THE EFFECT OF NATURAL TOXICANTS AND OTHER CHEMICALS ON THE KIDNEY

by

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To the Memory of my Parents

ALFONSO and LYLIA

and

ALICIA

ABSTRACT

1- Repeated administration of ochratoxin A (OTA) caused renal morphological dose-related changes, that were associated with proximal tubular and glomerular damage the latter showing oedema and prominent PAS staining suggestive of glomerular basement membrane thickening. On the other hand, the combined administration of repeated doses of OTA and aflatoxin B_1 (2.5 mg and 100 ug per kg, respectively), appeared to have a synergistic effect, characterized by severe disruption of proximal tubules and general morphological derrangement of the glomerulus, involving intense and faint staining nuclei (suggestive of cell necrosis) and cytoplasmic vacuolation, which was not seen with either toxin alone. When clinical biochemical parameters were measured after repeated administration of a low dose of OTA, enzymuria, glucosuria, polyuria and proteinuria were observed, with glucose and alkaline phosphatase as the most sensitive parameters.

2- Metabolic studies performed <u>in vitro</u> showed that isolated pig and rat glomeruli incorporate different amino acids linearly for several hours at different rates and perform oxidative metabolism of glucose and fatty acids to CO_2 also linearly.

For pig glomeruli the order of amino acid incorporation was LEU >> PRO = HIS > LYS > GLY and for rat glomeruli it was TRP >> PHE > TYR = LEU > PRO > HIS. The same amino acids were incorporated in a similar way in rat tubules, but the incorporation rate is 10-fold lower.

When <u>de novo</u> synthesis of protein by pig glomeruli exposed to different chemicals was assessed, using proline (PRO) as the precursor, adriamycin

(ADR) and ethacrynic acid (ETA) inhibited protein synthesis more than 2-bromoethanamine (BEA) and streptomycin (STR), and much more than puromycin aminonucleoside (PAN).

3- When isolated rat glomeruli were exposed to low concentrations of OTA, there was a generalized inhibition on de novo synthesis of protein from the six amino acids tested and the aromatic amino acids (TRP, TYR and PHE) were more sensitive to OTA effect than PRO.

Low concentrations of OTA (10 - 100 uM) enhanced glomerular and tubular glucose metabolism to CO_2 and only high concentrations of the mycotoxin (1000 uM) caused significant inhibition of glomerular and proximal tubular linolenic acid metabolism.

CONTENTS

CHAPTER 6

TABLE OF ABBREVIATIONS

AAP	-	Alanine aminopeptidase
ADP	-	Adenosine diphosphate
ADR	-	Adriamycin
ALP	-	Alkaline phosphatase
AT	-	Aflatoxin
ATB_1	-	Aflatoxin B_1
ATH_1	-	Aflatoxin H_1
ATM_1	-	Aflatoxin M_1
ATP	-	Adenosine triphosphate
ATPase	-	Adenosine triphosphatase
BEA	-	2-Bromoethanamine
BEN	-	Balkan Endemic Nephropathy
BSA	-	Bovine serum albumin
cAMP	-	Cyclic adenine monophosphate
EB	-	Ethidium bromide
ETA	-	Ethacrynic acid
FA	-	Folic acid
FDA	-	Fluorescein diacetate
GAG	-	Glycosaminoglycans
GBM	-	Glomerular basement membrane
GFR	-	Glomerular filtration rate
GGT	-	Gamma-glutamyl transpeptidase
GLY	-	Glycine
G6P	-	Glucose-6-phosphate
G6PDH	-	G6P-dehydrogenase
H&E	-	Haematoxylin and eosin
HIS	-	Histidine
HK	-	Hexokinase
ICA	-	Instituto Colombiano Agropecuario
LEU	-	Leucine
LYS	-	Lysine
MM	-	Mesangial matrix
MNP	-	2-methoxy-4(2'-nitrovinyl)-phenol
MNP-GLcNAc	-	MNP-2-acetamido-2-deoxy-beta-D-glucopyranoside
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide (reduced)
NAG	-	N-acetyl-beta-D-glucosaminidase
NBF	-	Neutral buffered formalin
OH-PRO	-	Hydroxyproline
OH-Lys	-	Hydroxylysine
OTA	-	Ochratoxin A
4-OH-OTA	-	4-Hydroxyochratoxin A
10-OH-OTA	-	10-Hydroxyochratoxin A
OTB	-	Ochratoxin B
OT-alpha	-	Ochratoxin-alpha
OT-beta	-	Ochratoxin-beta
OTC	-	Ochratoxin C
PAN	-	Puromycin aminonucleoside
PAS	-	Periodic acid-Schiff
PBN	-	Polybrene
PEPCK	-	Phosphoenol pyruvate carboxykinase

PHE	-	Phenylalanine
PheRS	-	Phenylalanine-tRNA synthetase
Pi	-	Inorganic orthophosphate
PRO	-	Proline
RPN	-	Renal papillary necrosis
STR	-	Streptomycin
TCA	-	Trichloroacetic acid
TRP	-	Tryptophan
TYR	-	Tyrosine
$_{32}^{P}$-UMP	-	Radiolabelled uridine monophosphate

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CHAPTER 1.

INTRODUCTION

Mycotoxins represent a very large number of chemicals that are ubiquitously distributed. They are secondary metabolites of toxigenic fungi that can be found on a variety of agricultural crops under field, harvest, as well as storage conditions. The accumulation of these toxic metabolites occurs under specific conditions of substrate, moisture and temperature. Insect and mechanical damage and improper drying and storage of grains are the main factors allowing fungal invasion with the subsequent mycotoxin production. Mycotoxins may also occur in finished foods and feeds since the ingredients destined for human or animal feeding may have been exposed at some point in time to mould contamination. In addition, more than one mycotoxin can occur simultaneously in foods and feedstuffs, since more than one species of moulds can grow on the same commodity, which may lead to the formation of more than one toxin (Lillehoj and Ciegler, 1975), and also some individual moulds may produce two or more different mycotoxins simultaneously. On the other hand, mixtures of various food or feed supplies can also be the source of more than one mycotoxin in the same commodity. Exposure to two or more mycotoxins introduces the possibility of interactions between these chemicals (Huff and Doerr, 1981).

The ingestion of mycotoxin contaminated feeds, has been responsible for acute and chronic diseases - called mycotoxicoses - in man and animals.

Some of the mycotoxins have been studied extensively. Because of the

diversity of chemical structures, the lesions produced by these compounds vary widely, but all of the major organs have been shown to be sensitive to the adverse effects caused by mycotoxins in experimental systems. These lesions are an accurate representation of what happens in animals and man with naturally occurring mycotoxicoses including those in which nephrotoxicity is an important problem.

While porcine and avian nephropathy are the most clearly identified renal lesions linked with a mycotoxin (most likely ochratoxin) ætiology, it is still not clear if Balkan endemic nephropathy (BEN) is caused by mycotoxins, although consensus currently suggests that ochratoxin A (OTA) is the most likely ætiological (or the most easily identifiable) factor. The nephrotoxicity of other mycotoxins in laboratory animals has been linked to other conditions of clinical significance. Indeed it has been suggested (Bach, P. personal communication) that mycotoxin exposure could be a key factor in the 50% of chronic renal failure and end stage renal disease that has no known ætiology. This is, however, speculation and may be untrue.

The problems associated with assessing the real role of mycotoxins in clinical nephropathies are extensive, and there needs to be a number of problems resolved before this question can be answered.

This thesis has aimed:

1- To identify the extent of glomerular involvement in the renal lesion caused by the acute administration of ochratoxin A <u>in vivo</u> and its interaction with aflatoxin B_1 .

2- To identify metabolic changes taking place in the isolated glomeruli

exposed to ochratoxin A.

The experimental work is presented in two parts, the first one deals with the renal clinical biochemical and morphological effects produced by the administration of single and repetitive different doses of ochratoxin A alone or in combination with small doses of aflatoxin B_1. In the second part, due to the findings of early glomerular changes after exposure of the animals to small repetitive doses of ochratoxin A, the studies were focused on the effect of this toxin *in vitro*. Glomeruli were isolated from rat kidneys by fractionated sieving and the effect of this mycotoxin was assessed on several metabolic pathways (i.e. *De novo* protein synthesis, and oxidative metabolism of glucose and fatty acids). For comparison proximal tubular fragments were also isolated and exposed to ochratoxin A.

CHAPTER 2.

MYCOTOXINS IN HUMAN AND ANIMAL HEALTH.

2.1 Mycotoxins in human and animal health.

2.1.1 Effects of Mycotoxins on Animal Health and Productivity

Mycotoxins can profoundly influence the health of most animal species. They can cause both acute and chronic primary diseases and in addition they can impair the animal's resistance and immunity to secondary diseases. In animal husbandry, the presence of those metabolites in feedstuffs has become a serious toxicological concern, since modern feeding practices in commercial organizations is aimed at a maximum growth or productivity, and therefore involve a high intake of concentrated feeds.

Administration of contaminated feeds to susceptible animals can cause substantial economic losses through increased mortality, slowed growth, and poor feed conversion reflected as lower productivity. In the field, outbreaks of mycotoxicosis are difficult to diagnose because of the complexity of the clinical syndrome, which can also resemble intoxication by other compounds. A causal relationship can only be demonstrated by the detection of the toxin in the feed, plasma or tissue, at concentrations sufficient to cause the toxic symptoms in the animal (Pier, et al 1975; 1977; Nelson and Christensen, 1976). Moreover, mild mycotoxin-induced disease syndromes can occur and can also be easily confused with diseases caused by pathogenic microorganisms, or by nutritional deficiencies and/or imbalances (Pier et al., 1980; Hamilton, 1977).

Individual mycotoxins can also affect more than one organ or metabolic system of an animal species, and different species may be affected in different ways (Table 2.1). On the other hand, mixtures of two or more mycotoxins can lead to a "multiple mycotoxicosis". These are much more complex and difficult to diagnose, since the symptom patterns of toxicity can differ from those that occur when only one of the mycotoxins is present, thus confusing the diagnosis.

2.1.2 **Public health aspects of mycotoxins.**

Some human diseases such as ergotism, alimentary toxic aleukia and acute cardiac beri beri, have been known for a long time, but it was some time after they were described that they were attributed to fungal toxins. However, apart from these rarely occurring human mycotoxicoses, it is difficult to determine the ætiology of most mycotoxicoses in man, because most of the information available on this matter has been derived from field or experimental animal studies and there is no direct evidence to show a causal relationship of fungal toxins in human disease. There is, however, some evidence such as epidemiological data and reports of isolated incidences believed to relate human disease, to mycotoxins.

Some reports have associated aflatoxin B_1 with acute outbreaks of disease in humans (Yadgiri et al., 1970; Serck-Hanssen, 1970; Krishnamachari et al, 1975; Jukes, 1978) resembling the acute effects of the toxin in animals. Epidemiological studies in some areas of Africa and Asia support the positive association of aflatoxin ingestion and liver cancer in man (Shank, 1976). Aflatoxin cannot be excluded as a contributing factor to some acute or chronic diseases in humans that have poorly defined aetiologies, although other factors might also have been associated with

Table 2.1 Responses of different species to various amounts of selected mycotoxins. (Pier et al., 1980)

Species	Mycotoxin	Quantity of toxin	Effect
Cattle	Aflatoxin	0.08 mg/kg [a]	Reduced weight gain (calves)
		0.2 mg/kg (B1)	Reduced weight gain coagulopathy (calves)
		0.7 ppm (B1) [b]	Reduced weight gain (steers)
		2 ppm	Reduced milk production
		0.5mg/cow/day	Detectable milk residue
		14 to 46 ppb (B_1)	
		Approx. feed (B_1)	
		milk (M_1)	
		ratio = 200:1	
		0.5 mg/kg	Death (calf); icterus haemorrhage; hepatic necrosis; coagulopathy
	Ochratoxin A	1.0 ppm	Death (steer)
		1.0 mg/kg	Depression; reduced weight gain; nephritis enteritis
Swine	Aflatoxin		
	20 kg pig	0.26 ppm	Decreased growth rate
	20 kg pig	0.86 ppm [c]	Impaired immunogenesis
	22 kg pig	2-4 ppm	Acute fatal toxicosis
	6.5 kg pig	0.62 mg/kg of body weight	Single oral dose LD_{50}
	Ochratoxin A		
	20-90 kg pigs	0.2 ppm	Chronic nephropathy
	3 kg pig	2.0 ppm [c]	Nephrosis, slow growth
	3 kg pig	10.0 ppm [c]	Acute fatal enteritis
Poultry	Aflatoxin	1-10 ppm	Acute death, hepatic necrosis and haemorrhage
		0.25 ppm	Impaired immunogenesis
		0.6-1.0 ppm	Reduced resistance
		1.5-2.5 ppm	Decreased gain
		2-8 ppm	Decreased egg production
	Ochratoxin	4-16 ppm	Acute disease, diarrhoea, death
		4 ppm	Toxic nephropathy
		2-4 ppm	Reduced gain
		2 ppm	Decreased egg production

[a] mg/kg= daily intake of toxin/kg of body weight.
[b] Feed content expressed as ppm or ppb.
[c] Level estimated from mg/kg dose and National Research Council Feed Consumption Data.

these conditions. For example, it has been also suggested that aflatoxin may play a role in the ætiology of the Reye's syndrome in children (Harwig et al., 1975). Therefore, there are still several aspects of the relationship between aflatoxin exposure and human disease that need to be clarified from more experimental and epidemiological data.

OTA has been found in foodstuffs of certain areas of the Balkans, and has been suggested to be involved in the potentially fatal disease known as Balkan Endemic Nephropathy (Krogh, 1979). The disease is characterized by renal disorders and occurs in certain very limited areas of Bulgaria, Romania and Yugoslavia. No direct proof of the association between ochratoxin and the disease has been demonstrated so far in man (see below).

2.1.3 **Natural occurrence of Ochratoxin A (OTA) and Aflatoxin B_1 (ATB_1).**

OTA can occur in a wide variety of feedstuffs. A natural occurrence of this toxin has been reported from Denmark, Sweden, Canada, USA and Yugoslavia (Table 2.2) and high concentrations have been found in sorghum, corn, wheat and barley, and have also been reported from green coffee beans, peanuts, and occassionally hay. Levels of approximately 27 ppm have been reported in Canadian wheat and Danish barley.

ATB_1 has been found in a great diversity of agricultural products in several countries (Table 2.3). As it can be seen, high concentrations of aflatoxins have been detected in corn, peanuts, sorghum and wheat. In some cases these high levels have been associated with diseases and death in animals, and man. Several other commodities have been found contaminated with aflatoxins, such as oats, soybeans, cottonseed and their subproducts,

Table 2.2 Natural ocurrence of ochratoxin A in foods and feeds of plant origin (Krogh and Nesheim, 1982)

Commodity	Country	No. of samples	% contaminated	Ochratoxin A level (ug/kg)
Foods				
Maize	USA	293	1.0	83-166
Maize (1973)	France	463	2.6	15-200
Maize (1974)	France	461	1.3	20-200
Wheat (red winter)	USA	291	1.0	5-115
Wheat (red spring)	USA	286	2.8	5-115
Barley (malt)	Denmark[d]	50	6.0	9-189
Barley	USA	182	12.6	10-29
Coffee beans	USA	267	7.1	20-360
Maize	Yugoslavia[a]	542	8.3[b]	6-140
Wheat	Yugoslavia[a]	130	8.5[b]	14-135
Wheat bread	Yugoslavia[a]	32	18.8[b]	
Barley	Yugoslavia[a]	64	12.5[b]	14-27
Barley	Czechoslovakia	48	2.1	3800
Bread	UK[c]	50	2.0	710
Flour	UK	7	28.5	490-2900
Rice	Japan[c]	2	(100)	230-430
Beans	Sweden	71	8.5	10-442
Peas	Sweden	72	2.8	10
Feeds				
Barley, wheat, oats, rye, maize	Poland	150	5.3	50-200
Mixed feed	Poland	203	4.9	10-50
Maize	Yugoslavia	191	25.7	45-5125
Barley, oats	Sweden	84	8.3	16-409
Wheat, hay	Canada[c]	95	7.4	30-6000
Wheat, oats, barley, rye	Canada[c]	32	56.3	30-27000
Barley, oats	Denmark[c]	33	57.6	28-27500

[a] From an area with endemic human nephropaty.
[b] Average values for a period of 2-5 years.
[c] All samples suspected of containing mycotoxins.
[d] Unpublished data.

Table 2.3 Levels of aflatoxin B_1 found in peanuts and cereals from several countries.

Product	Country	% contaminated	Level of ATB_1	Reference
Peanuts	USA	28.0	6-3.700	Petit and Taber (1968)
	Brazil	12.5	40-1.040	Fonseca et al (1982)
	Thailand	49.9	12.256[a]	Shank et al (1972)
Corn	USA		80-8.733[b]	Diener and Davis (1969)
	USA		83-101.000[c]	Hamilton (1971)
	USA		10.000[c]	Shank et al (1972)
	Australia		320.000[d]	Connole and Hill (1970)
	India		6.250-15.600[e]	Krishnamachari et al (1975)
	Thailand	35.0	400[f]	Shank et al (1972)
	Philipines	97.0	213[f]	Campbell and Stoloff (1974)
	Brazil	4.7	190-2.000	Fonseca et al (1982)
	Colombia	100.0	20-120	Martinez and Escobar (1981)
	Colombia	33.0	125-250	ICA-Colombia (1982) (Unpublished data)
Sorghum	Uganda	38.0	1-1.000	Alpert et al (1971)
	Australia		8.000	Connole and Hill (1970)
	Colombia	7.8	20-30	Jimeno et al (1980)
	Colombia	22.0	33-276	ICA-Colombia (1985) (Unpublished data)
Wheat	USSR	17.5	10-333	L'vova et al (1976)

[a] Aflatoxins B_1, B_2, G_1, and G_2.
[b] Associated with death in pigs.
[c] Associated with aflatoxicosis in birds.
[d] Associated with aflatoxicosis in pigs.
[e] Samples taken from a region where there were many human deaths.
[f] Average.

kidney beans, cocoa, green coffe beans, cassava, etc.

Simultaneous contamination of agricultural commodities with OTA and ATB_1 have been reported to be of frequent occurrence in India (Giridhar and Krishnamurthy, 1977; Rao et al., 1979) and mixtures of these toxins have been detected in Australian grains and animal feeds; in sorghum, the level for OTA was 100 ug/kg and for ATB_1 was 4.64 mg/kg; in animal feed, the concentration reported for OTA was 70 mg/kg, and ATB_1 levels ranged between 0.51 and 2 mg/kg (Connole et al., 1981). In both cases the possibility of an interaction between both toxins was considered in outbreaks of mycotoxicosis in pigs in the Queensland area (Ketterer et al., 1982). Both OTA and aflatoxin have also been reported as simultaneous contaminants of feed ingredients and finished feed in the USA (Huff and Doerr, 1981).

Whereas mycotoxin levels have been studied extensively in Europe and USA, there are few reports from South America on the occurrence of mycotoxins in foods and feedstuffs. In Brasil, peanuts is the most commonly surveyed agricultural commodity, and recent reports have indicated contamination with ATB_1 ranging from 40 - 1040 ug/kg. In corn, the levels found ranged from 190 - 2000 ug/kg for the same toxin (Fonseca et al., 1982). Acute outbreaks of mycotoxicoses caused by other mycotoxins have been reported, but there are no data available on the levels found in animal feeds.

In Colombia, despite the fact that under storage conditions, many agricultural commodities are susceptible to fungi development, the published information on this matter is scarce, as well as that on levels of mycotoxins and natural occurrence. Some recent surveys of grains and stock feed, revealed that low to moderate levels either of ATB_1 or OTA

have been detected, possibly associated with outbreaks of mycotoxicoses.

ATB_1 has been reported in different products of human and animal consumption. In corn the levels of ATB_1 ranged between 125 - 250 ug/kg (ICA (1982) unpublished results), and in sorghum from different regions of the country, the levels of the toxin fluctuated between 33 - 276 ug/kg (ICA (1983) unpublished results). In mixed poultry feed, the concentration of ATB_1 was between 32 - 128 ug/kg (Pena et al., 1982). These values are much lower than those reported in most of the countries (Table 2.3), but this can be explained by the little information that is available on this topic.

OTA has been found in coffee beans as well as in some cereal grains and mixed feeds. Simultaneous occurrence of ATB_1 and OTA in Colombian grains and animal feeds has been reported, but there is no published data on levels of contamination.

Although mycotoxins are mainly produced under natural conditions, in vegetable commodities, they can also be retained as residues in tissues and other products, from animals that have ingested contaminated food, and transferred to the human food chain in this way (Fig. 2.1).

2.2 **Aflatoxin B_1**

2.2.1 **Chemistry of ATB_1**

The term aflatoxin refers to the group of bisfuranocoumarin metabolites produced by strains of the Aspergillus flavus and Aspergillus parasiticus (Schindler et al., 1967; Diener and Davies, 1966). The major members are designated B_1, B_2, G_1 and G_2 (Fig. 2.2).

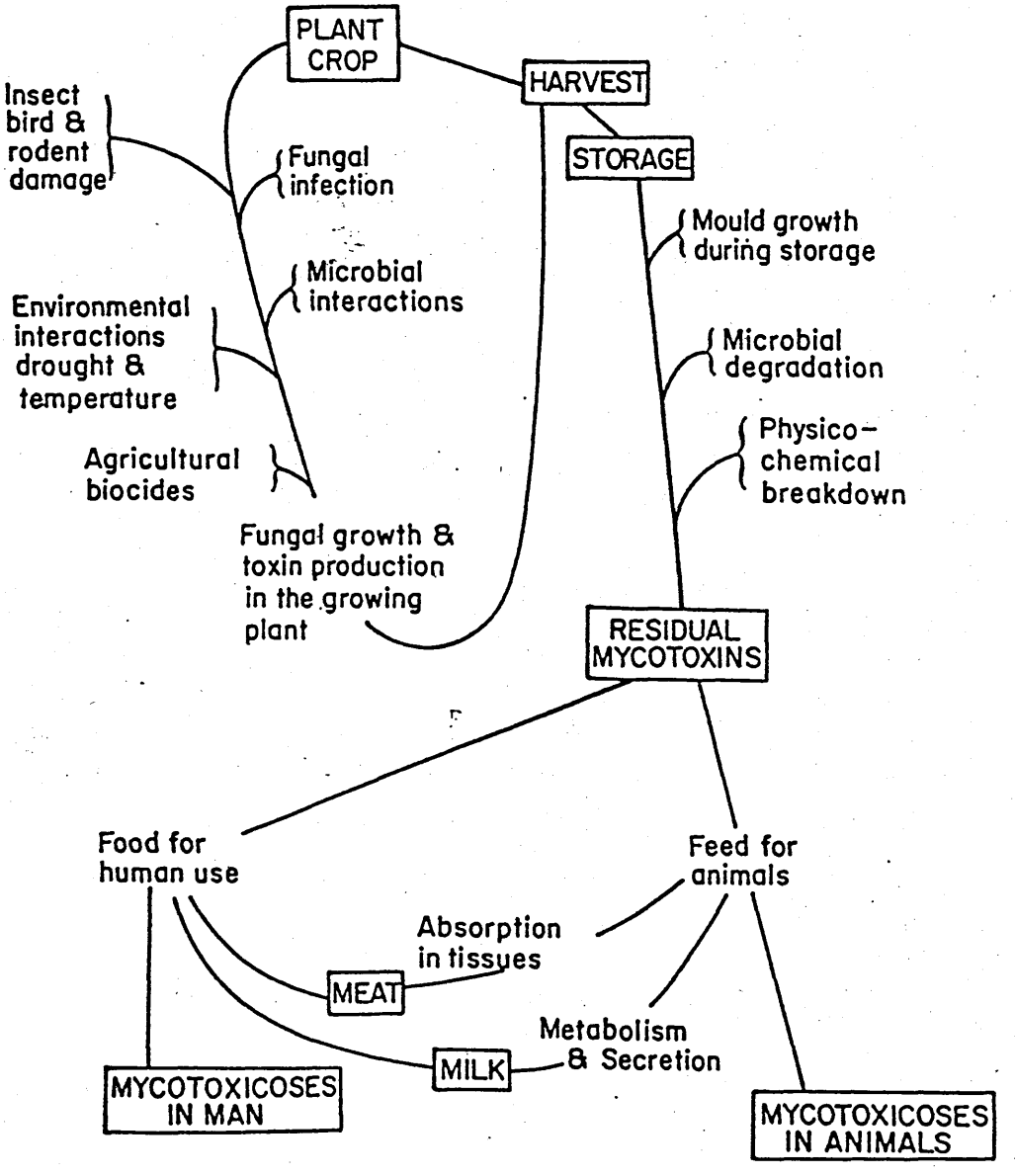
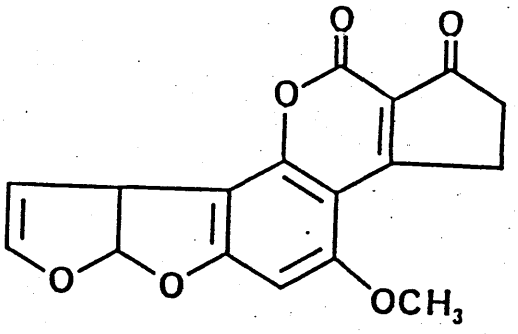
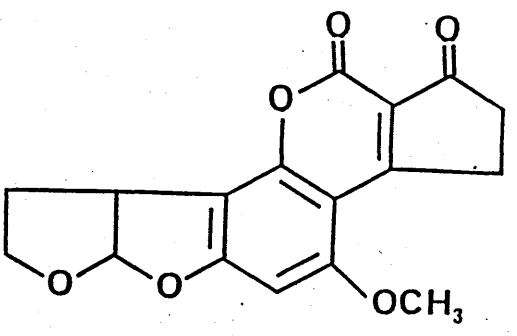


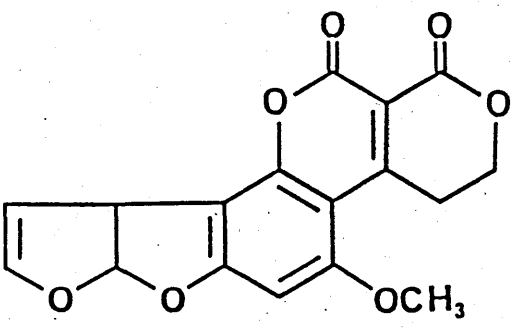
Fig.2.1. Factors influencing the occurrence of mycotoxins in human food and animal feed (from Smith and Moss,1985).



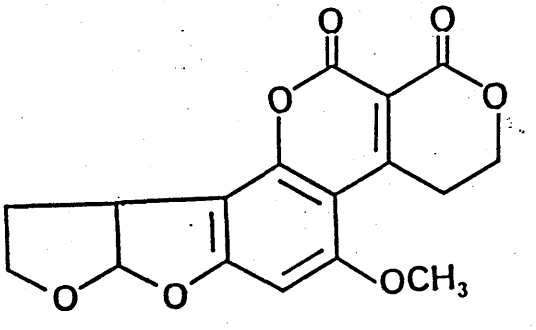
B_1



B_2



G_1



G_2

Fig. 2.2. The chemical structure of aflatoxins

The aflatoxins are crystalline compounds that fluoresce strongly in UV-light: B_1 and B_2 produce a blue fluorescence (thus the designation "B") whereas G_1 and G_2 produce a green (hence "G") fluorescence (Hartley et al., 1963).

ATB_1 is the most important of the group, due to the frequency and level of natural contamination as well as its toxic properties (Stoloff, 1976).

2.2.2 **Pharmacokinetics of ATB_1.**

2.2.2.1 Distribution.

The fate of ATB_1 was studied after a single ip dose of the radiolabelled toxin given to rats (Wogan et al., 1967). At 30 min after dosing, the kidneys contained approximately 5%, the liver 17% and the eviscerated carcass 27% of the recovered radioactivity. At 2 hr radioactivity in the kidneys and liver decreased rapidly so the kidneys and liver contained less than 1% and 10% respectively. The animals retained about 20% of the ^{14}C activity, 24 hr after administration. The liver had the highest concentration, which was equal to the content of the remainder of the carcass (5 - 8% of the recovered radioactivity). Plasma was not examined for ATB_1 content.

When pigs were fed rations containing aflatoxins at levels of 300 - 500 ug/kg for 4 months (Krogh et al., 1973), the highest concentrations of the toxin were also found in liver, with residual levels also detected in kidneys, muscle and adipose tissue. In poultry, following 8 weeks of ingestion of rations contaminated with 15 mg/kg of ATB_1 (Mintzlaff et al., 1974), the liver contained the greatest amount of the toxin, and there were also residues in muscle tissue.

In humans, under suspected natural exposure to ATB_1, the toxin has been detected in several tissues, including serum (Siraj et al., 1981; Wray and Hayes, 1980; Tsuboi et al., 1984) and liver (Siraj et al., 1981; Phillips et al., 1976).

ATB_1 binding to albumin in rats, is the principal interaction with plasma protein <u>in vitro</u> ($K = 30\ mM^{-1}$) and <u>in vivo</u> (Dirr and Schabort, 1986; Wild et al., 1986; Sabbioni et al., 1986; Dirr et al., 1987; Nassar, et al., 1982). The mycotoxin binds strongly with either bovine or human serum albumin <u>in vitro</u> (Bassir and Bababunmi, 1973). This will increase the half life of the toxin and the complex aflatoxin-albumin may act as a continuous endogenous source of the toxin to the liver.

2.2.2.2 **Metabolism**

In relation to ATB_1 metabolism it is believed to be transformed in the liver into three hydroxylated metabolites (aflatoxicol, aflatoxin Q_1 and aflatoxin P_1) that apparently may be conjugated with glucuronic acid and rapidly eliminated from the body via urine. Fig 2.3 shows the different pathways for the metabolism of ATB_1 in liver.

2.2.2.2.1 **Metabolic Activation**

Two routes for the metabolic activation of ATB_1 have been proposed. The first involves the metabolic conversion of ATB_1 to its hemiacetal ATB_{2a}, which is bound strongly to protein and has been detected in livers of animal species with susceptibility to acute aflatoxin poisoning (Patterson, 1971), and liver tumour induction (Hsieh et al., 1977). The second route, which is probably the more important form of metabolic activation is the formation of the 2,3-oxide. Although there is only indirect evidence for this, it has been shown that when ATB_1 is incubated

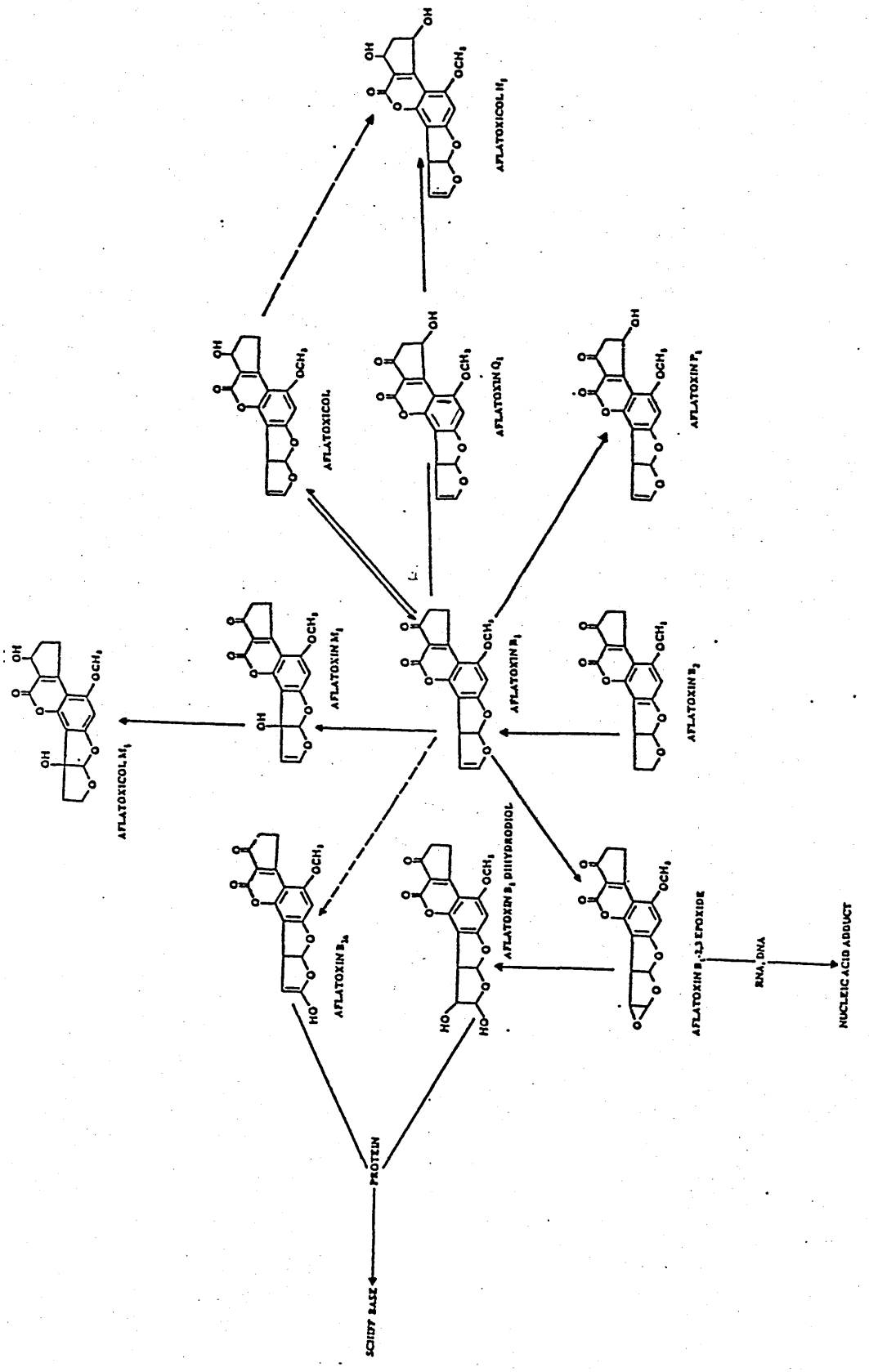


Fig. 2.3. Pathways for the metabolism of aflatoxin B_1 in the liver

in vitro with liver microsomes and RNA or DNA, or after *in vivo* ip injection of the toxin, a nucleic acid adduct was formed, which after mild acid hydrolysis, yielded the 2,3-dihydrodiol (Swenson et al., 1974; 1977). It is now generally accepted that the epoxide of ATB_1 (which is mutagenic) is also the proximal carcinogen. Conjugation with glutathione might provide a route for detoxification of the epoxide (Raj et al., 1975).

After a single ip dose of radiolabelled ATB_1 to rats, at least 80% of the dose was excreted within 24 hr of administration, and not less than 65% of the initial dose was excreted in fæces. The remaining amount was excreted in the urine (Wogan et al., 1967; Bassir and Osiyemi, 1967; Wogan, 1969). Aflatoxin is excreted conjugated or unchanged via the urine, fæces or milk. Elimination of ATB_1 in the milk of dairy cows is mainly through the excretion of the metabolite ATM_1 in a ratio 1/300 of the concentration of the toxin in the dairy ration (Rodricks and Stoloff, 1977). ATM_1 is almost as acutely toxic as ATB_1. ATH_1 has also been found in urine of cows, sheep and rats, after experimental administration of ATB_1 (Ueno and Ueno, 1978).

2.2.3 Biochemical effects of ATB_1

2.2.3.1 Mitochondrial effects

Studies on the effects of aflatoxins in isolated rat liver mitochondria showed that the compounds inhibited respiration, uncoupled phosphorylation and affected ATPase (Doherty and Campbell, 1973; Pai et al., 1975). Succinate dehydrogenase, which is associated with the inner membrane of the mitochondria, was inhibited in both liver and kidney of rats after ip administration of the toxin (Bai et al., 1977). In mice, however an elevation of this enzyme activity has been reported (Shankaran et al., 1970). This species-difference is reflected by the relative resistance of

mice to the hepatotoxic effects of aflatoxins. Other enzymes such as isocitrate dehydrogenase and malate dehydrogenase were not affected in rat liver up to 48 hr after injection of ATB_1, but decreased 3 to 5 days after administration (Clifford and Rees, 1967). In chick liver, these enzymes as well as succinate dehydrogenase were inhibited 24 hr after a single dose of the mycotoxin (Raj et al., 1974). Bai et al., (1977) observed no inhibition of isocitrate and malate dehydrogenase in rat liver, 48 hr after a single ip dose of ATB_1, but these enzymes were remarkably inhibited in kidney. Succinate dehydrogenase was inhibited in both liver and kidney. The mitochondrial effects of ATB_1 on kidney may be related to the nephrotoxic effects of the toxin and also with the synergism that has been found between ATB_1 and other toxins (eg. OTA).

2.2.3.2 **Protein and RNA synthesis**

Aflatoxins were observed to be <u>in vitro</u> inhibitors of amino acid incorporation in liver slice preparations (Clifford and Rees, 1967), and in tissue culture (McIntosh et al., 1976). Several studies have aimed at pin-pointing the exact mechanism of inhibition of protein synthesis and have concluded that the inhibition is early and due to the alteration of RNA synthesis (Moule, 1974). Nuclear RNA synthesis appeared to be a primary target for aflatoxin action. This biochemical process was the most affected by the toxin and it was amongst the more sensitive (Wogan, 1969).

2.2.3.3 **Carbohydrate metabolism**

In chicks, ATB_1 reduced hepatic glycogen, by inhibiting glycogen synthetase and glucose-6-phosphatase activities (Feuer et al., 1965; Shankaran et al., 1970). The reduction in the activities of the enzymes

may be attributed either to direct inhibition of the enzymes, or to reduction in the enzyme levels. Some dehydrogenases of the pentose shunt pathway showed increased activity (Shankaran et al., 1970), suggesting a stimulatory effect of ATB_1 on this metabolic route.

2.2.4 **Nephrotoxicity of ATB_1**

Although the focus of most studies on ATB_1 has been the liver, there are few reports describing toxic effects in the kidney, such as non-specific renal changes in ducklings given large doses (Asplin and Carnaghan, 1961). In monkeys that were fed daily with 1 mg of ATB_1 for 4 weeks (Madhavan et al., 1965), the kidneys were grossly enlarged and yellow and histological sections showed marked accumulation of fat in the tubular epithelial cells which were swollen and vacuolated, along with exudates in Bowman's spaces. Other changes included fatty liver and biliary fibrosis.

Acute aflatoxin poisoning in guinea pigs, after daily administration of 500 and 250 ug of the toxin per animal for 7 days, showed renal changes mainly in the proximal tubules, which were necrotic and often displaced into Bowman's spaces. There was also eosinophilic cytoplasmic degeneration and nuclear pyknosis in the epithelium of some proximal tubules. The necrotic changes were accompanied by separation of the epithelial segments from the basement-membrane and protrusion of the separated mass into tubular lumen (Madhavan and Rao, 1967). When rats and monkeys were given 500 ug of aflatoxin/animal daily for 3 weeks, the kidneys showed tubular necrosis and epithelial reflux, with accumulation of granular material in Bowman's space (Madhavan et al., 1965). As the tubular epithelial reflux was found in the presence of severe liver damage, it was difficult to decide whether the renal lesions were directly due to the aflatoxin or indirectly to the liver damage.

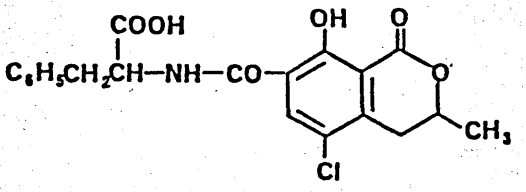
Alterations in kidney function can be induced in rats after administration of a single low dose of ATB_1, not capable of producing gross liver or kidney changes (Grosman et al., 1983, 1984). In vivo studies demonstrated that the toxin decreased glomerular filtration rate, tubular reabsorption of glucose, and tubular transport of p-aminohippurate. There was also increased urinary excretion of sodium and potassium and urinary gamma-glutamyl transferase content. Furthermore, renal cortex slices from the same rats had a diminished capacity to accumulate p-aminohippuric acid and showed alterations in water and electrolyte content.

Ketterer et al., (1982) described field cases of aflatoxicosis in pigs involving liver damage and acute renal tubular changes with abnormaly eosinophilic tubular epithelial cells and luminal dilatation with proteinaceous material. The cortex showed mild multifocal interstitial nephritis with infiltration of reticuloendothelial cells, plasma cells and fibroblasts. Diffuse interstitial infiltration was also seen in the medulla.

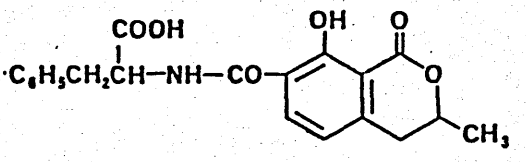
2.3 **Ochratoxin A (OTA).**

2.3.1 **Chemistry of OTA**

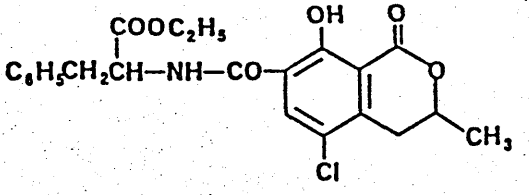
The ochratoxins constitute a group of related isocoumarin derivatives (Fig. 2.4), produced by several species of Aspergillus and Penicillium (Van der Merwe et al., 1965). The name ochratoxin was derived from A. ochraceous, and the first compound discoverd was OTA. Only this product and its dechloro-analogue, OTB have been found as natural contaminants in food, feed and their processed products. The remaining ochratoxins, including 4-OH-OTA, the methyl and ethyl esters, and the isocoumarin part of OTB (OT-Beta), have only been isolated from fungal



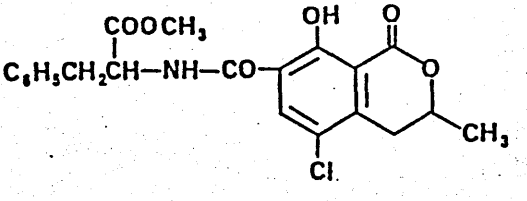
OCHRATOXIN A (OTA)



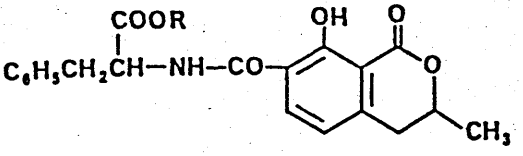
OCHRATOXIN B (OTB)



ETHYL ESTHER OF OTA



OCHRATOXIN C



R=CH_3 or C_2H_5

METHYL OR ETHYL ESTHER OF OTB

Fig. 2.4. The chemical structure of ochratoxins.

cultures under experimental conditions.

OTA is a colourless, crystalline compound, intensely fluorescent in UV-light, emitting green and blue fluorescence in acid and alkaline solutions respectively. The toxin is slightly soluble in water, soluble in diluted aqueous bicarbonate and polar organic solvents.

2.3.2 **Pharmacokinetics of Ochratoxin A**

2.3.2.1 **Absorption**

After oral administration to rats OTA (10 mg/kg) is absorbed by the stomach (Galtier, 1974b; Lillehoj et al., 1979), and distributed particularly to the liver, the kidneys and muscle. Although this is a high dose that may cause injury to the tissue, the site of absorption was confirmed by another study using lower concentrations of the toxin (288.8 ug/kg), similar to those encountered in naturally contaminated feed (Kane et al., 1986a).

In a study on intestinal absorption, the jejunum was regarded as a site of maximal absorption of OTA from the intestinal tract (Kumagai and Aibara, 1982) after injection of low doses of the toxin (1.4 mg/kg) which did not induce observable macroscopic damage to the alimentary tract. No comparison was made with higher doses of OTA in this study to see if the toxic effects on the gastrointestinal tract changed its absorption.

2.3.2.2 **Distribution**

After a single iv dose of OTA (2.5 mg/kg), the toxin is distributed into two compartments; a central compartment that includes the plasma and well perfused tissues, and a peripheral compartment (Galtier et al.,

1979). The two compartments differ in their relative accessibility to OTA and in the ease with which the toxin can leave them. After 1 hour, approximately 44% of the injected radioactivity was in the plasma, decreasing to 24% by 6 hr, and to about 9% by 48 hr. One hour after the injection, the liver contained 6% of the injected dose, followed by testes (5.7%), small intestine (4.3%), small intestine contents (4.2%), fat (1.8%), and kidneys (1.2%). However, at this time-point, muscle contained the largest percentage of radioactivity (11.6%), and only decreased to 8.4% at 48 hr after the injection. OTA exhibited a long biological half-life which was around 55 hr for either oral or iv administration; a finding strongly suggestive of tissue accumulation. When ^{14}C-OTA (highly labelled in its phenylalanine (PHE) moiety) was given to rats in a single ip dose of 1 mg of the toxin per animal (Chang and Chu, 1976), the levels of the toxin in serum, liver and kidney, 30 mins after the injection accounted for 90, 4.5 and 4.4% respectively of the dose. These decreased gradually to 56, 1.7 and 1.2% respectively, at 24 hours. No other tissues were examined for the presence of OTA. The serum half-life of the toxin was 12-18 hr which is considerably shorter than that reported by Galtier et al., (1979) for the same species when ^{14}C-OTA (uniformly labelled) was administered orally or iv. This discrepancy could be due to the different routes of administration, and/or characteristics of the radio labels used.

The distribution of radioactivity in rat tissues after oral administration of labelled OTA as studied by Lillehoj et al., (1979), showed preferential association with stomach tissue early after dosing, where a significant fraction remains 24 hr after the treatment. With the exception of stomach, kidney exhibited a higher count than other tissues during the first 3-12 hr after treatment, but this decreased during the

subsequent 12 hr. Other organs such as liver and intestine exhibited the greatest counts 18 hr after toxin exposure. Plasma was not examined for radioactivity.

The pharmacokinetic profile of OTA shows big differences among various animal species. When Galtier et al., (1981) administered the toxin orally, peak plasma concentrations were observed after 10, 1 and 0.33 hr in pig, rabbit and chicken respectively; and the fractions of the administered dose that were absorbed were 66, 56 and 40% respectively. The biological half-life of OTA was about 90 hr for the pig, 8 hr for the rabbit and 4 hr for the chicken. These results and the lower elimination rate constant obtained in the pig, provide evidence that OTA accumulates in the tissues of this animal, as had been previously demonstrated in the rat (Galtier et al., 1979). Large apparent volumes of distribution were found for the rabbit and chicken in contrast to the pig, which implied a wider tissue distribution and this, rather than accumulation, may account for problems of residues in these two species.

The observed species differences may be related to differences in the physiological processes that determine the disposition of the toxin in the animal, such as binding to serum albumin, digestive flow rate and biotransformation rates of the mycotoxin in the digestive tract. OTA has been found to interact with porcine serum albumin with higher affinity constants ($7.1 \times 10^4 M^{-1}$) than with chicken ($5.07 \times 10^4 M^{-1}$) or rat (4.01 $10^4 M^{-1}$) serum albumins (Galtier, 1979).

OTA has been reported to bind strongly to bovine serum albumin <u>in vitro</u>, with affinity constants of $3.17 \times 10^6 M^{-1}$ (Chu, 1971) and of $7.67 \times 10^5 M^{-1}$

(Uchiyama et al., 1985). Both observations showed that two molecules of the toxin were bound to one molecule of bovine serum albumin (BSA) at a common binding site, indicating that such binding may play an important role as a large mobile depot of the OTA.

The possibility of interaction of other ochratoxins with BSA has also been examined (Chu, 1974). OTB and OTC resembled OTA in their mode of interaction with bovine serum albumin, but in the case of OT-alpha and OT-beta only one molecule of either ochratoxin was bound to one molecule of BSA. The affinity of OTA for serum albumin is, however, not so high when compared to the binding of the toxin to other plasma constituents. Recent in vitro studies showed a specific and high affinity binding of OTA with a protein of molecular weight of 29,000 found in human and porcine sera (Stojkovic et al., 1984). The association constants were 2.3 x $10^{10}M^{-1}$ and 0.59 x $10^{10}M^{-1}$ respectively. Such low molecular weight protein bound with OTA can easily pass through normal glomerular membrane, before being taken up by proximal tubular brush border and broken down to amino acids and polypeptides, with the subsequent release of OTA. All thus filtered bound mycotoxin could accumulate in the kidney through this mechanism, and this selective delivery may explain the nephrotoxic effect in mammals. The same cannot happen with OTA bound to albumin, because it is not filtered unless there is a breakdown of the glomerular permselectivity. This specific low molecular weight, high-affinity ochratoxin A binding plasma macromolecule has not yet been identified. While binding of OTA to serum albumin has also been reported in vivo (Galtier, 1974a; Chang and Chu, 1977), there is no such data on the in vivo binding of the toxin to other plasma constituents.

2.3.2.3 **Metabolism of OTA**

Four metabolites of OTA are known so far (Fig 2.5). However no active forms of this toxin have so far been identified. OTA has been reported to be hydrolysed by proteolytic enzymes such as carboxypeptidase A and alpha-chymotrypsin, in vitro, with the formation of OT-alpha plus the amino acid PHE (Pitout, 1968). In vitro formation of OT-alpha by liver, small intestine and large intestine homogenates has also been reported by Doster and Sinnhuber (1972). This metabolite has also been found in vivo (Galtier et al., 1979). OT-alpha is much less toxic than the parent toxin in all acute in vivo and in vitro test systems assessed (Chu et al., 1972).

OTA is metabolized by rat liver microsomal fractions into more polar metabolites. Stormer and Pedersen (1980) identified a major metabolite (90% of the total product) as 4-OH-OTA. This metabolite has also been identified as a major urinary excretion product of OTA in rats.

4-OH-OTA has been shown to be non-toxic when single ip doses of 40 mg/kg were given to rats (Hutchinson and Steyn, 1971). Recently, human and pig liver microsomal fractions were shown to catalyze the formation of the isomers (4R)- and (4S)- OH-OTA (Stormer et al., 1981). The rates of formation for the two metabolites varied from species to species. In humans and rats the major metabolite was the (R) isomer; whereas in pigs it was the (S) isomer.

Studies on the metabolism of OTA by primary cultures of rat hepatocytes at 37^{o} showed the formation of the (4R) and (4S) hydroxylated metabolites (Hansen et al., 1982). The major product was the (4R) isomer, and 4.4% of the substrate added was converted to this metabolite. A longer

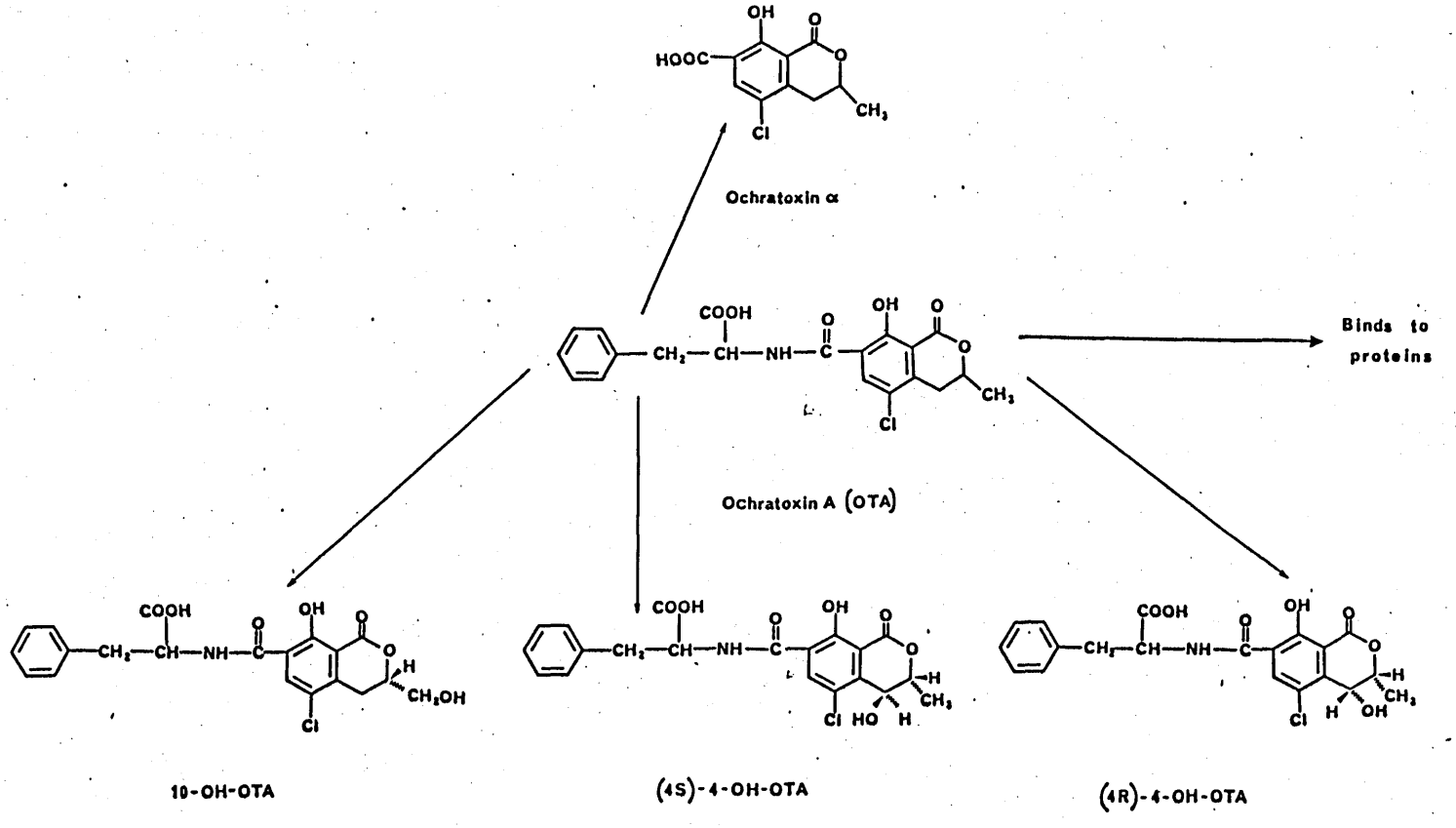


Fig. 2.5. Metabolites formed from ochratoxin A.

incubation time was needed for the detection of small amounts of (4S)-OH-OTA. A new metabolite, identified as 10-OH-OTA, has been reported to be produced by rabbit liver microsomes in addition to the (4R) and (4S) isomers (Stormer et al., 1983).

In comparing two rat strains phenotyped as extensive (Lewis) and slow metabolizers of debrisoquine, it was observed that the former strain also extensively metabolized OTA to its 4-OH metabolite, both in vivo and in vitro (Hietanen et al., 1986). It was not suggested whether the hydroxylation took place in the cis or trans position.

The formation of all hydroxylated metabolites in the different species, is inhibited by the addition of carbon monoxide and metyrapone to the incubation system and is stimulated in vivo when animals are pretreated with phenobarbital, suggesting that cytochrome P-450 catalyzes the reactions. This has been confirmed in a study where the metabolic transformation of OTA in the reconstituted cytochrome P-450 of the rat liver was investigated (Ueno, 1985). Cytochrome P-450 IIa fractionated from PCB-microsomes plays a major role in the formation 4(R)-4-OH-OTA whereas the formation of 4(S)-4-OH-OTA was the highest with P-450 I-C. As in the rat hepatic microsomal system, 4(R)-4-OH-OTA was the major metabolite in the reconsitituted cytochrome P-450 system.

2.3.2.4 **Excretion of OTA.**

OTA is excreted via fæces and urine. When ^{14}C-labelled OTA was administered in a single ip dose (10mg/kg) to rats (Chang and Chu, 1977), the toxin was excreted primarily as the parent compound in the urine, as 50% of the total radioactivity, during the first 24 hr. In the same period the excretion of OTA in the fæces was only 13%, and small proportions of

OT-alpha, the hydrolysed product of the mycotoxin, were found in urine and fæces. These findings are in agreement with previous studies (Purchase and Nel, 1967; Nel and Purchase, 1968; Van Walbeek et al., 1971), in which OTA and also small amounts of OT-alpha were reported to be excreted in the urine and fæces of rats that had been given the toxin.

After single oral or iv doses (2.5 mg/kg) of ^{14}C-labelled OTA given to rats, the radioactivity excreted via fæces (56%) was greater than via urine (32%) for up to ten days after the treatment (Galtier et al., 1979). The parent compound and OT-alpha were recovered in the fæces and urine, as the two main excretion products. Although 5 more metabolites were found in urine, they remained unidentified.

In another study, when OTA was administered to rats either ip or per os (6.6 mg/kg), the excretion of the toxin was mainly via urine (Storen et al, 1982), with OT-alpha as the main excretion product (26%), followed by OTA (6%). Small amounts of other metabolites were also found in urine, but not identified. Only traces of OTA and OT-alpha were found in fæces.

Few excretion studies have been performed in other species. OTA, OT-alpha and OT-beta were found in the urine and fæces of pregnant pigs (Shreeve et al., 1976; Patterson et al., 1976), that were given a mixture of OTA and OTB. In cows fed on OTA, OT-alpha was the main excretion product in the urine (Still, 1973). In another study in which high doses of OTA were administered to cows (Ribelin et al., 1978), the toxin was found in milk and urine, in spite of previous reports on the capability of ruminal flora to hydrolyze OTA into its non-toxic metabolite, OT-alpha, preventing the absorption of the toxin (Hult et al., 1976; Galtier and Alvinerie, 1976).

There is recent evidence to show that OTA undergoes enterohepatic circulation in the rat as well as in the mouse. Fuchs et al., (1988) observed that OTA was rapidly excreted into the bile, in the conjugated form, with the highest concentrations measured during the first 6 hr after oral dosing. When the bile from OTA treated animals was administered to untreated rats, either into the stomach or the duodenum, the toxin was found in plasma, suggesting the existence of enterohepatic circulation for OTA in rats. OTA measurements in plasma were performed 24 hr after the administration of the toxin in bile, and no other time points were assessed, nor were the levels in other tissues or fluids measured. There was therefore no confirmation by other means. Roth and coworkers (1988) observed that after im injection of ^{3}H-OTA to mice, the mycotoxin was rapidly absorbed and distributed in the organs, and found in serum, bile, intestinal contents and urine 30 min after administration. When measured at other time points, a cyclic pattern was shown, that corresponded to the absorption from the intestine to serum and re-excretion through bile into the intestine. OT-alpha plus PHE were the metabolites found in intestinal content. In bile OTA was found mainly in conjugated form, although the hydroxylated metabolites and a small proportion of free OTA were also detected. When cholestyramine, an inhibitor of the enterohepatic circulation was given prior to OTA, the elimination pattern of the toxin was completely changed, with smaller amounts of OTA being found in bile, indicating that the compound prevented OTA reabsorption from the intestine and confirming the enterohepatic circulation of OTA. Although PHE seemed to facilitate the absorption of OTA when both compounds were given simultaneously by gavage, the enterohepatic cycle of OTA appeared to be suppressed (Roth et al., 1988). This could possibly have happened through competition between the two compounds for the metabolism of OTA by the

intestinal microflora and by a competition for the intestinal reabsorption.

The urinary excretion of OTA has recently been shown to increase in mouse after simultaneous administration of ^{3}H-OTA and PHE (Roth et al., 1988). The toxin was eliminated mainly as OT-alpha and PHE, and in the OTA and PHE treated animals, 4OH-OTA was also found.

2.3.3 **Biochemical effects of OTA**

2.3.3.1 **Mitochondrial effects.**

OTA inhibited respiration to 55% of the control at the concentration of 2.1×10^{-6}M and completely blocked the respiration at 4.2×10^{-4}M. As the presence of 2,4-dinitrophenol overcame the effect of the toxin, it was concluded that OTA inhibited ADP-stimulated respiration by blocking some point in the mitochondrial respiratory chain beyond the action site of 2,4-dinitrophenol (Moore and Truelove, 1970). Furthermore it has been demonstrated that OTA not only inhibits succinate-supported respiration, but also acts as a competitive inhibitor of mitochondrial transport carrier proteins such as ADP and Pi exchange proteins (Meisner and Chan, 1974). However the exact site and action of the toxin in mitochondrial respiration was not found.

More recently, it was found that when OTA was added to isolated liver mitochondria respiration was decreased as the concentration of the toxin increased (Wei et al., 1985). Mitochondrial respiration was gradually uncoupled by the toxin when its concentration was raised above 1.2×10^{-6}M, and became fully uncoupled at 6.2×10^{-4}M. Oxidative phosphorylation was not damaged until the toxin concentration was higher than 9.3×10^{-5} M and at concentrations above 1.0×10^{-4}M OTA inhibited

the succinate dehydrogenase, succinate-cytochrome C reductase, and succinate oxidase activites of the respiratory chain. The activities of the latter two enzymes were decreased by 50% in the presence of 8.0×10^{-4}M and 6.2×10^{-4} M OTA, respectively. However, activities of either cytochrome oxidase or NADH dehydrogenase in the mitochondrial respiratory chain were not inhibited by the mycotoxin. OTA exerts its effect on mitochondrial respiration and oxidative phosphorylation through alteration of the mitochondrial membrane and inhibition of the succinate-supported electron transfer activities of the respiratory chain. Inhibition of mitochondrial respiration and oxidative phosphorylation will diminish the supply of ATP, which is essential for the active transfer of molecules by the proximal tubular cells, thus causing impairment to the tubular reabsorptive functions.

2.3.3.2 **Protein synthesis**

OTA inhibited protein synthesis from ^{3}H-leucine by 60% when 9×10^{-5} M of the toxin were incubated with cultured hepatoma cells (Creppy et al., 1979a). There was also a delayed inhibition of RNA synthesis, which began 2.5 hr after treatment, and was probably a consequence of the effect on protein synthesis. There was no inhibition of DNA synthesis up to 5 hr. OTA induced inhibition of protein synthesis can be completely prevented if PHE is present simultaneously in the cell culture medium at a concentration of 5×10^{-5} M (Creppy et al., 1979a). The reversal of inhibition could be interpreted as an impairment of entry of OTA into the cells by a competition with PHE for some cell receptors.

MDCK cells were much more sensitive to OTA effects (Creppy et al., 1986). Protein synthesis was inhibited by 94% and 50% when the cells were

incubated with 2.5 and 1.5 x 10^{-5} M of OTA respectively for 24 hr. Similar effects were obtained with the same concentrations of the toxin on RNA and DNA synthesis, suggesting that MDCK cells are very sensitive to the mycotoxin. With simultaneous addition of PHE and 2.5 x 10^{-5} M OTA, the inhibition of protein synthesis dropped from 94% only to 40%.

The metabolite (4R)-OH-OTA, which has been shown to be non-toxic in vivo (Hutchinson and Steyn, 1971), inhibited protein synthesis in hepatoma cell cultures within a few hours (Creppy et al., 1983). As this effect was also prevented by PHE and the metabolite OT-alpha had no effect it was suggested that the PHE moiety plays an important role in the toxicity of OTA.

The effects of OTA on protein synthesis have not been assessed in renal in vitro systems other than MDCK cells, such as isolated nephron segments, other cell lines, or isolated glomeruli or proximal tubular fragments.

In vivo, renal protein synthesis from ^{3}H-PHE or ^{3}H-leucine (LEU) is reduced by 30 - 40% in rats given 2 mg of OTA/kg/day, orally for two days (Meisner and Meisner, 1981), and a single ip dose of 1 mg/kg OTA caused a 68% inhibition of protein synthesis in mice kidney, 5 hr after administration of the toxin (Creppy et al., 1984). No other species have been assessed for the in vivo effect of OTA on protein synthesis in kidney.

OTA may act as an analogue of PHE with regard to aminoacylation of tRNA in bacteria and eucaryotes (Konrad and Roschenthaler, 1977; Nishino et al., 1979; Creppy et al., 1979b), a reaction that is catalysed by PHE-tRNA synthetase (PheRS) and is essential for all living organisms. OTA

competitively inhibited the formation of PHE-tRNA. The effect of OTA and its 4R epimer was tested in vitro on the catalytic action of PheRS (Creppy et al., 1983), and both compounds inhibited the reaction by about 70%. This would suggest that the toxicity may be related to the inhibitory effect on PheRS, perhaps due to the binding of OTA to the PHE sites of the enzyme.

2.3.3.3 **Carbohydrate metabolism**

The effect of OTA on carbohydrate metabolism in the liver has been investigated in rats by Suzuki et al., (1975a). The toxin caused depletion of hepatic glycogen, reduced activity of glycogen synthetase and increased serum glucose levels. By contrast, phosphorylase activity was increased. These effects were in part attributed to the inhibition of the active transport of glucose into liver tissue. In contrast to the OTA effect on carbohydrate metabolism in rats, Huff et al., (1979) found that in chickens, liver glycogen levels were increased, implying inhibition of glycogenolysis. It was suggested that in this species OTA primarily affects cAMP-dependent protein kinase which initiates the enzymatic cascade leading to glycogenolysis.

Regarding the kidney, which is considered the target toxicity organ, gluconeogenesis was inhibited in rats fed 2 mg/kg/day OTA for two days (Meisner and Selanik, 1979). Renal gluconeogenesis from pyruvate was decreased by 26%, and phosphoenol pyruvate carboxykinase (PEPCK) activity was lowered by about 55%. Hepatic PEPCK was unchanged or slightly increased; and hexokinase activity remained unaffected in both organs. The decrease in renal gluconeogenesis was therefore probably due to inhibition of PEPCK, which was decreased to a greater extent than gluconeogenesis.

Furthermore, renal cytosolic PEPCK activity was inhibited by OTA in a dose dependent pattern when the toxin was fed to rats (Meisner and Meisner, 1981). The enzyme's activity was inhibited 50% by a total OTA dose of 0.3 - 0.5 mg, and renal gluconeogenic capacity was reduced only after PEPCK activity had been inhibited by 50%. Hepatic PEPCK was unaffected. Other proximal tubular enzymes such as gamma-glutamyl transpeptidase, pyruvate carboxylase and Na, K-ATPase were also not affected. Renal PEPCK was also inhibited in swine fed OTA for one to four weeks (Meisner and Krogh, 1982).

Removal of the PHE group from the toxin, prevented the in vivo inhibition of PEPCK activity (Meisner and Meisner, 1981), suggesting that the PHE residue of OTA may play a role in the enzyme inhibition.

Apart of the gluconeogenic pathway, there is no published data on the study of other routes of carbohydrate metabolism in the kidney, with relation to OTA toxicity, either in vivo or in vitro.

2.3.3.4 **Effect of OTA on mRNA.**

To find out whether the reduction of renal PEPCK activity was associated with a reduction in the level of mRNA coding for this enzyme Meisner et al., (1983) measured the effect of OTA on the level of translatable mRNA for PEPCK in kidneys of rats fed the toxin for 2 days. It was found that OTA greatly reduced the concentration of the mRNA coding for PEPCK in kidney, while the rate of transcription of PEPCK-RNA was not affected. The role played by these biochemical events in the pathology of OTA lesion is still unknown. The effect of OTA on mRNA pool size is a distinguishing feature of the biochemical action of the nephrotoxin. The reduction has been attributed to a rapid turnover on mRNAs (Meisner et al., 1983).

A recent study in isolated kidney mRNA from rats fed OTA for 2 - 5 days, identified several mRNAs whose concentration were changed within 2 days by the toxin (Meisner and Polsinelli, 1986). The transcription rate of each mRNA, was measured in nuclei. The incorporation of ^{32}P-UMP into PEPCK mRNA and the synthesis of other RNAs were not affected, suggesting that the toxin must therefore affect the concentration of certain mRNAs by a post-transcriptional mechanism, which has not been defined yet.

2.3.4 **Nephrotoxic effects of OTA.**

The major effect of OTA in animals is damage to the kidneys, both in field intoxications and in experimentally dosed animals. OTA is a major determinant of porcine nephropathy, a disease occurring endemically in several countries.

2.3.4.1 **Porcine nephropathy.**

This peculiar renal disorder of spontaneous occurrence in swine, and initially designated as "mold nephrosis", has been known in Denmark for many years and more recently in Sweden, and other European countries (Elling and Moller, 1973; Rutqvist et al, 1978; Sandor et al, 1982). The disease is now associated with the ingestion of feed contaminated with OTA. The histopathological changes which have been described include tubular degeneration and atrophy, accompanied by interstitial fibrosis. The changes in renal function are characterized by impairment of proximal tubular function (Elling and Moller, 1973).

Several workers have tried to reproduce the disease symptoms in experimental animals. Pigs exposed to A. ochraceus cultures or pure OTA

in the range 0.2 to 2.0 mg/kg, developed renal damage within one to three weeks (Szczech, et al., 1973a). However, changes in other organs like gastrointestinal tract, liver and lymphoid tissues and progressive leukocytosis were also found, which may well be attributed to the high levels of OTA used, amounts which are unlikely to be encountered in naturally contaminated feed. Alterations in renal function resulted in polydipsia, polyuria, low urinary specific gravity, proteinuria, glucosuria, increased blood urea, and increased urinary excretion of lactate dehydrogenase, isocitrate dehydrogenase and glutamic oxaloacetic transaminase. Structurally necrosis of renal tubular epithelium was most frequent in the convoluted tubules and there were many dilated tubules.

Pigs fed naturally contaminated rations containing 0.2 - 4 ppm of OTA, or the pure toxin over four months, developed nephropathy identical to that in the spontaneously occurring cases, without changes in other organs or tissues different to the kidneys (Krogh et al., 1974; 1976). The toxin doses corresponded to those naturally occurring in animal feed. The changes in renal function was characterized by impairment of proximal tubular function, indicated by decrease in transport capacity for p-amino hippuric acid, reduction in the ability to concentrate urine and by increased urinary excretion of glucose, leucine amino peptidase and proteins. Structurally there was degeneration of proximal tubules, interstitial formation of connective tissue, and at later stages development of atrophied and sclerotic glomerular tufts. Long-term administration of the toxin induced progressive nephropathy in pigs, without reaching a state of terminal renal failure (Krogh et al., 1979), with functional and morphologic changes similar to those found in the previous studies with naturally contaminated feed given to pigs for three

months (Krogh et al., 1974), and to those in spontaneously occurring mycotoxic porcine nephropathy (Elling and Moller, 1973).

The examination of early structural changes in OTA-induced nephropathy after short-term exposure, identified a process of condensation of cellular material with disappearance of membranes and continuous desquamation in the lower part of the proximal convoluted tubules (Elling et al., 1985). This process appeared to be linked to enhanced levels of peroxisomal beta-oxidation in the soluble kidney fractions.

2.3.4.2 **Avian nephropathy**

Toxicity of OTA in poultry has also been observed. Spontaneous avian nephropathy associated with the ingestion of feed contaminated with OTA, has been reported in Denmark (Elling et al., 1975), with frequencies similar to those observed for porcine nephropathy in the same counties and during identical time periods. Structurally the lesions are characterized by degeneration of the tubular epithelium in the form of dilated tubules, "ballooning" and desquamation of epithelial cells. Giant nuclei are frequently seen in epithelial cells. These changes are accompanied by formation of interstitial fibrous tissue.

Avian nephropathy similar to spontaneously occurring cases was observed in chickens and hens following exposure to 0.3 and 1 ppm OTA feed during one year (Krogh et al., 1976). The histopathological changes were similar to those in the spontaneously occurring cases. The changes in renal function were characterized by impairment of glomerular and tubular function, as indicated by decreases in inulin clearance, maximum tubular secretion rate of p-amino hippuric acid and urine concentrating ability. The same alterations in renal function have been observed in hens fed OTA at levels

of 0.3 - 1 ppm OTA for 49 weeks (Svendsen and Skadhauge 1976).

Sublethal concentrations of OTA (2 - 4 ppm) fed to young broiler chicks (Dwivedi and Burns, 1984), mainly affected the proximal convoluted tubules of the kidney, causing severe distension, enlargement and hypertrophy, together with thickening of the glomerular basement membrane. There were changes in liver as well, such as vacuolation and glycogen accumulation in the hepatocytes. The toxin also caused regression of, and a drastic reduction in, the lymphoid cell population in the immunological organs. Nephrotoxicity, accompanied by hepatotoxic effects in OTA-fed chicks, have also been documented in other studies (Peckham et al., 1971.

Other effects of OTA in poultry have been described. The toxin causes impaired blood coagulation (Doerr et al., 1974), anæmia (Huff et al., 1979), bone abnormalities (Huff et al 1980) and type X glycogen storage disease (Warren and Hamilton 1980).

OTA has also been shown to produce nephropathy in controlled laboratory studies in rats and acute doses have shown that the female is more sensitive. Fatal doses of OTA, led to severe necroses of the renal tubules, in particular the proximal tubules (Purchase and Theron 1968). Necrosis of periportal cells in the liver was also seen. However, another study using sublethal doses of OTA, did not elicit any evidence of renal damage (Kanisawa et al., 1977), instead the administration of small repetitive doses were able to induce recognizable changes without killing the animal consisting of massive acidophilic degeneration with necrosis and desquamation of the epithelium in the proximal tubules. Lymphatic tissues were also sensitive to the toxin, and hepatic changes were

injected ip produced death, but this was not preceded by any alterations in renal function (Berndt and Hayes, 1979). On the other hand, daily administration of OTA to rats in doses of 0.75 and 2 mg/kg for five days caused functional renal changes characterized by a significant depression of urine osmolality and increased excretion of protein and glucose. The requirement for repeated doses of OTA to produce an alteration of renal function, suggested the necessity for accumulation of the toxin before any overt nephrotoxic effect was noted.

Following a single oral dose of OTA (15 mg/kg) urinary enzyme activities were measured (Obara et al., 1984). Urinary excretion of alanine aminopeptidase and leucine aminopeptidase was significantly increased by OTA . When nephron segments were isolated, both enzymes were localized in the proximal tubule, showing much higher activity in the straight portion than in the convoluted one. Enzyme release from this part in the presence of OTA revealed a dose-dependent relationship and was inhibited by probenecid, and it was suggested that OTA might enter the proximal tubule cell through the basolateral membrane and release enzymes such as alanine aminopeptidase and leucine aminopeptidase located in the luminal brush border membrane.

Biochemical changes in urine and tissues from rats treated with 5 consecutive daily doses of OTA (10 mg/kg) were studied (Ngaha, 1985) and found to consist of elevation of urine volume, urinary proteins, and enzymuria. Excretion of muramidase, was significantly raised 24 hr after the first dose, but increases in alkaline phosphatase, acid phosphatase, and glutamate dehydrogenase levels occurred very much later during the course of treatment with OTA. Kidney alkaline phosphatase and acid phosphatase, lactic dehydrogenase and glutamate dehydrogenase were reduced

seven days after the beginning of the treatment.

In a subchronic study, the activities of 5 enzymes in urine and renal tubules were measured, following oral administration of small doses of OTA to rats (145 ug/kg per day for 8 - 12 weeks (Kane et al., 1986b). Urinary activity of gamma-glutamyl transpeptidase, alkaline phosphatase and leucine amino peptidase were increased after one week of the treatment, and N-acetyl-beta-D-glucosaminidase and lactic dehyrogenase activities remained normal. All the enzymes showed their maximun increase in the fourth week, followed by a decrease in week 5 - 7, increasing again in the 8th week and suggesting a cycle of degeneration and regeneration of the cells. However, histological studies were not performed to support this assumption. In tubules the activity of all the enzymes decreased, but N-acetyl-beta-D-glucosaminidase exhibited less decrease than other enzymes, suggesting that the effect of low doses of OTA is essentially directed towards the brush border membrane and is slight on the lysosomal membrane. The study also showed that 20 ppm of phenylalanine partially prevented the nephrotoxic effects of OTA under subchronic conditions (Kane et al, 1986b).

In a subacute study in which rats were fed dietary levels of OTA (0.2 - 5 mg/kg) for three months (Munro et al., 1974), renal damage, including desquamation of cells in the proximal tubules and thickening of the basement membrane was observed at all dose levels. Administration of high levels of ochratoxin A (5 mg/kg/day) to rats for three days, demonstrated an acute toxic effect on renal function in rats consisting of decrease of both inulin and p-aminohippuric acid clearance (Suzuki et al., 1975b). On histological examination, thickening of the basement membrane was observed and attributed to be the cause of the tubular malfunction.

More recently the effects of low levels of OTA were investigated in partially nephrectomized rats (Stein et al., 1984). The changes in renal function were characterized by decreased urine osmolality and inulin, creatinine and p-aminohippurate clearances. The histopathological findings showed considerable renal tubular necrosis and subcellular damage. Although similar changes were observed in OTA-treated control animals, partially nephrectomized rats were more susceptible, not only with regard to renal function, but also in exhibiting enhanced morphological end points.

In spite of the fact that some studies have demonstrated that probenecid has a protective effect against nephrotoxicity of some compounds normally cleared by renal secretion (Tune, 1975; Berndt and Hayes 1982), such an effect against OTA-induced nephrotoxicity was not observed when probenecid was administered to rats before or simultaneously with the mycotoxin (Stein et al., 1985). On the contrary, the animals exhibited signs of increased nephrotoxicity which included significant decreases in urine osmolality, Na^+ and K^+ concentrations, body weight and increased OTA concentrations in the kidneys. However, histopathological data to support these findings were not reported.

The clinical and clinicopathologic features of experimental ochratoxicosis in dogs have been studied. The animals were exposed for one to three weeks to A. ochraceous cultures or crystalline OTA in the range to 0.2 to 3 mg/kg (Szczech et al., 1973 b,c.). Changes in renal fuction were described as characterized by polydipsia, polyuria, low urinary specific gravity, glucosuria, proteinuria, and increased urinary excretion of the following

enzymes: lactic dehydrogenase, isocitrate dehydrogenase, leucine aminopeptidase, glutamic-piruvic transaminase, glutamic-oxaloacetic transaminase. Renal structural changes consisted of necrosis and desquamation of epithelial cells mainly but not exclusively in proximal convoluted tubules. Additional findings were colitis, necrosis of lymphoid tissues and hepatic alterations. Ultrastructural changes were described primarily in the epithelial cells of renal proximal tubules (Szczech et al., 1974).

2.3.5 Effect of combined OTA and ATB_1 administration.

The effect of simultaneous administration of OTA and ATB_1 has been reported. In a chronic study in rats fed the toxins simultaneously, the collecting tubules showed degenerative changes in epithelial cells and some of these cells were anaplastic with hyperchromatic nuclei, after 4 months of treatment (Rati et al., 1981). These changes were not observed in animals fed either OTA or ATB_1, alone, suggesting that the combined action of the toxins might be synergistic.

In a study with chickens fed both OTA and ATB_1 simultaneously for three weeks, the interaction between the mycotoxins was manifested by nephropathy as the primary effect (Huff and Doerr 1980). No histopathological assessment supported this interaction.

2.4 OTA and Balkan Endemic Nephropathy (BEN).

Balkan Endemic Nephropathy is a chronic disease affecting the human population in localised areas of Yugoslavia, Bulgaria and Rumania. Although the condition has been recognized since the 1950s, the ætiology of the disease is still unknown.

The clinical picture is of a slowly progressive renal degeneration, including proteinuria and uræmia, leading to end stage renal disease. The pathology of BEN (Fig. 2.6) shows shrunken kidneys with atrophied tubules interstitial nephritis and marked glomerular basement membrane thickening and hyalinization (Hall and Dammin, 1978). BEN is also associated with a high frequency of carcinoma of the renal pelvis, ureter and urinary bladder (Ceovic et al., 1976; Chernozemsky et al., 1977; Nicolov et al., 1978).

Various factors such as bacterial infection, heavy metal toxicity, immunologic and genetic factors have been implicated in the ætiology of BEN, but as yet there is no conclusive evidence to definitively identify the causative agent(s). The hypothesis that mycotoxins may be involved in this disease, has emerged as the most probable (WHO, 1979; Krogh, 1972), in view of the higher frequency of OTA contamination that has been encountered in foods of endemic compared to disease-free areas (Krogh et al., 1977, Pavlovic et al., 1979) and of the higher levels of OTA bound to plasma proteins, that has been detected in inhabitants of endemics areas of Yugoslavia and Bulgaria than in control groups within or outside the endemic area (Hult, et al., 1982). Evidence for the involvement of OTA in BEN, is also based on the fact that the mycotoxin is a major disease determinant of porcine nephropathy (Krogh, 1978; 1979), in which the pathological and functional features resemble those of the human disease (Table 2.4). However the final determination as to whether OTA has a causal role in BEN, awaits further evidence.

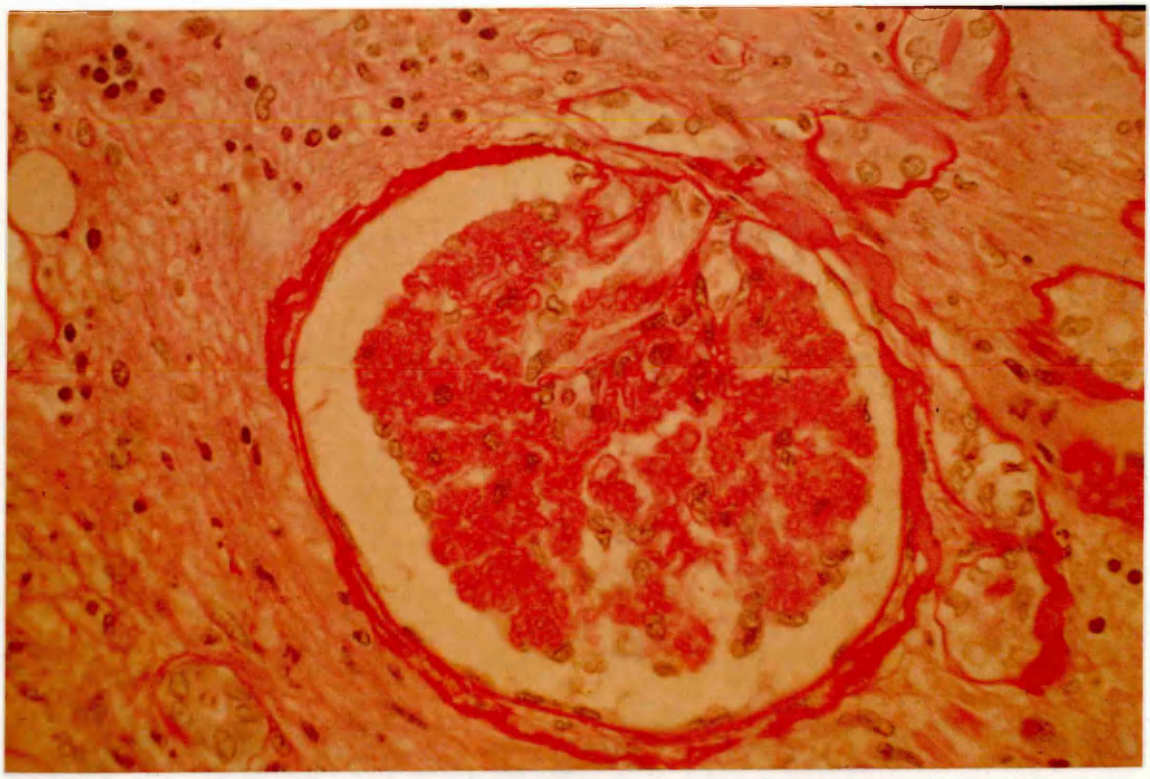


Fig. 2.6
Pas stained section of BEN tissue showing sclerosis and thickening of Bowman's capsule membrane (arrow) and the glomerular tuft together with fibrosis and tubular atrophy (x172).

Table 2.4 Endemic (Balkan) nephropathy and ochratoxin A-induced porcine nephropathy (Krogh, 1977).

	Common features
Renal function	Impairment of proximal tubular function (decreased Tm_{PAH}) Subsequent impairment of glomerular function (decreased C_{In})
Renal structure	Atrophy of tubules Hyalinization of glomeruli Interstitial fibrosis
Epidemiology	Endemic ocurrence Sex-specific distribution

CHAPTER 3

THE GLOMERULUS AS A SITE OF TARGET TOXICITY

3.1. Introduction

In spite of its small mass in relation to the body weight, the kidney receives approximately 25% of the resting cardiac output. Therefore, any drug or chemical capable of being excreted by the kidney, will be delivered to the organ in significant amounts from the systemic circulation, and will undergo concentration and dilution in different parts of the nephron.

The functional unit of the kidney is the nephron (Fig 3.1), which consists of the renal corpuscle (glomerulus), the proximal tubule, the thin limbs, the distal tubule and the connecting segment. The location of the renal corpuscles within the cortex, is the determining factor for three major types of nephrons: superficial, midcortical and juxtamedullary. There are clear differences in the size of glomeruli and rates of filtration; juxtamedullary nephrons having a greater filtration rate (Ulfendahl et al, 1981).

The focus of most toxicological studies has been the proximal tubule, because of the large number of chemicals that damage this region, while the glomerulus has not generally been considered as an area affected by many toxic chemicals. Therefore there are, so far few studies on the effect on glomerular metabolism of compounds known to cause injury to the glomerulus. There are also several compounds known to cause renal injury,

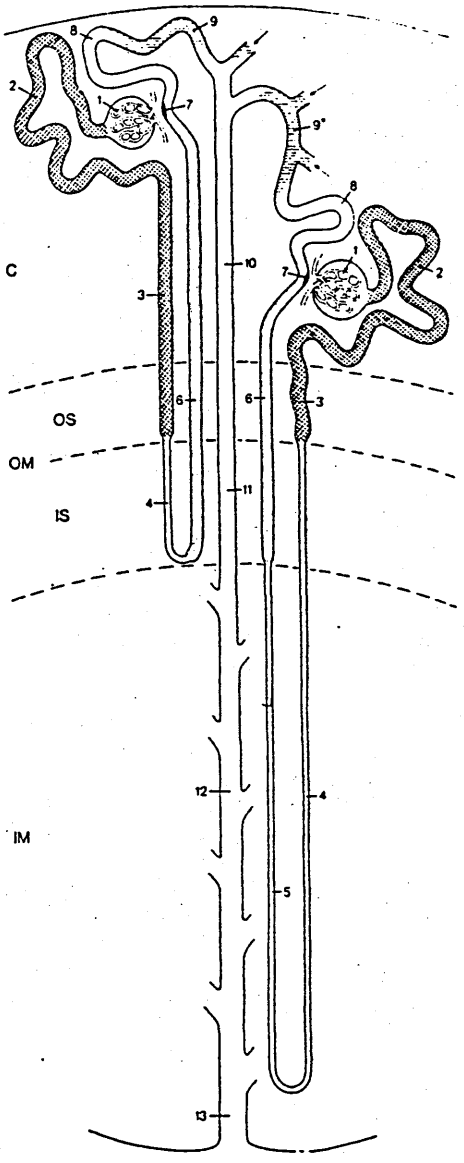


Fig. 3.1 Short looped nephron on the left side, long looped nephron on the right side. *1*, renal corpuscle; *2*, proximal convoluted tubule; *3*, proximal straight tubule; *4*, distal descending thin limb of the loop of Henle; *5*, ascending thin limb; *6*, thick ascending limb; *7*, macula densa; *8*, distal convoluted tubule; *9*, connecting tubule (*9**, arcade formed by connecting tubule); *10*, cortical collecting duct; *11*, outer medullary collecting duct; *12*, inner medullary collecting duct; *13*, papillary collecting duct. *Arrows* indicate joining of other nephrons. Zonal division of the kidney: *C*, cortex; *OM*, outer medulla with outer stripe *(OS)* and inner stripe *(IS)*: *IM*, inner medulla (From Bachmann et al, 1986)

whose effects have mainly been related to tubular lesions. Among these chemicals there are some mycotoxins such as ochratoxin A, which have been related to animal and human nephropathies, where there appear to be glomerular involvement. In the case of BEN, although morphological glomerular changes are present, these, and the clinical signs of the disease appear so late that it has not been possible to have an accurate picture of the early degenerative events that lead to this condition. Therefore, it is necessary to determine the metabolic role played by glomeruli on the functional and morphological lesion caused by compounds such as ochratoxin A and by possibly several other chemicals (in which glomerular involvement may have been overlooked), and this has been the aim of this study.

3.2. **Glomerular Metabolism**

The glomerulus (Fig 3.2) is the structural unit of the nephron whose function has been considered to be the passive filtration of molecules up to a certain size from the plasma into the tubules. However, its maintenance, regulation, and reaction to disease, suggests a more complex function, which involves the coordinated interaction among the different cell types that constitute it (Table 3.1).

The normal function of the glomeruli requires the constant integration of many complex biochemical processes. Toxic insult to one or more of these processes could alter or interrupt this integration and lead to a change in renal function and eventually to irreversible morphological changes.

3.2.1. **Methodological approaches**

Current knowledge on glomerular biochemistry and metabolism (Table 3.2)

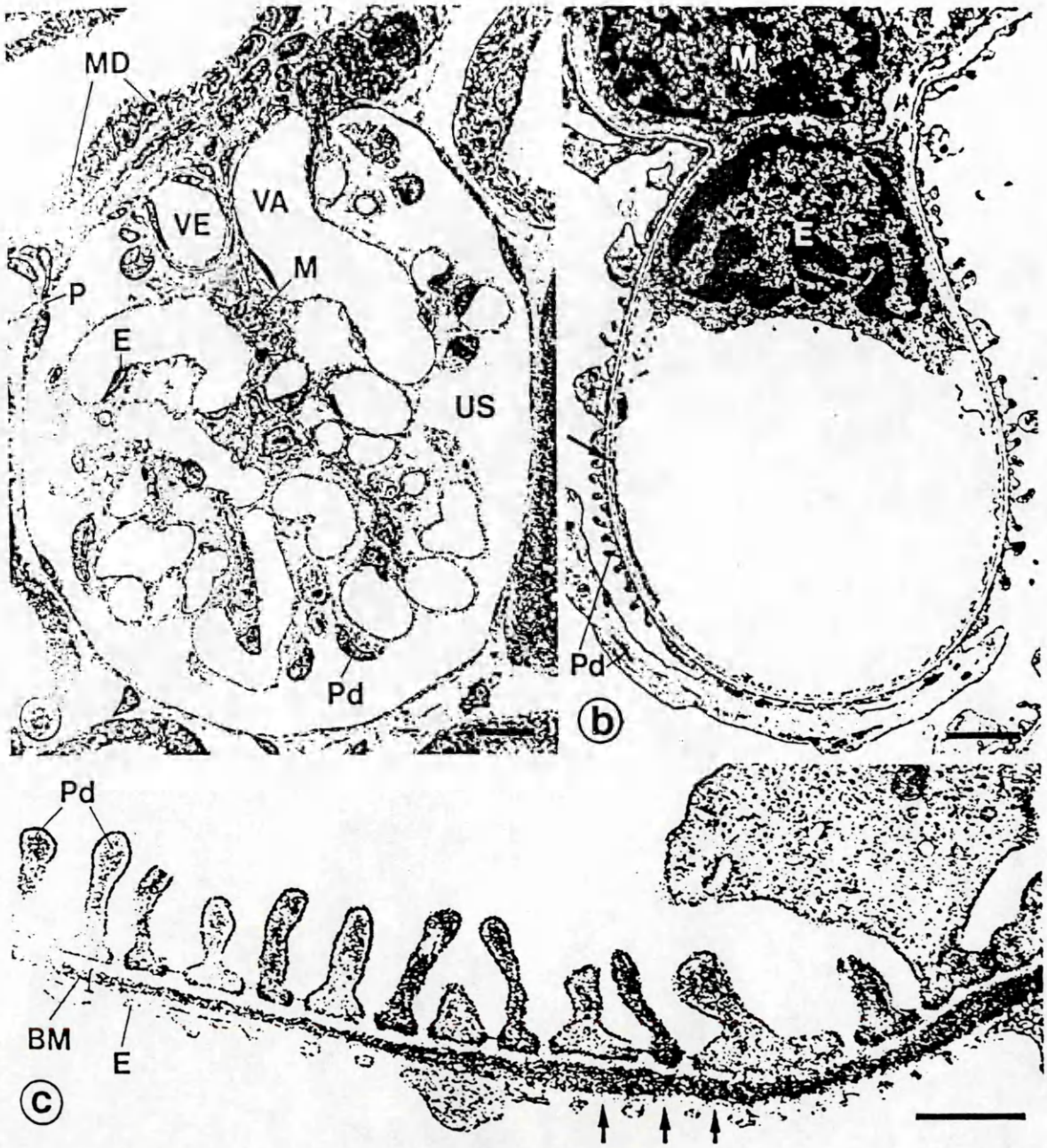


Fig. 3.2 The glomerulus. **a** The major components of a renal corpuscle are demonstrated. *P*, parietal epithelium; *Pd*, podocyte of the visceral epithelium; *E*, capillary endothelial cell; *M*, mesangial cells; *US*, urinary space; *VA*, afferent arteriole; *VE*, efferent arteriole. Next to the glomerular arterioles the juxta-glomerular apparatus is seen with extraglomerular mesangial cells and the macula densa *(MD)*. TEM, bar = 10 µm. **b** Cross section of a glomerular capillary. An endothelial cell *(E)* is seen in contact with the mesangial cell *(M)*. The basement membrane is indicated by the *arrow*; *Pd*, processes of podocytes. TEM, bar = 1 µm. **c** The filtration barrier. The fenestration of the endothelium *(E)* is open *(arrows)*. The basement membrane *(BM)* is three layered; the filtration slits between the podocyte foot processes *(Pd)* are bridged by slit diaphragms. TEM, bar = 0.5 µm

(From Bachmann et al, 1986)

TABLE 3.1. Summary of the Functions of the constituents of the filtration barrier*

	FILTRATION	OTHER
Endothelium:	'Valve' which controls access of filtrate to the GBM	SYNTHESIS OF GBM
Basement Membrane:	Main filter (size and charge)	
Epithelium:	Monitors filtrate and recovers lost protein (by endocytocis) Regulates hydraulic flux by providing "porous support" for GBM	SYNTHESIS OF GBM
Mesangium:	Phagocytosis of filtration residues (particles, immune complexes, etc.)	REMOVAL OF GBM CONTRACTION

*From Farquhar (1978)

TABLE 3.2. Summary of the Biochemical Characteristics of Glomeruli

1. Energy producing metabolism.

   Glycolysis
   Gluconeogenesis.
   Pentose phosphate shunt
   Lipid oxidation.

