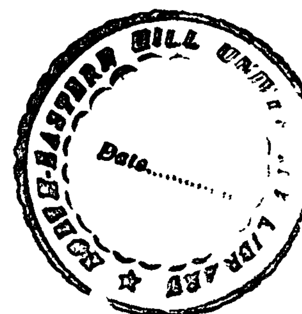
STUDIES ON THE EFFECT OF CISPLATIN ON MALIGNANT AND NORMAL CELLS: PRELIMINARY INVESTIGATIONS ON CISPLATIN COMBINATION CHEMOTHERAPY

ABSTRACT

By

Anirudha Giri
M Sc



THESIS

Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in Zoology

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A B S T R A C T

STUDIES ON THE EFFECT OF CISPLATIN ON MALIGNANT AND NORMAL CELLS: PRELIMINARY INVESTIGATIONS ON CISPLATIN COMBINATION CHEMOTHERAPY

by

ANIRUDHA GIRI

Studies on the tumor growth pattern and antitumor activity of cisplatin against ascites Dalton's lymphoma adapted to Swiss albino mice show that a regular increase in ascites tumor volume could be noted with time following tumor transplation. During the initial phase (2-12 days) of tumor growth ascites fluid increases more rapidly in the ascites tumor so that the ratio of ascites fluid to tumor cell pellet reached about two during the middle period of tumor growth.

Cisplatin treatment of the tumor-bearing hosts on the 10th day post-tumor transplantation resulted in a significant reduction in tumor volume indicating effective tumor regression.

Measurement of carbohydrate and protein contents in tumor supernantant, and glucose contents in serum as

well as tumor supernatant of the tumor-bearing hosts treated with or without cisplatin revealed that following 3-4 days of cisplatin treatment, the carbohydrate contents markedly increased in the ascites fluid of the tumor-bearing hosts. However, the protein contents in the ascites fluid decreased by about three fold following 2-4 days of cisplatin treatment. The serum glucose level of tumor-bearing hosts was found to be comparatively lower than the normal animals and 1-3 days of cisplatin treatment of the tumor-bearing hosts resulted in a significant increase in serum glucose level. In the tumor supernatant however, it is found that in the tumor-bearing hosts very low concentration of glucose was present which increased progressively following 1-4 days of cisplatin treatment.

It is suggested that since ascites fluid is the direct nutritional source to the tumor cells, the rapid increase in ascites fluid during tumor growth could possibly be a means to meet more nutritional requirements of tumor cells. This is evident from the rich carbohydrate and protein contents in the ascites fluid and the presence of numerous surface membrane ruffles and cytoplasmic processes all over the tumor cells which could play a role in nutritional exchange with ascites fluid. The observed lower serum glucose level in tumur-bearing hosts than the normal animals may indicate higher rate of glucose uptake by tumor cells. And

an increase in a carbohydrate contents in ascites fluid following 3-4 days of cisplatin treatment could be due to utilize the carbohydrates present in the ascites fluid as well as release of surface mucopolysaccharides and sialic acid moieties from tumor cells. Studies on the rate of glucose consumption by tumor cells and tumor cell sialic acid content also revealed a decrease in the rate of glucose consumption and also decrease in sialic acid content in the tumor cells.

Metaphase chromosome analysis of Daltion's lymphoma cells show that cisplatin (8 mg/kg b.w.) for 1-4 days resulted in a very high frequency (80-90%) of aberrant metaphases. Pulverized (severely damaged) cells were also frequently observed.

Light microscopical studies show that the percentage ratio of leukocytes (neutrophils, monocytes, lymphocytes; based on nuclear shape and size) to tumor cells increased about three times by 1-4 days of cisplatin treatment than the control animals. In control ascites tumor very few leukocytes were seen among tumor cells which were round in shape. After 8-96 hr of cisplatin treatment many leukocytes are found coming closer to the tumor cells and surround them finally resulting in the formation and shedding of membrane vesicles, and disintegration of plasmamembrane

leading to the lysis of tumor cells.

Measurement of Ca^{2+} concentrations in various tissues i.e., liver, kidney, brain, spleen and tumor cells show that following cisplatin treatment resulted in significant increase could be noted in tumor cells followed by kidney and brain tissues. The potassium concentration decreased significantly in the kidney and tumor cells following cisplatin treatment, whereas liver and spleen tissues showed no significant change. Significant variations in the rate of oxygen consumption (QO_2) were observed in the tumor cells following cisplatin treatment. Tumor cells showed about 40% increase in QO_2 following one day of cisplatin treatment. However, following 2-4 days of the treatment, QO_2 decreased steadily.

Scanning electron microscopic observations show that during tumor regression following cisplatin treatment definitie changes in the pattern of surface membrane ruffles/ blebs also occur along with the infiltration of leukocytes towards tumor cells and sharp decrease in ascites fluid. Control tumor cells showed the presence of fine ruffles/ blebs distributed evenly over the cell membrane. After 8 hr of cisplatin resulted in the infiltration of leukocytes surrounding tumor cells and forming connections with the latter. Also it leads to a definite movement of ruffles/

blebs from the top surface of the tumor cells towards the marginal areas. One day of cisplatin treatment showed formation of broader tumor cell-leukocyte connections and the appearance of fine microvilli like processes extending from tumor cells. At 2-3 days of the treatment, thick blebs are formed over the surface membrane of tumor cells and lysis starts. There is disappearance of thin cellular processes, formation of membrane vacuoles and breaking of plasmamembrane of tumor cells after 4 days of cisplatin treatment which leads to the lysis of tumor cells.

It is suggested that cisplatin has a definite effect on the cell surface membrane. And the disintegration of plasmamembrane of tumor cells surrounded/connected by leukocytes could be due to the release of some toxic factors from leukocytes.

Studies on the glucose-6-phosphatase enzyme activity in the liver tissues show that the normal animals exhibit higher activity than the tumor-bearing animals. Cisplatin treatment of the tumor-bearing hosts resulted in a steady increase in enzyme activity. In the kidney tissues the enzyme activity decreased till the 2nd day of cisplatin treatment and showed sign of recovery thereafter.

Studies on lactate dehydrogenase (LDH) enzyme acti-

vity show that except for liver all other tissues studies definite patterns of changes in enzyme activity could be noted. It is observed that the LDH activity in serum and liver of the tumor-bearing animals were comparatively higher than the normal animals. Following cisplatin treatment except for tumor cells, in other tissues an overall increase in enzyme activity could be observed. In the tumor cells however, a progressive decrease in LDH activity was noted following cisplatin treatment.

LDH isozyme analysis in serum, kidney, liver, tumor supernatant and tumor cells following cisplatin treatment of the tumor-bearing host *in vivo* show that in the serum and kidney tissues only all the 5 isozymes are present. In the other three tissues distinct variations are notable as regards to the number and nature of the various isozyme bands. In the liver tissue only 3 isozyme bands (LDH-3, LDH-4, and LDH-5) are seen. Following 1-4 days of cisplatin treatment of the tumor-bearing hosts. LDH-3 and LDH-4 showed marked variations in band intensities (decreasing) indicating changes in activity and at the 4th day of the treatment LDH-5 was found to be the only isozyme form. In the tumor supernatant besides LDH-5, LDH-4 isozyme could also be seen. But, in the tumor cells LDH-5 is the only isozyme form present.

It has also been noted that one extra band near the cathodic end could be found in the serum of tumor-bearing as well as cisplatin treated group which may also be seen in tumor supernatant and tumor cells; but absent from liver, kidney and serum normal animals.

Assay of Na^{+}+K^{+}-ATPase activity in the tumor cells as well as in tumor supernatant show that a gradual decrease in enzyme activity could be noted in the tumor cells following 2-4 days of cisplatin the enzyme activity increased upto 2nd day of the treatment, but; following 3-4 days of the treatment decreased to that of the control level.

5'-Nucleotidase (5'-ND) activity in liver of tumor-bearing hosts was found to be about 2.5 times higher than the normal animals which progressively decreased following cisplatin treatment. However, in the kidney tissues the enzyme activity increased following cisplatin treatment.

Studies on the activity of arginase in liver, kidney as well as in tumor supernatant revealed that tumor-bearing animals exhibit significantly lower enzyme activity than the normal animals which decreased further following 3-4 days of cisplatin treatment of the tumor-bearing hosts. An almost similar pattern of arginase activity was also observed in the kidney tissues. In the tumor supernatant

however, the arginase activity increased steadily following 8 hr to 96 hr (4 days) of cisplatin treatment of the tumor-bearing hosts.

Measurement of cathepsin activity in serum as well as in tumor supernatant revealed that following 2-4 days of cisplatin treatment of the tumor-bearing hosts, the activity of both cathepsin B and cathepsin H increased progressively and by 3-4 days of the treatment very high activity could be recorded for both the enzymes. Similar trends were also observed for cathepsin B and cathepsin H activity in tumor supernatant.

It is suggested that although DNA is considered as the primary target for cisplatin for its anticancer activity, however, it may have multilevel targets and act through changes in the activity of various enzymes and isozymes of metabolic importance.

Combination chemotherapeutic studies with subtherapeutical dose of cisplatin (4.0 mg/kg b.w.) and vitamin C (0.5% in drinking water) revealed that tumor-bearing hosts receiving either cisplatin or vitamin C alone show almost similar survival patterns and 50% animals survived upto 35 days.

The combined administration of 0.5% vitamin C in

drinking water from the first day and cisplatin (i.p., 4.0 mg/kg b.w.) on the 10th day resulted in 70% survivals upto 55 days and 40% of the treated mice were found to be tumor free. The increase in body weight in this group of mice was very slow indicating effective retardation of tumor growth. This combined treatment was found to be sequence dependent since cisplatin treatment first and vitamin C treatment started second did not result in any synergistic effect. Changing the host strain from C3H/He mice to Swiss albinomice did not alter the result thus indicating that the observed synergistic antitumor activity between vitamin C and cisplatin may not be host strain specific.

Studies on the effect on thymus and spleen weight, and tumor pH revealed that combined treatment of 0.5% vitamin C and cisplatin (4.0 mg/kg b.w.) significantly increased the weight of spleen and thymus, and the average tumor pH decreased to 6.27 as compared to 6.93 found in the controls.

It is also noted that tumor-bearing animals have a lower serum ascorbic acid level (7.11 µg/ml) which increased significantly (13.44 µg/ml) in the vitamin C treated group. The combined treatment of vitamin C and cisplatin also resulted in a more sustained increased in total leukocyte count in the blood as compared to the treatment of cisplatin as a single agent.

Agglutination studies on ascites Dalton's lymphoma (DL) using conconavalin A (Con A) show that control DL cells show high degree of Con A agglutination. However, combined treatment with vitamin C plus cisplatin markedly decreased the degree of agglutination as compared to that when these agents were treated separately.

Fluorescence labelling of DL cells with Con A fluorescentisothyocyanate (Con A - FITC) show uniformly distribed bright even fluorescence all over the surface. However, treatment with cisplatin or vitamin plus cisplatin for 15-60 min resulted in rearrangemtn/loss/removal of labelled Con A from the cell surface thus reducing fluorescence intensity. This effect was more rapid in case of the combined treated groups than the groups treated separately.

Studies on the effect of vitamin C on cisplatin induced mutagenicity revealed that treatment of the tumor-bearing hosts with 0.5% vitamin C prior to cisplatin treatment significantly reduced the incidence of chromosomal aberrations, micronuclei in bonemarrow cells and sperm head abnormalities in mice thus indicating that vitamin C may have a chemoprotective effect against cisplatin induced mutagenicity in the hosts.

Studies on the cisplatin induced nephrotoxicity

as observed from serum uric acid as well as serum urea level show that cisplatin causes nephrotoxicity which is dose dependent in mice. However, pretreatment with vitamin C significantly reduces the serum urea level thus suggesting that vitamin C may have some protective effect against cisplatin induced nephrotoxicity in Swiss albino mice.

It is concluded from the present study that:

i) Cisplatin treatment brings about definite changes in the ascites fluid as well as in tumor cells in terms of nutritional requirements, degree of infiltration of leukocytes towards tumor cells finally leading to the death of the tumor cells.

ii) Cisplatin has some definite effect on the arrangement and movement of ruffles/blebs over the surface of tumor cells and also leads to the formation of membrane vesicles/cellular vacuoles/thick blebs all of which ultimately favour the tumor cell death.

iii) The enzyme lactate dehydrogenase showed comparatively increased/decreased activity in tumor cells (↓), ascites fluid (↑), serum (↑) and kidney (↘) following cisplatin treatment. In addition to this the appearance of a new isozyme which is here named as LDH-T was noted in the serum and tumor cells of the tumor-bearing hosts.

iv) Vitamin C showed synergistic effect with cispla-

tin and it may be used with subtherapeutical dose of cisplatin in protecting the host against cisplatin induced nephrotoxicity without losing the therapeutic efficacy.

v) Along with the enhanced therapeutic efficacy of cisplatin by vitamin C, it may also protect the host against cisplatin induced mutagenicity.

vi) These studies further indicate the involvement of multistep and multilevel effects of cisplatin resulting the tumor regression in the host.

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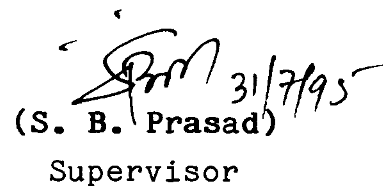
I certify that the thesis entitled **"Studies on the effect of cisplatin on malignant and normal cells: Preliminary investigations on cisplatin combination chemotherapy"** submitted by **Mr. Anirudha Giri**, for the **Degree of Doctor of Philosophy** of the North-Eastern Hill University, Shillong, embodies the record of original investigation by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the **Ph.D. Degree.** The work has not been submitted for any degree of any other University.



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SHILLONG **(ANIRUDHA GIRI)**

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I N T R O D U C T I O N

Perhaps no disease known to the modern civilization is viewed with as much concern as cancer. Cancer has become an unpredictable disease that strikes indiscriminately at rich and poor, fat and thin, old and middle-aged, as if it usually owed nothing to external causes. If that were true, our only hope of overcoming cancer would be to improve the treatment of the disease.

Considerable efforts are continually directed at improving the diagnosis and treatment of cancer. At present there are three categories of widely accepted treatment for cancer, i.e., surgery, radiotherapy and chemotherapy. Out of these three, chemotherapy has gained wide acceptance both as a separate treatment modality and also during/after radiation and/or surgery. In chemotherapy, cisplatin is now established to play a pivotal role both as a single agent as well as with other drugs against many cancers.

I. What Is 'Cancer':

The zygote divides and redivides through infancy and adolescence to form an adult, but, once adulthood is reached, the cells divide at a much slower rate reproducing only to maintain the status quo, that is, only to heal wounds and replace cells that have died. This cell division and growth are strictly regulated.

In abnormal cellular proliferation, termed neoplasia, growth occurs because cells are not removed fast enough from the dividing pool of stem cells. Tumors are strictly defined as neoplasms, although the term 'tumor' may be loosely applied to any swelling (Vincent, 1985). The terms 'neoplasm' and 'tumor' are commonly used interchangeably (Friedberg, 1986).

The transformed cell or tumor cell arises as a variant abnormal cell which escapes the host's natural control of growth and differentiation (Nicolson, 1979). Tumors always violate the basic homeostatic principle of the body and ideally fall into one of two categories: the slowly growing 'benign' (innocent) and the rapidly growing malignant forms which are invasive, disseminating and lethal (Vincent, 1985).

A tumor is generally considered benign if it remains similar in structure to the tissue from which it is derived, grows slowly by simple expansion and remains encapsulated

by a layer of connective tissue, and its cell nuclei divide almost normally with few abnormal chromosomes (Nicolson, 1979). The word 'benign' refers only to a form of behaviour characterised by inability to metastasize (Vincent, 1985). A malignant tumor on the other hand is atypical in tissue structure, grows rapidly and does not remain encapsulated; displays many abnormal nuclear divisions and chromosomes and invades the surrounding tissues shedding cells that have the ability to colonize new sites (Nicolson, 1979). Cancer is defined as a malignant neoplasm.

The word 'cancer' derives from the Latin for 'crab' and suggests its capacity to reach out and cling tenaceously to adjacent tissues (Vincent, 1985). A cancer/transformed cell probably starts out as a small normal cell that under the influence of various oncogenic/carcinogenic agents e.g., chemicals, viral, physical (X-rays, UV-rays) or hormonal undergoes a series of progressive changes. It is also considered to be a dynamic developmental disorder and disease of cellular differentiation (Rubin, 1985). Thus, a precancerous cell is suggested to be an intermediate or transitional stage between a normal and a cancer cell (Lupulescu, 1983). It has been found that normal cells have oncogenes which are the altered versions of normal genes and their expression can lead to cancer growth (Bishop, 1982).

Cairns (1986) classified cancers into three broad groups: carcinomas, sarcomas and leukemias/lymphomas. The carcinomas arise in the epithelia, the sheets of cells covering the surface and lining the various glands. The sarcomas arise in supporting structures such as fibrous tissues and blood vessels. The leukemias/lymphomas arise in the blood forming cells of the bone marrow and lymphnodes. However, cancers may also be classified by the organ in which they originate.

The spread of tumor cells from the primary organ or tissue in which the neoplasm initially occurs to secondary sites is called metastasis (Fidler and Hart, 1982). It is a multistep process and involves multiple and intricate cell-cell and cell-matrix interactions during metastatic dissemination that reflect the adaptability of the surface membrane of tumor cells and their capacity for survival in potentially hostile environments. Certain critical steps identified in metastasis can be summarised as: development of the primary tumor; vascularization of the primary tumor; invasion/breaching of normal tissue – blood barrier; transport in blood stream; arrest in capillaries or attachment of cancer cells to vascular endothelium; breaching of blood – tissue barrier; and host organ – malignant cell interactions with the growth of the secondary tumor (Gallagher, 1985).

Various common characteristics of a cancer cell fairly distinguishes a cancer cell from that of its normal counterpart in most of the cases. Some of these altered properties of transformed/malignant cells are observed to be related to (i) growth control - like unlimited life span, growth at high cell density, lower requirements for serum factors, loss of anchorage dependence for growth; (ii) morphology/ adhesion - rounded, convex in culture, poorly spread, reduced adhesion to substratum and loss of contact inhibition of movement and multilayering in culture; and (iii) others - like increased production of proteolytic enzymes, altered antigenicity, disorganization of cytoskeleton, increase in negative surface charge etc. (Hynes, 1979). These changes could lead to immortalization of cancer cells.

II. Cell Surface In Malignancy:

The cellular microenvironment plays a central role in regulating the growth and development of cells and the cell interacts with the extracellular matrix via the external surface of its plasmamembrane (Gallagher, 1985) probably by receiving and transmitting regulatory signals from the microenvironment. Cancer is a disease in which abnormalities both in cell growth and cell development are found. Defects in cell-cell recognition are thought to underlie the uncontrolled growth and motility which characterize neoplastic

transformation and metastasis (Nicolson *et al*., 1985). Cancer cells are capable of indefinite proliferation and are able to overcome some of the physiological and mechanical constraints that restrict the population growth and territorial expansion of normal cells. Alterations in the cell surface are involved in various altered behaviour of the tumor cell. Altered growth controls are thought to reflect altered responses to hormones which act on the cell surface binding to specific receptors (Fabricant *et al*., 1977), rate of transport of ions and nutrients (Rozengurt, 1979).

Lectins (Latin, 'leger' – to pick out or choose) are a group of plant proteins that have been extensively used as tools or probes to study and characterize the cell surface architectural features of a wide variety of cells. Cancer cells are reported to be highly agglutinated with lectins while normal cells do not agglutinate appreciably (Prasad and Sodhi, 1981). The lectin concanavalin A (Con A), binds to simple sugars and polysaccharides containing terminal non-reducing α-D-manose or α-D-glucopyronyl residues (Poretz and Goldstein, 1970). It has been reported by various authors that malignantly transformed cells agglutinate more readily by Con A whereas under similar conditions their normal counterparts show very little or no agglutination. Furthermore, treatment of cells with trypsin results in a gain of aggluti-

nability of normal cells with native Con A whereas the treatment reduces the agglutinability of transformed cells (reviewed in Brown and Hunt, 1978).

Yokoyama (1980) using Con A labelled either with ferritin or horse raddish perox;dase technique showed that lectin-induced redistribution and formation of clusters of the binding sites is more pronounced in the tumor cells than that of normal cells. He suggested that this uneven surface labelling and clustering might be due to membrane internalization and change in fluidity. An increased membrane fluidity has already been reported for cancer cells (Rule et al., 1979).

Warren et al. (1978) believe that changes in bound carbohydrates at the cell surface might result in persistent cell division, decreased intercellular adhesiveness, altered transport, altered/diminished/masked immunogenicity and other specialized functions accompanying malignant transformation. The widely distributed sialic acid moieties of glycoproteins are reported to have damping, protective and regulatory functions at the cell surface (Warren et al., 1978). Prasad (1986) reported that the agglutination behaviour of normal and malignant cells depends upon the sialic acid moieties present on the cell surface. It has also been demonstrated that the release of cell surface carbohydrates are associated with

the increase/decrease of Con A agglutinability of cisplatin treated thymocytes, splenocytes and lymphnode cells. Based on these studies he suggested that the release of carbohydrate moieties from the surface of these cells after cisplatin treatment may result in the unmasking or in a topographical rearrangement of the Con A binding sites making them more readily available to Con A for binding and agglutination. In a reconstitution study he further demonstrated that when cisplatin treated cells showing maximum agglutinability are incubated with three sugars such as D-glucose, D-xylose and sialic acid separately, the agglutinability of cells decreased and the maximal decrease was observed for sialic acid – incubated cells. He suggested that the binding sites of Con A may get masked again, thus decreasing the agglutinability (Prasad, 1989). Yogeeswaran and Salk (1981) reported that differences in surface charge resulting from differential expression of sialic acid may have a direct effect on such properties as cell-cell and cell-substrate adherence and in turn could have a direct effect on cellular behaviour *in vivo*.

Cell surface glycoproteins and glycolipids (gangliosides) are succeptible to such marked elevations as soon as a malignant growth starts to develop, metastasize or recurs, that they are referred to as 'tumor markers' (Stringou *et al.*,

1992). Sialic acid is the main structural component of gangliosides and the terminal carbohydrate chain of glycoproteins and glycolipids. It has been reported by a number of workers that sialic acid is an important biological tumor marker of high sensitivity and specificity in diagnosis and response to treatment of cancer (Chen et al., 1979; Shamberger, 1984; Pulcinsky et al., 1986; Stringou et al., 1992).

Thus, the cell surface components could play vital role in the malignant transformation of a variety of cells. And the study of some cell surface parameters like lectin agglutination and sialic acid in tumor cells treated with or without anticancer drugs may give some useful information to understand the chemotherapeutic mechanisms.

III. Enzymes And Cancer:

The enzymatic changes may reflect the overall changes in metabolism that occur in malignancy. Thus, changes in activity of ecto- as well as endoenzymes and appearance of isozymes and enzyme variants, and the various studies demonstrating significant changes both in content and molecular structure of enzymes in malignant diseases have been reviewed (Stefanini, 1985). Increased activity of lactate dehydrogenase (LDH) in most malignant tumors (Greengard et al., 1982; Carda-Abella et al., 1982), 5'-nucleotidase in cancers of stomach (Rogers et al., 1981), total acid phosphatase

in breast cancer (Filmus *et al.*, 1984), glucose-6-phosphate dehydrogenase in breast, prostate and lung cancers (Greengard *et al.*, 1982; Hilf *et al.*, 1982; Zampella *et al.*, 1982), glycosyl transferase in ovarian carcinoma (Chatterjee *et al.*, 1981), aldolase (isozyme A) in cancers of lung, stomach, colon and rectum (Greengard *et al.*, 1982) and hepatoma (Asaka *et al.*, 1980). While decreased activity of adenylate kinase in lung cancer (Greengard *et al.*, 1982) and 5'-nucleotidase in ovarian carcinoma (Chatterjee *et al.*, 1981) have also been noticed.

These changes which trigger biochemical processes aiding cancerous cells over normal surrounding tissue cells, may be related to the aggressiveness of the tumor as reported in the case of β-hexosaminidase activity in ovarian cancer and glucose-6-phosphate dehydrogenase in carcinoma of the prostate (Zampella *et al.*, 1982). It is postulated that anaplasia is accompanied by molecular adjustments, which precede morphologic changes in malignancy and may manifest themselves through changes in enzyme patterns, thus there is predominance of LDH-1 in normal colonic mucosa and LDH-5 in colonic cancer tissue (Carda-Abella *et al.*, 1982).

Rogers *et al.* (1981) demonstrated that the activity of LDH seems higher in malignant tissue and in gastric juice fluid of patients with carcinoma f the stomach. Leptomeningeal

infiltration by carcinoma is accompanied by increased activity of β-glucuronidase and LDH-5 in the cerebro-spinal fluid (Tietz, 1980).

Presence of enzyme variants in the serum of patients with a particular malignancy have been marked. A specific isozyme of acid phosphatase in serum was detected in metastatic adenocarcinoma of the prostate (Holyoke et al., 1981). A fast-moving 5'-nucleotide phosphodiestrase is elevated in metastases to liver from tumor of the breast and gastro-intestinal tract (Tsou et al., 1980; Tsou et al., 1982). Aldolase is present in three isozyme (A, B and C) forms. Aldolase A predominates in cancer tissue and is elevated in carcinomas of the gut and pancrease and in hepatomas (Asaka et al., 1980).

A) Lactate dehydrogenase (LDH):

LDH (LD, EC1.1.1.27) catalyses a reversible reaction of pyruvate to lactate in cells. It exists in five isozyme forms and is controlled by two genes (Cahn et al., 1962; Markert, 1963). Each gene is responsible for synthesis of a unique peptide. A single peptide unit combines with itself forming a tetramer representing the native LDH molecule. The two proteins (tetramers) thus formed are the electrophoretic extremes, that is LDH-1 (most anodic) and LDH-5 (most cathodic). Cahn et al., (1962) denote these enzymes as H_4

and M_4 respectively. 'H' represents the main cardiac enzyme (LDH-1) and 'M' the peptide from skeletal muscles (LDH-5). The intermediate isozymes LDH-2, LDH-3 and LDH-4 are hybrids formed by random association of H and M subunits into tetramers. Thus, LDH-2, -3 and -4 would contain H3M, H2M2 and HM3 subunits respectively. The accepted nomenclature for LDH isozymes is LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5 and will be used henceforth in the text. LDH-1 moves farthest toward the anode during electrophoresis and LDH-5 is the most cathodic enzyme. LDH-1 and LDH-2 are sometimes termed as the fast isozymes and LDH-4 and LDH-5 the slow isozymes. LDH-3 is an isozyme with intermediate electrophoretic mobility.

The use of LDH isozymes as a diagnostic aid has been amply documented (Wilkinson, 1970; Dito, 1973). Alterations in LDH isozyme pattern can be quite useful for evaluating tissue disease entities such as hemolytic anemia, megaloblastic anemia, leukemia, renal necrosis, pulmonary infarction and neoplasia (Starkweather *et al.*, 1966). Increased activity of LDH has also been reported in testicular cancer (Lippert and Javadpour, 1981), leukemias and lymphomas, Burkitt's lymphoma and metastatic tumors (Schneider *et al.*, 1980).

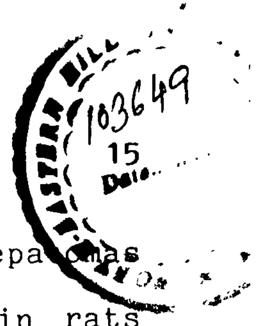
It has been reported that human malignant cells both lymphoid (Rambotti and Davis, 1981) as well as other organs

(Goldman *et al.*, 1964; Fleischer, *et al.*, 1981) possess isozyme patterns distinguished by lower H:M ratios. It is known that LDH-5 is active in anaerobic glycolysis (Dawson *et al.*, 1964). Predominance of LDH-5 in many malignant tissues has been observed (Goldman *et al.*, 1964). It has been suggested that this predominance reflects anaerobic metabolism of these tissues (Goldman *et al.*, 1964) since hypoxic conditions have been shown to exist in many tumors (Kallman, 1972).

B) Glucose-6-phosphatase:

Glucose-6-phosphatase or D-glucose-6-phosphatase (EC 3.1.3.9) is a phosphoestrase that acts preferentially upon glucose-6-phosphate. Under physiological conditions it catalyzes the unidirectional reaction with the equilibrium in favour of the formation of free glucose and inorganic phosphate (Cahill *et al.*, 1959). Werve (1989) reported that the glucose-6-phosphate system is modulated by changes in Ca^{++} concentration in the range of those occurring in liver cell upon hormonal stimulation. Although liver is the main source of glucose-6-phosphatase, it is also found to be present in kidney and other tissues (Nordlie and Sukalski, 1985; Waddell and Burchell, 1988).

The metabolism of tumor in relation to glucose-6-phosphatase activity have been studied by various workers. It has been reported that the activity of glucose-6-phospha-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tase decreases in the soluble fraction of primary hepatomas induced by nitrosamine (Kilderma et al., 1977) and in rats undergoing carcinoma by nitrosamine (Elitzo et al., 1977). Schull et al. (1956), however, reported an increase in the glucose -6-phosphatase activity in mice bearing corticotrophin secreting tumor.

C) Arginase:

Arginase (L-arginine ureohydrolase, EC 3.5.3.1) is the terminal enzyme of the urea cycle. Arginase catalyzes the hydrolysis of arginine to urea and ornithine. Ornithine produced during the reaction is used in the urea cycle. Arginase is an important enzyme being involved in the removal of toxic ammonia as urea. Devamanoharan et al., (1987) studied the activity of arginase in polychlorinated dibenzofuran (PCDF) fed rats and observed a decrease in the activity of liver arginase indicating decreased protein catabolism. Elevated levels of arginase activity has been observed in various pathophysiological conditions. Increased level of arginase activity has been reported during blastogenesis (Klein and Morris, 1978). Mammalian arginase enzyme is reported to have some antitumor activity against Walker 256 carcinosarcoma and Jensen sarcoma, two tumors that cannot grow on citrulline and has little effect on C_3HED lymphoma, Ehrlich ascites carinoma, Shimkim mammary tumor, or the tumors in the cancer

screen of the National Cancer Institute (Holcenberg, 1981). Arginase has also been reported to be involved in macrophage activation and tumoricidal activity (Roitt et al., 1985).

D) 5'-Nucleotidase and $Na^+ + K^+$ - ATPase:

The importance of surface membrane and its changed properties in malignancy has already been elaborated in the previous section. Therefore, studies on cell membrane enzymes are of great importance to evaluate the functional changes that occur during malignancy. 5'-Nucleotidase (5'-ND, NTD, EC 3.1.3.5) and ($Na^+ + K^+$) - activated adenosine triphosphatase (TAP - phosphohydrolase, EC 3.6.1.3) are cell membrane enzymes. It has been reported that malignant cells display very low level of 5'-ND activity (Raz et al., 1978). Chatterjee et al., (1981) have also found similar results in ovarian carcinoma and suggested that decreased level of 5'-ND may serve as a marker for the said tumor. Cell membranes from Yoshida hepatoma and Morris hepatoma 5123 tc contain respectively a six to eight fold and three fold lower specific activity of 5'-ND than normal liver (Graham, 1979). In contrast to these results Emmelot and Bos (1969) found that the activity of 5'-ND in the surface membrane from mouse hepatomas 147042, 4189 and 143066 were increased by two to five fold over that of mouse liver. Although the function of 5'-nucleotidase at the cell surface is not clear, however,

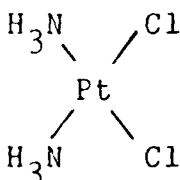
it may regulate the supply of nucleoside precursors for nucleic acid synthesis (Lopes et al., 1973).

Na^+ + K^+ - activated adenosine triphosphatase which catalyzes the active transport of Na^+ and K^+ through cell membrane, requires Mg^{2+} for the overall reaction (Post et al., 1975) and thus helps in maintaining the ionic balance across the cell membrane. Emmelot and Bos (1969) reported 25% and 50% decrease in Na^+ + K^+ - ATPase in hepatomas 147042 and 4189 respectively. A dramatic reduction in the specific activity of Na^+ + K^+ - ATPase in the plasmamembrane from 58.0 to 1.75 is reported for Morris hepatoma 5123 tc as compared to normal liver (Barclay and Terebus-Kekish, 1973). In contrast to these hepatomas, the Na^+ + K^+ - ATPase in the plasmamembrane of hepatoma 484 was similar to or significantly higher than that of liver (Emmelot and Bos, 1969).

Thus, no consistent change in the overall activity of these enzymes could be associated with malignant transformation. However, since membrane constituents are shed into the surrounding milieu at increasing rates when cells replicate more rapidly or are destroyed, hence enzymatic changes may reflect the overall change in metabolism that occur in malignancy and after therapy.

IV. Cisplatin And Experimental Tumors:

Cisplatin is a water-soluble, squireplanar co-ordination complex containing a central platinum atom surrounded by two chloride atoms and two ammonia moeities in cis-configuration.



The accidental discovery of *cis*-diammine-dichloroplatinum (II) (Cisplatin; DDP; Cis-Pt(II)$(NH_3)_2Cl_2$; Cis-Pt) as an antitumor agent in 1968 later proved to be effective against various experimental animal tumor model systems, for example - sarcoma-180 and leukemia L-1210 is mice (Rosenberg *et al*., 1969; Rosenberg and Van Camp, 1970), Dunning ascites leukemia and Walker 256 carinosarcoma in rats (Kociba *et al*., 1970), sarcoma-180 in Swis white mice (Sodhi and Aggarwal, 1974), L-1210 murine leukemia in BDF 1 mice (Speer *et al*., 1972), chemically induced mammary tumors in Sprague Dawley rats (Welsch, 1972), fibrosarcoma in Swiss white mice (Sarna and Sohi, 1978), Dalton's lymphoma in dba and Swiss white mice (Prasad, 1981), V-2 carcinoma in rabbit (Wright *et al*., 1985) and in clinical treatment of a variety of human cancers (Haskell, 1985) also in combination with other anticancer drugs (Barberi-Heyob *et al*., 1993).

Since the first observation of Harder and Rosenberg (1970) that cisplatin inhibits DNA synthesis at lower concentrations and then the sequential inhibition of DNA, RNA and protein synthesis occurs at higher concentrations, many reports have been published describing the interaction of cisplatin with DNA (Rosenberg, 1985; Pinto and Lippard, 1985; Reed, 1990; Sip et al., 1992). Roberts and Pascoe (1972) reported the cross-linking of guanine bases in complementary strands of DNA is responsible for the cytotoxic actions of bifunctional alkylating agents. Mancy et al. (1973) found that cisplatin and its trans-isomer readily bind to the ring nitrogen atoms of the purnies and pyrimidines with loss of chloride ion and suggested that cis-configuration with two neighbouring leaving groups may act as bifunctional while trans-forms act as monofunctional agents.

Roos and Arnold (1977) reported that cross-linking of platinum complexes with DNA increase with the guanine-cytosine (G-C) content of DNA. The formation of a closed ring chelate of the aquated cisplatin with both N7 and O6 of guanine has also been reported (Dehand and Jordanov, 1976). On the basis of electrophoresis, chemical probes and molecular mechanics modeling data Sip et al. (1992) proposed that cisplatin induces the cross-linking of two guanine N7 atoms on opposite strands within the sequence d(GC/CG) and produces

a kink of about 55° of the helix axis. However, because of differential repair capacities of normal and cancer cells, cisplatin has been suggested to preferentially kill cancer cells (Rosenberg, 1985).

Sodhi (1977) demonstrated that cisplatin treatment inhibits the process of cytokinesis and induces giant cell formation which could be due to inhibition of DNA replication. Heinen and Bassler (1976) found that high dose cisplatin provokes some inhibition of premitotic DNA synthesis whereas at lower doses DNA synthesis takes place and is completed but the majority of the cells cannot divide. However, RNA and protein synthesis were quite unaffected, thus, causing the cell volume to increase progressively. These cells have an abnormally high RNA and protein content whereas their DNA content remains at the normal premitotic level (unbalanced growth) (Heinen and Bassler, 1976). Studies performed *in vitro* using cisplatin in animal and human tumors revealed G2-blocks following transient accumulation of cells in S-phase after cisplatin administration (Kopf-Maier *et al.*, 1983). Jackel and Kopf-Maier (1991) have suggested that the hindrance of cell traversal through the S-phase obviously represents the main and significant cytokinetic event which indicates a potent antitumor effect of cisplatin that leads to pronounced tumor regression and the duration and magnitude of the G1/S

block seem to indicate the strength of the cytotoxic action. Studies using DNA electrophoresis have revealed that DNA synthesis is blocked at nucleotides at which platinum binds to nucleotides (Heyden et al., 1980). Moreover, Harder et al., (1976) demonstrated a reduced affinity of human DNA polymerases for a platinated templet and suggested that the observed inhibition of DNA synthesis is unlikely to be the consequence of platinum linkage to enzymes. This hypothesis has been supported by other findings which confirm that DNA is the most important macromolecular target for intracellular platinum binding (Pascoe and Roberts, 1974). The interaction of cisplatin with DNA involving various interstrand-, intra-strand- and DNA-protein cross-links has already been reported (reviewed in Pinto and Lippard, 1985).

Although DNA damage in tumor cells is caused by intra-strand cross-links of cisplatin with purine N7 at d(GpC) and d(ApG) sequences and thought to represent the main route leading to inhibition of tumor cell proliferation (reviewed in Johnson et al., 1989); evidences are growing in favour of other biochemical targets besides DNA which can contribute to cell growth inhibition and cytoxicity by cisplatin. Several observations like – (i) minimal fractions of the overall incorporated platinum are detected in coordination with DNA (Pooly et al., 1984) and (ii) because of repair, inhibition

of DNA synthesis should be transient and thus, may not be as critical for cisplatin-induced cytotoxicity as hitherto assumed (Sorenson and Eastman, 1988), seem to support this hypothesis. Among other evidences in favour of this hypothesis are – (iii) attempts to correlate the amount of cisplatin required for inhibition of DNA synthesis _in vitro_ with the concentration needed for inhibition of cell growth are indeed unsuccessful (Maquet _et al._, 1984), (iv) amino acid transport is impaired by cisplatin and certain cisplatin resistant cell lines display alterations in their plasmamembrane transport systems (Shionoya _et al._, 1986), (v) other alkylating agents that share common features with cisplatin in their mechanism of action, involve multiple cellular targets apart from DNA (Grunike _et al._, 1983), (vi) cisplatin at lower doses inhibits cell division while synthesis of DNA and protein continues at almost unperturbed and balanced rates leading to the appearance of giant cells in P388/D1 cell lines (Just and Holler, 1989), (vii) purine nucleoside 5'-triphosphates react with cisplatin in cell-free systems at rates as fast as observed for DNA (Just and Holler, 1991), (viii) reactions of cisplatin with several cellular components may be implicated in drug resistance, and such components have been summarized recently (Andrews and Howell, 1990). Just and Holler (1991) have demonstrated that rise in the concentration of cyclic AMP is an early effect (within 6 hr

after cisplatin treatment *in vitro*) and preceded the arrest of cell in the G2-phase of the cell cycle (6-12 hr) (Just and Holler, 1989). Cells with elevated cyclic AMP content show increased sensitivity to cisplatin (Andrews and Howell, 1990, and references therein) suggesting that pleiotypic effects could, at least, sensitize cells for treatment with platinum (II) complexes. Therefore, the function of DNA as the sole target seems unlikely in the face of the rapid responses of the cyclic AMP and Ap^{4}A nucleotides (Just and Holler, 1991), and the cell membrane should be considered as another important target for cisplatin (Tritton and Hickman, 1985).

Apart from the fact that DNA is considered to be the primary target for cisplatin in its cytotoxicity, however, it has also been suggested that a component of the antitumor activity of cisplatin may arise from a host immunological reaction against cisplatin treated cells (Rosenberg, 1980a). Mally *et al*. (1980) reported that cisplatin (but not its trans-isomer) when added directly to a spontaneous cell-mediated cytotoxicity assay by normal human peripheral blood lymphocytes, or treated the cells with cisplatin prior to assay, resulted in a small but significant enhancement of target cell lysis. Conran and Rosenberg (1972) have reported that mice treated with Zymozan (an immunostimulant) display increased ability

to reject tumors that were originally not very sensitive to cisplatin, while those mice receiving hydrocortisone (an immuno-suppressant) did not reject cisplatin-sensitive tumors as well as the control group. In another study, Rosenberg (1980b) demonstrated that cisplatin has a reduced antitumor activity in immunologically depressed (i.e., X-irradiated) mice. These findings, along with the observation that cisplatin treated cells show an increased appearance of surface antigens (Rosenberg, 1971), has led to the hypothesis that cisplatin treatment increases the expression of tumor cell-surface-associated antigens and thereby permits the immunological recognition and subsequent rejection of cisplatin treated cells (Collins and Kao, 1989). Kleinerman et al., (1980a) studied the effect of cisplatin on spontaneous monocyte-mediated cytotoxicity (SMMC) in vitro and demonstrated that a concentration 1,000-fold less than needed to create detactable DNA damage or produce cytotoxic effect in cultured malignant cells, cisplatin stimulated SMMC, a naturally occurring immune function. In another clinical study (Kleinerman et al., 1980b) reported increased SMMC in vivo and postulated that cisplatin and possibly other chemotherapeutic agents may act in vivo in two fashions: the first is a direct tumoricidal action and the second is an enhancement of a naturally occurring immune function causing destruction of tumor by the host's own activated defence system. Similar results have also been

reported for other chemotherapeutic agents such as L-phenylalanine mustard and adriamycin, which also enhance monocyte-mediated killing (Kleinerman and Muchmore, 1981).

Kociba et al. (1970) showed that a single intraperitoneal injection of 2-4 mg cisplatin/Kg given 24 h after the implantation of Dunning leukemia cells was not only sufficient to inhibit the development of Dunning leukemia in these animals but also protected them when those leukemia cells were reimplanted 30 days later. Sarna and Sodhi (1978) described similar effect in mice inoculated with cisplatin-treated fibrosacrcoma cells. Despite 50% tumor cell viability, the treated cells failed to produce any tumor when transplanted into a host animal; and upto 50% of these animals still did not develop tumor after a subsequent inoculation with untreated fibrosarcoma cells. Increasing the dose of cisplatin used to treat the malignant cells prior to inoculation did not improve the subsequent tumor rejection response, but in fact, lessened it. Thus it is unlikely that the immunity derived solely from the injection of dead tumor cells. When dead malignant cells were injected into mice no similar protective response observed (Marx, 1976). They further implied that some 20 days are necessary between cisplatin-treated tumor cell inoculation and untreated tumor cell challenge to obtain maximum rejection of the second inoculum. They

suggested that a time dependent response to first inoculation was necessary to optimize subsequent immunity (Sarna and Sodhi, 1978). Similar results have been obtained in other murine systems (Page *et al*., 1977).

Morphological evidence for immuno stimulation in cisplatin-treated animals also exists. Sodhi and Sarna (1979), Sodhi (1979) reported heavy infiltration of macrophages and a direct close contact between fibrosarcoma cells and macrophages after cisplatin treatment resulting in degeneration of tumor cells. Sodhi and Aggarwal (1974) also noted the appearance of lymphocytes and macrophages in the Sarcoma-180 mass 6 days after cisplatin treatment. Other effective chemotherapy, for example, the bifunctional alkylating agent sarcolysin did not produce a similar effect (Presnov *et al*., 1978).

Sodhi (1976) showed that treatment of Sarcoma-180 by cisplatin *in vivo* induces the release of virus-like particles and dissolves the plasma membrane. It is speculated that during the process of dissolution of plasma membrane many criptic antigenic components of the plasma membrane are exposed or released which in turn induces the specific immune response by the host. The depolymerisation of microfilaments, formation of giant multinucleate cells after cisplatin treatment have also been reported (Sodhi, 1976).

The effect of cisplatin on the plasma membrane and its dissolution in fibrosarcoma cells resulting in the formation of multinucleate cellular bodies has been reported (Sarna, 1979).

It has been suggested that cell surface components may be directly involved in the antigenic expression on tumor cells and enhancement of host's immune system after cisplatin treatment. Sarna and Sodhi (1978) suggested that removal of mucopolysaccharides from the surface of fibrosarcoma cells after cisplatin treatment might be involved in the unmasking of antigenic sites and increased antigenicity of tumor cells resulting in enhanced host's immune response against fibrosarcoma. Sarna (1979) hypothesized that immune enhancement by cisplatin treatment is rendered by a definite effect of cisplatin on the surface of tumor cells, thus, increasing their antigenicity and rendering them more susceptible to the cells of reticulo-endothelial system.

Sodhi *et al*. (1985) have reported that co-cultivation of spleenocytes with cisplatin-treated tumor cells generate cytotoxic spleenocytes, which when injected to normal mice, render them resistant to tumor challenge. Sodhi and Bhatia (1986) studied the effect of cisplatin on macrophage mediated cytotoxicity against Dalton's lymphoma cells and suggested that cisplatin treatment activates the macrophages, resulting in their increased capacity to lyse the target cells *in vitro*.

The release of various chemical mediators like H_2O_2, O^{-2} and interleukin-1 by activated macrophages after cisplatin treatment have been demonstrated suggesting their possible role in the tumor cell killing (Sodhi and Gupta, 1986; Gupta and Sodhi, 1987). Gupta and Sodhi (1988) also have reported the increased production of lysozyme, β-hexosaminidase, plasminogen activator and leucine aminopeptidase by murine macrophages treated with cisplatin and suggested that these substances could have some effects on the cell surface of both target cells and also the surface of macrophages. Based on these observations, they speculated that these may contribute to increase tumor cell lysis by macrophages either by enhanced direct contact and binding to the tumor cells or through the release of extracellular mediators. In another study, Singh and Sodhi (1988) demonstrated specific interaction between cisplatin-treated macrophages and Dalton's lymphoma cells morphologically and showed that macrophages and tumor cells interact with each other through definite cytoplasmic connections. They also reported the transfer of lysosomes from the cytoplasm of cisplatin-treated macrophages to the tumor cell cytoplasm through these cytoplasmic connections.

Bagasra _et_ _al_. (1985) have reported that mice treated with cisplatin mount an enhanced splenic plaque-forming cell response to sheep erythrocytes and pneumococeal polysaccha-

ride *in vitro*. An enhanced responsiveness to tumor cells by cisplatin has also been reported *in vivo* (Kleinerman and Zwelling, 1984) as well as *in vitro* (Schlaefli *et al.*, 1983). Collins and Kao (1989) demonstrated that tumor cells that are normally resistant to lysis by naturally occurring cytotoxic cells show an increased sensitivity to lysis mediated by these cells (both murine spleen cells and human peripheral blood monocytes and lymphocytes) *in vitro* in the presence of cisplatin. Based on their findings they suggested that naturally occurring cytotoxic cell activity may be a host antitumor defense mechanism and whose efficiency is also increased in the presence of cisplatin supporting the hypothesis that there is a host immunologic component involved in the antitumor activity of cisplatin.

V. Cisplatin And Host Toxicities:

Cisplatin is a cancer chemotherapeutic drug used widely against a variety of gynecological malignancies (Holland *et al.*, 1980; Yagoda, 1980). However, its therapeutic efficacy is limited due to significant side effects (Kociba and Sleight, 1971; Zwelling *et al.*, 1979; Hamilton *et al.*, 1989).

Cisplatin-induced neurological impairments is a significant clinical problem in cisplatin therapy. Cisplatin-induced neurological toxycity occurring in patients is limited

in most cases to peripheral neuropathy and ototoxicity (Hamers et al., 1991). However, despite the still unknown pathegonesis of cisplatin-induced neuropathy, some therapeutic options are available. Apart from the aminothiol WR-2721 [S-2-(3-aminophenyl amino) - ethylphosphorotiotic acid, ethiofors] which is believed to protect against cisplatin-induced neuropathy (Glover et al., 1989), the protective effect of an ACTH (4-9) analogue, Org 2766 is reported (Gerritsen van der Hoop et al., 1988).

The mutagenic potential of cisplatin has been demonstrated in bacterial systems and has been attributed to the induction of base-substitution and frameshift mutations (Beck and Brubacker, 1975; Brouwer et al., 1981). Cisplatin-induced chromosomal aberrations (Tandon and Sodhi, 1985) and mutations (Zwelling et al., 1979) in mammalian systems has also been reported.

VI. Cisplatin And Nephrotoxicity:

Development of nephrotoxicity during cisplatin treatment has been shown to be dose related both in animals and human (Madias and Harrington, 1978; Safirstein et al., 1987). The proximal tubules in the distal nephron segments in the kidney are reported to be affected during cisplatin nephrotoxicity (Gonzales-Vitale et al., 1977). Dobyan et al. (1980) have located the lesion in the rat to the S3 segment of the

proximal tubule in the outer stripe of the medulla. Prolonged weekly injections in rats causes tubular atrophy of cortical nephrons, cystic dialation of inner cortical or medullary tubules, and chornic renal failure due to tubulointerstitial nephrititis (Ward and Fauvie, 1976).

Over the years several reports have been published describing the nephrotoxicity of cisplatin. However, the mechanism(s) by which cisplatin causes renal tubular damage is yet to be fully understood. To reduce cisplatin-induced nephrotoxicity, an insight into the pathophysiological mechanism is of great importance and an important goal for future studies concerning cisplatin nephrotoxicity is therefore, to elusidate the mechanism(s) of the toxicity at the cellular level.

Daugaard (1990) reported that cisplatin nephrotoxicity is associated with reduced renal blood flow, the mechanism of which is not known; and a simultaneous severe increase in glomerular filtration rate which might be due to either a reduction in the filtration pressure or filtration coefficient. Phelps et al. (1987) obsrved a rapid decline of both ATP and K^+ ion following cisplatin administration in rabbit renal cortical slice model. They suggested that since ATP production is necessary for the activity of the Na^+ - K^+ - ATPase, the flux of Na^+ and K^+ ions would be disrupted

thereby explaining the decline of intracellular K^+. A change in mitochondrial respiration and calcium accumulation has also been reported to occur following cisplatin administration (Gordon and Gattone, 1986). On the other hand Safirstein et al. (1987) found that when isolated tubule suspensions were used and cisplatin concentrations were comparable to those observed in vivo, neither membrane associated Na^+ - K^+ - ATPase nor the mitochondria seemed to be important early pathogenic targets for cisplatin.

Levi et al. (1980) reported a reduction in free sulfhydrul groups following cisplatin treatment. Therefore, it is suggested that the high cystein - containing intracellular protein, metallothionine (MT), which is known to play an important role in the detoxification of cadmium (Cherian, 1980), may also reduce cisplatin nephrotoxicity. The induction of MT synthesis and binding of platinum to MT following cisplatin treatment into experimental animals has been reported (Litterst et al., 1986). However, Catherine et al. (1990) reported that following cisplatin injection to rats, there were neither any induction of MT synthesis nor binding of platinum with pre-existing MT could be observed. They also reported that pretreatment with Zn did not alter cisplatin-induced enzymuria or renal damage in rats (Catherine et al., 1990).

Glutathione (GSH), the major non-protein thiol present in the cell is reported to play an important role in the metabolism of a number of chemicals (Baggett and Berndt, 1986). Levi *et al*. (1980) observed a decrease in tissue thiol content following cisplatin treatment and suggested that GSH may also play a role in the binding of cisplatin. Litterst *et al*. (1986) reported that depletion of tissue GSH by pre-treatment of rats with dimethyl-maleate (DEM), a GSH depletor, resulted in an increase in the nephrotoxicity of cisplatin as monitored by the level of blood urea nitrogen. However, pretreatment with DL-buthionine-(S,R)-sulfoximine (BSO), another GSH depletor has been reprted to decrease the rise in blood urea nitrogen caused by cisplatin (Mayer *et al*., 1987). It is known that DEM is not a specific inhibitor of GSH synthesis and will reduce the total thiols and protein synthesis in the tissue (Catherine *et al*., 1990).

