EFFECT OF CARCINOGEN ON ARGINASE-AN ENZYME ASSOCIATED WITH THE REGULATION OF POLYAMINE LEVELS IN RAT LIVER

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T O T H E

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CERTIFICATE

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

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ABBREVIATIONS

BSA	Bovine serum albumin
cAMP	Adenosine 3',5'-cyclic phosphate
DEN	Di ethyl nitrosamine
MPG	2-Mercaptopropionyl glycine
ODC	Ornithine decarboxylase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-poly acrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethyl ethylene diamine

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INTRODUCTION

The metabolic cross roads centred upon ornithine is of interest because of the recognition that this amino acid acts as common substrate to a number of diverse pathways and, as such, the regulation of its disposition amongst these pathways could have significant effects on the metabolic patterns prevailing in the tissue (Weber *et al.*, 1972; Oka and Perry, 1974; Herzfeld and Raper, 1976). The most important of the enzymes directing the metabolism of ornithine are: Ornithine decarboxylase which initiates the pathway to polyamine synthesis and hence may possibly be involved in gene-expression; Ornithine transcarbomylase, which initiates urea synthesis and is therefore linked to ureogenesis and gluconeogenesis from amino acids; Ornithine keto transaminase, which is an initial step on the pathway to glutamate and protein synthesis; and arginase which is the enzyme generating ornithine.

The cellular functions of natural polyamines, putrescine, spermidine and spermine although not as yet unambiguously established, appear to be closely associated with the proliferation of animal cells

(Scalabrino et al., 1978). A great deal of attention has been paid to the occurrence and enhanced synthesis of polyamines in experimental tumors of animals such as Ehrlich ascites cells (Slimes and Janno, 1967; Bachoooh et al., 1967; Noguchi et al., 1976; Andersen and Heby, 1972; Kalio et al., 1977), transplantable hepatomas (Williams-Ashman et al., 1972) tumors of neural origin (Marton and Heby, 1974), rat sarcomas (Heish and Key, 1967) etc. But very few studies have dealt with the changes in polyamine metabolism taking place during preneoplastic stages in tissues undergoing carcinogenesis. The latter studies include a series of papers dealing with the early stimulation of polyamine biosynthesis during chemical induction of skin tumors in the mouse (O'Brien et al., 1975a,b; Yuspa et al., 1976; Verma and Boutwell, 1977; Verma et al., 1979; Mufson et al., 1979; Goto et al., 1980).

Interest in the metabolism of polyamines has been greatly increased following the proposal that measurement of extracellular polyamines may be used as a diagnostic marker in human malignancies (Russel, 1971). It seems reasonable to expect that the study of polyamines may not only provide insight into the

cellular and molecular basis for neoplasia but also contribute to practical application in cancer therapy.

Since the initial reports from industrial laboratories first revealed the dangers of handling N-nitroso compounds, an extensive literature has developed on their acute toxic (Heath and Magee, 1962), carcinogenic (Magee and Barnes, 1967) and mutagenic (Kehlman, 1966) properties. Several experimental studies have shown that a number of N-nitroso amines are such potent carcinogens that a single dose is often sufficient to lead to the development of malignant tumors in a variety of organs. One of these compounds, diethylnitrosamine (DEN), has been selected for the present investigation. In contrast to other hepatocarcinogens, feeding of DEN to rats results in the development of hepatocellular carcinomata only and there is practically no reaction of connective tissue or bile duct cells (Grundman and Sieburg, 1962). The tumor yield is reported to be almost 100 per cent and the dose response relationships are extraordinarily precise (Rajewsky, 1967). DEN is carcinogenic in all animal species tested: mice, rats, syrian golden, Chinese and

European hamsters, guineapigs, rabbits, dogs, gerbils, pigs, monkeys, hedgehogs, various fish, frogs and birds. Available information on occurance suggests that in the case of human beings, the general population may be exposed to low levels of N-nitrosodiethylamine (See IARC monographs, 1978).

The types of tumors and their frequency observed in the rat liver are largely similar to those found in the human liver (Schauer and Kunze, 1976). Hence the use of rat liver as a system for the process of carcinogenesis becomes important.

In addition to its well established role in urea cycle, another possible role for arginase is participation in the biosynthesis of polyamines (Oka and Perry, 1974). This possibility is attractive in the light of the presence of arginase in tissues, which, unlike liver, lack other enzymes of the urea cycle. Arginase participates in the polyamine biosynthetic pathway by cleaving arginine to generate urea and ornithine of which the latter is the substrate for ornithine decarboxylase (EC. 4.1.1.1.17) which is the initial and rate limiting enzyme in the polyamine biosynthesis.

2-Mercaptopropionyl glycine (MPG) is widely used for the treatment of various hepatic disorders, without reported side-effects. This sulphydryl compound is reported to be radio-protector and a hepatotrophic detoxicant. It is interesting to know whether this compound is having any effect on the carcinogen metabolism and action.

While it is difficult to link at present any specific patterns or alterations of proteins synthesis with neoplasia, it is interesting to look into the protein profile during the process of carcinogenesis. This is important since some cytoplasmic proteins may contribute directly or indirectly to the onset of neoplasia.

The several differences observed in comparative studies of normal and neoplastic tissues may simply be a reflection of the new proliferative state of the cell, the development of a new cell type, difference in the new chromosomal content, or the expression of endogenic non-mutagenic viruses and may not be directly related to the malignant transformation (Stein et al., 1976). Hence, it would be highly informative

if it were possible to study the cells initiated by carcinogens but that are in a very early preneoplastic state.

Keeping in view of the above observations, an attempt is made in the present study to look into the effect of DEN on rat liver within a short period after treatment. The parameters investigated are the effect on arginase activity and on the general profile of cytosol proteins. An attempt is made to know whether the effect of the carcinogen is modified or altered by HPO.

REVIEW OF LITERATURE

Variable gene activity against a constant cellular genome is the guiding concept upon which much of the current research in the field of normal cellular differentiation is directed. Cytodifferentiation is a very stable process when one considers that trillions of different types of cells in an organism originate from a single cell. Differentiation does not entail mutagenesis but rather epigenesis or the regulation of gene expression. Thus differential expression of gene activity which is very stable, could similarly represent the basis of the malignantly transformed phenotype (Braun, 1974). It has been postulated that cancer is a disease of gene regulation (Coggin et al., 1974; Markent, 1968; Weinhouse, 1972). This would imply that the abnormalities seen in cancer are due to the malfunctioning of the very complex and undefined mechanisms that dictate the gene expression of a given cell at the particular time of its life cycle. A malfunctioning of these mechanisms would allow for the derepression or repression of genes in a manner that is prohibited in the normal differentiated cell. Although the causative factors may be quite varied and unrelated, the manifestation of the disease would

be solely due to the disruption of these regulatory mechanisms (Stein et al., 1976). Thus understanding of the mechanism involved in neoplastic transformation is intimately linked with a better understanding of the regulation of gene expression in normal cells. Unfortunately, the tools which eukaryotic cells use to regulate gene activity and the mechanism of genetic regulation by these tools are obscure.

That the polyamines may act as general regulators of gene transcription has been suggested since the discovery that they enhance phosphorylation of non-histone chromosomal proteins from hog liver nuclei (Imai et al., 1975). Components of non-histone chromosomal proteins have been implicated as the specific regulators of gene expression in mammalian cells (See Stein et al., 1976).

The aliphatic polyamines, putrescine, spermidine and spermine are natural constituents of most living organisms - prokaryotes (bacteria and viruses) and eukaryotes (Tabor and Tabor, 1976). Prokaryotes contain only putrescine and spermidine whereas eukaryotes can also synthesize spermine,

the largest of the three polyamines (Bachrach, 1973). Although the discovery of spermine by Leuowenhock extends back to 1677, a more systematic research has been focussed on these natural bases only during the last two decades.

The increased interest in the metabolism of natural polyamines is mainly because of the appearance of large number of reports proposing important roles for polyamines in various growth processes. The idea that natural polyamines would be involved in proliferative processes is, among others, based on the following facts:

(i) These compounds occur in all living material;

(ii) Their polybasic structure leads them to react, even with certain specificity, with important intracellular polyanions such as nucleic acids;

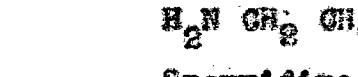
(iii) Polyamines have been reported to exert profound effects <u>in vitro</u> on various cellular reactions of central importance and finally,

(iv) The synthesis of these compounds is precisely regulated to meet the metabolic needs of the cell.

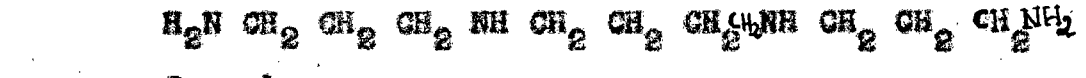
The structure of the natural polyamines, putrescine, spermidine and spermine are presented below:

1,4-Diaminobutane

(putrescine)



Spermidine

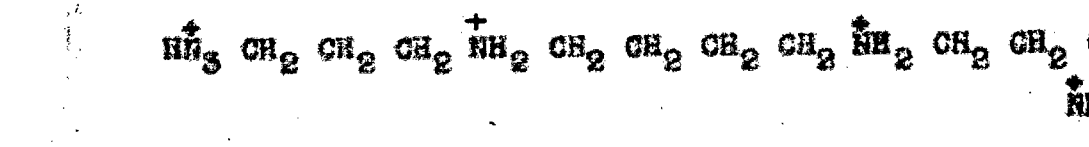


Spermine

The polycationic structure of putrescine, spermidine and spermine are represented as

Putrescine

Spermidine



Spermine

Production and Utilization of Ornithine - Synthesis of Polyamines:

As pointed out by Williams-Ashman, L-Ornithine cannot be regarded as a true building-block for protein biosynthesis (Williams-Ashman et al., 1969; Williams-Ashman et al., 1972). The action of arginase on L-arginine produces L-ornithine during the operation of the urea cycle in ureotelic species. The utilization of ornithine in the liver comprises several metabolic pathways. The following enzymes known to occur in mammalian tissue catalyze either the formation or utilization of L-ornithine, as summarized in Fig. (Williams-Ashman et al., 1969).

An important avenue for utilization of L-ornithine in higher animals is the biosynthesis of polyamines. The enzyme L-ornithine decarboxylase (EC.4.1.1.17) catalyzes the synthesis of putrescine and carbondioxide from L-ornithine (Pegg and Williams-Ashman, 1968). The sequential actions of arginase (EC.3.5.3.1) and Ornithine decarboxylase thus provide a pathway for the biological formation of putrescine from L-arginine. Arginase is, therefore, of potential importance in the biosynthesis of polyamines, as well as in the

Fig. 1. Reactions involved in the formation and utilization of L-ornithine in mammalian tissues. (Modified from Williams-Ashman et al., 1969). The numbered enzyme reactions represent:

1. Arginase:

   L-Arginine + H_2O ⟶ L-ornithine + urea.

2. L-Arginine: Glycine amidino-transferase:

   L-Arginine + glycine ⟺ L-ornithine + guanidino acetic acid

3. Ornithine carbamyl-transferase:

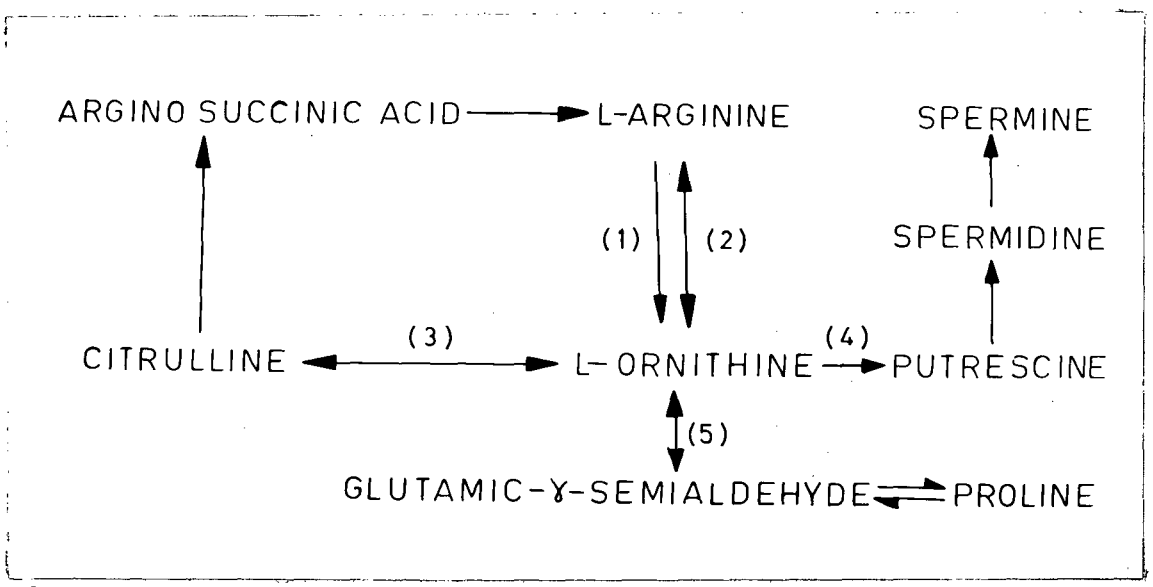
   L-ornithine + Carbamyl phosphate ⟺ Citrulline + Pi.

