Pharmaceuticals in the Environment: The Effects of Clofibric Acid on Fish

A thesis submitted for the degree of Doctor of Philosophy

By

Tamsin Runnalls

Institute for the Environment, Brunel University,
Uxbridge, Middlesex, UB8 3PH

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Declaration

The work submitted in this thesis was carried out between 2000 and 2004 at Brunel University (Uxbridge, Middlesex). This work was carried out independently and has not been submitted for any other degree.

ABSTRACT

Brunel University, Uxbridge, Middlesex, UB8 3PH
Institute for the Environment
Tamsin Runnalls

Pharmaceuticals in the aquatic environment is an emerging issue and the risks they pose are mostly unknown. They are used in large amounts throughout the world and can enter the environment, as the active metabolite or unmetabolised, through excretion by people and improper disposal. As these drugs are designed to have specific biological effects in a specific organism (as well as sometimes having other non-specific side effects), their potential to cause effects within the environment is great. Clofibric acid (the major metabolite of the lipid lowering drug, Clofibrate) is non-biodegradable, highly motile, very persistent and frequently detected at μg/l levels in the environment.

I studied possible effects of clofibric acid in fish, using different experimental approaches and endpoints. The studies involve two different species, and for one of these species, fish at different stages of development. The chapters within this thesis have presented the first evidence (albeit preliminary) of clofibric acid having effects on both adult and embryo fish.

When fathead minnow embryos were exposed to clofibric acid, the effects seen included changes in the eggshell, time to hatch, hatchability, mortality and viability. Adult fathead minnow were similarly exposed and significant effects on specific parameters were also observed. These included effects on lipid metabolism, steroidogenesis and spermatogenesis - thought to be via cholesterol transport - as well as significant effects on the expression of several genes involved in lipid metabolism and detoxification. Exposure of juvenile (sexually undifferentiated) bream also found significant differences in some endpoints. Other results suggested, less pronounced effects of clofibric acid on some other parameters.

The results from this research show that there are effects of clofibric acid in pathways which were not only unexpected in fish (for example, steroidogenesis, spermatogenesis and gene expression), but also at concentrations below those previously shown to have any biological effects on fish. These effects indicate that clofibric acid may potentially have an impact on fish fecundity, and even more worryingly, on human health for those people prescribed it.

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Abbreviations

AWQC	Ambient water quality criteria
BAF	Bioaccumulation factor
CASA	Computer Assisted Sperm Analysis
cDNA	Coding DNA
CPRG	Chlorophenol-red-β-D-galactopyranoside
CYP	Cytochrome p450
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
dpm	decays per minute
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuracil triphosphate
EC	Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
EE2	Ethinyloestradiol
EPA	Environmental Protection Agency
ETOH	Ethanol
G6PDH	Glucose-6-Phosphate Dehydrogenase
GSI	Gonadosomatic Index
HDL	High Density Lipoprotein
HSI	Hepatosomatic Index
IP	Intra Peritoneal
LCMS	Liquid Chromatography Mass Spectrometry
LDL	Low Density Lipoprotein
LOEC	Lowest Observed Effect Concentration
LPL	Lipoprotein Lipase
MB	Maximum Binding
MLT	Molecular Light Technologies
mRNA	Messenger RNA
NAD	Nicotinamide Adenine Dinucleotide
NBT	Nitro Blue Tetrazolium
NOEC	No Observed Effect Concentration
NSB	Non-Specific Binding
NTC	Non-Template Control
PCR	Polymerase Chain Reaction
PMS	Phenazine Methosulfate
PMSF	Phenylmethylsulphonylfluoride
RNA	Ribonucleic Acid
RT-PCR	Reverse -Transcriptase PCR
SEM	Standard error of the mean
STW	Sewage treatment works
TGRL	Triglyceride rich lipoproteins
VHDL	Very High Density Lipoprotein
VLDL	Very Low Density Lipoprotein
VTG	Vitellogenin
Zrp/ZP3	Zona radiata protein/3

Chapter 1: General Introduction

Anthropogenic contaminants are present worldwide in the aquatic environment. These pollutants range from those of historic concern, like organochlorine pesticides, polychlorinated biphenyls, polychlorinated dioxins and furans and polycyclic aromatic hydrocarbons, to emerging contaminants such as pharmaceuticals and personal care products (Petty, *et al*, 2004). During the last three decades, assessments of the impact of chemical pollution has focused almost exclusively on 'priority' pollutants, such as endocrine disrupting, carcinogenic and toxic chemicals. These are, however, only a small piece of a larger puzzle (Daughton, and Ternes, 1999).

Pharmaceuticals represent a large group of chemicals that have until recently received little attention; they are used in large amounts throughout the world and their usage is increasing exponentially. In the UK alone, for example, there are currently 3000 active compounds used as constituents of medicinal products, which comprise a wide range of chemical structures (Ternes, 2001, Ayscought *et al*, 2000). They enter the environment, some excreted unmetabolised, some as the active metabolite (after escaping degradation in waste water treatment), and some through improper disposal of industrial waste.

In more recent years, a number of research groups have reported the presence of these pharmaceuticals in the aquatic environment (Snyder *et al*, 2003). They include: steroids, anti-inflammatory, anti-cancer, lipid regulators, anti-epileptic, antibiotics and painkillers. Clofibric acid, a lipid lowering drug, was one of the first drug residues to be found in the environment, back in 1976, when US researchers analysed domestic sewage (ENDS, 2000). Little is currently known about the environmental impact of these drugs and there is currently no regulation or monitoring of their entry into the environment

(although as part of registration for new drugs, companies are now required to conduct environmental risk assessments (Ayscought, *et al*, 2000)). As these drugs are developed to have a biological effect, their potential to cause effects within the environment is great.

1.1 General pharmaceutical use in the UK

In the UK, records of drug use are kept by the Department of Health (for prescription drugs) and the Proprietary Association of Great Britain (for over-the-counter drugs), both of which only keep a record of drug use in terms of the number of prescription items issued. This type of data is therefore not very useful when trying to determine the amount of drugs (or their metabolites) present in the aquatic environment. It does, however, give indications of the groups of drugs that have the potential to enter the environment. The data in Table 1.1 below are likely to be an underestimation of total drug use, because they do not include over-the-counter drugs or illegally acquired drugs.

Compound name	Therapeutic use	Amount used per year (kg)
Paracetamol	Analgesic	390,954.26
Metformin hydrochloride	Anti-hyperglycaemic	205,795.00
Ibuprofen	Analgesic	162,209.06
Amoxycyllin	Antibiotic	71,466.83
Sodium valporate	Anti-epileptic	47,479.65
Sulphasalazine	Antirheumatic	46,430.43
Mesalazine (systemic)	Treatment of ulcerative colitis	40,421.72
Carbamazepine	Anti-epileptic	40,348.75
Ferrous sulphate	Iron supplement	37,538.52
Rantidine hydrochloride	Anti-ulcer drug	36,319.24
Cimetidine	H_2 receptor antagonist	35,654.20
Naproxen	Anti-inflammatory	35,065.98
Atenolol	β-blocker	28,976.55
Oxytretracycline	Antibiotic	27,195.11
Erythromycin	Antibiotic	26,483.78
Diclofenac sodium	Anti-inflammatory and analgesic	26,120.53
Flucloxacilin sodium	Antibiotic	23,381.47
Phenoxymethylpenicillin	Antibiotic	22,227.59
Allopurinol	Antigout drug	22,095.64
Diltiazem hydrochloride	Calcium antagonist	21,791.50
Gliclazide	Antihyperglycaemic	18,783.11
Aspirin	Analgesic	18,105.89
Quinine sulphate	Muscle relaxant	16,731.26
Mebeverine hydrochloride	Antispasmodic	15,497.35
Mefenamic acid	Anti-inflammatory	14,522.77

Table 1.1: The 25 most used pharmaceuticals by weight in England in 2000. Taken from Jones et al, (2002).

1.2 Pharmaceuticals present in environment

Pharmaceuticals have probably been around in the environment for as long as they have been in use. It was not until the early nineties that the analytical techniques needed to accurately detect these compounds in complex environmental samples such as sewage or river waters were developed. As methods improve (sensitivity of methods increases), lower and lower levels are detectable. Consequently, more and more of these compounds have been detected in the environment. Currently more than 80 have been detected.

The concentrations of drugs that have been found routinely in surface waters and sewage effluent vary, but are in trace amounts (at the ng/l level, or at the most the low µg/l level). Some studies (associated with older landfills over vulnerable aquifers) have also found them to be present in ground water and in drinking water (Heberer and Stan, 1996; Fielding *et al*, 1981). Currently, no quantitative data have been reported on the presence of pharmaceuticals in sewage sludge (probably due to analytical difficulties), but this is a highly likely route for the pharmaceutical substances to enter the terrestrial environment.

1.3 Routes of entry

The routes of entry of pharmaceuticals into the environment are summarised in Figure 1.1. The main routes for human pharmaceuticals entering the environment are expected to be through use by patients in hospitals, medical centres, or in the community, or through the disposal of unused or out-of-date drugs (via toilet or drain), landfills (domestic rubbish) or as licensed waste (Ayscought *et al*, 2000). Another route becoming increasingly important is aquaculture. Over-feeding, loss of appetite (by diseased fish) and poor absorption leads to the introduction of drugs via the feed, which are consequently discharged directly into the water (up to 70% of the drugs administered; Diaz-Cruz *et al*, 2003).

In humans, most pharmaceuticals, once administered, are degraded in the body, and may even become inactivated. Others, particularly those excreted renally or those not absorbed fully by the body, can leave in their active form. Drugs can be excreted as either the parent compound, water-soluble conjugates or as metabolites. These then enter the aquatic environment via the sewage system, where they can have their unknown

effects. The effects and side effects specified in humans may not automatically correlate to the effects that may occur in organisms exposed to the drugs in the environment.

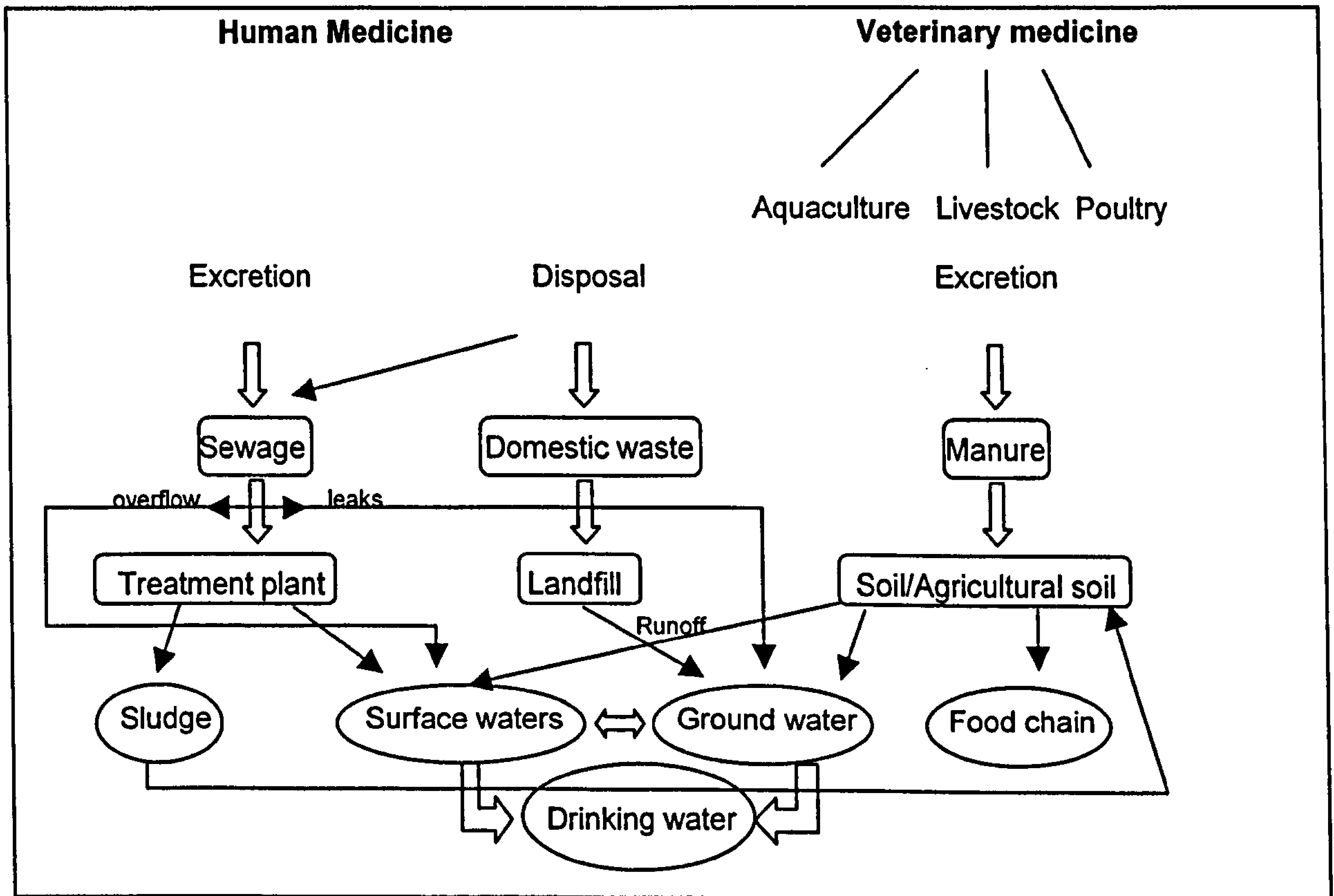


Figure 1.1: Principal routes into the environment for human and veterinary drugs (adapted from Diaz-Cruz *et al*, 2003).

1.4 Drugs found to be present in the environment

1.4.1 Introduction

Low levels of reproductive hormones, steroids, antibiotics and numerous prescription and non-prescription drugs as well as their metabolites have been detected in European and US waters (Erickson, 2002). Only a few have been shown to be completely degraded during the sewage treatment (ENDS, 2000; Daughton and Ternes, 1999) and generally most are lipophilic; these properties lead to the potential for bioaccumulation and persistence in the environment (Christensen, 1998). As little is known about their mode of action or effect on

non-target organisms, it is difficult to predict what health effects they may have individually or collectively on aquatic organisms that are exposed to them. Even apparently slight, non-significant influences on single parameters, which would probably not result in any acutely discernable effect, might ultimately affect a whole population by their negative consequences on fitness, disturbance of hormonal homeostasis (e.g. endocrine disruption), immunological status, signal transduction or gene regulation (Seiler, 2002). Daughton and Ternes (1999) also indicate that some drug residues in the environment could have subtle behavioural effects that could be undetectable or overlooked, but may nonetheless result in a population decline.

In general, antibiotics, prescription drugs and reproductive hormones have been found at similar detection frequencies and non-prescription drugs at higher frequencies. This is almost certainly because the latter have a higher annual use compared to the other pharmaceuticals (Kolpin *et al*, 2002). However, even at low levels, effects can occur: levels as low as <0.001µg/l of reproductive hormones (e.g. ethinylestradiol; Lange *et al*, 2001) have been shown to have deleterious effects in aquatic organisms. Also, it is not currently known what the effects of long-term exposure are, both on adult fish and during the different stages of the life cycle (for example, during development).

The occurrence of these pharmaceuticals in the environment has instigated numerous studies all over the world. Various classes of prescription drugs have been detected at up to µg/l levels in sewage, surface and ground water, and these results are discussed below.

1.4.2 Contraceptive steroids

Natural and synthetic oestrogens are amongst the most frequently used drugs and these contribute to the highly publicised oestrogenic effects of sewage effluents on fish (Purdom *et al*, 1994, Harries, *et al*, 1997) and other wildlife (Colborn *et al*, 1992, 1996). Many studies have consequently been carried out to look for effects of these oestrogenic chemicals. Effects reported have included feminisation in male fish (Jobling *et al*, 1998), skewed sex ratios (Sumpter and Pickering, 2005), oocytes in the testes of male fish living downstream from sewage treatment works (Nolan *et al*, 2001), male fish producing vitellogenin (Purdom *et al*, 1994), retardation of the growth and development of the testes in maturing male fish (Jobling *et al*, 1995), and reduction of the number of eggs oviposited in female fish (Harries *et al*, 2000; reviewed in Tyler *et al*, 1998).

Although generally low, significant levels (i.e. biologically active) of synthetic oestrogens such as ethinylestradiol (EE2) and mestranol have been found in sewage effluents (ENDS, 2000). These synthetic steroids are often prescribed as oral contraceptives, but because of their very high potency, the total amounts sold are relatively low (Heberer, 2002). In Germany, Ternes *et al* (1999) predicted that annual prescriptions only totalled 50kg EE2/year. This indicates that the levels expected in the environment would only be in trace amounts (low ng/l range), and this has generally been shown to be true. Synthetic oestrogens have been reported in UK sewage effluent (Desbrow *et al*, 1998, Aherne and Briggs, 1989) at levels from non-detectable to 7.0 ng/l, with the majority of the samples having concentrations below detection limits. Belfroid (1999) and Larsson (1999) identified Dutch and Swedish levels which were similar to those in the UK (7.5 ng/l and 4.5ng/l, respectively). In 1999,

however, Ternes *et al* reported quite high levels compared with the UK, with concentrations of EE2 from non-detectable to 42 ng/l in Canadian effluents (with the median being 9ng/l) and 15 ng/l in German effluents. Stumpf (1996) found 17ng/l (median) in German sewage effluents, with the maximum being 62ng/l. Generally, the levels of EE2 found in the UK, Germany, the Netherlands and US are usually between 1 and 3 ng/l (and usually lower – below detection limits) in both sewage effluent and influent (Heberer, 2002). However, although these synthetic hormones are found at trace levels, effects of these hormones have been shown to occur in fish *in vivo* at levels as low as 0.1ng/l (Purdom *et al*, 1994).

In rivers, the highest level of EE2 reported was by Kolpin *et al* (2002), who reported levels up to 830ng/l with a median of 73ng/l of EE2 in 139 USA rivers (although these levels were considered to be incorrect by Ericson *et al* (2002) based on usage levels, and the data were consequently found to be erroneously published and maximum values were actually 273ng/l with a median of 94ng/l). Belfroid *et al* (1999) reported that rivers and other water bodies in the Netherlands that received treated wastewater contained <0.1-4.3ng EE2/l. In Italy, concentrations of 0.04ng/l (less than the limit of detection) were reported, downstream from a sewage treatment plant, on the river Tiber (Baronti *et al*, 2000). In 1996, Stumpf *et al* detected a maximum concentration of 4.3ng/l, and in 1989, Aherne and Briggs reported 2-15ng/l in UK rivers.

A more worrying finding was that reported by Alder *et al* (2001), who reported a positive detection of EE2 in German ground and drinking water, with levels up to 2.4ng/l.

Mestranol has only been sporadically detected in the environment (sewage effluent) at concentrations up to 4ng/l (Ternes, 1998). Mestranol itself

is not oestrogenic, but it is metabolised in the body into EE2, which is (Christensen, 1998).

Along with the synthetic oestrogens, there has also been a significant amount of focus on natural oestrogens in recent years, including 17β-oestradiol, oestrone, oestriol, and their possible involvement with the endocrine disruption issue in wildlife. Natural steroid oestrogens are very potent (although not as potent as their synthetic counterparts) and have biological activity at low concentrations, including effects on spermatogenesis and regression of the testis (Billard, *et al*, 1981).

Levels of natural steroids found in sewage treatment effluents include reports of 17β-oestradiol from 1-50ng/l and oestrone from 1-80ng/l (Shore *et al*, 1993; Stumpf *et al* 1996; Desbrow *et al*, 1998). Ternes *et al* (1999) reported concentrations of oestrone and oestradiol at mean levels of 70ng/l and 3ng/l, respectively, in German treated effluents, and Canadian effluent at 48 ng/l and 64ng/l, respectively. Belfroid *et al* (1999) also detected levels in the Netherlands of 47ng/l and 1-12ng/l.

In surface waters, levels of oestrone (0.7-1.6ng/l) and 17β-oestradiol (5ng/l) have also been reported in German rivers and streams (Belfroid *et al*, 1999; Ternes *et al*, 1999). Although 17β-oestradiol is used therapeutically for hormone replacement, it is metabolised and excreted in the same way as endogenous 17β-oestradiol. Natural excretion of endogenous hormones is thought to be the main route of entry of the oestrogens into the environment (women excrete between 10 and 100μg of oestrogen daily, depending on the phase of their menstrual cycle, and pregnant women excrete even more).

The presence of these oestrogens (both natural and synthetic) is very important, as levels detected have been shown to produce effects in fish, and

consequently have the potential to affect wild populations of aquatic organisms (reviewed in Tyler *et al*, 1998).

1.4.3 Antidepressants

Antidepressants have been shown to be present in the environment and have also been shown to have effects on organisms living in it. The drugs found include selective serotonin reuptake inhibitors (SSRIs), which in people increase the levels of serotonin in the brain by preventing reabsorption. Because of the critical nature of the functions of serotonin, there is the potential for environmental SSRIs to alter appetite, the immune system, reproduction, as well as other behavioural functions, in organisms that receive exposure. There are therefore many potential effects of chronic exposure.

In surface waters and municipal effluents, fluoxetine (the active ingredient of Prozac) has been detected at levels of 0.012 and 0.540µg/l, respectively, although the magnitude and prevalence in the environment has not been fully explored (Brooks *et al*, 2003). Fluoxetine has been shown to induce spawning in mussels at levels below 1µg/l, increase egg size in crustaceans and alter sex organ maturation in crabs. It has also been shown to affect behaviour in animals such as squid and lobsters (Brooks *et al*, 2003). The fluoxetine metabolite, norfluoxetine, has not yet been reported in the environment.

1.4.4 Analgesics/Anti-inflammatories

Among the drugs found in the environment, the nonsteroidal, anti-inflammatory drugs (NSAIDs), including compounds used as analgesics, belong to one of the most important groups of pharmaceuticals world wide, with an

estimated annual production (based on the number of prescriptions) of several kilotons (Cleuvers, 2004). In addition to this, some of these drugs are bought without prescription, so the actual consumption is considerably higher. Of these, paracetamol (acetaminophen) and aspirin (acetylsalicylic acid - ASA) are the two most popular over-the-counter drugs. Webb (2000) reported that the drug used the most in the UK was paracetamol (2000 tonnes/annum) with aspirin next (770 tonnes/annum). ASA has been reported to be readily degraded by deacetylation into its more active form, salicylic acid, and into two other metabolites, ortho-hydroxyhippuric acid and the hydroxylated metabolite gentisic acid. Salicylic acid, ortho-hydroxyhippuric acid and gentisic acid were reported by Ternes (1998) to be present in sewage influent at concentrations up to 54, 6.8 and 4.6μg/l, respectively. He also observed that all three compounds were efficiently removed by the sewage treatment process, and only salicylic acid was detected at very low levels in sewage effluent and rivers. Heberer (in press), however, recently reported that residues of salicylic acid in the environment are not necessarily from ASA, they could also be from ketatolytic, dermatice, natural formation and food preservatives.

In 1996, Stumpf *et al* identified diclofenac (<1.59μg/l), ibuprofen (<3.35μg/l), and ASA (1.51μg/l) in sewage, with lower levels (0.01-0.5μg/l) in river waters. Diclofenac and ibuprofen have also been detected at very low (1-6ng/l) levels in drinking water. In Germany, diclofenac, ibuprofen and phenazone residues have also been detected at trace levels in a few drinking water samples (Heberer, 2002; Heberer *et al*, 2001; Ternes, 2001). Ternes (1998) also reported concentrations of diclofenac, ibuprofen, naproxen, ASA and other compounds (some at concentrations >1μg/l) in wastewater treatment plants and at lower concentrations in surface waters. Furthermore, in long term

monitoring programs of sewage and surface waters samples in Germany, Heberer *et al* (2002) reported diclofenac as one of the most important pharmaceutically active chemicals present in the water cycle. Average concentrations were found to be 3.02µg/l (influent) and 2.51µg/l (effluent). Additional findings of diclofenac and ibuprofen have been reported from Swiss lakes and rivers (Buser *et al*, 1998a,b), Brazil (Stumpf, *et al*, 1999), Spain (Farre *et al*, 2001), Greece and the United States (Heberer *et al*, 2001).

Other analgesics (4-aminoantiyrine, aminophenazone, codeine, fenoprofen, hydrocodone, indometacine, ketoprofen, mefenamic acid, naproxen, phenazone and propyphenazone) have also been detected in sewage and surface waters (Heberer, 2002; Heberer *et al*, 1997, 2001; Ternes, 1998; Stumpf, *et al*, 1996).

1.4.5 Anti-epileptics

Anti-epileptic drugs (such Carbamazepine and Primidone) represent the most dominant of all investigated drugs in well-treated domestic effluents in Arizona and California (Jos *et al*, 2003). They have been shown to be persistent (the removal of the drugs did not seem to occur during travel times of more than 6 years in the subsurface: Drewes *et al*, 2002). Carbamazepine has frequently been detected in municipal sewage plant effluents at concentrations up to 6.3µg/l (Ternes, 1998), in surface samples up to 1.075µg/l, and in ground water samples up to 1.1µg/l, and even in drinking water up to 30ng/l (Heberer, 2002). Primidone has also been found in samples from municipal sewage influents and effluents and in surface waters (up to 635ng/l) in Germany (Heberer, 2002; Heberer *et al*, 2001).

Jos *et al* (2003) state that there are very few reports on the effects of Carbamazepine on aquatic organisms. The results they presented were similar to those from Jones *et al* (2002) who reported that Carbamazepine is toxic for cnidaria and non-toxic for crustaceans and fish.

1.4.6 β-blockers

Another class of drugs present in the environment is the beta-adrenergic receptor-blocking compounds (β-blockers), which are extensively used to treat angina and hypertension (Ternes, 1998). These compounds act by binding and blocking the β receptor, without causing receptor activation or stimulation. There are different types of β-blocker, some specific e.g. metoprolol and atenolol, which act via for the β_1 receptor, and some non-specific, e.g. propranolol and nadolol, which have a similar affinity for both β_1 and β_2 receptors. In organisms that lack β adrenoreceptors, β blockers cannot pharmacodynamically affect them (Jones, 2002). Fish have been shown to have β receptors, including various types, in several of their tissues (Huggett *et al,* 2002), and thus in theory β-blockers could affect these organisms.

After ingestion, approximately 10% of the parent compound is excreted (although in the case of atenolol, almost all of the ingested drug is excreted unchanged). Consequently, several beta blockers (e.g. metoprolol, propranolol, betaxolol, nadolol and bisoprolol) have been detected in European sewage effluents at concentrations up to the µg/l level (Hirsch *et al*, 1988; Ternes, 1998; Sedlak and Pinkson, 2001). Of these, metoprolol, propranolol and bisoprolol have also been found in surface water samples (Hirsch *et al*, 1988; Ternes, 1998). Sacher *et al*, (2001) reported that sotalol was detected in three groundwater samples from Germany – to a maximum concentration of 560ng/l.

Huggett *et al* (2002) reported levels in the US to be comparable to those in Europe; propranalol in wastewater effluents was present at concentrations up to 1.9µg/l and metoprolol and nadolol 1.2 and 0.36µg/l, respectively. To date, β-blockers have not been reported in any drinking waters.

From exposure studies using fish, Huggett *et al* (2002) concluded that the levels found in the environment represent a significant hazard. Effects on steroid hormone concentrations, fecundity and egg viability were reported after a 2-week exposure to 1µg propranolol/l. It is thought that because of their low but persistent presence in the environment, these compounds are more likely to have chronic effects, such as behavioural changes, rather than acute toxic effects.