Among other sulphur containing compounds, sodium thiosulfate (STS) is reported to afford protection against side effects of cisplatin including that of nephrotoxicity (Howell *et al*., 1983). Using a two route chemotherapy model, Iwamoto *et al*. (1985) reported that STS reduced cisplatin toxicities by inactivating biologically active cisplatin in blood. On the contrary, Howell *et al*. (1983) have found that active platinum in blood stream after cisplatin treatment

was not reduced by STS administration. Thus, the therapeutic efficacy of cisplatin against the tumor also demands more clarification when STS is used in combination.

Jones and Basinger (1989) examined eighteen thiols and thioethers, and found that simultaneous administration of several of these with cisplatin as a single injection to rats bearing Walker 256 carcinosarcoma led to significant reduction in the nephrotoxicity with no apparent interference in its antineoplastic action. The most effective compounds in suppression of cisplatin nephrotoxicity were D-, L-methioninate, and N-acetyle-D, L-methionine (Jones and Basinger, 1989). The complex of cisplatin and L-methionine has been reported to have a reduced nephrotoxicity in comparison with cisplatin itself (Ormond et al., 1988). From these studies and those of Xu et al. (1984) who reported that the platinum (II) complex with GSH was itself active against Sarcoma-180 in mice, it seems apparent that probably the cytotoxicity of cisplatin in tumor cells and that of in kidney may be mediated through different mechanisms.

The ability of dithiocarbamate (DDTC) to control nephrotoxicity of intravenously administered cisplatin was first suggested by Borch and coworkers and subsequently investigated its mode of action in considerable details (Borch and Plesants, 1979; Borch et al., 1980). They postulated

that DDTC acts via competitive chelation and removal of platinum coordinated to protein-bound -SH groups of the kidney tubule cells (Borch and Plesant, 1979; Borch et al., 1980). However, Basinger et al. (1989) suggested that part of the renal protection obtained by the use of dithiocarbamates may be due to the shift of platinum excretion to the bile which obviates additional renal exposure to platinum. But, Reznik et al. (1991) using two hydroxyl containing dithiocarbamates could not find any correlation between renal platinum levels and the degree of renal damage.

The involvement of lipid peroxidation in cisplatin nephrotoxicity has been reported by various authors (Hannemann and Bauman, 1988; Zhong et al., 1990). Sugihara et al. (1987) suggested that cisplatin directly affects renal tissue and generates free radicals which may interact with membrane lipids causing the production of lipid peroxides that damage membrane functions. Superoxide dismutase and other antioxidants are reported to ameliorate cisplatin-induced nephrotoxicity in experimental animals (McGinness et al., 1978; Sugihara and Gemba, 1986).

Despite the numerous studies that have been reported so far, the mechanism of cisplatin-induced nephrotoxicity is yet to be fully understood. However, from the literature already cited in this section it seems reasonable to expect

that cisplatin-induced renal damage could probably be associated with formation of free radicals or peroxides and oxidative stress. And any method to control the adverse effects of cisplatin could only be advantageous if that do not result in the reduction of the antineoplastic activity of cisplatin, or allow the administration of a dose of cisplatin which will provide an antitumor effect equal or superior to that possible with cisplatin alone.

In the present study the effect of vitamin C (an antioxidant and a vitamin) on the anticancer activity and toxicities of cisplatin are examined.

VII. Vitamin C as a Chemotherapeutic Agent:

Vitamin C (L, 3-ketothreohexuronic acid lactone) is commonly referred to as ascorbic acid (L-ascorbic acid). Ascorbic acid is a one-electron carrier and acts as a reducing agent. In the presence of oxygen and iron, ascorbate catalyzes the hydroxylation of a variety of compounds. Vitamin C has multiple functions. It can behave as a reducing agent as well as a prooxidant depending upon the dose used. Vitamin C is also involved in collagen biosynthesis, cytochrome P-450 dependent hydroxylase activities, maintenance of polysomes, stimulation of chemotaxis, phagocytosis, protection against infection, detoxification processes, stimulation of the immune system, wound healing, prevention of thiol group oxidation etc. (Kallistratos and Faske, 1983).

Evidences continue to accumulate that vitamin C has numerous biological effects including some that may relate to prevention and treatment of cancer (Benedict et al., 1983; Kao et al., 1993). Since oxidation and free radicals are associated with carcinogenesis, the free radical scavenger and antioxidant properties of vitamin C holds promising prospect in cancer prevention and treatment.

Patients with malignant diseases have been reported to have low levels of tissue vitamin C (Krasner and Dymock, 1974). In experimental animals also Ghosh and Das (1985) reported that vitamin C levels were lower in mice with Sarcoma -180, Dalton's ascites lymphoma and Schwartz lymphoblastic leukemia than that of normal controls. Epidemiological studies revealed that vitamin C may modify cancer risk (Gey et al., 1987). In another study, Cook-Mozaffari (1979) reported an inverse relationship between oesophageal cancer and consumption of fresh fruits and calculated intake of vitamin C. An inverse relationship between consumption of vitamin C in fresh fruits and gastric cancer incidence has also been reported (Kolonel et al., 1981).

Several reports have been published describing the growth inhibitory effect of vitamin C on tumor cells in experimental animals. Logue and Frommer (1980) reported that vitamin C inhibited 1,2-dimethyl-hydrazine (DMH) - initiated

colon carcinogenesis. Inhibition of metaplastic, hyperplastic and neoplastic lesions in mice exposed to fibreglass dust (Morrison et al., 1981), pulmonary tumor development in amines, amides and nitrite fed mice (Mirvish et al., 1975), estrogen-induced renal carcinoma in Syrian hamsters (Liehr and Wheeler, 1983), ethylurea- and nitrite-induced tumors of the peripheral nervous system in the offsprings of pregnant hamsters (Rustia, 1975), benzo (a) pyrene (BP) - induced sarcoma in rats (Kallistratos and Fasske, 1980) as well as in rats with transplanted fibrosarcoma cells (Kallistratos and Fasske, 1983) by vitamin C has also been reported.

Pauling et al. (1985) reported that large quantities of dietary vitamin C decreased the incidence of and delayed the first appearance of spontaneous mammary tumors in RIII/Imr mice. When hairless mice were irradiated with ultraviolate light, dietary vitamin C delayed the formation of skin lesions, and lesions in mice fed with vitamin C were consistently smaller than those of the controls (Dunham et al., 1982). Inhibitory effect of vitamin C on solid sarcoma-180 in Swiss mice (Chakrabarti and Dasgupta, 1984), Ehrlich ascites carcinoma in Swiss or CF1 mice (Tewfic et al., 1982) and mammary carcinoma in Balb/Cf/Had/Se mice (Liotti and Tolesa, 1982) has also been reported.

Contrary to these observations, results of some animal studies indicate that vitamin C may increase tumor growth. Liotti *et al.* (1983) reported that administration of vitamin C potentiated the growth of transplanted solid tumors in mice. In another study, Pauling *et al.* (1985) reported that although vitamin C at high doses inhibited tumor growth in mice; but low doses accelerated tumor growth. In contrast, Migliozzi (1977) found that when tumor was induced in gunea pigs by injection of 20-methylcholanthrene, tumor regression occurred in animals maintained on a small amount of vitamin C (0.3 mg/kg bw/day), but tumors in animals receiving a very large amount of vitamin C (1 g/kg ow/day) grew without any sign of retardation of gwoth. Banic (1981) also reported that high dietary levels of vitamin C enhanced the appearance of MCA-induced sarcomas in gunea pigs.

Evidences suggest that vitamin C can inhibit tumors produced by nitrosamine in animals through inhibition of nitrosamine formation (Chen *et al.*, 1988). However, the extent of its effect against different cancers is quite variable because of various carcinogen induced cancers, various doses, different routes of administration and different species of animals (Migliozzi, 1977). Apart from that, large doses of carcinogens may owerwhelm the effect of vitamin C. In addition, the use of commercial diets containing potential

carcinogens and anticarcinogens may give different results when compared to studies using purified diets.

In *in vitro* studies, vitamin C has been shown to reduce the mutagenic actions of carcinogenic nitroso compounds (Guttenplan, 1977). Weishberger *et al.* (1980) reported that the formation of mutagens could be completely blocked by addition of vitamin C to reaction mixtures of Sanam fish extracts and nitrite in the *Salmonella typhimurium* assay. Benedict *et al.* (1982) reported that when vitamin C is added to culture medium, the transformation of C3H10T1/2 mouse embryo cells induced by exposure to MCA is inhibited. They found that vitamin C was effective even when it was added as late as 23 days after the MCA treatment. It was also possible under certain circumstances to add vitamin C to the culture medium and causes reversion of chemically transformed cells to normal-appearing morphological phenotypes.

Prasad *et al.* (1979) reported that sodium ascorbate potentiated the growth inhibitory effect of certain agents like 5-fluorouracil on cultured neuroblastoma cells. It has been reported that when vitamin C is given immediately preceding radiation, a significant reduction in the toxic effects of radiation in both skin and bonemarrow could be observed which, however, do not protect the tumor, thus resulting in a therapeutic gain (Henson *et al.*, 1991). Significant

reduction in doxorubicin-induced toxicity and prolongation of survival time in animals receiving vitamin C and its derivatives has also been reported. Experimentally, vitamin C prevented the elevation of lipid peroxide levels found in the heart following administration of doxorubicin (Henson *et al*., 1991).

From the available reports so far, it can be said that the chemopreventive and antineoplastic property of vitamin C is relatively more consistent in *in vitro* systems than in *in vivo* systems. This may be due to the fact that the number of other variables in the *in vitro* systems are far less than that of the *in vivo* systems. In addition, these studies also indicate a possible role of vitamin C as a chemo-adjuvant and hence may have therapeutic importance in combination chemotherapy of neoplastic diseases.

The mechanism of the observed effects of vitamin C is very poorly understood. Individual researchers propose various possible mechanisms depending upon the specific observations, but none of them is totally convinishing. Using two cultured tumor cell lines, i.e., Hep-2 and KB, Bishnu *et al*. (1978) found that vitamin C decreased the rate of DNA synthesis. Shoyab (1981) observed that vitamin C significantly reduced binding of DMBA to DNA in cultured murine epidermal cells and suggested that the antitumorigenic effect

of vitamin C may be related to its ability to inhibit the binding of the active metabolite of the carcinogen to cellular DNA. Studies of Tsao *et al.* (1992) on human mammary xenografts in mice have revealed that ascorbic acid, dehydroascorbic acid, the oxidation product of ascorbic acid and from among twelve other ascorbic acid derivatives tested, six of them had antitumor activity. They also found that D-isoascorbic acid, an isomer of ascorbic acid with 5% of the antiscorbutic potency and very high turnover rate, is similar to that of ascorbate, and they suggested that the anticancer activity of ascorbic acid is not due to metabolism of ascorbic acid as a vitamin, rather due to its chemical properties (Tsao *et al.*, 1992).

Vitamin C acts as a reducing compound in the aquous medium of the cell. As an antioxidant, vitamin C may also have potential anticancer and chemopreventive activity. It has been reported that vitamin C protects plasma lipids from oxidative damage. Of all the agents tested including protein thiols, bilirubin, uric acid, betacarotene and α-tocopherol, vitamin C was the most effective (Henson *et al.*, 1991). It has also been reported that ascorbate is the only antioxidant in the plasma that completely protects lipids from oxidation, and also prevents oxidative damage to cell membranes induced by aquous radicals (Henson *et al.*, 1991). Tocopherol and

ubiquinol are the primary scavengers of radicals within lipid membranes. Parker et al. (1979) reported that in the presence of vitamin C, tocopherol is regenerated from the oxidized form which in turn can continue to scavenge free radicals within membranes. Apart from that, stimulation of the cellular immune system by vitamin C is well documented (reviewed in Anderson, 1984).

In the present study, combination chemotherapy of cisplatin and vitamin C were undertaken to evaluate the effectiveness of subtherapeutical doses of cisplatin and vitamin C on Dalton's lymphoma in vivo in an attempt to evolve a nontoxic chemotherapy against cancers without losing the therapeutic efficacy of cisplatin. Apart from that, since the major side effect of cisplatin is severe nephrotoxicity, which however is probably due to the oxidative damage caused by cisplatin (Sugihara et al., 1987; Nakano and Gemba, 1989), in the present study the effect of vitamin C on cisplatin-induced nephrotoxicity was also studied.

In view of the above reports mentioned in various sections so far on the effects of cisplatin and vitamin C, the present cellular and biochemical studies on tumor as well as normal cells/tissues in vivo/in vitro were undertaken with an idea to understand:

i) the antitumor effect of cisplatin against murine ascites Dalton's lymphoma *in vivo*,

ii) the effect of cisplatin on the changes in some enzyme activities in normal and tumor bearing animals thereby showing their importance in metabolism,

iii) the possible mechanism(s) of cisplatin-induced nephrotoxicity, and

iv) the possible use of cisplatin in combination chemotherapy with reduced host toxicities.

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MATERIALS AND METHODS

1) ANIMALS AND TUMOR

Swiss albino mice in the age group of 8-10 weeks weighing 20-22 g of both sexes were used for the experiments. The animals were maintained in a closely inbred colony under conventional laboratory conditions. Four to five mice are usually kept in a single polypropylene cage with food (food pellets from Hindustan Live Ltd., New Delhi, India) and water provided *ad libitum*.

The tumor, ascites Dalton's lymphoma originally obtained from Gauhati University, India, is being maintained in Swiss albino mice for the last 5 years in the laboratory by srial intraperitoneal (i.p.) transplantations. After the transplantation of 1 x 10^7 tumor cells (0.25 ml vol., in phosphate buffered saline, PBS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4) the animals usually survive for 20-24 days.

2) CHEMICALS

Cisplatin was a gift from Prof. Litterst of N.I.H., U.S.A. L-asorbic acid (vitamin C) was purchased from HiMedia Laboratories, Bombay, India. Glucose-6-phosphate, L-arginine, diacetyl monoxime, NADH, nitroblue tetrazolium, sodium lactate and polyacrylamide were purchased from Sisco Research Laboratories, Bombay, India. Concanavalin A (Con A), concanavalin A-fluoresceinisothiocynate (Con A-FITC), phenazine methosulfate, bis-acrylamide and commercial diagnostic kits were obtained from 'Sigma' Chemical Company, U.S.A. Glutaraldehyde and sodium cacodylate were purchased from Agar Scientific Ltd., U.K. However, some specific chemicals obtained from other sources have been mentioned accordingly. All other chemicals used were of analytical grade. Various buffers, reagents, stains and solutions were always prepared in double glass distilled water.

During experiments cisplatin was suspended in 0.89% NaCl and mixed thoroughly in dark 10-15 min prior to use.

3) METHODOLOGY

A) Antitumor Studies

i) Tumor growth pattern

The mice were transplanted i.p. with ascites Dalton's lymphoma (1×10^7 tumor cells per animal in PBS, pH 7.4). The animals were randomly divided into three groups consist-

ing of 16 mice in each group. Starting from the 4th day through to the 22nd day post-tumor transplantation, one host mouse from each group was killed on every second day (i.e. on 6th, 8th, 10th, ... days) by cervical dislocation. The tumor was collected and the volume measured. Then the collected tumor was centrifuged at 2000 r.p.m. for 5 min at 4° C and the packed cell volume (ascites fluid supernatant : tumor cell pellet, v:v) was determined. On the 10th day post-tumor transplantation, 6 mice from each group were injected (i.p.) with cisplatin (8 mg/kg body weight) and the total tumor volume and its packed cell volume were calculated for each day. A point in the graph is the mean of three groups of experiments repeated twice.

Ascites fluid supernatants, tumor cells and serum collected from both controls and cisplatin treated animals were used for various biochemical estimations as well as light and scanning electron microscopical studies. Metaphase chromosome preparations from the tumor cells were also made following the air-drying technique as given in section 3.C.v.a.

ii) Protein estimation

Protein was estimated following the method of Lowry et al. (1951) in the tumor supernatants.

To 0.5 ml of the tissue sample (after appropriate dilutions) taken in duplicate, 2.5 ml of alkaline solution

[50 ml of 20 g/l Na_2CO_3 in 0.1 ml/l NaOH and 1 ml of copper sulphate-sodium potassium tartrate solution (5 g/l $CuSO_4$. $5H_2O$ in 10 g/l Na, K tartrate) prepared immediately before use] was added, mixed thoroughly and allowed to stand at room temperature for 10 min. Then 0.25 ml diluted (1:1, v/v) Folin-Ciocalteau reagent was added rapidly with immediate mixing. The solutions were kept at room temperature for 30 min and the optical density was measured at 750 nm against the appropriate blank. The protein concentrations were determined from a standard curve prepared in the same way by taking known concentrations of bovin serum albumin (fraction V).

iii) Carbohydrate estimation

Carbohydrate was estimated by anthrone reagent method as described by Plummer (1978a) in the tumor supernatants.

To 0.5 ml of the sample (after appropriate dilutions wherever necessary) taken in duplicate, 2 ml of anthrone reagent (2 g/l in concentrated H_2SO_4) was added and mixed rapidly. The tubes containing the mixtures were kept in a boiling water bath for 10 min with a marble on the top of each tube to prevent loss of water by evaporation. Then the tubes were taken out, cooled at room temperature and the optical density was measured at 620 nm against a reagent blank prepared simultaneously. the carbohydrate concentra-

tions were determined from a standard curve prepared similarly.

iv) Glucose estimation

Glucose was estimated in the tumor supernatans and blood sera using a dignostic kit obtained from 'Sigma' Chemical Company, U.S.A. The Sigma procedure followed the method of Trinder (1969) with some modification.

A series of test tubes were labelled as reagent blank, standard and sample in duplicates. To each tube 3.0 ml of glucose (Trinder) reagent was added and warmed to 37° C in a water bath. The glucose (Trinder) reagent contained 4-aminoantipyrine (0.4 mmol/l), p-hydroxybenzene sulfonate (20 mmol/l), glucose oxidase (15,000 u/l), horseradish peroxidase (10,000 u/l), and the solution was buffered at pH 7.0. The spectrophotometer was set at 505 nm and adjusted the absorbance reading was adjusted to zero with water as reference. Then at timed intervals, 10 µl of deionized water, standard and samples were added to appropriately labelled test tubes and mixed by gentle inversion. Each tube was incubated for exactly 10 min at 37° C. The absorbance (A) of all tubes were read and recorded at 505 nm. The change in absorbance (ΔA) was calculated by substracting the absorbance of blank from absorbance of standard and samples respectively. The glucose concentrations of samples

were calculated from the following equation:

Glucose concentration (mg/dl)

$$= \frac{\text{A sample} - \text{A blank}}{\text{A standard} - \text{A blank}} \times \text{Concentration of standard}$$

v) Sialic acid estimation

Sialic acid was estimated in the tumor cells following the method of Warren (1959).

Ascites Dalton's lymphoma tumor cells were collected from the tumor bearing hosts following cisplatin treatment after different intervals of time and centrifuged at 3000 r.p.m. for 15 min at 4° C. The supernatant was separated, the tumor cells were weighed and a 5% tissue homogenate in 0.1 N H_2SO_4 was prepared. The homogenates were then incubated for 1 hr in a water bath maintained at 80° C so as to release the bound sialic acid. The homogenates were then centrifuged at 8000 r.p.m. for 15 min and the clear supernatants were used for sialic acid estimation.

To 0.2 ml of the supernatant taken in duplicate, 0.1 ml of periodate solution (sodium periodate, 0.2 M in 9 M phosphoric acid) was added, mixed and allowed to stand at room temperature for 20 min. One ml of arsenite solution (sodium arsenite, 10% in a solution of 0.5 M sodium sulfate in 0.1 N H_2SO_4 was added to each tube and mixed until a

yellow-brown color disappeared. Then 3 ml of thiobarbituric acid (0.6% in 0.5 M sodium sulfate) was added, mixed thoroughly, capped with glass beads and heated in a vigorously boiling water bath for 15 min. The tubes were removed from the water bath and rapidly cooled in cold water. Then the chromophore was extracted in an equal volum (4.3 ml) of cyclohexanone by vigorously shaking and then centrifuging at 2000 r.p.m. for 10 min. The clear upper cyclohexanone phase containing the chromophores was removed carefully to another set of tubes without disturbing the lower aqueous phase. The optical density (O.D.) was read and recorded at 532 nm and 549 nm against a reagent blank.

The sialic acid concentrations were determined from the extinction coefficient using the following formula:

$$\text{Sialic acid (in } \mu\text{moles)} = 0.090 \times O.D._{549} - 0.033 \times O.D._{532}$$

vi) Light microscopy

On the 10th day post-tumor transplantation, host mice were treated (i.p.) with cisplatin (8 mg/kg body weight) or same volume of normal saline (controls). After 8 hr, 1, 2, 3 and 4 days of cisplatin treatment, the mice were sacrificed by cervical dislocation and ascites tumore was collected, centrifuged at 1000 r.p.m. for 5 min at 4° C and washed once with phosphate buffered saline (PBS, 0.15 M

NaCl, 0.01 M sodium phosphate buffer, pH 7.4). The cell pellet was suspended in PBS (1:4, v/v). A drop of the cell suspension was taken on a clean grease-free microscope slide and a thin smear was made with the help of another slide. The smear was air dried, fixed in absolute methanol for 15 min and stained with Leishman's stain in the following day.

The percentage ratio of leukocytes (neutrophils, eosinophils, monocytes, lymphocytes) to tumor cells was determined for each batch of mice. The morphological changes in the tumor cells as well as their association with leukocytes following cisplatin treatment were also noted and photographed.

vii) Scanning electron microscopy

The same cell suspensions from each batch (those used for light microscopic preparations) were used for scanning electron microscopical studies.

A drop of the cell suspension was taken on a clean glass stub (approximately 18 mm x 18 mm size) and allowed to stand for 5-10 min allowing the cells to settle down. Then the cells were fixed in 2.5% glutaraldehyde (prepared in 0.1 M sodium cacodylate buffer) for 30 min at 4° C. After fixation, the cells were washed in cacodylate buffer twice

for 10 min each dipping the whole stub containing the cells in the buffer in a cavity block. Then in a similar manner the cells were dehydrated with an ascending grade of acetone (30% - 50% - 70% - 80% - 90% - 95%, two changes of 10 min each) at room temperature and finally kept in dry acetone (prepared by adding $CuSO_4$ in excess to absolute acetone and filtering). The cells were then dried by critical point drying (CPD) method substituting dry acetone from the cells by carbondioxide. The cells were then coated with gold in a fine coat ion sputter, J.F.C. 1100 by mounting the glass stub containing the cells on a brass stub (32 mm diameter x 20 mm height) with electro-conducting paint. The cells were then thoroughly examined and photographed under Jeol scanning electron microscope operated at 12 KV and the working distance of 30 mm.

viii) Rate of glucose consumption

Ascites Dalton's lymphoma cells in the log phase of growth were collected from tumor bearing hosts and centrifuged at 1000 r.p.m. for 3 min. The cells were once washed with Dulbeco's modified Eagle tissue culture medium (containing 1 g/l glucose and supplemented with 1% BSA) and resuspended in fresh medium. The cell count was determined in a hemocytometer and the cell suspension was diluted to 2 x 10^6 cells/ml. A series of sterile tissue culture tubes were

labelled appropriately and 2 ml of the cell suspension was pipetted out to each tube under sterile conditions. Then to each of the tubes (except for controls) cisplatin was added (in a total volume of 50 µl) to a final concentration of 25 µg/ml of the cell suspension. The tubes were incubated in an incubator at 37° C with intermittent shaking. After every 30 min interval till the first 2 hr and then at 3 and 6 hr of treatment, one tube was removed from the incubator, centrifuged quickly at 1000 r.p.m. for 3 min, the cells were resuspended in fresh medium in a total volume of 2 ml and incubated for another 30 min. After the second incubation, the cells were quickly separated by centrifugation at 2000 r.p.m. for 5 min at 4° C and the supernatants were kept frozen.

At the end of the experiment, the cell supernatants were used for glucose estimation. Glucose was estimated using a diagnostic kit obtained from 'Sigma' Chemical Company, U.S.A. as described in section 3.A.iv. From the amounts of glucose present in the cell supernatants, the amounts consumed were calculated and expressed as a percentage of the control.

The cell viability checked at various times during the experiment by trypan blue exclusion test was always above 85%. The experiment was repeated thrice without much variations in the results.

ix) Measurement of oxygen consumption

Oxygen consumption by ascites Dalton's lymphoma cells following cisplatin treatment and control conditions was measured polarographically using Clark type oxygen sensors (Rank Brothers, Cambridge, U.K.) in water jacketed, continuously stirred chambers. Temperature in the sample chamber was regulated by a continuous flow water bath.

The tumor bearing hosts were sacrificed following 1, 2, 3 and 4 days of cisplatin treatment (8 mg/kg body weight on the 10th day post-tumor transplantation). The tumor was collected, centrifuged at 1000 r.p.m. for 3 min and the cells were suspended in Krebs-Ringer solution discarding the supernatants. The Krebs-Ringer solution was prepared by mixing 0.9% NaCl (100 ml), 1.15% KCl (4 ml), 1.22% $CaCl_2$ (3 ml), 2.11% KH_2PO_4 (1 ml), 3.82% $MgSO_4.7H_2O$ (1 ml), and 0.1 M phosphate buffer pH 7.4 (20 ml). The solution was gassed with oxygen for 10 min after mixing. The cell count was determined by hemocytometer and the cell suspension was diluted to 2 x 10^6 cells/ml.

The oxygen sensors were calibrated with oxygen saturated Krebs-Ringer solution at 37° C. Oxygen measurements were carried out on 3.0 ml volumes of Krebs-Ringer solution containing 2 x 10^6 tumor cells/ml. Oxygen consumption was continuously monitored and recorded. Oxygen consumption

per unit of time was calculated using the values of oxygen solubility given by Lessler (1982). The results are presented as a percentage of control.

x) Measurement of Ca^{2+} and K^{+} concentration

Calcium and potassium contents were determined by flameless atomic absorption spectrophotmetry (FAAS). The tumor bearing animals were treated with cisplatin (8 mg/kg body weight) on the 10th day post-tumor transplantation. After 4 hr, 12 hr and 1, 2, 3 and 4 days following cisplatin treatment, the animals were sacrificed by draining out of blood through the post-orbital vein. Controls having no tumor and tumor bearing hosts without any treatment were also taken simultaneously. The tissues were collected, minced into small pieces and kept in separate pre-cleaned and pre-weighed conical flasks. The tissues oven-dried over a period of 5 days at 50° C. Then the tissues were weighed and wet ashed for 24 hr at 40° C in a mixture of concentrated niric acid and perchloric acid (3:1, v/v) in a volume just enough to immerse the tissues in it. After digestion a clear solution was resulted. Then the samples were diluted with distilled and deionized water to a final volume keeping the acidity of approximately 10%. The samples were filtered and stored in polypropylene bottles. The amount of calcium and potassium was determined in a flameless atomic absorption

spectrophotometer after calibrating the instrument with the appropriate standard solutions.

B) Enzymatic Studies

i) Glucose-6-phosphatase assay

Glucose-6-phosphatase activity in tissues was assayed following the method of Plummer (1978b).

A reaction mixture containing 0.5 ml of cacodylate buffer (sodium cacodylate, 0.1 mol/l, pH 6.5), 0.1 ml EDTA (10 mmol/l buffered at pH 6.5) and 0.2 ml glucose-6-phosphate (50 mmol/l in buffer, pH 6.5) was incubated at 37° C for 10 min. To the preincubated reaction mixture, 0.2 ml of a 10% tissue homogenate was added and incubated for another 15 min at 37° C. The reaction was terminated by adding 1 ml of ice-cold 10% TCA. The resulting mixture was centrifuged after 10 min at 3000 r.p.m. for 10 min. The precipitate was discarded and the supernatant was used for free phosphate estimation following the method of Fiske and subbarow (1925) as follows:

To 0.25 ml of the supernatants taken in duplicate in test tubes, 2.5 ml distilled water, 0.5 ml of 5 N H_2SO_4, 0.5 ml ammonium molybdate (2.5%) solution and 0.25 ml of Fiske and Subbarow reducer (obtained from 'Sigma' Chemical Company, U.S.A.) was added and mixed thoroughly after each

addition. After 30 min, the optical density was measured at 660 nm in a spectrophotometer. The amount of inorganic phosphorus was determined from a standard curve prepared similarly taking know concentrations of KH_2PO_4 as standard.

The substrate and enzyme blanks were prepared simultaneously. The enzyme activity was calculated in units per minute per gram of itssue. One unit of the enzyme activitiy has been defined as the amount of enzyme necessary to catalyze the conversion of one micromole substrate per minute at 37° C and pH 6.5.

ii) Lactate dehydrogenase (LDH) assay

a) Quantitative determination of LDH

Lactate dehydrogenase (LDH) activity was measured in tissues using diagnostic enzyme determination kits obtained from 'Sigma' Chemical Company, U.S.A. following the method of Cabaud and Wroblewski (1958).

To 1 ml of pyruvate substrate (0.75 mmol/l, pH 7.5) contianing NADH (1 mg/ml), 0.1 ml of a six fold diluted serum or tumor supernatant (or 0.1 ml of a 10% homogenate in case of tumour cells, liver or kidney tissues) was added. The mixture was mixed gently and incubated exactly for 30 min at 37° C. Then 1 ml of 'Sigma color reagent' (2,4-dinitrophenylhydrazine, 20 mg/dl in 1 N HCl) was added, mixed by swir-

ling (stops reaction and starts color development) and allowed to stand at room temperature. Twenty minutes later, 10 ml of 0.4 N sodium hydroxide solution was added and mixed properly. After at least 5 min, but not more than 30 min, the absorbance was measured at 525 nm in a spectrophotometer. The change in absorbance in various treated groups was expressed as a percentage of control.

b) Electrophoretic analysis of LDH-isozymes

LDH isozyme analysis was done electrophoretically following the guidelines suggested by Rosalki (1974). LDH isozymes were separated by electrophoresis on polyacrylamide gels following the method of Davis (1964). The areas of enzyme activity were then visualized by histochemical staining.

Preparation of gels

Prior to use the gel tubes (0.5 cm x 7 cm) were washed with a detergent solution, rinsed with distilled water and finally dried in an oven. The tubes were kept vertically in a gel-casting stand with their lower ends tightly closed with rubber stoppers. Then 2 ml of small-pore gel containing 7% acrylamide, 0.18% N, N-methylene bis acrylamide, 0.06% N, N, N', N'-tetramethyle ethylene diamine (TEMED) and 0.08% ammonium persulphate was added to each tube. The surface of the small-pore gel was covered

with a few drops of water. After 30 min of polymerization the water layer was removed and the gel tubes were rinsed with the large-pore gel containing 2.5% acrylamide, 0.62% N, N-methylene bis acrylamide, 0.12% TEMED, 0.001% riboflavin and 20% sucrose. About 0.2 ml of this solution was transferred to each gel tube and the surface was covered with a few drops of water. The photopolymerization of the large-pore gel was effected by placing the tubes under a mercury lamp. After polymerization, the water layer was removed and the gel tubes were placed in the electrophoretic tank in such a way that the large-pore containing ends were in the upper chamber. Tris-glycine buffer, pH 8.2, I = 0.02, was added to the two chambers of the apparatus such that the lower ends of the gel tubes made contact with the buffer while the upper ends were not completely dipped in the buffer.

Sample preparation and loading

The same tissue samples used for colorimetric determination of LDH enzyme activity were used for electrophoretic analysis. 50 µl of the tissue sample was mixed with a small amount of glycerol and applied on the surface of the large-pore gel with the help of a syringe. Utmost care was taken so as not to disturb the gels.

Electrophoretic run

After the samples are loaded, few drops of the marker

dye bromophenol blue solution (0.01%) were added to the upper chamber and then the buffer was added in a volume such that the gel tubes were completely immersed in the buffer. Then electrophoresis was carried with an anodic current of 1.5 mA/tube for 15 min. Then the current was raised upto 3 mA/tube. The electrophoresis was continued for about 2.5 hr at 4° C till the bromophenol blue front migrated to the extreme bottom of the tubes. Then the instrument was switched off and the gel tubes were taken out of the electrophoretic tank.

Staining of the gels for LDH

At the end of electrophoresis the gels were carefully removed from the gel tubes and stained for LDH as follows:

The gels were kept in pre-labelled test tubes individually and LDH staining solution was poured into the test tubes so that the gels were completely immersed in it. The LDH staining solution contained 2.5 ml of 1 M tris-HCl buffer (pH 8.3), 0.5 ml of 1 N lithium lactate solution, 80 mg of nicotinamide adenine dinucleotide (NAD), 1.2 mg of phenazine methosulfate (PMS), 0.8 mg of p-nitroblue tetrazolium-chloride (NBT) and 47 ml of double distilled water. The gels were incubated for 15 min at 37° C in an incubator. LDH bands were violet in color. At the end of the incubation period the staining solution was removed and the gels were

rinsed once with fixative (7% glacial acetic acid) and stored in the fixative till photographed.

iii) $Na^+ + K^+$ - ATPase assay

The assay of $Na^+ + K^+$ - ATPase activity in tumor supernatant as well as in the single cell suspension of tumor cells (2×10^6 cells/ml in 0.89% NaCl) was performed following the method of Tamai _et al_. (1978). ATP (type V) and $MgCl_2$ were obtained from 'Sigma' Chemical Company, U.S.A.

To 0.8 ml of cell suspension or tumor supernatant, 0.8 ml of tris-HCl buffer (pH 7.4) containing 4 mmol $MgCl_2$, 10 mmol NaCl, 10 mmol KCl and 6 mmol ATP was added. The blanks omitting tissue samples or substrate (ATP) from the reaction mixture were also prepared. The mixtures were incubated for 30 min at 37° C. The enzymatic reaction was terminated by adding 0.2 ml of 50% TCA at 0° C for 10 min. Precipitates were centrifuged off (at 3500 r.p.m. for 10 min) and the amount of inorganic phosphorus liberated was determined in the clear supernatant by the method of Fiske and Subbarow (1925) as described in section 3.B.i.

The enzyme activity was expressed in units per 100 ml of the tissue sample. One unit of the enzyme activity has been defined as the amount of enzyme necessary to result in the formation of 1 mg of inorganic phosphorus per hour

per 100 ml of the tissue sample at 37° C.

iv) 5'-Nucleotidase assay

5'-nucleotidase (5'-ND) activity was assayed in the tissues using diagnostic enzyme determination kits obtained from 'Sigma' Chemical Company, U.S.A., following the method of Dixson and Purdom (1954).

The substrates used were adenosine phosphate substrate solution (adenosine-5-monophosphate, 250 µmol, buffered at pH 7.5) and glycerophosphate substrate (sodium β-glycerophosphate 160 µmol, buffered at pH 7.5). Tumor supernatant was used without any dilution and other tissues were used as 10% homogenates.

Into four centrigure tubes, labelled TEST A, BLANK A, TEST B and BLANK B, the reagents were pipetted out in the following manner.

	TEST A	BLANK A	TEST B	BLANK B
Adenosine phosphate substrate solution (ml)	4.8	4.8	-	-
Glycerophosphate substrate solution (ml)	-	-	4.8	4.8
Tissue sample (ml)	0.2	-	0.2	-

The solutions were mixed by gentle shaking and incubated for 2.5 hr at 37° C. At the end of the incubation, 1 ml of cold TCA (30%) was added to all tubes to terminate the reaction. Then to BLANK A and BLANK B only, 0.2 ml of tissue sample was added, mixed by inversion and allowed to stand for 10 min in an ice bath. Then all the tubes were centrifuged at 3000 r.p.m. for 10 min to obtain clear supernatans.

To a set of another four cubes labelled similarly, 4 ml of the supernatant was transferred from the corresponding tubes and phosphate was measured following the method of Fiske and Subbarow (1925) as described in section 3.B.i. The absorbance (A) of TEST A vs BLANK A as reference to obtain ΔA of TEST A, and TEST B vs BLANK B as reference to obtain ΔA of TEST B were determined.

ΔA TEST B (non-specific phosphatase activity) was deducted from ΔA of TEST A (apparent 5'-ND activity). The true 5'-ND activity was determined in terms of µg of inorganic phosphorus from the calibration curve by applying the difference in absorbance.

The enzyme activity was calculated in units, and one unity of 5'-ND activity has been defined as the amount of enzyme necessary to result the formation of 1 mg of inorganic phosphorus per hour per 100 ml of the tissue sample at 37° C.

v) Glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) assay

The activities of glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were assayed using diagnostic enzyme determination kits obtained from 'Sigma' Chemical Company, U.S.A., following the method of Reitman and Frankel (1957).

Aspartate- α -ketoglutaric acid (DL-aspartate, 0.2 mol/l, and α-ketoglutaric adic, 1.8 mmol/l, in phosphate buffer, pH 7.5) and alanine-α-ketoglutaric acid (DL-alanine, 0.2 mol/l, and α-kotoglutaric acid, 1.8 mmol/l, in phosphate buffer, pH 7.5) were used as substrates for GOT and GPT respectively.

To 0.5 ml of prewarmed (37° C) substrate solution, 0.1 ml tissue sample was added and incubated for 1 hr at 37° C. Then 0.5 ml 'Sigma' color reagent (2, 4-dinitrophenylhydrazine, approximately 20 mg/dl, in acid solution) was added (stops reaction and starts color development) and left at room temperature after mixing. Twenty minutes later, 5 ml of 0.4 N sodium hydroxide solution was added and mixed by inversion. After 5 min, the absorbance was read and recorded at 505 nm in a spectrophotometer against water as reference. The enzyme activity was calculated from a calibration curve and the enzyme activity was expressed in units/ml.

One unit of the enzyme has been defined as the amount of enzyme necessary to catalyse 1 μmol of substrate per minute at pH 7.5 and 37° C.

vi) Arginase assay

Arginase activity in various tissues was assayed following the method of Brown and Cohen (1959).

Briefly, 0.9 ml of $MnCl_2$ (0.5 μmole) and 0.1 ml of a 10% tissue homogenate (enzyme source) was pre-incubated at 37° C for 10 min. Then 1 ml of L-arginine (50 μmole prepared in tris-glycine buffer, pH 9.5) was added to the reaction mixture and incubated for 15 min at 37° C. The reaction was terminated by adding 1 ml of 10% perchloric acid and the mixture was centrifuged at 2000 r.p.m. for 10 min after 15 min. The supernatant was collected and used for urea estimation following diacetyl monoxime reagent method. The substrate and enzyme blanks, were also prepared simultaneously.

For urea estimation, 0.25 and 0.5 ml supernatants were taken in duplicate and distilled water was added to make the total volume to 1 ml. Then 2.5 ml of acid mixture (30 ml H_3PO_4 + 10 ml conc. H_2SO_4 + 39.8 ml H_2O + 0.2 ml of 0.1 M $FeCl_3$ + 0.0237 g $MnSO_4$) was added to each sample and mixed properly. Then 0.25 ml of diacetyl monoxime (2.5%

in distilled water) was added, mixed and kept in boiling water bath for 10 min. The tubes were cooled to room temperature and optical density was read at 478 nm in a spectrophotometer against the reagent blank. The concentration of urea was calculated from a standard curve prepared similarly by taking known concentrations of urea.

The enzyme activity was calculated in units per gram of tissue, and one unit of enzyme activity has been defined as the amount of enzyme necessary to catalyze the conversion of one micromole of substrate per minute at 37° C.

ii) Cathepsin assay

The activities of cathepsin B and cathepsin H were assayed following the method of Barrett (1981).

The substrates used were benzylarginyl-arginyl napthylamide (BANA) and leucine napthylamide for cathepsin B and cathepsin H respectively. 50 µl of the tissue sample was incubated with 450 µl of 20 mM phosphate buffer (pH = 6.5) containing 2 mM each of β-mercapto ethanol and EDTA, and 500 µl of the (1 mg/ml) at 37° C for 30 min. Then the reaction was stopped by adding 500 µl of 4 NHCl. The product β-napthylamine was estimated by diazotization with N-1-napthylethylenediamine-HCl dye. The optical density was measured at 540 nm in a UV-visible spectrophotometer and

compared between different experimental conditions as a measure of increase/decrease in enzyme activity.

C) Combination Chemotherapeutic Studies With Vitamin C

i) Tumor growth pattern and antitumor studies

1 x 10^7 tumor cells in PBS (phosphate buffered saline, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4) was transplanted (i.p.) into C3H/He mice. The animals were divided into five groups consisting of 10 mice each, according to randomized block design. The day of tumor transplantation was taken as day '0'. Two groups of mice were given vitamin C (0.25% and 0.5%; 4.75 - 5.5 mg and 8.4 - 9.5 mg/day/animal respectively) orally through drinking water from the first day of tumor transplantation and continued thereafter. On the 10th day post-tumor transplantation, the animals received a single i.p. injection of cisplatin (4 mg/kg b.w.). The third group of mice was given only 0.5% vitamin C from the first day till the end of experiment. The fourth group of mice received only cisplatin (4 mg/kg b.w.) on the 10th day. To the fifth group of mice serving as controls were injected with the same amount of 0.89% NaCl and kept on tap water without vitamin C. Animals were kept under standard laboratory conditions.

Animal deaths and body weights were monitored daily.

The antitumor activity was assessed from the mean survival time of the treated and control mice and from the change in average body weight in different groups indicating ascites tumor growth.

In other set of experiments, on the 5th day of tumor transplantation, the same dose of cisplatin was administered i.p. into the mice and vitamin C (0.5%) treatment was started from the 10th day. The host survival was monitored and compared with that of the other treated groups of mice.

Similar studies were also carried out using Swiss albino mice and similar doses of cisplatin (4 mg/kg b.w.) and vitamin C (0.25% and 0.5%; 4.5 - 5.5 mg and 8.5 - 9.5 mg/day/animal respectively) were given. The antitumor efficacy and drug efficacy were evaluated by determining the increase in life span (ILS) and growth inhibition rate (GIR) respectively. The ILS (%) is defined as the percentage $\frac{T}{C} - 1$, where T is the mean survival time of the drug treated group and C is the mean survival time of the control group. The GIR (%) is defined as the percentage $1 - \frac{V}{V_o}$, where V is the mean tumor weight of the drug treated group and V_o is the mean tumor weight of the control group.

The effects of vitamin C plus cisplatin on thymus and spleen weight, tumor pH and total leukocyte count were

also studied. As the combination of 0.5% vitamin C plus cisplatin treatment resulted in maximum host survival, tumor bearing mice were treated with 0.5% vitamin C and cisplatin as described above and on the 20th day of tumor transplantation mice were sacrificed; spleen and thymus from normal, control and treated mice were dissected out, weighed and compared. The pH of ascites tumor from control and treated mice was determined and compared. The total leukocyte count was determined using a hemocytometer. Apart from that the serum ascorbic acid level was also determined following the method of Omaye *et al*. (1979) as described below.

ii) Ascorbic acid estimation

Serum samples collected during various experimental conditions were used for ascorbic acid (vitamin C) estimation following the method of Omaye *et al*. (1979).

Serum was deproteinized by addition of an equal volume if ice-cold 10% metaphosphoric acid (H_3PO_4) and after through mixing, centrifuged for 20 min at 4000 r.p.m. at room temperature. The precipitate was discarded and 0.6 ml aliquot of the supernatant was used as the sample for a single analysis. Then 0.3 ml of citrate-acetate buffer (22 g of trisodium citrate dihydrate was placed in about 40 ml of distilled water, the pH was adjusted to 4.15 with glacial acetic acid, and the total volume is brought to 100 ml with

distilled water) was added to each sample and any turbid sample was centrifuged. From this point onwards the samples were analysed individually. Then 0.3 ml of dichlorophenolindophenol (DCIP, 0.1 mg/ml in distilled water) was added to the sample and the optical density (O.D.) was read and recorded at 520 nm against distilled water as reference exactly after 30 seconds. Then a few crystals of ascorbic acid was added to bleach the dye by reducing it completely, and the O.D. was measured again. This value was used as the blank for the sample. The change in absorbance (ΔA) due to reduction of the dye by ascorbic acid in the sample was calculated using the equation - $\Delta A = (RB-RBb)-(S-Sb)$, where RB is the absorbance of the reagent blank, RBb is the absorbance of the reagent blank after bleaching with ascorbic acid, S is the absorbance of the sample, and Sb is the absorbance of the sample after bleaching with ascorbic acid.

The concentration of ascorbic acid in the sample was determined by comparing ΔA with that of a standard curve prepared in the same way taking standards ranging between 0 and 20 µg of ascorbic acid per milliliter in 5% H_3PO_4.

iii) Agglutination study

a) Preparation of cell suspension

Dalton's lymphoma cells removed from the peritoneal

cavity of the tumor bearing mice were washed with phosphate buffered saline (PBS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4). The cells were then treated with 0.85% NH_4Cl for 15 min, washed again with PBS and resuspended in PBS to have a single cell suspension of these tumor cells. The final cell count was adjusted to 2 x 10^6 cells/ml. The PBS used was always Ca^{2+} and Mg^{2+} free.

b) Agglutination assay

Tumor cell suspension was incubated with cisplatin (25 µg/ml cell suspension), vitamin C (50 µg/ml cell suspension) or vitamin C plus cisplatin in sterile tissue culture tubes for 15, 30, 45 and 60 min at 37° C with gentle intermittent shanking. Controls were incubated without cisplatin or vitamin C. After the appropriate time of incubation 0.5 ml of cell suspension was removed in a shallow concavity slide and mixed with 0.1 ml of con A solution (2 µg/ml in PBS) at room temperature. After 15 min the pattern of cell agglutination for each batch of cells was examined under a light microscope at low magnification. Cell agglutination patterns were expressed by '+' signs. Single '+' was used to express the agglutination of 5 or more cells at a minimum of 5 places.

A point in the graph (Fig.26) showing the degree of agglutination is the mean value of the observations in two concavities of the three sets of experiments.

Cell viability checked with trypan blue exclusion test at different stages of agglutination studies, like the final cell suspension in PBS, after cisplatin, vitamin C or vitamin C plus cisplatin treatment and before agglutination assay was above 85%.

iv) Fluorescence microscopy

The multivalent lectin Con A can be covalently linked to fluorescein isothiocyanate (FITC) which in the conjugated form helps in the direct visualization of the topography of lectin receptor sites on the cell surface under the fluorescence microscope. FITC-conjugated form of Con A (Con A-FITC) was obtained from 'Sigma' Chemical Company, U.S.A.

The same batches of tumor cell suspensions as described above, section 3.C.iii, treated with cisplatin, vitamin C or vitamin C plus cisplatin were used for Con A-FITC labelling. After each incubation time 0.5 ml of cell suspension was removed and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 15 min at room temperature. Then the cells were washed twice with PBS and the final cell pellets were suspended in 0.5 ml of PBS containing Con A-FITC (100 μg/ml) and incubated at room temperature for 30 min. The cells were again centrifuged and washed once with PBS to remove any extra fluorochrome labelled lectins. The cells were finally suspended in 0.2 ml of PBS.

A drop of this cell suspension was taken on a clean slide, sealed with cover glass and wax, observed and photographed under Ziess fluorescence microscope. The intensity as well as pattern of fluorescence was marked and compared for different batches of cells.

v) Mutagenicity studies

a) Chromosome aberration assay

The animals were injected (i.p.) with colchicine (4 mg/kg body weight) 1.5 hr before they are sacrificed. Marrow cells were collected from humerus and femur by flushing in warm (37° C) sodium citrate (1%) solution with a hypodermic syringe, aspirated and incubated at 37°C for 10 min. The material was then centrifuged (1000 r.p.m.) and fixed in acetic acid/methanol (1:3). Centrifugation and fixation were repeated twice with a 30 min interval. Finally, immediately before preparation of slides, the fixed material was again centrifuged and resuspended in a small volume of fixative by gentle flushing until a cloudy suspension resulted. Then 2-3 drops of the suspension were dropped on a clean slide previously chilled in 50% ethanol, burnt on a flame for a while, air-dried, and stained the following day for 1.5 hr in 10% buffered Giensa (pH 7). One hundred good metaphase spreads were examined per animal and chro-

mosome aberrations were classified into the following general categories: breaks of chromosome and chromatid types, gaps of chromosome and chromatid types, exchanges of chromosome and chromatid types and sister chromatid unions. Gaps have not been considered for statistical analysis of the data due to their controversial genetic significance (Preston et al., 1981; WHO criteria 46, 1985).

b) Micronucleus assay

In this assay the animals were sacrificed by cervical dislocation 30 hr after the treatment of cisplatin. Bone marrow smears and staining were done following the method of Schmid (1976). Both the femora were removed and adhering muscles were cleared. Then the epiphyses were cut and the bone marrow was flushed into a centrifuge tube with 1% sodium citrate solution (20° C) from a syringe. The marrow was gently agitated and immediately centrifuged at 1000 r.p.m. for 5 min. The supernatant was decanted off and the precipitate was thoroughly mixed. A drop of the material was smeared on a clean slide, air-dried, fixed in absolute methanol for 15 min and stained the following day in Leishman's stain. Two thousand each of polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs) and immature white cells were scored per animal. The frequency of micronucleated PCEs was the end point used for statistical evaluation.

c) Sperm abnormality assay

In this test, only male mice were used, and the animals were sacrificed 10 days after the cisplatin treatment. Sperm collection was done from caudae epididymes in physiological saline (0.89%). Smears were made on clean slides taking a drop of the material, air-dried, fixed in absolute methanol for 15 min and stained in 0.1% aqueous eosin-Y solution in the following day. Five hundred sperms were scored per animal. Abnormalities were categorized using the criteria as close as possible to those established by Wyrobek and Bruce (1975) with modifications (Bhunya and Pati, 1988). Only the head morphology was examined. Abnormal sperms had forms readily recognizable as triangular, banana-shaped, amorphous, dwarf, hooked and rhomboid types.

vi) Nephrotoxicity studies

Serum uric acid and serum urea levels were used as parameters to assess nephrotoxicity. Blood was collected through the post-orbital vein from the animals under light ether anaesthesia. The blood drawn without any anticoagulant or fixative was allowed to clot and serum was separated by centrifugation. Serum uric acid and serum urea levels were estimated following the method of Henry *et al*. (1957) and Brown and Cohen (1959) respectively as described below.

a) Serum uric acid estimation

The tumor bearing mice were treated with cisplatin (8 mg/kg b.w.) on the 10th day of tumor transplantation. The day of treatment was taken as day 0 and thereafter in every three days intervals serum was collected as described above and used for uric acid estimation. Uric acid was estimated following the method of Henry et al. (1957) using diagnostic kits obtained from 'Sigma' Chemical Company, U.S.A.