4. Ornithine decarboxylase:

   L-ornithine ⟶ Putrescine + CO_2

5. Ornithine: Ketoacid aminotransferase:

   L-Ornithine + 2-ketoglutarate ⟺ Glutamic-γ - Semialdehyde + glutamate.


ARGINO SUCCINIC ACID
L-ARGININE
SPERMINE
(1)
(2)
SPERMIDINE
CITRULLINE
(3)
L- ORNITHINE
(4)
PUTRESCINE
(5)
GLUTAMIC-γ-SEMIALDEHYDE
PROLINE

urea cycle. Although an alternate pathway for putrescine synthesis from L-arginine involving the intermediary formation of the base agmentine is known to take place in certain bacteria and higher plants (Pegg and Williams-Ashman, 1968; Smith, 1971), this series of reactions do not seem to operate in mammalian tissues, from which L-arginine decarboxylase appears to be absent (Pegg and Williams-Ashman, 1968; Williams-Ashman et al., 1969; Williams-Ashman et al., 1972). Though the major function of liver arginase is the formation of urea as a component of the urea cycle, it is well known that arginase occurs in most extrahepatic mammalian tissues, which suggests other functional roles for this enzyme (Knox and Greengard, 1965).

Arginase, Ornithine Decarboxylase and Polyamines in Cellular Metabolism:

It has been shown that arginase activity is elevated during rapid cellular proliferation (Bach and Lasnitzki, 1947). Arginase has been found to be induced in rabbit skin by the papilloma viruses (Rogers, 1959). The arginase isolated from cells

infected with Shope papilloma virus has since been characterized. It differs significantly from the rabbit enzyme. The molecular information for its production apparently comes from the virus and not from the rabbit. The role hypothesized for this excess arginase activity in the exuberant cell proliferation which characterizes the papilloma is to deplete the cells of arginine. Formation of the arginine - rich nuclear histones might be reduced, and if histones suppress RNA function, the nuclear material would be freed for greater activity in growth processes. In support of this hypothesis it has been demonstrated that canavanine, an inhibitor of arginase, decreased the growth rate of papillomas (Damodaran and Narayanan, 1940; Rogers and Moore, 1963). It is of interest that human warts, which are also characterized by cellular proliferation, contain more arginase activity than normal skin (Van Scott, 1951).

On the other hand, there are reports of tumor regressions or a reduction of tumor growth after injection of arginase into tumor-bearing animals (Wiswell, 1951; Vrat, 1951; Irons and Boyd, 1952). Arginase has been found to be an antimitotic agent in tissue culture (Bach, 1953).

Arginase activity has been found to be considerably lower in hepatomas. The activity has been studied in both the precancerous stage and in the tumors resulting from giving the carcinogenic azo-dyes in diet (McLean et al., 1964). Arginase activity has been found to be considerably lower in hepatomas than in either normal liver or adjacent host liver. In the precancerous tissue there was an initial increase followed, after six weeks of treatment, by a significant fall in activity. These changes may be secondary to earlier changes. A decreased arginase activity has been observed in liver of fibrosarcoma-bearing mouse compared to normal liver (Sukumar and Nagarajan, 1978).

Arginase is a very active enzyme. If the enzyme activities are very high compared with the amounts of available substrate, then any substrate as it becomes available will react rapidly and the steady state concentrations of the intermediates will be very close to those of the equilibrium state (Krebs, 1963). It is one of the implications of this situation that the free energy changes occuring in the course of the intermediate steps are minimal -

almost zero - in that any losses of free energy are reduced to negligible quantities. The high activity of certain enzymes is thus understandable as a useful arrangement. It constitutes economy of energy and far from being wasteful it is a saving.

Ornithine decarboxylase (ODC) catalyzes the conversion of ornithine to the diamine putrescine, an apparent rate limiting step in the biosynthesis of the polyamines, spermidine and spermine. It is a pyridoxal - phosphate requiring enzyme, the coenzyme loosely bound to the apoenzyme (Rahiala et al., 1971). Molecular weight is 100,000 (Ono et al., 1972; Obenrader and Prouty, 1977). There apparently are no low molecular weight effectors of physiologic occurrence for ornithine decarboxylase, even though the enzyme shows a rather stringent requirement for thiol compounds (Janne and Williams-Ashman, 1971). Ornithine decarboxylase possesses the far shortest half life ever recorded for a mammalian enzyme. After cessation of protein synthesis, the activity of the enzyme swiftly decays following first order kinetics with an apparent half life of about 10-15 minutes (Russel and Synden, 1969; Obenvader and Prouty, 1977).

The induction of ODC is an early marked event in all growth systems studied to date. Several studies have indicated that in many systems cyclic AMP is involved in the induction of ornithine decarboxylase, mainly after the administration of various hormones that activate adenylate cyclase, or other agents that cause the increase in cellular cyclic AMP (Byus and Russel, 1975).

It has been postulated that AMP exerts its influence on cellular metabolism through the activation of cAMP dependent protein kinases (Kuo and Greengard, 1969). Induction of ODC in several systems seems to be more closely related to the activation of cAMP dependent protein kinase(s) than to the actual intracellular fluctuations of cAMP (Byus and Russel, 1974; Byus and Russel, 1975; Byus and Russel, 1976; Byus et al., 1976).

The rapid activation of cAMP dependent protein kinase followed by the transcriptional induction of ODC is consistantly observed in response to trophic stimuli. Alterations in cAMP dependent protein kinase isozyme activities and/or cAMP binding proteins

have been observed in several transformed cells. Recently, it has been reported that a single carcinogenic dose of diethylnitrosamine rapidly activated cAMP dependent protein kinase and induced ODC (Oslon and Russel, 1979).

There is now substantial evidence that ornithine decarboxylase is also an initiation factor for RNA polymerase I (Manen and Russel, 1975; Manen and Russel, 1977; Heddex and Russel, 1978). The accumulation of spermidine, a polyamine and ribosomal RNA are parallel in a variety of growth stimulated tissues that have been studied (Cohen, 1971; Russel, 1971; Bacharch, 1973; Russel and Durie, 1978).

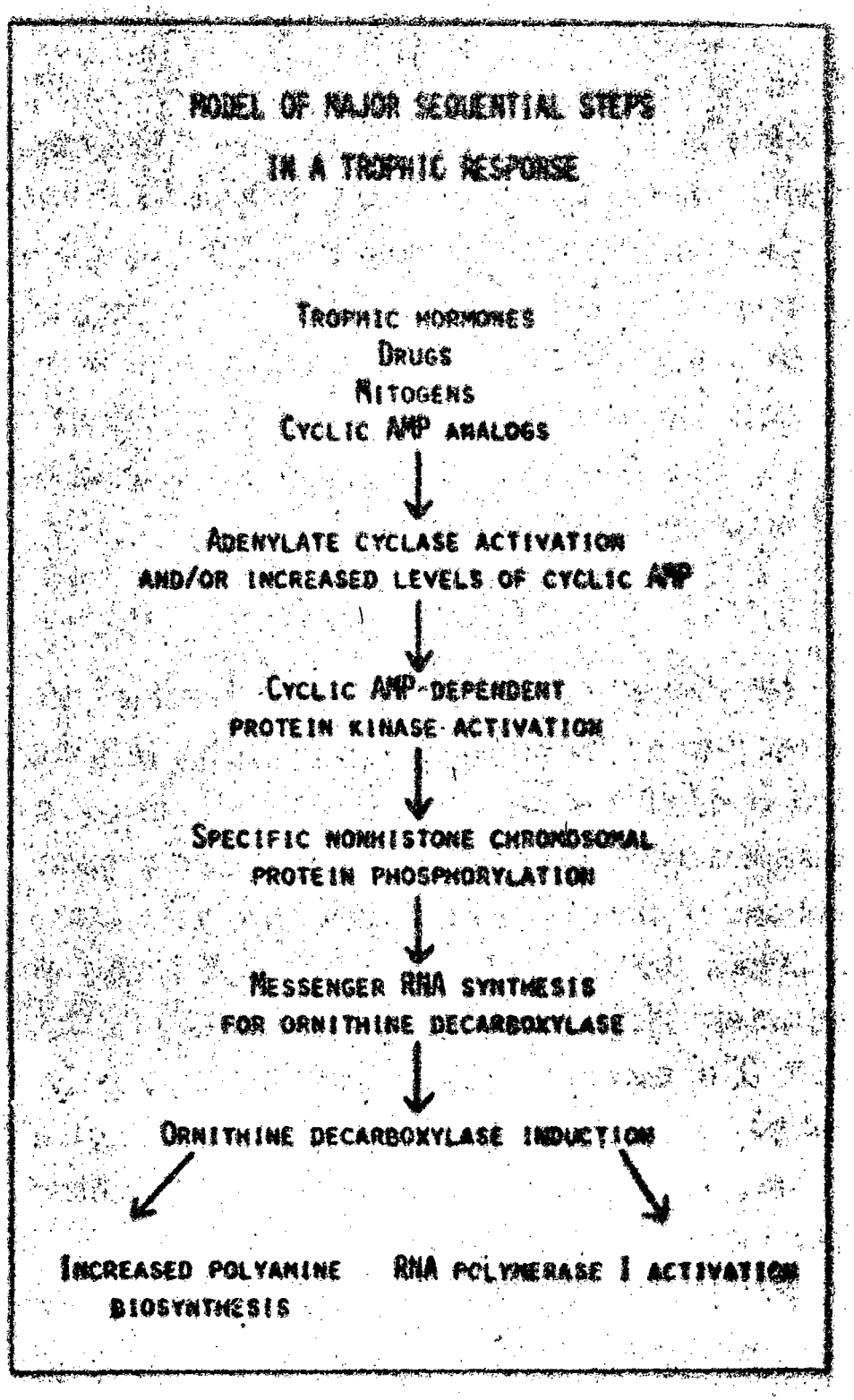
Studies of the requirements for increased protein synthesis suggest that new ribosomal RNA must be synthesized as a pre-requisite of stepped up protein synthesis (Aroggrois, 1974; Russel and Manen, 1976; Wheely et al., 1978). Drug-induced enzyme synthesis is coupled with an initial induction of ornithine decarboxylase and increased ribosomal RNA synthesis (Byus et al., 1976; Costa et al., 1976). Trophic hormones universally induce ornithine

decarboxylase and result in increased ribosomal RNA synthesis and accumulation (Russel et al., 1976; Russel and Dunic, 1978; Russel and Maddox, 1979).

Studies of cell cycle progression indicate that ornithine decarboxylase is a marker of G_1 progression (Russel and Stambrook, 1975; Fuller et al., 1977; Maddox, 1979). Its failure to be expressed in response to inhibitors results in a block of cells in the G_1 phase of the cycle (Russel and Maddox, 1979). Re-expression of ornithine decarboxylase activity is concomitant with movement of cells into the S phase. A model of the major steps in a trophic response or of G_1 progression (Fig. 2) has been proposed recently (Russel, 1980).

Extensive studies have been conducted on the effects of phorbol esters, particularly TPA, on mouse skin (O'Brien et al., 1975; O'Brien, 1976; O'Brien and Diamond, 1977; Verma et al., 1978; Kensler et al., (1978). TPA treatment results in a several hundred fold induction of ornithine decarboxylase in the skin and, if administered after a carcinogen such as 2-methylcholanthrene, acts as a tumor promotor.

Fig. 2; Schematic diagram of major sequential steps in a trophic response. (From Russel, 1980).


MODEL OF MAJOR SEQUENTIAL STEPS
IN A TROPHIC RESPONSE
TROPHIC HORMONES
DRUGS
NITOGENS
CYCLIC AMP ANALOGS
ADENYLATE CYCLASE ACTIVATION
AND/OR INCREASED LEVELS OF CYCLIC AMP
CYCLIC AMP-DEPENDENT
PROTEIN KINASE ACTIVATION
SPECIFIC NONHISTONE CHROMOSOMAL
PROTEIN PHOSPHORYLATION
MESSENGER RNA SYNTHESIS
FOR ORNITHINE DECARBOXYLASE
ORNITHINE DECARBOXYLASE INDUCTION
INCREASED POLYAMINE
BIOSYNTHESIS
RNA POLYMERASE I ACTIVATION

Inhibition of TPA-induced ornithine decarboxylase activity by Vitamin A analogs also inhibits the formation of papillomas (Verma et al., 1978).

