GASTROINTESTINAL ABSORPTION OF OCHRATOXIN A

GWYNEDD BARROWMAN

GASTROINTESTINAL ABSORPTION OF OCHRATOXIN A

by

Gwynedd Barrowman B.Sc

A thesis submitted to the

School of Graduate Studies

in partial fulfilment of the

requirements for the degree of

Master of Science

Department of Biochemistry

Memorial University of Newfoundland

1993

St. John's Newfoundland

ISBN 0-315-91611-7

ABSTRACT

The mycotoxin Ochratoxin A (OTA), is a widespread contaminant of cereals, beans and meat products. Its solubility in the aqueous phase depends on ionization of the phenolic hydroxyl group whose pK_a was found to be 7.05. Earlier researchers have demonstrated the importance of the portal venous route in OTA absorption. Absorption in the presence of lipids is of interest because lipids form a significant proportion of the diet in countries where OTA contamination occurs. When OTA was administered to conscious rats in corn oil rather than in sodium bicarbonate, the lymphatic route played a greater role in the transport of absorbed OTA. In the absence of bile, OTA transport in lymph was not increased even in the presence of corn oil, suggesting that OTA handling by the lymphatic route depends on micellar solubilization in mixed bile salt/lipid micelles. When carried in lymph, OTA reaches the general circulation without being subjected to "first-pass" hepatic metabolism.

Key words: ochratoxin A; nephrotoxin; lymphatic transport

For Jim

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. A. D. Rahimtula for his guidance, support and patience. Thanks are also due to the other members of my supervisory committee, Dr. J.T. Brosnan and Dr. P. Georghiou, whose encouragement and constructive criticism have been most helpful. I also wish to thank Gary Chernenko, Marie Codner and Laura Gray for skilled technical assistance, Dr. U. Balasooriya for statistical advice and Drs. E. A. Barnsley, J. C. Orr and P. Tso for helpful discussion. I am grateful to Rabeea Omar for his practical advice and help, to Nicholas Barrowman for designing computer programs used to record and plot data, to Gillian Megann-Barrowman for providing material from Greek and Roman sources, to Judy Gibson for assistance with proofreading and to all my family for their constant support and encouragement.

This research was made possible by generous financial assistance from the Kidney Foundation of Canada.

TABLE OF CONTENTS

3. ABSORPTION OF FOREIGN COMPOUNDS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS and SYMBOLS

ATP	Adenosine triphosphate
BEN	Balkan endemic nephropathy
B+	rats in experiment (5) given OTA in sodium bicarbonate, with bile reinfused to replace diverted bile
C+	rats in experiment (5) given OTA in corn oil, with bile reinfused to replace diverted bile
C—	rats in experiment (5) given OTA in corn oil, without replacement of diverted bile
FABP	Fatty acid binding protein
HDL	High density lipoprotein
HGL	Human gastric lipase
HPLC	High performance liquid chromatography
LDL	Low density lipoprotein
OTA	Ochratoxin A
Oα	metabolite of OTA which lacks the phenylalanine moiety
PEP	Phosphoenolpyruvate
SD	Standard deviation
TLC	Thin layer chromatography

Abbreviations continued:

UDP	Uridine diphosphate
UDPGA	Uridine diphosphate glucuronic acid
VLDL	Very low density lipoprotein

Introduction

The mycotoxin Ochratoxin A (OTA) is a widespread contaminant of cereals, beans and meat products. It poses a risk to the health of animals and humans, and has been linked to the fatal kidney disease, Balkan endemic nephropathy. This thesis addresses the mode of absorption of OTA and the effect of pH changes on its ionization state and solubility.

1. THE ROLE OF FUNGI [1,2,3]

1.1 Biology of Fungi Fungi are eukaryotic organisms which, unlike green plants, do not possess chlorophyll. They are unable to synthesize from CO_2 and H_2O the organic compounds they require for growth and reproduction, and therefore depend on other organisms to supply the nutrients they need. They establish themselves on a host, which may be living or dead, and either absorb compounds intact (in the case of monosaccharides or amino acids) or secrete extracellular enzymes which degrade nutrients to simpler constituents which can then be absorbed. Fungi are composed of threadlike tubular structures, hyphae, which form extensive anastomosing systems called mycelia.

There are approximately 50,000 species of fungi, ranging from unicellular yeasts, moulds, mildews, rusts and smuts to lichens and the larger fungi such as mushrooms. Classification of fungi is not straightforward: the initial distinction is made on the basis of presence or absence of a cell wall, and a further distinction is based on whether reproduction is sexual or asexual. The genera *Aspergillus* and *Penicillium* fall within a

group of Eumycota (fungi with walls) which reproduces almost entirely by means of asexual spores, known as conidia. These structures form externally on the hyphae, or on modified hyphal branches. *Aspergillus* and *Penicillium* are classified either under Deuteromycotina which only reproduce asexually by means of conidia, or under Ascotina, which occasionally will also undergo sexual reproduction with the formation of ascospores. When conditions are not favourable to a fungus, it may move into an essentially dormant phase, producing dry, spore-like structures called sclerotia. When conditions improve, the sclerotia germinate and the fungus resumes active metabolism.

In the life of fungi, there are several essential stages: spore germination; vegetative growth of the mycelium; development of the spore-bearing structures; and liberation of spores. Metabolism, using organic compounds derived from other organisms, generates energy in the form of adenosine triphosphate (ATP); provides reducing power for biosynthetic reactions, usually in the form of reduced co-enzymes; and provides intermediates for a range of biosynthetic pathways. Regulation of glucose metabolism is of major importance. It is achieved through key enzymes, in response to the availability of nutrients and trace elements such as zinc and manganese, and by feedback inhibition by end products.

1.2 Secondary metabolism and mycotoxins If excess carbohydrate is available after growth of the fungus has ceased due to shortage of a required nutrient (commonly a source of nitrogen), the fungus may switch into secondary metabolism. Certain strains

of fungi are particularly prone to make this switch. Relationships between the pathways of primary and secondary metabolism are shown in Figure 1.1. Secondary metabolites are derivatives of many different intermediates of primary metabolism, and can be classified according to five major sources: glucose-derived metabolites including some polysaccharides, peptidopolysaccharides and sugar alcohols; polyketides and phenolic compounds derived from the condensation of acetate from acetyl-coA in the acetate-malonate pathway of fatty acid biosynthesis; steroids and terpenes derived from the condensation of acetate from acetyl-coA in the mevalonic acid pathway; phenolic compounds derived from the shikimic acid pathway for the biosynthesis of aromatic amino acids; and various other pathways of amino acid synthesis. The amino acids involved in the synthesis of secondary metabolites originate from many stages in glycolysis and the Krebs cycle. The shikimic acid pathway uses erythrose phosphate from the hexose monophosphate pathway and phosphoenolpyruvate derived from glycolysis. The function of products of secondary metabolism has been the subject of much speculation. Some secondary metabolites play a role in the normal physiology of the fungus, *e.g.* compounds in the isoprenoid group behave as hormones influencing sexual reproduction in many fungi. It has been suggested that the secondary pathways are safety mechanisms which prevent accumulation of harmful intermediates of primary metabolism during periods of nutrient imbalance [4]. Some secondary metabolites are active as antibiotics against bacteria; this may provide a competitive advantage to the fungus. Many secondary metabolites possess toxic properties and are known as

Figure 1.1

Interrelationships between pathways of primary and secondary metabolism

The principle pathways of secondary metabolism are numbered (1) to (5)

(1) glucose-derived metabolites

(2) acetate-malonate pathway

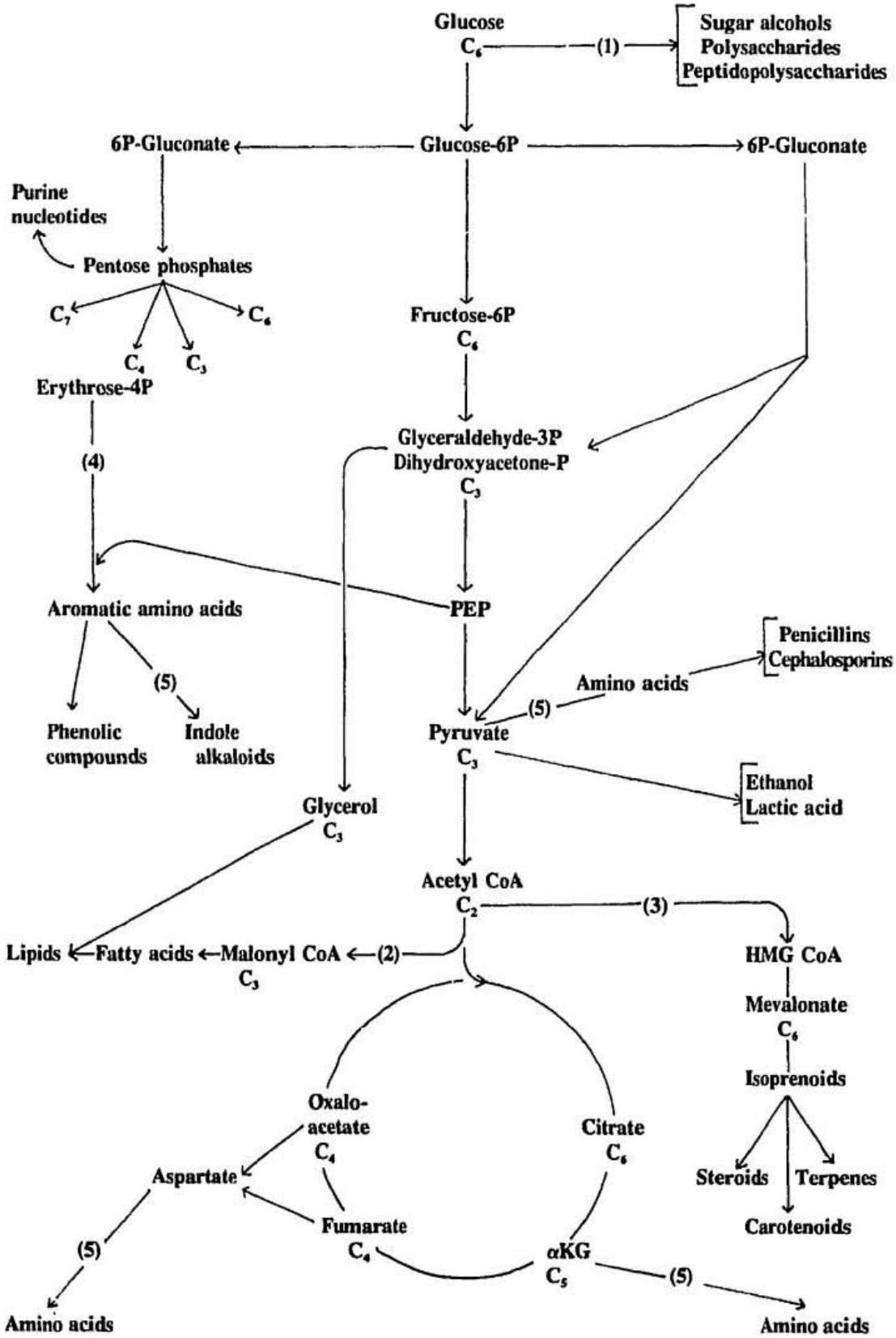
(3) mevalonic acid pathway

(4) shikimic acid pathway

(5) amino acid-derived pathways

For simplicity, many intermediates have been omitted, and arrows point in one direction only, although many of the reactions are reversible. The incorporation or production of CO_2 in some reactions is not shown but may be inferred from a change in the number of carbon atoms.

Adapted from Deacon J.W. [2] and Griffin D.H. [3]


Glucose
C6
(1)
Sugar alcohols
Polysaccharides
Peptidopolysaccharides
6P-Gluconate
Glucose-6P
6P-Gluconate
Purine
nucleotides
Pentose phosphates
C7
C6
C4
C3
Erythrose-4P
Fructose-6P
C6
(4)
Glyceraldehyde-3P
Dihydroxyacetone-P
C3
Aromatic amino acids
PEP
Penicillins
Cephalosporins
Amino acids
(5)
(5)
Phenolic
compounds
Indole
alkaloids
Pyruvate
C3
Ethanol
Lactic acid
Glycerol
C3
Acetyl CoA
C2
(3)
Lipids
Fatty acids
Malonyl CoA
C3
(2)
HMG CoA
Mevalonate
C6
Isoprenoids
Oxalo-
acetate
C4
Citrate
C6
Steroids
Terpenes
Carotenoids
Aspartate
Fumarate
C4
αKG
C5
(5)
(5)
Amino acids
Amino acids

mycotoxins: this term is usually reserved for toxins secreted by fungi which have invaded feed and foodstuffs.

The word mycotoxin has an interesting derivation. It comes from the Greek roots μυκης, meaning mushroom, and τοξικός, which originally referred to a bow or arrow and by association with arrows tipped with poison came to refer to the poison itself. The root "ochra" in ochratoxin comes from the Greek ὠχρα, meaning yellow or pale yellow, often related to corn.

1.3 Historical Background Fungal contamination of crops has been recognized for centuries. Mildew is mentioned several times in the Bible [5], and in the works of classical authors such as Xenophon [6], Plato [7] and Theophrastus [8]. Opinion was divided as to whether it was divine retribution for wrongdoing, or an accident due to disturbances in the motion of the stars. Pliny [9] claimed that such damage to crops was only caused when there was a full moon. Plato [10] regarded mildew [1] as an evil specific to grain. Lucius Junius [11] advised that certain crops, in particular barley, were better hoed when dry so that they would not be attacked by rust [2]. He also suggested that when the weather was unseasonably cold, the smoke from fires lit between

---

[1] ερύσίβη, mildew or rust in corn.

[2] Rubigo - inis (f): rust, in the agricultural sense. This word hints at a reddish quality (a meaning transferred from the rust of iron) but includes ideas such as blight, mildew, smut and mouldiness).

the vines would protect against fog and mildew [12]. These references give no information as to the effects on animals or humans of consumption of mouldy crops, and probably referred to a whole spectrum of fungal infections.

In 1928, Larsen linked kidney disease in swine with consumption of mouldy rye [13] and over the years "mould nephritis" has been recorded on a regular basis in Danish slaughterhouses [14]. Balkan Endemic Nephropathy (BEN) had been known in Bulgaria and other Balkan countries for years before Ivanov started animal experiments in 1950 to try to shed some light on the causes of the disease [15]. In 1956 Tanchev published the first formal description of BEN, describing the high incidence of renal disease in northwestern Bulgaria [16]. The first report of OTA, together with its structure and physical and chemical properties, is found in a letter to Nature in March 1965 from van der Merwe *et al.* [17]. They described the laboratory production of OTA using strains of *Aspergillus ochraceus* grown on moist corn meal, and noted that consumption of the mouldy meal caused rapid death in ducklings, mice and rats. Since that time many reports have been published, confirming the association of OTA contamination of food and animal feeds with the incidence of BEN, and noting the risks to human and animal health in many countries, including Germany, Poland, Romania, the former Yugoslavia, the U.S.A. and Canada. Other studies have focused on the distribution of ingested OTA, and its metabolic handling. In spite of more than thirty years work on this mycotoxin, its mode of action is only incompletely understood and it continues to pose a risk to human and animal health.

1.4 Factors which promote fungal infections in crops Fungal infections are a normal occurrence in crops but the profile and severity vary from year to year according to field and storage conditions, with complex interactions between temperature, humidity and availability of trace elements such as zinc, iron and copper. The presence of debris in stored grain interferes with drying, and damaged and immature seed is more susceptible to fungal damage. Crops infected by fungi deteriorate in nutritional quality and often become discoloured. There may be "hot spots" in stored grain, with a rise in temperature and a fall in pH [18]. The crops may smell mouldy and animals may find their feed less palatable.

1.5 Ochragenic Fungi Of the fungi which produce OTA, *Aspergillus* species tend to predominate at lower moisture levels (16% humidity) and a higher temperature range (8 - 37° Celsius, optimum for OTA production being 31 - 37°), whereas *Penicillium* species predominate at higher moisture levels (20% humidity) but lower temperatures (range 0 - 31° with maximum OTA production at 16 - 24° C) [14]. Sometimes fungi produce more than one mycotoxin, *e.g.* certain strains of *Aspergillus ochraceus* can produce both penicillic acid and OTA; the balance between the two depends on temperature [19] and humidity [20], lower temperature and moisture favouring production of penicillic acid. One laboratory study [21] comparing the mycotoxins produced by *A. alutaceus* (formerly *A. ochraceus)* and *P. verrucosum* growing on oilseeds (soybeans and peanuts) or grain crops (corn and wheat) showed that *A. alutaceus*

growing on oilseeds produced more OTA than it did when growing on grain, whereas *P. verrucosum* produced more OTA when it was growing on grain rather than oilseeds. Ochratoxin B, the non-toxic dechloro form, was only produced by *A. ochraceus* on oilseeds (particularly soybean) and another related mycotoxin, citrinin, was only found in this study when *P. verrucosum* grew on corn or wheat. Another factor influencing fungal growth is aeration. Fungi are aerobic organisms and alterations in levels of atmospheric gases, such as might be found in bulk storage conditions, affect the balance of metabolic processes. One study demonstrated relatively high levels of OTA production in a static submerged culture of *A. ochraceus*, but no toxin production in an aerated culture under otherwise identical conditions [22]. Table 1.1 lists species of storage fungi known to produce ochratoxins [23].

Table 1.1

Species of storage fungi known to produce Ochratoxin A

Aspergillus	*Penicillium*
alutaceus / ochraceus [b]	*verrucosum* [a b]
ostianus	*cyclopium* [a]
melleus	*chrysogenum*
petrakii	*commune*
sclerotium	*palitans*
sulphureus	*purpurescens*
alliacius	*variable*

[a] Common in western Canada
[b] Also produces citrinin
Taken from Marquadt, Frohlich and Abramson [23]

2. OCHRATOXIN A

2.1 Structure and Characteristics of Ochratoxin A. OTA is described chemically as 7-carboxy - 5-chloro - 8-hydroxy - 3,4-dihydro - 3R methylisocoumarin linked through the carboxyl group to L phenylalanine. The structure of OTA is shown in Figure 2.1. Other members of the ochratoxin group include the less toxic Ochratoxin B, which lacks the chlorine atom at position 5; Ochratoxin C, the ethyl ester, which appears to be just as toxic as OTA; Ochratoxin α, the non-toxic hydrolysis product of OTA, which lacks the phenylalanine moiety; and the hydroxylated metabolites, (4R) and (4S) 4-hydroxy OTA, and 10-hydroxy OTA. OTA, and rarely, Ochratoxin B, occur naturally in foods of plant origin. The hydroxy metabolites and Ochratoxin α have been found in urine of animals which have ingested OTA [24].

OTA is a colourless, crystalline compound of molecular weight 403.8. When obtained by crystallization from benzene, it has a melting point of approximately 90°C, but with drying at 60° for 1 hour, benzene of crystallization volatilizes and the melting point is then about 170°. It is soluble in chloroform, xylene, benzene, methanol and ethanol, and aqueous solutions at alkaline pH. The UV absorption spectrum varies with pH and solvent polarity: in ethanol, maxima are present at 213 nm (ϵ = 36.8 mM^{-1}) and 332 nm (ϵ = 6.4 mM^{-1}). In methanol, with excitation at 340 nm, the fluorescence emission maximum occurs at 465 nm [25].

The pK_a of the phenolic hydroxyl group at position 8 of OTA was identified as 7.1 by Pitout [26] who observed the shift in absorbance of OTA in phosphate buffer

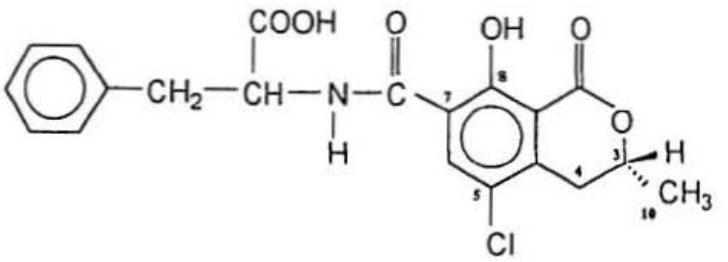


Figure 2.1 The structure of Ochratoxin A

with changing pH. Chu [27] used OTA in KCl/HCl solution and found the pK_a of this group to be 7.04. On the other hand, Galtier *et al.* reported two constants, pK_1 at 6.75 and pK_2 at 10.25, which they attributed to the carboxyl and phenolic groups respectively [28].

2.2 Biosynthesis of OTA. The mycotoxin which is the subject of this thesis is a secondary metabolite of strains of various species of *Aspergillus* and *Penicillium.* The biosynthesis of OTA was studied using [^{14}C]- and [^{13}C]-labeled precursors. It was shown that [^{14}C]-phenylalanine labelled at C_1 was incorporated into OTA by cultures of *A. ochraceus* [29]. Hydrolysis of the labeled OTA with 6N hydrochloric acid gave the isocoumarin moiety and L-phenylalanine. All of the [^{14}C] label was found in the amino acid. It was then shown that the isocoumarin moiety is formed *via* the acetate-malonate pathway from one acetate and four malonate units, and the carbonyl carbon atom (C_{12}) is derived from the C_1 pool [30]. Incubation of *A. ochraceus* in a medium containing [^{36}Cl]-labeled sodium chloride resulted in the incorporation of 0.75% of [^{35}Cl] into OTA [31], but it is not known at what stage the chlorine atom is introduced. An outline of the biosynthesis of OTA is shown in Figure 2.2 [32,33].

2.3 Crops at risk from ochragenic fungi Ochragenic fungi can infect a variety of crops including maize [34], wheat [35] and barley [36], rye [13,37] and sorghum [38], oil seeds, beans [39] and rice [40].

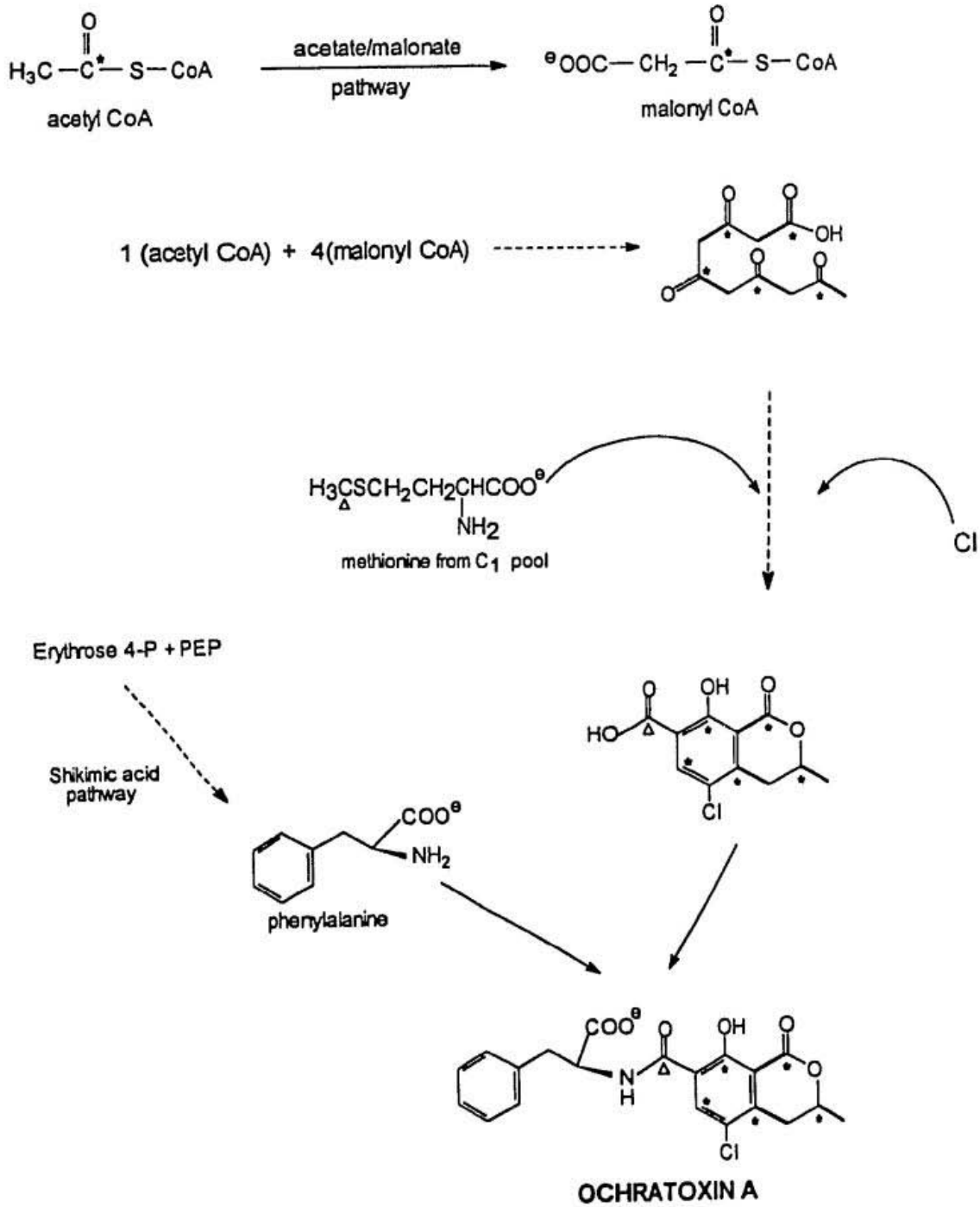


Figure 2.2 Biosynthesis of Ochratoxin A
Adapted from Yamazaki *et al.* [32] and Vlegaar and Steyn [33]

2.4 Ochratoxin A contamination of foodstuffs Sampling of foodstuffs is undertaken in two ways: either at random, or specifically directed towards clearly suspect items. The incidence and levels of OTA contamination reported are therefore likely to differ depending on sample selection. Tables 2.1 and 2.2 give examples of recent occurrences of OTA contamination in foods of plant and animal origin, with the criteria for sample collection made clear whenever possible. It is not possible from appearance alone to be certain that food products are free of contamination: if pigs or chickens are given feed contaminated with OTA, they may not necessarily exhibit symptoms of disease and there may be no obvious macroscopic lesions which would lead to rejection by health inspectors [41]. In spite of this, meat and meat products from these animals can contain measurable amounts of the toxin [42].

2.5 Stability of Ochratoxin A OTA is remarkably stable whether in cereals, beans or animal tissue, even after prolonged storage, processing or cooking [43,44]. Cleaning and milling of wheat and barley did not remove OTA in naturally contaminated samples [36]. Ammoniation (2% NH_3 at 45° for 6 weeks) effectively decomposed OTA without affecting the nutritive value of the grain, but rats found the grain unpalatable [45]. Although γ irradiation may have some application in food processing to reduce OTA levels in meat products [46], protection from the toxic effects of OTA is best achieved by preventing the damp storage conditions which favour its production [18,19]. In Denmark, only in normal or dry years can the level of OTA contamination in cereals be kept below 10 μg/kg [47].

Table 2.1

Recent* instances of natural occurrence of OTA in plant products

Product	Country	Incidence	[OTA](ng/g)	Ref.
Maize (1984 -1990)				
(from area with endemic human nephropathy)	Bulgaria	89/151	0.2 - 1418	[48]
(from non-endemic area)	Bulgaria	14/113	0.2 - 235	[48]
Beans (1984 -1990)				
(from endemic area)	Bulgaria	73/147	0.05 - 264	[48]
(from non-endemic area)	Bulgaria	13/113	0.20 - 285	[48]
Wheat and barley	Canada	5/440	10 - 51	[49]
Feeds and feed grain (suspect)	Canada	4/51	48 - 5,900	[50]
Beer	France	4/37	5 - 110	[51]
Barley	Germany	10/68	0.1 - 206	[52]
Wheat	Germany	8/64	0.1 - 137	[52]
Roasted coffee	Japan	5/68	3.2 - 17	[53]
Olives and olive oil	Morocco	8/163	40 - 80	[54]
Cereals	Poland	6/100	up to 1200	[55]
Wheat and rye flour	Poland	48/215	mean 4370	[56]
Corn oil	U.K.	2/7	11 - 50	[57]
Breakfast cereals	U.K.	12/243	< 10 - 50	[57]
Beans (endemic area)	Yugoslavia	4/50	17 - 53	[58]

* 1980 onwards

Table 2.2

Recent* instances of natural occurrence of OTA in meat and meat products

Product	Country	Incidence	[OTA](ng/g)	Ref.
Pig kidneys (all)	Denmark (1986 - 1990)	0.015% of 80×10^6	>25	[59]
Pig kidneys (nephropathic)	Denmark (1986 - 1990)	18.4% of 65.2×10^3	>25	[59]
Black pudding	Germany	20/125	0.1 - 3.4	[60]
Liver sausage	Germany	19/100	0.1 - 3.4	[60]
Pig kidneys	U.K.	43/278	1 - 44	[61]
Meat products	U.K.	7/33	up to 4	[62]
Smoked meats and sausages	Yugoslavia	12 - 29%	10 - 920	[63]

* 1980 onwards

2.6 Toxic Effects of Ochratoxin A. Toxicity of OTA depends on the presence of the phenylalanine moiety, and on the chlorine atom at position 5 (Figure 2.1). Ochratoxin B which lacks the Cl is much less toxic than OTA, and Ochratoxin α is not toxic. Ochratoxin C, the ethyl ester, which has not been shown to occur under natural conditions, is as toxic as the parent OTA.

OTA, the most abundant ochratoxin, is toxic to a wide variety of organisms from bacteria to mammals [64]. The acute toxicity to some species is listed in Table 2.3 [65,66]. OTA is nephrotoxic, hepatotoxic, carcinogenic, mutagenic, teratogenic and immunosuppressive [67]. It disturbs calcium homeostasis [68] and damages membranes by lipid peroxidation [69]. After oral ingestion, OTA has a long biological half-life in mammals, ranging from 39 hours in the mouse to 120 hours in the rat and 510 hours in the Rhesus monkey [70]. This is a consequence of the strong binding of OTA to plasma proteins which in mammals has been shown to be greater than 99%. Chu [27] used equilibrium dialysis and gel filtration studies to show that 2 moles of OTA were bound to 1 mole of bovine serum albumin. Stojković *et al.* [71] have proposed that OTA binds in a non-specific low affinity manner to serum albumin, but with much higher affinity (by several orders of 10) to a group of low molecular weight (approximately 20 kD) serum macromolecules. Albumin is present in the serum in high concentration, and so acts as a large mobile store of OTA. The serum concentration of the 20kD molecules is much lower than that of albumin, but specific binding of OTA to these smaller macromolecules would allow passage of bound OTA through the glomerular membrane, thus delivering OTA

Table 2.3

Acute toxicity of orally ingested Ochratoxin A

Species	LD_{50} mg/kg
mouse	46.0 - 58.3
rat	20.0 - 30.3
rat neonate	3.9
dog	0.2
pig	1.0
chicken	3.3

Based on literature compilations by Harwig *et al.* [65] and the National Institute of Occupational Safety and Health [66]

to its prime target organ, the kidney. OTA specifically damages the proximal tubule of the kidney cortex, impairing the anion transport system [41]. A measure of this damage is given by the T_{mPAH} / C_{in} ratio, which is reduced in OTA toxicity. T_{mPAH} represents tubular excretion of a typical anion, *para* aminohippuric acid, and C_{in} represents glomerular filtration, using inulin as a standard compound which in normal renal function is found in the filtrate. There is glycosuria, proteinuria and decreased ability to concentrate urine. Renal gluconeogenesis is impaired due to inhibition of phosphoenolpyruvate carboxykinase [72]. Inhibition of protein synthesis by OTA is seen in bacteria as well as higher organisms. Phenylalanyl tRNA synthetase is blocked by the OTA molecule; protein translation and synthesis are impaired [73,74]. The mechanism(s) whereby OTA depresses immune function are not known, but both the inhibition of protein synthesis and the immunosuppression caused by OTA are prevented or at least minimized by the concurrent administration of phenylalanine [75,76].