One test tube was labelled as TOTAL and one preweighed uricase vial (containing 0.1 unit from Candida utilis; one unit converts 1 μmol of uric acid to allantoin per minute at 25° C, pH 8.5) was labelled as RESIDUAL. To each of the tubes 0.25 ml of serum was added, mixed by lateral shaking and incubated at 25° C for 30 min. Then to each of the tubes, 8.5 ml of distilled water, 0.5 ml of 2/3 N H_2SO_4 and 0.5 ml of sodium tungstate solution (10%, W/V) were added in that order mixing well after each addition. Then the tubes were centrifuged at 3000 r.p.m. for 10 min to obtain protein-free supernatants. To a set of another test tubes labelled RESIDUAL, TOTAL AND BLANK respectively, 4.0 ml of the protein-free supernatants were pipetted out to the correspondingly labelled tubes. To the BLANK tube, 4.0 ml of distilled water was added. Then to all the tubes

1.5 ml of sodium carbonate (10%, W/V) solution and 1.0 ml of phosphotungstate reagent (sodium tungstate, 40 g/l in 85% phosphoric acid) were added in that order after mixing well following each addition. After 15 min, the absorbances of TOTAL and RESIDUAL were read at 700 nm in a spectrophotometer using BLANK as reference. The amount of uric acid was determined in mg/dl from a calibration curve prepared in the similar way. The serum 'true' uric acid was calculated in mg/dl by substracting the RESIDUAL amount from that of the TOTAL amount.

b) Serum urea estimation

The tumor bearing mice were treated with cisplatin (8 mg/kg b.w. or 4 mg/kg b.w. on the 10th day of tumor transplantation). vitamin C (0.5% in drinking water from the first day following tumor transplantation) or vitamin C (0.5 % in drinking water) from the first day and cisplatin (8 mg/kg b.w.) on the 10th day following tumor transplantation. The day of cisplatin treatment was taken as day 0. On the first day and every alternate day thereafter, blood of one mice from each group was collected and serum was separated as described above. The serum was used for urea estimation following the method of Brown and Cohen (1959) as described in section 3.B.vi. Normal and control values were also determined simultaneously.

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RESULTS

1) Antitumor Studies

Tumor growth and cisplatin treatment

A regular increase in ascites tumor volume was noted with time following tumor transplantation which reached about 12 ml by the 18th day of tumor growth (Fig. 3). Between days 18-22 the volume remained almost unchanged until the death of the host mice. Cisplatin (8 mg/kg b.w.) treatment of the hosts on the 10th day post-tumor transplantation, resulted in a significant reduction in tumor volume and by the 6th day following treatment very little (0.5-1 ml) ascites tumor could be collected indicating effective regression of the tumor (Fig. 3).

The value of packed tumor cell volume was about one at the beginning of tumor growth (Fig. 4). There was greater increase in the volume of ascites fluid to tumor cells so that its value reached approximately two during the middle period of tumor growth. However, following cispla-

tin treatment, the ratio of ascites supernatant to cell pellet decreased sharply and reached below 0.5 by the 6th day following cisplatin treatment (Fig. 4) when the tumor was collected in viscous form.

Carbohydrate and protein contents

Measurement of carbohydrate contents (Fig. 5) showed that following 3-4 days of cisplatin (8 mg/kg b.w.) treatment, the carbohydrate contents markedly increased in the ascites fluid of the tumor bearing hosts. However, the protein contents in the ascites fluid decreased by about three fold, following 2-4 days of cisplatin treatment (Fig. 5).

Glucose contents

The measurement of glucose concentrations in serum as well as tumor supernantants (Fig. 6 A,B) showed that tumor bearing animals had comparatively lower serum glucose level than those of the normal animals (Fig. 6A). Following 1-3 days of cisplatin (8 mg/kg b.w.) treatment of the tumor bearing hosts resulted in a significant ($p < 0.001$) increase in the serum glucose level. However, by the 4th day of the treatment, the serum glucose level was found to be similar to that found in the normal animals.

In the tumor supernantants (Fig. 6B) it was found that in the tumor bearing hosts (without any treatment)

very little amount of glucose could be detected. However, cisplatin (8 mg/kg b.w.) treatment of the tumor bearing hosts for 1-4 days, resulted in a progressive increase in the level of glucose in the tumor supernatants. On the 4th day of the treatment the glucose level in the tumor supernatants increased to 0.55 mmol/l which was significantly ($P < 0.001$) higher than the control value.

Rate of glucose consumption *in vitro*

Measurement of the rate of glucose consumption *in vitro* by the tumor cells (Fig. 7) showed that following cisplatin (25 μg/ml of 2×10^6 tumor cells/ml) treatment resulted in an increase in the rate of glucose consumption upto 1 hr of the treatment. However, the rate of glucose consumption decreased thereafter following 2-6 hr of the treatment. And following 6 hr of cisplatin treatment, about 40% decrease in the rate of glucose consumption *in vitro* by the tumor cells could be noted.

Metaphase chromosome analysis

Metaphase chromosome preparations of Dalton's lymphoma (DL) cells (Table 1) showed that in control DL cells, only 6% of the metaphases were aberrant with a per cell aberration of 0.02 ± 0.00 SD. However, cisplatin (8 mg/kg b.w.) treatment for 1, 2, 3 and 4 days dastrically affected the DL cells resulting in a very high frequency (80-92%)

of aberrant metaphases. Pulverized (severely damaged) cells were frequently observed which are included among the percentage of abnormal metaphases; but excluded from the calculations of the aberrations/cell. Following 24 hr of cisplatin treatment, as high as 88-55% of aberrant metaphases with an average per cell aberration of 5.07 ± 0.34 SD were observed. On the 4th day of the treatment also 81.66% of aberrant metaphases with an average per cell aberration of 3.72 ± 0.13 SD could be noted.

Sialic acid content

The sialic acid analysis in the Dalton's lymphoma cells (Fig. 8) revealed that cisplatin treatment resulted in a gradual decrease in the sialic acid contents in the tumor cells. In the control tumor cells the sialic acid content was found to be 1.53 ± 0.1 SD μmole/g wet weight. However, following 3-4 days of the treatment a significant decrease ($P < 0.02$ and $P < 0.01$ respectively) was noted.

Light microscopical studies

The percentage ratio of leukocytes (neutrophils, monocytes, lymphocytes; based on nuclear shape and size) to tumor cells increased about three times by 1-4th day of cisplatin treatment *in vivo* (Fig. 9). In control ascites tumor very few leukocytes were seen among tumor cells which were round in shape (Fig. 10A). After 8 hr of cisplatin

treatment leukocytes are noticed to be closer to the tumor cells while at 24 hr (1 day) of the treatment, many leukocytes surround the tumor cell and also small membrane vacuoles appeared in the plasmamembrane of tumor cells (Fig. 5C). After 2-4 days of cisplatin treatment an increase in the number of membrane vacuoles, formation and shedding of membrane vesicles, and disintegration of plasmamembrane leading to the lysis of tumor cells were observed (Fig. 10 D-F).

Calcium ion concentration

The calcium concentration in various tissues studied (Table 2) showed that except for the brain tissues no significant variation could be found between the normal and the tumor bearing animals. Following cisplatin (8 mg/kg b.w.) treatment, significant increase in the calcium content of kidney, brain and tumor tissues was noted which was more prominent in the tumor followed by kidney and brain tissues. However, no significant variation in the calcium content of liver and spleen tissues could be found in response to tumor growth or cisplatin treatment of the tumor bearing hosts.

Rate of oxygen consumption

Significant variations in the rate of oxygen consumption (QO_2) were observed in the tumor cells following cispla-

tin (8 mg/kg b.w.) treatment in vivo (Fig. 11). Tumor cells collected immediately following cisplatin treatment (day 0) showed a 10% increase in QO_2 over the control (untreated) value. Following 1 day of the treatment, further increase in QO_2 by the tumor cells was noted which was about 40% higher than the control value. However, following 2-4 days of the treatment, QO_2 decreased steadily and on the 4th day it was found to be about 78% and significantly ($P < 0.01$) lower than the control value.

Potassium ion concentration

The potassium concentration varied significantly in the kidney, tumor and brain tissues following cisplatin treatment, whereas liver and spleen tissues showed no significant change (Table 3). In kidney significantly ($P < 0.02$) higher potassium content was found in the tumor bearing mice than the normal animals. Following cisplatin treatment significant decreases in the potassium contents of kidney as well as tumor tissues were noted. However, in the brain tissues an increase was recorded till 24 hr (2nd day) of the treatment which however decreased to about the control level subsequently. In the other two tissues studied i.e., liver and spleén, no significant variation could be found (Table 3).

Scanning electron microscopical studies

Control tumor cells showed the presence of fine

ruffles/blebs distributed evenly over the cell membrane (Fig. 12A). 8 hr post-cisplatin treatment resulted in infiltration of leukocytes surrounding tumor cells and forming connections with the latter. Also, it leads to a definite movement of ruffles/blebs from the top surface of the tumor cells towards the marginal areas (Fig. 12B). One day of cisplatin treatment (day 11) showed formation of broader tumor cell-leukocyte connections and the appearance of fine microvilii like processes extending from tumor cells (Fig. 12C). At 2-3 days of the treatment, thick blebs are formed over the surface membrane of tumor cells and lysis starts (Fig. 12 D and E). There is disappearance of the thin cellular processes, formation of membrane vacuoles and breaking of plasmamembrane of tumor cells after 4 days of cisplatin treatment (Fig. 12F) which leads to the lysis of tumor cells.

2) Enzymatic Studies

Glucose-6-phosphatase assay

Glucose-6-phosphatase activity in the liver tissues (Fig. 13A) showed that in the normal animal, the enzyme activity (84.0 ± 4.0 SD units/g tissue) was maximum and was significantly ($P < 0.01$) higher than the enzyme activity in the tumor bearing animals (52.5 ± 5.5 SD units/g tissue). Cisplatin treatment of the tumor beearing hosts resulted

in a steady increase in the enzyme activity. After 3-4 days of the treatment, the enzyme activity increased upto 73.0 ± 5.0 SD unit/g tissue which is significantly ($P < 0.01$) higher than the tumor bearing control.

In the kidney tissues (Fig. 13 B) it has been found that the enzyme activity in the tumor bearing animals (32.6 ± 4.0 SD units/g tissue) was significantly ($P < 0.02$) lower than the normal animals (42.0 ± 2.6 SD units/g tissue). Following cisplatin treatment the enzyme activity decreased till the 2nd day of the treatment. However, following 3-4 days of the treatment, the enzyme activity increased almost to the level found in the tumor bearing animals but; remained significantly ($P < 0.02$) lower than the activity found in the normal animals.

Lactate dehydrogenase (LDH) assay

Quantitative determination of LDH

Lactate dehydrogenase (LDH) activity in the serum (Fig. 14 A) of tumor bearing animals was found to be more than two fold higher than that found in the normal animals. Cisplatin treatment resulted in a progressive increase in the enzyme activity following the first day of the treatment. At 2-3 days of the treatment the LDH activity was found increased sharply and on the 3rd day the enzyme activity was maximum which was about 2.75 fold higher than the tumor

bearing control value. However, on the 4th day of the treatment a small but significant ($P < 0.05$) decrease in LDH activity was noted.

In the liver tissues (Fig. 14 B) the LDH activity did not show any definite pattern. However, in the liver of the tumor bearing animals significant ($P < 0.02$) higher LDH activity was noted than the normal animals. Following one day of cisplatin treatment, the LDH activity decreased significantly ($P < 0.01$) which however increased by the 2nd day and subsequently decreased to about the normal level.

In the kidney (Fig. 14 C) of the normal as well as the tumor bearing animals, no significant variation could be found in LDH activity. However, following cisplatin treatment, an over all decrease in the LDH activity was noted, although at day 2 an increase in the enzyme activity was observed.

In the tumor supernatants (Fig. 14 D), the LDH activity was found to be very high which increased further following cisplatin treatment. At 1-2 days of the treatment the LDH activity was found to be more than 2 fold higher over the control value. However, following 3-4 days of the treatment, the enzyme activity decreased again and on the 4th day, the LDH activity was found to be almost similar to that of the control value.

Tumor cells (Fig. 14 E) on the other hand, showed a different pattern in LDH activity. In the control supernatant, the enzyme activity was highest which decreased steadily over the 3-4 days of post-treatment period. The lowest activity was noted on the 3rd day of the treatment which was about 50% of the control value.

LDH isozyme analysis

Studies on LDH isozyme patterns (Fig. 15) showed that in serum (Fig. 15 A) of both normal and tumor bearing mice with or without cisplatin treatment for different intervals of time, all the 5 isozymes of LDH (i.e., LDH - 1, LDH - 2, LDH - 3, LDH - 4 and LDH - 5) were present in varying intensities. Among all the isozyme forms, LDH - 5 was found to be maximally expressed and LDH - 1 was the least expressed isozyme form as indicated from the band intensities. In the serum of the normal animal (lane - 1), LDH - 2 and LDH - 3 are seen to be more prominently expressed than the tumor bearing (lane - 2) or cisplatin treated (lane - 3-6) hosts. Following cisplatin treatment of the tumor bearing hosts, LDH - 5 isozyme band activity (intensity) was found to be apparently increasing upto the 2nd day (lane - 4) of the treatment and remained more or less same till the 4th day (lane - 6).

In the kidney tissues (Fig. 15 B), LDH - 5 isozyme

form was the least active form while LDH - 2 and LDH - 3 isozyme bands are comparatively more dense and hence reflecting higher activity. As found in serum, all the 5 isozyme forms are also found in the kidney tissues. Following cisplatin treatment, no significant variations in the band intensities of various isozymes could be noted, although on the 4th day of the treatment (lane - 6) LDH - 4 and LDH - 5 isozyme bands seem to be some what less intense in comparison to the corresponding bands in cther groups.

In the other three tissues studied, i.e., liver, tumor supernatant and tumor cells (Fig. 15 C, D and E), distinct variations are notable in relation to the number and nature of the various isozyme bands. In the liver tissue (Fig. 15 C) only 3 isozyme bands (LDH - 3, LDH - 4 and LDH - 5) are seen, of which LDH - 5 seems to be the predominant form. The notable feature is that from the first day of treatment (lane - 3), DH - 3 and LDH -4 isozymes showed marked variations in band intensities indicating changes in activity, and by the 4th day of the treatment (lane - 6), LDH - 5 seems to be the only isozyme form present in the liver.

In the tumor supernatant (Fig. 15 D), LDH - 5 is the most active form and LDH - 4 is the other distinct form present, whereas other forms vary considerably. In the tumor

cells (Fig. 15 E), LDH-5 is the only conventional form present.

The most important notable feature is the presence of one extra band near the cathodic position in the serum of tumor bearing as well as cisplatin treated group (Fig. 15 A, lane - 2-6), which is also seen in tumor supernatant and tumor cells (Fig. 15 D, E); but absent from liver (Fig. 15 C), kidney (Fig. 15 B) and serum of normal animals (Fig. 15 A, lane - 1).

$Na^+ + K^+$ - ATPase assay

$Na^+ + K^+$ - ATPase activity in Dalton's lymphoma cells (Fig. 16 A) from the control animals was maximum (150 ± 6 SD units/100 ml). Cisplatin treatment resulted in a gradual decrease in the enzyme activity. Following 2-4 days of the treatment the enzyme activity significantly ($P < 0.01$) decreased as compared to the control value.

On the other hand, in the tumor supernatant (Fig. 16 B) it is found that cisplatin treatment resulted in an increase in the enzyme activity upto 2nd day of the treatment. However, during 3-4 days of the treatment, the enzyme activity decreased and was found to be marginally higher but statistically insignificant as compared to the control value.

5'-Nucleotidase assay

5'-Nucleotidase (5'-ND) activity in liver (Fig. 17 A) showed that the tumor bearing animals had the maximum activity (28.26 ± 1.12 SD units/100 ml) which was almost 2.5 times higher than the enzyme activity found in the liver of normal animals (10.75 ± 1.75 SD units/100 ml). However, following cisplatin treatment of the tumor bearing hosts, 5'-ND activity progressively decreased during 1-4 days of the treatment. On the 4th day of the treatment, the enzyme activity was found to be almost equal to that found in the liver of normal animals without tumor.

In the kidney tissues (Fig. 17 B) it is found that 5'-ND activity in the tumor bearing animals was significantly ($P < 0.01$) higher than the normal animals. Following cisplatin treatment the enzyme activity gradually increased till 2-3 days of the treatment. However, by the 4th day of the treatment, no significant variation could be noted in the enzyme activity as compared to the tumor bearing controls.

In the tumor cells (Fig. 17 C) it is found that the control tumor bearing mice showed the maximum 5'-ND activity (16.0 ± 1.9 SD units/100 ml). Cisplatin treatment resulted in a significant ($P < 0.02$) decrease in enzyme activity following one day of treatment which decreased

further till the 4th day of the treatment. On the 4th day of treatment, the enzyme activity was found to be less than half (7.2 ± 0.2 SD units/100 ml) of the control value.

In the control tumor supernatant (Fig. 17 D) the 5'-ND activity was found to be 4.4 ± 0.5 SD units/100 ml which increased significantly ($P < 0.001$) till the 2nd day following cisplatin treatment. However, following 3-4 days of the treatment a decrease in the enzyme activity was noted. On the 4th day of cisplatin treatment, no significant variation in the enzyme activity could be found as compared to the control value.

Glutamic oxalacetic transaminase assay

GOT activity in the serum (Fig. 18 A) of the tumor bearing animals (25.4 ± 0.9 SD units/ml) was significantly ($P < 0.001$) higher than the normal animals (16.3 ± 0.6 SD units/ml). Following cisplatin treatment for 1 day resulted in significant ($P < 0.01$) decrease in enzyme activity which however increased to about the tumor bearing control value by 2-3 days of the treatment. On the 4th day of the treatment, no significant variation could be found in the enzyme activity between the treated and the normal animals. In the tumor supernatants also a similar pattern of variation in GOT activity was noted following cisplatin treatment for 1, 2, 3 and 4 days (Fig. 18 B).

Glutamic pyruvic transaminase assay

GPT activity in the serum (Fig. 19 A) of tumor bearing animals (5.7 ± 0.6 SD units/ml) was marginally higher than the serum of normal animals (4.8 ± 0.3 SD units/ml). Following cisplatin treatment for 1-2 days, GPT activity decreased significantly ($P < 0.01$) being lowest (3.3 ± 0.4 SD units/ml) on day 2 of the treatment. However, on the 4th day of treatment, no significant difference in the enzyme activity was found between the treated group and the tumor bearing controls. Similar patterns in GPT activity was also found in the tumor supernatants following cisplatin treatment (Fig. 19 B).

Arginase assay

Arginase activity in the liver (Fig. 20 A) of normal mice was found to be maximum (530.0 ± 12.0 SD units/g) and was significantly higher ($P < 0.001$) than the tumor bearing animals (416.0 ± 8.0 SD units/g). Following cisplatin treatment of the tumor bearing hosts, a significant ($P < 0.01$) increase in the enzyme activity was noted. However, following 2-4 days of cisplatin treatment the enzyme activity decreased and on the 4th day (98 hr) of treatment the enzyme activity (378.0 ± 6.0 SD units/g) was significantly ($P < 0.01$) lower than the tumor bearing controls.

In the kidney tissues (Fig. 20 B), the maximum enzyme

activity (19.0 ± 1.0 SD units/g) was noted in the normal animals which was significantly ($P < 0.02$) higher than the arginase activity (15.0 ± 1.5 SD units/g) found in the tumor bearing hosts. Following 8 hr of cisplatin treatment, the enzyme activity increased to about the normal level which however decreased subsequently by 2-4 days at the treatment during which no significant variation between the treated group and the tumor bearing controls was found.

In the tumor supernatants (Fig. 20 C) the minimum enzyme activity (3.4 ± 0.6 SD units/ml) was found in the control animals. Cisplatin treatment for 8, 48 and 96 hr resulted in a steady increase in the arginase activity. On the 4th day (96 hr) of the treatment a significant ($P < 0.02$) increase in enzyme activity was noted in the tumor supernatant as compared to the control value.

Cathepsin assay

In the serum of tumor bearing animals both cathepsin B and cathepsin H (Fig. 21 A and C) activities were significantly ($P < 0.05$) lower than the respective normal counter parts. Cisplatin treatment for 1 day resulted in significant ($P < 0.05$ and $P < 0.01$ respectively) decrease in the activity of both the enzymes. But, following 2-4 days of the treatment, the activity of both cathepsin B and cathepsin H increased progressively and by 3-4 days of the treatment

very high activity was noted for both the enzymes.

In the tumor supernatants (Fig. 21 B and D) it is found that control tumor supernatants had lower cathepsin B and cathepsin H activities. Cisplatin treatment for 1-4 days resulted significant ($P < 0.01$) elevations in the cathepsin B activity in the tumor supernatants. Cathepsin H activity also showed a similar pattern but the change was more gradual than cathepsin B.

3) Combination Chemotherapeutic Studies

Tumor growth and vitamin C plus cisplatin treatment

Cisplatin (4.0 mg/kg b.w.) treatment of tumor bearing mice on the 10th day of tumor transplantation resulted in an increase in their mean survival time and more than 50% animals survived upto 35 days. The other group of mice receiving 0.5% vitamin C from the first day showed almost similar survival patterns as was observed in mice treated with cisplatin only (Fig. 22). The intake of vitamin C in 0.5% vitamin C treated group of mice was 8.5-9.5 mg/day/ animal.

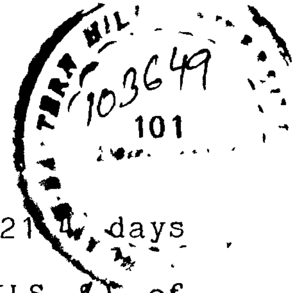
The mice receiving 0.25% vitamin C with drinking water from the first day and treated with cisplatin (i.p., 4.0 mg/kg b.w.) on the 10th day of tumor transplantation showed slow tumor growth and thereby a smaller increase

in body weight/tumor growth with increase in host survival upto 45-50 days in 50% of the animals (Fig. 23, 24).

The combined administration of 0.5% vitamin C in drinking water from the first day and cisplatin (i.p., 4.0 mg/kg b.w.) on the 10th day resulted in 70% survivals upto 55 days and 40% of the treated mice were found to be tumor free (Fig. 23). The increase in body weight in this group of mice was very slow indicating an effective retardation of tumor growth (Fig. 24).

In the other set of experiments when cisplatin (4.0 mg/kg b.w.) injection was given on the 5th day and 0.5% vitamin C treatment started from the 10th day after tumor transplantation, tumor regression was not as effective as was noted in the group of mice receiving 0.5% vitamin C continuously from the first day and treated with a single i.p. injection of cisplatin (4.0 mg/kg b.w.) on the 10th day after tumor transplantation. The host survival rates almost resembled to the groups treated with cisplatin alone (Fig. 23).

In the studies with ascites Dalton's lymphoma bearing Swiss albino mice, it was observed that cisplatin (4.0 mg/kg b.w.) treatment on the 10th day of tumor transplantation increased their mean survival time upto 34.3 days, while



the controls showed the mean survival time of 21.4 days registering a per cent increase in life span (ILS %) of 60.28. The other group of mice receiving 0.5% vitamin C (8.5-9.5 mg/day/animal) in drinking water from the first day of tumor transplantation showed an ILS (%) of 21-96 (Table - 4).

The third group of mice receiving 0.25% vitamin C (4.0-4.8 mg/day/animal) with drinking water from the first day and cisplatin (4.09 mg/kg b.w.) on the 10th day after tumor transplantation showed an ILS (%) of 143.45 over the controls (Tabel - 4). The fourth group in which the mice were treated with 0.5% vitamin C from the first day and cisplatin (4.0 mg/kg b.w.) on the 10th day of tumor transplantation showed a marked increase in the ILS (%) which was greater than 203.73 and the mean survival time was more than 65 days (Table - 4).

The drug efficacy determined in terms of per cent growth inhibition rate (GIR %) showed that the mean tumor weight in the control animals was 14.0 g on the 20th day of tumor transplantation, and cisplatin (4.0 mg/kg b.w.) treatment resulted in a decrease in the mean tumor weight to 5.2 g exhibiting a GIR (%) of 62.85 by the same time. However, the other group of animals receiving 0.5% vitamin C alone from the first day of tumor transplantation showed

slower tumor growth and thereby a smaller mean tumor weight of 12.1 g with a GIR (%) of 13.57 (Table 5).

The group of mice receiving 0.25% vitamin C from the first day and cisplatin (4.0 mg/kg b.w.) on the 10th day of tumor transplantation showed very effective tumor regression and the GIR (%) was 71.71 (Table - 5). However, the most effective regression of the tumor was noted in the group fo mice receiving 0.5% vitamin C from the first day and cisplatin (4.0 mg/kg b.w.) on the 10th day of tumor transplantation which showed a GIR (%) of 88.57 and the mean tumor weight was reduced to only 1.6 g by the 20th day of tumor transplantation (Table - 5).

Effect on thymus and spleen weight, and tumor pH

A decrease in the weight of spleen and thymus was noted in tumor bearing mice as compared to that of control animals. there was a significant increase in the weight of spleen ($P < 0.01$) as well as thymus ($P < 0.05$) of tumor bearing mice treated with 0.5% vitamin C and cisplatin (4.0 mg/kg b.w.) combination than the tumor bearing controls. Average tumor pH in control tumor bearing mice was 6.93 while it decreased to about 6.27 in treated animals (Table - 6).

Ascorbic acid content

Normal Swiss albino mice showed a mean serum ascorbic acid (vitamin C) level of 10.38 μg/ml. However, the tumor bearing mice had a comparatively lower serum ascorbic acid level of 7.11 μg/ml as determined on the 11th day of tumor transplantation (Fig. 25). On the other hand, mice receiving 0.5% vitamin C in drinking water from the first day of tumor transplantation showed a significantly ($P < 0.05$) higher serum ascobic acid level (13.44 μg/ml) than those of the tumor bearing mice and was marginally higher than the normal animals (Fig. 25).

Cisplatin (4.0 mg/kg b.w.) treatment of the tumor bearing mice on the 10th day of tumor transplantation failed to alter the ascorbic acid level significantly as determined following one day of the treatment. The combined treatment of 0.5% vitamin C from the first day and cisplatin (4.0 mg/kg b.w.) on the 10th day of tumor transplantation showed almost similar ascorbic acid level as was observed in the mice receiving 0.5% vitamin C alone (Fig. 25).

Total leukocyte count

Cisplatin (4.0 mg/kg b.w.) treatment of the tumor bearing hosts, on the 10th day of tumor transplantation showed an increase in the total leukocyte count (TLC) upto the 3rd day of the treatment, which however, decreased sub-

sequently and almost 50% decrease was noted on the 6th day of treatment (Table - 7). On the other hand, 8 mg/kg b.w. of cisplatin treatment showed an increase in the TLC for the first two days which decreased subsequently and the decrease was more rapid than the decrease in the group of mice receiving 4.0 mg/kg b.w. of cisplatin (Table - 7).

In the group of mice receiving 0.5% vitamin C from the first day, the TLC was marginally higher than the control animals. However, the group of mice receiving 0.5% vitamin C from the first day and cisplatin (4.0 mg/kg b.w.) on the 10th day of tumor transplantation, significantly higher number of leukocytes were noted upto the 5th day of cisplatin treatment (Table - 7).

Agglutination studies

Control Dalton's lymphoma cells showed high degree of Con A agglutination (Fig. 26). When cells were treated with cisplatin (25 μg/ml) or vitamin C (50 μg/ml) for 15, 30, 45 and 60 min, gradual decrease in the Con A agglutinability of cells was noted. Vitamin C treated cells showed less decrease in the agglutination as compared to that of cisplatin treated cells (Fig. 26). During 45-60 min of cisplatin treatment, Con A agglutinability decreased to about one-third of the controls. Combined treatments of vitamin C plus cisplatin decreased the cell agglutination maximally (Fig. 26).

Fluorescence microscopical studies

Dalton's lymphoma cells showing high degree of agglutination, showed bright even fluorescence intensity over the cells and 15 min of cisplatin treatment led to a decrease in the fluorescence intensity (Fig. 27 A and B). At 30 min of the treatment further decrease in fluorescence intensity was noted. However, there was the appearance of fluorescent grains over the cells following 45 min of the treatment which decreased sharply at 60 min (Fig. 27 C and D). The cells treated with vitamin C plus cisplatin for 15 min showed a decrease in fluorescence intensity with the appearance of distinct, sharp fluorescent grains over the cells (Fig. 27 b). At 30 min of the treatment further decrease in fluorescence intensity was noted but the granular fluorescence pattern was still present over the cells. At 45-60 min of the treatment very sharp decrease in fluorescence intensity with the disappearance of fluorescent grains was noted (Fig. 27 c and d).

Mutagenicity studies

Chromosome aberration assay

In metaphase analysis of bone marrow cells, both chromatid and isochromatid type gaps, chromatid deletions that include breaks and fragments of unknown origin, sister chromatid unions forming rings, and exchanges forming dicen-

trics were observed in the treated series. Chromatid breaks and gaps occurred more frequently than other type of abnormalities in majority of cases both in cisplatin treated as well as in vitamin C plus cisplatin treated groups (Table - 8). Assessment of the distribution of breaks and gaps tentatively revealed that both the long chromosomes and short chromosomes were equally vulnerable to cisplatin treatment and the distal regions were found to be comparatively more sensitive. Exchanges were the next frequently observed anomalies to those of chromatid breaks and gaps (Table - 8).

Significant variations were obtained among the results of the time-response both in the cisplatin as well as vitamin C plus cisplatin treated groups (Table - 8). Significantly different variations in the total number of aberrations as well as in the percentage abnormal metaphases among the two groups (cisplatin, and vitamin C plus cisplatin treated) was noted for the corresponding periods of the treatment. And the vitamin C plus cisplatin treated mice were almost always less affected than those treated with cisplatin alone for the same interval of time (Table - 8).

Micronucleus assay

The frequencies of micronucleated polychromatic erythrocytes (PCEs) in bonemarrow cells induced by cisplatin

were significantly higher than the controls with both the doses (4.0 mg and 8.0 mg/kg b.w.) used (Table - 9). Further, significant variations were noted between the micronucleated PCEs results of 4 versus 8 mg/kg b.w. ($P < 0.01$) cisplatin treatment. Cisplatin at both the doses also induced significantly higher frequencies of micronucleated normochromatic erythrocytes (NCEs) as well as other nucleated cells than the respective control values (Table - 9).

Treatment of 0.5% vitamin C alone from the first day of transplantation failed to induce any significant variation in the incidence of micronucleus than those of the control values in any of the three cell types studied. However, the combined treatment of 0.5% vitamin C and cisplatin (4.0 mg/kg b.w.) significantly decreased the incidence of micronucleated PCEs. ($P < 0.05$), NCEs ($P < .01$), and nucleated cells ($P < 0.05$) than the group treated with cisplatin (4.0 mg/kg b.w.) alone (Table - 9). The incidence of micronucleated PCEs and NCEs in the combined treated group were significantly different than those of the respective controls (Table - 9).

Sperm abnormality assay

In the sperm head abnormality assay it was found that amorphous types (56%) and hooked heads (31%) were more prevalent while banana-like heads (7%), triangular (3%)

and dwarf (3%) were less frequent. The results of all the treatment groups were significantly higher than the control value (Table - 10). Again, significant variations were observed between the results of cisplatin versus vitamin C plus cisplatin combined treated groups at 1 day ($P < 0.05$), 2 days ($P < 0.01$) and 10 days ($P < 0.05$) following the treatment (Table 10).

Nephrotoxicity studies

Serum uric acid content

The tumor bearing animals showed a 15% increase in the serum uric acid level than the normal animals (Fig. 30, day 0). Cisplatin (8 mg/kg b.w.) treatment of the tumor bearing hosts for 3, 6, 9 and 12 days resulted in a gradual increase in the serum uric acid level. Following 3 days of the treatment, the uric acid level increased by 26% over the tumor bearing control value (Fig. 30). The serum uric acid level further increased by 39, 49 and 56 per cents over the tumor bearing control value following 6, 9 and 12 days of cisplatin treatment respectively (Fig. 30).

Serum urea content

The results of the serum urea measurement (Fig. 31) showed that neither the tumor bearing controls nor the vitamin C (0.5% in drinking water from the first day of tumor transplantation) treated animals had any appreciable

variation in the serum urea level than the normal animals. Cisplatin (8 mg/kg and 4 mg/kg b.w.) treatment markedly increased the serum urea level which was dose dependent (Fig. 31). Following 8 mg/kg b.w. of cisplatin treatment, a peak serum urea level of about 95 µmole/ml was noted by the 5th day of the treatment whereas, in the 4 mg/kg b.w. cisplatin treated group, the peak urea level of about 65 µmole/ml was observed on the 7th day of the treatment (Fig. 31). However, the combined treatment of vitamin C (0.5%) and cisplatin (4 mg/kg b.w.) significantly decreased the serum urea level following 5 to 15 days of the treatment.

Fig. 1. Photograph showing a normal mouse without any tumor and a tumor (ascites Dalton's lymphoma) bearing mouse of the same age group. Note the unusually enlarged abdominal region of the tumor bearing mouse due to the presence of tumor. Present inside the cage is a tumor bearing mouse.

Fig. 1

Fig. 2. Photomicrographs of metaphase chromosome spreads of ascites Dalton's lymphoma cells with or without cisplatin treatment. A - Control tumor cell showing modal number (= 66) of chromosomes having one metacentric marker chromosome (arrow). B - Control tumor cell showing near diploid number (= 122) of chromosomes having two metacentric chromosomes (arrows). C - Control tumor cell showing highly heteroploid number (= 259) of chromosomes. D, E - Cisplatin treated tumor cells showing various types of chromosomal aberrations such as chromatid interchanges, chromatid gaps and chromatid breaks (arrows).

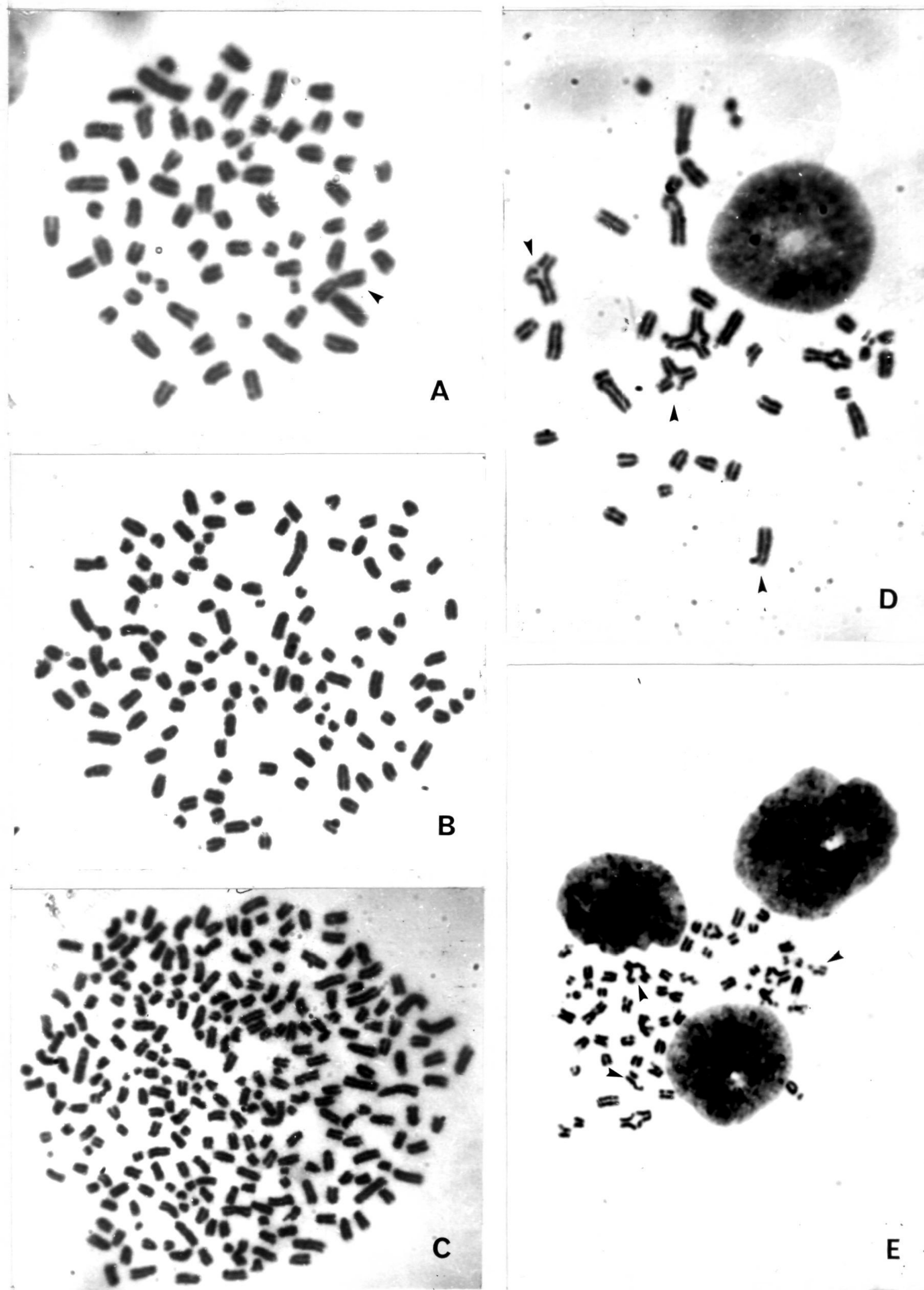


Fig. 2

Fig. 3. Graph showing the changes in the volume of tumor (ascites Dalton's lymphoma) after transplantation (O——O). Note the significant decrease in recoverable tumor volume from the hosts after cisplatin treatment (▲——▲) on 10th day post tumor transplantation with almost complete tumor regression after six days of the treatment.

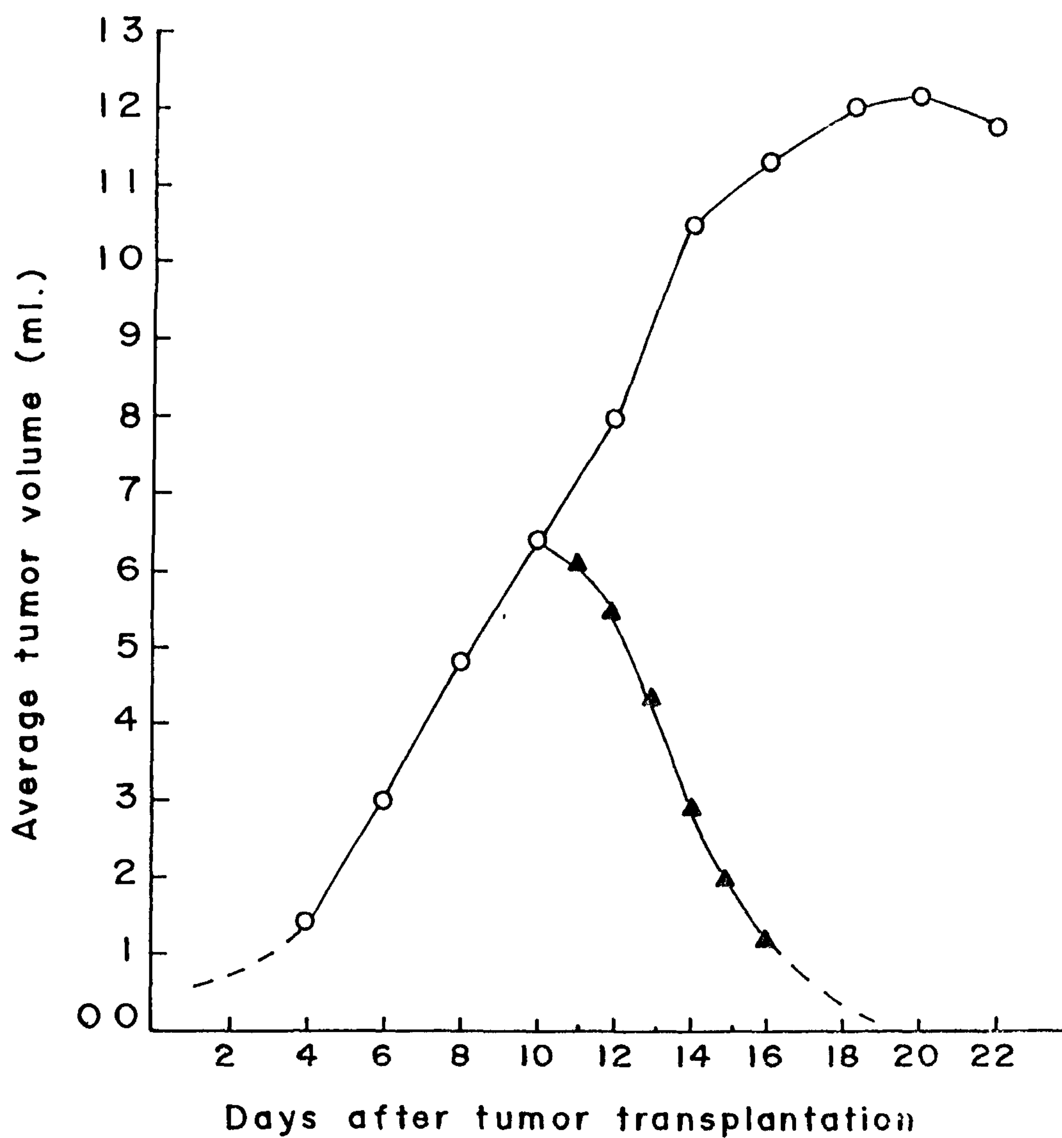


Fig. 3

Fig. 4. Graph showing the changes in the ratio of ascites fluid to tumor cell pellet (v/v) with and without cisplatin treatment *in vivo*. Note the steady increase in the ratio during tumor growth indicating more accumulation of ascites fluid to tumor (0——0) following transplantation. After cisplatin treatment (▲——▲) sharp decrease in ascites fluid is noted and the ratio falls below 0.5.

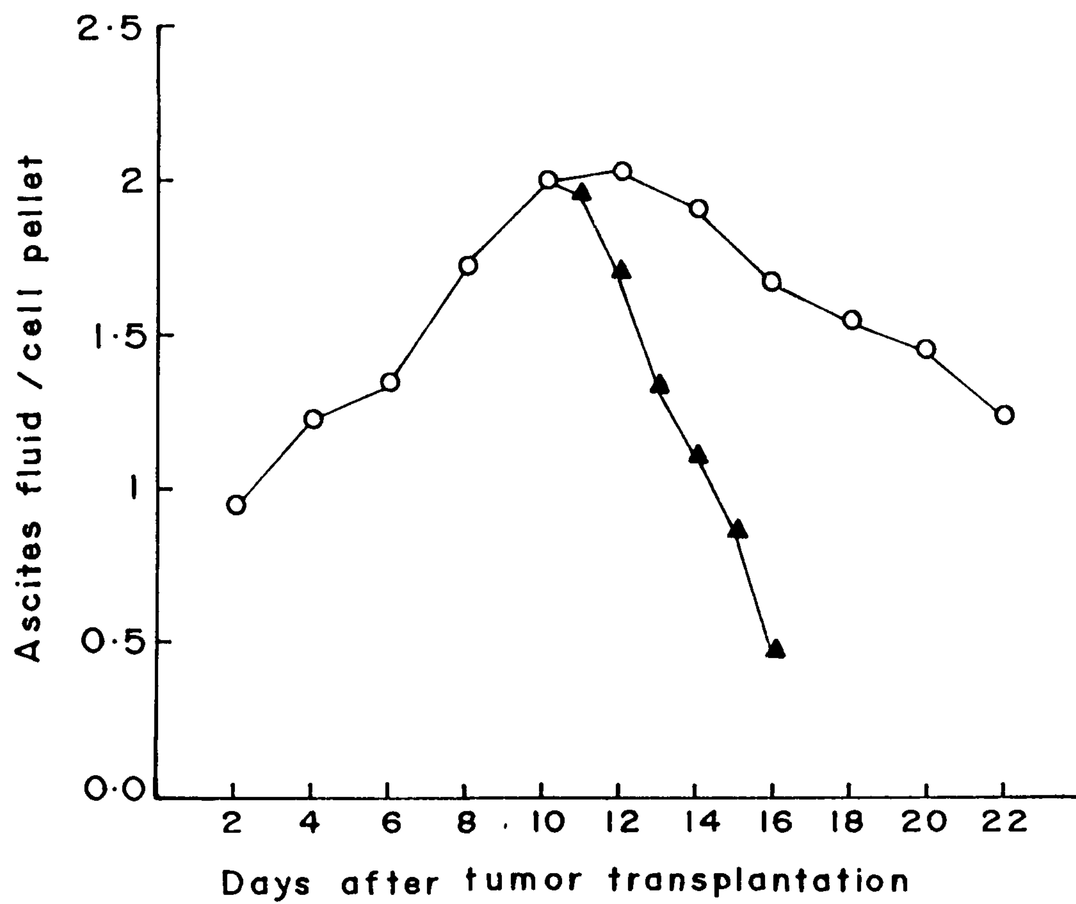


Fig. 4

Fig. 5. Graph showing the changes in protein (●——●) and carbohydrate (0——0) contents in ascites fluid of hosts treated with or without cisplatin. Note the decrease in protein contents (▲——▲) and increase in carbohydrate contents (Δ——Δ) in ascites fluid during 3-4 days of cisplatin treatment *in vivo*.

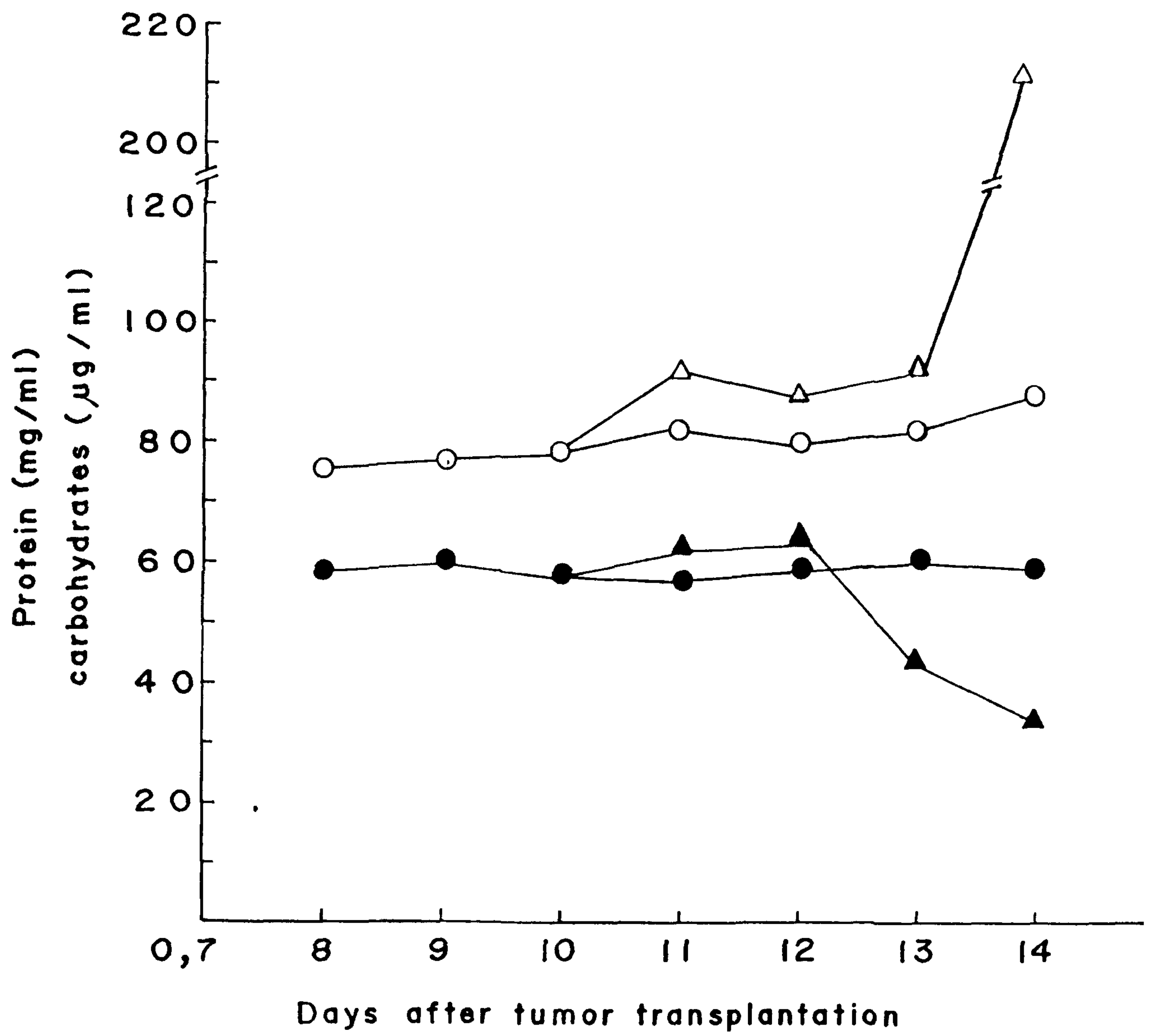


Fig. 5

Fig. 6. Historgram showing the changes in the concentration of glucose in serum (A) and tumor superntants (B). Note the significantly higher serum glucose concentration following 2-3 days of cisplatin treatment which however, decreased to about the normal level by the 4th day of the treatment. Also note the very low level of glucose in the tumor supernatant which increased sharply following 1-4 days of the treatment. N = Normal mice; TB = Tumor bearing mice; TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.02$; ** = $P < 0.001$.

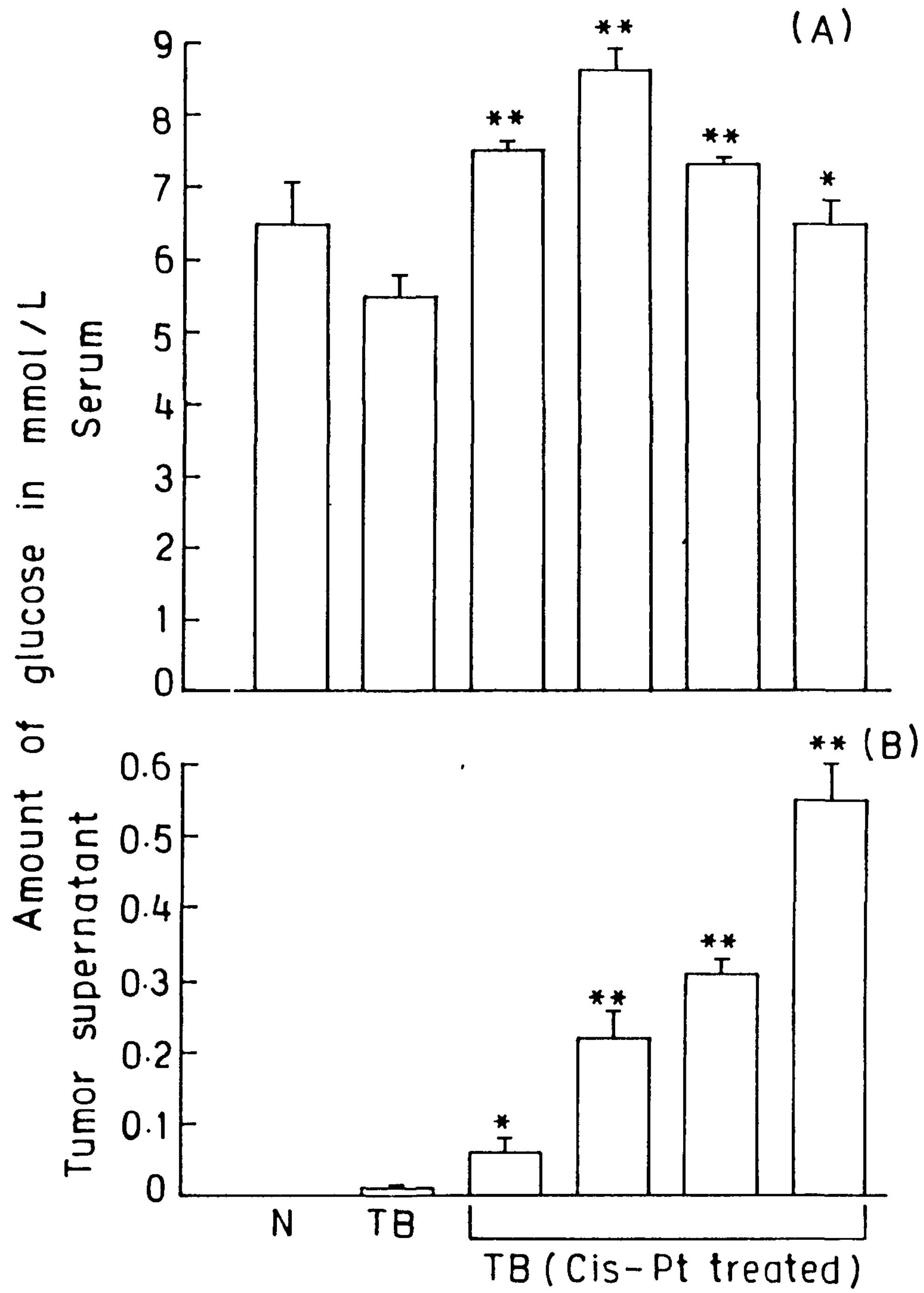


Fig. 6

Fig. 7. Graph showing the changes in glucose consumption by Dalton's lymphoma cells following cisplatin treatment _in vitro_. Note the significant increase in the rate of glucose consumption following 1 hr of the treatment. Also note the subsequent gradual decrease in glucose consumption following 2-6 hr of the treatment which is about 40% lower at 6 hr.

