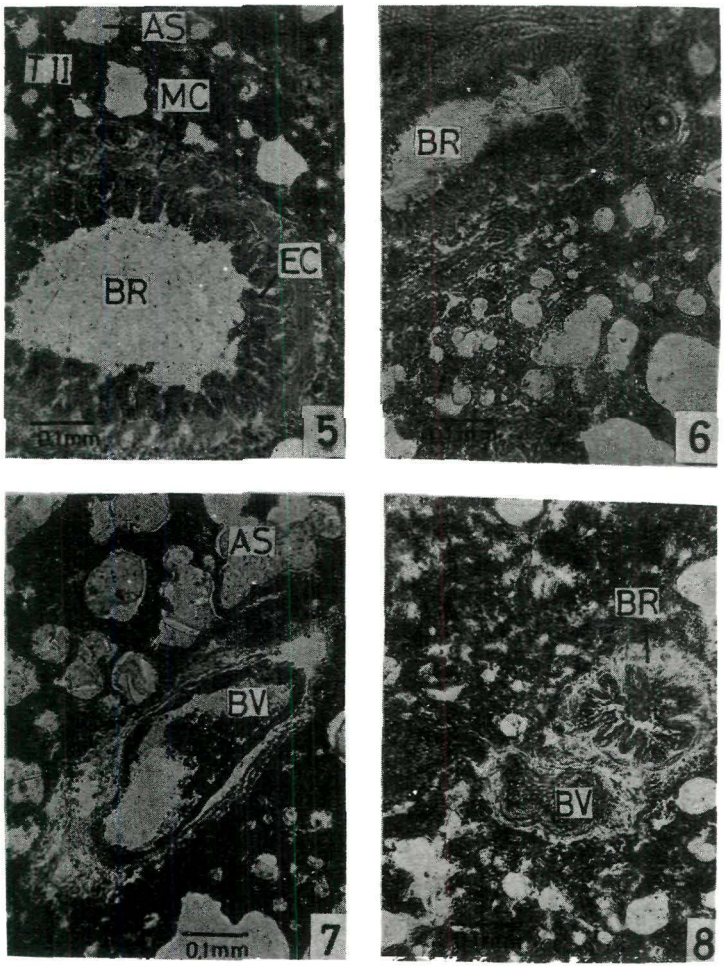

Fig.5. T.S. Lung of rat in C group showing lipid granules in various cells of respiratory bronchiole and alveoli. FCa-PC, SBB, X125.

Fig. 6. T.S. Lung of rat in T_1 group showing decrease in sites of lipids in Type II cells of alveolar epithelium. FCa-PC, SBB, X125.

Fig. 7. T.S. Lung of rat in T_2 group showing decrease in sites of lipids as compared to T_1 group. FCa-PC, SBB, X125.

Fig. 8. T.S. Lung of rat in R group showing broncho-constriction. Staining pattern of sites of lipids is almost same as that of T_2 group. FCa-PC, SBB, X125.

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SOME ASPECTS OF AGE AND GROWTH OF *LABEO CALBASU* (HAM.), *LABEO GONIUS* (HAM.) AND *PUNTIUS SARANA* (HAM.) FROM HARIKE WETLAND

M.S. Johal, J.R. Gupta* and K.K. Tandon

Department of Zoology, Panjab University, Chandigarh – 160 014.
Department of Zoology, D.A.V. College, Amritsar

Abstract

The paper describes the age and growth of *Labeo calbasu*, *Labeo gonius* and *Puntius sarana* from Harike Wetland. It has been seen that these fishes which constitute a considerable and an important part of the catch are overexploited and need conservation. It has been suggested that the legal size limit for harvesting be fixed in the case of these fishes as has been done in the case of major carps.

INTRODUCTION

Harike is a confluence of the rivers Beas and Sutlej (Longitude 74°57'E, Latitude 31°10'15"N) where a lake with an approximate area of 28 sq kms with varying depth (0.3-4.5 mts.) has been formed. On the sides of the rivers and lake, swamps with thick growth of aquatic weeds are common and are good source of fish like *Labeo rohita, L.calbasu, L.bata, L.gonius, Cirrhinus mrigala, Catla catla, Puntius sarana, Wallago attu, Aorichthys seenghala, Rita rita, Bagarius bagarius* and *Channa stiatus*. Growth studies are extremely important for the management of fisheries as they give us an idea about the age, catch composition of the fishable stock, its survival and mortality rates. The importance of such studies has been stressed by various workers [3,6,8&11]. The fishery of *L.calbasu, L.gonius* and *Puntius sarana* has been studied from that angle.

The literature regarding age and growth has been amply reviewed by Tandon and Johal[11] in their book entitled, 'Age and growth of freshwater fishes of India'.

MATERIALS AND METHODS

The scales of *Labeo calbasu, L.gonius* and *Puntius sarana* were collected at monthly interval from April 1988 – March 1990 from the commercial catches and were processed in the laboratory for further analysis. The growth parameters such as back-calculated lengths, growth compensation, harvestable size and ultimate size of the fishes under report have been calculated by the formulae described by Tandon & Johal[11].

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For age determination, the annulus formed during the months of June-August was considered valid, whereas any other type of mark present on the scales was ignored. For the study of scale VEB Dokukator Carl Zeiss was used.

OBSERVATIONS AND DISCUSSION

Scales of 411 specimens of *Labeo calbasu*, 348 of *L.gonius* and 295 of *Puntius sarana* have been studied for age and growth. The correction factor is 35mm in case of *L.calbasu*, 45mm in *L.gonius* and 11mm in *Puntius sarana*. (Figs. 1-3) Larval, false and true marks have been recorded on scales of these fishes. True marks were formed in the months of May-June in *L.calbasu*, June-July in *L.gonius* and April-May-June in *Puntius sarana*. For the sake of convenience 1st of August was taken as completion of each annulus.

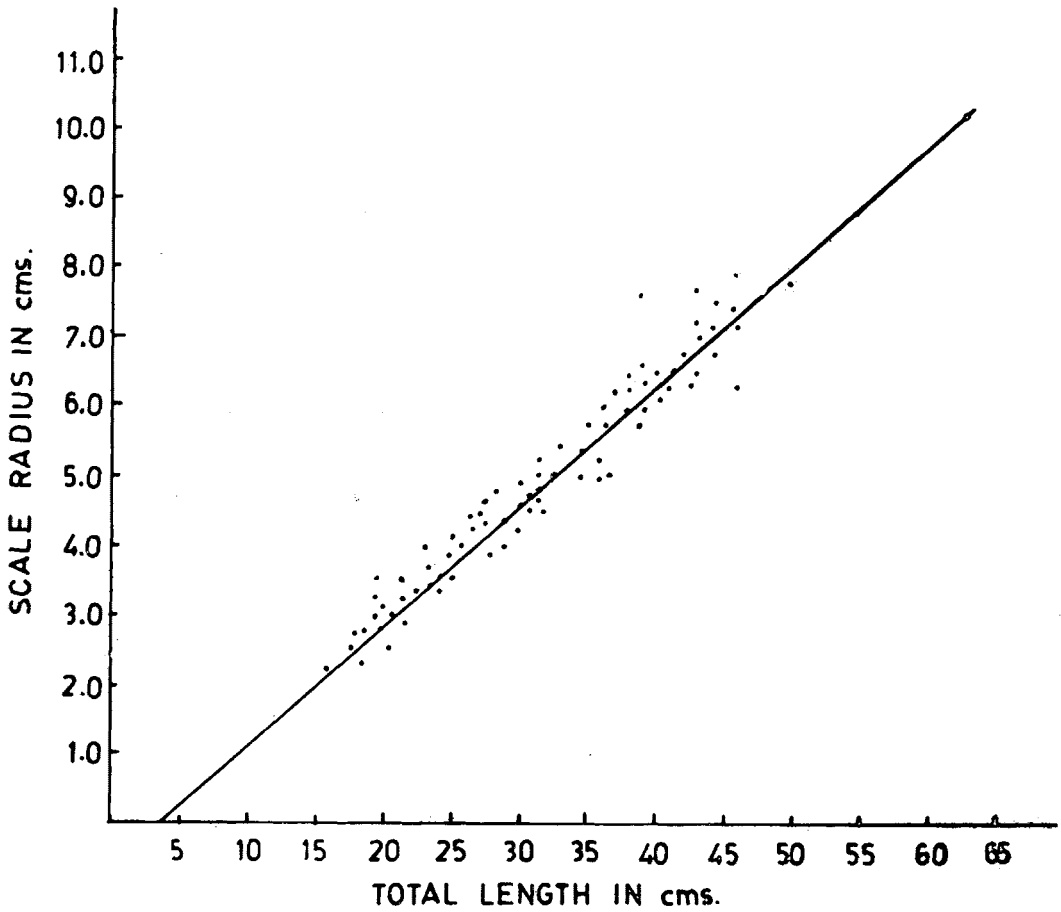

Fig. 1. Correction factor in *Labeo calbasu*.

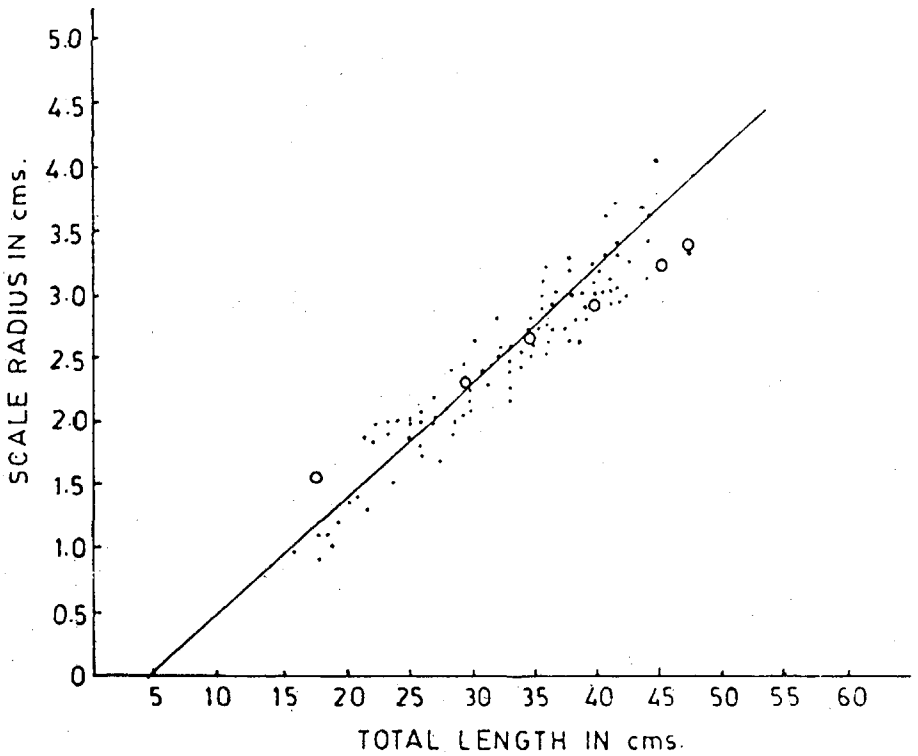

Fig. 2. Correction factor in *Labeo gonius*.

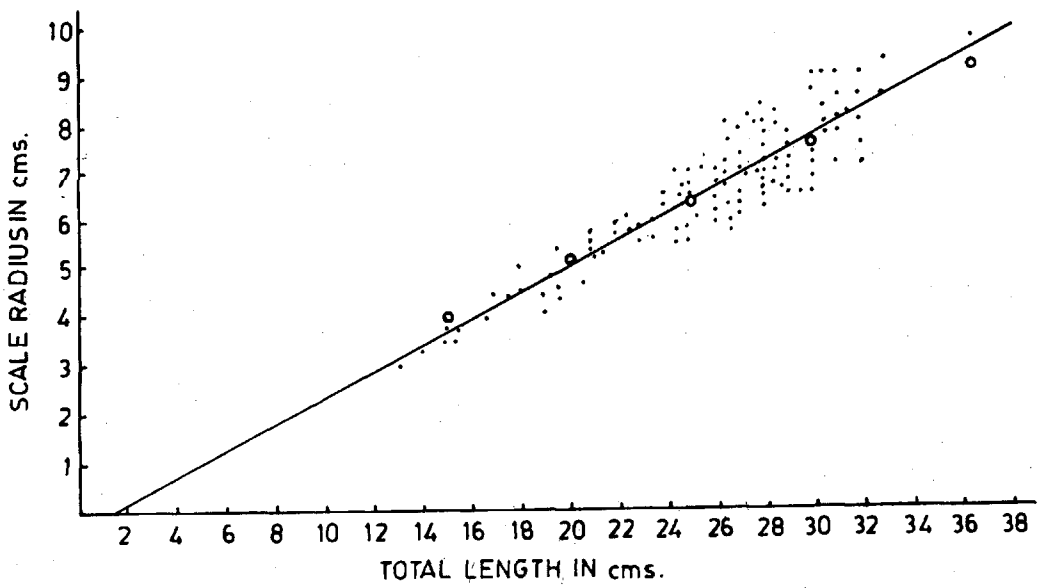

Fig. 3. Correction factor in *Puntius sarana*.

Labeo calbasu

During the period April 1988 to March 1990, scales of 411 specimens were examined and six age classes were deciphered. Maximum number of specimens were recorded in age classes III and IV (183 and 135 respectively) and minimum (5) in age class VI, indicating the maximum vulnerability of age classes III-IV. The older individuals either escape or there is natural mortality. The average back-calculated lengths at different ages are as under :-

l_1 = 15.88, l_2 = 22.17, l_3 = 28.58, l_4 = 35.77, l_5 = 42.10 and l_6 = 52.30

Growth compensation occurred at different steps. Specimens ranged from 15.70 cm to 63.00 cm.

I-VIII age classes have been reported from the river Yamuna[1] and Jaismand Lake and I to V from the river Ghaggar[10]. The growth rate as indicated by the index of species average size was better in these rivers and poor in reservoirs. One of the factors responsible for better growth of *L.calbasu* in the rivers is the benthic dwelling nature of the fish in which there is no paucity of space as well as by absence of interspecific competition for food.

Labeo gonius

From April 1988 to March 1990, 348 fish specimens were examined for scale studies. I-VI age classes were recorded. Age classes II and III were more vulnerable to fishing gear (145 and 116 respectively) because the use of gill-nets (mesh size 10-15cm) of specific size for commercial fishing. Age class VI was represented by a single specimen. The average back calculated lengths are as under :-

l_1 = 17.43, l_2 = 24.42, l_3 = 30.29, l_4 = 32.25, l_5 = 38.32 and l_6 = 39.79.

Phenomenon of growth compensation was not observed. The specimens ranged from 16.00-48cm in total length.

Puntius sarana

Scales from 295 specimens were examined during April 1988 to March 1990. Age classes I to V were deciphered. Age class IV was more vulnerable (147 specimens) as compared to age class III (80 specimens). Age class I was represented by 3 specimens whereas II and V by 21 and 44 specimens respectively. The average back-calculated lengths are :-

l_1 = 10.59, l_2 = 16.04, l_3 = 21.40, l_4 = 25.55 and l_5 = 28.62.

Growth compensation was not recorded. Specimens ranged between 13.00 and 36.5 cm in total length. Comparison of the growth rate with those of Ghaggar[7] and Sukhna lake[2] indicated that growth was less in specimens collected from Harike.

Growth parameters of *L.calbasu, L.gonius* and *P.sarana* have been studied according to Tandon and Johal[11]. It has been observed that specific rate of linear growth decreased with the increase in the age in the fishes studied except in *L.calbasu* between age classes V-VI.

In these carps the absolute weight increase showed increasing trend with the increase in age. However, decreasing values were recorded in age classes V and VI in *L.gonius*.

Interestingly the higher values of specific rate of linear growth and specific rate of weight increase in age class VI in *L.calbasu* corresponds with the occurrence of phenomenon of growth compensation in the respective age classes. The index of population weight growth intensity has been found to be 112.60, 79.20 and 152.00 in *L.calbasu, L.gonius* and *P.sarana*, respectively. The index of species average size has been found to be 8.72 cm in *L.calbasu*, 6.63 cm in *L.gonius* and 5.72 cm in *P. sarana*.

Growth constants and growth characteristics, too, have been calculated according to Tandon and Johal[11]. It has been seen that *L.calbasu* remains in active sexual phase for most of the time. The fish becomes sexually mature during second year. Old age has not been recorded in this fish. The low stocks of this fish may be due to its poor fecundity in the water body. A population from Jaismand Lake[6] shows that fish does enter old age.

L.gonius and *P.sarana* do not enter old age. *P.sarana* remains in 1st phase for more time whereas L.gonius spends major part in second phase. In *P.sarana* the most active part in life cycle lies between age classes III-IV but this cannot be said of other minor carps.

Existence of old age often results in abnormal growth patterns, hence presence of old age in catches is not desirable.

L.gonius and *P.sarana* show two distinct periods in their life history. Similar observations have been recorded by Johal and Tandon[2,3,4] and earlier workers [2-11].

Harvestable size calculated according to the methodology by Tandon and Johal[11] indicated that in *L.calbasu*, the point of intersection lies nearly at the end of IInd year of life (Fig.4). It is, thus evident that *L.calbasu* should be harvested after the completion of IInd year of life when it has attained the size of 26.00 cm total length. However, Tandon et.al.[10] described the harvestable size of this fish as 34.00 cm from the river Ghaggar at

Rang Mahal, Rajasthan. Singh[6] described that this fish should not be harvested below the size of 35 cm total length. Rao and Rao[5] reported first maturity of the fish in the IInd year of life. Since the harvestable size depends upon the growth rate from different water bodies, it is desirable that for this fish +30 cm size should be recommended.

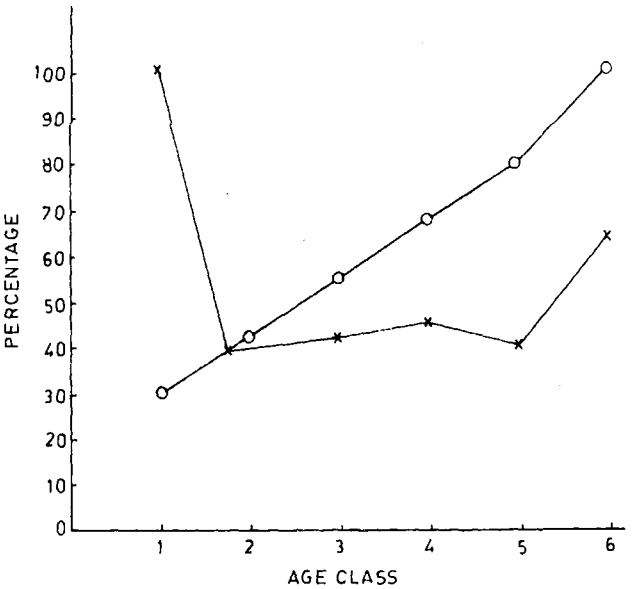

Fig. 4. Harvestable size in *Labeo calbasu.*

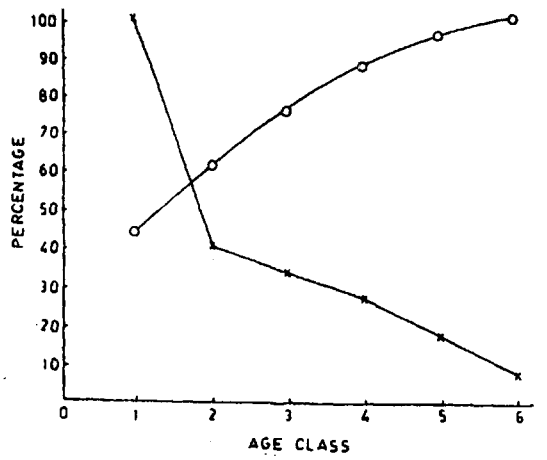

Fig. 5. Harvestable size in *Labeo gonius.*

Fig. 4,5,6 : O——O % of length first growth season in final growth season.

X——X % length increment in each age class in first year growth.