2. Metabolism of structural compounds.

   Proteosynthesis
   Synthesis of nucleic acids.
   Synthesis of membrane components.

   Metabolism of extracellular glomerular matrix (GBM and MM).

3. Metabolism of regulatory substances.
   Synthesis of chemical mediators:
   Prostaglandins.
   Renin
   Synthesis and catabolism of histamine.

has been obtained mainly from studies using isolated glomeruli.

Different procedures have been used to isolate glomeruli for metabolic or biochemical studies. Microdissection of fresh or frozen tissue (Imbert et al, 1974; Burch et al, 1980) gives a preparation of the highest purity, but the total amount of tissue obtained is so little that the method cannot be used routinely.

Larger-scale isolation of glomeruli has been performed by mechanical mincing and/or enzymatic digestion of the cortex, followed by sequential sieving and centrifugation (Misra, 1972), by perfusing the kidney with particulate iron oxide, followed by the harvesting of glomeruli with a magnet (Meezan, et al 1973), and by other modifications of these basic methods (Dousa, 1981). Highly purified preparations of intact glomeruli, most of them without Bowman's capsule, can be obtained by these methods with relative faility and no need of sophisticated or expensive apparatus. Although minor structural damage is caused to the glomeruli (Noorgard,1976), the preparations are generally well preserved and maintain many of their metabolic functions for several hours, and are therefore suitable for specific biochemical studies.

In addition, studies on isolated glomeruli offer several advantages over both the intact animal and other <u>in vitro</u> techniques such as perfusion, slices and isolated cells. Contrary to <u>in vivo</u> studies, considerable information can be obtained with the use of small numbers of animals and several different parameters can be assessed from the same tissue, allowing comparison of data, while minimizing tissue variability. Isolated glomeruli allow the study of glomerular characteristics, or functions as well as metabolic properties, in a defined environment and independent of

hæmodynamic changes or other extraglomerular factors. This permits the distinction of direct and indirect effects and the assessment of precise dose-effect and time-course relationships for a given chemical.

Studies with isolated glomeruli however, have some limitations, some of which are inherent to most in vitro systems, such as the short viability which restricts the study of morphological and biochemical effects to the investigation of acute responses following a toxic insult. In addition, due to the cellular heterogeneity it is not possible to correlate the observations to specific cell types. In the in vitro situation, the chemical is not presented to the glomeruli as part of the blood supply. There is no previous metabolism, no plasma binding, and no pharmacokinetic distribution of the compound. Therefore, the interpretation and extrapolation of data from observations performed on isolated glomeruli may present some difficulties and care must be taken in interpreting the results and in deciding what is or is not an artifact, as the conclusions have to be compatible with what occurs in vivo. Although histochemical studies of kidney tissue have also given useful information about the biochemistry of glomeruli (Burch et al, 1980; Dousa et al, 1977; Nevins and Michael, 1981) by providing precise information on the localization of specific biochemical characteristics, the major limitation of the method is that it can not be used to study dynamic metabolic processes.

Other studies on glomerular metabolism, have employed freshly isolated cells (Camazine et al, 1976), and different glomerular cell types in culture. Although the culture of glomerular cells has been limited by problems common to all tissue culture (dedifferentiation, loss of polarity, etc), and the origin and preserved differentiation of each cell

type are controversial (Foidart et al, 1981), this could be another useful approach to gaining knowledge on the glomerular cell metabolism. This topic has been reviewed elsewhere (Foidart et al, 1981; Kreisberg and Karnovsky, 1983; Striker and Striker, 1985).

3.2.2. **Intermediary Metabolism in Glomeruli**

3.2.2.1. Energy-producing metabolism

Studies on isolated glomeruli have shown that this part of the nephron has a high energy turnover. Oxygen consumption and production of CO_2 from different radiolabeled substrates have been the parameters measured to determine the rates of energy (ATP)-producing glomerular metabolism (Gregoire et al, 1976). The ATP content of glomeruli *in situ* is similar to (or only slightly lower than) that in the proximal tubular and medullary tubular segments i.e. 8 mmol/kg for glomeruli vs. 10 mmol/kg dry weight for proximal tubular segments (Burch et al, 1980), indicating a high metabolic activity.

A variety of intermediary substrates are known to be utilized by isolated glomeruli. For example, oxidation of carbohydrates, and fatty acids has been demonstrated in preparations of isolated glomeruli (Fong and Drummond, 1969; Brendel and Meezan, 1973), and the presence of tricarboxylic acid cycle activity was observed by Nowinski and Pigon (1967). However, in comparison with renal tubules, lower activities of some key tricarboxylic acid cycle enzymes, such as isocitrate and succinate dehydrogenase, are present in glomeruli (Schmidt and Guder, 1976), as it is the activity of glomerular glucose-6-phosphate dehydrogenase a key enzyme of the hexose monophosphate shunt.

Other *in vitro* studies have suggested that respiration of glomeruli is

also lower than in cortical tubules (Gregoire, 1975; Gregoire et al, 1976). However, the respiration rates measured in isolated glomeruli (Brendel and Meezan, 1973) and cortical slices (Cohen and Barac-Nieto, 1973) showed similar values, suggesting a high metabolic rate for glomeruli. Isolated rat glomeruli are also capable of gluconeogenesis at a rate comparable to that observed in tubules (Brendel and Meezan, 1973). Thus, there is evidence to suggest that glomeruli have a quite high metabolic rate, and it is believed that the energy released is utilized to build the specific cellular and extracellular structures required to accomplish their specialized functions.

3.2.2.2 Metabolism of structural components of glomerular cells

Isolated glomeruli are capable of incorporating aminoacids and glucosamine into macromolecules (Walker and Hulter, 1969; Brendel and Meezan, 1973), indicating that glycoproteins and glycosaminoglycans (GAG) are synthesised (Kornfeld and Ginsburg, 1966). Isolated glomeruli have been reported to incorporate ^{3}H-cytidine (Walker and Hulter, 1969) and ^{3}H-uridine (Brendel and Meezan, 1973) into RNA. This indicates that major biosynthetic pathways that are common to all tissues, involving protein, nucleic acids, and membrane components in general, are present in glomeruli.

The main function of the glomerulus as a refined and highly specialized ultrafilter relies on the metabolism of its complex extracellular matrix, which needs to be continually synthesized and maintained by the glomerular cells (Venkatachalam and Rennke, 1980). It appears that the metabolic processes involving synthesis and catabolism of glomerular basement membrane are unique to the glomeruli, or much more prominent here as compared with other parts of the nephron.

The biosynthesis of extracellular matrix is a very intricate process, since it involves the formation of complex macromolecular compounds such as collagen, glycoproteins and GAG (Kefalides et al, 1979; Lindahl and Hook,1978; Spiro, 1969).

Biosynthesis of components of the glomerular matrix has been studied *in vivo* and *in vitro* in isolated glomeruli (Brown et al, 1981), isolated GBM and tissue-cultured glomerular cells (Foidart et al, 1980a,b; Oberley et al., 1979). Several approaches have been used, like incorporation of radiolabelled precursors such as aminoacids, monosaccharides and sulphate, and the determination of post-transcriptional side-chain modifications such as the hydroxylation of proline and hydroxylysine, sulphatation of GAG and glycosylation of collagen. Labelled proline and lysine are incorporated in isolated glomeruli, and carbohydrate-rich proteins, containing OH-lysine and OH-proline, are synthesized, indicating the formation of collagen-like protein (Grant et al, 1975).

In vitro and *in vivo* studies on the biosynthesis of components of glomerular matrix indicate that non-collagenous glomerular proteins are rather quickly synthetized and incorporated, whereas collagenous components are deposited at a slower rate (Hjelle et al, 1979). The turnover of GBM collagen takes about 100 days which is slower than the turnover of non-collagen glomerular proteins which takes only 9 days (Price and Spiro, 1977).

In relation to the catabolism of GBM components, a role of the hydrolytic actions of lysosomal enzymes in the metabolism of glomerular extracellular matrix has been suggested (Davies et al, 1980a,b; Davies and Bonney, 1980;

Dousa et al, 1980; Matsuo and Yamada, 1980; Velosa et al, 1980; 1981).

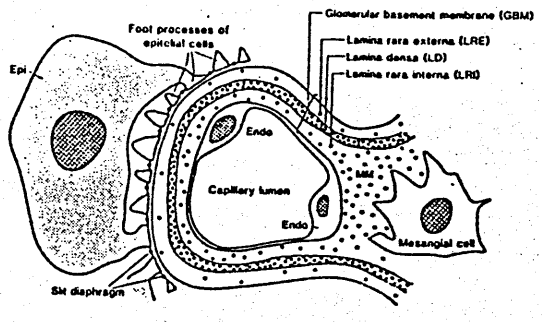
There is evidence that both epithelial and endothelial cells are involved in the synthesis of GBM components and that mesangial cells are active in removal and catabolism of extracellular matrix (Michael et al, 1980; Nayyar et al, 1980) as well as in its biosynthesis (Foidart et al, 1980b).

3.2.2.2.1 Composition of GBM

Collagens type IV and V have been localized to the mesangial matrix region of the glomerulus as well as to the peripheral capillary wall (Courtoy et al, 1980a; Scheinman, 1980; Houser et al., 1982). Type IV collagen is largely concentrated in the lamina densa of the GBM - Fig. 3.3A - (Roll et al, 1980). The collagenous component of the GBM is secreted in the form of procollagen and is deposited in the extracellular matrix without any further reduction in molecular size (Kefalides, 1978).

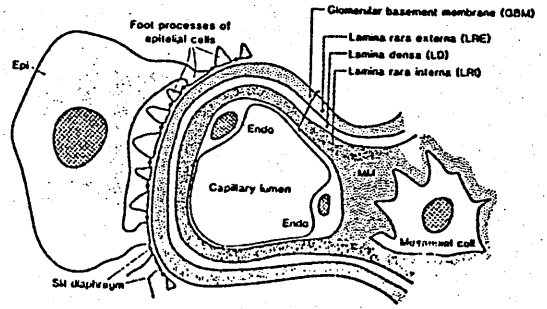
The procollagen molecule is composed of a collagen tripple-helix having non-helical, largely non-collagenous extensions at the carboxyl and amino termini (Fig. 3.4). The procollagen molecules are thought to interact with one or more glycoproteins, non-collagenous in nature. The relative proportion of procollagen in GBM is approximately 25% (Kefalides, 1981). The procollagen and non-collagen glycoproteins interact via hydrogen bonds and disulphide cross-linkages. The amino acids composition of the intact GBM (Table 3.3) shows a high content of the aminoacids proline and 3- and 4-hydroxyproline, of the non-polar amino acid glycine and the basic aminoacid hydroxylysine; resulting in high ratios of 3-hydroxyproline to total hydroxyproline, and hydroxylysine to lysine (Kefalides et al, 1979; Spiro, 1969 and 1973). The monosaccharides constitute approximately 10%

a



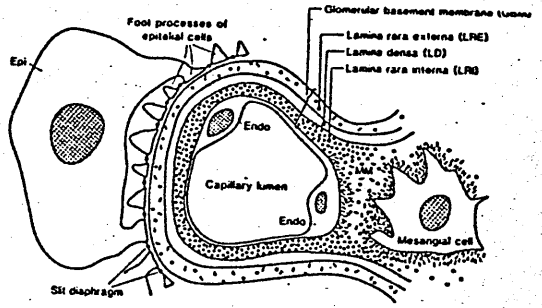
Distribution of glomerular collagen *(black dots)* type IV (with some collagen AB_3). The highest density of collagen is in lamina densa of GBM and in MM.

b



Distribution of acidic GAG (*stippled area*) as determined by staining techniques. The main localization includes lamina rara and MM.

c



Intraglomerular distribution of fibronectin *(dashes)* and laminin *(dots)*. Note that fibronectin is mainly localized in the MM in highest densities around mesangial cells and adjacent endothelial cells.

Fig. 3.3

(From Dousa, 1985)

Schematic cross section of glomerular capillary with attached visceral epithelial cell at the left side (Epi) and with endothelial cells (Endo) inside the lumen. On the left side, the capillary is in contact with mesangium, consisting of mesangial matrix (MM) and mesangial cell. Layers of GBM and other details of structures are indicated in the figure.

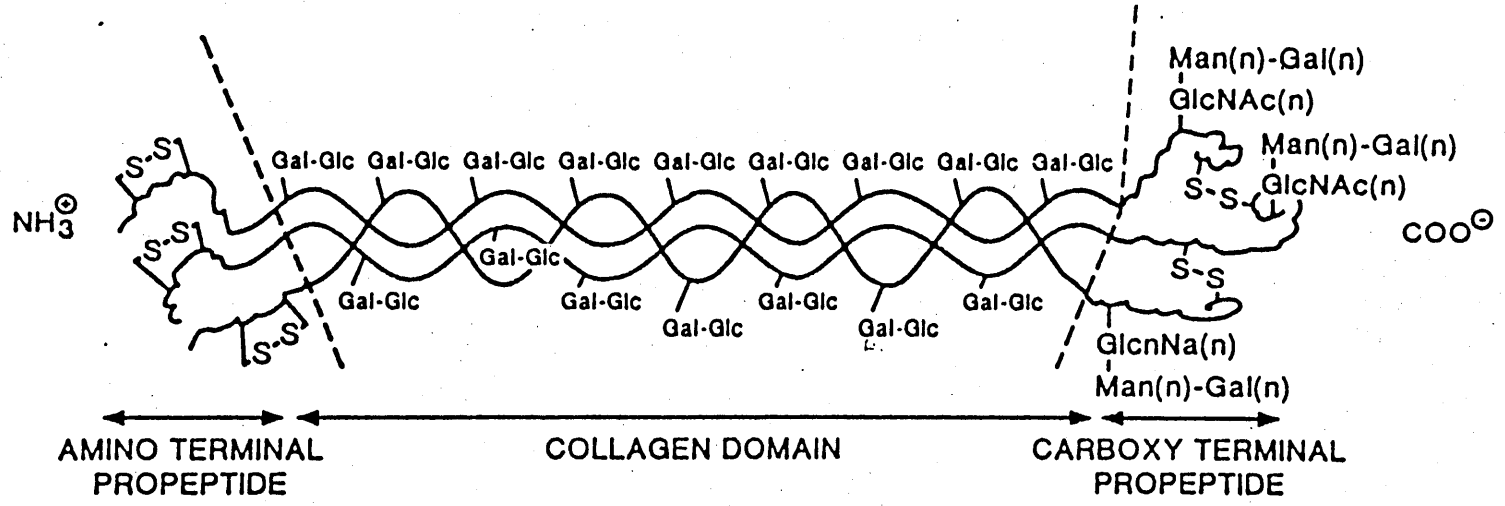


Fig. 3.4 Schematic outline of a molecule of procollagen (collagen type IV) from GBM. The procollagen molecule consists of a major triple-helical middle region ("collagen domain") that is typical for collagen molecules and has numerous glucosyl-galactosyl disaccharide units attached to hydroxylysine side chains. The amino-terminal and carboxy-terminal parts of the molecule are nonhelical and contain disulfide bonds. In the amino-terminal region *(left side)*, disulfide bonds connect to sites within a single polypeptide chain. On the other hand, the carboxy-terminal region *(right side)* has disulfide bonds forming a connection between different polypeptide chains. The carboxy-terminal region also has heterooligosaccharide side chains covalently attached to the polypeptide molecule. After synthesis and secretion, procollagen molecules (4 or 5) form microfibrils, which then aggregate by end-to-end, side-to-side interactions to form GBM. Abbreviations: S, sulfur; Glc, glucose; Gal, galactose; Man, mannose; GlcNAc, *N*-acetyl glucosamine; GlcnNa, glucosamine; the suffix (n) following the abbreviations indicates multiple monosaccharides in the chain. (Adapted from Kefalides, 1981.)

TABLE 3.3.

Amino acid and carbohydrate components of glomerular basement membranes[a].

Component	Human GBM[b]	Rat GBM[c]
Hydroxylysine	24.5	36.7
Lysine	26.0	19.9
Histidine	18.7	14.5
Arginine	48.3	42.7
3-Hydroxyproline	7.0	4.4
4-Hydroxyproline	66.0	91.7
Aspartic acid	65.0	60.2
Threonine	40.0	32.7
Serine	60.0	51.3
Glutamic acid	103.0	82.0
Proline	62.0	72.0
Glycine	227.0	240.3
Alanine	58.0	55.9
Half-cystine	23.0	13.2
Valine	36.0	37.7
Methionine	7.0	14.1
Isoleucine	28.0	27.3
Leucine	66.0	57.8
Tyrosine	14.5	15.2
Phenylalanine	28.0	27.8
Glucose	2.5	2.41
Galactose	2.6	2.15
Mannose	1.7	0.81
Fucose	0.7	0.20
Glucosamine	1.7	
Galactosamine	0.3	
Sialic acid	1.5	
N-Acetylneuraminic acid		1.00
N-Acetylgalactosamine		1.08
N-Acetylglucosamine		0.23

[a]Amino acids expressed as residues per 1000 residues; carbohydrates expressed as grams of sugar per 100 gm.
[b]Kefalides (1971)
[c]Koide et al (1981)

of GBM material, and is composed largely of galactose and glucose and amino sugars and other monosaccharides such as glucosamine, galactosamine, mannose, fucose and sialic acid, are present in significant amounts (Kefalides et al, 1979; Spiro, 1973; Wargo et al, 1982). The oligosaccharide units are linked to asparagine.

The non-collagenous components of the GBM include the sulphated glycosaminoglycans (GAG), fibronectin, laminin and entactin. The major sulphated GAG synthesized by cultured human and rat glomerular epithelial cells is heparan sulphate (Striker et al, 1980; Foidart et al, 1980b). This component of GBM contributes significantly to the total anionic charges of the GBM and of the entire glomerular capillary wall (Kanwar and Farquhar, 1979). The main localization includes lamina rarae and MM (Fig.3.3B). Heparan sulphate appears to be linked to the collagen backbone of the GBM by disulfide bonds (Parthasarathy and Spiro, 1981). It has a high content of uronic acids (glucuronic and iduronic), N-linked sulphur, xylose and serine. It has been suggested that the polysaccharide chains are attached to the polypeptide via xylose-serine covalent links (Kefalides, 1981).

The non-collagen glycoproteins are believed to play a vital role in the adhesion of the glomerular components. Fibronectin is known to be involved in cell-to-cell and cell-to-substrate attachment (Yamada and Olden, 1978) and it is located mainly in the mesangial matrix, at the interface between mesangial and endothelial cells, and to a lesser extent in the lamina raræ of the GBM - Fig. 3.3C- (Courtoy et al, 1980a,b; Houser et al, 1982; Oberley et al, 1979). Two subunits constitute the molecule of fibronectin which are connected by a disulfide bridge. Asparagine acid residues connect fibronectin oligosaccharide components to polypeptides (Yamada and

Olden, 1978), there are also sialic acids in the terminal positions of the oligosaccharides. The amino acid composition of fibronectin is shown in Table 3.4. Residues of aromatic amino acids such as TRP and TYR appear to play an important role in the secondary and tertiary structure of fibronectin. The intactness of these residues seems to be necessary for cell spreading, cytoagglutinating process, and therefore for adhesive activity (Yamada and Olden, 1978; Yamada, 1983).

Cultured glomerular epithelial and mesangial cells from humans and rats have been shown to be capable of producing fibronectin (Fish et al, 1975; Scheinman and Fish, 1978; Oberley et al, 1979; Foidart et al, 1980b). Cellular fibronectin is synthesized in the rough endoplamic reticulum (Yamada et al, 1980; Hedman, 1980) as a monomer with high mannose oligosaccharides, and rapidly becomes a disulfide-linked dimer, and after some molecular rearrangements, is secreted via the Golgi apparatus (Olden and Yamada 1977; Choi and Hynes, 1979; Olden et al, 1980; Uchida et al, 1980). Fibronectin undergoes protein turnover on the cell surface (Olden and Yamada, 1977). It has been suggested that fibronectin levels on the cell surface can be modulated by a variety of events such as altered growth conditions, transformation, hormonal stimulation, and embryonic differentiation (Yamada, 1983). This regulation may occur through altered synthesis, turnover or binding to the cell surface, but the mechanisms of these effects remain to be determined. These studies have been conducted on different cells other than glomerular ones, therefore it is necessary to perform studies on the role of fibronectin in glomeruli and the effect of chemical toxicity on this part of the nephron.

Laminin is a sialoglycoprotein similar to fibronectin, but it differs in

TABLE 3.4

Amino Acid and Carbohydrate Compositions of Fibronectin

Amino acid	Bovine[a]	Human[b]
	(g of amino acid/100 g of protein)	
Aspartic acid	9.1	9.3
Threonine	9.8	9.7
Serine	8.0	6.8
Glutamic acid	11.4	11.6
Proline	7.9	7.6
Glycine	9.1	8.0
Alanine	4.6	4.3
Cystine	2.0	2.6
Valine	7.6	8.1
Methionine	1.0	1.1
Isoleucine	4.3	4.4
Leucine	5.6	5.7
Tyrosine	4.0	4.5
Phenylalanine	2.2	2.7
Lysine	3.9	3.6
Histidine	1.8	2.1
Arginine	5.2	5.2
Tryptophan	1.8	2.8
Carbohydrate		
Hexose (%)	1.8	1.8
Hexosamine (%)	2.2	2.1
Sialic acid (%)	1.1	1.2
Total (%)	5.1	5.1

[a]From Iwanaga et al.(1978).
[b]From Mosesson et al. (1975).

its amino acid composition and immunologic reactivity (Timpl et al, 1979). This protein is involved primarily in attachment of epithelial cells onto collagenous substrates, such as collagen type IV (Courtoy et al, 1982). Laminin has been localized to the lamina raræ of the peripheral capillary wall in the rat and human glomerulus - Fig. 3.3C - (Courtoy et al, 1980a; Scheinman et al, 1980; Madri et al, 1980).

Entactin, a sulphated glycoprotein, has been localized to the lamina raræ of the rat GBM as well as the mesangium and may also be involved in cell adhesion (Bender et al, 1981; Carlin et al., 1981). Table 3.5 shows the amino acid composition of entactin.

3.2.3. **Glomeruli as site of synthesis of prostaglandins**

Another unique property of glomeruli is their ability to synthesize chemical mediators, such as prostaglandins which are related to glomerular functions and which are also believed to contribute to the major pathophysiological events leading to glomerular changes.

The prostaglandin-forming enzyme cyclooxygenase has been localized in the mesangial cells by immunofluorescence (Smith and Bell, 1978), its specific activity (per mg protein) is 10 - 40 times higher in glomeruli than in cortical tubules (Hassid et al, 1979), and it is also higher than in the renal medullary region suggesting an important role in glomerular physiology.

Subsequently, isolated rat glomeruli have been shown to convert arachidonic acid to prostaglandins and thromboxanes which accumulate in the incubation medium, (Folkert and Schlondorf, 1979; Hassid et al, 1979).

Prostacyclins and thromboxanes have also been detected. The glomeruli seem to be the major site of prostaglandin production in the cortex.

Glomerular prostaglandin synthesis may be modulated by kinins such as angiotensin II (Schlondorff et al, 1980) which has functional consequences on glomeruli, since prostaglandins can reduce the angiotensin II-induced reduction of glomerular surface area thus modulating contractile properties of the mesangium and therefore influencing GFR. Glomerular prostaglandin synthesis also increased by reactive oxygen species (Baud et al, 1981).

The role of prostaglandins in glomerular physiology appears to be related to the secretion of renin, and to the vasoreactivity of the glomerular capillaries and their permeability to water.

3.3 **Metabolic changes in glomeruli induced by toxic injury**

Glomerular metabolism may be changed by toxic insult or disease. Damage to glomeruli after toxic injury has been mainly assessed by morphological changes, and there is still a lack of knowledge on the early biochemical alterations which lead to the loss of the structural and functional properties of the filtration barrier.

Among the compounds that are recognized to cause injury to the glomeruli, puromycin aminonucleoside (PAN) has been the most extensively studied. In glomeruli isolated from rats with PAN-induced nephrotic syndrome, the oxidative metabolism of glucose and alanine was markedly decreased (Kaplan et al, 1974); while other substrates such as acetate and citric acid were unaffected. Histochemical studies on several enzymes showed increased activities of glucose-6-phosphate, lactate and malic dehydrogenase (Dubach

and Recant, 1960; Kissane and Hoff, 1962).

When incorporation of radiolabeled proline into glomerular protein was measured (Blau and Michael, 1971), increased incorporation was observed in glomeruli from PAN-treated rats, which was converted to hydroxyproline. Kefalides and Forsell-Knott (1970) reported that the GBM of PAN nephrotic rats contained lower amounts of hydroxylysine and hydroxyproline and higher amounts of lysine and proline than in the controls, suggesting that the compound could interfere with the hydroxylation of both amino acids by inhibiting the proline and lysine hydroxylase enzymes. However this has not been confirmed in other studies.

Although depletion of the sialoglycoprotein coat on the surface of glomerular epithelial cells of PAN-treated rats with consequent loss of the negative charges on the surface of glomerular epithelial cells, and decreased content of sialic acid in GBM has been observed in histochemical studies (Michael et al, 1970), there was an increase in the rate of sialic acid catabolism, with secondary enhanced synthesis and increased incorporation of ^{3}H-glucosamine into glycoproteins of the glomeruli (Blau and Michael, 1972). Repair of the anionic coat may account for this effect. By constrast no changes on sialic acid content were found by another group (Kefalides and Forsell-Knott, 1970). Methodological differences may have been responsible for the conflicting findings.

There was a decrease in GBM heparan sulphate content as shown by immunohistochemistry, after PAN treatment (Caulfield and Farquhar, 1978 Mynderse et al, 1983). However when the synthesis of GAG was assessed on isolated nephrotic glomeruli of PAN treated rats, it did not appear to be

a net change in the heparan content of the GBM, but increased synthesis of new, lower molecular weight heparan-sulphate proteoglycans was observed (Klein et al, 1984). This is in agreement with published observations on perfused kidneys of PAN treated rats (Lelongt et al, 1987), which describe a generalized increase in the synthesis of heparan sulphate-proteoglycan, as shown by the increase incorporation of ^{35}S-sulphate. However, no significant changes in the macromolecular composition of heparan sulphate-proteoglycan were detected. The differences in the techniques applied may account for the different species of the GBM component synthesised.

Regarding the catabolism of GBM components and the role played by the lysosomal enzymes, it is important to mention that in PAN nephrotic rat kidneys the number and sizes of lysosomes were found to be increased in glomerular epithelial and mesangial cells (Cauldfield et al, 1976; Grond and Elema, 1981). This suggests that the changes in the amount of acidic glycoproteins and acidic GAG of the glomerular matrix in aminonucleoside nephrosis could be due to an accelerated breakdown by lysosomal enzymes, or an accumulation blocking the turnover of other material.

Studies on glomerular epithelial cells in culture exposed to PAN, showed immediate and marked depression in the utilization of precursors of protein, sugar, and sialoprotein synthesis (Fishman and Karnovsky, 1985). Incorporation of these precursors returned to control levels after a brief or continuous exposure to PAN, suggesting that metabolic pathways other than those involved in the uptake of glycoprotein precursors must be injured in PAN exposure.

Oxidative metabolism of glucose and palmitic acid was studied in isolated glomeruli from rats pretreated with nephrotoxic serum (Fong and Drummond,

1969). There was marked increase in the oxidation rates of the two compounds. Although enzymatic activity was not measured, there exists the possibility of increased enzymatic activity in the tricarboxylic acid cycle (Dubach and Recant, 1960) that may account for this effect. It has also been reported that in nephrotic serum nephritis, both the turnover of glomerular sialic acid and hydroxyproline and the uptake of N-acetyl glucosamine were increased (Chow and Drummond, 1969).

The effects of adriamycin on glomeruli, have been studied mainly from the ultrastructural point of view, where thickening of basement membrane, fusion and detachment of foot processes and focal dilation of cisternae of the rough endoplasmic reticulum (Bertani et al, 1982). There are few studies concerning the effect of this compound in glomerular metabolism. Incorporation of ^{3}H-proline into isolated rat glomeruli exposed to a concentration of 32 uM adriamycin was decreased by 70%; of the total amino acid incorporated into glomeruli, 55% was shown to be incorporated into GBM. The incorporation of radioactive lysine and histidine were not affected by the same concentration of the drug (Ahmed et al, 1987). This suggests that proline incorporation into glomerular and GBM protein is sensitive to adriamycin and may be a selective index of glomerular toxicity.

Isolated glomeruli of nephrotic rats that had been administered adriamycin, showed abnormal arachidonic acid metabolism which led to a significant increase in thromboxane generation (Remuzzi et al, 1985). This may be in connection with alterations of GBM permeability to proteins.

Further metabolic studies in glomeruli affected by adriamycin toxicity,

are necessary to fully elucidate the mechanism of toxicity of the compound on this part of the nephron.

CHAPTER 4

MATERIALS AND METHODS

4.1. Materials and Instrumentation.

4.1.1. Chemicals

All chemicals used were analytical reagent grades or of the highest purity that is commercially available unless otherwise specified. They are listed according to the suppliers. Water for analysis was glass distilled.

Adriamycin was a gift from Carlo Erba (St Albans, Hertfordshire).

Gamma-glutamyl-p-nitroanilide, glycylglycine, naphtylethylene-diamine dihydrochloride, p-nitroanilide l-alanine-p-nitro aniline, ochratoxin A, aflatoxin B_1 HEPES, Earles salts, Tris, BSA, L-tryptophan, L-phenylalanine, L-tyrosine, L-leucine, L-histidine, glucose, collagenase, puromycin aminonucleoside, streptomycin, folic acid and ethacrynic acid, were purchased from the Sigma London Chemical Co Ltd (Poole, Dorset).

2-methoxy-4 (2′-nitrovinyl)-phenol (MNP); and MNP-2-acetamindo-2-deoxy-beta-D-glucopyranoside were obtained from Dr R Price, University of London, London, UK.

Alkaline phosphatase OPT KIT, Glucose Rapide Test and Protein Kit, were supplied by Roche (Welwyn Garden, Hertfordshire).

^{3}H-glycine, ^{3}H-histidine, ^{3}H-leucine, ^{3}H-lysine, ^{3}H-phenylalanine, ^{3}H-

proline, ^{3}H-tryptophan, ^{3}H-tyrosine, ^{14}C-glucose, ^{14}C-linolenic acid, ^{14}C-oleic acid, ^{14}C-stearic acid, ^{14}C-palmitic acid and ^{14}C-mevalonic acid, were purchased from Amersham International Plc (Aylesbury, Buckinghamshire).

2-bromoethanamine, methanol, dimethyl sulphoxide, DPX and fibrowax were obtained from British Drug House Ltd (Poole, Dorset).

Haematoxylin and eosin were supplied by Gurr/British Drug House.

OptiSafe scintillant liquid was purchased from LKB.

All the photographic material was supplied by Eastman Kodak Co. (Rochester, USA).

4.1.2. Instrumentation

Lambda Spectrophotometer (Perkin Elmer Corp, Norwalk, USA).

Cobas Bio Centrifugal autoanalyser (Roche Diagnostica, Roche Products Ltd, Welwyn Garden, Hertfordshire).

Histokinette Automatic Tissue Processor (British American Optical Co Ltd, Slough, Berkshire).

Rotary microtome (American Optical, P.A., U.S.A.).

1219 Rack beta scintillation counter (LKB, Wallak, Finland).

Leitz Dialux 400 microscope (E. Leitz Instruments Ltd, Luton, UK).

TJ-6 Centrifuge (Beckman Instruments, Paloalto, Cal., USA).

Phase Contrast light microscopy (Nikon Instruments UK Ltd, Telford, Shropshire).

Leitz Dialux 20 microscope (E. Leitz Instruments Ltd, Luton, UK) with attached Nikon UFX-II Photographic Camera (Nikon Instruments UK Ltd, Telford, Shropshire).

Stainless steel sieves (Endecotts, London).

Plastic scintillation vials, Plastic cuvettes, Pyrex conical flasks, G/FA glass-fibre discs (Whatman) and rubber Suba-Seal stoppers, were supplied by FSA Laboratory Supplies, Loughborough, Leics.

Plastic centre wells (Burkard, Scientific (Sales) Ltd, Uxbridge, Middx).

Paraffin wax embedding oven (R.A. Lamb, London).

4.1.3. Animals

Male Wistar rats, University of Surrey strain (Guildford, Surrey). Large White pig kidneys (Steve Chitty and Sons Ltd, Dorking, Surrey).

4.2. METHODS

4.2.1. In vivo experiments

4.2.1.1. Animals

Wistar rats, with an initial weight of approximately 200 g, were housed in plastic cages (North Kent Plastic Cages Ltd, GSI), with free access to food (Laboratory Animal Diet No. 1 pellets. Labsure Laboratory Animal Diets Poole, Dorset) and tap water. The animals were kept in a controlled environment, of 22^{o}C temperature and 40-50% humidity, and a cycle of 12:12 hours light:dark starting at 0700 hr GMT. For collection of urine for the single dose regime, the animals were kept in plastic metabolic cages (Techniplast. Code 1700872 Forth-Tech Services, Dalkeith) designed for separate collection of urine and faeces, and fed Lab Diet 2 Powdered Diet (Spratts, Barking). For the repeated doses regime, the animals were kept in metallic metabolic cages under 18-hr food restriction, and had access to food for 6-hr periods (1200-1800 hr daily). Tap water was supplied ad libitum.

4.2.1.2. Urinalysis

Urine samples were refrigerated for the entire period of collection, after which they were centrifuged at 3000 r.p.m. for 15 min, to separate debris and particulate matter. The supernatant of the urine was diluted as appropriate for each enzyme determination, and GGT, ALP and NAG activity were measured the same day of collection. Samples for protein and glucose were frozen at -20^{o}C and analysed within one week of collection.

4.2.1.2.1. Gamma-Glutamyl Transpeptidase (GGT)

The assay is based on the transfer of the glutamyl group from l-glutamyl-p-nitroanilide to glycylglycine catalyzed by GGT as follows (Naftaline et al, 1969):

L-glutamyl-p-nitroanilide + glycylglycine. GGT ----> p-nitroaniline + glutamyl glycylglycine.

The para-nitro aniline liberated is then diazotized:

p-Nitro aniline + $NaNO_2$ -----> acid diazo compound.

Diazo compound + N-(1-naphtyl)-ethylene diamine ----> pink azo-dye.

The absorbance of the pink azo-dye is measured at 540 nm, and is proportional to GGT activity.

Reagents

0.1 M Tris-HCl buffer, pH 9.0

Substrate solution: 4.6 mM L-gamma-glutamyl-p-nitroanilide.

0.1 M glycylglycine in 0.1 M Tris-HCl buffer, pH 9.0

0.1 % Sodium nitrate.

1 % Ammonium sulphamate.

2 mM Naphthylethylenediamine dihydrochloride.

0.1 M p-Nitroanilide.

1.7 N Acetic acid.

Method

Urine was assayed for GGT by adding 20 ul of the sample to 0.5 ml of a solution containing L-glutamyl-p-nitroanilide and glycylglycine and the mixture incubated at 37°C for 20 min. The reaction was stopped by adding 2 ml of acetic acid solution.

In time sequence, the following reagents were added: 1 ml of sodium nitrite and after 3 min, 1 ml of ammonium sulphamate solution and after 3 min 1.0 ml of naphthylethylene diamine solution. A blank for the reagents was also carried out. The absorbance of the azo-dye was measured at 540 nm and the enzyme activity was determined from a calibration curve, prepared using the diazo-compound. Results were expressed as Units/24 hr or Units/18 hr urinary volume.

4.2.1.2.2. N-Acetyl B-D Glucosaminidase (NAG)

The assay is based on the release of 2- methoxy-4(2'-nitrovinyl)-phenol (MNP) from the substrate MNP-2-acetamido-2-deoxy-beta-D-glucopyranoside (MNP-GLcNAc) catalyzed by N-acetyl-beta-D-glucosaminidase as follows (Yuen et al, 1982):

```
            NAG
MNP-GLcNAc ------> MNP
```

The absorbance of the product is measured at 505 nm.

Reagents

1.5 mM MNP-GLcNAc.

0.15 M Citrate-phosphate buffer pH 4.5.

0.5 M Carbonate-bicarbonate buffer pH 10.0

1 mM MNP.

A 0.1 ml aliquot of urine is added to 0.5 ml of incubation buffer (0.15M citrate-phosphate buffer) pH 4.5 and 1 ml of 1.5mM MNP-GLcNAc. The mixture

is then incubated for 30-45 min at 37°C.

The reaction is stopped by addition of 0.5 ml sodium carbonate-bicarbonate buffer, pH 10.0. The absorbance of the solution is read at 505 nm, and the enzyme activity is calculated from a calibration curve, prepared using MNP. The activity was expressed as nmol/ml/18 hr or 24 hr.

4.2.1.2.3. Alkaline Phosphatase (ALP)

Alkaline phosphatases catalyze the hydrolysis of p-nitrophenyl-phosphate to p-nitrophenol and phosphoric acid. The p-nitrophenol produced is proportional to the phosphate activity and is measured in alkaline media at 405 nm.

Reagents

1 M Diethanolamine buffer pH 9.8.

0.5 mM Magnesium chloride.

10 mM p-nitrophenyl phosphate.

The assay was performed kinetically on a Cobas Bio Centrifugal autoanalyser using alkaline phosphatase OPT KIT (Roche). The activity was expressed as nmol/ml/18 hr or 24 hr.

4.2.1.2.4. Alanine aminopeptidase (AAP)

AAP activity was assayed kinetically (Mondorf et al, 1978). The enzyme catalyzes the hydrolysis of L-alanine-p-nitro aniline to p-nitroanilide and L-alanine. The p-nitroanilide produced is proportional to the peptidase activity and is measured at 405 nm.

Reagents

L-alanine p-nitroanilide (1.66×10^{-2}M).

0.1 M Phosphate buffer pH 7.6.

A volume of 0.2 ml of urine was pipetted into plastic cuvettes, using 0.1 ml of substrate in 0.4 ml phosphate buffer and the absorbance was recorded against a water reference after 0 and 5 min. The enzymatic activity is expressed as nmol/ml/18 hr or 24 hr.

4.2.1.2.5. Glucose

Urinary glucose was measured enzymatically by the hexokinase (HK) method in which glucose is converted to glucose-6-phosphate (G6P) by the enzyme and then to the gluconate by G6P-DH in the presence of NAD:

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP}$$

$$\text{G-6-P} + \text{NAD} \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-P} + \text{NADH}^+ + \text{H}^+$$

The increase in the $NADH^+$ concentration is directly proportional to the glucose concentration and is measured at 340 nm.

Reagents

50 umol ATP.

50 umol NAD^+.

7 U HK.

8 U G6P-DH.

0.25 M Tri-ethanolamine pH 7.5.

The assay was performed on a Cobas autoanalyzer using Glucose Rapide Test (Roche). The concentration was expressed as mmol/18 h urinary volume.

4.2.1.2.6. Total protein

Protein was measured by the biuret method (Kingsley, 1939) in which a coloured complex is produced, in alkaline solution with copper salts (biuret reaction). The colour intensity is proportional to the protein concentration and is measured at 546 nm.

Reagents

Biuret reagent.

20 mM Potassium-sodium tartrate.

12 mM Cupric sulphate.

10 mM Potassium iodide.

0.2 M NaOH.

The reaction was performed in a Cobas autoanalyser using a protein kit (Roche). The results are expressed as mg of protein /18 hr urine volume.

4.2.1.3. Dosing regime of mycotoxins

Single and repetitive doses of ochratoxin A (OTA) and aflatoxin B_1 (ATB_1) and a combination of both mycotoxins were given i.p. to different groups of rats as shown in Tables 4.1 and 4.2. Those receiving single doses were killed and tissue used for histopathology at 24, 48, 72 and 96hr. From the 96 hr group one 24-hr control urine sample was collected before the injection of the mycotoxins and daily 24-hr urine samples were collected. Urinary volume was measured and the activities of GGT, NAG and ALP and AAP were assayed.

The groups receiving repetitive doses were given three daily doses of either toxin or a combination and killed 24hr after the last dose for histopathological examination.

A two week experiment was performed with rats receiving 2.5 mg OTA/kg, after which the animals were killed. One 18-hr control urine sample was collected before the first injection of OTA subsequent to which 2 daily injections were made. Daily 18-hr urine collections were made on each of the days of injection, and on each sample, volume, protein and glucose were measured and also NAG, GGT, AAP and ALP activities, were measured.

TABLE 4.1

SINGLE DOSE REGIME OF OTA AND ATB_1 ALONE OR IN COMBINATION

COMPOUND	DOSE OF COMPOUND	VEHICLE (ml)	NO. OF ANIMALS PER GROUP/TIME POINT
SINGLE MYCOTOXIN ADMINISTRATION			
OTA*	1	0.1	3
	5	0.5	5
ATB_1**	100	0.2	3
COMBINED ADMINISTRATION			
OTA*	1	0.1	3
ATB_1**	100	0.2	
OTA*	5	0.5	5
ATB_1**	100	0.2	

* mg/kg
** ug/kg
Vehicle for OTA : 0.1 M $NaHCO_3$
Vehicle for ATB_1 : DMSO

TABLE 4.2
REPETITIVE DOSE REGIME OF OTA AND ATB_1
ALONE OR IN COMBINATION

COMPOUND	DOSE TREATMENT	VEHICLE (ml)	NO. OF ANIMALS PER GROUP/TIME POINT
OTA* (alone)	7.5	1.50	3
	5.0	0.5	6
	2.5	0.25	3[a],5[b]
	0.5	0.05	3
ATB_1** (alone)	100	0.20	3
COMBINED ADMINISTRATION			
OTA*	5	0.50	3
ATB_1**	100	0.20	
OTA*	2.5	0.25	5
ATB_1**	100	0.20	
OTA*	0.5	0.05	3
ATB_1**	100	0.20	

[a] for histopathy
[b] for urinalysis and further pathology

* mg/kg
** ug/kg
Vehicle for OTA : 0.1 M $NaHCO_3$
Vehicle for ATB_1 : DMSO

A control group for each treatment was set up in which equal volume of the respective vehicle was injected.

4.2.1.4. Autopsy

The rat and all its tissue were allocated accession numbers. Rats were sacrificed under ether anaesthesia and the abdominal cavity was opened. Abnormal macroscopic findings such as abdominal fluid, enlarged organs, etc. were noted. The kidneys were removed and weighed. Samples of liver were also taken.

4.2.1.5. Histopathological Assessment

4.2.1.5.1. Fixation of tissues

Pieces of tissue taken immediately after the death of the animal were fixed in 10 % neutral buffered formalin (NBF), pH 7.4. The container was gently shaken to avoid adherence of the tissue to the bottom. The tissue was left for at least one week in the fixative before being embedded in wax (Lillie and Fulmer, 1976).

4.2.1.5.2. Preparation of paraffin-wax embedded sections (Culling, 1974).

Reagents

Methanol.

Paraffin-wax (Fibrowax).

Toluene.

Method

Fixed tissue was trimmed to pieces of approximately 5 mm thick, placed in a plastic processing container and covered with a metal lid. The containers were placed in a tissue basket which was then fitted to the Histokinette Automatic Tissue Processor. The process of dehydration,

clearing and impregnation with wax were carried out as follows:

Dehydration:	
70 % (v/v) Ethanol/water	1 hr
85 % (v/v) Ethanol/water	1 hr
95 % (v/v) Ethanol/water	1 hr
Absolute ethanol I, II and III	1 hr each
Clearing	
Toluene I and II	1 hr
Impregnation	
Paraffin Fibrowax at 58°C	1 hr each.

After this process the tissue containers were transferred to an embedding oven containing paraffin Fibrowax at 58°C and the tissue removed using a pair of electrically heated forceps and placed into a metal mould containing a small amount of soft wax. The tissue was orientated so that the surface to be cut rested on this layer of wax facing the base of the mould. The mould was then filled with molten wax covered with a plastic guard. After framing, the wax blocks were removed from the moulds. Excess wax was cut off the blocks and the block mounted into a Rotatory Microtome. The block was trimmed until the tissue was exposed and 7 um thick sections were cut.

The sections were floated on distilled water (50°C) to flatten them and then mounted onto single frosted edge slides The animal's accession number was clearly printed in the frosted edge of the slide. The slides were placed in a rack and dried in an oven at 50°C for 2-3 hr.

4.2.1.5.3. Dewaxing and Rehydration

The process is schematically described as follows:

Xylene I and II, to remove wax	2 min each.
Absolute methanol (Rehydratation process)	1 min.
80 % (v/v) Methanol/water	1 min.
60 % (v/v) Methanol/water	1 min.
Tap water (rinse)	1 min.

4.2.1.5.4. Haematoxylin and eosin staining (Culling, 1974).

Reagents

Harris haematoxylin.

Acid alcohol. 1 % HCl in 70 % methanol.

Scott's tap water. 0.2 % potassium bicarbonate,

2 % (w/v) Magnesium sulphate in tap water.

1 % (w/v) Eosin in distilled water.

D.P.X.

Slides were examined using a Leitz Dialux 400 microscope at X60, X100 and X250 magnification.

Method

The section was dewaxed and hydrated (4.2.1.5.3) and stained as follows:

Erlich's acid haematoxylin	15 minutes
Tap water (washing)	5 minutes
Differentiation in acid alcohol	5-10 seconds
Immersion in Scott's tap water	1-2 minutes
At this step the sections were examined to ensure proper differentiation of nuclei.	
Counter staining with 1 % (w/v) aqueous eosin	2 minutes
Tap water (rinse)	30 seconds

85 % Methanol/water (dehydration)	30 seconds
Absolute methanol I and II	30 seconds each
Xylene I and II	30 seconds each

The sections were mounted in D.P.X. by placing a drop on a clean coverslip, taking the slide directly from the xylene, inverting it over the coverslip and pressing it gently, so that the D.P.X. spread under the coverslip and air bubbles were removed.

4.2.1.5.5. Sections stained with periodic acid-Schiff (PAS) staining

(Culling, 1974)

Reagents.

1 % Periodic acid.

Schiff reagent for the detection of aldehydes.

Mayer's haemalum.

Xylene.

D.P.X.

Method

Two sections of the same block were passed through the dewaxing process and brought into water (4.2.1.5.3). One slide of each tissue was labelled as "control".

The slides were exposed to periodic acid for 5 min, washed gently in tap water and rinsed twice with distilled water after which they were transferred to Schiff reagent for 15 min, at the end of this period, the slides were washed again with distilled water and dehydrated in absolute ethanol for 1 min, cleared with xylene and mounted in D.P.X.

4.2.2. In vitro studies

A schematic representation of the procedures used for the in vitro studies is shown in Fig. 4.1.

4.2.2.1. Isolation of glomeruli and proximal tubular fragments from rat kidney.

Groups of ten 150g male University of Surrey Wistar rats were killed by cervical dislocation. The kidneys were disected free, decapsulated, and the papillary tissue removed. The freshly chopped cortical tissue was forced through a 250 um stainless steel sieve to be disrupted, tubules were harvested on the 150 um sieve and glomeruli were collected on the 75 um sieve, and suspended in Tyrodes phosphate buffered salt solution supplemented with glucose or Earles-HEPES buffer, pH 7.4. The glomerular and tubular fractions were then washed and centrifuged at 300 rpm for 3 min, and the pellet resuspended in 25 ml buffer.

4.2.2.2. Isolation of glomeruli from pig kidney

Pig kidneys collected from freshly slaughtered female Large White pigs, aged 16 - 24 weeks, were cleaned, decapsulated, the cortex sliced and chopped very finely, and then incubated with calcium-free Krebs-Henseleit or Earles-HEPES buffer and in the presence of EDTA, for 15 min. Subsequently, the tissue was rinsed with buffer plus calcium and incubated with collagenase in buffer plus calcium for another 15 min. Following this, the tissue was forced through a 250 um stainless steel sieve and then the glomeruli collected on a 90 um sieve, and suspended in buffer. The glomerular fragments were then washed and centrifuged for 3 times and resuspended in 50 ml of buffer.

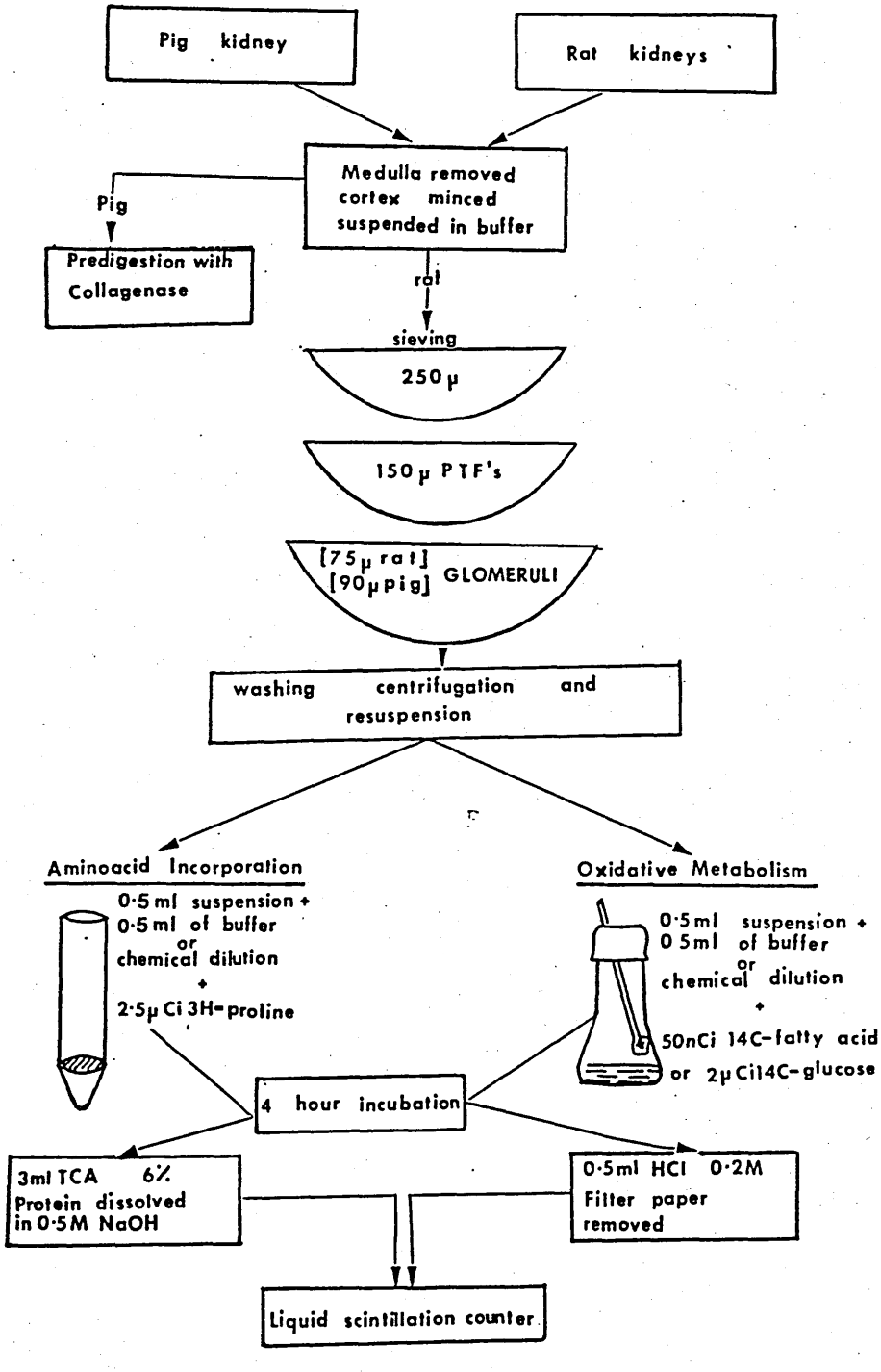


Fig. 4.1. Schematic representation of the procedures used in the in vitro studies.

4.2.2.3. De novo synthesis of protein

4.2.2.3.1. Incorporation of proline

The time course of ^{3}H-proline uptake and incorporation was followed by incubating 0.5 ml aliquots of the suspension of glomeruli at 37^{o}C in 0.5ml of incubation buffer, containing 2.5 uCi of the labelled amino acid, for 0-4 hr.

The effect of nephrotoxins on isolated rat or pig glomeruli and rat proximal tubular fragments (where applicable) was studied through the incorporation of radiolabelled proline, for 4 hr. Equal aliquots of glomerular suspension were incubated in the presence of several different concentrations of the nephrotoxins and ^{3}H-proline. As far as possible all compounds to be tested were dissolved in the corresponding incubation buffer. The chemicals tested (Table 4.3) were the following: adriamycin (ADR), puromycin aminonucleoside (PAN), streptomycin (STR), ochratoxin A (OTA), folic acid (FA), 2-bromoethanamine (BEA) and ethacrynic acid (ETA).

After incubation, the reaction was stopped with 3 ml of ice-cold 6% (w/v) trichloroacetic acid (TCA), containing 1 mM "cold" (non-radioactive) proline. The samples were centrifuged at 3000 rpm for 5 min and the supernatant removed. The precipitates were washed twice in cold 6% (w/v) TCA by centrifugation and aspiration of the supernatant to remove radioactivity. Washed samples were filtered onto glass-fibre discs (Whatmans) in a 25mm filter holder (stainless steel) and dried with absolute alcohol and residual TCA was removed with diethyl ether. The filter discs were transferred to plastic scintillation vials and the precipitate dissolved in 0.2 ml of 0.5M NaOH, after which 4 ml of scintillant OptiSafe were added and samples counted on a Rack Beta spectral scintillation counter.

TABLE 4.3

CHEMICALS USED TO STUDY IN VITRO TARGET TOXICITY

Compound	Range of concentrations	Assay	
		Protein	OXM*
ADR[a]	7.8 - 10000 uM	+	+
STR[a]	0.25 - 2 mM	+	+
PAN[a]	0.25 - 5 mM	+	+
BEA[a]	1.0 - 10 mM	+	+
ETA[a]	0.1 - 0.5 mM	+	+
FA[a]	1.0 - 10 mM	+	-
OTA[a]	1.6 - 3.2 mM	+	-
OTA[b]	10.0 - 1000 uM	+	+

[a] pig glomeruli

[b] rat glomeruli and tubular fragments

* Oxidative metabolism

4.2.2.3.2. Incorporation of other amino acids

The time course of other ^{3}H-labelled amino acids incorporation, (Table 4.4) was performed in the same way as for proline (see section 4.2.2.3.1).

4.2.2.4. Oxidative Metabolism

The time-course of $^{14}CO_2$ production from different ^{14}C-labelled substrates (Table 4.4) was followed by incubating 0.5 ml aliquots of the pig or rat glomerular suspension, or rat proximal tubular fragments containing 0.1 ml of the labelled substrate, in a closed reaction system for 0 - 4 hr, at 37°C in a shaking water bath, in 0.5 ml of Earle's-HEPES buffer, pH 7.4. The reaction and collection of $^{14}CO_2$ took place in a 10 ml conical flask with elongated neck (20 mm) tightly closed with a rubber Suba-Seal stopper to which a plastic center well had been attached (Fig. 4.1), so that the released CO_2 could be trapped into a filter paper strip embeded with 0.5 ml of 1M NaOH resting inside the well.

The effect of several toxins in the oxidative metabolism by pig glomeruli and rat proximal tubular fragments was studied by determining $^{14}CO_2$ release, at several different drug concentrations, for 4 hr. Equal aliquots of glomerular suspension were incubated in the presence of the nephrotoxins and either labelled glucose (2 uCi) or fatty acids (50 nCi), as shown in Table 4.4.

After the incubation the reaction was stopped with 0.5 ml of 0.2M HCl which acidified the buffer releasing any HCO_3^- as free $^{14}CO_2$ which was subsequently trapped as $NaCO_3$ by NaOH.

The flasks were then allowed to stand for 15 min at room temperature, following which the filter paper strips were transferred to a plastic

TABLE 4.4

RADIOLABELS USED TO STUDY IN VITRO GLOMERULAR AND PROXIMAL TUBULAR METABOLISM

Synthesis of Protein	Oxidative Metabolism
Amino acids[a]	Glucose[b,d,e]
Proline[d,e]	Fatty acids[c]
Histidine[d,e]	Oleic[d]
Leucine[d]	Linolenic[d,e]
Lysine[d]	Palmitic[f]
Glycine[d]	Stearic[f]
Phenylalanine[e]	Mevalonate
Tryptophan[e]	
Tyrosine[e]	

[a] specific activity 2.5 uCi/ml

[b] specific activity 2 uCi/ml

[c] specific activity 50 nCi/ml

[d] pig glomeruli

[e] rat glomeruli and proximal tubules

[f] rat proximal tubules

scintillation vial, the wells were washed twice with 0.5 ml of distilled water, to remove any residual radioactivity. Four ml of the scintillant were added and the radioactivity counted.

4.2.2.5. Determination of protein

Aliquots of the glomerular or tubular preparation (0.5 ml) were dissolved in 0.5M NaOH overnight, for protein analysis by the Coomassie blue dye-binding method (Read and Northcote, 1981), in which to 0.2 ml of the homogeneous sample were added 3.75 ml of the Coomassie blue reagent, the mixture allowed to stand for 10 min and the absorbance recorded at 595 nm.

The protein concentration was determined from a calibration curve prepared with different concentrations of BSA.

4.2.2.6. Assessment of Purity

4.2.2.6.1. The purity of the preparation is assayed by phase contrast light microscopy (using x10 or x20 lens), where a slide prepared using 50 ul of glomerular suspension is examined in a number of fields. The purity is presented as a percentage of tubular contamination. Fig. 4.2 shows the aspect of the glomerular preparation as seen under the microscope.

4.2.2.6.2. Glomerular viability

Two combined fluorescence probes (Rotman and Papermaster, 1966; Edidin 1970) were used to assess cell viability: fluorescein diacetate (FDA) and ethidium bromide (EB). FDA is a nonpolar compound that enters the cell membrane readily, where non-specific esterases convert it to fluorescein, which is retained by intact cells, giving a green/yellow fluorescence. In contrast EB which is a negatively charged molecule penetrates intact cells slowly, but rapidly enters damaged cells where it forms a bright red

fluorescent complex with nucleic material. A 25 ul of the glomerular suspension was incubated with 25 ul of a solution containing FDA dissolved in acetone (5 ug/ml) and EB dissolved in phosphate buffered isotonic saline (200 ug/ml), for 3 min. The preparation was then examined by fluorescent microscopy (590 nm). Viable cells presented a bright green fluorescent cytoplasm. In contrast non-viable cells showed a weekly green cytoplasm and a bright red fluorescent nucleous.

4.2.3 Statistical analysis

Results were analysed by t-test. Results are expressed as mean $\pm$ SD. Tables of results and statistics are located in Appendices 1 and 2.

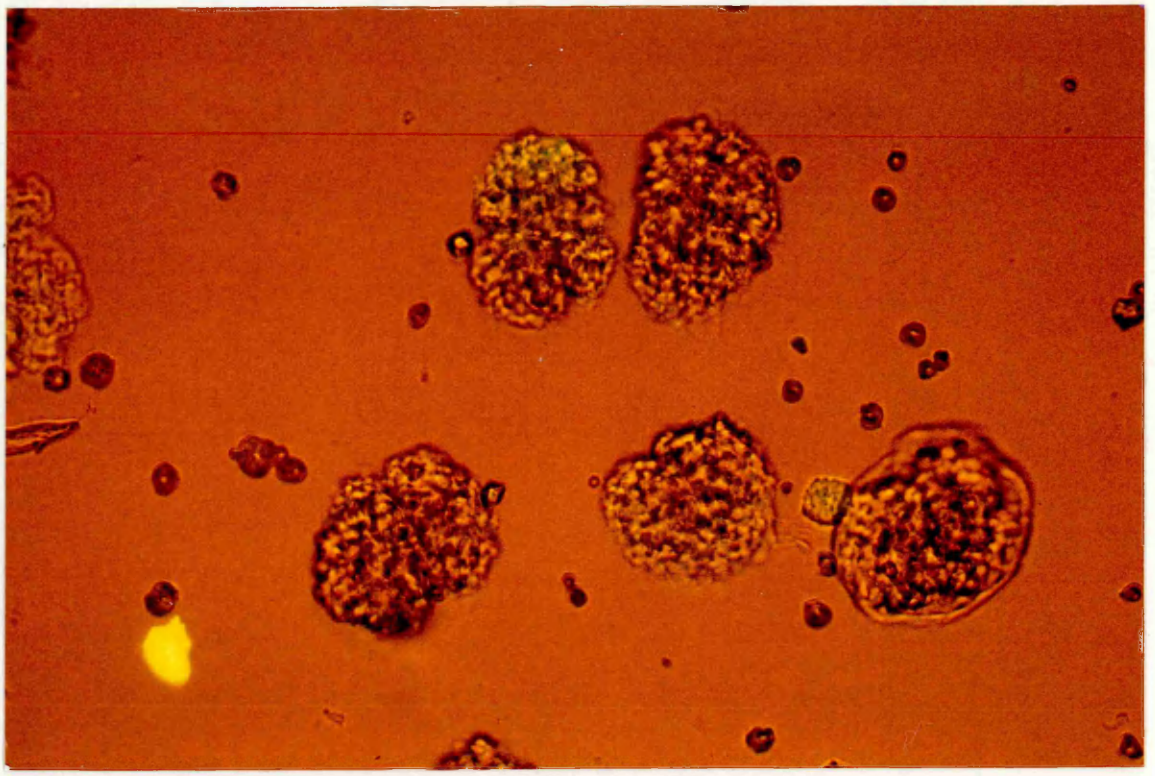


Fig. 4.2 Glomerular preparation as seen under the phase contrast light microscope.

CHAPTER 5

IN VIVO STUDIES: OCHRATOXIN A AND AFLATOXIN B_1 NEPHROTOXICITY

5.1. Urinalysis

5.1.1. Single dose regime

Urinary enzyme excretion was determined over four consecutive 24-hr periods, after a single injection of OTA alone, ATB_1 alone or the combined administration of both toxins. Histological findings of the kidneys, removed and fixed 96 hr after the injection are described in section 5.2.

5.1.1.1. OTA alone

When OTA was administered in a single ip dose of 1 mg/kg urinary ALP activity decreased on day 1 and 2, but was increased on day 3. On day 4 there was a statistically significant increase ($P \leq 0.05$), up to 272% of the control (Table 5.1.1, Appendix 1; Fig. 5.1.1A and B). Urinary AAP activity was decreased on day 1 and 2, the latter being significantly different from the control ($P \leq 0.05$, Table 5.1.2, Appendix 1; Fig. 5.1.2A and B) and was elevated on day 4 (to 143% of the control), although it was not statistically significant. There were no significant variations on the GGT and NAG urinary activity, as compared to the control (Tables 5.1.3 and 5.1.4, Appendix 1; Figs. 5.1.3A and B, and 5.1.4A and B) throughout this experiment. Urinary volume was higher for the control than for the treated group, (Table 5.1.5, Appendix 1; Figs. 5.1.5A and B) however there were not significant differences.

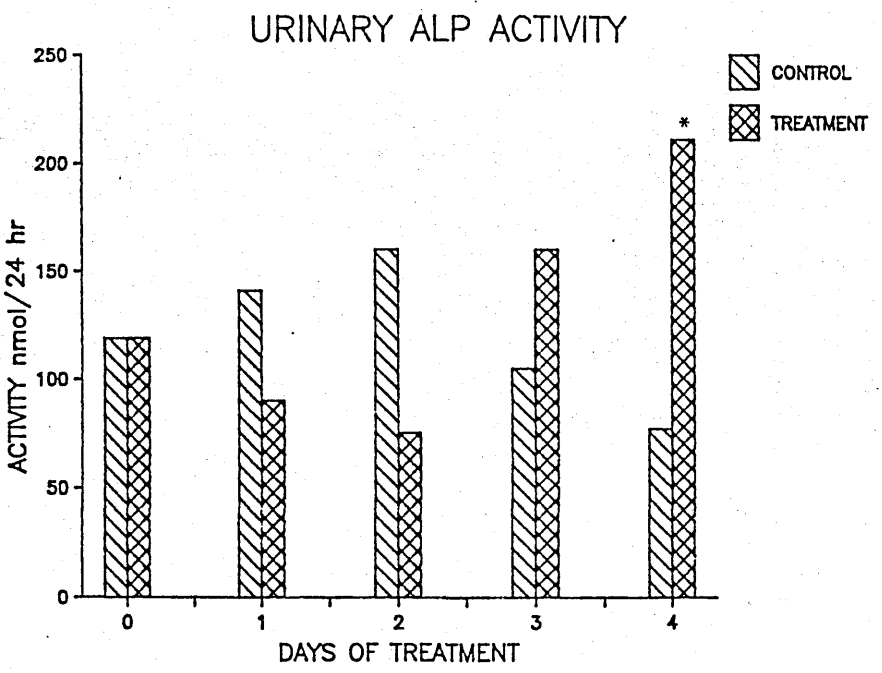


Fig. 5.1.1A

Mean level of alkaline phosphatase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (1 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant increase ($P \leq 0.05$) was seen on day 4 (*).

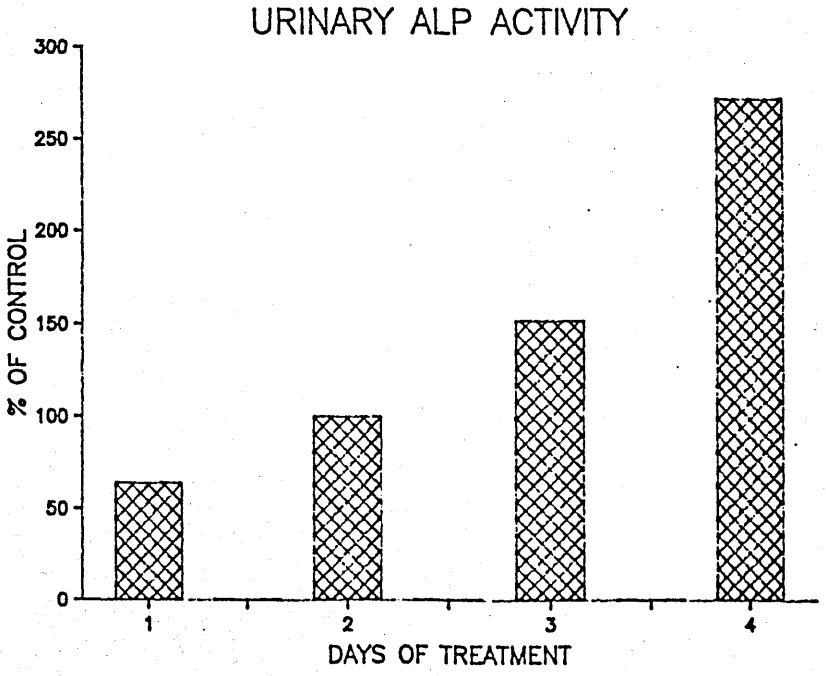


Fig. 5.1.1B

Urinary alkaline phosphatase excretion calculated as a percentage of the control group.

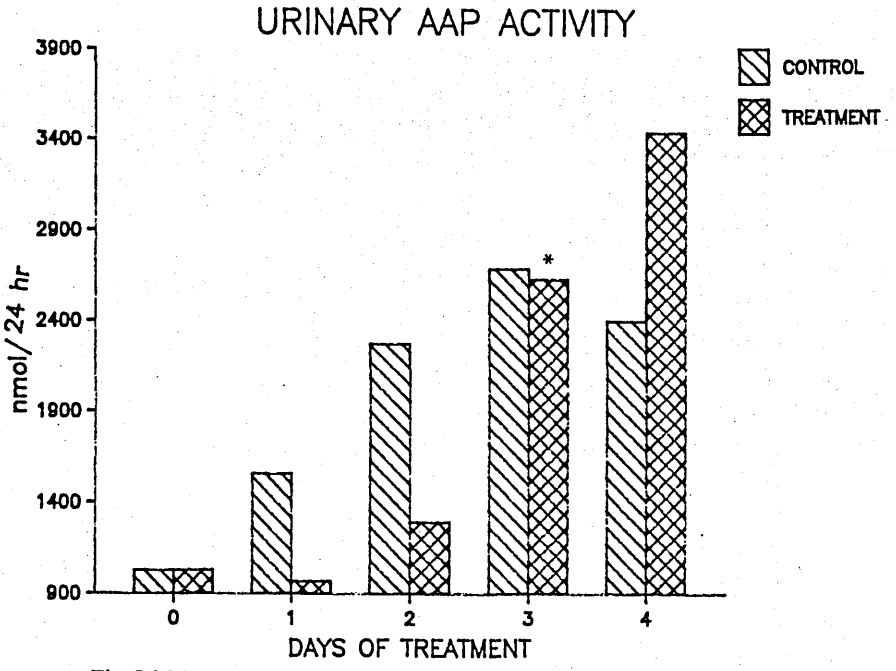


Fig. 5.1.2A

Mean level of alanine aminopeptidase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (1 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant decrease ($P \leq 0.05$) was seen on day 3 (*).

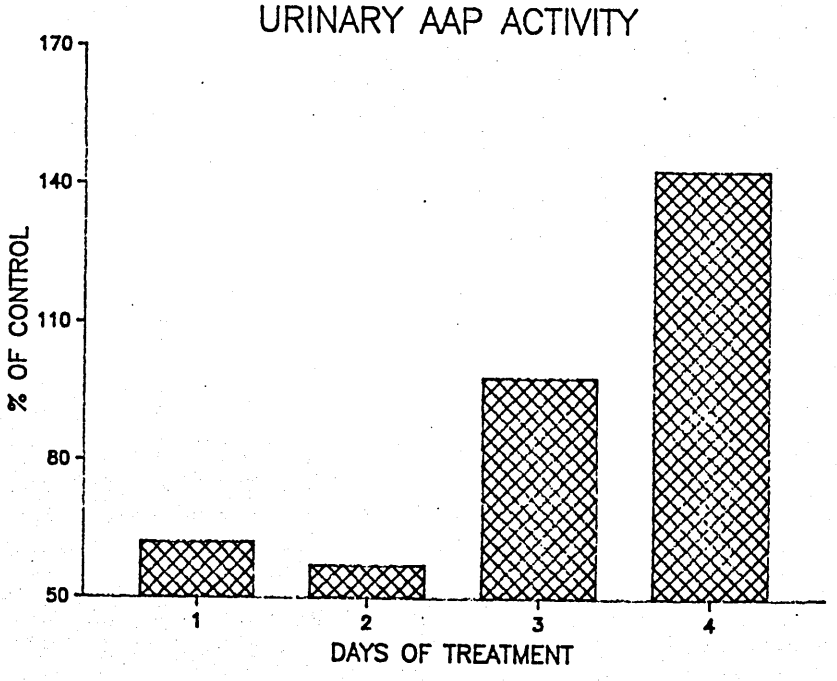


Fig. 5.1.2B

Urinary alanine aminopeptidase excretion calculated as a percentage of the control group.

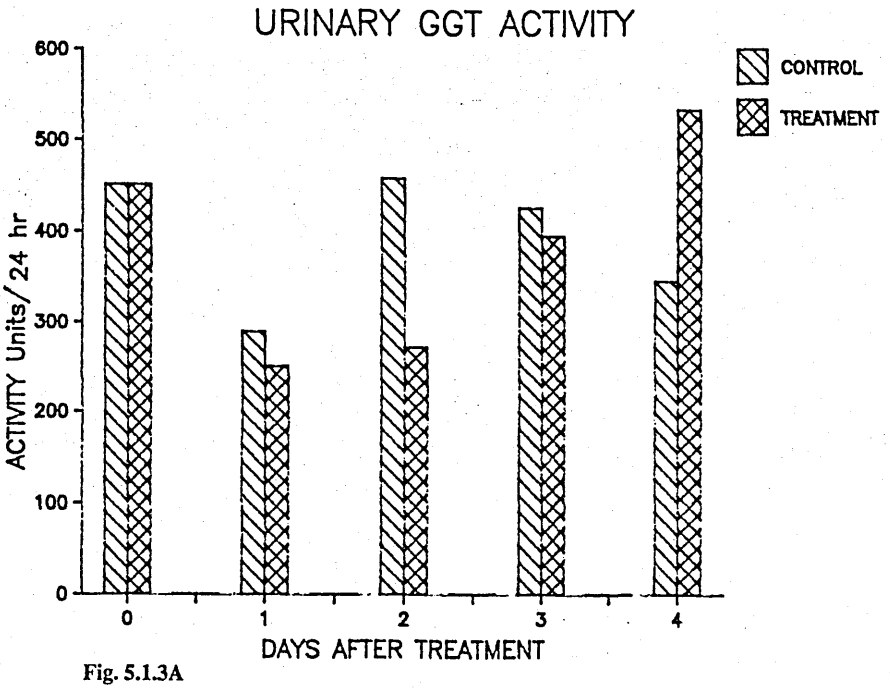


Fig. 5.1.3A

Mean level of gamma-glutamyl transpeptidase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (1 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3.

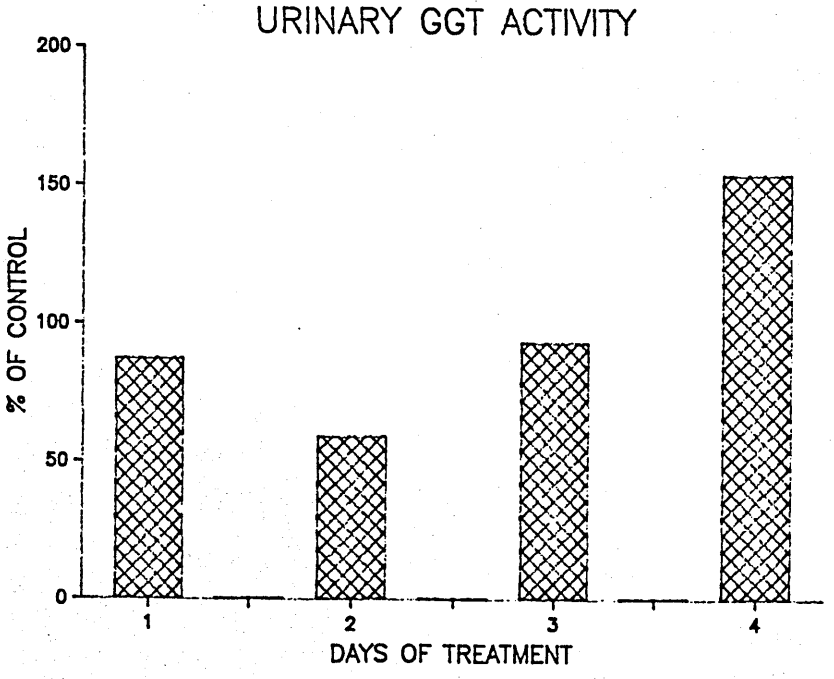


Fig. 5.1.3B

Urinary gamma-glutamyl transpeptidase excretion calculated as a percentage of the control group.

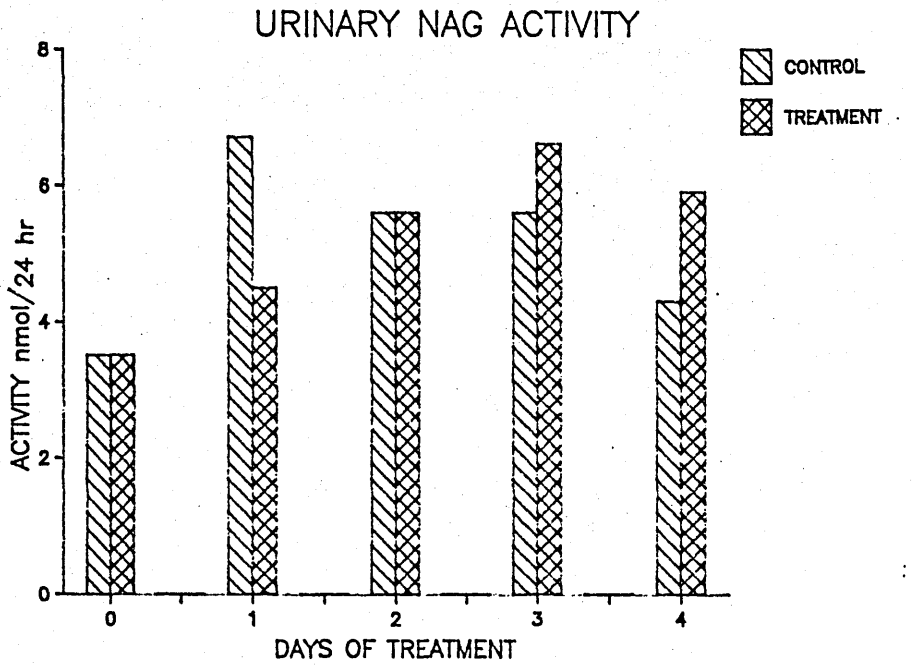


Fig. 5.1.4A

Mean level of N-acetyl-beta-glucosaminidase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (1 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3.

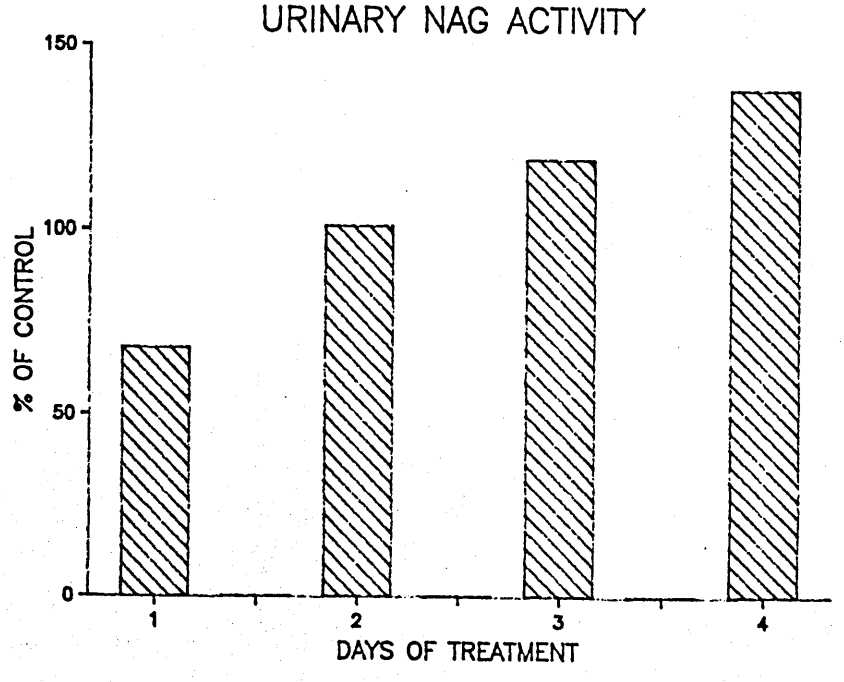


Fig. 5.1.4B

Urinary N-acetyl-beta-glucosaminidase excretion calculated as a percentage of the control group.

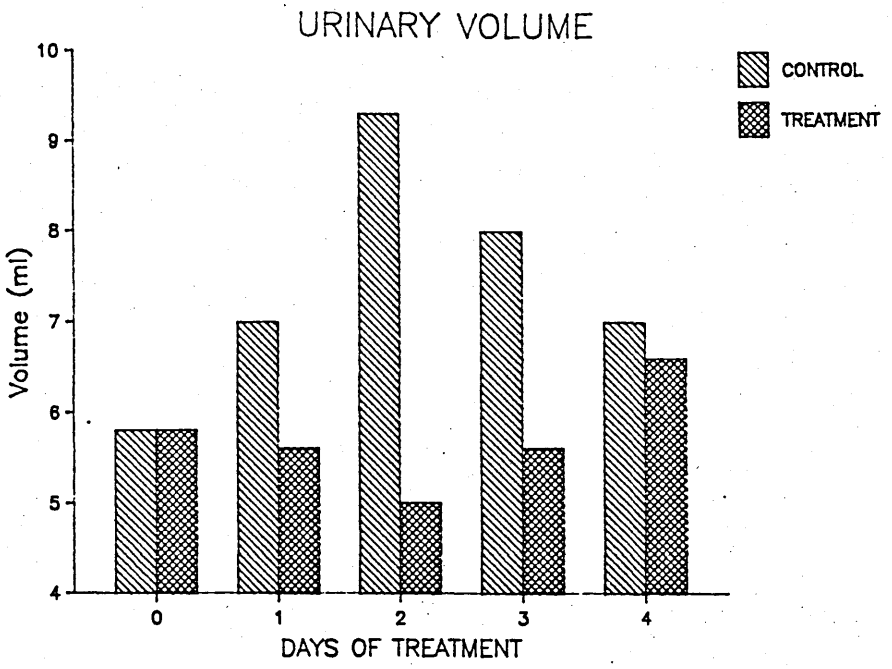


Fig. 5.1.5A

Mean volume in 24-hr urine before (Day 0) and after a single ip dose of ochratoxin A (1 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3.

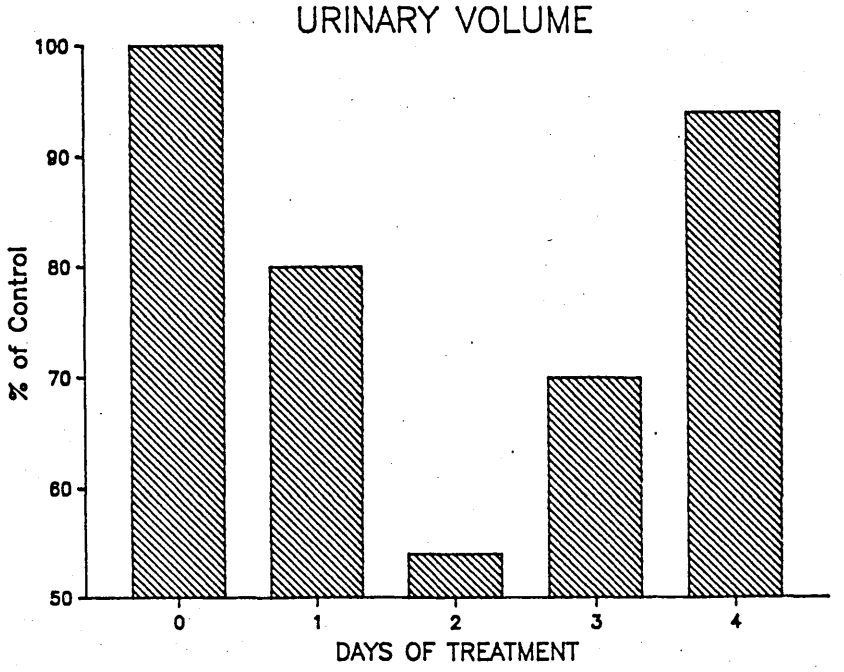


Fig. 5.1.5B

Urinary volume calculated as a percentage of the control group.

When the concentration of OTA was increased to 5 mg/kg ALP activity increased on day 2, showing significant differences from the control on day 3 and 4 (Table 5.1.6, Appendix 1; Figs. 5.1.6A and B). AAP activity first increased on day 2 and was significantly different ($P \leq 0.05$) from the control on days 3 and 4 (Table 5.1.7, Appendix 1; Figs. 5.1.7A), up to 209 and 196% (Fig. 5.1.7B). NAG activity was significantly higher than the control up to 184% ($P \leq 0.05$) on day 4 (Tables 5.1.8, Appendix 1; Figs. 5.1.8A and B). Urinary GGT activity did not show significant variations compared to the control (Table 5.1.9, Appendix 1; Figs. 5.1.9A and B) at any time point in this experiment. Urinary volume rose significantly ($P \leq 0,05$) compared to the control on day 3 (Table 5.1.10, Appendix 1; Figs. 5.1.10A and B).

Discussion. These data show that single low doses of OTA caused changes in the urinary excretion of several enzymes. At the lowest concentration of OTA tested, ALP showed a decrease on day 1 and 2 of the treatment, but increased on day 3 and 4, and with 5 mg/kg of the toxin, the enzyme activity was also increased on day 3 and 4. Both doses of the mycotoxin caused a rise in ALP activity, however this was not dose-related, since the increase on day 3 is similar for both concentrations, and on day 4 the lowest concentration caused the highest increase in the enzyme activity.

AAP showed an initial decrease and further slight elevation, at the low concentration of OTA, and when the concentration was increased, it showed a considerable increase towards day 3 and 4, suggesting a dose-response effect. Although GGT changes were not statistically significant at the low concentration of OTA, this enzyme followed a similar trend to ALP, with an initial decrease, and further elevation, and also a similar pattern to that of ALP is followed by GGT at the high concentration of the compound.

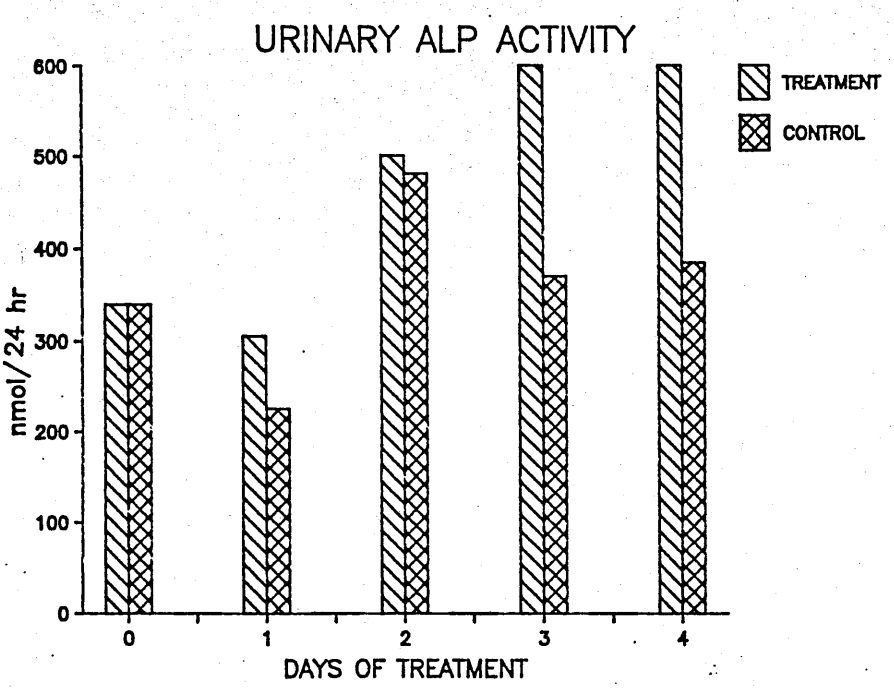


Fig. 5.1.6A

Mean level of alkaline phosphatase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (5 mg/kg), in comparison to a group injected with the vehicle only (control). The height for each bar is the mean value for n=4 in the control group and n=5 for the treated group.

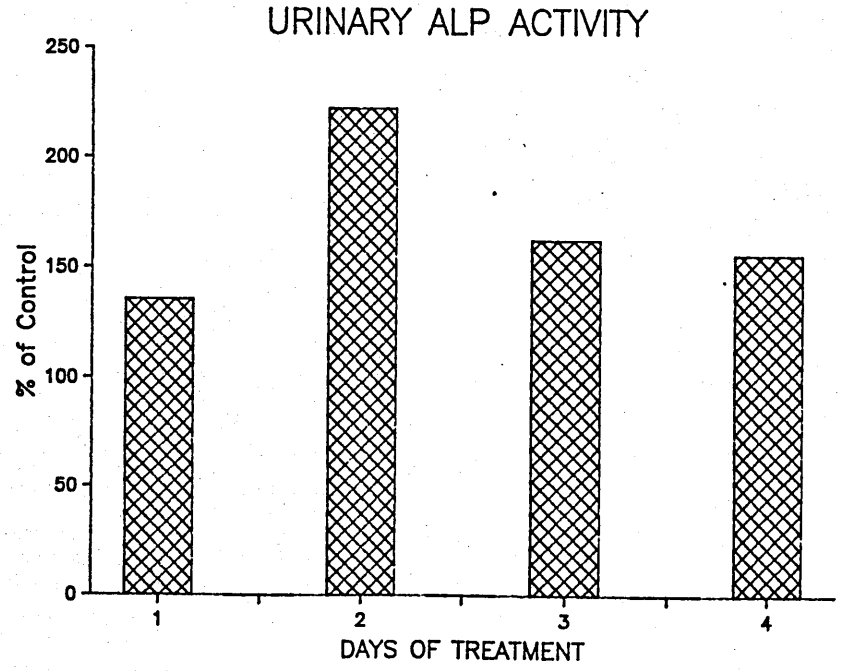


Fig. 5.1.6B

Urinary alkaline phosphatase excretion calculated as a percentage of the control group.

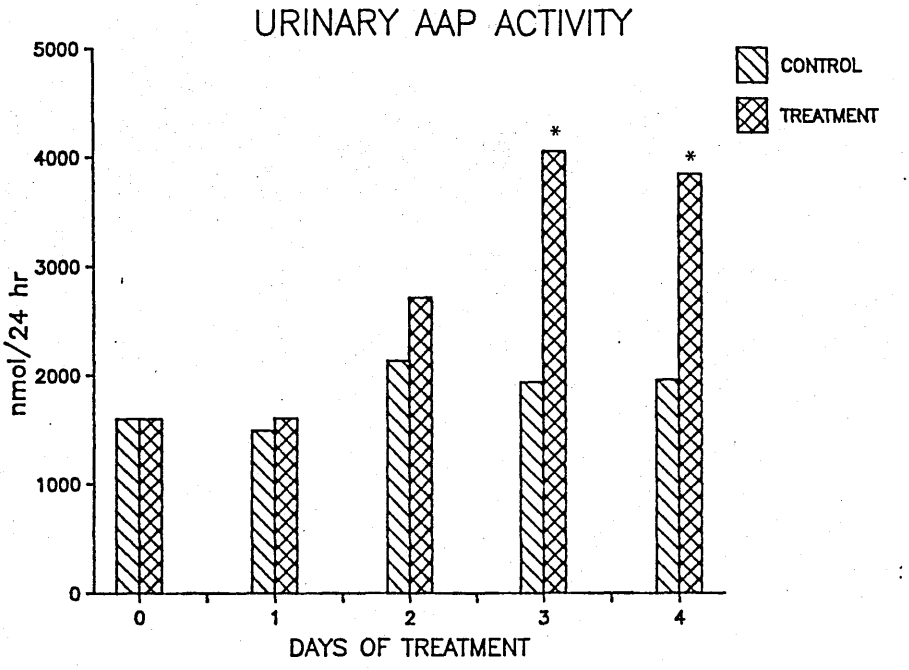


Fig. 5.1.7A

Mean level of alanine aminopeptidase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 in the treated group. Significant increases($P \leq 0.05$) were seen on days 3 and 4 (*).

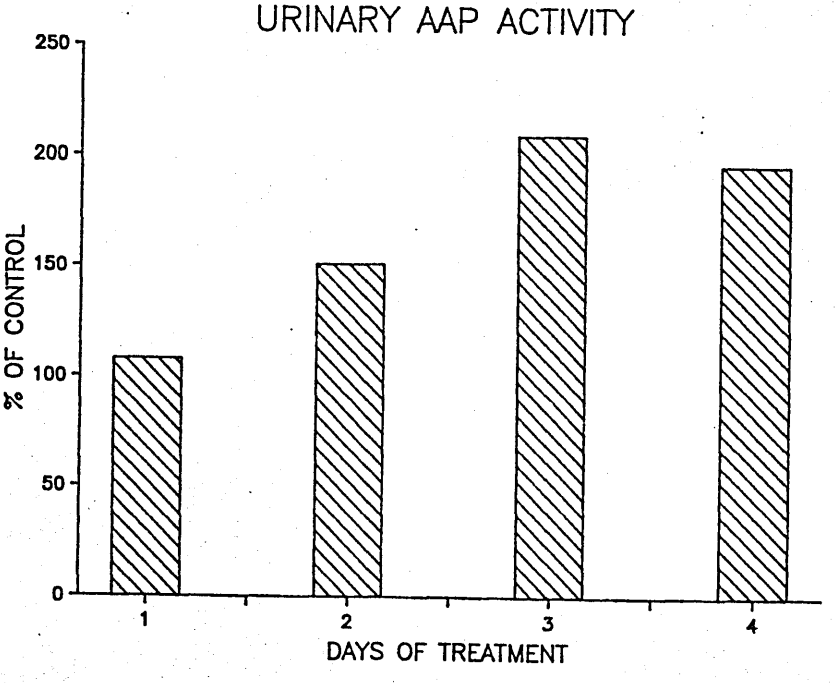


Fig. 5.1.7B

Urinary alanine aminopeptidase excretion calculated as a percentage of the control group.