Statistical analysis: Student's t-test. * = $P < 0.02$; ** = $P < 0.01$.

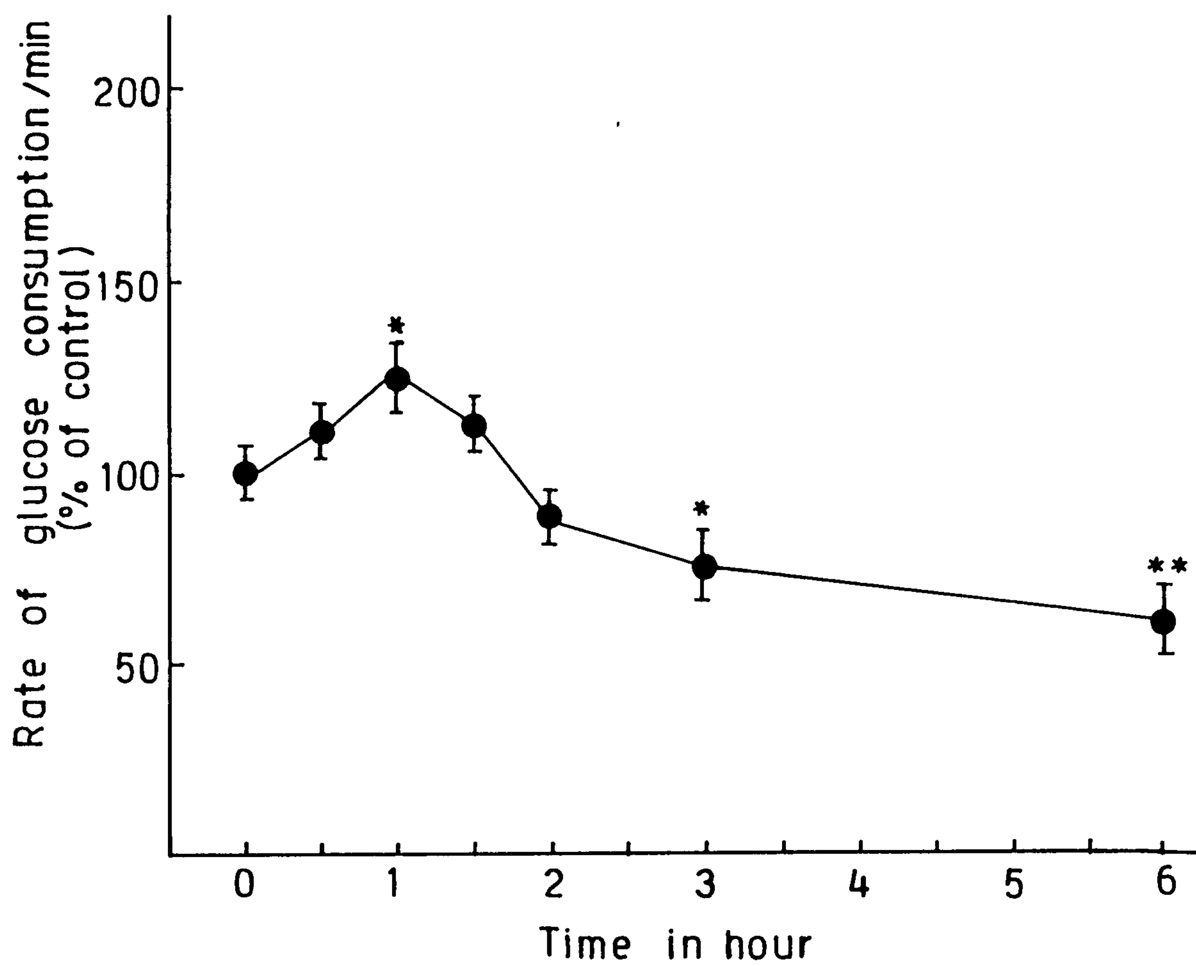


Fig. 7

TABLE 1 - Frequency of chromosomal aberrations in Dalton's lymphoma cells induced by cisplatin.

Dose (mg/kg)	Time after treatment (in hours)	No. of metaphases studied	Mean of anormal metaphases	Aberrations/ cell ± SD
0	Control	400	6.00	0.02 ± 0.00
8	24	300	88.55	5.07 ± 0.34
8	48	300	92.21	4.73 ± 0.20
8	72	300	92.22	4.21 ± 0.30
8	96	300	81.66	3.72 ± 0.13

Fig. 8. Histogram showing the changes in the sialic acid content of Dalton's lymphoma cells following cisplatin treatment *in vivo*. Note the significant decrease in sialic acid content following 3-4 days of the treatment. TB = Tumor bearing animal (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.02$; ** = $P < 0.01$.

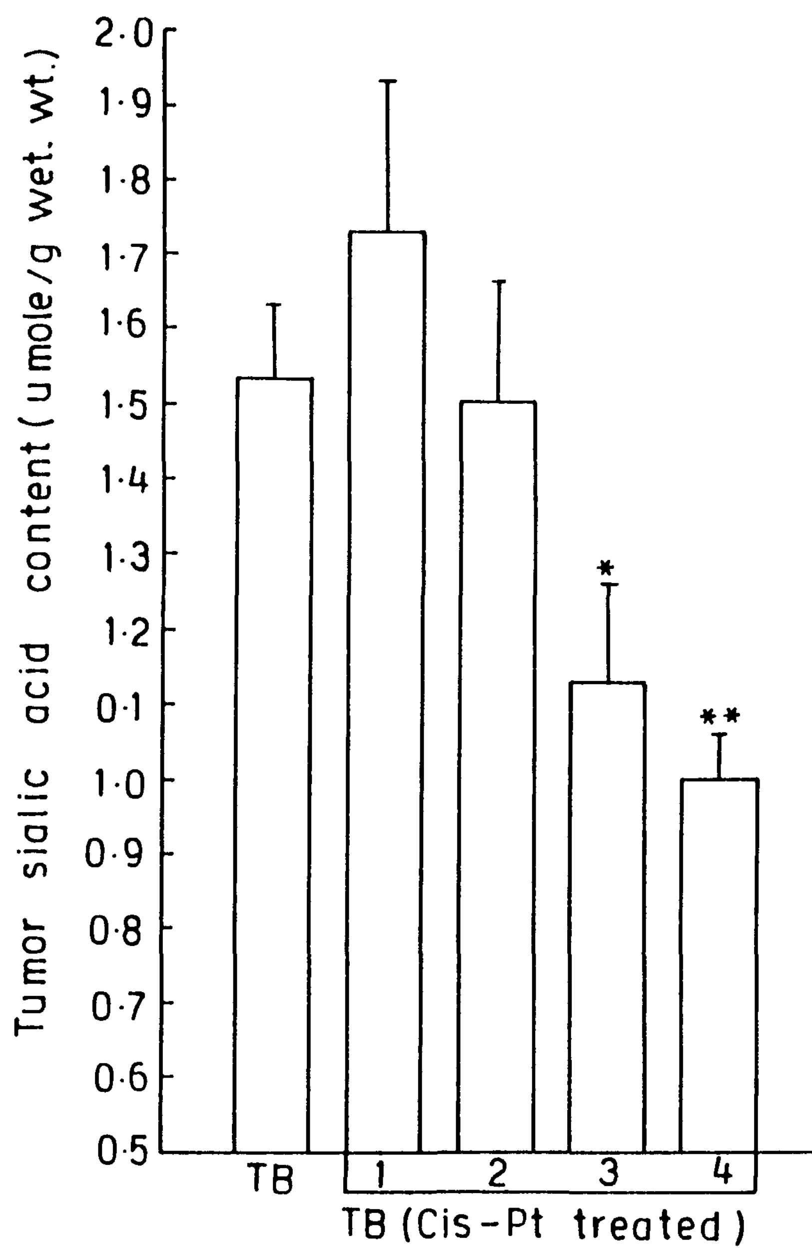


Fig. 8

Fig. 9. Graph showing the changes in the ratio of leukocytes to tumor cells with tumor growth (●——●) and after cisplatin treatment (Δ——Δ). Note that about three times increase in the ratio of leukocytes to tumor cells after cisplatin treatment.

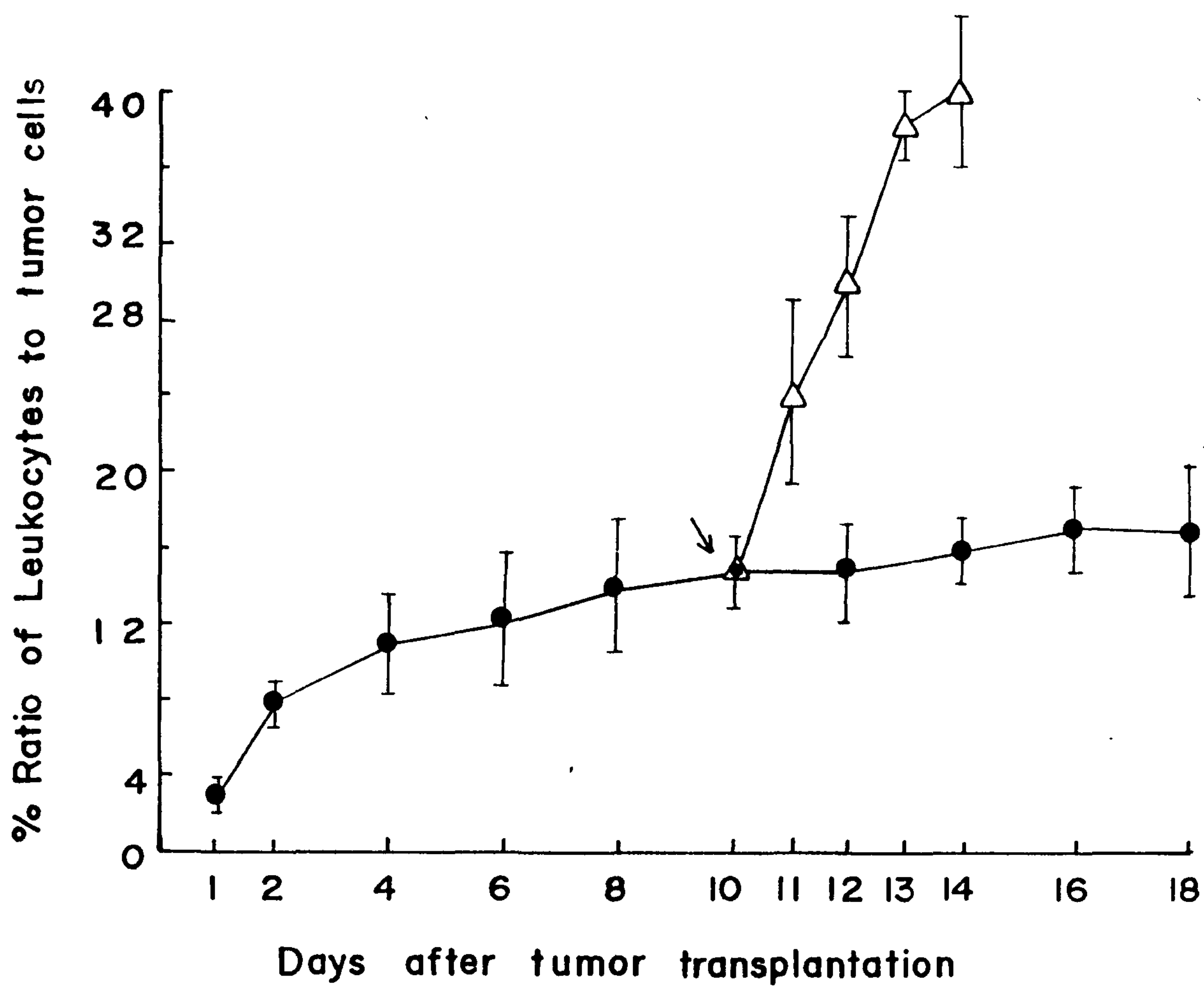


Fig. 9

Fig. 10. Light micrographs of tumor cells treated with or without cisplatin *in vivo*. A-Control ascites tumor cells round in shape having very few leukocytes among tumor cells. B-After 8 hr of cisplatin treatment leukocytes are noticed to be closer to tumor cells which at 1 day (C) of the treatment many leukocytes surround tumor cell and also small membrane vacuoles appear on the plasma membrane of tumor cells. D-2 days of cisplatin treatment showing an increase in the number of membrane vacuoles.

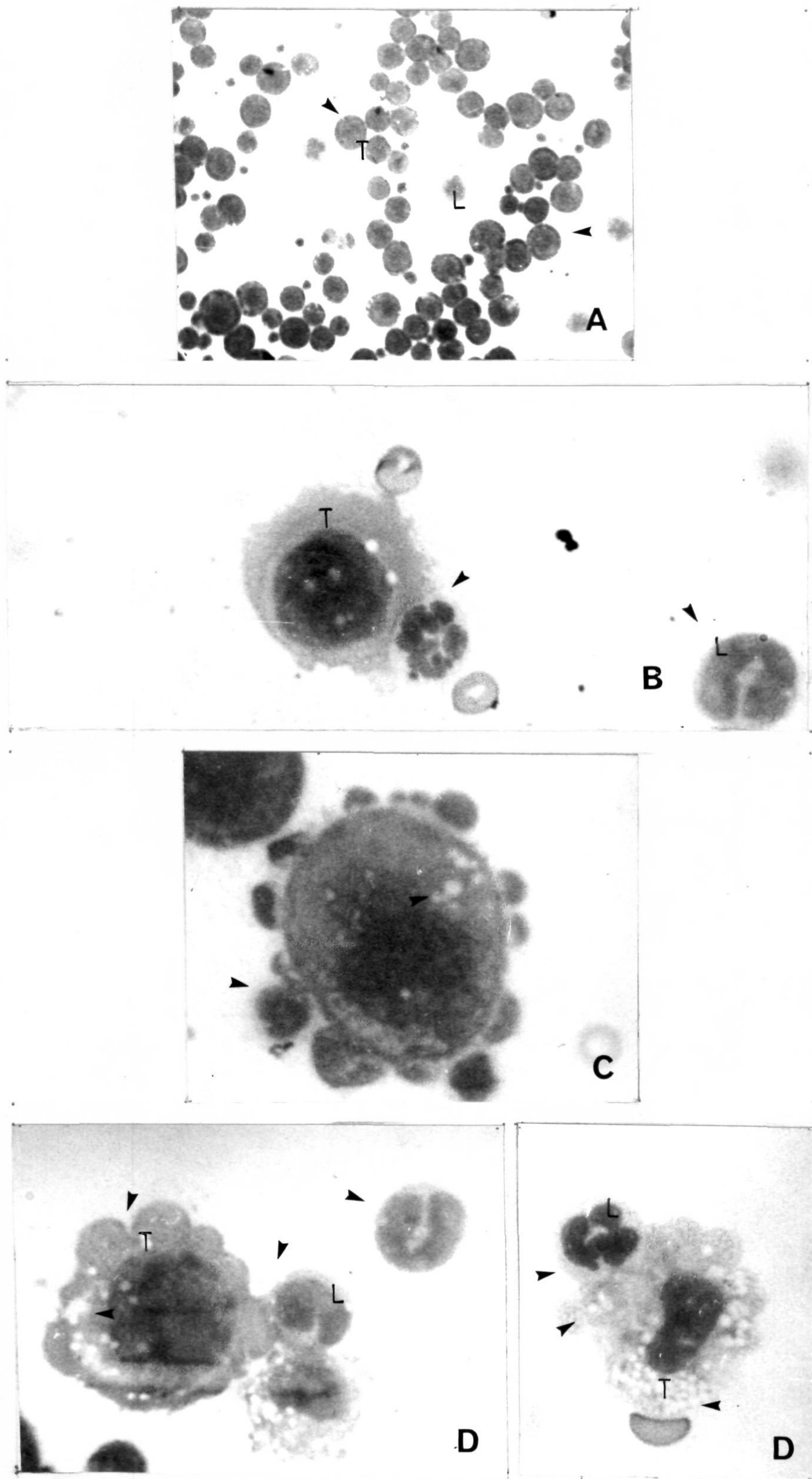


Fig. 10

Fig. 10. E & F-3 and 4 days of cisplatin treatment showing large number of membrane vacuoles, formation and shedding of membrane vesicles and disintegration of plasma membrane leading to the lysis of tumor cells. T = Tumor cell; L = Leukocyte. A = x 600; B = x 1600; C, D, E (Inset), F = x 2000; E = x 800. Arrows indicate the surface of the tumor cells, leukocytes, membrane visicles and vacuoles, disintegration in plasma membrane.

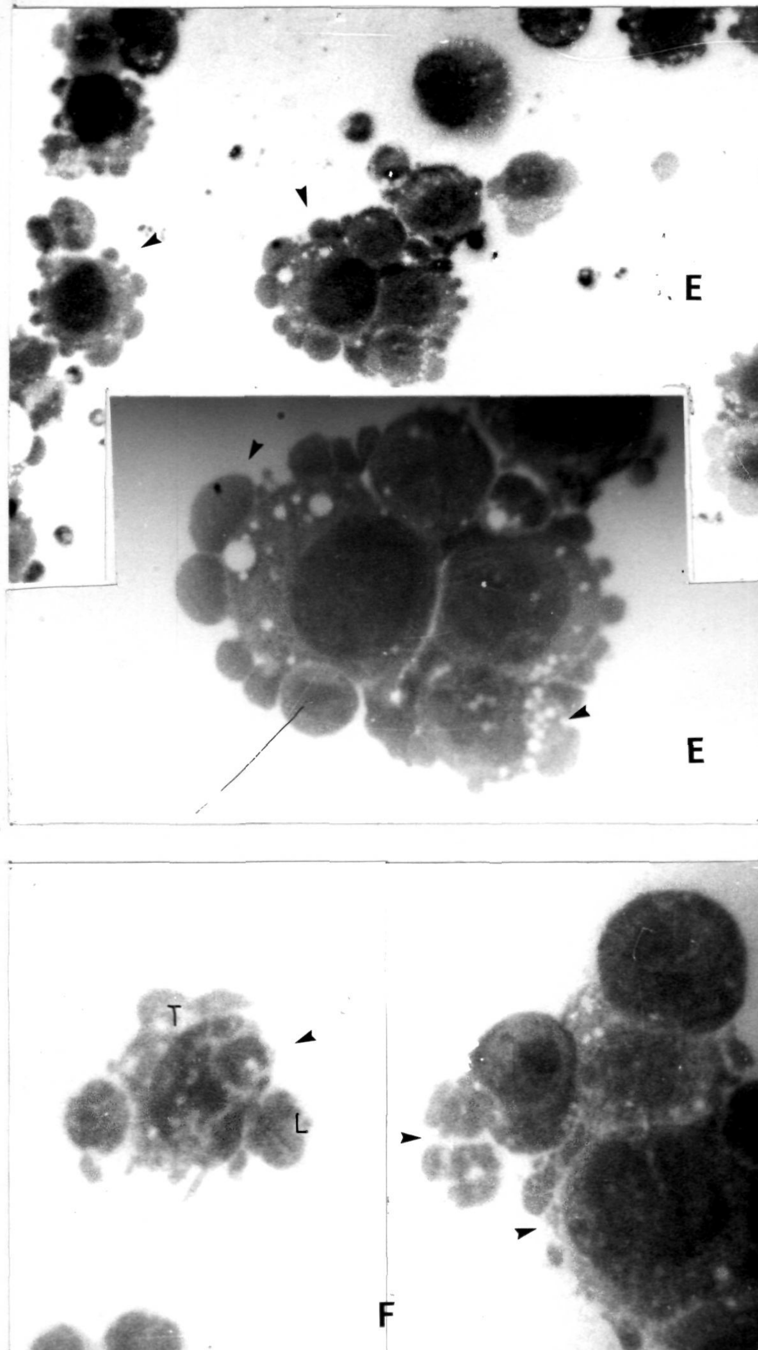


Fig. 10

TABLE 2 - Effect of ciplatin (8 mg/kg b.w.) on tissue calcium concentration.

Time after treatment (in hours)	Amount of Ca present in μg/g dry weight of tissue				
	Liver	Kidney	Spleen	Brain	Tumor
Normal*	202.17 (28.32)	239.41 (21.70)	445.95 (46.57)	554.30[a] (11.33)	-
Tumor-bearing	235.57 (17.04)	288.04 (26.33)	466.98 (23.26)	502.97 (18.00)	167.21 (9.71)
4 hours	242.55 (11.27)	337.15 (20.11)	488.72 (34.44)	542.10 (21.32)	189.96 (16.66)
12 hours	233.77 (13.33)	395.18[b] (18.22)	472.05 (27.18)	580.00[a] (23.33)	232.71[b] (10.46)
24 hours	284.38 (41.77)	404.67[b] (15.13)	439.27 (21.36)	605.45[b] (26.76)	255.25[c] (7.75)
48 hours	292.71 (21.73)	414.08[b] (13.55)	456.63 (34.17)	603.57[a] (37.42)	291.66[c] (18.34)
72 hours	267.35 (26.66)	435.22[b] (16.41)	426.72 (17.98)	639.20[b] (33.67)	320.88[c] (10.03)
96 hours	228.83 (18.33)	443.07[c] (9.66)	440.33 (11.77)	618.64[b] (23.03)	354.44[c] (11.53)

The results are mean 3-4 independent determinations. The figures in the parentheses are ± SD.

* = Normal mice without tumor or treatment.

Statistical analysis: Student's t-test. The data are compared with the tumor bearing mice as control. a = $P < 0.0.5$; b = $P < 0.01$; c = $P < 0.001$.

Fig. 11. Graph showing changes in the rate of oxygen consumption by Dalton's lymphoma cells following cisplatin treatment _in vivo_. Tumor cells showed significantly higher rate of oxygen consumption at 1 day of the treatment. Note the subsequent decrease following 2-4 days of the treatment.

Statistical analysis: Student's t-test. * = $P < 0.01$.

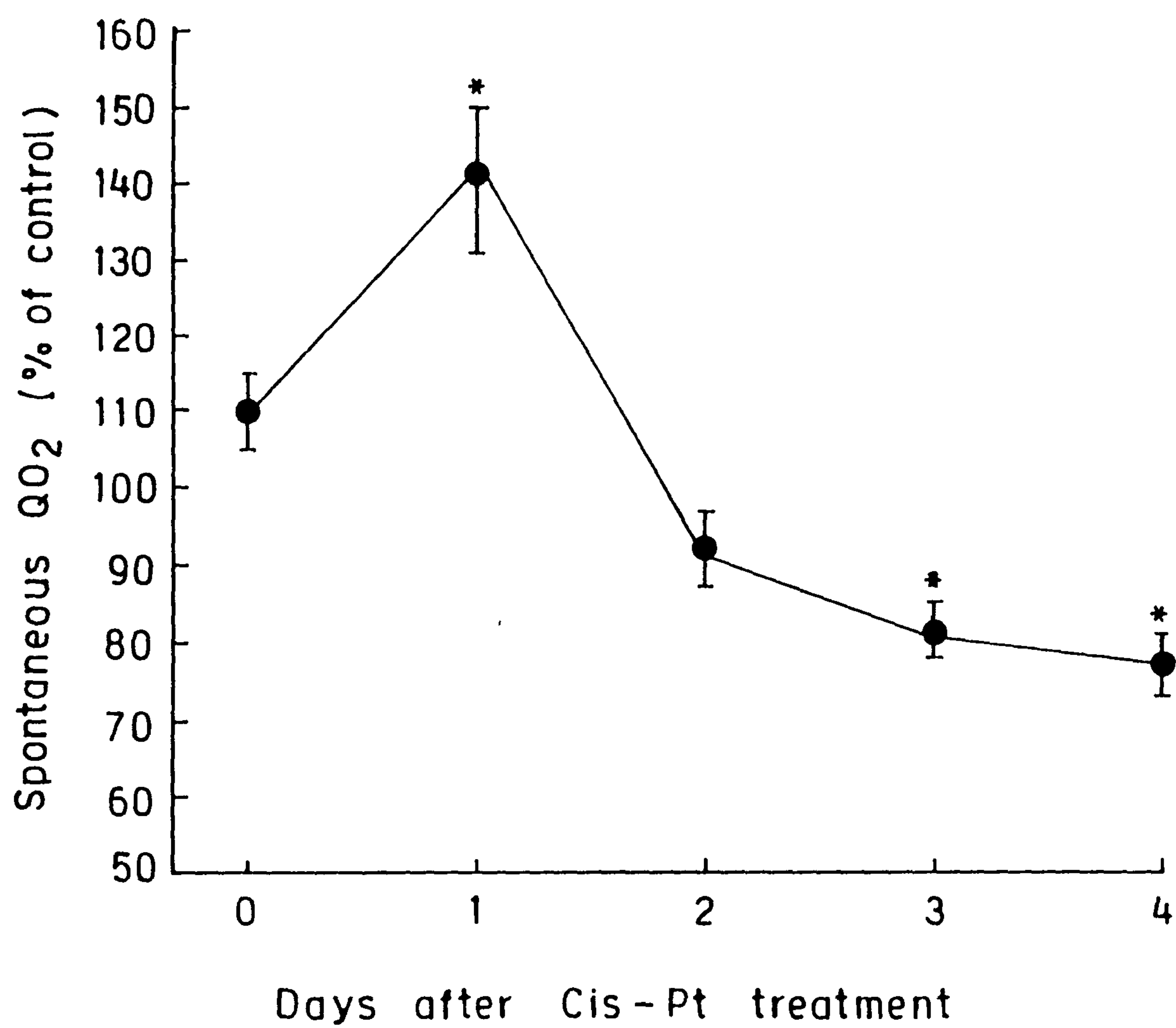


Fig. 11

TABLE 3 - Effect of cisplatin (8 mg/kg b.w.) on tissue potassium concentration.

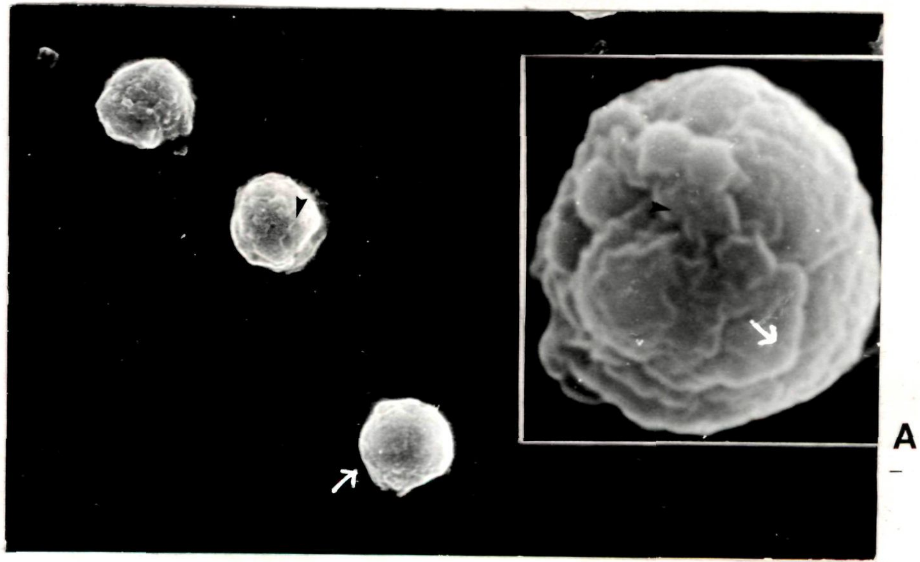
Time after treatment (in hours)	Amount of K present in µg x 10/g dry weight of tissue				
	Liver	Kidney	Spleen	Brain	Tumor
Normal*	1017.52 (42.73)	1018.76[b] (34.21)	1202.38 (18.77)	1272.15 (25.35)	
Tumor-bearing	1076.62 (53.06)	1126.81 (23.67)	1227.83 (42.21)	1250.00 (16.33)	2010.90 (23.37)
04 hours	1156.31 (31.17)	1096.02 (15.08)	1245.75 (21.35)	1368.42[b] (43.08)	1939.36 (38.44)
12 hours	1144.48 (44.76)	1083.13[a] (17.73)	1223.33 (27.06)	1337.20[c] (24.43)	1869.00[c] (23.33)
24 hours	1117.87 (23.33)	1036.27[c] (11.33)	1187.08 (31.12)	1322.61[b] (29.19)	1814.47[d] (13.56)
48 hours	1086.74 (81.41)	990.58[b] (17.09)	1156.40 (18.66)	1286.27 (18.83)	1637.22[d] (32.88)
72 hours	1019.53 (27.11)	956.25[d] (13.35)	1198.52 (23.41)	1289.77 (33.33)	1524.40[d] (36.33)
96 hours	1029.24 (18.67)	912.54[c] (16.66)	1131.08[a] (26.33)	1212.96 (51.37)	1366.53[d] (27.07)

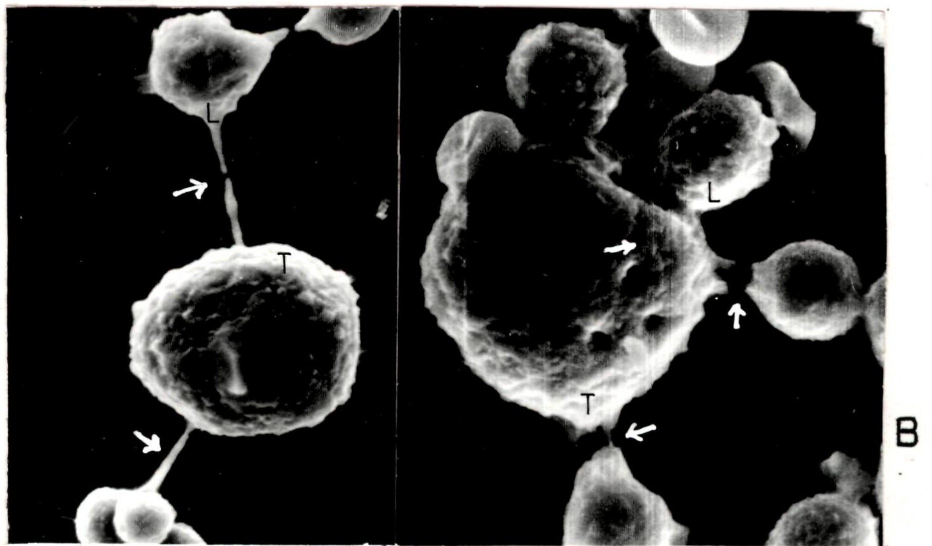
The results are mean of 3-4 independent determinations. The figures in parentheses are ± SD.

* = Normal mice without tumor or treatment.

Statistical analysis: Student's t-test. The data are compared with the tumor bearing mice as control. a = $P < 0.05$, b = $P < 0.02$; c = $P < 0.01$, d = $P < 0.001$.

Fig. 12. Scanning electron microscopic images of ascites Dalton's lymphoma treated with or without cisplatin _in vivo_. A-Control, showing the presence of fine ruffles/blebs distributed evenly over the cell membrane. B-8 hr of cisplatin treatment showing the inflitration of leukocytes towards tumor cells and forming connections with the latter. Also note the movement of ruffles/ blebs from the top surface of tumor cells to the marginal areas. C-One day of the treatment showed the formation of broader tumor cell-leukocyte connections and the appearance of fine microvilli-like processes extending from tumor cells.





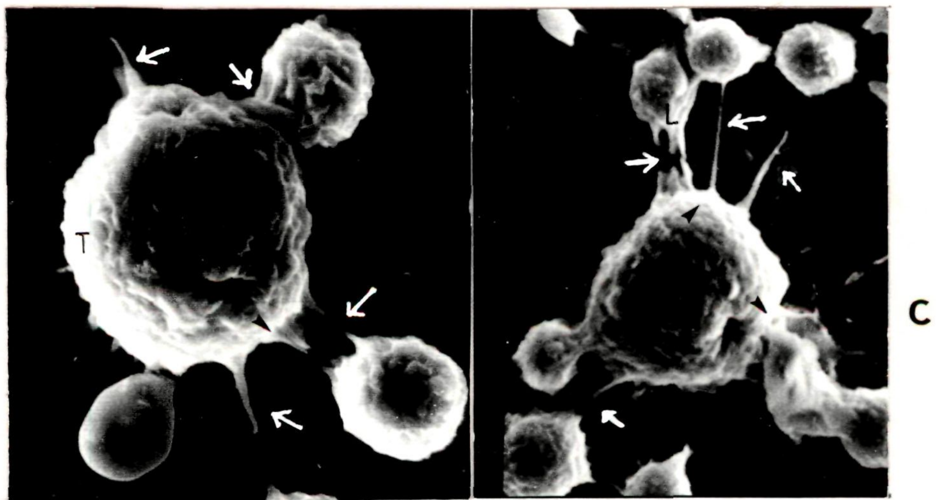
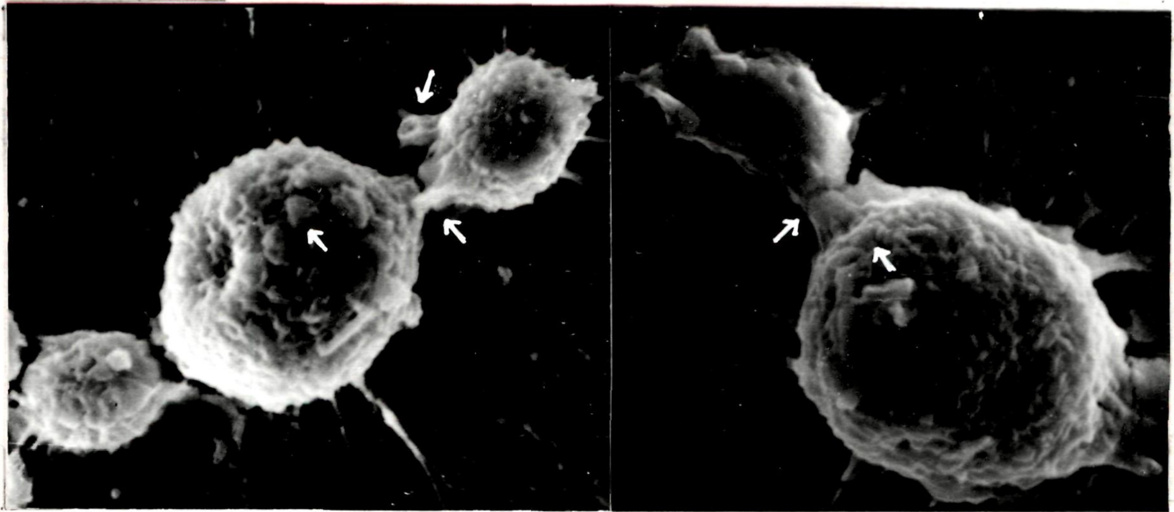
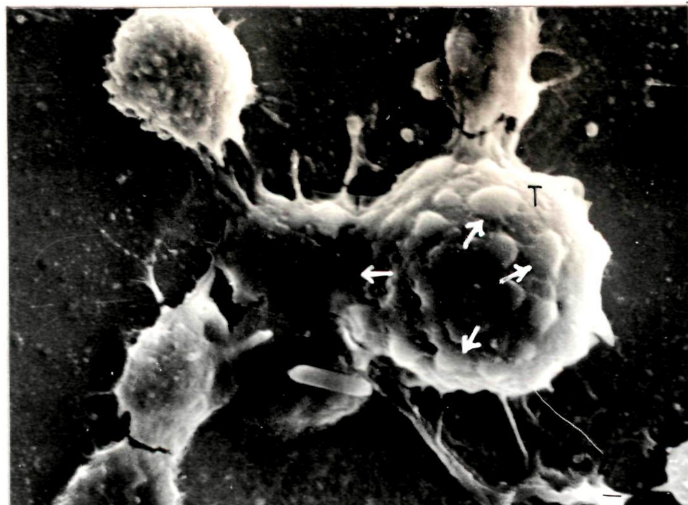


Fig. **12**

Fig. 12. D & E-2 to 3 days to the treatment leads to the formation of thick surface membrane blebs and starting of tumor cell lysis. F-4 days of cisplatin treatment shows the disappearance of thin processes, formation of membrane vacuoles, breaking of plasma membrane and lysis of tumor cells. Arrows indicate the ruffles/blebs, cellular processes/connections or disintegration of plasma membrane. T = Tumor cell; L = Leukocyte.





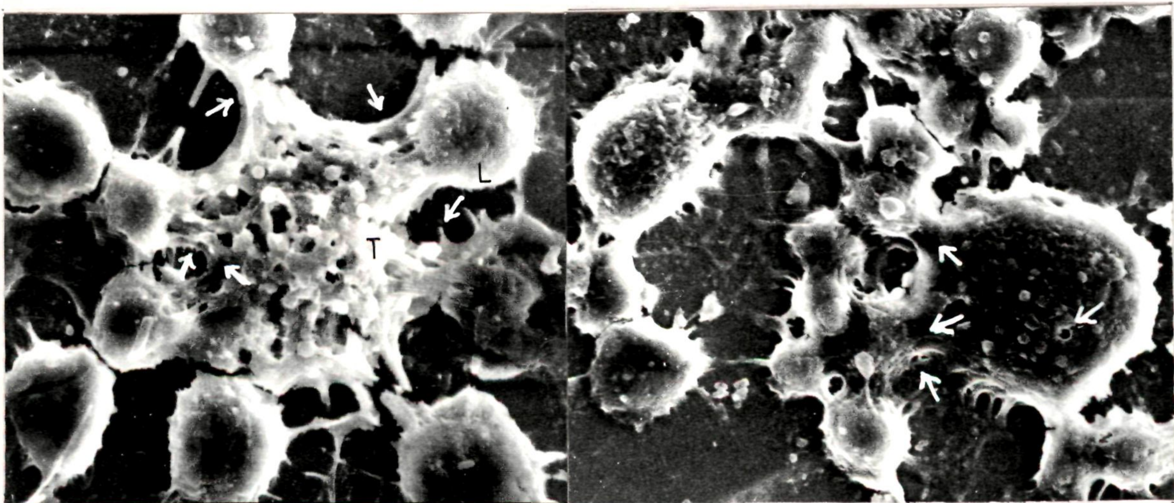


Fig. **12**

Fig. 13. Histogram showing the changes in glucose-6-phosphatase activity in liver (A) and kidney (B) treated with or without cisplatin *in vivo*. Note the significantly higher enzyme activity in both the tissues (A, B) of normal mice than the tumor bearing mice. Following 3-4 days of cisplatin treatment the enzyme activity significantly increased in the liver (A). But, the enzyme activity in kidney decreased significantly following 2 days of the treatment and subsequently showed an increasing trend. N = Normal mice; TB = Tumor bearing mice (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.02$; ** = $P < 0.01$. Results with similar superscripts differ significantly: a = $P < 0.05$; b = $P < 0.02$.

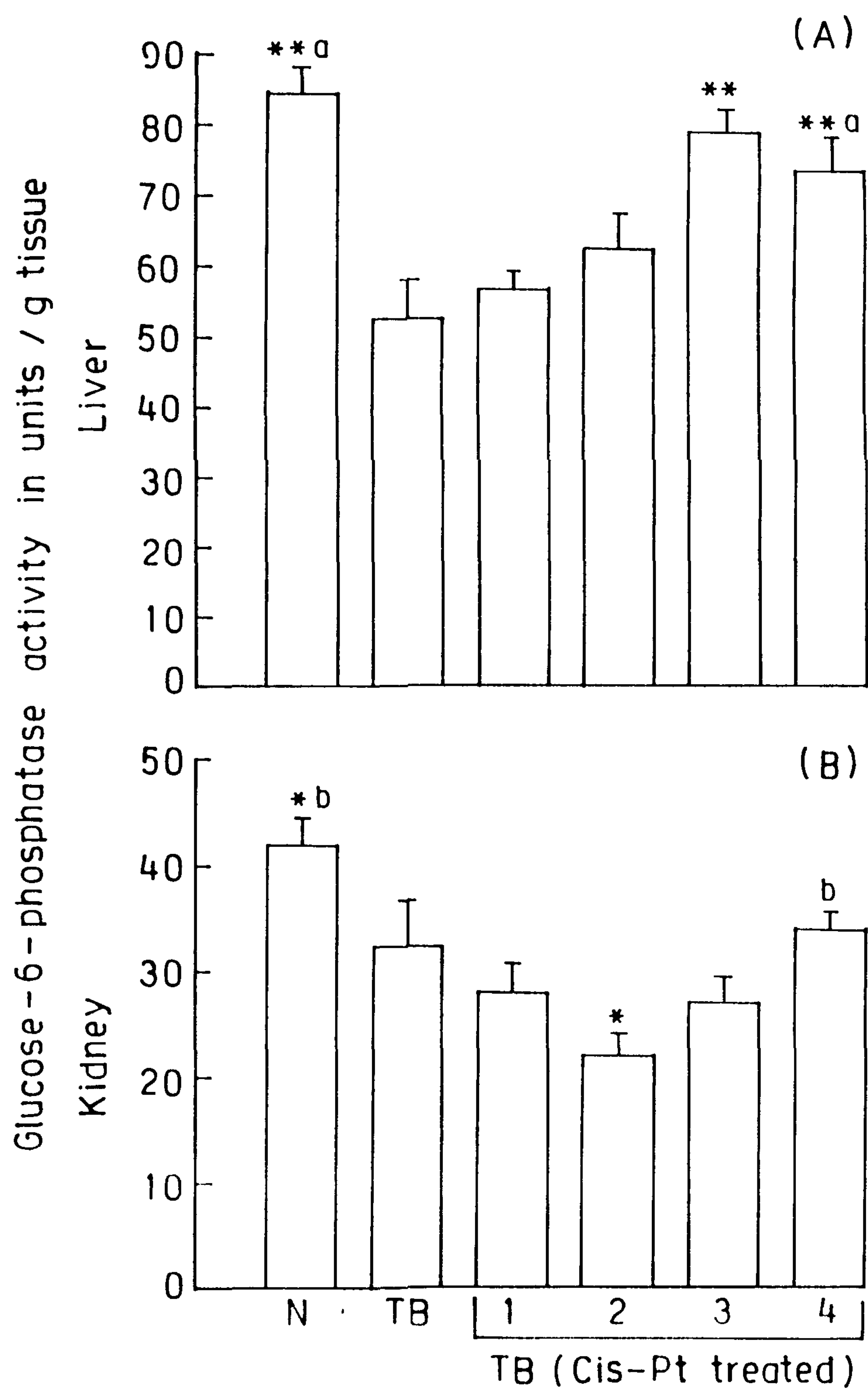


Fig. 13

Fig. 14. Histogram showing the changes in LDH activity in serum (A) treated with or without cisplatin. Note the more than two fold increase in LDH activity in the tumor bearing mice than the normal mice. Also note the significant increase in LDH activity following 2-4 days of the treatment.

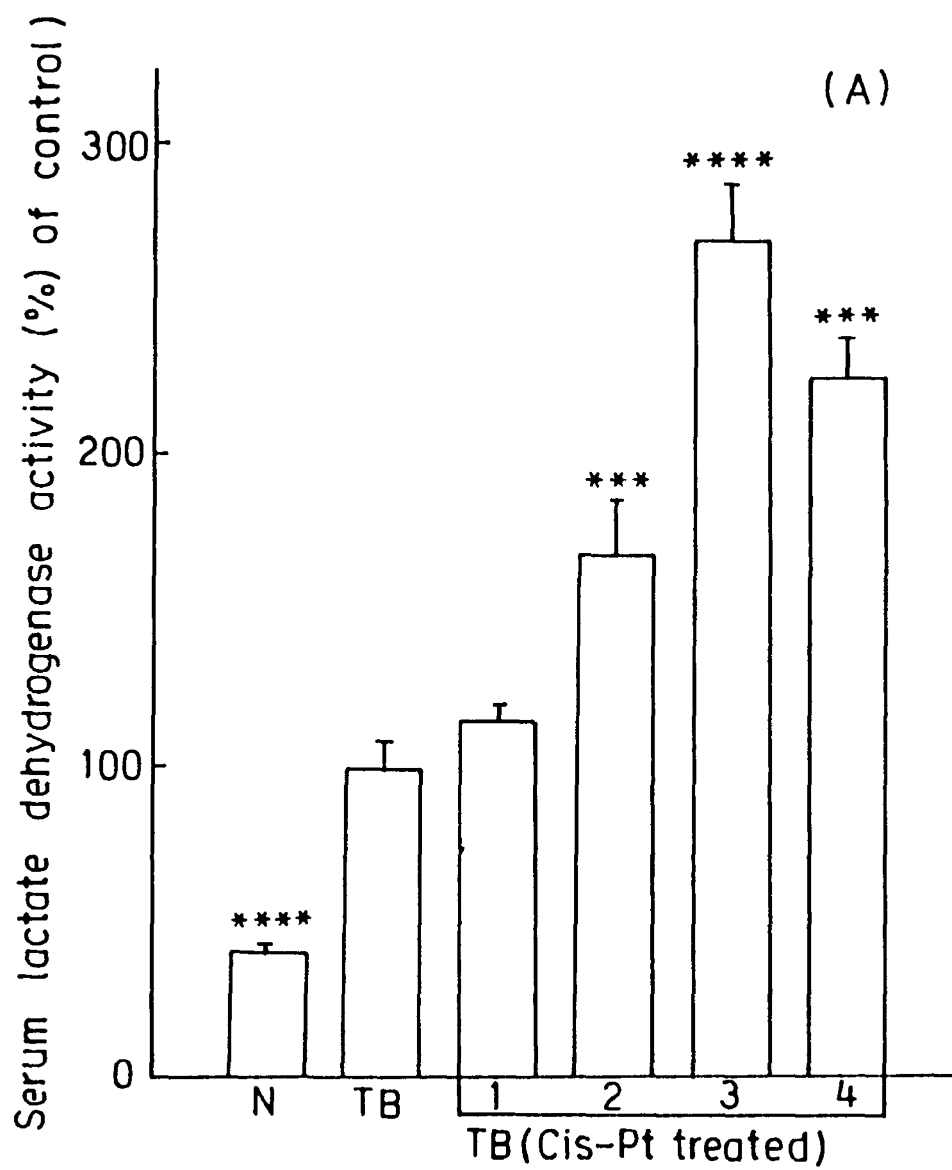


Fig. 14

Fig. 14. B and C show changes in LDH activity in liver and kidney respectively following treatment with or without cisplatin. Note the significantly higher LDH activity in the liver of tumor bearing mice than the liver of normal mice. In the kidney no significant difference could be observed between the normal and tumor bearing mice. Note the overall decrease in LDH activity in kidney following cisplatin treatment.

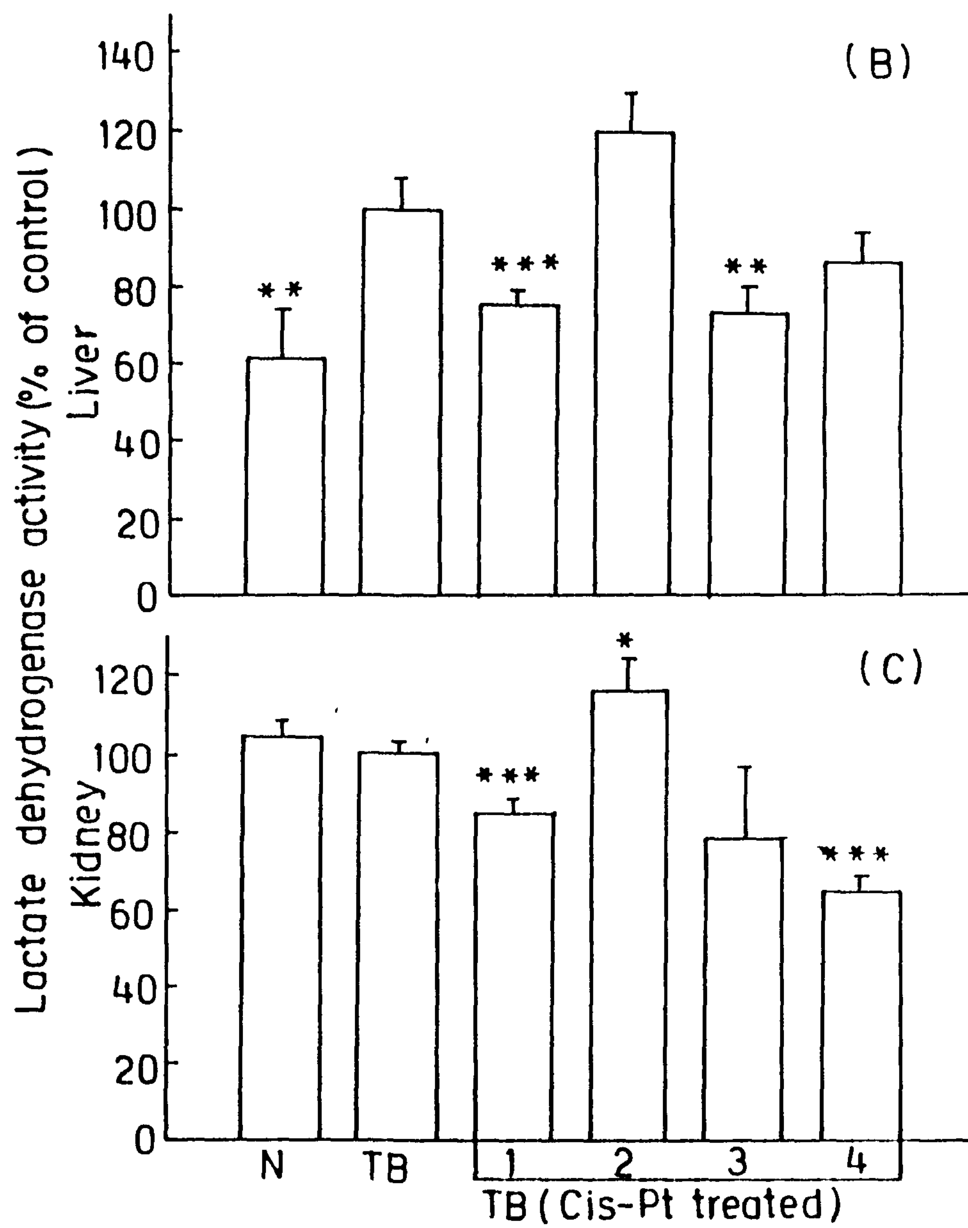


Fig. 14

Fig. 14. D and E show changes in LDH activity in tumor supernatant and tumor cells respectively following treatment with or without cisplatin. Note the sharp increase in LDH activity in the tumor supernatant (D) following 1-2 days of the treatment. Also note the steady decrease in the enzyme activity in the tumor cells (E) following 1-4 days of the treatment. N = Normal mice; TB = Tumor bearing mice (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.05$; ** = $P < 0.02$; *** = $P < 0.01$; **** = $P < 0.001$.

