NITROSAMINE CONTAMINATION OF SOME NIGERIAN INDIGENOUS BEVERAGES

A THESIS

PRESENTED BY

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D E D I C A T I O N

THIS THESIS IS DEDICATED TO MY MOTHER ISABELIA;
MY NIECE "LITTLE" FUNLOLA; AND TO THE WARM AFFECTION
OF MY CHARMING FUNLAYO.

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ABSTRACT

The possible role of carcinogenic nitrosamines in the aetiology of human cancer has been discussed by experts (O. Bassir, Pers. Comm.; Eisenbrand et al, 1969; Lijinsky and Epstein, 1970).

As a result of this apprehension an assessment of the level of contamination of Nigeria's local alcoholic beverages with nitrosamines was carried out. Using thin-layer chromatographic and colorimetric techniques dimethyl-nitrosamine and diethylnitrosamine were found to be present in Palm Wine, Burukutu, Pito, Oti Agbagba and Ogogoro in amounts ranging from 20 - 100 ug/litre.

Although these amounts are small, histopathological evidence is presented to show that the two nitrosamines found in the alcoholic beverages are potent liver carcinogens exhibiting a clear - dose response relationship.

Evidence is also presented to show that some of the early biochemical changes induced by dimethylnitrosamine in the course of liver damage include an impairment of the bile pigment metabolism, inhibition of protein synthesis, elevation of blood sugar and increased activity of Alkaline Phosphatase and serum glutamic oxalacetate transaminase in the blood.

The toxicity of dimethylnitrosamine is shown to be repressed by diets severely deficient in protein.

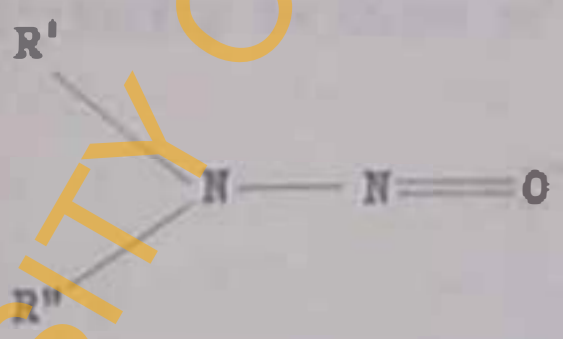
CHAPTER ONE

INTRODUCTION

1. Chemistry of the Nitrosamines.

The autopsy report of cirrhosis of the liver in three men working in a large industrial undertaking has brought into light a new group of chemical carcinogens under the group name of nitrosamines (Magee and Barnes, 1956). Many workers now accept nitrosamines as one of the most formidable and versatile groups of carcinogens yet discovered (Druckrey et al. 1965; Magee and Barnes, 1970), for they have proved effective in all animal species in which tests have been reported.

Nitrosamines can be represented by the general formula



They have in common the nitroso group and an alkyl group.

R' = $CH_3(CH_2)n$; (n = 0-4).

R" can be an alkyl,

$CH_3(CH_2)n$; (n = 0 - 4)

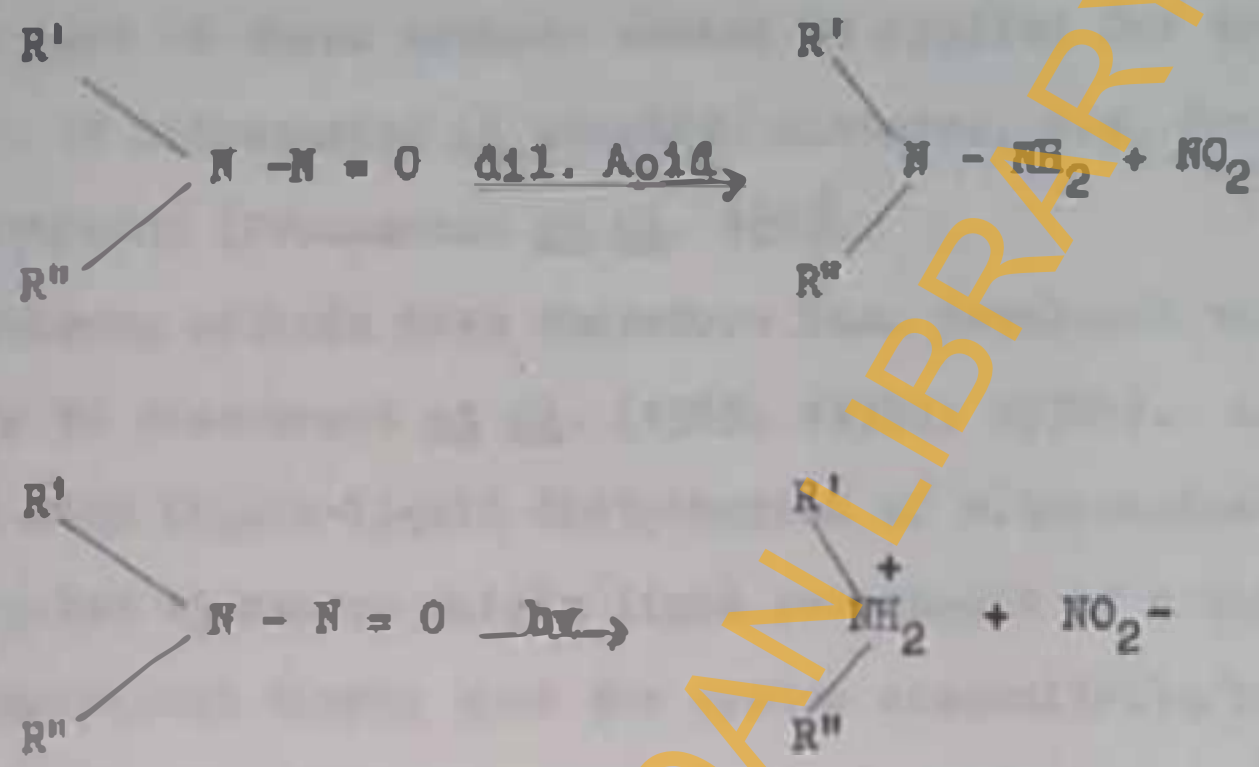
$COOC_2H_5$; $CONH_2$; or certain other groups.

The Nitrosamines are usually prepared from the respective alkylamine compounds by the action of nitrous acid (Sutton and Heath, 1956; Heath and Mattocks, 1961; Preussmann, 1962), although other methods can be used, (Walter et al. 1972).

$$R_2NH + HNO_2 \longrightarrow R_2N{-}N{=}O + H_2O.$$

Quite recently it has been shown that under a well defined pH condition nitrite and secondary amines in plants could react to form the corresponding nitrosamine (Sander, 1969). Also bacteria have been shown to nitrosate secondary amines, even under conditions that would not permit a spontaneous reaction between the two compounds, to form nitrosamines (Sander, 1968).

The nitrosamines are represented by solid and yellow oily substances which vary in their solubility. Some are miscible with water in all proportions, while others are only slightly soluble in water. The nitroso group is lost under acid conditions with reversion to the secondary amine and this group also confers the characteristic sensitivity to light of these compounds.



Nitrosamines can be roughly divided into two groups, one exemplified by N - methyl - N - nitrosourethane, which is unstable to alkali and which interacts at neutral pH with sulphydryl groups (Schoental, 1961) and the other exemplified by dimethylnitrosamine and its homologues, which are stable to alkali and do not react with sulphydryl groups.

The analyses of nitrosamines have been achieved via polorographic (Heath, 1962; Lyndersen and Nagy, 1967), iodometric (Gal et al. 1968) colorimetric (Griess, 1879; Daiber and Preussmann, 1964. Mohler and Mayrhofer 1968), infrared (Mohler and Mayrhofer 1968), fluorescent (Mohler and Mayrhofer, 1969), spectroscopic as well as acid-base and decomposition methods, (Asworth, 1964).

However most of these methods cannot be applied for trace analysis of nitrosamine in complex mixtures, e.g. food or plant extracts (Preussmann et al. 1967).

Clean-up methods have therefore been developed very recently by Eisenbrand et al. (1969, 1970a; 1970b). As a first step liquid-liquid distribution of nitrosamines was investigated to remove mainly lipid components of a mixture. Their experiment showed that the system acetonitrile/n-heptane is favourable for this purpose. In their second experiment Eisenbrand et al. (1970a) investigated the recovery of nitrosamines by steam distillation at neutral, alkaline and acid pH under reduced pressure and atmospheric pressure. Since many foodstuffs of animal or plant origin contain nitrite or nitrate as well as amino compounds they suggested distillation from an alkaline medium first to avoid the formation of N-nitrosamine artifacts at low pH. In the last part of their experiment thin-layer chromatography of nitrosamines was investigated as part of a clean-up step before quantitative estimation using appropriate colorimetric procedure.

11. Nitrosamines in Man's Environment.

Despite their being well established carcinogens very little information is yet available on the occurrence of nitrosamines in the environment. The question whether any human cancer can be attributed to their presence in the environment either naturally or as a result of unsuspected chemical reaction has prompted the quest for them in some food items.

A most remarkable example of unsuspected formation of nitrosamines in the environment, in this way was first indicated in Norway. During the years 1961 and 1962 there were in Norway outbreaks of toxic hepatosis in ruminants. Kopang et al. (1964) indicated a connexion between the disease and the feeding of a meal made from herring preserved with nitrite. This connexion was confirmed by Sakshang et al. (1965) who detected dimethylnitrosamine in the herring meal samples known to be toxic.

The high incidence of oesophageal cancer in Bantu people in localised areas of the Transkei, since 1940, has been related to signs of molybdenum deficiency in the leaves of their plants which result in accumulation of nitrate in these plants. These and secondary amines present in them might react to form nitrosamines (Druckrey et al. 1962).

The first positive result of the above hypothesis has been obtained with the detection of diethylnitrosamine in the fruit of solanaceous bush (Solanum incanum) by Dubeise (1966).

The question of the occurrence of nitrosamines in tobacco and tobacco smoke has been receiving increasing attention primarily due to the assumed existence of a causal relationship between cigarette smoking and lung cancer, incidence. Although Heuroth et al. (1965) claimed that nitrosamines in tobacco smoke are formed in a time dependent on chemical reaction occurring in the vapour phase and after the combustion zone, Druckrey (1964) asserted that the possibility cannot be entirely eliminated that nitrosamines in tobacco smoke originate at least in part from the the material of the tobacco plant.

Sorkestein and Burton, (1966), described evidence for the presence of nitrosamines in tobacco smoke condensate and detected three nitrosamines in the condensate.

Sorkestein and Smit, (1967), further described evidence for the occurrence of N-nitrosamines in tobacco and confirmed the existence of N-nitrosopiperidine.

The presence of nitrosamines in tobacco smoke has also been more recently studied by Johnsen et al. (1968), ; and Rhodes and Donald (1972).

Examination of neutral smoke condensates utilizing a highly selective gas chromatographic system revealed no detectable nitrosamine peaks.

Recent studies have shown that home-cure Malawi gin contains nitrosamines (Waldman et al. 1968) and the presence of trace amounts of nitrosamine in white flour has been claimed, (Marquardt, 1966); confirmed (Keller, 1967); and denied (Thevlis, 1967).

Helene Herrmann (1961) isolated and identified 4-methyl-nitrosaminebenzaldehyde as a metabolic product from a culture of Gilitocybe subvelaris which is an edible mushroom.

The diabetogenic antibiotic, streptozoin, isolated from Streptomyces arbuscensis, has been shown to be a nitrosamide capable of inducing kidney tumour in rats (Argus and Pendale, 1967).

Analysis of various food items, cheese, meat, fish and bacon for nitrosamine using clean up methods and mass spectrum have also been very recently carried by Crosby et al. (1972).

iii. Metabolism of Nitrosamines.

(a) Nature of the biologically active intermediate.

Several workers have shown that nitrosamines themselves are not toxic but that the active principle is a metabolite.

Discussions have been suggested to suit the theory of carcinogenesis by alkylation (Lijinski, 1961; Magee and Schoental, 1964).

The cytotoxic action of biological alkylating agents particularly the bifunctional compounds, is well known (Lawley and Brookes, 1965), and it seems possible that the acute cell-damaging action of the nitroso compounds may be attributed to alkylation. This suggestion as it relates to the acute hepatotoxic action of the nitrosamine has received considerable support from the work of Heath (1962). Alkylation may also explain the mutagenic and teratogenic actions of the nitroso compounds, since several biological alkylating agents are potent in both these actions (Loveless, 1966; Fave, A. 1964). The position with regard to mutagenesis, however, is far from being clear, and some evidence has been interpreted as indicating that the nitrosamines are mutagenic by mechanisms other than alkylation.

A reasonable objection to alkylation as the necessary and sufficient mechanism of carcinogenesis by the nitroso compounds is that many of the well known biological alkylating agents cannot be regarded as powerful carcinogens (Brookes and Lawley, 1964).

From the fore-going discussion it is apparent that alkylation is far from being established as the critical intracellular reaction responsible for the biological activity of the nitroso compounds.

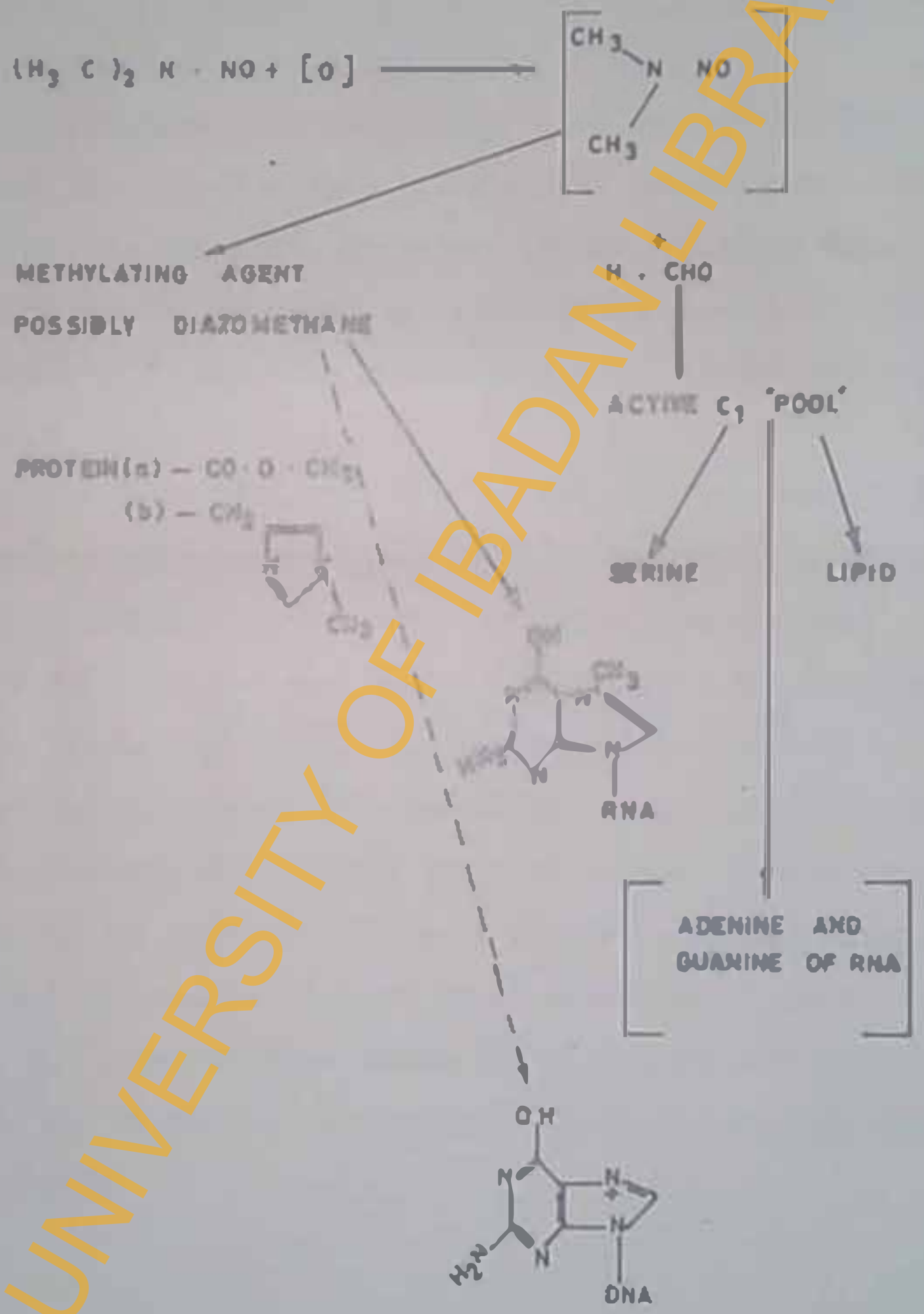
Nevertheless, there seems sufficient evidence from a variety of sources to justify the retention of alkylation as a working hypothesis to explain the activity of these compounds.

(b) Metabolic Pathways of Dimethylnitrosamine.

Dimethylnitrosamine is rapidly metabolised in rats but metabolism is faster in the mouse (Magee and Schoental, 1954). The compound is uniformly distributed in the body soon after injection and metabolism appears to occur mainly in the liver (Magee, 1956). Experiments with (^{14}C) dimethylnitrosamine showed a large fraction of the dose of radioactivity is expired as $^{14}CO_2$ during the first twenty-four hours after injection (Dutton and Heath, 1956). In animals given (^{35}S) dimethylnitrosamine (Heath and Dutton, 1958), subcellular fractions of liver, liver protein, nucleic acids and lipids, and acid-soluble fractions were evenly and lightly labelled. In the urine, free bases and urea nitrogen were heavily labelled. Tests of urine and acid soluble fractions of liver for hydrazine, hydrazide, methylamine, nitrite, hydroxilamine, hydroxamic acids, amides were negative except for traces of methylamine in liver and more in urine, some of which was derived from dimethylnitrosamine.

The metabolism of dimethylnitrosamine was studied by Magee and Vanderkar, (1958) using tissue slices and homogenates. Preparations from liver destroyed dimethylnitrosamine in the presence of oxygen but the other tissues tested were inactive with the exception of kidney slices; these showed barely detectable activity. The ability to destroy dimethylnitrosamine was found in the microsomal plus cell-sap fraction and there was a requirement for phosphopyridine nucleotide. The metabolism of dimethylnitrosamine and diethylnitrosamine was studied in greater detail by Emmelot and his colleagues, (1960). Microsomal preparation of rat liver produced formaldehyde when incubated with dimethylnitrosamine (Brouwers and Emmelot, 1960), and a similar enzyme catalysing the oxidative N-dealkylation of dimethylnitrosamine, with the formation of acetaldehyde, was reported by Mierahi and Emmelot, (1962). Pre-treatment with cysteine had a protective action against dimethylnitrosamine but not against diethylnitrosamine (Emmelot and Mierahi, 1961). Heath, (1962) studied the metabolism in female rats of dimethyl, diethyl, n-butyl-methyl and tert-butylmethylnitrosamine, using (^{14}C) - labelled and unlabelled compounds. From observations on the rates of expiration of labelled CO_2 and on the mutual inhibition of oxidation by different compounds, he concluded that the nitrosamines are not

SOME POSTULATED METABOLIC PATHWAYS OF DIMETHYLNITROSAMINE



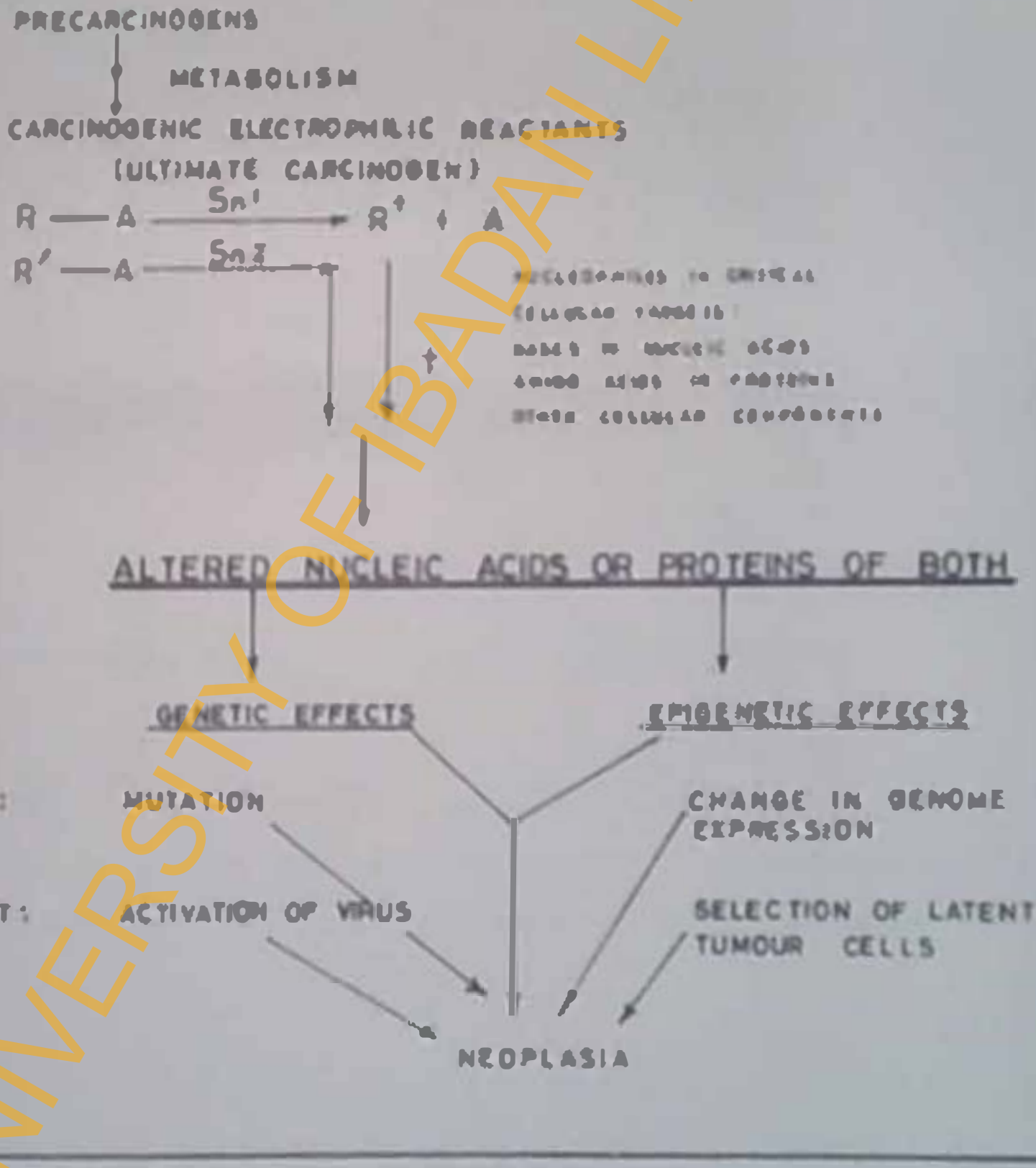
themselves toxic but that the toxic agents must be products of oxidation.

iv. Carcinogenesis by Nitrosamines

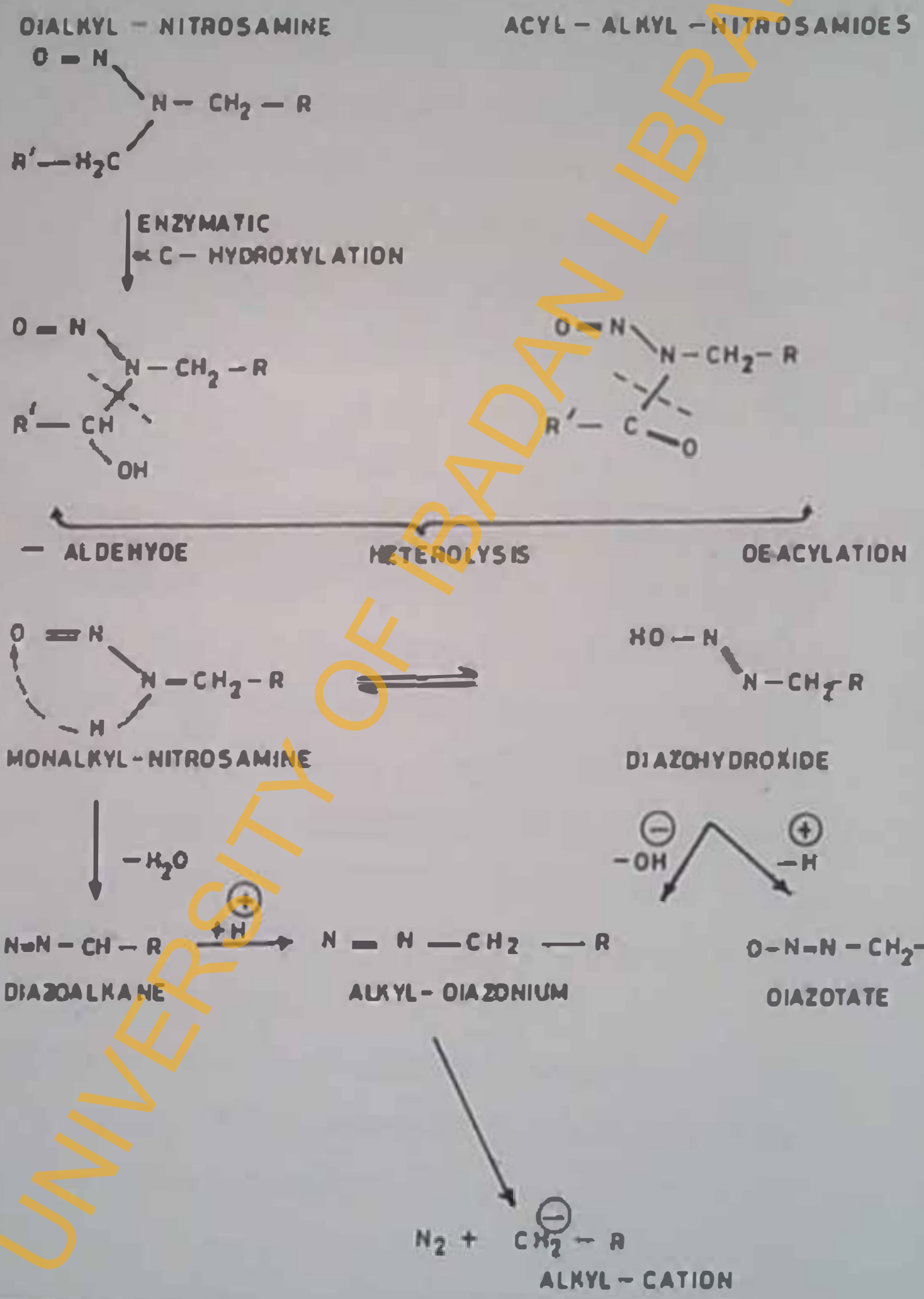
Nitrosamines have been shown to be really "pre-carcinogens" that are metabolished into the ultimate carcinogenic forms which are electrophilic reactants (Price and co-workers, 1969). Evidence for this conversion to alkylating agents has been discussed by Preussmann, (1969), Druckrey, (1969) and Magee, (1970).

The most critical cellular nucleophilic targets of carcinogenic nitrosamines appear to be the bases of nucleic acids and certain amino acids. The resultant altered DNA and RNA, and proteins would thus initiate the carcinogenic process (Druckrey, 1969). The subsequent events that would lead to the characteristic neoplastic state remain essentially uncharacterised. At present the four general mechanisms, two direct and two indirect listed in figure 4, appear to be the principal hypothesis that are under experimental tests. Any of these mechanism or combinations thereof may account for the way in which a carcinogenic agent operates (Miller and Miller, 1969).

A UNIFYING CONCEPT OF CHEMICAL CARCINOGENESIS
(Mills and Mills, 1969)



SCHEME FOR THE REACTIONS OF DIALKYLNITROSAMINES AND ACYLALKYLNITROSAMIDES EVENTUALLY LEADING TO ALKYLATING INTERMEDIATES. (Druckrey et al 1969)



(a) Dialkylnitrosamines.

Diethylnitrosamine has proved to be a very potent liver carcinogen (Schoental et al. 1960). Extensive quantitative studies (Druckrey et al. 1963) revealed clear dose-response relationship down to a daily dose of 0.75 mg/kg. body weight. Even in guinea pigs, formerly considered resistant to carcinogens, hepatocellular carcinoma have been produced with the same regularity as in rats (Druckrey and Steinhoff, 1962; Argus and Hoch-Ligeti, 1963).

All other symmetrical dialkylnitrosamine up to the diamyl compound, after continuous oral administration to rats have been shown to produce liver cancer with decreasing potency (Druckrey, 1967). The only exception was di-n-butylnitrosamine, which produced carcinomas of the urinary bladder. Biochemical studies revealed that soluble metabolites are excreted in the urine in high concentration, probably coupled hydroxylation products (Drunkrey et al. 1968).

Since hydroxylation occurred mainly in the liver Druckrey et al. (1969) attempted to avoid the liver by sub-cutaneous injections.

With dialkylnitrosamines, not one single local sarcoma was observed at the site of injections, during several experiments. Sub-cutaneous injections of dibutylnitrosamine produced bladder cancer in all treated rats. With the diamyl compound however, bladder cancer was never observed, but carcinomas of the lungs were seen (Druckrey and Preussmann, 1962).

The non-symmetric dialkylnitrosamines induced carcinomas of the oesophagus with high regularity (Druckrey et al., 1963). Similar results were reported by Weisburger and his colleagues (1966). Druckrey et al. (1969) showed in their experiments that the methylalkyl compounds were the most potent carcinogens in this group. The effect was completely independent of the route of administration.

With cyclic nitrosamines, again organotropic effects were observed. After oral administration N-nitrosopyrrolidine produced only liver cancer. The next higher homologues, nitrosopiperidine, as well as N. N' - dinitrosopiperidine, however, led to carcinomas of the oesophagus. After sub-cutaneous injections in rats both compounds produced carcinomas of the ethmoturbinalia and neuroepithelioma of the olfactory nerve (Druckrey et al.

— — 1964). The same tumours

have been observed by Harrold (1964) in golden hamsters after sub-cultaneous injections of diethylnitrosamine.

(b) Acylalkylnitrosamides.

After sub-cutaneous injections in rats, local sarcomas have been observed with several acylalkyl-nitrosamides (Druckrey et al. 1966). Additionally, the very unstable phenylnitrosourea was also active (Preus mann et al. 1968). Methylnitrosourea painted on the skin of mice, rats or hamsters proved to be a very strong topical carcinogen in experiments of Graffi et al., (1966, 1967). When given by oral route, several acylalkylnitrosamides produced carcinomas of the fore stomach in rats (Druckrey et al., 1961 and 1967). In guinea pigs, since the whole stomach is glandular, adenocarcinomas of the stomach and pancreas have been produced in high yield with methylnitrosourea and methylnitrosourethane by Druckrey and his colleagues (1968).

In order to avoid local carcinogenic effects, experiments were performed with intra-venous injections in rats. They revealed striking organotropic effects. Methylnitrosourethane, even at a dosage of 1 mg/kg. body weight once every two weeks, regularly produced carcinomas of the lungs (Druckrey et al., 1967). Methylnitrosourea, by contrast, proved to be a carcinogen highly specific to the brain and in some cases also to

the spinal cord (Druckrey et al., 1964; 1965).

This result was confirmed by Thomas and co-workers (1967), and by Stroobandt and Bruncher (1968) and in rabbits by Janish and Schreiber (1967).

Both compounds, methylnitrosoures and methylnitrourethane, after deacylation yield the same proximal carcinogen namely diazomethane and methyldiazonium (Druckrey et al., 1969). Therefore, the respective organotropic effects are to be attributed to the whole as the "transport form".

Neurotropic effects have also been found with ethyl- dimethyl-, and trimethylnitrosoures, producing malignant neuroepithelial tumours of the brain, the spinal cord, and the peripheral nervous system (Ivankovic et al. 1965).

v. Biochemical Effects on the Cell

(a) Methylation of Nucleic Acids

Dimethylnitrosamine has been shown to methylate liver proteins and nucleic acids in the intact rat and in rat and human liver slices using (^{14}C) dimethylnitrosamine (Magee and Farber, 1962; Magee and Hultin 1962). A large part of the radioactivity incorporated into proteins was present as methylated histidines, and most of the activity in the nucleic acids was 7-methylguanine,

this is consistent with methylation in vivo (Brookes and Lawley, 1964). Labelling of the ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) of the kidneys also occurred and was shown to be largely due to 7-methylguanine, but quantitatively incorporation was considerably lower than in the liver (Craddock and Magee, 1963). More recent work by Lawley and Brookes (1968) has demonstrated alkylation of nucleic acids on adenine and cytosine moieties. Methylation of RNA and DNA in rat liver and kidney reached maximum levels about six hours following injection of necrotizing and non-necrotizing doses, after which there was a sharp fall. These results suggested that very little if any of the incorporated methyl-group could remain for more than a week, but the persistence of minute amounts could not be excluded (Craddock and Magee, 1963). The typical methylation reaction also occured in mouse, hamster and guinea pig liver RNA, and the reaction can be demonstrated with (^{3}H) dimethylnitrosamine as well as with (^{14}C) dimethylnitrosamine. The distribution of methylation as measured by 7-methylguanine in RNA was studied in several organs of Wister rats and BALB/C mice, following injection with labelled dimethylnitrosamine. In both species the liver showed much the highest level of methylation; this represented about 1-2% of RNA

guanines into 7-methylguanine. In the rat the organ showing the next highest level was the kidney, followed by the lungs and then the spleen. The squamous stomach showed much less, while the pancreas and the small intestine showed almost no detectable methylation. In the mouse, the pattern of methylation was similar except that the lung RNA had the highest level of methylation after the liver and the kidney very much less. (Lee, Lijinsky and Magee, 1964). Ethylation of liver RNA occurred in rats treated with (^{14}C) diethylnitrosamine, and maximal incorporation of label from this compound occurred 24 hours after injection. Methylation of rat liver RNA followed treatment with n-butyl-(^{14}C)-methylnitrosamine but not with tert-butyl-(^{14}C)-methylamine and not with ethylnitrosamine (Magee and Lee, 1963, 1964).

(b) Inhibition of Protein Synthesis.

The inhibitory effect of dimethylnitrosamine on the incorporation of amino acids into proteins of rats liver in vivo was reported by Magee (1958). Incorporation of ^{14}C-amino acids into liver proteins was reduced by about 50% by three hours after a necrotising dose of dimethylnitrosamine, the extent of the reduction being the same in the different subcellular fractions of the liver.

Incorporation of amino acids into kidney and spleen proteins was unimpaired. The action of dimethylnitrosamine on protein synthesis was analysed further by Kultin et al. (1960) using in vitro technique. Incorporation of valine - ^{14}C into proteins of rat liver slices in vitro was inhibited by pre-incubation of the slices with dimethylnitrosamine at concentrations of about 0.1mM but inhibition was observed with kidney slices. Incorporation of adenine - ^{14}C into RNA of rat liver slices in vitro was also inhibited on pre-incubation with diethylnitrosamine. Brouwers and Emmelot (1960) also observed marked impairment of the amino acid incorporation system of the combined microsomal-soluble fraction of liver from rats treated with dimethylnitrosamine and they found further, no significant impairment of the reaction between leucine - ^{14}C. and soluble RNA (SRNA) in the presence of the pH-5 enzyme system of the supernatant fraction. This indicated that the impairment of protein synthesis involved transfer and the incorporation of the amino acid from the SRNA to the microsomal protein rather than at an earlier stage. Brouwers and Emmelot (1960) concluded that the inhibitory effects of dimethylnitrosamine on hepatic protein synthesis is rather specific since respiration,

glycolysis and a number of other enzymatic activities were not impaired under their experimental conditions. Emmelot and Nierhahi (1961) reported that subcutaneous injection of cysteine prolonged the livers of rats treated with dimethylnitrosamine and reduced the inhibition of protein synthesis from the liver. The group (Emmelot et al. 1962; Nierhahi and Emmelot, 1962, 1963), showed that cysteine does not protect against the toxicity or the inhibition of amino acid incorporation due to dimethylnitrosamine. Cysteamine exerted a protective effect against inhibition of protein synthesis by both dimethyl- and diethylnitrosamines, but this compound had no effect on the enzymes responsible for the metabolism of either compound.

The mechanism of the inhibition of incorporation of amino acids has been further analysed by Nierhahi and Emmelot, (1964), who showed that there could be loss of messenger RNA (mRNA) from the polyribosomes in the livers of dimethylnitrosamine-treated rats. This would be consistent with the appearance in the electron microscope of detached ribosomes lying in the cytoplasmic matrix and lacking the aggregate structure characteristic of polysomes.