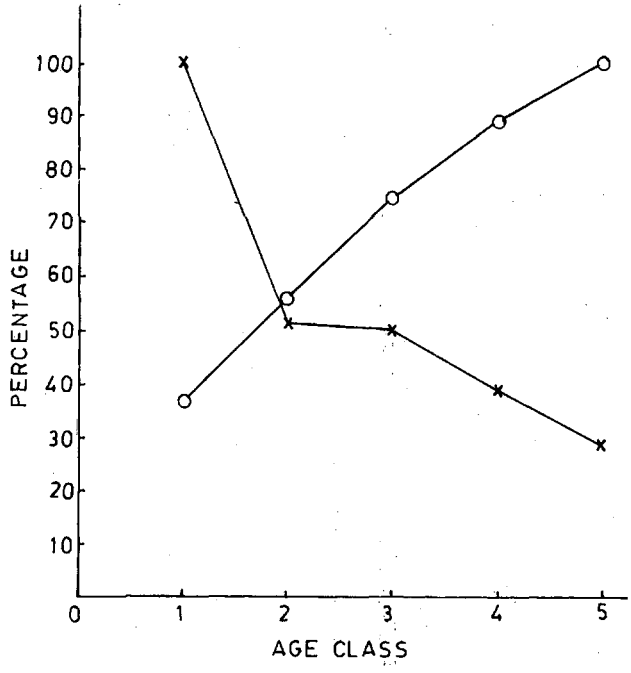

Fig. 6. Harvestable size in *Puntius sarana.*

In *L.gonius*, the harvestable size (Fig.5) has been estimated at 30 cm i.e. after the completion of IInd year.

Puntius sarana, according to Tandon and Johal[7] should be harvested when it is in second year. In the present context the harvestable size (Fig.6) lies at the completion of IInd year when the fish has attained a size of 20cm total length.

It looks imperative that the State Fisheries Department should enforce size limit for the minor carps also to avoid depletion of their stocks in the wetland, because percentage of mortality rates in age classes IV-V is 70-80 percent. Calculation of ultimate size, using Walford's graph as described by Tandon and Johal[11], has shown that *L.calbasu, L.gonius* and *P.sarana* attain the size of 64.50, 49.50 and 39.00 cm, respectively (Figs. 7,8,9) whereas in the sample, the recorded size based on scale studies, was found to be 60.67, 39.37 and 33.11 cm, respectively, indicating that the, methodology followed is correct.

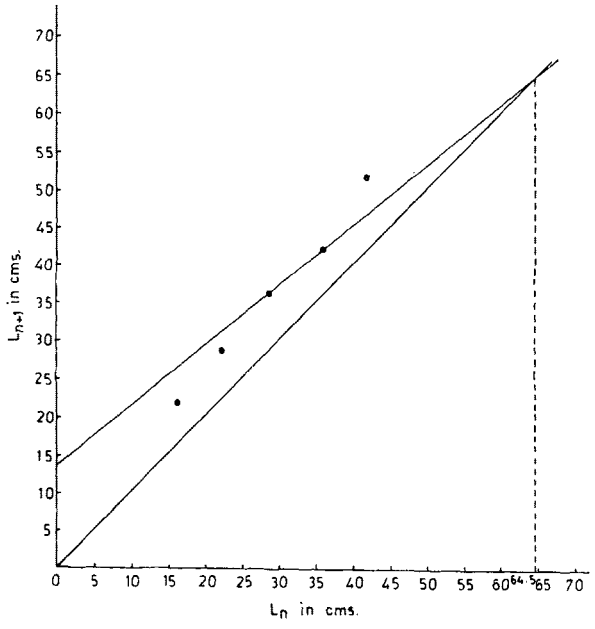

Fig. 7. Ultimate size in *Labeo calbasu*.

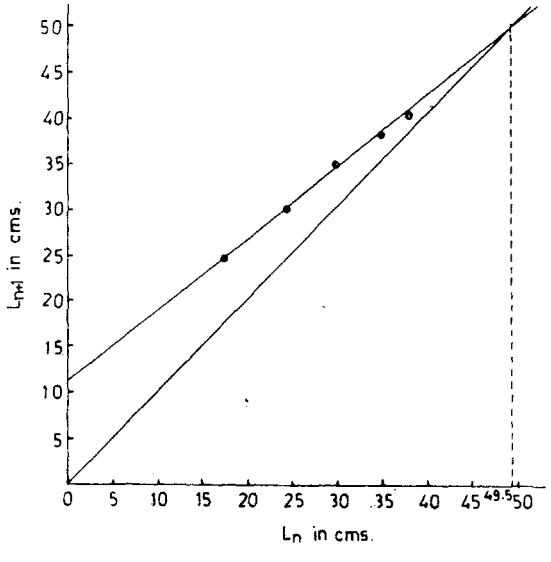

Fig. 8. Ultimate size in *Labeo gonius*.

Fig. 7.8,9 : L_n = Back-calculated length in each class.

L_{n+1} = Back-calculated length in the next age class.

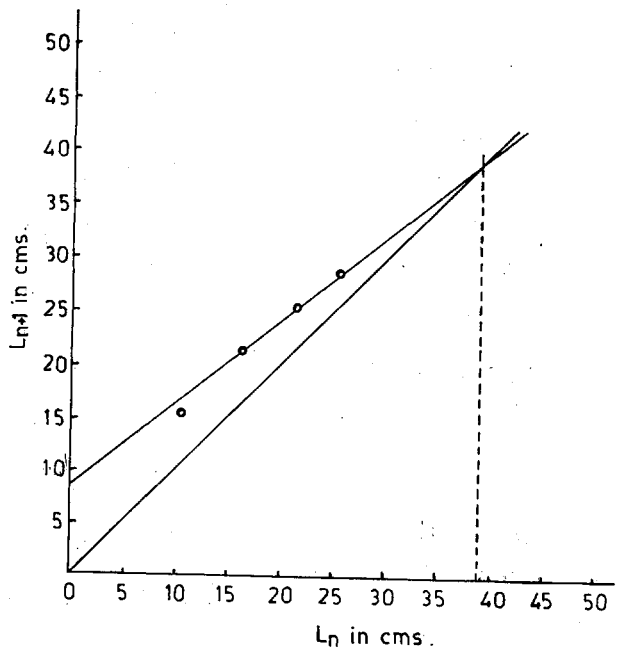

Fig. 9. Ultimate size in *Puntius sarana*.

SUMMARY

1. Harike is a confluence point of the rivers Sutlej and Beas and is important from the point of fishery resources.

2. *L.calbasu, L.gonius* and *P.sarana* are important constituents of the fishery.

3. Age and growth have been calculated from the scales of 411 specimens of *L.calbasu*, 348 of *L.gonius* and 295 of *P.sarana*.

4. Correction factor of 11mm has been calculated in *P.sarana*, 45mm in *L.gonius* and 35mm in *L.calbasu*.

5. Larval, false and true marks are recorded on the scales of these fishes.

6. 1st of August was taken as completion of each annulus.

7. Back-calculated lengths were calculated for each species separately based on the relationship : Total length-Lateral scale radius using correction factor.

8. Growth compensation has been observed only in *L.calbasu*.

9. Active sexual phases in each species have been described based on growth characteristics and growth constant studies.

10. *L.gonius* and *P.sarana* do not enter old age. *P.sarana* remains in 1st phase for most of the time whereas *L.gonius* spends major part in 2nd phase.

11. Harvestable size shows that the legal limit for these fishes should be fixed to avoid depletion of stocks in the wetland as the mortality rate is very high in the age classes IV-V.

12. Results of Walford graph confirm the authenticity of data i.e. the maximum size in the sample available is under calculated size.

ACKNOWLEDGEMENTS

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PURIFICATION AND CHARACTERISATION OF CATHEPSIN L. FROM GOAT BRAIN

Ramesh C.Kamboj*, Neera Raghav*, Rachna Sadana and Hari Singh

Department of Biochemistry, Kurukshetra University, Kurukshetra – 136 119
**Department of Chemistry, Kurukshetra University, Kurukshetra – 136 119*

Abstract

Cathepsin L (EC 3.4.22.15), a thiol protease was purified ~1800-fold with ~ 20% recovery to apparent homogeneity as judged by native PAGE by following acetonefractionation, homogenisation, acid-autolysis at pH 4.0, 30-70% $(NH_4)_2SO_4$ fractrionation, Sephadex G-100 column chromatography and ion-exchange chromatography on CM-Sephadex C-50 in presence of $HgCl_2$. Low concentrations of $HgCl_2$ helped in increasing the yield both by protecting the enzyme from autolysis as well as by enhancing the binding of the enzyme on CM-Sephadex. The enzyme had molecular mass of about 27 kDa when determined by SDS-PAGE. The enzyme hydrolyzed the synthetic substrate Z-Phe-Arg-NnapOMe maximally at pH 6.0 with K_m and V_{max} values of 6.6 x 10^{-5} M and 19.04 units mg^{-1} respectively. The thiol-blocking and alkylating agents were strong inhibitors. Leupenptin inhibited the enzyme very effectively with K_i value of 1.5 x 10^{-9} M. Glutathione in conjunction with EDTA was found to be best activator of cathepsin L. Cathepsin L was stable between pH 4.0-6.0 and upto 50°C. It had a temperature optima between 45-50°C with activation energy of 11.5 Kcal $mole^{-1}$.

INTRODUCTION

Cathepsin L (EC 3.4.22.15) has high degradative activity on cellular and matrix proteins[5,15,17,18] and thus assumes significance *vis-à-vis* other intracellular thiol proteases in lysosomal protein catabolism. This protease has been shown to be associated in diseases such as muscular dystrophy[19], peridontal disease[21], bone resorption[11],rheumatoid arthritis[9] and tumor invasion and metastasis[13]. Recently, cathepsin L has been proved to be a critical factor in tumor growth[16] by the inhibition studies. The pro-enzymic form of cathepsin L has been found to promote the proliferation[14] of immature thymocytes in the presence of IL-1,IL-7 and anti-CD_3 antibody. In brain, several neurotransmitters and hormones which are peptidyl in nature may be generated and activated/inactivated by the action of proteolytic enzymes[24]. Hence, it is important to study these proteolytic enzymes which may throw more light on the complex mechanism of action of brain. Though the presence of cathepsin L has earlier been reported in brain cortex[1] and rat brain[25], these studies involved lengthy and cumbersome purification procedures where only low activity yields were recovered. We report here a

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very rapid, economical and novel procedure for the purification of this enzyme from goat brain. The protease purified in its inactive Hg-form had very high yields. In addition, physico-chemical properties of the enzyme have also been determined.

MATERIALS AND METHODS

Chemicals : The chromogenic synthetic substrates like Z-Phe-Arg-NnapOMe, Z-Arg-Arg-NnapOMe, BANA, Z-Ala-Arg-Arg-NnapOMe, Z-Val-Lys-Lys-Arg-NnapOMe, Z-Arg-Arg-pNA, Z-Phe-Arg-pNA and other chemicals like DTE, DTT, TLCK, TPCK, E-64, Fast Garnet GBC and 2,2'-dipyridyl disulphide were purchased from Sigma Chemical Co., USA. Sephadex G-100, CM-Sephadex C-50 and molecular weight marker proteins were procured from Pharmacia Fine Chemicals, Uppsala, Sweden. Leupeptin, antipain, bestatin and pepstatin were obtained from Peptide Institute Osaka, Japan. All other chemicals and biochemicals were of purity. Goat brain, the source of enzyme, was obtained fresh from the local slaughter-house. The enzyme was concentrated through Amicon ultra-filtration cell model 8200 with YM 10 membrane filters. Shimadzu UV/visible and EC-digital (350-950 nm) visible spectrophotometers were used to monitor the enzyme protein.

Abbreviations used : SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; Z-, benzyloxycarbonyl-; -NnapOMe, -4-methoxy-2-naphthylamide; BANA, α-N-benzoyl-D, L-arginine-2-naphthylamide; -Nnap, -2-naphthylamide; -pNA, p-nitroanilide; DTE, dithioerythritol; DTT, Dithiothreitol; 2-ME, 2-mercaptoethanol; Fast Garnet GBC, o-amino-azotoluene diazonium sulphate; PCMB, p-chloromercuribenzoic acid; DTNB 5,5'-dithiobis-(2-nitrobenzoic acid);EDTA, ethylenediaminetetraacetic acid disodium salt; Antipain, [(S)-1-carboxy-2-phenylethyl]-carbamoyl-L-arginyl-L-valyl-L-argininal; Leupeptin, Acetyl-L-Leu-L-Leu-L-Argininal; Pepstatin, Isovaleryl-L-Val-AHMHA-L-Ala-AHMHA; AHMHA, 4-amino-3-hydroxy-6-methylheptanoic acid; E-64, L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane.

Enzyme assay : Cathepsin L was assayed using Z-Phe-Arg-NnapOMe as the substrate at pH 6.0. The assay mixture (2.0 ml) was prepared by mixing 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.0) containing 1.33 mM EDTA, 2.66 mM cysteine with 0.2 ml of enzyme solution and 0.25 ml water to make the volume 1.95 ml. This mixture was incubated at 40°C for 5 min. to activate the enzyme and the reaction was started by the addition of 50µl of substrate stock solution (Z-Phe-Arg-NnapOMe, 6 mg/ml in DMSO). After 10 min at 37°C, the reaction was stopped

by the addition of coupling reagent (2.0 ml), which was prepared by mixing equal volumes of Fast Garnet GBC dye (1 mg/ml in water) with a solution of 10 mM PCMB and 50 mM EDTA in water, pH 6.0. The released 4-methoxy-2-naphthylamine was coupled with Fast Garnet GBC, extracted in 4.0 ml n-butanol and the red color was read at 520 nm. One unit of the enzyme activity has been defined as the amount of enzyme which released 1μmole of 4-methoxy-2-naphthylamine from Z-Phe-Arg-NnapOMe substrate at the assay conditions. The assay of enzymes like cathepsin B[12], DPPI[3] and DPPII[10] were carried out using Z-Arg-Arg-NnapOMe, Gly-Arg-NnapOMe and Lys-Ala-NnapOMe, respectively as substrates. The azocasein degrading activity was determined by using the method of Barrett[3]. Cathepsin D activity was measured using denatured haemoglobin[4] at pH 3.5.

Protein Estimation : The protein content of the different samples was estimated by the method of Lowry *et al.*[22] using bovine serum albumin as the standard protein. The fractions emerging from columns were monitored for their protein content by measuring absorbance at 280 nm.

Purification of goat brain cathepsin L

All procedures were carried out at 4°C unless stated otherwise. The starting material, brain acetone powder, was prepared by homogenising the fresh and washed goat brain tissue in 10 volumes of chilled acetone and subsequent filtration under suction. The tissue powder was stored dry in a desiccator under reduced pressure . One hundred g of this acetone powder was homogenised in 3000 ml of 0.1 M sodium acetate buffer (pH 5.5) containing 0.2 M NaCl and 1 mM EDTA and allowed to stir for 30 min. The homogenate was centrifuged at 13000 x g for 30 min in a refrigerated centrifuge. The supernatant S_1 was decanted off, acidified by 1 N HCl to pH 4.2 and allowed to autolyse overnight at room temperature. The autolysed homogenate was then centrifuged at 13000 x g for 30 min. to collect supernatant S_2. The above supernatant was saturated to 30% by adding solid $(NH_4)_2 SO_4$ and centrifuged at 25000 x g for 30 min. The pellet was discarded and the resulting supernatant S_1 was further saturated to 70% by the addition of solid $(NH_4)_2SO_4$ and centrifuged at 25000 x g for 30 min to get the precipitated proteins as pellet P_4. The pellet P_4 was dissolved in minimum volume of 0.1 M sodium acetate buffer (pH 4.6) containing 0.2 M NaCl, 1 mM EDTA and 0.1 mM $HgCl_2$ and dialysed against the same buffer for 24 h and then

concentrated through Amicon Diaflo ultrafiltration device using YM 10 membrane. This sample was then fractionated on a Sephadex G-100 column (135 x 3.3 cm) using the above buffer as the eluent. The fractions active against Z-Phe-Arg-NnapOMe, BANA, Z-Arg-Arg-NnapOMe and azocasein were pooled together, concentrated and dialysed against 0.05 M sodium acetate buffer (pH 5.3) containing 0.1 mM $HgCl_2$ and 1 mM EDTA and put on a cation-exchange column CM-Sephadex C-50 (30 x 2.6 cm). After washing the column free from unadsorbed proteins, the bound proteins were eluted by applying a linear NaCl gradient (0.0-0.8 M NaCl). In this step, the cathepsin L.(Z-Phe-Arg-NnapOMe-degrading activity) was clearly separated from cathepsin B (Z-Arg-Arg-NnapOMe-degrading activity). The fractions eluted at 0.6 M NaCl concentration and having only Z-Phe-Arg-NnapOMe-hydrolysing activity were pooled and concentrated. The Z-Arg-Arg-NnapOMe-hydrolysing activity (cathepsin B) was eluted at 0.35 M concentration of NaCl.

Homogeneity of the enzyme

The purity of the enzyme was established by gel electrophoresis at pH 8.3[6], gel electrophoresis at pH 4.5[31] and electrophoresis in presence of SDS at pH 7.2[33].

Molecular weight determination

The molecular weight of the enzyme was determined by gel filtration on Sephadex G-75 column and SDS-PAGE using bovine serum albumin (68 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13 kDa), as standard marker proteins.

RESULTS AND DISCUSSION

Purification of cathepsin L

Cathepsin L from goat brain was purified ~1800-fold with ~20% recovery and had a specific activity of 17.40 units mg^{-1} protein with chromogenic substrate Z-Phe-Arg-NnapOMe (Table 1). The procedure adopted is very rapid and economical because it involves only one purification step i.e. CM-Sephadex C-50 cation-exchange chromatography at pH 5.3 after molecular sieve chromatography on Sephadex G-100. The proteins/proteases present in ammonium sulphate precipitated (30-70%) sample were separated by molecular sieve chromatography

on Sephadex G-100 column (Fig.1). In this step, the proteins/proteases were separated on the basis of their molecular weights while in the earlier procedures[26,27] used by Mason *et al.*[26] for purification of cathepsin L from human and rabbit liver, this fractionation was carried out after the cation-exchange chromatography, where it has limited advantage. The present method, therefore, becomes very economical particularly when several proteases like DPPI (EC 3.4.14.1), DPPII (EC 3.4.14.2), cathepsin B (EC 3.4.22.1) and cathepsin L are to be isolated simultaneously. The use of cation-exchange chromatography (Fig.2) separated cathepsin B and other related proteinases from cathepsin L where they were eluted at 0.35 and 0.6 M NaCl concentrations respectively. The protein eluted at 0.35 M concentration represented cathepsin B as revealed by the hydrolysis of Z-Arg-Arg-NnapOMe and BANA. Further, the protein eluted at 0.6 M NaCl concentration represented cathepsin L because it hydrolysed Z-Phe-Arg-NnapOMe. The substrates Z-Arg-Arg-NnapOMe and BANA were not hydrolysed at all by the protease in this peak and thus any contamination with cathepsin B is excluded. In this purification procedure for cathepsin L, a very good yield was obtained as compared to the yield reported by other workers[2,18,25,26,29,32], which varied from 1.5 to 11%. The main reason for this improvement in yield seems to be less number of steps involved in the total purification procedure. Moreover, the inclusion of 0.1 mM $HgCl_2$ in the purification steps of Sephadex G-100 and CM-Sephadex C-50 chromatographies, saved the enzyme from autolysis/inactivation by maintaining it in its inactive mercury form. In the present case also, the enzyme activity was poor when 0.1 mM $HgCl_2$ was excluded from the buffers during the purification procedure.

Table 1. Purification of cathepsin I from goat brain

PURIFICATION STEP	TOTAL ACTIVITY (Units)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY (units mg^{-1})	PURIFICATION FACTOR	YIELD (%)
Crude extract, supernatant, S_1	208.86	21665.0	0.0096	1.0	100.00
Acid fractionation, supernatant, S_2	207.35	10796..5	0.0192	2.0	99.28
30-70% (NH_2) SO_4 Pellet, P_4	206.42	624.0	0.3308	34.5	98.83
Sephadex G-100 pool	146.92	180.0	0.8162	85.0	70.34
CM-Sephadex C-50 pool	41.78*	2.4	17.4083	1813.4	20.0

Z-Phe-Arg-NnapOMe was used as substrate to measure the activity of cathepsin L. The acticity units are expressed as μmoles of 4-methoxy-2-naphthylamine liberated per min at 37°C. The assay procedures are listed under 'Materials and Methods'.