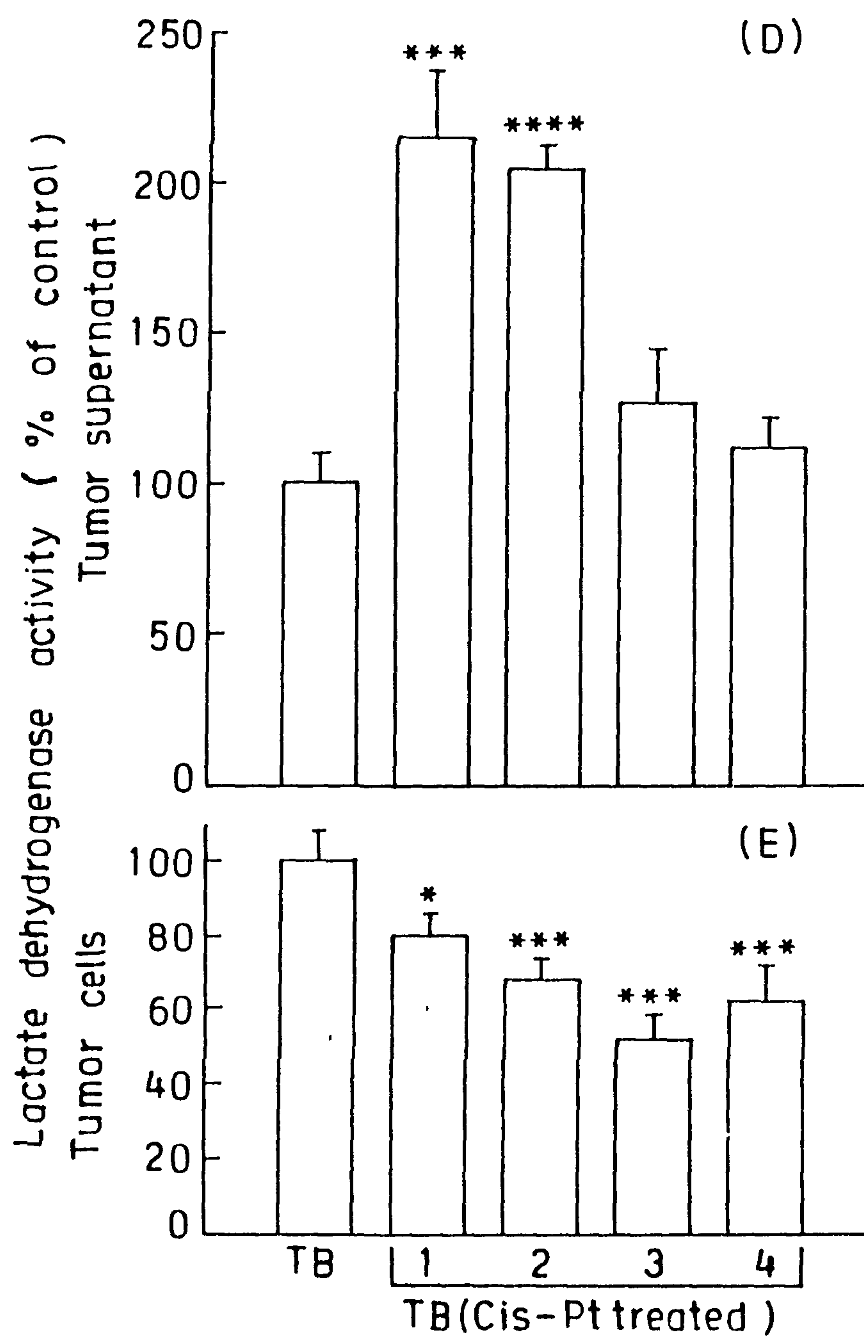


Fig. 14

Fig. 15. Photographs showing lactate dehydrogenase (LDH) isozyme patterns in various tissues with or without cisplatin treatment. A - LDH isozyme pattern in serum. Note the presence of all the five isozymes and predominance of LDH-5. B - LDH isozyme pattern in kidney. Note the relatively higher activity of LDH - 2 and LDH - 3 forms. C - LDH isozyme pattern in liver. Note the predominance of LDH - 5, and LDH - 4 and LDH - 3 as other isozyme forms. D, E - LDH isozyme patterns in tumor supernatant and tumor cells respectively. Note the high activity of LDH - 5 and absence of other isozyme forms in the tumor cells. In A, B and C lanes 1, 2, 3, 4, 5 and 6 represent normal, tumor bearing, and 24 hr, 48 hr, 72 hr and 96 hr of cisplatin treated animals respectively. In D and E lanes 1, 2, 3 and 4 represent tumor bearing, and 24 hr, 48 hr and 72 hr of cisplatin treated animals respectively. Note the presence of a new isozyme (LDH-T) in the serum of tumor bearing and cisplatin treated tumor bearing animals which can also be seen in tumor supernantant and tumor cells in varying intensities.

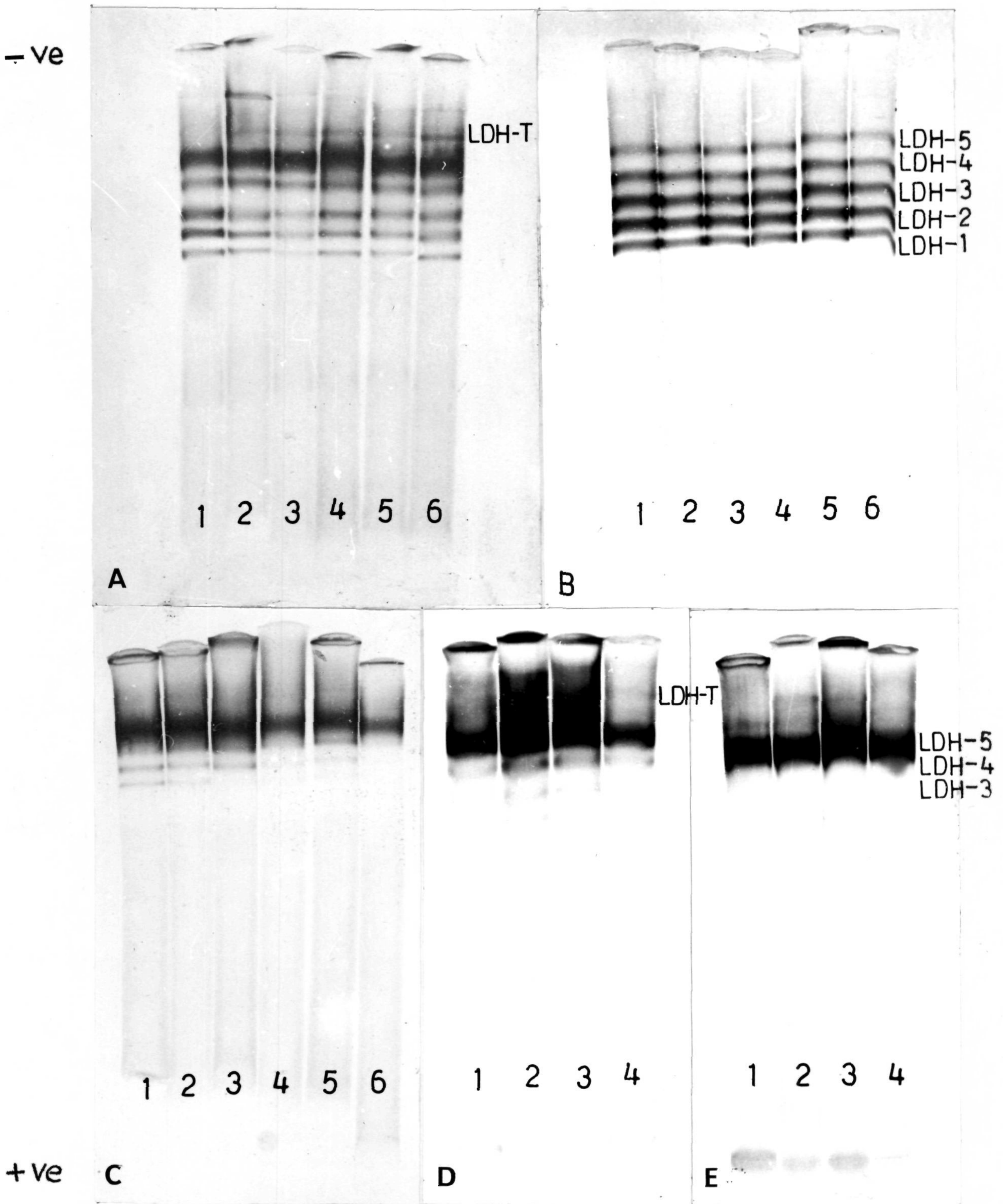


Fig. 15

Fig. 16. Histogram showing changes in Na^{+}+K^{+}-ATPase activity in tumor cells (A) and tumor supernatant (B) treated with or without cisplatin treatment. Note the gradual decrease in the enzyme activity in tumor cells (A) following 1-4 days of the treatment. Also note the increase in the enzyme activity in tumor supernatant (B) following 1-2 days of the treatment. TB = Tumor bearing mice (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.05$; ** = $P < 0.01$.

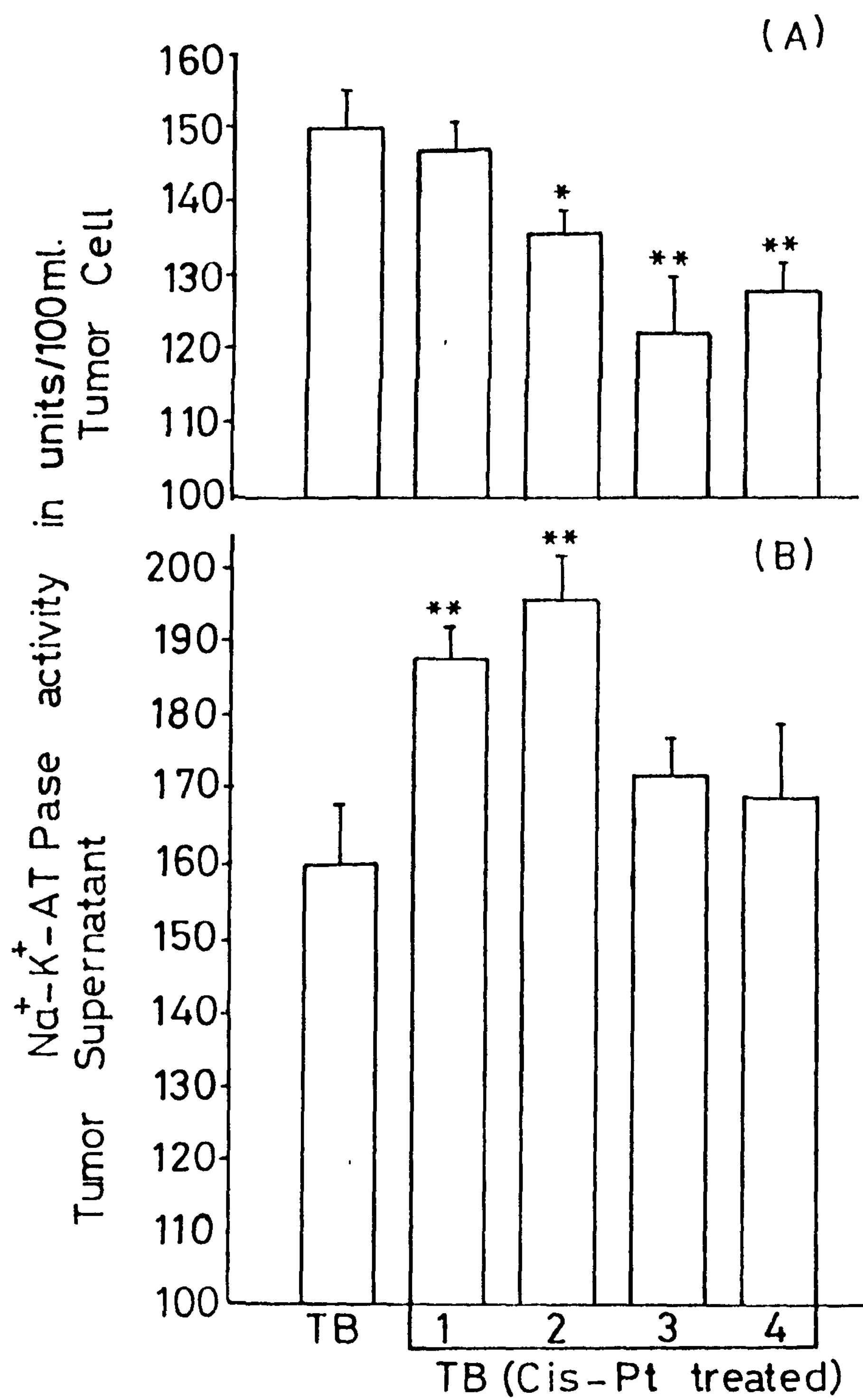


Fig. 16

Fig. 17. Histogram showing the changes in 5'-nucleotidase activity in liver (A) and kidney (B) following treatment with or without cisplatin. Note the significantly higher enzyme activity in the liver of tumor bearing mice which decreased significantly following 1-4 days of the treatment. Also note the significantly higher enzyme activity in the kidney of tumor bearing mice which increased gradually following 2-3 days of the treatment.

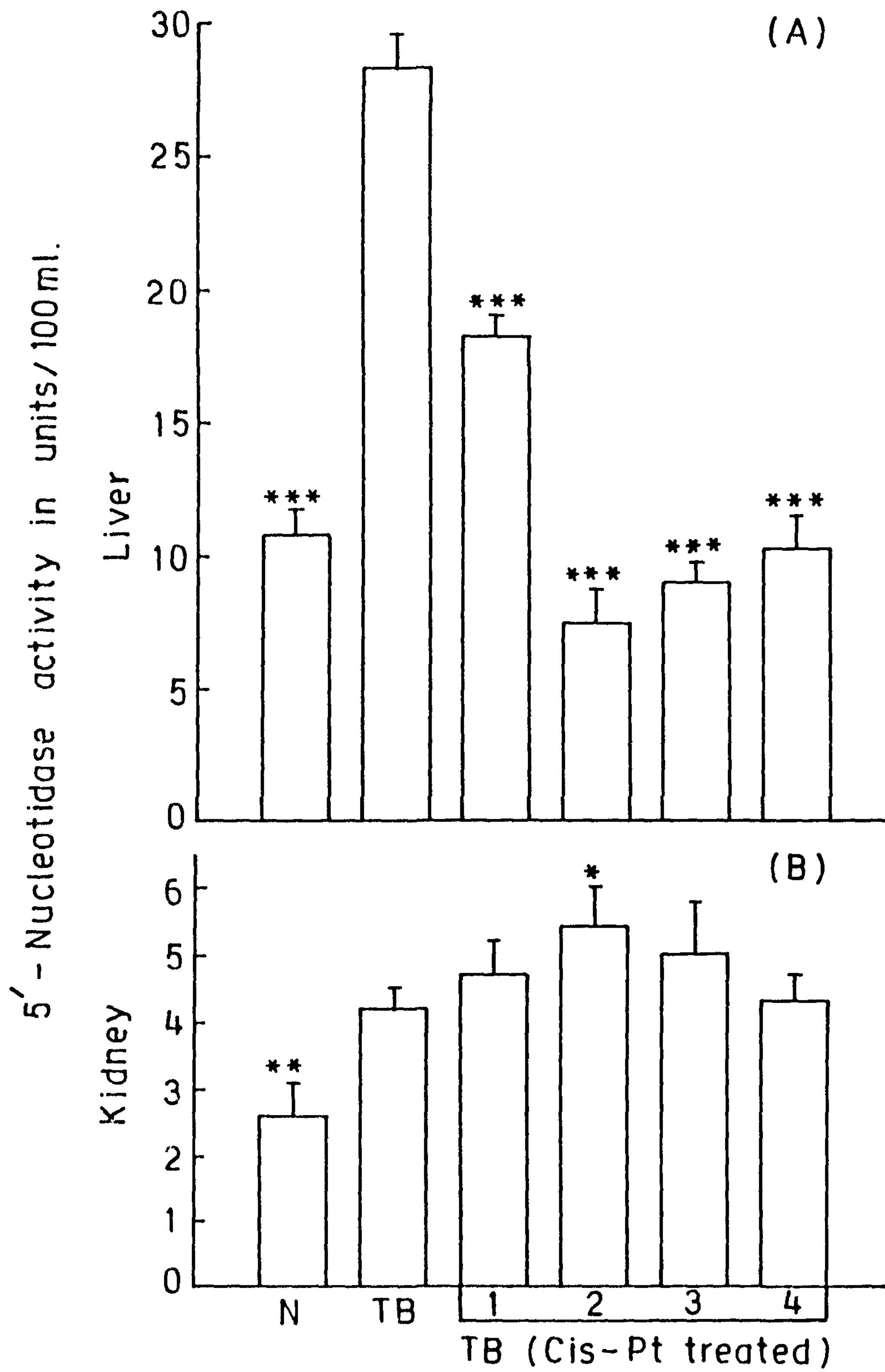


Fig. 17

Fig. 17. C and D showing changes in 5'-nucleotidase activity in tumor cells (C) and tumor supernatant (D) following treatment with or without cisplatin. Note the progressive decrease in enzyme activity in the tumor cells (C) following 1-4 days of the treatment. also note the significant increase in enzyme activity in the tumor supernatant following 1-3 days of the treatment. N = Normal mice; TB = Tumor bearing mice (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.02$; ** = $P < 0.01$; *** = $P < 0.001$.

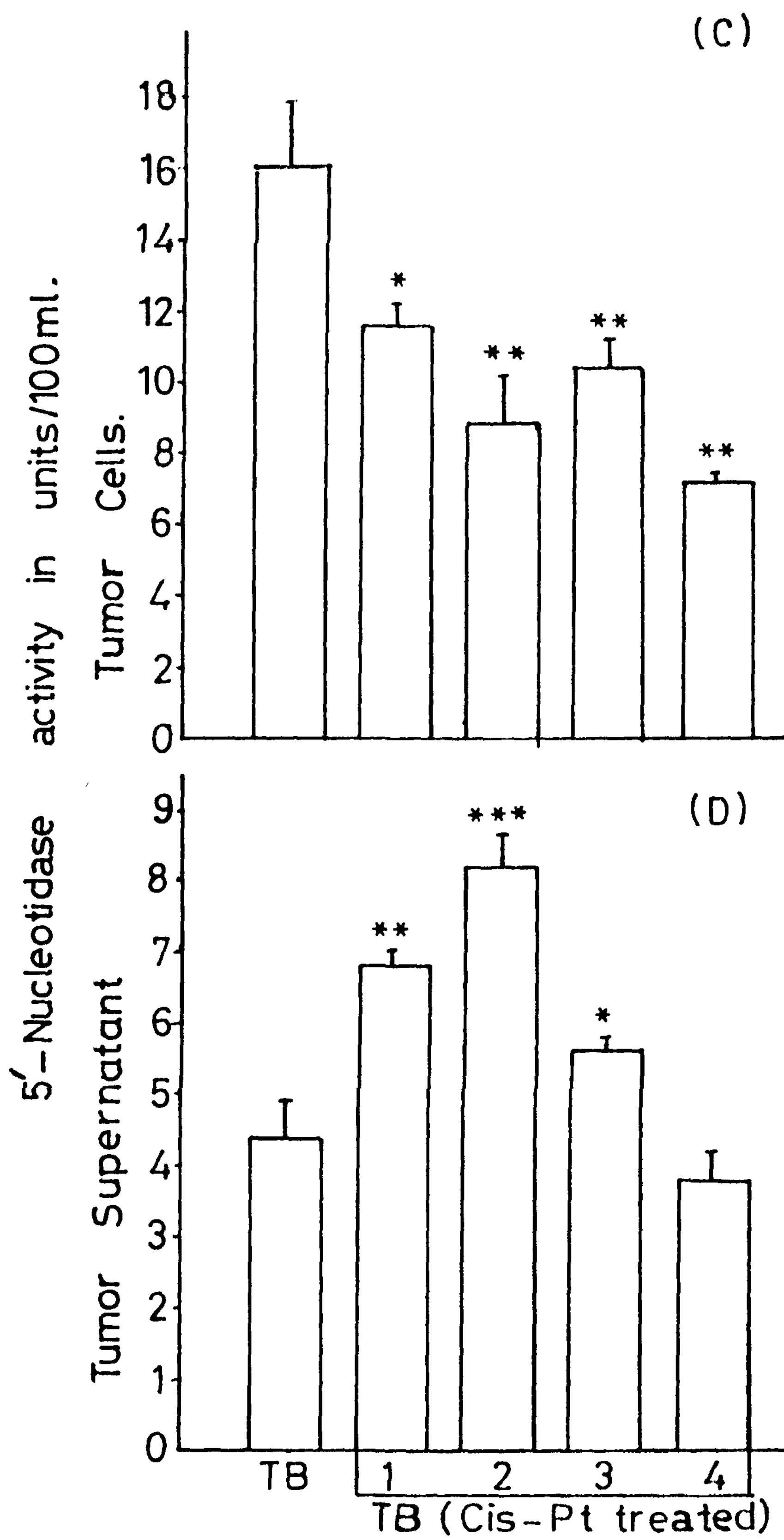


Fig. 17

Fig. 18. Histogram showing the changes in glutamic oxalacetic transaminase (GOT) activity in serum (A) and tumor supernatant (B) following treatment with or without cisplatin. Note the high level of GOT activity in the serum of tumor bearing mice than the normal animals which decreased significantly following 1 day of the treatment. But, following 2-3 days of the treatment the GOT activity registered an increase (A). In the tumor supernatant (B) also GOT activity decreased significantly following 1 day of the treatment; but increased activity could be found following 3-4 days of the treatment. N = Normal mice; TB = Tumor bearing mice (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student't t-test. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

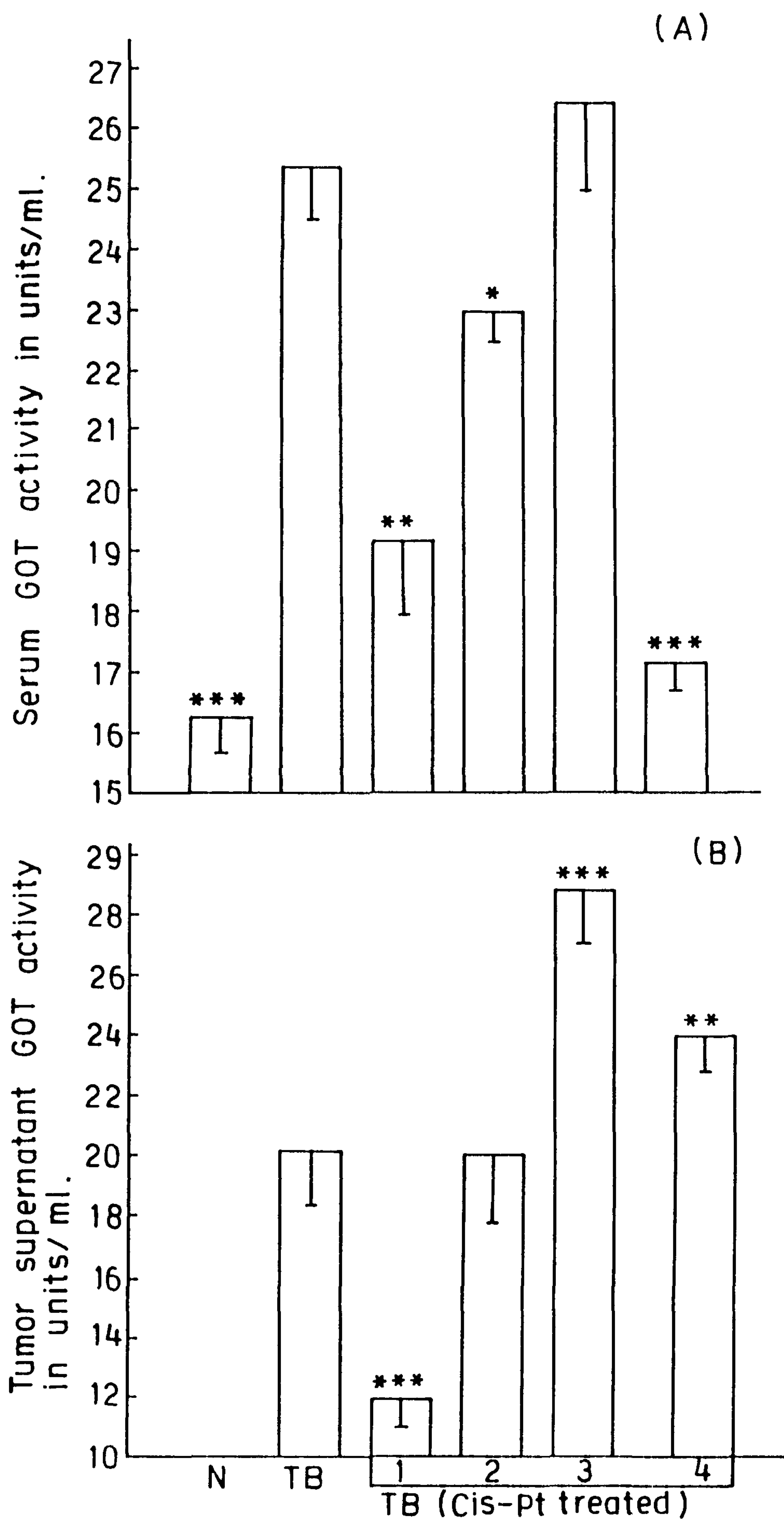


Fig. 18

Fig. 19. Histogram showing the changes in glutamic pyruvic transaminase (GPT) activity in serum (A) and tumor supernatant (B) following treatment with or without cisplatin. Note the decreased GPT activity following 1-2 days of the treatment which however showed an increasing trend at 3-4 days in the serum (A). Also note the similar pattern of change in GPT activity in the tumor supernatant (B) following cisplatin treatment. N = Normal mice; TB = Tumor bearing mice (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.02$; ** = $P < 0.01$.

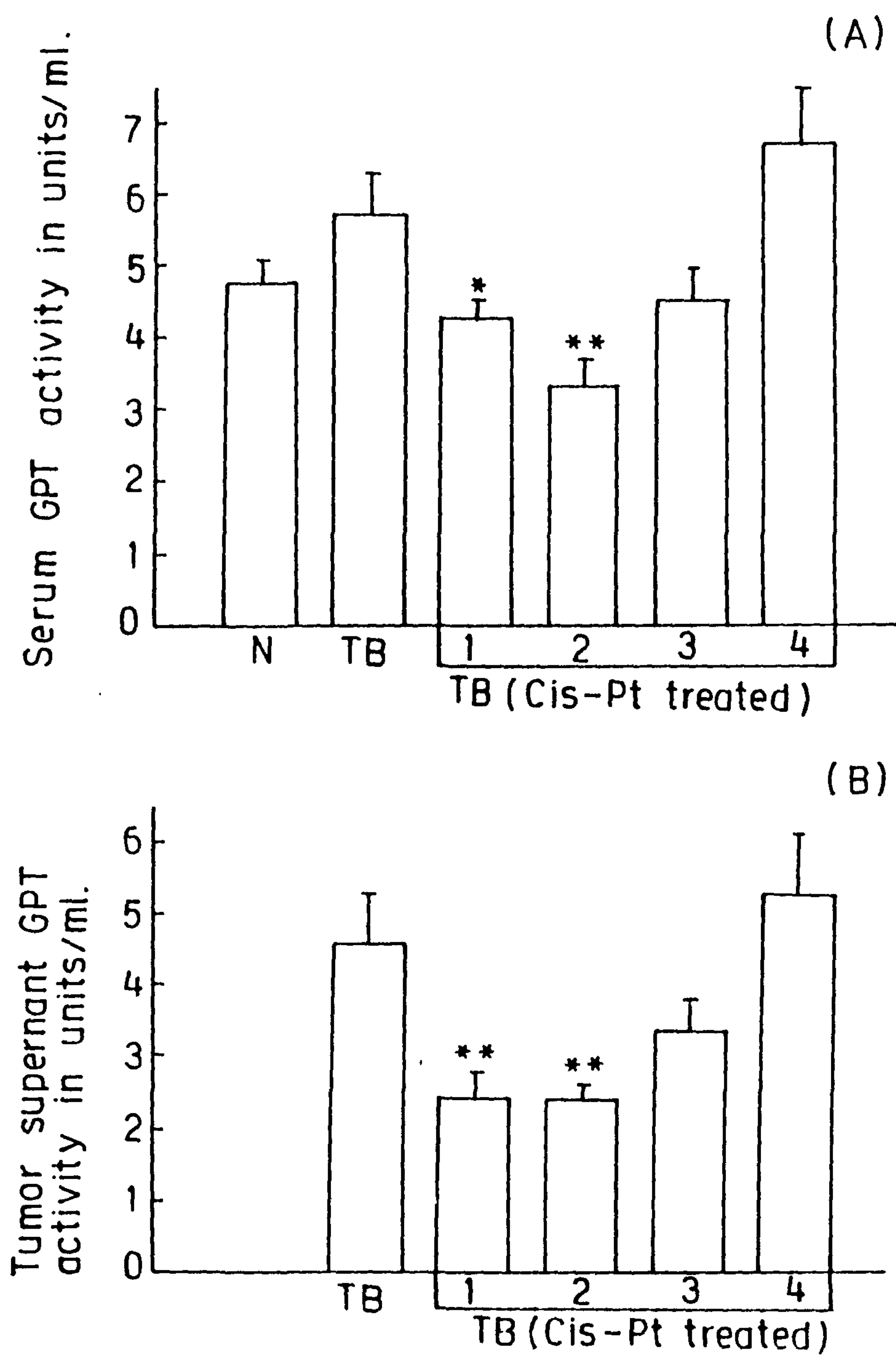


Fig. 19

Fig. 20. Histogram showing the changes in arginase activity in liver (A), kidney (B) and tumor supernatant (C) following treatment with or without cisplatin. In the liver of tumor bearing mice more than two fold lower enzyme activity is found than the normal mice. Following cisplatin treatment the enzyme activity significantly increased following 8-48 hr of treatment and by 96 hr significant decrease is noted (A). In the kidney (B) significantly lower enzyme activity is noted in the tumor bearing mice than the normal mice. Cisplatin treatment for 48-96 hr resulted an overall decrease in enzyme activity. Note the progressive increase in the enzyme activity in tumor supernatant following the treatment. NL = Liver of normal mice; TL = Liver of tumor bearing mice; TL (Cis-Pt) = Liver of tumor bearing mice treated with cisplatin for 8-48 hr; NK = Kidney of normal mice; TK = Kidney of tumor bearing mice; TS = Tumor supernatant.

Statistical analysis: Student's t-test. * = $P < 0.05$; ** = $P < 0.02$; *** = $P < 0.01$; **** = $P < 0.001$.

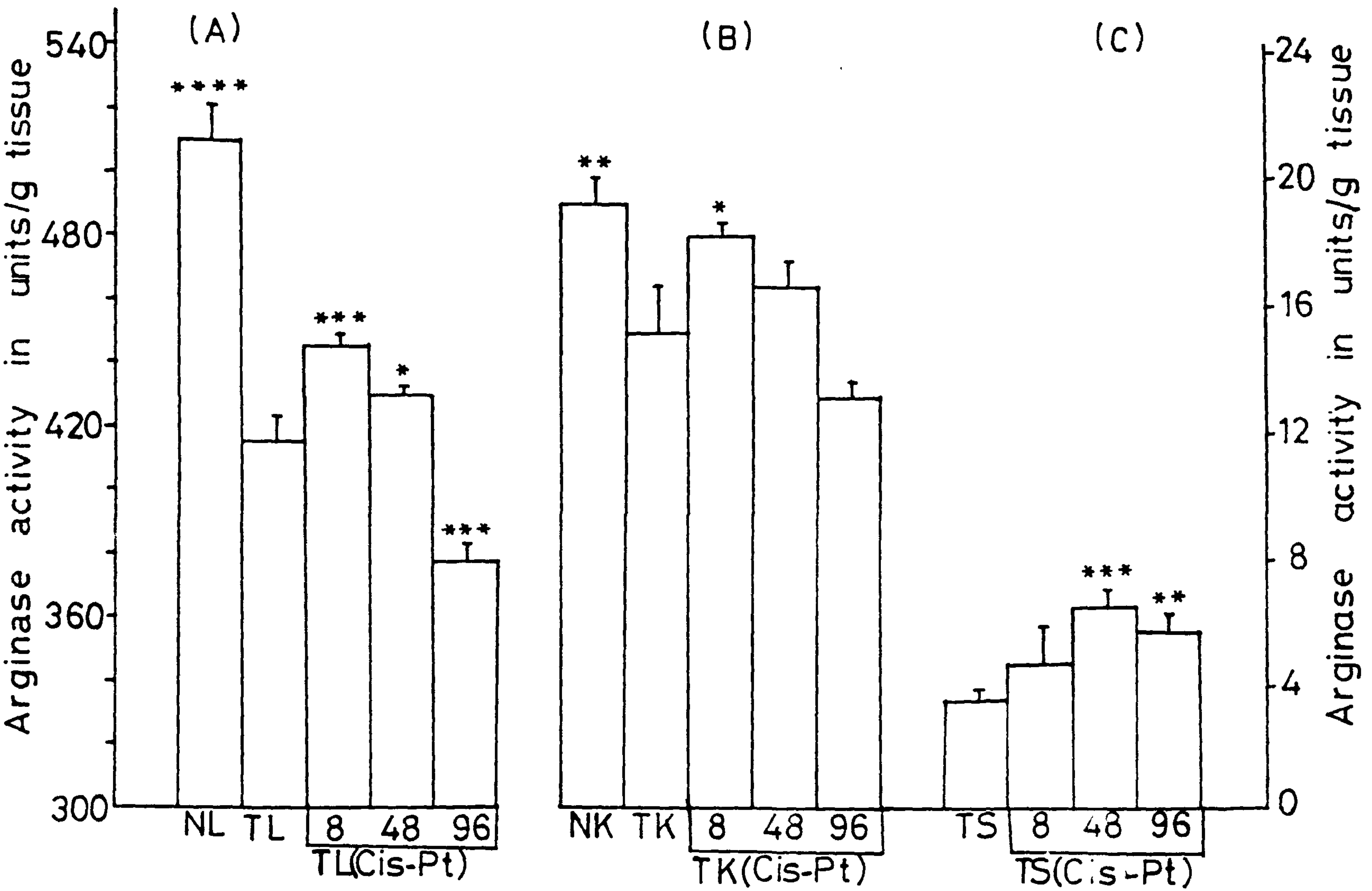


Fig. 20

Fig. 21. Histogram showing changes in cathepsin activity in serum (A, C) and tumor supernatant (B, D) following treatment with or without cisplatin. In the serum of normal mice cathepsin B (A) and cathepsin H (C) activity is significantly higher than the tumor bearing animals. Note the overall increase of both cathepsin B and cathepsin H following 2-4 days of the treatment (A,C). In the tumor supernatant (B,D) note the significant increase in both cathepsin B and cathepsin H activity following 1-4 days of the treatment. N = Normal [illegible]; TB = Tumor bearing mice (control); TB (Cis-Pt treated) = Tumor bearing mice treated with cisplatin for 1-4 days.

Statistical analysis: Student's t-test. * = $P < 0.05$; ** = $P < 0.01$.

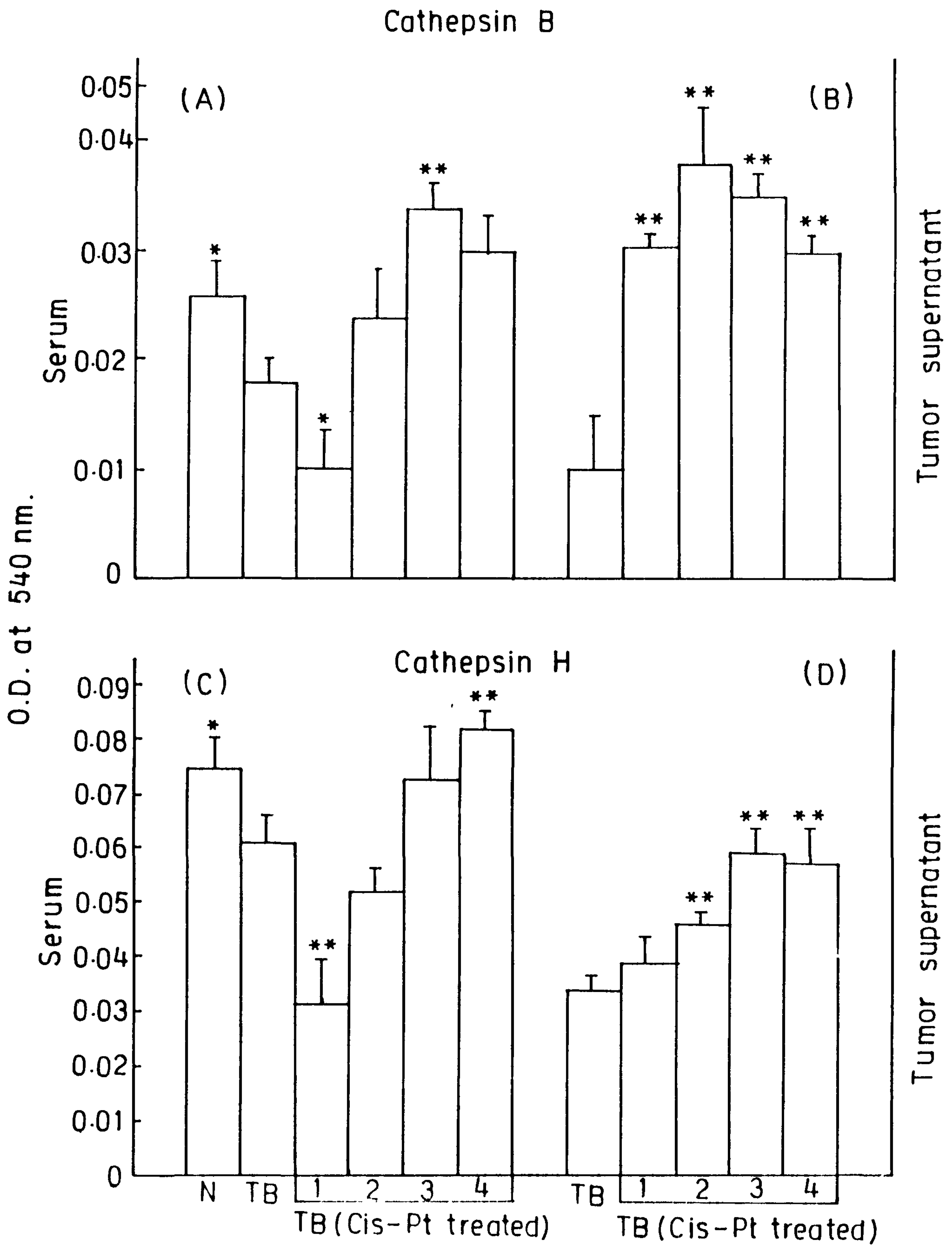


Fig. 21

Fig. 22. Graph showing the effect of subtherapeutical dose of cisplatin (4.0 mg/kg b.w.) and 0.5% vitamin C given separately on the survival of tumor-bearing mice. ●——● control, tumor transplanted mice without any treatment; ---- tumor transplanted mice receiving 0.5% vitamin C from the first day; ▲——▲ tumor transplanted mice receiving a single i.p. injection of cisplatin on the 10th day of transplantation. The experiment was repeated twice with 10 animals in each group.

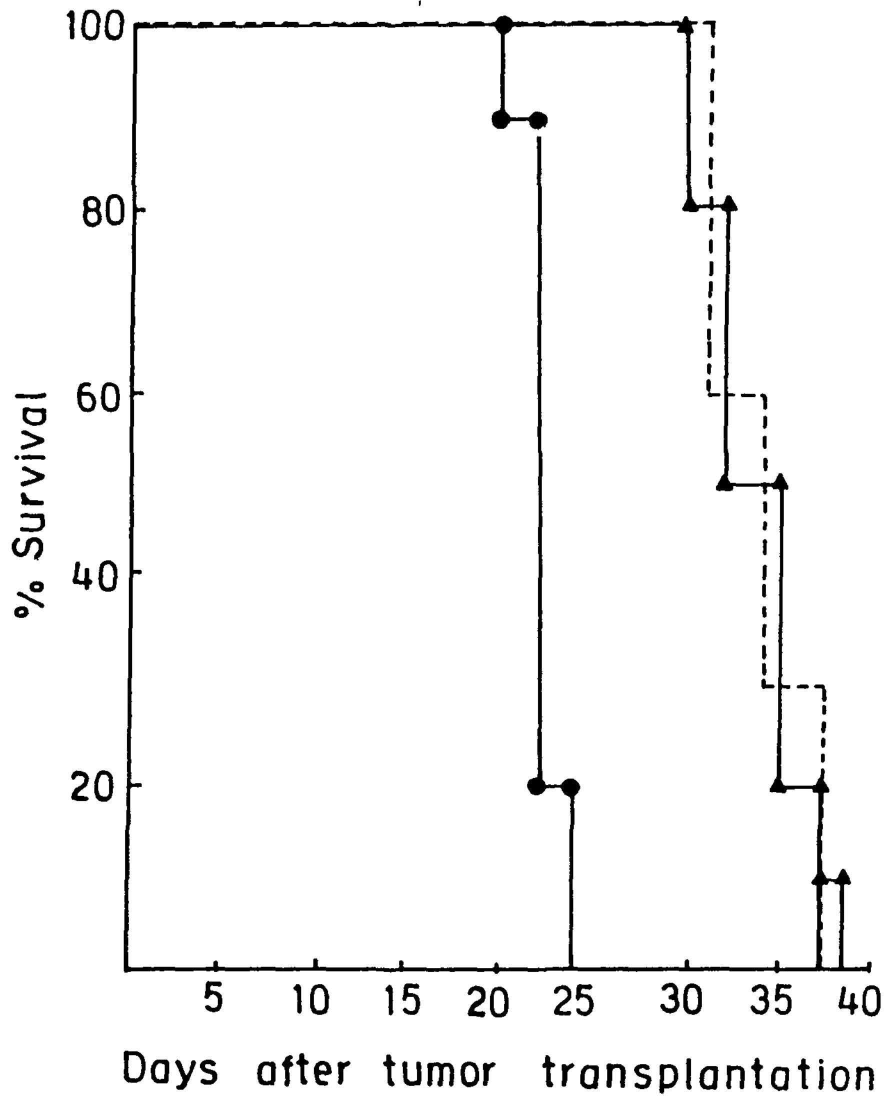


Fig. 22

Fig. 23. Graph showing the effect of subtherapeutic dose of cisplatin plus vitamin C on the survival of tumor-bearing mice. ●——● control tumor transplanted mice without any treatment; ▲——▲ tumor-bearing mice receiving cisplatin on the 5th day after tumor transplantation; ---- animals receiving 0.25% vitamin C from the first day and cisplatin injected on the 10th day ■——■ ; animals kept on 0.5% vitamin C from the first day and cisplatin injected on the 10th day after tumor transplantation.

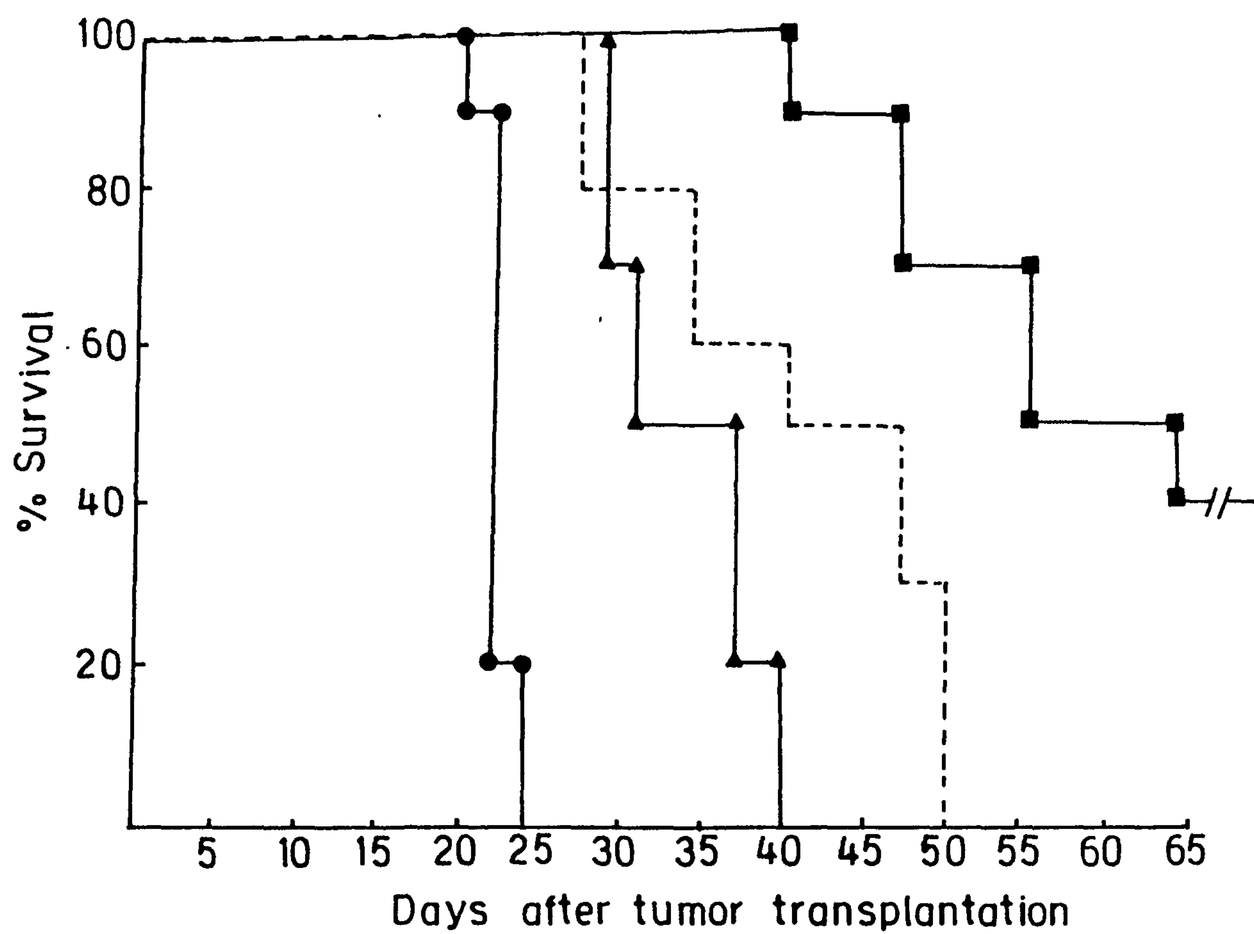


Fig. 23

Fig. 24. The graph showing the average increase in body weight of mice of different groups. A-Control, tumor transplanted mice without any treatment; B - Tumor-bearing mice receiving 0.5% vitamin C from the first day; C - Tumor-bearing mice treated with cisplatin only on the 10th day; D - Tumor-bearing mice kept on 0.25% vitamin C from the first day and given a cisplatin i.p. injection on the 10th day; E - Tumor-bearing mice kept on 0.5% vitamin C from the first day and receiving cisplatin on the 10th day after tumor transplantation; F - normal mice without tumor.

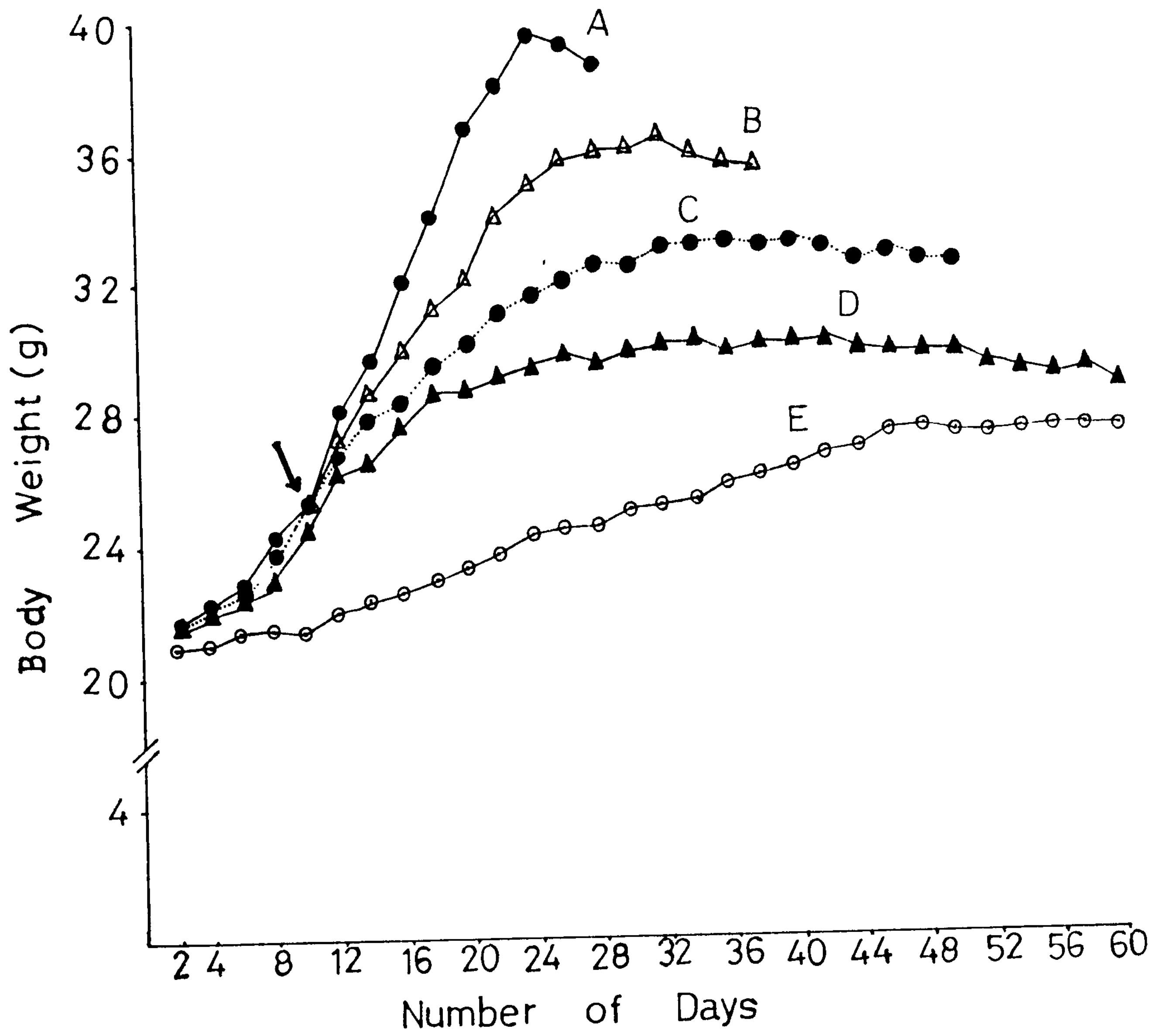


Fig. 24

TABLE 4 - **Antitumor activity of cisplatin plus vitamin C against murine ascites Dalton's lymphoma.**

Drug	Route of treat-	Day of treat-ment	Dose (mg/kg)	No. of mice	Survival (mean±SD) in days	ILS %
Control	-	-	-	10	21.4 ± 1.00	-
Vitamin C	oral	1-10[a]	8.5-9.5[b]	10	26.1 ± 2.50	21.96
Cisplatin	i.p.	10	4	10	34.3 ± 1.25	60.28
Vitamin C + Cisplatin	oral i.p.	1-10[c] 10	4.0-4.8[b] 4	10	52.1 ± 2.90	143.45
Vitamin C + Cisplatin	oral i.p.	1-10[a] 10	8.5-9.5[b] 4	10	> 65	>203.73

a = From the 1st day after transplantation till the 1 th day (0.5% vitamin C in drinking water).

b = Average amount consumed in mg/day/animal.

c = From the 1st day after transplantation till the 10th day (0.25% vitamin C in drinking water).

