LECTURE NOTES
ON
PHARMACOLOGY – III
(Subject Code: 15R00702)

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UNIT-I
COAGULANTS & ANTICOAGULANTS

LEARNING OBJECTIVES

• Define what are anticoagulants.
• Define what are coagulants.
• Classify the medicines in this group.
• Enumerate their salient features.
• Describe their mechanism of action.
• Enumerate when they should be used.
• Enlist when they should not be used.
• Enlist their bad effects.

COAGULANTS & ANTICOAGULANTS

LECTURE OUTLINE

WHAT IS BLOOD?

• Connective tissue.
• Balance between thrombosis & haemorrhage.
• Blood maintains haemostasis.

COAGULANTS:

Definition: An agent that produces coagulation (Coagulation is a complex process by which blood forms clots).

ANTICOAGULANTS

Definition: An anticoagulant is a substance that prevents coagulation; that is, it stops blood from clotting.
COAGULATION FACTORS:

- I  Fibrinogen.
- II Prothrombin.
- III Tissue factor.
- IV Calcium.
- V  Proaccelerin, labile factor.
- VI  Accelerin.
- VII Stable factor.
- VIII Anthemophilic factor.
- IX Christmas factor.
- X  Stuart-Prowers factor.
- XI  Plasma thromboplastin antecedent.
- XII Hageman factor.
- XIII Fibrin-stabilizing factor.

NATURAL ANTICOAGULANTS:

1. PGI-2.

2. Antithrombin.

3. Protein-C.

4. TFPI.

5. Heparin.

6. Fibrinolytic system.

CLASSIFICATION:

A) Anticoagulants.
B) Thrombolytic agents.
C) Antiplatelet agents.
ANTICOAGULANTS:

1. **Parenteral**
   - Heparin Sulphate.
   - Danaparoid.
   - Lepirudin.

2. **Oral**
   - Warfarin sodium.
   - Dicumarol.
   - Phenprocoumon.
   - Acenocoumarol.
   - Anisindione.

THROMBOLYTIC AGENTS:

- Streptokinase.
- t-PA.
- Urokinase.
- Alteplase.

ANTIPLATELET AGENTS:

- Aspirin
- Dipyridamole
- Ticlopidine
- Clopidogrel

HEPARIN SULPHATE:

- Glycosaminoglycan found in mast cells.
- UDP sugar precursors.
- Extracted from porcine intestinal mucosa or bovine lung.
- Available as USP units/mg.
- LMWH are 4500 daltons or 15 monosaccharide units.
- Isolated from standard heparin.
Mechanism of action:

• Acts via heparin co-factor or antithrombin III.
• Inhibits thrombin by PAI-1, protein-C inhibitor & protease nexin-1.
• Inhibits factor Xa by TFPI.
• Releases lipoprotein lipase enzyme.
• Interferes with platelet aggregation.

Indications:

WHEN THROMBI ARE FORMED IN

• Brain.
• Abdomen.
• Heart.
• Venous or arterial catheters.
• Performing procedures
  ➢ Coronary angiography.
  ➢ Haemodialysis.

Adverse effects

• Bleeding.
• Allergy.
• Increase in serum potassium level (Hyperkalaemia).
• Decrease in platelet count (Thrombocytopenia).
• Softening of bones (Osteoporosis).
• Loss of hairs (Alopecia).

Contraindications:

• Hemorrhagic disorders
• Thrombocytopenia (platelets < 80 x 10^9/L)
• Bleeding from esophagus, stomach, brain etc.
• Bleeding from any major trauma
• Hypersensitivity to heparin
ORAL ANTICOAGULANTS:

Warfarin sodium:

- Synthetic derivative of coumarin.
- Found naturally in plants e.g. woodruff.
- Act by inhibiting vitamin-K epoxide reductase.
- It recycles oxidated vitamin-K to its reduced form.
- Also known as vitamin-K antagonists.

Mechanism of action:

- Act by inhibiting the synthesis of vitamin-k dependent clotting factors that is II, VII, IX & X
- Inhibit synthesis of regulatory factors like protein C, S & Z
- Warfarin inhibits epoxide reductase & diminishes available Vit-K & its reduced form in the tissues.

BAD EFFECTS (Adverse effects):

- Haemorrhage
- Abortion
- Warfarin syndrome
- Itching of skin
- Purple toe.

USES (Indications):

- Thrombosis
- Embolism in pulmonary & coronary circulation.

Heparin:

- Heparin is a large polymer
- Given parenterally
- Acts in blood
- Rapid onset of action
- Activates anti-thrombin III
- Monitored by APTT
- Protamine sulphate
- Mostly used for acute purposes
- Used in pregnancy
Warfarin:

- Warfarin is a small lipid soluble molecule
- Given per orally
- Acts in liver
- Slow onset of action
- Impairs synthesis of factor II, VII, IX & X
- Monitored by PT/INR
- Vitamin-K & plasma
- Mostly used for chronic purposes
- Not used in pregnancy.

THROMBOLYTICS

Sources:

- Thrombolytic drugs are derived from *Streptomyces spp.*
- Recombinant technology.
- Manufactured by bacteria like streptokinase etc.

Mechanism:

- Work by activating the enzyme plasminogen
- Clears the cross-linked fibrin mesh
- Makes the clot soluble
- Followed by proteolysis
- Blood flow in the occluded blood vessels is therefore restored.

Adverse effects: Bleeding because of

- Local fibrinolysis.
- Systemic fibrinogenolysis.
- Destruction of coagulation factors V & VIII.

Antiplatelet agents:

- Decrease platelet aggregation.
- Inhibit thrombus formation.
- Effective in the arterial circulation.
- Used in primary and secondary prevention of thrombosis.
- E.g aspirin.
Coagulants:

- VITAMIN- K
- PLASMA FRACTIONS
- CRYOPRECIPITATE
- DESMOPRESSIN ACETATE
- FIBRINOLYTIC INHIBITORS
  1. Aminocaproic acid.
  2. Tranexamic acid.
- SERINE PROTEASE INHIBITORS
  1. Aprotinin.

VITAMIN -K

- Group of lipophilic, hydrophobic vitamins.
- Needed for the post-translational modification of coagulation proteins.
- Phylloquinone (vitamin K1) is the major dietary form of vitamin K.
- Vitamin K2 (menaquinone, menatetrenone) is produced by bacteria in the intestines.

2. PLASMA FRACTIONS:

a) Fresh frozen plasma.

b) Platelets.

c) Plasma concentrates.

d) Non-plasma recombinant factor concentrates.
UNIT II

DIURETICS

A. OVERVIEW OF THE CLINICAL USE OF DIURETICS

B. CLASSIFICATION OF DIURETICS

I. Based on the intensity of the diuretic effect: highly, moderately, and weakly effective diuretics

II. Based on effect on K⁺ excretion: K⁺ (and H⁺)-losing and K⁺ (and H⁺)-sparing diuretics

III. Based on the site and mechanism of diuretic action

C. SPECIFIC DIURETICS

I. Osmotic diuretics: mannitol (urea, glycerin, isosorbide)

II. Carbonic anhydrase inhibitors: acetazolamide (dichlorphenamide, metazolamide)

III. Loop diuretics: furosemide, bumetanide, torasemide, ethacrynic acid

IV. Thiazides, thiazide-like diuretics: (chlorothiazide), hydrochlorothiazide, clopamide, indapamide, chlorthalidone

V. Na⁺ channel antagonists: amiloride, triamterene

VI. Aldosterone antagonists: spironolactone, (canrenoate), eplerenone
A. OVERVIEW OF THE CLINICAL USE OF DIURETICS

The clinical use of diuretics is extensive (Table 1); they are important in treating various disease conditions.

1. **To decrease the expanded extracellular volume (edema)**
   a. **Systemic edemas** (thiazides, loop diuretics):
      - Cardiac edema: congestive heart failure (+ aldosterone antagonists)
      - Hepatic edema: liver cirrhosis (+ aldosterone antagonists)
      - Renal edema: chronic renal disease, nephrosis
   b. **Localized edemas** (acute and dangerous conditions):
      - Brain edema (mannitol infusion)
      - Pulmonary edema (furosemide i.v.)
      - Glaucoma (acute: mannitol or urea infusion, or isosorbide per os; chronic: acetazolamide per os/i.v.; dorzolamide or brinzolamide topically)

2. **To decrease the blood pressure in hypertensive patients**
   - Chronic hypertension: thiazides (e.g. HCTZ) + amiloride, aldosterone antagonists (eplerenone)
   - Acute hypertensive crisis: furosemide i.v.

3. **To increase urinary excretion of inorganic ions, such as**
   - Ca\(^{2+}\) in acute hypercalcemia: furosemide
   - K\(^+\) in acute hyperkalaemia: furosemide
   - Li\(^+\) in lithium intoxication: amiloride
   - Br\(^-\) in bromide intoxication: thiazides

4. **To prevent anuria in acute renal failure**: - furosemide i.v.
   - mannitol infusion (only if it produces diuresis)

5. **Other indications**:
   - Dialysis disequilibrium syndrome (mannitol inf. to correct hyposmolarity of the blood)
   - Calcium nephrolithiasis (thiazides to decrease Ca\(^{2+}\) excretion into urine)
   - Osteoporosis (thiazides to decrease Ca\(^{2+}\) excretion into urine)
   - Nephrogenic diabetes insipidus, i.e. ADH refractoriness (thiazides)*
   - Epilepsy (carbonic anhydrase inhibitors to increase CO\(_2\) concentration in brain)
   - Metabolic alkalosis (carbonic anhydrase inhibitors to increase NaHCO\(_3\) excretion)
   - Altitude sickness (carbonic anhydrase inhibitors)
   - Cystic fibrosis (inhalaion of Na\(^+\) channel inhibitor solution or of mannitol powder to dilute the bronchial secretion and thus promote the mucociliary clearance)
   - Cardiovascular diseases, e.g. congestive heart failure, cardiac infarct, hypertension (aldosterone antagonists: spironolactone, spirorenalolactone, spironolactone, steroidal aldosterone antagonist)

* Indomethacin (a NSAID) may also be useful in nephrogenic diabetes insipidus (ADH refractoriness). Desmopressin, a selective V2 receptor agonist ADH derivative, is effective only in neurogenic (or central) diabetes insipidus that is caused by ADH deficiency.
Classification of diuretics may be based on different properties:

I. Based on the intensity of the diuretic effect, diuretics can be listed as highly effective, moderately effective and weak diuretics.

<table>
<thead>
<tr>
<th>Highly effective diuretics</th>
<th>+25% of GFR may be voided</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop diuretics (furosemide, bumetanide, torasemide, ethacrynic acid)</td>
<td></td>
</tr>
<tr>
<td>Mannitol infusion (at a high rate)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Moderately effective diuretics</th>
<th>+6% of GFR may be voided</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiazides (chlorothiazide, hydrochlorothiazide = HCTZ)</td>
<td></td>
</tr>
<tr>
<td>Thiazide-like drugs (clopamide, indapamide, chlorthalidone)</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Weak diuretics</th>
<th>+3% of GFR may be voided</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonic anhydrase inhibitors (acetazolamide)</td>
<td></td>
</tr>
<tr>
<td>Na(^+) channel inhibitors (amiloride, triamterene)</td>
<td></td>
</tr>
<tr>
<td>Aldosterone antagonists (spironolactone, eplerenone, canrenoate)</td>
<td></td>
</tr>
</tbody>
</table>

II. Diuretics may differentially alter potassium excretion, although this effect is unwanted.

Some diuretics are potassium losing drugs (incidentally these drugs also increase H\(^+\) excretion), whereas others are potassium sparing diuretics (these are also H\(^+\) sparing drugs). The K\(^+\) and H\(^+\) losing diuretics can induce hypokalemia and alkalosis, whereas the K\(^+\) and H\(^+\) sparing drugs may cause hyperkalemia and acidosis. These opposite types of diuretics may be combined in order to mutually minimize their unwanted effects (e.g. fixed combinations of HCTZ and amiloride are available), or the K\(^+\) losing diuretics should be coadministered with K\(^+\) supplement to avoid hypokalemia.

<table>
<thead>
<tr>
<th>K(^+) (and H(^+)) losing diuretics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loop diuretics (furosemide, bumetanide, torasemide, ethacrynic acid)</td>
</tr>
<tr>
<td>Thiazides (chlorothiazide, hydrochlorothiazide)</td>
</tr>
<tr>
<td>Thiazide-like drugs (clopamide, indapamide, chlorthalidone)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>K(^+) (and H(^+)) sparing diuretics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone antagonists (spironolactone, canrenoate, eplerenone)</td>
</tr>
<tr>
<td>Na(^+) channel inhibitors (amiloride, triamterene)</td>
</tr>
</tbody>
</table>

Increased excretion of K\(^+\) and H\(^+\) (i.e. K\(^+\) and H\(^+\) loss) is secondary to increased delivery of Na\(^+\) to the collecting duct because increased reabsorption of Na\(^+\) from the distal nephron promotes there the secretion of K\(^+\) and H\(^+\).

Therefore, K\(^+\) and H\(^+\) loss is caused by diuretics that inhibit the reabsorption of Na\(^+\) upstream of the collecting duct, such as the loop diuretics and thiazides.

In contrast, K\(^+\) and H\(^+\) sparing is caused by diuretics that inhibit the reabsorption of Na\(^+\) in the collecting duct, because these secondarily decrease the secretion of K\(^+\) and H\(^+\) there. Such diuretics are the Na\(^+\) channel inhibitors and the aldosterone antagonists.

More detailed explanation is given under loop diuretics.

Note: Carbonic anhydrase inhibitors cannot be listed into either of these two groups, as they are weak K\(^+\) losing diuretics, but cause H\(^+\) „sparing” effect, because they decrease the tubular secretion of H\(^+\) – see p. 7.
III. A third way for classification of diuretics is based on the **site and mechanism of diuretic action**.

Diuretics may act at various segments of the nephron (see the figure in Appendix 1). Osmotic diuretics act partly before the kidney (in the systemic circulation) and partly all along the nephron. Carbonic anhydrase inhibitors act in the proximal convoluted tubules, the loop diuretics in the loop of Henle (within the thick ascending limb), thiazide diuretics in the distal convoluted tubules, whereas Na\(^+\)-channel antagonists and aldosterone antagonists (or mineralocorticoid receptor antagonists, MRA) in the collecting tubule. The table below lists diuretics according to their site of action in a descending order.

<table>
<thead>
<tr>
<th>DIURETICS</th>
<th>DRUGS</th>
<th>SITE OF ACTION</th>
<th>TARGET MOLECULE</th>
<th>EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSMOTIC DIURETICS</td>
<td>Mannitol</td>
<td>• Systemic: EC space</td>
<td>None</td>
<td>↓Intracellular water space ↑Extracellular water space</td>
</tr>
<tr>
<td></td>
<td>Urea</td>
<td>• Renal: leaky segments</td>
<td></td>
<td>↓Water reabsorption</td>
</tr>
<tr>
<td></td>
<td>Glycerin</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Isosorbide</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CARBONIC ANHYDRASE INHIBITORS</td>
<td>Acetazolamide</td>
<td>Proximal convoluted tubule (PCT)</td>
<td>Carbonic anhydrase (luminal and intracellular)</td>
<td>↓Na(^+)--H(^+) exchange</td>
</tr>
<tr>
<td></td>
<td>Brinzolamide*</td>
<td></td>
<td></td>
<td>↓NaHCO(_3) reabsorption → alkaline urine</td>
</tr>
<tr>
<td></td>
<td>Dichlorphenamide*</td>
<td></td>
<td></td>
<td>↑H(^+) secretion → systemic acidosis</td>
</tr>
<tr>
<td></td>
<td>Methazolamide*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOOP DIURETICS</td>
<td>Furosemide</td>
<td>Loop of Henle (thick ascending limb)</td>
<td>Na(^+) K(^+) 2Cl(^-) symporter</td>
<td>↓Na(^+), Cl(^-) reabsorption</td>
</tr>
<tr>
<td></td>
<td>Bumetanide</td>
<td></td>
<td></td>
<td>↓Ca(^{2+}), Mg(^{2+}) reabsorption</td>
</tr>
<tr>
<td></td>
<td>Torasemide</td>
<td></td>
<td></td>
<td>↑K(^+), H(^+) secretion in the DCT</td>
</tr>
<tr>
<td></td>
<td>Ethacrynic acid</td>
<td></td>
<td></td>
<td>(→ hypokalemia, alkalosis, hypercalcemia, hypomagnesemia)</td>
</tr>
<tr>
<td>THIAZIDES, THIAZIDE-LIKE DIURETICS</td>
<td>(Chlorothiazide)</td>
<td>Distal convoluted tubule (DCT)</td>
<td>Na(^+) Cl(^-) symporter</td>
<td>↓Na(^+), Cl(^-) reabsorption</td>
</tr>
<tr>
<td></td>
<td>Hydrochlorothiazide</td>
<td></td>
<td></td>
<td>↓Mg(^{2+}), ↑Ca(^{2+}) reabsorption</td>
</tr>
<tr>
<td></td>
<td>Clopamide</td>
<td></td>
<td></td>
<td>↑K(^+), H(^+) secretion in the DCT</td>
</tr>
<tr>
<td></td>
<td>Indapamide</td>
<td></td>
<td></td>
<td>(→ hypokalemia, alkalosis, hypercalcemia, hypomagnesemia)</td>
</tr>
<tr>
<td></td>
<td>Chlorothalidone</td>
<td></td>
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</tr>
<tr>
<td>Na(^+) CHANNEL ANTAGONISTS</td>
<td>Amiloride</td>
<td>Collecting duct, CD (principal cells)</td>
<td>Epithelial Na(^+)-channel</td>
<td>↓Na(^+) reabsorption</td>
</tr>
<tr>
<td></td>
<td>Triamterene</td>
<td></td>
<td></td>
<td>↓K(^+), H(^+) secretion in the CD</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(→ hyperkalemia, acidosis)</td>
</tr>
<tr>
<td>ALDOSTERONE ANTAGONISTS (MRA)</td>
<td>Spironolactone</td>
<td>Collecting duct, CD (principal cells)</td>
<td>Mineralocorticoid receptor</td>
<td>↓Na(^+) reabsorption</td>
</tr>
<tr>
<td></td>
<td>Canrenoate</td>
<td></td>
<td></td>
<td>↓K(^+), H(^+) secretion in the CD</td>
</tr>
<tr>
<td></td>
<td>Eplerenone</td>
<td></td>
<td></td>
<td>(→ hyperkalemia, acidosis)</td>
</tr>
</tbody>
</table>

* Used for topical treatment of glaucoma, not as a diuretic.

