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For my wonderful parents

About the Author

After undergraduate majors in chemistry and pharmacology, Phil Burcham completed a Ph.D. in biochemical toxicology in 1990 under the supervision of Dr. Andrew W Harman at the University of Western Australia. He then completed postdoctoral training in molecular toxicology under Lawrence J Marnett at Vanderbilt University. After teaching pharmacology and toxicology at the University of Adelaide for 12 years, he returned to Perth to establish a research group focused on studying noxious smoke constituents. His research efforts have led to 55 scientific publications. He has over 20 years of experience in teaching toxicology, pharmacology and drug metabolism to science, medicine, pharmacy, podiatry and dentistry students. His popular team-taught freshers course, *Drugs that Changed the World*, attracts over 400 enrolments. He currently serves on the Editorial Advisory Board of *Chemical Research in Toxicology* and the Editorial Board of *Toxicological Sciences*. He is also a member of the toxicology subsection of the *Faculty of 1,000 (Biology)*.

Preface

Some five or six decades ago, the normal perils of childbirth were shockingly escalated for an unsuspecting generation of mothers in Germany, Canada, Australia and the UK. In a ghastly epidemic that unfolded over several years beginning in the late 1950s, distraught parents were confronted by the birth of babies with severely disfigured upper and lower limbs. By the time the sedative drug thalidomide was identified as the culprit, some 10,000 infants were affected – assailed within the womb by a poison that condemned its victims to lives of struggle and significantly reduced horizons.

The thalidomide disaster was a jarring wake-up call to a generation of scientists, regulators and physicians, a sharp lesson concerning the need for extreme diligence when testing new drugs for safety and unexpected harmful effects. Inspired by the scope of the disaster, a whole branch of science blossomed into existence – *modern toxicology*. New scientific societies dedicated to studying the harmful effects of drugs and industrial chemicals promptly emerged. New journals appeared for toxicologists wishing to publish their research findings. Innovative scientific institutions as well as university departments offering toxicology training programmes were established all around the world.

In today's fast-moving research enterprise, five decades is a very long time, yet recent history suggests the societal need for toxicology expertise remains as strong as ever. Many significant toxicity-related episodes rocked the global community during the first decade of the new millennium. These included the withdrawal of the arthritis drug Vioxx due to cardiotoxicity concerns; the adulteration of infant formula with the protein-mimic melamine; the contamination of children's toys with lead-containing paints or the solvent 1,4-butanediol; the finding of high acrylamide levels in potato chips; and many deaths in Bangladesh and Nigeria due to respective outbreaks of arsenic and lead poisoning. In the USA, ongoing controversies over the human and environmental impact of the 2010 Deepwater Horizon oil spill as well as chronic exposure to high-volume synthetic chemicals such as the plasticiser Bisphenol A or the herbicide atrazine show that toxicological issues still arouse great public concern. In an era shaped by social media in which immediate, emotive reactions govern public responses to many issues, the need for rigorous, science-based investigation of chemically induced disease remains high.

This book seeks to provide a basic overview of modern toxicology. Due to its introductory scope, it does not endeavour to summarise the entire toxicological enterprise. For example, coverage of topics pertaining to chemical regulation and risk assessment is limited; hence, an apology is offered to any readers seeking to expand their knowledge of these vital topics.

A primary aim is to convey a basic appreciation of contemporary understandings of the *mechanisms* underlying chemically induced disease. This emphasis reflects the dramatic advancements over recent times to understand *how* chemicals inflict harm on cells and body organs. Special stress is placed on the main 'big idea' in modern toxicology – namely *bioactivation* – the ubiquitous phenomenon whereby many relatively harmless chemicals are converted to noxious metabolites within the body. We will see how this process helps explain a wide variety of chemically induced toxic syndromes. Since one can complete an entire undergraduate or graduate programme of biological chemistry, biochemistry or cell biology without ever meeting this key concept, bioactivation-dependent toxicity was made a conceptual centrepiece of this volume.

Our journey begins with a potted history of the emergence of modern toxicology (Chap. 1) before exploring some basic concepts relevant to chemically mediated disease (Chap. 2). The various toxicokinetic processes that control the behaviour of chemicals within the body receive attention in Chap. 3 before laying the foundations of a basic understanding of chemical bioactivation in Chap. 4. Various adaptive responses that occur in mammalian systems to counteract the harmful effects of foreign chemicals are introduced in Chap. 5. Separate chapters then explore the relevance of bioactivation to major toxic phenomena such as organ injury, birth defects and cancer (Chaps. 6, 7 and 8). Next, to show how toxicological paradigms can enrich medical understandings of major human diseases, the book closes with explorations of the mechanistic basis for the main disorders plaguing heavy users of two widely consumed 'chemical mixtures' – alcoholic beverages (Chap. 9) and tobacco smoke (Chap. 10).

This book attempts to convey some of the adventure and excitement that accompanied the growth of modern toxicology. Readers will also gain appreciation for the achievements of the thousands of researchers who have to date prevented repetition of poisoning episodes that mimic the appalling thalidomide disaster. If my efforts are in any way instrumental in inspiring some budding scientists to embark on a toxicology career, this writing project will have been worthwhile.

In addition to the many individuals who contributed to my own education as a toxicologist, I thank my humanities colleagues at UWA for helping to broaden my awareness of the long-standing interactions between humans and chemicals. I am especially indebted to Dr Philippa Martyr for her insights into the social and historical aspects surrounding alcohol use (Chap. 9). Any limitations in my brief surveys of either scientific or historical issues are certainly my own.

Perth, Western Australia, Australia
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Chapter 1
The Emergence of Modern Toxicology

Abstract Many ancient societies exhibited astute awareness of poisonous substances: their rudimentary 'toxicology communities' likely included physicians who used toxic plants and metals for therapeutic purposes as well as 'professional poisoners' who offered their services to political leaders for the elimination of unwanted rivals. Eventually, the emergence of modern chemistry began unleashing the economic and scientific powers of chemical substances, and evidence for their harmful effects accumulated as the Industrial Age unfolded. Incisive descriptions of occupational diseases by astute physicians such as Paracelsus, Pott and Rehn were of groundbreaking significance to toxicology. Soon after, pioneers such as Fontana, Orfila and Christison laid the foundations for experimental toxicology by studying chemical toxicity in animals. Yet no event was more important to the development of toxicology than the thalidomide disaster of the early 1960s. A flurry of activity saw toxicology-focussed societies, journals, regulatory agencies and research institutions established around the world. Toxicology today is a highly evolved, multidisciplinary endeavour that develops its own conceptual frameworks while also drawing upon advances in the chemical, medical and biological sciences.

Keywords Poisons • History • Paracelsus • Felice Fontana • Mathieu Orfila • Robert Christison • Percival Pott • Ludwig Rehn • Frances Kelsey • Rachel Carson

1.1 Introduction

Historians label the period following World War 2 the 'New Chemical Age' since it witnessed a dramatic increase in global chemical output. The number of chemicals in existence multiplied rapidly so that by early 2012, the Chemical Abstracts Service, an electronic register used by chemists to track molecules made by their peers, listed over 60 million unique substances (organic and inorganic). Most of these substances are used in small quantities and hence are only of concern to professional chemists. However, in terms of 'everyday' exposures relevant to the

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general public or workers in common occupations, an estimated 50,000–100,000 or so synthetic chemicals are typically in current use in most industrialised countries.

The use of chemicals brings important economic and health benefits to society, but prudence suggests the need for continual vigilance regarding the risks that might accompany their penetration into modern daily life. The task of evaluating the toxicity of new chemicals in humans and nonhuman species – and of clarifying the mechanisms underlying the harm chemicals cause under some circumstances – is the responsibility of the toxicology community, a global assembly of government, private and academic researchers who devote their careers to studying chemically induced disease.

Although modern toxicology is of recent origin, prior generations of humans were aware of problems caused by toxic substances. The fact that planet Earth is abuzz with plants and animals producing poisons to ward off predators and pests – and that the planetary crust is rich in toxic metals and minerals – ensures a long and fascinating history for toxicology.

1.2 The First Toxicologists

While there are no records of their discoveries, the first toxicological observations were likely made by early humans who found to their detriment that chewing berries from a particular plant elicited debilitating stomach cramps, headaches or diarrhoea. The written records left by ancient civilisations also reveal an early awareness of the toxicity of plants or other natural substances (Fig. 1.1). From such simple discoveries, aided by trial-and-error assessment of the medicinal potential of natural materials in their local ecosystem, the earliest societies assembled crude catalogues of noxious plants, insect toxins, animal venoms and poisonous minerals and metals. One of the oldest manuscripts of this kind, the *Ebers Papyrus*, purchased in 1874 from a Luxor antiquities dealer by the University of Leipzig Egyptologist Georg Ebers, includes references to such natural poisons as hemlock, opium, mercury and aconite.

Following the introduction of weapons, hunters discovered that smearing the tips of spears and arrows with animal venom enhanced their killing efficiencies, a practice that remains popular among Amazonian tribes and African pygmies. One reference to such practices appears in early editions of Homer's *Odyssey* (ca. 660 BC) and refers to Anchialos, King of the Taphians, supplying a man-killing poison to warriors for application to bronze-headed arrows. Perhaps a couple of centuries later, the ancient Hebrews displayed familiarity with this practice: in the Old Testament, the perpetually tormented Job cries out 'The arrows of the Almighty are in me, my spirit drinks in their poison' (Job 6:4, *New International Version*). In fact, the etymology of the term *toxicology* conveys the prevalence of such practices in the ancient world. The modern English term derives from the Latin *toxicum,* an abridgement of the earlier Greek name for arrow poison – *toxikon pharmakon* (*drug pertaining to the bow*). In the transition to English, *toxicum* became 'toxin', the study of which is 'toxicology'.

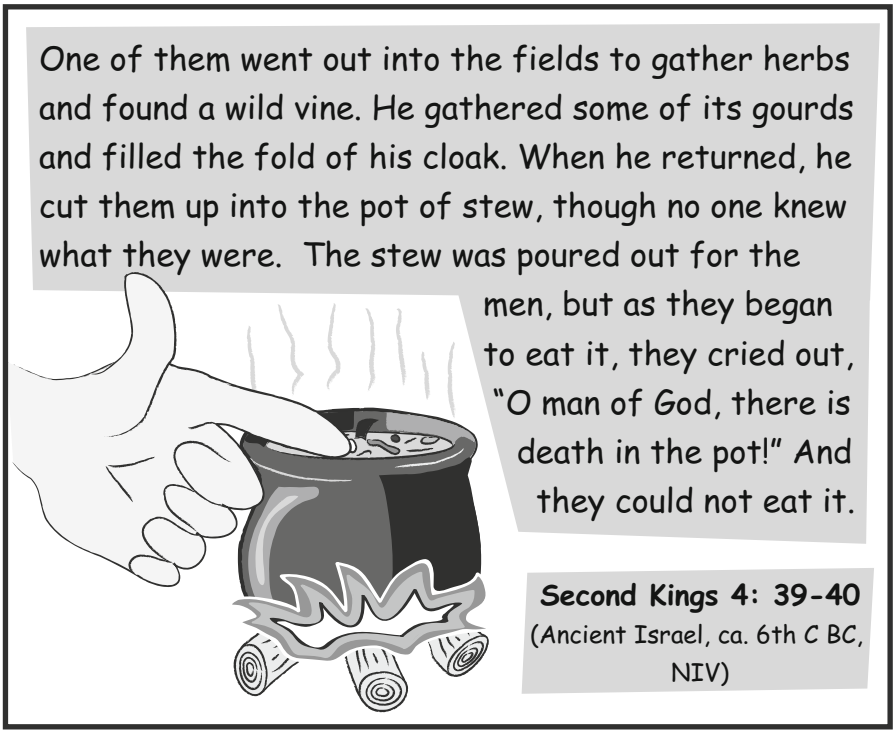

Fig. 1.1 Early awareness of plant poisons within ancient Hebrew literature

The origins of toxicology include sinister elements since a major motivation of early practitioners was the assistance of well-heeled customers who wished to poison adversaries or business rivals: as the option of assassinating opponents on evening news bulletins did not exist, ancient politicians hired poisoners to prepare concoctions of toxins for adding to foodstuffs and beverages. Famous poisoning victims of these practices include Socrates, Cleopatra and Claudius. Such practices meant politicians who desired long terms of office needed the services of competent food tasters. Another preventative strategy, at which King Mithridates VI of Pontus (134–64 BC) supposedly excelled, involved daily consumption of antidotes against common poisons. His cocktail of protective substances – the *Antidotum Mithridaticum* – was used for hundreds of years and even into the Renaissance era. Mithridates VI also conducted some of the first – albeit involuntary – clinical trials in recorded history, using state prisoners as guinea pigs to determine doses of antidotes needed to counteract ingested poisons. A century or so later, Emperor Nero (AD 37–68) probably used slaves to identify toxic mushrooms. When slaves became too expensive, rulers sometimes substituted the use of dogs, in effect performing the first animal studies in the history of toxicology.

Ancient toxicologists also appreciated the 'dose–response relationship', the recognition that the severity of toxicity elicited by a toxic substance is dictated by the

total dose received. According to Plato's famous account, the execution of Socrates required consumption of hemlock, a parsley-like herb used as the official State poison since it contained high concentrations of the neurotoxin coniine. As his execution neared, Socrates asked whether he could pour out some of the poison as a libation to a deity. Denying the request, the executioner famously replied, 'We only prepare, Socrates, just as much as we deem enough'.

1.3 The Early Modern Period

Although toxin use for criminal purposes continued, the medieval era saw little systematic advancement in toxicological knowledge. One exception appears in the writings of the Spanish-born North African rabbi–physician Moshe ben Maimon (Maimonides, AD 1135–1204). His treatise on *Poisons and their Antidotes* (AD 1198) was influential for centuries and contained treatments for snakebites and insect stings.

In the Renaissance period, powerful family dynasties in the Italian cities of Florence and Vienna raised political poisoning to an art form. Although modern historians temper the legends surrounding the de Medicis and Borgias by suggesting infectious disease claimed some of their victims rather than poisons, considerable proficiency in the use of arsenic and other toxic metals such as antimony certainly flourished in this era (Fig. 1.2).

Pope Alexander VI (AD 1431–1503), who with his son Cesare represented a definite low point in papal history, used poisons to eliminate wealthy rivals before confiscating their assets. This father-and-son team also learnt the value of

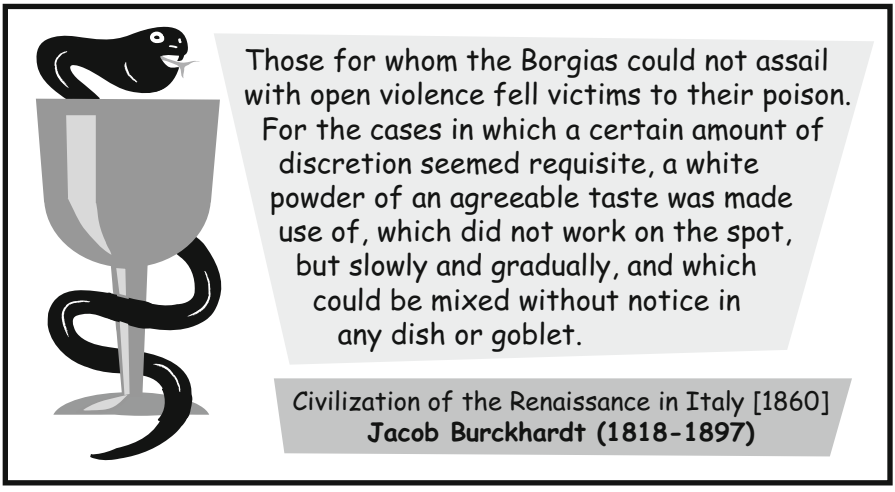

Fig. 1.2 Dynastic intrigue and the struggle for power during the Italian Renaissance saw a revival of the Greco-Roman art of political poisoning

evaluating poisons in animals before proceeding to human tests, with the bodies of unfortunate study subjects thrown into the Tiber River on trial completion. The infamous 'Cup of the Borgias' also derives from this era; it contained a hidden compartment from which poisons were dispensed during meals with unsuspecting victims. Alas, according to the traditional account, Alexander VI likely fell prey to his own scheming, consuming a bottle of adulterated wine intended for a rival cardinal. The poisons produced an unusually rapid deterioration of his remains: after seeing it lying in state at St. Peter's, one contemporary declared it 'the most loathsome and the most monstrous and frightful corpse that had ever been seen'.

1.3.1 Paracelsus

The sixteenth century produced many history-changing personalities but none are as significant to the history of toxicology as the Swiss-born physician–alchemist, Paracelsus (AD 1493–1541) – often denoted the 'Grandfather of Modern Toxicology'. Since the name assigned at his christening is taxing to undergraduate memories, history students thank him for following the popular sixteenth-century habit of adopting a Latin name: Paracelsus is easier to recall than Philippus Aureolus Theophrastus Bombastus von Hohenheim! A man of contradictions and a capacity for binge drinking, Paracelsus helped foster the emergence of the modern scientific culture not so much through his own empirical research but via his assault on the inherited Greco-Roman medical wisdom from Galen and Hippocrates that then dominated the medical curriculum. This endearing willingness to confront entrenched orthodoxies and powerful elites earned Paracelsus his traditional designation as the 'Martin Luther of Medicine'.

A chief quality was the capacity to rub people up the wrong way, a trait that came to the fore during a period as town physician and medical educator in Basel, a position Paracelsus secured in 1526. His strident advocacy of the use of mercury – a widely feared poison – in the treatment of syphilis was too much for the local medical establishment. In a hot-headed written response to his critics, Paracelsus made his famous justification for why even toxic substances, if used in small doses, might elicit curative actions; 'What is it that is not poison? All things are poison and none without poison. Only the dose determines that a thing is not poison'. This quotation – often in its Latin form ('Dosis sola facit venenum' – the dose alone makes the poison) has adorned the frontispiece of many toxicology textbooks to this day.

In spite of his courage and insights, as one might expect of a transitional figure in the evolution of modern medicine – a bridge between the medieval and modern eras – the alchemist worldview articulated by Paracelsus contained strange ideas that baffle contemporary readers. We can certainly applaud his awareness of the ability of poisons to undermine human health – he included *ens veneni* or poisoning and the ensuing imbalance in metabolism as one of the five *Enses* or 'active principles' that trigger disease. However, the most important of his five principles makes us scratch our heads in wonderment – *Ens astrorum* – the 'virtue of the stars'. In his

alchemistic fusion of astronomy with anatomy, the 'fact' that there were equal numbers of organs in the body as planets in the solar system reveals a clear influence of the latter on the former. Proficiency with a telescope is thus indispensable during the diagnosis and treatment of disease; 'The physician who does not understand astronomy cannot be a complete physician because more than half of all diseases are governed by the heavens' (*Astronomia magna* [1537–8]).

Paracelsus also authored a classic manuscript that is revered within the annals of occupational toxicology, the subdiscipline that concerns itself with the risks accompanying chemical exposure in the workplace. His posthumous *On the Miner's Sickness and Other Diseases of Miner's* (1567) was a groundbreaking analysis of the signs and symptoms of respiratory diseases that plagued workers in filthy and poorly ventilated mine sites (Fig. 1.3). The book derived from observations of ill health among Fuggers mineworkers during a trip to the mountainous Tyrolean region near the Italian–Austrian border. In addition to poor work conditions, long hours and low pay, miners struggled with a serious respiratory condition that prematurely ended their lives. The 'miner's sickness' likely comprised a combination of ailments that reflected long-term damage to the lung via microbes and airborne particulate matter (e.g. pneumonia, tuberculosis, bronchitis and fibrosis). By providing an early description of the condition, Paracelsus fostered the slow emergence of a scientific understanding of the environmental factors that harm workers in specific industries.

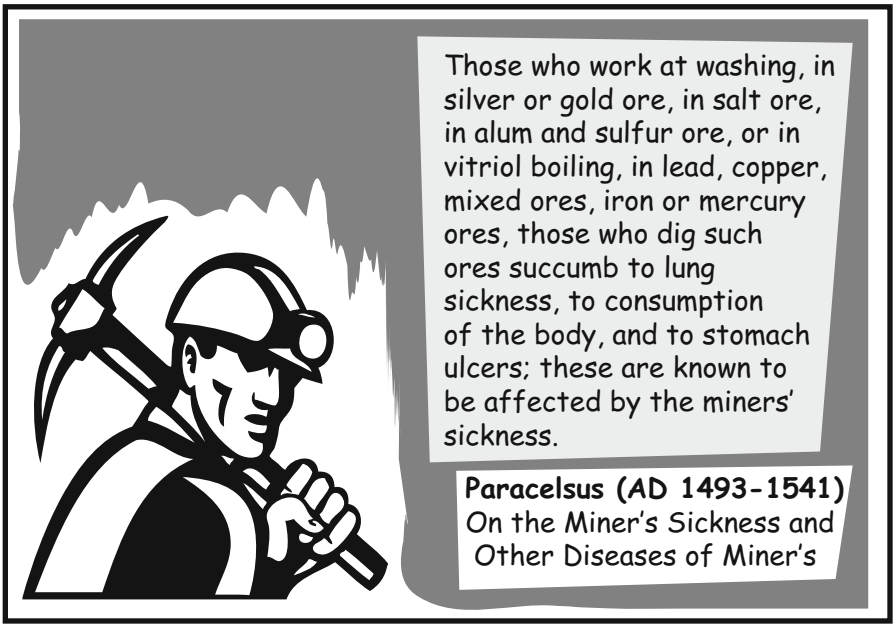

Fig. 1.3 In addition to his famous advocacy of toxic mercury amalgams in syphilis victims, Paracelsus made early clinical descriptions of disease syndromes that afflicted workers in the mining industry during the early modern era

1.3.2 Felice Fontana

For toxicology to evolve into a scientific discipline, experimentalists needed to dispel many entrenched wrong-headed ideas concerning the causes of disease. Up until the late seventeenth century, a belief that dominated thinking concerning the toxicity of snake venoms and other poisons was termed the 'sympathy theory'. It proposed that poisons triggered local 'impressions' or impulses that passed along nerves from the site of exposure to cause harm within secondary organs. This view largely rested upon the speed with which poisoning symptoms emerged after envenoming – it was thought that only fast transmission of impulses via the nervous system could explain the rapid onset of symptoms. An early toxicology practitioner who subjected this belief to experimental validation using snake venoms and laurel berry poisons was the Italian physicist and experimentalist Felice Fontana (1730–1805). A devout Catholic who performed experiments while dressed in clerical attire, his experimental findings made Fontana an early critic of the 'nerve sympathy' theory. Using animals as test subjects to whom he administered poisons via the veins, Fontana laid the foundations for the latter appreciation of the importance of absorption and distribution of chemicals via the circulatory system. This gifted Italian scientist anticipated many basic concepts we will explore in Chap. 3.

1.3.3 Mathieu Orfila

Few chemicals rank higher than arsenic on the list of preferred chemicals used with malicious intent down through the ages. The colourless and tasteless trivalent oxide of arsenic has proven especially popular since its can be added to wine, porridge or stews without detection. The favoured poison of the working classes, hundreds if not thousands of victims were sent to early graves throughout Europe alone, a strategy that proved popular with infamous serial killers such as Helene Jegado (1803–1854) and Mary Ann Cotton (1832–1873). Arsenic was also freely available due to its extensive use in diverse consumer and agricultural products. As historian Peter Bartrip noted, 'Curtains, furniture fabrics, lampshades, ornaments, artificial flowers, carpets, linoleum, children's toys and books were among the products which routinely contained arsenic as a colouring agent. The rooms of innumerable Victorian houses were lined with wallpaper coloured with arsenical dyes; their inhabitants wore clothes dyed with arsenical pigments; at night they lit their premises with candles containing arsenic. So extensive were the uses to which arsenic were put that "it was hard to escape exposure… in life or death"'. Chronic arsenic exposure likely contributed significantly to the burden of disease in Victorian times.

Advances in chemistry during the eighteenth and nineteenth centuries, together with legislative restraints on the sale of arsenic products, meant that the curtain was drawing on an era in which wanton killing by arsenic could be carried without fear

of reprisal. The emerging science of forensics assisted by developing methods to detect the toxic metal in suspected human poisonings. The 'Marsh test' invented by the British chemist James Marsh (1794–1846) was pivotal in the emergence of forensic toxicology: his method detected arsenic by bubbling hydrogen sulphide gas through urine or blood samples – formation of yellow arsenic sulphide precipitates was a telltale sign of arsenic intoxication.

A key figure who helped bring forensic chemistry to birth was Mathieu Joseph Bonaventure Orfila, born on the island of Minorca off the coast of Spain in 1787. If Paracelsus is the Grandfather of Toxicology, Orfila is surely its 'Father' due to his efforts to systematise the discipline in the early nineteenth century. In addition to his publishing efforts (see below), Orfila developed assay procedures to detect arsenic in autopsy samples and the exhumed corpses of poisoning victims. Orfila lectured on chemistry and anatomy at the University of Paris and later performed the unusual feat of securing twin professorial chairs in medical jurisprudence (1819) and chemistry (1823).

Orfila's magisterial 2-volume *Traitè des toxicology* (*A Treatise of Toxicology*) which appeared in 1813–1815 was a milestone in the emergence of scientific understandings of chemically induced disease. The first comprehensive toxicology text of modern times, the book systematically addressed the chemical, physiological and toxic effects of chemicals, combining case studies from clinical toxicology and animal tests with analytical chemistry and forensic science. The material was arranged into six categories of noxious substances that included corrosive, astringent, acrid, stupefying and septic poisons. Remarkably, Professor Orfila sensed that the practice of toxicology is an inherently multidisciplinary endeavour, stating in the Introduction to his famous book that 'it is not possible to investigate thoroughly any poisonous substance, without taking into consideration its relations with Chemistry, Natural History, Physiology, Pathology, and Morbid Anatomy'. Few nineteenth-century books did more to lay the foundations for experimental toxicology or to distinguish the discipline from the fields of pharmacology and therapeutics with which the study of poisons had traditionally been aligned.

1.3.4 Robert Christison

Toxicology entered a more mature phase of existence after its forerunner's 'disciples' carried the discipline forward into the future. Within the English-speaking world, one of Mathieu Orfila's most influential students was the Scottish physician, Robert Christison (1797–1882). A graduate of the University of Edinburgh, Christison spent time in Paris learning analytical chemistry and toxicology from Professor Orfila as well as the great French biochemist Pierre Jean Robiquet (1780–1840). Returning to Scotland to accept a position in medical jurisprudence, Christison served in this capacity for a decade before he secured appointment to the Chair of Materia Medica and Therapeutics which he held until 1877. In addition to service as President of the British Medical Association, Christison spent a notable career laying the foundations for modern toxicology in Great Britain, publishing a

major monograph in 1829 entitled *Treatise on Poisons in relation to medical jurisprudence, physiology, and the practice of physic*. This work reviewed emerging methods for the diagnosis and treatment of intoxications with oxalic acid, arsenic, lead, opium and other poisons.

As with Mathieu Orfila, Christison was disturbed by the easy availability of arsenic in nineteenth-century Europe and his book devoted much attention to this problem. Thanks to his sterling efforts and energetic promotion of toxicology, the *Arsenic Act* passed the House of Commons in 1851. For the first time in laissez-faire Victorian Britain, legislative restrictions were placed on the sale of a highly toxic substance. Christison also developed particular expertise in studying renal physiology as well as toxic responses of the kidney and for this reason is an acknowledged pioneer in medical nephrology.

1.4 Occupational Hazards in the Industrial Era

Paracelsus was ahead of his time in recognising the link between specific work practices and susceptibility to disease. Wider appreciation of this association would not be forthcoming until the Industrial Revolution was fully underway in the eighteenth and nineteenth centuries. By raising participation in the mining and chemical industries, the Industrial Revolution dramatically increased opportunities for occupational exposure to hazardous substances. Early clinical descriptions of health conditions in affected workers – usually made by astute physicians – empowered some of the most important discoveries in the emergence of modern toxicology.

1.4.1 Percival Pott

One physician who helped associate specific workplace exposures with disease outcomes was the English surgeon Percival Pott (1714–1788). On top of major contributions to orthopaedic surgery, Pott made astute clinical observations of adolescent London chimney sweeps: in 1775 he was the first to associate early-onset skin tumours of the scrotum with childhood exposure to coal soot (Fig. 1.4). The occupational practices of the day were appalling, with young boys sent up small chimneys in a state of near or total nudity that provided no protection against thick soot. Pott's brief report produced a flurry of supportive correspondence from clinicians mystified by the same patient cohort, thereby overturning the long-standing habit of diagnosing the unfortunate young men as victims of venereal disease. As Pott noted, 'it being taken, both by surgeon and patient, for venereal, and being treated with mercurials, is thereby soon and much exasperated'.

According to popular prejudices, the habit of taking daily baths explained the lower frequency of scrotal cancer among chimney sweeps on the Continent. The careful studies of the St. Bartholomew's laryngologist, Sir Henry Butlin, reported in three lectures to the Royal College of Surgeons in 1892, identified the protective

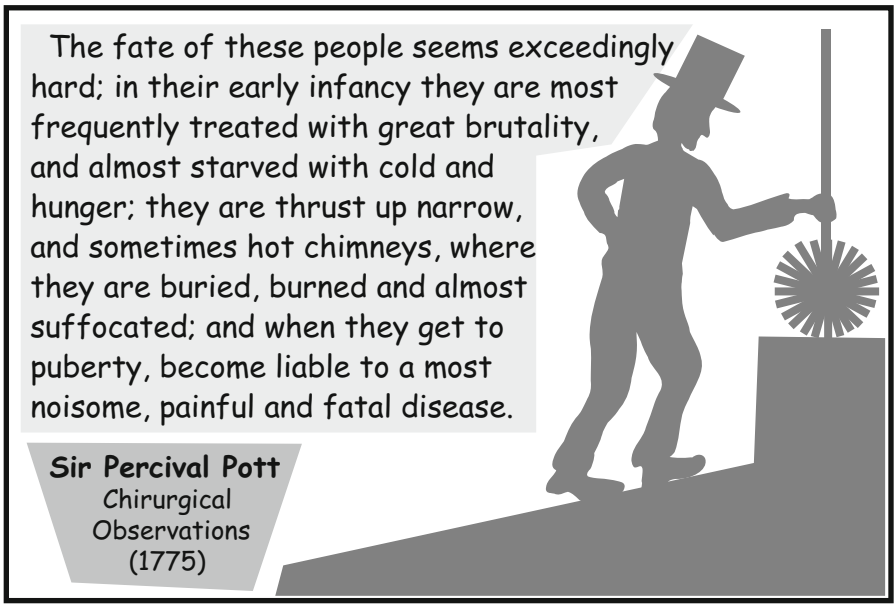

Fig. 1.4 Early medical descriptions of disease syndromes affecting workers in specific industries attracted scientists who began exploring the chemical and biochemical basis for the disease associations

clothing worn by Continental sweeps as a likelier explanation for the comparative rarity of scrotal cancer in Germany compared to Great Britain.

While Pott's findings strongly implicated coal combustion products in the development of scrotal tumours, this hypothesis was not proven until 1915 during groundbreaking animal experiments conducted by the Japanese researcher Katsusaburo Yamagiwa. By painting coal tar onto the ears of rabbits, Yamagiwa induced skin tumours that resembled those seen in the scrotum of chimney sweeps. Organic chemists at the Chester Beatty Research Institute (UK) led by Professor Ernest Kennaway soon identified multiple polycyclic aromatic hydrocarbons as the cancer-causing constituents of soot and coal tar. Since members of this chemical class also cause cancer in tobacco smokers, we will explore these compounds in more detail in Chap. 10 (Sect. 10.6.5.1). Furthermore, during pioneering epidemiological studies in the late nineteenth and early twentieth centuries, these noxious chemicals were tellingly associated with cancer causation in diverse work settings including German coal distillate handlers, Scottish shale workers and Lancashire cotton spinners.

1.4.2 Ludwig Rehn

The polycyclic aromatic hydrocarbons were not the only chemicals waging a silent war on unsuspecting Industrial Era workers. In April 1895, during the Congress of the

German Surgical Society held in Berlin, another observant physician, Ludwig Rehn (1849–1930), tried to convince sceptical colleagues that bladder tumours in a cluster of workers from a Hoechst chemical plant were caused by aniline dyes used in the textile industry. 'It all came to nothing', Rehn lamented, 'I had pointed quite clearly to the great importance of the aniline factor in view of the aetiology of cancer. It was in vain'. Despite the initial incredulity, posterity would remember Rehn as the discoverer of a new class of powerful carcinogens known as the 'aromatic amines'. Upon uptake into the body, these chemicals undergo conversion to cancer-causing metabolites within the liver, but it is following renal export to the bladder that they inflict greatest damage. Since related chemicals form during the combustion of tobacco, we will explore this compound class more carefully in Chap. 10 (Sect. 10.6.5.3).

The twentieth century witnessed many additional epidemics of occupational cancer. Leading examples include outbreaks of a deadly form of lung cancer, mesothelioma, in workers who participated in the mining and milling of asbestos. Although first hailed as a wonder material, asbestos quickly achieved the reputation of a villain. In Australia, for example, asbestos mining in the remote western town of Wittenoom produced an epidemic of mesothelioma in the 1960s and 1970s that ranks among the most serious disasters in mining history. The tragic use of tailings from the mine site as ground cover in housing estates within the arid outback town ensured the children of Wittenoom miners were also tragically affected by the epidemic.

The 1970s also saw the first association of exposure to vinyl chloride, a precursor of the widely used polymer PVC, with a rare yet deadly form of liver cancer, angiosarcoma. First seen in workers from a BF Goodrich PVC plant in Louisville, Kentucky, the disease was subsequently diagnosed in workers around the world. Subsequent improvements in plant design and work practices eliminated this disease.

Although few diseases invoke as much fear as cancer, occupational chemicals can cause other diseases that also take a substantial toll on human health. During the twentieth century, a steady growth in work-related morbidities occurred, ranging from correlations between lead exposure and brain injury; use of arsenical pesticides and skin disease; or solvent-induced liver disease in dry cleaning industry workers. Many examples of this kind will be encountered throughout this book.

The realisation that the workplace is potentially a hazardous place led to the recognition that industries carry an obligation to recompense workers and their families for disabilities incurred during their employment. The German Reichstag led the way by passing the Workingmen's Insurance Law in 1883. This groundbreaking legislation mandated the establishment of insurance funds to assist provision of health care and compensation to injured workers. The UK passed a similar Workman's Compensation Law in 1897. In the USA, growing awareness of occupational hazards led to the formation of the National Safety Council in 1911 and the Division of Industrial Hygiene 3 years later. Alice Hamilton (1869–1970), the first woman appointed to the faculty of Harvard Medical School, is widely recognised as having laid the foundations of industrial toxicology and occupational medicine in the USA. A physician and pathologist, she conducted extensive human research into the health effects of occupational chemicals. She also published several textbooks upon industrial toxicology.

1.5 Modern Toxicological Disasters

Paralleling these initiatives, the mid-twentieth century saw the emergence of modern toxicology as a self-sustaining, independent discipline. While rising awareness of occupational disease helped drive this process, several disasters involving pharmaceuticals gone awry also played catalytic roles.

1.5.1 Diethylene Glycol

A defining episode in late 1930s America involved release of an oral suspension of sulfanilamide, one of the first effective antibacterial drugs. Sulfanilamide was discovered as a metabolite of Prontosil, the groundbreaking medicine developed by the German scientist Gerhard Domagk (1895–1964). Although sulfanilamide was marketed in tablet or capsule forms as a 'wonder drug' by several US companies, their bitter taste deterred some consumers. Due to low solubility in common solvents such as water and alcohol, sulfanilamide was unavailable as a liquid formulation until the S.E. Massengill Company of Bristol, Tennessee, marketed a sweet, cherry-flavoured 'sulfanilamide elixir' in 1937. Disastrously, the firm's chemist chose to dissolve sulfanilamide in a high concentration of diethylene glycol (72 % on a weight per volume basis). While an excellent solvent used in commercial products ranging from paint strippers to antifreeze, the toxic properties of diethylene glycol render it entirely unfit for human consumption. By September 1937, 240 gal had been distributed in 1,304 shipments across the USA. The resulting epidemic of kidney disease claimed 105 victims over a 4-week period, with the average fatal dose estimated at 53 mL for children and 99 mL for adults. On learning the consequences of his actions, the distraught Massengill chemist committed suicide.

In the wake of the sulfanilamide disaster, President FD Roosevelt signed the *US Food, Drug and Cosmetic Act* into law in June 1938, a significant piece of legislation that stipulated an approval process for New Drug Applications (NDA) that involved submission to the Food and Drug Administration as well as new requirements concerning safety testing, drug labelling and advertising. The Act effectively brought to an end a chaotic era in US commercial history which featured numerous poisoning outbreaks caused by disreputable consumer products – the 'American Chamber of Horrors' as it was dubbed by one muckraking journalist. In addition to dispelling many therapeutic agents of questionable efficacy such as the *Wilhide Exhaler*, the Act provided a legislative framework for addressing problems raised by overtly toxic products such as *Lash Lure*, an eyelash dye containing aromatic amines which inflicted permanent injuries on user's eyes, and *Radithor*, a health tonic that condemned users to unpleasant chronic poisoning with radium.

1.5.2 TOCP

Another toxicological disaster unfolded on a large scale in 1930 across the Southern and Midwestern states. Desire for alcoholic beverages ran high following the stock market crash, creating strong demand for Ginger Jake, an ostensibly medicinal product made from pulverised ginger extract that had been sold in drugstores for decades as a 'cure-all' remedy for ill health. Supplying high alcohol content (80 %) in an easily concealed bottle, the amber liquid was popular throughout the Prohibition Era. On becoming aware of the abuse, in 1935 the Treasury Department mandated a doubling of the ginger content to make the concoction too bitter for casual consumption. Somewhat predictably, this change triggered many innovative adulteration efforts among producers seeking to make their own Ginger Jake products more palatable.

For reasons that are not entirely clear, two disreputable businessmen in Boston, Harry Gross and Max Reisman, hit upon the idea of adulterating their Ginger Jake product with the plasticiser tri-O-cresyl phosphate (TOCP), then manufactured by the Eastman Kodak company for use in lacquers and varnishes. Unaware of its toxic properties, Gross and Reisman purchased 135 gal of TOCP and added it to Ginger Jake batches that were used to fill hundreds of thousands of bottles. The product was then sold throughout the continental USA. The resulting delayed-onset neurotoxic syndrome seen in users of the product was nicknamed 'Jake Walk' due to the paralysing loss of leg muscle tone that progressed to the point where victim's feet flopped like those of a marionette (Fig. 1.5). Nationwide, around 40,000–50, 000 people were affected in a disaster that unfolded rapidly: in Wichita, Kansas, around 500 patients manifested signs of TOCP intoxication in a single night alone. Although partial recovery sometimes occurred, many victims were permanently incapacitated, spending the remainder of their lives in charitable institutions or county asylums. The epidemic also left its stamp on Southern popular culture, with at least a dozen references to 'Jake Walk' in commercial phonograph recordings by jazz musicians of the time.

1.5.3 Thalidomide

The most jarring toxicological disaster of the twentieth century unfolded on a global scale a generation or so after the Ginger Jake and sulfanilamide elixir epidemics. In 1950s societies across the developed world, a stressed-out generation of World War 2 survivors struggled amidst the fast-paced social and technological change of modern times. To help patients cope with sleeplessness and other stress-related symptoms, physicians wrote multitudes of prescriptions for barbiturate tranquilisers: in 1955 alone, the US pharmaceutical industry produced 26 barbiturate pills for every man, woman and child in the country. But problems with dependence and tolerance

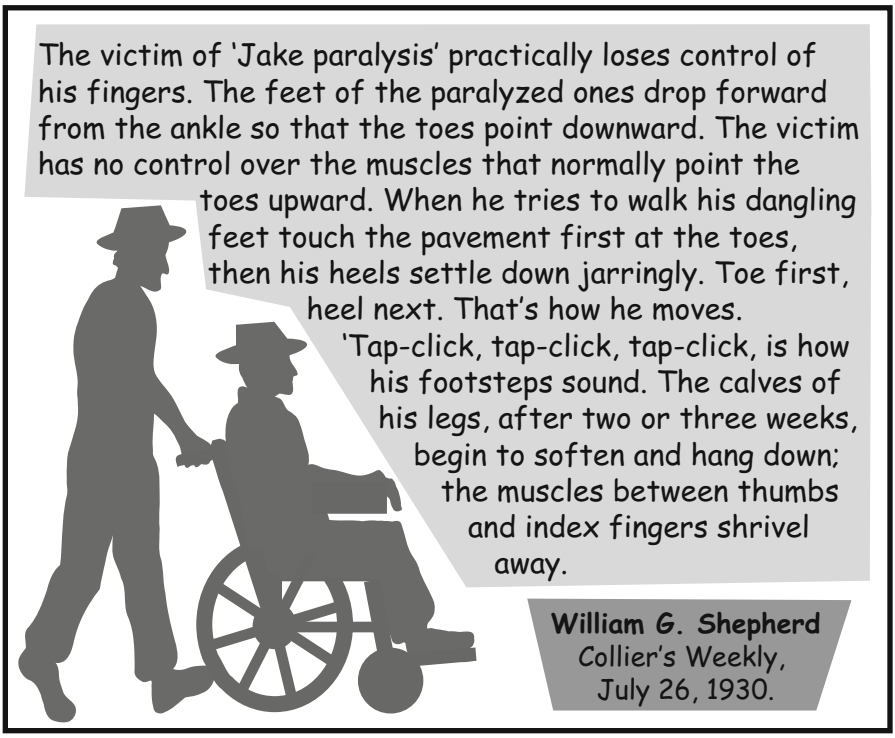

Fig. 1.5 Adulteration of alcoholic beverages with TOCP during the 1930s Prohibition Era in America led to a distressing epidemic of peripheral neuropathy

meant barbiturates were not ideal insomnia therapies, so hopes were raised when a fledgling German company, Chemie Grunenthal, began marketing a new hypnosedative – thalidomide – by claiming it lacked toxic effects and was safe for use in pregnancy. The grounds for the latter claim are questionable since little evidence exists for safety testing in pregnant animals or humans prior to thalidomide release. Reflecting the lax approval standards of the time, thalidomide was soon sold over the counter throughout Europe and the British Commonwealth before adequate evaluation of its pharmacological and toxicological properties took place. Due to an aggressive advertising campaign that touted its safety, thalidomide soon achieved stellar sales figures that in some countries were second only to those of aspirin. Thalidomide was especially popular among the elderly and with pregnant women struggling with disrupted sleep patterns and morning sickness.

During the late 1950s, evidence began emerging which suggested thalidomide was less safe than early marketing materials claimed. For example, long-term users developed a neurotoxic syndrome that in some ways resembled problems seen earlier in Ginger Jake victims: patients reported unpleasant tingling in their upper and lower limbs that sometimes progressed to numbness, muscle paralysis and difficulty in walking. During 1960 and early 1961, several case studies describing this

syndrome in isolated patients appeared in various medical journals. As a result, some hospitals in Germany and the UK began limiting thalidomide use.

On the other side of the Atlantic, an overbearing representative of the Richardson-Merrell company in Cincinnati was pressuring a cautious official at the Food and Drug Administration (FDA), Dr Frances Kelsey, to proceed with approving a US marketing application. A pharmacologist and clinician by training, as a research student at the University of Chicago, Frances had helped identify diethylene glycol as the culprit in the 'sulfanilamide elixir' epidemic. Her eyes opened to the need for thorough testing of human pharmaceuticals, Dr Kelsey and her handful of FDA associates were concerned by the paucity of data concerning the fate of thalidomide in animals within the marketing dossier submitted by Richardson-Merrell. Moreover, a case study implicating thalidomide in peripheral neurotoxicity that appeared in the December 1960 issue of the *British Medical Journal (BMJ)* had caught her attention, emboldening Dr Kelsey to stand her ground during negotiations with Richardson-Merrell. The courageous decision to block thalidomide approval largely spared US citizens from the tragedy that unfolded elsewhere, earning Dr Kelsey recognition as 'the heroine of the FDA' as well as a Presidential Medal from JF Kennedy. This outcome was ironic given that as a recent arrival at the FDA, the thalidomide dossier was assigned to Dr Kelsey in the belief it was a 'straightforward drug' that would help her 'learn the ropes'. The US populace is fortunate that she approached her first assignment with scientific rigour and moral integrity.

On the other side of the planet, the 1960 *BMJ* letter linking thalidomide to peripheral neuritis caught the eye of William McBride, a young Australian obstetrician at the Women's Hospital in Sydney. Supplied with samples of thalidomide pills by the local sales representative of Distillers Co., the UK company that was licensed to market thalidomide throughout the British Commonwealth, McBride was impressed by the hypnosedative actions of thalidomide after prescribing it to a pregnant woman under his care. Although that mother gave birth to a healthy child, a cluster of severe birth abnormalities in the offspring of three other women to whom he also gave thalidomide caused Dr McBride growing concern. All three babies died within a year of birth and displayed a peculiar reduction in the length of the forearm bones that was accompanied by malformations within internal body organs including the digestive tract. McBride promptly notified the Distillers Company of his concerns, and while they took little interest, he persuaded the chief of his hospital pharmacy to remove thalidomide from its shelves.

Eager to confirm his suspicions experimentally, Dr McBride initiated a round of animal studies in which thalidomide was administered to pregnant mice and rats. By a strange quirk of nature, the offspring of these two rodent species are resistant to thalidomide abnormalities, ensuring the winter of 1961 was frustrating for Dr McBride and his small team. In September, however, the birth of two affected babies at the Sydney clinic quickly dispelled his reservations concerning the prenatal toxicity of thalidomide. A letter published in a December 1961 issue of *The Lancet* earned Dr McBride high acclaim in the Australian media as well as in the international medical community. Although Dr McBride's reputation would be tarnished through his later involvement in a controversial case involving another drug – Debendox – he

deserves credit for being the first to implicate thalidomide in the plague of birth defects that was then breaking out all over the world.

Meanwhile in Germany, the epicentre of the evolving disaster, another vigilant clinician – Dr Widukind Lenz – was suspicious that thalidomide was causing the outbreak of severe limb malformations among babies born in his country. Spurred on by the father of an affected child, a lawyer named Karl Schulte-Hillen, Dr Lenz made a detailed study of the incidence of *phocomelia* – the clinical term for the peculiar reduction in the upper or lower limbs in newborns – in paediatric clinics in Hamburg. To his horror, he discovered that the condition was exceedingly rare in prior decades but that a significant increase in affected children had occurred in very recent times. Placing advertisements in several German newspapers, Lenz and Schulte-Hillen identified a small cohort of affected families, and upon interview of the mothers eventually identified thalidomide as a common factor in all cases. Although some Grunenthal executives initially attempted to discredit his findings, thanks to media publicity the company eventually withdrew thalidomide from German use in late November 1961. In subsequent days and weeks, national governments around the world placed bans on the drug.

Since many victims – perhaps 40 % or so – died in the early months of life, precise estimates of the number of children affected by thalidomide are hard to obtain, but a figure of 10,000 or so victims is commonly accepted. The then-largest medical damages court case in German history followed, but with the passage of time public interest in the case subsided. For thousands of families, however, the pain of living with the carnage inflicted by thalidomide would have a lasting impact.

1.6 The Discipline Emerges

If anything positive can be gleaned from the thalidomide episode, it might be the impetus it provided to the development of toxicology as a distinct scientific discipline. The peak of the disaster coincided with the founding of one of the world's largest toxicology organisations, the Society of Toxicology (SOT), a US-based body that held its first conference in Atlantic City in April 1962. A chief objective of SOT was to encourage universities to develop systematic toxicology curricula as well as research capabilities for the study of drug- and chemically induced toxicity. A decade later, a similar body formed in the UK which was later renamed the British Toxicology Society.

The 1960s and 1970s also witnessed growth in the establishment of journals dedicated to reporting research findings from studies of chemically induced toxicity (e.g. *Toxicology and Applied Pharmacology* [1959], *Toxicology* [1973], *Toxicology Letters* [1977], *Toxicological Sciences* [1981]). In 1988, growing interest in the chemical basis for toxicological phenomena prompted the world's largest scientific body, the American Chemical Society, to launch a significant new journal, *Chemical Research in Toxicology*.

The growing number of toxicology-themed journals reflected the growth in private and public funding to support research into chemically induced diseases. In the USA, expanding federal investment in human risk assessment and environmental health helped fuel the growth in modern toxicology research. In 1978, Joseph A. Califano, then Secretary of what is now the US Department of Health and Human Services, helped establish the National Toxicology Program (NTP), a body that coordinates the allocation of federal funding to support toxicology research in universities and research institutes within the USA and abroad. In addition to leadership by the FDA and US Environmental Protection Agency (US-EPA), the National Toxicology Program is overseen by the National Institute of Environmental Sciences which was established in North Carolina in 1966. The only NIH Institute located outside of Bethesda, Maryland, the NIEHS quickly became a leading international centre for toxicology research as it pursued its mission to 'reduce the burden of human illness and disability by understanding how the environment influences the development and progression of human disease'. Yet major advances in toxicology also occurred within other NIH laboratories, led by pioneers such as Bernard Brodie and Julius Axelrod and later continued by investigators such as JR Mitchell, D Jollow and JR Gillette. Breakthroughs by these Bethesda research teams helped secure our modern appreciation of the role of metabolism in chemical toxicity.

Another key event during the early 1970s occurred with the establishment of the National Center for Toxicological Research (NCTR) in Jefferson County, Arkansas. This facility remains the main toxicology research initiative of the US Food and Drug Administration, the world's largest pharmaceutical regulatory agency. The forerunner to the NCTR was part of the Pine Bluff Arsenal that conducted controversial Cold War research into biological pathogens and chemical warfare agents. In 1969, following public concern over Agent Orange use in the Vietnam War, President Nixon banned military research of this kind, leading the US military to transfer operation of Pine Bluff to the FDA. Under its new overseers, the NCTR became a major hub of research into the health effects of drugs and pollutants.

The 1970s also witnessed growing realisation within the US chemical industry concerning the need to study the health effects of their products. Cooperation between various chemical companies produced the Chemical Industry Institute for Toxicology (CIIT) in 1974, a successful venture that was later relaunched as the Hamner Institutes for Health Sciences (2007). This era also saw a growing focus upon toxicology within universities, leading to the establishment of strong research centres dedicated to investigating toxic phenomena. In Sweden, the Institute for Environmental Medicine at the Karolinska Institute, one of Europe's most prestigious medical universities, played a key role in uncovering the mechanisms underlying chemically induced toxic syndromes. In the UK, universities of Liverpool, Leicester, Surrey and elsewhere helped foster the discipline, while in the US entities such as the Center for Molecular Toxicology at Vanderbilt University and the Toxicology Program at the University of Kansas helped train new generations of toxicologists in modern research skills.

Towards the end of the twentieth century, fast-paced developments in biology and computer science began changing the face of toxicology and risk assessment. This

era saw the emergence of *computational toxicology* which applies insights from cheminformatics, high-throughput screening and structural biology to the development of virtual or computer-based toxicity prediction tools. These developments occurred in parallel with efforts to diminish dependence on mammalian species during toxicity testing by using nontraditional species such as molluscs, worms and zebrafish. Within the European Union, these trends were accelerated by the introduction of new regulations governing the use of synthetic chemicals known as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Conducted under the oversight of the European Chemicals Agency in Helsinki, Finland, the new REACH framework fosters growing use of alternative tests. Within the USA, the Office of Research and Development at the EPA has nurtured similar initiatives that encourage development of virtual and alternative test approaches. Thanks to assistance from the US-EPA, the Carolina Center for Computational Toxicology at the University of North Carolina became a hive of activity in this field.

The growth of toxicology was further spurred by awareness of the need for close attention to the environmental impact of the use of synthetic chemicals in modern industry and agriculture. The publication of *The Silent Spring* by Rachel Carson in 1962 helped forge this concern, as did a number of serious episodes involving plant failures or poor industrial plant management. The appalling Minamata Bay disaster that unfolded in mid-twentieth-century Japan took a heavy toll on both human and animal health following mercury contamination of the food chain subsequent to poor wastewater management at a chemical plant. Somewhat later, a 1976 accident at a chemical plant in Serveso in Northern Italy captured much public attention, this time due to the release of several kilograms of dioxin into the atmosphere. Although more effectively managed than Minamata, Serveso focussed attention on the need to consider geographical factors and proximity to settlements when building industrial plants. In the USA, the Love Canal controversy of the late 1970s, which focussed on the use of contaminated landfill sites for housing developments, further galvanised community concerns over the health impact of industrial chemicals. Within the toxicology community, these concerns fostered the establishment of journals and specialist societies devoted to environmental toxicology, a subdiscipline that continually grows in relevance due to the human and environmental impact of globalisation and industrialisation.

1.7 Global Toxicology

The growth of toxicology also required cultivation at the international level, a recognition that led to cooperation between various national societies to form the International Union of Toxicology (IUTOX) in 1980. In addition to aiding the maturation of toxicology in traditional centres of research strength, IUTOX assists its development throughout the developing world where chemical exposures due to expanding mining, manufacturing and agricultural industries create a need for applied toxicological knowledge.

In the late twentieth century, the growing industrial strength of the Asian economies helped spur the growth of toxicology and occupational health in Japan, Korea, Taiwan and elsewhere. Major legislative initiatives to protect the health of workers in these countries included implementation of the Japanese *Chemical Substances Control Law* in 1973, the Korean *Toxic Chemicals Control Act* in 1991 and the *Chinese Provision on Environmental Administration of New Chemical Substances* in 2010. Judging by the vitality of societies such as the Korean Society of Toxicology, the Chinese Society of Toxicology and the Toxicology Society of Taiwan, an increasing volume of toxicology research will be conducted outside of the Western world in coming decades. Thriving toxicology communities are also emerging in various African and Southeast Asian nations, evidenced by the rising profile of groups such as the Toxicology Society of South Africa (est. 2001) and the Cameroon Society for Toxicological Sciences (2006). In keeping with these trends, the attendees at major international toxicology conferences show increasing signs of ethnic and gender diversity.

As the twentieth century drew to a close, the trend towards the formation of 'continental toxicology blocs' accelerated as national societies sensed the need to collaborate on a supranational scale. EUROTOX – the Federation of European Toxicologists and European Societies of Toxicology – was launched in 1989 in an effort to unify toxicology societies in various European nations. EUROTOX traces its roots to the European Society for the Study of Drug Toxicity which was founded in Zurich. Following the collapse of the Berlin Wall in 1989, EUROTOX increasingly welcomed societies from several former Eastern bloc nations. Today, EUROTOX represents over 5,000 members within national societies in 33 nations from across Europe. Following the European lead, toxicology societies elsewhere began a similar process of heightened cooperation: the Asian Society of Toxicology (ASIATOX) was founded in 1994, while the Latin American Association of Toxicology (ALATOX) was formally organised in 1998.

1.8 The Breadth of Modern Toxicology

As modern toxicology grew and matured, a variety of subdisciplines emerged which focussed on particular concerns (Fig. 1.6). Just as the brass, strings and percussion instruments harmonise within an orchestra, the various subdisciplines cooperate to create a powerful discipline devoted to protecting public health via a better understanding of the harmful effects of chemicals on living systems.

1.8.1 Descriptive Toxicology

At a fundamental level, the edifice of toxicological knowledge rests on the foundation of knowledge of the specific toxicities that accompany exposure of a particular species to a given chemical. Information of this kind is obtained via careful study of model

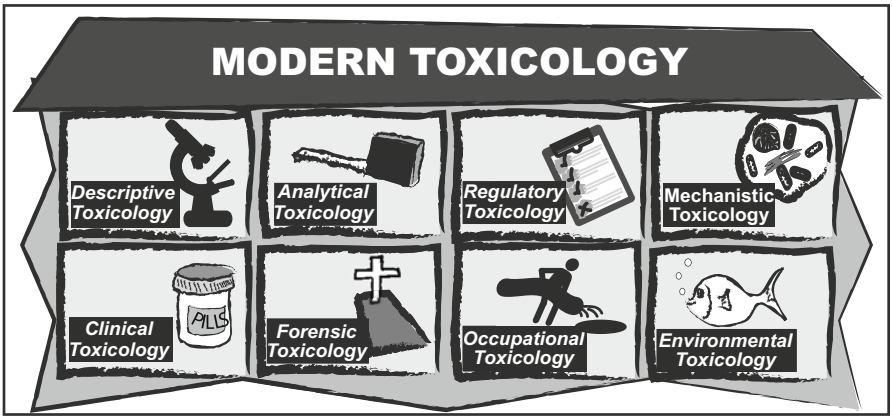

Fig. 1.6 Modern toxicology is a conglomerate of subdisciplines that cooperate to promote knowledge of chemical toxicity and overcome its potential to diminish individual and communal wellbeing

organisms following exposure to carefully controlled doses of the chemical in question, with meticulous records kept of the timing, magnitude and frequency of compound administration. Such studies are complex and costly hence it is no surprise that specialised companies as well as government institutions have emerged which specialise in performing toxicity tests of this kind. Since the welfare of the animals is of uppermost concern if quality data is to be obtained, exquisite attention must be given to the maintenance of inbred animal strains, bedding, cages, diet, housing conditions, etc.

Descriptive toxicologists working in these settings are expert in the design of toxicity tests using a range of model species including rats, mice, guinea pigs, dogs, primates and even zebrafish or invertebrates. Many different animal-based models are used to evaluate new chemicals, ranging from tests that examine short-term toxic responses as well as those that develop after months or even years of exposure (e.g. cancer). Tests in pregnant animals are also performed to assess the vulnerability of unborn animals to new chemicals. Yet the 'tools' used by descriptive toxicologists extend beyond live animals to include in vitro tests using primary or cultured cell lines.

Descriptive toxicology depends strongly on knowledge of cognate disciplines such as histology, anatomy, cell biology and pathology. In the modern setting, a strong background in the use of cell and tissue imaging technologies to detect the subtle effects of chemicals on the detailed architecture of body systems is also valuable.

1.8.2 Analytical Toxicology

Even if a particular chemical is implicated in the causation of disease or ill health in human populations, robust confirmation is required to confirm that toxicologically relevant concentrations of the substance are actually present within the body or

tissues of exposed individuals. In humans, correlations between blood concentrations of a toxic substance and the severity of pathological outcomes are invaluable if dose–response relationships for causality are to be established.

Analytical toxicologists specialise in the use of sensitive detection instrumentation to screen body samples collected from exposed individuals – most commonly blood or urine samples – to confirm the presence of suspected poisons. Nowadays, these methods are also used in industrial settings to detect use of banned substances by workers. Such analytical capabilities are also important during the evaluation of medicines as well as toxic substances by descriptive toxicologists in lab animals. Analytical data gained from such studies can reveal how much of an administered chemical gets into the circulating bloodstream and tissues of exposed animals under different routes of exposure (e.g. when the chemical is administered via the diet, drinking water or inhaled air of lab animals). Analytical toxicology as a subdiscipline draws strongly from the knowledge base provided by modern analytical chemistry; hence, a strong background in organic and inorganic chemistry is essential.

1.8.3 Mechanistic Toxicology

The observation of toxic responses to a particular chemical in an animal test need not necessarily imply the compound in question poses a threat to normal use in humans. Sometimes the administration of high doses of chemicals to rats or mice can trigger toxic responses that may not occur in humans who are exposed to lower doses. Indeed, toxicological history includes several examples of health scares caused by uncritical application of the assumption that a toxic response in rodents is automatically predictive of a comparable response in humans. One famous example involved the artificial sweetener saccharin, a widely used component of diet beverages and food additives. During the 1970s, much public concern erupted after a study reported bladder tumours in rats following treatment with high doses of saccharin. Once mechanistic toxicologists investigated the biological mechanisms underlying this response – which only occurred in male rats and not females – it was found that the bladder toxicity was due to a rat-specific protein named α-2u-globulin that has little relevance to humans. The work of Lois Leehman-McKeeman and others soon established that this troublesome protein was responsible for false alarms for renal tumours in male rats exposed to other substances including d-limonene and the fuel additive methyl tert-butyl ether. By associating renal tumours with a phenomenon known as hyaline droplet nephropathy which only occurred in male rats, this mechanistic research helped allay concerns to human health.

The saccharin scare and related episodes helped modern toxicology appreciate the need to thoroughly understand the mechanisms underlying the toxic responses of organisms – both human and nonhuman – to foreign chemicals. The mechanistic toxicologists who perform this work typically have strong backgrounds in such core biomedical disciplines as biochemistry, genetics, pharmacology, physiology,

immunology and molecular biology. In recent decades, such mechanistic insights have assumed greater importance during the risk assessment process as chemical regulators have appreciated the need to base their rulings upon rigorous, experimentally verified mechanistic understandings of chemically induced disease.

1.8.4 Regulatory Toxicology

Once the toxicological profile of a new chemical is established, the decision to proceed with its use within a particular sector of the economy is made via a review process that involves application by sponsoring chemical or pharmaceutical companies to appropriate regulatory bodies. In most jurisdictions the approval of new chemicals is subject to tight legislative control and usually involves specific regulatory bodies which focus on chemicals used within particular commercial settings, including pharmaceuticals, agrochemicals, veterinary products, industrial chemicals, etc. Regulatory toxicologists working within these public entities are typically trained in the evaluation of scientific literature and commercial trials which examine the fate and toxicity of chemicals under conditions that approximate their likely use within the 'real world'. Regulatory bodies are also responsible for setting 'safe' exposure levels for chemicals that are likely to contaminate the human food chain via their use in agriculture and food production. Since analytical chemists can frequently detect very low levels of synthetic chemicals in common food items, it is important to know what levels can be safely consumed. Preparation for a career in regulatory toxicology usually involves a broad background in toxicology and associated life sciences such as public health.

1.8.5 Clinical Toxicology

Recent decades has seen the emergence of a specialised subgrouping among medical practitioners who are interested in the diagnosis and management of chemically induced disease. This can include emergency medicine specialists who are experts in the treatment of acutely poisoned individuals such as victims of deliberate or accidental overdosing with drugs or workplace chemicals. Yet there is much more to clinical toxicology than poisoning management alone since the skills of trained clinicians are also essential in the medicolegal setting where it is important to judge if particular symptoms or diseases experienced by a patient are due to work-related chemical exposures or whether they result from pre-existing diseases or other kinds of exposures. Since these cases demand skills that extend beyond the knowledge of drug and chemical toxicity that is acquired in a typical medical training programme, medical graduates seeking a career in clinical toxicology usually complete additional specialised graduate programmes in pharmacology, toxicology and environmental health.

1.8.6 Occupational Toxicology

The use of synthetic chemicals within the workplace raises critical issues that are of special concern to occupational toxicologists. Recognising the natural duty of care employers bear towards their employees, modern democracies have enacted legislative frameworks that promote the safety and health of workers in specific occupational settings. Workplaces that involve repetitive use of noxious chemicals often receive extra scrutiny. For example, in addition to workers in the chemical industry, personnel within the mining industry and other primary industries are regularly exposed to hazardous substances. Occupational toxicologists are responsible for monitoring chemical hazards within these workplaces while also keeping abreast of ongoing toxicological research concerning the health effects of substances relevant to their industrial sector. Their responsibilities can include overseeing the use of personal and environmental monitoring devices to assess chemical concentrations within the workspace of workers as well as ensuring regular health checks assess health end points and biomarkers that are relevant to the chemicals used by each employee. Preparation for a career in occupational toxicology usually requires strong training in toxicology and chemistry together with occupational health and safety, public health and epidemiology.

1.8.7 Forensic Toxicology

Sometimes relations between workers and employers in particular work settings can deteriorate, leading to legal proceedings in which chemical exposures often take centre stage. In such cases, the complainants may be family members of workers who died of a disease that they suspect was work related. Such situations require the input of forensic toxicologists with appropriate medicolegal and analytical training to assist courts and juries establish the validity of such claims.

As we saw in the case of Mathieu Orfila in nineteenth-century France, forensic toxicologists also possess expertise concerning the detection of drugs and poisons within body fluids and autopsy tissues obtained from homicide victims and other cases of unexpected death. Such scenarios are often highly complex and subject to ambiguity concerning the effects of concurrent chemical exposures that include prescription medicines and recreational drugs such as alcohol, tobacco or cannabis. Forensic toxicologists also require training in topics unique to this branch of science, including study of the fate of chemicals with decaying corpses and their rates of degradation by the microorganisms that cannibalise the bodily remains of deceased individuals. Forensic toxicologists typically require a strong background not just in toxicology, microbiology and pharmacology but also training in forensic science, legal theory and court proceedings.

1.8.8 Environmental Toxicology

Environmental toxicology explores the broader environmental impact of chemical exposure, with a particular concern for the effect of chemicals on nonhuman target species including birds, terrestrial organisms and marine species. This complex field requires attention to the stability of pollutants within soil and water as well as the ability of chemicals to accumulate within the food chain. Working with ecologists, environmental toxicologists assess any role of agricultural chemicals in the extinction of native species or monitor changes in speciation patterns within the proximity of mines or industrial plants. Environmental toxicologists also work with regulatory toxicologists and environmental engineers to assess the effectiveness of remediation efforts at contaminated land sites.

The research tools used by environmental toxicologists are increasingly sophisticated and, in addition to the use of analytical technologies to measure levels of pesticides or herbicides in soil, air or water samples, now include the use of genomic fingerprinting to monitor the effect of agrochemicals and pollutants on populations of microflora and microfauna in soil samples collected from contaminated sites. In addition to training in general toxicology, a background in molecular biology, molecular genetics, ecology, environmental law and population biology provides a solid foundation for a career in environmental toxicology.

1.9 The Scope of Modern Toxicology Research

Over recent decades, toxicology research developed rapidly to accommodate investigators specialising in studying toxic phenomena at many levels of biological organisation. At one end of the spectrum, *chemical toxicology* uses the instrumental tools of modern chemistry to characterise the mechanistic aspects of toxicological phenomena, seeking descriptions of the chemistry occurring when reactive chemicals attack cellular macromolecules such as DNA or proteins to form abnormal species that are the ultimate drivers of pathological responses. To extend these insights, *molecular* and *cellular toxicology* studies the biochemical and cell signalling responses resulting from such chemical damage to cell macromolecules. *Organ toxicology* focusses on the effects of noxious chemicals upon the function of whole organs such as liver, kidney, brain or lung. At a still higher level of biological organisation, *systems toxicology* develops conceptual models which integrate genome-wide changes in gene expression within an exposed tissue with changes in the metabolome or metabolite profiles that might accompany chemical toxicity within biofluids such as blood or urine. *Human toxicology* draws on such knowledge together with insights from immunology, biochemistry and clinical medicine to understand chemical toxicity at the level of human patients. Finally, *population toxicology* explores the impact of chemical toxicity within groups of humans, seeking to understand how chemically induced disease alters the wellbeing of an entire workforce within a specific occupational setting or how environmental pollutants such as pesticides or herbicides affect disease frequencies and health end points in whole cities or even entire national groups (Fig. 1.7).

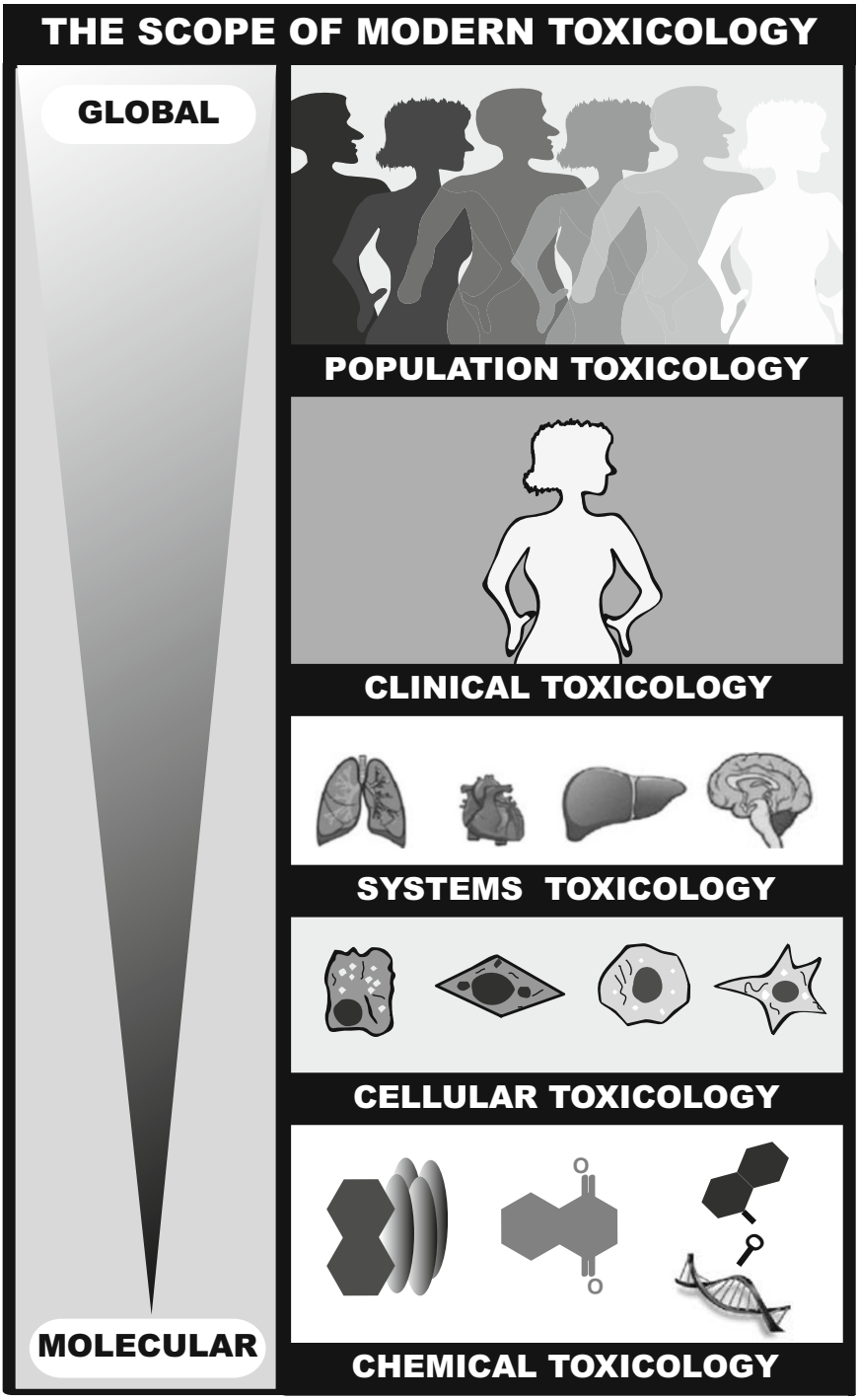

Fig. 1.7 Fully accounting for the toxicity of a noxious substance involves developing explanations that extend from the fundamental chemical level, through molecules, organelles, cells, organs and systems to individuals and whole populations

Since ongoing advances occur in human knowledge relevant to each of these areas, modern toxicology is a continually evolving enterprise. Understanding the toxicity of any toxic substance is never a 'closed book' situation. Since modern science is a massive global enterprise, the ongoing development of new technologies that allow fresh approaches to long-standing questions surrounding the health impact of chemicals perpetually opens new avenues of investigation to researchers. For the foreseeable future, toxic phenomena will continue to be actively investigated at many levels of biological organisation.

1.10 Conclusion

This chapter has conveyed a basic appreciation of how awareness of toxic substances is long-standing in human cultures while also providing a thumbnail sketch of how modern toxicology evolved in recent times. Due to space constraints, the latter account was highly potted and many key contributors were likely omitted. Nevertheless, the way is now clear to explore some basic ideas in toxicology that will supply a foundation upon which we can build a subsequent understanding of chemically induced disease. While an effort is made to sketch the broad contours of modern toxicological knowledge, it is inevitable that some areas receiver fuller treatment than others. Despite these limitations, we will learn that while much progress has been made in understanding the toxic effects chemicals exert on living tissues, since new chemicals continue to find their way into the human and natural environments, continued vigilance and investment in this crucial discipline is needed to maintain human and environmental wellbeing in coming decades. The advances of recent decades provide a solid basis for future progress within this crucial sector of the modern scientific enterprise.

Going Further

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Chapter 2
Core Concepts in Toxicology

Abstract Modern toxicology uses many distinctive terms and concepts during its efforts to explain toxic phenomena. Firstly, since a suitable umbrella term is needed for the types of substances that are of concern to toxicologists, this chapter explores linguistic nuances surrounding such words as poison, toxin, xenobiotic, endobiotic and toxicant. Next, the complications that can accompany study of toxicity due to differences in the duration of chemical exposure are explored as determinants of toxic responses. The importance of dose in governing the severity of toxicity after chemical exposure is surveyed, together with the role of individual factors in predisposing susceptible subpopulations to exaggerated toxic responses. The possibility of interactions between chemicals in complex 'real-world' exposure scenarios involving simultaneous exposure to more than one substance is also considered. The role of lifestyle factors in shaping individual susceptibility to chemical toxicity is also highlighted, as is the timing of the manifestation of toxicity following toxicant exposure.

Keywords Allergies • Dose • Idiosyncratic toxicity • Latent toxicity • Local toxicity • Obesogens • Poison • Toxicant • Systemic toxicity • Xenobiotic

2.1 Introduction

Although the mission statement of modern toxicology – to understand the full breadth and depth of chemically induced disease in humans and other species – seems straightforward, achieving this goal is complicated by several real-world considerations. This chapter explores some basic concepts and complicating factors that accompany the study of chemical toxicity. First, however, we will explore some basic terminology used within modern toxicology.

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2.2 The Terminology of Toxicology

As with other fields of knowledge, toxicology has its own distinctive vocabulary. Since the imprecise use of toxicological words can hinder effective communication of chemical hazards, some semantic issues that can confuse toxicology neophytes require our attention.

2.2.1 Poison

Most pressing perhaps is the umbrella term that best denotes the types of noxious substances that are of interest to toxicologists. The word *poison* is widely used for this purpose during everyday life, with its popularity traceable to ancient times. The Father of Modern Toxicology, Mathieu Orfila, also found the term suitable when writing the introductory chapter to his famous 1813 *General Treatise of Toxicology*, 'The name of poison is given any substance, which, taken inwardly, in a very small dose, or applied in any kind of manner to a living body, impairs health, or destroys life'.

Despite its longstanding pedigree, the term *poison* is not widely used as an umbrella term in modern toxicology because it may convey the misleading connotation that the world contains just two types of chemicals: 'poisons' and 'nonpoisons'. By implying we should be anxious about the subset of chemicals that are designated poisons, yet complacent about all the other 'nonpoisonous' chemicals in current use, the word *poison* is potentially misleading. Following Paracelsus, modern toxicology emphasises that *any* chemical substance might elicit toxicity under appropriate exposure conditions (e.g. at very high doses, even common table salt or drinking water is harmful). We will return to the 'dose–response' relationship for chemical toxicity below.

2.2.2 Toxin

We might instead use the word *toxin*, which is also encountered in daily speech. This word inhabits many alternative medicine websites that sell products which allegedly 'cleanse' ones liver or 'system' of these fearful entities. In toxicological circles, however, the term *toxin* is best reserved for harmful substances made by living organisms (such as poisonous marine organisms, infectious pathogens or venomous spiders). Strictly speaking, synthetic chemicals including most modern drugs as well as many industrial chemicals are not covered by this term.

2.2.3 Xenobiotic

One handy word often used by toxicologists is *xenobiotics*. It derives from the Greek word *xenos*, which denotes something *strangely unfamiliar* or *foreign*. A *xenobiotic*

is thus a chemical that enters the body from a foreign or external source. Such substances contrast with *endobiotics* – chemicals that form within the body during normal physiological processes (e.g. androgens, glucocorticoids, neurotransmitters, eicosanoids and metabolic waste products such as bilirubin). Endobiotics also include toxic chemicals that form within diseased tissues via pathological processes. In theory, *xenobiotic* excludes these endogenous substances and instead denotes the tens of thousands of synthetic chemicals in widespread use in today's world, including food additives, industrial pollutants, consumer products, medicines, recreational drugs, pesticides, herbicides and industrial reagents.

Note that in labelling a chemical a *xenobiotic,* we are reserving judgment as to whether it has noxious biological properties under normal conditions of human use or exposure. A xenobiotic may be toxic under some extreme exposure conditions but quite harmless under others. For example, with occasional use in appropriate doses, many synthetic drugs are sufficiently safe that they are available over the counter without a physician's prescription. The fact that these and other nonhazardous synthetic molecules are considered *xenobiotics* means the term does not entirely fit our need for a name which embraces chemicals with definite toxic potential under common conditions of use.

In addition to synthetic substances, the term *xenobiotic* covers naturally occurring chemicals to which humans are regularly exposed via consumption of plant-based foodstuffs, botanical beverages and herbal remedies. While many of these substances are likely harmless or even beneficial to human health, some xenobiotics of natural origin can be very harmful indeed. As a rule, modern toxicology does not concur with the popular belief that foreign or synthetic chemicals are inherently more toxic than naturally occurring substances or even endobiotics. Many of the most toxic substances known to toxicology are of natural origin – a point that will be reinforced throughout this book. Nevertheless, synthetic chemicals of human origin typically attract the greatest attention in modern toxicology simply because they are used on a vast scale in today's industrial societies. So while nature may produce some highly potent toxins, they are rarely produced on a comparable scale to modern synthetic substances. Another factor that maximises interest in synthetic xenobiotics is their frequent possession of physicochemical features that ensure they are long lived within biological systems or the wider environment. Since we have been exposed to natural chemicals throughout human history, our bodies are better adapted to coping with their presence compared to some synthetic substances of modern origin that may contain unusual chemical properties that render them resistant to metabolism.

Although it is handy to classify chemicals according to whether they are of natural or synthetic origin, this distinction is often artificial. With the development of sensitive analytical instruments for the detection and quantitation of chemicals in body fluids or tissues, we now know that many chemicals – even some we once assumed were entirely of synthetic origin and would only be encountered in the factory or industrial workplace – are actually formed at low levels within the body. Acrolein, for example, is a highly toxic carbonyl compound used during the manufacture of plastics and other synthetic chemicals (Fig. 2.1). It is also a major environmental pollutant, formed during the combustion of organic matter including tobacco, fossil fuels and forest vegetation. Acrolein also forms during cooking

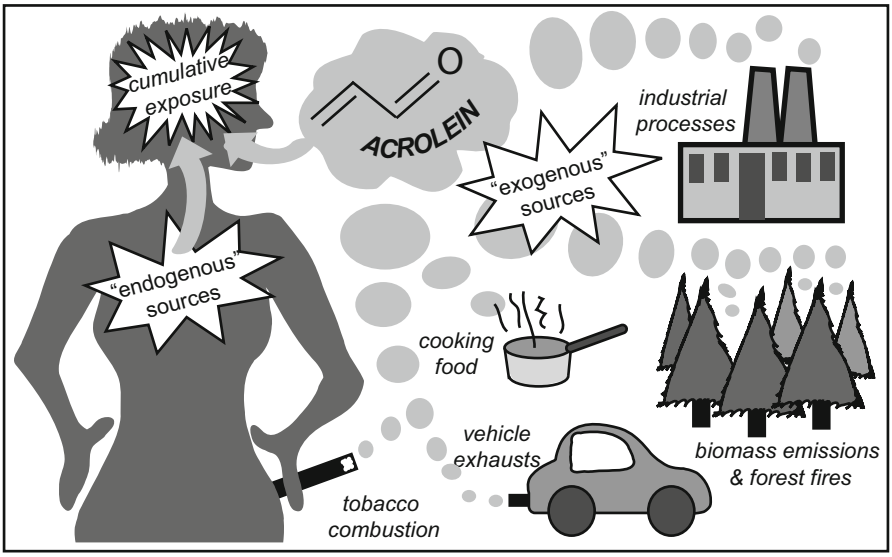

Fig. 2.1 Classifying chemicals via their origin (i.e. xenobiotics vs. endobiotics) is complicated for chemicals that arise from both sources. The airborne pollutant acrolein is a typical example: humans are exposed to exogenous acrolein that forms via combustion of diverse organic matter (e.g. tobacco, fossil fuels) but also to endogenous acrolein that forms via biochemical reactions within the body (e.g. lipid peroxidation)

processes and can attain high airborne concentrations in kitchens if deep fried foods are prepared over a poorly ventilated stovetop. Yet in recent decades, our assumption that acrolein is mainly ingested from these foreign sources has been overturned by the discovery that it forms endogenously via diverse biochemical processes, including a phenomenon termed *lipid peroxidation* which we will examine in Chap. 4 (Sect. 4.4.4). Some scientists suspect that endogenous acrolein participates in such degenerative diseases of old age as Alzheimer's dementia. This remains to be fully proven, and ongoing research is assessing the health significance of these endogenous exposures. It could well be that for some endogenous exposures, the high sensitivity of our modern analytical instruments leads us to overestimate their importance. Nevertheless, the fact that we are exposed to noxious substances from both external and internal sources poses a conceptual problem: should we categorise a substance like acrolein as a *xenobiotic*, an *endobiotic* or both (Fig. 2.1)?

2.2.4 Toxicant

The favoured catch-all within the toxicological lexicon for noxious chemicals of synthetic *and* natural origin is *toxicant*. This phrase denotes a chemical with a definite potential to induce cellular and tissue damage under commonly encountered

conditions of exposure, whether deliberate, accidental or unintentional. The main limitation with the term is that it rolls off the tongue less freely than other phrases, perhaps explaining why this scientifically accurate term has not penetrated into everyday usage – and why inappropriate use of 'toxins' and 'poisons' is still encountered even in the scientific literature. *Toxicant* is especially informative when coupled with a prefix that designates the site of toxic action for a given substance; alcohol, for example, is a *hepatotoxicant* since it causes liver damage in high doses, while cadmium is a *nephrotoxicant* because it targets the kidney. The phenomenon of organ-selective toxicity is addressed in Chap. 6 (Sect. 6.5).

2.3 Chemical Exposure Scenarios

The millions of diverse molecules in existence, both natural and synthetic – together with the rich complexity of living cells – suggest the likelihood of a wide array of pathological outcomes following exposure to chemicals. This biological complexity alone is enough to ensure that uncovering the molecular mechanisms underlying chemical toxicity is far from simple. Yet the study of toxic syndromes is further complicated by the nearly limitless possibilities concerning patterns of human exposure: for some chemicals, exposure may be intermittent in nature, occurring fleetingly over a timeframe of seconds, minutes or hours, while at the other extreme, some substances might be encountered continuously throughout much of a human lifespan. The risk assessment process needs to take into account individuals within the latter 'high exposure populations' who face heavy, frequent exposure to toxic substances beyond levels encountered by most individuals. The patterns of exposure to a given volatile solvent used in paint production, for example, are very different for spray painters who encounter the substance every day throughout much of a career, relative to hobby mechanics who handle it only sporadically within a home workshop. These factors require careful attention during the design of toxicity tests since it is essential to know whether the same chemical induces different toxic effects under divergent exposure scenarios.

To simplify the study of toxic phenomena, chemical exposures are usually assigned to one of four categories. Firstly, an *acute* exposure features chemical intoxications occurring over 24 h or less. These types of exposures are encountered in hospital emergency room situations where clinical toxicologists must manage patients who have accidentally or deliberately ingested high doses of drugs or other substances. Depending on the specific substance and the dose received, clinical signs of acute intoxication can involve such symptoms as restlessness, irritability, headache, delirium, confusion, vomiting, diarrhoea, muscle cramping, convulsions or even death.

Toxicities that manifest after repeated exposure to chemicals over several days or up to 1 month in duration are termed *subacute* intoxications. These types of syndromes are also relevant clinically since they can involve toxic responses to medicines that occur in the early stages of therapy, especially when patients have had no

prior exposure to the drug. Alternatively, *subchronic* exposures involve ongoing, repeated exposure to a chemical for periods of 1–3 months in total. This broad category covers both repeated single dosing with a substance (e.g. an antibacterial drug taken every day to treat a persistent urinary tract infection) as well as prolonged exposures to chemicals present in our diet as food additives or contaminants of drinking water.

Finally, *chronic* exposures are of 3 months or greater duration and resemble the previous category in that they can involve either repeated discrete dosing or long-term exposure to chemicals present in food, drinking water or air. This category also includes occupational exposures such as when print shop workers inhale ink solvents regularly over many years or even decades.

The fact that different toxic responses can occur to the same chemical depending on its patterns of exposure greatly complicates the toxicological assessment of new drugs and chemicals. This ensures that multiple study designs that mimic different patterns of exposure are included during routine toxicity testing of chemicals in rodent bioassays. Hence, some tests are designed to detect acute toxic responses following a limited number of doses, while at the other end of the spectrum, others involve chronic testing and examination of effects on animal health after a lifetime of exposure under controlled lab conditions. Issues surrounding the detection of chemically induced disease in rodents receive attention in subsequent chapters.

2.4 The Localisation of Toxicity

The human body is profoundly complex, constructed from well over 100 different cell types that are assembled into highly ordered three-dimensional tissue and organ architectures. A vast network of genetic and metabolic pathways enables the assembly and proper functioning of these systems. Since toxicants can exert complex and unexpected effects upon these systems, predicting the precise bodily location where toxicity is manifest is a challenging task. Our sketch of toxicological history in Chap. 1 hinted at the tendency of different chemicals to injure specific body organs – TOCP damaged nerve cells in the central nervous system, diethylene glycol produced kidney injury, while short-term use of thalidomide injured unborn children without necessarily harming their mothers. This tendency for particular toxicants to cause injury at distinct body sites raises the issue of the *locus* or *localisation* of toxicity. In general, toxicologists classify a given toxic syndrome as either *local* or *systemic* in nature (Fig. 2.2).

2.4.1 Local Toxicity

Firstly, some substances primarily induce *local* toxicity upon their initial contact with the body (Fig. 2.2). Such chemicals usually possess pronounced physicochemical

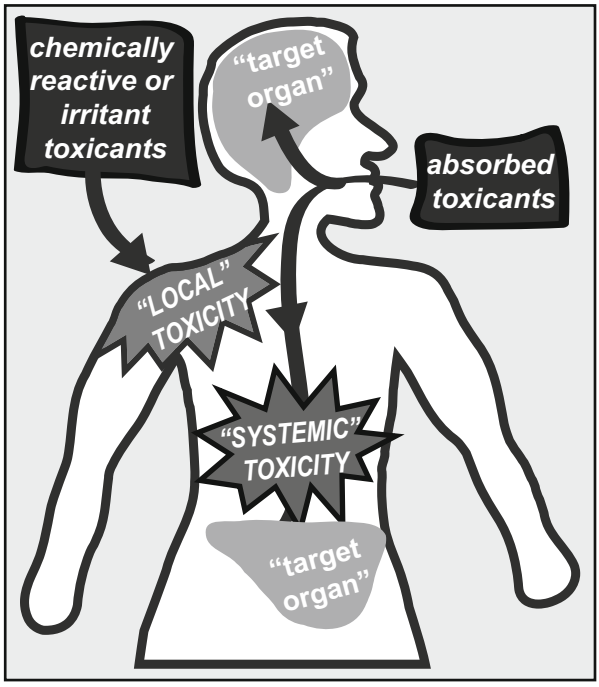

Fig. 2.2 While the toxicity of reactive or irritant chemicals is often expressed *locally* at the first point of contact with the body, most absorbed chemicals induce *systemic* toxicity that involves a handful of internal 'target organs'. In the example shown, the liver and brain are the relevant 'target organs'

properties such as a strongly acidic or alkaline character that ensures they injure any tissue they directly encounter. Body parts that interface directly with the external environment – such as skin, lungs, nasal cavity or the eyes – are obviously most vulnerable to locally acting toxicants. Exposure to such substances – especially if they are handled manually during the course of one's daily employment – can cause skin burns, rashes, contact dermatitis or blistering. For obvious reasons, chemicals with strong chemical reactivity and overt local toxicity profiles have sometimes attracted attention as chemical warfare agents – including vesicant gases such as phosgene, mustard gas and chlorine.

2.4.2 Systemic Toxicity (Target Organ Toxicity)

Although local toxicity is relevant to a subset of chemicals with extreme properties, most chemicals tend to induce *systemic toxicity*. Such substances require absorption into the body where they undergo dispersal throughout the blood stream, typically causing damage within one or more susceptible organs (Fig. 2.2).

Some target organs sustain injury because they accumulate high concentrations of respective toxicants. Examples include the eye toxicity that can accompany systemic use of the antimalarial drug chloroquine, or the life-threatening lung injury following accidental or deliberate ingestion of paraquat, a widely used herbicide. In

both instances, high jacking of cellular uptake processes leads to high intracellular concentrations of chloroquine and paraquat within their respective target tissues.

Other chemicals damage specific organs that are highly vascularised and well perfused with blood, thereby receiving a high proportion of the 'internal' or 'absorbed dose' of toxicants. The physicochemical properties governing the absorption of foreign chemicals into body tissues receive attention in Chap. 3. In general, the solubility of a chemical in nonpolar solvents predicts its absorption properties, since this property governs its membrane-penetrating abilities. Following oral ingestion, chemicals with lipophilic properties are efficiently delivered to the liver, thereby ensuring liver damage is a common outcome following exposure to toxic chemicals.

In addition to passive diffusion, recent research has uncovered a major role for miniature 'pumps' or 'membrane transporters' in controlling the transport of foreign chemicals across cell membranes, revealing that chemicals often accumulate in target tissues because they express high levels of influx pumps. Such factors often render the main excretory organs of the body, liver and kidney, highly vulnerable to ingested toxicants. For example, these tissues typically accumulate heavy metals prior to their permanent removal from the body, ensuring hepatic and renal levels of lead, cadmium and mercury are hundreds of times higher than tissue concentrations in other organs. This ability to sequester such substances comes at a cost; liver damage ('hepatotoxicity') and renal injury ('nephrotoxicity') are classic signs of intoxication with many heavy metals.

Very frequently, the toxicity of specific chemicals localises to a particular organ because that tissue expresses high levels of enzymes that convert the compound to toxic, cell-damaging metabolites. Termed *toxicological bioactivation*, this phenomenon contributes to numerous chemically induced pathological states. James and Elizabeth Miller at the University of Wisconsin laid the foundations for our modern appreciation of the role of bioactivation in chemical toxicity during the 1940s. This husband-and-wife research team established that the cancer-causing properties of azo dyes – a class of compounds resembling those that Rehn associated with bladder cancer in German textile workers during the nineteenth century – was not due to the parent compound but to toxic metabolites that attacked DNA and proteins in exposed cells. Bioactivation is so important to modern toxicology that Chap. 4 explores this topic at some length (Sect. 4.3).

Target organ toxicity is inherently interesting because it raises many productive research questions. For example, why does a particular chemical cause liver damage when a closely related substance targets the kidney? What happens to the chemical within the body – is it metabolised quickly or does it linger in specific tissues? If the latter, are those organs most vulnerable to toxicity, or does toxicity occur elsewhere? What are the dose–response relationships for the toxic syndrome – do the same toxic responses occur at high doses as at low doses, or do different problems emerge due to alternate routes of metabolism that follow saturation of low capacity pathways? What happens if the same total toxicant dose is administered in small fractionated amounts over an extended timeframe – does toxicity disappear, or do new toxicities emerge? How does the 'route of entry' affect toxic outcomes – is, for example, the substance as toxic following dermal application compared to oral absorption? What are the fundamental mechanisms underlying cellular injury

in affected tissues, and why are these actions not exerted on cells in unaffected tissues? How does the body cope with the presence of the chemical if exposure occurs over an extended period – can target tissues increase the levels of specific transporters or enzyme systems to protect themselves? Finally, are individuals from different genetic backgrounds equally vulnerable to the target organ toxicity?

The fact that target organ toxicity generates so many questions explains why these types of syndromes often attract greater research attention than those caused by compounds that damage the first site of contact with the body.

2.5 Dose: The Magnitude of Exposure

Our historical survey in Chap. 1 revealed that a common-sense appreciation of the importance of dose to poisoning outcomes is traceable to at least the time of Socrates (fifth century BC). Thousands upon thousands of studies performed by modern toxicology researchers have reinforced this age-old conviction: irrespective of the toxicity under consideration – whether cancer induction, hepatotoxicity, neurotoxicity or induction of skin rash – no factor exerts a stronger influence over the severity of toxic responses than the total amount of toxic substance received by an individual. Known simply as the *dose*, the quantity received by an individual is represented in units of mass or molar amount (e.g. milligrams, micromoles). Often it is more informative to express the dose in terms of recipient characteristics (e.g. body weight, body surface area): the relevant term in this instance is the *dosage* (e.g. milligrams/kg body weight, micromoles per m^2).

The relationship between the administered dosage and toxicity severity within an individual takes the form of the *graded response* shown in Panel A of Fig. 2.3. In this hypothetical case, a toxicant induces apoptotic cell death within circulating blood cells, with a greater proportion of cells exhibiting cell death with increasing dosage of the toxicant (Fig. 2.3a). The graph suggests the individual response to a toxicant is *graded* over a range of doses, with the severity of toxicity rising with escalating doses. Assuming a log scale is used to display the dosage on the *x*-axis, such representations resemble the S-shaped *sigmoidal curve* long known to classic pharmacology.

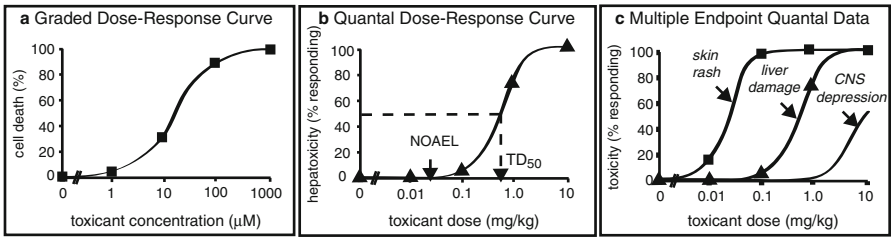

Fig. 2.3 Sigmoidal dose–response relationships for graded toxic responses in individuals (*Panel a*) and quantal responses in groups (*Panel b*). (*Panel c*) shows how the same dose-ranging toxicity assessment experiment can yield multiple dose–response curves for different toxic end points depending on their sensitivity to the toxicant (e.g. skin rash, hepatotoxicity)

The dose–response relationship is more commonly studied at the level of populations comprising multiple individuals, where a graphical representation of the administered dosage and the prevalence of the toxic response also yields the familiar sigmoidal curve (Fig. 2.3b). Data of this kind is known as quantal data since it describes the toxic response in terms of the percentage of affected individuals within an exposed population. A quantal dose–response curve supplies useful quantitative estimates that provide helpful insight into the toxicity of a given compound: for example, the dose eliciting the reported toxic response in 50 % of the population can be easily determined (i.e. TD_{50}), as can the *threshold dose* at which toxicity is first observed (Fig. 2.3b). A related concept is the *no adverse effect level* (NOAEL) which can be estimated from dose–response data of this kind. It must be kept in mind that these quantitative estimates are not absolute values that are carved in granite, for the actual number calculated for the threshold dose and TD_{50} is dependent upon the number of individuals within the population that is under investigation. A study designed to estimate the TD_{50} for a given food additive which is performed in a population of 20,000 mice will likely yield a different number to one obtained from just ten exposed mice.

Another feature of *quantal* representations of toxicological phenomena is that differences are frequently seen in the dose dependence of different toxic outcomes. In the hypothetical example shown in Fig. 2.3c, skin rashes are an especially sensitive indicator of intoxication with this toxicant, while hepatotoxicity occurs at higher doses, while still higher doses induce CNS depression.

As during the interpretation of dose–response curves in pharmacology, the shape and slope of dose–response relationships allow important comparisons between different toxicants (Fig. 2.4). Such curves reveal the *efficacy* of a toxicant, namely, the *effectiveness* with which it induces the toxic response of concern, as well as its *potency,* the doses required to elicit its characteristic toxic effects. In the hypothetical example shown in Fig. 2.4, several toxicants are compared for their effectiveness at inducing bladder cancer in a population of laboratory rats. Since toxicant A induces the highest yield of tumours at low doses (e.g. 0.1 μg/kg), it is said to exhibit greater carcinogenic *potency* than toxicants B or C. On the other hand, toxicant C exhibits lower tumourigenic *efficacy* than either toxicants A or B since it induces a lower proportional yield of tumours at high doses. Toxicants B and C exhibit comparable potency.

Before concluding our brief survey of dose–response data, we need to reinforce the importance of establishing this relationship whenever efforts are made to incriminate particular chemicals in specific pathological syndromes or disease outcomes in human populations. This can be a challenging task, given that most humans continually absorb diverse chemicals via our dietary habits, use of personal care products, consumption of alcohol or tobacco, ingestion of medicines or alternative therapies or even chemical handling within the home workshop or during other ‘after-hours’ hobby activities. Against this backdrop of normal xenobiotic exposure, seeking to tease out the involvement of individual workplace toxicants can be difficult indeed. In such circumstances, the strength of disease associations for a given toxicant is increased if the dose received by each member of the study population is quantified. This can

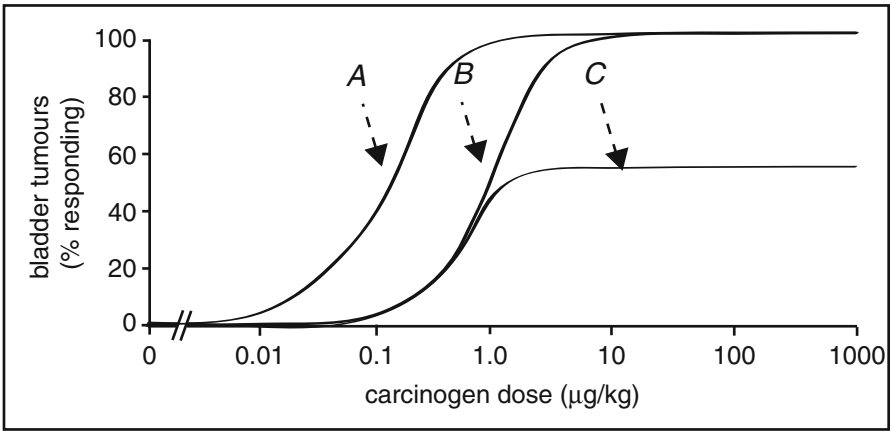

Fig. 2.4 Comparison of carcinogenic *potency* and *efficacy* of three hypothetical toxicants, *A*, *B* and *C*. Toxicant *A* has the highest potency since it increases tumour incidence most strongly at the lowest doses tested. The fact that toxicants *A* and *B* both induced a maximal tumour response at high doses indicates they possess comparable efficacy. Toxicant *C* has comparable potency to toxicant *B*, yet lower efficacy

include the wearing of personal exposure monitoring devices that estimate the amount of chemical to which a person is exposed during a working day. Alternatively, measuring the levels of individual chemicals and their metabolites within blood or urine samples collected from exposed shift workers can reveal the internal dose of substance received by each study participant. Characterising the dose–response relationships for toxic responses of concern for a given xenobiotic greatly enhances the quality of human risk assessment efforts. In recognition of this concern, modern toxicology devotes great effort to characterising exposure profiles of workers in particular occupational settings, including the use of personal monitoring devices that quantify concentrations of toxicants within the workspace of individual workers over an actual work day shift. Thanks to ongoing US-EPA initiatives, knowledge of individual exposure patterns in real time will become a distinct possibility with the impending availability of sensitive, affordable and mass-produced personal chemical sensors. By incorporating such devices into cell phones, the use of 'exposure apps' may allow individuals to monitor their personal toxicant exposure on a daily basis.

2.6 Unexpected Toxicant Sensitivity

Explorations of dose–response relationships typically proceed on the assumption that target populations are homogenous and comprise individuals who conform to bell-shaped Gaussian distributions in their sensitivity to toxicants. Within such a normally distributed group, a median toxicant dose is identifiable which induces toxicity of comparable severity within most individuals in the population (Fig. 2.5a).

However, the population also contains small numbers of individuals who show toxicity at relatively low exposures, together with relatively resistant individuals who only exhibit toxicity at high exposure levels (Fig. 2.5a). Since susceptibility is normally distributed within the population, the number of individuals at both poles of the continuum is comparable (Fig. 2.5a).

In the real world, studies of toxicant sensitivity within heterogenous human populations sometimes deviate from these expectations. Of special concern is the situation shown in Fig. 2.5b, where a subpopulation of individuals displays exaggerated sensitivity to a toxicant due to genetic or environmental factors (Fig. 2.5b). *Regulatory toxicologists* must consider such sensitive individuals when setting safe exposure levels for food additives and other chemicals (see below).