2.7 Geography of Ochratoxin A contamination Health consequences for humans are clearly seen in the Balkan countries Bulgaria (Vratza District), Romania (Banat region) and the former Yugoslavia (particularly districts in Serbia, Bosnia and Croatia, notably near the city of Slavonski Brod) [77,78,79] (Figure 2.3). Affected areas are located along tributaries of the Danube; most are at relatively low altitude (100 - 200 m) and are often flooded. Significant levels of OTA have also been detected in food of plant and animal origin in other countries (Tables 2.1, 2.2).

Figure 2.3 The Balkans and the river Danube

Adapted, with permission, from American Scientist [80]

2.8 Human Exposure and evaluation of risk It is clear that humans in many countries are exposed to OTA. Table 2.4 gives details of human serum OTA levels reported in recent studies in different countries including Canada. OTA has also been found in trace amounts in human milk in Germany [81] and Italy [82], and in human urine in Bulgaria [83]. Balkan endemic nephropathy, mentioned earlier in this review, is strongly linked to OTA contamination of crops [39,84]. Other mycotoxins such as citrinin may be also be involved, exerting synergistic effects with OTA [48].

Kuiper-Goodman and Scott [85] using data from the National Toxicology Program [86] study of carcinogenesis in rats applied a safety factor and arrived at estimates of 0.2 - 4.2 ng/kg body weight as the tolerable daily limit of consumption in humans. This is lower than levels identified as virtually safe with respect to nephropathic and teratogenic effects; in 1991 the Food and Agriculture / World Health Organization suggested a provisional weekly tolerable dose of 112 ng/kg body weight [87].

Table 2.4

Prevalence of OTA in human serum

Year(s)	Country	Detection limit ng/g	Positive samples	Range ng/g	Ref.
1984 - 1990	Bulgaria: individuals with B.e.n. or u.t.t.	1.0 - 2.	28/105	2.0 - 39.1	[88]
1984 - 1990	Bulgaria: healthy relatives of the above	1.0 - 2.0	18/119	2.0 - 20.0	[88]
1984 - 1990	Bulgaria: healthy people in affected villages	1.0 - 2.0	14/116	2.0 - 13.0	[88]
1984 - 1990	Bulgaria: healthy people in non-endemic area	1.0 - 2.0	9/125	2.0 - 15.0	[88]
1990	Canada: subjects with renal disease	< 0.5	28/69	< 0.5 - >20.0	[89]
1990	Canada: subjects without renal disease	< 0.5	35/90	< 0.5 - >20.0	[89]
1986 - 1988	Denmark	0.1	78/144	ND - 13.2	[90]
reported 1991	France: rural	0.2	22.0%	0.1 - 6.0	[91]
reported 1991	France: urban	0.2	20.15%	0.1 - 1.3	[91]
reported 1986	Germany: Bayern	0.1	56.5%	0.1 - 14.4	[90]
1983 - 1984	Poland	5.0	77/1065	ND - 40.0	[56]
1989	Sweden: urban, mainland	0.3	4.0 - 8.0%	0.3 - 6.8	[90]
1989	Sweden: Visby - island community	0.3	31.0%	0.3 - 6.0	[90]
1981 - 1989	Yugoslavia	5.0	200/13797	5.0 - 100.0	[92]

B.e.n. denotes Balkan endemic nephropathy, u.t.t., urinary tract tumours, ND - not detected

3. ABSORPTION OF FOREIGN COMPOUNDS [93]

3.1 Routes of absorption There are three main barriers which separate higher animals from their environment: the skin, the lungs and the gastrointestinal tract. To date, there have been no reports of OTA assimilation *via* the skin or the lungs, although in principle absorption by either of these routes might be feasible. This account will only consider absorption *via* the gastrointestinal tract. The major function of the digestive tract is to absorb nutrients and water, but non-nutritive compounds such as food additives, preservatives, drugs and contaminants are often ingested and absorbed along with the diet.

3.2 Toxic compounds in foods Toxic compounds often contaminate animal and human foodstuffs, sometimes naturally, sometimes by accident and sometimes by deliberate action [94]. A few examples will be given to illustrate some of the various ways in which toxins find their way into foodstuffs. Some plants naturally contain toxic compounds, *e.g.* cassava, a tuberous root vegetable found in South America, contains cyanide. Fungal infections of crops can result in contamination of foodstuffs with toxic metabolites: the most well known are the aflatoxins which are frequently found in mouldy corn and peanuts. Aflatoxin M_1 has been identified in the milk of cows whose feed contained aflatoxin B_1. Aquatic species such as shellfish may contain natural toxins originating from the plankton they consume. Contamination of foodstuffs can occur accidentally, as with pesticide residues, or

deliberately, *e.g.* the toluidine-contaminated oil sold in Madrid in the early 1980s [95]. Man-made pollution often contaminates food: industrial effluent discharged into some lakes and rivers has resulted in a dangerous mix of organic chemicals and heavy metals whose effects are seen in malformations in fish, and birth defects and neurological disease in humans [96,97]. Carcinogenic polycyclic aromatic hydrocarbons are ubiquitous contaminants wherever petroleum products are used. The surface layers of foods which have been smoked, or cooked at high temperatures over a barbecue contain a number of carcinogenic substances. Nitrates and nitrites used in preservation of meat products are ultimately carcinogenic, so while offering protection against bacterial spoilage they probably pose a longer-term hazard to health. Although generally speaking our food supply is safe and nutritious, these examples show that xenobiotics can and do contaminate both human and animal foodstuffs.

3.3 Absorption: general principles [98,99] Mechanisms of absorption vary with the chemical characteristics of each compound, in particular with size and lipophilicity. The majority of toxicants cross membranes by simple diffusion. Small hydrophilic molecules such as ethanol and cyanide pass through aqueous pores in membranes, and are rapidly absorbed from the stomach and intestine, but most toxic substances are larger organic molecules with differing degrees of lipid solubility. The more lipid-soluble a compound is, the more readily it will cross lipid membranes.

3.3.1 Weak acids and bases Many chemicals are weak acids or bases which ionize in solution according to their pK and the prevailing pH. This concept is expressed by the Henderson-Hasselbalch equation:

for acids: $pK_a - pH = \log$ [non-ionized] / [ionized]

and for bases: $pK_a - pH = \log$ [ionized] / [non-ionized]

By definition, when the pH is at the pK value, the non-ionized form of a weak acid or base is present in the same concentration as the ionized form. Depending on how far is the prevailing pH below the pK value, a weak acid will be more or less protonated and non-ionized; as the pH rises above its pK_a it will become further ionized and more soluble in aqueous media. The non-ionized form of weak acids and bases is to some extent lipid soluble, allowing it to diffuse across lipid membranes. We have not been able to find any reports concerning the solubility of OTA in lipids, but given its solubility in organic solvents, it is likely that it is at least partially soluble.

A weak acid such as benzoic acid (pK_a 4.0) diffuses more rapidly across a membrane if the prevailing pH is acidic, whereas a weak base such as aniline (pK_a 5.0) which is non-ionized above its pK_a diffuses more readily from an alkaline medium. Even so, because absorption is a dynamic process, if only 1% of a compound is non-ionized at a a given pH (*e.g.* benzoic acid at pH 6.0) some of that 1% will diffuse across the lipid membrane. If the compound is removed from the intracellular side of the membrane, a concentration gradient will exist and diffusion across the membrane will continue. The extent to which simple diffusion will take

place depends on surface area, permeability of the membrane, the prevailing pH and the physical characteristics of the compound. Because the small intestine has a large surface area, benzoic acid is well absorbed, even at a pH which might be considered unfavourable [94].

3.3.2 Other factors affecting absorption Gastrointestinal motility and the length of time food stays in various segments of the gut also influence absorption. The presence of fat in a meal delays gastric emptying; dilution of some compounds increases toxicity, probably because increased volume hastens gastric emptying, resulting in more rapid delivery to the duodenum and consequent exposure to a greater absorptive surface [100]. Laxatives may reduce absorption by speeding intestinal transit time [101]; a high fibre diet also speeds the passage of food through the gut. Fibre binds water, and adsorbs important minerals (calcium, iron, magnesium and zinc) and bile salts and lipids, thus reducing their bioavailability for absorption. This may lead to deficiencies of essential minerals and fat-soluble vitamins, and may also reduce absorption of some toxins.

3.3.3 Specialized carrier systems The mammalian gastrointestinal tract has specialized carriers for the absorption of monosaccharides, small peptides and free amino acids. There are also specialized transport systems for the absorption of calcium and ferrous iron. Some toxic substances may be absorbed by these systems, *e.g* lead and cobalt are transported by the calcium and iron transport systems. Most toxins however are absorbed passively.

3.4 The stomach Although small molecules such as water and ethanol are absorbed, and some important digestive processes start in the stomach, it is not primarily an organ of absorption. A striking feature of the stomach is the regulation of pH, which is achieved through neural and hormonal mechanisms. When HCl is secreted in response to a meal, the pH of the gastric juice is $\simeq$ 1.0. Food buffers the luminal pH, which may rise to 4.0 or even 5.0; if the pH in the distal portion of the stomach falls below 3.0, secretion of gastric juice is reduced. If acid reaches the upper small intestine, gastric secretion of HCl stops. Mucus provides a protective layer for the stomach mucosa, creating with basal secretion of bicarbonate a pH gradient between the surface mucosal cells ($\simeq$pH 6.5) and the contents of the lumen. Gastric emptying is regulated according to the composition and osmolarity of the food mixture (known as chyme). Liquids leave the stomach first, followed by carbohydrates, then proteins and lastly fats. Emptying is delayed in response to low pH, and the presence of long-chain fatty acids and their mono- and diglycerides. A mixed meal including fats probably stays in the human stomach for about 4 hours.

3.5 Digestion and absorption in the small intestine The small intestine presents a vast surface over which absorption takes place. It is divided arbitrarily into three segments: the duodenum, the jejunum and the ileum. Like the digestive tube in other regions of the gastrointestinal tract, the wall of the small intestine has four main layers: the serosa, the muscle layer, the submucosa and the mucosa (Figure 3.1).

3.5.1 The mucosa The mucosa will be described in detail because it is the site of absorption. It can be divided into three layers: the muscularis mucosae (a thin sheet of muscle), the lamina propria, and the epithelium. The mucosal epithelium consists of columnar absorptive cells known as enterocytes, with interspersed mucus-secreting goblet cells and endocrine cells. The surface area of the mucosa is made much greater by circular folds (in larger mammals only), by villi (Figure 3.1) and by microvilli (Figure 3.2). The villi project into the lumen at a density of ten to forty per square millimetre. Each absorptive cell has between 3,000 and 7,000 microvilli which make up the brush border seen on light microscopy. In the human, the microvilli are approximately 1 μm long and 0.1 μm broad. Figures 3.3, 3.4 and 3.5 are electron micrographs showing the intestinal epithelium and the microvilli. The plasma membrane of microvilli is a lipid bilayer, containing many specialized proteins such as enzymes, transporters and receptors. The enzymes of the brush border are glycoproteins, with the protein component to a greater or lesser extent embedded into the lipid membrane of the microvillus; the carbohydrate chains protrude into the lumen, forming the glycocalyx (Figures 3.2, 3.5). Next to this is a protective layer of mucus. Barriers to absorption of compounds are discussed in 3.5.11. The basolateral membrane of the absorptive cell is thinner and more permeable than the luminal membrane; passage across it occurs by diffusion.

Figure 3.1 Anatomy of the small intestine

The wall of the mammalian intestine is composed of four concentric layers. Three outer layers - the serosa, the muscularis (consisting of longitudinal and circular muscle strata), and the submucosa - surround the innermost layer, the mucosa. The surface area of the mucosal epithelium is vastly increased by villi and microvilli, to about 300 m^2 in the human. Inside each villus is a dense network of blood and lymph vessels to which nutrients are transported.

From: The Lining of the Small Intestine by Florence Moog [102].

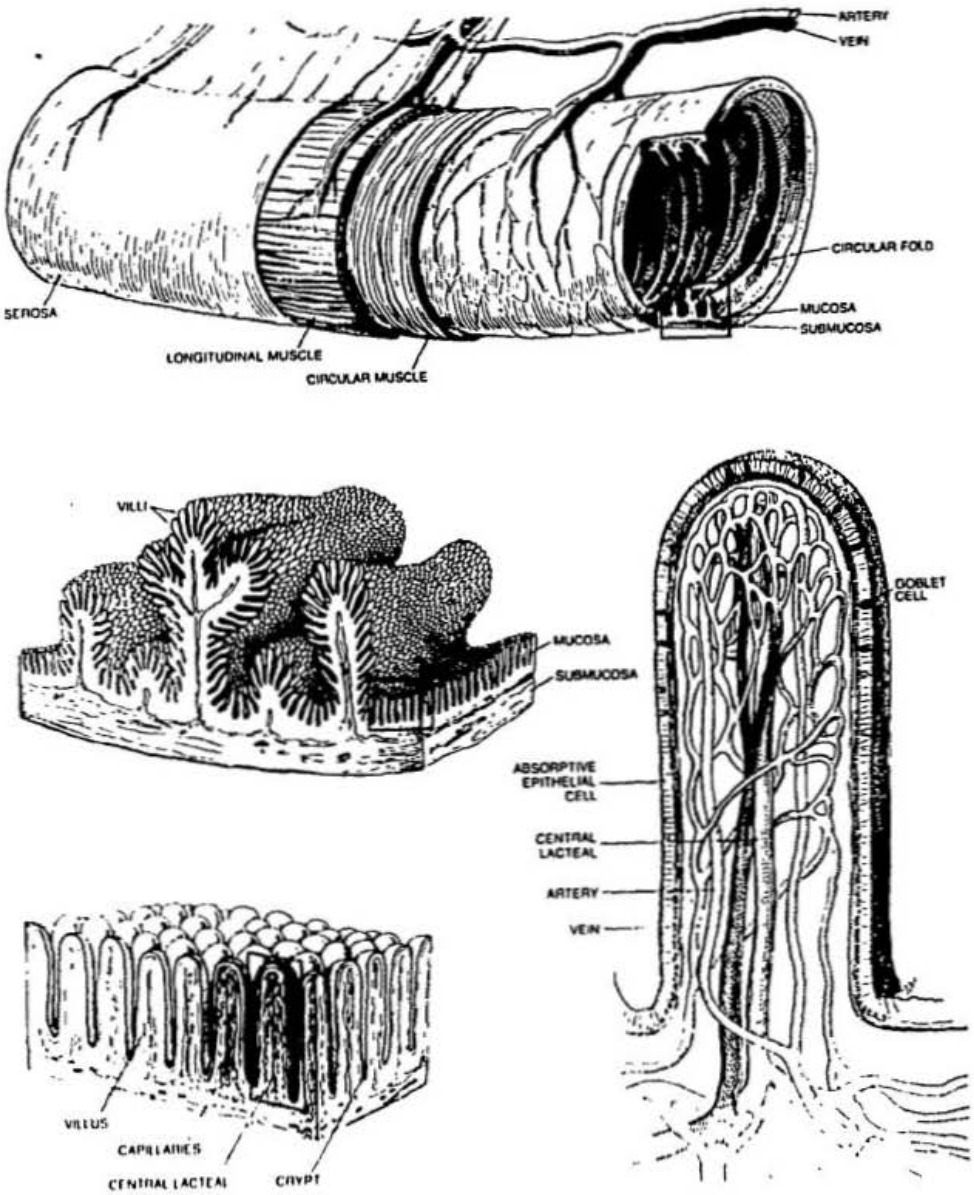

ARTERY
VEIN
CIRCULAR FOLD
SEROSA
MUCOSA
SUBMUCOSA
LONGITUDINAL MUSCLE
CIRCULAR MUSCLE
VILLI
MUCOSA
SUBMUCOSA
GOBLET CELL
ABSORPTIVE EPITHELIAL CELL
CENTRAL LACTEAL
ARTERY
VEIN
VILLUS
CAPILLARIES
CENTRAL LACTEAL
CRYPT

Figure 3.2 Structure of the absorptive cell

This figure shows diagramatically the important structures of the absorptive cell. The glycocalyx is composed of the carbohydrate chains of enzymes embedded in the microvillus membrane. The shape of the microvillus is maintained by protein fibres which run throughout its length; these fibres join with the terminal web, a mesh of fibres running parallel to the luminal surface of the cell.

From: The Lining of the Small Intestine by Florence Moog [102].

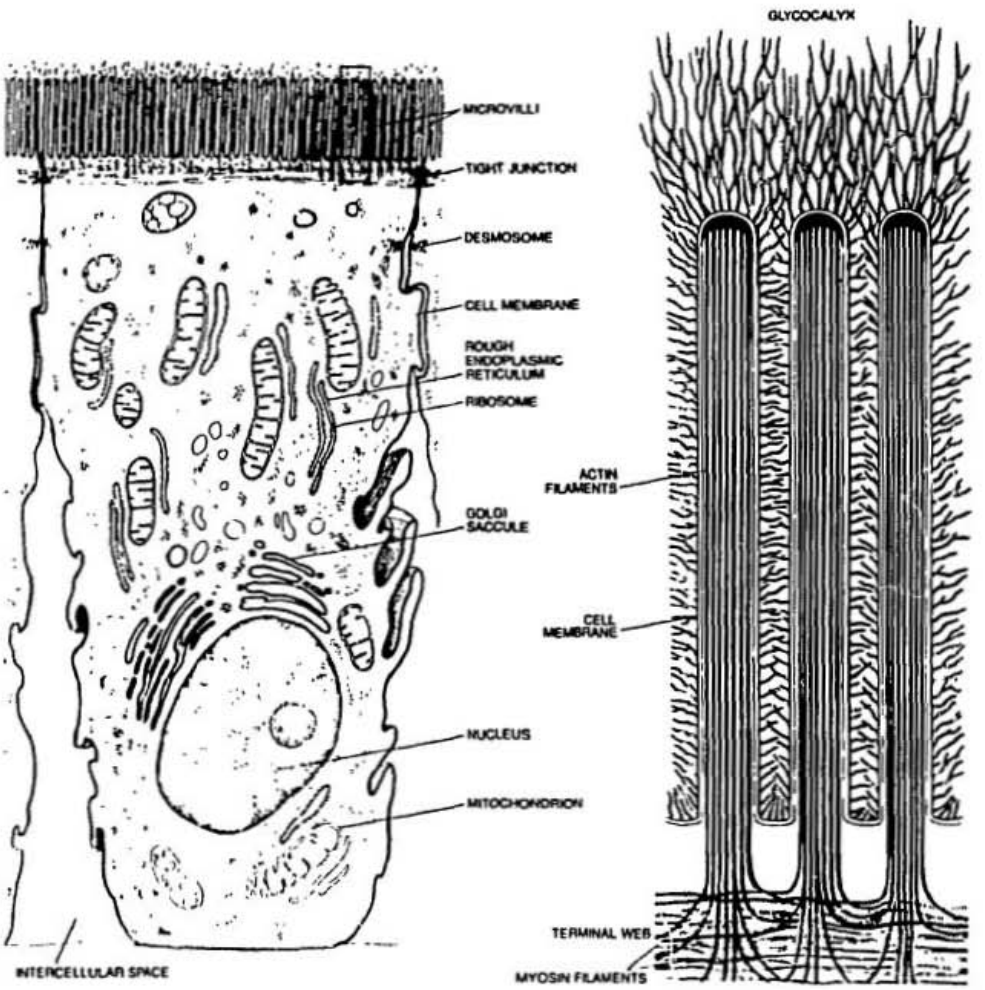

GLYCOCALYX
MICROVILLI
TIGHT JUNCTION
DESMOSOME
CELL MEMBRANE
ROUGH ENDOPLASMIC RETICULUM
RIBOSOME
ACTIN FILAMENTS
GOLGI SACCULE
CELL MEMBRANE
NUCLEUS
MITOCHONDRION
TERMINAL WEB
MYOSIN FILAMENTS
INTERCELLULAR SPACE

Figure 3.3

Electron micrograph of intestinal epithelial cells and a goblet cell

x 5000 The goblet cell **(G)** is packed with mucous secretory granules at the apex. Note the Golgi apparatus between the nucleus and the mucous granules.

By permission, Springer-Verlag New York Inc. [103]

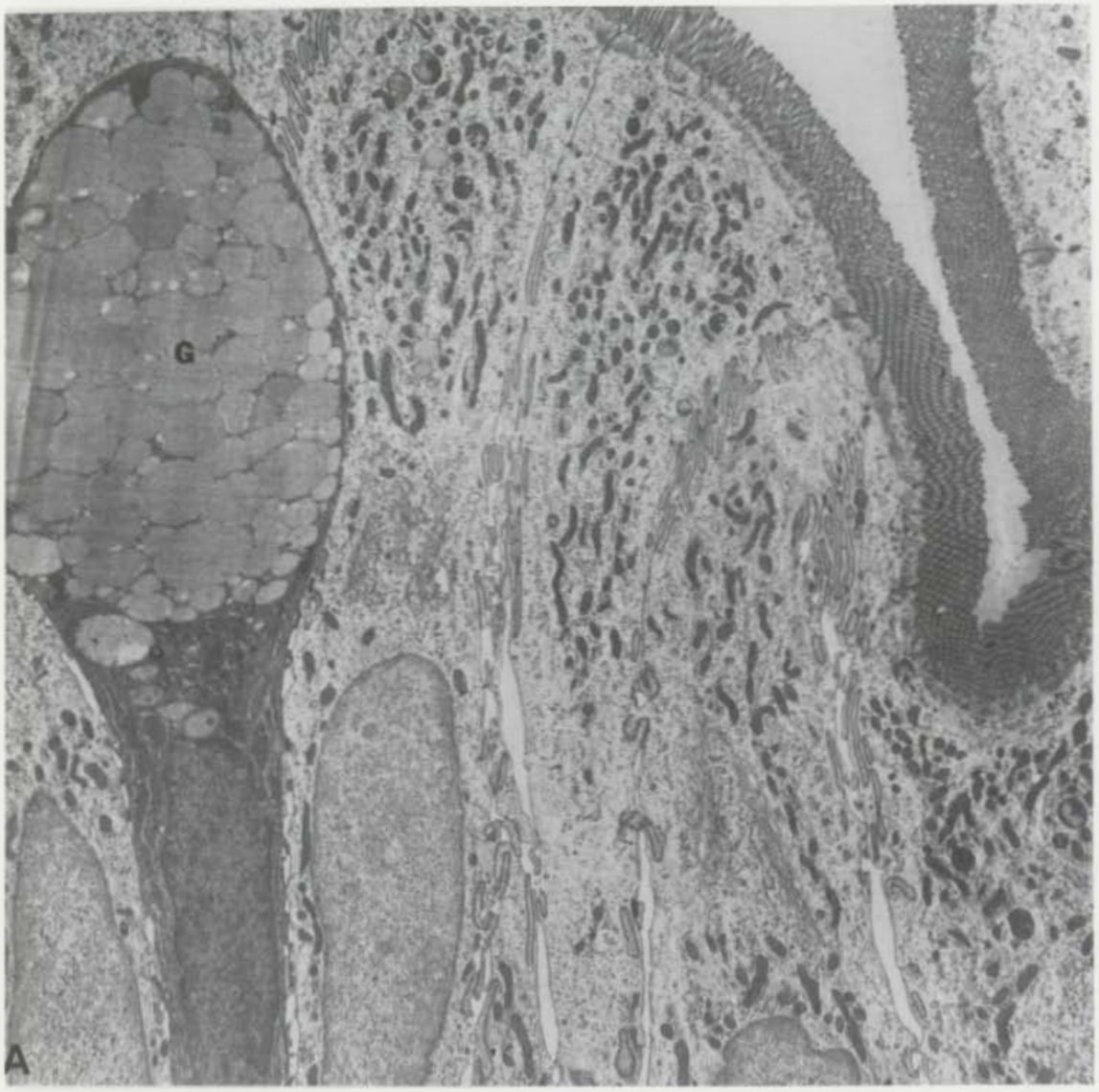

G
A

Figure 3.4

Electron micrograph of epithelial cells from the apical one third of a villus

x 10000 Note the tall columnar epithelial cells, nuclei **N**, brush border **B**, supranuclear Golgi complex **G**, intra-epithelial lymphocyte **I**, terminal web **T**.

By permission, Springer-Verlag New York Inc. [104]

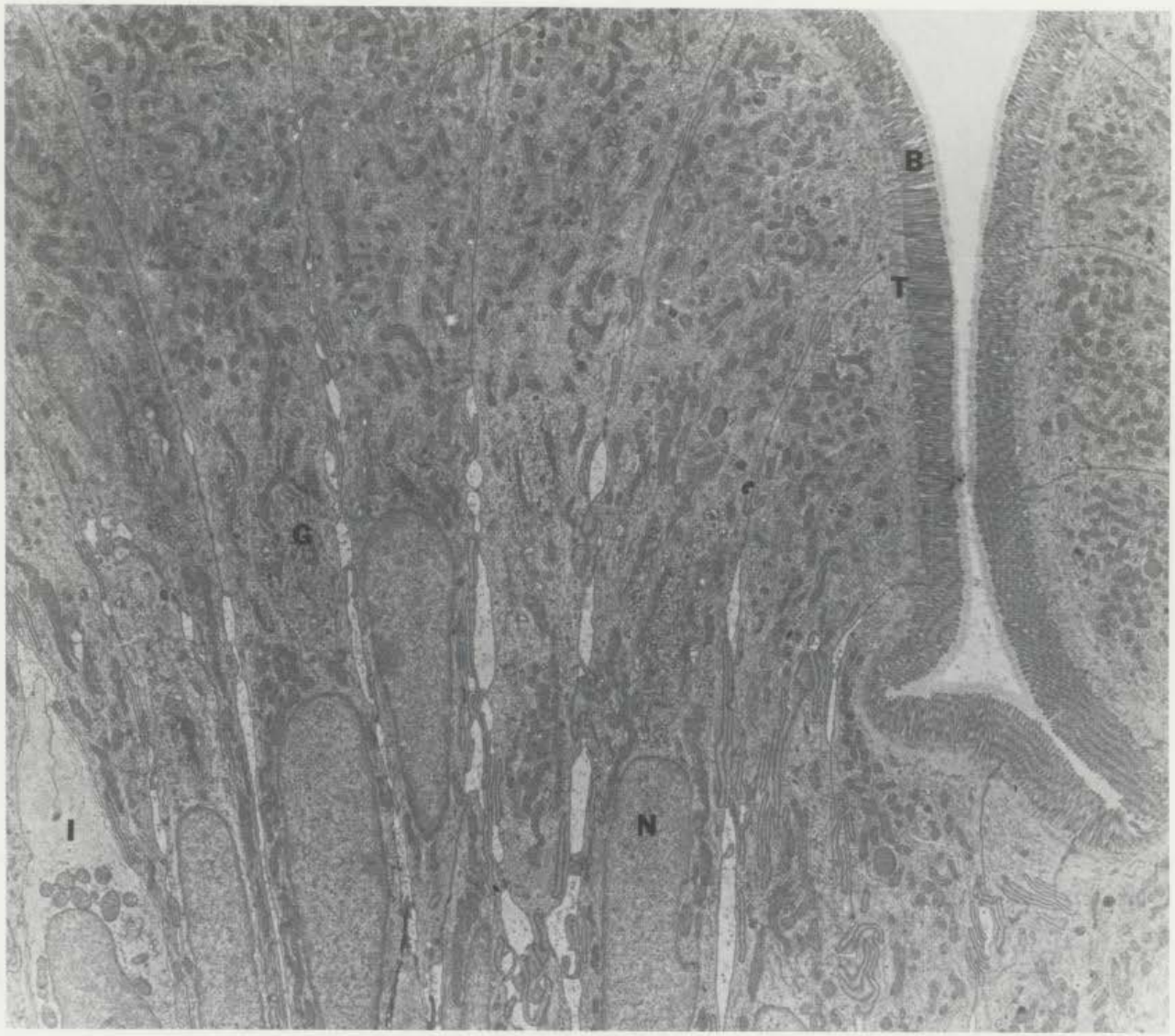
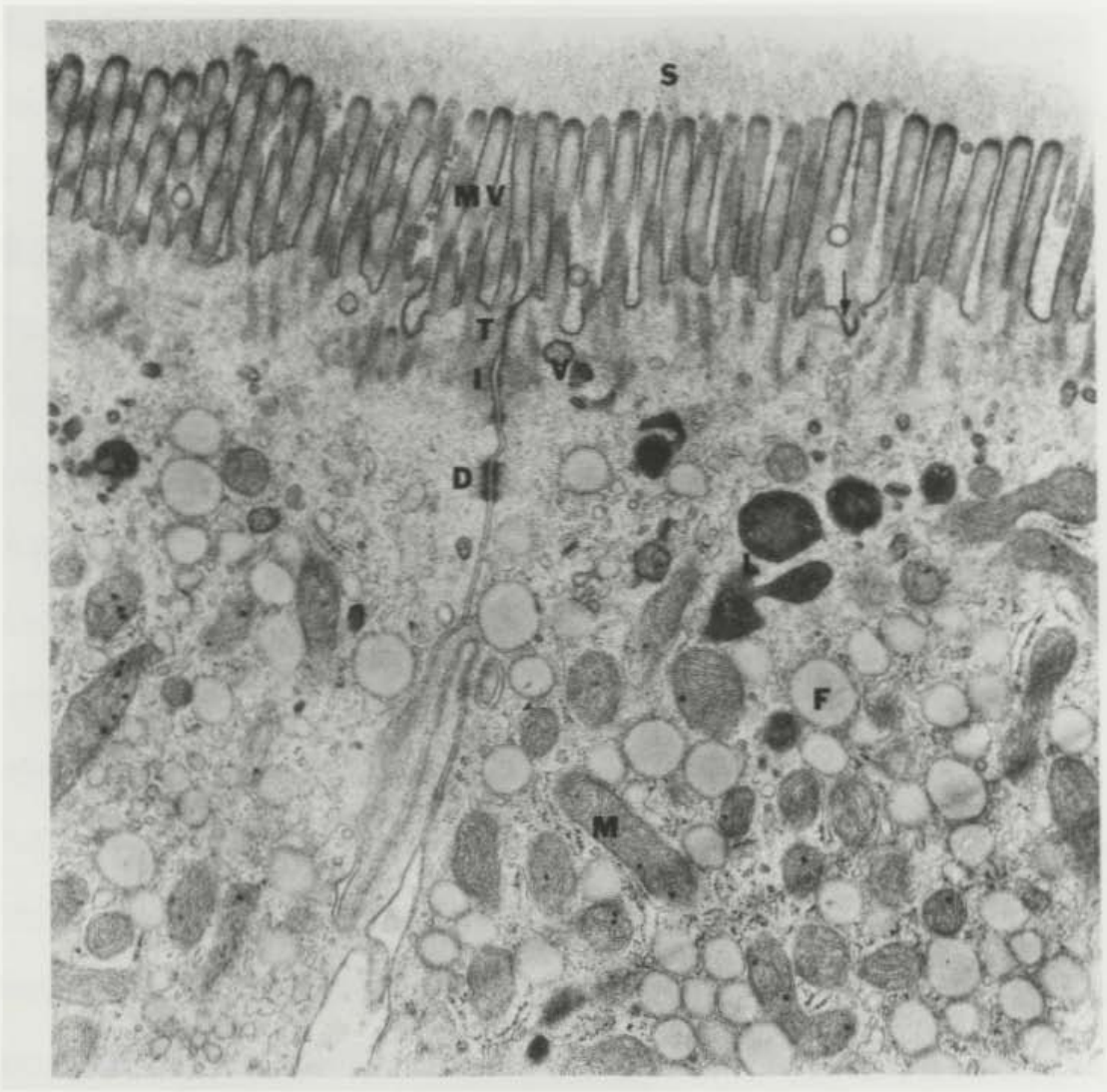

B
T
G
I
N

Figure 3.5

Electron micrograph of the apical portion of two adjacent absorptive cells during fat absorption x 25000 Note the surface coat (glycocalyx) **S**, microvilli **MV**, secretory vesicle **V**, absorbed lipid **F**, mitochondrion **M**, and the apical pit (arrowed) at the base of microvilli, where ferritin but not fat is absorbed.