Of the diuretics, the **loop diuretics are most effective** because the ascending limb of the loop of Henle (LOH) has a very high reabsorptive capacity: ~25% of the GFR is reabsorbed from the loop. Thus, under the effect of loop diuretics up to 25% of the GFR (~35 L urine/day) may be voided.

Diuretics acting only **upstream** of the LOH (i.e. in the proximal tubules) have **limited efficacy** because the thick ascending limb of the LOH with its huge reabsorptive capacity can reabsorb most of the rejectate coming from the proximal tubule.

Diuretics acting **downstream** of the LOH also have **limited efficacy** because normally only a small percentage of filtered Na\(^+\) load reaches the distal nephron and because these distal segments do not possess high reabsorptive capacity. Because of its small reabsorptive capacity, the distal nephron cannot rescue the flood of rejectate that arrives from the LOH in response to loop diuretics. This also explains why the loop diuretics are most effective.
In discussing the specific drugs, we are going to "travel" along the nephron, from the glomerulus to the collecting duct, “stopping” at sites where specific diuretics act.

**OSMOTIC DIURETICS:** mannitol (urea, glycerin, isosorbide)

1. **Chemical and pharmacokinetic properties of mannitol** (MANNITOL 10% inf., MANISOL A 10% inf., MANISOL B 20% inf.):
   - it is a small water-soluble molecule: a sugar alcohol with 6 C atoms and 6 OH groups
   - it is not readily permeable across the cell membrane; therefore, mannitol is not absorbed orally (it is an osmotic laxative; >20g per os) → given in i.v. infusion → distributed in the extracellular space
   - after being freely filtered in the renal glomeruli, it is not reabsorbed in the tubules
   - it is inert pharmacologically → can be given in large doses

2. **Mechanisms of action of osmotic diuretics** — *two-fold:*
   (1) After getting into the bloodstream and then into the extracellular water space, osmotic diuretics increase the osmolarity of the *plasma and the extracellular (EC) water*

   → osmotically extract water from the intracellular space
   → expand the extracellular fluid volume
   → ↑ the renal blood flow, i.e.:
     - ↑ the *glomerular* blood flow → ↑ GFR
     - ↑ the blood flow in *vasa recta*
       → NaCl in the interstitium of the medulla (carried there by Na⁺K⁺2Cl⁻ symporter of the *ascending* limb of the loop of Henle) is washed out
       → ↓ the medullary tonicity created by the *ascending* limb of the loop of Henle
       → ↓ water reabsorption from the leaky *descending* limb of the loop of Henle

   → **DIURESIS**

   (2) After being filtered in the glomeruli without being reabsorbed in the *renal tubules*, osmotic diuretics ↑ the osmolarity of the *tubular fluid*

   → ↓ the reabsorption of water from the "leaky" segments of the tubular system, i.e.

   - from the proximal convoluted tubule, i.e.:
   - from the descending limb of the loop of Henle
   - from the collecting duct → **DIURESIS**

Osmotic diuretics are - *primarily* diuretics: ↑ water excretion

- *secondarily* saluretics: ↑ salt excretion due to:
  - dilution of tubular fluid (→ ↓ salt reabsorption)
  - faster tubular fluid flow (→ ↓ salt reabsorption)

3. **Indications:** osmotic diuretics are used not only as diuretics!

   (1) *Prevention of anuria in acute renal failure (ARF)*

<table>
<thead>
<tr>
<th>Causes of ARF:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>~renal ischemia caused by circulatory collapse</td>
<td></td>
</tr>
<tr>
<td>~renal injury caused by ~ nephrotoxicants (aminoglycosides, cisplatin, Hg²⁺ salts)</td>
<td></td>
</tr>
<tr>
<td>~hemoglobinuria, myoglobinuria</td>
<td></td>
</tr>
</tbody>
</table>
(2) For treatment of acute cerebral edema and glaucoma

By raising the plasma osmolarity, osmotic diuretics extract water from the brain and the eyes (aqueous humor) → they lower the intracranial and intraocular pressure, respectively.

They are also used pre- and postoperatively in patients who require ocular surgery or brain surgery in order to prevent an increase in the intraocular pressure and to reduce cerebral edema, respectively.

(3) "Dialysis disequilibrium syndrome" – a complication of vigorous hemodialysis.

Hemodialysis → rapid removal of solutes from the extracellular (EC) compartment
→ the EC fluid becomes hypotonic, a condition similar to water intoxication

→ water moves into the intracellular (IC) space by osmosis – Consequences:
  • EC hypovolemia, hypotension
  • increased intracranial pressure (like in brain edema) with CNS symptoms (e.g. headache, nausea, restlessness, convulsion)

Mannitol corrects the osmolarity in the EC space and withdraws water from the IC space.

(4) Cystic fibrosis, CF: dry mannitol powder (300 mg) is given by inhalation. Acting osmotically, it dilutes the viscous bronchial fluid, thereby promoting the mucociliary clearance. (CF = loss-of-function mutation of an ATP-driven Cl⁻ transporter, causing impaired formation of secreted fluids; mucoviscidosis.)

4. Unwanted effects

If overdosed, mannitol causes overexpansion of EC fluid volume
→ increased load to the heart
→ heart failure (↓ left ventricular performance)
→ pulmonary edema. This is why furosemide and not mannitol is used in pulmonary edema!

5. Other osmotic diuretics: urea, glycerin and isosorbide

- Pharmacokinetic features:
  - Urea and mannitol are given exclusively i.v., whereas glycerin and isosorbide may also be given orally.
  - They are eliminated by urinary excretion, except for glycerin which is also metabolized by the liver.
  - They have short half-life ($T_{1/2}$ ≤ 1 h), except for isosorbide whose $T_{1/2}$ is ~6 hr.

- Clinical use:
  - For brain edema, use urea or mannitol.
  - For acute glaucoma, use urea or isosorbide as their ocular action is more rapid, although each osmotic diuretic is approved for this indication.

Note: The nitrous acid (HNO$_2$) esters of glycerin and isosorbide (i.e. glyceryl trinitrate and isosorbide mononitrate as well as dinitrate, in which the H atom of –OH groups is replaced with an NO$_2$ group) are metabolized to NO, and therefore they are potent antianginal vasodilators.

6. Contraindications

- All osmotic diuretics are contraindicated in anuria and heart failure,
  as they may cause EC volume expansion, overload of the heart, and thereby, pulmonary edema.
- Urea is contraindicated in hepatic cirrhosis. At high concentration, urea inhibits arginase and thereby impairs the elimination of NH$_3$ in the urea cycle.
- Glycerin is contraindicated in diabetes mellitus (as it is a gluconeogenic substrate).
II. CARBONIC ANHYDRASE INHIBITORS:

   Prototype: acetazolamide (HUMA-ZOLAMIDE 250 mg tabl., DIAMOX 125-250 mg tabl, 500 mg inj.)
   Others: brinzolamide, dichlorphenamide, methazolamide

2. Mechanism of action
   Acetazolamide avidly binds to and potently inhibits carbonic anhydrase (CA), a Zn-containing enzyme (IC ~10 nM). Renal CA is largely in the proximal tubular cells, both in the luminal membrane (facing the lumen) and the cytoplasm.

   Carbonic anhydrase catalyzes the following reversible reaction, i.e.
   dehydration of carbonic acid to form the diffusible CO₂ and hydration of CO₂ to form carbonic acid:

<table>
<thead>
<tr>
<th>CA-catalyzed processes in the lumen and in the cells of the proximal tubules (see Appendix 1):</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the lumen: H⁺ is secreted from the cell across the luminal membrane by the Na⁺–H⁺ exchanger</td>
</tr>
<tr>
<td>• Spontaneous reaction (association): H⁺ + HCO₃⁻ → H₂CO₃, then</td>
</tr>
<tr>
<td>• CA-catalyzed reaction (dehydration): H₂CO₃ → H₂O + CO₂ → diffusion into the cell</td>
</tr>
<tr>
<td>In the cell:   • CA-catalyzed reaction (hydration): CO₂ + H₂O → H₂CO₃</td>
</tr>
<tr>
<td>• Spontaneous reaction (dissociation): H₂CO₃ → H⁺ + HCO₃⁻</td>
</tr>
</tbody>
</table>

Thus, carbonic anhydrase promotes the reabsorption of NaHCO₃ and the secretion of H⁺ because:
• the luminal CA permits reabsorption of HCO₃⁻ by dehydrating H₂CO₃ to diffusible CO₂.
• the intracellular CA permits H⁺ secretion and Na⁺ reabsorption by providing H⁺ for the Na⁺–H⁺ exchanger.

3. Effects of acetazolamide
   (1) In the kidney:
      • ↓ NaHCO₃ reabsorption → weak diuresis; NaHCO₃-rich alkaline urine is voided.
         The urinary loss of HCO₃⁻ depletes extracellular HCO₃⁻ → less HCO₃⁻ is filtered in the glomeruli
         → the diuretic effect of CA inhibitor becomes terminated (i.e. CA inhibitors have self-limiting effect).
      • ↓ H⁺ secretion → metabolic acidosis in blood
   (2) In the eye, in the ciliary processes (like in proximal tubular cells), CA forms bicarbonate from CO₂:
      H₂O + CO₂ → H₂CO₃ → H⁺ + HCO₃⁻
      Secretion of bicarbonate contributes to formation of the aqueous humor.
      Acetazolamide: ↓ aqueous humor (AH) formation → ↓ intraocular pressure. Therefore, CA inhibitors are used in open-angle glaucoma (in combination with timolol, which also ↓ AH formation)
   (3) In red blood cells (like in proximal tubular cells), CA forms bicarbonate from CO₂:
      H₂O + CO₂ → H₂CO₃ → H⁺ + HCO₃⁻
      This is how CO₂ is transported by RBC to the lung (i.e. in the form of bicarbonate anion).
      Acetazolamide: ↑ CO₂ in tissues. In the CNS, CO₂ exerts a weak general anesthetic effect causing
      - somnolence, paresthesia (numbness and tingling in the fingers and toes), and
      - antiepileptic effect.
4. Pharmacokinetics of acetazolamide

- GI absorption and oral bioavailability: complete
- Binding to albumin in plasma (~97%) and to CA in RBC, plus low lipid solubility → low Vd: 0.25 L/kg
- Elimination: - Mech.: excreted unchanged in urine by the tubular secretion mechanism for organic acids.
  - Speed: T_{1/2} is 6-9 hr (due to its high binding to plasma protein and RBC).

5. Unwanted effects

- Somnolence, paresthesia (by ↑ CO₂ in the brain – see above)
- Formation of Ca₃(PO₄)₂-containing calculi in the urinary tract, because acetazolamide - ↑ phosphate excretion into urine (by an unknown mechanism)
  - ↑ phosphate ionization (because alkaline urine is produced)

6. Drug interactions

- By alkalinizing the tubular fluid, carbonic anhydrase inhibitors promote tubular reabsorption of basic drugs, such as amphetamine and its congeners, thus delaying their elimination.
- On the contrary, CA inhibitors decrease the reabsorption of acidic drugs, e.g. aspirin, phenobarbital, thus promoting their excretion.

Yet, administration of a CA inhibitor to promote excretion of salicylic acid (the major metabolite of aspirin) in aspirin intoxication is prohibited because carbonic anhydrase inhibitors cause systemic acidosis, which in turn would increase protonation of salicylate, thus promoting the diffusion of salicylic acid into the brain, which would aggravate the intoxication. To promote urinary excretion of salicylate, NaHCO₃ infusion should be used instead of a carbonic anhydrase inhibitor.

7. Indications

- CA inhibitors are rarely used as diuretics and never used as a sole agent.
- To combat metabolic alkalosis (i.e. ↓ H⁺ and ↑ HCO₃⁻ in the plasma)
  - in congestive heart failure which may be associated with metabolic alkalosis because of (a) RAAS activation, and/or (b) treatment with thiazides/loop diuretics (both a and b cause K⁺ and H⁺ loss)
  - together with diuretics that cause K⁺ and H⁺ loss with metabolic alkalosis (thiazides, loop diuretics)
- Open-angle glaucoma: acetazolamide p. os/i.v. + dorzolamide or brinzolamide topically (+ timolol)
- Epilepsy (in absence seizures and myoclonic seizures), as an adjuvant
- Altitude sickness (the symptoms appear to be caused by the low CO₂ levels and the resultant alkalosis) For prevention of altitude sickness, administer 250 mg acetazolamide twice daily.

III. LOOP DIURETICS: furosemide, bumetanide, torasemide (also called torsemide), ethacrynic acid

- These are the most effective diuretics: they can inhibit the reabsorption of as much as 25% of GFR.
- They are K⁺ (and H⁺)-losing diuretics.
- All are organic acids; some with two acidic groups (e.g. –SO₂NH₂ and –COOH groups in furosemide).

Ethacrynic acid (EA) gains the second acidic group by conjugation with glutathione (Glu-Cys-Gly), which is hydrolyzed, first by GGT to EA-Cys-Gly and then by a dipeptidase to EA-Cys.

EA-Cys is the active metabolite of EA. Note: Similar steps are involved in the conversion of LTC₄ (a glutathione conjugate) to LTD₄ (a Cys-Gly conjugate), and then to LTE₄ (a Cys conjugate).
1. Mechanism of action – 3 steps:

(1) They are secreted by the proximal convoluted tubule via the basolateral OAT1 → luminal OAT4 and MRP4 – see Appendix 4.
(2) Travel along the nephron to the thick ascending limb of the loop of Henle
(3) Bind to and inhibit the Na⁺ K⁺ 2Cl⁻ symporter in the luminal membrane of the tubular cells → The diuretic effect correlates with the urinary excretion rather than with the blood levels of these electrolytes.

The Na⁺ K⁺ 2Cl⁻ symporter moves 1 Na⁺, 1 K⁺ and 2 Cl⁻ from the lumen into the tubular cells. Then, these ions are exported into the interstitium via transporters/channels in the basolateral membrane, however, K⁺ is largely returned into the cells by the Na⁺K⁺-ATPase. This process has two consequences:

(1) The Na⁺ K⁺ 2Cl⁻ symporter creates a hypertonic interstitium because the ions are not followed by water here, as the thick ascending limb is not permeable for H₂O → The hypertonic interstitium drives the reabsorption of water by extracting water from the leaky descending limb of the loop.
(2) The Na⁺ K⁺ 2Cl⁻ symporter creates an interstitium-negative transepithelial potential difference because in effect 1 Na⁺ and 2 Cl⁻ moves from the lumen into the interstitium. This drives the reabs. of Ca²⁺ and Mg²⁺. Mutation of Na⁺ K⁺ 2Cl⁻ symporter causes the Barter’s syndrome = inherited hypokalemic alkalosis with salt wasting and hypotension (symptoms are similar to those in furosemide overdose).

Loop diuretics block the Na⁺ K⁺ 2Cl⁻ symporter (by binding to its Cl⁻-binding site)

→ the interstitium cannot become hypertonic (and negative)

2. Effects of loop diuretics

(1) Large increase (10-20-fold) in urine volume → volume depletion and hypotension may result!
(2) Increased urinary excretion of electrolytes:
   • Primarily Na⁺, Cl⁻ (due to inhibition of the Na⁺ K⁺ 2Cl⁻ symporter)
   • Secondarily:
     - ↑ Ca²⁺ and Mg²⁺ excretion (as reabsorption of Ca²⁺ and Mg²⁺ from the loop of Henle decreases because the interstitium-negative transepithelial potential difference is abolished)
     - ↑ K⁺ and H⁺ excretion by ↑ secretion in the collecting duct → K⁺-LOSING DIURETICS

Mechanism of K⁺ and H⁺ loss into urine:
More Na⁺ reaches the collecting duct because Na⁺ reabsorption had been inhibited upstream
→ more Na⁺ gets reabsorbed in the collecting duct through the Na⁺ channels (in principal cells)
   → the lumen-negative transepithelial potential difference increases in the collecting duct

(3) Other effects:
   a. Loop diuretics block the tubuloglomerular feedback (TGFb)
      by inhibiting NaCl transport into the macula densa cells.