**The steep decrease in activity actually represents the separation of cathepsin B which also hydrolyses Z- he-Arg-NnapOMe approximately to the same extent as cathepsin L does.*

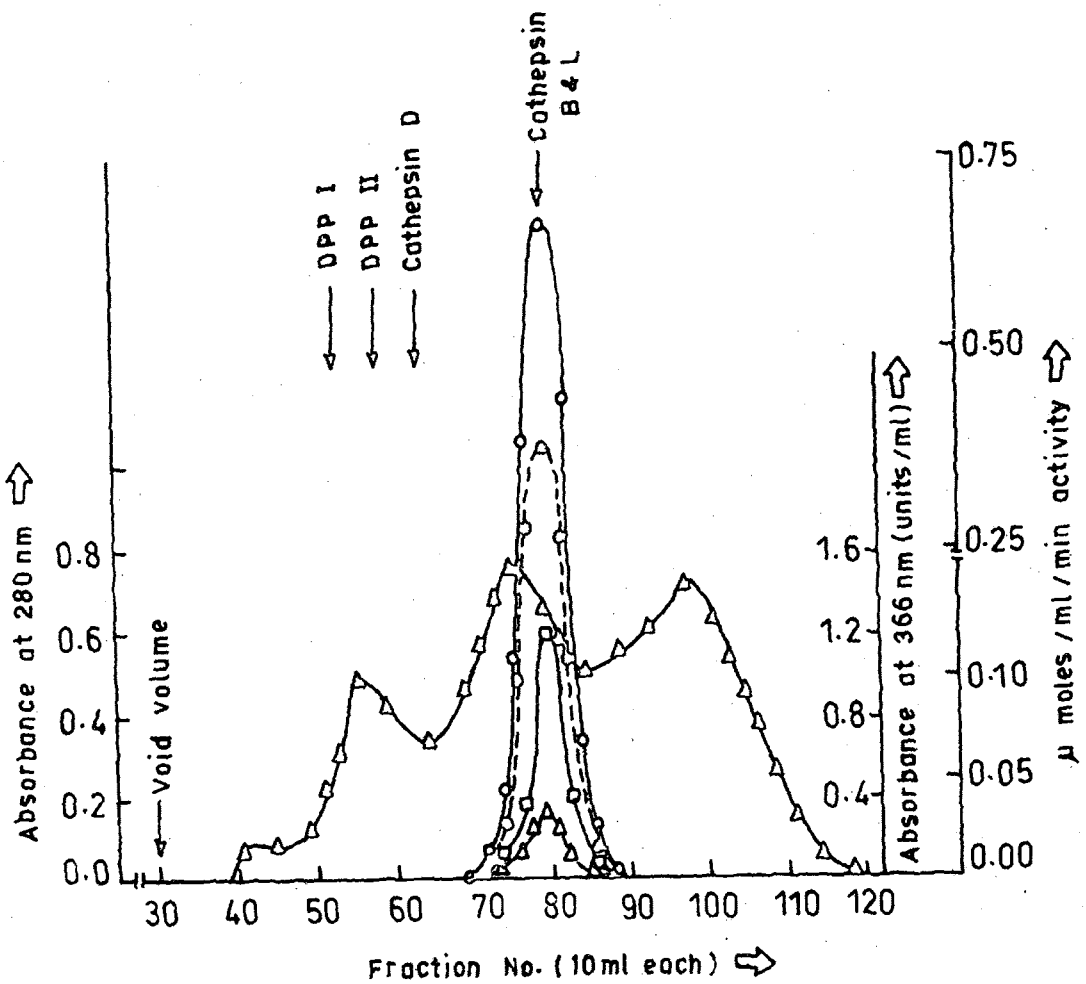

Fig. 1. **Gel filtration chromatography on Sephadex G-100 column.** Ammonium sulphate (30-70%) fraction (50 ml) of goat brain homogenate was loaded on Sephadex G-100 column (135 x 3.3 cm) and eluted with 0.1 M sodium acetate buffer pH 4.6 containing 0.2 M NaCl, 1 mM EDTA and 0.1 mM $HgCl_2$. Protein (△——△); Z-Phe-Arg-NnapOMe-hydrolysing activity (○——○), Z-Arg-Arg-NnapOMe-hydrolysing activity (○——○), BANA- hydrolysing activity (△——△) andazocasein hydrolyising activity (□——□).

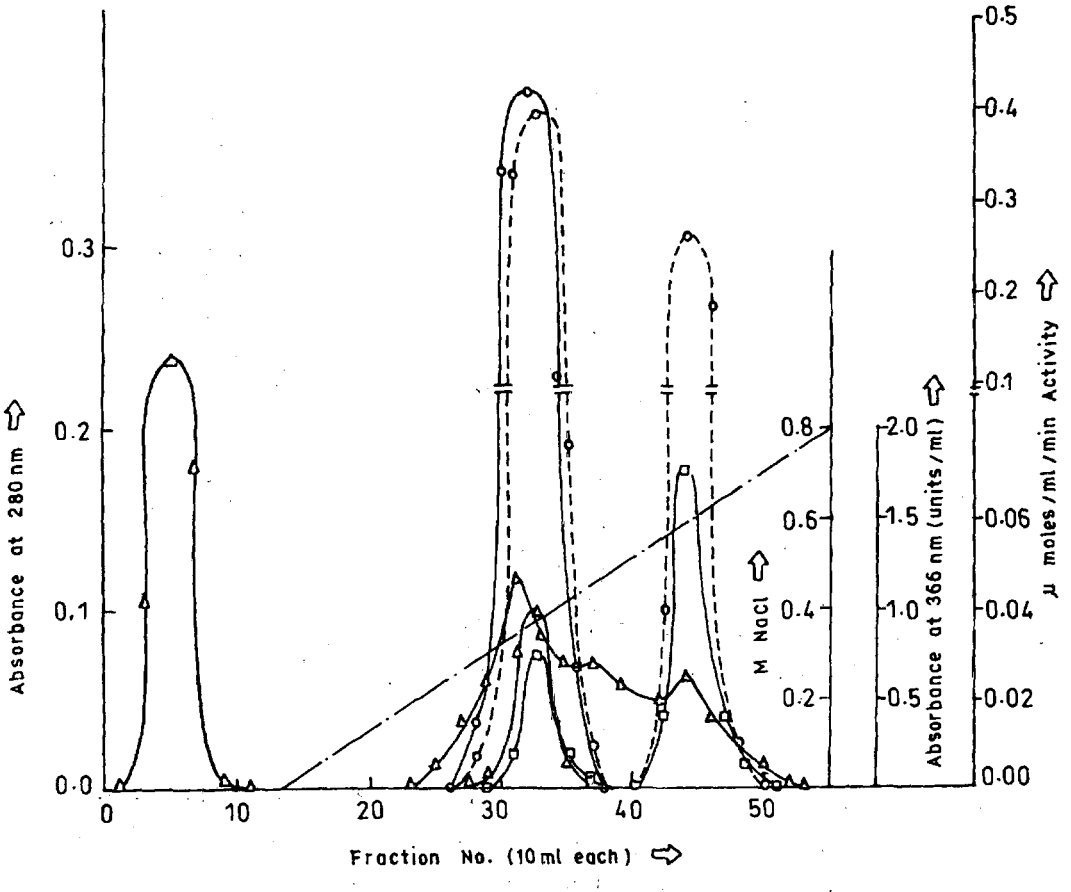

Fig. 2. **Cation-exchange chromatography onCM-Sephadex** C-50. The enzyme pool degrading Z-Phe-Arg-NnapOMe was loaded on CM-Sephadex column and eluted with 50 mM sodium acetate buffer pH 5.3 containing 1mM EDTA, o.1 mM $HgCL_2$ using 0.0-0.8 M Nacl gradient Protein (△—△); Z-Phe-Arg-Nnap-OMe-hydrolysing activity (○—○); Z-Arg-Arg-NnapOMe-hydrolysing activity (○——○); BANA-hydrolyzing activity (△——△) and azocasein degrading activity (□—□).

The enzyme obtained from goat brain by the above procedure was found to be electrophoretically pure (Fig.3) as evidenced by a single band at pH8.3, pH 4.5 and pH 7.2. In electrophoresis at pH 4.5 (cathepin L activity is not destroyed at this pH), the gels were also stained for enzymatic activity with the specific substrate Z-Phe-Arg-NnapOMe, where the activity band corresponded well with the protein band (Fig.3). The molecular weight estimations of goat brain cathepsin L by gel filtration and SDS-PAGE gave the values of 27 kDa and 25 kDa, respectively. The protein in SDS-PAGE moved as a single band even in highly reduced (with 2-ME) and denatured conditions. Dufour *et al.*[8], recently showed that chicken liver cathepsin L also existed as single polypeptide chain with molecular weight of 25 kDa. However, cathepsin L. from human kidney[20] and human liver[28] have been shown to consist of two chains : a heavy polypeptide chain of 25 kDa and a light polypeptide chain of 5 kDa as revealed under denaturing and reducing conditions though under non-reducing conditions these enzymes also showed a single polypeptide band of molecular weight of 30 kDa[20,28].

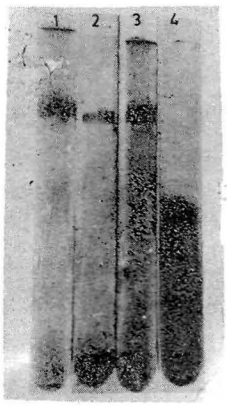

Fig. 3. **Homogeneity of purified cathepsin L by gel electrophoresis at various pHs :** Gel 1, Davis Gel electrophoresis at pH 8.3; Gel 2 and 3, Reisfeld gel electrophoresis (in gel 2, the activity stain was carried out by incubating the gel in assay buffer at pH 6.0 using Z-Phe-Arg-NnapOMe, where released NnapOMe was coupled with Fast Garnet GBC). Gel 4, SDS-PAGE in presence of 2-ME at pH 7.2. All the gels were loaded with 20µg of purified goat brain cathepsin L.Gels 1,3 and 4 were stained for protein with coomassie brilliant blue R 250.

Substrate specificity

Cathesin L exhibited very narow substrate specificity. Among the various substrates tested at pH 6.0, only Z-Phe-Arg-NnapOMe was hydrozed to an appreciable extent (17.4 units mg^{-1}). The specific substrates of cathepsin B[12] like Z-Val-Lys-Lys-Arg-NnapOMe and Z-Ala-Arg-Arg-NnapOMe were not hydrolyzed at all, whereas another substrate Z-Arg-Arg-NnapOMe of cathepsin B was hydrolyzed only negligibly (0.3 units mg^{-1}). No dipeptidyl peptidase and aminopeptidase substrate was hydrolyzed by goat brain cathepsin L. As regards the p-nitroanilide substrates, only Z-Phe-Arg-pNA was cleaved by goat brain enzyme (3.09 units mg^{1}) and was 5 times less sensitive than Z-Phe-Arg-NnapOMe. Though an extensive substrate specificity study of cathepsin L with –7-amino-4-methylcoumarinamide substrates [1,26,27] has been reported whereby the enzyme activity was quantitated fluorometrically, yet studies relating to chromogenic –2-naphthylamide and –4-methoxy-2-naphthylamide substrates *vis-à-vis* cathepsin L are non-existent. Hence, Z-Phe-Arg-NnapOMe has been established here as a good chromogenic substrate for cathepsin L. The K_m and V_{max} values of 6.6×10^{-5} M and 19.04 units mg^{-1} protein respectively were obtained for the hydrolysis of Z-Phe-Arg-NnapOMe by goat brain cathepsin L. The literature survey revealed the absence of any kinetic data with regards to action of cathepsin L on chromogenic substrates like Z-Phe-Arg-NnapOMe, which is reported here for the first time. The reported K_m values for Z-Phe-Arg-7-amino-4-methylcoumarinamide substrate of 5×10^{-6}M by Azaryan and Galoyan[1] and 1.25×10^{-6}M by Marks and Berg[25] could not, however, be compared.

Activation and Inhibition Studies

For goat brain cathepsin L, glutathione in conjunction with EDTA proved to be the best activator followed by cysteine, 2-ME, DTE, DTT and thioglycolic acid. Though the metal chelating agent EDTA and the thiol activators when used separately could activate brain cathepsin L only partially i.e. 50%, the full activating potential of these additives was revealed when thiol activators were used in conjunction with EDTA (>100% increase in activity). Thus EDTA was found to be essential to exploit the full potential of activators. Though different workers have measured cathepsin L activity in the presence of glutathione or cysteine[1,18,27] but the comparative study in respect of these activators does not exist. The thiol blocking and alkylating reagents like DTNB, p-hydroxy mercuriphenyl sulphonic acid, N-

ethylmaleimide, 2,2[1]-dipyridyl disulphide, iodoacetic acid, iodoacetamide, desylbromide and E-64 inhibited the activity of goat brain cathepsin L (Table 2). This behaviour of goat brain cathepsin L resembled rabbit liver[27] and bovine brain[1] cathepsin L. The enzyme was also inhibited by heavy metal ions like Zn^{2+}, Mn^{2+} and Hg^{2+}. The inhibition by Zn^{2+} and Mn^{2+} was reversed by EDTA, whereas the inhibition by Hg^{2+} was of permanent nature and was not reversed by EDTA. This inhibition by Hg^{2+} however could be reversed by thiol activators simply by reduction of mercuri-sulfide bond formed between Hg and enzyme active-site. The known elastase inhibitors[30] like Ac-$(Ala)_4$-CH_2Cl, Ac-$(Ala)_2$-Pro-Ala-CH_2Cl and Ac-$(Ala)_2$-Pro-Val-CH_2Cl were very good inhibitors and are being reported here as such for the first time. The microbial peptide inhibitors antipain and leupeptin were also good inhibitors for goat brain cathepsin L as reported for rabbit liver[27] cathepsin L but pepstatin and bestatin did not inhibit the activity at all. The inhibitors like egg-white trypsin inhibitor type II, soyabean trypsin inhibitors type-II, trasylol, puromycin, benzamidine, α_2-macroglobulin and α_1-antitrypsin were found to be poor inhibitors. The K_1 value obtained by the dose-dependent data using Dixon method[7] for the inhibition of goat brain cathepsin L by leupeptin was 1.5×10^{-9} M. The K_1 value for the brain enzyme compares well with K_1 values of 1.6×10^{-9} M and 1.8×10^{-9}M for human and rabbit liver 26,27 cathepsin L.

Table 2 : Effect Of Various Inhibitors On Brain Cathepsin L

Inhibitor	Final Concentration	% Inhibition
None		0.0
α_1-Antitrypsin	0.1 mg/ml	21.0
α_2-Macroglobulin	0.05 mg/ml	31.0
TLCK	0.001 mM	80.0
TPCK	0.01 mM	51.1
Leupeptin	0.00000 mM	91.0
Antipain	0.001 mM	95.0
Ac-$(Ala)_4$-CH_2Cl	0.001 mM	95.0
Ac-$(Ala)_2$-Pro-Ala-CH_2Cl	0.001 mM	88.0
Ac-$(Ala)_2$-Pro-Val-CH_2Cl	0.001 mM	96.0
E-64	0.001 mM	98.8
p-Chloromercuribenzoic acid	0.1 mM	93.1
2,2'-Dipyridyl disulphide	0.1 mM	94.0
Iodoacetic acid	0.01 mM	78.1
Iodoacetamide	1.0 mM	88.0
$MnSO_4$	1.0 mM	60.0
$MnSO_4$ + EDTA (1 mM)	1.0 mM	0.0
$ZnSO_4$	1.0 mM	98.0
$ZnSO_4$ + EDTA (1 mM)	1.0 mM	0.0
$HgCl_2$	0.01 mM	74.0
$HgCl_2$ + EDTA (1 mM)	0.01 mM	74.0

Cathepsin L. was first activated by incubating with 2 mM cysteine and 1 mMEDTA for 10 min. Then cysteine and EDTA were completely removed from the activated enzyme through extensive dialysis. This activated enzyme free from cysteine and EDTA was then pre-incubated with the respective inhibitor for 10 min and then the residual activity was measured by the usual assay procedure (the assay buffer did not contain any cysteine and EDTA) using Z-Phe-Arg-NnapOMe as substrate. The inhibition was taken as 0% when no inhibitor was added.

Catalytic activity

Cathepsin L obtained from goat brain hydrolyzed the synthetic substrate Z-Phe-Arg-NnapOMe maximally at pH 6.0. The activity of cathepsin L showed a sharp decline above pH 6.0. This pH optima of 6.0 is exactly same as the value for rabbit liver enzyme[27]. The enzyme cathepsin L was stable up to 47°C and beyond that there was a sharp inactivation. Up to 50% activity was lost at 55°C and there was hardly any activity left at 60°C. The enzyme was stable between pH 4.0-6.0. It was rapidly inactivated below pH 4.0 and above pH 6.0. At pH 3.5 only 50% activity was observed while at pH 3.0 there was no activity. Similarly at pH 7.0 only 14% of the activity remained. The optimum temperature for the hydrolysis of Z-Phe-Arg-NnapOMe by goat brain cathepsin L was between 45-50°C with activation energy of 11.5 Kcal mol^{-1} (Fig.4 and Fig.5). Typically, the activity of the enzyme increased linearly up to 40°C and there was sharp decrease above 50°C. The enzyme cathepsin L when stored in 50 mM sodium acetate buffer (pH 4.6) containing 1 mM EDTA, 0.2 M NaCl in presence of 1 mM $HgCl_2$ retained 100% activity up to at least four months. However, in presence of 1 mM 2-ME, it lost 90% activity only in one month.

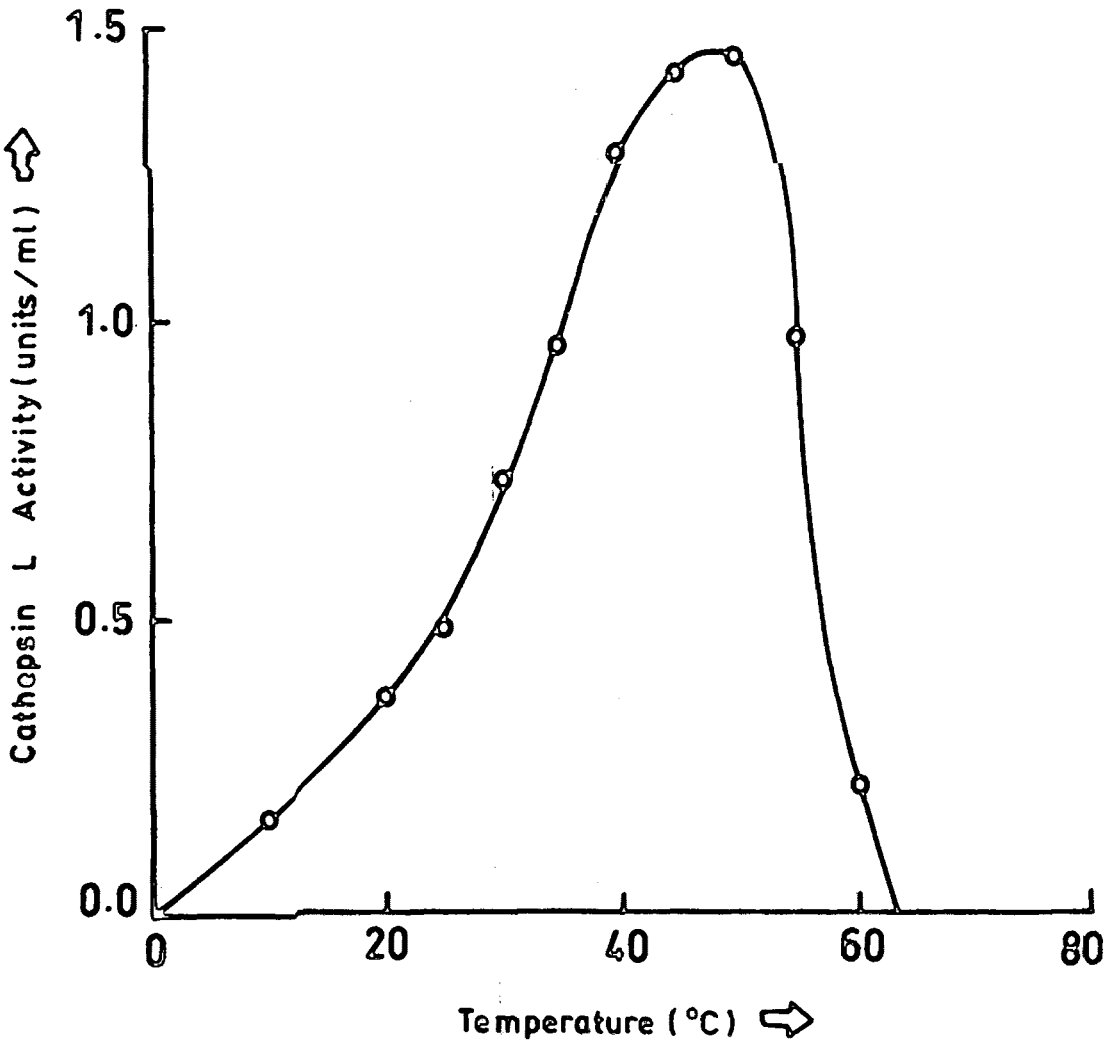

Fig. 4. **Temperature dependence of cathepsin L activity:** Activity of activated cathepsin L has been measured at different temperatures i.e. 0°, 10°, 20°, 25°, 30°, 35°, 40°, 45°, 50°, 55°, 60° and 70°C by usual assay procedure.