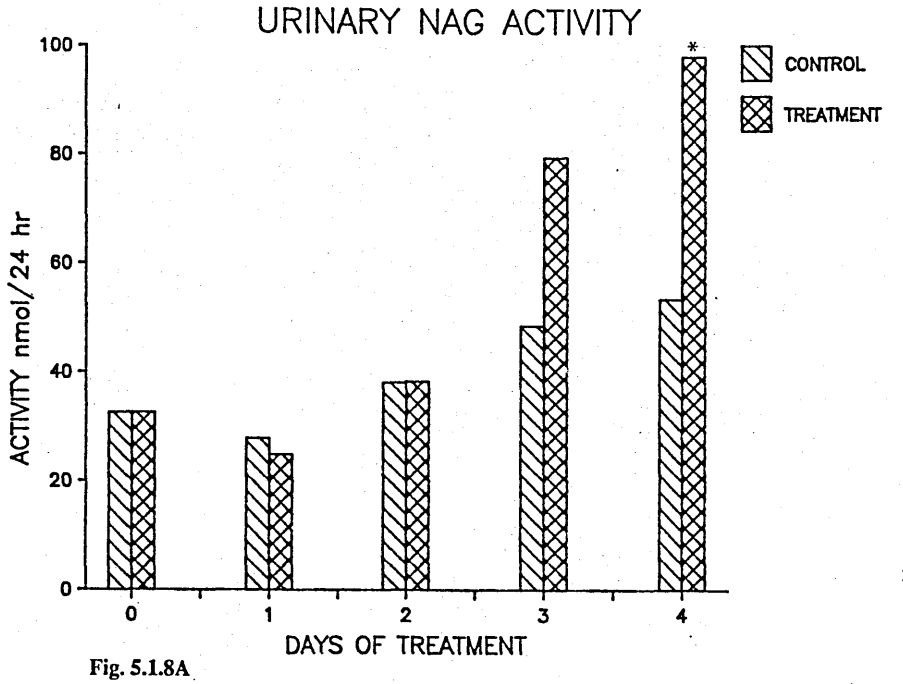


Fig. 5.1.8A

Mean level of N-acetyl-beta-glucosaminidase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 in the treated group. Signficant increase ($P \leq 0.05$) was seen on day 4 (*).

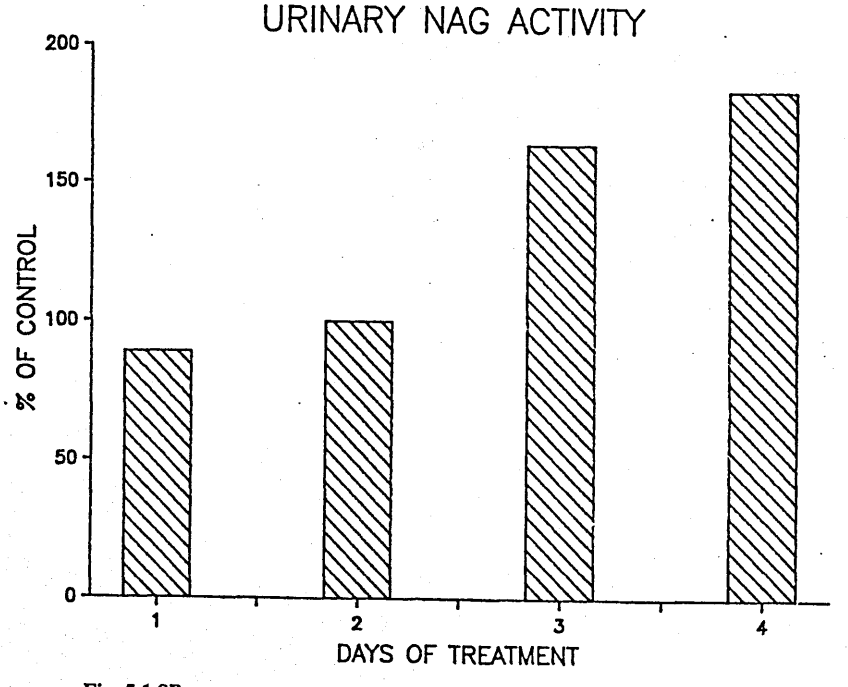


Fig. 5.1.8B

Urinary N-acetyl-beta-glucosaminidase excretion calculated as a percentage of the control group.

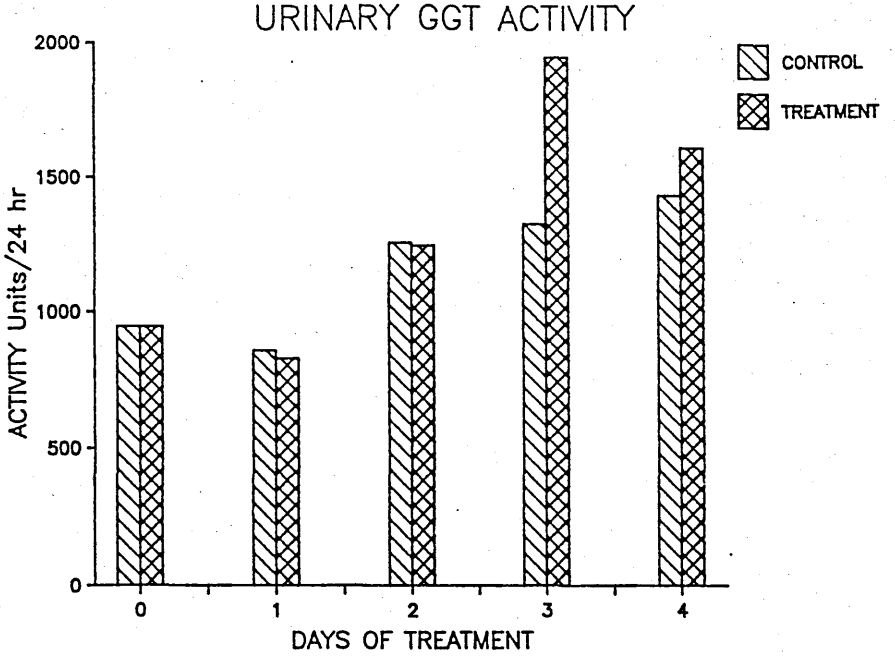


Fig. 5.1.9A

Mean level of gamma-glutamyl transpeptidase in 24-h urine before (day 0) and after a single ip dose of ochratoxin A (5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 for the treated group.

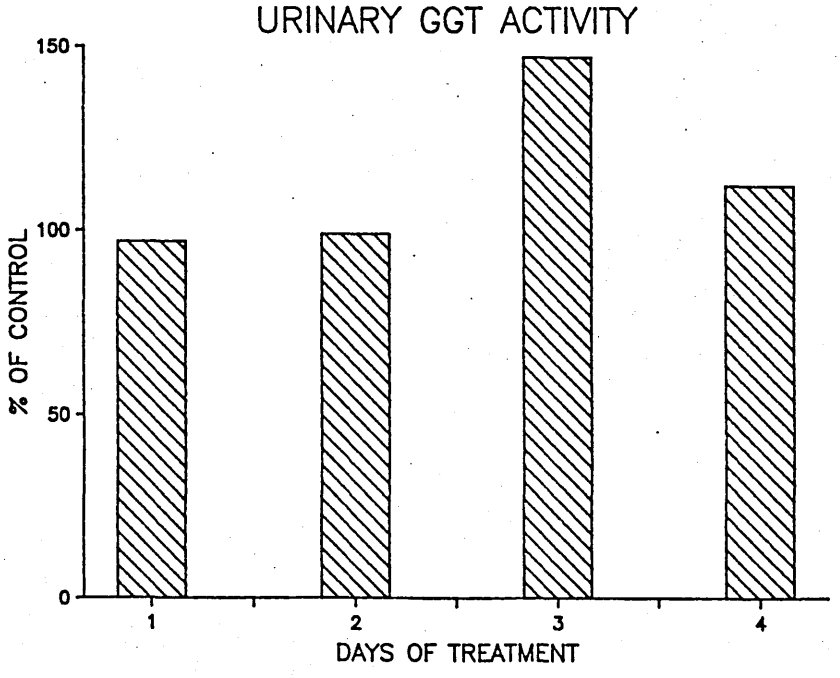


Fig. 5.1.9B

Urinary gamma-glutamyl transpeptidase excretion calculated as a percentage of the control group.

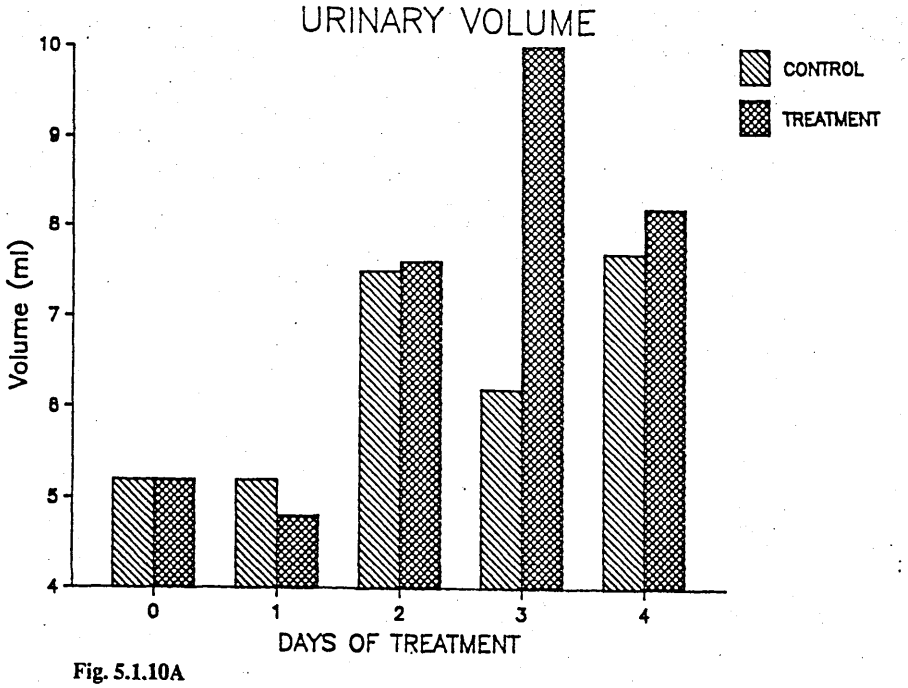


Fig. 5.1.10A

Mean volume in 24-hr urine before (Day 0) and after a single ip dose of ochratoxin A (5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 in the treated group. Significant increase ($P \leq 0.05$) was seen on day 3 (*).

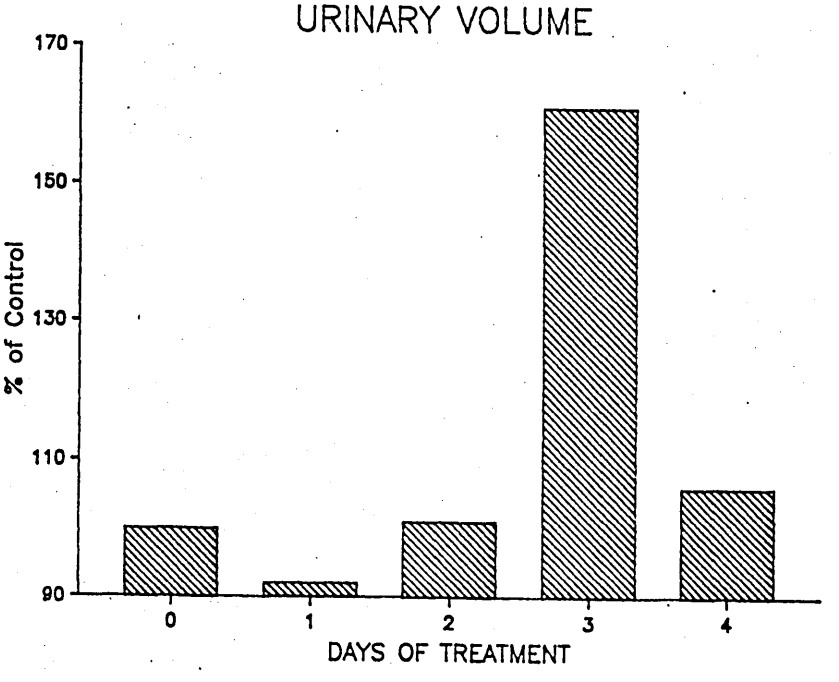


Fig. 5.1.10B

Urinary volume calculated as a percentage of the control group.

NAG excretion did not show any statistically significant changes relative to the control at the lowest concentration of OTA, but it decreased on day 1 and rose slightly over the control values, on days 3 and 4. When the concentration of the mycotoxin was 5 mg/kg, the enzyme activity was elevated on day 3 and 4, suggesting a dose-response effect.

The decreased activity of some enzymes, below the control values, is difficult to explain. A rise would be normally expected as a consequence of the tubular injury, with the subsequent enzymatic leaking, loss of the brush border and/or the exfoliation of renal tubular cells. One possible explanation for the lower enzymatic activity of the treated group may be either the presence of inhibitors in the urine samples, or the high variability seen between animals in both control and treatment group. All of these were on day 1 or 2, therefore may also represent renal functional changes involving decrease in the normal enzyme turnover.

5.1.1.2. ATB_1 alone

When a small dose of ATB_1 (100 ug/kg) was administered ip to the rats, AAP activity remained constant from days 1 - 3 and showed a significantly different decrease ($P \leq 0.05$) (Table 5.1.11, Appendix 1; Fig. 5.1.11A) to 78% of the control, on day 4 (Fig. 5.1.11B). Urinary GGT activity increased on day 2, and started to decrease on day 3, to show a significant decrease ($P \leq 0.05$) to 67% of the control on day 4 (Table 5.1.12, Appendix 1; Fig. 5.1.12A and B). NAG activity was significantly lower ($P \leq 0.05$) than the control on day 1 (Table 5.1.13, Appendix 1; Fig. 5.1.13A), to 85%. On day 2 there was a significant increase ($P \leq 0.01$) to 182% of the control (Fig. 5.1.13B). ALP activity did not show significant variations in relation to the control throughout the experiment (Table 5.1.14, Appendix 1; Fig. 5.1.14A and B). There were no singificant

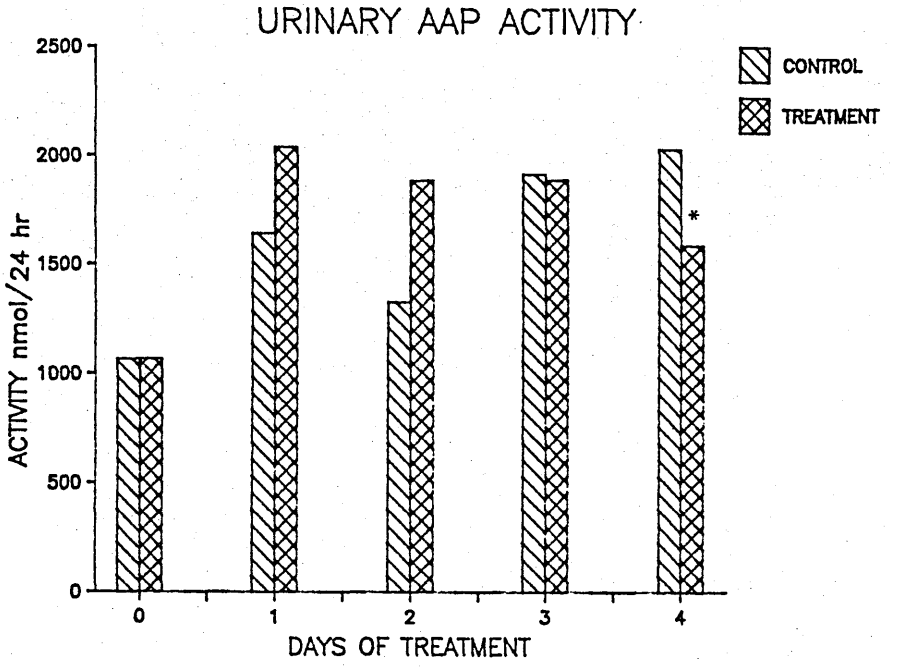


Fig. 5.1.11A

Mean level of alanine aminopeptidase in 24-h urine before (day 0) and after a single ip dose of aflatoxin B_1 (100 ug/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant decrease ($P \leq 0.05$) was seen on day 4 (*).

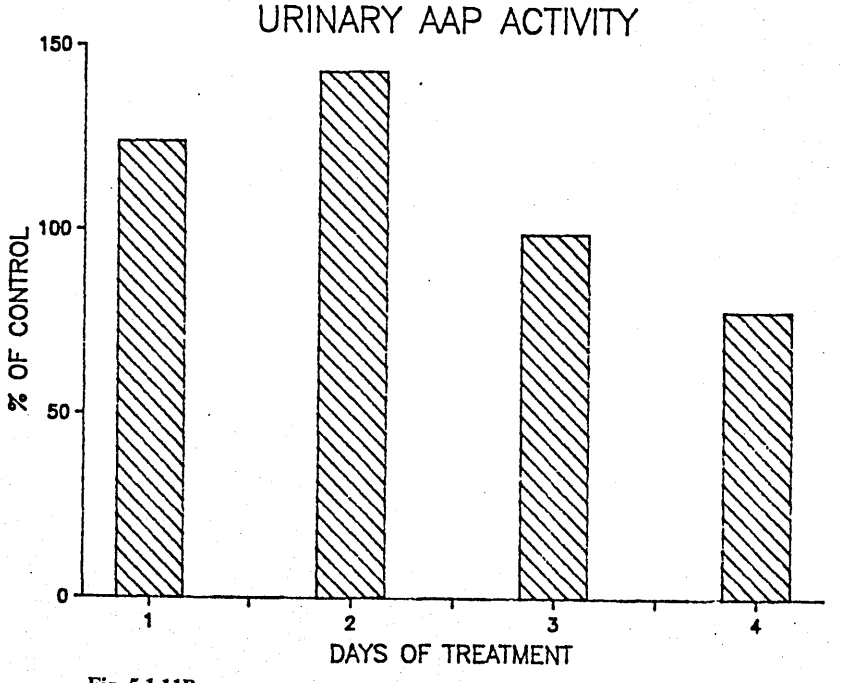


Fig. 5.1.11B

Urinary alanine aminopeptidase excretion calculated as a percentage of the control group.

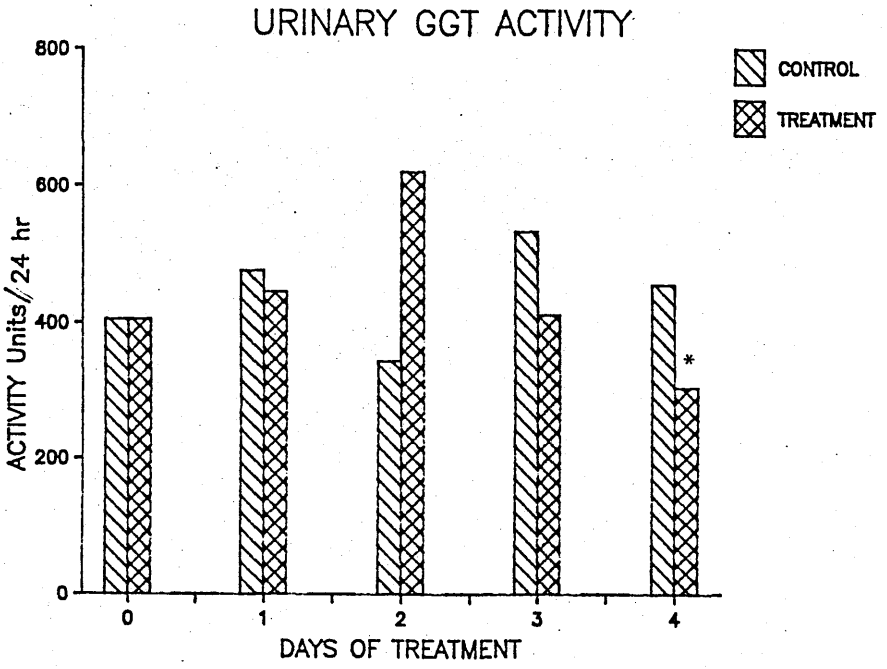


Fig. 5.1.12A

Mean level of gamma-glutamyl transpeptidase in 24-h urine before (day 0) and after a single ip dose of aflatoxin B_1 (100 ug/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant decrease ($P \leq 0.05$) was seen on day 4 (*).

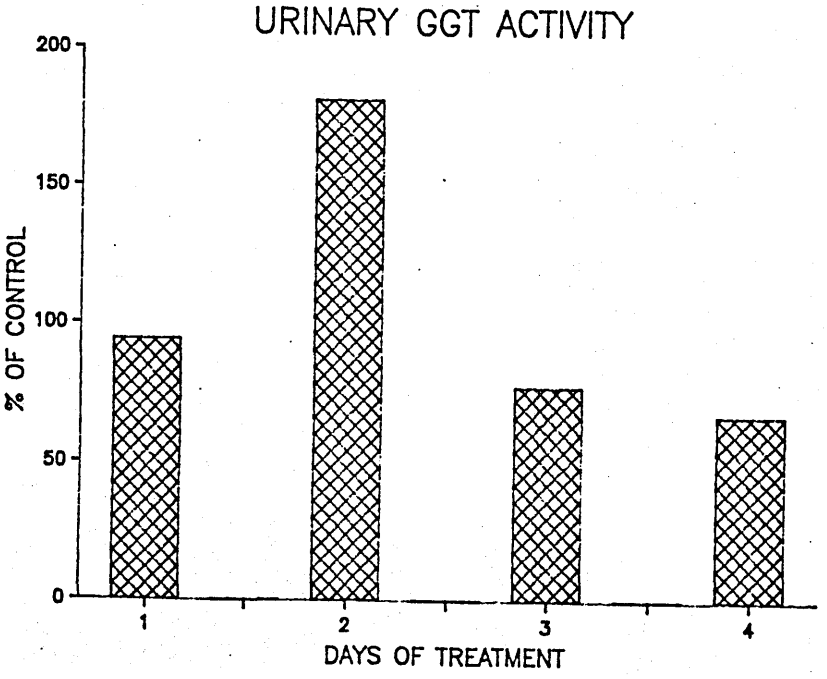


Fig. 5.1.12B

Urinary gamma-glutamyl transpeptidase excretion calculated as a percentage of the control group.

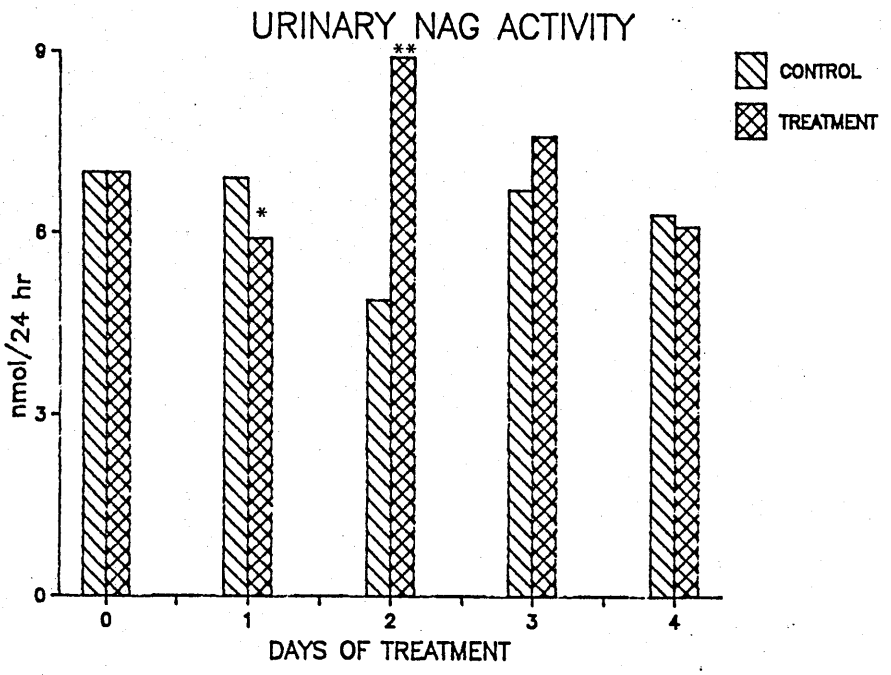


Fig. 5.1.13A

Mean level of N-acetyl-beta-glucosaminidase in 24-h urine before (day 0) and after a single ip dose of aflatoxin B_1 (100 ug/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant decrease ($P \leq 0.05$) was seen on day 1(*) and increase ($P \leq 0.01$) on day 2(**).

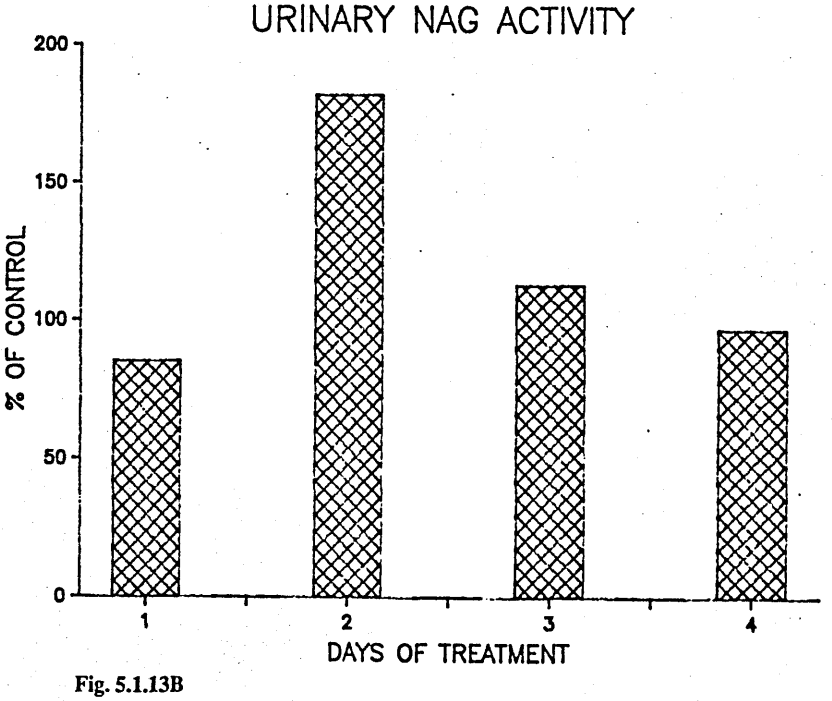


Fig. 5.1.13B

Urinary N-acetyl-beta-glucosaminidase excretion calculated as a percentage of the control group.

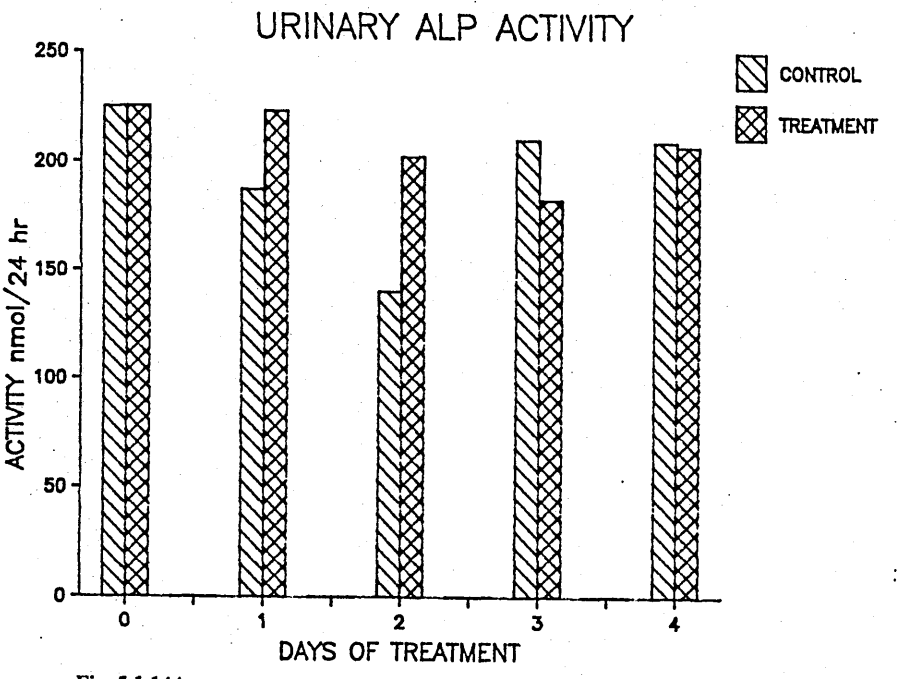


Fig. 5.1.14A

Mean level of alkaline phosphatase in 24-h urine before (day 0) and after a single ip dose of aflatoxin B_1 (100 ug/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3.

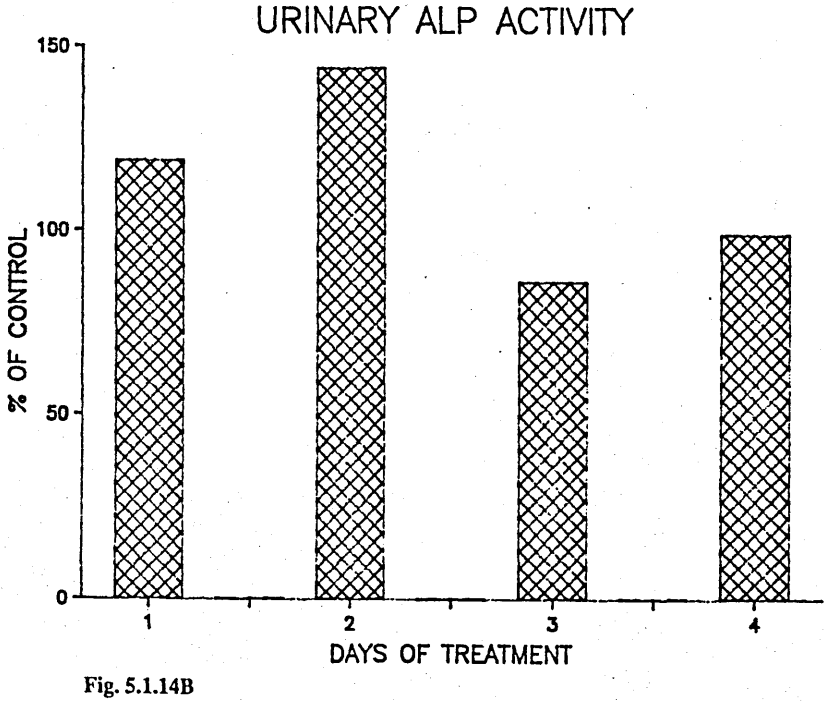


Fig. 5.1.14B

Urinary alkaline phosphatase excretion calculated as a percentage of the control group.

differences in urinary volume between the control and treated groups (Table 5.1.15, Appendix 1; Figs. 5.1.15A and B).

Discussion. These results showed that single low doses of ATB_1 caused some changes on the excretion of urinary enzymes such as ALP, AAP, GGT and NAG. ALP activity was below the control values on days 1 and 2, but none of the differences were statistically significant. AAP excretion was lower for the treated group on most of the days; on day 4 the decrease was statistically different from the control. GGT was increased maximally on day 2, and started to decrease on day 3, at which it was still significantly different from control. NAG values were significantly lower than the controls on day 1, and significantly higher on day 2, after which the activities remained within the control range.

There have been no published studies on the effect of single small doses of ATB_1 on urinary enzymes such as ALP, AAP and NAG; there is one report in which administration of a single dose of 100 ug ATB_1/kg is reported to have caused a significant increase in urinary GGT within 6 to 7 hr after injection (Grosman et al, 1983), after which the enzymic activity diminished, but remained higher than the control. The results in these studies are not consistent with that report, but several factors may account for this difference. These include the different timed period for the collection of urine samples, and also the wide variations between animals, and other factors such as purity of the sample and type of diet.

5.1.1.3. Combined administration of OTA and ATB_1

When OTA and ATB_1, were administered ip simultaneously (1 mg and 100 ug/kg, respectively), urinary ALP activity increased on day 2 and 3 (Table 5.1.16, Appendix 1; Fig. 5.1.16A), rose significantly ($P \leq 0.05$) to 208%

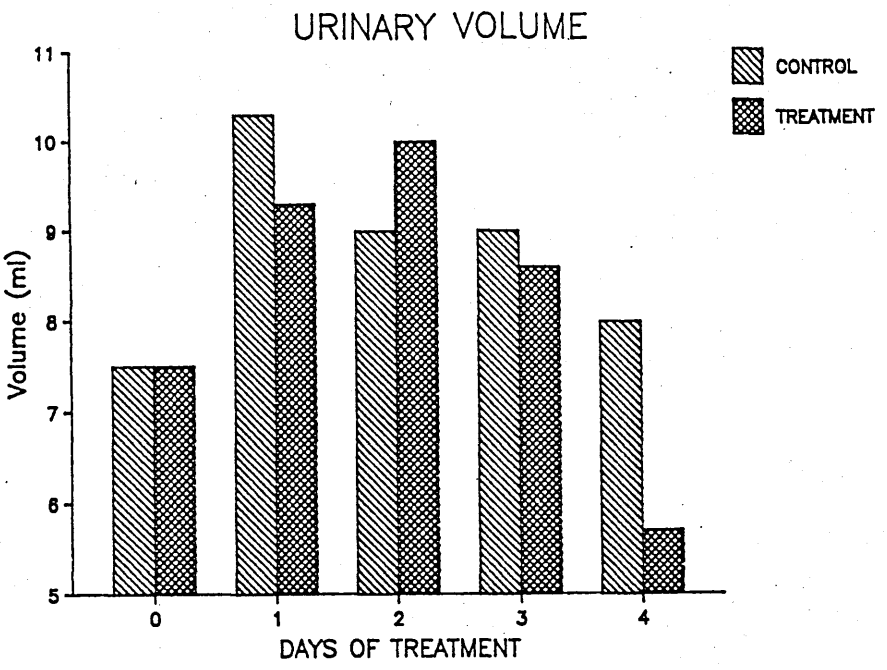


Fig. 5.1.15A

Mean volume in 24-hr urine before (Day 0) and after a single ip dose of aflatoxin B_1 (100 ug/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3.

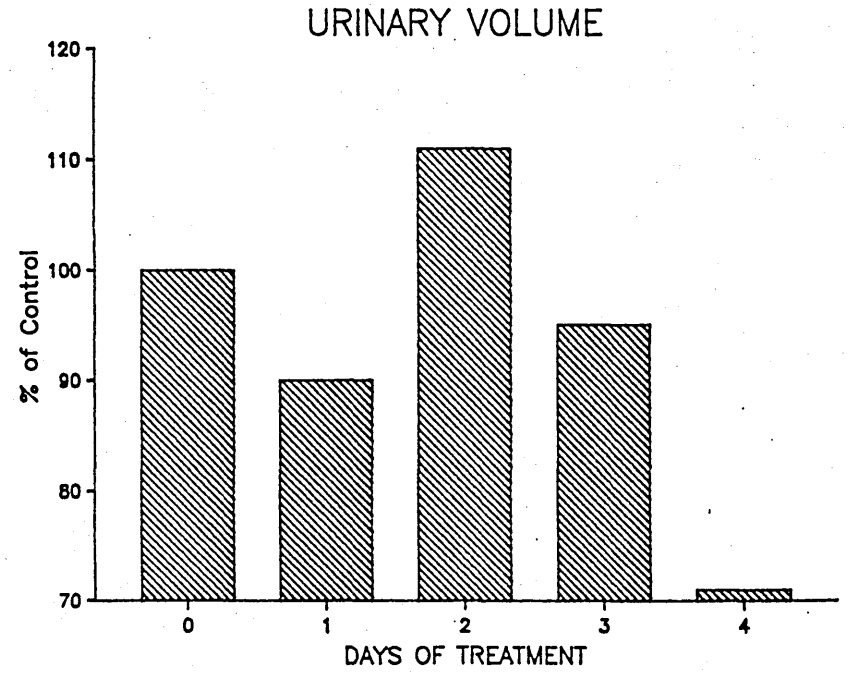


Fig. 5.1.15B

Urinary volume calculated as a percentage of the control group.

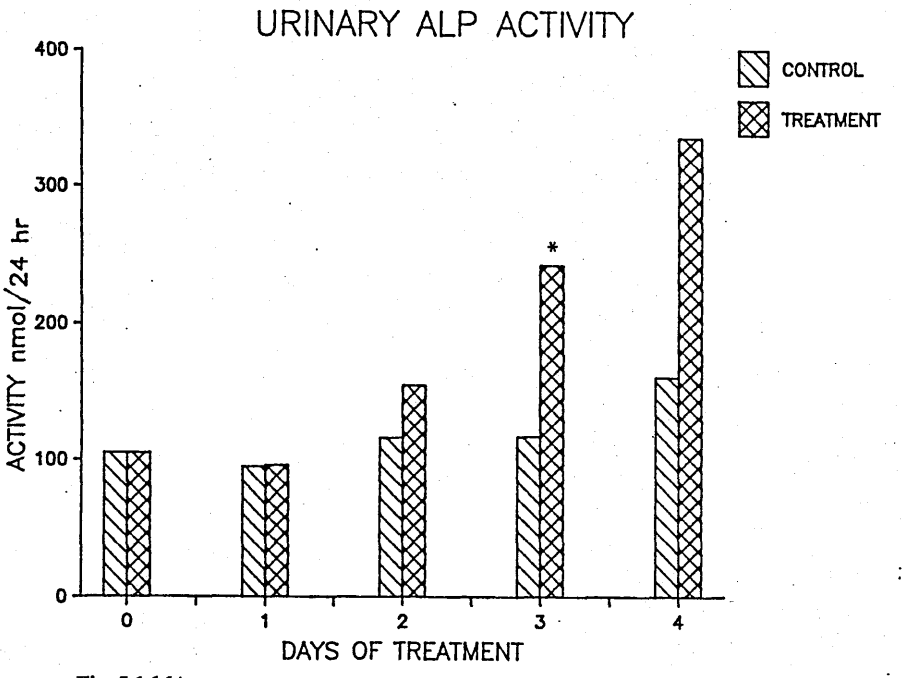


Fig. 5.1.16A

Mean level of alkaline phosphatase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (1 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant increase ($P \leq 0.05$) was seen on day 3 (*).

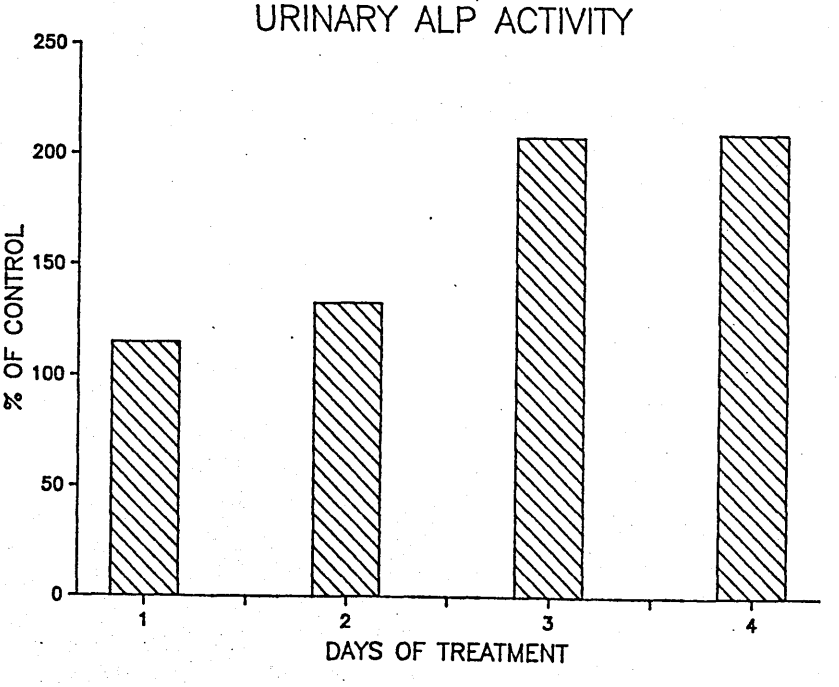


Fig. 5.1.16B

Urinary alkaline phosphatase excretion calculated as a percentage of the control group.

of the control (Fig. 5.1.16B). The enzyme activity continued to increase on day 4, but was not statistically significant due to the high variance. GGT activity decreased up to day 3 and then increased significantly ($P \leq 0.05$) from the control, on day 3 (Table 5.1.17, Appendix 1; Fig. 5.1.17A) by 33% (Fig. 5.1.17B). NAG activity, showed a significant elevation ($P \leq 0.05$) on day 1 (Table 5.1.18, Appendix 1; Fig. 5.1.18A) up to 161% of the control (Fig. 5.1.18B) and also on day 3, to 149% of the control. The enzyme activity was also increased on day 4, but not significantly. Although AAP activity of the experimental group was higher than the control on day 3, this was not statistically significant (Table 5.1.19, Appendix 1; Fig. 5.1.19A and B). Urinary volume rose significantly ($P \leq 0.05$) compared to the control on day 3 (Table 5.1.20, Appendix 1; Figs. 5.1.20A and B).

When OTA was increased to 5 mg/kg and administered simultaneously with 100 ug/kg of ATB_1, ALP activity was significantly decreased on day 1 ($P \leq 0.01$), to 71% of the control (Table 5.1.21, Appendix 1; Fig 5.1.21A and B), and increased on day 2, reaching a peak of 217% of the control on day 3 that was significant ($P \leq 0.05$) after which the enzyme activity decreased. GGT activity in the experimental group was lower than the controls throughout the experiment (Table 5.1.22, Appendix 1; Fig. 5.1.22A), having presented a significant decrease ($P \leq 0.05$) to 63% of the control, on day 1 (Fig. 5.1.22B). AAP activity remained lower for the treatment group than for the control on days 1 and 2, but there were no significant differences (Table 5.1.23, Appendix 1; Fig. 5.1.23A and B). NAG activity was lower for the treatment group on day 1 and after that the activity values remained very close to the controls (Table 5.1.24, Fig. 5.1.24A and B). There were no statistically significant differences. Urinary volume (Figs. 5.1.25A and B remained lower for the treated group

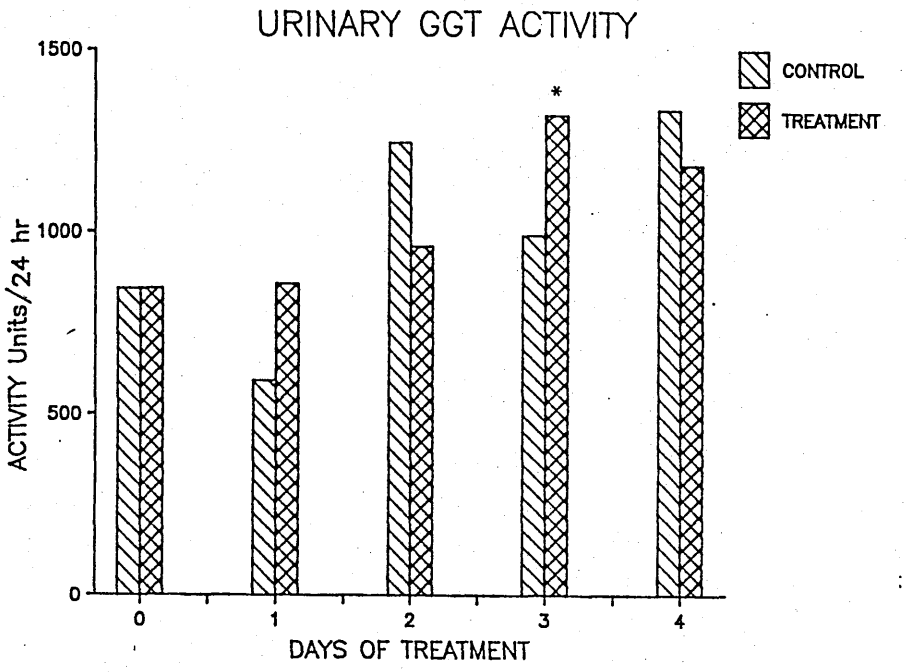


Fig. 5.1.17A

Mean level of gamma-glutamyl transpeptidase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (1 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant increase ($P \leq 0.05$) was seen on day 3 (*).

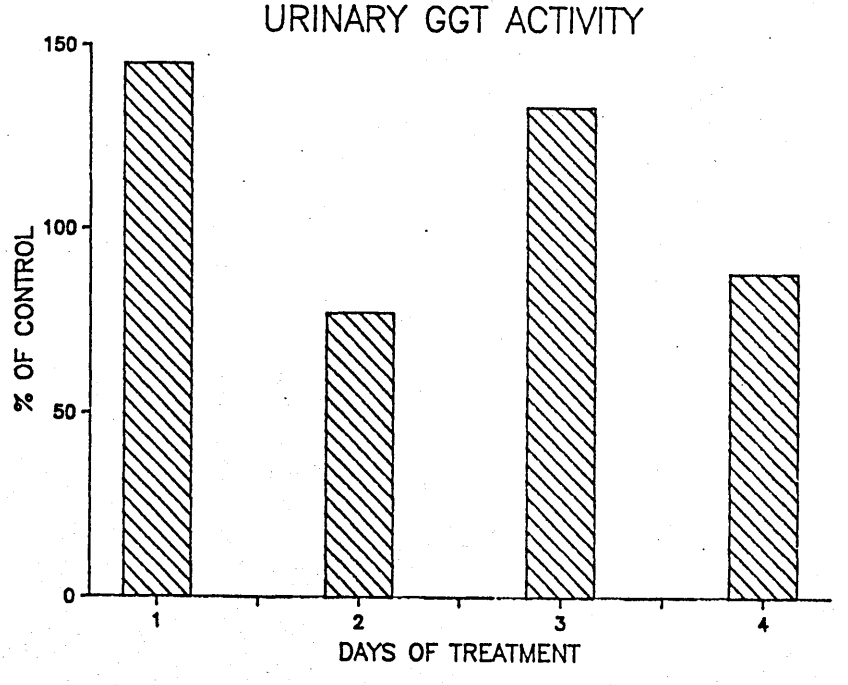


Fig. 5.1.17B

Urinary gamma-glutamyl transpeptidase excretion calculated as a percentage of the control group.

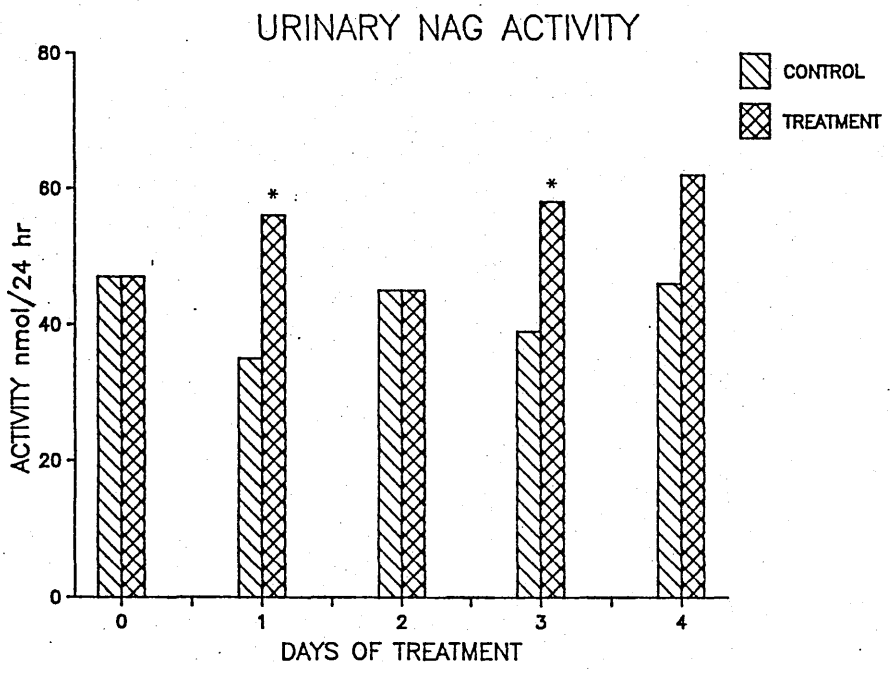


Fig. 5.1.18A

Mean level of N-acetyl-beta-glucosaminidase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (1 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant increases ($P \leq 0.05$) were seen on days 1 and 3 (*).

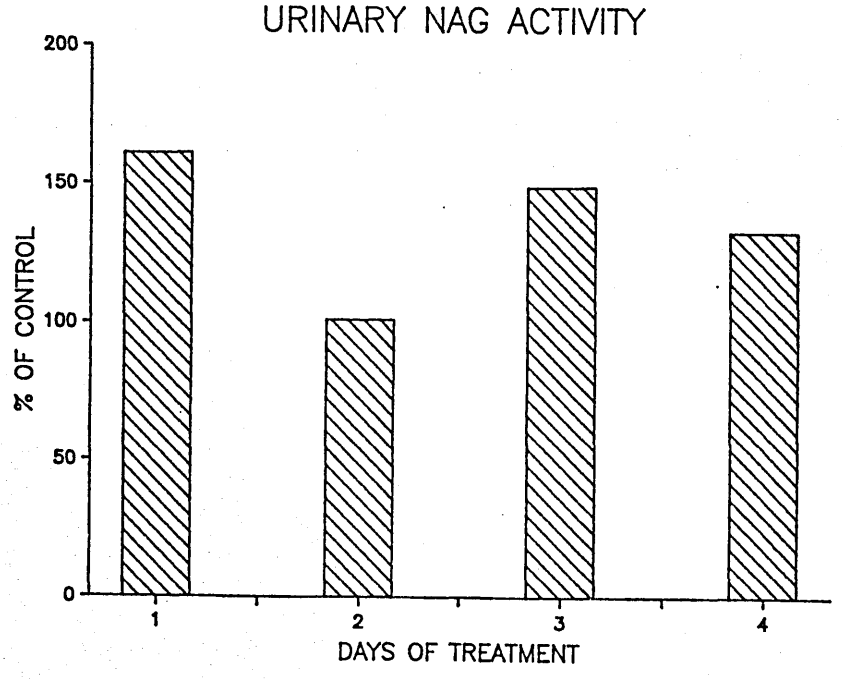


Fig. 5.1.18B

Urinary N-acetyl-beta-glucosaminidase excretion calculated as a percentage of the control group.

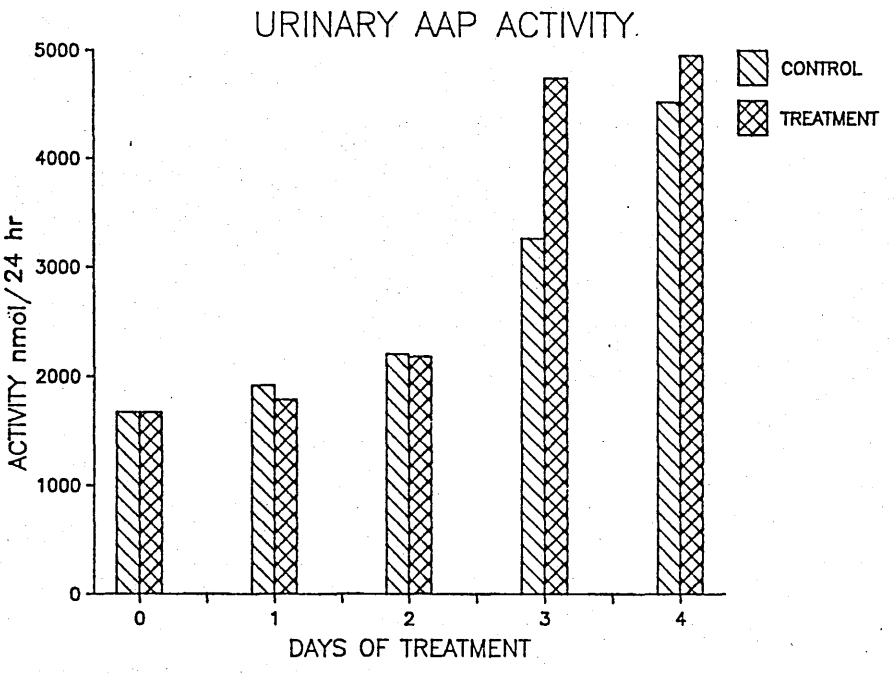


Fig. 5.1.19A

Mean level of alanine aminopeptidase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (1 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3.

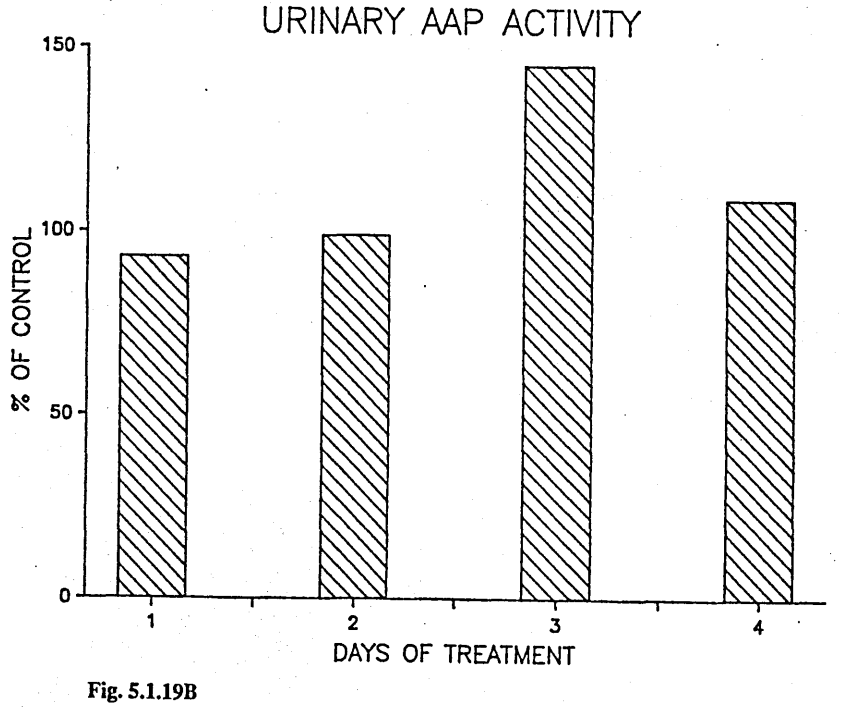


Fig. 5.1.19B

Urinary alainine aminopeptidase excretion calculated as a percentage of the control group.

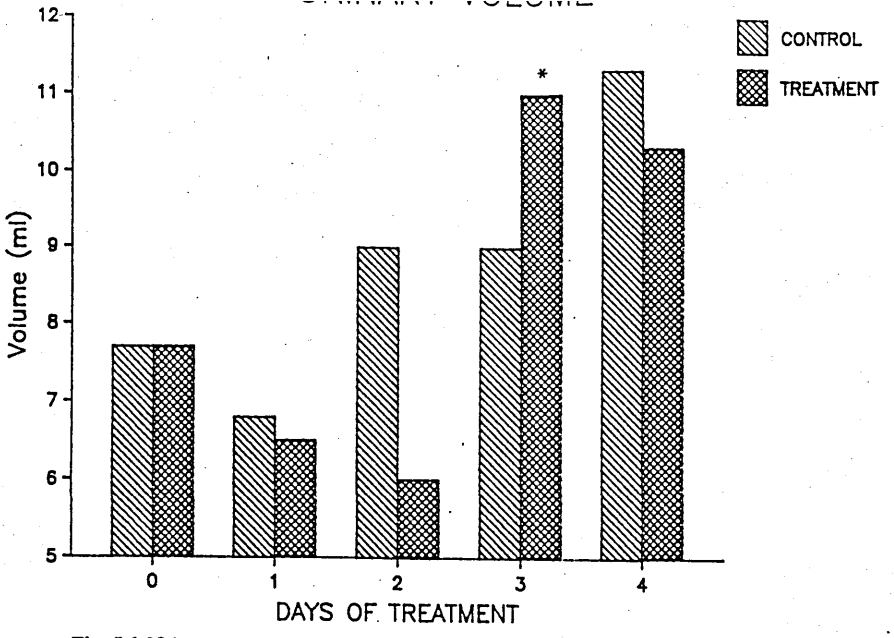


Fig. 5.1.20A

Mean volume in 24-hr urine before (Day 0) and after a single ip dose of ochratoxin A and aflatoxin B_1 (1 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=3. Significant increase ($P \leq 0.05$) was seen on day 3 (*).

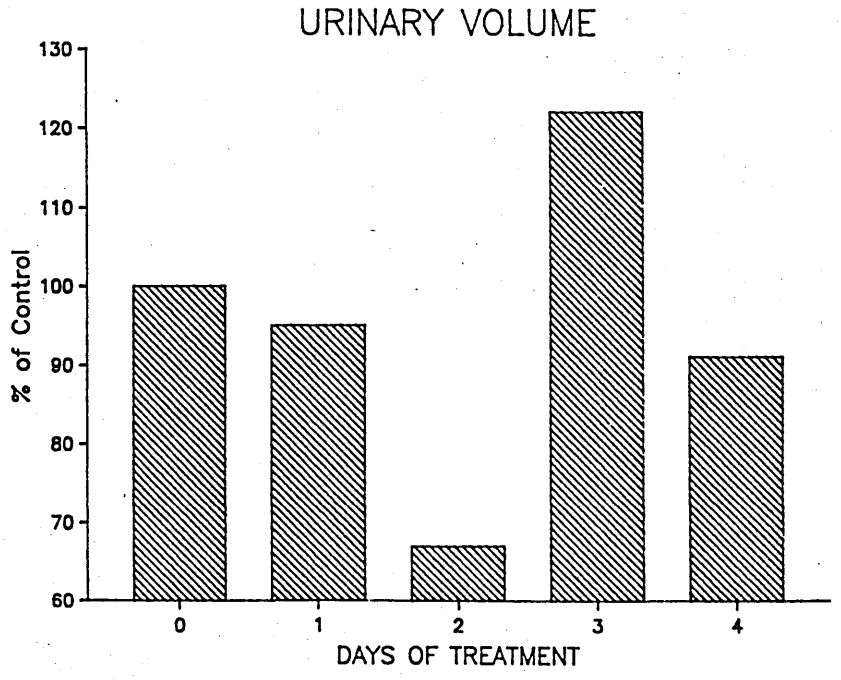


Fig. 5.1.20B

Urinary volume calculated as a percentage of the control group.

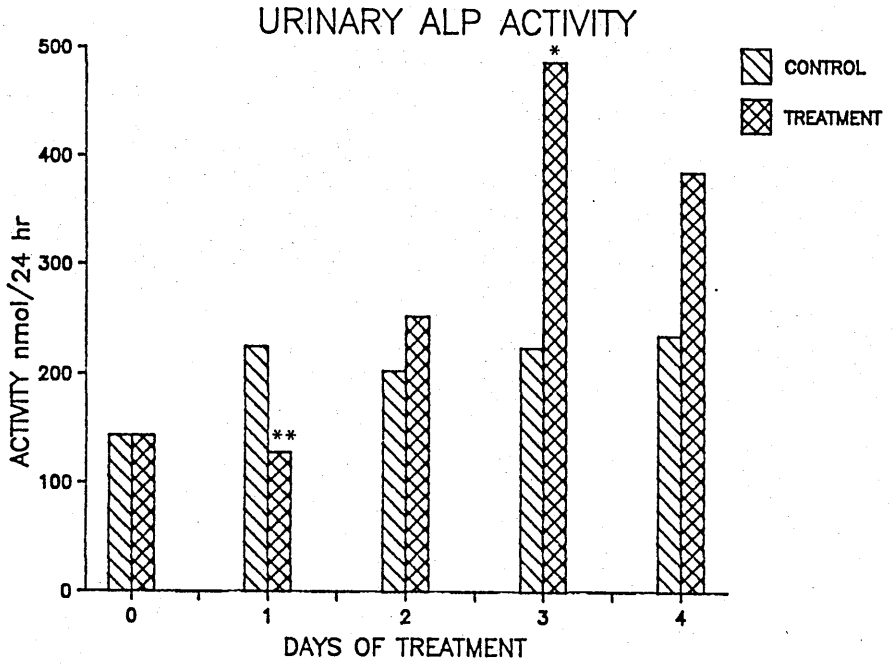


Fig. 5.1.21A

Mean level of alkaline phosphatase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (5 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 for treated group. Significant decrease ($P \leq 0.01$) was seen on day 1 (**) and increase ($P \leq 0.05$) on day 3 (*).

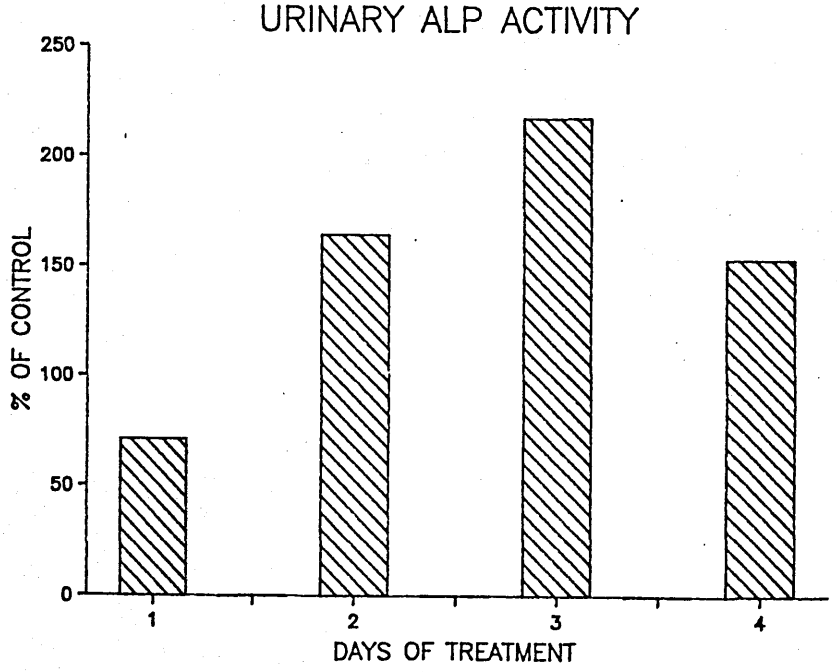


Fig. 5.1.21B

Urinary alkaline phosphatase excretion calculated as a percentage of the control group.

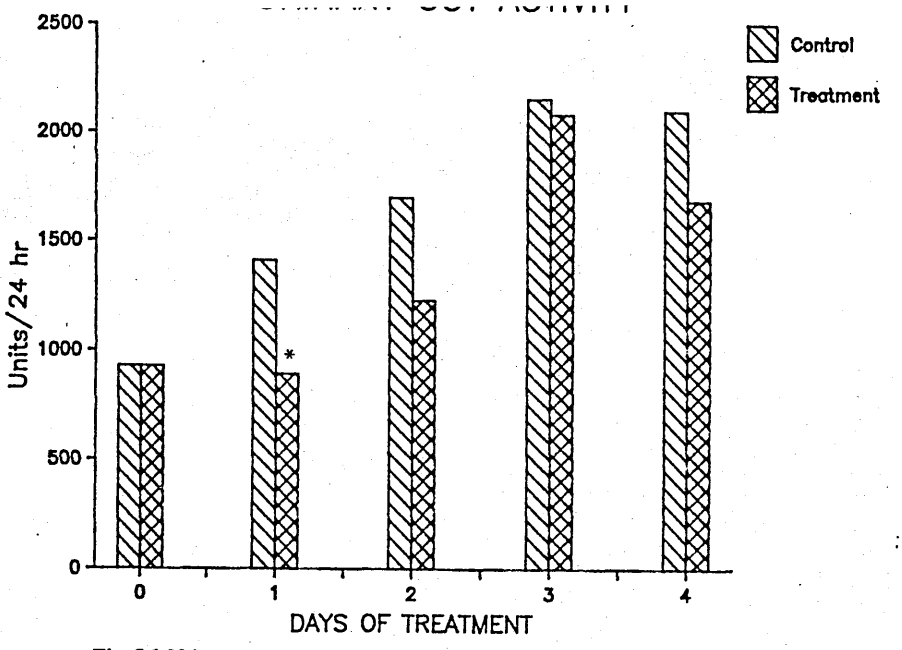


Fig. 5.1.22A

Mean level of gamma-glutamyl transpeptidase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (5 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 for the treated group. Significant decrease ($P \leq 0.05$) was seen on day 1 (*).

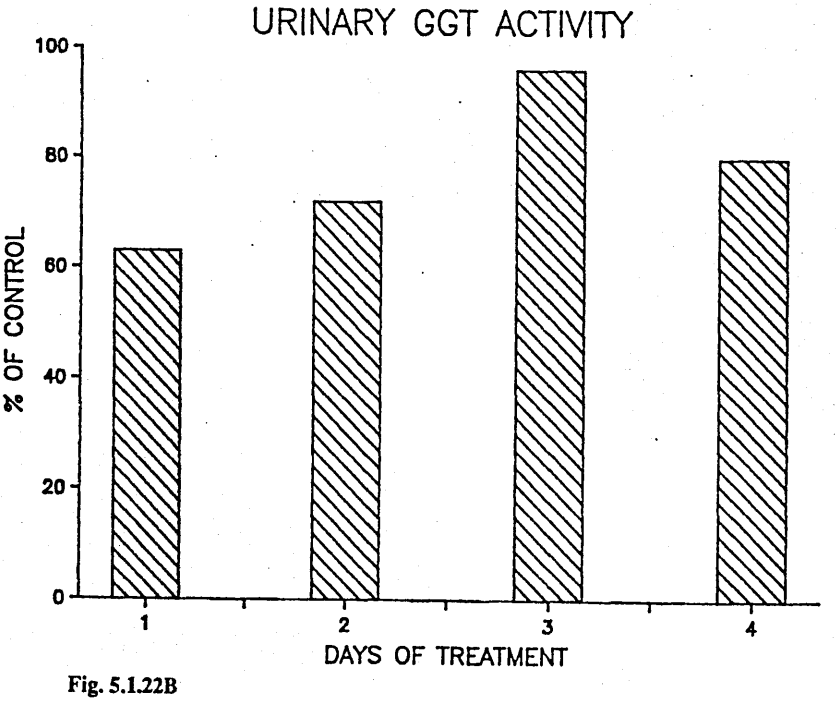


Fig. 5.1.22B

Urinary gamma-glutamyl transpeptidase excretion calculated as a percentage of the control group.

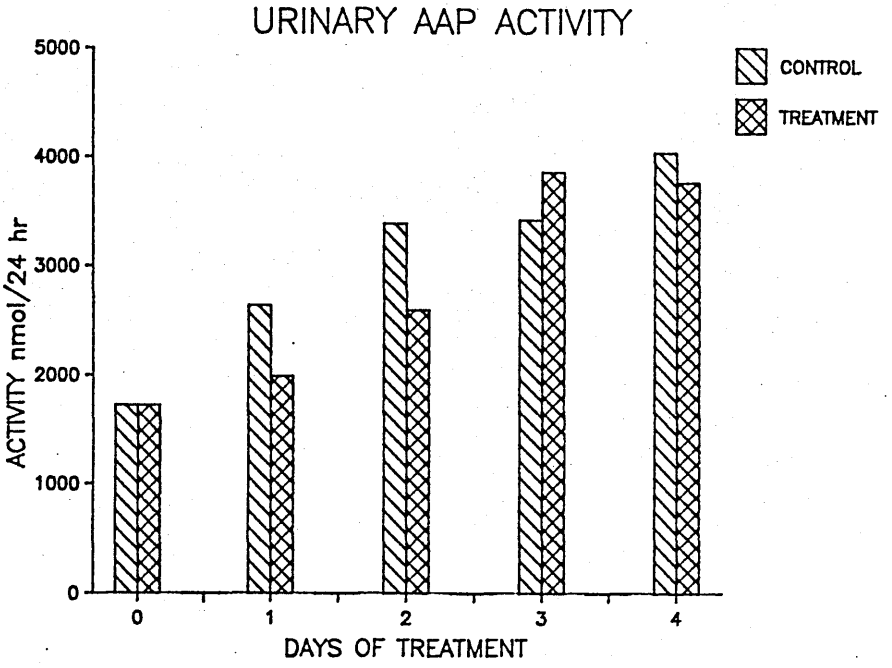


Fig. 5.1.23A

Mean level of alanine amino peptidase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (5 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 for the treated group.

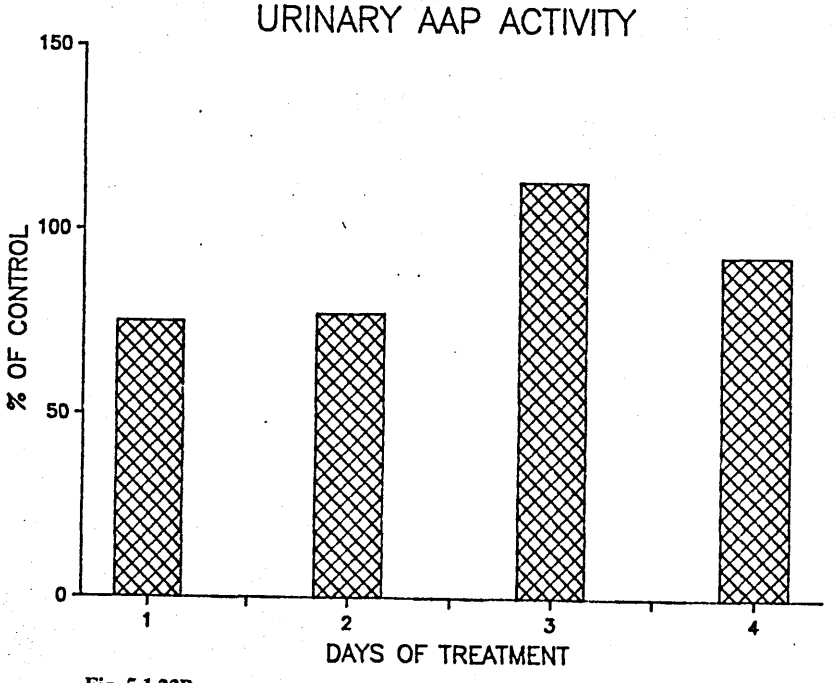


Fig. 5.1.23B

Urinary alanine amino peptidase excretion calculated as a percentage of the control group.

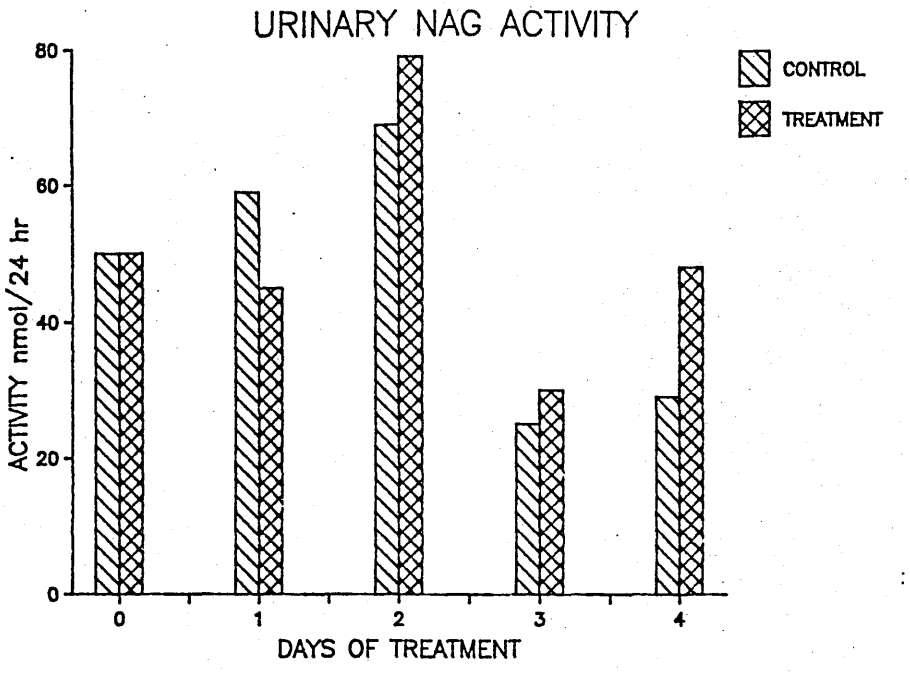


Fig. 5.1.24A

Mean level of N-acetyl-beta-glucosaminidase in 24-h urine before (day 0) and after combined administration of ochratoxin A and aflatoxin B_1 (5 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 for the treated group.

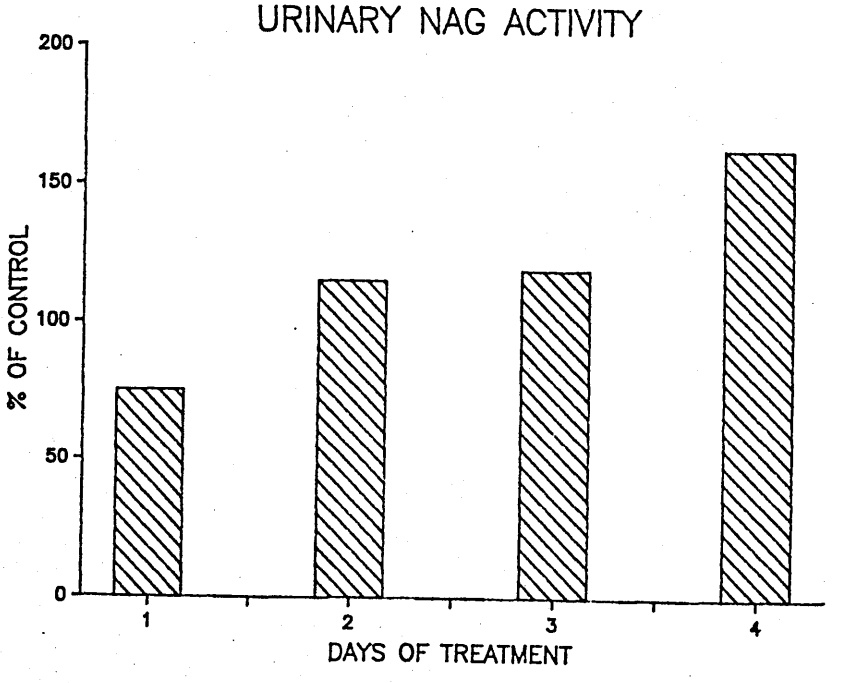


Fig. 5.1.24B

Urinary N-acetyl-beta-glucosaminidase excretion calculated as a percentage of the control group.

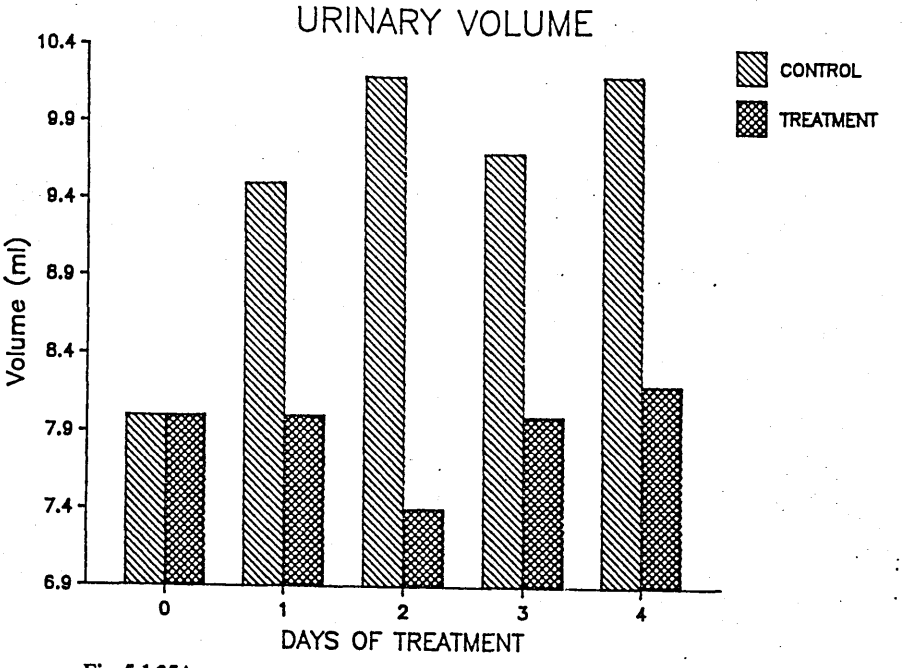


Fig. 5.1.25A

Mean volume in 24-hr urine before (Day 0) and after a single ip dose of (ochratoxin A and aflatoxin B_1 (5 mg and 100 ug/kg respectively), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=4 in the control group and n=5 in the treated group.

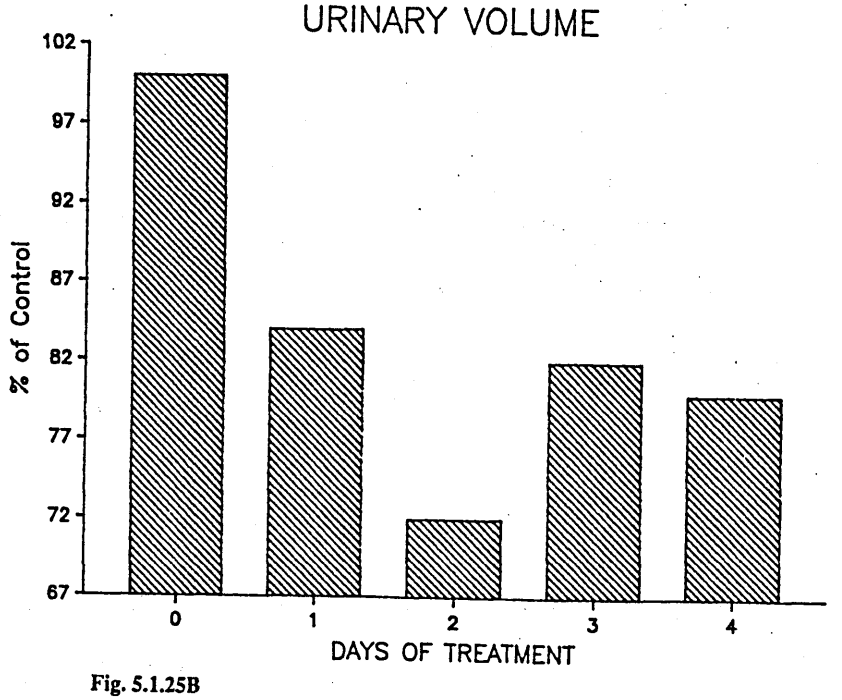


Fig. 5.1.25B

Urinary volume calculated as a percentage of the control group.

throughout the experiment, however there were no significant differences with the control group.

Discussion. These data show some changes on the excretion of the urinary enzymes tested. Among the enzymes tested, ALP seemed to be the most sensitive urinary enzyme marker, the activity of which started to increase from day 2 and and remained higher than the control group throughout the experiment.

The pattern of interaction for ALP, AAP, GGT and NAG is different and not consistent with the one seen when either ATB_1 or OTA were administered alone. Thus it can be concluded that there is an interactive effect caused by the simultaneous administration of the two mycotoxins. This cannot be classified as either additive or synergistic on the basis of excretion of those urinary enzyme markers tested after the combined administration of 1 mg OTA/kg and 100 ug ATB_1/kg to the rats.

The data on the effect of the combined administration of 5 mg OTA/kg and 100 ug ATB_1 show that ALP excretion was again the highest of the enzymes assessed. AAP and GGT activities showed lower values than the control at most time points tested, and ALP and NAG also decreased on day 1. ALP pattern of interaction was consistent with the one seen with the 5 mg OTA/kg alone, where the activity started to rise on day 2, reached a peak on day 3, which was higher in the combined administration and although started to decrease, remained high on day 4. However, once again it can not be said that a synergistic or additive effect took place, only that there was an interaction. The ALP pattern of interaction following both mycotoxins was different from the ATB_1 and OTA treatment. AAP, GGT and NAG pattern of interaction were also different from those obtained after

administration of either 5 mg OTA/mg or ATB_1 alone.

In general, it is difficult to draw final conclusions from the single doses studies. There were some difficulties, under the experimental conditions of this study, such as recovering the entire 24-hr urine sample, and avoiding contamination with faeces or food; although the metabolic cages were designed for separate collection of faeces and urine, sometimes some urine went into the faeces collection flask or some faeces went to contaminate urine. In addition, there were considerable animal-to-animal variations in the levels of enzyme excretion, and urinary volume in both control and treated group, the day-to-day values for the control groups showed also considerable variations. The urinary enzyme levels were adjusted to the urinary volume in 24-hr specimens, to have the total enzymatic activity per 24 hr; however the problem of wide variations in this parameter still persisted.

Therefore it can be concluded that for the study of the effect of low single ip doses of OTA and ATB_1 alone or after simultaneous administration, on renal function, the study of enzymes such as ALP, AAP, GGT and NAG, over 24-hr period is not a suitable sensitive test, and does not give a dose related response. It is possible that the measurement of urinary enzymes under the same regime give better results if performed at earlier time points than those performed in the present study. Other parameters of renal function should be assessed in parallel to help define the nature of the interaction between the two mycotoxins.

In order to decrease one factor that contributed to the variability of data that may be introduced by the contamination of urine samples with food, it was decided to collect 18-hr urine along with food restriction

for the same period of time. This was applied to the repetitive dose study detailed below.

5.1.2 Repetitive doses of OTA.

Urinary enzyme xcretion and other parameters of renal function where determined, over daily 18 hr periods following ip administration of 2.5 mg OTA/kg/day, on 3 consecutive days. From a dose-response histopathological assessment of a range of concentrations, this was the dose regime that showed the minimal morphological changes, and under which nephrotoxicity was exacerbated by the simultaneous administration of ATB_1. Histological findings on kidneys removed 24 hr after the last dose, and two weeks after the first dose, is described in section 5.2.

5.1.2.1 Enzymuria

ALP activity started to increase on day 3, and showed the highest elevations on day 6 and 7 (1616 and 1752% of the control), which due to the high variance, were not statistically significant (Table 5.1.26, Appendix 1; Fig. 5.1.26A). The activity elevated to 337% on day 8, and was highly significantly different ($P \leq 0.01$) from the controls (Fig. 5.1.26B). GGT activity started to increase from day 2 onwards, showing a significantly elevated difference ($P \leq 0.05$) on days 4 and 5 (Table 5.1.27, Appendix 1; Fig. 5.1.27A) to 184 and 196% of the control group, respectively (Fig. 5.1.27B). The enzyme activity remained elevated until day 8 and then started to decrease, although not significantly until day 11. From day 12 until the end of the experiment, the enzyme activity values remained very close to those in the controls. AAP activity started to rise from day 2 onwards, and showed a significant increase ($P \leq 0.05$) on days 4, 5, 6 and 8 (Table 5.1.28, Appendix 1; Fig. 5.1.28A),

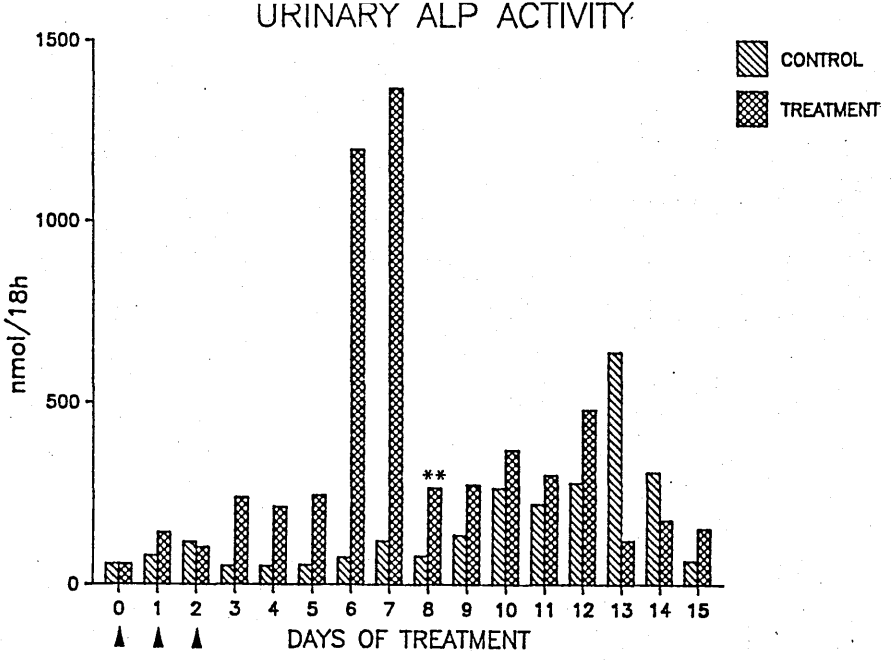


Fig. 5.1.26A

Mean level of alkaline phosphatase in 18-hr urine before (day 0) during (arrows) and after 3 consecutive daily ip doses of ochratoxin A (2.5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=5. Significant increase ($P \leq 0.01$) was seen on day 8 (**).

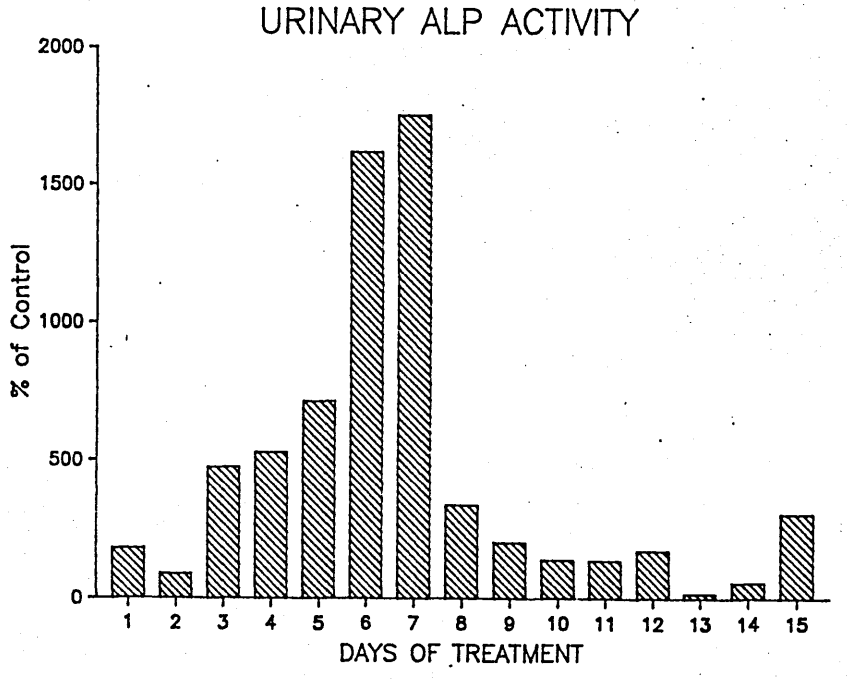


Fig. 5.1.26B

Urinary alkaline phosphatase excretion calculated as a percentage of the control group.

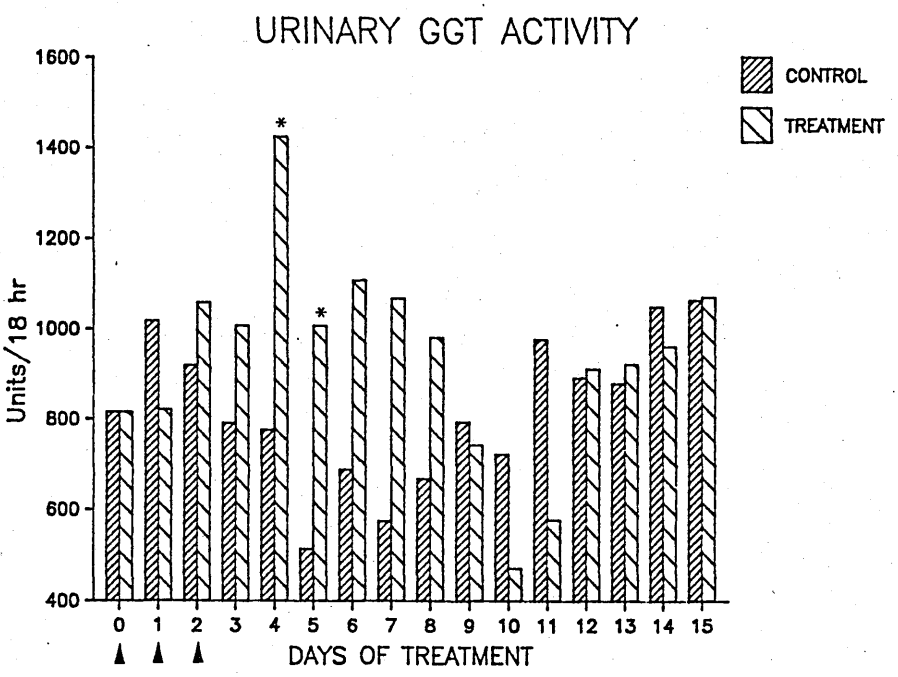


Fig. 5.1.27A

Mean level of gamma-glutamyl transpeptidase in 18-hr urine before (day 0) during (arrows) and after 3 consecutive daily ip doses of ochratoxin A (2.5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=5. Significant increases ($P \leq 0.05$) were seen on days 4 and 5 (*).

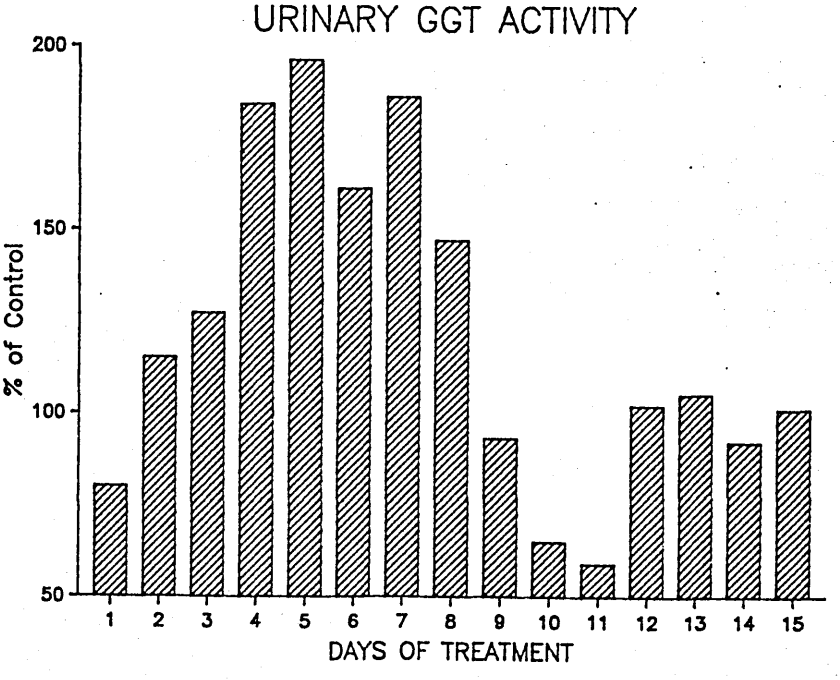


Fig. 5.1.27B

Urinary gamma-glutamyl transpeptidase excretion calculated as a percentage of the control group.

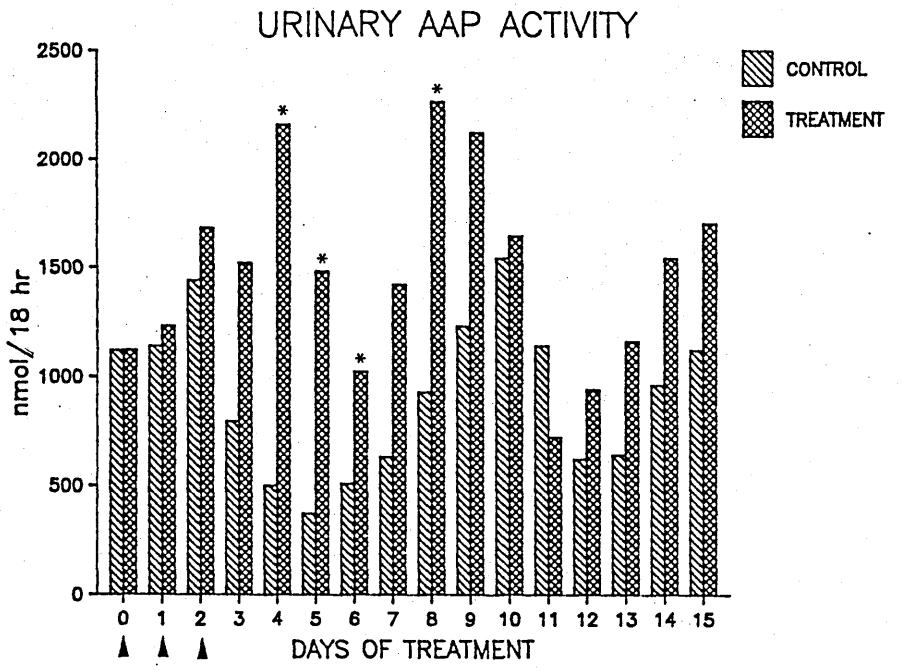


Fig. 5.1.28A

Mean level of alanine aminopeptidase in 18-hr urine before (day 0) during (arrows) and after 3 consecutive daily ip doses of ochratoxin A (2.5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=5. Significant increase ($P \leq 0.05$) were seen on days 4, 5, 6 and 8 (*).

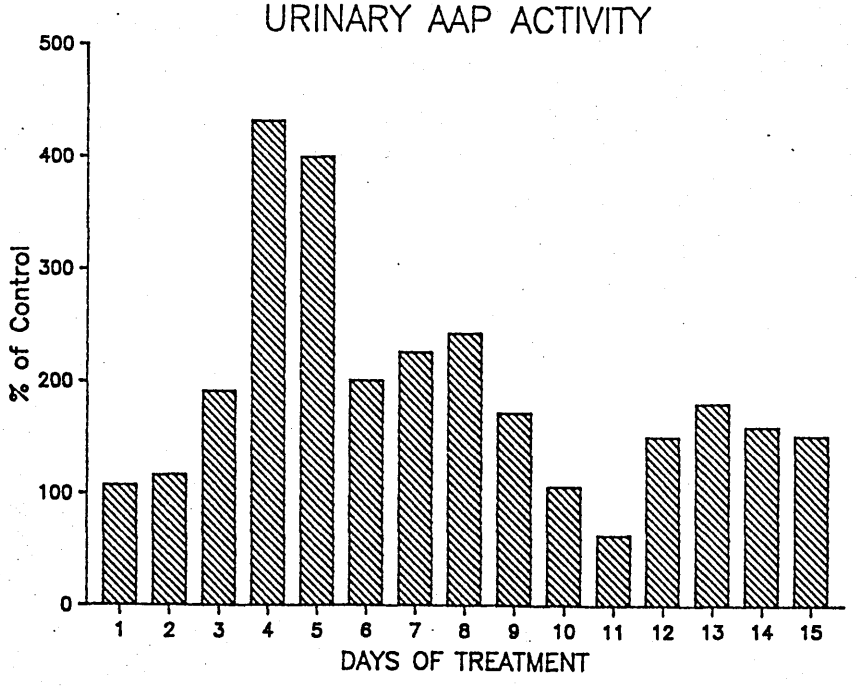


Fig. 5.1.28B

Urinary alanine aminopeptidase excretion calculated as a percentage of the control group.

corresponding to 432, 400, 201 and 243% respectively of the control group (Fig. 5.1.28B). With the exception of days 10 and 11, the enzymatic activity remained elevated through the rest of the experiment. The assessment of NAG activity was not possible, due to technical difficulties of unknown origin, where the blanks had higher values than the actual urine samples.