Vulnerable individuals exhibiting rare toxicities are of great concern to the pharmaceutical industry, since unusual toxic responses are often undetected in animal tests and clinical trials and only become obvious after large patient populations ingest newly marketed medicines. Since getting a molecule approved for human use involves investment of hundreds of millions of dollars, the withdrawal of a medicine

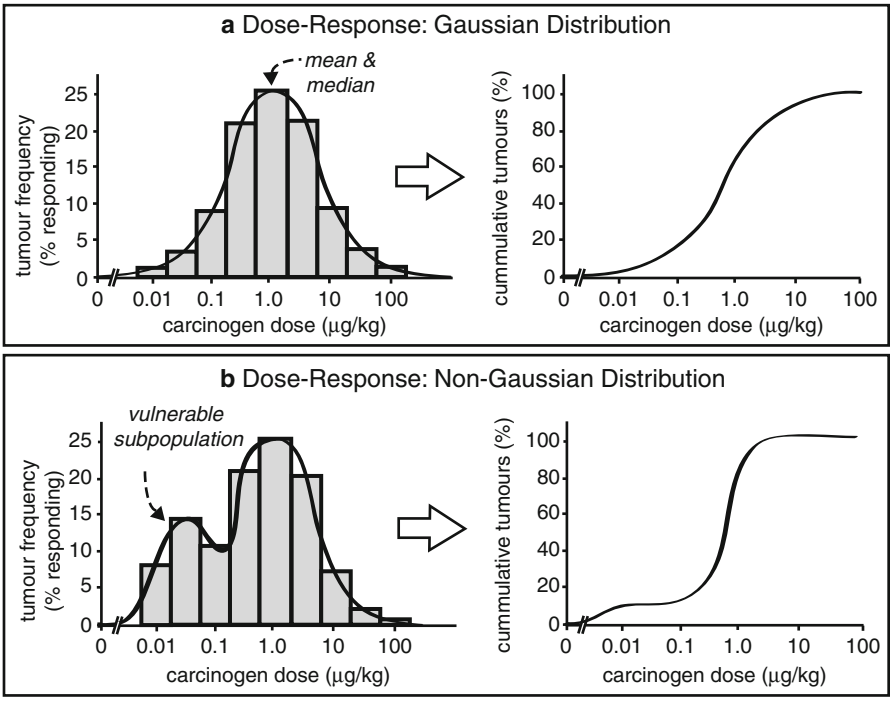

Fig. 2.5 The presence of individuals possessing heightened susceptibility to toxicants (*Panel b*) can skew dose–response relationships in exposed populations compared to normal Gaussian distributions (*Panel a*). In each left-hand graph, the toxic response is shown as a frequency of responding individuals within each exposure group (dose level), while the right hand panels show the cumulative incidence (i.e. the sum of all responding individuals within the study population as a whole)

due to unpredicted toxicity in susceptible subpopulations is a disaster in human terms for affected families and in economic terms for the industry.

Since they are rare events, classifying these unpredictable toxic syndromes is often difficult. To keep things simple, we will assume that rare toxic responses are classifiable as either *idiosyncratic* or *allergic* in nature. There is potentially much overlap between these categories, but to distinguish these complex phenomena we will use the term *idiosyncratic* to denote rare toxicities in which immune system involvement is not immediately obvious. Conversely, the term *allergic toxicity* denotes syndromes where immune system activation plays a clear and obvious role. This distinction is artificial since categorising a given case of unexpected toxicity as nonimmune is often uncertain given that absence of common symptoms suggesting activation of the immune system (e.g. eosinophilia) does not necessarily preclude immune involvement. Nevertheless, for the sake of clarity we will draw a tentative distinction between these categories.

2.6.1 Idiosyncratic Toxicities

Idiosyncratic toxicity occurs when an individual possesses a rare or unexpected trait that enhances their vulnerability to a particular toxicant or class of chemicals. *Idiosyncrasy* is a cognate term derived from several Greek words and denotes a *physical constitution peculiar to an individual.* In the case of pharmaceutical agents, serious idiosyncratic toxic reactions are thankfully rare, usually affecting between 1 person in a 1,000 or 10,000 (i.e. 1 in 10^3 or 10^4). Our understanding of such rare toxicities is often poor since they are hard to reproduce in lab animals, confounding efforts to clarify their mechanistic basis.

Early studies of idiosyncratic toxicity often assumed these phenomena exhibit dose *in*dependence. Thus, among individuals prone to idiosyncratic responses, toxicity would occur as frequently at low toxicant doses as at high doses. Revision of these assumptions occurred upon publication of a key 2008 study by Lammert and associates that examined the incidence of idiosyncratic hepatotoxicity towards prescribed medicines in the USA and Sweden. The study suggested that idiosyncratic toxicity was in fact dose related and rarely seen for medicines used at daily doses of less than 10 mg/kg.

Idiosyncratic sensitivity sometimes occurs because individuals express mutated or polymorphic versions of enzymes that cannot properly metabolise toxicants to facilitate their bodily elimination. In some ethnic populations, mutant xenobiotic-metabolising genes are so prevalent that they influence prescribing decisions by physicians. A famous example of this phenomenon involves the tuberculosis drug isoniazid, which causes liver damage in ~1 % of patients. The conjugative enzyme N-acetyl transferase 2 (NAT2) plays an important role in isoniazid metabolism, and studies in a variety of ethnic groups have associated a genetic deficiency in NAT2 (known as ‘slow acetylators’ due to their reduced ability to metabolise isoniazid and other xenobiotics) with an increased susceptibility to liver injury.

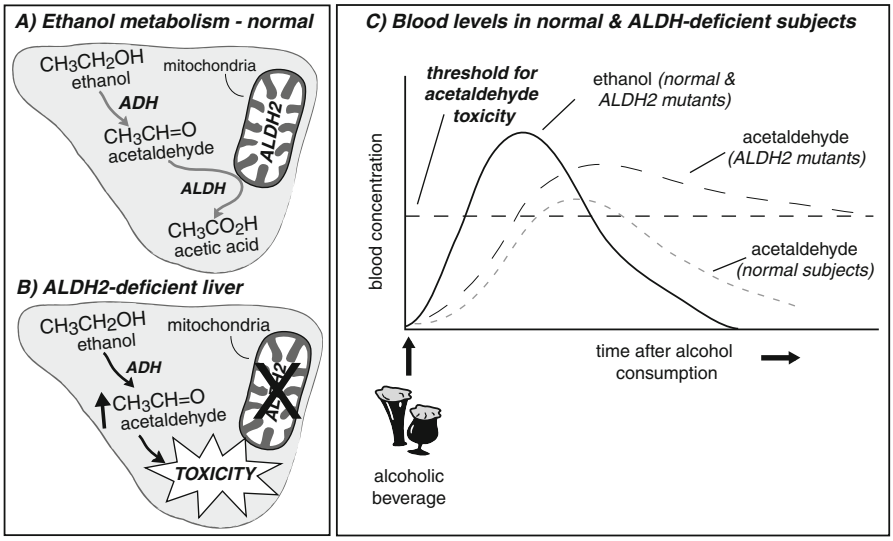

Fig. 2.6 A conspicuous ALDH2 deficiency confers heightened sensitivity to alcohol in some Asian populations. ALDH2 metabolises acetaldehyde that forms via ethanol oxidation by alcohol dehydrogenase in the liver (*Panel a*). Acetaldehyde causes many unpleasant symptoms that occur in the aftermath of binge drinking sessions (*Panel b*). The impaired detoxication of acetaldehyde in ALDH2-deficient individuals causes sustained 'hangover' symptoms since aldehyde levels remain above the toxic threshold for an extended duration (*Panel c*)

Another example of this phenomenon involves an aldehyde dehydrogenase gene (ALDH2) variant that confers a low tolerance of alcohol upon some Asian populations. The ALDH2 enzyme is located within hepatic mitochondria and is responsible for converting the acetaldehyde that forms during alcohol metabolism into acetic acid (Fig. 2.6a). In some Asian populations, a dominant mutation in the ALDH2 gene renders the enzyme largely inactive, ensuring affected individuals metabolise acetaldehyde poorly after consuming alcohol (Fig. 2.6b). Due to impaired clearance, blood concentrations of acetaldehyde remain above a toxic threshold for an extended timeframe following alcohol ingestion (Fig. 2.6c). Since acetaldehyde causes many unwanted 'hangover' symptoms in heavy drinkers, ALDH2 deficiency diminishes tolerance for wine, beer and other alcoholic beverages. Even consumption of low quantities of alcohol by ALDH2-deficient individuals triggers symptoms of acetaldehyde intoxication that include dizziness, nausea, hypotension and palpitations. These subjects thus display a permanent phenotype that resembles the effects of disulfiram treatment in alcoholics (i.e. Antabuse, the ALDH2-blocking drug that is used with varying degrees of success to counteract alcohol dependence in heavy drinkers).

NAT2 and ALDH2 are just two of hundreds of enzymes within excretory tissues that protect the body against foreign and endogenous toxicants. Researchers have described vast numbers of mutations in these pathways, spawning entire

scientific subdisciplines such as *pharmacogenetics* and *toxicogenetics*. Mutant genotypes within metabolic pathways influence the safety and effectiveness of many medicines but also govern susceptibility to environmental toxicants and pollutants.

In addition to mutations in xenobiotic-metabolising enzymes being a contributing factor to non-Gaussian patterns of chemical sensitivity, a role is also possible for inherited deficiencies in transporters that help pump toxicants across cell membranes. Since drug concentrations may exceed toxic thresholds within excretory organs that depend on normal transporter function (e.g. liver and kidney), individuals affected by these mutations can be vulnerable to drug toxicity.

In the main, however, the 'mutation in metabolic enzymes or transporters' theory has met with only modest success upon its application to rare toxic syndromes. One problem is that unusual susceptibility to toxicity is often much rarer than the frequency of a variant genotype, suggesting other factors contribute to the toxic susceptibility. The search for the molecular factors that predispose individuals to these rare toxic syndromes is far from complete.

2.6.2 Allergic Toxicities

Hypersensitivity toxicities proceed via xenobiotic-induced activation of the immune system. Although such toxicities are quite infrequent, certain types of chemicals are notorious for eliciting serious, life-threatening allergies. Within clinical medicine, the penicillin class of antibiotics is notorious for causing allergic responses in susceptible individuals. Typically, the allergenicity of such drugs is unrelated to their pharmacological mode of action; hence, their *chemical properties* are likely more important to immunotoxicity than any intrinsic biological actions. Since penicillins are chemically reactive β-lactams, their reactivity with cell proteins in a process known as *haptenisation* helps explain their allergic properties. In susceptible individuals, such reactions with self-protein(s) form neo-antigens that trigger an antibody response by the adaptive immune system. While penicillins typically possess inherent chemical reactivity, the *hapten* theory also applies to drugs that undergo conversion to reactive metabolites that attack cell proteins to trigger an antibody response by T-lymphocytes.

While the hapten theory helps explain some drug hypersensitivities (e.g. hydralazine, tienilic acid), several gaps in this theory have long puzzled researchers. For example, for allergenic drugs that depend upon bioactivation, given that routes of xenobiotic metabolism are relatively constant across most members of the population, it is unclear why allergic responses are so rare if the same reactive metabolites likely form in most patients. This suggests metabolism is not the sole requirement but that personal immunological factors dictate hypersensitivity responses. Such considerations may explain why allergic reactions often occur in tissues with low capacities to metabolise foreign chemicals (e.g. skin) or why they can occur for chemicals that are not known to undergo bioactivation of any kind.

Since the immune system works mainly against microbes and large protein toxins, the mechanisms underlying its activation by small molecule allergens long proved mysterious. The 2002 discovery of the role of human leukocyte antigen (HLA) allele HLA-B*57:01 in hypersensitivity to the anti-HIV drug abacavir was a crucial discovery in understanding these rare toxic syndromes. A highly effective antiretroviral medicine, abacavir causes serious rash, nausea and other allergic symptoms in small numbers of patients. Association of the HLA-B*57:01 allele with abacavir hypersensitivity was a key step towards personalised medicine since it allowed the development of genotyping tests to identify carriers of this allele who are at risk of abacavir hypersensitivity. These findings suggested the genotype of a susceptible individual is as important to hypersensitivity reactions as the chemical properties of a drug or its metabolites.

Over recent decades, several serious drug-induced hypersensitivities have been associated with specific HLA genotypes. The HLA designates a cluster of hundreds of genes on chromosome 6 that are synonymous with the human major histocompatibility complex (MHC). This genetic locus is highly polymorphic between individuals and encodes various cell surface markers, antigen-presenting molecules and other proteins which participate in immune function. HLA class I and II gene products are involved in antigen presentation to T-lymphocytes, with the former comprising gene clusters at three distinct loci in humans (HLA-A, HLA-B and HLA-C). While the heterogeneity of the HLA is a well-known barrier to successful organ transplantation, researchers have also associated HLA alleles with allergic responses to diverse drugs including the antiepileptic carbamazepine, the gout medicine allopurinol and several sulfonamide antibiotics. The various hypersensitivity responses associated with rare HLA alleles range from severe, rapid-onset skin reactions such as Stevens–Johnson syndrome/toxic epidermal necrolysis to milder, delayed onset skin rashes but also include single-organ toxicities (e.g. drug-induced hepatitis) and multi-organ hypersensitivities. Table 2.1 reports a number of HLA associations with hepatotoxic responses to several important drugs. The increasing use of genotyping approaches including genome-wide association studies is likely to uncover more HLA associations of this kind, although it is unlikely that all cases of drug hypersensitivities will be attributable to HLA variants since some likely involve other immune system pathways.

While it was once assumed that haptenisation must underlie allergic drug responses, the emerging *pharmacological interaction* theory proposes that the strong binding of a drug to a specific HLA protein variant need not involve covalent bonding but can simply involve reversible interactions with the target HLA protein. By promoting the presentation of drug–protein complexes by antigen-presenting cells to the T-cell receptor (TCR) which is expressed by antibody-producing T-cells, these interactions promote the cytotoxic immune response in patients carrying rare HLA alleles.

By identifying a key role for HLA alleles in triggering allergic drug reactions, these ongoing discoveries raise the prospect that similar mechanisms govern allergic responses to other nonmedicinal toxicants. Given the importance of drug allergies within the clinical setting, most attention to date has focussed on the role of HLA alleles in unusual drug reactions. It is likely future research in this area will address the role these loci play in allergic responses to toxic metals, synthetic chemicals and environmental pollutants.

Table 2.1 Genetic associations of HLA variants with idiosyncratic drug-induced liver injury

Gene	Associated HLA allele	Drug	Reference
HLA-A	*3303	Ticlopidine	Hirata K et al. (2008) Pharmacogenomics J 8:29–33
HLA-B	*5701	Flucloxacillin	Daly AK et al. (2009) Nat Genet 41:816–819
HLA-DRB1	*1501	Amoxicillin–clavulanate	Lucena MI et al. (2011) Gastroenterol 141:338–347
	*1501	Lumiracoxib	Singer JB et al. (2010) Nat Genet 42:711–714
	*0701	Ximelagatran	Kindmark A et al. (2008). Pharmacogenomics J 8:186–195
HLA-DQA1	*0201	Lapatinib	Spraggs CF et al. (2011). Clin Oncol 29: 667–673

2.7 'Chemical Mixtures': Chemical Exposure in the Real World

Much of our toxicological database concerning the health effects of chemicals – spanning from in vitro studies in cultured cells to in vivo studies in lab animals or even human studies in volunteers – involves observations of single toxicants. This is understandable since an appreciation of the inherent biological properties of any specific molecule is essential. Yet exclusive reliance on such data during the application of toxicological knowledge to 'real-world' exposure scenarios may create problems since the latter often involve simultaneous exposure to multiple toxicants, raising the possibility of interactions between chemicals during toxicity induction. This possibility is of great pharmacological importance since clinical researchers have documented hundreds of 'drug–drug interactions' (DDIs) in patients receiving multiple drugs simultaneously. Given that two or three thousand prescription medicines are in widespread use today, since the incidence of polypharmacy is increasing in most countries, clinical problems due to DDIs are likely to increase over coming decades.

Not all DDIs are of equal importance; many are of limited clinical significance and can be managed by simply reducing the dose of one of the offending drugs. On the other hand, some DDIs are so severe that they have fatal consequences, especially among elderly or very ill patients with impaired xenobiotic clearance capabilities who receive multiple medicines concurrently.

Study of the mechanistic basis for common DDIs has revealed most involve interactions during drug metabolism in the liver, such as when two drugs compete for the same CYP enzyme. Alternatively, DDIs may involve competition for the same membrane transporter that helps clear drugs from plasma or renal filtrate. Less commonly, interactions can occur at the pharmacodynamic level when two drugs might produce opposing pharmacological effects on the same receptor pathway within a given tissue.

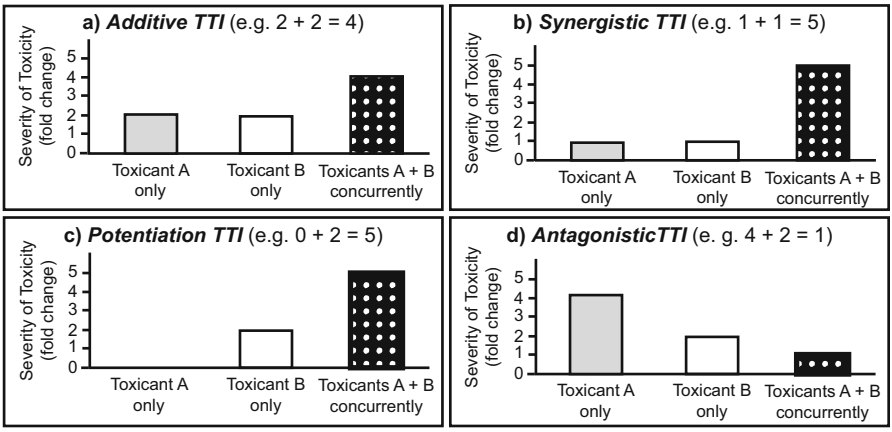

Fig. 2.7 The range of possible outcomes that accompany concurrent exposure to multiple toxicants can be envisaged by considering four possible TTI (toxicant–toxicant interaction) scenarios accompanying exposure to just two toxicants either alone or in combination, namely, *additive* (*Panel a*), *synergistic* (*Panel b*), *potentiation* (*Panel c*) and *antagonism* (*Panel d*)

In an analogous manner to DDIs, toxicological interactions can occur when an individual is exposed to two or more nonmedicinal toxicants simultaneously (i.e. a toxicant–toxicant interaction [TTI]). While the number of medicines taken concurrently by most patients – even very sick ones – is usually limited to fewer than half a dozen, no such limitations apply in the case of nonmedicinal toxicants since workers in many industries are regularly exposed to dozens of chemical entities. The range of possible outcomes accompanying simultaneous exposures is conceptualised by envisaging four scenarios involving just two chemicals (Fig. 2.7). In each scenario shown in Fig. 2.7, it is assumed that the doses of toxicants A and B are the same when tested alone or in combination.

In one common scenario, two toxicants induce additive toxic responses, producing a combined response that is equal in severity to the sum of the responses induced by each toxicant alone (Fig. 2.7a). A more troublesome TTI occurs when much greater toxicity accompanies combined exposure to two toxicants than is seen when the xenobiotics are tested independently (Fig. 2.7b). Such interactions can reflect the ability of a toxicant to boost a metabolic pathway that is involved in the bioactivation of the co-administered toxicant, thereby resulting in greater cell injury by the toxic metabolite. Another worrying scenario is termed a synergistic interaction, which occurs when a compound that is devoid of observable toxicity strongly enhances the toxicity of a co-administered toxicant (Fig. 2.7c). Outcomes of this kind can reflect impairment of metabolic or cellular repair pathways that otherwise protect cells and tissues against the co-administered substance. In the final example (Fig. 2.7d), one toxicant antagonises the adverse properties of a co-administered xenobiotic, with the combined toxicity significantly lower than that elicited by the two agents alone. In this instance, the co-administered agent may inhibit the conversion of the toxicant into its ultimate toxic metabolite, thereby suppressing the

induction of cell injury. Such interactions can be beneficial in the clinical setting, since one agent may be used as an antidote during the management of poisoning syndromes involving the other toxicants.

The problem of TTIs assumes special relevance for communities who live in proximity to chemical waste dumps that contain diverse potentially hazardous chemicals. With the number of synthetic xenobiotics used in modern economies numbering in the tens if not hundreds of thousands, the potential for concurrent exposures and thus unexpected TTIs is subject to an almost limitless number of permutations. Since some researchers fear that the extent of this problem could resemble a submerged iceberg, in recent years the toxicity assessment of 'chemical mixtures' has received growing research attention. This problem poses special challenges to research toxicologists charged with identifying important new TTIs: in a world where limited resources are available to support toxicological research, it is challenging to conduct adequate research into the effects of single toxicants let alone test them in seemingly limitless numbers of combinations with other substances. Clearly, such investigations should be guided by consideration of the chemical combinations likely to be encountered in the occupational setting.

2.8 Lifestyle Determinants of Toxic Susceptibility

In addition to genetic factors and concurrent exposures, toxic outcomes following xenobiotic exposure are also influenced by various lifestyle factors. For example, the dietary habits of an individual can alter the expression of xenobiotic-metabolising enzyme systems within the liver, producing changes in the bodily fate of ingested xenobiotics that are substrates for the affected pathways. The consumption of well-grilled, barbecued meat has received considerable attention due to likely exposure to cooking by-products that may alter the expression of xenobiotic-metabolising genes within the gut wall and liver. These topics receive further attention in Chaps. 3 and 5. Similarly, by altering the osmolarity of the gut lumen, the consumption of salty foods can upregulate the expression of biotransformation enzymes in the gut wall, most notably CYP3A family proteins that metabolise a huge range of exogenous chemicals (Chap. 3, Sect. 3.5).

Naturally occurring compounds ingested via dietary consumption of fruits and vegetables can also influence the body's ability to handle foreign chemicals. Following the chance observation over 20 years ago of high plasma concentrations of the drug felodipine in subjects who consumed grapefruit juice, much attention has focussed upon the chemical constituents of citrus products and their diverse effects upon xenobiotic-handling systems in the GI tract and liver. Growing attention has also focussed on likely benefits accompanying consumption of fresh cruciferous vegetables that contain isothiocyanates, a class of compounds that upregulate the expression of enzymes involved in conjugative xenobiotic metabolism (see Chaps. 3 and 5). A growing number of studies suggest such diets afford protection against cancer caused by DNA-damaging chemicals. Much research attention is

currently focussed on identifying specific chemopreventative food constituents that lower susceptibility to complex diseases such as cancer.

Few lifestyle factors exert a stronger influence over toxicant susceptibility than an individual's pattern of consumption of alcohol or tobacco. Both practices substantially alter the efficiency with which the liver and kidneys remove toxicants from the circulation. Heavy smoking and drinking strongly increase the expression of select biotransformation enzymes, boosting xenobiotic-metabolising capacity within the liver (see Chap. 5). At first glance this outcome seems only beneficial, yet xenobiotic metabolism in within the liver can often generate DNA-damaging metabolites or other noxious species that inflict injurious cellular effects. Since the toxicological impact of tobacco smoking and heavy alcohol consumption is enormous, this topic is addressed in detail within Chaps. 9 and 10.

2.9 The Timing of Toxicity

Another key issue complicating the study of chemically induced toxicity is the *latency* of the pathological response, namely, the duration of time elapsing between exposure to the toxicant and the subsequent manifestation of disease. TOCP neurotoxicity, considered in Chap. 1 (Sect. 1.5.2), is a classic example of a latent toxic response in that signs of intoxication (numbness in extremities, difficulty in walking) did not emerge until 7–10 days after consuming TOCP-contaminated Ginger Jake.

A much longer latency period occurs following human exposure to cancer-causing chemicals (carcinogens). Since tumour development proceeds via the sequential accumulation of multiple genetic alterations over many years, the time taken for the manifestation of cancer following carcinogen exposure can run into decades. These considerations are especially relevant to tobacco-related cancers, where a time lag of decades is commonly observed between the commencement of smoking and the diagnosis of tumours of the lung or other tissues (Chap. 10, Sect. 10.5.4).

A classic example of tumour latency involved thousands of women who received the synthetic oestrogen diethylstilbestrol as a putative remedy for miscarriages and other pregnancy complications during the 1950s and 1960s. Although later studies sadly revealed that these women received no therapeutic benefit from the drug, a 1971 paper in the *New England Journal of Medicine* reported an insidious latent effect in offspring who were exposed to diethylstilbestrol in utero. Exposure in the first 1–5 months of life was especially disruptive to reproductive system development, an effect that manifested in females as vaginal clear cell adenocarcinoma during their late teenage or early adult years. While the tumour incidence was not particularly high (approx. 0.1 % of exposed offspring), the unfortunate young women were also prone to other reproductive problems including breast cancer, prematurity, miscarriages and ectopic pregnancies. Male offspring also displayed signs of toxicity and tumour development within genitourinary tissues.

Subsequent research sadly revealed that diethylstilbestrol-exposed mothers were themselves at risk of breast cancer as they grew older. Yet some fear that even these findings do not close the sad chapter in toxicological history that diethylstilbestrol represents: the first decade of the twenty-first century witnessed a series of epidemiological studies that explored the health of the granddaughters of the original diethylstilbestrol-exposed women. These studies were motivated by mouse studies that suggested the third generation of exposed animals can manifest reproductive toxicity. The results from the human epidemiological studies are subject to debate, but some suggest a weak increase in ovarian cancer and cardiac abnormalities in the third generation. If such findings are confirmed, they will reinforce the likelihood that chemical toxicity in one generation can trigger lasting epigenetic changes that have outcomes for subsequent generations of descendants. As with the earlier thalidomide disaster, the diethylstilbestrol episode highlighted significant dangers that accompany the administration of unsafe drugs to pregnant women, revealing how drug safety assessment requires investigation across consecutive generations to detect rogue compounds that elicit delayed, slow-onset toxicity.

2.9.1 Obesogens and Latent Toxicity

Rising interest in the role of early epigenetic programming in the health of subsequent generations stems from the recognition that the cellular phenotype is influenced by factors other than changes in the underlying genetic sequence. The three major epigenetic determinants of prenatal gene expression are histone modification, DNA methylation and noncoding RNAs. Interest in these mechanisms was fostered by an influential 2005 Spanish study that identified epigenetic factors as determinants of the long-term health of identical twins. Although the monozygous twins were epigenetically indistinguishable in infancy, strong interindividual differences in gene expression became evident as the twins matured: striking disparities in the genomic distribution of 5-methylcytosine DNA and patterns of histone acetylation became increasingly apparent. Such findings made epigenetic programming a major concern during study of the influential ‘foetal origins hypothesis’ which traces various adult diseases to factors prevailing within the intrauterine environment.

Within the toxicology community, epigenetic programming is increasingly recognised as a key determinant of individual susceptibility to diverse chemical toxicities. On the basis of rodent data, particular interest focusses on the possibility that prenatal xenobiotic exposure disrupts normal foetal programming of energy homeostasis, conferring a lifelong predisposition towards weight gain. Termed ‘obesogens’, chemicals with these properties are objects of growing concern since animal data suggests prenatal exposure to polycyclic aromatic hydrocarbons, organochlorines and polychlorinated biphenyls can trigger adiposity and changes in adipose tissue structure while also induce chronic inflammatory states that predispose towards adult obesity and metabolic intolerance.

Some animal studies also suggest that prenatal exposure to obesogenic toxicants confers a predisposition to weight gain that is more obvious in subsequent generations than the initially exposed generation. While the mechanisms underlying this transgenerational toxic response await full clarification, epigenetic reprogramming of gamete cells within foetal reproductive tissues seems likely a contributing factor. In addition to better mechanistic understandings of these phenomena, ongoing studies will explore the relevance of obesogens to the rising incidence of obesity and metabolic syndrome within many human populations. Chemicals with these suspected properties include various persistent organic pollutants with endocrine-disrupting properties such as bisphenol A (see Chap. 8, Sect. 7.8.3.1). The role of epigenetics in latent toxic responses will likely be a major focus within toxicology research for the foreseeable future.

2.10 Conclusion

This chapter has surveyed a number of factors that influence the severity, timing and nature of toxicity following the exposure of humans and animals to toxic xenobiotics. In addition to introducing some core terminology used to describe the types of chemicals that induce toxic responses, we explored a number of factors that complicate the study of chemically induced toxic syndromes, including dose, duration of exposure, concurrent chemical exposures, latency of toxic response, lifestyle factors and pre-existing biological characteristics in an individual. With a basic appreciation of these factors, we are better placed to begin examining the fate of chemicals within the body, beginning with the types of physicochemical properties that determine whether chemicals even enter the body in the first instance.

Going Further

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Chapter 3
Toxicokinetics: The Behaviour of Chemicals in the Body

Abstract Four basic processes govern the concentrations toxicants achieve within vulnerable tissues – absorption, distribution, metabolism and excretion. These processes dictate the 'toxicokinetic fate' of a xenobiotic – describing how it penetrates cell barriers to enter tissues (i.e. absorption), whether it is dispersed to particular organs and tissue compartments (distribution), how it undergoes chemical transformation within the liver (metabolism) and whether the parent compound or its metabolites are permanently eliminated in urine, faeces or both (excretion). The behaviour of a xenobiotic during these processes is influenced by its basic physicochemical properties, including mass, charge and solubility in water and/or lipids. Study of the toxicokinetic fate of xenobiotics has confirmed the role of hundreds of xenobiotic-handling proteins – including enzyme catalysts and membrane transporters – in controlling the disposition of ingested chemicals. The possibility that one chemical might alter the ability of these proteins to metabolise or export another xenobiotic is also briefly considered (i.e. toxicant interactions).

Keywords Absorption • Distribution • Metabolism • Biotransformation • Elimination • Lipophilicity • Cytochrome P450 • Conjugation • Glucuronidation • Sulfonation • Glutathione conjugation • Membrane transporters

3.1 Introduction

A key concept applying to any toxic phenomena concerns the distinction between *toxicodynamics* and *toxicokinetics*. The former describes the harmful actions a chemical exerts on specific body components, organs or cell functions – simply put *toxicodynamics is what the toxicant does to the body. Toxicokinetics* on the other hand denotes the fate of foreign chemicals within the body – namely, *what the body does to the toxicant.* To fully understand toxic phenomena, each side of this coin must be studied: both the *toxicodynamic* and *toxicokinetic* dimensions of chemically induced toxicity need clarification.

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The acronym 'ADME' summarises the four main processes involved in the *toxicokinetic* phase of xenobiotic action. This mnemonic is borrowed from pharmacology where it describes the kinetic properties of drugs, but it equally applies to the bodily disposition of any foreign chemical. The acronym highlights four main processes which govern the behaviour of chemicals in the body: *absorption, distribution, metabolism* and *excretion* (Fig. 3.1).

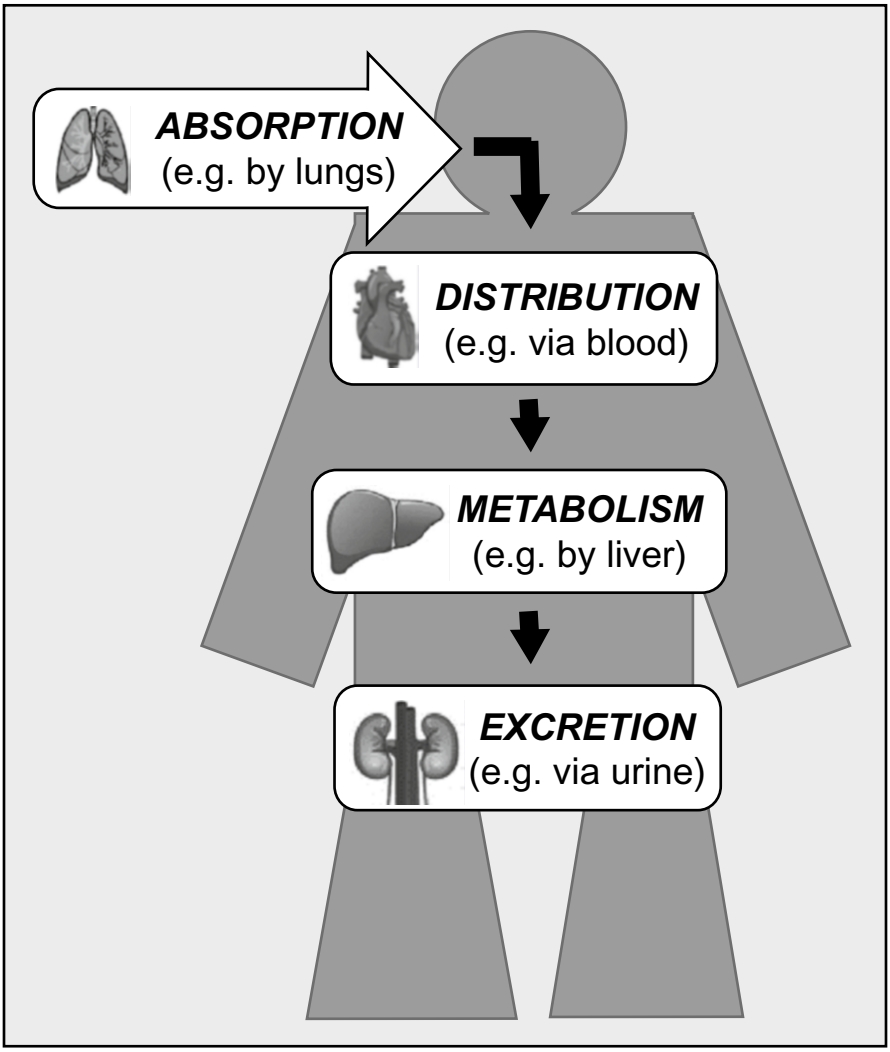

Fig. 3.1 The acronym ADME highlights four major toxicokinetic processes that control the fate of chemicals within the body: *absorption, distribution, metabolism* and *excretion*

3.2 Absorption

The 'A' in ADME denotes the readiness with which foreign chemicals penetrate tissue barriers and are *absorbed* into the body. Toxicant absorption occurs from many anatomical sites, but three routes are especially relevant for most chemicals during everyday life (Fig. 3.2). For volatile chemicals, gases and small airborne particles (e.g. smoke particulates), inhalation by the lungs is most important. The pulmonary route is very significant for occupational chemicals; since a typical worker inhales over 10 m^3 of air during a regular 8 h working day, ensuring significant lung intake can occur over a lifetime if workers handle the same types of substances regularly. Since the biological design of lung tissue facilitates rapid oxygenation of blood as it perfuses the alveolar spaces, lungs do not possess anatomical barriers restricting the accumulation of foreign airborne chemicals.

Skin by contrast possesses a protective layer known as the *stratum corneum*, which limits the dermal absorption of many chemicals. Those chemicals which penetrate the skin and result in significant exposure of internal organs are of particular concern in the workplace, such as when workers in specific industries recurringly handle the same chemicals (e.g. spray painters routinely using paint thinners or beauticians handling hair-colouring agents). Such workers can experience frequent

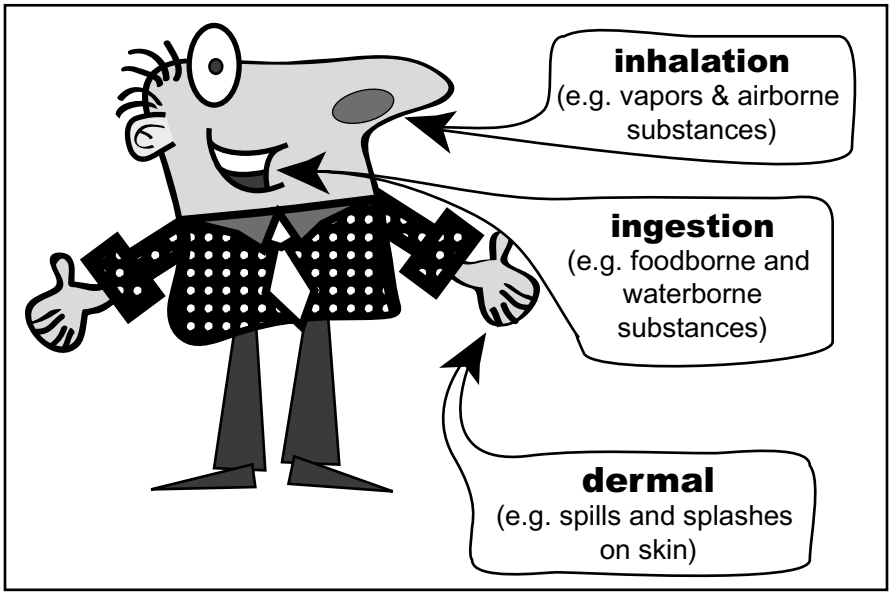

Fig. 3.2 Toxic substances enter the body via three main routes – *inhalation* of airborne substances into the lungs, *ingestion* into the gastrointestinal tract and *dermal absorption* via the skin. The pulmonary and dermal routes are most important for occupational chemicals, while oral ingestion is especially relevant for drugs and food additives

dermal exposure via splashes on uncovered body sites or contact through the fingers and palms during careless handling. Dermal uptake is also important for market gardeners or agricultural workers who handle pesticides regularly. Such workers may also experience appreciable dermal uptake from airborne substances, such as when sprays are used to deliver pesticides to fruit trees or grape vines. In addition to such workplace exposures, chemicals also enter the body via deliberate dermal application of facial creams, skin cleansers or lotions. According to some estimates, around 2 kg of chemicals enter the body of a typical Western woman every year via the dermal application of cosmetics and skin treatments.

For substances that enter by the oral route – via ingestion of chemical contaminants in drinking water and food-borne chemicals or the handling of food with contaminated hands in the workplace – the cells lining the gastrointestinal tract (GI-tract) pose a potential obstacle although many chemicals with appropriate physicochemical properties can enter the body by this route. In recent decades, much has been learnt concerning the gastrointestinal absorption of drugs thanks to the efforts of pharmaceutical industry and academic researchers seeking to optimise the oral usage of medicines. In general, absorption via the stomach and colon is limited since most toxicant absorption occurs in the upper intestinal tract due to the large surface area. The upper GI-tract is also highly vascularised, ensuring it receives a substantial blood flow.

In addition to the three routes shown in Fig. 3.2, chemicals can enter the body via several lesser pathways, including absorption via nasal passages, from the surface of the eye or, in the case of pharmaceutical products, administration via the intravenous or intramuscular routes.

3.2.1 Attributes of Absorbed Chemicals

Toxicokinetics essentially views the human body as a succession of membrane barriers that define a series of fluid-filled aqueous compartments. The membrane barriers comprise phospholipid bilayers which surround not only cells and tissues but also define intracellular compartments such as mitochondria, endoplasmic reticulum or Golgi apparatus. Within the membrane bilayer, phospholipids are oriented such that the polar ionised head is oriented towards the aqueous compartment while the hydrocarbon tail is aligned towards the hydrophobic core. These biological realities ensure several physicochemical factors govern the readiness with which chemicals are absorbed from most anatomical sites (Fig. 3.3).

3.2.1.1 Size

Firstly, the *size* or *mass* of a molecule strongly influences its absorption upon initial contact with the body. Well-absorbed chemicals are usually not too large, with a

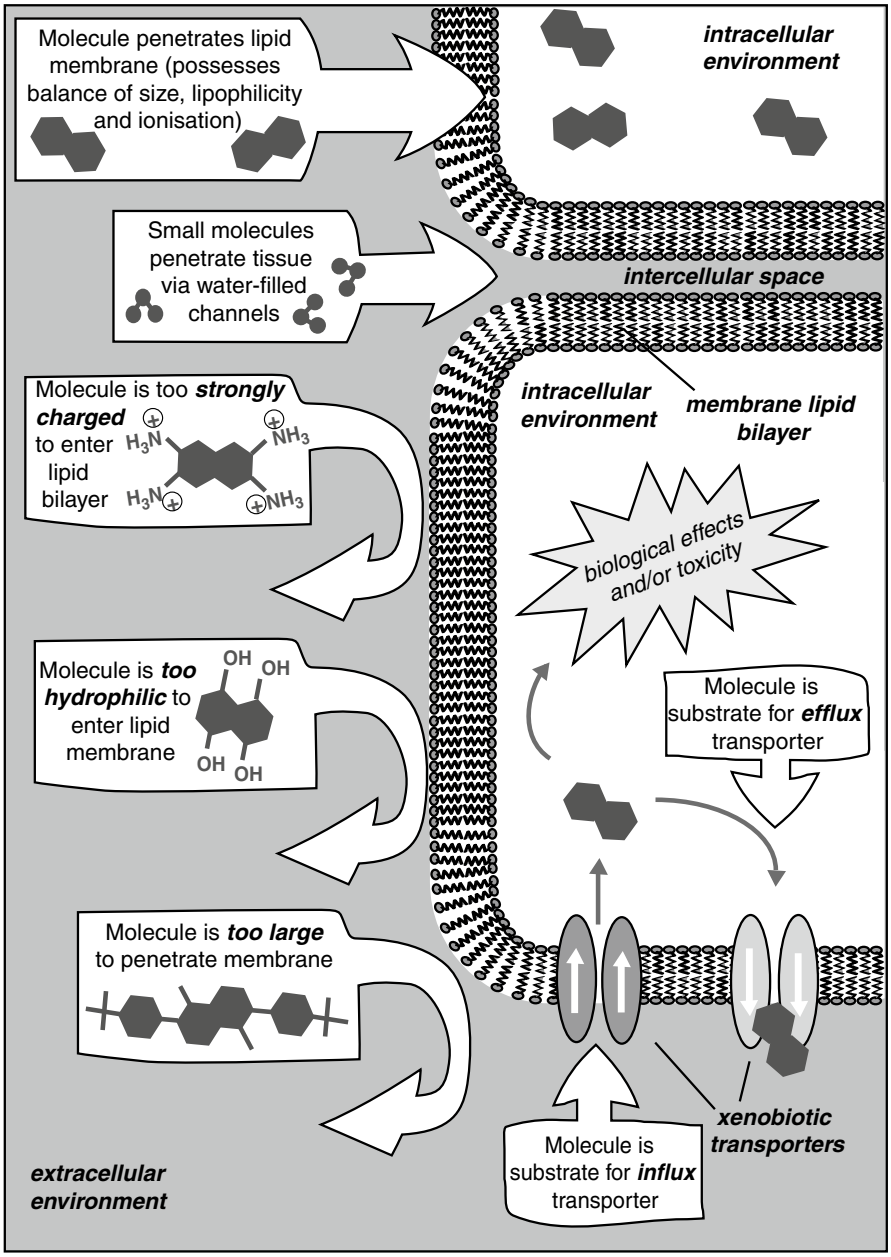

Fig. 3.3 Lipid-rich cell membranes act as barriers towards foreign chemicals. Key properties influencing membrane penetration by xenobiotics include mass, lipophilicity, hydrophobicity and ionisation status (charge). Membrane transporters also govern xenobiotic accumulation in many tissues

molecular mass of 500–600 Da representing an upper limit to absorption from most tissue sites. Xenobiotics falling below this mass threshold and that possess appropriate physicochemical properties enter the body by penetrating cell membranes in a process known as *transcellular* permeability (Fig. 3.3). In contrast, very small molecules (say, with a mass of 150 Da or less) that are highly water soluble may enter tissues via *paracellular permeability*, using water-filled intercellular spaces as aqueducts to penetrate epithelial barriers (3–10Å [10^{-10} m] in diameter) (Fig. 3.3). Such mechanisms permit the uptake of metal ions and small organic molecules such as alcohol.

3.2.1.2 Solubility

Secondly, chemicals entering the body require an ability to dissolve in hydrophobic, nonpolar organic solvents since they must traverse the lipid-rich membrane bilayers within 'sheets' of epithelial cells that line the inner surface of the GI-tract or lung airways. Since epithelial cells form tight boundaries or 'junctions', chemicals must possess 'lipophilicity' or 'fat-loving' character to penetrate this lipid-rich, nonaqueous organic barrier (Fig. 3.3). By contrast, hydrophilic or 'water-loving' chemicals typically dissolve poorly in a lipid environment. Such molecules often possess heteroatoms (e.g. N, O) or polarised functional groups that readily form hydrogen bonds with water molecules. Generally, while some chemicals inhabit either pole of the 'lipophilic ↔ hydrophilic' continuum, most toxicants that enter the body exhibit a mix of lipophilic and hydrophilic character.

3.2.1.3 Charge

Thirdly, the electric charge carried by a molecule – its *ionisation status* – is also crucial since only neutral (uncharged) species passively cross lipid bilayers (Fig. 3.3). Many molecules contain functional groups that accept or donate protons and therefore, depending on the prevailing pH, carry either a positive or negative charge. Since a significant pH gradient exists throughout the gastrointestinal tract (e.g. from pH 1–2 in the stomach to pH 5–6 in the duodenum and pH 8 in the ileum and colon), the ionisation state of ingested molecules frequently changes as they transit through this organ. The pKa of the molecule dictates its ionisation behaviour within a particular pH environment, with stronger organic acids (i.e. low pKa) typically nonioinised within the acidic gastric environment, while organic bases are nonionised within the neutral pH environment prevailing within the small intestine. Yet while we might expect that absorption would only occur in zones where the molecule assumes a neutral pH, for most molecules the duodenum remains the main site of GI-tract absorption due to its large surface area and high blood flow. Thus, while the ionisation equilibrium may favour the formation of charged species, sufficient nonionised form is present in the duodenum to allow substantial uptake from this site.

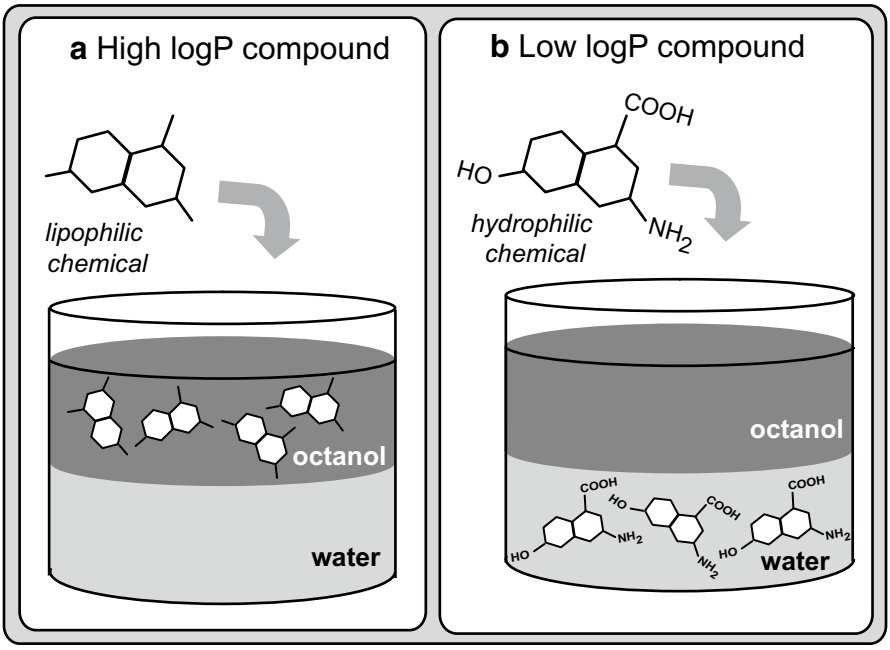

Fig. 3.4 The distribution of dissolved chemicals into the two layers of biphasic water/octanol systems predicts their ability to penetrate lipid bilayers. Highly fat-soluble ('lipophilic') chemicals will distribute to the organic layer (*Panel a*), while hydrophilic molecules prefer the water layer (*Panel b*). The 'logP' is the logarithm of the ratio of toxicant concentrations in the octanol layer relative to the aqueous layer

3.2.2 Predicting Permeability

An ability to predict the membrane permeability of xenobiotics is important during the evaluation of toxicity. Since it is especially important during the oral absorption of drugs, medicinal chemists have developed a wide range of tools for predicting membrane permeability.

3.2.2.1 LogP

The physicochemical properties of chemicals are assessed by estimating the 'oil/water' or 'octanol/water' partition coefficient. Briefly, the substance of interest is added to a vessel containing equal volumes of water and a nonmiscible organic liquid such as *n*-octanol or a biotic lipid or vegetable oil. The vessel is sealed and shaken vigorously until the compound is dissolved. Since the organic solvent is usually less dense than water, it rises to form an upper layer (Fig. 3.4). Once the system achieves equilibrium, the xenobiotic concentration in each layer is measured using an appropriate analytical method: typically, hydrophobic compounds concentrate in

the organic layer while hydrophilic molecules distribute to the water layer (Fig. 3.4). The ratio of toxicant concentrations in the two layers – the *partition coefficient* – can predict the readiness with which chemicals are absorbed across biological membranes. This value is usually expressed as a base-10 logarithm (i.e. the 'logP'). Nowadays, useful online calculators can reliably estimate the 'logP' before molecules are synthesised in the 'real world'. In general, the relationship between logP and membrane permeability is nonlinear or essentially shaped like an inverted 'U': molecules with either a very low or very high logP value will cross membranes poorly. In the former instance, poorly lipophilic molecules fail to enter the lipid membrane in the first place, while in the latter, strongly lipophilic molecules partition within membranes and do not exit into adjacent aqueous cell or tissue domains.

3.2.2.2 Polar Surface Area (PSA)

To better predict chemical absorption, newer tools extend beyond simple logP values to include descriptors relating to the size and shape of molecular structures, flexibility of internal chemical bonds, hydrogen-bonding capabilities or the surface properties of molecules. Calculating the balance of hydrogen bond donors and acceptors within a molecule is also useful since this predicts the affinity for water molecules, thereby influencing water solubility and the release of water molecules during the process of *desolvation* that occurs when hydrated molecules encounter a lipid bilayer. The polar surface area (PSA) is also useful – this is estimated by viewing molecules as a sum of fragments containing polar atoms (i.e. atoms such as O or N which contain unequally distributed electronic charge). In general, the PSA correlates negatively with the readiness of molecules to undergo absorption from the GI-tract or penetrate the blood–brain barrier: the more highly polar the surface of a molecule is, the lower its solubility in membrane bilayers.

3.2.3 Membrane Transporters and Absorption

While physicochemical properties govern the *passive* diffusion of molecules through biological barriers, absorption from some anatomical sites is further complicated by the actions of membrane transporters (Fig. 3.3). These can take the form of either *importer pumps* that facilitate xenobiotic accumulation or *exporter pumps* that actively return absorbed toxicants to the biofluids from which absorption occurred (Fig. 3.3). Since these processes are relevant to each of the four processes that govern the toxicokinetic fate of xenobiotics in the body, they are discussed separately at the end of this chapter. Here it will suffice to say that the high prevalence of efflux transporters within the gut wall epithelium plays a key role in minimising the absorption of orally ingested chemicals. Even if a xenobiotic possesses excellent physicochemical properties that facilitate its absorption across epithelial membranes within the GI-tract, like an energetic bouncer ejecting unruly guests

from a saloon, diligent efflux transporters can return these molecules to the intestinal lumen, thereby minimising exposure of internal tissues to ingested compounds. While this capability is welcomed by toxicologists, it is problematic for pharmaceutical scientists wishing to maximise the oral effectiveness of ingested medicines.

3.3 Distribution

Once within the bloodstream, absorbed chemicals enter the second stage of the toxicokinetic process – 'D' for *distribution*. Just as physicochemical properties govern the absorption of chemicals on their initial contact with the body, they also influence their distribution behaviour within the body. After penetrating the biological barriers that interface with the external environment, chemicals encounter many additional membrane barriers as they penetrate deeper and deeper into body tissues.

3.3.1 Blood-Borne Chemicals

Very different patterns emerge upon studying the dispersal of individual toxicants around the body, with some tending to accumulate in specific tissues such as body fat, skeletal muscle or liver, while others remain mainly in the bloodstream. Toxicants exhibiting the latter tendencies often exhibit strong binding affinity for blood proteins such as albumin or various serum glycoproteins. Highly water-soluble (hydrophilic) chemicals also display a preference for the blood compartment during the distribution phase. Since they often achieve high concentrations within the proximal tubules, hydrophilic toxicants can be associated with kidney damage (see Chap. 6). On the other hand, lipophilic chemicals that penetrate deep into body tissues usually leave only negligible concentrations within the bloodstream.

3.3.2 Accumulation in Body Tissues

Molecules that penetrate deep into body tissues often possess strong affinity for tissue binding sites (e.g. in skeletal muscle), or they may possess strong lipophilic character that facilitates accumulation within lipid-rich tissues including adipose tissue deposits. The latter property greatly extends their 'transit time' in the body, a factor that allows detection of lipophilic drugs such as the marihuana constituent tetrahydrocannabinol or anabolic steroids in the urine of elite athletes weeks after their previous use of the offending drug. The tendency for lipophilic environmental pollutants such as organochlorine pesticides or polychlorinated biphenyls to accumulate in body fat greatly complicates assessment of their health effects in humans and other species since these compounds can accumulate as the food chain is

ascended. Deposition of lipophilic xenobiotics in body fat also raises the prospect of acute intoxication in individuals who mobilise their fat reserves during 'crash diets', thereby accelerating the release of chemicals into the systemic circulation.

Toxic metals or inorganic species such as lead, fluorine or strontium greatly extend their residence time in the body by accumulating in the skeleton. Once deposited within mineralised tissue by osteoblasts, the slow metabolic turnover of bone ensures the release of these inorganic toxicants is slow. At high levels of exposure, the bone itself may sustain toxicity, resulting in susceptibility to fractures and skeletal deformities. But the slow leeching of lead and other inorganic toxicants from bone into circulating blood can ensure blood levels remain high over extended periods, allowing chronic exposure of susceptible internal organs to these toxic metals (e.g. the CNS in the case of lead). There are many examples of workers from lead smelters and related lead-based industries showing signs of chronic lead intoxication long after retirement from the workforce. Among individuals, considerable variability is seen in susceptibility to lead toxicity due to differences in bone turnover caused by dietary practices, different rates of ageing and disease.

3.3.3 Distribution and Drug Responsiveness

Although the distribution phase influences the toxicity of all chemicals, since it is especially relevant to the pharmacological effects of medicinal agents, much of what we know concerning xenobiotic distribution is based on studies of the fate of medicines in human volunteers or patients. Pharmacokinetic properties often guide the choice of drugs in particular patients and take on extra relevance in specific clinical situations. For example, during the treatment of infectious diseases with antibiotic drugs, a drug that distributes extensively into skeletal muscle or body fat may be unhelpful in patients suffering from blood-borne infections where a hydrophilic antibiotic with plasma protein binding properties is more suitable. Similarly, distribution properties strongly influence the selection of anaesthetic drugs for patients facing surgical procedures, since a drug that penetrates deep into body tissues may produce more prolonged anaesthesia than one that is confined to circulating blood.

For a given drug, the distribution phase is subject to considerable variability between individuals. For example, significant age-related changes can occur in the distribution of xenobiotics in elderly individuals compared to young healthy adults. For example, elderly individuals may have diminished skeletal muscle reserves which are a major reservoir for some toxicants. Likewise the amount of body fat an individual carries can confer interindividual differences in the distribution of some compounds, with elderly individuals again likely to carry more body fat than young individuals. Proportional differences in body fat also give rise to subtle gender differences during the disposition of lipophilic xenobiotics. In women of childbearing age, pregnancy can strongly alter the distribution of hydrophilic toxicants due to expansion of the blood volume as the pregnancy progresses. The distribution of

xenobiotics can also be altered in lactating women, especially for molecules that possess ionisable groups that ensure they are trapped in milk (e.g. cocaine).

3.3.4 The Volume of Distribution

The need for a numerical value to simply summarise the behaviour of a chemical within the body led to the introduction of the *volume of distribution* (V_{dist}). This crude but useful tool plainly reveals how effectively a toxicant is distributed into body tissues during the distribution phase. The V_{dist} is routinely estimated for medicinal agents and is calculated by simply dividing the dose of xenobiotic administered to the subject by the concentration the substance achieves in blood:

$$V_{dist}\left(\mathrm{L}\right) = \frac{\text{administered dose}\left(\mathrm{mg}\right)}{\text{blood concentration}\left(\mathrm{mg/L}\right)}$$

By relating the total amount of xenobiotic within the body to the concentration achieved in blood, the V_{dist} is sometimes thought of as a dilution factor for the chemical substance (Fig. 3.5). In the case of human pharmaceuticals, the V_{dist} is ideally calculated after administering drugs to subjects via the *intravenous* route, thereby allowing estimation of precise 'starting' (time = 0 or 'T_0') plasma concentrations that are not complicated by inefficiencies in drug absorption or metabolism within the GI-tract or liver. The resulting V_{dist} is reported in volume units such as litres and denotes the *apparent* volume of blood needed to accommodate all the xenobiotic in the body at the actual concentration found in the blood. Although an essentially imaginary concept, the V_{dist} conveys insight into the behaviour of xenobiotics within the body. A highly lipophilic substance, for example, will readily partition into body fat, leaving low concentrations in blood. Dividing the administered dose by a low blood concentration ensures the V_{dist} is enormous – for some drugs, values of over 10,000 l are obtained. While no body compartment occupies such a massive volume, the high V_{dist} nonetheless tells us something valuable – the molecule at hand has a pronounced ability to penetrate into nonvascular tissues (e.g. Fig. 3.5c). At the other extreme, a highly polar, hydrophilic toxicant with a strong affinity for plasma proteins may display little tendency to leave the vascular compartment, thereby yielding a V_{dist} of just a 4 or 5 l (e.g. Fig. 3.5a). Many chemicals occupy the middle ground between these extremes, achieving significant concentrations in tissues and blood (Fig. 3.5b). Note that the examples shown in Fig. 3.5 assume that the body behaves as a simple two-compartment entity, with toxicants distributing into either blood or the extravascular tissues, with the latter behaving as a single homogeneous compartment. This oversimplifies the distribution behaviour of most substances, since after entering the blood, many compounds partition unequally into different tissues depending on blood flow factors, tissue protein binding and the *logP*. Analysing toxicokinetic data for such chemicals often requires the use of complex multicompartmental models.

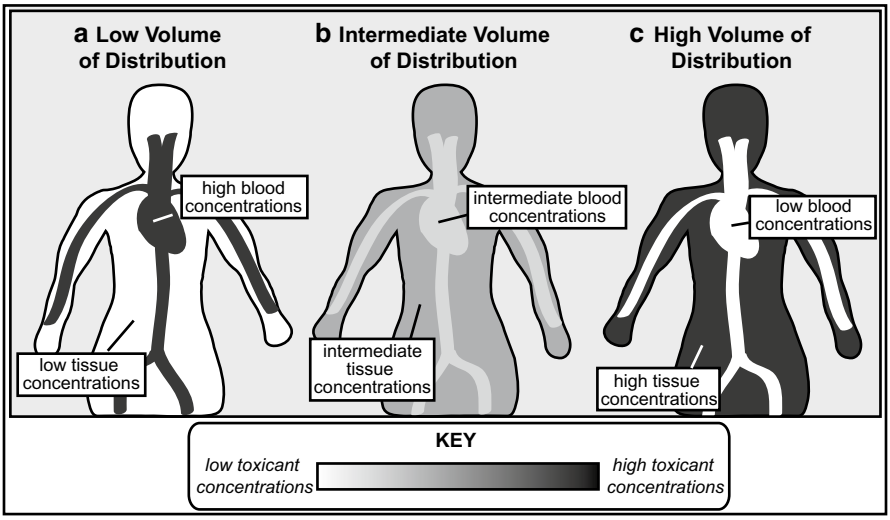

Fig. 3.5 The volume of distribution (V_{dist}) reveals the distribution of absorbed toxicants within the body. For the sake of clarity, the body is represented as comprising only the vascular and extravascular compartments. The shading intensity indicates toxicant concentrations in the respective compartments (see Key). In *Panel a*, a xenobiotic with a low V_{dist} has strong affinity for plasma proteins in blood and therefore remains mainly within the vascular compartment. At the other extreme is a drug with high affinity for tissue reservoirs (*Panel c*). Many chemicals inhabit the intermediate space, penetrating tissues to some extent while also retaining significant concentrations in plasma (*Panel b*)

The V_{dist} concept is also used for risk assessment purposes when assessing the biological uptake of toxicants and environmental chemicals, although for obvious reasons, studies of this kind are typically made in laboratory animals rather than humans. In general good concurrence is seen between animal and human toxicokinetic estimates, provided interspecies 'scaling' factors are taken into account that include differences in body weight, surface area and maximum lifespan. Some industrial chemicals such as lipophilic organic solvents yield surprisingly high V_{dist} estimates, indicating a strong tendency to partition into body fat and other extravascular tissues. Toluene (methylbenzene), for example, a common constituent of paints, fuels and cleaning fluids, exhibits a V_{dist} close to 1,400 l in humans (Table 3.1).

3.4 Metabolism

Since the body readily accumulates chemicals that are sufficiently lipophilic and are not too large or highly charged, continuing exposure to chemicals of this kind raises an intriguing problem: since many body tissues are lipid rich, ongoing exposure could cause them to accumulate to toxic levels. Clearly, the body needs ways to

Table 3.1 Volume of distribution estimates calculated for select toxicants in adult male human subjects

Toxicant	Major industrial uses	V_{dist} (L)	Exposure route in study	Reference
Methanol	Solvent, chemical synthesis, fuel additive	30	Inhaled vapours (100 ppm)	Ernstgard et al. (2005) *Toxicol Sci* 88: 30–38
Paraquat	Contact herbicide, crop desiccant, defoliant, harvest aid	120[a]	Oral ingestion (poisoning victims – various dosages)	Houze et al. (1990) *Hum Exp Toxicol* 9: 5–12
2-Propanol	Disinfectant, personal care products, cleaning agents	38	Inhaled vapours (150 ppm)	Ernstgard et al. (2003) *Toxicol Appl Pharmacol* 193: 158–167
Toluene	Paint solvent, chemical synthesis, fuel additive	1,380	Inhaled vapours (100 ppm)	Pierce et al. (1996) *Toxicol Appl Pharmacol* 139: 49–61
m-Xylene	Paint and varnish thinner, cleaning agent, chemical synthesis	420[b]	Inhaled vapours (50 ppm)	Ernstgard et al. (2003) *Toxicol Appl Pharmacol* 193: 147–157

[a]Estimated for a typical 75 kg male from reported values
[b]The value reported for the peripheral compartment is shown

remove these substances or at least ways to overcome their lipophilicity and tendency to accumulate in body tissues. This problem brings us to the 'M' of the ADME acronym – *metabolism*. Given the ubiquitous presence of xenobiotics in nature, it comes as no surprise to learn that the human genome contains hundreds of genes which encode enzymes that carry out sophisticated chemical modifications on foreign substances. Following such structural alterations, chemicals are often more easily eliminated from the body. This phenomenon is supremely important to toxicology, and we return to this topic throughout this book.

Human cells, and especially those within the liver, express many enzymes which carry out a huge range of chemical transformations on ingested xenobiotics. Although these catalysts share many features with the enzymes that have long preoccupied biochemists with their cofactor requirements, kinetic properties, temperature preferences, etc., a significant difference underlies the structure and function of xenobiotic-metabolising proteins: while most enzymes are very fussy in their substrate preferences, xenobiotic-metabolising enzymes are willing to metabolise hundreds or even thousands of substrates. This lack of fussiness long puzzled enzymologists, but the growing availability of detailed structures of these protein complexes thanks to the power of X-ray crystallography has helped clarify the distinctive molecular features of these enzymes. By obtaining detailed snapshots of xenobiotic-metabolising proteins embedded within crystals in the presence or absence of preferred substrates, structural biologists have described their

remarkable molecular features. Unlike many enzymes which possess a constrained 3D structure in the presence of substrate, the active site of many xenobiotic-metabolising enzymes displays unusual plasticity, allowing stretching and distortion in order to accommodate large, structurally diverse substrates. Indeed, the active site of some such enzymes is sufficiently elastic to accommodate multiple substrates simultaneously. This feature explains a key function of these pathways: xenobiotic biotransformation pathways cooperate with the immune system to provide comprehensive defence against incoming bodily invaders. While the immune system copes best with large invading pathogens, viruses and protein-based toxins, xenobiotic-metabolising pathways intercept and inactivate low-molecular-weight molecules that 'fly under the radar' of the immune system.

Typically although not invariably, the metabolism of foreign chemicals renders them more water soluble and better substrates for excretory transporters in the kidney or liver, thereby hastening their bodily elimination. We will return to the latter processes later in this chapter. The importance of metabolism in clearing lipophilic molecules from the body is seen in the case of lipophilic molecules that are resistant to metabolism on chemical grounds. This includes fat-soluble organochlorine molecules such as chlordane, DDT, dioxins and certain polychlorinated biphenyls (PCBs) which are very stable in chemical terms. Since such molecules resist oxidative metabolism by liver enzymes, their duration of residence within fatty tissues is often very long indeed.

While many tissues metabolise xenobiotics to some extent, the key metabolic organ is the liver. Comprising just one-fortieth to one-fiftieth of an adult's body mass, this highly compact yet remarkably proficient chemical factory performs thousands of chemical modifications on highly diverse xenobiotics. Clearly, the huge numbers of substrates which enter these pathways, the biochemical diversity of the enzymes involved and the wide range of chemical modifications sustained by xenobiotics as they transit the liver means that a classification system is needed to comprehend them. For many years, pharmacologists and toxicologists followed a metabolic classification scheme based on a binary system proposed by the pioneering Welsh researcher RT Williams in his groundbreaking book *Detoxication Mechanisms* (1947). According to this model, xenobiotics typically underwent a *Phase 1* transformation event (e.g. oxidation) followed by a *Phase 2* reaction to form a highly soluble metabolite which was subsequently eliminated from the body. Although this model proved very useful during subsequent decades of toxicological research, a growing number of inconsistencies highlighted the need for a revised classification system.

3.4.1 Reductive Metabolism

A more satisfying classification system proposed by David Josephy, Fred Guengerich and John Miners sorts the chemical alterations sustained by foreign substances within the body into four groups instead of two as per the Williams model (Fig. 3.6). Fidelity to the actual chemical modification occurring during the enzymatic transformation is the primary concern in the Josephy system. The first class involves

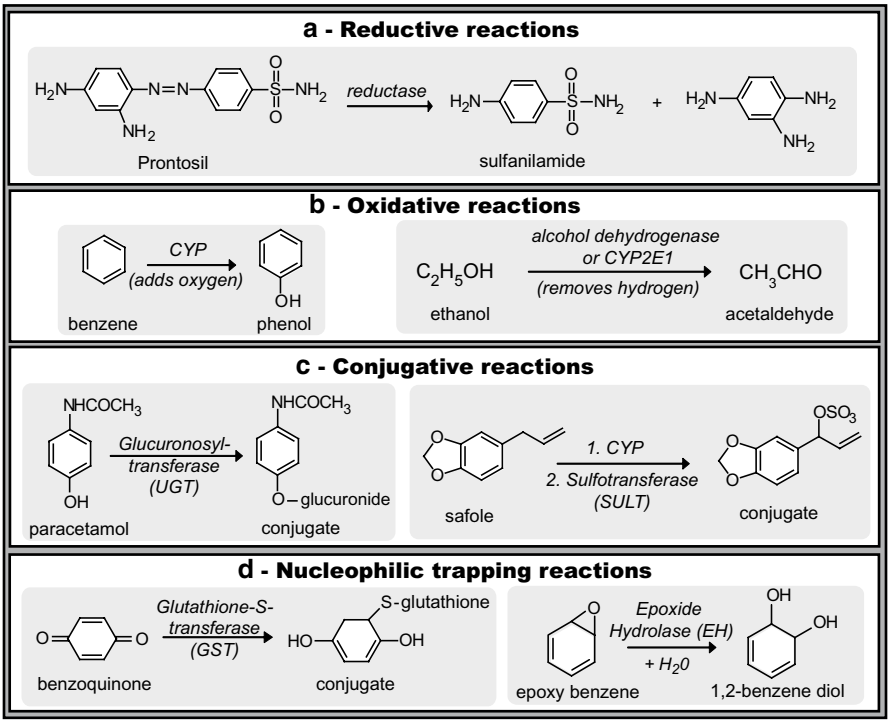

Fig. 3.6 A modern classification system developed by Josephy and colleagues recognises four broad types of metabolic fates for foreign chemicals. For the examples shown (e.g. paracetamol, benzene, safrole), the reactions depicted are only a subset of the total number of metabolites formed from the respective compounds in the body

reductive metabolism, a type of transformation that involves the addition of extra hydrogen atoms to a molecule (or the removal of oxygen atoms) (Fig. 3.6a). A famous example is Prontosil which undergoes reductive metabolism to form sulfanilamide (Fig. 3.6a), the drug that when mixed with diethylene glycol triggered the 1930s nephrotoxicity epidemic in the USA (Chap. 1, Sect. 1.5.1). Reductive metabolism is not especially common in the big scheme of things, but it can inadvertently convert some important chemicals into toxic, DNA-damaging species. One class of suspected cancer-causing chemicals, the nitrotoluenes, undergo just this type of reductive metabolism during their conversion to harmful metabolites – a topic we explore in Chap. 6 (Sect. 6.2).

3.4.1.1 The Gut Microbiome

Since reductive reactions are favoured within low oxygen environments, they are relatively uncommon in the liver which is normally well supplied with oxygenated blood. In contrast, oxygen levels are low within the lower colonic reaches of the

human GI-tract, allowing thriving populations of anaerobic microorganisms to carry out novel reductive biotransformations. Performing a census on the bacterial populations within the lower digestive tract was laborious using traditional microbiology methods, but the availability of molecular methods to identify specific bacteria by analysing a single gene such as the 16s rRNA is allowing detailed surveys of the 'gut microbiome'. According to stool sample analyses, the average human GI-tract accommodates around 200 microbial strains, with individual microbial fingerprints significantly influenced by dietary practices and cohabitation with other humans or companion animals.

Although the gut microbiome plays only a minor overall role in human drug metabolism, its relevance to the pharmacological properties of select drugs is substantial. A classic example is the nonsteroidal anti-inflammatory sulindac, which exerts its pharmacological effects via an active sulfide metabolite. Although this metabolite forms in various tissues, pharmacokinetic studies in animals and humans suggest intestinal microflora perform most sulfoxide reduction during sulindac clearance. Such reductive capacities are also important during the metabolism of DMSO, an organosulfur solvent sometimes used to facilitate the dermal uptake of topical pharmaceuticals.

In addition to sulfoxide reduction, colonic bacteria perform other reductive and conjugative metabolic transformations. These capabilities likely influence the in vivo bioavailability of many xenobiotics, with an important role likely for dietary polyphenolics present within fruits, vegetables and alcoholic beverages. In novel work, researchers recently uncovered a central role for intestinal microbes in two of the most serious toxicological disasters of recent times, namely, the melamine food contamination scares of 2007 and 2008. While in these cases the biotransformation affected by microbes did not involve reductive metabolism, they nevertheless underscore the strong influence gut microbes can exert over toxic phenomena.

3.4.1.2 The Melamine Disaster

Melamine is used during the production of laminates, coatings, plastics and kitchenware. In 2007 and 2008, melamine was associated with respective outbreaks of life-threatening kidney injury in animals and humans due to adulteration of pet foods and infant formula. The latter crisis affected an estimated 294,000 Chinese infants, with over 50,000 hospitalisations and at least six known deaths. In both instances, the motives for the adulteration were likely similar: melamine interferes with standard assays used to measure the protein content of foodstuffs; hence, melamine-adulterated foods appear to contain more protein than is actually the case. Melamine adulteration thus allows unscrupulous manufacturers to sell substandard food items at a higher price.

In susceptible individuals, the kidney damage caused by melamine involves widespread deposition of 'stones' in the kidney, ureter and bladder. Why some infants were highly vulnerable to such pathology is mysterious, although recent findings concerning the efficient bacterial biotransformation of melamine to

cyanuric acid provides a hint that interindividual differences within the gut microbiome may underlie variations in melamine susceptibility. *Klebsiella terrigena* appears especially efficient at carrying out melamine deamination. Due to strong hydrogen bonding between melamine and cyanuric acid, extensive crystalline deposits form within kidney tissue. The role of intestinal bacteria was confirmed in rat experiments where a 4-day antibiotic pretreatment (which killed off intestinal *Klebsiella*) strongly protected against melamine-induced renal injury. These findings will likely open the door to future studies of the role of the gut microbiome in other toxic syndromes.

3.4.2 Oxidative Metabolism

A second – and critically important – class of metabolic transformations are the *oxidative* reactions. These molecular transformations are familiar to organic chemists and are analogous to the alterations one produces in a test tube by treating organic compounds with an oxidising agent such as potassium permanganate or osmium tetroxide. In the body, such oxidative reactions are enzyme catalysed and typically proceed by adding oxygen to a foreign compound, as when a hydroxyl group is inserted in benzene to form phenol (Fig. 3.6b). Alternatively, oxidation can proceed by removing hydrogen atoms from a molecule, such as when ethanol from alcoholic beverages is converted to acetaldehyde by alcohol dehydrogenase (Fig. 3.6b). The most important catalysts of xenobiotic oxidations in the body are the cytochrome P450 (commonly abbreviated CYP or simply P450) enzymes, arguably the most highly researched family of proteins known to biology. Due to their profound importance to toxicology, the CYP family is discussed separately below.

3.4.3 Conjugative Metabolism

Next to oxidative reactions, in terms of the sheer numbers of xenobiotics that undergo these reactions, the *conjugative reactions* are of high importance to toxicology (Fig. 3.6c). A defining characteristic is the formation of chemical bonds between foreign chemicals and hydrophilic substances already present in the liver, thereby forming a diverse class of metabolites known as *conjugates*. The first reaction of this kind was discovered in 1824 by the German researcher Friedrich Wohler, who administered benzoic acid to dogs and recovered hippuric acid from their urine (conjugate with glycine). Many foreign chemicals are directly metabolised by conjugative pathways, while others require oxidative metabolism before entering these pathways. Note that most of the conjugative pathways discussed below are not exclusively involved in the metabolism of foreign substances, since they also metabolise many endobiotics. In many cases, inherited genetic defects in these genes have been associated with clinical syndromes of varying degrees of severity, most of which manifest as alterations in the metabolism of key endogenous substrates.

3.4.3.1 Glucuronidation

The most commonly utilised pathway of conjugative transformation in humans involves glucuronidation reactions, facilitated by a family of enzymes known as *glucuronosyltransferases*. As is normally the case in biochemistry, their name reveals their primary function – glucuronosyltransferases transfer a glucuronic acid group from a 'cofactor' in the liver (UDP-glucuronic acid, where UDP = uridine diphosphate) onto a nucleophilic foreign chemical, forming a *glucuronide* conjugate. These reactions are analogous to the nucleophilic substitution reactions known to chemists. These enzyme catalysts are commonly abbreviated as UGTs, or UDP-glucuronosyltransferases.

Within liver cells, UGT proteins are commonly – but not exclusively – located within the endoplasmic reticulum, a lipid-rich subcellular compartment which is also home to the CYP enzymes. Since the endoplasmic reticulum acts as a miniature conveyer belt to deliver lipophilic xenobiotics to the catalytic chamber of CYP proteins, this location ensures UGT enzymes are well supplied with substrates. In addition to competing with CYP for the 'first bite' at some xenobiotics, UGT proteins metabolise many products of CYP-catalysed reactions.

A substrate for glucuronidation is usually lipophilic while possessing a nucleophilic oxygen, sulfur or nitrogen atom(s). Such substrates are termed *aglycones*. Since glucuronic acid is highly polar, tacking this sugar onto a lipophilic xenobiotic ensures the resulting conjugate is more water soluble than the parent molecule. Normally, such conjugates lack significant biological activity, thus glucuronidation usually results in *pharmacological* or *toxicological deactivation* of the parent molecule. An example of such an outcome is paracetamol (acetaminophen), since approximately 50 % of a normal adult dose (1 g) is converted to an inactive glucuronic acid conjugate (Fig. 3.6c). In addition to their high levels in liver, UGP proteins are highly expressed in nasal tissue where they help deactivate inhaled airborne odorant molecules. UGT proteins are also expressed in the lungs and kidneys and other tissues.

The human genome encodes multiple UGT isoforms which differ in terms of their sequence, substrate specificity, tissue distribution and expression patterns throughout the human lifespan. The term *isoform* rather than *isoenzyme* is used to denote individual members of most biotransformation enzyme families: while isoenzyme denotes multiple variants of an enzyme that carries out the same basic chemical transformation (e.g. lactate dehydrogenase isoenzymes all convert lactate to pyruvate, although they differ in their kinetic properties, tissue expression, etc.). By contrast, isoforms are related enzymes which carry out broadly similar chemical transformations yet differ significantly in their amino acid sequences and specific substrate preferences.

Some 20 human UGT isoforms have been cloned and expressed in model systems, allowing study of their substrate preferences and enzymological properties. Some of these are expressed exclusively within liver – the hepatic UGTs – while others exhibit broader 'extrahepatic' expression patterns (Table 3.2). The highly conserved genetic locus of UGT1A family members is unusual compared to other

Table 3.2 Some representative UGT enzymes within the human genome and their preferred substrates

Isoform	Tissue	Substrates (examples)
UGT1A1	Liver	Xenobiotics + endobiotics (e.g. bilirubin, oestrogens)
UGT1A4	Liver	Mainly xenobiotics (e.g. aromatic amines)
UGT1A6	Liver	Mainly xenobiotics (e.g. small phenolics)
UGT1A7	GI-tract	Mostly endobiotics (e.g. steroids, bilirubin)
UGT2B4	Liver + GI-tract	Xenobiotics + endobiotics
UGT2B17	Liver + GI-tract	Xenobiotics + endobiotics

xenobiotic metabolism genes since multiple family members are spliced from a common precursor mRNA transcript. mRNA editing of this long transcript yields mRNA species for each UGT1A isoform which contain four common exons from the constant region as well as a unique exon 5 from the variable region which confers the distinctive substrate preferences on each isoform. This efficient method of storing genetic information is unfortunately prone to inactivation by frameshift mutations that cause deleterious changes to the reading frame of multiple isoforms. UGT1A promoter mutations of this kind accompany Crigler–Najjar syndrome, a fatal congenital condition which results in severely impaired clearance of the neurotoxic endobiotic bilirubin.

While glucuronide conjugates normally lack biological activity, some important exceptions exist, most famous of which is morphine, the powerful painkiller extracted from the opium poppy. Much of the analgesic effectiveness of morphine is actually due to its metabolite morphine-6-glucuronide. Yet another conjugated metabolite, morphine-3-glucuronide, probably produces such unwanted effects in morphine recipients as CNS excitation and mental confusion.

3.4.3.2 Sulfonation

Sulfonation is an important class of conjugative reactions(Fig. 3.6c) which involve the transfer of a sulfonate group ($-SO_3$) from a cofactor (PAPS – 3′-phosphoadenosine-5′-phosphosulfate) onto a nucleophilic xenobiotic. These reactions are often called *sulfation* pathways, due to the formation of a sulfate group during sulfonation of O-containing substrates (e.g. phenols). Yet since sulfonation also occurs on many nitrogen-containing substrates to form N-sulfonates, *sulfation* is an inadequate name for these reactions.

These reactions are catalysed by the *sulfotransferases (SULT),* a diverse family of enzymes which are expressed strongly in the GI-tract, liver, kidneys, platelets and brain. Unlike many other biotransformation genes, SULT genes are strongly expressed in foetal tissues. Over 50 SULT genes are present within the human genome, which are further classified into 10 main families (Table 3.3).

SULT enzymes reside almost exclusively within the cytosolic fraction of cells, and the products of these reactions are generally very water soluble. In addition to

Table 3.3 Some representative SULT enzymes within the human genome and their preferred substrates

Isoform	Tissue	Substrates (examples)
SULT1A1	Mainly liver	Very important in toxicology and human drug metabolism. Active site is highly 'plastic' – i.e. stretches to accommodate large hydrophobic xenobiotics. Metabolises some endobiotics (e.g. thyroid hormones)
SULT1A3	Mainly liver	Similar sequence to SULT1A1 but metabolises few xenobiotics. Main substrate is dopamine (endobiotic neurotransmitter)
SULT1B1	Placenta, uterus, prostate	Mainly endobiotics (e.g. sex hydroxysteroids)
UGT1A7	GI-tract	Mostly endobiotics (e.g. steroids, bilirubin)
UGT2B4	Liver + GI-tract	Xenobiotics + endobiotics
UGT2B17	Liver + GI-tract	Xenobiotics + endobiotics

metabolising thousands of xenobiotics, SULTs also convert important endobiotics such as dopamine and bile acids into water-soluble species. Compared to glucuronidation pathways which possess an insatiable appetite and can metabolise repeated doses of xenobiotics without experiencing saturation, sulfonation pathways have a low capacity due to limited reserves of PAPS (hepatic levels of UDP-glucuronic acid are typically much higher). Since their substrate preferences frequently overlap, UGT and SULT proteins often compete for the same xenobiotic substrate, with SULT-derived metabolites usually predominating at low xenobiotic concentrations.

Sulfonation of a xenobiotic usually abolishes its biological activity, but some noteworthy exceptions exist. Safrole, a naturally occurring flavouring in nutmeg and cinnamon, causes cancer in laboratory animals via a mechanism that involves oxidative metabolism followed by sulfonation to anoxious, DNA-damaging metabolite (Fig. 3.6c). Whether such reactions are relevant to human cancer is unclear. A more benign example of sulfonation generating a biologically active metabolite involves minoxidil, a heart drug which was introduced in the 1970s as a remedy for high blood pressure. Surprisingly, minoxidil caused unexpected hair growth in some patients, an effect that was due to a sulfonated metabolite.

3.4.3.3 Other Conjugative Pathways

Although glucuronidation and sulfonation are the most common routes of conjugative xenobiotic metabolism, other important pathways exist, including acetylation, methylation and conjugation with amino acids such as glycine or glutamine. While they are important to the toxicity of some select chemicals, these reactions lack the universal importance to xenobiotic metabolism as glucuronidation and sulfonation pathways.

3.4.4 Nucleophilic-Trapping Reactions

The final class of biotransformations under Josephy's naming system are the *nucleophilic-trapping reactions* (Fig. 3.6d). These are of great importance in toxicology since they protect the liver against chemically reactive, toxic metabolites that inadvertently form during the oxidative metabolism of some chemicals. Damaging metabolites of this kind are often unstable, electron-deficient species which attain chemical equilibrium by reacting with electron-dense centres in other molecules. Unfortunately for cells, certain amino acids in proteins, together with the nitrogen bases in DNA, contain electron-rich sites that are reactive with electrophiles. Interactions of this kind generate new covalent bonds between the metabolite and the macromolecule and the formation of a protein or DNA *adduct*. This process is fundamental to chemical toxicity and is explored further in Chaps. 4 and 8.

3.4.4.1 Glutathione-S-Transferase

Tissues are not entirely at the mercy of electrophilic metabolites since the presence of small, electron-dense scavenger molecules within cells can intercept these species before they damage proteins or DNA. The most important nucleophilic-trapping reactions involve glutathione, a small peptide comprising just three amino acids (glutamate, cysteine and glycine). Although small peptides are quickly degraded by peptidases within most cells, glutathione resists this fate since glutamate is attached to cysteine through an unusual γ-carboxyl linkage (c.f. the α-linkage in conventional peptide bonds) ensuring glutathione resists proteolysis. As a result the intracellular concentrations of glutathione are high in many tissues, in the range of 5–10 mM. By contrast, glutathione levels within extracellular fluids are typically within the low micromolar range or below (i.e. 1/1,000th or less).

The possession of a strongly nucleophilic thiol group ensures glutathione reacts rapidly with many electrophiles as well as free radicals (Fig. 3.6d). As the thiol group readily forms disulfide bonds (–S–S–), the formation of glutathione disulfide (GSSG) accompanies cellular exposure to many prooxidants, electrophiles and reactive oxygen species. Under normal physiological conditions, glutathione reductase (GR) maintains glutathione predominantly within the reduced form (i.e. GSH, ~98 % of total cellular glutathione). The remaining ~2 % is present as mixed protein disulfides due to reactions with cysteine thiol groups in proteins (i.e.to form GS-S-protein) as well as the disulfide form of glutathione (GSSG). In addition to direct oxidation of GSH, GSSG also forms during the enzymatic detoxication of inorganic and organic peroxides by glutathione peroxidise. During oxidative stress conditions in which GSSG formation is increased, GR helps restore the cellular thiol redox balance (i.e. GSH:GSSG) to normal values.

The nucleophilic properties of the thiol group are most relevant to the cytoprotection GSH provides against electrophiles during xenobiotic biotransformation. While they usually proceed at a measurable rate in the absence of enzyme, electrophile

trapping by GSH is accelerated by glutathione-*S*-transferases (GST), a ubiquitous enzyme family which protects cells against endogenous electrophiles and reactive intermediates formed during xenobiotic metabolism. GSTs are smaller than most xenobiotic-metabolising enzymes, exhibiting a typical monomeric mass of ~25 kDa, although they usually exist as cytosolic homodimers. These highly expressed proteins can comprise over 5 % of total protein in some cells, including the liver. The human genome contains at least 17 genes for cytosolic GSTs which are assigned to eight major classes, namely, A, K, M, P, S, T, Z and O. Each GST class typically contains multiple isoforms. In addition to their roles in xenobiotic detoxication, GST proteins play broad roles in the regulation of apoptosis, oxidative stress, cell proliferation, inflammatory responses, metabolic processes and the fine-tuning of many cell signalling pathways.

Although most GSTs are mainly present in cytosol, some isoforms sustain post-translational modifications that alter their subcellular distribution, as in the case of GSTA4-4 which undergoes phosphorylation followed by redistribution to mitochondria. Cytosolic GST isoforms that are also found in the nucleus, ER or plasma membrane are termed 'echoproteins'. Some isoforms such as GSTA1-1 and GSTM1-1 are widely expressed in liver, lung, kidney, GI-tract and testis where they provide broad protection against electrophilic xenobiotics and reactive metabolites. Other isoforms such as GSTA4-4 mainly trap endogenous electrophiles and play key roles in diabetes, cardiovascular disease and neurological disorders such as Alzheimer's and Parkinson's neurodegeneration. Other isoforms such as GST P1-1 and T1-1 are upregulated in tumour cells and mediate multidrug resistance by accelerating the detoxication of cytotoxic chemotherapy drugs. A range of polymorphisms have been identified for some GST isoforms including GST T1 and GST M1, ensuring considerable attention has been devoted to determining whether individuals with deficient GST activities are vulnerable to chemical toxicities or tumour responses.

In the example of a glutathione-dependent reaction shown in Fig. 3.6d, the chemically reactive benzene metabolite benzoquinone is trapped by glutathione to form a conjugate that is exported from the liver. Benzoquinone is just one of several toxic metabolites formed following the initial CYP-catalysed oxygenation of benzene to form phenol: it likely forms via subsequent oxidation of phenol by peroxidases within bone marrow. Although readily detoxicated by GSTs, glutathione-dependent protective pathways can be overwhelmed during chronic benzene exposure – such as occurred among gas station attendants who manually 'pumped' gasoline in a bygone era. Benzene is added to automobile fuel to promote efficient combustion due to its 'anti-knock' properties, yet workers who inhaled benzene fumes over an extended timeframe were sadly vulnerable to leukaemia, a serious form of haematopoietic cancer caused by DNA-damaging benzene metabolites.

Although electrophile-trapping reactions initially generate glutathione-S-conjugates, due to further metabolic processing, these species are often undetectable within the urine of animals following exposure to bioactivation-prone xenobiotics. Subsequent to GST-catalysed trapping reactions, membrane transporters typically export glutathione-S-conjugates out of hepatocytes into extracellular fluids (Fig. 3.7). The plasma membrane of many epithelial cells and especially those within renal tissues express various enzymes that further process glutathione conjugates in an effort to recycle amino acid components of the tripeptide (Fig. 3.7). In

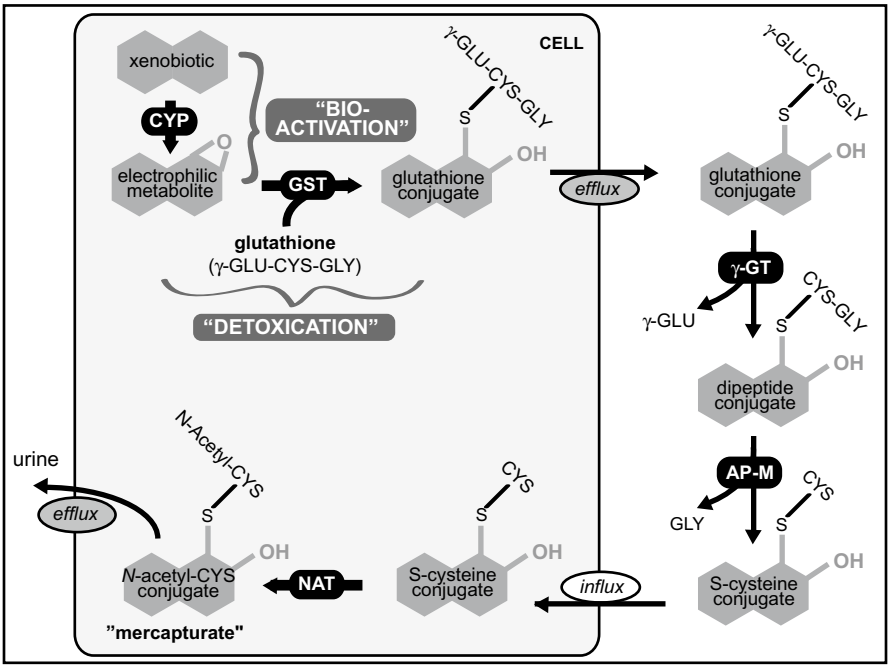

Fig. 3.7 Detoxication by glutathione-S-transferases is a common fate for electrophilic metabolites (e.g. epoxides) which form during CYP-mediated metabolism. Following export by membrane transporters, glutathione-S-conjugates undergo proteolytic processing and *N*-acetylation to form mercapturic acid metabolites that appear in urine (*N*-acetyl-cysteine conjugates)

the first step, membrane-associated γ-glutamyltranspeptidase cleaves the glutamate residue to form a cysteinyl-glycine conjugate. The resulting product is then processed by dipeptidases such as aminopeptidase-M to release glycine, forming an *S*-cysteine conjugate. Within kidney tissues, *S*-conjugates are converted to *N-acetyl-cysteine-S*-conjugates by *N*-acetyl transferases (NAT). These metabolites are otherwise known as mercapturates. These metabolites are telltale signs of the formation of noxious electrophilic metabolites within the liver and can be detected in urine collected from animals and humans following exposure to many environmental pollutants, industrial chemicals and dietary constituents. Mercapturate detection during studies of the in vivo metabolism of candidate drugs during the drug discovery process is often a red flag precluding further preclinical development of the molecule(s).

3.4.4.2 Epoxide Hydrolase

Another nucleophilic trapping reaction that is highly important to toxicology involves the enzymatic hydration of epoxides, a class of electrophiles that can form during the CYP-dependent oxygenation of xenobiotics that contain

aromatic rings or unsaturated groups. The catalysts of these detoxication reactions, epoxide hydrolases (EH), effectively cleave the carbon–oxygen bond within the oxirane ring possessed by their substrates, using H_2O as their cosubstrate (Fig. 3.6d).

Two broad forms of EH are expressed in mammalian cells, including a microsomal EH (mEH) with very broad substrate preferences and a soluble predominantly cytosolic EH (cEH) that specialises in metabolising endogenous substrates. The microsomal form is encoded by the EPHX1 gene which is inducible by ligands that activate 'xenosensors' such as PXR and CAR as well as the cytoprotective transcription factor Nrf2 (see Chap. 5, Sect. 5.3.4). Containing 455 amino acids, mEH attaches to the ER membrane via an N-terminal anchor sequence.

Within modern toxicology, CYP-catalysed epoxidation chemistry attracts much attention for its role in forming promutagenic epoxy metabolites from such foreign carcinogens as vinyl chloride, 1,3-butadiene and polycyclic aromatic hydrocarbons (see Chaps. 8 and 10). The ability of EH enzymes to protect the genome against these damaging electrophiles strongly influences cancer outcomes in smokers and chemical industry workers. Despite this interest, it is evident that not all epoxy metabolites possess chemical reactivity, including various epoxidated fatty acids that form via oxidation of arachidonic acid and other lipids. By controlling tissue levels of these species, EH enzymes play broad roles that extend beyond xenobiotic detoxication and include physiological regulation of the levels of endogenous signalling molecules.

3.5 The Cytochrome P450 Superfamily

Most oxidative xenobiotic metabolism within the liver is catalysed by cytochrome P450 (CYP). Nicknamed 'nature's blowtorch', this remarkable enzyme family converts a huge range of organic molecules into oxidised metabolites. The P450 system is very important to pharmacology since it catalyses some three-quarters of all human drug biotransformations. The human genome contains 57 CYP genes, although this includes several inactive pseudogenes, species which are exclusively involved in endobiotic metabolism, and 'orphans' with as yet undiscovered substrates. The human liver makes a dozen or so CYP isoforms which accomplish xenobiotic metabolism, yet the workload is not shared equally among members of this subgroup since just five CYP isoforms likely account for 90 % of human drug metabolism – CYP1A2, -2C9, -2C19, -2D6 and -3A4. Yet even within this select group, the workload is unevenly distributed: CYP3A4 likely metabolises around one-half of medicinal agents in current use (Fig. 3.8). Yet toxicologists would insist on adding another elite CYP family member to form a Gang of Six: CYP2E1. While 2E1 plays a minor role in human drug metabolism, its contribution to the biotransformation of nontherapeutic xenobiotics is substantial.

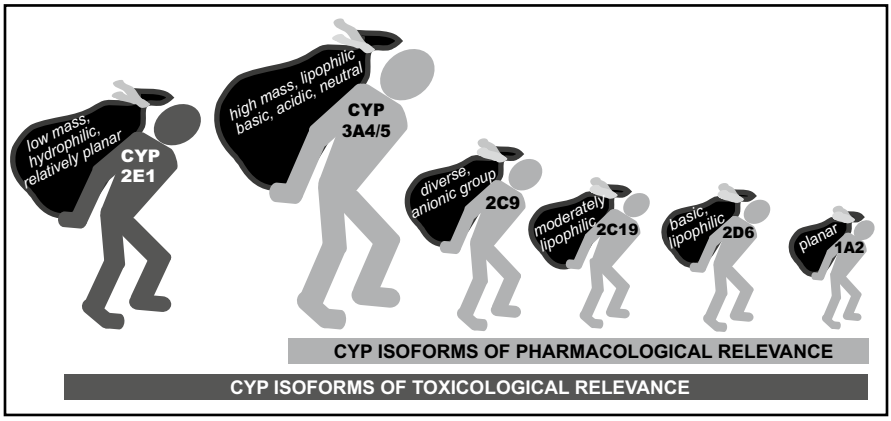

Fig. 3.8 Half a dozen CYP450 isoforms do most of the 'heavy lifting' during oxidative xenobiotic metabolism in humans, although the workload is shared unequally. CYP3A4/5 is most important during the metabolism of pharmaceuticals, while 2E1 metabolises many synthetic chemicals of relevance to toxicology. The types of substrates preferred by each CYP isoform are shown in italicised text. The relative size of each figure reflects estimates of their contribution to xenobiotic metabolism (Reprinted (adapted) with permission from FP Guengerich, Cytochrome P450 and Chemical Toxicology (*Chem Res Toxicol*, 21, 70–83). Copyright (2008) American Chemical Society)

3.5.1 Molecular Aspects of CYP450 Action

CYP isoforms are mid-sized proteins comprising approximately 500 amino acids and with a typical mass of 48–56 kDa. Members of this family are most strongly expressed in liver, but strong expression of CYP3A proteins also occurs in the gut wall. Other CYP-expressing tissues include the kidneys, lung, skin, testes and brain. The catalytic chamber represents the heart of the CYP structure since it contains the crucial heme group which is anchored to the protein near its carboxy-terminus. The hydrophobic amino terminus tethers the structure within the lipid membranes of the endoplasmic reticulum. The iron atom within the heme is where the crucial redox chemistry occurs during the oxygenation of substrate molecules – after binding the substrate and molecular oxygen (O_2), the heme undergoes a rapid series of sequential redox reactions which are driven by the supply of reducing equivalents obtained from the cofactor NADPH by *NADPH/cytochrome P450 reductase*. Like a power plant providing electricity to an adjacent town, the *reductase* is an essential ancillary protein that is located in close proximity to the CYP complex, with each reductase likely supplying 'reducing equivalents' to up to 30 individual CYP proteins.

The growing availability of crystal structures of CYP isoforms has provided valuable insights into the mechanistic basis for the oxidation of xenobiotics (Fig. 3.9). For a given xenobiotic to undergo oxidation by a CYP, it needs to be accommodated within the hydrophobic catalytic chamber, which it accesses via an access channel. The substrate must fit into the chamber in a manner allowing

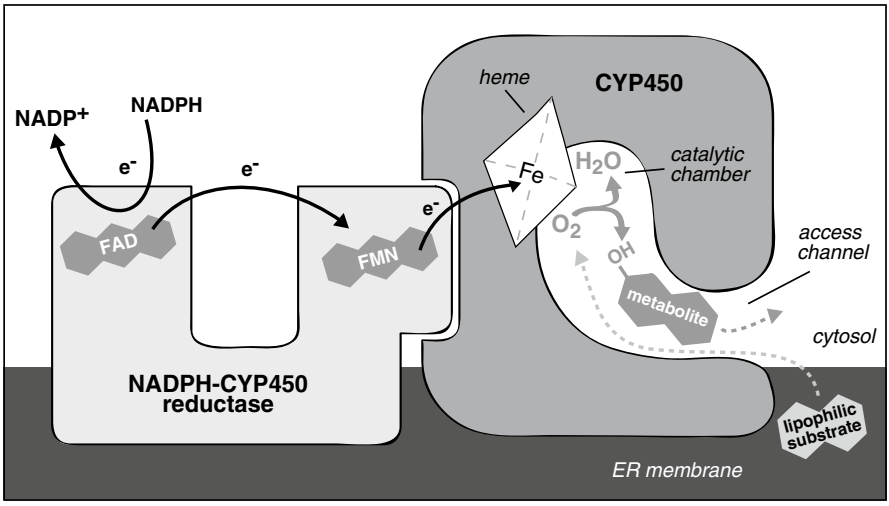

Fig. 3.9 Cooperation by CYP450 and NADPH/CYP450 reductase during xenobiotic oxidation. As a monooxygenase, CYP typically incorporates one oxygen atom (O) from O_2 into the substrate to form a hydroxylated metabolite. The other O atom is converted to H_2O using reducing equivalents donated by the flavoprotein NADH/CYP oxidoreductase. Substrate oxidation is enabled via redox changes in the central iron (Fe) atom in the heme group

interactions between the molecule and critical amino acid residues which surround the active site. Several chemical forces contribute to these interactions, including hydrogen-bonding interactions with key amino acid residues and Van der Waals interactions which are highly significant for xenobiotics containing aromatic rings. Ideally, the molecule must bind such that its most oxidation prone site is oriented towards the heme group, thereby allowing the high-valence iron complex that forms transiently during the CYP catalytic cycle – the perferryl complex ($Fe^{V}=O$) – to rapidly insert an oxygen atom, forming a metabolite which then diffuses out of the CYP complex.

While our knowledge of 3-dimensional CYP crystal structures has improved substantially, forecasting the types of oxidations sustained by substrates using docking software – techniques that have worked well in studies of more conventional enzymes – has proved difficult. This is partly because the active sites of CYPs are surprisingly malleable, able to stretch and bloat to accommodate diverse molecules. Despite these ongoing challenges, structural biology has clarified the importance of the size of the active site in dictating the oxidative capabilities of individual CYP isoforms: as a rule, the larger the catalytic chamber, the greater the number of xenobiotics an individual CYP can metabolise. CYP3A4 – the isoform with the most voracious appetite for xenobiotics – has the largest active site of 1,440 cubic angstroms, compared to just 585 cubic angstroms in the fussier CYP2E1. These differences help explain why 2E1 prefers to metabolise small organic substrates. Most of the remaining CYPs occupy the middle ground between these extremes. The huge active site of 3A4 has further consequences for xenobiotic biotransformation – not only can it contain more than one substrate molecule simultaneously, it can also

accommodate many substrates in different orientations, presenting different parts of the molecules to the all-important heme group. This capability explains why multiple oxidised metabolites can form from the same xenobiotic (e.g. oxygen atoms are inserted at multiple positions in an aromatic ring).

The five CYP isoforms which metabolise human drugs are very important to the pharmaceutical industry which spends large sums each year investigating these pathways. Long before a new drug candidate is administered to humans, it will be screened for its susceptibility to oxidation by the major human CYPs. Many promising drug candidates are discarded because they are too quickly metabolised by CYPs: if hepatic metabolism is too extensive, a drug's effectiveness is diminished since a high proportion of an orally administered dose is destroyed in the liver before it accesses a remote tissue to elicit its therapeutic effect. Second, a rapidly metabolised drug might need to be taken 3 or 4 times a day to ensure a consistent therapeutic response. The need for frequent dosing could lead to patient noncompliance but also might cause the drug to fail in the marketplace where it competes against rival drugs which are metabolised more slowly and need be taken just once or twice a day.

While CYP3A enzymes can effectively metabolise many pharmaceuticals, CYP2E1 seems oddly intended to metabolise the sort of industrial substances that interest modern toxicologists, such as benzene, acetone, styrene or vinyl chloride. Given the prevalence of alcohol consumption across human cultures, the most important CYP2E1 substrate is ethanol, which CYP2E1 readily converts to acetaldehyde (Fig. 3.6b). The role of CYP2E1 within the broader context of alcohol metabolism is explored in Chap. 9 (Sect. 9.5).