TABLE 5 - Effect of vitamin C plus cisplatin on the growth of Dalton's lymphoma *in vivo*.

Drug	Route of treat-	Day of treat-ment	Dose (mg/kg)	No. of mice	Tumor weight 20(g) on day (mean±SD)	GIR % on day 20
Control	-	-	-	10	14.00 ± 0.96	-
Vitamin C	oral	1-10[a]	8.5-9.5[b]	10	12.10 ± 0.67	13.57
Cisplatin	i.p.	10	4	10	5.20 ± 0.37	62.85
Vitamin C + Cisplatin	oral i.p.	1-10[c] 10	4.0-4.8[b] 4	10	3.96 ± 0.31	71.71
Vitamin C + Cisplatin	oral i.p.	1-10[a] 10	8.5-9.5[b] 4	10	1.6 ± 0.35	88.57

a = From the 1st day after transplantation till the 10th day (0.5% vitamin C in drinking water).

b = Average amount consumed in mg/day/animal.

c = From the 1st day after transplantation till the 10th day (0.25% vitamin C in drinking water).

TABLE 6 - Influence of vitamin C plus cisplatin on spleen and thymus weight and on tumor pH under different experimental conditions.

Group	Spleen Weight (g)	Thymus weight (g)	Tumor pH
Normal mice	0.328 ± 0.018[a*]	0.043 ± 0.010[¶]	
Tumor-bearing mice (control)	0.264 ± 0.011	0.027 ± 0.008	6.93 ± 0.16
Vitamin C plus cisplatin treated tumor bearing mice	0.354 ± 0.021[a*]	0.047 ± 0.007[b¶]	6.27 ± 0.09[a]

Statistical analysis: Student's t-test. a = P 0.01; b = P 0.05.
Results having similar superscripts do not differ significantly.

Fig. 25. Histogram showing the changes in serum ascorbic acid content under different experimental conditions. Note the decrease in ascorbic acid content in the tumor bearing mice than the normal mice. Also note the significant increase in ascorbic acid content of the tumor bearing mice following vitamin C supplementation. N = Normal animal; TB = Tumor bearing animal; VT = Tumor bearing mice provided with 0.5% vitamin C in drinking water from the 1st day after transplantation; PT = Tumor bearing mice treated i.p. with cisplatin (4 mg/kg b.w.); VP = Tumor bearing mice treated with vitamin C (0.5%) plus cisplatin (4 mg/kg b.w.).

Statistical analysis: Student's *t*-test. Results having the same superscript differ significantly. a = $P < 0.05$.

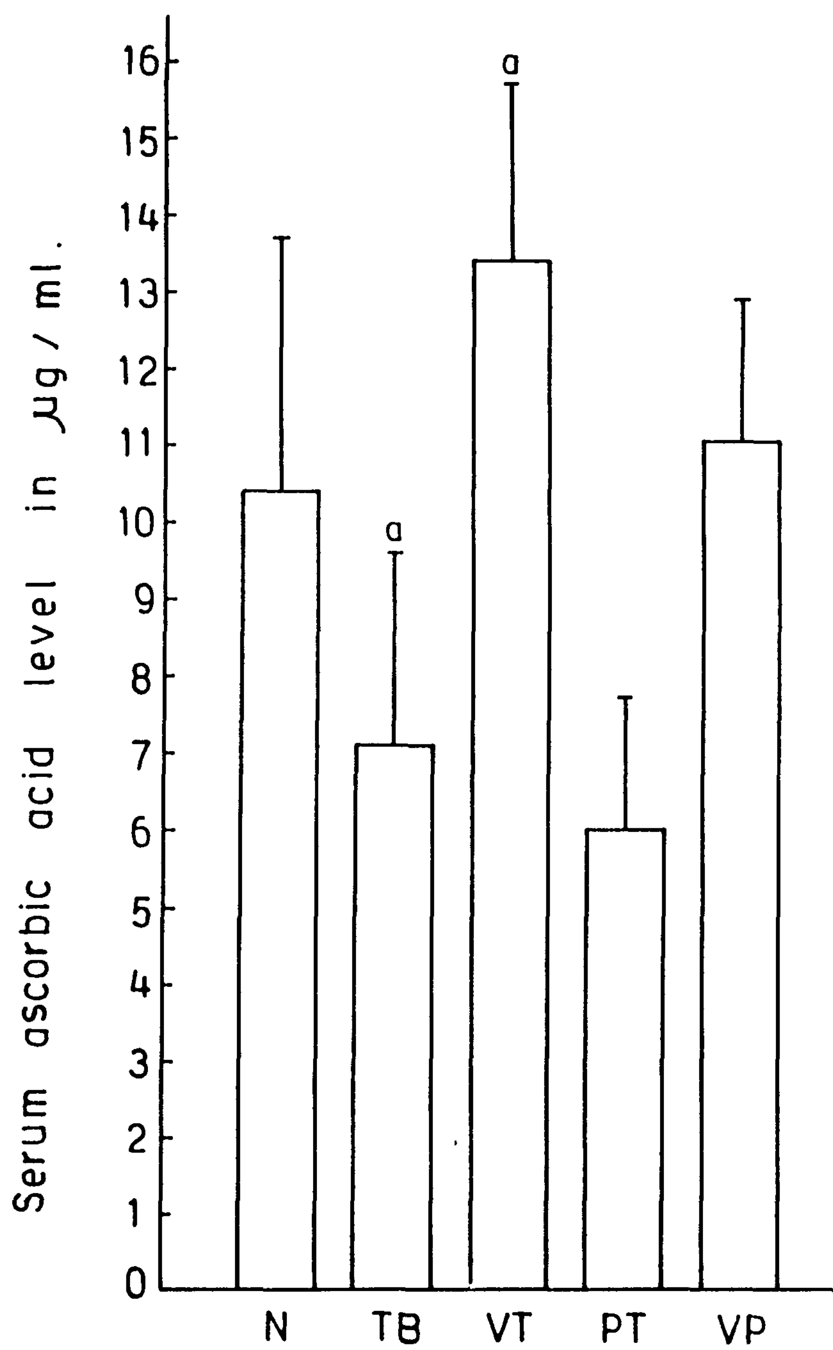


Fig. **25**

TABLE 7 - **Effect of cisplatin, vitamin C and cisplatin plus vitamin C on total leukocyte count in Dalton's lymphoma bearing mice.**

Treatment and dose	Total leukocyte count (in x10^3 cells/ml)					
	Days after treatments					
	1	2	3	4	5	6
Tumor bearing (Control)	10433* (603)	ND	ND	ND	ND	ND
Cisplatin 4 mg/kg b.w.	14500^b (683)	15866^c (650)	11600 (915)	10233 (850)	7833^a (874)	6800^b (600)
Cisplatin 8 mg/g b.w.	13200^b (600)	14400^b (872)	10500 (1300)	7233^a (1266)	6066^b (850)	5066^c (902)
Vitamin C	11500 (954)	ND	ND	ND	ND	ND
Vitamin C + Cisplatin 4 mg/kg b.w.	14266^b (945)	15733^c (702)	14333^b (1416)	13333^b (611)	12800^a (693)	10533 (945)

Vitamin C, 0.5% was given in drinking water from the first day after tumor transplantation.

* On the day of treatment

ND = Not determined.

Statistical analysis: Student's t-test. a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$.

Fig. 26. Graph showing the Con A agglutination pattern of ascites Dalton's lymphoma cells under different experimental conditions *in vitro*. Control tumor cells (Δ——Δ) show high degree of agglutination. Note the gradual decrease in the agglutination of cells treated with vitamin C (O——O) or cisplatin (●----●) for 15-60 min. Also note the maximal decrease in the agglutination in the vitamin C plus cisplatin combined treated group (O----O).

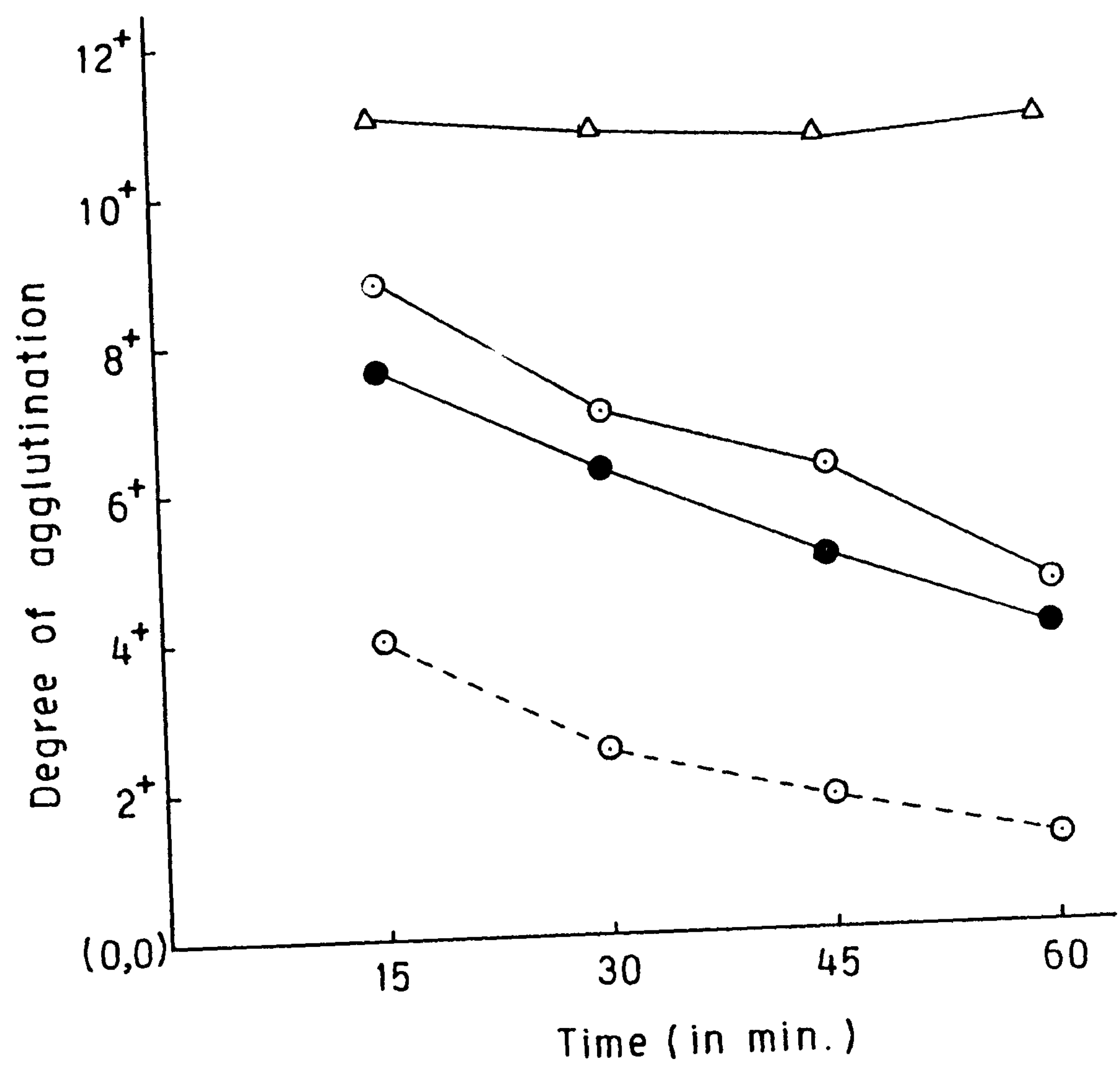


Fig. 26

Fig. 27. Con A-FITC fluorescence pattern of Dalton's lymphoma cells under different experimental conditions *in vitro*. A-Control tumor cells showing bright even fluorescence. B-15 min of the treatment showing decrease in fluorescence intensity. C-Decrease in fluorescence intensity is followed with the appearance of fluorescent grains at 45 min of the treatment. D-Weak fluorescence and decrease in fluorescent grains at 60 min of treatment. b-15 min of treatment of vitamin C and cisplatin showing decrease in fluorescence and appearance of sharp, distinct granular fluorescence over the cells. c-30 min of treatment results further decrease in fluoresence intensity. d-At 45 min of the treatment further decrease in fluorescence intensity with the disappearance of fluorescent grains is noted.

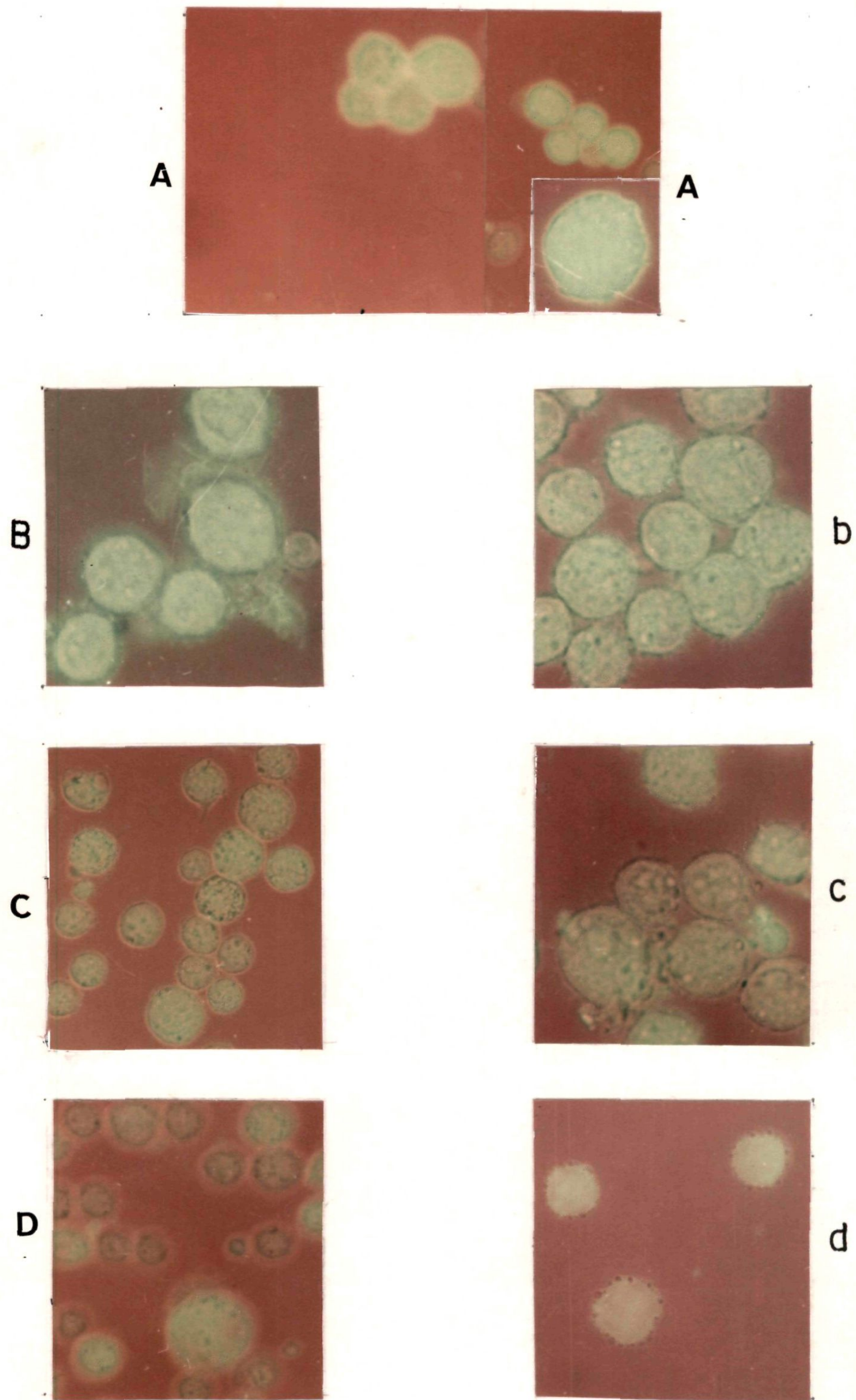


Fig. 27

Fig. 28. Photomicrographs of bone marrow metaphase spreads showing different types of chromosal aberrations induced by cisplatin. A - Control bone marrow cell showing normal complement (2n = 40) of chromosomes. B, C - Cisplatin treated bone marrow cells showing chromatid gaps, chromatid breaks, acentric fragments and chromatid exchanges (arrows). D - A pulverized (highly damaged) cell showing extensive DNA destruction. An artifact in the form of a black dot appearing in all the photo plates due to camera error should be taken note of.

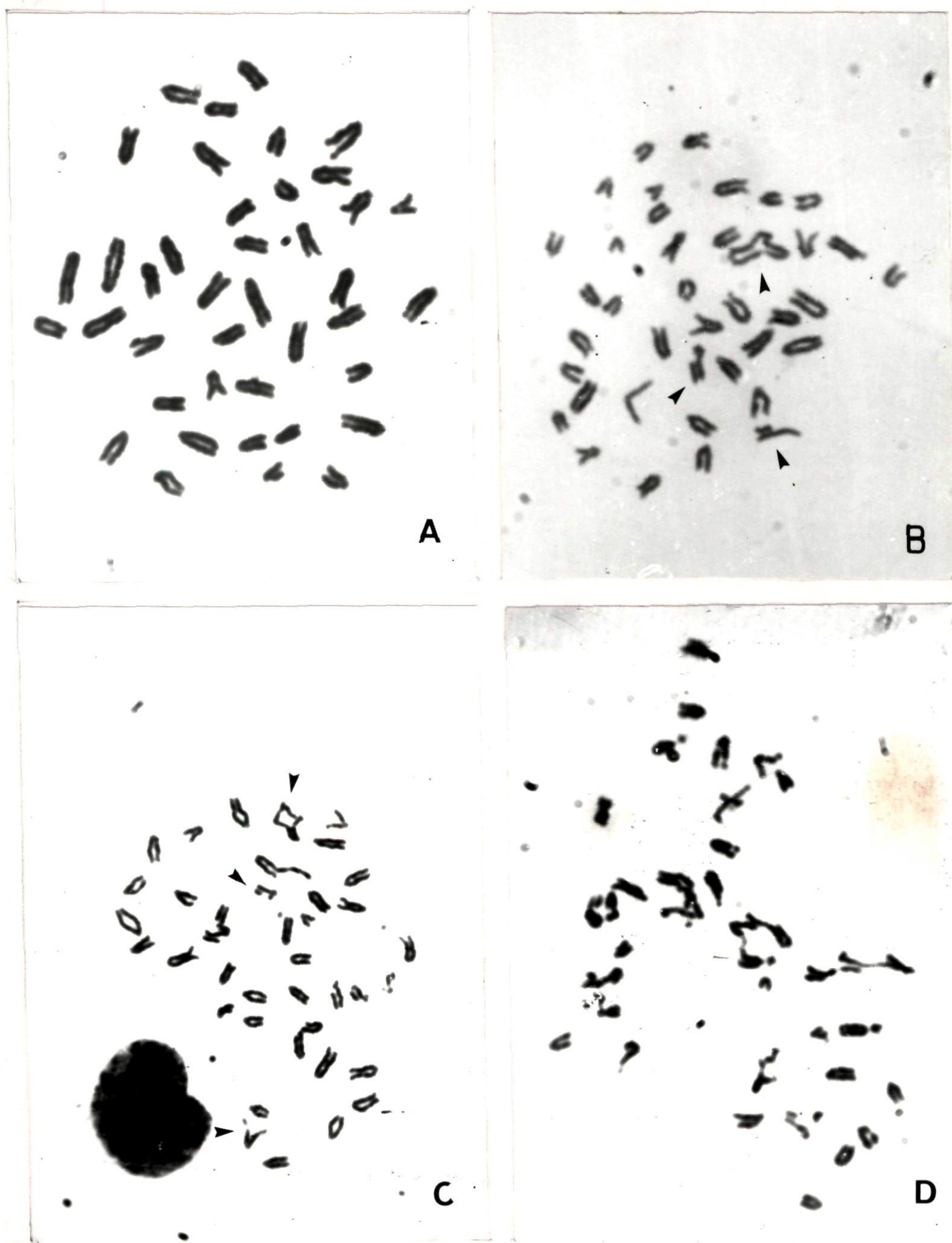


Fig. 28

TABLE 8 - Effect of vitamin C on the frequency of chromosomal aberrations in bonemarrow cells induced by cisplatin.

Treatment conditions	Total No. metaphases	Mean % of abnormal metaphases	% of Chromatid		% of exchanges	% of isochromatid		Mean % SCU	Total % of abnormalities (Mean ± SD)*
			Break	Gap		Break	Gap		
Control	600	0.83	0.16	0.33	0.16	-	-	-	0.66 ± 0.51
CDDP 24 hr	300	40.00	50.33	12.00	5.33	2.33	4.00	1.66	59.66 ± 5.03
CDDP 48 hr	400	22.50	19.50	8.50	3.75	2.00	1.75	2.50	27.75 ± 3.59[a]
CDDP 72 hr	500	14.60	7.21	5.60	2.80	0.40	0.20	2.00	12.40 ± 1.67[b]
CDDP 96 hr	400	6.25	4.00	1.75	2.25	0.50	0.25	0.25	7.25 ± 1.25[c]
Vit. C + CDDP (24 hr)	300	26.33	48.33	7.00	3.33	2.33	3.33	0.66	54.66 ± 7.02
Vit. C + CDDP (48 hr)	300	12.00	9.33	4.00	3.66	1.00	1.00	3.00	17.33 ± 2.51[a]
Vit. C + CDDP (72 hr)	300	8.66	2.66	2.33	2.66	0.66	2.00	1.33	7.33 ± 2.30[b]
Vit. C + CDDP (96 hr)	300	3.00	1.33	0.66	1.00	0.40	0.33	0.33	3.33 ± 0.57[c]

Cisplatin was given i.p. (4 mg/kg b.w.) and vitamin C (0.5%) in drinking water from the first day after tumor transplantation.

SCU = Sister chromatid union.

* Values are excluding gaps.

Statistical analysis: Student's t-test. Results with same superscripts differ significantly from each other. a = $P < 0.02$; b = $P < 0.05$; c = $P < 0.02$

Fig. 29. Photomicrographs showing micronucleated bone marrow cells induced by cisplatin. A-D: Poly chromatic erythrocytes (PCEs) with micronuclei. E, F: Normochromatic erythrocytes (NCEs; normocytes) with micronuclei. G-I: Nucleated cells with micronuclei.

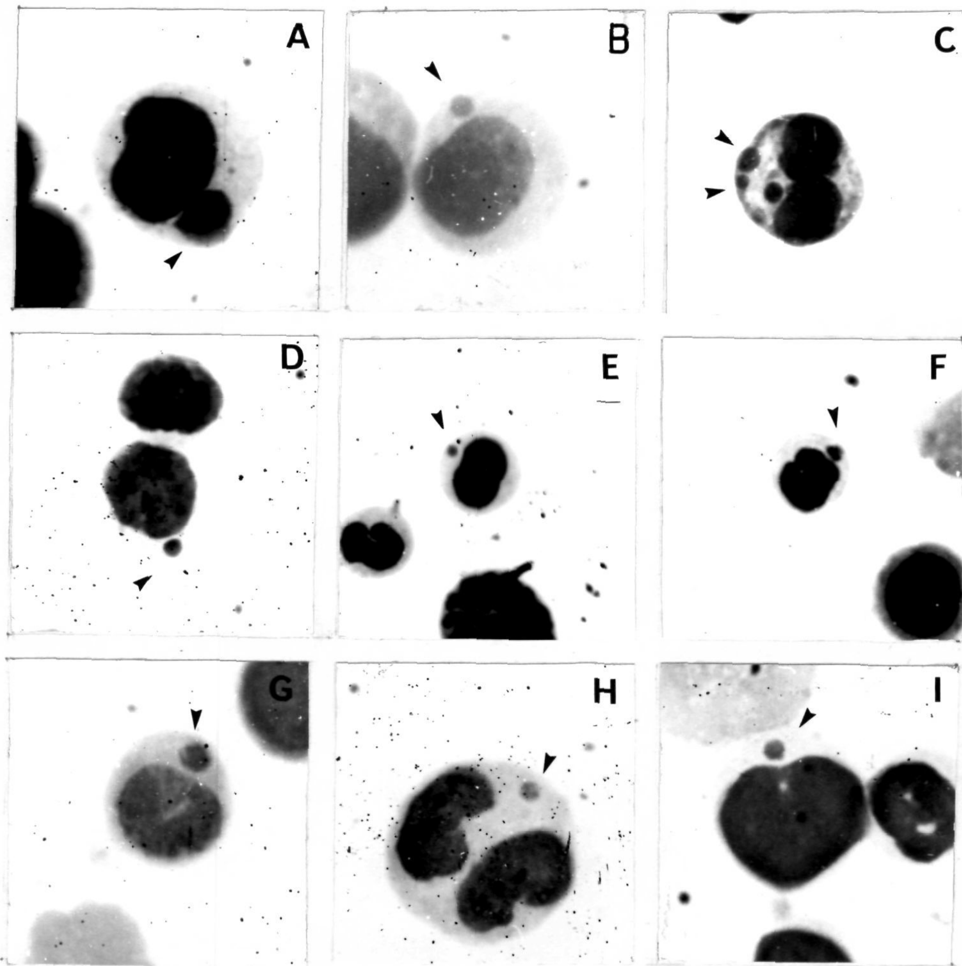


Fig. 29

TABLE 9 - **Effect of vitamin C on the incidence of micronucleated bone marrow cells induced by cisplatin in mice. (Values are mean ± SD).**

Treatment conditions	Total No. of cells studied	% PCEs with MN	% NCEs with MN	% Nucleated cells with MN
Control	3000	0.13 ± 0.05	0.06 ± 0.05	0.16 ± 0.05
Cisplatin (8 mg/kg)	3000	$1.26 \pm 0.05^{c¶}$	0.53 ± 0.15^{b}	0.50 ± 0.10^{b}
Cisplatin (4 mg/kg)	3000	$0.56 \pm 0.15^{b¶\#}$	$0.46 \pm 0.05^{c¶}$	$0.30 \pm 0.05^{a\#}$
Vitamin C*	3000	0.16 ± 0.05	0.13 ± 0.05	0.10 ± 0.05
Vitmin C* + Cisplatin (4 mg/kg)	3000	$0.30 \pm 0.05^{a\#}$	$0.23 \pm 0.05^{a¶}$	$0.16 \pm 0.05^{\#}$

* = Vitamin C, 0.5% in drinking water from the first day after tumor transplantation. PCE = Polychromatic erythrocyte; NCE = Normochromatic erythrocyte; MN = Micronucleus.

Statistical analysis: Student's t-test. a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$. Values in the same column having similar superscripts are statistically different: ¶ = $P < 0.01$; # = $P < 0.05$.

TABLE 10 - Effect of vitamin C on the incidence of cisplatin induced sperm abnormality in mice.

Treatment conditions	No. of sperms studied/animal	Total No. of abnormal sperms	Mean % ± SD
Control	3000/3	66	2.20 ± 0.36
Cisplatin (1 day)	1500/3	221	7.33 ± 0.50[a]
Cisplatin (2 days)	1500/3	447	14.86 ± 2.61[b]
Cisplatin (10 days)	1500/3	298	9.93 ± 1.70[c]
Vitamin C + Cisplatin (1 day)	1500/3	174	5.80 ± 0.80[a]
Vitamin C + Cisplatin (2 days)	1500/3	230	7.66 ± 0.41[b]
Vitamin C + Cisplatin (10 days)	1500/3	198	6.60 ± 0.60[c]

Cisplatin was given i.p. (4 mg/kg b.w.) and Vitamin C (0.5%) in drinking water from the first day after tumor transplantation.

Statistical analysis: Student's t-test. Results with same superscripts differ significantly from each other. a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.05$.

Fig. 30. Graph showing the changes in serum uric acid concentration following treatment with or without cisplatin. Note the gradual increase in the serum uric acid level following 3-12 days of cisplatin treatment of the tumor bearing mice. ---- = Normal level of serum uric acid.

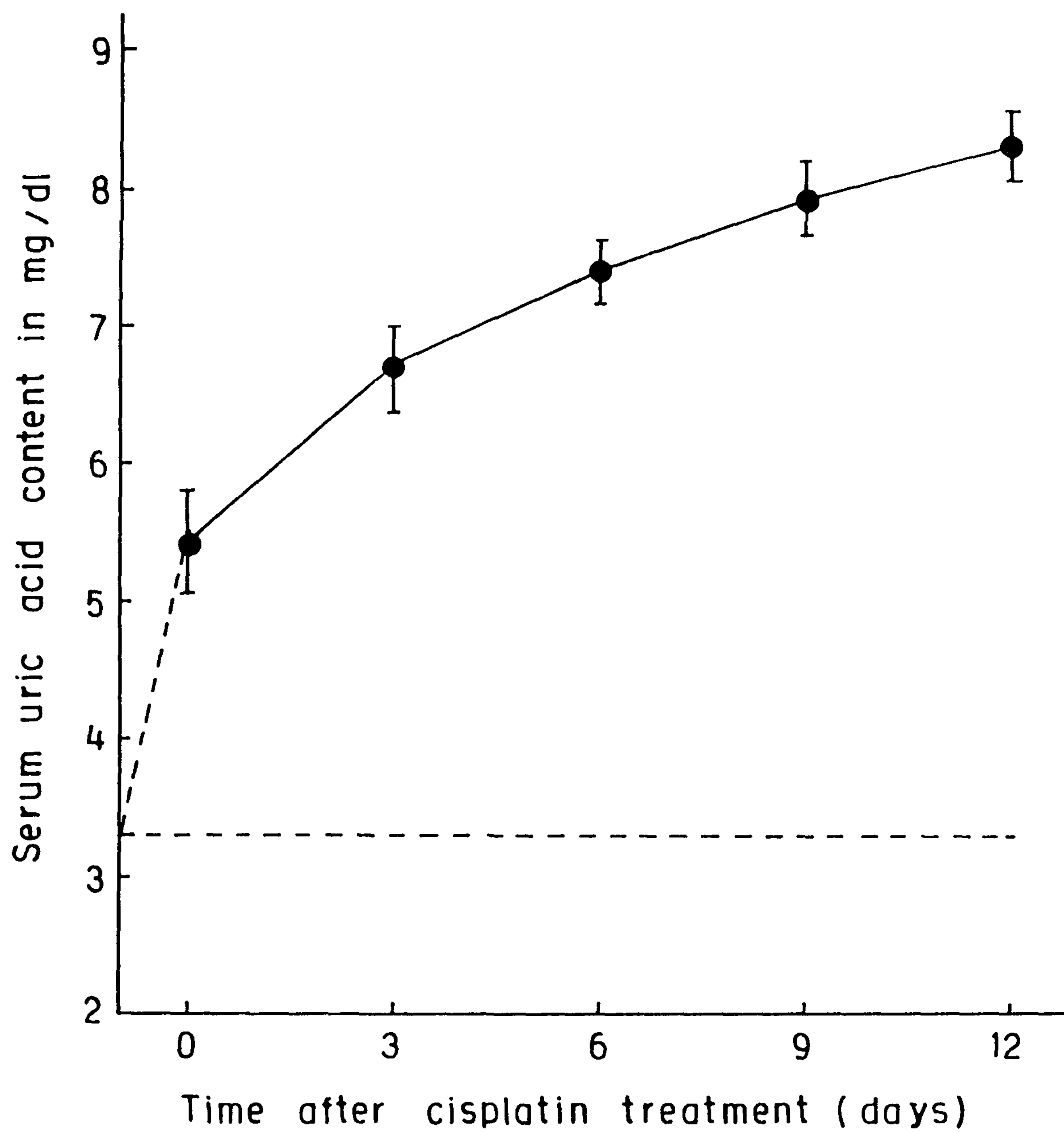


Fig. 30

Fig. 31. Graph showing the changes in serum urea level under different experimental conditions. ●——● control, tumor transplanted mice without any treatment; Δ——Δ tumor transplanted mice receiving 0.5% vitamin C from the first day showing no significant change in serum urea level; Θ----Θ tumor transplanted mice treated with cisplatin (8 mg/kg b.w.) showing marked increase in serum urea level; Θ——Θ tumor transplanted mice treated with cisplatin (4 mg/kg b.w.) shwoing elevations in serum urea level; ▲——▲ tumor bearing mice treated with vitamin C plus cisplatin showing significantly lower serum urea level than the groups treated with cisplatin alone; ---- normal range of serum urea level.

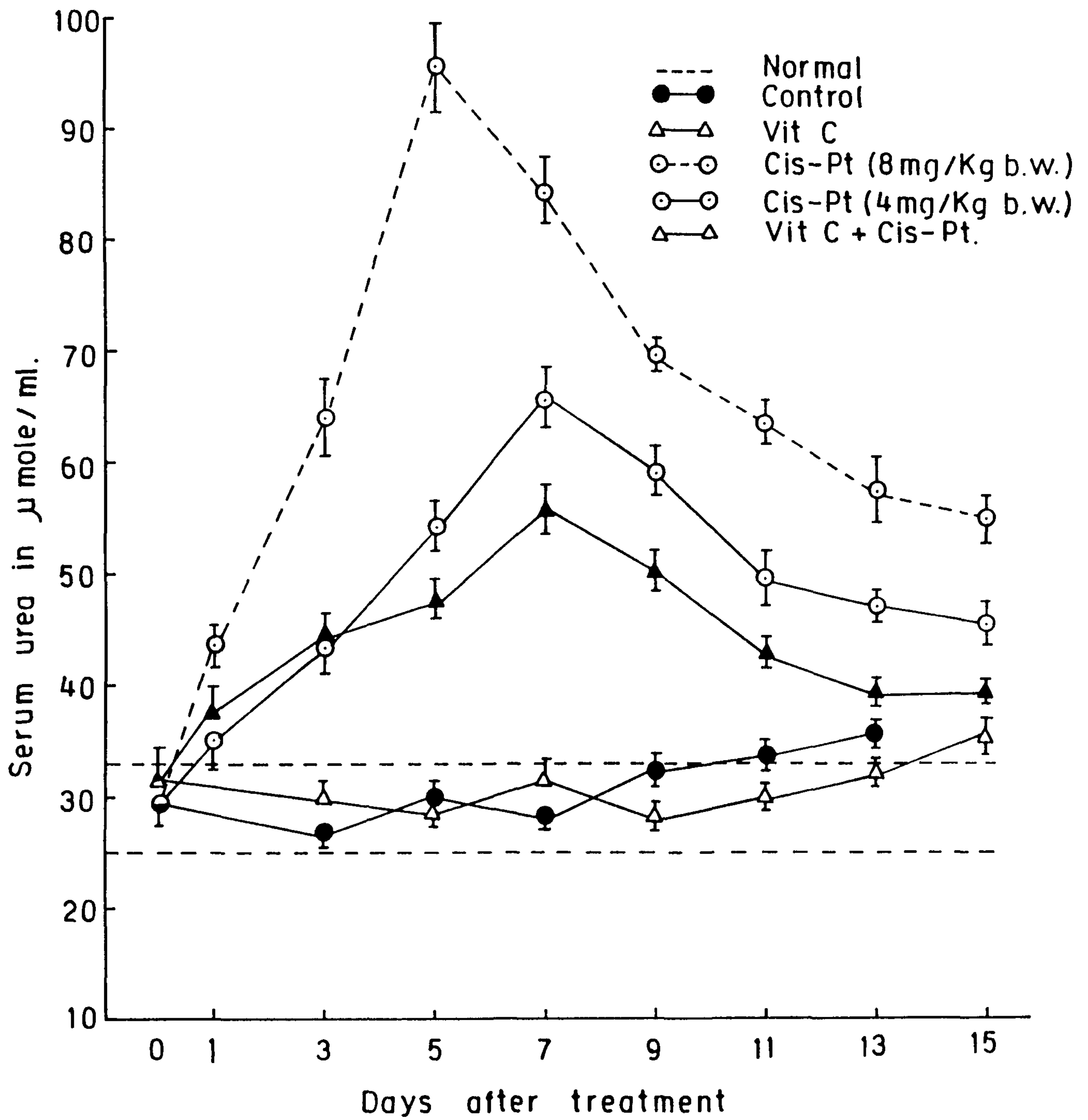


Fig. 31

D I S C U S S I O N

The tumor, Dalton's lymphoma was originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, MD, in 1947. Subsequently an ascites form was developed by repeated intraperitoneal transplantation of the tumor (Goldie and Felix, 1951). The ascites tumor used in the present study was collected from Gauhati University, India, and is being maintained by serial intraperitoneal transplantations since 1990 in 8-10 weeks old Swiss albino mice.

Chakraborty _et al_. (1984) studying on the clonal evaluation and C-heterochromatin distribution in Dalton's lymphoma have reported that the modal number was 71 and only one rb-marker. However, after successive transplantation _in vivo_ they reported the appearance of a new clone of cells which gradually predominated having two rb-markers and the modal number reduced to 70. In the present study however, it is found that the modal number is 66 with only one rb-

marker (Fig. 2). It has been reported that the cells adopted to growth *in vitro* often show karyotypic changes when compared with the karyotype of the *in vivo* parent line (Dowjat and Kawiak, 1979). The changes are usually characterized by the appearance of new marker chromosome(s) and/or the disappearance of the preexisting one(s). Therefore, the observed difference in the modal number in the present study and that reported earlier by Chakraborty *et al.* (1984) may be due to differential adaptability of Dalton's lymphoma to different heterogeneous hosts. This difference may also be attributed to the fact that the tumor cells used in the earlier study (Chakraborty *et al.*, 1984) was being maintained in Swiss albiono mice all through, whereas, the tumor used in the present study was recently adopted to Swiss albino mice and earlier was being maintained in C_3H/He mice for several successive passages *in vivo* at the Gauhati University.

From the studies on LDH isozyme patterns in tumor as well as various tissues, it is found that a new isozyme/ enzyme variant of LDH is preferentially expressed in the tumor tissues which can also be detected from the serum of the tumor bearing hosts (Fig. 15). This new isozyme specifically associated with this tumor may be expressed as a marker for Dalton's lymphoma. The significance of this new

isozyme variant in relation to tumor cell metabolism will be discussed in a later section.

Cisplatin, *cis*-dichlorodiammine platinum II has been shown to be effective either alone or in combination with other agents against a wide variety of malignant tumors (Kociba *et al.*, 1970; Rosenberg and Van Camp, 1970; Wright *et al.*, 1985). In the present study the anticancer activity of cisplatin has been investigated against Dalton's lymphoma adopted to Swiss albino mice, on various cellular and biochemical parameters along with some studies on the combination chemotherapy using cisplatin with vitamin C.

Present studies demonstrate that cisplatin is effective against murine ascites Dalton's lymphoma. After the tumor transplantation (1×10^7 cells in PBS), a regular increase in ascites tumor volume was noted which reached about 12 ml by the 18th day of tumor growth. Between days 18-22 the volume remained unchanged until the death of the mice (Fig. 3). However, cisplatin treatment of the tumor bearing hosts resulted in a significant reduction in tumor volume and by the 6th day following the treatment very little (0.5 - 1.0 ml) ascites tumor could be collected indicating effective regression of the tumor (Fig. 3). Average survival time of the untreated tumor bearing host is about 22 days with gradual increase in tumor volume. The volume of the

ascites fluid increased more rapidly in the ascites tumor during initial phase (2-12 days) of tumor growth so that the ratio of ascites fluid to tumor cell pellet reached about 2 during the middle period of tumor growth (Fig. 4). Ascites fluid is the direct nutritional source to tumor cells and faster increase in ascites fluid during tumor growth could possibly be a means to meet more nutritional requirements of tumor cells. This is evident from the rich carbohydrate and protein contents in the ascites fluid (Fig. 5) and the presence of numerous surface membrane ruffles and cytoplasmic processes all over the tumor cells which could play a role in nutritional exchange with ascites fluid. Porter et al., (1973) reported the presence of unusual ruffles on several virally and spontaneously transformed Balb/C 3T3 cells and showed that ruffles occupy a significant part on the surface of cells, a feature probably related to the known capacity of tumor cells to phagocytose their environment.

An increase in the carbohydrate contents in the ascites fluid during 3-4 days post-cisplatin treatment as reported in the present study (Fig. 5) could be due to cell mortality and that most dying cells are not able to utilize the carbohydrates present in the ascites fluid as well as due to the release of surface mucopolysaccharides (Sodhi

and Prasad, 1981; Prasad and Sodhi, 1982) and sialic acid moieties (Prasad and Sodhi, 1981; Prasad, 1986) from tumor cells. Measurement of glucose concentrations in serum and tumor supernatants also provide parallel indices (Fig. 6). The comparatively lower serum glucose level in tumor bearing hosts than the normal animals noted in the present study may indicate higher rate of glucose uptake by tumor cells. Continuous fall in serum glucose level during tumor growth has been reported (Fung *et al.*, 1986). They have also noted that Ehrlich ascites tumor cells depend primarily on glycolysis and consume glucose at a prodigious rate by increasing the surface density of glucose transporter molecules in Ehrlich ascites tumor cells. Similarly, the very low level of glucose in the tumor supernatant of untreated mice (Fig. 6 B) could be due to higher uptake and utilization of glucose by tumor cells. However, the subsequent increase in glucose level in the tumor supernatants following cisplatin treatment may indicate reduced utilization of glucose by the dying tumor cells (Fig. 6 B). Reduced uptake/utilization of glucose *in vitro* by Dalton's lymphoma cells is noted in the present study following 2 hr of cisplatin treatment (Fig. 7).

Increased release of carbohydrates and decrease in protein content in the supernatant of normal and tumor cells treated with cisplatin has been reported (Prasad and

Sodhi, 1981; Prasad, 1989). Similarly the present study has shown that the protein contents decreased in ascites fluid following 3-4 days of cisplatin treatment (Fig. 5). The inhibitory effect of cisplatin on DNA, RNA and protein synthesis has been demonstrated earlier in vitro in mammalian cells (Harder and Rosenberg, 1970).

The primary mechanism of the anticancer activity of cisplatin probably resides in its ability to inhibit DNA synthesis (Zwelling and Kohn, 1979; Pinto and Lippard, 1985). In the present chromosomal studies on the effect of cisplatin on Dalton's lymphoma, also reveals the same facts as after cisplatin treatment, about 14 fold increase in the number of aberrant metaphases have been observed (Fig. 2, Table - 1). In the control tumor cells 6% of the metaphases were found aberrant with a per cell aberration of 0.02, whereas cisplatin treatment resulted in 80-90% aberrant metaphases with per cell aberrations of 3.72 to 5.07 (Table - 1).

However, it has also been suggested that a component of the antitumor activity of cisplatin may arise from a host immunological reaction against cisplatin treated cells (Rosenberg, 1980a; Kleinerman and Zwelling, 1982; Collins and Kao, 1989; Mizutani et al., 1993). Conran and Rosenberg (1972) have reported that mice treated with zymosan (an

immunostimulant) showed increased ability to reject tumors, originally not very sensitive to cisplatin while those mice receiving hydrocortisone (an immuno suppressant) did not reject cisplatin sensitive tumors as well as the control group. It has also been reported that cisplatin has a reduced antitumor activity in immunologically depressed (i.e., X-irradiated) mice (Rosenberg, 1980b).

Sialic acid constitutes the common terminal sugar constituent of cell surface oligosaccharide side chains (Abercrombie and Ambrose, 1962). It has been reported that sialic acid influences many properties of the cell surface such as (i) the determination of the cell surface negative charge (Bohn et al., 1977) and the loss of contact inhibition during malignancy (Abercrombie and Ambrose, 1962), and antigen-masking agent (Currie and Bagshawe, 1960). In the present study it is noted that the sialic acid content of Dalton's lymphoma cells gradually decreased during 2-4 days of cisplatin treatment (Fig. 8). Cisplatin-induced removal of cell surface sialic acids may expose new cryptic antigens for immune recognition which however might have been masked by the presence of sialic acid. In vivo studies of Prasad (1989) showed that removal of cell surface sialic acids decreased the agglutinability of Dalton's lymphoma cells. Increased sensitivity of human and murine tumor cells to natural

cytotoxic (NC) cell mediated lysis after cisplatin treatment has been reported (Collins and Kao, 1989). This is supported from the observation that the per cent ratio of leukocytes to tumor cells increased significantly from 1-4 days of cisplatin treatment (Fig. 9). Increase in the number of leukocytes in tumor cell population after cisplatin treatment *in vivo* suggests the infiltration of many leukocytes towards tumor cells which surround the tumor cells (Fig. 10). Infiltration of many lymphocytes and macrophages to murine fibrosarcoma tumor cells has been noted after *in vivo* cisplatin treatment with different degrees of tumor cell degeneration adjacent to lymphocytes and macrophages (Sodhi and Sarna, 1979).

A characteristic series of changes in cell surface morphology consisting of the appearance of protrusions at discrete regions of the plasma membrane, known as plasma membrane blebbing, have been considered as a fairly general cell response during toxic oxidative and anoxic injury (Jewell *et al.*, 1982; Lemasters *et al.*, 1982; Lemasters *et al.*, 1987). The formation of membrane vesicles and vacuoles on the tumor cells following cisplatin treatment (Fig. 10 D and E) could be an indication of tumor cell lysis. And it is thus, conceivable to hypothesize that cisplatin treatment brings about definite changes in the cell surface

membrane resulting in extensive blebbing and eventually causes cell death.

Several aspects of cell injury, including the generation of sustained increase in cytosolic free calcium, the occurrence of cytoskeletal damage have been postulated to be involved in bleb formation (Jewell et al., 1982; Mirabelli et al., 1989). The observed sustained increase in the calcium level following cisplatin treatment in the tumor cells and kidney but; not in liver, spleen and brain (Table - 2) may therefore, influence the selective cytotoxicity of cisplatin in the tumor cells and kidney. Cisplatin is known to cause severe nephrotoxicity (Madias and Harrington, 1978; Natochini et al., 1987).

The results of the cell respiration experiments show that following 1 day of cisplatin treatment of the tumor cells in vivc, the spontaneous oxygen consumption (spontaneous QO_2) increases by about 40% than the control tumor cells (Fig. 11). It has been suggested that cisplatin shares many common features with other alkylating agents in its mode of action (Grunik et al., 1983), therefore, it may act like a classical uncoupler of mitochondrial oxidative phosphorylation. The subsequent time dependent fall in QO_2 during 2-4 days of cisplatin treatment may indicate mitochondrial injury. Brady et al. (1990) observed

similar fall in spontaneous QO_2 and a progressive loss of cytosolic K^+ in renal proximal tubule of rabbit, and an overall reduction in cell ATPase activity including $Na + K^+$ - ATPase activity. Here also it is noted that the total K^+ content in the tumor cells and kidney decreases progressively following 12-96 hr of cisplatin treatment (Table - 3). A reduction in the $Na^+ + K^+$ - ATPase activity is also observed in the tumor cells (Fig. 16A). The present data therefore support the hypothesis that the fall in intracellular K^+ level may result from inhibition of $Na^+ + K^+$ - ATPase activity at the cell membrane (Brady et al., 1990). The fall in $Na^+ + K^+$ - ATPase activity would therefore be expected to result in an inhibition of all secondary active transport processes causing marked disruption of vectorial solute transport across the tumor cell membrane.

The formation and shedding of membrane vesicles has been reported to occur during T-lymphocyte mediated cytolysis of tumor cells (Liepins and de Harven, 1982; Liepins, 1983). Hence, taken together the data presented here and those already reported, it is clear that formation of membrane vesicles/blebs in an important event during tumor cell lysis following cisplatin treatment which may arise from oxidative stress induced during drug metabolism (Lemasters et al., 1987) and/or immune cell mediated killing (Liepins, 1983).

Scanning electron microscopic observations show that during tumor regression following cisplatin treatment, definite changes in the pattern of surface membrane ruffles/ blebs also occur along with the infiltration of leukocytes towards tumor cells and sharp decrease in ascites fluid.

Ruffles are plasma membrane bound sheets of cytoplasm which contain microtrabecular lattice (MLT) usually found at the leading edge of migrating cells while blebs are seen on the top surface of cells resulting from the alterations of microfilament net work (Liepins and de Harven, 1982). Many transformed cells which have been reported to have large number of ruffles/blebs on the surface of interphase cells include mouse Sarcoma - 180, rat Sarcoma - 4337, mouse hepatoma - 129, mouse lymphoma cells (Porter and Fonte, 1973) and malignant melanoma A 375 cells (Gonda *et al.*, 1976).

Dalton's lymphoma cells also show the presence of many evenly distributed ruffles/blebs over the plasma membrane (Fig. 12A). Movement of surface membrane ruffles/ blebs from the top surface to the marginal areas and the appearance of leukocytes around the tumor cells and forming connections with the latter was observed after 8 hr of cisplatin treatment (Fig. 12B). By 1-2 days of the treatment, tumor cell-leukocyte connections become broader with deep

surface membrane folds and more thin cellular processes arise from the tumor cells towards the adjacent leukocytes (Fig. 12 C and D). Murine peritoneal macrophages treated in vitro with cisplatin showed increased binding to Dalton's lymphoma cells through distinct cytoplasmic extensions which transfer the lysosomes from the cytoplasm of macrophages to the tumor cell cytoplasm (Singh and Sodhi, 1988). Cisplatin treatment (3-4 days) resulted in the formation of thick blebs with membrane vacuoles and disintegration in the plasma membrane of tumor cells (Fig. 12 E and F). Lymphocytes attack cancer cells and the death of the cell is indicated by deep folds on its surface membrane. It has been suggested that lymphocytes kill tumor cells by the release of toxic factors that disrupt cell membrane (Old, 1977; Young and Cohn, 1991). In the present observations also the disintegration in the plasma membrane of tumore cells surrounded/connected by leukocytes could be due to the release of some toxic factors from leukocytes. In vitro cisplatin treatment of murine peritoneal exudate cells (macrophages) has shown the increased secretion of lysozymes (Gupta and Sodhi, 1988), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) (Sodhi and Gupta, 1986) and interleukin - 1 (Gupta and Sodhi, 1987). The present data suggest a definite effect of cisplatin on the cell surface membrane. Prasad and Sodhi (1982) reported that cisplatin treatment removes the cell surface sialic

acid moieties and acid mucopolysaccharides which may enhance the antigenicity of tumor cells by exposing new cryptic epitopes and thus permitting immunological recognition and tumor cell lysis. The elevations in the per cent ratio of leukocytes in the tumor tissue establishing definite connections with the tumor cells after cisplatin treatment (Fig. 9 and 12) may also support similar conclusions.