Discussion. These data show that all 3 enzymes assessed, increased considerable from the control value, during the course of the experiment. Although AAP and GGT activities started to increase earlier than ALP, this enzyme maximum value was 15 fold the control, reached on day 5, in contrast to AAP and GGT maximum increases (4- and 2-fold respectively), on days 4 and 5. Therefore, ALP seemed to be the most sensitive enzyme in terms of amount released, (as was the case for single dose studies) and AAP showed its maximum release at an earlier time than the two other enzymes. This means that low repetitive doses of OTA (2.5 mg/kg) caused major elevations of urinary enzymes such as ALP, AAP and GGT, which may be reflecting early and minimal injury to the proximal tubule.

Although there are no reports in the literature, on the effect of small repetitive doses of OTA, such as those used in this experiment, on urinary enzymes, these findings are consistent with reports on elevation of these parameters, after administration of five consecutive daily sc doses of 10 mg/kg (Ngaha, 1985), in which ALP became elevated and remained so throughout the experiment. In spite of the length of the treatment and large doses of the toxin used by Ngaha (1985) the maximun increase of the enzyme activity was much lower than that in this study (6-fold vs. 15-fold). ALP and GGT activity has also been reported to increase after subchronic oral administration of small doses of OTA to rats (Kane et al,

1986a). It is difficult to establish comparison between different treatments, unless it is done on very general bases, since there is evidence to show that the pattern of urinary excretion is not only dependent on the type of toxin, but also on the manner in which it is administered (Cottrell et al, 1976). Also probably the sex and strain of test animals, other dietary factors and technical considerations such as food and fæcal contamination may be source of differences between treatments.

Amongst the enzymes tested in this study, ALP appeared to be the most sensitive indicator of proximal tubular injury caused by small repeated doses of OTA, although GGT and AAP showed a lower effect, they also reflected proximal tubular injury.

The measurement of urinary enzyme activity appeared to be a sensitive means of monitoring nephrotoxicity caused by small repetitive doses of OTA, but the considerable variability seen restricts it usefulness to massive effects. The variance of the enzymatic urinary data did seem to have improved under the 18-hr food restricted urine collection. This probably helped avoid factors that increased variability such as those introduced by the contamination of the samples with food. However, the problem of the wide variations remained, not only for enzyme activity, but for the other parameters measured (see below). Lower values for the treated group, than for controls were also seen for ALP and GGT activities towards the end of the experiment. The specific enzymatic activity was adjusted for variation in urine flow, but this parameter was also very variable, it is possible that this can be the real cause for lower enzyme activities found in the treated groups. When adjustment for other factors

was done (ie. creatinine), there was no improvement in the variability (data not shown).

5.1.2.2. Other urinary parameters.

a. Glucose. After presenting values similar to the control group, urinary glucose in the treated group, increased on day 5, which became significantly different ($P \leq 0.05$) on day 6 when it reached a peak and also on day 7 (Table 5.1.29, Appendix 1; Fig. 5.1.29A), accounting for 8789 and 5932% of the control (Fig. 5.1.29B). On day 10, urinary glucose decreased significantly ($P \leq 0.01$) to 61% of the control, and on days 11, 12, and 13 the values remained very close to those for the controls to get a further highly significant ($P \leq 0.01$) decrease on day 14 to 40% of the controls value.

Discussion. These data show that the excretion of glucose was strongly affected, having started to rise on day 5, reaching a maximum value on day 6, which was 90-fold the control values. This means that the administered dose of OTA, was able to impair considerably the mechanism of reabsorption of the proximal tubule. This is in agreement with previous reports by Krogh et al (1976) and Berndt and Hayes (1979), who found increased glucose excretion in pigs and rats treated with OTA.

b. Urinary protein. This parameter was elevated from day 1 through to day 7, showing significant differences from the controls ($P \leq 0.05$) on days 2 and 4 (Table 5.1.30, Appendix 1; Fig. 5.1.30A) up to 200 and 537% (Fig. 5.1.30B). From day 8 onwards, the urinary protein was decreased, but not significantly, until the end of the experiment.

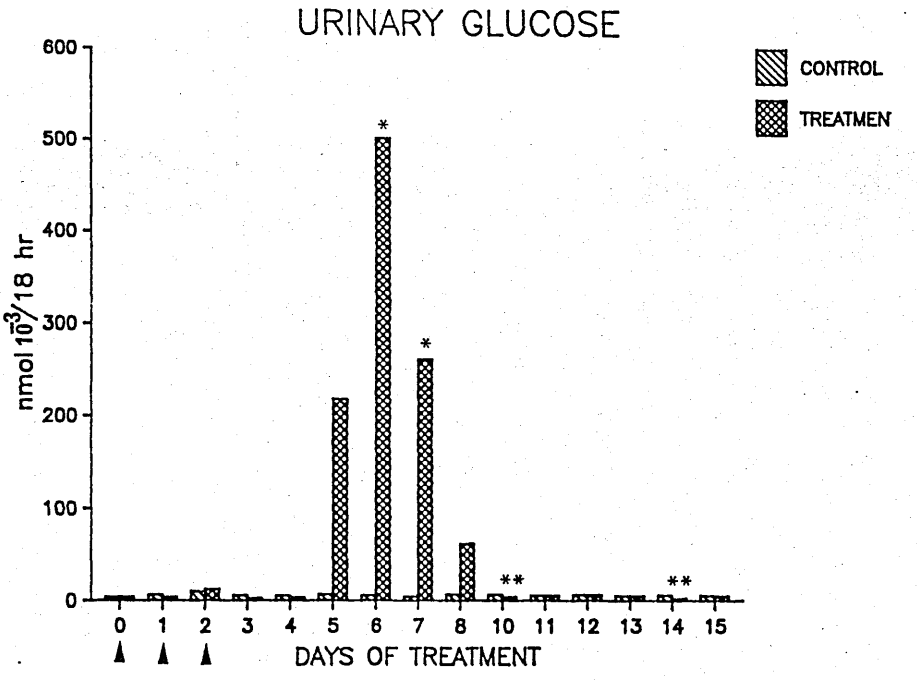


Fig. 5.1.29A

Mean level of glucose in 18-hr urine before (day 0) during (arrows) and after 3 consecutive daily ip doses of ochratoxin A (2.5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=5. Significant increases ($P \leq 0.05$) were seen on days 6 and 7 (*) and significant decreases ($P \leq 0.01$) on days 10 and 14. (**)

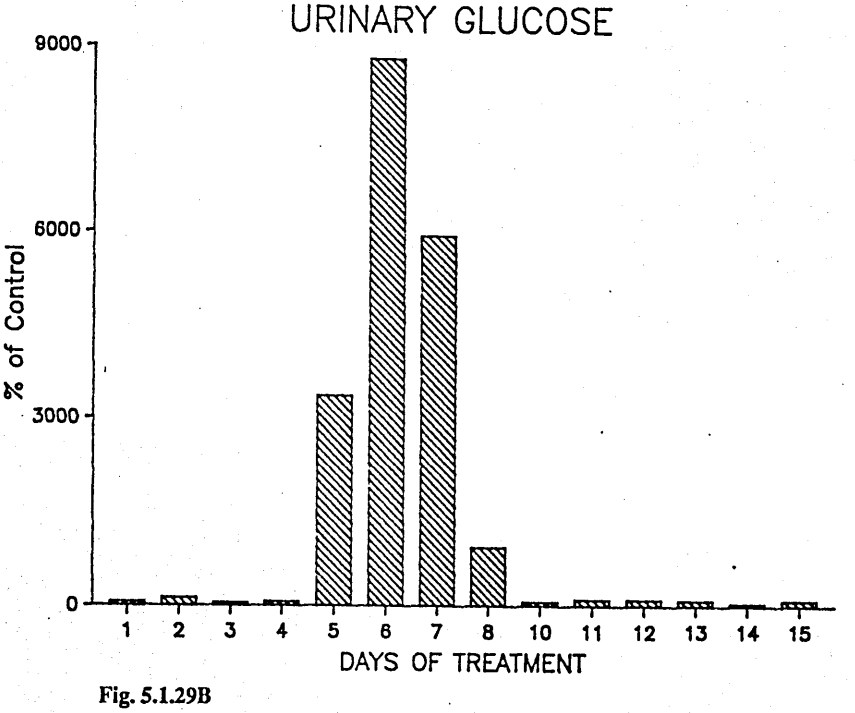


Fig. 5.1.29B

Urinary glucose excretion calculated as a percentage of the control group.

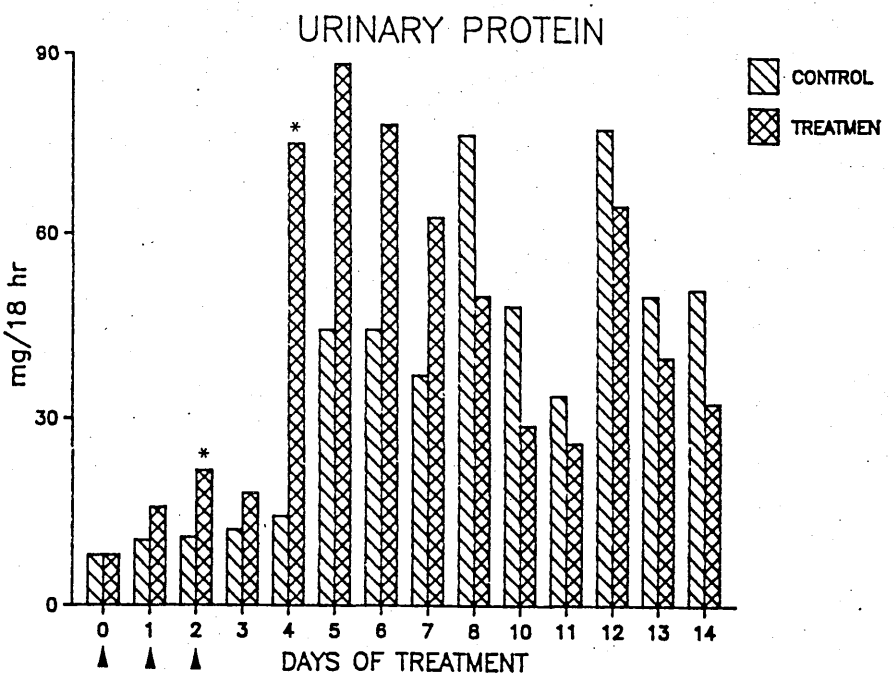


Fig. 5.1.30A

Mean level of protein in 18-hr urine before (day 0) during (arrows) and after 3 consecutive daily ip doses of ochratoxin A (2.5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=5. Significant increases ($P \leq 0.05$) were seen on days 2 and 4 (*).

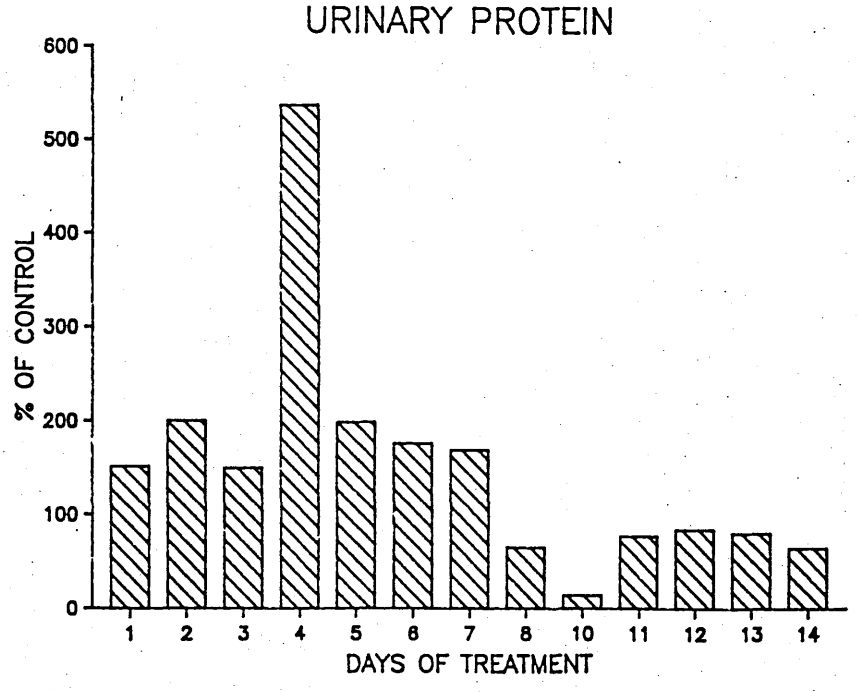


Fig. 5.1.30B

Urinary protein excretion calculated as a percentage of the control group.

Discussion. These data show that excretion of protein was increased with the administration of OTA. It started to increase on day 2 and reached a maximum value on day 8 which was 7-fold the control value.

According to several studies, an increase of the total urinary protein excretion is a sensitive indicator of renal damage induced by some toxic agents (Balazs et al, 1963, Nomiyama et al, 1973; Sharrat and Frazer, 1963), however, this parameter on its own fails to provide information concerning the site and nature of the lesion.

The findings on proteinuria means either that protein macromolecules have been allowed to cross the glomerular barrier and therefore appeared excreted in urine, which would suggest glomerular involvement or that small molecules of protein were not reabsorbed by the tubules, therefore appearing excreted in urine, indicating tubular damage. Both renal changes may also have occurred simultaneously. In addition, enzymuria can also contribute to the increase in total urinary protein.

Fractionation of urinary protein on the basis of molecular size would allow us to determine the origin of proteinuria (Balant and Fabre, 1979). Proteinuria of glomerular origin differs from tubular proteinuria by a larger albumin to low molecular weight protein ratio in the urine.

These findings are in agreement with the proteinuria reported previously in rats and pigs (Berndt and Hayes, 1979; Szczech et al., 1973a). Krogh, et al. (1974) found increased amounts of albumin in the urine of pigs fed OTA for three months, which suggests glomerular involvement. Histopathological studies performed on different species treated with OTA have shown glomerular changes (Albassam et al, 1987; Dwivedi and Burns,

1984; Krogh et al, 1974; 1976) and these have become a focus of several major studies in this project detailed below (Section 5.2 and Chapter 6).

c. Urinary volume. This parameter started to increase from day 1 and remained higher for the treated group than for the control throughout the experiment. On days 5 - 8 the elevated levels were significantly different ($P \leq 0.05$) from the control values up to 216, 222, 273 and 276% (Table 5.1.31, Appendix 1; Fig. 5.1.31A and B).

Discussion. These data show that reabsorption of water is decreased by OTA, causing an increase in the urinary volume, which started after the first dose of the toxin, reaching a peak value of 3 fold the control, on day 8. This also indicates impairment of the reabsorptive properties of the proximal tubules, caused by administration of OTA. This is in agreement with findings by Berndt and Hayes, 1979, who reported a slight increase in the urinary volume, and consistent with the finding of polyuria by the chronic and acute administration of OTA to pigs (Krogh et al, 1974; Szczech et al, 1973a), but not with Suzuki et al. (1975b) who found decreased urine volume in rats treated with 3 daily doses of 5 mg OTA/kg. Increased urine volume may have a number of causes ranging from RPN to acute tubular necrosis and are therefore this parameter is of little direct mechanistic value.

Although in these experiments the histopathological changes were minimal at the two time points measured, renal function was considerably disrupted, following the administration of three consecutive daily doses of 2.5 mg OTA/kg. The most sensitive parameters were glucose and ALP excretion.

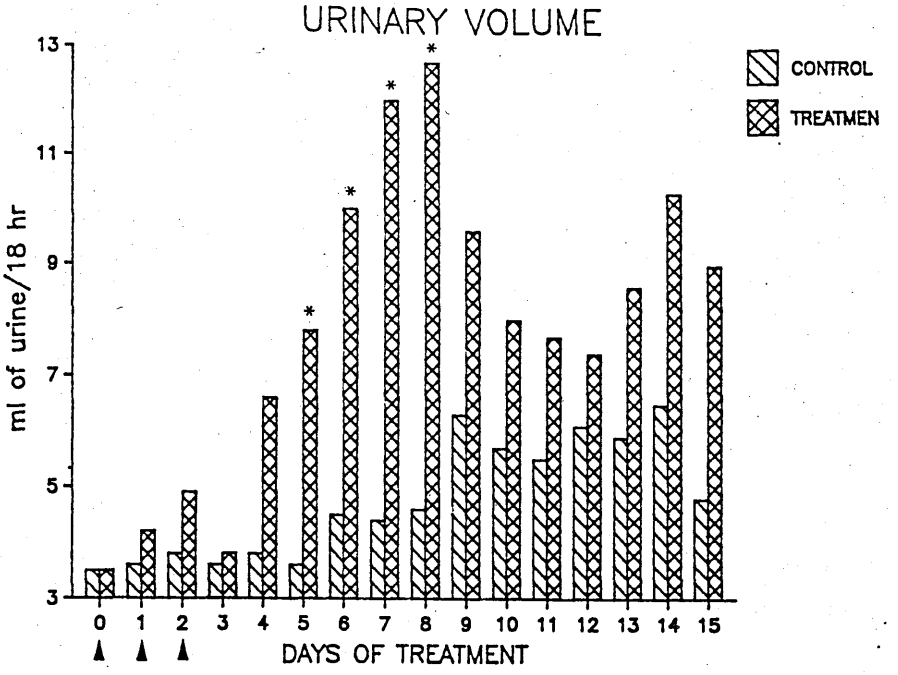


Fig. 5.1.31A

Mean volume in 18-hr urine before (day 0) during (arrows) and after 3 consecutive daily ip doses of ochratoxin A (2.5 mg/kg), in comparison to a group injected with the vehicle only (control). The height of each bar is the mean value for n=5. Significant increases ($P \leq 0.05$) were seen on days 5 to 8 (*).

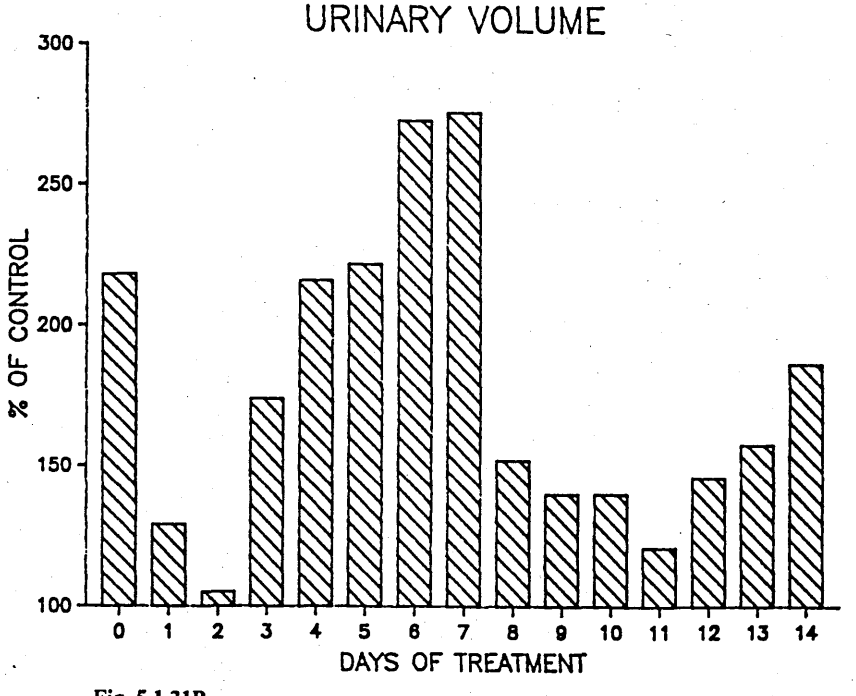


Fig. 5.1.31B

Urinary volume calculated as a percentage of the control group.

5.2. Histopathological assessment

5.2.1 Single doses of OTA alone or in combination with ATB_1

There were no detectable changes on the kidneys assessed histopathologically by H&E and PAS staining at the end of all experiments in which single doses of OTA and ATB_1 either alone or in combination were administered to the rats.

The fact that single doses of the different concentrations of OTA tested (1 and 5 mg/kg), did not produce histological changes, is in agreement with reports that low, single, nonlethal doses of the toxin do not cause morphological changes (Berndt and Hayes, 1979), and that only single lethal doses caused mild proximal tubular alterations (Kanisawa et al, 1977) or severe tubular necrosis (Purchase and Theron, 1968). With regard to ATB_1, which did not cause renal histological alterations, at the concentration tested, this is also in agreement with reports where only large doses of the toxin caused non-specific renal changes in ducklings (Asplin and Carnagham, 1961), and only repeated or chronic administration of ATB_1 was able to induce renal morphological changes. Combined doses of OTA and ATB_1 did not produce microscopic renal changes, suggesting also the necessity for accumulation of the toxins. However another factor may have contributed to the lack of sensitivity of the histopathological assessment, in which the tissues were embedded in wax with subsequent H&E and PAS staining only. It is possible that the use of semithin resin embedded sections, and other more sensitive techniques, such as enzyme and immunohistochemistry will allow the detection of renal changes caused by low single doses of the mycotoxins assessed.

5.2.2 Repeated doses of OTA.

Figs. 5.2.1A, 5.2.1B and 5.2.1C show outer and deeper cortex and medulla respectively, from control rat kidneys.

The kidneys of animals dosed with 7.5 mg/kg for 3 days, showed microscopic changes such as patchy tubular damage (Fig. 5.2.2A), consisting of disruption of the proximal tubular brush border, shrunken nuclei, large vacuoles and cytoplasmic extrusion. Consequently there was a large amount of cellular debris within the proximal tubular lumen. There was dilatation of the loops of Henle and distal tubules with PAS positive staining casts in the lumen. There was proliferation of the interstitial cells in the corticomedullary junction. In the superficial cortex, glomeruli looked abnormal, with no Bowman's space and very wide interstitial spaces; partial glomerular sclerosis and thickening of basement membrane were also observed (Fig. 5.2.2B).

The kidneys of animals administered 5 mg OTA/kg for two days and sacrificed 24 hr after the last dose, showed patchy proximal tubular disruption of brush border, vacuolation of cytoplasm (Fig 5.2.3), occasional extruded nuclei in lumen, and an occasional mitotic figure present.

The kidneys of animals dosed with 5 mg OTA/kg for 3 days showed patchy proximal tubular damage in the superficial cortex, consisting of cytoplasmic vacuolation, blebbing and extrusion with detachment of cells into the lumen. There was also interstitial cell proliferation around distal tubules and collecting ducts (Fig. 5.2.4). There were very large, and rounded nuclei in dilated distal tubules. There were some basophilic areas around the distal tubules. The glomeruli showed prominent nuclei.

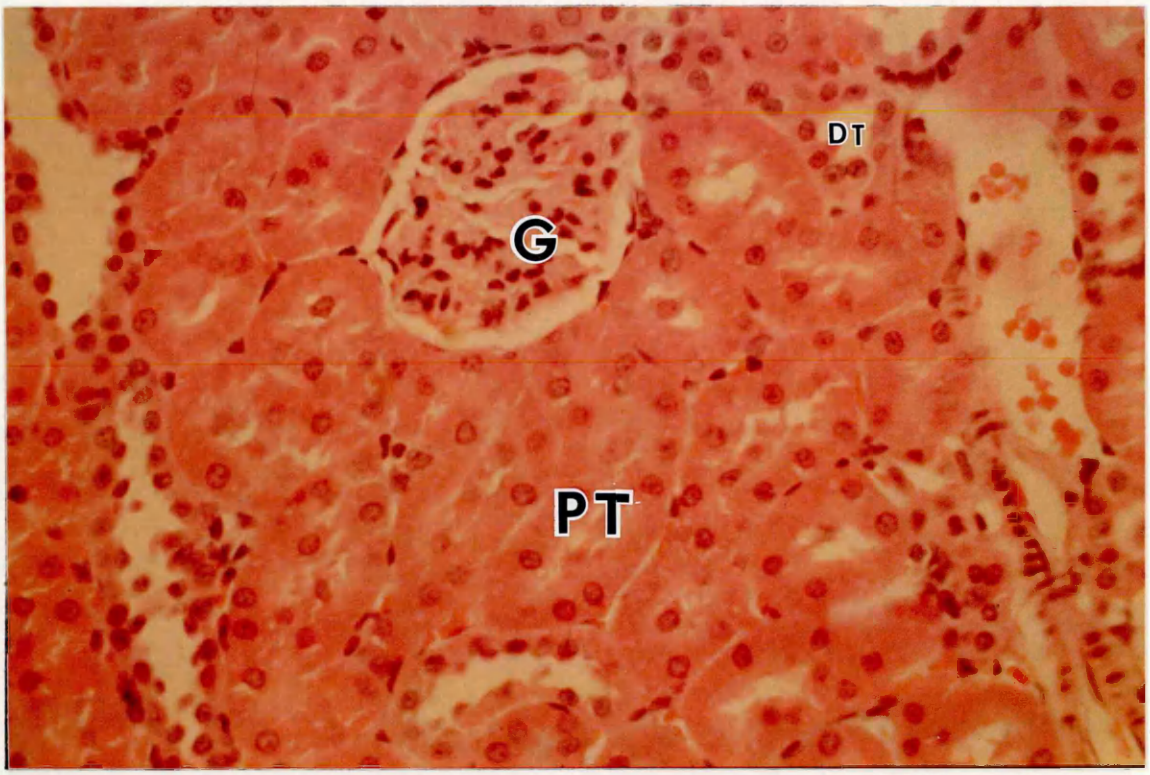


Fig. 5.2.1A
Section of control rat kidney outer cortex showing typical appearance of proximal tubules (PT), glomerulus (G) and distal tubules (DT). H&E (x172).

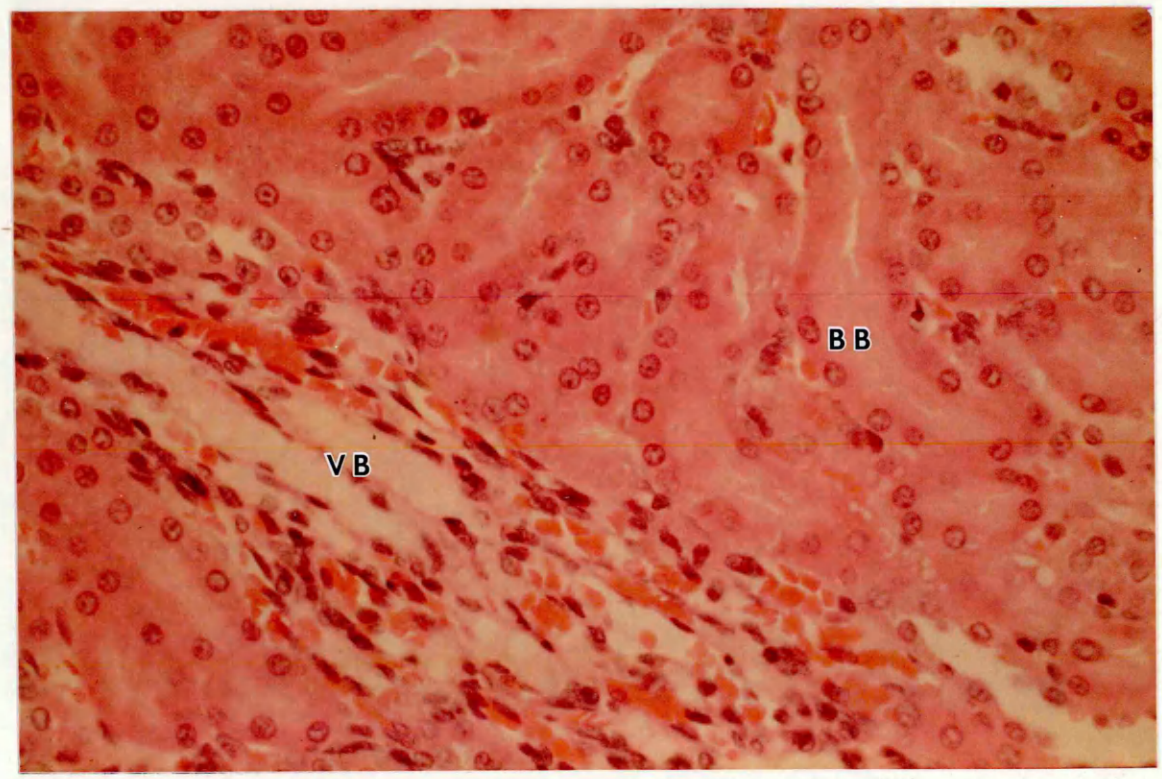


Fig. 5.2.1B
Section of control rat kidney deeper cortex showing proximal tubules, S_3 segment with distinct brush borders (BB) and vascular bundle (VB). H&E (x172).

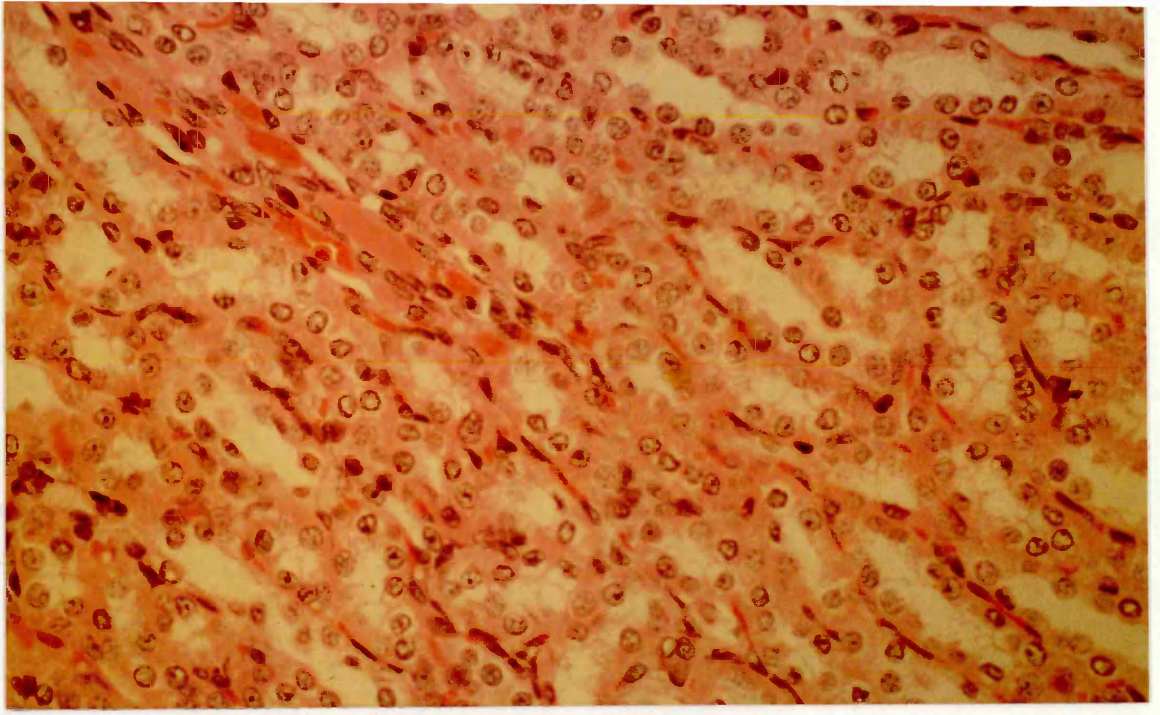


Fig. 5.2.1C
Section of control rat kidney showing typical appearance of medulla. H&E (x172).

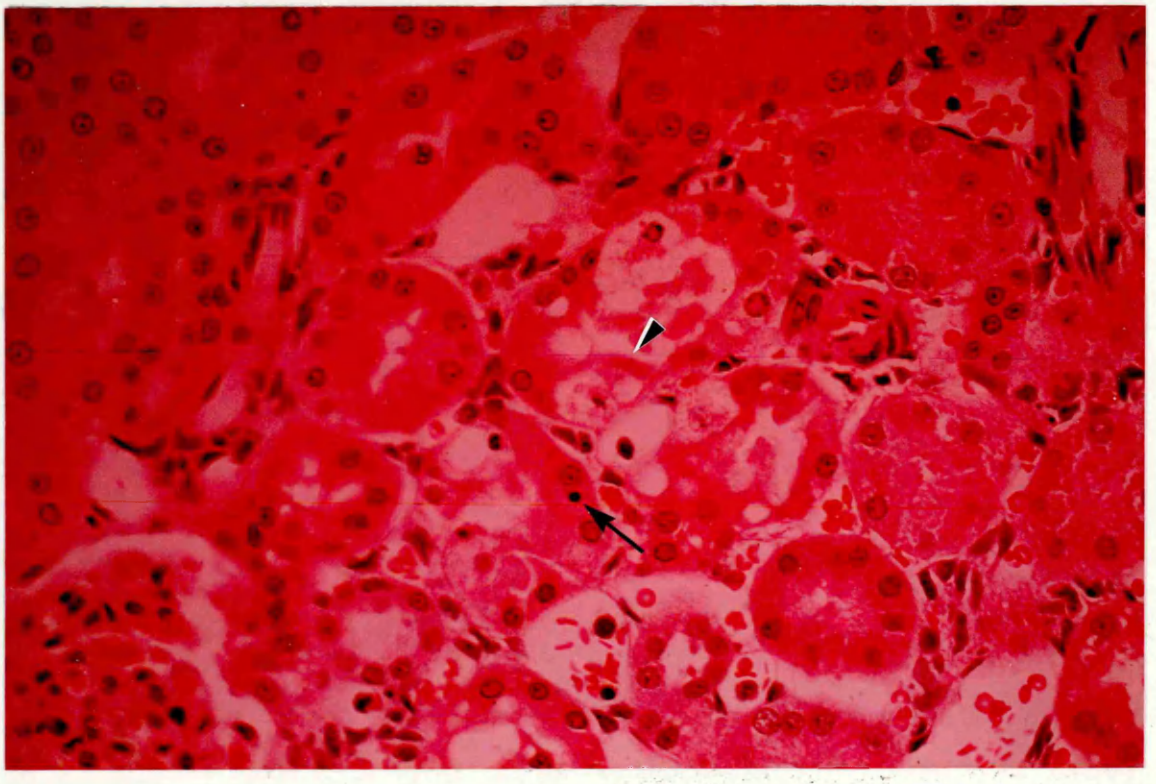


Fig. 5.2.2A
OTA 7.5 mg/kg for 3 days. Section showing proximal tubular necrosis (arrow head), pycnotic nuclei present (arrow). H&E (x172).

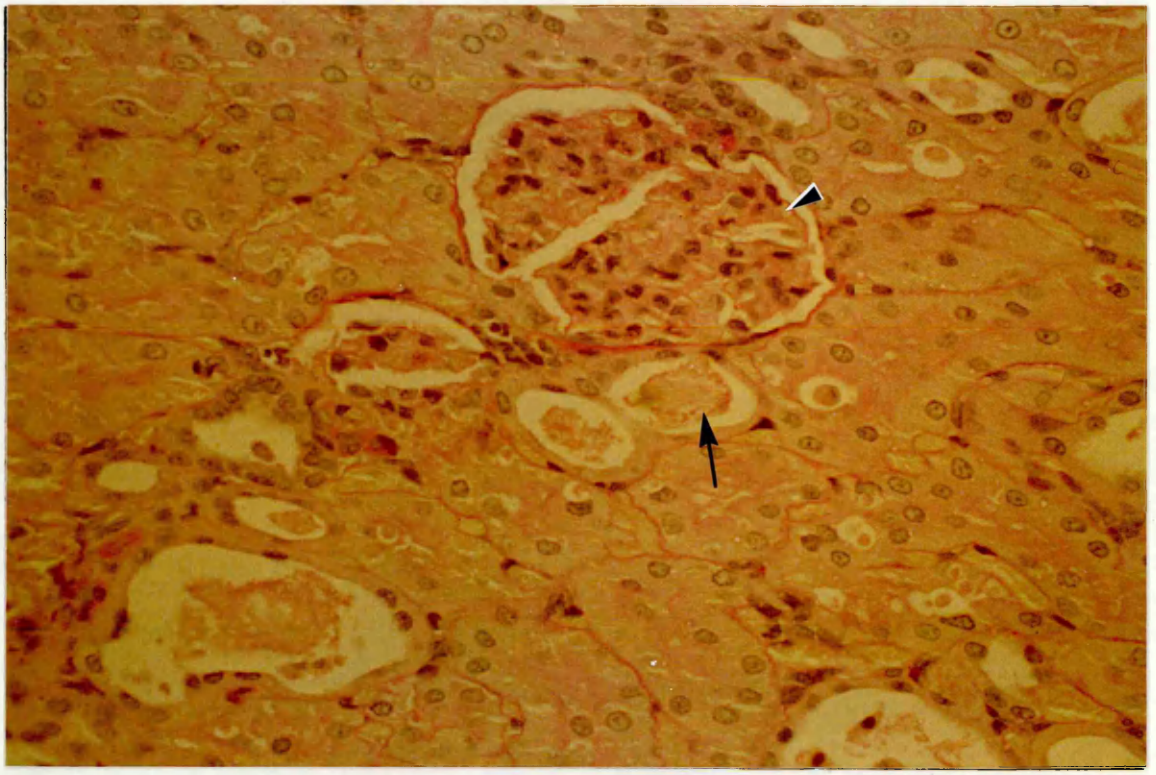


Fig. 5.2.2B
OTA 7.5 mg/kg for 3 days PAS staining casts in dilated distal tubules (arrow), glomerular sclerosis and thickening of basement membrane (arrow head). x107.

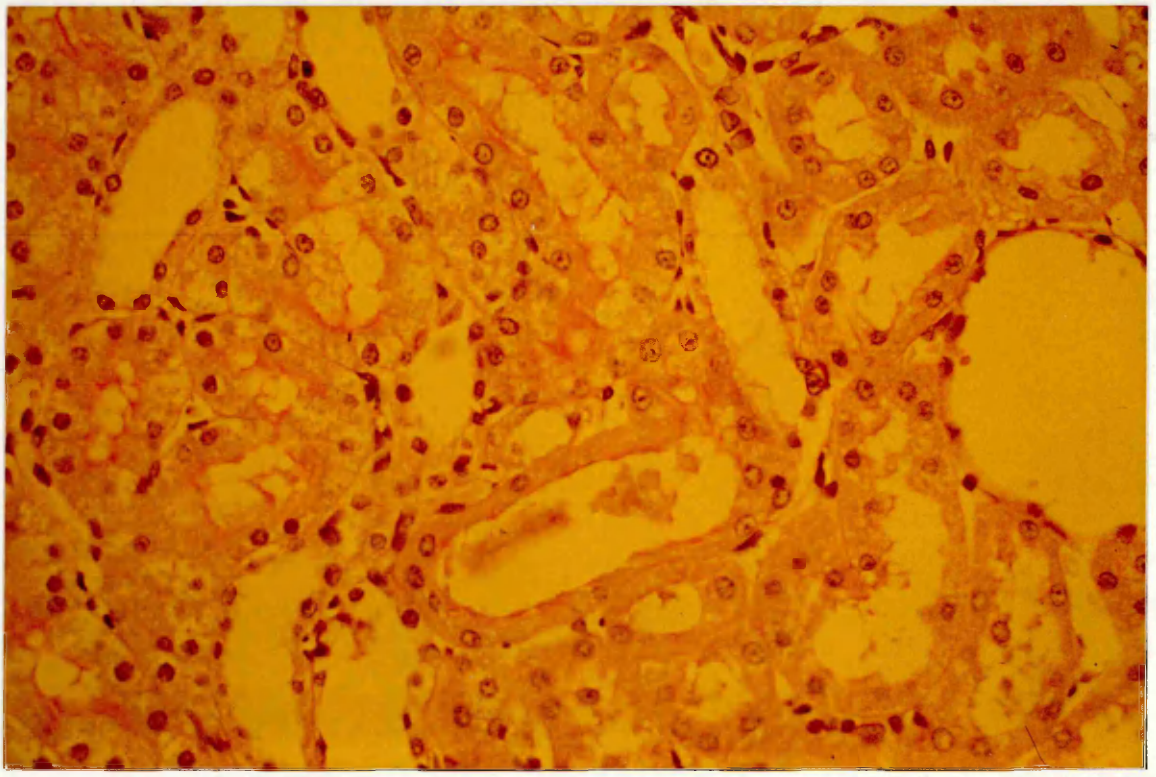


Fig. 5.2.3
OTA 5mg/kg for 2 days. Section showing proximal tubular disruption of brush borders and cytoplasm. PAS (x172).

The kidneys of animals dosed with 2.5 mg OTA/mg for three days, and sacrificed 24 hr after the last dose, showed diffuse changes including some disruption of the proximal tubular brush borders (Fig. 5.2.5A), in addition, interstitial cell proliferation was evident, particularly close to glomeruli and medullary rays in the corticomedullary junction (Fig. 5.2.5B). Some glomeruli looked edematous, with wide interstitial spaces, and in some cases prominent PAS staining basement membrane was observed (Fig. 5.2.5C).

When the animals were sacrificed two weeks after the last dose, the proximal tubules and nuclei showed enlargement (Fig. 5.2.6), and some nuclei were displaced towards the luminal edge of the cell. There was also diffuse interstitial cell proliferation with basophilic nuclei. Some nuclei of distal tubules were also displaced to the centre luminal edge of the cell.

5.2.3 Repeated doses of ATB_1.

The kidneys of animals dosed with 100 ug/kg showed slight microscopic changes, such as diffuse interstitial cell enlargement between proximal tubules. Some proximal tubules showed slight necrotic changes, with nuclei displaced towards the lumen. There was also disruption of the brush border, and a few mitotic figures were present in the proximal tubules (Fig. 5.2.7). In the distal tubules, nuclei were enlarged and rounded and displaced towards the centre of the tubules.

5.2.4 Repeated and combined administration of OTA and ATB_1.

With the aim to study the effect of the interaction between OTA and ATB_1, after simultaneous administration of repeated doses of both compounds, different doses of OTA (5, 2.5 and 0.5 mg/kg), were administered in

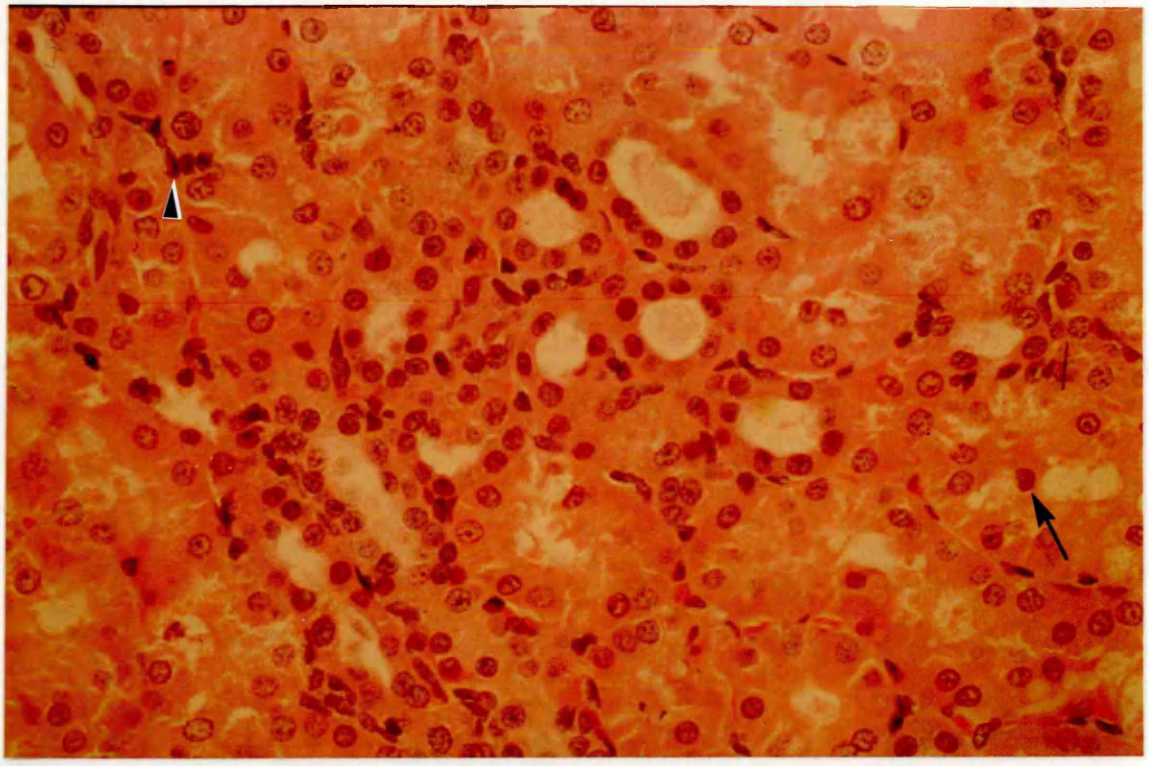


Fig. 5.2.4
OTA 5 mg/kg for 3 days. Proximal tubular cytoplasmic disruption and extrusion of nuclei (arrow). Prominent interstitial cells (arrow head) adjacent to distal tubules and collecting ducts. H&E (x172).

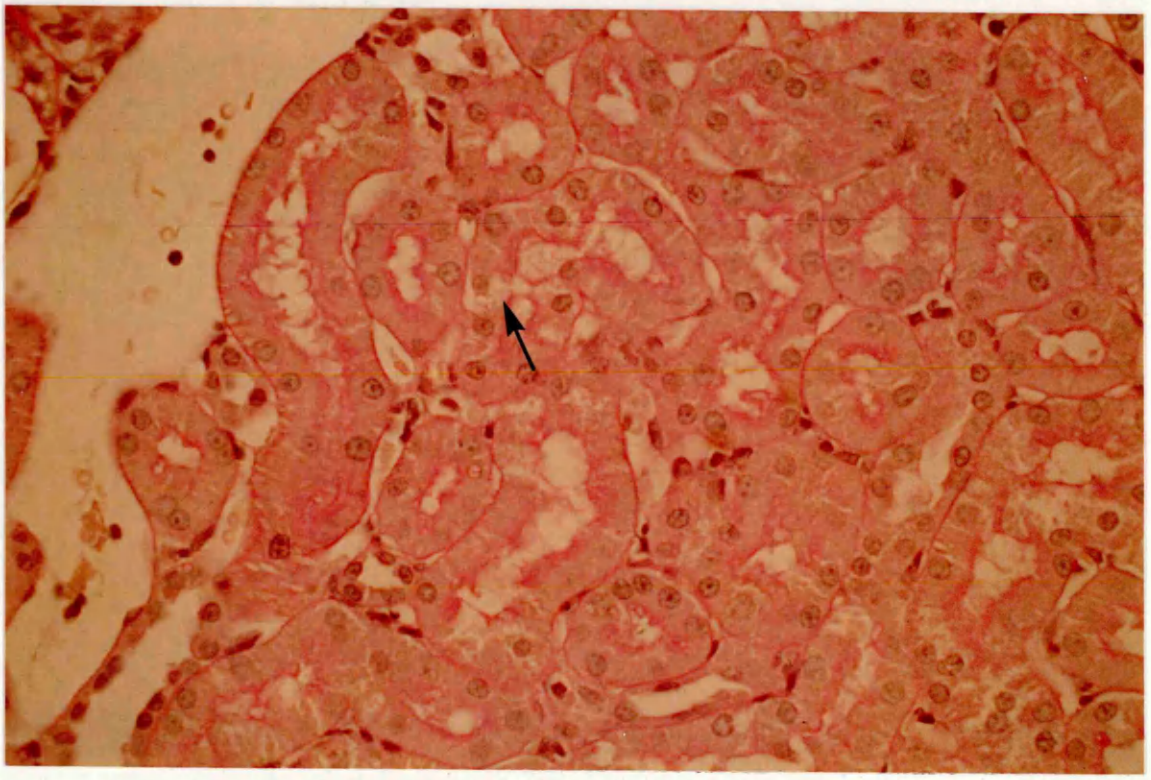


Fig. 5.2.5A
OTA 2.5 mg/kg for 3 days. PAS stained section showing disruption of brush border and vacuolation of cytoplasm (arrow). x172.

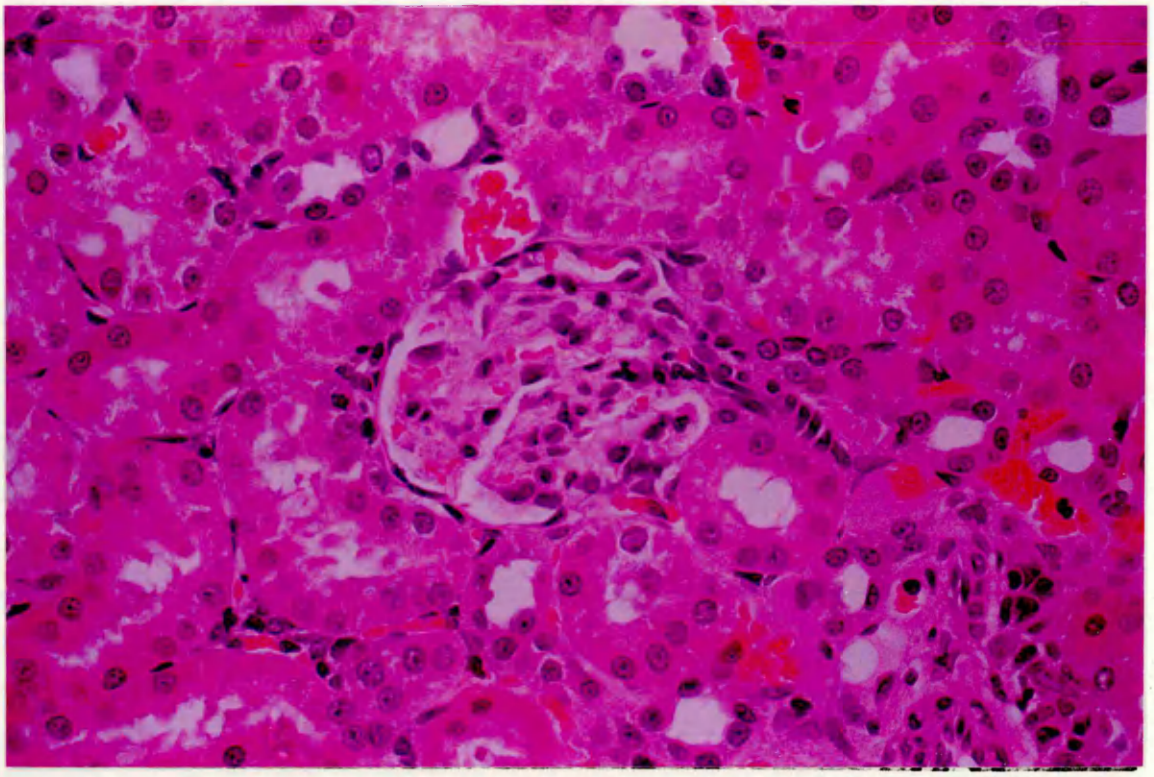


Fig. 5.2.5B
OTA 2.5 mg/kg for 3 days. Section showing interstitial cell proliferation. H&E (x107).

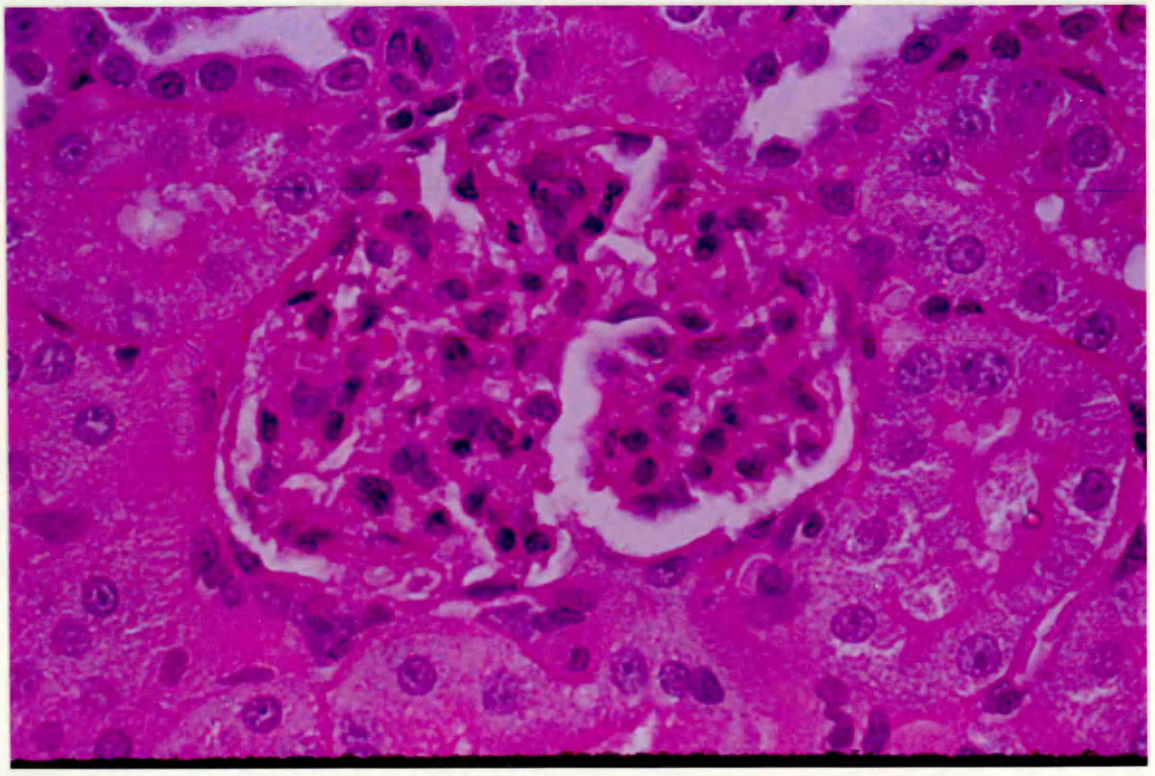


Fig. 5.2.5C
OTA 2.5 mg/kg for 3 days. Edematous glomeruli with prominent PAS staining basement membrane (x172).

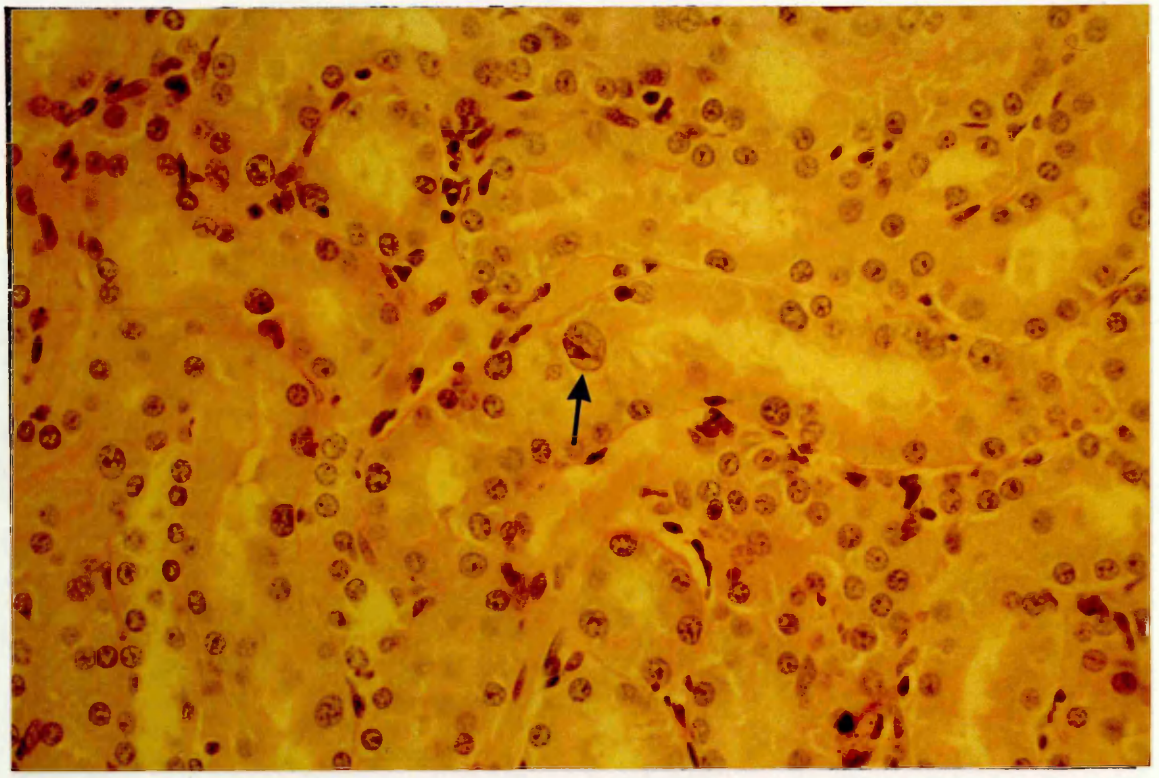


Fig. 5.2.6
OTA 2.5 mg/kg for 3 days. Enlarged nuclei within proximal tubules (arrow) after two weeks respite. PAS (x172).

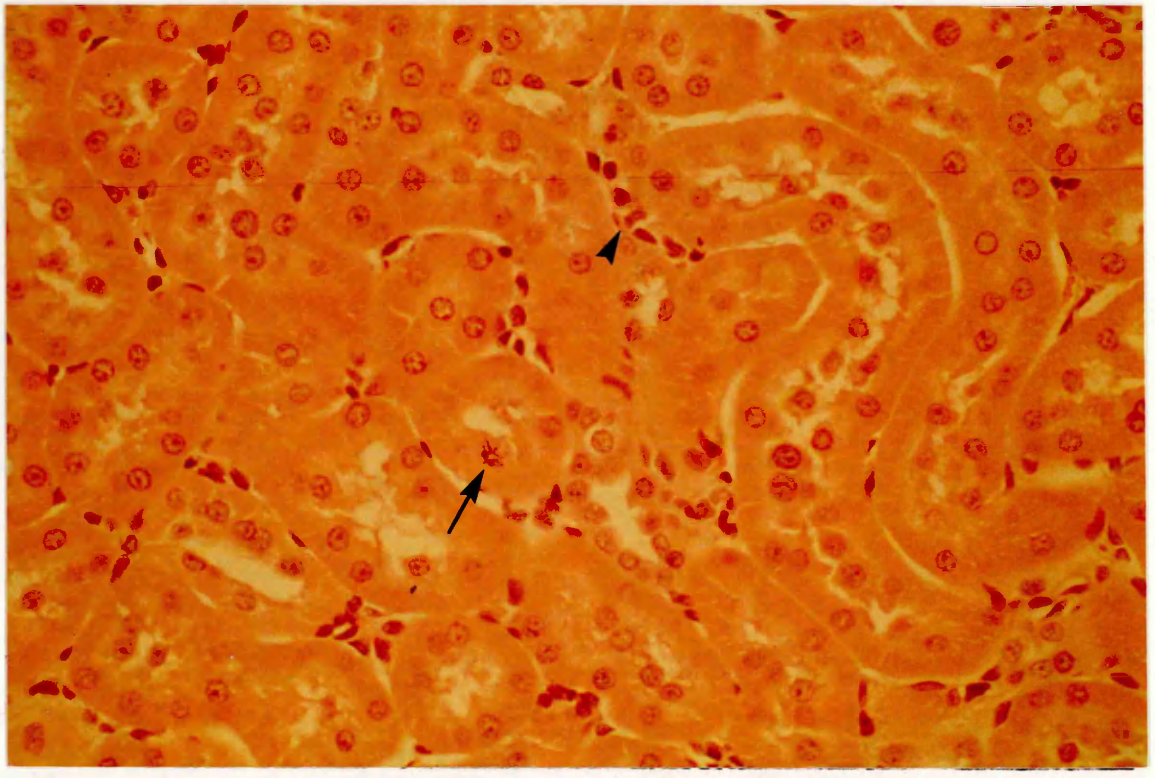


Fig. 5.2.7
ATB_1 100 ug/kg for 3 days. Section of cortex showing extrusion of proximal tubular nuclei also mitotic figures (arrow) and prominent interstitial cells (arrow). H&E (x172).

combination with one low, dose of ATB_1 (100 ug/kg).

The kidneys of animals administered 5 mg OTA plus 100 ug of ATB_1/kg for three days, showed severe and extensive damage of proximal tubules, with swelling and necrosis.

The kidneys of animals dosed with 2.5 mg OTA plus 100 ug ATB_1/kg for two days, showed generalized cytoplasmic swelling, with vacuolation and blebbing. The nuclei were dark and basophilic, and some were displaced towards the lumen. There were some casts within distal tubules. The presence of monocytes, could be indicative of a necrotic change taking place within the tissue.

The kidneys of animals dosed with 2.5 mg OTA plus 100 ug ATB_1/kg for three days, showed severe disruption of the S_3 segment of the proximal tubule with cellular swelling, vacuolation of cytoplasm and necrosis (Fig. 5.2.8A). The PAS staining of brush border was less intense than in the controls. Some glomeruli showed disruption of the general morphology (Fig. 5.2.8B), including edematous appearance, intense and faint staining nuclei, and cytoplasmic vacuolation, possibly indicative of necrosis. Tubular and glomerular changes appeared to be more marked than with OTA (See Fig. 5.2.5A) or ATB_1 alone.

The kidneys of animals dosed with 0.5 mg OTA plus 100 ug ATB/kg for three days, showed minimal changes, involving occasional cytoplasmic disruption and displaced nuclei in proximal tubules.

Discussion. In the present study in rats, repeated ip doses of OTA, induced microscopic renal changes that were dose related. The

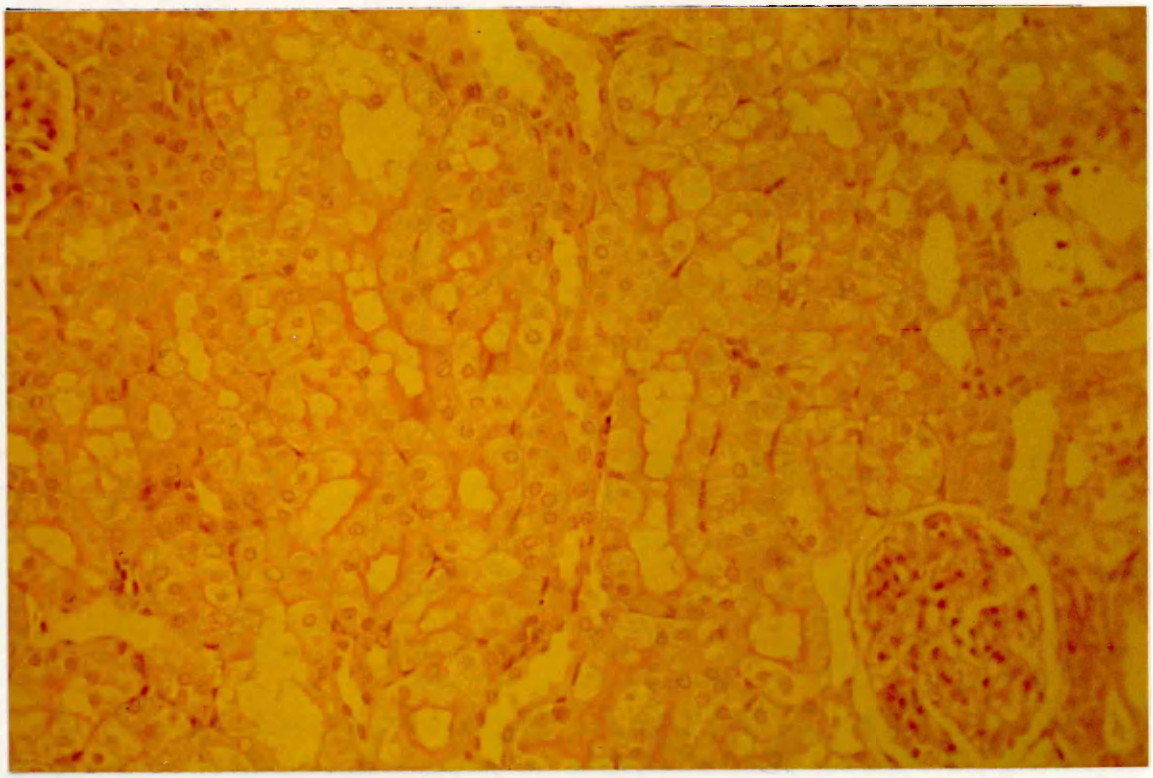


Fig. 5.2.8A
OTA 2.5 mg/kg and ATB_1 100 mg/kg for 3 days. Section showing severe disruption of the S_3 segment of the proximal tubule with cellular swelling, vacuolation of cytoplasm and necrosis. Compare to Figs. 5.2.5A and B. PAS (x107). Yellow colour is due to lack of filter during photographing.

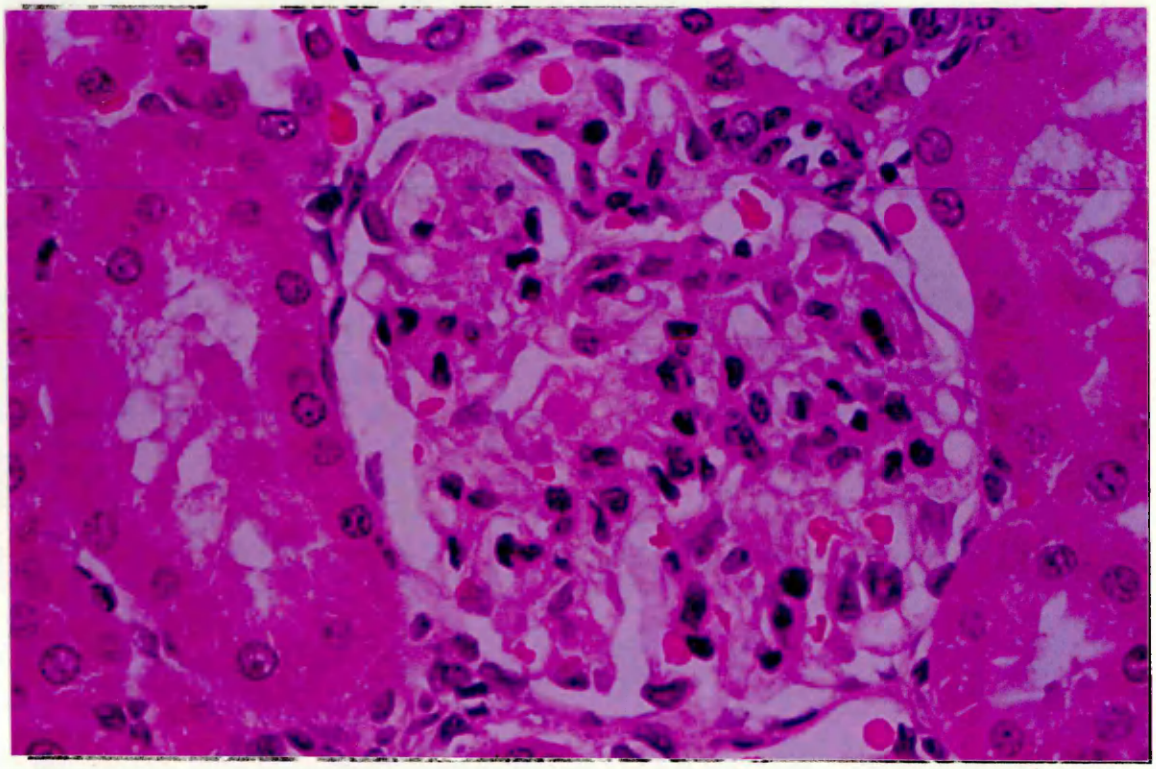


Fig. 5.2.8B
OTA 2.5 mg/kg and ATB_1 100 ug/kg for 3 days. Section of glomerulus showing intense and faint staining nuclei and cytoplasmic vacuolation. H&E (x172)

nephrotoxicity of OTA is associated here mainly with proximal tubular damage, which is in agreement with previous studies (Suzuki et al, 1975). But most important, after routine histology, there is evidence of glomerular injury, such as edema and prominent PAS staining basement membrane, which has not been previously described in the literature, following such low doses of OTA. Damage to glomeruli has been reported in pigs fed the toxin for three months (Krogh, 1976), and after the administration of massive single doses to rats (Albassam, 1987).

Repeated doses of ATB_1 caused only slight microscopic renal changes. Renal lesions have been previously described, only after relatively high doses of the mycotoxin were administered for several days (Madhavan and Rao, 1967; Madhavan et al, 1965). But the most important feature in this work is the interactive effect that such low doses of ATB_1 had when applied in combination with repetitive doses of OTA (2.5 mg/kg).

The combined administration of 2.5 mg OTA/kg and 100 ug/kg to rats for 3 consecutive days, caused severe proximal tubular damage that was not seen with OTA or ATB_1 alone. But a distinct feature is the glomerular injury detected in the form of oedema, general cellular derrangement and faint staining nuclei, possibly indicative of cell necrosis, which has not been described previously in the literature. There is a report in which the effect of simultaneous administration of OTA and ATB_1 to rats during four months has been described as synergistic with degenerative changes, anaplasia and hyperchromatic nuclei in epithelial cells, not present when either toxin was administered alone (Rati et al, 1981). In another report the effect of OTA and ATB_1 in chickens fed both toxins simultaneously for three weeks was also described as synergistic, based on gross changes observed in kidney (Huff and Doerr, 1980). However acute effects due to

the combined administration of OTA and ATB_1 have not been reported previously, neither in proximal tubules, nor in glomeruli of any species.

From the staining methods it is difficult to specify the type of glomerular cell and change being undergone, therefore these data still need to be confirmed by high resolution microscopy, histochemistry, immunohistochemistry and electron microscopy, in order to be able to describe these changes in greater detail.

5.3 Summary and conclusions

5.3.1 Summary

5.3.1.1 Biochemical parameters

1- Single low doses of OTA and ATB_1 alone or in combination caused some changes on the excretion of urinary enzymes such as ALP, AAP, GGT and NAG.

2- Low repetitive doses of OTA (2.5 mg/kg) caused major elevations of urinary enzymes such as ALP, AAP and GGT. ALP activity showed the highest increase of all the enzymes (1752% on day 7) and AAP showed its maximum release at an earlier time point (432% on day 4).

3- Low repetitive doses of OTA (2.5 mg/kg) remarkably affected the excretion of glucose which reached a maximum value on day 6, which was 90-fold the control values. Protein and urinary volume were also increased (537% on day 4 and 276% on day 8 respectively).

4- There was great animal-to-animal variability in all biochemical parameters measured.

5.3.1.2 Histopathological assessment

1- There were no detectable changes on the kidneys assessed histopathologically by H&E and PAS staining at the end of all experiments in which single doses of OTA and ATB_1 either alone or in combination were administered to the rats.

2- Repeated ip doses of OTA, induced microscopic renal changes that were dose related. The nephrotoxicity of OTA is associated here with proximal tubular damage and glomerular changes.

3- Repeated doses of ATB_1 caused only slight microscopic renal changes.

4- The histopathological changes observed after the combined administration of different doses of OTA and a fixed low dose of ATB_1 for 3 consecutive days were not dose-related.

5- The combined administration of 2.5 mg OTA/kg and 100 ug/kg to rats for 3 consecutive days, caused severe proximal tubular damage that was not seen with OTA or ATB_1 alone. Glomerular injury was also detected in the form of oedema, general cellular derrangement and faint staining nuclei, possible indicative of cell necrosis.

5.3.2 Conclusions

5.3.2.1 Biochemical parameters

1- The study of urinary enzymes such as ALP, AAP, GGT and NAG, over 24-hr period, after low single ip doses of OTA and ATB_1 administered alone or simultaneouly is not a sensitive test, and does not give a dose related response.

2- Although the histopathological changes were minimal when assessed on days 4 and 15, renal function was considerably disrupted, following the administration of three consecutive daily doses of 2.5 mg OTA/kg. The most sensitive parameters were glucose and ALP excretion.

3- The administered dose of OTA, was able to impair considerably the mechanism of glucose reabsorption in the proximal tubule.

4- The findings on proteinuria mean either that protein macromolecules were allowed to cross the glomerular barrier and therefore appeared excreted in urine, suggesting the possibility of glomerular involvement or that tubular reabsorption of low molecular weight proteins was impair. Both possibilities can also have occurred; however this needs further elucidation.

5.3.2.2 Histopathological assessment

1- The interactive effect observed between OTA and ATB_1 after combined administration of both toxins seems to be synergistic.

2- The use of more sensitive techniques such as, high resolution microscopy, histochemistry, immunohistochemistry and electron microscopy, might allow the detection of renal changes caused by low single doses of the mycotoxins assessed and would also help to confirm and describe the glomerular changes in greater detail.

CHAPTER 6

IN VITRO STUDIES ON ISOLATED GLOMERULI AND PROXIMAL TUBULAR FRAGMENTS.

6.1 De novo protein synthesis

Because work was being done with isolated pig and rat glomeruli, at the time when these experiments were started, the same conditions were maintained (ie. incubation with Krebs and Tyrodes buffer respectively) for the initial experiments. However, in order to be able to undertake further metabolic studies in isolated and cultured glomeruli, it was necessary to change the buffer system. Brendel and Meezan (1973) used Earles-HEPES to study glucose and fatty acid metabolism, and there was the need to set up similar experimental conditions, to establish the same reference point.

6.1.1 Amino acid incorporation into pig glomeruli incubated with Krebs buffer.

The incorporation of several different tritiated amino acids into isolated pig glomeruli was measured. The results are shown in Table 6.1.1 of the Appendix 2 and Figure 6.1.1. The levels of statistical significance are shown in Table 6.1.2 of Appendix 2. The results indicate that LEU had the highest rate of incorporation into macromolecules of all the amino acids tested. The rates of incorporation increased almost linearly with time. With the exception of the incorporations at 3 and 2 hr, the uptake of LEU was significantly different between each of the time points compared. PRO and HIS had very similar rates of incorporation at about 50% of the rate of LEU and also followed a linear pattern. For both amino acids the incorporations were significantly different between all time

points studied up to 2 hr of incubation, LYS showed a similar rate of incorporation to PRO and HIS, after which it decreased. GLY was the least well incorporated amino acid with very low rates up to 4 hr. There were significant differences in the uptake of the amino acid between most of the time points compared for GLY. At 4 and 3 hr of incubation the incorporation of the different amino acids were significantly different with the exception of PRO and HIS which showed very similar values. At 2 hr of incubation with the exception of PRO and LYS, PRO and HIS and LYS and HIS, all other amino acids showed statistically significant differences. At 1 hr of incubation there were statistically significant differences in the incorporations between LEU and GLY ($P \leq 0.05$), LEU and LYS ($P \leq 0.05$), LEU and PRO ($P \leq 0.05$), LEU and HIS ($P \leq 0.01$) and HIS and PRO ($P \leq 0.05$). After 4 hr of incubation all five amino acids could be ranked according to the amount of amino acid incorporated as follows: LEU >> PRO = HIS > LYS > GLY.

The time course of the incorporation of PRO between different experiments (Fig. 6.1.2, Table 6.1.3 Appendix 2) was very similar and, in most of them, the maximum amino acid uptake (after 4 hr of incubation) was in the range of 1-2 pmol/mg of protein. Only one experiment showed a higher PRO uptake at all time points, reaching 5.6 pmol/mg of protein at 4 hr of incubation. The rate of incorporation of PRO was statistically significantly different for all time points studied for most of the experiments (Table 6.1.4 Appendix 2).

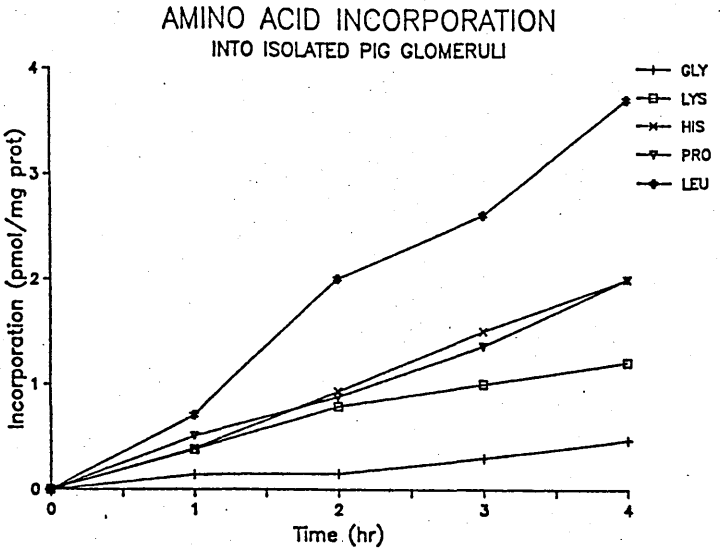


Fig. 6.1.1

Kinetics of de novo protein synthesis by isolated pig glomeruli, incubated with Krebs buffer. 0.5 ml of buffer containing 2.5 uCi of each of the ^{3}H labelled amino acids (GLY, HIS, LEU, LYS, and PRO) was added separately at 0 time to 0.5ml of the glomerular preparation. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

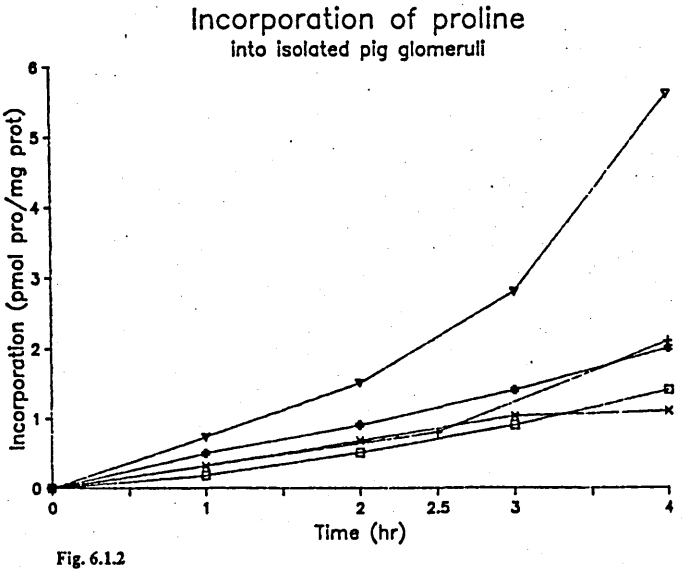


Fig. 6.1.2

Time-course of the incorporation of ^{3}H-proline into total glomerular protein. 0.5 ml of buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquot of pig glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

6.1.2 Comparison of the incorporation of PRO by isolated glomeruli incubated with two different buffers.

6.1.2.1 Pig glomeruli.

Incubation with Krebs buffer (pH 7.4) gave a much lower incorporation of PRO than Earles-HEPES (pH 7.4) at all time points studied, as can be seen in Fig. 6.1.3 and Table 6.1.5 Appendix 2. At 2 hr of incubation with Krebs, the incorporation of PRO was significantly lower ($P \leq 0.05$) than with Earles-HEPES. After 4 hr of incubation in Earles-HEPES the incorporated PRO was 10 times more than in Krebs and the difference was highly significant ($P \leq 0.001$).

6.1.2.2 Rat glomeruli.

A comparison was undertaken on the incorporation of PRO into isolated rat glomeruli incubated with Tyrodes and Earles-HEPES.

Fig. 6.1.4 and Table 6.1.6 Appendix 2 show the inorporation of PRO when rat glomeruli was incubated either with Tyrodes buffer or Earles-HEPES buffer. Incubation with Tyrodes gave a much lower incorporation of PRO than Earles-HEPES at all time points studied, and after 4 hr of incubation, the incorporation of the amino acid was twice higher with Earles-HEPES than with Tyrodes.

While the effects of chemicals on glomeruli in Krebs was studied in the early part of this work (Section 6.1.1), Earles-HEPES was then chosen as the incubation buffer for all further experiments (Sections 6.1.3-4).

Other conditions were also set up, as follows:

6.1.2.3 Influence of non-radioactive PRO on the incorporation of the labelled precursor into isolated pig glomeruli.

While some workers favour adding only the tracer molecule such as in the

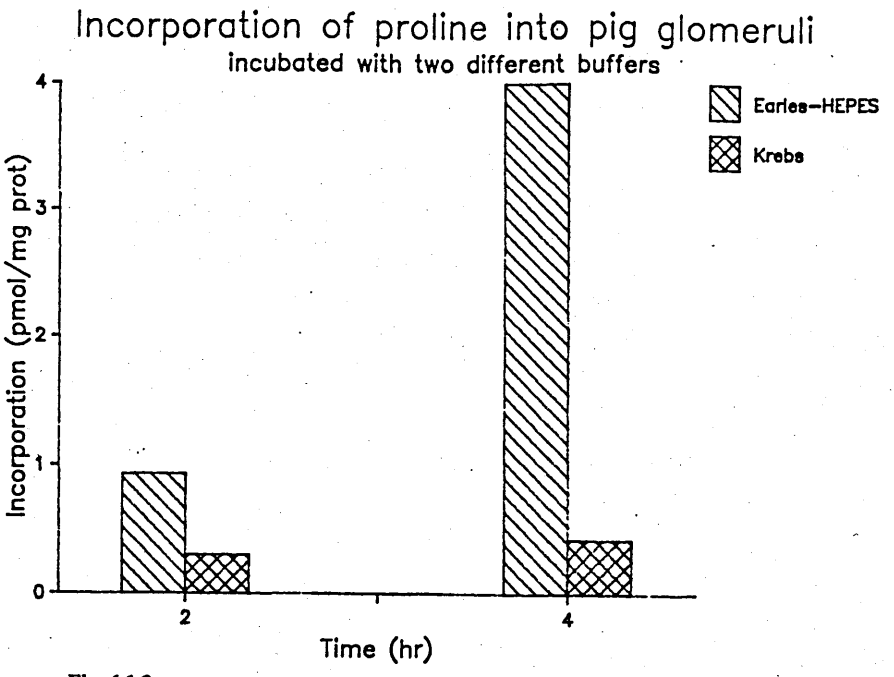


Fig. 6.1.3

Isolated pig glomeruli were suspended either in Krebs or Earles-HEPES buffers, and incubated with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline at 0, 2 and 4 hr.

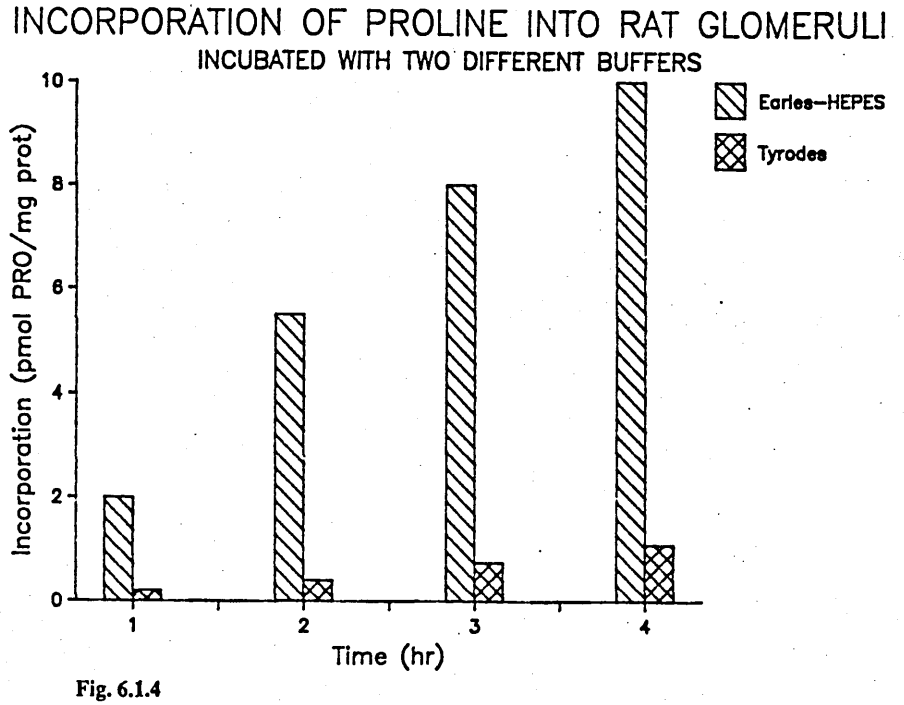


Fig. 6.1.4

Isolated rat glomeruli were suspended either in Tyrodes or Earles-HEPES buffers, and incubated with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline at 0, 1, 2, 3 and 4 hr.

assay system used above, others have added carriers to saturate non-specific binding sites. The effect of non-labelled precursor was therefore assessed on ^{3}H-PRO incorporation.

When ^{3}H-PRO incorporation was assessed in the presence of different concentrations of the non-labelled amino acid there were no variations at the minimum concentration used, in relation to the control (Figs. 6.1.5 and Table 6.1.7 Appendix 2). However, as long as the concentration of non-label increased (100 - 1000 uM), there was a dose-related decrease in the incorporation of ^{3}H-PRO (to 69 - 55%). Only the incorporation of PRO in the presence of 1000 uM of the non-radioactive aminoacid was significantly lower ($P \leq 0.05$) than the control.

6.1.2.4 Influence of non-radioactive analogues of PHE on the incorporation of this radiolabelled amino acid into isolated rat glomeruli.

The effect of different concentrations of non-labelled amino acids, and analogues of PHE on the incorporation of this one as glomerular protein was studied (Fig. 6.1.6 and Table 6.1.8 Appendix 2). Cold or non-labelled TYR and especially TRP, in the range of 0.025 - 2.5 mM, inhibited radiolabelled PHE incorporation in a dose-related pattern. Low concentrations of non-labelled HIS (50 uM), caused a slight and non-significant increase on PHE uptake, but at higher concentrations of the amino acid (ie. 0.5 and 2.5 mM), PHE incorporation was decreased significantly ($P \leq 0.05$ and 0.01 respectively), with similar values to those obtained with non-labelled TRP.

6.1.3 Incorporation of PRO into pig glomeruli incubated with Earles-HEPES.

Thus a new set of experiments was started using optimized experimental

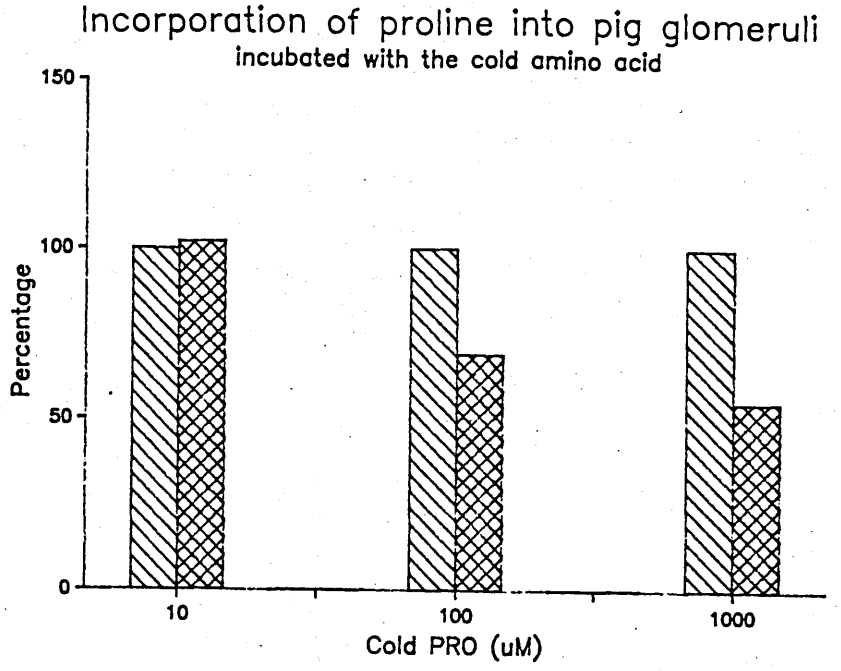


Fig. 6.1.5

0.5 ml aliquots of pig glomeruli suspended in Earles-HEPES were incubated with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of the "cold" amino acid for 4 hrs.

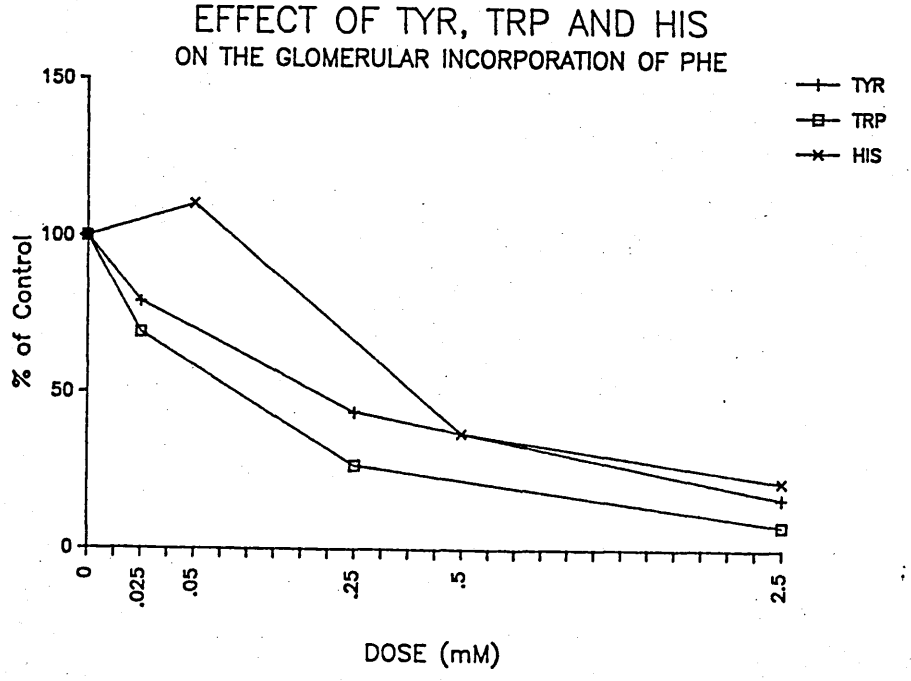


Fig. 6.1.6.

0.5 ml aliquots of rat glomeruli suspended in Earles-HEPES buffer were incubated with 0.5 ml of buffer containing 2.5 uCi ^{3}H-phenylalanine and different concentrations of "cold" HIS, TRP and TYR for 4 hrs.

conditions such as incubation with Earles-HEPES buffer.

Incorporation of PRO into isolated pig glomeruli incubated with Earles-HEPES buffer was compared between different experiments (Fig. 6.1.7 and Table 6.1.9 Appendix 2). In most of the cases, the incorporation follows a similar pattern, with maximum values at 4 hr of incubation, ranging from 1.3 - 3 pmol/mg of protein. Two of the experiments had much higher incorporation rates after 4 hr of incubation (5.1 and 7.6 pmol/mg protein). The incorporation of PRO showed statistically significant differences between all time points studied for each of 6 experiments (Table 6.1.10 Appendix 2), with the exception of one where the uptake of the amino acid did not show statistically significant differences between 3 and 4 hr.

6.1.4 Amino acid incorporation into rat glomeruli using Earles-HEPES as the incubation buffer.

Radiolabelled PRO and PHE were incubated with rat glomeruli and Earles-HEPES buffer, and their incorporation into de novo synthesis of protein was compared (Fig. 6.1.8). The amino acids uptake was linear with time, but PHE rate of incorporation was twice that of PRO (Table 6.1.11 Appendix 2).