1.4.7 Contrast media

Contrast media are not strictly pharmaceuticals, in that they are not given to patients to treat illness; instead, they are administered during the diagnosis of disease. Iodinated X-ray contrast media are some of the most frequently applied compounds in medicine. They have been identified by Gartiser *et al* (1996) as the main contributor to the loads of total adsorbable organic halogens in clinical wastewaters. In all countries where there is a developed medical care system, it can be expected that X-ray contrast media are present at appreciable quantities in sewage effluents and hence contamination of receiving waters is possible. Iodinated contrast agents are very persistent in the environment and also easily leach into the groundwater aquifers. In sewage effluents, ground and surface waters they have been detected at concentrations up to µg/l, and in some cases they have also been found in drinking water (Heberer, 2002).

1.4.8 Cytostatic drugs

Cytostatic drugs are frequently used in chemotherapy and, therefore mainly originate from hospital applications. Hospital sewage has been shown to contain concentrations of these drugs up to low µg/l levels (Steger-Hartmann *et al*, 1996). In effluents from municipal sewage treatment plants receiving and purifying hospital effluents, they have been found at trace concentrations, mostly at the low ng/l level. They have not yet been detected in surface or drinking waters.

Due to their pharmacological potency, cytostatic drugs often exhibit carcinogenic, mutagenic or embryotoxic properties and consequently their presence in the environment could be potentially hazardous.

1.4.9 Antibiotics

Antibiotics are also an important group of pharmaceuticals, due to the scale of their ubiquitous use worldwide. In addition to the treatment of human infections, they are also widely used in veterinary medicine (Hirsch *et al*, 1999) and sometimes incorporated in the feed as growth promoters in farming livestock. They are probably the most discussed of the pharmaceuticals because of their potential role in the spread and maintenance of (multiple) resistance of bacterial pathogens. This poses a serious threat for public health, as more and more infections can no longer be treated with current antibiotics. Epidemic diseases in hospitals are often caused by resistant strains and at present, more than 70% of all bacteria are insensitive against at least one antibiotic, and many show multiple resistance.

Some 5,400 tonnes of antibiotics were used in human medicine in the EC in 1997 (ENDS, 2000), whilst veterinary use totalled 5,100 (including 3500

tonnes of medicines and 1600 tonnes of growth promoters). Although not very persistent in the environment (these drugs have half lives of a few days or weeks), they are very prevalent, especially in surface waters, as they are soluble and are washed out of the soil. Macrolide antibiotics (e.g. clarithromycin, dehydro-erythromycin [a metabolite of erythromycin], roxithromycin, lincomycin), sulfonamides (sulfamethoxazole, sulfadimethoxine, sulfamethazine, and sulfathiazole), fluoroquinolones (ciprofloxacin, norfloxacin, and enrofloxacin), chloramphenicol, tylosin, and trimethroprim have been found at concentrations up to the low µg/l level in sewage and surface waters (Heberer, 2002; Golet, *et al*, 2001, 2002). However, when monitoring investigations of various sewage, surface and ground water samples in Germany were carried out by Hirsch *et al* (1999), they did not detect penicillin or tetracycline. This result was not surprising, as penicillins are easily hydrolysed and tetracyclines readily precipitate with cations such as calcium and hence tend to accumulate in sewage sludge or sediment.

The environmental concerns of antibiotics are not only based on the potential of resistance but also on their unfavourable ecotoxicological profile (Halling-Sorensen, 2000). There are no publications to date on the issue of the development of resistance of bacteria to the levels of antibiotics present in the aquatic environment.

1.4.10 Lipid lowering drugs

The first ever pharmaceutical detected in the aquatic environment was a lipid lowering drug, first detected in 1976. Since then, bezafibrate, gemfibrozil, clofibric and fenofibric acids (the metabolites of clofibrate and fenofibrate) have all been detected at concentrations up to the µg/l level in sewage effluents and

surface waters (Ternes, 1998; Stumpf *et al*, 1999; Heberer *et al*, 2001; Stan and Heberer, 1997). Bezafibrate and gemfibrozil have also been found in ground water samples at maximum concentrations of 190 and 340ng/l, respectively (Ternes, 2001; Heberer, 2002).

The first lipid lowering drug, cholestyramine, was approved for use in 1964. Clofibrate and dextrothyroxine were released in 1967, followed by colestipol and probucol in 1977 (Clinical pharmacology online, 2000 - www.cponline.gsm.com). Hence, these drugs have probably been present in the environment for the last few decades.

Antilipidemic agents can be subdivided into bile acid sequesterants (cholestyramine, colestipol), HMG-CoA reductase inhibitors (fluvastatin, lovastatin, pravastatin and simvastatin), fibric acid derivatives (clofibrate and gemfibrozil), and some miscellaneous agents (dextrothyroxine, nicotinic acid and probucol). They act in humans either by reducing the production of lipoproteins or by increasing their removal from the blood. The overall effect is to decrease plasma cholesterol levels.

Although there are several chemically unrelated classes of lipid lowering drugs, they all reduce lipoprotein fractions by interfering with the absorption, synthesis and/or metabolism of cholesterol and lipoproteins. Eghdamian and Ghose (1998) state that lipid lowering drugs may be effective in reducing lipids and lipoproteins by one of the following ways:

1. by reducing the synthesis of VLDLs and LDLs
2. by enhancing VLDL clearance
3. by enhancing LDL catabolism
4. by inhibiting the HGM-CoA reductase enzyme

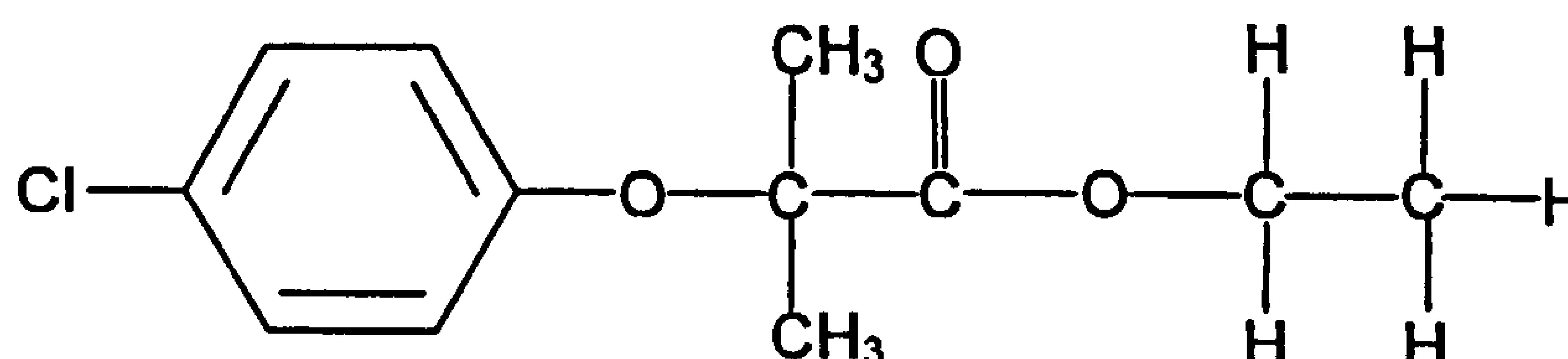
As this thesis focuses on the possible ecotoxicological effects of clofibric acid, this drug will be discussed in greater depth.

1.5 Clofibrate and Clofibric acid

1.5.1 Background

Clofibrate (ATROMID-S) is an orally administered antilipemic agent; the parent compound was discovered during the screening of aryloxyacetic acid derivatives. It was licensed for human use in 1962 and given FDA approval in 1967 (Catapano, 1995; Clinical pharmacology, 2000). Since then, second, third and fourth generation compounds have been developed with higher potency and fewer side effects.

Figure 1.2: The structure of Clofibrate.



Clofibric acid (Figure 1.3) is the major metabolite of the lipid lowering drugs Clofibrate (Figure 1.2), Etofibrate and Etofyllinclofibrate. It is structurally similar to the herbicide Mecoprop.

Figure 1.3: The structure of Clofibric acid.

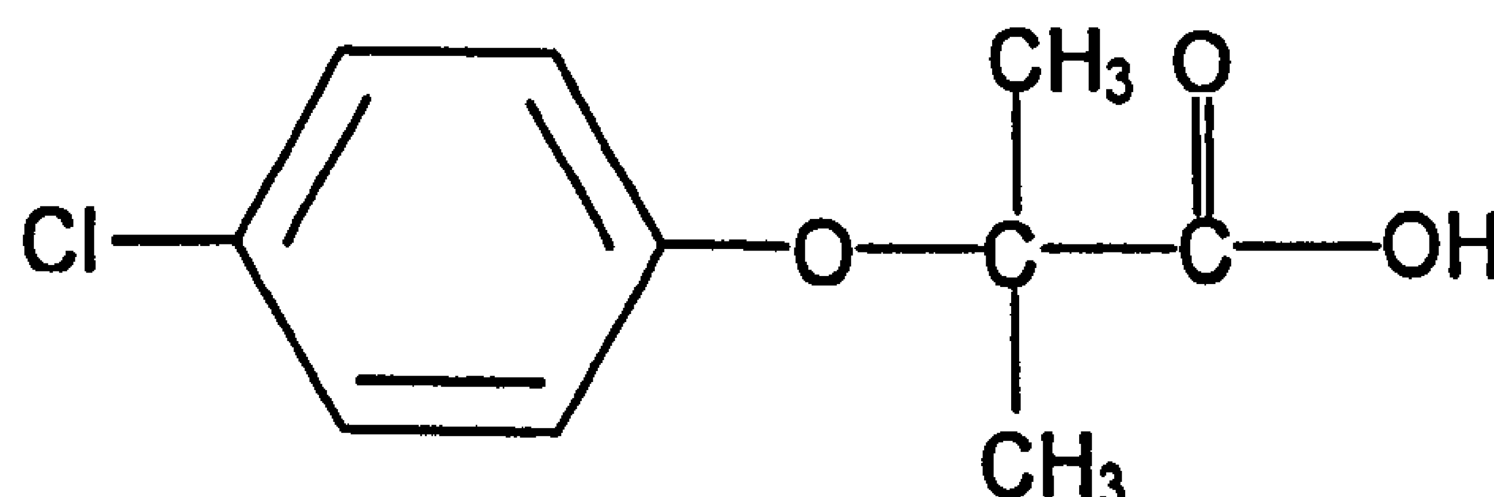


Table 1.2: Physical properties of Clofibrate and Clofibric acid.

Name	Chemical formula	Molecular weight	Solubility	Log K_{ow}	CAS RN
Clofibrate	$C_{12}H_{15}ClO_3$	242.7	Insoluble in water, soluble in DMSO or ETOH	3.62 (Sanderson *et al*, 2003)	637-07-0
Clofibric Acid	$C_{10}H_{11}ClO_3$	214.04	582.5 mg/l (Ferrari *et al*, 2003)	2.57 (Ferrari *et al*,2003) 2.84 (Sanderson *et al*, 2003)	882-09-7

As already mentioned, clofibric acid was the first prescription drug metabolite ever reported in sewage influent and effluent in Kansas City, USA in 1976 (Hignite and Azaznoff, 1977). It was detected in the environment, as a consequence of the routine analysis for acidic pesticides (Ayscought, *et al*, 2000), and is still the most frequently detected drug in sewage effluents, ground water, surface and drinking water all over the world (Koutsouba *et al*, 2003).

1.5.2 Motility

Clofibric acid has been found to be highly motile and very persistent in the environment (a half life of 21 years has been reported), because it is non-biodegradable (Richardson and Bowron, 1985). It is still detected in lakes and rivers, even though Clofibrate is rarely prescribed nowadays as other drugs have replaced it (Zuccato, 2000; IARC, 1996). It has been found to have a water residence time of 1–2 years (Buser and Muller, 1998), whereas in human plasma the half-life is about 17 hours, from where it is excreted in the urine, predominantly in the form of a glucuronide conjugate.

The presence of clofibric acid in the environment is summarised in Table 1.3, which shows that most measurements have been made in Germany. In addition to these records, it has been detected down to depths of 125m below

sewage farm areas in Germany (Heberer, 2002). The results are relatively consistent: clofibric acid is often detected in aqueous samples, usually at concentrations in the high ng/l range, but occasionally in the low µg/l range.

In laboratory experiments using soil columns, Scheytt *et al* (2001) found that clofibric acid did not show any significant sorption. It leached almost tracer-like through the soil columns without retardation (Heberer, 2002). This is confirmed by other studies, where clofibric acid was reaching water supply wells without being removed in the subsoil (Heberer *et al,* 2001). Concentrations of bezafibrate, on the other hand, have been found to be easily attenuated during bank filtration (Heberer *et al,* 2001).

Table 1.3: Concentrations of Clofibric acid present in the environment. Adapted from Schulman, 2002. Where ND – Not detected; NA – Not available; LOD – Limit of detection; * - Median, 90 percentile; *** - maximum; ** - median maximum; + - positive identification but concentration not quantified.

Compound	Medium	Number of samples	Conc. detected (ng/l)	LOD (ng/l)	Country	Reference	Year
Clofibrate	Sewage	20	ND	100	Germany	Ternes	1998
Clofibrate	•	5	ND	1	Germany	Kalbfus	1997
Clofibric acid	•	7	2540-9740	140	US	Hignite & Azarnoff	1977
Clofibric acid	•	NA	3μg/l	NA	USA	Garrison	1976
Clofibric acid	•	NA	Up to 4550	NA	Germany	Stan & Heberer	1997
Clofibric acid	•	NA	ND-2050	250	Germany	Stan & Heberer	1997
Clofibric acid	•	NA	ND-1030	NA	Germany	Stan & Heberer	1997
Clofibric acid	•	37	270,660,1560*	50	Germany	Stumpf *et al*	1996
Clofibric acid	•	NA	450,680	NA	Germany	Heberer *et al*	1998
Clofibric acid	•	49	360,720,1600*	40	Germany	Ternes	1998
Clofibric acid	•	NA	102,1030**	50	Brazil	Stumpf *et al*	1999
Clofibric acid	•	18	ND	NA	Canada	Metcalf	2003
Clofibric acid	•	5	420***	1	Germany	Kalbfus	1997
Clofibrate	Surface	36	ND	30	Germany	Ternes	1998
Clofibrate	•	10	ND	0.5	Germany	Kalbfus	1997
Clofibrate	•	NA	40	5-10	UK	Richardson & Bowron	1985
Clofibric acid	•	NA	ND-1750	1	Germany	Stan & Heberer	1997
Clofibric acid	•	NA	140-180	NA	Germany	Stan & Heberer	1997
Clofibric acid	•	NA	ND-300	1	Germany	Stan & Heberer	1997
Clofibric acid	•	NA	180***	5	Germany	Stumpf *et al*	1996
Clofibric acid	•	28	ND-875	NA	Germany	Heberer *et al*	1998
Clofibric acid	•	43	66,210,550*	10	Germany	Ternes	1998
Clofibric acid	•	NA	19-222	1-10	Germany	Stan, Heberer *et al*	1994
Clofibric acid	•		0.01-1.35, 18		Germany/ North sea	Weigel *et al*	2002, 2003
Clofibric acid	•	NA	ND-9	NA	Switzerland	Buser *et al*	1998
Clofibric acid	•	10	ND	1	Germany	Kalbfus	1997
Clofibric acid	•	NA	90***	10	Brazil	Stumpf, Ternes *et al*	1999
Clofibric acid	•	NA	30	NA	Italy	Herberer & Stan	1997
Clofibric acid	•	NA	ND	1.5	Italy	Zuccato *et al*	2000
Clofibrate	Ground	3	ND	0.5	Germany	Kalbfus	1997
Clofibric acid	•	17	70-7300	NA	Germany	Heberer *et al*	1997
Clofibric acid	•	NA	4000	NA	Germany	Herberer & Stan	1997
Clofibric acid	•	3	ND	0.5	Germany	Kalbfus	1997
Clofibric acid	•	NA	300-1600	NA	Germany	Bouwer	1982
Clofibric acid	•	18	~50-2900	NA	Germany	Heberer *et al*	1997
Clofibrate	Drinking	3	ND	0.5	Germany	Kalbfus	1997
Clofibric acid	•	NA	+	NA	UK	Fielding *et al*	1981
Clofibric acid	•	25	1,24,70*	1	Germany	Stumpf *et al*	1996
Clofibric acid	•	NA	165***	1-10	Germany	Stan *et al*	1994
Clofibric acid	•	3	ND	0.5	Germany	Kalbfus	1997
Clofibric acid	•	NA	ND-170	NA	Germany	Herberer & Stan	1997
Clofibric acid	•	NA	270***	1	Germany	Herberer & Stan	1996
Clofibric acid	•	NA	ND-5.3	1.5	Italy	Zuccato *et al*	2000

1.5.3 Usage

The recommended human clinical dose of Clofibrate is 2g daily, delivered as 2-4 doses (Boyd, 2001; IARC, 1996); the lowest clinically active dose is reported to be 500mg/day (Perlemuter *et al*, 1975). Currently in the UK, it is difficult to obtain a reliable estimate of the use of clofibrate in tonnes per year, as it is not regularly prescribed, although it has been reported that in 1995 it was 1.5 ton/annum. In Germany, however, in 1992-3 it was 30 ton per year. The Ends report (2000) states that clofibric acid loads imposed on typical sewage works are estimated in the order of kilograms per day. The removal rate in sewage treatment works (STW) has been found to be 15-51% (Schowanek and Webb, 2002). Clofibric acid has a lower bioaccumulation factor (BAF) (372) than Clofibrate (4170), therefore the ambient water quality criteria (AWQC) is an order of magnitude higher (1.93 and 0.22mg/l, respectively; Schulmann, 2002).

Although no longer used in the UK (as other lipid lowering drugs have replaced it), Clofibrate is still widely used in France (Laville, 2003). Having said this, it is reported (Ferrari, 2003) that Clofibric acid has not recently been detected in effluents from Lyon in France, perhaps because it has been replaced with fenofibrate, bezafibrate or gemfibrozil in that country.

1.5.4 Mode of action

In humans, Clofibrate has been found to reduce elevated plasma concentrations of triglycerides by 30-60%, by up-regulation of lipoprotein lipase (LPL) and, to a lesser extent, cholesterol by 20-25% (Blane, 1987). It has also been associated with a moderate decrease in LDL cholesterol and an increase in HDL cholesterol (Staels *et al*, 1998). It is not effective in patients with high

plasma cholesterol but a normal triglyceride concentration (Eghdamian and Ghose, 1998).

The wide spectrum of lipid modulating actions of fibrates is mediated by their capacity to mimic the structure and biological functions of free fatty acids: they interfere with fatty acid synthesis, and stimulate hepatic fatty acid oxidation, thus reducing the amount of fatty acid available to the liver for triglyceride synthesis. They can inhibit cholesterol biosynthesis by blocking incorporation of acetate into cholesterol; they induce the hepatic uptake of cholesterol from plasma and enhance its elimination in the bile as bile acids and they also increase lipoprotein lipolysis by inducing liver LPL expression and lowering Apo CIII production (Guo *et al*, 2001).

Fibrates are known to act at the molecular level by altering transcription of genes encoding for proteins involved in lipid metabolism (Staels *et al*, 1998). This is facilitated by them activating specific transcription factors, the Peroxisome Proliferator Activated Receptors (PPAR), which are members of the nuclear hormone receptor family of transcription factors (see Figure 1.4). These bind specifically to response elements in the regulatory regions of a spectrum of target genes, causing them to be up or down regulated (Chapman, 2003; see Figure 1.4 and Table 1.4).

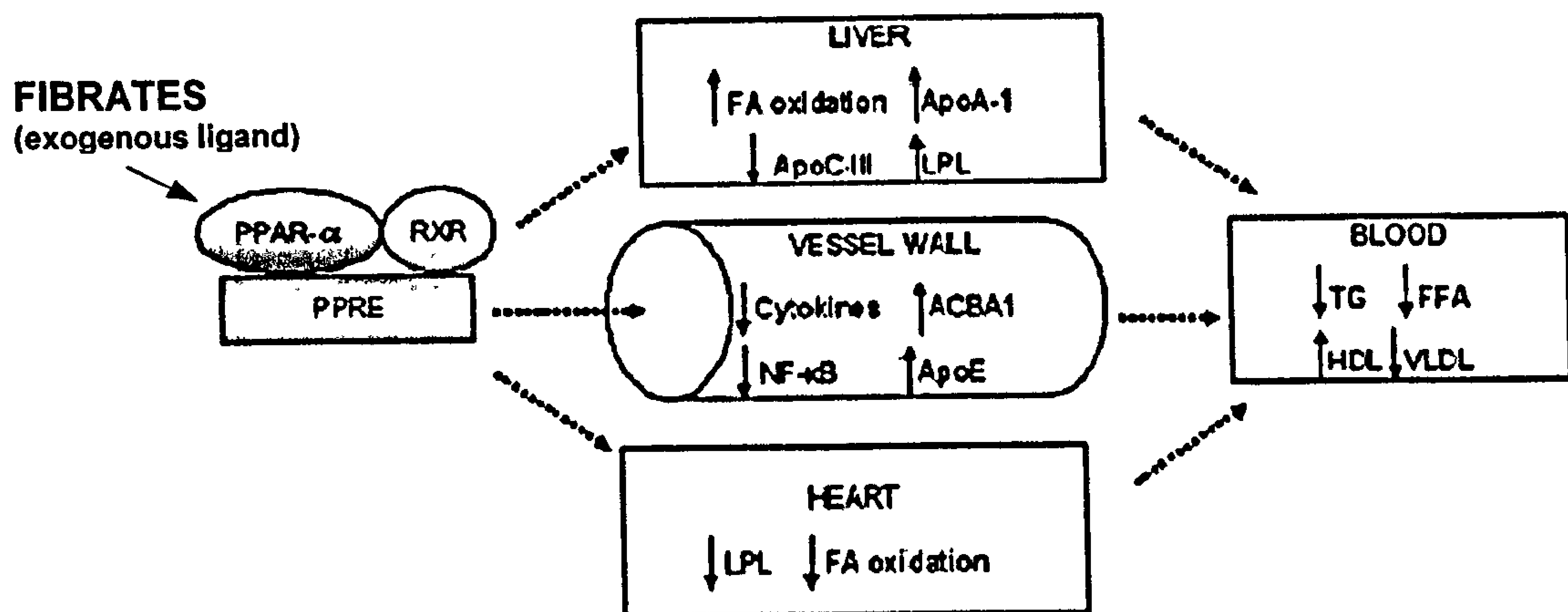


Figure 1.4: Mechanisms of action of fibrates. PPARα exerts its multiple effects in the liver, heart and vessel wall of humans and rodents by regulating expression of corresponding genes (Kota *et al*, 2004). Fibrates reduce triglyceride concentrations by changing the transcription rates of different genes (PPAR: Peroxisome Proliferator-Activated Receptor; PPRE: Peroxisome Proliferator Response Element; RXR: Retinoid X Receptor; TG: Triglyceride).

Table 1.4: Major genes implicated (in humans) in the PPAR-mediated action of fibrates on lipid metabolism (adapted from Chapman, 2003).

Target Gene	Function of gene product	Gene expression
Apolipoprotein CIII	Inhibitor of VLDL clearance	↓
Lipoprotein lipase	Lipolysis, TGRL	↑
Apo AI	HDL formation	↑
Apo AII	HDL formation	↑
Fatty acid binding protein	Cellular fatty acid uptake	↑
Acyl CoA synthase	Fatty acid activation; acyl CoA esters	↑
β-oxidation pathway	Fatty acid oxidation	↑
Acetyl CoA carboxylase	Fatty acid synthesis	↓

PPARs are known to be key messengers in the translation of nutritional, metabolic and pharmacological stimuli into changes in gene expression (Schoonjans *et al.*, 1997), and are primarily involved in energy balance, modulating fatty acid oxidation in liver and lipid accumulation in adipose tissue (Latruffe and Vamecq, 1997). PPAR genes have been previously identified in Atlantic salmon and plaice (Ruyter *et al.*, 1997; Leaver *et al.*, 1998) and thus probably also have a role in regulating lipid metabolism in fish. In fact, Perkins

and Schlenk (1998) suggested that after a 24hr waterborne exposure to Clofibric acid (0, 10,50, 100, 225mg/l), catfish display a PPAR response similar to that observed in mammals. Chapter 6 goes into further detail on PPARs.

1.5.5 Toxicology studies/Side effects in humans

In humans the side effects of treatment with clofibrate include: nausea, headaches, dizziness, fatigue, drowsiness, weakness, fever, dry skin, breast tenderness in men, brittle hair, rash, alopecia, pruritus, toxic epidermal necrolysis, and erythema (AHFS, 2000). A decreased libido and impotence have also been reported (Martindale, 1999) and treatment has also been associated with hepatic tumours and certain cardiovascular disorders.

In a study in 2001, Dietrich reported that the acute toxicity of clofibric acid in rat, mouse and human is between 800 and 1200 mg/kg. He also found that the chronic toxicity (found from a 2 year rat study) of clofibric acid produced a no observed adverse effect level (NOAEL) of 17-220 g/kg based on the cumulative dose. Along with this he also stated that chronic toxicity of clofibric acid induces promotion of liver tumours in rats, by the interaction of clofibric acid with the PPAR receptor.

In reproduction studies it has been previously documented that doses approximately two times the maximum recommended human dose of clofibrate instigates the arrest of spermatogenesis in dogs and monkeys (RxList, 2002 - www.RxList.com), whereas Schulman *et al* (2002), suggest that this same effect occurs at four to six times the maximum recommended dose.

Peroxisome proliferators (such as phthalates) have previously been shown to induce pathological changes in the reproductive system in rodents (and exert subtle effects on fertility and litter size). As fibrates are known

peroxisome proliferators, their effects may well be similar. Treatment with ciprofibrate has been shown to alter gene expression in the Leydig cells of rats, however no changes were seen in the seminiferous tubule or in peroxisomal volume. The treatment of Clofibrate has been found to alter cell growth, gene expression and inhibit progesterone synthesis in human cell lines (Gazouli *et al*, 2002).

It has also been shown by Gazouli *et al* (2002), in mouse cell lines, that peroxisome proliferators (Bezafibrate) exhibit antiandrogenic properties through the inhibition of the hormone-induced rate-delimiting step in steroid biosynthesis, the transport of cholesterol into the mitochondria. They found that the testicular Leydig cell is one of the target cells of peroxisome proliferators. Alterations of Leydig cell function and testosterone formation could lead to developmental defects in androgen-dependant tissues of the male reproductive system, directly affecting testicular function, spermatogenesis, and fertility in adults.

1.5.6 Ecotoxicological information on Clofibric acid

The majority of previous research that has been carried out to examine the effects of clofibric acid on aquatic organisms has been based on standard ecotoxicological tests for risk assessment. Henschel *et al* (1997) reported the results of toxicological studies carried out using clofibric acid (Table 1.5). They reported that cell cultures (derived from bluegill sunfish), fish embryos (zebrafish) and algae were all sensitive to clofibric acid, with EC_{50} values of 14, 86 and 89 mg/l, respectively. Pulse rate tests carried out with *Daphnia*, fish embryos and ciliate proliferation found EC_{50}s of around 100mg/l. Kopf (1995) found similar results with acute/short term tests with clofibrate and clofibric acid,

with lowest endpoint effects seen at 12mg/l and an EC_{50} of 89mg/l. Ferrari *et al* (2003) also carried out ecotoxicological bioassays to determine the effects of clofibric acid on six species (covering bacteria, algae, microcrustaceans and fish). They found that their 48hr EC_{50} values were more than 200mg/l for clofibric acid in daphnid and ceriodaphnid acute toxicity tests, but in chronic toxicity tests using rotifers, a no observed effect concentration (NOEC) of 0.25mg/l was found, indicating they are far more sensitive and suggesting that non-target organism can be affected by clofibric acid.