3.5.2 Inhibition of CYP Pathways

Knowing which individual CYP isoform metabolises a drug of interest can help predict drug interactions within the body: if two co-administered drugs compete for the same hepatic CYP isoform, they may disrupt each other's removal from the body, leading to life-threatening outcomes. Many clinically significant 'DDIs' (drug–drug interactions) are directly attributable to interactions at the level of hepatic CYP isoforms. In the normal situation in which individuals are exposed to a single CYP substrate, molecules have unhindered access to the active site of a CYP (Fig. 3.10a). CYP inhibitory scenarios typically involve two main mechanisms, the most common of which involves two xenobiotics competing for the same active site of a CYP isoform (Fig. 3.10). The molecule with the strongest affinity acts as a *competitive inhibitor*, blocking access by the other molecule (substrate) and preventing the oxidation chemistry from occurring (Fig. 3.10b). Competitive inhibitors and substrates often share structural similarities that facilitate competition for the same CYP isoform. *Non-competitive inhibitors*, by contrast, are less likely to possess structural similarities and involve the inhibitor binding to an allosteric or modulatory site on the CYP molecule that is structurally and spatially distinct from the active site accessed by the substrate (Fig. 3.10c). While physical competition does not occur, these binding events change the structure of the CYP

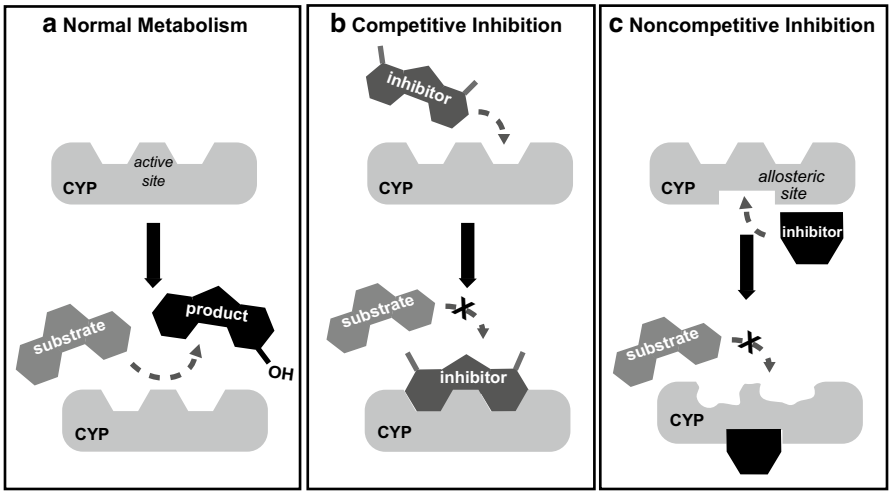

Fig. 3.10 In the normal situation, exposure to a single substrate allows unhindered access to the CYP catalyst (*Panel a*). Toxicological interactions between foreign substances often involve competition between structurally related molecules for the same CYP isoform, with one toxicant acting as a competitive inhibitor which blocks access by a competing substrate (*Panel b*). Less commonly, a toxicant may bind to a spatially distinct allosteric site, producing a change in the geometry of the active site that diminishes substrate affinity (*Panel c*)

and disrupt the geometry of the active site, thereby diminishing its affinity for substrates (Fig. 3.10c). The catalytic efficiency with respect to product formation is thereby decreased.

3.5.3 Induction of CYP Pathways

The capacity of the liver and other tissues to metabolise xenobiotics varies under the influence of diet, age, smoking and drug exposure. At the molecular level, prolonged xenobiotic exposure can boost levels of hepatic CYP enzymes due to a phenomenon termed *induction*. Since such changes represent key adaptive responses to xenobiotics, CYP induction is considered at greater depth in Chap. 5.

3.5.4 Polymorphisms in CYP Pathways

Genetic diversity in xenobiotic biotransformation pathways is a major cause of interindividual differences in the toxicokinetic properties of drugs and other xenobiotics. Genotypic variants within genes for xenobiotic metabolising enzymes that differ in a particular amino acid or possess other sequence differences can potentially alter an individual's susceptibility to toxic agents. The branch of toxicology

that investigates these phenomena is *toxicogenetics* or *toxicogenomics*. These subdisciplines devote considerable effort to clarifying the toxicological significance of gene polymorphisms, those genetic variants within xenobiotic biotransformation genes that are present within more than 1 % of the population. Single-nucleotide substitutions in the genomic DNA sequence (also known as SNPs) are the most common form of polymorphisms. Other sequence variations include insertion of deletion of runs of deoxynucleotides, gene copy variations (e.g. duplications) and gene conversions (e.g. via chromosomal recombination). The phenotypic impact of polymorphisms varies according to the gene and position of the mutated residue within the gene product: some polymorphisms exert minimal effects upon enzyme function, while others lead to completely non-functional protein products. In between these two poles are many polymorphic variants that can alter the metabolic fate of specific xenobiotics, sometimes in a highly significant manner.

Polymorphisms have been identified for genes involved in virtually all aspects of toxicokinetics, ranging from xenobiotic transporters to enzyme catalysts involved in oxidative metabolism, glucuronidation, sulfonation, acetylation or glutathione conjugation pathways. Polymorphisms have also been reported for xenosensor proteins that regulate the transcription of various biotransformation gene products (see Chap. 5). Polymorphisms within key conjugative pathways such as N-acetylation and glutathione conjugation have been investigated in hundreds of studies in efforts to associate particular alleles with particular cancer outcomes or other toxic responses to xenobiotics. In the case of CYP, documented polymorphisms reported in the literature are categorised by the Human Cytochrome P450 Allele Nomenclature Committee (see website at www.cypalleles.ki.se). The nomenclature followed when naming CYP polymorphisms usually indicates a polymorphic variant with an asterisk followed by a number and perhaps a letter (e.g. CYP2C19∗3A). By definition, the normal wild-type allele is designated with a '1'.

Historically, much attention has been directed to CYP2D6 polymorphisms, due to the early discovery of patient subgroups that display exaggerated responses to the cardiovascular drugs debrisoquine and sparteine. The inability to metabolise debrisoquine was linked to a 2D6 polymorphism that was found to vary in its prevalence in different ethnic groups (e.g. 5–10 % of Caucasians are 'poor metabolisers (PM)', while the incidence in Asian populations is ~1 %). Using such techniques as restriction fragment length polymorphism, PCR and gene sequencing, over 110 polymorphisms were subsequently identified in the CYP2D6 gene. Genetic variants that exist at the same chromosomal locus are termed *alleles*. Although the number of 2D6 alleles is unusually large, allele numbers are typically high for most xenobiotic biotransformation genes compared to other genetic loci.

Discovery of the wide range of CYP2D6 alleles allowed segregation of humans into distinct phenotypic groups, with at least four groups currently recognised, including poor (PM), intermediate (IM), normal (NM) and ultra-metabolisers (UM). Individuals within the 'PM' phenotype often possess a G→A transition mutation at an intron/exon boundary that disrupts splicing of RNA transcripts, impairing the production of functional CYP2D6 mRNA. These individuals usually fail to produce any 2D6 protein. CYP2D6 polymorphisms within the IM group can involve mutations

within the protein coding sequence, leading to enzymes with altered activity towards xenobiotic substrates. The UM phenotype is often due to gene duplication, with 13 copies of the CYP2D6 gene observed in some members of a Swedish family caused by a base change that promotes gene duplication. A recent large study in the USA identified the UM genotype in around 1.5 % of the study population. Among the >110 known allelic CYP2D6 variants however, only a subset are prevalent or have major phenotypic consequences during pharmacological and toxicological phenomena.

Promising to help clinicians during the selection of drugs for individual patients, the era of 'personalised medicine' began in 2004 when the US Food and Drug Administration approved the Roche *AmpliChip CYP450 Test,* the first microarray-based diagnostic test for the detection of CYP mutations in human subjects. The test is able to detect 29 CYP2D6 polymorphisms and two CYP2C19 gene polymorphisms. Knowledge of 2D6 status may improve patient responses to anticancer drugs such as tamoxifen, while knowing a patient's 2C19 genotype can help minimise toxic responses to the blood thinner warfarin. While the promise of personalised medicine seemed high initially, clinical opinion is currently divided concerning the actual benefits such diagnostic approaches bring to patient care. Such pharmacogenomic tools also hold promise during investigations of the factors that predispose individuals to toxicity caused by specific xenobiotics that are cleared by specific CYP pathways. However the fact that CYP2D6 and 2C19 play relatively minor roles in toxicant detoxication or bioactivation may require the development of new arrays that assess polymorphisms in a wider range of CYP isoforms than the *AmpliChip CYP450 Test* allows.

3.6 Excretion

Altering the structure of lipophilic xenobiotics represents a short-term solution to their tendency to accumulate in the body: eventually, both the metabolites and any remaining unmetabolised compound require permanent removal from the body. This process – the 'E' for excretion in ADME – is the critical final stage in the toxicokinetic fate of all chemical substances. Typically, most foreign compounds and their metabolites are removed via the kidney and/or the liver. However for some chemicals, other minor routes including the lungs (for volatile substances such as alcohol), breast milk (important for basic drugs such as some antidepressants) or even sweat (relevant to some metallic substances such as nickel) can participate in toxicant elimination. In the main however, excretion via urine and/or faeces represents the ultimate destination for most foreign chemicals that transit the human body.

3.6.1 Bile or Urine?

The factors that determine whether a given xenobiotic is excreted via urine or bile have long fascinated researchers. In a classic 1975 study conducted by researchers

at St Mary's Hospital in London, comparison of the excretory fates of 30 xenobiotics in rats revealed that the molecular weight of a compound determined whether it is excreted in urine or bile. The compounds essentially fell into three groups depending upon their molecular mass: the first group comprised molecules with a mass of 350 g/mol or less which were predominantly eliminated via the urine. A second set of molecules exhibited a mass of between 450 and 850 g/mol and were excreted predominantly in bile. When the bile duct was ligated via surgical intervention, alternative routes could not compensate for the loss of this pathway; hence these compounds accumulated to toxic levels in blood and tissues. A third group of xenobiotics comprised mid-sized molecules possessing a mass range of between 350 and 450 g/mol that were eliminated extensively in both urine and bile.

Later work revealed that whether a xenobiotic is an organic acid or base also influences its excretory fate. In general, small, hydrophilic molecules are primarily excreted by the kidneys, whereas large, amphipathic organic compounds are mainly excreted by the bile. For many years, the mechanisms underlying these different excretory destinies were obscure, but thanks to modern knowledge concerning the molecular biology and tissue expression of xenobiotic transporters, these processes now less mysterious (see below).

3.6.2 Renal Excretion

The elimination of small, water-soluble substances including conjugates formed during xenobiotic biotransformation is performed by the one million or so nephrons in each adult kidney. Nephrons are highly vulnerable to chemical toxicity since these crucial structures only form during the foetal period of prenatal development, ensuring there is little capacity for the replacement of injured nephrons during the later stages of life. These crucial structures are constantly at work, with the entire blood volume passing through the kidneys once every 4 or 5 min. The basic functional unit of the kidney, a nephron comprises a glomerulus that acts as a filter to retain cells and large proteins within circulating blood. The resulting glomerular filtrate then drains into the long tubular structure in which the complex process of urine formation occurs. Anatomically and functionally distinct regions of the tubule are discernible and include the loop of Henle and distal tubule. After completing their migration through the nephron, the concentrated body wastes are delivered to the collecting duct from where they ultimately flow to the bladder (Fig. 3.11).

Three main processes control the efficiency with which foreign chemicals are excreted via the kidneys (Fig. 3.11). First, most xenobiotics, with the exception of very large protein toxins, undergo filtration at the glomerulus, the part of the nephron that interfaces directly with substances as they enter the kidney from the circulation. Since only unbound molecules are filtered in this manner, the extent of glomerular filtration is limited by any binding to plasma proteins such as albumin.

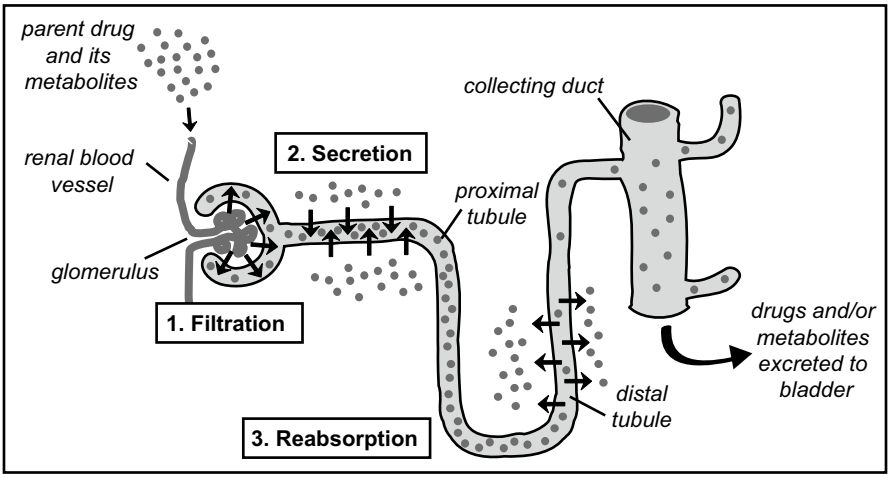

Fig. 3.11 Three processes occurring at kidney nephrons control the efficiency with which foreign chemicals and their metabolites are excreted into urine: *glomerular filtration*, *active secretion* and *passive reabsorption*. The diagram is simplified and does not show the blood vessels that supply the proximal and distal tubules

A second contributing mechanism to renal excretion occurs in the proximal tubules – the end of the tubule directly adjacent to the glomerulus – and involves the energy-dependent transport of chemicals from blood directly into the renal filtrate. This process varies greatly from one chemical to another due to the complex expression of xenobiotic transporters within the proximal tubules. Some transporters are especially good at 'pumping' positively charged molecules out of the blood (i.e. organic cation transporters), while others only transport negatively charged molecules (i.e. organic anion transporters). We will briefly survey the main categories of membrane transporters and their roles in xenobiotic clearance below.

Finally, lipophilic nonionised chemicals that undergo filtration at the glomerulus may return to the bloodstream via the passive reabsorption that occurs throughout the kidney nephron but is especially obvious in distal tubules (Fig. 3.11). Passive reabsorption is due to the dramatic reduction in fluid volume that occurs as renal filtrate proceeds through the nephron: in a healthy kidney, just a few mL of urine results from every 100 mL of blood that is filtered at the glomerulus, reflecting the effectiveness with which the kidneys salvage H_2O and precious blood constituents. During this process, the progressive reduction in filtrate volume ensures that tubular concentrations of many toxicants increase to higher levels than those in circulating blood. If the chemical is sufficiently lipophilic and carries no ionic charge, it will likely diffuse down its concentration gradient back into the general circulation. For such chemicals, conversion to hydrophilic metabolites during a subsequent pass through the liver likely facilitates their eventual elimination.

3.6.3 Biliary Excretion

While the kidneys are very important in terms of the total number of xenobiotics they excrete from the body, the ability of the liver to remove chemicals via bile is important to bodily defences against large, lipophilic molecules as well as conjugated metabolites formed from bulky molecules. Once again, molecular pumps or transporters present within the canalicular membranes of liver cells – those regions of liver cell membranes that interface with the structures that eventually drain into the bile duct – facilitate the excretion of chemicals into bile. Substances cleared by this route are returned to the gut and, together with any unabsorbed molecules (at least for orally ingested substances), are permanently eliminated during the expulsion of faecal matter. We will further consider the role of transporters in the hepatic excretion of xenobiotics below.

3.7 Xenobiotic Transporters

Our survey of the uptake of foreign chemicals across biological barriers emphasised physicochemical properties such as size, lipophilicity and ionisation as determinants of the effectiveness with which substances penetrate body tissues (see Sect. 3.2.1). For many decades, these processes were assumed to exert primary influence over chemical uptake, but in recent years it has become obvious that membrane transporters also govern these outcomes. Molecular genetics has identified hundreds of genes that encode membrane transporters, most of which belong to two superfamilies: the ATP-binding cassette ('ABC') transporters or the solute carrier ('SLC') family. In humans, the former class comprises 49 genes which typically encode ATP-dependent membrane pumps that transport substrates in one direction only. These ATP-dependent protein systems are especially important as efflux transporters, removing xenobiotics and their metabolites from cells. The second class, the SLC transporters, is more numerous, with around 300 genes present within the human genome. These pumps are typically bidirectional, able to facilitate substrate transport either into or out of cells. Rather than utilising ATP, SLC transporters utilise the electrochemical potential differences that exist across cell membranes (e.g. Na^+ gradients). The transporting efficiency of SLC transporters is usually much slower than that of ABC complexes. These systems have broad physiological functions that include the transport of such endobiotics as hormones, nutrients, metabolites, cofactors and eicosanoids.

In pharmacology and toxicology, most attention has focussed on transporter roles in four settings: the oral absorption of xenobiotics from the gut lumen, the handling of foreign chemicals by the liver, the renal elimination of xenobiotics and their metabolites and the penetration of the 'blood–brain barrier' by CNS-acting chemicals. Other specialised physiological settings in which transporters play key roles include the placenta, the testes–blood barrier and the tumour cell where they frequently confer resistance to chemotherapy drugs.

Xenobiotic transporters fulfil two roles of high toxicological relevance. Firstly, *uptake transporters* facilitate the accumulation of xenobiotics by tissues, often ensuring the accumulation of chemicals that would not normally undergo passive diffusion due to unconducive physicochemical properties such as ionic character (e.g. anions and cations). A second important function involves *efflux transporters* that facilitate cellular removal of metabolites formed via oxidative and conjugative metabolism. Since the goal of hepatic metabolism is the conversion of lipophilic xenobiotics to more polar, hydrophilic species, these metabolites might be trapped inside cells unless they could be exported back across lipid membranes. Since this capability is closely associated with xenobiotic transformation, in keeping with the spirit of the historic Williams classification scheme, the efflux of metabolites by membrane transporters was denoted 'Phase 3 metabolism'. Since no further change in the structure of a xenobiotic occurs during the handling of metabolites by membrane transporters, this nomenclature was never particularly apt.

3.7.1 Transporters and Intestinal Xenobiotic Absorption

Within the context of clinical pharmacology, transporter contributions to intestinal drug absorption receive extensive research attention since they significantly influence the oral bioavailability of medicines. Many negatively charged drugs, for example, are substrates for organic anion-transporting polypeptides (OATPs) that facilitate their accumulation from the gut lumen. Yet for many such drugs, their ability to reach the portal circulation is counteracted by the activity of *efflux* transporters such as the p-glycoprotein (P-gp) that diligently return ingested xenobiotics back to the lumen. A key determinant of the bioavailability of an orally ingested xenobiotic – the proportion of an oral dose that ultimately enters the systemic circulation – is thus whether an individual expresses high levels of functional P-gp.

P-gp is the prototypical ABC transporter in that it utilises cellular energy to expel bulky chemicals from enterocytes back into the gut lumen. It belongs to a broad family of related pumps that includes such noteworthy members as multidrug resistance-associated protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (BCRP or ABCG2). Researchers initially discovered these proteins due to their role in protecting tumour cells against chemotherapy drugs, but P-gp was subsequently found to efflux many diverse xenobiotics and even to exert a strong influence over gastrointestinal drug absorption.

Together with the CYP3A4 isoform which is expressed strongly in the gut wall, P-gp is part of an elaborate defence system that protects the body against ingested xenobiotics by minimising their gastrointestinal absorption. The close cooperation between these pathways manifests in the extensive overlap between substrates for these processes: many CYP3A4 substrates are transported by P-gp while many P-gp substrates are oxidised by CYP3A4 (i.e. termed 'bi-substrates'). When exploring the voluminous literature surrounding P-gp, it helps to keep in mind that this protein is often known by its alternate names MDR1 and ABC-B1.

How P-gp functions at the molecular level long puzzled researchers, although the recent crystal structure of a murine P-gp homologue clarified this issue. The protein complex comprises 1,280 amino acids arranged into two homologous halves that resemble a tiny membrane-embedded baseball mitt (Fig. 3.12). Each half of the P-gp complex comprises six membrane-spanning domains together with an ATP-binding domain. The extracellular domains are decorated with branching glycosyl moieties, explaining why P-gp is designated a glycoprotein. Although the molecular aspects of the dynamic cycle are not fully clarified, lipophilic molecules appear to diffuse into the inner P-gp chamber via a slit that transiently opens during the transport cycle, followed by a contraction of the structure that forcefully ejects the molecule back to the lumen (Fig. 3.12). However the precise mechanisms remain conjectural since X-ray crystallography images essentially represent a snapshot that overlook dynamic changes in the lipid membrane, substrate translocation, ATP binding and hydrolysis as well as protein side-chain contortions.

As with many CYPs, the levels of P-gp in the GI-tract increase during prolonged exposure to some xenobiotics, a finding that focussed great interest upon the

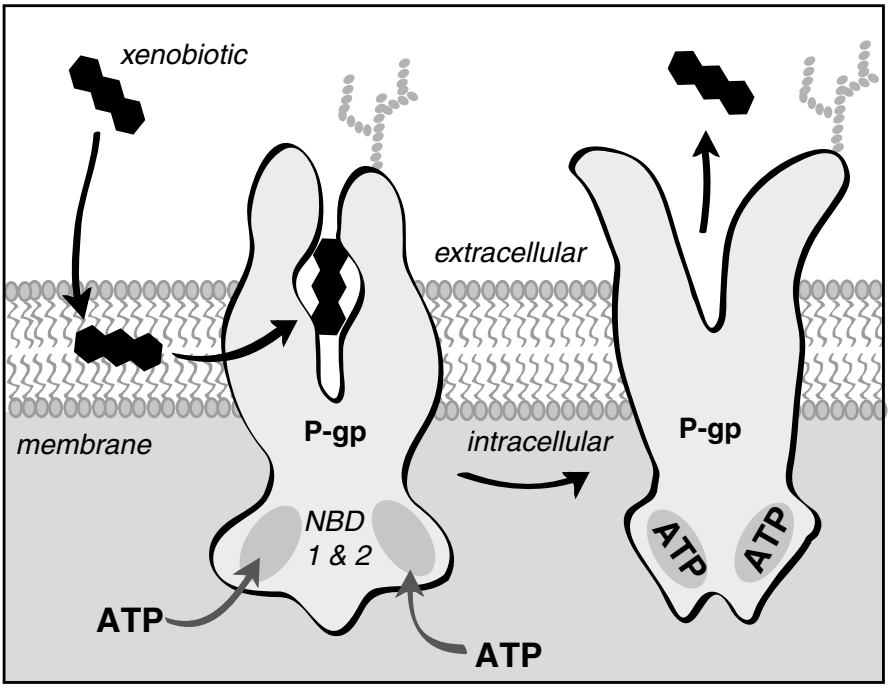

Fig. 3.12 The P-glycoprotein (ABCB1) is a membrane-embedded ABC xenobiotic transporter that limits the cellular accumulation of lipophilic xenobiotics. *P-gp* substrates typically span a mass range of 200–1,000 Da. The dynamic cycle likely involves diffusion of xenobiotic substrates through a slit within the *P-gp* structure. *ATP* binding to the nucleotide-binding domains (*NBD*) likely promotes rapid twisting of the complex to expel the xenobiotic back into the extracellular domain

transcription factors that drive its expression in the gut wall and other tissues. The xenosensor PXR (see Chap. 5, Sect. 5.2.1.4.) is one significant driver of P-gp expression as are various inflammatory cytokines that target inflammation-responsive sequences within the P-gp gene promoter (e.g. NF-κB, C-EBPβ). Polymorphisms within the P-gp promoter also influence the amount of functional protein expressed within the gut wall and thus alter intestinal drug absorption in some individuals. Interindividual variability in responses to the same drug can often reflect wide differences in the expression, inducibility or activity of P-gp within the gut wall.

3.7.2 Transporters and the Liver

Following absorption at the gut wall, xenobiotics enter the distribution phase during which membrane transporters can further influence their dispersal around the body. On entering the liver, xenobiotics are processed by hepatocytes that are highly polarised in terms of membrane transporter expression patterns on either the basolateral/sinusoidal membranes or the apical/canalicular membrane. Firstly, SLC transporters within basolateral membranes control the accumulation of many xenobiotics from hepatic circulation, with key roles for members of the organic anion-transporting peptide subfamily such as OATP1B1 and OATP2B1 as well as organic cation transporters such as OCT 1. The basolateral membranes also express major *efflux* transporters such as MRP1 and MRP2 which may excrete polar metabolites formed via oxidative and conjugative metabolism back into the blood to allow their eventual excretion by the kidneys. Finally, high mass substances are often excreted by canalicular membrane ABC transporters which deliver molecules into bile for eventual drainage into the GI-tract. P-gp participates strongly in this process, although many other ABC transporters are similarly expressed at high levels in canalicular membranes.

Knowledge of hepatic xenobiotic transport was long limited to studies on either canalicular or basolateral membrane vesicles prepared from donor livers. These studies were useful in clarifying the kinetics of xenobiotic transporters and for studying interspecies differences in these processes and revealed that for some xenobiotics hepatic transport depended on the availability of sodium ions or a proton motive force. Yet full identification of the molecular participants awaited the advent of molecular genetics and the ability to clone genes that encoded anionic and cationic transporters as well as ABC transporters within the liver. Table 3.4 contains a partial listing of transporters that govern the fate of xenobiotics in the liver. The reading list at the end of this chapter specifies reviews that explore this field in detail.

3.7.3 Transporters and the Kidney

The active transport of xenobiotics within the kidneys is enabled by strong expression of SLC and ABC transporters on the apical and basolateral membranes of renal

Table 3.4 Some select xenobiotic transporters involved in the transportation of xenobiotics within liver cells

Transporter	Localisation	Uptake or efflux	Representative substrates
BSEP (bile salt export pump)	Canalicular membrane	Efflux	Bile salts
MRP2 (ABCC2)	Canalicular membrane	Efflux	Bilirubin glucuronide
MRP1 (ABCC1)	Basolateral	Efflux	Many drugs
OAT2	Basolateral	Uptake	Many drugs
OCT1	Basolateral	Uptake	Drugs: metformin, acyclovir, etc.
OATP1B1	Basolateral	Uptake	Drugs: statins, sartans, antibiotics, etc.

epithelial cells. Sometimes, the efficiency of these excretory pathways greatly exceeds that of glomerular filtration, ensuring active secretion dominates the overall renal clearance of such xenobiotics. Furthermore, while glomerular filtration cannot filter protein-bound xenobiotics, membrane transporters efficiently clear both free and bound toxicants from circulating blood. Such capabilities often achieve high toxicant concentrations within proximal tubules, rendering them vulnerable to injury, a problem to be explored in Chap. 6.

The basolateral membrane expresses transporters that import xenobiotics from blood into epithelial cells. One key basolateral cationic transporter, OCT2, transports relatively small organic cations such as the antidiabetic drug metformin and the anti-influenza drug amantadine. Major organic anion transporters on the basolateral membrane such as OAT1 and OAT3 show overlapping preferences for a variety of drugs and select toxicants including various polycyclic aromatic hydrocarbons.

The apical membrane of renal epithelial cells expresses the anionic transporter OAT4 as well as the cationic transporters OCTN1 and OCTN2 which pump their preferred substrates into tubular fluid. The apical membrane also expresses key ABC transporters such as P-gp and MDR1. These transporters actively excrete large, bulky substrates that otherwise exhibit low membrane permeability.

Due to the importance of anion and cation transporters in the renal excretion of many medicines, toxic interactions can occur when two co-administered drugs compete for the same clearance mechanism. While such episodes can contribute to serious drug–drug interactions, problems of this kind are less prevalent than interactions between drugs at CYP enzymes in the liver. Although it is used less frequently during the treatment of congestive heart failure than was once the case, the cardiac glycoside digoxin is a common culprit in transporter-mediated drug–drug interactions since it is exclusively cleared at proximal tubules by P-gp. Concurrent use of digoxin and such drugs as ritonavir or ranolazine can lead to dangerous increases in plasma concentrations of digoxin due to inhibitory effects on the P-gp. Since digoxin has a narrow margin of safety, this can lead to serious cardiotoxicity in affected patients.

3.8 Conclusion

This chapter has surveyed the key factors that control the entry of chemicals into the body, their dispersal to different tissues, as well as their metabolism and eventual elimination. While awareness of the importance of these processes to toxicological phenomena is long standing, their molecular basis was long mysterious. Assisted by insights from molecular genetics and biochemistry, modern toxicology possesses a far richer understanding of the mechanistic basis for these essential toxicokinetic phenomena that was possessed by researchers even a generation ago. With this basic appreciation of the main physiological and chemical processes that govern the fate of xenobiotics within the body, we are better placed to explore the mechanistic events underlying the toxic responses that chemicals elicit within human tissues.

Going Further

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Chapter 4
Toxicodynamics: How Chemicals Harm Cells

Abstract Understanding how small molecules comprising just a few dozen or less atoms inflict permanent harm upon complex living cells – which contain thousands upon thousands of metabolites, genes, proteins and interconnected signalling networks – is a central concern in modern toxicology. While toxicologists were long restricted to observational descriptions of morphological changes in dying cells, modern developments in chemistry and molecular biology opened the door to new mechanistic understandings of toxicity. One useful concept emerging from this effort proposes noxious xenobiotics bind selectively to 'toxicity receptors' in much the same way medicinal agents target 'drug receptors' to elicit a pharmacological response. An even more influential insight recognises that instead of inducing toxicity via transient interactions with receptor proteins, many toxic xenobiotics instead undergo enzyme-catalysed conversion to electrophilic metabolites that react chemically with cell macromolecules. This chapter explores the basic properties of reactive metabolites that influence their toxicological properties together with the major deleterious consequences of reactive metabolite formation within tissues, including adduct formation, calcium dyshomeostasis, oxidative stress, lipid peroxidation, apoptosis and kinase activation.

Keywords Direct-acting toxicants • Metabolism-dependent toxicants • Receptors • Computational toxicology • Metabolite stability • Electrophiles • Covalent binding • Calcium dyshomeostasis • Oxidative stress • Lipid peroxidation • Apoptosis • Kinase signalling

4.1 Introduction

If *toxicokinetics* considers the *fate* of chemicals within the body, *toxicodynamics* attempts to describe *how* they cause tissue damage. This concern is the natural domain of *mechanistic toxicology,* a dynamic branch of modern science since our understanding of the mechanisms underlying chemical toxicity is continually enriched by fast-paced advances occurring in biology and biomedical science.

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Modern toxicology has also embraced the use of new 'omics' technologies in its attempt to understand the molecular basis for chemically induced toxicity. This chapter will survey this rapidly expanding body of knowledge to provide an overview of current understandings of how toxicants inflict damage on living tissues.

4.2 Direct-Acting or Metabolism-Dependent?

To fully understand any chemically induced toxic syndrome, researchers must clarify the chemical form of the toxicant that drives the expression of toxicity: is the original toxicant causing the toxicity, or does it undergo enzymatic conversion to toxic metabolites within the body? (Fig. 4.1). In the former instance, direct-acting chemicals usually bind to specific proteins in a cell or tissue, triggering a sequence of events that lead to cell death or other toxic outcomes (Fig. 4.1). Metabolism plays little or no role since the parent molecule directly mediates cell dysfunction. Table 4.1 shows a select handful of such direct-acting toxicants together with their main molecular targets and the toxic syndromes they elicit. The list includes chemicals of both natural and synthetic origin.

4.2.1 Receptors and Drug Responses

The effects of many direct-acting toxicants can be understood by borrowing the concept of a *receptor* from modern pharmacology. In the late nineteenth century, the

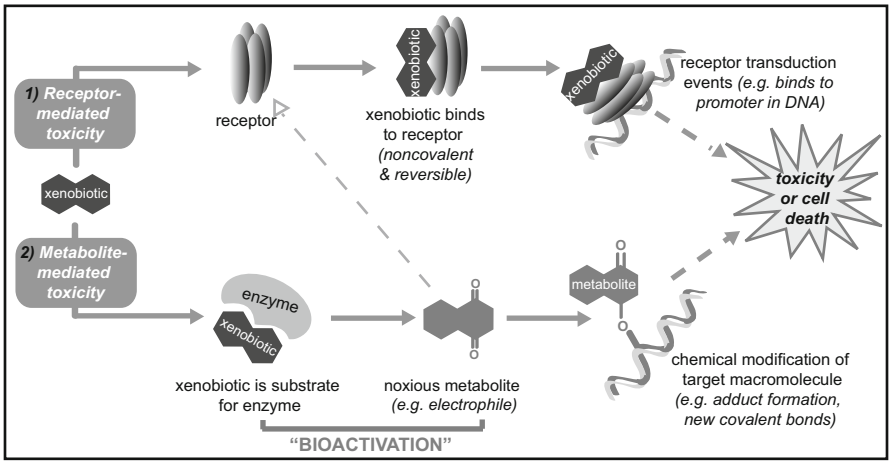

Fig. 4.1 Many chemically induced toxic syndromes can be understood as either mediated by direct interactions of chemicals with target receptors (Scenario 1) or involving toxic metabolites formed by enzymatic processing of the xenobiotic in the process known as bioactivation (Scenario 2). While reactive metabolites that form adducts are of special concern, less reactive metabolites may also induce toxicity via noncovalent interactions with receptors (*dashed line*)

German researcher Paul Ehrlich (1854–1915) proposed tissues possess 'side chains' or 'receptors' that are targeted by toxins released from infectious pathogens. The receptor concept appealed to pioneering pharmacologists such as John Langley (1852–1925) who in 1905 proposed that medicinal agents might also specifically interact with *receptive substances* in the body. This proposal initially met with resistance, but elegant theoretical and experimental work by mid-twentieth-century British pharmacologists such as Clarke and Gaddum reinforced the receptor theory by providing mathematical models that explained differences in the pharmacological potency of individual drugs. In the 1970s, isolation of the first protein receptors from animal tissues placed the theory beyond doubt. According to this theory, transient interactions between drugs and their receptors within cell membranes trigger changes in receptor structure that activate downstream signalling pathways that alter cellular function, ultimately triggering physiological responses. Receptor theory also helped explain the effects of *antagonist* drugs that block the effects of

Table 4.1 Some direct-acting chemicals that target specific cell proteins to elicit toxicity

Toxicant	Natural or synthetic origin?	Toxic effects	Likely target ('receptor')
Capsaicin	Chilli peppers	Eye, skin and lung irritation	TRPV1 vanilloid receptors (sensors for painful stimuli)
Di-(2-ethylhexyl) phthalate (DEHP)	Synthetic (widely used plasticiser during the production of PVC)	Peroxisome proliferation, reproductive tract defects, cancer (rodents)	Peroxisome proliferator-activated receptor (PPARα). Wider PPAR family has broad roles in lipid metabolism, immunity, cardiovascular function, etc.
Diethylstilbestrol	Synthetic oestrogen once used to prevent miscarriages	Tumours in reproductive tissues (offspring of exposed mothers)	Oestrogen receptor (ERα)
Dioxin	Synthetic (by-product of 2,4,-D defoliant synthesis)	Persistent acne ('chloracne'), foetal abnormalities, cancer	*Ah* (Aryl hydrocarbon) receptor (transcription factor)
Hydrogen cyanide	Synthetic	Respiratory failure	Cytochrome c oxidase (mitochondrial protein)
Microcystin	Cyanobacteria (blue-green algal blooms)	Liver damage	Protein phosphatases 1 and 2A (regulate protein activity by removing phosphate groups)
Thevetin A	Yellow oleander	Cardiac stimulation	Na^+, K^+-ATPase (ion pump that maintains voltage gradients in nerve cells)
Strychnine	Ignatia beans	CNS excitation (convulsions)	Glycine receptor (aids chloride ion movement across neuronal membranes)

naturally occurring ligands or neurotransmitters at their preferred receptors: such inhibitory molecules could bind to critical domains within the target protein, yet fail to elicit conformational changes needed for a physiological response.

4.2.2 Receptors and Toxic Responses

The receptor theory also provided an explanation for chemically induced toxic syndromes by proposing that toxicants could bind to cell proteins and elicit downstream subcellular responses that elicit tissue dysfunction. This conceptual approach has provided especially valuable insights into the toxicity of dioxin, an organochlorine contaminant of the Agent Orange defoliant that was used extensively during the Vietnam War (~1959–1975). Strictly speaking, dioxin is more accurately known by its proper name TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), since the name dioxin simply denotes the nonchlorinated parent compound. By stripping jungles of undergrowth, Agent Orange supposedly increased the visibility of guerrilla bands. Yet these questionable practices also exposed US soldiers, their allies and opponents alike to TCDD, a highly toxic by-product of the synthesis of 2,4-D and 2,4,5-T defoliants, the main ingredients of Agent Orange. The health effects of TCDD in war veterans caused sustained political controversy in the USA and elsewhere during the 1970s and 1980s. In addition, TCDD likely had harmful effects upon the offspring of women in Vietnamese villages. Against this socially charged backdrop, toxicologists conducted crucial research into the molecular mechanisms underlying the toxic effects of TCDD.

A key breakthrough involved the identification of a protein known as the *Ah*R (aryl hydrocarbon receptor) as the subcellular target for dioxin. This receptor is also activated by various natural and synthetic toxicants that share chemical properties with TCDD: possession of multiple aromatic rings, a planar structure and hydrophobic character. The AhR became the prototype for understanding a wide range of receptor-driven toxic responses. Like other xenobiotic receptors, the AhR acts as a transcriptional activator to elicit broad transcriptional responses within the nucleus of exposed cells. The mechanisms whereby *AhR* mediates cellular responses to dioxin are explored in Chap. 5 (Sect. 5.2.1.2).

While the receptor paradigm has enriched our understanding of many toxic phenomena, the theory has required some modifications from classic drug receptor interactions studied by pharmacologists. For example, while many drug-induced receptor interactions occur rapidly over a time frame of milliseconds to seconds, the toxic effects elicited by receptor-targeting toxicants such as dioxin involve changes in gene expression that can take hours, days or even weeks to elicit observable toxic responses. Furthermore, most interactions between drugs and their receptors are transient in nature, increasing in intensity as drug concentrations rise in blood following their ingestion and then recede upon their subsequent removal from the circulation. In contrast, many toxicological phenomena are not explicable in terms of freely reversible interactions between xenobiotics and receptors within tissues and

instead involve *irreversible* interactions between chemicals and their subcellular targets. To understand these phenomena, mechanistic toxicologists must traverse terrain that is unexplored by classical pharmacology theory. By finding that many chemicals cause toxicity via reactive metabolites which inflict irreversible damage upon cell targets, toxicologists have had to develop new modes of thinking while also using concepts borrowed from organic chemistry and biochemistry.

4.2.2.1 Computational Toxicology

As the twenty-first century unfolds, growing awareness of the role of specific protein targets in mediating toxicity combined with developments in computer-based modelling has fuelled the emergence of computational toxicology. The expanding availability of detailed descriptions of protein target structures provided by X-ray crystallography helped cultivate this field. Computational toxicology is also aided by *cheminformatics* – the modern discipline that draws on computer science and chemistry to develop software tools for storing, managing and interrogating large sets of chemicals ('compound libraries') within a computer-based ('in silico') environment. Typically, computational toxicologists develop software tools that allow large compound libraries to be screened in silico for their binding affinity towards select protein targets of known toxicological significance.

One example of the approaches possible in computational toxicology is typified by the *VirtualToxLab*. This powerful Swiss project uses in silico tools to predict the toxicity of drugs, chemicals and natural products by simulating their binding affinity towards 16 proteins that participate in chemically induced toxic responses (e.g. nuclear receptors, CYP proteins, transcription factors, ion channels). The chemical structures for chemicals of interest can be uploaded within the VirtualToxLab environment to study interactions with toxicity receptors. This model has been used to predict the abilities of over 2,500 chemicals to elicit such toxic responses as metabolic disruption, carcinogenicity and cardiotoxicity: results obtained from this initiative can be seen at www.virtualtoxlab.org. The growing popularity of virtual screening approaches within influential regulatory bodies such as the US Environmental Protection Agency illustrates that the toxicologist of tomorrow will require competency in such areas as cheminformatics and bioinformatics in addition to training in experimental biology and toxicology.

4.3 Metabolism-Dependent Toxicity

Toxicological bioactivation – the process whereby innocuous compounds are metabolised to toxic metabolites – underpins the toxicity of many xenobiotics (Table 4.2). This phenomenon intrigues researchers wishing to clarify the metabolic changes individual molecules undergo within the liver, trying to distinguish between those achieving detoxification and those causing toxicity.

Table 4.2 Select chemically induced pathologies mediated by reactive metabolites formed in the liver or other organs

Foreign chemical and its usage or relevance	Target organ and toxic syndrome	Metabolic transformation and toxic metabolite	Key cellular target
Acrylamide (chemical industry reagent and starchy food contaminant)	Liver cancer (rodent tests only – equivocal in humans – see Chap. 8)	Oxidation by CYP450 (epoxide)	Genetic material (DNA)
Benzo[*a*]pyrene (tobacco combustion product)	Lung cancer (plus other organs)	CYP450 (×2) and epoxide hydrolase ('diol epoxide')	Genetic material (DNA)
Bromobenzene (solvent and engine oil additive)	Liver (hepatic necrosis)	CYP450 (epoxide)	Cellular proteins
Hexachlorobutadiene (intermediate in rubber production)	Nephrotoxicity (renal necrosis) + other organs	Glutathione-S-transferase + peptidases ('cysteine conjugates')	Cellular proteins
2-Napthylamine (discontinued dye and rubber constituent)	Bladder (tumours in the cellular lining)	CYP450 and sulfotransferases and/or N-acetyl transferases	Genetic material (DNA)

4.3.1 Determinants of Metabolite-Mediated Toxicity

Bioactivation is especially damaging when it generates reactive metabolites that attack DNA or proteins. Such 'reactive intermediates' attract particular research interest since they can exhibit intriguing diversity in their reactions with cell macromolecules. In general, the type of toxicity inflicted by such metabolites depends upon several key factors.

4.3.1.1 Metabolite Stability

The *chemical stability* dictates the lifetime of a metabolite within the cellular environment, which in term determines how far it can travel within the biological environment before undergoing reactions with cell macromolecules, enzymatic detoxication or spontaneous decay. The time taken for their intracellular concentrations to fall by 50 % – the metabolite half-life ($T_{½}$) – is especially useful when predicting the damage inflicted by bioactivation products (Fig. 4.2).

Normally, if a stable metabolite forms during the catalytic cycle of CYP450, it diffuses quickly away from the enzyme, ultimately undergoing excretion from the cell. The situation can be quite different for reactive bioactivation products, with at least four broad outcomes possible. As Scenario 1 in Fig. 4.3 shows, some CYP-derived metabolites possess ultrashort half-lives (e.g. in the microsecond range). The pronounced reactivity of these ultra-unstable metabolites ensures they attack

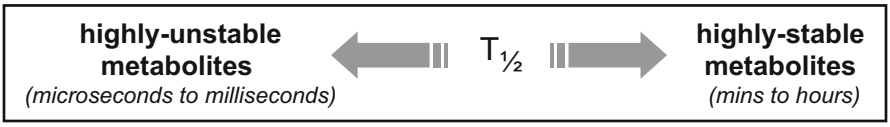

Fig. 4.2 Bioactivation of different chemicals generates metabolites that vary in their chemical stabilities and $T_{½}$ values

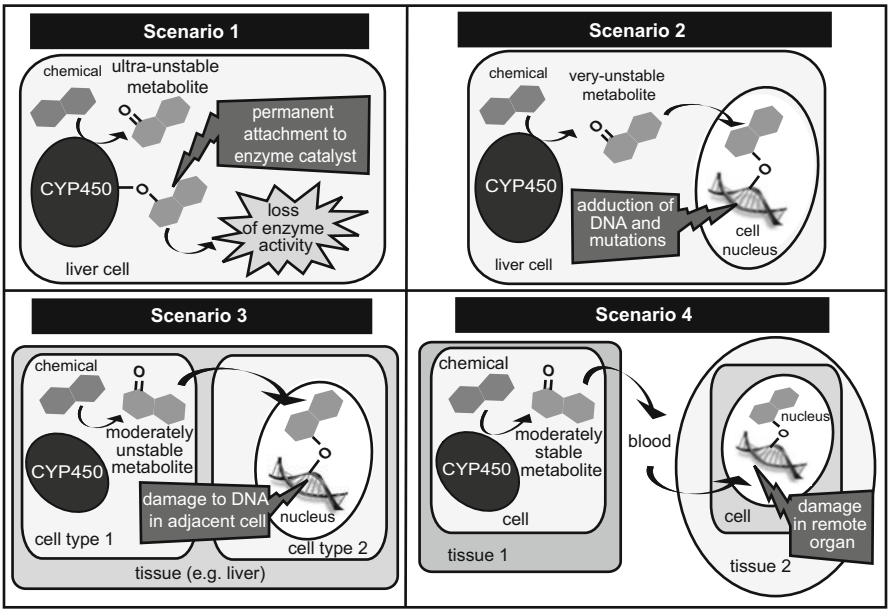

Fig. 4.3 The chemical stability of reactive metabolites influences the localisation of tissue damage during the metabolism of xenobiotics by CYPs

CYP before diffusing away from the active site of CYP. These 'suicide inactivation' reactions can 'kill' the CYP enzyme by attacking the heme group or destroying its ability to metabolise additional xenobiotic molecules. Such outcomes accompany therapy with dihydralazine, a blood-pressure-lowering drug that is restricted in a number of countries due to concerns over liver toxicity. Following conversion to an ultra-unstable metabolite that attacked CYP, dihydralazine can trigger life-threatening immunoallergic hepatotoxicity in susceptible patients.

Another possibility arises if the reactive metabolite is sufficiently stable to escape the CYP catalytic chamber but cannot exit the cell in which it formed (Scenario 2 in Fig. 4.3). Such outcomes are probably rare since liver cells are very small entities; hence, reactive metabolites that are sufficiently long lived to escape their enzyme environment can usually traverse multiple cell diameters even in a brief lifetime.

Reactive metabolites possessing half-lives of a few seconds or more can likely damage neighbouring cells in the same organ (Scenario 3, Fig. 4.3). This situation

is quite common and reflects the fact that most organs contain a diversity of cell types which differ in their abilities to metabolise xenobiotics or detoxicate reactive metabolites. A classic example is vinyl chloride-induced hepatic angiosarcoma, a problem we encountered in Chap. 1 due to its importance during the history of occupational toxicology (Sect. 1.4). In liver cells, vinyl chloride is converted to a reactive metabolite that damages adjacent endothelial cells within hepatic blood vessels. The latter contain low levels of glutathione and are highly vulnerable to toxic vinyl chloride-derived metabolites. The mechanisms underlying vinyl chloride carcinogenicity are explored in Chap. 8 (Sect. 8.7.1).

Another common situation arises when reactive metabolites survive for a few minutes or more, providing sufficient time to escape the organ in which they formed (Scenario 4, Fig. 4.3). On entering the bloodstream, the metabolites migrate throughout the body, causing damage in remote tissues. This phenomenon is very important in smoking-related cancer, since the chemicals in tobacco smoke can undergo bioactivation to reactive metabolites either in the lungs or liver before dispersing around the body. In addition to lung cancer, smokers face a high risk of cancer in peripheral organs such as the pancreas, kidney and bladder.

4.3.1.2 Macromolecular Selectivity

Rather than attacking all cell constituents indiscriminately, reactive metabolites often react differently with DNA compared to proteins. Identifying the preferred macromolecular targets for a reactive metabolite can help explain the toxic responses occurring upon exposure to the parent toxicant.

For metabolites displaying pronounced reactivity with DNA, long-term consequences can include cancer and other types of genetic disease. During pregnancy, DNA-damaging metabolites can cause embryonic malformations or increase the risk of childhood cancers such as leukaemia. Once a reactive metabolite is known to damage DNA, the question emerges as to which sequences within the genome are susceptible to attack. Damage within the promoter sequence of a gene can suppress the expression of the protein product, causing enzyme deficiency or a loss of normal protein functions. Alternatively, damage within key coding regions of a gene target can trigger mutations that lead to abnormal, mutated proteins. Events of this kind are well described for critical growth regulatory genes such as p53 or K-*Ras* which are major targets for tobacco smoke carcinogens. Pinpointing chemical damage to specific mutation-prone exons within these genes has helped incriminate specific carcinogens in particular forms of occupational- or smoking-related cancer (see Chaps. 8 and 10).

Metabolites showing a preference for cell proteins are often associated with organ dysfunction or allergic toxic reactions. Once it is known that a given xenobiotic forms a protein-damaging metabolite, the quest begins to identify the precise protein targets that sustain damage within vulnerable ‘target organs’. Knowing if a metabolite preferentially damages proteins involved in cell death regulation, cell proliferation or inflammatory response pathways provides valuable insight into the

toxicological profile of the parent xenobiotic. Since most cell proteins exist in multiprotein complexes or function via transient partnerships with other proteins, knowing how damage to a specific protein alters the behaviour of global cellular networks is emerging as a major topic of interest in modern toxicology.

4.3.1.3 Electronic Properties

Many reactive metabolites are 'electrophiles', electron-deficient species that seek to fill up their electron shells via reactions with electron-donating reagents ('nucleophiles'). Proteins are common targets since they are prevalent within the cellular environment and contain electron-rich atoms on amino acid side chains. DNA also contains many nucleophilic heteroatoms such as N and O within purine and pyrimidine bases as well as O atoms on the sugar–phosphate backbone. According to the 'HSAB theory' (hard–soft acid–base), metabolites formed during xenobiotic metabolism behave as either 'hard' or 'soft' electrophiles during reactions with cell macromolecules. 'Hard' electrophiles carry formal positive charge and possess valence electrons that are not easily delocalised within the metabolite structure. 'Soft' electrophiles on the other hand carry a partial charge and electrons are delocalised across the metabolite.

According to the HASAB theory, nucleophiles also are either 'hard' or 'soft', depending on the electron distribution within the atom. 'Hard' nucleophiles within cells include O and N atoms on pyrimidine and purine bases in DNA and RNA and N atoms on lysine, histidine and arginine within cell proteins. Thiol groups on cysteine side chains in proteins as well as the thiol group possessed by the tripeptide glutathione are 'soft' nucleophiles.

Knowing the electronic properties of a reactive metabolite is helpful since according to the HSAB theory of toxicity, soft electrophiles react with soft nucleophiles, while hard electrophiles react with hard nucleophiles. This insight helps explain why different metabolites show preference for proteins or DNA: as a general rule, soft electrophiles (e.g. N-acetyl-p-quinoneimine metabolite formed from paracetamol) mainly damage proteins and are thus typically associated with organ damage (e.g. hepatotoxicity, nephrotoxicity), while hard electrophiles (e.g. diol epoxides formed from polycyclic aromatic hydrocarbons – see Chap. 10) tend to damage DNA and cause cancer.

4.4 How Reactive Metabolites Harm Cells

The discovery that reactive intermediates form during the metabolism of a drug or foreign chemical opens the door to many experiments. The list of questions raised by such findings is lengthy. For example, we should determine whether the reactive metabolites are 'trapped' by protective cell molecules such as glutathione. The preferred cellular targets also await identification: do the metabolites damage cell

proteins, and if so, which ones are most vulnerable? Does such damage compromise key protein functions or trigger cell death? Whether the metabolites attack DNA is also critical; does any such damage induce genetic mutations during DNA replication? Any outcomes of this kind are taken seriously since such DNA damage might induce cancer in humans. The discovery of bioactivation potential for a candidate drug or xenobiotic also raises questions concerning the implications for exposure during pregnancy; is reactive metabolite formation in the embryo, foetus or maternal placenta likely to harm prenatal development?

A final question concerns how reactions between metabolites and their target(s) trigger cell death in exposed tissues, an issue that fascinates many researchers. In the remainder of this chapter, we will focus on some major theories that address this question. As we briefly explore each mechanism, bear in mind that they rarely occur in isolation. For many chemicals that undergo bioactivation, it is likely several of the following mechanisms contribute to cell injury. Indeed, for some chemicals it is possible all of the highlighted mechanisms participate in their toxic effects on living tissues.

4.4.1 The 'Covalent Binding' Hypothesis

Since it involves permanent sharing of electrons, covalent bonds are the most stable bonds that form during reactions between two molecules. The formation of new covalent bonds during reactions of reactive metabolites with DNA or protein (called 'covalent binding' by toxicologists) occurs because electrophilic metabolites are attacked by electron-rich nucleophiles in cell macromolecules. Early pioneers such as the Millers in the USA and McGee in the UK were quick to realise that the intracellular environment is loaded with nucleophilic molecules. The reaction of an electrophilic metabolite with a cellular macromolecule forms a stable covalent bond, with the resulting altered DNA base termed a 'DNA adduct' (a modified amino acid in a protein target is a 'protein adduct').

When James and Elizabeth Miller first hypothesised that the carcinogenicity of many chemicals involves reactive metabolites that attack cell macromolecules, the research technology available for detecting adducts was highly rudimentary. In the early years, the Millers studied highly chromogenic carcinogens that were detectable in animal tissues using a simple spectrophotometer (e.g. DAB (N,N-dimethyl-4-aminoazobenzene) an 'azo dye' liver carcinogen which forms highly coloured metabolites). This simple approach exhibited poor sensitivity since it could barely detect the low level of DNA adducts formed in animals following treatment with carcinogens. Another problem was that due to the chemistry of DNA damage, DNA adducts formed by azo dyes do not always retain their coloured properties.

A key advance occurred in the late 1940s when researchers at the Division of Medical Physics at the University of California Berkley devised a way to make 'radioactive' versions of carcinogenic molecules. By incorporating a radioactive atom ('radiolabel'), a xenobiotic can be tracked in tissues or biofluids collected

from lab animals or human subjects. For example, protein or DNA can be extracted from body tissues and the amount of radioactivity 'covalently' incorporated into the macromolecules could be measured with a radioactivity counter. In the early years, the β-particle emitters tritium and carbon-14 were commonly used radiolabels. Using chemical carcinogens that contained these radiolabels, researchers confirmed that an irreversible introduction of radioactivity into DNA and protein accompanied carcinogen exposure in rats or mice.

This approach also facilitated study of other toxic responses. From the 1960s to 1980s when such approaches were widely used, researchers proved the formation of electrophilic metabolites from numerous radiolabelled toxicants, including the liver-selective agent carbon tetrachloride, the lung-selective toxicant 4-ipomeanol and the kidney-selective toxicant bromobenzene. The relationship of bioactivation to toxicity was inferred on finding that more radiolabelled toxicant was incorporated into proteins or DNA in metabolically competent 'target organs' relative to noninvolved tissues. For example, much higher protein-associated radioactivity was detected within the livers of ^{14}C-labelled carbon tetrachloride-treated rats than in lung, heart or brain tissue – organs that showed little or no toxicity to this toxicant. These findings confirmed that tissues with the greatest capacity to generate reactive metabolites are usually most vulnerable to injury (Scenario 3 in Fig. 4.8).

Associations of protein adduction with chemical toxicity were strengthened by the finding that treating animals with chemicals that abolished hepatic CYP (e.g. cobalt chloride) protected them against carbon tetrachloride toxicity. Much less radioactivity was incorporated into liver proteins in cobalt chloride-pretreated animals while, on the other hand, treating animals with phenobarbital – which increases CYP expression in the liver – had the opposite effect on carbon tetrachloride hepatotoxicity: liver injury and protein-associated radioactivity both increased.

Such findings, made especially by researchers such as Gillette, Brodie, Jollow and Mitchell at the National Heart and Lung Institute in Maryland, provided strong support for the 'covalent binding' hypothesis. The theory essentially proposed that the ability of chemicals to induce organ-selective toxicity involved the formation of electron-deficient metabolites that attacked proteins or DNA in tissues to form covalently modified macromolecules (adducts). Tissues in which little covalent binding occurred would not display toxicity. Many impressive papers coming from the Maryland researchers and their collaborators in the 1970s applied this hypothesis to diverse toxicological end points including cancer, organ necrosis, hemolytic anaemia, hypersensitivity reactions and even prenatal toxicity.

With time, new research discoveries necessitated refinement of the 'covalent binding hypothesis'. For example, the 1980s saw advances in the development of cell culture technology, allowing researchers to isolate liver cells from human or lab animal tissues and maintain them in an incubator. This assisted study of the mechanisms underlying toxicant-induced hepatotoxicity, with some findings in hepatocytes suggesting the need for a more nuanced understanding of the 'covalent binding' hypothesis. For example, high levels of covalent binding could be achieved by incubating cells briefly with toxicants such as 4-ipomeanol or bromobenzene. Then, before cell death ensued, toxicant-containing culture media could be removed

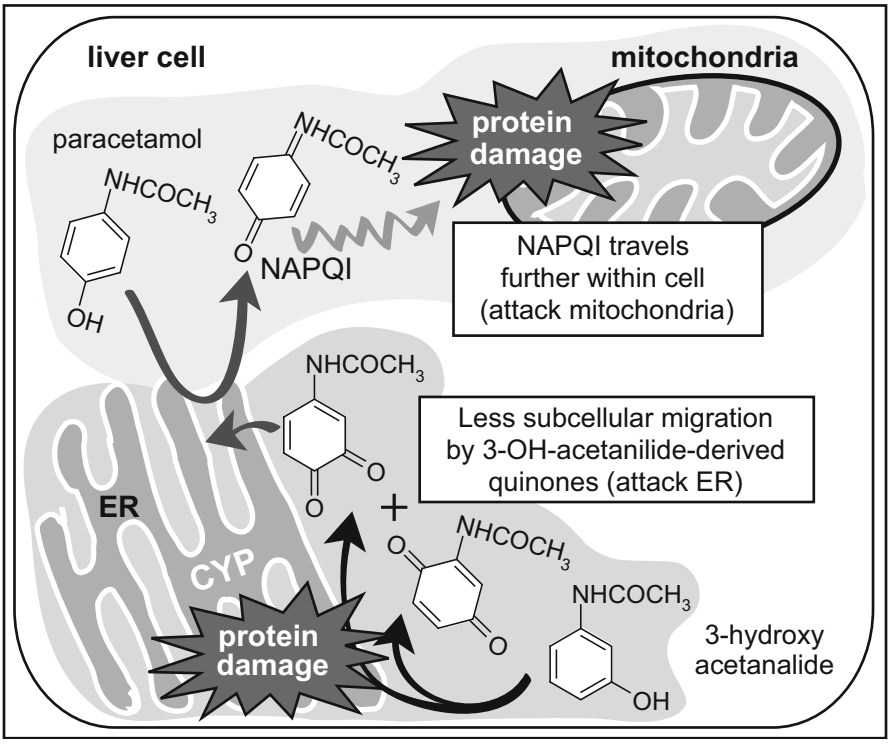

Fig. 4.4 Reactive metabolites formed from paracetamol and its nontoxic analogue 3-hydroxyacetanilide both attack liver cell proteins, yet only the reactive metabolite formed from paracetamol, NAPQI, is sufficiently long lived to migrate through the cell environment to attack key mitochondrial proteins

before treating cells with various protective drugs or antioxidants that 'rescued' cells from cell death. Despite harbouring a high level of protein adducts, cell death did not occur.

Other researchers established that it was possible to make close chemical analogues of toxicants that underwent covalent binding yet did not necessarily cause cell death. This was shown for 3-hydroxyacetanalide, a close chemical relative of paracetamol that possesses a similar molecular structure (Fig. 4.4). Paracetamol is safe at low doses yet induces severe liver damage in overdose victims (see Chap. 6 (Sect. 6.4.1.1)). Although 3-hydroxyacetanalide also undergoes extensive CYP-mediated covalent binding in the liver, it does not cause overt liver damage (Fig. 4.4). Such observations reinforced the view that the biological events underlying chemically induced toxicity are complex and that the sum concentrations of covalently bound adducts in macromolecules is not the sole determinant of toxicity per se.

Some researchers realised that the 'covalent binding theory' could be improved if the identity of individual protein targets for electrophiles was known, concluding that metabolites that attack nonessential proteins will be less toxic than those that

damage highly important targets. The development of new technologies helped researchers test this new version of the 'covalent binding theory'. By the late 1980s, several laboratories including those led by Stanley Cohen in Connecticut and Jack Hinson and Lance Pohl in Arkansas were using antibody-based methods to detect adducted proteins in the livers of toxicant-treated animals. These important studies confirmed that reactive metabolites typically damage only a small subset of proteins within the cell and that toxicity might depend on damage to relatively few crucial proteins.

The arrival of new types of mass spectrometers in the 1990s gave researchers unprecedented power to study protein damage caused by reactive metabolites. Mass spectrometry typically works by bombarding proteins with high-energy particles, assessing the mass and charge of resulting peptide fragments and working backwards to deduce the structure and identity of the original protein. These approaches were of limited value until 'soft ionisation' methods for generating protein ions such as electrospray ionisation and matrix-assisted laser desorption became available. These new tools also allowed mapping of sites within the protein that contain chemically modified amino acids (i.e. adducts), showing that electrophiles often displayed reactivity with particular amino acids within particular secondary features of proteins.

The power of mass spectrometry to resolve mysteries in toxicology was demonstrated by Al Burlingame and associates at the University of California San Francisco in the late 1990s. They compared patterns of covalent binding to proteins in the livers of mice treated with high doses of ^{14}C-labelled versions of paracetamol or its nontoxic relative 3-hydroxyacetanilide (Fig. 4.4). As expected, 3-hydroxyacetanilide generated comparable levels of 'total covalent binding' to paracetamol, but the identities of its target proteins were quite different. Intriguingly, metabolites from the nontoxic analogue not only damaged fewer proteins, they tended to target proteins close to their site of formation in the CYP-rich endoplasmic reticulum. Paracetamol on the other hand tended to damage proteins in mitochondria, the all-important 'cellular powerhouse'. Since mitochondria are major regulators of a form of cell death known as apoptosis (see below), these findings provided a key mechanistic rationale for the pronounced hepatotoxicity of the drug. Thus, although both paracetamol and 3-hydroxyacetanilide underwent bioactivation to reactive metabolites that produced comparable levels of 'total covalent protein binding', closer examination via mass spectrometry-based approaches revealed key differences between these toxicants.

For many reasons, mass spectrometry soon proved the most important technological development in the history of toxicology. Within the context of mechanistic studies, these technologies allowed identification of target proteins that sustain damage by reactive metabolites in a large number of toxic syndromes. The Target Protein Database maintained by the Hanzlik lab at the University of Kansas is a useful repository of information concerning protein targets for reactive metabolites that currently includes over 400 protein targets for some 48 toxicants (available at http://tpdb.medchem.ku.edu:8080/protein_database/index.jsp). The database includes information gained during in vivo studies conducted in four species as well as cell culture-based observations. Despite these advances, however, the biological

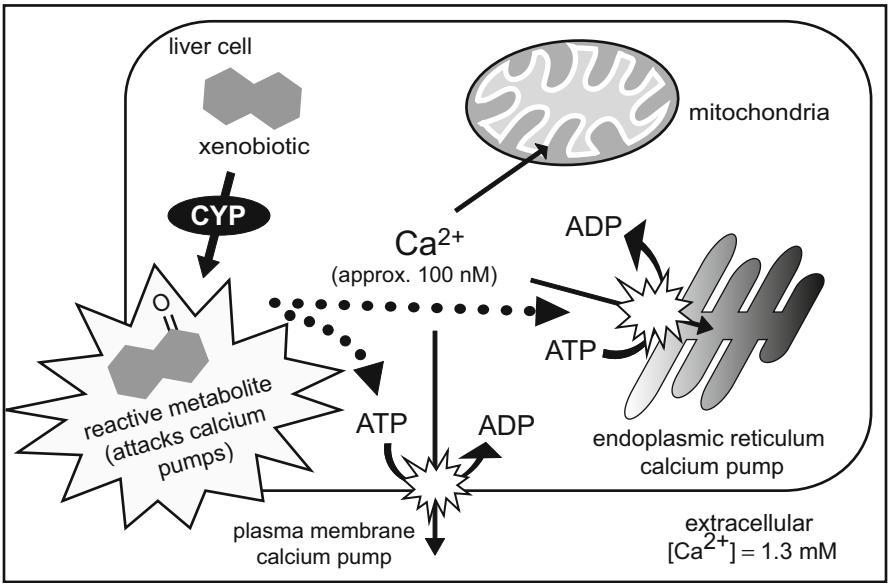

Fig. 4.5 ATP-utilising pumps in the plasma membrane, ER and other intracellular compartments keep 'free' calcium levels low in normal cells. According to the 'calcium theory', reactive metabolites attack these pumps, impairing their function and leading to unregulated calcium levels. The resulting activation of calcium-dependent proteases and nucleases digests cells from within

mechanisms linking chemical damage within individual protein targets to the loss of cell viability are often unclear. This area remains an active field of research as investigators seek to fill in the gaps linking protein adduction by a particular xenobiotic with subsequent toxicological outcomes.

4.4.2 Loss of Calcium Control

Other researchers drew on advances in calcium biochemistry during their search for the fundamental cell processes that are perturbed by reactive metabolites. During the twentieth century, biochemists and physiologists uncovered a key role for calcium ions (Ca^{2+}) in controlling many aspects of cell function. Due to its diverse regulatory roles, intracellular levels of 'free' Ca^{2+} are kept very low relative to its much higher concentrations in extracellular fluids (Fig. 4.5). Yet cells must also increase Ca^{2+} concentrations transiently to allow activation of needed biochemical functions, while also being able to return levels to baseline once the need subsides. Cells use a complicated range of ion pumps, calcium-binding proteins and ion channels to control the movement of Ca^{2+} across the cell membrane and between intracellular compartments including mitochondria, the nucleus and endoplasmic reticulum (Fig. 4.5).

The toxicological relevance of calcium emerged from the discovery that an uncontrolled increase in intracellular free Ca^{2+} can have broad harmful effects on many key cell functions. First, unregulated Ca^{2+} can activate degradative enzymes that digest proteins, nucleic acids and membrane lipids (e.g. calpains, nucleases and lipases, respectively), leading to broad perturbation of their normal functions. Increased Ca^{2+} levels can also activate a controlled form of cell death known as *apoptosis* (*see below*). Finally, increased intracellular Ca^{2+} may alter the activity of nuclear transcription factors, dysregulating the expression of genes involved in many biochemical processes including cell growth and cell death signalling.

During the 1980s, research led by Sten Orrenius and Peter Moldeus at the Karolinska Institute in Stockholm formed the basis for the 'calcium dyshomeostasis theory' of cell death. These labs established that reactive metabolites formed during toxicant metabolism could attack critical proteins or pumps that maintain low cytosolic Ca^{2+} concentrations (Fig. 4.5), precipitating a lethal activation of degradative enzymes and gene dysregulation. In a sequence of elegant publications, Orrenius and associates showed that the toxicity of paracetamol and various electrophilic quinones involved damage to 'calcium pumps' in the plasma membrane or endoplasmic reticulum. Subsequent work then confirmed that other toxicants disrupt intracellular calcium control indirectly by targeting receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs), to promote high cellular levels of inositol 3-phosphate (IP), a potent inducer of Ca^{2+} release from the endoplasmic reticulum. This mechanism could thus explain the toxicity of chemicals that do not undergo conversion to reactive intermediates yet nevertheless promote a loss of intracellular Ca^{2+} homeostasis.

The 'calcium theory' has been applied with varying degrees of success to many chemically induced toxic phenomena within the liver and kidney but is of greatest relevance to disease settings within the CNS. Calcium regulates many neuronal functions, and a loss of Ca^{2+} homeostasis likely mediates neuronal wasting in brain disorders as diverse as epilepsy, Alzheimer's disease and Parkinson's disease. Unregulated neuronal Ca^{2+} levels are especially relevant during 'glutamate excitotoxicity' which occurs in the brains of epileptics and other patients due to excessive release of the excitatory neurotransmitter glutamate. Some neurotoxic chemicals also induce glutamate-mediated brain injury, ensuring toxicology researchers will continue to study the role of calcium in neuronal death in a range of chemically induced disease settings.

4.4.3 Free Radical Production

The survival of aerobic organisms requires O_2 availability to support mitochondrial respiration, a process involving stepwise addition of four electrons to O_2 to form H_2O. The possibility that this process is not fully efficient and that a small percentage of O_2 might 'leak' from mitochondria as partially reduced species was confirmed in the late 1960s by the discovery of superoxide dismutase by McCord and Fridovich

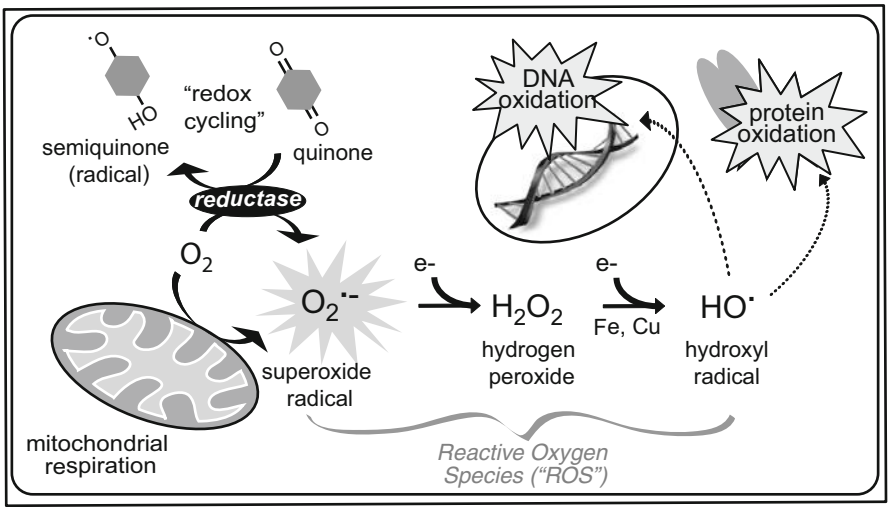

Fig. 4.6 Reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals form via normal metabolic processes (e.g. mitochondrial respiration) or upon exposure to certain chemicals (e.g. redox-cycling quinones or redox-active transition metals such as copper [Cu] and iron [Fe]). The resulting macromolecular oxidation contributes to the onset of cell death

at Duke University. The fact that aerobic cells produce enzymes to detoxicate superoxide – the product of the one-electron reduction of O_2 – provided confirmation that this noxious species forms during routine cell metabolism. Researchers soon established that cells possess multiple forms of SOD, including a cytosolic iron – and zinc-containing form as well as a mitochondrial manganese-containing form.

Superoxide is not the only reactive species formed during the incomplete reduction of O_2 (Fig. 4.6). Adding a second electron produces O_2^{2-}, which in biological systems exists in its protonated form as hydrogen peroxide [H_2O_2]. Adding a third electron generates one of the most reactive species known to biological chemistry, the hydroxyl radical (HO•), while a fourth reduction step generates water (H_2O). Collectively, the various harmful products of oxygen metabolism are termed reactive oxygen species (ROS), and their overproduction within biological systems is termed 'oxidative stress', among the most widely researched phenomena in modern biomedical science.

The fact that SOD converts two molecules of superoxide to H_2O_2 initially seems puzzling: since H_2O_2 is an oxidant, why should cells convert one noxious species into another? The discovery of a second class of enzymes that rapidly detoxify H_2O_2 resolved this question, showing that antioxidant defences against ROS involve the sequential activity of distinct enzymes that convert superoxide to H_2O via the intermediate H_2O_2. Subsequent research uncovered many enzymes in aerobic cells that rapidly detoxify H_2O_2, including catalases, glutathione peroxidases and peroxiredoxins.

Cells take such precautions to detoxicate superoxide and H_2O_2 because these species are precursors to the hydroxyl radicals that are highly damaging to cells. In

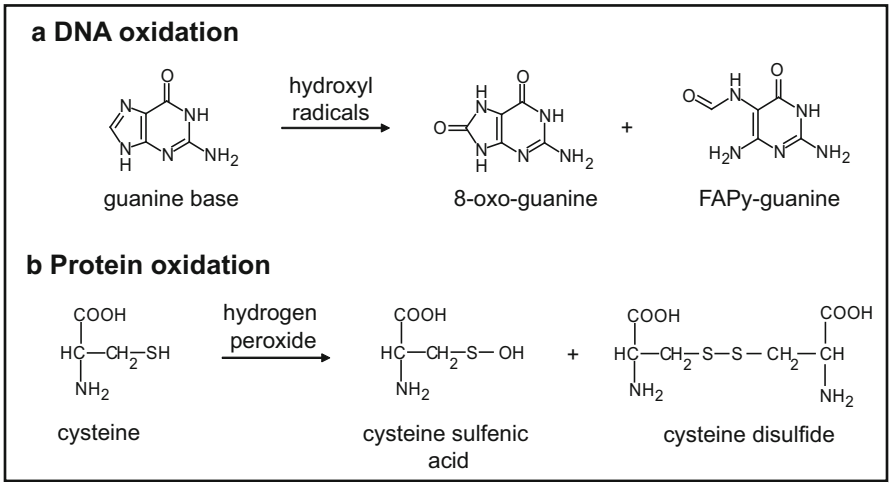

Fig. 4.7 Reactive oxygen species produce diverse damage products upon attacking DNA (*Panel a*) or proteins (*Panel b*). Some prevalent products are shown, but dozens of other oxidation products are omitted. For simplicity, the structures are shown as free bases or amino acids

the classic Fenton reaction, the presence of iron and copper ions ensure H_2O_2 rapidly fragments to form hydroxyl radicals. Since these metal ions are commonly associated with the sugar–phosphate backbone of DNA and the active site of proteins, hydroxyl radicals can form if H_2O_2 diffuses within reach of metal-containing macromolecules. Hydroxyl radicals that form via these routes rapidly attack oxidation-sensitive, nucleophilic amino acids in proteins as well as nitrogen bases in DNA. Exhibiting a half-life of barely a few nanoseconds, hydroxyl radicals are very difficult to trap; hence, the best way for cells to suppress hydroxyl radical-induced oxidative DNA or protein damage is to remove their precursor H_2O_2 via catalase and other protective enzymes.

The chemical damage accompanying exposure of DNA to ROS closely resembles that produced by ionising radiation such as X-rays or γ-rays. In the 1960s and 1970s, radiation chemists discovered dozens of chemical modifications to DNA bases that form upon irradiation of cellular DNA. When biochemists later began studying ROS-induced DNA damage in human tissues, they were surprised to discover oxidised DNA bases that were already known to radiation chemists. This suggested hydroxyl radicals formed via water radiolysis within irradiated tissues mediate the genetic damage caused by ionising radiation.

Among the DNA bases, guanine is most vulnerable to oxidative damage, undergoing conversion to 8-oxoguanine as well as a ring-opened species, FAPY-guanine (Fig. 4.7). These species form spontaneously in human cells due to ongoing ROS production by the ‘leaky’ mitochondrial electron transport chain and other redox processes. Several thousand 8-oxoguanine and FAPY-guanine adducts likely form in a typical human cell every day, indicating high-level ‘background’ genetic

damage occurs in the absence of heightened exposure to radiation or prooxidant xenobiotics. While such damage is repairable by cells, mutations can occur if the damaged DNA is replicated prior to repair, a factor that contributes strongly to spontaneous mutation rates in aerobic organisms.

Many amino acids in cell proteins also sustain damage by hydroxyl radicals. The sulfur-containing amino acids methionine and cysteine are especially vulnerable, with cysteine undergoing conversion to cysteine sulfenic acid and cysteine disulfide (Fig. 4.7). Other amino acids including tryptophan, phenylalanine, tyrosine and histidine also sustain damage by ROS, forming products that serve as useful 'markers' of oxidative protein damage. Measurement of such species in proteins from healthy tissues has shown that, as with DNA oxidation, a high level of oxidative protein damage accompanies normal aerobic metabolism. Some estimates suggest 10 % of proteins in youthful human cells contain an oxidised amino acid, increasing to 20–30 % in elderly individuals. Although exceptions exist, cells generally do not 'repair' oxidised proteins. Instead, they prefer to 'trash' damaged proteins by tagging them for degradation by cellular sanitation systems such as the proteasomal complex.

The fact that oxidative stress occurs in normal tissues is relevant to many diseases, especially those that afflict ageing individuals such as atherosclerosis, Alzheimer's neurodegeneration, diabetes and cancer. An accrual of oxidatively damaged macromolecules is evident in aged skin and the lens of the eye, suggesting oxidative damage contributes to the functional deterioration of these structures during the ageing process. In addition, modern toxicology devotes considerable attention to free radical production by toxic xenobiotics, and a large body of data confirms that many toxic chemicals induce damage of this kind.

The mechanisms underlying induction of oxidative stress during chemical toxicity vary from one compound to another. Some chemicals – and this includes redox-active metals such as iron or copper – simply catalyse the fragmentation of H_2O_2 to release hydroxyl radicals (Fig. 4.6). Since metals are common contaminants of many materials, such Fenton reactions contribute to diverse pathological conditions. Asbestos fibres, for example, the cause of life-threatening lung diseases such as asbestosis and mesothelioma (Chap. 8, Sect. 8.7.4), contain high levels of iron, accounting in part for the chronic oxidative damage that accompanies their deposition in lung tissue. Iron also contaminates the microscopic airborne particles that comprise smog and air pollution, ensuring the lungs of people in high-density urban areas experience chronic oxidative stress.

Other molecules generate radicals via a chemical process known as 'redox-cycling' (Fig. 4.6). Quinones are prone to such reactions since they undergo one-electron reduction to semiquinones by ubiquitous enzymes known as reductases. Semiquinones readily donate their spare electron to O_2 to form superoxide radicals that inflict cell damage after conversion to H_2O_2 or hydroxyl radicals. Such chemistry is exploited when treating cancer patients since important chemotherapy drugs such as adriamycin undergo redox-cycling in solid tumours to produce ROS that help kill tumour cells.

Tobacco smoke is a major source of avoidable exposure to redox-cycling quinones. Containing a toxic quinone–semiquinone–hydroquinone complex, the tar

deposit that accumulates in the lungs of smokers is an efficient source of superoxide and hydroxyl radicals. Autopsied lung tissue from smokers contains elevated levels of 8-oxoguanine and other DNA oxidation products, a factor that contributes to their increased risk of emphysema, lung cancer and other respiratory conditions. The poor diet consumed by many smokers – one that is comparatively deficient in antioxidant-rich vegetables and fruits – likely exacerbates their susceptibility to oxidative damage. The molecular toxicology of tobacco receives attention in Chap. 10.

The 'oxidative stress theory' of cell injury makes clear sense if plausible chemical mechanisms can be envisaged to explain an overproduction of ROS by a given toxicant (e.g. the toxicant of interest undergoes redox-cycling). Yet the theory may also apply to chemicals that lack such obvious properties since recent research has confirmed that oxidative stress can feature prominently in the toxicity of chemicals that undergo conversion to electrophilic protein-damaging metabolites. The accumulation of adducted proteins can stimulate the immune system by activating immune cells such as macrophages and lymphocytes as well as promoting the recruitment of neutrophils to sites of tissue damage. The cell membrane of many immune cells contains a protein complex known as NADPH oxidase that on activation produces superoxide radicals in a phenomenon termed the 'respiratory burst'. This response normally protects tissues against bacteria and other infectious agents, yet following its activation subsequent to the formation of reactive metabolites and protein adducts, an inflammatory response amplifies tissue injury by flooding cells with ROS and other noxious mediators. In addition to superoxide and H_2O_2, the barrage of noxious oxidants produced by neutrophils includes hypochlorous acid (HOCl), the versatile antiseptic constituent of common household bleach. HOCl forms via the metabolism of H_2O_2 by a green-tinged, copper-containing enzyme known as myeloperoxidase (the presence of myeloperoxidase-containing neutrophils in mucous and pus gives these body secretions their distinctive coloration).

Another damaging species released by activated immune cells is peroxynitrite ($ONOO^-$), a strong oxidising agent which forms during a rapid reaction between superoxide radicals and nitric oxide (NO). Peroxynitrite oxidises critical cysteine residues that are needed for normal protein function. Such damage torments sufferers of diseases that involve chronic inflammation in knee or hip joints (e.g. rheumatoid arthritis), but similar reactions likely occur in target tissues following intoxication with chemicals that form reactive metabolites and ROS.

4.4.4 Lipid Peroxidation

The most vulnerable cellular targets for ROS are a subset of oxidation-prone fatty acids in lipid membranes. The cell membrane is a complex entity, with about 50 % of its mass due to lipids, while the remainder is mostly protein. Most membrane lipids exist as phospholipids that comprise a hydrophilic head to which are attached two hydrophobic lipid tails. Within aqueous environments, phospholipids spontaneously form bilayers in which the hydrophilic heads aggregate into sandwich-like

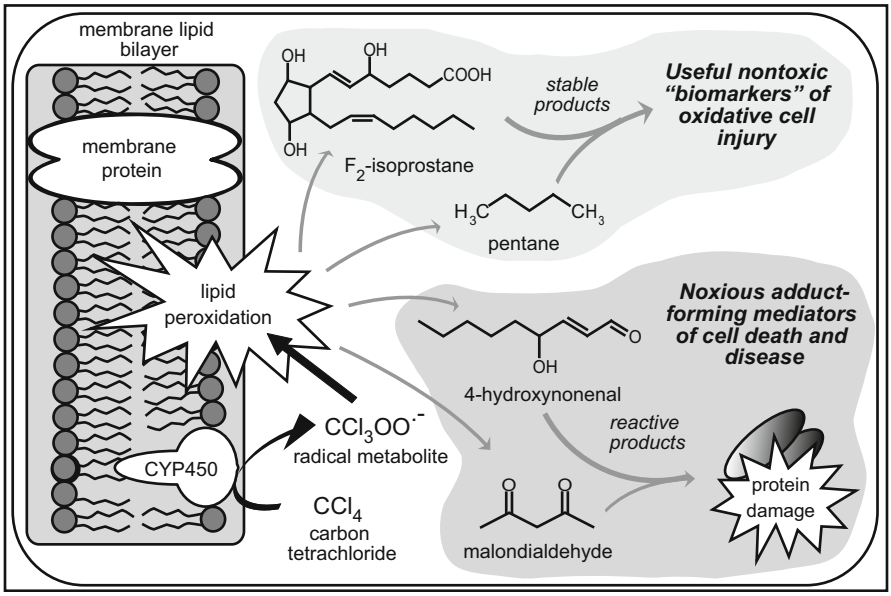

Fig. 4.8 Polyunsaturated lipids in membrane bilayers are vulnerable to damaging radicals formed during the oxidation of carbon tetrachloride by CYP450. The resulting lipid peroxidation cascade produces many products, some of which are reactive and mediate additional tissue injury. Other stable products such as pentane and isoprostanes are also useful biomarkers of oxidative stress

planar structures in which the hydrophobic tail is buried internally (Fig. 4.8). This ability to form fluid bilayers confers flexibility on many cellular structures.

Most phospholipids contain two hydrophobic fatty acid tails comprising between 14 and 24 carbon atoms. One fatty acid is usually saturated (i.e. it contains no double bonds, such as the 18-carbon fatty acid stearic acid). The other is typically unsaturated, containing one or more double bonds (e.g. a *monounsaturated* fatty acid such as oleic acid [18 carbon atoms and 1 double bond] or a polyunsaturated fatty acid such as arachidonic acid [20 carbons and 4 double bonds]). The unsaturated fatty acids confer fluidity upon cell membranes, since lipids become increasingly rigid in inverse proportion to their number of double bonds.

The membrane fluidity conferred by unsaturated lipids comes at a cost, since their multiple double bonds makes polyunsaturated lipids vulnerable to radicals. Richard Recknagel in Cleveland and Trevor Slater in Middlesex first grasped the toxicological importance of the oxidative vulnerability of unsaturated lipids. These researchers used gas chromatography to quantify individual lipids in animal tissues before and after intoxication with prooxidant xenobiotics. Strikingly, although the levels of saturated fatty acids such as stearic acid were similar in both groups, levels of polyunsaturated lipids such as arachidonic acid were much lower in target organs of intoxicated animals. These researchers had rediscovered a phenomenon known to chemists in the rubber industry – *lipid peroxidation (LPO),* a ubiquitous degradative

process causes the deterioration of rubber tyres on cars and bicycles. LPO is also important to the food industry since it causes rancidity in edible fats and oils.

LPO often accompanies oxidative stress and is highly damaging to cell membranes due to its *autocatalytic* or self-propagating nature. After LPO is initiated by reactions of a free radical with an unsaturated lipid, new radicals form which amplify membrane damage by attacking bystander lipids. This explains the dramatic loss of arachidonic acid in the livers of carbon tetrachloride-poisoned rodents – the toxicant is oxidised by CYP to free radical metabolites that trigger LPO by attacking oxidation-sensitive arachidonic acid 'tails' in neighbouring phospholipids (Fig. 4.8). As the chain reactions gain momentum, free radicals fragment the long phospholipid tails into dozens if not hundreds of products. Some of these lack significant toxic properties, including volatile organic molecules such as alkanes, ketones and alkanals. A traditional method for monitoring 'whole body' LPO involves quantifying two volatile alkanes – ethane and pentane – in exhaled human breath (Fig. 4.8).

Another important class of relatively stable LPO products are the isoprostanes. Chemically, these species resemble the prostaglandins, a class of endogenous molecules that exert powerful physiological actions. Prostaglandins form via oxidation of arachidonic acid by the cyclooxygenases (COX-1 and COX-2). COX-2 is strongly upregulated during inflammation, whereas COX-1 is expressed constitutively. Prostaglandin overproduction causes many unpleasant symptoms in inflamed tissue, including pain, swelling and heat. In contrast to these enzymatic products of arachidonic acid oxidation, *isoprostanes* form via random, nonenzymatic attack on membrane lipids by free radicals. Oxidised lipids are released from membranes via the hydrolytic action of phospholipases. Intriguingly, the levels of isoprostanes in circulating human blood are often much higher than the concentrations of prostaglandins, suggesting ROS and other endogenous oxidants take an ongoing toll on cell membranes (analogous to the surprisingly high 'baseline' levels of spontaneous DNA and protein oxidation). Since they can be accurately measured using mass spectrometry-based methods, isoprostanes are arguably the best LPO biomarkers available to modern researchers. They are often measured during studies of the effect of smoking, antioxidant therapy, or fruits or vegetable consumption on 'baseline' levels of oxidative tissue injury.

Toxicological interest in LPO also focusses on the production of chemically reactive species during membrane oxidation. Such reactive products include free radicals (e.g. lipid peroxyl radicals) and nonradical electrophiles. Hermann Esterbauer at the University of Graz was among the first researchers to realise that various unsaturated aldehydes are highly important mediators of cell damage during LPO. This group showed that one product of LPO, the 9-carbon aldehyde 4-hydroxynonenal, is a key 'toxicity mediator' due to its strong reactivity with cell macromolecules and ability to cause cell death at low concentrations. As an α,β-unsaturated aldehyde, 4-hydroxynonenal possesses two electrophilic centres in close proximity (i.e. a carbonyl group immediately adjacent to a double bond). This structural feature heightens the electrophilicity of the molecule, facilitating reactivity with nucleophilic targets. Following formation in oxidised membranes, 4-hydroxynonenal can diffuse throughout the cell to damage enzymes in other cell

compartments (e.g. mitochondria) or DNA within the cell nucleus. Nevertheless, cells possess multiple defences against this noxious electrophile since several enzyme pathways rapidly convert 4-hydroxynonenal to inert metabolites that are safely excreted from cells (e.g. glutathione conjugation, oxidation to carboxylic acid). The fact that protein and DNA adducts formed by 4-hydroxynonenal are often detected in normal cells indicates, these protective metabolic pathways are not fully efficient.

Many other α,β-unsaturated aldehydes also form during LPO, including numerous short-chain molecules that comprise just a few carbon atoms such as crotonaldehyde, acrolein and malondialdehyde. As with 4-hydroxynonenal, these electrophiles attack cell macromolecules to trigger diverse deleterious outcomes. Together with 4-hydroxynonenal, these molecules receive much research attention due to their role in many degenerative diseases.

4.4.5 Programmed Cell Death (Apoptosis)

In the early 1960s, discovery of a novel, controlled form of cell death by a young Australian pathologist provided a boost to many biomedical fields including toxicology. John Kerr was studying changes that occur in animal livers upon interruption of blood flow and astutely described two distinct types of cell death. *Necrosis* was already familiar to pathologists since it involved the death of many cells within significant areas of liver tissue. An infiltration of immune cells was conspicuous during necrosis, leading to long-term tissue scarring.

A second form of death occurred in isolated cells scattered throughout the circulation-starved liver lobe. Whereas cells undergoing necrosis tended to swell, Kerr noted that these dying cells usually became smaller. Small blobs of membrane-bound cytoplasm (‘apoptotic bodies’) containing DNA and intact mitochondria were also noted. Unlike necrosis, little or no inflammation occurred since macrophages quickly cleared tissues of the apoptotic bodies. While nineteenth-century pathologists had noted this second type of cell death, Kerr described it in greater scientific detail. He also identified heliotrine, a plant-derived toxin that causes hepatotoxicity in sheep, as a novel inducer of this controlled form of cell death. In 1972, Kerr applied a name to this phenomenon that captured the attention of many researchers: *apoptosis*. This form of cell death was soon found to participate in diverse phenomena including embryonic development, heart disease, AIDS-related tissue wasting and cancer chemotherapy. Research interest accelerated after geneticists began uncovering the molecular events that mediate apoptotic cell death: unlike the seemingly uncontrolled nature of necrosis, apoptosis involved defined steps that led to ‘cellular suicide’.

An early finding involved the discovery that specific nucleases are activated during apoptosis. These ‘genetic scissors’ mediate controlled DNA digestion, snipping the genetic material at regular intervals to form fragments of consistent size. Analogous to how cotton thread is wound around a spool, nuclear DNA is tightly

packaged into nucleosomal structures. Using a blade to cut along one side of a spool would release cotton 'fragments' of regular length dictated by the spool diameter. Similarly, internucleosomal digestion of DNA via nuclease activation during apoptosis generates predictably sized fragments that can be detected by analysing DNA via gel electrophoresis. In this simple technology, DNA fragments are separated by passing a current through a horizontal agarose gel containing DNA loaded into small wells. The negatively charged DNA migrates to the positive electrode, with small fragments moving through the gel more quickly than large fragments. In DNA from cells undergoing apoptosis, a distinct 'laddering' of DNA fragments due to defined nuclease cleavage is seen, while in necrotic cells the distribution of DNA is 'smeared' due to random, uncontrolled DNA digestion. The 'DNA laddering' assay approach is widely used to detect apoptotic DNA fragmentation in cells and tissues.

A breakthrough in understanding of apoptosis occurred with the identification of *Bcl*-2, a protein that was initially identified in human tumour cells (B-cell lymphoma, hence Bcl-2). In 1992, *Bcl*-2 was shown to inhibit apoptosis in *C. elegans* worms, a discovery that made this humble worm a popular tool in cell death research. Subsequent research uncovered dozens of Bcl-2 family members, some of which like Bcl-2 itself block apoptosis, while others such as Bax and Bak are strongly proapoptotic. These proteins exert complex effects on cell death but mainly control the permeability of mitochondrial membranes, with their role in the *mitochondrial permeability transition* (MPT) of vital importance to the induction of apoptosis. Cell survival depends on the balance of proapoptotic versus anti-apoptotic Bcl-2 family proteins within the immediate vicinity of mitochondrial membranes: under normal conditions the anti-apoptotic proteins Bcl-2 and Bcl-X normally keep cytochrome c safely incarcerated within mitochondria. During the induction of cell death, proapoptotic proteins such as Bax and Bak congregate at the outer mitochondrial membrane, overwhelming Bcl-2 and Bcl-X and triggering the formation of channels in mitochondrial membranes that release pro-death proteins such as cytochrome c (Fig. 4.9).

Known to biochemists since the 1920s, cytochrome c is an electron carrier that is essential for mitochondrial respiration. However, these discoveries uncovered a dark side to cytochrome c – during apoptosis it surges out of mitochondria into cytosol where it associates with several proteins to initiate a sequence of events that mediate cell death. In particular, cytochrome c facilitates the self-assembly of the apoptosome, the infamous *wheel of death* which comprises seven 'spokes' containing the protein Apaf-1 (apoptotic protease activating factor-1) with cytochrome c molecules assembled to the tips of each spoke (Fig. 4.9). This complex attracts procaspase 9 to the hub of the wheel that is promptly cleaved to form caspase 9, a key 'initiator caspase' during apoptosis.

Caspases are a unique family of proapoptotic proteases that mediate the cellular disaggregation that accompanies apoptosis. In the case of caspase 9, the targets are other procaspases that upon proteolytic activation release 'executioner' proteases such as caspase 3, 6 and 7. These proteases initiate the final phase of apoptosis, digesting key cell components such as the cytoskeletal proteins vimentin and actin to trigger morphological changes that are typical of apoptosis.

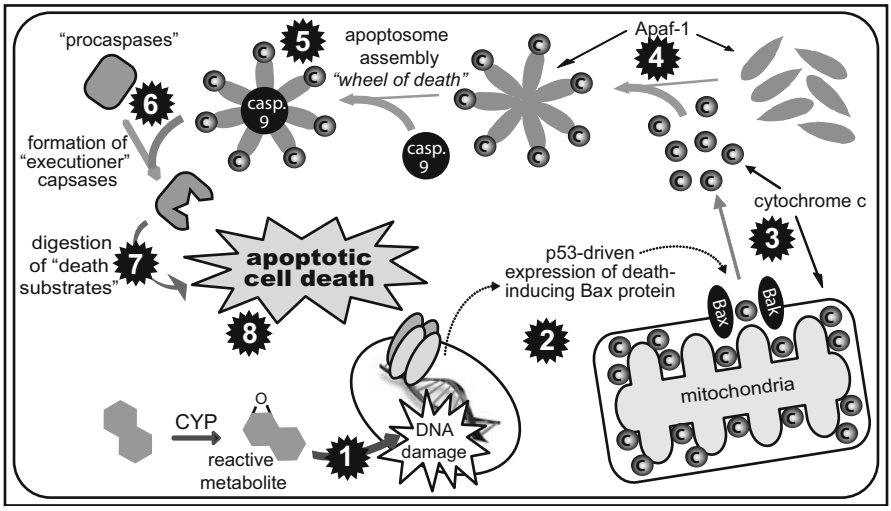

Fig. 4.9 A simplified overview of apoptosis (intrinsic pathway only). Induction of DNA damage by free radicals and reactive metabolites (Step *1*) triggers p53 activation leading to increased expression of the pro-death Bcl-2 family member Bax (Step *2*). In concert with Bak, Bax facilitates the permeabilisation of mitochondrial membranes, releasing cytochrome c into cytosol (Step *3*). Cytochrome c spontaneously associates with Apaf-1 (Step *4*) and caspase 9 to form the apoptosome (*'wheel of death'*) (Step *5*) which then activates the 'executioner' caspases 3, 6 and 7 (Step *6*). The digestion of target proteins as well as the cell cytoskeleton by these caspases produces changes in cell shape and function (Step *7*) that lead to cell death (Step *8*)

How can this knowledge explain the toxicity of chemicals that form electrophilic, cell-damaging metabolites? How might such species activate apoptotic cell signalling? This is a complex question and much remains unknown, but one pathway involving the multifunctional p53 protein deserves special mention. This 'guardian of the genome' was discovered in 1979 as a member of the tumour suppressor gene family that prevents the conversion of normal cells to tumour cells. p53 is a nuclear transcription factor that is present at low levels in healthy cells and plays little role in the 'normal' apoptosis that accompanies such routine physiological processes as erythropoiesis. Yet during cellular emergencies such as those accompanying exposure to cell-damaging threats, p53 assumes a major executive role by deciding whether individual cells survive or undergo apoptosis. Due to this crucial role, p53 must be inactivated if a tumour cell is to successfully grow – we will touch upon in this topic in Chap. 8.

The p53 pathway can activate apoptosis upon detecting cells that contain DNA damage inflicted by such diverse stresses as ionising radiation, free radicals, UV light and reactive metabolites. These diverse threats activate p53 via complicated means but in essence increase the stability of the p53 protein by preventing its rapid turnover by ubiquitin-dependent degradation. In normal cells, p53 has a half-life of just 20 min, yet this increases dramatically when p53 degradation is blocked in the presence of toxic cell stresses. The major controller of p53 degradation, Mdm2, normally tethers newly

made p53 protein within the nucleus and promotes its tagging by ubiquitin. The tagged p53 is then exported from the nucleus to the cytosol where it is degraded. This pathway is disrupted upon exposure to cell stresses, since damage-sensing kinases can phosphorylate p53, reducing its affinity for the Mdm2 protein. Rather than binding Mdm2, an assembly containing four p53 molecules forms which acts as a potent transcriptional activator to switch on the expression of diverse proapoptotic genes, including boosted production of the death-inducing Bax protein. The end result is the promotion of apoptotic cell death, especially within cells that contain substantial DNA damage.

If cells contain less substantial DNA damage, p53 can put the brakes on cellular division, allowing time for enzymatic repair of damaged DNA. p53 is thus a powerful intracellular operator that governs cellular destinies by choosing between repair, replication or apoptosis of cells that contain damaged genomes. We will explore these destinies in more detail when we examine chemical carcinogenesis in Chap. 8.

The apoptotic pathway we have briefly sketched is termed the *intrinsic pathway* since it is activated via the release of intracellular inducers such as cytochrome c. Although the intrinsic pathway has attracted much attention as a mechanism of cell damage by toxic chemicals, an alternative *extrinsic* pathway also contributes to many toxicological syndromes. The extrinsic pathway is dependent upon interactions of extracellular death-inducing proapoptotic ligands such as the Fas ligand (Fas-L) or TNFα with their respective membrane-embedded 'death receptors', Fas and tumour necrosis factor-receptor 1 (TNF-R1). Activation of these receptors triggers apoptosis by activating downstream endonucleases and various caspases. We previously saw that p53 can activate apoptosis by inducing the expression of proteins involved in the intrinsic pathway: it can also activate the extrinsic pathway by upregulating the expression of membrane receptors for apoptosis-inducing proteins such as Fas-L. For many chemicals, inducing the expression of these cell surface receptors is sufficient to induce apoptosis: death-inducing receptor ligands are abundant within the extracellular environment of many cells; hence, simply increasing the presentation of death receptors on the plasma membrane is adequate to activate the extrinsic pathway.

Ongoing discoveries have uncovered considerable complexity in the regulation of apoptosis, and our treatment above overlooked regulatory input from many proteins in cytosol and other cell compartments. Nevertheless, even our cursory treatment revealed how better understandings of apoptosis have provided new tools whereby toxicologists could study the harmful effects of chemicals on cells. Measurement of DNA fragmentation and detection of increased levels of cytochrome c in the cytosol of toxicant-exposed cells are common ways of detecting chemically induced apoptosis. Similarly the activity of initiator or executioner caspases in exposed cells can be monitored by following the cleavage of fluorescence-tagged protein substrates using a flow cytometer or, alternatively, via Western blotting to detect cleavage of endogenous caspase substrates. Alternatively, the effects of chemical exposure on the abundance of pro- and anti-apoptotic genes can be monitored via the polymerase chain reaction (PCR) or using gene microarrays. These approaches show how an improved understanding of the molecular basis for cell death has dramatically enriched our modern descriptions of how chemicals or their reactive metabolites cause cell injury.

As researchers studied apoptosis, they soon realised that this phenomenon is more similar to necrosis than was once assumed. If they occur in many cells simultaneously, mechanisms that trigger apoptosis might instead lead to necrosis. The mitochondrial permeability transition is one example of a process that accompanies both apoptosis and necrosis, and the number of cells that undergo this process determines which type of death predominates in a given tissue. On this understanding, since it occurs in isolated cells, apoptosis might even represent a form of tissue repair: by removing isolated cells that have sustained moderate DNA damage, the tissue may be protected against the emergence of clusters of mutated cells that eventually form tumours. Apoptosis is especially useful in tissues that can replace missing cells via regeneration, such as liver, bone marrow and lung. In tissues that lack this regenerative capability, such as myocytes in the heart or nerve cells in the brain, the activation of apoptosis by xenobiotics may be very detrimental.

4.4.6 Stress-Responsive Kinase Signalling (MAPK)

Toxic xenobiotics that form reactive intermediates frequently elicit complex changes in cell signalling pathways. Alterations in the activity of protein kinases – a large family of proteins that regulate diverse cell processes by phosphorylating downstream protein targets – are commonly associated with chemical toxicity. Many kinase pathways exist within cells, most of which conform to a common pattern whereby membrane-spanning receptor proteins couple with intracellular signalling targets via phosphorylation cascades that involve multitiered kinase activation. One class that is often involved in chemical toxicity are the mitogen-activated protein kinases (MAPKs), which were first discovered via their contribution to the proliferative effects of growth factors. Three distinct MAPK classes are recognised, namely, the extracellular signal-regulated kinase 1 and 2 pathway (ERK1/2), the c-Jun N-terminal kinase (JNK) pathway and the p38 MAPK pathway. Each group typically possesses multiple family members. The MAPK proteins act in the lower levels of cascades that follow their activation by MAPK kinases (MAPKK). The MAPKK are in turn activated by MAPKK kinases (MAPKKK).

Due to considerable crosstalk between these pathways generalisations are risky, yet as a rule activation of ERK family members triggers cellular proliferation or differentiation, while JNK and p38 MAPK are stress-responsive MAPKs that modulate apoptotic cell death. The extent to which these MAPKs are activated by xenobiotics is often variable, influenced by the toxicant concentration, cell type and experimental model used.