By permission, J. B. Lippincott and Co. [104]


S
MV
T
I
V
D
F
M

3.5.2 The lamina propria The lamina propria consists mainly of connective tissue which supports the epithelium, and forms the core of each villus. Within the villus is a central lymph collecting vessel, the lacteal, surrounded by a network of capillaries, figure 3.1. Also found in the lamina propria are small lymphocytes and lymphoid nodules. These form part of the GALT system - the "gut-associated lymphoid tissue" - which is a major subdivision of the immunological defences of the body.

3.5.3 Pancreatic Juice Pancreatic juice contains enzymes and electrolytes, notably bicarbonate. When stimulated, the pancreas secretes sufficient bicarbonate to neutralize all the titratable acid reaching the duodenum from the stomach, thus providing a pH suitable for the digestive enzymes active in the lumen.

3.5.4 Digestion and absorption of carbohydrates Complex carbohydrates are hydrolysed enzymatically to simple sugars which are transported by carrier-mediated systems across the cell membrane. From the enterocyte they pass by facilitated or passive diffusion into capillaries of the portal circulation.

3.5.5 Digestion and absorption of proteins The final products of protein digestion are small peptides and free amino acids which are transported across the lipid membrane by specific carrier mechanisms. Four different sodium-dependent systems have been described, specific for the transport of neutral, dibasic and dicarboxylic L-amino acids, and the imino acids (proline and hydroxyproline) and glycine. Cytosolic enzymes degrade the di- and tripeptides to amino acids, which can diffuse passively across the basolateral membrane into the portal blood.

3.5.6 Absorption of vitamins Water-soluble vitamins are absorbed in the jejunum and ileum, by diffusion which may be facilitated or active. Fat-soluble vitamins are primarily absorbed in the proximal small intestine by solubilization with fats in bile salt micelles.

3.5.7 Bile Bile is an alkaline aqueous solution of bile acids which is secreted into the duodenum. Electrolyte concentrations are similar to those in plasma, with the exception that bicarbonate whose concentration in bile may be double that in plasma. Other constituents of bile are phospholipids, cholesterol and small amounts of plasma proteins. Bile may also contain lipids, steroid hormones, end-products of metabolism of drugs and other foreign compounds, and traces of heavy metals such as lead. Conjugated with glycine or taurine, bile acids are ionized at the pH of bile and exist as salts of cations, *e.g.* Na taurocholate. The bile acid pool (about 3 g in adult humans) circulates two or three times per meal and up to twelve times each day, in an enterohepatic circulation. Bile salts are actively reabsorbed in the ileum and carried back to the liver in the portal circulation. The yellow colour of bile is due to bilirubin, a breakdown product of haemoglobin, which is conjugated in the liver with glucuronide and to a lesser extent with sulfate.

3.5.8 The gall bladder In the rat and the whale, bile drains directly into the duodenum, but in most species, including humans, about half of the bile formed in the liver is stored in the gall bladder. Here water and some bicarbonate and chloride are resorbed, and so the pH of gall bladder bile is lower and the

bile salt concentration is higher than that of bile fresh from the liver. Other constituents of bile, including drugs and other foreign compounds are also concentrated in the gall bladder, so that when gall bladder contents are ejected into the duodenum in response to a meal, a bolus of highly concentrated chemicals is delivered. By contrast, in animals which do not have a gall bladder, delivery of bile and any other compounds it contains is a smoother process.

3.5.9 Digestion and absorption of fats Triglyceride digestion is initiated by preduodenal lipase; human gastric lipase (HGL) has a pH optimum of ~ 4.5 - 5.5 [105]. Bile salts activate HGL *in vitro* and it is possible that preduodenal lipases continue to act in the duodenum [106], in cooperation with pancreatic lipase [107]. Peristaltic action in the small intestine promotes emulsification of fats; this is assisted by the presence of monoglycerides, fatty acids, lecithin and protein, and is much more efficient in the duodenum than it is in the stomach. Emulsification provides a large surface area for the action of pancreatic lipase, which can only operate at the oil/water interface. Pure pancreatic lipase is inhibited by bile salts [108], and in order to be active it requires the presence of colipase [109], a small protein which anchors pancreatic lipase to the oil/water interface [110]. *In vitro*, the pH optimum of pancreatic lipase alone is close to 8.0, but in the presence of bile salts and colipase there is a downward shift to more acid values (6.0 - 6.5) [111]. Hydrolysis of triglycerides at positions 1 and 3 by pancreatic lipase yields diglycerides and then the 2-monoglyceride and two fatty acids.

These products of lipolysis are removed from the interface by solubilization in bile salt micelles (see below), allowing the reaction to continue.

3.5.10 Micelles and micellar solubilization of lipids Bile acid molecules are amphipathic: their hydroxyl groups are arranged on one side of the molecule, and the water-insoluble part faces the other way. Above the critical micellar concentration (which varies from ~ 0.7 mM [112] to ~ 12 mM [113] according to the properties of individual bile salts, the ionic composition of the medium, the temperature, pH, and the presence or absence of lipids) bile acids in aqueous solution tend to aggregate to form micelles. The hydrophilic aspect of each molecule faces outward to the aqueous medium, and the nonpolar aspect faces inward. The hydrophobic centre provides a milieu where lipid-soluble compounds such as cholesterol can be carried. Simple micelles containing only bile salts are found *in vitro*, but *in vivo* phospholipids and cholesterol from bile are incorporated, forming mixed micelles, Figure 3.6. Also incorporated into mixed micelles are the products of digestion of dietary fats, and lipid-soluble compounds. In addition to mixed micelles, there may also be other lipid aggregates in the aqueous phase, whose structure will change during the course of absorption and digestion, depending on the relative concentrations of mixed lipids and bile salts [114].

3.5.11 Barriers to the absorption of digestive products There are three barriers that digestion products in the small intestine must cross before they pass into the enterocyte. The first is the unstirred water layer which exists close to the absorptive

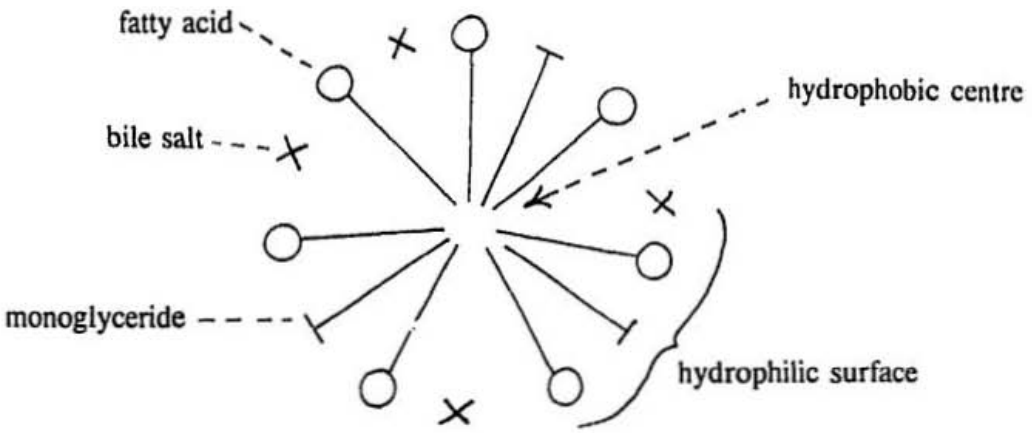


Figure 3.6 The structure of a mixed micelle

surface; the second is the mucus coat covering the brush border; and the third is the lipid membrane and glycocalyx. The first two barriers present little difficulty to water-soluble compounds. Micellar solubilization enhances solubility of lipids and lipid-soluble compounds in the aqueous phase by a factor of about 10^3, enabling them to cross the unstirred water layer. This layer is thought to be between 100 - 200 μm thick in highly stirred systems *in vitro*, and as much as 400 - 800 μm in the intestine perfused *in vivo* [115]; confusion has arisen because some authors have referred to "functional thickness" of the unstirred water layer when it seems they were including the layer of mucus overlying the brush border [116]. Mucus is composed of mucopolysaccharide which acts as an ampholyte. It possesses many ionizable groups of different pK_a values and is therefore able to maintain the acidic microclimate of the mucosal membrane in spite of the luminal pH, from which it may differ by > 1.5 pH units. In the jejunum, *e.g.*, the luminal pH is 6.8 - 7.2, while that of the mucosal surface is about 5.4 [117]. The lower pH close to the mucosal membrane enhances both the diffusion of micelles and their dissociation, so that micellar contents carried across the water and mucus layers are then released in monomeric form, adjacent to the absorptive surface [118]. Short- and medium-chain fatty acids and their triglycerides are sufficiently soluble in the aqueous phase that they can diffuse across the unstirred layer without being associated with micelles, but for the long-chain fatty acids which make up 90% of ingested triglycerides, micellar solubilization is essential. The third barrier to digestive products, the

mucosal membrane, is a phospholipid bilayer with a high protein to lipid ratio ($\simeq$ 1.7:1). Some of its many proteins are enzymes; others act as specific carriers for the products of carbohydrate and protein digestion. Lipid and lipid-soluble molecules move through the membrane by passive diffusion. In comparison with many other biological membranes, the mucosal membrane is less fluid [119].

3.5.12 Water and electrolyte absorption Water transport in the intestine occurs passively. It varies according to the region, depending on the blood-lumen osmotic pressure difference and on solute transport. Sodium is conserved very effectively throughout the intestine by a number of mechanisms. Potassium is absorbed mainly in the jejunum, chloride in the jejunum and ileum. Bicarbonate is secreted into the duodenum; in the jejunum, it is rapidly reabsorbed while H^+ ions are secreted, giving rise to the acid microclimate close to the jejunal mucosa. In the ileum, bicarbonate is secreted in exchange for chloride ions. Calcium is absorbed in the duodenum and jejunum; magnesium is absorbed throughout the small intestine, mostly proximally. About 90% of the fluid which enters the human large intestine is absorbed. Sodium and chloride are conserved, while bicarbonate and usually potassium are secreted.

3.6 Pre-systemic metabolism: Phase I and Phase II reactions [120] The liver is the major site of xenobiotic biotransformation but biotransformation also occurs to a significant extent in the mucosal tissue of the gastrointestinal tract. Biotransformations are classified as phase I or phase II reactions. Phase I reactions

generally alter the molecular structure of compounds by cytochrome P450-mediated oxidation or hydrolysis, whereas phase II reactions involve conjugation with groups such as glucuronide, sulfate and glutathione. Many fat-soluble compounds are to some extent resistant to biotransformation. Metabolized compounds are usually transported in the portal vein to the liver where further biotransformation can take place, but a proportion may be exported directly from the intestinal tissue into the gut lumen. The metabolites formed are generally more water-soluble than the parent compounds; they bind less easily to plasma and tissue proteins, and are usually found in lower concentrations in body storage depots. Biotransformation usually results in metabolites which are less toxic than the parent compound, but occasionally more toxic compounds are formed. Increased aqueous solubility facilitates excretion in bile or urine; compounds excreted in bile may then be subject to enterohepatic circulation.

3.6.1 Modification of intestinal biotransformation Many dietary components can alter the extent of biotransformation: indoles present in brussel sprouts and excess cholesterol have been shown to increase the activity of enzymes of oxidation and conjugation; high intake of unsaturated fats decreases the activity of enzymes of biotransformation, as does deficiency of selenium or cholesterol. Glucuronosyl-transferase activity can be induced by oral administration of inducing agents such as phenobarbital or 3-methylcholanthrene.

3.6.2 Gastrointestinal flora [121] Both anatomically and functionally, the wall of the gastrointestinal tract is part of an animal's external surface. No part of the gut

is normally sterile. Acidity keeps the stomach nearly free of bacteria, but the intestine contains large numbers of microbial organisms of which 95% are strictly anaerobic. Bacterial counts in the human intestine range from 10^2 - 10^3/mL in the proximal small intestine to 10^{11}/mL in the ileum and colon. There is usually a balance between the various bacterial species, but this can be upset, *e.g.* by antibiotic therapy, which can cause serious side effects. Foreign compounds are metabolized both by body tissues and by gut flora. Cytochrome P450-mediated oxidation reactions require oxygen, but most intestinal bacteria are anaerobic. Mammalian bio-transformations are generally oxidation and conjugation reactions, whereas reactions carried out by intestinal bacteria are mainly reductions and hydrolyses.

3.7 Intracellular processing of lipid digestion products: esterification of fatty acids Within the enterocyte, long chain fatty acids are re-esterified into triglycerides. A fatty acid-binding protein (FABP) [122] which preferentially binds long-chain unsaturated fatty acids plays a major role in transporting fatty acids to the endoplasmic reticulum where re-esterification takes place [123]. It is probable that binding to FABP is necessary for re-esterification. Medium-chain fatty acids (smaller than 12 carbons) do not bind with FABP which might explain why they are largely absorbed in the unesterified form *via* the portal system [124]. Two metabolic pathways are involved in the synthesis of triglycerides in the enterocyte, the monoglyceride pathway and the α-glycerophosphate pathway. The monoglyceride

route is the major pathway: activated fatty acids are added to monoglyceride in the smooth endoplasmic reticulum, in reactions catalyzed by acyl CoA acyltransferases. The glycerophosphate pathway, which is associated with the rough endoplasmic reticulum, normally plays a minor role in triglyceride handling, probably because in energy terms the monoglyceride pathway is more efficient, requiring four high energy phosphate bonds rather than the ten needed by the glycerophosphate pathway [124]. Based on studies using intestinal microsomes and intestinal slices, some authors have proposed that the first reaction in the glycerophosphate pathway conversion of glycerol 3-phosphate to phosphatidic acid, is inhibited by high levels of monoglyceride [125]. Studies using everted sacs of rat intestinal mucosa do not support this theory [126]. During fasting, when monoglyceride is not present in the gut lumen, the glycerophosphate pathway becomes more important: endogenous fatty acids, and glycerol 3-phosphate derived from intracellular glucose metabolism are used to synthesize glycerophospholipids. During fat absorption, triglyceride droplets, often containing esterified cholesterol, accumulate within the smooth endoplasmic reticulum. The particles receive a coating of apoprotein, cholesterol and phospholipid in the rough endoplasmic reticulum and move to the Golgi apparatus where carbohydrate moieties are added. Packaged vesicles, the lipoprotein particles are delivered to the lateral cell membrane and released into the intercellular space. They then pass through the basement membrane, across the lamina propria and into the central lacteal through gaps in the endothelial lining of the lymph vessel.

3.8 Lipoprotein particles [127] Lipoproteins are complexes of lipid and protein which transport lipids in the circulation. They are classified according to density, which is inversely related to lipid content. Table 3.1 shows the principal lipoproteins and their composition [128]. Chylomicrons are synthesized in the intestine and carry dietary triglyceride. Very low density lipoproteins (VLDL), which are synthesized in both liver and intestine, are the major lipoprotein found during fasting; this suggests that the lipid they contain is largely derived from endogenous sources. Low density lipoproteins (LDL) are derived from VLDL and deliver cholesterol to the tissues. High density lipoproteins (HDL) are synthesized in a disc-like form in the liver and small intestine; they scavenge excess cholesterol from chylomicron remnants and VLDL. The inner hydrophobic core of lipoproteins contains triglyceride and cholesterol ester plus a little unesterified cholesterol; the surface is made up of a phospholipid monolayer with small amounts of free cholesterol and apoproteins. Apoproteins play a crucial role in lipoprotein transport and metabolism: some are recognized by ligands and thus target the lipoproteins to specific sites ; others bind and activate specific enzymes, *e.g.* LCAT (lecithin-cholesterol acyl-transferase) is activated by apo A-I. Apoprotein B is necessary for the export of chylomicrons from the cell: in abetalipoproteinemia (the absence of apoprotein B) lipid particles accumulate within the enterocyte and chylomicrons do not appear in lymph.

Table 3.1

Composition of the principle lipoprotein particles

	Chylomicra	VLDL	LDL	HDL
Density	< 1.006	0.95 - 1.006	1.006 - 1.063	1.063 - 1.210
Chemical composition %				
Triacylglycerol	85	50	10	4
Free cholesterol	1	7	8	2
Cholesterol ester	3	12	37	15
Phospholipid	9	18	20	24
Protein	2	10	23	55

Adapted from Kritchevsky, D. [128]

3.9 Lymph composition and flow Lymph contains all of the proteins found in plasma, generally in lower concentrations. In the dog, the gastric lymph : plasma ratio for total protein was found to be 0.51. Specialized transport proteins such as ferritin are also seen in lymph. Among non-protein constituents are low molecular weight substances (< 10,000) such as glucose, which are found in similar concentrations in lymph and plasma. Substances which are protein-bound in plasma are found in lymph in concentrations determined by the relative concentrations of the binding proteins in plasma and lymph. Foreign substances transported in lymph include toxins and bacteria. Toxins of high molecular weight are selectively carried in lymph, but lower molecular weight toxins are transported in both blood and lymph. The composition of lymph is modified as fluid filters out and as the different lymphatic branches empty into the main trunk. Passage through lymph nodes allows some exchange of small molecules between blood and lymph, as well as the addition of lymphocytes to lymph. The rate of formation of lymph is determined by the amount of interstitial fluid which forms; this is a function of blood capillary permeability and perfusion pressure. Intestinal lymph flow is strongly influenced by digestion and absorption. Contraction of gut musculature propels lymph onwards in the central lacteals; contraction of smooth muscle fibres in the walls of medium and large lymph vessel move the lymph on towards the thoracic duct and so to the systemic blood circulation. Variation of thoracic pressure with respiration also promotes lymph flow. Valves in the lymph vessels prevent the back flux of lymph.

3.10 Transport from the gut After absorption into enterocytes (the absorptive cells of the intestinal epithelium) compounds are transported from the gut either *via* the portal venous system, or if they are lipid-soluble, *via* the lymphatics. Some compounds partition between the two routes. The portal vein carries compounds to the liver where many are extensively metabolized before being excreted in bile or exported into the systemic circulation. This "first-pass" hepatic metabolism of compounds reduces the percentage of unchanged parent compound reaching the general circulation. By contrast, because the lymphatic system drains into the jugular vein, compounds carried in lymph bypass the liver and are therefore not subjected to first-pass metabolism. These fat-soluble compounds are less easily excreted and tend to accumulate in body stores.

4. RATIONALE AND OBJECTIVES OF THIS STUDY

In many studies of OTA absorption, OTA was administered in sodium bicarbonate [129,130,131,132,133] but under natural conditions ingestion of OTA commonly occurs in the presence of lipids. Livestock commonly ingest OTA in mouldy cereals such as corn and barley and therefore OTA is accompanied by plant fibre and a small percentage of cereal oils, of the order of 3% [134]. Human ingestion of OTA may occur with consumption of contaminated meat or meat products, often with a high fat content, bread or other cereal products, beans, or even human milk. Experimental studies of the toxic effects of OTA have occasionally involved administration of the toxin supplemented by, or dissolved in, corn oil [135,136], but details of absorption were not discussed. The purpose of this study was to compare the effect of the vehicle

- sodium bicarbonate or corn oil - on the absorption of OTA,

and to investigate the effect of changing pH on the ionization state of OTA and its partition between aqueous and organic media.

5. MATERIALS AND METHODS

5.1 Chemicals OTA was isolated and purified as described by Omar (137). It was shown to be 98% pure on HPLC, using a Partisil 10 ODS column (0.46 cm x 25cm) and a solvent system consisting of a 65:35 mixture of (a) acetonitrile:methanol, 1:1 v/v, and (b) 5 mM sodium acetate:acetic acid, 500:14 v/v. [^{3}H]OTA [3] purchased from Amersham U.K. was purified by TLC, using benzene:methanol:acetic acid, 90:5:5 v/v as running solvent, then eluting the OTA with benzene: acetic acid, 4:1 v/v. [^{3}H]OTA was added to unlabelled OTA and dissolved in 50 mM sodium bicarbonate or in corn oil to give a concentration of 180 μg/mL, 25 μCi/mL. Chemicals and solvents used were of the purest grade available. Mazola corn oil (Best Foods Canada Inc), for culinary use, was purchased locally and stored in the dark at 4°C. To dissolve OTA in corn oil, it was first dissolved in a small volume of chloroform which was added to corn oil and thoroughly vortexed. The chloroform was removed by evaporation under vacuum for at least 4 hours. In one set of experiments, [^{14}C]trilinolein [3] purchased from Dupont, Boston MA U.S.A., was added to the corn oil, 2:3 v/v, to give 2.0 μCi/mL.

5.2 Surgical supplies Xylazine was purchased from Haver, Bayvet Division, Chemagro Ltd., Etobicoke, Ontario. Ketamine was purchased from MTC Pharmaceuticals, Cambridge, Ontario. Clay Adams 7401 PE 10 and 50 polyethylene

---

[3] For location of [^{3}H] and [^{14}C] labels, please see Appendix, pp. 153, 154.

tubing was used for cannulation of the bile duct and intestinal lymph vessel. A size 5 French infant nasogastric tube was used as the gastric or duodenal cannula.

5.3 Surgery Male Sprague-Dawley rats (270 - 300 g) purchased from Charles River, Canada Ltd. were anaesthetized by intramuscular injection of a mixture of ketamine (40 mg/kg) and xylazine (5 mg/kg up to weight 300 g, and above that weight, up to 6 mg/kg). After midline laparotomy, the duodenal loop was reflected to one side to expose the bile duct, which was cannulated proximal to the pancreas. The main intestinal lymph vessel was cannulated where it runs alongside the superior mesenteric artery, as described by Bollman *et al.* [138], Figure 5.1. A cannula was inserted into either the stomach or duodenum, for administration of 5% glucose/saline and the single dose of OTA. Tubing was brought to the exterior through individual stab wounds, and the incision was closed in two layers. In one set of experiments, the bile duct was not cannulated; only lymph was collected.

After surgery, rats were restrained in Bollman cages and received 5% glucose/saline at a rate adequate to ensure good hydration, initially 2.3 - 2.4 mL/hour. During a post surgery recovery period of 16 - 24 hours, bile was collected from all animals with a bile duct cannula.

5.4 Bile duct cannulation Cannulation of the bile duct diverts all the bile which in natural circumstances flows into the duodenum. Normal absorption of medium and long chain fatty acids depends on the presence of bile, so in order to promote normal absorption, it is necessary to replace the bile diverted for collection. In most of the

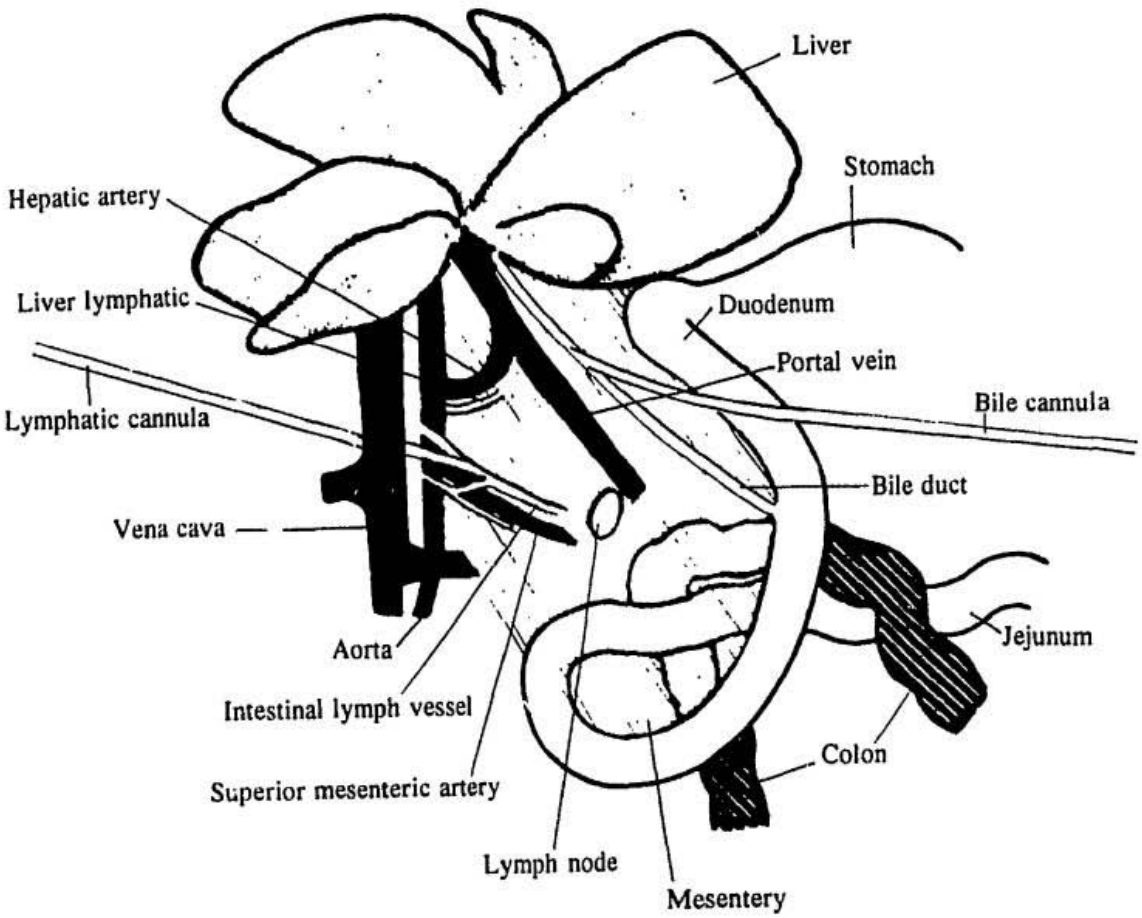


Figure 5.1
Diagram to show cannulation of the bile duct and main intestinal lymph vessel
Adapted from Lambert [139]

experiments in which the bile duct was cannulated, bile collected during the post-surgical recovery period was mixed with glucose/saline (1 mL bile : ≈ 5 mL glucose/saline) and reinfused at 3.1 mL/hour *via* the gastric or duodenal cannula, starting about one hour before the dose of OTA was given. The proportion of bile : glucose/saline was calculated so that at the flow rate chosen to give adequate hydration, sufficient bile was reinfused to replace losses, due to bile diversion, over the time course of the experiment. Ideally, reinfused bile should in all cases have been delivered to the duodenum, but in the experiments in which OTA was administered intragastrically, bile was infused into the stomach. It is probable that at the pH prevailing in the stomach, mechanisms of OTA absorption would not be significantly affected by the presence of bile.

5.5 *In vivo* experiments [4] A summary of *in vivo* experiments is given in Table 5.1. [^{3}H]OTA was given in a single bolus dose, usually 288.8 μg/kg, 40 μCuries/kg (in one instance, double the dose was used) in either 50 mM sodium bicarbonate or corn oil (1.67 mL/kg) *via* the gastric or duodenal cannula, (or in intact animals by oral gavage). This dose approximated two days' intake of OTA of rat chow contaminated by 4 ppm OTA [140]. This is similar to concentrations sometimes found in naturally contaminated livestock feed [25]. In one experiment, a dose of

---

[4] Experiments were performed under the guidelines and with the approval of the Animal Care Committee of Memorial University of Newfoundland.

Table 5.1 Summary of *in vivo* experiments

		Surgery: Cannulation		Dose μg/kg	Vehicle	± infused bile	Route	Duration	#
Exp	n	Bile	Lymph	[^{3}H]OTA					
(1)	1	✓	-	288.8	$NaHCO_3$	+	intrad	12 hr	b, u, o
	1			577.6					
(2)	4	✓	-	288.8	$NaHCO_3$	☼	intrad	6 hr	s, b,
	4	✓	-	288.8	$NaHCO_3$	☼	intrad	12 hr	u,
	5	✓	-	288.8	$NaHCO_3$	☼	intrad	18 hr	k,
	4	✓	-	288.8	$NaHCO_3$	☼	intrad	24 hr	liv
(3)	5	✓	✓	288.8	$NaHCO_3$	+	intrag	7 hr	s, l,
	3	✓	✓	288.8	corn oil	+	intrag	7 hr	b, u
(4)	4	✓	✓	288.8 ± Phe	$NaHCO_3$	+	intrag	7 hr	s, l,
	2	✓	✓	288.8	$NaHCO_3$	+	intrag	7 hr	b, u
(5)	5	✓	✓	288.8	$NaHCO_3$	+	intrad	6 hr	s,
	8	✓	✓	288.8	corn oil	+	intrad	6 hr	l, b,
	4	✓	✓	288.8	corn oil	-	intrad	6 hr	u

(6)	4	-	✓		288.8	[^{14}C] corn oil	n/a	intrad	6 hr	s, l, gut c
(7)	4	-	-		288.8	$NaHCO_3$	n/a	oral	6 hr	u
	4*	-	-		288.8	$NaHCO_3$	n/a	oral	6 hr	s
	4	-	-		288.8	corn oil	n/a	oral	6 hr	gut t, c
	3	-	-		288.8	$NaHCO_3$	n/a	oral	24 hr	liv,
	4	-	-		288.8	corn oil	n/a	oral	24 hr	k
(8)	6	-	-		3.0 mg/kg	$NaHCO_3$	n/a	oral	6 day	u,s

± The bile duct was cannulated at surgery and bile was collected from then on. In most animals bile collected during the overnight recovery period was mixed with glucose/saline and reinfused via the gastric or duodenal cannula (+ infused bile) in order to replace losses due to bile diversion during the course of the experiment. In some animals (- infused bile) no bile was reinfused. Animals were hydrated with glucose/saline only.