The response of the post-mitochondrial fraction, (12,000g. supernatent), and of ribosomes from a control and dimethylnitrosamine-treated rats to synthetic messenger, polyuridylic acid, was therefore tested. The incorporation of phenylalanine-^{14}C was stimulated to a greater extent in both types of preparation from the treated rats than from the controls, suggesting that available sites for exogenous messenger RNA were saturated at lower concentrations of poly U in the control than in the treated preparations. The incorporation pattern of the ribosomes from the dimethylnitrosamine treated rats resembled that of normal ribosomes after pre-incubation or treatment with ribonuclease, conditions known to convert polyribosomes to smaller aggregates and single ribosomes through the loss of mRNA. Further evidence that mRNA is lost from the treated preparations was obtained by sucrose gradient centrifugation of ribosomal components. The number and size of the ribosomal aggregates were decreased and there was a corresponding increase in the number of smaller aggregates and ribosomal monomers (80S) in the dimethylnitrosamine treated rat liver, as compared with the control.

In their later work Mizrahi and de Vries (1965) observed that a further breakdown of polyribosomes from livers or rats treated five hours previously with dimethylnitrosamine occurred during incubation in experiments on amino acid incorporation into protein. No significant inhibition of incorporation of P^{32} into nuclear or cytoplasmic RNA was found in the livers of treated rats.

Villa-Trevino (1965) observed progressive breakdown of the ribosomal aggregates which was detected one hour after administration of dimethylnitrosamine; the extent of breakdown was proportional to the degree of inhibition of protein synthesis. This breakdown of microsomal aggregates was not accompanied or preceded by inhibition of incorporation of orotate into nuclear RNA, therefore two hours after administration of dimethylnitrosamine, when in vivo incorporation of leucine was decreased by 48%, no significant difference was observed in orotate incorporation. The greater stimulation of poly U of incorporation. of phenylalanine - ^{14}C by ribosomes from dimethylnitrosamine-treated rat livers was confirmed by Mager *et al.* (1965a), who suggested that this reflects an increased availability of the ribosomal surface for interaction with exogenously supplied coding agent (i.e. poly U).

They presumed that the destruction of messenger RNA bound to ribosomes and consequent unmasking of normally screened combining sites can account both for the excessive affinity of the system for poly U and the concomitant decline of its intrinsic amino acid incorporation activity. These findings (Mizrahi and Emmelot, 1964, Mizrahi and de Vries, 1965; Villa-Trevino, 1965; Mager et al. 1965), led to the suggestion that there may be accelerated breakdown of messenger RNA in the livers of treated animals. This might be a result of alkylation of messenger RNA, a possibility that received support from the demonstration of Villa-Trevino (1965) that purified liver nuclear RNA is methylated in the dimethylnitrosamine treated rat.

(c) Effect on Liver Glycogen.

Six hours after a necrotising dose of dimethyl-nitrosamine to rats Magee (1958) observed a reduction in the level of the liver RNA but not of DNA or total phospholipid phosphorus. As the liver lesions developed there was an increase in stainable and chemically determined lipid and marked loss of glycogen. Three hours after a necrotising dose, however, the level of liver glycogen was only slightly reduced and the difference from the control levels was not statistically

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significant (Hultin et al, 1960). Emmelot and Benedetti (1960, 1961) reported progressive loss of glycogen from livers of rats treated with dimethylnitrosamine. The loss of liver glycogen could be very largely prevented by prior treatment of the animals with cysteine (Emmelot and Mizrahi, 1961). On the other hand, cysteine gave no protection against the glycogenolysis induced by diethylnitrosamine (Mizrahi and Emmelot, 1962) but cysteamine treatment greatly reduced the loss of liver glycogen; this effect was counteracted by the nitrosamines, suggesting a mutual elimination of sulphydryl compounds and nitrosamine metabolite (Mizrahi and Emmelot, 1963).

(d) Fatty Liver.

The mechanism of production of fatty liver by dimethylnitrosamine in the rat was investigated by Rees and Shotlander (1963). They found no significant change in total lipid, triglyceride, cholesterol, or phospholipid in the livers of rats five hours after dimethylnitrosamine (100mg/kg. body weight) but at 22 hours the total lipid was about twice the level of the controls. The increase was due to a 5-fold rise in triglyceride of cholesterol or phospholipid. The authors concluded that inhibition of protein synthesis by dimethylnitrosamine preceded inhibition of secretion of triglyceride from the

liver by several hours but that the accumulation of fat is not solely due to reduction in lipoprotein synthesis.

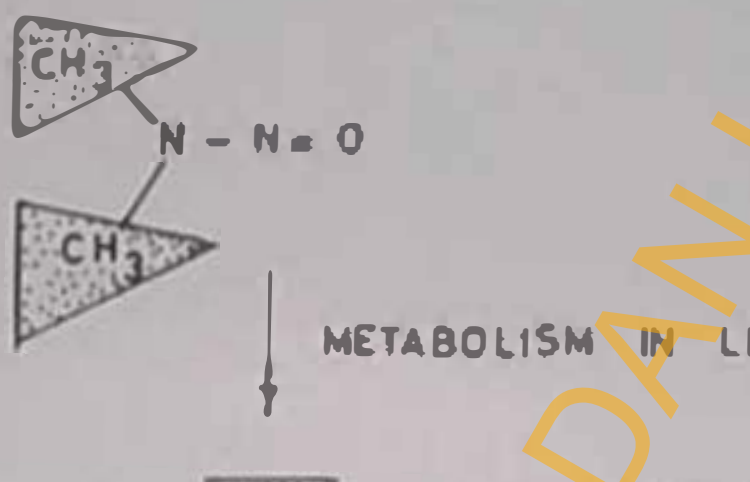
(e) Enzyme Activity.

Rose et al. (1962) studied the leakage of liver enzymes into the serum of rats poisoned with diethylnitrosamine 100 mg/kg. body weight. Serum levels of isocitric dehydrogenase were raised at six hours and continued to rise up to 24 hours. Glutamic dehydrogenase showed little change until 24 hours, when it also rose. These changes in serum enzyme content were reflected by losses of isocitric and malic dehydrogenase activity of the liver homogenate and they were mainly due to losses from the extramitochondrial cytoplasmic fractions with little mitochondrial loss. The increase in liver lipid induced by dimethylnitrosamine was confirmed. Neither previous adrenalectomy nor treatment with the antihistamine drug phenergan prevented the leakage of the enzymes into the serum or the development of liver necrosis. Release of lysosomal enzymes was not observed in the early prenecrotic stage of liver injury by dimethylnitrosamine and it was concluded that the lysomes probably play no role in the early development of liver necrosis but they may be involved in the later scavenging processes,(Slatter and Greenbaum, 1965).

Heise and Gorlich (1964) studied changes in metabolic rate and activity of the glycolytic enzymes in rat liver during carcinogenesis by feeding diethylnitrosamine for 120 days. Enzyme activities were expressed per liver weight and milligrams of protein in the homogenate. The liver weight increased during feeding but there was little damage in respiration of slices of homogenates of liver prepared at intervals of two weeks. Aerobic glycolysis increased in liver slices after the rats had received about 600 mg. diethylnitrosamine and then remained on a steady level, but in homogenates there was little change during the period. Anaerobic glycolysis only became definitely raised in slices toward the end of the feeding period while the homogenates showed an early fall in level followed by a slight rise during the last week.

The induction of enzymes in the liver during carcinogenesis by feeding N-nitrosomorpholine was studied by Kroger and Greuer (1965), who also made a parallel histological study. The animals received 10 mg. per kg. body weight of the carcinogen daily in the drinking water. Substrate induction of tyrosine-2-oxogluterate transaminase was reduced after about twenty days on the carcinogenic regime and that of tryptophan oxygenase after 70 days.

MODIFICATION OF DEOXYRIBONUCLEIC ACID BY METABOLITES OF THE CARCINOGEN DIMETHYLNITRO-SAMINE



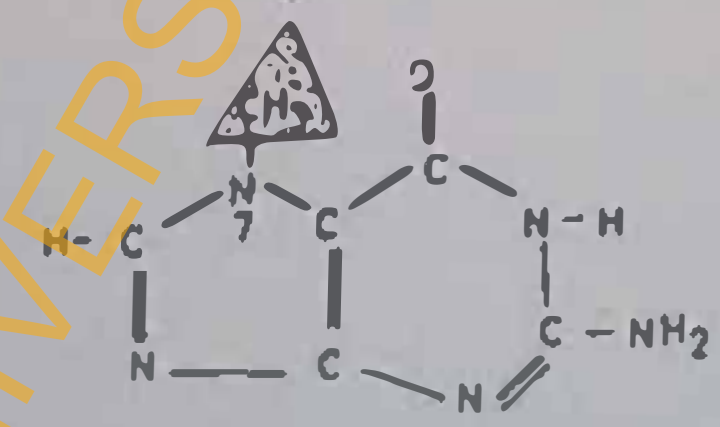
METABOLISM IN LIVER

HIGHLY REACTIVE INTERMEDIATE

REACTS WITH DNA IN THE CELL

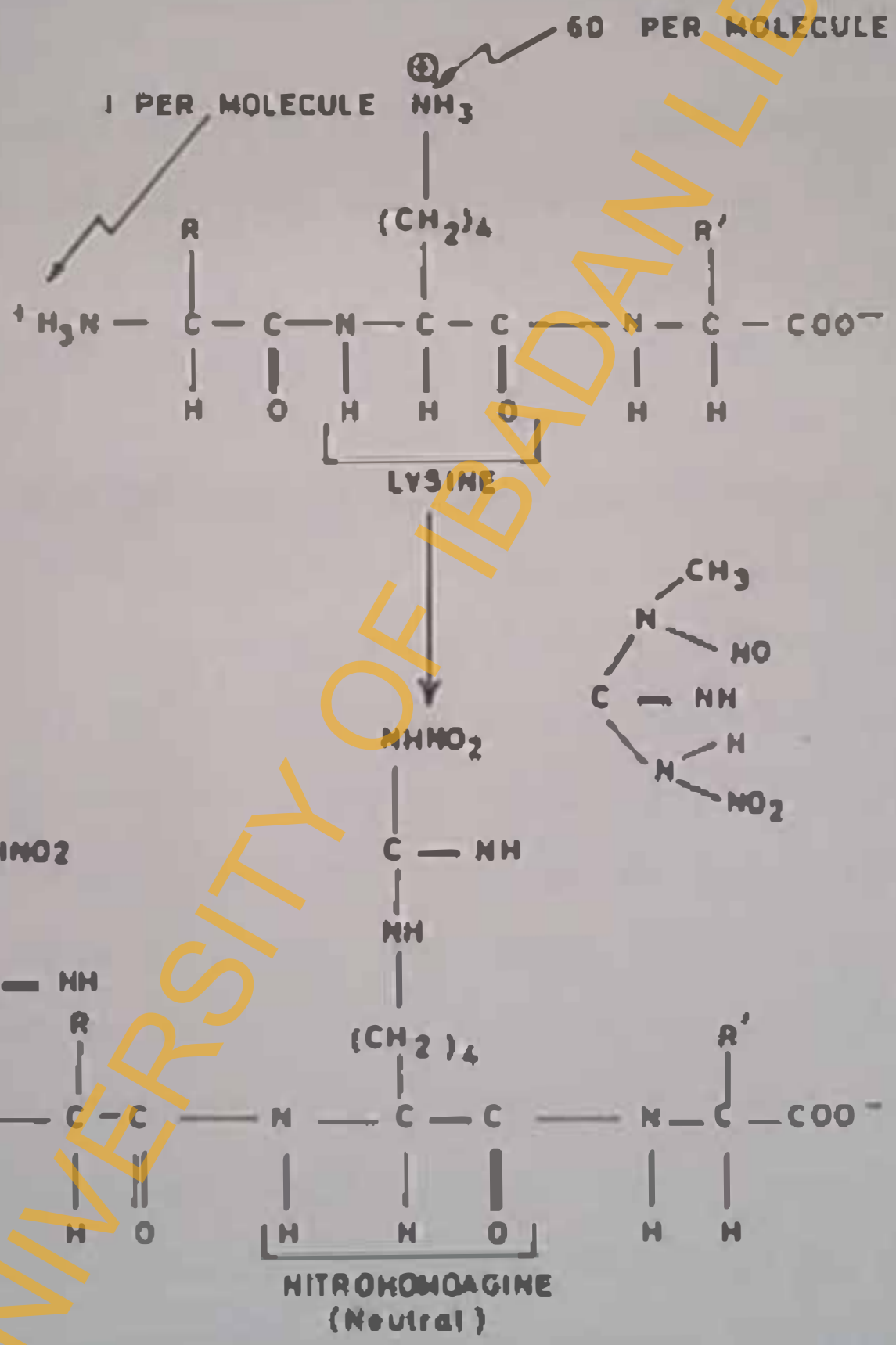
MODIFIED DNA

CHEMICAL BREAKDOWN OF DNA



MODIFIED GUANINE
(7 - METHYLGUANINE)
FROM DNA

REACTION OF N-METHYL-N'-NITRO-N-NITROSO-GUANIDINE WITH A LYSINE RESIDUE OF A PROTEIN MOLECULE (Lawley, 1965)



vi. Pathological Effects

On the whole there is remarkably little published work on the acute lesions produced by the carcinogenic nitrosamines, but some detailed studies of the liver in animals given dimethyl - and diethylnitrosamine have been made.

In rats after 20 mg/kg body weight dimethylnitrosamine a pallor of the cells in the centrilobular and mid-zone region of the liver developed and progressed so that by 18 hours, the cytoplasm was amorphous and vacuolated and the nuclei was pale and irregular. Necrosis of these cells was completed by 24 hours and confluent areas were frequently haemorrhagic. The haemorrhage was more pronounced by 48 hours and polymorph infiltration was prominent. By 72 hours the repair processes were in full swing and the necrotic areas contracting, and within 3 weeks repair and restoration of the liver tissue was almost complete (Magee and Barnes, 1956). The acute lesion in the liver of the dog, mouse, and rabbit showed a similar pattern. A veno-occlusive lesion involving the hepatic veins might also be seen in rats 10 days after an approximate LD50 dose (Nolan et al. 1965).

Under electron microscope changes could be seen in the endoplasmic reticulum of some liver cells within

3 hours, after a dose of 50 mg/kg. body weight dimethylnitrosamine intravenously. The endoplasmic reticulum was swollen and ribonucleoprotein particles were detached from the membranes. The nuclei, mitochondria, microbodies, and golgi apparatus were unaffected. These changes progressed markedly within the next 13 hours (Emmelot and Benedetti, 1960, 1961). These changes in endoplasmic reticulum induced by dimethylnitrosamine have been confirmed by Mukherjee et al., (1963), who correlated the electron microscopic changes with stimulation and depression of amino acid incorporation produced by different doses of dimethyl-nitrosamine. De Mann (1964) was able to show that cortisone could protect the endoplasmic reticulum to some extent from the damage produced by dimethylnitrosamine.

vii. Mutagenesis.

Many carcinogenic nitroso compounds have been shown to be mutagenic as well. The nitrosamides (e.g. nitrosourethane) are active in Vicia faba (Kihlmann, 1960), Ophiostoma, (Zetterberg, 1960), and Drosophilla (Rapoport, 1948; Pasternak, 1963). Methylnitrosourethane proved more active in Saccharomyces than ethylnitro-sourethane (Marquardt et al. 1963). Dimethyl - and Diethylnitrosamine are mutagenic in Drosophila,

(Pasternak 1962; 1963), but inactive in bacteria (Geissler, 1962) and in Neurospora (Marquardt, Schwaier and Zimmermann, 1963). Diazomethane itself is also known to be mutagenic (Rapoport, 1949).

The mechanism of mutagenesis by nitroso compounds has been discussed by several authors. Pasternak (1964) suggested that an identical molecular mechanism may account for both the carcinogenic and mutagenic activity of the nitroso compounds. Marquardt et al. (1964) also concluded that the mutation induced in Saccharomyces cerevisiae by nitrosamides is likely to be due to methylation resulting in the formation of 7-methylguanine. Rahn et al. (1966) compared the mutagenic activity of diethylnitrosamine and nitrosourea in Drosophilla. Although there was a broad similarity in the mutagenic mode of action of the two compounds, more detailed analysis of their results revealed differences which were difficult to explain by simple ethylation of genetic material. They suggested that either the compounds themselves or products of their metabolism, other than diazomethane, must be playing a role in the initiation or subsequent stabilisation of certain mutations. They pointed out that the metabolic production of aldehyde and reduction

Products such as the corresponding hydrazines or hydroxylamines, all of which are known to be mutagenic in some systems, may be important and should not be excluded.

viii. Teratogenesis.

N-nitrosomethylurea (Kreybig, 1965) and N-nitrosoethylurea (Druckrey et al, 1966) are potent teratogens, and both can induce tumours in the progeny of rats treated during pregnancy. The high incidence of tumours of the central and peripheral nervous system induced by N-nitrosoethylurea was remarkable. By contrast, dimethylnitrosamine was reported not to have teratogenic action in the rat but to induce renal and other tumours in the progeny of pregnant females treated during the third week of pregnancy (Alexander, 1968). A possible explanation of these results was advanced by Magee (1969). The developing embryo, at the stage when it is most susceptible to teratogenesis, lacks the metabolic capacity to decompose the nitrosamine, but at a later stage metabolism of the nitrosamine may occur to yield sufficient carcinogenic products to induce tumours. The new born rat is capable of metabolising dimethyl-nitrosamine during the first twenty-four hours after birth (Lee and Spencer, 1964; Terracini and Magee, 1964).

ix. Inhibitors of Nitrosamine toxicity.

Aminoacetonitrile has been reported to prevent the inhibition of protein synthesis induced by dimethylnitrosamine (Fuime, 1964). This lathyrogenic agent partially protects the liver against the necrosis induced by the caroinogen (Fuime, 1962). Injection of aminoacetonitrile daily for two days before and on the same day as dimethylnitrosamine abolished the inhibitory effect on protein synthesis. This antedotal effect on protein synthesis was further studied by Moger et al. (1965) who found that the protective action could be obtained when the aminoacetonitrile was injected 12-20 hours before the dimethylnitrosamine but not when injected two hours before or simultaneously with the nitrosamine. Addition of aminoacetonitrile to the in vitro preparations did not reverse the inhibition. The behaviour of the in vitro system was determined by the origin of the microsomes and was independent of the source of the supernatant fraction indicating that the site of both the intracellular injury and the protective effect are in the microsomal particles thus confirming the observations of Mallia et al, (1960). Pre-treatment with aminoacetonitrile also reduced the response of the microsomes from the nitrosamine liver to the addition of Poly U.

The authors interpreted their results to imply a "stabilising" effect of aminoacetonitrile on the ribosomal particle. Fuime and Raffia (1965) have reported that aminoacetonitrile inhibits metabolism of dimethylnitrosamine.

It has been reported that a protein-free diet protect rats against acute dimethylnitrosamine poisoning (Mclean and Verschuurens, 1969). The LD_{50} was almost doubled and indices of the liver damage show the same trend. Dimethylnitrosamine toxicity has also been shown not to be enhanced by starvation and there is evidence of light reduction in the toxic effects in starved animals (Mclean and Verschuurens, 1969). Protein-free diet reduces the rate of dimethylnitrosamine metabolism both in vivo in the whole animal and in vitro in liver slices (Swann and Mclean, 1968). The reduction of dimethylnitrosamine toxicity after feeding a protein-free diet might be attributed to this reduced rate of catabolism in the liver. However, the failure of DDT or phenobarbitone to reverse the "no protein" effects (Mclean and Verschuurens, 1969) suggests either that the rate of dimethylnitrosamine metabolism is not affected by these inducers of microsomal hydroxylating enzyme activity or else that liver damage does not depend on the rate of dimethylnitrosamine metabolism.

The first seems possible though unlikely in view of the finding of Orrenius, et al. (1965) that phenobarbitone injections increase microsomal oxidations using dimethylnitrosamine as substrate. The second and more likely explanation is that neither the rate, (Heath , 1962) nor the amount of dimethylnitrosamine metabolised in the liver is the predominant factor in dimethylnitrosamine liver damage. Dimethylnitrosamine, after conversion to a toxic metabolite such as a carbonium ion must attack cell sites which become accessible, or are protected, depending on the previous diet. The nature of the cell site is not clear. It has not been shown which of the many alterations in the cell produced by feeding a protein-free diet, is the one that has produced a protection against dimethylnitrosamine (Lijinsky et al. 1968).

x. Incidence of Cancer in Nigeria.

It has been considered appropriate at this point to review the cancer situation in Nigeria in an attempt to further justify the need for this research.

Some of the earliest survey of the incidence of cancer in Nigeria was carried out by Smith and Elmes (1934) who reported a large series of cases of malignant diseases. They analysed 500 tumours which had been received and histologically examined at the Medical

Research Institute, in Lagos, over the eight years prior to 1934. Their analysis went no further than a classification into morphological types and according to regions of the body, with a short note on age distribution. They found that primary liver carcinoma had constituted 6.4 percent and tumours of the female genitalia 6.8 percent and tumours of the skin 18.8 percent.

Smith and Elmore also recorded forty melonotic sarcomas, thirty of them being situated on the foot, and ten cases of Kaposi's sarcoma.

These findings are not only valuable indicators of the tumours seen in Southern Nigeria forty years ago, but provided at the same time a preview of the present situation.

No account of the Nigerian situation appeared in literature again until 1947 when Elmes and Baldwin published an analysis of 1000 tumours diagnosed in Lagos. The period covered from 1935-1944, and the analysis was once more based upon biopsy specimens sent in from all over the country, with the addition of a number of local autopsy examinations. This series did not differ materially from the earlier one in terms of the relative frequency of the major types.

Once again, the prominence of primary liver cancer in Nigeria was apparent (8.1% percent of the total and melanoma 6.2% percent. 122 of the tumours (12.2%) had been squamous epitheliomas of the skin and the association of this common tumour with chronic leg ulceration was noted. 100 carcinomas of the female genitalia were diagnosed on the whole (uterus, ovaries, vulva and vagina, and chorionepithelioma).

A more detailed survey was carried out by Edington and Molean (1960-1963) in an attempt to assess the incidence of malignant diseases in Ibadan, the then capital of Western Nigeria. The result of the survey showed high frequency for the incidence of carcinoma of the liver, stomach, cervix and breast; and in the case of liver cancer the incidence was much higher than would be expected for a similar population in American white and non-white males.

In 1964, C. O. Bery, from the Regional pathology laboratory in Kaduna, Northern Nigeria, published a brief summary of the cases of malignant disease diagnosed during the year 1963-1964. There had been 296 malignant tumours in all age groups in this year. The commonest among these was various types of skin tumours (22.9 percent).

Maligmant lymphomas were next in order of frequency (19.8 percent.) and then carcinoma of the breast (5.4 percent). Liver cell carcinoma had not featured largely in this biopsy series, constituting only 2.4 percent.

The commonest form of maligmant disease in the age group 0 - 15 years was Burkitt Tumour.

Oettle (1964) in his report on the incidence of cancer in Africa made mention of the incidence of certain types of cancer in Nigeria. Among these were multicullular lymphoma, oesophageal, Bladder and liver cancer. Incidence of cancer of the penis, he claimed was relatively low.

Edington and Eremon (1965) reported the occurence of cancer of the alimentary tract in Ibadan, Western Nigeria. Oesophageal cancer was found to be rare, the relative frequency of the carcinoma was only 0.4% in 1,920 tumours recorded. The relative frequency of the caroinoma of the stomach in males and females in Ibadan was similar to the incidence expected in U.S. whites and non-white until ages fifty and then is considerably less. No reason could be advanced for this falling off in the incidence of cancer in the older age groups.

Genetic and sociological factors (including lack of medical care for the aged) have been considered but further work is required on this problem before conclusions can be reached.

Odebiyi, T. (1972) in her cancer rate survey in Ibadan patients registered at the University College Hospital showed that an average of 200 cancer patients a year were admitted to the hospital from 1965-1970. She also recorded the prominence of carcinoma of the liver, stomach and oesophagus.

From the various surveys one can conclude that the cancer types of common occurrence in Nigeria are liver cancer, oesophageal cancer, carcinomas of the stomach, skin and bladder.

Several speculations have been made in an attempt to account for the aetiological factors of these cancer types in Nigeria.

A causal relationship has been postulated between smoking habit, (especially the smoking of locally grown tobacco which is also chewed and used as snuff) and lung cancer (Edington and Lesson, 1965).

Drinking has been shown to be more common in oesophageal cancer patients than in control subjects in Ibadan (Edington, 1963).

Herbal remedies are still widely used in Nigeria and a possible relationship between this habit and gastric carcinoma has been speculated (Edington, 1964).

Whilst speculations and correlations of these types could be suggestive of some aetiological factors of the cancer types in Nigeria, there is a dire need for the establishment of the causative agents in the environment.

CHAPTER TWO

MATERIALS

1. The Local Alcoholic Beverages.

Five local alcoholic beverages commonly consumed in Nigeria were investigated. These are Palm Wine, which is the fermented sap of the palm tree (Elaies spp.); Burukutu, a fermentation product of millet (Pennicetum spp.); Pito, a fermented beverage from maize (Zea mais); Oti Agbagba, obtained from the fermentation of overipe plantain (Musa sapientia); and Ogogoro, a distilled spirit from Palm Wine.

ii. Palm Sap

This is the fresh unfermented palm wine (Bassir, 1962) collected under sterile conditions from local palm trees (Elaies guineneis).

iii. Sterile Filter

This is the gallenkamp FD-370 with accessories. It was used to get rid of the bacteria and fungi population in Palm Wine.

iv. Standard Nitrosamine

Six authentic samples of nitrosamines were prepared in our laboratory. These are: Dimethyl-nitrosamine, Diethyl-nitrosamine, Nitrosopiperidine, N-nitrosodibenzylamine, Diphenylnitrosamine and Nitrosomorpholine. They were used

as reference materials in the analysis for nitrosamines in the alcoholic beverages.

v. Quickfit Distillation Rate

These were manufactured by A. Gallenkamp and Co. Limited, London, with the appropriate heating mantles. Various sizes were used for the various distillation experiments.

vi. Thin-film Rotary Evaporator

Gallenkamp, EU-100. This equipment was used for concentrating extracts. It is provided with a high vacuum pump and a thermostirrer as a source of heat.

vii. Thin-layer Chromatographic Equipment

This is the "Shadon" unoplan type. A complete outfit for carrying out thin layer chromatographic experiments, designed for use with glass plates 20 x 20 cm. which can be processed in batches of five.

Accessories:

(a) Spreader
(b) Plate leveller
(c) Applicator.
(d) Plate Rack
(e) All glass spray.

The equipment was used for qualitative and quantitative estimation of nitrosamines present in the alcoholic beverages.

viii. Silica Gel G.

This was manufactured by Merck in Germany. It was the adsorbent of choice for coating glass plates in thin-layer chromatographic experiments.

ix. Oven

This is the Gallenkamp-BS. The equipment is thermostat-controlled and was used for activating thin-layer chromatographic plates.

x. Solvent Systems.

Hexane-Ether-dicholoromethane	4: 3: 2
Hexane-Ether-dichloromethane	5: 7: 10
Acetone-Benzene-Pet. Ether	2:99: 99
Ethyl Acetate - Petroleum Ether	20: 80:
Carbon tetrachloride-dichloromethane	3: 2.

Volume by volume.

These were variously used in the analysis of nitrosamines on thin-layer plates.

xi. Nitrosamine Detectors

(a) Preussmann's Reagent: Diphenylamine - Palladium chloride (a 4: 1 mixture of (1) 1.5% solution of diphenylamine in ethanol and (2) 0.1% palladium chloride in 0.2% saline.

(b) Griess Reagent: A 1:1 mixture of (a) 1% sulphanilic acid in 30% Acetic acid and (2) a 0.1% solution of a-naphthylamine in 30% Acetic Acid.

xii. Ultraviolet Lamp

This is the Gallenkamp LH-330, portable UV lamp with peak absorption at 254 nm. The lamp was used for the detection of nitrosamines on thin-layer plates.

xiii. Spectrophotometers.

(a) Perkin-Elmer UV-137 Spectrophotometer

The ultraviolet absorption characteristic of the nitrosamine standards were established using this instrument.

(b) Eye Unicam SP 600

This instrument was used in the quantitative estimation of serum proteins, blood sugar; Urine Urobilinogen, serum alkaline phosphatase and serum glutamic oxalacetate transaminase.

(c) Pye Unicam SP 500 Spectrophotometer

This instrument was used in the quantitative estimation of nitrosamines present in the alcoholic drinks.

Hitachi Mass Spectrometer.

This is the RMU-6E model. It was used to determine the molecular weights of the standard nitrosamines, as a purity check.

xiv. The Experimental Animals

These are littermates of white albino rats imbred in the departmental animal house. They were used to assess the pathological and physiological properties of nitrosamines. The weight ranges were 145 - 150g; and 99 - 102 g. respectively.

xv. Metabolic Cages

These are made of wire-mesh to house each rat separately, and with facilities for separate collection of faeces and urine.

xvi. Reagents and Equipments for Histological Studies

(a) Formal Saline

Formaline	10 ml
Sodium Chloride	0.9 g.
Water, to	100 ml.

This was used to fix specimen tissues.

(b) Ethyl Alcohol

(i) 50% Ethanol
(ii) 70% Ethanol
(iii) 96% Ethanol
(iv) 100% Ethanol.

These were used to dehydrate specimen tissues.

(c) Paraffin Wax

Paraffin Wax, m.p. 54-60°C was used to embed the specimen tissues.

(d) Wax Embedding Oven, 100°C.

This is the Gallenkamp, Hi - 100; electrically heated with twelve embedding pots and a fitted angle thermometer. It was used for embedding specimen tissues in wax.

(e) Leuckart's L - pieces

These are two L-shaped pieces of metal (brass) on a metal plate to form an oblong. They were used for casting specimen tissues in paraffin wax.

(f) Minot Microtome, Leitz - 1212

This instrument allows for vertical object movement, automatic object feed, and is operated by a hand wheel. It was used for cutting thin uniform slices of specimen tissues embedded in paraffin wax.

(g) Paraffin Section Mounting Water Bath

This bath is fitted with a thermostat to control temperature between 40° and 80°C. It was used for flattening out paraffin wax sections before mounting on microscope slides.

(h) Glycerin Albumin

This was used to fix sections on microscope slides.

(i) Xylol

This chemical was employed to remove wax from sections before stainning.

(j) Ehrlich's Alum Haematoxylin

Haematoxylin	6 g.
Absolute Alcohol	300 ml.
Water	300 ml.
Glycerol	300 ml.
Glacial Acetic Acid	30 ml.
Potassium Alum	in excess.

This was one of the stains used to differentiate the cellular organisation of the specimen tissues.

(k) 1% Alcoholic Eosin

Eosin	1 g.
95% Ethyl Alcohol	100 ml.

This was used for the same purpose as in (j) above.

(l) Celestine Blue

Celestine blue B	0.5 g.
Glycerin	14 ml.
Distilled water	

This was also used as a stain.

(m) Canada Balsam

This was used to mount cover slips on microscope slides.

(n) Slide Drying Plate

This is the Gallenkamp HJ - 620, electrically heated with a smooth enamelled steel top fitted with a thermostat giving temperature control up to 60°C. This was used to dry mounted slides.

(o) Microscopes

(i) Olympus Model KHC

This model has an inclined binocular head rotable through 360° with interlocular distance adjustable from 56 mm. to 74 mm. The base of the microscope incorporates a precentred 20W lamp. Vertical movement of the stage is by coarse and fine focusing controls. This instrument was used for visual assessment of the cellular damaged induced by the nitrosamines.

(ii) "Abi Bi P03"

This is a photographic research microscope and was used to take photographic impressions of the cellular

damage induced by the nitrosamines.

xvii. Composition of the Diets

(a) Basal Diet

Sucrose	15%
Cassava Starch	65%
Corn oil	10%
Non-Nutritive cellulose	5%
Salts Mixture USPXV	4%
Vitamin Mixture	1%

(b) Test Diets

The test diets were as follows:

Diet No. 1	0%	Protein (Protein-free)
" " 2	5%	"
" " 3	10%	"
" " 4	15%	"
" " 5	20%	"
" " 6	25%	"

The protein was incorporated into the basal diet at the expense of the cassava starch. Nutritional casein was the source of protein. The diets were used to study the effects of graded dietary protein levels on the toxicity of nitrosamine.

(c) Composition of the USPXV Salt

Sodium chloride "Analar Grade "	139.0g.
Potassium hydrogen Sulphate "Analr Grade	389.0g.
Magnesium sulphate (Anhydrous) " "	57.3g.
Calcium Carbonate " "	380.0g.
Ferrous Sulphate " "	27.0g.
Manganese sulphate " "	4.0g.
Zinc Sulphate " "	0.5g.
Potassium Iodide " "	0.8g.
Copper Sulphate " "	0.47g.
Cobalt Chloride " "	0.02g.

(d) Composition of the Vitamin Mixture

Vitamin A	1000 International Unit (I.U.)
Vitamin D	100 I. U.
Vitamin E	10 I.U.
Vitamin K (Menadione)	0.5mg.
Thiamine	0.5mg.
Riboflavin	1.0mg.
Pyridoxine	0.04mg.
Niacin	200mg
Cholin	25mg
Inositol	10mg.
Para-aminobenzoic Acid	2mg.
Vitamin B_{12}	0.02mg
Folic Acid	0.2mg.

xviii. Automatic Mixer

This is the "Rotamixer" type with perspex containers which spins as it rotates. The instrument was made by Forster Equipment, Leicester, England. It was used to ensure thorough mixing of the various diet preparations used in the feeding trials.

xix. Grinding Mill

This is the "Disintegrator-type" laboratory mill size 8" manufactured by Christy Norris, Chelmsford, England. It was used to process dried cassava starch into a fine powder.

xx. Reagents for Determination of Serum Protein

(a) Stock Biuret Reagent

Solution A.

Sodium Potassium tartrate "Analar Grade "		45g.
0.2M NaOH	"	400 ml.
Copper Sulphate	"	15g.
Potassium Iodide	"	5g.
0.2M NaOH, to	"	1 litre.

Solution B.

Potassium iodide, "Analar Grade"	0.5%
in Sodium hydroxide	0.2N.

(b) Working Biuret Reagent

Solution A.	50ml
Solution B.	200ml.

xxi. Reagents for Determination of Serum Bilirubin

(a) Diazo Reagent 'A'

Sulphanilic Acid	1g.
Cono. HCl	15 ml.
Water	to 1 litre.

(b) Diazo Reagent 'B'

Sodium Nitrite, 0.5% solution in distilled water.

(c) Diazo Reagent Working Solution

Diazo Reagent 'A'	5 ml.
Diazo Reagent 'B'	0.15 ml.

(d) Diazo Blank

Hydrochloric Acid	1.5% (v/v) in distilled water.

(e) Benzoate-Urea Solution

Sodium benzoate	10 g.
Urea	10 g.
Distilled Water, to	100 ml.

(f) Methyl Red Standard

(i) Stock Standard

Methyl Red	0.290 g.
Glacial Acetic Acid, to	100 ml.

(ii) Working Standard

Stock Standard	1. 0 ml.
Glacial Acetic Acid "Analar Grade"	5.0 ml.
Sodium Acetate " "	14.4 g.
Water, to	100 ml.

xxii. Reagents for Estimation of Blood Sugar

(a) Isotonic Sodium Sulphate-Copper Sulphate Solution

Sodium Sulphate crystals "Analar Grade"	30g.
Copper Sulphate " " "	6g.
Water, to	1000ml.

(b) Sodium Tungstate

Sodium Tungstate crystals "Analar Grade"	10g.
Water, to	100ml.

(c) Alkaline Tartrate

Sodium Potassium Tartrate crystals "Analar Grade"	12g.
Sodium Carbonate crystals "Analar Grade"	20g.
Sodium Bicarbonate " " "	25g.

Potassium Oxalate 18g.

Water, to 1000ml.

(d) Arseno-Molybdate

Ammonium molybdate crystals "Analar Grade" 50g.

Water 900ml.

Conc. H_2SO_4 " " 42ml.

Arseno-molybdate 6g./50 ml.

(e) Stock Glucose Solution

Glucose "Analar Grade" 1000 mg.

Water 100 ml.

(f) Working Glucose Solution

Glucose "Analar Grade" 1000 mg.

Saturated Benzoic Acid 100 ml.

xxiii. Reagents for Alkaline Phosphatase Determination

(a) 0.5M glycine buffer, pH 10.5; 0.0005. $MgCl_2$

(b) 0.5M glycine buffer, pH 10.5; 0.0005M. $MgCl_2$,
0.0055M nitrophenylphosphate, sodium salt.

xxiv. Reagents for the Estimation of Urine Urobilinogen

(a) Saturated Sodium Acetate.

(b) Ehrlich's Reagent

Paradimethylaminobenzaldehyde "Analar Grade"		0.7g.
Con. Hydrochloric Acid	" "	150 ml.
Water		100 ml.

(c) 10% Barium Chloride

Barium Chloride "Analar Grade"	10 g.
Water	100 ml.

(d) Stock Standard

(i) Pontacyl Carmine 2B	100 g.
Acetic Acid 0.5%	500 ml.
(ii) Pontacyl Violet 6R	0.095 g.
Acetic Acid 0.5%	till dissolv
Pontacyl Carmine (Solution 1)	25 ml.
Acetic Acid 0.5%, to	1000 ml.