Toxicity	Test	Species	Exposure time	Assessment Endpoint	EC_{50} (µg/l)
Acute	Bacteria	*V. fischeri*	30 mins	Luminescence	100,000
	Ciliates	*T. pyriformis*	48hr	Growth	175,000
	Daphnids	*D. magna*	48hr	Mobility	106,000
	Fish embryos	*D. rerio*	48hr	Mortality	86,000
Chronic	Algae	*P. subcapitata*	96hr	Growth	89,000
In vitro	Fish cells	BF-2 cells	48hr	Cytotoxicity/ proliferation	14,000

Table 1.5: Ecotoxicological effects of Clofibric acid. Taken from Henschel *et al* (1997).

A small number of chronic tests using algae and fish embryos have also been carried out (Ferrari *et al*, 2003), and these organisms have been shown to be less sensitive than invertebrates. The low sensitivity of fish may be explained by the procedure used, in particular the age of the embryo (as the permeability of the chorion affects the uptake of the chemicals and consequently their viability (see Chapter 3)). It is possible that current chronic tests may not necessarily detect specific or sensitive deleterious effects, as the protocols involve measuring only defined endpoints. More sensitive endpoints

need to be determined and used, rather than the traditional toxicological endpoints.

In summary, the very limited amount of information currently available on the ecotoxicological effects of clofibric acid suggests that the drug is relatively non-toxic in acute studies (concentrations causing effects were very high – in the mg/l range). Almost nothing is really currently known about the chronic toxicity of clofibric acid, but this could be because present methods do not necessarily involve monitoring specific or more sensitive endpoints.

1.6 Metabolism

It is very important, when exploring the effects of any pharmaceutical drugs on an organism, to firstly look at the mechanisms that are involved in processing that drug in humans, as they are usually the ones that the drug has been designed to treat. It is therefore important to consider briefly the basic metabolism in humans, and then compare relevant areas with those that are also known in fish.

Metabolism is the sum of the physical and chemical changes that take place in living organisms. These changes include both synthesis (anabolism) and breakdown (catabolism) of body constituents. In a narrower sense, it is the physical and chemical changes that take place to a given chemical substance within an organism. It includes the uptake and distribution within the body of chemical compounds, the biotransformations undergone by such substances, and the elimination of the compounds and their metabolites.

Metabolism also enables absorbed products of digestion (glucose, fatty acids and glycerol, and amino acids) to be processed, and is essentially dependant upon the nature of the basic diet (carbohydrate, lipid and protein).

All of the products of digestion are processed by their respective pathways to a common product (acetyl-CoA), which is then completely oxidised by the citric acid cycle.

1.6.1 Carbohydrates

Carbohydrate in the diet is processed to glucose, which is then metabolised to pyruvate and lactate by glycolysis. This produces ATP, thus glucose is the major fuel of many tissues. It also takes part in other processes, including:

1) storage to glycogen,
2) the pentose phosphate pathway (see Section 1.6.1.1.),
3) the glycerol moiety
4) the synthesis of the carbon skeletons for the synthesis of amino acids – acetyl CoA is the building block for long chain fatty acids and cholesterol – the latter being the precursor of all steroids synthesised in the body.

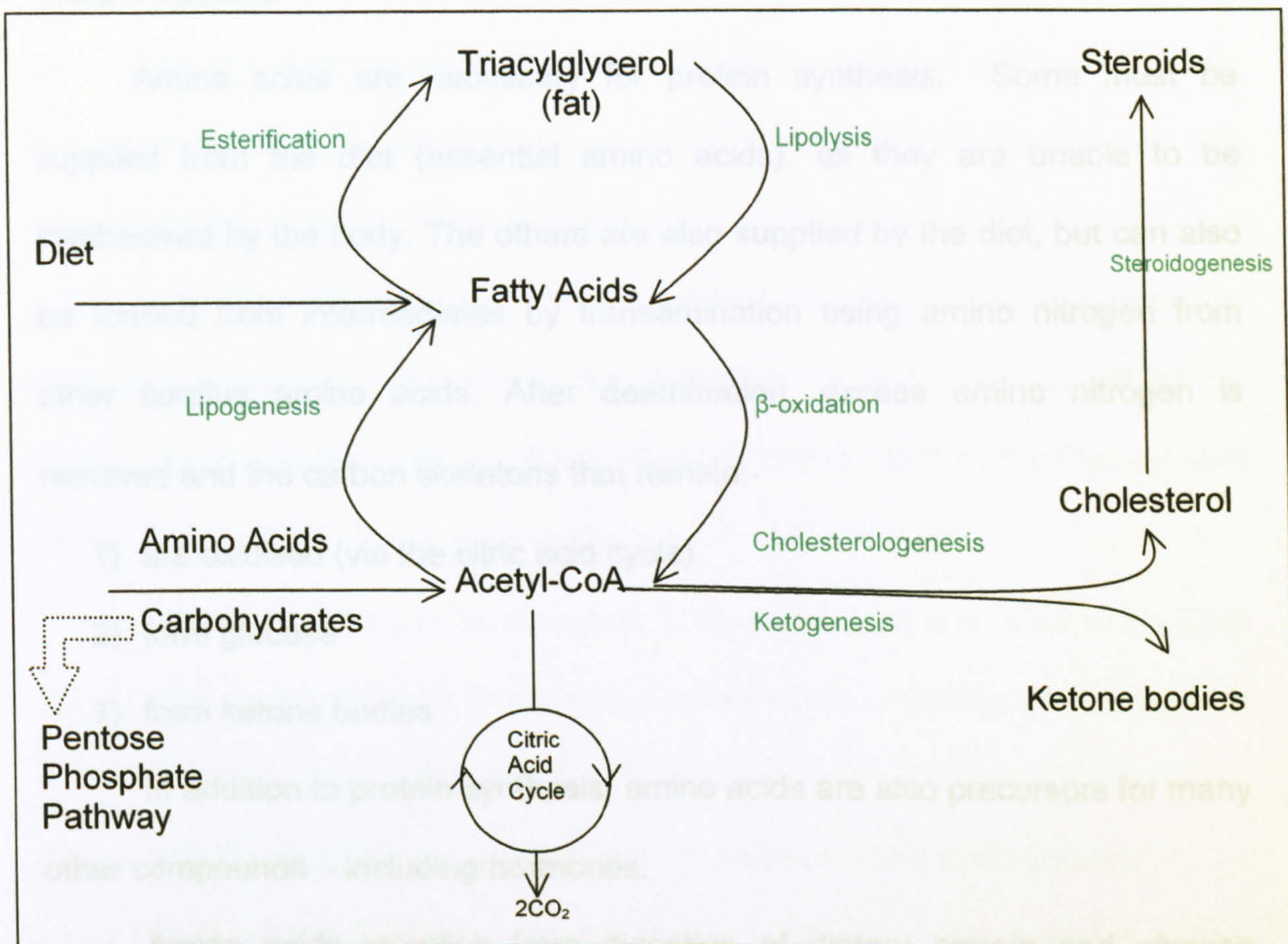


Figure 1.5: An overview of fatty acid metabolism, showing the major pathways and end products (adapted from Murray *et al*, 1993).

1.6.1.1 The Pentose Phosphate Pathway

The pentose phosphate pathway is an alternative route for the metabolism of glucose. It does not generate ATP, but instead has 2 major functions: 1) to generate NADPH for reductive syntheses (such as fatty acid synthesis and steroid biosynthesis), and 2) to provide ribose residues for nucleotide and nucleic acid biosynthesis (see Figure 6.1). Along with these major functions, it also has the very significant function of excretion of metabolites and foreign chemicals (xenobiotics) via the uronic acid pathway. In humans the pentose phosphate pathway is active in the liver, adipose tissue, adrenal cortex, thyroid, erythrocytes, testis and lactating mammary gland (Murray *et al*, 1993). Further details follow in Chapter 6.

1.6.2 Proteins

Amino acids are necessary for protein synthesis. Some must be supplied from the diet (essential amino acids), as they are unable to be synthesised by the body. The others are also supplied by the diet, but can also be formed from intermediates by transamination using amino nitrogen from other surplus amino acids. After deamination, excess amino nitrogen is removed and the carbon skeletons that remain:-

1) are oxidised (via the citric acid cycle)
2) form glucose
3) form ketone bodies

In addition to protein synthesis, amino acids are also precursors for many other compounds – including hormones.

Amino acids resulting from digestion of dietary protein and glucose resulting from the digestion of carbohydrates are directed to the liver. The glucose is converted into glycogen by glycogenesis or to fat by lipogenesis (Figure 1.5). The liver draws on these glycogen stores between meals to replenish glucose in the blood.

1.6.3 Lipids

Lipids in the diet are represented mainly by triacylglycerols, which upon digestion form monoacylglycerols and fatty acids. These are then recombined with proteins and released into the circulation as lipoproteins (chylomicrons). All hydrophobic, lipid soluble products of digestion form lipoproteins, which facilitates their transport in the plasma between tissues. Unlike amino acids and glucose, chylomicrons are not taken up directly by the liver, but are first metabolised by extra-hepatic tissues, by lipoprotein lipase (LPL), (further details

follow in Chapter 6), which hydrolyses the triacylglycerol, releasing the fatty acids that are incorporated into tissue lipids or oxidised as fuel. Surplus triacylglycerol arising from both lipogenesis and free fatty acid is secreted into the circulation as VLDL, where it undergoes a similar fate to chylomicrons.

In the tissues, fatty acids may be oxidised to acetyl–CoA (β-oxidation) or esterified to acylglycerols where, as triacylglycerol (fat), they constitute the body's main calorific reserve. Acetyl–CoA formed by β-oxidation has several fates:

- Acetyl–CoA derived from carbohydrate is oxidised and used in the citric acid cycle, where it is used to produce ATP, providing the tissue with energy.
- It is a source of the carbon atoms in cholesterol and other steroids
- In the liver it forms ketone bodies (water soluble fuel for certain conditions, e.g. starvation)

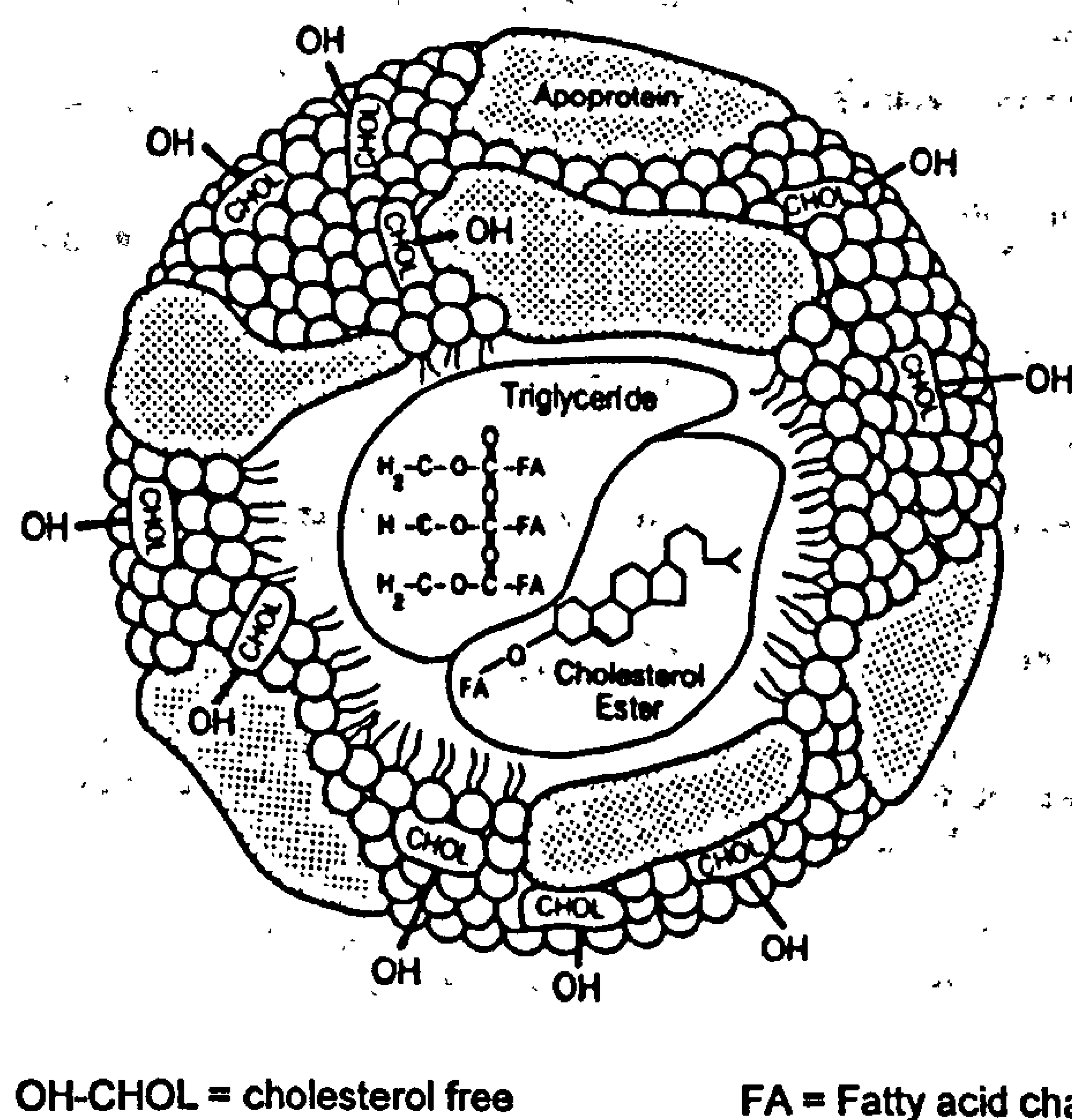


Figure 1.6: Structure of a plasma lipoprotein (from Thain and Hickman, 2000).

Lipids are insoluble in water, and hence they have to be transported in the blood as lipoprotein complexes. These are defined as a micellar complex of protein and lipids. The protein component of these complexes (apolipoproteins) transport otherwise insoluble lecithin, triglyceride and cholesterol in blood plasma. The lipoprotein particle comprises an outer lipid bilayer with specific conjugated protein components, within which the transport molecules are either free or esterified to bilayer fatty acids (Figure 1.6; Thain and Hickman, 2000).

Fish, unlike mammals, utilise lipids in preference to carbohydrate as their main source of energy (Wantanabe, 1982), and therefore tend to have high levels of lipoprotein in their plasma, reflecting this high utilisation of lipid. They are capable of accumulating considerable amounts of lipid in muscle and liver, as well as adipose tissue, for their energy reserves (Sheridan, 1988).

Metabolic studies of plasma lipids and lipoproteins in fish remain limited; nevertheless, existing data suggest that the process and mechanisms involved in their metabolism have features in common with those of mammals. Fish lipoproteins are separated into four classes, which are similar to those of other vertebrates (Babin and Vernier, 1989). They consist of very low-density lipoproteins (VLDL), intermediate lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Santulli *et al*, 1996). Babin and Vernier (1989) and Wallaert and Babin (1994) state that, in fish, the percentage of total lipids in each class of lipoproteins is comparable to that observed for humans, although HDLs predominate in the lipoprotein profile of fish (Ando, 1996).

When compared to standards for mammals, and humans in particular, fish are classified as hyperlipidemic and hypercholesterolemic (with elevated LDL levels) and in female oviparous species vitellogenin (a VHDL) causes the

fish to appear lipoproteinemic. Rainbow trout have been shown to carry three times (and sometimes up to a maximum of twelve times) more lipid and cholesterol in the blood than fed rats (Babin and Vernier, 1989). Most of the cholesterol is present in the plasma of most fish species in the esterised form.

There are very little data concerning the quantitative changes of fish lipoproteins in response to varying external factors. A large body of work, however, has shown that the plasma concentration of lipids, particularly cholesterol and triglycerides, in fish is highly dependant on their nutritional or physiological state, their sex and on their developmental stage (Babin and Vernier, 1989; Iijma *et al*, 1985; Santulli *et al*, 1991). Factors such as age and growth, sex, temperature, salinity or certain endocrine factors (such as concentrations of plasma steroid hormones), can also affect plasma cholesterol concentrations in fish (Babin and Vernier, 1989). It is therefore very important that fish entering any laboratory experiment are all of a similar physiological and nutritional condition and have all been treated in exactly the same way before beginning any experiment.

1.7 The Reproductive system of fish

The effects of peroxisome proliferators (such as fibrates) on reproductive endpoints in humans and rodents have been mentioned previously. It is therefore important for me to describe the basic reproductive biology of fish, as various reproductive parameters were considered during experimental protocol in this thesis.

1.7.1 Basic reproductive endocrinology

The endocrine system of fish, just as in mammals, consists of a complex interaction of external stimuli, hypothalamic and pituitary hormones, gonadal hormones and deactivation of hormones by the liver (Kime, 1999). Sexual maturation and reproduction in fish are complex biological processes which are regulated by endogenous hormones, and synchronised by exogenous factors such as photoperiod and temperature, thus ensuring reproduction occurs at the optimal time for survival of the offspring.

Fish differ from mammals in that the major testicular androgen is 11-keto-testosterone rather than testosterone (Kime, 1993), and whilst oestradiol is the ovarian oestrogen, it acts very differently than it does in mammals. Its major target tissue is the liver, which is stimulated by oestradiol to produce the yolk protein vitellogenin, which is incorporated into the oocyte under the influence of the gonadotrophin GtH-1 (Tyler *et al*, 1991), which is now usually called FSH, as in mammals.

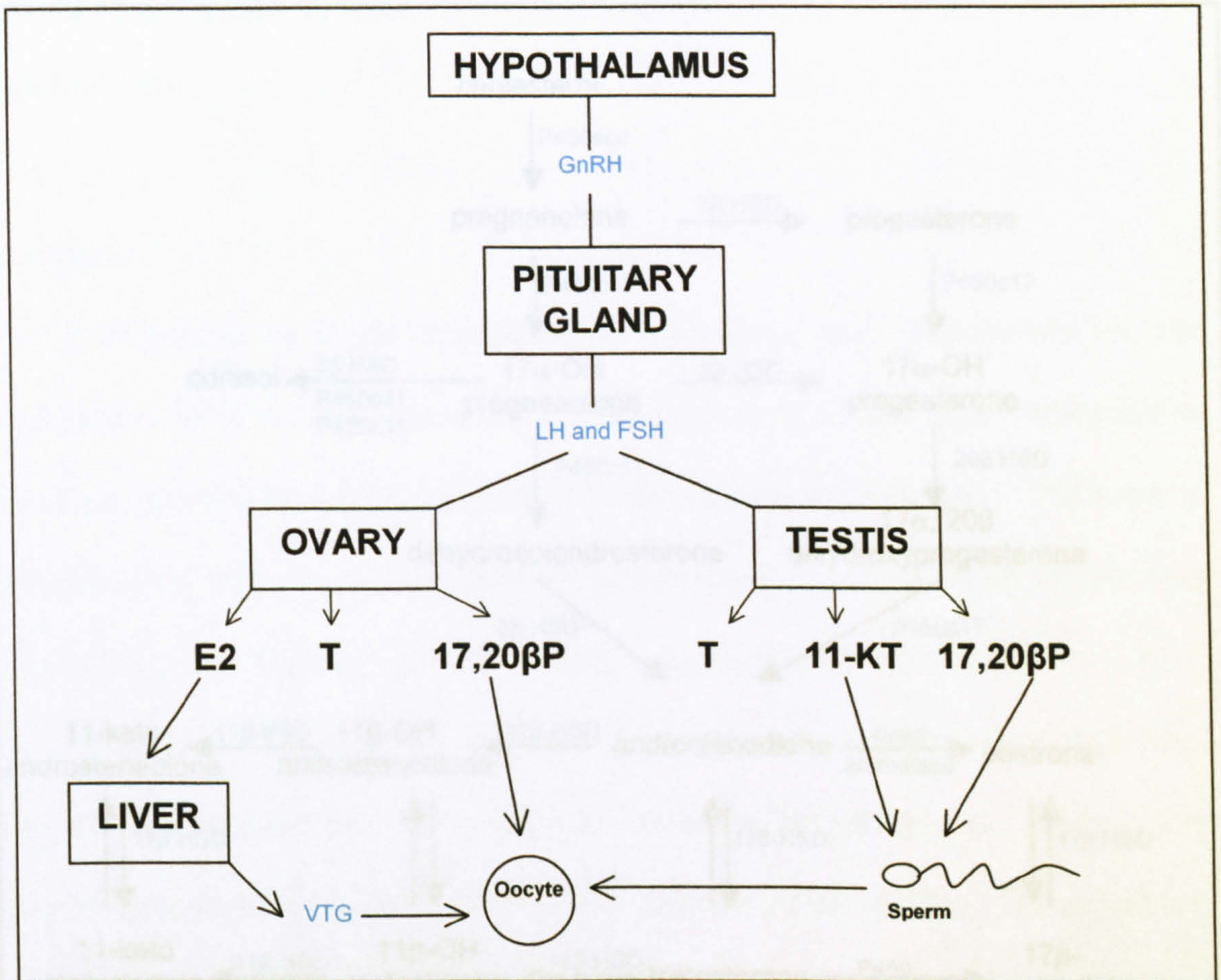


Figure 1.7: Schematic diagram of the reproductive system in fish (GnRH – gonadotrophin releasing hormone, E2 – oestradiol, T – Testosterone, 17,20βP – 17α,20β-dihydroxy-4-pregnen-3-one, 11-KT- 11-Ketotestosterone; LH – leuteinising hormone; FSH – Follicle stimulating hormone). Adapted from Kime, 1999.

1.7.2 Steroid hormones

The synthesis of steroid hormones is initiated by the conversion of acetate to cholesterol (Figure 1.5). This is the precursor of all steroid hormones. Cholesterol is transformed to pregnenolone (see Figure 1.8), which is itself metabolised by a sequence of enzymatic reactions to form the steroid hormones. As in rodents, HDL appears to be the main exogenous source of cholesterol for steroidogenesis in fish (Gilman *et al*, 2003). Cholesterol, testosterone and 17β-estradiol have relatively minor differences in chemical structure. These seemingly minor changes, however, result in profound difference in biological responses.

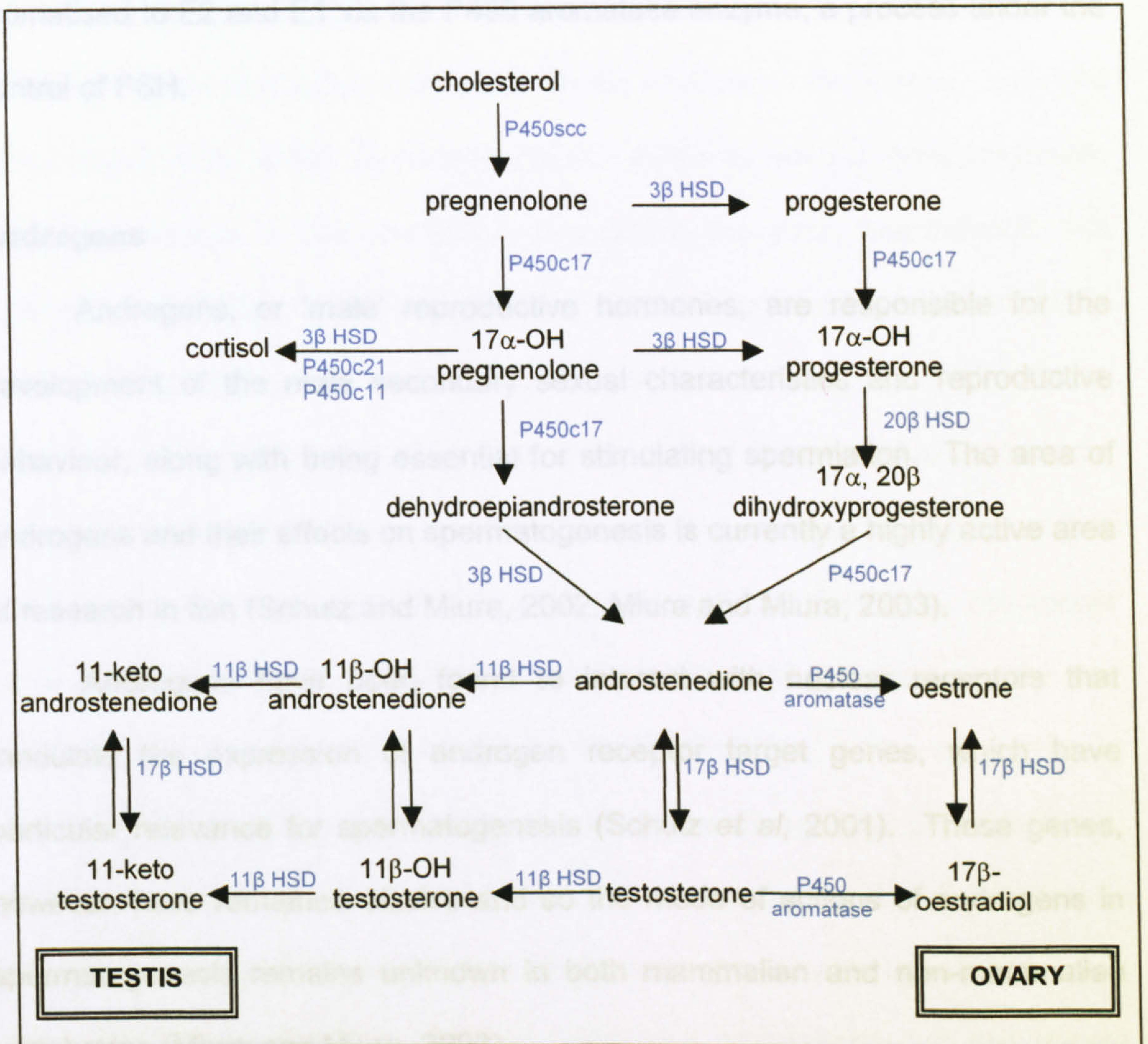


Figure 1.8: The principal pathways of steroidogenesis in fish. Where HSD = hydroxysteroid dehydrogenase.

Oestrogens

The most important of the 'female' reproductive hormones are 17β-oestradiol (E2) and oestrone (E1) (Kime *et al*, 1995). They are secreted by the ovaries and are responsible for the control of sexual differentiation, secondary sexual characteristics and the oestrous cycle in mammals. In non-mammalian oviparous vertebrates, they play a vital role in oocyte development by inducing the synthesis of vitellogenin (Ng and Idler, 1983). The production of oestradiol in teleosts is based on a two-cell mechanism. The theca layer of the oocytes is stimulated by LH to produce androgens (Kagawa *et al*, 1982). These are then transported to the granulosa layer, where testosterone and androstenedione are

aromatised to E2 and E1 via the P450 aromatase enzyme, a process under the control of FSH.

Androgens

Androgens, or 'male' reproductive hormones, are responsible for the development of the male secondary sexual characteristics and reproductive behaviour, along with being essential for stimulating spermiation. The area of androgens and their effects on spermatogenesis is currently a highly active area of research in fish (Schulz and Miura, 2002; Miura and Miura, 2003).

Androgens have been found to interact with nuclear receptors that modulate the expression of androgen receptor target genes, which have particular relevance for spermatogenesis (Schulz *et al*, 2001). These genes, however, have remained elusive and so the mode of actions of androgens in spermatogenesis remains unknown in both mammalian and non-mammalian vertebrates (Miura and Miura, 2003).