☼ The bile duct was cannulated and tubing looped back into the duodenum, so that bile drained without interruption into the duodenal lumen. For the last six hours of each experiment, the loop of tubing was cut and bile was collected for analysis.

* pre-treated for 5 days with phenobarbital

intrag indicates that an intragastric cannula was used for hydration and for giving the test dose.
intrad indicates that an intraduodenal cannula was used for hydration and for giving the test dose.

# denotes samples collected: s denotes serum, b bile, u urine, l lymph,
o organs, k kidneys, liv liver,
gut c,t gut contents and tissues

3 mg [^{3}H]OTA/kg body weight was given orally to intact rats. After the test dose was given, hydration was resumed, operated animals receiving glucose/saline ± bile *via* the cannula, and orally-dosed rats having water *ad lib.*

5.5.1 In the first experiment, the bile duct was cannulated, and bile and urine were collected over ice, protected from light, for twelve hours after intraduodenal administration of [^{3}H]OTA in sodium bicarbonate, to observe the appearance of [^{3}H] label in bile and urine and to determine an appropriate time frame for subsequent experiments. In order to identify those organs in which OTA was deposited, organs were excised, blotted and weighed, and weighed samples of liver, kidney, lungs, pancreas, spleen and epididymal fat, and also intestinal contents and feces, were solubilized in NCS tissue solubilizer (Amersham, Canada Ltd., Oakville, Ontario) and when dissolved, duplicate 80 μL aliquots were taken and neutralized with glacial acetic acid before counting of radiolabel.

5.5.2 In experiment (2), liver and kidney samples were taken from four groups of rats, six, twelve, eighteen and twenty-four hours after intraduodenal dosing with [^{3}H]OTA in sodium bicarbonate. The bile duct was cannulated in the usual manner and the polyethylene tubing was brought through the stab wound to the exterior but then looped back into the abdominal cavity to drain into the duodenum, so that initially bile was not collected. For the last six hours of the experiment, the exteriorized loop of tubing was cut so that bile could be collected, *e.g.* in the six hour experiment, bile was collected immediately after the dose of OTA was given, until the

rats were sacrificed at six hours post dosing; in the twenty-four hour experiment, bile collection started at eighteen hours.

5.5.3 In experiment (3), [^{3}H]OTA was administered intragastrically in either bicarbonate or corn oil, to see if the vehicle used affected the route of absorption.

5.5.4 This experiment involved intragastric administration of phenylalanine with OTA in bicarbonate (in a molar ratio, phenylalanine : OTA, approximately 10:1, *i.e.* 4:1 by weight), to see if simultaneous administration of phenylalanine either enhanced gastric absorption of OTA, as suggested by some authors [130], or reduced it, as proposed by others [141].

5.5.5 In experiment (5), [^{3}H]OTA was administered to three groups of rats intraduodenally, to eliminate any differences resulting from variable timing of gastric emptying. These rats were designated group B+ (those rats given OTA in bicarbonate, with bile reinfused), group C+ (those rats given OTA in corn oil, with bile reinfused) and group C- (OTA in corn oil, without reinfused bile).

5.5.6 In a subsequent experiment, the intestinal lymph vessel was cannulated but not the bile duct; additional bile was not reinfused. [^{3}H]OTA was administered intraduodenally in a single dose, 288.8 μg/kg, 40 μCi/kg, in [^{14}C]corn oil (1.67 mL/kg, 3.33 μCi/kg). It was then possible to follow concurrently the absorption and transport of [^{14}C]lipid and [^{3}H]OTA. Intestinal tissues and contents were extracted, in order to ascertain how much of the dose remained in the digestive tract when each animal was sacrificed, six hours after dosing. Duplicate aliquots of

lymph collected in this experiment were taken for counting of radioactive labels, then the lymph collected from each rat over the first two hours and the fourth and fifth hours was centrifuged as described in 5.6.2, to separate chylomicra and VLDL from infranatant.

5.5.7 In experiment (7), intact rats were given [^{3}H]OTA, 288.8 μg/kg, in bicarbonate or corn oil 1.67 mL/kg in a single dose, by oral gavage. Four of the bicarbonate-dosed rats were pre-treated for five days with phenobarbital, concentration 0.1% in drinking water. Phenobarbital treatment induces the cytochrome P450 enzymes which are responsible for hydroxylation of OTA to 4-hydroxyochratoxin A (4-OH-OTA). One set of rats had free access to chow and water before and after dosing with OTA. Other rats were fasted for either eighteen or twenty-four hours prior to dosing, and for the six or twenty-four hours after dosing. All rats had water *ad lib.* throughout. Six or twenty-four hours after receiving OTA, rats were sacrificed. Blood was taken from the abdominal aorta; weighed liver and kidney samples, and gut tissues and contents were taken for extraction of OTA as described under 5.6.4.

5.5.8 In experiment (8), intact rats were given [^{3}H]OTA 3.0 mg/kg in bicarbonate, by oral gavage. After dosing, urine was collected for six days, to observe the clearance of OTA in urine. Duplicate aliquots were taken for counting of [^{3}H] label, and the remainder of each collection was extracted immediately in chloroform as described in 5.6.4, or frozen at - 20°C for extraction later.

5.6 Sample Collections Pre-dose collections of urine, bile and/or lymph, and in one experiment, blood, were made. After OTA administration, bile and/or lymph were collected on ice, every 15 minutes for the first 2 hours, and at ½ then 1 hour intervals thereafter, for 6 or 7 hours. In the experiment in which serum levels of OTA were measured, 6 drops of blood, total volume approximately 150 - 200 μl, were collected from a femoral arterial line at each time point. Urine was collected as it was produced. When all bile and lymph collections had been completed, rats were anaesthetized as described previously, the abdominal cavity was opened by midline incision and a sample of blood was obtained by left ventricular puncture or from the abdominal aorta. Blood was allowed to stand at room temperature for at least one hour, then centrifuged for 10 minutes at 250 g to give serum as the supernatant.

5.6.1 Counting of radioactive label [^{3}H] and in one experiment [^{14}C] were counted in order to follow [^{3}H]OTA and [^{14}C]trilinolein. Scintillation fluid (Scintiverse II, Fisher Scientific) was added to duplicate aliquots of bile, lymph, urine, serum and solubilized or extracted gut contents and tissue samples. Where tissue solubilizer had been used, glacial acetic acid was added to solubilized tissue, 1 μL acid to 10 μL sample, to neutralize the alkaline solution. After cooling overnight in the dark, sample aliquots were counted in a Beckman LS 1801 scintillation counter.

5.6.2 Centrifugation of lymph Fresh lymph, from experiment (6) in which rats were given [^{3}H]OTA in [^{14}C]corn oil, was overlaid with 1.006 g/L density solution [5]

---

[5] For preparation of 1.006 g/mL density solution, see Appendix, p. 154.

and centrifuged for 40 minutes at 177,700 g (7.1×10^6) at room temperature in a Beckman L5 50B ultracentrifuge, to separate chylomicra. After slicing the tube, the chylomicra were withdrawn by pipette. The remaining solution was transferred by pipette to another centrifuge tube, overlaid with 1.006 g/L density solution, and centrifuged again at 177,700 g for 18 hours (1.9×10^8) to separate VLDL from infranatant. Duplicate aliquots of chylomicrons, VLDL and the infranatant were taken for counting of [^{3}H] and [^{14}C].

5.6.3 Estimation of gut contents and tissues Gut contents from two rats dosed with [^{3}H]OTA in [^{14}C]corn oil were collected in order to assess how much of the dose had been absorbed during the course of the experiment. The stomach was tied off from the oesophagus at the level of the diaphragm, and from the small intestine, close to the pylorus. The intestine was separated from mesentery and tied off distally at the ileocaecal junction. The caecum and colon were excised in one piece. After excision, the small intestine was divided by ligatures and cut into four segments of equal length. Each gut segment was rinsed carefully three times with 0.5% sodium taurocholate in normal saline; from the washings 500 μL was taken for counting of [^{3}H] and [^{14}C] label. When gut tissues and contents from intact rats were extracted, gut segments were excised and divided as described, but were rinsed only twice with 0.5% sodium taurocholate in normal saline, then twice with 50 mM bicarborate. Gut contents and rinsings were then extracted as described in 5.6.4. Tissues were chopped finely then acidified and homogenized in a Waring blender before extraction.

5.6.4 Extraction of OTA Samples were brought to pH 2.0 by addition of HCl, then extracted three times in chloroform. Duplicate aliquots of aqueous extracts were taken for counting of [^{3}H] label; chloroform extracts were dried under nitrogen and dissolved in methanol, then aliquots were taken for counting of [^{3}H] label. Aqueous extracts were freeze dried then dissolved in methanol or methanol:water, 80:20.

5.6.5 HPLC analysis The chloroform and aqueous extracts were filtered through a 0.45 μm HPLC filter prior to analysis by HPLC. A Perkin Elmer ISS 100 autosampler and an LS-5 fluorescence spectrophotometer were used with a Supelcosil LC 18 column, 3.3 cm x 4.6 mm x 3.0 μM. The solvent system used was methanol:water, 80:20 or 64:36, with a flow rate of 1 mL/minute. Samples were detected by fluorimeter, with excitation set at 340 nm and emission at 465 nm. HPLC was used to clarify whether OTA was present in extracted samples in the parent form or as a metabolite.

5.7 *In vitro* experiments

5.7.1 Partition between organic and aqueous media Partition of [^{3}H]OTA between chloroform and phosphate buffer, and between corn oil and phosphate buffer, was studied at a range of pH from 2.5 to 8.5 by comparing [^{3}H] label and by observing fluorescence under ultraviolet light in the two phases. In the chloroform/phosphate buffer experiment, [^{3}H]OTA was dissolved in potassium phosphate buffer (0.1 M) to give a final concentration 13.3 μM (0.125 μCi/mL) and 1:1 mixtures of chloroform

and OTA in buffer (2 mL total) were vortexed prior to centrifugation at 1000 g for ten minutes. In the corn oil/phosphate buffer experiment, OTA was dissolved in corn oil to give a final concentration 13.3 μM (0.05 μCi/mL). 1:1 mixtures of buffer and OTA in corn oil (2 mL total) were vortexed, then one of each duplicate was shaken, and the other rotated at 22 rpm, at room temperature for 19 hours, protected from light, prior to centrifugation and separation of phases.

5.7.2 Absorbance scan at different pH Protonation of the phenolic group at C_8 was investigated by scanning between 300 and 400 nm the absorbance of solutions of OTA in phosphate buffer at a range of pH between 5.0 and 10.5, as described by Pitout [26].

5.7.3 Standard curves: known amounts of OTA and Oα were run on HPLC and the peak areas were plotted against mass in ng so that quantities of OTA and Oα in samples could be estimated. 4(R) and 4(S) 4-OH OTA were assumed to have the same fluorescence coefficient as OTA.

5.8 Statistical analysis Data from rats whose lymph flow was less than 3 ml during the first 2 hours after OTA administration were not included in the statistical analysis. *In vivo* data were analyzed by the Wilcoxon rank-sum test, taking an α value of 0.05 as significant.

6. RESULTS

6.1 *In vivo* studies

6.1.1 The aim of the first experiment was to demonstrate in general terms the absorption, disposition and excretion of OTA, and to determine an appropriate time frame for subsequent experiments. [^{3}H]OTA in 50 mM sodium bicarbonate was given intraduodenally to two rats, one receiving 288.8 μg/kg body weight, and the second double that dose, 577.6 μg/kg. Bile and urine were collected over the course of the experiment, and when the animals were killed twelve hours after dosing, organ samples and intestinal contents and feces were solubilized. Duplicate aliquots of all samples were taken for counting of [^{3}H] label. Table 6.1 shows the twelve hour recovery of [^{3}H] label from the two rats, expressed as a percentage of dose administered. Percentage recovery was similar in the two animals, except that recovery in urine, intestinal contents and liver was higher in the rat given the larger dose. Only ~ 30% of the administered dose was recovered: it is probable that a further 10% may have been in the serum, and perhaps as much as 30% in skin, muscle and intestinal tissues, which were not sampled. Only excreted urine was collected: urine in the bladder was not recovered.

From the profile of [^{3}H] appearance in bile (data not shown), it was seen that if bile was collected, *i.e.* withdrawn from the system, so that enterohepatic circulation of the toxin was prevented, 65% of the twelve hour total of OTA had already appeared in six hours. Based on percentage recovery in the different organs, and

Table 6.1

Twelve hour recovery of [^{3}H] label from two rats given [^{3}H]OTA intraduodenally in sodium bicarbonate

Dose [^{3}H]OTA	288.8 μg/kg	577.6 μg/kg
Bile	20.49	20.56
Urine	5.80	8.60
Liver	2.20	3.15
Kidneys	0.48	0.50
Lungs	0.41	0.44
Pancreas	0.27	0.23
Spleen	< 0.10	< 0.10
Epididymal fat	n/a	0.11
Intestinal contents	0.60	1.57
Feces	0.06	0.05

[^{3}H]OTA was administered intraduodenally in 50 mM sodium bicarbonate, 1.67 mL/kg. Recovery is expressed in terms of the whole organ or collection, as a percentage of dose administered.

literature reports of pathology caused by ingestion of OTA, it was decided that in future experiments, only liver and kidneys would be harvested.

6.1.2 The second experiment was set up to observe how much of the administered dose persisted in rat serum, liver and kidneys at 6, 12, 18 and 24 hours after intraduodenal administration of OTA in bicarbonate, and to give an idea of how much OTA was circulating in bile.

Figure 6.1 shows that recovery of [^{3}H] label in samples decreased with time: twelve-hour samples had significantly lower percentage recovery of OTA than the six-hour samples ($p \leq 0.05$ for serum, bile and liver, and $p = 0.01$ for kidney); eighteen-hour serum, bile and liver samples had significantly lower levels in terms of administered dose than the twelve-hour samples ($p \leq 0.05$). Although the total recovery of [^{3}H]OTA from liver was greater than that from kidney, the recovery/g tissue was higher in the kidney at each time point, as shown in Table 6.2.

6.1.3 There has been some discussion as to the importance of the stomach in the absorption of OTA: Kumagai and Aibara isolated various segments of the gastro-intestinal tract of the rat and demonstrated that the primary site of OTA absorption is the small intestine, with the most efficient absorption occurring in the jejunum [128]; Galtier [142] reported that gastric absorption played a major role, and Roth (working with mice) noted rapid absorption of OTA from the stomach [129]. These authors used OTA in sodium bicarbonate. We therefore gave OTA intragastrically to two groups of rats, using sodium bicarbonate as the vehicle for the first group and,

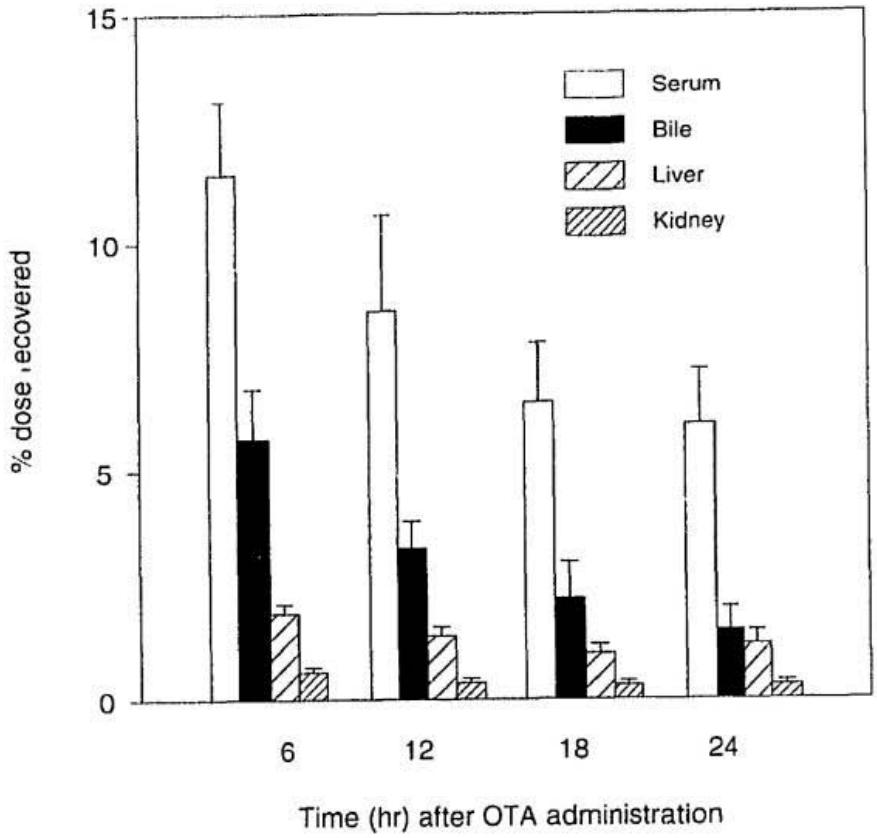


Figure 6.1 Recovery of [^{3}H]OTA in serum, bile, liver and kidneys after intraduodenal administration of [^{3}H]OTA in sodium bicarbonate

OTA was administered at a dose of 288.8 μg/kg. Overnight bile was reinfused. Recovery is expressed as a percentage of dose administered.
Values shown are means $\pm$ SD. Calculation of OTA serum levels was made on the basis that serum volume is 0.55 blood volume, which in turn, expressed in mL, is 0.07 mass of the rat expressed in g; thus total serum volume is taken as 0.0385 mass of the rat. Bile was initially allowed to drain into the duodenum, but for the last six hours of each experiment it was collected. Levels in bile are plotted to show OTA recovery during the six hour collection.

Table 6.2

Recovery of [^{3}H]OTA in liver and kidney

Time in hours	Recovery of [^{3}H] label, % dose/g tissue	
	Liver	Kidney
6 (n = 4)	0.18 ± .02	0.28 ± .05 *
12 (n = 4)	0.14 ± .03	0.16 ± .03
18 (n = 4)	0.09 ± .02	0.11 ± .05
24 (n = 7)	0.11 ± .02	0.13 ± .03 *

[^{3}H]OTA was administered at a dose of 288.8 μg/kg, in 50 mM sodium bicarbonate, 1.67 mL/kg. Recovery is expressed as percentage of administered dose, per g wet (blotted) tissue. Values shown are means ± SD.

* * indicate significant differences p = 0.01, 0.05, between liver and kidney in recovery of [^{3}H]/g tissue

recognizing that lipid is often present when OTA is ingested, corn oil for the second. [^{3}H] counts were seen in lymph, serum and bile within 15 minutes, and reached peak values within 1 hour of OTA administration. Figure 6.2 shows the cumulative recovery of [^{3}H]OTA in lymph and bile in these animals. From 15 minutes up to 3 hours, significantly more OTA was recovered in bile when bicarbonate rather than corn oil was used as vehicle ($p = 0.01$ from 30 minutes to 1¼ hours after dosing, and $p = 0.05$ from 1¼ hours to 3 hours; thereafter, the difference was insignificant). The results of this experiment are summarized in Table 6.3, which shows the recovery of [^{3}H] label in bile, lymph, serum and urine, expressed as a percentage of dose administered, seven hours after dosing with [^{3}H]OTA. Recovery in bile over seven hours was more when the vehicle was bicarbonate, ($16.7 \pm 5.9\%$, $n = 5$), than when corn oil was used ($10.4 \pm 2.7\%$, $n = 3$). Less [^{3}H] label was recovered in lymph when OTA was given in bicarbonate ($10.6 \pm 4.0\%$) rather than in corn oil (17.7 ± 5.2 %). These differences were not significant. In this experimental system, in which lymph was collected from the main intestinal lymphatic vessel, it was assumed that no OTA could reach the general circulation by the lymphatic route. OTA appearing in bile must first have been absorbed *via* the portal vein, transported to the liver and thence exported into bile (and serum). Recovery of OTA in bile therefore gives an indication of portal venous uptake, whereas OTA recovery in lymph is a direct measure of lymphatic transport.

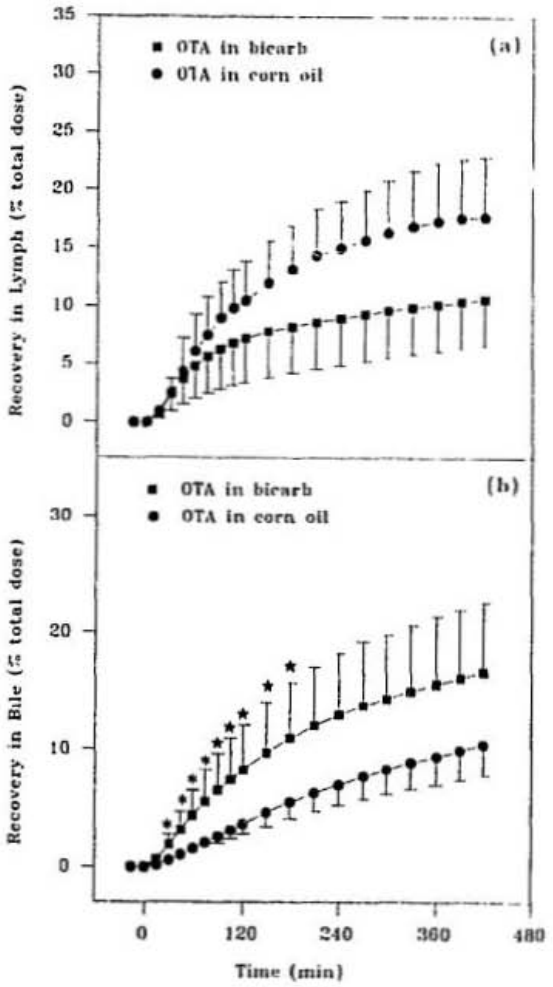


Figure 6.2

Cumulative recovery of [^{3}H] label in lymph and bile of rats given [^{3}H]OTA intragastrically in sodium bicarbonate or corn oil

OTA was administered at a dose of 288.8 μg/kg. Overnight bile was reinfused. Recovery is expressed as a percentage of dose administered. Values shown are means $\pm$ SD, where n(bicarb) = 5, n(corn oil) = 3 (*) (⋆) denote significant differences, p = 0.01 and 0.05 respectively between the two groups in recovery of [^{3}H] label.

Table 6.3

Seven hour recovery of [^{3}H] label after intragastric administration of [^{3}H]OTA

	OTA/$NaHCO_3$ + bile n = 5	OTA/corn oil + bile n = 3
Bile	16.7 ± 5.9	10.4 ± 2.7
Lymph	10.6 ± 4.0	17.7 ± 5.2
Serum [1]	6.8, 6.4 (n = 2)	8.1 ± 4.7
Urine	5.1 ± 0.8	2.0 ± 0.64 *

OTA was administered at a dose of 288.8 μg/kg. Bile was reinfused. Recovery is expressed as a percentage of dose administered. Values shown are means ± SD.

Key: (*) denotes a significant difference ($p = 0.01$)
from the group of rats given OTA in $NaHCO_3$.

[1] Serum OTA was calculated by assuming that serum volume is 0.55 blood volume, which in turn, expressed in mL, is 0.07 mass of the rat, expressed in g. Serum volume is therefore 0.0385 mass of the rat.

6.1.4 Roth *et al.* [129] also reported that gastric absorption in mice was enhanced by simultaneous administration of phenylalanine, whereas Delacruz and Bach [138] suggested that OTA may be transported into the absorptive cell by the Na^+-dependent carrier system specific to the L-isomers of neutral amino acids [143]. With its L-phenylalanine group, OTA might well be recognized by this system. We examined the effect of simultaneously administered phenylalanine on the appearance of OTA in lymph and bile, to determine whether phenylalanine affected OTA absorption. Table 6.4 shows the recovery of [^{3}H] label in bile and lymph when OTA in bicarbonate was given intragastrically with or without phenylalanine. Simultaneous intragastric administration of phenylalanine minimally depressed recovery of OTA in bile and had no effect on recovery in lymph.

6.1.5 Knowing that bile is important in fat absorption, we wondered if it plays any role in the absorption of OTA. We therefore compared the appearance of [^{3}H]OTA in lymph and bile when OTA in corn oil was administered either with replacement of bile lost as a result of bile diversion (group C+), or without reinfused bile (group C−). A third group of rats (group B+) received OTA in sodium bicarbonate. To eliminate differences due to variable timing of gastric emptying, the dose of OTA was administered intraduodenally. Plots showing recovery/minute (Figure 6.3) and cumulative recovery of label in lymph and bile (Figure 6.4) had typical profiles, which varied according to the vehicle used. Bile flow was consistent, whereas lymph flow was always variable (Figure 6.5).

Table 6.4

Seven hour recovery of [^{3}H] label in bile and lymph after intragastric administration of [^{3}H]OTA in sodium bicarbonate ± phenylalanine

	OTA/$NaHCO_3$ + Phe + bile (n = 4)	OTA/$NaHCO_3$ - Phe + bile (n = 2)
Bile	19.4 ± 2.2	22.0, 22.9
Lymph	13.4 ± 1.2	14.2, 12.9

[^{3}H]OTA was administered at a dose of 288.8 μg/kg. Phenylalanine was given concurrently at a dose of 1.155 mg/kg, *i.e.* 4-fold by weight, 10-fold by molar concentration in comparison with OTA. Bile was reinfused. Recovery is expressed as a percentage of OTA administered. Values shown are means ± SD (n = 4) or individual values (n = 2).

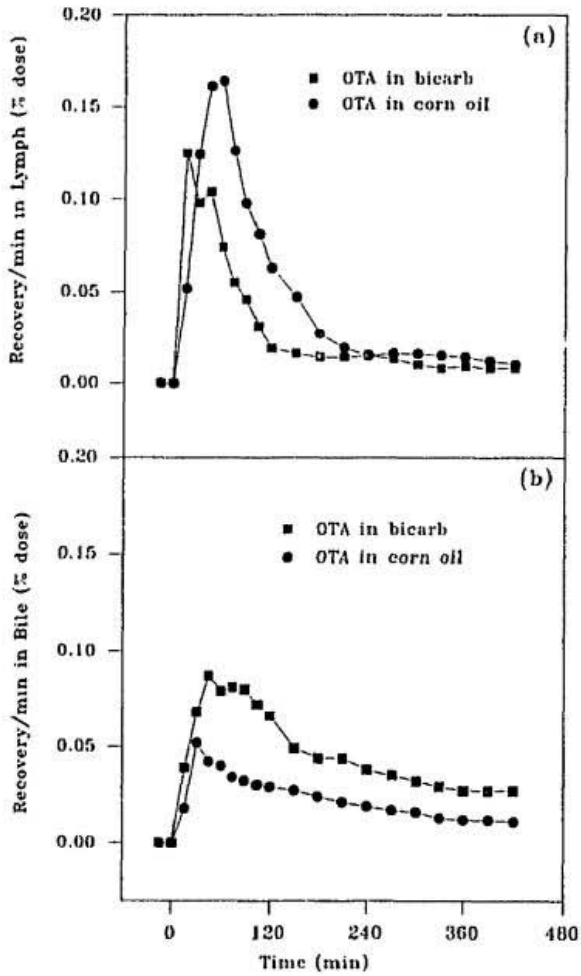


Figure 6.3 Recovery of [^{3}H] label/minute in lymph and bile of two rats after intraduodenal administration of [^{3}H]OTA in sodium bicarbonate or corn oil

[^{3}H]OTA was administered in bicarbonate (■), or corn oil (•), with overnight bile reinfused, at a dose of 288.8 μg/kg body weight. Recovery/minute in (a) lymph and (b) bile is expressed as a percentage of dose administered.

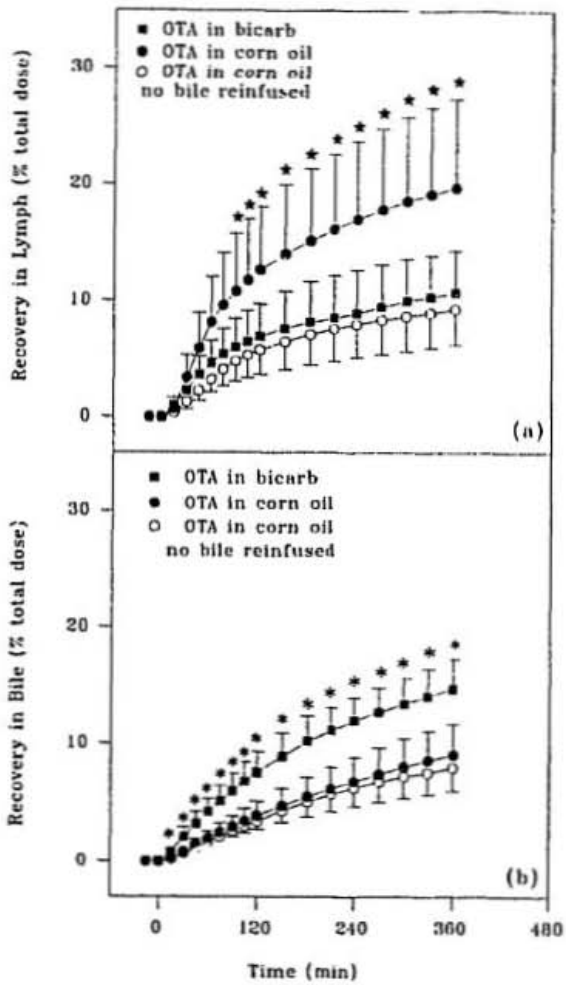


Figure 6.4

Cumulative recovery of [^{3}H] label in lymph and bile after intraduodenal administration of [^{3}H]OTA in sodium bicarbonate or corn oil

[^{3}H]OTA was administered in $NaHCO_3$ (▪) (B+), or corn oil (•) (C+), with overnight bile reinfused, or in corn oil without reinfusion of bile (○) (C-), at a dose of 288.8 μg/kg body weight.
Values shown are means $\pm$ SD, where n(B+) = 5, n(C+) = 8 and n(C-) = 4.
(*) (⋆) denote significant differences, $p < 0.01$ and ≤ 0.05 respectively between B+ and C+ in recovery of [^{3}H] label.