(e) Working Standard

Stock Standard (d)	10.2 ml.
Acetic Acid 0.5%, to	100 ml.

xxv. Reagents for SGO-T Determination

(a) SGO-T Substrate

a - aspartic Acid	200 mM
a - keto-glutaric Acid	85 ml.
in 0.1M phosphate buffer at	pH 7.4.

(b) Ehrlich's Reagent

Paradimethylaminobenzaldehyde "Analar Grade"		0.7g.
Con. Hydrochloric Acid	" "	150 ml.
Water		100 ml.

(c) 10% Barium Chloride

Barium Chloride "Analar Grade"	10 g.
Water	100 ml.

(d) Stock Standard

(i) Pontacyl Carmine 2B	100 g.
Acetic Acid 0.5%	500 ml.
(ii) Pontacyl Violet 6R	0.095 g.
Acetic Acid 0.5%	till dissolved
Pontacyl Carmine (Solution 1)	25 ml.
Acetic Acid 0.5%, to	1000 ml.

(e) Working Standard

Stock Standard (d)	10.2 ml.
Acetic Acid 0.5%, to	100 ml.

xxv. Reagents for SGO-T Determination

(a) SGO-T Substrate

a - aspartic Acid	200 mM
a - keto-glutaric Acid	85 ml.
in 0.1M phosphate buffer at	pH 7.4.

(b) 2:4 Dinitro Phenylhydrazine Reagent

2:4 dinitrophenylhydrazine	200 mg.
Concentrated Hydrochloric Acid	85 ml.
Water, to	1000 ml.

(c) 0.4N Sodium Hydroxide

Sodium Hydroxide	16 g.
Water	1000 ml.

(d) Aniline Citrate Reagent

Citric Acid	50 g.
Water	50 ml.
Aniline	50 ml.

(e) M/15 Phosphate Buffer pH. 7.5

Sodium phosphate (anhydrous)	7.95 g.
Potassium Phosphate (anhydrous)	1.5 g.
Water, to	1000 ml.

xxvi. pH Meter

This is the portable model No.6877 "Doran" pH meter made by Doran Instruments Co. Ltd., Glos, England. It is equipped with a calomel electrode and was used to determine the pH of buffer solutions.

xxvii. Antibiotics

Neomycin sulphate, tetracycline hydrochloride and bacitracin sulphate were purchased and used for the antibiotic treatment of the experimental rats.

CHAPTER THREE

METHODS

1. Preparation of Nigeria's local alcoholic beverages.

(a) Palm Wine

Palm wine is the fermented sap of the palm tree. There are two main sources of palm wine in Nigeria:

(i) The fermentable sap of the Raphia palms among which R. vinifera and R. hookeri are very popular.

(ii) The fermentable sap of the Oil palm, Elaies guineensis.

The process of making palm wine starts with the tapping of the palm trees. The trees are tapped erect (tapping of the felled tree is now obsolete). The method of tapping is described as either "inflorescence tapping or "stem tapping" i.e. tapping at the base of the male inflorescence in the former case or tapping at the base of the terminal bud in the latter case.

The palm tree is cleared of the older leaves to expose the point of tapping and a triangular hole is then cut either at the base of the male inflorescence or at the stem a little way below the terminal bud depending on the method in mind. This hole is roughly about an inch deep and the area is enlarged as tapping progresses. The sap starts to ooze out after about 24 - 36 hours. A funnel which invariably is made of bamboo is thrusted into the

incision and the other end of it is fitted into a collecting vessel which could be a gourd affixed to the tree by a piece of rope.

The sap is inoculated naturally by yeast cells which accumulate in millions in exudates on the flower stalk. These fungi have been shown to be mostly Saccharomycos cerevisiae and Schizosaccharomycos pombe, (Bassir, 1968). The sap is contaminated with bacteria as it drops from the incision. The bacteria most commonly found are Lactobacillus plantarum and Leuconostoc mesenteroidea. The biochemical activities of these microorganisms resulting in the formation of palm wine has been studied by Faparusi (1967).

At each visit to the palm tree, the tapper slightly enlarges the hole with a pen-knife in an attempt to clear the hole of mucoid substances which tends to block the xylem vessels from which the sap proceeds. A tree is usually not tapped for more than 12 days.

(b) Oti Agbo

The main ingredients for the preparation of this alcoholic beverage is plantain (Musa sapientes).

Very soft and overiped plantains are peeled and the "flesh" is chopped into small bits and placed in an earthen ware pot of convenient size. Some ground red pepper is added and water is poured in to soak the content of the pot. After mixing

thoroughly the pot is covered and allowed to stand for 3 - 4 days, at the end of which the content of the pot. is filtered and a sweet stimulating alcoholic drink is the product. The fermenting organisms are not known but it is claimed that the pepper helps to give the drink a sharp taste.

(c) Pito

Pito is a fermenting beverage from maize, sorghum or a mixture of both. It is an important food in the Mid-western, Western, West-central, Benue Plateau and North-Central States of Nigeria.

A widespread procedure for the preparation of this alcoholic beverage among the people is to wash and soak the cereal grains employed in water for 2 days, after which they are malted by leaving for five days in baskets lined with moistened banana leaves. The malted grains are ground, mixed with water and cooked. The mash is allowed to cool and is filtered through a fine mesh basket. The residue is used as animal feed. The filtrate is then left, usually overnight, until it tastes sour. It is then concentrated by boiling. A small quantity of the "starter" (sediment from a previous brew) is added to the cooled concentrate and left overnight. The product is pito, a dark-brown liquid with taste varying from sweet to bitter. Some detailed study of the biochemical activities of the fermenting organisms has

been carried out by Ekundayo (1970).

(d) Ogogoro

Ogogoro is a distillation product of palm-wine.

The fresh palm wine as brought down from the palm tree is immediately filtered into a clean gourd and is allowed to ferment for 2 days or until fermentation is complete. The palm wine is then emptied into a large drum - usually the 44-gallon drum - if production is on a large scale. The brew is then distilled from the drum over a fire. The tubes used for distillation and condenser system may be old car exhaust pipes, water pipes or any other convenient form of pipe available. The condenser is immersed in a water bath which can be emptied and refilled when the water gets warm. The other end of the condenser is placed in a funnel on a collecting vessel. To get a rough idea of the strength of the spirit or when to stop fermentation a piece of cotton wool is used at intervals to collect some of the distillate. This is ignited and from the colour of the flame and rapidity of burning the brewer makes his decision.

The product of distillation, which is called Ogogoro, is a clear liquid with a powerful fruity odour.

(e) Burukutu

Burukutu is an alcoholic beverage made from guinea corn grains (Sorghum vulgare).

The procedure for preparation starts with the grains which are steeped in water overnight. Excess water is then drained off the soaked seeds using a basket, and the grains are spread out on mats under shade, and are covered with leaves. During this malting period, the grains are occasionally turned over. Germination is allowed to continue for about four days after which the malt is spread in thin layers in the sun to dry for 1 or 2 days depending on how hot the day is, and the malt is later ground.

The ground malt is poured into a pot of cold water and in some cases gari is added to increase the viscosity of the liquid. Gari is a starchy powder produced from the tuber of the cassava plant and the course textured variety is prefered. The resulting mixture (gari - malt powder - water) is roughly in the ratio 1: 2: 6 by volume.

A small quantity of the left-over of the last production is added. It is claimed that this helps to maintain a unique aroma for the drink produced in a particular stall. The mixture is then left for 2 days and boiled for a few hours thereafter. The brew is then allowed to mature for 2 days, and is filtered though a white piece of cloth, sewed in the form of a pillow-case, to allow for squeezing into another pot. The filtrate which tak the form of a suspension of some particles in a creamy liquid is the Burukutu and it is sour to the taste. It can keep for

7 days.

11. Preparation of Nitrosamines.

(According to Vogel, 1968)

(a) Dimethylnitrosamine

A 100 ml. distilling flask was fitted with a condenser for downward distillation. 50 g. of dimethylamine hydrochloride was dissolved in 25 ml. of water and dilute sulphuric acid was added until acid to congo red paper. The resulting solution was placed in the distilling flask and a solution of 45 g. of pure sodium nitrite in 50 ml. of hot water was added. The mixture was distilled rapidly to dryness, when the nitrosamine passed over (although it was not visible as a separate layer) together with a little of the base as dimethylamine nitrite. The latter was removed by redistilling the distillate with a ltle more dilute sulphuric acid. Excess solid potassium carbonate was added to the distillate to separate out the nitrosamine which appeared as a yellow oil. This was separated and treated with more solid potassium carbonate until no further action occured. Finally the liquid was dried over fresh anhydrous potassium carbonate in a small flask. The liquid was then distilled from a 100 ml. flask and the dimethylnitrosamine was collected at 150° - 151°c.

(b) Diethylnitrosamine

(b) Diethylnitrosamine

50 ml. of diethylamine was added slowly to a calculated quantity of carefully standardised 5N-hydrochloric acid cooled in ice. The solution of the hydrochloride was introduced into a solution of 39 g. of sodium nitrite in 45 ml. of water and distilled rapidly to dryness. The yellow upper layer of the nitrosamine was separated from the distillate. The aqueous layer was saturated with solid potassium carbonate and the nitrosamine which separated was removed and added to the main product. The lot was dried over anhydrous potassium carbonate and redistilled. The diethylnitrosamine was collected at 172°c.

(c) N-nitrosopiperidine

This was prepared following the procedure described for diethylnitrosamine using piperidine in place of diethylamine.

(d) Diphenylnitrosamine

8.5 g. of pure diphenylamine was dissolved in 70 ml. of warm alcohol. 4 g. of sodium nitrite was dissolved in 6 ml. of water. Each solution was cooled in ice until the temperature fell to 5°C. 6 ml. of conc. hydrochloric acid was added slowly with stirring to the diphenylamine solution and immediately the sodium nitrite solution was poured rapidly into the well-stirred mixture. The diphenylnitrosamine crystallised out as the temperature rose to 20-25°c. The mixture was cooled in ice water for 15 - 20 minutes and was filtered through a

Buchner funnel, washed with water to remove sodium chloride, and pressed well with a wide glass stopper. The diphenylnitrosamine was recrystallised from methylated spirit. Pure pale yellow crystals of diphenylnitrosamine were obtained. M.P. 68°c.

(e) Nitrosodibenzylamine

Dibenzylamine hydrochloride was used in place of Dimethylamine hydrochloride and the procedure for the preparation of dimethylnitrosamine described in (a) was followed.

(f) N-nitrosomorpholine

100 ml. of morpholine was added slowly to a calculated quantity of carefully standardised 5N-hydrochloric acid cooled in ice. The solution of the hydrochloride was introduced into a solution of 78 g. of sodium nitrite in 90 ml. of water contained in a 250 ml. distilling flask. The mixture was distilled rapidly to dryness. The yellow upper layer of the nitrosamine was separated from the distillate. The aqueous layer was saturated with solid potassium carbonate and the nitrosamine which separated was removed and added to the main product. The lot was dried over anhydrous potassium carbonate and distilled. The N-nitrosomorpholine was collected and stored in a brown bottle.

The synthesis of authentic samples of nitrosamines has been successfully carried out from available purity check using various solvent systems. The results obtained in the mass spectrum and ultraviolet absorption spectrum of the

nitrosamines supplied additional evidence for their purity. These results were in agreement with those of other workers cited in literature, (Vogel, 1968, Haseldine and Jander, 1954, Druckrey et al. 1967).

Table 1 Rf Values of the prepared Nitrosamines in Various Solvent Systems

	n-Hexane		Diethyl Ether		Dichloromethane	
Nitrosamines	4: 3: 2		5: 7: 10		10: 3: 2	
Dimethylnitrosamine	0.24		0.35		0.12	
Diethylnitrosamine	0.48		0.55		0.25	
Diethylnitrosamine	0.80		0.82		0.60	
Dibenzylnitrosamine	0.85		0.92		0.63	
Nitrosomorpholine	0.40		0.47		0.22	
Nitrosopiperidine	0.63		0.52		0.24	

Fig. 7

Ultraviolet absorption spectrum of the prepared Dimethylnitrosamine showing absorption peaks in ethanol.

Fig. 8

Ultraviolet absorption spectrum of the prepared Diethylnitrosamine showing absorption peaks in ethanol.

Fig. 9

Ultraviolet absorption spectrum of the prepared Diphenylnitrosamine showing absorption peak in ethanol.

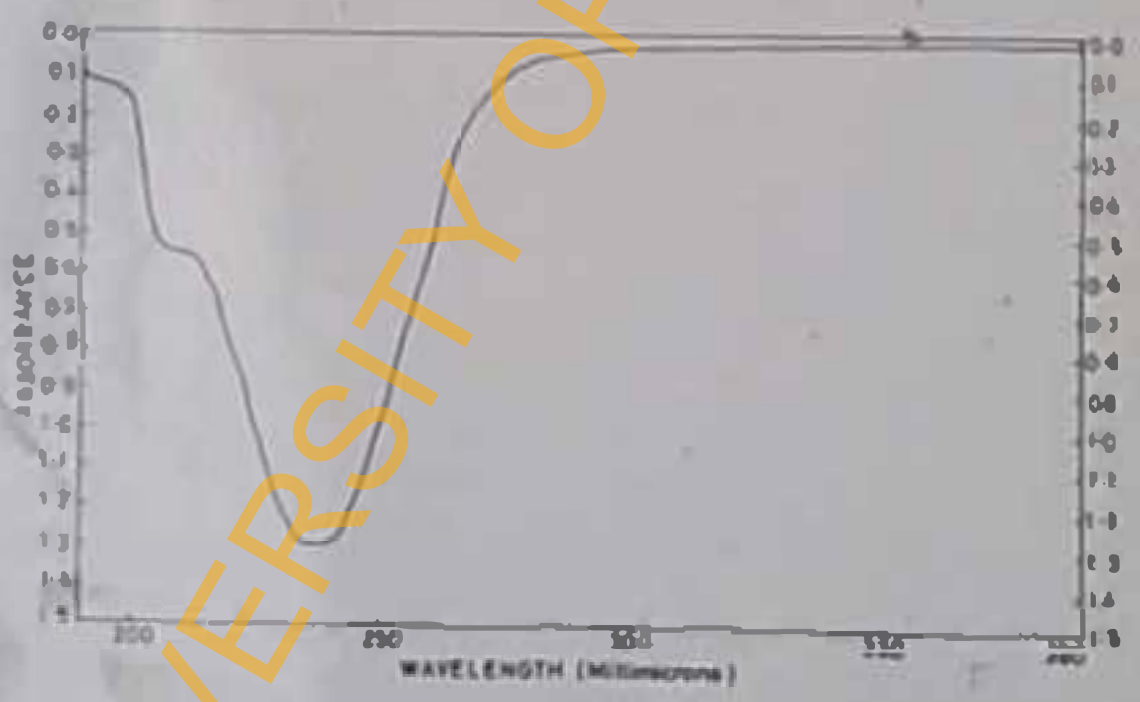


Fig. 10

Ultraviolet absorption spectrum of the prepared Nitrosopiperidine showing absorption peaks in ethanol.

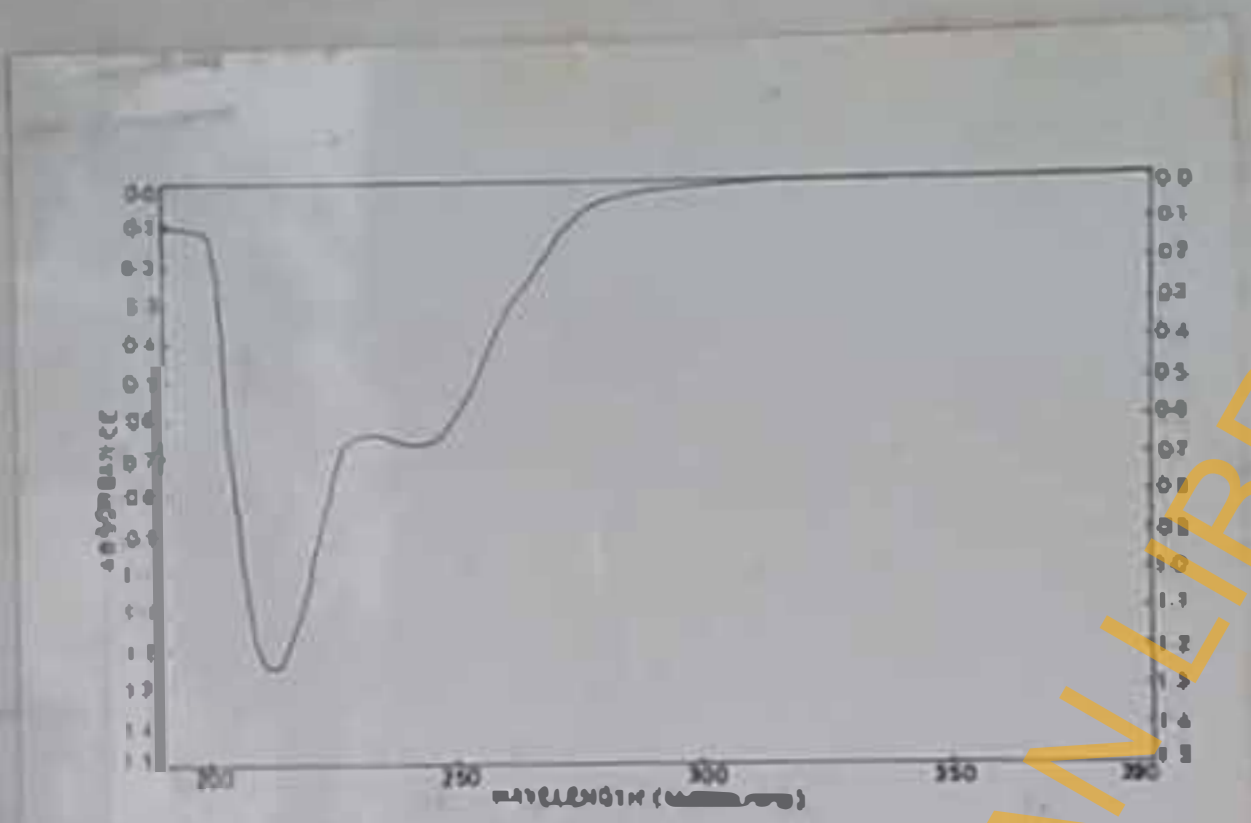


Fig. 11

Ultraviolet absorption spectrum of the prepared Nitroso-dibenzylamine showing absorption peaks in ethanol.

Fig. 12

Ultraviolet absorption spectrum of the prepared Nitrosomorpholine showing absorption peaks in ethanol.

Fig. 13

Determination of the molecular weight of the prepared Dimethylnitrosamine using the Mass spectrometer.

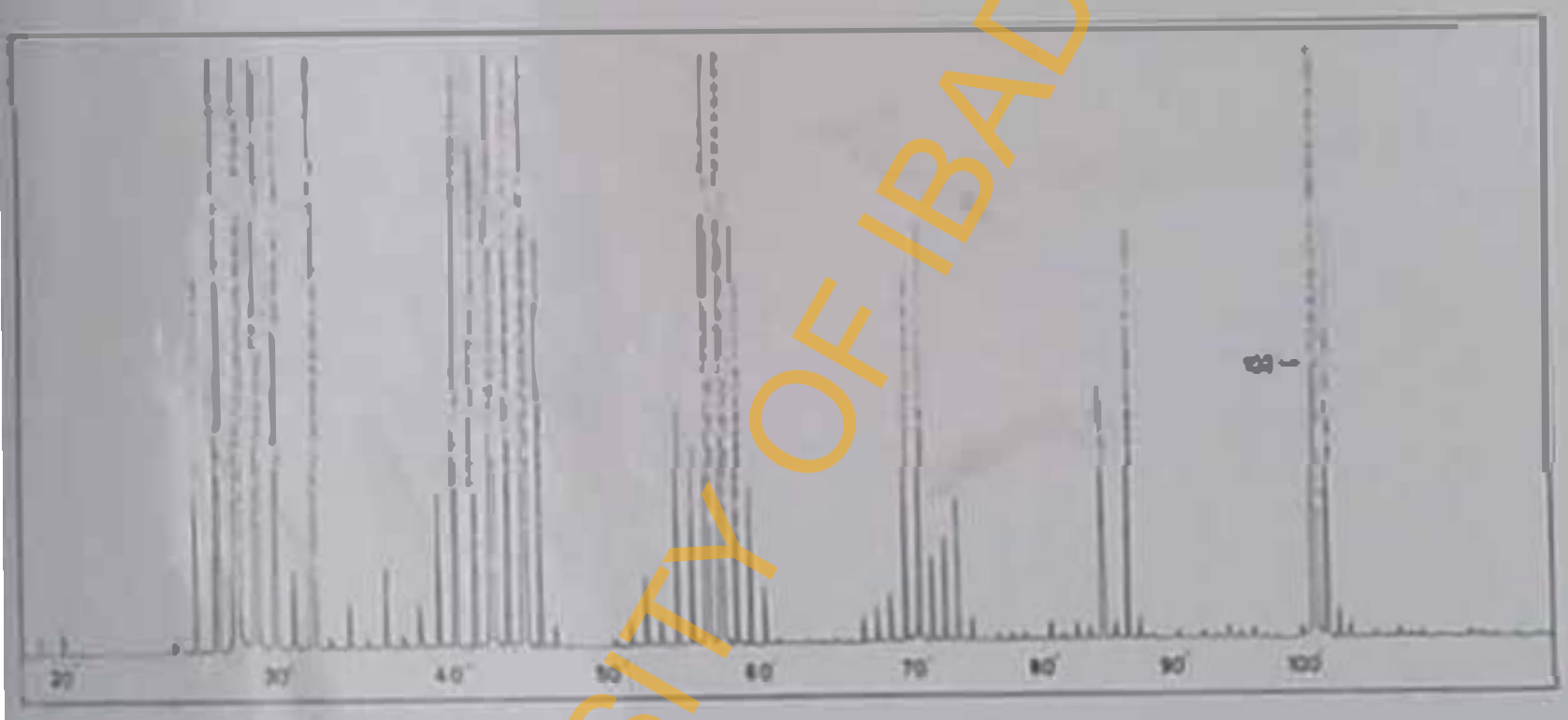


Fig. 14

Determination of the molecular weight of the prepared Diethylnitrosamine using the mass spectrometer.

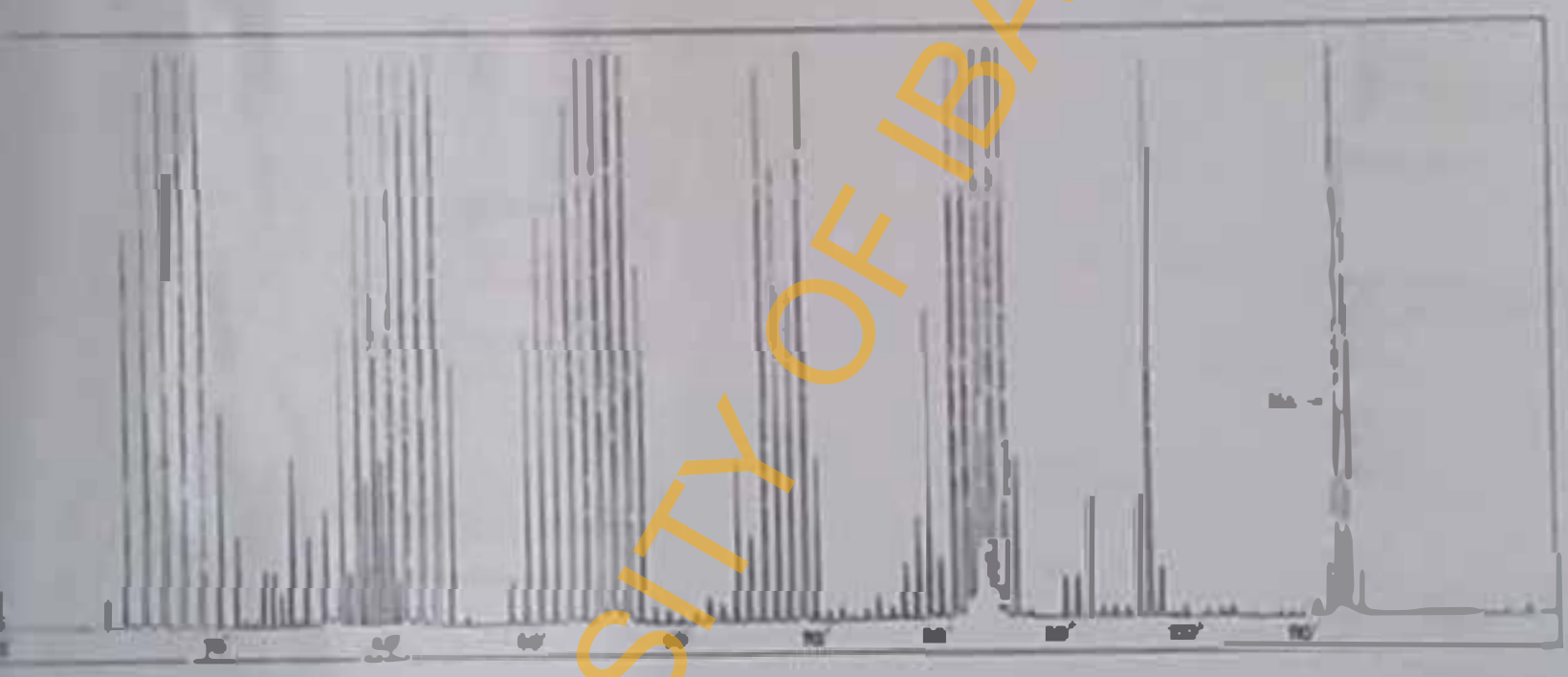


Fig. 15

Determination of the molecular of the prepared Nitrosopipe-dine using the Mass Spectrometer.

Fig. 16

Determination of the molecular weight of the prepared Nitrosomorpholine sulphate the Mass Spectrometer.

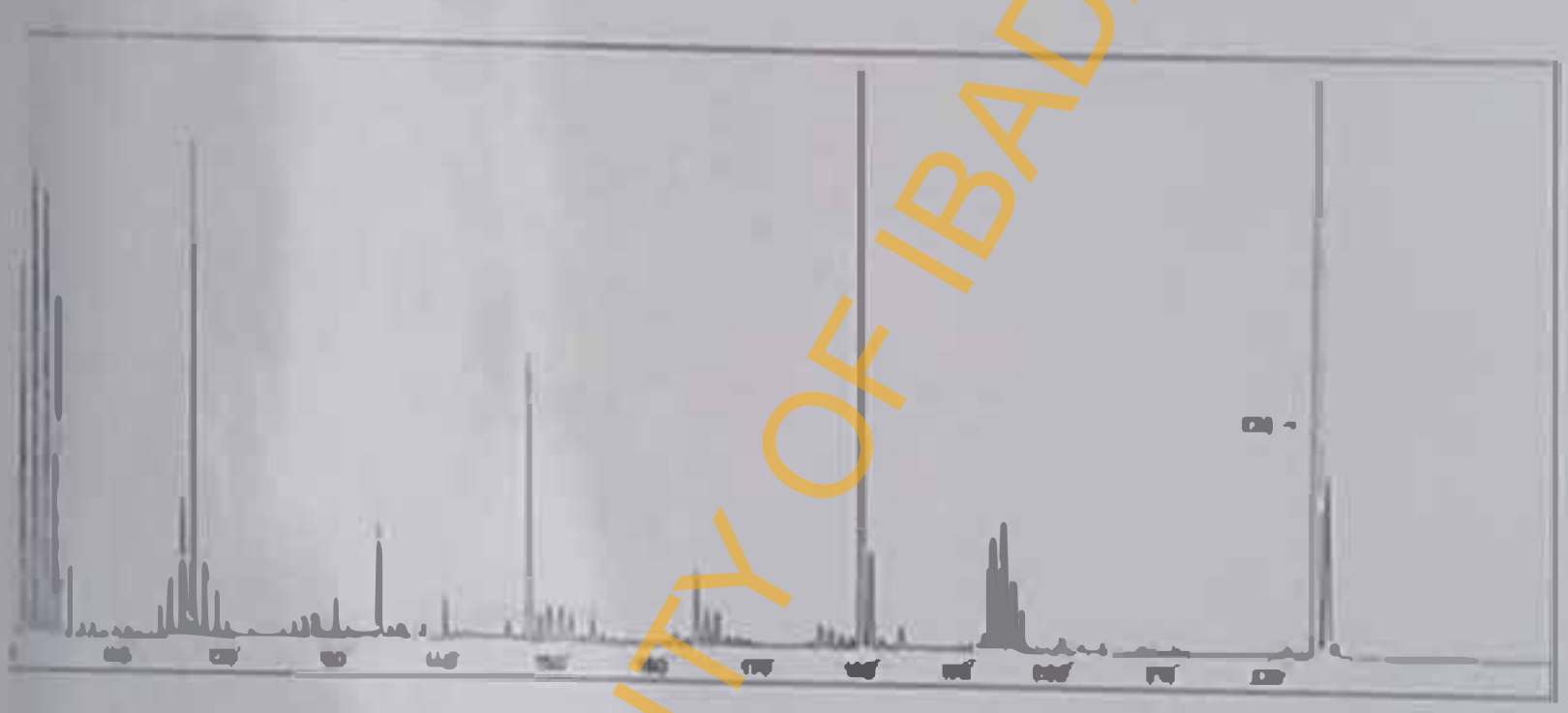


Fig. 17

Determination of the molecular weight of the prepared Dibenzylnitrosamine using the Mass Spectrometer.

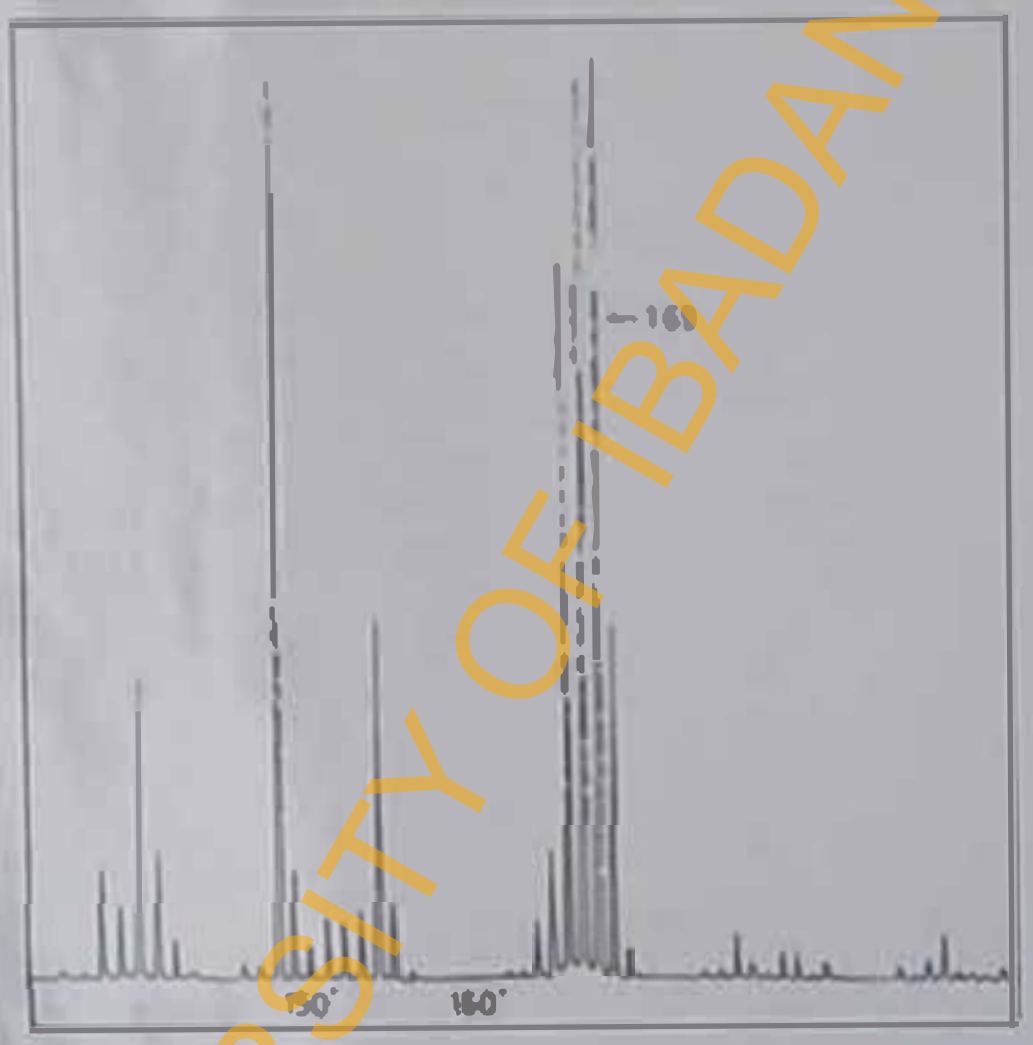


Fig. 18

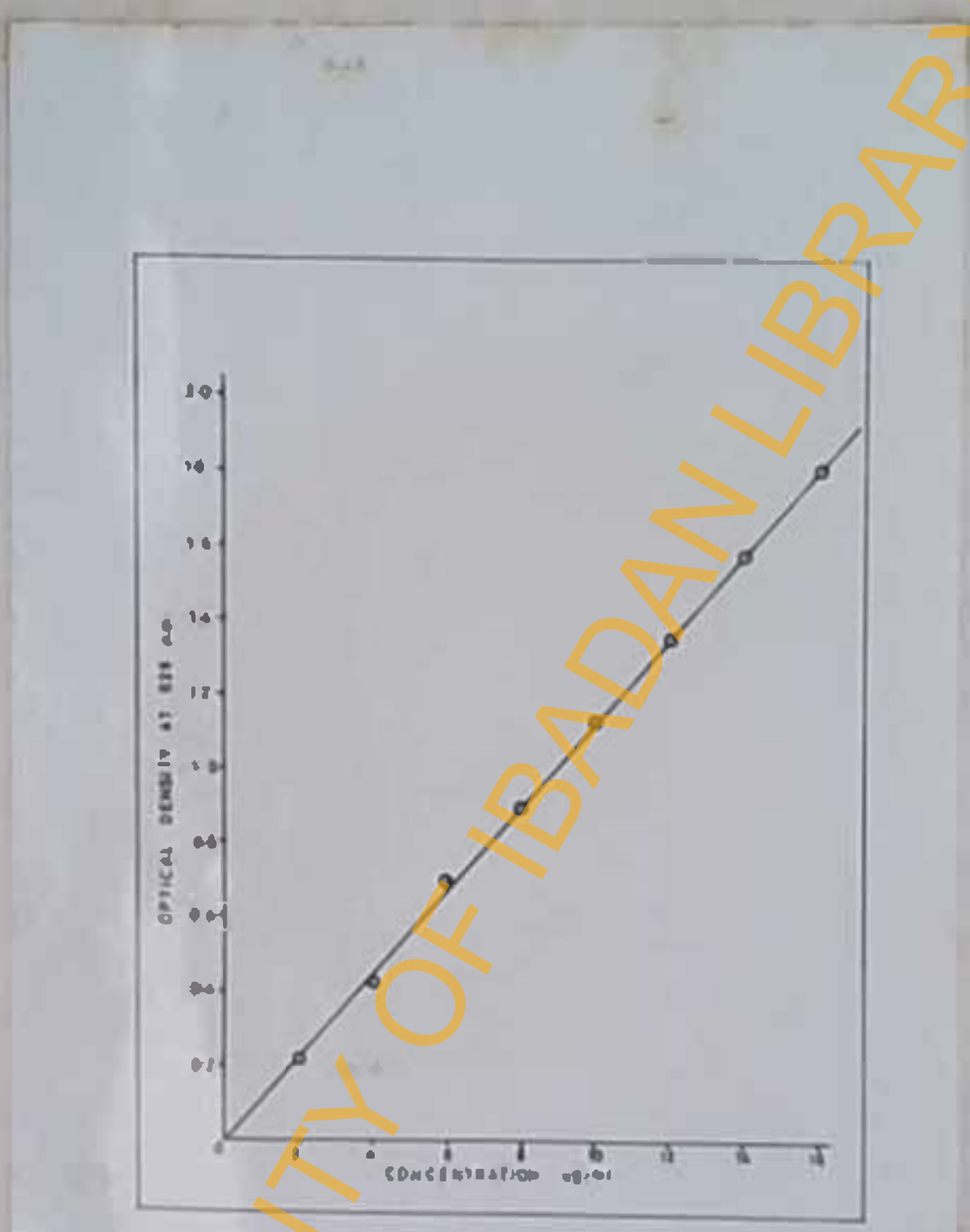
Determination of the molecular weight of the prepared diphenylnitrosamine using the Mass Spectrometer.