The most active androgens in mammals are dihydrotestosterone and testosterone whereas in teleost fish it is 11-ketotestosterone (11-KT) (Kime, 1993, 1999). These are synthesised interstitially in the gonad, by the Leydig cells, (via the activation of the LH receptor) and are then metabolised to the more active compound in the Sertoli cells. As a male matures, gonadotrophin-releasing hormone (GnRH) from the hypothalamus stimulates the synthesis and release of leuteinising hormone (LH) and follicle stimulating hormone (FSH) (see Figure 1.7). LH acts on small groups of Leydig cells to stimulate the production of testosterone. Together with FSH, testosterone acts on the seminiferous tubules and promotes spermatogenesis (Kime *et al*, 1999; Schulz *et al*, 2002).

Testosterone and sperm production are controlled by negative feedback: as testosterone production increases, GnRH production decreases, and vice versa (Kime *et al*, 1999; Ostrander, 2000). In some mature male mammals, including humans, which reproduce throughout the year, testosterone and sperm production are continuous. In other species reproduction is seasonal. In these species, changes in day length and temperature affect the hypothalamus, growth and descent of the testis and the onset of the reproductive season. During the reproductive season, testosterone and sperm are controlled as they are in humans, but after the reproductive season GnRH is no longer synthesised, testosterone production falls and the testis become smaller and inactive until needed for the next season (Sherazi, 2000).

Testosterone is also present in female teleost fish at high concentrations; in fact, it is often seen at higher levels than in the males (Borg, 1994). In females it is unclear what the role of testosterone is, although it has been suggested that it may be concerned with the maintenance of the oocyte maturation processes following vitellogenesis (Kime, 1993).

As with oestradiol, testosterone is derived from cholesterol through a series of steps that are dependant on cholesterol availability and biosynthetic activity (see Figure 1.8). This biosynthetic activity is stimulated by LH from the pituitary. As shown in Figure 1.8, the key enzymes responsible for the synthesis of androgens are P450 cholesterol side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD) and P450 17α-hydroxylase, 17-20-lyase (P450c17).

11-KT is derived from testosterone (shown in Figure 1.8), and in some fish 11-KT has been shown to induce the entire process of spermatogenesis, from spermatogonial proliferation to sperm formation (Miura and Miura, 2003)

as well as the quantity of milt produced (Yamazaki and Donaldson, 1969). It has been shown to activate Sertoli cells to stimulate production of a spermatogonial proliferator, although exact mechanisms of actions are not fully identified (Todo *et al*, 1999). It is thought that this action is mediated by factors produced by Sertoli cells in which the androgen receptor exists (Ikeuchi *et al*, 2001).

Progesterones

In mammals, progesterone is mainly produced by the ovaries, with minor secretions by the testes and adrenal glands – it is primarily responsible for maintenance of the placenta during pregnancy. In teleost fish, progesterone is of little consequence, but from this other progesterones are synthesised (e.g. 17α,20β-dihydroxy-4-pregnen-3-one), and these are involved in the final stages of maturation of the oocyte and instigate germinal vesicle breakdown prior to spawning (Yoshikuni and Nagahama, 1991; Kime, *et al*, 1993).

Plasma steroid concentrations are ultimately a combination of the rate of gonadal secretion and rate of hepatic deactivation and excretion. Low plasma steroid concentrations could be the result of either inhibited synthesis or elevated hepatic catabolism. A wide range of pollutants can stimulate hepatic mixed function oxidase (MFO) activity, which is involved in steroid catabolism. Such elevated activity could result in more rapid oxidation if the enzyme was available for steroid catabolism, but might equally well decrease metabolism if the pollutant was preferentially used as substrate (Kime, 1999).

1.7.3 Vitellogenin (VTG)

Vitellogenin is a VHDL (phospholipoglycoprotein), and is the precursor of the major egg-yolk proteins of oviparous organisms (Mouchel, 1996). In maturing female teleost fish, VTG is the dominant blood protein that is under oestrogen control. It is secreted by the liver into the blood stream, and taken up by oocytes via receptor-mediated endocytosis. VTG comprises primarily precursors of the yolk proteins (lipovitellin and phosvitin), but it also contains a proportion of lipid (≈20%) (Babin and Vernier, 1989).

Vitellogenesis is the process by which yolky eggs are produced; it entails both the synthesis of vitellogenin by the liver and its uptake by growing oocytes, where it is stored as yolk to serve subsequently as the food reserve of the developing embryos (Tyler and Sumpter, 1996). The plasma vitellogenin concentration rises steadily during sexual maturation of a female fish, simultaneously with increasing 17β-oestradiol concentrations (Scott and Sumpter, 1983), to reach tens of milligrams per millilitre in some species, at which point vitellogenin is the major blood protein. Such high concentrations are required by the female to grow an ovary that will contain thousands of large yolky oocytes. The plasma vitellogenin concentration consequently increases around one million-fold during the seasonal reproductive cycle.

Vitellogenin has been widely used as a biomarker in the detection of oestrogenicity of environmental contaminants in fish (Mouchel *et al* 1996; Orn *et al* 2003; Van den Belt, 2003), and also as an indicator of the oestrogenic activity of effluents and the aquatic environment (Purdom *et al*, 1994; Harries *et al*, 1997; 1999).

1.7.4 Gamete production

Normal development of the gametes requires a specific temporal pattern of hormone secretion, which is determined by signalling from the gonad itself to the pituitary. Such signalling may be of particular importance in indicating that the gamete has reached a sufficient stage of maturity for the synthetic pathway to switch from 11KT/estradiol to progesterones. In the ovary, oestradiol synthesis must be sufficient to induce the liver to produce vitellogenin which can be incorporated into the oocyte. Decreased oestradiol may result in either decreased number of eggs, or the normal number of smaller eggs (Tyler *et al* 1996). Both of these factors may affect the production of potential viable offspring.

1.7.4.1 Sperm

The specific cues for controlling the process of spermatogenesis are modulated by the endocrine system which have been previously well documented by Scott and Sumpter, (1989) and Schulz and Miura, (2002). Androgens and FSH are the most important reproductive hormones with regards to its regulation (Schulz and Miura, 2002).

Spermatogenesis is a developmental process with a mitotic, a meiotic, and a spermiogenic phase (Schulz *et al* 2001), which takes place within the testicular lobules. The undifferentiated germ cells, which are termed spermatogonia, begin to divide mitotically at the onset of gonadal recrudescence. The daughter cells of this first division then divide, and so on, for a specified number of division cycles, so that a clone of spermatogonia is produced from each original spermatogonium. Some differentiation occurs as well as mitosis. The cells that result from the final mitotic division and

differentiation in the series are called primary spermatocytes, and these are the cells that will undergo the first meiotic division of spermatogenesis.

Not every spermatogonium becomes a primary spermatocyte. At an early point, one of the cells of each clone 'drops out' of the mitosis-differentiation cycle and reverts to being a primitive spermatogonium that, at a later time, will enter into its own full sequence of divisions. In turn, one cell of the clone it produces will do likewise, and so on. Thus the supply of spermatogonia does not deplete. Each primary spermatocyte changes in size and undergoes the first meiotic division to form two spermatids (Grier, 1981; Schulz and Miura, 2002).

The final stage to spermatogenesis is the differentiation of the spermatids into mature spermatozoa. This process involves extensive cell remodelling, including elongation, but no further cell divisions. In spermatozoa, the entire cell is almost exclusively occupied by the nucleus containing the DNA that encodes the genetic information (Schulz and Miura, 2002).

Sperm of teleost fish differs in a number of aspects from those of mammals. They are immotile upon ejaculation and only become motile on contact with water (Schulz and Miura, 2002, Miura and Miura, 2003). They only remain motile for a few minutes at most and enter the egg via the micropyle rather than through an acrosomal reaction (Kime, 1999). The first minute after induction of motility is therefore crucial for it to succeed in fertilising an egg (Kime, 1999).

1.7.4.2 Eggs

Fish populations, both farmed and wild, are dependant on the production of good quality eggs. Egg quality can be measured by mortality and fertilisation

rates, time to hatch, and the proportions reaching the 'eyed' stage and first feeding (Bromage, *et al* 1992). Although these give ultimate measures of egg quality, they provide no information on the factors affecting egg quality. The factors which can affect quality are determined by the intrinsic properties of the egg itself, and the environment in which the egg is fertilised and subsequently incubated. Once ovulated, fish eggs take up very little, if any, nutrients: only water, and chemicals in the water, are known to pass into an ovulated egg (reviewed in Brooks *et al*, 1997)

In females, LH and FSH are involved in controlling ovarian functions. LH stimulates the theca cells of the oocyte to produce androgens, which the adjacent granulosa cells, under stimulation of FSH, use to proliferate and convert into oestradiol, which is excreted. Oestradiol levels then rise and provide positive feedback, leading to an LH surge and ovulation.

Also crucial to successful oocyte development is the production of the Zona radiata proteins (Zrp), fish vitelline envelope proteins, which assist in the development of the eggshell. This provides protection for the developing embryo against mechanical disturbances during the first fragile period. The vitelline envelope is composed of a small number of proteins, which are usually synthesised in the teleost liver in response to an oestrogen signal (Arukwe *et al*, 1997). Some teleosts however, synthesise these proteins in the ovary itself. These glycoproteins are termed ZP1, ZP2 and ZP3, and have molecular masses around 200, 120 and 80kDa, respectively. Transcription of the ZP3 gene takes place in the ovary and begins in early oogenesis in female fish; very little, if any, Zrp can be detected in male or juvenile fish. The eggshell is shed when hatching is induced by developmental and environmental signals. Small changes in Zrp synthesis may cause the thickness and mechanical strength of

the eggshell to be altered, thus causing a loss in its ability to protect against mechanical disturbances during development (Arukwe *et al*, 1997).

There is considerable evidence that fish oocytes are particularly sensitive to environmental pollutants and that their quality can be adversely affected by them (Brooks *et al*, 1997). For example, fish have a 100-fold greater sensitivity compared with mammals to induction of specific mutations during oogenesis (Walker and Streisinger, 1983). Malformations and impaired development and viability have been shown to result from exposure to a variety of environmental contaminants including insecticides, organochlorinated biphenyls and polychlorinated biphenyls (Brooks *et al*, 1997). Exposure may occur when the oocytes are developing in the ovary or subsequently when the eggs are released into the aquatic environment. Lipophilic chemicals particularly may be transported with lipid reserves into the developing oocyte, leading to increased rates of mortality and deformities (Knickmeyer and Steinhart, 1989; Miller and Amrhein, 1995).

Pollutants can affect sperm and egg development indirectly via disturbance of the correct hormonal environment, but if such pollutants have hormonal activity themselves, they can also directly affect the local hormonal environment in which the gamete develops. Many chemicals that are described as having oestrogenic or other hormonal activity may, like non-hormonal chemicals, also have toxic effects. These toxic effects can disrupt gamete development both indirectly through toxicity to the endocrine system, or directly through toxicity to the gametes themselves (Kime, 1999). The key point about any environmental toxicant is that it can have an effect which disrupts reproduction at levels below that which disrupt the general health of the fish –

consequently the fish is able to live a normal life, but has decreased fertility, for example.

1.8 Previous relevant research

Clearly, when pharmaceuticals are present in the environment, the organisms that live in the aquatic environment will be particularly vulnerable. Fish have the potential to take up toxins via several pathways, and these toxins may in turn be subject to bioconcentration by virtue of the fact that the fish may be permanently surrounded by the contaminated water. Water passes over the gills, which are extremely efficient at removing chemicals from the water. These chemicals would then enter the blood stream and reach most organs before passing through the liver, where they may be metabolised. Also, as many species of fish are relatively near the top of the food chain, there is a high chance of them receiving exposure via eating contaminated food (the chemicals may be found at high concentrations in them due to biomagnification).

Concentrations of pharmaceuticals found in the environment are in the ng/l range, or occasionally low µg/l, and this is at least 3 to 4 orders of magnitude lower than those producing a pharmacological effect in humans. The levels presently found in the environment are therefore not considered to be significant when compared to therapeutic doses in humans, although they must be considered further with regard to subtle long term or chronic effects of lifetime exposure within the aquatic environment.

To date, there are only a handful of studies which have reported on the effects of clofibrate or clofibric acid on fish. In 1998, Perkins and Schlenk exposed catfish to 0, 10, 50, 100 and 225mg clofibric acid per litre, for 24hrs in a static tank water system. They showed that treatment with water-borne

clofibric acid caused a dose-dependant increase in the 51-kDa CYP2 protein and also a dose-dependant increase (although not significant) in HSI. The highest concentration (225mg/l) caused death and 100mg/l caused haemorrhaging to the tail and pectoral fins. Concentrations of 10 and 50 mg/l caused dose response but over those concentrations effects decreased to those similar to the controls.

Pretti *et al* (1999), looked at the effect of 35 or 70mg/kg Clofibrate on enzymes in liver, kidney and gill of 4-year-old sea bass after 2 weeks daily IP injection. They also found no significant changes in cytosolic, microsomal, mitochondrial or peroxisomal enzyme activities compared to the controls. They did, however, find induction of hepatic glutathione S-transferase activity. They concluded that adult sea bass are essentially refractory to a classical peroxisome proliferator such as Clofibrate. In trout, salmon and medaka hepatocytes, however, the induction of peroxisome proliferator marker enzymes in response to clofibrate has been observed (Yang *et al*, 1990, Scarano *et al*, 1994, Donohue *et al*, 1993, Ruyter *et al*, 1997). These support conclusions found in mammalian models.

Haasch (1998), exposed male blue gill to clofibrate via IP injection and found significant cytochrome p450 induction. In 2004, Kazeto *et al* looked at endocrine effects of clofibrate on juvenile zebrafish, including at the levels of expression of the CYP19 genes - the key steroidogenic enzyme responsible for the conversion of androgens to oestrogens. They found no significant changes in expression of the CYP19 genes after exposure to clofibrate, however there was a tendency for a decrease in abundance by 60-70% in some cases (so a possible trend). When they exposed zebrafish to EE2 and clofibrate together, they found that there was no anti-oestrogenic effect. They concluded that there

were no oestrogenic effects and no anti-oestrogenic effects at concentrations of Clofibrate of 0.01-10µM during 3 days of exposure. They also exposed adult zebrafish to clofibrate at the same concentration and determined that it was effective in inducing a PPAR gene response after 3 days exposure.

1.9 Aims of the PhD

This project is concerned with the presence of pharmaceutical drugs, in particular clofibric acid, in the aquatic environment and their effects on fish. The aim is to investigate the effects of these drugs on fish at environmentally relevant concentrations, in order to determine whether the amounts of these drugs in the environment are great enough to cause a deleterious effect at the individual or whole population level in fish.

There is a plethora of information which has recently been disseminated in the scientific press regarding levels of pharmaceutical drugs (and their metabolites) identified in the aquatic environment. Very little information, however, is available for pharmaceutical exposure, and any consequent effects in aquatic organisms.

The aims of this thesis are:

- To summarise the data available on occurrence and detection of pharmaceuticals.
- Determine if effects on fish occur, using an open ended and varied approach, beginning with a number of preliminary experiments to guide subsequent experiments. These involved assessing possible effects, on both embryos and adult fish, and investigating parameters ranging from physiological and biochemical ones, through to effects at the molecular level.

Chapter 2: Material and Methods:

Methods employed in determining the effects of a pharmaceutical drug metabolite, clofibric acid, on fish.

As so little is known about the effects of pharmaceutical drugs on fish, the methods employed to determine if any effects occur must be diverse and take into account the effects that occur in humans taking the drug. However, just because a drug has a specific mode of action in humans, it may not act in the same way in a different organism. It may cause novel adverse reactions in normal functions in other organisms, possibly even at concentrations lower than those normally applied to humans. Deciding on which particular endpoints to employ for the research reported in this thesis was therefore very difficult.

2.1 Dosing Experiments

It was decided that *in vivo* studies would be the best approach for detecting pharmaceutical effects in fish, because this approach might enable us to identify whole body effects. The basic methodology employed is included below. In subsequent chapters, the specific details relevant to the particular experiments reported in that chapter are provided.

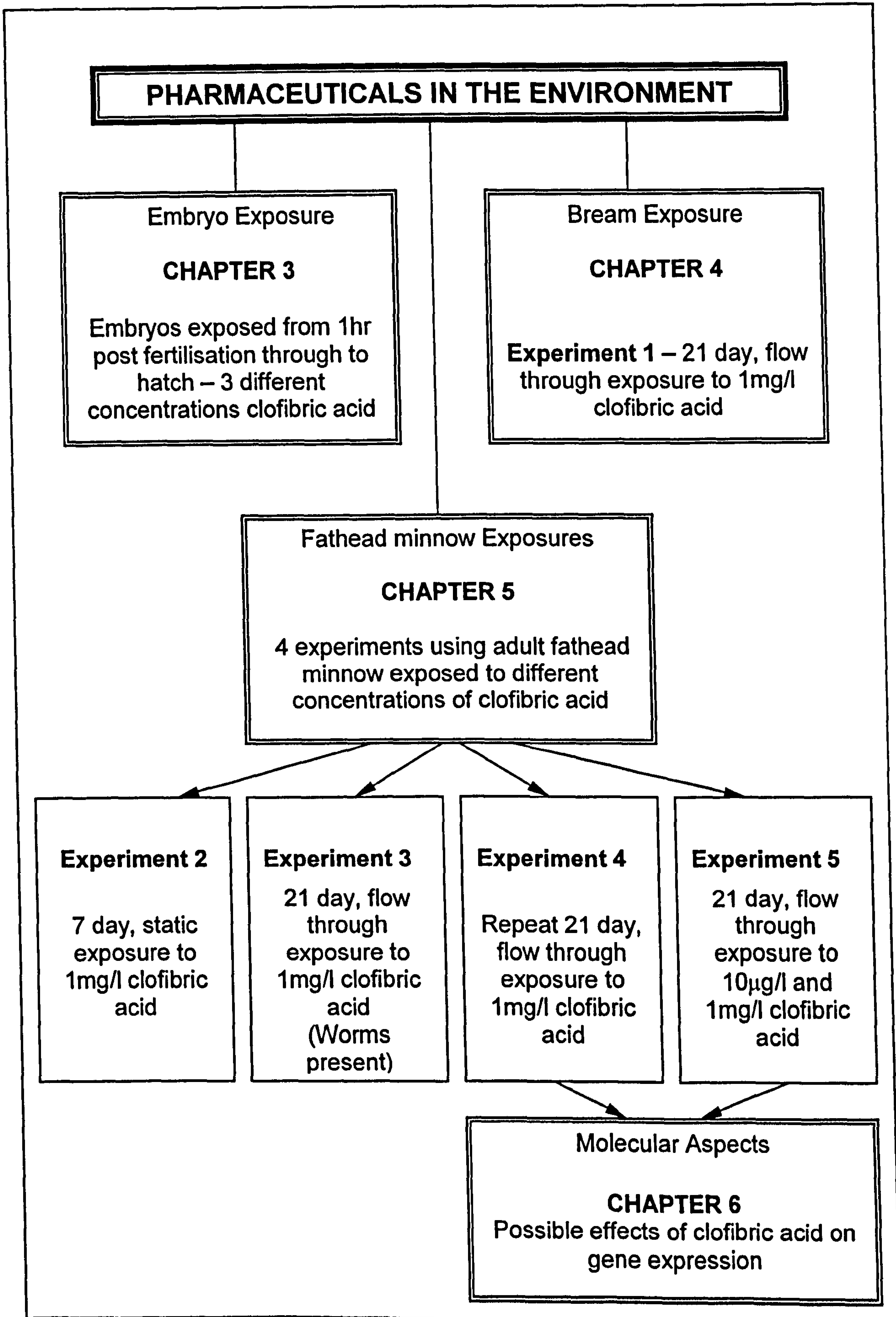


Figure 2.1: Structure of the research reported in this thesis - An overview of experiments carried out using Clofibric acid.

2.1.1 Test Organisms

Two species of fish were used for all the exposure experiments, the Bream (*Abramis brama*) for the preliminary adult study (Chapter 4) and the fathead minnow (*Pimephales promelas*) for the embryo study (Chapter 3) and for subsequent adult experiments (Chapters 5 and 6). Initially, the Bream was chosen for the adult experiment because individuals of this species are large (larger than the fathead minnow), and consequently blood could be taken pre and post exposure from the same fish, thus enabling within-fish data analysis to be conducted.

The Bream (*Abramis brama*)

The common bream (*Abramis brama*) is a common cyprinid fish that lives in large numbers in the lower reaches of slow flowing, large rivers, lowland reservoirs and lakes. It has a high, laterally flattened body with a lead blue back and silvery sides. They can grow to a length of 30cm, a weight of 6kg, reach sexual maturity at 2+ years, and can live naturally for up to 25 years.

The fathead minnow (*Pimephales promelas*)

The fathead minnow (*Pimephales promelas*) is a representative of the ecologically important and ubiquitous Cyprinidae family. It has been extensively used in chronic life cycle and early life stage survival and development tests in support of regulatory programs, both in Europe and North America (Jensen *et al*, 2001). It is a small freshwater fish, with adults reaching an average length of 50mm and a weight of 5g. Sexually mature males have a striking appearance, and are easily distinguished from the females - they are larger and darker in colour with vertical bands on the body and head (Figure 2.2). They also have

tubercles on the face and a prominent fatpad on the top of the head (hence the name fathead). In contrast, the female is silvery/olive in colour, and has a more pointed head. She has an ovipositor and a swollen ventral contour associated with the presence of mature eggs.

Figure 2.2: Photograph of the adult male and female fathead minnow (*Pimephales promelas*).

Under optimal conditions, the fathead minnow reaches sexual maturity at about 4-5 months post hatch. They are fractional spawners and under favourable conditions the females can produce clutches of 50-100 eggs every 3 to 5 days (Harries *et al*, 2000). The incubation period is about 4 days at 25°C, after which newly hatched fry of about 5mm in length hatch out.

The fathead minnow is relatively easy to culture in a laboratory setting using standard methods (EPA, 1987), and control of its reproduction cycle is readily achieved through alterations in temperature and photoperiod.

A continuous flow-though system (Figure 2.3) was employed for all except one (Experiment 2) of the exposure experiments, using 30 litre glass tanks, ensuring a complete water change of dechlorinated tap water (5 and 10µm carbon filtered) every 3hrs. Parameters monitored within the test tanks throughout the studies were: temp (25±1°C) and dissolved oxygen level (8±1mg/l), and the photoperiod was maintained at 16h light: 8hr dark throughout, incorporating 20 min dawn:dusk transition periods.

Figure 2.3: The dosing set up used to expose fish to Clofibric acid.

2.1.2 Preparation of stock solutions and dosing of tanks

Stock solutions of clofibric acid were freshly prepared in 4 litre amber bottles prior to each experiment. Clofibric acid (CAS No: 882-09-7, purity >97%) was dissolved in double distilled water by stirring vigorously overnight and heating gently (to ~ 40°C). The stock solution (see individual chapters for nominal tank concentrations) was dosed at 12ml/hr (0.2ml/min), using a Watson Marlow (Cornwall, UK) Multi channel, peristaltic pump, into glass mixing vessels (aspirator bottles), where it mixed with dilution water (at 10l/hr) before delivery to each of the test tanks. All tubing within the system was medical grade silicon. The chemical was left dosing for a week prior to fish being put into the tanks, to allow the system to equilibrate. For numbers and sex of fish per exposure experiments, see details of individual experiments in subsequent chapters. Flow rate and stock solution dosing were monitored daily.

2.1.3 Sampling fish

At the end of each experiment (apart from bream exposure - see Chapter 4), fish were anaesthetised using MS-222 (Sigma, Poole, UK) at 40-100ppm to obtain complete anaesthesia (Ostrander, 2000), blood sampled and killed using standard procedures approved by the UK Home Office.

Blood Sample collection and storage from fathead minnow

For lipoprotein analysis, blood was collected via a tail cut (through the caudal peduncle), into 75µl capillary tubes (with no heparin*), then decanted into eppendorf tubes and kept on ice. The blood samples were then left to coagulate, after which they were spun in a centrifuge at 12,000x g for about 15 seconds. Serum was then taken off carefully and stored at 4°C (for a maximum of three days) before lipoprotein assays were carried out (Section 2.2.2).

Plasma samples for analysis of triglyceride, cholesterol, sex steroid and VTG levels were collected similarly, although the capillary tubes used to collect the blood contained heparin and the eppendorf tubes contained the enzyme inhibitor aprotinin (Sigma). Blood samples were kept on ice until plasma was separated by centrifugation for 4 minutes at 14,000x g, and this was then stored at –20°C ready for analysis (see Sections 2.2.3, 2.2.4 and 2.2.5).

Blood Sample collection and storage from bream

Blood samples from bream for lipoprotein analysis (Chapter 4) were taken using a 5ml syringe and needle. Blood samples were kept on ice until they coagulated and then briefly spun at 12,000x g for 15 seconds. Serum was

* Heparin must not be used during collection of blood for lipoprotein analysis as it alters the lipoprotein profile (Wayne Brown, Hillingdon Hospital, Personal Communication, 2001).

then taken off carefully and stored at 4°C (for a maximum of three days) before lipoprotein assays were carried out (Section 2.2.2).

Plasma samples for analysis of triglyceride, cholesterol, and VTG levels were collected similarly, although the syringes used to collect the blood contained heparin and the eppendorf tubes contained aprotinin (Sigma). Blood samples were kept on ice until plasma was separated by centrifugation for 4 minutes at 14,000x g, and this was then stored at –20°C ready for analysis (see Sections 2.2.3, 2.2.4 and 2.2.5).

2.1.4 Dissection of fish

After blood was taken, the fish were measured (fork length, in millimetres) and weighed (grams) and, depending on the experiment, gonad and liver were either removed and weighed using RNase free conditions, snap-frozen in liquid nitrogen and stored at –80°C until needed for analysis or, if the testis was being used for determination of sperm count, it was not snap-frozen.

The **gonadosomatic index** (GSI) expresses the size of the gonad in relation to the body weight. It was calculated by using the following formula:

GSI = gonad wt (g)/body wt (g) x 100

The **hepatosomatic index** (HSI) expresses the size of the liver in relation to the body weight. It was calculated by using the following equation:

HSI = liver wt (g)/body wt (g) x 100

2.2 Endpoint Methods

2.2.1 Sperm count

Sperm counts were not initially considered as an endpoint for the exposure experiments, but were carried out as a pilot study in Experiment 4. When interesting results were obtained, it was decided that for the final exposure experiment (Experiment 5), it should be an endpoint. Sperm counts were determined using an established method (Katrien VanLook, personal communication; 2003; WHO, 1992). Testes were removed from the fish and weighed, and a known amount was then placed into an eppendorf tube (Alpha Labs, Hampshire) with 200µl catfish extender (pH 7.58, NaCl 94mM 5.52g/l, KCL 27mM 200g/l, Tris HCL 15mM 2.42g/l, Glycine 50mM 3.75g/l, made in nanopure water. These samples were then immediately stored at 4°C until analysed (within 1 week of sampling).

The testis was homogenised thoroughly, using a clean pipette tip, and 1µl of the homogenate was then added to 99µl of trypan blue and left in a 'damp box' for 10 minutes. Counting of mature viable (Figures 2.4 a and b) and non-viable sperm was carried out using a Neubauer haemocytometer (Weber, UK) (depth 0.1 mm) containing 20µl of the sample, under x40 magnification. Viable sperm were identified by their movement and their ability to exclude the trypan blue, whereas dead sperm were identified by their uptake of the trypan blue and non-movement.