Programmed cell death or apoptosis is a form of cell death in which DNA digestion occurs at an early stage by non-lysosomal endonucleases (Eastman, 1990). Studies with dextramethasone treated thymocytes suggest that the degradation of DNA in this svstem is mediated by Ca^{2+}, Mg^{2+} - dependent endonuclease that is present in an inactive form in the cell nuclei (Yoshihara _et al_., 1975). In the present study it is noted that cisplatin treatment causes regular and sustained increase in the tissue calcium content in tumor and kidney tissues (Table - 2). It has been reported that the use of calcium ionophores can experimentally increase Ca^{2+} and induce DNA digestion, whereas intracellular chelation of Ca^{2+} can inhibit DNA digestion (McConky _et al_., 1989) and this requirement for a sustained increase in Ca^{2+} level contrasts with the transient increases that are associated with normal intracellular signal transduction pathways (Eastman, 1990). Thus the selective increase of

calcium in tumor and kidney but not in liver, spleen and brain after cisplatin treatment may correlate with the selective toxicity of cisplatin in tumor and kidney.

The involvement of a multilevel action during tumor regression following cisplatin treatment has been suggested by many workers (Prestayko _et al_., 1980; Prasad and Sodhi, 1982; Bahadur _et al_., 1984; Pinto and Lippard, 1985). Various results from the present studies also provide good support in this direction of the action of cisplatin in tumor cell killing.

There are extensive literatures available on various biological actions/properties of cisplatin. However, very less studies have been undertaken on the various enzymes of metabolism and their possible involvement in the regression of malignant tumors after cisplatin treatment.

The effect of some platinum complexes on some cellular enzymes have been studied and in most of the cases concluded that enzyme inhibition by platinum complexes is primarily due to reaction with essential sulfhydryl groups of these enzymes (Friedman _et al_., 1975; Aull _et al_., 1979). Aull _et al_. (1979) studied the effect of K_2PtCl_4, cisplatin and its transiosomer on the activities of seven enzymes. All the enzymes which are thought to have essential sulfhydryl

groups (glyceraldehyde-3-phosphate dehydrogenase, aldolase and glucose-6-phosphate dehydrogenase) were significantly inhibited by K_2PtCl_4. The other four enzymes (dihydrofolate reductase, catalase, tyrosinase and peroxidase) not having the sulfhydryl groups were not significantly affected by the platinum compounds.

In the present study eight enzymes i.e., glucose-6-phosphatase, lactate dehydrogenase, Na^+ + K - ATPase, 5'-nucleotidase, glutamic oxalacetic transaminase, glutamic pyruvic transaminase, arginase and cathepsin have been studied in one or more of the following tissues: liver, kidney, tumor cells, tumor supernatant (ascites fluid) and serum of normal and tumor bearing mice with or without cis-platin treatment and compared.

Glucose-6-phosphatase is a key enzyme in gluconeogenesis in liver, and blood in order to supply a continuous source of energy to the remote organs; also functions in the process of active resorption of glucose in the intestine and kidney tubules (Stelten and Goldsmith, 1976). Werve (1989) reported that glucose-6-phosphatase system is modulated by changes in Ca^{2+} concentration in the range of those occurring in the liver cell upon hormonal stimulation. The tendency of decreasing activity of glucose-6-phosphatase in tumor bearing mice noted in the present study (Fig. 13A)

may reflect a metabolic shift favourable for the tumor progression in the animal. Stefanini (1985) has discussed the changes in various enzymes, isozymes and enzyme variants in patients in malignancies and suggested that enzymatic changes may indicate the overall changes in metabolism that occur in malignancy. The glucose-6-phosphatase activity in the soluble fraction of primary hepatomas induced by nitrosamine was found to be decreased by 6 fold than the normal activity (Kilderma et al., 1977). The results here also showed decreasing activity of glucose-6-phosphatase in the tissues of tumor bearing mice which in turn suggests a decreased gluconeogenesis, thus increasing the rate of glycolysis (Fig. 13). The higher rate of glycolysis in tumor bearing hosts has been suggested to trigger biochemical processes aiding cancerous cells over normal surrounding tissue cells and it may be related to the aggressiveness of the tumor (Yesher, 1978). Elitto et al., (1977) studied the activity of glucose-6-phosphatase in rats undergoing carcinoma by diethyl nitrosamine and have also observed a decreased activity of the enzyme. Cisplatin treatment of the tumor bearing mice for 1, 2, and 4 days showed an increase in glucose-6-phosphatase activity in the liver (Fig. 13A). In the kidney on the other hand, the enzyme

activity decreased till the 2nd day of the treatment and showed a slow recovery during the later periods (Fig. 13B). The increased glucose-6-phosphatase activity in the liver after cisplatin treatment may indicate a shift in carbohydrate metabolism in the opposite direction thereby making less favourable condition for tumor growth. On the other hand, it might also be possible that since the demand for glucose by the dying tumor cells decreases with time, the increased glucose-6-phosphatase activity in the liver may favour the metabolism towards increased gluconeogenesis in the liver. The observed increase in blood glucose level (Fig. 6A) may indicate towards the second hypothesis.

Lactate dehydrogenase (LDH) is the terminal enzyme in the anaerobic type of glycolysis. Hill and Levi (1954) were among the first investigators to study serum LDH, who reported marked elevations in LDH activity in many patients with leukemia. Elevation of serum LDH activities has also been reported in cases of hepatocellular necrosis and metastatic carcinoma (Mac Donald *et al.*, 1957). Elevations of LDH in gastric juice of gastric cancer (Smyrniotis *et al.*, 1962), in stomach cancer (Faulk *et al.*, 1972) have also been reported. In the present study also more than two-fold increase in serum LDH activity has been noted in the Dalton's lymphoma bearing Swiss albino mice than the

normal animals (Fig. 14A). Tumor cells are known to exhibit increased level of glycosis and rapid turnover of malignant cells are excepted to release ecto- and endoenzymes into the blood stream (Stefanini, 1985). In the present study higher level of LDH activity is observed in tumor and liver tissues of the tumor bearing, mice, reflecting a higher rate of anaerobic glycolysis (Fig. 14 B and E).

After cisplatin treatment, LDH activity in liver, kidney and tumor cells decreased by the 1st day of the treatment. However, in liver and kidney it showed a slight increase over the control value while in the tumor cells the LDH activity progressively decreased following 2 days of cisplatin treatment (Fig. 14 B, C and E). Following 3-4 days of the treatment an overall decrease in the LDH activity was noted in liver, kidney and tumor cells as compared to the tumor bearing animals which may indicate decreased synthesis and/or increased leakage from the cells due to injury. The second possibility may be strongly implicated here since a sustained increase in LDH activity was noted in the serum (Fig. 14 A). At the same time, the continuous decrease in the enzyme activity in tumor cells and the parallel increase in the tumor supernatant (Fig. 14D) is a fair indication of altered membrane permiability of tumor cells for LDH and cell injury. A correlation between cytotoxicity and

LDH release has been demonstrated and used as parameters of cytotoxicity by many workers (Takema et al., 1991).

It has been postulated that anaplasia is accompanied by molecular adjustments, which preceded morphological changes in enzyme patterns thus, there is predominance of LDH-1 in normal colonic mucosa and LDH-5 in colonic cancer tissues (Carda-Abella et al., 1982). Tietz (1980) reported that leptomeningeal infiltration by carcinoma is accompanied by increased activity of LDH-5 in the cerebro-spinal fluid. The use of LDH isozymes as a diagnostic aid has been amply documented (Wilkinson, 1970; Dito, 1973). LDH isozyme analysis has also been proved valuable in determining the effect of carcinostatic agents upon certain tumors (Starkweather et al., 1966).

Studies on LDH isozyme patterns showed that in serum of both normal and tumor bearing mice with or without cisplatin treatment for 1-4 days, all the 5 isozymes, i.e. LDH -1, LDH-2, LDH-3, LDH-4 and LDH-5 were present in varying intensities (Fig. 15A). In all the serum samples LDH-5 was found to be maximally expressed starting from the normal animal (lane-1) till the 2nd day of the treatment (lane-4) and remains more or less constant following 3-4 days of the treatment (lane-5 and 6). Another notable feature is that in the serum of normal animals both LDH-2 and LDH-3

are maximally expressed than those of tumor bearing as well as ciplatin treated group (Fig. 15A). On the other hand, in kidney (Fig. 15B) LDH-5, was found to be the least active form whereas LDH-2 and LDH-3 are the relatively more active forms. While all the 5 isozymes are expressed in serum and kidney, in the other three tissues, i.e., liver, tumor and tumor supernatant, distinct variations are notable in relation to the number and nature of the various isozyme patterns. In the liver tissue, only 3 isozyme bands (LDH -3, LDH-4 and LDH-5) are seen (Fig. 15C) of which LDH-5 seems to be the predominant form. The notable feature is that from the first day of cisplatin treatment (lane-3), LDH-3 and LDH-4 isozymes show marked variations in band intensities suggesting changes in activity, and following 4th day of treatment (lane-6) LDH-5 seems to be the only isozyme form present in the liver (Fig. 15C). In the tumor supernatant (Fig. 15D) LDH-5 is the most active form and LDH-4 is the other distinct form, whereas other forms vary considerably. On the other hand, in the tumor cells LDH-5 was the only conventional isozyme form found (Fig. 15E). The most important notable feature is the presence of a new band near the cathodic end in the serum of tumor bearing as well as cisplatin treated group (Fig. 15A) which is also seen in tumor supernatant and tumor cells (Fig. 15D and E) but; absent in kidney and liver (Fig. 15B and C).

It has been reported that human malignant cells both lymphoid (Rambotti and Davis, 1981) as well as other organs (Goldman et al., 1964; Fleischer et al., 1981) possess isozyme patterns distinguished by lower H:M ratios (i.e., predominance of LDH-5). In the present study also it is found that LDH-5 is the most predominant form in all the tissues except for kidney, and is the only available form present in the tumor tissues. It has been suggested that the predominance of LDH-5 reflects anaerobic metabolism of tissues (Goldman et al., 1964) since hypoxic conditions have been shown to exist in many tumors (Kallman, 1972). The present findings in this tumor model also goes in well accordance with the earlier reports discussed for other types of cancers and suggest that there may exist a similar hypoxic condition in Dalton's lymphoma also. The increased expression of LDH-5 following cisplatin treatment in the tumor cells therefore, suggests that cisplatin treatment may further aggravate the hypoxic condition in the tumor.

The observation of a new isozymes band in Dalton's lymphoma which is also reflected in the serum in the present study may indicate the presence of a new isozyme, and it has been tentatively designated as LDH-T for being present in tumor. Although the exact physiological significance of this new isozyme cannot be explained at present, it could

be a physiological adaptation of tumor cells to the general hypoxic conditions that exist in the tumor tissues or could be due to a gene mutation, and it may serve as an useful marker for Dalton's lymphoma *in vivo*. Lippert and Javadpour (1981) have observed that LDH is a fairly sensitive marker for solid neoplasma.

The cellular environment plays a central role in regulating the growth and development of cells and the cell interacts with the extracellular matrix via the plasma membrane (Gallagher, 1985). Two cell membrane enzymes, i.e., Na^+ - K^+ - ATPase and 5'-nucleotidase (5'-ND) have been investigated in the present study in an attempt to evaluate the functional alterations that may occur during malignancy and their response to cisplatin treatment and possible role in the anticancer activity/cytotoxicity of cisplatin.

Emmelot and Bos (1969) observed 25% and 50% decrease in Na^+ + K^+ - ATPase activity in hepatoma 147042 and 4189 respectively. Similar results have been obtained by Barclay and Terebus-Kekish (1973) for Morris hepatoma 5123 tc. Na^+ + K^+ - ATPase regulates the Na^+ and K^+ ion balance within the cell and also involved in the vectorial transport of many solutes across the cell membrane. Therefore, any change in its activity will dastrically affect the cell function.

It is observed in the present study that following cisplatin treatment $Na^+ + K^+$ - ATPase activity progressively decreased from the tumor cell surface (Fig. 16A). However, in the tumor supernatant in contrast to tumor cells $Na^+ + K^+$ - ATPase activity increased following cisplatin treatment (Fig. 16B). The inhibition of $Na^+ + K^+$ - ATPase activity on the tumor cell surface could be due to loss from the cell surface or some other mechanism, but a direct interaction of cisplatin with the enzyme is less likely. This is also supported from the observations of Brady et al. (1990) that $Na^+ - K^+$ - ATPase activity measured in lysed renal tubular cells did not decrease in contrast to the intact cells following cisplatin treatment. The fall in $Na^+ + K^+$ - ATPase activity would therefore, be expected to result overall changes in other secondary active transport processes across the tumor cell membrane which may facilitate cisplatin cytotoxicity.

Although the exact function of 5'-ND at the cell surface is not very clear, it is suggested that it may regulate the supply of nucleoside precursors for nucleic acid synthesis (Lopes et al., 1973). It has been reported that malignant cells display very low levels of 5'-ND activity (Raz et al., 1978; Chatterjee et al. 1981). In contrast to these reports Emmelot and Bos (1969) found that the

activity of 5'-ND on the surface membrane from mouse hepatoma 147042, 4189 and 413066 were increased two to five fold over that of normal liver. Graham (1979) concluded that there appears to be no consistent pattern of alterations in 5'-ND activity associated with transformation.

It is noted that 5'-ND activity in the liver of tumor bearing mice shows a more than 2.5 fold increase over that of the normal liver (Fig. 17A) and so also in the kidney (Fig. 17B). However, cisplatin treatment brings about a decrease in 5'-ND activity both in liver and tumor cells (Fig. 17 A and C), but in kidney and tumor supernatant the 5'-ND activity increased following the treatment (Fig. 17B and D). Although in the liver enzyme activity showed a gradual recovery following 3-4 days of the treatment, in the tumor it decreased further.

The reduction in 5'-ND activity may add to the anti-tumor activity of cisplatin enhancing cell death. This suggestion is based on the fact that adenosine, the product of 5'-ND is a powerful vasodialator (Osswald, 1983) except for in renal circulation (Thurau, 1964; Osswald, 1983). The decrease in 5'-ND activity significantly below the control value could therefore, abolish the vasodialatory effect upon the circulation to the tumor tissue thus reducing the blood flow and thereby creating a more hypoxic condition

within the tumor mass leading towards cell death. On the other hand, in the liver, since the level of 5'-ND did not fall much below the normal level, the harmful effect could be minimal to the liver tissues.

Serum contains many different transaminases. The two most frequently determined are glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). These enzymes catalyze the transfer of α-aminogroups from the specific amino acids to α-ketoglutaric acid to yield glutamic acid and oxalacetic acid or pyruvic acid. The glutamate thus formed are catabolised via the urea cycle to arginine which however, may be hydrolysed to urea, the end product of amino acid metabolism.

In the present study it is noted that serum GOT activity is markedly increased in Dalton's lymphoma bearing mice (Fig. 18A). This indicates an increased synthesis and release into the body fluids. The exact significance of these changes is not clearly explainable. Stefanini (1985) has suggested that the changes in content and molecular structure of various enzymes that accompany transformation may result from genetic reprogramming to malignant behaviour, a likely strategy for survival of tumors. Following 1 day of cisplatin treatment, GOT activity significantly decreased and by 2-3 days of the treatment showed increasing activity

both in serum and tumor supernatant (Fig. 18A and B). However, by the 4th day of the treatment, the GOT activity in serum has been found to be almost similar to the normal animals, but in the tumor supernatant the enzyme activity remained significantly higher than the control value (Fig. 18). On the other hand, no significant variation could be found in the serum GPT activity between normal and tumor bearing animals (Fig. 19A). However, following 1-2 days of cisplatin treatment the GPT activity decreased and during 3-4 days of the treatment an increasing trend in the GPT activity was noted both in serum and tumor supernatant (Fig. 19A and B). Waldman and Borman (1959) reported increased GOT activity following lead absorption and suggested that transaminase levels be used as guide line for physicians in control of poisoning. Increased activity of GOT has also been reported following carbon tetrachloride poisoning (Wroblewski and La Due, 1955). The decreased levels of both GOT and GPT following one day of cisplatin treatment may indicate altered amino acid metabolism. Cisplatin induced impairements in amino acid metabolism has been reported (Gross and Scanlon, 1986). The subsequent increase in the enzyme activities may be due to increased release from the dying and injured cells.

In the present study it has been found that arginase

activity in the liver and kidney of tumor bearing mice is significantly lower than the normal mice (Fig. 20A and B). Following 8 hr of cisplatin treatment the enzyme activity was noted to be increased than the tumor bearing animals which however decreased continuously afterwards. In the tumor supernatant on the other hand, a small amount of enzyme activity was observed which increased significantly following cisplatin treatment (Fig. 20C). The initial increase in the arginase activity following cisplatin treatment could be directly involved in the regression of the tumor by activating the macrophages. The activated macrophages have been reported to show tumoricidal activity through arginase (Roitt et al., 1985). As many leukocytes were observed to form close contacts with tumor cells (Fig. 10 and 12), their tumoricidal activity may be facilitated by this mode of action also. It has been reported that cisplatin treatment of tumor bearing mice resulted in infiltration of large number of macrophages into the tumor tissue, and showed definite sequential interaction with tumor cells (Sodhi and Aggarwal, 1974; Sodhi and Sarna, 1979; Sodhi, 1979). Activated macrophages selectively and effectively kill a wide array of neoplastic cells in a contact-dependent non-phagocytic process (Higuchi et al., 1990). Macrophage activation for target cell lysis following cisplatin treatment is further supported from the sustained increase in the activity

of both cathepsin H and cathepsin B in tumor supernatant and serum (Fig. 21). Cathepsin is lysosomal in origin and the increased levels in the serum and tumor supernatant may indicate release by activated macrophages and the dying tumor cells. *In vitro* cisplatin treatment of murine peritoneal exudate cells (macrophages) have shown increased secretion of lysosomal enzymes (Gupta and Sodhi, 1988).

The increased GOT and GPT activity along with the decreased arginase activity as observed in the present study is likely to increase the concentration of arginase. Kusenda *et al*. (1992) have shown that the tumoricidal activity of activated macrophages is L-arginine dependent. Therefore, it could be possible that the resultant increase in arginine concentration in the tumor supernatant due to inhibition of arginase and increased activity of GOT and GPT could be metabolised oxidatively producing reactive nitrogen intermediates (RNI). Enhanced cytolytic activity by activated macrophages and its dependence on a surplus of L-arginine has been reported (Kusenda *et al*., 1992).

It has been suggested that L-arginine dependent effector systems inhibit the enzymes of the mitochondrial electron transport chain (Drapier and Hibbs, 1988, Hibbs *et al*., 1987; Granger *et al*., 1980). The reduced oxygen

consumption by tumor cells following cisplatin treatment (Fig. 11) may indicate towards similar conclusions. Thus, from the data presented and those reported earlier, it may be suggested that arginase might play an important role in macrophage-mediated tumor cell lysis thus potentiating the antitumor activity of cisplatin.

The therapeutic dose of cisplatin against a wide variety of malignant tumor, has been established to be about 8-9 mg/kg body weight. However, the full exploitation of this anticancer drug is limited due to its major side effect, nephrotoxicity (Krakoff, 1979; Rosenberg, 1985) and moreover, other reports indicating a carcinogenic risk and development of secondary tumors in patients/animals treated with therapeutic dose of cisplating (Kempf and Ivankovic, 1986 a, b). This evoked the idea of evaluating the effectiveness of subtherapeutical doses of cisplatin alone and in combination with low doses of another chemopreventing agent, vitamin C, against the transplantable tumor, Dalton's lymphoma in C_3H/He as well as Swiss albino mice to evolve a nontoxic chemotherapy against cancers without loosing the efficacy of cisplatin. Ghosh and Das (1984, 1985) showed that treatment of tumor bearing mice with 1% ascorbic acid orally reduces the tumor growth. Therefore, in the present studies vitamin C was used in concentrations below 1% with subtherapeutical dose of cisplatin.

It has been reported that sodium ascorbate at a nontoxic concentration potentiates the growth inhibitory effect of 5-fluorouracil, bleomycin sulfate, Ro 20-1724 (an inhibitor of cyclic nucleotide phosphodiesterases) and sodium butyrate on neuroblastoma cells in culture (Prasad et al., 1979) and it also reduces the cytotoxic effect of methotrexate and DTIC (Dacrabazine) on neuroblastoma cells (Prasad and Rama, 1983). Ascorbic acid has been reported to be effective as a protectant against a variety of toxic chemical agents including heavy metals (Holloway and Peterson, 1984). However, the extent of modification of the effect of various aspects by ascorbic acid depends upon the type of tumor cells and the type of pharmacological agents (Prasad and Rama, 1983).

Host survival data in the present study, showed a significant increase in survival time of tumor bearing mice treated with vitamin C and cisplatin combination as compared to that of the group of mice treated with either agent alone (Fig. 22 and 23). The survival pattern of tumor bearing mice receiving 0.5% vitamin C alone was almost the same as that of tumor bearing mice receiving cisplatin alone (Fig. 22). The combination of 0.5% vitamin C and cisplatin treatment proved to be very effective as it doubled the host's survivality and about 40% of the treated mice were noticed to be tumor free as no ascites tumor could be reco-

vered from them (Fig. 22). The synergistic antitumor effect brought about by vitamin C and cisplatin, which were administered via different routes into the animal, is noted to be sequence dependent. This is supported from the host survival data of other set of experiment in which mice were first treated with cisplatin on the 5th day of tumor transplantation and vitamin C treatment was started on the 10th day. It showed that tumor regression was not as effective as was observed in the group of tumor bearing mice receiving vitamin C first for few days followed by cisplatin. The host survival rates resembled those of the groups treated with cisplatin alone (Fig. 23). Treatment of tumor bearing mice with vitamin C first could be helpful in developing suitable conditions in the host potentiating cisplatin's effect and successful tumor regression. Peters and Stuart (1990) reported that synergistic effect of 4-hydroperoxycyclophosphamide (4-HC) and cisplatin is also sequence dependent: it was only present when the tumor cells were exposed to 4-HC first. Potentiation of antitumor activity of cisplatin by 3-aminobenzamide and nicotinamide in mice have also been reported (Chen and Pan, 1988).

The changes in total body weight of tumor bearing mice noted under different experimental conditions reveal definite changes in tumor growth and relate with the changes

in host survivality pattern. Tumor bearing mice in the control group showed a steady increase in body weight due to rapid growth of tumor until they die (Fig. 34). However, in cisplatin alone and vitamin C plus cisplatin treated tumor bearing mice, less increase in body weight indicates a retardation in the tumor growth rate leading to an increase in the survival time of these mice. The group of tumor bearing mice treated with 0.5% vitamin C plus cisplatin, which showed maximum survivality, showed the least increase in body weight/tumor weight due to effective regression of tumor growth (Fig. 24).

Similar results in tumor growth pattern and host survivality have been found in the studies with Dalton's lymphoma bearing Swiss albinomice as was observed for Dalton's lymphoma bearing C_3H/He mice. In this study same doses of vitamin C and cisplatin was used. The antitumor activity and drug efficacy show the synergistic effect of vitamin C and subtherapeutical dose of cisplatin. It has been noted that the ILS% in the combined treated group with 0.5% vitamin C and 4 mg/kg b.w. cisplatin was >203.73 whereas, it was in the order 143.45, 60.28 and 21.96 for 0.25% vitamin C plus cisplatin, cisplatin alone and vitamin C (0.5%) treated groups respectively (Table - 4). The GIR% also showed a value of 88.57 in the vitamin C (0.5%) plus cisplatin

treated group whereas, it was in the order 71.71, 62.85 and 13.57 for 0.25% vitamin C plus cisplatin, cisplatin alone and vitamin C (0.55) treated groups respectively (Table - 5). These results indicate that the observed synergistic antitumor effect between vitamin C and cisplatin may not be host strain specific.

Although at the moment it is difficult to arrive at a definite conclusion on the mechanism of the sequence dependent synergistic antitumor action by these two drugs, the observations on the increase in the average weight of spleen and thymus of vitamin C plus cisplatin treated tumor bearing mice suggest the possibility of the involvement of host's immune response in regression of the tumor after the treatment (Table - 6). The stimulation of host's own defense system is further supported from the increased leukocyte count in blood following vitamin C plus cisplatin treatment (Table - 7). It has been observed that higher dose of cisplatin (8 mg/kg b.w.) resulted in an early myclosuppression whereas at a lower dose (4 mg/kg b.w.) shoed a more sustained increase in the total leukocyte count in the blood. The combined treatment of vitamin C plus cisplatin evoked a prolonged increase in leukocytes in the blood (Table - 7). Enhancement of the host's naturally occurring immune function by very low doses of cisplatin has been reported (Kleinerman,

et al., 1980a). Vitamin C (Firasen *et al.*, 1981, Yonemoto, 1983) and cisplatin (Bahadur *et al.*, 1984, Collins and Kao, 1989) have been reported to enhance host's immune response in their antitumor activity. Vitamin C has been reported as an immunostimulatory agent involving various components of the immune system in the host (Anderson, 1984).

Tumor bearing mice have lower serum ascorbic acid than that observed in normal mice and apparently decreases following cisplatin treatment (Fig. 25). Vitamin C treatment increased the serum ascorbic acid level than the normal value (Fig. 25). Decreased level of serum ascorbic acid in tumor bearing hosts has been reported (Ghosh and Das, 1984, 1985). The decrease in tumor pH of tumor bearing mice treated with 0.5% vitamin C plus cisplatin could also be involved to have had some effect in enhancing the antitumor activity, resulting in an increase in the surival time with regression of the tumor growth. Low tumor pH induced by hyperglycemia has been reported to enhance the antitumor activity of subtherapeutical dose of cisplatin with glucose against Dalton's lymphoma and it has been suggested that cisplatin potentiated that effect at low pH maintained by regular glucose injections (Sarna and Bhola, 1987, 1989).

It has been suggested that normal cells contain cryptic lectin binding sites that are exposed upon trans-

formation or by treatment with proteolytic enzymes (Sela _et al_., 1970). It has also been reported that cisplatin has definite effect on the surface of tumor and normal cells, brings about changes in the lectin agglutinability of cells (Prasad and Sodhi, 1981; Prasad, 1989) and lectin binding sites (Sodhi and Prasad, 1981; Prasad and Sodhi, 1982; Prasad, 1987). In the present study it has been found that vitamin C and cisplatin have definite effect on the Con A agglutinability of Dalton's lymphoma (DL) cells involving changes in the pattern of Con A binding sites. DL cells showed high degree of Con A agglutination (Fig. 26) and also bright even fluorescence intensity (Fig. 27A) suggesting the evenly distributed readily available Con A binding sites which bind to Con A leading to high degree of cell agglutination. High agglutination of tumor cells with lectins has been attributed to the altered dynamics of cell surface lectin receptor sites (Barnett _et al_., 1974).

Gradual decrease in agglutination and fluorescence intensity after cisplatin or vitamin C treatment is coupled with the appearance of granular/patchy fluorescence at 45 min which indicates a change in the pattern of Con A binding sites in such a way that they could be less available to Con A, resulting a decrease in cell agglutination (Fig. 26). The cells treated with vitamin C plus cisplatin for 15 min

only showed a decrease in fluorescence intensity with distinct appearance of fluorescent grains, which in turn may suggest that the combined effect of vitamin C plus cisplatin on DL cells is more rapid as compared to the separate treatments. This observation is supported from the findings of the agglutination studies where Con A agglutinability of the DL cells is observed to be about half after treatment with vitamin C plus cisplatin for 15 min as compared to that of separate treatments (Fig. 26).

The decrease in Con A agglutination of DL cells could be due to topographical rearrangement/removal/destruction of Con A binding sites over the cells. However, it seems likely that before the final removal/destruction of the binding sites (showing very weak fluorescence at 60 min) there are definite changes in the topographical arrangement of cell surface Con A binding sites. Earlier studies (Prasad and Sodhi, 1982) using Con A - ferritin labelling on splenocytes and DL cells have shown that the Con A - ferritin patches are arranged in a regular homogeneous pattern. The sharpness as well as the number of ferritin particles, indicating Con A binding sites, decreases gradually from 10 to 60 min of cisplatin treatment with the rearranged clusters of Con A - ferritin particles at 30 min of the treatment (Prasad and Sodhi, 1982).

In the present studies it is clear that the combined treatment of vitamin C plus cisplatin leads to the removal/ destruction of Con A binding sites more rapidly than that of individual treatments. It has been found that the degree of agglutination of transformed cells is reduced after trypsin treatment (Inbar and Sachs, 1969). Marquez (1976) using ^{125}I-Con A binding assays has reported that binding of Con A to normal HEF (hamster embryo fibroblast) cells could be increased by trypsin treatment only upto fixed interval of time (here 6 min) and after that the amount of bound Con A decreased rapidly which was explained due to the destruction of Con A binding sites.

It is quite clear from these observations that vitamin C enhances cisplatin-induced effects on the Con A agglutinability of DL cells and also the pattern of Con A binding sites. It may therefore, be suggested that the observed synergistic effect of the combined treatment of vitamin C plus cisplatin in the tumor regression could involve the enhancing effect of vitamin C on cisplat -induced changes on the surface of DL cells _in vivo_ hence increasing their succeptibility for naturally occurring cytotoxic/killer/ macrophage mediated cytolysis. The observations that cisplatin causes a myelosuppression following 5-6 days of the treatment (Table - 7) further suggest that a time dependent

enhancement of cisplatin's effect on cell surface by vitamin C could result in more effective tumor cell killing by activated cytotoxic leukocytes before their number falls in the blood which may be due to general cytotoxic effect of cisplatin. On the other hand, the sustained increase in leukocyte count in the combined treated group may indicate the protective effect of vitamin C against the myelosuppression observed in the groups treated with cisplatin alone during later periods. Cisplatin induced bonemarrow toxicities have been reported (Kociba and Sleight, 1971). These observations also strongly support the possible involvement of the host's naturally occurring immune function in the synergistic effect of vitamin C and cisplatin. Ascorbic acid is known to enhance the phagocytic functions of leukocytes (Shilotri, 1977).

Cisplatin is known to induce chromosomal aberrations (Wieneke *et al.*, 1979; Bocian *et al.*, 1983; Tandon and Sodhi, 1985) and mutations in cultured mammalian cells (O'Neill *et al.*, 1977). Others have reported a carcinogenic risk and development of secondary tumors in patients/animals treated with therapeutic doses of cisplatin (Kempf and Ivankovic, 1986a, b; Leopold *et al.*, 1979).

In the present study cisplatin is shown to induce various types of chromosomal aberrations (Fig. 28). Chromatid

breaks were more frequent than any other type of aberrations noted and chromatid gaps follow next (Table - 8). Since no such gaps or very negligible number of gaps are observed in the control mice, the fairly large number of gaps are likely to be induced by the clastogenic effect of cisplatin and cannot be explained as artifacts as was thought by Buckton and Pike (1964). Tandon and Sodhi (1985) also observed gaps induced by cisplatin. Assessment of chemically induced chromosomal damage has been reported by Gebhart (1977) and suggested that the gaps may be associated with chemical mutagenesis. Highly damaged chromosomal plates observed at 24 hr of cisplatin treatment and at the same time the lack of higher frequency of chromosome (isochromatid) type damage at later hours suggest that a post-replication repair process might be operating which results in recovery from cisplatin induced damage to DNA. The possible involvement of a post-replication repair process has been reported (Sorenson and Eastman, 1988). It has also been suggested that although G_2 arrest appears to be a prerequisite for cell death (except for very high drug concentrations), all such arrested cells do not die and at minimally toxic concentrations of cisplatin, cells may ventually bypass the block and return to normal cycling (Eastman, 1990). It is known that cisplatin preferentially react with the G-C rich regions of DNA (Stone _et al._, 1974) forming a close ring chelate

of the aquated cisplatin with both N_7 and O_6 of guanine (Dehand and Jordanov, 1976). Rosenberg (1985) proposed that cisplatin lesions on O_6 of guanine in normal cells are repaired before replication, while in cancer cells, which are so because of a deficiency in this repair process, the lesions are not removed and the burden of mutations increase beyond the limits of survivality. Thus, this might explain the fairly high frequency of aberrations observed in the tumor cells following 24 to 96 hr of cisplatin treatment (Table - 1). Defective repair of alkylated DNA by human tumor and SV-40 transformed human cell strains has been reported (Day *et al.*, 1980).

On the other hand, the data obtained on chromosomal aberrations both in frequency and pattern (Table - 8) strongly suggests the protective effect of vitamin C against cisplatin induced mutagenicity in mice. This is further supported from the micronucleus (MN) test and sperm head abnormality assays.

Schmid (1976) reported that MN test is comparable to or even more sensitive and reliable than metaphase analysis for chromosomal aberrations. The incidence of higher percentage of MN over the controls following cisplatin treatment (Table - 9) indicate the mutagenic effect of cisplatin. However, the significant decrease in the percentage

of MN in vitamin C plus cisplatin treated group than the group receiving cisplatin alone suggests the chemopreventive effect of vitamin C against cisplatin induced mutagenicity in mice.

Sperm abnormalities are thought to arise primarily from point mutations (Wyrobek and Bruce, 1975). Some specific kinds of sperm abnormalities induced by radition have been shown to be heritable (Hugenholtz and Bruce, 1977). In the present study the high incidence of sperm abnormality induced by cisplatin (Table - 10) may be a measure of the genetic damage caused in the germline cells. Cisplatin induced toxicities in the testis have been reported (Loehrer and Einhorn, 1984; Pogach et al., 1989). The significant decrease in the frequency of abnormal sperms in the vitamin C plus cisplatin treated group than the group receiving cisplatin only, indicate the protective effect of vitamin C against cisplatin induced mutagenicity in the testis. Ascorbic acid is known to protect against endogenous oxidative DNA damage in human sperm (Fraga et al., 1991).

Although the mechanism of the protective effect of vitamin C against cisplatin induced mutagenicity cannot be explained definitively; however, it might be possible that vitamin C probably stimulates the cell's own repair system for a quicker removal of the DNA lesions thus reducing

the chromosomal aberrations in the bonemarrow cells as has been observed (Table 8).

Independent of the nature of molecular interactions that occur, or the mechanism(s) involved, the present data however, clearly indicate that vitamin C effectively ameliorates cisplatin induced mutagenicity in the bonemarrow cells of mice. Also, it is apparently likely that the observed synergistic antitumor effect of vitamin C plus cisplatin may not be at the DNA level, and an enhancement of the host's immune function could be involved. The immunostimulatory (Anderson, 1984) and enhancement of phagocytic functions of leukocytes (Shilotri, 1977) by ascorbic acid have been reported.

Cisplatin has been reported to cause severe nephrotoxicity which is dose related both in animals and human (Madias and Harrington, 1978; Goldstein _et al._, 1981; Natochini _et al._, 1987). The increased uric acid (Fig. 30) and urea level in the blood (Fig. 31) further support these findings.

In most mammals, uric acid is oxidized in the liver into allantoin. Increased serum uric acid levels are reported to be found in acute and chronic nephrititis (Hepler, 1957). The observed time depend increase in serum uric acid level

following cisplatin treatment (Fig. 30) could possibly be due to decreased renal excretion indicating renal insufficiency. This can be further supported from the serum urea content, which closely parallels the glomerular function (Provoost and Molenaar, 1980). Following 8 mg/kg b.w. of cisplatin treatment, serum urea level markedly increased and remained elevated even on the 15th day of the treatment (Fig. 31). Lower dose (4 mg/kg b.w.) of cisplatin also caused significant elevation in serum urea level, however, the elevation was lower than the 8 mg/kg b.w. of cisplatin treated group (Fig. 31). These results suggest that cisplatin induces nephrotoxicity in 'mice which is dose dependent.

In cisplatin nephrotoxicity as in other forms of renal damage, reduced renal blood flow is observed (Daugaard, 1990), the mechanism of which is unknown. As suggested by him, the simultaneous severe decrease in glomerular filtration rate might be due to either a reduction in the filtration pressure or filtration coefficient. Independent of the mechanism of action, the clinical importance of the problem has necessitated the search for an early and effective antidote for cisplatin induced nephrotoxicity. Quite a large number of reports so far have been published in this direction with different degrees of success and has been reviewed (section VI, page 30). Most of these studies

have employed various antioxidants/free radical scavengers of which a major proportion are thiols or thiol containing compounds, with various degrees of success having advantages and disadvantages of their own.

In the present investigation the effect of vitamin C on cisplatin induced nephrotoxicity has been studied. Vitamin C is an excellent antioxidant and free radical scavenging nutrient, and protects the cells from damage by oxidants (reviewed in Machlin and Bendich, 1987). It has been found that pretreatment with vitamin C markedly decreased the serum urea level than the group in which cisplatin was administered alone (Fig. 31) indicating improvement of renal function and reduced nephrotoxicity. These findings suggest that vitamin C may to some extent protect cisplatin induced nephrotoxicity in mice.

Despite the observed chemopreventive action of vitamin C against cisplatin induced nephrotoxicity, the mechanism, however, is not understood. Superoxide dismutage and antioxidants have been reported to ameliorate cisplatin induced nephrotoxicity (McGinness _et al_., 1978; Sugihara and Gemba, 1986). Cisplatin has been shown to increase lipid peroxidation in renal slices _in vitro_, and the enhanced formation of lipid peroxides is prevented by antioxidants (Sugihara _et al_., 1987; Hanneman and Bauman, 1988). It may

therefore possible that a component of the protective effect of vitamin C against cisplatin induced nephrotoxicity may reside on its antioxidant property.

From the various aspects of studies covered and discussed, following main conclusions may be derived:

(i) Cisplatin treatment brings about definite changes in the ascites fluid as well as in tumor cells in terms of nutritional requirements and degree of infiltration of leukocytes towards tumor cells finally leading to the death of the tumor cells.

(ii) Cisplatin has some definite effect on the arrangement and movement of ruffles/blebs over the surface of tumor cells and also leads to the formation of membrane vesicles/cellular vacuoles/thick blebs all of which ultimately favour the tumor cell death.

(iii) The enzyme lactate dehydrogenase showed comparatively increased/decreased activity in tumor cells (↓), ascites flud (↑), serum (↑) and kidney (↘) following cisplatin treatment. In addition to this the appearance of a new isozyme which is here named as LDH-T was noted in the serum and tumor cells of the tumor-bearing hosts.

(iv) Vitamin C showed synergistic effect with cisplatin and it may be used with subtherapeutical dose of cisplatin in protecting the host against cisplatin induced nephrotoxicity without losing the therapeutic efficacy.

(v) Along with the enhanced therapeutic efficacy of cisplatin by vitamin C, it may also protect the host against cisplatin induced mutagenicity.

(vi) These studies further indicate the involvement of multistep and multilevel effects of cisplatin resulting the tumor regression in the host.

REFERENCES

Abercrombie, A. and Ambrose, E.J. (1962). The surface properties of cancer cells: A review. Cancer Res.; 22: 525.

Anderson, R. (1984). The immunostimulatory, anti-inflammatory and anti-allergic properties of ascorbate. Adv. Nutr. Res.; 6: 19-45.

Andrews, P. and Howell, S.B. (1990). Cellular pharmacology of cisplatin: Perspectives on mechanisms of acquired resistance. Cancer Cells; 2: 35-43.

Asaka, M., Nagase, K., Shiraishi, T. and Miyazaki, T. (1980). Diagnosis of cancer by radioimmunoassay of muscle type aldolase. Gann.; 71: 433-440.

Aull, J. L., Allen, R. L., Bapat, A. R., Daron, H. H., Friedman, M. E. and Wilson, J. F. (1979). The effect of platinum complexes on seven enzymes. Biochem. Biophys. Acta.; 57: 352-358.

Bagasra, O., Currao, L., De Souza, L. R., Oosterhuis, J. W. and Damjanov (1985). Immune response of mice exposed to cis-diamminedichloroplatinum. Cancer Immunol. Immunother.; 19: 142-147.

Baggett, J. and Berndt, W. O. (1986). The effect of depletion of non-protein sulfhydryls by diethyl maleate plus buthionine sulfoxime on renal uptake of mercury in the rat. Toxicol. Appl. Pharmacol.; 83: 556-562.

Bahadur, A., Sarna, S. and Sodhi, A. (1984). Enhanced cell mediated immunity in mice after cisplatin treatment. Pol. J. Pharmacol. Pharm.; 36: 441-448.

Banic, S. (1981). Vitamin C acts as a carcinogen to methylcholanthrene in guinea pigs. Cancer Lett.; 11: 239-242.

Barberi-Heyob, M., Griffon, G., Merlin, J. L. and Weber, B. (1993). Sequence-dependent growth-inhibitory effects of the in vitro combination of fluorouracil, cisplatin and dipyridamol. Cancer Chemother. Pharmacol.; 33: 163-170.

Barclay, M. and Terebus-Kekish, O. (1973). Enzyme activities and extrinsic proteins in plasmamembrane from normal liver and Morris hepatoma 5123 tc. J. Natl. Cancer Inst.; 51: 1709-1710.

Barnett, R. E., Furcht, L. T. and Scott., R. E. (1974). Differences in membrane fluidity and structure in contact inhibited and transformed cells. Proc. Natl. Acad. Sci. (USA); 71: 1992-1994.

Barrett, A. J. (1981). Cystatin, the egg white inhibitor of cysteine proteinases. Methods Enzymol.; 80: 771-778.

Basinger, M.A., Jones, M. M., Gilbreath IV, S. G., Walker, E. M., Fody, E. P. and Mayhue, M. A. (1989). Dithiocarbamate-induced biliary platinum excretion and the control of cis-platinum nephrotoxicity. Toxicol. Appl. Pharmacol.; 97: 279-288.

Beck, D.J. and Brubaker, R. R. (1975). Mutagenic properties of cis-platinum (II) diamminedichloride in Escherichia coli. Mutat. Res.; 27: 181-189.

Benedict, W. F., Wheatly, W. L. and Jones, P. A. (1982). Differences in anchorage-dependent growth and tumorigenicities between transformed C3H 10T1/2 cells with morphologies that are or are not reverted to a normal phenotype by ascorbic acid. Cancer Res.; 42: 1041-1045.

Benedict, W. R., Wheatley, W. L. and Jones, P. A. (1983). The use of ascorbic acid for selection of transformed cells with differences in tumorigenecities and anchorage-independent growth: implications for chemoprevention. In: Modulation and Mediation of Cancer by Vitamins. Karger, Basel; pp. 114-118.

Bhunya, S. P. and Pati, P. C. (1988). Genotoxic effects of a synthetic pyrethroid insecticide cypermethrin in mice in vivo. Toxicol. Lett.; 41: 223-230.

Bishnu, N., Basu, T. K., Metcalfe, S. and Williams, D. C. (1978). The effect of ascorbic acid on two tumor cell lines in culture. Oncology; 35: 160-162.

Bishop, J. M. (1982). Oncogenes. In: Readings from Scientific American. Cancer Biology, Freeman, W. H. and Company, New York; pp. 66-76.

Bocian, E., Laverick, M. and Nias, A. H-W. (1983). Sister chromatid exchanges induced by two radiosensitizing platinum compounds cis-dichlorobis-isopropylamine-trans-dihydroxyplatinum IV (CHIP) and cis-platin metronidazole C/2 (FLAP) in CHO cells in vitro. Br. J. Cancer; 48: 803-807.

Bohn, B., Thies, C. and Brossmer, R. (1977). Cell surface changes, sialic acid content and metabolic behaviour of two tumor sublines. A comparative study. Eur. J. Cancer; 13: 1145-1150.

Borch, R. F. and Pleasants, M. E. (1979). Inhibition of cis-platinum nephrotoxicity by diethyldithiocarbamate in a rat model. Proc. Natl. Acad. Sci. (USA); 76: 6611-6614.

Borch, R. F., Katz, J. C., Lieder, P. H. and Pleasants, M. E. (1980). Effect of diethyldithio-carbamate rescue on tumor response to cis-platinum in a rat model. Proc. Natl. Acad. Sci. (USA); 77: 5441-5444.

Brady, H. R., Kone, B. C., Stromski, M. E., Zeidel, M. L., Giebisch, G. and Gullans, S. R. (1990). Mitochondrial injury: An early event in cisplatin toxicity to renal proximal tubules. Am. J. Physiol.; 258: F1181-F1187.

Brouwer, J. P., Vande, P., Fichtinger-Schepman, A.M.J. and Reedijk, J. (1981). Base pair substitution hotspots in GAG and GCG nucleotides sequences in E. coli K_2 induced by cis-dichlorodiammine platinum (II). Proc. Natl. Acad. Sci. (USA); 78: 7010-7014.

Brown, G. J. Jr. and Cohen, P. P. (1959). Comparative biochemistry of urea synthesis. I. Method for the quantitative assay of urea cycle in liver. J. Biol. Chem.; 234: 1769-1774.

Brown, J. C. and Hunt, R. C. (1978). Lectins. Int. Rev. Cytol.; 52: 277-349.

Buckton, K. A. and Pike, M. C. (1964). Time in culture; An important variable in studying in vivo radiation-induced chromosome damage in man. Int. J. Rad. Biol.; 8: 439-452.

Cabaud, P. G. and Wroblewski, F. (1958). Calorimetric measurement of lactic dehydrogenase activity of body fluids. Am. J. Clin. Pathol.; 30: 34-240.

Cahill, G. F. Jr., Anhmore, J., Renold, A. E. and Hasting, A. B. (1959). Am. J. Med.; 26: 264-269.

Cahn, R. D., Kaplan, N. O., Levine, L. and Zwelling, L. E. (1962). Nature and development of lactic dehydrogenases. Science; 136: 962-969.

Cairns, J. (1986). The cancer problem. In: Readings from Scientific American. Cancer Biology, Freeman, W. H. and Company, New York; pp. 4-14.

Carda-Abella, P., Perez-Cuadrado, S., Lara-Barique, L., Gil-Grande, L. and Nunez-Puertas, A. (1982). LDH isozyme patterns in tumors, polyps and uninvolved mucosa of human cancerous colon. Cancer; 49: 80-93.

Catherine, A. M., Suzuki, and Cherian, M. G. (1990). The interactions of cis-diamminedichloroplatinum with metallothionein and glutathione in rat liver and kidney. Toxicology; 64: 113-127.

Chakrabarti, R. N. and Dasgupta, P. S. (1984). Effects of ascorbic acid on survival and cell-mediated immunity in tumor bearing mice. IRCS Med. Sci.; 12: 1147-1148.

Chakrabarti, S., Chakrabarti, A. and Pal, A.K. (1984). Chromosome analysis of Dalton's lymphoma adapted to Swiss mouse: Clonal evaluation and C-heterochromatin distribution. Cancer Genet. Cytogenet.; 11: 417-423.

Chatterjee, S. K., Bhattacharya, M. and Barlcw, J. J. (1981). Evaluation of 5'-nucleotidase as an enzyme marker in ovarian carcinoma. Cancer; 47: 2648-2653.

Chen, G. and Pan, Q. (1988). Potentiation of antitumor activity of cisplatin in mice by 3-aminobenzamide and nicotinamide. Cancer Chemother. Pharmacol.; 22: 303-307.

Chen, L. H., Boissnneault, G. A. and Glauert, H. P. (1988). Vitamin C, Vitamin E and cancer. Anticancer Res.; 8: 739-748.

Chen, Y. Q., Zhou, Y. Q. and Yu, S. Y. (1979). Studies on the correlation between alterations in serum sialic acid levels and remission, metastasis, and recurrence of disease in patients with cancer. Zhonghua Zhongliu Zazhi; 1: 29-34.

Cherian, M. G. (1980). The synthesis of metallothionein and cellular adaptation to metal toxicity in primary rat kidney epithelial cell cultures. Toxicology; 17: 225-232.

Collins, J. L. and Kao, M. S. (1989). The anticancer drug, cisplatin, increases the naturally occurring cell-mediated lysis of tumor cells. Cancer Immunol. Immunother.; 29: 17-22.

Conran, P. B. and Rosenberg, B. (1972). The role of host defense in the regression of sarcoma-180 in mice treated with cis-dichlorodiammineplatinum (II). In: Antimicrobial and Antineoplastic Chemotherapy. Semonesky, M., Hejzlar, M. and Masal, S. (eds.), University Park Press, New York; pp. 235-236.

Cook-Mozaffari, P. (1979). The epidemiology of cancer of the oesophagus. Nutr. Cancer; 1: 51-60.

Currie, G. A. and Bagshawe, K. D. (1969). Tumor specific immunogenicity of methylcholanthrene induced sarcoma cells after incubation in neuraminidase. Br. J. Cancer; 22: 141-149.

Daugaard, G. (1990). Cisplatin nephrotoxicity: Experimental and clinical studies. Danish Med. Bull.; 37: 1-12.

Davis, B. J. (1964). Disc electrophoresis: II: Method and application to human serum proteins. Ann. N. Y. Acad. Sci.; 121: 404-427.

Dawson, D. M., Goodfriend, T. L. and Kaplan, N. O. (1964). Lactic dehydrogenases: functions of the two types. Science; 143: 929-933.

Day, R. S. III., Ziolkowski, C. H. J., Scudiero, D. A., Meyer, S. A., Lubiniecki, A. S., Girardi, A. J., Galloway, S. M. and Bynum, G. D. (1980). Defective repair of alkylated DNA by human tumor and SV 40-transformed human cell strains. Nature; 288: 724-727.

Dehand, J. and Jordanov, J. (1976). Interaction of cis-diaminotolueneplatinum (II) with nucleosides: Evidence for guanosine O(6).N(7) chelation by platinum. JCS. Chem. Comm.; 598-599.

Devamanoharan, P. S., Sankar, R. and Shyamala Devi, C. S. (1987). Levels of arginase and transaminase in polychlorinated divenzofuran (PCDFs) fed rats. Curr. Sci.; 56: 601-603.

Dito, W. R. (1973). A simple time-saving method for interpretative report generation. I. Lactate dehydrogenase isozymes. Am. J. Clin. Pathol.; 59: 439.

Dixon, T. F. and Purdon, M. (1954). Serum 5'-nucleotidase. J. Clin. Pathol.; 7: 341-348.

Dobyan, D. C., Levi, J., Jacobs, C., Kosek, J. and Weiner, M. W. (1980). Mechanism of cis-platinum nephrotoxicity: II. Morphologic observations. J. Pharmacol. Exp. Ther.; 213: 551-556.

Dowjat, K. and Kawiak, J. (1979). Karyotype analysis of two L 1210 murine leukemia lines growing in vivo and in vitro. Cytologia; 44: 927-934.

Drapier, J. C. and Hibbs, J. B. Jr. (1988). Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells result in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in macrophage effector cells. J. Immunol.; 140: 2829-2838.

Dunham, W. D., Zuckerkandl, E., Reynolds, R., Willoughby, R., Marcuson, R., Earth, R. and Pauling, L. (1982). Effects of intake of L-ascorbic acid on the incidence of dermal neoplasms induced in mice by ultraviolet light. Proc. Natl. Acad. Sci. (USA); 79: 7532-7536.

Eastman, A. (1990). Activation of programmed cell death by anticancer agents: Cisplatin as a model system. Cancer Cells; 2: 275-280.

Elitzo, M., Jung, A. and Jackiseh, R. (1977). Chem. Biol. Interact.; 18: 295.

Emmelot, P. and Bos, C. J. (1969). Studies on plasma membranes. X. A survey of enzyme activities displayed by plasma membranes from mouse liver and three mouse hepatoma strains. Int. J. Cancer; 4: 723-734.

Fabricant, R. N., Delarco, J. E. and Todaro, G. J. (1977). Nerve growth factor receptors on human melanoma cells in culture. Proc. Natl. Acad. Sci. (USA); 74: 365-569.

Faulk, W. P., Rider, J. A. and Swader, J. I. (1972). Lactate dehydrogenase in gastric juice: Diagnostic adjunct in human stomach cancer. Lancet; 2: 1115.

Fidler, I. J. and Hart, I. R. (1982). Biological diversity in metastatic neoplasms. Science; 217: 998-1003.