111. Recovery of known amounts of Nitrosamine added to (a) water, and (b) Palm wine.

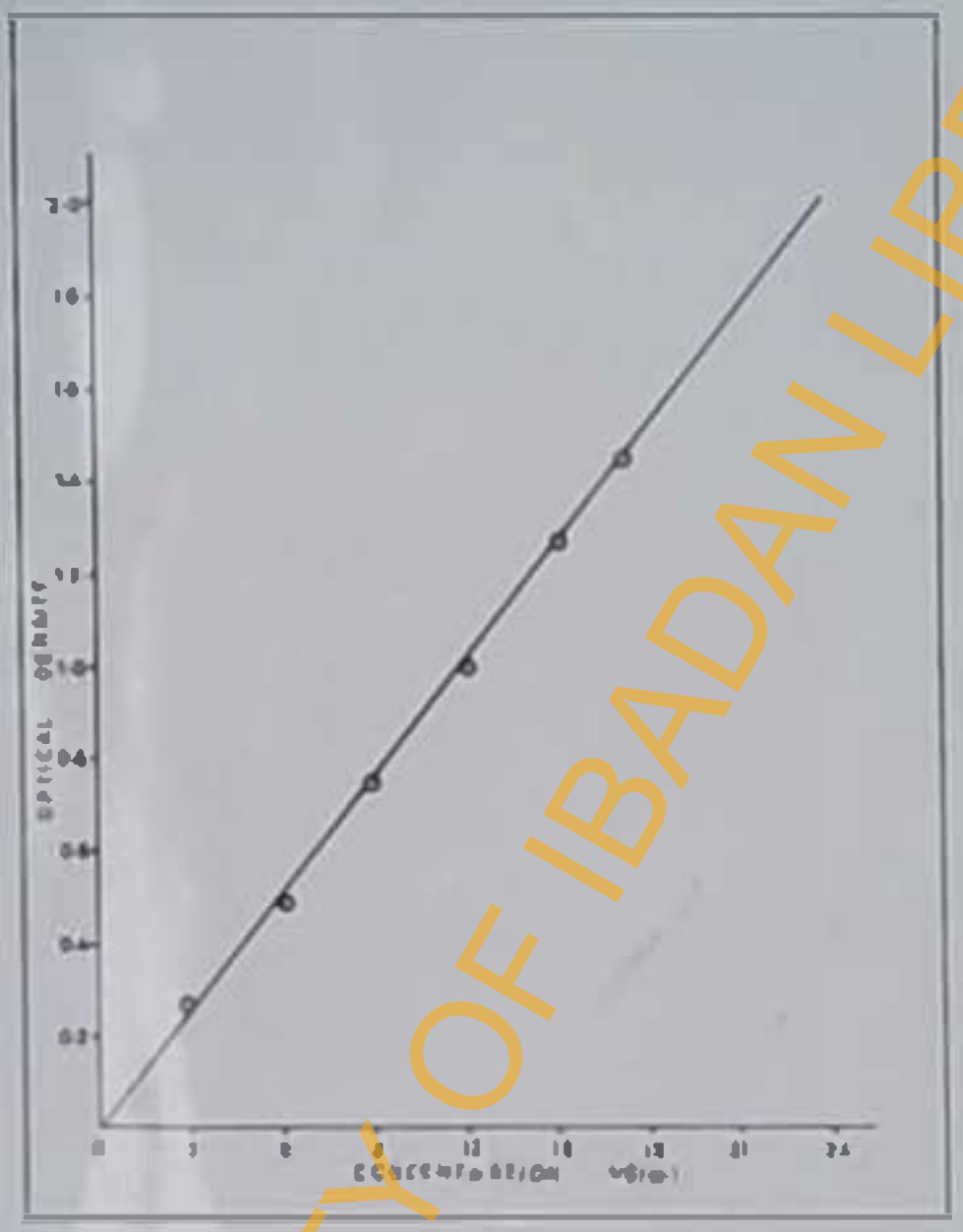
(a) Calibration curves

Solutions of dimethylnitrosamine in water were prepared into nine 10 ml. volumetric flasks, such that the strength of the solutions were 5ug/ml; 10ug/ml; 20ug/ml; 25ug/ml; 30 ug/ml; 35ug/ml; 40ug/ml; and 50ug/ml; respectively. 1 ml. of each of these stock solutions was placed in a test-tube. 0.5 ml. of 5% Na_2CO_3 was added and the mixture was irradiated for 15 minutes with UV light at 230 nm. 3 ml. of griess reagent was added and the mixture was allowed to stand for 15 minutes, after which the optical densities of the various solutions were recorded at 525 nm. using the sp 600.

With diphenylnitrosamine graded concentrations were prepared as above with methanol. 1 ml. of each stock solution was placed in a test tube and treated with 0.2% Na_2CO_3 in 80% methanol in water. This was followed by 15 minutes irradiation after which 3 ml. of griess reagent was added. The mixture was allowed to stand for 15 minutes and the optical density was recorded at 525 nm. In both cases (for dimethylnitrosamine and diphenylnitrosamine) the various optical densities were plotted against the concentrations to obtain the calibration curves.



Standard curve for dimethylnitrosamine in Griess regent.



Standard curve for diethylnitrosamine in Griess reagent.

(b) Contamination of water and Palm wine with known amounts of Nitrosamines

Standard solutions of Dimethylnitrosamine in water containing 10 ug/ml. 20ug/ml 50ug/ml. 100ug/ml. 500ug/ml 1000ug/ml respectively were prepared in six 50 ml. volumetric flasks.

One ml. of each standard solution was added to 100ml. of distilled water and 100 ml. of Palm wine respectively, to contaminate the water and Palm wine with 10 ug/ml. 20 ug/ml. 100 ug/ml. 500 ug/ml. and 1000 ug/ml. dimethylnitrosamine respectively.

The above contamination was repeated using Diphenylnitrosamine.

(c) Analytical procedures

The analytical procedures employed for the recovery of these known amounts of nitrosamines from water and palm wine are described in sections iv, v, and vi of this chapter.

iv. Extraction procedures for Nitrosamines in the alcoholic beverages

(a) Extraction of volatile water-soluble Nitrosamines

1000 ml. of the alcoholic beverage sample (except ogogoro) were mixed carefully with 20 ml. of ethanol and the mixture was transfered into a 3-litre round-bottom quickfit flask. Sufficient solid NaOH was added to make the liquid 0.2N with respect to this reagent. Using a few glass beads to prevent bumping about $\frac{1}{3}^{rd}$ of the original volume was distilled through a splash-head and Liebig condenser. The distillate was collected in a graduated tube cooled in an ice-bath, care being taken not to let the distillate freeze as this increases the risk of losing some nitrosamine by re-evaporation.

A second distillation was carried out from an acid medium by making the distillate from above 0.2N with respect to sulphuric acid.

The distillations were carried out at atmospheric pressure and the temperature of the heating mantle was kept at 170° - 180°c. After each distillation the splash-head and condenser were rinsed into the receiving flask with water.

The distillate collected was treated with solid potassium carbonate with cooling. The neutral components that separated out (the bulk of which is ethanol) was

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The distillate collected was treated with solid potassium carbonate with cooling. The neutral components that separated out (the bulk of which is ethanol) was

recovered in a separating funnel and the aqueous layer was treated with more solid potassium carbonate until no further action occured. The neutral layer was removed and added to the main product. Finally the lot was dried over fresh anhydrous potassium carbonate, filtered through a sintered funnal and concentrated under vacuum in a thin-film rotary evaporator at a temperature below 37°c. The nitrosamines were extracted from the concentrate with methylene chloride.

(b) Extraction of Non-volatile water in-soluble Nitrosamines.

1000 ml. of the alcoholic beverage sample were mixed thoroughly with 200 ml. of pure ether. The ether extract was recovered in a separating funnel and filtered. The filtrate was shaken with 20 ml. portion of 5% aqueous NaOH. The resulting alkali extracted ether solution was washed with 20 ml. distilled water. The ether solution was recovered and treated with 20 ml. of 5% HCl. The ether layer was retained, dried with anhydrous magnesium sulphate. The ether was distilled off and the residue was extracted with dichloromethane.

v. Thin-layer Chromatographic Analysis

(a) Preparation of thin-layer chromatographic plates

Stahl's technique for preparing thin-layer plates was adopted. Silica gel G was the adsorbent of choice

and glass plates (20 x 20 cm) served as the firm support. The glass plates were washed with water and a detergent, drained and dried and subjected to a final wash with acetone. They were then arranged on a unoplan instrument for coating with silica gel.

60 grams of silica gel was made into a slurry by shaking for about a minute with 120 ml. water. The slurry was poured into the spreader on the unoplan and coating was accomplished by pulling the spreader (0.5 mm slit) over the glass plates. After coating the plates were allowed to dry in air for 20 minutes and were activated in an oven at 110°C for 2 hours.

(b) Running

A starting line was marked at the edge of the plate with a sharp pencil and the finishing line was drawn right across the plate (the pencil removes a fine line of adsorbent down to the glass, and the solvent flow is forced to stop when the solvent front reaches the line). The edges of the plates were rubbed clear with a piece of cotton wool, before spotting, to a width of about 0.5 cm. to give a sharper edge to the adsorbent layer.

The test solutions were spotted on the thin-layer plates alongside standard nitrosamine solutions in

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The test solutions were spotted on the thin-layer plates alongside standard nitrosamine solutions in

dichloromethane using a micro-pipette. The spots were arranged about 2 cm. centre to centre and individual spotting positions were noted.

When the spots have dried the plates were placed vertically in a suitable tank with their lower edges immersed in the selected mobile phase (Hexane-diethyl-ether-dichloromethane 4: 3: 2) to a depth of 0.5 - 1.0 cm. to obtain an ascending chromatographic separation.

At the end of the run the solvent was allowed to evaporate from the plate.

(c) Location

Location of the nitrosamine rich spots on the plate was by the methods of Daiber and Preussmann (1964) and Preussmann et al. (1964). The detectors used were as enumerated in chapter 2, section xi.

(d) Identification of the Nitrosamines

The nitrosamines in the extracts were identified with respect to the positions of the standards on the same plate. The Rf values for each nitrosamine was recorded.

vi. Quantitative Estimation of Nitrosamine Fractions

The test solutions were separated into components on thin-layer chromatographic plates. Applications of the test solutions for this purpose were in bands. Using the Rf values obtained in the previous experiment

as guide, the nitrosamine rich bands were scraped off and extracted with methylene chloride.

Quantitative estimation of the nitrosamines was by the colorimetric method of Daiber and Preussmann (1964).

The determination was carried out by treating the extracts as stated in section 3 for the stock solution and reading the absorbance on SP 500 (spectrophotometer) at 525 nm. The corresponding concentration was estimated from the calibration curves prepared.

vii. Dosing of Rats with Nitrosamines:

Solutions of the appropriate nitrosamines were made in water such that each contained 500 ppm. 200 ppm, 100 ppm, 50 ppm, 12.5 ppm and 5 ppm. of the nitrosamine respectively. The contaminated water was then served to the rats as their drinking water.

viii. Histopathological Studies

(a) Fixation of specimen tissues

The tissues for histological examinations were carefully dissected out and cut into convenient pieces (7 mm. thickness) for fixation. Formal saline was the fixative of choice. The cut tissues were immersed in specimen bottles containing the formal saline for 24 hours.

(b) Dehydration

Following fixation, the tissues were placed in tissues baskets and labelled appropriately. Dehydration was then carried out in 50%, 70%, 90% and 100% alcoholic baths in screw-capped glass jars. The jars were occasionally agitated to speed up the process of dehydration. The tissues were then laid on a piece of filter paper and lightly blotted to remove excess fluid before clearing in xylol.

(c) Impregnation with Wax

After blotting lightly with filter paper the tissues were transfered from the clearing agent to molten paraffin wax, in the wax embedding oven.

(d) Casting

Fresh molten wax was poured from the stock jug into the mould. With forceps previously warmed to prevent wax setting on them the tissues were lifted from the final wax pot on the embedding oven and each specimen was placed in the bottom of the mould. The labels were fixed in position by pressing one edge against the solidifying wax at one corner of the mould. When the block has cooled sufficiently to form a skin on

the surface it was immersed in cold water to cool it more rapidly. The block of wax, having set quite hard, was removed. from the mould and trimmed ready for cutting.

(e) Sectioning

After cutting the blocks their labels were attached by pressing them on the side with a hot searing iron so as to embed them slightly in the block. For sectioning, the blocks were fixed on the block-holder on the microtome. The microtome was then operated to cut thin uniform sections of the specimen tissues.

(f) Fixing Sections to Microscope Slides

During cutting, the sections became slightly compressed and creased. Before attaching the sections to slides these creases were removed by floating the sections on a warm water-bath after dividing the sections with a scapel into convenient lengths that would go on microscope slides. The sections were then lifted into clean albuminised microscope slides from the bath, being guided into position with a dissecting needle. The slides were then positioned upright on a wooden rack to drain. All the slides were subsequently arranged on a slide drying tray and left overnight in the incubator at 37°C

(g) Staining

(1) Removal of Wax

The sections were placed in xylol for 1 - 2 minutes to dissolve the wax.

(2) Hydration

The sections were taken out of xylol and transferred to absolute alcohol for 1 minute, when they became opaque. The sections were then removed from absolute alcohol, drained, and placed in 90% alcohol for 1 minute.

(3) Staining

The slides were transferred from 90% alcohol, after draining, to haematoxylin, where they were left for 30 - 40 minutes. After draining off excess haematoxylin the slides were transferred to the slide-washing tray, and washed in water until the sections became blue. The sections were then placed in acid alcohol where they were agitated for a few seconds and were returned to the slide-washing tray until blue again.

The sections were then transferred to 1% eosin for 2 - 4 minutes to counter stain them.

(4) Dehydration

After draining, the sections were transferred from the slide-washing tray to 90% alcohol where they were agitated for 10 - 15 seconds. From 90% alcohol they were placed in absolute alcohol I, where they were

agitated for 10 - 15 seconds. The slides were then taken into absolute alcohol II for 30 seconds.

(5) Clearing

The sections were taken from absolute alcohol II into xylol I and II and left until completely clear. This took about 15 seconds. The sections were again cleared in xylol II from which they were mounted.

(h) Mounting

The cover slips were cleaned and laid in rows on a pad of blotting paper. The slides were then removed from xylol and one or two drops of canada balsam were placed on each section. The slides were quickly inverted over the cover-slips being guided into place with a dissecting needle.

ix. Preparation of Blood Serum

Blood from a decapitated rat was passed through a clean and perfectly dry glass funnel into a clean and perfectly dry centrifuge tube. After about 5 minutes when a firm clot had formed, the content of the tube was centrifuged and the supernatant serum was collected.

x. Estimation of serum Bilirubin in the Test Rats (According to Powell et al. 1965).[11]

Into two 6" x ¾" test tubes were placed the following materials in the order and amounts set out below:

Materials	Test (Tube 1)	Blank (Tube 2)
Serum	0.4 ml.	0.4 ml.
Diazo reagent	0.2 ml.	-
Diazo blank	-	0.2 ml.
Benzoate urea solution	3.4 ml.	3.4 ml.
Distilled water	-	-

The tubes were allowed to stand at room temperature for 10 minutes and the optical density was read in an SP 600 spectrophotometer at 520 nm. wavelength using the blank to set the instrument to zero.

Standard

The optical density of the working standard was read in an SP 600 spectrophotometer at 520 nm. using distilled water to set the instrument to zero.

Calculation

$$\frac{\text{Optical Density of the test}}{\text{Optical Density of the Working Standard}} \times 4 = \text{mg. Bilirubin per 100 ml. serum.}$$

(4 = calculated strength of the Working Standard).

xi. Estimation of Total Blood Sugar
(According to Nelson, 1944)

Into four 6" x ¾" test tubes were placed the following materials in the order and amount arranged in the table below:

Materials	Test (Tube 1)	Low standard Tube 2)	High standard Tube (3)	Blank (Tube 4)
Isotonic sodium sulphate	3.6 ml.	3.6 ml	3.6 ml.	3.8 ml.
Blood	0.2 ml.	-	-	-
Low Standard	-	0.2 ml.	-	-
High Standard	-	-	0.2 ml.	-
10% Sodium Tungstate	0.2 ml.	0.ml.	0.2 ml.	0.2 ml.

The contents of each tube were mixed and centrifuged. 1 ml. of the blank, test and standards were introduced into a Folin and Wu tube and 1 ml. of the alkaline tartrate solution was added. The tubes were plugged with cotton wool and placed in a boiling water bath for 10 minutes.

The tubes were cooled and 3 ml. of Arsono-molybdate reagent was added to each. The tubes were left to stand for 5 minutes and the volumes were carefully made up to 25 ml. with distilled water. The contents of the tubes were mixed

carefully by inversion. The optical densities of the tests and standards were read in an SP.600 spectrophotometer at 680 nm., setting the instrument to zero with the blank.

Calculation

mg. glucose per 100 ml. of blood, =

$$\frac{\text{Optical Density of the test}}{\text{Optical Density of the Low standard}} \times 100$$

or

$$\frac{\text{Optical Density of the test}}{\text{Optical Density of the High standard}} \times 200$$

The standard nearest the reading of the test was used.

xii. Estimation of Urine urobilinogen

(According to Watson et al. 1944.)

To 5 ml. fresh urine was added 5 ml. of 10% $BaCl_2$ with shaking. This was filtered.

Into each of two 5 ml. Erlenmeyer flasks was added 2.5 ml. of the bile free urine (from above). 5 ml. of sodium acetate solution was added to one portion with shaking. 2.5 ml. of modified Ehrlich's reagent was added slowly with shaking. The content of the flask was then emptied into a 19 x 105 mm cuvet and the spectrophotometer (Sp 600) was set to zero absorbance at 565 nm. with it.

To the other portion of urine was added 2.5 ml. of modified Ehrlich's reagent with shaking. 5 ml. of sodium acetate solution was added. The absorbance was immediately read

against the blank.

Calculation

Reading of test x factor*

= Amount of urobilinogen in Ehrlich's unit.

Working Standard

10.2 ml. of the stock standard was measured into a 100 ml. volumetric flask, and diluted to volume with 0.5 per cent. acetic acid. This solution represents a urobilinogen concentration of 1.2 Ehrlich's units. A series of standards was prepared in cuvets as follows:

ml. of working standard	ml. of 0.5% Acetic Acid	Urobilinogen Ehrlich's Units
10	0	1.20
6	4	0.72
4	6	0.48
2	8	0.24
1	9	0.12

Absorbance readings were made in an SP 600 spectrophotometer against a water blank. The factor* was calculated by dividing each concentration by its respective reading and averaging the figures.

xiii. Estimation of total Serum Proteins (According to Watson et al. 1947)

Into a 6" x ½" test tube was placed 0.1 ml. of serum. 2.9 ml. of distilled water was added. Into another test tube was placed 3 ml. of distilled water (blank). 3 ml. of working biuret solution was added to each tube and the tubes were allowed to stand in a 37°C water bath for 10 minutes. The optical density of the test solution was read at 540 nm. in an SP 600 spectrophotometer by setting the instrument to zero with the blank. The protein concentration in g./100 ml. was obtained from the calibration curve.

The calibration curve was prepared with vercotol. The vercotol was reconstituted as directed on the vial and various graded concentrations were prepared as follows: 3.5g/100 ml., 4.0 g./100 ml., 4.5 g./100 ml., 5.0 g./100 ml., 5.5g./100 6.0 g./100 ml., 6.5g./100 ml., 7.0 g./100ml., and 7.5 g./100., A curve of absorbance at 540 nm. against the various concentration was then prepared.

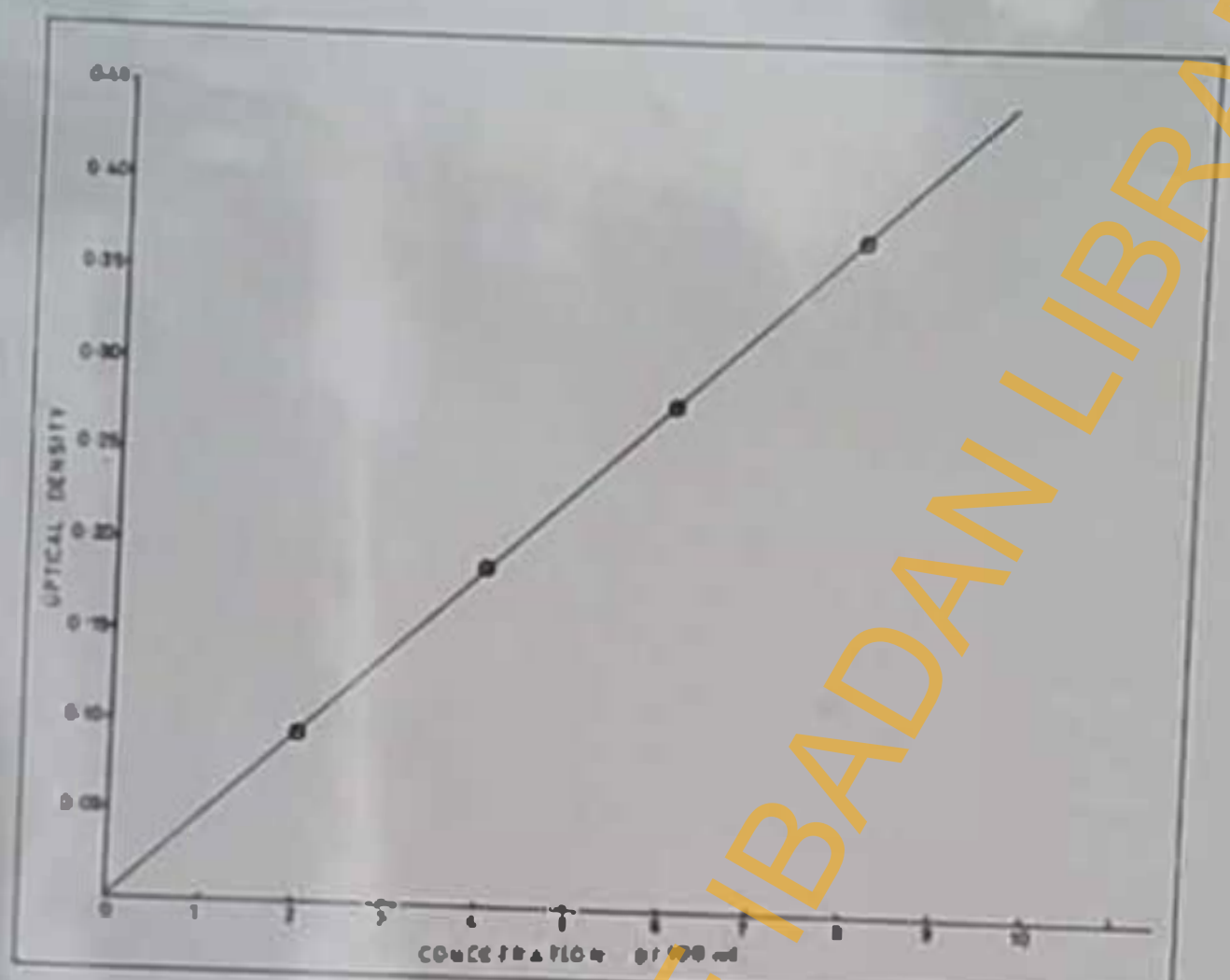

Fig. 19 Calibration curve for the estimation of total serum protein.

xiv. Estimation of serum Alkaline Phosphatase
(Method as recorded in BDH Enzyme Assay set 3 for Alkaline phosphatase)

Into 6" x ¾" test tubes were placed the following materials in order set out below:

Material	Sample	Blank
Solution 2	1.00 ml.	1.00 ml.
Serum	0.10 ml.	-

The test tubes were incubated in a water bath at 37°C for exactly 30 minutes and the following were mixed:

Sodium hydroxide	10.00 ml.	10.00 ml.
Serum	-	0.10 ml.

The solutions were poured into cuvettes and the optical densities were read in an SP.600 spectrophotometer at 405 nm. and the alkaline phosphatase concentration in the serum was calculated thus:

E405 nm. x 200 = milli. - units

I = U = 0.06 = mole Bassey - Lowry Units.

xv. Estimation of Serum glutamic Oxaloacetate transaminase (Method as recorded in BDH Enzyme Assay note 2 for SGO-T).

(a) Calibration curve

A series of tubes were set up as follows:

Tube No.	Water	2mM Standard	Sub-strate	mMoles Pyruvate per min. per lit.	Int. Units SGO-T
1	0.2 ml.	0 ml.	1.0 ml.	Blank	-
2	0.2 ml.	0.05ml	0.95ml.	8.5	6.0
3	0.2 ml.	0.10ml	0.90ml.	16.5	13.0
4	0.2 ml.	0.15ml	0.85ml.	25.0	22.0
5	0.2 ml.	0.2ml.	0.80ml	33.5	30.5
6	0.2 ml.	0.25ml	0.75ml	41.5	39.0
7	0.2 ml.	0.30ml	0.70ml	50.0	51.0

The standards and blank were incubated at 37°C for 30 minutes, 1 ml. of 2, 4-dinitrophenylhydrazine reagent was added to each tube and the tubes were incubated for a further 20 minutes. The tubes were removed from the water bath and 10ml. of 0.4m. sodium hydroxide was added to each tube with shaking. The tubes were allowed to stand for 10 minutes and the optical densities were recorded at 515mu. using an SP 600 spectrophotometer from the optical densities a calibration curve was plotted for the reagents in the set.

(b) <u>Determination of the enzyme Glutamic Oxalacetate transaminase in the serum</u>

Two test tubes were labelled "sample" and control respectively. Into each tube was placed 1 ml. of substrate. The temperature was brought to 37°C by placing the tubes in a water bath at this temperature. After noting the time, 0.2 ml. of serum was added to the "sample" tube, and both tubes were activated again at 37°C for 60 minutes. With the tubes still in the water bath 1 drop of aniline citrate reagent was added to each tube; after 5 minutes 1 ml. of 2, 4-dinitrophenylhydrazine solution was added to the control tube. Incubation was continued for a further 20 minutes after which the tubes were removed from the water bath and 10 ml. of 0.4 n. sodium hydroxide was added to each tube. The contents of the tubes were mixed by inversion and allowed to stand for 10 minutes. The optical densities was recorded at515 mu in the SP 600. The values for SGO-T in the International units was read from the calibration curve.

xvi. <u>Antibiotic treatment of animals</u>

Neomycin sulphate	100 mg
Tetracycline hydrochloride	50 mg
Bactracin sulphate	50 mg

A mixture of the above three antibiotics was administered orally to the rats twice daily two days before the experiment. Dosing was then repeated every other day during the experiment.

CHAPTER FOUR

EXPERIMENTS AND RESULTS.

INVESTIGATION ONE

A survey for nitrosamines in palm wine samples being hawked for sale in Ibadan, Western State of Nigeria.

Materials

(a) Palm Wine.

Four samples of palm wine being hawked for sale were purchased from each of seventeen areas of Ibadan, the capital of Western State of Nigeria. The seventeen sample areas were selected in such a way as to cover a large part of the City.

(b) Reagents and Equipments

Analytically pure reagents and very clean glasswares were used. Other materials and equipments used in this investigation are enumerated in Chapter II, sections (iv - xiii).

Methods

The analytical procedures relevant to this investigation have been described in detail in Chapter III, sections iii - vi. The analysis has been carried out by a combination of thin-layer chromatographic and colorimetric techniques.

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Analytically pure reagents and very clean glasswares were used. Other materials and equipments used in this investigation are enumerated in Chapter II, sections (iv - xiii).

Methods

The analytical procedures relevant to this investigation have been described in detail in Chapter III, sections iii - vi. The analysis has been carried out by a combination of thin-layer chromatographic and colorimetric techniques.

Spot tests were carried out with extracts on thin-layer plates prior to identification and quantitative estimation.

Results

In the spot tests only extracts for volatile, water-soluble nitrosamines gave indication of the presence of nitrosamine. Extracts for non-volatile nitrosamine gave negative results.

Separation on thin-layer chromatographic plates showed the presence of two kinds of nitrosamines. The nitrosamine spots had Rf values 0.24 and 0.48 respectively in Hexane - diethylether-dichloromethane (4:3:2), and were identified as dimethylnitrosamine and diethylnitrosamine respectively. Ultraviolet spectrum of the separated extract showed characteristic absorption maximum of nitrosamine in the region 230 - 240 nm; and in griess reagent the characteristic maximum of 525 nm.

The amounts of each kind of nitrosamine in palm wine from each sample area of Ibadan are shown in tableV.

TABLE IV

IDENTIFICATION OF NITROSAMINES IN PALM WINE EXTRACTS BY THIN-LAYER CHROMATOGRAPHY.

STANDARD NITROSAMINE	TESTS ON STANDARD NITROSAMINES			TESTS ON PALM WINE EXTRACTS			NITROSAMINE IN PALM WINE EXTRACT IDENTIFIED AS -
	*Rf x 100	SPOT COLOUR DETECTOR(A)	DETECTOR(B)	*Rf x 100	SPOT COLOUR DETECTOR(A)	DETECTOR(B)	
DIPHENYLNITROSAMINE	80	Blue-Violet	Pink-Red				
DIMETHYLNITROSAMINE	24	"	"	24	Blue-Violet	Pink-Red	DIMETHYLNITROSAMINE
DIETHYLNITROSAMINE	48	"	"	48	Blue-Violet	Pink-Red	DIETHYLNITROSAMINE
NITROSPIPERIDINE	63	"	"				
DIBENZYLNITROSAMINE	85	"	"				
NITROSOMORPHOLINE	40	"	"				

* DEVELOPING SOLVENT: n - Hexane-Diethyl Ether - Dichloromethane (4:3:2).

DETECTOR (A) : Pronosmam's Reagent.

DETECTOR (B) : Griess Reagent.

TABLE V.

AMOUNTS OF NITROSAMINES IN PALM WINE BEING HAWKED FOR SALE IN IBADAN.

SOURCE OF PALM WINE	NO. OF SAMPLES	NO. OF SAMPLES CONTAINING NITROSAMINE	KINDS AND MEAN AMOUNTS OF NITROSAMINE		TOTAL AMOUNTS OF NITROSAMINE (ug/litre.
			DIMETHYLNITROSAMINE	DIETHYLNITROSAMINE	
X 1 - Odo Ona	4	4	20.3	14.6	34.9
X 2 - Oio	4	4	16.0	10.4	26.4
X 3 - Unibadan	4	4	18.3	12.0	30.3
X 4 - Unife	4	4	14.3	9.8	24.1
X 5 - Bodija	4	4	17.5	12.2	29.7
X 6 - Barracks	4	4	17.0	12.7	29.7
X 7 - Kloyelo	4	4	18.6	12.4	31.0
X 8 - Agodi	4	4	20.0	10.7	30.7
X 9 - Jericho	4	4	15.5	12.5	28.0
X10 - Cat.Rest House	4	4	16.3	10.3	26.6
X11 - Jericho Hosp.	4	4	20.7	12.6	33.3
X12 - C.C.I.	4	4	20.0	10.9	30.9
X13 - Alaghon	4	4	20.2	10.7	30.9
X14 - New G.R.A.	4	4	18.3	12.9	31.2
X15 - Oke-Ado	4	4	17.4	10.1	27.5
X16 - Isale Ijebu	4	4	18.3	11.0	29.3
X17 - Kuleti	4	4	17.1	9.8	26.9

Conclusion

On the basis of the experiments carried out in this investigation, it can be stated that Palm Wine being hawked for sale in Ibadan, the capital of Western State of Nigeria, contains minute amounts of two kinds of nitrosamine namely, Dimethylnitrosamine and Diethylnitrosamine. The level of contamination being 20ug/litre and 10ug/litre respectively with a mean total level of 30ug/litre. The physiological role of this amounts of nitrosamine is the subject of another investigation in this thesis.

INVESTIGATION TWO.

An extensive survey for Nitrosamines in Palm Wine as hawked for sale in other parts of the Western State and some parts of Lagos State of Nigeria.

In this investigation a survey for nitrosamines in Palm Wine was carried out with samples purchased from fifteen towns in the Western and part of Lagos States in this country.

The pilot areas now include, Oyo, Ife, Abeokuta, Ondo, Akure, Okitipupa, Ijebu-Ode, Ogbomosho, Shagamu, Oshogbo, Otta and Ore in the Western State, and Badagri, Ikorodu and Epe in the Lagos State. In this way palm-wine samples have been collected from a large coverage of the States.

Materials

(a) Reagents and Equipments

Analytical grade reagents and solvents were used. Other materials and equipments used in this investigation are as enumerated in Chapter II, sections ii - xiii.

(b) Palm Wine

Twenty random samples of stale palm wine being hawked for sale were purchased from each of the towns mentioned above.

Methods

The analytical procedures relevant to this investigation have been described in detail in Chapter III, sections III - VI.

Results

The spot tests gave positive indication of the presence of volatile nitrosamines only.

Separation on thin-layer plates showed the presence of two kinds of nitrosamines, with the two detectors. These had Rf values of 0.24 and 0.48, respectively, and were identified as dimethylnitrosamine and diethylnitrosamine respectively. UV spectrometry of the separated extract showed characteristic maximum for nitrosamines in the region 230 - 240 nm; and in griess reagent the characteristic maximum of 525 nm.

The mean amounts of each kind of nitrosamine in palm wine from each sample area are shown in table.

Statistical analysis of the results for the various towns show that except in two places, Akure and Ondo, in the Western State, the mean amounts of nitrosamine in palm wine from each town do not differ significantly from the overall mean. This is an estimate of the expected amount of nitrosamine in a litre of palm wine.

The mean for Akure is just significant at the 5% point of T. There is a real difference here, which may be traceable to peculiarities of the areas in relation to palm wine production.

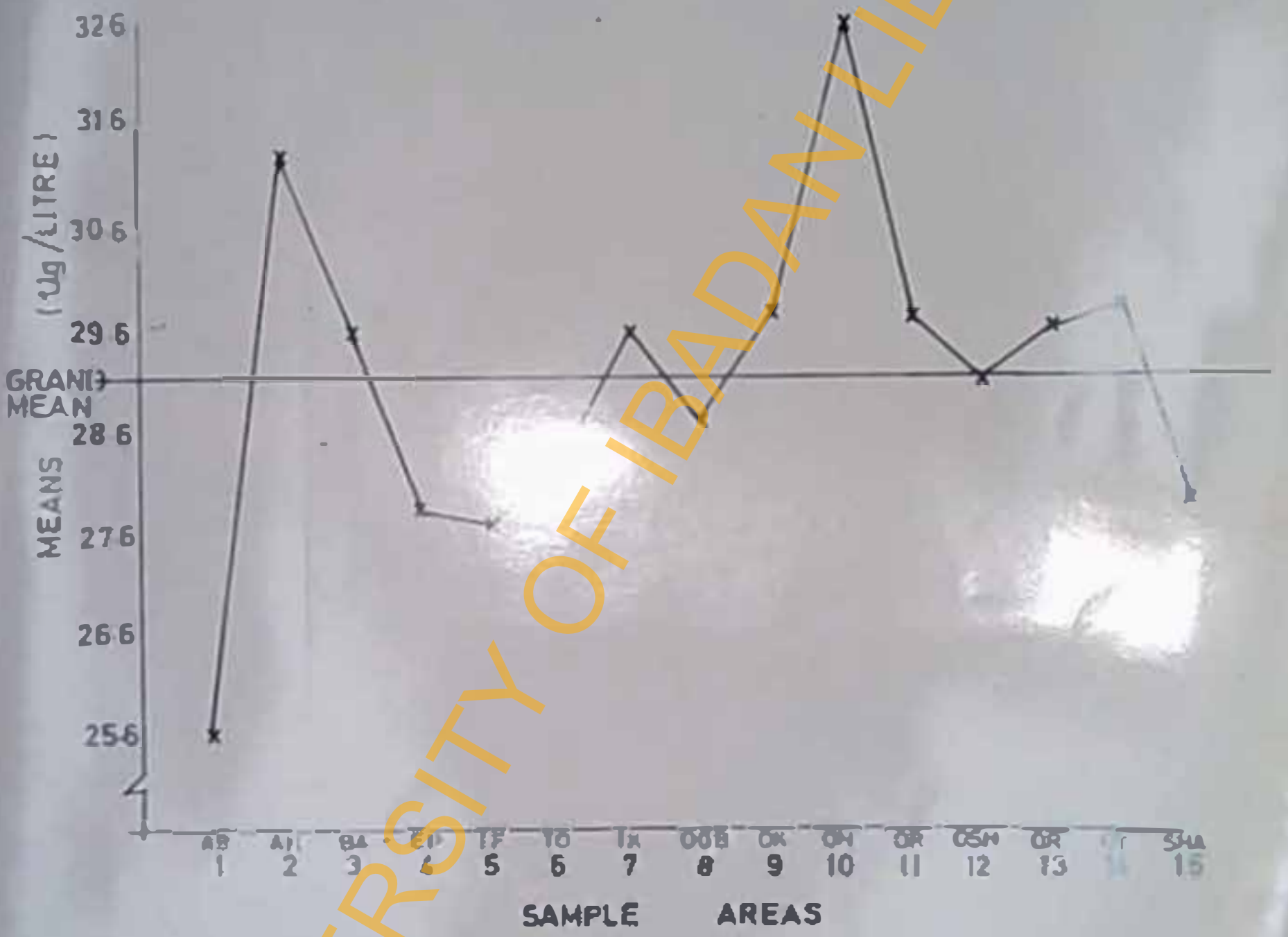
A positive correlation was found between the amounts of dimethylnitrosamine and diethylnitrosamine in the palm wine samples, suggesting that the distribution of the two compounds in palm wine follows a definite pattern.