Cell counting rules were used as follows:

- In the first grid, if <10 mature sperm - count all 25 squares
- If 10-40 mature sperm, count 10 random squares
- If >40 mature sperm, count 5 random squares

These results were then analysed taking into account gonad weight, and the number of sperm per mg gonad was calculated.

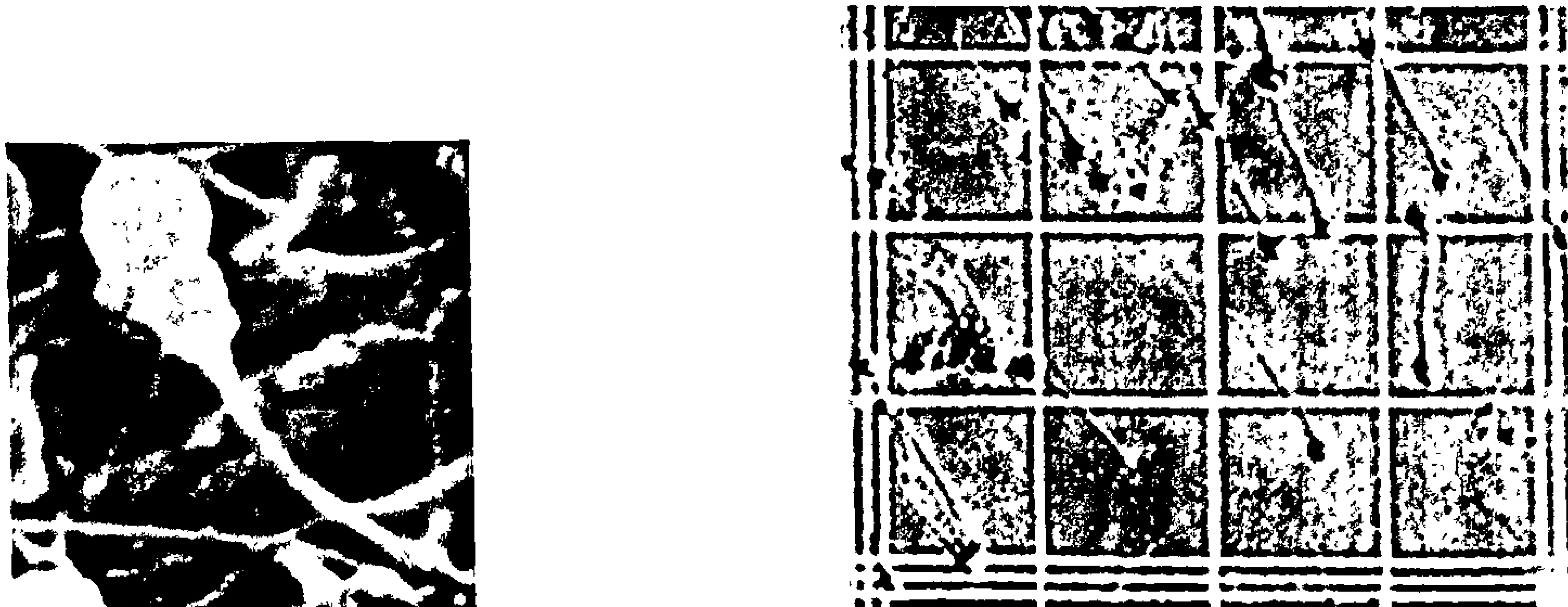


Figure 2.4: a) Mature sperm (picture kindly supplied by Krishen Rana, University of Stirling) and b) sperm on a haemocytometer.

2.2.2 Measurement of Cholesterol within Lipoproteins

Traditionally, lipoproteins were separated by ultracentrifugation, and in fish this approach has been carried out on a number of species, including Rainbow trout, Pacific sardines, and Catfish (Babin, 1989). More recently, a method involving separation according to their electrophoretic properties has been used. This is based on their electrophoretic migration on agarose gels (e.g. Babin, 1989; Santulli *et al*, 1989).

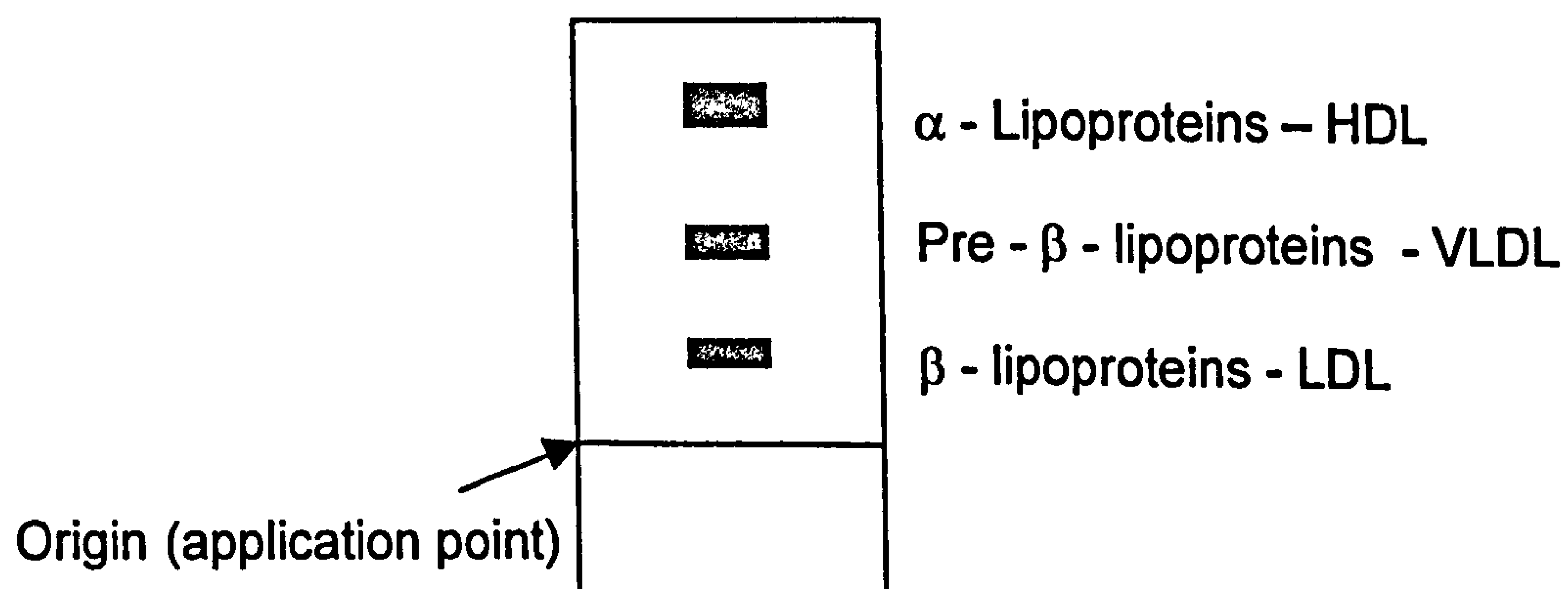
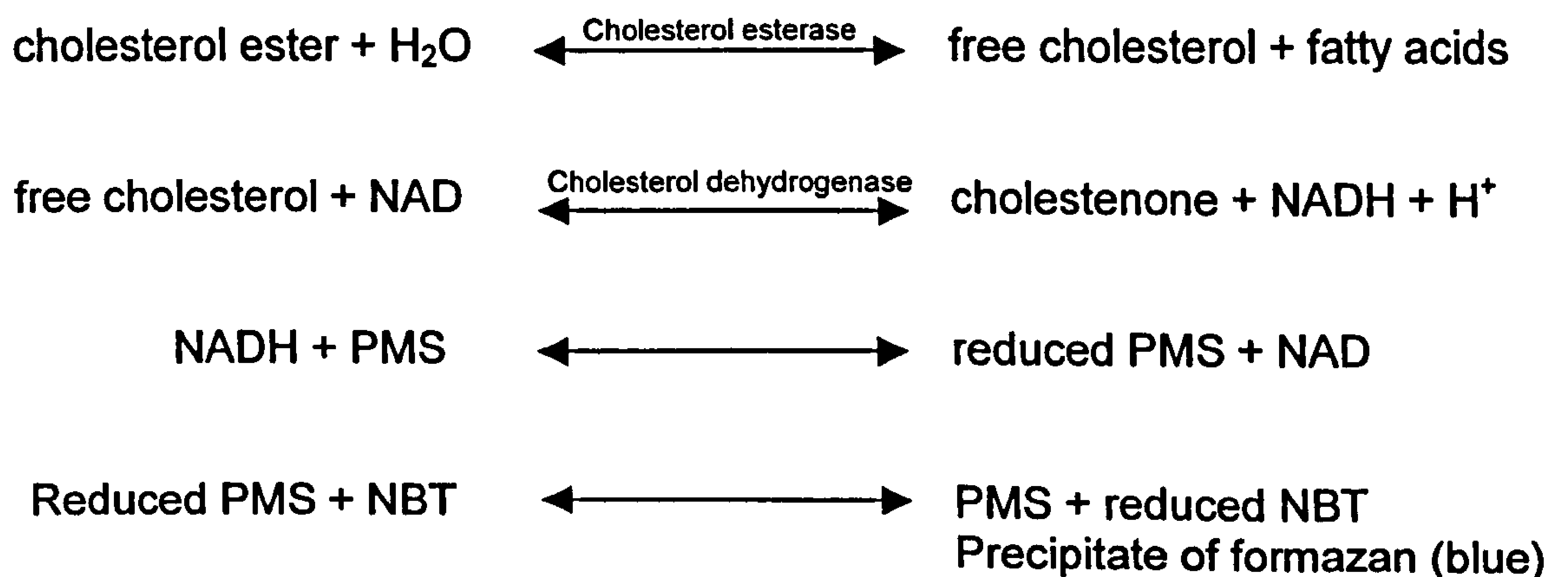


Figure 2.5: Separation of plasma lipoproteins by electrophoresis on agarose gel (adapted from Murray *et al*, 1993).

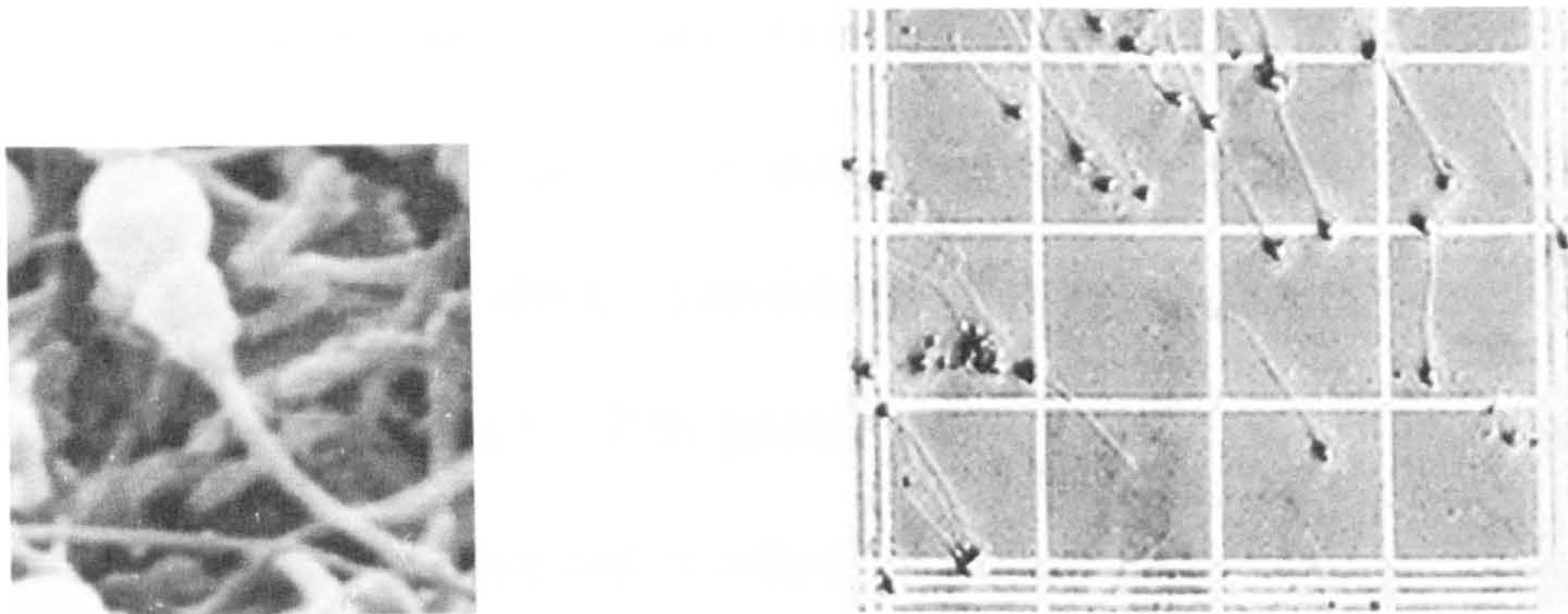
Serum was analysed using a method based on the HYDRAGEL LDL/HDL CHOL direct kit (Sebia, France). This kit is routinely used in clinical biochemistry labs for the quantification of cholesterol within lipoproteins in human serum, and involves electrophoresis on alkaline-buffered (pH 9.1) agarose gels. The system works by assaying for the cholesterol content of the high-density lipoproteins (HDL) low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL). The process is carried out in two stages; firstly, electrophoresis on agarose gel is used to separate the VLDL, LDL and HDL, followed by visualization of the cholesterol within each of the lipoprotein fractions using a sensitive and cholesterol-specific enzymatic method, based on the following reactions:



These stained gels can be evaluated by densitometry to obtain relative quantification of individual zones (bands). The amount of resulting formazan precipitate is proportional to the cholesterol concentration. The assay has been shown previously to have good sensitivity and to be linear in the entire range studied.

Serum samples (previously stored at 4°C and used within 3 days) were applied (6µl of serum + 4µl buffer) to the gel (agarose, 0.8g/dL; alkaline buffer, pH 9.1) via an applicator and left for 15 minutes. Six serum samples were run

These results were then analysed taking into account gonad weight, and the number of sperm per mg gonad was calculated.



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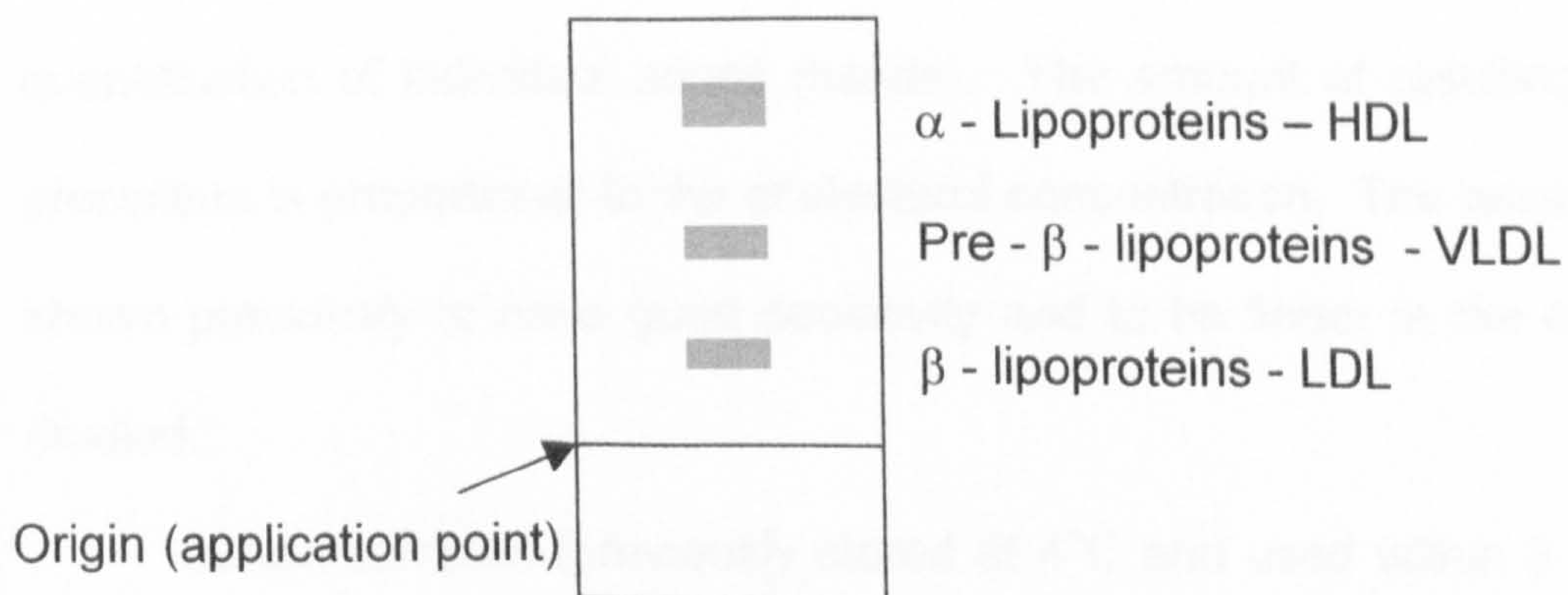

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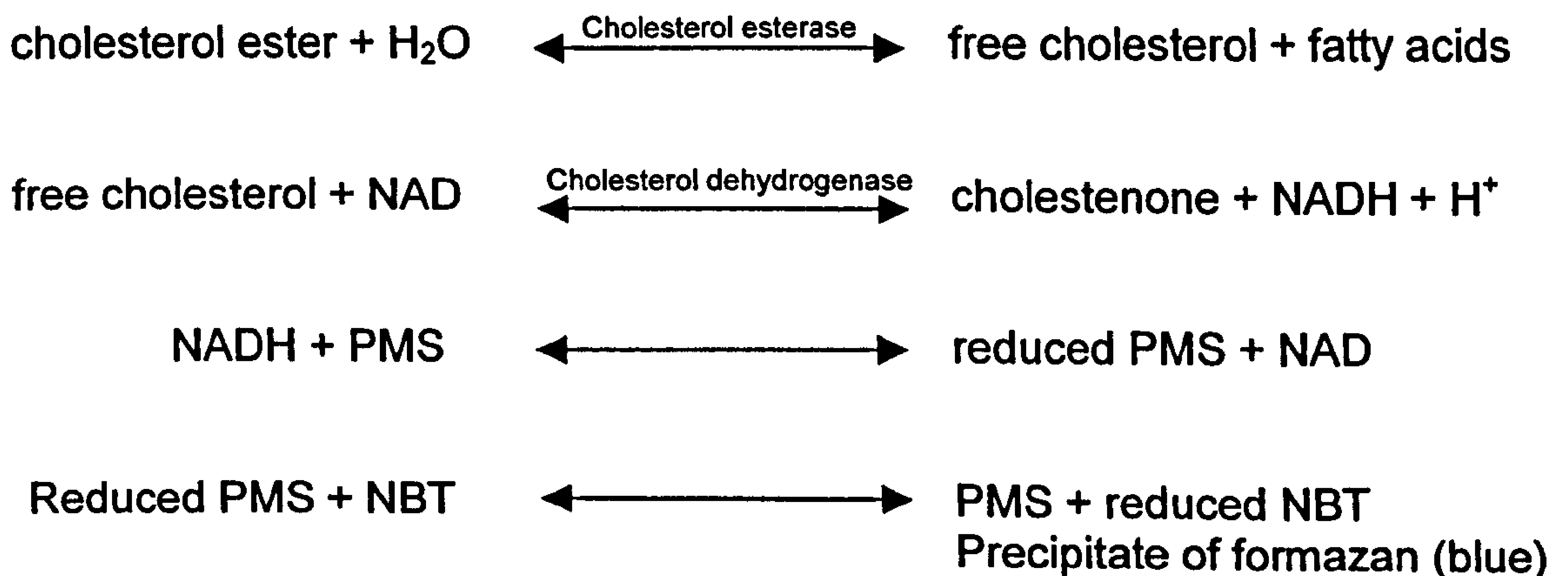


These stained gels can be evaluated by densitometry to obtain relative quantification of individual zones (bands). The amount of resulting formazan precipitate is proportional to the cholesterol concentration. The assay has been shown previously to have good sensitivity and to be linear in the entire range studied.

Serum samples (previously stored at 4°C and used within 3 days) were applied (6µl of serum + 4µl buffer) to the gel (agarose, 0.8g/dL; alkaline buffer, pH 9.1) via an applicator and left for 15 minutes. Six serum samples were run

on each gel, plus one standard which was run on all gels. The gel was then placed carefully into an electrophoresis chamber using the following conditions: 150ml tris-barbital buffer (2.45% barbital, 13.73% sodium barbital, 0.13% sodium azide) pH 9.2) per compartment (300ml total), initial current (per gel) of 10±2 mA, and a constant voltage of 60V.

After running the gel for one hour, visualisation solution (2ml NaCl; added to the enzyme vial supplied with the kits, which contained cholesterol esterase, cholesterol dehydrogenase, nicotinamide adenine dinucleotide and 0.5ml chromogen (nitroblue tetrazolium and phenazine methosulphate)) was added to the surface of the gel using an applicator and the gel left for 30 minutes at room temperature. The solution was removed, a blocking solution was added and the gel was then left for 10 minutes at room temp. Filter paper was applied on top of the gel and it was placed in an incubator for 15 minutes at 51°C. The gel was subsequently submerged in destaining solution for 20 minutes, then removed and completely dried at 51°C.

The resulting gels were scanned using an Alpha Imager 1220 and analysis system V5.5. It was set up using filter 2, white light table, white transilluminator and reflective white light, and an image was obtained (Section 5.2.1). Densitometry was then carried out on these images and the intensity of each band was calculated using the area-under-the-curve function in the analysis software (Figure 5.2 in Chapter 5). These results were then analysed, comparing control and exposed fish, after standardising each area to the internal standard.

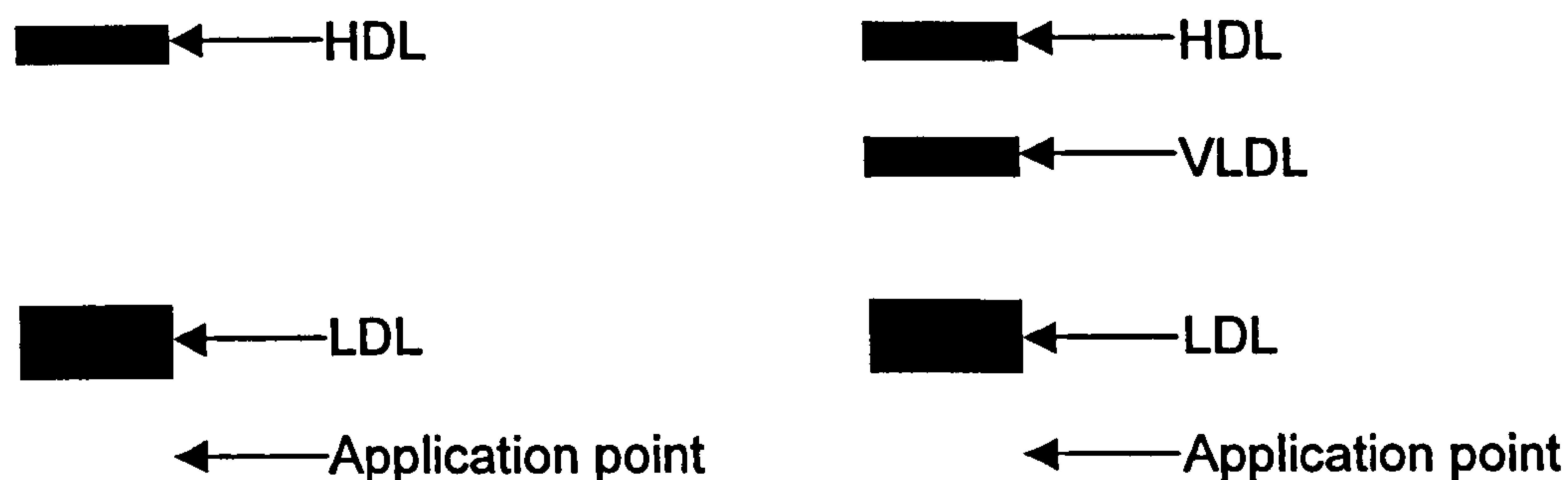


Figure 2.6: Migration patterns for human lipoproteins on Sebia gels. Depending on the sample composition, either one of two patterns can be observed. In fish however, HDL dominates within the plasma (Babin and Vernier, 1989), and consequently the top band is the main one visualised. In contrast, when human serum was run as a standard, LDL is the main band.

2.2.3 Manual methods for measurement of cholesterol and triglyceride levels.

Enzymatic methods were used to quantify both cholesterol and triglyceride levels in the serum sample obtained from some of the experiments. Plasma levels of triglyceride and total cholesterol were measured using a colorimetric assay kit purchased from ThermoTrace DMA (Victoria, Australia). Reagent volumes were modified from those used with human plasma, using a method adapted by Jensen (2002) to accommodate the small volumes of plasma collected from small fish and the lipid-rich nature of the plasma.

All assays were performed in non-absorbent Titertek 96-multiwell flat-bottomed plates (Flow labs, Irvine). For the standards, 35µl 0.9% saline was added to each well, followed by 15µl of each standard. Sample wells received 49µl of 0.9% saline followed by 1µl plasma, and blanks contained 50µl of 0.9% saline.

Cholesterol Assay

A non-absorbent 96 well plate (Titertek, Flow labs, Irvine) was prepared, containing blanks (50µl 0.9% saline), cholesterol standards (Sigma C0534), (an example is shown in Figure 2.7), at 0.03, 0.06, 0.09, 0.12, 0.15, 0.18, 0.24 and 0.3 µg/µl made up to 50µl with 35µl 0.9% saline, and samples (1µl serum plus 49µl 0.9% saline). The plate reader (Molecular Devices, Spectra Max 340pc) was turned on prior to preparation of the plate to ensure 37°C had been reached. 150µl of cholesterol reagent (liquid stable - cholesterol oxidase, cholesterol esterase, peroxidase (horseradish), 4-aminoantipyrine, HBA, buffer and surfactants – pH 6.7±0.1 at 20°C) was then added to all wells, and the plate shaken and incubated in the plate reader at 37°C for 5 minutes. Just before reading, the plate was again shaken, and it was then read at an absorbance of 500nm. Calculations were carried out using SOFTmaxPRO version 3.0 (which used the regression equation from the standard curve) to determine actual concentrations in the samples.