After an acute tubular injury, the TGFb decreases filtration pressure in the glomeruli and lowers the GFR. TGFb (although is to compensate for tubular dysfunction) may lead to anuria and acute renal failure.

Therefore, loop diuretics are useful to combat anuria in conditions leading to acute renal failure (shock, nephrotoxicant exposure, hemoglobinuria, myoglobinuria).

b. Loop diuretics have venodilator action which precedes their diuretic effect. This is beneficial in congestive cardiac failure: dilation of veins → ↓ venous pressure → ↓ preload to the heart.
   Mechanism: furosemide induces COX2 locally → ↑ PGI₂ synthesis. Therefore, the venodilator action of furosemide is counteracted by NSAIDs, which inhibit COX enzymes.
3. Pharmacokinetics

- Oral bioavailability: - furosemide: incomplete (~50% in the average) and highly variable (10-90%) - bumetanide, torasemide and ethacrynic acid: near complete (80-100%)
- Plasma protein binding: extensive (>98%) for each → low Vd (~0.2 L/kg bw). In nephrosis sy, binding to proteins in the tubular fluid prevents loop diuretics from binding to the Na⁺K⁺2Cl⁻ symporter.
- Elimination mechanism:
  - Furosemide, bumetanide: mainly by renal tubular secretion (OAT1 → OAT4/MRP4; see Append 4), partly (~30%) by glucuronidation at the COOH group (“ester glucuronide”)
  - Ethacrynic acid: mainly by renal tubular secretion, partly (~30%) by glutathione conjugation (→ Cys-conjugate, the active metabolite)
  - Torasemide: mainly by C-hydroxylation (CYP2C9) → further oxidation into the inactive -COOH acid
- Elimination T½: torasemide ~5 hr, others ~2 hr (the effect of furosemide lasts SIX hours → LASIX)

4. Unwanted effects

(1) Hypovolemia → hypotension, haemoconcentration → risk for thromboembolisation
(2) Hypokalemia (K⁺ loss) → muscle weakness, cramps; → ↑ risk for intoxication with digitalis and class III antiarrhythmic drugs
(3) Hypomagnesemia → risk for arrhythmias (Hypomagnesemia impairs the Na⁺K⁺-ATPase activity → delays myocardial repolarization → increases the risk for torsade-type arrhythmias.)
(4) Hyperuricemia (in the prox. tubules the loop diuretics are secreted by the luminal AOT4 transporter in exchange for urate → they promote the tubular reabsorption of urate; see App. 5) → risk for gout
(5) Hyperglycemia (they open the Kₐtp channels in ß-cells → hyperpolarization → ↓ insulin secretion) → they may convert latent diabetes to manifest diabetes
(6) Hypercholesterolemia (↑ LDL-cholesterol) – due to reflex sympathetic and RAAS activation?
(7) Ethacrynic acid especially → ototoxicity: hearing impairment (deafness); vertigo (dizziness) → avoid coadministration with other ototoxic drugs (e.g. aminoglycosides, vancomycin)

5. Indications – in all acute cases furosemide is used:

(1) Acute pulmonary edema caused by acute heart failure: inject furosemide i.v., because it - rapidly and profoundly ↓ the circulatory volume → ↓ the afterload to the heart
  - exerts venodilatory effect → ↓ the preload to the heart
  In chronic edemas (cardiac, renal, hepatic), loop diuretic or other (e.g. thiazide) is given p. os. In cirrhotic edema, the dose of torasemide should be reduced because torasemide is cleared by the liver (CYP2C9).

(2) Acute hypertensive crisis: inject furosemide i.v. (Alternatives: urapidyl, labetalol, enalaprilate i.v.)
  In chronic hypertension, loop diuretics are given orally in low daily doses, if thiazides are not effective.
  Torasemide (2.5-5 mg daily) is preferred because of its longer effect.

(3) Acute renal failure (ARF): inject furosemide i.v. in order to convert oliguric ARF to non-oliguric ARF.
  Give a high dose, because in the failing kidney diuretics barely reach their site of action!

(4) Acute hypercalcemia: inject furosemide i.v. in order to ↑ urinary excretion of Ca²⁺. In addition, infuse isotonic saline to prevent volume depletion! Alternatives: calcitonin, etidronate.

(5) Acute hyperkalemia: furosemide i.v. in order to ↑ urinary excretion of K⁺. In addition, infuse isotonic saline to prevent volume depletion! – Alternative: polystyrene sulfonate (Kayexalate®, Resonium A® powder) per os – a cation-exchange resin, which binds K⁺ in the gut, thus decreasing K⁺ absorption.
6. Drug interactions

(1) Pharmacokinetic interactions:
   a. Loop diuretics are strongly plasma protein bound (~ 99%) and have low Vd → displace highly protein-bound drugs, e.g. coumarin anticoagulants (warfarin) → risk of bleeding
   b. Acidic drugs that undergo extensive tubular secretion (e.g. probenecid, salycilates, some NSAIDs) inhibit the tubular secretion of loop diuretics → the loop diuretics do not reach the loop of Henle at effective concentration → decreased diuretic effect

(2) Pharmacodynamic interactions:
   a. NSAIDs have antidiuretic effect and diminish the diuretic effect of loop diuretics. Mechanism: NSAIDs ↓ the formation of vasodilatatory PGs (PGE1, PGI2) in the kidney
      → ↓ renal blood flow, including the flow in vasa recta
      → the hypertonicity of the interstitium (generated by NaCl reabsorption) is not washed out
      → the hypertonic interstitium causes ↑ water reabsorption → antidiuretic effect
   Thus, NSAIDs may diminish the effect of diuretics both by
      - pharmacokinetic interaction (i.e. by lowering their concentration at the site of action), and
      - pharmacodynamic interaction (i.e. by counteracting their action).
   b. Loop diuretics → ↓ K+ → potentiates the effect of digitalis → risk for digitalis intoxication
      → ↓ Na+ → promotes Li+ reabsorption in the prox. tubules → risk for Li+ toxicity
      → ↓ Mg2+ → increases the risk of torsade-type arrhythmia, e.g. by quinidine, sotalol

7. Preparations

- Furosemide: FUROSEMID inj 20 mg (for acute conditions – see above), tabl 40 mg
  Another trade name, LASIX, is derived from the fact that its effect L.Asts for SIX hours.
- Bumetanide: BUMEX tabl 0.5-1-2 mg (it is the most potent → lowest dose)
- Torasemide: DEMADEX tabl 5-10-20 mg (it has the most prolonged effect → for chronic hypertension)
- Ethacrynic acid: UREGYT inj, tabl 50 mg (rarely used nowadays due to its ototoxicity)

IV. THIAZIDES, THIAZIDE-LIKE DIURETICS

Classified as moderately effective diuretics, and as K+ (and H+)-losing diuretics

1. Chemical properties: All are sulfonamides (= aminosulfonic acids with acidic –SO2NH2 group)
   - May contain a thiazide ring = thiazides: chlorothiazide (no longer used), hydrochlorothiazide
   - Others are not thiazides but act similarly = thiazide-like drugs:

2. Mechanism of action – 3 steps:
   (1) They are secreted in the proximal convoluted tubules (like the loop diuretics; OAT1 → OAT4, MRP4)
   (2) Travel along the nephron down to the distal convoluted tubule (DCT; the site of action)
   (3) Inhibit Na+Cl- symporter in the luminal membrane of DCT cells (by binding to its Cl- -binding site)
Effects

(1) Diuretic effect: moderate, because only ~5% of the GFR is reabsorbed in the DCT.
   Normally 1-2% of GFR is excreted as urine. In response to thiazides 1-2% + 5% = 6-7% of GFR is
   voided. That is, the urine flow may increase as much as 3-6 fold, up to 9 L/day.

(2) Increased excretion of electrolytes
   • Primarily: Na⁺ and Cl⁻
   • Secondarily: K⁺ and H⁺ (this is due to ↑ delivery of Na⁺ to the collecting duct → ↑ Na⁺ reabsorption
     → ↑ lumen-negative transepithelial potential difference → ↑ secretion of K⁺ and H⁺)

3. Pharmacokinetics
   • Oral bioavailability: good (70%) for HCTZ and chlorthalidone, near complete for clopamide and
     indapamide (due to their high lipid solubility)
   • Plasma protein-binding: moderate (60-80%)
   • Distribution: In general, even – in the total body water (Vd ~0.8 L/kg)
     Peculiarity: chlorthalidone is concentrated 70-80 fold in red blood cells – see Appendix 3
   • Elimination: - Mechanism: HCTZ and chlorthalidone by renal excretion; indapamide: biotransformation
     by CYP3A4: hydroxylation (at the arrow) and dehydrogenation (at the asterisk)
     - T₁/₂: HCTZ 6-9 hr, clopamide 10 hr, indapamide 20 hr, chlorthalidone 40 hr

4. Unwanted effects
   a. Most are similar to those of the loop diuretics:
      (1) Hypovolemia → hypotension
      (2) Hypokalemia (due to K⁺ loss), metabolic alkalosis (due to H⁺ loss)
      (3) Hypomagnesemia (but not hypocalcemia!)
      (4) Hyperuricemia (by promoting urate reabsorption via OAT4, see Appendix 5)
      (5) Hyperglycemia (↓ insulin secretion by the pancreatic β-cells)
      (6) Hypercholesterolemia (↑ LDL-cholesterol and triglyceride levels, indapamide is an exception)
   b. Unlike loop diuretics, thiazides may cause:
      (1) Hypercalcemia
         Mechanism: thiazides ↓Na⁺ concentration in the DCT cells
         → ↑Na⁺ import and ↑Ca²⁺ export (= reabsorption) via the Na⁺–Ca²⁺ exchanger.
         This effect can be exploited in the treatment of patients with:
         • Ca²⁺-nephrolithiasis (to prevent the growth of Ca²⁺-containing calculus)
         • Osteoporosis (to elevate Ca²⁺ in blood, and in turn, to diminish parathyroid hormone secretion)
      (2) Erectile dysfunction – indapamide is an exception (allegedly).

5. Indications
   (1) Hypertension
      • Mechanisms: - ↓ ECV → ↓ cardiac output
        - ↓PVR – Mech.: ↓Na⁺ conc. in the vasc. smooth m. → ↑Na⁺ import and ↑Ca²⁺ export
          via the Na⁺–Ca²⁺ exchanger → ↓Ca²⁺ in the vascular smooth m. → ↓PVR
      • For hypertension, thiazides are given in relatively low doses (e.g. 25 mg/day HCTZ)
   (2) Generalized edemas: cardiac, hepatic, renal (but not pulmonary – thiazides are not effective enough)
   (3) Calcium nephrolithiasis, osteoporosis (thiazides ↓ Ca²⁺ excretion)
   (4) Nephrogenic diabetes insipidus (paradoxically, thiazides ↓ urine formation by 50% in NDI)
   (5) Bromide intoxication (Thiazides ↓ Br⁻ reabsorption, like they ↓ Cl⁻ reabsorption.)

6. Preparations
   • Hydrochlorothiazide – typically in fixed combination with the K-sparing amiloride:
     AMILORID COMP or AMILOZID = HCTZ 50 mg + amiloride 5 mg
   • Chlorthalidone: HYGROTON tabl 25-50 mg
   • Clopamide: BRINALDIX tabl 10-20 mg
   • Indapamide: APADEX or RAWEL tabl 1.5 mg; COVEREX = indapamide + perindopril (ACEI)
V. Na⁺ CHANNEL INHIBITORS: amiloride and triamterene

Classified as weak diuretics and K⁺ sparing diuretics

1. Chemical properties: Basic compounds with amino groups that can be protonated. Triamterene is a prodrug; its active metabolite is 4-hydroxy-triamterene-sulfate.

2. Mechanism of action – 3 steps:
   (1) Amiloride and triamterene, as organic cations, are secreted by the organic cation secretory mechanism into the proximal convoluted tubules (OCT2 → MATE; see Appendix 4).
   (2) They travel along the nephron to the collecting duct (the site of action).
   (3) They block Na⁺ channels in the apical membrane of the principal cells in the collecting duct.
      These Na⁺ channels are called epithelial Na⁺ channels; they are different from the voltage-gated Na⁺ channels that are present in the plasma membrane of excitable cells.

3. Effects
   • Primary: ↓Na⁺ (and Cl⁻) reabsorption
      → weak diuresis (because only 2% of filtered Na⁺ and GFR is reabsorbed in the coll. duct)
   • Secondary: ↓lumen-negative transepithelial potential diff. (by decreasing the reabsorptive Na⁺ flux)
      → ↓K⁺ secretion (via K⁺ channels in principal cells) → K⁺ sparing effect
      → ↓H⁺ secretion (via the H⁺-ATPase in the type A intercalated cells)
      → metabolic acidosis

4. Pharmacokinetics
   • Amiloride: well absorbed orally, eliminated by urinary excretion in unchanged form, T₁/₂ ~ 6-9 hr (like for HCTZ)
   • Triamterene: moderately absorbed, eliminated partly by renal excretion and largely by hydroxylation then by sulfation (see figure) to form the active metabolite 4-hydroxy-triamterene sulfate. T₁/₂ is ~1-2 hr for the parent compound and 3 hr for the sulfate ester (given

For those interested: The sulfate-conjugates of drugs (e.g. paracetamol-sulfate) are almost always highly water-soluble, inactive and rapidly excreted. It is quite exceptional when such a conjugate is pharmacologically active and relatively slowly excreted, like 4-hydroxy-triamterene sulfate.

Explanation: The deprotonated (anionic) sulfate group reacts with the protonated (cationic) amino group in the molecule, forming an inner salt (also called zwitter ion). This process neutralizes the anionic sulfate group, therefore the water solubility of this metabolite decreases and so does its urinary excretion rate. A second consequence: at pH <5.5, formation of the poorly water-soluble inner salt is facilitated because of increased protonation of the amino group. This may lead to precipitation of 4-hydroxy-triamterene sulfate in the tubules (crystalluria).
5. Adverse effects

(1) Hyperkalemia; therefore Na\(^+\) channel inhibitors
- should not be combined with ACEIs and aldosterone antagonists (which decrease K\(^+\) secretion and also tend to cause hyperkalemia),
- may be dangerous in patient with renal impairment (due to K\(^+\) retention)
(2) Gastrointestinal disturbances: nausea, vomiting, diarrhea
(3) Triamterene only:
- Megaloblastic anemia after prolonged treatment with triamterene, which is a weak folic ac antagonist, a DHFR inhibitor, as it is a pteridine-containing compound.
- Crystalluria (4-OH-triamterene-sulfate is poorly water-soluble), interstitial nephritis
- Photosensitization (as UV light converts triamterene into an allergen)

6. Indications

(1) As diuretics; Na\(^+\) chan. inhib. are often combined with thiazides or loop diuretics to ↓ their K\(^+\) losing effect. Fixed combinations of HCTZ and amiloride are available (AMILORID COMP, AMILOZID).
(2) Cystic fibrosis (due to mutation of CFTR gene): aerosolized amiloride solution is given by inhalation.
   It blocks Na\(^+\) channels in bronchial mucosa → ↓ Na\(^+\) and water reabsorption from the bronchi
   → the bronchial secretion becomes dilute → the mucociliary clearance improves
(3) Li\(^+\) intoxication: Na\(^+\) channel blockers ↓ Li\(^+\) reabsorption via the Na\(^+\) channels → ↑ Li\(^+\) excretion
(4) Liddle syndrome: an inherited (autosomal dominant) a gain-of-function mutation of epithelial Na\(^+\) channels with hypertension, hypokalemia and alkalosis (↑Na\(^+\) reabsorb. → ↑K\(^+\) and H\(^+\) secretion).

7. Preparations

- Amiloride: AMILORID COMP or AMILOZID = hydrochlorothiazide 50 mg + amiloride 5 mg This is an ideal combination pharmacokinetically because HCTZ and amiloride have similar T\(_{1/2}\) (6-9 hr).
- Triamterene: DYRENIUM caps 50-100 mg

VI. ALDOSTERONE ANTAGONISTS (or MR ANTAGONISTS)

1. Mechanism of action

The effects of aldosterone (the most potent mineralocorticoid produced by the suprarenal gland):

(1) The renal effects of aldosterone – physiological effects:
- It acts on i.c. mineralocorticoid receptors (MR) in the principal cells of the distal convoluted tubule (DCT) and the collecting duct (CD)
- It increases the expression of: - the Na\(^+\) channels – in the luminal membrane of principal cells
  - the Na\(^+\)K\(^+\)ATPase – in the basolateral membrane of principal cells.
- Effects:
  - Primary: ↑ Na\(^+\) reabsorption from the DCT and the CD (via Na\(^+\) channel → Na\(^+\)K\(^+\)ATPase)
  - Secondary: ↑ lumen-negative transepithelial potential difference, which promotes:
    - K\(^+\) secretion (via K\(^+\) channels in principal cells)
    - H\(^+\) secretion (via H\(^+\)-ATPase in type A intercalated cells).