Precisely how reactive metabolites trigger JNK and p38 activation is poorly defined, although some electrophilic species appear to form adducts directly on the kinase proteins, triggering conformational changes that promote changes in their phosphorylation status and activity (Fig. 4.10). Another possibility is that reactive metabolites inactivate the phosphatase enzymes that ‘switch off’ phosphorylation signalling by dephosphorylating the MAPK protein. This proposal concurs with the

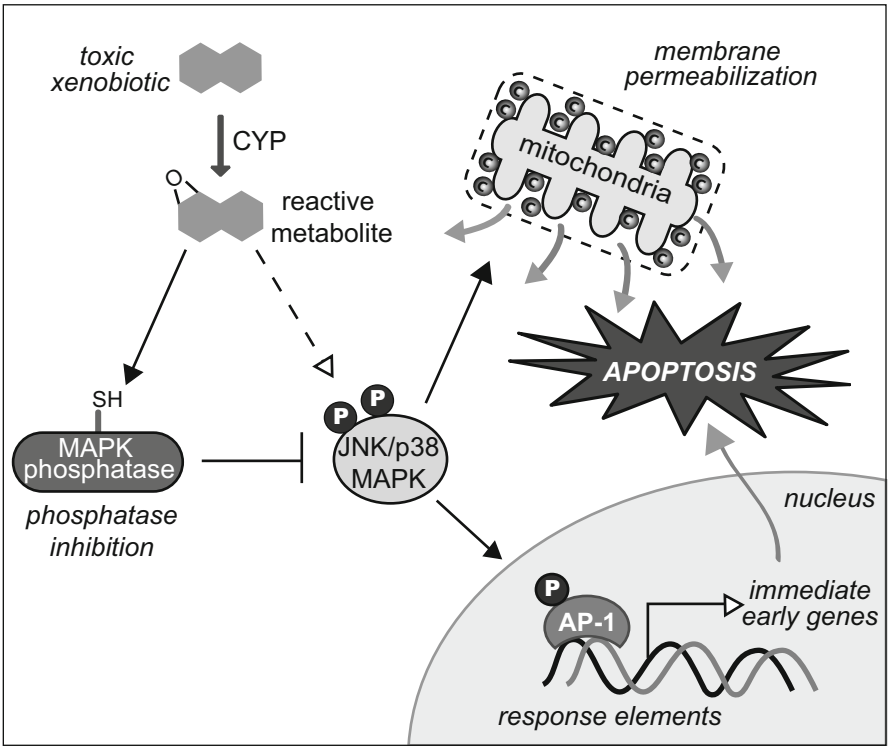

Fig. 4.10 Activation of the stress-responsive mitogen-activated protein kinases (*MAPK*) JNK and p38 is a common cellular response to bioactivation-prone xenobiotics. The MAPK may be activated by via changes in phosphorylation due to direct adduction or inhibition of phosphatase activity. The induction of apoptosis likely involves effects on mitochondrial membrane permeability as well as transcriptional changes related to the expression of apoptosis genes (Reprinted (adapted) with permission from West JD, Marnett LJ, Endogenous reactive intermediates as modulators of cell signaling and cell death. *Chem Res Toxicol* 19:173–194. Copyright (2006) American Chemical Society)

catalytic dependence of MAPK phosphatases on cysteine thiol groups that are likely targets for reactive electrophiles. By blocking the ability of phosphatases to suppress MAPK signalling, adduct-forming toxicants may induce apoptosis via unrestrained MAPK activation.

Precisely how stress-activated MAPKs induce apoptosis during toxicant exposure is not entirely clear. One possibility is that the MAPKs phosphorylate prosurvival Bcl-2 family members such as Bcl-2 and Bcl-xL, impairing their ability to maintain mitochondrial integrity and prevent cytochrome c release. Alternatively, MAPK-mediated phosphorylation of proapoptotic Bcl-2 family members such as Bim and Bad may boost their ability to promote mitochondrial permeabilisation. According to both scenarios, distinct phosphorylation events on Bcl-2 family members may either activate or suppress apoptotic signalling. Ongoing experimental work in this area will likely shed light on these possibilities.

4.5 Mechanistic Toxicology in the 'Omics' Era

Mechanistic toxicology has undergone a revolution over recent decades under the impact of fast-paced technological advancements. Ever since the discoveries of Watson and Crick unlocked the genetic code in 1953, modern biology has grown rapidly in its knowledge of the molecular basis for life. The awareness that living tissues represent complex chemical and biochemical conglomerates under the control of genes fuelled an energetic effort to understand these processes at a basic level. While progress was initially slow due to technological constraints that often allowed biological study of only a single gene, protein or metabolite at once, in recent decades technological advances in chemistry, computing hardware, software systems, robotics and bioinformatics fostered the emergence of new disciplines that study biological phenomena from a global or 'bird's-eye perspective'. These 'omics' approaches allow concurrent study of tens of thousands of molecular participants in biological processes.

Since global technologies can provide unprecedented insight into toxic phenomena, modern toxicology has embraced new technologies such as genomics, transcriptomics, proteomics and metabolomics during the study of cancer, birth defects, organ injury and reproductive toxicity (Fig. 4.11). One clear advantage of these approaches is that they are largely 'bias free': depending on the technological capabilities of the instrumentation and sample preparation methods, they can potentially

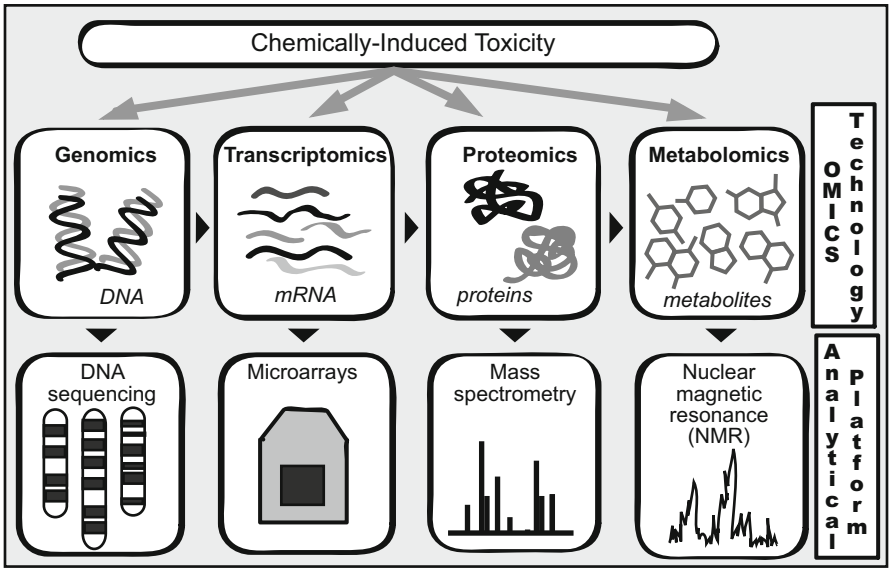

Fig. 4.11 Systems toxicology integrates data obtained during study of the effects of toxicant exposure on gene functions (*genomics*), mRNA transcripts (*transcriptomics*), protein expression (*proteomics*) and metabolite profiles (*metabolomics*)

reveal toxicant-induced changes within the levels of any gene target or metabolite within a biofluid or cell. This is unlike traditional approaches that depend upon the hypotheses embraced by the researcher who selected particular genes or targets for investigation based on their personal beliefs concerning the key steps in disease pathogenesis. The advent of 'omics' technologies opened the door to 'hypothesis-free' research in which the effects of toxicants can be studied on thousands of pathway participants, without prior filtering due to the beliefs of the investigator. These new approaches can often reveal exciting new roles for proteins or genes in toxicant-induced syndromes. One problem is that the researcher is confronted with the 'needle in a haystack' problem, of trying to identify important disease mediators among hundreds or even thousands of altered products. The use of bioinformatics tools to analyse 'omics' data sets is crucial during these ventures.

In addition to enabling study of the interactions of xenobiotics with thousands of defined molecular targets, modern approaches provide amazing high-throughput capability – namely, the capacity to screen thousands of chemicals for specific toxic properties very quickly. Since they assist the rapid identification and characterisation of hazardous substances, these approaches are quickly reshaping the domain of regulatory toxicology. These capabilities are of particular relevance in a world that is seeking safe alternatives to chemicals that have long been used in various industrial and commercial settings but are increasingly viewed with concern over their impact at low doses (e.g. DEHP, bisphenol A, pesticides, metals).

4.5.1 The Transcriptome and Toxicity

The availability of technologies allowing precise placement of thousands or even millions of gene probes on glass slides – known as microarrays – revolutionised toxicology since they allowed monitoring of the up or downregulation of multitudes of genes simultaneously. In a remarkable demonstration of lateral thinking, the methods used to spot gene probes on a small glass slide were developed from inkjet printer technologies. Other technologies that assisted this field included methods for extracting RNA from samples and confirming its structural integrity. An ability to make complementary DNA molecules containing fluorescent labels from each mRNA gene transcript within a cell extract was also essential. After hybridising these to the microarray slides followed by extensive washing, laser scanners are used to monitor levels of individual genes. By allowing the emergence of toxicogenomics – the branch of toxicology that specialises in monitoring transcriptional responses to chemicals – these microarray technologies provided powerful insights into chemical toxicity.

After extracting RNA from toxicant-exposed cells or tissues, microarrays permit comparison of the expression of thousands of genes within control tissues and toxicant-exposed samples. Identifying clusters of genes that are down- or upregulated during toxicity is helpful since they can reveal genes within whole pathways that are disrupted by noxious bioactivation products. For example, our preceding

discussion of apoptosis highlighted the role of the p53 transcriptional activator during the induction of apoptosis since it regulates the expression of many pro- and anti-apoptotic genes. Yet cell death and cell viability pathways represent only a small subset of genes that typically change during the onset of chemically induced toxicity. For example, microarrays commonly detect altered expression of genes involved in innate or adaptive immune responses within poisoned tissues. In addition, xenobiotic toxicity is often accompanied by boosted expression of genes for CYP enzymes as well as other biotransformation pathways including SULTs, UGTs, NATs and GSTs. Upregulation of genes involved in stress responses including antioxidant pathways or chaperone functions is also common. Microarray analysis often reveals that toxicants activate tissue regeneration genes or those involved in the synthesis of extracellular matrix or in cell proliferation to replace unrepairable cells. Finally, telltale signs of cancer causing properties may be seen in altered expression of DNA damage and DNA repair pathways, cell cycle genes or DNA replication pathways.

The ability to interrogate complex microarray data to identify important changes within signalling pathways that reveal a particular toxic response is very useful in today's toxicology laboratory. Much attention has been devoted to identifying 'gene signatures' – sets of toxicant-responsive mRNA molecules – that are characteristic of specific toxic responses in a particular tissue (e.g. gene sets that distinguish between fibrotic, necrotic and cholestatic responses in the liver or cancer, fibrosis or asthma in the lung) Once gene signatures that reveal particular toxic responses are confirmed and validated, they can be used to create 'focussed arrays' that contain genes that reveal particular toxic responses of concern to particular researchers. For example, an 'inflammation array' or 'apoptosis array' can be very useful in experiments where researchers are interested in the role of these processes in xenobiotic toxicity.

The pharmaceutical industry makes particular use of toxicogenomic approaches since these approaches can reduce the need for laborious drug testing in animals. Identifying gene sets that predict drug-induced toxicity in rodents is demanding since the specificity and sensitivity of these approaches must be compared to traditional toxicity assessment methods (e.g. histological evaluation, biochemical methods such as blood enzymes). For example, researchers may explore whether a mouse liver gene signature that responds to high doses of a hepatotoxic drug 72 h after drug administration also respond to subtoxic doses after 12, 24 or 48 h? Can the gene set detect early liver injury by other compounds that elicit similar toxic responses? The specificity of the biomarkers is also investigated: is the gene set affected by non-hepatotoxic chemicals that produce injury in other organs such as kidney, heart or lungs? Does it behave the same way in other strains of mice or in other species such as rats and rabbits? Can it detect human hepatotoxicity in clinical trials?

In addition to their use in animal studies, toxicogenomic studies find wide application in toxicity testing in cell culture models. Microarrays are also increasingly used in novel 3D cell culture systems that permit recreation of essential in vivo organ characteristics in an architectural environment that overcomes the limitations of 2D monolayers in conventional in vitro settings. The combined use of toxicogenomic tools together with these emerging 'organ on a chip' approaches ensures the future is bright for mechanistic toxicology.

4.5.2 The Proteome and Toxicity

Although toxicants often alter gene transcript levels, proving the functional significance of changes in an individual mRNA requires confirmation of changes in the expression of the corresponding protein product. Once again, while researchers once monitored changes in levels of a few select proteins via traditional methods, improvements in mass spectrometry now allow detection of hundreds or thousands of proteins simultaneously within tissues.

Proteomics is a broad term covering various mass spectrometry-enabled technologies that find special use in toxicology due to their usefulness in detecting altered protein abundance during xenobiotic toxicity. As with the use of mRNA transcripts in toxicity prediction, toxicant-induced changes within protein clusters can be diagnostic of pathological effects in specific tissues such as the liver or kidneys. Once again, issues surrounding biomarker specificity and sensitivity are pertinent during such studies, as are technical issues accompanying the extraction of proteins from different tissues. Some proteins including low copy number proteins or integral membrane proteins are hard to extract reliably from many tissues. To avoid the latter concern, some researchers prefer using the 'plasma proteome' as an indicator of chemical toxicity, since tissue-specific proteins may appear within circulating blood during injury to tissues of origin.

Proteomic technologies also allow characterisation of the 'adductome' for particular bioactivation-dependent toxicants. The adductome simply comprises the subset of proteins within cells that sustain adduction by particular electrophiles. As highlighted in Sect. 4.4.1 above, the tendency for toxic chemicals to form reactive metabolites that attack multiple protein targets in vulnerable tissues was deduced during investigation of the 'covalent binding hypothesis'. Once their identity is established, bioinformatics tools allow study of any functional links between adducted proteins in particular tissues. Such knowledge can reveal whether electrophiles damage multiple proteins within a particular metabolic pathway, such as lipid metabolism, the TCA cycle or cell death regulation. Yet adduct formation is not the only post-translational modification that occurs upon exposure to noxious chemicals: mass spectrometry also allows study of global toxicant-induced changes in phosphorylation or ubiquitination status within the cell proteome. These approaches are especially useful for toxicants that activate MAPK and related stress-responsive kinase pathways.

4.5.3 The Metabolome and Toxicity

For proteins that function as enzymes to control the intracellular levels of low-molecular-weight metabolites, toxicant-induced disruption of protein expression can change associated metabolite concentrations within blood or other biofluids with which the tissue interacts. The unbiased global survey of metabolites in cells,

tissues or biofluids such as blood or urine is termed *metabolomics*. Global changes in metabolite concentrations within tissues and biofluids can yield powerful insights into an individual's nutritional wellbeing, disease status, microbiome, genetic background, epigenetic programming or xenobiotic exposure. As with the proteome, the metabolome is chemically diverse and often requires different methods to achieve analytical coverage of most compounds within a complex mixture. The two main analytical platforms used in metabolomics analysis are NMR spectroscopy and mass spectrometry, although strengths and weaknesses apply to each methodology. NMR is suitable for sugars, amines and large substances, but is relatively insensitive and likely requires large sample volumes. While gas chromatographic responses are useful during some metabolomic studies, ultrahigh performance liquid chromatography mass spectrometry is an optimal technology in this field due to its capacity for high throughput and ease of sample preparation. In general, some 1,500 metabolites likely provide insights into important biological events within biofluids and tissues. A remaining challenge in this area is the identification of novel metabolites, a problem that is complicated by the use of different technology platforms in competing laboratories. The growing availability and quality of online metabolomics databases is helping to resolve these issues.

As with other 'omics' technologies, the use of metabolomics in toxicology seeks to identify 'metabolite signatures' that reveal injury within a specific organ. In addition, the high power of metabolomic technologies is also useful during studies of xenobiotic metabolism within humans and lab animals. In recent years, use of such technologies identified interesting and unexpected metabolites for drugs and toxicants that we assumed the metabolism was already well known. Since they facilitate detection of minor routes of metabolism, use of isotope-labelled xenobiotics can assist these efforts. For example, recent work by the Gonzalez metabolomics group at the National Institutes of Health identified several previously unknown metabolites of paracetamol in mouse serum, including a dimerised species likely formed via reactions between radicals that arise via one-electron oxidation of paracetamol. While this species has been observed in 'test tube' experiments, its discovery in mice that received hepatotoxic doses of paracetamol suggests a role for free radicals in the hepatotoxicity of this drug.

4.5.4 The 'Omics' Revolution in Regulatory Toxicology

Our discussion has highlighted how 'omics' technologies helped mechanistic toxicology improve its understanding of how chemicals injure tissues. Others sensed that these technologies could revitalise the screening of chemicals for toxic potential, an area some believe is too heavily dependent on whole animal testing. In 2008, the director of the US National Institutes of Health, Francis S Collins, penned an influential article that exhorted the toxicology community to embrace modern technologies during its evaluation of chemicals for regulatory purposes. This viewpoint echoed the vision outlined in a persuasive document published by the US National

Academies in 2007, *Toxicity Testing in the twenty-first Century*. These perspectives energised some very exciting developments, including several large-scale screening initiatives that promise to provide unprecedented insight into the toxic properties of thousands of xenobiotics. Initiatives such as *Tox21* (Toxicology for the twenty-first Century) – a cooperative US venture involving the US-EPA, NIH, NTP and others – is a leading example of the powerful approaches available within the contemporary robotic era.

Initiated in 2008, the ongoing *Tox21* programme is set to screen over 12,000 chemicals under standardised conditions in more than 1,000 human cell lines. In the initial preliminary phase, some 2,800 compounds were evaluated in over 75 cell-based tests that assessed diverse toxic properties including the ability to elicit cytotoxicity, CYP induction, xenosensor activation, cytokine production, stress responses, ion channel dysfunction or epigenetic reprogramming. To facilitate gathering of structure-activity data for each toxic response, compounds are tested at 15 different concentrations spanning over five orders of magnitude (low nanomolar to high micromolar range). During the second phase, the yield of data for each compound will increase by using 'multiplexing' approaches to monitor numerous toxic responses within the same cell population simultaneously.

The flood of data from these and related studies will provide a powerful boost to computational toxicology and permit the development of better predictive tests for toxicity. The scale of these approaches, the volume of data they generate and the multiplicity of mechanistic insights they provide necessitate that the regulatory toxicologist of tomorrow will require strong training in bioinformatics, toxicogenomics and related fields. Systems toxicologists – researchers who can extract information from Tox21 data sets while also possessing skills required to study chemically induced perturbations to transcriptomes, adductomes, proteomes and metabolomes – will likely underpin many toxicological advances in coming years.

4.6 Conclusion

This wide-ranging chapter has explored a broad swath of terrain with the intention of conveying a basic appreciation of the progress made during a sustained research effort to understand the mechanisms underlying chemical toxicity. Unfortunately, space limitations necessitated that our coverage was very concise, with the contributions of many important researchers either glossed over or neglected. To facilitate our journey, particular attention was devoted to six main mechanisms in chemical toxicity, namely, covalent binding, calcium dyshomeostasis, oxidative stress, lipid peroxidation, programmed cell death and kinase activation. Summarising the field this way should not convey the impression that individual chemicals cause toxicity exclusively by a particular mechanism. In reality, most reactive chemicals elicit a broad range of cellular effects, with overlapping effects on many cell pathways. The challenge for toxicologists in coming years will be to develop an integrated understanding of chemical toxicology that incorporates knowledge of the chemical

properties of reactive metabolites with a detailed knowledge of their effects on diverse cell signalling pathways.

Thanks to progress in many fields, the production of electrophilic metabolites, an event that first intrigued James and Elizabeth Miller over 60 years ago, is now known to trigger a complex sequence of events that can alter the expression of hundreds or even thousands of genes and their associated protein products. Figuring out how all these changes fit together will yield new theoretical insights while also supplying innovative screening methods to allow prediction of the precise toxicological properties of new drugs and chemicals. The ongoing search for the critical molecular changes that underlie the often unpredictable toxicity of foreign chemicals represents one of the great intellectual pursuits in modern science. An early discovery from this research was the recognition that cells and tissues are not at the full mercy of noxious substances but that they possess elaborate defence systems that afford protection during times of heightened xenobiotic exposure. These crucial adaptive capabilities receive consideration in the next chapter.

Going Further

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Chapter 5
Fight Back: Adaptive Responses to Toxicant Exposure

Abstract The popularity of barbiturate sedatives in bygone days helped physicians discover an unusual property of these medicines: on repeated use, patients experienced a dramatic loss of effectiveness, with higher and higher doses required to elicit the original drug response. This capacity for physical tolerance alerted researchers to the phenomenon of enzyme induction: upon sustained exposure, the liver and other tissues simultaneously boost the expression of enzymes that convert xenobiotics to water-soluble metabolites and membrane transporters that expel metabolites via bile or urine. These outcomes reflect the actions of xenosensors – bifunctional proteins that detect ingested xenobiotics and then activate broad transcriptional responses that facilitate their clearance from the body. While such adaptive changes protect the body by altering the toxicokinetic fate of xenobiotics, transcriptional changes also occur at the subcellular level to protect tissues against cell-damaging reactive metabolites. Upon detecting cell or protein damage caused by electrophiles, cells mount strong transcriptional responses to suppress the toxicodynamic properties of xenobiotics via a number of pathways including the heat shock response, the antioxidant response, the unfolded protein response and the NFkB pathway.

Keywords Induction • Xenosensors • Pregnane X receptor (PXR) • Aryl hydrocarbon receptor (AhR) • Constitutive androstane receptor (CAR) • Peroxisome proliferator-activated receptor (PPAR) • Antioxidant response • Nrf2 • Endoplasmic reticulum stress • Unfolded protein response • Heat shock proteins • Heat shock factor-1 • NFkB

5.1 Introduction

The ability to metabolise foreign toxicants and eliminate any resulting metabolites is crucial to the health of all mammalian organisms. Yet researchers soon discovered that the hepatic capacity to detoxicate xenobiotics is not static: upon repeated exposure, hepatocytes boost the expression of biotransformation and excretory pathways

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in efforts to eliminate foreign substances from cells. Such discoveries focused great interest on the transduction mechanisms underlying these capabilities: how precisely might a small molecule such as a barbiturate drug selectively increase the expression of nuclear genes encoding proteins with xenobiotic-handling properties? Thanks to work on this problem, recent decades saw the identification of numerous 'xenosensor' proteins that mediate adaptive responses to xenobiotics. This chapter will first explore how these mechanisms protect the body during the *toxicokinetic* phase of toxicant action by upregulating xenobiotic metabolism and transporter expression. We will subsequently consider adaptive *toxicodynamic* responses that counteract harmful mechanisms elicited by toxic xenobiotics at the subcellular level.

5.2 Adaptive Toxicokinetic Responses

Upon repeated exposure to a xenobiotic, the body can alter its toxicokinetic fate by diminishing the absorption of the toxicant, limiting distribution, boosting metabolism or accelerating excretion of the parent compound or metabolites. The capability to boost these processes depends on the presence of sensors that detect trespassing xenobiotics and mount transcriptional responses that accelerate toxicant elimination from the body. Although research historically focussed upon the mechanisms whereby the cellular capacity for xenobiotic metabolism is boosted upon repeated exposure, the same sensing systems that mediate these changes usually concurrently upregulate membrane transporters that control the absorption, distribution and elimination of xenobiotics. This integrated response makes biological design sense: any sustained increase in metabolite formation within the body is potentially counterproductive unless the ability to permanently excrete these species is also enhanced.

5.2.1 CYP450 Induction

The capacity of the liver and other tissues to metabolise xenobiotics varies under the influence of diet, age, smoking and drug exposure. At the molecular level, prolonged xenobiotic exposure boosts the levels of individual CYPs via a phenomenon termed *induction*. This increased capacity for oxidative biotransformation is usually benign since it is intended to accelerate xenobiotic clearance from the body, although harmful consequences can occur if toxicants are made more toxic via inducible CYP pathways. CYP induction also has significant consequences for drug therapy and is a major cause of drug–drug interactions (DDIs) in patients who receive multiple medicines simultaneously. Since drug concentrations within blood may fall due to increased clearance by the liver, CYP induction robs patients of clinical benefit from their medicines. Since time is required for the synthesis of new CYP proteins, clinical problems caused by CYP inducers often require 1 week or so to become obvious (i.e. after a premedicated patient commences therapy with an

additional drug). This is in contrast to DDIs caused by CYP inhibition that typically manifest a little more quickly.

CYP induction was discovered in the 1950s when barbiturate hypnosedatives were widely used sleep aids. Despite responding well initially, many recipients reported a loss of effectiveness or *tolerance* upon repeated use. Studies in lab animals revealed a kinetic basis for such barbiturate tolerance, with a four- to fivefold elevation in total hepatic CYP detected following sustained exposure to these drugs. Since the barbiturates are metabolised more quickly, higher doses are needed to attain sedative responses, leading to physical dependence within patients. Following these discoveries, the popularity of barbiturates declined significantly.

Initially, techniques for detecting CYP induction in tissues depended upon the classical procedure for estimating total CYP (i.e. bubbling liver microsome extracts with carbon monoxide (CO) gas followed by the addition of a reducing agent to form a species that absorbs light at 450 nm). When using this method to measure 'total CYP' in livers of barbiturate-treated animals, observant researchers noticed that the absorption maximum of the reduced CO–heme pigment was actually 448 nm compared to 450 nm in controls. These findings suggested different CYP subpopulations might respond unequally to individual inducers.

Researchers eventually gained powerful tools for measuring individual CYP proteins in cells and tissues, including specific antibodies that bind to each major CYP isoform. These reagents are widely used during 'Western blotting' procedures which allow comparisons between cells from control animals and inducer-exposed tissues. The development of methods for detecting mRNA transcripts for individual CYPs (e.g. Northern blotting and later RT-PCR and microarrays) greatly assisted the study of CYP induction. Collectively, these approaches revealed that most inducers affect the levels of individual CYP isoforms differently, with often just a few or even a single isoform most strongly affected. By suggesting different inducers might act via distinct transduction pathways, these findings spurred researchers to clarify the mechanisms underlying CYP induction.

5.2.1.1 Role of Xenosensors in CYP450 Induction

One explanation for CYP induction proposed that inducing ligands bind to DNA-targeting receptors, eliciting transcriptional responses that increase the abundance of specific CYP isoforms in target tissues. This hypothesis proved very fruitful, and subsequent decades saw several receptors identified as targets for CYP inducers (Table 5.1). Known as *xenosensors*, these ligand-activated receptors protect the body by 'sensing' ingested foreign compounds before activating the synthesis of protein catalysts that facilitate xenobiotic clearance. All xenosensors discovered to date are ligand-responsive nuclear transcription factors that upregulate the expression of CYP and related biotransformation genes as well as membrane transporters. Although their normal distribution can sometimes be controversial depending on the cell types under experimental observation, as a rule most xenosensor proteins exist in cytosol until ligands promote their redistribution to the nucleus (Fig. 5.1).

Table 5.1 Major xenosensor proteins that activate the expression of human CYP isoforms in response to ingested xenobiotics

Xenosensor name	Representative CYP isoforms affected	Typical inducers
*Ah*R (aryl hydrocarbon receptor)	CYP1A1, CYP1A2	Polycyclic aromatic hydrocarbons, aromatic amines, dioxin, tryptophan metabolites (e.g. kynurenine), some drugs (e.g. omeprazole, phenothiazine)
CAR (constitutive androstane receptor)	CYP2A6, CYP2B6, CYP2B10, CYP2C9, CYP2C19, CYP3A4	Same as for PXR + clotrimazole; 3α, 5α-androstanol; 5β-pregnane-3, 20-dione, etc.
PPAR (peroxisome proliferator-activated receptor – multiple isoforms)	CYP4A1	*Endogenous*: fatty acids, prostaglandins, leukotrienes. *Exogenous*: fibrate drugs (e.g. clofibrate), trichloroethylene, phthalate plasticisers
PXR (pregnane X receptor)	CYP1A1, CYP1A2, CYP2B6, CYP2B10, CYP2C8, CYP2C9, CYP2C19, CYP3A4	Many: dexamethasone, hyperforin, lithocholic acid, phenobarbital, PCN, rifampicin, SR12813, taxol, etc.

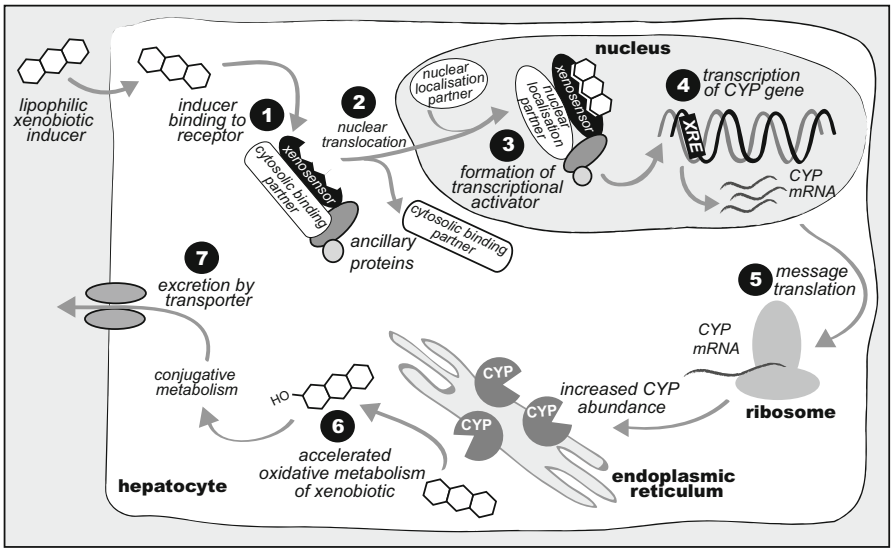

Fig. 5.1 Generalised representation of key events in the induction of CYP expression via ligand binding to xenosensors such as AhR, PPAR, PXR and CAR. Note that the identity of the partner nuclear receptor protein involved in dimerisation (Step 3) varies according to the xenosensor pathway (e.g. retinoid X receptor and Arnt). The resulting transcriptional complex binds to specific DNA sequences within the promoters of target genes known as xenobiotic response elements (*XRE*)

5.2.1.2 The Aryl Hydrocarbon Receptor

The first xenosensor discovered was the aryl hydrocarbon receptor (AhR), a transcriptional activator that controls the expression of two closely related CYPs, CYP1A1 and CYP1A2. The latter is expressed in liver, while CYP1A1 is expressed at low levels in many extrahepatic tissues including epithelial layers in the lung, skin and GI-tract. While CYP1A1 metabolises comparatively few drugs, CYP1A2 oxidises some major human pharmaceuticals including clozapine, naproxen, theophylline and caffeine. Both CYP1A1 and 1A2 are important in toxicology since they metabolise a wide range of foreign toxicants, with particular roles in the bioactivation of polycyclic aromatic hydrocarbons. Both CYP1A1 and 1A2 are strongly induced by TCDD ('dioxin'), a ubiquitous organochlorine pollutant that attracted much research attention due to its role in the health complaints that afflicted Vietnam War veterans (see Chap. 4, Sect. 4.2.2). TCDD also contributed to a number of industrial accidents that occurred during the latter half of the twentieth century (e.g. Seveso, Italy, 1976). Exposure to this highly toxic substance is associated with various cancer syndromes in humans and rodents as well as other toxic responses including developmental abnormalities in the unborn, immunosuppression and a disfiguring skin disorder known as chloracne.

The Canadian toxicologist Allan Okey discovered the *Ah*R xenosensor in 1979 when he was working within Daniel Nebert's laboratory at the National Institutes of Health in the USA. The methods used to isolate the *Ah*R protein seem heroically laborious from today's standpoint yet represented a major milestone in the emergence of modern toxicology. Whereas researchers had searched fruitlessly for steroid receptors as TCDD targets, the finding that cells possessed a distinct receptor for toxic xenobiotics raised the status of the entire xenobiotic biotransformation field.

The *Ah*R protein belongs to the basic helix–loop–helix (bHLH) family of dimeric transcription factors, members of which possess a distinctive structural motif comprising two α-helices connected by a loop. One helix typically mediates binding to specific DNA sequences, while the other facilitates interactions with partner proteins. Upon activation, the *Ah*R targets xenobiotic response elements (XRE) within the promoters of TCDD-responsive genes. The *Ah*R also contains other structural motifs that are vital for its action, including the PAS-A and PAS-B domains that participate in protein dimerisation and ligand binding. In recent years, researchers have also uncovered the interaction of the *Ah*R with novel TCDD-unresponsive genetic elements that differ from classic XREs. While their role is somewhat unclear, they appear to mediate novel responses to nonclassical AhR activators and likely include a number of endogenous ligands.

Normally, inactive *Ah*R is sequestered within the cytosol of hepatocytes via binding to several partner proteins including heat shock protein-90 (hsp90), c-SRC, p23 and XAP2. Upon TCDD binding, hsp90 is displaced and nuclear relocation is fostered via formation of a complex with the aryl hydrocarbon receptor nuclear translocator (ARNT), another bHLH/PAS transcription factor family member. The resulting transcriptional complex activates the expression of key TCDD-responsive

genes, including genes for enzymes involved in oxidative (CYP1A1, CYP1A2, CYP1B1, ALDH3A1), reductive (NQO1) and conjugative xenobiotic metabolism (e.g. UGT1A2 and GSTA). Ligand-induced *Ah*R activation thus broadly increases the metabolic capacity of tissues towards circulating xenobiotics. In recent years, the use of microarrays to detect TCDD-responsive genes has identified scores upon scores of altered genes within many cellular pathways that extend beyond xenobiotic biotransformation and include cell communication, signal transduction, inflammation, cell cycle control, cell proliferation and differentiation. While these findings seem directly relevant to many toxic responses that accompany TCDD exposure, significant variability in TCDD-induced transcriptional responses between different species and strains of experimental animals complicates clarification of their biological roles. Some hints of *Ah*R roles beyond regulation of xenobiotic metabolism emerged following the creation of *Ah*R-knockout mice which often show deficiencies in cardiovascular function, fertility and growth regulation. While these animals are resistant to TCDD toxicity, these other deficits suggest broad endogenous roles for the AhR in normal physiology.

In addition to mediating responses to TCDD, the *AhR* is activated by many other foreign toxicants including organochlorine pesticides, aromatic amines and polycyclic aromatic hydrocarbons. A number of pharmaceutical agents also act as AhR inducers, including omeprazole, flutamide and leflunomide. Several endogenous molecules including various oxidised tryptophan metabolites such as kynurenine, indigo and indirubin as well as heme metabolites likely represent naturally occurring ligands for the *Ah*R, but their roles within the physiological setting remain to be fully clarified. Recent investigations of the biological properties of TCDD-insensitive *Ah*R homologue genes within model invertebrate species such as *C. elegans* hold promise for revealing noncanonical biological roles of the *Ah*R.

The discovery of *Ah*R intensified efforts to identify receptors for other CYP inducers. With time, xenosensors were linked to most CYP isoforms that participate in human drug metabolism, although CYP2D6 remains an important exception to this rule. While CYP2D6 contributes to the metabolism of one in every six or seven drugs in current clinical use, no transduction mechanisms likely exist for this CYP isoform.

5.2.1.3 Peroxisome Proliferator Receptors

Soon after discovering the *Ah*R, researchers identified a similar family of xenobiotic-responsive receptors that are activated by a diverse class of chemicals known as *peroxisome proliferators*. These xenobiotics share an ability to increase the size and number of peroxisomes within liver cells, the key subcellular organelles that assist the metabolism of lipids, peroxides and cholesterol. This broad ligand family includes lipid lowering drugs (e.g. fibric acid analogues such as clofibrate), chlorinated solvents (e.g. trichloroethylene) and phthalate plasticisers (e.g. DEHP, di-2-ethylhexyl phthalate). The health effects accompanying human exposure to DEHP have aroused considerable debate since it is a high volume plasticiser used in the

production of medical products and consumer goods (the addition of DEHP to polyvinylchloride (PVC) polymers increases their malleability). Such uses are controversial since surgical tubing containing DEHP is associated with several toxic outcomes in newborn babies that receive intensive care within neonatal units.

The finding that DEHP induces liver cancer in rodents conferred much interest on the peroxisome proliferators, fostering concern that this ubiquitous compound might be fuelling a hidden epidemic of liver cancer. These concerns abated somewhat after researchers identified the PPARs (peroxisome proliferator-activated receptors), the family of nuclear receptors that act as cellular receptors for peroxisome proliferators. One family member, PPARα, attracted special attention since it appears to 'drive' cancer development in DEHP-exposed rodents (PPARα knockout mice are resistant to DEHP-induced tumours). Activation of the PPARα also strongly induces the expression of CYP4A1, a CYP isoform that participates in lipid metabolism but not in human xenobiotic metabolism.

Subsequent research by mechanistic toxicologists revealed that while humans possess a functional PPARα, significant differences exist between humans and mice in terms of downstream signalling and cellular changes that follow PPARα activation. Unlike mouse and rat hepatocytes, PPARα activation by DEHP does not stimulate cell proliferation in human hepatocytes, implying human livers are less susceptible to DEHP carcinogenesis than their rodent counterparts. An ability to increase mitogenesis is a common property of many so-called nongenotoxic carcinogens (see Chap. 8, Sect. 8.5.2). Since DEHP lacked mitogenic potency in PPARα-expressing liver cells, this lessened concerns that DEHP acts as a nongenotoxic liver carcinogen in humans. By diminishing this possibility, these mechanistic findings helped divert research attention to more pressing needs, including, for example, evaluation of DEHP-associated tumour outcomes in tissues other than the liver. Since these issues remain under active investigation, DEHP toxicology is far from a closed book.

5.2.1.4 The Master Xenosensors: PXR and CAR

The xenosensors PXR and CAR possess enormous relevance to human pharmacology (Table 5.1). Belonging to a separate class of transcription factors to *Ah*R, PXR and CAR resemble 'classic' nuclear receptors that mediate the biological effects of such endogenous ligands as thyroid hormones, cholesterol, oestrogen and bile acids. Key structural motifs shared by CAR and PXR include a DNA-binding domain (DBD) which is very similar among family members and typically comprises two zinc finger motifs, a structural feature possessed by many DNA-binding proteins. One zinc finger also contains a D-box enabling dimerisation with other nuclear receptor partner proteins. Another typical feature of classic nuclear receptors is the ligand-binding domain (LBD) which exhibits structural variability due to the need for each receptor to bind distinct ligands.

Upon ligand activation, nuclear receptors form dimers that recognise specific sequences in target genes: family members that are activated by endogenous ligands

usually form homodimers comprising the same family member, while those that are activated by exogenous ligands (e.g. PXR, CAR, PPAR) usually heterodimerise with the retinoid X receptor (RXR).

PXR, the pregnane X receptor, is very relevant to human pharmacology since it regulates the expression of a host of CYPs that perform major roles in drug metabolism, includingCYP1A, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5 and CYP3A7. PXR also regulates the expression of many UDP-glucuronosyltransferase, sulfotransferase and glutathione-S-transferase family members plus a number of xenobiotic transporters. Classic PXR inducers include drugs such as dexamethasone, clotrimazole and rifampicin as well as numerous environmental pollutants including DDT, di-n-butyl phthalate (DBP), chlordane, dieldrin and endosulfan. PXR is also activated by various phytochemicals present within herbal medicines such as St John's wort. Binding of these ligands to PXR promotes the formation of a heterodimer involving the retinoid X receptor (RXR) as well as other ancillary proteins such as steroid receptor coactivator 1 (SRC-1). The resulting transcriptional complex targets its respective xenobiotic response elements within the 5′ promoter region of responsive genes. Following transcription, mRNA molecules are exported via nuclear pores to allow ribosomal processing and the synthesis of new CYP proteins that subsequently facilitate the formation of more readily excreted metabolites as per Fig. 5.1.

While major xenosensors such as PXR and CAR share many similarities to the glucocorticoid class of nuclear receptors, they typically require higher ligand concentrations for transcriptional activation to occur (e.g. micromolar concentrations compared to the nanomolar hormone concentrations that activate glucocorticoid receptors). Concurring with these considerations, structural studies have revealed much greater plasticity within the ligand-binding domain of PXR compared to glucocorticoid receptors, allowing the former to accommodate a large number of bulky, structurally dissimilar ligands. These properties help explain why PXR is arguably the least discriminating member of the xenosensor family, able to drive CYP expression following exposure to a wide range of structurally diverse xenobiotics.

5.2.1.5 Detecting Xenosensor Activators

PXR and CAR attract much interest within the pharmaceutical industry due to their role in drug-induced CYP induction, a phenomenon that can cause serious drug–drug interactions in patients receiving multiple medicines simultaneously. Since CYP3A4 inducers frequently disrupt the metabolism of co-administered drugs, discovering a drug candidate that is a potent PXR or CAR ligand is often a 'red flag' during the preclinical investigation of new medicines. To address this problem, various high-throughput screening methods allow rapid evaluation of compounds for their ability to activate nuclear receptors. A common approach uses cultured cells co-transfected with a reporter vector and an expression vector for PXR, CAR or AhR that are porter vector containing response elements for each respective xenosensor. The easily monitored luciferase reporter gene is commonly used in these models. Other approaches evaluate the ability of test compounds to displace radiolabelled model ligands from xenosensor proteins.

Another promising approach involves development of predictive models that identify PXR or CAR-activating molecules within an in silico or computer-based software environment. This approach remains experimental in nature: the fact that PXR contains a highly malleable ligand-binding domain means developing such virtual tools is not easy. Perseverance with this strategy is worthwhile since the availability of predictive in silico models would likely extend beyond clinical pharmacology and drug development. Virtual tools for the identification of CYP-inducing xenobiotics would likely assist the toxicological assessment of many xenobiotics with broad industrial and environmental relevance.

5.2.1.6 Other Roles of Xenosensors

Although the term *xenosensor* underscores their role in pharmacological and toxicological phenomena, these multifaceted proteins likely play diverse physiological roles. For example, ligand-activated transcription factors help control circulating levels of such endogenous molecules as the heme metabolite bilirubin as well as thyroid hormones and steroid hormones. The study of the wider biological roles of xenosensors is complicated by interspecies differences in the ligand-binding preferences of xenosensors and the genes induced upon ligand binding. Caution is needed when extrapolating findings concerning inducer properties in one species to another.

5.2.1.7 Xenosensor-Independent CYP Induction

Although xenosensor-driven transcriptional responses account for most CYP induction during xenobiotic exposure, other mechanisms apply for some chemicals. For example, receptor-independent CYP2E1 induction during chronic alcohol consumption involves increased 2E1 protein abundance due to protein stabilisation against proteolysis rather than increased 2E1 gene transcription, although the latter may occur on exposure to massive ethanol doses. CYP2E1 protein stabilisation accompanies numerous altered physiological states and pathological syndromes (e.g. fasting, diabetes, obesity, consumption of a high fat diet). The mechanisms underlying alcohol-induced CYP2E1 induction involve stabilisation against proteolytic turnover due to suppression of kinase-dependent phosphorylation of serine residues in 2E1 that normally flag proteosomal degradation. The normally short half-life of CYP2E1 protein is substantially increased upon alcohol exposure, from around 7 h in controls to ~47 h in ethanol-treated rats. The effect of alcohol on CYP2E1 abundance is not selective for liver since comparable induction occurs in other tissues including lymphocytes, the placenta and oesophagus. Substrate stabilisation against 2E1 proteolysis also occurs with other 2E1 substrates such as the anti-tuberculosis drug isoniazid and various industrial chemicals including acetone, trichloroethylene, styrene and toluene. The ability of substrates to suppress CYP proteolysis may also extend to other CYP isoforms since modest 3A4 induction also occurs upon prolonged alcohol exposure.

5.2.2 Induction of Conjugative Metabolism

Response elements for xenosensors such as AhR and PXR are located in the promoters of genes that encode many enzymes that mediate conjugative metabolism such as UGTs, SULTs, GSTs and NATs. Consequently, exposure to classic CYP inducers such as TCDD, rifampicin, phenobarbital or dexamethasone frequently increases cellular capacities for conjugative metabolism. While exploring these phenomena, researchers were surprised to learn that induction of UGTs and GSTs also accompanies exposure to xenobiotics that are not ligands for classic xenosensor proteins yet nevertheless exhibit reactivity as electrophiles. Conjugative enzyme inducers of this kind include various synthetic phenol compounds such as butylated hydroxyanisole (BHA) and *t*-butylhydroquinone (tBHQ) as well as dietary phytochemicals including the green tea constituent (−)-epigallocatechin-3-gallate (EGCG). Various isothiocyanate constituents of cruciferous vegetables such as phenethyl isothiocyanate and sulforaphane also strongly induce conjugative enzyme expression and for this reason attract great interest as anticancer agents (i.e. as constituents of 'chemopreventative' diets). Work in this area was pioneered by the NIH researcher Michael Sporn, the widely acknowledged 'father of chemoprevention'. By activating the transcription factor Nrf2 which mediates the antioxidant response, chemopreventative electrophilic compounds likely confer cytoprotection against noxious oxidants (see Sect. 5.3.2 below).

5.2.3 Induction of Membrane Transporters

Increased metabolite concentrations due to enhanced capacity for oxidative and conjugative biotransformation following inducer exposure place stresses on cellular capacities to export these species from cytosol. To offset these risks, the expression of numerous membrane transporters such as P-gp, multidrug resistance-associated proteins (MRPs), organic anion-transporting polypeptide 2 (OATP2) and the organic cation transporter OCTN2 is controlled by key xenosensors such as PXR and CAR. By boosting the cellular ability to remove metabolites at the same time as the capacity to perform metabolism is enhanced, these xenosensor proteins function as master coordinators of cellular responses to xenobiotics, helping maintain homeostasis by boosting various defences against ingested xenobiotics.

5.2.4 Deleterious Consequences of Induction

While an enhanced ability to metabolise xenobiotics is clearly beneficial during exposure to noxious chemicals that are detoxicated in the liver, this capability is a mixed blessing if organisms with a boosted metabolic capacity are exposed to

bioactivation-dependent toxicants. For toxicants undergoing complex metabolism in vivo, untangling the effect enzyme induction has upon their overall toxicity profile can be difficult, especially when the xenobiotic enters competing detoxication and bioactivation pathways.

Such issues are especially pressing during exposure to complex chemical mixtures, the classic example of which is tobacco smoke. Since smoke released from a burning cigarette contains many AhR inducers, smoking induces CYP1A isoforms that bioactivate tobacco carcinogens such as the polycyclic aromatic hydrocarbons or the tobacco-specific nitrosamines. Consistent with the formation of DNA-damaging metabolites during these reactions, high levels of DNA adducts form within the lungs of smokers. Yet tobacco smoke also contains potent inducers of enzymes that detoxicate CYP1A1-derived electrophilic metabolites. For example, Nrf2 upregulation in response to tobacco smoke electrophiles can induce the expression of epoxide hydrolase which detoxicates epoxides formed during the CYP1A1-catalyzed bioactivation of benz[a]pyrene. Nrf2 also boosts the expression of GSTs that detoxicate electrophilic diol epoxides that form via CYP1A1-catalyzed PAH metabolism. Since individuals vary in the extent to which they induce these responses, teasing out the role of particular pathways in an individual's susceptibility to smoking-related disease is complicated. The toxicological aspects of tobacco smoking are discussed in Chap. 10.

5.3 Adaptive Toxicodynamic Responses

Chapter 4 reviewed several pathways whereby electrophilic metabolites induce cell damage. A common mechanism underlying their toxicity was the tendency to attack cell macromolecules, forming adducts that disrupt core biological functions. Alongside these discoveries, as toxicologists used molecular tools to investigate cell responses to electrophiles, they learnt that cells are not fully defenceless against these damaging species. In particular, use of microarray technologies to study alterations to mRNA gene transcripts revealed that cells mount broad cytoprotective responses involving hundreds of genes to counteract chemical toxicity. In conceptual terms, these changes represent attempts by cells to counteract the toxicodynamic effects of toxicants, rather than alter their toxicokinetic fate (e.g. reduce the intracellular concentrations of the parent compound). Major cell signalling responses that counteract the toxicodynamic effects of reactive chemicals are briefly reviewed below. Note that in some instances, certain adaptive responses to reactive metabolites can be deleterious to cell function, while at others they are clearly cytoprotective. Distinguishing between these polar opposite outcomes is sometimes difficult, a consideration that is especially pertinent to the transcription factors Nrf2 and NFκB. Due to space constraints, the choice of pathways for consideration below is selective, with transcriptional responses of clear toxicological significance highlighted for attention. Note that pathways focused on repairing DNA damage during exposure to electrophiles are covered in Chap. 8 (Sect. 8.5.1.3).

5.3.1 The Heat Shock Response

The reaction of electrophiles with proteins generates adducts that, if they target critical residues that help maintain protein structure, promote protein unfolding and the exposure of motifs and domains that are normally buried within the protein structure. By diminishing the affinity for ancillary proteins that routinely bind to the target protein, such changes disrupt its 'interactome', namely, the set of proteins that normally interact with a given protein as it fulfils its usual biological functions. Other potentially deleterious outcomes occur when the formation of adducts in a target protein exposes hydrophobic domains via protein unfolding or other subtle changes in protein structure. Within the crowded intracellular environment which is crammed with proteins, newly exposed hydrophobic domains act as Velcro patches, presenting 'sticky' surfaces to other proteins, triggering protein clumping and aggregation.

Since uncontrolled protein aggregation disrupts protein functions in diverse metabolic pathways, cells counteract protein-damaging stresses by mobilising chaperone proteins that mediate the heat shock response. First discovered in *Drosophila* salivary glands as a general response to rising temperature, ongoing research revealed that the heat shock response mediates responses to diverse cell stresses that include oxidants and electrophiles. The role of heat shock proteins in protecting the liver of heavy alcohol drinkers is especially significant, although similar transcriptional heat shock responses to those elicited by alcohol's noxious metabolite, acetaldehyde, are mounted against other electrophilic compounds.

The defining feature of the heat shock response is the upregulated expression and mobilisation of many heat shock proteins (Hsps), a broad family that includes over 100 members within the human genome. These proteins typically possess an ability to refold misfolded and denatured proteins. Rapid intracellular Hsp mobilisation is observed in response to a wide range of proteotoxic stresses.

Rather than being able to refold any protein in the cell, most Hsps show preferences for particular substrates. Hence, much interest is devoted to defining the 'interactome' of a given Hsp family member. Such substrate selectivity explains why individual Hsps are more important in responding to some toxicants than others (e.g. Hsp110 helps suppress ethanol toxicity but plays little role in protecting against heavy metals). In addition to Hsp110, major human Hsps include Hsp90, −70, −60, −40, −27 and −10. Most of these proteins represent subfamilies comprising multiple members that are localised in specific cellular settings where they play specific roles. For example, Hsp90 attracts much attention during the search for new cancer drugs due to its role in cell proliferation. Hsp90 belongs to a subfamily that includes such isoforms as TRAP (expressed in mitochondria) and GRP-94 (expressed in the endoplasmic reticulum). In Sect. 5.2.1.2, we saw how Hsp90 sequesters AhR within cytosol until inducing ligands such as TCDD displace the chaperone to allow interaction with nuclear translocating signals. Such roles are typical for Hsp90: this chaperone often restricts the activity of partner proteins until an appropriate substrate or hormone becomes available.

Hsp70 is of particular importance to chemical toxicity. In addition to chaperone roles in protein refolding and the clearance of aggregated proteins, Hsp70 suppresses the ability of cells to undergo apoptotic cell death. These actions are exerted at many levels, including blocking the formation of apoptosomes by sequestering Apaf-1, a key constituent of the 'wheel of death' (see Fig. 4.9 in the preceding chapter). Hsp70 further inhibits apoptosis by binding protein mediators that can activate stress-responsive mitogen-activated protein kinase (MAPK) pathways (see Fig. 4.10 in preceding chapter). Most likely, the pronounced Hsp70 mobilisation that accompanies cellular exposure to proteome-modifying toxicants can suppress cell death via multiple mechanisms.

During sustained electrophile exposure and associated protein folding, cells must manufacture more heat shock proteins to cope with growing demands on this pathway. In most cells, basal Hsp expression is low, with their expression boosted to counteract protein aggregation during cellular stress. When demand on these pathways increases, upregulated Hsp expression is driven by the transcription factor HSF-1 (heat shock factor-1). HSF-1 is normally bound within an inactive cytosolic complex that involves other chaperones such as Hsp40, Hsp90 and Hsp70. As with the xenosensors we have considered, HSF-1 contains sequences allowing binding to specific heat shock elements (HSEs) in target genes. The protein also contains an oligomerisation domain allowing the formation of a transcriptionally active complex comprising three HSF-1 molecules within a trimeric assembly. The *regulatory domain* possessed by HSF-1 is very important to its role as a stress sensor since this domain restrains the transcriptional activity of HSF-1 under normal conditions while allowing rapid changes upon the detection of protein damage by facilitating transition from a monomer-to-trimer pro-transcriptional state.

The classic hypothesis for heat shock response activation proposes that upon exposure to electrophiles and other proteotoxic stresses, Hsps that normally bind HSF-1 are released to allow their recruitment to sites of protein damage (Fig. 5.2). The liberated HSF1 then migrates to the nucleus where it undergoes trimerisation and hyperphosphorylation. These changes confer potent DNA-binding ability, forming an active transcriptional complex that drives the transcription of Hsp genes (Fig. 5.2). The resulting boost in Hsp expression typically increases Hsp abundance from between 1 % and 2 % of total cell protein to as high as 6 % in stressed cells.

Since Hsp overexpression accompanies exposure to many toxicants, this capability likely represents an attempt by cells to withstand exposure to reactive metabolites that form during enzymatic processing of the parent compound. To date, however, since much of our knowledge concerning HSF-1 function was derived from models that involve subjecting cells to acute stresses such as heat shock treatment, there is much that is unknown concerning the precise role of mobilised heat shock proteins during chemically induced toxicity. Despite these uncertainties, it seems likely that an enhanced ability to cope with unfolded and damaged proteins is a vital adaptive response during chemical toxicity.

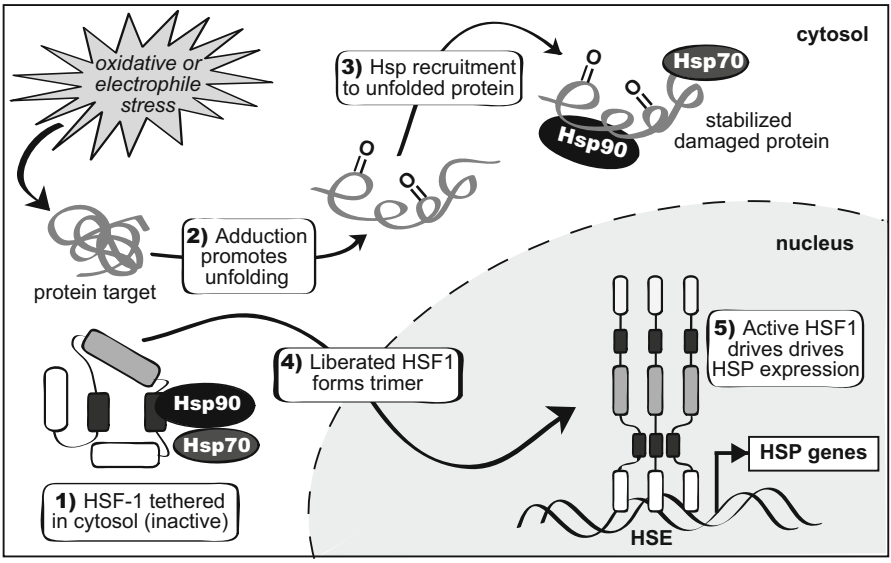

Fig. 5.2 The expression of heat shock proteins is regulated by the transcription factor heat shock factor-1 (*HSF-1*). The numbered text boxes specify the likely order in which events occur during HSP upregulation: Normally sequestered in cytosol by chaperone binding, thermal and chemical stresses promote protein damage, causing the recruitment of Hsp90 to unfolded proteins. The resulting liberation of HSF-1 allows trimerisation to form an active transcriptional activator that drives heat shock protein expression

5.3.2 The Antioxidant Response

Reactive oxygen species (ROS) production accompanies many toxic phenomena due the prooxidant properties of some xenobiotics or their ability to activate ROS production by immune cells. While cells possess many antioxidant defences including low-molecular-weight radical scavengers and a host of antioxidant proteins, in recent years attention has focused on an inducible response that confers protection against oxidants and participates in many toxic syndromes as well as diverse health disorders. Researchers surmised the existence of this pathway from long-standing studies on inducible transcription factors such as OxyR that protect bacteria against hydrogen peroxide and other endogenous oxidants. An analogous mammalian pathway was uncovered following the discovery of the role of the NF-E2-related factor 2 (Nrf2) transcription factor in the inducible expression of cytoprotective enzymes during oxidative stress. The efficacy of this response is suggested by experiments in which researchers overexpressed Nrf2-inducible target genes in cultured cells *prior* to treatment with hydrogen peroxide or electrophilic compounds: such experiments revealed significant suppression of cell death, confirming that the Nrf2 pathway is clearly cytoprotective.

Subsequent work revealed that a broad transcriptional response accompanies Nrf2 activation by toxic xenobiotics, conferring protection against electrophilic

metabolites in part by boosting cellular capacities for conjugative metabolism. This inducible antioxidant response also boosts pathways that minimise damage to DNA, proteins and lipids or, following induction of such damage, facilitate the repair or removal of damaged macromolecules. The spectrum of Nrf2-driven genes also includes proteins that help restore normal biological function by initiating tissue renewal via mitogenesis.

The antioxidant response signalling cascade depends upon Nrf2, an NF-E2-related transcription factor belonging to the large basic leucine zipper protein (bZIPs) family. Other Nrf2 family members also modulate the expression of xenobiotic metabolising enzymes (e.g. Nrf1), although their potency is weaker than that of Nrf2. As with the xenosensors already considered, Nrf2 contains a basic region next to the leucine zipper region that possesses DNA-binding activity. Nrf2 also contains a cap 'n' collar region, which is homologous to a protein found in *Drosophila*. As with most bZIP transcription factors, Nrf2 must form a dimer in order to activate transcription. Yet rather than partnering with other Nrf proteins, Nrf2 forms heterodimers with Jun family members, such as c-Jun, or with small Maf proteins, a family of transcription factors that lack transactivation domains yet exert actions at many gene promoters (Fig. 5.3). The Nrf2 complex specifically targets genes containing the antioxidant response element (ARE) within their promoters – also known as the electrophile response element – which includes over 200 genes in humans. The consensus core sequence within the ARE/ERE is '9-TGACnnnGC-3'.

Unlike xenosensors such as PXR or *Ah*R, Nrf2 is not a true receptor since it lacks a ligand-binding domain. Instead, Nrf2 resembles an 'on–off' switch that is either involved in nuclear transcription or tethered out of harm's way within cytosol. The mechanisms whereby Nrf2 is translocated to the nucleus upon oxidant or electrophile exposure have received much attention. Under normal conditions, Nrf2 activity is repressed via binding to the inhibitory binding protein Keap1 that tethers Nrf2 to the actin cytoskeleton. Rather than simply acting as a tent peg to retain Nrf2 within cytosol, to minimise Nrf2-driven gene expression Keap1 also promotes proteasomal degradation of Nrf2 by facilitating the attachment of multiple ubiquitin molecules which flag Nrf2 for proteolytic degradation.

The precise mechanisms whereby Nrf2 escapes Keap1 during heightened exposure to oxidants and electrophiles are subject to debate, but it is likely Keap1 sustains modifications to several cysteine residues that trigger Nrf2 release (Fig. 5.3). Dissociation from Keap1 confers a longer half-life on Nrf2 and facilitates relocation to the nucleus (the Nrf2 protein contains a nuclear localisation signal (NLS) within its sequence). After reaching the nucleus, Nrf2 forms complexes with other bZIP proteins, forming an active transcriptional complex that regulates the expression of a battery of genes that protect against oxidative injury. Note that in addition to this classic pathway, Nrf2 can also be upregulated via phosphorylation by cell kinases including a number of protein kinase C isoforms.

Among the hundreds of ARE-containing genes that are Nrf2 targets, many clearly provide cytoprotection against oxidative stress and electrophile-induced cell injury. The role of Nrf2 in inducing conjugative biotransformation pathways was

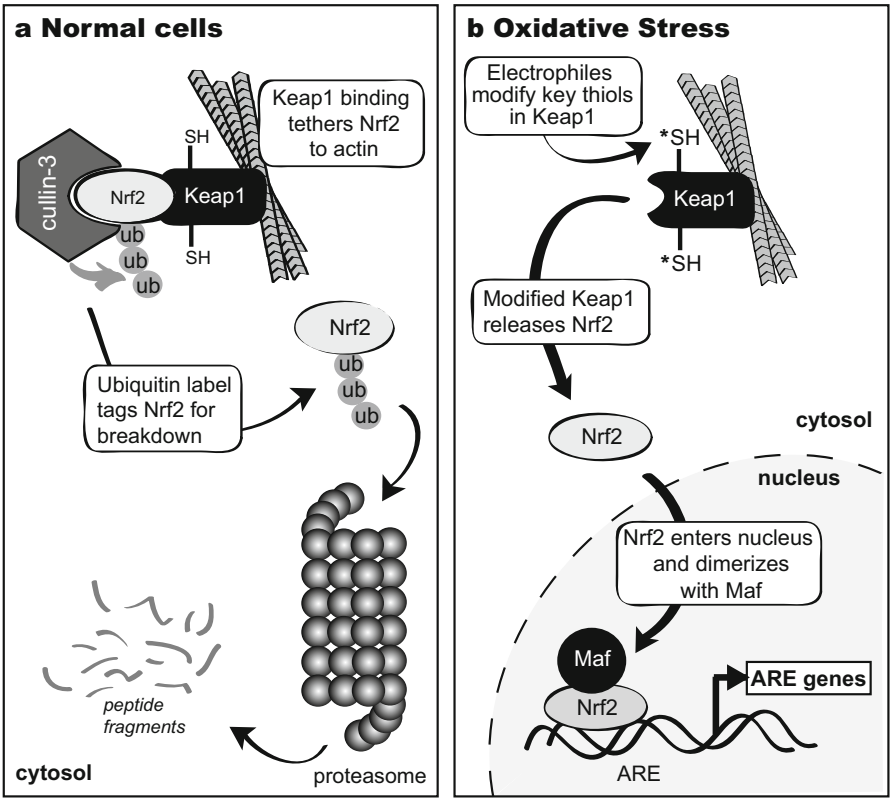

Fig. 5.3 A common transcriptional response to oxidants and electrophiles involves the transcription factor Nrf2. Normally tethered to actin via Keap1, reactive intermediates promote the release of Nrf2, allowing its entry to the nucleus where it binds to antioxidant response elements (*ARE*) within over 200 cytoprotective genes. The presence of an asterisk on the thiol group (−*SH) indicates the presence of a chemical modification within critical redox-sensitive cysteine residues within Keap1

highlighted in Sect. 5.2.2 above. Other key Nrf2-inducible gene products include the flavoproteins NADP(H) quinone oxidoreductases (NQO1 and NQO2) which detoxicate redox-cycling quinones by reducing them to hydroquinones, bypassing the formation of partially reduced semiquinones which otherwise may form damaging superoxide radicals. Nrf2-driven induction of NQO1 and NQO2 likely reduces intracellular exposure to ROS by redox-cycling xenobiotics.

Other Nrf2-inducible genes either enhance the cellular ability to manufacture the cytoprotective tripeptide glutathione or help glutathione better trap damaging electrophiles. Such upregulated gene products include glutathione-S-transferase Ya subunit (GSTYa), an efficient scavenger of reactive intermediates, and glutamate cysteine ligase (GCL), an essential enzyme catalyst of glutathione biosynthesis. Other Nrf2-inducible proteins help maintain the cellular thiol–disulfide redox balance, including glutathione peroxidase which protects cells against hydrogen

peroxide and lipid hydroperoxides formed during lipid peroxidation. Upregulation of the selenoprotein thioredoxin reductase 1 (TrxR1) gene also helps maintain the ability of thioredoxin to reduce deleterious protein disulfides that are early products of oxidative cell injury.

A major Nrf2-activated gene is heme oxygenase 1 (HO-1), which helps prevent accumulation of heme groups during oxidative stress. Although iron-containing heme groups are important prosthetic groups in many proteins, the release of heme groups via protein modification is damaging to cells since free heme catalyses production of free radicals from inorganic and organic hydroperoxides. Boosted expression of heme oxygenase 1 (HO-1) likely affords multitiered protection against oxidative injury: this enzyme rapidly degrades heme into three products, carbon monoxide (CO), biliverdin and free iron. Carbon monoxide possesses anti-apoptotic actions, while biliverdin is rapidly converted to bilirubin, an efficient radical-scavenging antioxidant. Even the availability of free iron may benefit cells since it stimulates the biosynthesis of the iron-binding protein ferritin. These benefits flowing from HO-1 induction likely confer a range of anti-inflammatory, anti-apoptotic and antiproliferative cellular benefits.

Finally, to help cells cope with proteins that are irreversibly damaged by oxidants or electrophiles, the Nrf2-driven ARE response facilitates their proteolytic clearance by upregulating the expression of proteasomal subunits needed to assemble these cellular 'garbage disposal' complexes. Collectively, the broad range of cytoprotective pathways boosted upon Nrf2 activation show how this transcriptional response strongly assists cells during exposure to prooxidants and electrophiles.

5.3.3 The ER Stress Response

We have already considered how exposure to damaging oxidants and electrophiles fosters the accumulation of destabilised, misfolded proteins within cells. Such damage particularly targets proteins within the lumen of the endoplasmic reticulum (ER), the subcellular setting to which newly made proteins are transported following their release from ribosomes. Within the ER, nascent proteins are finally processed to form functional, mature proteins via several post-translational processes including protein folding, attachment of glycosyl groups and formation of disulfide bridges that lock proteins into a final tertiary conformation. ER processing is especially important for secreted proteins and membrane proteins.

Since the ER takes active concern in the quality of its handiwork, only properly folded proteins are packaged into ER vesicles for eventual export or display on cell membranes. Cellular exposure to protein-damaging reagents places inevitable stresses upon these capabilities. By exceeding the capacity of the ER to process misfolded proteins, accumulation of abnormal, unfolded proteins induces a deleterious situation known as 'ER stress'. ER stress also occurs when electrophiles deplete cell stores of the nucleophilic amino acid L-cysteine, producing a cellular environment paralleling nutrient deprivation.

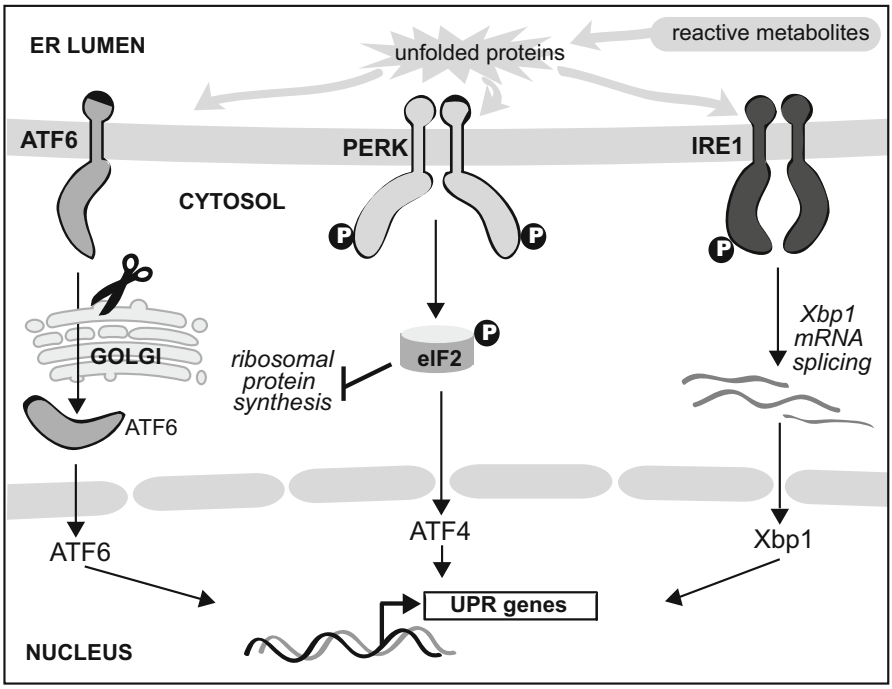

Fig. 5.4 The accumulation of damaged, unfolded proteins within the lumen of the endoplasmic reticulum (*ER*) triggers a deleterious condition known as ER stress. Several sensor systems respond by sending messages to the nucleus, promoting the expression of genes involved in the unfolded protein response (*UPR*). The end result of UPR is an increased ER volume, thereby increasing the cellular capacity to cope with unfolded proteins. Alternatively, if excessive ER stress is detected, the UPR can orchestrate the induction of cell death (Adapted by permission from Macmillan Publishers Ltd: EMBO Rep. 10: 1206–1210, 2009, Cyr and Hebert (2009 Protein quality control-linking the unfolded protein response to disease)

In the face of ER stress, cells mount a broad compensatory and cytoprotective response known as the 'unfolded protein response' (UPR) (Fig. 5.4). In terms of its basic biological rationale, the UPR brings several benefits to stressed cells. First, due to several protein sensors that detect ER stress onset, cells can activate a transcriptional response that boosts the production of key ER components such as the ER chaperone BiP/GRP78. The increased availability of these constituents allows expansion of the ER compartment to help cells overcome an overload of unfolded proteins. Other cellular outcomes during the UPR include a cessation of nonessential protein synthesis by ribosomes, as well as suppression of the cell cycle to delay further cellular proliferation. Note that the UPR also includes a 'safety valve' which, in the event capacities to process unfolded proteins are overwhelmed, initiates apoptosis in excessively damaged cells. Tissues would rather see cells die than risk the establishment of rogue cell populations displaying low standards of quality control over protein production. The finding that the UPR is activated in tissues during exposure to diverse xenobiotics indicates ER stress accompanies many chemically induced toxic syndromes.

The accumulation of unfolded proteins within ER lumen increases the nuclear activity of not one but three stress-responsive bZIP transcription factors: activating transcription factor 4 (ATF4), X-box binding protein 1 (XBP1) and ATF6. Activation of each involves distinctive signalling pathways that originate in the ER and converge on the nucleus. Each branch of the UPR is initiated by distinct signalling molecules within ER membranes: IRE1 (inositol requiring enzyme-1), PERK [double-stranded RNA-activated protein kinase (PKR)-like ER kinase] and ATF6 (activating transcription factor 6).

The first branch of the UPR involves the transcription factor ATF6 that exists as an ER membrane-spanning proprotein which is normally bound to the ER chaperone BiP/Grp78 (Fig. 5.4). Upon sensing the presence of unfolded proteins in the ER lumen, ATF6 is translocated via pinched-off ER membrane vesicles to the Golgi apparatus where it is processed by proteases form a mature, functional transcription factor. The mechanisms whereby ATF6 is committed to this relocation are poorly understood yet likely involve dissociation of the partner protein BiP. The internal (luminal) domain of ATF6 also contains intra- and intermolecular disulfide bonds that likely monitor the ER environment as redox sensors (in a similar fashion to the role played by Keap1). Following its formation in the Golgi, ATF6(N), the liberated N-terminal cytosolic fragment, is transported to the nucleus where it binds to ER stress response elements (ERSE) in target genes. Important ATF6-responsive genes encode various ER-resident molecular chaperones such as BiP/Grp78 and glucose-regulated protein 94 (Grp94, an Hsp90 family member). ATF6(N) also drives the synthesis of the ER stress-signalling transcription factor XBP1.

Activation of the second arm of the UPR involves the ER-resident transmembrane kinase PERK, which, upon sensing ER stress, forms oligomeric complexes that trigger phosphorylation of itself and the ubiquitous translation initiation factor eIF2a (Fig. 5.4). Since the latter inactivates eIF2, an essential requirement for ribosomal protein synthesis, PERK activation suppresses cellular protein production, reducing the migration of newly made proteins to the ER and thereby alleviating ER stress. The suppression of protein synthesis is not entirely effective, however, since cells continue to manufacture the transcription factor ATF4. The latter drives the transcription of numerous genes including one that plays a major role in cellular responses to ER stress, namely, CHOP (transcription factor C/EBP homologous protein), a transcription factor that controls genes which participate in apoptosis. ATF4 also drives the expression of proteins that regulate apoptosis. These capabilities ensure that while activation of PERK is cytoprotective under conditions of modest ER stress, high levels of unfolded proteins can trigger apoptosis via the ATF4-mediated pathway. The ATF4-driven arm of ER stress signalling is of special toxicological significance since this pathway is commonly activated by xenobiotics that form reactive intermediates.

Activation of the third arm of the UPR involves IRE1, a multifunctional transmembrane kinase/endoribonuclease that transmits UPR signals to the nucleus via an unusual mechanism that involves mRNA splicing (Fig. 5.4). Upon sensing ER stress, IRE1 oligomerises within the ER membrane, eliciting conformational changes that unleash its latent ribonuclease (RNase) activity. The activated IRE1

then doubly cleaves the mRNA for a UPR-specific transcription factor, XBP1 (X-box binding protein 1). The cleaved exons are then joined by uncharacterised ligases to form a spliced mRNA that is translated to generate the transcription factor XBP1. This transcription factor helps drive the biosynthesis of enzymes needed to manufacture new ER membranes.

Even the above simplified account of UPR pathways reveals cells devote considerable effort to achieving high-quality outcomes during the synthesis of secretory and membrane proteins. The fact that UPR pathways are activated during many chemically induced toxic syndromes confirms cells go to comparable lengths to counteract protein damage caused by reactive intermediates.

5.3.4 The NFκB Response

The broad transcription-activating system involving members of the nuclear factor-κB (NFkB) family is swiftly induced upon cellular exposure to diverse stresses including toxic xenobiotics, free radicals, microbial pathogens, inflammatory cytokines, phorbol esters and calcium-disrupting agents. First identified as the *rel* oncogene during studies of retrovirus-induced B-cell lymphomas in poultry, this family of NFkB transcription factors now includes five mammalian members. NFkB proteins typically exist in dimeric form within cytosol, with a heterodimer comprising a p65 and a p50 subunit most abundant in human cells.

The mechanisms underlying NFkB activation are complex, involving both canonical and noncanonical pathways. According to the former or classic pathway, NFkB mainly exists in a transcriptionally silent or inactive state due to its cytosolic association with an inhibitory polypeptide known as IκB (Inhibitor of κB) (Fig. 5.5). IκB proteins contain multiple copies of an amino acid sequence called ankyrin repeats which mediate protein–protein interactions. These protein-binding domains enable IκB to mask nuclear localisation signals (NLS) possessed by NFkB proteins, effectively retaining them within cytosol in an inactive state.

In response to activating signals, IκB is rapidly phosphorylated, marking it for ubiquitin tagging and proteolytic degradation (Fig. 5.5). The phosphorylation step is catalysed by IκB kinase (IKK). Upon eluding its inhibitor, NFkB migrates to the nucleus where it activates several hundred genes, including many immediate-early genes that are rapidly expressed in cells following diverse stresses. In keeping with other regulatory responses, NFkB targets specific response elements within the 5′-promoters of its target genes. The biological effects resulting from NFkB-induced transcriptional responses are complex and vary according to cell type and activating ligand, but in general result in inflammation, mitogenesis and cell survival.

The ability of NFkB to promote cell survival can confer cytoprotection against damaging electrophiles formed during xenobiotic metabolism. NFkB primarily upholds cell viability by inhibiting the onset of apoptosis: NFkB activation thus strongly activates the expression of numerous anti-apoptotic genes. Chief among the upregulated proteins is cIAP (cellular inhibitor of apoptosis), which can

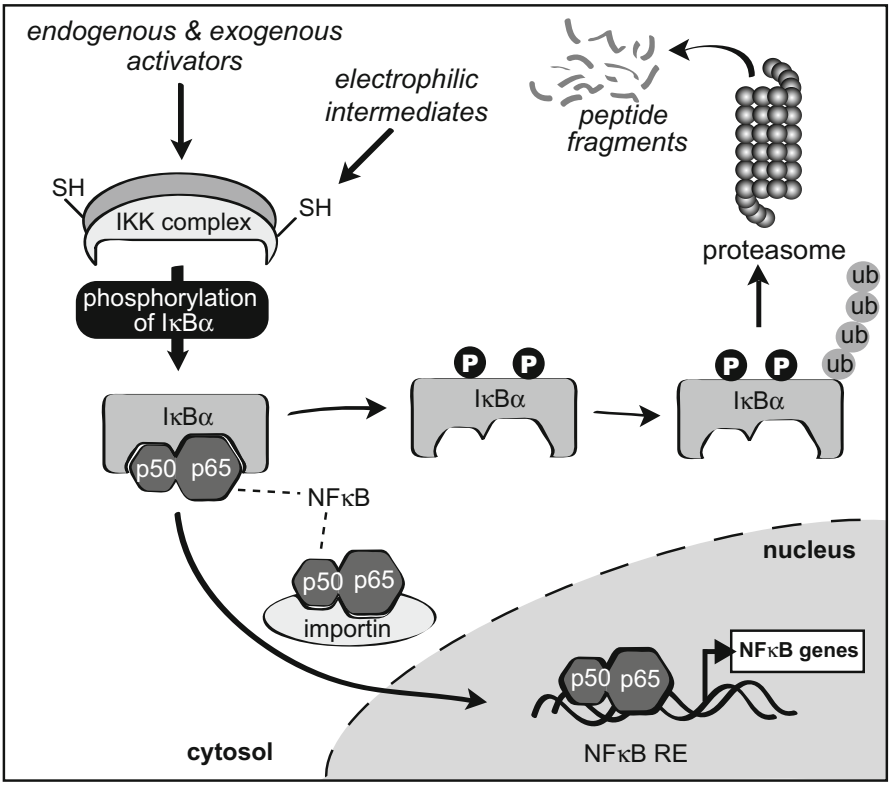

Fig. 5.5 The NF**κ**B system participates in many inflammatory conditions and has wide significance to human health. Activation the classic pathway proceeds via IKK-dependent phosphorylation of the NF**κ**B inhibitor, **IκBα**, promoting its degradation by the proteasome. The liberation of NFkB allows its importation to the nucleus where it targets response elements in over 200 genes. Activation of this pathway during chemical toxicity has many cellular consequences, although the inhibition of apoptosis is considered a key determinant of whether cells survive exposure to noxious xenobiotics

suppress cell death by directly inhibiting caspase activity although it also exerts other cell preserving actions. NFkB also increases transcription of anti-apoptotic Bcl-2 family members such as Bcl-XL and Bfl-1, thereby limiting the release of cytochrome c during the induction of cell death via the intrinsic apoptotic pathway (see Chap. 4, Sect. 4.4.5). NFkB also likely increases expression of several proteins that interfere with the extrinsic or death receptor-induced pathway of apoptosis. Finally, NFkB activation may suppress apoptosis by expressing genes that counteract mediators of stress-responsive MAPK signalling.

The ability of NFkB to block apoptotic cell death is problematic in some contexts, especially within the cancer setting where this mechanism is used by some tumour cells to prolong their lifetime or evade steps taken by tissues to kill abnormal cells. Within the context of chemical toxicity, the NFkB system helps secure cellular survival during exposure to noxious xenobiotics. Indeed, its importance is

reinforced by the finding that some highly toxic substances promote cell damage by preventing the ability of NFkB to preserve cell viability in the face of oxidant or electrophile exposure. Subverting the cell survival that accompanies NFkB upregulation is thus a common effect of toxic chemicals that possess electrophilic character such as the smoke constituent acrolein, the lipid peroxidation product 4-hydroxynonenal and various prooxidant xenobiotics. Such species may block NFkB upregulation by forming adducts on IKK, preventing the phosphorylation of the NFkB inhibitor IκB and attenuating the ability of this pathway to promote NFkB liberation and migration to the nucleus. The likelihood that xenobiotics known to form reactive intermediates can disable this crucial cellular survival pathway is under investigation in many laboratories.

5.4 Conclusion

Our preceding chapter (Chap. 4) examined several mechanisms that are thought to mediate toxicity during exposure to xenobiotics, with particular emphasis placed upon chemicals that undergo conversion to reactive, macromolecule-modifying intermediates. We saw that due to the chemical complexity and macromolecular diversity of the intracellular environment, many reactive intermediates attack numerous cellular targets, thereby eliciting broad changes in a sweep of cell pathways. In this chapter, we surveyed evidence that cells are not at the full mercy of these toxic invaders, since they possess diverse sensing systems that detect circulating xenobiotics and mount elaborate transcriptional responses that act during both the toxicokinetic and toxicodynamic phase of toxicant action. With this appreciation of the basic mechanisms underlying the deleterious effects of toxicants upon biological systems, and an awareness of the capabilities cells possess to counteract such damage, we are better placed to explore the types of toxic responses that chemicals elicit in specific body organs.

Going Further

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Chapter 6
Target-Organ Toxicity: Liver and Kidney

Abstract Accidental or deliberate overdose with the analgesic paracetamol can cause life-threatening liver damage. Likewise, extensive use of nonsteroidal anti-inflammatory drugs such as ibuprofen causes kidney damage in vulnerable patients (e.g. the elderly). In addition to these medicinal risks, liver and kidney damages accompany exposure to synthetic chemicals in the workplace or as environmental pollutants. Still other chemicals that harm the excretory organs are consumed as food contaminants. The vulnerability of the liver and kidney to xenobiotic toxicity raises the question as to why such chemicals often display 'organ selectivity' when inducing toxicity. This chapter explores the factors that render the liver and kidney susceptible to toxicity, while also surveying typical toxic responses in these organs (e.g. steatosis, fibrosis, cancer). The diverse mechanisms underlying excretory organ toxicity are illustrated for such classic toxicants as azidothymidine, carbon tetrachloride, paracetamol, troglitazone, cylindrospermopsin and trichloroethylene.

Keywords Target-organ toxicity • Hepatotoxicity • Fatty liver (steatosis) • Cholestasis • Liver fibrosis • Paracetamol • Azidothymidine • Troglitazone • Carbon tetrachloride • Thioacetamide • Aflatoxin B_1 • Cylindrospermopsin • Microcystin-LR • Nephrotoxicity • Aminoglycosides • Chloroform • Trichloroethylene

6.1 Introduction

After absorption into the systemic circulation and distribution throughout the body, few toxicants equally damage each of the 60 trillion or so cells possessed by a grown human: instead, their noxious effects are usually manifest within select *target organs* (Fig. 6.1). This chapter explores some basic considerations that govern the localisation of toxicity in distinct tissues, with special focus upon the main excretory organs of the body, the liver and the kidneys. These organs are chosen for attention as they are more commonly involved in overt organ toxicity than most other tissues. Our survey will reveal that while some toxicity is manifest in

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DOI 10.1007/978-1-4471-5553-9_6,

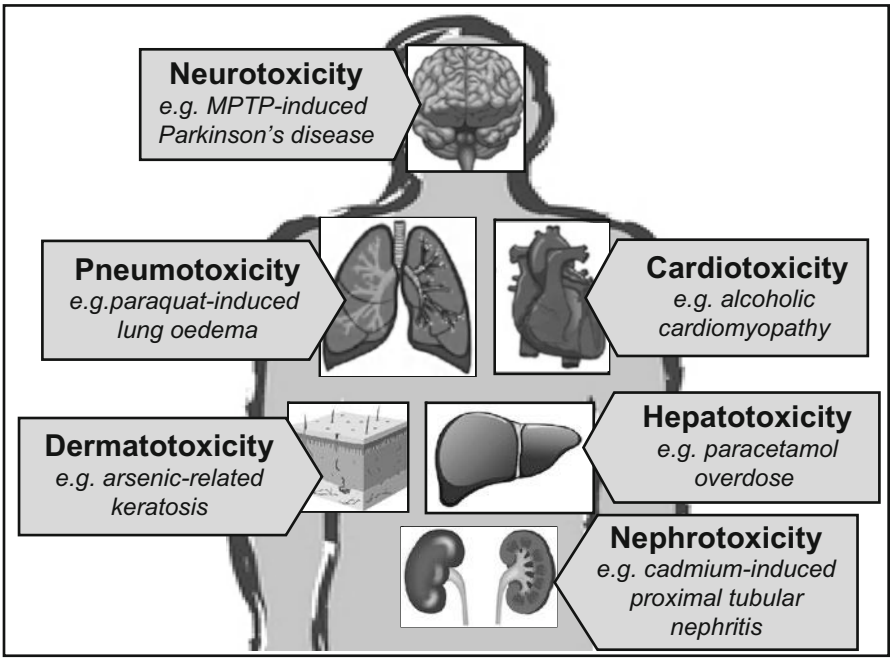

Fig. 6.1 When eliciting tissue damage, many toxicants preferentially injure a handful of *target* organs. Some representative chemically induced target-organ toxicities are shown

excretory organs because it disrupts a distinctive capability of the target tissue (e.g. cessation of bile flow in the liver, impairment of proximal tubular function in the kidney), other pathologies resemble toxicant-induced responses that can occur in any tissue (e.g. cancer, inflammation, fibrosis). If space permitted, a broader range of tissues (e.g. brain, lungs, heart) could be considered since many important toxic syndromes also involve these organs. The reading list at the end of Chap. 2 should be consulted by readers seeking comprehensive introductions to target-organ toxicity within non-excretory organs.

6.2 Factors Influencing Susceptibility

Predicting the target organ(s) that endures the most overt chemically induced toxicity is never easy and is rarely fully predictable without conducting toxicity tests in a complex biological system (i.e. a living animal or human subject). While in vitro systems such as cultured cell lines derived from different organs are useful for comparative toxicity testing or investigating some mechanisms of cell damage, the in vivo organ selectivity of toxicants is rarely predicted using cultured cells alone. For example, the finding that a renal cell line shows greater vulnerability to a given

chemical under in vitro test conditions relative to cultured hepatocytes need not imply the kidney will incur more damage than the liver in whole animals or human subjects.

Firstly, the complexity accompanying xenobiotic distribution within the whole body can hamper prediction of target-organ toxicity. Frequently, specific organs sustain damage because toxicokinetic factors ensure they accumulate especially high concentrations of a xenobiotic. In theory, concentrations of toxicants within target tissues primarily determine the severity of acute and chronic toxic syndromes; hence, tissues with highly active uptake processes are often vulnerable to toxicity. Well-perfused organs or tissues – those that receive strong blood flow – can also readily accumulate blood-borne toxicants. These considerations are especially important for the liver and kidneys – which as major excretory organs necessarily receive a high blood flow while also strongly expressing many xenobiotic transporters.

Another factor complicating prediction of in vivo target-organ toxicity is the metabolic fate of a chemical. While in silico or computational models for the prediction of xenobiotic metabolism are under development in the pharmaceutical industry and academic laboratories, these approaches rarely fully predict the complex metabolism many xenobiotics undergo within the whole body setting. Many xenobiotics generate dozens upon dozens of distinct metabolites during processing by the liver and other body tissues. Usually, most such metabolites lack toxic potential and their formation typically facilitates removal of the parent xenobiotic from the body. Yet sometimes one or more metabolites may possess strongly toxic character. Such metabolites may be of minor significance within the overall toxicokinetic profile of the xenobiotic (i.e. make a minor contribution to total body clearance) yet may rate highly in terms of toxicological significance due to effects upon one or more target organs. Moreover, such toxic metabolites often form via highly unpredictable routes. For example, a toxicant may undergo one or more rounds of metabolic processing in the gut wall or colonic contents to form benign metabolites that are later converted to the ultimate toxic species within excretory organs.

A classic example of 'multi-organ' participation intoxicant bioactivation is seen with 2,6-dinitrotoluene (2,6-DNT), a chemical reagent used during the manufacture of dyes, explosives and synthetic polymers. 2,6-DNT belongs to the *nitroaromatic* class of toxicants which notoriously induce liver tumours in rodents while displaying little or no activity in classic in vitro assays for mutagenicity (i.e. the Ames Salmonella test – see Fig. 8.10 in Chap. 8). This anomalous outcome reflects the cooperative role of the liver and GI-tract microfloral enzymes during the in vivo bioactivation of 2,6-DNT to its ultimate toxic metabolites (Fig. 6.2). The in vivo bioactivation of nitroaromatic compounds thus involves a complex interplay between two organs *and* the gut microbiome. We previously considered how such factors contributed to a major toxic disaster early in the twenty-first century, the melamine contamination of infant formula and pet food episodes (see Chap. 3, Sect. 3.4.1.2). Other examples of target-organ toxicity involve noxious metabolites that form via convoluted routes of this kind. Since predicting or reproducing the full

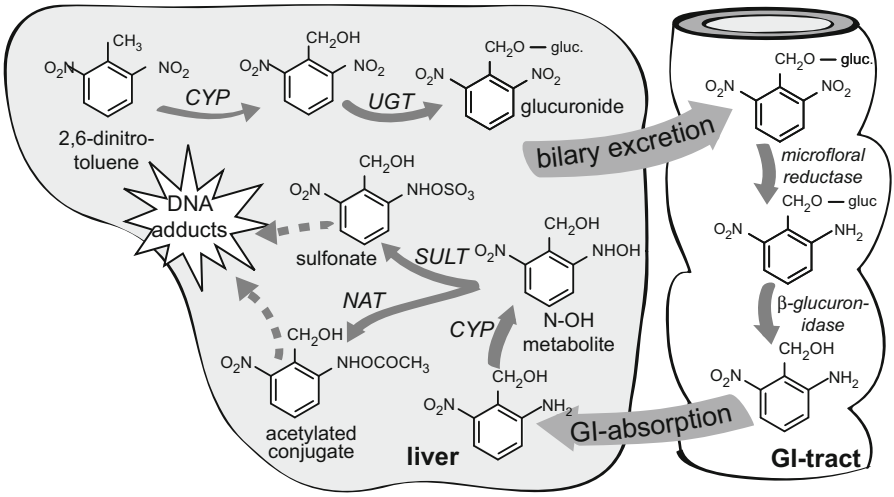

Fig. 6.2 Cooperative role of liver and GI-tract during the metabolic activation of 2,6-dinitrotoluene (2,6-DNT) to its DNA-damaging metabolites. Due to the necessary contribution of intestinal microflora to the bioactivation of nitroaromatic compounds in vivo, members of this class are inactive within in vitro test systems that cannot generate the full range of metabolites that form in whole animals (With permission Taylor and Francis Group LLC Books, Hayes (Ed), Principles and Methods of Toxicology, J Donald deBethizy and Johnnie R. Hayes, Chapter 3, Metabolism: A Determinant of Toxicity, 2001)

metabolic profile of such toxicants in cultured cell systems is often difficult, exclusive reliance upon data obtained from in vitro test systems that do not reproduce the multi-organ dimensionality of in vivo toxicity is inappropriate for some xenobiotics.

Very often, high-level expression of xenobiotic-metabolising enzymes in the liver and kidney explains why these organs are targets for bioactivation-dependent toxicants. Since many unstable metabolites cannot survive for extended periods within a biological environment, toxicity is most overt in the tissues that generate them. Such considerations are very important to many chemically induced hepatotoxicity and nephrotoxicity syndromes (see below).

Yet the role of metabolism in target-organ toxicity extends beyond bioactivation and includes cytoprotective pathways that detoxicate reactive metabolites (e.g. glutathione-S-transferases (GSTs) which inactivate electrophilic CYP-derived metabolites). Since tissues differ in the expression of individual GST isoforms, chemical toxicity may localise in tissues that cannot detoxicate a particular electrophile or other reactive species such as free radicals. For example, the low antioxidant capacity of a given tissue can confer vulnerability to redox-cycling xenobiotics or those that induce oxygen radical production via effects upon mitochondrial electron transport pathways. The unborn child is susceptible to damage of this kind since many embryonic tissues express low levels of GST proteins and antioxidant enzymes.

6.3 Hepatotoxicity

The liver's status as the largest organ in the body reflects its key roles in many physiological processes, ensuring its undisputed position as 'metabolic coordinator' of the entire body (Table 6.1). Due to the organ's importance to many body functions, any tendency for a chemical to damage the liver is taken very seriously in modern toxicology and risk assessment.

Several factors predispose the liver to xenobiotic toxicity. Firstly, for chemicals entering the body via the oral route, anatomical proximity to the GI-tract ensures the liver is the 'first port of call' for ingested xenobiotics. Secondly, chemicals and nutrients are not the only substances that enter portal blood as it perfuses the intestines: it also accumulates products of the degradation of intestinal microorganisms such as inflammogenic lipopolysaccharide components of the bacterial cell wall (i.e. endotoxin). Since endotoxin delivery may increase during xenobiotic intoxication, immunological responses to co-absorbed endotoxin can exacerbate the hepatotoxicity of ingested chemicals (this consideration is especially relevant to heavy alcohol drinkers – see Chap. 9, Sect. 9.6.4.1). Thirdly, in addition to entry via the portal circulation, chemicals can access the liver via arterial blood that mixes with venous blood in the hepatic sinusoids. For example, inhaled tobacco constituents that enter via the lungs are efficiently delivered to the liver via the arterial route. Fourthly, the vast metabolic capacities of the liver also paradoxically heighten its vulnerability to chemical toxicity: by functioning as a miniaturised chemical factory that performs many diverse chemical modifications on foreign molecules, CYPs and other hepatic enzymes can inadvertently generate noxious metabolites that induce 'bioactivation-dependent' hepatotoxicity.

6.3.1 Basic Liver Structure

At the level of gross anatomy, the human liver is separated into four unequally sized lobes which are encased by a thin capsule made of connective tissue known as Glisson's capsule. The human liver has a dual blood supply, with some 80 % of the blood entering the liver via the portal vein that drains poorly oxygenated circulation from the intestines. Orally ingested xenobiotics encounter the liver via this route. The remainder of the blood supply comprises well-oxygenated arterial blood that is delivered by the hepatic artery. These blood vessels enter at the porta hepatis where

Table 6.1 Core physiological roles of the healthy human liver

Production of bile acids	Regulation of blood clotting via production of multiple clotting factors
Fat transport around body	Metabolism of urea, drugs and foreign chemicals
Glucose metabolism and glycogen storage	Production of immune factors
Iron storage and heme synthesis	Bacterial clearance from blood

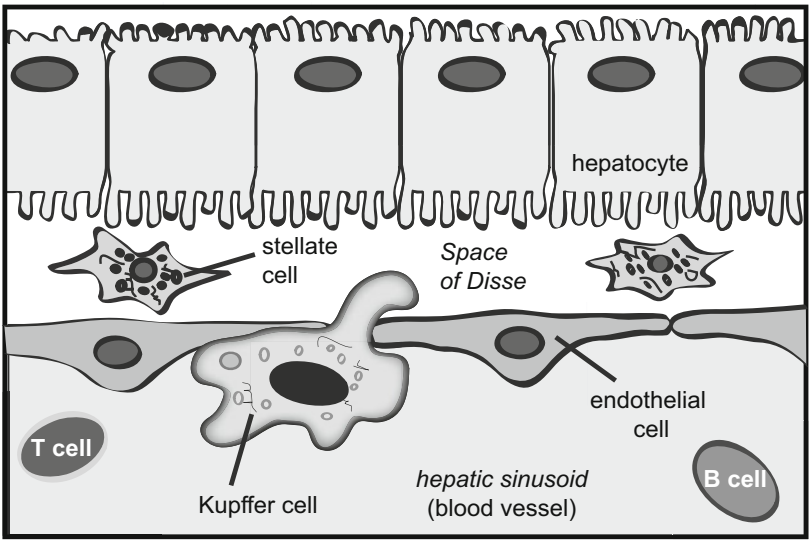

Fig. 6.3 Cross section through a hepatic sinusoid (blood vessel) shows a layer of hepatocytes running parallel to SECs (sinusoidal endothelial cells) within the vascular wall. Other liver cell types that participate in hepatotoxic responses include the Kupffer cells and the stellate cells

efferent bile ducts as well as lymphatic vessels also exit the organ. The venous drainage of the liver occurs through the inferior vena cava.

6.3.1.1 Hepatocytes

The liver comprises numerous cell types that orchestrate many chemically induced hepatotoxic syndromes. The most prevalent cell is the hepatic parenchymal cell or hepatocyte that contributes some four-fifths of the organ volume (Fig. 6.3). The unrivalled workhorses of the liver, hepatocytes carry out many essential liver functions (Table 5.1): they extract nutrients from blood, manufacture bile to aid food digestion and fat absorption, secrete metabolic waste into the bile, synthesise proteins for hepatic and nonhepatic use and store energy-rich products such as fat and glycogen. Hepatocytes also facilitate the excretion of endogenous waste products and foreign chemicals across the canalicular membrane, the region of these polarised cells that merges with the interconnecting network of minute intercellular channels known as the *bile canaliculi*. These networks meander between adjacent hepatocytes, receiving the bile secretions that subsequently drain through short bile ductules and eventually merge with bile ducts.

From the perspective of toxicology, a central function of hepatocytes concerns their ability to detoxicate lipophilic xenobiotics: hepatocytes thus express high levels of key xenobiotic-metabolising enzymes including CYPs, UGTs,

SULTs, NATs and GSTs. These properties ensure hepatocytes dictate the toxicological fate of many xenobiotics: no matter what novel pharmacological properties a candidate drug may exhibit in pharmacological bioassays, any tendency to undergo conversion to noxious metabolites during metabolic processing by hepatocytes is often a 'kiss of death' for its prospects as a human pharmaceutical.

The invention and optimisation of methods for culturing hepatocytes from lab animals and human liver samples were of major significance in the historical development of toxicology. With the use of appropriate culture media and suitable supplements, primary hepatocyte cultures can perform a wide range of xenobiotic biotransformations that reproduce most metabolic alterations the liver performs on the same compounds in vivo. Hepatocyte cultures are widely used research and screening tools during studies of xenobiotic toxicity.

6.3.1.2 Sinusoids

Within the intact liver, hepatocytes are organised in layers or laminae that are interconnected to form a distinctive three-dimensional lattice. This mass of parenchymal tissue is inter-penetrated by a network of tiny blood vessels known as sinusoids that nourish hepatocytes and carry away metabolic wastes (Fig. 6.3). Due to the complexities of hepatic perfusion, the blood carried by sinusoids comprises a mixture of venous and arterial blood which varies according the contractility of supplying blood vessels. The sinusoids are lined by sinusoidal endothelial cells (SECs) that comprise nearly half of the sinusoidal volume. The SECs form an unusually 'leaky' barrier which acts as a sieve to limit the interaction of erythrocytes and circulating immune cells with hepatocytes while allowing rapid hepatocellular uptake of small blood-borne substances including lipophilic xenobiotics.

6.3.1.3 Lobules and Metabolic Zonation

The arrangement of cellular structures within the liver lattice is classically conceptualised in terms of the *liver lobule*, a polygonal structure that is bounded at its periphery by terminal branches of the hepatic artery and portal vein, while the lobular centre is defined by the central venule. Within the hepatic lobule, hepatocytes are distributed in a linear fashion along the sinusoidal tracts, an arrangement that leads to spatial variations in oxygenation and metabolic function whereby hepatocytes inhabit three main microenvironments, namely, centrilobular, midlobular or periportal zones. Significant heterogeneity in terms of xenobiotic metabolism occurs across these settings, with the expression of CYPs and UGTs typically highest in centrilobular hepatocytes while SULTs are mainly expressed in periportal liver cells. The differences in CYP expression are reflected within the morphology of the smooth endoplasmic reticulum in centrilobular versus periportal

hepatocytes. Differences in oxygen tension across the liver lobule also influence the toxicity of some chemicals. These factors ensure many hepatotoxicants tend to damage cells in particular zones: allyl alcohol and cocaine mainly injure periportal hepatocytes, while toxicants requiring CYP-catalysed bioactivation (e.g. carbon tetrachloride (CCl_4) and paracetamol [acetaminophen]) typically induce centrilobular damage.

6.3.1.4 Stellate Cells

About one-fifth of the cell volume occupied by the sinusoids comprises hepatic stellate cells (HSC) that feature prominently in chronic toxic responses to chemicals such as ethanol. These fat-storing cells (also known as Ito cells) are located in the small perisinusoidal gap between sinusoids and hepatocytes known as the space of Disse (Fig. 6.3). Stellate cells comprise about 5 % of the total cells in the liver and normally exist in a quiescent state. Their physiological functions while in this dormant state are unclear but likely include roles in the storage of vitamin A and other fat-soluble vitamins as well as functions in the immune system. Stellate cells also help manufacture the *extracellular matrix*, a key form of connective tissue that 'glues' liver cells into position within the overall organ structure. Upon exposure to noxious chemicals including alcohol, stellate cells enter a dangerous activated state in which they become highly proficient factories dedicated to collagen production. The deposition of collagenous scar tissue is thus characteristic of liver fibrosis, a common accompaniment of chronic exposure to many hepatotoxicants including heavy metals, alcohol and dietary mycotoxins.

6.3.1.5 Kupffer Cells

Another prominent cell type within the liver are the resident macrophage-like immune cells, the Kupffer cells. Numbers of Kupffer cells in the liver typically exceed resident phagocytic cell counts in other organs and are typically higher in periportal zones than in centrilobular areas. Since Kupffer cells express Toll-like surface receptors that are activated by ingested endotoxins, they help phagocytise blood-borne toxicants and particulates received from the portal circulation. Upon activation, Kupffer cells release a variety of mediators that affect the function of neighbouring cells, including prostaglandins, cytokines, interferons, platelet-activating factor, lysosomal enzymes and reactive oxygen species. In many toxic syndromes, these changes create a persistent inflammatory state in which neighbouring tissue is continually exposed to damaging oxidants and inflammatory cytokines. This unrelenting barrage often leads to stellate cell activation and a sustained fibrotic response to hepatotoxicants. Yet Kupffer cells are more than paratroopers that attack invading pathogens or absorbed toxicants during times of stress – a large body of data suggests they also participate in many beneficial host defence pathways.

6.3.2 Major Toxic Responses of the Liver

Traditionally, chemicals that cause liver injury were labelled *intrinsic hepatotoxicants* if they induced predictable, dose-related liver injury in a majority of exposed individuals. *Idiosyncratic hepatotoxicants* on the other hand typically elicit hepatotoxicity in a small minority (approximately <1 in 100,000) of exposed individuals. The severity of the hepatic response to an idiosyncratic hepatotoxicant is often assumed to bear little relation to the administered dose, although recent studies suggest this is not necessarily always the case for drug-induced allergic hepatotoxicity.