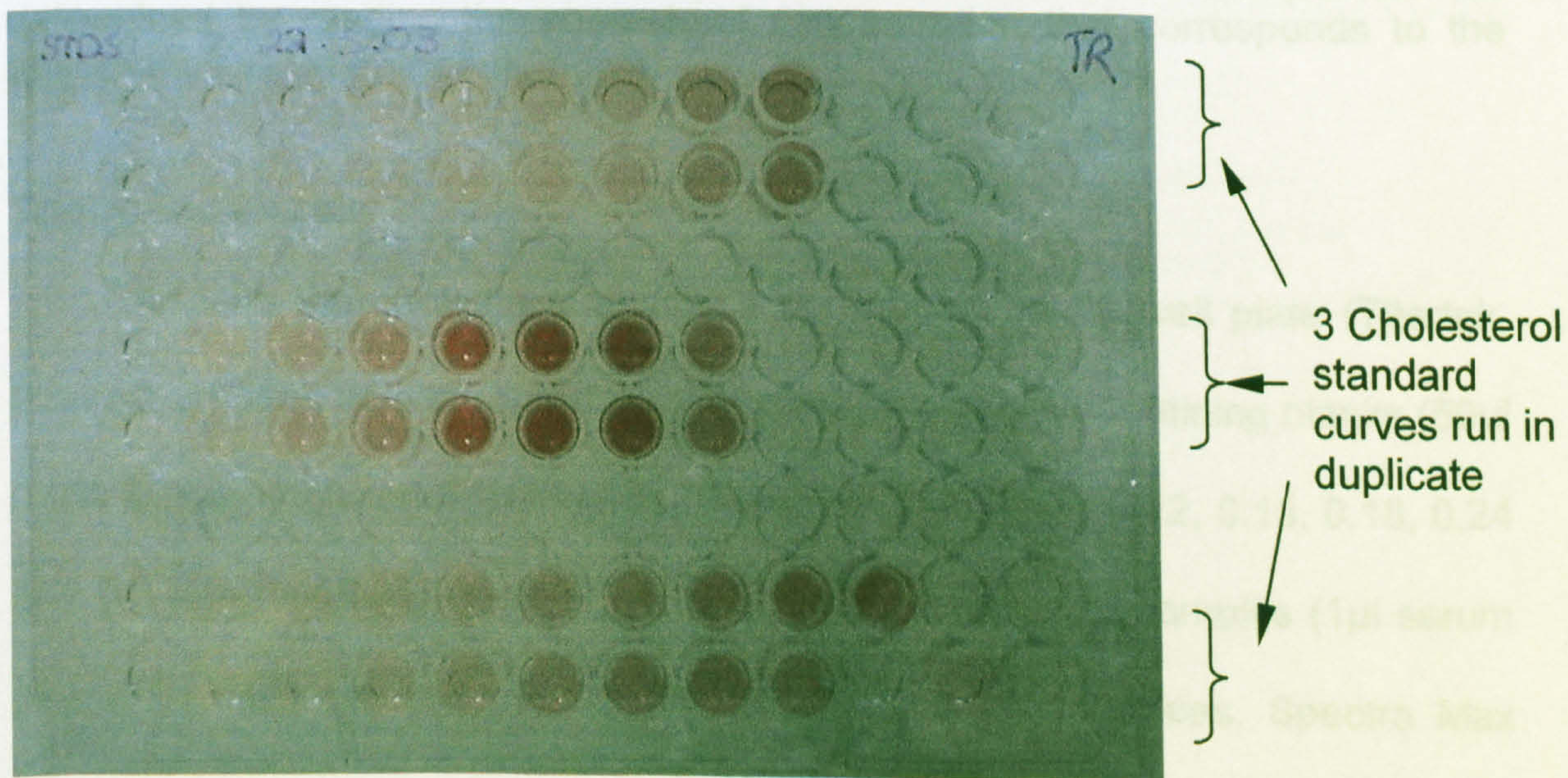
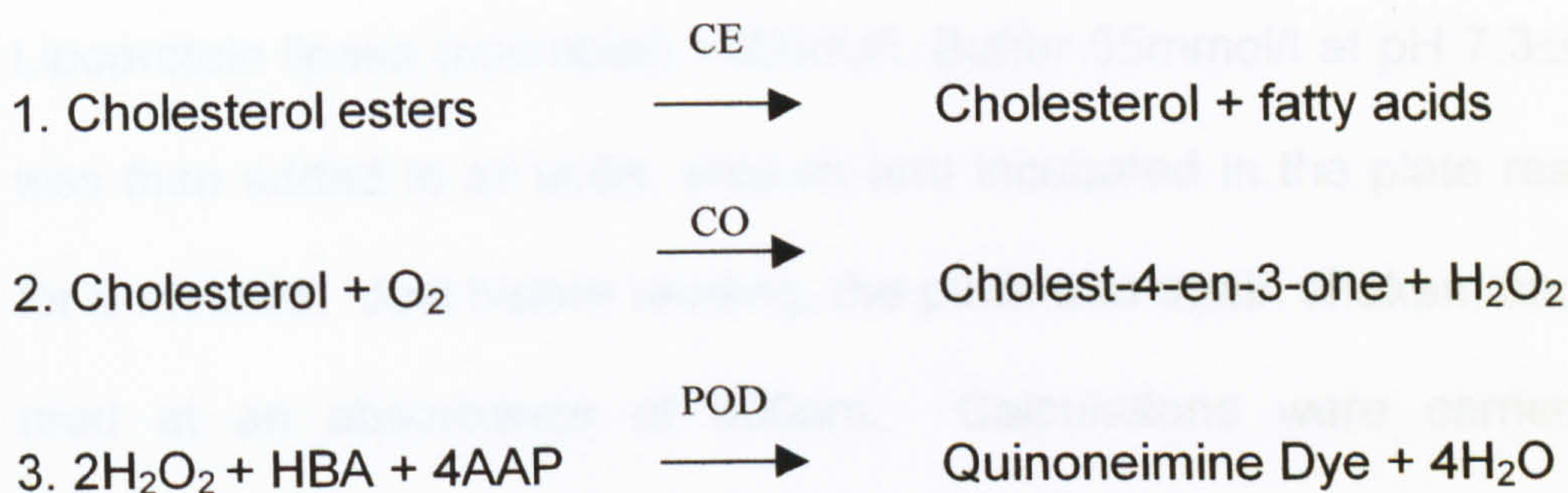


Figure 2.7: An example of a 96-well plate used for the manual cholesterol assay - showing three cholesterol standard curves (at different dilutions) which were all run in duplicate.

The reactions involved in the assay are as follows:



Where: CE = cholesterol esterase, CO = Cholesterol oxidase, HBA= Hydroxybenzoic acid, 4AAP = 4-aminoantipyrine, POD = Peroxidase.

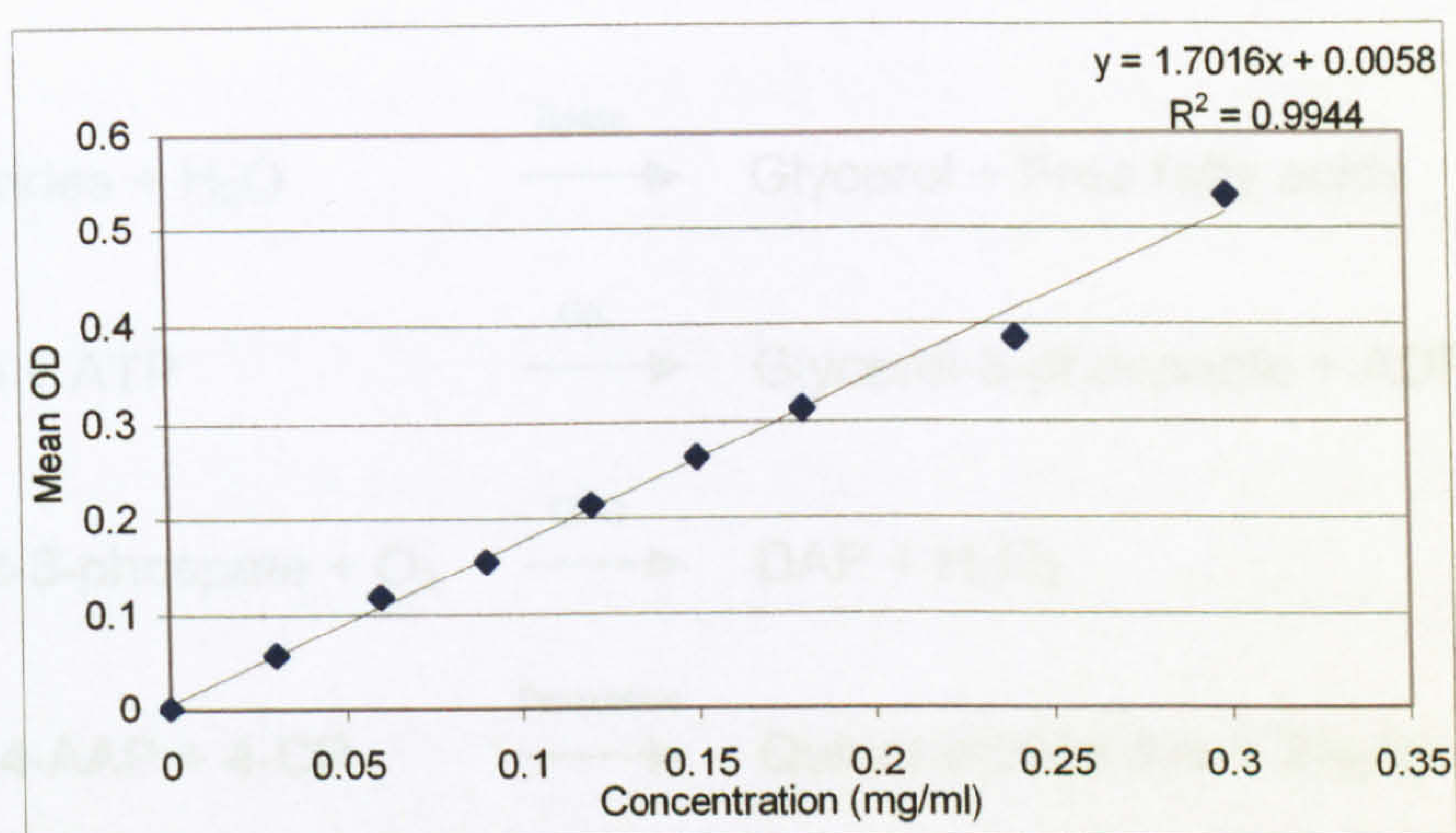


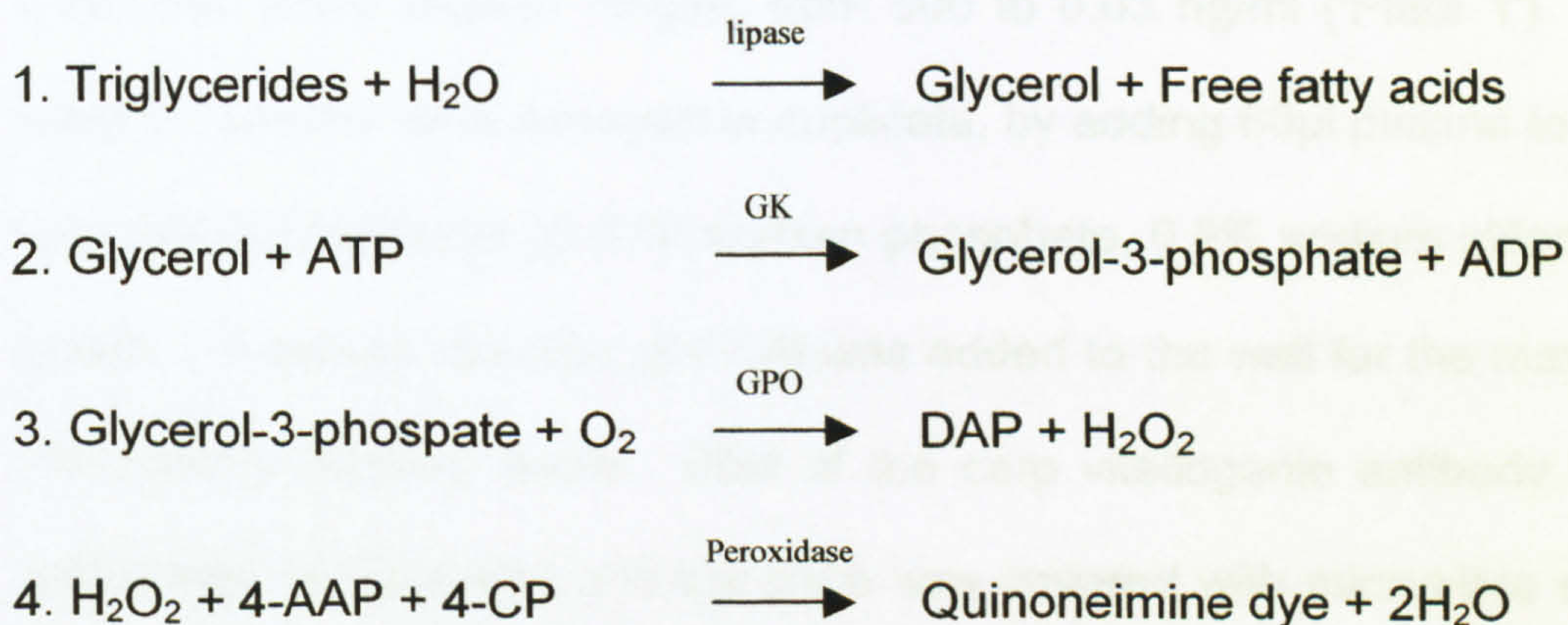
Figure 2.8: An example of a typical standard curve produced for the cholesterol assay. The cholesterol level of the unknown sample can be determined by reading the cholesterol concentration that corresponds to the absorbance value of that unknown.

Triglyceride Assay

As with the cholesterol assay, a non-absorbent 96 well plate (Titertek, Flow labs, Irvine) was prepared as described previously, containing blanks (50µl 0.9% saline), triglyceride standards (Sigma) at 0.03, 0.06, 0.12, 0.15, 0.18, 0.24 and 0.3 µg/µl made up to 50µl with 35µl 0.9% saline, and samples (1µl serum plus 49µl 0.9% saline). The plate reader (Molecular Devices, Spectra Max 340pc) was turned on prior to preparation of the plate to ensure 37°C had been reached. 150µl of triglyceride reagent (liquid stable; ATP 2.5mmol/l, Mg^{2+} 2.5mmol/l, 4-Aminoantipyrine 0.4mmol/l, 4-Chlorophenol 2mmol/l, Peroxidase

(Horseradish) >2000U/l, GK (microbial)>600U/l, GPO (Microbial) >6000U/l, Lipoprotein lipase (microbial) >3000U/l, Buffer 55mmol/l at pH 7.3±0.1 at 20°C) was then added to all wells, shaken and incubated in the plate reader at 37°C for 5 minutes. Just before reading, the plate was again shaken, and it was then read at an absorbance of 500nm. Calculations were carried out using SOFTmaxPRO version 3.0 (which used the regression equation from the standard curve) to determine actual concentrations in the samples.

The reactions involved in the assay are as follows:



Where: ATP - Adenosine triphosphate, GK - Glycerol kinase, ADP - Adenosine diphosphate, DAP – Dihydroxyacetone phosphate, 4AAP-4-aminoantipyrine, 4CP – 4-chlorophenol.

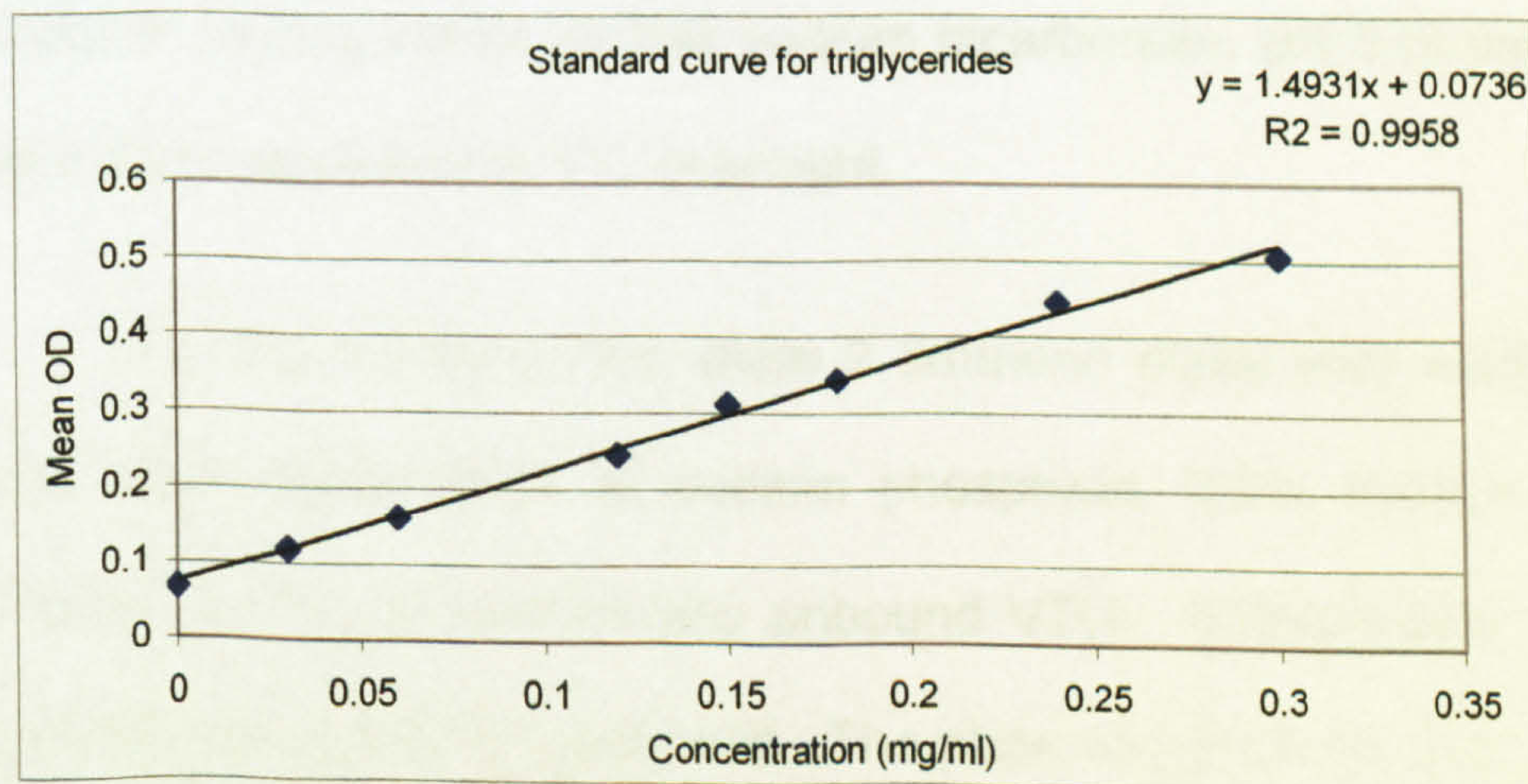


Figure 2.9: An example of a typical standard curve for the triglyceride assay. The triglyceride level of the unknown sample can be determined by reading the triglyceride concentration that corresponds to the absorbance value of that unknown.

2.2.4 Vitellogenin ELISA

The VTG Enzyme-Linked-Immunosorbant-Assay (ELISA) based on carp vitellogenin (*Cyprinus carpio*), previously validated for use in a wide range of other species by Tyler *et al*, (1996) was used (see Chapter 4) to determine concentrations of vitellogenin present in the plasma of bream before and after exposure to clofibric acid. The protocol was as follows:-

Day 1: A VTG (carp-VTG) standard curve was prepared in duplicate in a non-absorbent Titertek 96-multiwell flat-bottomed plate (Flow labs, Irvine), with a two-fold serial dilution ranging from 500 to 0.03 ng/ml ('Plate 1'). Unknown plasma samples were assayed in duplicate, by adding 60µl plasma to each well. 60µl of blocking buffer (0.01M sodium phosphate, 0.9% sodium chloride, 0.05% tween, 1% bovine albumin, pH 7.4) was added to the well for the maximum and non-specific binding levels. 60µl of the carp vitellogenin antibody (1:50 000) was added to each well and the plate was covered with microplate sealing film (Anachem, Bedfordshire). In parallel, wells in an immunsorbant plate – 'plate 2' (Nunc™ maxisorp microtitre plate, GibcoBRL) - were coated with 60 ng of carp-VTG (in 100µl), except for those used to determine non-specific binding, to which 100µl of coating buffer (0.05M sodium bicarbonate, pH 9.6) was added. Both plates were incubated at 4°C overnight.

Day 2: On the following day, plate 2 (immuno plate) was washed three times with wash buffer (0.01 M sodium phosphate, 0.9% sodium chloride, 0.05% Tween, pH7.4) to remove any unbound VTG. Subsequently, 150µl of blocking buffer was added to each well. The plate was then incubated at 37°C for 30 minutes, together with plate 1, containing the standards and unknown samples. Following the incubation, plate 2 was washed as before and 100µl of

standard/samples were taken from each well on plate 1 and transferred to plate 2. Plate 1 was then discarded and plate 2 incubated for 60 min at room temperature. Plate 2 was then washed, as before, and 125µl of second antibody (goat-anti-rabbit horse-radish peroxidase-linked; 1:2000 dilution; Sigma) was added to each well. The plate was then incubated at 37°C for 120 minutes. Following this, 125µl of substrate buffer (0.05M dibasic sodium phosphate, 0.024M citric acid, pH 5.0; and just before use, the addition of O-phenylene diamine (0.05mg/ml) and 0.5µl/ml hydrogen peroxide) was added to each well and the plate incubated in the dark at room temperature for 50 min. The reaction was stopped by adding 30µl of 3M sulphuric acid to each well. The plate was then incubated for a further 10 minutes in the dark and the absorbance was read at 450nm using a plate reader (Molecular Devices, Spectra Max 340pc). The level of maximum binding was calculated according to the formula below:

$$\frac{\text{Average of duplicate} - \text{average of NSB values}}{\text{Average maximum binding} - \text{non specific binding}} \times 100$$

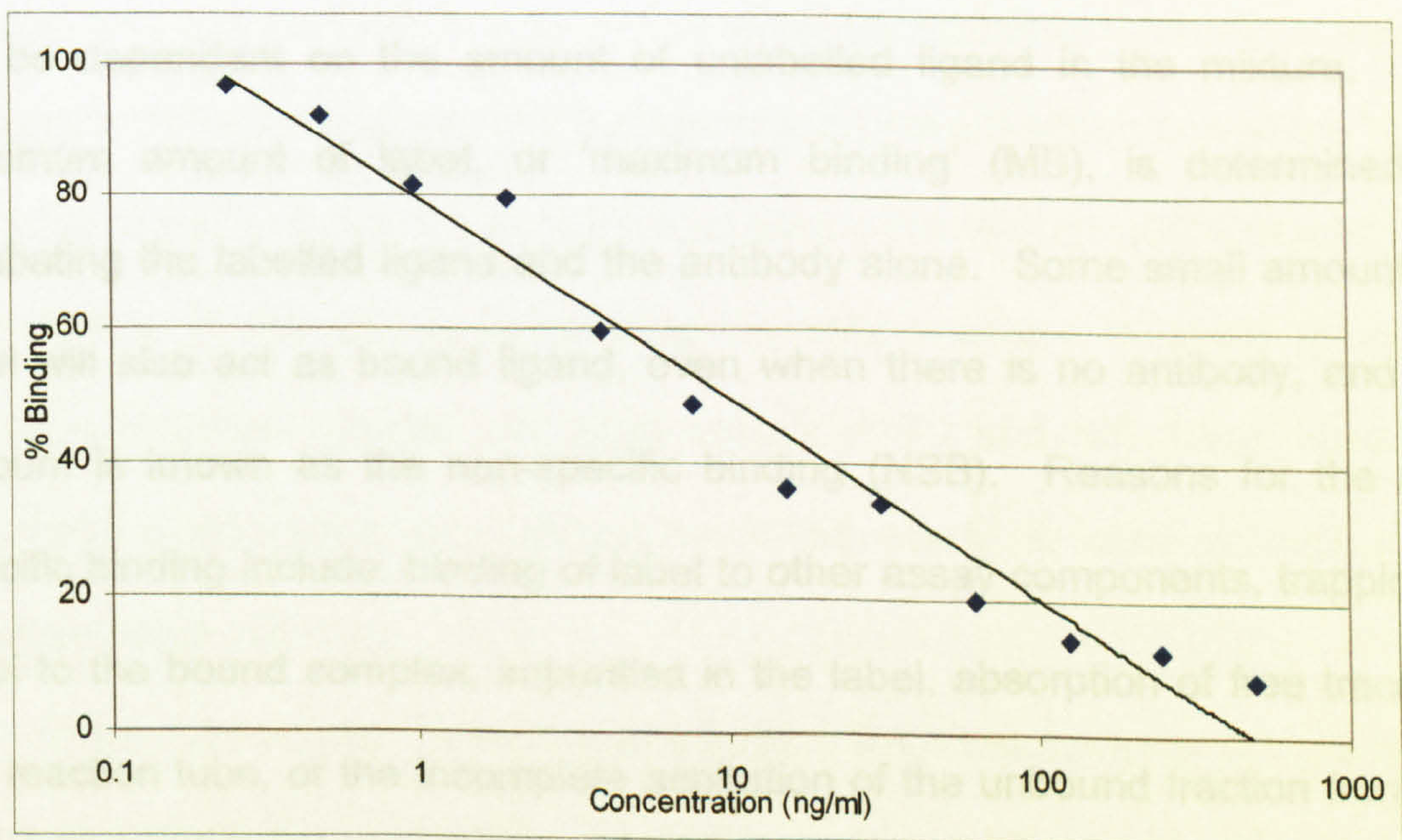


Figure 2.10: An example of a standard curve produced from the vitellogenin ELISA. Data from the unknown samples can then be read off the standard curve to give the concentration of vitellogenin in ng/ml.

The standard curve was plotted (semi logarithmically) (Figure 2.10), by plotting percentage binding against VTG concentration. VTG concentrations of the unknowns were then read from this (unknown concentrations are generally taken from between 20-80% binding, as this is the straightest part of the curve).

2.2.5 Sex steroid Radioimmunoassays

Plasma testosterone and 11-Ketotestosterone concentrations were not initially intended as endpoints for any of the experiments within this study, but after obtaining the sperm count data it was decided that it would be very interesting to determine them (see Section 5.5.4.1 for results). These assays were kindly carried out by Miss Susan Maddix.

The principal of the radioimmunoassay is based upon the equal competition between a radiolabelled ligand and an unlabelled ligand for binding to a specific antibody. The amount of the radiolabelled ligand and antibody used in an assay are kept constant so that, when the mixture of reagents has been allowed to equilibrate, the amount of radiolabelled ligand bound to the antibody will be dependant on the amount of unlabelled ligand in the mixture. The maximum amount of label, or 'maximum binding' (MB), is determined by incubating the labelled ligand and the antibody alone. Some small amounts of label will also act as bound ligand, even when there is no antibody, and this amount is known as the non-specific binding (NSB). Reasons for the non-specific binding include: binding of label to other assay components, trapping of label to the bound complex, impurities in the label, absorption of free tracer to the reaction tube, or the incomplete aspiration of the unbound fraction from the tube. The values represented by the MB and NSB are in effect the upper and lower limits of the assay. Within each assay, a standard curve is included to

determine the concentration range over which the amount of unknown ligand can be quantified. The assay is set up with fixed amounts of antibody and label and serially diluted concentrations of known amounts of unlabelled standard. The NSB value is subtracted from the MB value and the corrected MB is taken as 100%. The remaining values for the standard tubes are also corrected for NSB and are expressed as a percentage of MB. The data are plotted on a logarithmic scale and a sigmoidal curve is produced. The central region of the curve is where extrapolations of sample counts to sample concentration can be made (the straight region in general lies between 20 and 80% of maximum binding).

At the end of the radioimmunoassay, the free unbound label needs to be separated from the antibody-ligand complex. In steroid assays, this separation involves the use of activated charcoal, to which small molecules will be absorbed. Unlabelled ligand will thus be adsorbed and this complex will be precipitated out by centrifugation. The antibody (with bound labelled or unlabelled hormone attached) remains in the supernatant, which is decanted directly into beta scintillation vials and retained for counting. Albumin or dextran is used to fill the mesopores of the charcoal, to prevent adsorption of the bound antibody-ligand complex into these larger sites. The timings of the reaction and temperature of the assay tubes must be kept constant to avoid excessive variation between assays. In large assays, NSB and MB tubes were also included in each batch of samples within the assay, to allow adjustment for batch-to-batch variation.