The cellular functions of natural polyamines, the products of ornithine decarboxylase, although not as yet unambiguously established, appear to be closely associated with the proliferation of animal cells (Scalabrino et al., 1978). The idea that these compounds do not simply act intracellularly as non-specific polycations, is supported by a wide variety of effects exerted by polyamines that cannot entirely be ascribed to the polycationic structure of these compounds. Whatever be the role of polyamines, there is a fundamental difference between divalent cations and natural polyamines in the living cell. This difference relies upon the fact that latter compounds can be synthesized so as to meet the ambient metabolic requirements of the cell. This kind of regulation, even considering compartmentalisations would hardly occur in vivo as a rapid response of the inorganic cations to attend metabolic needs of the cell (Janne et al., 1977).

Various reports suggest a role of polyamines in cellular metabolism through cyclic AMP and protein

kinases (Murray et al., 1970; Manen and Russel, 1975; Shah et al., 1975; Atmar et al., 1976; Shoth et al., 1976; Taki et al., 1976; Jungman and Russel, 1977; Maenpaa, 1977; Bacharch et al., 1978; Hochman et al., 1978; Clo et al., 1979).

Polyamines are reported to enhance phosphorylation of non-histone chromosomal proteins (Imai et al., 1975) and protoins of nuclei and nucleoli of the slime mold Physarum polycephalum (Atmar et al., 1978), thereby suggesting the possibility that polyamines may have a role in regulation of gene transcription.

Recently, it has been conjectured that the fluctuations in cellular polyamine concentrations and/or ratios found in proliferating and neoplastic cells alter chromatin structure and cause variations in the ADP-ribosylation of chromosomal proteins, thereby affecting nuclear function (Perella and Lee, 1979). Polyamines are found to stimulate the Herpes Simplex Virus DNA polymerase purified from infected Baby Hamster kidney cells (Walkee et al., 1980).

Spatial models for the complexes of spermine (or spermidine) with the B form of DNA were suggested

in the studies of several workers (Tsuboi, 1964; Liquori et al., 1967; Sawalsky, et al., 1969). The significance of these works is that a correspondence of distances between the positive charges of the polyamines and the negative charges of the DNA phosphates was emphasized.

The double-stranded DNA can exist in different forms depending on the relative humidity, the nature and concentration of counterions (Arrot, 1970) as well as on the nucleotide sequence (Brom, 1971). These forms, which may be divided into two forms ($\bar{B}$ and $\bar{A}$) differ, in particular, by the distances between the phosphate residues both within one chain and in the opposite chains (Zurkin et al., 1978). It has been reported that while spermine and spermidine facilitated the $\bar{B}$ to $\bar{A}$ transition, and thereby prefers the A-like forms, putrescine and cadaverine prefer B-like form (Minyat et al., 1978). It is suggested that since the distance between the phosphates of the same chain is almost invariant with each of the families being $1A^{o}$ shorter in the forms of $\bar{A}$ family, the propyl diamine (but not butylene diamine) part of the spermine or

spermidine) molecule is responsible for the stabilisation of the $\bar{A}$ forms.

The effect of stabilization of $\bar{A}$ form by polyamines is significant biologically.

DNA replication is known to start with the synthesis of an oligonucleotide which forms a hybrid with one DNA chain (Sugino et al., 1972). Since the DNA-RNA hybrid can have only the $\bar{A}$ conformation (Arnott et al., 1968), then $\bar{B}$ to $\bar{A}$ shift could be important in replication, and the activating effect of polyamines on replication may be due to this circumstance (Florentiene and Ivanov, 1970).

It has been proposed that transcription must be accompanied by a local $\bar{B}$ to $\bar{A}$ transition in the DNA template under RNA polymerase (Beabealashvily, et al., 1972).

Based on this, later a detailed stereochemical model for the RNA polymerase operation was suggested (Wang et al., 1979). Although there are many ways in which polyamines might activate transcription, a direct influence of the polyamine on the DNA template conformation is highly possible (Minyat et al., 1978).

Recently, it is suggested that complexions such as polyamines or specific binding proteins might favour the transition from B form to the left handed helical form of DNA which is called Z-DNA (Wang et al., 1979; Davies and Zimmerman, 1980).

These observations are highly significant in view of the altered patterns of gene expression during neoplastic transformation.

Besides the large number of reports pouring in, indicating the importance of polyamines in various cellular functions, general interest in the metabolism of polyamines has been greatly increased following reports promising the value of polyamines in early diagnosis of cancer and in cancer therapy. There may be certain applications for a clinical monitoring of polyamine levels, especially in short-term follow-up of the therapeutic efficacy of cytostatic medication (Russel, 1977; Cohen, 1977).

The value of applied knowledge of polyamine metabolism in cancer therapy seems to be promising in the light of several observations that the use of selective inhibitors of polyamine synthesis result in a profound inhibition of cell proliferation under

a variety of experimental conditions (Inoue et al., 1975; Otani et al., 1974; Fillingame et al., 1975; Boynton et al., 1976; Krokan and Eriksen, 1977; Sunkara et al., 1977; Morris et al.,1977). Methylglyoxal-bis (guanylhydroazone) a cytostatic drug that has been used in the treatment of human malignancies in the early sixties and later withdrawn from clinical use mainly due to an unacceptable toxicity has been found to affect the metabolism of natural polyamines and vice versa (Seppanen et al., 1980). Inhibition of polyamine accumulation and cell proliferation by derivatives of diamino propane has been obtained in Ehrlich Ascites cells grown in culture (Alhanen-Hongisto et al., 1979). Selective killing of transformed cells has been attained by exploitation of their defective cell cycle control by polyamines (Rupniak and Paul, 1980). Elevation of serum polyamines has been observed in malignant lymphomas and acute myeloid leukemia (Hospattankar et al., 1980).

The mounting evidences for the role of polyamines in cellular growth, proliferation and neoplastic transformation point to the importance of further investigation on various aspects of these

biomolecules for understanding the mechanisms - molecular and cellular - involved in neoplastic transformation as well as for exploiting the avenues of their practical utility in cancer detection and therapy.

2-Mercaptopropionylglycine (MPG)

Sulphydryl compounds are widely distributed in animals, plants and microorganisms participating in various enzymatic reactions, peptide hormone activities and other important biological phenomena (Funae et al., 1971). Mercaptopropinylglycine, also known as Thiola, consists of glycine and 2-Mercaptopropionic acid as a liver-intensifying agent. The chemical structure of MPG is as follows:

$$CH_3 - \underset{\underset{SH}{|}}{CH} - CONH - CH_2\ COOH.$$

It has comparatively less side effects than other SH-compounds and is regarded to be effective for the detoxication of heavy metals and general organic compounds (Fujimura et al., 1964). It is also known to be effective for radiation protection (See Sugahara and Srivastava, 1976) and

for the treatment of liver (Kaito et al., 1972; Milazza et al., 1972; Shiota et al., 1972). MPG has been found to have protective effect on paracetamol-induced hepatic necrosis. It is suggested that binding of the reactive metabolite of paracetamol to hepatocyte macromolecules need not lead to hepatic necrosis provided that sulphhydril groups are present in liver to prevent the deleterious effects of such binding and that MPG might have such a sort of protective action (Labraderios et al., 1977). MPG can form complexes with many metals (Funae et al., 1971). Recently it has been proposed that MPG may act at the level of mitochondrial energy coupling (Zimmer, et al., 1978).

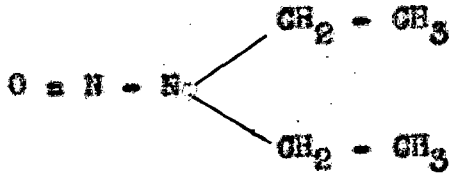
Except for a recent one (Aparna, 1981) there are no reports on the effect of MPG on the action of chemical carcinogens.

Neither the biochemistry nor the mechanism of action of MPG is clearly known.

Diethyl Nitrosamine: A member of Carcinogenic N-Nitroso amines:

The current interest in the carcinogenic and

other biological actions of N- Nitroso compounds arose following the reports of the hepatotoxicity (Burnes and Magee, 1954)and carcinogenicity(Magee and Barnes, 1956) of the simplest nitrosamine N-nitroso dimethylamine (dimethyl nitrosamine) and the subsequent observations on the carcinogenic action of the nitrosamides, N-methyl-N-nitrosourethane and N-methyl-N-nitrosourea (Druckery et al., 1961; Schoental, 1960). About 100 nitroso compounds are known to be carcinogenic in experimental animals (Magee and Schoental, 1964; Magee and Barnes, 1967). In addition to their toxic and carcinogenic properties many N-nitroso compounds are mutagenic and some are teratogenic in experimental animals (Magee and Barnes, 1967).



Structural and molecular formulae and weight.

$C_4H_{10}N_2O$ Mol. Wt. 102.1.

N-Nitroso diethylamine (diethylnitrosamine) is found to be carcinogenic in all animal species tested. It induces benign and malignant tumours after its

administration by various routes, including ingestion, parenteral injection, inhalation and rectal instillation. The major target organs are the liver, respiratory and upper digestive tracts and kidney. It is carcinogenic following its administration prenatally and in single doses. In several studies dose-response relationships were established (see IARC monographs, 1978).

DEN is reported to be present in air, tobacco, smoke, water, food and feed (Cheese, vegetables and vegetable oils, cereal products, fish, meat products) and alcoholic beverages. The in vitro formation of DEN was observed when diethylamine and sodium nitrite were incubated with gastric juice from rats, rabbits, cats, dogs and man (Sen et al., 1969). Human and rabbit gastric juices (pH 1.3-2) produced more nitrosamine than juice from the rat (pH 4.4-4.6). The nitrosation reaction was also demonstrated in vivo in cats and rabbits after feeding diethylamine hydrochloride and sodium nitrite. Nitrates and, to a lesser extent, nitrites are widely distributed in nature. The major human intake of nitrates in food stuffs comes from vegetables or water supplies or from nitrates used as additives in the meat curing

processes. Spinach, beets, lettuce, radishes, egg plant, celery and turnips are vegetables with the highest concentration of nitrates.

Although there is no firm proof, the available data on experimental animals shows that it is highly probable that nitrosamines are also carcinogenic in man. It is now well established that carcinogenic nitrosamines may be present in minute quantities in certain foods for human consumption. Carcinogenic nitrosamines may also be formed from secondary or tertiary amines and nitrites in the body, particularly in the acid conditions of the stomach after simultaneous ingestion. Secondary and tertiary amines occur in some foods, and a number of drugs and other environmental chemicals have secondary or tertiary amino structures. Humans also are exposed to secondary amines and nitrosating agents in various industries.

Unfortunately, little is known about the mechanism of action of N-nitrosamines. It is believed that they require metabolic activation to produce their effect (Heath, 1961). It is currently accepted that they are metabolically degraded by microsomal mixed function oxidases (Montesano and Bartsch, 1976)

to an alkldiazohydroxide (R-N = N-OH) which subsequently decomposes to a carbocation intermediate (Park et al., 1977) responsible for the alkylation of DNA (Margison and Montesano, 1976).

Presently, no direct experimental evidence is available which demonstrates the involvement of carbonium ions in the alkylation mechanism of nitrosamines. Calculations based on theoretical framework suggest that nitrosamines do not alkylate the DNA molecule via carbocation intermediates. The most likely alternative is, then, direct nucleophilic attack (S_N2) on the diazonium ion by basic sites on the DNA molecule (Andreozzi and Klopman, 1980). From a mechanistic point of view, this suggests that carcinogenicity is related to the ease of nucleophilic attack at the alkyl group of N-nitrosamines and not to the stability of carbenium ions they can generate.

MATERIALS AND METHODS

Animals:

Adult male rats of Wistar Strain were obtained from the Animal House of Maulana Azad Medical College, New Delhi. The weight of the animals was 135 ± 22.6 g.

Chemicals:

Diethyl Nitrosamine, N_2-Mercaptopropionyl-glycine, glycine, L-Arginine, Isonitrosopropiophenone, Urea, Trizma base, Sodium dodecyl sulphate, 2-mercapto-ethanol and Bromophenol blue, Bovine Serum Albumin and Standard proteins for electrophoresis (Albumin, Ovalbumin, Trypsinogen, B-lactoglobulin and Lysozyme) were from Sigma Chemical Company, USA.

N,N'-bis methylene acrylamide, TEMED and coomassie brilliant blue - R - 250 were products of Biorad Laboratories, USA.

Acrylamide (3 x cryst.) and Folin-phenol reagent were purchased from SISCO Research Laboratories Pvt. Ltd., Bombay.

Sodium chloride, Manganous chloride, Perchloric acid, hydrochloric acid, sulphuric acid, cupric sulphate,

glycerol were of 'Anlar' grade from BDH, India; Phosphoric acid, Sodium carbonate, Sodium hydroxide were of 'Guaranteed reagent' grade from Sarabhai M. Chemicals, India; Ammonium persulphate (AR) from IDPL, India; Glacial acetic acid (AR) from BDH, India or Polypharm, Bombay, India. Methanol was Anlar or Laboratory Reagent grade product of BDH, India. Potassium Sodium tartrate (Purified) was a product of E. Merck (India) Pvt. Ltd.