Hepatotoxic responses can often be distinguished on the grounds of whether they can be reproduced in rodent species: as a rule, intrinsic hepatotoxicants usually induce a comparable hepatotoxicity in lab animals, whereas idiosyncratic toxicity is often hard to reproduce in rodents. Historically, chemicals inducing intrinsic hepatotoxicity were often assumed to inflict hepatotoxicity via non-immunologic mechanisms, while idiosyncratic toxicity was typically attributed to immune-mediated responses such as antibody production and T-cell activation.

While these broad categorisations retain some use during the classification of hepatotoxic chemicals, the assumption that the presence or absence of immune mechanisms is of defining importance is no longer valid. Immunologic mechanisms are now thought to contribute to the pathogenesis of many hepatotoxic syndromes, including acute intrinsic hepatotoxic syndromes that were once thought to exclude immunological pathways.

Studies of the effects of hepatotoxicants upon liver histology have revealed a surprisingly constrained cluster of toxic responses. Thus, toxic responses to hundreds of chemically diverse hepatotoxicants typically feature a handful of well-defined pathological responses.

6.3.2.1 Fatty Liver

An early sign of liver injury upon exposure to diverse hepatotoxicants is *steatosis*, a reversible condition in which small or large fatty vesicles(i.e. micro- or macrovesicular droplets) emerge within the cytoplasm of parenchymal cells throughout the liver lobule. These typically comprise triglyceride-filled droplets that are coated with phospholipids and specific lipid droplet-associated proteins, most notably members of the PAT protein family that assist triglyceride storage in adipocytes. Ongoing exposure to hepatotoxicants that induce droplet formation often triggers the emergence of more overt liver pathology.

Diagnosing xenobiotic-associated steatosis is challenging for clinicians since fatty liver occurs in a wide range of common health disorders, including nonalcoholic fatty liver disease (NAFLD) which afflicts patients suffering from obesity and metabolic syndrome, as well as diverse steatotic conditions that occur in patients with micronutrient deficiencies or chronic viral infections.

The mechanisms underlying fatty droplet accumulation in intoxicated liver are complex. Long-standing biochemical explanations attributed fat deposition to toxicant-induced shifts in the hepatocellular redox state that favour the accumulation of fatty acids rather than their oxidation, but this mechanism is hard to prove for all steatogenic compounds. Newer insights gained from microarray studies of steatotic livers have identified changes in the expression of numerous proteins involved in hepatic lipid metabolism, including dysregulation of transcription factors that control fatty acid synthesis (e.g. SREBP, sterol regulatory element binding protein) as well as fatty acid oxidation (e.g. PPAR, peroxisome proliferator-activated receptor). Such microarray studies of steatogenesis suggest a transcriptional shift towards an 'adipogenic' state in which the liver boosts its capacity for fatty acid synthesis and simultaneously downregulates fatty acid oxidation and secretion of very-low-density lipoproteins (VLDLs). These explanations may not represent the full story, however, and Chap. 9 will explore a new mechanism involving lipid peroxidation products that helps explain the onset of fatty liver in heavy drinkers (Sect. 9.7.1.1).

6.3.2.2 Cell Death

Cell death within the liver typically proceeds via either necrosis or apoptosis. Apoptotic cell death involves small clusters of hepatocytes and proceeds via a tightly orchestrated sequence of molecular events that involve the controlled digestion of cellular components by cell death enzymes such as caspases (briefly explored in Sect. 4.4.5). Alternatively, heavier intoxication with hepatotoxicants can induce a more overt, uncontrolled form of cell death known as *hepatic necrosis*. An irreversible process involving the death of many hepatocytes, necrotic liver injury may be 'focal' (i.e. localised to specific zones as in the case of centrilobular or periportal necrosis) or 'fulminant' (rapid in onset and affecting a majority of liver cells). Although its early stages involve reversible cell swelling, events quickly progress to irreversible necrotic tissue destruction.

Typical signs of cell death by necrosis include loss of membrane integrity, swelling of mitochondria and other intracellular organelles, ATP depletion and loss of calcium homeostasis secondary to calcium influx. The latter change activates calcium-dependent endonucleases, proteases and phospholipases that begin digesting key cell components, leading to cytoskeletal derangement and cell blebbing. Cell lysis and striking changes in the organisation of nuclear DNA (e.g. pyknosis, karyorrhexis and karyolysis) are also conspicuous in necrotic liver.

Hepatic necrosis occurs upon intoxication with many noxious substances including medicinal agents, natural substances and synthetic chemicals. In industrialised nations, the leading cause of hepatic necrosis in emergency department patients is often paracetamol intoxication (see below). Although the liver has a remarkable regenerative capacity that ensures rapid regrowth after acute intoxication with paracetamol and other hepatotoxicants, some types of hepatic necrosis trigger the formation of persistent scar tissue.

Since liver necrosis releases hepatocellular constituents into the bloodstream, measuring the levels of common liver enzymes such as alanine transaminase (ALT) or aspartate transaminase (AST) within blood samples is commonly used during the evaluation of xenobiotic-exposed patients. The greater the liver injury sustained by a patient, the higher the ALT and AST levels in their blood, with up to 1,000-fold elevations seen in serious cases of hepatic injury. Some caution is needed when interpreting clinical 'liver transaminase' data since these enzyme markers are not entirely liver specific; their expression in skeletal and cardiac muscle ensures damage to these tissues can also elevate blood transaminase levels. Moreover, modest elevations in plasma transaminases are not necessarily predictive of a progressive hepatotoxic response: for patients receiving some drugs such as the Alzheimer's medication tacrine, doctors may tolerate a modest 'asymptomatic' elevation in ALT levels.

Plasma markers can sometimes provide subtle insights into the nature of the hepatic insult: elevations in alkaline phosphatase and γ-glutamyltranspeptidase (GGT), for example, can indicate impaired biliary excretion rather than hepatocellular necrosis. Measuring an array of enzyme markers can thus clarify the nature of the hepatic damage occurring in poisoned patients.

Recent years have witnessed testing of new diagnostic approaches to detect chemically induced liver toxicity. Changes in the abundance of circulating microRNAs – short pieces of RNA that help regulate the expression of gene networks – represent one attractive possibility. Animal experiments suggest that hepatotoxic doses of model toxicants such as acetaminophen or carbon tetrachloride up- or downregulate particular clusters of microRNA molecules in both blood and urine samples. While these approaches seem promising, whether their sensitivity or specificity in intoxicated human subjects is better than traditional enzymological approaches largely awaits future clarification.

6.3.2.3 Impaired Bile Flow

As the largest gland in the body, the bile-secreting capacity of the liver rids the circulation of diverse endogenous waste products as well as foreign xenobiotics and their metabolites. Bile also contains cholesterol-derived detergent-like substances that help emulsify ingested fats. Some hepatotoxic chemicals disrupt these core hepatic functions by eliciting *cholestasis,* namely, a partial or complete arrest of bile flow. This has immediate physiological consequences since the bile is a major elimination route for bilirubin and biliverdin, toxic chromogenic pigments which form during the degradation of heme-rich red blood cells. During normal liver function, bilirubin and biliverdin are actively exported into the tubular canalicular network that eventually drains into the bile duct. Since some hepatotoxic chemicals inhibit the membrane transporters that export these pigments across canalicular membranes, the impaired hepatic clearance of biliverdin and bilirubin causes their deposition in blood and body tissues, with their accumulation in dermal layers conferring a yellow tinge to the patient's skin. Jaundice – a hyperbilirubinaemic state – is also common in newborn babies due to poor conjugative metabolism within neonatal

liver. Jaundice also accompanies some drug-related hepatotoxicity syndromes (e.g. it is particularly common among heavy users of anabolic steroids). Unresolved jaundice can precipitate a medical emergency since the accumulation of unconjugated bilirubin in body tissues and particularly the brain can elicit cellular injury.

6.3.2.4 Liver Fibrosis

Continuing exposure to hepatotoxicants frequently promotes excessive deposition of extracellular matrix proteins such as collagen, leading to the serious condition known as liver fibrosis. Although the histology accompanying fibrotic responses to structurally diverse hepatotoxicants is often similar, the mechanisms involved are frequently dissimilar and complex. Activated hepatic stellate cells (HSCs) are the main effectors of fibrosis, although myofibroblasts play a substantial supporting role, especially in the deposition of collagen fibres throughout the canalicular tracts of the liver lobule. Fibroblasts originating in extrahepatic tissues including bone marrow may also assist fibrogenesis.

The molecular factors that trigger fibrosis have received much attention. Although a complex cocktail of cytokines and other mediators can arouse HSCs from their normal quiescent state to become profibrogenic factories, platelet-derived growth factor (PGDF) released from activated Kupffer cells is likely most important. Other mediators assist by suppressing the normal apoptotic death of HSCs, thereby prolonging their duration of fibrogenic activity. The complexity of the biology underlying fibrosis unfortunately means few effective therapies are available for this condition, beyond cessation of xenobiotic exposure. In the case of alcoholic fibrosis, continued drinking achieves transition to the cirrhotic phase of alcoholic liver disease, a terminal condition in which uncontrolled fibre deposition and widespread hepatocellular death leaves the liver a shrunken mass of dysfunctional tissue (see Chap. 9).

6.3.2.5 Liver Cancer

Primary liver cancer is relatively uncommon in Western nations but more prevalent in African and eastern Asian populations. Variations in contamination of the food supply with fungal carcinogens likely contribute to these geographical differences (see below). Liver cancer arises from tumour cell clusters that are typically *monoclonal* in origin, meaning they originate when cells acquire genetic changes that confer growth advantages upon their descendants. Within the liver, tumours can arise in cells of the bile duct or hepatic blood vessels, although they most commonly originate in hepatocytes. The prognosis following the detection of liver cancer is usually dire since it is typically a highly metastatic, aggressive form of cancer. The disease is often highly advanced by the time of diagnosis. Liver tumours can accompany chronic exposure to alcohol and a wide range of occupational hazards including the toxic metal arsenic and the industrial reagent vinyl chloride. Chapter 8 devotes special attention to the mechanisms underlying cancer induction by xenobiotics.

6.4 Major Hepatotoxicant Classes

Many structurally diverse chemicals have been associated with hepatotoxicity in humans. These include chemicals that are used as medicinal agents, reagents that are employed during particular occupational practices in the workplace and hepatotoxicants that arise from natural sources such as plants and fungi. A growing contribution to the global burden of human liver injury reflects the rising popularity of herbal remedies and dietary supplements. Due to the diversity of substances that induce liver injury, geographical differences are seen in the relative importance of different causative agents: antibiotics, anticonvulsants and psychotropic drugs are leading causes of hepatotoxicity in Western societies, whereas in Asia, 'herbs' and 'health foods or dietary supplements' represent a leading cause. Discussion of the most widely consumed human hepatotoxicant – alcohol – is reserved until Chap. 9.

6.4.1 Hepatotoxicity and Drugs

Since the oral route is the preferred route of delivery for human pharmaceuticals, the liver is exposed to high concentrations of ingested drugs, ensuring hepatotoxicity features prominently among drug-induced adverse responses. Despite several decades of intense investigation, these problems remain a significant challenge within clinical medicine and the pharmaceutical industry alike.

Drug-induced liver injury (DILI) can occur under several exposure scenarios, although the boundaries defining each category are not watertight. Firstly, for some drugs such as paracetamol, hepatotoxicity mainly occurs after ingesting large 'supratherapeutic' doses such as occurs during a suicidal 'overdose' attempt. Still other types of DILI occur upon chronic, ongoing exposure to a drug following months or even years of continual use, such as occurs in epileptic patients who take anticonvulsant drugs such as carbamazepine or valproic acid to prevent seizures or in HIV-infected patients who consume a lifetime cocktail of antiretroviral drugs to suppress AIDS onset. For still other drugs, hepatotoxicity involves an allergic component, and DILI only manifests unpredictably in sporadic cases with variable latency, often following resumption of the use of a drug that the patients might have taken years previously without unwanted effects. Each of these different types of drug-induced hepatotoxicity responses is of interest to clinicians, toxicologists and pharmaceutical innovators alike. Indeed, within the latter context of the drug development enterprise, discovery of hepatotoxic properties during routine testing of candidate drugs usually sounds the death knell for that compound. Also, since rare forms of DILI only become obvious upon the use of drugs in large, genetically diverse populations, hepatotoxicity concerns have led either to many drugs being removed from the market entirely or to their use being sharply curtailed (Table 6.2).

Table 6.2 Select pharmaceutical agents known to cause liver damage in humans

Drug	Drug use	Toxic mechanism	Current status
Pemoline	Attention-deficit hyperactivity disorder	Allergic hypersensitivity (immune system dependent)	Withdrawn 2005 (USA)
Rosiglitazone	Diabetes	Allergic hepatitis	Withdrawn 1997 (UK)
Didanosine	AIDS	Toxic to liver cell mitochondria	In use (liver monitoring)
Amoxicillin–clavulanate	Antibacterial	Cessation of bile formation	In use (warnings)
Bromfenac	Anti-inflammatory	Fulminant hepatic necrosis	Withdrawn 1998 (USA)
Tolcapone	Parkinson's disease	Acute hepatotoxicity (rare and reversible)	Restricted use (USA)

6.4.1.1 Paracetamol: Liver Injury on Acute Overdose

Few drugs in current use exhibit a more conflicted 'Dr Jekyll and Mr Hyde' personality than paracetamol (acetaminophen): freely obtainable from supermarkets and gas stations on account of its remarkable safety at low doses yet exhibiting overt, life-threatening hepatotoxicity under overdose conditions. Paracetamol became increasingly popular as an alternative to aspirin in the aftermath of World War 2, and for many years, few clinicians knew of the drug's unappealing dark side. This changed in 1966 when Thompson and Prescott published their famous case report in the *British Medical Journal* describing severe liver damage in a 54-year-old Scotsman who overdosed on 70 paracetamol tablets. Thanks to publication of similar episodes, the popularity of paracetamol in suicidal poisonings increased to the extent that it became a leading cause of admission for acute liver damage in hospital emergency departments in many countries.

The multiple personalities possessed by paracetamol are entirely due to metabolism (Fig. 6.4). As a phenolic compound, around 90 % of a low dose is effectively cleared via conjugative metabolism to form O-sulfonate and O-glucuronide metabolites. Only a small proportion undergoes renal clearance to appear unmetabolised in urine. The final metabolite, which comprises just 2–3 % of a conventional adult dose, forms via 2-electron oxidation by CYP2E1 or 3A4 to form a highly reactive quinoneimine metabolite, NAPQI (Fig. 6.4). Under normal conditions, NAPQI is detoxicated via conjugation with glutathione in a classic GST-catalysed reaction.

Upon paracetamol overdose, the various conjugative pathways are overwhelmed due to depletion of the cofactors PAPS and UDPGA (required by SULT and UGT, respectively). Consequently, a greater proportion of paracetamol is oxidised by CYP, forming dangerously high levels of NAPQI that deplete hepatic glutathione stores and leave the hepatocellular proteome vulnerable to attack. According to the Target Protein Database, some 36 proteins are known targets for NAPQI within the paracetamol-poisoned liver (http://tpdb.medchem.ku.edu:8080/protein_database/index.jsp, accessed April 2013). Although attempts have been made to surmise the biochemical basis for paracetamol hepatotoxicity from the identity of the proteins

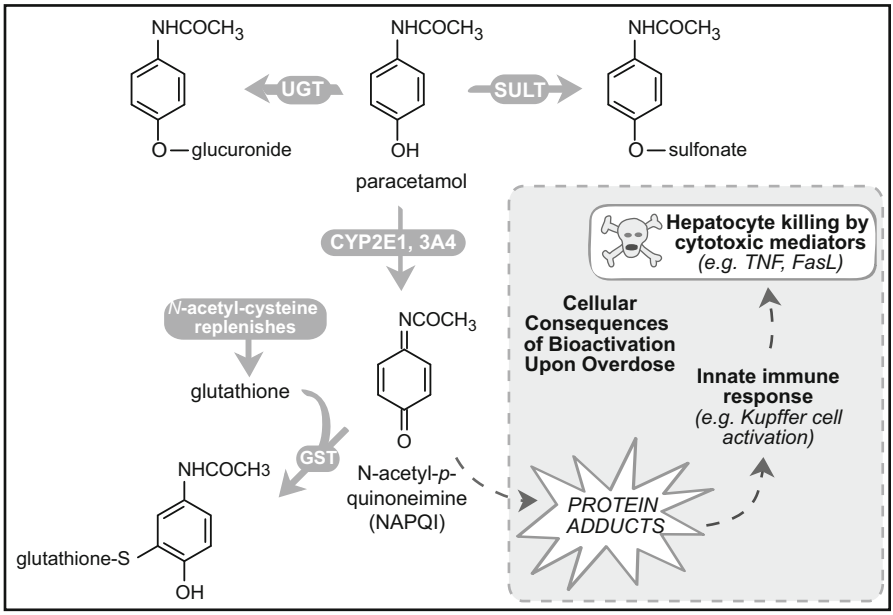

Fig. 6.4 At normal doses, paracetamol is converted to nontoxic O-sulfonate and O-glucuronide metabolites, with only a small proportion of the drug undergoing oxidation to the toxic quinoneimine NAPQI. These low levels of NAPQI are efficiently detoxicated via glutathione conjugation. Upon overdose, conjugative metabolism is overwhelmed and NAPQI causes extensive adduction of hepatocellular proteins, triggering liver cell death via a combination of pathways that include activation of the innate immune response

that are targeted by the electrophilic metabolite, these efforts have met limited success. A modern understanding of paracetamol hepatotoxicity instead invokes activation of the innate immune system following widespread protein damage and glutathione loss as the main determinant of liver damage (Fig. 6.4). The extent and severity of hepatotoxicity likely depends on whether anti-inflammatory or proinflammatory immune responses dominate within the liver of an individual paracetamol-poisoned patient.

Early discovery of the role of NAPQI in paracetamol hepatotoxicity helped identify glutathione-replenishing drugs for use as antidotes in poisoned patients (Fig. 6.4). This discovery was assisted by the fact that susceptible mouse strains were quickly identified as useful models of paracetamol hepatotoxicity in humans, thereby allowing testing of various antidote drugs. Compounds that mimicked the effects of glutathione within the liver proved especially attractive, with N-acetyl cysteine showing particular effectiveness in mice. However, while N-acetyl cysteine is effective in most patients, the drug carries its own safety concerns that include a propensity to serious allergies in small numbers of patients.

Due to uncertainty concerning the actual dose of paracetamol ingested by a patient or the precise timing of ingestion, the triage of paracetamol-poisoned

patients – the identification of patients at risk of hepatotoxicity – is often challenging. While determination of paracetamol concentrations in poisoned patients has long been the mainstay of patient management, these approaches are not effective in all patients. The ability to assess the probability of toxic responses within the liver of an actual patient would represent a major advance. Intriguingly, the recent use of toxicogenomic approaches (e.g. microarrays) to monitor changes in gene expression within blood cells collected from paracetamol-poisoned patients has yielded promising results that suggest particular clusters of mRNAs may serve as 'signatures' to predict toxicity severity in a given patient. In an era of competition for hospital beds due to rising health-care costs, any improvements in the identification of paracetamol-poisoned patients that are at genuine risk of life-threatening outcomes will likely be beneficial.

6.4.1.2 Azidothymidine: Liver Injury on Extended Use

In one of the great achievements of modern pharmacology, within 25 years of the discovery of the human immunodeficiency virus (HIV) as the cause of AIDS, 25 antiretroviral drugs were available for this devastating condition. Later, the finding that AIDS suppression is achievable by administering several anti-HIV drugs concurrently was a key breakthrough in the mid-1990s. Although the spectre of antiviral resistance remains of growing concern, these so-called HAART protocols (highly active antiretroviral therapy) ushered in an era in which HIV infection became a manageable chronic condition for most patients.

Unfortunately, it soon emerged that success in AIDS prevention came at a cost to patients who daily ingest a powerful drug cocktail: HAART recipients displayed such an elevated risk of DILI that liver disease mortality became a leading cause of death in HIV patients. Given the multiplicity of anti-HIV drugs available and the plethora of prescribing permutations, HAART-related hepatotoxicity is a nebulous and variable syndrome comprising both acute and chronic disorders. Some cases involve symptoms that are typical of rare allergic DILI syndromes, while others involve hepatic steatosis with lactic acidosis, while still others resemble viral hepatitis and can be confused with comorbidities due to hepatitis B or C injection. Given the profound benefits that accompany suppression of HIV replication, clinicians involved in the long-term management of these patients face a prescribing dilemma when confronted by signs of nonspecific liver injury (e.g. an asymptomatic elevation in blood transaminase levels): if viral loads are successfully suppressed with the existing drug, should a prescribing switch be made to a newer anti-HIV drug with lesser hepatotoxic potential?

Most research into HAART-related hepatotoxicity has focussed on the nucleotide/nucleotide reverse transcriptase inhibitor (NRTI) family of HIV drugs. Ever since the 1985 discovery of the first member of this class, azidothymidine (AZT), NRTIs have formed a mainstay of anti-HIV therapy. NRTIs inhibit a key viral protein needed for HIV reproduction, reverse transcriptase (RT), a member of the atypical RNA-dependent family of DNA polymerases. NRTIs typically act as nucleotide mimics, undergoing phosphorylation on their 5′-hydroxy group by cellular kinases

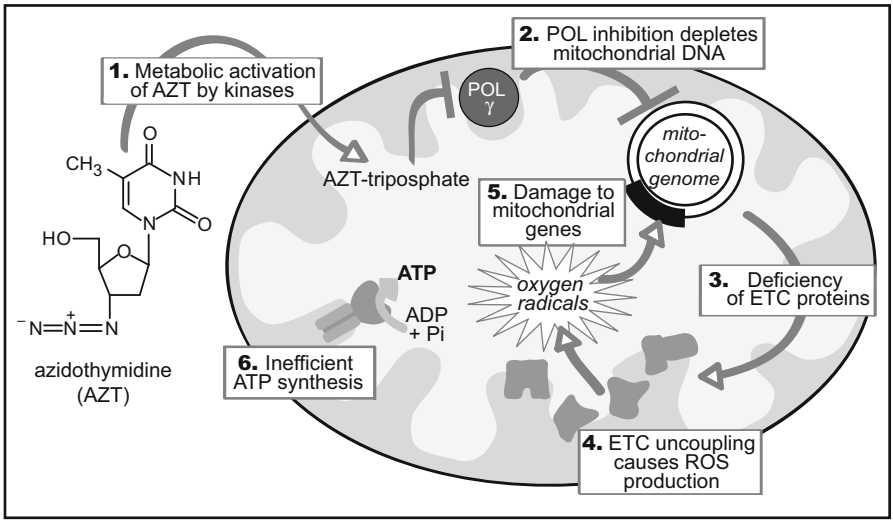

Fig. 6.5 Liver injury in HIV-infected patients receiving the NRTI azidothymidine (*AZT*) involves mitochondrial toxicity due to inhibitory effects of AZT-triphosphate on Pol **γ**. The resulting depletion of electron transport chain (*ETC*) proteins leads to overproduction of reactive oxygen species (due to overutilisation of Complex II). The subsequent oxidative DNA damage promotes further loss of ETC proteins

before they are incorporated into DNA by RT. The lack of a free 3′-hydroxy group prevents extension by DNA polymerase, thereby terminating further DNA synthesis. Despite the success of this approach within HIV-infected patients, a number of limitations plague the clinical use of NRTIs including emergence of drug-resistant RT mutants and host toxicity including neuropathy and, of particular concern, hepatotoxicity.

Although several mechanisms may participate, NRTI hepatotoxicity is mainly due to inhibition of human mitochondrial DNA polymerase γ (Pol γ). Pol γ, a member of the A family of DNA polymerases, closely resembles HIV RT in structural terms, ensuring Pol γ is more likely than other human DNA polymerases to utilise NRTIs as enzyme substrates during DNA replication. Since Pol γ helps copy the mitochondrial genome, inhibition of this enzyme by NRTI leads to side effects that are consistent with a loss of mitochondrial function within the liver of drug-treated patients (Fig. 6.5). Impaired production of respiratory chain proteins leaves the NRTI-treated liver ATP deficient and vulnerable to mitochondrial uncoupling and associated overproduction of damaging free radicals (Fig. 6.5). While the liver is most vulnerable to such toxicity, other tissues can exhibit mitochondrial pathology during long-term AZT therapy (Table 6.3). Recognition of the role of Pol γ inhibition in NRTI side effects inspired the development of alternative NRTIs where such problems are less pronounced, but the low cost of AZT relative to newer drugs ensures this drug is still popular in some countries, especially within the developing world where HIV infection is of great concern.

Table 6.3 Toxic syndromes accompanying use of NRTIs in HIV-infected patients (With kind permission from Springer Science+Business Media: Iatrogenic Mitochondriopathies: A Recent Lesson from Nucleoside/Nucleotide Reverse Transcriptase Inhibitors, 2012, George P. H. Leung)

NRTI	Year approved	Hepatotoxicity (incl. steatosis)	Peripheral neuropathy	Skeletal myopathy	Pancreatitis
Azidothymidine	1985	√	√	√	√
Didanosine	1991	√	√	√	√
Stavudine	1994	√	√	√	↔
Zalcitabine	1992	√	√	√	↔
Lamivudine	1995	↔	↔	Rare	Rare

6.4.1.3 Troglitazone: Liver Injury in Susceptible Individuals

The approval of troglitazone in several countries in 1997 was celebrated as a significant advance in the treatment of type 2 diabetes, a serious metabolic disorder featuring inadequate control of blood sugar levels. Although the prevalence of diabetes has risen in recent decades, little innovation had occurred in this field of pharmacology for many years until troglitazone became the first member of the new thiazolidinedione class of antidiabetic medicines. As a ligand for PPARγ, a member of the peroxisome proliferator-activated receptor family, troglitazone acted as an 'insulin sensitiser' to help body tissues remove glucose from blood more effectively following meals. Despite these promising properties, alarm bells rang within a few months of troglitazone release onto the market as doctors in Japan, Britain and the USA began reporting life-threatening idiosyncratic hepatotoxicity. The manufacturers withdrew the drug from the market a few years later. In total, some 60–70 deaths were attributed to troglitazone worldwide. As with most idiosyncratic toxicities, troglitazone-induced liver disease was quite unpredictable, with serious hepatotoxic injury estimated to occur in approximately 1 in 10,000–20,000 patients.

The mechanisms underlying troglitazone hepatotoxicity are likely complex. The parent drug itself may play a role since it can directly induce apoptosis in both hepatocytes and nonhepatic cells. However, a contribution for metabolism and bioactivation is also likely, since the unusual vitamin E-like chroman ring possessed by troglitazone undergoes CYP-catalysed oxidation to a cocktail of toxic intermediates including ring-opened electrophiles, quinone metabolites, phenoxy radicals and epoxides (Fig. 6.6). Since troglitazone is a ligand for the xenosensor protein PXR, a potent inducer of CYP3A expression, it is likely troglitazone increased the formation of reactive metabolites by inducing its own CYP-dependent bioactivation.

A key role for reactive metabolites in toxicity was suggested by Japanese researchers who studied genetic polymorphisms in xenobiotic biotransformation pathways as risk factors in patients who developed troglitazone hepatotoxicity. Their findings suggested patients who carried twin null mutations in two key glutathione-S-transferase genes (GSTT1 and GSTM1) were significantly overrepresented among at-risk patients. Since these mutations likely compromise the cellular

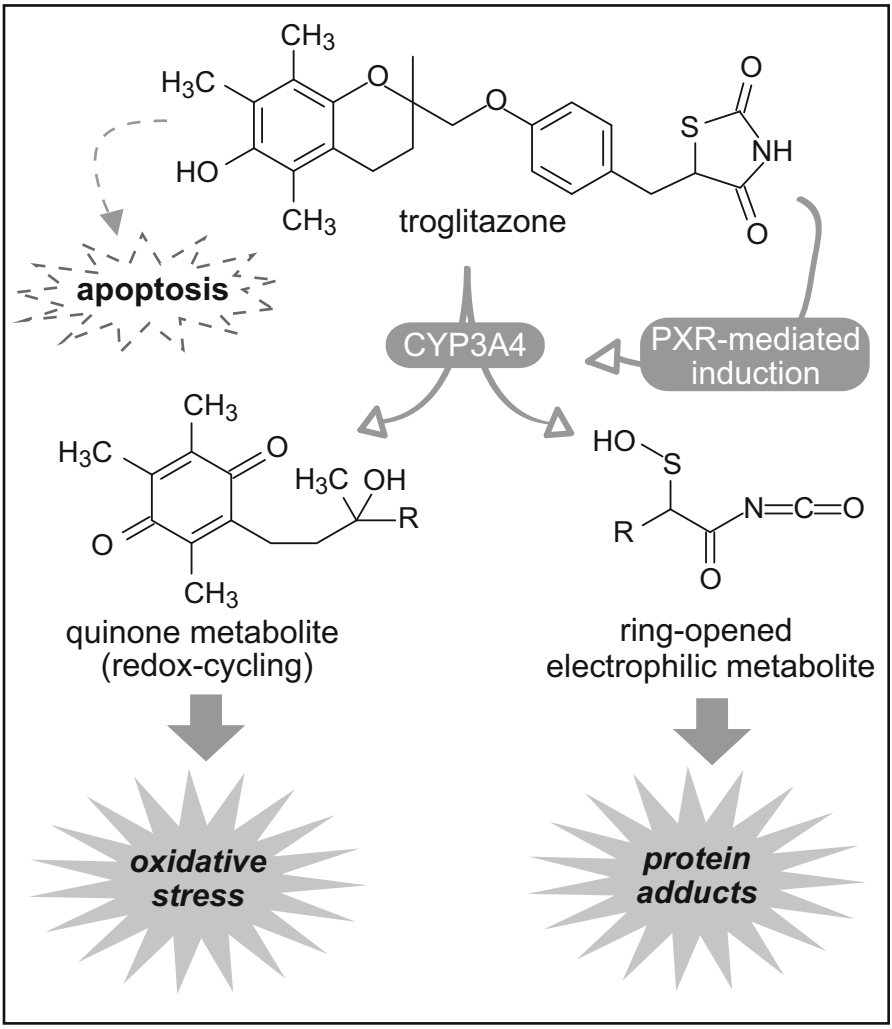

Fig. 6.6 Postulated pathways for the induction of hepatotoxicity in patients receiving the antidiabetic drug troglitazone. For the sake of simplicity, some noxious species are omitted (e.g. phenoxy radicals and epoxy metabolites)

ability to detoxicate electrophilic metabolites (e.g. epoxides and quinones), these findings strengthen the proposed role of reactive metabolites in the pathogenesis of at least some cases of troglitazone hepatotoxicity (the strength of the association was not sufficiently strong to confirm the involvement of this pathway in every individual case). Research on the mechanisms involved in troglitazone hepatotoxicity is ongoing, since any insights that aid the identification of at-risk patients for rare idiosyncratic toxic responses of this kind will benefit our understanding of other drug-related syndromes.

6.4.2 Hepatotoxicity and Industrial Chemicals

For chemicals used in occupational or industrial settings, the discovery of hepatotoxic potential usually ensures heavy restrictions are placed on their commercial use, ranging from outright bans or the mandatory use of strict engineering controls to high-grade protective clothing.

6.4.2.1 Carbon Tetrachloride

The organic liquid carbon tetrachloride (CCl_4) possesses excellent solvent properties that once underpinned its widespread use in fire extinguishers, degreasing products, dry cleaning solutions and during the production of fluorocarbon refrigerants. While CCl_4 usage declined strongly following the discovery of liver disease in exposed workers during the 1960s and 1970s (e.g. among dry cleaning shop attendants), CCl_4 still finds use in some industrial settings. CCl_4 is also used in toxicology labs due to its ability to induce dose-dependent, intrinsic hepatotoxicity and lipid peroxidation in diverse species. CCl_4 is also a significant environmental pollutant via its presence in soil, drinking water and ambient air. As a highly volatile substance, most occupational exposure occurs via the inhalational route, although cases of accidental domestic intoxication also occur and typically involve CCl_4 ingestion via the GI-tract.

Although the kidneys may show some injury upon acute or chronic exposure, the liver is the predominant target for CCl_4. Upon acute intoxication, the liver quickly assumes an enlarged appearance, while at the cellular level, the hepatocytes appear swollen and steatotic, with the most pronounced changes evident within centrilobular cells. Large fat droplets appear in affected zones together with signs of necrotic cell death, likely due to impairment of hepatic protein synthesis by CCl_4 metabolites and subsequent downregulation of transport proteins that normally export fatty acids, very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) from hepatocytes. The impaired protein synthesis likely reflects changes within the rough endoplasmic reticulum where dissociation of polyribosomal structures occurs within 10 min of administering CCl_4 to rodents. Destruction of many enzymatic activities also occurs early in CCl_4 intoxication, including a loss of CYP450 and calcium pump activities in hepatocellular membranes. Centrilobular haemorrhaging and immune cell infiltration are also conspicuous within necrotic zones.

CCl_4 suffers a complex metabolic fate within the liver, undergoing conversion to multiple degradation products that include phosgene, chloroform and carbon monoxide. The most important route to hepatotoxicity proceeds via a homolytic cleavage of a carbon–chlorine bond by CYP2E1 to form the damaging trichloromethyl radical ($Cl_3C^{\bullet}$) (Fig. 6.7). This species undergoes fast covalent binding to hepatocellular proteins but also reacts rapidly with O_2 to form damaging trichloromethylperoxyl radicals ($Cl_3COO^{\bullet}$). These radicals attack numerous macromolecular targets to form radical species within proteins, DNA and lipids via classic H-abstraction

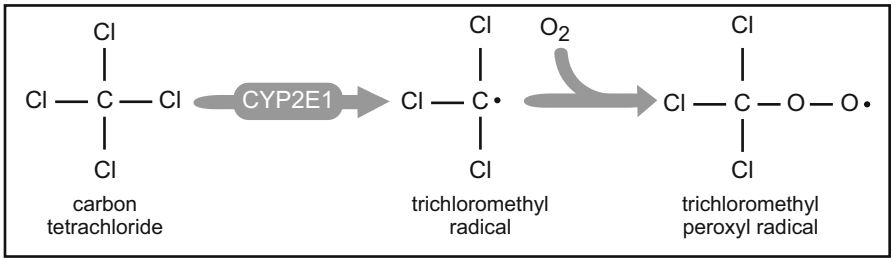

Fig. 6.7 The hepatotoxicity of carbon tetrachloride involves CYP2E1-catalysed conversion to damaging free radicals that initiate lipid peroxidation by attacking polyunsaturated lipids in cell membranes

chemistry. Polyunsaturated lipids in cell membranes are most vulnerable to $Cl_3COO^{\bullet}$, where H-abstraction triggers deleterious lipid peroxidation cascades which are a distinctive feature of CCl_4 hepatotoxicity. The formation of a plethora of lipid peroxidation products including volatile hydrocarbons such as ethane and pentane; toxic unsaturated aldehydes such as malondialdehyde, acrolein and 4-hydroxynonenal; and multiple lipid hydroperoxides in addition toperoxyl and hydroperoxyl free radicals exposes the CCl_4-intoxicated liver to a cocktail of noxious products. The highly reactive and cytotoxic mediator 4-hydroxynonenal likely plays a major role in triggering hepatic responses to CCl_4, serving as a 'toxicological second messenger' that can diffuse through tissues and attack neighbouring cells.

In addition to inducing cytotoxicity, lipid peroxidation products can activate hepatic stellate cells (HSCs) during chronic CCl_4 intoxication to initiate the pronounced deposition of collagen fibres that is characteristic of CCl_4 liver fibrosis. Liver cancer is also a long-term risk following occupational CCl_4 exposure, although the causative mechanisms remain subject to debate since there is little evidence that either $Cl_3C^{\bullet}$ or $Cl_3COO^{\bullet}$are especially mutagenic. Yet such conclusions are inherently tentative since studying the toxicological properties of CCl_4 and its metabolites is complicated by the volatility of the parent toxicant (e.g. CCl_4 is rapidly lost via evaporation from conventional in vitro mutagenicity assay systems). Due to the limited evidence for a genotoxic pathway, some researchers favour a nongenotoxic mechanism of CCl_4 carcinogenesis.

6.4.2.2 Thioacetamide

Thioacetamide was identified during the 1940s as an effective treatment against fungal deterioration of citrus crops. It continues to find use as a sulfur donor during the manufacture of diverse synthetic compounds ranging from pharmaceuticals to pesticides, while its sulfide-releasing properties in aqueous solutions ensure wide use in analytical chemistry and during the cleaning of sewerage systems. Agricultural use of thioacetamide declined following its identification as a strong liver toxicant

and carcinogen; a discovery that traces to 1950s concerns over the consumption of fruit juice made from thioacetamide-treated oranges.

Nowadays, thioacetamide remains a useful research tool since it induces reproducible centrilobular liver necrosis and cirrhosis in experimental animals. Its toxicity depends on a 2-step bioactivation sequence which is catalysed by various CYP450 isoforms as well as the FAD-containing monooxygenase (FMO). The initial oxidative metabolite, thioacetamide S-oxide (TASO), is subsequently converted to the chemically reactive metabolite, thioacetamide S,S-dioxide ($TASO_2$). The S,S-dioxide behaves as a hard electrophile by attacking the free amine possessed by either lysine side chains in proteins or phosphatidylethanolamine fatty acids.

Although the protein targets for $TASO_2$ within the livers of exposed animals are unknown, a recent in vitro study from the Hanzlik laboratory detected adduction of 321 proteins in cultured rat hepatocytes following exposure to radiolabelled TASO. The main route to protein adduction likely involved tautomerisation of $TASO_2$ to form an iminosulfinic acid species that then reacted with protein lysine groups. Proteins within mitochondria, endoplasmic reticulum and cytosol all sustained damage, including enzymes involved in intermediary metabolism, xenobiotic biotransformation and protein folding and/or the stress response. Since it likely alters key cellular pathways by disrupting protein–protein interactions, the researchers postulated that damage to chaperone proteins (e.g. heat shock proteins) is important to thioacetamide toxicity in liver cells.

6.4.3 Hepatotoxicity and Naturally Occurring Chemicals

Many liver-selective toxicants are present within nature, since diverse direct-acting or metabolism-dependent hepatotoxicants are present in plants and microorganisms or as constituents of minerals within the Earth's crust.

6.4.3.1 Aflatoxin B_1

The mycotoxin family – so named as a composite of the Greek word for fungus (*mykes*) and the Latin word for poison (*toxicum*) – contains many toxic substances that are produced during secondary fungal metabolism. The term is usually reserved for toxins that are made by fungal species that colonise crops for human consumption within the field or after harvesting. Depending on the fungal species, mycotoxin contamination of foodstuffs occurs both in tropical areas and within temperate regions. Many common foods are prone to mycotoxin outbreaks, including cereals, nuts, coffee, cocoa, spices, beans and fresh or dried fruit.

Mycotoxins are very important in veterinary toxicology due to their toxicity towards livestock. Due to consumption of fungus-infested fodder, human mycotoxin exposure can occur via consumption of contaminated meat or other animal products (e.g. eggs, cheese). Since most mycotoxins are chemically stable, they

typically survive common food processing procedures including exposure to high temperatures (e.g. during the baking of flour-based products such as cakes and bread). Due to concerns over their toxicity, many national food authorities stipulate legal limits for mycotoxin concentrations in common foodstuffs (e.g. peanut butter). Many such bodies also perform routine mycotoxin testing upon imported foods. Ongoing vigilance is required since fungal outbreaks vary significantly according to prevailing climate conditions; hence, food imports from the same region can vary from one season to the next.

In addition to concerns over liver damage, many mycotoxins exhibit diverse toxicological properties that include carcinogenicity, genotoxicity, nephrotoxicity and immunotoxicity. Hepatotoxicity is of particular concern for the aflatoxins, a class of difuranocoumarin compounds produced by the common moulds *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins were discovered in 1960 following a major outbreak of hepatotoxicity in British poultry that was traced to feedstock that contained mouldy peanut meal. Four aflatoxins were soon isolated from the offending fungus – two of which fluoresced blue and were named B_1 and B_2, with the former attracting great attention due to its strong hepatotoxic and carcinogenic potency. Major crops that are prone to aflatoxin B_1-producing *Aspergillus* infestations include peanuts, pistachios, corn, wheat and rice. Although aflatoxin outbreaks occur throughout the world, they are of special prominence in food-producing economies in Southeast Asia and Sub-Saharan Africa.

While chronic intoxication has attracted most research attention, acute intoxication with aflatoxin B_1 can also occur ('aflatoxicosis'). In lab animals, acute aflatoxin B_1 intoxication elicits profound biochemical and molecular changes within the liver, inducing haemorrhage, hepatocyte necrosis and bile duct hyperplasia. A human aflatoxicosis outbreak in Kenya during 2004 and 2005 claimed at least 125 lives. Although these acute poisonings are of concern, most interest in aflatoxin B_1 toxicology has focussed upon the liver cancer risks accompanying chronic exposure to contaminated foods including peanut butter and other foodstuffs.

Aflatoxin B_1 is rapidly metabolised in the liver via diverse oxidative, reductive, hydrolytic and conjugative reactions, some of which represent bioactivation reactions while others achieve aflatoxin B_1 detoxication. Significant interspecies variability is evident concerning the prevailing metabolic pathways, thereby explaining strong differences in species susceptibility to cancer and other toxic outcomes. Despite the diversity of metabolites formed, the carcinogenic potency of aflatoxin B_1 mainly depends upon the CYP-catalysed epoxidation of the furan ring to form highly reactive 8,9-epoxides which readily attack DNA (Fig. 6.8). A mixture of 8,9-epoxide isomers form in human cells, with the strongly mutagenic *exo* isomer formed by CYP3A5 and CYP1A2, while CYP3A4 forms a weakly mutagenic isomer. While rodents detoxicate AFB_1 epoxides via glutathione conjugation, the human capacity for this pathway is limited, a factor which may explain human susceptibility to AFB_1-mediated liver cancer. Epoxide hydrolase (EPHX1) may also protect human hepatocytes against toxic epoxidated AFB_1 metabolites.

The genotoxic 8,9-epoxide attacks DNA to form several damage products, although the most strongly mutagenic adduct forms via reaction at the N^7 of

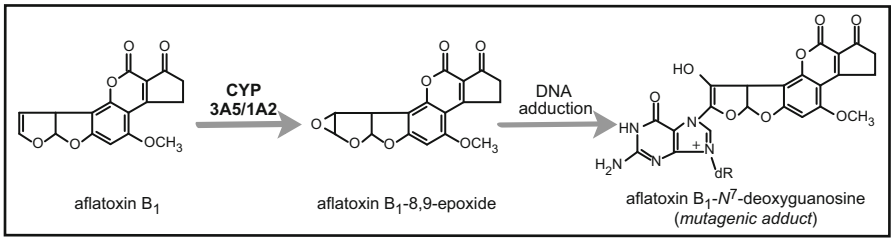

Fig. 6.8 The hepatotoxic mycotoxin aflatoxin B_1 undergoes complex hepatic metabolism to form a large number of metabolites. The cancer response is likely driven by an electrophilic 8,9-epoxy metabolite (*exo* isomer) which forms mutagenic adducts

guanine. N^7-guanine adduct levels within the livers of aflatoxin B_1-treated animals correlate closely with tumour yields, with a linear relationship observed over several orders of magnitude of administered dose of the mycotoxin. Although the human evidence is not definitive, these adducts are suspected of inducing G→T transversions and G→A transitions within critical codons in mutated growth regulatory genes in liver tumours in regions of the world where food contamination with aflatoxin B_1 is substantial. The role of DNA adducts in chemical carcinogenesis is explored in Chap. 8 (Sect. 8.5.1).

6.4.3.2 Cyanobacterial Toxins

Fungi are not the only microorganisms causing serious liver injury in the unwary: the consumption of water contaminated with cyanobacterial toxins represents a significant public health problem within many geographical locales. Blooms of these photosynthetic bacteria can confer an unsightly 'pea soup' appearance upon waterways especially during warm summer months. Although dozens of cyanobacterial species are detectable in healthy lakes, reservoirs and rivers, a combination of favourable weather conditions and suitable water levels of acidity, nutrients and fertilisers can allow rapid cyanobacterial overgrowth and clumping. Cyanobacteria produce many powerful neurotoxins and hepatotoxins which undergo lytic release upon the death of a bloom, ensuring the contaminated water is highly toxic to livestock and other animals including fish.

Cyanobacterial contamination of human water supplies has long been associated with liver disease outbreaks, with the deaths of soldiers due to drinking water from a green-coloured lake famously reported by a Chinese military commander around 1,000 years ago. In recent times, health problems accompanying exposure to cyanobacterial toxins attracted research attention after a mass poisoning episode involving indigenous inhabitants of Palm Island in northeastern Australia in 1979. In this instance, consumption of water affected by *Cylindrospermopsis raciborskii* caused an outbreak of serious liver toxicity in over 100 school children. The epidemic occurred several days after copper sulfate was added to a local reservoir to control

a recurring ‘algal’ bloom, an intervention that likely lysed the cells and released cyanobacterial hepatotoxins into the town drinking water.

Once methods for culturing *C. raciborskii* were perfected, a novel tricyclic alkaloid containing a guanidinium group and hydroxymethyluracil moiety named cylindrospermopsin was isolated (Fig. 6.9). Following its discovery, cylindrospermopsin was identified in other cyanobacterial strains along with other structurally related toxins. An association with the Palm Island episode was strengthened after cylindrospermopsin was shown to cause massive centrilobular necrosis in mice. During studies in cultured hepatocytes, low concentrations of cylindrospermopsin produced inhibition of protein synthesis, oxidative stress and induction of apoptosis. Cylindrospermopsin is also believed to form DNA adducts although the chemistry has yet to be definitively proven. The precise role of metabolism in cylindrospermopsin toxicity has proven difficult to establish, and it is likely the parent compound contributes significantly to toxicity.

Another major class of cyanobacterial toxicants are the microcystins, a family of unique cyclic heptapeptides that includes over 80 members. The best-studied member is microcystin-LR, a powerful inducer of cholestasis in laboratory rodents (Fig. 6.9). Microcystin-LR exerts many toxic effects upon liver cells including induction of apoptosis and oxidative stress, although the primary toxic action likely involves inhibition of protein phosphatases 1 and 2A, a class of enzymes that de-phosphorylate serine groups on many hepatocellular proteins. Serine phosphorylation regulates the activity of proteins involved in many cell functions including proliferation, metabolism and cell death. Dysregulation of these diverse pathways due to protein hyperphosphorylation likely underlies the broad cytotoxic actions of microcystin-LR in hepatocytes. In particular, altered intermediate filament phosphorylation likely mediates many deleterious cellular responses to microcystins, including MAPK activation and other biochemical changes.

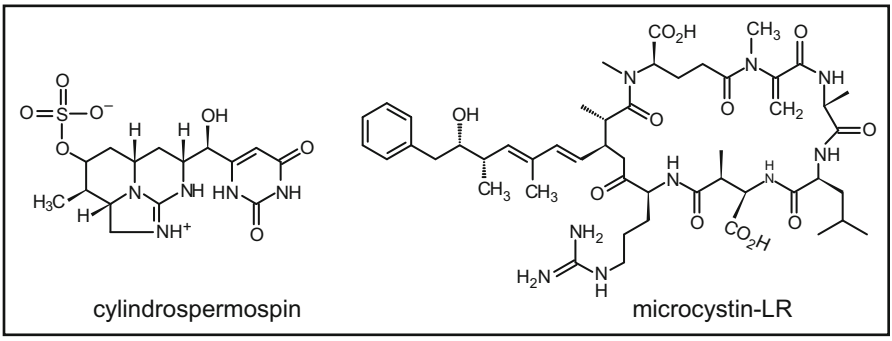

Fig. 6.9 Due in part to ongoing climate change, contamination of human water supplies with cyanobacterial blooms is an emerging public health problem in temperate regions, potentially exposing human populations to potent hepatotoxins such as cylindrospermopsin and microcystin-LR

6.5 Nephrotoxicity

Chemically induced nephrotoxicity is closely related to hepatotoxicity in that these two excretory organs often incur simultaneous damage by the same toxicant. For example, upon human exposure to cadmium – a heavy metal constituent of batteries, paints and plastics – the balance between nephrotoxicity and hepatotoxicity varies according to the magnitude and duration of exposure: the liver typically sustains damage by large, acute doses of cadmium, while the kidneys are vulnerable during extended exposure to low doses. Since the kidney lacks the regenerative capacity of liver, nephrotoxic episodes that diminish the number of functional nephrons often condemn victims to either long-term renal dialysis or renal transplants.

Several physiological considerations predispose the kidneys to chemical toxicity. The strong expression of xenobiotic transporters within the luminal membranes of the renal nephron renders the kidneys highly vulnerable to *nephrotoxicants* since it means local toxicant concentrations can significantly exceed their levels in circulating blood. The large surface area of the luminal membranes and strong expression of xenobiotic metabolising enzymes in proximal tubules is a further exacerbating factor. The vulnerability of the renal vasculature to vasoactive compounds also predisposes the kidneys to injury, since blood flow changes can further maximise local xenobiotic concentrations within renal tissue. These collective factors ensure human exposure to nephrotoxic substances is of high clinical relevance. Indeed, xenobiotic intoxications contribute to around one-half of acute and chronic renal failures, while between 10 % and 15 % of intensive care unit admissions involve acute renal failure. Since mortality rates for acute renal failure have barely budged in the past 50 years, the need for basic and clinical research in this area remains high.

As with the liver, normal kidney function underpins the wellbeing of many physiological systems, with important roles in the maintenance of electrolyte homeostasis via regulation of the volume and ionic composition of total body fluid (e.g. levels of water, sodium, potassium or hydrogen ions). The kidneys also produce crucial hormones that regulate blood pressure (e.g. rennin), red blood cell production (erythropoietin) and blood calcium levels (calcitrol). The renal capacity to excrete foreign chemicals as well as metabolic waste products (e.g. urea, ammonia and uric acid) is also crucial to bodily health.

6.6 Basic Renal Structure

The adult human kidneys process some 180 l of blood every day, an amazing feat for two small organs that comprise approximately the same size as a typical computer mouse. Each kidney is surrounded by a renal capsule, a clear membrane pouch that protects against infections and trauma. Viewed in transverse section, each kidney comprises an outer renal cortex and an inner renal medulla. Each kidney is

perfused by blood from a single renal artery that originates in the aorta, while blood exits the organ via a renal vein that drains into the inferior vena cava. The urine released by each kidney is delivered to the bladder via a single ureter.

6.6.1 The Renal Nephron

The basic functional unit of the kidney is the nephron, a long tubular structure that filters the blood and exchanges bodily wastes (refer to Fig. 3.11 in Chap. 3). Each kidney contains some one million nephrons that are assigned to various subclasses depending on the location of its glomerular apparatus (e.g. midcortical and juxtamedullary). Considerable heterogeneity is thus evident within the spatial distribution of different nephron subtypes within the kidney and in the morphology of epithelial cells within each region of the nephrons. The harmful effect of individual nephrotoxicants upon specific renal functions thus reflects the anatomical complexity of the organ that ensures different chemicals tend to target distinct segments of the renal nephron. The tendency for chemicals to accumulate in different kidney regions further influences nephrotoxic syndromes.

6.6.2 The Glomerulus

As the filtering unit of the kidney, the glomerulus represents the initial interface between circulating blood and the filtered biofluid that eventually becomes the urine. Each glomerulus is perfused by blood delivered by several capillary loops, with blood entering via afferent arterioles and exiting by efferent arterioles. The core glomerular structure is assembled from three main components, namely, endothelial cells, the glomerular basement membrane and visceral epithelial cells. Upon entering the glomerulus, blood is processed to form an ultrafiltrate within Bowman's space which comprises water and small solutes. In healthy individuals with an intact glomerular membrane, circulating blood cells and large negatively charged proteins do not enter the ultrafiltrate.

Renal ultrafiltration is driven by hydrostatic pressure within the glomerulus which depends upon the relative vasoconstriction (tone) of the afferent and efferent arterioles. Multiple inputs control renal vascular tone, including sympathetic nerves and mediators such as endothelin, angiotensin II, vasopressin, prostaglandins and cytokines. The extent of filtration at the glomerulus – the glomerular filtration rate (GFR) – is constant in healthy adults and can be estimated by monitoring the excretion of model compounds that are filtered but not reabsorbed or actively excreted (e.g. inulin). Blood concentrations of endogenous molecules such as creatinine, urea or cystatin C that are normally cleared by glomerular filtration can be used to detect renal toxicity within the clinical setting, with rises in their plasma levels indicative of renal dysfunction.

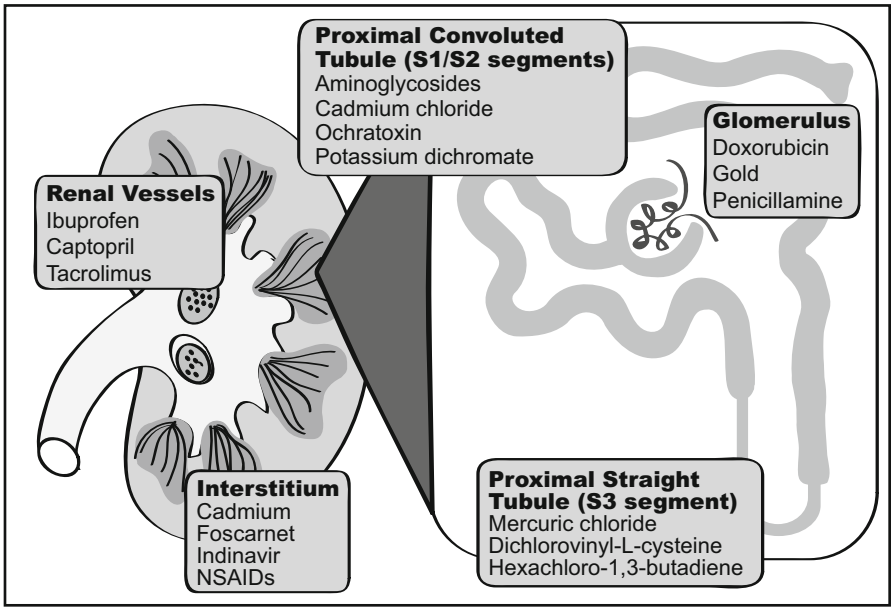

Fig. 6.10 Nephrotoxicants often display selectivity when causing kidney injury by either damaging specific regions within the renal nephron (insert), causing vasoconstriction within specific renal zones, or inducing prolonged inflammatory damage within the interstitium (interstitial nephritis)

Since blood-borne xenobiotics are delivered to the glomerulus in high concentrations, glomerular damage may accompany exposure to diverse nephrotoxicants (Fig. 6.10). Since renal biopsies are rarely performed in the emergency room setting to confirm glomerular injury, its contribution to acute human nephrotoxicity is likely underestimated. In addition to acute injury, chronic exposure to environmental pollutants such as heavy metals and hydrocarbons can induce *glomerulonephritis* which involves a sharp reduction in blood-filtering capacity and urine formation. Reactive oxygen species and cytokines released by neutrophils and macrophages trapped within the inflamed glomerulus are likely mediators of such immune-mediated renal injury.

6.6.3 Proximal Tubules

The proximal tubule comprises distinctive epithelial cells that are highly polarised, possessing a basolateral membrane zone that interfaces with blood vessels and a highly invaginated membrane known as the brush border. At least two-thirds of solute and water within the ultrafiltrate undergoes reabsorption at the proximal tubules, assisted by aquaporin-1 water channels within proximal tubular membranes. Extensive transporter-mediated recovery of amino acids and glucose also occurs at the proximal tubules. These structures are of high toxicological importance since most renal CYP expression localises to the proximal tubules. These structures also

strongly express multidrug resistance proteins (MRP1–5) and multiple organic anion-transporting proteins within the basolateral (e.g. OAT4 and OATPA) and brush border membranes (e.g. OAT1-3). Various organic cation transporters are also strongly expressed in both apical and basolateral proximal tubule membranes. The strong capacity for membrane transport underlies the tendency to accumulate high levels of circulating toxicants, which, coupled to the high metabolic activity of this tissue, renders proximal tubules highly vulnerable to nephrotoxicity (Fig. 6.10).

Tubular toxicity often proceeds via toxic metabolites which induce cell rupture and the release of protein aggregates that promote tubular obstruction. Other nephrotoxicants such as the immunosuppressant cyclosporine impair urine production by inducing *interstitial nephritis*, a chronic condition involving inflammation within the space between the renal tubules (Fig. 6.10).

The proximal tubules comprise three distinct segments, S_1, S_2 and S_3. S_1 epithelial cells are highly metabolically active, containing many mitochondria and possessing an extensive vacuolar lysozyme system that helps cells clear small proteins from ultrafiltrate. Some 600 mg of blood proteins are likely recovered from the ultrafiltrate per day in healthy adults, ensuring urinary protein levels are normally low. S_1 cells have extensive capacity for active secretion of xenobiotics and their metabolites, further increasing their vulnerability to nephrotoxicants. S_2 cells possess similar properties, but their metabolic and excretory capacities are more limited than those of their S_1 neighbours. S_3 cells are cuboidal and possess long brush borders as well as high numbers of peroxisomes.

6.6.4 Loop of Henle, Distal Tubules and Collecting Duct

The terminal regions of the renal nephron comprise several structurally and functionally distinct segments. The descending and ascending arms of the loop of Henle exhibit clear differences in water permeability, respectively showing high and low expression of aquaporins needed for water permeability. The water permeability of the distal tubules is limited although extensive transport of sodium ions occurs in these segments. The collecting duct strongly influences the final composition of urine due to its hormone-responsive regulation of the reabsorption of solute, water and hydrogen ions. Due to limited CYP expression in the distal tubules or collecting duct, chemically induced toxicity towards these cells is less common than toxicity towards the glomerulus or proximal tubules (Fig. 6.10).

6.7 Functional Consequences of Nephrotoxicity

As a rule, functional changes accompanying toxicant-induced nephrotoxicity is *acute* in nature, developing rapidly following initial exposure to a noxious drug or chemical, or *progressive*, developing slowly and insidiously during chronic exposure to nephrotoxicants. For still other toxic substances, renal cancer is a major long-term toxic outcome.

Xenobiotic-induced renal injury often makes its presence known via changes in the quantity or quality of urine: symptoms can involve overproduction of dilute urine (*polyuria*), excretion of low quantities of urine (*anuria*) or the passage of blood-stained urine (*haematuria*). Alterations in the composition of urine that are indicative of renal damage include the appearance of glucose (*glucosuria*) or blood proteins in urine (*proteinuria*). Levels of specific proteins such as albumin (*albuminuria*) are useful indicators of nephrotoxicity in the clinical setting. Unfortunately, renal injury is often far advanced before such changes become obvious to patients, a situation that drives a search for early markers of drug- or toxicant-induced nephrotoxicity. Specific urinary markers that pinpoint kidney damage to particular renal zones are of particular interest since relying upon gross urinary changes to detect nephrotoxicity is often unreliable (e.g. both glomerular and tubular toxicity can alter urine volumes).

In recent decades, researchers within the pharmaceutical industry and academic laboratories have studied specific proteins within urine as markers of injury to specific renal structures. Since these approaches initially used traditional biochemical approaches with limited scope, their clinical uptake in diagnostic settings was typically low. More recently, the use of broad-based metabolomic and proteomic methods for monitoring large numbers of molecules in urine has identified many promising nephrotoxicity biomarkers (Table 6.4). While most of these markers are still undergoing validation in animal-based studies and human trials, they may have a significant clinical impact in coming years.

6.8 Major Human Nephrotoxicants

Development of a unified mechanistic understanding of chemically induced nephrotoxicity has been hampered by the dissimilarities between nephrotoxicants in terms of their chemical structures and physicochemical properties. Thus, while the clinical signs accompanying kidney damage are often similar, few chemical or

Table 6.4 Potential urinary markers of acute kidney injury identified during proteomic or metabolomic investigations

Urinary marker	Form of injury detected	Example of nephrotoxicant	Representative study
α-GST (α-glutathione-S-transferase)	Proximal tubules	Hexachloro-1, 3-butadiene	Swain et al. (2012)
Collagen type I and II fragments	Many – arise by ↑ECM[a] turnover	Gentamicin, cisplatin	Rouse et al. (2012)
KIM-1 (kidney injury marker-1)	Proximal tubular injury	Cadmium, many	Prozialeck et al. (2007)
NGAL (neutrophil gelatinase-associated lipocalin)	Tubular injury	Polycyclic aromatic hydrocarbons	Lacquaniti et al. (2012)
Vanin-1	Tubular injury	Gentamicin, cisplatin	Hosohata et al. (2012)

[a]Extracellular matrix

structural similarities exist between toxicants as diverse as the heavy metal mercury, the mycotoxin fumonisin B_1, the immunosuppressant cyclosporine A or the aminoglycoside gentamicin. Rather than seeking to cover a comprehensive range of xenobiotic-induced nephrotoxic syndromes, we will examine several that highlight different mechanisms underlying the toxic response.

6.8.1 Nephrotoxic Drugs

A small number of human pharmaceuticals are overrepresented in drug-related human kidney failure. Two commonly involved drug classes deserve particular attention.

6.8.1.1 Aminoglycosides

Despite serious concerns over renal injury and hearing loss, due to their value in treating serious Gram-negative bacterial infections, aminoglycoside antibiotics such as gentamicin, tobramycin and amikacin remain in modern use. As highly ionised polycations, these drugs are poorly absorbed via the oral route and are typically administered as intravenous injections. Even though plasma concentrations may be monitored and kept within the therapeutic range, a significant number of patients will develop renal injury. Aminoglycoside nephrotoxicity is due to efficient active transport of these drugs by renal transporters in proximal tubules. Some members of this drug class may be less nephrotoxic than others, although debate surrounds clinical comparisons of this sort. The risk of renal injury is likely higher in the elderly and in patients receiving multiple drugs. In laboratory animals, aminoglycoside nephrotoxicity features progressive epithelial cell necrosis within the S1 and S2 segments of proximal tubules. As the renal injury progresses, tubular cell mitochondria become increasingly swollen and distorted with accompanying structural abnormalities (e.g. cristae and matrix loss). Lysosomal abnormalities and formation of myeloid bodies are also conspicuous. While the molecular mechanisms underlying aminoglycoside nephrotoxicity are not fully clear, they likely involve interference with the transport of ions and nutrients at the apical and basolateral membrane, combined with inhibitory effects upon mammalian protein synthesis at the high concentrations achieved in the proximal tubular cells.

6.8.1.2 NSAIDs

The nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs in current use, with some members of this broad class such as aspirin, ibuprofen and naproxen frequently available as over-the-counter medications. The popularity of the NSAIDs reflects their effectiveness as inhibitors of cyclooxygenase,

a class of enzymes that mediate pain and inflammation in various rheumatological and musculoskeletal conditions. Cyclooxygenases oxidise arachidonic acid to powerful eicosanoids including multiple prostaglandins and prostacyclins, some of which help maintain key renal functions such as glomerular filtration, water and ion transport and blood vessel tone. Although NSAID-induced renal toxicity is rare in healthy individuals, their use by patients with pre-existing renal impairment (e.g. the elderly and other susceptible patient groups) carries a risk of serious nephrotoxicity. By suppressing the normal role of cyclooxygenase-derived eicosanoids in maintaining renal function, NSAIDs elicit an abrupt drop in urine output that in some patients signals acute renal failure. While such toxic outcomes are the predictable consequence of the pharmacological effects of NSAIDs (i.e. COX inhibition), other less predictable renal toxicities can accompany chronic NSAID use. Acute tubular necrosis within long-term aspirin users in addition to the rare interstitial nephritis that accompanies extensive NSAID use exemplifies the serious nephrotoxic syndromes these drugs can induce.

6.8.2 Occupational Nephrotoxicants

Chronic exposure to halogenated solvents has attracted considerable attention as a cause of kidney injury in workers. Due to the numerous members of this diverse chemical class, only two culprits are briefly considered here.

6.8.2.1 Chloroform

Chloroform finds many industrial applications due to its excellent solvent properties, although its use in developed countries is declining as industry adopts cleaner technologies based on ‘green chemistry’ synthetic principles. Chloroform was used extensively during the high volume production of the refrigerant chlorodifluoromethane (‘R-22’), although this usage is discontinued in many countries due to its role in atmospheric ozone depletion. In laboratory rodents, chloroform induces proximal tubular damage involving overt tubular swelling and necrosis. Accompanying signs of functional impairment include elevated blood urea nitrogen as well as proteinuria and glucosuria. Comparable changes occur in human workers following occupational chloroform exposure.

While chloroform nephrotoxicity is known to be metabolism dependent, uncertainty surrounds the identity of the culprit metabolites. One low-affinity CYP-catalysed pathway of reductive metabolism generates the dichloromethyl radical which likely elicits damage to phospholipids in cell membranes (Fig. 6.11). Of greater importance in oxygenated mammalian tissues is the CYP2E1-catalysed pathway that proceeds via a hydroxylated metabolite (trichloromethanol) to form phosgene, the chemical warfare agent of World War 1 infamy (Fig. 6.11). Although subject to spontaneous hydrolysis and detoxication by glutathione, ongoing

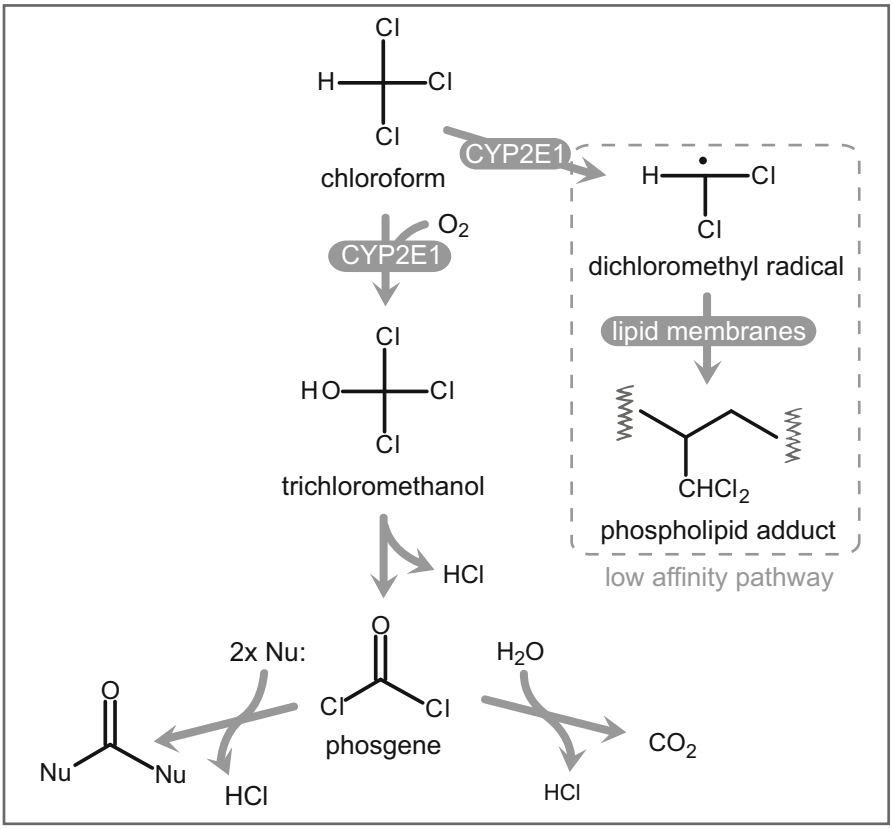

Fig. 6.11 Chloroform nephrotoxicity likely involves CYP2E1-catalysed conversion to phosgene, although other metabolic fates may also contribute. Phosgene suffers multiple fates, including spontaneous hydrolysis or reaction with nucleophiles such as glutathione or protein. At high concentrations under hypoxic conditions, chloroform may be oxidised to a free radical species which attacks cell membranes

formation of this strong electrophile causes adduction of renal cell proteins while also reacting with cellular phospholipids. The protein targets for phosgene are poorly characterised although nuclear histone proteins are likely targets in renal epithelial cells. While similar routes of chloroform bioactivation can occur in both liver and kidney, the possibility that noxious metabolites formed in liver are exported to the kidney is suggested by some studies.

6.8.2.2 Trichloroethylene

One of many nephrotoxic substances within the broad haloalkene class of reagents, 1,1,2-trichloroethylene is a widely used solvent and component of degreasing

products. Trichloroethylene attracts significant concern as an environmental pollutant due to contamination of chemical dumps and its widespread presence as an environmental pollutant in water, air and soil samples. In addition to liver injury, trichloroethylene is associated with acute and chronic renal injury in lab animals as well as kidney and liver tumours as well as non-Hodgkins lymphoma in humans. Trichloroethylene has also attracted significant attention as a peroxisome proliferator in rat liver. High occupational and environmental exposure to trichloroethylene is also associated with immunotoxicity and autoimmune disorders in humans.

Trichloroethylene nephrotoxicity likely depends upon a complex, multistep pathway of bioactivation that begins with CYP-catalysed oxidative conversion to a range of toxic species including chloral (Cl_3CCHO), dichloroacetic acid and oxalic acid. Significant debate has centred upon whether trichloroethylene epoxidation is essential for the formation of electrophilic species, and the Guengerich laboratory has described an alternative pathway whereby chloral forms via chloride migration from an oxygenated CYP enzyme intermediate. Various acyl halides formed upon hydrolysis of TCE-oxide such as dichloroacetic chloride and formyl chloride are also likely contributors to cell damage.

While these electrophiles likely contribute to liver damage and cancer outcomes, the main mediators of kidney damage probably form via an alternative pathway of glutathione-dependent conjugative metabolism (Fig. 6.12). In quantitative terms, the conjugative pathway is less pronounced than the oxidative pathway, although its toxicological significance within the kidney is high. The initial metabolite formed via the conjugative route, S-(1,2-dichlorovinyl)glutathione (DCVG), likely forms in liver before it is exported to the kidney where it undergoes further metabolic

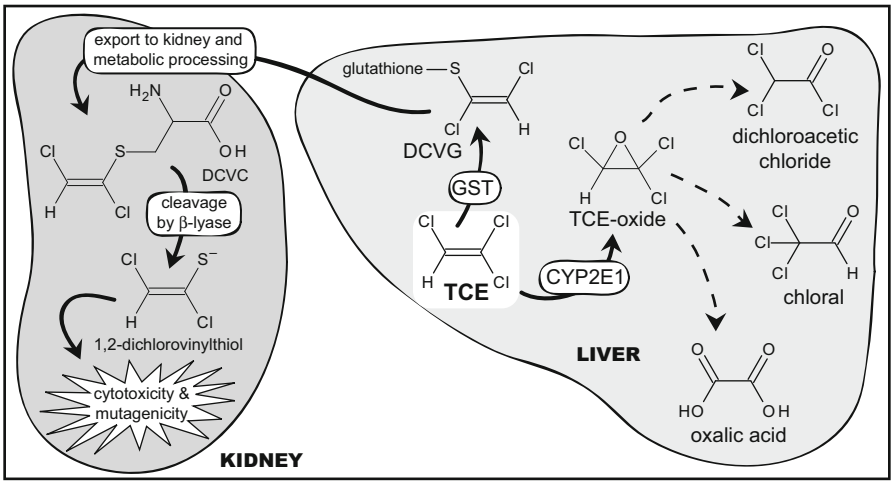

Fig. 6.12 For a small molecule, trichloroethylene generates a complex array of metabolites in vivo, some of which are shown here. While the prevailing pathway of oxidative metabolism likely causes liver injury, kidney damage is mediated by thiol metabolites formed during metabolic processing of S-glutathione conjugates. See Lash et al. (2000) and Guengerich (2005) for more details

processing within renal proximal tubules to form *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC). The latter is cleaved by β-lyase to form 1,2-dichlorovinylthiol, a reactive intermediate which is implicated in covalent modification of proteins and other cellular targets (Fig. 6.12).

The potential for trichloroethylene to induce renal injury is of concern in work settings involving heavy use of this solvent, and the early detection of the onset of renal injury in workers has long proved problematic. A recent study of urinary levels of the nephrotoxicity marker KIM-1 (Table 6.3) in trichloroethanol-exposed Chinese factory workers has raised hopes that this marker can identify at-risk workers within industrial settings.

6.8.3 Naturally Occurring Nephrotoxicants

Strongly nephrotoxic compounds are widely distributed within the plant kingdom. Over the past decade, significant progress was made in solving the contribution of weed-derived nephrotoxicants to renal nephropathy and tumourigenesis that was long epidemic to the Balkans – a topic to be addressed in Chap. 8 (Sect. 8.7.2). Other episodes of human nephrotoxicant exposure arise via contamination of agricultural products with moulds – with particular concern focussed upon citrinin, a nephrotoxic mycotoxin produced by *Penicillium*, *Aspergillus* and *Monascus* mould strains. *Monascus* strains have found extensive use in China as a source of food dyes. Other citrinin-producing moulds cause problems during the production of cereal crops such as corn, wheat, rice, oats and barley.

Studies of citrinin nephrotoxicity are subject to some variability depending on the experimental species used, but long-standing rodent studies indicate strong potential to induce proximal tubular necrosis and deterioration of brush border membranes. Although citrinin undergoes oxidative metabolism, the parent compound can directly induce apoptotic cell death and oxidative stress. Since citrinin-producing moulds often produce another potent nephrotoxic mycotoxin, ochratoxin A, distinguishing the proximal tubular injury caused by each toxicant is difficult. Ochratoxin A has received extensive attention due to its ability to induce hepatic tumours as well as malignant renal tumours.

6.9 Conclusion

This chapter has explored how chronic and acute exposure to toxicants can cause organ-selective injury to specific body organs. Due to the importance of such injury to the pharmaceutical and chemical industry, special research attention has been devoted to chemicals that damage the two main excretory organs, the liver and kidney. A tendency to damage these organs is viewed with special concern during the development of new medicines, and it is unsurprising that the history of

pharmaceutical innovation is littered with the corpses of failed drugs that caused unwanted nephrotoxicity or, more commonly, hepatotoxicity. The development of sensitive predictive tools allowing early detection of compounds with these rogue properties poses an ongoing challenge to the toxicology community.

To reiterate the point made in Sect. 6.1, due to space constraints within an introductory volume, this chapter was unable to explore important target-organ toxicity in other tissues (e.g. CNS, lungs, heart or reproductive organs). Interested readers seeking insight into other target toxicities should consult comprehensive toxicology texts cited in the Chap. 2 reading list. In the following chapter, attention is given to chemically induced toxicity within the developing child, a topic of increasing concern in the wake of rising contemporary awareness of the role of the intrauterine environment in dictating the lifelong health of mammalian species including humans.

Going Further

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Chapter 7
Chemicals and the Unborn

Abstract The thalidomide epidemic dispelled the complacent belief that the placenta is an impenetrable barrier protecting the foetus against all toxicants within a mother's blood. In subsequent decades, knowledge of the risks accompanying exposure to developmental toxicants increased substantially. According to long-standing observations, substances that harm the unborn typically produce a combination of three toxic outcomes, namely, growth retardation, embryolethality and congenital abnormalities. Study of affected offspring revealed the influence of the precise timing of toxicant administration, with 'windows of prenatal susceptibility' identified for many developmental toxicants. While knowledge of the molecular basis for prenatal toxicity in animals has advanced significantly, extending these findings to humans is rarely straightforward. This chapter explores basic principles that apply during study of chemically induced birth defects, with particular focus on the mechanisms underlying the prenatal toxicity of drugs such as thalidomide and valproate as well as workplace toxicants such as cadmium and organic solvents.

Keywords Embryolethality • Teratogenicity • Growth retardation • Epigenetic teratogens • Cadherins • Teratogenicity testing • Thalidomide • Cadmium • Toluene • Bisphenol A • Endocrine disruptors

7.1 Introduction

Throughout history, the birth of malformed offspring has evoked feelings of horror, hysteria or hostility. In antiquity, deformed children were feared as portents from the gods; with this mindset, superstitious explanations were preferred over any search for environmental factors that might perturb prenatal development. Such attitudes proved persistent, and by the early seventeenth century, their influence is seen within Shakespeare's famous drama, *King Lear*. At one point in the script, an outbreak of birth defects affecting the craniofacial region (*harelip*), lower limbs (*the pin*) and interdigital spaces (*the web*) is attributed to the nocturnal exploits of a 'foul

P.C. Burcham, *An Introduction to Toxicology*,
DOI 10.1007/978-1-4471-5553-9_7,

fiend' named Flibbertigibbet: '*He begins at curfew, and walks till the first cock; he gives the web and the pin, squints the eye, and makes the hare-lip; mildews the white wheat, and hurts the poor creature of earth'* (iii,4). Even as the nineteenth century unfolded and the Western medical mind became increasingly open to the idea that some human diseases might originate in exposure to chemical toxicants, a reluctance to apply this paradigm to birth defects proved surprisingly deep seated. The complacent idea that the embryo developed within a uterine sanctuary that was impregnable to toxic invaders was hard to dispel.

After World War 2, modern epidemiology began uncovering the clear influence of dietary, vocational, cultural and socioeconomic factors upon pregnancy outcomes, thereby eroding confidence in the barrier properties of the placenta. Yet, it took the worst medical technology failure of modern times – the thalidomide disaster of the early 1960s – to vanquish the notion that unborn children are invulnerable to ingested toxicants. In the wake of the epidemic, developmental toxicology emerged as a vigorous branch of modern toxicology, inventing a raft of experimental protocols that guided the testing of new pharmaceuticals and other chemicals for toxicity towards the unborn with a rigour that was absent before the 1950s. In spite of these successes, the need remains for an improved mechanistic understanding of chemically induced prenatal toxicity to ensure the testing methods used by developmental toxicologists keep abreast of modern understandings of human biology. This chapter will explore some key concepts that surround chemical toxicity in the unborn while also considering the mechanistic aspects underlying the noxious effects of some select developmental toxicants.

7.2 Core Terminology and Concepts

The branch of toxicology specialising in chemical toxicity towards the unborn child is *teratology* – a name derived from the Greek word for monster, *teras*. Initially, teratologists mainly conducted observational studies and developed anatomical descriptions of the effects of chemicals on the developing organism. More recently, however, drawing upon improved knowledge of the molecular basis of human development, the field has focussed upon the biochemical mechanisms underlying chemically induced birth defects.

Since chemically induced prenatal toxicity can differ significantly from other toxic syndromes, this field has necessarily evolved a distinctive terminology. First, the term *conceptus* denotes the entire product of conception during the whole prenatal period and thus embraces the fertilised egg, embryo and foetus as well as the sac, cord and placenta. A *teratogen* is a substance eliciting an observable and irreversible change in the morphology of the unborn child. Exposure to such chemicals can harm the conceptus at any stage of the pregnancy yet is of special concern during the first trimester. Exposure to an *embryolethal toxicant* is incompatible with prenatal life, with the timing of lethality determining whether the outcome involves resorption (as during the preimplantation phase), spontaneous abortion (during the

embryonic stage) or stillbirth (during the foetal period). A *developmental toxicant* elicits toxicity towards the conceptus at any time throughout the prenatal period until birth. Unlike the observable structural changes elicited by teratogens, developmental toxicants may elicit biochemical and molecular alterations that are unseen to the unaided or assisted human eye. In theory, a true developmental toxicant or teratogen affects prenatal development at doses that do not undermine the mother's health. Finally, a *growth retardant* typically suppresses histogenesis and the growth of the conceptus, leading to the birth of low birth weight or 'runted' offspring. This outcome is especially associated with chemical intoxication during the final two trimesters. A major consequence of growth retardation is a suppression of the functional maturation of body systems that are growing and developing in the final trimester. The brain, for example, is highly vulnerable to chemicals during this period.

Much of the knowledge base upon which developmental toxicology rests derives from studies in multiparous mammalian species such as rats, rabbits and mice. Litters born to toxicant-treated mothers can exhibit a combination of the three classic effects of developmental toxicants – malformations, growth retardation and embryolethality – with individual toxicants typically inducing a particular spectrum of outcomes within a given litter (Fig. 7.1a). The first scenario (Fig. 7.1b) depicts a strongly teratogenic compound that produces high yields of structural malformations and growth suppression at low doses yet only induces embryolethality at high doses. Such an agent would arouse significant alarm during the risk assessment process. For such compounds, the molecular events mediating birth defects likely differ from those causing embryonic death. The second scenario is more common in that a given toxicant induces a mixture of all three toxic responses within the same litter at a given dose (Fig. 7.1c). For such compounds, each toxic response likely reflects a single, shared mechanism. The final toxicant lacks teratogenic potency of any kind and only induces growth suppression and embryolethality (Fig. 7.1d).

7.3 Timing of Exposure and Toxicity

Prenatal toxicology employs comparable concepts to those applied to other chemically induced phenomena: first, the dose of toxicant delivered to the unborn is considered the primary determinant of the severity of toxic outcomes. Second, understanding how chemicals interact with specific molecular targets is thought essential to understanding prenatal toxic responses. Third, the genotype of the mother and unborn child likely strongly influences prenatal toxic outcomes.

A key concept distinguishing developmental toxicity from most toxicological responses in mature organisms is that the *timing* of exposure uniquely influences the main toxic outcomes. Typically, whatever body systems or organ components are undergoing active development at the time of exposure will likely incur the most overt damage. This differs from the situation for most toxic phenomena in mature organisms: upon paracetamol overdose, for example, liver damage is the main toxic outcome observed irrespective of whether the intoxicated individual is a young adult,

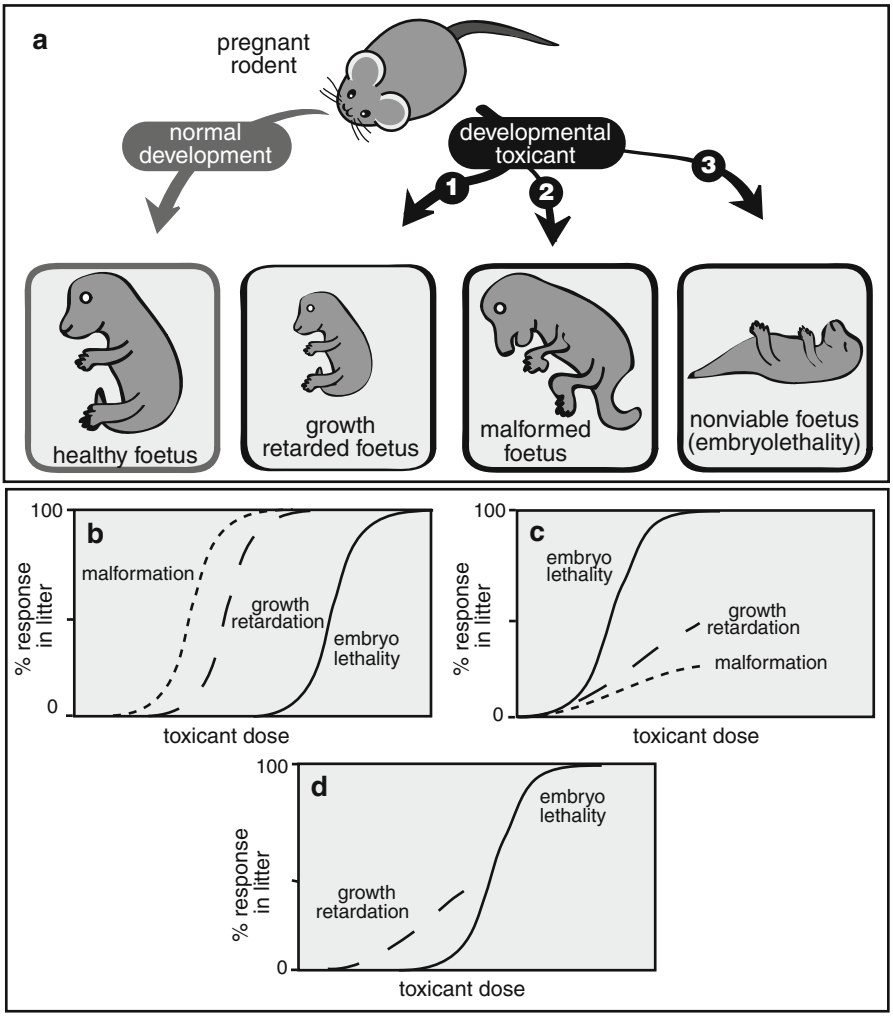

Fig. 7.1 Littermates of a multiparous species often exhibit a mixture of three classic responses to developmental toxicants, namely, growth suppression, malformations and embryolethality (*Panel a*). The lower panels depict various outcomes that accompany prenatal exposure to developmental toxicants that elicit different combinations of these three toxic responses, with the compound in *Panel b* of particular concern due to a tendency to induce birth defects at low doses. Some true developmental toxicants induce all 3 responses at any given dose (*Panel c*), although nonteratogenic agents may elicit growth retardation and embryolethality only (*Panel d*). (The graphs in the lower panels are with kind permission from Springer Science + Business Media: Neubert et al. (1980) Drug-induced damage to the embryo or fetus (molecular and multilateral approach to prenatal toxicology). Curr Top Pathol. 1980;69:241–331)

a middle-aged individual or a geriatric patient. While differences in the severity of toxicity may occur between such individuals, the main manifestation of toxicity (i.e. liver damage) is essentially similar throughout the postnatal human lifespan.

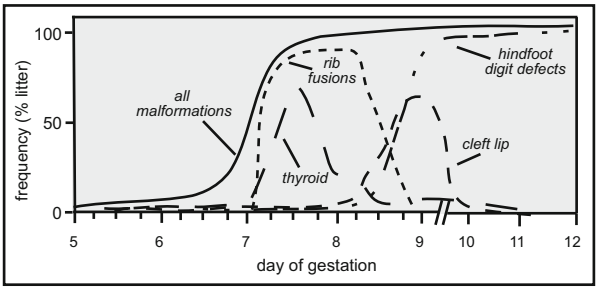

Fig. 7.2 Classic studies by Ray Shenefelt confirmed that the timing of teratogen exposure determines the precise nature of the malformations seen in offspring. Pregnant hamsters received a single LD_{50} dose of retinoic acid at one of 20 carefully defined points within the gestation period. Only a select set of malformations are shown – see original manuscript for more information (With permission from R.E. Shenefelt, (2010) Morphogenesis of malformations in hamsters caused by retinoic acid: Relation to dose and stage at treatment. *Birth Defects Research Part A: Clinical And Molecular Teratology* 2010: 88(10):847–862, Copyright © 2010 Wiley-Liss, Inc.)

Prenatal toxicity is inherently different in that the equivalent dose of the same teratogen can produce very different effects depending on the prenatal age of the conceptus. As a rule, the human conceptus is most vulnerable to malformations during the embryonic period that spans weeks 3–8 of pregnancy. During this crucial 2-month window the most profound and complex events required for constructing the human form occur: new limbs and internal organs begin forming as embryonic cells are committed to a tightly orchestrated programme of proliferation, migration and differentiation. Within this interval, each organ or structure exhibits its own critical period of teratogenic susceptibility, often coinciding with the period in which the rudiments of each organ begin forming. The length of the critical period for each body system varies according to how long it takes for cellular differentiation, migration and extension to occur: for organs such as the heart, this period lasts for just 3 or 4 weeks, while for the brain it lasts for at least 6 months.

The corollary of this concept is that the same dose of a given teratogen can elicit different toxic responses depending on the precise timing of prenatal exposure (Fig. 7.2). This principle was established in classic work in pregnant hamsters conducted by Ray Shenefelt at the University of Cincinnati over 40 years ago. A clinical pathologist, Shenefelt was mystified by variable malformation patterns in paediatric patients who received the same drug. To systematise these observations, he administered an equivalent dose of retinoic acid at one of 20 different stages throughout the 12-week gestational period in hamsters. Retinoic acid is a water-soluble analogue of vitamin A, an essential dietary requirement that causes embryonic malformations at high doses. Some 623 foetuses were scored for malformations in internal body organs as well as the head, neck and spinal cord. To allow correlation of drug-induced damage with normal events in embryonic development, careful examination was also made of 200 time-matched control foetuses. Researchers also tracked the number of foetuses per litter.

This classic study revealed a clear ‘window of susceptibility’ for many teratogenic outcomes, with retinoic acid eliciting profound toxicity towards one body component at one stage in development yet having no effects whatever on the same body component if given several weeks later (Fig. 7.2). With time, experiments with other teratogens in other species provided similar proof of the ‘window of susceptibility’ phenomenon. Such findings helped improve the performance of animal testing methods for developmental toxicity since researchers realised the importance of timing toxicant administration in relation to gestational age. These findings also had ramifications for clinical practice since some potentially teratogenic drugs are reluctantly administered to pregnant mothers suffering from ‘breakthrough’ diseases such as epilepsy that require therapeutic intervention throughout pregnancy. Also, with trends towards older maternal age evident in many countries, a rising incidence of pregnancy-related cancer in mature mothers is necessitating the use of toxic anticancer drugs in pregnant women. Defining the ‘window of susceptibility’ for such drugs in humans is essential to minimising adverse developmental outcomes in their offspring.

Before concluding our brief review of the classic studies by Shenefelt, it is worth noting that he was among the first to conclude that the susceptibility of a given embryonic organ to toxicity was not fully predictable from observed developments within control embryos at the same gestational age. A particular body component, for example, might not be visible in control embryos until day 8, yet its formation could be disrupted by teratogen administration on days 5 or 6, well before the structure was physically visible. Shenefelt proposed that teratogens might disrupt early biochemical events in susceptible body parts that are initially unseen by the naked eye yet subsequently manifest as observable anatomical structures. These crucial insights helped focus attention upon the biochemical and molecular mechanisms underlying the induction of prenatal toxicity. Nowadays powerful ‘omics’ technologies assist studies of this kind by assessing changes in the levels of thousands of gene transcripts, intermediary metabolites or individual protein markers during the onset of teratogen-induced embryonic injury. By identifying clusters of responsive genes and disrupted developmental programmes, these studies may reduce the need for testing new chemicals in detailed and expensive animal studies.

7.4 Foetal and Embryonic Metabolism

The complex interplay between xenobiotic metabolism in maternal tissues, the placenta and unborn child greatly complicates mechanistic study of teratogenic syndromes. Indeed, the precise teratogenic contributions are unknown for any specific metabolites for virtually all of the several dozen or so proven human teratogens. The situation is likely better in animal studies, but an unfortunate element of vagueness surrounds the precise role of particular metabolites in the human teratogenicity of even well-studied toxicants such as thalidomide. The fact that the xenobiotic-metabolising capacity of the conceptus changes continually throughout pregnancy

further complicates implication of specific metabolites in teratogenic outcomes. Finally, significant interspecies differences in the maturation of xenobiotic-metabolising enzymes between commonly used lab species and the human conceptus present additional obstacles to progress in this field.

For some teratogens, most bioactivation likely occurs within the maternal liver; hence, whether toxic metabolites can evade detoxication in that organ before entering the systemic circulation strongly influences toxicity in the unborn. The teratogenicity of polycyclic aromatic hydrocarbons (PAH) – ubiquitous environmental pollutants and major tobacco smoke carcinogens (see Chap. 10) – likely conforms to this scenario by involving bioactivation within the maternal liver. Yet, the likelihood that the conceptus may also bioactivate xenobiotics complicates these conclusions, since repeated PAH exposure strongly boosts the expression of placental CYP1A1 which can efficiently convert PAH to damaging metabolites. Dissecting the contribution of maternal bioactivation relative to foetal metabolism to teratogenic outcomes is rarely easy, even in well-defined animal models.

As a rule, the human embryo oxidises xenobiotics poorly. The embryonic liver originates from mesoderm and endoderm during the fourth week of gestation, and its development proceeds throughout the first trimester. Since the smooth endoplasmic reticulum develops slowly during this process, the expression of most major CYP isoforms (e.g. CYP1A2, -2C9, -2D6, -2E1 and -3A4) is low or undetectable in human embryonic liver. CYP3A7 defies this trend since its embryonic expression is often high. Since it can bioactivate many noxious xenobiotics (e.g. PAHs and aflatoxin B_1), CYP3A7 is likely a key villain in human teratogenic syndromes.

Although significant interindividual variability is observed, expression of most CYP isoforms rises as pregnancy proceeds through the second and third trimesters. Rising expression of CYP2E1, for example, the main isoform that converts alcohol to its toxic acetaldehyde metabolite, is likely a key factor in the foetal alcohol syndrome (see Chap. 9, Sect. 9.5). Despite rising CYP expression as birth approaches, the hepatic levels of most human CYP isoforms will not approach adult levels until an infant completes its first year of postnatal existence. This diminished capacity for oxidative metabolism often guides the selection of medicines during the postnatal care of young infants (i.e. medicines requiring oxidative metabolism for clearance may be avoided).

The ability of the conceptus to carry out conjugative metabolism varies according to the pathway under consideration. The expression of glutathione-S-transferases is typically low in the embryonic period, thus enhancing vulnerability to cell damaging electrophiles. The embryonic capacity for replenishing cellular glutathione via biosynthesis is also low. The glucuronidating capacity of the conceptus is also limited until midgestation, with UDP-glucuronosyltransferase expression generally rising from that point until birth. In clear contrast, prenatal liver exhibits a strong ability to sulfonate xenobiotics, although embryonic patterns of expression of individual SULT isoforms can differ significantly.

Although CYP expression is limited in the embryonic liver, electrophilic metabolites may still form via alternative routes such as peroxidase-catalysed oxidative activation. Such pathways can expose the conceptus to oxidants and free radicals,

thereby triggering oxidative cell injury due to low embryonic expression of key antioxidant enzymes such as catalase and superoxide dismutase. The vulnerability of embryonic tissues to oxidative stress likely exacerbates many teratogenic syndromes.

7.5 The Role of the Placenta

A crucial interface between the maternal and foetal circulation that forms via union of uterine mucous membranes with foetal blood vessels, the placenta enables the successful completion of pregnancy by nourishing the growing foetus and removing its metabolic wastes. Its critical constituent is the syncytiotrophoblast that forms an epithelial layer covering the placental villi that penetrate the uterine wall to access the maternal circulation. The underlying placental substratum comprises the cytotrophoblast layer.

7.5.1 Placental Metabolism

A complex and dynamic structure that grows as pregnancy proceeds, the placenta is itself targeted by some toxicants. Its toxicological significance stems from three main roles: regulating the flow of oxygenated blood to the foetus, enabling the bidirectional transport of blood-borne substances and facilitating xenobiotic metabolism. The main placental CYP isoform in humans is CYP1A1, although its levels are normally low in the absence of circulating CYP inducers (e.g. tobacco combustion products, drugs such as dexamethasone or environmental pollutants such as polychlorinated biphenyls). For most other important CYPs (e.g. 2C9, 2D6, 3A4/7 or 2E1), researchers have typically shown the presence of respective mRNAs in human placenta yet have failed to detect expressed protein or associated catalytic activity. CYP1A1 is thus likely the primary catalyst of oxidative xenobiotic metabolism within human placenta. The capacity for conjugative metabolism is considerably greater, with functionally active isoforms of SULT, UGT and GSTs typically detectable in most full-term placenta.

7.5.2 Placental Transporters

While researchers once assumed that chemicals cross the placenta via passive diffusion, evidence suggesting a major role for xenobiotic transporters has accumulated. The cells comprising the syncytiotrophoblast layer are highly polarised, comprising a distinct apical brush-border membrane that faces the maternal circulation and a basolateral membrane that interacts with foetal capillaries. Expression of xenobiotic

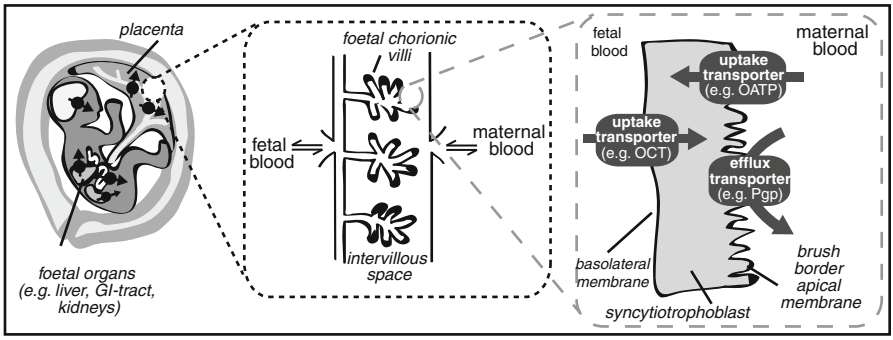

Fig. 7.3 Strong expression of xenobiotic transporters in foetal organs and placental membranes protect against toxicants in the maternal circulation while also clearing metabolic waste products from the foetus

transporters is strong on both membranes, including both ATP-binding (ABC) and solute carrier (SLC) classes (Fig. 7.3). Transporter expression is dynamic and varies with advancing gestation. In terms of functional roles, efflux transporters in the apical membrane appear to minimise foetal accumulation of xenobiotics from the maternal blood, with major roles likely for the ABC transporters P-gp (ABCB1) and BCRP in this context. Efflux membrane transporters in the basolateral membrane may assist the accumulation of desired solutes by the foetus or, alternatively, limit the export of foetal waste products back to the maternal blood (Fig. 7.3).

7.6 Teratogenic Mechanisms

Many xenobiotics that disrupt fundamental biological processes such as DNA synthesis or mitochondrial respiration predictably cause toxicity in the unborn. A major challenge facing modern teratology is the development of mechanistic explanations for the effects of rogue teratogens that do not disrupt basic cell processes of this kind. In some respects, this task grew simpler in recent decades with improved understandings of the molecular basis for normal prenatal development, thanks in part to knockout mouse technology that allowed study of the role of individual genes in embryonic development. Subsequently the availability of green fluorescent protein marker technology allowed visualisation of specific gene products during embryonic development in lab animals. Methods allowing overexpression of genes in embryonic tissue also assisted studies of prenatal development. While such advances in mechanistic embryology aided the study of chemical teratogenesis, progress in understanding the precise events whereby major human teratogens induce prenatal toxicity has proved difficult. A major source of frustration is that while powerful theories are testable in animal models, problems arise when applying such insights to the human situation. In particular, uncertainty concerning the

actual teratogenic metabolites formed within embryonic tissues during the 'window of susceptibility' for a given toxic response hinders proper understandings of many teratogenic syndromes. Nevertheless, with the proviso that the human relevance of mechanistic insights sometimes awaits future proof, in recent decades animal studies have supplied many valuable insights into teratogenic mechanisms. As a rule, most teratogenic substances damage the conceptus via either receptor-mediated or bioactivation-dependent mechanisms. Note that these are not watertight categories since for some human teratogens experimental evidence for both mechanisms is suggested by animal studies.

7.6.1 Receptor-Mediated Teratogenesis

For some chemically induced teratogenic syndromes, the disruption of prenatal development proceeds via transient, reversible interactions between teratogens and receptors within embryonic tissues. While the ligand is usually the parent xenobiotic, metabolism can conceivably convert an innocuous molecule into a receptor-binding ligand with teratogenic potency. While the nature of the target macromolecule varies according to the teratogen, a common outcome of receptor activation is an observable dysregulation of gene expression in embryonic tissues. Receptor-driven transcriptional changes within key developmental pathways can be monitored using microarrays, PCR or other analytical approaches.

Known receptors or protein targets for human teratogens typically belong to several main classes. In some instances, the target protein possesses enzymatic activity; hence, xenobiotic binding may interfere with production of associated metabolites or, in other cases, stimulate enzyme catalysis. A classic human teratogen with these properties is the anticonvulsant valproate, a known inhibitor of histone deacetylase (HDAC) enzymes that regulate chromatin behaviour during embryonic development (see below). More recently, a breakthrough in our understanding of thalidomide teratogenicity occurred when Ito and associates identified a novel thalidomide-binding protein which they named cereblon (see below). This target belongs to a family of E3 ubiquitin ligases that regulate the proteolytic turnover of transcription factors. Drug-induced interference with these enzymatic processes can disrupt many signalling pathways.

Other chemicals target transcriptional factors directly, in which case toxicant binding alters interactions with accessory proteins and coactivators which disrupt binding of the transcription complex to promoters or other gene regulatory elements. Most attention in this regard has centred on transcription factors known as homeobox (HOX) genes that guide the assembly of body parts by controlling head-tail patterning during embryonic development. HOX factors contain a highly conserved 61-amino acid DNA-binding domain known as a *homeodomain*. The human genome contains 39 HOX genes organised in four genomic clusters, forming a network of transcription factors that control embryonic development while also regulating key processes in mature cells. Many teratogens likely disrupt HOX function,

including retinoic acid, valproate and endocrine disruptors as well as toxic metals such as cadmium. Since several HOX genes (HOXA9-A13) are under the transcriptional control of nuclear hormone receptors such as the oestrogen receptor, estrogenic xenobiotics likely induce birth defects by disrupting these HOX pathways in developing tissues.

A final mechanism of receptor-mediated teratogenic action proceeds via interactions of agonists or antagonists with key physiological receptors. The best understood of such pathways involve teratogen binding to nuclear receptors for glucocorticoids (e.g. GR), retinoids (e.g. RAR) or aryl hydrocarbons (e.g. AhR). Such interactions are difficult to avoid in some obstetric settings, such as when the GR ligand dexamethasone is administered to women at risk of premature delivery in an effort to foster foetal lung maturation. Studies in pregnant rats suggest such uses of dexamethasone may alter forebrain development, leading to long-term disruptions of neural wiring within the brain.

Another area where receptor-driven teratogenic responses are of major clinical significance is during the treatment of severe acne vulgaris, a condition that can afflict women of childbearing age. The use of isotretinoin (e.g. Accutane), a significant human teratogen, is best avoided but may be acceptable if contraception therapy is co-administered and the drug is used under the care of a senior dermatologist.