A few samples (about 5 in all) showed no detectable amounts of nitrosamine.

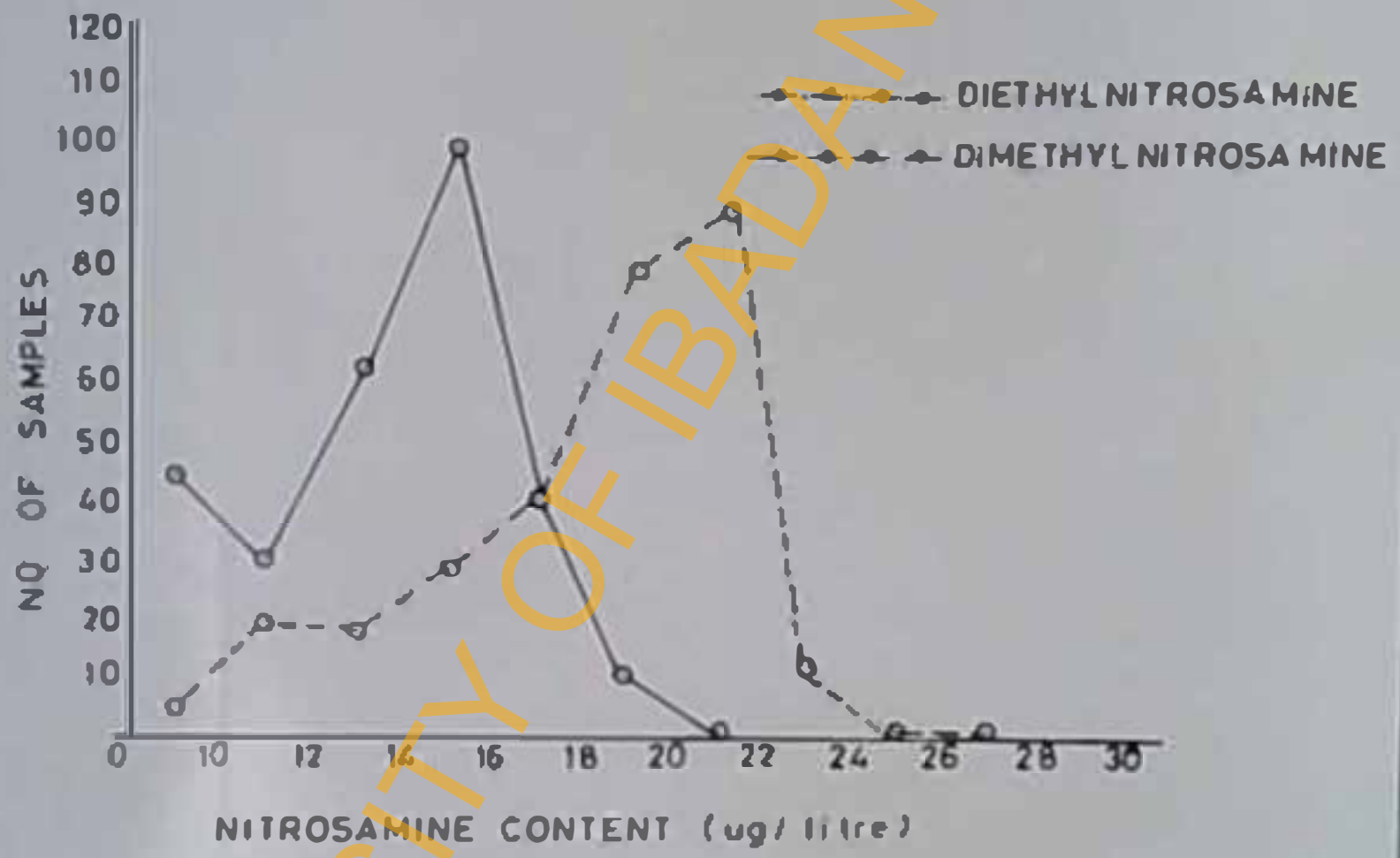
TABLE VI: AMOUNTS OF NITROSAMINES IN STALE PALM WINE FROM THE SAMPLE AREAS AND STATISTICAL ANALYSIS.

SOURCE OF PALM WINE	NO. OF SAMPLES	NO. OF SAMPLES CONTAINING NITROSAMINE	KINDS AND MEAN AMOUNTS OF NITROSAMINE		TOTAL AMOUNTS OF NITROSAMINE ug/litre.	STANDARD ERROR (S.E.)	t = id1 S.E.	SIGNIFICANCE.
			DIMETHYLNITROSAMINE ug/litre.	DIETHYLNITROSAMINE ug/litre.				
Abeokuta	20	20	14.60	11.00	25.60	± 2.2166	1.629	
Akure	20	20	18.15	13.00	31.15	± 0.9040	2.146	*
Badagri	20	20	17.20	12.50	29.70	± 1.6028	0.306	
Epe	20	20	15.95	12.00	27.95	± 2.4531	0.514	
Ibadan	20	20	16.90	12.00	28.90	± 2.5000	0.124	
Ife	20	20	16.20	11.60	27.80	± 2.2775	0.619	
Ijebu-Ode	20	19	16.42	11.88	28.30	± 2.4753	0.368	
Ikorodu	20	19	17.30	12.30	29.60	± 2.1156	0.184	
Ogbomosho	20	19	16.89	12.01	28.90	± 2.5100	0.124	
Okitipupa	20	20	17.00	13.05	30.05	± 1.7462	0.481	
Ondo	20	20	18.30	14.30	32.60	± 0.8777	3.862	**
Onhogbo	20	20	16.70	12.45	29.15	± 2.0905	0.029	
Otta	20	20	17.10	12.50	29.60	± 1.9217	0.203	
Oyo	20	19	17.60	12.40	30.00	± 2.5361	0.312	
Shagamu	20	20	16.50	11.45	27.95	± 2.2507	0.560	

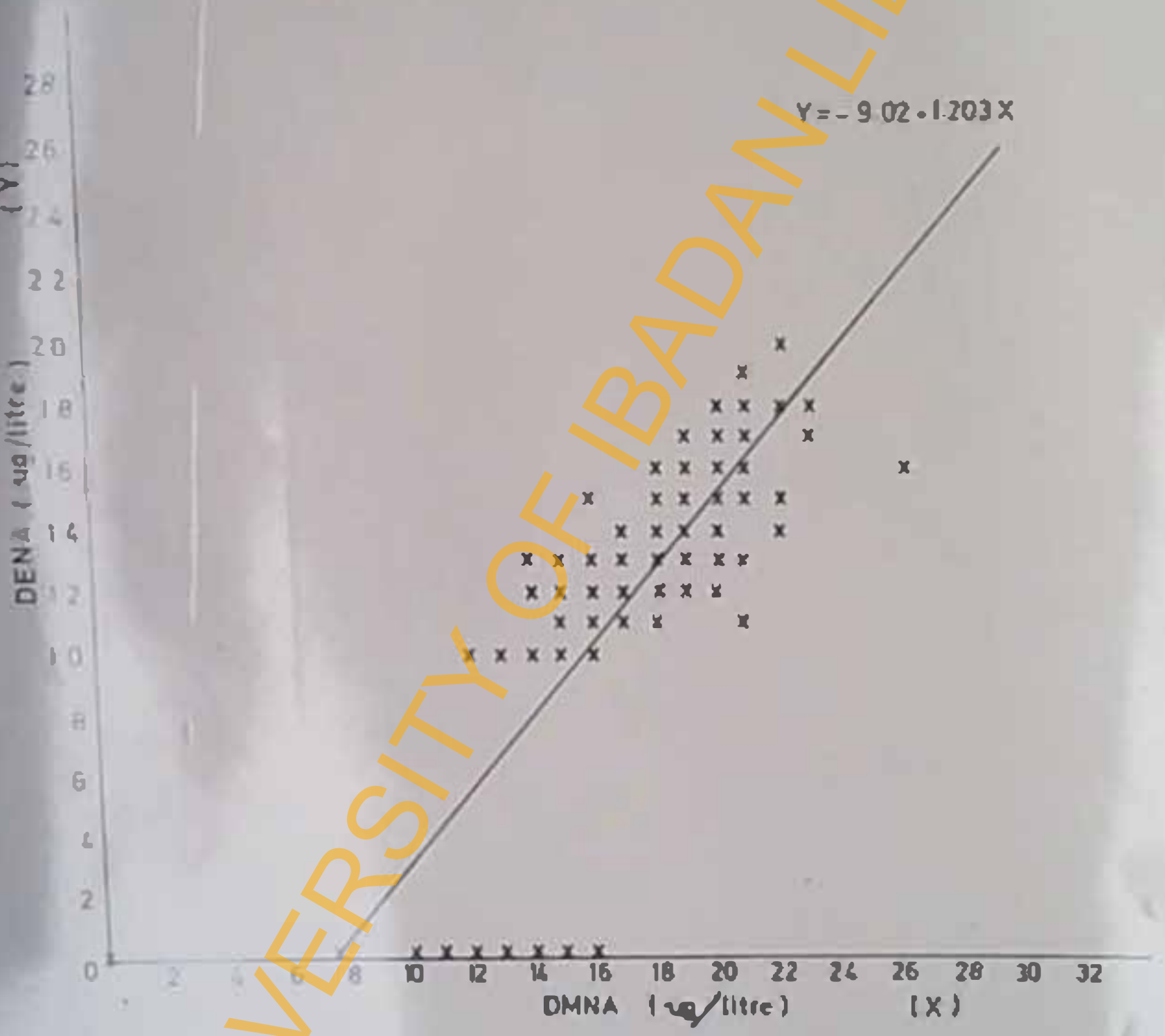
THE DISTRIBUTION OF THE MEAN AMOUNTS OF NITROSAMINE IN PALM WINE FROM VARIOUS SOURCES AROUND THE OVERALL MEAN



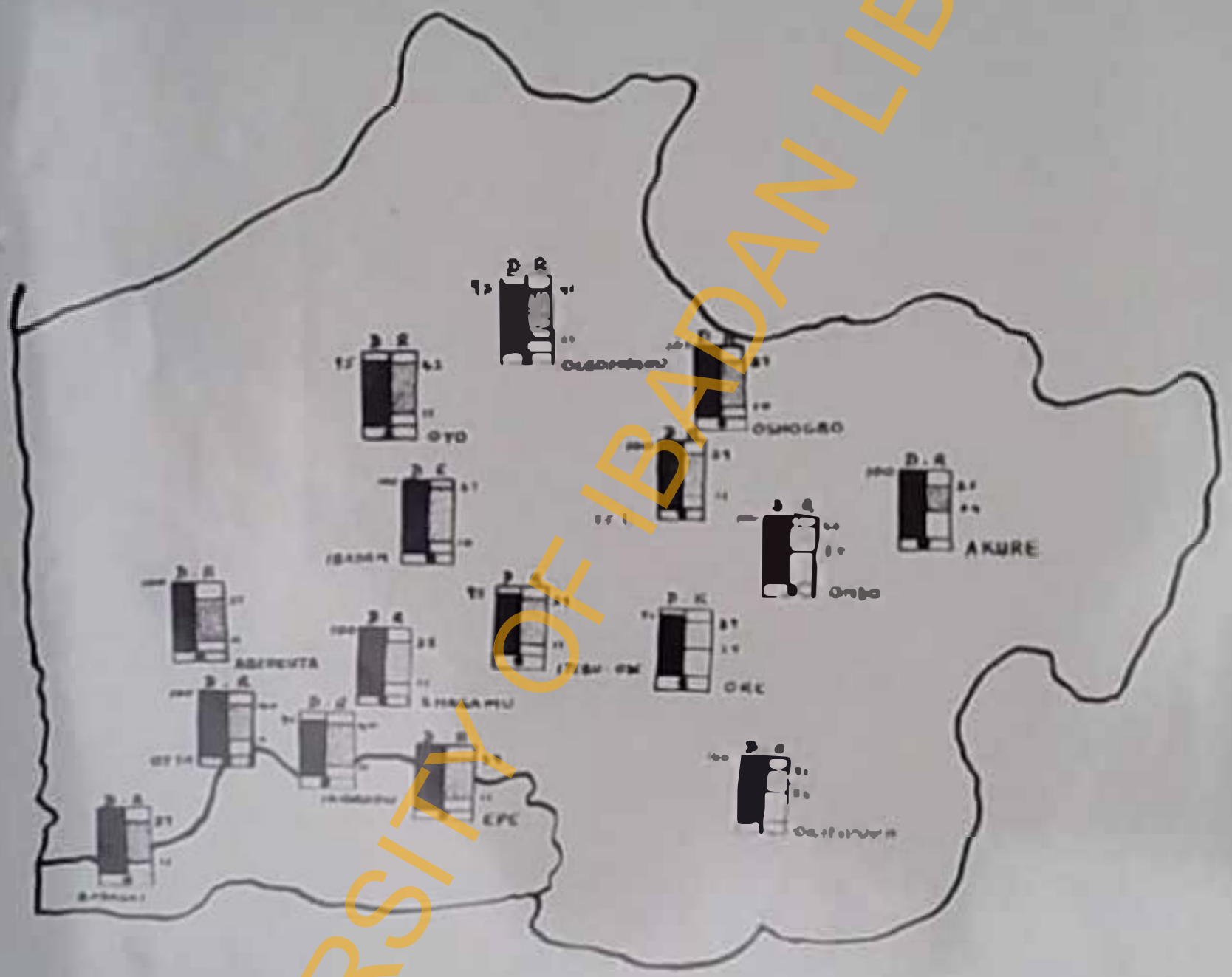
RELATIVE AMOUNTS OF NITROSAMINE IDENTIFIED
IN STALE PALM WINE FROM SAMPLE AREAS



SCATTER DIAGRAM SHOWING THE ASSOCIATION BETWEEN THE AMOUNTS OF DIMETHYLNITROSAMINE AND DIETHYLNITROSAMINE IN PALM WINE



MAP SHOWING DISTRIBUTION (%) AND RANGE (ug) OF NITROSAMINES IN PALM WINE FROM THE SAMPLE AREAS



HIGHEST DISTRIBUTION (%) = 100 (SOME SAMPLES CONTAIN ONLY ONE OF THE TWO KINDS DISCOVERED)

OVERALL RANGE = 10 —— 47 ug litre (EXCLUDING SAMPLES WITH NO NITROSAMINE)

Conclusion

This study has shown that palm wine as served for sale in the various towns of Western and Lagos States of Nigeria is contaminated with nitrosamine to a level of 30ug/litre.

Conclusion

This study has shown that palm wine as marketed for sale in the various towns of Western and Lagos States of Nigeria is contaminated with nitrosamine to a level of 30ug/litre.

INVESTIGATION THREE

A survey for Nitrosamines in Ogogoro, Burukutu, Pito, and Oti Agbagba.

In this investigation the amount of nitrosamine in Ogogoro, a local gin distilled from palm wine; Burukutu, a fermented beverage from millet grains; Pito, a fermentation product of maize or a mixture of maize and sorghum grains; and Oti Agbagba, a local alcholic beverage produced by the fermentation of overipe plantains, was determined.

The purpose of this part of the work was to assess the level of contamination of other locally available alcoholic beverages with nitrosamine.

Materials

(a) Reagents and Equipments

Analytical grade reagents and solvents were used. The equipments and glass-wares which found use in this investigation have been described in Chapter II Sections ii - xiii.

(b) The Alcoholic Beverages

Twenty random samples of Ogogoro, and of each of the other alcoholic beverages refered to above were purchased from various hawkers in Ibadan and nearby villages. Each sample was analysed in triplicate for nitrosamine.

Methods

The analytical procedures relevant to this investigation are as described in Chapter III, Sections (iii - vi).

Results

The spot tests gave indication of the presence of volatile nitrosamine. Extracts for water insoluble nitrosamine showed no detectable amount of nitrosamine.

Separation on thin-layer chromatographic plates indicated the presence of two kinds of nitrosamines in the extracts from Ogogoro, Burukutu, and Pito, using the two spray reagents. These have Rf values 0.24, and 0.48 respectively with Hexane-Ether-dichloromethane (4: 3: 2) as the developer, and they were identified as dimethyl-nitrosamine and diethylnitrosamine, respectively. In the case of Oti Agbagba only one spot corresponding to dimethylnitrosamine was observed.

The table shows the mean value of each kind of nitrosamine found in each brew and the graphs show the pattern of distribution of the nitrosamine types.

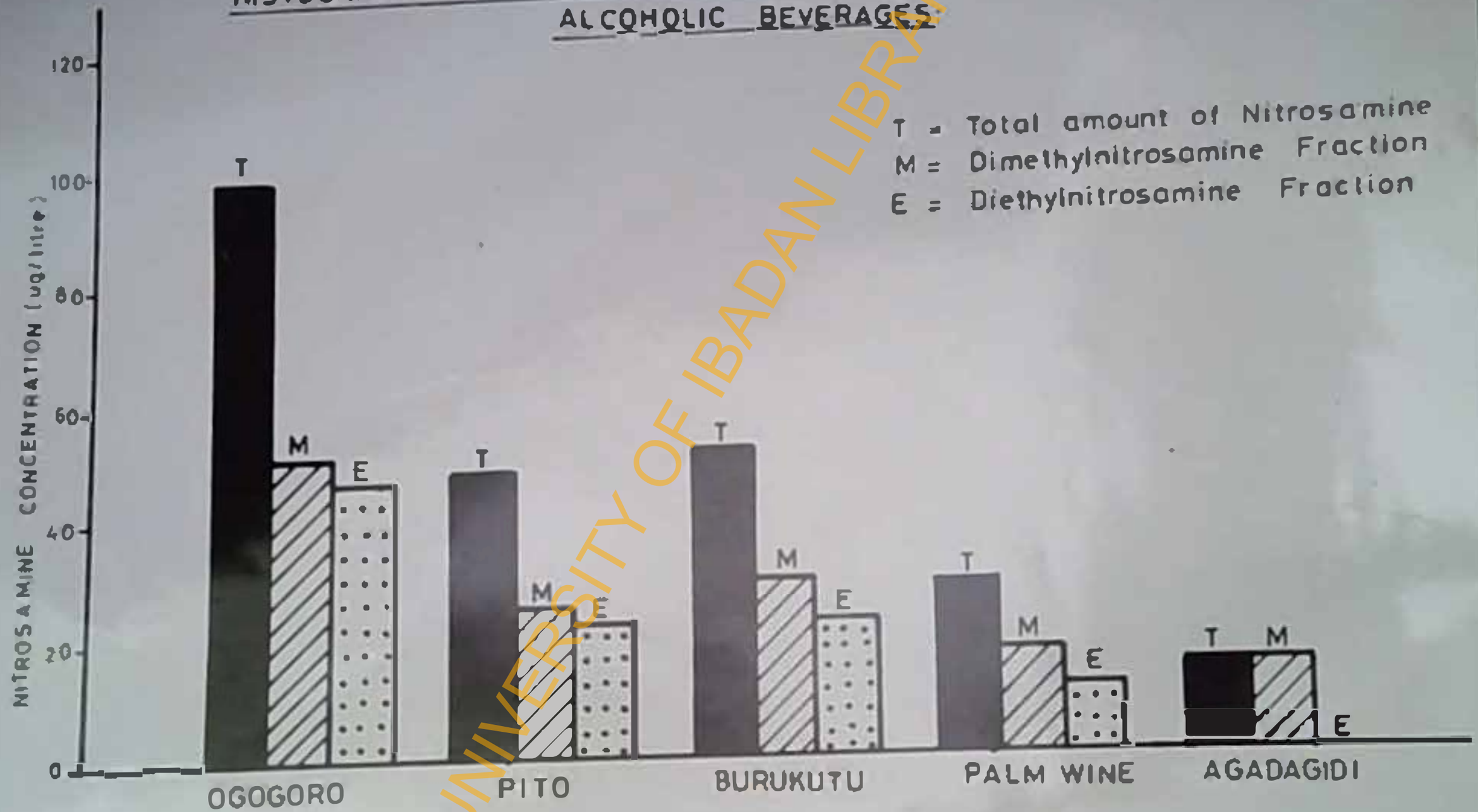
TABLE VII:

KINDS AND AMOUNTS OF NITROSAMINES IN OGOGORO, BURUKUTU, PITO AND OTI AGBAGBA (AGADAGIDI).

ALCOHOLIC BEVERAGE.	NUMBER OF SAMPLES	NUMBER OF SAMPLES CONTAINING NITROSAMINE	DISTRIBUTION PERCENT SAMPLE	KINDS OF MEAN AMOUNTS OF NITROSAMINE		TOTAL AMOUNTS OF NITROSAMINES (ug/litre.
				DIMETHYLNITROSAMINE (ug/litre.)	DIETHYLNITROSAMINE (ug/litre.)	
Ogogoro	20	20	100	52	46	± 98 ± 2.7312
Burukutu	20	20	100	29	22	51 ± 2.4753
Pito	20	20	100	25	23	48 ± 3.0411
Oti Agbagba (Agadagidi)	20	16	80	14	-	14 ± 1.1315

HISTOGRAM SHOWING THE DISTRIBUTION OF NITROSAMINE IN THE ALCOHOLIC BEVERAGES

T = Total amount of Nitrosamine
M = Dimethylnitrosamine Fraction
E = Diethylnitrosamine Fraction



Conclusion

The results of the experiments carried out in this investigation have demonstrated the presence of dimethylnitrosamine and diethylnitrosamine in Ogogoro, Burukutu and Pito. The total level of contamination of these drinks with nitrosamine being about 100 ug/litre for Ogogoro; 50ug/litre for Burukutu; 48ug/litre for Pito.

Only one kind of nitrosamine - dimethylnitrosamine was found in Oti Agbagba; the level of contamination of this drink with nitrosamine was only 14 ug/litre.

Since nitrosamines were found in all the samples of Ogogoro, Burukutu and Pito, it can be stated that these alcoholic drinks generally contain nitrosamine.

INVESTIGATION FOUR

A study of the biological production of nitrosamines in Palm Wine

This investigation is concerned with the assessment of the role of the palm wine fermenting organisms in the production of nitrosamine in this alcoholic beverage; having regard to the fact that bacteria could nitrosate secondary amines in the presence of nitrite ions (Sander , 1968).

Materials

(a) Reagents and Equipments.

The reagents and solvents used in this investigation were of analytical grade. Glass-wares were thoroughly washed with a detergent prior to a final wash with acetone. Other materials and equipments used in this investigation are as enumerated and described in Chapter II, sections ii - xiii.

(b) Palm Wine

The palm wine samples were purchased from hawkers in Ibadan, in desired quantities.

(c) Palm Sap

This is the fermented sap of the palm tree. It was collected in sterile flasks by a specially commissioned palm wine tapper.

Methods

The investigation was carried out in seven experiments.

Experiment I Palm Wine samples were purchased as hawked for sale in Ibadan. Each batch of four samples were left to continue fermenting until 6, 12, 18, 24, 30, 36, 42, and 48 hours after purchase respectively, by placing a litre of each sample in a 3 litre conical flask. The mouths of the flasks were plugged lightly with cotton wool and the flasks were placed in a fine wire-mesh cage to keep off flies and other insects. After the palm wine samples had fermented for the appropriate number of hours, analysis for nitrosamines in them were carried out. The experiment was carried out in quadruplicate.

Experiment II Palm sap samples were collected in sterile flasks and the samples were allowed to stand for the various number of hours as described for palm wine in experiment I. After the samples had also fermented for the appropriate number of hours analyses of their nitrosamine contents were carried out. The experiment was carried out in quadruplicate.

Experiment III Palm wine samples as available for sale were purchased in batches of four. One litre of each sample was placed in a plastic bag and allowed to freeze. The frozen samples were left under this condition for the same period of time that their counterparts were allowed to ferment in experiment I of this investigation; i.e. 6, 12, 18, 24, 30, 36, 42, and 48 hours respectively after which they were analysed for nitrosamine. The experiment was carried out in quadruplicate.

Experiment IV Palm sap samples were treated as above for palm wine. The experiment was carried out in quadruplicate.

Experiment V Palm wine samples were purchased and refluxed in a water bath for 30 minutes at 50°C. After this pre-treatment each batch of four samples were allowed to stand (ferment) for the number of hours refered to earlier prior to analyses for nitrosamine in the samples. Pre-treatment of the samples as stated above was to kill the fungi population in the palm wine. The experiment was carried out in quadruplicate.

Experiment VI Palm wine samples as tapped for sale were obtained and filtered through a sterile filter to remove all yeast and bacteria present in them.

After this pre-treatment, each batch of four samples were allowed to stand for the 6, 12, 18, 24, 30, 36, 42, and 48 hours respectively after which they were analysed for nitrosamine.

Experiment VII Palm sap samples were treated as described for palm wine in Experiment VI. The experiment was carried out in quandruplicate.

Results

The nitrosamine contents of the variously treated palm wine and palm sap samples are shown in Table VIII. The relationship between the various results are depicted in the graphs (figures 25 - 28).

Experiment I The results show a linear relationship suggesting that as fermentation progresses the level of nitrosamine in palm wine increases.

Experiment II A similar trend as above was observed when palm sap was used. The rise in nitrosamine content with fermentation time here was however of a lower magnitude.

Experiment III There were no increases in nitrosamine content of palm wine with time. This was because the samples were frozen and as such the activities of the yeast and bacteria in the palm wine samples were markedly reduced if not totally arrested.

Experiment IV The results of this experiment show a somewhat similar trend as the results of experiment III. However the amounts of nitrosamine content of the frozen palm sap were smaller than those recorded for frozen palm wine.

Experiment V The purpose of this experiment was to kill the yeast cells present in the palm wine samples and to observe the potential role of the bacterial population in the production of nitrosamine in palm wine. In this experiment increases in nitrosamine content were still observed with fermentation time although the amounts detected were smaller than the amounts detected when the activities of both the bacteria and fungal population were undisturbed.

Experiment VI The removal of the fungal and bacterial population in the palm wine samples resulted in the distortion of the linear relationship observed in experiment I where these organisms were retained in the samples.

Experiment VII When palm sap was treated as for palm wine in experiment VI, a similar trend was observed but again the magnitude of the nitrosamine content recorded was smaller than it was for palm wine.

TABLE VIII:

NITROSAMINE CONTENTS OF THE VARIOUS PALM WINES AND PALM SAP SAMPLES.

SAMPLE AND TREATMENT	OBSERVATION TIME IN HOURS/NITROSAMINE CONTENT IN mg/litre.								
	0	6	12	18	24	30	36	42	48
EXPERIMENT I	23	27	32	35	40	43	27	50	52
EXPERIMENT II	15	20	24	27	30	35	39	42	46
EXPERIMENT III	21	23	22	25	26	26	25	27	26
EXPERIMENT IV	13	15	16	19	16	17	18	18	19
EXPERIMENT V	20	24	27	31	35	38	42	44	44
EXPERIMENT VI	18	20	21	22	20	19	21	22	20
EXPERIMENT VII	12	12	14	11	13	13	11	12	12

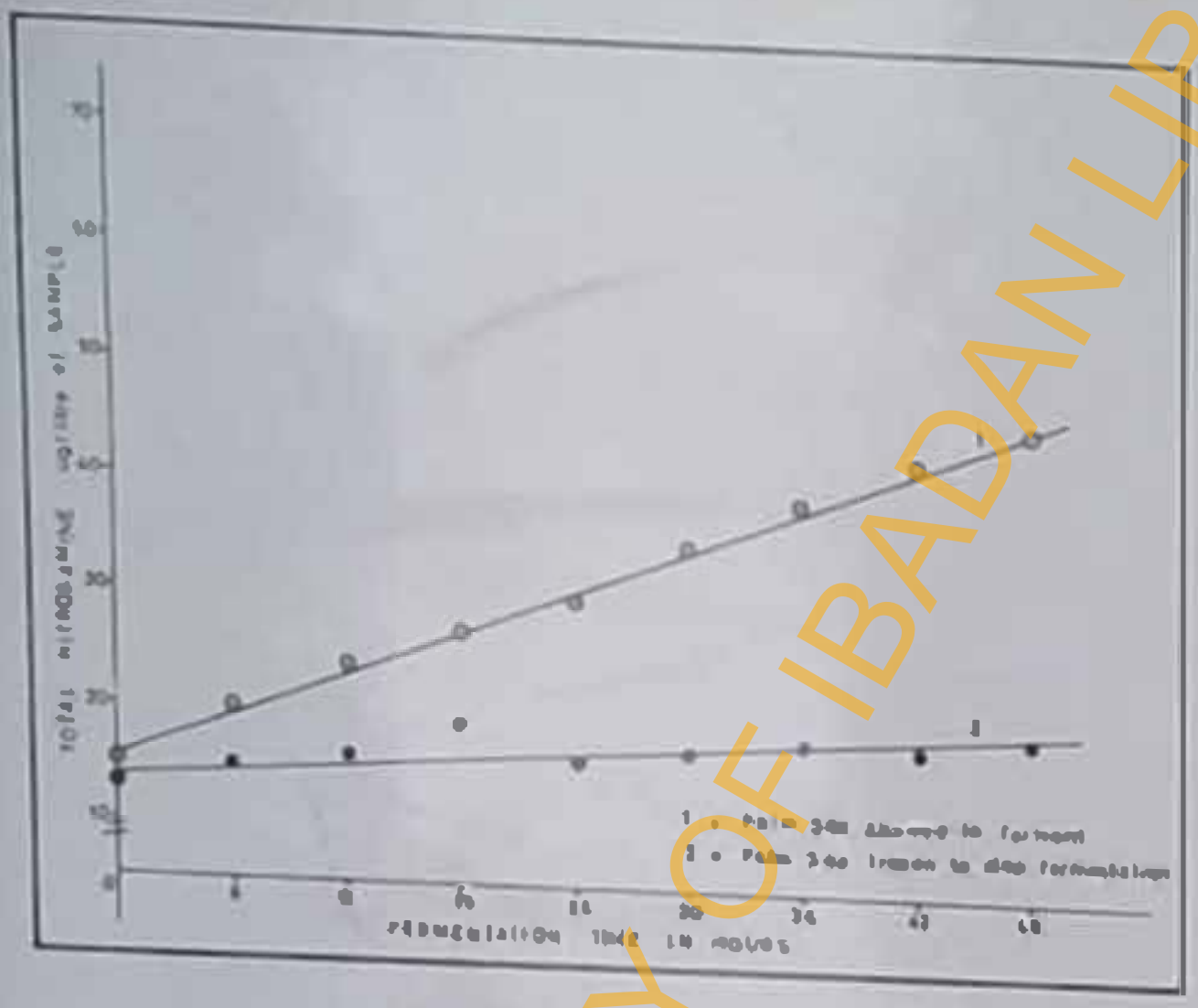


Fig 25

Variation in Nitrosamine content of Palm Sap with Fermentation Time.

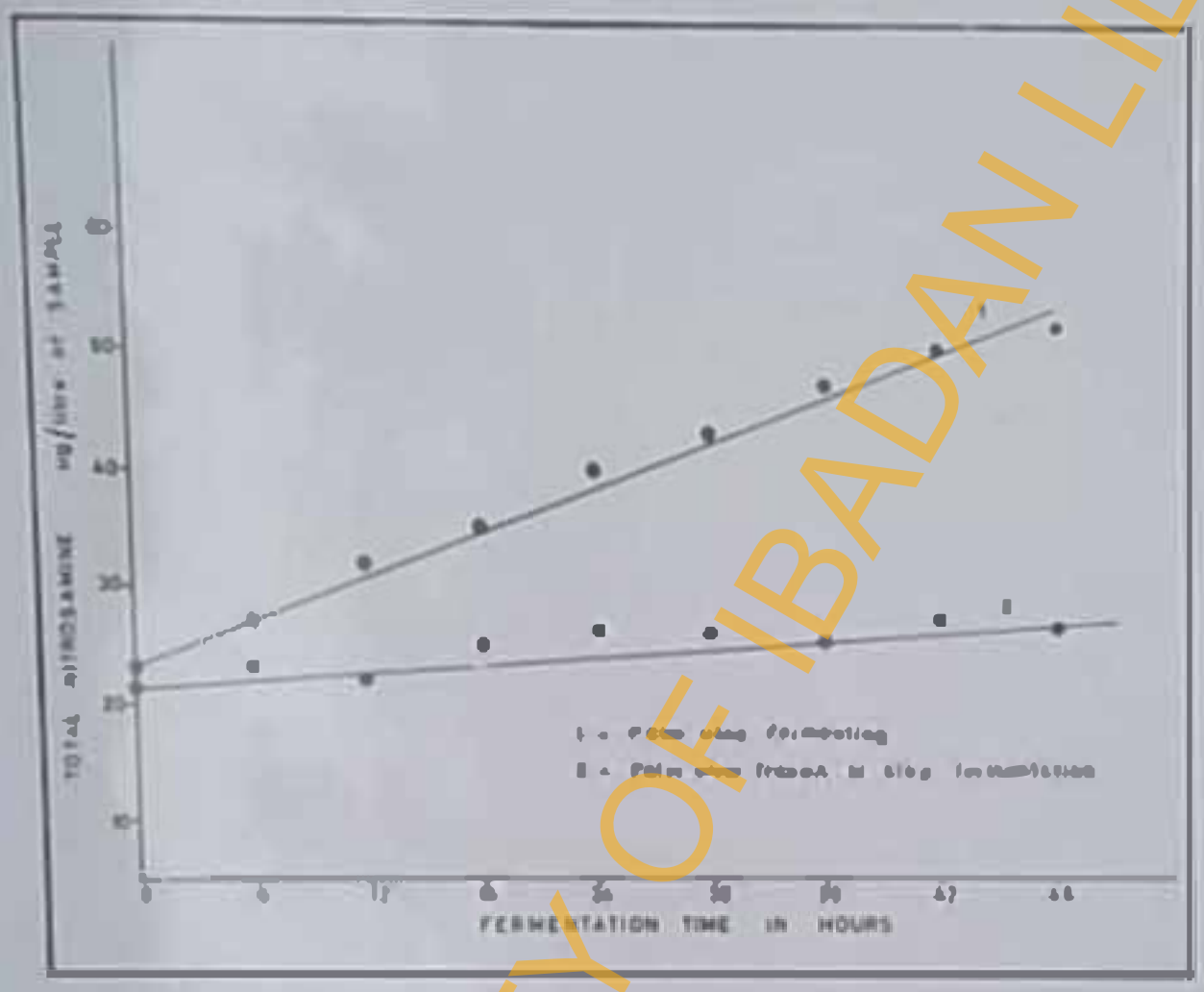


Fig 26

Variation in Nitrosamine content of Palm Wine with Fermentation time.

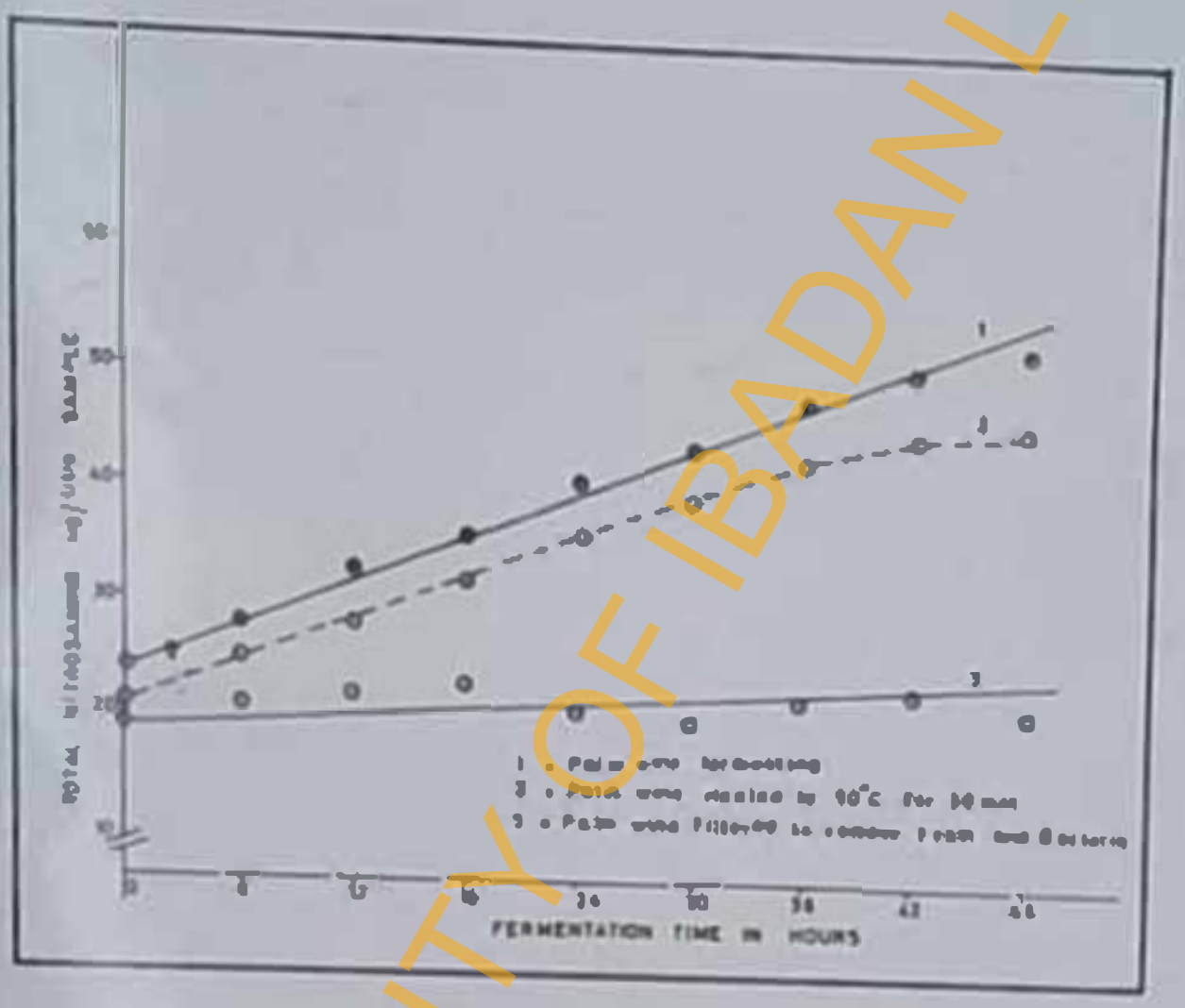


Fig. 27

Nitrosamine content of Palm Wine variously treated.