The rats were divided into 4 groups:

Group I comprised of control animals.

DEN was gavaged (dose: 200 mg DEN per kg body wt. with 10 ml tap water per kg. body wt.) to the animals of group II under mild ether anaesthesia.

To the group III animals MPG was administered at a dose of 0.25 mg/gb.wt/day in drinking water commencing 8 to 10 hrs before DEN gavaging and continued till the time of sacrifice.

The animals of group IV were given MPG in drinking water at the same dose as that used for group III.

The animals of group I and IV were gavaged with tap water (volume same as that of DEN gavaged to group II and III) under anaesthesia.

The animals were sacrificed at the intervals of 24 hr, 48 hr and one week. The animals were sacrificed between 2 PM and 5 PM by cervical dislocation and liver was excised and chilled immediately.

Preparation of homogenate:

The homogenising medium was 0.9% Saline. One gm tissue was homogenized in 20 ml saline by using a Potter Elvehjem type homogeniser fitted with a teflon plunger. The homogenate was centrifuged at 20,000 g for 20 minutes at 4°C. The supernatant was collected and centrifuged at 105,000 g for 60 minutes at 4°C. The supernatant (soluble fraction) was used for the estimation.

Assay of Arginase:

Arginase was assayed by the procedure as described by Schimke (1970) with some modifications. Arginase was first activated by incubating 0.9 ml of 1:200 homogenate at 37°C in the presence of 0.1 ml of 0.1 M $MnCl_2$ for 10 minutes.

The assay mixture contained the following:

Activated extract : 0.1 ml.

1.25 M glycine : 0.4 ml.

0.25 M Arginine (pH 9.7) : 0.4 ml.

0.01 M $MnCl_2$: 0.1 ml.

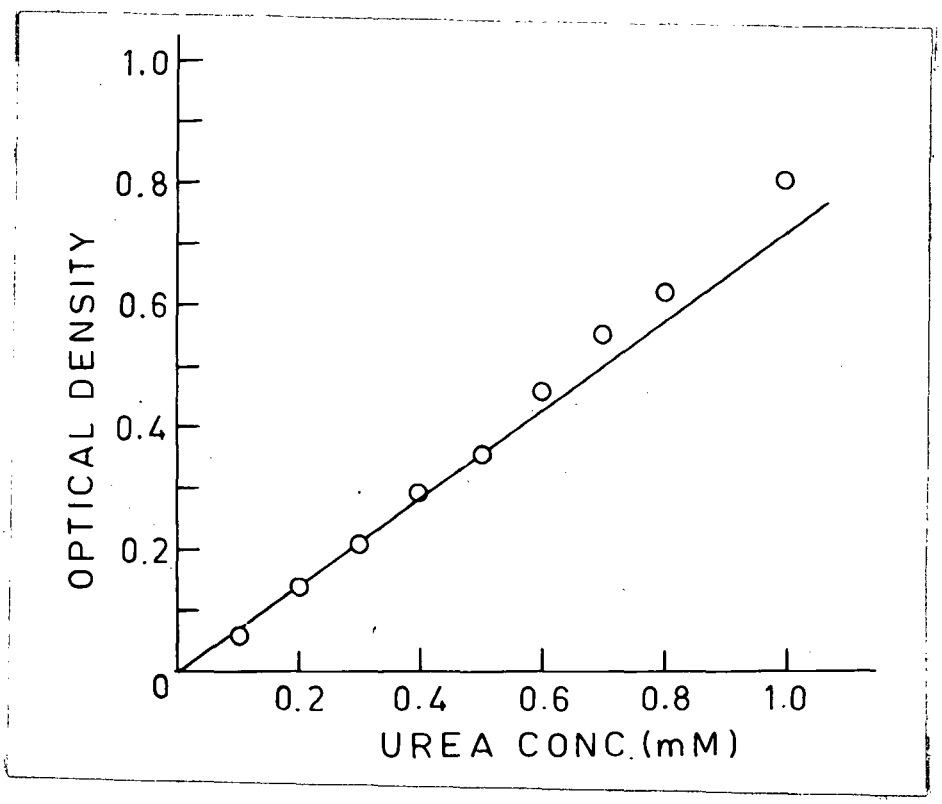
This mixture was incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 2.0 ml of 1N Perchloric acid.

Tubes with assay mixture and 2 ml of 1 N Perchloric acid were also included for incubation.

After incubation the mixture is centrifuged at about 3,000 RPM in a Remi centrifuge. 1.0 ml of the supernatant was taken and to it 3.0 ml of H_2So_4/H_3Po_4 mixture (acid mixture) and 0.1 ml of isonitroso propiophenone was added. The samples were heated in dark in a boiling water bath for 30 minutes. The tubes were cooled in darkroom temperature and optical density was read at 540 nm using an appropriate blank on a Carl Zioss Spectrophotometer.

Urea standards in the range of 0.1 to 1.0 µmoles were run and the data was plotted to obtain a standard curve.

Fig. 3. Standard curve for urea.


OPTICAL DENSITY
1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
UREA CONC.(mM)

Protein estimation:

Protein in the soluble fraction was estimated by the method of Lowry et al. (1951) as modified by Cooper (1977). The optical density measurements were done on Schimadzu UV-240 Spectrophotometer.

Units of Enzyme Activity:

Arginase activity was expressed as μmoles of urea formed at 37°C in one minute per g fresh weight of tissue or μmoles of urea formed at 37°C in one minute per mg protein.

Polyacrylamide gel electrophoresis in presence of SDS:

Sodium dedecyl sulphate polacrylamide gel electrophoresis was performed by the method of Laemmli (1970) with some modifications.

The samples contained the final concentration (final "sample buffer"): 0.0625m Tris HCl (pH 6.8)
2% SDS
10% glycerol
5% mercaptoethanol and
0.001% bromophenol blue as the dye.

The proteins were completely dissociated by immersing the sample for 1.5 minutes in boiling water.

7 cm gels were prepared in glass tubes of a total length of 9.0 cm and with an inside diameter of 5 mm.

Gels containing 7.5% (separation gels) and 3% (stacking gels) acrylamide were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N'-bis-methylene acrylamide. The separation gel contained a final concentration of 0.375 M Tris HCl (pH 8.8) and

0.1% SDS.

The stacking gels of 3% acrylamide and a length of 1 cm contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS.

The gels were polymerized chemically by the addition of 0.025% by volume of TEMED and 0.025% by weight of ammoniumpersulfate.

The electrode buffer (pH 8.3) contained:

0.025 M Tris.

0.192 M glycine and

0.1% SDS.

150 to 280 mg protein was loaded on each gel. 5 standard proteins were run. 100μl(5 μg) of each protein and 100μl(33 μg approximately) of the mixture of 5 proteins were loaded. Electrophoresis was carried out with a current of 3 mA pergel until the marker dye had reached about 1 cm from the bottom of the gel tube (about $1\frac{1}{2}$ hrs).

The gels were stained for about 10 hr for protein with coomasie Brilliant Blue R-250 (1.25 gm in a mixture of 227 ml distilled water, 227 ml methanol and 646 ml glacial acetic acid. After the dye was dissolved, it was filtered through Whatman No. 1 filter paper) and destained in the destaining solution (875 ml glass distilled water with 50 ml methanol and 75 ml glacial acetic acid).

The results were recorded by both photographing and scanning the gels.

The relative mobility of protein was calculated by using the equation:

$$\text{Mobility} = \frac{(\text{Distance of protein migration}) \times (\text{gel length before staining})}{(\text{Distance of dye migration}) \times (\text{gel length after staining})}$$

The mobilities obtained were plotted as a function of the molecular weights on semi-logarithmic coordinates.

RESULTS

1. Mortality:

There was no mortality noticed during the entire course of experiment.

2. Biochemical alterations:

(1) Arginase

Table 1 shows the enzyme activity (μmoles of urea formed at 37°C per minute per g fresh wt. of tissue) under different modes of treatment and after different time intervals. In the case of DEN-treated animals (Group II) arginase shows some increased activity but this increase does not significantly deviate from control value (Fig. 4). This could be due to error variance obtained. At 48 hr the enzyme activity does not show any significant alteration (Fig. 4). In this case also the enzyme activity has increased slightly over that at 24 hr, but the error variance is high. At 1 week arginase registers a significant increase in activity as compared to that of control ($P \leq 0.05$).

In the case of animals treated with MPC and DEN arginase activity registers a significant decrease at 24 hr ($P \leq 0.05$), but shows an increase by 48 hr ($P \leq 0.025$) -

TIME AFTER TREATMENT	24 hr	48 hr	1 week
GROUP			
I (Control)	263.70±11.38(5)	-	-
II (DEN)	311.12±45.94(4)	328.20±46.8(4)	298.16±14.57(3)*
III (MPC+DEN)	208.90±32.37(3)*	314.90±19.12(5)**	307.44±28.10(4)
IV (MPC)	213.50±22.26(5)*	302.50±15.8(4)*	272.31±19.70(5)

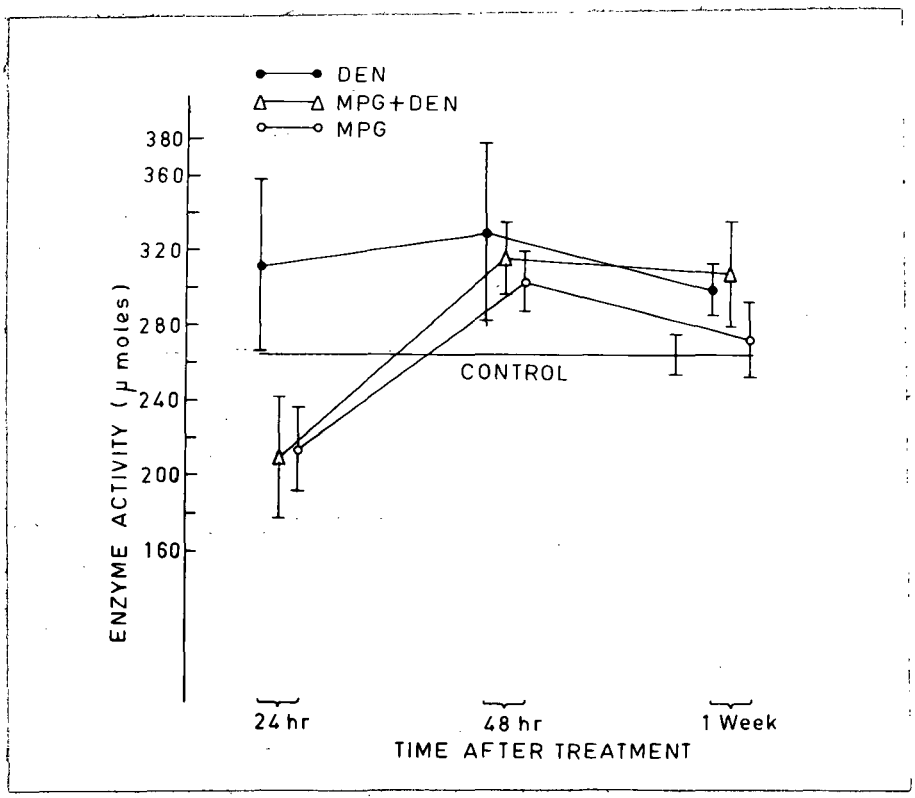
Table - 1. Changes in arginase activity (μmoles of urea formed at 37°C in one minute per g. fresh wt. of tissue). The figures represent Mean±S.E.M. Numbers in parentheses represent the number of animals.

* $P \leqslant 0.05$

** $P \leqslant 0.025$

(For details of groups, see 'Methods')

Fig. 4. Alterations in Arginase activity (activity expressed in terms of μmoles urea formed at 37°C per minute per g. fresh wt. of tissue) at different time intervals after different modes of treatment.


DEN
MPG+DEN
MPG
380
360
320
280
240
200
160
ENZYME ACTIVITY (μ moles)
CONTROL
24 hr
48 hr
1 Week
TIME AFTER TREATMENT

and at 1 week the activity attains near-normal value. Actually, as shown in Fig. 4 the activity at one week is slightly lower than that at 48 hr and well above that of control. But the value does not show significant deviation from control value because of high error variance.

MPG-treated animals (Group IV) showed a significant arginase activity at 24 hr ($P \leq 0.05$) but an increase above control level is observed at 48 hr ($P \leq 0.05$). At 1 week, however, the activity comes to the level of control. Here again, from Fig. 4, it is seen that, at 1 week, the enzyme activity is slightly higher than that of control even though it is below significant level.