7.6.2 Bioactivation-Dependent Teratogenesis

The enzymatic conversion of a pro-teratogenic xenobiotic into a chemically reactive species that attacks cellular targets in embryonic tissues helps explain several teratogenic syndromes. As we saw in Chap. 4 (Sect. 4.3.1.3), the toxicity of these reactive intermediates is governed by such factors as their chemical stability and preference for ‘hard’ versus ‘soft’ nucleophiles. Similar considerations apply within the context of human teratogenesis where exposure to ‘hard’ electrophiles and formation of miscoding DNA adducts within foetal tissues confers an increased risk of childhood tumours in surviving offspring.

Alternatively, reactive intermediates that behave as ‘soft’ electrophiles favour reactions with cell proteins that are rich with ‘soft’ nucleophilic residues (e.g. cysteine groups). Although their role in human teratogenesis remains to be proven, electrophilic epoxy metabolites and reactive quinones formed during phenytoin metabolism are possible contributors to the craniofacial abnormalities seen in infants following in utero exposure to this anticonvulsant drug (a condition termed foetal hydantoin syndrome, FHS). A role for electrophilic epoxides in the induction of birth defects in the offspring of phenytoin-treated mothers is suggested by observations concerning a protective role for placental microsomal epoxide hydrolase (*EPHX1*) in protecting against craniofacial abnormalities, but little progress has been made in identifying protein targets for this electrophilic species. So while these metabolites may behave as ‘soft’ electrophiles during their reaction with embryonic proteins, this awaits experimental confirmation. At present, the biology

underlying phenytoin teratogenicity remains unclear, with animal data suggesting complex contributions by a range of deleterious mechanisms including hypoxia, osmotic imbalances, cardiac arrhythmia, free radicals and electrophilic metabolites.

7.6.3 Epigenetic Teratogenesis

In addition to mutations triggered by DNA adducts and other kinds of genetic damage, some developmental toxicants disrupt prenatal development by producing epigenetic changes within developing embryos. Such toxicants induce changes in gene activity and transcription without inducing underlying DNA sequence alterations. Interest in epigenetic mechanisms has grown in recent decades, fuelled in part by epidemiological data that uncovered the powerful influence of the intrauterine experience of the foetus in governing susceptibility to adult disease – a field of study that explores the 'foetal origins hypothesis'. Using animal models to test this theory, biomedical researchers dismissed a major role for DNA mutations as the link between maternal malnutrition or other prenatal stresses and subsequent adult ill health. After the search for alternative explanations identified novel epigenetic mechanisms, toxicologists quickly grasped their potential to explain the long-term effects of prenatal chemical exposure. In addition to helping explain the effects of toxicants on an exposed embryo, animal toxicity studies as well as emerging epidemiological data have suggested that epigenetic reprogramming can extend the noxious effects of chemicals over several generations. Addressing these possibilities is an emerging research concern within twenty-first-century toxicology.

Developmental toxicants likely disrupt prenatal development via one of three major epigenetic mechanisms, including changed patterns of DNA methylation at cytosine residues in CpG dinucleotides, altered post-translational modifications on the amino-terminus of histone proteins or disrupted regulation of gene expression by microRNAs. The latter recognition followed the identification of various microRNAs that are under strong epigenetic control. The ongoing development of focussed arrays to monitor changes in microRNA abundance during teratogen exposure will assist future work in this area.

Although our attention in this chapter is restricted to toxicants that disrupt prenatal development in utero, it is important to note that epigenetic mechanisms also mediate the effects of *reproductive toxicants* that target meiotic gamete production within reproductive organs. Since such events can reprogramme ovarian function within the unborn female child, toxicant-induced epigenetic changes can have long-term intergenerational consequences.

Although research in this area is still expanding, some significant chemicals have attracted attention for their ability to elicit developmental and reproductive toxicity via epigenetic mechanisms. These include the environmental pollutant dioxin (e.g. prostate, thymus abnormalities), the plasticiser bisphenol A (e.g. testicular, brain and mammary gland dysmorphology) and toxic hydrocarbons (such as the aviation

fuel JP-8). Experimental findings suggesting that epigenetic changes occur at lower doses of toxicants than elicit ultrastructural or biochemical changes in targeted cells underscore the importance of this research endeavour and ensure that work in this crucial area will expand in coming years. The role of epigenetic mechanisms in the prenatal toxicity of valproate teratogenicity receives attention below (Sect. 7.8.1.1).

7.6.4 Disruption of Cell Adhesion

A growing embryo represents a highly complex agglomerate of cells that is assembled via a dynamic yet tightly orchestrated genetic script. From a tissue engineering perspective, the success of this venture depends on the cellular ability to form and maintain appropriate three-dimensional attachments with neighbouring cells within emerging tissue structures. Over recent decades, developmental biologists have gained useful insights into the genetic basis for these 'patterning' processes within growing embryos, revealing that cell surface proteins function as tiny pieces of Velcro to maintain an orderly tissue architecture by holding cells together in an appropriate spatial array. Particular attention has focussed on two broad families of cell adhesion molecules, namely, the cadherins and immunoglobulin cell adhesion molecules (IgCAM). Growing awareness of their role during normal embryonic development raised the possibility that teratogens might disrupt cell adhesion pathways during embryonic development. This hypothesis soon proved very fruitful: many important teratogens now seem likely to elicit their destructive effects upon the developing child by disrupting cell adhesion pathways.

Human cadherins comprise a diverse class of over 80 family members that belong to three or four main subgroups. Their role during the early stages of prenatal development is especially important, extending beyond cell adhesion alone to include functions in cell sorting, cell recognition and cell movement. Changes in the expression of individual cadherins occur throughout embryogenesis, leading to a phenomenon known as *cadherin subtype switching* during neurulation where N-cadherin (neural cadherin) is strongly expressed on the invaginating neural plate while the overlying ectoderm expresses E-cadherin (epithelial cadherin).

Cadherins also participate during the growth and maturation of foetal tissues, including regulation of neuronal and glial cell adhesion and migration during formation of the nervous system. Chemical-induced disruption of these functions by teratogens can have broad and long-lasting neurological consequences for the developing child.

Teratogens and developmental toxicants may disrupt cadherin function via several routes (Fig. 7.4). Firstly, an indirect route can involve downregulation of their expression via effects on cell signalling pathways or transcription factors that regulate cadherin synthesis. Downregulation of mRNA transcripts for various cadherin isoforms is detected upon microarray analysis of RNA extracted from rodent embryos following exposure to many developmental toxicants. Since cadherin expression is regulated via promoter methylation, these epigenetic mechanisms may also mediate teratogen-induced changes in cadherin expression in the unborn.

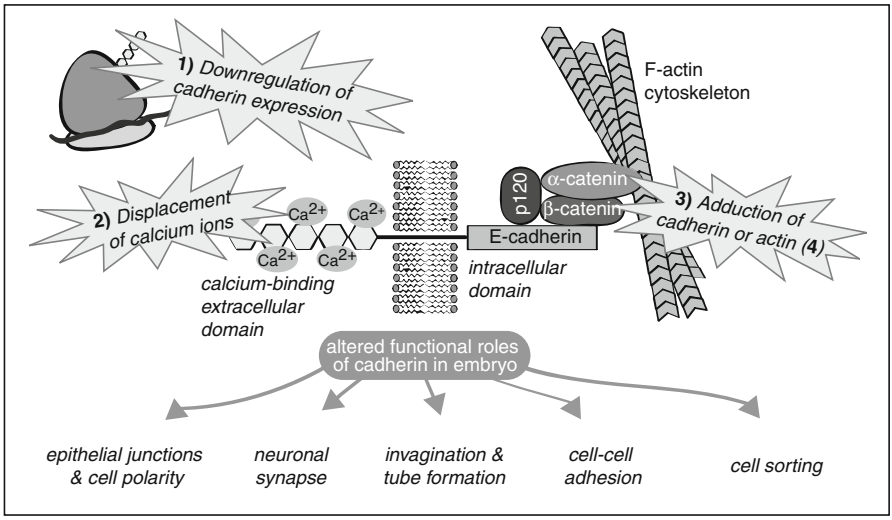

Fig. 7.4 A common mechanism of teratogenicity involves interference with the expression or function of cadherin molecules that are key constituents of adherens junctions and other cell adhesion structures. See text for discussion of four potential mechanisms for cadherin disruption

A second route to altered cadherin function involves direct interaction of teratogens with the cell surface structure itself. The adhesive properties of cadherins require maintenance of their normal molecular conformation via binding of calcium ions to extracellular calcium-binding repeat domains that are core constituents of any cadherin assembly. Some important human developmental toxicants such as lead and cadmium likely perturb prenatal development via 'calcium mimicry' in which calcium ions are displaced from their binding sites on the cadherin structure. We will briefly return to the problem of cadmium teratogenicity below.

Thirdly, although this mechanism has received most attention within contexts other than developmental toxicity, it is conceivable that an electrophilic metabolite might disrupt cell attachment by attacking the cadherin protein directly, forming adducts that erode intercellular adhesive strength in vulnerable embryonic structures. This mechanism seems worthy of investigation for teratogens which form reactive intermediates within the womb.

Finally, since the functions of cadherins depend on close partnerships with actin filaments, the strength of cell–cell adhesion complexes may be undermined following damage to these cytoskeletal components by reactive metabolites. As highly expressed proteins, actins are frequent targets for reactive chemicals within many cell types.

Although the above coverage focussed on cadherin disruption, developmental dysfunction can also proceed via disruption of adhesion structures other than cadherin-dependent adherens junctions, such as gap junctions and tight junctions. A number of developmental toxicants likely disrupt the functions of occludin and ZO-1 within tight junctions or promote connexion-43 dysfunction within gap junctions. The key developmental role of adhesion structures implies disruption of these processes has deleterious consequences for many body systems.

7.7 Testing for Developmental Toxicity

The thalidomide disaster as well as other 1960s teratogenic outbreaks focussed considerable attention on the need for improved testing procedures to protect unborn humanity against rogue xenobiotics. While lab animals formed the mainstay of early teratogenicity testing, over subsequent decades these approaches were supplemented by newer methods that reflect growing biomedical knowledge. In addition to reflecting community concerns over animal wellbeing, increasing use of alternatives to traditional rodent-based testing also reflects pressures from both economic reality and scientific necessity.

7.7.1 Testing in Pregnant Animals

In retrospect, it was inevitable that the black shadow of thalidomide would hang heavily over developmental toxicology during its initial development. For example, the unusual species susceptibility to thalidomide strongly influenced regulatory requirements established by government health bodies to guide the testing of new drugs and xenobiotics. By a strange quirk of nature, the offspring of pregnant mice, rats and hamsters are entirely resistant to thalidomide, while rabbits and chickens responded to massive doses of the drug. Based on this knowledge, many regulatory agencies stipulated the use of rabbits and one other rodent species during routine prenatal testing of drugs, pesticides, herbicides, solvents and other chemicals. Regulators quickly realised that when designing studies of this kind, the timing of toxicant administration needs careful attention in relation to the timing of key developmental events in the target species.

Testing procedures in pregnant animals are typically laborious since they are carefully designed to assess the effects of chemicals not only during the embryonic and foetal periods but also to detect reproductive toxicity throughout the entire phase of mammalian development. Hence, to detect deleterious effects on gamete production, test chemicals are often administered to male and female animals for between 2 and 6 weeks in duration prior to mating. After mating and the confirmation of pregnancy, exposure is often continued throughout the gestational interval as well as following delivery and the period of lactation (i.e. until weaning). Indeed, the most demanding and sensitive developmental toxicity protocols involve continuous exposure of rats to test chemicals throughout three generations. This allows detection of subtle toxicant-induced effects on physiological end points such as body weight and food consumption as well as numerous reproductive end points including fertility, timing of delivery, litter size, weight gain in newborns and age of puberty. In recent years, studies of this kind have uncovered the effects of a diverse class of chemicals known as ‘obesogens’: substances that disrupt lipid metabolism within the developing embryo and confer a lifelong tendency towards weight gain. These outcomes are especially relevant to endocrine disrupting chemicals such as bisphenol A (see below).

Animal tests of this kind are expensive, requiring scrupulous attention to protocols surrounding every aspect of animal breeding, housing, climactic control, food and water quality, toxicant delivery, animal monitoring and maternal wellbeing. Finally, at the completion of the study, detailed histopathological analysis is performed upon the mothers and their offspring. The body weight, rump length and other morphological end points are determined, with particular care taken to measure the craniofacial dimensions of the neonates. The skeleton is also subjected to intense scrutiny since disruption of bone formation is a highly sensitive teratogenic indicator. For drugs, pesticides and other chemicals with likely effects upon neurological processes, the offspring are subjected to extensive neurobehavioural testing to identify subtle disruption of CNS development. If the compound under investigation warrants extra attention, study of its toxicokinetic behaviour or its metabolism is included in the study design. Mechanistic investigations may also be performed, such as the incorporation of microarray evaluation of embryos, offspring or mothers at defined end points within the study.

Since many confounding factors can influence study outcomes, the interpretation of data from prenatal toxicity testing in rodents requires considerable sophistication. The gender balance of offspring in control and toxicant-treated groups requires thoughtful attention. Sometimes a toxicant produces a reduction in birth weight that might suggest growth retardation, but upon closer examination, the apparent reduction might reflect an over-representation of smaller females in some litters. Different litter sizes between control and toxicant-treated groups also require attention, since small litters often comprise heavier foetuses, thereby compounding detection of growth retardation. Retarded growth also needs careful analysis to weed out confounding effects of reduced consumption of unpalatable food or water (a problem if the animals disliked the taste of high doses of test chemicals). Since male rodents are likely less fussy than females in this regard, sex ratios need careful consideration when interpreting toxicity data obtained from studies of this kind. If the toxicant possesses physical bulk, growth reduction secondary to undernutrition at high doses can reflect lower-calorie contents per gram of food.

Despite such confounding factors, traditional methods using pregnant rodents offer many benefits: on the one hand, they are obviously effective since they have prevented the recurrence of large-scale teratogenic episodes that come anywhere near replicating the horror of the thalidomide epidemic. The sustained use of these methods over many decades has also generated a rich database of knowledge for use as a benchmark during the assessment of new chemicals. The use of animals also allows detailed investigation of teratogenic mechanisms, dose–response relationships and the window of susceptibility for a given developmental toxicant. Due to these benefits, animal-derived teratogenicity data is given significant weighting during the classification of human pharmaceuticals and other xenobiotics by regulatory agencies. Many agencies such as the US Food and Drug Administration assign drugs to one of five classes that span from ‘category A’ (safest for use in pregnancy) to ‘category X’ (unsafe for use in pregnancy since risks outweigh benefits). Drugs are assigned to these categories on the basis of human evidence for prenatal toxicity, although animal data is also given significant weighting during the review process.

Other national regulatory bodies use similar classification systems to convey information to physicians during the selection of drugs for use in pregnant mothers.

Although animal-based tests will continue for the foreseeable future, economic realities and changing attitudes to drug testing have inspired modern toxicology to develop alternatives to these methods. Due to their low capacity for throughput, a backlog comprising thousands of xenobiotics awaits evaluation in pregnant rodents. Another concern is that developmental toxicity data is sometimes gained at toxicant doses that are of questionable relevance to humans. At high doses, distinguishing between primary toxicity towards the conceptus and secondary toxicity due to maternal pathology can confound interpretation of test findings. The shrinking pool of experienced lab facilities that can perform rodent tests competently is also driving the search for new approaches.

7.7.2 Alternative Tests

Toxicologists have evaluated hundreds of tests as alternatives to conventional animal testing. An obvious feature of many simplified test systems is the lack of such maternal body components as the placenta or liver. Opinions differ as to whether such absence is beneficial or detrimental to the performance of test systems. On the one hand, the absence of a placenta may increase the sensitivity of the test to toxicants, especially if placental membrane transporters otherwise limit foetal accumulation of the test substance in the whole animal. The counterargument mounted by critics of in vitro tests is that this may ensure these approaches are unduly conservative, incriminating too many chemicals as developmental toxicants unnecessarily. A similar divergence of opinion prevails concerning the value of alternative test systems on account of their absence of maternal toxicity: for some, the ability to attribute developmental toxicity to secondary toxicity in the mother is a strength of traditional tests in pregnant animals, for others, removing this confounding factor strengthens in vitro approaches.

A widely used in vitro test that has been refined in recent decades is the cultured rodent embryo. First developed in the 1950s, whole-embryo culture techniques soon found wide use in developmental biology and toxicology. While full embryonic development requires a placenta and other maternal inputs, cultured mouse and rat embryos can be used to study toxicant-induced effects during the midgestational period (i.e. embryonic days (E) 7–12 in the mouse or 9–15 in the rat). Ongoing improvements in culture techniques are likely extending the timeframe of rodent embryo studies. When combined with microarray and other ‘omics’ technologies, these methods can detect a wide range of toxicant-induced developmental responses. Other rodent-derived models include the use of cultured embryonic limb buds, embryonic stem cells or single cells from specific organs or tissue slices. Recent testing of a battery of several hundred environmental chemicals revealed that mouse embryonic stem cells exhibit highly focussed transcriptional changes that predict teratogenic potency for some chemicals.

In addition to mammalian models, recent years have seen growing use of non-mammalian vertebrates such as zebrafish embryos to study developmental toxicity. Related innovations study the effects of toxicants on early development in invertebrate species such as molluscs. The lack of placental structures in these models is a limitation to these approaches as well as a potential strength. Some pharmaceutical companies are now using these nonmammalian tests as early 'toxicity' screens to guide the selection of safer lead compounds for further development. The pharmaceutical industry also makes growing use of computational models that allow predictive analysis of teratogenic potential of compound libraries within an in silico environment. In principle, such high-throughput screening approaches should reduce the need for large-scale in vivo testing in pregnant animals. The latter more expensive traditional methods are thus reserved for short-listed candidates that require additional examination to satisfy commercial development and regulatory requirements.

7.8 Major Human Teratogens

The list of known human teratogens includes natural toxins, synthetic environmental pollutants, occupational toxicants and, most commonly, human pharmaceutical agents. Unfortunately, the list includes many drugs that are used to treat chronic conditions that require clinical management during pregnancy, such as epilepsy and cancer. Due to space constraints, we will explore a small group of mechanistically diverse chemicals below.

7.8.1 Drugs

7.8.1.1 Valproate

Valproate is an effective anticonvulsant that controls epileptic seizures for some patients who respond poorly to other medications. The drug also assists the clinical management of bipolar manic-depressive disorder as well as migraine. Standard dosing schedules recommend the use of comparatively high daily doses of between 1 and 3 g. In spite of the beneficial properties, valproate can cause hepatotoxicity in some patients, and special concerns accompany its use in pregnancy due to significant toxic potential towards unborn children. For example, the probability of neural tube defects rises to around 1 in 20 babies in mothers who ingest valproate during the first trimester. During prenatal development, the neural tube develops into the spinal cord and brain via shaping, folding and midline fusion of the neural plate in a complex process termed *primary neurulation*. Spontaneous defects within this process are common, and spina bifida is a general term applied to a range of neural tube malformations, which most commonly feature caudal lesions affecting the spinal cord, vertebrae and skin. In the USA, the incidence of spina bifida is about 1 in 1,500

births. The seriousness of spina bifida varies from mild conditions that are correctable by simple surgery, through to serious conditions requiring long-term care.

While neural tube defects have received most attention as a toxic response to valproate, prenatal exposure to this drug is also associated with neurological and cognitive deficits, craniofacial defects, heart abnormalities and skeletal malformations. The neurocognitive deficits are especially worrying and include changes in verbal intelligence and communication skills that resemble those accompanying some forms of autism. Although other antiepileptic drugs such as phenytoin and carbamazepine carry significant risks of prenatal toxicity, US data suggests the risk of malformations in epileptic women receiving valproate alone (i.e. monotherapy) is nearly fourfold greater than corresponding outcomes in neonates exposed to other anticonvulsant agents.

Reproduction of valproate-induced neural tube deficits in various lab animal species has allowed study of potential teratogenic mechanisms. Only limited progress occurred until researchers at the University of Pennsylvania identified valproate as a strong inhibitor of histone deacetylase (HDAC) activity in 2001. HDAC enzymes regulate chromatin remodelling during embryonic development by controlling the balance between histone acetylation and deacetylation. Acetylation of the N-terminus of histones diminishes their intrinsic positive charge, preventing electrostatic interactions with negatively charged DNA and allowing unhindered access by transcription factors to their respective promoter sequences (Fig. 7.5). By augmenting histone acetylation, valproate disrupts the transcription of genes that mediate many embryonic events, including the key process of neurulation. The role of HDAC inhibition in the induction of teratogenic outcomes has been investigated using a series of valproate analogues, such as the highly potent teratogen (S)-2-pentyl-4-pentynoic acid which is more teratogenic in mice than VPA and the nonteratogenic analogue 2-ethyl-4-methylpentanoic acid (2-Et-4-Me-Penta). Use of animal models to compare the in vivo teratogenicity of dozens of valproate analogues has revealed a close correlation between the induction of neural tube deficits and potency as a HDAC inhibitor. Analogues lacking HDAC inhibitory potency are typically devoid of teratogenic activity.

Once the role of HDAC inhibition and associated epigenetic changes in the induction of neural tube defects by valproate was established, the search began for the subset of dysregulated genes that disrupt neural tube development. In a recent study by Uppsala University researchers, microarrays containing over 22,000 gene probes were used to study gene expression changes in undifferentiated mouse embryonic stem cells following exposure to either valproate or its analogues. A brief exposure (6 h) to valproate and its highly teratogenic analogue strongly disrupted the expression of hundreds of genes involved in embryonic morphogenesis, while the nonteratogenic analogue elicited a narrower transcriptional response. Considerable overlap was seen between the genes that were disrupted by valproate and its toxic analogue, suggesting the two agents shared a common mechanism of action: both teratogens upregulated 755 genes in common while downregulating 636 shared genes. Gene ontology analysis of the responding gene sets revealed that over one-third of the upregulated genes participate in prenatal development and morphogenesis, while around one-sixth were involved in cell communication and signal transduction. Around 10 % of the overexpressed genes participated in cell

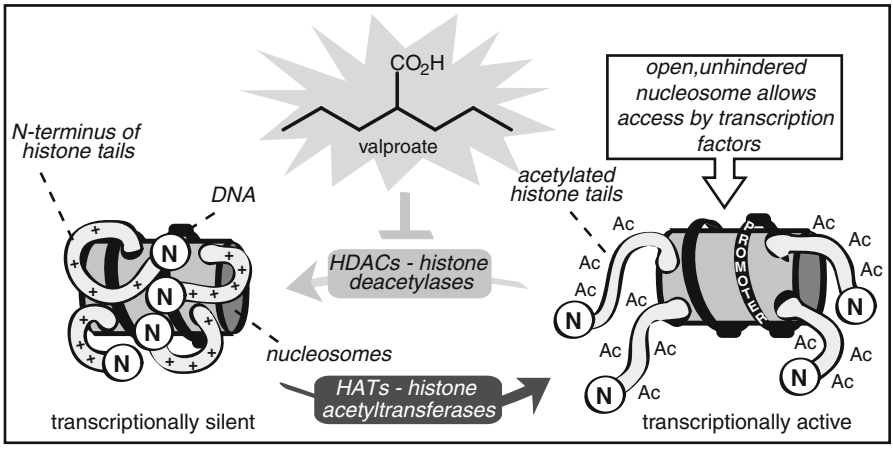

Fig. 7.5 The teratogenic potency of valproate and its analogues depend on their potency as inhibitors of histone deactylases (HDACs). Histone proteins participate in nuclear DNA winding via positively charged lysine-rich tails that bind to negatively charged DNA. Although histone binding promotes gene inactivity (*left*), in an open, transcriptionally active state, lysine residues are neutralised via acetylation (*right*), thereby losing their affinity for DNA and allowing access by transcription factors. By blocking HDACs, valproate induces a hyperacetylated, transcriptionally overactive state (The image of promoter acetylation with kind permission from Springer Science and Business Media, I.C.G. Weaver (2011) Epigenetic Programming of Stress Responses and Trans-Generational Inheritance Through Natural Variations in Maternal Care)

death regulation. Genes with vital roles in embryonic development and stem cell function such as mitogen-activated protein kinase signalling and the transforming growth factor-β signalling pathway were heavily represented among the teratogen-responsive genes. Overall, the gene changes observed implied that the major effect of the teratogens was the suppression of cell division while boosting cell differentiation. These trends were evident as a very early response, occurring long before morphological changes in the mouse embryos were observable.

While the Swedish researchers attempted to correlate gene expression changes with those caused by other teratogens such as thalidomide and carbamazepine, the data did not support the expectation that diverse teratogens might disrupt a common subset of developmentally important genes in mouse embryo stem cells. While studies of this kind reveal growth in our understanding of valproate teratogenicity, the identification of common genetic markers of teratogenicity that will facilitate screening of unrelated chemicals for these noxious properties remains an ongoing objective.

7.8.1.2 Thalidomide

Recent gains have clarified the mechanisms underlying the pronounced teratogenicity of thalidomide, the cause of the most serious medical technology disaster of the twentieth century (Chap. 1, Sect. 1.5.3). The hunt for the relevant teratogenic pathways began immediately following the disaster, with an estimated 30 theories

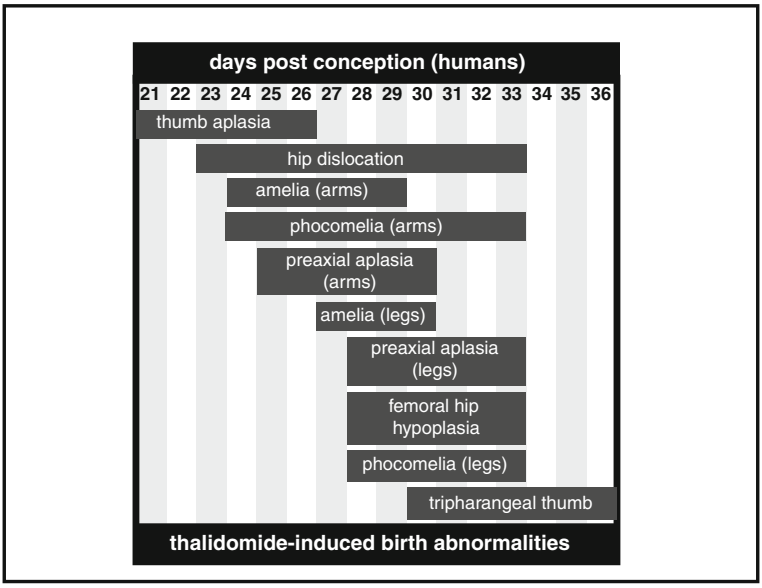

Fig. 7.6 Critical 'windows of susceptibility' for different body parts during thalidomide-induced neonatal toxicity in humans (Modified with permission from Kim and Sialla, Thalidomide: The Tragedy of Birth Defects and the Effective Treatment of Disease *Toxicol Sci.* 122(1):1–6, copyright Oxford University Press 2011)

proposed over the following three or four decades. Some of these led to distinct dead ends, such as the theory that the teratogenicity of thalidomide was due to its S-isomer (thalidomide exists as two isomeric forms, S(−) and R(+)). The subsequent finding that the isomers rapidly interchange in solution and that both are likely teratogenic undermined this theory. While many early mechanisms provided useful general insights into thalidomide toxicity (e.g. its ability to disrupt folic acid and glutamate metabolism), none satisfactorily explained the most obvious teratogenic property of the drug: why were growing limbs uniquely sensitive, and why was the 'window of susceptibility' to teratogenic outcomes so clear-cut? (Fig. 7.6). Any adequate theory also needed to explain the pronounced differences in species susceptibility to thalidomide, explaining why humans are highly sensitive while rats and mice are refractory, while rabbits are somewhere in between.

The search for underlying toxic mechanisms grew 'hotter' throughout the 1990s and subsequent decade. In the early 1990s, researchers at the Free University of Berlin uncovered a novel response to thalidomide that involved downregulation of a cluster of cell surface receptors including integrins, a family of proteins that attach cells to the extracellular matrix. These findings implied thalidomide might disrupt the attachment and migration of cells during the growth and maturation of limb buds. Soon after, in 1994, researchers at Harvard University identified thalidomide as a strong inhibitor of blood vessel growth in rabbits. By suppressing angiogenesis, perhaps thalidomide starved limb buds of growth factors and nutrients needed for proper maturation. In 1998, a Canadian group led by Peter Wells

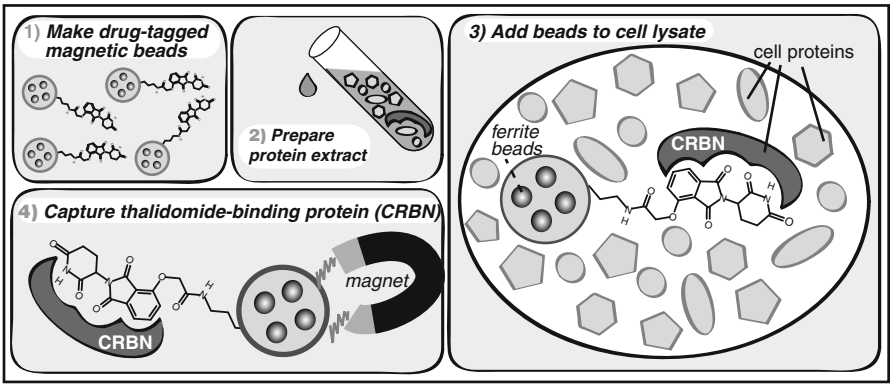

Fig. 7.7 Key steps in the ligand capture strategy used by Japanese researchers to identify a thalidomide-binding proteins in human cells. See text for details (Image redrawn with permission from Ito et al. (2012) Deciphering the mystery of thalidomide teratogenicity. *Congenital Anomalies*. 52(1):1–7, John Wiley and Sons© 2011 The Authors. Congenital Anomalies © 2011 Japanese Teratology Society)

identified a likely role for oxidative stress in thalidomide teratogenicity, showing that a radical-sequestering 'spin trap' reagent blocked the induction of limb abnormalities in rabbits. During the same decade, other labs reported immunomodulatory and pro-apoptotic actions of thalidomide, suggesting that the drug might exert a combination of deleterious actions on the growing limb. Yet despite these advances, there was little awareness of which mechanism was most important to teratogenesis, and specific protein targets for thalidomide in embryonic cells remained unknown.

A significant event in 50 years of thalidomide research occurred in 2010 during investigations led by Hiroshi Handa at the Tokyo Institute of Technology. This team of researchers used a novel protein capturing approach to identify a previously unknown thalidomide target (Fig. 7.7). Similar to how an angler attaches bait to a hook, the researchers created capturing beads containing thalidomide as a 'bait' that was attached via a linker to magnetic microspheres. This reagent was then used to 'fish' for protein targets in several human cell lines, using a magnet to recover beads from cell lysates (Fig. 7.7). Surprisingly, only one protein was consistently 'caught' by the beads, a thalidomide-binding subunit of E3 ubiquitin ligase that the researchers named cereblon. Other members of the E3 ubiquitin ligase complex include Cul4A and DDB1. Cereblon ordinarily attaches ubiquitin to various transcription factors that regulate developmental pathways, thereby earmarking them for degradation via proteasomal proteolysis. One regulator of limb development that is depleted upon cereblon binding by thalidomide is fibroblast growth factor 8 (Fgf8), a key participant in many embryonic processes that plays vital roles in limb bud growth. The loss of Fgf8-driven input to limb buds during critical periods of embryonic development likely causes the profound reduction in the length of the long bones in thalidomide-exposed infants.

To test this mechanism, Dr Handa and colleagues created a cereblon-knockout zebrafish embryo and were amazed to find the fish failed to form fins as they grew. Exposing zebrafish embryos to thalidomide induced similar fin malformations. On the other hand, zebrafish embryos carrying a mutated cereblon protein that lacked binding affinity for thalidomide resisted the teratogenicity of the drug.

As occurs following any significant breakthrough, researchers began exploring other avenues of cereblon biology and soon identified a key role for this protein in diverse settings including memory and learning within the brain. Cereblon also assists the survival of myeloma cells, a type of tumour that originates in certain white blood cells. These and other findings concerning the effectiveness of thalidomide against body wasting in leprosy patients helped fuel a cautious clinical revival of thalidomide, and a class of related drugs known as immunomodulatory drugs (IMiDs) are now used to treat myeloma as well as solid tumours (e.g. lenalidomide).

While the discovery of cereblon represents a significant achievement in modern toxicology, more work is needed to confirm the relevance of this pathway in human and other mammalian systems. One thorny issue awaiting clarification concerns the role of thalidomide metabolites in cereblon-mediated teratogenic responses. Thalidomide likely undergoes conversion to over a dozen metabolites, including a number of CYP-derived hydroxylation products. While some animal studies suggest a role for teratogenic metabolites, their precise role or the chemical identity of the key metabolites is poorly resolved. For example, in addition to CYP-derived electrophiles, some animal studies suggest a role for peroxidase-catalysed thalidomide bioactivation to free radical metabolites. How these competing mechanisms contribute to thalidomide teratogenicity is presently unclear. Now that a key protein target has been identified, we are better placed to reinvestigate the role of metabolism in toxic responses to this ghastly drug.

7.8.2 Occupational Teratogens

Rising workplace participation by women during their reproductive years has focussed growing attention on health outcomes for children exposed in utero to chemicals their mothers encounter during their work practices.

7.8.2.1 Cadmium

Cadmium is a malleable blue-white metal that also exists as compounds possessing an oxidation state of +2. Its many desirable properties once fostered wide industrial usage, including excellent corrosion resistance, low melting temperature, high ductility and high thermal and electrical conductivity. While industrial use has declined due to concern over environmental persistence and toxicological properties including nephrotoxicity, hepatotoxicity, carcinogenicity and teratogenicity, cadmium remains in use during the manufacture of batteries, pigments and colouring agents,

coatings and platings, polymer stabilisers and during the production of semiconductors and photovoltaic devices. Significant environmental exposure also occurs since cadmium naturally contaminates many metal commodities such as zinc, lead and copper; iron and steel products; fossil fuels such as coal, oil, gas, peat and wood; and many cement products and phosphate fertilisers. Small quantities of atmospheric cadmium are also released during the combustion of tobacco and other organic matter (Chap. 10, Sect. 10.6.4). The environmental impact of cadmium released from recycled materials such as batteries and scrap metals is also cause for concern. Rising concern over elevated blood cadmium levels in children of workers at the world's largest centre of electronic waste recycling ('e-waste') in Guiyu, China, is attracting growing attention to the developmental toxicology of this metal.

Evidence from lab animals confirms disruption of numerous body systems following in utero exposure to cadmium. Effects begin in the preimplantation phase, where exposure of animal oocytes to cadmium reduces the chance of fusion with sperm, while some studies show that cadmium may also suppress the progression of fertilised eggs to the blastocyst stage. Cadmium also disrupts the implantation process as well as the formation of the early embryo. While the precise spectrum of defects varies according to species, dose and timing of administration, exposure of animals to cadmium during the latter embryonic period results in diverse deficits that include craniofacial malformations (e.g. cleft palate), skeletal defects (e.g. malformed ribs, upper and lower limbs) and soft tissue abnormalities (e.g. eye disorders, neural tube defects, hernia). An increased risk of embryolethality and growth retardation also occurs under some exposure conditions.

Many harmful biochemical processes likely contribute to cadmium teratogenesis in lab animals, including induction of apoptosis, oxidative DNA damage and disruption of ion homeostasis. Most commonly, however, the wide spectrum of developmental deficits induced by cadmium reflects disrupted expression or localisation of a swathe of cell adhesion molecules, including various connexions, cadherins and zonula occludens (ZO)-1. Altered formation of gap, occludens and adherens junctions likely contributes to the broad teratogenic effects of this noxious metal. In addition to downregulation of cadherin expression, the displacement of calcium ions by cadmium likely mediates these outcomes (Fig. 7.4).

How relevant are these animal-based findings concerning cadmium toxicity to the human situation? While direct evidence of cadmium teratogenicity in humans is limited, epidemiological evidence associating exposure to this metal with the birth of underweight, growth-restricted babies has emerged from several studies. Given the wealth of data suggesting strong disruption of prenatal development in several animal species, steps to minimise maternal exposure to this noxious substance during pregnancy are highly prudent.

7.8.2.2 Organic Solvents

Organic solvents comprise a diverse group of chemicals that are widely used in industry and household consumer products. Common solvent classes include

aromatic hydrocarbons (such as toluene and xylenes which are present in adhesives and cleaners), aliphatic hydrocarbons (such as butane and propane which are constituents of fuels and lighter fluids) and alkyl halides (such as 1,1,1-trichloroethane and methylene chloride which are used as degreasing products, paint strippers and aerosol propellants). Other common volatile organics include the aliphatic nitrites, ketones and acetone. With proper use, exposure to such products likely carries minimal risk. However, poor work practices or accidents can cause high-level exposure, as can deliberate solvent abuse in some sectors of the population.

Although concern over the developmental toxicity of toluene historically centred on workplace exposures, the growing popularity of 'sudden sniffing' of propellants released from aerosol cans has focussed attention on risks to the children of women who engage in these risky practices. Toluene-containing products are abused within this setting due to their intoxicating and rewarding effects upon the brain: one recent survey of drug abuse during pregnancy incredibly ranked toluene-based solvents among the most commonly used psychoactive substances in the USA. The spectrum of adverse outcomes in affected offspring is labelled 'foetal solvent syndrome', a term that invokes comparisons with the constellation of developmental problems accompanying alcohol use in pregnancy (see 'Foetal Alcohol Syndrome' in Chap. 9, Sect. 9.7.4).

Distinguishing the effects of solvents and alcohol within affected infants is difficult for clinicians. The spectrum of physical and behavioural deficits in solvent-affected children includes prematurity and growth retardation, undersized heads, small palpebral fissures, a thin upper lip, abnormal hair patterning on the scalp, mouth malformations, urinary tract abnormalities and structural malformations of the eye and ear. As the infants grow, they display serious behavioural problems including neurocognitive deficits, hyperactivity and developmental delay. Due to co-exposure to other solvents including alcohol, whether such outcomes are fully attributable to toluene is often unknown, although studies of toluene-exposed rodent offspring do suggest subtly different toxic effects to those induced by alcohol. Studies in pregnant rats, for example, suggest the developing brain is highly vulnerable to toluene. While the mechanistic basis for these effects remains unclear, some studies suggest the possibility that toluene alters cell adhesion pathways within the developing brain.

7.8.3 Environmental Pollutants

With advances in the sensitivity in analytical technologies, recent decades have allowed quantification of a wide range of environmental pollutants within cord blood and placental samples collected upon completion of human pregnancies. The near-ubiquitous presence of these substances within these human materials confirms that exposure to environmental pollutants is an unfortunate aspect of modern life. A major challenge facing modern toxicology is the need to better understand, quantify and mitigate the risks accompanying these exposures.

7.8.3.1 Endocrine Disruptors and Bisphenol A

Within the USA, Europe and elsewhere, few compounds have attracted greater media attention in recent years than the widely used plasticiser bisphenol A (BPA). BPA is a high-volume chemical used in the production of polycarbonate plastics during the manufacturing of food and beverage packaging, compact discs, safety equipment and medical devices. BPA is also used during the production of epoxy resins that are applied as coatings to food cans, bottle tops and water piping. In 2009, the global production of BPA exceeded 2.2 million tons.

Due to the wide use in food manufacturing and processing, environmental BPA exposure is an unavoidable consequence of modern life. Consequently, BPA metabolites are detected in urine samples from an overriding majority of adults alive today. The primary source of human exposure is dietary, due to low-level contamination of foodstuffs and beverages via leaching of BPA from storage containers, plastic tableware and bottles. BPA thus belongs to a cluster of compounds that are collectively termed Food Contact Materials. Minor uptake may also occur via dermal contact with low-level BPA in water during bathing and showering. The European Food Safety Authority and other national regulatory bodies have accepted a tolerable daily BPA intake of 0.05 mg/kg, although some environmental and consumer advocacy groups seek to lower this threshold. Due to the potential for infant exposure to BPA, many manufacturers have restricted its use in baby bottles and other consumer products for children.

Most concerns over BPA revolve around its weak potency as an 'endocrine disrupter' and ability to alter hormone-signalling pathways in the body. Although the interpretation of the scientific and risk assessment database for this diverse class of compounds is highly contested, endocrine disrupters are implicated in a wide range of human health problems, including cancer, diabetes, obesity, heart disease, infertility, and juvenile developmental disorders. In addition to BPA and other plasticisers such as the phthalates, the list of suspected endocrine-disrupting chemicals includes many structurally diverse compounds of both natural and synthetic origin, including pharmaceuticals, dioxin-like organochlorine compounds, polychlorinated biphenyls, DDT and other pesticides (Fig. 7.8a). While the toxicology of such diverse compounds is rarely reducible to a single biological property, their effects upon endocrine tissues are in large degree predictable from their potency and efficacy as ligands at steroid hormones, the main cellular targets for endocrine-disrupting chemicals (Fig. 7.8b, c).

The endocrine toxicity of BPA and related environmental compounds raises the possibility that combined exposure to multiple compounds with weak oestrogenic activity might produce additive toxic outcomes. This concept receives some support from animal studies, although substantiating a hypothesis of this kind in humans is confronted by many difficulties. Moreover, while nuclear oestrogen receptors have received greatest attention as cellular targets for BPA and other endocrine disruptors, recent research has explored nonconventional mechanisms including disruption of epigenetic programming, nonsteroid receptors, transcriptional coactivators and steroid biosynthesis pathways. In adult animals, endocrine disruptors elicit

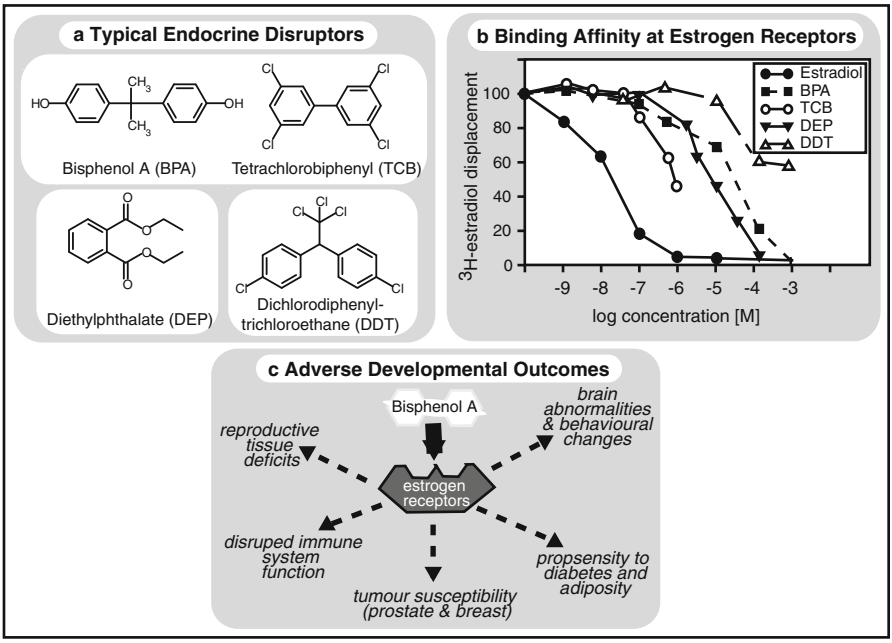

Fig. 7.8 *Panel a:* Structures of environmental pollutants with suspected activity as endocrine disruptors. *Panel b:* Potency of various environmental pollutants compared to estradiol as ligands for the oestrogen receptor. The competitive displacement of specific [^{3}H]-estradiol binding was assessed in cytosolic extracts prepared from amphibian livers. *Panel c:* Actions at oestrogen receptors likely drive toxicity in the developing conceptus (Data in *Panel b* is Reprinted from Lutz and Kloaz, (1999) Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding. *Sci Total Environ.* 225(1–2):49–57 with permission from Elsevier)

dysfunction in virtually all of the major endocrine organs of the body, although significant debate surrounds extrapolations to the human situation of toxic effects seen in animals exposed to moderately high doses of oestrogenic substances.

The effects of BPA and other endocrine disruptors on prenatal development are of special concern to the public and regulators alike. After reviewing over 500 scientific studies of BPA toxicology, the Center for the Evaluation of Risks to Human Reproduction (CERHR) affiliated with the National Toxicology Program in the USA released an influential study in 2008. The CERHR was commissioned to assess risks to human health that might accompany 'current' levels of human exposure to BPA. The final report concluded that many of the toxic effects of BPA on prenatal development are subtle in nature, noting considerable uncertainty surrounding the extrapolation of animal-derived findings to the human setting. After stating that it had 'negligible concern' over mortality or malformations in newborns, the CERHR report noted it had 'some concern' over developmental toxicity in the brain, behaviour and prostate gland of exposed foetuses. Some parties contested

these cautious conclusions, and ongoing debate over BPA safety as well as litigation involving environmental groups, consumer advocacy groups, federal agencies and chemical industry bodies shows few signs of abating. The BPA saga illustrates the significant scientific, social, regulatory and commercial complexities that attend the application of mechanistic toxicology data within a complex, changing and uncertain 'real world'. Nevertheless, to ensure the wellbeing of future generations, given epidemiological trends suggesting a rising incidence of endocrine-related diseases and disorders among children within the USA and other industrialised nations, the need for perseverance and continued research into the human health effects of this challenging class of compounds is far from optional. Growing awareness of this obligation within the toxicology community is focussing more attention upon the long-term health risks accompanying chronic, low-level prenatal exposures to multiple synthetic chemicals that are present within the social and physical environment of humans within industrialised nations the world over.

7.8.3.2 Pesticides

The heavy agricultural use of synthetic pesticides has conferred considerable toxicological attention upon these compounds. As a diverse group of chemicals with distinct mechanisms of action and dissimilar chemical properties, it is difficult to draw collective conclusions about their health impact. For example, the high lipophilicity and resistance to metabolism of many organochlorine pesticides ensures their spectrum of toxicity differs from those accompanying exposure to most organophosphate pesticides. Some 40 or so organophosphate pesticides are in current global use as replacements for organochlorine insecticides, and members of this class represent the most heavily used pesticides in current use.

While organochlorine use has declined in many developed countries, these substances remain persistent organic pollutants within rural, semirural, urban and suburban environments alike. Recent documentation of impaired neurodevelopmental outcomes in children of immigrant workers in Californian market gardens suggests that the harmful effects of pesticides are shared unequally across the socioeconomic spectrum. In addition to their human impact, pesticides exert a long-term environmental impact that exceeds those attending their initial use in farms or orchards: these include the continuing presence of residues in ground and surface water supplies, their ability to contaminate soil over extended timeframes or exert biological effects on nontarget soil microfauna, insects, reptiles, amphibian and mammalian species.

Assessing the human health impact of pesticides is complicated by divergent use patterns for members of this class of compounds, with some used sporadically within narrow agricultural settings to treat 'boutique crops' on so-called yuppie farms. On the other hand, some pesticides are used heavily in massive quantities over extended periods during the production of high-volume crops. Toxicologists and risk assessors charged with protecting public health must take into account human exposures resulting from very different exposure scenarios. These can range

from acute or subacute intoxications in farm workers handling pesticides in large quantities on either a recurring or intermittent basis; inhabitants of rural communities who sustain chronic environmental exposures via drinking water, inhaled dust or childhood ingestion of soil samples; to end consumers of agricultural products in urban settings who consume foodstuffs containing low-level residues over a lifetime.

While cancer outcomes following pesticide exposure were long of primary concern, pesticide effects upon the developing brain attract rising attention. This shift in emphasis partly reflects the availability of imaging technologies allowing study of brain function in real time in living subjects. While it rarely permits definitive incrimination of individual toxicants, such technology can uncover subtle differences in brain structure between control subjects and those incurring regular pesticide exposures.

The widely used organophosphate insecticide chlorpyrifos attracts particular attention for its neurodevelopmental impact on the brain. First introduced in 1965, chlorpyrifos is a broad-spectrum agent used to control pest outbreaks in large-scale crops such as cotton, maize, oranges, bananas and apples. Chlorpyrifos is also applied to the coat of some animals to control pests (e.g. as a sheep dip additive). Chlorpyrifos also finds wide usage during the extermination of pests in equine stables, dog kennels, farm buildings and crop storage bins. Due to concern over neurodevelopmental toxicity, many countries prohibit the use of chlorpyrifos for termite eradication in domestic dwellings. Chlorpyrifos is available in several formulations including granules, powder and dusts as well as sprays.

As an organophosphate, chlorpyrifos characteristically acts as a cholinesterase inhibitor, phosphorylating the acetylcholinesterase (AChE) enzyme in nerve cell endings within target insect pests, producing lethal overstimulation of the nervous system via accumulation of undegraded acetylcholine.

On administration to pregnant rodents, chlorpyrifos elicits several neurodevelopmental deficits and neurobehavioral abnormalities in offspring. The initial assumption that the neurodevelopmental toxicity of chlorpyrifos was due to AChE inhibition was subverted by the finding of brain alterations at insecticide doses below the threshold for AChE-mediated systemic toxicity. Rather than proceeding via AChE inhibition, chlorpyrifos may impair foetal brain development by inducing apoptotic neuronal death and disrupting core neuronal functions such as replication, differentiation, axon formation, synaptogenesis and wiring of neural circuits. A spectrum of subtle yet deleterious ultrastructural changes are also elicited within the developing rodent brain, including altered cortical thickness and changed ratios of neuronal versus glial cells in brain zones that regulate mood, behaviour and cognition (e.g. the hippocampus, septal nucleus and somatosensory cortex). Early signs of hippocampal glial scarring and astrogliosis also accompany prenatal exposure to chlorpyrifos, with these effects also observed at lower pesticide doses than those causing systemic toxicity.

Due to differences between the chlorpyrifos doses used in animal studies and those encountered by humans, considerable debate has attended this area of toxicological investigation. To circumvent this issue, researchers have increasingly

assessed pesticide-exposed human populations for signs of neurotoxicity. While segregating the effects of chlorpyrifos from other organophosphates is rarely possible in these studies, prenatal exposure to this family of pesticides has been associated with a range of adverse neurological outcomes including growth retardation, reduction in cranial circumference and impairment of neonatal reflexes. As exposed children mature, multiple neurodevelopmental problems have been noted including diminished performance in psychometric tests for IQ and attention span as well as decreased learning competence. The most useful epidemiological studies have associated such neurodevelopmental deficits with levels of organophosphate metabolites in biofluids collected from study participants (e.g. umbilical cord blood or urine samples from mothers or their offspring).

In a recent study by Columbia University researchers, use of magnetic resonance imaging revealed significant chlorpyrifos-related morphological abnormalities in the cerebral surface of children from low socioeconomic status urban areas that experienced high prenatal pesticide exposure. When analysed via MRI at ~8 years of age, changes in brain structure correlated with chlorpyrifos levels in cord blood samples collected at their birth. MRI also revealed striking cerebral changes in regions that contribute to key cognitive and emotional capabilities such as language processing, social cognition, reward, emotion and inhibitory control. The brain changes were more overt in children in a 'high-exposure' group for prenatal chlorpyrifos. Collectively, such human studies are providing disturbing evidence for a consistent and lasting pattern of cognitive and behavioural impairment following prenatal organophosphate exposure within both agricultural and urban populations.

7.9 Conclusion

Preserving the health and safety of future generations by ensuring a safe intrauterine environment for the growing human is among the most important goals in modern toxicology. While our understanding of the mechanisms whereby major teratogens disrupt prenatal development has steadily grown, substantial uncertainty still surrounds the application of such knowledge to the human setting. On top of this challenging situation, the strong emotional and cultural factors surrounding human reproduction and the birth of newborn humans make developmental toxicity a highly charged area of modern toxicology. Clearly, in the face of ongoing chemical innovation, the need for improved and less costly screening protocols that display high predictive accuracy during the evaluation of new chemicals for prenatal toxic potential will remain strong for the foreseeable future.

Finally, one sobering reality remains glaringly obvious even after five decades of applying the powerful approaches of molecular biology and genetics to the study of human birth abnormalities: after much global effort, we still cannot identify actual causes of more than 65–70 % of the congenital defects that afflict newborn infants. The possibility that subtle interactions between environmental inputs such as xenobiotics and maternal and foetal genes contribute to these poorly understood toxic

responses remains high. So long as this high proportion of congenital malformations stubbornly commands assignment to the 'unknown aetiology' category, the need for ongoing toxicological investigation in this area will remain as strong as ever.

Going Further

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Chapter 8
Chemicals and Cancer

Abstract Cancer is a deadly yet diverse condition featuring the emergence of cell subtypes that escape normal restraints on proliferation, forming cellular masses possessing unstable genomes and aggressive tendencies to invade local tissue or metastasise to remote sites. Since the accumulation of mutations in critical genes typically underpins these harmful capabilities, modern toxicology devotes much effort to identifying chemicals that cause mutations. Particular attention focusses on the mechanisms whereby bioactivation-dependent carcinogens form DNA-reactive metabolites that generate abnormal bases within the genome. Such 'DNA adducts' are central to chemical carcinogenesis since they can either generate mutations during processing by DNA polymerases, trigger apoptosis or undergo enzymatic repair. DNA adducts are also useful biomarkers of carcinogen exposure in humans and animals. These concepts are reinforced by studying the toxicology of known human carcinogens (e.g. vinyl chloride, asbestos and aristolochic acid) as well as chemicals with a still unclear role in human cancer (e.g. acrylamide).

Keywords Acrylamide • Ames test • Angiogenesis • Asbestos • Aristolochic acid • Biomarkers • Cancer hallmarks • Cancer testing • Carcinogenesis • DNA adducts • DNA repair • Genotoxicity • Mutagenesis • Vinyl chloride

8.1 Introduction

In 1971, President Nixon fired the opening salvo in the 'War on Cancer' by signing the National Cancer Act into US law, thereby unleashing a massive effort to identify the causes of cancer and develop better anticancer therapies. Today, opinions differ concerning how much ground has been won in this War, with critics pointing to such problems as the ineffectiveness of many cancer remedies, their lack of selectivity and serious side effects. Yet while cancer may not be vanquished anytime soon, the War on Cancer has certainly advanced human understanding of the chemical causes of tumour emergence. In recent decades, modern toxicology has made

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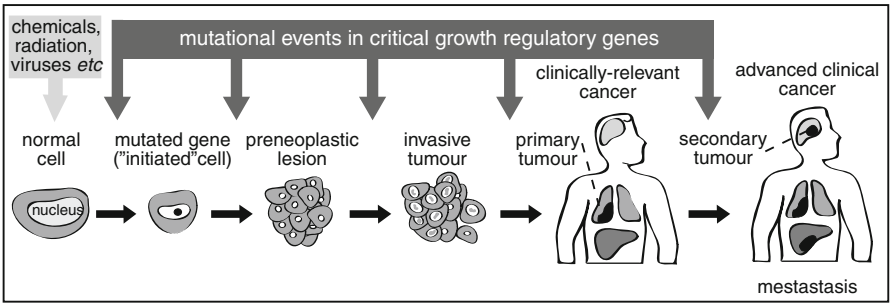

Fig. 8.1 The classical model of cancer development proposes a monoclonal origin for tumours, with minor modifications arising as a single cell starts acquiring new capabilities via mutational events (Figure reprinted by permission from Macmillan Publishers Ltd: Hussain et al. (2007) TP53 mutations and hepatocellular carcinoma: insights into the aetiology and pathogenesis of liver cancer. Oncogene 26: 2166–2176)

substantial progress in identifying the major classes of cancer-causing chemicals as well as the mechanisms whereby they induce cancer formation.

8.2 The Biology and Terminology of Cancer

Cancer is an umbrella term covering over 100 neoplastic diseases, all of which feature the emergence of genetically altered cells that exhibit a growth advantage over their neighbours or disregard the normal restraints tissues place on cellular behaviour (Fig. 8.1). The cell mass resulting from unregulated growth is a *tumour* or *neoplasm*. Whereas *tumour* denotes a local mass of aberrant tissue, *cancer* is a broader term embracing the full spectrum of clinical responses within a tumour-bearing patient. Ongoing research has shown that tumours are highly heterogeneous in terms of their genetic mutations. Their cellular phenotype also becomes messier as tumour growth proceeds, partly due to infiltration by bone marrow-derived immune cells such as macrophages and neutrophils.

From the beginnings of nineteenth-century histopathology, researchers began classifying tumours into two broad categories based on their biological properties. A *benign* tumour tends to grow locally and does not invade adjacent tissues, while a *malignant* tumour invades local tissue *and* metastasises to form dangerous colonies in remote body sites. Metastatic cells typically travel through blood or lymph to reach their destinations. The ability to invade and destroy surrounding tissue is termed ‘invasiveness’. Malignant tumours are generally more harmful to a patient’s health than most benign tumours and typically account for 90 % of cancer deaths. Cancer biologists also distinguish between ‘solid tumours’ and ‘liquid tumours’. The former manifest as lumps in tissues, while the latter involve changes in circulating blood cells (e.g. uncontrolled overproduction of white blood cells in leukaemia victims).

While tumours may conceivably arise in any of the over 100 tissue types in the human body, most common tumours originate within the sheets of epithelial cells that line the walls of organ cavities or, in the case of skin, encase the whole body. Termed *carcinomas,* such tumours cause ~80 % of cancer deaths. *Squamous cell carcinomas* originate in the protective layers of epithelium that line or cover underlying tissues, while *adenocarcinomas* originate in specialised epithelial cells that release secretions into ducts or cavities. Some tissues such as the lung and uterus often exhibit both adenocarcinomas and squamous cell carcinomas within the same neoplastic mass.

Tumours originating in nonepithelial tissues command their own terminology: a *sarcoma*, for example, originates in one or more connective tissue types within the body. Some more common nonepithelial tumours originate in haematopoietic or blood-forming tissues: *leukaemia* (literally 'white blood') can arise within several haematopoietic lineages and feature an overgrowth of nonpigmented cells that can quickly dominate the blood compartment. Tumours arising in the lymphoid lineages typically congregate in nodes of the lymphatic system, forming masses known as *lymphomas*. A third class of uncommon nonepithelial tumours arises from cells in the central or peripheral nervous system, such as *gliomas, glioblastomas* and *neuroblastomas*.

8.3 The Molecular Biology of Cancer

A fruitful concept in tumour biology proposes that cancers are *monoclonal* in *origin*: they are assumed to develop from a single cell that begins behaving abnormally and gives rise to offspring that become more dangerous as the tumour progresses. The mechanistic events underlying this risky transformation were long mysterious, but as the twentieth century progressed, sequential breakthroughs greatly enriched our understanding of cancer development. The detailed molecular insights into the abnormal genetic and biochemical wiring that occurs as cells become tumourigenic rank among the greatest achievements of modern science.

While it was long thought tumours arise via stepwise accumulation of mutations in key growth regulatory genes (e.g. Fig. 8.1), insights from new technologies have revolutionised our knowledge concerning the number of mutations occurring in human tumours. Increasing use of genome-wide sequencing (GWS) to catalogue the somatic mutations that accumulate within the tumour genome of cancer victims revealed that the number of mutations in a typical tumour cell genome is much higher than once thought. For example, common solid tumours such as those targeting the colon, breast, brain or pancreas typically exhibit an average of 33–66 somatic mutations that disrupt the function of associated protein products. Still higher numbers of mutations are detected in tumours from tissues such as lung or skin that incur exposure to external carcinogens: over 200 mutations have been found in some tumour types within these tissues, confirming the high importance of exposure to DNA-damaging species such as tobacco smoke carcinogens or UV radiation.

Most of the mutations that accumulate in a tumour genome – perhaps over 95 % of them – are unimportant to the pathogenesis of cancer. Rather, depending on the tumour type, as few as 2–8 *driver* mutations appear to confer a distinct growth advantage or some other dangerous capability on emerging tumour cells. Most common tumours require 20–30 years to accumulate the number of driver mutations needed for a fully malignant state.

What kinds of mutational events drive the emergence of tumours? To help the human mind come to grips with the myriads of molecular changes that occur in cancer, much thinking has gone into developing conceptual models that convey a holistic understanding of the disease. The fact that tumours display genetic instability – an increasing propensity to randomly accumulate mutations – complicates this difficult task. One compelling approach is the 'hallmarks of cancer' model proposed by Hanahan and Weinberg in 2000 and in a revised form in 2011. On their view, progression of a normal cell to a cancerous state involves the acquisition of up to 10 'hallmarks' or biological traits and enabling characteristics that confer tumourigenic and malignant/metastatic properties. This model was assembled by sifting through the complex circuits of cellular signalling pathways to find particular nodes or branches that are reprogrammed or 'rewired' in cancer (Fig. 8.2). Disruption of

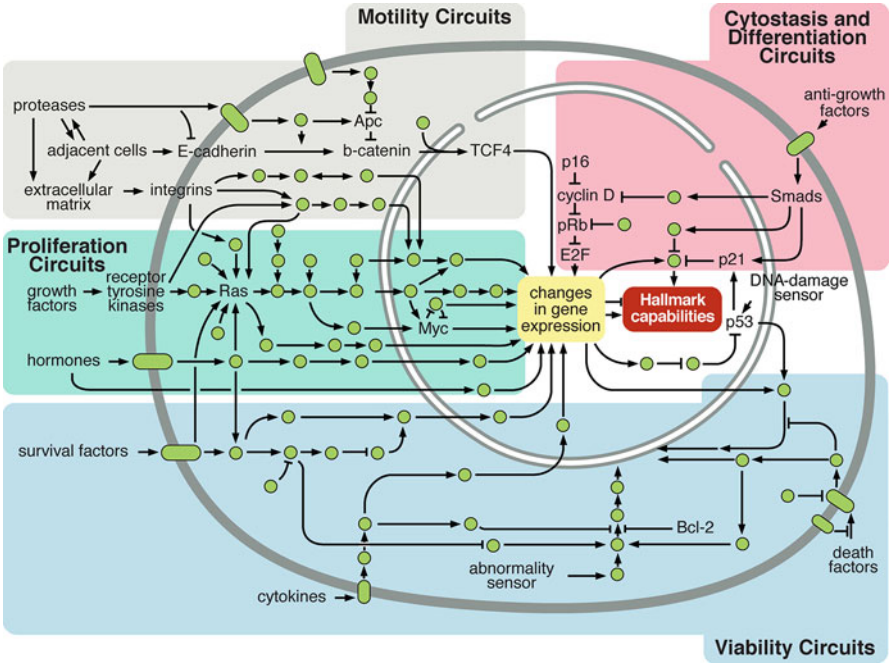

Fig. 8.2 The emergence of a tumour cell involves acquisition of mutations that disrupt intracellular signalling networks. Within the context of chemical carcinogenesis, the formation of DNA adducts in growth regulatory genes generates mutations that drive aberrant reprogramming of cellular circuits, facilitating cancer onset (Image used with permission from Hanahan D and Weinberg RA (2011), Hallmarks of cancer: the next generation, *Cell*, 144: 646–674)

these circuits via the acquisition of mutations within critical signalling pathways fosters the emergence of a tumourigenic state. Note that current understandings do not propose a direct link between specific mutations and hallmarks since it is recognised that the same tumour capability can be attained by a slew of different mutational events: in tumour biology, the destination is likely more important than the route.

8.3.1 Self-Sufficiency in Growth Signals

Understanding tumourigenesis is aided by the metaphor of driving a car: to move a stationary vehicle forward, the accelerator pedal must be depressed. This ability to move forward – to sustain itself via chronic proliferation – is a key characteristic of cancer cells. In the normal quiescent state, cells remain unproliferative until receiving promitogenic growth-stimulatory inputs from other cells. Such growth factors usually target transmembrane receptors to 'switch-on' a proliferative state. Gaining liberation from such dependence on external growth factors is a key step in tumourigenesis: instead of requiring external inputs, tumour cells produce their own pro-oncogenic signals which short-circuit signalling pathways linking cell surface receptors to intracellular replicative machinery. One important oncogenic pathway is the Ras–Raf–MAP kinase pathway which is activated in around one-quarter of all human tumours.

8.3.2 Insensitivity to Growth Inhibitors

To revisit our automotive analogy, even if the accelerator pedal is depressed, a car will not budge until the handbrake is disengaged. Another key characteristic of cancer involves disabling the normal brakes upon cellular proliferation. Just as cars have multiple braking mechanisms (e.g. foot and hand brakes), cells possess many tight controls upon replication. Within the tissue environment, cells receive many antiproliferative inputs, including soluble growth inhibitors as well as insoluble inhibitors that are immobilised in the extracellular matrix. By targeting cell surface receptors, these negative signals suppress cell replication by two main mechanisms. Firstly, antigrowth signals may redirect cells out of an active proliferative cycle into a resting (G_0) state. Secondly, antiproliferative signals may force cells to undergo irreversible differentiation. The first antiproliferative pathway of this kind, the Rb protein, was discovered via its role in retinoblastoma, a devastating tumour that causes childhood blindness. A key growth-suppressing input into this pathway is TGFβ. A second important tumour-suppressing pathway involves the transcription factor p53. p53 and Rb are vital gatekeepers in suppressing cell growth, and mutational loss of these controls accompanies many human cancers.

8.3.3 Able to Evade Cell Death

On becoming too costly to maintain, cars are sent to automotive recycling factories for destruction. Likewise, upon sensing cells that are behaving abnormally, healthy tissues quickly mark upstarts for elimination by apoptosis. Successful tumours must evade this destiny; hence, a key characteristic of cancer cells is the ability to ignore signals that consign aberrant cells to premature deaths. The molecular pathways involved in the sensor and effector arms of apoptosis are complex; hence, the mechanisms whereby tumour cells outwit these pathways are multifaceted. One pathway to apoptosis evasion involves mutational loss of the p53 tumour suppressor gene, since this key multifunctional protein acts as a DNA damage sensor to activate apoptosis in cells possessing damaged genomes.

8.3.4 Cellular Immortality

A hypothetical car that extends its lifespan indefinitely by self-repairing malfunctioning engine parts captures the essence of a fourth characteristic of tumour cells, namely, the ability to offset mechanisms that restrict the number of times cells can divide. The cellular 'counting device' used to monitor replicative events works by adding several thousand repeats of a short 6-base-pair sequence to the ends of chromosomes. Known as 'telomeres', these genetic sequences are maintained by telomerase, a remarkable enzyme that replaces the 50–100 base-pairs that are lost from telomeres during every cell division. The eventual loss of telomeres results in genetic abnormalities and cell death. To avoid this consequence of unrestrained cell division, tumours fight back by boosting the expression of telomerase, a feature seen in 85–90 % of human tumours. More recently, this enzyme has gained renewed attention due to newly discovered 'noncanonical' or 'telomere-independent' capabilities.

8.3.5 Access to Blood Vessels

Just as cars require gasoline to fuel their forward motion, growing tumours demand increasing supplies of nutrients and O_2 while also using blood to remove metabolic wastes. Successful tumour cells should be located within 100 μm of a blood vessel; otherwise, the growing tumour mass risks becoming hypoxic and necrotic. If they are to continue expanding in size, tumours must acquire the ability to promote blood vessel growth ('angiogenesis'). This highly complex process involves positive and negative regulatory inputs. One significant pro-angiogenic molecule is VEGF (vascular endothelial growth factor), which is released from many oxygen-depleted tumour cells and can target transmembrane tyrosine kinase receptors on endothelial tissue to promote blood vessel sprouting.

8.3.6 Become Invasive and Metastatic

Tumour *invasiveness*, the tendency to expand abnormally within the tissue of origin, and *metastasis,* the ability to colonise remote tissues, are entirely pathological processes since normal cells are always restricted to their usual location. On acquiring these capabilities, tumours enter the most dangerous phase of their existence.

Due to anatomical realities, tumour cells metastasising from a given tissue are often delivered to a particular organ. For example, since the portal circulation drains the intestinal circulation to the liver, tumours originating in the GI-tract are often found as bizarre overgrowths within hepatic tissue. In female breast cancer victims, secondary tumours often proliferate within the brain.

At the genetic and biochemical level, invasiveness and metastasis are closely related phenomena. One simplistically assumes that particular mutations must convert a primary tumour to an invasive tumour, just as additional mutations convert invasive cells into metastatic tumours. Yet pinpointing genetic alterations that drive such key transformational events has proven challenging, partly due to complex interactions between tumour cells and other cell types such as macrophages, neutrophils and mesenchymal stem cells. Mutations targeting proteins such as integrins that normally tether cells tightly to the extracellular matrix are commonly seen in metastatic tumours, as are mutations in cell–cell adhesion molecules such as the cadherins. Beyond these long known pathways, the biology gets more murky.

8.3.7 Evading Immune Destruction

When Hanahan and Wienberg revised their conceptual model in 2011, they included two ‘emerging hallmarks’, the first of which proposed that a successful tumour needs to evade destruction by the immune system. This recognition reflected growing awareness of the extraordinary immunological complexity of most human tumours.

Initially, tumours were assumed to comprise a relatively homogeneous community of tumour cells sharing similar genetic features, but more careful histological studies uncovered a complex ‘tumour microenvironment’. In this understanding, the macrophages, neutrophils and other invading cells of the innate and adaptive arms of the immune system are not innocent bystanders but somehow drive the emergence of a neoplastic state. These suspicions of accessory roles by immune cells are consistent with epidemiological findings suggesting a reduction in tumour risk with long-term consumption of anti-inflammatory medicines such as aspirin.

Yet the new understanding also affirms the older ‘immunosurveillance’ paradigm that emphasised the role of the immune system in finding and eradicating aberrant tumour cells: tumours that can circumvent these anticancer actions of the immune system are more likely to succeed. This idea is partly informed by results from animal studies: immunodeficient mice which lack key components of the immune system (e.g. CD8+ cytotoxic T lymphocytes, NK cells) are considerably more vulnerable to

chemical carcinogens than normal 'immunocompetent' animals. Similar studies also showed that tumours originating in immunodeficient mice are less metastatic when transplanted into immunocompetent animals. These findings concur with the idea that an ability to evade immune destruction is a key feature of successful tumours.

8.3.8 Reprogramming of Energy Metabolism

The final 'emerging hallmark' draws from insights into aberrant carbohydrate metabolism in cancer cells developed by Otto Warburg in the 1930s. In normal cells, glucose is metabolised via glycolysis to pyruvate, with further oxidation by the mitochondrial respiratory chain generating high yields of ATP. Surprisingly, many tumour cells make little use of mitochondrial oxidative phosphorylation and instead fuel their energy needs by boosting glycolysis. Since mitochondrial oxidative phosphorylation achieves 18-fold higher yields of ATP from each molecule of glucose compared to glycolysis, this unusual energy preference has long puzzled cancer researchers. While the answer remains speculative, one long-standing possibility is that high rates of glycolysis allow diversion of carbon atoms into building blocks needed by growing tumour cells, such as amino acids and deoxynucleotides. Another view is that subpopulations of tumour cells which utilise lactate as their energy source may benefit from lactate release as a glycolytic waste by other tumour cells.

In parallel with new insights into tumour biology, much progress has been made in understanding how chemical carcinogens accelerate cancer onset. Understanding these properties is a major achievement of modern toxicology.

8.4 Chemicals and Cancer

Much effort has been devoted to identifying the causes of the mutations that accumulate in human cancer cells. Very often, the mutations that drive cancer arise spontaneously, caused in part by endogenous DNA-damaging chemicals such as free radicals and electrophiles that form during normal metabolism. On the other hand, evidence from epidemiology and occupational toxicology reveals that exogenous carcinogens also play important causative roles in many human tumours, especially those plaguing workers who handle particular chemicals during their daily employment. Table 8.1 contains a partial list of human tumours that have been associated with occupational exposure to specific carcinogens.

In addition to endogenous and occupational sources, human carcinogen exposure also occurs via tobacco smoke, lifestyle practices, use of medicinal agents and absorption of environmental pollutants (Table 8.2). While occupational carcinogens are of particular concern in toxicology, their overall contribution to human cancer is not especially high: in their classic 1981 review of cancer causation, the British epidemiologists Doll and Peto attributed around 1–2 % of human cancer to occupational exposures. Other epidemiologists raise this figure to between 5 % and 8 % of total cancers. Part of the difficulty in obtaining precise estimates of occupational

Table 8.1 Some human occupational cancers and their known chemical causes

Tumour type	Occupational chemical
Scrotal carcinomas	Coal soot (PAHs)
Liver angiosarcoma	Vinyl chloride
Acute leukaemia	Benzene
Nasal adenocarcinoma	Hardwood sawdust
Skin carcinoma	Arsenicals
Mesothelioma	Asbestos

Table 8.2 Estimated percentage of total cancers avoidable through established nongenetic causes of cancer (Used with kind permission from Springer Science + Business Media, Cancer Causes and Control, Commentary: eight ways to prevent cancer: a framework for effective prevention messages for the public, 23, 4, 2012, Hank Dart)

Risk factor	Percentage of cancers (%)
Tobacco	29
Adult diet/obesity	25
Viruses/other biologic agents	8
Sedentary lifestyle	5
Family history of cancer	5
Reproductive factors	5
Prescription drugs and medical procedures	5
Alcohol	4
Environmental pollution	4
Ionising/ultraviolet radiation	2

cancer risks relates to the dynamic nature of modern economic activity, with technological innovation continually fuelling the emergence of new industries that foster distinct occupational exposures, while older technologies become progressively obsolete. Thus, compared to the workplaces of yesteryear, the modern workforce is increasingly heterogeneous in terms of carcinogen exposures. A recent in-depth study of the current cancer burden in modern workplaces conducted by the British Occupational Cancer Burden Study Group identified workers in the construction and mining industries as showing the highest incidence of occupational cancer.

Quantifying cancer risks is complicated by the likelihood that many tumours develop via a multifactorial process involving synergistic interactions between exogenous and endogenous chemicals, lifestyle factors and consumption of a calorie-rich diet. Yet while debate concerning the causes of human cancer is likely to continue for the near future, toxicologists have in the meantime made much progress in understanding the mechanisms underlying cancer induction by known carcinogens. Insight from these efforts is helping to improve evaluation of new chemicals that emerge via ongoing technological improvements by modern industries.

8.5 Classifying Carcinogens

A key concept in chemical carcinogenesis is that of *genotoxicity*. Throughout this book, this term denotes chemicals that induce *chemical damage* to DNA, such as base modifications, base loss, strand breaks, cross-linking or alkylation of the sugar–phosphate backbone. In this restricted use of the word, genotoxic chemicals

are not necessarily mutagenic, since some forms of genetic damage do not necessarily disrupt the information-storing capacity of DNA. However, readers should note that the term *genotoxicity* is sometimes used more broadly to denote the ability of a chemical to induce DNA mutations (i.e. heritable sequence changes). For example, the Ames Salmonella test is sometimes called a genotoxicity assay, whereas strictly speaking it is a *mutagenicity* assay (i.e. assesses the ability of carcinogens to induce sequence mutations in the histidine operon of *Salmonella – see below*). Since the chemical aspects of DNA damage are distinguishable from the loss of genetic information that may or may not occur during the biological processing of such lesions, it is best to use distinct terms for these phenomena.

Chemicals that cause cancer via chemically induced DNA damage are termed *genotoxic carcinogens*. This important class includes the *alkylating agents*, a miscellaneous group which includes methylating and ethylating agents such as 1,2-dimethylhydrazine as well as methyl and ethyl halides. Alternatively, a *non-genotoxic carcinogen* causes cancer *without* inducing chemical damage to DNA. These carcinogens typically induce cancer by triggering receptor-mediated mitogenic responses or eliciting epigenetic changes.

8.5.1 Genotoxic Carcinogens

While some genotoxic carcinogens possess intrinsic chemical reactivity, most require bioactivation by CYP or other enzymes before attacking DNA. The basic features governing the reactivity of metabolites with cell macromolecules such as DNA were surveyed in Chap. 4. Chemicals that form ‘hard’ electrophilic metabolites are especially relevant to carcinogenesis since DNA contains numerous ‘hard’ nucleophiles, including the ‘ring’ nitrogens (e.g. N3 and N7) possessed by guanine and adenine as well as the exocyclic oxygens on guanine (O^6), thymine (O^2 and O^4) and cytosine (O^2) (Fig. 8.3). Some ‘hard’ electrophiles also attack oxygen atoms in the phosphodiester bonds within the sugar–phosphate backbone, although such damage likely plays minimal role in chemical mutagenesis. The accessibility of a nucleophilic

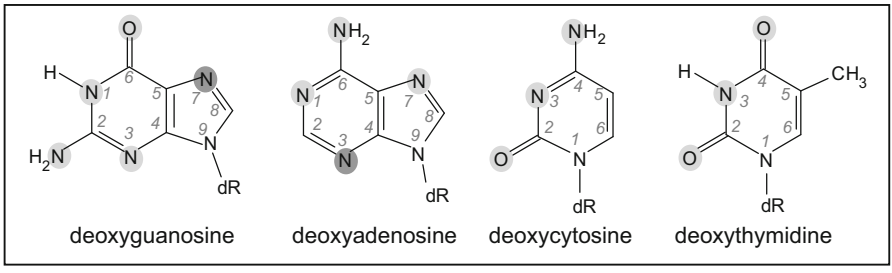

Fig. 8.3 Nucleophilic centres in the four nitrogen bases within DNA are key targets for adduct-forming electrophiles

centre also influences its reactivity with electrophiles. For example, the N7 of guanine is highly exposed within the major groove of the double helix, maximising reactions with electrophiles that diffuse into this domain. Although it is equally nucleophilic, the N3 of adenine is positioned within the less accessible minor groove.

8.5.1.1 Carcinogen Bioactivation Pathways

While some genotoxic carcinogens possess intrinsic reactivity allowing spontaneous adduction of DNA bases, most require metabolic activation by one or more biotransformation enzymes (see survey of relevant systems in Chap. 3). Among the hundreds of xenobiotic-metabolising enzymes expressed within the liver, which ones are most responsible for forming genotoxic metabolites? This question is not just of academic interest since knowing which pathways account for carcinogen bioactivation can assist the design of chemoprevention strategies (e.g. consumption of CYP-modulating foods to alter pathways of carcinogen metabolism in human tissues).

To address these questions, Rendic and Guengerich recently conducted a detailed literature survey of the enzyme catalysts responsible for 713 carcinogen bioactivation reactions (Fig. 8.4a). In keeping with expectations, CYP enzymes accounted for two-thirds of carcinogen bioactivation steps, while the conjugative pathways involving NAT and SULT together activated one in five carcinogens (Fig. 8.4a). The aldo-ketoreductase family of enzymes also activated some 8 % of carcinogens.

Tallying the contribution of individual CYPs to carcinogen bioactivation revealed quite different results to their roles in the metabolism of pharmaceutical agents considered in Chap. 3 (i.e. Fig. 3.7; c.f. Fig. 8.4b). Just six CYPs carried out 77 % of published carcinogen bioactivations (1A1, 1A2, 1B1, 2A6, 2E1 and 3A4), with major roles for several CYPs that generally play minimal roles in human drug metabolism (e.g. 1A1, 2A6, 1B1). Conversely, compared to the dominant role of CYP3A4 in human pharmacology (metabolises ~50 % of marketed drugs), its

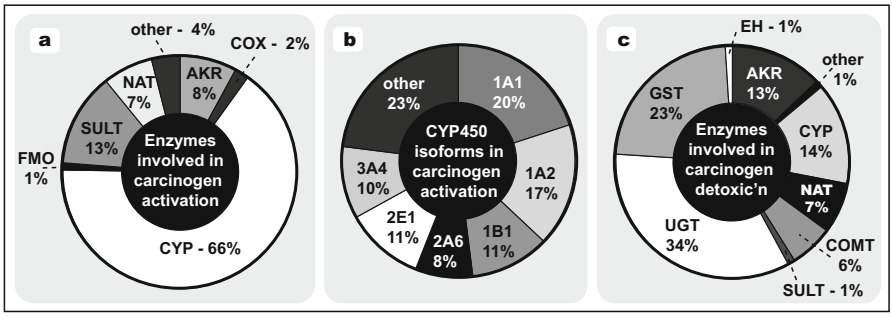

Fig. 8.4 Contribution of various biotransformation pathways to 713 carcinogen bioactivation reactions (*Panels a and b*) or 281 detoxication reactions (*Panel c*). Estimates are based on published studies (Reprinted (adapted) with permission from data presented in Rendic and Guengerich (2012) *Chem Res Toxicol*, 25: 1316–1383, (Copyright (2012) American Chemical Society)

contribution to carcinogen bioactivation is likely more modest (~10 % of carcinogens) (Fig. 8.4b). Likewise, despite a significant role in human drug clearance (Fig. 3.7), CYP2D6 is a minor contributor to carcinogen bioactivation.

Consideration of the enzymes responsible for *carcinogen detoxication* yielded quite different outcomes (Fig. 8.4c). For some enzyme and carcinogen combinations, distinguishing detoxication versus bioactivation outcomes is not necessarily easy. Despite this caveat, when Rendic and Guengerich reviewed 281 published biotransformation reactions, they identified major roles for glucuronidation (UGT) and glutathione conjugation (GST) in carcinogen detoxication (i.e. 57 % of reactions – see Fig. 8.4c).

8.5.1.2 DNA Adducts

Since nitrogenous bases confer the Watson–Crick base-pairing properties of DNA, chemical damage to these species can have serious cellular consequences. The products of reactions between electrophilic species and DNA bases are termed 'DNA adducts', and their formation is central to cancer causation by genotoxic carcinogens. Some DNA adducts formed by major human carcinogens are shown in Fig. 8.5. As the most reactive base in DNA, guanine is a particularly common participant in adduct formation by exogenous and endogenous genotoxicants (Fig. 8.6).

Since biological responses to individual DNA adducts vary considerably, their toxicological properties have attracted intense research attention. For many years, drawing firm conclusions about the mutagenic properties of specific adducts was hampered by a lack of well-defined research reagents. Simply treating cells with genotoxic carcinogens or reactive metabolites before screening for mutations can confirm mutagenic potential, but such studies rarely clarify the mutational efficacy of individual adducts. Chemical damage to other cell components such as proteins, RNA or lipids further complicates studies of this kind. Since DNA is a quantitatively minor constituent of mammalian cells, treating cells with a reactive chemical inflicts collateral damage upon many cell components, often ensuring cytotoxicity confounds the observation of mutagenicity.

Significant advances occurred in the 1980s when researchers developed the ability to synthesise short stretches of DNA – termed oligodeoxynucleotides – that contain specific DNA adducts at defined sites within the genetic sequence. This approach requires advanced skills in synthetic organic chemistry in order to position adducts precisely within a run of deoxynucleotides. In addition to avoiding collateral damage to proteins, the availability of such reagents overcame another inherent limitation in traditional approaches to studying chemical mutagenesis: virtually all electrophilic chemicals (e.g. reactive metabolites) produce multiple types of DNA adducts, complicating precise clarification of the mutagenic properties of an individual species.

In the first instance, the availability of short pieces of synthetic DNA containing adducts at a defined site within a genetic sequence allowed careful study of

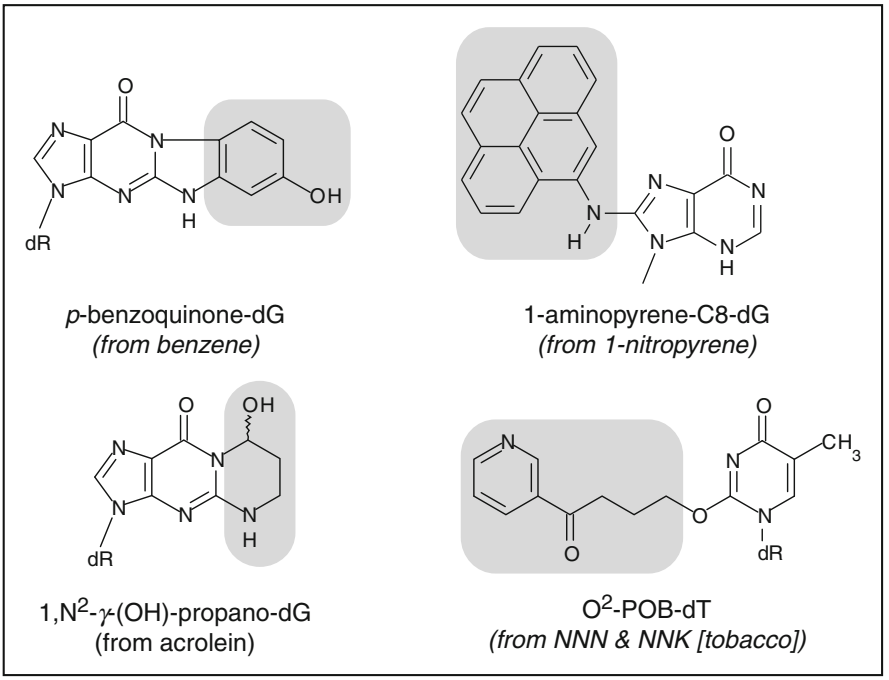

Fig. 8.5 Representative DNA adducts formed by known human carcinogens including the fossil fuel constituents benzene and 1-nitropyrene and the tobacco combustion products acrolein and N-nitrosamines NNN and NNK (see Chap. 11). The *shaded boxes* highlight the adduct portion contributed by the carcinogen

the structural distortions that adducts introduce when the double helix is required to accommodate bulky lesions. Such studies on the chemistry of DNA damage typically involve sophisticated analytical approaches such as two-dimensional nuclear magnetic resonance spectroscopy (2D-NMR). Moreover, the stability of DNA duplexes formed during different mutational events can be evaluated by annealing various complementary oligodeoxynucleotides to the DNA such that different bases are positioned opposite the adduct site. For example, for a DNA molecule containing an adducted G residue, researchers can test the thermal stability of duplexes containing G, C, A or T opposite the adduct in the complementary DNA strand. Measuring the 'thermal stability' of these oligodeoxynucleotide duplexes – the temperature at which the double helix 'melts' or dissociates into two single-stranded molecules of complementary DNA – is a simple way to assess the effects of DNA adducts on the entire DNA duplex. As a rule, a low melting point for a DNA duplex reveals destabilisation of the double helix.

In addition to these physical studies, adduct-bearing DNA molecules allow exploration of the mutagenic properties of adducts within simple cell-free systems or intact prokaryotic or eukaryotic cells (see below).

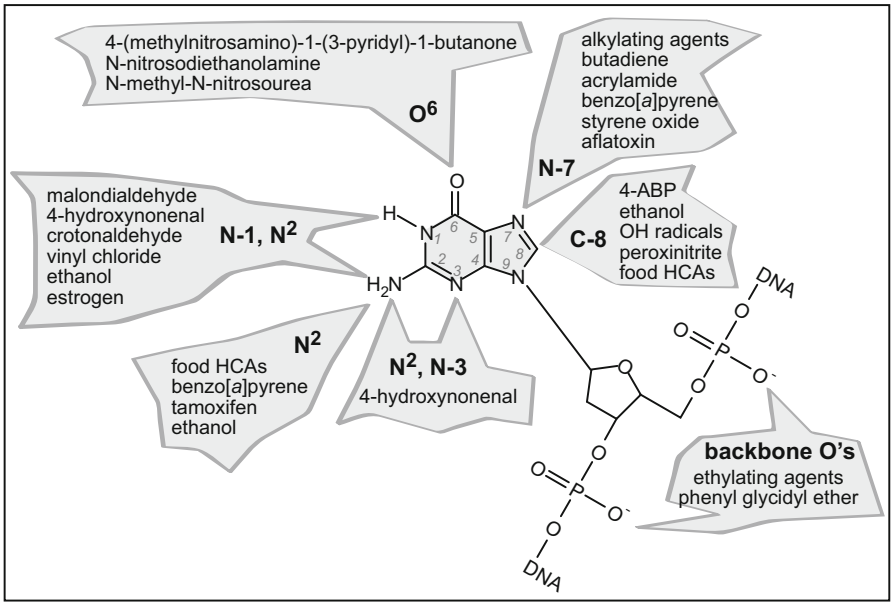

Fig. 8.6 Guanine bases in DNA contain multiple nucleophilic sites that are adducted by diverse endogenous and exogenous genotoxicants. (HCAs = food derived heterocyclic amines such as IQ, PhIP; 4-ABP = 4-aminobiphenyl) (From Singh and Farmer, Liquid chromatography-electrospray ionization-mass spectrometry: the future of DNA adduct detection. Carcinog (2006) 27:178–196 by permission of Oxford University Press)

8.5.1.3 DNA Adducts and Mutations

Many DNA adducts are biologically inert until they are processed by DNA polymerases and introduce sequence errors in the genome. The tumourigenic significance of adduct-driven mutations is confirmed by strong correlations between DNA adduct levels and mutation frequencies in target tissues of carcinogen-treated animals (see below). In recent decades, knowledge concerning the mutagenic properties of specific DNA adducts has advanced dramatically, thanks in large part to the use of DNA reagents containing specific DNA adducts during biological experiments. In addition to the physical studies using 2D-NMR mentioned above, these reagents have allowed exploration of the interactions of adducts with purified DNA polymerases in simple cell-free experimental systems as well as intact cells (Fig. 8.7).

Since mammalian cells express multiple DNA polymerases (e.g. α, β, κ, μ), in vitro studies can be designed to clarify the accuracy with which different DNA copying proteins process the same DNA adduct (Fig. 8.7a). For example, the frequency with which a given polymerase inserts each of the various bases opposite the adduct site can be investigated, thereby yielding a 'mutational spectrum' for the adduct of interest (Fig. 8.7a). Alternatively, the kinetics of base misinsertion as well as the readiness with which DNA primers are extended past the adduct can be

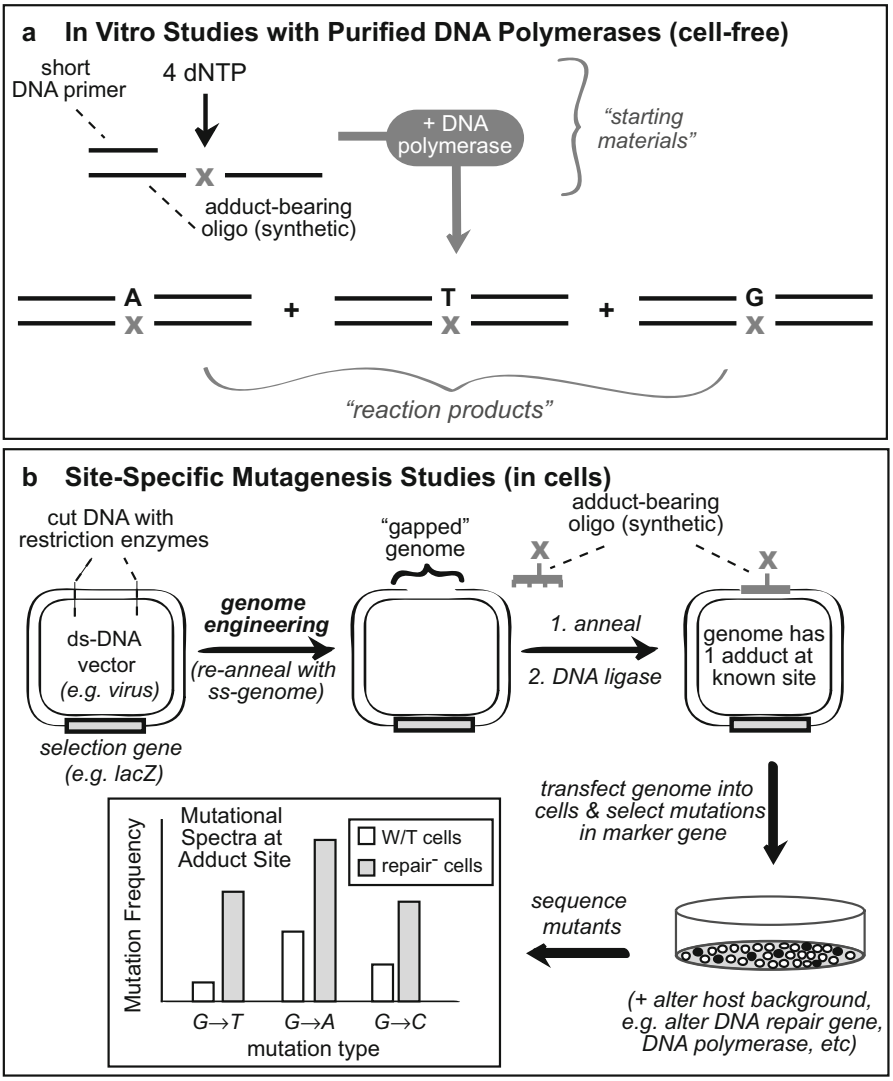

Fig. 8.7 DNA oligodeoxynucleotides containing a single DNA adduct are used in both in vitro (**a**) and in vivo (**b**) studies. The DNA adduct is shown with an '*X*'. (**a**) In vitro studies can use purified DNA polymerases to explore the frequency with which different deoxynucleotides are inserted opposite specific adducts. See Guengerich (2006) for a thorough description of this approach. (**b**) 'Site-specific' mutagenesis experiments in transfected cells define the mutational spectra for adducts carried on viral vectors following replication under different genetic backgrounds in host cells (e.g. BER-deficient cells)

investigated with different polymerases. In addition to detecting base misinsertion, these experiments reveal whether an adduct causes slippage of the DNA template during processing by polymerases, triggering errors within runs of bases (e.g. frameshift mutations).

Even more powerful insights into the mutagenicity of individual adducts within intact cells emerge upon incorporating adduct-bearing DNA into a viral genome or shuttle vector (Fig. 8.7b). This 'site-specific mutagenesis' approach was pioneered by the Essigmann lab at the Massachusetts Institute of Technology. (Not to be confused with the 'site-directed mutagenesis' approach used by molecular biologists to study the effect of amino acid substitutions upon the structure or function of gene products.) Following the Essigmann strategy, after transfecting an adduct-bearing genome into either prokaryotic or eukaryotic cells, time is allowed for viral replication before mutations are scored within viral progeny to assemble a mutation spectrum for the adduct (Fig. 8.7b). Transfecting the adducted genome into cells that are deficient in particular DNA polymerase genes, or lack specific DNA repair or DNA damage response capabilities, can provide further clarity concerning adduct properties. The local DNA sequence within which adducts are positioned can also be varied, allowing study of the effect of genetic context on the mutagenicity of genetic lesions. An entire mutation-prone sequence from a proto-oncogene or tumour suppressor gene can be introduced into a viral vector, allowing testing of hypotheses concerning 'mutation hotspots' within specific human genes.

Explaining why DNA adducts induce particular mutations during processing by DNA polymerases is challenging since the mechanisms involved vary according to the chemico-biological properties of each adduct. In the early days of DNA adduct research, it was believed the thermodynamic properties of a given base-pair combination strongly influenced the types of mutations occurring. This is likely true for some adducts formed by classic methylating carcinogens present in tobacco smoke (see Chap. 10, Sect. 10.6.5.2). However, subsequent research revealed that the difference in free energies between the 'correct' base-pairs (G:C, A:T) and alternative arrangements between DNA adducts and inappropriate bases cannot explain the frequency of mutational events. Other factors contributing to mutational outcomes include vertical 'base stacking' with adjacent bases in the DNA helix as well as the overall shape, size and volume of the base-pair combination.

Sometimes either in vitro studies with purified DNA polymerases or cellular studies with adducted viral genomes reveal that a specific adduct elicits a total block to DNA replication. This results in the synthesis of truncated DNA molecules. Since this outcome is deleterious to cells, to allow DNA synthesis to resume under these emergency conditions, DNA polymerases often follow the so-called A-rule, blindly inserting deoxyadenosine opposite the adduct. Any benefits this brings to organisms comes at a potential cost since any failure to repair mismatched bases that are introduced under the 'A-rule' can have deleterious consequences for gene function. The cell follows this practice by gambling that most mutations are inconsequential since they occur in noncritical codons.

8.5.1.4 DNA Adduct Repair

Ideally, cells repair or remove DNA adducts before they are processed by DNA polymerases. Recent decades have uncovered an elaborate cellular network of

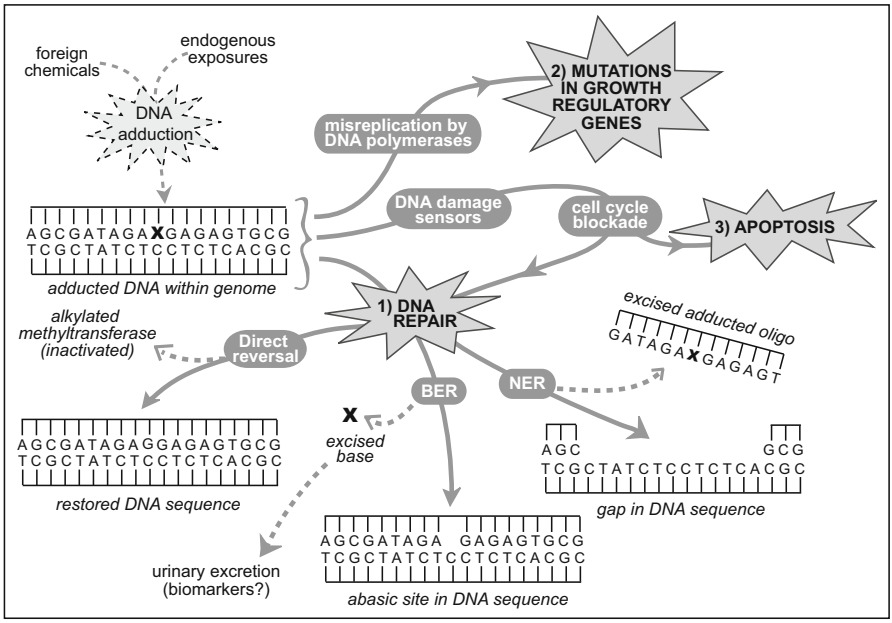

Fig. 8.8 Three main destinies await DNA adducts ('*X*') formed during reactions of toxic metabolites with DNA: repair, misreplication or apoptosis. See text for details concerning molecular participants

DNA repair enzymes that offset chemically induced genotoxicity by restoring the correct structure of damaged DNA (Fig. 8.8). Since knockout mice lacking specific DNA repair pathways are often prone to chemical carcinogens, this capacity is clearly of high toxicological importance. Modern GWS studies revealing a high prevalence of base substitutions in tumours from patients with inherited DNA repair defects further underscore the importance of repair to the maintenance of normal genetic integrity.

Depending on the type of DNA adduct, repair can proceed via reversal of the chemical modification, removal of the adducted base or excision of a run of nucleotides adjacent to and including the DNA adduct (Fig. 8.8). An example of the first type of repair occurs when O^6-methylguanine DNA methyltransferase (encoded by the MGMT gene in humans) repairs O^6-methylguanine, a type of adduct that forms in the lungs of cigarette smokers due to the presence of methylating reagents in tobacco smoke (Chap. 10, Sect. 10.6.5.2). If unrepaired by methyltransferases, O^6-methylated guanine adducts can generate mismatch mutations in target genes (see Chap. 10, Fig. 10.8). These 'single-use' repair enzymes efficiently transfer the methyl group from the O^6 of guanine to a cysteine residue on the methyltransferase protein, restoring the normal structure of DNA (Fig. 8.8). This pathway can also slowly remove ethyl, propyl and butyl moieties from modified O^6-guanine.

In the second instance, *base excision repair* (BER) enzymes such as the DNA glycosylases excise damaged bases from the DNA structure (Fig. 8.8). These pathways are especially adept at repairing small, non-distorting DNA adducts. Classic members of this family include uracil DNA glycosylase, formamidopyrimidine DNA glycosylase and N-methylpurine DNA glycosylase. By cleaving the N-glycosyl bond linking the adducted base to the sugar–phosphate backbone, BER proteins generate an *abasic* site in DNA (also known as *apurinic* or *apyrimidinic* sites depending on the absent base). These noninstructional lesions are subsequently cleaved by apurinic/apyrimidinic (AP) endonucleases. The correct DNA sequence is then restored by the sequential actions of DNA polymerase and DNA ligase enzymes. A BER enzyme with strong relevance to chemical carcinogenesis is N-methylpurine DNA glycosylase that excises various alkylated DNA bases as well as mutagenic 'etheno' adducts (see below).

Thirdly, the less efficient but versatile *nucleotide excision repair* (NER) pathway removes entire runs of nucleotides from sites of damaged DNA (Fig. 8.8). In eukaryotes NER involves over 30 proteins and helps cells withstand a range of stresses including UV radiation, polycyclic aromatic hydrocarbons and oxidative stress. Rather than recognising specific DNA adducts, NER processes respond to distortions or bulges in the double helix that accompany adduct formation, thereby permitting repair of damage caused by diverse genotoxic agents. Distinct endonucleases appear to cut the DNA backbone on either side of a damaged site, such as the ERCC1/XPF protein that cleaves on the 3′ side of the adduct, and XPG that cleaves on the 5′ side. The liberated adduct-containing oligodeoxynucleotide typically contains 24–32 bases. The resulting gap is filled by DNA polymerases before DNA repair is completed by DNA ligase. Note that different NER proteins repair DNA adducts within the transcribed strand of actively expressed genes (i.e. *transcription-coupled repair*) compared to damage elsewhere in the genome (termed *general genome repair* [GGR]). Significant health complications occur in patients suffering functional deficits in NER systems (e.g. Xeroderma pigmentosum, Cockayne syndrome).

8.5.1.5 DNA Adducts and Apoptosis

Cells containing many DNA adducts must decide whether their repair capacity is sufficient to prevent too many mutations. If not, it may be in the organism's best interest to sacrifice the cell via apoptosis (Fig. 8.8). Alternatively, cells may slow down cellular replication by activating cell cycle checkpoints, allowing time for repair enzymes to restore the DNA sequence. Choosing between these fates involves efficient sensing systems that detect DNA adducts and relay signals via kinases to 'executor proteins' that mediate effects on the cell cycle or cell death. Key sensors within the DNA damage response (DDR) system are ATM (ataxia–telangiectasia mutated), ATR (ATM and Rad3 related) and DNA-PK. These sensors are mainly activated by stalled DNA replication forks that form when the DNA replication apparatus encounters replication-blocking adducts or double-strand breaks. By

phosphorylating numerous downstream proteins, the activated DDR sensors regulate the activity of key decision point proteins such as the transcription factor p53 to determine whether cells should survive, allow time for repair or initiate cell death. The final outcome of p53 activation – cell cycle inhibition, DNA repair or apoptosis – likely depends on the nature of the DNA-damaging compound and the number of DNA adducts present in the genome of the target cell.