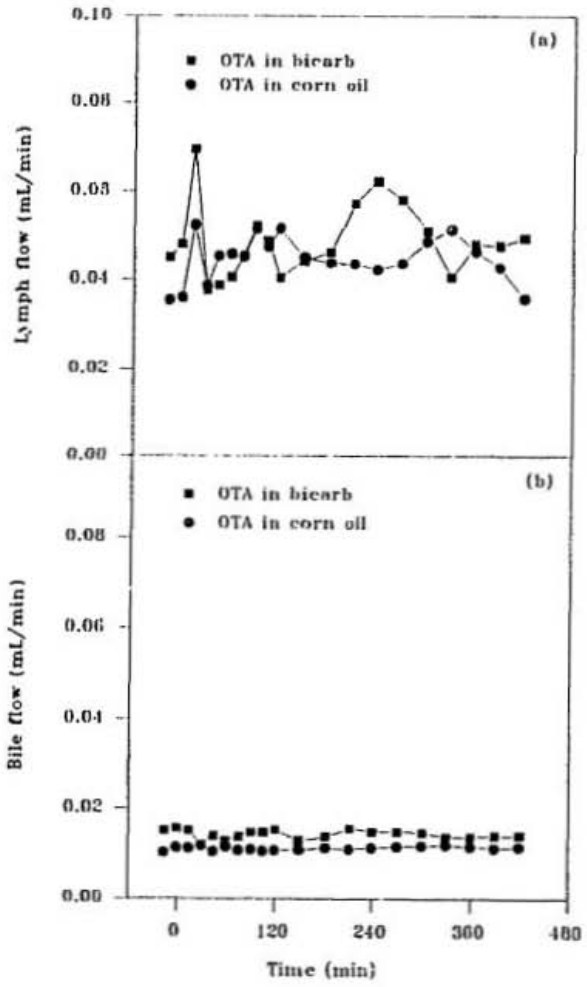


Figure 6.5

Lymph and bile flow in two rats after intraduodenal administration of [^{3}H]OTA in sodium bicarbonate or corn oil

[^{3}H]OTA was administered in $NaHCO_3$ (▪), or corn oil (•), with overnight bile reinfused, at a dose of 288.8 μg/kg body weight. Lymph and bile flow is expressed in mL/minute

Data reported in Figure 6.4 and Table 6.5 show that intraduodenal administration of OTA in corn oil (C+) enhanced uptake of OTA by the lymphatic route. Over the first 1¼ hours there was no significant difference between groups B+ and C+; thereafter, significantly more OTA was taken up in lymph in group C+, $p \leq 0.05$. Administration in bicarbonate (B+) significantly increased cumulative recovery in bile, $p < 0.01$, at all time points. Table 6.6 shows the relationship between lymph flow and OTA uptake in lymph. There was a significant difference between groups B+ and C+ with respect to [^{3}H]OTA uptake/mL of lymph. In all three groups, lymph flow increased significantly from basal levels in response to administration of OTA; the increase was significantly greater in oil-dosed rats than in bicarbonate-dosed animals (Table 6.7). Between groups C+ and C−, the increase in lymph flow was not significant, but the C+ group had significantly greater lymphatic uptake of [^{3}H]OTA than the bile-deficient C− group ($p < 0.05$, at all time points after the first 15 minutes); recovery of [^{3}H] label in bile was essentially the same whether or not bile was reinfused. No chylomicrons were seen unless both corn oil and bile were present. Of the rats given bile infusions, total [^{3}H] recovery over six hours was significantly less ($p < 0.05$) in the C+ group ($39.0 \pm 9.7\%$) than in the B+ group ($47.8 \pm 5.1\%$). Total recovery in the C− group was much less ($25.7 \pm 6.67\%$), $p = 0.01$ by comparison with B+, and $p < 0.05$ when compared with C+.

Table 6.5

Six hour recovery of [^{3}H] label after intraduodenal administration of [^{3}H]OTA

	B+ (n = 5)	C+ (n = 8)	C- (n = 4)
Bile	14.8 ± 2.5 *	9.1 ± 2.7	8.0 ± 2.0
Lymph	10.7 ± 3.6 *	19.8 ± 7.7	9.3 ± 3.1 *
Serum	19.8 ± 1.3 * (n = 3)	8.8 ± 3.2 (n = 7)	4.5 ± 0.5 *
Urine	3.2 ± 0.7 (n = 4)	3.4 ± 2.1	2.6 ± 1.5

OTA was administered at a dose of 288.8 μg/kg. Bile was reinfused. Recovery is expressed as a percentage of dose administered. Values shown are means ± SD. (*) (⋆) denote a significant difference ($p \leq 0.01$) ($p < 0.05$) from group C+. There is also a significant difference ($p = 0.01$) in biliary recovery between groups B+ and C-.

Table 6.6

Lymph flow and lymphatic uptake of [^{3}H]OTA

Time (hr)	Group	n	Lymph flow (mL)	OTA uptake (% dose)	Uptake / flow (% dose / mL)
0 - 2	B+	5	4.81 ± 1.46	6.93 ± 2.83 ☼	1.04 ± 0.62 ★
	C+	8	4.75 ± 1.07	12.66 ± 5.42	2.91 ± 1.60
0 - 6	B+	4	14.95 ± 4.76	10.72 ± 3.63 ★	0.79 ± 0.48 ★
	C+	8	15.42 ± 4.38	19.75 ± 7.68	1.39 ± 0.68
0 - 2	C-	4	5.63 ± 1.84	5.76 ± 2.11 ★	1.10 ± 0.58 ★
	C+	8	4.75 ± 1.07	12.66 ± 5.42	2.91 ± 1.60
0 - 6	C-	4	17.51 ± 5.54	9.32 ± 3.09 ★	0.58 ± 0.31 ★
	C+	8	15.42 ± 4.38	19.75 ± 7.68	1.39 ± 0.68

OTA was administered at a dose of 288.8 μg/kg. Bile was reinfused. Recovery is expressed as a percentage of dose. Values shown are means ± SD.
(☼) (★) denote a significant difference ($p = 0.05$) ($p < 0.05$) from group C+.

Table 6.7 Increase in lymph flow, and lymphatic uptake of Ochratoxin A

Time (hr)	Vehicle	Reinfused bile	n	Increase in lymph flow: % above basal	p	OTA uptake † in lymph	p
0 - 2	B	+	5	4.7 ± 2.1	0.01	3.46 ± 1.42	0.05
	C	+	8	18.9 ± 9.0		6.33 ± 2.71	
0 - 6	B	+	5	7.8 ± 6.5	0.01	1.79 ± 0.6	0.01
	C	+	8	24.6 ± 11.3		3.30 ± 1.28	
0 - 2	C	+	8	18.9 ± 9.0	–	6.33 ± 2.71	< 0.05
	C	–	4	20.7 ± 4.1		2.88 ± 1.06	
0 - 6	C	+	8	24.6 ± 11.3	–	3.30 ± 1.28	< 0.05
	C	–	4	25.5 ± 8.0		1.56 ± 0.52	
0 - 2	B	+	5	4.7 ± 2.1	0.01	3.46 ± 1.42	–
	C	–	4	20.7 ± 4.1		2.88 ± 1.06	
0 - 6	B	+	5	7.8 ± 6.5	< 0.05	1.80 ± 0.70	–
	C	–	4	25.5 ± 8.0		1.56 ± 0.52	

Data given are for three groups of rats, B+ (bicarbonate + bile), C+ (corn oil + bile) and C− (corn oil — bile), for the first two hours and for the six hours after OTA administration. † OTA uptake is expressed as percentage of administered dose taken up in lymph, per hour.

6.1.6 In order to understand more fully how the presence of lipids affects OTA absorption, we added [^{14}C]trilinolein to corn oil, and this was used as the vehicle for [^{3}H]OTA. It was then possible to follow the appearance of both labels in lymph, and after centrifugation, in chylomicra and very low density lipoproteins (VLDL). Extraction of gut contents enabled us to ascertain how much OTA and corn oil remained in the digestive tract when animals were sacrificed six hours after dosing.

Figure 6.6 shows the cumulative appearance of [^{3}H] and [^{14}C] label in lymph of five rats after intraduodenal [^{3}H]OTA administration in [^{14}C] corn oil. [^{3}H] label was seen within fifteen minutes of dosing; most of the OTA transported in lymph was recovered within the first two hours. By contrast, [^{14}C] uptake started slowly and continued throughout the six hour experiment. Figure 6.7 shows the percentage distribution of [^{3}H] and [^{14}C] label in chylomicrons, VLDL and infranatant after centrifugation of the lymph collected in the first two and the fourth and fifth hours after OTA was given. These results show clearly that OTA in the lymph is not carried in chylomicrons or VLDL. Table 6.8 shows the recovery of [^{3}H] and [^{14}C] from intestinal contents of two animals in this experiment. Approximately 30% of the dose administered was recovered in luminal washings, mostly in the distal part of the small intestine and the cecum and colon. Recovery of [^{3}H] label from the distal parts of the gastrointestinal tract does not distinguish between OTA which has not been absorbed, and OTA which has been absorbed and subsequently excreted in bile. On the other hand, [^{14}C] label found in the lumen six hours after dosing mostly (because of dilution of absorbed lipid with the endogenous lipid pool before excretion in bile) represents unabsorbed lipid.

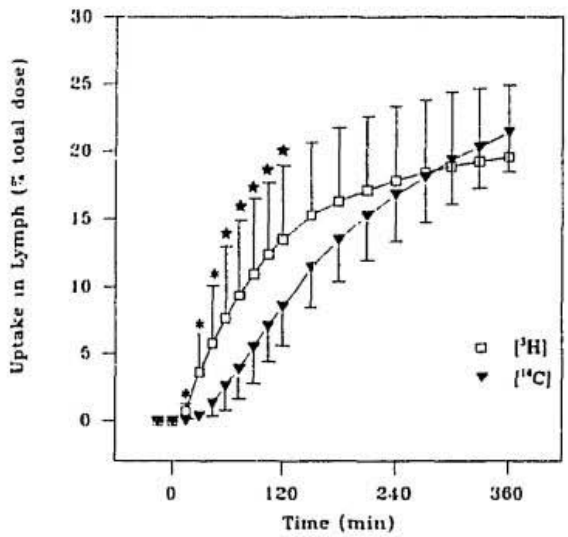


Figure 6.6

Cumulative recovery of [^{3}H] and [^{14}C] label in lymph of rats given [^{3}H]OTA in [^{14}C]corn oil intraduodenally

[^{3}H]OTA was administered (n = 5) at a dose of 288.8 μg/kg in [^{14}C]corn oil, 1.67 mL/kg. Recovery is expressed as a percentage of dose administered.
(•) (*) denote significant differences in cumulative percentage recovery of [^{3}H] and [^{14}C], p = 0.01 and 0.05 respectively.

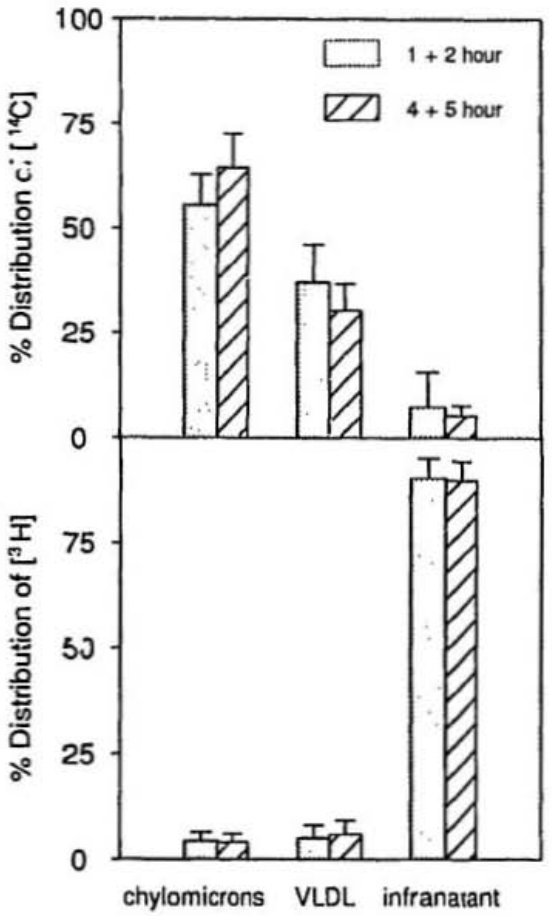


Figure 6.7 Percentage distribution of [^{3}H] and [^{14}C] label in chylomicra, VLDL and infranatant

[^{3}H]OTA was administered to the five rats described in Figure 6.6, in [^{14}C]corn oil, 1.67 mL/kg. Lymph collected over the first two hours and the fourth and fifth hours was overlaid with 1.006 g/mL density solution, centrifuged at 177.7 x 10^3 g for 40 minutes to give chylomicra, then overlaid with more 1.006 g/mL solution, and centrifuged at 177.7 x 10^3 g for 18 hours, to give VLDL and infranatant.

Table 6.8

Recovery of [^{3}H] and [^{14}C] label from luminal contents of two rats six hours after intraduodenal administration of [^{3}H]OTA in [^{14}C]corn oil

	Luminal Contents	
	[^{3}H]	[^{14}C]
Stomach	0.0, 0.7	0.0, 0.1
Intestine I	0.4, 0.5	0.1, 0.1
Intestine II	0.5, 0.7	0.1, 0.2
Intestine III	1.9, 0.6	0.5, 0.2
Intestine IV	21.6, 3.6	5.8, 0.7
Cecum + colon	9.1, 23.1	20.8, 22.8
Total Recovery	33.5, 29.2	27.3, 24.1

[^{3}H]OTA was administered in [^{14}C]corn oil, 1.67mL/kg. Gut segments were excised and tied off, then rinsed in 0.5% sodium taurocholate in normal saline. Duplicate aliquots of the rinsings were taken for counting of [^{3}H] and [^{14}C] label. Recovery is expressed as a percentage of dose.

These results suggested that the dose of 1.67 mL corn oil/kg, given intraduodenally, exceeded the absorptive capacity of the small intestine.

6.1.7 In intact animals, gastric emptying controls the amount of lipid reaching the small intestine, hence absorptive mechanisms in the small intestine are not usually overwhelmed even by meals rich in fat. In order to examine OTA absorption in a situation less artificial than that of experiments (1) to (6), rats were given OTA by oral gavage. They had free access to water but were deprived of food starting 18 or 24 hours prior to dosing. When rats starved for 18 hours were given [^{3}H]OTA in corn oil, most of the dose was found in the stomach six hours later (66.9, 72.8%); in rats starved for 24 hours, less (32.6 and 20.4%) was found in the stomach, probably because gastric emptying occurred sooner. In four rats given OTA in bicarbonate, whether starved for 18 or 24 hours, essentially no [^{3}H]OTA was found in the stomach, six hours post dose (0.15 $\pm$ 0.08%). Pretreatment with phenobarbital slowed intestinal transit; results in these rats were difficult to interpret. Figure 6.8 shows the HPLC plots of chloroform extracts of stomach contents from (a) and (b) bicarbonate-dosed, (a) pretreated with phenobarbital, and (c) oil-dosed rats; (d) is standard Oα, 4(S) and 4(R) 4-hydroxy OTA and OTA. In oil-dosed animals, 5 - 10% of the OTA recovered from the stomach was in the form of Oα [6], most likely formed by acid hydrolysis of OTA. Serum levels at six hours were lower in

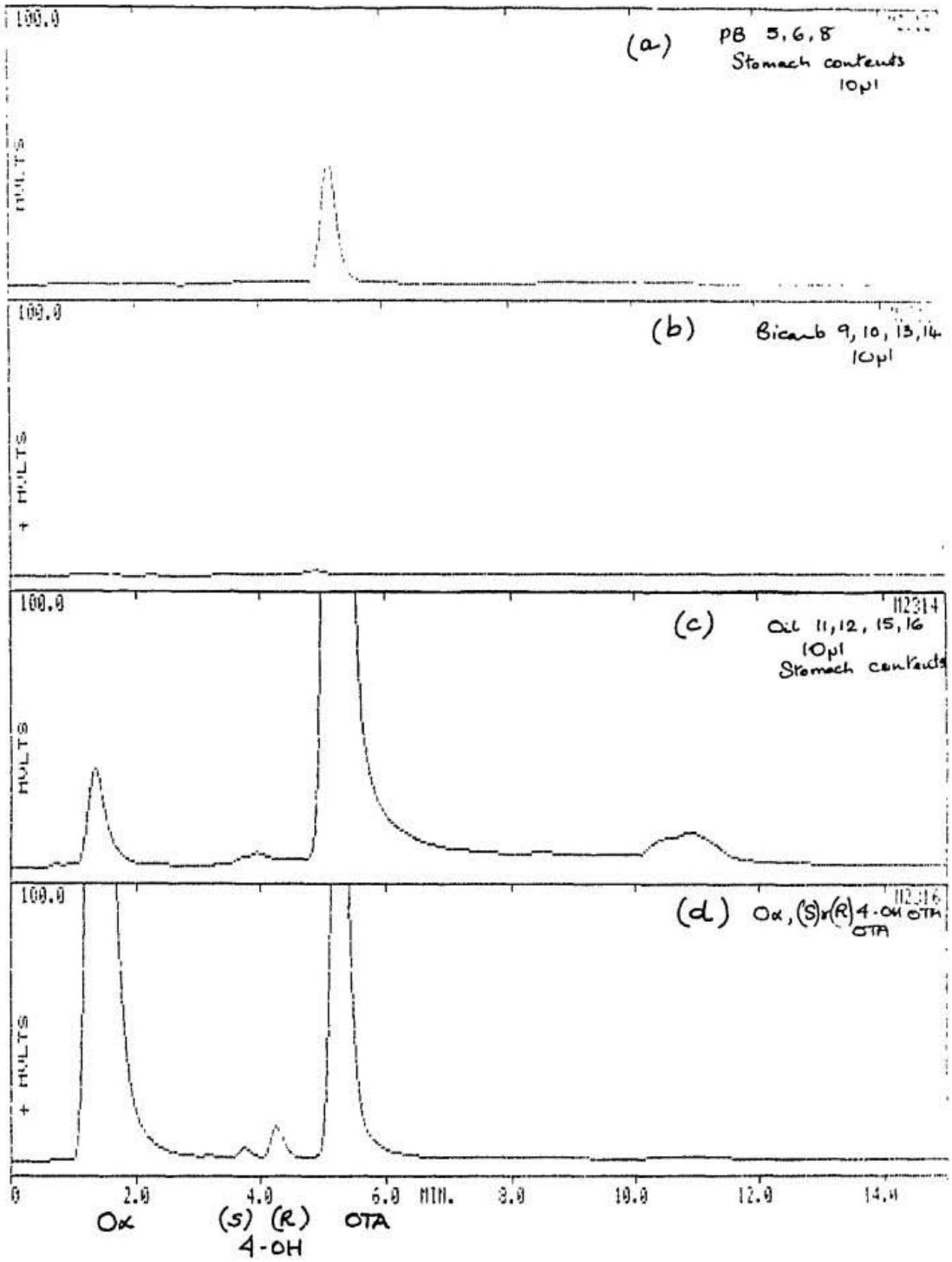
---

[6] Standard curves for OTA and Oα, showing concentration against peak area with the HPLC system used, are given in the Appendix on p. 155.

Figure 6.8

HPLC traces showing chloroform extracts of stomach contents six hours after rats were given OTA in sodium bicarbonate or corn oil

Rats were fasted for eighteen hours before receiving OTA, 288.8 μg/kg, by oral gavage, in sodium bicarbonate groups (a) and (b), (a) pretreated with phenobarbital, and group (c) in corn oil. Water was provided *ad lib.* Stomach contents were extracted three times in chloroform, dried under N_2, picked up in methanol and filtered through a 0.45 μm filter prior to HPLC analysis. A Supelcosil LC 18 column, 3.3 cm x 4.6 mm x 3.0 μm was used with a solvent system methanol:water, 80:20, and flow rate 1 mL/minute. Samples were detected by fluorimeter, excitation 340 nm, emission 465 nm. Trace (d) shows standard Oα, (4S) and (4R) 4-hydroxy OTA, and OTA.


100.0
(a) PB 5,6,8
Stomach contents
10µl
MULTS
100.0
(b) Bicarb 9,10,13,14
10µl
+ MULTS
100.0
(c) Oil 11,12,15,16
10µl
Stomach contents
MULTS
100.0
(d) Oα, (S)+(R) 4-OH OTA
OTA
+ MULTS
0
2.0
4.0
6.0 MIN.
8.0
10.0
12.0
14.0
Oα
(S) (R)
4-OH
OTA

the 18-hour fasted oil-dosed rats (6.7, 5.1%) than in the 24-hour fasted oil-dosed rats (16.5, 15.2%), or the bicarbonate-dosed rats (14.4 ± 2.1%). Recovery of [^{3}H] label at six hours in the distal small intestine and cecum plus colon was much higher in the bicarbonate group (17.2 ± 4.9%) and the 24 hour-fasted oil-dosed rats (16.8,12.6%) than in the oil-dosed 18-hour fasted rats (7.3, 5.7%). Figure 6.9 shows the HPLC plots of chloroform extracts of cecum plus colon contents in these animals: 6.9(a) refers to rats pretreated with phenobarbital, (a) and (b) were given OTA in bicarbonate, (c) were given OTA in oil; (d) shows standard OTA and metabolites. Less than 2% of OTA recovered from cecum and colon contents was in the hydroxylated 4-OH form. Twenty-four hours after administration of [^{3}H]OTA, less than 1% of the dose was found in the stomach of either bicarbonate-dosed or oil-dosed rats; less than 2% was found in contents of the small intestine. Recovery of [^{3}H]OTA in cecum and colon contents, and in feces and urine is shown in Table 6.9. The 24 hour recovery of OTA in cecum and colon contents, and in urine was significantly more in bicarbonate-dosed than in oil-dosed rats ($p = 0.05$).

6.1.8 The kidney is known to be the prime target organ of OTA, although it is not known with certainty whether OTA itself, or one or more of its metabolites is ultimately responsible for the toxic effects seen. It therefore seemed worthwhile to examine OTA excreted in urine, and to confirm the identity of metabolites described by other workers. Figure 6.10 shows HPLC traces of aqueous and organic extracts of urine from a rat given [^{3}H]OTA, 288.8 μg/kg, in sodium bicarbonate, 1.67 mL/kg.

Figure 6.9

HPLC traces showing chloroform extracts of cecum and colon contents six hours after rats were given OTA in sodium bicarbonate or corn oil

Rats were starved for eighteen hours before receiving OTA, 288.8 μg/kg, by oral gavage, in sodium bicarbonate, groups (a) and (b), (a) pretreated with phenobarbital, and group (c) in corn oil. Water was provided *ad lib.* Cecum and colon contents were extracted three times in chloroform, dried under N_2, picked up in methanol and filtered through 0.45 μm filters prior to HPLC analysis. A Supelcosil LC 18 column, 3.3 cm x 4.6 mm x 3.0 μm was used with solvent system methanol:water, 80:20, flow rate 1 mL/minute. Samples were detected by fluorimeter, excitation 340 nm, emission 465 nm. Trace (d) shows standard Oα, (4S) and (4R) 4-hydroxy OTA, and OTA.

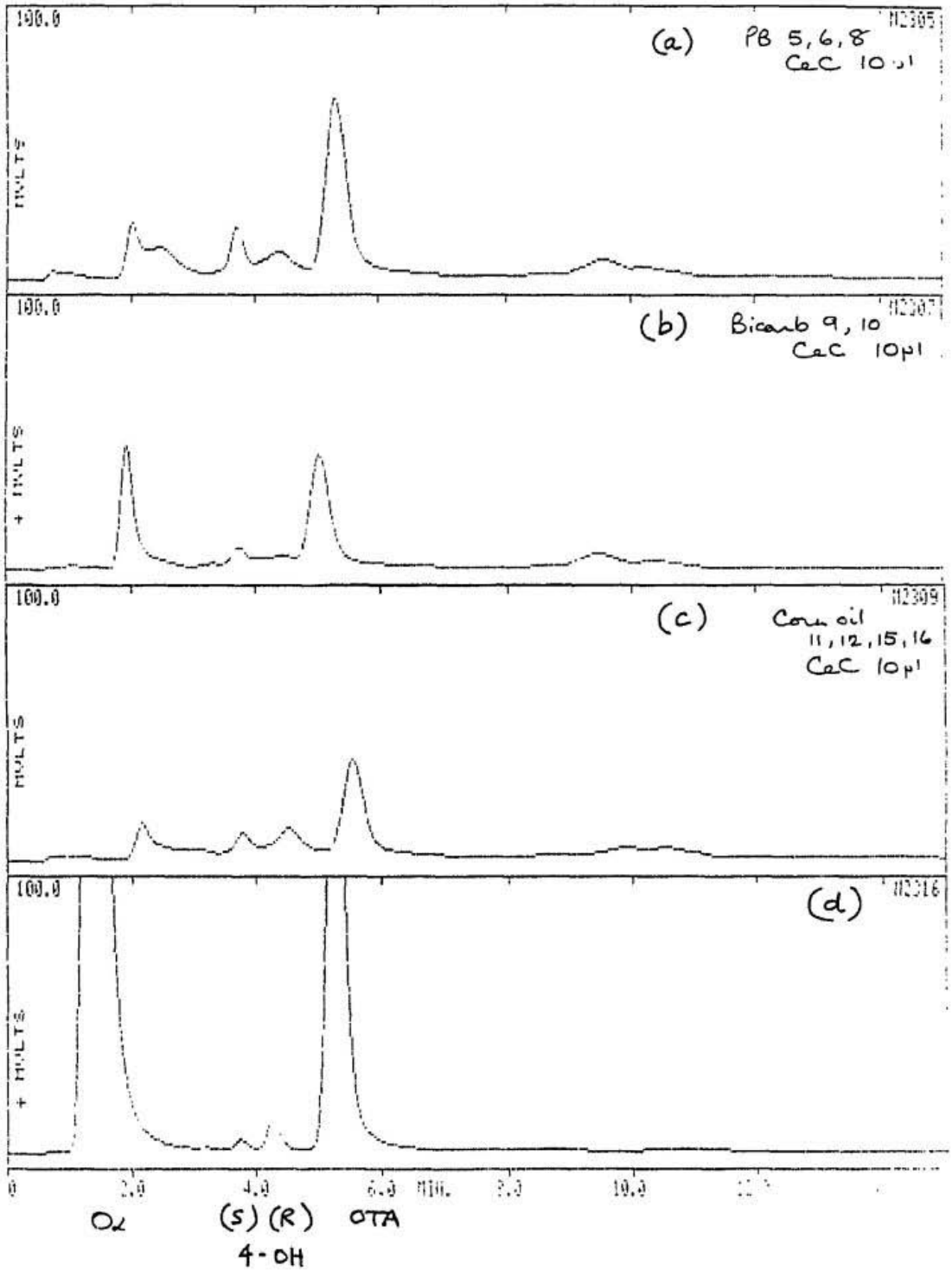

100.0
(a) PB 5,6,8
CeC 10 µl
MULTS
100.0
(b) Bicarb 9, 10
CeC 10µl
+ MULTS
100.0
(c) Corn oil
11, 12, 15, 16
CeC 10µl
MULTS
100.0
(d)
+ MULTS
0
2.0
4.0
6.0
MIN.
8.0
10.0
Oα
(S) (R) OTA
4-OH

Table 6.9

Recovery of [^{3}H]OTA from contents of large intestine, and from feces and urine in the 24 hours after oral administration of OTA

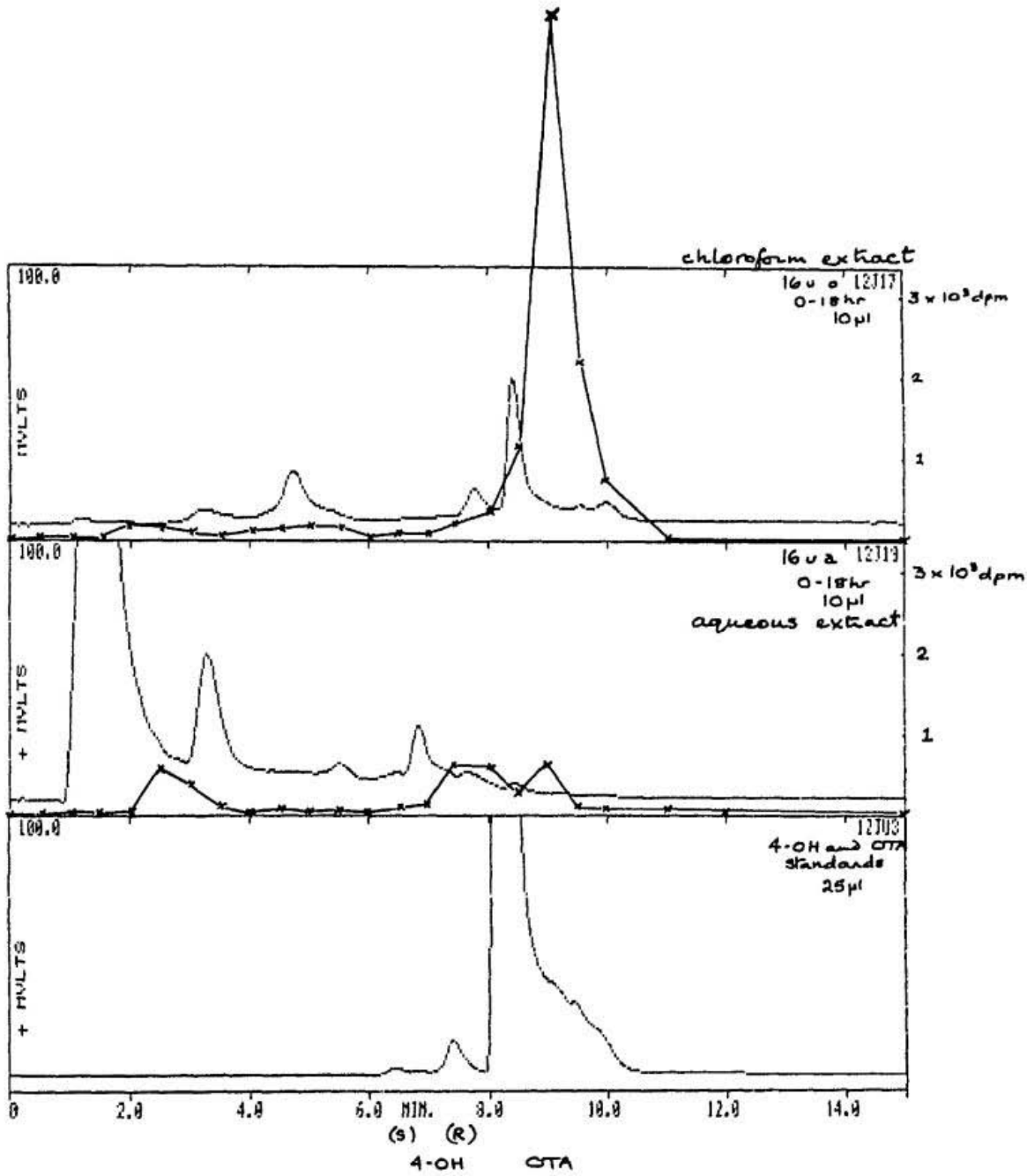
[^{3}H]OTA vehicle	bicarbonate n = 3	corn oil n = 4
Recovery of [^{3}H] label (expressed as % dose)		
Cecum and colon contents	11.4 ± 1.5% *	6.1 ± 1.2%
Feces	8.9 ± 5.2%	7.3 ± 3.6%
Urine	7.6 ± 2.5% *	3.1 ± 0.9%
Total excretion in feces and urine	16.5 ± 2.9%	10.4 ± 4.3%

[^{3}H]OTA was administered orally in sodium bicarbonate or corn oil, at a dose of 288.8 μg/kg, ~ 2.3 μCi/kg. Contents were gently squeezed out of the cecum and colon, which were then rinsed x 2 with 1 mL 0.5% sodium taurocholate in normal saline, then x 2 with 1 mL 50 mM sodium bicarbonate. Contents and washings were homogenized, brought to pH 2.0 and extracted x 3 in chloroform. Feces were similarly extracted. Recovery is expressed as a percentage of dose. Values given are means ± SD.
* denotes a significant difference, p = 0.05 in recovery of [^{3}H] label between rats given OTA in bicarbonate and those given OTA in corn oil.