In view of the differences found between PRO and PHE, it was decided to compare the incorporation of several other amino acids (Table 6.1.12 Appendix 2 and Fig. 6.1.9A and B) into rat glomeruli. The highest rate of incorporation was that of TRP. After 4 hr of incubation, 117 - 134 pmol of amino acid/mg protein had been incorporated. This was 4 times higher ($P \leq 0.01$) than PHE which was ranked in second place with a range from 25 - 26.3 pmol of amino acid/mg protein. PHE incorporation showed the best

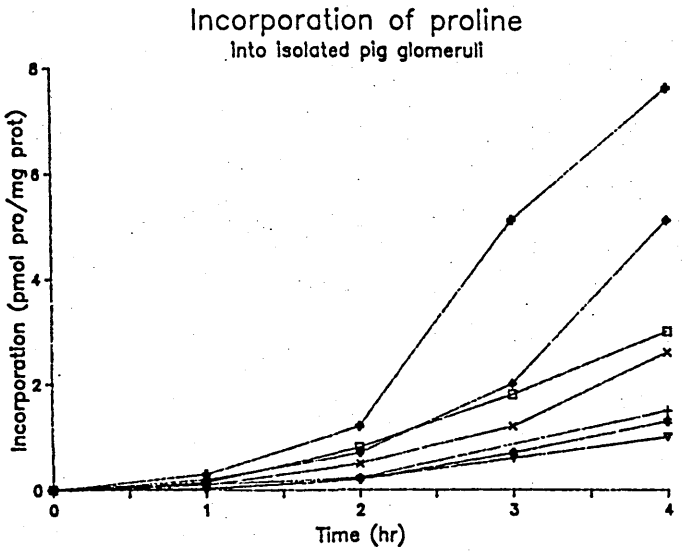


Fig. 6.1.7

Time-course of the incorporation of ^{3}H-proline into total glomerular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of pig glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

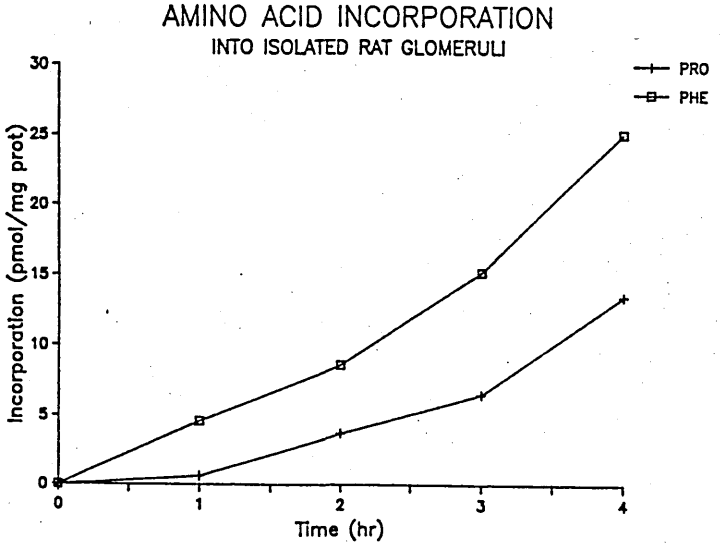


Fig. 6.1.8

Kinetics of de novo protein synthesis by isolated rat glomeruli incubated with Earles-HEPES buffer. 0.5 ml of buffer containing 2.5 uCi of each of the ^{3}H-labelled amino acids (PHE and PRO) was added at 0 time to 0.5 ml of the glomerular preparation. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each time point is the result of triplicate incubations.

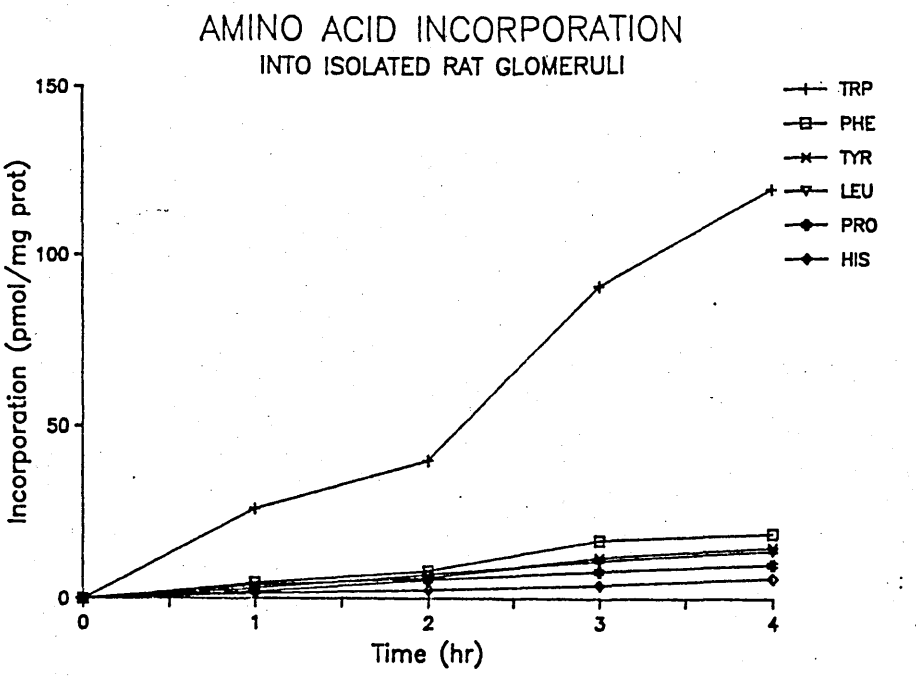


Fig. 6.1.9A

Kinetics of de novo protein synthesis by isolated rat glomeruli, incubated with Earles-HEPES buffer. 0.5 ml of buffer containing 2.5 uCi of each of the ^{3}H-labelled amino acids (HIS, LEU, PHE, PRO, TRP and TYR) was added at 0 time. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each time point is the result of triplicate incubations.

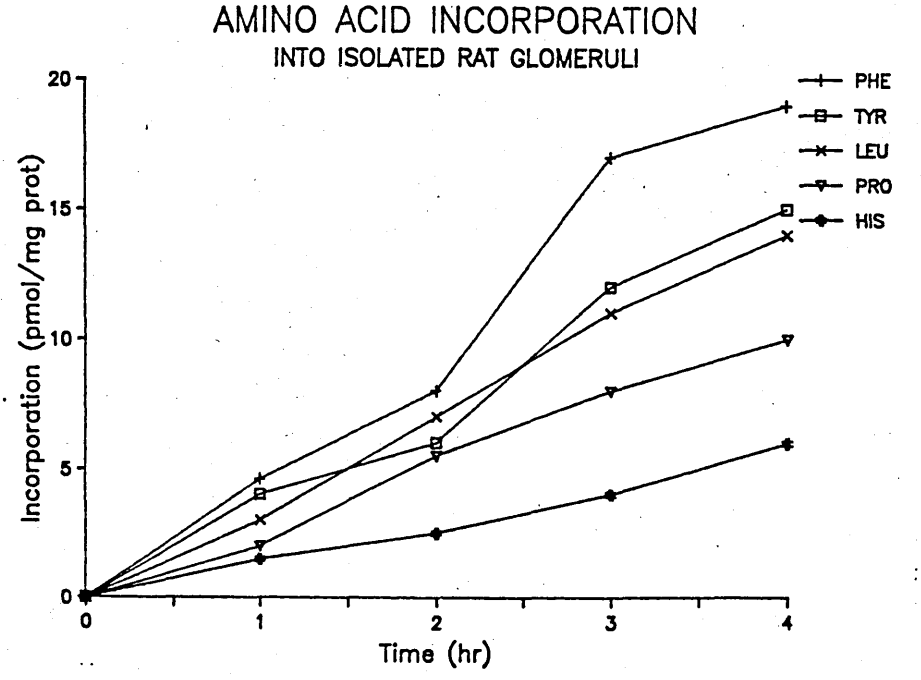


Fig. 6.1.9B

Enlarged view of the incorporation of HIS, LEU, PHE, PRO and TYR from Fig. 6.1.9A.

inter-experimental reproducibility with very small variance values at every time point that was studied for each of 4 experiments. The incorporation of this amino acid was significantly higher than LEU ($P \leq 0.05$) and the rest of the aminoacids ($P \leq 0.01$). LEU incorporation was ranked third and PRO and TYR had similar incorporations after 4 hr. TYR incorporation was significantly higher ($P \leq 0.01$) than HIS, and HIS was the least actively incorporated. Despite the inter-experimental variability in some of the cases (Figs. 5.1.1 - 6 of Appendix 2), the amino acid incorporation followed a similar, almost linear pattern. As can be seen from the tables, coefficients of variance were normally low.

6.1.5 Discussion.

Amino acid incorporation has been extensively used as a marker of de novo synthesis of protein. These data represents de novo protein synthesis based on the fact that:

a- Macromolecules were TCA-precipitated and the TCA solution contained small amounts of the respective cold amino acid, in order to avoid non-specific adsortion.

b- There were differences in the rates of amino acid incorporation.

c- The incorporation of a specific amino acid was inhibited by its analogues.

The data here show that isolated pig and rat glomeruli incorporate different amino acids, linearly for several hours, at different rates. This means that isolated glomeruli maintain their viability for a reasonable period of time (ie. > 4 hr), and also some of their biochemical properties, such as de novo synthesis of protein.

The effect of buffer on glomerular de novo protein synthesis. The

incorporation of PRO into isolated pig and rat glomeruli was compared by incubation with two different buffers (Earles-HEPES vs. Krebs for pig and Earles-HEPES vs. Tyrodes for rat). The rate of amino acid incorporation was greater when the incubation was performed with Earles-HEPES buffer for both types of glomeruli, although in the assays with pig, the absolute values of amino acid incorporation were far greater for Earles-HEPES than was the case of rat glomeruli. This means that the amount of de novo synthesised protein can vary with the composition of the incubating media. It appears that Earles-HEPES buffer offers better control of pH than Krebs and Tyrodes, the buffering properties of which relay on the amount of bicarbonate present. Unless a constant supply of CO_2 is provided, Tyrodes and Krebs loose their buffering capability with time. Earles-HEPES seems to be especially appropriate for those studies involving prolonged incubation out of a CO_2-incubator and without constant supply of CO_2, and also for trapping $^{14}CO_2$ from ^{14}C labelled precursors and Earles-HEPES is also useful as the incubation buffer for cultured cells. Thus because CO_2 gassing was not used in the previous experiments a non-gassing system was chosen.

Earles-HEPES was therefore chosen as the incubation buffer for all further experiments with isolated pig and rat glomeruli, and also for tubular fragments.

The effect of addition of non-labelled precursors to the incubation media. Addition of increasing concentrations of non-radiolabelled PRO, decreased the incorporation of the radioactive amino acid. This is probably due to the "dilution" of the radiolabelled compound by competitive inhibition, therefore decreasing the chance of the labelled molecule been used as the

only source of precursor for protein synthesis. In view of the observed effect of the non-radiolabelled PRO, it was decided not to add it to the incubation media, in order to get higher radioactive counts and therefore increased sensitivity of the method.

Incorporation of different radioactive amino acids. Pig glomeruli incorporated LEU at a much higher rate than PRO and the other amino acids. The order of incorporation being LEU >> PRO = HIS > LYS > GLY.

In rat glomeruli TRP was incorporated at the highest rate, with PHE and TYR ranking next. The ranking of incorporation was TRP >> PHE > TYR = LEU > PRO > HIS.

These data show that isolated rat glomeruli incorporate a high proportion of aromatic amino acids into protein and that this incorporation is at a higher rate than for PRO, which is a novel observation.

The incorporation of PRO and other different labelled amino acids into isolated piglet or rat glomeruli has been reported to be linear for several hours (Grant et al, 1975; Krisko and Walker, 1974), and when equimolar amounts of radioactive PRO and LYS, were added to isolated rat glomeruli, LYS was incorporated more than PRO (Krisko and Walker, 1976), which is in agreement with recent studies by Ahmed et al. (1987) who found the ranking of incorporation being LYS > PRO > HIS. However no comparison on the rates of incorporation between other different amino acids, nor interspecies comparison have previously been reported. Thus, the data presented in this thesis, appear to be the first that documents the incorporation of aromatic amino acids into isolated rat glomeruli and also shows differences in the basal <u>de novo</u> protein synthesis in pig and rat

glomeruli.

The data on PRO incorporation in Krebs buffer as compared between different experiments show that the amino acid is incorporated linearly over 4 hr with similar rates of incorporation for most of the cases. Only one out of 6 experiments showed much higher rates of the PRO incorporation which can be interpreted as a factor of individual variation within the different pigs, as discussed in section 6.2.2.

The data on the incorporation of PRO into pig glomeruli incubated in Earles-HEPES buffer, show a pattern of incorporation similar to the one when Krebs buffer was used, was also linear and the rate of amino acid incorporation increased with time. The values of the incorporation of PRO from Earles-HEPES, for several different experiments were distributed in three different populations with a total range going from 1.0 to 7.6 pmol/mg protein after 4 hr of incubation. This indicates a 7.6-fold variability, which may reflect the individual differences existing in the pigs sampled, as suggested above.

From these studies on de novo synthesis of protein it is difficult to explain the specific fate of the different amino acids within either pig or rat glomeruli. Amino acids such as LEU are known to be present in all proteins, therefore its use does not allow differentiation between the synthesis of specific proteins of the glomerulus. PRO is incorporated into all proteins, but its subsequent hydroxylation occurs exclusively in GBM collagen, thus allowing differentiation between GBM and non-GBM protein metabolism. However our study did not distinguish whether the label once incorporated into macromolecules was present as PRO or OH-PRO. LYS is also used in GBM, and like PRO, it undergoes hydroxylation. Thus OH-LYS is also

present in large amounts in GBM, being indicative of the presence of collagenous components. TYR is an amino acid present in small amounts in basement membranes. This amino acid together with TRP have been found to make part of the non-collagen GBM protein fibronectin, and localized in folding regions, that seem to be important for adhesive activity and cellular spreading (Yamada, 1983).

Although GLY appears to be a major constituent of GBM, in our study in isolated pig glomeruli, it had the lowest incorporation into total glomerular protein. GLY incorporation has also been reported to be low in proximal tubular fragments (Kwizera and Bach 1987). GLY appears to be largely incorporated into GBM protein as it was shown by Hjelle et al (1979), who compared the incorporation of GLY, LYS and PRO and found GLY to be the most incorporated in rat GBM, being followed by LYS and then PRO. The fact that our study measured the incorporation of GLY into total glomerular protein and not into isolated GBM, does not allow to compare the proportion in which GLY was incorporated in these two different systems; furthermore, it has been shown above that the use of a different buffer can change the rate at which an amino acid is incorporated; this could also account for the differences in the incorporation of GLY into total glomerular protein and GBM. There are no previous reports on the incorporation of GLY into total glomerular protein.

Observations by Price and Spiro (1977), suggest that only a small proportion of the protein synthetic activity in the cells of glomeruli is dedicated to GBM synthesis. Therefore, a small proportion of amino acids incorporated into GBM-collagen, in these experiments with isolated glomeruli is expected. In studies involving total glomerular protein

synthesis, the amino acids are more readily incorporated in either non-collagen or non-GBM proteins which have a faster turnover within the glomerulus (ie. 9 days vs 100 days for GBM-collagen). Therefore is difficult to extrapolate from this data to de novo synthesis of GBM protein. However, indirect information can be obtained from the biochemistry of the cells that produce and maintain this extracellular matrix, assuming that changes in the biochemical functions of the cells which are in contact with the GBM, take place before or in parallel to any modification in the structure or composition of the GBM. On the other hand, the component of GBM that has been the focus of attention for studies in composition and turnover rate is the glycoprotein collagen (Kefalides et al, 1979), probably because of the difficulties in isolating non-collagenous material, with the traditional methods. However, glomerular extracellular matrix is also composed of other non-collagenous proteins with higher turnover rates, which have been more recently detected (eg. fibronectin) and are not yet as well characterized structurally and functionally within the glomeruli. Although fibronectin has been extensively studied in other cells and tissues, further studies are needed to characterize the properties of this protein in glomeruli.

6.1.6 Summary and conclusions.

6.1.6.1 Summary

1- Isolated pig and rat glomeruli incorporate different amino acids, linearly for several hours, at different rates.

2- Pig glomeruli incorporates LEU at a much higher rate than PRO and other amino acids. The order of incorporation being LEU >> PRO = HIS > LYS > GLY.

3- In rat glomeruli TRP is incorporated at the highest rate, with PHE and TYR ranking next. The ranking of incorporation was TRP >> PHE > TYR = LEU > PRO > HIS.

4- Increasing concentrations of non-radiolabelled amino acids decrease the incorporation of the respective labelled precursor.

5- Increasing concentrations of non-labelled amino acids, such as HIS, TRP and TYR, decrease the incorporation of radiolabelled PHE.

6- Incubation of isolated rat and pig glomeruli with Earles-HEPES buffer increases the incorporation of PRO more than Tyrodes and Krebs.

6.1.6.2 Conclusions.

1- Isolated rat and pig glomeruli maintain their viability for a reasonable period of time (ie. > 4 hr), and also some of their biochemical properties, such as de novo synthesis of protein.

2- Isolated rat glomeruli incorporate a high proportion of aromatic amino acids into protein and that this incorporation is at a higher rate than for PRO, which is a novel observation.

3- The amount of de novo synthesised protein can vary with the composition of the incubating media.

4- Only a small proportion of the amino acids incorporated into total glomerular protein is expected to be incorporated into GBM-collagen.

5- The fact that isolated rat glomeruli incorporate aromatic amino acids (ie. TRP and TYR) at higher rates than PRO, suggests that a glomerular macromolecule other than GBM-collagen with a high turnover, is being synthesised de novo .

6.2 Effect of different chemicals on de novo protein synthesis.

6.2.1 Adriamycin (ADR)

6.2.1.1 Effect of ADR on the incorporation of different amino acids by isolated pig glomeruli, using Krebs as the incubation buffer.

The effect of ADR assayed on de novo protein synthesis using different amino acids it is shown in Fig. 6.2.1 and Table 6.2.1 and 2 of Appendix 2). PRO and LEU were similarly affected, with very close IC_{50} (Concentration of chemical that inhibits protein synthesis to 50% of the control values) (13 and 12.7 uM) as shown in Table 6.2.1, They showed a dose-related response. The incorporation of the amino acids in the presence of 7.8 uM of ADR, was significantly different from the control at $P \leq 0.01$ for LEU and $P \leq 0.05$ for PRO. Increasing concentrations of the drug caused an inhibitory effect on the incorporation of both LEU and PRO, which was significantly different from the respective controls ($P \leq 0.01$). Although the IC_{50} for HIS (15.6 uM) was slightly higher than those for PRO and LEU, there was a dose-related response. The incorporation of amino acid at ADR concentrations of 7.8 and 15.6 uM were significantly different from the control ($P \leq 0.01$) and higher concentrations depressed protein synthesis more ($P \leq 0.001$).

In spite of the low level of protein synthesis showed by GLY, in comparison to the other amino acids, its incorporation was markedly affected by ADR, having an IC_{50} of 13.6 uM, and showed statistically significant reductions at all concentrations tested ($P \leq 0.05$ for concentrations of 7.8, 15.6, and 31.2 uM, and $P \leq 0.01$ at 62.5 uM of ADR). However, this was not a dose-related effect, since higher concentrations did not depress the synthesis of protein below 40% of control.

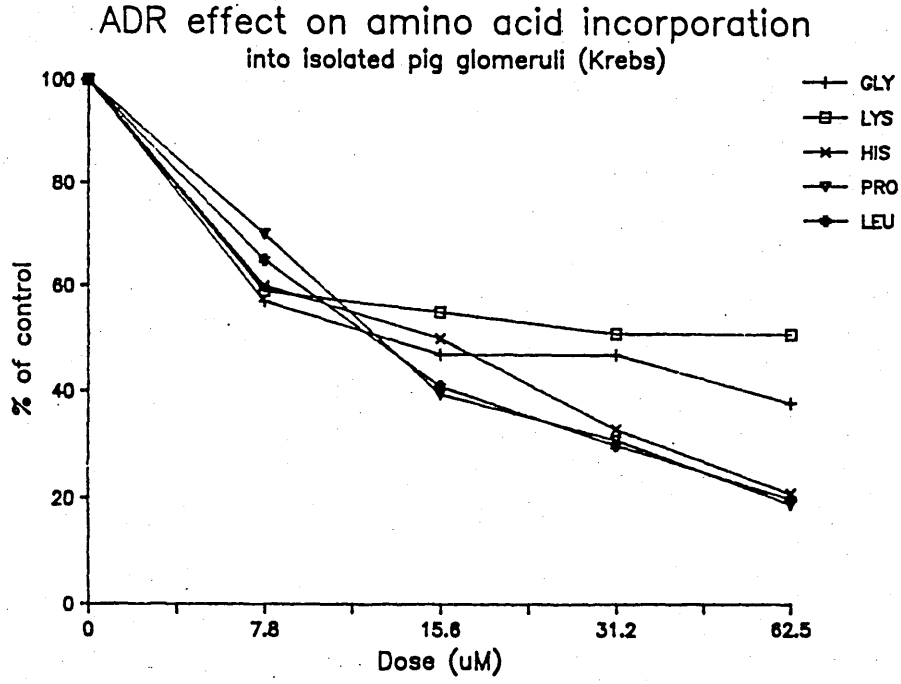


Fig. 6.2.1

0.5 ml aliquots of pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi of various ^{3}H-amino acids (GLY,LYS, HIS, PRO and LEU), and different concentrations of adriamycin, for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total amino acid incorporation into isolated pig glomeruli incubated without the chemical(control). Each point is the result of triplicate incubations.

TABLE 6.2.1
RELATIVE TOXICITY OF ADR ON THE INCORPORATION OF DIFFERENT AMINO ACIDS INTO ISOLATED PIG GLOMERULI

AMINOACID	MAXIMUM INCORPORATION[+]	IC_{50} (uM)
LEUCINE	3.7 ± 0.38	12.7
PROLINE	2.0 ± 0.2	13.0
HISTIDINE	2.0 ± 0.17	15.6
LYSINE	1.2 ± 0.01	*
GLYCINE	0.5 ± 0.1	13.6

[+] pmol/mg protein.

* LYSINE DID NOT REACH THE IC_{50} POINT.

From all amino acids studied, LYS was the least sensitive to ADR effect, none of the concentrations tested produced an inhibition of the incorporation that reached 50%. However, the reduction in the incorporation was significantly different ($P \leq 0.01$) from the control value at 7.8 and 15.6 uM of ADR, and highly significant ($P \leq 0.001$) at higher concentrations.

Effect of ADR on PRO incorporation. The effect of different concentrations of ADR was assessed on the incorporation of PRO and compared in several experiments. Fig. 6.2.2A shows the results as percentage of control, it can be seen that the effect of ADR was reproducible between experiments and very similar for all of them. In all of the experiments, the concentrations of ADR (7.8 - 250 uM) assayed caused a statistically significant reduction on PRO incorporation. The degree of significances is shown in Table 6.2.3 of Appendix 2. The relative toxicity of the drug, presented as IC_{50} (Table 6.2.2) was in the range 11.7 - 27.3 uM which represents an inter-experimental variation of less than 3-fold. At the maximum incorporation of PRO, the dose of the compound needed to cause a 50% inhibition of amino acid incorporation was less than when the de novo synthesis of protein was lower. Fig. 6.2.2B shows the general trend of ADR effect as an average of several experiments.

6.2.1.2. Effect of ADR on PRO incorporation, using Earles-HEPES as the incubation buffer.

The effect of ADR on PRO incorporation into isolated pig glomeruli incubated with Earles-HEPES buffer was assessed in several experiments (Figs. 6.2.3A and 6.2.3B). All concentrations of ADR had a significant inhibitory effect on PRO incorporation, in all experiments, as can be seen

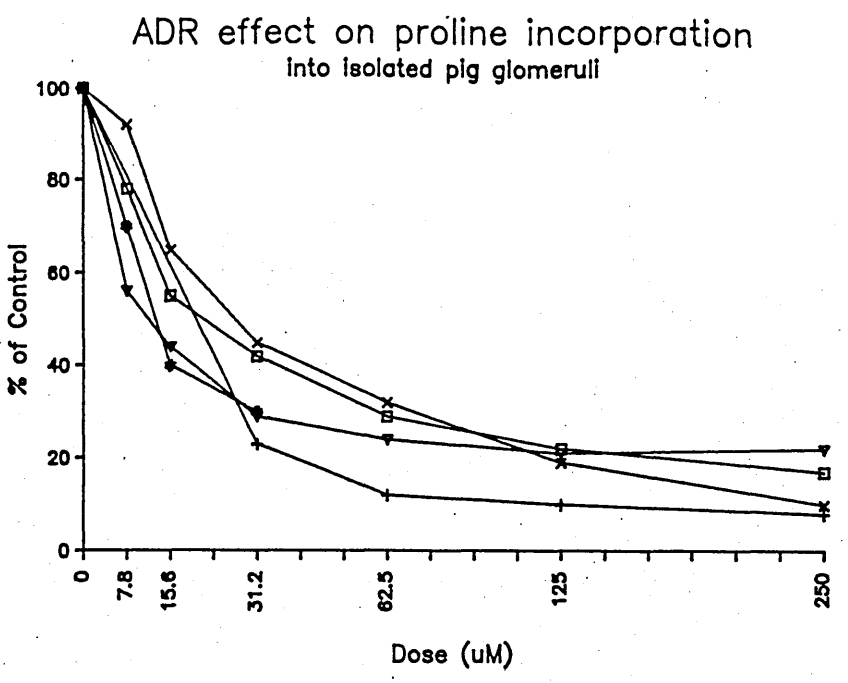


Fig. 6.2.2A

0.5 ml aliquots of isolated pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of adriamycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

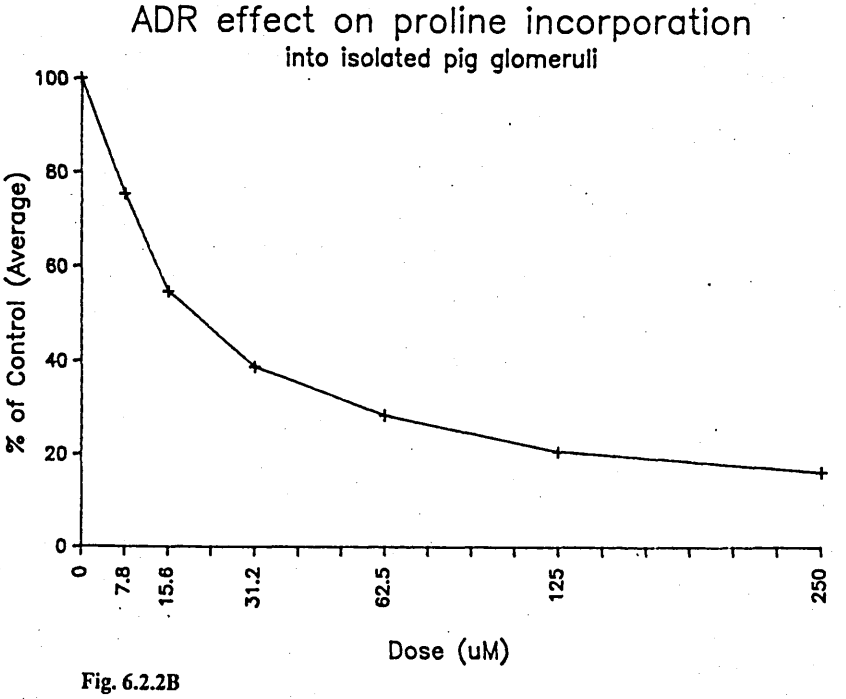


Fig. 6.2.2B

Data presented as the average of five experiments.

TABLE 6.2.2
RELATIVE TOXICITY OF ADR ON PROLINE INCORPORATION INTO ISOLATED PIG GLOMERULI

EXPERIMENT	MAXIMUM INCORPORATION[+]	IC_{50} (uM)
1	2.10	18.7
2	1.4	20.3
3	1.1	27.3
4	5.6	11.7
5	2.0	13.0

[+] pmol proline/mg protein

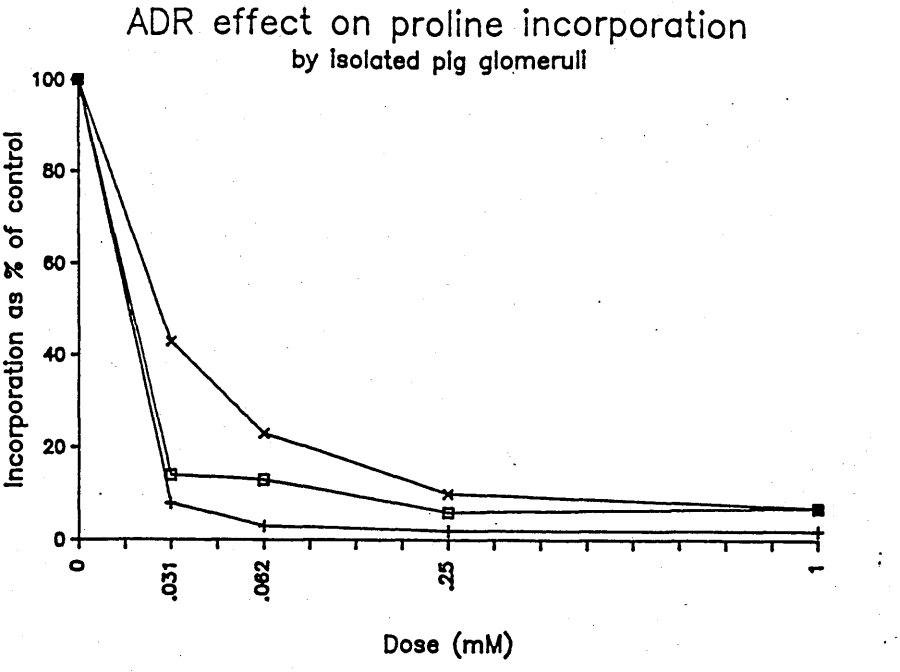


Fig. 6.2.3A

0.5 ml aliquots of isolated pig glomeruli suspended in Earles-HEPES buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of adriamycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

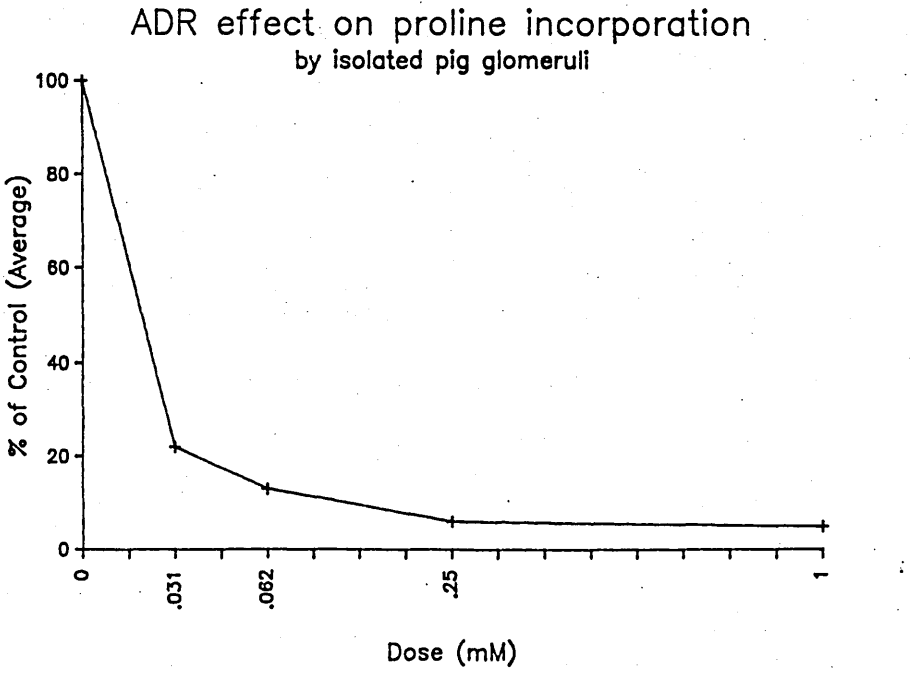


Fig. 6.2.3B

Data presented as the average of three experiments.

in Table 6.2.4 Appendix 2. The estimated IC_{50} was < 26 uM.

These data show that PRO incorporation into glomeruli exposed to ADR, and assayed in Earles-HEPES buffer, was markedly inhibited as it was when Krebs buffer was used. There was little inter-experimental variability. Although no smaller concentrations than 31.2 uM were assessed, the estimated concentration to cause an inhibition of 50% relative to the control value, was very similar to the one obtained using Krebs buffer (26 vs. 22 uM).

6.2.1.3 ADR effect on PRO incorporation into isolated rat glomeruli.

The effect of ADR on de novo protein synthesis into isolated rat glomeruli using PRO as the precursor is shown in Fig. 6.2.4A and B. All concentrations of the chemical caused significant inhibition on the incorporation of the amino acid. The degree of statistical significance is shown in Table 6.2.5 of Appendix 2.

6.2.1.4 Discussion.

The results on the effect of ADR on the incorporation of several amino acids into pig glomeruli, show that the drug depressed considerably the incorporation of all amino acids, with LEU and PRO, being the most sensitive and showing a similar effect, and LYS being the least sensitive. This means that de novo synthesis of glomerular protein from different amino acids on pig glomeruli is inhibited by ADR, and that some aminoacids (ie. LEU and PRO) are more affected than others (ie. GLY, HIS and LYS).

The data on the inhibitory effect of ADR on PRO incorporation into glomeruli incubated in Krebs buffer, as compared in several different experiments are very similar and showed an inter-experimental variability

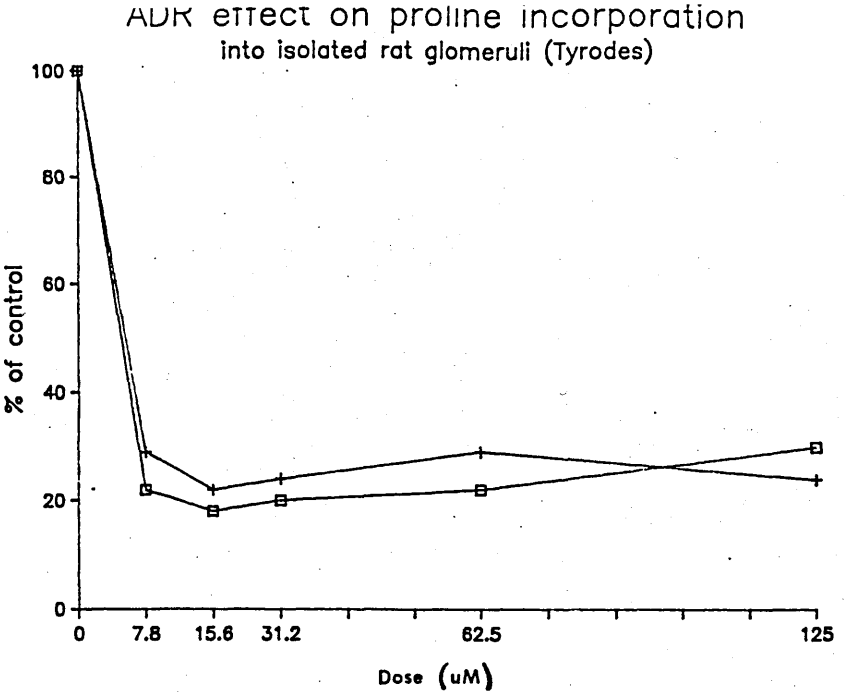


Fig. 6.2.4A

0.5 ml aliquots of isolated rat glomeruli suspended in Tyrodes buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of adriamycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

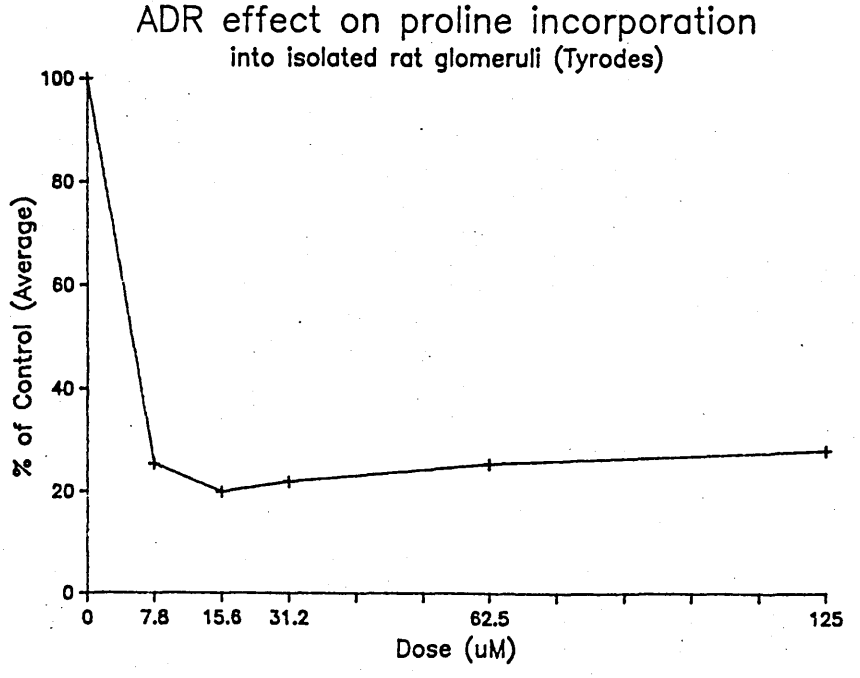


Fig. 6.2.4B

Data presented as the average of two experiments.

of less than 3-fold, which is very small, considering that some characteristics of the sampled pigs, such as sub-strain, age, sex, diet, status of health, hydration, exposure to nephrotoxins, and time between slaughtering and harvesting the tissue can differ and account for individual variations in the different metabolic processes.

PRO incorporation was the most effectively inhibited parameter and is therefore considered to be a sensitive index of ADR glomerular cytotoxicity in pig glomeruli. This is in agreement with previous studies performed in glomeruli isolated from the rat (Ahmed et al., 1987).

The fact that ADR decreased considerably the incorporation of PRO (and other amino acids) into total glomerular protein, is consistent with the in vivo findings that ADR damages glomeruli in rats (Bertani et al, 1982), and can also be related to the fact that the drug causes nucleolar alterations in renal epithelial cells (Hayashi et al 1984), similar to those reported to occur in a variety of in vitro and in vivo cell systems, secondary to the action of various compounds such as actinomycin D, 4-nitroquinoline 1-oxide, Aflatoxin B_1, amanitin and proflavin. All these compounds possess the biochemical property of forming complexes with DNA, suggesting that after interaction of ADR with DNA, transcription may rapidly be inhibited, with subsequent inhibition of translation. Inhibition of glomerular protein synthesis in vitro may be related to the changes produced by the toxin on GBM-collagen synthesis by glomerular epithelial cells; the target cell in vivo (Bertani et al, 1982).

Although it has been reported previously that ADR inhibit synthesis of protein in isolated rat glomeruli (Ahmed et al., 1987), no data has so far shown the effect of the drug in the synthesis of protein by isolated pig

glomeruli. Therefore, these data presented above appear to be the first reporting such in vitro effect on glomeruli from pigs. The extrapolation of these in vitro data to the whole animal seems likely.

The information on the in vivo toxicity of ADR in pigs is scarce, so far there is only one report (Van Fleet et al, 1979), where renal microscopic lesions in tubules and glomeruli were observed, after chronic iv administration of the drug, but no data on the clinical chemistry was provided.

For further experiments, PRO was chosen as a sensitive index with which to study glomerular toxicity for several reasons:

i- It is regarded as a very important constituent of GBM, being part of the collagen-like regions, as such, and also in the hydroxylated form (Kefalides et al, 1979). GBM is of considerable physiological and pathological interest because of its role as the main filter in the filtration process, and its apparent modification in a variety of degenerative renal conditions (Glassock, 1978).

ii- PRO incorporation into rat glomerular protein has been previously reported to be a sensitive parameter of glomerular toxicity caused by ADR (Ahmed et al., 1987) which has also been confirmed by this work.

iii- The good performance of PRO between the different experiments, such as linearity, and low inter-experimental variability.

6.2.2 Puromycin aminonucleoside (PAN).

6.2.2.1. Effect of PAN on PRO incorporation into pig glomeruli incubated with Krebs buffer.

Having shown that protein synthesis from PRO is a sensitive indicator of

ADR glomerular cytoxicity, the effect of other compounds on the incorporation of the amino acid into pig glomeruli was investigated. Fig. 6.2.5A shows the effect of PAN on the incorporation of PRO as a percentage of the control and Fig. 6.2.5B as an average of several experiments.

There was a certain degree of inter-experimental variation at the lower concentrations assayed, for most of the experiments, PAN caused an 50% inhibitory effect at high concentrations (> 2.5 mM). The degree and level of statistical significance is shown in Table 6.2.6 Appendix 2.

6.2.2.2 Effect of PAN on PRO incorporation with Earles-HEPES as the incubation buffer.

Fig. 6.2.6A shows the effect of PAN on the incorporation of PRO as percentage of control. There is a certain degree of inter-experimental variation at the lowest concentration assayed. The average IC_{50} estimated for the drug was 1 mM (Table 6.2.3). The degree and level of statistical significance is shown in Table 6.2.7 Appendix 2. Fig. 6.2.6B shows the data as an average of several experiments.

6.2.2.3 PAN effect on PRO incorporation into isolated rat glomeruli using Tyrodes as the incubation buffer.

Fig. 6.2.7A and B show the effect on PAN on the incorporation of PRO as a percentage of the control and average of two experiments respectively. For both experiments, low doses of PAN caused an increase in the incorporation of PRO, and the dose of the compound needed to cause a 50% inhibition in the amino acid incorporation was greater than 2.5 mM. Table 6.2.8 Appendix 2 shows the degree of statistical significance.

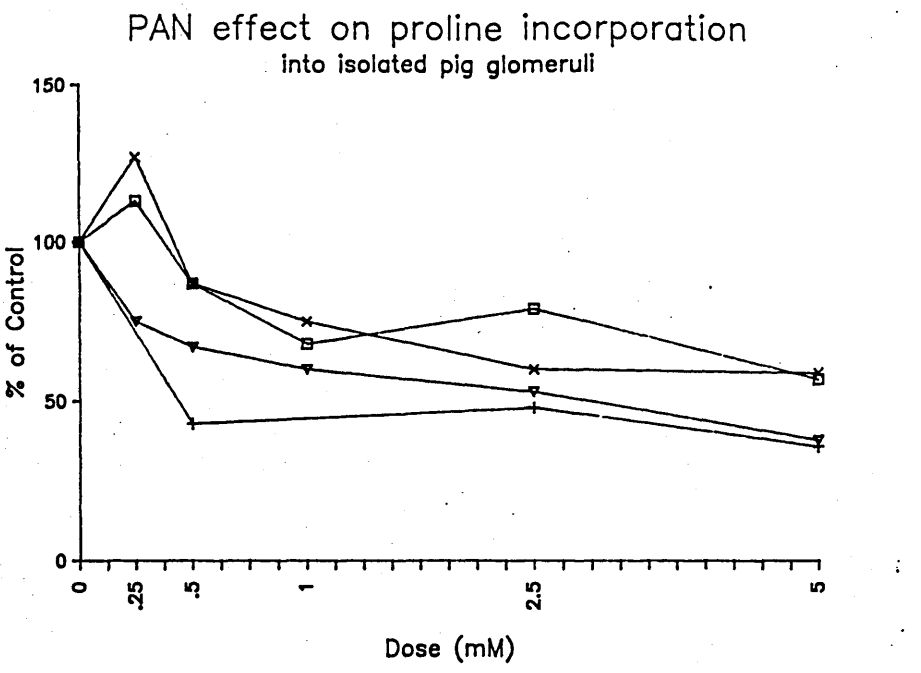


Fig. 6.2.5A

0.5 ml aliquots of isolated pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of puromycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

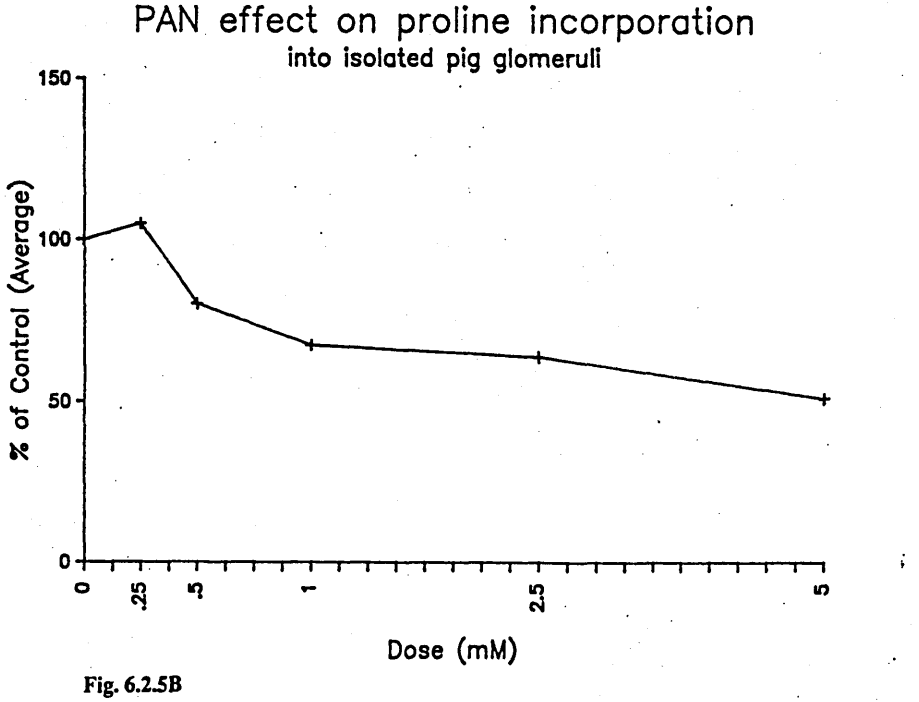


Fig. 6.2.5B

Data presented as the average of four experiments.

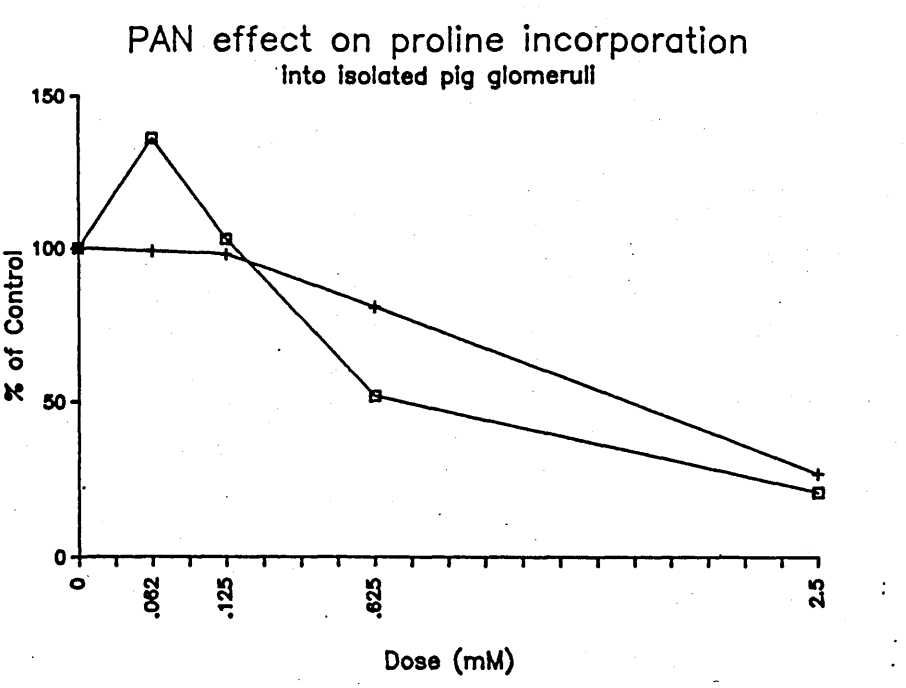


Fig. 6.2.6A

0.5 ml aliquots of isolated pig glomeruli suspended in Earles-HEPES buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of puromycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

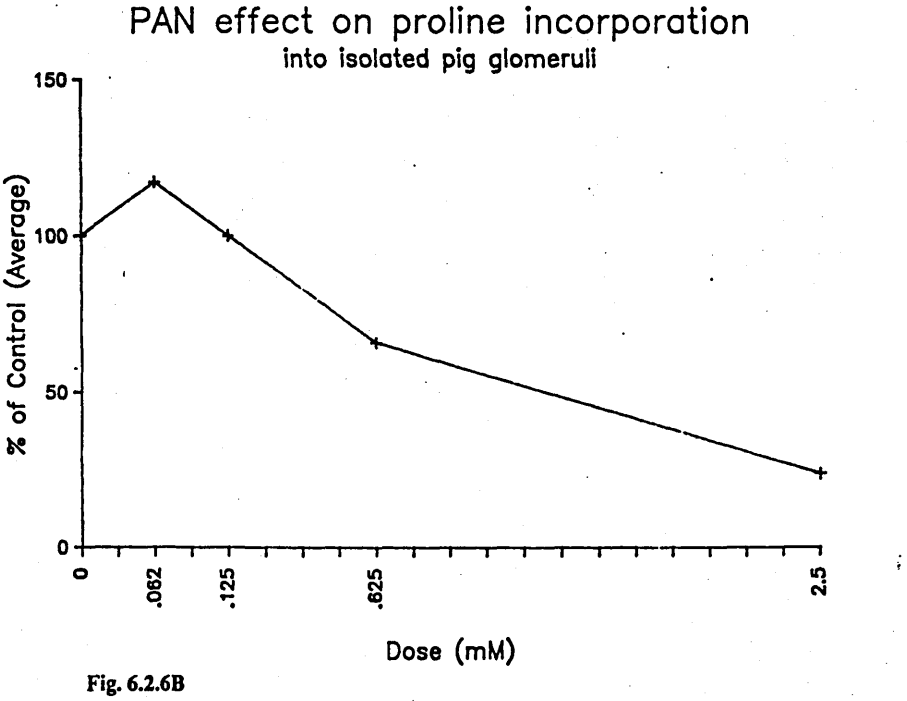


Fig. 6.2.6B

Data presented as the average of two experiments.

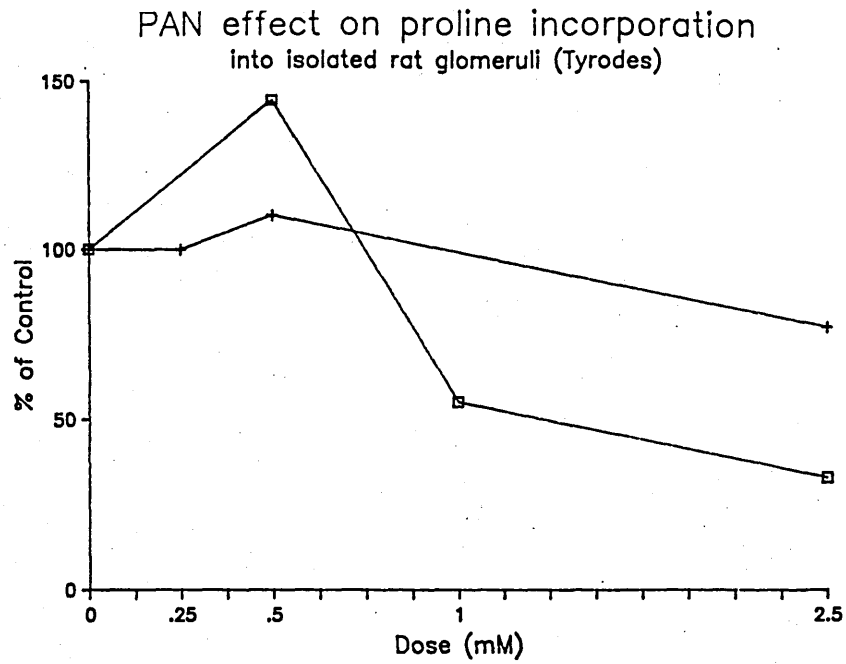


Fig. 6.2.7A

0.5 ml aliquots of isolated rat glomeruli suspended in Tyrodes buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi 2.5 uCi ^{3}H-proline and different concentrations of puromycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

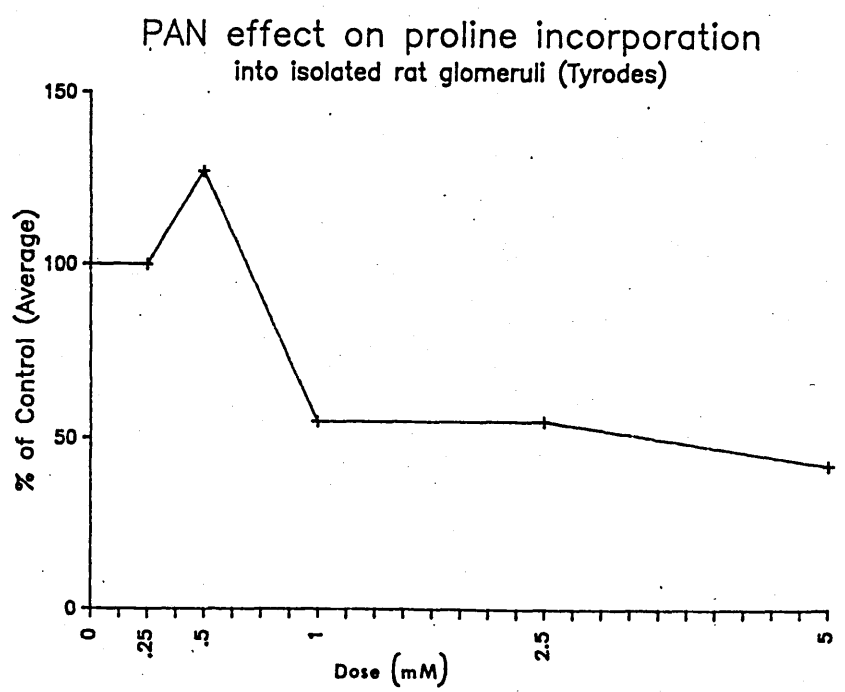


Fig. 6.2.7B

Data presented as the average of two experiments.

TABLE 6.2.3 Relative toxicity (IC_{50}) of different chemicals on PRO incorporation into isolated pig and rat glomeruli incubated with different buffers.

Buffer	Krebs	Tyrodes	Earles-HEPES	
Species	Pig	Rat	Pig	Rat
CHEMICAL	IC_{50} (mM*)			
ADR[a]	0.022	<7.8 uM**	0.026	-
PAN[a]	> 2.5	>2.5	1	-
STR[e]	< 0.5	<0.5	0.5	-
ETA[f]	-	-	0.05	-
BEA[c]	>10	10	< 1	-
FA[d]	5	-	>10	-
OTA[b]	>3	-	-	<19 uM**
PBN[a]	<0.125	<0.125	-	

* M x 10^{-3}
** M x 10^{-6}
a Target for glomeruli
b Target for proximal tubule
c Target for papilla
d Target for distal tubule
e Least nephrotoxic of the aminoglycosides
f Therapeutic agent without known nephrotoxic properties
\- No data

6.2.2.4 Discussion.

When Krebs was used as the incubation buffer, PAN affected PRO incorporation only at high concentrations (IC_{50} > 2.5 mM), but when the amino acid and glomeruli were incubated with Earles-HEPES buffer, the concentration of the drug that decreased the amino acid incorporation to 50% of the control was lower (1mM) than when the mixture was incubated with Krebs. This suggest the possibility of an increase in the sensitivity of the test, perhaps due to the increase of the rate of the incorporation of the amino acid, can account for this difference. However, the trend that PAN is not as potent in vitro glomerulotoxin as ADR, is maintained. This means that de novo synthesis of protein from PRO into isolated pig glomeruli exposed to the compound, is not as sensitive as it was for ADR, and in spite of the fact that PAN is regarded as a potent glomerulotoxin in vivo (Kreisberg and Karnovsky, 1983). The compound did not show the effect that would be expected in vitro, despite the fact that PAN shows a similar histopathological lesion to that caused by ADR. There was also a certain degree of inter-experimental variability that was not seen with the ADR studies in vitro.

When isolated rat glomeruli exposed to PAN was incubated with Tyrodes buffer, a moderate effect on PRO incorporation is obtained at high concentrations (IC_{50} = 2.5 mM) of the chemical. This is consistent with the observations in pig glomeruli, using Krebs as the incubation buffer. In previous studies (Norgaard, 1979), PAN did not show a potent effect when the morphology of isolated pig glomeruli exposed to the drug was examined, the compound did not have an acute effect on the epithelial cells, as shown by the staining with colloidal iron which was not altered by a short term exposure to PAN. The author suggested that the influence of PAN on glomerular morphology may be due to a long term effect of the

compound, but in our case it is more likely that it is not the compound per se, but a metabolite that exerts the effect in vivo or that a different biochemical mechanism is affected. PRO incorporation into glomeruli of rats that had been treated with PAN was reported to have decreased (Krisko and Walker, 1976), although the degree to which PRO was deppressed was not stated in the report. Studies on amino acid incorporation into GBM, have shown that the rate of incorporation of ^{3}H-PRO and its conversion to OH-PRO was increased in PAN treated animals (Blau and Michael, 1971). According to another report, however, the GBM in PAN-nephrotic rats showed decreased amounts of OH-PRO, OH-LYS and GLY (Kefalides and Forsell-Knott, 1970). At this stage it is not possible to relate these findings with those in the literature, because detailed studies of GBM were not performed. On the other hand, most of the observations on the effects of PAN on glomerular metabolism, have been undertaken on isolated glomeruli from nephrotic rats, this is generally done several days after PAN treatment, therefore the renal degenerative effect has been caused in vivo most likely by a metabolite, and not by the parent compound. By contrast glomeruli isolated from normal rats and subsequently exposed to PAN, appear to lack the metabolic components for activation or bioconversion. Regarding the in vitro effect of PAN on protein synthesis, so far there is a report where LEU incorporation into cultured glomerular epithelial cells was transiently decreased (Fishman and Karnovsky, 1985), after either a brief or continuous exposure. Although our findings are consistent, in so far as there was a decrease in the incorporation of PRO, at this stage it is not clear whether there was an earlier and greater decrease, followed by a recovery, and therefore the low inhibition produced. Further studies should be conducted to elucidate the effect of PAN metabolites on isolated glomeruli.

6.2.3 Streptomycin (STR).

6.2.3.1 Effect of STR on protein synthesis in isolated pig glomeruli incubated with Krebs buffer.

PRO incorporation into isolated pig glomeruli exposed to STR and incubated with Krebs is significantly decreased ($P \leq 0.01$) at all concentrations tested. The lowest concentration of the chemical (0.5 mM) depressed de novo synthesis of protein to 19% of the control values (Table 6.2.9 Appendix 2; Fig 6.2.8). The concentration of the drug required to produce a 50% inhibition from the control value was less than 0.5 mM.

PRO incorporation into isolated pig glomeruli exposed to STR is decreased at all concentrations tested, Fig. 6.2.9A and B. The results and levels of statistical significance are shown in Table 6.2.10 of Appendix 2. The concentration of the drug required to produce a 50% inhibition from the control value, was between 0.5 and 1.0 mM. The inter-experimental variability is only of 2-fold.

6.2.3.3 Effect of STR on protein synthesis into isolated rat glomeruli incubated with Tyrodes.

STR caused a remarkable and significant decrease ($P \leq 0.01$) at all concentrations tested. The lowest concentration assessed, inhibited protein synthesis from PRO almost completely (by 94% of the control). Table 6.2.11 Appendix 2 and Fig. 6.2.10).

6.2.3.4 Discussion

The data on STR effect on PRO incorporation into isolated pig glomeruli, using Krebs as the incubation buffer show a greater effect than in Earles-HEPES. In rat glomeruli, the effect of the drug on the incorporation of

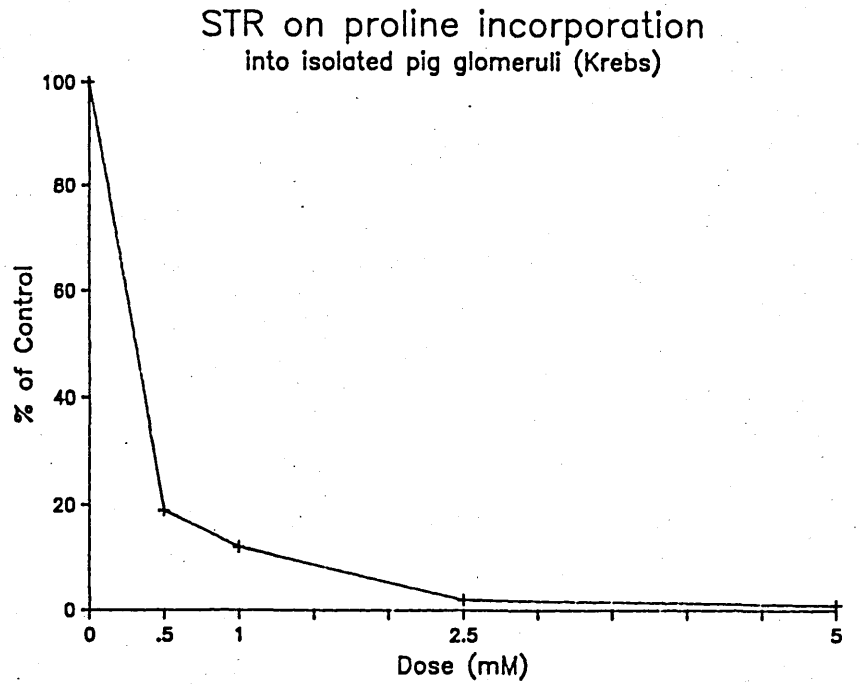


Fig. 6.2.8

0.5 ml aliquots of isolated pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of streptomycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

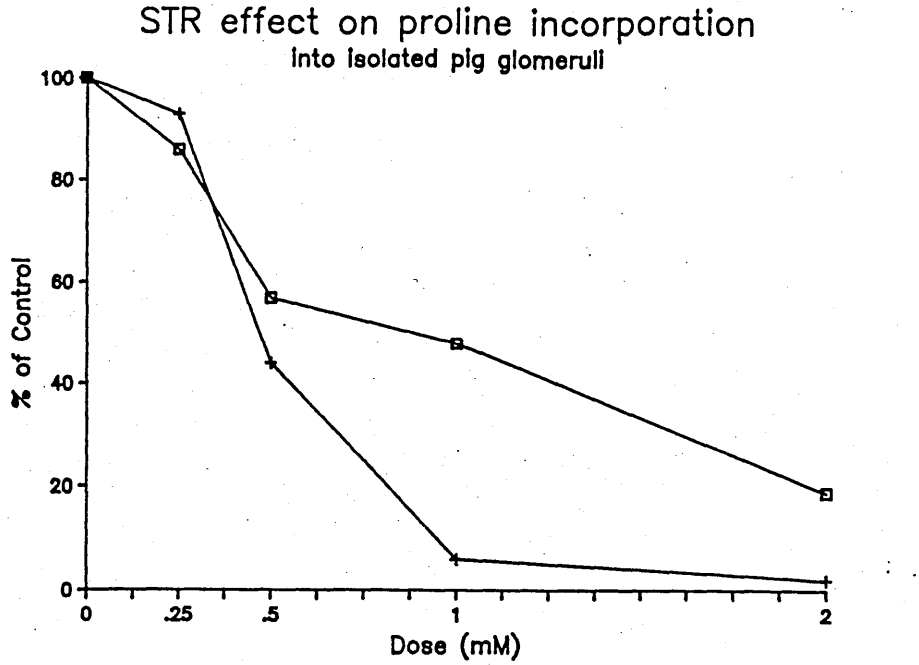


Fig. 6.2.9A

0.5 ml aliquots of isolated pig glomeruli suspended in Earles-HEPES buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of streptomycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

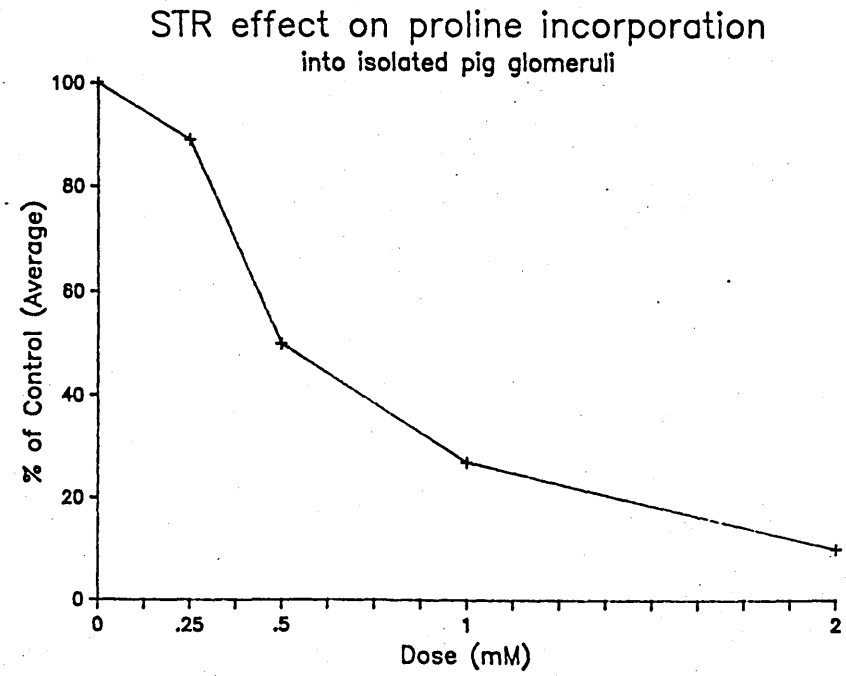


Fig. 6.2.9B

Data presented as the average of two experiments.

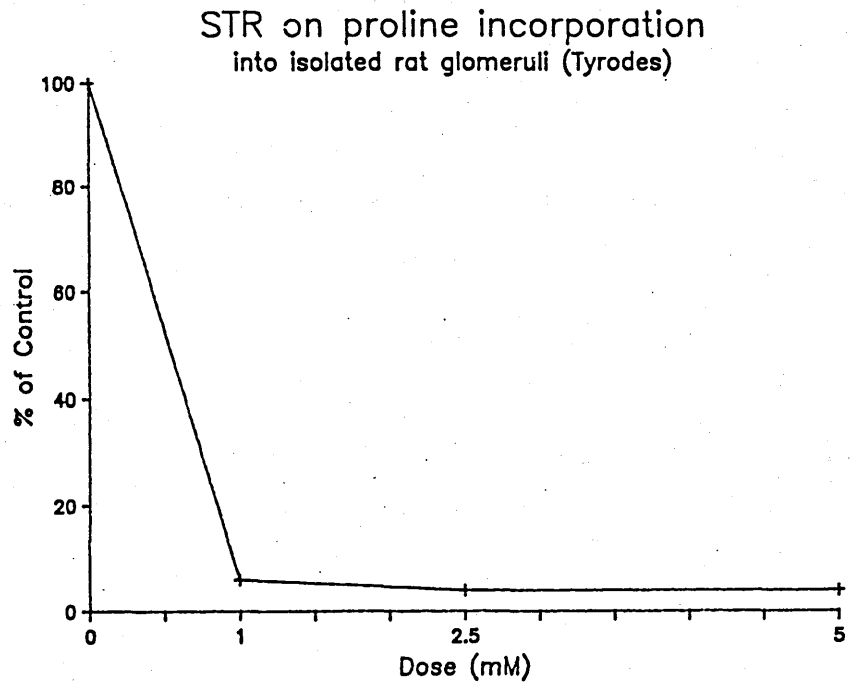


Fig. 6.2.10

0.5 ml aliquots of isolated rat glomeruli suspended in Tyrodes buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of streptomycin for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

the amino acid incubating with Tyrodes, was similar to that on pig glomeruli using Krebs. At this stage it is difficult to draw any conclusion from these data, and more work need to be done on STR toxicity on isolated glomeruli in order to obtain a proper and more consistent dose-response relationship.

6.2.4 Ethacrynic acid (ETA).

6.2.4.1 Effect of ETA on protein synthesis into isolated pig glomeruli incubated with Earles-HEPES.

PRO incorporation was strongly inhibited ($P \leq 0.001$) by increasing concentrations of ETA, as shown in Table 6.2.12 of Appendix 2 and Fig. 6.2.11.

6.2.4.2 Discussion

These data show that PRO incorporation was markedly depressed by the lowest concentration (0.1 mM) assayed. This means that ETA is a potent inhibitor of <u>de novo</u> synthesis of glomerular protein and the concentrations required to produce this effect are comparable to those for ADR. ETA is known to interact strongly with sulfhydryl groups of protein (Duggan and Noll, 1965), and the strong inhibition shown by the compound on <u>in vitro</u> protein synthesis by pig glomeruli may be related to this interaction.

ETA effects on fatty acid and glucose metabolism have been previously shown in isolated rat glomeruli by Meezan and Brendel (1973). However, there appear to be no previous reports on the effect of the compound on protein synthesis by isolated pig glomeruli, therefore this appears to be a novel finding.

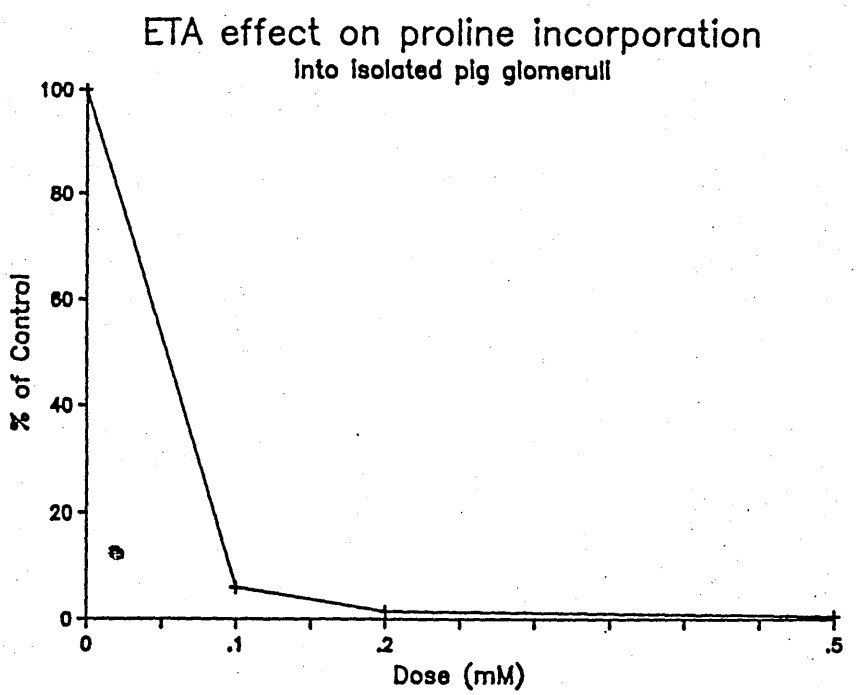


Fig. 6.2.11

0.5 ml aliquots of isolated pig glomeruli suspended in Earles-HEPES buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of ethacrynic acid for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

In view of the potent effect of the compound on glomerular protein synthesis and oxidative metabolism, and the fact that ETA has not been considered a toxic drug, several questions are raised, such as the importance of ETA effects on glomerular metabolism in vitro, as related to glomerular function in vivo. Whether the inhibition of protein synthesis is a secondary effect, or a direct effect. Possible GBM structural and compositional changes caused by ETA. Studies with ETA-related compounds and metabolites such as the saturated derivatives on in vitro glomerular protein synthesis may help to elucidate the mechanism of action.

6.2.5 2-Bromoethanamine (BEA)

6.2.5.1 Incorporation of PRO into isolated pig glomeruli exposed to BEA and incubated with Krebs.

At the maximum concentration tested of 10 mM (Figs. 6.2.12) BEA effect on PRO incorporation did not reach an IC_{50}. Table 6.2.13 of Appendix 2 shows the degree of statistical significance.

6.2.5.2 Incorporation of PRO into isolated pig glomeruli exposed to BEA and incubated with Earles-HEPES.

The effect of BEA on PRO incorporation is shown in Table 6.2.14 Appendix 2 and Figs. 6.2.13A and B. At the lower concentration tested (1mM), the amino acid incorporation was reduced significantly to 10% of the control value, and at increasing concentrations of the drug this was practically nil.

6.2.5.3 Incorporation of PRO into isolated rat glomeruli exposed to BEA and incubated with Tyrodes.

Low concentrations of this compound caused a slight and not significant increase on PRO incorporation. The dose of the compound needed to cause a

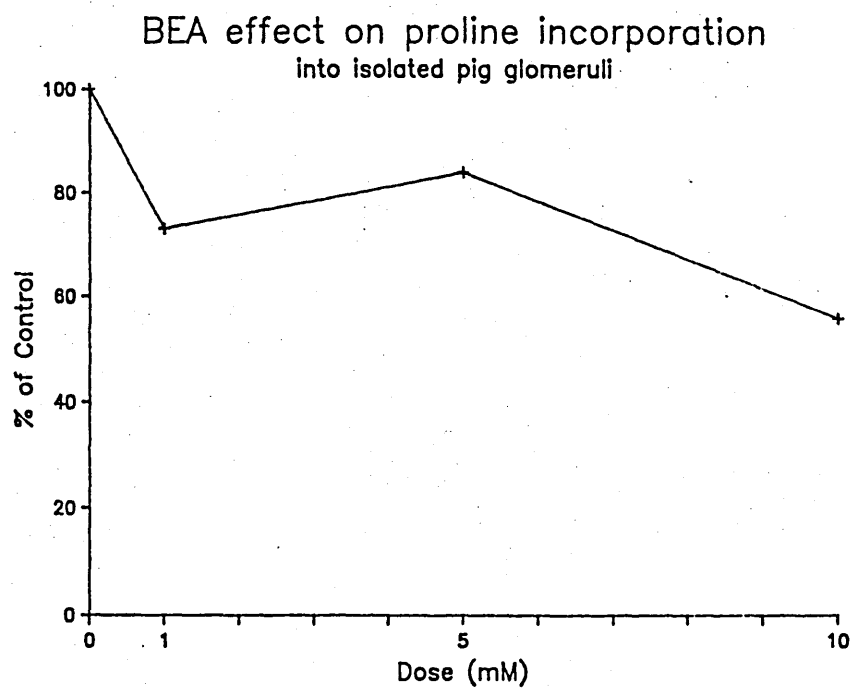


Fig. 6.2.12

0.5 ml aliquots of isolated pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of 2-bromoethanamine for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

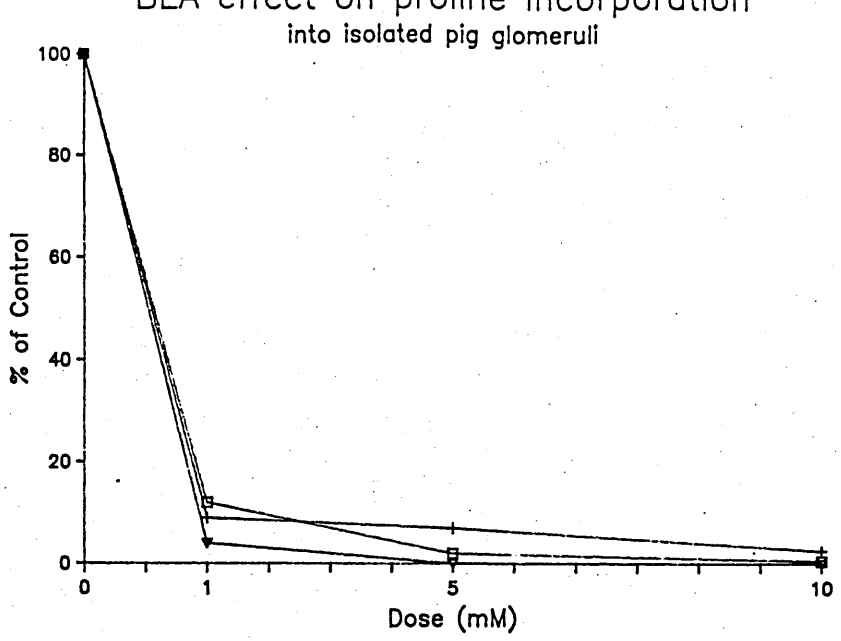


Fig. 6.2.13A

0.5 ml aliquots of isolated pig glomeruli suspended in Earles-HEPES buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of 2-bromoethanamine for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

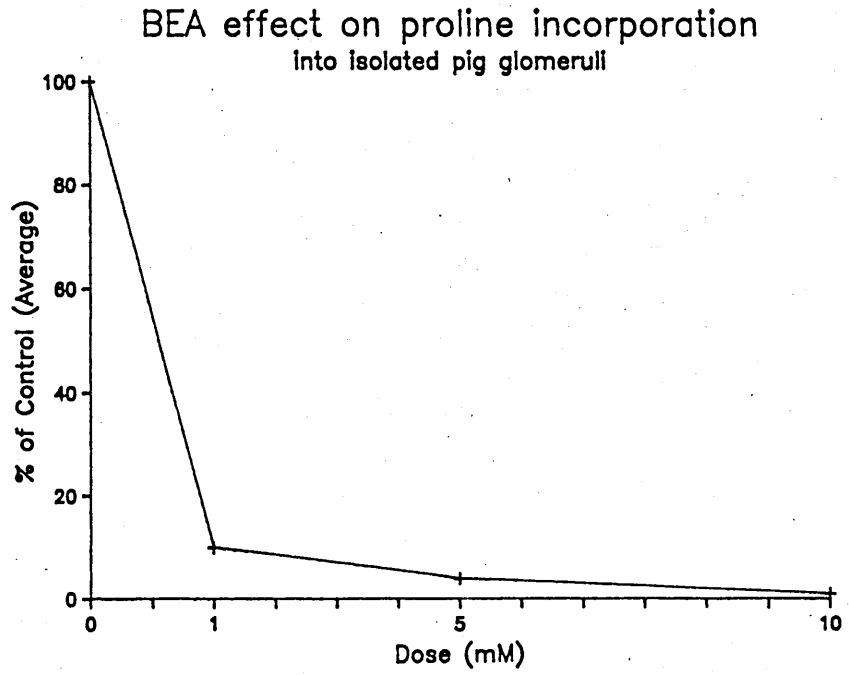


Fig. 6.2.13B

Data presented as the average of three experiments.

50% inhibitory effect ($P \leq 0.05$) was near 10 mM, under the conditions of this experiment (Fig. 6.2.14 and Table 6.2.15 Appendix 2)

6.2.5.4 Discussion.

The data on BEA effect on PRO incorporation using Krebs as the incubation buffer show that even at large concentrations (10 mM) BEA failed to cause an inhibition of 50% on the incorporation of PRO. However, the data on PRO incorporation using Earles-HEPES as the incubation buffer, show that 1 mM BEA depressed PRO incorporation to 10% of the control value. This variation in the behavior of the compound can be due to an increase in sensitivity with the second buffer. As no concentrations lower than 1mM of the compound were assessed, it is difficult to draw conclusions and compare this effect with the glomerular cytotoxicity of ADR and ETA. To estimate an IC_{50} value it is necessary to perform a dose-response curve, with concentrations below 1 mM. Due to the selective papillotoxicity shown by the drug in vivo, a strong effect of the drug on glomeruli would not be expected. However, BEA is regarded as a potential alkylating agent (Dermer and Ham, 1969), and therefore inhibition of protein synthesis could occur as a secondary effect.

6.2.6 Other toxins.

6.2.6.1 Folic acid

Folic acid caused a dose-related decrease on PRO incorporation into isolated pig glomeruli, when incubated with Krebs, which reached the IC_{50} at 5mM (Fig. 6.2.15; Table 6.2.16 Appendix 2). At 1 mM, there was a significant reduction ($P \leq 0.01$) from the control value by 25%, higher concentrations produced a highly significant reduction ($P \leq 0.001$) from the control value.

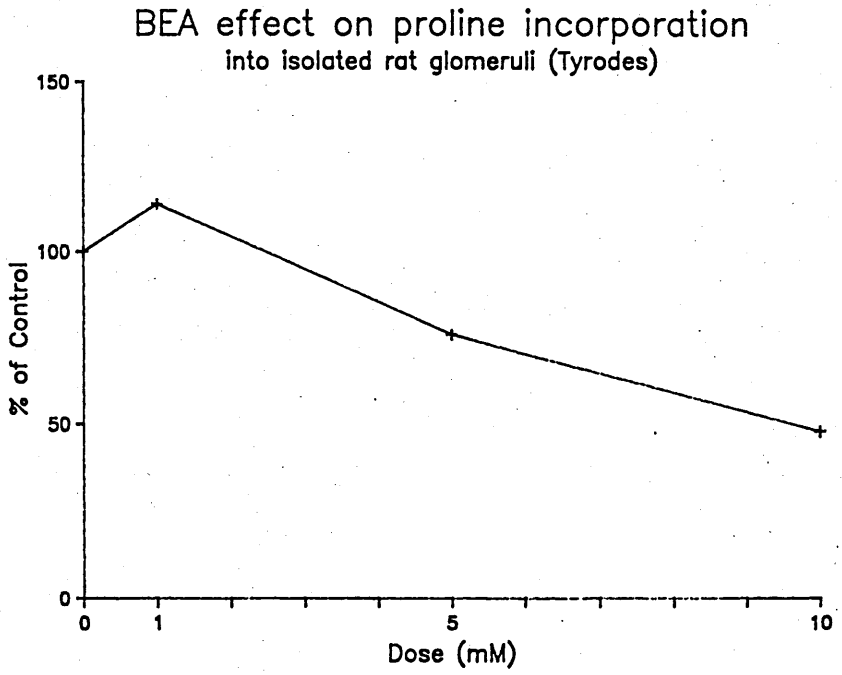


Fig. 6.2.14

0.5 ml aliquots of isolated rat glomeruli suspended in Tyrodes buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of 2-bromoethanamine for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

Low concentrations of folic acid only caused little effect on PRO incorporation by isolated rat glomeruli when incubated with Tyrodes (Fig. 6.2.16; Table 6.2.17 Appendix 2), which even did not reach a IC_{50} at the highest concentration tested (10 mM).

6.2.6.1.1 Discussion.

PRO incorporation into isolated pig glomeruli exposed to folic acid is reduced to 50% of the control by higher concentrations of the compound than in the case of ADR or even PAN. The in vitro effect of folic acid seems to correlate with the fact that it is another part of the nephron the one that is affected by this compound - i.e. distal tubule -.

The data on rat glomeruli show that de novo synthesis of protein is slightly inhibited by large concentrations of folic acid, and from the data on pig glomeruli it can be assumed that rat glomeruli are less sensitive to the effect of the compound. However, it is necessary to confirm this using the same buffer system (ie. Earles-HEPES) for both types of glomeruli.

6.2.6.2 Ochratoxin A (OTA).

The effect of ochratoxin A on PRO incorporation into isolated pig glomeruli was assayed at two concentrations (Table 6.2.18 Appendix 2 and Figs. 6.2.17) and neither caused an inhibitory effect of 50% on the amino acid incorporation. The reduction in de novo protein synthesis caused by 3.2 mM OTA, was significantly different from the control value ($P \leq 0.01$).

6.2.6.2.1 Discussion.

The data on the effect of OTA show that none of the concentrations tested caused a reduction of 50% on the incorporation of PRO. This means that

OTA, a mycotoxin regarded as targeting for proximal tubule, does not have a selective inhibitory effect on de novo synthesis of protein when glomeruli have been exposed to it in the present in vitro system.

6.2.6.3 Polybrene.

All concentrations of polybrene, assayed on isolated pig and rat glomeruli, significantly depressed PRO incorporation as can be seen in Figs. 6.2.18 and 6.2.19A and B and Tables 6.2.19 and 20 of Appendix 2.

6.2.6.3.1 Discussion.

These data show polybrene as being a strong inhibitor of de novo synthesis of protein in isolated rat glomeruli. This confirms previous observations under the same experimental conditions (Ahmed et al., 1987). The effect of this compound on glomerular protein synthesis may be in connection to the changes in GBM and glomerular epithelial cells after the administration of the compound in vivo (Hunsicker et al, 1981).

It is necessary to perform a dose-response curve for polybrene, with a lower range of concentrations, in order to estimate the IC_{50} value.

6.2.7 Summary and conclusions

6.2.7.1 Summary.

1- ADR considerably depressed the incorporation of all amino acids into isolated pig glomeruli, with LEU and PRO, being the most sensitive and showing a similar effect, and LYS being the least sensitive.

2- PAN and STR affected PRO incorporation into isolated glomeruli only at high concentrations ($IC_{50} \geq$ 1mM and $\geq$ 0.5 respectively) as can be seen in Fig. 6.2.20.

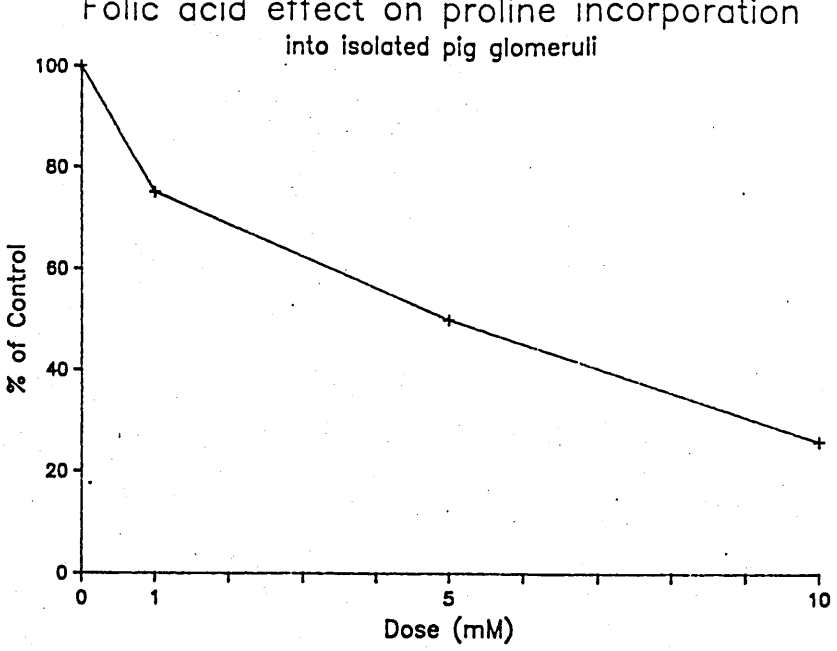


Fig. 6.2.15

0.5 ml aliquots of isolated pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of folic acid for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

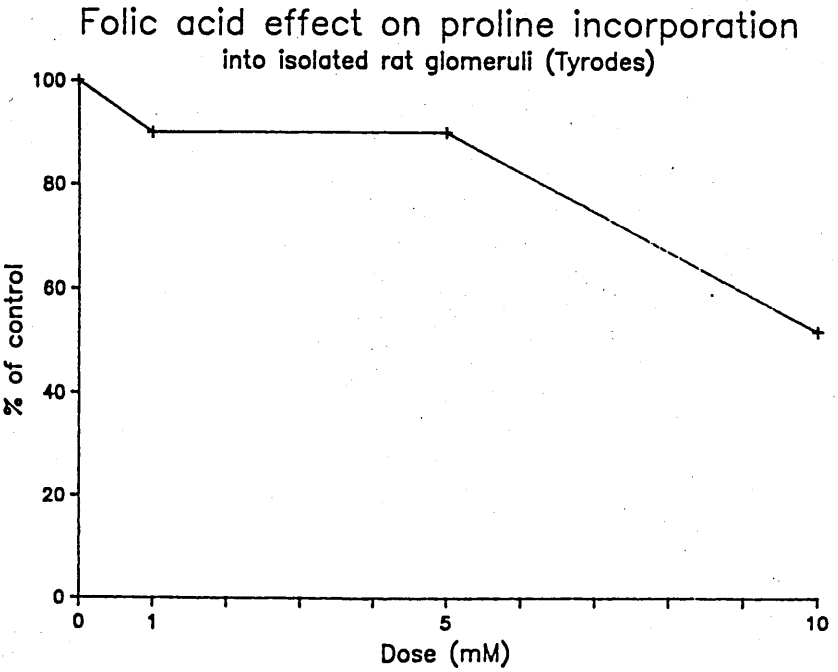


Fig. 6.2.16

0.5 ml aliquots of isolated rat glomeruli suspended in Tyrodes buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of folic acid for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

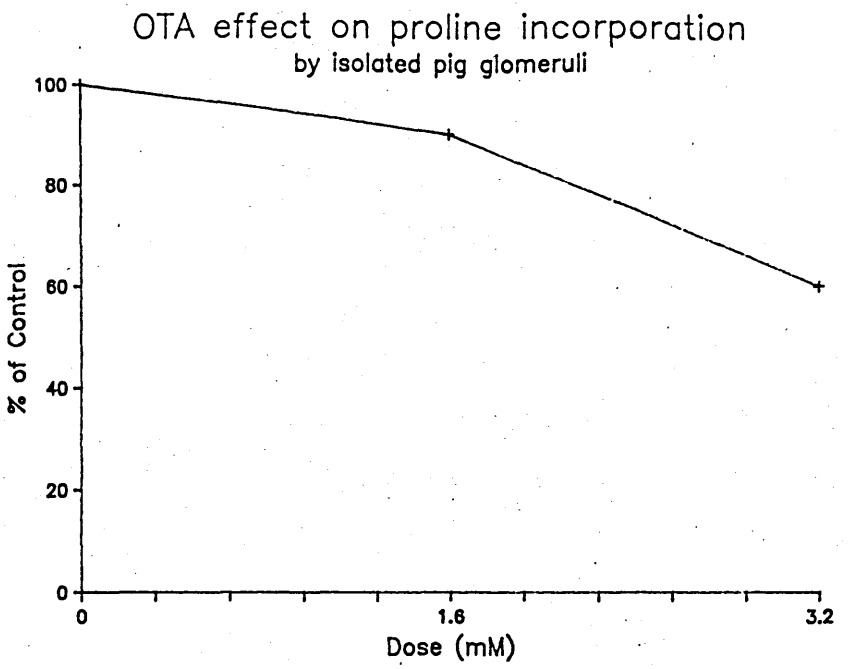


Fig. 6.2.17

0.5 ml aliquots of isolated pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of ochratoxin A for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

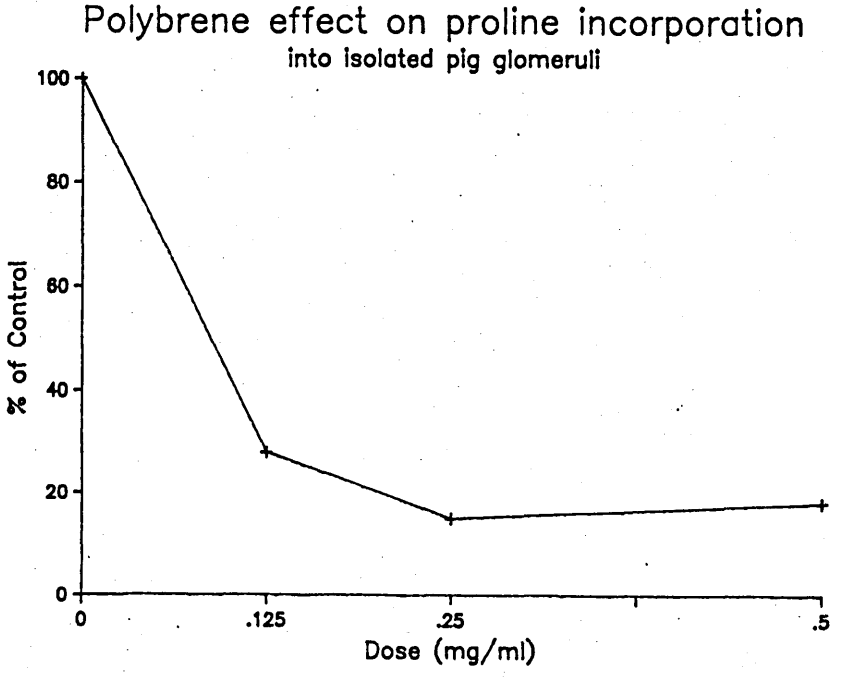


Fig. 6.2.18

0.5 ml aliquots of isolated pig glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of polybrene for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

3- PRO incorporation into isolated pig glomeruli was markedly depressed by ETA (IC_{50} = 0.05 mM).

4- The effect of STR and BEA on PRO incorporation into isolated pig glomeruli incubated with different buffers varies (IC_{50} with Earles-HEPES = 0.5 mM for STR and < 1mM for BEA; with Krebs << 0.5 for STR and > 10 mM for BEA).

6.2.7.2 Conclusions.

1- De novo synthesis of glomerular protein from different amino acids in pig glomeruli is inhibited by ADR; some amino acids (ie. LEU and PRO) are more affected than others (ie. GLY, HIS and LYS).

2- From the amino acids assessed, PRO incorporation appeared to be the most sensitive index of cytotoxicity in pig glomeruli caused by ADR, as has already been shown in rat.

3- De novo synthesis of protein from PRO into isolated pig glomeruli exposed to PAN, is not as sensitive as it was for ADR, and in spite of the fact that PAN is regarded as a potent glomerulotoxin in vivo.

4- STR and BEA effect on protein synthesis needs further elucidation.

5- ETA is a potent inhibitor of de novo synthesis of glomerular protein and the concentrations required to produce this effect seem comparable to those for ADR.

7- ETA effect on protein synthesis by isolated pig glomeruli appears to be a novel finding.