(2) The cardiovascular effects of aldosterone – pathophysiological effects:
- Activation of the RAAS (which occurs in congestive heart failure and cardiac infarct, for example) causes several adverse cardiovascular effects. These include high blood pressure, cardiac and vascular remodeling (i.e. hypertrophy and fibrosis), renal injury with magnesium loss, baroreceptor sensitization, ventricular arrhythmias, and increased mortality in patients with heart failure. The effects of aldosterone antagonists
(1) **The renal effects of aldosterone antagonists – diuretic action**

- **Mechanism:** they competitively inhibit the binding of aldosterone to the MR
  
  → ↓ expression of Na⁺ channels in the luminal membrane of the principal cells
  → ↓ expression of Na⁺K⁺ATPase in the basolateral membrane of the principal cells

- **Effects:**
  - **Primary:** ↓ reabsorption of Na⁺ (and Cl⁻) → diuresis
  - **Secondary:** ↓ lumen-negative transepithelial potential difference → ↓ K⁺ secretion = "K⁺ sparing"
    → ↓ H⁺ secretion = acidosis

The diuretic effect of aldosterone antagonists:
- develops after a few days when the presynthesized Na⁺ channels and Na⁺K⁺ATPase become depleted.
- is weak because solute and water reabsorption from the collecting duct amounts to only 2% of GFR.

(2) **The cardiovascular effects of aldosterone antagonists**

*Aldosterone antagonists* increase the beneficial cardiovascular effects of *ACE inhibitors* and *angiotensin receptor antagonists* (i.e. antihypertensive effect, reversal of cardiovascular remodeling). Although these latter drugs lower aldosterone secretion initially, later aldosterone blood levels become normalized or even elevated above normal despite continued therapy with an ACE inhibitor or angiotensin receptor antagonist ("aldosterone escape"). This explains the clinical benefit of additional therapy with aldosterone antagonists.

2. **Adosterone antagonist drugs:** spironolactone and eplerenone

**Spironolactone** (VEROSPIRON 25-50-100 mg tabl, HUMA-SPIROTON 25-50 mg tabl)

- Spironolactone (SPL) is a *non-specific* aldosterone antagonist, because it acts on:
  - Mineralocorticoid receptors → diuretic action, cardiovascular affects
  - Other steroid receptors (androgen rec antagonist, progesterone rec agonist) → endocrine effects

- **Pharmacokinetics of spironolactone:**
  - Orally absorbed (F ~0.7), highly protein-bound in the plasma
  - Rapidly and extensively biotransformed into active metabolites: 7-thio-SPL, 7-thiomethyl-SPL, canrenone (see figure on p.16)
  - SPL is rapidly eliminated (T₁/₂ ~1 hr) by thiolesterase (that forms 7-thio-SPL), however, its active metabolites are eliminated much more slowly (the T₁/₂ of canrenone is ~16 hr), therefore once daily administration of SPL is sufficient to maintain its clinical effect.

- Potassium canrenoate (the potassium salt of canrenoic acid) has also been used as a drug.
  - Poorly absorbed → given i.v.
  - In the body, canrenoate lactonizes into canrenone (see fig.), a more active and persistent metabolite.

- **Unwanted effects of spironolactone:**
  (1) Hyperkalemia, especially when combined with other drugs that also cause ↑ in plasma K⁺ level, e.g.:
    - K⁺ supplement, high K⁺ diet, K⁺-containing drugs, e.g. parenteral penicillin G potassium
    - ACE inhibitors (↓ angiotensin formation → ↓ aldosterone secretion)
    - Angiotensin antagonists (e.g. losartan)
    - NSAIDs: by ↓ synthesis of renal vasodilatatory PGs, NSAIDs may cause oliguria, Na⁺ and K⁺ retent.

  (2) Metabolic acidosis (by ↓ H⁺ secretion; H⁺ sparing)

  (3) Steroid effects:
    - Sex steroid effects:
      > In men (antiandrogenic effects): Gynecomastia, breast pain, erectile dysfunction, testicular atrophy
      > In women (progesterone rec agonist effect): menstrual irregularities
    - Glucocorticoid effects (SPL counters the negative feedback control on ACTH secretion → ↑ACTH)
      > Gastric bleeding, peptic ulcer
      > CNS effects: drowsiness, lethargy
Clinical use of spironolactone:

1. **As a diuretic**, together with thiazides or loop diuretics (to decrease their the K⁺- and H⁺-losing effects) for edema (especially in hepatic edema because hepatic cirrhosis causes sec. hyperaldosteronism)
   - hypertension

2. **As an aldosterone antagonist**:
   a. In hyperaldosteronism:
      - Primary hyperaldosteronism: in adrenal adenoma or hyperplasia
      - Secondary hyperaldosteronism, e.g.:
        > in cardiac failure (↑ aldosterone secretion caused by RAAS activation)
        > in hepatic cirrhosis (↓ aldosterone elimination in the liver by reduction and glucuronidation)
   b. In cardiovascular diseases (hypertension, congestive heart disease, acute myocardial infarction)
      - to lower blood pressure
      - to diminish cardiac and vascular hypertrophy and fibrosis (i.e. remodeling),
        which is caused in part by aldosterone secreted upon overactivation of the RAAS.

3. **As an androgen antagonist**: for treatment of hirsutism, acne and seborrhea in females
**Eplerenone** (INSPRA, 25 mg tabl) – differs in several respects from spironolactone

- Eplerenone – due to its epoxide group – is a specific aldosterone antagonist, not acting on other steroid receptors. (Unlike in many other epoxides – e.g. the toxic and carcinogenic benzpyrene epoxide, aflatoxin epoxide – the epoxide group in eplerenone is sterically hindered, therefore is non-reactive.)

- Pharmacokinetics of eplerenone:
  - Orally absorbed (F ~0.7), moderately protein-bound in the plasma
  - Elimination:
    > Mechanism: CYP3A4-catalyzed hydroxylation into inactive metabolites (see figure)
    > Speed: moderate (T_{1/2} ~ 6 hr). CYP3A4 inhibitors (e.g. erythromycin, itraconazole, cyclosporine A) delay the elimination of eplerenone.

- Unwanted effects of eplerenone: partly similar to those of spironolactone (i.e. hyperkalemia, metabolic acidosis); however, eplerenone is devoid of sex steroid effects.

- Clinical use of eplerenone:
  Eplerenone is primarily used with **cardiovascular indications**, hypertension, congestive heart disease and acute myocardial infarction:
  - to lower blood pressure,
  - to diminish cardiac and vascular hypertrophy and fibrosis (i.e. remodeling),
  which is caused in part by aldosterone secreted upon overactivation of the RAAS.

Preparation: INSPRA 25 mg filmtabl.
At present, eplerenone is over 10 times more expensive than spironolactone, which limits its clinical use.
Mechanism and site of action of diuretics
UNIT-III

TETRACYCLINE HYDROCHLORIDE - tetracycline hydrochloride capsule

Rx only

To reduce the development of drug-resistant bacteria and maintain the effectiveness of tetracycline hydrochloride and other antibacterial drugs, tetracycline hydrochloride should be used only to treat or prevent infections that are proven or strongly suspected to be caused by bacteria.

DESCRIPTION

Tetracycline is a yellow, odorless, crystalline powder. Tetracycline is stable in air but exposure to strong sunlight causes it to darken. Its potency is affected in solutions of pH below 2 and is rapidly destroyed by alkali hydroxide solutions. Tetracycline is very slightly soluble in water, freely soluble in dilute acid and in alkali hydroxide solutions, sparingly soluble in alcohol, and practically insoluble in chloroform and in ether. The chemical name for tetracycline hydrochloride is 4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,-12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecar-boxamide monohydrochloride. Its structural formula is as follows:

Each capsule, for oral administration, contains 250 mg or 500 mg tetracycline hydrochloride.

Inactive Ingredients: Lactose, light mineral oil, and magnesium stearate.

The 250 mg and 500 mg capsule shells contain D&C yellow no. 10, FD&C blue no. 1, FD&C yellow no. 6, gelatin, and titanium dioxide.

The imprinting ink for the 250 mg and 500 mg capsules contains D&C yellow #10, FD&C blue no. 1, FD&C blue no. 2, FD&C red no. 40, iron oxide black, pharmaceutical shellac glaze, propylene glycol and n-butyl alcohol.
CLINICAL PHARMACOLOGY

Tetracyclines are readily absorbed and are bound to plasma protein in varying degrees. They are concentrated by the liver in the bile and excreted in the urine and feces at high concentrations in a biologically active form.

Microbiology

Tetracyclines are primarily bacteriostatic and exert their antimicrobial effect by the inhibition of protein synthesis by binding to the 30S ribosomal subunit. Tetracycline is active against a broad range of gram-negative and gram-positive organisms. The drugs in the tetracycline class have closely similar antimicrobial spectra, and cross-resistance among them is common.

Gram-negative Bacteria

- *Acinetobacter* species
- *Bartonella bacilliformis*
- *Brucella* species
- *Campylobacter fetus*
- *Enterobacter aerogenes*
- *Escherichia coli*
- *Francisella tularensis*
- *Haemophilus ducreyi*
- *Haemophilus influenzae*
- *Klebsiella* species
- *Klebsiella granulomatis*
- *Neisseria gonorrhoeae*
- *Shigella* species
- *Vibrio cholerae*
- *Yersinia pestis*
Gram-positive Bacteria

*Bacillus anthracis*

*Streptococcus pyogenes*

*Streptococcus pneumoniae*

*Staphylococcus aureus*

*Listeria monocytogenes*

Anaerobes

*Bacteroides* species

*Clostridium* species

*Fusobacterium fusiforme*

*Propionibacterium acnes*

Other Bacteria

*Actinomyces* species

*Borrelia recurrentis*

*Chlamydophila psittaci*

*Chlamydia trachomatis*

*Rickettsiae*

*Treponema pallidum*

*Treponema pallidum subspecies pertenue*

Parasites

*Entamoeba* species

*Balantidium col*
INDICATIONS AND USAGE

To reduce the development of drug-resistant bacteria and maintain the effectiveness of tetracycline hydrochloride and other antibacterial drugs, tetracycline hydrochloride should be used only to treat infections that are proven or strongly suspected to be caused by susceptible bacteria. When culture and susceptibility information are available, they should be considered in selecting or modifying antibacterial therapy. In the absence of such data, local epidemiology and susceptibility patterns may contribute to the empiric selection of therapy.

Tetracycline is indicated in the treatment of infections caused by susceptible strains of the designated organisms in the conditions listed below:

- Upper respiratory tract infections caused by *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Hemophilus influenzae*. Note: Tetracycline should not be used for streptococcal disease unless the organism has been demonstrated to be susceptible.

- Lower respiratory tract infections caused by *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* (Eaton agent, and Klebsiella sp.)

- Skin and soft tissue infections caused by *Streptococcus pyogenes*, *Staphylococcus aureus*. (Tetracyclines are not the drugs of choice in the treatment of any type of staphylococcal infections.)

- Infections caused by rickettsia including Rocky Mountain spotted fever, typhus group infections, Q fever, rickettsialpox.

- Psittacosis caused by *Chlamydophila psittaci*.

- Infections caused by *Chlamydia trachomatis* such as uncomplicated urethral, endocervical or rectal infections, inclusion conjunctivitis, trachoma, and lymphogranuloma venereum.

- Granuloma inquinale caused by *Klebsiella granulomatis*.

- Relapsing fever caused by *Borrelia sp.*

- Bartonellosis caused by *Bartonella bacilliformis*.

- Chancroid caused by *Hemophilus ducreyi*.

- Tularemia caused by *Francisella tularensis*.

- Plaque caused by *Yersinia pestis*.

- Cholera caused by *Vibrio cholerae*.
- Brucellosis caused by *Brucella* species (tetracycline may be used in conjunction with an aminoglycoside).
- Infections due to *Campylobacter fetus*.
- As adjunctive therapy in intestinal amebiasis caused by *Entamoeba histolytica*.
- Urinary tract infections caused by susceptible strains of *Escherichia coli*, *Klebsiella*, etc.
- Other infections caused by susceptible gram-negative organisms such as *E. coli*, *Enterobacter aerogenes*, *Shigella sp.*, *Acinetobacter sp.*, *Klebsiella sp.*, and *Bacteroides sp.*
- In severe acne, adjunctive therapy with tetracycline may be useful.

When penicillin is contraindicated, tetracyclines are alternative drugs in the treatment of the following infections:

- Syphilis and yaws caused by *Treponema pallidum* and *pertenue*, respectively,
- Vincent’s infection caused by *Fusobacterium fusiforme*,
- Infections caused by *Neisseria gonorrhoeae*,
- Anthrax caused by *Bacillus anthracis*,
- Infections due to *Listeria monocytogenes*,
- Actinomycosis caused by *Actinomyces* species,
- Infections due to *Clostridium* species.

**CONTRAINDICATIONS**

- This drug is contraindicated in persons who have shown hypersensitivity to any of the tetracyclines.

**WARNINGS**

**Tooth Development**

- The use of drugs of the tetracycline-class during tooth development (last half of pregnancy, infancy and childhood to the age of 8 years) may cause permanent discoloration of the teeth (yellow-gray-brown). This adverse reaction is more common during long-term use of the drugs but it has been observed following repeated short-term courses. Enamel hypoplasia has also been reported. Tetracycline drugs should not be used in this age group, except for anthrax, unless other drugs are not likely to be effective or are contraindicated.

**Clostridium difficile** Associated Diarrhea
• *Clostridium difficile* associated diarrhea (CDAD) has been reported with use of nearly all antibacterial agents, including tetracyclines, and may range in severity from mild diarrhea to fatal colitis. Treatment with antibacterial agents alters the normal flora of the colon leading to overgrowth of *C. difficile*.

• **ADVERSE REACTIONS**

• **Gastrointestinal:** anorexia, nausea, epigastric distress, vomiting, diarrhea, glossitis, black hairy tongue, dysphagia, enterocolitis, and inflammatory lesions (with *Candida* overgrowth) in the anogenital region.

• Esophagitis and esophageal ulceration have been reported in patients receiving particularly the capsule and also the tablet forms of tetracyclines.

• Most of the patients were reported to have taken medication immediately before going to bed (see DOSAGE AND ADMINISTRATION).

• **Teeth:** permanent discoloration of teeth may be caused during tooth development. Enamel hypoplasia has been reported (see WARNINGS).

• **Skin:** maculopapular and erythematous rashes. Exfoliative dermatitis has been reported. Onycholysis and discoloration of the nails have been reported. Photosensitivity is discussed in WARNINGS.

• **Renal Toxicity:** an increase in BUN has been reported and is dose related.

• **Liver:** hepatotoxicity and liver failure have been observed in patients receiving tetracycline and in tetracycline-treated patients with renal impairment.

• **Hypersensitivity Reactions:** urticaria, angioneurotic edema, anaphylaxis, anaphylactoid purpura, pericarditis, exacerbation of systemic lupus erythematosus, and serum sickness-like reactions, as fever, rash, and arthralgia.

• **Blood:** hemolytic anemia, thrombocytopenia, thrombocytopenic purpura, neutropenia and eosinophilia have been reported.
UNIT IV
Immunosuppressants

The importance of the immune system in protecting the body against harmful foreign molecules is well recognized. However, this protection can result in serious problems. For example, rejection of the transplanted tissue. Transplantation of organs and tissues (for example, kidney, heart, or bone marrow) has become routine due to improved surgical techniques and better tissue typing. Also, drugs are now available that more selectively inhibit rejection of transplanted tissues while preventing the patient from becoming immunologically compromised. Earlier drugs were nonselective, and patients frequently succumbed to infection due to suppression of both the antibody-mediated (humoral) and cell-mediated arms of the immune system. Today, the principal approach to immunosuppressive therapy is to alter lymphocyte function using drugs or antibodies against immune proteins. Because of their severe toxicities when used as monotherapy, a combination of immunosuppressive agents, usually at lower doses, is generally employed. [Note: Immunosuppressive therapy is also used in the treatment of autoimmune diseases. For example, corticosteroids can control acute glomerulonephritis.] Immunosuppressive drug regimens usually consist of anywhere from two to four agents with different mechanisms of action that disrupt various levels of T-cell activation. Immunosuppressive drugs can be categorized according to their mechanisms of action: 1) Some agents interfere with cytokine production or action; 2) others disrupt cell metabolism, preventing lymphocyte proliferation; and 3) monoclonal and polyclonal antibodies block T-cell surface molecules.

II. SELECTIVE INHIBITORS OF CYTOKINE PRODUCTION AND FUNCTION

Cytokines are soluble, antigen-nonspecific, signaling proteins that bind to cell surface receptors on a variety of cells. The term cytokine includes the molecules known as interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), transforming growth factors, and colony-stimulating factors. Of particular interest when discussing immunosuppressive drugs is IL-2, a growth factor that stimulates the proliferation of antigen-primed (helper) T cells, which subsequently produce more IL-2, IFN-γ, and TNF-α (Figure 40.2). These cytokines collectively activate natural killer cells, macrophages, and cytotoxic T lymphocytes. Clearly, drugs that interfere with the production or activity
of IL-2, such as cyclosporine, will significantly dampen the immune response and thereby decrease graft rejection.