Table 2 shows the alterations in arginase activity under the same experimental conditions explained above but here the enzyme activity is expressed as specific activity, i.e. μmoles urea formed at 37°C per minute per mg protein. The alterations in activity are found to be non-significant in the case of all groups and at all time intervals except for the animals treated with MPG which showed a significantly decreased arginase activity only at one week.

TIME AFTER TREATMENT / GROUP	24 hr	48 hr	1 week
I (Control	5.16±0.51(5)	-	-
II(DEN)	5.13±0.64(4)	5.69±0.51(4)	4.64±0.65(5)
III (EPO+DEN)	4.34±0.52(3)	5.19±0.34(5)	3.93±0.56(4)
IV (EPO)	4.60±0.57(5)	5.11±0.42(5)	3.99±0.28(5)*

Table - 2. Changes in arginase activity (μmoles of urea formed at 37°C in one minute per mg. protein). The figures represent mean±S.E.M. Numbers in parentheses represent the numbers of animals.

* $P \leq 0.05$.

(For details of groups see 'Methods').

Fig. 5.- Alterations in Arginase activity (activity expressed in terms of μmoles urea formed at 37°C per minute per mg. protein) at different time intervals after different modes of treatment.

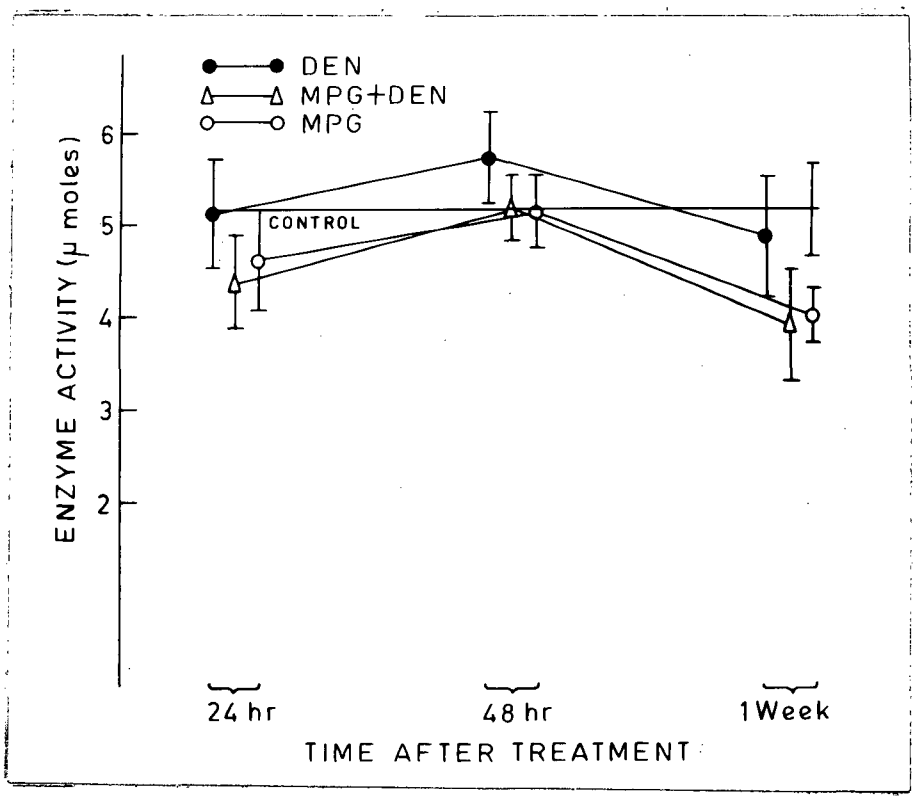

DEN
MPG+DEN
MPG
CONTROL
ENZYME ACTIVITY (μ moles)
6
5
4
3
2
24 hr
48 hr
1 Week
TIME AFTER TREATMENT

Fig. 5 shows that all groups behave almost in same pattern as far as arginase activity is concerned. But there are slight alterations. In the case of DEN-treated animals the activity is almost at the control level, but it increases slightly at 48 hr, again to decrease slightly below control level at 1 week. Group III, i.e. animals treated with MPG and DEN shows a decrease in arginase activity at 24 hr, but activity is slightly elevated above the control level at 48 hr and again considerably decreased, as compared to that of control at 1 week. The MPG-treated animals also show almost the same pattern.

(ii) Total protein content of 'soluble fraction'

Table 3 summarises the alterations in total protein content of the soluble fraction. At 24 hr, protein content is significantly altered in none of the experimental groups. But from Fig. 6, it appears that in DEN treated animals (Group II) the protein level is slightly higher than that of the control at 24 hr. At 48 hr, protein content increases in animals treated with MPG and DEN ($P \leq 0.05$) and also in those treated with MPG alone ($P \leq 0.025$), the amount being higher in latter than in the former one as is evident from Fig. 6. In

TIME AFTER TREATMENT / GROUP	24 hr	48 hr	1 week
I (Control)	53.00±5.33(5)	-	-
II (DEN)	56.63±8.64(5)	58.16±2.72(5)	65.71±6.54(5)
III (MPG+DEN)	43.79±4.2(4)	62.66±2.85(6)*	68.24±11.48(4)*
IV (MPG)	52.64±10.7(6)	70.93±6.20(4)**	68.57±3.93(5)*

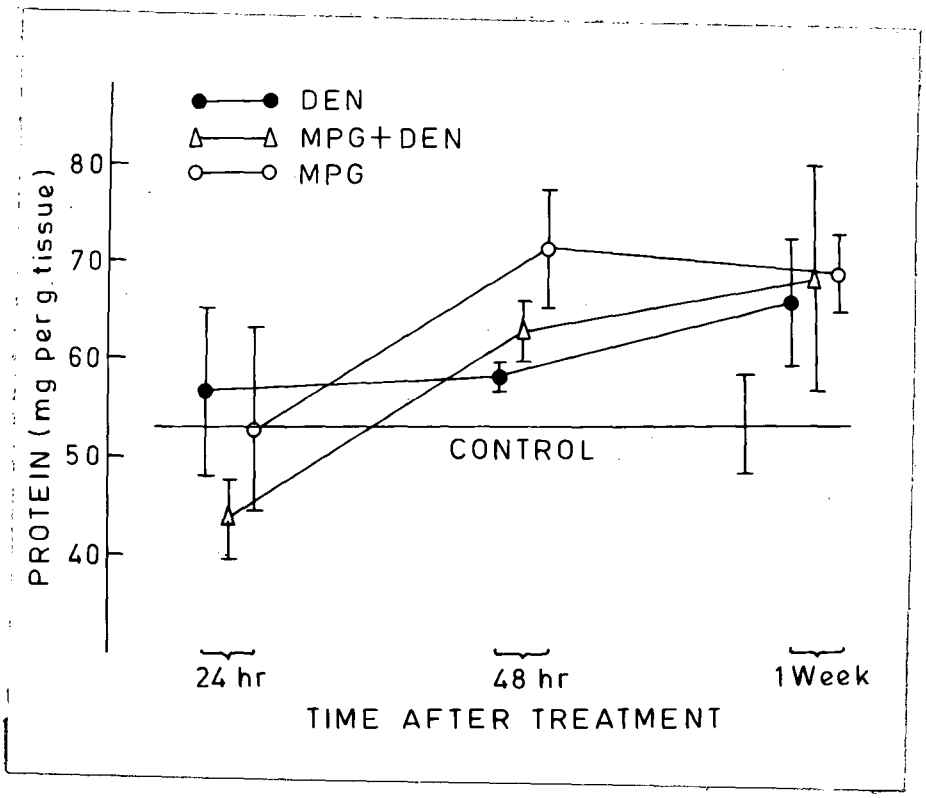
Table - 3. Changes in total protein of soluble fraction (mg. per g. fresh tissue weight). The figures represent Mean±S.E.M. Numbers in parentheses represent the number of animals.

* $P \leq 0.05$

** $P \leq 0.025$

(For details of groups see 'methods').

Fig. 6. Changes in total protein content (mg protein per g. tissue) at different time intervals after different modes of treatment.


DEN
MPG+DEN
MPG
PROTEIN (mg per g tissue)
80
70
60
50
40
CONTROL
24 hr
48 hr
1 Week
TIME AFTER TREATMENT

the case of DEN-treated animals, the protein content at 48 hr is increased above the control level and slightly above that at 24 hr (Fig. 6). But this alteration is non-significant statistically. By 1 week the protein content of DEN-treated animals is increased from the control level as well as from that at 48 hr. In animals treated with MPG + DEN, the protein content goes up from that at 48 hr ($P \leqslant 0.05$) but in MPG-treated animals, the amount of protein in the soluble fraction remains almost the same as that at 48 hr and it is well above control level ($P \leqslant 0.05$).

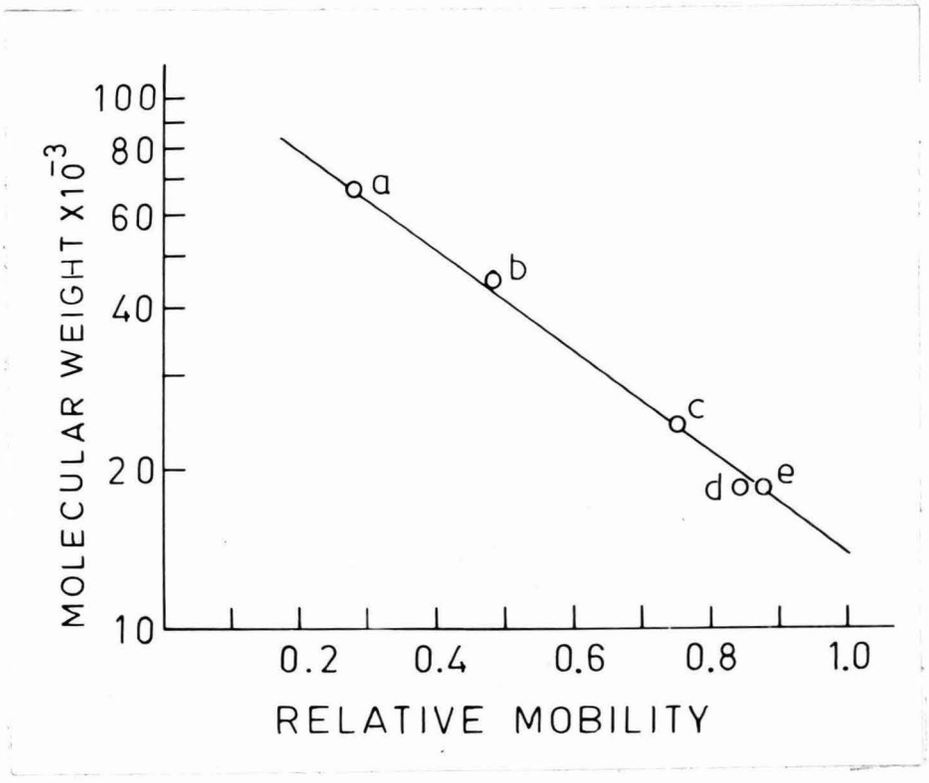
(iii) Protein profile in SDS-PAGE

The protein profiles can be observed and compared among different groups from the gel photographs and respective scan-diagrams.

At 24 hr, it is seen (Figs. 8, 9) the protein band A_1 (Mol. wt. > 66,000 daltons) present in the control is missing conspicuously in the case of DEN-treated animals (Group II) where as it is present in the case of animals treated with MPG + DEN and MPG alone (Group III and IV). The band B_1 (Mol. wt. 66,000 approximately) which is very thick in control and group III and IV, is very light in group II (DEN-treated). Band C_1

Fig. 7. A. Separation of standard proteins using SDS acrylamide gel electrophoresis. The standards are (a) Albumin (Bovineplasma) (66,000), (b) ovalbumin (45,000), (c) Trypsinogen (24,000), (d) β-lactoglobulin (18,400), (e) Lysozyme (14,300).

B. Coomassie Brilliant Blue staining of the acrylamide gels from which these data were obtained.


MOLECULAR WEIGHT X10^{-3}
100
80
60
40
20
10
a
b
c
d
e
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

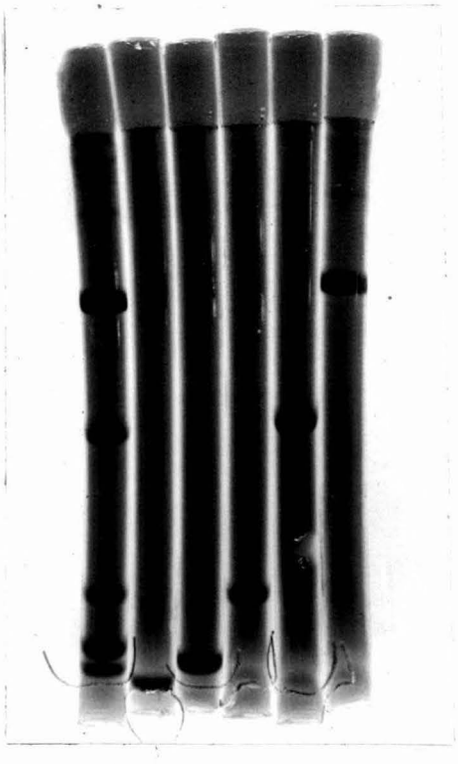


Fig. 8. SDS-PAGE: Profile of cytosol proteins of liver from animals of group I (control); group II (DEN); Group III (MPG+DEN) and group IV (MPG) at 24 hr after treatment.