Figure 6.10

HPLC traces of organic and aqueous extracts of urine from a rat given [^{3}H]OTA, in sodium bicarbonate

[^{3}H]OTA, 288.8 μg/kg, in sodium bicarbonate, 1.67 mL/kg, was given intraduodenally. Overnight bile was reinfused. Urine was acidified, then extracted three times in chloroform. The organic extract was dried under N_2, and the aqueous extract was freeze-dried. Samples were picked up in methanol and passed through a 0.45 μm filter prior to HPLC analysis. A Supelcosil LC 18 column, 3.3 cm x 4.6 mm x 3.0 μm, was used with solvent system methanol:water, 80:20, and flow rate 1 mL/minute. Samples were detected by fluorimeter, excitation 340 nm, emission 465 nm. 0.5 mL fractions eluting from the fluorimeter were collected for counting of [^{3}H] label. Radioactivity is shown —— superimposed on the HPLC trace.


chloroform extract
100.0
16 v o 12317
0-18hr
10µl
3 x 10³dpm
2
1
MVLTS
100.0
16 v a 12319
0-18hr
10µl
aqueous extract
3 x 10³dpm
2
1
+ MVLTS
100.0
12303
4-OH and OTA
standards
25µl
+ MVLTS
0
2.0
4.0
6.0 MIN.
8.0
10.0
12.0
14.0
(S) (R)
4-OH OTA

Recovery of [^{3}H] label from 0.5 mL fractions of the eluant from the fluorimeter is shown superimposed on the HPLC plot. There are many fluorescent pigments in urine, and it proved difficult to use a volume of extracted urine small enough to give sharp peaks on HPLC while ensuring that adequate amounts of [^{3}H] label were present in the fractions collected. Urine acidified to pH 2 was extracted three times in chloroform: when urine was first produced, 80% of the OTA excreted was in the form of water-soluble metabolites. If the urine was incubated at 37°C for thirty minutes or allowed to remain at room temperature for an hour, the percentage of water-soluble metabolites dropped to 70%, and if left at - 4°C overnight, to 60%, presumably because conjugates of OTA underwent spontaneous hydrolysis. Extraction and freeze-drying of aqueous samples probably resulted in some loss of water-soluble metabolites.

Elimination of OTA occurs by excretion in urine and feces, so to quantify the efficacy of urinary excretion in rats, a dose slightly over ten times that previously used, was given to Wistar and Sprague-Dawley rats, two of each strain. Figure 6.11 shows the six day recovery of [^{3}H]OTA in the urine of these animals, after receiving a single oral dose of [^{3}H]OTA, 3 mg/kg, in bicarbonate 1.67 mL/kg. Approximately 2.6% was excreted in urine in the first six hours after dosing. In six days, about 13.7% of the administered dose was excreted in urine.

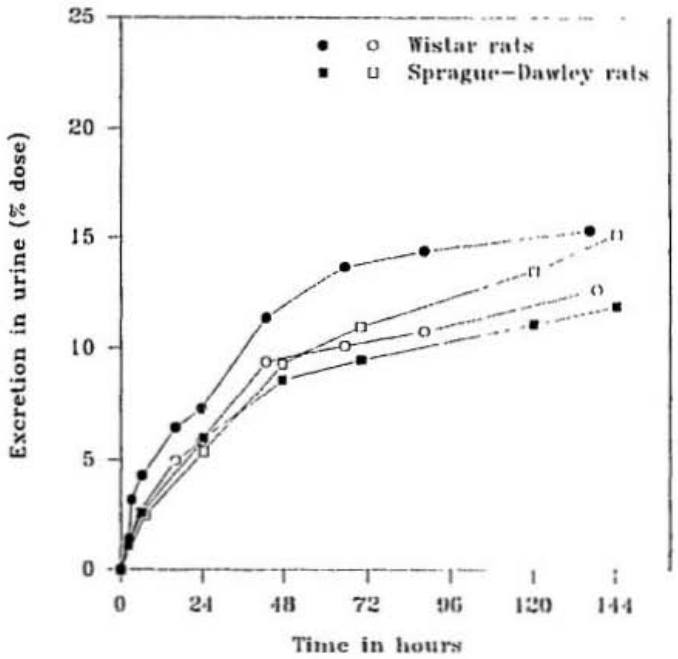


Figure 6.11

Six day recovery of [^{3}H]OTA in urine of four rats given a single dose of [^{3}H]OTA in sodium bicarbonate

Rats were given [^{3}H]OTA, 3 mg/mL, in sodium bicarbonate, 1.67 mL/kg, by oral gavage. Urine was collected for six days.

6.1.9 When lymph, serum and bile samples were acidified prior to extraction, protein, associated with [^{3}H] label ($\geq$ 70% in the case of lymph), precipitated at the aqueous/organic interface. HPLC analysis of extracted samples showed that OTA was carried in the parent form in lymph, Figure 6.12, and serum, Figure 6.13.

6.2 *In vitro* studies

6.2.1 To gain a better understanding of how OTA partitions between the aqueous and lipid phase in gut contents, we examined partition of [^{3}H]OTA between chloroform or corn oil and potassium phosphate buffer, as a function of pH. The percentage of OTA in the two phases (1 mL of each) was equal at $\simeq$ pH 7.1 when chloroform was used as the organic solvent, and 6.4 when corn oil was used, Figure 6.14.

The characteristic fluorescence of OTA under ultraviolet light was clearly seen in the organic or aqueous phase, depending on pH. Counting of [^{3}H] label confirmed the partition as observed visually, and measurement of fluorescence (excitation 340 nm, emission 465 nm) in the aqueous phase of the potassium phosphate buffer/corn oil partition followed the same trend as the radioactivity measurements.

6.2.2 To confirm the pK_a of the phenolic hydroxyl group at position 8, OTA solutions in phosphate buffer were scanned between 300 and 400 nm, as shown in Figure 6.15. The peak at 332 nm represents the protonated phenolic hydroxyl group, and the peak at 380 nm, the unprotonated form, plotted as a function of pH. The extinction coefficient of the unprotonated form of the phenolic hydroxyl group was

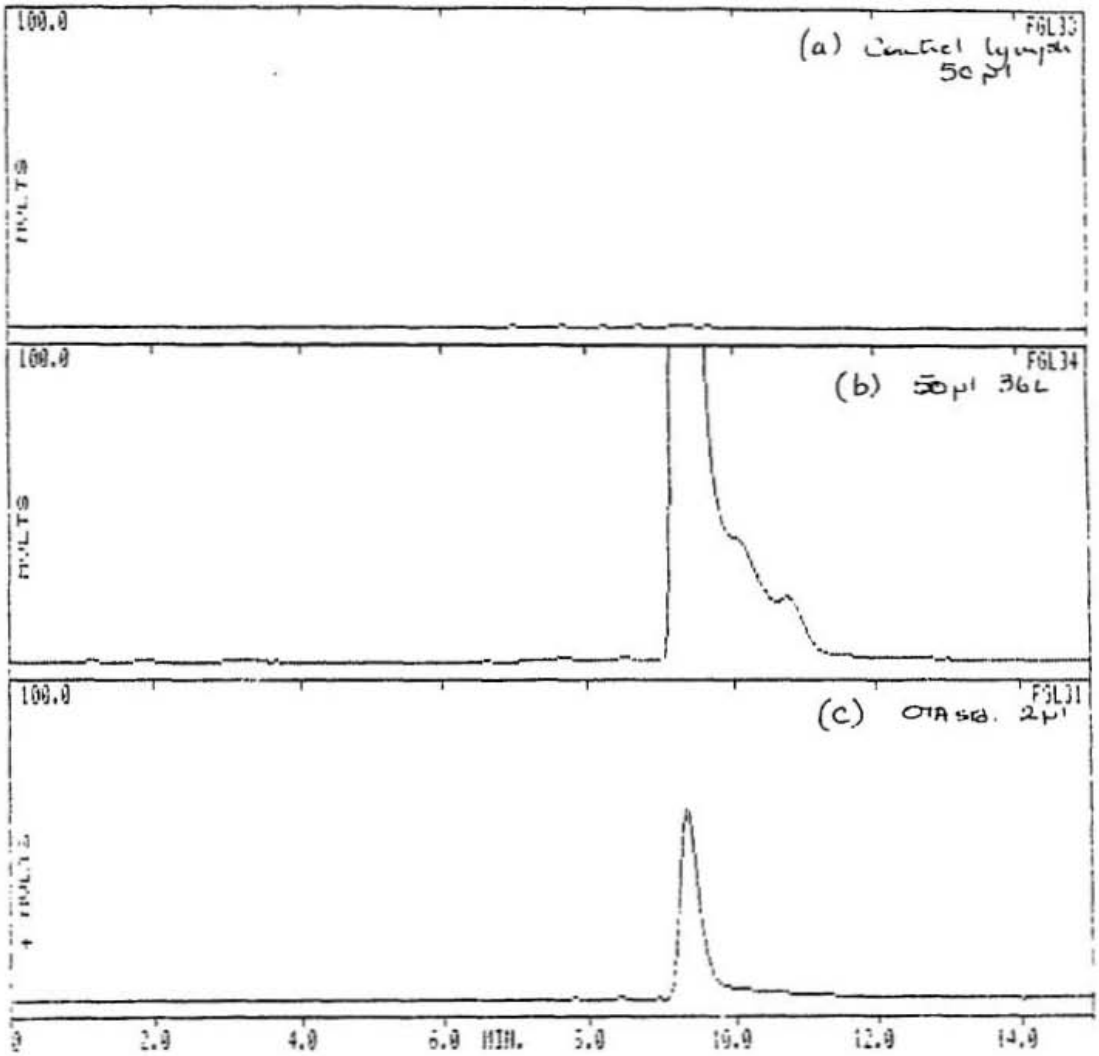


Figure 6.12

HPLC trace of chloroform extract of lymph

Rats were given OTA intraduodenally, 288.8 µg/mL,in corn oil 1.67 mL/kg. Lymph was brought to pH 2, and extracted three times in chloroform. It was dried under N_2, picked up in methanol and filtered through a .45 µm filter prior to HPLC analysis. An LC 18 3.3 cm x 4.6 mm x 3.0 µm column was used with a solvent system of methanol:water, 80:20, flow rate 1mL/minute. Samples were detected by fluorimeter, excitation 340 nm, emission 465 nm. (a) shows a sample of lymph from a control rat; (b) shows lymph from the rat given OTA; (c) is standard OTA.

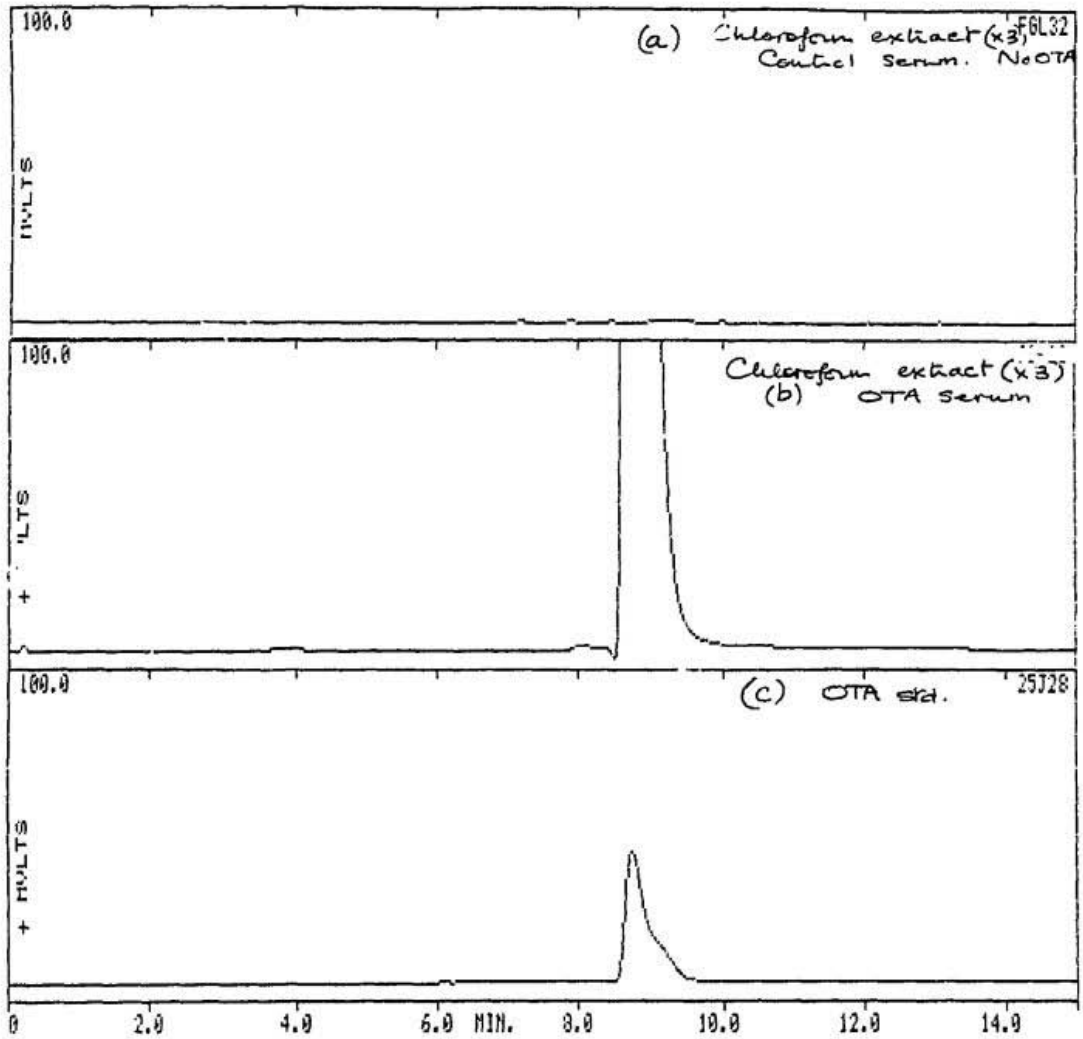


Figure 6.13
HPLC trace of chloroform extract of serum

Rats were given OTA by oral gavage, 288.8 μg/mL,in corn oil 1.67 mL/kg. Serum was obtained by allowing blood drawn from the descending aorta to stand at room temperature for one hour, then centrifuging it for 10 minutes at 250 g to precipitate red blood cells. Serum was then withdrawn by pipette. It was brought to pH 2, and extracted three times in chloroform, dried under N_2, picked up in methanol and filtered through a .45 μm filter prior to HPLC analysis. An LC 18 3.3 cm x 4.6 mm x 3.0 μm column was used with a solvent system of methanol:water, 80:20, flow rate 1mL/min. Samples were detected by fluorimeter, excitation 340 nm, emission 465 nm.
(a) serum from a control rat; (b) serum from a rat given OTA; (c) OTA standard

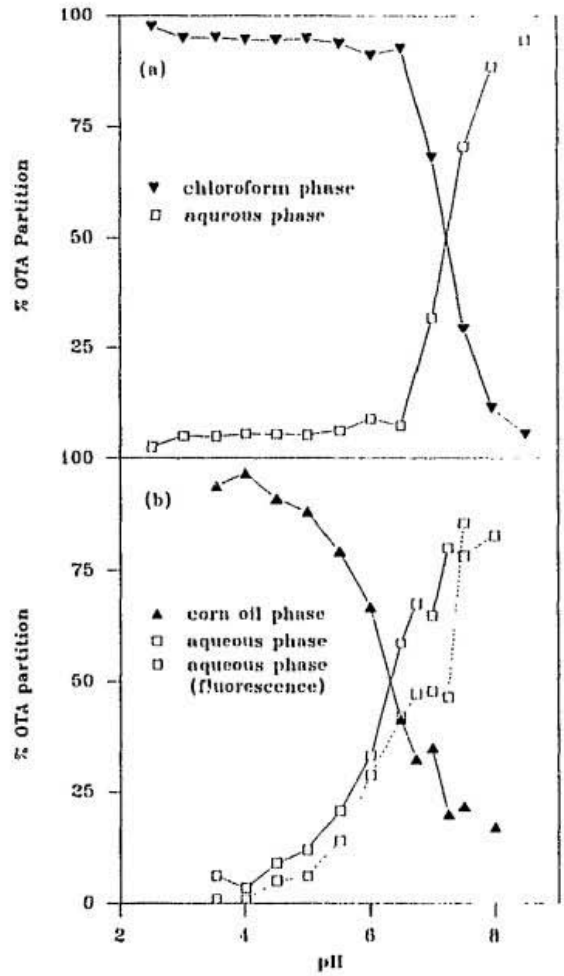


Figure 6.14

Partition of [^{3}H]OTA between aqueous and organic solvents as a function of pH

[^{3}H]OTA was partitioned between chloroform (▾) or corn oil (▴) and potassium phosphate buffer (□) at a range of pH from 2.5 to 8.5 Details are given in Materials and Methods. Chloroform/buffer partition is shown in 6.14.a, and corn oil/buffer partition in 6.14.b. Values shown are [^{3}H] counts in the aqueous and organic phases, expressed as a percentage of total [^{3}H] label recovered, where n = 2 at each pH value. Fluorescence values ----□---- (excitation 340 nm, emission 465 nm) in the aqueous phase (diluted x 5) of the corn oil/buffer partition are shown on the ordinate on a scale of 0 - 200.

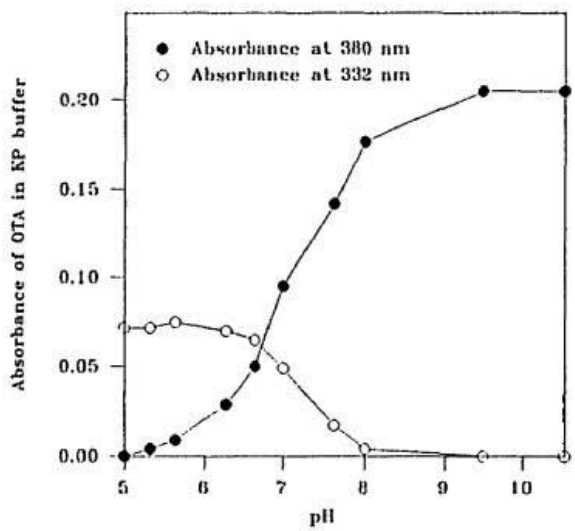


Figure 6.15

Absorbance of OTA in phosphate buffer at 332 and 390 nm

OTA, 8.3 μM, was dissolved in potassum phosphate buffer, 0.1 M, at a range of pH from 5.0 to 10.5. Solutions were scanned between 300 and 400 nm.

nearly three times as great as that of the protonated form, and therefore values for the unprotonated form, given by absorbance readings at 380 nm, were used to calculate the pK. This was identified as being approximately 7.15, the pH at which the height of the peak was half its maximum value. This value is slightly higher than that recorded by Pitout [26], who took the pH (7.1) at which the two peaks were of equal height, or Chu [27], who reported an average value (7.04).

6.2.3 It seemed possible that OTA in bile and urine might be conjugated with glucuronide, so attempts were made to synthesize OTA glucuronide, using UDPGA with UDP-glucuronosyl transferase present in liver microsomes. Although the system worked well to glucuronidate *p*-nitrophenol, there was no evidence of OTA glucuronide formation. When urine and bile were incubated with β-glucuronidase, no difference was seen on thin layer chromatography between extracts of samples treated with enzyme and the control samples without enzyme. Phenolphthalein glucuronide in the same system was readily hydrolysed to liberate phenolphthalein.

6.3.1 Summary of *in vivo* experiments:

	Objective	Result
1.	To observe uptake in organs 12 hours after [^{3}H]OTA in $NaHCO_3$ was given to rats intra-duodenally, with bile.	[^{3}H]OTA uptake was seen in liver (~ 2.7% of dose), kidneys (~ 0.5%), lungs (~ 0.4%0), pancreas (~ 0.3%) and epididymal fat (0.1%). Over the 12 hours, 7.2% of dose was excreted in urine and 20.5% was secreted in bile.
2.	To observe the time-dependent load of [^{3}H]OTA in serum, bile, liver and kidneys 6, 12, 18 and 24 hours after OTA in $NaHCO_3$ was given intra-duodenally with bile.	Recovery of [^{3}H]OTA in serum and organs decreased with time, as did the amount collected in bile over the last 6 hours of each experiment. Although the total amount of [^{3}H]OTA was higher in liver than in kidneys, uptake/g tissue was higher in kidney.
3.	To observe [^{3}H] appearance in bile and lymph over 7 hours after [^{3}H]OTA in $NaHCO_3$ or corn oil was given intragastrically.	[^{3}H] label appeared in bile and lymph within 15 minutes of [^{3}H]OTA administration, and reached peak values within one hour. More [^{3}H]OTA was recovered in bile when $NaHCO_3$ was the vehicle, and conversely, more in lymph when the vehicle was corn oil.
4.	To determine whether phenylalanine given intragastrically at the same time as [^{3}H]OTA affects OTA absorption.	Recovery of [^{3}H]OTA in bile was minimally depressed in the presence of phenylalanine. Uptake of [^{3}H]OTA in lymph was not affected by simultaneously administered phenylalanine.

5.	To note any differences in uptake of [^{3}H]OTA depending on site of administration (stomach or duodenum); to observe the effect of vehicle ($NaHCO_3$ or corn oil) when OTA was given intraduodenally, and when corn oil was used, to investigate the role of bile.	As with (3), recovery of [^{3}H] label in bile was higher when the vehicle was $NaHCO_3$; with intraduodenal administration, this difference was significant at all time points. Similarly, recovery of [^{3}H] label in lymph was enhanced by administration in corn oil; the difference was significant from 1¼ hours onwards. When [^{3}H]OTA was given in corn oil without bile reinfusion, uptake of [^{3}H] label in lymph was not greater than that seen when $NaHCO_3$ was the vehicle; there was no difference in the appearance of [^{3}H] label in bile when corn oil was the vehicle whether or not bile was reinfused. The increased uptake in lymph when corn oil was the vehicle may have been partly due to increase in lymph flow, but micellar solubilization in mixed bile salt/lipid micelles was most likely the principal mechanism enhancing uptake in lymph
6.	To observe the uptake of [^{14}C] and [^{3}H] in lymph of rats given [^{3}H]OTA in corn oil enriched with [^{14}C]trilinolein, and following centrifugation of lymph, to observe the distribution of radio-labels in chylomicrons, VLDL and infranatant. To ascertain how much of the dose remains within the digestive tract 6 hours after administration.	[^{3}H] label appeared in lymph within 15 minutes of dosing, and by two hours most of the [^{3}H]OTA transported in lymph had been recovered. [^{14}C] uptake started slowly and proceeded throughout the six hour experiment. Chylomicrons appeared later than expected, at ~ one hour. Less than 5% of [^{3}H]OTA was found in either chylomicrons or VLDL: 90% was seen in the infranatant. Approximately 60% of [^{14}C] was in chylomicrons, and ~ 34% was in VLDL. Usually, > 80% of unsaturated long-chain fatty acid is found in chylomicrons, so this unusual ratio suggests that lipid handling by the monoglyceride pathway has been impaired. 30% of administered [^{3}H] and 22% of [^{14}C] was found in the distal gut: some of this may have been absorbed and secreted back into the gut, but some may have passed through the gut without being absorbed.

7.	To observe the effect of corn oil on the rate of gastric emptying of an oral dose of [^{3}H]OTA, and to measure [^{3}H]OTA in different regions of the gut lumen and tissues following oral administration of [^{3}H]OTA in either $NaHCO_3$ or corn oil.	Corn oil markedly delayed gastric emptying. Six hours after dosing, no OTA remained in the stomach when $NaHCO_3$ was the vehicle, but between 25 and 70% of the dose remained (depending on duration of fasting prior to dosing) when corn oil was used. In 18 hour-fasted, corn oil rats, in whom 70% of dose was found in the stomach, serum levels were low, suggesting that absorption from the stomach, if it does occur, takes place slowly. Recovery of [^{3}H] label from the distal intestine of these rats was $<$ ½ of that seen in either 24 hour-fasted oil-dosed rats in whom 25% of dose remained, or of the bicarbonate-dosed rats, in whom no OTA was found in the stomach. 5 - 10% of OTA found in stomachs of oil-dosed animals was in the form of Oα. 24 hours after OTA was given, no OTA was found in the stomach of any rat. Of OTA found in $CHCl_3$ extracts of cecum and colon contents, less than 2% was 4-OH-OTA.
8a	To examine the excretion of OTA in urine, and to confirm the identity of metabolites seen in urine. To relate [^{3}H] label in chloroform and aqueous extracts of urine of rats given [^{3}H]OTA to fluorescent peaks seen on HPLC (excitation 340 nm, emission 465 nm).	When urine at pH 2 was immediately extracted in chloroform, 80% of OTA was in the form of water-soluble metabolites; if extraction of samples was delayed, the percentage of OTA which appeared in the aqueous fraction was lower, probably because OTA conjugates underwent spontaneous hydrolysis. HPLC analysis of the chloroform extract gave peaks corresponding with standard OTA and 4(R) 4-OH OTA, as well as an earlier unidentified peak. In the aqueous extract, peaks corresponding with Oα and 4(S) and 4(R) 4-OH OTA standards were seen, and some small unidentified peaks. [^{3}H] label in HPLC effluent agreed with parent OTA peaks in organic and aqueous extracts, and with Oα and 4(R) 4-OH OTA standards in the aqueous extract.
8b	To quantify urinary excretion of OTA in Wistar and Sprague-Dawley rats given [^{3}H]OTA in $NaHCO^3$.	There was no difference in rates of excretion of OTA between Wistar and Sprague-Dawley rats. Approximately 2.6% of dose administered was excreted in the first six hours, but the rate of excretion fell after that so that cumulative excretion in urine at 6 days was 13.6%.
9.	To examine the form in which OTA is carried in lymph and serum.	OTA was tightly bound to protein in both lymph and serum. HPLC analysis showed that unbound OTA was only found in the chloroform extract, and was only seen in the parent form.

6.3.2 Summary of *in vitro* experiments

1.	To examine the partition of OTA between organic and aqueous media as a function of pH.	At low pH, OTA was found in the organic phase, and as the pH rose, it was increasingly found in the aqueous phase. The percentage of OTA was equal in the chloroform and aqueous phases at ~ pH 7.1, and in the corn oil and aqueous phases at ~ 6.4. Fluorescence matched radioactivity.
2.	To observe protonation of the phenolic group of OTA, by scanning its absorbance between 300 and 400 nm.	The pK_a of this group, based on half the maximum peak height of the unprotonated form of the phenolic hydroxyl group, was found to be approximately 7.15.

7. DISCUSSION

The physical characteristics of OTA play a role in both absorption and excretion. The pK_as of the phenylalanine carboxyl group and the phenol are critical in determining the degree of ionization of OTA in the different parts of the gastrointestinal tract. *In vivo*, pH is regulated by secretion of HCl into the stomach, and bicarbonate into the duodenum; in the jejunum, bicarbonate is absorbed while H^+ ions are pumped into the lumen of the gut. Thus pH is maintained in a dynamic fashion at levels characteristic of each region. It was not possible to recreate this system with HCl and bicarbonate *in vitro* and therefore phosphate buffer was used to provide a stable range of pH. Such stability, while useful in demonstrating the effect of pH, lacks the flexibility of the physiological system.

Based on the shift in the absorbance peak of OTA with varying pH, in the experiment described in 5.7.2 and 6.3.2(2), the pK of the phenolic group of OTA was found to be 7.15; this value is slightly higher than that found by Chu [27], who identified the pK of this group as 7.04 (using KCl, and adjusting the pH with HCl), and Pitout [26], who found 7.1 (using phosphate buffer). On the other hand, Galtier *et al.* reported two constants, pK_1 at 6.75 and pK_2 at 10.25, which they attributed to the carboxyl and phenol groups respectively [28]. The accepted pK value for the carboxyl group of phenylalanine alone is 2.58 [144], and it seems improbable that its pK in OTA could be modified by more than four pH units as a result of the linkage of phenylalanine with methylisocoumarin. It appears more likely that Galtier's pK_1 refers to the phenolic group. It may be that the pK_2 they described reflects the opening of the methylisocoumarin lactone ring, which would be expected

at high pH. Titration of OTA against acid and alkali, with the intention of identifying the pK_a of the carboxyl group, was unsuccessful. OTA is only sparingly soluble in aqueous media at acid pH, when it is largely protonated and non-ionized. In the experiment described in 5.7.1 and 6.3.2(1), 50% partition of OTA between chloroform and phosphate buffer, and between corn oil and phosphate buffer, occurred at pH 7.1 and 6.4 respectively (Figure 6.15). The partition constant at any pH is the ratio between the protonated form dissolved in the organic phase, and that dissolved in the aqueous phase, of which some is ionized and some protonated, depending on the pK_a.

Partition of OTA in a two-phase system such as phosphate buffer and corn oil is relevant to its distribution in stomach contents, but less so when partition in duodenal contents is considered. Given the pH prevailing in the stomach, OTA in a meal which contains lipid will to a large extent be found in the oily phase. When food reaches the duodenum and the pH starts to rise, OTA will increasingly partition into the aqueous phase; in the presence of bile, the aqueous phase will contain a variety of lipid aggregates, including mixed bile salt/lipid micelles and bilayer vesicles. Depending on the relative concentrations of bile salts, phospholipids and lipids, various lipid phases may co-exist, and these can be expected to change as digestion and absorption proceed [114]. It is probable that OTA is associated with some or all of the lipid/bile salt aggregates; we have shown that the presence of bile enhances uptake into lymph when corn oil was used as the vehicle for OTA.

In vitro, partition of polycyclic hydrocarbons in simulated intestinal contents has been examined by adding the xenobiotics to a sonicated mixture of buffered solutions of bile salt with triglyceride, monoglyceride, fatty acids and phospholipid. Solubility in the aqueous phase was attributed to true micellar solubilization [145]. It would be interesting to observe the distribution of OTA in such a system.

The importance of the pk_a of the phenolic hydroxyl group at C_8 is related to toxicity of the OTA molecule, which appears to be dependent on ionization of this group [146]. The chlorine atom at C_5 enhances ionization of the hydroxyl group at C_8 by delocalizing electrons of the isocoumarin ring; ochratoxin B, the ochratoxin which lacks the chlorine atom, is at least ten times less toxic than OTA.