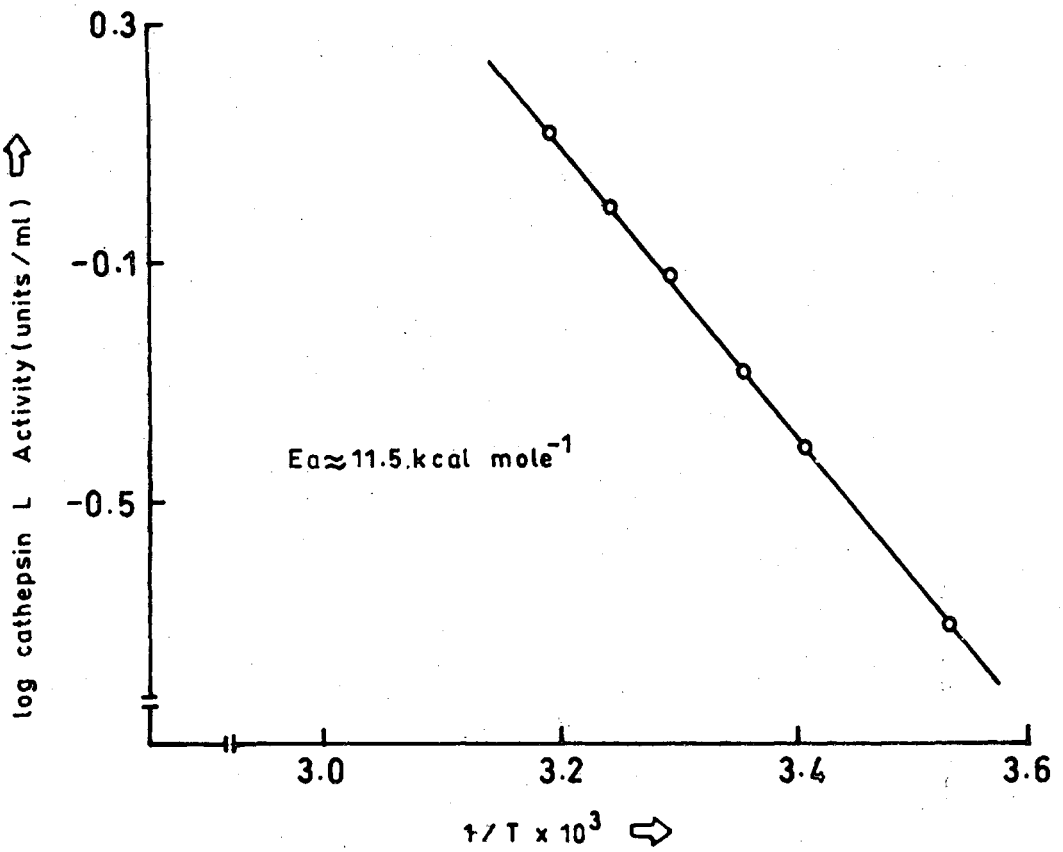

Fig. 5 . Arrhenius plot for cathepsin L : Activation energy, E_a has been determined by plotting log of cathepsin L activity versus $1/T \times 10^3$ (where T is absolute temperature).

The availability of highly purified cathepsin L in high yield will facilitate the production of antibodies and detection of the enzyme in intact tissues and cells by immunoprecipitation method. Such method can also be utilized as a probe to detect/measure the diseased conditions occurring in the mammalian tissue. This will also stimulate the interest to study the action of cathepsin L on naturally occurring peptides/polypeptides including the bioactive peptides present in brain.

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DANAID BUTTERFLIES (NYMPHALIDAE : DANAINAE) OF BUXA TIGER RESERVE, JALPAIGURI, WEST BENGAL

Sumana Saha and Dinendra Raychaudhuri

Entomology Laboratory, Department of Zoology, University of Calcutta, 35, Ballygunge Circular Road, Kolkata – 700 019.

Abstract

The paper deals with the taxonomy, patterns, associations and sequences of danaid butterflies of Buxa Tiger Reserve, a typical forest of West Bengal. Seven species belonging to 7 patterns recognized for the north-east Indian fauna[1] have been recorded. *Idea agamarschana agamarschana* though distributed in Chittagong, Bangladesh is absent in the forest.

INTRODUCTION

The subfamily Danainae recognized by the possession of a pair of specialized brush-like organs, the 'hair pencils' pushed out from the abdominal tip to disperse the female attractants in male, and clubbed ankylosed fore tarsi in female belong to the family Nymphalidae or the 'tetrapods'. Danaine fauna of North Eastern region of India has poorly been worked out. However, 22 species are known to occur in this region. Buxa forest more commonly referred to as Buxa Tiger Reserve, a typical of north-east India has received extremely poor attention from the standpoint of biodiversity. This is evident from the publication which listed 11 butterfly species only from the forest. Our sustained survey for the 3 consecutive (1994-97) years has helped recording 97 species of butterflies. We have been able to record representatives of all the 7 recognized patterns[1]. It is concluded that the forest appears to be the representative of entire north-eastern Indian region with extreme degree of biodiversity potential.

TAXONOMY

1. *Danaus (Salatura) genutia (Cramer)* (Fig. : 1)

   Common name : The Striped Tiger/The Common Tiger

   Diagnosis : Brown or orange, 'African Queen' pattern (7^1)

Received August 18, 2000; Accepted September 10, 2001

Status : Fairly common[2].

Distribution : Australian, Malayan, Oriental.

Period of record : Summer, Winter.

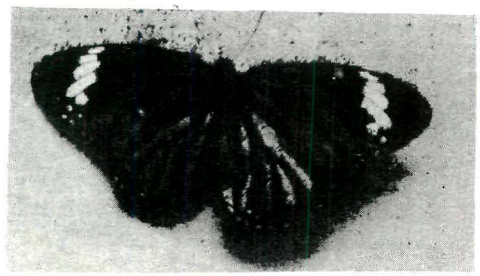

Fig. 1. *Danaus (Salatura) genutia* (Cramer)

2. *Euploea core* (Cramer) (Fig. : 2)

Common name : The Common Indian Crow

Diagnosis : Dark, with pale marginal spots and submarginal spots/splashes (1^1).

Status : Very common[2].

Distribution : Australian, Malayan, Oriental.

Period of record : Summer, Autumn, Winter.

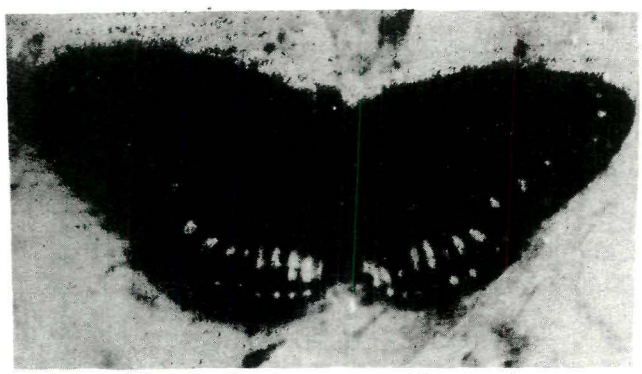

Fig. 2. *Euploea core* (Cramer)

3. *Euploea mulciber* (Cramer) (Figs. : 3-4)
Common name : The Striped Blue Crow

Diagnosis : □ : Dark, violet forewing with or without pale spots (1/2[1]); □ : Dark, forewing part violet, hindwing'tiger' pattern (2/3a[1]).

Status : Common in north-east India and Burma, rare elsewhere[2].

Distribution : Malayan, Oriental.

Period of record : Summer, Winter.

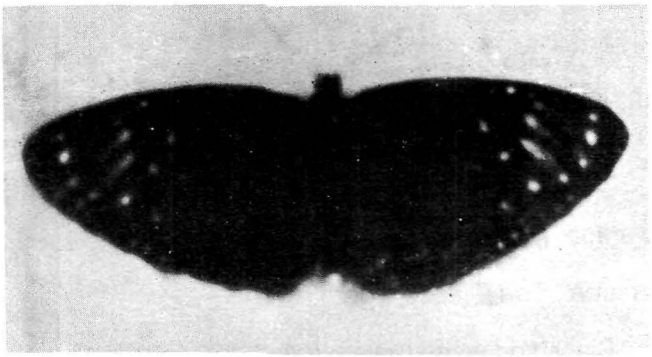

Fig. 3. *Euploea mulciber* (Cramer) □

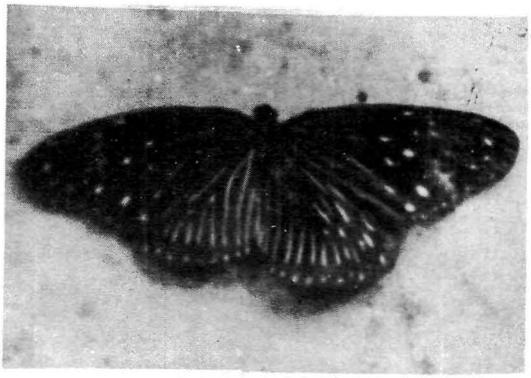

Fig. 4. *Euploea mulciber* (Cramer) □

4. *Euploea* radamanthus (Fabricius) (Fig. : 5)

Common name : The Magpie Crow

Diagnosis : Dark, forewing part violet (sometimes only in □), white bar at apex of forewing, hindwing white splashed (2/3[1]).

Status : Common[2].

Distribution : Malayan, Oriental.

Period of record : Summer.

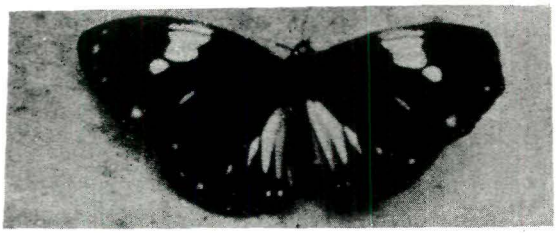

Fig. 5. *Euploea radamanthus* (Fabricius)

5. *Parantica aglea* (Stoll) (Fig. : 6)

Common name : The Glassy Tiger

Diagnosis : Black and white (pale blue), 'tiger' pattern (3[1]).

Status : Fairly common[2].

Distribution : Oriental.

Period of record : Summer, Autumn, Winter.

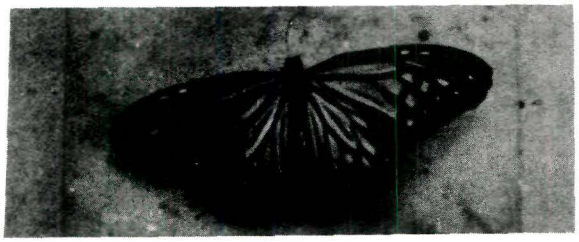

Fig. 6. *Parantica aglea* (Stoll)

6. *Parantica melaneus* (Cramer) (Fig. : 7)

Common name : The Chocolate Tiger

Diagnosis : Black hyaline and brown (6^1).

Status : Common[2].

Distribution : Malayan,Oriental.

Period of record : Winter.

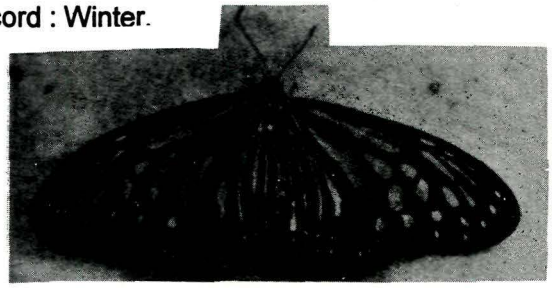

Fig. 7. *Parantica melaneus* (Cramer)

7. *Tirumala limniace* (Cramer) (Fig. : 8)

Common name : The Blue Tiger

Diagnosis : Black and white (pale blue), 'tiger' pattern (3^1).

Status : Very common[2].

Distribution : Malayan, Oriental.

Period of record : Summer, Winter.

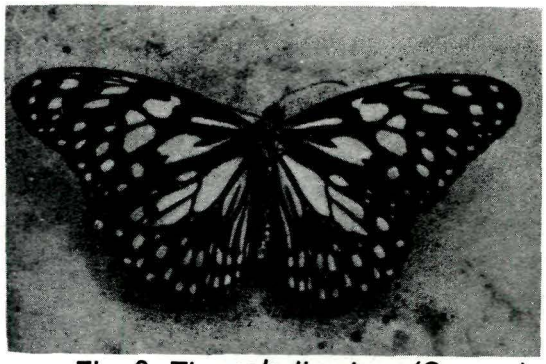

Fig. 8. *Tirumala limniace* (Cramer)

DISCUSSION

A careful look at the distribution of the recorded danaine species in the forest reveals that the area Jayanti shows maximum species richness as compared to 19 other localities (Table 1). This is followed by Bhutanghat, Nimati and South Raydak wherefrom 4 species could be recorded. Poorest situation is there in Buxaduar, Cheko and Hatipota. Incidentally it is mentioned that the first named area is at an altitude of 1917m, the second a reclaimed forest area while the last one is experiencing dolomite mining. It appears, therefore, that largely the human factors and to some extent the altitudinal factors are responsible for species congregation in the different localities of the forest. It is needless to mention that Jayanti provides an absolutely pristine forest area with Assam Himalaya at its back. Again, all these 7 species have their distribution in north-eastern Indian zone, 5 in peninsular India, 4 in western zone (Table 2). Ackery & Vane Wright listed 22 species from north-eastern zone including Bangladesh while presenting the patterns, associations and sequences of the danaine fauna. They (op. cit.) recognized 8 such patterns of the danaines of the Indian subregion. Of these, patterns 5 and 8 are absent in the north-eastern Indian zone. Our finding is in absolute conformity with earlier report[1]. However, we could not record the species *Idea agamarschana agamarschana* (distributed in Chittagong : Bangladesh) belonging to the pattern 4[1]. Absence of the said pattern in the forest area appears rational. Broadly the said pattern is altogether absent in the geographically defined area of the north-eastern Indian zone.

It appears that the forest can be regarded as miniature of north-eastern Indian zone with regard to the danaine fauna since the representatives of each of the patterns is there in the forest. The forest, therefore, should be attributed as a highly rich ecosystem promoting optimal diversity.

We are also inclined to support the remark of Ackery & Vane Wright who experienced difficulty in fitting the south-east Asian danaine fauna into the 6 discrete patterns. Our samples firmly support the colour patters,1, 1/2, 2/3, 2/3a, 3, 6 and 7 of Ackery & Vane Wright.

Table 1. Showing distribution of the butterfly species within buxa tiger reserve

DS	BG	BD	CK	DM	GD	HP	JY	KG	NL	NM	PB	PK	PR	RM	RK	SK	ST	SB	SR	TG
1			+				+			+								+	+	
2	+						+					+			+		+	+	+	
3	+					+	+				+	+		+	+				+	
4											+									
5	+	+					+			+	+			+				+		
6	+						+			+				+					+	
7							+			+		+								

BG -Bhutanghat, BD – Buxaduar, CK – Cheko, DM – Damanpur,

GD – Gadadhar, HP – Hatipota, JY – Jayanti, KG – Kumargram,

NL – Newland, NM – Nimati, PB – Panbari, PK – Phaskhawa,

PR – Poro, RM – Raimatang, RK – Rajabhatkhawa, SK – Sankosh,

ST – Santrabari, SB – South Bholka, SR – South Raydak,

TG – Tashigaon

DS – Danaine Species, 1. Danaus (Salatura) genutia (Cramer), 2. Euploea core (Cramer), 3. Euploea mulciber (Cramer), 4.Euploea *radamanthus* (Fabricius), 5. *Parantica aglea* (Stoll), 6. *Parantica melaneus* (Cramer), 7. *Tirumala limniace* (Cramer).

Table 2. Showing distribution of the butterfly species in India

Danaine Species	Eastern	Western	Southern	Northern
1.*Danaus (Salatura) genutia* (Cramer)	+			+
2. *Euploea core* (Cramer)	+	+	+	+
3. *Euploea mulciber* (Cramer)	+		+	+
4. *Euploea radamanthus* (Fab)	+			+
5. *Parantica aglea* (Stoll)	+	+	+	+
6. *Parantica melaneus* (Cramer)	+	+	+	+
7. *Tirumala limniace* (Cramer)	+	+	+	+

Further, of the 22 clades recognized[1], the forest harbours members of the clades 1, 11, 111, 1111; 12,121, 122, 122.2, 122.21; 2, 21, 211. It also speaks about proportionately greater density of the danine fauna in the forest as compared to the global one.

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BLEACH-BOOSTING OF EUCALYPTUS KRAFT PULP USING COMBINATION OF XYLANASE AND PECTINASE FROM *STREPTOMYCES* SP. QG-11-3

Qasim Khalil Beg[1], Mukesh Kapoor[1], R.P. Tiwari and G.S. Hoondal*

[1]*Department of Microbiology, Delhi University, Delhi - 110 021*

Department of Microbiology, Panjab University, Chandigarh – 160 014.

Abstract

The boosting effect of eucalyptus kraft pulp bleaching was attained when a combination of xylanase and pectinase from *Streptomyces* sp. QG-11-3 was used for the purpose of biobleaching. The 'xylanase-pectinase' combination, each at 3.5 U/g for moisture free pulp, reduced the kappa number and viscosity of treated pulp by 30% and 31%, respectively and increased the tensile strength and burst factor up to 61% and 8% respectively, while the brightness of the pulp sheet was increased by 8% ISO (International Standard Organization). When the enzymatically treated pulp was subsequently treated with 4.5% chlorine the brightness of the pulp increased from 25% ISO (control) to 54% ISO. These pulp properties were superior to those achieved after pulp treatment with either xylanase or pectinase alone.

INTRODUCTION

Pulp biobleaching involves use of microorganisms and their enzymes. It relies on the ability of microorganisms to depolymerize lignin directly and on the degradation of hemicellulosic components[1,13]. A number of microbial enzymes are known to assist in processing a range of pulps in paper industries. Pectinases are used for enzymatic debarking[1] and lipases in resolving pitch problems[1,12]. Xylanases have been shown to play the main role in pulp prebleaching[13]. However, synergism with other hemicellulolytic enzymes such as mannanases[5,12] and oxidase enzymes such as laccases and manganese peroxidases have been successfully used for prebleaching[7], leading to a significant reduction in amount of chlorine consumption. Recently, pectinases have been shown to assist in lowering the cationic demand in paper making after peroxide treatment stage[11].

The strain *Streptomyces* sp. QG-11-3 that produced a thermostable alkaline xylanase and a thermostable pectinase active in both acidic and alkaline range[3] was used in the present study. We have already reported the potential of xylanase from *Streptomyces* sp. QG-11-3 in biobleaching of kraft pulp[2]. Here a bleach-boosting effect of synergism

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between pectinase and xylanase from *Streptomyces* sp. QG-11-3 has been reported for prebleaching of eucalyptus kraft pulp.

MATERIALS AND METHODS

Chemicals and pulp samples:

Birch-wood xylan, citrus pectin, 3,5-dinitrosalicylic acid and glutaraldehyde were purchased from Sigma (St. Louis, USA). All other analytical reagents and media components were purchased from SRL, Qualigens or Hi-Media, India. Unbleached eucalyptus kraft pulp, used in the present study, was kindly provided by Ballarpur Paper Industries Limited (BILT), Yamunanagar, India. The composition of pulp was as follows: eucalyptus, 29.56%; veneer waste, 34.56%; hardwood scrap, 7.8%; kokat wood, 3.96%; and bamboo, 24.22%. The kraft pulp was thoroughly washed before use and after each treatment step until the water attained neutral pH. All the studies were performed at 6% pulp consistency, pH 8.5-9.0 at 50°C, unless otherwise mentioned.

Enzyme production and enzyme assays:

The xylanase and pectinase from *Streptomyces* sp. QG-11-3 were produced in xylan-Horikoshi and pectin-Horikoshi medium respectively and both the enzymes were assayed under similar conditions as described previously[2,3]. Birchwood xylan and citrus pectin were used as the assay substrates for xylanase and pectinase respectively. The reaction mixture for each enzyme assay contained 250 µl of 1% of respective substrates prepared in glycine NaOH buffer, pH 8.6, and 250 µl of appropriately diluted enzyme and was incubated at 60°C for 10 min. The enzyme activity was determined by measuring the release of reducing sugars during the enzyme-substrate reaction using Miller's method[8]. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the release of one µ mole of reducing sugar from the respective substrates in one min under the standard assay conditions. All the experiments were carried out independently in triplicates and results presented here are the mean of three. The standard deviations were within 15%.