8.5.1.6 DNA Adducts and Gene Dysfunction

Depending on the normal function of the affected gene, the induction of mutations during DNA adduct replication can facilitate the emergence of a neoplastic state. For example, gene mutations that result in either a gain or loss of function may confer one or more of the eight distinctive 'hallmarks' of cancer mentioned earlier. On the one hand, *activating mutations* in oncogenes such as *ras* or *myc* family members represent gain of function events that can confer a cellular capacity for unregulated cell growth. Alternatively, common targets for *mutational inactivation* during chemical carcinogenesis include tumour suppressor genes such as *p53*, FHIT or *rb*. Inactivating mutations can also impair DNA repair capacity, leaving cells prone to genetic instability. Study of the biological consequences of adduct-driven mutations has revealed wide disparities in the importance of different sequence changes. While many mutations are likely of minimal relevance, others are highly damaging and move cells dangerously towards a neoplastic state.

The ability to recover mutated genes from chemically induced tumours in animals or smoking-related cancers in humans has provided strong support for the role of carcinogen-induced mutations in cancer formation. For example, sequence analysis of tumour genes recovered from human or animal tumours can reveal the presence of 'hotspots' within particular codons in which mutations strongly alter the biological properties of the gene product. In the example shown in Fig. 8.9, sequence analysis of over 2,300 mutated p53 genes from tobacco-associated human lung tumours reveals clustering of mutations in particular codons within the gene (e.g. 157, 158, 248, 273). These tools allow tentative association of exposure to particular carcinogens with specific 'fingerprint' mutations in hotspots within cancer genes such as *p53* and *KRAS*. The role of specific tobacco smoke carcinogens in the induction of 'diagnostic mutations' in growth regulatory genes is explored in Chap. 10.

8.5.1.7 DNA Adducts as Biomarkers of Chemical Exposure

In addition to their role in tumour induction, DNA adducts are useful markers of an individual's exposure to carcinogenic substances. Following their excision from the genome in a target tissue, excised adducts may be excreted as either free bases or intact deoxynucleotides within urine (Fig. 8.6). Sensitive analytical methods using mass spectrometry to quantify urinary DNA adduct excretion can provide useful insights into workplace or lifestyle exposures to genotoxic carcinogens.

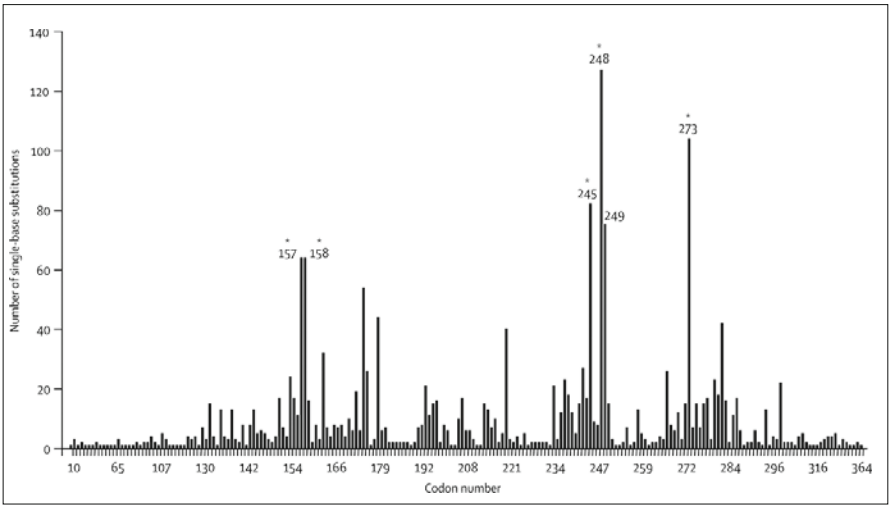

Fig. 8.9 Codon distribution of mutations within the p53 tumour suppressor gene in smoking-associated lung cancer in human subjects ($n = 2{,}340$). The image is used with permission from Besaratinia and Pfeifer (2008) and is based upon the p53 mutation database maintained by IARC (http://www-p53.iarc.fr). Entries with confounding exposure to asbestos, mustard gas or radon were excluded. The *asterisks* highlight codons containing methylated CpG sequences

Alternatively, use of these technologies to analyse DNA extracted from blood samples (e.g. leukocytes) collected from test subjects also clarifies an individual's exposure to DNA-damaging carcinogens.

One classic method for gaining semiquantitative insight into adduct levels within an individual's DNA was provided by the ^{32}P-postlabelling assay. In this procedure, DNA recovered from a carcinogen-exposed individual is phosphorylated with radioactive ATP before the samples are separated using thin layer chromatography. While normal unadducted deoxynucleotides are quickly eluted from the chromatography gel, adducted-deoxynucleotides move more slowly depending on their size and physicochemical properties. The latter adducts are then detected via exposure to X-ray radiography film. Developed by Kurt Randerath, this highly sensitive method is especially suitable for the detection of low levels of bulky DNA adducts within DNA samples recovered from smokers or workers in foundries and other work settings that involve exposure to complex hydrocarbon mixtures or fossil fuels.

Although the use of DNA adducts as 'molecular dosimeters' of exposure to genotoxic carcinogens can be very powerful, complications can arise when similar adducts form as a result of background exposure to endogenous genotoxicants. The presence of these adducts was surmised during early ^{32}P-postlabelling studies by Kurt Randerath many years ago when he described unidentified 'I-compounds' (I = indigenous adducts) within DNA samples from control individuals that were not knowingly exposed to carcinogens. Randerath astutely noted that I-adduct levels varied according to the age of the DNA donor and their dietary habits.

Table 8.3 Steady-state concentrations of select endogenous DNA adducts within the genomes of healthy control subjects (From Swenberg et al. [2011]. Endogenous versus Exogenous DNA Adducts: Their Role in Carcinogenesis, Epidemiology, and Risk Assessment, Toxicological Sciences, 120, suppl 1, 130–45, by permission of Oxford University Press)

Endogenous DNA lesions	Number per cell
Abasic sites	30,000
N7-(2-hydroxyethyl)guanine (7HEG)	3,000
8-hydroxyguanine	2,400
7-(2-oxoethyl)guanine	1,500
Formaldehyde adducts	960
Acrolein-deoxyguanosine	120
Malondialdehyde-deoxyguanosine	60
N2,3-ethenoguanine (eG)	36
1,N2-ethenodeoxyguanosine (1,N2-edG)	30
1,N6-ethenodeoxyadenosine (1,N6-edA)	12
	Total: 38,118

With time, the availability of improved analytical methods based on mass spectrometry began identifying these mysterious endogenous adducts. These tools confirmed that high levels of endogenous DNA adducts are present in DNA from healthy individuals, suggesting the genome continually encounters many endogenous electrophiles that form via normal cellular metabolism (Table 8.3). The presence of endogenous adducts complicates the risk assessment process for workplace chemicals such as vinyl chloride that generate the same types of DNA adducts as form spontaneously in healthy individuals (*see below*).

8.5.2 Nongenotoxic Carcinogens

While chemicals that cause cancer via DNA adduct formation receive special attention within modern toxicology, *nongenotoxic* carcinogens that promote tumour growth in the absence of clear genetic damage are also of great concern. The miscellaneous members of this diverse class promote tumourigenesis via diverse mechanisms, but a shared response involves disrupting normal proliferative control pathways to permit the outgrowth of preneoplastic lesions. In the traditional multistage model of cancer induction, derived from observations in the classic mouse skin tumourigenesis bioassay, chemicals were classified as either ‘initiators’ or ‘promoters’, categories that were loosely analogous to the contemporary distinction between genotoxic (i.e. DNA-damaging) and nongenotoxic carcinogens. In the older understanding, tumour promoters accelerated the clonal expansion of cells containing DNA mutations but did not induce mutations themselves. This older model was weakened by findings that several classic tumour promoters (e.g. phorbol esters) were themselves carcinogenic in long-term animal studies, even in the absence of prior exposure to ‘tumour initiators’. Nevertheless, despite these revisions, the concept that chemicals can accelerate cancer by promoting clonal expansion or driving cellular proliferation remains relevant to current understandings of

chemical carcinogenesis. Nongenotoxic mechanisms contributing to uncontrolled cell growth can include suppression of apoptosis, receptor-stimulated cellular proliferation and epigenetic mechanisms including disrupted DNA methylation or histone acetylation. Alternatively, some nongenotoxic xenobiotics may drive tumour development by stimulating the growth of blood vessels to nourish the growing tumour mass.

8.6 Testing Chemicals for Cancer-Causing Potency

Due to the complex sequence of events involved in tumour development, traditional tests for cancer-causing potential in animals are costly and time-consuming since they involve exposure of animals to chemicals throughout the duration of their normal lifespan. Toxicologists have long laboured to develop alternative approaches for identifying rogue cancer-causing chemicals. The most useful procedures focus on detecting chemicals that induce mutations in model cell lines.

8.6.1 In Vitro Tests

Since cancer formation is a complex, multistep process, it is virtually impossible to fully reproduce these phenomena in a test tube setting. One approximation to the in vivo setting is provided by so-called cellular transformation assays that evaluate the ability of xenobiotics to induce anchorage-independent cell growth in soft agar (Fig. 8.10a). These procedures use cell lines that only grow via attachment to a substratum: when suspended in soft agar they fail to proliferate since they cannot form the attachments needed for normal proliferation. In the presence of cancer-causing chemicals, they undergo cellular transformation, gaining the ability to grow uncontrollably throughout soft agar in an *anchorage-independent* manner. Cell transformation assays reproduce some stages of in vivo cancer development such as morphological transformation and acquired cell immortality. The fact that transformed cells obtained in these systems often form tumours upon transplantation into experimental animals further underscores their relevance to in vivo carcinogenesis. While cell transformation assays have been available for over 50 years, in the past decade they gained popularity within contract research organisations, regulatory bodies, university laboratories and chemical, agrochemical, cosmetic and pharmaceutical industries. The availability of high-throughput assay versions that use agar-containing 96-well plates and automated plate readers to monitor colony growth has overcome the laboriousness of traditional versions of these assays.

Predictive tools for the in vitro evaluation of chemicals with cancer-causing potential typically focus on detecting mutagenic activity. Some assays rely on the observation of macroscopic mutations such as chromosomal abnormalities (e.g. chromatid deletions, chromatid exchange), while others assess the generation of microlesions that are cytologically invisible. A key requirement of an in vitro mutagenicity assay that detects micromutations (e.g. base-pair substitutions) is the

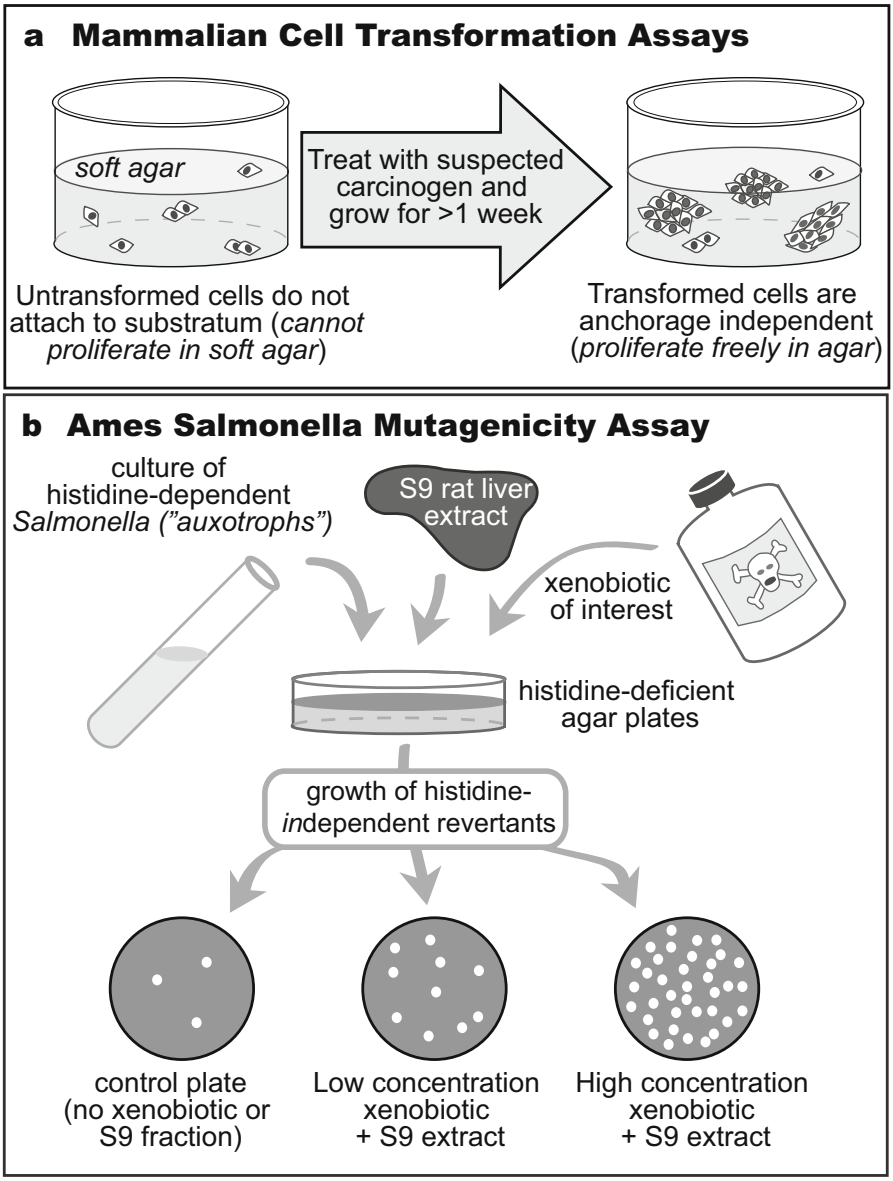

Fig. 8.10 Common predictive methods for assessing chemicals for cancer-causing potential include the cell transformation assay (*Panel a*) and the Ames *Salmonella* mutagenicity assay (*Panel b*)

possession of a cell line containing a target gene that when subjected to a chemically induced mutation generates an *observable* phenotypic change.

The most widely used short-term mutational bioassay is the Ames Salmonella assay developed by the University of California researcher Bruce Ames (Fig. 8.10b). The bacterial cells used are histidine auxotrophs (*his*$^{-}$) which contain a mutated gene otherwise used to synthesise the amino acid histidine: these bacteria grow

poorly on histidine-free agar. Typically, the assay employs several histidine-dependent *Salmonella* strains, allowing detection of mutagens that induce different types of reverse mutational events (e.g. base-pair substitutions, frameshift mutations). The Ames test assesses the ability of mutagenic chemicals to revert the cells back to a histidine-independent state (his^+). Such revertants manufacture their own histidine and thus grow in histidine-free media. Most mutagens increase the number of revertant colonies per plate in a dose-related manner. The Ames test is usually performed in the presence of a CYP-containing extract prepared from the livers of CYP-inducer exposed rats (so-called S9 or postmitochondrial fraction), thereby improving detection of chemicals that require metabolic activation.

Since its invention four decades ago, the Ames test has undergone many improvements. The so-called Ames II test offers six *Salmonella* tester strains that detect all six of the possible base-pair substitutions that can arise during the replication of DNA adducts. Another variation known as the 'fluctuation test' does away with agar plates by performing the Ames test in a liquid environment in multiwell culture plates (e.g. 96 well plates), allowing faster testing of more compounds. Thanks to these improvements, the Ames test is widely used as an initial screen to assess the mutagenicity of new food additives, consumer chemicals, pollutants, pesticides and drugs.

The Ames test is just one of many in vitro methods used during the evaluation of chemical mutagenicity. Due to concerns over the eukaryotic relevance of mutational responses in *Salmonella*, a positive result in the Ames test is usually followed by compound testing in mammalian mutagenicity assays such as the mouse lymphoma assay. This test resembles the Salmonella assay since it assesses the ability of compounds to induce reverse mutations in the *hprt* gene target. While the mouse lymphoma assay addresses concerns over the relevance of Ames test data to the mammalian situation, as an in vitro assay, it can potentially 'miss' chemicals that require complex, interorgan metabolism to cause DNA damage. Due to this concern, animal testing is still required for many drugs and chemicals.

8.6.2 In Vivo Testing

In vivo screening methods include classic tests such as the micronucleus assay that detects macroscopic genetic lesions in bone marrow cells from carcinogen-exposed mice. Yet the 'gold standard' of in vivo cancer testing is the rodent bioassay that is used by the US National Toxicology Program (NTP) and other government agencies. A great deal of thought has gone into the design and execution of these studies over recent decades. The results they provide are often invaluable during the risk assessment process for suspected carcinogens.

A typical NTP cancer bioassay involves lifetime exposure of both genders of two rodent species (usually Fischer 344 rats and B6C3F1 mice) to suspected carcinogens. These tests employ significant numbers of animals since each sex-species group comprises three xenobiotic-exposed groups (i.e. three dose groups) and one

control (unexposed) group, with between 50 and 60 animals per exposure group. The test substances are typically delivered via the food or drinking water, although dermal and inhalational routes may be preferred for some substances. Each of the three xenobiotic-exposed groups receives escalating doses of the test substance. Much of the controversy surrounding the interpretation of cancer bioassay data pertains to the doses used: as a rule, the top dose is the maximally tolerated dose (MTD) which is chosen via a dose-ranging pilot study as the dose inducing no more than a 10 % loss of body weight during a brief pilot study (e.g. 12 weeks). The other two doses are fractions of the MTD (e.g. 0.1, 0.25, 0.5X MTD). Usually, chemical exposure is continued throughout much of the normal lifespan of the animals (e.g. 18–24 months). Upon study completion the animals are sacrificed and a carefully prescribed list of tissues and organs are taken for detailed histological/pathological evaluation.

Although the rodent bioassay has come under considerable criticism, significant benefits can result from these procedures. For example, the cancer bioassay can reveal the carcinogenic potency of test substances: compounds that elicit cancer at low doses are invariably viewed with greater seriousness than agents requiring high doses to induce tumours. Rodent studies also provide useful insight into the body organs that are most vulnerable to the carcinogenicity of a given compound while also clarifying the types of tumours that can occur: compounds that elicit benign tumours arouse less concern than agents that induce malignant neoplasias. Furthermore, confirmation of carcinogenic potency in rodents can often strengthen suspicions surrounding human cancer risks for a particular xenobiotic which are fuelled by epidemiological studies.

While acknowledging these advantages, critics often point to a number of shortcomings with rodent bioassays. For example, the interpretation of bioassay data is often complicated, especially if tumours only occur in the top dose group, or if the tumour response is accompanied by observable toxicity in the target organ. Critics charge that the use of high xenobiotic doses in these tests may saturate protective biotransformation and/or DNA repair processes or induce tumours via mechanisms that have little relevance to human exposures. Rodent bioassays are also expensive, especially if the study design includes investigation of the toxicokinetic properties of the test substance, or microarray studies if mechanistic data is needed to clarify tumour responses. Since the typical NTP cancer bioassay takes 5 or more years to complete, their high cost ensures they are only performed upon carefully selected chemicals.

In recent decades the availability of genetically modified mice has helped improve rodent cancer bioassays. The sensitivity of animals to genotoxic carcinogens can be increased by deleting one copy of a tumour suppressor allele or by introducing an activated oncogene into the mouse genome. Animals can also be ‘humanised’ by introducing human genes of toxicological relevance into the mouse genome, such as human genes for CYP, UGT, SULT, DNA repair enzymes or receptors for inflammatory mediators (e.g. cytokines). By increasing animal sensitivity to carcinogens, these genetic modifications can decrease the number of animals needed, accelerate animal studies, decrease costs and allow the use of lower carcinogen doses.

One valuable application of transgenic technology to cancer testing involved the introduction of bacterial mutation marker genes into the mouse genome. These modified animals combine in vivo mammalian metabolism and toxic responses with simple prokaryotic mutation detection systems, thereby facilitating the detection of genotoxic carcinogens. Two commercially available test systems are the 'Big Blue Mouse' (Stratagene) and the 'Muta Mouse' (Hazleton Laboratories) which contain the bacterial transgenes lacI and cII in every animal tissue. Since the phage DNA marker is easily recovered and quickly scored for mutations in bacteria, toxicant-induced mutations within the introduced transgene are more easily detected than mutations in 'endogenous' mouse genes. These animals are also available on modified genetic backgrounds (e.g. inactivated tumour suppressor gene or activated oncogene). A growing number of mutational spectra are available for diverse carcinogens in these experimental systems, thereby providing a useful database for comparative purposes. The popularity of these models highlights the powerful benefits molecular techniques can bring to toxicity testing. Thanks to these innovations, the toxicological insights achievable via animal-based testing have significantly improved over recent years.

8.7 Representative Human Carcinogens

The International Agency for Research on Cancer (IARC) was formed in 1965 by the World Health Organization to facilitate global cancer prevention. In addition to promoting cancer research, the IARC performs comprehensive reviews on known and suspected human carcinogens. While individual chemicals were of main concern in its early decades, with time, the IARC categories of cancer hazards have expanded to include complex mixtures, multifaceted occupational or environmental exposures, cultural or behavioural practices, biological organisms and physical agents.

Depending on the strength of the evidence linking particular agents to cancer, IARC reviewers assign substances to one of five main categories (Table 8.4). As a rule, epidemiological evidence derived from human cancer studies receives a higher weighting than animal-based data. The severity of the tumour response is also taken into account, with chemicals inducing malignant tumours in several organs viewed more seriously than those inducing a benign tumour in one tissue only. Suspected carcinogens also receive higher rankings if cancer induction is rationalised by plausible mechanistic considerations, even if human epidemiological associations are weak. Signs of dose-dependent carcinogenicity in either animals or humans also cause heightened concern.

Due to space constraints, we will briefly explore a small number of carcinogenic agents from the 'Group 1' IARC category, with agents chosen to illustrate the diverse mechanisms whereby chemical substances can induce cancer. Additional Group 1 carcinogens are considered in Chaps. 9 and 10 (alcohol and tobacco). Since it helps clarify the complexity confronting the interpretation of animal carcinogenicity data, the Group 2A carcinogen acrylamide is briefly considered.

Table 8.4 Carcinogen classification groups used by the International Agency for Research on Cancer (IARC)

Category	Group descriptor	No. agents (2013)	Select examples
Group 1	Carcinogenic to humans	111	Aristolochic acid, asbestos, arsenic, benzene, bis(chloromethyl)ether, cyclophosphamide, diethylstilbestrol, formaldehyde, melphalan, nickel compounds, polychlorinated biphenyls, tamoxifen
Group 2A	Probably carcinogenic to humans	65	Acrylamide, adriamycin, chloramphenicol, 1,2-dimethylhydrazine, 1-nitropyrene, 2-nitrotouene, styrene-7,8-oxide, vinyl bromide
Group 2B	Possibly carcinogenic to humans	274	Acrylonitrile, antimony trioxide, benzofuran, caffeic acid, carbon tetrachloride, chloroform, DDT, 1,2-dichloroethane, furan, heptachlor, hydrazine, isoprene, methyleugenol, nitrobenzene, oxazepam, phenobarbital, safrole, styrene, toluene diisocyanates, vinyl acetate
Group 3	Not classifiable as to its carcinogenicity to humans	504	Acrolein, aldrin, ampicillin, benzoyl peroxide, bisulfites, caffeine, chloroethane, chromium, diazepam, 1,2-dichloropropane, dieldrin, ethylene, furosemide, gemfibrozil, hexachlorophene, isopropanol, jet fuel, methyl glyoxal, nitrofurantoin, parathion, phenol, rifampicin, toluene
Group 4	Probably not carcinogenic to humans	1	Caprolactam

8.7.1 Vinyl Chloride

Vinyl chloride (VC) is a high volume petrochemical used to manufacture polyvinylchloride (PVC) for the fabrication of plastic pipes and fittings, tubes, wire and cable insulation, doors, floorings and storage containers. VC also serves as a synthetic intermediate during the production of many organic chemicals. As of 2011, global production was growing at an annual rate of 4.5 %, suggesting VC has continuing relevance to occupational toxicology. While usually transported as a refrigerated liquid, since VC is a flammable gas at room temperature, it poses a major inhalational risk to unprotected workers. Pulmonary VC absorption is highly efficient in humans: due to ignorance concerning its cancer risks during the 1940s and 1950s, workers charged with cleaning PVC polymerisation vats encountered very high concentrations of airborne VC. Barbers and hairdressers also encountered dangerous levels due to the popularity of VC-containing hairsprays during the 1960s and 1970s.

Since VC is also a significant environmental pollutant, its levels in water supplies require monitoring by national environmental protection agencies. The US-EPA estimates that ~0.3 % of the US population encounters VC in drinking water at concentrations of 5 μg/L or higher. Accidents during VC transportation also cause significant environmental contamination: in late 2012, a train derailment in Paulsburg, New Jersey, discharged 25,000 gal of VC into the air and hospitalised over 20 victims.

The main tumour concern following VC exposure is an unusual liver cancer, hepatic angiosarcoma, which is very rare in the absence of occupational VC exposure. Its initial clinical description in 1974 featured three PVC plant workers. The tumour originates in the endothelium of hepatic blood vessels and, given its immediate proximity to the bloodstream, metastasises readily throughout the body during its final stages. VC exposure also increases the incidence of tumours that originate in hepatic parenchyma. Human evidence for tumours at other sites including the lung, connective tissue, breast and brain is contentious. VC is mutagenic in a range of in vitro mutagenicity assays such as the Ames test and the mouse lymphoma assay. It also induces macroscopic genetic lesions such as chromosomal aberrations and micronuclei formation in cultured cells. VC is consistently carcinogenic in most test animal species, usually inducing hepatic angiosarcoma and less frequent tumours at other sites.

VC is a classic genotoxic carcinogen, undergoing rapid oxidation by hepatic CYP2E1 to a highly reactive epoxide, chloroethylene oxide (Fig. 8.11). The epoxide and its rearrangement product chloroacetaldehyde attack DNA to form several mutagenic etheno adducts (εA, εC and N2,3-εG) which possess an additional ring due to cyclisation reactions involving nucleophilic nitrogen bases. An acyclic N7-(2-oxoethyl)guanine adduct also forms in high yields (Fig. 8.11). Since they are highly persistent within the mammalian genome, etheno adducts are likely of major relevance to tumourigenesis. Intriguingly, researchers can detect low levels of etheno adducts in the genome of unexposed controls due to reactions with endogenous lipid peroxidation products (Table 8.3).

Etheno adducts exhibit high miscoding potential during site-specific mutagenesis experiments, with the εA adduct inducing A → G and A → T mutations, εC inducing C → A and C → T substitutions and εG yielding G → A transitions. These base-pair substitutions are common in mutated p53 tumour suppressor genes and H-*ras* oncogenes recovered from VC-induced tumours of human and animal origin, although the precise identity of the adducts that drive human mutagenesis during VC exposure are debated. Humans are not at the full mercy of VC metabolites since detoxication by glutathione followed by renal processing generates two main urinary metabolites, N-acetyl-S-(2-hydroxyethyl) cysteine and thiodiglycolic acid. The latter is a useful biomarker of VC exposure in PVC plant workers.

8.7.2 Aristolochic Acid

In the past decade, the powerful methodologies of modern toxicology helped solve a long-standing medical mystery by identifying the causative agent responsible for

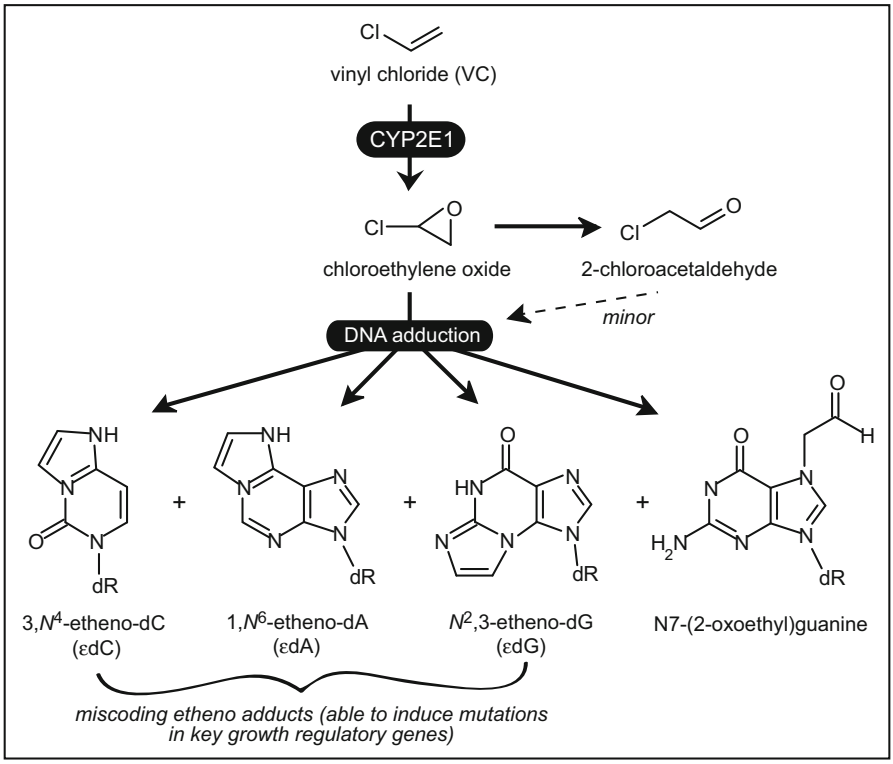

Fig. 8.11 CYP2E1-catalysed bioactivation of vinyl chloride to a damaging epoxide metabolite leads to multiple DNA adducts, with several etheno adducts attracting greatest attention on account of their mutagenic potential and resistance to repair

an epidemic of kidney disease that has long plagued rural populations within the northern Balkan Peninsula. According to parish death records in churches throughout the area, inhabitants in affected regions of Croatia, Serbia, Bosnia/Herzegovina and Bulgaria have long endured a highly debilitating kidney disease. First reported in the scientific literature in the 1950s, this progressive renal disease afflicts villagers in farming communities who have long traditions of making bread from locally grown wheat.

The main features of Balkan endemic nephropathy include chronic tubulointerstitial nephritis (progressive fibrosis) and accompanying carcinomas of the upper urinary tract (UUC). Although the histopathology of this disease resembles drug- and chemical-induced nephritis, recurrent efforts to associate known nephrotoxicants such as heavy metals and ochratoxin A with Balkan nephropathy long met with failure. Likewise, groundwater contamination by polar polycyclic aromatic hydrocarbons leeching from coal deposits in the region failed to explain the geographic distribution of the disease. Genetic studies ruled out inherited gene mutations as major causes of the condition.

In the late 1950s, Jelaković drew attention to numerous cases of veterinary nephrotoxicity in horses that ingested hay contaminated with *Aristolochia clematitis*, a weed that grows extensively in the Danube region. Remarkably, the nephrotoxicity of *A. clematitis* had been proven in rabbits decades earlier by Pohl. Later, in 1967, Ivić reported that crop contamination with *A. clematitis* seeds occurred during wheat harvesting and proposed that a toxic phytochemical might thereby taint home-baked bread. Villagers also reported finding yellow petals from the plant in locally made loaves. Despite these concerns, little investigation of the role of plant-derived toxicants in Balkan nephropathy occurred until the opening years of the twenty-first century.

With time, natural product chemists isolated a family of nitrophenanthrene carboxylic acids from *A. clematitis*, principally aristolochic acid I (AA-I) and aristolochic acid II (AA-II). Administering mixtures of these compounds to rodents reproduced the main features of Balkan nephropathy, including renal cancer induction. AA-I and AA-II also proved highly toxic towards cultured cells due to the formation of a reactive nitrenium intermediate via enzymatic nitro reduction (Fig. 8.12). Several enzyme systems may catalyse this reaction, including NAD(P)H/quinone oxidoreductase, xanthine oxidase, CYP1A2, CYP1A1, NADPH/CYP reductase and renal prostaglandin H synthase. The resulting nitrenium intermediate is highly genotoxic, readily attacking the exocyclic amino groups of adenine and guanine bases to form aristolactam DNA adducts (Fig. 8.12). These adducts are resistant to DNA repair and likely persist for years within the renal cortex. Beginning in 2005, a team of US researchers led by Arthur Grollman at Stony Brook University used ^{32}P-postlabelling/polyacrylamide gel electrophoresis and subsequently multistage tandem mass spectrometric analysis to definitively confirm the presence of deoxyadenosine-aristolactam DNA adducts in the tissues of nephropathy patients from affected Balkan regions.

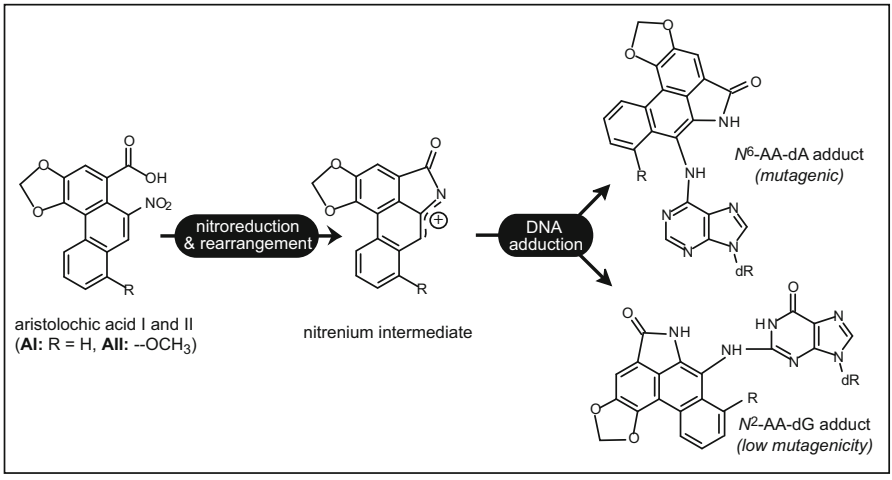

Fig. 8.12 Study of the toxicological basis for endemic Balkan nephropathy identified aristolochic acid (*AA*) as the causative agent in the induction of urothelial tumours. Several enzyme systems can catalyse the initial nitroreduction step (see text)

Dr Grollman's team secured the association of aristolochic acid with endemic nephropathy when they compared the spectrum of mutations in the *p53* tumour suppressor gene of affected subjects with the spectrum of mutations known to accompany translesion DNA synthesis past deoxyadenosine-aristolactam DNA adducts. Site-specific mutagenesis experiments in which plasmids containing deoxyadenosine-aristolactam DNA adducts were replicated in mouse cells yielded A → T transversions as the main mutations. In strong agreement with these findings, sequence analysis of mutated *p53* genes from Balkan urothelial carcinoma samples revealed a high prevalence of A:T → T:A transversions, a class of mutations which is rare in the global database of *p53* mutations which contains close to 30,000 sequences from human cancers.

The speed with which the Grollman group confirmed aristolochic acid as the causative agent in Balkan's nephropathy highlights the power of modern toxicology research techniques. For example, in addition to mutational analysis, the ability to quantify persistent DNA adducts within human tissue samples helped explain the long, 30-year latency applying to the emergence of urothelial tumours in Balkan nephropathy patients. While these findings will clearly benefit inhabitants of the Balkan Peninsula, this work also suggests the sobering possibility that residents of nations such as China and Taiwan – where *Aristolochia* use is an established part of traditional herbal medicine – may be facing a future epidemic of renal disease and urothelial cancer.

8.7.3 Acrylamide

While the *Aristolochia* episode underscored the power of modern research tools to solve long-standing medical mysteries, the past decade also witnessed their use to study emergent carcinogenicity concerns. One toxicant that remains under active investigation is acrylamide, a synthetic reagent used in many industrial settings including the production of paper, cardboard and electrophoresis gels. The neurotoxicity of acrylamide has long been of concern and received renewed attention following a major poisoning episode in southwestern Sweden in late 1997. In an attempt to control water leaks while boring a railroad tunnel, construction workers injected 1,400 t of an acrylamide-containing sealant into porous rocks. The resulting contamination of ground and surface water led to outbreaks of cattle paralysis on surrounding farms as well as a loss of fish in local waterways. Tunnel workers also displayed signs of neurotoxicity.

To identify susceptible workers, Swedish toxicologists began measuring levels of acrylamide-haemoglobin adducts within human blood samples and were surprised to find very high levels in some control subjects. The hunt for the source of their acrylamide exposure soon revealed dietary origins. A groundbreaking Swedish study in 2002 reported the presence of acrylamide in such starchy foods as potato chips, French fries, biscuits and crackers, with lower levels also detected in breads, breakfast cereals and corn chips. The acrylamide likely formed via reactions

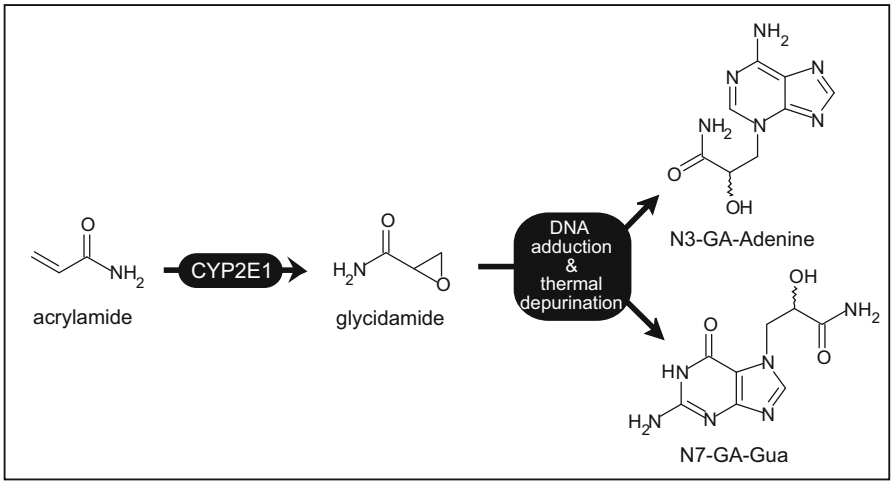

Fig. 8.13 Acrylamide is a genotoxic rodent carcinogen that induces mutations via adducts formed via its epoxidation product glycidamide. The relevance of these mechanisms to the human situation awaits clarification

between glucose and asparagine during high-temperature cooking. The accompanying storm of media attention made acrylamide toxicology a topic of household conversation everywhere: *Dinner to die for* – ran a headline in *The Sydney Morning Herald,* reflecting the angst the findings generated all around the world.

The cancer risks accompanying consumption of acrylamide-containing food are of particular interest since the carcinogenic potency of acrylamide is well established in lab rodents. For example, chronic acrylamide exposure in B6C3F1 mice induced pulmonary adenomas and carcinomas, forestomach squamous cell tumours, mammary gland tumours and skin neoplasms. Lung tumours have also been observed in other mouse strains, while in rats, high doses of acrylamide induced tumours of the mammary gland, testes, oral cavity, thyroid, etc.

Tumour responses to acrylamide involve a clear genotoxic component. Acrylamide is readily oxidised by CYP2E1to glycidamide, an electrophilic epoxide which attacks DNA to form adducts which upon thermal hydrolysis release an N7-guanine adduct (N7-GA-Gua) and a N3-adenine species (N3-GA-Ade) (Fig. 8.13). A role for glycidamide-derived adducts in tumourigenesis is suggested by findings in *Big Blue* mice which showed that mutagenic responses to glycidamide closely resemble those induced by acrylamide. Glycidamide is also more mutagenic than acrylamide in the Ames test.

In recent work at the National Center for Toxicology Research in Arkansas, researchers used a neonatal mouse model to compare the genotoxicity of acrylamide and glycidamide. Newborn mice lack hepatic CYP2E1 activity; hence, no glycidamide formation is likely to occur in acrylamide-treated neonates. Intriguingly, the researchers found that only the epoxide (i.e. glycidamide) induced mutations in critical codons (12, 13 and 16) of the H-*ras* oncogene. The spectrum of base-pair

substitutions closely concurred with the mutagenic properties displayed by glycidamide-derived DNA adducts in vitro. The researchers also confirmed dose- and time-dependent formation of N7-GA-Gua and N3-GA-Ade in glycidamide- and acrylamide-treated mice. However, this promising scenario was undermined by the detection of DNA adducts in many mouse tissues, including those that do not develop tumours during chronic acrylamide dosing. This observation sowed the seeds of doubt concerning the tumourigenic potency of acrylamide-derived DNA adducts.

Another issue surrounding these animal-derived mechanistic insights concerned the size of the acrylamide doses employed since they likely significantly exceed those encountered by humans from dietary sources. In early studies of DNA adduct levels in acrylamide-treated mice, the acrylamide dose used (10 mg/kg) would likely require an 80 kg human to consume some 1,100 kg of French fries, 620 loaves of bread or 4,100 bags of potato chips to receive an equivalent acrylamide dose to the mice (on a mg/kg body weight basis). While subsequent studies have used 1/10th of these doses, they remain high compared to human intakes. While some groups have explored the use of high-sensitivity accelerator mass spectrometry to quantify acrylamide-derived DNA adducts at low-level exposures in mice, this technology has not been applied to actual human samples.

Assessing the cancer risks accompanying dietary acrylamide exposure is further complicated by the paucity of high-quality epidemiological data: most human investigations of this kind have yielded inconclusive results. Knowing the levels of acrylamide-derived DNA adducts within the tissues or blood cells of subjects in epidemiological studies would greatly assist the risk assessment process, yet data of this kind is also limited in nature. Due to these shortcomings, the extent to which dietary consumption of acrylamide alters levels of DNA adducts or mutations in humans is currently unknown. In the face of such uncertainty, it seems prudent to follow the advice of international food safety agencies and practise sensible moderation during the consumption of foodstuffs that contain high levels of acrylamide.

8.7.4 Asbestos

Asbestos is a general term assigned to a family of fibrous hydrated mineral silicates. Goods containing asbestos became popular in the nineteenth century due to their tensile strength, heat stability and insulating properties. During peak usage of the ‘miracle mineral’, the estimated 3,000 asbestos-containing goods in use included products as diverse as paints, tiles, brake linings, cement, plastics, paper, gaskets, textiles and filters. The name *asbestos* designates a variety of six naturally occurring fibrous silicate minerals that are assigned to two groups on morphological grounds: the *serpentine* class (lit. ‘snake-like’) which mainly comprises chrysotile asbestos (‘white asbestos’) and the larger *amphibole* class (lit. ‘uncertain grouping’) of hard, rigid fibres which includes amosite (‘brown asbestos’), crocidolite (‘blue asbestos’)

and tremolite. The serpentine asbestos chrysotile was used in over 95 % of asbestos-containing products in the USA. In the four decades that commenced in 1940, an estimated 27 million Americans encountered asbestos in their daily work, with workers in the construction and shipyard industries most vulnerable to exposure. Despite drastic curtailment of asbestos use, the US Occupational Safety and Health Administration ('OSHA') estimates that over one million US workers remain at risk of exposure today.

The most important route of exposure involves the lungs, with airborne asbestos resulting from both natural processes (e.g. rock weathering) and human activities (e.g. mining, milling, automotive industry, demolition of asbestos-containing buildings). Low levels of asbestos are usually detected within outdoor air in rural locations (~10 fibres/m^3), with typical concentrations in urban settings some 10-fold higher (these can be much higher if industrial sources are nearby).

Occupational exposure to airborne asbestos elicits several deleterious respiratory syndromes. These complex diseases are strongly influenced by the physical diversity of different asbestos fibres, including such factors as particle dose, dimension, biopersistence, surface reactivity and the genetic background of the subject. Some asbestos-related respiratory conditions are largely benign (e.g. pleural disease), but individuals with significant work-related exposure often progress to more serious conditions.

Asbestosis is a chronic respiratory condition with a long latency involving breathlessness, chest pain, coughing and diminished respiratory function. The condition occurs frequently in miners, millers, textile workers and insulation handlers. The disease histopathology involves diffuse interstitial fibrosis (collagen deposition) accompanied by the pulmonary deposition of asbestos bodies. The latter comprise fibrous structures (20–200 μm in length and 2–6 μm in width) containing a core of asbestos particles encased by mucopolysaccharides and iron-rich proteins such as ferritin and haemosiderin.

Asbestosis is a complex condition involving chronic pulmonary inflammation, aberrant cytokine signalling and oxidative stress. Reactive oxygen species likely form via redox reactions on the iron-rich surface of asbestos fibres or by activated polymorphonuclear leukocytes and macrophages. The pattern of inflammation is dose-related: high asbestos doses over short periods promote an acute neutrophil-dominated response, whereas low doses and prolonged exposures induce a chronic inflammatory state dominated by alveolar macrophages. Macrophage activation triggers an intense fibrogenic response with key signalling roles for transforming growth factor β (TGF-β), platelet-derived growth factor, tumour necrosis factor α (TNF-α) and interleukin-1β (IL-1β). Activation of the inflammasome Nalp3 underlies many of the pulmonary effects of asbestos. The resulting burst of fibroblast proliferation leads to widespread deposition of collagen fibres within respiratory bronchioles and alveolar ducts.

A risk of lung cancer also accompanies asbestos exposure, currently accounting for 5–7 % of all lung cancers worldwide. Asbestos-induced lung carcinomas typically develop in the upper or lower respiratory tract. While chronic inflammation is a clear risk factor, lung cancer may also develop in individuals who do not exhibit classic asbestosis symptoms. A major cancer concern is malignant *pleural* mesothelioma, a fatal, rapidly progressing cancer of the thin membrane that surrounds the

lung. *Peritoneal* mesothelioma can also occur due to extensive trafficking of asbestos fibres to the pleura under heavy exposure conditions. Tobacco smoking has a strong synergistic impact upon asbestos-related lung cancer and mesothelioma risks. Since the huge volume of epidemiological data is complicated by significant geographical variation in the types of fibres to which workers are exposed, drawing firm conclusions concerning the carcinogenic potency of different asbestos types is difficult. Based on experimental observations, elongated thin fibres are generally more tumourigenic than short, thick fibres.

A long latency of 20–40 years is observed between asbestos exposure and cancer detection. Many molecular changes accumulate during this time, including chronic inflammation, epigenetic changes and mutations in growth regulatory genes. Microarray analyses of asbestos-induced tumours have detected over 2,500 dysregulated genes, with transcriptional pathways controlled by p*53* featuring prominently in the responses. The mechanisms underlying these transcriptional responses are complex, but a role for fibre-mediated oxidative DNA damage is suggested by the finding that workers with inherited deficiencies in base excision repair pathways (e.g. mitochondrial human 8-oxoguanine DNA glycosylase I) are more prone to asbestos-related malignancies. Asbestos fibres may also induce macroscopic genetic lesions by binding directly to the mitotic apparatus.

8.8 Conclusion

Our survey of the role of chemicals in cancer causation was highly selective, focussing on major carcinogens with the potential to inflict chemical damage on the genome. This survey highlighted that since DNA adducts are central to cancer induction by genotoxic xenobiotics, modern toxicology devotes considerable effort to describing the molecular properties and biological fate of individual DNA adducts. The ability to quantify such species within tumour tissue – as in the case of aristolochic acid exposure – provides powerful support for suspected cancer associations, while the inability to detect such modifications (e.g. in the case of dietary acrylamide exposure) can help defuse anxiety and direct attention towards more important carcinogen risks.

Despite these advances, many significant challenges remain in the area of chemical carcinogenesis, including a pressing need for faster, cheaper and more accurate screening methods for identifying rogue carcinogens. By extending the terrain already won since the outbreak of hostilities in the War on Cancer, future decades will likely see significant improvements in our understanding of chemical carcinogenesis.

Since this chapter has developed a basic awareness of the role of reactive intermediates and DNA damage in chemically induced cancer, we are better placed to consider how these conceptual insights illuminate common syndromes accompanying exposure to two widely consumed xenobiotic mixtures: alcoholic beverages (Chap. 9) and tobacco smoke (Chap. 10).

Going Further

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Chapter 9
Everyday Toxicology I: Alcohol

Abstract The potential benefits to cardiovascular health accompanying moderate wine consumption are undermined in heavy drinkers who risk harming multiple body systems. The resulting health disorders are classified according to whether they are 'partly' or 'wholly' attributable to alcohol. The former include hypertension and breast cancer, while those in the latter category, which typically attract most toxicological attention, include alcoholic liver disease, alcoholic myopathy, alcoholic neuropathy and foetal alcohol syndrome. Alcohol suffers a complex metabolic fate in vivo, with a key pathogenetic role likely for acetaldehyde, the primary oxidative metabolite of ethanol. Protein and DNA adduction feature prominently in the toxicity of this toxic electrophile. Alcohol also undergoes nonoxidative metabolism to form such conjugative metabolites as ethyl-glucuronide, ethyl-sulfate and fatty acid ethyl esters. These metabolites find growing use as biomarkers of alcohol intake and appear especially useful during the diagnosis of foetal alcohol syndrome.

Keywords Acetaldehyde • Alcoholic liver disease • Cardiac myopathy • Cirrhosis • CYP2E1 • Endotoxin • Ethyl-glucuronide • N^2-ethyl-dGuo • Ethyl-sulfate • Fatty acid ethyl esters • Foetal alcohol syndrome • Hybrid adducts • Peripheral neuropathy • Protein carbonylation

9.1 Introduction

Human awareness that fermentation of rotting fruit yields beverages with euphoric properties is long-standing, traceable to ancient Egypt and written records left by Sumerian and Babylonian populations in the Fertile Crescent. Over subsequent millennia, alcohol cemented its popularity to claim the impregnable status as the most widely consumed xenobiotic on the planet. This was not entirely due to its inebriating properties, since throughout much human history, alcoholic beverages posed a healthy alternative to poor-quality, microbe-contaminated drinking water. Moreover,

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within traditional cultures, fermented beverages found uses beyond recreational purposes that included cultural and religious contexts and even economic applications as a unit of currency. In addition, as in today's world, ancient politicians often raised state revenue via alcohol taxation: during the Ptolemaic dynasty in ancient Egypt, Cleopatra VII financed her military campaigns via a tax on alcohol production. Much more recently, the twentieth-century Soviet state underpinned a significant part of its military expansion by taxing the sale of vodka.

Alongside ancient evidence for alcohol production, the historian frequently finds awareness that social harm plagues its unrestrained use. For example, the ancient Egyptian religious text, *The Maxims of Ani*, contains warnings from an old man to his son concerning the dangers accompanying alcohol use: 'Frequent not the house where men drink beer, for the words that fall from thy mouth will be repeated, and it is a bad thing for thee not to know what thou didst really say. Thou wilt fall down, thy bones may be broken, and there will be no one to give thee a hand'. Ancient records also reveal attempts to curtail the negative impact of alcohol: the Babylonians, for example, likely imposed the first legislative restrictions upon the operations of taverns. Early writings from Greek, Roman, Hebrew and Hindu cultures also convey a frank awareness of the potential harm accompanying alcohol abuse. Based no doubt on personal experiences after his famous binges, King Solomon from ancient Israel displayed a particularly acute awareness of alcohol toxicology: 'Do not gaze at wine when it is red, when it sparkles in the cup, when it goes down smoothly! In the end it bites like a snake and poisons like a viper' (Proverbs 23: 31–32).

Due to the substantial contribution of alcohol to chronic ill health in the modern world, alcohol-related toxicities are among the most intensively studied xenobiotic-induced toxic syndromes of our time. This chapter will review current toxicological insights into alcohol toxicity, with particular emphasis given to the deleterious effects of alcohol on the liver, nervous system, heart and unborn child. By exploring the likely molecular mechanisms underlying these toxic outcomes, this chapter will put flesh on general toxicological concepts introduced in earlier chapters.

9.2 Alcohol and Human Disease

While the social consequences accompanying alcohol use engage public health researchers, modern toxicologists seek to understand the damage it inflicts on the body systems of individual users. From the latter perspective, the cardiovascular benefits accompanying moderate alcohol consumption need balancing against the substantial long-term pathology accompanying heavy intake. Worldwide, an estimated 2.5 million individuals die from alcohol-related causes every year – the equivalent of a Rome, Toronto or Beirut. In the USA, alcohol-related deaths are the third leading preventable cause of death. The economic impact of alcohol abuse is also substantial, comprising an estimated $257 billion in 2006 in the USA alone. The prevalence of problematic drinking patterns is also high, with typically 1 in 20 Americans exhibiting signs typical of alcoholism, including impaired control over their intake, preoccupation with alcohol, denial of alcohol use or continued use in

Table 9.1 Adverse health effects accompanying heavy alcohol intake in humans (Reprinted from Clin Liver Dis.;16(4):659–66, Schwartz and Reinus, Prevalence and natural history of alcoholic liver disease, Copyright (2012), with permission from Elsevier)

Pancreatitis (acute and chronic)	Epilepsy	Prostate cancer
Cardiomyopathy	Oesophageal cancer	Psoriasis
Polyneuropathy	Foetal alcohol syndrome	Spontaneous abortion
Chronic liver disease	Gastrointestinal bleeding	Stroke
Myopathy	Hypertension	Supraventricular tachyarrhythmia
Psychosis	Ischaemic heart disease	–
Breast cancer	Oropharyngeal cancer	–

the face of obvious ill effects. Although the USA has likely devoted the greatest research attention to understanding the toxicology of alcohol, problematic drinking is significantly more prevalent in other nations such as the Russian Federation, Kazakhstan, Mexico, South Africa or the Ukraine.

According to the Global Information System on Alcohol and Health maintained by the World Health Organization (WHO), alcohol has a causal role in over 60 diseases, some of which are listed in Table 9.1. This includes disorders that are wholly attributable to alcohol and to which the label 'alcoholic' is attached to the respective clinical syndrome. Since they are especially relevant to toxicology, disorders within this category will occupy our attention in this chapter.

In addition to 'wholly attributable' conditions, the global burden of disease includes conditions in which alcohol only partly contributes to disease pathogenesis as an exacerbating risk factor. This includes chronic disorders such as various infectious diseases as well as acute conditions that include injuries and falls. When all such conditions are considered, nearly 4 % of the global burden of disease is attributable to alcohol, a figure that resembles the health impact of tobacco and hypertension. So important is its role in human disease that an anonymous sage once wisely observed that *to know alcohol is to know medicine*.

Studying associations between human disease incidence and alcohol consumption is complicated by substantial variations both within and between human populations in terms of drinking patterns and differences in the quantity and quality of alcoholic beverages consumed. Further complicating these efforts is the changing tastes of rising generations coupled with product innovation by alcohol manufacturers that ensure relationships between alcohol exposure and associated diseases are rarely simple and never static.

9.3 Alcohol Products

Technological advances in liquor production ensure contemporary alcoholic beverages differ from those found in ancient societies or in traditional indigenous cultures today. Beers in the Ancient world, for example, were quite distinct from modern ales, stouts or lagers: they were typically thick and fibrous and could easily be

mistaken for an alcohol-impregnated porridge. As during indigenous beer preparation today, the fermentation process was commonly initiated by chewing or spitting into the brew. In Zulu villages today, such techniques are still used to prepare beer from sorghum or corn.

In contrast to the use of cereal grains during beer production, wine is made from fruit such as berries, stone fruits or, most commonly, grapes. Use of a longer fermentation process compared to beer production typically achieves higher alcohol contents. Historically, wine was produced in temperate fruit-growing regions throughout Europe that included the lower Caucasus, the Balkans and the Mediterranean rim. With European settlement of the New World, viticultural districts in North America (e.g. Napa), South America (e.g. Mendoza), Southern Africa (e.g. Stellenbosch) and Australia (e.g. Barossa) began producing quality, affordable wines that competed with vintage from traditional regions in France, Spain and Italy.

The production of spirits via distillation is technically challenging, and hence, these practices emerged as a later development, perhaps around the time of Christ. Spirits were made by boiling a fermented product using a condenser apparatus to enrich the alcohol content, adapting technologies that were already used to produce medicines and perfumes. Later, in the early modern era, the growth of shipping created a need for alcoholic beverages that withstood spoiling during long sea voyages. Countries with strong maritime economies such as Spain and Portugal thus perfected the preparation of fortified wines by adding spirits to wines as preservatives. Major fortified wines include such long-lived favourites as Port (wine + neutral grape spirit), Sherry (wine + brandy) and Vermouth (wine + neutral spirit + oils, herbs, etc.).

In recent decades, mass production capabilities coupled with an expansion of global trade have ensured unprecedented availability of affordable alcoholic beverages in modern societies. While the opportunity to broaden the palate entices many drinkers, this growth in alcohol availability has had a downside in societies where unprecedented access to low-cost, high-alcohol beverages is increasing the prevalence of alcoholism, binge drinking and associated health problems.

9.4 The Toxicokinetics of Alcohol

Possessing just two carbon atoms, an oxygen atom and a handful of hydrogen atoms, the diminutive size of ethanol belies the powerful effects it exerts upon many physiological processes. Compared to many drugs with complex structures and nuanced physicochemical properties, the toxicokinetics of alcohol are relatively straightforward. The ethanol molecule is amphipathic in nature, possessing a polar end that is attracted to water and a nonpolar end that is repelled by an aqueous environment. This mix of hydrophilic and hydrophobic properties ensures ethanol is effectively absorbed following oral ingestion. The membrane permeant properties of ethanol also reflect its small size and ability to penetrate intercellular spaces (see

Fig. 3.3 in Chap. 3). Since it is freely absorbed, the alcohol concentration within gut lumen is the main determinant of blood concentrations. Practices such as binge drinking that involve ingesting a single high dose of alcohol thus achieve higher concentrations within blood than if the same total dose is ingested as a series of smaller doses. Consuming alcohol on an empty stomach may also yield higher blood concentrations since many food constituents – including fats, proteins and complex carbohydrates – likely slow the oral absorption of alcohol. This factor is partly offset by the higher rate of metabolism occurring in the fed compared to fasted state.

The high water solubility of alcohol ensures ready distribution via the blood, with little binding to plasma proteins to complicate its kinetic properties. The disposition of alcohol into body organs and tissues depends on the fat and water content of respective tissues, with peak concentrations correlating closely with the tissue water content. Interindividual differences in body fat composition also contribute to variable sensitivity to alcohol. This factor accounts for significant gender differences in alcohol toxicokinetics, since the volume of distribution is often lower in women due to a higher percentage of body fat compared to men. Such factors contribute to the higher incidence of alcoholic liver disease in women: the lower volume of distribution results in higher plasma concentrations per unit of alcohol ingested, increasing exposure of internal organs to the toxicant.

Most ethanol removal from the body proceeds via metabolism, although as a relatively volatile substance, about 10 % of an absorbed dose is excreted unchanged within exhaled breath, sweat and urine. Ethanol release from blood as it passes through the lung is indicative of plasma concentrations, explaining the widespread use of breathalysers for law enforcement purposes. Since appreciation of its metabolism is crucial to understanding alcohol toxicology, we will explore this topic in a little detail.

9.5 Alcohol Metabolism

Alcohol, like any ingested xenobiotic, requires removal from the body, with the main responsibility for this task falling upon the liver. A typical 70 kg adult metabolises ~170–240 g alcohol per day, corresponding to ~7–10 g per hour (i.e. ~1 standard drink per hour). The hepatic metabolism of ethanol proceeds via both nonoxidative and oxidative routes, with the latter of greatest quantitative significance (Fig. 9.1). On surface appearances, the oxidative metabolism of alcohol is unremarkable, proceeding via oxidation to acetaldehyde in a reaction normally catalysed by one of seven alcohol dehydrogenase (ADH) isoforms present within human tissues. These enzymes normally detoxicate endogenous alcohols formed by microbes present within the GI-tract. Although expressed strongly within the liver and GI-tract, ADH is also present within nasal mucosa, the uterus and testes. Acetaldehyde formed by ADH is subsequently oxidised by aldehyde dehydrogenase (ALDH) to acetic acid that is further converted to acetyl-CoA. The

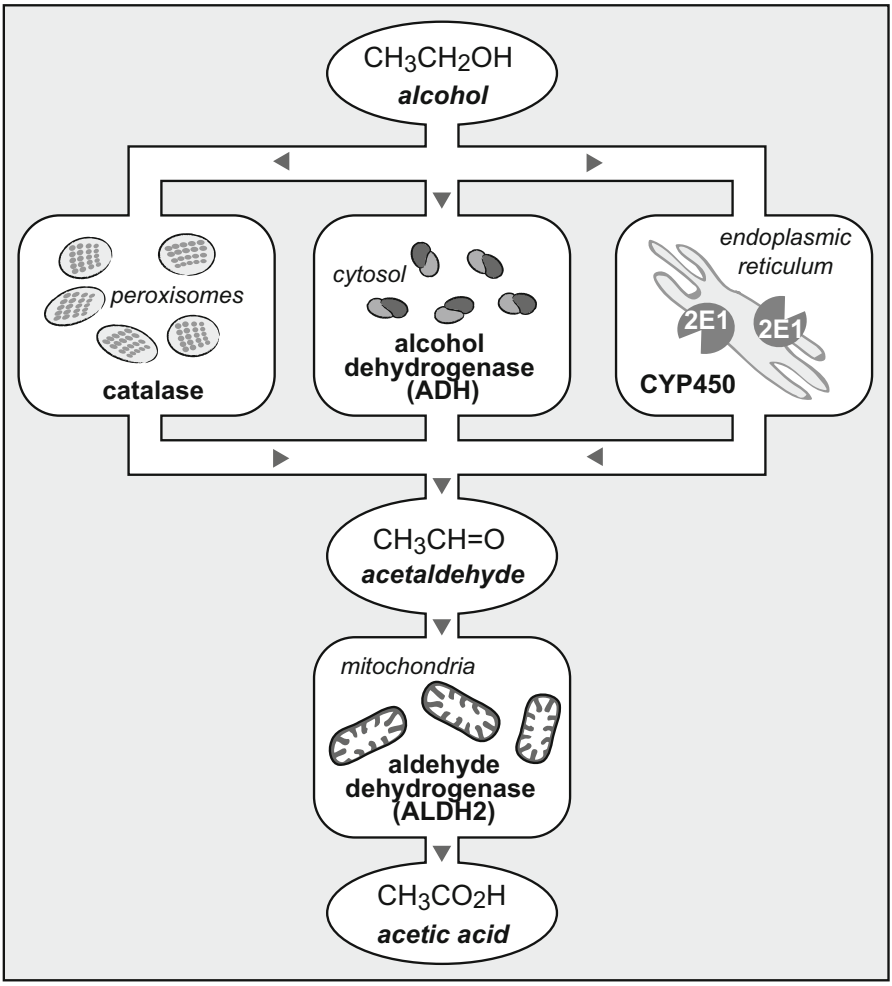

Fig. 9.1 The main metabolic fate of alcohol within the liver involves oxidation to acetaldehyde via one of three main enzyme systems followed by conversion to acetic acid

niacin-derived cofactor NAD accepts reducing equivalents during both the ADH- and ALDH-catalysed steps of oxidative ethanol metabolism. Many members of the ALDH family exist within the human genome (approx. 30 genes), yet most acetaldehyde oxidation in human liver is catalysed by the mitochondrial protein ADLH2. Acetic acid formed via this reaction is converted to acetyl-CoA, a shared substrate in many metabolic pathways. Alcohol thus can suffer a range of enzymatic fates depending on the prevailing nutritional environment and energetic status of the liver, including further oxidation to CO_2 and subsequent elimination in exhaled breath, or enzymatic conversion to fatty acids, ketone bodies or even cholesterol.

Compared even to the metabolism of glucose, the oxidative metabolism of ethanol releases a considerable amount of energy. Alcoholics, for example, can receive in the order of ~1,400–2,100 kcal/day from their alcohol consumption alone (~5,900–8,800 kJoules/day). An individual quaffing a more socially acceptable quantity of two glasses of wine per day can still receive 10 % of their daily calorie intake from alcohol.

The metabolism of ethanol to acetaldehyde is not left entirely to ADH since CYP2E1 also catalyses this reaction (Fig. 9.1). The Michaelis–Menten coefficient (K_M) (approx. 10 mM) is some tenfold higher than for ADH, ensuring CYP2E1likely makes little contribution to ethanol clearance in light drinkers, although its contribution increases as alcohol concentrations rise. This contribution can increase, however, since CYP2E1 abundance within hepatocytes is induced up to tenfold in heavy drinkers via a xenosensor-independent pathway (see Sect. 5.2.1.7). The increased role of CYP2E1 in ethanol oxidation within alcoholics comes at a cost since poor coupling during the catalytic cycle of this isoform releases substantial quantities of superoxide radicals within the alcohol-intoxicated liver. Such oxidative stress is a key driver of hepatocellular injury during alcohol abuse (see below).

High expression of catalase within the peroxisomes also helps during the conversion of ethanol to acetaldehyde (Fig. 9.1). These organelles also convert ethanol to a novel series of esterified fatty acid derivatives known as fatty acid ethyl esters (FAEE) (Fig. 9.2). These reactions represent a pathway of nonoxidative ethanol metabolism that likely makes minimal contribution to the overall toxicokinetic fate of ethanol yet nevertheless is of high toxicological significance due to their roles as either disease mediators or biomarkers of alcohol intake (Fig. 9.2). The formation of FAEE is analogous to the esterification of glycerol with lipids to form the mono-, di- and triglycerides that are well known to classical biochemistry, except that in the case of ethanol, possession of a single hydroxyl group ensures the formation of monoesterified products only. The peroxisomal enzymes that perform these reactions, FAEE synthases, typically conjugate ethanol to multiple lipids including linoleic, arachidonic, palmitic, oleic and stearic acids. Enzymes with comparable capabilities are also present in cytosol and microsomes. While these pathways are quantitatively minor contributors to overall alcohol clearance from the body, FAEE likely mediates a range of deleterious outcomes within the alcoholic liver, including induction of mitochondrial and lysosomal damage, inhibition of cell replication and suppression of protein synthesis. Since levels of FAEE increase with rising blood alcohol concentrations, they are useful biomarkers of chronic alcohol ingestion.

A small proportion of alcohol also undergoes conjugative metabolism to form ethyl-sulfate and ethyl-glucuronide (Fig. 9.2). The former is potentially detectable in body fluids, tissue and hair samples for several days after alcohol ingestion, ensuring this metabolite is increasingly used as a biomarker of alcohol consumption in humans during medicolegal and forensic investigations as well as clinical trials. Compared to other markers, ethyl-glucuronide concentrations within 3 cm lengths of hair samples provide a noninvasive and reliable indication of heavy alcohol use during the preceding 3-month period. This metabolite is typically undetectable in hair samples collected from social drinkers.

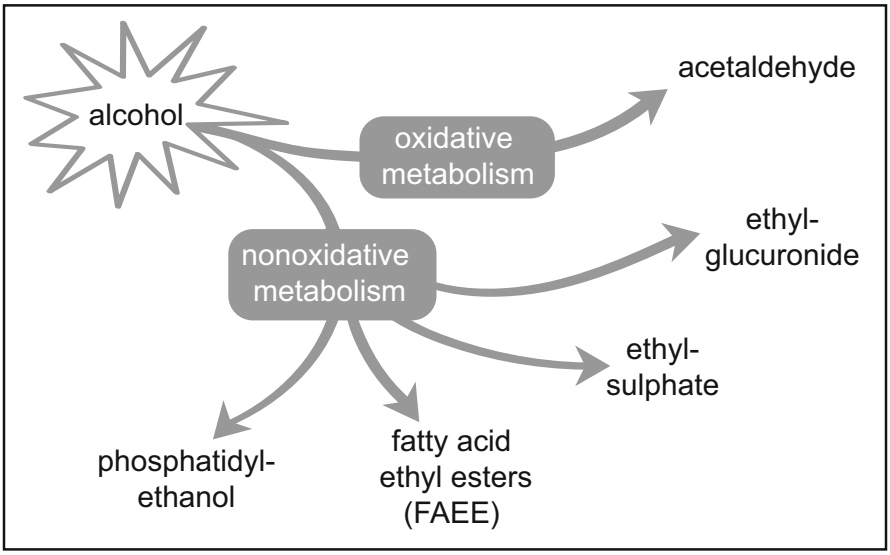

Fig. 9.2 Several routes of minor nonoxidative metabolism generate various conjugated ethanol metabolites that are useful biomarkers in diverse clinical and forensic contexts

Due to the broad routes of oxidative and nonoxidative metabolism within the gut wall and liver, a substantial proportion of ingested alcohol is metabolised before it reaches the systemic circulation. Such first-pass metabolism is typically most significant within the stomach and to a lesser extent within the duodenum. In addition to the physical effects of food on alcohol absorption highlighted above, the higher blood concentrations observed when alcohol is consumed in the fasted state may partly reflect less extensive first-pass metabolism within the stomach due to faster transit.

The ability to metabolise alcohol is constant throughout the human lifespan, although it declines somewhat among the elderly. The unborn child also metabolises alcohol poorly due to a general deficiency in the expression of xenobiotic-metabolising enzymes within foetal liver. This factor likely contributes to foetal alcohol syndrome and related disorders that receive attention below.

9.6 Molecular Toxicology of Alcohol

Alcohol is the archetypal bioactivation-dependent toxicant in that its main toxic effects are mediated by reactive metabolites formed within the body. The liver and other tissues of heavy drinkers incur unrelenting exposure to multiple electrophilic species that readily attack cellular macromolecules such as DNA and protein. Yet as we saw in Chap. 4, while the formation of macromolecular adducts is fundamental to many chemically induced toxic syndromes, identifying the precise molecular events whereby such chemical damage triggers toxicity is rarely easy.

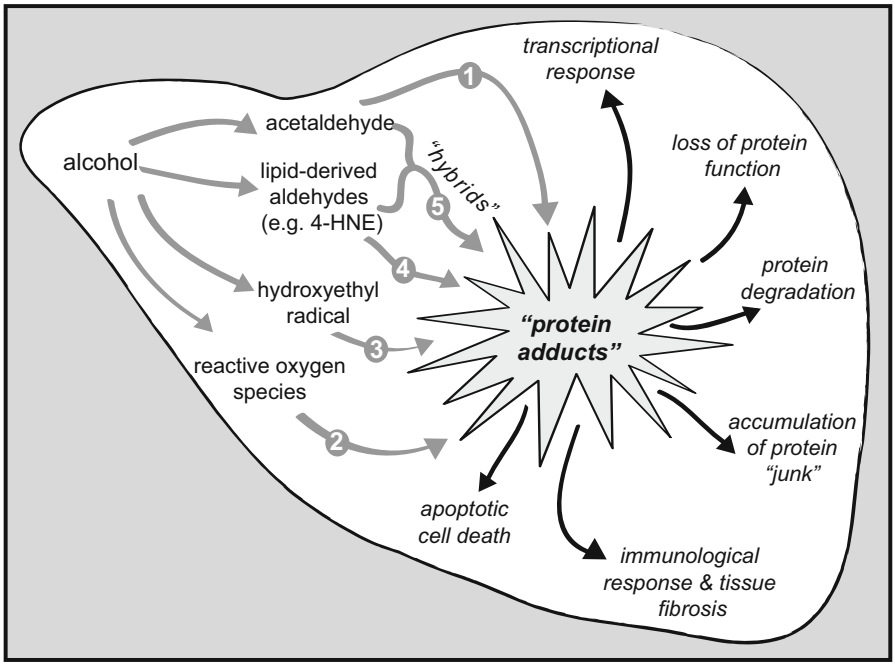

Fig. 9.3 Ethanol metabolism generates various reactive intermediates that inflict damage upon the hepatic proteome via five main routes. The numbers designate the order in which each pathway is discussed in the text

9.6.1 Protein Adducts

During alcohol metabolism, protein damage occurs via at least five discernible pathways, ensuring a heavy drinker's hepatic proteome is a veritable 'chemical soup' of protein modifications (Fig. 9.3). Due to limitations in current technologies, it is difficult to quantify the full range of protein adducts simultaneously within the liver of an alcohol-exposed individual; hence, questions persist concerning which of these chemical routes to protein damage is most important during the pathogenesis of alcohol-related disease.

A major route to protein adduction is mediated by acetaldehyde that forms during the oxidation of ethanol by ADH, CYP2E1 or catalase (labelled with a '1' in Fig. 9.3). Although acetaldehyde is not an especially reactive electrophile, as the primary product of ethanol oxidation, it forms in huge quantities within the livers of heavy drinkers and so its abundance overrides its lesser reactivity compared to other biogenic aldehydes. Acetaldehyde reacts directly with nucleophilic lysine groups in cell proteins to form Schiff base adducts that likely undergo chemical rearrangements to form more stable species. Upon heavy alcohol exposure, such adducts accumulate in tissues that exhibit alcohol-related pathology, including liver, muscle and heart. Acetaldehyde may also form stable adducts at the N-terminal primary amine of target proteins.

Knowledge of this chemistry raises the possibility that measuring acetaldehyde adducts in circulating blood proteins might show how much alcohol an individual has consumed in recent weeks or months. Given that self-reporting by alcoholics is notoriously unreliable, such 'objective' biomarkers of alcohol consumption could be very useful in clinical studies. While some researchers have explored these possibilities, the results have not been especially encouraging partly due to high variability in acetaldehyde adduct levels between individuals. Future work in this area using modern analytical instrumentation may resolve these problems. In the meantime, conjugated ethanol metabolites will likely find growing use as markers of alcohol intake (Fig. 9.2).

A second route to protein damage in alcohol-intoxicated tissues involves oxygen radical by-products of CYP2E1-catalysed ethanol metabolism (Fig. 9.3). In the presence of transition metal catalysts, reactive oxygen species such as hydrogen peroxide are degraded to highly damaging hydroxyl radicals. This powerful oxidant inflicts broad damage upon proteins, one general form of which is termed *protein carbonylation* that occurs when hydroxyl radicals attack amino acid side chains to form carbonyl-retaining adducts. Protein carbonylation is conspicuous within proteins extracted from the livers of alcoholics. In addition to serving as biomarkers of cell damage during mechanistic studies, protein carbonyls are potentially reactive species that facilitate disease progression by reacting further to form cross-linked proteins and high-mass protein aggregates.

A third form of protein damage during CYP2E1-catalysed ethanol metabolism involves hydroxyethyl radicals that form via 1-electron oxidation of the ethanol molecule. While the formation of these carbon-centred radicals was initially demonstrated under test tube conditions, use of 'spin trap' reagents and electron spin resonance spectroscopy subsequently confirmed their presence in bile samples collected from alcohol-treated lab animals. As highly reactive species, hydroxyethyl radicals react with multiple sites in proteins to form adducts that are detectable via immunoblotting using antibodies directed against the modified amino acid.

A fourth significant route to protein adduction involves reactive aldehydes formed during free radical attack upon cell membranes (Chap. 4, Sect. 4.4.4). Such reactive species include α,β-unsaturated aldehydes such as malondialdehyde (MDA), acrolein (ACR) and 4-hydroxynonenal (4-HNE)which likely act as diffusible 'toxicity mediators', migrating through cell membranes to attack proteins in neighbouring cells. The unsaturated bond possessed by these electrophiles is readily attacked by nucleophilic residues in target proteins via Michael addition chemistry to form carbonyl-retaining adducts. Alternatively, the carbonyl group can react with nitrogen-containing nucleophilic amino acids to form Schiff base adducts. In comparison to acetaldehyde, these lipid-derived aldehydes tend to be 'soft' electrophiles which react extensively with cysteine groups, whereas acetaldehyde typically prefers 'harder' nucleophiles such as lysine groups. Using classical antibody-based methods, adducts formed by lipid-derived aldehydes are readily detected in liver biopsies from alcoholic patients. Alternatively, mass spectrometry-based techniques allow accurate quantification of these adducts within alcohol-exposed tissues. Recent insights from animal models gained via the latter approach suggest that proteome damage by lipid-derived aldehydes participates directly in the pathogenesis of alcoholic liver disease (see below).

A fifth key route to protein damage involves formation of hybrid adducts ('MAA adducts') via synergistic protein damage by malondialdehyde and acetaldehyde. Discovered by toxicologists at the VA Alcohol Research Center in Omaha, Nebraska, this novel pathway is favoured because the presence of one aldehyde enhances the reactivity of the other, generating hybrid adducts that differ from those formed by individual aldehydes alone. MAA adducts are detected in diverse tissues from alcohol-treated rodents and human drinkers. They seem especially immunogenic and may trigger the abnormal deposition of fibrotic tissue in alcoholic liver (see below).

Documenting these diverse routes to protein adduction in the liver proteome during alcohol intoxication represents a major achievement, but how might such chemistry facilitate the progression towards a pathological state? Identifying the specific proteins that sustain adduction may clarify this issue, since such knowledge can reveal the biochemical defects that might develop in the alcohol-injured tissue. Identifying these protein targets is less challenging today due to the wide availability of proteomic technologies. After new proteins are identified as targets for damage during alcohol metabolism, the question becomes whether such damage is of toxicological relevance to disease progression. Thankfully, modern researchers also possess powerful tools for evaluating the toxicological importance of damage to particular proteins by reactive alcohol-derived metabolites. For example, tissue-specific gene-ablation technology can allow creation of transgenic mouse strains that lack the target protein within the tissue of interest. If pathology occurring within the gene-deficient target organ resembles that caused by alcohol, then the biological significance of damage to that protein during alcohol intoxication is likely high.

Commonly, however, efforts to identify proteins that sustain adduction by reactive metabolites often make the unexciting observation that the most heavily damaged proteins are simply the most abundant proteins in a given tissue. The most common targets for acetaldehyde and related aldehydes within many tissues are often ubiquitous proteins such as actins, tubulins, albumin, globulins and collagen. While these proteins are essential biochemical workhorses, we might be sceptical that damage to these abundant species will carry profound toxicological consequences. Yet, the significance of such damage should not be prematurely dismissed: in addition to direct implications of protein adduction, protein adducts within abundant proteins may indirectly cause tissue injury by triggering dangerous activation of the immune system. Alcohol-related adduction of abundant proteins such as collagen likely plays a key role in activating the innate and acquired arms of the immune system during alcoholic tissue injury (see below).

9.6.2 DNA Adducts

While the higher cellular prevalence of proteins compared to nucleic acids ensures reactive electrophiles formed during alcohol metabolism are more likely to attack proteins rather than DNA, a growing body of data confirms that DNA adducts involving guanine form in many tissues under conditions of high-alcohol exposure. One such acetaldehyde-derived adduct, N^2-ethyl-dGuo, is emerging as a very useful

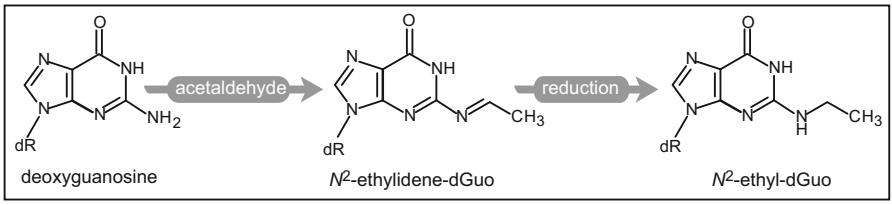

Fig. 9.4 Reaction of acetaldehyde with the exocyclic primary amine of guanine (on N-2) generates an unstable adduct which is reduced in vivo or in vitro to N^2-ethyldeoxyguanosine (*N2-ethyl-dGuo*). N^2-ethyl-dGuo is a useful tissue biomarker of alcohol-related genetic damage

biomarker of alcohol-related genetic damage (Fig. 9.4). In a recent US study, the levels of this adduct increased ~100-fold within DNA extracted from cells scraped from the inner cheek of volunteers 4 h after ingesting a dose of vodka that achieved a blood alcohol concentration of 0.03 %. Levels of this adduct also increase within the oral cavity of lab rodents fed alcohol in drinking water. Since cancers of the head and neck are common in heavy drinkers, such findings implicate acetaldehyde as a likely mediator of alcohol-related carcinogenesis. Although N^2-ethyl-dGuo is repaired and ultimately subjected to urinary excretion, this adduct is best measured in DNA samples from exposed tissues rather than urine: since N^2-ethyl-dGuo is detectable in urine collected from teetotallers, its usefulness as a urinary biomarker of alcohol-induced genetic damage is uncertain.

Formation of N^2-ethyl-dG and other acetaldehyde-derived adducts within target tissues likely participate in the induction of mutations within growth regulatory genes during the pathogenesis of cancers in alcoholics. The mutagenicity of *N2*-ethyl-dGuo has been studied in human cells using site-specific mutagenesis strategies in which the adduct was positioned within a viral vector (see Fig. 8.8B in Chap. 8). Although these studies have not revealed strong mutagenic properties for this adduct, it does strongly block DNA replication by DNA polymerases. These properties may mediate induction of frameshift deletion mutations in ethanol-exposed cells.

In addition to forming DNA adducts, acetaldehyde also produces DNA cross-links which can generate mutations during imperfect repair of these lesions. Cross-links likely account for the induction of sister chromatid exchange and 'macroscopic' chromosomal aberrations observed in alcohol-exposed cells. The high concentrations of acetaldehyde required to induce these genetic macrolesions implies such damage is most relevant to heavy drinkers who regularly abuse alcohol.

Although acetaldehyde is likely the main contributor to genetic damage during alcohol intoxication, most of the various pathways to proteome modification in alcoholic liver shown in Fig. 9.3 may also promote DNA damage. DNA oxidation products, lipid peroxidation-induced DNA adducts and modified bases formed by hydroxyethyl radicals all likely participate in the loss of genetic stability within the alcoholic liver.

9.6.3 Transcriptional Responses

The availability of microarray technology and other techniques for monitoring global changes in gene expression is helping to clarify the cellular effects of heavy alcohol consumption. The broad transcriptional changes induced by alcohol likely represent cellular responses to macromolecular damage caused by reactive metabolites (i.e. protein and DNA adducts). One possible route to altered gene expression involves adduction at critical residues on transcription factors, thereby disrupting binding to promoter sequences within target genes or hampering the binding of coactivator proteins needed for a functional transcription complex. Direct damage to promoters via the formation of DNA adducts may also disrupt gene transcription events.

Although debate continues concerning the model species that are most relevant to humans, new insights are emerging from studies of changes in mRNA profiles during alcohol toxicity in animal models. Such approaches are often more technically feasible than using human samples since the quality of biopsy tissue recovered from alcoholic liver is often poor due to overt tissue destruction (see below). Use of microarray technology to monitor mRNA profiles during alcoholic liver injury in baboons, rats and mice has revealed considerable similarities in alcohol-induced transcriptional responses irrespective of species or method of alcohol administration, suggesting high-dose alcohol exerts a common hepatotoxic mode of action across multiple species. Commonly upregulated genes in alcoholic liver include those participating in different pathways of ethanol metabolism. Various wound healing pathways are also upregulated, suggesting ethanol intoxication triggers an effort of the damaged liver to regenerate itself. Strong changes also occur within pathways that promote remodelling of the extracellular matrix, a further sign of boosted liver regeneration. From a clinical standpoint, transcriptional changes suggesting strong activation of the immune response within the livers of alcohol-intoxicated rodents and humans alike seem highly significant (see below).

More recently, researchers have studied changes in microRNA expression in various alcohol-related disorders, leading to the identification of alcohol-responsive microRNAs that serve as master switches during cellular responses to alcohol. While alcohol-responsive microRNAs initially were studied within the brain during the development of tolerance and alcohol addiction, their contribution to overt toxicity in peripheral tissues is attracting growing attention. Recent research implicates changes in alcohol-responsive microRNAs to toxic outcomes as diverse as foetal alcohol syndrome, GI-tract leakiness, alcoholic liver disease, enterohepatic tumourigenesis and even skeletal toxicity and bone fragility. It is hoped ongoing work on these mechanisms may open the door to new therapeutic options for the treatment of alcohol-related pathological syndromes.

9.6.4 Immunological Responses

The role of the immune system is clear in the liver where it takes centre stage during the *induction* and the *progression* of alcoholic liver disease. Disruption to both major arms of the immune system occurs during chronic alcohol intoxication.

9.6.4.1 Innate Immune Responses

The innate immune system provides an immediate and nonspecific defence against incoming pathogens. It lacks memory capacity and essentially represents an automatic response to ubiquitous antigens such as endotoxin, the lipopolysaccharide component of the Gram-negative bacterial cell wall that participates in many serious diseases (e.g. sepsis, organ failure). A major effector of the innate response within the liver is the Kupffer cell, a class of resident macrophages that phagocytise bacteria and mount an oxidative and cytokine assault upon antigens. In heavy drinkers, Kupffer cell activation proceeds via increased delivery of bacterial endotoxin to the liver via the portal circulation due to leakiness of the gut wall caused by tissue injury accompanying continual alcohol exposure (Fig. 9.5). Changes in the gut microbiome within heavy drinkers, including strong overgrowth of some bacterial species, likely exacerbate chronic endotoxin leakage into the portal circulation. Epidemiological data suggests exacerbation of these problems in premenopausal women who display high susceptibility to alcoholic liver disease, likely due to increased gut wall permeability caused by high levels of circulating oestrogen.

On reaching the liver, endotoxin assembles a complex that includes the co-receptor CD14 that then binds to Toll-like receptor-4 (TLR4), a membrane-embedded pattern recognition receptor that regulates cytokine production by Kupffer cells (Fig. 9.5). Cytokines facilitate communication between the many cell types within the immune system and form a broad family including tissue-damaging proinflammatory species such as tumour necrosis factor (TNF-α) and interleukin (IL)-1β. With a strong ability to induce apoptosis in hepatocytes, TNF-α is a central mediator of alcoholic liver disease. Another arm of the innate immune system that is activated in heavy drinkers involves release of complement C3 and C5. These proteins also target Kupffer cells by binding to complement receptors on these cells, with resultant activation of the complement pathway further contributing to TNFα overproduction and the induction of liver cell death.

Proinflammatory cytokines also activate hepatocyte production of other acute-phase inflammatory mediators such as IL-6 and the chemoattractant IL-8 that attract other innate response cells such as neutrophils and natural killer (NK) cells. By activating these responses, Kupffer cells act as sentinels to recruit other immune cells to help the liver defend itself invading bacterial endotoxin during prolonged alcohol intoxication. Via a dose-related phenomenon, overactivity of this response in heavy drinkers causes extensive organ damage.

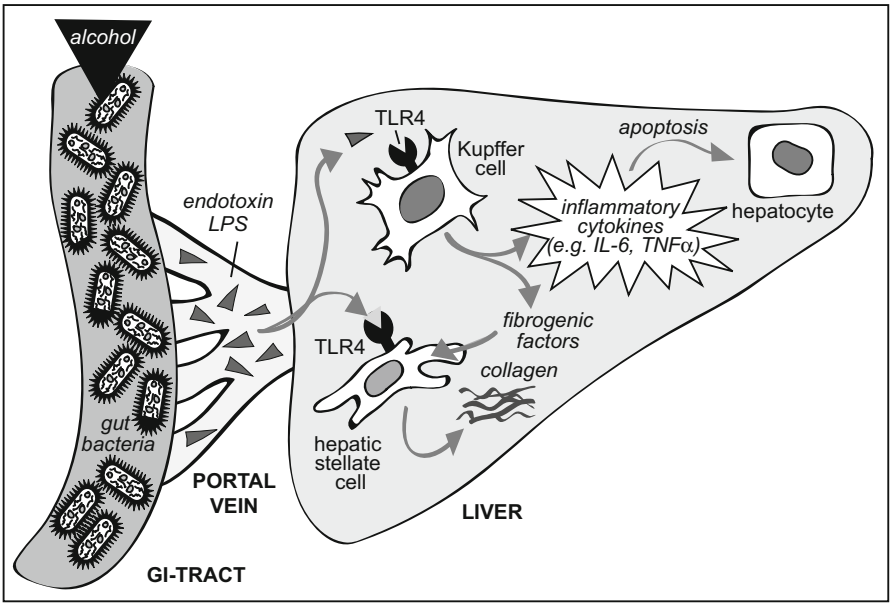

Fig. 9.5 Following the release of bacterial endotoxin into the portal circulation, overt liver injury follows activation of the innate immune response by Toll-like receptor-4 (TLR4)

Further evidence for a key role in alcoholic liver injury includes the long-standing finding that pretreating rodents with chemicals that selectively deplete Kupffer cells (e.g. gadolinium chloride) significantly blunts the severity of hepatotoxicity. Gadolinium pretreatments also reduce oxidative stress within the alcohol-intoxicated liver, indicating that an oxidative burst within activated immune cells contributes to ROS production in alcoholic liver (i.e. additive with free radical leakage during CYP2E1-catalysed ethanol metabolism). Activated Kupffer cells generate large quantities of superoxide radicals by activating the NAPDH oxidase system during efforts to kill and digest pathogens. Consistent with a role for this pro-oxidative system in ALD, knockout mice lacking a key regulatory component of the NADPH oxidase system, p47phox, are less vulnerable to alcoholic liver injury.

9.6.4.2 Acquired Immune Responses

Unlike the immediate response of the innate system, the acquired immune response is intended to facilitate memory of prior antigen exposures, thereby ensuring organisms are better placed to cope with subsequent exposures to antigens. In the analogy of a defence system, the adaptive response needs time to handcraft weapons for defensive use against specific antigens. The effects of alcohol upon acquired immune pathways are complex since they sometimes involve downregulation of

these responses, eliciting a protracted immunodeficient state in some individuals. On the other hand, patients with alcoholic hepatitis can display high levels of circulating antibodies against lipid peroxidation adducted proteins (i.e. reactions 4 and 5 in Fig. 9.3) as well as high numbers of T-cells in the liver, suggesting adaptive immune system responses do contribute to alcohol-induced liver injury.

In advanced alcoholic liver disease, clinicians have few options other than performing organ transplants. Intriguingly, transplanted livers obtained by former alcoholics often display heightened vulnerability to alcohol-induced toxicity, suggesting permanent activation of the memory function of the adaptive immune system in alcoholics which responds strongly if individuals resume their prior drinking habits. Despite these broad indications that the acquired immune system participates in liver injury, the precise immunological mechanisms await future clarification. The problem of alcoholic liver disease is discussed in more detail below.

9.7 Major Toxic Outcomes

Alcohol exerts broad and complex effects on human health. In addition to its addictive properties, alcohol is a foetal teratogen, a neurotoxicant and an inducer of hepatic and cardiovascular disease. Even the skeleton is affected during chronic alcohol intoxication, with alcoholics showing a heightened susceptibility to bone loss and fractures. We will briefly explore some select toxic outcomes that arguably take the greatest toll on the health of individuals and societies.

9.7.1 Alcoholic Liver Disease

Since the liver is the first internal organ to encounter ingested ethanol, this organ sustains significant damage in heavy drinkers. In many populations, liver disease accounts for over 50 % of alcohol-related fatalities. The prevalence of alcoholic liver disease (ALD) correlates closely with per capita rates of alcohol consumption and is typically highest in Eastern Europe, Southern Europe and the British Isles. Three or four distinct phases are typical of alcoholic liver injury:

9.7.1.1 Fatty Liver (‘Steatosis’)

Fatty liver is a highly prevalent condition which represents the ‘entry stage’ of ALD in that everyone who develops full-blown disease passes through this stage. Also known as *alcoholic steatosis,* fatty liver typically occurs in heavy drinkers who consume 60 or more grams of ethanol per day (i.e. 5–6 ‘standard drinks’ daily). Clinical symptoms include abdominal pain and discomfort secondary to liver enlargement. These effects are largely reversible if individuals reduce their alcohol

consumption upon diagnosis. The distinctive features of alcoholic steatosis include a build-up of fat within the liver due to the deposition of 'fatty droplets' in centrilobular hepatocytes. The droplets are initially small ('microvesicular' – some 1–2 μm in diameter) but over a period of days can coalesce to form 'macrovesicles' (~20 μm). In the earliest stages, fat droplets are digestible by lipases, but the larger forms resist this process and can thus persist for months even in the absence of continued drinking. Initially, the fat deposition is unaccompanied by inflammation or necrosis.

Fatty liver has long fascinated researchers who have explored many causal mechanisms. At the most fundamental level, fat accumulation reflects the abundant release of energy during alcohol metabolism: if the mitochondrial capability to metabolise acetyl-CoA formed during ethanol metabolism is exceeded, this metabolic 'building block' is diverted to the production of fatty acids via the ligation of acetate units. Other adipogenic changes accompanying acute and chronic alcohol intoxication include an induction of mitochondrial damage and subsequent lowering of hepatic levels of the lipoprotein ApoB100, a key member of low-density lipoprotein particles that assists lipid transport around the body. Other contributing mechanisms include changes in the levels of transcription factors such as SREBP-1 and PPARα that regulate the biosynthesis and degradation of fatty acids. In recent years, researchers have also studied the possibility that autophagy, the cellular process that delivers unwanted cell components to the lysozymes for degradation, is inhibited in the alcoholic liver, leading to fat accumulation via poorly understood mechanisms.

Recent work by researchers at the University of Colorado has provided fascinating new insights into alcoholic steatosis. Recall that carbonylation of liver cell proteins commonly accompanies hepatic alcohol metabolism (Fig. 9.3). Although such knowledge is long-standing, the identity of the liver proteins that sustain carbonylation during alcohol intoxication was largely unknown. To revisit this long-standing problem, Dennis Petersen and associates used a modern technique known as 'biotin tagging' to selectively extract carbonylated proteins from the livers of alcohol-treated mice. The mice received a 6-week dose-escalating regimen whereby at the conclusion of the dosing period they ingested one-third of their total calories via alcohol, similar to the intake profile of alcoholics. At the end of the exposure, mouse livers were analysed for carbonylated proteins using the biotin/hydrazide tagging method followed by enrichment and analysis via two-dimensional liquid chromatography–tandem mass spectrometry (2D LC-MS/MS).

Intriguingly, some 414 protein targets were carbonylated within mouse livers, including 50 targets that were also damaged in control livers, 84 that were only damaged in alcohol-treated livers and 280 that were carbonylated in both control and alcohol-treated animals. Although multiple adduction chemistries participated in protein damage, lipid-derived aldehydes such as 4-hydroxynonenal, 4-oxononenal and acrolein were prominent contributors (i.e. pathway 4 in Fig. 9.3). The study established that a subset of proteins sustained damage under conditions of low aldehyde production, then, as alcohol intake progressed, these targets were saturated and damage to other proteins occurred.

Fascinating biological insights emerged when a systems toxicology approach was used to identify functional linkages between targets for protein carbonylation in alcohol-intoxicated liver. The total set of 414 target proteins was investigated via the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes) which explores linkages between biochemical networks and signalling circuits. The KEGG analysis by Petersen and associates revealed that proteins involved in drug metabolism, oxidative phosphorylation, peroxisome proliferator-activated receptor (PPAR) signalling, amino acid metabolism and the TCA cycle were commonly damaged in the alcoholic liver.

The most fascinating outcome from the KEGG analysis was the high number of targets that participate in fatty acid metabolism (the fold enrichment was 12.20, indicative of a strongly affected pathway). Damage to this cluster of proteins was suggested to cause the global disruption of fatty acid metabolism in alcohol-intoxicated liver, triggering a deposition of hepatic lipid droplets that represents the initiating step in ALD. Since the heightened lipid content increases the pool of oxidation-prone unsaturated lipids within the liver, the ongoing formation of free radicals via CYP2E1-catalysed ethanol metabolism leads to further production of reactive aldehydes, eliciting cascading damage throughout the hepatocellular proteome. Rather than acting as uninvolved bystanders, these findings suggest lipid peroxidation products are key drivers of liver pathology during heavy alcohol exposure. Future confirmation of these conclusions in human ALD could point the way to new pharmacological strategies allowing early intervention in alcoholic liver injury.

9.7.1.2 Alcoholic Hepatitis

Without curtailed alcohol consumption, around one-third of heavy drinkers progress to a second, more serious phase of ALD. For poorly understood reasons, some drinkers remain at the steatotic stage despite continuing heavy alcohol intake. Progression to the secondary hepatitis stage is typically dose-dependent, requiring regular consumption of 80–160 g of alcohol by susceptible individuals.

With the onset of hepatitis, the liver begins shrinking and symptoms increasingly resemble those accompanying viral inflammation of the liver. The patient is likely to feel unwell and typically seeks medical assistance at this stage. Typical signs include a protracted rise in body temperature as well as generalised fatigue, nausea and anorexia. Vomiting and gastric pain may also occur. Jaundice is increasingly evident to onlookers, involving yellowing of the skin due to subcutaneous accumulation of bilirubin, a metabolic waste product that undergoes hepatic elimination in healthy individuals. A majority of these patients will progress to the more severe third stage of ALD, although a lucky minority (perhaps 10 % of patients) will recover completely upon cessation of drinking during the hepatitis stage.

At the histological level, although the fatty deposits often recede during the hepatitis phase, the overall liver histopathology grows much more abnormal and disordered. Scattered cellular foci show extensive ‘ballooning degeneration’ in which massively swollen or oncotic hepatocytes display overt cell injury. The shrinking of the liver during this phase reflects declining cell volume control due to falling cellular

ATP and an attendant rise in calcium levels. The cytoskeleton is increasingly disorganised, and cells display extensive vacuolation and karyolysis (DNA disintegration) while also releasing cell components. During the hepatitis phase, scattered cells also accumulate tangled aggregates of keratins, heat shock proteins and ubiquitins, giving rise to characteristic structures known as Mallory bodies. Although once viewed as neutral disease participants, Mallory bodies act as magnets to attract neutrophils and likely assist the onset of inflammatory hepatocellular injury. Rising accumulation of neutrophils ('neutrophilia') within liver parenchyma as ALD progresses is especially harmful to heavy drinkers since upon activation these cells release many cytotoxic mediators. Together with the flux of noxious substances released by Kupffer cells considered earlier (Sect. 9.6.4.1), the barrage of neutrophil-derived oxidants, proteases and proapoptotic cytokines such as TNFα triggers the widespread hepatocellular death that is conspicuous during the inflammatory phase of ALD.

9.7.1.3 Cirrhosis

With continued drinking, 10–20 % of alcoholic hepatitis sufferers progress to the end-stage cirrhotic phase every year. Upon diagnosis, the 5-year survival of cirrhosis patients is just 50 %. The defining pathological feature of liver cirrhosis is the irreversible tissue scarring which follows years of alcohol abuse. The release of inflammatory mediators during the hepatitis phase, and ongoing endotoxin-mediated activation of the Toll receptor pathway, promotes the activation of hepatic stellate cells, the fibroblast-like species that can manufacture prodigious amounts of collagen. The wide-scale deposition of fibrotic tissue gradually replaces hepatocytes with scar tissue, causing ongoing organ contraction until it represents a brown, shrunken mass of 1 kg or less. Desperate attempts of surviving hepatocytes to regenerate the organ produce scattered nodules of cell masses, conferring a 'hobnail' appearance upon the organ surface.

As cirrhosis progresses, the hepatic capacity to sustain core physiological functions recedes, ensuring the final months of victims lives are not pleasant. Failing clotting factor production promotes tissue bruising and bleeding disorders, while impaired immune function leaves patients vulnerable to opportunistic pathogens. Symptoms of multiple organ failure also rise in intensity due to declining renal and pulmonary function. Impaired liver perfusion also causes blood to 'back up' within the portal circulation, triggering hypertension. Worst of all, the mental wellbeing of patients declines due to rising blood levels of ammonia and other neurotoxic waste products. During their sad final days, liver cirrhosis patients exhibit mental confusion, cognitive disturbance and, eventually, coma and death.

9.7.1.4 Cancer

For some unfortunate individuals, the end-stage of ALD becomes even worse due to liver cancer. Perhaps 3–10 % of ALD patients progress to hepatocellular

carcinoma, leading some researchers to identify liver cancer as an additional, final stage of ALD. The risk of developing hepatocellular carcinoma is dose-dependent, and while it occurs most commonly in individuals who consume more than 80 g of alcohol per day for at least a decade, an elevated risk can be detected among moderate drinkers who consume just two standard drinks per day. In humans, studying the association of alcohol with liver cancer is complicated by strong synergy with underlying or pre-existing diseases (e.g. co-morbidities such as hepatitis B, hepatitis C) as well as obesity or smoking. The synergistic effect of viruses is especially strong, with HCV and HBV infection significantly increasing progression from cirrhosis to hepatocellular carcinoma.

In addition to liver cancer risks, the International Agency for Research on Cancer assigns the 'carcinogenic to humans' classification to alcoholic beverages due to clear epidemiological associations between alcohol consumption and various malignancies of the oral cavity, head and neck. The association with oral cancer is especially clear, with large population studies revealing that mortality due to these tumours rises by 0.26 per 100,000 people for every litre of spirits consumed annually. Alcohol use is also associated with tumours of the oesophagus, colorectum and female breast.

Recognition of the carcinogenic potency of alcohol was long delayed by difficulties in reproducing tumour responses in rodents. At the high doses of alcohol required, rats and mice typically become sedated and anorexic, making long-term testing and detection of slow-onset tumours problematic. In the absence of clear animal data, the toxicology community remained uncertain concerning the cancer potential of alcohol.

The carcinogenic potential of alcohol was finally placed on a firmer footing following completion of studies in which rodents were exposed to acetaldehyde for extended periods. Some studies of this kind involved acetaldehyde administration to rodents via drinking water, while others exposed animals to acetaldehyde vapours. Following study completion, animals received full pathological examination for the scoring of tumours. Intriguingly, high doses of acetaldehyde that did not impair the general health of the animals (e.g. caused no changes in body weight, food or water consumption, behaviour, lifespan) significantly increased tumour incidence in both male and female animals, inducing nasal and oral cavity cancers resembling those seen in heavy drinkers. During animal testing, observation of tumours in the same tissues for which a given chemical is suspected to cause cancer in humans significantly strengthens disease associations.