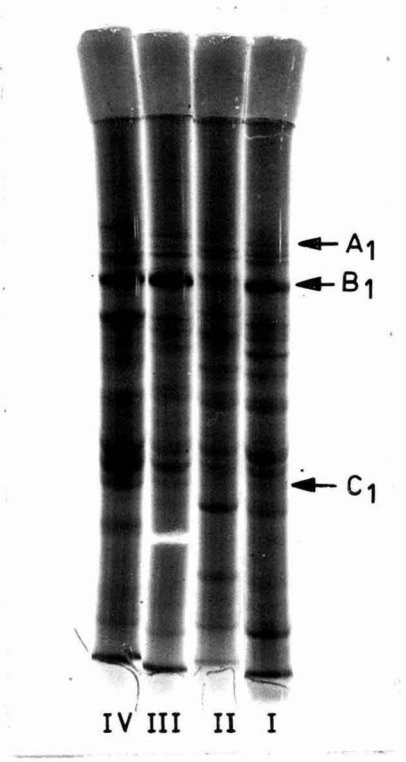
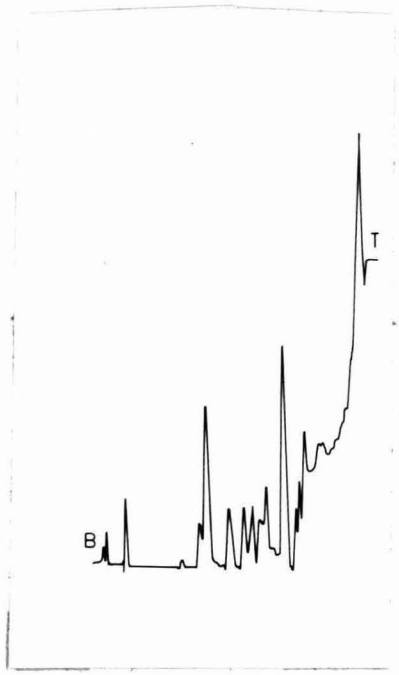
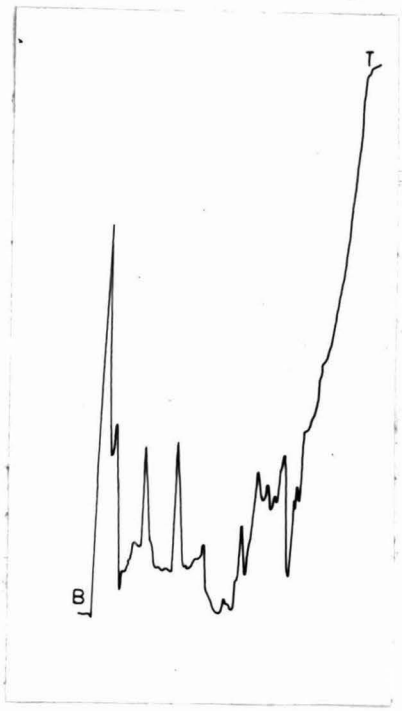
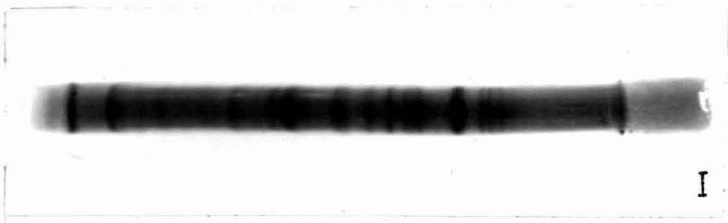

A1
B1
C1
IV III II I

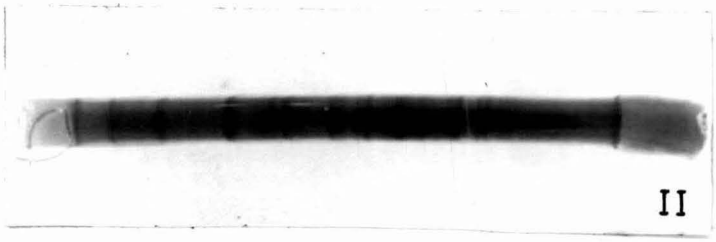
Fig. 9. SDS-PAGE: Profile of cytosol proteins of liver from animals of group I (control); group II (DEN); group III (MPG+DEN); group IV (MPG) at 24 hr after treatment (gel photographs shown separately along with respective scans).



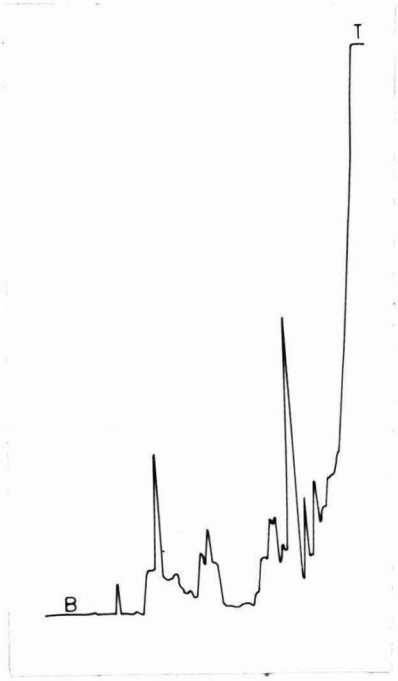


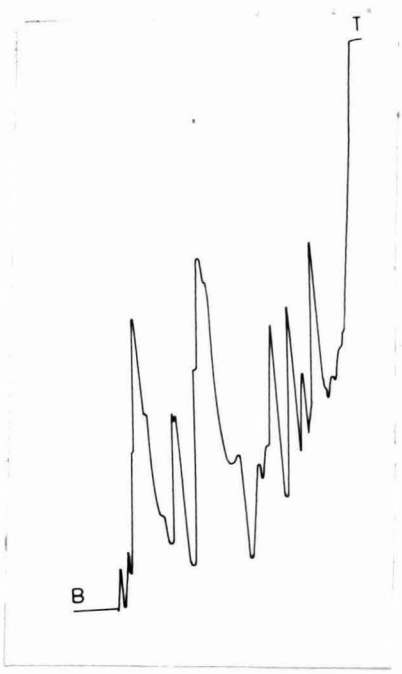


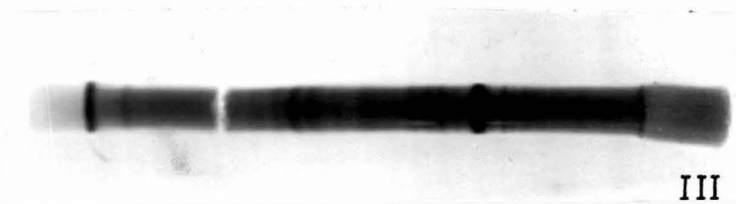
I



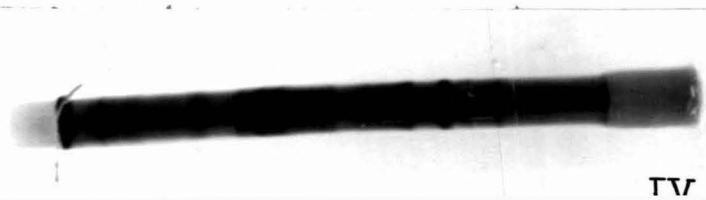
II







III



IV

(Mol. wt. 45,000 approximately) is observed both in the case of Group III and IV but is absent in I and II.

At 48 hr (Figs. 10, 11) a protein of molecular wt. falling in between 18,000 and 24,000 (designated as A_2) makes its appearance in Group II and III but is absent in I (control) and IV (MPG-treated).

At 1 week, differences in several protein bands were observed among different groups (Figs. 12, 13). Several high molecular weight subunits (mol. wt. >66,000) and low molecular wt. sub units present in the (mol. wt. <45,000) control were missing in group II, III and IV. The protein band A_3 (Mol wt. > 66,000) is very light in Group II, III and IV as compared to that in control.

Fig. 10. SDS-PAGE: Profile of cytosol proteins of liver from animals of group I (control); group II (DEN); group III (MPG+DEN); and group IV (MPG) at 48 hr after treatment.

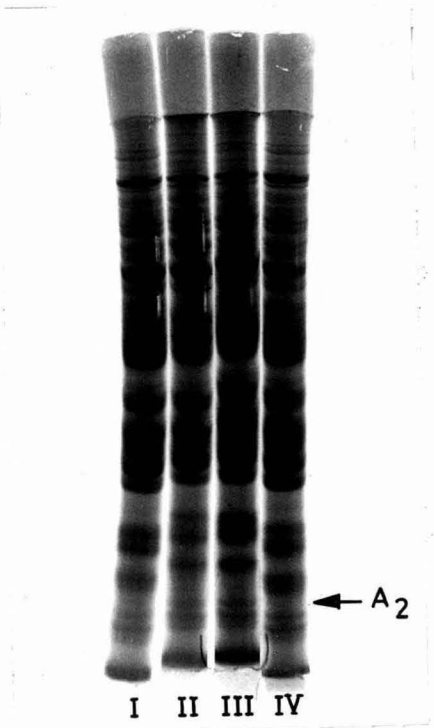
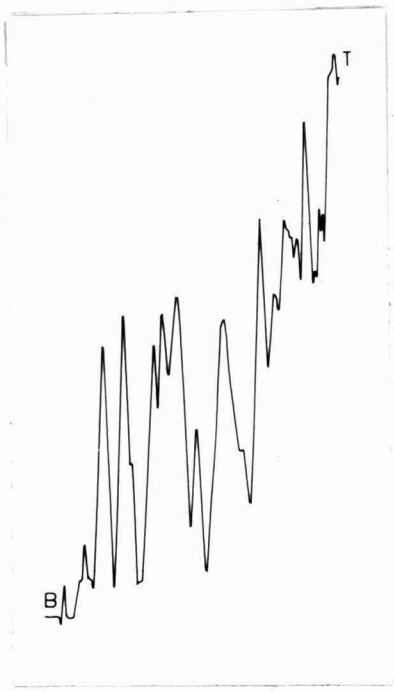
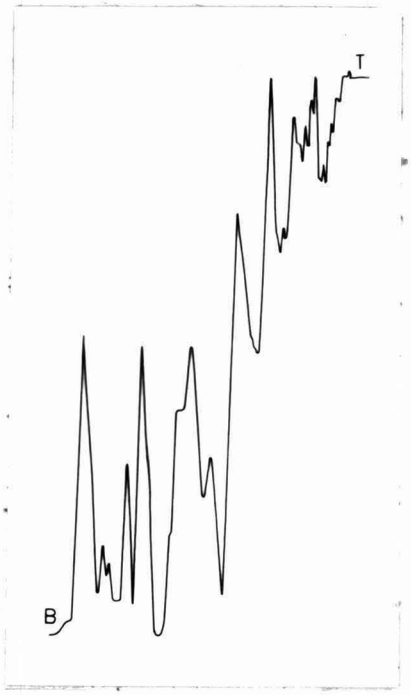
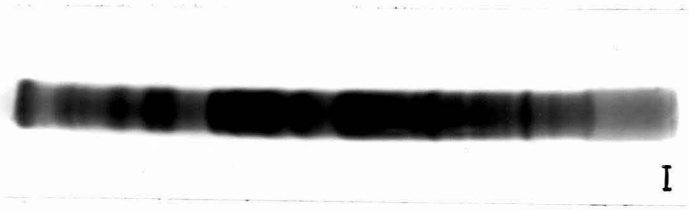

A2
I
II
III
IV

Fig. 11; SDS-PAGE: Profile of cytosol proteins of liver from animals of group I (control); group II (DEN); group III (MPG+DEN); group IV (MPG) at 48 hr after treatment. (gel photographs shown separately along with respective scans).