A. Cyclosporine

Cyclosporine is a lipophilic cyclic polypeptide. The drug is extracted from the soil fungus Beauveria nivea. Cyclosporine is used to prevent rejection of kidney, liver, and cardiac allogeneic transplants. Cyclosporine is most effective in preventing acute rejection of transplanted organs when combined in a double-drug or triple-drug regimen with corticosteroids and an antimetabolite such as mycophenolate mofetil. Cyclosporine is an alternative to methotrexate for the treatment of severe, active rheumatoid arthritis. It can also be used for patients with recalcitrant psoriasis that does not respond to other therapies, and it is also used for - erophthalmia.

1. Mechanism of action: Cyclosporine preferentially suppresses cell-mediated immune reactions, whereas humoral immunity is affected to a far lesser extent. After diffusing into the T cell, cyclosporine binds to a cyclophilin (more generally called an immunophilin) to form a complex that binds to calcineurin. The latter is responsible for dephosphorylating NFATc (cytosolic Nuclear Factor of Activated T cells). Because the cyclosporine-calcineurin complex cannot perform this reaction, NFATc cannot enter the nucleus to promote the reactions that are required for the synthesis of a number of cytokines, including IL-2. The end result is a decrease in IL-2, which is the primary chemical stimulus for increasing the number of T lymphocytes.

2. Pharmacokinetics: Cyclosporine may be given either orally or by intravenous (IV) infusion. Oral absorption is variable. Interpatient variability may be due to metabolism by a cytochrome P450 (CYP3A4) in the gastrointestinal (GI) tract, where the drug is metabolized. Cyclosporine is also a substrate for P-glycoprotein (P-gp), a drug efflux pump, which limits cyclosporine absorption by transporting the drug back into the gut lumen. About 50 percent of the drug is associated with the blood fraction. Half of this is in the erythrocytes, and less than one tenth is bound to the lymphocytes. Excretion of the metabolites is through the biliary route, with only a small fraction of the parent drug appearing in the urine.
Adverse effects: Many of the adverse effects caused by cyclosporine are dose dependent. Therefore, it is important to monitor blood levels of the drug. Nephrotoxicity is the most common and important adverse effect of cyclosporine, and it is critical to monitor kidney function. Reduction of the cyclosporine dosage can result in reversal of nephrotoxicity in most cases, although nephrotoxicity may be irreversible in 15 percent of patients. Hepatotoxicity can also occur, liver function should be periodically assessed. Infections in patients taking cyclosporine are common and may be life-threatening. Viral infections due to the herpes group and cytomegalovirus (CMV) are prevalent. Lymphoma may occur in all transplanted patients due to the net level of immunosuppression and has not been linked to any one particular agent. Anaphylactic reactions can occur on parenteral administration. Other toxicities include hypertension, hyperlipidemia, hyperkalemia (it is important not to use K+-sparing diuretics in these patients), tremor, hirsutism, glucose intolerance, and gum hyperplasia.

B. Tacrolimus

Tacrolimus (originally called FK506) is a macrolide that is isolated from the soil fungus Streptomyces tsukubaensis. Tacrolimus is approved for the prevention of rejection of liver and kidney transplants and is given with a corticosteroid and/or an antimetabolite. This drug has found favor over cyclosporine, not only because of its potency and decreased episodes of rejection, but also because lower doses of corticosteroids can be used, thus reducing the likelihood of steroid-associated adverse effects. An ointment preparation has been approved for moderate to severe atopic dermatitis that does not respond to conventional therapies.

1. Mechanism of action: Tacrolimus exerts its immunosuppressive effect in the same manner as cyclosporine, except that it binds to a different immunophilin, FKBP-12 (FK-binding protein).

2. Pharmacokinetics: Tacrolimus may be administered orally or IV. Tacrolimus is subject to gut metabolism by CYP3A4/5 isoenzymes and is a substrate for P-gp. Together, both of these mechanisms limit the oral bioavailability of tacrolimus. Absorption is decreased if the drug is taken with high-fat or high-carbohydrate meals. Tacrolimus is from 10- to 100-fold more potent than cyclosporine. It is highly bound to serum proteins and is also concentrated in erythrocytes. Like cyclosporine, tacrolimus undergoes hepatic metabolism. At least one metabolite of tacrolimus has been shown to
have immunosuppressive activity. Renal excretion is very low, and most of the drug and its metabolites are found in the feces.

Adverse effects: Nephrotoxicity and neurotoxicity (tremor, seizures, and hallucinations) tend to be more severe in patients who are treated with tacrolimus than in patients treated with cyclosporine. Development of posttransplant, insulin-dependent diabetes mellitus is a problem. Other toxicities are the same as those for cyclosporine, except that tacrolimus does not cause hirsutism or gingival hyperplasia. Compared with cyclosporine, tacrolimus has also been found to have a lower incidence of cardiovascular toxicities, such as hypertension and hyperlipidemia. Anaphylactoid reactions to the injection vehicle have been reported.

C. Sirolimus

Sirolimus is a macrolide obtained from fermentations of the soil mold Streptomyces hygroscopicus. The earlier name is rapamycin. Sirolimus is approved for use in renal transplant- tion, to be used together with cyclosporine and a corticosteroids, allowing lower doses of those medications to be used, thereby lowering their toxic potential. The combination of sirolimus and cyclosporine is apparently synergistic because sirolimus works later in the immune activation cascade. The antiproliferative action of sirolimus has found use in cardiology. Sirolimus-coated stents inserted into the cardiac vasculature inhibit restenosis of the blood vessels by reducing proliferation of the endothelial cells.

1. Mechanism of action: Sirolimus and tacrolimus bind to the same cytoplasmic FK-binding protein, but instead of forming a complex with calcineurin, sirolimus binds to mTOR. Binding of sirolimus to mTOR blocks the progression of activated T cells from the G1 to the S phase of the cell cycle and, consequently, the proliferation of these cells. Unlike cyclosporine and tacrolimus, sirolimus does not owe its effect to lowering IL-2 production but, rather, to inhibiting the cellular responses to IL-2.

2. Pharmacokinetics: The drug is available only as oral preparations. Although it is readily absorbed, high-fat meals can decrease the drug’s absorption. Sirolimus has a long half-life (57 to 62 hours) compared to those of cyclosporine and tacrolimus, and a loading dose is recommended at the time of initiation of therapy, but only requires once daily dosing. Sirolimus also increases the drug concentrations of cyclosporine, and careful blood level monitoring of both agents must be done to avoid harmful drug toxicities. The parent drug and its metabolites are predominantly eliminated in feces.
Although the administration of sirolimus and tacrolimus appears to be less nephrotoxic, sirolimus can still potentiate the nephrotoxicity of tacrolimus, and drug levels of both must be monitored closely. Other untoward problems are headache, nausea and diarrhea, leukopenia, and thrombocytopenia. Impaired wound healing has been noted with sirolimus in obese patients and those with diabetes.

D. Everolimus

Everolimus (another mTOR inhibitor) was recently approved by the U.S. Food and Drug Administration for use in renal transplantation in combination with low-dose cyclosporine and corticosteroids. It was originally approved in 2009 for second-line treatment in patients with advanced renal cell carcinoma.

1. Mechanism of action: Everolimus has the same mechanism of action as sirolimus. It inhibits activation of T cells by forming a complex with FKBP-12 and subsequently blocking mTOR.

2. Pharmacokinetics: Everolimus differs from sirolimus in its pharmacokinetic profile. Everolimus is rapidly absorbed, attaining maximal concentrations in 1 to 2 hours post dose, but absorption is decreased with high-fat meals. Everolimus is a substrate of CYP3A4 and P-gp and, thus, is subject to the same drug interactions as previously mentioned immunosuppressants. Everolimus avidly binds erythrocytes, and monitoring of whole blood trough concentrations is recommended. It has a much shorter half-life than does sirolimus at 30 ± 11 hours and requires twice-daily dosing. Everolimus increases drug concentrations of cyclosporine, thereby enhancing the nephrotoxic effects of cyclosporine, and is, therefore, recommended to be used with reduced doses of cyclosporine.

3. Adverse effects: Everolimus has similar side effects to sirolimus, including hyperlipidemia, impaired or delayed wound healing following transplantation, and enhanced nephrotoxicity in combination with higher doses of cyclosporine. An additional adverse effect noted with everolimus is angioedema, which may increase with concomitant use of angiotensin-converting enzyme inhibitors. There is also an increased risk of kidney arterial and venous thrombosis, resulting in graft loss, usually in the first 30 days posttransplantation.
Immunosuppressive antimetabolite agents are generally used in combination with corticosteroids and the calcineurin inhibitors, cyclosporine and tacrolimus.

A. Azathioprine

Azathioprine was the first agent to achieve widespread use in organ transplantation. It is a prodrug that is converted first to 6-mercaptopurine (6-MP) and then to the corresponding nucleotide, thioinosinic acid. The immunosuppressive effects of azathioprine are due to this nucleotide analog. The drug has little effect on suppressing a chronic immune response. Its major toxicity is bone marrow suppression. Concomitant use with angiotensin-converting enzyme inhibitors or cotrimoxazole in renal transplant patients can lead to an exaggerated leukopenic response. Allopurinol, an agent used to treat gout, significantly inhibits the metabolism of azathioprine. Therefore, the dose of azathioprine must be reduced by 60 to 75 percent. Nausea and vomiting are also encountered.

B. Mycophenolate mofetil

Mycophenolate mofetil has, for the most part, replaced azathioprine because of its safety and efficacy in prolonging graft survival. It has been successfully used in heart, kidney, and liver transplants. As an ester, it is rapidly hydrolyzed in the GI tract to mycophenolic acid. This is a potent, reversible, uncompetitive inhibitor of inosine monophosphate dehydrogenase, which blocks the de novo formation of guanosine phosphate. Thus, like 6-MP, it deprives the rapidly proliferating T and B cells of a key component of nucleic acids. Mycophenolic acid is quickly and almost completely absorbed after oral administration. Both mycophenolic acid and its glucuronidated metabolite are highly bound (greater than 90 percent) to plasma albumin. The glucuronide metabolite is excreted predominantly in urine. The most common adverse effects include diarrhea, nausea, vomiting, abdominal pain, leukopenia, and anemia. Higher doses of mycophenolate mofetil (3 g/day) were associated with a higher risk of CMV infection. [Note: mycophenolic acid is less mutagenic or carcinogenic than azathioprine.] Concomitant administration with antacids containing magnesium or aluminum, or with cholestyramine, can decrease absorption of the drug.
C. Enteric-coated mycophenolate sodium

In an effort to minimize the GI effects associated with mycophenolate mofetil, enteric-coated mycophenolate sodium was developed. The active drug (mycophenolic acid) is

IV. ANTIBODIES

The use of antibodies plays a central role in prolonging allograft survival. The names of monoclonal antibodies conventionally contain “muro” if they are from a murine (mouse) source and “xi” or “zu” if they are chimerized or humanized, respectively. The suffix “mab” (monoclonal antibody) identifies the category of drug. The polyclonal antibodies, although relatively inexpensive to produce, are variable and less specific, which is in contrast to monoclonal antibodies, which are homogeneous and specific.

A. Antithymocyte globulins

They are primarily used, together with other immunosuppressive agents, at the time of transplantation to prevent early allograft rejection, or they may be used to treat severe rejection episodes or corticosteroid-resistant acute rejection. The antibody-bound cells are phagocytosed in the liver and spleen, resulting in lymphopenia and impaired T-cell responses. The antibodies are slowly infused intravenously, and their half-life extends from 3 to 9 days. Because the humoral antibody mechanism remains active, antibodies can be formed against these foreign proteins. [Note: This is less of a problem with the humanized antibodies.] Other adverse effects include chills and fever, leukopenia and thrombocytopenia, infections due to CMV or other viruses, and skin rashes.

B. Muromonab-CD3 (OKT3)

Muromonab-CD3 is a murine monoclonal antibody that is synthesized by hybridoma technology and directed against the glycoprotein CD3 antigen of human T cells. MuromonabCD3 is used for treatment of acute rejection of renal allografts as well as for corticosteroid-resistant acute allograft rejection in cardiac and hepatic transplant patients. It is also used to deplete T cells from donor bone marrow prior to transplantation.

Adverse effects: Anaphylactoid reactions may occur. Cytokinerelease syndrome may follow the first dose. The symptoms can range from a mild, flu-like illness to a life-threatening, shock-like reaction. High fever is common. Central nervous system effects, such as seizures, encephalopathy, cerebral edema, aseptic meningitis, and headache.
C. IL-2-receptor antagonists

Basiliximab is said to be “chimerized” because it consists of 25 percent murine and 75 percent human protein. Daclizumab is 90 percent human protein, and is designated “humanized.” Both agents have been approved for prophylaxis of acute rejection in renal transplantation in combination with cyclosporine and corticosteroids. They are not used for the treatment of ongoing rejection. In late 2009, daclizumab was withdrawn from the U.S. market by the manufacturer due to a diminished demand for the product.

1. Mechanism of action: Both compounds are anti-CD25 antibodies and bind to the α chain of the IL-2 receptor on activated T cells. They thus interfere with the proliferation of these cells. Basiliximab is about 10-fold more potent than daclizumab as a blocker of IL-2 stimulated T-cell replication. Blockade of this receptor foils the ability of any antigenic stimulus to activate the T-cell response system.

2. Pharmacokinetics: Both antibodies are given IV. The serum half-life of daclizumab is about 20 days, and the blockade of the receptor is 120 days. Five doses of daclizumab are usually administered, the first at 24 hours before transplantation, and the next four doses at 14-day intervals. The serum half-life of basiliximab is about 7 days. Usually, two doses of this drug are administered, the first at 2 hours prior to transplantation, and the second at 4 days after the surgery.

3. Adverse effects: Both daclizumab and basiliximab are well tolerated. Their major toxicity is GI. No clinically relevant antibodies to the drugs have been detected, and malignancy does not appear to be a problem.

D. Alemtuzumab

Alemtuzumab, a humanized monoclonal antibody, exerts its effects by causing profound depletion of T cells from the peripheral circulation. This effect may last for up to 1 year. Alemtuzumab is currently approved for the treatment of refractory B-cell chronic lymphocytic leukemia. Although it is not currently approved for use in organ
transplantation, it is being used in combination with sirolimus and low-dose calcineurin inhibitors in corticosteroid-avoidance protocols at many transplant centers. Preliminary results are promising, with low rates of rejection with a prednisone-free regimen. Side effects include first-dose cytokine-release syndrome, requiring premedication with acetaminophen, diphenhydramine, and corticosteroids. Adverse effects include neutropenia, anemia, and, rarely, pancytopenia. Intermediate term results have shown an increase in B-cell mediated rejection and development of autoimmune disorders in a small number of patients and, thus, this agent should be used with caution.

CORTICOSTEROIDS

The corticosteroids were the first pharmacologic agents to be used as immunosuppressives both in transplantation and in various autoimmune disorders. They are still one of the mainstays for attenuating rejection episodes. For transplantation, the most common agents are prednisone or methylprednisolone, whereas prednisone or prednisolone are used for autoimmune conditions. The steroids are used to suppress acute rejection of solid organ allografts and in chronic graft-versus-host disease. In addition, they are effective against a wide variety of autoimmune conditions, including refractory rheumatoid arthritis, systemic lupus erythematosus, temporal arthritis, and asthma. The exact mechanism responsible for the immunosuppressive action of the corticosteroids is unclear. The T lymphocytes are affected most. The steroids are able to rapidly reduce lymphocyte populations. They bind to the glucocorticoid receptor. The complex passes into the nucleus and regulates the translation of DNA. The use of these agents is associated with numerous adverse effects. For example, they are diabetogenic and can cause hypercholesterolemia, cataracts, osteoporosis, and hypertension with prolonged use.
UNIT-V

ACUTE, SUBCHRONIC, AND CHRONIC TOXICITY

SCOPE AND LIMITATIONS

The acute, subchronic, and chronic toxicology of the chlorinated dioxins, dibenzofurans, biphenyls, and related compounds have been reviewed extensively in recent years. This chapter summarizes knowledge on the toxicology of tetrachlorodibenz-\(p\)-dioxin (TCDD), but also includes references to other dioxin-like compounds when relevant data are available. Included are selected various data that are considered to be of importance to risk assessment, particularly experimental animal data. Immunotoxicity, reproductive/developmental toxicity, carcinogenicity, toxicity to humans, and epidemiology are all covered in other chapters. Ecotoxicology is not covered in this chapter, but examples from mammalian and avian laboratory species are included.