Filmus, J. E., Podhaycer, O. L., Moreso, E., Guman, N. and Mordoh, J. (1984). Acid phosphatase in human breast cancer tissue. Cancer; 53: 301-305.

Firasen, R. C., Pavlovic, S., Kurahara, C. G., Murata, A., Peterson, N. S., Taylor, K. B. and Feigen, G. A. (1981). The effect of variation in vitamin C intake on the cellular immune response in guinea pig. Am. J. Clin. Nutr.; 33: 839-847.

Fiske, C. H. and Subbarow, Y. (1925). The colorimeter determination of phosphorus. J. Biol. Chem.; 66: 375-400.

Fleisher, M. Wasserstorm, W. R., Schold, S. C., Schwartz, M. K. and Posner, J. B. (1981). Lactic dehydrogenase isozymes in the cerebral fluids of patients with systemic cancer. Cancer; 47: 2654-2659.

Fraga, C. G., Motchnik, P. A., Shigenaga, M. K., Helbock, H. J., Jacob, R. A. and Ames, B. N. (1991). Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc. Natl. Acad. Sci. (USA); 88: 11003-11006.

Friedberg, E. C. (1986). Cancer: the nature of the problem. In: Readings from Scientific American. Cancer Biology, Friedberg, E. C. (ed.), Freeman, W. H. and Company, New York; p. 1.

Friedman, M. E., Otwell, H. B. and Teggins, J. E. (1975). Protection of the active site of mitochondrial malate dehydrogenase from inhibition by potassium tetrachloroplatinate. Biochem. Biophys. Acta.; 391: 1-8.

Fung, K. P., Choy, Y. M., Chan, T. W., Lam, W. P. and Lee, C. Y. (1986). Glucose regulates its own transport in Ehrlich ascites tumor cells. Biochem. Biophys. Res. Commun.; 134: 1231-1237.

Gallagher, T. J. (1985). The cell-surface membrane in malignancy. In: The Molecular Basis of Cancer. Farmer, P. B. and Walker, J. M. (eds.), Croom Helm Ltd., Sydney, pp. 37-69.

Gebhart, E. (1977). Experimentelle beitrage zum problem der lokalen achromasien (Gops.), Human Genet.; 13: 98-107.

Gerritsen van der Hoop, R., De Koning, P., Boven, E., Neijt, J. P., Jennekens, F. G. I. and Gispen, W. H. (1988). Efficacy of neuropeptide Org 2766 in the prevention and treatment of cisplatin-induced neurotoxicity in rats. Eur. J. Cancer Clin. Oncol.; 24: 637-642.

Gey, K. F., Brubacher, G. B. and Stahelin, H. B. (1987). Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. Am. J. Clin. Nutr.; 45: 1368-1377.

Ghosh, J. and Das, S. (1984). Effect of vitamin C supplementation on some murine tumors. Ind. J. Cancer Chemother.; 6: 17-20.

Ghosh, J. and Das, S. (1985). Evaluation of vitamin A and C status in normal and malignant conditions and their possible role in cancer prevention. Jpn. J. Cancer Res.; 76: 1174-1178.

Glover, D., Grabelsky, S., Fox, K., Weiler, C., Cannon, L. and Glick, J. (1989). Clinical trials of WR-2721 and cis-platinum. Int. J. Radiat. Oncol. Biol. Phys.; 16: 1201-1204.

Goldie, H. and Felix, M. D. (1951). Growth characteristics of free tumor cells transformed serially in the peritoneal fluid of the mouse. Cancer Res.; 11: 73-80.

Goldman, R. D., Kaplan, N. C. and Hall, T. C. (1964). Lactic dehydrogenase in human neoplastic tissues. Cancer Res.; 24: 929-935.

Goldstein, R. S., Noordeweier, R., Bond, J. T., Hook, J. B. and Mayor, G. H. (1981). Cis-Dichlorodiammineplatinum nephrotoxicity: Time course and dose response of renal function impairment. Toxicol. Appl. Pharmacol.; 60: 163-178.

Gonda, M. A., Aaronson, S. A., Elimore, N., Zeve, V. H. and Nagashima, K. (1976). Ultrastructural studies of surface features of human normal and tumor cells in tissue culture by scanning and transmission electron microscopy. J. Natl. Cancer Inst.; 56: 245-263.

Gonzales-Vitale, J. C., Hayes, D. M., Cvitkovic, E. and Sternberg, S. S. (1977). The renal pathology in clinical trial of cis-platinum (II) diamminedichloride. Cancer; 39: 1362-1371.

Gordon, J. A. and Gattone, V. H. (1986). Mitochondrial alterations in cisplatin induced acute renal failure. An. J. Physiol.; 250: F991-F998.

Graham, J. M. (1979). Surface membrane enzymes in neoplasia. In: Surfaces of Normal and Malignant Cells. Hynes, R.O. (ed.), John Wiley and Sons Ltd., New York; pp. 199-246.

Granger, D. L., Taintor, R. R., Cook, J. L. and Hibbs, J. B. Jr. (1980). Injury of neoplastic cells by murine macrophages leads to inhibition of mitochondrial respiration. J. Clin. Invest.; 65: 357-370.

Greengard, O., Head, J. F., Goldberg, S. L. and Kirschern, P. A. (1982). Enzyme pathology and the histologic categorization of human lung tumors: The continuum of quantitative indices of neoplasticity. Cancer; 49: 460-467.

Gross, R. B. and Scanlon, K. J. (1986). Amino acid membrane transport properties of L 1210 cells resistant to cisplatin. chemioterapia; 5: 37-43.

Grunicke, H., Grunewald, K., Hellinger, W., Scheidl, F., Wolff-Schreiner, E. and Puschendorf, B. (1983). Inhibition of tumor growth by an alkylation of the plasmamembrane. Adv. Enzyme Regul.; 29: 21-30.

Gupta, P. and Sodhi, A. (1987). Increased release of interleukin-1 from mouse peritoneal macrophages in vitro after cisplatin treatment. Int. J. Immunopharmacol.; 9: 385-388.

Gupta, P. and Sodhi, A. (1988). Effect of cisplatin on release of lysozyme, plasminogen activator, leucine aminopeptidase and β-hexosaminidase by murine peritoneal macrophages in vitro. Ind. J. Exp. Biol.; 26: 679-684.

Guttenplan, J. B. (1977). Inhibition by L-ascorbate of bacterial mutagenesis induced by two N-nitroso compounds. Nature; 268: 368-370.

Hamers, F. P. T., Gispen, W. H. and Neijt, J. P. (1991). Neurotoxic side-effects of cisplatin. Eur. J. Cancer; 27: 372-376.

Hamilton, R. C., Bliss, J. M. and Horwich, A. (1989). The late effects of cis-platinum on renal function. Eur. J. Cancer Clin. Oncol.; 25: 185-189.

Hannemann, J. and Baumann, K. (1988). Cisplatinum induced lipid peroxidation and decrease of gluconeogenesis in rat kidney cortex: Different effects of antioxidants and radical scavengers. Toxicol.; 51: 119-132.

Harder, H. C. and Rosenberg, B. (1970). Inhibitory effects of anti-tumor platinum compounds on DNA, RNA and protein synthesis in mammalian cells in vitro. Int. J. Cancer; 6: 207-216.

Harder, H. C., Smith, R. G. and Leroy, R. F. (1976). Template primer inactivation by cis- and trans-dichlorodiammineplatinum for human DNA polymerase α, β, and Rauscher murine leukemia virus reverse transcription, as a mechanism of cytotoxicity. Cancer Res.; 36: 3821-3829.

Haskell, C. M. (1985). Drugs used in cancer chemotherapy-cisplatin. In: Cancer Treatment. Haskell, C. M. (ed.), Washington Sq., Sounders, W. B. Company, II edition; pp. 88-90.

Heinen, E. and Bassler, R. (1976). Mode of action of cis-dichlorodiammine platinum (II) on mouse Ehrlich ascites tumor cells. Biochem. Pharmacol.; 25: 1871-1875.

Helper, O. E. (1957). Manual of Clinical Laboratory Methods. Thomas, Springfield (IL); p. 285.

Henry, R. J., Sobel, C. and Kim, J. (1957). A modified carbonate-phosphotungstate method for the determination of uric acid and comparison with the spectrophotometric uricase method. Am. J. Clin. Pathol.; 28: 152-162.

Henson, D. A., Block, G. and Levine, M. (1991). Ascorbic acid: Biological functions and relation to cancer. J. Natl. Cancer Inst.; 83: 547-550.

Heyden, H. W. von, Schroder, M. and Achterrath, W. (1980). Chemotherapie von tumoren in Kopf-Halsbereich mit cisplatin. In: Cisplatin-derzeitiger stand und neue entwicklungen in der chemotherapie maligner neoplasien, Seber, S., Schmidt, C. G., Nagel, G. and Achterrath, W. (eds.), Karger, Basel; pp. 127-130.

Hibbs, J. B., Vavrin, Z. and Taintor, R. R. (1987). L-arginine is required for expression of the activated macrophage's effector mechanism causing selective metabolic inhibition in target cells. J. Immunol; 138: 550-565.

Higuchi, M., Higashi, N., Taki, H. and Osawa, T. (1990). Cytolytic mechanisms of activated macrophages: Tumor necrosis factor and L-arginine-dependent mechanisms of activated macrophages. J. Immunol.; 144: 1231-1425.

Hilf, R., Fledsten, M. L., Gibson, S. L. and Savlov, E. D. (1982). A logistic model based on enzyme activities for the prediction of response of breast cancer patients to chemotherapy. Cancer; 50: 1734-1738.

Hill, B. R. and Levi, C. (1954). Elevations of serum component in neoplastic diseases. Cancer Res.; 14: 513-522.

Holcenberg, J. S. (1981). Therapy of neoplasia with other non-essential amino acid degrading enzymes. In: Enzymes as Drugs. Holcenberg, J. S. and Roberts, J. (eds.), John Wiley and Sons, New York; pp. 25-61.

Holland, J. F., Bruckner, H. W., Cohen, C. J., Wallach, R. C., Gusberg, S. B., Greenspan, E. M. and Goldberg, J.

(1980). Cisplatin therapy of ovarian cancer. In: Cisplatin, current status and new developments. Prestayko, A., Crooke, S. T. and Carter, S.K. (eds.), Academic Press, Inc.; pp. 383-391.

Holloway, D. F. and Peterson, F. J. (1984). Ascorbic acid in drug metabolism. In: Drugs and Nutrients. Roe, D. A. and Campbell, T. C. (eds.), Marcel Dekker, Inc., New York; 21: 225-295.

Holyoke, E. D., Block, G. E. and Jemsen, E. et. al. (1981). Biological markers in cancer diagnosis and treatment. In: Current Problems in Cancer. Hickey, R. C. (ed.), Year Book Medical Publisher, Chicago.

Howell, S. B., Pfeile, C. E., Wung, W. E. and Olshen, R. A. (1983). Intraperitoneal cis-diamminedichloroplatinum with systemic thiosulfate protection. Cancer Res.; 43: 1426-1431.

Hugenholtz, A. P. and Bruce, W. R. (1977). Transmission of radiation induced elevations in abnormally shaped murine sperms. In: Abstracts of the 8th Annual meeting, Environmental Mutagen Society, Denvor, Colorodado; p. 75.

Hynes, R. D. (1979). Tumorigenicity, transformation and cell surfaces. In: Surface of Normal and Malignant Cells. Hynes, R. D. (ed.), John Wiley and Sons, New York; pp. 1-19.

Inbar, M. and Sachs, L. (1969). Interaction of the carbohydrate binding protein concanavalin A with normal and transformed cells. Proc. Natl. Acad. Sci. (USA); 63: 1418-1425.

Iwamato, Y., Kawano, T., Ishizawa, M., Aoki, K., Kuroiwa, T. and Baba, T. (1985). Inactivation of cis-diamminedichloroplatinum (II) in blood and protection of its toxicity by sodium thiosulfate in rabbits. Cancer Chemother. Pharmacol.; 15: 228-232.

Jäckel, M. and Köpf-Maier, P. (1991). Influence of cell-cycle progression in xenografted human head and neck carcinomas. Cancer Chemother. Pharmacol.; 27: 464-471.

Jewell, S. A., Bellome, G., Thor, H., Orrenius, S. and Smith, M. T. (1982). Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. Science; 217: 1257-1259.

Johnson, N. P., Botour, J. -L., Wimmer, F. L., Defais, M., Pierson, V. and Brabec, V. (1989). Metal antitumor components: The mechanism of action of platinum complexes. Prog., Clin. Biochem., Med.; 10: 1-24.

Jones, M. M. and Basinger, M. A. (1989). Thiol and thioether suppression of cis-platinum induced nephrotoxicity in rats bearing the Walker 256 carcinosarcoma. Anticancer Res.; 9: 1937-1942.

Just, G. and Holler, E. (1989). Platinum incorporation and differential effects of cis- and trans-diamminedichloroplatinum (II) on the growth of mouse leukemia P388/D1. Cancer Res.; 49: 7072-7078.

Just, G. and Holler, E. (1991). Enhanced levels of cyclic AMP, adenosine (5') tetraphospho (5') adenosine and nucleoside 5'-triphosphates in mouse leukemia P388/D1 after treatment with cis-diamminedichloroplatinum (II). Biochem. Pharmacol.; 42: 285-294.

Kallistratos, G. and Fasske, E. (1980). Inhibition of benzo (a)pyrene carcinogenesis in rats with vitamin C. J. Cancer Res. Clin. Oncol.; 97: 91-96.

Kallistratos, G. and Fasske, E. (1983). The effect of vitamin C on transplanted fibrosarcoma cells in rats. J. Med. Sci.; 1: 9-12.

Kallman, R. F. (1972). The phenomena of oxygenation and its implications for fractionated radiotherapy. Radiology; 105: 135.

Kao, T. -L., Mayer, W. J. III and Post, F. M. (1993). Inhibitory effects of ascorbic acid on grwoth of leukemic and lymphoma cell lines. Cancer Lett.; 70: 101-106.

Kempf, S. R. and Ivankovic, S. (1986a). Chemotherapy-induced malignancies in rats after treatment with cisplatin (CDDP) as single agent and in combination: preliminary results. Oncology; 43: 187-191.

Kempf, S. R. and Ivankovic, S. (1986b). Carcinogenic effect of cisplatin (cis-diamminedichloroplatinum-II, CDDP) in BD IX rats. J. Cancer Res. Clin. Oncol.; 111: 133-136.

Kilderma, L. A., Teras, L. E. and Lond, M. E. (1977). Vopr. Med. Khim.; 23: 496-506.

Klein, D. and Morris, D. R. (1978). Increased arginase activity during lymphocyte mitogenesis. Biochem. Biophys. Res. Commun.; 81: 199-204.

Kleinerman, E. S. and Muchmore, A. V. (1981). Effect of various cancer chemotherapeutic agents on naturally occurring human spontaneous monocyte-mediated cytotoxicity (SMMC). Proc. Am. Assoc. Cancer Res.; 22: 1101.

Kleinerman, E. S. and Zwelling, L. A. (1982). The effect of cis-diamminedichloroplatinum (II) on immune functions in vitro and in vivo. Cancer Immunol. Immunother.; 12: 191-196.

Kleinerman, E. S. and Zwelling, L. A. (1984). Effects of cisplatin, bleomycin and DTIC on immune function in vitro and in vivo. Clin. Immunol. Allergy; 4: 279-294.

Kleinerman, E. S., Zwelling, L. A. and Muchmore, A. V. (1980a). The enhancement of naturally occurring spontaneous monocyte-mediated cytotoxicity by cis-diamminedichloroplatinum (II). Cancer Res.; 40: 3099-3104.

Kleinerman, E. S., Zwelling, L. A., Howser, D., Barlock, A., Young, R. C., Decker, J., Bull, J. M. and Muchmore, A. V. (1980b). Defective monocyte killing in patients with malignancies and restoration of function during chemotherapy. Lancet; 2: 1102-1205.

Kociba, R. J. and Sleight, S. D. (1971). Acute toxicologic and pathologic effects of cis-diamminedichloroplatinum (NSC-119875) in the male rat. Cancer Chemother. Rep.; 55: 1-16.

Kociba, R. J., Sleight, S. D. and Rosenberg, B. (1970). Inhibition of Dunning ascitic leukemia and Walker 256 carcinosarcoma with cis-diamminedichloroplatinum (NSC-119875). Cancer Chemother. Rep.; 54: 325-328.

Kolonel, L. N., Hankin, J. H., Lee, J., Chu, S. Y., Nomura, A. M. Y and Hinds, M. W. (1981). Nutrient intakes in relation to cancer incidence in Hawaii. Br. J. Cancer; 44: 332-339.

Köpf-Maier, P., Wagner, W. and Liss, E. (1983). Induction of cell arrest at G_1/S and in G_2 after treatment of Ehrlich ascites tumor cells with metallocene dichlorides and cis-platinum in vitro. J. Cancer Res. Clin. Oncol.; 106: 44-52.

Krakoff, I. H. (1979). Nephrotoxicity of cis-dichlorodiammineplatinum (II). Cancer Treat. Rep.; 633: 1523-1525.

Krasner, N. and Dymock, I. W. (1974). Ascorbic acid deficiency in malignant diseases: A clinical and biochemical study. Br. J. Cancer; 30: 142-145.

Kusenda, J., Kalafut, F., Klobusicka, M. and Novotna, L. (1992). Tumoricidal properties of rat peritoneal macrophages activated with various activators depend on nitrogen oxide synthesis. Neoplasma; 39: 15-28.

Lemasters, J. J., Giuseppi, J. D., Nieminen, A. L. and Herman, B. (1987). Blebbing, free calcium and mitochondrial membrane potential preceding cell death in hepatocytes. Nature; 325: 78-80.

Leopold, w. R., Miller, E. C. and Miller, J. A. (1979). Carcinogenicity of antitumor cis-platinum (II) coordination complexes in the mouse and rat. Cancer Res.; 39: 913-918.

Lessler, M. A. (1982). Adaptation of polarographic oxygen sensors for biochemical assays. In: Methods of Biochemical Analysis. Glick, D. (ed.); 28: 175-199.

Levi, J., Jacobs, C., Kalman, S. M., McTigue, M. and Weiner, M. W. (1980). Mechanism of cis-platinum nephrotoxicity. 1. Effects of sulfhydryl groups in rat kidneys. J. Pharmacol. Exp. Ther.; 213: 545-550.

Liehr, J. G. and Wheeler, W. J. (1983). Inhibition of estrogen-induced renal carcinoma in Syrian hamsters by vitamin C. Cancer Res.; 43: 4638-4642.

Liepins, A. (1983). Possible role of microtubules in tumor cell surface membrane shedding, permeability and lympholysis. Cellular Immunol.; 76: 120-128.

Liepins, A. and de Harven, e. (1982). Effects of cyclic nucleosides on the shedding of tumor cell surface membranes. Exp. Cell Res.; 139: 265-273.

Liotti, F. S. and Talesa, V. (1982). Ascorbic acid and tumor growth. Convivia Medica Suppl.; 3: 81-96.

Liotti, F. S., Talesa, W. and Menghini, A. R. (1983). Absence of accumulation phenomena in normal and tumoral tissues of mice treated with ascorbic acid. Int. J. Vit. Nutr. Res.; 53: 251-257.

Lippert, M. C. and Javadpour, N. (1981). Lactic dehydrogenase in the monitoring and prognosis of testicular cancer. Cancer; 48: 2274-2278.

Lippert, M. C., Papadopoulos, N. and Javadpour, N. (1981). Role of lactate dehydrogenase isozymes in testicular cancer. Urology; 18: 50-53.

Litterst, C. L., Bertolero, F. and Uozumi, J. (1986). The role of glutathione and metallothionein in the toxicity and subcellular binding of cisplatin. In: Biochemical Mechanisms 'of Platinum Antitumor Drugs. McBerien, D. C. H. and Slater, T. F. (eds.), IRL Press, Oxford; pp. 227-254.

Loehrer, J. P. and Einhorn, L. H. (1984). Cisplatin. Ann. Int. Med.; 100: 704-713.

Logue, T. and Frommer, D. (1980). The influence of oral vitamin C supplements on experimental colorectal tumor induction. Austr. N. Z. J. Med.; 10: 588.

Lopes, J., Zucker-Franklin, D. and Silber, B. (1973). Heterogeneity of 5'-nucleotidase activity in lymphocytes in chronic lymphocytic leukemia. J. Clin. Invest.; 52: 1297-1300.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Foline phenol reagent. J. Biol. Chem.; 193: 265-275.

Lupulescu, A. (1983). Hormonal regulation of pre-cancerous and cancerous cell populations. In: Hormones and Carcinogens; pp. 46-88.

MacDonald, R. P., Simpson, J. R. and Nossal, E. (1957). Serum lactic dehydrogenase - A diagnostic aid in myocardial infarcation. JAMA; 165: 35-42.

Machlin, N. and Bendich, A. (1987). Free radical tissue damage: Protective role of antioxidant nutrients. FASEB. J.; 1: 441-445.

Madias, N. E. and Harrington, T. (1978). Platinum nephrotoxicity. Am. J. Med.; 65: 307-314.

Mally, M. B., Taylor, R. C. and Callewaart, M. (1980). Effects of platinum antitumor agents on in vitro assays of human antitumor immunity. Chemotherapy; 26: 1-6.

Mancy, S., Rosenberg, b. and Thomsson, A. J. (1973). Binding of cis- and trans-dichlorodiammine platinum (II) to nucleosides. I. Location of the binding sites. J. Amer. Chem. Soc.; 95: 1633-1640.

Maquet, J. P., Botour, J. L., Johnson, N. P., Razaka, H., Salles, B., Vieussens, C. and Wright, M. (1984). Is DNA the real target of antitumor platinum compounds? Dev. Oncol.; 17: 27-38.

Markert, C. L. (1963). Lactate dehydrogenase isozymes. Dissociation and recombination of subunits. Science; 140: 1329-1330.

Marquez, E. D. (1976). Binding of concanavalin A by normal, herpes virus transformed and trypsin treated hamster embryo fibroblasts. Exp. Cell Res.; 101: 425-429.

Marx, J. L. (1976). chemotherapy: Renewed interest in platinum compounds. Science; 192: 774-775.

Mayer, R. D., Lee, K. and Cockett, A. T. K. (1987). Inhibition of cisplatin-induced nephrotoxicity in rats by buthionein sulfoximine, a glutathione synthesis inhibitor. Cancer Chemother. Pharmacol.; 20: 207-210.

McConkey, D. J., Nicotera, P., Hartzell, P., Bellomo, G., Wyllie, A. H and Orrenius, S. (1989). Glucocorticoids activate a suicidal process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. Arch. Biochem. Biophys.; 269: 365-370.

McGinness, J. E., Proctor, P. H., Demopoulos, H. B., Hokanson, J. A. and Kirkpatrick, D. S. (1978). Amelioration of cis-platinum nephrotoxicity by orgotein (superoxide dismutase). Physiol. Chem. Phys.; 10: 267-277.

Migliozzi, J. A. (1977). Effect of ascorbic acid on tumor growth. Br. J. Cancer; 35: 448-453.

Mirabelli, F., Salis, A., Vairetti, M., Bellomo, G., Thor, H. and Orrenius, S. (1989). Cytoskeletal alterations in human platelets exposed to oxidative stress are mediated by oxidative and calcium-dependent mechanisms. Arch. Biochem. Biophys.; 270: 478-488.

Mirvish, S. S., Cardesa, A., Wallcave, L. and Shubik, P. (1975). Induction of mouse lung adenomas by amines or ureas plus nitrite and by N-nitroso compounds. Effect of ascorbate, gallic acid, thiocyanate and caffaine. J. Natl. Cancer Inst.; 55: 633-636.

Mizutani, Y., Bonavida, B., Nio, Y. and Yoshida, O. (1993). Enhanced susceptibility of cis-diamminedichloride - teated K 562 cells to lysis by peripheral blood lymphocytes and lymphokine activated killer cells. Cancer; 71: 1313-1321.

Morrison, D. G., Daniel, J., Lynd, F. T., Moyer, M. P., Esparza, R. J., Moyer, R. C. and Rogers, W (1981). Retinyl palmitate and ascorbic acid inhibit pulmonary neoplasms in mice exposed to fiberglass dust. Nutr. Cancer; 33: 81-85.

Nakano, S. and Gemba, M. (1989). Potentiation of cisplatinum-induced lipid peroxidation in kidney cortical slices by glutathione depletion. Jpn. J. Pharmacol.; 50: 87-92.

Natochini, Y. V., Myazina, E. M., Reznik, L. V., Brovtsyn, V. K., Bakhteeva, V. T. and Ivanov, V. B. (1987). Use of organic acids and bases for the prevention of renal function disorders after cisplatin injection. Pathol. Physiol. Exp. Ther. (Russ.); 2: 65-68.

Nicolson, G. L. (1979). Cancer metastasis. Sci. Am.; 240: 50-60.

Nicolson, G. L., Dulski, K., Basson, C. and Welch, D. R. (1985). Preferential organ attachment and invasion in vitro by B16 melanoma cells selected for differing metastatic colonization and invasive properties. Invas. Metas.; 5: 144-158.

Nordlie, R. C. and Sukalaski, K. A. (1985). In: The enzymes of biological membranes. Martonosi, A. N. (ed.), Plenum Publishing Co., New York; 2: 349-398.

Old, L. J. (1977). Cancer immunology. In: Cancer Biology, Readings from Scientific American. W. H. Freeman and Co., New York; pp. 125-137.

Omaye, S. T., Turnbull, J. D. and Sauberlich, H. E. (1979). Selected methods for the determination of ascrobic acid in animal cells, tissues and fluids. Methods Enzymol.; 62: 3-5.

O'Neill, J. P., Couch, D. B., Machanoff, R., San Sebastian, J. R., Brimer, P. A. and Hsie, A. W. (1977). A quantitative assay of mutation induction at the hypoxanthine-guanine phosphorybosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system). Utilization with a variety of mutagenic agents. Mutat. Res.; 45: 103-109.

Ormond, T., Pralt, I. and Ryan, M. P. (1988). Reduced nephrotoxicity in vivo and in vitro of cis-platinum - methionine complex. Br. J. Pharmacol.; Suppl. 95: p. 584.

Osswald, H. (1983). Adenosine and renal function. In: Regulatory Function of Adenosine. Berne, R. M., Rall, T. W. and Rubio, R. (eds.); The Hague, Boston, London, Martunus Nijhoff; pp. 399-415.

Page, R. H., Tally, R. W. and Liversmore, D. H. (1977). The effects of cis-diamminedichloroplatinum (II) and cyclophosphamide on immune response and tumor rejection in BALBC and PL/JAX mice. J. Clin. Hematol. Oncol.; 7: 105-113.

Parker, J. E., Slater, T. F. and Willson, R. L. (1979). Direct observation of a free radical interaction between vitamin E and vitamin C. Nature; 278: 737-738.

Pascoe, J. P. and Roberts, J. J. (1974). Interactions between mammalian cell DNA and inorganic platinum compounds: I. DNA interstrand crosslinking and cytotoxic properties of platinum (II) compounds. Biochem. Pharmacol., 23: 1345-1357.

Pauling, L., Nixon, J. C., Stiff, F., Marcuson, R., Dunham, W. B., Barth, R., Eensch, K., Herman, Z. S., Elaisdell, B. D., Tsao, C., Prender, M., Andrews, V., Willoughby, R. and Zuckerkandl, E. (1985). Effect of dietary ascorbic acid on the incidence of spontaneous mammary tumors in R III mice. Proc. Natl. Acad. Sci. (USA); 82: 5185-5189.

Peters, R. H. and Stuart, R. K. (1990). Synergism between 4-hydroperoxycyclophosphamide and cisplatin: Importance of incubation sequence and measurement of cisplatin accumulation. Biochem. Pharmacol.; 39: 607-609.

Phelps, J. S., Gandolfi, A. J., Brendel, K. and Dorr, R. I. (1987). Cisplatin nephrotoxicity: in vitro studies with precision-cut rabbit renal cortical slices. Toxicol. Appl. Pharmacol.; 90: 501-512.

Pinto, A. L. and Lippard, S. J. (1985). Binding of the antitumor drug cis-diamminedichloroplatinum (II) (cisplatin) to DNA. Biochem. et Biophys. Acta; 780: 167-180.

Plummer, D. T. (1978a). Estimation of carbohydrates by the anthrone method. In: An Introduction to Practical Biochemistry. McGraw-Hill Book Co., New York; pp. 183-184.

Plummer, D. T. (1978b). Assay of glucose-6-phosphatase. In: An Introduction to Practical Biochemistry. McGraw-Hill Book Co., New York; pp. 343-344.

Pogach, L. M., Lee, Y., Giglio, W., Naumoff, M. and Huang, H. F. S. (1989). Zinc acetate pretreatment ameliorates cisplatin-induced sertoli cell disfunction in Sprague-Dawley rats. Cancer Chemother. Pharmacol.; 24: 177-180.

Pooly, A. C. M., Van Dijk, M. and Lohman, P. H. M. (1984). Induction and repair of DNA cross-links in Chinese hamster ovary cells treated with various platinum coordination compounds in relation to platinum binding to DNA, cytotoxicity, mutagenicity, and antitumor activity. Cancer Res.; 44: 2043-2051.

Poretz, R. D. and Goldstein, I. J. (1970). An examination of the topography of the saccharide binding sites of concanavalin A and of the forces involved in complexation. Biochemistry; 9: 2890-2896.

Porter, K. R. and Fonte, V. G. (1973). Observations on the topography of normal and cancer cells. In: Scanning Electron Microscopy, Part III. Proc. Workshop on SEM in Path, Chicago; pp. 683-688.

Porter, K. R., Todaro, G. J. and Fonte, J. J. (1973). A scanning electron microscope study of surface features of viral and spontaneously transformants of mouse Balb/C 3T3 cells. J. Cell Biol.; 59: 633-642.

Post, R. L., Toda, G. and Rogers, F. N. (1975). Phosphorylation by inorganic phosphate of sodium plus potassium ion transport adenosine triphosphatase. J. Biol. Chem.; 250: 691-701.

Prasad, A. (1981). Chemo-immunotherapeutic and histochemical studies on transplantable tumor in mice with cis-dichlorodiammine platinum (II). Ph.D. thesis, Banaras Hindu University, Varanasi, India.

Prasad, K. N. and Rama, B. N. (1983). Modification of the effect of pharmacological agents on tumor cells in culture by vitamin C and vitamin E. In: Modulation and Mediation of Cancer by Vitamins. Karger, Basel; pp. 244-257.

Prasad, K. N., Sinha, P. K., Ramanujan, M. and Sakamoto, A. (1979). Sodium ascorbate potentiates the growth inhibitory effects of certain agents on neuroblastoma cells in culture. Proc. Natl. Acad. Sci. (USA); 76: 829-832.

Prasad, S. B. (1981). Biochemical fluorescence and electron microscopical studies on the effect of cis-diammine dichloro platinum (II) on the surface of tumor and normal cells. Ph. D. thesis, Banaras Hindu University, Varanasi, India.

Prasad, S. B. (1986). Studies on the sialic acid in tumor and normal cells using cisplatin as a probe. Curr. Sci.; 55: 651-654.

Prasad, S. B. (1987). Effect of cisplatin and trypsin on fibrosarcoma cells by Con A-FITC labelling. Curr. Sci.; 56: 1295-1298.

Prasad, S. B. (1989). Effect of cisplatin on con A agglutinability of different lymphoid cells of Swiss albino rats. Neoplasma; 336: 171-177.

Prasad, S. B. and Sodhi, A. (1981). Effect of cis-diammine dichloroplatinum (II) on the agglutinability of tumor and normal cells with concanavalin-A and wheat germ agglutinin. Chem. Biol. Interact.: 36: 355-367.

Prasad, S. B. and Sodhi, A. (1982). Effect of cis-dichlorodiammineplatinum (II) on surface of tumor and normal cells: Biochemical, fluorescence and electron microscopical studies. Ind. J. Exp. Biol.; 20: 559-571.

Presnov, M. A., Konovalova, A. L., Romanova, L. F., Sofina, Z. P. and Stetsenko, A. I. (1978). Chemotherapy of transplantable tumors with cis-dichlorodiammine platinum (II) alone and in combination with sarcolysin. Cancer Treat. Rep.; 62: 705-712.

Prestayko, A. W., Crooke, S. T. and Carter, S. K. (eds.) (1980). Cisplatin: current status and new developments. Academic Press, New York.

Preston, R. J., Au, W., Bender, M. A., Brewen, J. G., Carrano, A. V., Heddle, J. A., McFee, A. F., Wolff, S. and Wassom, J. S. (1981). Mammalian in vivo and in vitro cytogenetic assays: A report of the USEPA's Gene-Tox Programme. Mutat. Res.; 87: 143-188.

Provoost, A. P. and Molenaar, J. C. (1980). Changes in the glomerular filtration rate after unilateral nephrectomy in rats. Pflügers Archiv; 385: 161-165.

Pulcinski, M., Riley, M., Prorok, J. and Alhadeff, J. (1986). Total and lipid associated serum sialic acid levels in cancer patients with different primary sites and differing degrees of metastatic involvement. Cancer; 58: 2860-2865.

Rambotii, P. and and Davis, S. (1981). Lactic dehydrogenase in normal and leukemia lymphocyte subpopulations: Evidence for the presence of abnormal T cells and B cells in chronic lymphocytic leukemia. Blood; 57: 324-327.

Raz, A., Collard, J. G. and Inbar, M. (1978). Decrease in 5'-nucleotidase activity in malignant transformed and normal stimulated cells. Cancer Res.; 38: 1258-1262.

Reed, E. (1990). Cisplatin. In: Cancer Chemotherapy and Biological Response Modifiers Annual II. Pinedo, H. M., Chabner, B. A. and Longo, D. L. (eds.), Elsevier Science Publishers, B. V.; pp. 90-96.

Reitman, S. and Frankel, S. (1957). A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol.; 28: 56-62.

Reznik, L. V., Myazina, E. M., Shakohmatova, E. I., Gambaryan, S. P., Brovtsyn, V. K., Natochini, Y. V. and Jones, M. M. (1991). The prevention of cisplatin-induced renal dysfunction by hydroxyl-containing dithiocarbamates. Br. J. Cancer; 63: 234-236.

Roberts, J. J. and Pascoe, J. M. (1972). Cross linking of complementary strands of DNA in mammalian cells by antitumor platinum compounds. Nature; 235: 282-284.

Rogers, K., Roberts, G. M. and Williams, G. T. (1981). Gastric-juice enzymes: An aid in the diagnosis of gastric cancer? Lancet; 1: 1124-1125.

Roitt, I. M., Brostoff, J. and Male, D. (1985). Cell-mediated immunity. In: Immunology. Grower Medical Publishing Ltd., London, New York;, pp. 11.1-11.8.

Roos, I. A. G. and Arnold, M. C. (1977). Interaction of an antitumor platinum complex with DNA. J. Clin. Hematol. Oncol.; 7: 374-390.

Rosalki, S. B. (1974). Standardisation of isozyme assays with special reference to lactate dehydrogenase isozyme electrophoresis. Clin. Biochem.; 7: 29-40.

Rosenberg, B. (1971). Some biological effects of platinum compounds: New agents for the control of tumors. Plat. Met. Rev.; 15: 3-12.

Rosenberg, B. (1980a). Enhanced antigenicity as a possible mode of action of platinum antitumor drugs. In: Advances in Antimicrobial and Antineoplastic Chemotherapy. Semonsky, M., Hejzlar, M. and Masak, S. (eds.), University Park Press, Baltimore; 2: 101-102.

Rosenberg, B. (1980b). Cisplatin: Its history and possible mechanism of action. In: Cisplatin: current status and new developments. Academic Press, New York; pp. 9-20.

Rosenberg, B. (1985). Fundamental studies with cisplatin. Cancer; 55: 2303-2316.

Rosenberg, B. and Van Camp, L. (1970). The successful regression of large solid sarcoma-180 tumors by platinum compounds. Cancer Res.; 30: 1799-1802.

Rosenberg, B., Van Camp, L., Trosko, J. E. and Mansour, V. H. (1969). Platinum compounds: a new class of potent antitumor agents. Nature; 222: 385-386.

Rozengurt, E. (1979). Early events in growth stimulation. In: Surfaces of Normal and Malignant Cells. Hynes, R. O. (ed.), John Wiley and sons, New York; pp. 323-325.

Rubin, H. (1985). Cancer as a dynamic developmental disorder. Cancer Res.; 45: 2935-2942.

Rule, G. S., Kruuv, J. and Lepock, J. R. (1979). Membrane lipid fluidity as rate limiting in the concanavalin A - mediated agglutination of py BHK cells. Biochem. Biophys. Acta; 556: 399-407.

Rustia, M. (1975). Inhibitory effect of sodium ascorbate on ethyl urea and sodium nitrite carcinogenesis and negative findings in pregnancy after intestinal inoculation of precursors into pregnant hamsters. J. Natl. Cancer Inst.; 55: 1389-1394.

Safirstein, R., Winston, J., Moel, D., Dikman, S. and Guttenplan, J. (1987). Cisplatin nephrotoxicity: Insights into mechanism. Int. J. Androl.; 10: 325-346.

Sarna, S. (1979). Ultrastructural and chemo-immunotherapeutical studies of fibrosarcoma in Swiss white mice, with cis-dichlorodiammine platinum (II). Ph.D. Thesis, Banaras Hindu University, Varanasi, India.

Sarna, S. and Bhola, R. K. (1987). Antitumor effect of cisplatin and glucose in mice bearing Dalton's lymphoma. Curr. Sci.; 56: 1251-1253.

Sarna, S. and Bhola, R. K. (1989). Combination therapy of transplantable tumor in mice with cisplatin and glucose. Curr. Sci.; 58: 231-233.

Sarna, S. and Sodhi, A. (1978). Chemo-immunotherapeutical studies on a fibrosarcoma with cis-dichlorodiammine platinum (II). Ind. J. Exp. Biol.; 16: 1236-1239.

Schlaefli, E., Ehrke, M. J. and Mihich, E. (1983). The effect of dichloro-trans-dihydroxy-bisisopropylamine- platinum IV on the primary cell mediated cytotoxic response. Immunopharmacology; 6: 107-122.

Schmid, W. (1976). The micronucleus test for cytogenetic analysis. In: Chemical Mutagens: Principles and Method for Their Detection. Hollaender, A. (ed.), Plenum, New York; 4: 31-53.

Schneider, R. J., Seibert, K. and Passe, S. et. al. (1980). Prognostic significance of serum lactate dehydrogenase in malignant lymphoma. Cancer; 46: 139-143.

Schull, K. H., Cahill, G. F. J., Gadsden, F. L. and Mayer, J. (1956). Biol. Chem.; 222: 415-420.

Sela, B., Lis. H., Sharon, N. and Sachs, L. (1970). Different locations of carbohydrate-containing sites in the surface membrane of normal and transformed mammalian cells. J. Membr. Biol.; 33: 267-269.

Shamberger, R. J. (1984). Serum sialic acid in normals and in cancer patients. J. Clin. Chem. Clin. Biochem.; 22: 647-651.

Shilortri, P. G. (1977). Phagocytosis and leukocyte enzymes in ascorbic acid deficient guinea pigs. J. Nutr.; 107: 1513-1516.

Shionoya, S., Lu, Y. and Scanlon, K. J. (1986). Properties of aminoacid transport systems in K562 cells sensitive and resistant to cis-diammine-dichloroplatinum (II). Cancer Res.; 46: 3445-3448.

Shoyab, M. (1981). Inhibition of the binding of 7, 12-dimethylbenz(a)anthracene to DNA of murine epidermal cells by vitamin A and vitamin C. Oncology; 38: 187-192.

Singh, S. M. and Sodhi, A. (1988). Interaction between cisplatin-treated macrophages and Dalton's lymphoma cells in vitro. Exp. Cell Biol.; 56: 1-11.

Sip, M., Schwartz, A., Vovelle, F., Ptak, M. and Leng, M. (1992). Distortions induced in DNA by cis-platinum interstrand adducts. Biochemistry; 31: 2508-2513.

Smyrniotis, F., Schenker, S., O'Donnell, J. and Schiff, L. (1962). Lactate dehydrogenase activity in gastric juice for the diagnosis of gastric cancer. Am. J. Dig. Dis.; 7: 712-718.

Sodhi, A. (1976). Ultrastructural changes of sarcoma-180 cells after treatment with cis-dicholorodiammine platinum (II) in vivo and in vitro. Ind. J. Exp. Biol.; 14: 383-390.

Sodhi, A. (1977). Origin of giant cells in regressing sarcoma-180 after cis-dichlorodiammine platinum (II) treatment: A fine structural study. J. Clin. Hematol. Oncol.; 7: 569-579.

Sodhi, A. (1979). Ultrastructural observations on the effect of cis-dicholrodiammine platinum (II) on the cells of ascites fibrosarcoma in mice. I. Interaction of macrophages with fibrosarcoma cells. Ind. J. Exp. Biol.; 17: 623-627.

Sodhi, A. and Aggarwal, S. K. (1974). Effect of cis-dichlorodiammine platinum (II) in the regression of sarcoma-180: A fine structural study. J. Natl. Cancer Inst.; 53: 85-101.

Sodhi, a. and Bhatia, P. (1986). In vitro activation of murine macrophages and their increased capacity to lyse target cells after cisplatin treatment. Ind. J. Exp. Biol.; 24: 565-572.

Sodhi, A. and Gupta, P. (1986). Increased release of hydrogen peroxide (H_2O_2) and superoxide anion (O^-) by murine macrophages in vitro after cisplatin treatment. Int. J. Immonopharmacol.; 8: 709-714.

Sodhi, A. and Prasad, S. B. (1981). Ultrastructural and fluorescence microscopical observations on the effect of cis-dichloro diammineplatinum (II) on the surface of tumor and normal cells. Ind. J. Exp. Biol.; 19: 328-332.

Sodhi, A. and Sarna, S. (1979). Ultrastructural observations on the effects of cis-dichlorodiammine platinum (II) on the cells of fibrosarcoma in Swiss white mice. Ind. J. Exp. Biol.; 17: 1-8.

Sodhi, A., Tandon, P. and Sarna, S. (1985). Adoptive transfer of immunity against solid fibrosarcoma in mice with splenocytes and peritoneal exudate cells obtained after in vitro sensitization and in vivo immunization with cis-dichlorodiammine platinum (II) treated fibrosarcoma cells. Arch. Geschwulstforch; 55: 47-61.

Sorenson, C. M. and Eastman, A. (1988). Mechanism of cis-diamminedichloroplatinum (II)-induced cytotoxicity: Role of G_2 arrest and DNA double-strand breaks. Cancer Res.; 48: 4484-4488.

Speer, R. J., Ridgway, H. and Hill, J. M. (1972). Therapy of leukemia L-1210 with cis-platinous diammno-dichloride (PDD). Wadley Med. Bulletin; 2: 52-60.

Starkweather, W. H., Green, R. A., Spencer, H. H. and Schock, H. K. (1966). Alterations of serum lactate dehydrogenase isoenzymes during therapy directed at lung cancer. J. Lab. Clin. Med.; 68: 314-318.

Stefanini, M. (1985). Enzymes, isozymes, and enzyme variants in the diagnosis of cancer. Cancer; 55: 1931-1936.

Stelten, M. R. and Goldsmith, P. K. (1976). Biochem. Biophys. Acta; 444: 835.

Stone, P. J., Kelman, A. D. and Sinex, F. M. (1974). Specific binding of antitumor cis-Pt $(NH_3)_2Cl_2$ to DNA rich in guanine and cytosine. Nature; 251: 736-737.

Stringou, E., Chondros, K. Kouvaris, J., Kakari, S. and Papavassilious, K. (1992). Serum sialic acid (TSA/LSA) and carcinoembryonic antigen (CEA) levels in cancer patients undergoing radiotherapy. Anticancer Res.; 12: 251-256.

Sugihara, K. and Gemba, M. (1986). Modification of cisplatin toxicity by antioxidants. Jpn. J. Pharmacol.; 40: 353-355.

Sugihara, K., Nakano, S. and Gemba, M. (1987). Effect of cisplatin in vitro production of lipid peroxides in rat kidney cortex. Jpn. J. Pharmacol.; 44: 71-76.

Takema, M., Inaba, K., Uno, K., Kakihara, K. -I., Tawara, K. and Muramatsu, S. (1991). Effect of L-arginine on the retention of macrophage tumoricidal activity. J. Immunol., 146: 1928-1933.

Tamai, Y., Kuwata, S., Yamasaki, N. and Takakuwa, M. (1978). Effect of Ig G on membrane-bound enzyme activity of S-180 cells. Biochem. Biophys. Acta.; 542: 209-213.

Tandon, P. and Sodhi, A. (1985). Cis-Dichlorodiammine platinum (II) induced aberrations in mouse bonemarrow chromosomes. Mutat. Res.; 156: 187-193.

Tewfik, F. A., Tewfik, H. H. and Riley, E. F. (1982). The influence of ascorbic acid on the growth of solid tumors in mice and on tumor control by X-irradiation. Int. J. Vit. Nutr. Res.; 233: 257-263.

Thurau, K. (1964). Renal hemodynamics. Am. J. Med.; 36: 689-719.

Tiez, N. W. (1980). Present and future trends in selected areas of clinical enzymology. J. Clin. Chem. Clin. Biochem.; 18: 763-769.

Trinder, P. (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. Clin. Biochem.; 6: 24-27.

Triton, T. R. and Hickman, J. A. (1985). Cell surface membranes as a chemotherapeutic target. In: Experimental and Clinical Progress in Cancer Chemotherapy. Muggia, F. M. (ed.), Martinus Nijhoff, Boston; pp. 81-131.

Tsao, C. S., Dunham, w. B. and Leung, P. Y. (1992). Effect of ascorbic acid and its derivatives on the growth of human mammary tumor xenografts in mice. Cancer J.; 5: 53-59.

Tsou, K. C., Lo, K. W., Herberman, R. B. and Schutt, A. J. (1980). Detection of liver metastases with 5'-nucleotide phosphodiestrase isozyme-V in gastrointestinal cancer patients. Oncology, 37: 381-385.

Tsou, K. C., Lo, K. W., Rosato, E. F., Yuk, A., Enterline H. and Schwegman, B. C. (1982). Evaluation of 5'-nucleotide phosphodiestrase isozyme-V as a predictor for liver metastases in breast cancer patients. Cancer; 50: 191-196.

Vincent, M. D. (1985). The clinical problem. In: The Molecular Basis of Cancer. Farmer, B., and Walker, J. M. (eds.), Croom Helm Ltd., Australia; pp. 1-35.

Waddell, I. D. and Burchell, A. (1988). The microsomal glucose-6-phosphatase enzyme of pancreatic islets. Biochem. J.; 255: 471-576.

Waldman, R. K. and Borman, E. K. (1959). A note on serum transaminase activity after lead absorption. Arch. Indust. Health; 19: 431.

Ward, J. N. and Fauvie, K. A. (1976). The nephrotoxic effect of cis-diamminedichloroplatinum (II) (NSC-119875) in male F 344 rats. Toxicol. Appl. Pharmacol.; 38: 535-547.

Warren, L. (1959). The thiobarbifuric acid assay of sialic acids. J. Biol. Chem.; 234: 1971-1975.

Warren, L., Buck, C. A. and Tuszynski, G. P. (1978). Glycopeptide changes and malignant transformation. A possible role for carbohydrate in malignant behaviour. Biochem. Biophys. Acta; 516: 97-127.

Weisburger, J. H., Marquardt, H., Mower, H. F., Hirota, F. and Mori, H. (1980). Inhibition of carcinogenesis: Vitamin C and the prevention of gastric cancer. Prev. Med.; 9: 352-361.

Welsch, C. W. (1972). Cis-platinum diamminedichloride (II) induced regression of carcinogen induced rat mammary tumors. In: Advances in Antimicrobial and Antineoplastic Chemotherapy. University Park Press, Baltimore; 2: p. 231.

Werve, G. V. (1989). Liver glucose-6-phosphatase activity is modulated by physiological intracellular cation concentration. J. Biol. Chem.; 264: 6033-6036.

WHO (1985). Guidelines for the study of genetic effects in human population: Environmental health criteria, 46, WHO, Geneva.

Wieneke, J. R., Cervenka, J. and Paulus, H. (1979). Mutagenic activity of anticancer agent cis-dichlorodiammine platinum (II). Mutat. Res.; 68: 69-77.

Wilkinson, J. H. (1970). Isozymes. Lippincott, Philadelphia, pp. 134-203.

Wright, K. C., Carrasco, C. H., Wallace, S. and Stephens, L. C. (1985). Treatment of rabbit V-2 carcinoma with intralesional cisplatin. Chemotherapy; 31:60-67.

Wroblewski, F. and La Due, J. S. (1955). SGOT activity as an index to liver cell injury. Ann. Intern. Med.; 43: 345-351.

Wyrobek, A. J. and Bruce, W. R. (1975). Chemical induction of sperm abnormalities in mice. Proc. Natl. Acad. Sci. (USA); 72: 4425-4429.

Xu, S. J., Dou, P. Y., Yu, L., Wang, K. and Ji, X. (1984). Some platinum (II) complexes of cysteine, methionine and glutathione and their anticancer activity. Fenzi Kuxhe Yu Huaxue Yanjiu; 4: 537-542.

Yagoda, A. (1980). Cisplatin regimens in the treatment of bladder and penile cancer. In: Cisplatin: Current status and new developments. Prestayko, A., Creoke, S. T. and Carter, S. K. (eds.), Academic Press, Inc., New York; pp. 361-374.

Yesher, A. (1978). Spectrum of lung cancer and ectopic hormones. Pathobiol. Annu.; 13: 217-240.

Yogeeswaran, G. and Salk, P. L. (1981). Metastatic potential is positively correlated with cell surface sialylation of cultured murine tumor cell lines. Science; 212: 1514-1516.

Yokoyama, M. (1980). An electron microscopic study on the lectin-binding sites and their mobility in human and rat urologic tumor cells. Acta. Histochem; 13: 139-153.

Yonemoto, R. H. (1983). Alteration of cellular response with ascorbic acid. In: Modulation and Mediation of Cancer by Vitamins. Karger, Basel; pp. 334-339.

Yoshihara, K., Tanigawa, Y., Burzio, L. and Koide, S. S. (1975). Evidence for adenosine diphosphate ribosylation of Ca^{2+}, Mg^{2+} - dependent endonuclease. Proc. Natl. Acad. Sci. (USA); 72: 289-293.

Young, J. D. -E. and Cohn, Z. A. (1991). Immunology: Recognition and response. In: Readings from Scientific American. W. H. Freeman and Co., New York; pp. 83-93.

Zampella, E. J., Bradley, E. L. and Pretlow, T. D. II. (1982). Glucose-6-phosphate dehydrogenase: A possible indicator for prostatic carcinoma. Cancer; 49: 384-387.

Zhong, L. F., Zhang, J. G., Zhang, M., Ma, S. L. and Xia, Y. X. (1990). Protection against cisplatin-induced lipid peroxydation and kidney damage by procaine in rats. Arch. Toxicol.; 68: 599-600.

Zwelling, L. A. and Kohn, K. W. (1979). Mechanism of action of _cis_-dichlorodiammineplatinum (II). Cancer Treat. Rep.; 63: 1439-1444.

Zwelling, L. A., Bradley, M. O., Sharkey, N. A., Anderson, T. and Kohn, K. W. (1979). Mutagenicity, cytotoxicity and DNA crosslinking in V79 Chinese hamster cells treated with _cis_- and _trans_-Pt(II) diamminedichloride. Mutat. Res.; 67: 271-280.

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