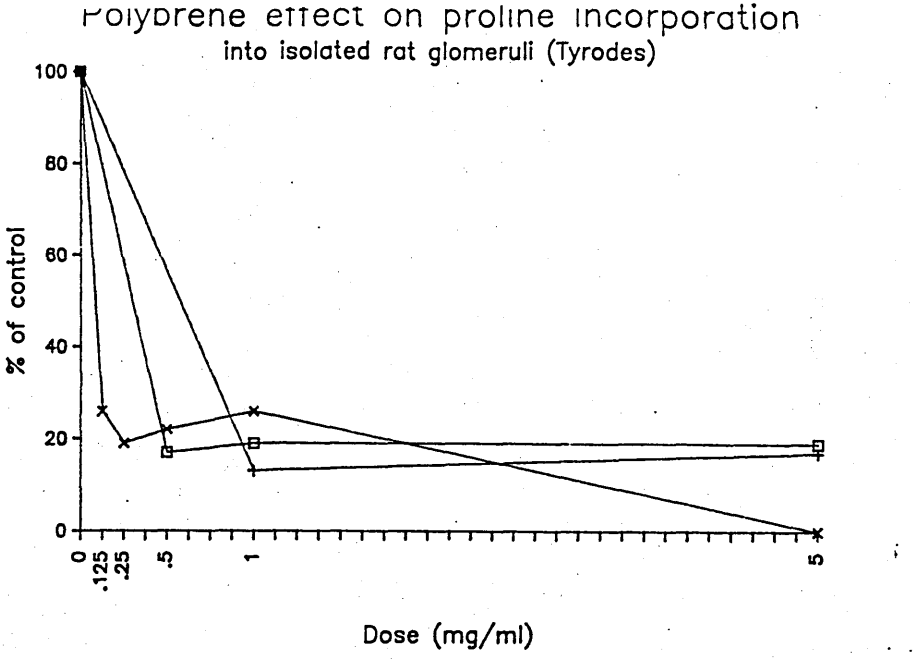


Fig. 6.2.19A

0.5 ml aliquots of isolated rat glomeruli suspended in Tyrodes buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline and different concentrations of polybrene for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total incorporation of ^{3}H-proline into isolated pig glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

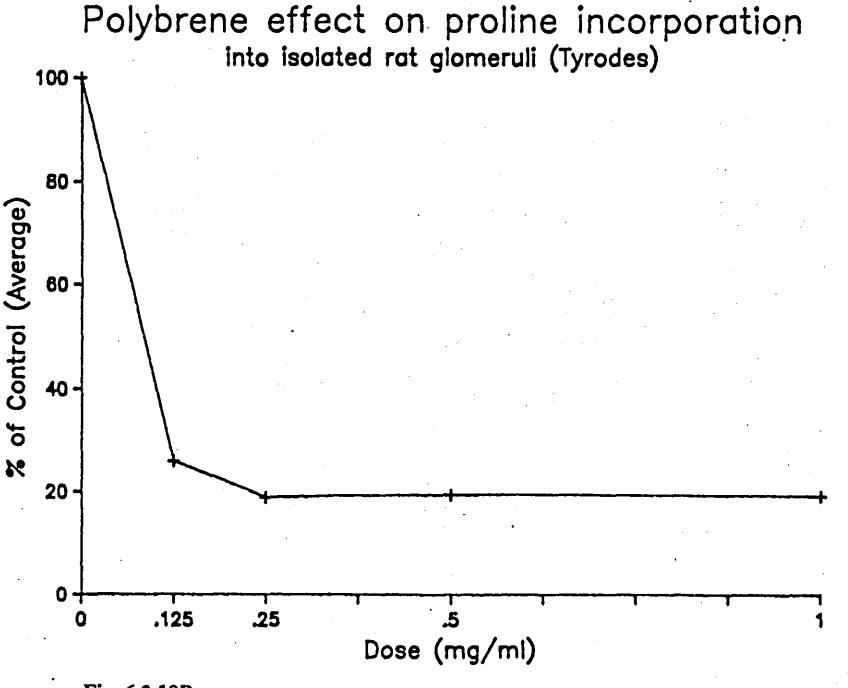


Fig. 6.2.19B

Data presented as the average of three experiments.

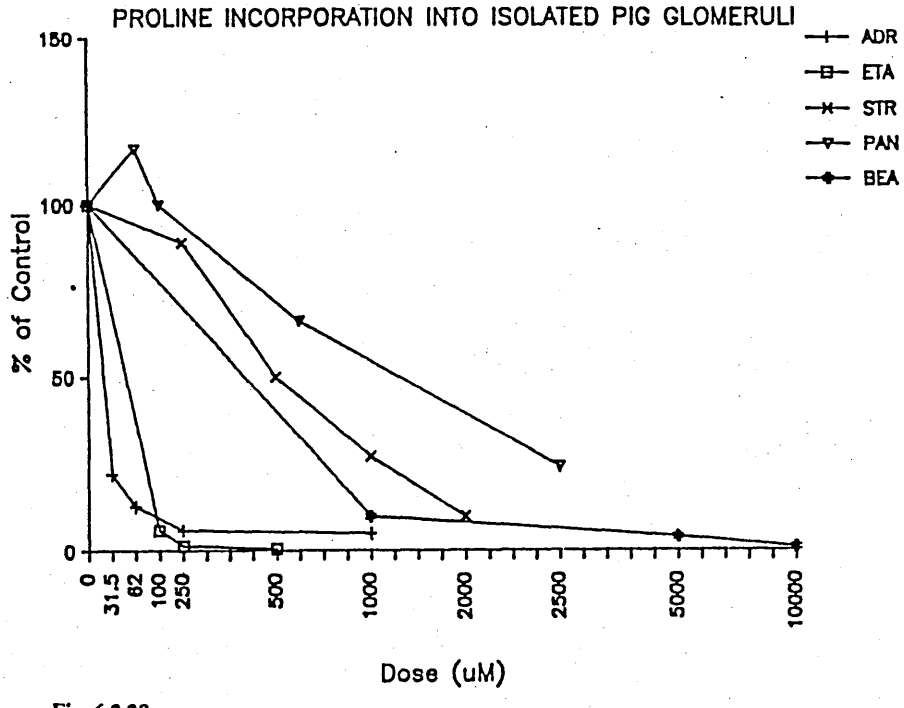


Fig. 6.2.20

Dose-response of proline incorporation into isolated pig glomeruli exposed to different chemicals. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi of ^{3}H-proline and different concentrations of the chemical. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the activity incorporated into isolated pig glomeruli incubated without the chemical (control). Each time point is the result of triplicate incubations.

6.3 Oxidative Metabolism.

6.3.1 Setting of the conditions to perform oxidative metabolism

6.3.1.1 Influence of non-radioactive substrates on the metabolism of the labelled substrate.

For the study of oxidative metabolism by isolated pig glomeruli the effect of non-radioactive glucose, linolenic and oleic acid when incubated together with the respective labelled substrates on the release of $^{14}CO_2$ is shown in Figs. 6.3.1 and Table 6.3.1 Appendix 2. Low concentrations of glucose (10 - 100 uM) did not cause an effect but at 1000 uM there was a highly significant decrease ($P \leq 0.001$) to 76% of the control. On the other hand all concentrations of the non-labelled linolenic acid caused a dose-related inhibitory effect on the metabolism of the radioactive compound, which was highly significantly different ($P \leq 0.001$) from the control. A similar effect was shown by increasing concentrations of non-radioactive oleic acid on the labelled substrate. At concentrations of 10 uM of non-radioactive oleic acid, the metabolism was significantly lower ($P \leq 0.05$) than the control. At 100 uM the metabolism was significantly reduced ($P \leq 0.01$) by 49% relative to the control, and at 1000 uM the reduction was highly significantly different from the control ($P \leq 0.001$), by 85%.

6.3.1.2 Effect of shaking.

The effect of shaking during incubation of glomeruli on the oxidative metabolism of glucose, linolenic acid and oleic acid was tested (Fig. 6.3.2 and Table 6.3.2 Appendix 2). Linolenic and oleic acid release of $^{14}CO_2$ under shaking conditions (120 oscillations/min) was highly significantly higher ($P \leq 0.001$) than when the samples were not agitated, while glucose values of $^{14}CO_2$ release under shaking conditions were

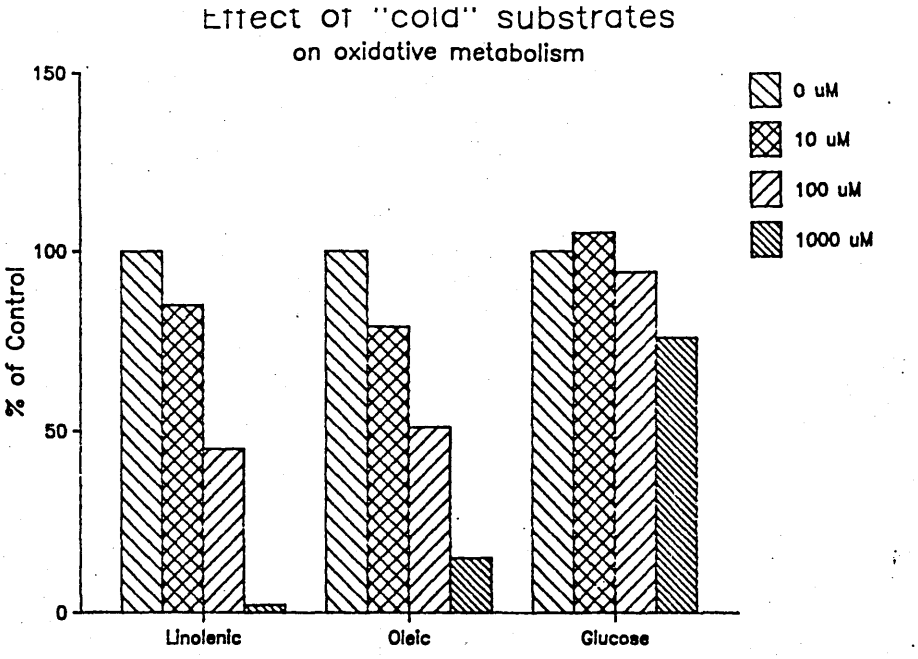


Fig. 6.3.1

0.5 ml aliquots of pig glomeruli suspended in Earles-HEPES buffer were incubated with 0.5 ml of buffer containing either ^{14}C- glucose (2.0 uCi) or linolenic or oleic acid (50 nCi), and different concentrations of each of the respective "cold" substrates for 4 hrs. The heights of the bars represents the proportion of $^{14}CO_2$ released, calculated as a percentage of the total $^{14}CO_2$ released by isolated glomeruli incubated only with the radiolabelled substrate (control).

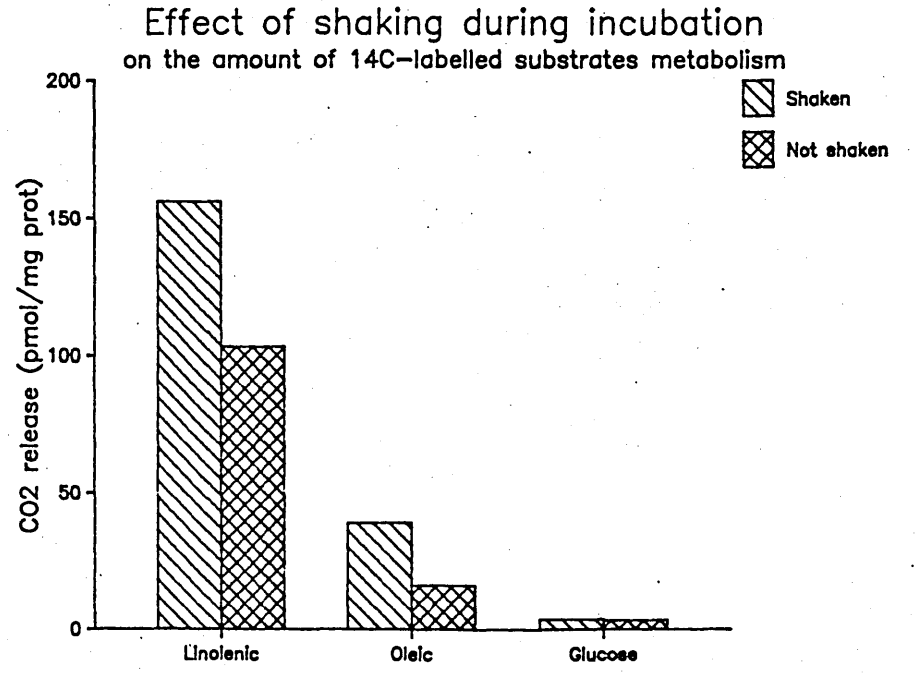


Fig. 6.3.2

0.5 ml aliquots of pig glomeruli suspended in Earles-HEPES buffer were incubated with 0.5 ml of buffer containing either ^{14}C- glucose (2.0 uCi) or linolenic or oleic acid (50 nCi), and $^{14}CO_2$ release assessed with and without agitation during incubation. The heights of the bars represents the proportion of $^{14}CO_2$ released.

significantly different from the non-agitated samples at the 5% level.

6.3.2 Glucose

6.3.2.1 Isolated pig glomeruli.

The generation of $^{14}CO_2$ from ^{14}C-glucose oxidation, using glomeruli incubated in Earles-HEPES buffer, was compared between several experiments. In most of the cases $^{14}CO_2$ release followed a linear pattern with time (Fig. 6.3.3, Table 6.3.3 Appendix 2) and the values ranged from 63 - 83 pmol CO_2/mg protein.

Two out of seven experiments had much higher $^{14}CO_2$ generated at all time points. After 4 h of incubation the values were 127 and 189 pmol CO_2/mg protein. In most cases the inter-experimental variability was low, with coefficient of variance $\leq$ 10%. The CO_2 release from glucose showed statistically significant differences within all time points studied in each experiment as can be seen in Table 6.3.4 of Appendix 2, with the exception of one in which there were no significant differences between CO_2 release at 3 and 4 hr.

6.3.2.2 Isolated rat glomeruli.

The rate of $^{14}CO_2$ release from labelled glucose was found to be linear when incubated for the first 3 hr, and at 4 hr, the rate of oxidation was the same as that at 3 hr (Fig. 6.3.4). The rate of CO_2 formation is shown in Table 6.3.5 Appendix 2.

6.3.2.3 Discussion.

The effect of shaking and addition of non-labelled substrates to the incubation media. The data on the comparison on the effect of shaking on oxidative metabolism show that it increases with agitation, supposedly

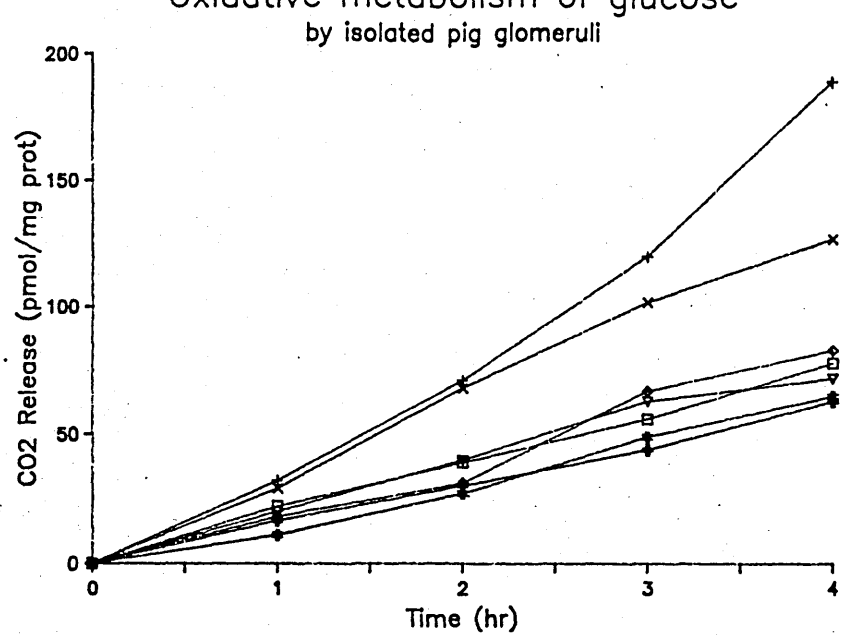


Fig. 6.3.3

Kinetics of glucose oxidation by isolated pig glomeruli. 0.5 ml of Earles-HEPES buffer containing 2 uCi of ^{14}C-glucose was incubated with 0.5 ml of the glomerular preparation for up to 4 hr. The ordinate represents specific activity of $^{14}CO_2$ trapped into NaOH. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

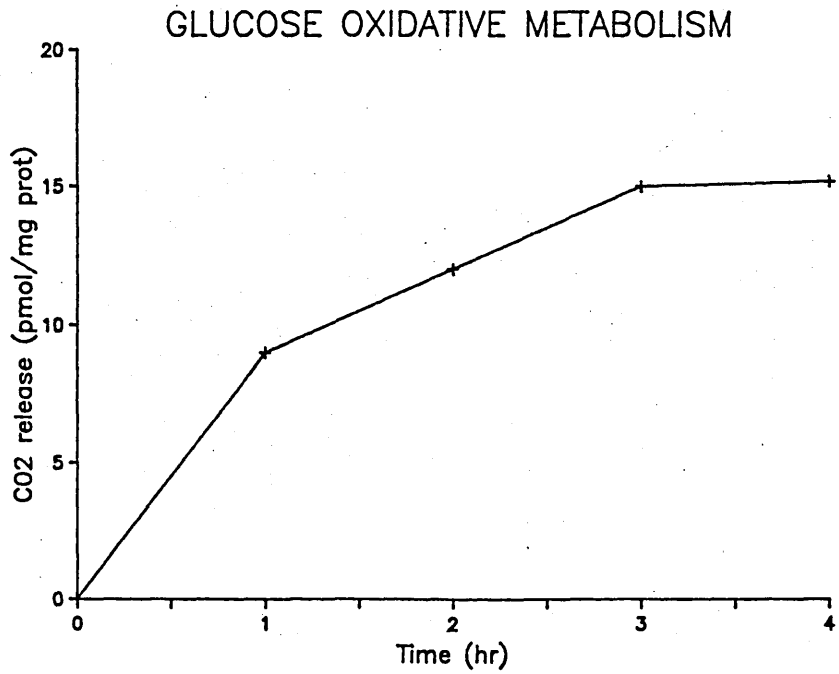


Fig. 6.3.4

Kinetics of glucose oxidation by isolated rat glomeruli. 0.5 ml of Earles-HEPES buffer containing 2 uCi of ^{14}C-glucose was incubated with 0.5 ml of the glomerular preparation for up to 4 hr. The ordinate represents specific activity of $^{14}CO_2$ trapped into NaOH. Each time point is the result of triplicate incubations.

because the the composition of the mixture is maintained homogeneous and also because aggregation of glomeruli is avoided, increasing in this way the surface that is exposed to the incubation mixture.

Addition of increasing concentrations of the non-radiolabelled precursor, decreased the respective radioactive yield, of oxidative metabolism. This is probably due to the "dilution" of the radiolabelled compound by competitive inhibition, therefore decreasing the chance of the labelled molecule being used as the only source of substrate for oxidative metabolism. In view of the observed effect of the non-radiolabelled substrates, it was decided not to add them to their respective incubation media, in order to get higher radioactive counts and therefore increased sensitivity of the method.

Glucose metabolism. Isolated pig glomeruli glucose metabolism was linear over 4 hr of incubation. The values of CO_2 release fell into two different populations, one having higher values after 4 hr of incubation (119 - 189 pmol $^{14}CO_2$/mg protein). However, most of the experiments fell in the lower values (63 - 83 pmol $^{14}CO_2$). The differences with the other one can be explained by the different origin of the pigs sampled.

Isolated rat glomeruli glucose oxidation seemed to begin to decrease the release of $^{14}CO_2$, after 3 hr. The possibility that may account for this effect, is a decrease in the viability of the glomerular preparation, and not a species difference, since the findings by Meezan and Brendel (1973), show a linear increase of the rate of glucose oxidation in their glomerular preparation.

6.3.3. Fatty acid metabolism

6.3.3.1 Metabolism of linolenic acid.

Pig glomeruli. A comparison on the release of CO_2 between different experiments was performed. In most of the cases there was a linear release of CO_2 with time (Fig. 6.3.5, Table 6.3.6 Appendix 2). Although there was a high inter-experimental variability, the variability within experiments and triplicates was low (≤10%). Table 6.3.7 Appendix 2 shows the level of statistical significance between different time points for each experiment.

Linolenic acid metabolism by isolated rat glomeruli. Oxidative metabolism of labelled linolenic acid was measured by the release of $^{14}CO_2$ over 4 hr, which was linear during the first 3 hr (Fig. 6.3.6, Table 6.3.8 Appendix 2) and presented a decrease at 4 hr.

6.3.3.2 Oleic acid metabolism by isolated pig glomeruli. For most of the cases the oxidative metabolism of oleic acid followed a very similar pattern to that of linolenic acid. $^{14}CO_2$ release was linear with time (Fig. 6.3.7, Table 6.3.9 Appendix 2) as for linolenic acid. The variability within triplicates performed in the same sample was around 10% in most cases. $^{14}CO_2$ release from linolenic and oleic acid showed statistically significant differences between all time points studied for each experiment (Table 6.3.10 Appendix 2). Only in one experiment, there were not a significant difference between the release of $^{14}CO_2$ at 3 and 4 hr and in another one at 1 and 2 hr.

6.3.3.3 Discussion.

These data show that isolated pig glomeruli metabolize oleic and linolenic acid linearly over a period of 4 hr. The maintenance of good rates of

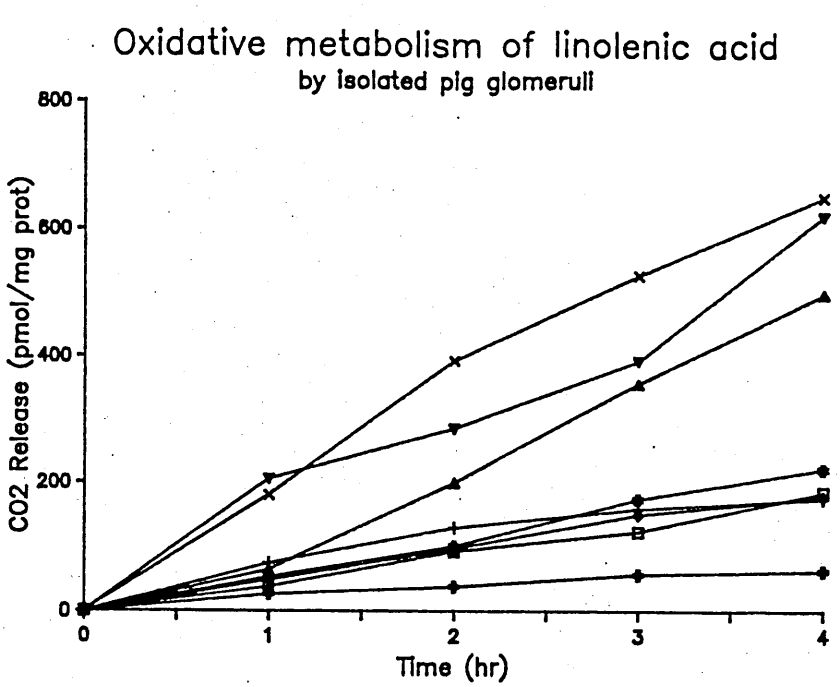


Fig. 6.3.5

Kinetics of linolenic acid oxidation by isolated pig glomeruli. 0.5 ml of Earles-HEPES buffer containing 50 nCi of ^{14}C-linolenic acid was incubated with 0.5 ml of the glomerular preparation for up to 4 hr. The ordinate represents specific activity of $^{14}CO_2$ trapped into NaOH. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

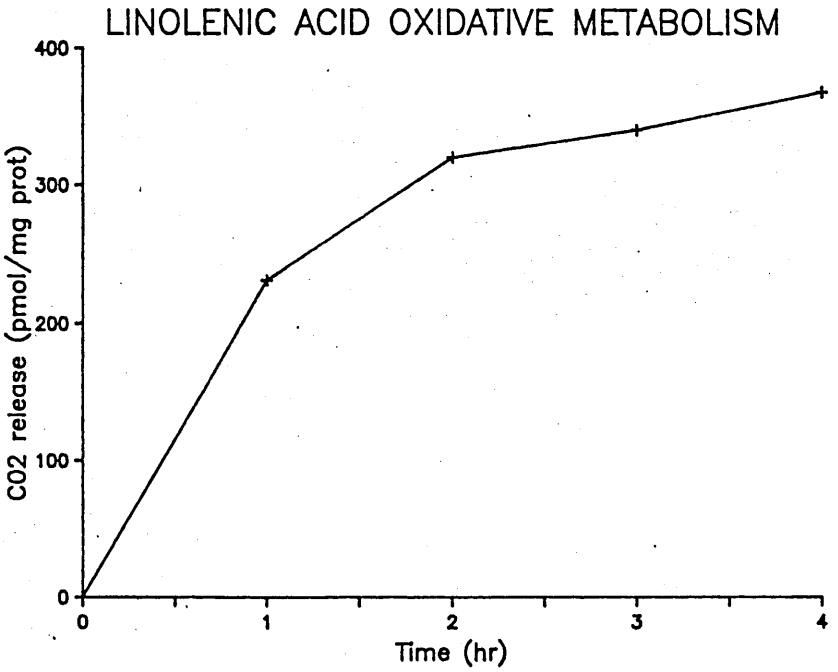


Fig. 6.3.6

Kinetics of linolenic acid oxidation by isolated rat glomeruli. 0.5 ml of Earles-HEPES buffer containing 50 nCi of ^{14}C-linolenic acid was incubated with 0.5 ml of the glomerular preparation for up to 4 hr. The ordinate represents specific activity of $^{14}CO_2$ trapped into NaOH. Each time point is the result of triplicate incubations.

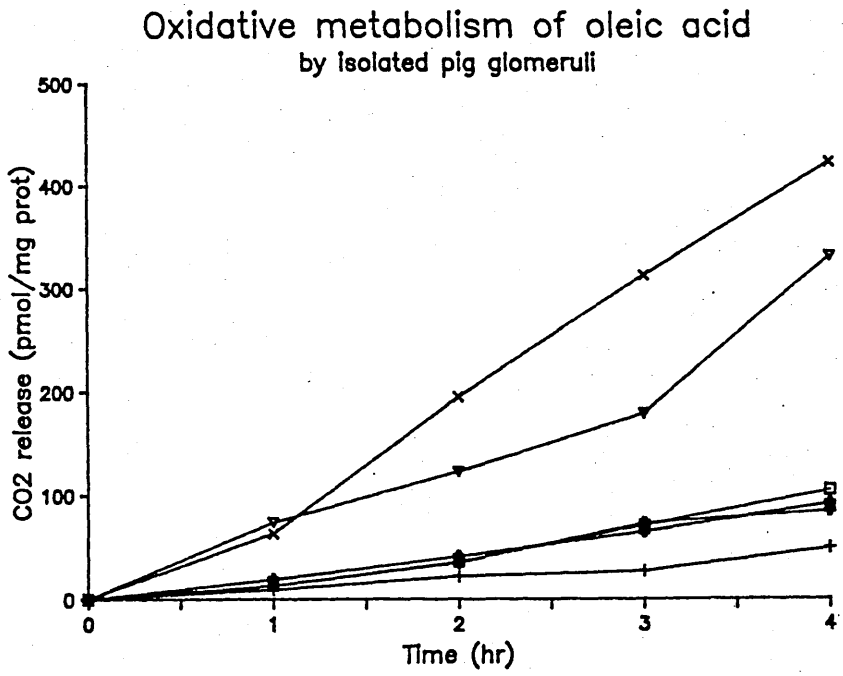


Fig. 6.3.7

Kinetics of oleic acid oxidation by isolated pig glomeruli. 0.5 ml of Earles-HEPES buffer containing 50 nCi of ^{14}C-oleic acid was incubated with 0.5 ml of the glomerular preparation for up to 4 hr. The ordinate represents specific activity of $^{14}CO_2$ trapped into NaOH. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

oxidation of fatty acids, for several hours is another indication of the viability of isolated pig glomeruli.

Fatty acid oxidation is performed mainly by the beta-oxidative system, which takes place in the mitochondria and is a major source of energy for the cell, this energy is generated as ATP by means of the oxidative phosphorylation. The oxidation of fatty acids is an important source of energy in the kidney (Hohenegger, 1976), being higher in the cortex than in the medulla.

There is also beta-oxidation in the peroxisome, a subcellular organelle, capable of respiration. The function of peroxisomal beta-oxidation is still poorly understood and its contribution to fatty acid oxidation is still controversial. Although peroxisomes of the kidney have not been comprehensively studied, they have been histochemically identified in some renal regions with the exception of glomeruli (Novikoff and Goldfisher, 1969), but catalase activity has been found in this part of the nephron, suggesting the presence of functional peroxisomes (Beard et al, 1985).

Further studies are necessary to confirm the presence of peroxisomes in glomeruli, and to establish the contribution made by peroxisomal beta-oxidation to fatty acid oxidation in glomeruli and tubules.

6.3.4 Summary and conclusions.

6.3.4.1 Summary.

1- This research has shown that pig glomeruli metabolize glucose and fatty acids (linolenic and oleic acid) linearly for several hours.

2- Glucose oxidative metabolism is much lower than fatty acid oxidation for both species assessed.

3- Glucose metabolism is lower in rat than in pig glomeruli.

4- Oleic acid rate of oxidation is lower than linolenic acid.

6.3.4.2 Conclusions.

1- Isolated rat and pig glomeruli have the ability to oxidize radiolabelled glucose and fatty acids (oleic and linolenic acid) to $^{14}CO_2$.

2- The higher rates of $^{14}CO_2$ production from fatty acids than glucose, suggests that fatty acids are a more important source of energy in glomeruli.

3- The maintenance of good rates of oxidation of glucose, linolenic and oleic acid shows the metabolic integrity of the glomerular preparation in isolated pig glomeruli.

4- Further studies are necessary to find out whether the lower rates of rat glomerular oxidative metabolism in comparison to pig glomeruli, corresponds to a species differences or loss of the viability of the glomerular preparation.

6.4 Effect of different chemicals on oxidative metabolism by isolated pig glomeruli.

6.4.1 Effect of ADR on glucose and fatty acid metabolism.

6.4.1.1 Glucose

When the effect of several concentrations of ADR (0.031 - 1 mM) was assessed on the release of $^{14}CO_2$ from glucose, the highest concentration of the drug (0.25 and 1 mM), caused a significant inhibition, as can be seen in Table 6.4.1 of Appendix 2 and in Figs. 6.4.1 and B.

6.4.2.2 Fatty acids

a)- Linolenic acid. All concentrations of ADR (0.031 - 1 mM), reduced significantly the release of $^{14}CO_2$ from linolenic acid (Table 6.4.2 Appendix 2), with the exception of 0.031 mM which caused a slight, but not significant decrease in one of the experiments. Figs. 6.4.2A and B show the release of $^{14}CO_2$ as percentage of the control and as an average of 2 experiments.

b)- Oleic acid. Oleic acid metabolism was markedly depressed by all concentrations of ADR assessed (0.031 - 1 mM). The reduction was highly significantly different ($P \leq 0.001$) from the control values as is shown in Table 6.4.3 Appendix 2. $^{14}CO_2$ release as percentage of the control, is shown in Fig. 6.4.3.

6.4.1.3 Discussion

Effect of ADR on glucose oxidation. The data show that ADR inhibited $^{14}CO_2$ release from glucose to a certain extent, however this inhibition is not as marked as in the case of protein synthesis in which the concentration required to cause a 50% inhibition is 3-fold lower than for inhibition glomerular glucose metabolism. Glucose oxidative metabolism is

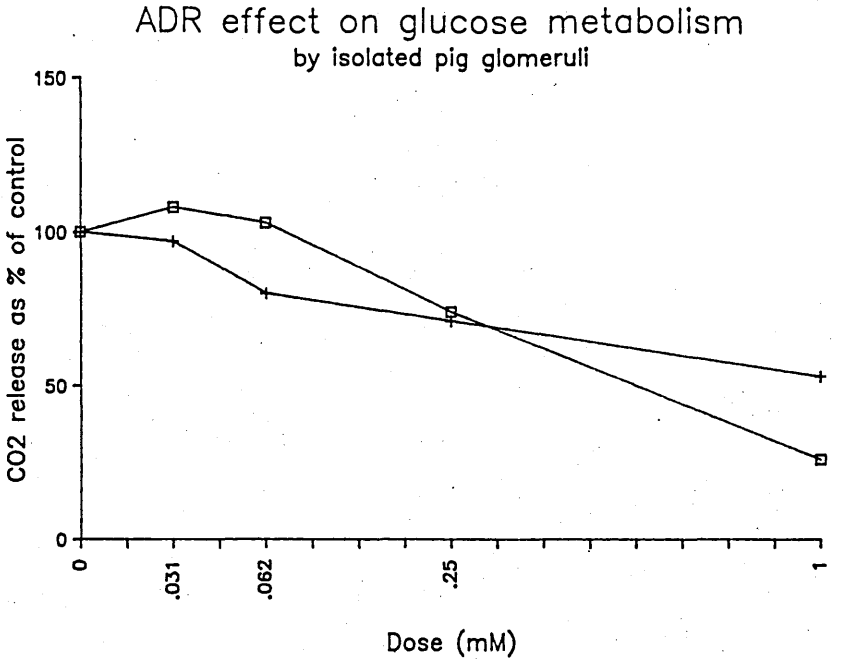


Fig. 6.4.1A

Dose-response of glucose oxidation by isolated pig glomeruli exposed to adriamycin. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C glucose an different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

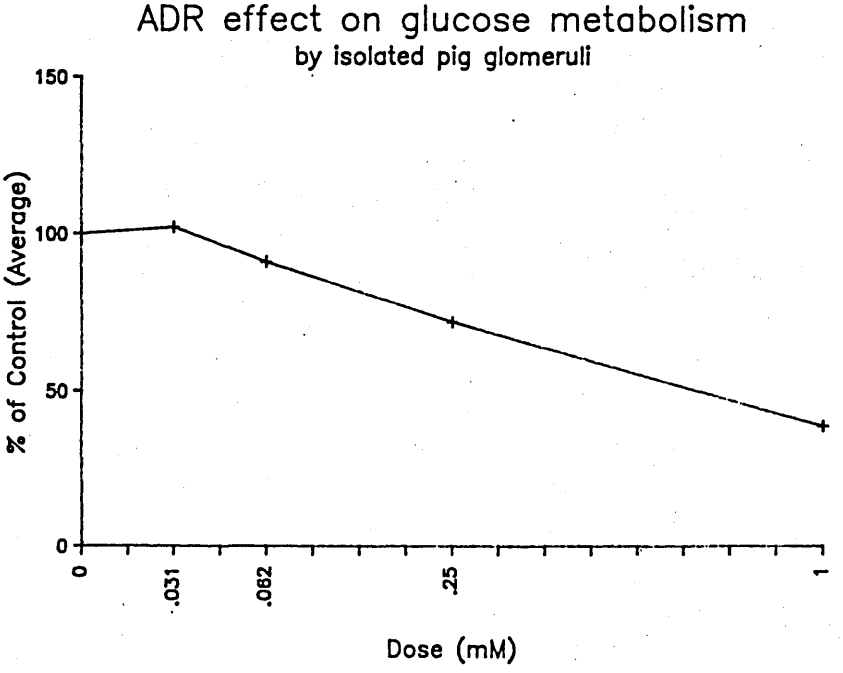


Fig. 6.4.1B

Data presented as the average of two experiments.

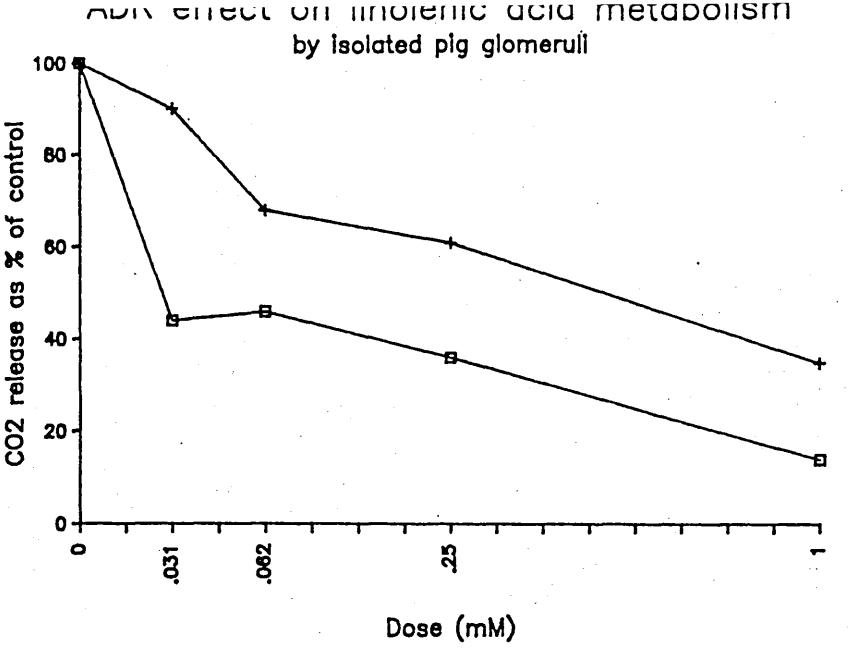


Fig. 6.4.2A

Dose-response of linolenic acid oxidation by isolated pig glomeruli exposed to adriamycin. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-linolenic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

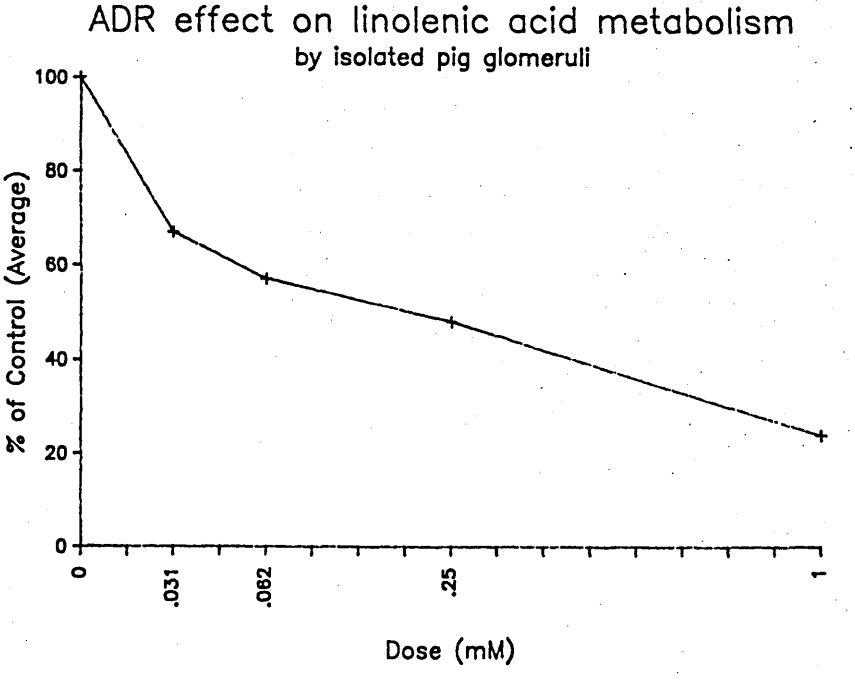


Fig. 6.4.2B

Data presented as the average of two experiments.

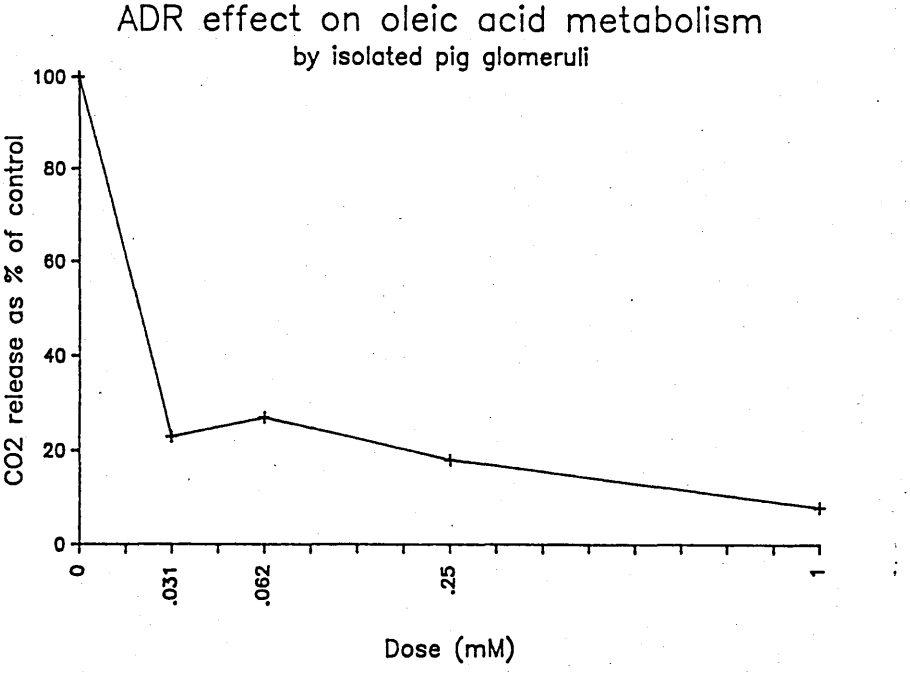


Fig. 6.4.3

Dose-response of oleic acid oxidation by isolated pig glomeruli exposed to adriamycin. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-oleic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-oleic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

also less sensitive to ADR than fatty acid metabolism.

Effect of ADR on fatty acids. These data show that ADR markedly inhibited the oxidative metabolism of oleic acid (IC_{50} < 31.5 uM) and although linolenic acid was inhibited in a dose related fashion, this effect was only moderate (IC_{50} = 0.21 mM). There are no previous observations on the effect of ADR on fatty acid metabolism of isolated glomeruli of any species. These findings are however consistent with observations of the inhibition of the oxidation of palmitic acid to CO_2 by renal mitochondria of ADR treated (Bizzi et al, 1983), although the type of cell responsible for this effect was not specified in this report.

A dose-response curve is needed to follow the effect of ADR on oleic acid oxidation at concentrations lower than 31.2 uM.

ADR effect on glucose and fatty acid metabolism may be of importance in connection with the degenerative changes described in renal mitochondria in vivo, using ultrastructural methods (Bertani et al, 1982).

6.4.2 Effect of PAN on oxidative metabolism.

6.4.2.1 Glucose.

The effect of PAN on glucose metabolism is shown in Figs. 6.4.4A and B Although the compound did not show a strong effect on glucose metabolism, this was decreased significantly at all concentrations tested as can be seen in Table 6.4.4 of Appendix 2, but the highest concentration (2.5 mM) did not reduced glucose metabolism below 48% of control.

6.4.2.2 Linolenic acid.

As is shown in Figs. 6.4.5A and B and Table 6.4.5 of Appendix 2, PAN

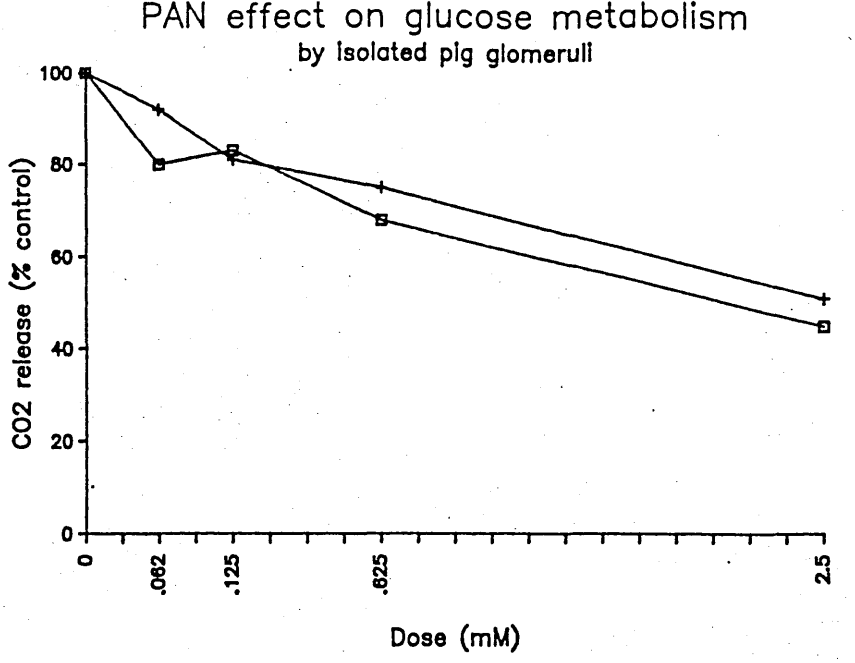


Fig. 6.4.4A

Dose-response of glucose oxidation by isolated pig glomeruli exposed to puromycin. 0.5 ml aliquots of glomeruli suspended in Krebs buffer were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C-glucose and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

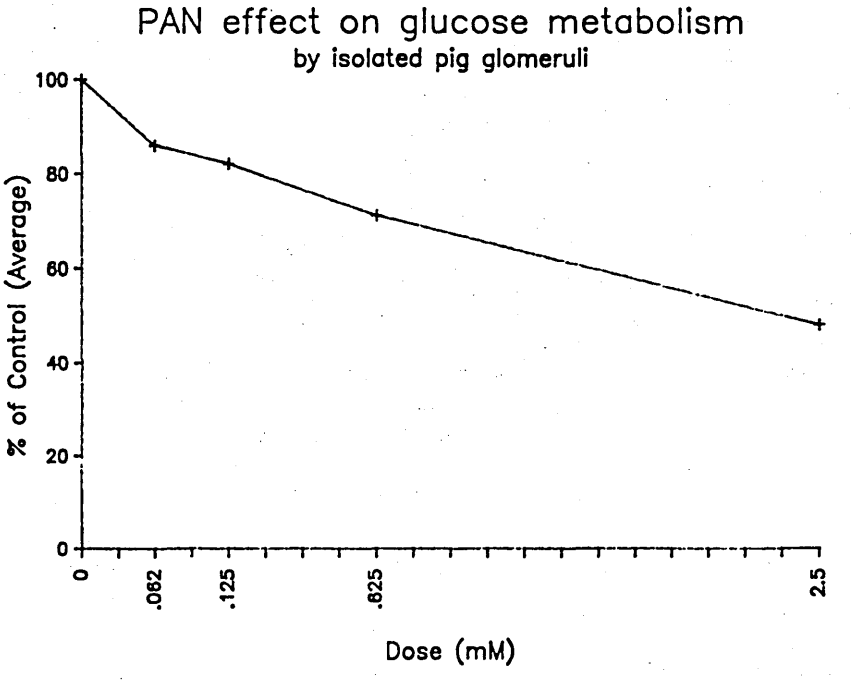


Fig. 6.4.4B

Data presented as the average of two experiments.

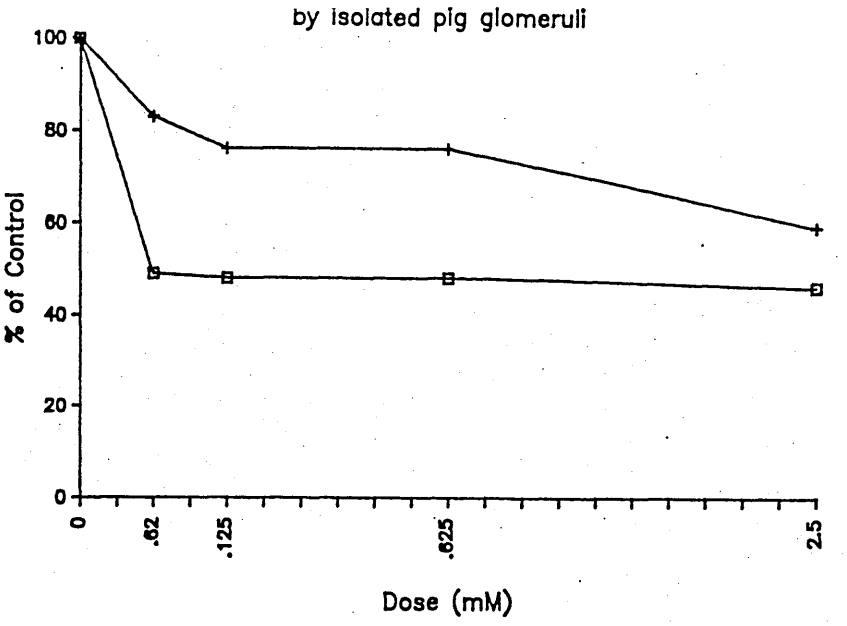


Fig. 6.4.5A

Dose-response of linolenic acid oxidation by isolated pig glomeruli exposed to puromycin aminonucleoside. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-linolenic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

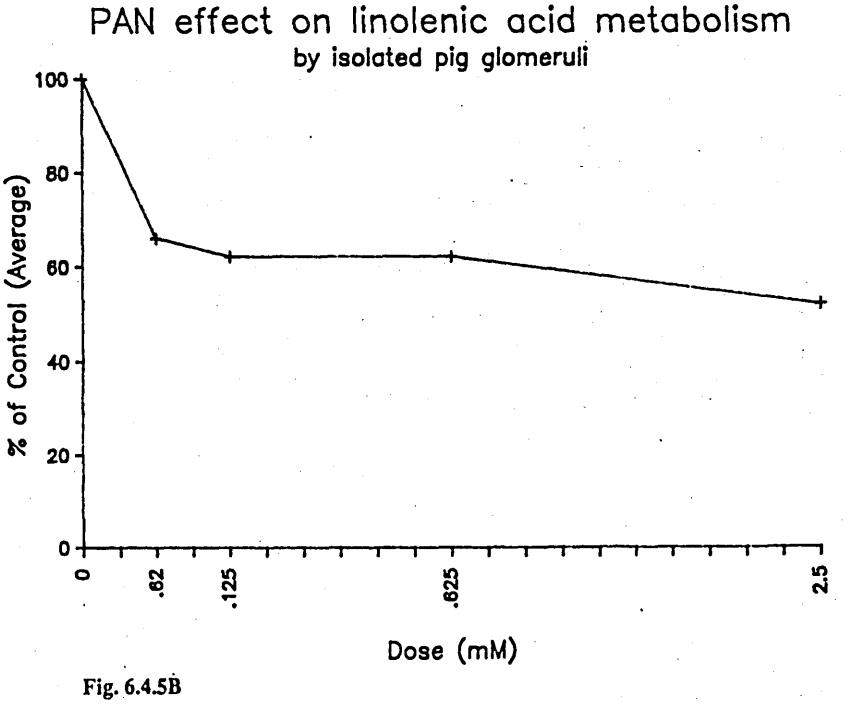


Fig. 6.4.5B

Data presented as the average of two experiments.

moderately but significantly decreased oxidative metabolism of linolenic acid. As there was a high degree of inter-experimental variability, it its difficult to draw conclusions about the effect of the drug on the oxidative metabolism of linolenic acid. However, in general terms it can be said that high doses of PAN such as 2.5 mM, did not decrease the release of $^{14}CO_2$ from this fatty acid, below 46% of the control.

6.4.2.3 Oleic acid.

The lowest concentration of PAN assessed (0.062 mM) caused a significant decrease to the $^{14}CO_2$ release from oleic acid, to 36% relative to the control value (Table 6.4.6 Appendix 2; Fig. 6.4.6). Increasing concentrations of the drug, did not produce a dose-related effect, and the highest concentration tested (2.5 mM), depressed oleic acid metabolism significantly to 26% of the control.

6.4.2.4 Discussion.

PAN inhibited glucose metabolism by pig glomeruli reproducibly for the two different experiments, but this was not a very strong effect, since the concentration of the drug needed to cause a 50% inhibition was slightly lower than the highest concentration assayed (2.5 mM). The utilization of glucose by PAN-nephrotic rat glomeruli has been reported to have progresively decreased to 50% of the control, between 1 and 7 days of the treatment (Kaplan et al, 1974). Although this research is in agreement in that there was a decrease in glucose metabolism of isolated glomeruli exposed to PAN, it is not possible to establish a comparison because of the difference between Kaplan's studies, where the chemical was applied <u>in vivo</u>, and this experiments, where glomeruli were exposed to PAN <u>in vitro</u>.

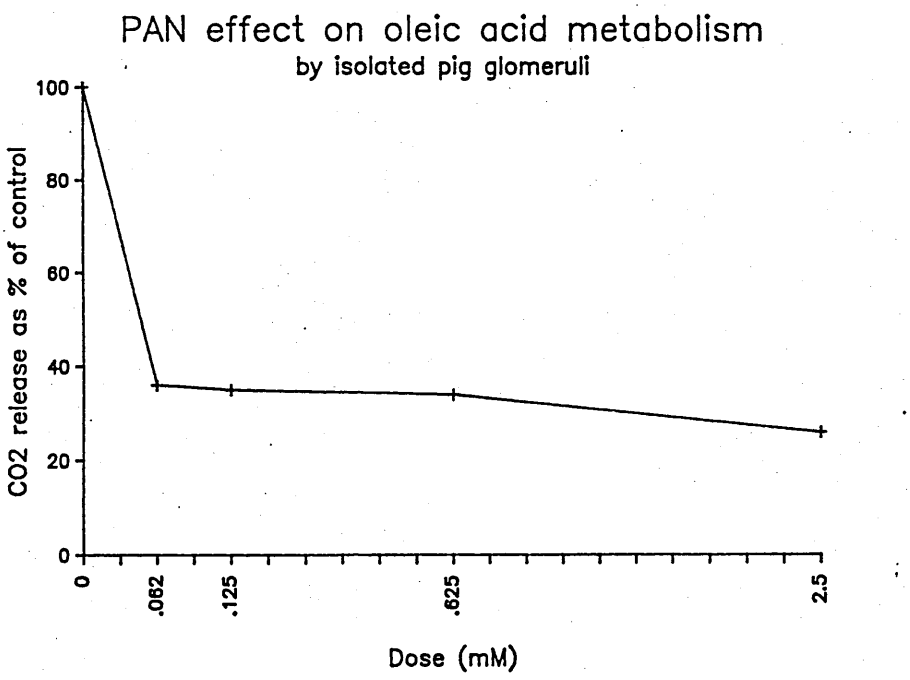


Fig. 6.4.6

Dose-response of oleic acid oxidation by isolated pig glomeruli exposed to puromycin aminonucleoside. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-oleic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-oleic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

Fatty acids. Due to the variability shown between experiments, in the case of linolenic acid metabolism, it is difficult to draw any conclusion. Therefore it is also difficult to conclude from the data of a single experiment using oleic acid.

6.4.3 STR effect on oxidative metabolism

6.4.3.1 Glucose.

Figs. 6.4.7A and B show the effect of STR on glucose oxidation by isolated glomeruli. The results and statistical analysis are shown in Table 6.4.7 Appendix 2.

6.4.3.2 Linolenic acid.

Fig. 6.4.8 and Table 6.4.8 Appendix 2 show the effect of STR on linolenic acid metabolism. There was low inter-experimental variability. The results on linolenic acid metabolism show that only the highest concentration of STR tested (2 mM), decreased the release of $^{14}CO_2$ from the fatty acid, to 50% of the control value. This means that although the aminoglycoside inhibited linolenic acid metabolism significantly, at various concentrations tested (1 - 2 mM), it had little effect on linolenic acid metabolism compared to other chemicals such as ADR and ETA.

6.4.3.3 Oleic acid.

Fig. 6.4.9 and Table 6.4.9 Appendix 2, show that none of the concentrations of STR inhibited significantly the release of $^{14}CO_2$ from the fatty acid below 80% of the control value. This means that STR effect on oleic acid oxidation, was minimal.

6.4.3.4 Discussion.

These data show that STR had little effect on the oxidative metabolism compared to ADR and ETA. In the case of glucose oxidation, although the results from the two experiments show some degree of variability, none of

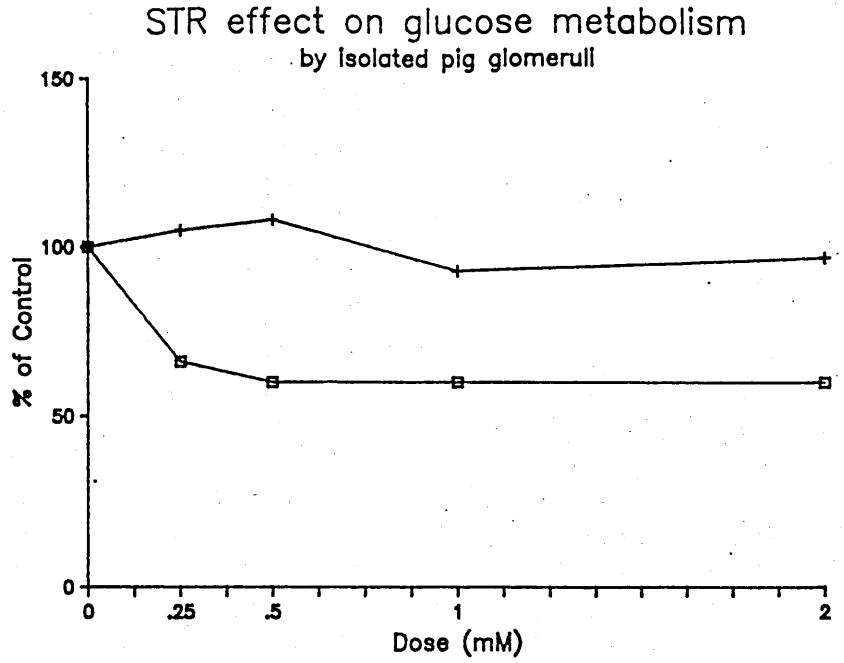


Fig. 6.4.7A

Dose-response of glucose oxidation by isolated pig glomeruli exposed to streptomycin. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C-glucose and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each line corresponds to a different experiment and each point is the result of triplicate incubations.

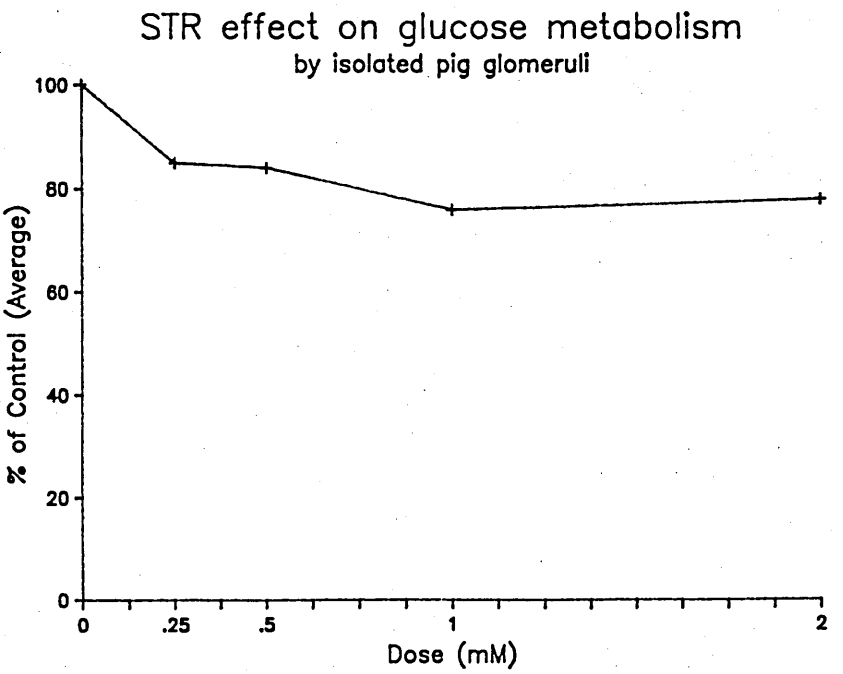


Fig. 6.4.7B

Data presented as the average of two experiments.

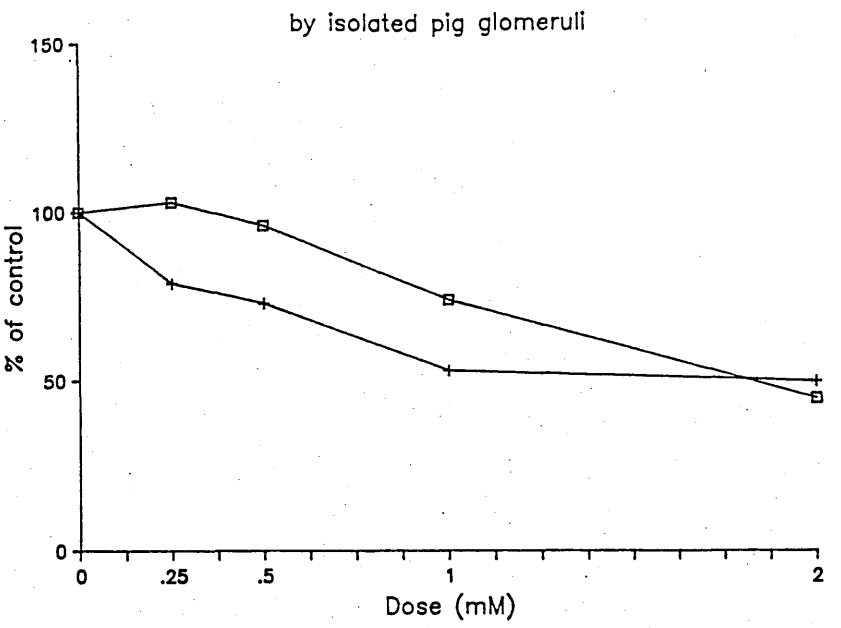


Fig. 6.4.8A

Dose-response of linolenic acid oxidation by isolated pig glomeruli exposed to streptomycin. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-linolenic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

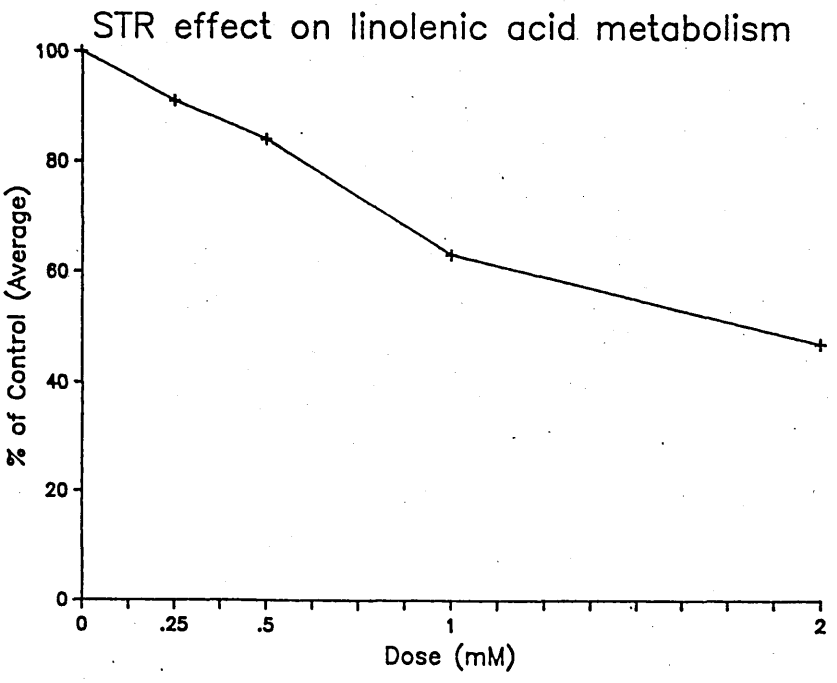


Fig. 6.4.8B

Data presented as the average of two experiments.

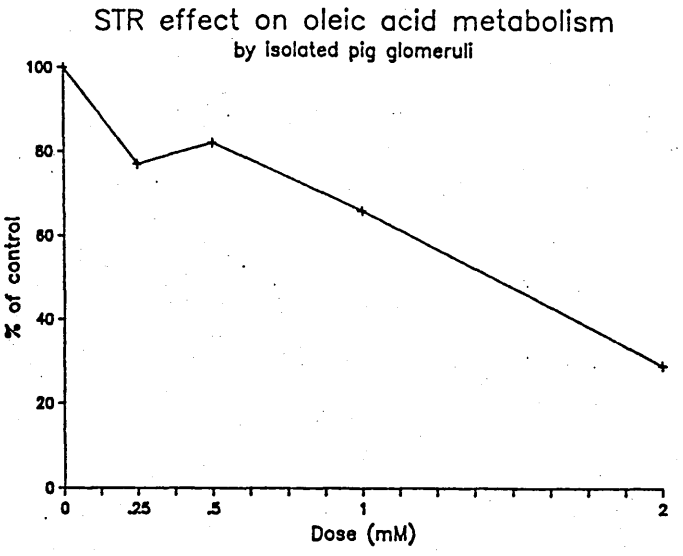


Fig. 6.4.9

Dose-response of oleic acid oxidation by isolated pig glomeruli exposed to streptomycin. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-oleic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-oleic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

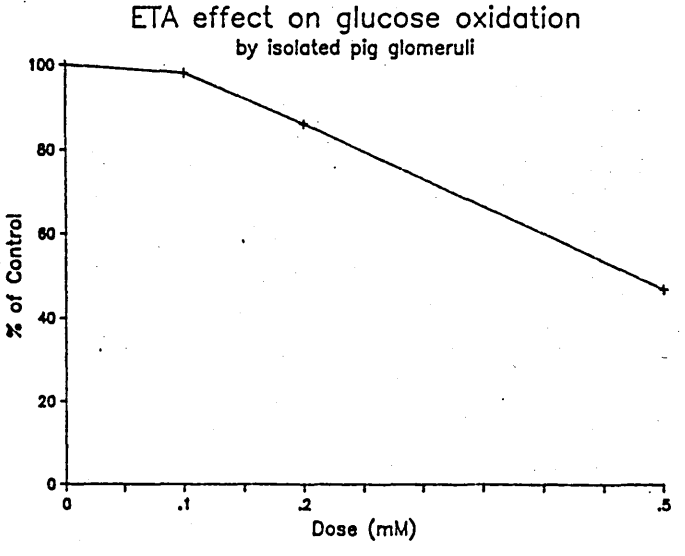


Fig. 6.4.10

Dose-response of glucose oxidation by isolated pig glomeruli exposed to ethacrynic acid. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C-glucose and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

the concentrations assessed, caused an inhibition below 60% of the control value.

The little effect seen on the oxidative metabolism of glucose and fatty acids, is also consistent with the fact that STR is not considered as a potent nephrotoxin, and that the aminoglycoside have shown little effect on cortical mitochondria respiration in vitro (Weinberg et al, 1980).

6.4.4 ETA effect on oxidative metabolism

6.4.4.1 Glucose

Fig. 6.4.10 and Table 6.4.10 Appendix 2 show the effect of ETA on glucose metabolism. The highest dose of ETA assessed (0.5 mM) depressed glucose oxidation no more than 47% of the control ($P \leq 0.01$).

6.4.4.2 Linolenic and oleic acid.

The effect of ETA on the oxidative metabolism of linolenic and oleic acid is shown in Figs. 6.4.11 and 6.4.12 and Tables 6.4.11 and 6.4.12 of Appendix 2). There was a highly significant inhibition ($P \leq 0.001$) on the metabolism of both fatty acids at all concentrations tested, and with 0.5mM ETA oleic acid metabolism was depressed completely.

6.4.4.3 Discussion.

Glucose. The effect of ETA on the $^{14}CO_2$ release from glucose is not strong, and the concentration that causes an inhibition of 50% in relation to the control is about 10-fold the dose required to inhibit the protein synthesis to the same extent. The effect of ETA on the pig glomerular metabolism of glucose in this study is in agreement with previous observations on rat glomeruli (Meezan and Brendel, 1973). The inhibition of glycolysis by ETA has also been reported in kidney slices

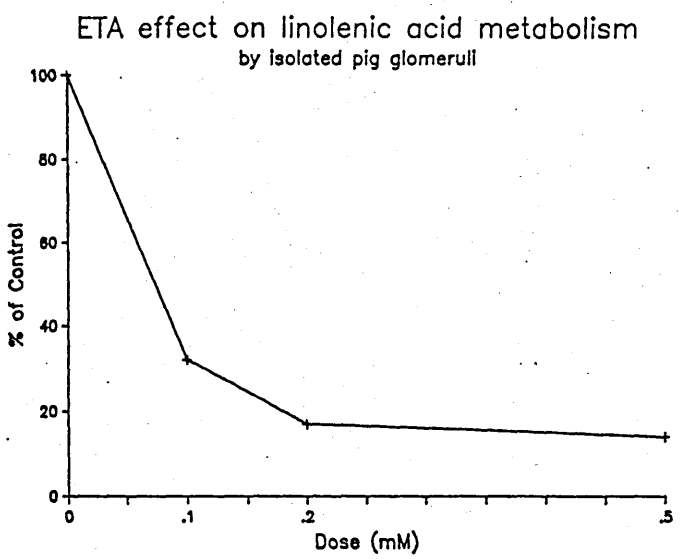


Fig. 6.4.11

Dose-response of linolenic acid oxidation by isolated pig glomeruli exposed to ethacrynic acid. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-linolenic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

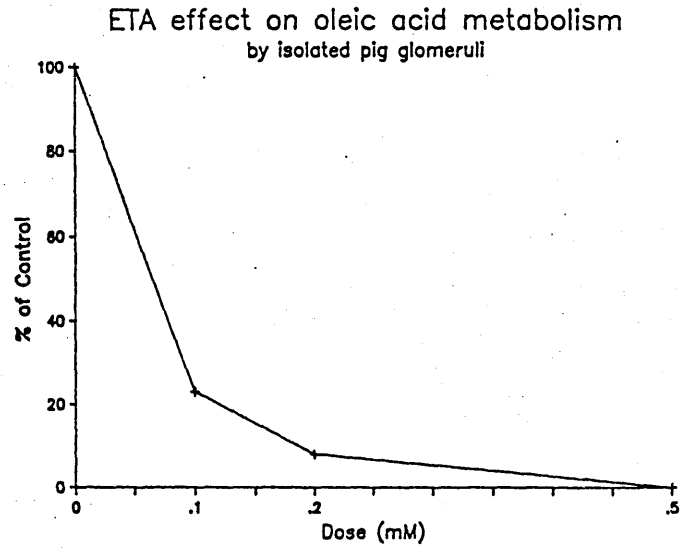


Fig. 6.4.12

Dose-response of oleic acid oxidation by isolated pig glomeruli exposed to ethacrynic acid. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-oleic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-oleic acid incubated with isolated pig glomeruli without the chemical (control). Each point is the result of triplicate incubations.

(Jones and Landon, 1967).

Fatty acids. The data on the effect of ETA on linolenic and oleic acid metabolism show that the compound markedly inhibited the metabolism of the two fatty acids, at the lowest concentration tested (0.1 mM). This means that ETA is a potent inhibitor of fatty acid metabolism in pig glomeruli, although the effect on protein synthesis is greater. This finding is in agreement with studies on isolated rat glomeruli by Brendel and Meezan (1973), who reported a marked inhibition of oleic acid oxidation in isolated glomeruli from rat kidney at ETA concentrations of 0.1 mM and greater. They suggested that an essential feature in the inhibitory effect of oxidative metabolism of glomerular cells is the presence of the double bond in the ETA molecule which determines it sulphydryl reactivity with proteins and enzymes. Previous studies had reported inhibition of respiration in kidney cortical slices, and rat kidney mitochondria (Jones and Landon, 1967; Poat et al, 1970; Landon and Fitzpatrick, 1972).

6.4.5 BEA effect on oxidative metabolism

6.4.5.1 Glucose.

The effect of BEA on glucose metabolism is shown in Fig. 6.4.13A and B and Table 6.4.13. All concentrations asessed (1, 5 and 10 mM) enhanced significantly the glomerular metabolism of glucose.

6.4.5.2 Linolenic and oleic acid.

The effect of BEA on linolenic and oleic acid is shown in Figs. 6.4.14 and 6.4.15 and Table 6.4.14 and 15 of Appendix 2. Fatty acid metabolism by glomeruli exposed to BEA show a high degree of inter-experimental variability for both linolenic and oleic acid.

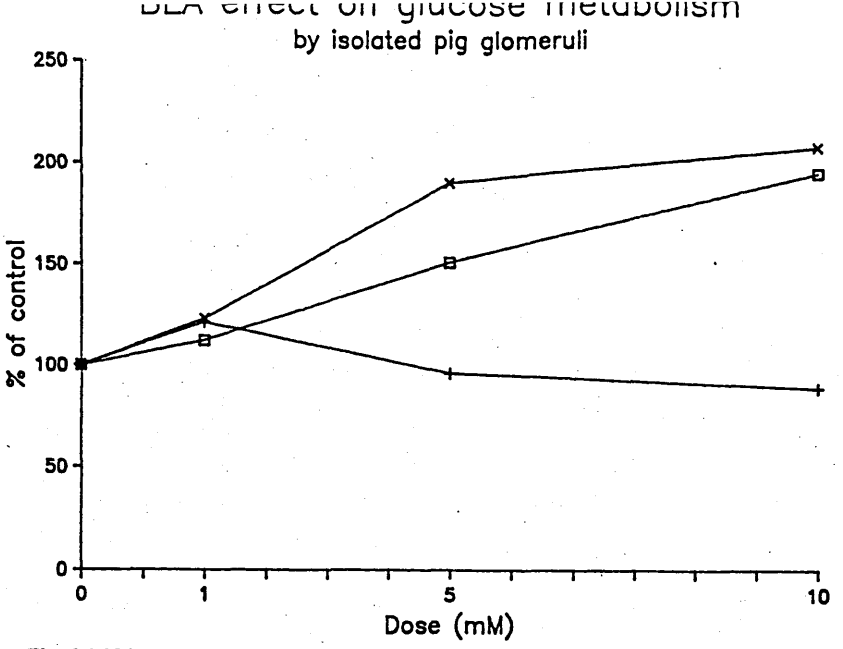


Fig. 6.4.13A

Dose-response of glucose oxidation by isolated pig glomeruli exposed to bromoethanamine. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C-glucose and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each line corresponds to a different experiment and each point is the result of triplicate incubations.

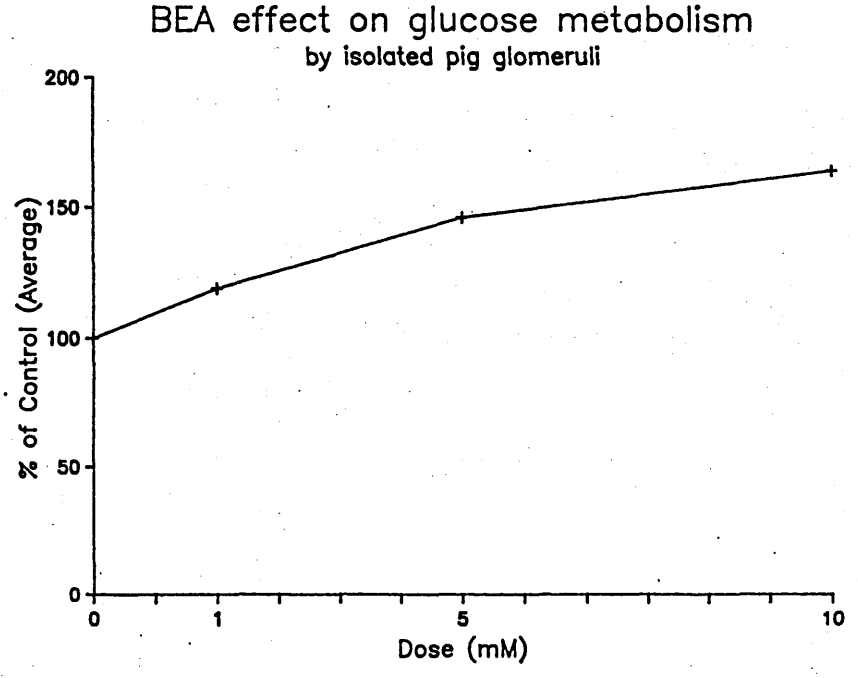


Fig. 6.4.13B

Data presented as the average of three experiments.

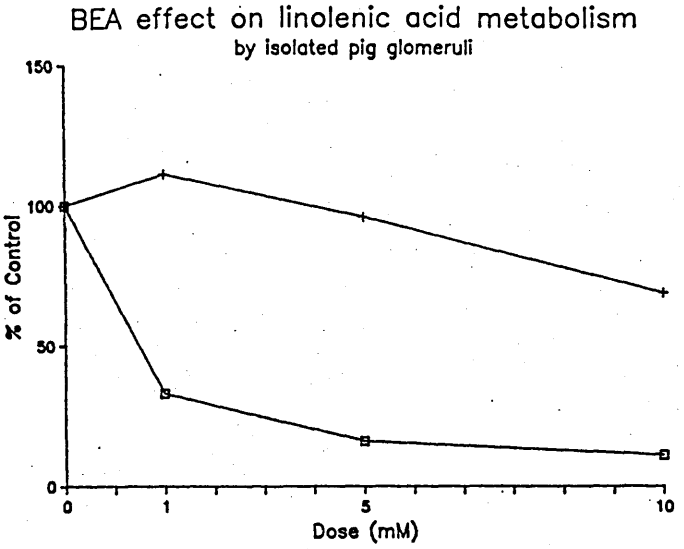


Fig. 6.4.14

Dose-response of linolenic acid oxidation by isolated pig glomeruli exposed to 2-bromoethanamine. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-linolenic acid incubated with isolated pig glomeruli without the chemical (control). Each line corresponds to a different experiment and each point is the result of triplicate incubations.

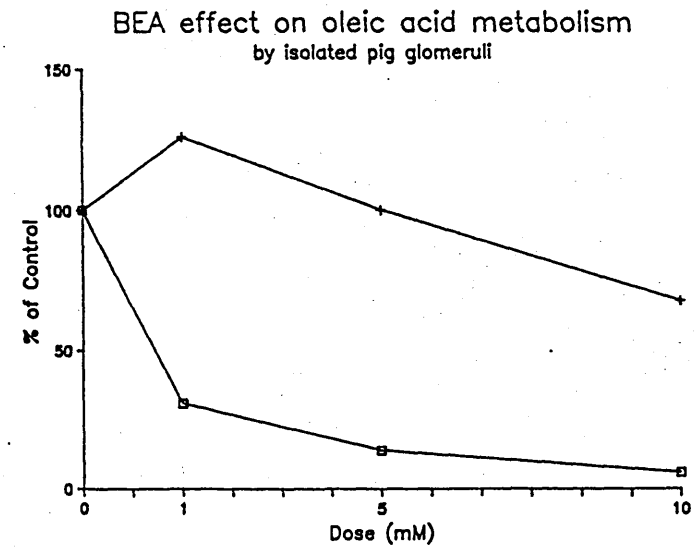


Fig. 6.4.15

Dose-response of oleic acid oxidation by isolated pig glomeruli exposed to 2-bromoethanamine. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-oleic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-oleic acid incubated with isolated pig glomeruli without the chemical (control). Each line corresponds to a different experiment and each point is the result of triplicate incubations.

6.4.5.3 Discussion.

The data on glucose metabolism show that BEA increases the $^{14}CO_2$ release from glucose at all concentrations tested. This means that BEA had a stimulating effect on glomerular oxidative metabolism of glucose. This effect is unexpected and was considered to be beyond the scope of this research project and still needs to be elucidated.

Due to the high degree of inter-experimental variability seen when the effect of BEA was assessed on fatty acid metabolism, it is difficult to draw any conclusion or to allow any comparison, and more experiments need to be done.

6.4.6 Summary and conclusions.

6.4.6.1 Summary.

1- Figs. 6.4.16 - 18 summarize the results on oxidative metabolism by isolated pig glomeruli.

2- ADR markedly inhibited the oxidative metabolism of fatty acids, such as linolenic and oleic acid (IC_{50} = 0.21 mM), but it only inhibited $^{14}CO_2$ release from glucose moderately (IC_{50} = 0.75 mM).

3- PAN had only a slight effect on glucose and linolenic acid metabolism by pig glomeruli. The concentration of the drug needed to cause a 50% inhibition on the metabolism of linolenic acid is higher than the highest concentration assayed (2.5 mM). The effect on oleic acid metabolism was more pronounced with an estimated IC_{50} = 62 uM.

4- STR had little effect on the oxidative metabolism of glucose (IC_{50} > 2mM) and oleic and linolenic acid (IC_{50} < 2 mM).

5- The effect of ETA on the $^{14}CO_2$ release from glucose is moderate (IC_{50} = 0.46 mM)).

6- ETA markedly inhibited linolenic and oleic acid metabolism at the

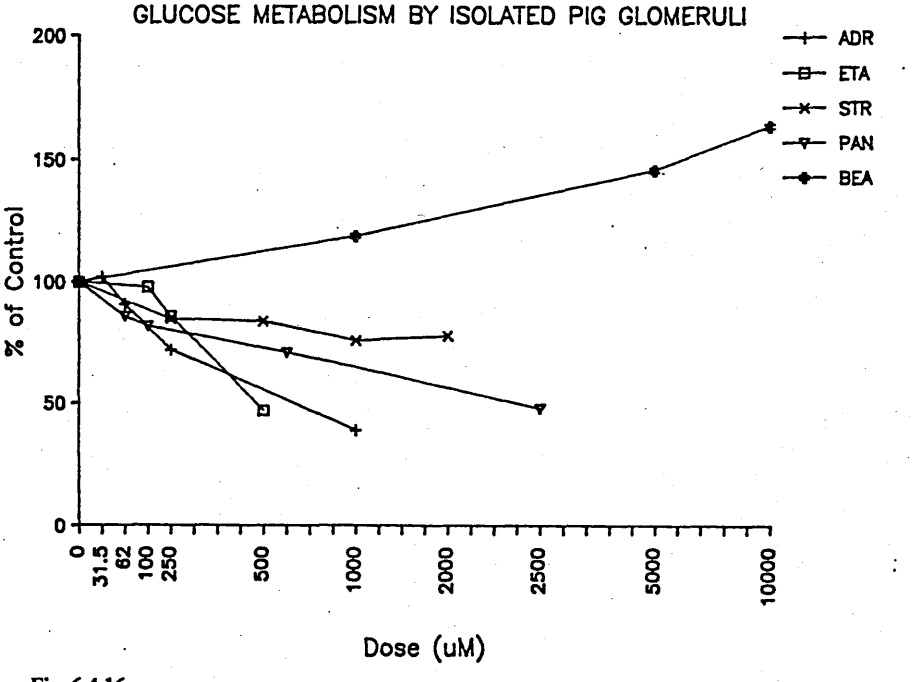


Fig. 6.4.16

Dose-response of glucose oxidation by isolated pig glomeruli exposed to different chemicals. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C glucose and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each time point is the result of triplicate incubations.

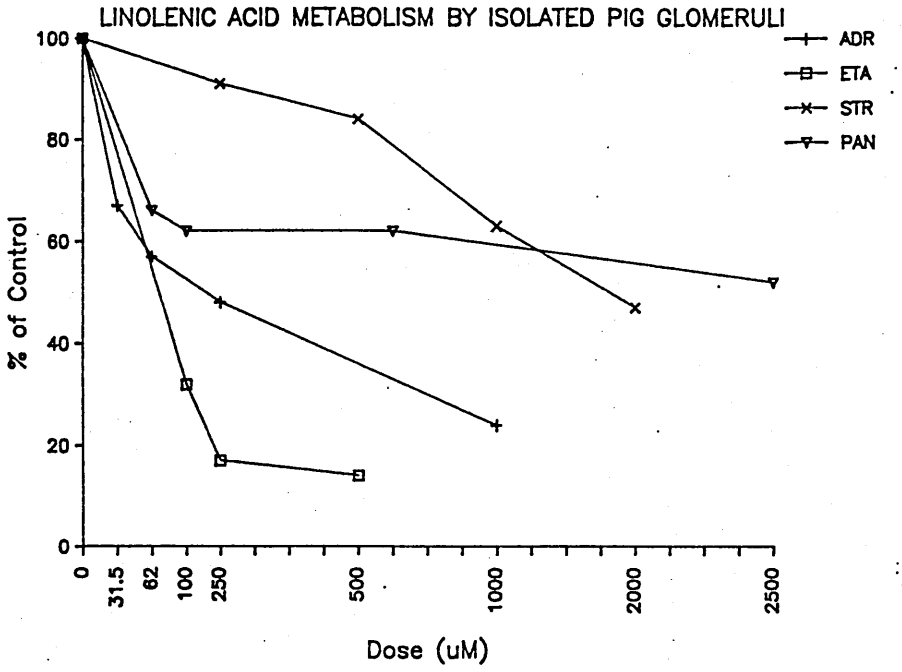


Fig. 6.4.17

Dose-response of linolenic acid oxidation by isolated pig glomeruli exposed to different chemicals. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each time point is the result of triplicate incubations.

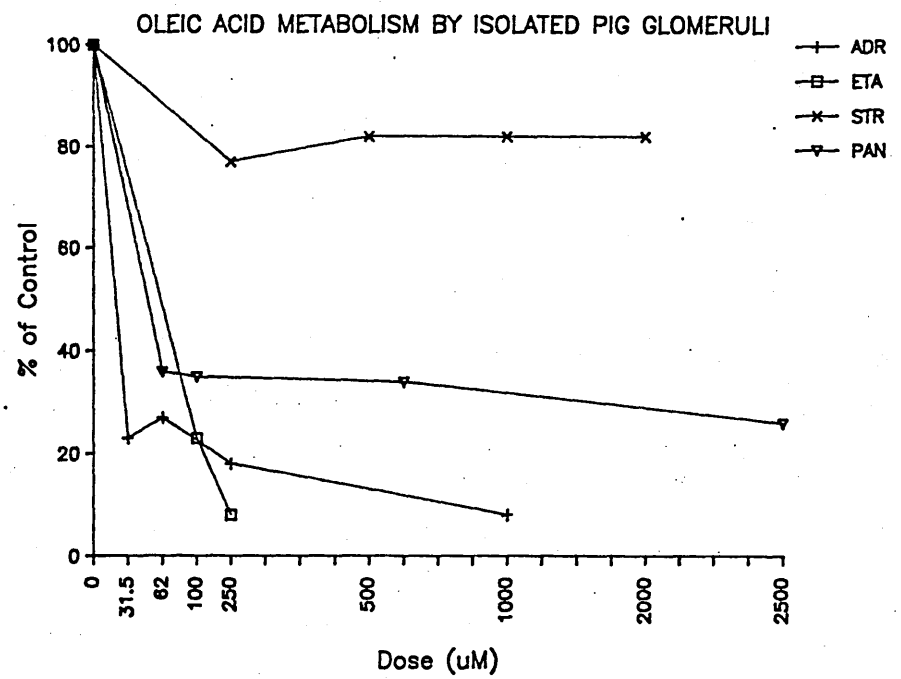


Fig. 6.4.18

Dose-response of oleic acid oxidation by isolated pig glomeruli exposed to different chemicals. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C oleic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated pig glomeruli without the chemical (control). Each time point is the result of triplicate incubations.

lowest concentration tested (0.1 mM).

7- BEA stimulated the $^{14}CO_2$ release from glucose oxidation at all concentrations tested.

6.4.6.2 Conclusions.

- Glucose oxidative metabolism is less sensitive to ADR than fatty acids.
- Of all compounds assessed for glucose and fatty acid metabolism, PAN and STR had the least inhibitory effect.
- ETA is a potent inhibitor of fatty acid metabolism by isolated pig glomeruli.
- The stimulating effect of BEA on glomerular oxidative metabolism of glucose is unexpected and needs further elucidation.
- Due to the high degree of inter-experimental variability seen when the effect of BEA was assessed on fatty acid metabolism, it is not possible to draw any conclusion or to allow any comparison, and more experiments need to be done.
- The ability of isolated pig and rat glomeruli to perform the different biochemical reactions leading to the oxidation of subtrates to CO_2 offers a useful metabolic system with which to study target selective toxicity.

6.5 Isolated glomeruli as an *in vitro* system for the study of OTA toxicity

6.5.1 Effect of OTA on amino acid incorporation into isolated rat glomeruli

The effect of OTA on the incorporation of several different amino acids into isolated rat glomeruli, was assessed over 4 hr. OTA markedly inhibited the incorporation of all the amino acids tested. The lowest concentration of the toxin assayed (10 uM) significantly decreased protein synthesis from LEU and TYR to 28% ($P \leq 0.01$ and 0.001 respectively), as is shown in Table 6.5.1 and 2 of Appendix 2, and Fig. 6.5.1, with an estimated IC_{50} of 5 uM. PHE incorporation was also decreased significantly ($P \leq 0.05$) to 44% ($IC_{50} = 7.8$), and TRP to 50% ($IC_{50} = 10$). Although the least affected amino acid was HIS, this showed a significant decrease of protein synthesis to 62% of the control. Higher concentrations of the OTA caused further decreases in protein synthesis, all statistically significant in relation to the control. Table 6.5.1. shows the relative toxicity of OTA on amino acid incorporation.

Discussion

These data show that OTA markedly depressed the incorporation of all amino acids, with very small doses of the toxin (ranging from 4.7 to 25 uM) required to decrease it to 50% of the control. This means that *de novo* protein synthesis from different amino acids, is strongly inhibited and that LEU and the aromatic amino acids are the most sensitive. The effect of OTA on protein synthesis has been previously reported in other *in vitro* systems such as cultured hepatoma cells (Creppy et al, 1979a), in which 90 uM of the toxin inhibited the incorporation of LEU by 60% and MDCK cells in which this parameter was inhibited to 50% with 15 uM OTA (Creppy et al,

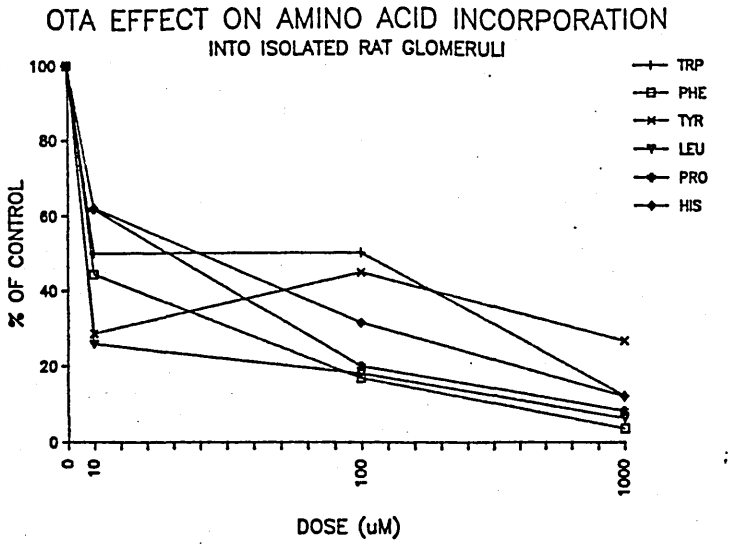


Fig. 6.5.1

0.5 ml aliquots of isolated rat glomeruli suspended in Earles-HEPES buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi of various ^{3}H-amino acids (HIS, LEU, PRO, PHE, TRP and TYR) and different concentrations of ochratoxin A, for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total amino acid incorporation into isolated rat glomeruli incubated without the chemical (control). Each point is the result of triplicate incubations.

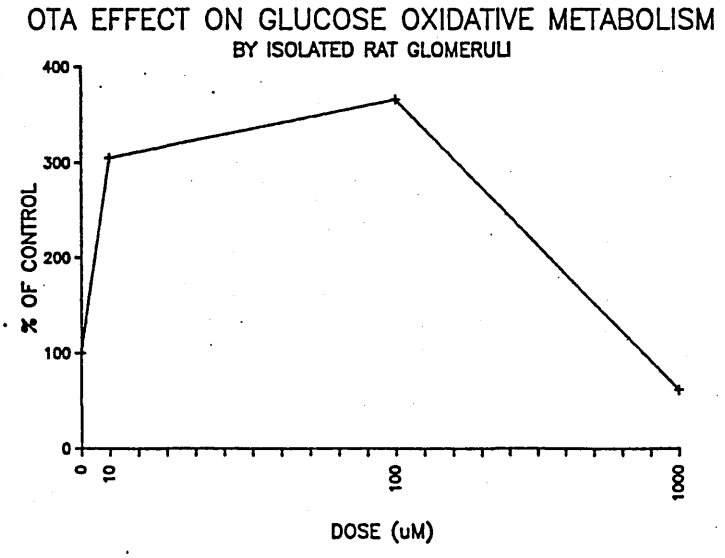


Fig. 6.5.2

Dose-response of glucose oxidation by isolated rat glomeruli exposed to ochratoxin A. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C-glucose and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with the glomerular suspension without the chemical (control). Each point is the result of triplicate incubations.

TABLE 6.5.1
RELATIVE TOXICITY OF OCA ON THE INCORPORATION OF SEVERAL AMINO ACIDS INTO ISOLATED RAT GLOMERULI

AMINO ACID	IC_{50}* (uM)
HIS	25.0
PRO	19.0
TRP	10.0
PHE	7.8
TYR	5.0
LEU	4.7

* Estimated concentration that inhibits protein synthesis to 50% of the control value.

1986). There are also reports on the reduction of in vivo renal protein synthesis from PHE and LEU by 30 - 40% in rats fed the toxin (Meisner and Meisner, 1981).

OTA has been also reported to decrease protein in liver and kidney of mice that had been administered the toxin (Creppy et al., 1984). In the case of these investigations, OTA inhibited more the incorporation of PHE than PRO and HIS.

The sensitivity of the aromatic amino acids compared to PRO, suggests the involvement of non-collagen components of the glomerular extracellular matrices, but to confirm this further studies with GBM, need to be done. There are no other reports on the incorporation on PHE and other amino acids into glomerular protein, but a protective effect of PHE against OTA toxicity has been reported in vitro and in vivo (Creppy et al, 1979a; 1984); and OTA inhibition of protein synthesis has been attributed to the competition between the drug and PHE for the binding site on the phenylalanyl-tRNA synthetase (Creppy et al, 1983).

The relevance of this findings in relation to the in vivo lesion produced by OTA is not yet known.

The different behaviour shown in the previous experiments with isolated pig glomeruli exposed to OTA incubated in Krebs buffer (Section 6.2.6.2), where large concentrations of the toxin (3.2 mM) did not produce a 50% inhibition relative to the control, could be due to a species difference, but it is more likely due to the different buffering system used. This still needs further elucidation using the same conditions as for rat glomeruli to allow a better comparison between the two species.

From this work, several questions have been raised, such as the relationship between OTA effect on glomerular protein to GBM structure and composition. Electrophoretic and autoradiographic studies on glomerular protein synthesised from different amino acids as affected by OTA, will allow one to follow the fate of the precursors, and the effect of the mycotoxin on the composition of glomerular protein (see Chapter 7).

6.5.2 Effect of OTA on the oxidative metabolism of glucose and linolenic acid

6.5.2.1 Glucose

CO_2 release from glucose was enhanced significantly ($P \leq 0.001$) up to 345% by low concentrations of OTA (10 - 100 uM) as can be seen in Fig. 6.5.2 and Table 6.5.3 of Appendix 2. On the other hand, higher concentrations of the toxin (ie. 1000 uM), inhibited significantly ($P \leq 0.001$) glucose metabolism to 59%.