ACUTE TOXICITY

The range of doses of TCDD that are lethal to animals varies extensively with both species and strain, as well as with sex, age, and the route of administration within a single strain (Table 3-1). One of the characteristics of TCDD-induced toxicity is delayed manifestation of lethality after acute exposure, with the time to death after exposure being several weeks. Death usually occurs as a consequence of loss of body weight (wasting syndrome) from TCDD-induced inhibition of gluconeogenesis and appetite suppression. Deaths within the first week after exposure—an unusually rapid course for TCDD toxicity—have been observed in guinea pigs (Schwetz et al., 1973), rabbits (Schwetz et al., 1973), and Syrian Golden hamsters (Olson et al., 1980). A more than 8,000-fold difference exists between the dose of TCDD reported to cause 50% lethality (LD\(_{50}\)) in male Hartley guinea pigs, the most sensitive species tested (Schwetz et al., 1973), and the LD\(_{50}\) dose in male Syrian Golden hamsters (Henck et al., 1981). Another animal with extremely high sensitivity is the mink (\textit{Mustela vision}); for the female, the calculated 28-day LC\(_{50}\) value is 0.264 µg TCDD/kg bw/day (Hochstein et al., 1998), which is an order of magnitude less than the 28-day LD\(_{50}\) of 4.2 µg TCDD/kg bw/day for male mink (Hochstein et al., 1988).

The rat seems to be the third most sensitive species among experimental animals, although there is a >300-fold variability in LD\(_{50}\) values among different strains. The Han/Wistar (H/W) Kuopio strain of rat has been shown to be particularly resistant to TCDD exposure (Pohjanvirta and Tuomisto, 1987). Among the five-rats-per-dose group (0, 1,500, 2,000, 2,500,
or 3,000 \mu g TCDD/kg bw), only one animal died within the 40-day observation period. The DBA/2 male mouse has also been shown to have a high resistance to TCDD toxicity (Chapman and Schiller, 1985).

Data on gender differences in sensitivity to the lethal effects of TCDD are conflicting. The gender differences in the acute toxicity of TCDD are likely due to differences in toxicokinetics, i.e., higher tissue concentrations and longer half-life in females than in males (Li et al., 1995). Acute toxicity data that address the effect of age at the time of exposure to TCDD are scarce, and comparisons are hampered by either the absence or inadequacy of information on the age and body weight of the tested animals. As demonstrated with other chemicals, the acute toxicity of TCDD may vary several-fold depending on the vehicle used or the presence of other substances that affect uptake.

Differences in sensitivity toward TCDD among various strains of mice have been shown to depend on a genetic variability in the Ah locus (see Chapter 2). In two strains of male C57B/6J mice that differ only at the Ah locus, Birnbaum et al. (1990) found LD_{50} values of 159 and 3,351 \mu g/kg for wild-type mice (Ah^{bb}) and congenic mice (Ah^{dd}), respectively. The mean time to death, 22 days, was independent of dose and genotype. Signs of toxicity were similar in the two strains, and it was concluded that the spectrum of toxicity is independent of the allele at the Ah locus. The relative dose needed to bring about various acute responses, however, is -8-24 times greater in congenic mice homozygous for the “d” allele than in the wild-type mice carrying two copies of the “b” gene.

The DBA/2 mouse strain requires 10 to 20 times higher doses of 2,3,7,8-TCDD than does the C57BL/6 strain for lethality (Chapman and Schiller, 1985). The reason for this difference between the two strains is the low TCDD-binding affinity to the Ah receptor in the DBA/2 strain (Okey et al., 1994). The difference in ligand-binding affinity, associated with susceptibility to TCDD-induced lethality that segregates with the Ah locus (Chapman and Schiller, 1985), is due to a point mutation (translated as alanine to valine) in the ligand-binding domain of codon 375 (Poland et al., 1994; Ema et al., 1994).

Wasting, hemorrhage, and anemia are the three primary causes for dioxin-induced lethality in rats, and a body weight loss of 25% is considered to be the minimum threshold to assign the presence of wasting syndrome for rats (Viluksela et al., 1997a,b, 1998).

1,2,3,4,5,6,7,8-HeptaCDD (HpCDD)-induced dose-response for wasting and hemorrhage overlap in female Sprague-Dawley rats (Rozman, 1999). Death from wasting and hemorrhage occur within the first few weeks of exposure. Animals that did not exhibit wasting or hemorrhage died from anemia, which did not start before day 126 postexposure (Rozman, 1999). Furthermore, unlike rats dying of wasting syndrome, the ones dying of anemia or hemorrhage had fat depots in the body, suggesting that increased body fat may aid them in surviving beyond
the 30-day mark (Rozman, 1999), only to succumb later to hemorrhage and anemia. Long-Evans (L-E) Turk AB strain rats are around 1000-fold more sensitive to TCDD-induced acute lethality (LD$_{50}$ about 10 µg/kg) than H/W Kuopio strain rats (LD$_{50}$ > 9,600 µg/kg) (Pohjanvirta et al., 1999). This feature of the H/W rat being highly resistant to acute TCDD toxicity, yet sensitive to enzyme induction, may be due in part to differences in AhR types between rat strains. The H/W strain has point mutations at exon 10 and at the first invariant nucleotide at the 5' end of intron 10 in the AhR gene. These cause alterations in AhR protein structure, leading to loss and alteration of multiple amino acid sequences at the carboxyl terminal region of the transactivation domain (Pohjanvirta et al., 1998). The homozygous AhR$^{hw/hw}$ type fails to mediate some endpoints of TCDD toxicity that parallel lethality. At lethal doses, H/W rats show only slight changes in bilirubin and body weight, while L-E rats show a five-fold increase in bilirubin and a 20% to 30% decrease in body weight as early as 6 days postexposure (Unkila et al., 1994a). Furthermore, at lethal doses H/W rats manifest only slight or transient inhibition of daily food intake and body weight gain, whereas in L-E rats progressive decrease in daily feed intake and body weight gain occur within 4 to 7 days postexposure (Unkila et al., 1994a).

Tuomisto et al. (1999) suggested that an uncharacterized gene, other than AhR, determines resistance of H/W Kuopio rats to TCDD-induced acute toxicity.

Geyer et al. (1990) utilized both their own and other data to determine a correlation between total body fat content and acute toxicity in various species and strains of laboratory mammals. They found a correlation of 0.834, and suggested that the reason for this correlation was that an increased total body fat content (TBF) may enhance the capacity to remove TCDD from the systemic circulation. This factor may be important, but it almost certainly does not explain all of the interspecies differences. Geyer et al. (1997) have determined that there is a linear relationship in mammals independent of strain and species between the logarithm of the oral 30-day LD$_{50}$ in units of µg/kg bw and the mammal’s TBF in percent via the regression equation:

$$\log \text{LD}_{50} = 5.30 \times \log \text{TBF} + 3.22$$

Data from studies of H/W Kuopio rats, which are extremely resistant to TCDD-induced lethality (Pohjanvirta and Tuomisto, 1987), were not included in this equation.

In chickens, acute toxicity is characterized by clinical signs such as dyspnea, reduced body weight gain, stunted growth, subcutaneous edema, pallor, and sudden death (chick edema disease). The disease first gained attention in 1957, but the causal agents were not identified as CDDs until much later (Firestone, 1973). Chick edema occurred in birds given oral doses of 1 or 10 µg TCDD/kg/day or 10 or 100 µg hexaCDD/kg/day, but it was not observed in chicks maintained on a diet containing 0.1% or 0.5% OCDD (Schwetz et al., 1973).
supplemented with 0, 0.001, 0.01, 0.1, 1, 10, or 100 ppb TCDD for up to 125 days. Feed consumption was significantly depressed in the 10 and 100 ppb groups beginning in weeks 4 and 3, respectively. When adjusted for body weight (g food intake/100 g bw/day), the feed intake in TCDD-exposed groups was not significantly different from the control, except in the 10 ppb-dosed group during week 5. Significant body weight loss associated with classic symptoms of wasting syndrome resulting in mortality was observed in the 1, 10, and 100 ppb-dosed groups, respectively, from the third, second, and first week of exposure. Mortality reached 12.5%, 62.5% and 100% by day 28 in the 1, 10, and 100 ppb-dosed groups, respectively. By day 125, mortality increased to 62.5% and 100% in the 1 and 10 ppb groups. Based on the average feed intake of 5.5 g/100 g bw/day for the control mink, the dietary LC$_{50}$ values of 4.8 and 0.85 ppb approximate 0.264 and 0.047 µg TCDD/kg bw/day, respectively, for 28 and 125 days of exposure.

**Signs and Symptoms of Toxicity**

TCDD affects a variety of organ systems in different species. It should be noted that much of the comparative database is derived from high-dose effects. The liver is the organ primarily affected in rodents and rabbits, while atrophy of the thymus and lymphatic tissues seems to be the most sensitive marker of toxicity in guinea pigs (WHO/IPCS, 1989; U.S. EPA, 1984, 1985). It is not possible to specify a single organ whose dysfunction accounts for lethality. Dermal effects are prominent signs of toxicity in nonhuman primates, and changes in epithelial tissues dominate both cutaneously and internally. This is most apparent in nonhuman primates in which the TCDD-induced cutaneous lesions closely mimic the chloracne and hyperkeratosis observed in humans. The histopathological alterations observed in epithelial tissues include hyperplastic and/or metaplastic alterations, as well as hypoplastic responses. The toxic responses of various species to TCDD are summarized in Table 3-2.

Loss of body weight, or wasting syndrome, is a characteristic sign observed in most animals exposed to TCDD. The weight loss usually manifests itself within a few days after exposure, and results in a substantial reduction of the adipose (Peterson et al., 1984) and muscle tissue (Max and Silbergeld, 1987) observed at autopsy. With sublethal doses of TCDD, a dose-dependent decrease in body weight gain occurs.

The greatest species-specific differences in toxicity concern pathological alterations in the liver. Administering lethal doses to guinea pigs does not result in liver damage comparable to the liver lesions observed in rabbits and rats, or to the liver changes observed in mice (McConnell et al., 1978a; Moore et al., 1979; Turner and Collins, 1983). In the hamster, manifest liver lesions do not occur even after fatal doses of TCDD; however, the ED$_{50}$ for increased hepatic weight is only ~15 µg/kg (Gasiewicz et al., 1986). Liver-related enzyme
activities in serum are elevated in those animal species where liver damage is a prominent sign of TCDD toxicity. In animal species where hepatotoxicity is not as apparent, such as monkeys and guinea pigs, these enzyme activities are nearly normal.

Thyroid atrophy has also been found in all animal species given lethal doses of TCDD. Treatment with TCDD inhibits bone marrow hematopoiesis in mice, both in vivo and in vitro, by directly altering the colony growth efficiency of stem cells (Chastain and Pazdernik, 1985; Luster et al., 1980, 1985).

Among other signs and symptoms that have been demonstrated in various species, the following should be noted: hepatic porphyria, hemorrhages in various organs, testicular atrophy, reduced prostate weight, reduced uterine weight, increased thyroid weight, lesions of the adrenal glands, inhibited bone marrow hematopoiesis, decreased serum albumin, and increased serum triglycerides and free fatty acids. The details of all underlying studies for these observations have been extensively reviewed (U.S. EPA, 1984, 1985; WHO/IPCS, 1989).

Effects on heart muscle have also been observed in guinea pigs and rats (Brewster et al., 1987; Kelling et al., 1987; Canga et al., 1988). Five days after a single lethal dose of TCDD (10 µg/kg intraperitoneally) was administered, a significantly decreased beta-adrenergic responsiveness was observed in the right ventricular papillary muscle of the guinea pig (Canga et al., 1988). In the TCDD-treated animals, a decrease in the positive inotropic effects of isoproterenol at 0.03-0.3 µM, but not at 0.1-10 nM, was also demonstrated. Additionally, enhanced responsiveness to low-frequency stimulation and increases in extracellular calcium were observed in these animals. Based on these findings, the authors suggest that the heart may be a major target for TCDD lethality at acutely toxic doses.

In the monkey, several additional symptoms have been registered, such as periorbital edema, conjunctivitis, and thickening of the meibomian glands followed by loss of the eyelashes, facial hair, and nails (McConnell et al., 1978b). These symptoms are similar to those observed in cases of human intoxication, such as from occupational exposure, the Seveso incident, and the Yusho and Yu-Cheng toxic oil intoxications, the latter involving exposure to PCBs and CDFs (see Chapter 7).

**Studies In Vitro**

Over 30 cell types, including primary cultures and cells from established and transformed cell lines derived from various tissues of at least six animal species, have been examined for their general cellular responses to TCDD (Beatty et al., 1975; Knutson and Poland, 1980a; Niwa et al., 1975; Yang et al., 1983a). The effects studied were changes in viability, growth rate, and morphology. Overall, there were few effects documented on these general cellular parameters in early studies.
Other in vitro studies, using more specific endpoints of toxicity, have clearly indicated effects of TCDD at comparatively low concentrations. For example, several studies have shown that TCDD affects cultured epidermal keratinocytes through interactions with differentiation mechanisms, and that this effect may be regulated by the modulation of epidermal growth factor (EGF) binding to the cells (Hudson et al., 1986). Additionally, in epithelial cells of human origin, TCDD has been shown to alter differentiation (Hudson et al., 1985), while aryl hydrocarbon hydroxylase (AHH) and 7-ethoxyresorufin O-deethylase (EROD) activity have been induced in vitro (see Section 3.5.4).

TCDD was found to inhibit high-density growth arrest in human squamous carcinoma cells in culture (Hebert et al., 1990a). Wiebel et al. (1991) identified a cell line (H4IIEC3-derived 5L hepatoma cells) that responds with decreased proliferation at low TCDD concentrations. In this cell line, half-maximum inhibition of proliferation occurs at a concentration of 0.1-0.3 nM. The onset of the effect is fairly rapid, manifesting itself as early as 4-8 hours after treatment. Further studies demonstrated that insensitive variants of this cell line were deficient in cytochrome P-4501A1 activity and lacked measurable amounts of the Ah receptor (Göttlicher et al., 1990). In addition, 3,3′,4,4′-TCB inhibited proliferation in the sensitive cell line, although at higher concentrations.

**Appraisal**

Numerous studies of acute toxicity in various mammalian species have demonstrated dramatic species- and strain-specific differences in sensitivity. However, most species and strains respond at some level with a spectrum of symptoms that is generally the same, although species differences do exist.

Lethality is typically delayed by several weeks, and there is a pronounced wasting syndrome in almost all laboratory animals. Studies in congenic mice differing in their Ah responsiveness indicate that the sensitivity to acute toxicity of TCDD segregates with the Ah locus. Furthermore, studies of other CDDs, CDFs, and coplanar PCBs demonstrate that the potency for inducing lethality correlates with their ability to bind to the Ah receptor. In contrast, studies in various other species, including various strains of rats, have demonstrated a wide range of sensitivities regardless of rather comparable levels of the Ah receptor. This in no way obviates the necessary, but not sufficient, role of AhR.

**SUBCHRONIC TOXICITY**

Available studies on the subchronic toxicity of TCDD have been reviewed by the U.S. EPA (1984, 1985) and WHO/IPCS (1989). Overall, the signs and symptoms observed are in agreement with those observed after administration of single doses.
The study of Kociba et al. (1976) is of special interest, as it has been used for comparisons of the relative toxicities of other CDDs and CDFs (Plüess et al., 1988a,b). Adult male and female SD rats, in groups of 12, were given 0, 0.001, 0.01, 0.1, and 1.0 µg TCDD/kg bw by gavage 5 days/week for 13 weeks. At the end of the treatment period, five rats of each sex were sacrificed for histopathological examination. The remaining animals were observed for postexposure effects. The highest dose caused five deaths among the females, three during the treatment period and two after, while two deaths occurred in males in the posttreatment period. The rats given 0.01 µg TCDD/kg did not differ overtly from the controls except for a slight increase in the mean liver-to-body weight ratio.

A 13-week dietary study of SD rats given 1,2,3,4,8-PeCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, or 1,2,3,6,7,8-HxCDF demonstrated that both subchronic toxicity and the depletion of hepatic vitamin A followed the rank order of the ability of the compounds to bind to the Ah receptor and to cause induction of AHH (Plüess et al., 1988a,b; Håkansson et al., 1990). Direct comparisons of the effects are hampered, however, by differences in the toxicokinetic behavior of the compounds. Slightly different relationships with regard to toxicity were observed in a tumor promotion study, where an initial loading dose (subcutaneous) of 2,3,4,7,8-PeCDF was given, followed by repeated lower doses (subcutaneous), in order to obtain a steady-state concentration (Wärn et al., 1991a). Both of these studies support the assumption that most signs and symptoms obtained may be mediated through the Ah receptor.

In another study primarily aimed at investigating TCDD-induced porphyria (Goldstein et al., 1982), groups of eight female SD rats were exposed to 16 weekly oral doses of 0, 0.01, 0.1, 1.0, and 10.0 µg TCDD/kg bw. The animals were killed and studied 1 week after the last treatment. Additional groups of rats received doses of 0 or 1.0 µg/kg/week for 16 weeks and were allowed to recover for 6 months. The high-dose level was lethal to all animals within 12 weeks, while the only other gross sign of toxicity was a decrease in body weight gain in the group receiving 1.0 µg/kg/week. After 16 weeks of exposure to TCDD, liver porphyrins were elevated ~1,000-fold in 7 of 8 animals receiving 1.0 µg/kg/week. Only 1 of 8 animals in the 0.1 µg/kg/week group had elevated porphyrin levels. The no-effect dose for porphyria was 0.01 µg/kg/week. After a 6-month recovery period, the porphyrin level in animals exposed to 1.0 µg/kg/week was still 100-fold higher than the values in the control group. A similar pattern was observed for urinary excretion of uroporphyrin. A 6-month recovery period was not sufficient for complete reversal of TCDD-induced porphyria.