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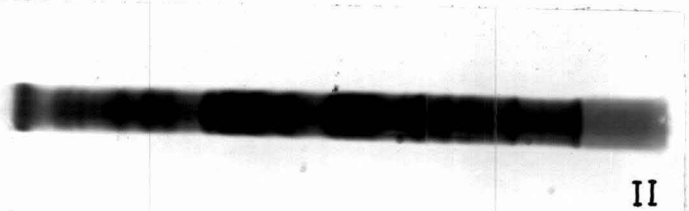

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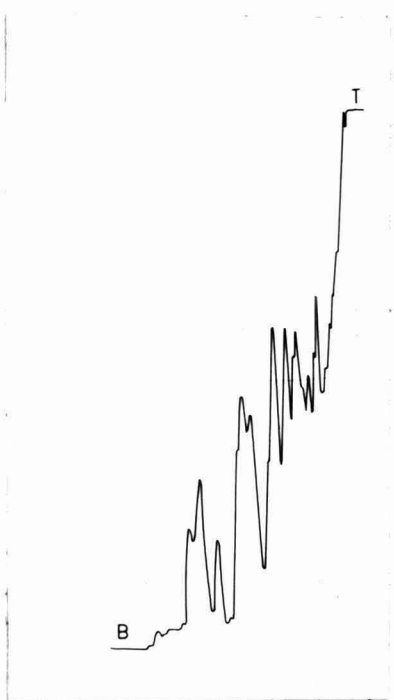

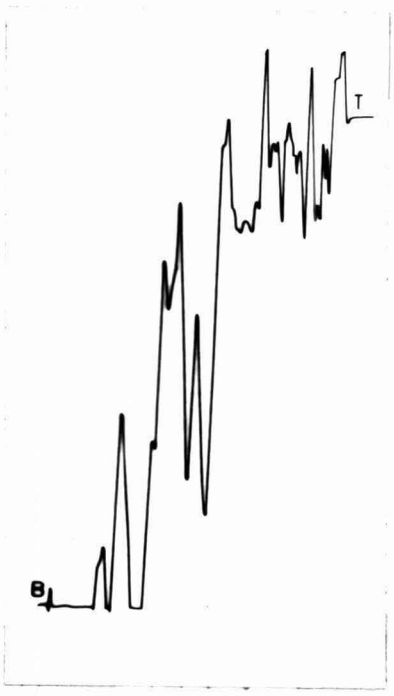

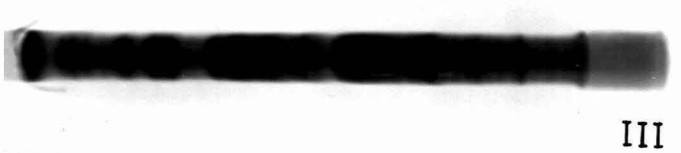

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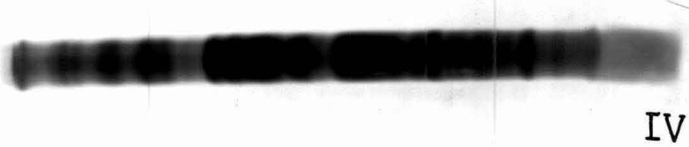
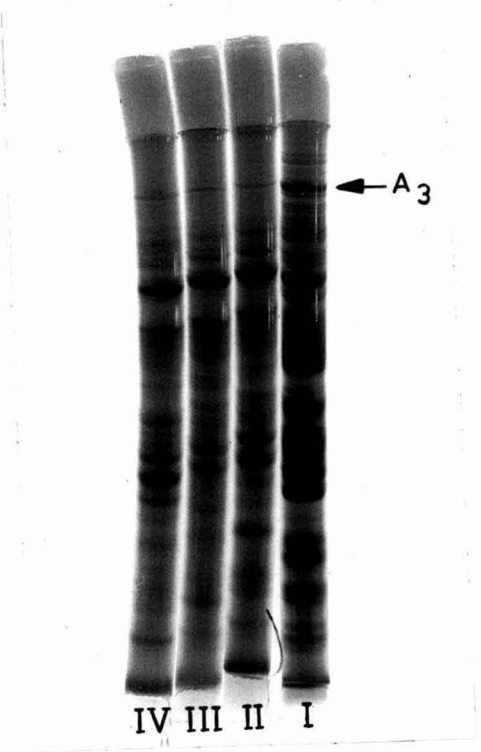


IV

Fig. 12. SDS-PAGE: Profile of cytosol proteins of liver from animals of group I (control); group II (DEN); group III (MPG+DEN); and group IV (MPG) at 1 week after treatment.


A3
IV III II I

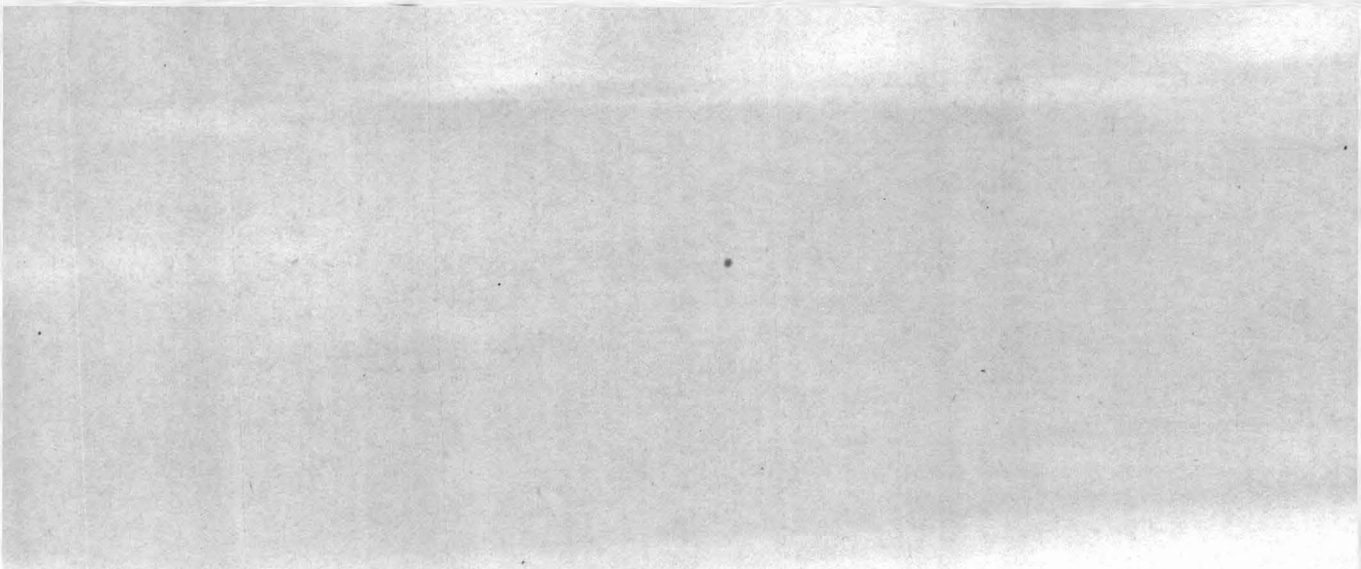
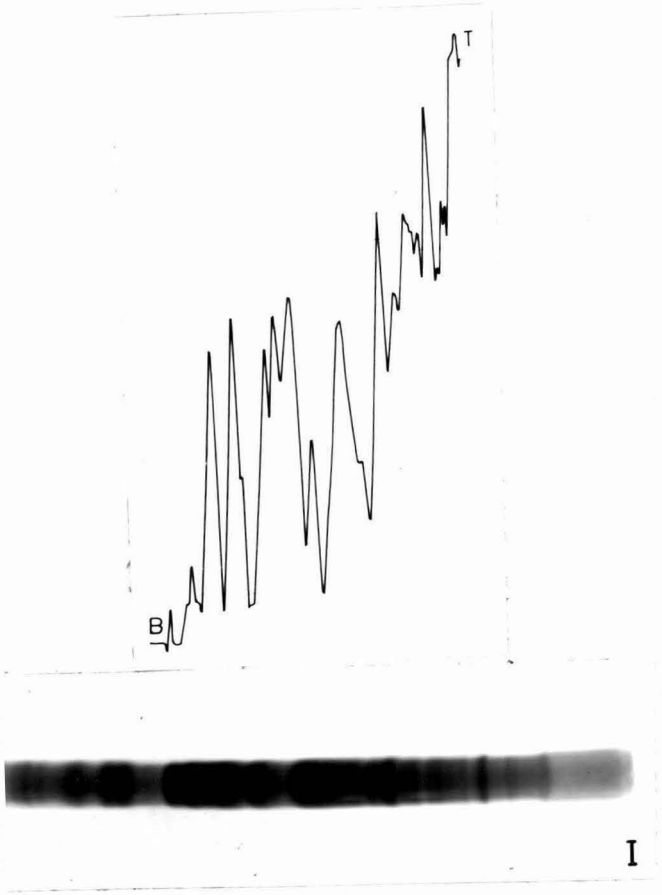


Fig. 13. SDS-PAGE: Profile of cytosol proteins of liver from animals of group I (control); group II (DEN); group III (MPG+DEN); and group IV (MPG) at 1 week after treatment. (gel photographs shown separately along with respective scans).



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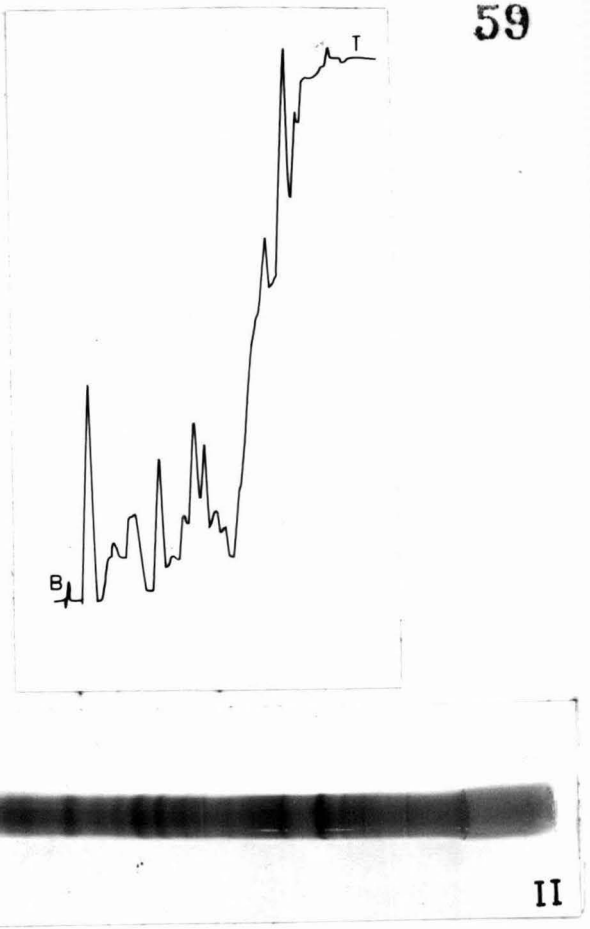

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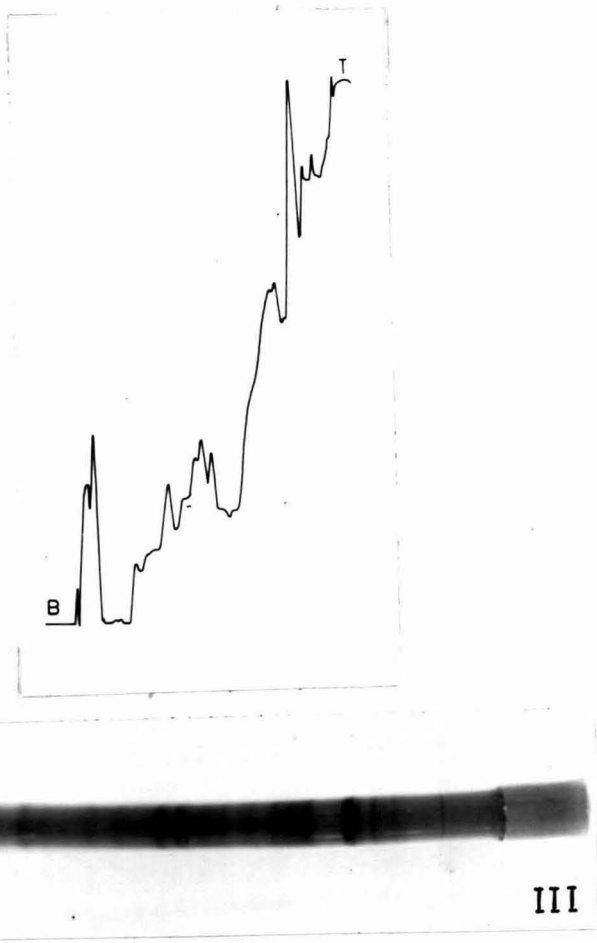
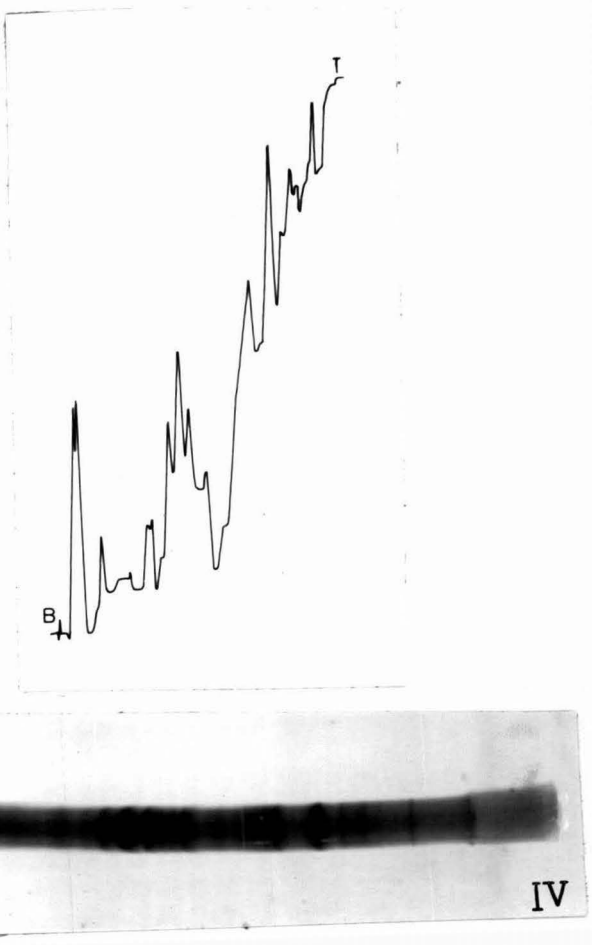