One remaining question from the epidemiological and animal studies concerned the mechanisms underlying tumour development following alcohol exposure – how precisely does alcohol induce carcinogenesis? In early work, the use of immunological techniques to analyse biopsy samples collected from precancerous and carcinoma lesions within the oral cavity of heavy drinkers confirmed the strong presence of protein adducts formed by acetaldehyde and the lipid peroxidation products malondialdehyde and 4-hydroxynonenal (Fig. 9.3). This suggested these reactive aldehydes might progress oral cavity tissue towards a cancerous state by damaging proteins that normally suppress tumour development (e.g. DNA repair

enzymes or anti-oncogenic transcription factors). Such findings also raised the prospect of concurrent DNA damage, and eventually the main DNA adduct formed by alcohol, N^2-ethyl-dGua, was detected within tumour prone tissues from heavy drinkers such as the oral cavity and upper GI-tract. These findings firmly positioned alcohol within the category of chemicals that trigger carcinogenesis via a genotoxic mechanism (see Sect. 8.5.1). While research is still underway to clarify the extent to which epigenetic mechanisms contribute to alcohol carcinogenicity, a role for genetic damage seems secure.

9.7.2 Peripheral Neuropathy

Peripheral neuropathy occurs in some 50–60 % of alcoholics and involves persistent sensory, motor and autonomic dysfunction. The most problematic symptoms involve the loss of motor function in the lower extremities, although as the disease progresses, the upper limbs are also affected. A tendency to sustain repeated falls and associated head trauma ensures this condition greatly complicates the clinical management of alcoholism. Impaired ability to sense painful stimuli via skin receptors also accompanies this syndrome. Patients also experience excruciating pain in their lower limbs, an effect that is likely due to the actions of alcohol on neuronal protein kinase C and protein kinase A signalling pathways. Upon histopathological analysis of alcoholic neuropathy patients, clear signs of distal axonal degeneration or dying off of the longest fibres in the legs is common, as are abnormalities to the insulating myelin sheath including segmental demyelination.

Whether alcoholic neuropathy is a true toxicological phenomenon has attracted much debate. A long-standing consensus attributed neuropathy to nutritional deficiencies resulting from the poor dietary habits of alcoholics. Since clinical signs somewhat resemble the ‘beriberi neuropathy’ seen in thiamine-deficient patients, alcoholic neurotoxicity was frequently attributed to inadequate thiamine intake. Indeed, distinguishing these syndromes proved challenging since heavy alcohol ingestion actually induces thiamine deficiency due to impaired oral absorption of thiamine, lowering of hepatic storage of thiamine and suppression of kinase-dependent activation of thiamine. Although these factors led many to believe alcoholic neurotoxicity was due to nutritional deficiencies, the ineffectiveness of thiamine supplementation against neuropathy cast doubts on the dietary deficiency hypothesis. With time, improvements in clinical study design yielded diagnostic criteria that drew clear distinctions between alcoholic neuropathy and thiamine deficiency.

The development of suitable animal models also confirmed the neurotoxicity of alcohol and its metabolites. Despite initial failed attempts, rodent models involving chronic exposure to high-dose alcohol eventually demonstrated dose-dependent axonal damage. Animal models also reproduce the myelin abnormalities that are typical of alcoholic neuropathy. Acetaldehyde again appears a major culprit since it elicits concentration-dependent cell death in neuronal cultures via a mechanism

likely involving protein adduction. In an analogous manner to the formation of Mallory bodies in alcoholic liver, changes in the neuronal distribution of key cytoskeletal components such as neurofilament- and microtubule-associated proteins suggest the cytoskeleton sustains acetaldehyde adduction. To date, however, limited studies of protein adduction during alcohol neurotoxicity in rodents have mostly focussed upon the brain rather than peripheral neurons. A role for acetaldehyde in human neuropathy is supported by findings suggesting alcoholics of Asian ethnicity who detoxicate acetaldehyde poorly due to the ALDH2*2 polymorphism are vulnerable to neurotoxicity. Collectively, these studies indicate a likely role for ethanol metabolites in alcoholic neuropathy, although differences in regional dietary practices, drinking behaviour and genetic endowment ensure this syndrome is harder to diagnose than other alcohol-related toxicities.

9.7.3 Cardiac Myopathy

The complexities surrounding association of pathological outcomes with alcohol consumption are especially obvious within the cardiovascular system. Under conditions of light to moderate consumption, alcohol may reduce the risk of coronary heart disease (CHD) and ischaemic stroke. On the other hand, during heavy chronic drinking or binge drinking, alcohol is clearly toxic to the heart and cardiovascular system. In recent decades, a consensus has emerged that alcoholic cardiomyopathy is a wholly alcohol-attributable condition plaguing between 20 % and 40 % of heavy drinkers. Although less well studied than other alcohol-related pathologies, recognition of this condition is long-standing, traceable to descriptions published by two nineteenth-century German physicians (e.g. ‘Tubingen Wine Heart’ [1877] and ‘Munich Beer Heart’ [1884]).

As with other alcohol-related disorders, wide interindividual variability accompanies alcoholic cardiomyopathy. In susceptible individuals, left ventricular dysfunction can be an early sign of alcohol intoxication in drinkers who consume 90 g alcohol per day for at least 5 years, with heart damage often manifesting at a relatively young age during the fifth decade of life. A history of heavy alcohol consumption is important to diagnosis, and physicians must exclude other cardiomyopathic risk factors such as exposure to cardiotoxic drugs (e.g. cocaine, doxorubicin).

Although alcohol-induced cardiac changes are initially asymptomatic, if drinking continues unabated, the condition progresses to the familiar signs and symptoms of congestive heart failure. Typical features include dilation or enlargement of all four chambers of the heart, diminished cardiac output and normal or decreased left ventricular wall thickness. Co-morbidities such as cirrhosis significantly worsen the prognosis for alcoholic cardiomyopathy sufferers, particularly if heavy drinking continues. Such patients are especially vulnerable to supraventricular arrhythmias and sudden cardiac death during bouts of increased drinking – the so-called holiday heart syndrome known to cardiologists.

Progress in understanding the mechanistic basis for alcoholic cardiomyopathy is hampered by difficulties in obtaining relevant biopsy material. Typically, histological analysis of heart tissue from alcohol-exposed rodents and human autopsy samples reveal a conspicuous loss of myocytes within the myocardium. Evidence for the involvement of apoptosis and necrosis is suggested by experimental studies although the relative importance of each in humans remains unclear. Subcellular changes in the sarcoplasmic reticulum and mitochondria are conspicuous and together with changes in myofilament function secondary to altered calcium signalling appear to underlie the loss of myocardial contractility.

Limited studies of protein adduction within heart tissue from alcohol-intoxicated rats suggest acetaldehyde plays a key role in the pathogenesis of cardiac injury. Stronger evidence linking acetaldehyde to alcoholic cardiomyopathy emerged from transgenic mouse studies in which ethanol metabolism is altered via either overexpression of ADH or ALDH2. Typically, pronounced myocardial damage occurs in ALDH2-deficient transgenic mice in which acetaldehyde detoxication is impaired, while ADH-overexpressing animals are similarly more vulnerable to cardiac myopathy. While the extent to which the various pathways to proteome modification highlighted in Fig. 9.3 mediate myocardial damage during alcohol intoxication are unknown, ongoing improvements in proteomic technologies will likely reveal their significance in the future.

9.7.4 Foetal Alcohol Syndrome

Of all alcohol-attributable human diseases, none have greater potential to cause long-term social or individual harm than toxicity towards the unborn child. The highly complex patterns of neural development occurring during the foetal period leave the developing brain particularly vulnerable to neurotoxic substances such as alcohol. Fascinating glimpses of long-standing awareness that alcohol use during pregnancy can harm the unborn child are found in many cultures. Some scholars have detected such awareness within the divine injunction offered to the mother of Samson, the Hebrew warrior who resisted Philistine tyranny in ancient Israel: ‘You will become pregnant and have a son. Now then, drink no wine or other fermented drink and do not eat anything unclean’ (Judges 13:7). Another famous quotation from the Greek philosopher Aristotle (384 BC–322 BC) is less ambiguous: ‘Foolish, drunken and hare-brained women most often bring forth children like unto themselves: morose and languid’.

Much later, during the eighteenth-century Gin Craze, the College of Physicians warned the British House of Commons that alcohol is ‘too often the cause of weak and feeble, distempered children’. Early clinical descriptions of the impact of alcohol on the unborn emerged in 1899 when William Sullivan published his studies of the offspring of alcoholic incarcerated women. Incredibly, of 600 infants under his observation, 56 % were stillborn or died in the first 2 years of life. In the grim opinion of Dr Sullivan, those children who survived into adulthood displayed persistent

signs of alcohol exposure and *were not productive members of society.* Despite this study, the foetal toxicity of alcohol only received sustained attention in the English-speaking world after Smith and Jones from the University of Washington in Seattle published their 1973 description of 'foetal alcohol syndrome' (FAS) in *The Lancet*. By carefully describing the signs and symptoms in eight affected infants drawn from three ethnicities, the authors concluded, 'The data are sufficient to establish that maternal alcoholism can cause serious aberrant fetal development'. The authors also credited an earlier European study published in French by Lemoine in1968. Although the name FAS is still applied to the full-blown syndrome, the umbrella term foetal alcohol spectrum disorders is used in modern literature to acknowledge the complexity accompanying diagnosis of alcohol-induced developmental toxicity in humans.

From the outset, researchers identified three main toxic outcomes in FAS victims: craniofacial abnormalities, growth reduction and neurodevelopmental deficits. While this basic categorisation remains intact, subsequent research added extra layers of subtlety to the developmental toxicity of alcohol such that FAS now includes malformations of various internal organs as well as foetal death. Moreover, while the classic triad of alcohol-induced endpoints is clear within the offspring of heavy drinkers, detecting less severe cases remains challenging for neonatal paediatricians. Misdiagnosis of FAS in children with unrelated genetic conditions sadly appears quite common.

A further problem complicating FAS diagnosis is the likely influence of indirect maternal factors such as poor diet or vascular disease. To improve FAS diagnosis, increasing use is made of biomarkers to confirm maternal alcohol consumption. Recent findings suggest the levels of metabolites of nonoxidative ethanol metabolism such as ethyl-glucuronide and FAEE (Fig. 9.2) within the first stool sample collected from a newborn infant may identify children requiring heightened care. Meconium samples represent a useful catalogue of foetal xenobiotic exposure since both parent compounds and their metabolites deposit in the GI-tract via bile secretion or foetal swallowing of amniotic fluid. Meconium formation commences during the 12th week of gestation and continues until birth. Xenobiotics detected within meconium include illicit drugs, nicotine metabolites, prescription medications, food additives and environmental toxicants. In the case of ethyl-glucuronide, a level above 2 mmol/g of neonatal stool mass indicates likely maternal drinking during pregnancy.

9.7.4.1 Facial Dysmorphology

A cluster of craniofacial changes represents the most obvious sign of in utero alcohol exposure (Fig. 9.6). Epidemiological studies suggest infants are vulnerable to craniofacial abnormalities during the second half of the first trimester. The pattern of abnormalities typically includes a diminished head circumference, alterations to the appearance of the eye including narrowed openings and an extra fold of skin at the eye fissure, a flat middle face and a lowered nasal bridge. Changes within the lip

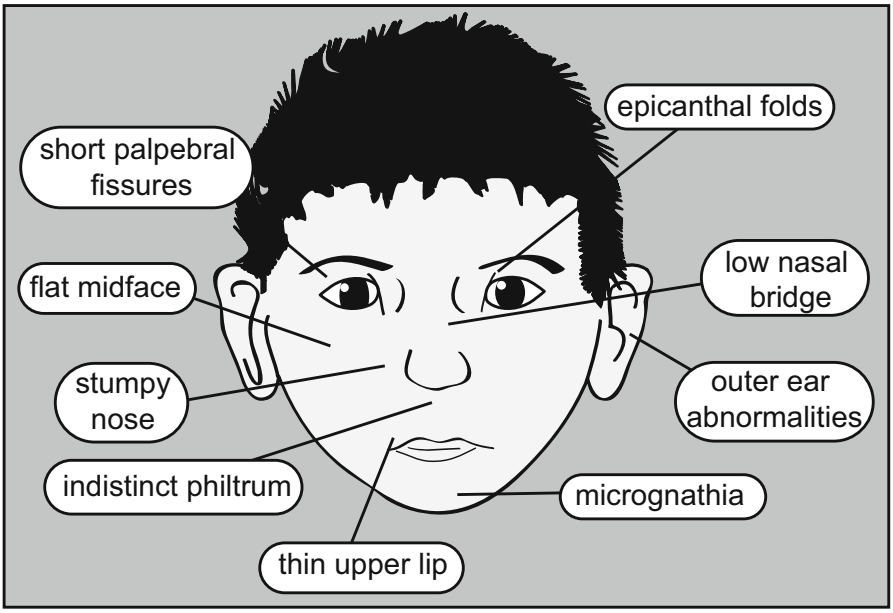

Fig. 9.6 Typical craniofacial abnormalities seen in the FAS-affected offspring of alcoholic mothers

region include missing vertical grooves between the nose and mouth (philtrum) together with a thin upper lip. The expected demarcation between the lip and adjacent normal skin is also less distinct (i.e. vermillion border). The jaw is also smaller and exhibits less prominent features. Finally, the morphology of the outer ear can display subtle alterations, the most conspicuous of which is the 'railroad track' appearance of the outmost rim of tissue. The severity of these changes is usually dose-related.

Experiments in pregnant animals allow detailed study of alcohol-induced craniofacial abnormalities. In susceptible mouse strains, the administration of high-dose alcohol to pregnant animals within a window comprising gestational days 7–14 stunts the growth of the snout, palate, mandible, ears and eye. While the mechanisms await full clarification, a role for acetaldehyde in disrupting normal developmental patterns of gene expression within the palate and associated craniofacial tissues is likely.

9.7.4.2 Growth Retardation

A further sign of prenatal alcohol intoxication is the birth of small, growth-retarded babies that are consistently undersized in terms of birth weight, body length and head circumference. During the postnatal period, alcohol-affected infants display a persistent 'failure to thrive' indicative of a delayed developmental programme.

Given that significant tissue growth and histogenesis occurs during the third trimester of pregnancy, alcohol-induced growth retardation likely reflects a general suppression of foetal cell growth and proliferation. Nevertheless, recent findings that growth retardation accompanies consumption of alcohol in any stage of pregnancy suggest these long-standing assumptions may require revision.

9.7.4.3 Cognitive Deficits

Changes in facial morphology and body weight are physically observable effects of alcohol on early human development, yet by far the most devastating damage occurs silently beneath the human skull within the brain. Such is the sensitivity of the developing brain to alcohol that subtle changes in brain function occur in the absence of craniofacial alterations or significant growth retardation. In utero alcohol exposure likely represents the leading contributor to avoidable neurocognitive impairment in the modern world.

The complex mechanisms underlying the foetal neurotoxicity of alcohol may vary according to the anatomical site within the brain but likely include glutamate excitotoxicity, formation of free radicals and lipid peroxidation products and neuroinflammatory damage resulting from TLR4-mediated activation of the innate immune system. Within the developing hippocampus, alcohol-related changes include disrupted expression and function of glutamate receptors, NMDA receptors, L-type Ca^{2+} channels, $GABA_A$ receptors and G-protein coupled inwardly rectifying potassium channels. Prenatal alcohol exposure also alters neuronal signalling pathways, including phosphorylation cascades regulated by protein kinase A and protein kinase C. Although animal findings suggest acetaldehyde involvement in triggering these changes, its role in FAS neurological defects in humans awaits confirmation.

The children of alcoholic mothers exhibit broad neurological effects that include a reduced IQ (average ~ 70) and brain processing deficits that include lower perceptional organisation, slowed processing speed and impaired working memory. These brain changes result in such adverse behavioural outcomes as hyperactivity, poor impulse control, low problem-solving skills, impaired abstract reasoning, lack of trial-and-error learning and educational underachievement. Alcohol neurotoxicity also includes various adverse socialisation outcomes in FAS children such as impaired maternal bonding, diminished communication skills, lowered levels of motor skills and coordination, difficulty linking actions to consequences, inability to 'read' environments and adapt accordingly, higher risks of psychiatric illness and a tendency to display inappropriate sexual behaviours. These broad cognitive and psychological abnormalities may persist lifelong in affected individuals and confer heightened vulnerability to anxiety, depression and other stress-related conditions. A proclivity towards alcohol dependence is also transmitted to the offspring of FAS-affected individuals.

Many subtle neurodevelopmental effects of prenatal alcohol exposure were identified after brain imaging technology was used to assess affected infants. Compared to healthy controls, alcohol-induced changes are obvious within cortical grey matter

regions that are crucial for the development of intelligence. Correlations between facial abnormalities and defects in cortical brain areas suggest alcohol is most damaging during the early stages of pregnancy when the baby's face undergoes formation. The neuroanatomical abnormalities persist through childhood and the teenage years into early adulthood, revealing significant divergence from normal patterns of fluctuations in cortical white and grey matter volumes. These observations suggest prenatal alcohol exposure disrupts fundamental brain processes that mediate brain plasticity and the formation of neural trajectories as well as alterations in synaptic pruning that accompany normal brain maturation.

Modern discoveries concerning the broad and persistent effects of alcohol upon the structure and function of the developing brain, coupled to the wide range of deleterious behavioural, social and neurological outcomes described in affected children, underpin the prevailing view in many jurisdictions that any amount of alcohol consumption should be avoided during pregnancy. Until the unlikely event that new findings mitigate this conclusion, for the near future, the pathway of caution seems highly judicious. Recent discoveries concerning the efficacy of behavioural interventions as well as efforts to improve the environmental conditions in which alcohol-affected infants are reared provide grounds for optimism that the devastating effect of alcohol on the young human brain can be at least partly offset.

9.8 Conclusion

The use of contemporary research tools and the paradigms of modern toxicology to study the multifaceted health problems accompanying heavy alcohol use have supplied a valuable body of scientific knowledge concerning the effects these beverages exert upon human health. As a popular mood-altering substance with pleasurable and reinforcing properties, it is unlikely that growing knowledge of the harm alcohol inflicts on human health will elicit substantial changes in human drinking behaviours. Nonetheless, it is likely ongoing research will improve our ability to identify individuals who, due to their genetic background and drinking habits, are most vulnerable to alcohol-induced toxicity. The use of this knowledge to develop pharmacological, dietary and behavioural strategies for suppressing the burden of alcohol-attributable disease may reinforce the value of toxicological investigations in this crucial area of human health.

Going Further

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Chapter 10
Everyday Toxicology II: Tobacco

Abstract The chemical complexity of tobacco combustion ensures smokers daily inhale a toxic cocktail comprising thousands of xenobiotics. Unsurprisingly, many health disorders accompany this habit including various cancers plus noncancer conditions such as emphysema. The smoke constituents causing lung injury have received much attention, with special interest focussed on carcinogens that drive the induction and progression of lung cancer. Major carcinogens within tobacco smoke include the polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines and volatile organics such as 1,3-butadiene. Due to their strong carcinogenic potency, the tobacco-specific nitrosamines NNN and NNK are of particular concern. Both form via nitrosation of nicotine, the neurostimulatory Nicotiana constituent that confers the highly addictive properties upon cigarette smoke. As with other tobacco smoke carcinogens, NNN and NNK undergo CYP-catalysed bioactivation to DNA-damaging metabolites. DNA adducts formed by these and other noxious metabolites drive the accumulation of mutations in growth regulatory genes within the smokers' lung.

Keywords Aromatic amines • Benzo[a]pyrene • 1,3-Butadiene • Chronic obstructive pulmonary disease • Free radicals • Irritants • Lung cancer • Metals • Microarrays • Tobacco production • Tobacco combustion • Tar

10.1 Introduction

In October of 1492, members of Christopher Columbus's crew had a momentous encounter with some gift-bearing South American inhabitants; 'The natives', Columbus wrote in his journal, 'brought fruit, wooden spears, and certain dried leaves which gave off a distinct fragrance'. Although initially discarding the leaves, astute crew members noted that the indigenous society valued tobacco highly and therefore took an active interest in its mysterious properties. Ramon Pane, a member of Columbus's second voyage, is typically credited with introducing *Nicotiana* to Europe upon returning to Spain. His journals include lengthy descriptions of the

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tobacco plant and its various uses as snuff and in pipes for smoking. These innocent observations initiated a fateful association between European cultures and tobacco that would subsequently claim millions of lives.

With time, sea-faring Europeans carried the plant to Africa, Japan and Asia to establish tobacco plantations in more conducive climatic conditions, although the industry initially grew slowly since tobacco enjoyed little usage beyond maritime workers. With time, due to its alleged medicinal powers for the treatment of toothache, worms and other ailments, tobacco steadily gained popularity. Its production became central to colonial economies within the American South such as Virginia and the Carolinas. New uses also steadily emerged: pipe smoking became popular in the seventeenth century, followed by the eighteenth-century Age of Snuff and then the nineteenth-century Cigar Era.

During the Crimean War, British troops adopted the Turkish practice of smoking paper-wrapped tobacco, a habit that grew explosively after James Bonsack invented the cigarette-making machine in 1881. Together with James Duke, Bonsack built a factory that made ten million cigarettes in its first year. Five years later, the American Tobacco Company could produce one billion cigarettes per year, selling their products in small wooden boxes that also contained collectable baseball cards. World War 1 and 2 both saw significant expansion in cigarette smoking due to its popularity among military personnel. Women also began smoking in large numbers during this era. The growing popularity of cigarettes displaced the use of cigars, pipes, snuff and chewing tobacco (Fig. 10.1).

Governments have long maintained an uneasy relationship with tobacco, having quickly seen the potential to raise revenue by taxing the highly addictive substance. 'I will certainly forbid it at once', Napoleon III asserted, 'as soon as you can name a virtue that brings in as much revenue'. In modern times, the collection of excises and tariffs from tobacco sales coexists in uneasy tension with government-funded smoking abatement efforts. Such tensions fuel the smuggling of untaxed or illegal tobacco in many countries. In general, however, evidence from around the world suggests that increased taxation does deter some young consumers from adopting the smoking habit. Reduction in advertising and bans upon the sponsoring of sporting events by cigarette manufacturers has also diminished tobacco use in many countries, as has the addition of graphic images and health warnings to cigarette packages. Together with restrictions upon public smoking due to growing evidence for risks accompanying exposure to second-hand cigarette smoke, tobacco use has fallen by 20–40 % in many nations over recent decades. Nevertheless, despite such progress, smoking remains the leading avoidable cause of death in many countries.

10.2 The Burden of Tobacco-Related Disease

Tobacco smoking takes an enormous toll on individual and societal health (Table 10.1). Currently, close to 15,000 tobacco-related deaths occur daily around the world, a statistic that is made grimmer by the knowledge that an estimated 100,000 people join the ranks of the approximately 1.3 billion current smokers each day. In the USA alone, some 400,000 current and former smokers die annually from

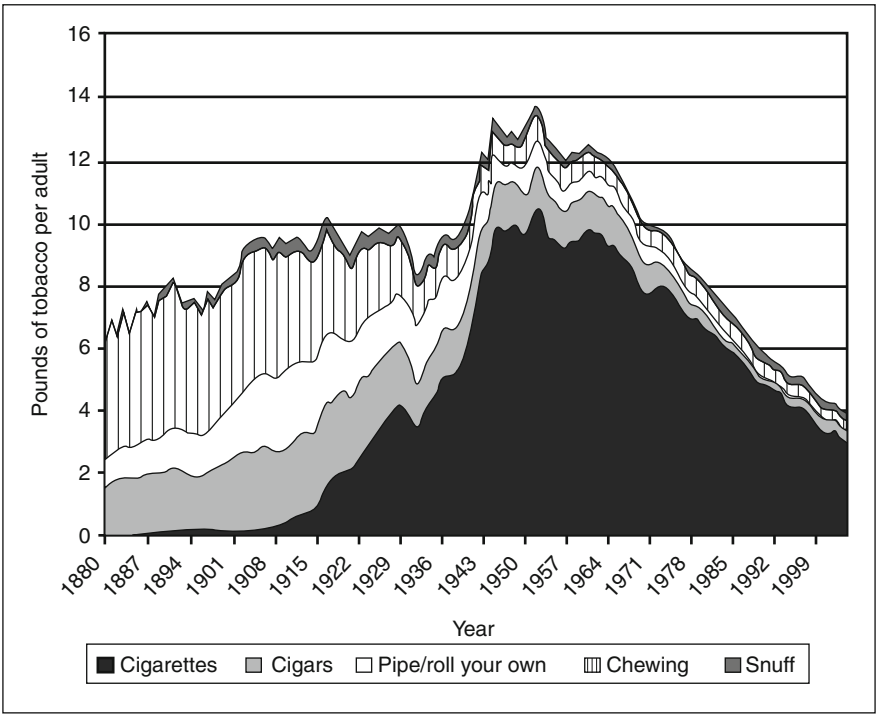

Fig. 10.1 Changes in the US per capita consumption of different tobacco products due to the availability of mass produced cigarettes, 1880–2004 (Reprinted from *Am J Prev Med*.;33(6 Suppl):S318–26, G.A. Giovino, The Tobacco Epidemic in the United States, 2007, with permission from Elsevier)

Table 10.1 Diseases and conditions that afflict tobacco smokers (Reprinted from *Am J Prev Med*.;33(6 Suppl):S318–26, G.A. Giovino, The tobacco epidemic in the United States Copyright (2007), with permission from Elsevier)

Disease	Health conditions and effects
Malignant neoplasms	Cancers of the lung, larynx, mouth, oesophagus, urinary bladder, pancreas, kidney, uterus, stomach and acute myeloid leukaemia
Cardiovascular diseases	Coronary heart disease, cerebrovascular disease, atherosclerosis and aortic aneurysm
Respiratory diseases in adults	Chronic obstructive pulmonary disease, pneumonia, early age-dependent decline in lung function, major symptoms of respiratory dysfunction (e.g. coughing, phlegm, wheezing, dyspnoea), poor asthma control
Respiratory diseases in young people	Impaired lung growth and asthma-related symptoms (e.g. wheezing) in childhood and adolescence, early onset of declining lung function in late adolescence and early adulthood
Reproductive and perinatal conditions	Sudden infant death syndrome, reduced fertility in women, foetal growth restriction, low birth weight, premature rupture of the membranes, placental abnormalities, preterm delivery and shortened gestation, respiratory distress syndrome
Miscellaneous	Cataracts, hip fractures, low bone density, peptic ulcer disease in *H. pylori*-positive patients, diminished general health (i.e. increased absenteeism from work, increased use of medical services), poor surgical outcomes due to impaired wound healing and respiratory complications

smoking-related disease, while nearly 40,000 nonsmoking Americans also die annually via involuntary exposure to second-hand tobacco smoke. Although ischemic cardiovascular disease and lung cancer account for most smoking-related deaths, at least 20 fatal diseases are causally linked to the habit. Serious chronic disease caused by smoking also currently diminishes the lives of some nine million Americans. According to some estimates, around 50 % of all smokers, especially those who adopted the habit as teenagers, will die from tobacco-related causes. Of these, around 50 % will die during middle age, robbing them of 20–25 years of the expected human lifespan.

For all smoking-related deaths, approximately 39 % are due to cardiovascular disease of which ischemic heart disease is the main contributor. Another 37 % are due to malignancies, most commonly tumours of the trachea, bronchus or lung. Twenty-three percent of smoking-attributable deaths involve noncancer respiratory diseases such as chronic airway obstruction, bronchitis and emphysema. In terms of the overall burden of disease in US adults, around 18 % of deaths due to cardiovascular disease, 30 % of malignant cancers and 79 % of chronic respiratory diseases are attributable to smoking. Due to this enormous toll upon the health of modern societies, developing a mechanistic understanding of the adverse health effects of tobacco is a major concern within modern toxicology.

10.3 Tobacco Production

The availability of diverse strains of tobacco that grow best within distinct agricultural conditions ensures there is no such thing as a simple, standard cigarette. The most common form of tobacco, *Virginia* (also known as 'bright' or 'flue-cured' tobacco), is high in sugar content and low in oils. Since the leaves on *Virginia* mature from the bottom-up, the lowest leaves are picked first. As they can be machine-harvested, *Virginia* leaves are popular with growers and are used extensively in tobacco blends for cigarettes, accounting for about 40 % of global tobacco production. China, the USA and Brazil are major producers of *Virginia* tobacco.

Oriental tobacco contains less nicotine and accounts for around 16 % of global production. Exhibiting a strong, distinctive aroma upon combustion, *Oriental* tobacco is popular in Russia, Turkey and Mediterranean countries such as Italy and Greece. *Burley* tobacco contains higher nicotine concentrations and a high sugar content and conveys a nutty flavour. Grown in the USA, Italy, Korea and South America, *Burley* tobacco is popular in many cigarette blends. During harvesting, the entire stalk is cut by hand before the plant is air-cured under natural conditions over an 8-week period. *Maryland* tobacco has a low nicotine content, is light and fluffy with a mild flavour and is especially popular in Swiss cigarettes.

Although most attention focusses on toxicants that form during tobacco combustion, many toxic chemicals can contaminate cigarettes due to the use of pesticides, growth regulators, herbicides and fungicides during tobacco cultivation. Various

organochlorine compounds such as endosulfan and chloropicrin are of special concern as tobacco contaminants since these agents notoriously resist spontaneous degradation. The filter attached to most cigarettes rarely traps more than half of the pesticides released from a burning cigarette.

After harvesting, tobacco leaves are typically *cured*, a 3-step process that involves yellowing, leaf dehydration and stem drying. Several approaches are available, including air curing, flue curing, fire curing or bulk curing, with the best approach determined by the type of tobacco under consideration. Compared to slow, traditional methods, the curing of *Virginia* leaves is nowadays achieved within a week or so within a 'bulk barn' using fan-forced heating and accelerated airflow. The first step in the curing process destroys chlorophyll within the leaves, giving bright tobacco its characteristic yellow-brown colour. The subsequent curing steps remove water from the plant and convert starches into sugar. Cured tobacco is then compressed into bales and trucked to receiving stations where it is graded and sold to buyers from cigarette manufacturers.

Tobacco leaves are then processed at a stemmery where rehydration is performed to facilitate stalk removal. The leaves are next compressed into boxes or vats known as 'hogsheads' which are stored for up to 2 years in special warehouses to allow ageing and mellowing of the leaves. The next step in tobacco manufacture involves treating cured leaves with a 'sauce' that contains various additives to improve the aroma and flavour of smoke released from burning cigarettes. Common additives include menthol and humectants such as glycerol in an effort to offset the irritant or 'dry' properties of tobacco smoke. Other additives exert pharmacological effects upon lung airways to maximise nicotine delivery and uptake (e.g. theobromine in cocoa and glycyrrhizin in licorice act as bronchodilatory agents).

The addition of sugar-containing additives such as corn syrup, molasses, honey or fruit juice is also common. Such innocuous-sounding practices are controversial since sugars generate high levels of toxic carbonyls such as acrolein, one of the most abundant and damaging constituents of tobacco smoke. In a recent study, the yield of acrolein from a burning cigarette increased from 118 to 215 μg when tobacco was treated with 16 % sucrose during the curing step. The humectant glycerol also makes a minor contribution to acrolein yields.

As one might expect, the precise additives used by cigarette manufacturers are closely guarded commercial secrets since they help achieve brand recognition or maximise product appeal within niche consumer groups. Yet, the fact that additives influence the toxicity of tobacco smoke ensures commercial factors must not override the obligation manufacturer's face to make their cigarettes as safe as possible.

The modern cigarette is a surprisingly complex creation, with numerous synthetic chemicals used during its manufacture. For example, the thin, permeable paper that encases chopped tobacco contains such additives as inks, whitening compounds and binding agents. Adhesives such as polyvinyl acetate secure the longitudinal seam of a cigarette while also attaching it to the 'tipping paper' which encases the cellulose acetate filter. Some manufacturers also add charcoal to the filter to increase absorption of organic substances.

10.4 Tobacco Combustion

Although lighting a cigarette seems a trivial task performed billions of times daily around the world, each ignition event sets in train a complex series of chemical reactions that generate a rich cocktail of toxicants. In essence, tobacco smoke is a complex aerosol comprising condensed liquid droplets and the particulate fraction ('tar') suspended in a mixture of volatile and semi-volatile compounds and combustion gases (the gas fraction). Incriminating individual smoke-borne toxicants in specific smoking-related diseases is difficult since the chemical composition of smoke differs according to whether the cigarette is simply smouldering (i.e. releasing sidestream smoke) or is actively burning due to airflow through the cigarette (i.e. mainstream smoke) (Fig. 10.2). As a smoker inhales, the cigarette coal rises from a smouldering temperature of approx. 600 C to in excess of 900°C. Due to less efficient combustion at the cooler temperature, levels of toxic constituents in sidestream smoke can exceed those in mainstream smoke by an order of magnitude or more. In one study, concentrations of the carcinogen 4-aminobiphenyl were 32-fold higher in sidestream smoke than in mainstream smoke. Such factors ensure sidestream smoke shows three to four times more activity than mainstream smoke during toxicity bioassays such as the Ames

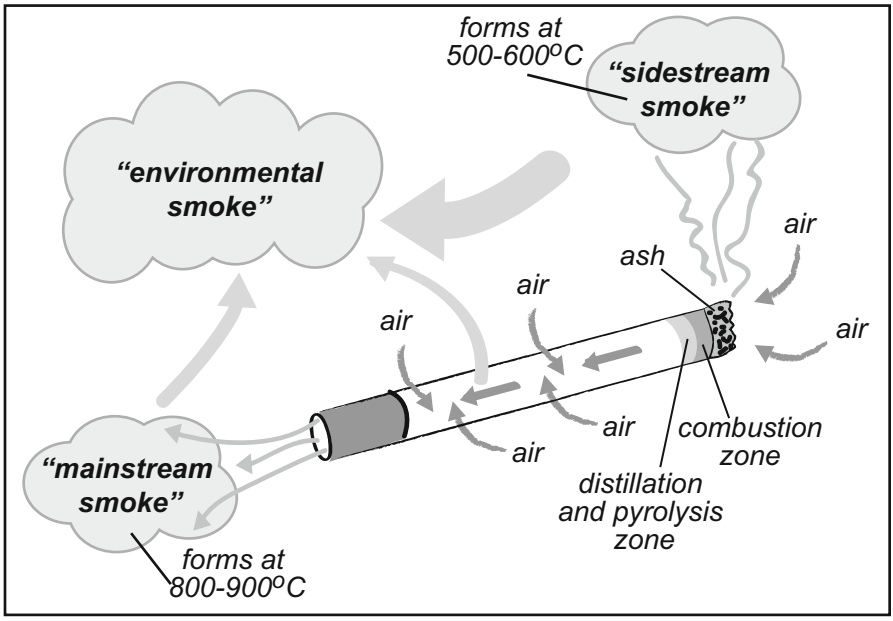

Fig. 10.2 Environmental smoke accumulating in an enclosed space during tobacco combustion comprises a mixture of exhaled mainstream smoke (minor component) and sidestream smoke (major component) together with a contribution by volatile substances that diffuse through the cigarette paper during puffs

Salmonella test for mutagenicity. These considerations have underpinned legislative efforts in recent decades to limit the exposure of nonsmokers to environmental tobacco smoke.

Another way of thinking about tobacco smoke involves recognition of first-, second- and third-hand smoke. First-hand smoke is analogous to mainstream smoke, representing the smoke inhaled through a burning cigarette by an individual smoker. Second-hand smoke is more complex and essentially equals the environmental smoke comprising a mixture of sidestream smoke as well as smoke constituents that diffuse through the paper sheath of the cigarette plus smoke that is exhaled from the lungs of a smoker. Third-hand smoke represents the residue of tobacco combustion products deposited on walls, furniture and furnishings in a room during the smoking of a cigarette. These deposited materials convey the characteristic stale odour of a smoker's automobile or domestic residence. Physical contact with these contaminated surfaces can expose smokers and nonsmokers alike to tobacco combustion products via dermal exposure.

Another factor complicating assessment of tobacco smoke is that O_2 is rapidly consumed during the combustion process, forming a hot, oxygen-poor zone adjacent to the coal (Fig. 10.2). The thermal decomposition of tobacco within this *pyrolysis* or *distillation zone* generates distinct reaction products including volatile and semi-volatile monocyclic aromatics formed via pyrolysis of amino acids, fatty acids and sugars. Most of the 5,000 or more chemicals within tobacco smoke and much of the nicotine that is released into smoke emanate from this zone.

Since a burning cigarette exhibits a complex and dynamic pattern of combustion, the environmental tobacco smoke that accumulates within an enclosed space is far from static: due to ageing and deposition of particles and nicotine on surfaces, the composition of smoke within a poorly ventilated room exhibits dynamic changes over a period of minutes to hours.

10.5 Major Toxic Outcomes

As smokers daily inhale thousands of toxicants into their lungs, an organ that presents few anatomical barriers to xenobiotic absorption, it is unsurprising that many adverse health outcomes accompany this habit. Accordingly, public awareness of the harmful effects of tobacco smoke is long-standing – as seen in early slang references to cigarettes as 'coffin nails'. Yet, it is only in recent decades that the extent of the health risks accompanying tobacco smoking – and of the biological and chemical mechanisms underlying these disorders – has clearly emerged. Indeed, thanks to recent discoveries, it is scarcely an exaggeration to conclude that there is hardly a chronic health disorder known to modern medicine for which smoking does not increase its risk or severity. Rather than explore all such possible associations, a handful of select disorders that are of particular relevance to toxicology will receive our attention.

10.5.1 Cardiovascular Disease

Epidemiologists long ago uncovered smoking-related risks for such common cardiovascular diseases as stroke, atherosclerosis and coronary arterial disease. Initially, these associations were studied within the context of risks experienced by individual smokers, yet more recent epidemiological studies have explored the role of environmental or second-hand smoke in triggering coronary heart disease in nonsmokers. For example, hospital admission data collected in many urban settings suggest exposure to second-hand smoke increases the risk of coronary heart disease in nonsmokers by 25–30 %. Several pro-atherogenic mechanisms likely underlie these observations, including promotion of thrombogenesis, oxidative damage to low-density lipoproteins, decreased exercise tolerance, impaired vasodilatation, proinflammatory changes and disrupted vascular repair. These discoveries helped fuel legislative efforts to achieve smoke-free public environments in cities all around the world – efforts that demonstrably achieved 15–20 % reductions in the incidence of emergency department admissions for acute coronary events. These findings underscore the value of tobacco abatement efforts for the promotion of societal health.

10.5.2 Type 2 Diabetes

Human data suggests long-term smoking modestly increases the risk of type 2 diabetes, with a dose–response relationship evident in which diabetic risk rises with the number of cigarettes smoked. Several mechanisms may underlie this disease association, including possible differences in body fat disposition between smokers and nonsmokers as well as the antioestrogenic actions of tobacco smoke. Animal studies also revealed that nicotine triggers apoptosis and dysfunction within pancreatic insulin-secreting β cells. The major tobacco smoke carcinogen NNK also induces pancreatic toxicity, impairing the regulation of blood sugar levels. These associations suggest reductions in smoking rates may attenuate the rising global tide of type 2 diabetes and the metabolic syndrome.

10.5.3 Chronic Obstructive Pulmonary Disease (COPD)

As the port of call for inhaled tobacco combustion products, the human lung is vulnerable to numerous smoking-related health conditions. Among the noncancer pulmonary risks, chronic obstructive pulmonary disease (COPD) is of particular significance. COPD involves a worsening loss of lung function due to progressive airflow obstruction and chronic respiratory inflammation. An increased risk of concurrent morbidities also accompanies COPD (e.g. heart failure, muscle weakness, diabetes, anxiety, bacterial infection). Due to permanent remodelling of lung tissue,

discontinuing smoking only partly reverses COPD symptoms. COPD is currently the 4th leading cause of death worldwide and according to the World Health Organization will be of growing global significance due to rising smoking rates in developing countries. While COPD most commonly plagues tobacco smokers, similar syndromes accompany chronic exposure to other forms of respiratory injury including childhood asthma, childhood lung infections and occupational exposure to dusts and fumes.

The pulmonary pathophysiology of COPD is essentially an exaggerated manifestation of the low-grade infiltration of inflammatory cells to the bronchi and peripheral lung that occurs in 'normal' smokers. In the minority of smokers who develop COPD, this process is amplified and accompanied by a tissue-remodelling process accompanied by mucus hypersecretion, small airways obstruction, alveolar damage and pulmonary hypertension. The airflow limitation and 'breathlessness' that is characteristic of COPD mainly reflects permanent enlargement of distal respiratory air spaces upon destruction of alveolar walls.

While the pathobiology of COPD is complex, the transition from the 'normal' inflammatory response seen in most smokers to an abnormal innate and adaptive immune response is likely driven by disparities in the protease–anti-protease and oxidant–antioxidant balance attending the influx of neutrophils, macrophages and lymphocytes. Together with proinflammatory cytokine production and other tissue responses, these processes trigger apoptosis and failure of repair mechanisms, eliciting alveolar destruction and remodelling of small airways. Although many tobacco smoke constituents likely contribute to COPD pathogenesis, the irritant acrolein likely triggers much of the mucus hypersecretion, proinflammatory cytokine production and metalloproteinase activation during the early stages of COPD.

10.5.4 Lung Cancer

According to the famous British Doctors' Study – the pioneering prospective cohort study conceived by Sir Richard Doll that provided unprecedented epidemiological proof for the long suspected link between smoking and lung cancer – the single most important determinant of lung cancer risk is the number of years of regular smoking. The data suggested that the excess incidence of lung cancer increased ~100-fold in men who had smoked for 45 years relative to those who had smoked for 15 years. Also, since the age at commencement strongly influenced the duration of practicing smoking, early adoption was sharply associated with lung cancer risk in both male and female smokers. The importance of smoking duration to lung cancer risk was reinforced by subsequent studies that demonstrated a strong reduction in cancer risk among former smokers who stopped at 50 years of age or, particularly, at 30 years of age. While former smokers had higher risks than men who had never smoked, they experienced substantially lower lifelong risks than those who continued to smoke.

Population studies also revealed that the number of cigarettes smoked each day influences lung cancer outcomes, although the strong increase in risk with duration of smoking still holds true for light and heavy smokers alike. The depth of smoke inhalation, number of puffs taken per cigarette and smoke retention time within the lung also influence lung cancer outcomes.

Lung cancer is an umbrella term that covers a handful of distinct histological forms of neoplastic disease. Epidemiological studies have associated smoking with all the major types of lung cancer including squamous-cell carcinoma, adenocarcinoma, large-cell carcinoma and small-cell undifferentiated carcinoma. Dose-related increases in susceptibility to each type of lung cancer have also been described (i.e. risk increases in heavy smokers relative to moderate smokers). Despite these long-standing findings, it is surprising that many smokers continue to partake in this risky practice: around the world each day, some 3,000 deaths occur due to smoking-related lung cancer. In the USA today, 90 % of lung cancer deaths are attributable to tobacco smoking. The role of individual tobacco carcinogens in lung cancer will receive further attention below.

10.5.5 Bladder Cancer

Although the association is not as strong as for lung cancer, epidemiological evidence confirms a link between smoking and various cancers of the 'lower urinary tract', including the bladder in particular but also the ureter, renal pelvis and urethra. Tobacco-related tumours at these sites typically originate within the urothelium and are mostly transitional-cell carcinomas or squamous-cell carcinomas. While the slope of the dose–response curve for bladder cancer is less steep than for lung cancer, epidemiological studies generally reveal a three- to fivefold increase in relative risk for male smokers who consume 20 or more cigarettes/day, although substantial variability is evident between different populations and ethnicities. In terms of smoke constituents that induce urothelial tumours, aromatic amines seem especially important (see below).

10.5.6 Other Cancers

The International Agency for Research on Cancer has identified causal links between smoking and 13 types of cancer (including tumours of the respiratory and urogenital systems highlighted above). Although the risk varies among different study populations, human studies suggest an association between smoking and tumours at such diverse anatomical sites as the oral cavity, larynx, sinonasal cavity, nasopharyngeal tissues, oesophageal tissues, pancreas, colorectal tissues and the liver.

10.6 Major Tobacco Smoke Constituents

While the popularity of tobacco smoking mainly reflects the *pharmacological* actions of a single highly addictive constituent – the alkaloid nicotine which typically comprises from 0.5 % to 5 % of the dry weight of tobacco leaves – the *toxicological* properties of tobacco smoke reflect complex contributions by diverse mixtures of organic and inorganic substances including gases, volatile organics, metals and particulate matter.

10.6.1 Particulates

Each cubic centimetre of inhaled tobacco smoke contains about 10^{10} particles spanning a size range of between 0.1 and1.0 μm in diameter. The size distribution of sidestream smoke particulates differs from those in mainstream smoke, with the former comprising smaller particles that more likely penetrate deep into alveolar spaces. Smoke released from a single cigarette exposes the respiratory tract to 10–40 mg of particulate matter. Upon deep inhalation, pulmonary penetration of particulates deposit over 1 g of 'tar' daily within the lungs of heavy smokers. Much of this pulmonary material remains throughout the lifetime of a smoker, explaining why a risk of some respiratory diseases persists after smoking cessation.

To study their chemical and toxicological properties, tobacco smoke particulates can be trapped using a Cambridge filter. The resulting condensate or 'tar' is rich in toxic metals as well as cancer-causing organic chemicals such as the polycyclic aromatic hydrocarbons and tobacco-specific nitrosamines. Study of lung biopsies from smokers suggests tissue damage is maximal in close proximity to tar deposits. Typically, the greatest deposition of carbonaceous material occurs within bronchial lymph nodes and within first-generation lung bronchioles. In addition to cell damage by leeched toxicants, deposited particulate matter likely initiates lung injury by promoting iron dyshomeostasis in neighbouring cells, triggering oxidative stress and activating the innate immune system. The appearance of particulate-laden macrophages is thus a highly conspicuous feature of tobacco-related lung injury. Animal studies reinforce a role for these materials in lung injury since stripping particulate matter from tobacco smoke prior to inhalational exposure significantly attenuates pulmonary damage.

10.6.2 Free Radicals

Oxidative stress features prominently in the pathogenesis of smoking related disease, due in part to the high yield of free radicals released during tobacco

combustion. Tobacco smoke contains two distinct populations of free radicals, both of which are present at concentrations greatly exceeding their levels in other forms of air pollution (e.g. smog).

Firstly, tar deposits contain a high density of relatively stable radicals (~10^{16}–10^{17} per gram), the best understood constituent of which is a polymeric quinone, semiquinone and hydroquinone species which generates oxygen radicals via redox-cycling. Tar-derived oxidants such as hydroxyl and superoxide anion radicals inflict significant cellular and genetic damage within the respiratory tract of smokers, causing the elevation in levels of oxidised DNA bases such as 8-oxo-deoxyguanosine within pulmonary tissues. High levels of isoprostanes – sensitive biomarkers of oxidative membrane damage – are detectable in sputum, breath condensate, blood, bronchiolar lavage fluid and lung tissue collected from smokers. Since these biomarkers remain at elevated levels after smoking cessation, pro-oxidants within lingering tar deposits likely take a lasting toll upon the respiratory tract in ex-smokers.

The second free radical population comprises comparatively long-lived species present within the gas phase. For example, the gas phase of newly produced cigarette smoke contains up to 600 μg of nitric oxide (NO). NO reacts rapidly with superoxide radicals to form the damaging oxidant peroxynitrite, a key mediator of smoking-related lung injury. Yet, NO is just one type of radical present within the gas phase: according to some estimates, a single puff of a cigarette delivers approx. 10^{15} gas-phase radicals to the human respiratory tract. The chemical identity of these species is complex since they likely represent 'descendents' of ultrashort lived radicals present within the flame of the burning cigarette tip. First-generation radicals do not survive passage through the body of the cigarette and react with other combustion products to form progressively more stable and damaging species. While the exact identity of the dominant species is subject to debate, likely culprits include carbon-centred radicals as well as diverse oxygen-centred radicals including alkoxyl and peroxyl radical species. Due to propagating reactions between these species and NO and isoprene, concentrations of gas-phase radicals actually increase as tobacco smoke ages. Oxidative injury to lung cells by both tar- and gas-phase radicals takes a heavy toll on the health of smokers, a problem that is sometimes compounded by diminished intake of dietary antioxidants due to a poor diet.

10.6.3 Irritants

The rich smoke cocktail formed during tobacco combustion includes a diverse set of chemicals that are hard to group collectively on any other basis than their shared tendency to exert irritant actions on lung tissue. These include diverse substances with electrophilic properties (e.g. acrolein, formaldehyde) as well as acidic substances and volatile organic irritants (e.g. naphthalene, styrene). The organic acids formic, acetic and propionic acids as well as tussive irritants such as citric acid and cyclohexanone likely mediate much of the acute irritation of respiratory tissues that

accompanies smoke inhalation by inexperienced smokers. These irritants exert complex effects upon chemosensory and somatosensory neural pathways to promote respiratory tract secretions, bronchoconstriction, coughing and oedematogenesis.

The pulmonary irritation caused by acetic acid and cyclohexanone proceeds via interactions with acid-sensing ion channels (ASICs), TRPV1 receptors and other sensory receptors. To suppress these respiratory responses, the 'counterirritant' menthol has been used during commercial cigarette production for nearly 100 years: mentholated cigarettes comprise the majority of brands sold in many countries. The presence of menthol strongly influences 'smoking topography' – the actual smoking behaviour of individual smokers – by improving perceptions of taste, smoothness and lung irritation, thereby determining the level of satisfaction smokers receive from particular cigarette brands.

The most toxic tobacco smoke irritant is acrolein, one of a broad class of reactive smoke-borne aldehydes that includes formaldehyde and crotonaldehyde. Acrolein readily targets TRPA1 irritant receptors to activate chemosensory nerves within the lung, triggering the coughing reflex and neurogenic inflammation. TRPA1 is a Ca^{2+}-permeant non-selective ion channel, the activation of which with selective ligands activates the cough reflex in human volunteers and experimental animals. The induction of coughing via TRPA1 differs from the 'classic' cough reflex which is induced by capsaicin acting at TRPV1 receptors.

The extreme chemical reactivity of acrolein ensures it exerts actions on lung tissue that extend beyond the typical effects of smoke irritants. In addition to receptor-driven responses, acrolein is a strong electrophile that reacts readily with cell macromolecules including DNA and proteins (Fig. 10.3). The release of up to 100 micrograms or more acrolein from a burning cigarette has focussed attention on acrolein-derived DNA adducts as biomarkers of exposure to tobacco smoke. The major DNA adducts formed by acrolein – a pair of hydroxylated 1,N2-propanodeoxyguaosine species formed via a 2-step reaction with deoxyguanosine – have been detected within blood cell and lung cell DNA collected from smokers (Fig. 10.3). The major form – the γ-hydroxy species – is likely more mutagenic than its α-hydroxy adduct, although it is also more effectively repaired. In recent years, significant controversy has surrounded the role of acrolein adducts in smoking-related lung cancer, with contradictory findings reported concerning the mutagenic properties of acrolein-derived DNA adducts. The fact that acrolein-dG levels do not change strongly in lung or blood DNA in response to smoking habits may undermine a major role for acrolein in tobacco-related lung carcinogenesis, an outcome that likely reflects the efficiency with which the aldehyde is detoxicated by glutathione in human tissues (Fig. 10.3). The resulting metabolite, 3-hydroxypropyl-mercapturic acid (3-HPMA), is a useful urinary biomarker of tobacco smoking since its levels in smokers are substantially elevated over those in nonsmoking controls and change as a function of smoking behaviour (e.g. cessation).

Acrolein-derived protein adducts – especially those formed on lysine side chains (Fig. 10.3) or cysteine residues in target proteins – are likely key contributors to the toxicity of inhaled smoke. Acrolein attacks a huge swathe of proteins in respiratory tract cells – one recent proteomics study identified over 750 damaged proteins in

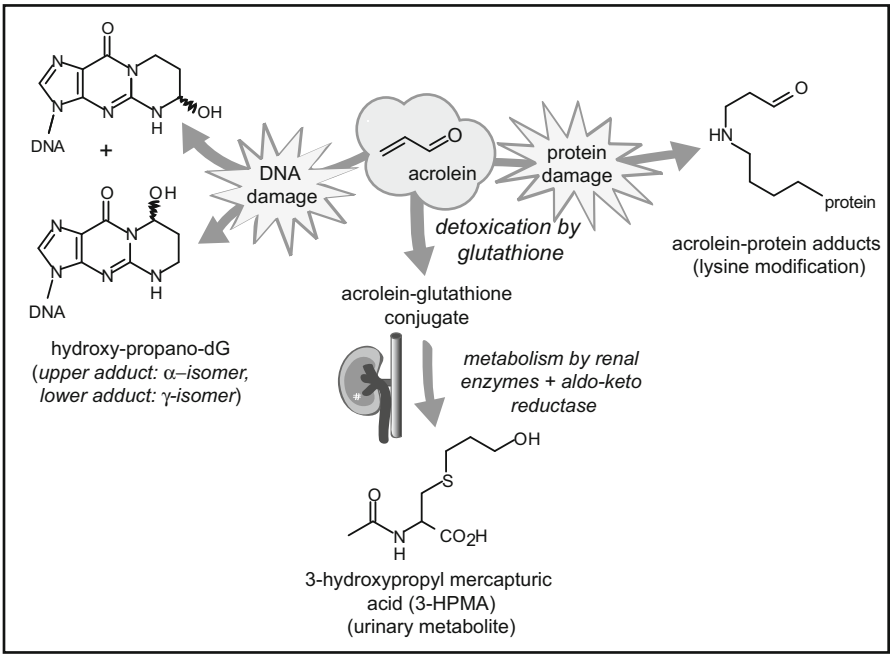

Fig. 10.3 In addition to activating TRPA1 receptors, the combustion product acrolein contributes to smoke-related pathophysiology by forming multiple DNA and protein adducts. Detoxication of acrolein by glutathione-S-transferase leads to the urinary metabolite 3-hydroxypropyl mercapturic acid (3-HPMA), a useful biomarker of tobacco smoking. 3-HPMA forms via the processing of glutathione conjugates by γ-glutamyl-cysteinyl-glycine, cysteinylglycinase, N-acetyl transferase and aldo-keto reductase

human bronchiolar epithelial cells following acrolein exposure. The full mechanistic relevance of this damage is still being unravelled, but disrupted expression of hundreds of genes is a likely consequence that alters the activity of many cellular pathways including those involved in cell growth, cell death and inflammatory responses. Damage to proteins which regulate macrophage responses to inhaled irritants (e.g. c-Jun, JNK2) suggests acrolein may contribute to chronic immunosuppression in the lungs of some long-term smokers.

10.6.4 Metals

Due to their ubiquitous presence as soil and water contaminants in tobacco-producing agricultural regions, toxic metals such as cadmium, chromium, arsenic, nickel, copper and lead are important tobacco contaminants that are retained during leaf processing and cigarette manufacture. The extent of metallic contamination varies according to geographical factors but is especially significant where the use

of animal waste as crop fertilisers (rich in excreted heavy metals) and synthetic phosphate fertilisers (possess metal-chelating properties) is an established part of tobacco production. The levels of toxic metals in some cigarette brands are surprisingly high, ensuring heavy smokers subject themselves to chronic metal intoxication that affects multiple organs. Smoke-borne metals appear especially important during the pathogenesis of inflammatory lung disorders such as COPD and asthma, triggering airways hyperresponsiveness, inflammation and sensitisation to inhaled chemical and biological allergens.

The heavy metal cadmium is likely the most significant metallic tobacco contaminant. During a classic risk assessment of tobacco smoke constituents by Fowles and Dybing, cadmium ranked fourth most important in terms of its adverse consequences for respiratory health (the three more highly ranked chemicals all belonged to the aldehyde class of smoke constituents, with acrolein ranked as by far the most important followed by acetaldehyde and formaldehyde). Cadmium levels in tobacco vary with the country of manufacture, but according to classic studies on US smokers performed by Lewis and associates, smokers daily absorb 1–3 μg of cadmium if they smoke one cigarette pack a day, which is approximately equivalent to the total daily intake via vegetable consumption in nonsmokers. With a very long half-life in body tissues (e.g. 20–30 years), cadmium is a suspected contributor to such diverse tobacco-related disorders as pancreatic cancer, diabetes, periodontal disease and several respiratory disorders. Analysis of autopsied lung tissues has revealed significant cadmium accumulation within the various lobes of the lung in smokers. 'Leeching' of cadmium during lung perfusion by normal blood flow likely prolongs exposure of remote body organs in heavy smokers.

Since heavy metal levels in tobacco leaves reflect agricultural practices that are largely unavoidable, options for mitigating human exposure to these substances are limited. Cessation of smoking is the major means of reliably reducing chronic metal intoxication in tobacco smokers.

10.6.5 Carcinogens

Tobacco smoke contains around 72 or so compounds that are currently recognised as human or animal carcinogens by the International Agency for Research on Cancer (IARC). Since some carcinogens are only present at trace levels, their relevance to cancer induction within the lung or at other body sites is uncertain. Nevertheless, for many of the most potent carcinogens in tobacco smoke, even the release of nanogram quantities can be very significant in heavy smokers since lifelong smoking habits deliver cumulative doses that mimic the milligram quantities known to induce tumours in lab animals. According to one recent estimate by the US toxicologist Stephen Hecht, mainstream smoke released from a single cigarette contains about 600 trillion molecules of strong carcinogens belonging to the PAH and tobacco-specific nitrosamine classes, a total that could be 10,000 times larger if all known 72 carcinogens are included.

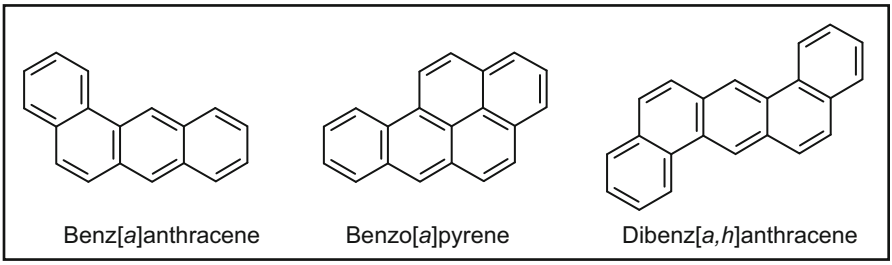

Fig. 10.4 Of the hundreds of polycyclic aromatic hydrocarbons produced during tobacco combustion, only a dozen or so possess likely carcinogenic potency. Several PAHs that are designated as group 2A carcinogens by IARC are shown

10.6.5.1 Polycyclic Aromatic Hydrocarbons (PAH)

Hundreds of multi-ring aromatic hydrocarbons are present within the particulate phase of tobacco smoke, a select few of which are shown in Fig. 10.4. Members of this class are also ubiquitous within the natural environment due to their formation during forest fires, presence in crude oil and other types of organic matter. High occupational exposure to PAHs occurs among workers in aluminium smelters, iron foundries, fossil fuel processing plants, roof coating, road paving and other industries. Transport workers such as truck drivers and locomotive engineers also encounter high levels of PAH via diesel exhaust fumes. Scientists have detected around 550 individual PAHs within tobacco smoke, with the yield of an individual PAH influenced by the total number of carbon atoms within its molecular structure. The formation of specific tobacco-derived PAHs involves complex chemistry and is strongly influenced by tobacco type and the temperature at which combustion proceeds, a factor which helps explain differing PAHs yields in sidestream versus mainstream smoke. PAHs form via thermal degradation and pyrolysis of organic constituents within the tobacco plant to yield small reactive molecules and/or free radicals that rapidly combine to form larger structures. Major tobacco leaf precursors to PAH formation include tobacco cell wall components and structural biopolymers such as lipids, cellulose, hemicellulose and lignin.

Due to the structural diversity within the PAH class, the metabolic fate, CYP-inducing efficacy, carcinogenic potency and tissue selectivity of individual family members can vary considerably. Of the hundreds of PAHs within tobacco smoke, only 12 are recognised as human or animal carcinogens by the IARC. Of these, only a few PAHs are categorised by the IARC as group 2A carcinogens (Fig. 10.4). The fact that most PAHs lack cancer-causing potential complicates formulation of structure–activity relationships for these compounds. Likewise, the probability that the toxicological properties of an individual PAH when studied alone might differ from its behaviour within complex mixtures comprising multiple PAHs further complicates study of these substances. As a rule, complex mixtures of PAHs (e.g. coal tar extracts) exhibit greater cancer-causing potency than individual purified compounds, yet the same is not necessarily true for the degree of DNA binding. This implies PAHs exert tumour-promoting actions beyond their classic roles as adduct-forming, genotoxic species. In a nongenotoxic mechanism seen in cultured cells, PAHs strongly inhibit gap junction-mediated cellular communication, an effect that is shared with

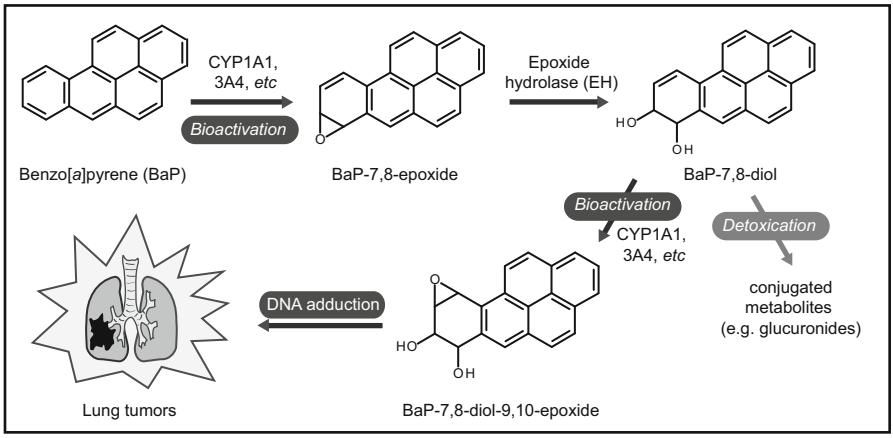

Fig. 10.5 Key steps in the activation of the carcinogenic PAH benzo[*a*]pyrene to its ultimate DNA adduct-forming diol-epoxide metabolite

classic tumour promoters such as the phorbol esters. Showing the relevance of these mechanisms within the whole animal or human setting is more difficult.

A key member of the PAH class, benzo[*a*]pyrene (BaP) (Fig. 10.4), attracts much attention due to its ubiquitous distribution within the environment and its broad toxicological properties (e.g. teratogenicity, mutagenicity, carcinogenicity and immunotoxicity). The isolation of BaP from coal tar in 1930, and the demonstration that it initiates tumours when repeatedly painted on mouse skin, was a key milestone in the emergence of experimental toxicology. The evidence incriminating BaP as a major tobacco carcinogen is especially strong in relation to the lung – low BaP doses strongly and reproducibly induce lung cancer when delivered via inhalational, dermal or oral routes in a range of animal species.

BaP is a prototypical genotoxic carcinogen in that the formation of reactive DNA-damaging metabolites is obligatory for its cancer-causing efficacy. Following pulmonary absorption from inhaled smoke, BaP is converted to its ultimate genotoxic metabolite via a classic 3-step PAH bioactivation process. In the first step, CYP1B1 inserts an oxygen atom into BaP to form epoxy species that are subsequently converted to 1,2-dihydroxy metabolites (diols) by microsomal epoxide hydrolase EPHX1 (Fig. 10.5). A second CYP-catalysed step forms the ultimate genotoxic metabolite, 7,8-9,10-diol-epoxide (BPDE). While multiple epoxidation events can occur, the most significant involve oxygenation of the same benzo ring as contains the diol group to form BP 7,8-diol-9,10-epoxides (BPDEs). These reactive electrophiles participate strongly in cancer causation. Recent investigation of the kinetics of the formation of diol epoxides in human smokers reveals that concentrations in blood peak within 15–30 min of smoking a cigarette. In the case of BaP, stereochemical considerations ensure that four different isomeric 7,8–9,10-diol-epoxides may form, of which only two exhibit mutagenic potency in the Ames Salmonella test while just one induces tumours in the mouse skin tumour bioassay.

Reactions of diol-epoxide metabolites with DNA primarily target the exocyclic amine ($-NH_2$) group possessed by 2′-deoxyguanosine (N^2) or 2′-deoxyadenosine

(N^6). These adducts are efficiently converted into base-pair substitution mutations during DNA replication (i.e. G→T and A→T mutations occur at a high frequency in BPDE-treated cells). Although bulky PAH adducts can sometimes block replication, polymerases lacking proofreading activity may bypass these lesions, allowing introduction of errors into the DNA sequence (i.e. 'error-prone bypass'). The yield of mutations is further influenced by neighbouring nucleotides within the DNA sequence in which the PAH adduct is positioned. Carcinogenic PAHs frequently cause mutations within 'hotspots' in growth regulatory proto-oncogenes or tumour suppressor genes. PAH-induced mutations in hotspots within *Ras* family members (e.g. codon-12 in K-*Ras*) or the p53 tumour suppressor (e.g. codon-249) are especially important in tobacco carcinogenesis in the lung.

Adduct formation ensures the DNA double helix undergoes substantial contortion to accommodate the large PAH moiety. The resulting distortion of the DNA molecule triggers recognition by nucleotide excision repair (NER) proteins that patrol the genome, although their efficiency in removing lesions is partly governed by the stereochemistry of the adduct and the neighbouring sequence in which it resides. PAH adducts may also trigger growth arrest and suppress the transition of replicating cells from the G1 to S phase of the cell cycle. The growth-arrested state buys time for the repair of DNA adducts, or, if extensive DNA damage is detected, cells may be eliminated via apoptosis. The tumour suppressor p53 is a major orchestrator of the fate of cells that contain DNA adducts, exerting complex actions that regulate both apoptotic and anti-apoptotic pathways, NER pathways and growth arrest mediators. The stress-activated kinase, p38 MAPK, also regulates the fate of cells containing PAH-adducted genomes.

Another route to PAH-induced mutations proceeds via depurination events that generate 'noninformational' abasic sites within DNA. These lesions likely arise via a different metabolic route to the classic diol-epoxide pathway. Other minor routes of PAH metabolism involve formation of redox-cycling *ortho*-quinones as well as 1-electron oxidised radicals. For example, radical metabolites of PAH can form unstable adducts at deoxyguanosine or deoxyadenosine residues, weakening the N-glycosyl bond that attaches bases to the sugar–phosphate DNA backbone. The resulting loss of the adducted base via spontaneous depurination forms 'abasic sites' which cannot perform normal template roles during replication by DNA polymerases. The latter typically follow the 'A-rule' when processing an abasic site, i.e. inserting a deoxyadenosine to allow DNA synthesis to proceed. This risky endeavour depends upon mismatch repair pathways to subsequently restore the correct sequence. Depurination events of this kind are key contributors to the induction of mutations by PAH compounds.

10.6.5.2 Tobacco-Specific N-Nitrosamines (TSNA)

While nicotine is the primary addictive component within tobacco smoke, it is also a chemical precursor to several highly toxic chemicals, including an important class of well-studied carcinogens, the *N*-nitrosamines (Fig. 10.6). NNN forms via a 2-step

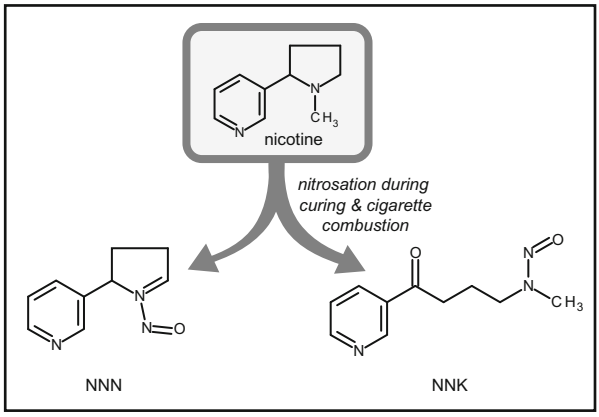

Fig. 10.6 Nitrosation of nicotine during tobacco processing and cigarette combustion generates carcinogenic tobacco-specific nitrosamines such as N-nitrosonornicotine (NNN) and 4-methylnitrosamino (NNK)-1-(3-pyridyl)-1-(butanone)

sequence that begins with the N-demethylation of nicotine (i.e. to yield nornicotine) followed by nitrosation of the pyrrolidine-ring nitrogen. NNK on the other hand forms via a direct nitrosation step that results in ring-opening of the N-methyl pyrrolidine ring (Fig. 10.6).

Tobacco smoke also contains 'generic' *N*-nitrosamines such as N-nitrosodiethylamine that can cause lung cancer in some rodent species, yet their levels in cigarette smoke are low relative to the *tobacco-specific nitrosamines* (TSNA) which derive from nicotine. TSNA concentrations in tobacco products usually greatly exceed concentrations of generic N-nitrosamines in other foodstuffs. TSNAs are also present in various 'smokeless' tobacco products, with especially high levels reported for some brands of moist snuff sold in the USA and Europe, although industry initiatives have diminished the scope of this problem in some markets.

Multiple TSNAs are present in tobacco (e.g. NNN (N-nitrosonornicotine), NNK ((4-methylnitrosamino)-1-(3-pyridyl)-1-butanone), NAB (1-nitrosoanabasine) and NAT (*N*-nitrosoanatabine)), although NNN and NNK have attracted greatest attention due to their pronounced carcinogenic potency. Both compounds and NNK, in particular, are potent lung carcinogens in many species, including rats, mice and hamsters. NNK is arguably the most potent and reproducible lung carcinogen known to modern toxicology. Only modest doses of NNN and NNK are required to induce lung cancer in animals (i.e. tumours occur at dosages that with appropriate species-scaling resemble those achieved by human smokers under normal smoking conditions). Moreover, unlike some putative carcinogens, NNN and NNK induce tumours in 'wild-type' lab animals without the need for genetic manipulations to boost the sensitivity to carcinogens (e.g. deletion of DNA repair genes or tumour suppressor genes). NNN and NNK are also multi-organ carcinogens, inducing cancer beyond the lungs in organs such as nasal tissue, liver and pancreas – sites that are vulnerable to tumours in human smokers.

TSNAs are present at low levels within freshly picked tobacco and mainly form during drying of the leaves, processing and curing as well as cigarette combustion.

Tobacco processing procedures that significantly lower TSNA yields are available, yet it is unclear whether these safer methods are widely used during commercial tobacco production. As a rule, flue-cured tobacco leaves contain higher TSNA levels relative to air-cured products. TSNA yields also vary according to the type of tobacco strain, and variants with a diminished capacity for nicotine demethylation of nicotine typically produce low TSNA yields.

TSNA formation during tobacco combustion can be quite unpredictable and significant variations are evident in mainstream and sidestream smoke depending on the country of origin of the cigarette. Generally, levels of smoke-borne NNN exceed those of NNK. International comparisons of NNN levels in different cigarettes have revealed significant variations in NNN and NNK levels depending on the country of origin.

The metabolism of NNN and NNK has been intensively studied in humans and other species. Numerous metabolites have been reported, including quantitatively minor products which form via denitrosation pathways, N-oxidation or hydroxylation of the pyridine ring. In the case of NNK, a major fate involves reduction of the carbonyl group to form NNAL (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol). NNAL then undergoes conjugative metabolism to form various NNAL-glucuronides. NNAL and its glucuronidated metabolites are widely used biomarkers of tobacco smoking. Detection of NNAL-glucuronide in blood and urine samples from nonsmokers (e.g. children and family members in same household as smokers, or patrons in casinos, bars and restaurants) was instrumental in the curtailment of smoking in public spaces in some countries. A related compound, *iso*-NNAL, is a useful biomarker of exposure to smoke residues on surfaces (i.e. so-called 'third-hand' tobacco smoke).

From the perspective of carcinogenesis, the key metabolic fate of NNK involves α-hydroxylation by CYP enzymes. These oxygenation reactions target either the methyl or methylene groups which are attached to the nitrosamine group in the open-ring side chain possessed by NNK (Fig. 10.7). These distinct hydroxylation events are important since they generate the two major classes of adducts that form on proteins (e.g. haemoglobin) or DNA in tissues during exposure to NNK, namely, methyl adducts that form via products of the α-methylene hydroxylation pathway and pyridyloxobutyl adducts that form via the α-methyl hydroxylation pathway (Fig. 10.7).

In the first instance, α-methylene hydroxylation of NNK generates an unstable α-hydroxy metabolite that decomposes to form methanediazohydroxide, a reactive methyl-donating reagent which methylates DNA and proteins to form stable adducts (Fig. 10.7). DNA adducts formed by this pathway include 7-methyl-deoxyguanosine, O^6-methyl-deoxyguanosine and O^4-methyl-deoxythymidine, the formation of which is demonstrable in both NNK-treated human cells and animal tissues. If uncorrected by DNA repair proteins, adducts such as O^6-methyl-deoxyguanosine may undergo inappropriate 'pairing' with deoxythymidine during replication by DNA polymerases, ensuring G→A transitions occur commonly in daughter DNA molecules (Fig. 10.8). Although such mutations are repairable by mismatch repair pathways, the high prevalence of these base-pair substitutions in activated oncogenes in lung tumours of tobacco smokers indicates that not all methylated DNA base lesions are efficiently repaired.

The alternative pathway involving α-methyl hydroxylation of NNK generates an unstable electrophile that attacks DNA and protein to form various pyridyloxobutyl

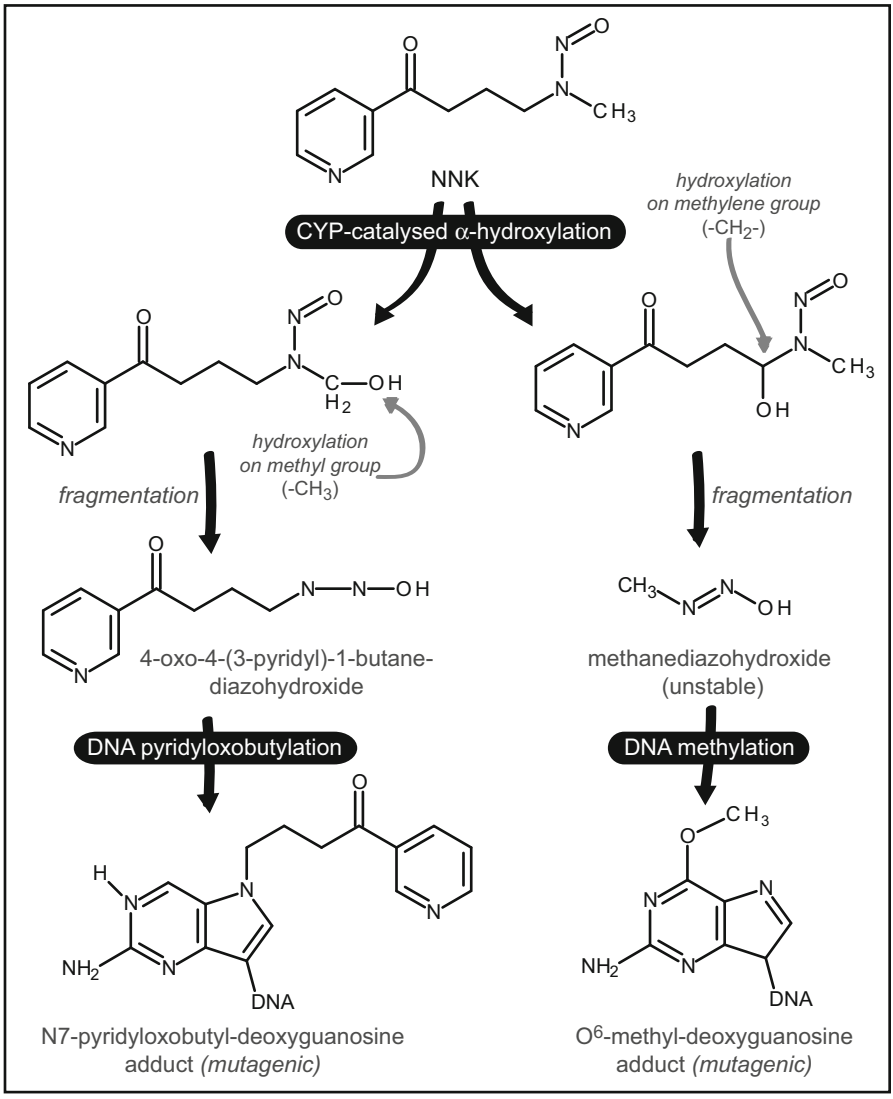

Fig. 10.7 The formation of DNA-damaging metabolites from the tobacco carcinogen NKK proceeds via CYP-catalysed hydroxylation of either the methyl or methylene group adjacent to the nitrosamine group. These represent two distinct adduct-yielding pathways: the 'DNA methylation pathway' (formed via oxidation of the methylene group) and the 'DNA pyridoxobutylation pathway' (formed via oxidation of the methyl group). DNA adducts from each pathway likely contribute to lung carcinogenesis

adducts (Fig. 10.7). During reaction with DNA, pyridyloxobutyl adducts form via reactions with deoxyguanosine, deoxycytosine and deoxythymidine (Fig. 10.7 only shows the N-7guanine adduct). While the role of these species in carcinogenesis is less well established than for the methylated bases discussed above, several lines of evidence suggest pyridyloxobutyl DNA adducts do participate in tobacco

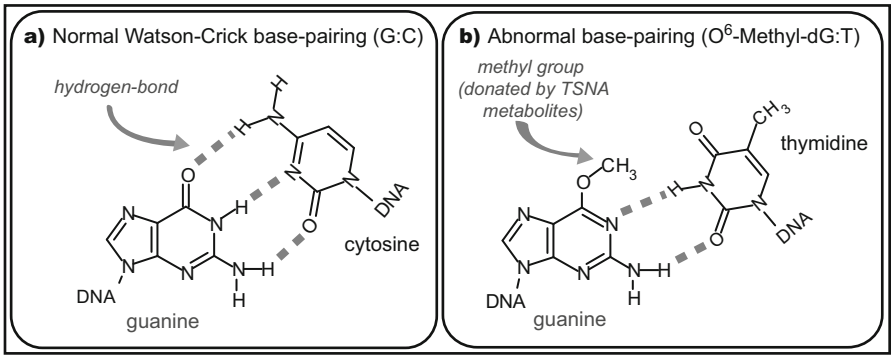

Fig. 10.8 Methanediazohydroxide formed via CYP-catalysed NKK metabolism induces mutations by adding a methyl group to the crucial O^6 atom of guanine. By increasing the thermodynamic stability of a mispaired base involving thymidine (*Panel b*) instead of cytosine (*Panel a*), the methylated base promotes mutations (G→A transitions) in daughter DNA molecules

carcinogenesis. For example, pyridyloxobutyl DNA adducts have been detected as persistent lesions in DNA extracted from tumour-prone tissues of NNK-treated rodents. Reagents that form pyridyloxobutylated adducts are also mutagenic in the Ames Salmonella test or analogous mammalian assays. Pyridyloxobutyl adducts also inhibit methyltransferase enzymes that repair methylated bases in damaged DNA, thus exerting a synergistic effect upon NNK mutagenicity. Finally, human studies have revealed higher levels of pyridyloxobutyl DNA adducts in tissue of smokers with lung cancer compared to smokers without lung cancer.

Although NNN is not susceptible to the α-methylene oxidation that generates the methyl-donating reagents formed via NNK metabolism, it does undergo CYP-catalysed hydroxylation on the pyrrolidine ring to form reactive hydroxy-NNN metabolites. One reactive α-hydroxynitrosamine formed via this route (2′-OH-NNN) is able to damage DNA and proteins in a manner analogous to the pyridyloxobutylating pathway described for NNK. NNN thus is a likely contributor to the formation of pyridyloxobutyl DNA and protein adducts detected in tobacco smokers.

10.6.5.3 Aromatic Amines

Our opening survey of toxicology history highlighted Ludwig Rehn's role in identifying bladder cancer as a risk accompanying occupational exposure to aromatic amines in nineteenth-century German textiles factories (Chap. 1, Sect. 1.4.2). Due to these observations and other investigations, the synthesis and use of aromatic amines was curtailed during the twentieth century. For example, a prototypical member of this chemical class, 4-aminobiphenyl (4-ABP), was once widely used during the manufacture of rubber tyres and synthetic dyes, although its US production was abolished in the 1950s. Despite these efforts, its presence within tobacco smoke ensures human exposure to 4-ABP persists in today's world. Also, since

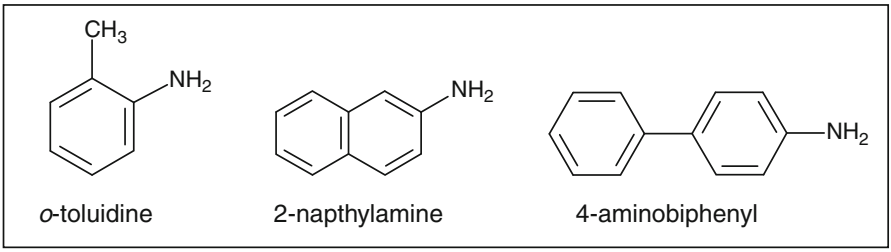

Fig. 10.9 Bladder cancer-causing aromatic amines within tobacco smoke

4-ABP concentrations in sidestream smoke from a smouldering cigarette greatly exceed those in mainstream smoke, the potential for exposure via second-hand smoke is substantial. Thus, classic studies by Hoffman and associates established that 4-ABP levels in mainstream smoke are in the range of 2.4–4.6 ng per cigarette without a filter and 0.2–23 ng per cigarette with a filter, whereas sidestream smoke contained up to 140 ng per cigarette. In just 1 month alone, a nonsmoker exposed to environmental tobacco smoke within a domestic dwelling can absorb a comparable 4-ABP dose as if they smoked a standard pack of 20 cigarettes. Since tobacco smoke contains other carcinogenic aromatic amines in addition to 4-ABP (e.g. *ortho-toluidine* and 2-naphthylamine – see Fig. 10.9), this compound class likely contributes strongly to smoking-related bladder cancer.

The induction of bladder cancer by aromatic amines involves a complex web of metabolic alterations. In addition to its role in bladder cancer and other toxic outcomes, these metabolic transformations attract attention due to their ability to generate blood-derived biomarkers such as DNA adducts (e.g. in white blood cell DNA) and protein adducts (e.g. haemoglobin or albumin). These useful molecular dosimeters of tobacco exposure have assisted studies of the impact of genetic background on susceptibility to bladder or liver cancer (e.g. comparisons of tumour risks in 'slow' versus 'fast' acetylators that possess different N-acetyl transferase genotypes).

The main route to 4-ABP bioactivation commences with the CYP1A2-catalysed hydroxylation of the exocyclic primary amine to form an N-hydroxy or hydroxylamine metabolite (Fig. 10.10). These metabolites react to some degree with proteins but typically display limited reactivity with DNA; hence additional metabolic processing is required to form the ultimate mutagenic species. The resulting *N*-hydroxy metabolite may undergo one of several conjugative reactions that differ in their carcinogenic significance: while *N*-acetylation by *N*-acetyl transferase achieves detoxication of the *N*-hydroxy metabolite, *O*-conjugation with acetate, sulfonate or glucuronic acid all represents bioactivation steps. However, the short half-lives of the acetate and sulfonate *O*-conjugates ensure these species mainly cause damage within the tissue of origin: only the *O*-glucuronide conjugate survives excretion from the liver to circulate around the body and undergo final excretion by the kidneys.

Within acidic urine, the 4-ABP *O*-glucuronide is hydrolysed to release a highly electrophilic nitrenium cation that is the primary mediator of bladder cancer. These

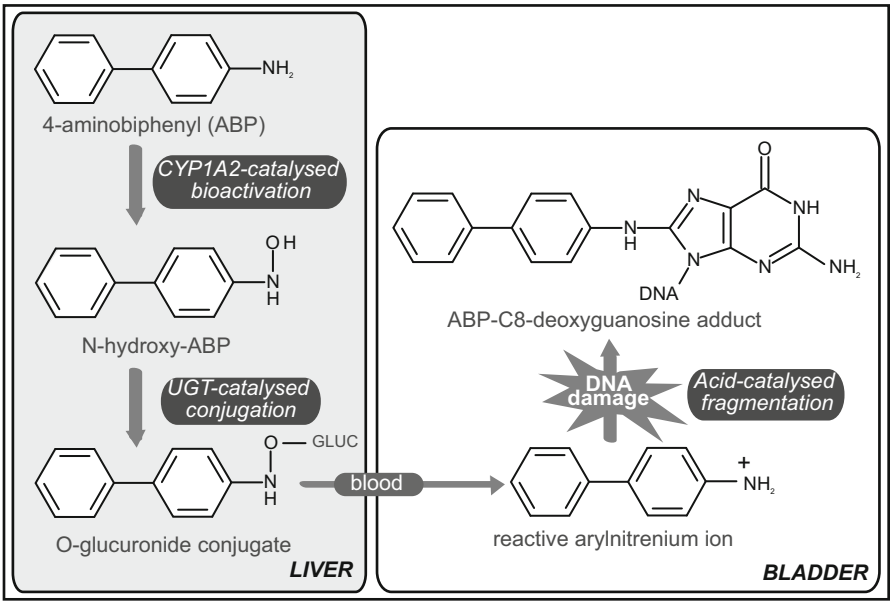

Fig. 10.10 Key steps in the formation of DNA-damaging metabolites from 4-aminobiphenyl. Note that this scheme is highly simplified and does not show the role of N-conjugates (e.g. acetylated or glucuronidated species) or other classes of O-conjugates (e.g. sulfonates)

species attack DNA within urothelial cells at the C-8 position of guanine to form the poorly repaired mutagenic adduct *N*-(deoxyguanosine-8-yl)-4-ABP. In recent work by Canadian researchers, use of *BigBlue*™ transgenic mice to screen *cII* transgene mutations following six weekly treatments with 4-ABP, followed by a 6-week recovery period, identified bladder as most prone to mutations among a range of tissues examined. Compared to control tissues, where G→A transitions were most prevalent, the most common mutations within bladder urothelium were G→T transversions, consistent with the known miscoding properties of the main 4-ABP-derived adduct, N-(deoxyguanosine-8-yl)-4-ABP. The finding that mutations were detected long after the final dose of 4-ABP concurs with the persistent properties of these adducts in mammalian tissues.

10.6.5.4 1,3-Butadiene (BD)

According to a risk assessment of smoke constituents by Fowles and Dybing, the highest increase in cancer risk due to any particular constituent upon smoking just one cigarette per day involves 1,3-butadiene (BD), one of many small volatile substances present in tobacco smoke (others include ethylene oxide, acrylonitrile, acetamide and benzene). The high rank assigned to BD partly reflects its comparatively high concentration in tobacco smoke compared to other tobacco

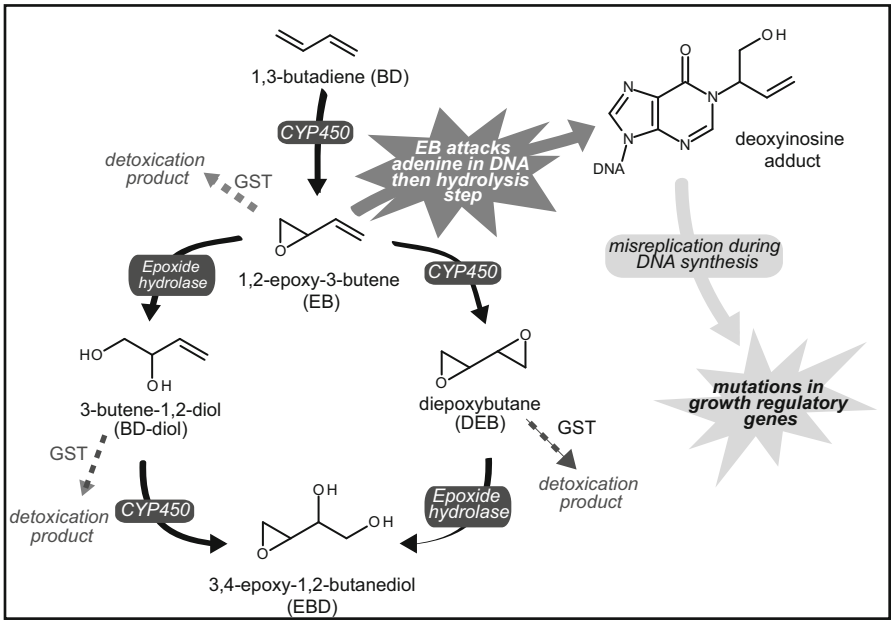

Fig. 10.11 Key steps in the bioactivation of 1,3-butadiene to form DNA-damaging metabolites that promote formation of promutagenic adducts at deoxyadenosine residues in DNA. Note that the scheme is highly simplified and that each of the epoxy metabolites (EB, DEB and EBD) likely participate in DNA adduct formation

carcinogens – levels ranging from 16 to 75 μg/cigarette have been measured in mainstream smoke and 205–361 μg/cigarette in sidestream smoke. These values are some 10^3- to 10^4-fold higher than corresponding levels of PAHs or aromatic amines.

BD is a colourless gas with a gasoline-like odour used in the chemical industry during the manufacture of plastic polymers and rubber products. Due to formation during fuel combustion, BD is also present in car exhausts and urban air, ensuring BD exposure is widespread. Analytical studies have established that BD is present within blood and urine samples of the general populace including nonsmokers, although levels are typically two or three times higher in smokers. When administered to laboratory rodents, BD induces diverse tumour responses including lymphocytic lymphoma and solid tumours of the heart, mammary gland, ovary, liver and lungs. Together with epidemiological findings suggesting an increased risk of leukaemia in occupationally exposed workers, BD is ranked a group 1 'human carcinogen' by the IARC.

The carcinogenic properties of BD are likely due to CYP2E1 and -2A6-catalysed conversion to the electrophilic epoxide metabolite, 1,2-epoxy-3-butene (EB). A simplified overview of BD biotransformation is shown in Fig. 10.11 (not all metabolites are shown). EB can be hydrolysed by epoxide hydrolase to 3-butene-1,2-diol (BD-diol), which can be further epoxidised by CYP to form 3,4-epoxy-1,2-butanediol (EBD). Another fate for EB involves a second oxidation catalysed by

CYP2E1 or -2A6 to yield diepoxybutane (DEB). DEB also undergoes epoxide hydrolase-catalysed conversion to the epoxy-diol metabolite (Fig. 10.11). Based on findings in in vitro test systems using both bacteria (e.g. Ames test) and mammalian cells (e.g. CHO cells), DEB is the most strongly mutagenic BD metabolite, although EB and EBD may also contribute to DNA adduction and mutagenic outcomes.

Multiple DNA adducts have been identified for the three main electrophilic metabolites of BD, many of which possess miscoding or replication-blocking properties. The most mutagenic DNA adducts formed from BD include various deoxyinosine derivatives which form upon deamination after reaction of EB with the N1 position of deoxyadenosine. These highly mutagenic adducts induce very high mutation frequencies during DNA replication. Deoxyinosine adducts likely contribute to the high frequency of base-pair substitutions at A residues within the *cII* transgene of BD-exposed *BigBlue*™ transgenic mice.

Electrophilic BD metabolites also attack blood proteins such as haemoglobin, forming adducts that serve as useful biomarkers of human exposure in smokers and chemical plant workers. A complicated array of glutathione conjugates also form via metabolic processing of electrophilic BD-derived metabolites within the liver and other tissues. The importance of glutathione in detoxicating BD metabolites is suggested by higher levels of haemoglobin adducts in BD-exposed workers who are genetically deficient in glutathione-conjugating pathways (e.g. GSTM1 and GSTT1 null genotypes)

10.7 Mutations in Target Genes

Quantitative analysis of adducts within human DNA samples has provided proof that tobacco combustion-derived toxicants inflict significant genetic damage upon the lungs of smokers. As the most reactive base in DNA, the most common tobacco-related adduction events involve damage to guanine (Fig. 10.12). Measurement of guanine adducts in lung biopsy or autopsy material from smokers or, more commonly, in surrogate tissue samples such as white blood cells has supplied strong chemical proof for contributions by diverse genotoxic pathways to DNA damage in smokers. Yet, demonstrating the mere existence of DNA adducts within the lungs of smokers does not fully prove the biological significance of such damage to cancer onset: additional work is needed to establish mechanistic links between chemical damage to DNA and the ‘early biological response’. Knowledge concerning the spectrum of mutations accumulating in target genes in the lungs of smokers is needed to prove that DNA adducts formed by tobacco combustion products actually drive carcinogenesis. The availability of rapid sequencing technology allowing large-scale studies of mutations in human cancers is proving useful to this endeavour.

Most early studies of this kind focussed on the tumour suppressor gene p53 and the oncogene K-Ras since mutations in these key genes are common in smoking-related lung cancer. Continually updated databases of human gene mutations are

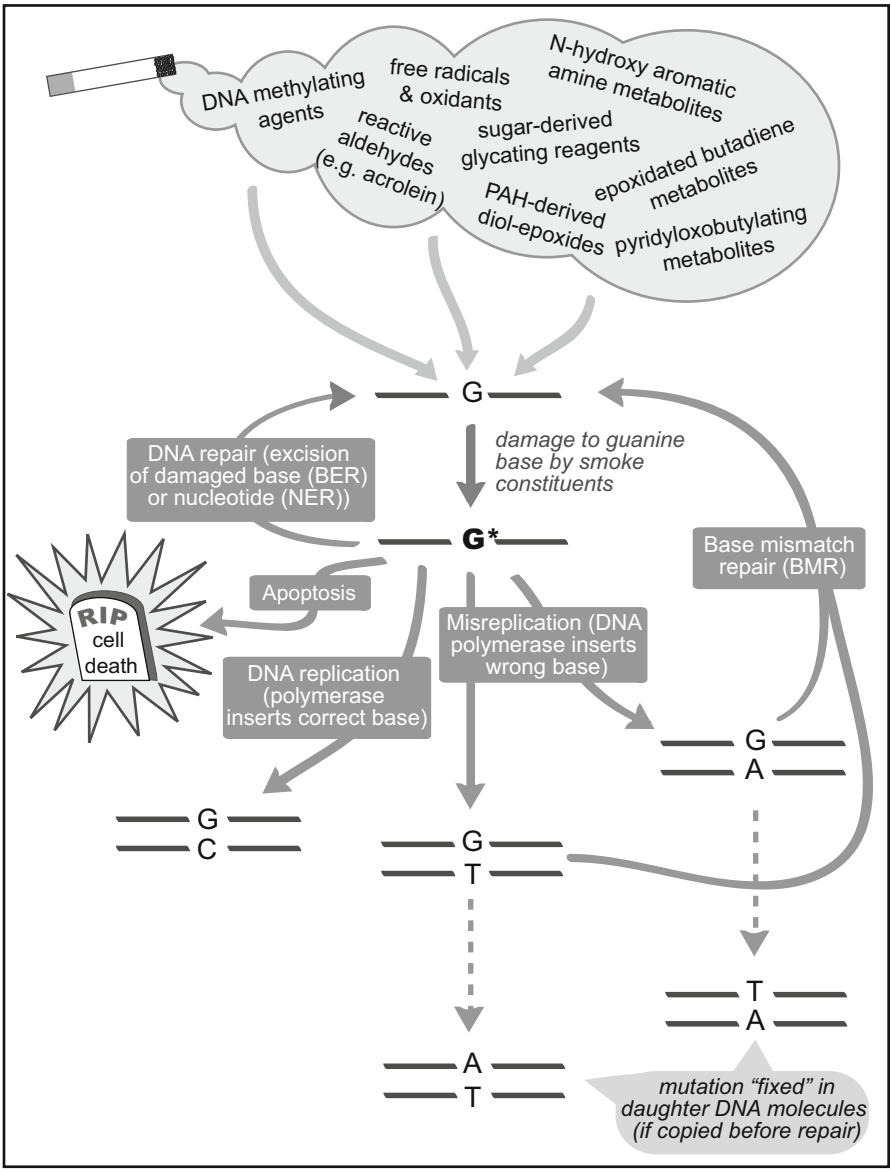

Fig. 10.12 Guanine bases (G) in lung cell DNA incur diverse types of chemical damage during exposure to tobacco smoke, ensuring the smokers lung genome contains a cocktail of DNA adducts formed by PAH–diol epoxides, pyridyloxobutylating metabolites derived from NNK and NNN, hydroxylamines formed from aromatic amines and adducts formed by direct-damaging species such as acrolein, crotonaldehyde or oxidants. The fate of individual adducts is complex and can include induction of cell death, mutagenesis or repair by either base excision (BER) or nucleotide excision repair (NER) pathways. Persistent adducts may miscode during replication by DNA polymerases, causing mutations (e.g. G→T transversions and G→A transitions) in target genes such as p53 or K-Ras

available online (e.g. COSMIC database for K-Ras mutations available at www.sanger.ac.uk/genetics/CGP/cosmic/ and the p53 database available at www-p53.iarc.fr). These databases reveal that the same mutations that accompany exposure to tobacco smoke carcinogens in cellular studies, such as G→T transversions and G→A transitions, are very prevalent in mutated genes recovered from human lung cancer. In keeping with expectations concerning the role of guanine-derived DNA adducts in driving p53 and K-ras mutations in smokers, G→T transversions are considerably more common in smokers than in nonsmokers.

Expectations have run high concerning the possibility that the distribution of mutations within cancer genes might reflect tendencies for specific carcinogens to induce 'fingerprint mutations' in particular codons within target genes (refer to Fig. 8.9 in Chap. 8). For example, mutation 'hotspots' in the p53 gene in smoking-related lung cancers occur at codons 157, 158, 245, 248, 249 and 273. Various techniques are used to confirm preferential reactivity of electrophilic metabolites with guanine bases within these mutation-prone codons. For example, use of the UvrABC nuclease incision method as well as mass spectrometry has confirmed that diol-epoxide metabolites of PAH react preferentially at p53 hotspots which show a high frequency of G mutations in smoking-related lung cancers. These results strongly support the involvement of PAH in lung cancer aetiology in smokers.

Studies of the distribution of K-Ras mutations in human lung cancers have also confirmed a likely role for adducts formed by PAH and NNK in the induction of G→T transversions and, to a lesser extent, G→A transitions within commonly mutated sites such as codon 12. G→T transversions at the first position of codon 12 in K-ras are clearly associated with PAH exposure, while G→A transitions within this codon may be induced by NNK or other TSNAs. However, while such associations are intriguing, some caution is required during the interpretation of human mutation data since multiple genotoxic carcinogens have been shown to mutate codon 12 of K-Ras. Yet, while the question of which carcinogen is specifically responsible for individual human mutations is hard to definitively answer given the multiplicity of pathways to guanine damage in the smoke-exposed lung, it is safe to conclude tobacco smoke carcinogens account for most guanine mutations in abnormal growth regulatory genes recovered from human lung cancers.

10.8 Toxicogenomic Responses

The rich chemical complexity of tobacco smoke ensures that relying on a limited number of cellular responses – such as a mutation in a solitary gene – as representative of the toxic effects of tobacco is risky. The availability of 'omics' technologies allowing the behaviour of hundreds or thousands of mRNA transcripts, miRNAs, proteins or cytokines and chemokines to be monitored simultaneously using various array platforms is ideally suited to the toxicological evaluation of highly complex

mixtures such as tobacco smoke. Newer technologies such as transcriptome sequencing (RNA-Seq) may also provide powerful insights into complex cell responses to tobacco smoke by permitting measurement of both coding and noncoding transcripts.

Array-based approaches can allow a range of questions to be addressed which are unanswerable using traditional methodologies. For example, microarrays recently allowed investigation of the dose dependence of transcriptional responses to smoke released from different brands of cigarettes in mouse lung epithelial cells. These studies revealed that patterns of gene dysregulation induced by tobacco smoke are highly dose responsive, with effects at low doses dominated by compensatory changes that include upregulation of antioxidant defence pathways, stimulation of cell growth and boosted expression of xenobiotic-metabolism genes (e.g. CYPs). Following exposure to higher concentrations of smoke extracts, the transcriptional response was dominated by cell death activation, cell cycle arrest, activated DNA repair and DNA damage response signalling.

Gene microarrays also allow study of the effects of tobacco smoking under more complex exposure scenarios including rodents exposed to whole tobacco smoke or tissue samples collected from human smokers either as lung autopsy or biopsy samples. The decreasing cost and rising availability of microarray approaches is also allowing the use of these approaches to analyse mRNA transcript profiles in cells extracted from whole populations of active smokers. Recent studies of gene expression changes in cells extracted from the buccal cavity of smokers hold great promise for the identification of biomarkers that predict the impact of pharmacological or dietary interventions in smokers.

10.9 Conclusion

The achievements surveyed in this chapter show how core concepts that first emerged within the context of modern toxicology – such as bioactivation, macromolecular adduction and toxicity signalling – can powerfully assist scientific investigations of major human diseases that take enormous toll on the health of individuals and whole societies. By summarising advances made during ongoing efforts to understand the chemical and biochemical mechanisms underlying the ravaging effects of inhaled tobacco smoke on the human body, it became apparent that the major toxicological responses to tobacco are likely driven by a relatively select subset of noxious chemicals. Recent decades witnessed major improvements in our understanding of the detailed metabolic changes tobacco-derived carcinogens undergo in the body and of the types of cellular and genetic damage they elicit. By uncovering new disease markers and molecular participants in the pathogenesis of tobacco-related disease, these improved insights may assist the development of clinical interventions for reversing the ravaging effects of tobacco smoke upon human tissues.

Going Further

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