Discussion

These data show that small concentrations of OTA stimulated considerably glucose metabolism. The interpretation of this effect is difficult, and at this stage it is not possible to say if the enhanced production of CO_2 from glucose was due to an stimulation either of the complete oxidation of the carbohydrate to CO_2 or the pentose phosphate pathway activities or both, although the first possibility does not fit with findings from previous studies where small concentrations of OTA are reported to inhibit mitochondrial respiration in the isolated rat liver mitochondria (Moore and Truelove, 1970; Meisner and Chan, 1974; Wei et al, 1985). However, the metabolism of glucose in glomeruli exposed to OTA has not been focus

of any previous published studies. More data on this topic needs to be obtained to be able to reach definite conclusions.

6.5.2.2. Linolenic acid

CO_2 release from linolenic acid showed a slight increase at OTA concentrations between 10 - 100 uM (Table 6.5.4 of Appendix 2 and Fig. 6.5.3), by 17% and 19% of the control respectively, which was significantly different ($P \leq 0.05$). However, 1000 uM of the toxin markedly depressed it, to 7.5% of the control ($P \leq 0.001$).

Discussion.

These data show that low concentrations of OTA caused a small, but definite stimulation of linolenic acid oxidation. A stimulatory effect on fatty acid metabolism in isolated glomeruli, could be linked to the observations of Elling and coworkers (1985) who found enhanced levels of peroxisomal beta-oxidation in soluble fractions of subcellular components of pig kidney, after <u>in vivo</u> short-term exposure to OTA.

The fact that 1000 uM OTA reduced remarkably fatty acid metabolism, may be in connection with the deteriorative changes of the mitochondria, and decreased respiration that have been reported to be caused by the toxin <u>in vitro</u>.

There are no previous reports on the study of fatty acid metabolism in isolated glomeruli of any species, exposed to OTA.

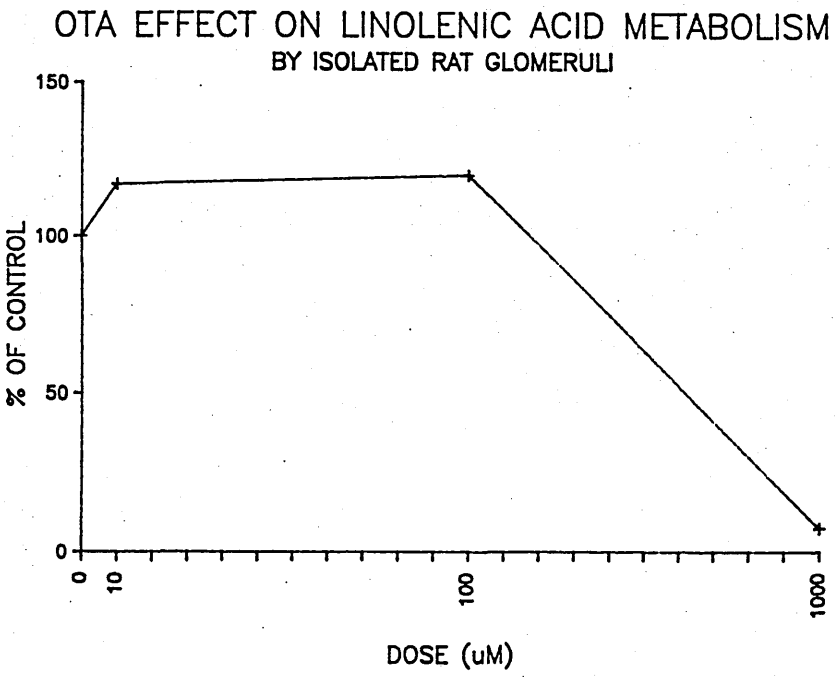


Fig. 6.5.3

Dose-response of linolenic acid oxidation by isolated rat glomeruli exposed to ochratoxin A. 0.5 ml aliquots of glomeruli suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-linolenic acid incubated with the glomerular suspension without the chemical (control). Each point is the result of triplicate incubations.

6.5.3. Summary and Conclusions

6.5.3.1. Summary

1- OTA markedly inhibited de novo synthesis of protein by isolated rat glomeruli from different amino acids.

2- The relative toxicity of OTA on the incorporation of several amino acids into isolated rat glomeruli ranked as follows:
LEU > TYR > PHE > TRP >> PRO > HIS.

3- Low concentrations of OTA (10 and 100 uM) enhanced glucose metabolism to CO_2.

4- Only high concentrations of OTA (1000 uM) caused significant inhibition on linolenic acid metabolism.

6.5.3.2. Conclusions

1- Protein synthesis from aromatic amino acids such as PHE and TYR appears to be a sensitive parameter of OTA toxicity.

2- The sensitivity of the aromatic amino acids to the effect of OTA (compared to proline) suggests that a glomerular macro-molecule other than GBM-collagen may be affected. This could be other functional and structural proteins of the glomeruli.

3- The inhibition of the incorporation of aromatic amino acids into glomerular macromolecules by OTA suggests that the synthesis of specific proteins may be central to the mechanism of target selective toxicity

towards the kidney.

4- The stimulation of glomerular glucose metabolism by OTA is still not clear.

5- The findings on OTA toxicity may be relevant to porcine endemic nephropathy and to the human condition BEN.

6.6. Metabolic studies on isolated rat proximal tubular fragments.

With the aim to compare some metabolic characteristics between rat isolated glomeruli and proximal tubular fragments, studies on the latter were carried out.

6.6.1 Amino acid incorporation into isolated rat tubules.

6.6.1.1 Comparison on the incorporation of PRO incubated with two different buffers.

Incubation with Earles-HEPES buffer gave a significantly higher incorporation of the amino acid than Tyrodes at all time points studied (Fig. 6.6.1; Table 6.6.1 Appendix 2), and after 4 hr of incubation the values for Earles-HEPES were three times higher ($P \leq 0.01$), therefore this buffer was also chosen for all experiments performed with proximal tubular fragments.

6.6.1.2 Incorporation of different amino acids.

The incorporation of several tritiated amino acids into rat proximal tubular fragments was measured. The highest rate of incorporation was shown by TRP (Fig. 6.6.2A; Table 6.6.2, Appendix 2). After 4 hr of incubation 18 - 26 pmol of amino acid/mg of protein were incorporated (Fig. 6.6.2 of Appendix 2), seven times higher ($P \leq 0.001$) than PRO whose incorporation was ranked second (2.7 pmol/mg of protein). Although TYR and PHE had similar values of incorporation after 4 hr of incubation (1.3 and 1.2 pmol/mg of protein), they were significantly different ($P \leq 0.05$). HIS and LEU were the least incorporated of all amino acids tested (0.9 and 0.7 pmol/mg protein), as can be seen in Fig. 6.6.2B). Although the pattern of amino acid incorporation was almost linear in all cases, there was a certain degree of variability when the amino acid

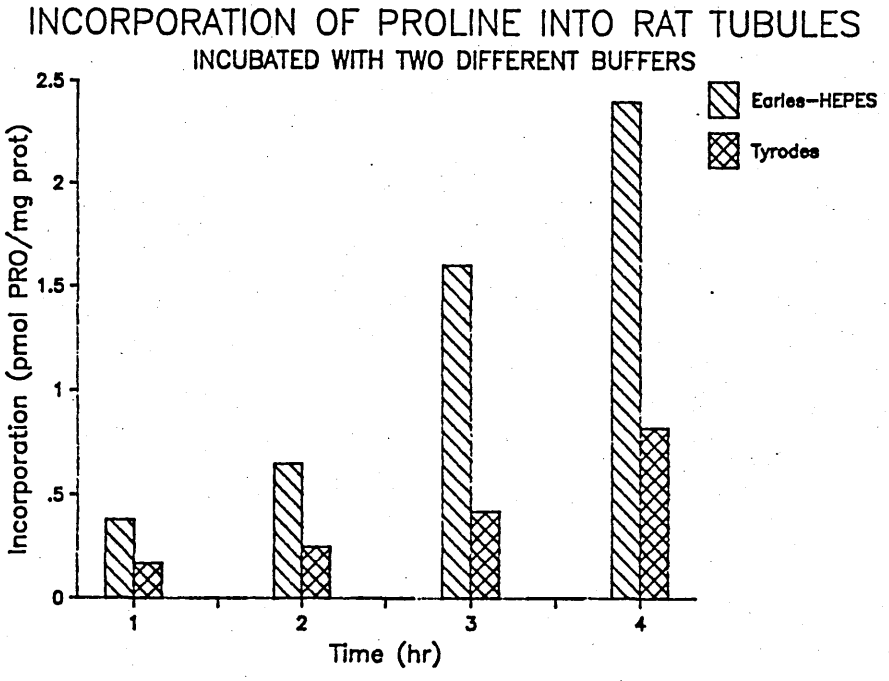


Fig. 6.6.1

Isolated rat proximal tubular fragments were suspended either in Tyrodes or Earles-HEPES buffers, and incubated with 0.5 ml of buffer containing 2.5 uCi ^{3}H-proline for 0, 1, 2, 3 and 4 hr.

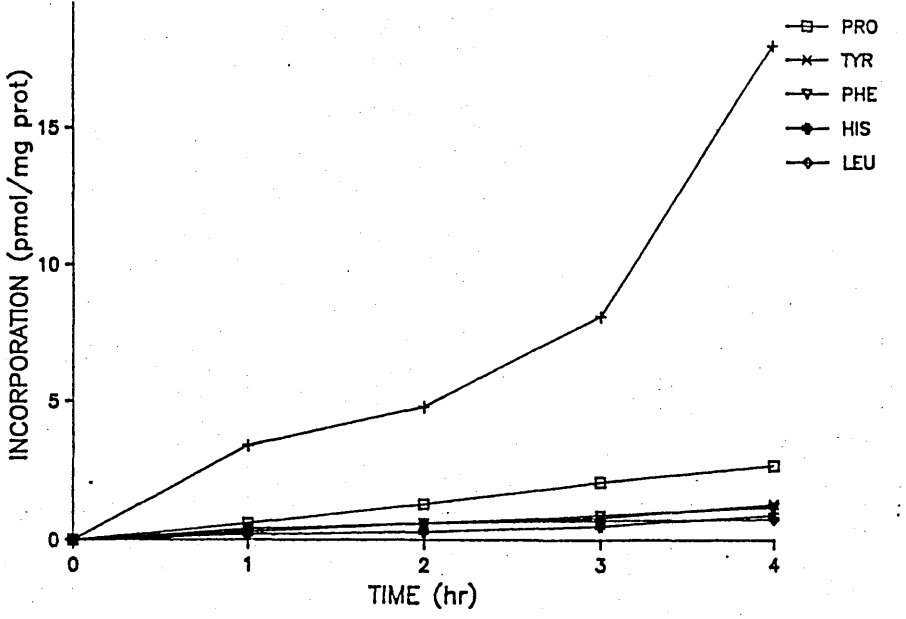


Fig. 6.6.2A

Kinetics of de novo protein synthesis by isolated rat proximal tubular fragments, incubated with Earles-HEPES buffer. 0.5 ml of buffer containing 2.5 uCi of each of the ^{3}H-labelled amino acids (HIS, LEU, PHE, PRO, TRP and TYR) was added at 0 time. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each time point is the result of triplicate incubations.

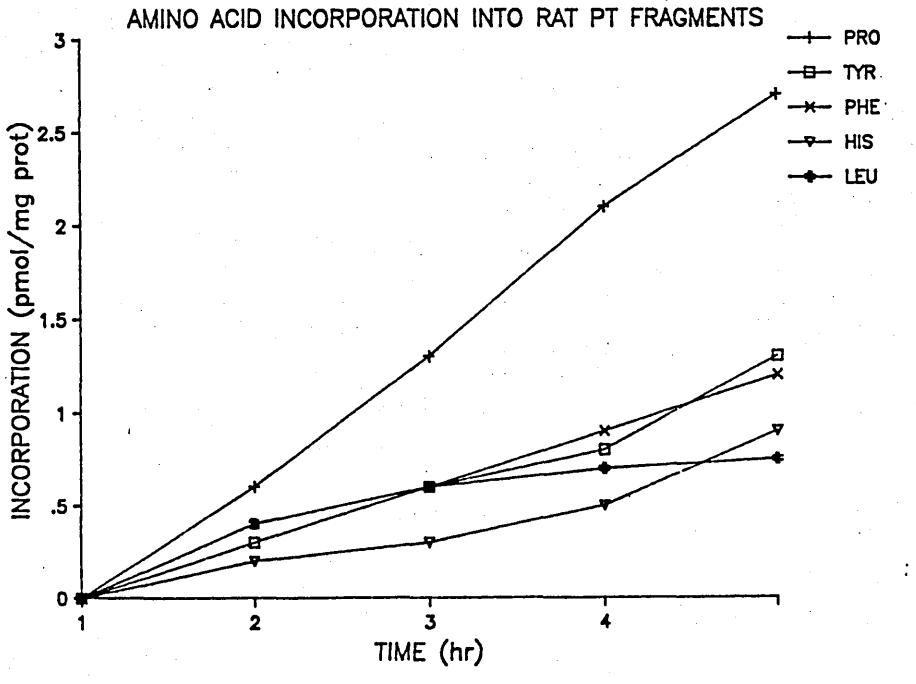


Fig. 6.6.2B

Enlarged view of the incorporation of HIS, LEU, PHE, PRO and TYR from Fig. 6.6.4A.

incorporation was tested in different experiments. PHE showed a 5-fold inter-experimental variability in its uptake having values ranging between (1.2 - 6 pmol/mg protein) as can be seen in Fig. 6.6.1 of Appendix 2. The other amino acids also presented some degree of inter-experimental variability (Figs. 6.6.3 - 6.6.6 Appendix 2). Standard deviations were normally low. The degree of statistical significance between different time points for each amino acid is shown in Table 6.6.3 Appendix 2, and for the different amino acids at the same time point in Table 6.6.4 Appendix 2.

6.6.1.3 Discussion

These data show that the incorporation of amino acids such as proline into rat proximal tubular fragments can vary with the composition of the incubating media in a similar way to isolated rat glomeruli, and the possible reasons for these differences are discussed in section 6.1.5. Proximal tubular fragments also incorporate different amino acids linearly for several hours, at different rates. The pattern of incorporation is similar to that seen for isolated glomeruli, but when the rate of incorporation was calculated per mg of protein, this is 10-fold lower for the proximal tubular fragments than for glomeruli (Table 6.6.1), suggesting that glomeruli have a higher metabolic rate for the synthesis of protein than tubules. This is consistent with the fact that glomerular cells have to produce and maintain the extracellular matrices such as GBM and other structural and functional proteins, therefore requiring higher activities of protein synthesis.

The incoporation of PRO and other different amino acids has been reported to be linear for several hours (Kwizera and Bach, 1987) but no comparison has bee: performed between the incorporation of different aromatic amino acids nor

TABLE 6.6.1.
RATE OF INCORPORATION OF SEVERAL AMINO ACIDS INTO ISOLATED RAT GLOMERULI (G) AND TUBULES (T)

AMINO ACID	INCORPORATION RATE (pmol/mg prot/hr)	
	G	T
TRP	27.3	3.20
PHE	4.7	0.67
TYR	3.7	0.33
LEU	3.5	0.31
PRO	2.4	0.27
HIS	2.5	0.21

between their rates of incorporation into isolated glomeruli and proximal tubules.

6.6.2 Oxidative metabolism

6.6.2.1 Glucose oxidative metabolism into isolated rat proximal tubular fragments

The rate of $^{14}CO_2$ release from labelled glucose was linear for 2 hr, after which it levelled off (Fig. 6.6.3; Table 6.6.5 Appendix 2). The degree of statistical significance between different time points is shown in Table 6.6.7 Appendix 2

6.6.2.2 Fatty acid metabolism into isolated rat proximal tubular fragments

A comparison of the time course of the oxidative metabolism of various ^{14}C-labelled fatty acids was performed. Oxidation of linolenic acid was the highest (Fig. 6.6.4; Table 6.6.6 Appendix 2), followed by oleic. Metabolism of stearic and palmitic acid was the lowest, and the blanks without tubules gave half of the counts that were obtained when tubular fragments were present. Mevalonic acid which was also assessed, did not undergo metabolism under the conditions of this experiment. The degree of statistical significance between different time points of each fatty acid is shown in Table 6.6.8 of Appendix 2.

6.6.3 Effect of Ochratoxin A

6.6.3.1 Effect of OTA on amino acid incorporation into isolated rat tubules

OTA markedly inhibited the incorporation of all the amino acids tested (Fig. 6.6.5; Table 6.6.9 Appendix 2). The lowest concentration of the

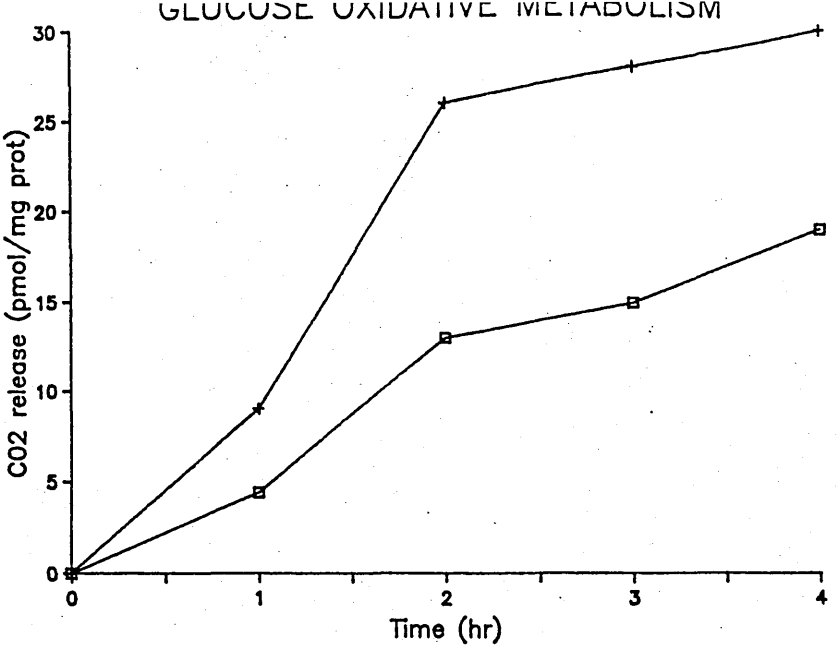


Fig. 6.6.3

Kinetics of glucose oxidation by isolated rat proximal tubular (PT) fragments. 0.5 ml of Earles-HEPES buffer containing 2 uCi of ^{14}C-glucose was incubated with 0.5 ml of the PT preparation for up to 4 hr. The ordinate represents specific activity of $^{14}CO_2$ trapped into NaOH. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

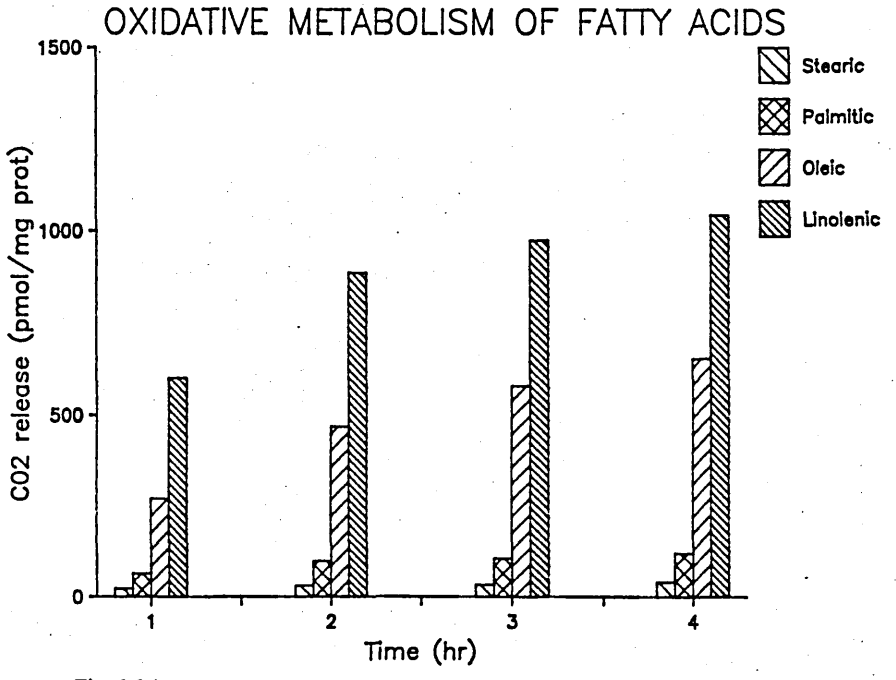


Fig. 6.6.4

Comparison of the time-course of the oxidative metabolism of various ^{14}C-labelled fatty acids by isolated rat proximal tubular (PT) fragment. 0.5 ml of Earles-HEPES buffer containing 50 nCi of either radiolabel was incubated with 0.5 ml of the PT preparation for up to 4 hr. The ordinate represents specific activity of $^{14}CO_2$ trapped into NaOH. Each time point is the result of triplicate incubations.

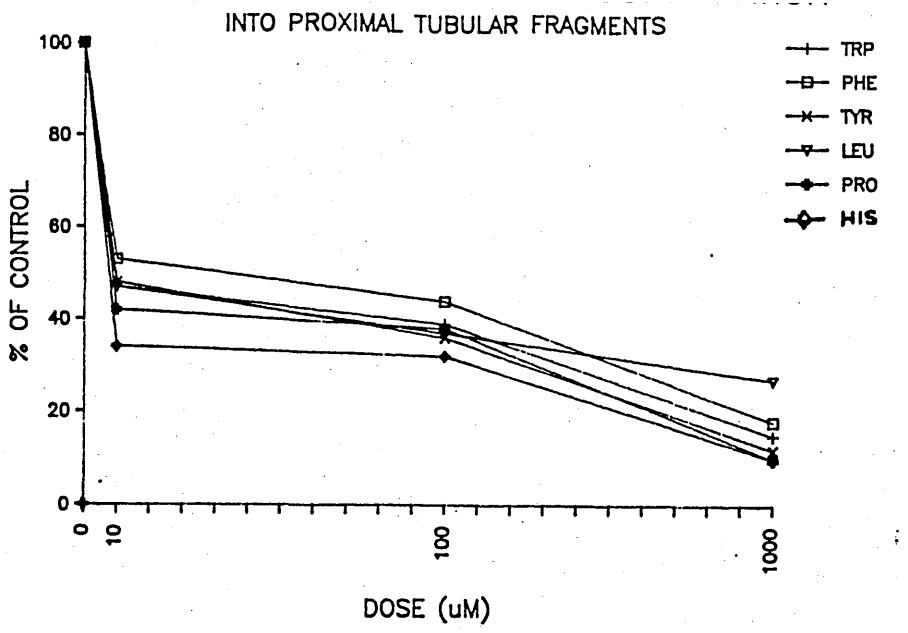


Fig. 6.6.5

0.5 ml aliquots of rat proximal tubular fragments (PTF) suspended in Earles-HEPES buffer were incubated at 37°C with 0.5 ml of buffer containing 2.5 uCi of various ^{3}H-amino acids (HIS, LEU, PRO, PHE, TRP and TYR) and different concentrations of ochratoxin A, for 4 hr. The ordinate represents the proportion of incorporated amino acid calculated as a percentage of the total amino acid incorporation into isolated PTF incubated without the chemical (control). Each point is the result of triplicate incubations.

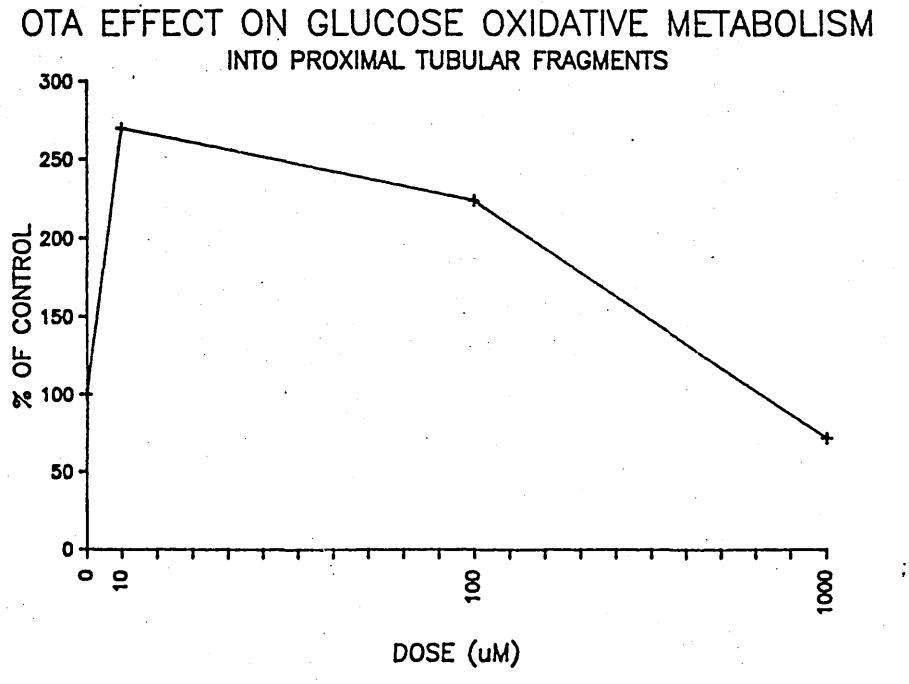


Fig. 6.6.6

Dose-response of glucose oxidation by isolated rat proximal tubular fragments (PTF) exposed to ochratoxin A. 0.5 ml aliquots of PTF suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 2 uCi of ^{14}C-glucose and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-glucose incubated with isolated PTF without the chemical (control). Each point is the result of triplicate incubations.

toxin assayed (10 mM) decreased significantly ($P \leq 0.001$) protein synthesis from HIS to 34%, PRO to 41%, LEU, TRP and TYR to 47%, and PHE to 53% (Table 6.6.10 Appendix 2). Higher concentrations of OTA caused further significant decreases ($P \leq 0.001$) relative to the control values. Although the estimated IC_{50} values were very close for most of the amino acids, HIS appeared to be the most sensitive (IC_{50} = 5.8 uM) and PHE the least (IC_{50} = 21 uM) as can be seen in Table 6.6.2.

Discussion

As in isolated glomeruli, OTA remarkably decreased de novo protein synthesis by isolated tubular fragments, but this time the ranking of OTA toxicicity towards the different amino acids was different from isolated glomeruli with PHE appearing as the less sensitive and HIS as the most. It is necessary to extend the range of concentrations between 0 - 100 uM to see if greater differences between the amino acids can be picked up.

6.6.3.2. Effect of OTA on the oxidative metabolism of glucose

Glucose metabolism was enhanced significantly ($P \leq 0.001$ and 0.01 respectively) to 270 and 224% of the control by 10 and 100 uM of OTA (Fig. 6.6.6; Table 6.6.10). On the other hand, higher concentrations of the toxin (ie. 1000 uM), inhibited glucose metabolism to 72%.

Discussion

These data show that OTA at low doses (10 and 100 uM) stimulates glucose oxidation by isolated rat proximal tubular fragments. The pattern of interaction is similar to that shown by isolated glomeruli. Therefore the interpretation of those results (Section 6.5.2.1.) also applies for these findings on proximal tubular fragments.

TABLE 6.6.2.
RELATIVE TOXICITY OF OCA ON THE INCORPORATION OF SEVERAL AMINO ACIDS INTO ISOLATED RAT GLOMERULI (G) AND TUBULES (T)

AMINO ACID	IC_{50}* (uM)	
	G	T
HIS	25.0	5.8
PRO	19.0	7.2
TRP	10.0	8.6
PHE	7.8	21.0
TYR	5.0	9.2
LEU	4.7	8.6

* Estimated concentration that inhibits protein synthesis to 50% of the control value.

6.6.3.3. Effect of OTA on the oxidative metabolism of linolenic acid

Linolenic acid metabolism was increased significantly ($P \leq 0.01$ and 0.05 respectively) by 43 and 33% at OTA concentrations of 10 and 100 uM respectively (Fig. 6.6.7; Table 6.6.11 Appendix 2). Higher concentrations of the toxin markedly depressed it ($P \leq 0.001$), to 11% of the control.

Discussion

These data show that low concentrations of OTA also increased moderately but significantly the oxidative metabolism of linolenic acid. The interaction is again similar to that seen for isolated glomeruli (Section 6.5.2.2) and the same interpretation applies.

In all two oxidative pathways assessed, it is necessary to extend the range of concentrations between 0-100 uM, for a proper dose-response relationship.

6.6.4 Summary and Conclusions

6.6.4.1 Summary

6.6.4.1.1. Proximal Tubular Metabolism

1- Isolated rat proximal tubules incorporate different amino acids, at different rates. The order of incorporation being the same as in glomeruli TRP >> PHE > TYR > LEU > PRO > HIS. However, the rates of incorporation were 10-fold lower for the proximal tubular fragments.

2- Incubation of isolated rat proximal tubules with Earles-HEPES buffer increases the incorporation of PRO more than Tyrodes and Krebs.

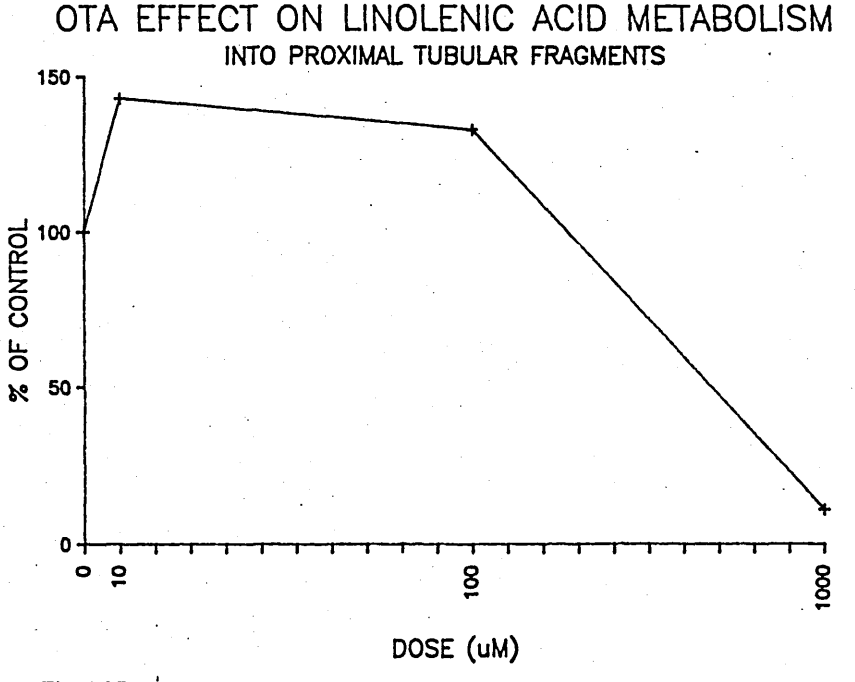


Fig. 6.6.7

Dose-response of linolenic acid oxidation by isolated rat proximal tubular fragments (PTF) exposed to ochratoxin A. 0.5 ml aliquots of PTF suspended in Earles-HEPES were incubated at 37°C with 0.5 ml of buffer containing 50 nCi of ^{14}C-linolenic acid and different concentrations of the chemical. The ordinate represents the proportion of $^{14}CO_2$ calculated as a percentage of the total $^{14}CO_2$ released from ^{14}C-linolenic acid incubated with isolated PTF without the chemical (control). Each point is the result of triplicate incubations.

3- Isolated rat proximal tubular fragments metabolize ^{14}C-glucose and ^{14}C-fatty acids (linolenic, oleic, palmitic and stearic) to $^{14}C_2$ linearly for two hours.

4- Glucose oxidative metabolism is much lower than fatty acid oxidation, in proximal tubular fragments, as in isolated glomeruli.

5- Among the fatty acids linolenic showed the highest oxidation rate.

6- The rate of fatty acids oxidative metabolism is much lower for isolated rat proximal tubular fragments when calculated per mg of protein.

7- Rat proximal tubular fragments glucose oxidative rate is similar to that in isolated rat glomeruli.

6.6.4.1.2. OTA Toxicity

1- OTA markedly inhibited de novo synthesis of protein by isolated proximal tubular fragments from different amino acids.

2- The relative toxicity on OTA on the incorporation of several amino acids into isolated proximal tubules ranked as follows:

HIS > PRO > LEU > TYR >> PHE.

3- Low concentrations of OTA (10 and 100 uM) enhanced glucose metabolism to CO_2 by isolated rat proximal tubular fragments, similarly to isolated rat glomeruli.

4- Only high concentrations of OTA (1000 uM) caused significant inhibition

on linolenic acid metabolism.

6.6.4.2. Conclusions

1- Isolated proximal tubular fragments maintain their viability for several hours as manifested for the maintenance of linear rates of amino acid incorporation.

2- The metabolic activity of isolated proximal tubular fragments per mg of protein (de novo protein synthesis, and oxidative metabolism) appears to be lower than in isolated glomeruli.

3- The amount of de novo synthesized protein can vary with the composition of the incubating media, similarly to isolated glomeruli.

4- Contrary to isolated rat glomeruli, PHE appears to be the least sensitive amino acid, and HIS the most sensitive (I_{50} 21 vs. 5.8 uM, respectively) to the toxicity of OTA.

5- OTA effect on glucose and fatty acid oxidative metabolism by isolated proximal tubular fragments is still not clear.

CHAPTER 7.

FINAL DISCUSSION

The problems associated with assessing the real role of mycotoxins in clinically relevant nephropathies are extensive, and there needs to be a number of problems resolved before this question can be answered.

In the first place, end stage renal disease is a long-term consequence of multifactorial events, it may be impossible to identify the initiating event. Secondly, when mycotoxins appear to be involved, it is difficult to determine the levels of exposure, because it will depend on the seasonal variation and whether it has been a pulse dose rather than a continuous exposure, or a combination of both. The nephropathies associated with mycotoxins may also involve more than one compound, with the possibility of interactions between them. The mycotoxins OTA and aflatoxin B_1 (ATB_1) have been detected in a great diversity of agricultural products, they can also occur simultaneously, due either to the growing of more than one species of mould on the same commodity or to the mixtures of various food or feed supplies. The combined ingestion of OTA and ATB_1 may be synergistic, with nephropathy as the primary effect. The possibility of interactions between a mycotoxin and other nephrotoxicant bring about further complications.

In addition, the target cell within the kidney needs to be defined accurately. In the case of OTA, the target has been identified as the proximal tubule, but there is evidence to suggest early glomerular

involvement. In the case of BEN, the clinical signs of the disease appear too late to have an accurate picture of the cascade of early degenerative events that lead to this condition.

This thesis has attempted to address some of these problems. First of all, the *in vivo* work aimed to study early renal clinical and morphological changes, caused by the administration of different repetitive doses of ochratoxin A alone or in combination with small doses of aflatoxin B_1 and tried to identify the extent of glomerular involvement.

Using clinical biochemistry, features relating to proximal tubular damage, such as enzymuria, glucosuria and polyuria, were observed. Most important, a proteinuria which could have been of glomerular origin was found. Routine histology showed besides the tubular lesion, morphological changes in glomeruli of animals treated with repeated doses of OTA, such as edema and prominent PAS staining, suggesting thickening of basement membrane. On the other hand repeated doses of OTA and ATB_1, applied simultaneously, appeared to have a synergistic effect, characterized by severe disruption of proximal tubules, but most important, a general morphological derangement of the glomerulus was seen involving intense and faint staining nuclei (possibly indicative of cell necrosis) and cytoplasmic vacuolation, which was not seen with either toxin alone. These findings have not been previously described in the literature, following such low dose regime of OTA alone or in combination with ATB_1. These *in vivo* data need to be confirmed by high resolution microscopy, histochemistry, immunohistochemistry and electron microscopy, in order to describe these changes in greater detail and follow the time-course at the ultrastructural level, to help understand molecular changes.

Due to the findings of early glomerular changes after exposure of the animals to small repetitive doses of OTA, as well as the relative high cost and time-consuming task of the *in vivo* experimentation, it was decided to focus studies on the use of *in vitro* techniques (ie. isolated glomeruli). The complex and highly organized structure of the kidney makes it difficult to study the various specialized functions separately in the intact organ. Therefore, studies on isolated glomeruli offer several advantages over both the intact animal and other *in vitro* techniques such as perfusion, slices and isolated cells. Glomeruli are easily isolated from the rest of the nephron in an intact form. More important, their anatomical characteristics are such that they can not be confused with other regions of the nephron. The preparation of isolated glomeruli is a relatively simple and quick procedure, with no need of sophisticated or expensive apparatus, and the glomerular preparation maintain many of their metabolic functions for several hours. Contrary to *in vivo* studies, considerable information can be obtained with the use of small number of rats. Several different parameters can be assessed from the same tissue, allowing comparison of data, while minimizing tissue variability. Isolated glomeruli allow the study of glomerular characteristics, or functions as well as metabolic properties, under controlled conditions and independent of hæmodynamic changes and other renal or extrarenal factors; this facilitates the differentiation of direct and indirect effects and allows an assessment of a precise dose-effect and time-concentration relationship of a specific chemical.

The disadvantages of studying isolated glomeruli are mainly those inherent to most *in vitro* systems, such as the short viability which restricts the study of morphological and biochemical effects to the investigation of

short responses such as acute changes following a toxic insult. Furthermore, due to the cellular heterogeneity, it is not possible to correlate the observations with a specific cell type within the glomeruli. Other disadvantage is that opposite to the in vivo situation, a chemical is presented to the glomeruli from outside the blood supply, it is not plasma bound, and it is not under pharmacokinetic changes in concentration. Therefore, the interpretation and extrapolation of data derived from observations performed on isolated glomeruli may require some care. However, most of the knowledge on glomerular biochemistry and metabolism has been obtained from studies in isolated glomeruli.

Useful information on the properties of the glomerular basement membrane (GBM) can also be obtained from observations in isolated glomeruli and therefore, after toxic injury the assessment of changes in the biochemical functions of the cells which produce and maintain this extracellular matrix can also be performed, based on the supposition that they take place before or in parallel with any modification in the structure or composition of the GBM. It is possible that some of these changes can be measured by studying selected parameters of glomerular metabolism. Isolated glomeruli also provide a suitable system to study the mechanism of action of nephrotoxic compounds either from one compound alone, its metabolites or after multichemical exposure, and also has the potential to help the development of a reliable in vitro system for the screening of chemicals for glomerular toxicity. The method can be applied to the study of different species such as rat, pig and man, allowing interspecies comparison. The work with human tissue does, however present a number of difficulties because of the fibrous nature of human kidney available from nephrectomies and cadavers, interindividual variability and association with biohazard risks such as hepatitis and AIDS. In addition, human

kidneys are not conveniently available from cadaver material (where the biohazard risk is more difficult to assess) and although surgical nephrectomy material provides a better basis for studies and easier biohazard assessment, the kidney may have, of necessity, pathological changes. In contrast, the pig kidney is amply available from abattoirs, which provides a source of disease-free tissue from an organ that is anatomically almost identical to that from man. One pig kidney provides a considerable amount of tissue, equivalent to that of more than 25, 150g rats.

In this thesis, de novo synthesis of protein has been studied using a variety of radiolabelled amino acids as precursors and extending the area of biochemical assessment by following the oxidative metabolism of glucose and linolenic acid, by isolated rat and pig glomeruli. In addition, the changes in the metabolism of isolated glomeruli (ie. protein synthesis and oxidative metabolism) have been investigated, after exposure to different chemicals (ie. Adriamycin, Puromycin aminonucleoside and Streptomycin), some of which are known to damage this part of the nephron in vivo. These effects were also compared to those compounds that target specifically for other parts of the nephron, such as Ethacrynic acid, 2-Bromoethanamine and Folic acid. Studies on the effect of OTA on de novo synthesis of protein and oxidative metabolism by isolated rat glomeruli and tubules were also carried out. The data presented in this thesis, appear to be the first that document differences in the pig and rat glomerular metabolism.

Isolated pig and rat glomeruli and rat tubules incorporated different amino acids linearly for several hours, at different rates. Pig glomeruli incorporated leucine at a much higher rate than the other amino acids,

and proline was ranked second. The order of incorporation being leucine >> proline = histidine > lysine > glycine. In rat glomeruli tryptophan was incorporated at the highest rate, with phenylalanine and tyrosine ranked next. The order of incorporation was tryptophan >> phenylalanine > tyrosine = leucine > proline > histidine. The rate of incorporation of amino acids into rat glomeruli was higher than that in tubules, however the same pattern of amino acid incorporation was followed.

Proline and the aromatic amino acids were chosen to assess de novo synthesis of protein because they are believed to play an important role as structural constituents of some GBM proteins. Proline is regarded as a major constituent of the collagen-like structure, and its conversion to the hydroxylated amino acid takes place specifically in GBM. This structural integrity seems to play an important role in the cell-matrix interactions. Fibronectin, a non-collagen GBM protein, is thought to be formed by floppy, asymetric molecules with localized regions of folding in which aromatic amino terminals (ie. the TRP and TYR terminals) appear to have the function of keeping this structural characteristic which seems to be important for intercellular adhesion and to promote cellular spreading.

It is important to point out the higher rate of incorporation of the aromatic amino acids into rat glomeruli, compared to proline, suggesting a higher turnover in glomerular proteins other than GBM-collagen (ie. fibronectin). This is consistent with previous observations in vivo in which the turnover rate of GBM-collagen was found to be about 100 days, while the turnover of non-collagen glomerular proteins took about 9 days only (Price and Spiro, 1977).

Isolated pig and rat glomeruli and rat tubules metabolized glucose to CO_2 linearly for several hours. In both species, the glucose oxidative glomerular metabolism, was much lower than fatty acid oxidation. Rat glomeruli metabolized glucose at a higher rate than tubules. Isolated pig and rat glomeruli and rat tubules also oxidize fatty acids to CO_2 linearly, for several hours.

When various chemicals that target in vivo for different parts of the nephron were assessed, Adriamycin exerted a strong inhibition on de novo synthesis of protein, from proline, in isolated pig glomeruli. Although it has been reported previously that Adriamycin inhibits synthesis of protein in isolated rat glomeruli (Ahmed et al., 1987), no data has shown the effect of the drug in the synthesis of protein by isolated pig glomeruli. Therefore, the data presented above appear to be the first reporting such effect in this species in vitro.

Adriamycin markedly inhibited the oxidative metabolism of fatty acids, such as linolenic and oleic acid. There are no previous observations on the effect of Adriamycin on fatty acid metabolism of isolated glomeruli of any species. The findings reported in this thesis are however consistent with observations on the inhibition of the oxidation of palmitic acid to CO_2 by renal mitochondria following Adriamycin treatment to rats (Bizzi et al, 1983), although the type of cell responsible for this effect could not be specified in this paper, as it used whole renal homogenates.

Adriamycin also inhibited the oxidative metabolism of glucose but to a lesser degree than fatty acids.

Puromycin aminonucleoside effects on de novo synthesis of protein and oxidative metabolism of isolated pig glomeruli, are weak compared to Adriamycin, suggesting that either it is not the compound per se, but a metabolite that may exert the toxic effect, or that de novo synthesis of protein is not sensitive and a different biochemical pathway is affected as part of the mechanism of injury.

Although moderate, the inhibition of glucose metabolism is consistent with observations on isolated glomeruli of Puromycin aminonucleoside nephrotic rats, where there was a decrease in glucose metabolism.

Ethacrynic acid showed the strongest inhibitory effect on de novo protein synthesis in isolated pig glomeruli but fatty acid and glucose metabolism were also remarkably decreased. The effect of the drug on fatty acid and glucose metabolism has been previously shown in isolated rat glomeruli by Meezan and Brendel (1973). However, there appear to be no reports on the effects of ethacrynic acid on glomerular synthesis of protein by isolated pig glomeruli.

2-Bromoethanamine showed a moderate inhibitory effect on protein synthesis of isolated pig glomeruli. Although, due to the selectivity of the compound for the renal papilla, a lesser effect on glomeruli would be expected, its potential alkylating properties may have been responsible for the inhibitory effect on protein synthesis.

2-Bromoethanamine also enhanced glucose oxidative metabolism in isolated pig glomeruli, at all concentrations tested.

OTA

The mycotoxin remarkably decreased de novo protein synthesis by isolated rat glomeruli and tubules. This is consistent with findings that OTA inhibited the synthesis of protein in several in vivo and in vitro systems. However, no one has ever looked at the biochemical processes taking place in glomeruli exposed to the action of OTA in vitro.

Although OTA showed a remarkable generalized depression on the incorporation of the six amino acids tested, there were differences in their sensitivity to OTA effect. All three aromatic amino acids, phenylalanine, tyrosine and tryptophan were more sensitive to the effects of OTA than proline, suggesting a great involvement of GBM non-collagen proteins in OTA toxicity in isolated rat glomeruli.

Small doses of OTA stimulates glucose oxidative metabolism. This can be due to an increase in either glycolysis or pentose phosphate shunt. There appears to be no report in the literature about the effect of OTA on this metabolic pathway in isolated glomeruli or other systems.

Low concentrations of OTA also increase moderately the oxidative metabolism of linolenic acid. This can be due to an increase in either mitochondrial Beta-oxidation or peroxisomal Beta-oxidation. This is consistent with findings of enhancement of peroxisomal Beta-oxidation in cortex of pig kidney after administration of OTA, although the type of cell responsible for this effect, was not specified.

In conclusion the experiments detailed above have highlighted:

1- The use of isolated glomeruli a)- as a system which allows the study of characteristics that are difficult to study in the intact organ.

b)- For screening of chemicals for glomerular toxicity.

c)- To study the mechanism of action of nephrotoxic compounds.

2- The differences in pig and rat glomerular metabolism.

3- The glomerular effects of OTA *in vivo* and *in vitro*.

4- The potential role of glomeruli in the toxic lesion caused by neprotoxicants.

Conclusions:

The following conclusions can be drawn from the present data:

1- Isolated rat and pig glomeruli maintain a range of metabolic fuctions (protein synthesis, glucose and fatty acid oxidation) for several hours.

2- The incorporation of a high proportion of aromatic amino acids into isolated rat glomeruli macromolecules is a novel observation.

3- From the metabolic pathways assessed in isolated pig glomeruli, proline incorporation appeared to be the most sensitive index of glomerular cytotoxicity caused by Adriamycin.

4- Protein synthesis from aromatic amino acids such as phenylalanine and tyrosine appears to be a sensitive parameter of OTA toxicity.

5- The sensitivity of the aromatic aminoacids to the effect of OTA (compared to proline) suggests that glomerular macromolecules other than GBM-collagen may be affected. These could include other functional and

structural proteins.

6- The inhibition on the incorporation of aromatic amino acids into glomerular macromolecules by OTA suggests that the synthesis of specific proteins may be central to the mechanism of target selective toxicity of OTA towards the kidney.

7- The findings on OTA toxicity may be relevant to porcine endemic nephropathy and to the human condition BEN.

8- The ability of isolated pig and rat glomeruli to synthesise protein and to perform different biochemical reactions involved in the oxidation of substrates to CO_2, offers a series of useful metabolic systems with which to study target selective toxicity.

Future research directions:

In common with most other research, some questions have remained unanswered and a score of vital questions has been opened:

- It is necessary to relate proline behaviour, in protein synthesis to GBM structural and chemical changes after toxic injury. Studies on the incorporation of proline into GBM, as well as its conversion to OH-proline will provide more specific information on GBM changes.

- The findings on the high incorporation of aromatic amino acids into glomerular protein, have raised the question on the possible role of other constituents (ie. aromatic amino acids vs. proline) of the glomerular extracellular matrix as related to glomerular injury, and the possibility that they can be used as sensitive indices of toxicity.

- It is necessary to follow the fate of the precursors within the different glomerular proteins and also to identify changes on the composition of specific glomerular proteins.

- The weak effects shown by Puromycin aminonucleoside on isolated glomeruli, raise the question of how and why this chemical is such a potent glomerulotoxin *in vivo*, but has only a moderate effect when incubated with glomeruli *in vitro*. One attractive hypothesis is that puromycin aminonucleoside is not the proximate toxin.

Further studies are necessary to elucidate the *in vivo* - *in vitro* effect, on the incorporation of proline in animals treated with puromycin aminonucleoside at early and different time points. It is also necessary to compare the effect of the parent compound and its metabolites on protein synthesis and other metabolic pathways.

- In view of the potent effect of ethacrynic acid on glomerular protein synthesis and oxidative metabolism, and the fact that this compound is a widely used drug considered to have few nephrotoxic effects, several questions are raised. These include the importance of ethacrynic acid effects on glomerular metabolism *in vitro*, as related to glomerular function *in vivo*. Whether the inhibition of protein synthesis is a secondary effect, or is it a direct effect. What are the possible GBM structural and compositional changes caused by the compound. Studies with ethacrynic acid-related compounds and metabolites such as the saturated derivative on *in vitro* glomerular protein synthesis will help to elucidate the mechanism of action and the relevance, if any, of the glomerular effect to the pharmacological action of the drug.

- It is necessary to establish a dose-response for 2-Bromoethanamine with a lower range of concentrations (0 - 1 mM), for protein synthesis, and to elucidate the mechanism responsible for the inhibitory effect. The effect of 2-bromoethanamine on glucose metabolism is unexpected and it is necessary to determine which of the glucose metabolic pathways is enhanced by the compound: glycolysis or pentose phosphate shunt, and the site of enhancement.

- With regard to OTA, expanding the range of OTA concentrations between 0 - 100 uM, will establish if greater differences in the rate of incorporation between the different amino acids can be identified at lower concentrations than the ones assessed in these studies.

It is also necessary to establish which is the route of glucose metabolism enhanced by OTA. Is it the pentose phosphate shunt or glycolysis and which of the beta- oxidative pathways (mitochondrial or peroxisomal) is affected by OTA. This should be measured over the range of concentrations between 0 - 100 uM, in order to obtain a dose response relationship.

- The relationship between the <u>in vivo</u> and <u>in vitro</u> effect of OTA on glomerular proteins needs to be related to GBM structure and composition. Electrophoretic and autoradiographic studies of glomerular protein synthesised from different labelled amino acids in the presence and absence of OTA, could help follow the fate of the precursors and the effect of the mycotoxin on the composition of glomerular protein. It would also be useful to establish where different labelled amino acids (especially the aromatic compounds) are distributed in glomeruli using

autoradiography at the light and electron microscopy levels.

- In vivo studies such as micropuncture of individual nephrons, will allow to distinguish between tubular and glomerular changes following toxic insult with OTA, and also to correlate functional with metabolic in vitro changes, but this techniques can only be undertaken with specialised equipment.

- It has been shown that glomeruli are vulnerable to the effect of OTA, but because of their cellular heterogeneity, from the observations on isolated glomeruli, the changes detected can be assessed as specific for glomeruli (in relation to other renal structures), but not specific for the various populations of glomerular epithelial and mesangial cells. The application of cell culture of glomerular cells will offer a unique means of assessing their individual response to the toxic injury such as changes in their metabolic properties and morphology.

- The fact that glomeruli from superficial and juxtamedullary zones of the kidney differ, has led our group to start studying the sensitivity of these two different populations of glomeruli. This is based on using isolated glomerular fragments that have been obtained from surgically separated superficial and juxtamedullary regions of the rat kidney respectively. The two populations of glomeruli are then exposed to adriamycin and have been shown to be different. The same approach could also be used to assess whether there are metabolic differences in the response of isolated superficial and juxtamedullary glomeruli from rats, the pig and man exposed to OTA.

- Immunohistochemical techniques at a light microscopy and electron microscopy will allow early glomerular changes taking place over the time course following administration of OTA, to be highlighted. These might no have been seen when general non-specific histopathological methods were used.

- It will be important to assess the effect of simultaneous exposure to more than one mycotoxin on isolated glomeruli.

- The relationship between the biochemical effects on isolated glomeruli and the pathology of OTA lesion still needs to be investigated.

These questions offer an interesting challenge to find out more about the role of glomeruli in nephrotoxicity and how mycotoxins may affect the kidney in animals and man exposed to these environmental chemicals with so much potential to cause major diseases.

REFERENCES

Ahmed, I., Lock, E.A. and Bach, P.H. In: Proceedings of the Third International Symposium on Nephrotoxicity, Guilford, 1987 (Abstract).

Albassam, M.A., Yong, S.I., Bhatnagar, R., Sharma, A.K., and Prior, M.G. Vet. Pathol. 24:427-435 .1987.

Alpert, M. E., Hutt, M.S.R., Wogan, G.N. and Davidson, C.S. Cancer.28: 253-260. 1971.

Appel, G.B. and Neu, H.D. N. Engl. J. Med. 296:722-728. 1980.

Asplin, F.D. and Carnaghan, R.B.A. Vet. Rec. 73: 1215-1218. 1961.

Bachman, S., Sakai, T. and Kriz, W. In:(Jones, T.C., Mohr, W. and Hunt, R.D. eds.) Urinary System. pp.3-24. Springer-Verlag, Berlin, 1986.

Bai, N.J., Pai, M.R. and Venkitasubramanian, T.A. Indian J. Biochem. Biophys. 14: 347-349. 1977.

Balant,L. and Fabre,J. In: (Hans Huber,ed.) Diagnostic Significance of Enzymes and Proteins in Urine. pp.216. Berne,1979.

Balazs,T., Hatch,A., Zawidzka,Z. and Grice,H.C. Toxicol.Appl.Pharmacol. 5:661-674. 1963.

Bassir,O. and Osiyemi,F. Nature. 215:882. 1967.

Bassir, O. and Bababunmi,E.A. Biochem. Pharmacol. 22:132-134. 1973.

Baud, L., Nivez, M.P., Chansel, D. and Ardadillou, R. Kidney Int. 20:332-339. 1981.

Beard,M.E., Baker,R., Conomos,P., Pugatch,D. and Holtzman,E. J. Histochem. Cytochem. 33:460-464. 1985.

Bender, B.L., Jaffe, R., Carlin, B. and Chung A.E. Am. J. Pathol. 103:419-426. 1981.

Berndt,W.O. and Hayes,A.W. Toxicology.12: 5-17. 1979.

Berndt,W.O., Hayes,A.W. and Philips,R.D. Kidney Int. 18: 656-664. 1980.

Berndt,W.O. and Hayes, A.W. Toxicol. Appl. Pharmacol. 64: 118-124. 1982.

Bertani, T, Poggi, A, Pozzoni, R, B.Sci., Delaini,F, Sacchi, G, Thoua, Y, Mecca, G, Remuzzi, G and Donati, M. Lab.Invest. 46(1): 16. 1982.

Bizzi, L., Ceriani, M., Gerundino, A. Spina, M.T., Tacconi, E. and Veneroni. Toxicol.Lett. 18: 291-300. 1983.

Blau,E.B. and Michael,A.F. J. Lab. Clin. Med. 77:97-109. 1971.

Blau, E.B. and Michael, A.F. Proc. Soc. Exp. Biol. Med. 141:164-172. 1972.

Brendel, K and Meezan, E. II. Metabolism. J. Pharmacol. Exp. Ther. 187:342-351. 1973.

Brown,D.M., Michael, F. and Oegema, T.R. Biochim. Biophys. Acta. 674:96-104. 1981.

Burch, H.B., Cole, B., Alney, T.R. and Dence, C. Int. J. Biochem. 12: 37-40. 1980.

Camazine, S.M., Ryan, G.B., Unanue, E.R. and Karnovsky, M.J. Lab. Invest. 35:315-326. 1976.

Campbell, T.C. and Stoloff, L. J. Agr. Fd. Chem. 22:1006-1015. 1974.

Carlin, B., Jaffe, R. and Chung, A.E. J. Biol. Chem. 256:5209-5214. 1981.

Cauldfield, J.P., Reid, J.J. and Farquhar, M.G. Lab. Invest. 34:43-59. 1976.

Cauldfield,J.P. and Farquhar,M.G. Lab. Invest. 39:505-512. 1978.

Ceovic,S., Grims,P. and Mitar,J. Lig. Vjes. 98: 301-304. 1976.

Chang,F.C. and Chu,F.S. Fd. Cosmet. Toxicol. 15, 199-204. 1977.

Chernozemsky, I.N., Stoyanov,I.S., Petkova-Bocharova,T.K., Nokolov,I.G., Draganov,I.V., Stoichev,I., Tanchev,Y., Naidenov,D. and Kalcheva, N.D. Bulg.J.Cancer. 19:1-11. 1977.

Choi, M.G. and Hynes, R.O. J. Biol. Chem. 254:2050-55. 1979.

Chow, A.Y.K. and Drummond K.N. Lab. Invest. 20:213-218. 1969.

Chu, F. S. Arch. Biochem. Biophys. 147: 359-366. 1971.

Chu, F. S., Noh, I. and Chang, C. C. Life.Sci. 11 (Part 1): 503-508. 1972.

Chu, F. S. Biochem. Pharmacol. 23: 1105-1113. 1974.

Clifford,J.I. and Rees,K.R. Biochem.J., 102: 65-75. 1967.

Cohen, J.J. and Barac-Nieto, M. In: (Berliner, R.W. and Orloff,J.,eds.). Handbook of Physiology, Section 8: Renal Physiology. pp.909. American Physiological Society, Washington,D.C. 1973.

Connole, M.D., Blaney, B.J. and McEtwan, T. Austral. Vet. J. 57: 1981.

Connole, M.D. and Hill, M.W.M. Austral. Vet. J. 46: 503-505. 1970.

Cottrell,R.C., Agrelo,C.E., Gangoli,S.D. and Grasso,P. Fd. Cosmet. Toxicol. 14: 593-598. 1976.

Courtoy, P.J., Kanwar Y.S., Timpl, R., Hynes, R.O. and Farquhar, M.G. J. Cell Biol. 87:2,(abstract). 1980a.

Courtoy, P.J., Kanwar, Y.S., Hynes, R.O. and Farquhar, M.G. J. Cell

Biol. 87:691-699. 1980b.

Courtoy, P.J., Timpl, R. and Farquhar,M.G. J. Histochem. Cytochem., 30:874-886. 1982.

Creppy,E.E., Lugnier,A.A.J., Beck,G., Roschenthaler,R. and Dirheimer,G., FEBS Lett.104(2):287-290. 1979.

Creppy,E.E., Lugnier,A.A.J., Fasiolo,F., Heller,K., Roschenthaler,R. and Dirheimer,G. Chem. Biol. Interact. 24, 257-261. 1979.

Creppy,E.E., Atormer,F.C., Kern,D., Roschenthaler,R. and Dirheimer,G., Chem. Biol. Interact. 47: 239-247. 1983.

Creppy,E.E., Roschenthaler,R. and Dirheimer,G. Fd. Chem. Toxicol. 22: 883-886. 1984.

Creppy, E.E., Kane, A., Giessen-Crouse, E., Roth, A., Roschenthaler, R. and Dirheimer, G. Arch. Toxicol. Suppl.9: 310-314. 1986.

Culling, C.F.A. Handbook of Histopathological and Histochemical Techniques. Butterworths, London. 1974.

Davies, M., Coles, G.A. and Hughes, K.T. Renal Physiol. 3:106-111. 1980a.

Davies, M., Hughes, K.T. and Thomas G.J. Renal Physiol. 3:116-119. 1980b.

Davies, P. and Bonney, R.J. In: (Weissmann, G.,ed.) The Cell Biology of Inflammation. pp. 497-542. Elsevier, Amsterdam. 1980.

Dermer,O.C. and Ham,G.E. Ethylenimine and other Aziridines. Academic Press. New York, 1969.

Diener, U.L. and Davis, N.D. Phytopathol. 56: 1390-1393. 1966.

Diener, U.L. and Davis, N.D. In: (Goldblatt, L.A., ed.). Aflatoxin: Scientific background, control and implications.p.55. Academic Press, New York. 1969.

Dirr,H.W. and Schabort,J.C. Biochim. Biophys. Acta. 881: 383-390. 1986.

Dirr H.W., and Schabort J.C. Biochim.Biophys.Acta. 913: 300-307. 1987.

Doerr,J.A., Huff,W.E., Tung,H.T., Wyat,R.D. and Hamilton,P.B. Poult. Sci. 53:1728-1734. 1974.

Doherty, W.P. and Campbell, T.C. Chem. Biol. Interact. 7: 63-77. 1973.

Doster, R.C. and Sinnhuber, R.O. Fd. Cosmet. Toxicol. 10: 389-394. 1972.

Dousa, T.P. In: (Seldin, D.W. and Giebish, G., eds.) The Kidney: Physiology and Pathophysiology.pp.645-667. Raven Press, New York.1985.

Dousa, T.P., Barnes L.D., Ong S.H. and Steiner A.L. Proc. Natl. Acad. Sci.USA. 74:3569-3573. 1977.

Dousa, T.P., Shah, S.V. and Abboud, H.E. Adv. Cycl. Nucleot. Res. 12:285-299. 1980.

Dousa, T.P. In: (Yoshida, H., Hagihara, Y. and Ebashi, S, eds.). Advances in Pharmacology and Therapeutics II. pp. 295-300. Pergamon, Oxford. 1981.

Dubach, U.C. and Recant, L. J. Clin. Invest.39:1364-1371. 1960.

Duggan, D.E. and Noll, R.M.. Arch. Biochem. Biophys.109: 388-396. 1965.

Dwivedi,P and Burns,R.B. Res.Vet.Sci., 36: 117-121. 1984.

Dwivedi,P. and Burns,R.B. Res.Vet.Sci.36: 92-103. 1984.

Edidin, M. J. Immunol. 104:1303-1306. 1970.

Elling, F. and Moller, T. Bull. Wld. Hlth. Org. 49: 411-418. 1973.

Elling,F., Hald,B., Jacobsen,Chr. and Krogh,P. Acta Path. Microbiol. Scand. 83:739-741. 1975.

Elling,F. Nielsen,J.P., Lillehoj,E.B., Thomassen,M.S. and Stormer,F.C. Toxicon. 23:247-254. 1985.

Farquhar, M.G. In: (Kefalides A.N.,ed.). Biology and Chemistry of Basement Membranes. pp.44-80. Academic Press, New York. 1978.

Feuer, G., Golberg, L. and Lepelley, J.R. Fd. Cosmet. Toxicol. 3:235-249.1965.

Fishman J.A. and Karnovsky M.J. Am. J. Pathol. 118:398-407. 1985.

Foidart, J.B., Pirard, Y.S., Winand, R.J. and Mahieu, P.R. Renal Physiol. 3:169-173. 1980a.

Foidart, J.M., Foidart, J.B. and Mahieu, P.R. Renal Physiol. 3:183-192. 1980b.

Foidart, J., Sraer, J., DeLarue, F., Mahieu, P. and Ardaillou, R. FEBS Lett. 121:333 - 339. 1980c.

Foidart, J.B., Dechenne, C., Dubois, C., Deheneffe, J. and Mahieu, P. In: (Hamburger J., Crosnier J., Grunfeld, J.P. and Maxwell,M.H.,eds.). Advances in Nephrology. pp. 267-292. Year Book, Chicago. 1981.

Folkert,V.W. and Schlondorff,D. Prostaglandins.17:79-86. 1979.

Fong, J.S.C. and Drummond, K.N. Lab. Invest. 29: 512-515. 1969.

Fonseca, H., Nogueira, J.N., Graner, M., Oliveira, A.J., Caruso, J.G.B., Boralli, C., Calori, C.A. and Khatounian, C.A. In: Proceding of the International IUPAC symposium on mycotoxins and Phycotoxins. pp.122-125. IUPAC, Vienna. 1982.

Fuchs,R., Radic,B., Peraica,M., Hult,K., and Plestina,R. Period. Biol. (In Press).1988.

Galtier, P. Ann. Rech. Vet.5:311-318. 1974a.

Galtier, P. Ann. Rech. Vet. 5:319-328. 1974b.

Galtier, P. and Alvinerie,M. Ann. Nutr. Alim. 31:911-918.1976.

Galtier, P. Etude toxicologique et pharmacocinetique d'une mycotoxine, l'ochratoxine A. These d'Etat es Sciences Pharmaceutique, Toulouse. 1979.

Galtier, P., Charpenteau, J.L., Alvinerie, M. and Labouche, C. Drug Metab. Dispos. 7(6). 1979.

Galtier, P., Alvinerie, M. and Charpenteau, J.L. Fd. Cosmet. Toxicol. 19: 735-738. 1981.

Giridhar, N. and Krishnamurthy, G.V. J. Fd. Sci. Technol. 10:84-85. 1977.

Glassock, R.J. In: (Kefalides A.N.,ed.). Biology and Chemistry of Basement Membranes. pp.421-441. Academic Press, New York. 1978.

Grant, M.E., Harwood, R. and Williams, I.F. Eur. J. Biochem. 54:531-540. 1975.

Gregoire, F. Kidney Int. 7:86-93. 1975.

Gregoire, F., Askenasi, R. and Potvliege, P. In: (Hamburger, J., Crosnier, J., Maxwell, M.H.,eds.). Advances in Nephrology. pp 165-181. Year book, Chicago. 1976.

Grond, J. and Elema, J.D. Lab. Invest. 45:400-409. 1981.

Grosman,M.E., Elias,M.M., Comin,E.J. and Garay,E.A.R. Toxicol. Appl. Pharmacol. 69, 319-325. 1983.

Grosman,M.E., Elias,M.M., Comin,E.J. and Garay,E.A.R. Toxicol. Lett. 21, 263-270. 1984.

Hagelberg,S., Hult,K. and Fuchs,R. J. Appl. Toxicol. (In press). 1988.

Hall, P.W. and Dammin, G.J. Nephron. 22: 281-300. 1978.

Hamilton, P.B. Poultry Sci. 50: 1880-1882. 1971.

Halmiton, P.B. Federation Proc. 36: 1899-1902. 1977.

Hansen,C.E., Dueland,S., Drevon, C.A., and Stormer,F.C. Appl. Environ. Microbiol. pp. 1267-1271. 1982.

Hartley, R.D., Nesbitt, B.F. and O'Kelly,J. Nature,London. 198: 1056-1058. 1963.

Harwig, J., Przybylski, W. and Moodie, C.A. Canadian Med. Ass. J. 1:113-281. 1975.

Hassid, A., Konieczkowski, M. and Dunn, M. Proc. Natl. Acad. Sci. USA. 76:1155-1159. 1979.

Hayashi, Y., Imazawa, T., Kokubo, T., Kurokawa, Y. and Takahashi, M. Toxicol. Lett. 20:105-110. 1984.

Hedman, K. J. Histochem. Cytochem. 28:1233-41. 1980.

Hietanen, E., Malaveille, C., Camus, A.M., Bereziat, J.C., Brun, G., Castegnaro, M., Michelon, J., Idle, J.R. and Bartsch, H. Drug Metabol. Dispos. 14:118-126. 1986.

Hjelle, J.T., Carlson, E.C., Brendel, K. and Meezan, E. Kidney Int. 15:20-32. 1979.

Hohenegger, M. In: (Schmidt,U. and Dubach,U.C., eds.). Renal metabolism in relation to renal function. pp.99-107. Huber, Bern. 1976.

Houser, M.T., Scheinman, J.I., Basgen, J., Steffes, M.W. and Michael, A.F. J. Clin. Invest. 69:1169-1175. 1982.

Hsieh,D.P.H., Wong,Z.A., Wong,J.JH., Michas,C. and Reubner,B.H. In: (Rodericks,J.V., Hesseltine,C.W. and Mehlman,M.A.,eds.). Mycotoxins in Human and Animal Health. pp.37-50. Pathotox, Park Forest,South,IL. 1977.

Huff, W.E., Doerr, J.A. and Hamilton, P.B. Appl. Environ. Microbiol. 37:122-126. 1979.

Huff, W.E., Doerr, J.A., Hamilton, P.B., Hamann, D.D., Peterson, R.E. and Ciegler, A. Appl. Environ. Microbiol. 40:102-107. 1980.

Huff, W.E. and Doerr, J.A. Poult. Sci. 60:550-555. 1981.

Hult, K., Plestina, R., Habazin-Novak, G., Radic, B. and Ceovic, S. Toxicol. 51:313-321. 1982.

Hunsicker L.G, Shearer T.P., and Shaffer S.J. Kidney Int. 20:7-17. 1981.

Hutchinson, R.D. and Steyn, P.S. Tetrahedron Lett. 43:4033-4036. 1971.

Imbert, M., Chabardes, D. and Morel, F. Mol. Cell. Endocrinol. 1:295-304. 1974.

Iwanga, S., Suzuki, K. and Hashimoto, S. Ann. N.Y. Acad. Sci. 312:56-73. 1978.

Jimeno, M. de, Ruiz, N. and Pena, N. Revista ICA (Colombia). 15:129-134. 1980.

Jones, V.D., and Landon, E.J. Biochem. Pharmacol. 16:2163-2169. 1967.

Jukes, T.H. Nature,London. 271-499. 1978.

Kane, A., Creppy, E.E., Roschenthaler, R. and Dirheimer, G. Toxicology. 42:233-243. 1986a.

Kane, A., Creppy, E.E., Roth, A., Roschenthaler, R. and Dirheimer, G. Arch. Toxicol. 58: 219-244. 1986b.

Kanisawa, M., Suzuki, S., Kozuka, Y. and Yamazaki, M. Toxicol. Appl.

Pharmacol. 42:55-64. 1977.

Kanwar,Y.S. and Farquhar,M.G. J.Cell Biol. 81:137-153. 1979.

Kaplan, B.S., Renaud, L. and Drummond, K.N. Kidney Int. 6:170-176. 1974.

Kefalides, N.A. and Forsell-Knott. Biochim. Biophys. Acta. 203:62-66. 1970.

Kefalides, N.A. Biochem. Biophys. Res. Commun. 45:226-234. 1971.

Kefalides, N.A., Alper, R. and Clark, C.C. In: (Bourne, G.H., Danielli, J.F. and Jeon, K.W., eds.). International Review of Cytology. pp. 167-228. Academic Press, New York. 1979.

Kefalides, N.A. Renal Physiol. 4:57-66. 1981.

Ketterer, P.J., Blaney, B.J., Moore, C.J., McInnes, I.S. and Cook, P.W. Austral. Vet. J. 59:113-117. 1982.

Kingsley, G.R. J. Biol. Chem. 131:197-200. 1939.

Kissane, J.M. and Hoff, E. J. Histochem. Cytochem. 10:259-268. 1962.

Klein, D.J., Dehnel, P.J., Oegema, T.R. and Brown, D.M. Lab. Invest. 50:543-551. 1984.

Koide, H., Soeda, N. and Ohno H. J. Renal Physiol. 4:102-107. 1981.

Konrad, I. and Roschenthaler, R. FEBS Lett. 83:341-347. 1977.

Kornfeld, J. and Ginsburg, B. Exp. Cell Res. 41:592-600. 1966.

Kreisberg, J.I. and Karnovsky M.J. Kidney Int. 23:439-447. 1983.

Krishnamachari, K.A.V.R., Bhat. R.V., Nagarajan, V. and Tilak, T.B.G. Indian J. Med. Res. 63:1036-1048. 1975.

Krisko, I. and Walker, W.G. Proc. Soc. Exp. Biol. Med. 146:942-947. 1974.

Krisko, I. and Walker, W.G. Biochim. Biophys. Acta. 446:506-514. 1976.

Krogh, P. In: (Puchlev,A.,ed.). Endemic nephropathy. Proceedings of the second international symposium on endemic nephropathy.pp.266-270. Bulgarian Academy of Sciences, Sofia. 1972.

Krogh, P., Hald, B., Hasselager, E., Madsen, A., Mortensen, H.P., Larsen, A.E. and Campbell, A.D. Pure Appl. Chem. 35:275-282. 1973.

Krogh, P., Azelsen, N.H., Elling, F., Gyrd-Hansen, N. Hald, B., Hyldgaard-Jensen, J., Larsen, A.E., Madsen, A., Mortensen, H.P., Moller, T., Petersen, O.K., Ravnskov, U., Rostgaard, M. and Aalund, O. Acta Pathol. Microbiol. Scand. Suppl. 246: 1-21. 1974.

Krogh, P., Elling, F., Gyrd-Hansen, N., Hald, B., Larsen, A.E., Lillehoj, E.B., Madsen, A., Mortensen, H.P. and Ravnskov, U. Acta Path. Microbiol. Scand. 84:429-434. 1976.

Krogh, P. and Neisheim, S. In: (Egan, H. Stoloff, L., Castegnaro, M., Scott, P., O'Neill, I.K. and Bartsch, H., eds.). Environmental carcinogens: Selected Methods of Analysis, vol. 5, Some Mycotoxins. pp.247-253. I.A.R.C., France. 1982.

Krogh, P. In: (Rodricks, J.V., Hesseltine C.W. and Mehlman, M.A.,eds.). Mycotoxins in Human and Animal Health. pp.489-498. Pathotox Publishers, Inc. Illinois, USA. 1977.

Krogh, P. Acta Pathol. Microbiol. Scand. Sect. A 269:1-28. 1978.

Krogh, P. In: (Strahinjic, S. and Stafanovic, V. eds.). Endemic Balkan Nephropathy. Proceedings of the 4th symposium on Endemic (Balkan) Nephropathy, NIS. 1979.

Krogh, P., Elling, F., Friis, Chr., Hald, B., Larsen, A.E., Lillehoj, E.B., Madsen, A., Mortensen, P., Rasmussen, F. and Ravnskov, U. Vet. Pathol. 16:466-475. 1979.

Kumagai, S. and Aibara, K. Toxicol. Pharmacol. 64:94-102. 1982.

Kwizera, E.N. and Bach, P.H. In: Proceedings of the Third International Symposium on Nephrotoxicity, Guilford, 1987, (abstract).

Landon, E.J. and Fitzpatrick, D.F. Biochem. Pharmacol. 21:1561-1568. 1972.

Lelongt, B., Makino, H. and Kanwar, Y.S. Kidney Int. 31:1299-1310. 1987.

Lillehoj,E.B. and Ciegler,A. In: (Schlessinger, D.,ed.). Microbiology pp.344-358. American Society for Microbiology,Washington DC. 1975.

Lillehoj, E.B., Kwolek, W.F., Elling, F. and Krogh, P. Mycopathologia 68:175- 177. 1979.

Lillie, R.D. and Fulmer,H.M. Histopathologic technique and practical histochemistry. Butterworths, London. 1976.

Lindahl, U. and Hook, M. Annu. Rev. Biochem. 47:385-417. 1978.

L'vova, L.S., Sosedov, N. I., Garall, W., Schwarman, M.I., Shatilova, T.I. and Shulgina, A.P. Prikladnaya Biochimia i Microbiologia. 12:741-749. 1976.

Madhavan, T.V., Tulpule, P.G. and Gopalan, C. Arch. Path. 79:466-469. 1965.

Madhavan, T.V. and Rao, K.S. J. Path. Bact. 93:329-331. 1967.

Madri, J.A., Roll, F.J., Furthmayr, H., and Foidart, J.M. J. Cell Biol. 86:682-687. 1980.

Martinez, G.P. de, and Escobar, L.A. de. Revista Gallescencia (Colombia). 1:79-88. 1981.

Matsuo, E. and Yamada, K. Renal Physiol. 3:97-105. 1980.

McIntosh, P.E., Evans, I.M. and Rabin, B.R. Br. J. Cancer 33:440-449. 1976.

Meezan, E., Brendel, K., Ulreich, J. and Carlson EC. J. Pharmacol. Exp. Ther. 187:332-341. 1973.

Meezan, E. and Brendel, K. J. Pharmacol. Exp. Ther. 187:352-364. 1973.

Meisner, H. and Chan, S. Biochemistry. 13:2795-2800. 1974.

Meisner, H. and Selanik, P. Biochem. J. 180:681-684. 1979.

Meisner, H. and Meisner, P. Arch. Biochem. Biophys. 208:146-153. 1981.

Meisner, H. and Krogh, P. In: Proceedings of the V International IUPAC Symposium on Mycotoxins and Phycotoxins. pp.342-345. Vienna. 1982.

Meisner, H., Cimbala, M.A. and Hanson, R.W. Arch. Biochem. Biophys. 223:264-270. 1983.

Meisner, H. and Polsinelli, L.Biochem. Pharmacol. 35:661-665. 1986.

van der Merwe,K.J., Steyn,P.S., Fourie,L., Scott De B. and Theron,J.J. Nature, London. 205:1112-1113. 1965.

Michael, A.F., Blau, E. and Vernier, R.L. Lab. Invest. 23:649-657. 1970.

Michael, A.F., Keane W.F., Raij l., Vernier R.L., Mauer S.M. Kidney Int. 17:141-154. 1980.

Mintzlaff, H.J., Lotzsch, R., Tauchmann, F., Meyer, W. and Leistner, L. Fleischwirtschaft. 54:774-778 . 1974.

Misra, R.P. Am. J. Clin. Pathol. 58:135-139. 1972.

Mondorf, A.W., Hendus,J., Scherberich,J.E., Mackenrodt,G., Shah,P.M. Stille,W. and Schoeppe,W. Europ. J. Pharmacol. 13:133-142. 1978.

Moore, J.H. and Truelove, B. Science. 168:1102-1103. 1970.

Moule,Y. Ann. Nutr. Aliment. 28:375-384. 1974.

Munro, I.C., Moodie,C.A., Kuiper-Goodman, Scott,P.M. and Grice,H.C. Toxicol. Appl. Pharmacol. 28:180-188. 1974.

Mynderse, M.S., Hassel, J.R., Kleinman, H.K. and Martinez-Hernandez, A. Lab. Invest. 48:292-302. 1983.

Naftalin, L., Sexton, M., Whitaker, J.F. and Tracey, D. Clin. Chim. Acta 26:293 . 1969.

Nassar, A.Y., Megalla, S.E., Abd El-Fattah, H.M., Hafez, A.H. and El-Deap,T.S. Mycopathologia 79:35-38. 1982.

Nayyar, R.P., Volini F.I. and Borke J.L. Renal Physiol. 3:212-219. 1980.

Nel, W. and Purchase, I.F.H. J. S. Afr. Chem Inst. 21:87. 1968.

Nelson, G.H. and Christensen C.M. Mod. Vet. Pract. 57:529-532. 1976.

Nevins T.E. and Michael A.F. Kidney Int. 19:553-563. 1981.

Ngaha, E.O. Enzyme 33:1-8. 1985.

Nicolov, I.G., Chernozemsky, I.N., Petkova-Bocharova, T., Stoyanov, I.S. and Stoichev, I.I. Europ. J. Cancer 14:1237-1242. 1978.

Nishino, T., Gallant, J., Shalit, P., Palmer, L. and Wehr,T. J. Bacteriol. 140:671. 1979.

Nomiyama, K., Sato, C., and Yamamoto, A. Toxicol. Appl. Pharmacol. 24:625-635. 1973.

Norgaard J.O.R. Kidney Int. 9:278-285. 1976.

Norgaard J.O.R. Lab. Invest. 41:224-236. 1979.

Nowinski, W.W. and Pigon, A. J. Histochem. Cytochem. 15:32-37. 1967.

Novikoff, A.B. and Goldfischer, S. J. Histochem. Cytochem. 17:675-680. 1969.

Obara, T., Akiba, I., Ueno, Y. and Endou, H. Jpn. J. Pharmacol. 36:193P. 1984.

Oberley, T.D., Deane, F.M., Mosher, M.D. and Mills, M.D. Am. J. Pathol. 96:651-662. 1979a.

Olden, K. and Yamada, K.M. Cell 11:957-69. 1977.

Olden, K., Hunter, V.A. and Yamada, K.M. Biochem. Biophys. Acta 632:408-16. 1980.

Pai, M.R., Bai, N.J. and Venkitasubramanian, T.A. Chem.-Biol. Interactions, 10:123-131. 1975.

Parthasarathy,N. and Spiro,R.G. Biol. Chem. 256:507-513. 1981.

Patterson, D.S.P. Biochem. J. 125:19p-20p. 1971.

Patterson, D.S.P., Roberts, B.A. and Small, B.J. Fd. Cosmet. Toxicol. 14:439-442. 1976.

Pavlovic, M., Plestina,R. and Krogh,P. Acta Pathol. Microbiol. Scand. Sect. B. 87:243-246. 1979.

Peckham, J. C., Doupnik, B. Jr., and Jones, O. H. Jr. Appl. Microbiol. 21:492-494. 1971.

Pena, N., Villamil, L. and Herrera, A.I.L. Revista Acovez (Colombia) 6:5-14. 1982.

Petit, R.E., Taber, R.A. Appl. Microbiol. 16:1230-1234. 1968.

Phillips, D.L., Yourtee, D.M. and Searles, S. Toxicol. Appl. Pharmacol. 36:403-406. 1976.

Pier A.C., Cysewski S.J. and Richard J.L. In: Proceedings of the 18th Annu. Meeti Assoc. Vet. Lab. Diag. pp 419-432. 1975.

Pier A.C., Cysewski S.J., Richard J.L. and Thurston J.R. In: (Rodricks JV, Hesseltine, C.W., Mehlman M.A.,eds.). Mycotoxins in Human and Animal Health. pp.745-750. Pathotox Inc, Park Forest South. III. 1977.

Pier,A.C., Richard,J.L. and Cysewski,S.J. J. Am. Vet. Med. Assoc. 176:719-724. 1980.

Pitout, M.J. Toxicol. Appl. Pharmacol. 13:299-306. 1968.

Poat, P.C., Poat, J.A. and Munday, K.A. Comp. Gen. Pharmacol. 1:401-408. 1970.

Price, R.G. and Spiro, R.G. J. Biol. Chem. 252:8597-8602. 1977.

Purchase, I.F.H. and Nel, W. In: (Metales, R.I. and Wogan, G.N., eds.). Bichemistry of some food-borne Microbial Toxins. pp.153-156. M.I.T. Press, Cambridge Mass., U.S.A. 1967.

Purchase, I.F.H. and Theron, J.J. Fd. Cosmet. Toxicol. 6:479-483. 1968.

Raj, H.G. and Venkitasubramanian, T.A. Z. Lebensm. U. Forsch. 154:201. 1974.

Raj, H.G., Santhanam, K., Gupta, R.P. and Venkitsubramanian, T.A. Chem.-Biol. Interact. 11:301-305. 1975.

Rao, E.R., Basappa, S.C. and Murthy, V.S. J. Fd. Sci. Technol. 16:113-114. 1979.

Rati, E.R., Basappa, S.C., Murthy, V. Sreenivasa, R.H.P., Ramesh, B.S. and Singh, G.B. J. Fd. Sci. Technol.18:176-179. 1981.

Read, S.M. and Northcote, D.H. Anal. Biochem. 116:53-64. 1981.

Remuzzi, G., Imberti, L., Rossini, M., Morelli, C., Carminati, C., Cattaneo, G.M. and Bertani, T. J. Clin. Invest. 75 :94-101. 1985.

Ribelin, W.E., Fukushima, K. and Still, P.E. Can. J. Comp. Med. 42:172-176. 1978.

Rodricks, J.V. and Stoloff, L. In: (Rodricks, J.V., Hesseltine, C.W. and Mehlman, M.A., eds.). Mycotoxins in human and animal health. pp.67-69. Pathotox Publishers, Park Forest South, 111. 1977.

Roll, F.J., Madri, J.A., Albort, J. and Furthmayr, H. J. Cell Biol. 85:597-616. 1980.

Roth, A., Chakor, K., Creppy, E., Kane, A., Roschenthaler, R. and Dirheimer, G. Toxicology 48:293-308. 1988.

Rotman, B. and Papermaster, B.W. Proc. Nat. Acad. Sci. 55:134-141. 1966.

Rutqvist, L., Bjurklund, N-E., Hult, K., Hukby, E. and Carlsson, B. Appl. Environ. Microbiol. 36: 920-925. 1978.

Sabbioni, G., Skipper, P.L. and Tannebaum, S.R. Carcinogenesis 27:86. (Abstract). 1986.

Sandor, G., Glavits, R., Vajda, L., Vanyi, A. and Krogh, P. In: Proceedings of the V International IUPAC Symposium on Mycotoxins and Phycotoxins. pp. 349-352. Vienna. 1982.

Scheinman, J. I. and Fish, A.J. Am. J. Pathol. 92:125-146. 1978.

Scheinman, J.I., Fish, A.J., Matas, A.J. and Michael, A.F. Am. J. Pathol. 90:71-84. 1978.

Scheinman, J.I., Foidart, J.M., Gehorn-Robey, P.G., Fish, A.J. and Michael, A.F. Clin. Immunol. Immunopathol. 15:157-159. 1980.

Schindler, A.F., Palmer, J.G., and Eisenberg, W.V. J. Assoc. Offic. Anal. Chem. 51:911-912. 1967.

Schlondorff, D., Roczniak, S., Satriano, J.A. and Folkert, V.W. Am. J. Physiol. 238:F486-F495. 1980.

Schmidt, U. and Guder, W.G. Kidney Int. 9: 233-242. 1976.

Serck-Hanssen, A. Arch. Environm. Health 20:729-731. 1970.

Shank, R.C.; Wogan, G.N.; Gibson J.B.; Nondasuta, A. Fd. Cosmet. Toxicol. 10:61-69. 1972.

Shank, R.C. In: (J.V Rodricks, ed.). Mycotoxins and other fungal related food problems. Advances in Chemistry Series, Number 149. pp.51-57. American Chemical Society, Washington, D.C. 1976.

Shankaran, R., Raj, H.G. and Venkitasubramanian, T.A. Enzymology 39:371-378. 1970.

Sharratt, M. and Frazer, A.C. Toxicol. Appl. Pharmacol. 5:36-48. 1963.

Shreeve, B.J., Patterson, D.S.P., Pepin, G.A., Roberts, B.A. and Wrathall, A.E. Br. Vet. J. 1976.

Shreeve, B.J., Patterson, D.S.P. and Roberts, B.A. Fd. Cosmet. Toxicol. 17:151-152. 1979.

Siraj, M. Y., Hayes, A. W., Unger, P. D., Hogan, G. R., Ryan, N. J. and Wray, B.B. Toxicol. Appl. Pharmacol. 58:422-430. 1981.

Smith, W.L. and Bell, G. Am. J. Physiol. 235:F451-F457. 1978.

Smith, J.E. and Moss, M.O. (Eds.) Mycotoxins: Formation, Analysis and Significance. p. 48. John Wiley and Sons. Chichester. U.K. 1985.

Spiro, R.G. N. Engl. J. Med. 281:991-1001, 1049-1056. 1969.

Spiro, R.G. Seminars in Medicine, Beth Israel Hospital, Boston, 288:1337-1342. 1973.

Stein, A.F., Phillips, T.D., Kubena, L.F. and Harvey, R.B. J. Toxicol. Envirnon. Health 16:593-605. 1985.

Stein, A.F., Geerling, S., Mollenhauer, H.H., Kubena, L.F., Heidelbaugh, N.D. and Phillips, T.D. J. Toxicol. Environ. Health 14:535-550. 1984.

Still, P.E. Mycotoxins as possible causes of abortion in dairy cattle. PhD. Dissertation, University of Wisconsin, Madison, Wisc. 1973.

Storen, O., Holm, H. and Stormer, F.C. Appl. Environ. Microbiol. 44:785-789. 1982

Stormer, F.C., Storen, O., Hansen, C.E., Pedersen, J.I. and Aasen, A.J. Appl. Environ. Microbiol. 45:1183-1187. 1983.

Stormer, F.C., Hansen, C.E., Pedersen, J.I., Hvistendahl, G. and Aasen, A.J. App. Environmen. Microbiol. 42:1051-1056. 1981.

Stormer, F.C. and Pedersen, J.I. Appl. Environ. Microbiol. 39:971-975. 1980.

Stojkovic, R., Hult, K., Gamulin, S. and Plestina, R., Biochem. Int. 9:33-38. 1984.

Stoloff, L. In: (Rodricks, J.V. ed.). Mycotoxins and other fungal related food problems. pp.23-50. Am. Chem. Soc.,Washington. 1976.

Striker, G.E. and Striker, J. Lab. Invest. 53:122-131. 1985.

Suzuki, S., Satoh, T. and Yamazaki, M. Toxicol. Appl. Pharmacol. 32:116-122. 1975a.

Suzuki, S., Kozuka, Y., Satoh, T. and Yamazaki, M. Toxicol. Appl. Pharmacol. 34:479-490. 1975b.

Svendsen, C. and Skadhauge, E. Acta Pharmacol. Toxicol. 38:186-194. 1976.

Swenson, D.H., Miller, E.C., and Miller, J.A. Biochem. Res. Comm. 60:1036-1043. 1974.

Swenson, D.H., Lin, J.K., Miller, E.C. and Miller, J.A. Cancer Res. 37:172-181. 1977.

Szczech, G.M., Carlton, W.W., Tuite, J. and Caldwell, R. Vet Path 10: 347-364. 1973a.

Szczech, G.M., Carlton, W.W. and Tuite,J. Vet. Path. 10:135-154. 1973b.

Szczech, G.M., Carlton, W.W. and Tuite, J. Vet. Path. 10:219-231. 1973c.

Szczech, G.M., Carlton, W.W. and Hinsman, E.J. Vet. Path. 11:385-406. 1974.

Timpl, R., Rhode, H., Robey, P.G., Rennard, S.I., Foidart, J.M. and Martin, G.R. J. Biol. Chem. 254:9933-9937. 1979.

Tsuboi, S., Nakagawa, T., Tomita, M., Seo, T., Ono, H., Kawamura, K. and Iwamura, N. Cancer Res. 44:1231-1234. 1984.

Tune, B.M. J. Infect. Dis. 132:189-194. 1975.

Uchida, N., Smilowitz, H., Ledger, P.W. and Tanzer, M.L. J. Biol. Chem. 255:8638-44. 1980.

Uchiyama, S., Sito, Y. and Uchiyama, M. Shokuhin Eiseigaku Zasshi 26:651-7. 1985.

Ueno, Y. and Ueno, I. In: (Uraguchi, K. and Yamazaki, M., eds.). Toxicology, Biochemistry and Pathology of Mycotoxins. pp. 107-188. Halsted Press, N.Y, U.S.A. 1978.

Ueno, Y. Maikotokishin 22:28-30. 1985.

Ulfendahl, H.R., Ericson, A.C., Goransson, A., and Sjoquist, M. Proc. 8th. Int. Congr. Nephrol. pp. 176-182. Athens, 1981.

Van Fleet, J.F., Greenwod, L.A. and Ferrans, V.J. Am. J. Vet. Res. 40:1537-1552. 1979.

Van Walbeek, W., Moodie, C.A., Scott, P.M., Harwig, J.W. and Grice, H.C. Toxicol. Appl. Pharmacol. 20:439-441. 1971.

Velosa,J.A., Shah,S.V., Ou,S-L., Abboud,H.E. and Dousa,T.P. Renal Physiol. 3:120-125. 1980.

Velosa, J.A., Shah, S.V., Ou, S.L., Abboud, H.E. and Dousa, T.P. Lab. Invest. 45:522-526. 1981.

Venkatachalam M.A. and Rennke H.G. In: (Leaf A., ed.). Renal Pathophysiology. pp. 43 - 56. Raven Press, New York. 1980.

Walker, W.G. and Hulter, H.N. Trans. Amer. Clin. Climatol. Ass. 81:174-183. 1969.

Wargo, A.A., Slotkoff, L.M., Jose', P.A., Pelayo, J. and Eisner, G.M. Nephron 32:165-169. 1982.

Warren,M.F. and Hamilton,P.B. Appl. Environ. Microbiol. 40:522-525. 1980.

Wei,Y-H., Lu,C-Y., Lin,T-N., and Wei,R-D. Toxicology 36:119-130. 1985.

Weinberg, J.M., Simmons, C.F. Jr. and Humes, D. Res. Comm. Chem. Pathol. Pharmacol. 27:521-531. 1980.

WHO. Environmental Health Criteria 11, Mycotoxins, Published under the joint sponsorship of the United Nations Environmental Programme and the World Health Organization, Geneva. 1979.

Wild, C.P., Garner, R,C., Montesano, R., and Tursi, F. Carcinogenesis 7:853-858. 1986.

Wogan, G.N. In: (Goldblatt., L. A., ed.). Aflatoxin: Scientific background, control and implications. pp.151-186. Academic Press, New York. 1969.

Wogan, G.N., Edwards, G.S. and Shank, R.C.. Cancer Res. 27:1729-1736. 1967.

Wray, B.B. and Hayes, A.W. Environ. Res. 22:400-403. 1980.

Yadgiri, B.,Reddy, V., Tulpule, P.G., Srikantis, S.G. and Gopalan, C. Am.J. Clin. Nutr. 23:94-98. 1970.

Yamada, K.M. Ann. Rev. Biochem. 52:761-99. 1983.

Yamada, K.M. and Olden, K. Nature, London. 275:179-184. 1978.

Yamada, S.S., Yamada, K.M., and Willingham, M.C. J. Histochem. Cytochem. 28:953-60. 1980.

Yuen, C. T., Price, R. G., Chatagoom, L., Richardson, A. C. and Praill, P. F. G. Clin. Chim. Acta 124: 195-204, 1982.