Treatment of pulp samples:

The eucalyptus kraft pulp was treated with optimized xylanase and pectinase enzyme dose of 3.5 U/g dry pulp and a combination of xylanase and pectinase at 50^{0}C for 2 h in sealed polythene bags[2]. The enzymatically-prebleached pulp, thus obtained, was given 4.5% chlorine treatment (pH 2.0) at 45^{0}C for 1 h, and pulp properties were determined, thereafter. In another experiment, the kraft pulp was first chemically bleached with

chlorine followed by the enzymatic treatment with different combinations at 50^{0}C for 2 h and pulp properties determined

Physical and chemical characterization of kraft pulp:

The treated pulp was washed and hand-sheets were prepared by standard TAPPI (Technical Association of Pulp and Paper Industries, Atlanta, USA) methods. The pulp properties such and kappa number (T236 cm-85), copper number (T430 om-94), burst factor (T403 cm-50), tensile strength (T231 cm-96), viscosity (T230 om-94) and brightness (T452 om-87) were determined by standard protocols of TAPPI, Atlanta. Release of reducing sugars was determined by DNSA method[8] and release of chromophores (A_{237nm}) and hydrophobic compounds (A_{465nm}) were determined by measuring the absorbance of the pulp free samples[9].

Scanning electron microscopy of pulp fibre:

Samples for scanning electron microscopy were processed following the method described elsewhere[2]. In brief, the fibres were washed thrice with deionized water and were fixed for 1 h in 2.5% glutaraldehyde (dissolved in phosphate buffer, pH 7.2). Fibres were separated from glutaraldehyde and washed thrice with same buffer and were gradually dehydrated with acetone gradient between 30-90% and finally suspended in 100% acetone. Small pieces of fibers were air dried and placed on the stubs mounted with silver tape and sputter coated with gold using fine coat, JEOL ion sputter, Model JFC-1100. The samples were examined at 20KV under scanning electron microscope (Model JSM 6100, JEOL) at various magnifications.

RESULTS AND DISCUSSION

Prebleaching of eucalyptus Kraft pulp using xylanase and pectinase from *Streptomyces* sp QG-11-3 when used in combination at 3.5 U each/ g moisture free pulp had the maximum effect. The treatment reduced the kappa number by 30% and viscosity by 31% with an appreciable increase in tensile strength, brightness and burst factor by 61%, 8%(ISO) and 8% respectively (Table 1) with higher release of reducing sugars, chromophore (A_{237nm}) and hydrophobic compounds (A_{465nm}). However, prebleaching with xylanase alone, could reduce the kappa number and viscosity by 22% and 19% respectively and increased the tensile strength, brightness and burst factor by 37%, 5%(ISO) and 5% respectively (Table 1). The correlation between the release of chromophores (A_{237nm}) and hydrophobic compounds (A_{465nm}) and the reduction in kappa

Table 1. Physicochemical properties of enzyme (xylanase, pectinase and 'xylanase + pectinase'), chemical, and 'enzyme + chemical' treated eucalyptus kraft pulp.

Bleaching process	Pulp Properties									
	Kappa Number	Tensile Strength (Nm/g)	Brightness (% ISO)	Copper Number	Burst Factor (KN/g)	Viscosity (Poise)	Final PH	Reducing Sugars (mg/g pulp)	A_{237nm}	A_{465nm}
Untreated (control)	16.08	10.20	25.50	1.42	1.06	17.67	N.D.	2.80	0.346	0.058
X	12.48	14.00	30.26	1.44	1.11	14.25	7.93	14.80	1.563	0.241
P	15.48	12.12	26.40	1.41	1.08	17.66	8.04	3.42	0.813	0.141
X+P	11.30	16.46	33.12	1.49	1.15	12.14	7.20	15.80	2.226	0.266
C	12.00	19.31	39.50	1.45	1.18	10.16	1.96	12.61	1.264	0.164
CX	11.62	19.68	43.75	1.43	1.21	10.12	6.42	11.30	1.374	0.064
C(X+P)	11.00	20.18	45.80	1.50	1.28	9.54	6.26	12.14	1.436	0.038
XC	9.25	22.92	50.40	1.53	1.20	9.40	1.67	10.02	1.034	0.056
(X+P)C	8.68	24.16	53.80	1.51	1.24	9.15	1.70	8.26	0.912	0.045

X : Xylanase treatment with 3.5 U/g moisture free pulp (50^{0}C for 2 h; pH 8.5–9.0)
P : Pectinase treatment with 3.5 U/g moisture free pulp (50^{0}C for 2 h; pH 8.5–9.0)
X+P : 'Xylanase + Pectinase' treatment with 3.5 U/g moisture free pulp, each (50^{0}C for 2 h; pH 8.5–9.0)
C : Chlorine treatment with 4.5% Cl_2 (50^{0}C for 45 min; pH 2.0–2.5)
A_{237nm} : Absorbance at 237nm
A_{465nm} : Absorbance at 465nm
N.D : Not determined

number coupled to the release of reducing sugars suggested the dissociation of lignin-carbohydrate complex from the pulp fibres. Release of chromophores also correlates with reduction in chemical demand during bleaching. A reduction in chlorine consumption by 8.8% was observed in bleached kraft pulp when both the enzymes were used in combination as compared to 8% reduction in chlorine consumption when only xylanase was used.

When we compared the "(X+P) C" treatment (xylanase plus pectinase' treatment followed by chemical treatment) with "C(X+P)" (chemical treatment followed by 'xylanase plus pectinase' treatment), it was found that the "(X+P)C" treated kraft pulp exhibited 15% higher reduction in kappa number, and 39% and 31% higher improvement in tensile strength and brightness respectively compared to "C(X+P)" treatment (Table 1). There was not much difference in the burst factor and viscosity change factor in both the treatments. However, the XC treated kraft pulp exhibited 14% higher reduction in kappa number and 31% higher improvement in both, tensile strength and brightness, compared to CX treatment. These results indicate that enzymatic prebleaching could have facilitated an increase in pulp fibrillation, water retention and restoration of bonding in fibers and increased freeness in fibres.

The present study showed that addition of enzymes, either before or after chemical bleaching stage resulted in enhanced pulp properties. The target substrates of both xylanase and pectinase prebleaching and post-chemical treatment may have similar features because there was a release of chromophores and lignin compounds (indicated by decrease in kappa number). In all the bleaching stages, it was observed that enzymatic-prebleaching has a major role to play in opening up the pulp structure to be accessible to chlorine and other chemicals to be used in later treatment stages. Therefore, enzymatic prebleaching of kraft pulp can be established as the most suitable step to facilitate bleach boosting of pulp. The application of xylanases as prebleaching agent in pulp bleaching for pulp improvement has been reported by several workers using xylanases from *Streptomyces lividans*[10], *Streptomyces* sp. TUB B-12-2[4] and *Staphylococcus* sp. SG-13[6].

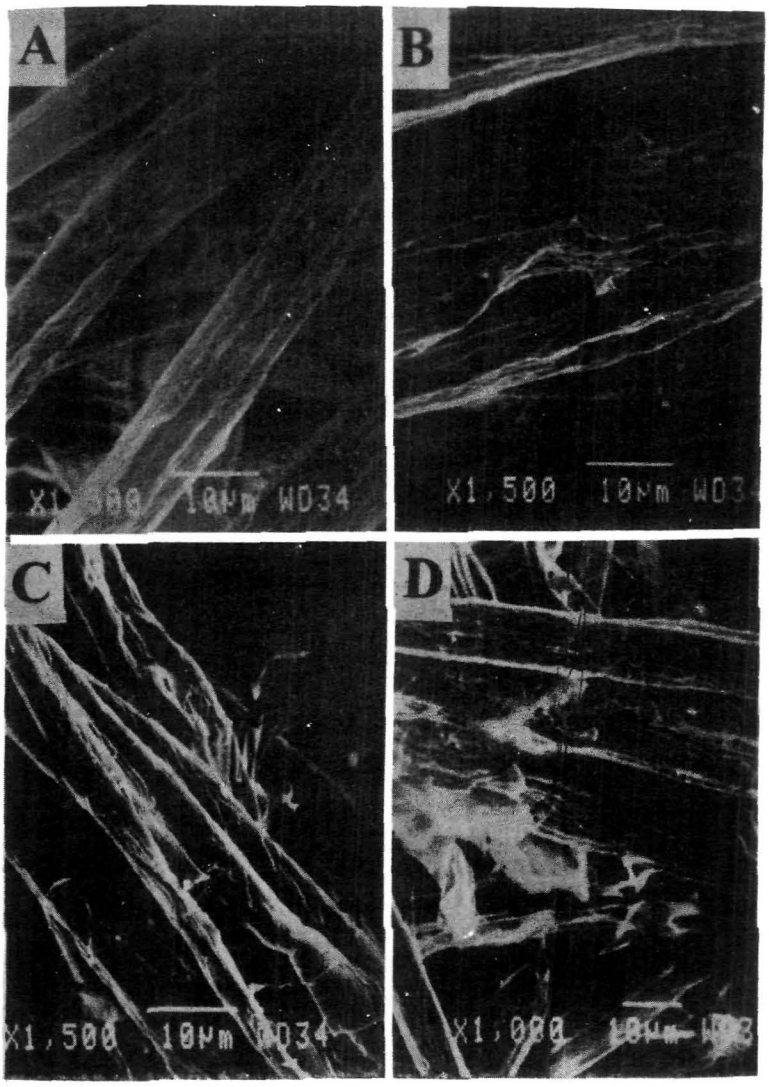

Fig.1. Scanning electron micrographs of control and treated eucalyptus kraft pulp

A: Untreated eucalyptus kraft pulp (control)

B: Xylanase treated (3.5 U/g) eucalyptus kraft pulp

C: "Xylanase+Pectinase" treated (3.5 U/g, each) eucalyptus kraft pulp

D: Eucalyptus kraft pulp treated with "Xylanase+Pectinase" followed by chemical treatment (4.5% Cl_2)

The scanning electron microscopic studies clearly show that enzymatic prebleaching of kraft-pulp using xylanase alone (Figure 1B) or in combination with pectinase (Figure 1C) opens the pulp structure causing separation of the pulp microfibrils as compared to smooth surfaces of the untreated pulp (Figure 1A), making it more accessible to subsequent treatment with chlorine and other chemicals (Figure 1D). These observations are also suggestive of additive effects of xylanase and pectinase in rendering the pulp fibers more accessible to chemical bleaching agents and thus resulting in bleach boosting of kraft pulp.

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BILATERAL ASYMMETRY OF LIMB DIMENSIONS AMONG THE PUNJABI ADOLESCENTS

Kewal Krishan* and J.C.Sharma**

**Department of Forensic Medicine, Government Medical College Hospital, Chandigarh*
***Department of Anthropology, Panjab University, Chandigarh*

Abstract

The study seeks to investigate bilateral limb asymmetry in a right handed Punjabi population of Chandigarh. The anthropometric data were collected on 145 males and 63 females belonging to a mixed Punjabi population of Chandigarh city and a nearby village. Seven anthropometric measurements were directly taken on the left and right sides of the subjects, and six dimensions were derived. Asymmetry was evaluated using paired t-tests. Most of the upper extremity measurements are significantly asymmetric in favour of the right side, whereas lower extremity measurements favour the left side. Total upper extremity length in both the sexes, upper arm length in males and hand length in females are not significantly asymmetric. In the upper extremity of males, asymmetry increases with the advancement of age, and in the lower extremity, asymmetry declines with the advancement of age. However, in females, there is no such relationship between age and asymmetry. Limb asymmetry is weakly correlated to body weight in both the sexes.

INTRODUCTION

Bilateral asymmetry in human morphology is demonstrable in various body parts and structures. Asymmetries of anthropometric dimensions like skulls, teeth, lengths of bones, foot and hand length, skeletal maturation, and dermatoglyphic patterns have already been reported [2,5,7-9,14,15,17,21-25,27,29,30,32].

Human upper limb bones are more bilaterally asymmetric in length and weight than lower limb bones[10,11,13,16,23,27,31] Asymmetries tend to be more pronounced in males than in females [2,20,23,25]

Many authors have also directed their attention to the study of asymmetry of growth by measuring size differences between bones in the two hands [1-4]. Several studies suggest that participation in sports may result in bilateral asymmetry in the growth of certain bones [6,12,33]. However, not all studies attribute environmental and physical activity related explanations to bilateral asymmetry. Asymmetries independent of the physical activity levels were reported by Schultz [26] and Garn et al.[8].

The objective of the present study is to evaluate bilateral asymmetry in commonly measured limb dimensions in the Punjabi adolescents and how this asymmetry is related to age, sex and body mass.

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MATERIALS AND METHODS

The cross-sectional sample consists of 145 males and 63 females 13 to 18 years of age from a mixed Punjabi population of Chandigarh city and a nearby village, Mullanpur Garibdass in northwest India. The subjects were randomly drawn from Government Senior Secondary Schools and were physically normal and adequately nourished. Seven anthropometric measurements were taken independently on left and right sides with an anthropometer to the nearest 0.1 cm. The measurements include acromiale height, radiale height, stylion height, dactylion height, iliocristale height, tibiale height and sphyrion height beside body weight. All measurements were taken according to the procedures recommended by Weiner and Lourie [34] . On females, the iliocristale height could not be measured, hence the lower extremity length in females could not be derived

All measurements were made by one researcher (KK) in a well-lighted room. Subjects were weighed with minimum possible clothes on a spring loaded weighing machine; results were recorded to the nearest 0.1 kg. Six dimensions were derived as follows :

Total upper extremity length (Total arm length)	= Acromiale height- Dactylion height
Upper arm length	= Acromiale height- Radiale height
Forearm length	= Radiale height- Stylion height
Hand length	= Stylion height- Dactylion height
Total lower extremity length	= Iliocristale height- 7.50% of iliocristale height [19]
Lower leg length	= Tibiale height – Sphyrion height

Bilateral asymmetry was defined as the difference between measurements on left and right sides (left-right) taking into consideration the sign of the difference. It was calculated for each individual and for each of the six dimensions. Significance of asymmetry was tested by using paired t-tests.

Intra-individual error or measurement error variance was calculated by measuring an individual twice, but at different times for all seven anthropometric measurements and is derived as the square root of the sum of the squared deviations divided by twice the sample size ($S^2=\sqrt{\Sigma d^2/2n}$)[25]. The same formula was applied to left-right differences and the value of 'F' was derived. For judging the statistical significance of the F-Value, a 'p' value of 0.05 was accepted as the level of significance.

RESULTS

Results are presented in Tables 1 through 3. Table 1 presents the means and standard deviations of the intra-individual difference between left and right (left-right) limbs of the

two sexes. In males, mean left - right differences in total upper extremity length and its segments are negative, indicating larger right dimensions. In females, however, these differences are somewhat erratic in the sense that although, right total upper extremity length, is longer in females, its two segments, upper arm length and hand length taken individually are longer on the left side. For total lower extremity length, the left dimensions are uniformly longer in both sexes. However, left-right differences are significant for lower leg length only.

Table 2 presents the correlation coefficients between age and asymmetry (left-right) and body weight and asymmetry, in both sexes. In males left-right asymmetries in upper extremity length, upper arm length and hand length are significantly and positively correlated with age ($p<0.05$, $p<0.01$), whereas lower extremity asymmetries show significant negative correlations ($p<0.05$) with age. However, in females, none of the correlation coefficients is significant suggesting that there is no relationship between age and degree of asymmetry. Asymmetry (left-right) of total upper extremity length in males and lower leg length in both sexes, upper arm length and forearm length in females have significant correlation with body weight ($p<0.05$). Asymmetry in all dimensions except total upper extremity length in females are negatively correlated with the body weight. In males, however, the correlation coefficients are negative only for total lower extremity length and lower leg length.

Table 1. Means and standard deviations of intra-individual differences (left-right) in the limb dimensions of males and females.

Anthropometric dimension (cm)	MALES			FEMALES		
	Mean (Left-Right)	S.D.	T	Mean difference (Left-Right)	S.D.	t
Total upper extremity length	-0.278	1.655	-0.607	-0.249	1.104	-1.792
Upper arm length	-0.264	1.188	-1.707	0.283	1.035	2.167**
Forearm length	-0.612	1.214	-3.954**	-0.667	1.018	-5.197**
Hand length	-0.283	1.244	-2.744**	0.135	1.260	0.850
Total lower extremity length	0.266	2.084	1.540	-	-	-
Lower leg length	0.240	1.031	2.801**	0.383	0.979	3.102**

**$p<0.01$

Table 2. Correlation coefficients of asymmetry (left-right) with age and body weight for various limb dimensions of males & females.

Anthropometric dimension (cm)	MALES				FEMALES			
	Mean difference (Left-Right)	n	r (Age & Asymmetry)	r (Weight & Asymmetry)	Mean difference (Left-Right)	n	r (Age & Asymmetry)	r (Weight & Asymmetry)
Total upper extremity length	-0.278	145	0.255**	0.202*	-0.249	63	-0.068	0.031
Upper arm length	-0.264	145	0.298**	0.157	0.283	63	-0.061	-0.168*
Forearm length	-0.612	145	0.089	0.154	-0.667	63	-0.032	-0.202*
Hand length	-0.283	145	0.164*	0.156	0.135	63	-0.136	-0.052
Total lower extremity length	0.266	145	-0.173*	-0.131	-	-	-	-
Lower leg length	0.240	145	-0.183*	-0.169*	0.383	63	-0.013	-0.159*

*p<0.05 **p<0.01

Table 3 shows the technical error variances of the measurements and their ratios (values of 'F') for the seven anthropometric measurements. Some of the measurements show statistically significant values. The bilateral variation is several times larger than the measurement error indicating that the error variances associated with measurement technique contributes little to the left-right differences.

Table 3. Technical error variance (S^2) of measurement within and between sides.

Measurement	S^2 Within side	S^2 Between side	F Ratio
Acromion height	0.1760	0.5668	3.2318*
Radiale height	0.1161	0.6017	5.1826**
Stylion height	0.1303	0.6249	4.7958**
Dactylion height	0.2549	0.7416	4.7997**
Iliocristale height	0.2549	0.7416	2.9093
Tibiale height	0.0975	0.2765	2.8358
Sphyrion height	0.0836	0.2674	3.19886*

*p<0.05
**p<0.01

DISCUSSION

The salient finding of the present study is that in Punjabi population also, significant bilateral asymmetry exists in both upper and lower limbs and their segments. The degree of asymmetry differs not only from one dimension to the other, but also between the sexes.

Asymmetry of upper extremity, *i.e.*, total upper extremity length, upper arm length, forearm length and hand length in males, and total upper extremity length and forearm length in females favour the right side. However, lower extremity dimensions, *i.e.*, total lower extremity length in males and lower leg length in both sexes, are larger on the left side. Results from the present study generally agree with previous investigations[14,17,25,28]. This pattern probably reflects the tendency to favour the right side for power activities involving the upper limbs, *e.g.*, throwing for distance, while the left lower limb provides the firm base of support as in movement requiring opposition of movements[17]. On the other hand, the right lower limb is commonly preferred for activities requiring a greater degree of motor control, *e.g.*, kicking, dancing, etc. Watson found significantly higher bone-mineral content (BMC) in the dominant humeri of young baseball players while the pattern of asymmetry in the bones of forearm was inconsistent[33]. The young baseball players (8-9 years) studied by Watson appeared to show greater than normal humeral asymmetry than a small control and also showed an increase in bilateral asymmetry with age. However, Schultz found marked asymmetry in the lengths of femur and foot in human fetuses as young as four months before any significant activity related stress are operative [26].

The present investigation shows a significant positive correlation between age and asymmetry in the upper extremity in males, suggesting that as the age advances, asymmetry increases. This is consistent with the study of van Dusen [32]. On the other hand, the correlations are negative in lower extremity length suggesting that asymmetry declines with age. In females, none of the correlations between age and asymmetry is significant suggesting that there is no relationship between age and degree of asymmetry. The present investigation also shows that body mass has a weak correlation with asymmetry of the upper and lower limb dimensions in both sexes (Table 2).

The determinants of asymmetric development is not clear, but it could be accounted for by differential rates of growth of the limbs and their segments. In addition, genetic factors and the intrauterine environment such as nutrition or placement of the fetus in the womb may also contribute to bilateral asymmetry.

Usefulness of such studies is especially visualized in the field of growth and development in view of more recent revelations that level of asymmetry of a person's hands and the length of his or her fingers is a fair indicator of fertility level. Dr John Manning and his

colleagues at the University of Liverpool found that men with least symmetrical hands had lowest sperm count and the lowest sperm motility[18]. They explained that the " digit asymmetry predicts the number of sperm per ejaculation or more the asymmetry, fewer the sperms ". According to them " since the bilateral asymmetry in hand is developing in the fetal stage itself , it might also shed light on the developmental disorders such as autism".