The radioactivity, or beta rays, emitted from the samples are measured using a scintillation counter. These rays are particulate and not highly penetrative, so cannot pass through the plastic tube. To get around this

problem, photons of light are generated within the vial, by a liquid scintillate which is activated by the radiation. The photons pass through the vials and can be detected by photomultipliers with the scintillation counter.

Testosterone and 11-Ketotestosterone radioimmunoassays were carried out using plasma samples obtained from Experiment 5. The samples were extracted using ethyl acetate (which separates the steroids from their sex steroid binding proteins) prior to the assay using a method modified for use with the small amounts of plasma obtained from the fathead minnow.

Method in detail

10µl of plasma was transferred to a polypropylene tube, followed by the addition of 250µl ethyl acetate. The tube was capped, shaken and stored at –20°C. On the day of the assay the samples (plasma plus ethyl acetate) were spun at 2500g for 2 minutes to separate the ethyl acetate (top layer) from the plasma (lower layer). The ethyl acetate, containing the extracted steroid hormones, was decanted into another tube. The ethyl acetate was then removed using a gyrovap (Howe, London, UK) and the steroid remaining was re-dissolved in 100µl buffer (0.05M phosphate buffered saline (PBS, Oxford), gelatine (Sigma), sodium azide). 200µl buffer was added to the total counts and NSB tubes; 100µl was added to each standard tube except the first pair. Standard was serially diluted from the second to the last standard, along an entire row, before serially diluting a duplicate row of standards.

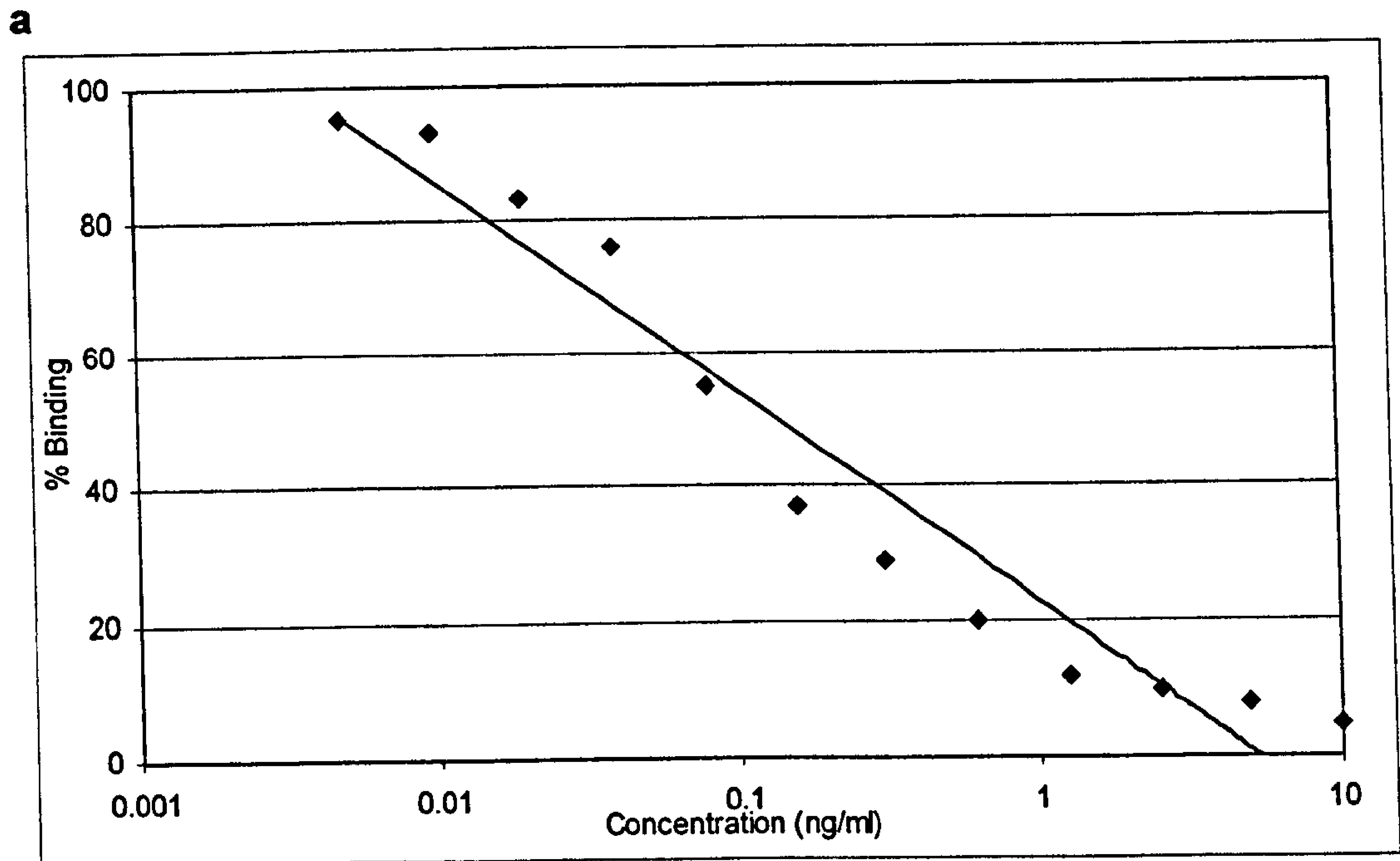
100µl of labelled (tritiated) hormone (approximately 6000 dpm for both testosterone and 11KT RIAs) was added to each tube. 100µl of antibody (diluted 1:50,000 for both testosterone and 11KT; kindly supplied by D.Kime of Sheffield University), was added to all tubes except the total counts and NSB

tubes. Tubes were then vortexed and centrifuged briefly to ensure the contents were in the base. The tubes were then covered and incubated overnight at 4°C.

Day 2: As previously mentioned, activated charcoal was used to separate the bound complex from the unbound label led hormone. Dextran-coated charcoal was prepared (0.5% activated charcoal; 0.05% dextran in buffer) 15 minutes prior to use. 500µl buffer was then added to the total count tubes. 500µl activated charcoal mixture was added to each of the remaining tubes (one batch at a time), the tubes vortexed and incubated on ice for 10 minutes prior to centrifuging at 2500g for 15 minutes, at 4°C. The supernatant (containing the bound fraction) was removed and transferred to scintillation vials to which 4 ml scintillation fluid (Liquiscint, National Diagnostics) was added. The tubes were then shaken and counted in a β-counter for 5-10 minutes.

Calculations were carried out by hand using mean dpm for each pair of tubes and applying the following equation: ((unknown-NSB)/(MB-NSB))x100. The standard curve was plotted on log-normal paper, and the unknown values were read from the linear part of the curve, usually between the 20 and 80% binding (Figures 2.11, a and b). The testosterone assay is highly specific and sensitive; the detection limit was 0.033ng/ml. The detection limit for the 11-KT assay on the other hand was 0.28ng/ml.

a



b

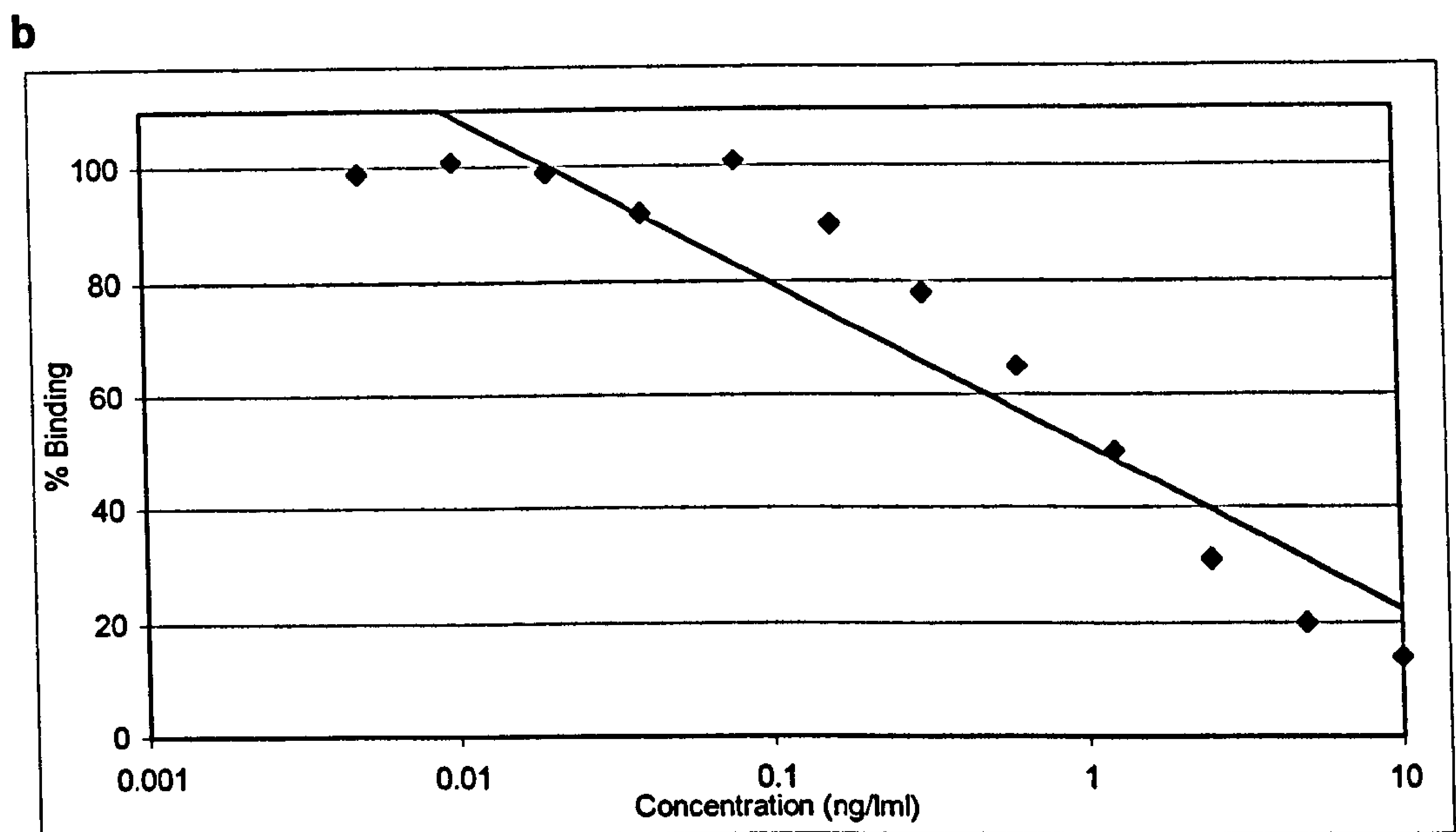
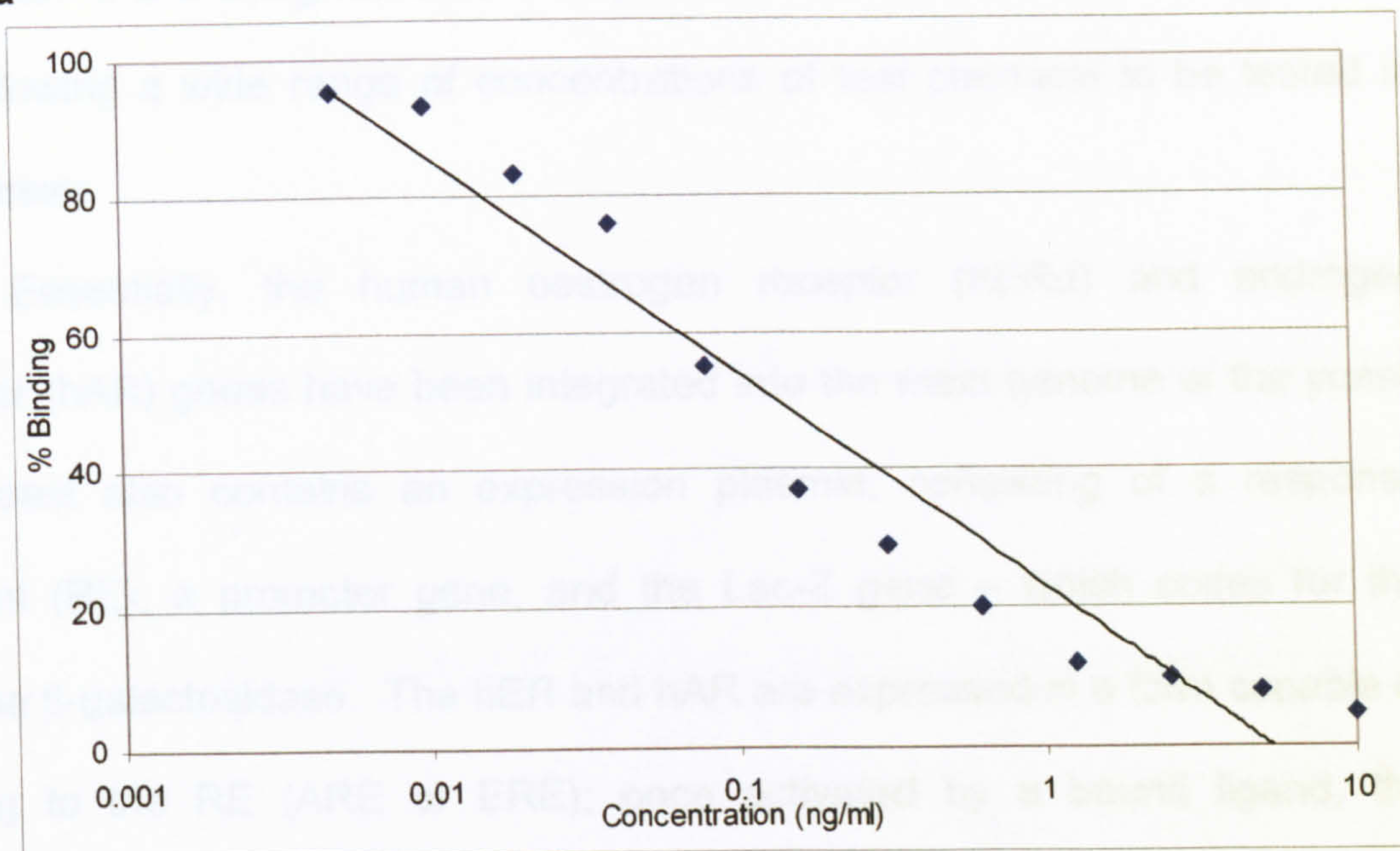


Figure 2.11: Typical standard curves for the a) testosterone and b) 11-ketotestosterone radioimmunoassay. Where concentrations of T and 11-KT in the unknowns can be read from the standard curve by extrapolating the percentage binding on the y-axis and reading down to the x-axis to give a concentration of ng/ml.

2.2.6 Yeast oestrogen assay

Clofibric acid was kindly analysed by David Hala using both the yeast oestrogen and androgen screens, using the protocols described by Routledge and Sumpter (1996) and Sohoni and Sumpter (1998). These assays are commonly utilised for the detection of oestrogen and androgen activity in test

a



b

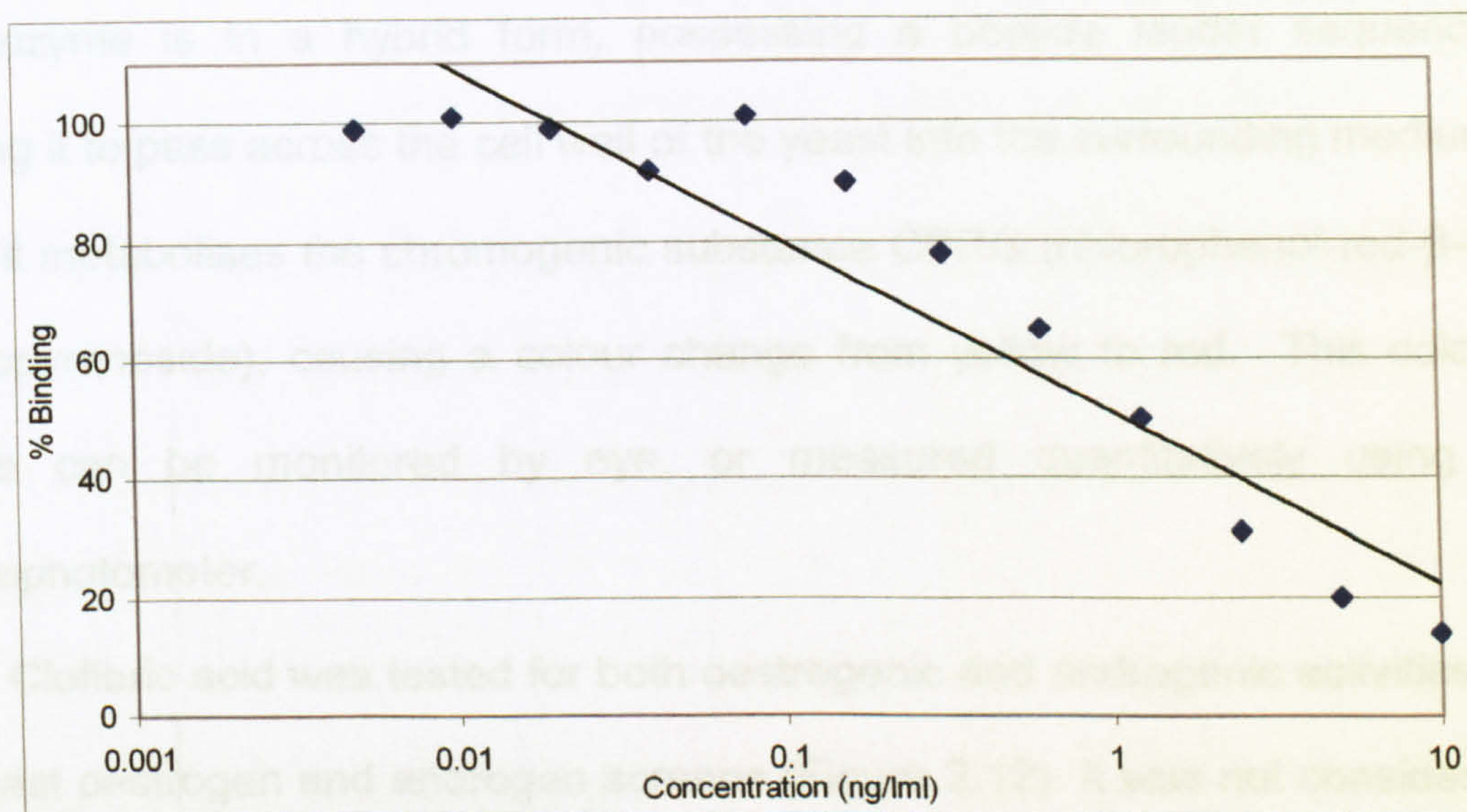


Figure 2.11: Typical standard curves for the a) testosterone and b) 11-ketotestosterone radioimmunoassay. Where concentrations of T and 11-KT in the unknowns can be read from the standard curve by extrapolating the percentage binding on the y-axis and reading down to the x-axis to give a concentration of ng/ml.

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chemicals. It is a straightforward assay carried out in a 96-well microtitre plate, thus allowing a wide range of concentrations of test chemical to be tested in each assay.

Essentially, the human oestrogen receptor (hERα) and androgen receptor (hAR) genes have been integrated into the main genome of the yeast. The yeast also contains an expression plasmid, consisting of a response element (RE), a promoter gene, and the Lac-Z gene – which codes for the enzyme β-galactosidase. The hER and hAR are expressed in a form capable of binding to the RE (ARE or ERE); once activated by a bound ligand, the expression of Lac-Z is triggered, resulting in the production of β-galactosidase. This enzyme is in a hybrid form, possessing a peptide leader sequence, enabling it to pass across the cell wall of the yeast into the surrounding medium, where it metabolises the chromogenic substance CPRG (chlorophenol-red-β-D-galactopyranoside), causing a colour change from yellow to red. This colour change can be monitored by eye, or measured quantitatively using a spectrophotometer.

Clofibric acid was tested for both oestrogenic and androgenic activities in the yeast oestrogen and androgen screens (Figure 2.12). It was not considered that clofibric acid would be oestrogenic, as Ashby *et al* (1997) stated that Clofibrate (the parent compound) was inactive as an oestrogen in the yeast oestrogen assay. Oestrogenic effects are also unexpected based upon the chemical structures of Clofibrate and clofibric acid. Kazeto *et al*, (2004) also found clofibric acid to be non-oestrogenic and non anti-oestrogenic.

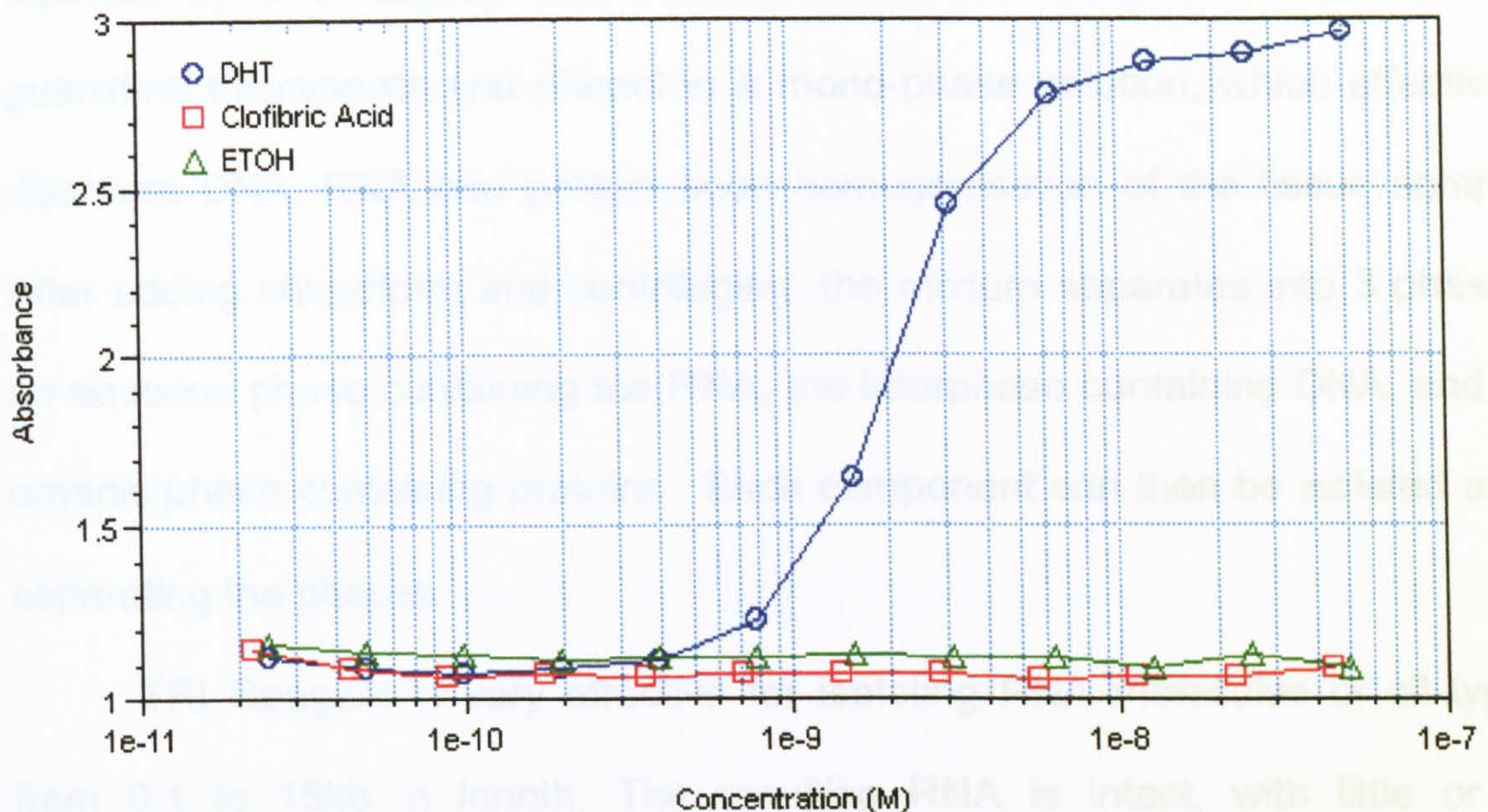

Figure 2.12: Lack of activity of Clofibric acid in the yeast androgen screen. Note that whereas DHT (the standard androgen) produced the expected dose-response curve, clofibric acid, at all concentrations, had absolutely no effect – the absorbance did not rise above the negative control (ethanol – ETOH).

In both assays clofibric acid was inactive; that is, it showed no oestrogenic or androgenic properties. Figure 2.12 shows an example of the results obtained when clofibric acid was tested in the yeast androgen screen when run along-side DHT.

2.2 Molecular Techniques

Effects of clofibric acid in the fathead minnow at the molecular level are investigated in Chapters 5 and 6. The routine molecular methods employed are described here, with more specific details provided subsequently.

2.3.1 RNA extraction

TRI Reagent (Sigma) is a single step liquid phase separation that results in the simultaneous isolation of RNA, DNA and protein. This method is an improvement on the previous single step method for total RNA isolation

reported by Chomczynski and Sacchi, 1987. The reagent is a mixture of guanidine thiocyanate and phenol in a mono-phase solution, which effectively dissolves DNA, RNA and protein upon homogenisation of the tissue sample. After adding chloroform and centrifuging, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after separating the phases.

TRI Reagent is very effective for isolating RNA molecules of all types from 0.1 to 15kb in length. The resulting RNA is intact, with little or no contamination from DNA or protein. The RNA can be used for Northern blots, mRNA isolation, RNase protection assays, cloning and polymerase chain reactions (PCR).

When preparing total RNA from tissue, it is important to inhibit as quickly and efficiently as possible the endogenous ribonucleases which are present in all living cells. Tissue samples were removed from the –80°C freezer and put on dry ice, before being homogenised in TRI Reagent (1ml/ 50-100mg of tissue) in eppendorf tubes (Alpha labs, Hampshire) using mini homogenisers. The homogenates were then left at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. 0.2ml chloroform per ml of TRI Reagent used was then added, and all the tubes covered tightly and shaken vigorously for 15 seconds. After standing for 2-15 minutes at room temperature, the resulting mixture was centrifuged at 12,000x g for 15 minutes at 4°C. This separated the mixture into 3 phases: the red organic phase (containing protein), the interphase (containing DNA), and a colourless upper aqueous phase (containing the RNA). This aqueous phase was then transferred to a fresh tube and 0.5ml isopropanol per ml of TRI Reagent used

was added. This was then mixed and allowed to stand for 5-10 minutes at room temperature, before centrifuging at 12,000x g for 10 minutes at 4°C. The RNA precipitate formed a pellet on the side and bottom of the tube. The supernatant was then removed and the RNA pellet washed by adding 1ml (minimum) 75% ethanol per ml of TRI Reagent used and mixed by vortexing. This was then centrifuged at 7,500x g for 5 minutes at 4°C. The RNA pellet was subsequently air-dried for approximately 10 minutes and dissolved in an appropriate amount of double autoclaved, sterile deionised water, by flicking the tube until dissolution occurred.

2.3.1.1 Quantification of RNA

RNA is quantified by measuring its absorbance in a spectrophotometer; the optical density (OD) is measured at its maximum absorbance wavelength of 260nm. One OD unit is equivalent to 40μg/ml of RNA. Contamination of RNA preparations with substantial amounts of protein is demonstrated by measuring the OD at a wavelength of 280nm, because proteins have a maximum absorbance at this wavelength. A 260/280 absorbance ratio of 1.8–2.0 is indicative of pure RNA.