The pH in the stomach is approximately 2.0, probably a little higher if buffered by food. OTA will therefore be in the protonated form, and the main mechanism of transfer into the absorptive cell, like that of other weak electrolytes, is likely to be passive diffusion of the non-ionized form across the lipid membrane [147]. Six hours post oral OTA administration, higher levels of OTA were found in stomach tissue than in intestinal tissue (5.5.7 and 6.1.7). Higher OTA levels in stomach tissue were also noted by Galtier [139] who concluded that gastric absorption of OTA is of major importance. It is more likely that OTA found in gastric tissue reflects a slow process occurring over a relatively small area; in quantitative terms gastric absorption is probably less important than absorption through mucosa of the small intestine. In two oil-dosed animals, given OTA by oral gavage after fasting for

18 hours, about 70% of the dose was found in the stomach six hours after dosing; in six hours following OTA administration in these animals, only 30% of the dose had left the stomach, either by absorption or by gastric emptying; approximately 7% of this was found in the distal gut lumen, and 6% was carried in the serum. In two oil-dosed animals starved for 24 hours before dosing, approximately 73% of the administered dose had passed out of the stomach; 15% was recovered from the distal lumen, and approximately 16% was found in serum. In four bicarbonate-dosed animals, 99% of the dose had left the stomach, 17% was found in the distal lumen and serum OTA accounted for 12.1 $\pm$ 2.4%. Excretion in urine was similar in all three groups: 1.8%, 2.1%, 2.0%. These results could be explained by slow absorption in the stomach and faster absorption occurring over the greater surface area of the small intestine. This would be in agreement with the findings of Kumagai and Aibara who demonstrated that the primary site of OTA absorption is the small intestine, with maximum absorption occurring in the jejunum [128].

Food entering the duodenum is initially acid but is soon neutralized by bicarbonate; OTA will thus become increasingly ionized and soluble in the aqueous phase, depending on the prevailing pH. In the proximal jejunum, the pH of the luminal contents varies from 6.8 - 7.2, but a more acid microclimate exists close to the mucosal surface [117], because H^+ ions are secreted as bicarbonate is reabsorbed. The low pH of this region close to the mucosa is maintained by a mucopolysaccharide coating, approximately 700 μm deep, which is secreted by epithelial cells [116].

Kumagai showed increased uptake of OTA by the everted jejunum as the pH of the incubation medium was reduced from 8.8 to 7.4 to 6.8 [148], suggesting that increased protonation of OTA consequent on the fall in pH enhanced uptake in this model. In the ileum, where bicarbonate is actively secreted into the lumen in exchange for chloride ions and the pH is 7.0 - 8.0, OTA is ionized and therefore not so readily absorbed across the lipid membrane.

Intestinal absorption of neutral amino acids has been shown to involve specific carrier-mediated energy- and Na^+-dependent systems, which show specificity for the L-isomers [140]. OTA might be transported in part by this means, in which case one might expect competitive inhibition of OTA absorption by phenylalanine, as suggested by Delacruz and Bach [138]. On the other hand, Roth *et al* [129] reported that gastric absorption of OTA in mice was enhanced in the presence of phenylalanine. The experiment described in 5.5.4 and 6.1.4 offers no evidence to support either of these hypotheses: phenylalanine concurrently administered intragastrically in a molar concentration 10 times that of OTA did not significantly affect OTA uptake in Sprague-Dawley rats. It is possible that the low molar concentrations used in this experiment (0.4 mM OTA and 4.0 mM phenylalanine, in 0.5 mL vehicle) did not approach the capacity of the carrier mechanism, and therefore one cannot rule out OTA transport by this means.

The absorption of foreign compounds is influenced by a number of factors, including the rate of passage through the gut. The presence of fat markedly delays

stomach emptying: in intact rats, a dose of OTA which left the stomach fairly quickly when it was given in bicarbonate took between 12 and 24 hours when it was administered in corn oil (5.5.7 and 6.1.7). The profiles of [^{3}H] uptake in lymph in operated rats hydrated at 3.1 mL/hour were similar whether OTA in corn oil was given into the stomach or the duodenum (experiments 5.5.3 and 5.5.5, with results described in 6.1.3 and 6.1.5, and Figures 6.4 and 6.5); it is probable that stomach emptying occurred more rapidly in these well-hydrated rats than in orally-dosed intact rats allowed free access to fluid.

Solubility in lipids enhances transport *via* the lymphatic system, particularly if the vehicle, like corn oil, consists of triglycerides containing long chain unsaturated fatty acids [149]. On the other hand, the enterocyte may modify lipid-soluble parent compounds to produce more polar metabolites which are more readily carried in portal venous blood. The physical form of a compound or its vehicle may affect both the rate and extent of absorption: many studies of fat absorption have used an emulsified test dose, thus immediately providing a large surface area for lipase action. OTA in the experiments described here was dissolved in non-emulsified corn oil, and therefore lipolysis would start more slowly. Recovery of 25% of [^{14}C] label in the distal lumen after intraduodenal administration (5.5.6, 6.1.6 and Table 6.7) points to the inability of the small intestine to absorb a bolus of lipid which, had it been delivered over some hours in an emulsified form, would have presented no difficulty. The maximum capacity of the small intestine for fatty acid absorption is about

500 μmol/hour [150], equivalent to 0.88 g trilinolein over six hours.
Of the 29% of administered [^{3}H] label recovered in distal intestinal contents, some had passed through the intestinal tract without being absorbed, and some had been absorbed and then returned to the intestine by biliary excretion. Based on biliary recovery of [^{3}H] label from rats given [^{3}H]OTA in corn oil in experiments 5.5.3 and 5.5.5, (see Tables 6.3 and 6.5) ~ 10% of the administered dose had been excreted in bile. In retrospect, it is clear that the administration of a bolus dose of OTA in non-emulsified corn oil resulted in lower absorption of both lipid vehicle and toxin, whereas administration in sodium bicarbonate maximized absorption. This should be borne in mind when recoveries of OTA are compared.

Blood flow in the portal vein is much greater than mesenteric lymph flow (by a factor of about 500) [151], so if a substance is partitioned between portal venous blood and lymph, as is the case with OTA, the lymphatic route is quantitatively less important. In spite of these considerations, it is clear that the lymphatic route plays a role in the transport of absorbed OTA. When OTA was administered intraduodenally in bicarbonate, 11% of the dose was recovered in lymph over 6 hours, by comparison with 20% when administered in corn oil (experiment 5.5.5, Table 6.5).
Total recovery of label in lymph, bile, serum and urine was somewhat lower (41% compared with 49%) when corn oil was the vehicle, but the important difference lay in the route of absorption. The enhanced lymphatic transport of OTA when it is administered in corn oil may be due in part to the increased lymph flow which occurs

when lipids are ingested, but Tables 6.6 and 6.7, which show the relationship between lymph flow and OTA uptake, suggest that other mechanisms may also be involved.

In the absence of reinfused bile, recovery of [^{3}H]OTA in lymph was not increased even in the presence of corn oil (Figure 6.4, Table 6.5). Thus both lipid and bile are necessary for maximum transport of OTA in lymph. This suggests the incorporation of OTA in mixed bile salt/lipid micelles [152] as an important means of OTA transport to the enterocyte. The dual polar/non-polar structure of OTA would be well suited to micellar solubilization, with the isocoumarin ring oriented towards the hydrophobic centre of the micelle. Micelles facilitate passage of lipid products, particularly long-chain fatty acids and cholesterol, across the unstirred water layer adjacent to the brush border. This unstirred water layer appears to be part of the same phenomenon as the acid microclimate [117] and mucopolysaccharide coating [116] described by Lucas and Shiau. The pH of this layer, thought to be about 5.4, favours both micellar diffusion through the aqueous medium and micellar dissociation as fatty acids within the micelle become more protonated and therefore less soluble in bile salt micelles [118]. Lipid products are released close to the membrane of the absorptive cell, and can diffuse into the membrane and so be absorbed. If OTA is carried in mixed lipid/bile salt micelles, similar considerations might apply. In the absence of dietary lipid, bile salts if present above the critical micellar concentration aggregate with biliary phospholipid and cholesterol to form micelles and other similar structures smaller than the mixed bile salt/lipid micelles

[114]; OTA is probably to some extent associated with these aggregates. In the absence of bile, approximately 10% of the administered dose still appeared in lymph (Table 6.5), so it seems that OTA can also traverse the unstirred water layer by itself, unassociated with micelles. Micellar solubilization in mixed bile salt/lipid micelles appears to increase the amount carried in lymph by 9 - 10%, *i.e* to double it, at the expense of portal venous transport. OTA carried in association with lipid in mixed bile salt micelles seems destined to appear in lymph. It is possible that the higher uptake of OTA/mL of lymph when both lipid and bile are present is to some extent due to increased efficiency of OTA transport across the unstirred water layer.

In spite of the association of OTA with lipid during absorption into the enterocyte, it is not exported into lymph with lipid in chylomicra or VLDL, but is clearly associated with the heavier components of lymph, presumably serum proteins (experiment 5.5.6, results described in 6.1.6 and Figure 6.7). Probably because it was administered in non-emulsified corn oil, OTA uptake occurred at the onset of lipolysis, rather than during the steady state phase of lipid processing (Figure 6.6). The profile of centrifuged lymph shows a distribution of [^{14}C]trilinolein in chylomicrons and VLDL was different from that expected (> 80% in chylomicrons following infusion of emulsified long-chain unsaturated fatty acid) with only 55% and 65% in the (1 + 2) and (4 + 5) hour samples respectively. This may reflect to some extent the slow onset of lipolysis when a bolus dose of non-emulsified corn oil was given after 24 hours of fasting, with consequent prolongation of activity of the

α-glycerophosphate pathway of lipoprotein (VLDL) synthesis. Alternatively, it may indicate that OTA impairs triglyceride handling by the monoglyceride (chylomicron-synthesizing) pathway. The delayed chylomicron appearance time (greater than one hour, by comparison with the 13.6 minutes noted by Tso [153]) might be in keeping with this possibility.

An indication of OTA transport *via* the portal vein is given by measurement of [^{3}H] label in bile. In the experimental preparation described here, when lymph was collected from the main intestinal lymphatic vessel, it was assumed that no OTA could reach the general circulation by the lymphatic route, *i.e.* that the contribution of minor mesenteric lymphatic vessels was negligible. The only route by which OTA could reach the liver and the general circulation was *via* the portal vein. Portal venous transport can therefore be estimated by taking the sum of OTA recovery in serum, bile and organs. Twelve hours after intraduodenal administration of OTA in $NaHCO_3$ to bile fistula rats (experiment 5.1.1), we found approximately 2.2% of the dose in the liver, 0.5% in the kidneys and 0.4% in the lungs, (Table 6.1). Galtier [127] demonstrated substantial uptake of [^{14}C] label in muscle (12.0%), skin (11.1%) and fat (1.7%), with 2.6% in the liver, 0.6% in the kidneys and 0.6% in the lungs, (total 28.6%) 6 hours after intravenous administration of [^{14}C]OTA in $NaHCO_3$. Bile and lymph were not withdrawn from the rats in his experiments. Even allowing for differences in experimental design, it is clear that both skin and muscle are important depots of OTA. Organ uptake is related to serum levels: where Galtier gave 24% as

the six hour (plasma) OTA load, we found 19.8 and 8.8% in the B+ and C+ groups respectively (Table 6.5). A conservative estimate of OTA distributed to organs, including muscle and skin, in our system would be ~ 20% and 10%. When OTA was given in sodium bicarbonate 38% of [^{3}H] label was recovered in bile, serum and urine (Table 6.5), plus (assumed) 20% in tissues. 11% was recovered in lymph, so that for the [^{3}H]OTA recovered (~ 70% of dose), the ratio of portal venous to lymphatic transport was ~ 5:1. When corn oil was the vehicle, 21.5% was recovered in bile, serum and urine, plus 10% in tissues; 20% was recovered in lymph. Thus for the 50% of dose recovered, portal:lymphatic ratio for oil-dosed rats was ~ 3:2. No account has been taken of unabsorbed [^{3}H]OTA in the C+ group.

Kumagai and Aibara studied OTA absorption in rats by isolating different regions of the gut *in situ*, and injecting the test dose directly into the lumen [128]. They demonstrated that OTA absorption occurred most effectively from the jejunum. They then studied the appearance of OTA in lymph and portal venous blood after injection of OTA (18 - 85 μg in 0.3 mL Krebs-Ringer solution) into the proximal jejunum. At the highest dose, fifty times as much OTA was taken up in the portal venous system as in lymph, but at the lowest dose there was only a four-fold difference. Kumagai and Aibara concluded that the primary route of OTA absorption is the portal vein, but the lower the luminal concentration of OTA, the greater the role played by the lymphatic route in absorption. The amount of OTA appearing in lymph over the first thirty minutes after OTA was given was less than 2% of the

administered dose. Mean lymph flow in their series of 41 rats was 246 μL/10 minutes, whereas in our lymph uptake studies, rats with lymph flow less than 3.0 mL over the first two hours (250 μL/10 minutes) were not included in analysis of data. It is possible that in the experimental system used by Kumagai and Aibara lymph flow was to some extent obstructed.

The mechanism of absorption of OTA *via* the portal vein is not clear. When OTA is given in corn oil, recovery of [^{3}H] in bile was not affected by the presence or absence of reinfused bile (experiment 5.5.5, results reported in 6.1.5 and Table 6.5). Thus it is probable in this case that a mechanism other than micellar solubilization is responsible for the transport of OTA *via* the portal vein. It is known that a substantial proportion of unsaturated long-chain fatty acids is transported from rat intestine in portal venous blood [154,155], particularly when absorption rates are low. OTA might diffuse across the unstirred water layer in the ionized form, as do short and a proportion of medium chain fatty acids. Passage across the lipid membrane of absorptive epithelial cells would depend on the permeability coefficient and the concentration of OTA on the two sides of the membrane, assuming this is a passive process. It is possible (but, based on chemical structure, unlikely) that OTA might be bound in the cell by the fatty acid binding protein [122] or a similar carrier, or it could diffuse in the free form, to pass out of the cell into blood capillaries which drain into the portal venous system and so to the liver. In the circulatory system, macromolecules in the serum bind OTA very efficiently and thus create the gradient

necessary to facilitate passage of OTA across the membrane.

Elimination of foreign organic compounds and their metabolites occurs through the complementary pathways of biliary and urinary excretion. In the rat, organic anions of relatively low molecular weight (< 300) are poorly excreted in bile (< 10% of dose), whereas those of higher molecular weight (> 500) are predominantly excreted in bile. Organic anions of intermediate molecular weight (350 - 450) such as OTA (m.w. 404) are excreted extensively *via* both pathways [156]. In humans, appreciable biliary excretion of organic anions only occurs when the molecular weight is above about 500 [157], so it is probable that OTA is to a large extent excreted in urine, with a smaller proportion excreted in conjugated form in bile. The general rule holds true that urinary excretion is greatest for compounds of low molecular weight, and decreases as molecular weight rises and biliary excretion becomes more extensive. Biliary output of OTA reflects the amount reaching the liver; initially this is a function of portal venous transport but subsequently, because OTA persists in the serum long after the OTA has been absorbed, there will be continuous low-level excretion in both bile and urine, gradually depleting the level of circulating OTA. In the normal situation, biliary secretions are subject to enterohepatic circulation, so that OTA and metabolites excreted in bile are recirculated. Hydrolysis of OTA metabolites by anaerobic gut flora in the cecum and colon would explain the high levels of parent OTA in these samples. Recovery of [^{3}H] label from the distal parts of the gut represents both

unabsorbed OTA and OTA which has been absorbed and subsequently excreted in bile.

The disparity of serum OTA levels (21% or 9%) apparently dependent on whether OTA was given in bicarbonate or corn oil is most likely a consequence of the experimental design (5.5.5, Table 6.5). When OTA is given intraduodenally in corn oil, the lymphatic route assumes greater importance than when OTA is administered in bicarbonate, and withdrawing all the mesenteric lymph rather than allowing it to reach the circulation clearly has a marked impact on serum levels.

The significance of lymphatic transport of substances absorbed from the gastrointestinal tract is that xenobiotic compounds are delivered directly into the general circulation *via* the thoracic duct, thus avoiding "first-pass" hepatic metabolism. OTA binding to plasma proteins ensures a long half-life. Stojković *et al.* [71] proposed that binding of OTA to low molecular weight (20,000) plasma constituents might be a means of delivery of OTA to the primary target organ, the kidney, because molecules of this size can pass through the glomerular membrane where albumin could not. Busbee *et al.* suggested in the context of benzo[a]pyrene metabolism that mesenteric lymph node cells and circulating lymphocytes in the thoracic duct could be exposed to transiently high concentrations of xenobiotic compounds transported in the lymphatic system [158]. This may contribute to the immunosuppressive effects of OTA [76,159]. The increased lymphatic transport which accompanies lipid intake may thus enhance the toxicity of OTA.

CONCLUSION

This study points out the role of fat in influencing the absorption of xenobiotics. Increased fat consumption is associated with an increased likelihood of developing certain cancers, *e.g.* of the colon and perhaps also the breast.

Ingestion of OTA in corn oil rather than sodium bicarbonate doubles the proportion of absorbed OTA carried in lymph. This increased lymphatic transport of OTA may contribute to the immunosuppressive effects of OTA by exposing mesenteric lymph nodes to transiently high concentrations of the toxin. Lymphatic transport bypasses the metabolizing enzymes of the liver, and unmetabolized OTA is delivered directly into the circulation where it binds extremely strongly to serum proteins, so prolonging the serum half-life. The toxicity of OTA may therefore be enhanced by ingestion in the presence of lipid.

BIBLIOGRAPHY AND REFERENCES

1. Ingold CT. *Biology of Fungi*, 5th edition. Hutchinson, London, 1984.
2. Deacon JW. *Introduction to Modern Mycology*. Blackwell Scientific Publications, Oxford, U.K., 1980.
3. Griffin DH. *Fungal Physiology*, pp. 95 - 98. John Wiley and Sons, Toronto, 1981.
4. Weinberg ED. Biosynthesis of secondary metabolites; role of trace metals. *Adv Microb Physiol* **4**: 1 - 44, 1970.
5. **I** *Kings* 8: 37 (10th century B.C.), *Amos* 4: 9 (8th century B.C.), *Deuteronomy* 28: 22 (7th century B.C.), *Haggai* 2: 17 (6th century B.C.), In: *The Bible*, King James Version, 1611.
6. Xenophon. *Oeconomicus* 5: 18, approximately 5th or 4th century B.C. In : *Opera omnia*, (Ed. EC Marchant), Clarendon Press, 1900.
7. Plato. *Symposium*, 188B, 5th and 4th centuries B.C. (Ed. WRM Lamb), William Heinemann, London, 1925.
8. Theophrastus. *De Causis Plantarum* (Enquiry into Plants) **VIII**, 3: 2, 4th and 3rd centuries B.C., William Heinemann, 1916.
9. Pliny (the elder). *Historia Naturalis* (Natural History), **XVIII**, 68: 275 - 69: 279. (Ed. H Rackham), William Heinemann Ltd., London, 1938.
10. Plato. *Republica* (The Republic), Book **X**, 609A, 5th and 4th century B.C. (Ed. P Shorey), Loeb, **2**, William Heinemann, London, 1930.
11. L. Junius Moderatus Columella. *De Re Rustica* (On Agriculture) Book **II**, 11: 5, approximately 50 A.D. (Ed. HB Ash), Harvard University Press, 1954.

12. L. Junius Moderatus Columella. *De Arboribus* (On Trees) 12: 13. (Ed. ES Forster), Harvard University Press, 1954.

13. Larsen S. Om kronisk degeneration av nyrerne (Nefrose) hos svin foraasaget af skimlet rug (Chronic degenerative changes seen in kidneys of pigs fed with mouldy rye). *Dan Maanedskr Dyrlæg* **40**: 259 - 284, 289 - 300, 1928. Original not seen, cited in reference (14).

14. Lillehoj E and Elling F. Environmental conditions that facilitate ochratoxin contamination of agricultural commodities. *Acta Agric Scand* **33**: 113 - 128, 1983.

15. Ivanov LM. Studies on the etiology of renal diseases in the district of Vlatza. *Sovrem Med* **7**(9): 30 - 34, 1956. Original not seen, cited in reference (16).

16. Tanchev Y and Dorossiev D. The first clinical description of Balkan Endemic Nephropathy (1956) and its validity 35 years later. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Pleština, G Dirheimer, I Chernozemsky and H Bartsch), pp. 21 - 28. IARC Scientific Publications No. 115, Lyon, 1991.

17. van der Merwe KJ, Steyn PS, Fourie L, Scott de B and Theron JJ. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus*. Wilh. *Nature*, **205**: 1112 - 1113, 1965.

18. Abramson D, Sinha RN and Mills JT. Mycotoxin formation and quality changes in granary-stored corn at 16 and 21% moisture content. *Sci Aliments* **5**: 654 - 663, 1985.

19. Ciegler A. Bioproduction of ochratoxin A and penicillic acid by members of the *Aspergillus ochraceus* group. *Can J Microbiol* **18**: 631 - 636, 1971.

20. Bacon CW, Sweeney JG, Robbins JD and Burdick D. Production of penicillic acid and ochratoxin A on poultry feed by *Aspergillus ochraceus:* temperature and moisture requirements. *Appl Microbiol* **26**(2): 155 - 160, 1973.

21. Madhyastha SM, Marquadt RR, Frohlich AA, Platford G and Abramson D. Effects of different cereal and oilseed substrates on the growth and production of toxins by *Aspergillus alutaceus* and *Penicillium verrucosum.* *J Agric Food Chem* **38**: 1506 - 1510, 1990.

22. Sansing GA, Davis ND and Diener UL. Effect of time and temperature on Ochratoxin A production by *Aspergillus ochraceus. Can J Microbiol* **19**: 1259 - 1263, 1973.

23. Marquadt RR, Frohlich A and Abramson D. Ochratoxin A: an important western Canadian storage mycotoxin. *Can J Physiol Pharmacol* **68**: 991 - 999, 1990.

24. Støren O, Holm H and Størmer F. Metabolism of ochratoxin A by rats. *Appl Environ Microbiol* **44**(4): 785 - 789, 1982.

25. Kuiper-Goodman T and Scott PM. Risk assessment of the mycotoxin Ochratoxin A. *Biomed Environ Sci* **2**: 179 - 248, 1989.

26. Pitout MJ. The effect of Ochratoxin A on glycogen storage in the rat liver. *Tox Appl Pharmacol* **13**: 299 - 306, 1968.

27. Chu FS, Interaction of Ochratoxin A with bovine serum albumin. *Arch Biochem Biophys* **147**: 359 - 366, 1971.

28. Galtier P, Baradat C and Alvinerie M. Etude de l'élimination d'ochratoxine A par le lait chez la lapine. *Ann Nutr Alim* **31**: 911-918, 1977.

29. Searcy JW, Davis WD and Diener UL. Biosynthesis of Ochratoxin A. *Appl Microbiol* **18**(4): 622 - 627, 1969.

30. Steyn PS, Holzapfel CW and Ferreira NP. The biosynthesis of the ochratoxins, metabolites of *Aspergillus ochraceus*. *Phytochem* **9**: 1977 - 1983, 1970.

31. Wei R-D, Strong FM and Smalley EB. Incorporation of chlorine-36 into Ochratoxin A. *Appl Microbiol* **22**(3): 276 - 277, 1971.

32. Yamazaki M, Maebayashi Y and Miyaki K. Biosynthesis of ochratoxin A. *Tetrahedron Letters* **25**: 2301 - 2303, 1971.

33. Vlegaar R and Steyn P. Ochratoxins and Related Dihydroisocoumarins. In: *The Biosynthesis of Mycotoxins: a study in secondary metabolism*, pp. 397 - 399. (Ed. P. S. Steyn), Academic Press, New York, 1980.

34. Shotwell O, Hesseltine CW and Goulden ML. Note on the natural occurrence of Ochratoxin A. *A J Ass Off Oil Chem* **50**: 366 - 370, 1969.

35. Scott PM, van Walbeek W, Harwig J and Fennell DI.
Occurrence of a mycotoxin, Ochratoxin A, in wheat and isolation of Ochratoxin A and citrinin producing strains of *Penicillium viridicatum*.
Can J Plant Sci **50**: 583 - 585, 1970.

36. Chelkowski J, Golinski P and Szebiotko K. Mycotoxins in cereal grain. Part II. The fate of Ochratoxin A after processing of wheat and barley grain.
Die Nahrung **25**(5): 423 - 426, 1981.

37. Frank HK. Food contamination by Ochratoxin A in Germany.
In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours*
(Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 77 - 81. IARC Scientific Publications No. **115**, Lyon, 1991.

38. Srinivasan M. Ochratoxins A and B occurrence in sorghum - analytical studies.
International Mycotoxin Conference, Cairo, Egypt, Abstract No. 28, 1983. Original not seen, cited in reference (25).

39. Petkova-Bocharova T and Castegnaro M. Ochratoxin A contamination of cereals in an area of high incidence of Balkan endemic nephropathy in Bulgaria.
Food Add Contam **2**(4): 267 - 270, 1985.

40. Yamazaki M, Maebayashi Y and Miyaki K. Production of Ochratoxin A by *Aspergillus ochraceus* isolated in Japan from moldy rice.
Appl Microbiol **20**: 452 - 454, 1970.

41. Cooper P. The kidney and Ochratoxin A. *Food Cosmet Toxicol* **17**: 406 - 408, 1979.

42. Jelinek CF, Pohland AE and Wood GE. Worldwide occurrence of mycotoxins in foods and feeds - an update. *J Assoc Anal Chem* **72**(2): 223 - 230, 1989.

43. El-Banna AA and Scott PM. Fate of mycotoxins during processing of foodstuffs. III. Ochratoxin A during cooking of Faba beans (*Vicia faba*) and polished wheat. *J Food Prot* **47**(3): 189 - 192, 1984.

44. Josefsson B and Möller T. Heat stability of Ochratoxin A in pig products. *Sci Food Agric* **31**: 1313 - 1315, 1980.

45. Chelkowski J, Szebiotko K, Goliński P, Buchowski M, Godlewska B, Radomyska W and Wieworówska M. Mycotoxins in cereal grain. V. Changes of cereal grain biological value after ammoniation and mycotoxins (ochratoxins) inactivation. *Nahrung* **26**: 1 - 7, 1982b.

46. Kostecki M, Goliński, Uchman W and Grabarkiewicz-Szczęsna J. Decomposition of ochratoxin A by heat and γ-irradiation. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 109 - 111. IARC Scientific Publications No. **115**, Lyon, 1991.

47. Tholstrup B and Rasmussen G. Ochratoxin A in cereals 1986 - 1989. (Publication No. **199**) Copenhagen, Statens Levnedsmiddelinstitut, p. 50, 1990. Original not seen, cited in reference (59).

48. Petkova-Bocharova T, Castegnaro M, Michelon J and Maru V. Ochratoxin A and other mycotoxins in cereals from an area of Balkan endemic nephropathy and urinary tract tumours in Bulgaria. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 83 - 87. IARC Scientific Publications No. **115**, Lyon, 1991.

49. Sinha R, Abramson D, Mills J. Interrelations between ecological variables in stored cereals and associations with mycotoxin production in the climatic zones of western Canada. *J Food Prot* **49**: 608 - 614, 1986.

50. Abramson D, Mills J and Boycott B. Mycotoxins and mycoflora in animal feedstuffs in western Canada. *Can J Comp Med* **47**: 23 - 26, 1983.

51. Payen J, Girard T, Gaillardin M and Lafont P. Sur la présence de mycotoxines dans des bières. *Microbiol Aliments Nutr* **1**: 143 - 146, 1983.

52. Bauer J and Gareis M. Ochratoxin A in der Nahrungsmittelkette. *Z Veterinärmed* B **34**: 613 - 627, 1987.

53. Tsubouchi H, Terada H, Yamamoto K, Hisada K and Sakabe Y. Ochratoxin A found in commercial roast coffee. *J Agric Food Chem* **36**: 540 - 542, 1988.

54. Tantaoui-Elaraki A and Le Tutour B. Contamination éventuelle des olives et dérivés par les mycotoxines: Le point. *Oléagineux* **40**: 451 - 454, 1985.

55. Chelkowski J and Goliński P. Mycotoxins and toxigenic fungi in mixed feeds and their cereal components. In: *Proceedings, V International IUPAC Symposium. Mycotoxins and Phycotoxins*, Vienna, Austria, pp. 68 - 71, Austrian Chem Soc, 1982.

56. Goliński P, Grabarkiewicz-Szczęsna J, Chelkowski J, Hult K and Kostecki M. Possible sources of Ochratoxin A in human blood in Poland. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 153 - 158. IARC Publications, No. **115**, Lyon, France, 1991.

57. Norton D, Toule G, Cooper S, Partington S and Chapman W. The surveillance of mycotoxins in human food. In: *Proceedings, Fourth Meeting on Mycotoxins in Animal Disease*. (Eds. G Pepin, D Patterson, D Gray), pp. 77 - 81. Ministry of Agriculture, Fisheries and Food, U.K., 1982.

58. Pepeljnjak S. Mycotoxic contamination of haricot beans in nephropathic areas in Yugoslavia. *Microbiol Aliment Nutr* **2**: 331 - 336, 1984.

59. Hald B. Porcine nephropathy in Europe (Danish figures quoted with permission from the Danish Veterinary Services.) In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 49 - 56. IARC Scientific Publications No. **115**, Lyon, 1991.

60. Scheuer R and Leistner L. Occurrence of Ochratoxin A in slaughter pigs and meat products. *Mitt Bundesanst Fleischforsch* **88**: 6436 - 6439, 1985.

61. Steering Group on Food Surveillance, *Mycotoxins*. Ministry of Agriculture, Fisheries and Food, Her Majesty's Stationery Office, London, U.K., 1987.

62. Cooper S, Wood G, Chapman W and Williams A. Mycotoxins occurring in mould-damaged foods. In: *Proceedings, V International IUPAC Symposium. Mycotoxins and Phycotoxins*, Vienna, Austria, pp. 64 - 67. Austrian Chem Soc, Vienna, 1982.

63. Pepeljnjak S and Blažević N. Contamination with moulds and occurrence of Ochratoxin A in smoked meat products from endemic nephropathy region of Yugoslavia. In: *Proceedings, V International IUPAC Symposium. Mycotoxins and Phycotoxins*, Vienna, Austria, pp.102 - 105. Austrian Chem Soc, Vienna, 1982.

64. Dirheimer G and Creppy EE. Mechanism of action of Ochratoxin A. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 171 - 186. IARC Scientific Publications No. **115**, Lyon, France, 1991.

65. Harwig J, Kuiper-Goodman T and Scott PM. Microbial food toxicants: Ochratoxins. In: *Handbook of Foodborne Diseases of Biological Origin* (Ed. M Rechcig), pp. 193 - 238, CRC Press, Boca Raton FL, 1983.

66. National Institute of Occupational Safety and Health, Registry of toxic effects of chemical substances - microfiche edition. NIOSH, Cincinnati, Ohio, July 1986.

67. World Health Organization. Selected ochratoxins, 1. Ochratoxin A. *Environmental Health Criteria* **105**: 13 - 69, 183 - 232. WHO, United Nations Environment Programme and the International Labour Organizations, Geneva, 1990.