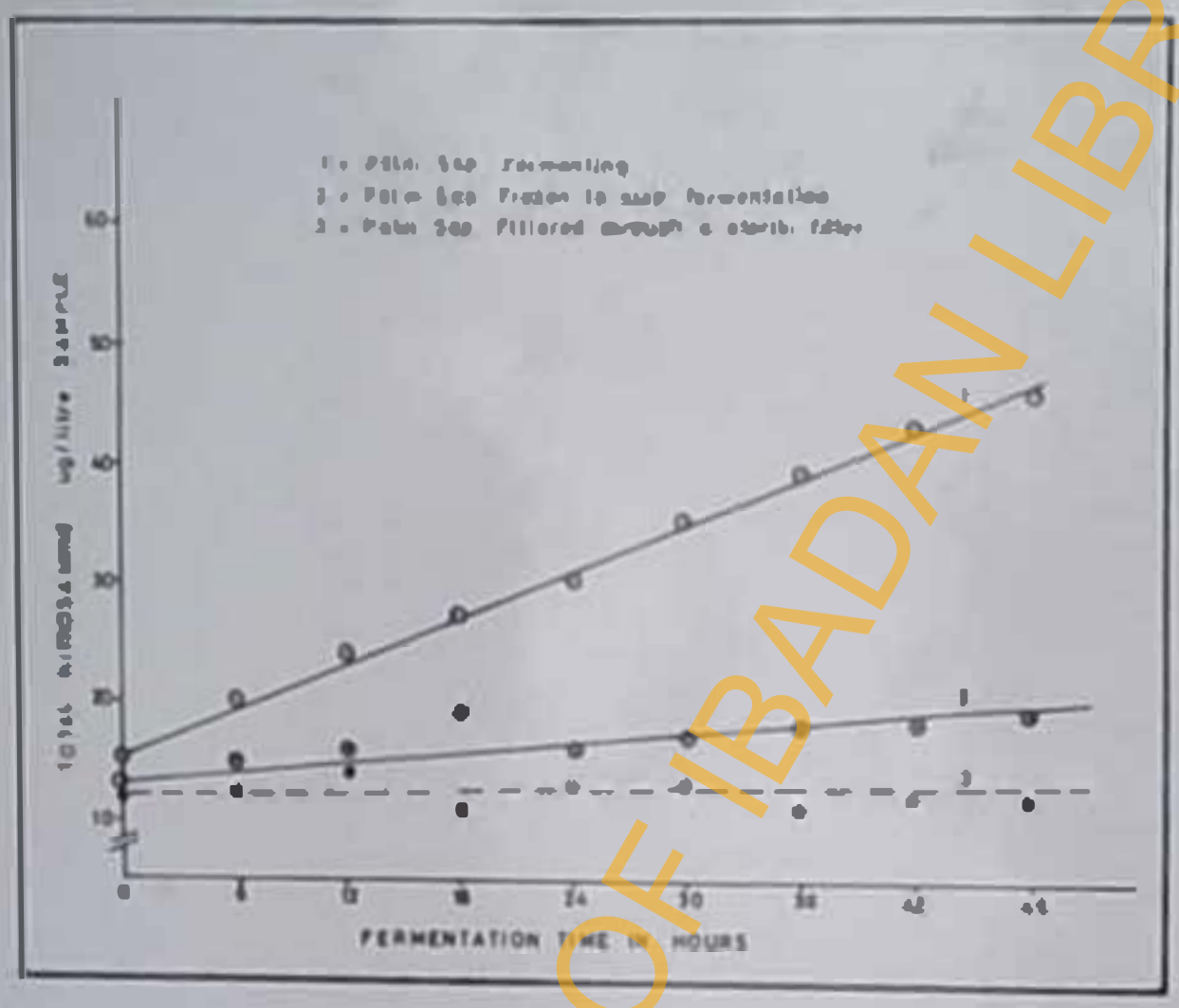


Fig 28

Nitrosamine content of Palm Sap variously treated.

Conclusions

Since progress in fermentation is dependent on the activities of the fungal and bacterial population in palm wine (Bassir, 1968), it might be possible to relate the increase in nitrosamine content of the palm wine samples with fermentation time, to the activities of these organisms.

The similar trend observed when palm sap was allowed to ferment, is an additional evidence in support of the direct involvement of the fermenting organisms in the formation of nitrosamine in palm wine.

Conclusive evidence is drawn from the control experiments where fermentation was arrested, (1) by freezing the samples, and (2) by sterilising the samples. No direct relationship suggesting increase in nitrosamine content with fermentation time was observed then, and it would appear that the nitrosamine found in these samples after treatment as above, would be that which had been formed, in the samples prior to treatment.

The detection of some nitrosamine in the frozen and particularly in the sterilised palm sap suggests that some nitrosamine is formed in the sap whilst within the plant. This would confirm an earlier finding by various workers including Dupleiss (1969) that a spontaneous reaction does occur in plants between secondary amines and nitrite to form nitrosamines.

An explanation of the role of the fermenting organisms would be the production of nitrosamine precursors during their diverse metabolic processes and a possible interaction between these and nitrite ions to form nitrosamines.

That only dimethylnitrosamine and diethylnitrosamine have been detected in palm wine suggests that either dimethylamine and diethylamine are the two most readily formed secondary amines during the activities of the fermenting organisms or that they are the two most readily available secondary amines for nitrosation under the prevailing conditions.

INVESTIGATION FIVE

2. INVESTIGATION 5a.

A study of some of the Biochemical effects of nitrosamine.

Previous experiments by various workers have demonstrated unequivocally that dimethylnitrosamine is primarily a potent hepatotoxin, (Magee and Barnes, 1956, 1963, 1967, 1970; Druckrey et al 1960; Preussmann et al. 1965, 1966). The purpose of this investigation is therefore to assess the level of alterations in some of the biochemical functions of the liver which in consequence contribute to the histopathological lesions characteristic of dimethylnitrosamine poisoning.

Materials

(a) Experimental Animals.

Litter mates of white albino rats inbred in the departmental animal house were used in this investigation. The rats selected were all males with weights ranging from 99 - 102 grams.

(b) Diet. The diet of the experimental rats was the stock diet for rats purchased from Livestock Feeds Nig. Ltd.

(c) Nitrosamine.

Graded amounts of dimethylnitrosamine were fed to the rats.

Procedure.

(a) Arrangements of the Experimental Rats

Four rats were placed on each treatment but each rat was housed in a separate metabolic cage.

(b) Dosing with Nitrosamine

Dimethylnitrosamine was used. The levels administered were as enumerated below and the route of administration was oral via the drinking water.

(1) 100ppm DMNA in daily drinking water.
(2) 50ppm " " " " "
* (3) 0.1ppm " " " " "
(4) Palm Wine in place of drinking water.
(5) Normal drinking water.

*Nitrosamine level in Ogogoro.

(c) Environmental conditions

The experiments were carried out in the departmental Animal house where the rats were exposed to similar environmental conditions, i.e. temperature, relative humidity, light, etc.

(d) Assay Period

Assay period was for 15 days on each "drug" treatment.

(e) Analytical techniques

The analytical techniques relevant to this investigation are described in detail in Chapter III, sections 9 - 15.

At the end of the 15 day trial period. The rats were decapitated and serum was collected from their blood. Serum of animals on the same trial were pooled before the various determinations were made.

Criteria for Judgement

(a) Influence of the various graded levels of dimethylnitrosamine on the growth (weight gains or losses) of the experimental rats.

(b) Biochemical effects of the various "drug" levels as estimated from the following liver function tests:-

PHYSIOLOGIC BASIS FOR TEST	TEST	SIGNIFICANCE
1. Bile Pigment Metabolism	(a) Serum Bilirubin	Hyperbilirubinemia indicates failure of the liver to excrete bilirubin produced in the reticulo-endothelial tissue from the catabolism of the heme pigment.
	(b) Urine Urobilinogen	Increase in urine urobilinogen may occur in complete obstruction of the bile duct and in hemolytic jaundice. Also increased blood destruction from any cause urine urobilinogen level. Urine urobilinogen may also be increased in damage to the hepatic parenchyma.

PHYSIOLOGIC BASIS FOR TEST	TEST	SIGNIFICANCE
2. ENZYME ACTIVITY	(a) Serum Alkaline Phosphatase	Since alkaline phosphatase is normally excreted by the liver, these values are increased in obstructive jaundice. In a purely haemolytic jaundice there is no rise. Various other factors such as hepatic damage also affect alkaline phosphatase activity in the serum.
	(b) Serum Glutamic Oxaloacetate transaminase (SGO-T)	Injury of the hepatic tissue is accompanied by elevations in serum glutamic oxaloacetate transaminase.
3. METABOLIC FUNCTIONS (a) PROTEIN SYNTHESIS	Serum Protein level.	Hypoprotenemia may be due to inadequate dietary protein intake. In addition, in acute and chronic liver diseases e.g. cirrhosis, there is a

PHYSIOLOGIC BASIS FOR TEST	TEST	SIGNATURE
		general tendency to hypoproteneamia. The severity of hypoproteneamia may serve as a criterion of the degree of damage.
b. CARBOHYDRATE METABOLISM.	Blood Sugar.	The characteristic sugar of the blood and of tissues fluids is glucose. The liver is the organ primarily responsible for the regulation of blood glucose concentration. A rise or fall in normal blood sugar level is an indication of some abnormality in the functioning of the liver.

Results:

From the observations made on the growth of the experimental rats, a level of contamination of 100ppm and 50ppm dimethylnitrosamine impressively retarded growth of the experimental rats over the experimental period.

a level of contamination of 0.1ppm (i.e. level of nitrosamine found in Ogogoro), while not supporting growth did not cause appreciable fall. Rats on palm wine (for level of nitrosamine in Palm Wine), manifested good growth and showed better weight gains than the control, probably because of the vitamins and other growth promoting factors in pale wine.

The liver function tests show that some of the biochemical effects of dimethylnitrosamine poisoning are a reduction in serum protein level; an impressive rise in the level of blood sugar; increase in the concentration serum bilirubin coupled with a slight rise in urine urobilinogen. There was also a sharp increase in the level of serum glutamo oxalacetate transaminase. The magnititude of these concentration changes were proportional to amount of toxin administered.

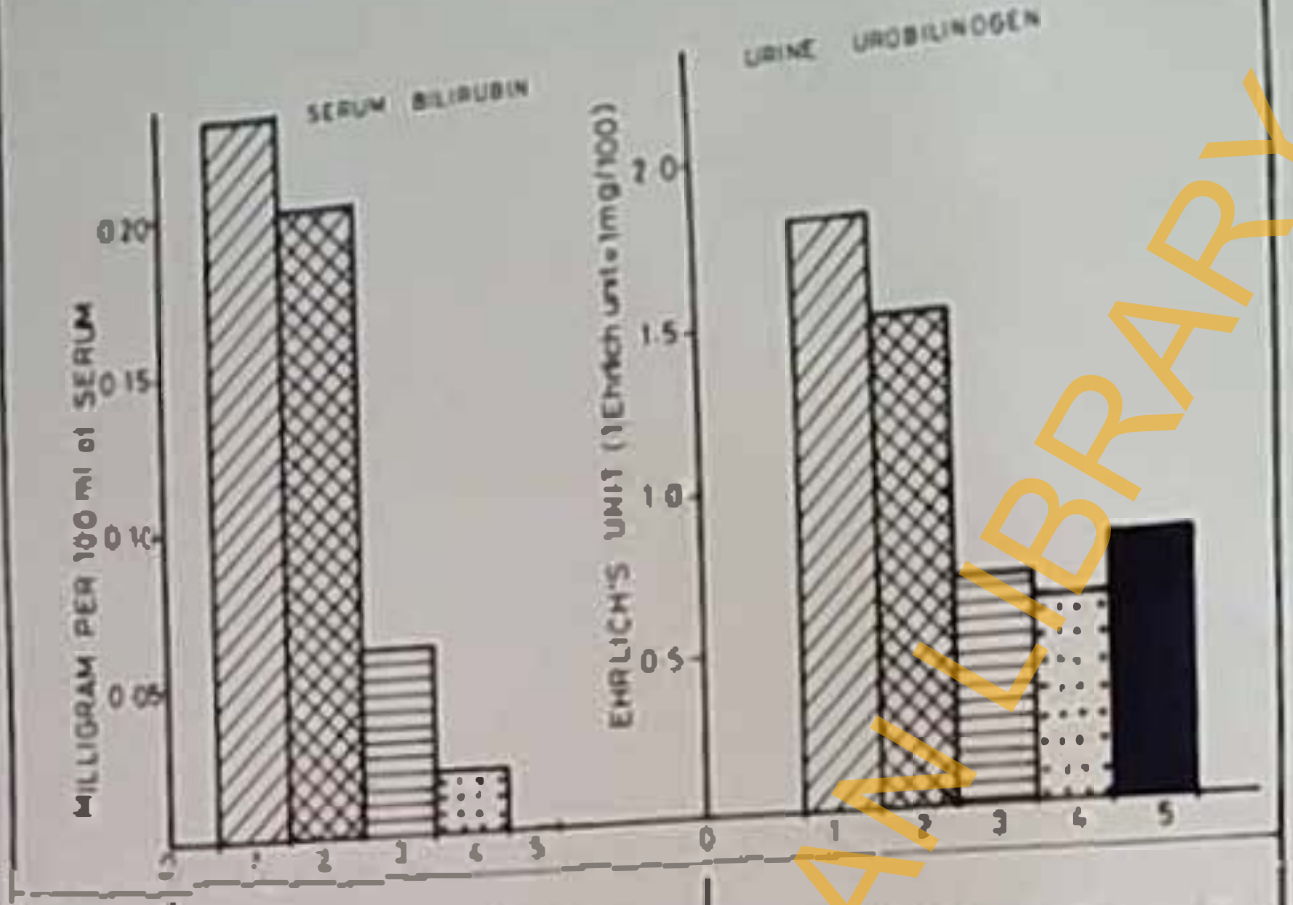
The overall results are shown in table IX and the graphs show the trend of the various results.

TABLE II.

THE EFFECT OF NITROSAMINE POISONING ON LIVER FUNCTION.

TREATMENT	MEAN START WEIGHT OF RATS (GMS)	MEAN LAST WEIGHT OF RATS (GMS)	MEAN WEIGHT GAIN OR LOSS (GMS)	LIVER FUNCTION TESTS					
				TOTAL SERUM PROTEIN (G/100ml SERUM)	BLOOD GLUCOSE mg/100ml BLOOD	SERUM BILIRUBIN mg/100ml SERUM	URINE UROBILINOGEN (EHRLICH'S UNITS)	ALKALINE PHOSPHATASE B.L. UNITS	SERUM GLUTAMIC OXALACETATE TRANSAMINASE. INT. UNITS.
1/100ppm Dimethylnitrosamine in daily drinking water for 15 days.	100.1	114.5	+14.4	5.0	141.4	0.26	1.8	6.10	26.9
1/30ppm Dimethylnitrosamine in daily drinking water for 15 days.	100.5	116.1 ± 12	+15.6	5.2	134.2	0.20	1.5	7.50	22.9
0.1ppm Dimethylnitrosamine in daily drinking water for 15 days.	101.0	126.5 ±10.8	+25.5	6.7	87.8	0.06	0.70	4.70	11.0
Pala replaces drinking water for 15 days.	100.9	130.9 ± 7.5	+30.0	6.7	89.4	0.02	0.65	3.60	8.6
Normal, uncontaminated drinking water for 15 days.	100.2	129.8	+29.6	6.8	86.0	0.00	0.60	3.90	8.2

CHANGES IN SERUM BILIRUBIN, SERUM ALKALINE PHOSPHATASE, SERUM GLUTAMIC OXALACETIC TRANSAMINASE, SERUM PROTEIN AND BLOOD SUGAR LEVEL OF RATS ON VARIOUS DOSES OF DIMETHYLNITROSAMINE



Conclusion

On the basis of the experiments carried out in this investigation, some of the early biochemical changes induced by dimethylnitrosamine in the course of progressive liver damage are (1) an impairment of bile pigment metabolism as exhibited by increases in serum bilirubin and urine urobilinogen; (2) inhibition of protein synthesis resulting in a fall in serum protein level; (3) disruption of the blood sugar regulatory mechanism leading to a sharp rise in blood sugar; (4) A considerable leakage into the blood of the enzymes primarily produced in the liver e.g. alkaline phosphatase and serum glutamic oxaloacetate transaminase. These effects are not limited to high doses of dimethyl-nitrosamine as rats on 0.1ppm (i.e. level of nitrosamine found in Ogogoro) also showed the same trend, although in a much less remarkable manner.

INVESTIGATION 5b.

A study of the pathological effects of Nitrosamine in the Rat.

Procedure

(a) Arrangement of the Rats. A group of ten white albino rats was placed on each treatment. The arrangement was repeated with both sexes, respectively.

(b) Treatment. The rats were placed on stock diet ad libitum. Dosing with nitrosamine was oral via the drinking water. The doses administered were graded as follows:

Group	(1a)	Rats placed on drinking water containing 500ppm	DMNA
"	(1b)	" " " " " "	DMNA
"	(2a)	" " " " " 200	" DMNA
"	(2b)	" " " " " "	" DMNA
"	(3a)	" " " " " 100	" DMNA
"	(3b)	" " " " " "	" DMNA
"	(4a)	" " " " " 50	" DMNA
"	(4b)	" " " " " "	" DMNA
"	(5a)	" " " " " 25	" DMNA
"	(5b)	" " " " " 12.5	" DMNA
"	(6a)	" " " " " "	" DMNA
"	(6b)	" " " " " "	" DMNA
"	(7a)	" " " " " 0.1	" DMNA
"	(7b)	" " " " " "	" DMNA
"	(8)	Normal drinking water.	

Fresh solutions were made each day.

Results.

The responses of the various groups of rats to the various levels of contamination of their drinking water with nitrosamines were as follows:

Group 1a: Rats on 500ppm Diethylnitrosamine in their daily drinking water.

Behavioral Changes.

Food intake of the rats on this treatment in the first four days was 6 grams as compared with 10 grams for the control group. However there was a sharper loss of appetite, and food intake fell to a daily average of 3 grams in the second week, and 0.3 grams in the third week, by the end of which all the rats had died. The rats started losing weight from their second day on this treatment and this was the picture throughout the experiment. By the third week the mean weight of the rats had fallen from the mean starting weight of 150.7 grams to 116.0 grams. There was also a marked reduction in water intake. By the sixth day all the rats appeared ill, sitting quietly with ruffled hair coat, wet especially around the ear orifice. Those that attempted to walk had a wobbly gait. The most striking external defect was the marked emaciation of flesh.

Results.

The responses of the various groups of rats to the various levels of contamination of their drinking water with nitrosamines were as follows:

Group 1a: Rats on 500ppm Diethylnitrosamine in their daily drinking water.

Behavioral Changes.

Food intake of the rats on this treatment in the first four days was 6 grams as compared with 10 grams for the control group. However there was a sharper loss of appetite, and food intake fell to a daily average of 3 grams in the second week, and 0.5 grams in the third week, by the end of which all the rats had died. The rats started losing weight from their second day on this treatment and this was the picture throughout the experiment. By the third week the mean weight of the rats had fallen from the mean starting weight of 150.7 grams to 116.0 grams. There was also a marked reduction in water intake. By the sixth day all the rats appeared ill, sitting quietly with ruffled hair coat, wet especially around the sex organs. Those that attempted to walk had a wobbled gait. The most striking external defect was the marked emaciation of flesh.

the first rat to die on this treatment did so on the seventh day after an accumulated intake of 29.1 mg. dimethyl-nitrosamine. And the last rat died after a total dose of 40.6 mg. Female rats responded more or less similarly to this treatment as the males.

Pathological Changes.

This level of dimethylnitrosamine proved to be acutely toxic to the experimental rats. At autopsy all the rats on this treatment showed extreme emaciation and body fat was completely absent. The rats also showed the presence of small amounts of blood stained fluid in the peritoneal cavity. In some rats this fluid had a colour and consistency approaching that of pure blood. In some others it was orange or almost colourless. The source of these haemorrhages into both the peritioneal cavity and lumen of the gut was not apparent. The livers were small and pale, but regular in outline. The pancreas was oedematous with a jellylike appearance. At cellular level the liver was intersected by irregularly shaped bands of tissue composed of hepatic cells in various degrees of degeneration. Bile duct proliferation was occasionally seen.

The lungs were somewhat congested and in some rats showed small haemorrhagic areas. The kidneys were normal but the spleen was shrunken and had a markedly reduced red-cell content.

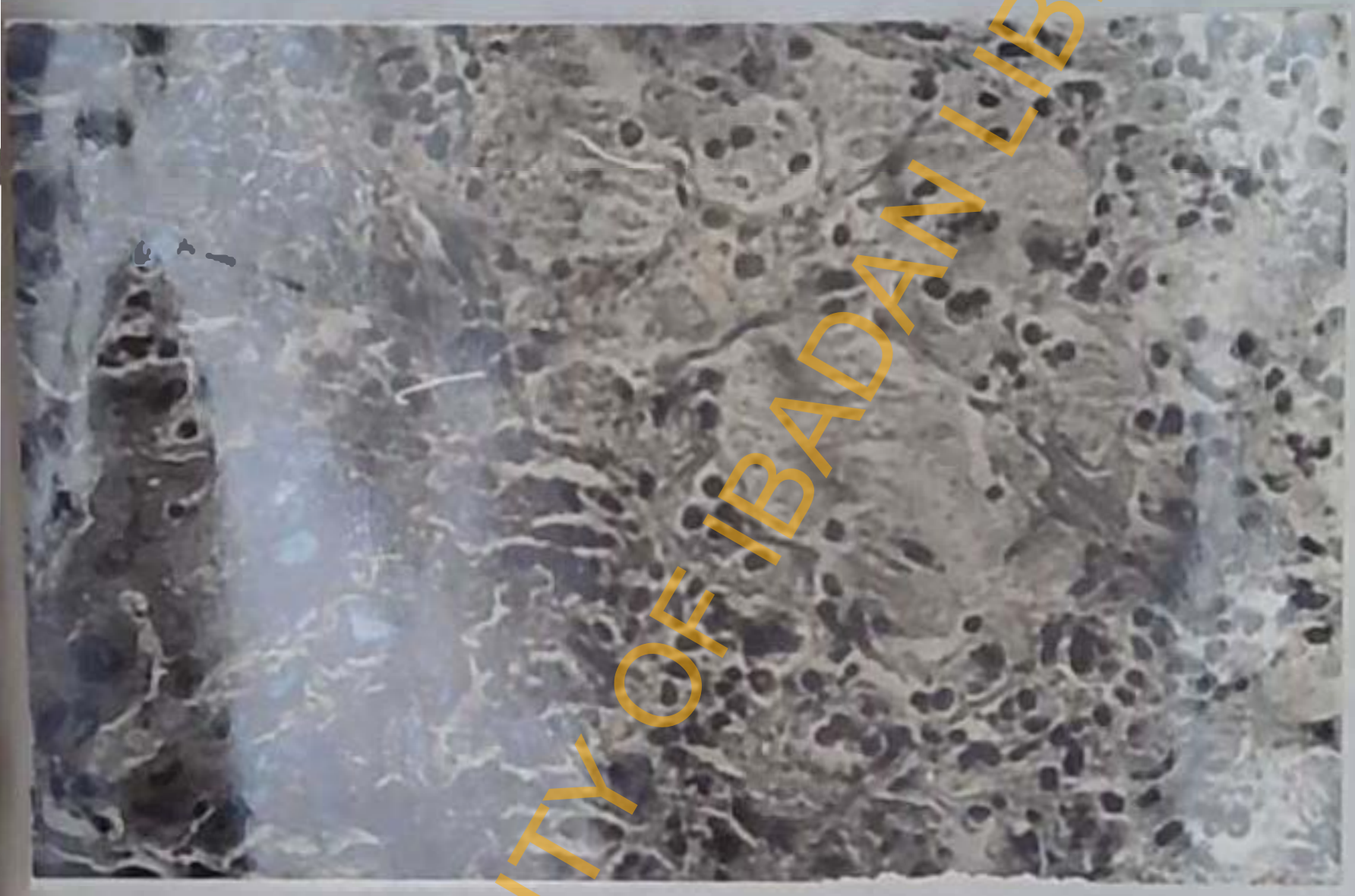


FIG.30 Rat liver after intake of drinking water contaminated to a level of 500ppm with Dimethyl-nitrosamine for 3 weeks. Irregular formation of fibrous tissue H and E X 30 c.

Group 1b: Rats on 500ppm Dimethylnitrosamine in their daily drinking water.

Behavioral Changes.

Food intake of rats on this treatment followed the same trend as described for their counterparts on an equivalent dose of dimethylnitrosamine. Marked emaciation of flesh was again a prominent feature, but the first rat to die on this treatment did so after an accumulated intake of 60mg on the 18th day. Again male and female rats exhibited similar behavioral responses.

Pathological Changes:

Severe damage to the gross composition of the internal organs was not observed. However the liver appeared small and congested and in all the rats the pancreas was oedematous. Cellular damage was again restricted to the liver and the red pulp of the spleen was replaced by large pale cells with vacuolated granular cytoplasm. Acutd hyperplasia was the striking feature of hepatic damage.

Group 2a: Rats on 200ppm Dimethylnitrosamine in their daily drinking water.

Behavioral Changes:

Food intake and growth of the rats on this treatment was on the decline throughout the experiment. Similar changes in external features were noted as for rats in group one. However the rats on this treatment survived for a longer time - six weeks as compared with less than three weeks recorded for rats in group one. By the time the first rat died the mean weight of the group had fallen from the mean starting weight of 150.3 grams to 91.4 grams for female rats and from 150.6 grams to 94.4 grams for the males.

Pathological lesions:

In those rats dying after five weeks on this treatment the liver was small, pale and somewhat irregular in outline. The edges of the lobes were folded but no tumour was observed. Histological changes in the liver included necrosis of the hepatocytes with condensation of the reticulum, bile duct proliferation, cell hyperplasia and thickening of the upper capsule with an increase in reticular fibres. Five of the rats on this treatment had haemorrhage into the gut. The amount of this was again variable and in one rat it was 15ml.

In all the rats the pancreas was either oedematous or prominently white or opaque. There was a marked reduction in the number of red cells in the spleen.

In those rats whose stomachs were distended histological examinations showed no detectable damage to the various parts of this organ. There was no visible damage to the gross structure of the kidney and its fine structure revealed no abnormality. All the other organs were also intact.

Group 2h: Rats on 200ppm Diethylnitrosamine in their daily drinking water.

Behavioral Changes:

There was a gradual fall in food intake and growth was retarded. Both sexes were affected as in the other groups. The death rate was however lower on this treatment than on the last treatment. The last rat to die on this treatment did so after the 8th week, with an accumulated diethylnitrosamine intake of 110mg.

Changes in external features were observed by the fifth week. The hair coat was ruffled and the rats were weak. By the 7th week the rats had lost much flesh that they appeared bony and repulsive.

Pathological Lesions:

No major abnormality was observed on the gross conformation of the various organs and even the liver was only small and pale.

In all the rats there were evidences of haemorrhage into the gut. The pleural surfaces of the lungs showed a number of discrete brown stains scattered over them. Histologically the liver showed acute liver injury with considerable variation from animal to animal. The bands of necrotic tissue showed variation in the proportion of its different constituents - degenerating hepatic cells, red cells and red cell debris, macrophages, fibroblasts and young connective tissue.

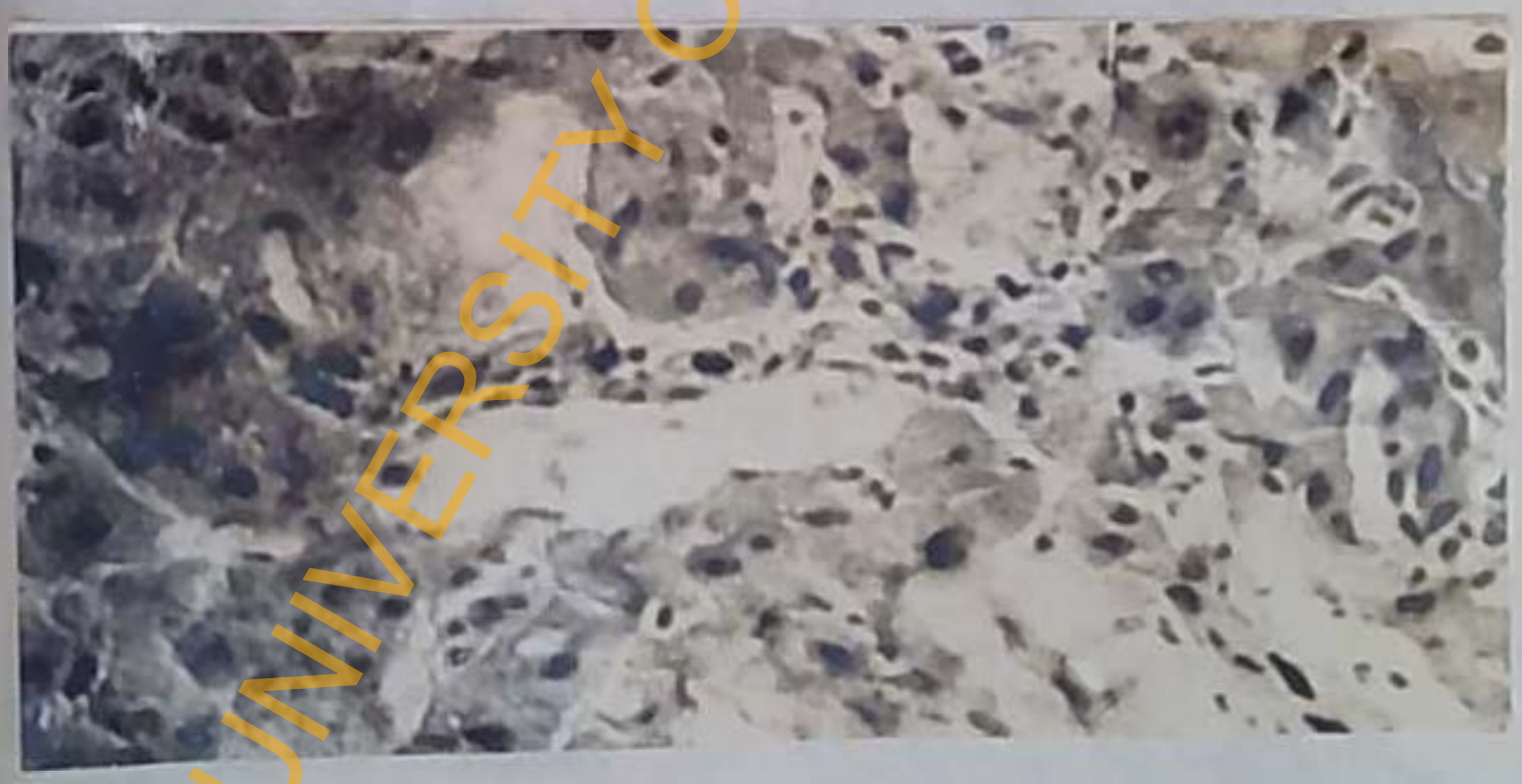


FIG. 31 Rat liver after intake of drinking water contaminated with 200ppm Diethylnitrosamine for 7 weeks - showing extremely severe structural change. H & E X. 300.

Group 3a: Rats on 100ppm Dimethylnitrosamine in their daily drinking water.

Behavioral Changes:

Growth of animals on this treatment was on the decline throughout the experiment as was the case with the earlier groups discussed. There were however some increases in weight although the overall picture was that of growth retardation. By the fourth week the rats appeared puffy and food intake had started to fall. At 60 days the mean weight of the group was only 65% of that of the control. All the rats died between the 62nd and the 90th day on this treatment.

Pathological Lesions:

This dose also proved actually toxic. Emaciation of flesh was again apparent but some body fat was present. Seven of the rats had haemorrhage into the gut. In one rat the amount of this was 30ml. This in fact was the highest amount recorded throughout the experiments. The livers were smaller than those of the control, but they were regular in outline and some had fatty lobes. In all the rats the pancreases was again oedematous. No tumour was observed on any organ and the liver was characterised by hyperplastic condition.

In some rats the spleens were quite small while in others they were rather large. This organ had a reduded number of red blood cells. The kidneys were haemorrhagic. The testis were smaller than those of the control but their fine structure revealed no abnormality of any form. The ovaries too were intact. The characteristic oedematous feature of the pancreas was again prominent.

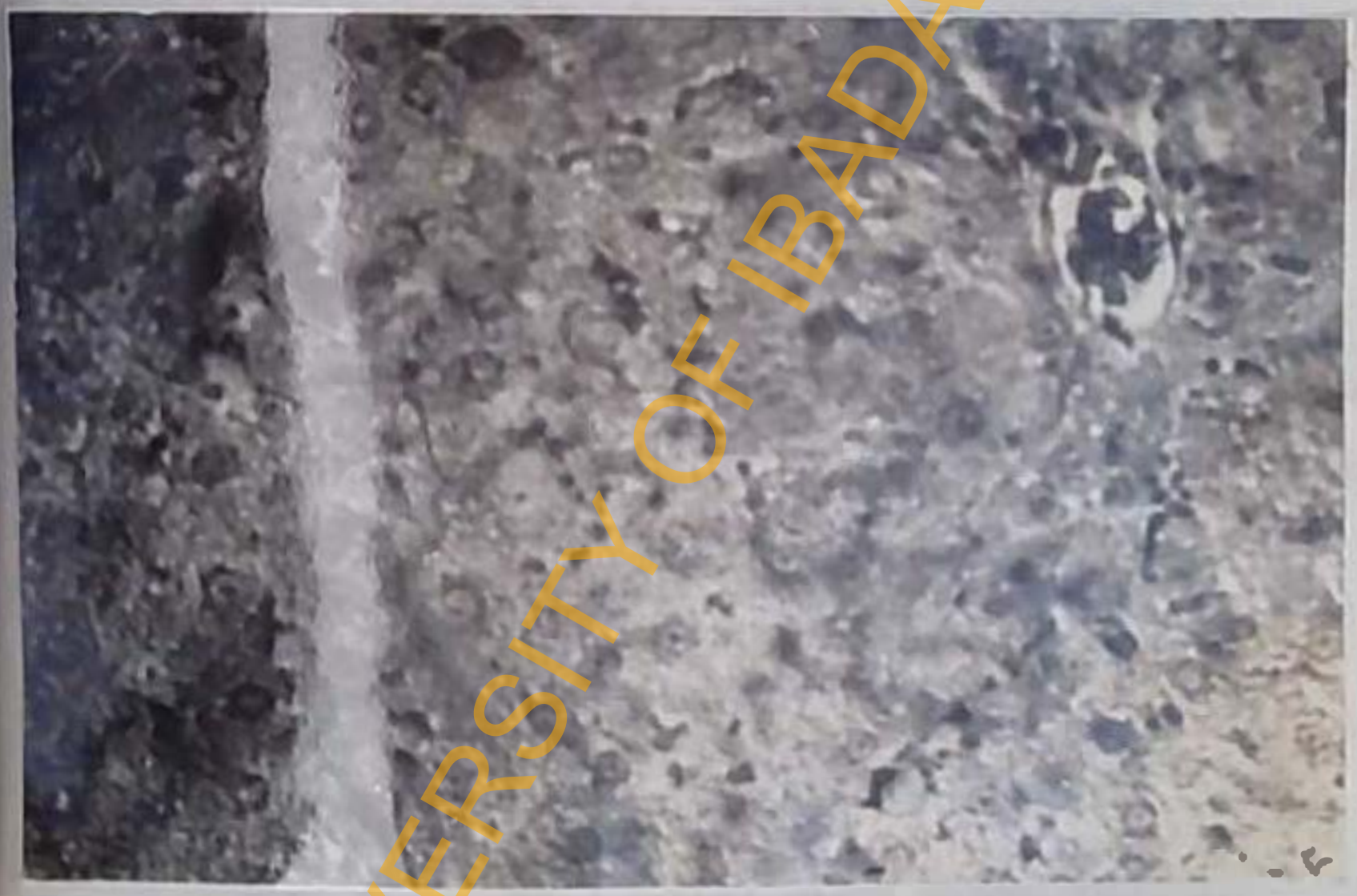


FIG 32 Rat liver after intake of drinking water contaminated with 100ppm D'TA - Showing bile duct proliferation.. H & E X. 300.