Two studies were conducted (Harris et al., 1973; Vos et al., 1973) in which four weekly oral doses of 0.2, 1, 5, or 25 µg TCDD/kg bw were given to male C57Bl/6 mice in corn oil. No effects were noted at 1 µg/kg/week, which corresponds to -0.1 µg/kg bw/day.
hepatic porphyrin in rats at levels comparable with those found in human milk and fat samples. Coadministration of TCDD with 2,2',4,4',5,5'-PCB (PCB 153) resulted in elevated hepatic porphyrin levels not observed in TCDD cotreated with 3,3',4,4',5-PCB (PCB126) or 2,3,3',4,4',5-PCB (PCB156) groups. In this experiment, LOAELs for hepatic porphyrin accumulation for TCDD, PCB126, and PCB 156 were found to be 0.047, 3.18, and 365 µg/kg/d, respectively. van Birgelen et al. (1996b) have further extended the observation on hepatic porphyrin activity in female B6C3F1 mice after subchronic exposure to individual PCDD, PCDF, and PCB congeners. A dose-response relationship with potencies, relative to TCDD, for increased hepatic porphyrin accumulation was observed for all of the individual congeners studied. The relative potencies of PCDDs and PCDFs tested, based on hepatic porphyrin and enzymatic activities associated with hepatic CYP1A1 and CYP1A2, were found to be in a comparable range.

A 90-day TCDD feeding study of male and female Hartley guinea pigs was performed by DeCaprio et al. (1986), in which surviving animals were subjected to extensive pathologic, hematologic, and serum chemical analyses. The diets contained 0, 2, 10, 76, or 430 ng TCDD/kg bw. The two lowest doses, 2 and 10 ng/kg, produced no dose-related alterations. Based on this study, a no-observed-adverse-effect level (NOAEL) of 0.6 ng TCDD/kg bw/day in guinea pigs was estimated. At the highest dose, severe body weight losses and mortality were observed. No dose-related mortality occurred at 76 ng/kg.

A cumulative dose of 0.2 µg TCDD/kg bw, which was divided into nine oral doses 3 times/week during days 20-40 of gestation, produced no clinical signs of toxicity in pregnant rhesus monkeys (Macaca mulatta) (McNulty, 1984). Signs of toxicity such as body weight loss, epidermal changes, and anemia did occur, however, in monkeys that received cumulative doses of 1.0 and 5.0 µg TCDD/kg bw over the same time period.

3.3.1. Appraisal

Utilizing the above data, subchronic no-observable-adverse-effect levels (NOAELs) for rats, mice, and guinea pigs are estimated to be 1 ng, 100 ng, and 0.6 ng TCDD/kg bw/day, respectively. These studies cannot be directly compared with each other, however, and these subchronic NOAELs cannot be used for extrapolating human risk. None of the studies utilized initial loading doses and, due to the long half-life of TCDD, steady-states may not have been reached in the animals except toward the end of the study periods. Distribution between tissues in the animals depends on both time of exposure and dose level (see Chapter 1), which further complicates any comparisons.

In spite of this, the limited data available seem to indicate that signs and symptoms of subchronic toxicity follow the same rank order as Ah receptor-mediated effects, such as induction of AHH.
3.4. CHRONIC TOXICITY

The results of chronic toxicity studies performed on laboratory animals exposed to TCDD are summarized in Table 3-3. Details have been reviewed by the U.S. EPA (1984, 1985) and WHO/IPCS (1989).

The most important study in rats is the chronic toxicity study of Kociba et al. (1978, 1979). Groups of 50 male and 50 female SD rats were fed diets providing daily doses of 0.001, 0.01, and 0.1 µg TCDD/kg bw for 2 years. Control rats, 86 males and 86 females, received diets containing the vehicle alone. Increased mortality was observed in females given 0.1 µg/kg/day, while increased mortality was not observed in male rats at this dose or in animals receiving doses of 0.01 or 0.001 µg/kg/day. From month 6 to the end of the study, the mean body weights of males and females decreased at the highest dose and, to a lesser degree, in females given 0.01 µg/kg/day. During the middle of the study, lower-than-normal body weights were also occasionally recorded in the low-dose group, although during the last quarter of the study the body weights were comparable with those of the controls.

Increased urinary coproporphyrin and uroporphyrin were noted in female rats, but not in males, given TCDD at a dose rate of 0.01 and 0.1 µg/kg/day. Analyses of blood serum collected at terminal necropsy revealed increased enzyme activities related to impaired liver function in female rats given 0.1 µg TCDD/kg/day. Necropsy examination of the rats surviving TCDD exposure until the end of the study revealed that effects in the liver constituted the most consistent alteration in both males and females. Histopathological examination revealed multiple degenerative, inflammatory, and necrotic changes in the liver that were more extensive in females. Multinucleated hepatocytes and bile-duct hyperplasia were also noted. Liver damage was dose related, and no effect was observed at the low-dose rate. The NOAEL was estimated to be 0.001 µg/kg/day. At the end of the study, the fat and liver concentration of TCDD at this dose was 540 ppt.

In male Swiss mice, weekly oral doses of 0, 0.007, 0.7, and 7.0 µg TCDD/kg bw for 1 year resulted in amyloidosis and dermatitis (Toth et al., 1979). The incidence of these lesions was 0 of 38, 5 of 44, 10 of 44, and 17 of 43 in the control-, low-, medium-, and high-dose groups, respectively. The LOAEL in this study was estimated to be 0.001 µg/kg/day (=1 ng/kg/day).

In the National Toxicology Program (NTP, 1982) gavage study of B6C3F1 male and female mice, no adverse effects were seen at the lowest dose tested (0.01 and 0.04 µg/kg bw/week for males and females, respectively; corresponding to -1.4 and 6 ng/kg bw/day).

The limited studies (9-20 months) available in rhesus monkeys (Allen et al., 1977; Barsotti et al., 1979; Schantz et al., 1979) revealed signs and symptoms similar to those recorded
in more short-term studies. Adverse effects were noted down to the lowest dose tested (-2-3 ng/kg bw/day for 20 months) (Schantz et al., 1979).

3.4.1. Appraisal

From different long-term studies on TCDD, it can be estimated that the NOAEL for the rat is 1 ng/kg bw/day, corresponding to a fat and liver concentration (NOAEL) of 540 ppt. For the male Swiss mouse, dermatitis and amyloidosis in 5 of 44 animals were noted at the lowest dose tested (the LOAEL was 1 ng/kg bw/day). NOAELs of 1.4 and 6 ng/kg/day were obtained for male and female B6C3F1 mice, respectively. The reported studies on rhesus monkeys are problematic for use in such a determination, because adverse effects were observed at the lowest dose tested, -2-3 ng/kg bw/day.

SPECIFIC EFFECTS

Wasting Syndrome

TCDD at high doses (lethal or near lethal) causes a starvation-like effect, or wasting syndrome, in several animal species. In young animals, or following a sublethal dose to adults, this response is manifested as a cessation of weight gain. Animals exposed to near lethal or higher doses characteristically lose weight rapidly. Numerous studies utilizing pair-feeding, total parenteral nutrition, and everted intestinal sacs have been performed to elucidate the mechanisms behind the wasting syndrome (U.S. EPA, 1984, 1985; WHO/IPCS, 1989), but no single explanation has been obtained thus far. No generalized impairment of intestinal absorption seems to occur.

Peterson et al. (1984) conducted behavior experiments and suggested a model for the TCDD-induced wasting syndrome that is based on the hypothesis, advocated by Keesey and Powley (1975, 1986), that body weight in rats is regulated to an internal standard or hypothalamically programmed set-point. According to this hypothesis, the body weight at a given age is constantly being compared to this set-point value and, if differences occur, feed consumption is adjusted. When TCDD lowers this set-point, reduction in food consumption results as the rat attempts to reduce its weight to a new lower level. This hypothesis has been tested in several experiments under carefully controlled feeding conditions. Repeated studies have demonstrated that reduction of feed intake due to increased food spillage is not sufficient to account for the loss of body weight in TCDD-treated SD rats. Additionally, TCDD-treated rats maintain and defend their reduced weight level with the same precision that ad libitum-fed control rats defend their normal weight level (Seefeld and Peterson, 1983, 1984; Seefeld et al., 1984a,b). The percentage of the daily feed intake that is absorbed by the gastrointestinal tract of
TCDD-treated and control rats is similar (Potter et al., 1986; Seefeld and Peterson, 1984).

Reduced appetite as a result of inhibition of tryptophan-2,3,-dioxygenase causes gradual development of eventual lethal hypoglycemia in TCDD-induced wasting syndrome in rats (Weber et al., 1994). No reduced appetite associated with gradual body weight loss and no tryptophan effects are observed in TCDD-exposed mice, although appetite and body weight loss are observed in mice at the terminal stage of wasting syndrome. Hypophagia was the major cause of adipose and lean tissue loss in male Fischer 344 rats, C57Bl/6 mice, and albino guinea pigs when exposed to a calculated LD$_{50}$ dose of TCDD. Body weight loss followed a similar time-course in TCDD-treated and pair-fed control animals of all three species (Kelling et al., 1985).

Body weight loss appears to contribute to lethality in a species- and strain-dependent fashion, but weight loss appears to play a greater role in causing death in SD rats and guinea pigs than it does in Fischer 344 rats and C57Bl/6 mice. Loss of body weight and loss of appetite are also prominent signs of thyroid dysfunction. However, some data indicate that the effect of TCDD on thyroid hormones cannot explain the TCDD-induced decrease in body weight gain.

Reduced gluconeogenesis due to inhibition of phosphoenol pyruvate carboxykinase (PEPCK) by TCDD has been suggested as one of the primary contributing factors to a gradual development of an eventual lethal hypoglycemia in wasting syndrome in rats (Stall et al., 1993) and mice (Weber et al., 1995).

TCDD-induced wasting is associated with reduction of adipose tissue mass, hypertriglyceridemia, redistribution of fatty acids (Gasiewicz and Neal, 1979; Chapman and Schiller, 1985; Brewster and Matsumura, 1988), and diabetic-like symptoms (Brewster and Matsumura, 1988). Carbohydrate and lipid metabolism are severely impaired in the liver and adipose tissue by TCDD. Glucose transport systems play vital roles in controlling the rate of energy utilization in adipose tissues. TCDD also affects lipoprotein lipase (LPL) activity. The rate of fat storage is determined by LPL, which controls the serum level of triglycerides. Brewster and Matsumura (1984) found that LPL activity was decreased in guinea pigs to 20% of the value of ad libitum-fed controls after 1 day, and that this effect persisted throughout the study (10 days). The authors suggest that TCDD irreversibly reduces adipose LPL activity, thus making the animals less capable of adapting to nutritional changes and needs. In the pancreas, LPL regulates the production and release of insulin, and in the liver it controls glucose metabolism and fatty acid synthesis. From their observations on the significant reduction of glucose-transporting activity in adipose tissue and pancreas in guinea pigs by TCDD at a very low dose (single IP injection of 0.03 µg/kg), Enan et al. (1992a,b) concluded that the reduction in glucose transporters is one of the major causes of TCDD-induced wasting syndrome in this species.
Phenanthroline, an Ah receptor blocker, prevents the effect of TCDD on glucose uptake, suggesting that TCDD-induced downregulation of functional glucose transporter proteins is mediated through the Ah receptor.

The insulin-recruitable form of glucose transporter Type 4 (GLUT4) provides energy to the cell by supplying glucose to the muscle and tissue tissues. Impairment of GLUT4 in adipose tissue, liver, and pancreas (Enan et al., 1992a,b), and reduction of PEPCK in liver (Viluksela et al., 1995), could play important roles in the pathogenesis of TCDD-induced diabetes.

In a series of studies on Wistar rats, Lakshman et al. (1988, 1989, 1991) demonstrated that single intraperitoneal injections of TCDD (from 1 µg/kg) caused a dose-dependent inhibition of fatty acid synthesis in the liver and adipose tissue. Adipose tissue was found to be more sensitive than the liver. They also found an increased mobilization of depot fat into the plasma compartment, accompanied by an increase in plasma free fatty acid concentrations.

In vitro studies of isolated heart mitochondria have indicated that a TCDD concentration of 1.5 nmol/mg in mitochondrial protein affects oxygen activation associated with cell respiration. Superoxide radicals and H$_2$O$_2$ were indicated to be involved in the development of the observed effects (Nohl et al., 1989).

Loss of muscle tissue, accompanied by a decreased glucocorticoid receptor-binding capacity and an increased glutamine synthetase activity, has been observed in male Fischer 344N rats given a single oral TCDD dose of 100 µg/kg (Max and Silbergeld, 1987).

Another biochemical effect associated with TCDD-induced wasting syndrome is the decrease in hepatic vitamin A storage in TCDD-exposed animals (Thunberg et al., 1979; Håkansson et al., 1989a, 1991). Vitamin A is necessary for growth; vitamin A deficiency will result in depressed body weight gain and reduced food intake. However, in contrast to TCDD-treated animals, the vitamin A-deficient animals continue to eat and grow, though body weight gain is less than normal (Hayes, 1971).

The hypothesis that decreased feed intake could be a result of a direct TCDD effect on the brain was initially indicated by Pohjanvirta et al. (1989), although contradictory information has been provided by other studies (Stall and Rozman, 1990). The intraperitoneal administration of TCDD at 50 µg/kg to male SD rats (~LD$_{50}$ level) caused a significant decrease in the serum concentration of prolactin, detectable after 4 hours, compared with pair-fed vehicle controls and noninjected controls (Jones et al., 1987). Further studies have demonstrated that the effect of TCDD was reversed by pimozide, a dopamine receptor antagonist, and [that the rate constant of dopamine depletion after "-methyl-p-tyrosine and the turnover rate were significantly elevated.] This suggests a hypothalamic site of TCDD action in their experiments (Russell et al., 1988), a finding supported by additional data on changes to central thermoregulation by dioxin in golden hamsters (Gordon et al., 1996) and rats (Gordon and Miller, 1998).
Changes in intermediary metabolism have been demonstrated in TCDD-treated experimental animals. Conflicting data on how TCDD affects serum glucose and hepatic glycogen levels have been reported earlier (WHO/IPCS, 1989). Several studies have suggested that the ultimate cause of death in some mammalian species may be a progressive hypoglycemia (Ebner et al., 1988; Gorski and Rozman, 1987; Gorski et al., 1990). Serum glucose levels in the guinea pig, however, were not affected by treatment of the animals with TCDD (Gasiewicz and Neal, 1979). Slight reductions in serum glucose levels were noted in both L-E and H/W rats (Pohjanvirta et al., 1989). Rozman et al. (1990) have suggested that the subchronic and chronic toxicities of TCDD are related to the inhibition of key enzymes of gluconeogenesis. They demonstrated that the induction of appetite suppression was preceded by the inhibition of PEPCK, which caused a reduction in gluconeogenesis. This was followed by a progressive increase in plasma tryptophan levels that was suggested to cause a serotonin-mediated reduction of the feed intake. In SD rats, TCDD in doses of 25 and 125 µg caused a rapid decrease (50%) in PEPCK activity 2 days after dosing, followed by a dose-dependent decrease in glucose-6-phosphatase activity 4 to 8 days after exposure. Both appetite suppression and reduced PEPCK activity occurred in the same dose range (Weber et al., 1991). TCDD-induced impairments of carbohydrate synthesis have also been suggested by studies in chick embryos (Lentnek et al., 1991).

Numerous studies have measured serum levels of free fatty acids, cholesterol, and triglycerides in various species after TCDD treatment (WHO/IPCS, 1989), but no pronounced qualitative differences have been observed between species or strains of mice.

The wasting syndrome seems to be a generalized effect, elicited in all species and strains, but at various dosages (single or repeated administration). Specific studies have not been performed to elucidate the extent to which this syndrome is elicited through the interaction of TCDD with the Ah receptor, although the binding affinities of various CDDs and CDFs to the Ah receptor, as well as those of related PCBs, have been shown to strongly correlate with their potency to induce the wasting syndrome in both rats and guinea pigs (Safe, 1990).

**Hepatotoxicity**

Even at sublethal doses, TCDD induces hyperplasia and hypertrophy of parenchymal cells and, thus, hepatomegaly in all species investigated. There is, however, considerable variation in the extent and severity of this lesion among the species tested. Other liver lesions are more species-specific. Lethality following the administration of TCDD cannot be explained by these liver lesions alone, although they may be a contributing factor in the rat and rabbit. The morphological changes in the liver are accompanied by impaired liver function characterized by liver enzyme leakage, increased microsomal monooxygenase activities, porphyria, impaired

Hepatotoxic reaction in various strains of rats given lethal doses of TCDD is characterized by degenerative and necrotic changes including the appearance of mononuclear cell infiltration, multinucleated giant hepatocytes, increased numbers of mitotic figures and pleomorphism of cord cells, an increase in the hepatic smooth endoplasmic reticulum, and parenchymal cell necrosis. The histological findings are accompanied by hyperbilirubinemia, hypercholesterolemia, hyperproteinemia, and increased serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) activities, which further indicate damaged liver function (WHO/IPCS, 1989). These lesions may be severe enough to be a contributing factor in death. The lesions observed after sublethal doses are qualitatively almost identical to those observed after lethal doses.