68. Khan S, Martin M, Bartsch H and Rahimtula AD. Perturbation of liver microsomal calcium homeostasis by ochratoxin A. *Biochem Pharmacol* **38**: 67 - 72, 1989.

69. Omar RF, Rahimtula AD and Bartsch H. Role of cytochrome P-450 in ochratoxin A-stimulated lipid peroxidation. *J Biochem Toxicol* **6**(3): 203 - 209, 1991.

70. Hagelberg S, Hult K and Fuchs R. Toxicokinetics of ochratoxin A in several species and its plasma-binding properties. *J Appl Toxicol* **9**: 91 - 96, 1989.

71. Stojković R, Hult K, Gamulin S, Plěstina R. High affinity binding of Ochratoxin A to plasma constituents. *Biochem Int* **9**(1): 33 - 38, 1984.

72. Krogh P, Gyrd-Hansen N, Hald B, Larsen S, Nielsen J, Smith M, Ivanoff C, Meisner H. Renal enzyme activities in experimental ochratoxin A-induced porcine nephropathy: diagnostic potential of phosphoenolpyruvate carboxykinase and gamma-glutamyl transpeptidase activity. *J Toxicol Environ Health* **23**: 1 - 14, 1988.

73. Konrad I and Röschenthaler R. Inhibition of tRNA synthetase from *Bacillus subtilis* by Ochratoxin A. *FEBS Letter* **83**(2): 341 - 347, 1977.

74. Bunge I, Dirheimer G, Röschenthaler R. *In vivo* and *in vitro* inhibition of protein synthesis in *Bacillus stearothermophilus* by Ochratoxin A. *Biochem Biophys Res Commun* **83**(2): 398 - 405, 1978.

75. Creppy E, Röschenthaler R and Dirheimer G. Inhibition of protein synthesis in mice by Ochratoxin A and its prevention by phenylalanine. *Food Chem Toxicol* **22**: 883 - 886, 1984.

76. Haubeck HD, Lorkowski G, Kölsch E and Röschenthaler R. Immunosuppression by Ochratoxin A and its prevention by phenylalanine. *Appl Environ Microbiol* **41**(4): 1040 - 1042, 1981.

77. Chernozemsky IM. Balkan endemic nephropathy and the associated tumours of the urinary system: a summary of epidemiological areas in Bulgaria. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 3,4, IARC Scientific Publications No. **115**, Lyon, 1991.

78. Čeović S, Pleština R, Miletić-Medved M, Stavljenić A, Mitar J and Vukelić M. Epidemiological aspects of Balkan endemic nephropathy in a typical focus in Yugoslavia. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 5 - 10. IARC Scientific Publications No. **115**, Lyon, 1991.

79. Pavlovic M, Plěstina R and Krogh P. Ochratoxin A contamination of foodstuffs in an area with Balkan (endemic) nephropathy. *Acta Pathologica Microbiologica Scandinavica*, Section B, **87**: 243 - 246, 1979.

80. Pringle C, Vellidis G, Heliotis F, Bandacu D and Cristofor S. Environmental problems of the Danube delta. *Am Sci* **81**(4): 350 - 361, 1993.

81. Gareis M, Märtlbauer E, Bauer J and Gedeck B. Bestimmung von Ochratoxin A in Muttermilch. *Z Lebensm Unters Forsch* **186**(2): 114 - 117, 1988.

82. Micco C, Ambruzzi M, Miraglia M, Brera C, Onori R and Benelli L. Contamination of human milk with Ochratoxin A. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky I and H Bartsch), pp. 105 -108. IARC Scientific Publications No. **115**, Lyon, 1991.

83. Castegnaro M, Maru V, Petkova-Bocharova T, Nikolov I and Bartsch H. Concentrations of Ochratoxin A in the urine of endemic nephropathy patients and controls in Bulgaria: lack of detection of 4-hydroxyochratoxin A. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 165 -169. IARC Scientific Publications No. **115**, Lyon, 1991.

84. Castegnaro M and Chernozemsky I. Endemic Nephropathy and Urinary Tract Tumors in the Balkans. *Cancer Res* **47**: 3608 - 3609, 1987.

85. Kuiper-Goodman T. Risk assessment of Ochratoxin A residues in food. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours*, (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 307 - 320. IARC Publications, No. **115**, Lyon, France, 1991.

86. National Toxicology Program (1989). NTP Technical Report on the Toxicology and Carcinogenesis Studies of Ochratoxin A (CAS No. 303-47-9) in F344/N Rats (Gavage Studies) (Ed. G Boorman), NIH Publication No. **89-2813**. U.S. Department of Health and Human Services, National Institutes of Health, Research Triangle Park, NC, 1989.

87. WHO Evaluation of Certain Food Additives and Contaminants. *Thirty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives* (WHO Technical Report Series **806**), pp. 28 - 31, Geneva, 1991.

88. Petkova-Bocharova T and Castegnaro M. Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary tract tumours in Bulgaria. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 135 - 137. IARC Scientific Publications No. **115**, Lyon, 1991.

89. Frohlich AA, Marquadt RR and Ominski KH. Ochratoxin A as a contaminant in the human food chain: a Canadian perspective. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 139 - 143, IARC Scientific Publications No. **115**, Lyon, 1991.

90. Hald B. Ochratoxin A in human blood in European countries. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 159 - 164, IARC Scientific Publications No. **115**, Lyon, 1991.

91. Creppy EE, Betbeder AM, Gharbi A, Counord J, Castegnaro M, Bartsch H, Moncharmont P, Fouillet B, Chambon P and Dirheimer G. Human ochratosis in France. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 145 - 151. IARC Scientific Publications No. **115**, Lyon, 1991.

92. Fuchs R, Radić B, Čeović S, Šoštarić B and Hult K. Human exposure to ochratoxin A. In: *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* (Eds. M Castegnaro, R Plěstina, G Dirheimer, I Chernozemsky and H Bartsch), pp. 131 - 135. IARC Scientific Publications No. **115**, Lyon, 1991.

93. *Casarett and Doull's Toxicology. The Basic Science of Poisons.* (Eds. MO Amdur, J Doull and CJ Klaassen), pp. 50 - 87. Pergamon Press, New York, Oxford and Toronto, 4th edition, 1991.

94. Hall JL, Collins LA, Barrowman G, Barrowman J. Food-associated toxicants. *Prog Food Nutr Sci* **12**: 1 - 43, 1988.

95. Tabuenca JM. Toxic-allergic syndrome caused by ingestion of rapeseed oil denatured with aniline. *Lancet* : 567, 568. September, 1981.

96. Cobb CE. The Great Lakes' Troubled Waters. *National Geographic* **172**(1): 2 - 31, 1987.

97. Irukayama K. Case history of Minamata. In: *Minamata Disease*, (Eds. T Tsubaki and K Irukayama), Elsevier Scientific Publishers, New York, 1977.

98. Granger DN, Barrowman JA and Kvietys PR. *Clinical Gastrointestinal Physiology*. WB Saunders Company, Toronto, 1985.

99. Guyton AC. *Textbook of Medical Physiology*, eighth edition. WB Saunders Company, Harcourt Brace Jovanovich, Inc., 1991.

100. Borowitz JL, Moore PF, Yim GK and Miya TS. Mechanism of enhanced drug effects produced by dilution of the oral dose. *Toxicol Appl Pharmacol* **19**: 164 -168, 1971.

101. Levine RR. Factors affecting gastrointestinal absorption of drugs. *Am J Dig Dis* **15**: 171 - 188, 1970.

102. Moog F. The lining of the small intestine. *Sci Am* **245**(5): 154 - 173, 1981.

103. Dobbins III WO. In: *Diagnostic Pathology of the Intestinal Mucosa*, pp. 24,40, Springer-Verlag New York Inc., New York, U.S.A., 1991.

104. Dobbins III WO. Morphological and functional correlates of intestinal brush borders. *Amer J Med Sci* **258**: 150, 1969b.

105. Carrière F, Gargouri Y, Moreau H, Ransac S, Rogalska E and Verger R. Gastric Lipases: cellular, biochemical and kinetic aspects. In: *Lipases: Their Biochemistry, Structure and Applications.* (Ed. P Woolley), Cambridge University Press, England, 1992.

106. Gargouri Y, Piéroni G, Lowe P, Sarda L and Verger R. Human gastric lipase: the effect of amphiphiles. *Eur J Biochem* **156**: 305 - 310, 1986.

107. Gargouri Y, Piéroni G, Rivière C, Lowe P, Saunière J, Sarda L and Verger R. Importance of human gastric lipase for intestinal lypolysis: an *in vitro* study. *Biochim Biophys Acta* **879**: 419 - 423, 1986.

108. Morgan RGH, Barrowman JA and Borgström B. The effect of sodium taurodeoxycholate and pH on the gel filtration behaviour of rat pancreatic proteins and lipases. *Biochim Biophys Acta* **175**: 65 - 75, 1969.

109. Borgström B and Erlanson C. Pancreatic juice colipase: physiological importance. *Biochim Biophys Acta* **242**: 509 - 513, 1971.

110. Vandermeers A, Vandermeers-Piret M, Rathé J and Christophe J. Effect of colipase on adsorption and activity of rat pancreatic lipase on emulsified tributyrin in the presence of bile salt. *FEBS Lett* **4**: 334 - 337, 1975.

111. Borgström B and Erlanson C. Pancreatic lipase and colipase. Interactions and effects of bile salts and other detergents. *Eur J Biochem* **37**: 60 - 68, 1973.

112. Duane WC. The intermicellar bile salt concentration in equilibrium with the mixed-micelles of human bile. *Biochim Biophys Acta* **398**:275 - 286, 1975.

113. Small DM. The physical chemistry of cholanic acids. In: *The Bile Acids, Chemistry, Physiology and Metabolism.* (Eds. PP Nair and D Kritchevsky), pp. 249 - 350. Plenum Press, New York, 1971.

114. Borgström B. The micellar hypothesis of fat absorption: must it be revisited? *Scan J Gastroenterol* **20**: 389 - 394, 1985.

115. Westergaard H, Holtermüller KH and Dietschy JM. Measurement of resistance of barriers to solute transport *in vivo* in rat jejunum. *Am J Physiol* **250**: G727 - 735, 1986.

116. Shiau Y-F, Fernandez P, Jackson M and McMonagle S. Mechanisms maintaining a low-pH microclimate in the intestine. *Am J Physiol* **248**: G608 - G617, 1985.

117. Lucas ML, Lei FH and Blair JA. The influence of buffer pH, glucose and sodium on the acid microclimate in rat proximal jejunum *in vitro*. *Pflugers Arch Eur J Physiol* **385**: 137 - 142, 1980.

118. Shiau Y-F and Levine G. pH dependence of micellar diffusion and dissociation. *Am J Physiol* **239**: G177 - G182, 1980.

119. Schachter D and Shinitzky M. Fluorescence polarization studies of rat intestinal microvillus membranes. *J Clin Invest* **59**, 536 - 548, 1977.

120. Laitinen M and Watkins J. Mucosal biotransformations. In: *Gastrointestinal Toxicology.* (Eds. K Rozman and O Hänninen), pp. 169 - 192. Elsevier, New York, 1986.

121. Pelkonen K and Hänninen O. Interactions of xenobiotics with the gastrointestinal flora. In: *Gastrointestinal Toxicology* (Eds. K Rozman and O Hänninen), pp. 193 - 212. Elsevier, New York, 1986.

122. Ockner RK and Manning JM. Fatty acid binding protein in small intestine: Identification, isolation and evidence for its role in cellular fatty acid transport. *J Clin Invest* **54**: 326 - 338, 1974.

123. Ockner RJ and Manning JA. Fatty acid binding protein: Role in esterification of absorbed long-chain fatty acid in rat intestine. *J Clin Invest* **58**: 632 - 641, 1976.

124. Johnston JM. Esterification reactions in the intestinal mucosa and lipid absorption. In: *Disturbances in Lipid and Lipoprotein Metabolism* (Eds. JM Dietschy, AM Gotto and JA Ontko), pp. 57 - 68, American Physiological Society, Baltimore, 1978.

125. Polheim D, David JSK, Schultz FM, Wylie MB and Johnston JM. Regulation of triglyceride biosynthesis in adipose and intestinal tissue. *J Lipid Res* **14**: 415 - 421, 1973.

126. Breckenridge WC and Kuksis A. Triacylglycerol biosynthesis in everted sacs of rat intestinal mucosa. *Can J Biochem* **53**: 1184 - 1195, 1975.

127. Ganong WF. *Review of Medical Physiology*, 11th edition, p. 243. Lange Medical Publishers, Los Altos, CA, 1983.

128. Kritchevsky D. Atherosclerosis and nutrition. *Nutrition International* **2**: 290 - 297, 1986.

129. Chang FC and Chu FS. The fate of ochratoxin A in rats. *Fd Cosmet Toxicol* **15**: 199 - 204, 1977.

130. Galtier P, Charpenteau J-L and Labouche C. The pharmacokinetic profile of ochratoxin A in the rat after oral and intravenous administration. *Drug Metab Dispos* 7(6): 429 - 434, 1979.

131. Kumagai S and Aibara K. Intestinal absorption and secretion of ochratoxin A in rat. *Toxicol Appl Pharmacol* **64**: 94 - 102, 1982.

132. Roth A, Chakor K, Creppy EE, Kane A, Röschenthaler R and Dirheimer G. Evidence for an enterohepatic circulation of ochratoxin A in mice. *Toxicol* **48**: 293 - 308, 1988.

133. Støren O, Helgerud P, Holm H and Størmer FC. Formation of (4R)-4-hydroxyochratoxin A and ochratoxin α from ochratoxin A by rats. In: *Proceedings, Vth International IUPAC Symposium. Mycotoxins and Phycotoxins*, Vienna, Austria, pp. 321 - 324. Austrian Chem Soc, Vienna, 1982.

134. Farnworth ES and Neish GA. Analysis of corn seeds for fungi and mycotoxins. *Can J Plant Sci* **60**: 727 - 731, 1980.

135. van Walbeek W, Moodie C, Scott P, Harwig J and Grice H. Toxicity and excretion of ochratoxin A in rats intubated with pure ochratoxin A or fed cultures of *Penicillium viridicatum*. *Toxicol Appl Pharmacol* **20**: 439 - 441, 1971.

136. Boorman G. Technical report on the toxicology and carcinogenesis studies of ochratoxin A in F334/N rats. *NIH Publication* No. **88**-28/3. National Toxicology Program, US Department of Health and Human Services, Research Triangle Park, NC, 1988.

137. Omar RF. *M.Sc. Thesis,* pp. 32 - 38. Memorial University of Newfoundland, 1990.

138. Bollman JL, Cain JC and Grindlay JH. Techniques for the collection of lymph from liver, small intestine and thoracic duct in the rat. *J Lab Clin Med* **33**: 1349 - 1352, 1948.

139. Lambert R. *Surgery of the digestive system in the rat.* Charles C. Thomas, Springfield, Illinois, 1965.

140. Kane A, Creppy E, Roth A, Röschenthaler R and Dirheimer G. Distribution of the [^{3}H]-label from low doses of radioactive ochratoxin A ingested by rats, and evidence for DNA single-strand breaks caused in liver and kidneys. *Arch Toxicol* **58**: 219 - 224, 1986.

141. Delacruz L and Bach P. The role of ochratoxin A metabolism and biochemistry in animal and human nephrotoxicity. *J Biopharm Sci* **1**(3): 277 - 304, 1990.

142. Galtier P. Devenir de l'ochratoxine A dans l'organisme animal. II. Distribution tissulaire et élimination chez le rat. *Ann Rech Vét* **5**: 319 - 328, 1974b.

143. Gray GM and Cooper HL. Protein digestion and absorption. *Gastroenterology* **61**(4): 535 - 544, 1971.

144. Rawn JD. *Biochemistry*, p. 59. Neil Patterson Publishers, Carolina Supply Company, Burlington, NC, 1989.

145. Laher JM and Barrowman JA. Polycyclic hydrocarbon and polychlorinated biphenyl solubilization in aqueous solutions of mixed micelles. *Lipids* **18**: 216 - 222, 1983.

146. Chu FS. Studies in ochratoxins. *CRC Crit Rev Toxicol* **2**(1): 499 - 524, 1974.

147. Jackson MJ, Williamson AM, Dombrowski WA and Garner DE. Intestinal transport of weak electrolytes. Determinants of influx at the luminal surface. *J Gen Physiol* **71**: 301- 327, 1978.

148. Kumagai S. Effects of plasma ochratoxin A and luminal pH on the jejunal absorption of ochratoxin A in rats. *Food Chem Toxicol* **26**(9): 753-758, 1988.

149. Palin KJ, Wilson CG, Davis SS and Phillips AJ. The effect of oils on the lymphatic absorption of DDT. *J Pharm Pharmacol* **34**: 707 - 710, 1982.

150. Bennet Clark S, Lawergren B and Martin J. Regional intestinal absorptive capacities for triolein: an alternative to markers. *Am J Physiol* **225**(3): 574 - 585, 1973.

151. Barrowman JA and Tso P. Gastrointestinal Lymphatics. In: *Handbook of Physiology, The Gastrointestinal System I (2),* pp. 1733 - 1777. The American Physiological Society, Bethesda, MD, 1989.

152. Hofmann A and Borgström B. The intraluminal phase of fat digestion in man: the lipid content of the micellar and oil phases of intestinal content obtained during fat digestion and absorption. *J Clin Invest* **43**: 247 - 257, 1964.

153. Tso P, Pitts V and Granger DN. Role of lymph in intestinal chylomicron transport. *Am J Physiol* **249**: G21 - G28, 1985.

154. McDonald GB, Saunders DR, Weidman M and Fisher L. Portal venous transport of long-chain fatty acids absorbed from rat intestine. *Am J Physiol* **239** (Gastrointest Liver Physiol **2**): G141 - G150, 1980.

155. Mansbach II CM, Dowell RF and Pritchett D. Portal transport of absorbed lipid in rats. *Am J Physiol* **261** (Gastrointestinal Liver Physiol **24**): G263 - 269, 1986.

156. Hirom PC, Millburn P and Smith RL. Bile and urine as complementary pathways for the excretion of foreign organic compounds. *Xenobiotica* **6**(1): 55 - 64, 1976.

157. Rollins DE and Klaassen CD. Biliary excretion of drugs in man. *Clin Pharmacokinet* **4**: 368 - 379, 1979.

158. Busbee DL, Joe C, Rankin PW, Ziprin RL and Wilson RD. Benzo[a]pyrene uptake by lymph: a possible transport mode for immunosuppressive chemicals. *J Toxicol Environ Health* **13**: 43 - 51, 1984.

159. Prior MG and Sisodia CS. The effects of Ochratoxin A on the immune system of Swiss mice. *Can J Comp Med* **46**: 91 - 96, 1982.

160. Smith DA. Species differences in metabolism and pharmacokinetics: are we closer to an understanding? *Drug Metab Rev* **23**(3,4): 355 - 373, 1991.

161. Coulston F and Serrone DM. The comparative approach to the role of nonhuman primates in evaluation of drug toxicity in man: a review. *Ann N Y Acad Sci* **162**(1):681 - 704, 1969.

162. Chabra RS and Eastin WC. Intestinal absorption and metabolism of xenobiotics in laboratory animals. In: *Intestinal Toxicology*, pp. 145 - 168, (Ed. CM Schiller), Raven Press, New York, 1984.

163. Castegnaro M, Bartsch H, Béréziat JC, Arvela P, Michelon J and Broussolle L. polymorphic ochratoxin A hydroxylation in rat strains phenotyped as poor and extensive metabolizers of debrisoquine. *Xenobiotica* **19**(2): 225 - 230, 1989.

164. Kahn GC, Rubenfeld M, Davies DS, Murray S and Boobis A. Sex and strain differences in hepatic debrisoquine 4-hydroxylase activity of the rat. *Drug Metab Disp* **13**(4): 510 - 516, 1985.

165. Wilson JT and Frohman LA. Concomitant association between high plasma levels of growth hormone and low hepatic mixed-function oxidase activity in the young rat. *J Pharm Exp Ther* **189**(1): 255 - 270, 1974.

166. Hietanen E, Malaveille C, Camus A-M, Béréziat J-C, Brun G, Castegnaro M, Michelon J, Idle JR and Bartsch H. Interstrain comparison of hepatic and renal microsomal carcinogen metabolism and liver S9-mediated mutagenicity in DA and Lewis rats phenotyped as poor and extensive metabolizers of debrisoquine. *Drug Metab Disp* **14**(1): 118 - 126, 1986.

167. Borowitz SM and Ghishan FK. Maturation of jejunal phosphate transport in rat brush border membrane vesicles. *Pediatric Res* **19**: 1308 - 1312, 1985.

168. Corazza GR, Frazzoni M, Gatto MRA and Gasbarrini G. Ageing and small bowel mucosa: a morphometric study. *Gerontology* **32**: 60 - 65, 1986.

169. Katz D, Hollander D, Said HM and Dadufalza V. Ageing-associated increase in intestinal permeability to polyethylene glycol 900. *Dig Dis Sci* **32**: 285 - 288, 1987.

170. Rall DP. Laboratory animal toxicity and carcinogenesis testing. Underlying concepts, advantages and constraints. *Ann N Y Acad Sci* **534**: 78 - 83, 1988.

171. Calabrese EJ. Gastrointestinal and dermal absorption: Interspecies differences. *Drug Metabolism Reviews* **15**(5,6): 1013 - 1032, 1984.

172. Moreau H, Gargouri Y, Lecat D, Junien JL and Verger R. Screening of pre-duodenal lipases in several mammals. *Biochim Biophys Acta* **959**: 247 - 252, 1988b.

173. Regus H, Dittbrenner M and Diaz J. Comparative histochemistry of the gastric mucosa: A survey of common laboratory animals and man. *Anat Rec* **150**: 179, 1964.

174. Coleman R. Biochemistry of bile secretion. *Biochem J* **244**: 249 - 261, 1987.

175. Abou-El-Makarem MM, Millburn P, Smith RL and Williams RT. Biliary excretion of foreign compounds: species differences in biliary excretion. *Biochem J* **105**: 1289 - 1293, 1967.

176. Gregus Z and Klaassen C. Enterohepatic circulation of toxicants. In: *Gastrointestinal Toxicology*, pp. 57 - 118, (Eds. K. Rozman and O. Hänninen). Elsevier Science Publishers Co. Inc., New York, 1986.

177. Chang FC and Chu FS. Preparation of [^{3}H]-labeled ochratoxins. *J Label Comp Radiopharm* **XII**(2): 231 - 239, 1976.

178. Hatch FT and Lees RS. Practical Methods for Plasma Lipoprotein Analysis. E. Preparative Ultracentrifugation. *Adv Lipid Res* **6**: 33,34, 1968.

179. van der Merwe KJ, Steyn PS and Fourie L. The constitution of ochratoxin A, B and C, metabolites of *Aspergillus ochraceus* Wilh. *J Chem Soc* 7083 - 7088, 1965.

APPENDIX

10.1 Species variability of absorptive mechanisms The choice of rat as the experimental animal was dictated by its convenient size, and because of familiarity with the anatomy.

Extrapolation of results and conclusions to other species should be made with caution because different species may vary in their ability to absorb specific xenobiotics. Metabolic handling [160], binding to serum proteins [70] and sensitivity to toxic insult [161] vary from one species to another, and sometimes from one strain to another. Activities of many enzymes vary with species [162], strain [163], sex [164], and age [165]; these differences are often associated with differing susceptibilities to mutagens [166]. Table 2.3, p.19 shows that pig and dog are much more sensitive than rat to the acute effects of OTA ingested orally. The activity of cytochrome P450 enzymes is greater in male rats, being influenced by the balance of sex hormones. In human females, biotransforming ability can vary during the menstrual cycle and with use of oral contraceptives. Maturation and ageing are associated with changes in intestinal morphology and enzyme activity which may affect absorption of specific substances [167,168,169]. Studies of toxicity and carcinogenesis in animals have been extrapolated to predict risk in humans [170], but it is recognized that estimation of human risk from animal studies is an imperfect science.

Interspecies variation in sensitivity to xenobiotics has been largely attributed to

differences in metabolic handling; differences in absorption have received less attention. In a thoughtful discussion of absorption of foreign compounds, Calabrese [171] describes some of the factors underlying interspecies differences: variations in anatomy and physiology of the gastrointestinal tract, in enterohepatic circulation, and in the spectrum and extent of colonization by gut flora.

There are marked anatomical variations in the gastrointestinal tracts of ruminants and monogastric animals, and between various monogastric animals such as rat and pig. Cellular localization of some enzymes, *e.g.* preduodenal lipases [172] and acid phosphatase [173], may vary. The pH in different regions of the gut varies, *e.g.* the rabbit stomach is very acid. This affects the degree of ionization of weak electrolytes and thus the extent to which they are lipid-soluble.

Urine and bile are complementary pathways in the excretion of organic compounds [156]; compounds excreted primarily in bile are polar, and their molecular weights exceed a threshold value which depends on species: 325 (rat), 400 (guinea pig), 475 (rabbit) and 500 (human) [174]. Biotransformation by phase I and phase II reactions converts many compounds by conjugation with glucuronide or glutathione to metabolites which meet these requirements. In rats, compounds of molecular weight below 350 are excreted predominantly in the urine, those with molecular weights between 350 and 450 are eliminated by both routes, and compounds with molecular weights greater than 450 are mostly excreted in bile [156]. Biliary excretion [175] and enterohepatic circulation of various drugs vary from one

species to another, and are influenced by age and sex. In a comprehensive review of entero-hepatic circulation of foreign compounds, Gregus and Klaassen discuss the significance of interspecies variations [176]. Rat, dog and hen tend to be more efficient biliary excretors than rabbit and guinea pig; biliary excretion of acetominophen and chloramphenicol is ten- and twenty-fold greater in rats than in humans. Biliary excretion and entero-hepatic circulation of OTA are probably less in humans than in rats, and therefore the load of OTA which must be handled by human kidneys is likely to be greater.

Variations in intestinal flora, related to differences in pH in the intestinal tract, and to diet, may result in differences in biotransformation of biliary metabolites. Glucuronides are poorly absorbed, whereas hydrolysed to the parent compound they are more easily absorbed. β-glucuronidase activity of cecal microflora differs greatly from one species to another, being very high in fowl, high in rat and sheep, and low in cow, pig, cat and horse; it is higher in humans on a diet rich in meat, and therefore high meat intake enhances reabsorption of xenobiotics excreted in bile. High meat intake probably correlates with slower intestinal transit, whereas transit is faster in individuals with a high percentage of fibre in the diet.

Conclusions from these studies of gastrointestinal absorption of OTA in male Sprague-Dawley rat are not necessarily applicable to female rats, to neonatal or old rats, or to other strains or species, but the conclusion that a lipid-rich diet influences the route of absorption is likely to be valid for humans as well as for rats.

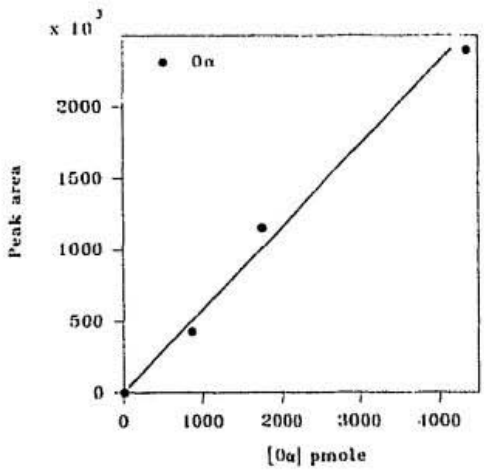
10.2 Radiolabeled Ochratoxin A [^{3}H]OTA was purchased from Amersham UK. Tritiated toxin was prepared by exposing OTA overnight to tritiated water in the presence of a platinum catalyst, at approximately 80°C. This results in a general exchange of tritium with hydrogen, which has been shown [177] to occur mainly (81%) in the phenylalanine residue, and less (19%) in the Oα moiety.
The tritiated toxin appeared to be quite stable both during storage at -20°, -4° and at room temperature over the time frame of the experiments, both in terms of its radioactivity and chemical structure. Care was taken at all times to protect it from light as it has been suggested that exposure to light may cause some degradation of OTA to phenylalanine and Oα.

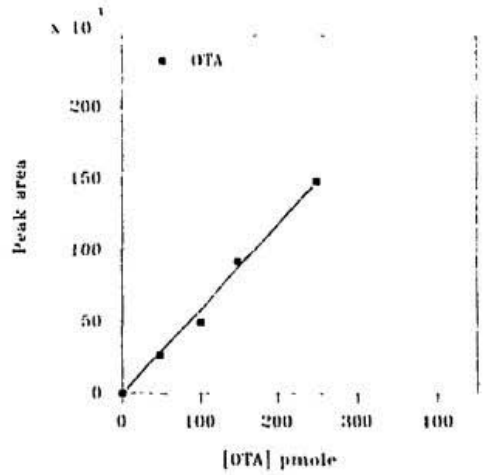
Lymph, serum, bile, and urine collected from rats given [^{3}H]OTA was extracted in chloroform, and subjected to HPLC analysis as described on p.63. OTA in lymph and serum was only seen in the parent form; estimation of OTA using HPLC with known standards correlated fairly well with estimation by measurements of radioactivity. Up to 70% of the radioactivity in lymph and serum samples was lost during extraction, much of it associated with protein which precipitated when the samples were acidified. When extracts of bile and urine were subjected to HPLC analysis, several fluorescent peaks were observed, corresponding with standard samples of Oα, (L) and (R) 4-OH OTA and parent OTA. There was good correlation between the OTA peak and radioactivity counts of HPLC effluent, but the correlation was less clear with Oα and 4-OH (see p.97).

10.3 Radiolabeled trilinolein [^{14}C]trilinolein purchased from Dupont, Boston MA was labelled with [^{14}C] in the carboxyl group of the linoleic acid residues.

10.4 Preparation of 1.006 g/mL density solution: [178]

Weigh 11.4 g NaCl and 0.1 g EDTA-Na_2, transfer to a 1L volumetric flask, and add 500 mL H_2O, 0.2 g Na azide and 1 mL N NaOH. Fill to 1L mark, then add 3 mL H_2O. [NaCl] = 0.195 M.





10.5 Oα and OTA standard curves

based on peak area reported on HPLC. Oα was obtained by refluxing OTA with 6N HCl for 30 hours, as described by van der Merwe *et al.* [179]. It was extracted in chloroform, dried over anhydrous sodium sulphate, and chloroform was removed by evaporation under reduced pressure. Dissolved in methanol it gave a single peak on HPLC. [OTA] was calculated using the absorption coefficient at 332 nm (6.33 mM^{-1}) [134]. Standards were made up in methanol. A Supelcosil LC 18 column, 3.3 cm x 4.6 mm x 3.0 μm, was used with solvent system methanol:water, 80:20, and flow rate 1 mL/minute. Samples were detected by fluorimeter, excitation 340 nm, emission 465 nm.