Group 4g: Rats on 50ppm Dimethylnitrosamine in their daily drinking water.

Behavioral Changes:

This dose proved more tolerable than the previous ones, as food intake was normal for a longer time, and only started to decrease after the seventeenth week. Weight gains were also recorded in the first five weeks and when the animals started to lose weight this was very gradual. By the twelfth week however loss in weight had become appreciable and the rats were looking quite ill, sitting quetly for most of the time. Later they started to shi .ver, and breathing became difficult. Some of the rats had sores around the throat and the areas around the sex organs were wet. The hair coat was brown instead of the characteristic glossy cream colour. By the nineteenth week the rats had become so weak that they were unreactive to teasing and food and water intake had become impressively low.

Pathological Lesions:

Rats dying after twenty weeks showed gross abnormality in the liver structure. This organ had its lobes swollen and tense and was distorted by a large rubbery mass on its proximal surface resembling a sarcoma. Four of the rats also had tumours on their liver lobes.

These outgrowths were whitish and rounded structures protruding just away from the edge of the lobes. Histological examination of the tumour showed no defined cellular organisation. The parts of the liver where there were no tumour showed much less general cellular damage. However there was a generalised increase in cell size with large nuclei and nucleoli.

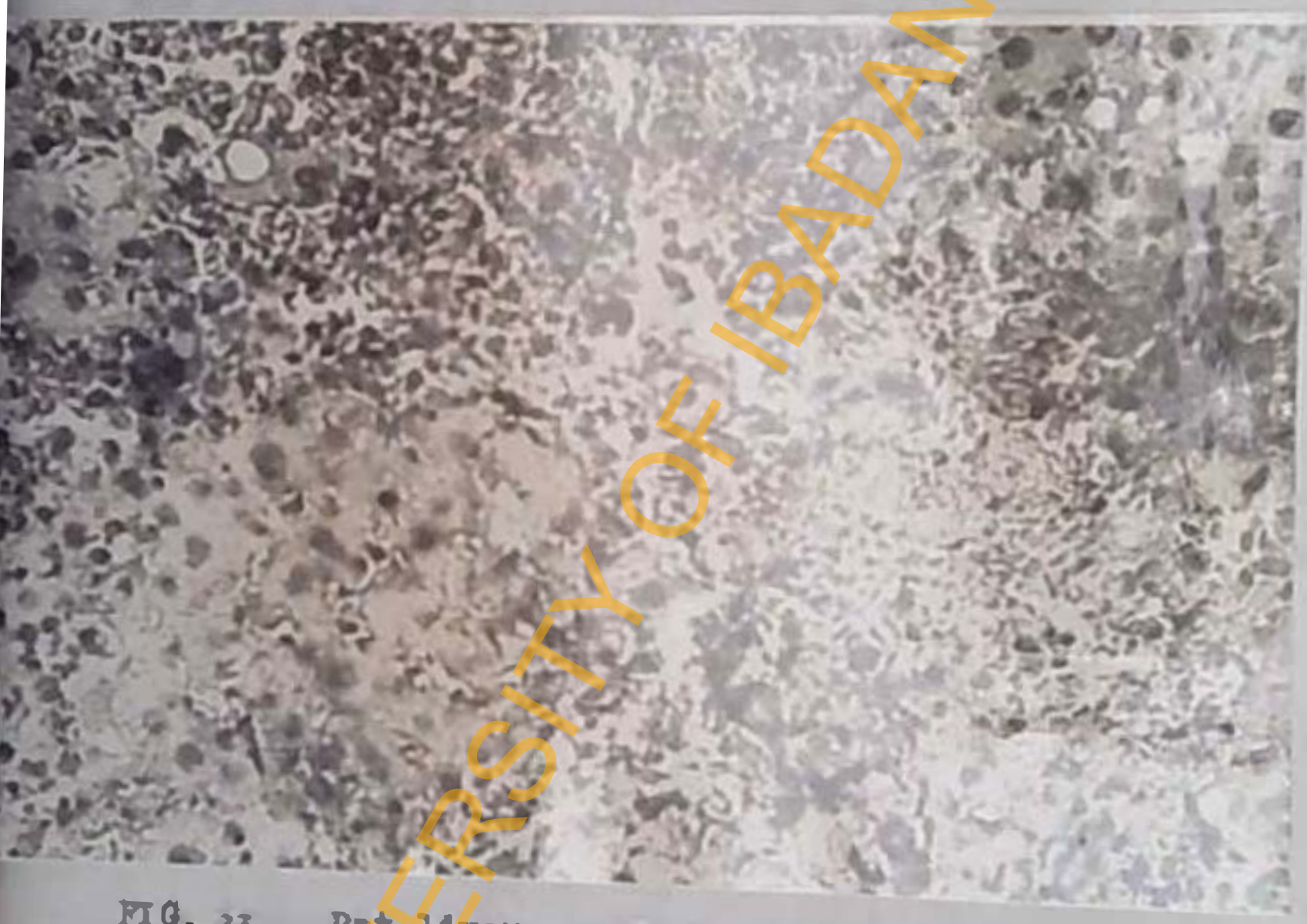


FIG. 33 Rat liver - Part of a tumour showing extreme anaplasia [redacted]. H & E x 300

These outgrowths were whitish and rounded structures protruding just away from the edge of the lobes. Histological examination of the tumour showed no defined cellular organisation. The parts of the liver where there were no tumour showed much less general cellular damage. However there was a generalised increase in cell size with large nuclei and nucleoli.

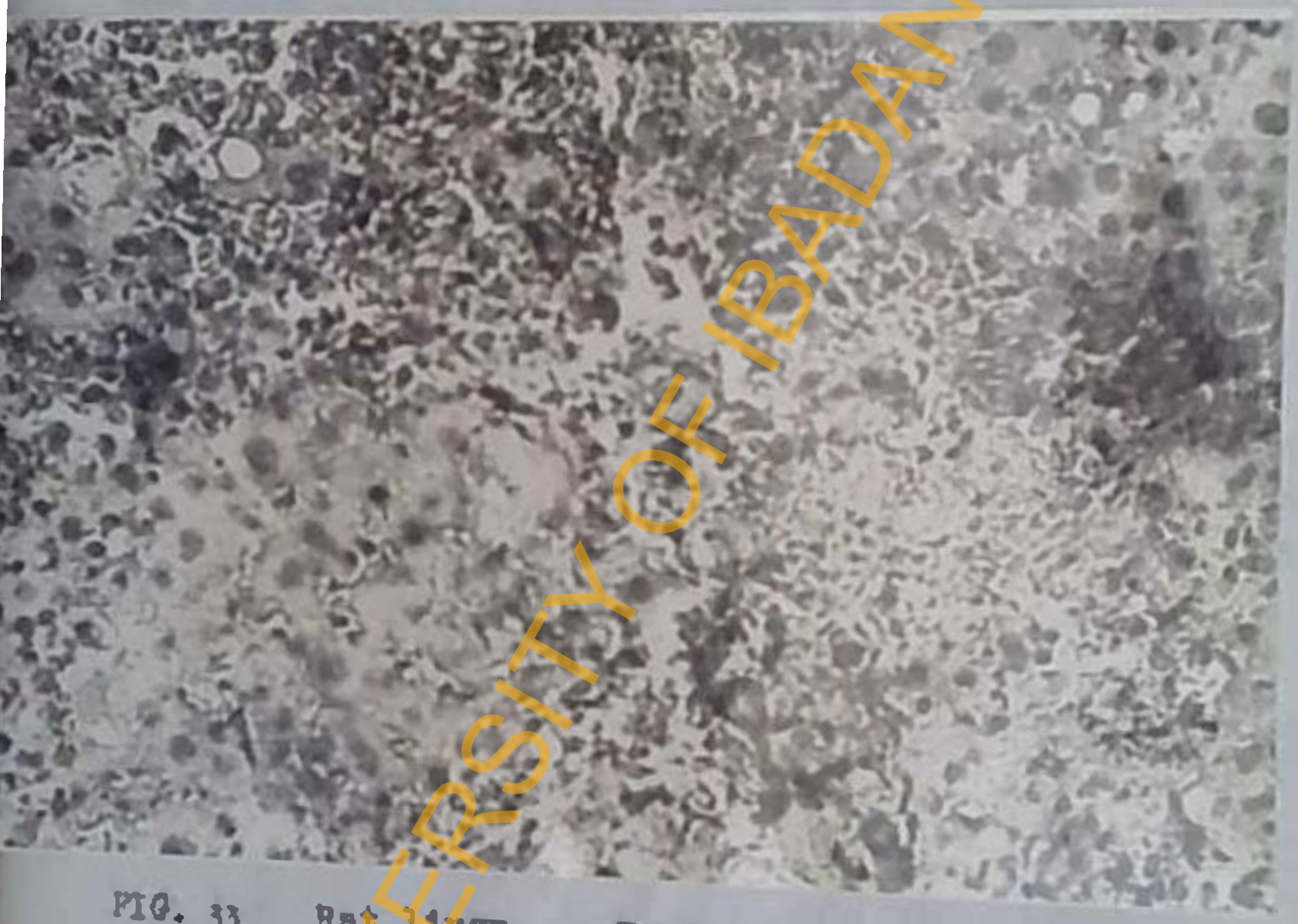


FIG. 33 Rat liver - Part of a tumour showing extreme anaplasia [redacted]. H & E x 300

Group 4b: Rats on 50ppm Diethylnitrosamine in their daily drinking water.

Behavioral Changes:

By the 120th day on treatment the mean weight of the group was significantly lower than that of the control group. Food intake had also fallen remarkably. The rats had abdominal swelling due to fluid accumulation in the peritoneal cavity. This fluid probably masked the detection of an early loss of weight due to emaciation. By the 170th day of treatment all the rats had died.

Pathological Changes:

At necropsy there was a variable quantity of fluid in the peritoneal cavity and the pancreas was oedematous. Sub-cutaneous odema over the abdominal wall was observed in some rats. The liver was swollen, dark and mottled. No tumour was observed on this organ. Aside from a little clear pericardial and pleural exudate in some rats there were no other lesions visible to the naked-eye.

Histological examination showed variable and less extensive damage to the liver.

The hyperplastic liver cells had very large nuclei with large multiple nucleoli. No histological evidence of damage to any other organ was observed.

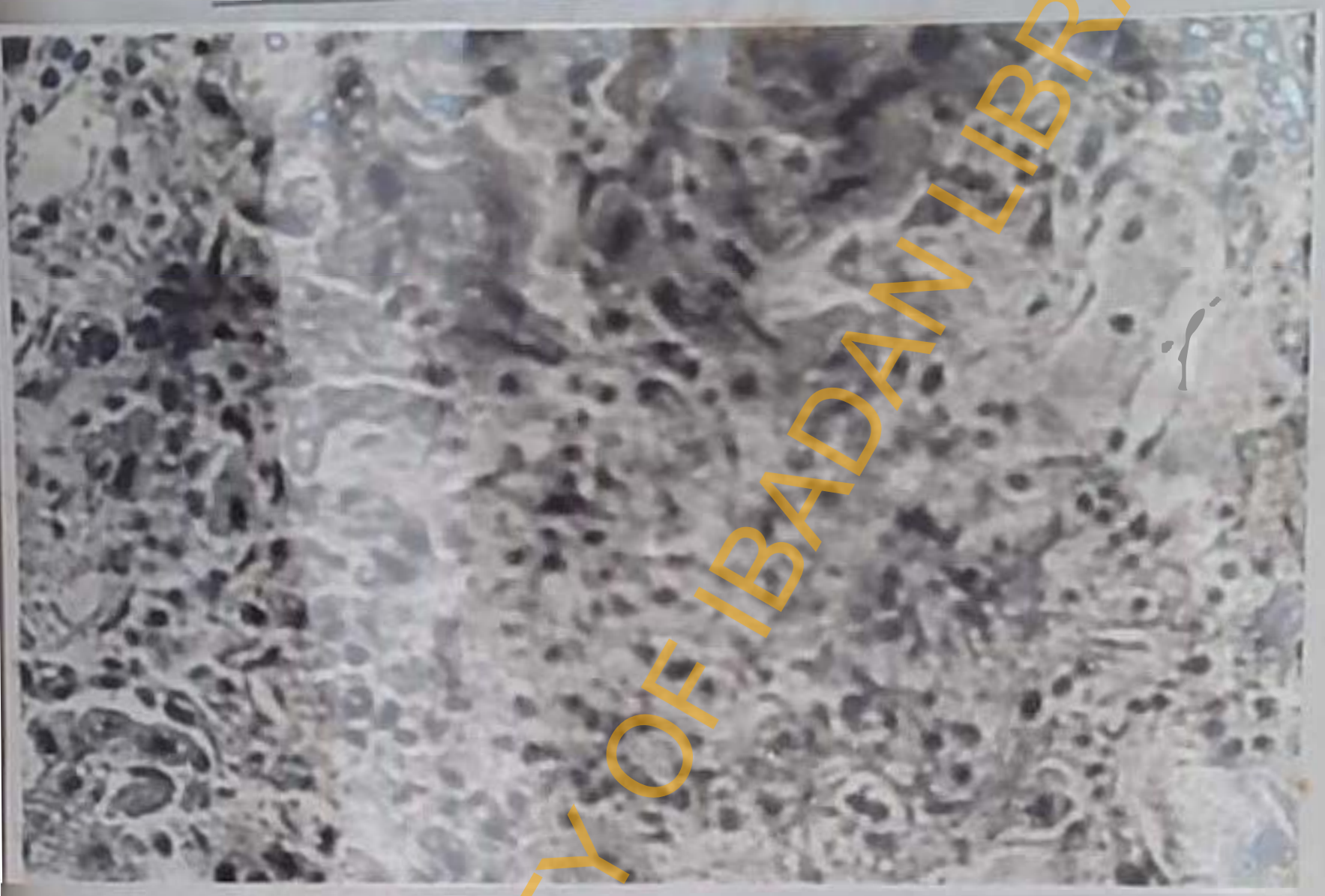


FIG. 34 Rat liver after intake of drinking water contaminated with 50 ppm DENA - Showing early centrilobular necrosis.

H & E X. 200

Group 5a: Rats on 25ppm Diethylnitrosamine in their daily drinking water.

Behavioral Changes

Growth and food intake quite normal and the effect of the treatment was not manifested until the 25th week when growth and food intake started to fail.

Pathological Lesions.

Six of the rats developed tumours which were localised in the liver. No other organs aside from this was involved in the neoplastic process.

The liver involved were grossly enlarged and had multiple, pale elevated nodules. Occasionally the tumours appeared cystic with a dark yellow coat.

Group 5b: Rats on 25ppm Diethylnitrosamine in their daily drinking water.

Behavioral Changes:

Growth followed a similar trend as for rats in group 5a. There was not much to be seen in the external features of the rats.

Pathological Changes:

The main organ affected was again the liver and six of the rats showed incidence of tumours in their livers. In some rats the appendix was distended just as the stomach, and the pancreas was oedematous.

In those parts of the liver where there were no tumour, the normal architecture of the liver was not much altered.

Group 6a: Rats on 12.5ppm Dimethylnitrosamine in their daily drinking water.

Behavioral Changes:

The rats were quite active throughout the experiment and their growth curve was quite normal except for a slight drop from the 32nd week to the 39th, when the rats started to die.

Pathological Lesions:

All the rats on this treatment developed tumour in their livers. In some rats the stomachs were enlarged and the pancreas was oedematous. The kidney and the rest of the gastro-intestinal tract were normal.

Group 6b: Rats on 12.5ppm Diethylnitrosamine in thier daily drinking water.

Behavioral Changes:

Not much could be noticed in the external features of rats in this group. Their growth rate and food intake were more or less similar to those of the control animals. The rats in this group were also very active throughout the experiment.

Pathological Changes:

The characteristic liver damage here was again tumour growth. The kidney and the other organs were intact except the pancreas which was again oedematous.

Group 7a: Rats on 0.1ppm Dimethylnitrosamine in their daily drinking water.

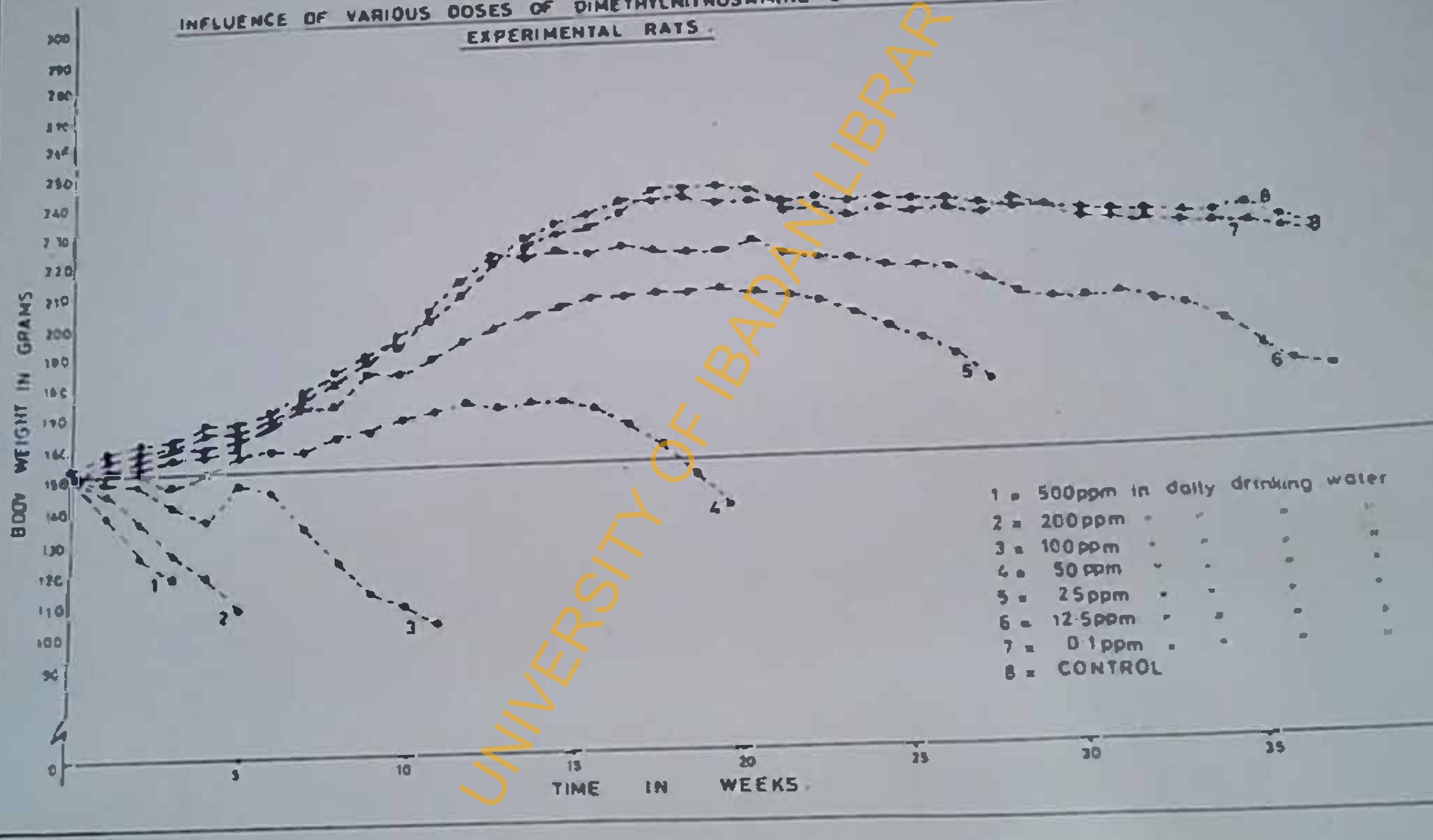
Behavioral Changes:

There was hardly any difference in the growth, food intake and external features of rats on this treatment with those of the control.

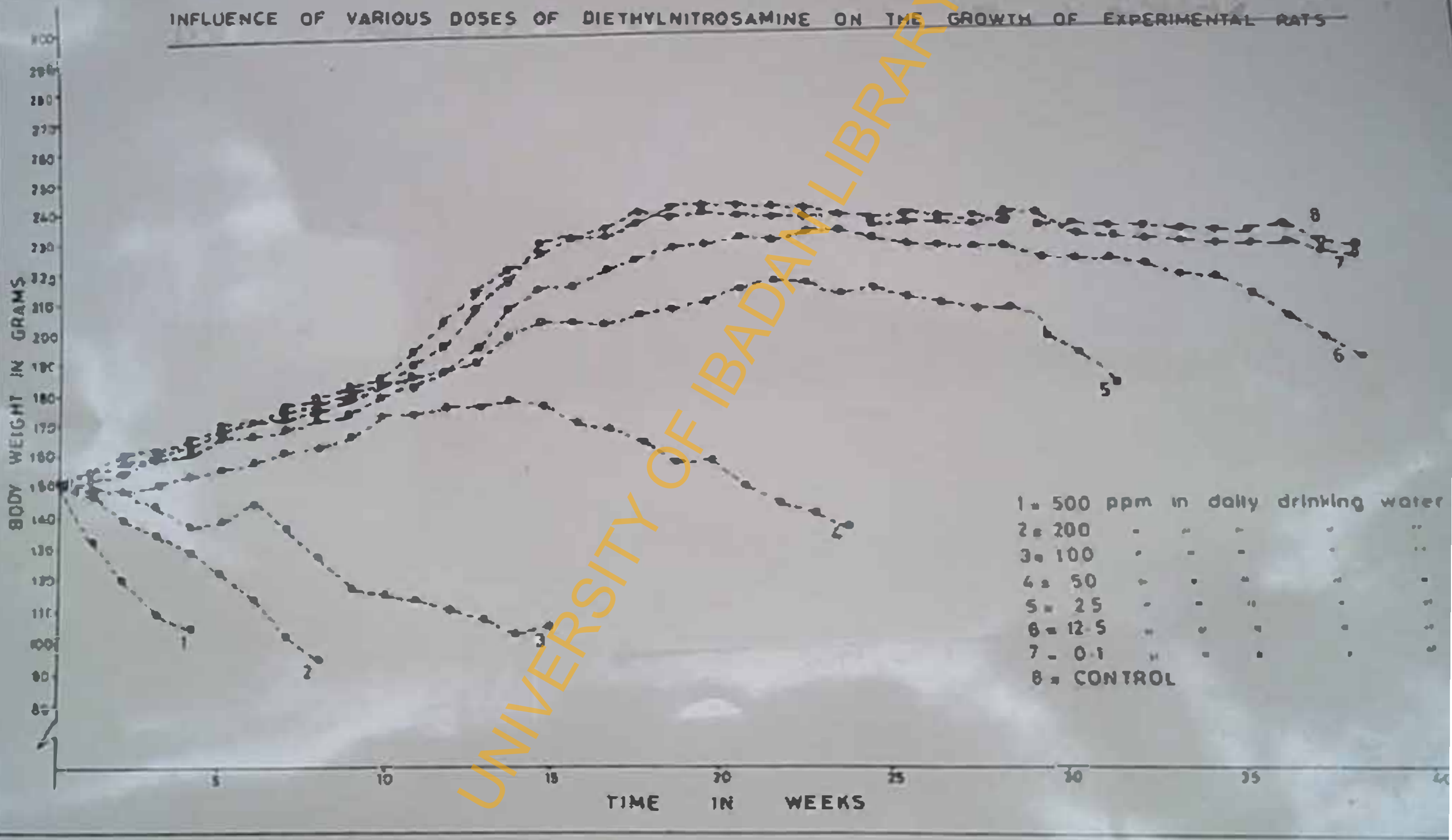
Fig. 35 Dose response curve for survival time of rats on graded levels of Nitrosamine in their daily drinking water.

INFLUENCE OF VARIOUS DOSES OF DIMETHYLNITROSAMINE ON THE GROWTH OF EXPERIMENTAL RATS.

INFLUENCE OF VARIOUS DOSES OF DIMETHYLNITROSAMINE ON THE GROWTH OF EXPERIMENTAL RATS.



INFLUENCE OF VARIOUS DOSES OF DIETHYLNITROSAMINE ON THE GROWTH OF EXPERIMENTAL RATS



Conclusion:

Dimethylnitrosamine and Diethylnitrosamine are very potent liver carcinogens, producing severe liver necrosis in rats. Both sexes are more or less equally susceptible and the results of the various experiments revealed a clear dose-response relationship.

An outstanding feature of the necrosis induced by dimethylnitrosamine and diethylnitrosamine is its very haemorrhagic character as the liver lesion was frequently accompanied by bleeding into the gastro-intestinal tract. This feature is however more prominent in rats poisoned with dimethylnitrosamine than in those poisoned with diethylnitrosamine. This difference in prominence is probably related to structural differences between the two compounds. The tendency to haemorrhage in the rat may be the result of the action of the poisons on blood vessels.

Changes in the fine structure of rat liver cells during the development of the acute necrotic lesion include swelling and vacuolisation of the endoplasmic reticulum followed by progressive accumulation of fat.

Not one single oral tumour was observed suggesting that irrespective of the local site of administration, dimethylnitrosamine and diethylnitrosamine are hepato-specific.

Lastly, it would appear that the tendency toward tumour growth is of a higher probability when the doses are small, for high doses 50ppm - 500ppm tend to kill the rats too early for the on set of tumour.

INVESTIGATION SIX

Effects of different planes of Nutrition on the toxicity of Dimethylnitrosamine in the rat.

The purpose of this study is to determine how the effects of some levels of dimethylnitrosamine on liver functions are influenced by various planes of dietary protein.

Experimental Procedures:-

(a) Arrangement of the Experimental Rats

Four rats were placed on each treatment and each rat was housed in a separate metabolic cage.

(b) Dosing with Nitrosamine

Dimethylnitrosamine was used. The levels administered were as enumerated below and the route of administration was oral, via the drinking water.

(1) 100 ppm Dimethylnitrosamine in daily drinking water
(2) 50 ppm " " " "
*(3) 0.1ppm " " " "
**(4) Palm wine in place of drinking water
(5) Normal drinking water.

*Nitrosamine level in Ogogoro

** For Nitrosamine level in palm wine.

INVESTIGATION XIX

Effects of different planes of Nutrition on the toxicity of Dimethylnitrosamine in the rat.

The purpose of this study is to determine how the effects of some levels of dimethylnitrosamine on liver functions are influenced by various planes of dietary protein.

Experimental Procedures:-

(a) **Arrangement of the Experimental Rats**

Four rats were placed on each treatment and each rat was housed in a separate metabolic cage.

(b) **Dosing with Nitrosamine**

Dimethylnitrosamine was used. The levels administered were as enumerated below and the route of administration was oral, via the drinking water.

(1) 100 ppm Dimethylnitrosamine in daily drinking water
(2) 50 ppm " " " " "
*(3) 0.1ppm " " " " "
**(4) Palm wine in place of drinking water
(5) Normal drinking water.

*Nitrosamine level in Ogogoro

** For Nitrosamine level in palm wine.

(c) Environmental conditions

The experiments were carried out in the departmental Animal House where the rats were exposed to similar environmental conditions, i.e. temperature, relative humidity, light, etc.

(d) Assay period

The Assay period was 15 days on each "drug"/dietary treatment.

(e) Dietary Arrangement

The diets were variously prepared from a basal diet such that they had graded protein content as follows:-

Diet No. 1	0%	Protein (i.e. Protein-free)
" No. 2	5%	"
" No. 3	10%	"
" No. 4	15%	"
" No. 5	20%	"
" No. 6	25%	"

The compositions of the basal diet, and the salt and vitamin mixtures are given in Chapter II, section xvii.

The Distribution of Diet and Drug in Relation to each Group of Rats were as fo

100 ppm Dimethyl-nitrosamine 50 ppm " 0.1 " " Palm Wine Normal water	% Dietary Protein Level	100 ppm Dimethyl-nitrosamine 50 " " 0.1 " " Palm wine Normal water	5% Dietary Protein Level	100ppm Dimethyl-nitrosamine 50ppm " 0.1" " Palm wine Normal water
100ppm Dimethyl-nitrosamine 50ppm " 0.1" " Palm wine Normal water	14% Dietary Protein Level	100ppm Dimethyl-nitrosamine 50ppm " 1.0" " Palm wine Normal water	20% Dietary Protein Level	100ppm Dimethyl-nitrosamine 50ppm " 0.1" " Palm wine Normal water

Results

From the observations made on the performance of the experimental animals on the various dietary protein levels, and normal drinking water a protein-free diet or one with a 5% or 10% dietary protein level would not support growth.

A dietary protein level of 15% while supporting growth would appear to be just adequate for maintenance. Rats on dietary protein levels of 20% and 25% respectively showed signs characteristic of normal healthy growth. However the efficiency of utilisation of dietary protein by the experimental rats begins to decline above a dietary protein level of 20% i.e. "diminishing returns" sets in above this level.

The group of animals on palm wine showed a somewhat similar trend in growth as their counterparts on normal water. But on all the dietary protein levels (0%, 5%, 10%, 20 and 25%) the rats showed statistically significant improvement over rats on ordinary water.

Growth response of rats on 0.1 ppm dimethylnitrosamine was not significantly affected on all the dietary protein levels, to suggest any marked effect of this level of contamination on growth.

Growth was however, appreciably depressed in rats on 100 ppm and 50ppm dimethylnitrosamine and the various dietary protein levels. The growth pattern of the rats were not much affected when dietary protein were 0%, 5% and 10% respectively in comparison with the control group, whereas one would have expected the "drug" to further amplify the emaciation of tissue caused by these poor diets. This suggests that the effect of the carcinogen was not fully felt by the rats on protein deficient diets.

The resistance of rats on 0%, 5% and, to some extent 10% dietary protein levels, to the toxicity of dimethyl-nitrosamine was amply confirmed by the results of the liver function tests.

With rats on 20% and 25% dietary protein levels, the hepatoxicity of dimethylnitrosamine at levels of 100ppm and 50ppm over the experimental period was manifested by a marked elevation of the activity of alkaline phosphatase in the serum, appreciable rise in serum glutamic oxaloacetate transaminase; a marked depression of serum total protein, and an elevation of blood sugar level, and slight increases in serum bilirubin and urine urobilinogen, above the normal limits. With lower dietary protein levels, these various effects were reduced as can be seen on the graphs.

With a level of 0.1 ppm the effects on liver functions over the experimental period were just detectable.

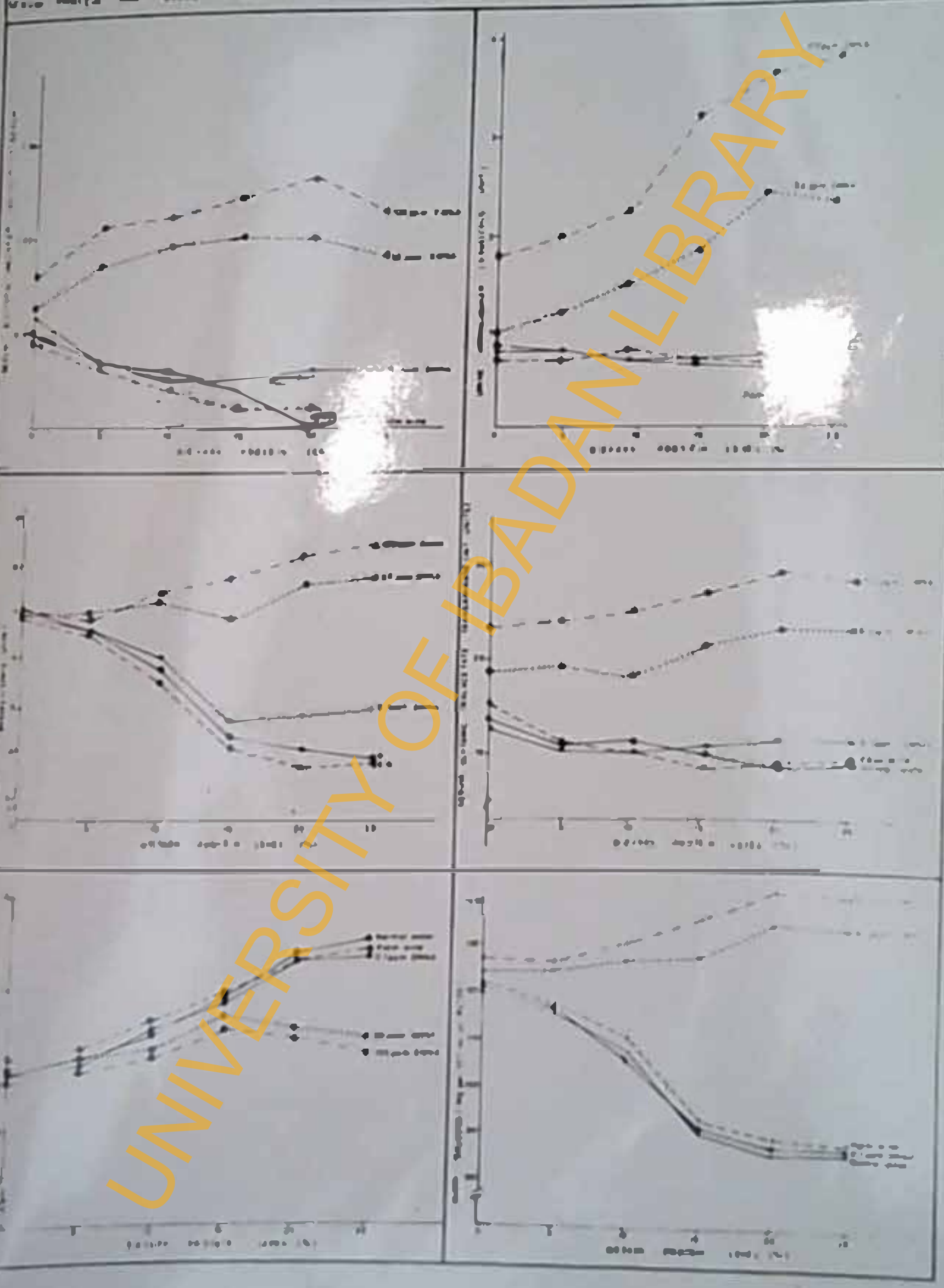
TABLE I.

EFFECT OF DIFFERENT PLANES OF NUTRITION ON SOME BIOCHEMICAL CHANGES INDUCED BY DIMETHYLNITROSAMINE

DIET	ADDITIONAL TREATMENT	MEAN STARTING WEIGHT (G.)	MEAN LAST WEIGHT (G.)	MEAN WEIGHT GAIN OR LOSS(G)	LIVER FUNCTION TESTS. TOTAL SERUM PROTEIN (G/100mg)	BLOOD SUGAR (mg/100ml)	SERUM BILIRUBIN (mg/100ml.)	URINE UROBILINOGEN (EHRLICH UNIT)	ALKALINE PHOSPHATASE B.L.UNITS	SGO-T INT.UNITS)
20% PROTEIN FREE	Normal Water	100.2	51.5	-48.7	4.30	120.5	0.85	6.82	6.82	13.5
	Palm Wine	100.4	52.6	-47.7	4.50	122.5	0.09	0.70	6.76	14.3
	0.1ppm DMNA	100.0	51.4	-48.5	4.30	120.7	0.12	0.83	6.69	12.5
	50ppm DMNA	100.1	51.0	-49.0	4.20	124.1	0.12	1.00	6.80	18.6
	100ppm DMNA	100.4	46.0	-54.4	4.00	127.5	0.16	1.80	6.97	23.4
5% PROTEIN LEVEL	Normal Water	100.2	59.8	-40.4	4.60	115.1	0.09	0.80	6.50	10.9
	Palm Wine	100.6	61.0	-39.6	4.80	117.0	0.06	0.70	6.43	11.1
	0.1ppm DMNA	99.8	60.0	-39.8	4.46	116.0	0.07	0.82	6.50	10.0
	50ppm DMNA	100.4	56.0	-44.4	4.60	124.0	0.12	1.21	1.21	6.85
	100ppm DMNA	100.6	53.0	-41.6	4.30	126.1	0.16	2.00	6.76	24.0
10% PROTEIN LEVEL	Normal Water	100.4	82.2	-18.2	5.10	5.10	1.07	0.70	5.52	11.0
	Palm Wine	100.6	83.1	-17.6	5.40	110.0	0.04	0.80	5.40	9.8
	0.1ppm DMNA	101.5	81.5	-20.0	5.20	105.5	0.05	0.72	5.92	10.1
	50ppm DMNA	102.2	82.5	-12.7	4.80	120.3	0.19	1.50	7.10	17.9
	100ppm DMNA	100.2	75.0	-25.2	4.60	130.0	0.22	2.25	7.30	25.0

TABLE X. (CONTD.)