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FLUORESCENCE BANDING OF MITOTIC CHROMOSOMES OF *PARASARCOPHAGA HIRTIPES*

V. Rai

Department of Biotechnology, VBS Purvanchal University, Jaunpur

Abstract

The heterochromatin of metaphase chromosomes of *Parasarcophaga hirtipes* is characterized by quinacrine (Q-), Hoechst 33258 (H-) banding. All autosomes display very characteristic fluorescent blocks of heterochromatin. The entire metacentric X and submetacentric Y chromosomes show differential fluorescence.

INTRODUCTION

Introduction of fluorescent DNA binding dyes of different specificities has made it possible to explore the heterogeneity of heterochromatin in different organisms e.g. in *Drosophila* (1,5,11,12,18,19) in human (8,13) . The fluorochromes—Quinacrine, an acridine derivative and Hoechst 33258 (2-[2-4(4-hydroxyphenyl 1)-6-benzimidazoly 1]-6-(1-methyl-4-piperazy 1)-benzimidazole) are known to impart bright fluorescence to AT-rich DNA in solution. In cytological preparations the chromosome regions which are rich in AT-base pair fluoresce brightly with quinacrine and Hoechst 33258 (2,6,7,9,10,15,18).

According to Comings et al. (1975), quinacrine interacts with DNA by an intercalation of the acridine nucleus of the dye in the small groove of the double helix forming an ionic binding of the diaminoalkane side chain to the phosphate group and Hoechst 33258 binds to DNA by an external attachment to the major groove of the double helix (2,10).

MATERIALS AND METHODS

For mitotic chromosomes, neural ganglia of colchicinized third instar larvae were dissected out in insect saline. The tissue was incubated in an aqueous solution of sodium citrate (0.45%) for 10-15 minutes and subsequently fixed in freshly prepared aceto-alchohol (one part acetic acid and three part ethanol). For air-dried preparations, fixed neural ganglia are squashed in 60% acetic acid under a clear coverslip. The slides were left overnight in an alcohol vapour bath. Next day the coverslips were removed with the help of a sharp razor blade and slides were airdried.

Q-banding was carried out according to the procedure of Lee and Collins (1977). The air-dried preparations were hydrated to water in an ethanol series comprising 95%, 70%, 35% ethanol followed by distilled water. The slides were stained in 0.5% solution of quinacrine dihydrochloride in 45% acetic acid by flooding for 10 minutes. Slides were rinsed in 95% alcohol followed by rinsing in absolute alcohol and mounted in 1:1 buffer-glycerol mixture.

Hoechst 33258 staining was done according to the method of Das et al. (1979). Airdried mitotic chromosomes were stained in 0.5% µg/ml solution of Hoechst 33258 in Sorenson's

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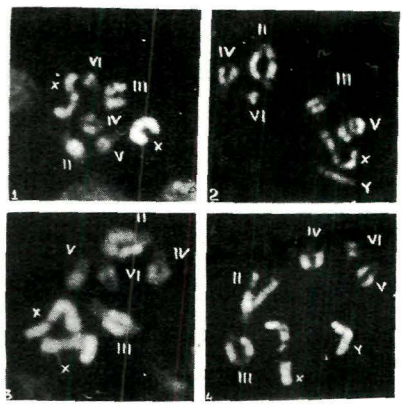
phosphate buffer (pH 5.6) for 20-25 minutes. Slides were rinsed thoroughly in Sorenson's phosphate buffer (pH 5.6) and then mounted in 1:1 buffer-glycerol mixture.

The fluorochrome-stained slides were viewed and photographed in Carl Zeiss Standard 14 Flurescence Microscope fitted with MC 63 automatic photomicrographic camera using Kodak Tri-X Pan black and white films. For quinacrine dihydrochloride and Hoechst 33258 filter sets BP 436/8. LP 475, FT 460 and G 365, LP 420, FT 395 respectively were used.

RESULTS AND DISCUSSION

The mitotic complement of P. hirtipes consists of five pairs of metacentric autosomes and a pair of sex chromosomes. The Y chromosome occupies a position between autosome pairs III and IV in the karyotype (14).

Mitotic chromosome preparations stained with quinacrine dihydrochloride or Hoechst 33258 display very characteristic fluorescent blocks of heterochromatin in the pericentromeric regions of all the autosomes on the basis of which individual autosome pair can be identified. Large almost symmetrical pericentric Q++/ H++ bands characterize chromosome pairs II,III,IV and VI while the pericentric fluorescent band of chromosome V is displaced towards the short arm. The entirely fluorescent metacentric X chromosome and submetacentric Y chromosome exhibit differential fluorescence. The X chromosome has two bright Q++/ H++ bands in the long arm. The entire Y chromosome is Q++/ H++ except a single moderate Q+/ H+ band in the long arm (Figs. 1-4).



Figs.1-4 Q and H-stained metaphase plates of *P.hirtipes.*

Fig.1 Q. stained female metaphase

Fig.2 Q stained male metaphase.

Fig.3 H stained Female metaphase

Fig.4 H stained Female metaphase

All the metacentric autosomes of P.hirtipes are characterized by the presence of very large blocks of C-band positive heterochromatin in the centromeric regions and the metacentric X chromosome and submetacentric Y chromosomes are C-band positive all along their length (14). Since quinacrine dihydrochloride and Hoechst 33258 stains belong to that group of flurochromes which specifically bind to AT-base pairs (2,6,7,10,18) and all Q++/ H++ regions coincide with C-band positive regions, thus the constitutive heterochromatin of P. hirtipes is C+ O+/ H+ i.e. all constitutive heterochromatin is rich in AT-base pairs (14,16,17).

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IDENTIFICATION OF ADHERENCE FACTOR IN *Escherichia coli* 0157:H7

Preeti Aghi and Sanjay Chhibber

Department of Microbiology, Panjab University, Chandigarh-160 014

Abstract

E.coli 0157:H7 demonstrated adherence to chicken epithelial cells *in vitro*. Attempts were made to look for adhesion molecule responsible for this adherence.In an earlier study by Karch et al., [1987], it has been reported that *E.coli* 0157:H7 carries non haemagglutinating pili. Since this strain showed negative haemegglutination, attempts were still made to isolate adhesion molecule following methodology which has earlier been used for isolating pili. The pure protein obtained after purification by sephorse 6B column showed a molecular weight of 16 Kda on SDS-PAGE. Antisera raised against this preparation reacted with protein on immunoblot. Moreover this antiserum-blocked adhesion of the bacteria to the chicken epithelial cells in a complementary binding assay suggesting its possible role in the binding.

INTRODUCTION

Escherichia coli is a major facultative anaerobic constitute of the normal flora of human intestine; particular *E coli* strains, however, are associated with diarrhoea. On the basis of distinct clinical syndromes and virulence properties diarrhoeagenic *E. coli* have been classified into five categories (Sack, 1976; Levine et al., 1986). Verotoxin producing *E.coli* (VTEC) strains of serotype 0157:H7 also known as enterohaemmorhagic *E. coli* which are associated with bloody diarrhoea (hemmorrhagic colitis), non bloody diarrhoea, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Riley et al., 1983). Adherence of diarrhoeagenic *E.coli* to intestinal mucosal surface is a critical step in the pathogenesis of diarrhoeal diseases (Beachey, 1981 and Peterson et al., 1989).

E.coli 0157:H7 demonstrate intimate adherence to enterocytes in animal models of human disease (Sherman et al., 1981) showing characteristic lesions in the intestine of gnotobiotic piglets, rabbits, chickens and in selected cell lines *in vitro* (Karch et al., 1987 and Knutton et al., 1997). Adherence to mucosal epithelium can be mediated by various cell surface antigens including pili, outer membrane proteins, capsular polysaccharide and lipopolysaccharide (Beachey ,1981). However, the surface antigens which mediate adherence of *E. coli* 0157:H7 to epithelial cells have not been defined. Karch et al., (1987) reported that 60 megadalton plasmid encodes for non haemagglutinating pili in *E. coli* 0157:H7 that mediates attachment to Henle 407 intestinal cells. On the contrary, Sherman and Soni (1988) reported absence of pili in majority of the isolates suggesting that surface

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structures other than pili may be responsible for attachment of serotype 015:H7 bacteria to epithelial cells. These workers were of the view that outer membrane proteins may function as bacterial attachment factors (adhesins) for *E. coli* 0157:H7. In the present study, an attempt was made to identify the adhesin molecule in *E. coli* 0157:H7 which mediated attachment to chicken epithelial cells.

MATERIALS AND METHODS

Bacteria: Enterohemorrhagic *E. coli* 0157:H7 was procured from National *Escherichia coli* Centre, Kasauli, India. The strain was identified biochemically and stored in nutrient agar stabs at 4°C.

(a) **Adherence to chicken epithelial cells:** This was studied according to the method of Beery et al., (1985). Adhesion of organisms to chicken epithelial cells was checked by mixing 0.5 ml of bacterial cell suspension (10^8 cells/ml) to 0.5 ml of intestinal cell suspension (10^9 cells/ml). Mean number of adhering bacterial/cell was calculated.

(b) **Haemagglutination pattern:** The presence of pili was investigated by performing haemagglutination using human blood group A, sheep and chicken erythrocytes according to the method of Duguid and Gillies (1957).

(c) **Purification of adhesion molecule:** Methodology for isolation of pili as proposed by Korhonen et al., (1980) with slight modification was used in this study. The organism was grown statically at 37°C in 2 liters of brain heart infusion (BHI) broth for 48 hours. The cells were harvested, washed and suspended in phosphate buffer (pH 7.2). The bacterial cells were removed by centrifugation at 7,000 rpm for 20 min. The supernatant was further centrifuged at 27,000g for 30 min. The resultant supernatant was centrifuged at 2,27,000g for 2 hrs and pellet was suspended in 6M urea.

The protein was loaded on a sepharose 6B column (Pharmacia) and eluted with 0.1m phosphate urea buffer (pH 7.2). The first peak fractions were pooled, dialyzed and lyophilized .The purified protein was resolved on 10% SDS gel according to method of Laemmli (1970).The sample before loading was heated at 100° C for 5 min at pH below 2.0. The second heating was done in presence of SDS (2%) at 100° C for 5 min.

Immunological Studies : Antiserum was prepared by giving intramuscular injection of antigen in rabbit at 2 weeks interval. One week after the last injection, the rabbit was bled

and antiserum obtained. The presence of antibodies in the serum was checked by immunoblot following the method of Towbin et al., 1979).

Adherence Assay: Complementary binding assay was done to determine whether the adhesion molecule functioned as colonization factor for *E.coli* 0157:H7.The organisms were treated with antisera in equal amounts, 250 µl each for 1 hour at 37°C. Following this 250 µl of above mixture was added to 250 µl epithelial cell suspension. The contents were incubated at 37°C for 1 hr. The following procedure remained same as described under adherence.

RESULTS

No heamagglutination was observed with either of the red blood cells used in this study. The adhesion molecule was extracted according to the method of Korhonen et al., (1980) with slight modifications and the final crude preparation was purified by gel chromatography. The results in Fig. 1 show the elution pattern obtained on sepharose 6B column. Fractions of peak 1 were pooled, dialysed and run on SDS-PAGE. The coomassie blue stained gel showed presence of adhesin molecule of 16 Kda (Fig 2). The antiserum was raised against this pure preparation in rabbit. The presence of antibodies was checked by immunoblot and results confirmed the presence of antibodies to the 16 Kda purified adhesin molecule preparation (Fig 3). The antiserum was further checked for adhesion inhibition to chicken epithelial cells. The results showed that antibody was able to block the adhesion of bacteria to epithelial cells.

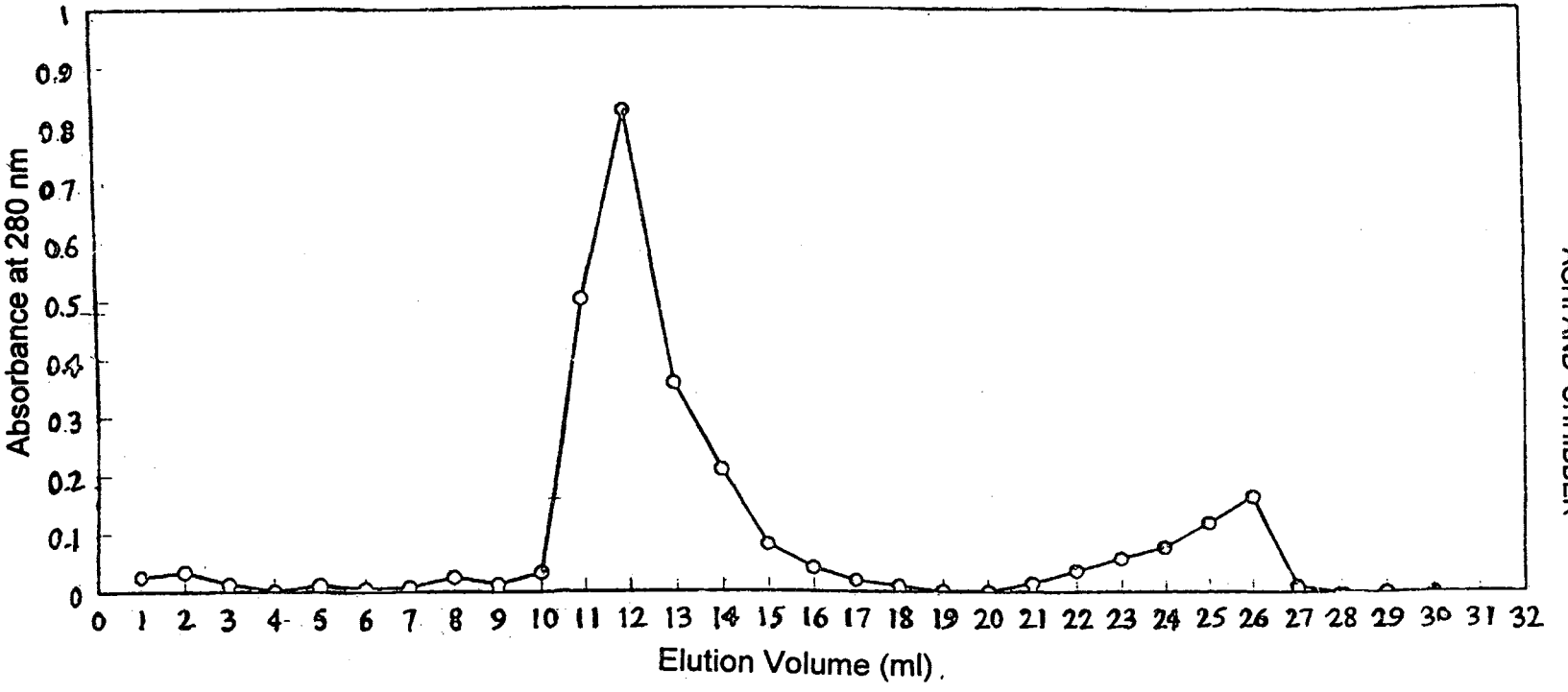

Fig. 1 : Chromatographic pattern of crude pili on sepharose 6B Column

Column size : 50 x 1 cm
Eluent : 6 M urea phosphate buffer pH 7.2
Flow rate : 30 ml/h
Fraction size: 2 ml

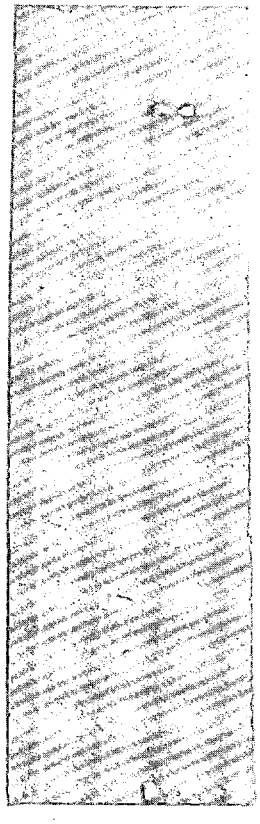

Fig.2. Commassie blue stained SDS-PAGE gel showing purity of adhesion molecule (Lane 2 & 3), standard Lysozyme marker (lane 1).

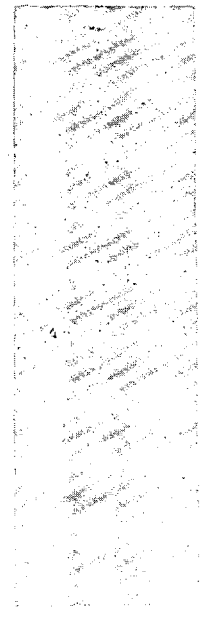

Fig. 3. Immunoblot developed with antisera to purified protein.

DISCUSSION

Binding of bacterial pathogens is a primary step in the pathogenesis of disease because it promotes internalization and invasion of invasive organisms (Finlay et al., 1989). Although adherence of *E.coli* 0157:H7 to gastrointestional epithelial cells has not been demonstrated in human disease, the organisms demonstrate adherence to ileum, caecum and colon of orally infected animals (Wadolkowski et al., 1990). In general pili are known to mediate the initial or early attachment of organisms to intact microvilli. In the present study attempts to demonstrate presence of pili by haemagglutination with human chicken and sheep red blood cells showed absence of pili. Karch et al., (1987) demonstrated that adherence can be

mediated by plasmid encoded, non haemugglutinating pili. In addition, outer membrane proteins have been suggested as possible bacterial attachment factors (adhesins) for *E.coli* 0157:H7 adherence to epithelial cell surfaces (Shermon et al.,1981). Intimin encoded is the best characterized *E.coli* 0157:H7 adherence molecule. It is a 97 KDa OMP that serves for attachment of bacteria to epithelial cells (Mckee and O'Brien.,1996). It mediates the attaching and effacing lesions caused by *E.coli* 0157:H7 and is an important component of pathogenicity (Kaper , 1998). Recently, Tarr et al., (2000) described Iha, an OMP of *E.coli* 0157:H7, that is sufficient to confer the adherence phenotype upon non-adherent laboratory *E.coli.*

In the present study, by employing the technique used for isolating pili we have identified a 16 Kda protein, antisera to which inhibited binding of bacteria to chicken epithelial cells. However, it remains to be ascertained that whether this adhesion molecule is a non haemagglutinating pili or low molecular weight outer membrane protein. The molecular weight of the protein obtained in this study however, is more suggestive of presence of pili as molecular weight of pilus proteins of *E. coli* has been found to be in this range (Korhonen et al,. 1980 , Hanson et al.,1988). The possibility of the presence of non-haemagglutinating pili in *E.coli* 0157:H7 may further be explored in future studies.

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GENETIC ANALYSIS OF ISOENZYME VARIATIONS IN CILICIAN FIR *(Abies cilicica* Carr.)

Ibrahim Turna, Ali Omer Ucler and Zeki Yahyaoglu

Karadeniz Technical University, Faculty of Forestry, Trabzon, TURKEY 61080

Abstract

Isozyme variation in six natural populations of Cilician fir *(Abies cilicica* Carr.) was investigated using starch gel electrophoresis. Isozyme patterns of four enzyme systems (PGI, PGM, 6PGDH, IDH) were studied. Analysis of megagametophyte tissue demonstrated that the allozyme variants were coded by a total of twenty alleles at eight loci. On an average, the expected heterozygosity was 0.218 and 56.25% loci were polymorphic. The mean number of alleles per locus was 1.645. The mean total genetic diversity was calculated 0.364. The mean genetic diversity within populations was determined as 0.218 and the average among populations was found to be 0.165. The relative magnitude of differentiation among subpopulations was measured as 0.409 indicating that only 40.9% of the total genetic variation was among populations. Two alleles in Kahramanmaras-Baskonus population and one allele in Antalya-Imrasangedigi population were detected, which were not present in other populations. No genetic differences were found between population 1 and 2 and population 3 and 6 for PGM. According to genetic distance, Baskonus population was different from others.

INTRODUCTION

Genetic variation is the fundamental component, which ensures survival and thus the stability of forest ecosystems as its quantity and quality determines the potential of populations to adapt to changing environmental condition. This is particularly important with changing populations and climatic condition and when the long-term stability of forest ecosystems is increasingly threatened by environmental stress. Thus, a genetic characterization of natural forest resources is the first step necessary for a better understanding of genetic resources for implementation of *in-situ* and *ex-situ* conservation activities.

There have been some studies on the genetic variation in forest tree species including *Abies equi-trojani* Aschers[9]. However, a genetic characterization of provenances of Cilician fir *(Abies cilicica* Carr.) has not yet been attempted, nor has, to our knowledge, any study dealing with environment independent markers been published.