APPENDIX 1

APPENDIX 1

TABLE 5.1.1

URINARY ALP ACTIVITY AFTER IP ADMINISTRATION OF OTA TO RATS (1 mg/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	119 ± 74	119 ± 74	100
1	141 ± 30	90 ± 46	64
2	160 ± 55	75 ± 26	100
3	105 ± 20	160 ± 80	152
4	77 ± 20	211 ± 46*	272

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.2

URINARY AAP ACTIVITY AFTER IP ADMINISTRATION OF OTA TO RATS (1 mg/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	1027 ± 619	1027 ± 619	100
1	1554 ± 93	967 ± 422	62
2	2268 ± 234	1289 ± 115*	57
3	2683 ± 464	2626 ± 987	98
4	2395 ± 808	3433 ± 1147	143

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.3

URINARY GGT ACTIVITY AFTER
IP ADMINISTRATION OF OTA TO RATS (1 mg/kg)

	ACTIVITY		
	Units/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	451 ± 251	451 ± 251	100
1	289 ± 73	250 ± 104	87
2	457 ± 156	271 ± 57	59
3	425 ± 146	394 ± 179	93
4	345 ± 126	532 ± 155	154
	n=3	n=3	

TABLE 5.1.4

URINARY NAG ACTIVITY AFTER
IP ADMINISTRATION OF OTA TO RATS (1 mg/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	35 ± 16	35 ± 16	100
1	67 ± 19	45 ± 20	68
2	56 ± 14	56 ± 20	101
3	56 ± 11	66 ± 20	119
4	43 ± 22	59 ± 24	138
	n=3	n=3	

TABLE 5.1.5

URINARY VOLUME AFTER IP
ADMINISTRATION OF OTA TO RATS (1 mg/kg)

	VOLUME		
	ml/24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	5.8 ± 2.9	5.8 ± 2.9	100
1	7.0 ± 2.6	5.6 ± 2.5	80
2	9.3 ± 3.0	5.0 ± 1.0	54
3	8.0 ± 2.0	5.6 ± 1.5	70
4	7.0 ± 3.6	6.6 ± 1.1	94

n=3

TABLE 5.1.6

URINARY ALP ACTIVITY AFTER
OTA ADMINISTRATION TO RATS (5 mg/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	340 ± 100	340 ± 100	100
1	225 ± 50	305 ± 100	135
2	480 ± 160	500 ± 200	222
3	370 ± 120	600 ± 200	162
4	385 ± 50	600 ± 100	156
	n=4	n=5	

TABLE 5.1.7

URINARY AAP ACTIVITY AFTER
OTA ADMINISTRATION TO RATS (5 mg/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	1600 ± 625	1600 ± 625	100
1	1491 ± 426	1605 ± 588	108
2	2133 ± 341	2715 ± 1142	151
3	1936 ± 654	4050 ± 973*	209
4	1956 ± 455	3843 ± 857*	196
	n=4	n=5	

* Significantly different from control at $P \leq 0.05$

TABLE 5.1.8

URINARY NAG ACTIVITY AFTER
IP ADMINISTRATION OF OTA TO RATS (5 mg/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	32.5 ± 12	32.5 ± 12	100
1	27.8 ± 7	24.8 ± 6	89
2	38.0 ± 5	38.2 ± 12	100
3	48.5 ± 14	79.5 ± 13	164
4	53.4 ± 11	98.1 ± 22*	184
	n=4	n=5	

* Significantly different from control at $P \leq 0.05$

TABLE 5.1.9

URINARY GGT ACTIVITY AFTER IP ADMINISTRATION OF OTA TO RATS (5 mg/kg)

	ACTIVITY		
	Units/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	947 ± 297	947 ± 297	100
1	859 ± 195	830 ± 297	97
2	1257 ± 72	1246 ± 611	99
3	1327 ± 476	1947 ± 775	147
4	1436 ± 378	1611 ± 347	112
	n=4	n=5	

TABLE 5.1.10

URINARY VOLUME AFTER IP ADMINISTRATION OF OTA TO RATS (5 mg/kg)

	VOLUME		
	ml/24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	5.2 ± 1.7	5.2 ± 1.7	100
1	5.2 ± 1.9	4.8 ± 1.8	92
2	7.5 ± 2.4	7.6 ± 4.0	101
3	6.2 ± 1.7	10.0 ± 1.6	161
4	7.7 ± 1.7	8.2 ± 1.2	106
	n=4	n=5	

* Significantly difference from control at $P \leq 0.05$.

TABLE 5.1.11

URINARY AAP ACTIVITY AFTER IP ADMINISTRATION OF ATB_1 TO RATS (100 ug/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	1066 ± 442	1066 ± 442	100
1	1640 ± 257	2037 ± 772	124
2	1326 ± 265	1903 ± 318	143
3	1911 ± 256	1885 ± 170	99
4	2029 ± 132	1586 ± 191*	78

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.12

URINARY GGT ACTIVITY AFTER IP ADMINISTRATION OF ATB_1 TO RATS (100 ug/kg)

	ACTIVITY		
	Units/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	405 ± 95	405 ± 95	100
1	476 ± 103	445 ± 87	94
2	343 ± 46	620 ± 222	181
3	531 ± 64	411 ± 105	77
4	456 ± 28	304 ± 61*	67

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.13

URINARY NAG ACTIVITY AFTER IP ATB_1 ADMINISTRATION TO RATS (100 ug/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	70 ± 30	70 ± 30	100
1	69 ± 2	59 ± 3*	85
2	49 ± 4	89 ± 3**	182
3	67 ± 10	76 ± 4	113
4	63 ± 10	61 ± 16	97

* Significantly different from control at $P \leq 0.05$ (n=3)
** Significantly different from control at $P \leq 0.01$ (n=3)

TABLE 5.1.14

URINARY ALP ACTIVITY AFTER IP ADMINISTRATION OF ATB_1 TO RATS (100 ug/kg)

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	225 ± 76	225 ± 76	100
1	187 ± 26	223 ± 46	119
2	140 ± 44	202 ± 31	144
3	210 ± 37	182 ± 6	86
4	209 ± 11	207 ± 55	99
	n=3	n=3	

TABLE 5.1.15

URINARY VOLUME AFTER IP ADMINISTRATION OF ATB_1 TO RATS (100 ug/kg)

	VOLUME		
	ml/24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	7.5 ± 1.0	7.5 ± 1.0	100
1	10.3 ± 1.1	9.3 ± 1.1	90
2	9.0 ± 3.6	10.0 ± 3.0	111
3	9.0 ± 1.7	8.6 ± 2.3	95
4	8.0 ± 0	5.7 ± 2.5	71

(n=3)

TABLE 5.1.16

URINARY ALP ACTIVITY AFTER COMBINED ADMINISTRATION OF 1 mg OTA AND 100 ug ATB_1/kg

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	105 ± 38	105 ± 38	100
1	95 ± 12	96 ± 41	115
2	116 ± 14	154 ± 33	133
3	117 ± 13	242 ± 43*	208
4	160 ± 22	335 ± 150	210

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.17

URINARY GGT ACTIVITY AFTER COMBINED ADMINISTRATION OF 1 mg OTA AND 100 ug ATB_1/kg TO RATS

	ACTIVITY		
	Units/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	847 ± 440	847 ± 440	100
1	593 ± 345	858 ± 199	145
2	1244 ± 170	960 ± 384	77
3	990 ± 160	1320 ± 1*	133
4	1336 ± 163	1182 ± 206	88

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.18

URINARY NAG ACTIVITY AFTER COMBINED ADMINISTRATION OF 1 mg OTA AND 100 ug ATB_1/kg

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	47 ± 10	47 ± 10	100
1	35 ± 9	56 ± 6*	161
2	45 ± 3	45 ± 2	101
3	39 ± 5	58 ± 1*	149
4	46 ± 3	62 ± 10	133

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.19

URINARY AAP ACTIVITY AFTER COMBINED ADMINISTRATION OF 1 mg OTA AND 100 ug ABT_1/kg TO RATS

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	1672 ± 440	1672 ± 440	100
1	1918 ± 869	1787 ± 1649	93
2	2199 ± 464	2175 ± 525	99
3	3262 ± 511	4743 ± 1024	145
4	4524 ± 505	4955 ± 150	109

(n=3)

TABLE 5.1.20

URINARY VOLUME AFTER COMBINED ADMINISTRATION OF 1 mg OTA and 100 ug ATB_1/kg

	VOLUME		
	ml/24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	7.7 ± 1.5	7.7 ± 1.5	100
1	6.8 ± 2.9	6.5 ± 2.5	95
2	9.0 ± 1.0	6.0 ± 3.4	67
3	9.0 ± 1.0	11.0 ± 0.0*	122
4	11.3 ± 0.6	10.3 ± 0.6	91

* Significantly different from control at $P \leq 0.05$ (n=3)

TABLE 5.1.21

URINARY ALP ACTIVITY AFTER COMBINED ADMINISTRATION OF 5 mg OTA AND 100 ug ATB_1/kg

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	143 ± 52	143 ± 52	100
1	225 ± 56	128 ± 52**	71
2	203 ± 67	252 ± 143	124
3	224 ± 112	486 ± 131*	217
4	235 ± 66	385 ± 129	163
	n=4	n=5	

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$

TABLE 5.1.22

URINARY GGT ACTIVITY AFTER COMBINED ADMINISTRATION OF 5 mg OTA and 100 ug ATB_1/kg

	ACTIVITY		
	Units/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	926 ± 235	926 ± 235	100
1	1407 ± 510	890 ± 281*	63
2	1695 ± 978	1226 ± 757	72
3	2152 ± 1014	2080 ± 856	96
4	2097 ± 610	1682 ± 216	80
	n=4	n=5	

* Significantly different from control at $P \leq 0.05$.

TABLE 5.1.23

URINARY AAP ACTIVITY AFTER COMBINED ADMINISTRATION OF 5 mg OTA AND 100 ug ATB_1/kg

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	1720 ± 486	1720 ± 486	100
1	2634 ± 698	1987 ± 1136	75
2	3379 ± 1248	2590 ± 1547	77
3	3412 ± 989	3853 ± 1703	113
4	4036 ± 1515	3761 ± 694	93
	n=4	n=5	

TABLE 5.1.24

URINARY NAG ACTIVITY AFTER COMBINED ADMINISTRATION OF 5 mg OTA AND 100 ug ATB_1/kg

	ACTIVITY		
	nmol/ 24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	50 ± 7.6	50 ± 7.6	100
1	59 ± 2.8	45 ± 12.2	75
2	69 ± 27.0	79 ± 42.0	115
3	25 ± 4.6	30 ± 12.7	119
4	29 ± 3.0	48 ± 6.1	163
	n=4	n=5	

TABLE 5.1.25

URINARY VOLUME AFTER COMBINED ADMINISTRATION OF 5 mg OTA and 100 ug ATB_1/kg

	VOLUME		
	ml/24 hr		% OF CONTROL
DAYS OF TREATMENT	CONTROL	TREATMENT	
0	8.0 ± 4.2	8.0 ± 4.2	100
1	9.5 ± 0.6	8.0 ± 4.5	84
2	10.2 ± 1.9	7.4 ± 7.6	72
3	9.7 ± 2.4	8.0 ± 4.5	82
4	10.2 ± 2.4	8.2 ± 2.2	80
	n=4	n=5	

TABLE 5.1.26

URINARY ACTIVITY OF ALP AFTER 3 IP DAILY ADMINISTRATION OF OTA (2.5 mg/kg) TO RATS

	ACTIVITY		
	nmol/ 18 hr		% OF CONTROL
TIME	CONTROL	TREATMENT	
0	56 ± 32	56 ± 32	100
1	78 ± 35	142 ± 73	182
2	116 ± 60	102 ± 70	88
3	50 ± 35	237 ± 340	474
4	50 ± 50	211 ± 190	527
5	54 ± 30	243 ± 160	714
6	74 ± 35	1196 ± 1740	1616
7	119 ± 83	1367 ± 1350	1752
8	78 ± 46	263 ± 50**	337
9	134 ± 70	272 ± 129	203
10	263 ± 90	367 ± 110	139
11	218 ± 130	298 ± 110	137
12	277 ± 60	479 ± 58	173
13	638 ± 610	120 ± 77	18
14	307 ± 300	176 ± 20	57
15	65 ± 50	153 ± 60	306

** SIGNIFICANTLY DIFFERENT AT $P \leq 0.01$ (n=5)

TABLE 5.1.27

URINARY ACTIVITY OF GGT AFTER 3 IP DAILY ADMINISTRATION OF OTA (2.5 mg/kg) TO RATS

	ACTIVITY		
	Units/ 18 hr		% OF CONTROL
TIME (DAYS)	CONTROL	TREATMENT	
0	816 ± 318	816 ± 318	100
1	1017 ± 209	821 ± 370	80
2	919 ± 266	1058 ± 243	115
3	791 ± 288	1007 ± 362	127
4	775 ± 171	1423 ± 160*	184
5	513 ± 175	1006 ± 258*	196
6	687 ± 322	1107 ± 301	161
7	573 ± 144	1067 ± 510	186
8	667 ± 228	980 ± 314	147
9	793 ± 272	741 ± 211	93
10	721 ± 197	471 ± 141	65
11	976 ± 421	577 ± 165	59
12	891 ± 216	911 ± 176	102
13	878 ± 318	921 ± 156	105
14	1049 ± 312	961 ± 169	92
15	1063 ± 379	1070 ± 276	101

* SIGNIFICANTLY DIFFERENT FROM CONTROL AT $P \leq 0.05$ (n=5)

TABLE 5.1.28

URINARY ACTIVITY OF AAP AFTER 3 IP DAILY ADMINISTRATION OF OTA (2.5 ml/kg) TO RATS

	ACTIVITY		
	nmol/ 18 hr		% OF CONTROL
TIME (DAYS)	CONTROL	TREATMENT	
0	1120 ± 520	1120 ± 520	100
1	1140 ± 340	1232 ± 300	107
2	1440 ± 480	1680 ± 920	116
3	794 ± 440	1520 ± 1000	191
4	500 ± 176	2160 ± 600*	432
5	370 ± 140	1480 ± 500*	400
6	508 ± 220	1020 ± 220*	201
7	628 ± 220	1420 ± 760	226
8	928 ± 340	2260 ± 620*	243
9	1230 ± 540	2120 ± 1140	172
10	1540 ± 320	1640 ± 460	106
11	1140 ± 540	720 ± 280	63
12	620 ± 240	940 ± 200	151
13	640 ± 320	1160 ± 200	181
14	960 ± 460	1540 ± 480	160
15	1120 ± 540	1700 ± 660	152

* SIGNIFICANTLY DIFFERENT FROM CONTROL AT $P \leq 0.05$ (n=5)

TABLE 5.1.29

URINARY GLUCOSE AFTER ADMINISTRATION OF 3 IP REPETITIVE DOSES OF OTA (2.5 mg/kg) TO RATS

	GLUCOSE		
	mmoles/ 18h		% CONTROL
TIME (DAYS)	CONTROL	TREATMENT	
0	3.9 ± 1.8	3.9 ± 1.8	100
1	6.2 ± 1.2	3.5 ± 3.0	56
2	9.8 ± 7.0	11.9 ± 5.0	121
3	5.5 ± 1.5	2.5 ± 5.0	45
4	5.3 ± 2.0	3.3 ± 3.6	62
5	6.5 ± 2.4	218.0 ± 234.0	3354
6	5.7 ± 2.0	501.0 ± 187.0*	8789
7	4.4 ± 2.6	261.0 ± 129.0*	5932
8	6.6 ± 2.2	62.0 ± 70.0	939
10	6.3 ± 1.0	3.9 ± 1.0**	61
11	5.3 ± 2.3	5.4 ± 3.0	102
12	6.0 ± 1.5	6.0 ± 1.0	100
13	5.3 ± 2.0	4.7 ± 1.0	89
14	6.0 ± 1.0	2.4 ± 2.0**	40
15	5.6 ± 2.0	5.0 ± 2.6	89

* SIGNIFICANTLY DIFFERENT FROM CONTROL AT $P \leq 0.05$ (n=5)

** SIGNIFICANTLY DIFFERENT FROM CONTROL AT $P \leq 0.01$ (n=5)

TABLE 5.1.30

URINARY PROTEIN AFTER 3 IP DAILY ADMINISTRATION OF OTA (2.5 mg/kg) TO RATS

	PROTEIN		
	mg/18h		% CONTROL
TIME (DAYS)	CONTROL	TREATMENT	
0	8.0 ± 3.1	8.0 ± 3.1	100
1	10.4 ± 3.3	15.7 ± 5.2	151
2	10.8 ± 2.2	21.6 ± 6.4*	200
3	12.1 ± 3.4	18.0 ± 5.4	149
4	14.3 ± 5.6	75.2 ± 34.6*	537
5	44.5 ± 32.8	88.5 ± 29.0	199
6	44.5 ± 39.3	78.4 ± 21.2	176
7	37.1 ± 19.0	62.9 ± 11.5	169
8	76.7 ± 32.5	50.1 ± 25.0	65
10	48.4 ± 47.4	29.0 ± 7.8	14
11	33.9 ± 16.0	26.2 ± 1.5	77
12	77.8 ± 50.9	65.1 ± 63.0	84
13	50.3 ± 29.9	40.2 ± 23.5	80
14	51.4 ± 47.3	32.8 ± 8.6	64

* SIGNIFICANTLY DIFFERENT FROM CONTROL AT $P \leq 0.05$ (n=5)

TABLE 5.1.31

URINARY VOLUME AFTER 3 IP DAILY DOSES OF OTA (2.5 mg/kg)

	VOLUME		
	ml/18h		% OF CONTROL
TIME (DAYS)	CONTROL	TREATMENT	
0	3.5 ± 1.4	3.5 ± 1.4	100
1	3.6 ± 2.0	4.2 ± 2.5	218
2	3.8 ± 1.5	4.9 ± 2.6	129
3	3.6 ± 1.3	3.8 ± 2.1	105
4	3.8 ± 1.0	6.6 ± 3.0	174
5	3.6 ± 1.0	7.8 ± 2.1*	216
6	4.5 ± 1.5	10.0 ± 3.0*	222
7	4.4 ± 1.0	12.0 ± 3.8*	273
8	4.6 ± 1.3	12.7 ± 3.2*	276
9	6.3 ± 2.2	9.6 ± 3.4	152
10	5.7 ± 1.3	8.0 ± 2.1	140
11	5.5 ± 1.7	7.7 ± 2.5	140
12	6.1 ± 1.1	7.4 ± 2.6	121
13	5.9 ± 0.8	8.6 ± 2.1	146
14	6.5 ± 2.0	10.3 ± 2.4	158
15	4.8 ± 1.4	9.0 ± 3.0	187

* SIGNIFICANTLY DIFFERENT FROM CONTROL AT $P \leq 0.05$ (n=5)

APPENDIX 2

APPENDIX 2

TABLE 6.1.1

AMINO ACID INCORPORATION INTO ISOLATED PIG GLOMERULI AT DIFFERENT TIME POINTS

TIME (Hr)	INCORPORATION (pmol of aminoacid/mg protein)				
	GLYCINE	LYSINE	HYSTIDINE	PROLINE	LEUCINE
0					
1	0.14 ± 0.3	0.38 ± 0.09	0.39 ± 0.05	0.51 ± 0.012	0.71 ± 0.1
2	0.15 ± $5x10^{-3}$	0.79 ± 0.02	0.93 ± 0.14	0.88 ± 0.070	2.00 ± 0.24
3	0.30 ± 0.05	1.00 ± 0.11	1.50 ± 0.12	1.36 ± 0.05	2.60 ± 0.29
4	0.47 ± 0.10	1.21 ± 0.01	2.00 ± 0.17	2.00 ± 0.20	3.70 ± 0.38

TABLE 6.1.2 T VALUES AT LEVELS OF 5%-0.1% OF PROBABILITY FOR THE INCORPORATION OF DIFFERENT LABELLED AMINO ACIDS INTO ISOLATED PIG GLOMERULI INCLUBATED WITH KREBS BUFFER AT VARIOUS TIME POINTS

AFTER 4 HR OF INCUBATION

	GLY	LYS	PRO	LEU
LYS	12.7***	-		
PRO	11.8***	6.8**	-	
LEU	14.2***	11.3***	6.8**	-
HIS	13.4***	8.0**	0	7.0**

AFTER 3 HR OF INCUBATION

	GLY	LYS	PRO	LEU
GLY	-			
LYS	10.0***	-		
PRO	26.0***	5.1**	-	
LEU	13.5***	8.0***	7.3**	-
HIS	16.0***	5.3**	1.9	6.1**

AFTER 2 HR OF INCUBATION

	GLY	LYS	PRO	LEU
GLY	-			
LYS	54.8***	-		
PRO	18.0***	2.1	-	
LEU	13.3***	8.7***	7.7**	-
HIS	9.6***	1.7	0.5	6.7**

APPENDIX 2

TABLE 6.1.2 (CONTINUED)

AFTER 1 HR OF INCUBATION

	GLY	LYS	PRO	LEU
GLY	-			
LYS	1.3	-		
PRO	2.1	2.5	-	
LEU	3.1*	4.2	3.4*	-
HIS	1.4	0.16	4.0*	4.9**

* $P \leq 0.5$
** $P \leq 0.1$
*** $P \leq 0.01$

APPENDIX 2

TABLE 6.1.3 INCORPORATION OF ^{3}H-PROLINE INTO ISOLATED PIG GLOMERULI INCUBATED WITH KREBS BUFFER.

	TIME (Hr)	INCORPORATION (pmol/mg PROTEIN)
EXPERIMENT 1:	0	0
	2.5	0.8 ± 0.07
	4	2.1 ± 0.27
EXPERIMENT 2:	0	0
	1	0.18 ± 0.02
	2	0.51 ± 0.05
	3	0.90 ± 0.07
	4	1.4 ± 0.2
EXPERIMENT 3:		
	0	0
	1	0.32 ± 0.03
	2	0.68 ± 0.02
	3	1.03 ± 0.16
	4	1.1 ± 0.16
EXPERIMENT 4:		
	0	0
	1	0.73 ± 0.10
	2	1.5 ± 0.07
	3	2.8 ± 0.12
	4	5.6 ± 0.4
EXPERIMENT 5:		
	0	0
	1	0.5 ± 0.012
	2	0.9 ± 0.07
	3	1.1 ± 0.05
	4	2.0 ± 0.2

TABLE 6.1.4 T VALUES AT LEVELS OF 5%-0.1% OF PROBABILITY FOR THE INCORPORATION OF ^{3}H-PROLINE INTO ISOLATED PIG GLOMERULI INCUBATED WITH KREBS BUFFER AT VARIOUS TIME POINTS IN DIFFERENT EXPERIMENTS

Hr of Incubation	1	2	3
1	-		
2	10.6***	-	
3	17.1***	7.8**	-
4	10.5***	7.5**	4.1*

Hr of Incubation	1	2	3
1	-		
2	17.3***	-	
3	7.5**	3.7*	-
4	8.3**	4.5**	0.12

Hr of Incubation	1	2	3
1	-		
2	10.9***	-	
3	22.9***	16.2***	-
4	20.4***	17.5***	11.6***

* $P \leq 0.5$
** $P \leq 0.1$
*** $P \leq 0.01$

TABLE 6.1.5
INCORPORATION OF ^{3}H-PROLINE INTO PIG GLOMERULI INCUBATED WITH TWO DIFFERENT BUFFERS^{+}

TIME (Hr)	INCORPORATION (pmol/mg prot) EARLES-HEPES^{+}	KREBS^{+}
0	0	0
2	0.93 ± 0.1*	0.3 ± 0.03
4	4.00 ± 0.7***	0.42 ± 0.074

* Significantly different from control at $P \leq 0.05$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.1.6
INCORPORATION OF ^{3}H-PROLINE INTO RAT GLOMERULI INCUBATED WITH TWO DIFFERENT BUFFERS^{+}

TIME (Hr)	INCORPORATION (pmol/mg prot) EARLES-HEPES^{+}	TYRODES^{+}
1	2.0 ± 0.10	0.20 ± 0.04
2	5.5 ± 0.03	0.40 ± 0.04
3	8.0 ± 0.50	0.75 ± 0.08
4	10.0 ± 1.00	1.10 ± 0.10

TABLE 6.1.7
EFFECT OF DIFFERENT CONCENTRATIONS OF NON-RADIOACTIVE PROLINE ON THE INCORPORATION OF ^{3}H-PROLINE INTO ISOLATED PIG GLOMERULI

NON RADIOACTIVE PROLINE (uM)	INCORPORATION (pmol /mg prot)	% OF CONTROL
0	4.2 ± 0.74	100
10	4.3 ± 0.14	102
100	2.9 ± 0.8	69
1000	2.3 ± 0.5*	55

* SIGNIFICANTLY DIFFERENT FROM CONTROL AT $P \leq 0.05$

TABLE 6.1.8

EFFECT OF TYR, TRP AND HIS ON THE GLOMERULAR INCORPORATION OF THE PHE INTO ISOLATED RAT GLOMERULI

DOSE (mM)	INCORPORATION pmol/mg prot.			INCORPORATION % OF CONTROL		
	TYR	TRP	HIS	TYR	TRP	HIS
0.000	26.3 ± 6.7	26.3 ± 6.7	26.3 ± 6.7	100.0	100.0	100.0
0.025	20.7 ± 2.6	18.1 ± 4.3	-	78.7	68.8	-
0.050	-	-	29.0 ± 3.4	-	-	110.2
0.250	11.5 ± 2.4*	7.0 ± 0.7**	-	43.7	26.6	-
0.500	-	-	9.7 ± 1.9*	-	-	36.8
2.500	4.3 ± 0.9**	2.0 ± 0.2**	5.7 ± 0.1**	16.3	7.6	2.6

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$

TABLE 6.1.9 INCORPORATION OF ^{3}H-PROLINE INTO ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER

TIME (Hr)	INCORPORATION OF PROLINE (pmol/mg protein)
EXPERIMENT 1:	
2	0.23 ± 0.084
4	1.5 ± 0.140
EXPERIMENT 2:	
1	0.15 ± 0.02
2	0.8 ± 0.06
3	1.8 ± 0.09
4	3.0 ± 0.07
EXPERIMENT 3:	
1	0.1 ± 0.004
2	0.5 ± 0.04
3	1.2 ± 0.08
4	2.6 ± 0.05
EXPERIMENT 4:	
1	0.035 ± 0.004
2	0.2 ± 0.03
3	0.6 ± 0.014
4	1.0 ± 0.184
EXPERIMENT 5:	
1	0.03 ± 0.008
2	0.2 ± 0.014
3	0.7 ± 0.090
4	1.3 ± 0.193
EXPERIMENT 6:	
0	0
1	0.2 ± 0.02
2	0.7 ± 0.07
3	2.0 ± 0.2
4	5.1 ± 0.6
EXPERIMENT 7:	
0	0
1	0.3 ± 0.06
2	1.2 ± 0.2
3	5.1 ± 0.5
4	7.6 ± 2

TABLE 6.1.10 T VALUES AT LEVELS OF 5%-0.1% OF PROBABILITY FOR THE INCORPORATION OF ^{3}H-PROLINE INTO ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER AT VARIOUS TIME POINTS, IN DIFFERENT EXPERIMENTS

EXPERIMENT 1:

	1	2	3	4
1	-			
2	17.8***	-		
3	31.0***	16.0***	-	67.8***
4	67.8***	41.3***	18.2***	-

EXPERIMENT 2:

	1	2	3
1	-		
2	17.2***	-	
3	23.8***	13.5***	-
4	86.3***	56.8***	25.7***

EXPERIMENT 3:

	1	2	3
1	-		
2	5.7**		
3	23.1***	20.9***	
4	9.1***	7.4**	3.7*

EXPERIMENT 4:

	1	2	3
1	-		
2	18.3***	-	
3	12.8***	9.5***	-
4	11.4***	9.8***	4.9

EXPERIMENT 5:

	1	2	3
1	-		
2	10.5***	-	
3	15.5***	10.4***	-
4	14.1***	12.6***	8.5**

TABLE 6.1.10 (CONTINUED)

EXPERIMENT 6:

	1	2	3
1			
2	7.5**	-	
3	16.5***	12.5***	-
4	6.3**	5.5**	2.1

* $P \leq 0.5$
** $P \leq 0.1$
*** $P \leq 0.01$

TABLE 6.1.11 TIME-COURSE OF PRO AND PHE INCORPORATION INTO ISOLATED RAT GLOMERULI

TIME (Hr)	INCORPORATION (pmol/mg protein) PRO	PHE
1	0.6 ± 0.07	4.6 ± 0.3
2	3.8 ± 0.40	8.6 ± 0.5
3	6.6 ± 0.87	15.2 ± 1.3
4	13.5 ± 2.00	25.1 ± 3.0

TABLE 6.1.12
AMINO ACID INCORPORATION INTO ISOLATED RAT GLOMERULI

Time (Hr)	Incorporation (pmol/mg protein) TRP	PHE	TYR	LEU	PRO	HIS
1	26 ± 4.0	4.6 ± 0.8	4 ± 0.3	3 ± 0.6	2.0 ± 0.10	1.5 ± 0.1
2	40 ± 0.9	8.0 ± 2.0	6 ± 1.0	7 ± 0.9	5.5 ± 0.03	2.5 ± 0.2
3	91 ± 1.0	17.0 ± 0.8	12 ± 0.5	11 ± 1.5	8.0 ± 0.50	4.0 ± 0.4
4	120 ± 19.0	19.0 ± 2.0	15 ± 1.0	14 ± 1.5	10.0 ± 1.00	6.0 ± 0.7

TABLE 6.1.13

T values at levels of 5% - 0.1% of probability for the time-course of the incorporation of different amino acids into isolated rat glomeruli.

INCORPORATION OF TRP INTO ISOLATED RAT GLOMERULI

	1	2	3
1	-		
2	5.6**	-	
3	26.5***	5.7***	-
4	2.0	0.22	9.2***

INCORPORATION OF PHE INTO ISOLATED RAT GLOMERULI

	1	2	3
1	-		
2	2.8*	-	
3	18.2***	7**	-
4	4.7	1.5	5.1**

INCORPORATION OF TYR INTO ISOLATED RAT GLOMERULI

	1	2	3
1	-		
2	2.5	-	
3	23.8***	6.9**	-
4	6.9**	4.6*	28.8***

INCORPORATION OF LEU INTO ISOLATED RAT GLOMERULI

	1	2	3
1	-		
2	6.2**	-	
3	8.2**	3.8*	-
4	3*	1.5	4.5

INCORPORATION OF PRO INTO ISOLATED RAT GLOMERULI

	1	2	3
1	-		
2	53.1***	-	
3	19.4***	8.6***	-
4	0.68	10.4***	13.5***

INCORPORATION OF HIS INTO ISOLATED RAT GLOMERULI

	1	2	3
1	-		
2	7.3**	-	
3	15.5***	14.2***	-
4	2.2	9.5**	15.8***

* P<0.5
** P<0.1
*** P<0.01

TABLE 6.1.14

T values at levels of 5% - 0.1% of probability for the incorporation of different amino acids into isolated rat glomeruli, at various times.

AFTER 4 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO
PHE	4.7^{**}	-			
TYR	6.2^{**}	6.9^{**}	-		
LEU	5.5^{**}	3.4^{*}	4.4^{*}	-	
PRO	6.1^{**}	6.7^{**}	0.3	$< 4.1^{*}$	-
HIS	6.4^{**}	7.9^{**}	6.1^{**}	$< 6^{**}$	$< 4.1^{*}$

AFTER 3 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO
PHE	83.2^{**}	-			
TYR	98.7^{***}	8.4^{**}	-		
LEU	70.2^{***}	5.9^{**}	1.2	-	
PRO	103.8^{***}	15.6^{***}	10	3.3^{*}	-
HIS	70.1^{***}	3^{*}	2.3	2.6	7.1^{**}

AFTER 2 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO
PHE	24.7^{**}	-			
TYR	34.5^{***}	1.3	-		
LEU	43.8^{**}	0.7	1	-	
PRO	65.2^{***}	2.2	0.9	3.3^{*}	-
HIS	69.3^{***}	4.8^{**}	4.5^{*}	8.8^{***}	25.7^{***}

TABLE 6.1.14 (CONTINUED)

AFTER 1 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO
PHE	8.7**	-			
TYR	9.1***	1	-		
LEU	9.4***	2.2	2.1	-	
PRO	9.9***	4.9**	9.8***	2.8*	-
HIS	10.2***	6.6**	13.8***	5.1**	8.4**

* P<0.5
** P<0.1
*** P<0.01

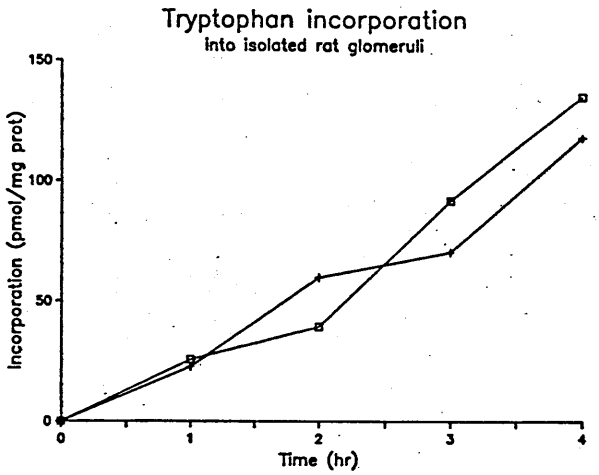


Fig. 6.1.1

Time-course of the incorporation of ^{3}H-tryptophan into total glomerular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

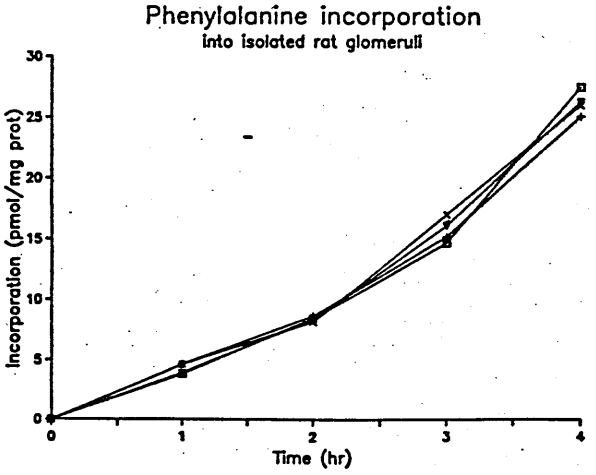


Fig. 6.1.2

Time-course of the incorporation of ^{3}H-phenylalanine into total glomerular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

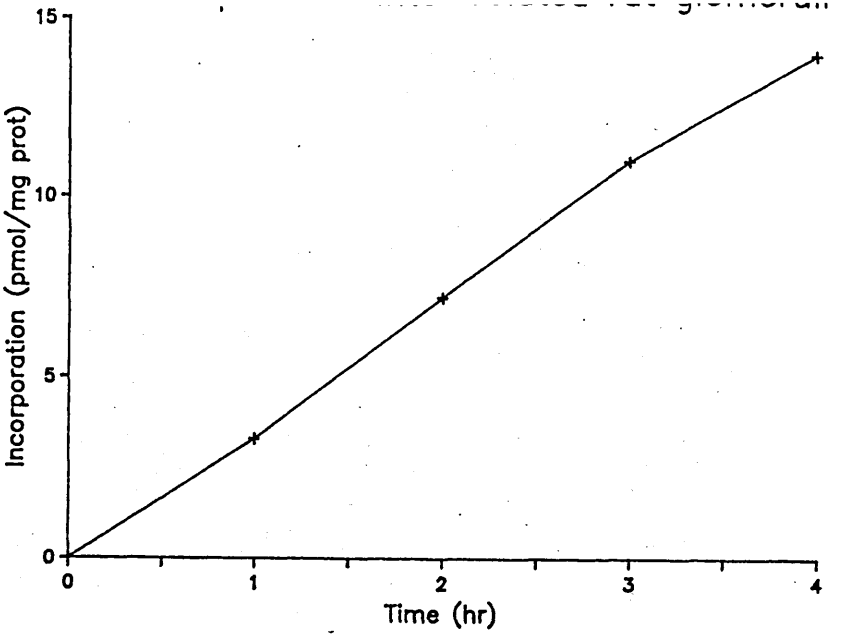


Fig. 6.1.3

Time-course of the incorporation of ^{3}H-leucine into total glomerular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each time point is the result of triplicate incubations.

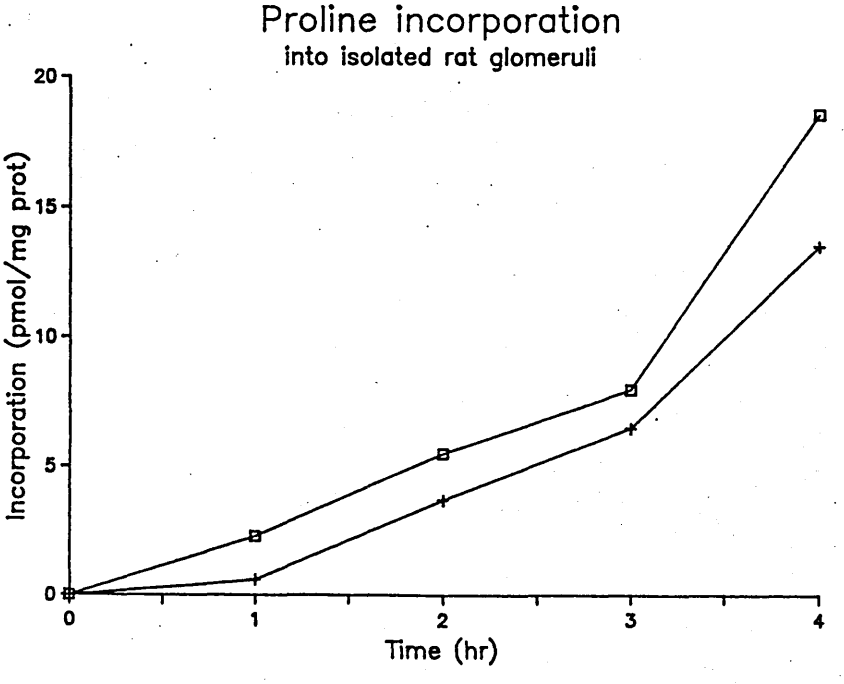


Fig. 6.1.4

Time-course of the incorporation of ^{3}H-proline into total glomerular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

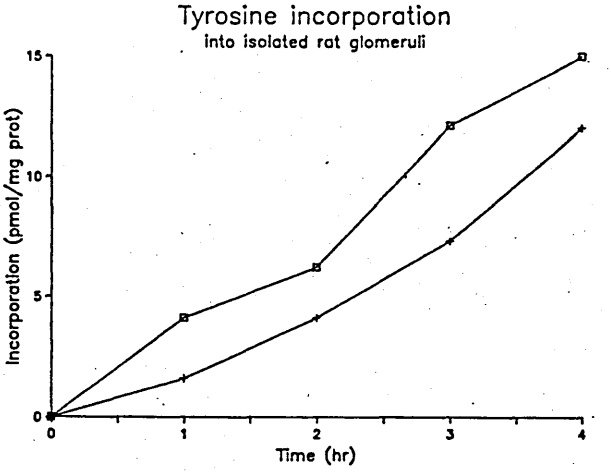


Fig. 6.1.5

Time-course of the incorporation of ^{3}H-tyrosine into total glomerular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

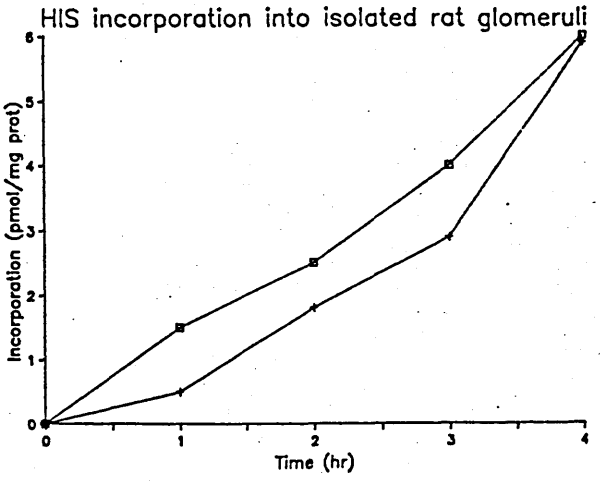


Fig. 6.1.6

Time-course of the incorporation of ^{3}H-histidine into total glomerular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat glomerular suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

TABLE 6.2.1

EFFECT OF ADRIAMYCIN ON AMINO ACID INCORPORATION INTO ISOLATED PIG GLOMERULI

DOSE (uM)	INCORPORATION (pmol of aminoacid/mg protein)				
	GLYCINE	LYSINE	HISTIDINE	PROLINE	LEUCINE
0	0.47 ± 0.10	1.21 ± 0.01	2.00 ± 0.17	2.00 ± 0.20	3.70 ± 0.38
7.8	$0.27 \pm 0.03^{*}$	$0.71 \pm 0.14^{**}$	$1.20 \pm 0.10^{**}$	$1.40 \pm 0.25^{*}$	$2.40 \pm 0.08^{**}$
15.6	$0.22 \pm 0.03^{*}$	$0.67 \pm 0.10^{***}$	$1.00 \pm 0.20^{**}$	$0.79 \pm 0.09^{***}$	$1.53 \pm 0.19^{***}$
31.2	$0.22 \pm 0.03^{*}$	$0.62 \pm 0.06^{***}$	$0.66 \pm 0.08^{***}$	$0.62 \pm 0.07^{***}$	$1.12 \pm 0.09^{***}$
62.5	$0.18 \pm 10^{-3*}$	$0.62 \pm 0.02^{***}$	0.42 ± 0.06	$0.38 \pm 9\text{x}10^{-3***}$	$0.74 \pm 0.03^{***}$

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.2.2

EFFECT OF ADRIAMYCIN ON AMINO ACID INCORPORATION INTO ISOLATED PIG GLOMERULI

DOSE (uM)	INCORPORATION AS % OF CONTROL				
	GLYCINE	LYSINE	HISTIDINE	PROLINE	LEUCINE
0.0	100	100	100	100	100
7.8	57	59	60	70	65
15.6	47	55	50	39.5	41
31.2	47	51	33	31	30
62.5	38	51	21	19	20

TABLE 6.2.3

INCORPORATION OF PROLINE INTO ISOLATED PIG GLOMERULI EXPOSED TO ADR

	DOSE (mM)	INCORPORATION[+]	% OF CONTROL
EXPERIMENT 1:			
	0	2.10 ± 0.02	100
	31.2×10^{-3}	0.48 ± 0.02***	23
	62.5×10^{-3}	0.25 ± 0.04***	12
	0.125	0.20 ± 0.01***	10
	0.25	0.17 ± 0.006***	8
	0.5	0.23 ± 0.014***	11
EXPERIMENT 2:			
	0	1.4 ± 0.2	100
	7.8×10^{-3}	1.08 ± 0.2**	78
	15.6×10^{-3}	0.77 ± 0.1**	55
	31.2×10^{-3}	0.58 ± 0.08**	42
	62.5×10^{-3}	0.40 ± 0.04**	29
	0.125	0.30 ± 0.02***	22
	0.25	0.24 ± 0.02***	17
EXPERIMENT 3:			
	0	1.1 ± 0.16	100
	7.8×10^{-3}	1.04 ± 0.22	92
	15.6×10^{-3}	0.74 ± 0.03*	65.5
	31.2×10^{-3}	0.51 ± 0.06**	45
	62.5×10^{-3}	0.36 ± 0.01**	32
	0.125	0.22 ± 0.01***	19.5
	0.25	0.11 ± 0.004***	10
EXPERIMENT 4:			
	0	5.6 ± 0.4	100
	7.8×10^{-3}	3.2 ± 0.37**	56
	15.6×10^{-3}	2.5 ± 0.31***	44
	31.2×10^{-3}	1.7 ± 0.15***	29.5
	62.5×10^{-3}	1.4 ± 0.03***	24.5
	0.125	1.2 ± 0.13***	21.1
	0.25	1.25 ± 0.03***	22
EXPERIMENT 5:			
	0	2.0 ± 0.2	100
	7.8×10^{-3}	1.4 ± 0.25*	70
	15.6×10^{-3}	0.8 ± 0.09***	40
	31.2×10^{-3}	0.6 ± 0.07***	30
	62.5×10^{-3}	0.4 ± 0.00***	20

* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$
+ pmol proline/mg protein

TABLE 6.2.4
EFFECT OF ADR ON PROLINE INCORPORATION
INTO ISOLATED PIG GLOMERULI

DOSE (mM)	INCORPORATION pmol/mg protein			% OF CONTROL			AVERAGE
0	7.619 ± 2.136	1.477 ± 0.006	3 ± 0.07	100	100	100	100
0.031	0.576 ± 0.138**	0.211 ± 0.041***	1.3 ± 0.2***	8	14	43	22
0.062	0.223 ± 0.023**	0.200 ± 0.046***	0.7 ± 0.03***	3	13	23	13
0.250	0.126 ± 0.005**	0.087 ± 0.002***	0.4 ± 0.01***	2	6	10	6
1.0	0.144 ± 0.010**	0.101 ± 6.006***	0.3 ± 0.04***	2	7	7	5

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$

TABLE 6.2.5 EFFECT OF ADR ON THE INCORPORATION OF PROLINE INTO ISOLATED RAT GLOMERULI

	Dose (uM)	Incorporation	
		pmol/mg prot	as % of control
Exp. 1	0	0.9 ± 0.3	100
	31.2	0.25 ± 0.08*	28
	62.5	0.28 ± 0.03*	31
	125	0.33 ± 0.05*	37
	250	0.31 ± 0.02*	34
	500	0.28 ± 0.03*	31
Exp. 2	0	0.41 ± 0.1	100
	7.8	0.12 ± 0.04**	29
	15.6	0.09 ± 0.03**	22
	31.2	0.10 ± 0.02**	24
	62.5	0.12 ± 0.01**	29
	125	0.10 ± 0.05**	24
	250	0.18 ± 0.01*	44
	500	0.09 ± 0.02**	22
Exp. 3	0	0.5 ± 0.05	100
	7.8	0.11 ± 0.005***	22
	15.6	0.09 ± 0.014***	18
	31.2	0.10 ± 0.04***	20
	62.5	0.11 ± 0.016***	22
	125	0.15 ± 0.07**	30

* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$

TABLE 6.2.6
INCORPORATION OF ^{3}H-PROLINE INTO ISOLATED PIG GLOMERULI EXPOSED TO PAN INCUBATED WITH KREBS BUFFER

	DOSE (mM)	INCORPORATION	% OF CONTROL
EXPERIMENT 1:			
	0	2.10 ± 0.27	100
	0.5	0.91 ± 0.16**	43
	2.5	1.01 ± 0.19**	48
	5	0.75 ± 0.05***	36
EXPERIMENT 2:			
	0	1.4 ± 0.2	100
	0.25	1.6 ± 0.24	113
	0.5	1.2 ± 0.15	87
	1	0.94 ± 0.05*	68
	2.5	1.10 ± 0.16	79
	5	0.79 ± 0.03**	57
EXPERIMENT 3:			
	0	1.1 ± 0.16	100
	0.25	1.44 ± 0.20	127
	0.5	0.98 ± 0.08	87
	1	0.85 ± 0.08	75
	2.5	0.68 ± 0.015*	60
	5	0.67 ± 0.06*	59

TABLE 6.2.6 (CONTINUED)

EXPERIMENT 4:		
0	5.6 ± 0.42	100
0.25	4.2 ± 0.57*	75.5
0.5	3.8 ± 0.33**	67
1	3.4 ± 0.28**	60
2.5	3.0 ± 0.39**	53
5	2.1 ± 0.24***	38

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.2.7
EFFECT OF PAN ON PROLINE INCORPORATION INTO ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER

DOSE	INCORPORATION[+]		% OF CONTROL		AVERAGE OF %
0	7.619 ± 2.13	1.477 ± 0.006	100	100	100
0.062	7.597 ± 0.22	2.012 ± 0.320*	99	136	117
0.125	7.459 ± 0.10	1.530 ± 0.330	98	103	100
0.625	6.205 ± 0.66	0.764 ± 0.017***	81	52	66
2.5	2.052 ± 0.16*	0.315 ± 0.038***	27	21	24

[+] pmol proline/mg protein.

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$

TABLE 6.2.8 EFFECT OF PAN ON THE INCORPORATION OF PROLINE INTO ISOLATED RAT GLOMERULI INCUBATED WITH TYRODES BUFFER

	Dose (mM)	Incorporation	
		pmol/mg prot	as % of control
Exp. 1	0	0.9 ± 0.3	100
	0.5	1.3 ± 0.28	144
	1.0	0.5 ± 0.10	55
	2.5	0.3 ± 0.05*	33
	5.0	0.3 ± 0.06*	33
Exp. 2	0	0.31 ± 0.05	100
	0.25	0.31 ± 0.09	100
	0.50	0.34 ± 0.006	110
	2.50	0.24 ± 0.003	77
	5.0	0.16 ± 0.002**	52

TABLE 6.2.9 STR EFFECT ON PROLINE INCORPORATION INTO ISOLATED PIG GLOMERULI INCUBATED WITH KREBS BUFFER

DOSE (mM)	INCORPORATION	
	pmol/mg protein	% OF CONTROL
0.0	5.60 ± 0.40	100
0.5	1.06 ± 0.11**	19
1.0	0.70 ± 0.11**	12
2.5	0.10 ± 0.03**	2
5.0	0.06 ± 0.01**	1

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.2.10 EFFECT OF STR THE INCORPORATION OF PROLINE BY ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER

DOSE (mM)	INCORPORATION pmol/mg protein		% OF CONTROL		AVERAGE OF %
0	5.101 ± 0.594	1.05 ± 0.18	100	100	100
0.25	4.753 ± 0.428	0.90 ± 0.17	93	86	89
0.5	2.260 ± 0.518**	0.60 ± 0.12*	44	57	50
1.0	0.306 ± 0.030***	0.50 ± 0.07**	6	48	27
2.0	0.007 ± 0.002***	0.20 ± 0.05**	2	19	10

TABLE 6.2.11 STR EFFECT ON PROLINE INCORPORATION INTO ISOLATED RAT GLOMERULI INCUBATED WITH TYRODES BUFFER

DOSE (mM)	INCORPORATION pmol/mg protein	% OF CONTROL
0.0	2.12 ± 0.60	100
1.0	0.14 ± 0.01**	6
2.5	0.09 ± 0.01**	4
5.0	0.09 ± 0.03**	4

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.2.12 ETA EFFECT ON PROLINE INCORPORATION INTO ISOLATED PIG GLOMERULI

DOSE (mM)	INCORPORATION (pmol/mg prot)	% OF CONTROL
0	6.10 ± 0.500	100.0
0.1	0.36 ± 0.040***	6.0
0.2	0.10 ± 0.020***	1.5
0.5	0.04 ± 0.002***	0.6

TABLE 6.2.13 INCORPORATION OF ^{3}H-PROLINE INTO ISOLATED PIG GLOMERULI EXPOSED TO BEA (KREBS)

DOSE (mM)	INCORPORATION (pmol/mg protein)	% OF CONTROL
0	5.6 ± 0.4	100
1	4.1 ± 0.4*	73
5	4.7 ± 0.5	84
10	3.1 ± 0.5**	56

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.2.14

EFFECT OF BEA ON PROLINE INCORPORATION INTO ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER

DOSE (Mm)	INCORPORATION						
	pmol/mg prot			% OF CONTROL			AVERAGE
0	5.101 ± 0.594	7.619 ± 2.130	1.055 ± 0.184	100	100	100	100
1	0.438 ± 0.157***	0.936 ± 0.056**	0.045 ± 0.004***	9	12	4	10
5	0.344 ± 0.146***	0.119 ± 0.006**	0	7	2	0	4
10	0.130 ± 0.040***	0.040 ± 0.005**	0	2.5	0.5	0	1

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.2.15 EFFECT OF BEA ON THE INCORPORATION OF PRO INTO ISOLATED RAT GLOMERULI INCUBATED WITH TYRODES

Dose (mM)	Incorporation	
	pmol/mg prot	% of control
0	2.1 ± 0.6	100
1	2.4 ± 0.0001	114
5	1.6 ± 0.04	76
10	1.0 ± 0.16*	48

* Significantly different from control at $P \leq 0.05$

TABLE 6.2.16 INCORPORATION OF PRO INTO ISOLATED PIG GLOMERULI EXPOSED TO FOLIC ACID

DOSE (mM)	INCORPORATION (pmol/mg protein)	% OF CONTROL
0	5.6 ± 0.4	100
1	4.2 ± 0.3**	75
5	2.8 ± 0.3***	50
10	1.5 ± 0.16***	26

** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.2.17 EFFECT OF FOLIC ACID ON THE INCORPORATION OF PRO INTO ISOLATED RAT GLOMERULI

Dose (mM)	Incorporation	
	pmol/mg prot	% of control
0	2.1 ± 0.6	100
1	1.9 ± 1.6	90
5	1.9 ± 0.2	90
10	1.1 ± 0.12*	52

* Significantly different from control at $P \leq 0.05$

TABLE 6.2.18 EFFECT OF OTA ON THE INCORPORATION OF PRO INTO ISOLATED PIG GLOMERULI

DOSE (mM)	INCORPORATION (pmol/mg protein)	% OF CONTROL
0	2.0 ± 0.20	100
1.6	1.7 ± 0.07	90
3.2	1.2 ± 0.06**	60

** Significantly different from control $P \leq 0.01$

TABLE 6.2.19
EFFECT OF POLYBRENE ON PROLINE INCORPORATION
INTO ISOLATED PIG GLOMERULI

DOSE (mg/ml)	INCORPORATION	
	pmol PRO/mg prot	% of Control
0	1.12 ± 0.16	100
0.125	0.32 ± 0.08	28
0.250	0.17 ± 0.12	15
0.500	0.21 ± 0.02	18

TABLE 6.2.20 EFFECT OF POLYBRENE ON THE INCORPORATION OF PRO INTO ISOLATED RAT GLOMERULI

	Dose (mg/ml)	Incorporation	
		pmol/mg prot	% of control
Exp. 1	0	0.9 ± 0.3	100
	1	0.12 ± 0.02*	13
	5	0.15 ± 0.04*	17
	10	0.16 ± 0.05*	17
Exp. 2	0	0.41 ± 0.05	100
	0.5	0.07 ± 0.002***	17
	1.0	0.08 ± 0.006***	19
	5.0	0.08 ± 0.001***	19
	10.0	0.06 ± 0.002***	15
Exp. 3	0	0.31 ± 0.05	100
	0.125	0.08 ± 0.01**	26
	0.25	0.06 ± 0.005***	19
	0.5	0.07 ± 0.004**	22
	1	0.08 ± 0.001**	26

* Significantly different from control at $P \leq 0.05$
** Significantly different from control at $P \leq 0.01$
*** Significantly different from control at $P \leq 0.001$

TABLE 6.3.1.

EFFECT OF DIFFERENT CONCENTRATIONS OF THE RESPECTIVE "COLD" SUBSTRATE ON THE OXIDATIVE METABOLISM OF ^{14}C-LINOLENIC AND OLEIC ACID AND GLUCOSE BY ISOLATED PIG GLOMERULI AFTER 1 HOUR OF INCUBATION

Concentration (uM)	$^{14}CO_2$ Release					
	Linolenic acid		Oleic acid		Glucose	
	a	%	a	%	a	%
0	156 ± 2.0	100	39 ± 3.0	100	3.7 ± 0.15	100
10	133 ± 4.0***	85	31 ± 1.0*	79	3.9 ± 0.06	105
100	70 ± 5.0***	45	20 ± 5.0**	51	3.5 ± 0.15	94
1000	3 ± 0.4***	2	6 ± 0.3***	15	2.8 ± 0.06***	76

a pmol of ^{14}C-substrate/mg protein.
% percentage of control.
* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$

TABLE 6.3.2.
EFFECT OF SHAKING DURING INCUBATION ON THE AMOUNT OF METABOLISM OF ^{14}C LABELLED SUBSTRATES BY PIG GLOMERULI AFTER 1 HOUR INCUBATION

	$^{14}CO_2$ Release[a]		
	LINOLENIC	OLEIC	GLUCOSE
NOT SHAKING	103 ± 0.9	16 ± 0.9	3.3 ± 0.1
SHAKING	156 ± 2***	39 ± 3***	3.7 ± 0.15*

a pmol/mg protein
* significantly different from control at $P \leq 0.05$
*** significantly different from control at $P \leq 0.001$

TABLE 6.3.3.
OXIDATIVE METABOLISM OF ^{14}C GLUCOSE BY ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER

TIME (H)	$14CO_2$ RELEASE (pmol/mg protein)
EXPERIMENT 1:	
1	32 ± 1
2	71 ± 3
3	120 ± 6
4	189 ± 14
EXPERIMENT 2:	
1	22 ± 3
2	39 ± 0.6
3	56 ± 2
4	78 ± 2
EXPERIMENT 3:	
1	38 ± 3
2	66 ± 7
3	119 ± 2
EXPERIMENT 4:	
1	29 ± 4
2	68 ± 7
3	102 ± 2
4	127 ± 11
EXPERIMENT 5:	
1	20 ± 1
2	40 ± 0.4
3	63 ± 3
4	72 ± 6
EXPERIMENT 6:	
1	11 ± 0.4
2	27 ± 0.7
3	49 ± 1
4	65 ± 4

TABLE 6.3.3 (CONTINUED)

EXPERIMENT 7:

0	0
1	18.1 ± 1.7
2	31 ± 2.4
3	67 ± 2.6
4	83 ± 7.0

EXPERIMENT 8:

0	0
1	16.4 ± 4
2	30 ± 1
3	44 ± 1
4	63 ± 7

TABLE 6.3.4

T VALUES AT LEVELS OF 5%-0.1% OF PROBABILITY FOR THE OXIDATIVE METABOLISM OF ^{14}-C GLUCOSE BY ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES, BETWEEN DIFFERENT TIME-POINTS IN SEVERAL EXPERIMENTS

EXPERIMENT 1:

	1	2	3
1			
2	21.4***		
3	25.0***	12.6***	-
4	19.4***	14.3***	7.8**

EXPERIMENT 2:

	1	2	3
1	-		
2	9.6***		
3	29.1***	14.1***	-
4	26.9***	32.3***	13.5***

TABLE 6.3.4 (CONTINUED)

EXPERIMENT 3:

	1	2	3
1			
2	6.4**	-	
3	38.9***	12.6***	-

EXPERIMENT 4:

	1	2	3
1	-		
2	8.4**	-	
3	28.2***	8.1**	-
4	14.5***	7.8**	3.9*

EXPERIMENT 5:

	1	2	3
1	-		
2	32.2***	-	
3	23.5***	13.1***	-
4	14.8***	9.2***	2.3

TABLE 6.3.4 (CONTINUED)

EXPERIMENT 6:

	1	2	3
1	-		
2	34.4***	-	
3	61.1***	31.2***	-
4	23.3***	16.2***	6.7**

EXPERIMENT 7:

	1	2	3
1			
2	7.6**		
3	27.3***	17.6***	-
4	15.6***	12.2***	3.7*

EXPERIMENT 8:

	1	2	3
1			
2	5.7**		
3	11.6***	17.1***	-
4	10.0***	8.1**	4.6**

* $P \leq 0.05$
** $P \leq 0,01$
*** $P \leq 0.001$

TABLE 6.3.5

OXIDADIVE METABOLISM OF ^{14}C-GLUCOSE
BY ISOLATED RAT GLOMERULI

TIME (hr)	CO_2 RELEASE (pmol/mg prot)
1	9.0 ± 0.32
2	12.0 ± 0.40
3	15.0 ± 0.60
4	15.2 ± 0.35

TABLE 6.3.6.
OXIDATIVE METABOLISM OF ^{14}C-LINOLENIC BY ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER

	TIME (H)	$^{14}CO_2$ RELEASE (pmol/mg protein)
EXPERIMENT 1:		
	1	74 ± 2
	2	129 ± 6
	3	159 ± 7
	4	176 ± 18
EXPERIMENT 2:		
	1	38 ± 4
	2	92 ± 9
	3	123 ± 3
	4	186 ± 8
EXPERIMENT 3:		
	1	180 ± 19
	2	392 ± 21
	3	527 ± 26
	4	651 ± 29
EXPERIMENT 4:		
	1	205 ± 21
	2	100 ± 7
	3	174 ± 12
	4	222 ± 21
EXPERIMENT 5:		
	1	53 ± 3
	2	100 ± 7
	3	174 ± 12
	4	222 ± 21
EXPERIMENT 6:		
	1	48 ± 4
	2	96 ± 18
	3	150 ± 16
	4	179 ± 5

TABLE 6.3.6. (CONTINUED)

EXPERIMENT 7:

1	26 ± 5
2	38 ± 3
3	57 ± 3
4	63 ± 3

EXPERIMENT 8:

1	64 ± 3
2	199 ± 14
3	356 ± 32
4	498 ± 33

TABLE 6.3.7.
T VALUES AT LEVELS OF 5%-0.1% OF PROBABILITY FOR THE OXIDATIVE METABOLISM OF ^{14}-C LINOLENIC ACID BY ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER BETWEEN DIFFERENT TIME POINTS IN SEVERAL EXPERIMENTS

EXPERIMENT 1:

	1	2	3
1			
2	15.4***	-	
3	20.2***	5.6**	-
4	9.7***	4.3*	1.5

EXPERIMENT 2:

	1	2	3
1	-		
2	9.5***		
3	24.4***	5.6**	-
4	28.7***	13.5***	12.8***

EXPERIMENT 3:

	1	2	3
1			
2	13.0***		
3	18.7***	7.0**	-
4	23.5***	12.5***	5.5**

TABLE 6.3.7. (CONTINUED)

EXPERIMENT 4:

	1	2	3
1	-		
2	2.4	-	
3	7.3**	2.9*	-
4	25.4***	10.5***	9.1***

EXPERIMENT 5:

	1	2	3
1	-		
2	10.7***	-	
3	16.9***	9.2***	-
4	13.8***	9.5***	3.4*

EXPERIMENT 6:

	1	2	3
1	-		
2	4.5*	-	
3	10.7***	3.9*	-
4	35.4***	7.7**	3.0*

TABLE 6.3.7. (CONTINUED)

EXPERIMENT 7:

	1	2	3
1	-		
2	3.5*	-	
3	9.2***	7.7**	-
4	11.0***	10.2***	2.4

EXPERIMENT 8:

	1	2	3
1	-		
2	16.3***	-	
3	15.7***	7.8**	-
4	22.7***	14.4***	5.3**

* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$

TABLE 6.3.8

OXIDATIVE METABOLISM OF ^{14}C-LINOLENIC ACID BY ISOLATED RAT GLOMERULI

TIME (hr)	CO_2 RELEASE (pmol/mg prot)
1	231 ± 5
2	320 ± 9
3	340 ± 18
4	368 ± 30

TABLE 6.3.9.
OXIDATIVE METABOLISM OF ^{14}C-OLEIC BY ISOLATED PIG GLOMERULI INCUBATED WITH EARLES-HEPES BUFFER

TIME (H)	$^{14}CO_2$ RELEASE (pmol/mg protein)
EXPERIMENT 1:	
1	9 ± 1
2	22 ± 3
3	27 ± 0.5
4	49 ± 3
EXPERIMENT 2:	
1	13 ± 1
2	36 ± 2
3	71 ± 4
4	105 ± 3
EXPERIMENT 3:	
1	63 ± 10
2	195 ± 29
3	313 ± 58
4	423 ± 27
EXPERIMENT 4:	
1	74 ± 16
2	123 ± 1
3	179 ± 15
4	332 ± 43
EXPERIMENT 5:	
1	19 ± 0.3
2	41 ± 10
3	65 ± 6
4	92 ± 9
EXPERIMENT 6:	
1	29 ± 1.3
2	79 ± 15
3	144 ± 8
4	182 ± 19
EXPERIMENT 7:	
1	13 ± 1.8
2	35 ± 8
3	73 ± 2
4	85 ± 4

TABLE 6.3.10.
T VALUES AT LEVELS OF 5%-0.1% OF PROBABILITY FOR THE OXIDATIVE METABOLISM OF ^{14}C OLEIC ACID BY ISOLATED PIG GLOMERULUS INCUBATED WITH EARLES-HEPES BUFFER, BETWEEN DIFFERENT TIME POINTS IN SEVERAL EXPERIMENTS

EXPERIMENT 1:

	1	2	3
1			
2	7.1**	-	
3	27.9***	2.84*	-
4	21.9***	11.0***	12.5***

EXPERIMENT 2:

	1	2	3
1			
2	17.8***	-	
3	24.4***	13.5***	-
4	50.4***	33.1***	11.8***

EXPERIMENT 3:

	1	2	3
1	-		
2	7.4**	-	
3	7.3**	3.1*	-
4	21.6***	10.0***	3.0*

TABLE 6.3.10. (CONTINUED)

EXPERIMENT 4:

	1	2	3
1	-		
2	5.3**	-	
3	8.3**	6.4**	-
4	9.7***	8.4**	5.8**

EXPERIMENT 5:

	1	2	3
1	-		
2	3.8*	-	
3	13.3***	3.6*	-
4	14.0***	6.6**	4.3*

EXPERIMENT 6:

	1	2	3
1	-		
2	5.7**	-	
3	24.6***	6.6**	-
4	13.9***	7.4**	3.2*

TABLE 6.3.10. (CONTINUED)

EXPERIMENT 7:

	1	2	3
1	-		
2	4.6**	-	
3	38.6***	8.0**	-
4	28.4***	9.7***	4.6**

* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$

TABLE 6.4.1. EFFECT OF ADRIAMYCIN ON THE OXIDATIVE METABOLISM OF ^{14}C-GLUCOSE BY ISOLATED PIG GLOMERULI

DOSE (mM)	CO_2 RELEASE pmoles/mg protein		% OF CONTROL		AVERAGE OF %
0	63 ± 7	65 ± 4	100	100	100
0.031	61 ± 2	70 ± 0	97	108	102
0.062	51 ± 4	67 ± 3	80	103	91
0.250	45 ± 3*	48 ± 3**	71	74	72
1.0	34 ± 3**	17 ± 5***	53	26	39

TABLE 6.4.2. EFFECT OF ADRIAMYCIN ON THE OXIDATIVE METABOLISM OF ^{14}C-LINOLENIC ACID BY ISOLATED PIG GLOMERULI

DOSE (mM)	CO_2 RELEASE pmoles/mg protein		% OF CONTROL		AVERAGE OF %
0	179 ± 5	63 ± 3			
0.031	161 ± 16	28 ± 2***	90	44	67
0.062	122 ± 10***	29 ± 3***	68	46	57
0.250	110 ± 5***	23 ± 5***	61	36	48
1.0	62 ± 3***	9 ± 3***	35	14	24

TABLE 6.4.3. EFFECT OF ADRIAMYCIN ON THE OXIDATIVE METABOLISM OF ^{14}C-OLEIC ACID BY ISOLATED PIG GLOMERULI

DOSE (mM)	CO_2 RELEASE pmoles/mg protein	% OF CONTROL
0	85 ± 4	
0.031	20 ± 2***	23
0.062	23 ± 4***	27
0.250	15 ± 1***	18
1.0	7 ± 3***	8

* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$

TABLE 6.4.4 EFFECT OF PUROMYCIN AMINONUCLEOSIDE ON THE OXIDATIVE METABOLISM OF ^{14}C-GLUCOSE BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein		% OF CONTROL		AVERAGE OF %
0	63 ± 3	65 ± 3			
0.062	58 ± 1	52 ± 4*	92	80	86
0.125	51 ± 2**	54 ± 3*	81	83	82
0.625	47 ± 2**	44 ± 2***	75	68	71
2.5	32 ± 7**	29 ± 2***	51	45	48

TABLE 6.4.5. EFFECT OF PUROMYCIN AMINONUCLEOSIDE ON THE OXIDATIVE METABOLISM OF ^{14}C-LINOLENIC ACID BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein		% OF CONTROL		AVERAGE OF %
0	179 ± 5	63 ± 3			
0.062	148 ± 15*	31 ± 3***	83	49	66
0.125	137 ± 11**	30 ± 2***	76	48	62
0.625	136 ± 15**	30 ± 1***	76	48	62
2.5	105 ± 5***	29 ± 2***	59	46	52

TABLE 6.4.6. EFFECT OF PUROMYCIN AMINONUCLEOSIDE ON THE OXIDATIVE METABOLISM OF ^{14}C-OLEIC ACID BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein	% OF CONTROL
0	85 ± 4	
0.062	31 ± 1***	36
0.125	30 ± 2***	35
0.625	29 ± 3***	34
2.5	22 ± 5***	26

* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$

TABLE 6.4.7. EFFECT OF STREPTOMYCIN ON THE OXIDATIVE METABOLISM OF ^{14}C-GLUCOSE BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein		% OF CONTROL		AVERAGE OF %
0	83 ± 7	65 ± 4	100	100	100
0.25	87 ± 4	43 ± 2**	105	66	85
0.5	90 ± 13	39 ± 2***	108	60	84
1.0	77 ± 6	39 ± 1***	93	60	76
2.0	86 ± 8	39 ± 3***	97	60	78

TABLE 6.4.8. EFFECT OF STREPTOMYCIN ON THE OXIDATIVE METABOLISM OF ^{14}C-LINOLENIC ACID BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein		% OF CONTROL		AVERAGE OF %
0	498 ± 33	179 ± 5			
0.25	396 ± 54*	185 ± 10	79	103	91
0.5	362 ± 61*	172 ± 17	73	96	84
1.0	265 ± 15***	133 ± 6***	53	70	63
2.0	250 ± 55**	81 ± 8***	50	45	47

TABLE 6.4.9. EFFECT OF STREPTOMYCIN ON THE OXIDATIVE METABOLISM OF ^{14}C-OLEIC ACID BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein	% OF CONTROL
0	182 ± 19	
0.25	140 ± 20	77
0.5	149 ± 14	82
1.0	150 ± 30	82
2.0	152 ± 1	82

* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$

TABLE 6.4.10. ETA EFFECT ON ^{14}C-GLUCOSE OXIDATION BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein	% OF CONTROL
0	51 ± 9	100
0.1	50 ± 5	98
0.2	44 ± 7	86
0.5	24 ± 2**	47

TABLE 6.4.11. ETA EFFECT ON ^{14}C-LINOLENIC ACID METABOLISM BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein	% OF CONTROL
0	63 ± 3	100
0.1	20 ± 2***	32
0.2	11 ± 2***	17
0.5	9 ± 3***	14

TABLE 6.4.12. ETA EFFECT ON ^{14}C-OLEIC ACID METABOLISM BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein	% OF CONTROL
0	85 ± 1	100
0.1	20 ± 4***	23
0.2	7 ± 1***	8
0.5	0	0

* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$

TABLE 6.4.13. EFFECT OF BEA ON THE OXIDATIVE METABOLISM OF ^{14}C-GLUCOSE BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein			% OF CONTROL			AVERAGE OF %
0	72 ± 6	83 ± 7	51 ± 9				
1	87 ± 3*	93 ± 6	63 ± 10	121	112	123	119
5	69 ± 6	126 ± 13**	97 ± 14**	96	151	190	146
10	64 ± 5	162 ± 27**	106 ± 13**	89	195	208	164

TABLE 6.4.14 EFFECT OF BEA ON THE OXIDATIVE METABOLISM OF ^{14}C-LINOLENIC BY ISOLATED PIG GLOMERULI

DOSE (mM)	$^{14}CO_2$ RELEASE pmol/mg protein		% OF CONTROL	
0	498 ± 33	224 ± 20	100	100
1	555 ± 90	73 ± 6***	111	33
5	477 ± 10	36 ± 5***	96	16
10	343 ± 20	24 ± 3***	69	11

TABLE 6.4.15. EFFECT OF BEA ON THE OXIDATIVE METABOLISM OF ^{14}C-OLEIC BY ISOLATED PIG GLOMERULI

DOSE (mM) OF %	$^{14}CO_2$ RELEASE pmol/mg protein		% OF CONTROL	
0	182 ± 19	92 ± 9	100	100
1	229 ± 18*	29 ± 4***	126	31
5	182 ± 23	13 ± 1***	100	14
10	123 ± 19*	6 ± 0.8***	68	6

* significantly different from control at $P \leq 0.05$
** significantly different from control at $P \leq 0.01$
*** significantly different from control at $P \leq 0.001$

TABLE 6.5.1.

EFFECT OF OTA ON THE INCORPORATION[+] OF DIFFERENT AMINO ACIDS INTO ISOLATED RAT GLOMERULI

DOSE	TRP[+]	PHE[+]	TYR[+]	LEU[+]	PRO[+]	HIS[+]
0	38 + 10	10.5 + 2	2.4 + 0.3	5.8 + 1.30	2.5 + 0.5	1.3 + 0.09
0.1	19 + 4.5[*]	4.7 + 0.7[**]	0.7 + 0.2[***]	1.5 + 0.30[**]	1.6 + 0.3	0.8 + 0.11[**]
0.10	19 + 3.9[*]	1.8 + 0.3[**]	1.1 + 0.04[**]	1.1 + 0.10[**]	0.5 + 0.1[**]	0.4 + 0.01[***]
1.00	4.7 + 1.1[**]	0.4 + 0.1[***]	0.7 + 0.08[***]	0.4 + 0.07[**]	0.21 + 0.03[**]	0.5 + 0.005[***]

[+] Incorporation measured as pmol of amino acid/mg glomerular protein
[*] Significantly different from the control value at $P < 0.05$.
[**] at $P < 0.01$
[***] at $P < 0.001$

TABLE 6.5.2.

EFFECT OF OTA ON THE INCORPORATION[+] OF DIFFERENT AMINO ACIDS INTO ISOLATED RAT GLOMERULI

DOSE (mM)	TRP[+]	PHE[+]	TYR[+]	LEU[+]	PRO[+]	HIS[+]
0.	100	100	100	100	100	100
0.01	50	44	29	25.8	61.8	62.2
0.10	50	17	45	18.1	20.1	31.6
1.00	12	4	27	6.4	8.3	12.1

[+] Incorporation measured as percentage of control values.

TABLE 6.5.3 EFFECT OF OTA ON GLUCOSE METABOLISM BY ISOLATED RAT GLOMERULI

DOSE (mM)	CO_2 RELEASE	
	pmol CO_2/mg protein	% of Control
0	15.2 ± 0.3	100
0.01	45.8 ± 6.6**	301
0.10	52.5 ± 5.3***	345
1.00	9.1 ± 0.2***	60

* SIGNIFICANTLY DIFFERENT FROM THE CONTROL GROUP AT $P \leq 0.01$
*** AT $P \leq 0.001$

TABLE 6.5.4 EFFECT OF OTA ON LINOLENIC ACID METABOLISM BY ISOLATED RAT GLOMERULI

DOSE (mM)	CO_2 RELEASE	
	pmol CO_2/mg protein	% of Control
0	325.0 ± 18	100
0.01	380.2 ± 17*	117
0.10	389.6 ± 34*	120
1.00	24.4 ± 1.2***	7.5

* SIGNIFICANTLY DIFFERENT FROM THE CONTROL GROUP AT $P \leq 0.05$
*** AT $P \leq 0.001$

APPENDIX 2

TABLE 6.6.1.

INCORPORATION OF ^{3}H-PROLINE INTO RAT TUBULES USING TWO DIFFERENT INCUBATION BUFFERS

	INCORPORATION (pmol/mg prot)	
TIME	TYRODES	EARLES-HEPES
1	0.17 ± 0.008	0.38 ± 0.03***
2	0.25 ± 0.017	0.65 ± 0.09**
3	0.42 ± 0.0006	1.60 ± 0.17***
4	0.82 ± 0.046	2.4 ± 0.33**

** Significantly different from Tyrodes at $P \leq 0.01$
*** Significantly different from Tyrodes at $P \leq 0.001$

TABLE 6.6.2
INCORPORATION OF DIFFERENT AMINO ACIDS INTO RAT PROXIMAL TUBULAR FRAGMENTS

TIME (Hr)	INCORPORATION (pmol/mg prot)					
	TRP	PRO	TYR	PHE	HIS	LEU
1	3.4 ± 0.1	0.6 ± 0.01	0.3 ± 0.01	0.4 ± 0.01	0.2 ± 0.01	0.40 ± 0.03
2	4.8 ± 0.3	1.3 ± 0.03	0.6 ± 0.03	0.6 ± 0.10	0.3 ± 0.04	0.60 ± 0.03
3	8.1 ± 0.4	2.1 ± 0.2	0.8 ± 0.12	0.9 ± 0.04	0.5 ± 0.05	0.70 ± 0.12
4	18.0 ± 1.3	2.7 ± 0.3	1.3 ± 0.06	1.2 ± 0.04	0.9 ± 0.05	0.75 ± 0.06

TABLE 6.6.3
T VALUES AT LEVELS OF 5%-0.1% OF PROBABILITY FOR THE INCORPORATION OF DIFFERENT AMIN ACIDS AT VARIOUS TIME-POINTS

INCORPORATION OF TRP INTO ISOLATED RAT TUBULE INCUBATED WITH HEPES

	1	2	3
1	-		
2	6.62**	-	
3	17.8***	9.9***	-
4	19.2***	16.7***	12.2***

INCORPORATION OF PHE INTO ISOLATED RAT TUBULE INCUBATED WITH HEPES

	1	2	3
1	-		
2	3.7*	-	
3	20.5***	5**	-
4	31.0***	9.3***	8.3**

INCORPORATION OF TYR INTO ISOLATED RAT TUBLE INCUBATED WITH HEPES

	1	2	3
1	-		
2	22.1***	-	
3	11.2***	5**	-
4	34.4***	23.5***	9.0***

TABLE 6.6.3 (CONTINUED)

INCORPORATION OF LEU INTO ISOLATED RAT TUBULE

	1	2	3
1	-		
2	8.5**	-	
3	9.5***	4.1*	-
4	4.1*	1.4	0.6

INCORPORATION OF PRO INTO ISOLATED RAT TUBULE

	1	2	3
1	-		
2	35.6***	-	
3	14.9***	8.2**	-
4	10.1***	6.8**	2.5

INCORPORATION OF HIS INTO ISOLATED RAT TUBULE

	1	2	3
1	-		
2	5**	-	
3	10***	4.9**	-
4	24.4***	17.0***	11.0***

* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$

TABLE 6.6.4
T VALUES AT LEVELS OF 5%-0.1% PROBABILITY FOR THE
INCORPORATION OF DIFFERENT AMINO ACIDS INTO ISOLATED RAT TUBULE

AFTER 4 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO	HIS
TRP	-					
PHE	22.2***	-				
TYR	22***	3.8*	-			
LEU	22.8***	6.3**	7.9**	-		
PRO	19.5***	7.3**	6.7**	9.3**	-	
HIS	22.6***	6.8**	9.5**	3.1	8.6**	-

AFTER 3 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO	HIS
PHE	27.7***	-				
TYR	27.7***	1.5	-			
LEU	28.2***	4.3**	1.7	-		
PRO	21.7***	11.9***	11.8***	13.2***	-	
HIS	29.3***	11.6***	6.4**	5.5**	15.9***	-

TABLE 6.6.4 (CONTINUED)

AFTER 2 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO
PHE	19.6***	-			
TYR	20.4***	0.17	-		
LEU	20.4***	0.50	0.96	-	
PRO	17.0***	11.4***	33.6***	29.4***	-
HIS	21.7***	4.8**	11.2***	9.3***	34.3***

AFTER 1 HR OF INCUBATION

	TRP	PHE	TYR	LEU	PRO
PHE	51.3***	-			
TYR	53.3***	5.5**	-		
LEU	50.4***	0.96	2.8	-	
PRO	47.6***	20.1***	42.5***	15.3***	-
HIS	54.8***	12.8***	11***	9.1***	36.4***

* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$

TABLE 6.6.5
OXIDATIVE METABOLISM OF GLUCOSE
BY ISOLATED RAT PROXIMAL TUBULAR FRAGMENTS

TIME (Hr)	CO_2 RELEASE (pmol/mg protein)	
1	9.3 ± 1.1	4.4 ± 0.4
2	27.0 ± 0.5	13.0 ± 1.3
3	30.0 ± 4.4	15.0 ± 2.5
4	26.0 ± 2.9	19.0 ± 2.0

TABLE 6.6.6
OXIDATIVE METABOLISM OF FATTY ACIDS+
BY ISOLATED RAT PROXIMAL TUBULAR FRAGMENTS

TIME	STEARIC*	PALMITIC*	OLEIC	LINOLENIC
1	23.2 ± 1.3	65 ± 4	271 ± 4	602 ± 5
2	31.5 ± 3.4	100 ± 12	470 ± 17	887 ± 67
3	34 ± 3.4	108 ± 9	581 ± 16	975 ± 2
4	41.5 ± 7.0	122 ± 7	655 ± 20	1043 ± 11

* Blanks without tubules gave half of the counts obtained with tubules

\+ Mevalonic acid did not undergo metabolism under the conditions of this experiment

TABLE 6.6.7
T VALUES AT LEVELS 5%-0.1% OF PROBABILITY FOR THE OXIDATIVE METABOLISM OF GLUCOSE BY ISOLATED RAT TUBULAR FRAGMENTS

	GLUCOSE					
	1	2	3	1	2	3
2	10.9***	-		24.1***	-	
3	7.2**	1.2	-	7.8**	1.3**	-
4	12.4***	4.3*	2.2	9.2***	0.5***	1.3

* P ≤ 0.05
** P ≤ 0.01
*** P ≤ 0.001

TABLE 6.6.8
T VALUES AT LEVELS 5%-0.1% OF PROBABILITY FOR THE OXIDATIVE METABOLISM OF FATTY ACIDS BY ISOLATED RAT TUBULAR FRAGMENTS

STEARIC

	1	2	3
2	3.9*	-	
3	5.1**	0.9	-
4	4.4*	2.2	1.7

PALMITIC

	1	2	3
2	4.8**	-	
3	7.6**	0.9	-
4	12.2***	2.7	2.1

OLEIC

	1	2	3
2	19.7***	-	
3	32.5***	8.2**	-
4	32.6***	12.2***	8.2**

LINOLENIC

	1	2	3
2	0.9	-	
3	1.0	0.4	-
4	0.5	0.03	0.11

* $P \leq 0.05$
** $P \leq 0.01$
*** $P \leq 0.001$

TABLE 6.6.9
EFFECT OF OTA ON AMINO ACID INCORPORATION INTO RAT PROXIMAL TUBULAR FRAGMENTS

DOSE (mM)	INCORPORATION (pmol/mg prot)					
	TRP	PRO	TYR	PHE	HIS	LEU
0	18.0 ± 1.3	2.7 ± 0.30	1.30 ± 0.05	1.20 ± 0.04	0.90 ± 0.05	0.75 ± 0.06
0.01	8.4 ± 0.3***	1.1 ± 0.01***	0.60 ± 0.05***	0.64 ± 0.07***	0.33 ± 0.06***	0.33 ± 0.03***
0.10	7.1 ± 0.8***	1.0 ± 0.09***	0.50 ± 0.07***	0.52 ± 0.08***	0.30 ± 0.01***	0.26 ± 0.03***
1.00	2.8 ± 0.4***	0.3 ± 0.04***	0.16 ± 0.01***	0.22 ± 0.03***	0.09 ± 0.01***	0.18 ± 0.02***

*** Significantly different from control values at $P \leq 0.001$

TABLE 6.6.10
EFFECT OF OTA ON AMINO ACID INCORPORATION INTO RAT PROXIMAL TUBULAR FRAGMENTS EXPRESSED AS PERCENTAGE OF CONTROL

	PERCENTAGE OF CONTROL					
DOSE (mM)	TRP	PRO	TYR	PHE	HIS	LEU
0	100	100	100	100	100	100
0.01	47	42	48	53	34	47
0.10	39	38	36	44	32	35
1.00	15	10	12	18	10	24

TABLE 6.6.11
EFFECT OF OCA ON GLUCOSE METABOLISM BY ISOLATED RAT TUBULES

DOSE (mM)	CO_2 RELEASE	
	pmol CO_2/mg protein	As % of Control
0 (CONTROL)	26 ± 2.9	100
0.01	69 ± 5.2***	266
0.10	58 ± 7.0**	224
1.00	19 ± 1.6*	72

* SIGNIFICANTLY DIFFERENT FROM THE CONTROL GROUP AT $P \leq 0.05$
** AT $P \leq 0.01$
*** AT $P \leq 0.001$

TABLE 6.6.12
EFFECT OF OCA ON LINOLENIC ACID METABOLISM
BY ISOLATED METABOLISM BY ISOLATED RAT TUBULES

DOSE (mM)	CO_2 RELEASE	
	pmol CO_2/mg protein	As % of Control
0 (CONTROL)	87.9 ± 7.1	100
0.01	125.8 ± 4.3**	143
0.10	116.6 ± 10.4*	133
1.00	9.8 ± 0.6***	11

* SIGNIFICANTLY DIFFERENT FROM THE CONTROL GROUP AT $P \leq 0.05$,
** AT $P \leq 0.01$, *** AT $P \leq 0.001$

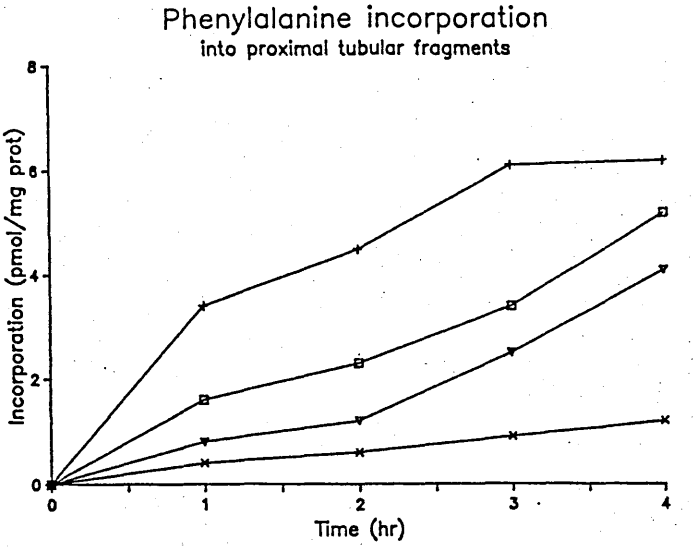


Fig. 6.6.1

Time-course of the incorporation of ^{3}H-phenylalanine into total proximal tubular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat PTF suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

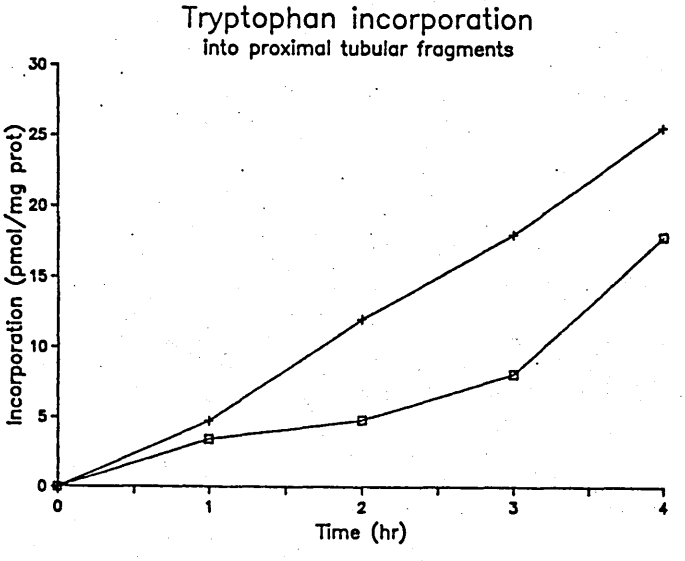


Fig. 6.6.2

Time-course of the incorporation of ^{3}H-tryptophan into total proximal tubular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat PTF suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

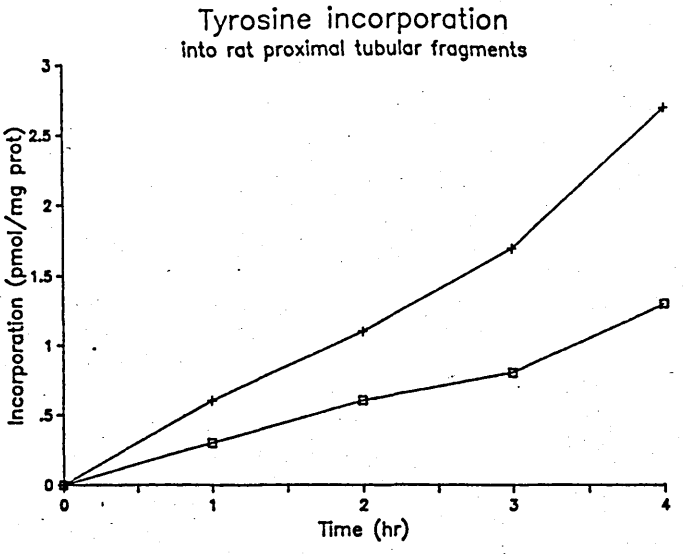


Fig. 6.6.3

Time-course of the incorporation of ^{3}H-tyrosine into total proximal tubular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat PTF suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

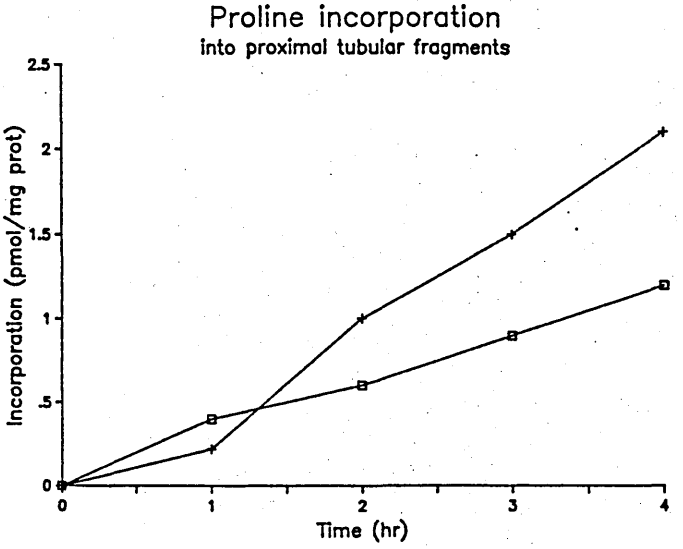


Fig. 6.6.4

Time-course of the incorporation of ^{3}H-proline into total proximal tubular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat PTF suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.

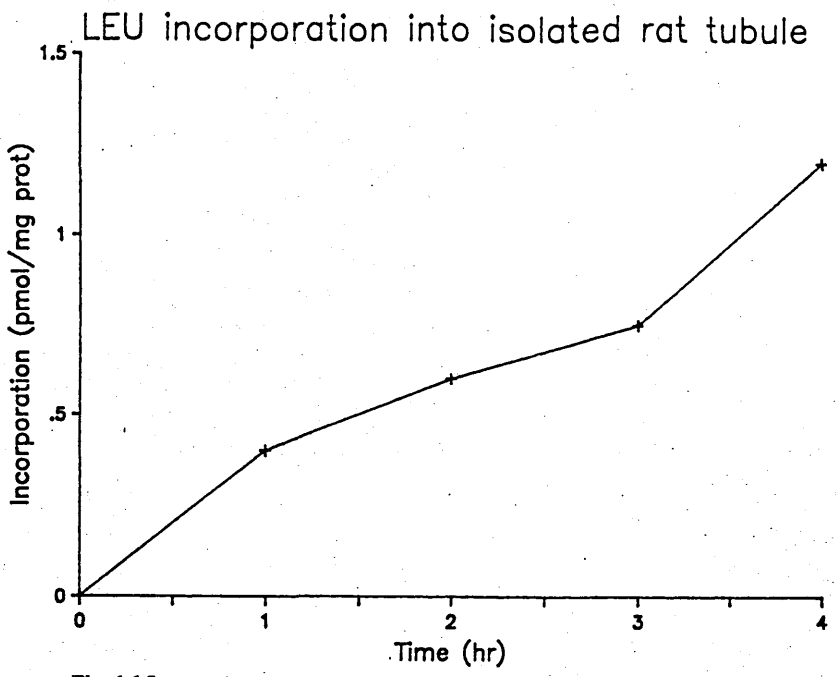


Fig. 6.6.5

Time-course of the incorporation of ^{3}H-leucine into total proximal tubular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat PTF suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each time point is the result of triplicate incubations.

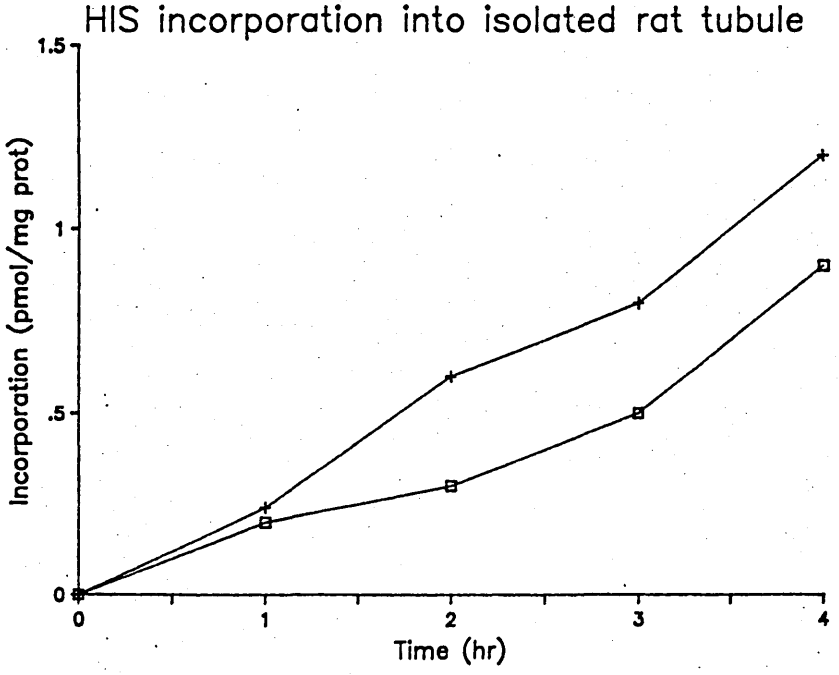


Fig. 6.6.6

Time-course of the incorporation of ^{3}H-histidine into total proximal tubular protein. 0.5 ml of Earles-HEPES buffer containing 2.5 uCi of the labelled amino acid were added at time 0 to 0.5 ml aliquots of rat PTF suspension and incubated up to 4 hr. The ordinate represents specific activity incorporated into TCA-precipitable labelled material. Each line corresponds to a different experiment and each time point is the result of triplicate incubations.