DIET	ADDITIONAL TREATMENT	MEAN STARTING WEIGHT (G.)	MEAN LAST WEIGHT (G.)	MEAN WEIGHT GAIN OR LOSS(G)	LIVER FUCTION TESTS. TOTAL SERUM PROTEIN (G/100ml	BLOOD SUGAR (mg/ 100ml)	SERUM BILIRU- BIN (mg/ 100ml)	URINE UROBILI- NOGEN. (EHR- LICH'S UNIT)	ALKALINE PHOSPHA- TASE (B.L.UNITS)	SGO-T INSE. UNITS.
15% PROTEIN LEVEL	Normal Water	100.9	113.5	+12.6	5.90	90.2	0.04	0.65	4.27	9.6
	Palm Wine	100.0	115.0	+15.0	6.10	93.1	0.02	0.70	4.00	8.4
	0.1ppm DMHA	100.0	112.2	+12.2	5.77	91.2	0.05	0.68	4.60	10.5
	50ppm DMHA	100.3	109.5	+ 9.2	5.50	127.1	0.20	1.85	7.30	21.3
	100ppm DMHA	100.4	101.0	+ 4.6	5.20	135.2	0.24	3.22	7.60	26.7
20% PROTEIN LEVEL	Normal Water	100.2	129.8	+29.6	6.80	86.0	0.00	0.63	3.98	8. 2
	Palm Wine	100.9	130.9	+30.0	6.70	89.4	0.02	0.66	3.60	8.6
	0.1ppm DMHA	101.0	126.5	+25.5	6.70	87.8	0.06	0.70	4.70	11.0
	50ppm DMHA	100.5	116.1	+15.6	5.20	133.4	0.20	2.40	7.50	22.0
	100ppm DMHA	100.1	111.5	+14.4	5.00	141.4	0.26	3.60	8.10	28.9
25% PROTEIN LEVEL	Normal Water	100.7	134.5	+33.8	7.10	86.3	0.00	0.65	3.80	8.5
	Palm Wine	100.5	138.8	+38.3	6.90	88.3	0.00	0.59	3.70	8.5
	0.1ppm DMHA	99.5	130.2	+30.7	6.80	88.0	0.06	0.71	4.80	10.7
	50ppm DMHA	100.8	120.3	+19.5	5.00	133.0	0.18	2.30	7.60	22.7
	100ppm DMHA	100.1	108.0	+23.9	4.70	142.2	0.22	3.75	8.30	28.0



Conclusion

The experiments carried out showed that a protein-free diet or one with a poor protein content reduces the toxic effects of dimethylnitrosamine in rats. This information was gathered from the growth responses of the rats and the results of the liver function tests. This observation therefore confirms the finding of Mclean and Mclean (1970), and Swan and Mclean (1970), who reported that rats on a protein-free diet developed some resistance to the toxicity of dimethylnitrosamine. An explanation for this sparing effect was advanced by Mclean (1971) who reported that the metabolism of dimethylnitrosamine in the liver was reduced by half when a protein-free diet was fed to the poisoned rats. This investigation therefore further supports the view that dimethylnitrosamine itself is not toxic but that the active principle is a metabolite.

and 50ug tetracycline hydrochloride in water was administered to the rate twice daily two days before the experiment and once every other day during the experimental period.

(d) Environmental Conditions

The experiment was carried out in the departmental animal house where the rats were exposed to similar environmental conditions, i.e. temperature, relative humidity, light, etc.

(e) Assay Period

Assay period was for 15 days on each treatment.

(f) Criteria for Judgement

This was as in investigation 6,i.e. the effects of the various treatments on some functions of the liver, viz. Bile pigment metabolism, metabolic functions (Protein and carbohydrate metabolism), and regulation of enzyme activity in the serum.

Results

The results obtained in this investigation show that in germ-free rats, just as in conventional rats, an intimate relationship exists in the toxicity of dimethylnitrosamine and dietary protein levels. In both rat types the toxic effect was wilder on protein deficient diets.

TABLE XII. EFFECT OF DIFFERENT PLANES OF NUTRITION OF THE TOXICITY OF DIMETHYLNITROSAMINE IN GERM-FREE RATS.

(A = CONVENTIONAL RAT
(B = GERM-FREE RAT

DIET	NITROSAMINE TREATMENT	MEAN STARTING WEIGHT (G.)		MEAN LAST WEIGHT (G.)		LIVER FUNCTION TESTS. TOTAL SERUM PROTEIN (G/100ml)		BLOOD SUGAR (Mg/100ml)		SERUM BILIRUBIN (Mg/100ml)		URINE UROBINOGEN (EHLICH UNITS)		ALKALINE PHOSPHATASE		SGO-T (LFT-UNITS)	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Protein Free	Normal Water	99.8	100.4	52.5	50.6	4.30	3.00	120.0	121.7	0.10	0.20	0.80	0.81	6.82	5.70	9.6	10.2
	0.1ppm DMNA	100.6	100.4	52.1	50.1	4.40	4.00	120.0	121.1	0.12	0.09	0.79	0.80	6.70	5.72	10.5	10.3
	50ppm DMNA	100.1	100.4	50.3	49.8	4.10	3.90	125.1	120.9	0.10	0.12	1.00	0.90	6.90	5.60	21.3	18.2
	100ppm DMNA	100.4	100.9	47.4	47.2	4.00	3.70	126.9	0.14	0.20	1.80	1.60	6.87	6.81	5.81	26.7	24.9
5% Protein level	Normal Water	100.2	100.7	62.2	60.3	4.52	4.40	114.4	0.10	0.12	0.86	0.71	6.50	6.60	5.50	8.5	9.1
	0.1ppm DMNA	100.5	100.2	60.1	60.7	4.47	4.00	118.0	116.3	0.09	0.10	0.84	0.75	6.95	5.47	12.0	10.2
	50ppm DMNA	100.0	100.3	54.7	53.0	4.67	3.90	124.7	125.7	0.13	0.17	1.25	1.10	6.76	5.92	22.0	20.1
	100ppm DMNA	100.4	99.7	49.3	48.25	4.90	107.5	27.8	127.8	0.18	0.24	2.00	1.79	6.64	5.50	28.9	26.5
10% Protein level	Normal Water	100.5	99.6	88.4	85.4	5.31	4.90	10.0	105.4	110.0	.08	0.05	0.70	5.40	5.50	2.5	11.2
	0.1ppm DMNA	100.1	100.5	86.1	87.2	5.20	4.40	28.1	107.3	0.04	0.05	1.50	1.02	5.72	5.50	2.7	11.1
	50ppm DMNA	100.7	100.1	83.1	81.1	4.67	4.10	51.9	125.9	0.20	0.19	2.25	2.02	7.10	5.67	21.2	21.8
	100ppm DMNA	99.3	100.3	80.1	77.6	4.60	5.66	92.4	128.1	0.22	0.20	0.65	0.66	6.50	5.10	26.5	24.2

TABLE XII. (CONTINUED)

DIET	NITROSAMINE TREATMENT	MEAN STARTING WEIGHT (G.)		TOTAL SERUM PROTEIN (G/100ml)		LIVER FUNCTION TESTS: BLOOD SUGAR mg/100ml		SERUM BILIRUBIN mg/100ml		URINE UROBILINOGEN (EHRLICH UNIT) (mg/100ml)		ALKALINE PHOSPHATASE B.L. UNITS		SGO-T (KFT-UNITS)	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B
15% Protein level	Normal Water	100.9	100.4	112.1	111.0	5.70	5.70	90.4	92.6	0.07	0.05	0.71	0.63	4.25	4.20	13.0	14.0
	0.1ppm DENA	100.5	100.7	115.0	111.3	5.77	5.00	127.1	93.1	0.05	0.04	1.95	1.53	4.60	4.39	12.5	12.30
	50ppm DENA	99.1	100.6	112.7	109.7	5.00	4.90	136.1	130.6	0.20	0.24	3.40	2.81	7.31	7.10	18.6	17.90
	100ppm DENA	100.3	100.5	105.9	102.1	5.40	6.50	86.0	136.5	0.29	0.23	0.60	0.54	7.60	7.34	23.5	19.04
20% Protein level	Normal Water	100.4	100.7	129.8	128.9	6.50	6.50	87.9	90.1	0.10	0.05	0.70	0.68	3.98	4.07	11.0	10.1
	0.1ppm DENA	100.4	100.2	128.7	126.8	6.20	5.30	134.6	89.7	0.06	0.09	2.10	2.00	4.78	4.70	10.0	11.2
	50ppm DENA	100.3	120.5	120.1	117.1	5.00	4.80	142.1	137.2	0.20	0.27	3.40	3.10	7.50	7.43	20.0	19.4
	100ppm DENA	100.7	100.4	112.3	113.2	5.13	5.00	86.0	143.4	0.28	0.26	3.67	3.15	7.57	7.8	24.0	22.7
25% Protein level	Normal Water	100.7	100.3	136.2	130.2	7.31	6.80	88.1	90.1	0.05	0.10	0.72	0.70	3.80	3. 78	11.01	10.9
	0.1ppm DENA	100.5	100.4	135.6	129.1	7.00	6.50	88.9	87.5	0.07	0.09	0.80	0.75	4.85	4.71	10.1	10.4
	50ppm DENA	100.3	100.3	121.9	120.3	5.32	4.90	133.0	141.2	0.20	0.24	2.30	2.10	7.63	7.90	17.9	16.1
	100ppm DENA	100.8	100.6	109.9	106.5	4.60	4.50	140.3	147.3	0.21	0.28	3.61	3.25	8.46	7.80	25.0	23.6

TABLE XIII. STATISTICAL ANALYSIS OF THE RESULTS OF NITROSAMIDE POISONING IN SERUM-FREE RATS (X = CONVENTIONAL RAT)
(Y = SERA-FREE RAT)

PHYSIOLOGICAL TEST.	X	Y	SE(X-Y)	t	CONCLUSION
Serum Protein Level	5.21	4.90	0.2308	1.343	DIFFERENCE NOT SIGNIFICANT
Blood Sugar Level	116.0	117.8	5.362	0.336	"
Serum Bilirubin	0.12	0.15	0.02345	1.279	"
Urine Urobilinogen	1.49	1.13	0.2644	1.362	"
Alkaline Phosphatase	6.35	6.22	0.3542	0.367	"
Serum Glutamic Oxaloacetate Transaminase	16.88	16.12	1.899	0.4002	"

Conclusion

On the basis of the differences obtained on the effects of dimethylnitrosamine poisoning and plane of nutrition in conventional rats and in germ-free rats it would appear that absence of gut bacteria by pre-treating rats with antibiotics results in a slight lowering of the toxic effects of dimethyl-nitrosamine especially when dietary protein level was adequate using changes in liver functions as criteria for judgement. While this slight lowering effect was not statistically significant, it may be of scientific significance.

The milder effects of dimethylnitrosamine poisoning in germ-free rats could mean that the interplay between gut bacteria and nutrients (especially protein) in the gut result in the production of nitrosamine precursors namely secondary amines and nitrites which might react in vivo to form nitrosamine. This would then suggest that the effect of any level of dimethylnitrosamine poisoning is in fact the overall effect of the administered dose plus the effect of the amount produced in the gut where this happens.

Conclusion

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CHAPTER FIVE

D I S C U S S I O N

1. Implications of the presence of nitrosamines in Nigeria's local alcoholic beverages

There are two main reasons for studying cancer-producing agents, whether chemical, physical or viral. First, a knowledge of the nature and source of a carcinogen is an essential part of modern biological technology required to ensure that such substances are not unwittingly introduced into our environment. The recent recognition that most cancers are not inevitable but are caused by environmental factors has therefore had important practical results in directing increased research towards the identification of sources of cancer-inducing agents, especially chemical agents, in our environment.

Second, a thorough understanding of the mechanism of cancer induction, whereby a cell is transformed into a cancer cell is an important tool in the development of cancer chemotherapy. Progress along this line has developed from fundamental studies on the chemistry and mode of action of cancer-inducing agents. The initial belief that only a

few substances with specific properties would have the capacity to induce cancer has not been realised.

Since the discovery of the carcinogenic properties of diethylnitrosamine by Magee in 1956, various attempts have been made to assess the dangers posed to man by the presence of nitrosamine in the environment, especially now that they have been recognised as one of the most versatile and formidable group of carcinogens yet discovered. Thus an assessment of the level of contamination of wheat flour (Margaret, 1966), smoked fish (Panis et al. 1971), and tobacco and tobacco smoke (Serfontein and Burter, 1966), with carcinogenic nitrosamine have been carried out. Analysis of home-made wine for N.NO in areas of Kenya where oesophageal cancer is common has also been carried out by mass spectrometry (Diller, 1972). Collis (1972) was able to demonstrate a positive correlation between oesophageal cancer and the drinking of 'beer' made from cereals.

The analytical survey for the assessment of the level of contamination of Nigeria's indigenous alcoholic beverages with carcinogenic nitrosamine, carried out in the present work, is therefore in line with the current practical steps towards the prevention of cancer in man.

The results of the surveys have demonstrated the presence of dimethylnitrosamine and diethylnitrosamine in Nigeria's local alcoholic beverages, namely Palm wine, Burukutu, Pito, Oti Agbo, and also in a local gin called Ogogoro.

Dimethylnitrosamine are proven potent liver carcinogens producing severe liver necrosis.

in all animal species in which tests have been reported (Druckrey et al. 1964; Argus and Koch Ligetti, 1963). There is also evidence for their metabolism into active carcinogenic products by human liver slices (Magee, 1970).

Disregarding the amounts of nitrosamine found in the beverages for the time being, their presence at all in a common food item as our alcoholic beverages is of some concern. Much of this concern is related to the fact that in our country about twenty million people consume these alcoholic beverages and over one-third of this number do so with considerable regularity. Many are even problem drinkers, and some have progressed to classic alcoholism where there is not only a psychological dependence, but also a physiological dependence, which has been brought about by the individual's increased tolerance for alcohol and his physical craving for the substance. The presence of caroinogenic nitrosamine in our local alcoholic beverages therefore call to question the wholesomeness of these beverages especially with relation to cancer incidence in the country.

Previously, our local alcoholic beverages, especially palm wine which is the most popular, have only been casually related to cancer incidence in Nigeria. Other habits too, such as smoking, the use of snuff, and herbal

remedies, have been blamed. Edington, (1964), in his report to the International Union Against Cancer, was just able to demonstrate a higher incidence of cancer of the various parts of the alimentary tract in natives who drink these local brews than in those people who do not. This type of observation could at best be only cause suggestive. As a matter of fact, any other correlation can be established between cancer incidence and some other totally unrelated habit. For example it is possible to establish a correlation between cancer incidence and attendance at Church!

The discovery of nitrosamine in our local alcoholic beverages has therefore gone much further in identifying an established group of chemical carcinogens with these alcoholic beverages. While the nitrosamines may not be the only cancer inducing agents in palm wine and the other beverages investigated, they form at least a group by which the role of these food items in the aetiology of human cancer in Nigeria may be partly assessed.

It is convenient at this point to consider the actual level of contamination of our local alcoholic beverages with carcinogenic nitrosamine in other to appreciate the potential role of such levels in our environment.

The total amounts of nitrosamine found in palm wine, Borukutu, Pito, Oti Agbagba and Ogogoro are 30 ug/litre (0.03ppm), 58ug/litre (0.058ppm) 50ug/litre (0.05ppm), 21 ug/litre (0.02ppm) and 100 ug/litre (0.1ppm) respectively.

Histopathological studies carried out showed that groups of rats whose drinking water had been contaminated with graded levels of nitrosamine, showed a clear dose-response relationship characteristic of nitrosamine poisoning up to a contamination level of 12.5ppm. Rats on much lower levels of contamination, including a level of 0.1ppm found in Ogogoro, grew well and histological examination of their livers and other organs showed no apparent difference from those of the control animals.

However, biochemical studies of the early alterations caused to rat liver functions by graded levels of diethyl-nitrosamine contamination, revealed that a level of 0.1ppm found in Ogogoro could result in a slight impairment of the bile pigment metabolism, elevation of blood sugar and an increased activity of alkaline phosphatase, and serum glutamic oxaloacetate transaminase in the blood.

Therefore, on the basis of this biochemical study the level of contamination of Ogogoro with carcinogenic nitrosamine is harmful to the rat.

If the same effect is expected in man, then those people who make a regular habit of excessive intake of Ogogoro need to be cautioned. For example if a person takes a litre of Ogogoro everyday, he would be ingesting 100 ug nitrosamine a day. Assuming this habit was formed at the youthful age of 20 years, by the time the individual is 50 years he would have taken a total of 1095 mg. The progressive effect of this could terminate in cancer.

We have to realise too, that our alcoholic beverages are not the only channel through which our system could come in contact with carcinogenic nitrosamines. The possibility of other sources of this group of chemical carcinogens in the environment lies in the ready distribution of secondary amines and nitrites in plant materials which might react to form nitrosamines (Druckrey et al. 1967).

Moreover, carcinogenic nitrosamines are not the only chemical carcinogens present in our environment. A host of others are scattered all around us. The dangers from coaltar, soot and oil and the aromatic amines of the dyestuff and rubber industries have preoccupied the attention of many workers for a long time. Chemicals such as herbicides, insecticides, fertilizers, antibiotics,

detergents, metals and the products of fungi and mould contaminate our food materials. The potential contribution of the latter are particularly well illustrated by the story of the aflatoxins. In addition to contaminants, food additives of increasing complexity are now used as colouring or flavouring agents, artificial sweeteners, preservatives, and emulsifying agents. Therefore, rather than dismiss the level of contamination of our local alcoholic beverages with carcinogenic nitrosamines as insignificant, we must think of it as an additional source of the cancer-inducing agents in our environment. As a matter of fact, the combined effects of these various chemical carcinogens at seemingly harmless concentrations may be the riddle behind the high incidence of cancer in the world today.

2. Mode of action of the nitrosamine types present in the alcoholic beverages

a. Pathological effects

For a proper judgement of the potential role of chemical substances in human carcinogenesis, a thorough understanding of the interaction of these chemical substances and biological systems is essential. For example it is essential to know whether cancer can be induced in every organ of experimental animals, and whether the neoplastic changes are comparable to corresponding tumours in man. The

answer to this questions requires systematic studies and it is an important tool in the second major "cancer Problem" which is therapy.

Systematic studies carried out on the pathological effects of the nitrosamine types found in our local alcoholic beverages proved that both dimethylnitrosamine and diethylnitrosamine are very potent liver carcinogens producing extensive liver necrosis with high regularity. This result therefore confirms the earlier findings of other workers including Magee and Barnes (1956), Baile and Christie (1959), Druckrey et al. (1964) who showed that the typical acute lesion induced by the dialkylnitrosamine is necrosis of the liver.

Not one single local carcoma was observed in the mouth, the route of administration. Since hydroxylation occurs mainly in the liver (Druckrey, 1964), the result also alludes to the fact that nitrosamines are non-carcinogenic per se, but become so only after metabolic activation in the liver. Evidence for this conversion has been discussed extensively by Preussmann (1969), Druckrey, (1969), and Magee, (1970).

A clear dose- response relationship was observed in the livers of rats on graded levels of dimethylnitrosamine treatments respectively, up to a contamination level of

12.5 ppm in the daily drinking water. However, extensive quantitative studies by Druckrey and Steinhoff (1962), and Argue and Hoch - Ligetti (1963) have revealed a dose-response relationship down to a daily dosage of 0.075 mg/kg body weight.

Death rate was also related to dose in the rat supporting an earlier observation by Magee and Barnes (1956).

At autopsy, rats on 500ppm, 200ppm and 100ppm levels of contamination respectively showed extreme emaciation and complete absence of body fat.

No tumour was observed on any organ of the rats on these same levels of contamination. Some rats however developed tumours of the liver on 25 ppm and 12.5 ppm levels of contamination respectively. The induction of liver tumour in the rats has also been shown by Schmahl and Preussmann (1959) and Magee and Barnes (1956) only with low levels of contamination. No tumour was observed on any other organ even at concentrations inducing liver tumour. However, Magee and Barnes (1962) have induced kidney tumours in rats by feeding high dose levels for short periods. The tumours were not clinically apparent until a year or longer after the treatment was stopped.

The liver necrosis induced by both dimethylnitrosamine and diethylnitrosamine was also accompanied by haemorrhage into the gastrointestinal tract and lungs. This observation

was first made by Magee and Barnes in 1956.

Male and female rats responded similarly to dimethylnitrosamine and diethylnitrosamine poisoning with the same regularity, confirming an earlier observation by Magee and Barnes (1956) that sex differences do not influence the toxicity of dimethylnitrosamine and diethylnitrosamine.

B. Effects of dimethylnitrosamine on some biochemical systems

Much work has been carried out by various workers on the effect of dimethylnitrosamine poisoning on some biochemical processes in the body. One of earliest, and most extensively studied is the effect of nitrosamine poisoning on the nucleic acids. Thus dimethylnitrosamine has been shown to methylate nucleic acids in the intact animal and in rat liver slices (Magee and Farber, 1962; Magee and Hultin, 1962) using ^{14}C - dimethylnitrosamine. Most of the activity in the nucleic acids was 7 - methylguanine. Labelling of the RNA and DNA of the kidneys also occured, and was shown to be largely due to 7 - methylguanine, but quantitative incorporation was considerably lower than in the liver (Craddock and Magee, 1963). More recently, Lawley and Brookes (1968) demonstrated alkylation of nucleic acids on

adenine and cytosine moieties.

The biochemical studies carried out in the present work was aimed at finding out more about the early biochemical changes resulting in the overall histopathological lesion characteristic of dimethylnitrosamine poisoning. The results show that changes do occur in rat liver functions in the course of the development of the necrotic lesion induced by dimethylnitrosamine. Among the earliest of these changes is the inhibition of protein synthesis. This was manifested by low serum protein levels in rats treated with dimethylnitrosamine, irrespective of dietary protein level. This result is consistent with the observation of other workers using various other criteria. Rages (1958) showed that incorporation of ^{14}C - amino acids into liver proteins was reduced by about 50% by three hours after a necrotising dose of dimethylnitrosamine, the extent of the reduction being the same in the different subcellular fractions of the liver; and in isolated microsome + cell sap preparations incorporation of amino acids was impaired. These workers have therefore suggested that the initial damaging action of dimethylnitrosamine on the liver cell may be in the microsome structures. This view has also received support from the work of Villa-Trevino (1965), who observed progressive breakdown of the ribosomal aggregates one hour

after administration of dimethylnitrosamine, the extent of breakdown being proportional to the degree of inhibition of protein synthesis.

The early inhibition of protein synthesis in the liver may be related to the accumulation of lipids in the parenchymal cell, through the inhibition of synthesis of plasma lipoproteins, which are the vehicle for transport of triglyceride away from the liver.

Blood sugar level was also remarkably elevated in rats whose drinking water had been contaminated with dimethylnitrosamine. Since the liver is involved in the removal of sugars by glycogenesis, this result suggests that the blood sugar regulatory mechanism is distorted by dimethylnitrosamine poisoning. Rapelot and Minraki (1961) have also reported progressive loss of glycogen from livers of rats treated with dimethylnitrosamine.

Serum bilirubin level was slightly increased in rats on dimethylnitrosamine treatment showing that bile pigment metabolism is impaired during the course of hepatic damage induced by dimethylnitrosamine. This view is also supported by the rise in the urine urobilinogen level of the experimental rats.

Considerable leakage of the enzymes alkaline phosphatase and serum glutamic oxaloacetate transaminase into the serum

study the role of these conditions in cancer induction.

It has been reported that a protein-free diet protected rats against acute dimethylnitrosamine poisoning (Nolsen and Verschnurens, (1969). As a follow-up, a study on the effects of graded dietary protein levels on the toxicity of dimethylnitrosamine was made an aspect of the present study. This part of the work has been prompted by the fact that people who might be exposed to dimethylnitrosamine poisoning are under different planes of nutrition, especially as it concerns dietary protein levels.

Changes observed in the results of the liver function tests, when graded dietary protein levels were fed to the rats have proved convincingly that protein-free diets or diets low in protein content relax the toxic effect of dimethylnitrosamine in rats. When dietary protein level was high (20% and 25% levels) indices of dimethylnitrosamine toxicity were manifested by a low serum protein level, elevation of blood sugar, impairment of bile pigment metabolism and high levels of alkaline phosphatase and serum glutamic oxalacetate transaminase in the serum as reported earlier. With diets lacking or low in protein these same effects were observed but the magnitude was reduced by about half. These results therefore show intimate relationship between the diet and the carcinogenic and toxic effects of dimethylnitrosamine.

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The reduction of dimethylnitrosamine toxicity after feeding diets low in protein might be attributed to a reduced rate of metabolism of the carcinogen in the liver.

However, the failure of DDT or phenobarbitone to reverse the "no protein" effects (Swan, 1968), suggests either that the rate of dimethylnitrosamine breakdown is not affected by these inducers of microsomal hydroxylating enzyme activity or else that liver damage does not depend on the rate of dimethylnitrosamine metabolism. The first seems possible though unlikely in view of the finding of Orrenius et al. (1965) that phenobarbitone injections increased microsomal oxidations using dimethylnitrosamine as substrate. The second and more likely explanation is that neither the rate, nor the amount of dimethylnitrosamine metabolised in the liver is the predominant factor in dimethylnitrosamine liver damage. Dimethylnitrosamine after conversion to a toxic metabolite such as a carbonium ion must attack cell sites which become accessible, or are protected, depending on the previous diet. The nature of the cell site is not clear. It is not known yet which of the many alterations in the cell produced by feeding a diet deficient in protein is capable of protecting the cell

against dimethylnitrosamine poisoning. However, the change in the activity of the enzyme system metabolising dimethylnitrosamine in the livers of rats fed a protein-deficient diet is definitely the result of lack of protein, rather than the high content of carbohydrate in the diet, as gathered from the reverse to the full toxic action as dietary protein levels fed to the rats increased.

Absence of gut bacteria by pretreating experimental rats with antibiotics resulted in a slight but statistically insignificant lowering of the toxic effects of dimethylnitrosamine, irrespective of dietary protein level, using changes in liver functions as criteria for judgement. This slight change probably suggests that the involvement of gut bacteria in the catabolism of protein might result in the production of nitrosamine precursors which do react to form nitrosamine in vivo so that the effect of any level of nitrosamine is the overall effect of the level administered plus the production by gut bacteria. The possibility of this in vivo formation has been discussed by Sander, (1967).

3. Valuable properties of Nigeria's indigenous alcoholic beverages

Having appreciated the dangers associated with the discovery of nitrosamine in our local alcoholic beverages all that is left now is to decide on the future of these drinks in our society.

The decision to allow or disallow a substance for human use is not always clear cut, but must depend upon a consideration of its potential value in other situations. For instance many food materials also contain natural chemical constituents which may be potentially harmful to man and his animals. The subject of naturally occuring toxicants in foods has been reviewed by several authors. Substances which have the ability to inhibit the proteolytic activity of certain enzymes are very common in legumes (Irvin, et al. 1969). The extracts of many plants have the property to agglutinate red blood cells caused by some remarkable proteins called "lectins" (Tobiska, 1964). goitrogens have been isolated from certain plants (Greer, 1950). Cyanide in trace amounts is almost ubiquitous in the plant kingdom and occurs mainly in the form of cyanogenetic glucosides. The alkaloid dioscorine has also been found in the tubers of D. hispida (Finder, 1953).

Under these circumstances evaluation of the risk that may arise from the use of these food items must involve an assessment of the balance between benefits from nutrition point of view and the overall risk during life expectancy.

This therefore brings us to a consideration of other valuable properties of Nigeria's local alcoholic beverages so that we can reach a balanced judgement between the "negative" contribution of the nitrosamins in them and the "positive" contribution of those other values. It is only in this way that we can make a reasonable decision on the future of these drinks in our society.

(a) Nigeria's local alcoholic beverages as a source of food for the general population

One highly significant impact of our alcoholic beverages on the well-being of the people resides in their contribution to nutrition.

Consumer surveys of dietary patterns of intakes for certain parts of Nigeria have been reported by Collis, Dema and Lesi (1962); Dema, (1967) and UNICEF Fellows (1966). These reports indicate that the food consumed in this part of the world is largely derived from domestic peasant sources and that the diets are inadequate and

ill-balanced with regard to protein, calories and certain other essential nutrients.

Also, the Food and Agricultural Organisation (FAO, 1966) showed that in 1963/64, available crude protein per caput, per day, in the former Northern, Western (including Lagos and the Mid-West), and Eastern Regions of Nigeria were 79.3, 39.5 and 32.2g. respectively; while available calories per day were 2719, 1,909 and 1,774 calories, respectively. These data further show that inadequate protein and calorie intakes are widespread phenomena in Nigeria.

On the medical side, the evidence on morbidity and mortality from protein - calorie malnutrition and from other clinically manifestable nutrient deficiences, is strong in many areas of the country.

An enormous amount of palm wine is consumed in the Southern part of Nigeria and that about six million people drink a litre a day may not be an over estimation.

A litre of good potable palm wine provides approximately 500 calories from sugars and alcohol. This means that the calorie level of palm wine would contribute a fair share to the calorie requirement of the adults. It

would be Particularly desirable in the case of manual labourers.

As protein requirement is hardly met in most parts of the country any additional ecurce of protein that would improve dietary protein levels must be cherished rather than abused. With a protein level of 0.5 - 2 gms/ litre in palm wine (Bassir, 1968), about 1/30th of the normal daily protein requirement of the adult is met.

As far back as 1939, Moore found in Nigeria a constant association in the consumption of diets based largely on cassava and the incidence of sores in the angles of the mouth blistered tongues, dry itchy rash, especially of the external genitalia region and, in more advance cases blurred vission. Later Monekosso (1963) reported, from the cassava eating districts of Epe and Ijebu in the then Western Region, the incidence of ataxia concomitant with defective vision and lip and tongue changes attributable to deficiency of B Group of vitamins. in the diet.

Fresh palm wine has been shown to contain a maximum of 35.5 mg. vitamin B_2 per litre, B_1 - 25-150mg/litre; and B_6 - 4-18mg/litre (Bassir, 1968). A judicious

regular intake of ripe palm wine would therefore make good the dietary deficiency of these vitamins at a cost within the reach of an average worker.

In addition, the intake of minerals from a litre of good palm wine would be as follows: 2.0 to 2.5 mg of iron; 0.18 to 0.19g. of sodium; 0.10 to 0.13 g. of potassium; 0.12 to 0.15 g. of calcium and 6.2 to 7.1 mg. of phoophorous (Bassir, 1968).

The nutritional value of our local alcoholic beverages is not restricted to palm wine. Burukutu, Pito, and Oti Agbagba possess most of the nutritional qualities of palm wine.

b. Influence of Nigeria's local alcoholic beverages on the social life of the general population

A discussion of the values of Nigeria's local alcoholic beverages will definitely not be complete without a word or two on their influence on the social life of the people.

The traditional role of our local alcoholic drinks is strongest in the villages less so in the fairly developed towns and least in the capital cities where the established influence of the Europeans has popularised imported drinks.

The custom of drinking our local alcoholic beverages in this country is very much more than merely an excuse to get drunk. As refreshing drinks they provide a good start to relaxation after a hard day's work especially on the farms. In this way the drinks afford the desired amusement to the farmers and serve as the chief break in the monotony of their village life.

The habit of drinking these beverages is also, on most occasions, an essential way of fulfilling social obligations. They are carried to Chiefs as tribute, used to reward labour or given as offering to spirits. Abundance of these beverages, especially palm wine which is the most popular, is the glory of a villager's hospitality. Without them tribal councils cannot be held and marriage or an initiation ceremony loses its pomp. The drink also come in handy during settlement of quarrels between local heads where two or more villages are involved in a dispute.

Collective work is the striking feature of our rural communities in Nigeria. The collective effort is initiated with the drinking of our alcoholic beverages, although it is the compulsion of custom and etiquette that exerts the real force. With the 'beer' available the work is done quickly and cheerfully, and the 'beer' itself

fortifies the endurance of the workers.

Our local alcoholic beverages also contribute their own fair share to the economy of the nation. Their economic importance lies primarily in their providing employment for a sizeable fraction of our rural and urban population. This contribution is well appreciated if we realise that in the Western State alone about three million people are involved in trading activities with our alcoholic beverages.

4. Reconciliation between the presence of nitrosamine in our local alcoholic beverages and their valuable properties.

Taking the levels of contamination of our alcoholic beverages with carcinogenic nitrosamine and the dangers attributable to these levels, and comparing this with the benefits obtainable from the beverages, it will not be good science in my opinion to prohibit the drinking of our much cherished beverages on the ground that they contain these amounts of nitrosamine. This is because by doing so our nutritional status would get worse and the bulk of our population would become unnecessarily sober and forced to withdrew into an isolatedly dull life.

Neither will abruptly making the sale of these

beverages illegal go any way to improve the lot of the people. Rather it would generate disastrous effects on the social behaviour of the community of a far greater magnitude than anything attributable to the physiological upset that could result from such levels of nitrosamine in the beverages. Afterall a state of health(WHO, 1970), is an overall assessment of complete physical, mental, and social well-being of the people and not merely the absence of disease or infirmity.

To the eyes of some people, (even before this work on nitrosamine contamination) the consumption of our local beverages has always been undesirable - the habit may lead to penury, cirrhosis of the liver, obesity, and an increased mortality in road accidents to cite a few of their criticisms. These impressions have been gathered from the abusive use of these refreshing drinks by classic alcoholics who form a class of regular drunks by their excessive, uncontrolled and addictive drinking. The fore-going conclusion does not wish to tolerate this group of people for their over-indulgence produces more problems within the society than any other known disease. However, while we should not tolerate these hardened drinkers we could prevent their number from increasing by educating the people that they get more value from judicious intake

of our local alcoholic beverages than from uncontrolled and excessive intake.

GENERAL SUMMARY

By a combination of thin-layer chromatographic and colorimetric techniques described, the presence of dimethylnitrosamine and diethylnitrosamine in Nigeria's indigenous alcoholic beverages has been demonstrated. Palm wine has been shown to be contaminated by a total nitrosamine level of 30ug/litre (or 0.03 ppm), while Burukutu, Pito and Oti Agbagba are contaminated by a level of 58 ug/litre (0.058 ppm); 50 ug/litre (0.05 ppm), and 21 ug/litre)(0.02 ppm) respectively. Ogogoro, a distilled spirit from palm wine has the highest level of contamination being 100 ug/litre or 0.1 ppm.

A study of the biological production of nitrosamine in one of these alcoholic beverages, namely palm wine, revealed the active involvement of the biochemical activities of the palm wine fermenting micro-organisms.

Biochemical evidence is presented to show that an intimate relationship exist between dietary protein levels and the toxicity of dimethylnitrosamine.

Histopathological evaluation of the toxicity of dimethylnitrosamine and diethylnitrosamine in the rat showed that both nitrosamines act primarily as liver poisons, producing severe liver necrosis. Dimethylnitrosamine appeared to be quicker in action than diethylnitrosamine. This difference may be related to the difference in the structure of the two compounds.

The haemorrhagic peritoneal exudate and bleeding into the lumen of the gut are however striking features of poison by both nitrosamines in the rat. Low doses (25 ppm downwards) tend to promote hepatic tumour growth over a long period of constant intake.

Effects of dimethylnitrosamine toxicity on some biochemical functions of rat liver revealed that some of the early alterations to rat liver functions in the course of progressive liver damage are: impairment of the bile pigment metabolism; inhibition of protein synthesis; mutilation of the blood sugar regulatory mechanism resulting in elevated blood sugar level; and a considerable leakage into the blood of the enzymes primarily produced in the liver, such as alkaline phosphatase and serum glutamic oxaloacetate transaminase.

The results of the various pathological and biochemical experiments revealed a clear dose - response relationship.

Levels of contamination of Palm wine, Burukutu, Pito and Oti Agbagba do not appear high enough to cause the rat any discomfort over its life span. However, biochemical studies revealed that the level of contamination of Ogogoro with nitrosamine could be toxic.

A review of other benefits attainable from our local alcoholic beverages as against the presence of minute amounts of carcinogenic nitrosamine in them, suggest that judicious intake of these local brews is more advantageous than indiscriminate indulgence in them.

CONTRIBUTION TO KNOWLEDGE

The present study has made the following important contributions to the knowledge of Nutritional Biochemistry and Environmental carcinogenesis.

1. Nigeria's local alcoholic beverages namely, Palm Wine, Burukutu, Pito, Oti Agbogbo and Ogogoro, have been shown to be contaminated with carcinogenic nitrosamine.

2. A study of the biological production of nitrosamine in palm wine has been investigated.

3. Some of the early biochemical changes induced by dimethylnitrosamine in the course of progressive liver necrosis have been shown to include an impairment of the bile pigment metabolism, inhibition of protein synthesis, mutilation of the blood sugar regulatory mechanism, and an increased activity of the enzyme alkaline phosphatase and serum glutamic oxaloacetate transaminase in the blood.

4. An intimate relationship has been shown to exist between the toxicity of dimethylnitrosamine and dietary protein levels.

5. The role of gut - microflora in the toxicity of dimethylnitrosamine has been assessed.

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