Cilician fir, one of the four fir species native to Turkey, occurs on the Taurus mountains from Bucak in the west to Kahramanmaras in the east, and on the Amanos mountains from Osmaniye (province of Adana) to Antakya. The natural distribution range of this species covers an area of 337.437 ha in the Mediterranean region[2] where it forms mixed forest stands with cedar (*Cedrus*

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libani A. Rich.) and Anatolian black pine *(Pinus nigra* Arnold subsp. *pallasiana* Lamb. Holmoboe.).[1] The purpose of this study is to determine genetic differences among six populations of Cilician fir by using four different enzyme systems. No allozyme studies have been previously reported in this species.

MATERIALS AND METHODS

For the present study, wind-pollinated bulk seed lots were used from six Cilician fir populations located in the Taurus mountain of Southern Turkey (Table 1). Research material was supplied by Forest Tree and Seeds Improvement Institute in Turkey. A total of 10 sample trees from each population and 5 seeds from each sample trees were used.

Table 1. Description of the populations studied

Population	Pop. No.	Latitude (N)	Longitude (E)	Elevation (m)
Antalya-Ugurlu-1	1	37° 19' 52"	30° 37' 10"	1200
Antalya-Akseki	2	37° 06' 51"	31° 46' 52"	1350
Antalya-Imrasangedigi	3	37° 06' 20"	31° 48' 30"	1560
Antalya-Ugurlu-2	4	37° 19' 42"	30° 37' 41"	1000
Kahramanmaras-Baskonus	5	37° 35' 03"	36° 34' 43"	1200
Kahramanmaras-Yenicekale	6	37° 32'00"	36° 35' 00"	1500

Seed collection areas were laid down from east through west of Taurus mountains in which the west border of *Abies cilicica* is Bucak region (Uğurlu-population number 1 and 4) and the east border is Yenicerkale and Baskonus forest of Kahramanmaras region. Populations 2 and 3 chosen from Antalya region cover very large areas along the Mediterranean Coast of the Taurus mountains. Due to large, scattered and patchy distribution of *Abies cilicica* in the region, demarcation was based on geographical units and land lines (*i.e.*, rivers and ridges). According to the land lines, populations were taken from (A/1), (A/2), (A/3), (B/5) and (B/6) population 1 and 4, 3, 2, 5 and 6, respectively.

Enzymes Systems : Genetic analysis was focussed on those enzyme systems, which exhibited the greatest phenotypic variability and clearly visible enzyme phenotypes. The selected systems were : phosphoglucose isomerase (PGI, E.C. 5.3.1.9), phosphoglucomutase (PGM; E.C. 2.7.1.9),6-phosphoglunate dehydrogenase (6 PGDH; E.C. 1.1.1.44) and isocitrate dehydrogenase (IDH; E.C.

1.1.1.42). The first two systems (PGI and PGM) were stained in one and the latter two (6PGDH and IDH) in another buffer system.

Electrophoretic Methods: The enzymes were extracted from endosperm tissue, which is the female gametophyte, a haploid tissue, a very convenient material for genetic studies. Before undertaking the assays, it was tested whether or not the enzyme phenotypes in the gels in fact refer to the enzyme systems under consideration. The respective enzyme substrates were omitted in one gel slice which served as the control and the phenotypes obtained were compared with those in the experimental second slice stained similarly.

Haploid endosperm tissue dissected from single seed was homogenized in an extraction buffer system. For the homogenisation of gametophytes to isolate phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), 6-phosphoglunate dehydrogenase (6PGDH) and isocitrate dehydrogenase (IDH) enzymes, 20 ml Tris-HCl taken from a stock solution consisting of 100 ml distilled water and 800 mg Tris-HCl at pH 7.5 + 60 mg polyvinyl-pyrrolidone (PVP) + 1 saccharose + 10 mg ethylenediamine tetraacetic acid (Na-EDTA) + 20 mg bovine serum albumin (BSA) were used[15]. The crude homogenates were subjected to horizontal starch gel electrophoresis. The gels were stained for various enzyme systems (Table 11). From each population 50 seeds were analysed per enzyme system. Details of the electrophoresis procedures, staining recipes and formal genetic analysis are explained in Conkle et al.[4] and Cheliak and Pitel[3].

Data Analysis : Allozyme frequency data were used to calculate, on a population basis, single locus measures of genetic diversity. The data was subjected to statistical treatment to calculate proportion of polymorpbhic loci (P), mean number of alleles per locus (A), mean expected heterozygosity (He), gene diversity within populations (Hs), total gene diversity (Ht), gene diversity among populations (Dst), relative degree of genetic differentiation (Gst) and genetic distance (Do) see references[8,12,13]).

RESULTS AND DISCUSSION

Inheritance of Isoenzyme Patterns : Eight loci were identified from the 4 enzyme systems analysed. The enzyme systems of PGI, PGM, 6PGDH and IDH showed at most two enzymograms of the endosperms. Two zones of PGI activity were observed. PGI-A exhibited two variants; PGI-B exhibited three variants. Two zones of activity were detected on PGM gels. GM-A had two variants (A1, A2) and PGM-B also exhibited two variants (B1, B2). Gels stained for 6PGDH had two zones of activity. 6PGDH-A had two variants (A1, A2) and 6PGDH-B had three variants (B1, B2, B3). Two zones of activity occurred on gels stained for

IDH : the upper, less intensely stained zone IDH-A, had three variants, the lower zone (IDH-B) was stained more intensely and had three variants. In other conifers too the same enzyme activity has usually been reported[11].

Genetic Variation: Allele frequencies for 8 loci in 6 populations of *Abies cilicica* are given in Table 2. It is interesting to note that while some alleles were not found in some populations, they were frequent in others. For instance, PGM-A1 allele was only found in population 4, and 6PGDH-B3 and IDH-A3 alleles were present only in population 5. On the other hand, while PGI-B3 allele was present only in populations 1 and 2, IDH-A1 allele was found in populations 4 and 5.

Table 2. Estimates allele frequencies for eight allozyme loci in *abies cilicica*

Enzyme System	Locus	Allele	Population Number					
			1	2	3	4	5	6
PGI	PGI-A	A1	0.70	0.00	0.70	0.50	0.00	0.00
		A2	0.30	1.00	0.30	0.50	1.00	1.00
	PGI-B	B1	0.30	0.10	0.70	0.50	0.00	0.90
		B2	0.40	0.00	0.50	0.50	1.00	0.10
		B3	0.30	0.90	0.00	0.00	0.00	0.00
PGM	PGM-A	A1	0.00	0.00	0.00	0.20	0.00	0.00
		A2	1.00	1.00	1.00	0.80	1.00	1.00
	PGM-B	B1	0.00	0.00	0.10	0.00	0.90	0.10
		B2	1.00	1.00	0.90	1.00	0.10	0.90
6PGDH	6PGDH-A	A1	0.30	0.00	0.50	0.00	0.40	0.00
		A2	0.70	1.00	0.50	1.00	0.60	1.00
	6PGDH-B	B1	0.00	0.20	0.50	0.00	0.40	0.60
		B2	1.00	0.80	0.50	1.00	0.20	0.40
		B3	0.00	0.00	0.00	0.00	0.40	0.00
IDH	IDH-A	A1	0.00	0.00	0.00	0.10	0.20	0.00
		A2	1.00	1.00	1.00	0.90	0.30	1.00
		A3	0.00	0.00	0.00	0.00	0.50	0.00
	IDH-B	B1	0.00	0.60	0.00	0.00	0.20	0.20
		B2	0.30	0.00	0.20	0.90	0.00	0.30
		B3	0.70	0.40	0.90	0.10	0.80	0.50

Mean expected heterozygosity values *(He)* and average number of alleles (A) *and polymorphic loci(P)* for studied populations are listed in Table 3. According to these values, the average percentage of polymorphic loci was 56.25% with a maximum of 75.0% (Imrasangedigi) and a minimum of 37.5% (Akeseki). The average number of alleles per locus was calculated as 1.645 and it ranged from 1.375 (Akseki) to 1.875 (Baskonus).

One of the most important parameters of the genetic diversity is expected heterozygosity. The expected heterozygosity *(He)* per population ranged from 0.123 Akseki to 0.280 (Baskonus) with a mean of 0.218. In previous studies on other conifers, the expected proportion of heterozygosity*(He)* values was found to indicate generally small differences. This value is 0.165 for *Picea abies*[6], between 0.174-0.290 for *Abies cephalonica*[5], 0.263 for *Pinus brutia*[10] and 0.284 for *Picea orientalis*[16]. The rate of expected heterozygosity in our study agrees well with the values obtained in the previous studies.

Two unique alleles detected in Baskonus population and one unique allele in Ugurlu-2 population were not present in other populations. Genetic "richness" as measured by the number of alleles indicates that population 5 (Baskonus) is richer than other populations. Expected heterozygosity value of this population is also higher than others. From this point of view the Baskonus and Uğurlu-2 populations should be considered for a gene conservation program.

Analysis of Genetic Diversity : Total gene diversity *(Ht)*, gene diversity within populations *(Hs)* and among populations *(Dst)*, and mean values were all very different for single loci (Table 4). So, the genetic differentiation among populations *(Gst)* was very high (40.09%). The organisation of gene diversity in Cilician fir is comparable to that observed on other conifers. For example, *Gst* values reported for black pine[14] were 6% and for Turkish red pine 5.3%[10], and for norway spruce 4.2%[6]. It is clear that the *Gst* values obtained from *Abies cilicica* are well above the values for other coniferous species. This can be ascribed to two things : (1) that populations in the present study are geographically isolated, (2) that genetic diversity within and among populations is negatively affected by excessive manipulations. As it has been pointed out, these ecosystems and habitats are very fragile and there has been an increasing human pressure on forest and forest resources in the region. Also, uncontrolled grazing and illegal cutting greatly contribute to genetic degenerations.

Table 3. Genetic diversity parameters for populations studied

Population	Mean Number of Loci	Mean Expected Het.(He)	Mean Number Of alleles	Population of Polymorphic loci
Antalya-Ugurlu	8	0.2400	1.625	50.00
Antalya-Akseki	8	0.1225	1.375	37.50
Antalya-Imrasangedigi	8	0.2750	1.750	75.00
Antalya-Ugurlu	8	0.2100	1.625	622.50
K. Maras-Baskonus	8	0.2800	1.870	62.50
K. Maras-Yenicekale	8	0.1825	1.625	50.00
Mean	8	0.2183	1.645	56.20

Table 4. Genetic diversity analysis in *abies cilicica*

W/L	Hs	Ht	Dst	Gst
PGI-A	0.223	0.433	0.210	0.485
PGI-B	0.324	0.639	0.315	0.493
PGM-A	0.053	0.064	0.011	0.172
PGM-B	0.090	0.299	0.209	0.699
6PGDH-A	0.233	0.320	0.287	0.272
6PGDH-B	0.323	0.493	0.170	0.345
IDH_A	0.133	0.239	0.106	0.444
IDH-B	0.367	0.580	0.213	0.367
Mean	0.218	0.383	0.165	0.409

Genetic distance: According to genetic distance, population 5 showed more important differences than other populations for PGM-B enzyme system (Table 5). No genetic differences were found between populations 1 and 2 and populations 3 and 6 for PGM enzyme system. However, the genetic differences among some other populations were very high. Genetic distance showed very high values between some populations for 6PGDH and IDH enzyme systems. A cluster analysis based on Nei's[13] unbiased genetic distance *(Do)* and using the UPGMA algoritham reveals high levels of genetic differences between the populations at the 95% significance level (Figure 1).

Table 5. Mean genetic distance among the populations studied

Pop No.	1	2	3	4	5
2	0.300				
3	0.287	0.387			
4	0.212	0.350	0.325		
5	0.512	0.500	0.450	0.600	
6	0.312	0.225	0.237	0.325	0.437

Averaged over all loci, the mean genetic distance between populations was 0.364. This value is rather high as compared with other coniferous tree species. According to the literature, the average genetic distance is 0.065 for *Picea abies* in Italian populations[6] and 0.003 to 0.012 in Latvian populations[7], 0.035 for *Pinus nigra* in European populations[14], and 0.176 for *Picea orientalis* in Turkish populations[16]. Therefore, it could be concluded that the genetic differentiation among natural populations of *Abies cilicica* in Turkey is very high when compared to other species indicated in this research.

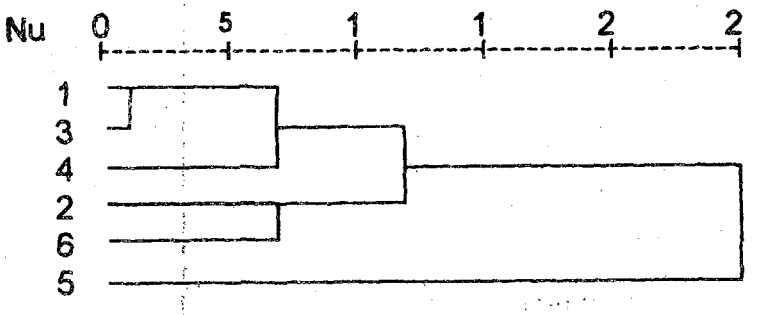

Fig.1. Dendrogram showing the clustering of the six natural populations of *Abies cilicica* based on NEI's genetic distance coefficient.

Given the results obtained from the study, it can be suggested that all populations, especially the population 5, be considered for a gene conservation program. Also, future studies are necessary to provide deeper insights into the subject.

Kahramanmaras-Baskonus forests are currently classified as research forests. The plant cover composition consists of the main forest tree species of *Abies cilicica, Pinus nigra* subsp. *pallasiana, Cedrus libani, Pinus brutia,* and minor

species of *Quercus cerris, Acer monspessulanum, Arceuthos drupacea* etc. and many mixed stands of broad-leaved and evergreen trees.

High values obtained in terms of genetic variability between populations may be explained by the fact that cilician fir populations are scattered across wide areas. Even though the populations in Antalya region are close to each other, they are grouped differently according to the land line and geographical characteristics. Due to this different grouping, populations are chosen from the sub-groups. However, no comparisons could be made since, to our knowledge, no scientific studies about the groups exist. Sustainability between the populations in terms of gene transfer might not be observed because the populations are composed of patched and scattered structures. It may also be the reason as to why the genetic distance values were found to be high among the populations.

Further studies are required to obtain precise information on linkage relation and to calculate chromosomal map distance in Cilician fir. The results in this study demonstrate that such interactions between some of the identified gene loci exist.

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REGENERATION STUDIES ON SOME MOSSES

Kukku Jain* and Anju Rao**

**Department of Botany, University of Rajasthan, Jaipur-302 004*
***Department of Botany, Panjab University Chandigarh – 160 014*

Abstract

Regeneration in three mosses, namely *Gymnostomum calcareum,* Nees and Hornsch., *Timmiella dimunata* (C. Muell.) Chen and *Pogonatum* junghuhnianum (Dozy. and Molk.) Dozy and Molk has been studied. In all the species, gametophytic parts regenerate readily while sporophytic tissues do not show positive response. The new leafy buds developed either directly from the plant tissue (more commonly in *P. junghuhnianum*) or on the protonemal filaments, formed as a result of regeneration. It has been suggested that multistratose leaves or leaves with lamellae possess some substance needed for bud initiation.

Gametophytic parts of most of the mosses have got a remarkable power to regenerate; similar but to lesser extent is the case with sporophyte. A good deal of work has been done on this subject by several workers[3-10, 12-21] etc. The aim of the present investigation is to determine the capacity of *Gymnostomum calcareum*, *Timmiella dimunata* and *Pogonatum junghuhnianum* to regenerate.

MATERIALS AND METHODS

Gymnostomum calcareum was collected from near the canal Headworks at Sri Ganganagar (Rajasthan) in first week of July. *Timmiella dimunata* was collected from Mount Abu (Rajasthan, India) on the west side of Nakki lake near Raghunath temple and *Pogonatum junghuhnianum* from Nainital (U.P.) on way to China peak, in the month of September. The material was kept in dry paper packets after removal of the excess of soil. Both gametophytic and sporophytic parts were tried for regeneration in different media in different concentrations. In the gametophyte, complete plant, leaves excited from the stem and stem without leaves were tried. In the sporophyte, complete sporogonium, capsule and seta separately were placed for regeneration. The nutrient media used for regeneration and germination were as follows:

1. Modified Beneck's solution (Bold, in Johansen[11]).
2. Modified Knop's solution (Bold in Johansen[11]).

In each case, the nutrient was used in solution form and as solid substrate. Solid substrate was prepared using 2% Agar in nutrient solution. Full, half and quarter concentrations were taken in each case. The media were sterilized under 10 1b per square inch pressure for half an hour. The parts of the plant used for experiments were

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surface sterilized with 2% sodium hypochlorite for two minutes and then thoroughly washed with sterile distiled water. The pH of medium was adjusted to 5.8 before autoclaving. Constant illumination of 820 lux was provided with the help of two tube lights of 40 Watts each and a bulb of 100 Watts from a distance of 16 inches. The temperature was maintained at 20°C±1°C.

To keep the cultures wet few drops of distilled water were added at regular intervals of 5-7 days. The temporary preparations for study were prepared in 10% glycerine. The leaves bearing regenerated plants in case of *Pogonatum junghuhnianum* were fixed in fromalin acetic alcohol and morphological studies were made using paraffin sections. Sections were cut 8μm thickness and the slides stained with safranin and fast green. The microphotographs were taken on monocular Olympus microscope using Pentax SLR camera with 35 mm, 125 ASA film.

OBSERVATIONS

(i) *Gymnostomum calcareum Nees and Hornsch.*

Regeneration

The sporophytic parts do not show any response, whereas, the gametophytic parts regenerate readily. The separated leaves, the axis and the axis with leaves, all show regeneration within 4 to 6 days in all concentrations (i.e. one fourth, half and full) of knop's and Beneck's nutrients. However, best growth of protonema is observed in one fourth concentration of nutrient as compared to half and full concerntration. Rhizoids also show regeneration and grow to form protonemal filaments.

Cells from any part of the stem or leaf may grow out and show regeneration. However, this tendency is more in the basal costal region and at the basal cut end of leaf. It takes about 8 to 10 days for the leafy buds to develop. Buds may develop on the protonema or directly from the stem in its basal region. More buds are observed to be developing directly from the stem than on the protonema. Bud formation is also better in terms of frequency in Beneck's medium as compared to higher concentrations.

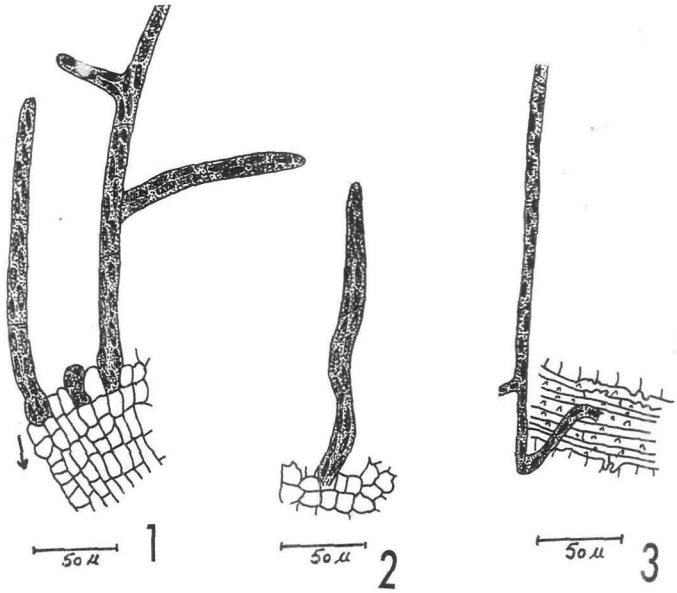
In solid substrate prepared with 2% agar with half knop's nutrient medium, the regenerated protonema develops many gemmae on it. The gemmae are usually formed terminally. From any part of leaf, a cell (e.g. marginal cell, surface cell of costa, cell at the cut end of leaf etc.) protrudes out and divides by several transverse divisions to give

rise to a multicellular uniseriate branched prostrate protonema (Figs. 1-3). After the prostrate growth of protonema, erect filaments are developed as described by Allsop and Mitra[1,2]. Erect filaments have restricted developments in comparison to the prostrate filaments which show unlimited growth. Among the cellular characteristic, it has been noted that the prostrate filaments are stouter than the erect ones. As the protonemal filament grows older, the prostrate system shows brown cell walls, oblique cross walls and it becomes deficient in chloroplasts. After the formation of protonema, some leafy buds develop on it. However, as stated earlier, buds are more frequently developed directly from the stem surface (Fig.4). The bud develops as a protuberence which grows and becomes almost ovoid or globose in outline and acts as a bud initial. Apical cell is formed by laying down of three successive oblique intersecting walls. Now this apical cell cuts off segments and forms young bud (Fig.5).

Development of 'Gemmae' or tubers on protonema formed after regeneration

The gemmae have been observed to have developed on half knop's solid substrate and quarter knop's liquid medium. The gemmae in fact develop when the cultures become relatively drier implying that the concentration of the nutrient go higher, may be more than full knop's. When these cultures are moistened with distilled water, the gemmae again develop into protonemal filaments.