All RNA samples were quantified by 260/280nm spectrophotometry (Genequant, Pharmacia, Cambridge, UK) and their integrity checked visually by agarose gel electrophoresis (see Section 2.3.1.3). Samples were then stored at –80°C until required. Samples were heated to 55-60°C just before dilution for assay, to ensure that the RNA was fully in solution.

2.3.1.2 DNase treatment

When carrying out RNA extraction, there is no guarantee that there is complete removal of DNA. DNAse I digests single and double stranded DNA -

so predigesting RNA with this enzyme eliminates any contaminating DNA prior to hybridisation or RT-PCR amplification. This was only carried out in preliminary studies (Section 5.2.2.1), because the results showed that it was not necessary for the analyses conducted in my project.

2.3.1.3 Gel electrophoresis using 1.2% Agarose gels

1.2g of agarose (BioRad, Hemel Hempstead, UK) was added to 100ml of 1x TBE buffer (90mM Tris-borate (Amresco), 2mM EDTA (pH 8.0) (Amresco)) and heated in a microwave until it had completely melted. After cooling to 60°C, 5µl of ethidium bromide (Sigma; final concentration 0.5µg/ml) was added to the agarose and the flask swirled to mix the contents. The gel was then poured into the assembled slide, and after cooling the comb was removed and the gel placed into the electrophoresis apparatus (BioRad, Hemel Hempstead, UK).

Before running, 4µl loading buffer (20% Ficoll 400 (Sigma), 1% SDS (Amresco), 1mM EDTA (pH8.0), and 0.25% bromophenol blue (BDH, Poole, UK), 0.25% xylene cyanol (Sigma) was added to each sample, which were then loaded into the wells. The gel was then run in a gel tank at 78V for 1¼ hours. It was then visualised using the Alpha Imager 1220 Doc and analysis system V5.5.

2.3.2 Hybridisation Protection Assays

These kits are quantitative hybridisation assays which can measure specific fathead minnow mRNAs. They were kindly supplied by Molecular Light Techniques, Cardiff. An oligonucleotide probe, labelled with a chemiluminescent acridinium ester, was introduced into a sample of extracted RNA, where it hybridised with any of the complementary target present. The hybridisation is

followed by a selection step, in which label attached to free probe is hydrolysed to a nonchemiluminescent derivative, while label attached to hybridised probe is protected from such hydrolysis. After the selection step, the chemiluminescence is measured over a 5 second interval using a luminometer (MLT Smartlight Luminometer). The light intensity is proportional to the concentration of the specific mRNA in the sample.

RNA resuspended in nuclease-free water, which had been previously prepared by TRI Reagent (described above) and stored at –80°C until needed, was used in the assays. The concentration was 0.01, 0.001/0.1ug/µl dependant on sex (to ensure samples fitted onto the standard curve). Samples were set up in duplicate, as shown in Figure 2.13, in 75x12mm assay tubes, using nuclease-free water and RNase-free conditions throughout.

	Calibrator 1	Calibrator 2	Calibrator 3	Calibrator 4	Test sample
RNA sample	-	-	-	-	50µl
Kit calibrator	50µl	50µl	50µl	50µl	-
Reconstituted probe	50µl	50µl	50µl	50µl	50µl
Final volume	100µl	100µl	100µl	100µl	100µl

Table 2.1: Reaction mixtures for HPA assays.

Four calibrators and three internal standards (synthetic target oligonucleotide) were also integrated (with the calibrators first, the standards at the beginning, middle and end of the samples) into each assay. 50µl of sample RNA (or calibrator or standard) were added to the tubes, followed by 50µl of probe (synthetic oligonucleotide labelled with acridinium ester). The tubes were then covered with laboratory tape, agitated, and incubated in a water bath at 60°C for 30min. The samples were again agitated, 300µl selection reagent (detergent solution) added to each tube and these were again agitated, before placing the tubes once more in the water bath at 60°C for 15 minutes. The

tubes were then taken out of the water bath and put straight on ice/water slurry for 2 minutes, and then at room temperature for a further 2 minutes before measuring the chemiluminescence associated with each tube, in order, on a luminometer (MLT Smartlight Luminometer) using two detection reagents (1: 0.032 M Hydrogen peroxide, 0.001 M Nitric acid and, 2: 1.575 M Sodium hydroxide).

The concentration of mRNA (both ZP3 and VTG) for each sample was then determined using a luminometer to measure the chemiluminescence associated with each sample. The light intensity is proportional to the concentration of mRNA in the samples.

The Calibration Curve

With each assay, a calibration curve was included to determine the concentration of mRNA in each sample (Figure 2.13). The light intensity (RLU) is proportional to the concentration of mRNA.

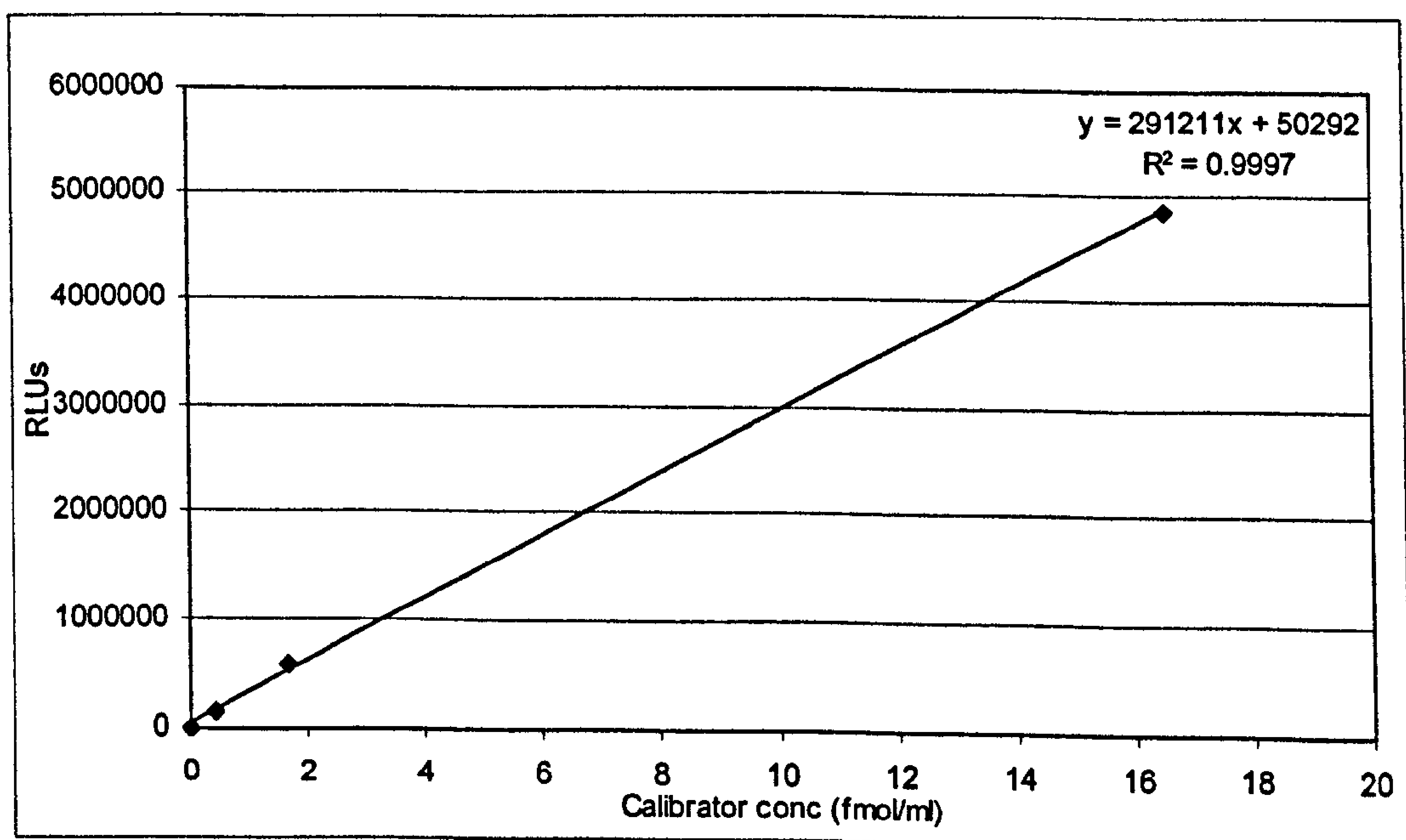

Figure 2.13: An example of a typical calibration curve obtained using a hybridisation protection assay. The concentration of the required mRNA in the 'unknown' sample can be extrapolated from reading the relative light units (RLU) on the y-axis and the calibrator concentration on the x-axis.

2.3.3 Polymerase Chain Reaction

In the 1980's, the Polymerase Chain Reaction (PCR) was invented (Saiki *et al* 1985). It is an enzymatic method for making multiple copies of a selected segment of DNA. The process employs synthetic oligonucleotide primers, a thermostable DNA polymerase (such as *Taq* polymerase) and four deoxyribonucleoside triphosphates acting on the template DNA. When this is denatured and cooled, the primers anneal to their targets on the single stranded templates. The DNA polymerase then extends the primers at the 3' ends. As the 3' ends of the primers point towards each other, the repeated cycles of heating and cooling leads to a chain reaction – an exponential synthesis of copies of the specific segment bound by the two primers.

PCR uses DNA, not RNA, as template – when amplifying using RNA as starting material, it has to be first converted into DNA (complementary or cDNA). This is done using a retroviral enzyme called reverse transcriptase (RT). This transcribes cDNA from mRNA using either a oligo-(dT) or random hexamer primers. The cDNA produced is then used as a template in the PCR reaction. Both steps together are referred to as RT-PCR.

2.3.4 Quantitative Real time RT-PCR

Quantitative RT-PCR was used to determine levels of expression of selected genes from the fathead minnow after 3 weeks exposure to clofibric acid (see Chapter 6 for specific method).

PCR-based techniques allow us to obtain genetic information through the specific amplification of nucleic acid sequences, starting from a very low number of target copies. These reactions are characterised by a logarithmic amplification of the target sequences; that is, an increase of PCR copies

followed by a plateau phase showing a rapid decrease to zero of copy number increment per cycle. The concept of 'real time' PCR consists of the detection of these PCR products as they accumulate by using either labelled probes of primers or a fluorescent DNA dye such as Syber Green. Detection of PCR products in real-time allows the extrapolation of initial target concentration to be calculated; that is, it is a quantification method.

The full details of primer design and construction, and the methods adopted for real-time PCR, are described in Chapter 6. Primers were optimised by performing PCR on a gradient block thermocycler to determine optimal annealing temperature ranges for each primer pair (data not shown). The following give examples of the type of results obtained when carrying out real time RT-PCR.

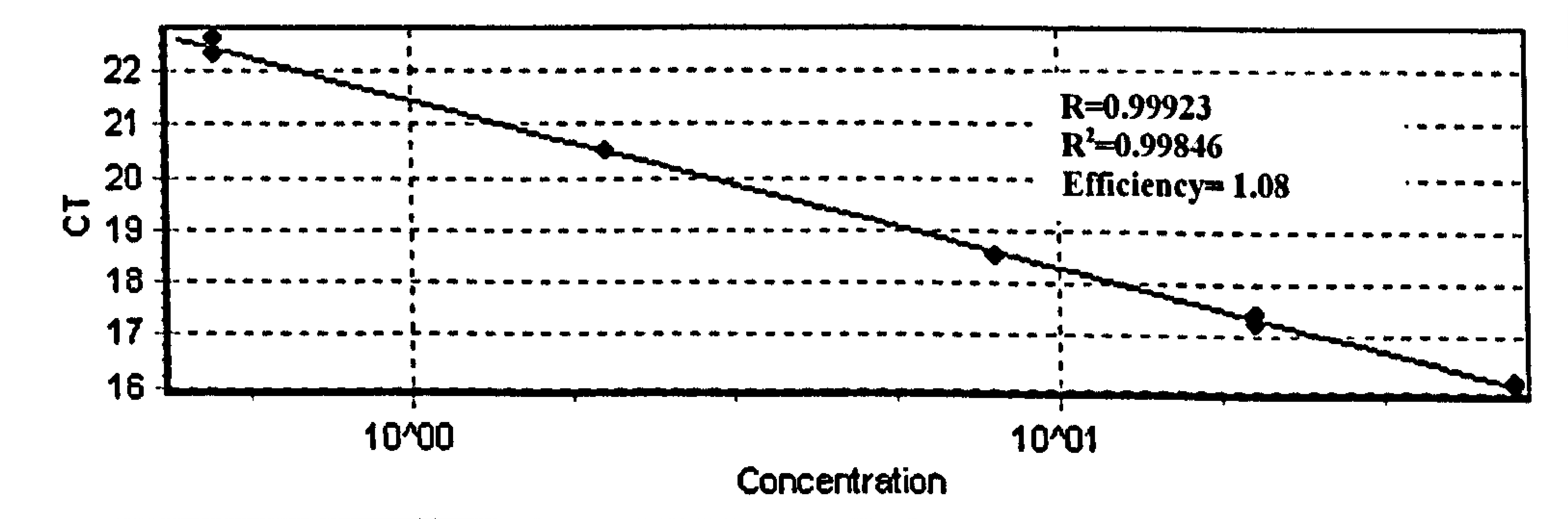

Figure 2.14: An example of a standard curve used for real time RT-PCR– using a dilution of sample 100 (used as a standard) for relative quantification of the expression of certain genes.

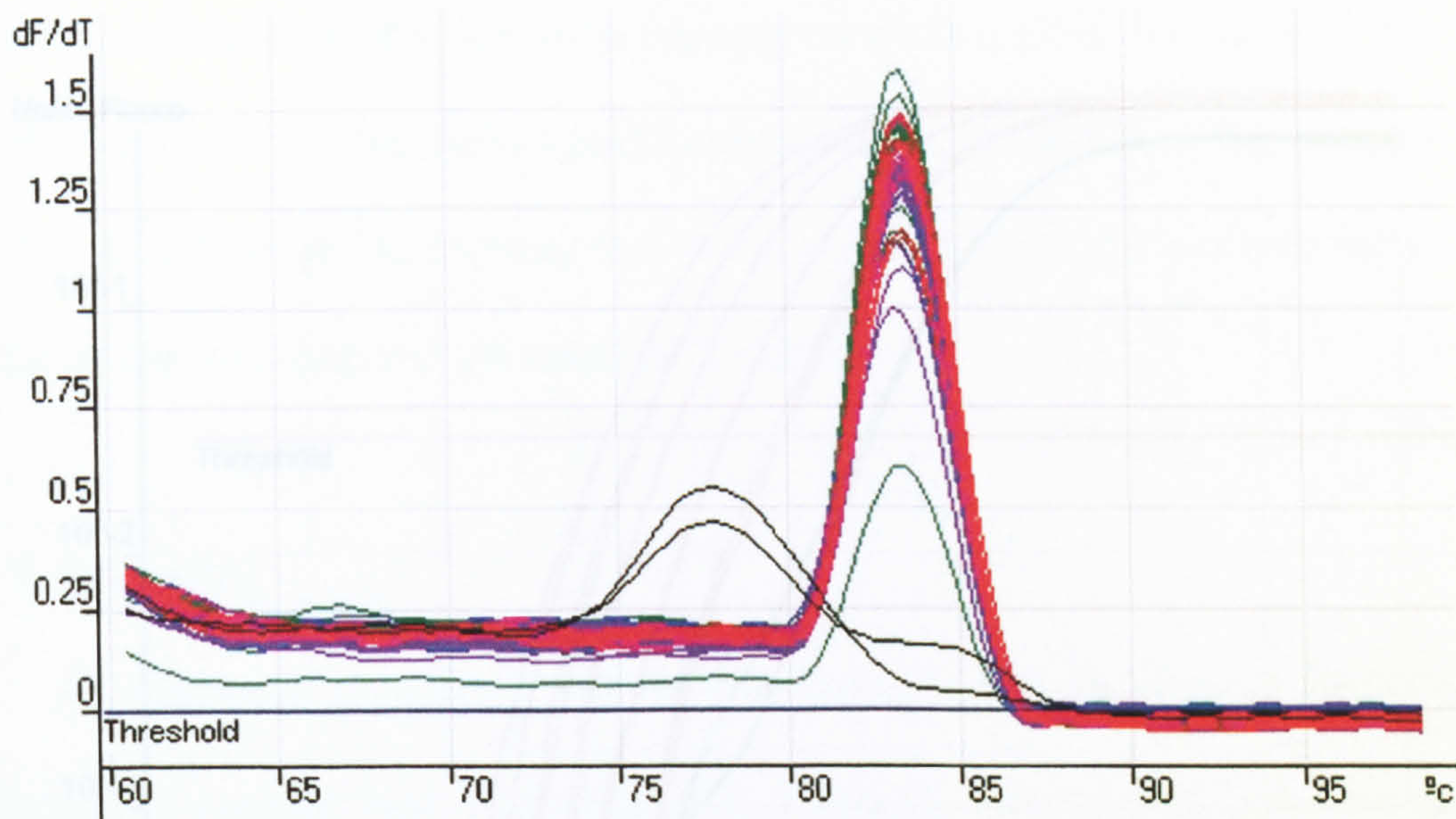

Figure 2.15: An example of a melt curve produced using the Rotorgene (see Chapter 6). The main peaks (red - standards, blue/green - samples) result from the specific amplification products; other peak results from formation of primer-dimers (seen in NTC (non-template controls) - black), which melt at a lower temperature.

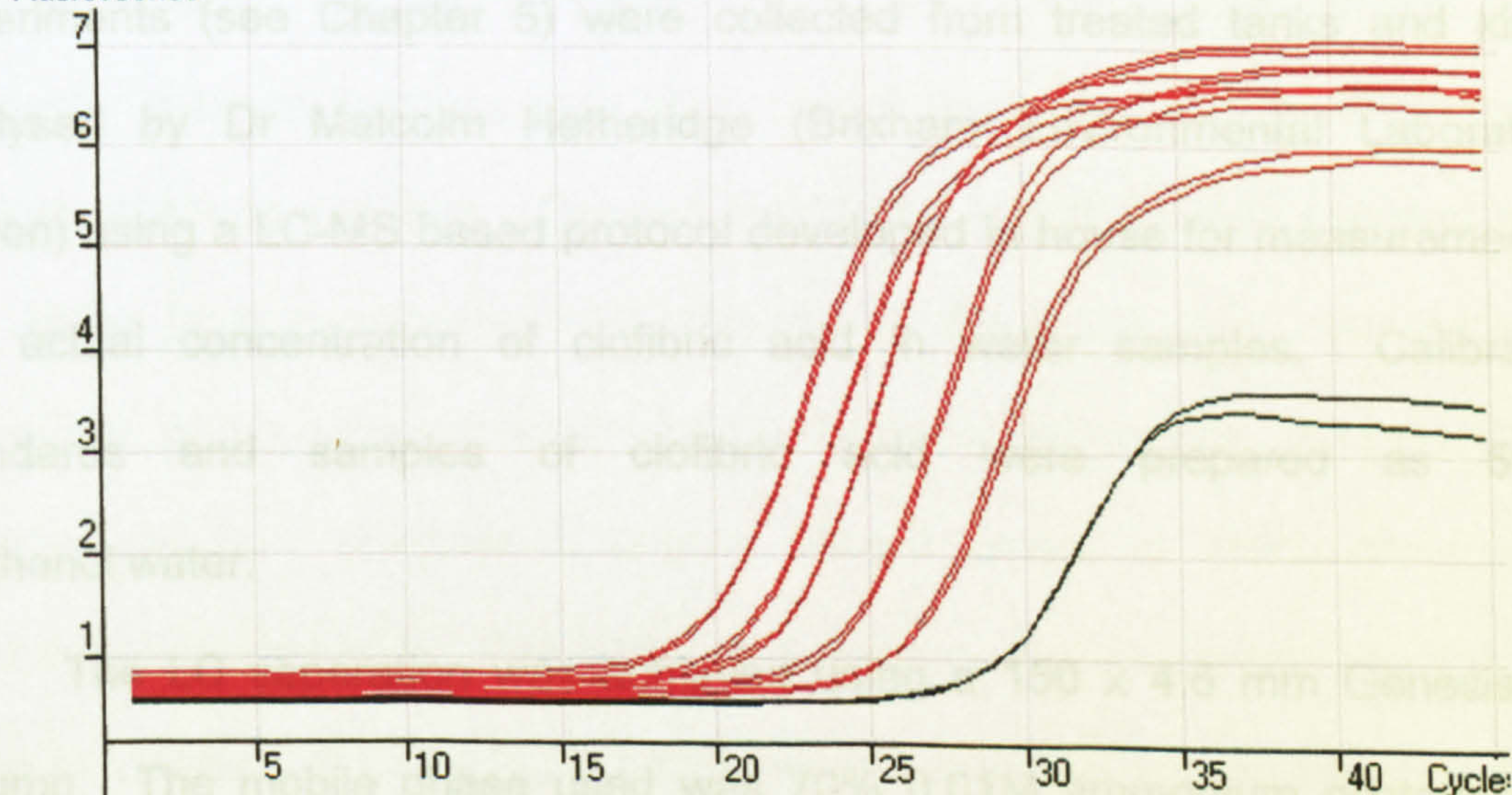

Figure 2.16: An example of some raw data showing an increase in fluorescence (and hence PCR product) with each cycle (using a serial dilution of sample 100 as template).

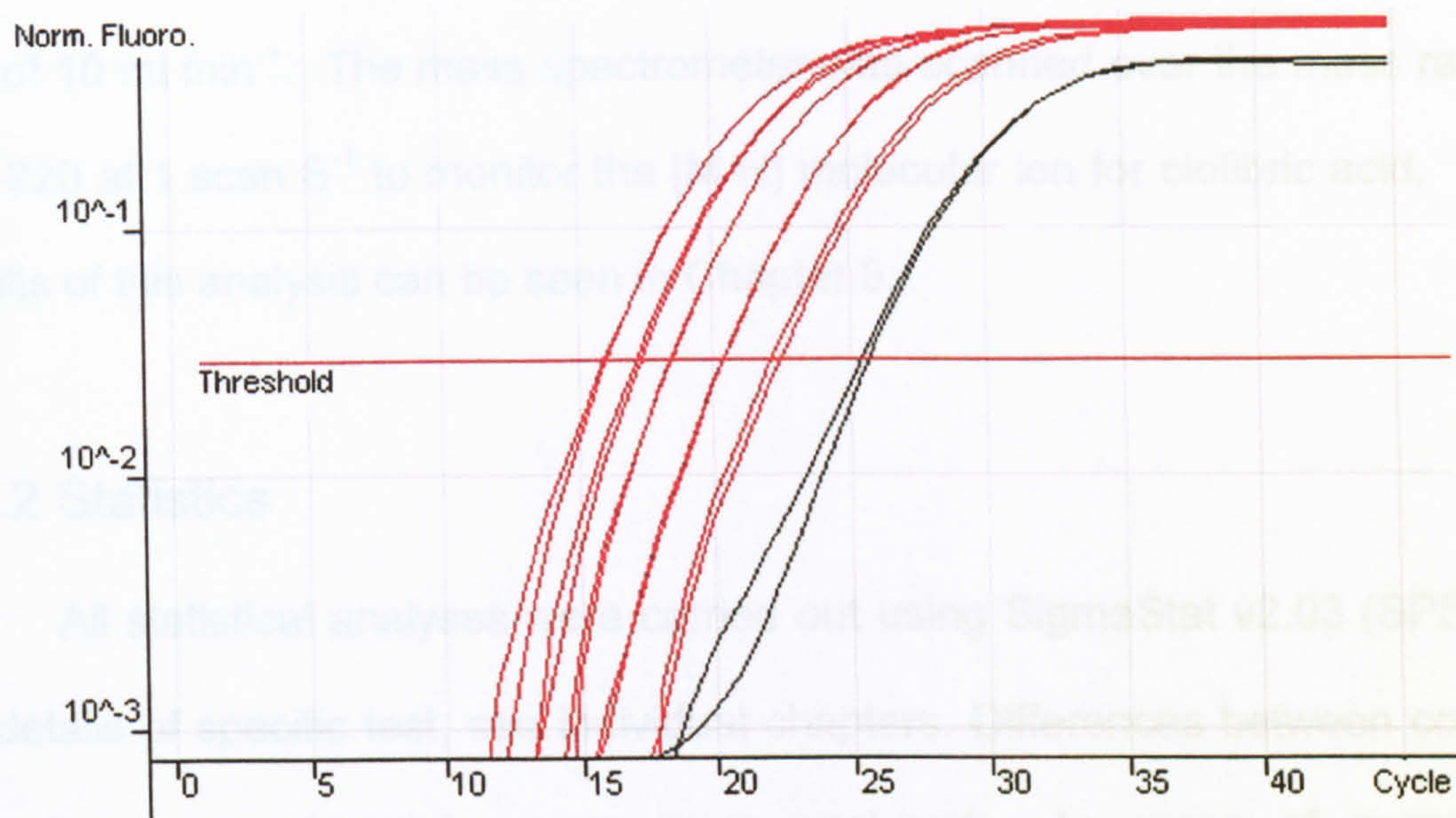

Figure 2.17: Amplification plot (Ct curves): each curve shows change in fluorescence over each cycle. These curves obtained from a serial dilution of sample 100 as template, used to construct a standard curve (Figure 2.14). Threshold set automatically to give best standard curve.

2.4 Other analyses

2.4.1 Water analysis

Water samples from representative samples from some of the experiments (see Chapter 5) were collected from treated tanks and kindly analysed by Dr Malcolm Hetheridge (Brixham Environmental Laboratory, Devon) using a LC-MS based protocol developed in house for measurement of the actual concentration of clofibric acid in water samples. Calibration standards and samples of clofibric acid were prepared as 50:50 methanol:water.

The LC separation was achieved using a 150 x 4.6 mm Genesis AQ column. The mobile phase used was 70% 0.01M ammonium acetate: 30% methanol at a flow rate of 0.7 ml min^{-1} and an injection volume of 50 μl. The TSQ-700 mass spectrometer with electrospray ionisation interface (Thermo) was operated in negative ionisation mode, using a capillary temperature of

245°C, spray voltage of 4 kV, with nitrogen sheath gas of 65 psi and auxiliary gas of 10 ml min^{-1}. The mass spectrometer was scanned over the mass range 210-220 at 1 scan S^{-1} to monitor the [M-H] molecular ion for clofibric acid. The results of this analysis can be seen in Chapter 5.

2.4.2 Statistics

All statistical analyses were carried out using SigmaStat v2.03 (SPSS) - for details of specific test, see individual chapters. Differences between control and dosed experimental groups were analysed. In cases of normality, differences were determined by a t-test. A t-test is a parametric statistical test used to determine if there is a difference between two groups that is greater than what can be attributed to random sampling variation. It is based on estimates of the mean and standard deviation parameters of the normally distributed populations from which the samples were drawn.

Where normality was not met, data were analysed using a suitable non-parametric test. The Mann-Whitney Rank Sum Test is used to test for a difference between two groups that is greater than what can be attributed to random sampling variation. The null hypothesis is that the two samples were not drawn from populations with different medians. It is a nonparametric procedure, which does not require normality or equal variance. It ranks all the observations from smallest to largest without regard to which group each observation comes from. The ranks for each group are summed and the rank sums compared. If there is no difference between the two groups, the mean ranks should be approximately the same. If they differ by a large amount, you can assume that the low ranks tend to be in one group and the high ranks are in

the other, and conclude that the samples were drawn from different populations (i.e., that there is a statistically significant difference).