III



IV

DISCUSSION AND CONCLUSION

Ornithine decarboxylase, the initial enzyme in the polyamines biosynthetic pathway is induced in target tissues in response to a variety of trophic agents including polypeptide and amine trophic hormones, cyclic AMP analogs, drugs and trophic steroid hormones (see Russel, 1980). It has become the prototype of inducible enzymes. The substrate for this rate-limiting enzyme in polyamine biosynthesis, compounds which are the only known organic cations of the cell, is ornithine which is formed by the action of arginase on arginine. Since the major biosynthetic pathway of polyamines in mammalian cells involves the following steps, i.e. Arginine → Ornithine → putrescine → spermidine → spermine, the behavior of enzymes which catalyze each of these steps during carcinogenesis is interesting to look into. The present study on the effect of carcinogen on arginase activity within a short period after exposure, is a preliminary step towards this venture.

Arginase activity and its role during carcinogenesis as well as in preneoplastic and

neoplastic tissues remains controversial. Decrease in arginase activity has been reported in azo-dye induced hepatomas (Greenstein et al., 1941; Takahasi, 1954; McLean et al., 1964) and in precancerous rat liver (McLean et al., 1964). On the other hand there are reports of elevated arginase activity in connection with rapid cellular proliferation (Bach and Lasnitzki, 1961), in cells infected by virus (Rogers, 1959) and in human warts (Van Scott, 1951). Tumor regression or a reduction of tumor growth has been observed after injection of arginase into tumor bearing animals (Vrat, 1951; Wiswell, 1951; Irons and Boyd, 1952).

In our present experiments, at 24 hr after DEN treatment, an increase in arginase is observed (Table 1 and Fig. 4). A still higher activity is observed at 48 hr after treatment with DEN. But these values are statistically non-significant due to high error variance. At one week after treatment the arginase activity is lower than those observed at 24 hr and 48 hr, but it is interesting to note that this value is significantly above the control value ($P \leqslant 0.05$). It should be noted that the above

mentioned pattern was obtained when the enzyme activity was expressed in terms of μmoles per gm fresh weight of tissue. When the activity was expressed as μmoles per mg protein (Table 2 and Fig. 6) all the above mentioned values come closer to the control level and in fact none of them are statistically significant. This observation may mean that the increased arginase activity observed when the activity was expressed per g fresh tissue wt. is nothing but a consequence of increased protein content in the cytosol. In fact, in the present study, there are strong indication of an elevated total cytosol protein content after DEN treatment (Table 3 and Fig. 6) even though the high error variance makes them statistically non-significant. The increased protein content can reflect

(i) an increase in several proteins including arginase;

(ii) an increase in arginase alone, or

(iii) an increase in other proteins except arginase.

Of these the second one is a remote possibility. In the light of the above three possibilities,

both ways of expression of the enzyme activity, i.e. in terms of μmoles per g fresh tissue weight or μmoles per mg protein, has their own drawbacks in presenting a real picture of arginase level. This problem is not restricted to this enzyme alone, but is a common one in biochemistry. Solution is not easy even though use of labelled precursors may be helpful to some extent.

If we take the enzyme activity expressed in μmoles per g tissue wt. as the basis of interpretation, it is possible that increase in arginase activity after DEN treatment may be due to altered metabolic pattern brought about by DEN in the cells. The increased arginase activity may be due to the altered pattern brought about by DEN at any or all of the following: (a) transport through plasma membrane, nuclear and/or mitochondrial membranes, (b) transcription, (c) translation & (d) degradation. But the mechanism of interference of these cellular functions by DEN is not known.

In the case of animals treated with MPG and DEN, at 24 hr arginase activity decreases as compared

to that in control (Table 1 and Fig. 5), but increases above control value by 48 hr and maintains almost the same activity at 1 week. Of these the values at 24 and 48 hr are statistically significant. It is very interesting to note that MPG alone also brings about the same pattern.

Arginase requires Mn^{++} for its activity (see Dixon and Webb, 1979). 2-Mercaptopropionyl glycine, a sulphydryl compound, is a ligand of novel type bearing - SH, - CONH - and COOH groups. Like many other sulfhydryl compounds, MPG also has been found to be chelating ligand (Funae et al., 1971). MPG may be chelating the Mn^{++} in the exposed cells thereby making Mn^{++} unavailable in sufficient quantity for arginase activity. The initial decrease in arginase activity at 24 hr could be due to this cellular depletion of Mn^{++}. This depletion of Mn^{++} may be a temporary initial consequence of the presence of MPG inside the cell.

MPG has been reported to bring about an increase in condensed configuration of mitochondria and ATP production has been found to be increased in isolated liver mitochondria (Zimmer et al., 1978).

In the light of this observation, one can conjecture that ATP formation is increased in liver cells by MPG and this may cause a spurt in protein synthesis. In the present experiment we did find a significant ($P \leqslant 0.025$) increase in total cytosol protein content of the liver of both groups III (MPG+DEN) and IV (MPG) at 48 hr after treatment (Table 3 and Fig. 6). Thus, this effect of MPG on protein synthesis could be able to explain the increased arginase activity in both groups III and IV at 48 hr after treatment.

At 1 week, the enzyme activity registers a sharp decrease (as compared to that at 48 hr) in the case of group IV and maintains almost the same activity (as that at 48 hr) in the case of group III (the values are statistically non-significant due to high error variance). This increase could simply be the consequence of the variation in total protein synthesis or protein degradation at 1 week, as is evident from the study on total proteins (Table 3 and Fig. 6).

The effect of DEN and MPG+DEN on total cytosol protein content is interesting. At 24 hr after treatment a decrease in protein content is observed in the animals belonging to group III. One possible reason

for this could be that the initial interaction between MPG and DEN may be somehow causing a decreased protein synthesis. By 48 hr MPG overcomes the effect of DEN possibly due to carcinogen's metabolisation or excretion and thus the pattern of protein alteration in group III becomes similar to that of group II. DEN-treated animals (group II) registers a high enzyme activity at 24 hr; this level is maintained at 48 hr also. This may be due to the compensatory growth to counter the toxic effect of DEN on liver. By one week the protein content shows a further increase. The increase although variable may be attributed to the repair processes going on in the liver cells.

When the arginase activity is expressed as µmoles per mg. protein, the alteration in activity are found to be non-significant in the case of all groups and at all time intervals except for the animals treated with MPG which showed a significantly decreased arginase activity only at one week. Here the changes in the amount of other proteins of the cytosol might be masking the subtle changes that might have occured to arginase activity. Changes in

environmental conditions, influencing enzyme levels, are as likely to affect degradation rates as synthetic rates (Dixon and Webb, 1979). It could be possible that MPG might be contributing to the degradation of arginase by activating degradation enzymes or affecting arginase structure in such a way that it becomes more vulnerable to degradation. Another possibility is the one mentioned earlier, i.e. the continuous presence of MPG in cellular environment makes the cells incapable of substituting effectively the Mn^{++} depleted by coordination bonding property of MPG.

The literature relative to amino acid and protein metabolism in neoplastic tissues is extensive. As Weinhouse stated (Weinhouse, 1973), "there is a common thread interwoven throughout a large body of recent literature pointing to a common biochemical lesion in cancer; namely, a misprogramming of genetic expression, manifested by aberrations of protein synthesis." Most of the studies in this area are restricted to comparative studies between normal and tumorous tissues. Whether the altered protein pattern is decisive in the onset of neoplasia or it is a

consequence of neoplastic condition is yet to become clear. Very few studies have been done to know at what stage in the process of neoplastic transformation this "misprogramming of genetic expression, manifested by aberrations of protein synthesis" occurs.

Present study is a very preliminary attempt to see if there are any changes in the cytosol protein profiles during the very early phase after exposure to carcinogen. By using the SDS-PAGE technique, we have found differences in the banding patterns in acrylamide gels.

At 24 hr, it is seen (Figs. 8, 9) that a protein band designated as A_1 (Mol. wt. > 66,000) present in the control is missing conspicuously in the case of DEN treated animals (group II). It is present in group III and IV. Another difference noted is band B_1 (Mol. wt. 66,000 approximately), very conspicuous in group I, III and IV, appears very light in group II (DEN treated group). This may be due to the decreased rate of its synthesis or increased rate of degradation brought about by DEN. The band denoted C_1 (Mol. wt. 45,000 approximately) is observed both in the case of group III and IV but not

in I and II. This may indicate that probably (i) the synthesis of a protein produced in very meagre quantity which escapes detection in gel, is increased by MPG, possibly via its effect on mitochondria and ATP production; (ii) it is possible that the proteins might have got perturbed by MPG (Zimmer and Maink, 1977); (iii) a protein subunit highly labile to degradative enzyme is stabilised by the sulfhydryl group containing MPG. This is in line with the pattern of enzyme degradation proposed by Katunuma (Katunuma, 1972).

At 48 hr (Figs. 10, 11) a protein of molecular weight in between 18,000 and 24,000 (designated as A_2) makes its appearance in group II and III but is absent in I and IV. The appearance of this protein subunit in group II (DEN) and III (MPG+DEN) suggest that this may be the result of the action of DEN.

Differences in several proteins were observed at 1 week among different groups (Figs. 12, 13). Several high molecular weight subunits (mol. wt. 66,000) and low molecular weight subunits (mol. wt. 45,000) were found to be missing in group II, III and IV. It is reported that, in rat liver, large polypeptides seem to be degraded more rapidly than small ones but this

is likely to be only one factor influencing catabolic rates (Dice and Goldberg, 1975). This type of degradation aided by DEN and MPG may explain the disappearance of several high molecular weight proteins in groups II, III and IV.

This type of degradation may be attributed as one of the causes to the cases of alteration of high molecular weight proteins noted earlier. The alterations observed in low molecular weight proteins may be due to alterations in transport, synthesis and/or degradation.

Since our immediate interest was only to see if there are any changes in total protein profiles, and in not looking into any specific proteins, the exact molecular weight of the observed proteins were not determined. Only the approximate range of molecular weight is considered. For a better and clearer understanding of the significance of the changes in protein pattern, further detailed analysis of the specific proteins is needed.

The interpretations given for various observations should be taken in the light of certain important uncertainties regarding carcinogenesis.

The development of cancer following exposure to chemical carcinogens is almost invariably slow and prolonged. Although the process can be initiated by a brief exposure to a carcinogenic stimulus, there is no evidence that target cells so altered are cancer cells (Slot and Farber, 1976). Rather, there is abundant indirect evidence from many systems that what is induced is an altered cell or cell population from which malignant neoplasia can gradually develop or evolve. Neoplastic development resembles a chain reaction, triggered by exposure to a carcinogen in which the links are new populations with altered organisational, structural and biochemical properties. These slowly proliferative new lesions are characteristically focal in distribution, implying that only a small proportion of the original target cell population in any organ or tissue participates.

In the present experiments, the very short period considered may not be qualified to include as any major links in the chain process mentioned above. So the observations made may be treated as just some indications of how to approach and analyse the chain of events leading to neoplasia.

Moreover, as reported earlier (McLean et al., 1964) it is difficult to assess the exact significance of arginase, since the enzyme is extremely active in normal liver, and a very small contamination with normal liver cells would mask the subtle changes in activity of the enzyme. This problem becomes more acute when the study is being done (as in the present experiments) during the very early period after exposure to carcinogen. Moreover, the exact mode of action of DEN including the involvement of microsomal enzymes (Nagour and Nievel, 1971; Ferelly, 1980) etc. are yet to be understood clearly. As far as NPC is concerned except for a very few reports the biochemistry and mode of action are still shrouded in mystery. In short it will be too premature to drew any definite conclusion from the present study.

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