Gemmae are developed in large numbers. A gemma develops on the protonema terminally. The terminal cell of the filament enlarges and divides by a transverse wall to form a short stalk and a body cell. The body cell divides further transversely and vertically to form body of gemma (Fig.6). Sometimes stalk is altogether absent and the gemma is directly joined to the parent filament.



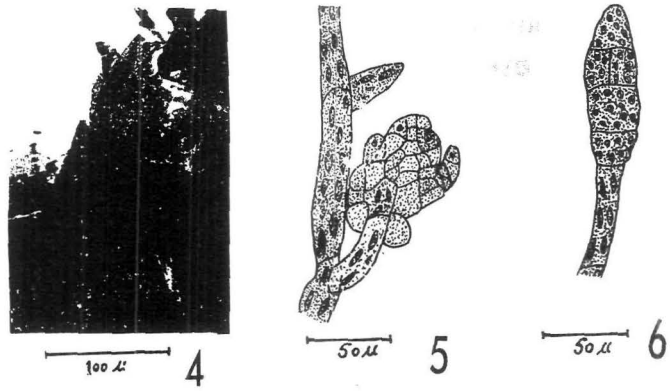


PLATE-I

Gymnostomum calcareum

Fig.1-3. Development of protonemal filaments from basal cells of leaf, injured leaf and basal costal region of leaf respectively. X310

Fig.4. Photograph showing young bud developed directly from stem. X225

Fig.5. A part of protonemal filament with young bud. X310

Fig.6. A gemma developed at the tip of protonemal branch. x 310

(ii) ***Timmiella dimunata* (C.Muell.) Chen.**

In this species only gametophytic parts *i.e.* stem and leaves were tried for regeneration. The experiments were set up in Beneck's and knop's nutrient solutions using quarter, half and full strength.

The regeneration took place in 7 to 9 days. In the knop's nutrient solution, percentage of regeneration is more in the quarter concentration than in half or full concentration, where as, in Beneck's nutrient solution, percentage of regeneration is more in full strength and less in half and quarter strength. In half and quarter strength of Beneck's medium, frequency of regeneration is equal.

Although regeneration may take place from any surface cell of stem or leaf, yet the basal portion of stem and cut ends of leaves show more regeneration as is the case in *Gymnostomum calcareum*. Some rhizoids too grow further forming protonemal filaments. After regeneration growth of protonema is more extensive in liquid medium than on moist solid substrate and bud formation is more in solid substrate than in the liquid medium. Buds may be formed directly also from the leaf or stem. Development of protonema is on the same lines as in *Gymnostomum calcareum*.

(iii) ***Pogonatum junghuhnianum* (Dozy. and Molk) Dozy. and Molk.**

Both sporophytic and gametophytic tissues were kept for regeneration in full, half and quarter strengths of knop's and Beneck's medium. Unfortunately, sporophytic parts did not show any positive response. The gametophytic tissues, both stem and leaves, showed regeneration, but it took longer time as compared to *Gymnostomum calcareum* and *Timmiella dimunata*. It took about a month's time for the regeneration to occur.

Full knop's solution shows better results as compared to half and quarter concentration in similar conditions. The regeneration occurs from the lamella covered part of the leaf, particularly the central region. The non-lamellate lamina seldom regenerates. However, regeneration is not uncommon from basal sheathing portion of leaf. Although protonema is also formed as a result of regeneration, yet leafy buds are mostly developed directly from the leaf surface. A single regenerating leaf may bear more than one leaf buds.

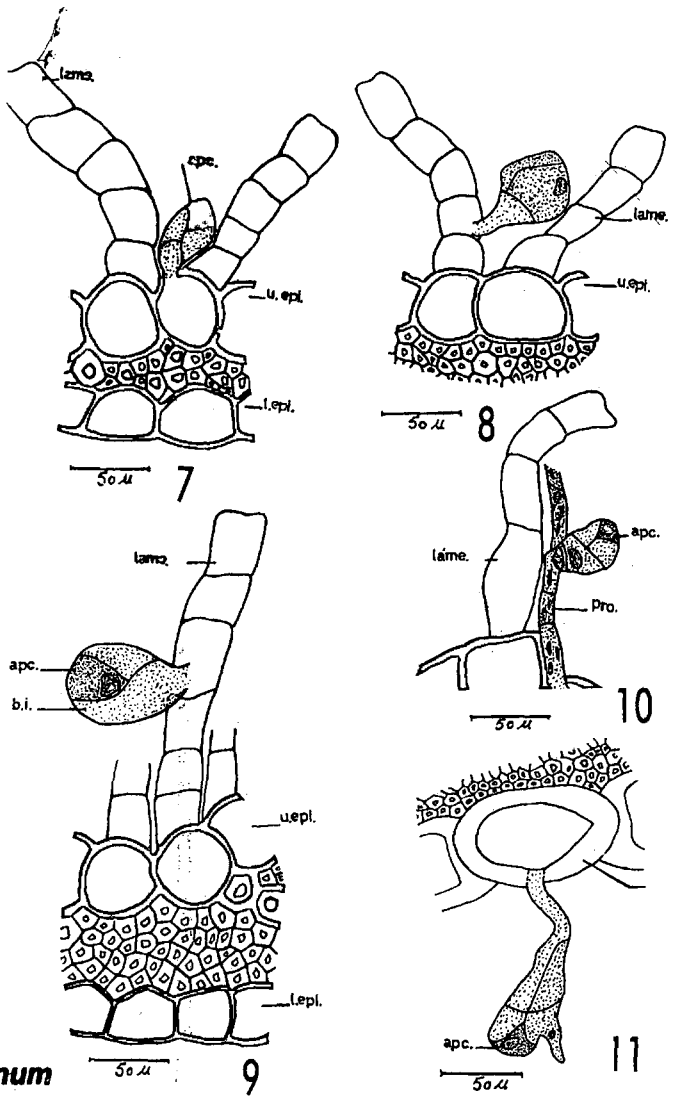


PLATE-II

Pogonatum junghuhnianum

Figs.7-11. Transverse sections of the regenerated leaves showing young buds developed from different regions. X310.

Fig.7. From upper epidermal cell

Figs.8&9. From lamellar cells.

Fig.10. A protonemal filament developed from upper epidermal cell and bearing a bud laterally.

Fig.11 Small protonemal filament with terminal bud developed from lower epidermal cell.

The development of the bud follows a pattern similar to the one described in *Pogonatum perichaetiale, Pogonatum microstomum* and *Oligotrichum similamellatum*[7,8]. Any cell of the upper epidermis of lamella bearing part of leaf (Fig.7) or a cell of the lamella itself may protrude out and form a club-shaped bud initial (Figs.8,9). In this bud initial are laid obliquely intersecting walls to establish an apical cell (apc) with three cutting faces. It is with the help of this apical cell that new leafy plant is formed.

Occasionally this small protrusion developed from the epidermal cell or the lamellar cell may grow out to form a small protonemal filament. On this protonemal filament then develops a young bud either laterally (Fig.10) or terminally (Fig.11). Sometimes the lower epidermal cell may also regenerate (Fig.11).

DISCUSSION

Previous studies reveal that there are two positions as far as the bud formation is concerned. Mostly as a result of regeneration protonema is formed on which the buds are developed later. Gemmal[9], while discussing regeneration in *Atrichum undulatum* has called the bud forming primordium a "protonemal filament". The filament is distinct from other cases since it directly passes into a bud. While studying regeneration in *Pogonatum microstomum* and *Oligotrichum semilamellatum*, Chopra and Sharma[8] found that in most of the cases the new buds were formed directly from the cells of the upper epidermis or the basal cells of the lamellae. They remarked that formation of a terminal bud on a short "protonemal filament" may be a condition intermediate between the formation of a well developed protonema which later bears leafy buds and the direct bud formation found in members of Polytrichaceae in which the leaf lamina is multistratose with lamellae on the surface. It seems probable that some substance which is required for the initiation of the buds on protonema and which in the normal course is supplied by protonema is already there in members of Polytrichaceae in the thick leaf midrib or the lamellae present on the surface.

In the present investigations of *Pogonatum junghuhnianun*, it is observed that the buds mostly arise directly either from the cells forming the upper surface layer of the leaf or from the cells of lamellae. Formation of protonemal filaments is also quite common, however, bud formation on these filaments is rare. This may represent a condition

intermediate between cases where new plants arise directly and those where an extensive protonema is developed from a leaf as noted by Kachroo[12], Chopra and Sharma[8] and Chopra and Bhandari[6].

Noguchi and Furuta[18] have pointed out that in case of *Merceya ligulata* the cells producing protonemata are restricted to the lower most portion of the leaf while in *M.gedeana* these cells are scattered all over the surface. Gemmal[9] has stated that the top third of the leaf in *Atrichum undulatum* has a greater tendency to regenerate and he has attributed this fact to the lesser number of lamellae towards that part and that the shoots are developed from the surface cells of the midrib region where they are not bearing any lamellae. Present study based upon the serial sections of a leaf from the base towards the apex reveals that there is no co-relation between the number of lamellae and the power to regenerate as described by Gemmal and the regenerating cells are not strictly restricted to any region as in *Merceya ligulata.* The new shoots are developed at any place on the leaf where the lamellae are present but the central region of the leaf possesses comparatively better capacity to generate.

Gemmal[9] has stated that the leaves attached to the stem do not regenerate. Noguchi and Furuta[19] have also stated that leaves with a fragment of the stem do not produce any protonemata while the fragments of the stem readily do so.

Chopra and Sharma[8] on the basis of their studies on *Pogonatum microstomum* and *Oligotrichum semilamellatum* stated that the upper epidermal cells and basal cells of lamellae regenerate to form buds. In *Pogonatum junghuhnianun*, it has been observed that as far as lamellae are concerned, regeneration is not restricted to their basal cells, rather any cell of lamellae may do so (Figs. 6,7).

Chopra and Sharma[8] also stated that wounding does not have a significant effect on the regeneration. As observed in the present study, it appears that wounding does increase the frequency of regeneration.

ACKNOWLEDGEMENTS

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ABBREVIATIONS USED

Apc.—apical cell; b.i.—bud initial; lame-lamella;l.epi.—lower epidermis; pro.—protonema; U. epi.—Upper epidermis.

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HENSELIZATION OF COMMUTATIVE RING

Ram Avtar

Department of Correspondence Studies,, Panjab University, Chnadigarh-160014

The study of valuations was initiated by Hensel. He was the first one to study metrics different from the usual one arising out of ordinary absolute value in the field of rational numbers. Kurschak and Ostrowski continued this study in greater detail. Later Krull generalized the notion of valuations with values in an arbitrary ordered abelian group.

Once we have the notion of a valuation on a field, then there is a natural topology induced by this valuation in the field and with respect to this topology ,the field becomes a topological field. So we can talk of the completions of a field with valuations. These fields have some very nice properties. For example, there is a structure theorem (due to Cohen) for complete discrete rank 1 valued fields. The complete fields have a role to play in the study of prolongations of valuations to any algebraic extension of it.

While looking at the prolongations to algebraic extensions, we noticed that it is not the completion which is used but the irreducibility criterion of Hensel known as Hensel's lemma. Ostrowski defined a field to be Henselian if (roughly speaking) Hensel's lemma holds. For example complete rank 1 valued fields are already Henselian. However, for rank more than 1 completeness need not imply that the field is Henselian.

The main thing which we wish to stress here is that most of the algebraic properties that are true for complete fields, holds for Henselian fields. For instance ,

Prestal (math.zeit .1975) has shown that if K is an arbitrary field of characteristics different from 2 then a quadratic form q is weakly isotropic over K if and only if q is weakly isotropic with respect to all henselizations at real places and real closures to all archimedean orderings of K. Grothendieck and Neukirch proved independently that Br. $\hat{K}$ = Br. (K^h) for discrete rank 1 valued field K where $\hat{K}$ is the completion of K and K^h is the henselization of K.

It is well known that if (K, v) is a complete discrete rank 1 valued field with k as its residue class field then W (K) the witt ring of quadratic forms over K is given by

$$W(K) = \frac{W(k)[t]}{[t^2-1]}$$

We proved an analogous result on witt ring of quadratic forms over Henselian field of arbitrary but finite rank [1].In a similar way ,we extend the result of Scharlau on Milnor fields [2]

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In the study of higher ramification groups ,there is an interesting theorem due to Hasse and Arf.. The statement is as follows :

Let L be a finite abelian extension of a complete discrete rank 1 valued field K, then the jumps of the ramification series in Herbrand's upper numbering occur at integers We proved an analogous result to this for Hensel fields in [3].

Ribenboim (Math Annalen 155 (1964) proved that if $(\hat{K}, \hat{v})$ is a completion of (K,v) with respect to the topology induced by v such that $\hat{K}$ is a finite dimensional K -vector space then $\hat{K}$ = K .We prove here a similar result for the henselization K^h of K.

Proposition : Let R be a commutative ring and *m* be an ideal contained in the radical of R. R^h the henselization of R at *m*. If R^h is a finite torsion free R-module then $R^h = R$.

Proof : Since $R/m = R^h/m.R^h$

$$\therefore R^h = R + m.R^h$$

As R^h is a finitely generated R-module and $m \subset$ Rad. (R) then by Nakayama's lemma,

we have $R^h = R$

For the field theoretic situation we denote by R the valuation ring of K and R^h the valuation ring of K^h. Whenever K^h is finite over K, then R^h is a finitely generated R - module for $K^h = K \underset{R}{\otimes} R^h$.

If v has rank 1 then clearly $m \subset$ Rad.(R) and by proposition $R^h = R$.

So that $K^h = K \underset{R}{\otimes} R^h = K \underset{R}{\otimes} R = K$.

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ON THE NOTION OF 'VALUE BASE' FOR FIELDS WITH VALUATION

N. Sankaran and Ram Avtar*

**Department of Correspondence Studies, Panjab University, Chandigarh- 160014.*

Recently, Hiroshi Matsuyama [1] introduced the notion of order basis of an ordered field K on a subfield L. In this note we introduced an analogous concept for fields with valuation. Our main result resembles the theorem of Steinitz on the transcendence base of a field K over some subfield L of K.

The main ingredients needed for our study are :

(i) a field K with a valuation v,

(ii) the value group Γ of K^* under v which we take to be totally ordered abelian group with addition as its operation ,

(iii) an arbitrary but fixed subfield L of K, and

(iv) convex subgroups of $\square$

An element x in K is said to be value independent over L if v(x) is outside the least convex subgroup containing the group of values of $L^*(= L\setminus(0))$. In other words, in case x is in the valuation ring R of K, then $v(x) > v(a)$ for all a in L^*. A set of elements $X \square K$ is called a value basis of K over L if (i) X is contained in R, (ii) each $x \square X$ is value independent over L (S(x)) where $S(x) = \{y \in X \mid v(y) < v(x)\}$, and (iii) the least convex subgroup that contains v (L(X)) in $\square$ is itself.

In the sequel, we shall show that (i) value basis for K over a subfield L exists. (ii) any two value basis have the same number of elements, and (iii) value basis of K over L remains unaltered on passage to algebraic closure of L in K ,or of completion or henselization of K with respect to v. Before this ,we shall give some examples.

1. Let K = Qp the field of p - adic numbers and L = Q . Now the value group for both these fields is Z so that there are no value independent elements in K over L.

2. If K = Fp ((t)) the field of Laurent series in t over a finite field Fp and L = Fp ,then the valuation v on K is given by

$$v\left(\square\ a_i t^i\right) = -n \text{ if } a_{-n} \square\square 0.$$

$$i = -n$$

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The value group of K is isomorphic to Z ,while the valuation induced on Fp is trivial . In this case any non constant formal power series in t will form a value basis for K over L.

3. If (K,v) is a field with valuation and L is a subfield of K such that K is algebraic over L, then K has only the empty set as its value basis over L.

Proposition: If X □ K is a value independent subset of K over L then X is algebraically independent over L.

Proof : First observe that if x □ K is algebraic over L, then it cannot be value independent over L. For if x were value independent, then v (x) and -v(x) are outside the convex subgroup □ generated by {v(a) | a ⊔ L} . Without loss of generality, we may suppose that v(x) > v(a) for all a in L, a □ 0. As x is algebraic over L, there exist $a_1, a_2, \ldots\ldots\ldots, a_n$ in L such that $x^n + a_1 x^{n-1} + \ldots\ldots\ldots\ldots + a_n = 0$. Therefore there exist i , j with $i \neq j, 1 \leq i, j \leq n$ with $v(a_i x^{n-i}) = v(a_j x^{n-j})$ and this yields $v(x) = v(a_i/a_j)^{1/i-j}$. This is a contradiction. In case X is non- empty, let x_1 □ X be such that $v(x_1)$ is least among the values □v (x) | x □ X□. This x_1 will be transcendental over L as it is value independent over L. As x_2 is value independent over $L(x_1)$. We have x_1 and x_2 are algebraically independent over L. Should there be an algebraic relation among the elements of X, let x_n □ X, be such that $v(x_n)$ is the largest among the values of x_i □□X that occur in the algebraic relation. Then x_n is algebraic over $L(S(x_n))$ and hence can not belong to the value independent set X of K over L. This proves the proposition.

Note that by a straight forward application of Zorn's lemma, we can extend any value independent subset of K over L into a value basis.

Remark : Let X be a value base for K over a subfield L and Y be an initial segment of X. If M = L (Y), then Y is an order basis of M over L and (X - Y) will be an order basis of K over M . In fact we may take the algebraic closure of L (Y) in K and the statement remains valid for if Y were not an order basis of M, then there can be found an element Z □. M such that Y U Z is value independent over L. Now Z is algebraic over L (Y) while v (Z) lies outside the convex subgroup containing the value group of L (Y) . This is not possible.

Lemma: Let X be a value basis of K over L and a in K be such that v(a) lies outside the least convex subgroup containing v (L^*). Then there exist a unique element x in X such that $v(x) < v(a^m) < v(x^n)$ for suitable integers m and n.

Proof : Exchange a with a-1, if need be, we may assume v(a) >0. Set S = □z ∈ X | v

(z) < v (a)}. If v(a) does not belong to the least convex subgroup □□containing v (L (S)*), then S U □a□ is value independent over L and X \ S contains an element x such that v(x) is in every convex subgroup of □/ □. Now X U □a□ cannot be value independent over L as X is a value basis and as such amaximal value independent set in K over L. As v (a) belongs to the least convex subgroup containing the value group of L(S U □a□), we see that v (a) and v (x) are in the least convex subgroup (≠ 0) in □/ □, say v(a), v (x) □ □□, where □⊂□□□□ □ and □□/ □ is archimedean. The result is now obvious.

Uniqueness of x. Suppose x_1, x_2 □ X be such that

$v(x_1) < v(a^m) < v(x_1^n)$ and $v(x_2) < v(a^r) < v(x_2^s)$

Then $v(x_1) < v(a^m) < v(a^{rm}) < v(x_2^{ms})$

and $v(x_2) < v(a^r) < v(a^{rm}) < v(x_1^m)$ so that $v(x_1)$ and

$v(x_2)$ can not be value independent over L.

Theorem : Any two maximal value independent subsets of K over L have the same number of elements.

Proof : Let X and Y be two maximal order independent subsets of K over L. If X is empty then so is Y since the least convex subgroup containing v(L(X)*) = v (L*) = □ = v (L (Y)*). Let then neither X nor Y is empty. By the above lemma, to each y □ Y we can assign a unique x in X and vice versa. Set □ : Y □ X and □□: X □ Y be the maps which assign to each y in Y (x □ X) a unique element □ (y) in X (□ (x) in Y).

If y_1, y_2 □ Y with $v(y_1) < v(y_2)$ in Γ then

$v(□(y_1)) < v(y_1^m) < v(□(y_1^n))$

and $v(□(y_2)) < v(y_2^r) < v(□(y_2^s))$.

Therefore, $v(□(y_1)) < v(□(y_2^t))$. Thus □ preserves the order. Likewise □ also preserves the order.

As $v(y_1^m) < v(\sigma(y_1^n)) < v(\sigma(y_1^{mn}))$, we get □ □□ $(y_1) = y_1$ so that □ □□ is the identity on Y and similarly □ □ □ is identity on X. Thus we see that □ and □ are 1-1 and onto order preserving maps of X to Y (and Y to X). Therefore X and Y are equicardinal.

We shall call the number of elements in any maximal value independent set of K over L as the order dimension of K over L. Thus the order dim. of Qp over Q is 0, of K over Fp in example 2 is 1.

These authors wishes to thank Prof. R. Wiegandt for having his attention to the paper of Matsuyama.

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