Early studies in mice found similar effects. More recently, Shen et al. (1991) reported a comparative study on the hepatotoxicity of TCDD in Ah-responsive and Ah-nonresponsive mice (C57BL/6J and DBA/2J, respectively). The C57BL/6J mice given a single dose of 3 µg/kg TCDD developed mild to moderate hepatic lipid accumulation but no inflammation or necrosis. Severe fatty change, mild inflammation, and necrosis occurred at 150 µg/kg. The DBA/2J mice given 30 µg/kg developed hepatocellular necrosis and inflammation but no fatty change. Lipid accumulation was only slight after 600 µg/kg. The authors concluded that the Ah locus may be involved in determining the steatotic effects of TCDD. This is consistent with the findings of Birnbaum et al. (1990) on the differential toxicity of TCDD in C57BL/6J mice congenic at the Ah locus. In this study, wild type mice were 8- to 24-fold more sensitive than congenic mice deficient at the Ah locus for a spectrum of effects, including increased liver weight, hepatocellular cytomegaly, fatty change, bile duct hyperplasia, and serum liver enzyme changes.

The guinea pig shows less severe morphological alteration in the liver than other species, although ultrastructural changes of the liver are found. Likewise, the hamster exhibits little or no liver damage even after a fatal dose, but liver lesions have been observed after prolonged periods following the administration of nonlethal doses.

Several parameters relating to disturbed hepatic plasma membrane function have been studied (U.S. EPA, 1984, 1985; WHO/IPCS, 1989). Adenosine triphosphatase (ATPase) activities were depressed and protein kinase C activity was increased in rats, but not in guinea pigs, treated with TCDD (Bombick et al., 1985). TCDD also induced a decrease in the binding of EGF. The relative doses of TCDD needed to suppress EGF binding to 50% of the control level were 1, 14, and 32 µg/kg for the guinea pig, the SD rat, and the Syrian Golden hamster, respectively (Madhukar et al., 1984). A single intraperitoneal dose of 115 µg TCDD/kg bw
decreased the EGF binding by 93.1%, 97.8%, and 46.0% in C57Bl/6, CBA, and AKR mice, respectively, 10 days after treatment (Madhukar et al., 1984).

Further studies on the interaction of TCDD with the EGF receptor have been performed in congenic mice of the strain C57BL/6J (Lin et al., 1991a,b). The ED$_{50}$ for the TCDD-induced decrease in maximum binding capacity of the EGF receptor was 10 times higher in the Ah-nonresponsive mice than the Ah-responsive animals. This study supports the hypothesis that the effects of TCDD on EGF receptor ligand binding are mediated by the Ah receptor.

The effects of TCDD on biliary excretion of various compounds have also been studied. Of special interest are studies on the excretion of ouabain, a model compound for neutral nonmetabolized substrates such as estradiol, progesterone, and cortisol, which was depressed in a dose-related manner by a single oral dose of TCDD in rats (Yang et al., 1977, 1983b). The available data suggest that the hepatic membrane transport of ouabain may be selectively impaired by TCDD. Peterson et al. (1979a,b) have indicated that changes in ATPase activities are not responsible for reduced ouabain excretion.

TCDD administration stimulates the accumulation of porphyrins in the liver and an increase in urinary porphyrin excretion (Goldstein et al., 1973, 1976, 1982). Indeed, during manifest porphyria, accumulation of porphyrins occurs not only in the liver but also in the kidney and spleen of rats (Goldstein et al., 1982).

Contradictory results on species variations have been published. It seems clear that porphyria can be produced in both mice and rats, but the condition is always the result of subchronic or chronic administration. Exposure to single doses has not been demonstrated to produce porphyria. The mechanism underlying the induction of porphyria has not been elucidated. Cantoni et al. (1981) exposed rats orally to 0.01, 0.1, and 1 µg TCDD/kg bw/week for 45 weeks. Increased coproporphyrin levels were observed at all dose levels. A marked porphyrinemic state appeared only at the highest dose tested, after 8 months of exposure.

Induction of aminolevulinic acid (ALA)-synthetase, the initial and rate-limiting enzyme involved in heme synthesis, does not seem to be a necessary event in TCDD-induced porphyria. Despite porphyria being evident, mice exposed to 25 µg TCDD/kg bw/week for 11 weeks were not found to have any increased ALA activity (Jones and Sweeney, 1980). A more likely suggestion is that decreased hepatic porphyrinogen decarboxylase is the primary event in porphyria induced by halogenated aromatics (Elder et al., 1976, 1978). TCDD depresses this enzyme activity in vivo in the liver of mice (Cantoni et al., 1984a,b; Elder and Sheppard, 1982; Jones and Sweeney, 1980), but not in vitro (Cantoni et al., 1984b). Of interest, too, are the results reported in van Birgelen et al. (1996a), where the porphyrinogenic effects of TCDD were correlated with CYP1A2 induction, and demonstrated a strong synergistic relationship with coadministered PCBs.
A comparative study of TCDD-induced porphyria has not been conducted in responsive and nonresponsive mice. In a study on Ah-responsive (Ah\(^b\)) and Ah-nonresponsive (Ah\(^d\)) C57BL/6J female mice, however, the urinary excretion of porphyrins was examined after treatment of the animals with hexachlorobenzene for 17 weeks (Hahn et al., 1988). After 15 weeks of treatment with 200 ppm hexachlorobenzene in the diet, the excretion of porphyrins was 200 times higher in the Ah\(^b\) mice than the controls. In contrast, the Ah\(^d\) mice only showed a sixfold increase. Induction of P-450c(1A1) was observed only in Ah\(^b\) mice, while induction of P-450d(1A2) was observed in both strains, but to a lesser degree in the Ah\(^d\) mice.

**Epidermal Effects**

Chloracne and associated dermatological changes are common responses to high exposures to TCDD in humans. However, this type of toxicity is expressed only in a limited number of animal species (e.g., rabbits, monkeys, cows, and hairless mice).

In a rabbit ear bioassay, a total dose of 80 ng TCDD gave a chloracnegenic response, while no response was obtained when the total dose applied to the ear was 8 ng (Jones and Krizek, 1962; Schwetz et al., 1973). The application of TCDD in various vehicles has been demonstrated to markedly decrease this response (Poiger and Schlatter, 1980). The hairless mouse is a less sensitive model for chloracnegenic response than the rabbit ear bioassay (Knutson and Poland, 1982; Puhvel et al., 1982). Following repeated applications of -0.1 µg TCDD over several weeks, however, an acnegenic response was noted in the hairless mouse strains, SkH:HR1 and HRS/J. An acnegenic response was also caused by repeated applications of 2 mg of 3,4,3\(\mathrm{N},4\mathrm{\textit{N}}\)-TCB (Puhvel et al., 1982). Female HRS/J hairless mice have also been used to test the dermal toxicity and skin tumor-promoting activity of 2,3,7,8-TCDD, 2,3,4,7,8-PeCDF, and 1,2,3,4,7,8-HxCDF (Hebert et al., 1990b). All of the tested compounds induced coarse, thickened skin with occasional desquamation. These effects were more severe after the application of PeCDF and HxCDF.

Keratinocytes, the principal cell type in the epidermis, have been utilized as an in vitro model for studies of TCDD-induced hyperkeratosis both in human- and animal-derived cell cultures. The response to TCDD is analogous to the hyperkeratinization observed in vivo.

A TCDD-induced keratinization response in vitro was first demonstrated in a keratinocyte cell line derived from a mouse teratoma (XB cells). The keratinization was doserelated (Knutson and Poland, 1980b). Late-passage XB cells (termed XBF cells) lost their ability to respond by keratinization after TCDD treatment. Both XB cells (keratinization assay) and XBF cells (flat-cell assay) have proven to be useful in in vitro bioassays to determine the dioxin-like activities of both environmental samples and pure isomers (Gierthy and Crane, 1985a,b; Gierthy et al., 1984).
Several continuous lines of human keratinocytes, derived from neonatal foreskin or squamous cell carcinomas, have been shown to respond to TCDD in nM concentrations, with a variety of signs indicating alterations in the normal differentiation process (WHO/IPCS, 1989). The responses include decreased DNA synthesis, decreased number of proliferating basal cells, decreased binding of EGF, and an increase in the state of differentiation (Osborne and Greenlee, 1985; Hudson et al., 1986). The responses were also obtained with TCDF, but not with 2,4-diCDD (Osborne and Greenlee, 1985). TCDD has also been shown to inhibit high-density growth arrest in human squamous carcinoma cell lines. Indeed, the minimum concentration for increases in cell proliferation was 0.1 nM in the most sensitive cell line (SCC-15G). This effect is not due to modulation of the transforming growth factor-$\beta$ binding (Hebert et al., 1990b,c).

**Enzyme Induction**

TCDD has repeatedly been found to increase the activities of various enzymes. While observations of enzyme inhibition have also been made, enzyme induction has been one of the most extensively studied biochemical responses produced by TCDD. The mixed-function oxidase (MFO) system is the most thoroughly investigated, and AHH and EROD (as markers for CYP1A1 induction) are the most frequently assayed enzyme activities. The induction of MFO activities might potentiate the toxicity of other foreign compounds that require metabolic transformation by the MFO system before they can exert their toxic effects. Furthermore, increased MFO activities might adversely affect important metabolic conversions of endogenous compounds. TCDD also affects a variety of other enzymes (e.g., uridine diphosphate-glucuronosyltransferase [UDPGT] and glutathione-s-transferase [GST]) that are components of multifunctional enzyme systems involved in the conjugation, biotransformation, and detoxification of a wide variety of endogenous and exogenous compounds.

Several investigators have studied the relative potency of various halogenated dioxins, dibenzofurans, and biphenyls to induce AHH or EROD activities (Safe, 1990). An apparent structure-activity relationship was found between the location of the halogen atoms on the dibenzo-p-dioxin molecule and the ability to induce AHH activity both in vivo and in vitro. Isomers with halogens at the four lateral ring positions produced a greater biological response than those with halogens at three lateral ring positions. Two lateral halogen atoms seemed to be insufficient to produce a biological response. Numerous studies have indicated that there is very strong agreement between the Ah-binding affinity of various CDDs, CDFs, and related PCBs and their potency to induce AHH, both in vivo and in vitro (Safe, 1990). Structure-activity studies have also demonstrated a clear correlation between the toxicity and induction potency of a series of CDDs, CDFs, and coplanar PCBs (Poland and Glover, 1973; Safe, 1990). This is discussed in Chapter 2 of this report.
On a molecular basis, TCDD is the most potent MFO-inducing compound known, and MFO induction seems to be the most sensitive biochemical response produced. Measurements of the induction of AHH or EROD (mediated through CYPlA1) are considered to be very sensitive markers of TCDD-induced enzyme induction. According to Kitchin and Woods (1979), induction in the rat takes place at doses as low as 0.002 µg TCDD/kg bw. The NOAEL for a single administration to rats seems to be 1 ng/kg, while a single dose of 3 ng/kg causes a detectable induction of AHH or EROD (Kitchin and Woods, 1979; Abraham et al., 1988). For more detailed dose-response information, see Chapter 8 of this report. Enzyme induction has also been observed in the offspring of various species after prenatal and postnatal (milk) exposure to TCDD (Lucier et al., 1975; Korte et al., 1990; Wærn et al., 1991b).

The effect of TCDD on enzyme activities has been most frequently investigated in the rat (WHO/IPCS, 1989). TCDD has been shown to increase both the contents of cytochrome P-450Ia1 and cytochrome P-450Ia2 in the liver, as well as other microsomal enzyme activities involved in the oxidative transformation and conjugation of xenobiotics (e.g., aniline hydroxylase, AHH, biphenyl hydroxylase, 7-ethoxycoumarin-O-deethylase [ECOD], EROD, and UDPGT) (U.S. EPA, 1984, 1985; WHO/IPCS, 1989).

TCDD also affects some other hepatic enzymes not related to the MFO system, including aldehyde dehydrogenase, *-ALA synthetase DT-diaphorase, transglutaminase, ornithine decarboxylase, transaminases (L-alanine aminotransferase [ALT] and L-aspartate aminotransferase [AST]), plasma membrane ATPases, porphyrinogen carboxylase, prostaglandin synthetase, enzymes involved in testosterone metabolism, and RNA polymerase (U.S. EPA, 1984, 1985; WHO/IPCS, 1989).

Studies of different species have also revealed that enzyme induction due to TCDD exposure varies with both species and strain. Pohjanvirta et al. (1988) studied enzyme induction in the L-E and H/W (Kuopio) rat strains (LD₅₀ -10 and >3,000 µg/kg, respectively). Differences in the inducibility of EROD, ECOD, or ethylmorphine N-demethylase were not found, nor were there any differences with regard to the amount of available Ah receptor or the amount of cytochrome P-450 in the hepatic microsomal fractions. Similarly, differences regarding possible induction of UDPGT were absent (Pohjanvirta et al., 1990).

Enzyme induction studies on mice have been performed mainly with strains that are genetically different at the Ah locus, thus making them responsive or nonresponsive to the induction of hepatic cytochrome P-450Ia1-related enzyme activities. Qualitatively, and in general, the same responses can be obtained in both strains, but there may be more than one order of magnitude difference with regard to the doses required to elicit a response. TCDD is thus 10-fold more potent in inducing hepatic cytochrome P-450Ia1 and the related AHH activity in C57BL/6J mice (Ah-responsive) than in DBA/2 mice (Ah-nonresponsive) (Poland and
Knutson, 1982; Nebert, 1989) and C57BL/6L mice congenic at the Ah locus (Birnbaum et al., 1990).

Although the guinea pig is the most sensitive species to the toxic effects of TCDD, it does not respond to the administration of TCDD with liver toxicity or extensive enzyme induction. Indeed, even at lethal doses, the induction of MFO, as measured by AHH activity, is only very slight (Beatty and Neal, 1977; Håkansson et al., 1994). The data on enzyme induction in rabbits are rather limited and somewhat conflicting with regard to increases in the amount of cytochrome P-450 (Hook et al., 1975; Liem et al., 1980). Similarly, hepatic enzyme induction has been only partially studied in Syrian Golden hamsters. When hamsters were given a lethal dose of TCDD, increased hepatic GST and glutathione reductase activities were found. The ED$_{50}$ values for the induction of hepatic ECOD and reduced NADP:menadione oxidoreductase activities and cytochrome P-450 content in male Syrian Golden hamsters were 1.0, 2.0, and 0.5 µg TCDD/kg bw, respectively (extremely low doses, compared with doses that produce tissue damage and lethality in this species) (Gasiewicz et al., 1986).

In a comparative study of EROD induction in guinea pigs, rats, C57BL/6 and DBA/2 mice, and Syrian Golden hamsters, the animals were given single doses that were intended to be equitoxic (i.e., 1, 40, 100, 400, and 400 µg TCDD/kg, respectively) compared with the acute toxicity for the respective species and strain. EROD induction was noted in all species except for the hamster. During the observation period (112 days), the EROD induction dropped to more or less normal values in all rats and mice, while the induction (albeit low compared with the other species) was sustained for the whole period in the guinea pig (Håkansson et al., 1994). This might be due to higher half-life of TCDD in guinea pigs than rats or mice.

The N-demethylation of caffeine has been applied as a noninvasive method for studying enzyme induction in vivo. Studies on the marmoset monkey (Callithrix jacchus), utilizing $^{14}$C-labeled caffeine and measuring $^{14}$CO$_2$ exhalation by a breath test, has indicated a NOAEL of 1 ng/kg and a LOEL of 3 ng/kg (Kruger et al., 1990). Studies by Butler et al. (1989) and others indicate that this reaction is dependent on cytochrome P-450IA2.

In the chick embryo, both AHH and $^*$-ALA synthetase have been reported to be extremely sensitive to the inductive effects of TCDD and related compounds (Poland and Glover, 1973; Brunström and Andersson, 1988; Brunström, 1990).

Although TCDD is relatively nontoxic in cell cultures, it is a very potent inducer of AHH or EROD activities in in vitro systems, including lymphocytes and primary hepatocytes, as well as established and transformed cell lines.

The ED$_{50}$ values for AHH induction by TCDD have been determined in 11 established cell lines and in fetal primary cultures from 5 animal species and cultured human lymphocytes. The values ranged from 0.04 ng/mL medium in C57BL/6 mouse fetal cultures and 0.08 ng/mL in
the rat hepatoma H-4-II-E cell line to >66 ng/mL in the HTC rat hepatoma cell line (Niwa et al., 1975). Several cultured human cells or cell lines have been shown to be inducible for AHH activity by TCDD including lymphocytes (Atlas et al., 1976), squamous cell carcinoma lines (Hudson et al., 1983; Hebert et al., 1990a), breast carcinoma cell lines (Jaiswal et al., 1985), and lymphoblastoid cells (Nagayama et al., 1985).

TCDD was demonstrated to be the most potent AHH inducer of 24 chlorinated dibenzo-p-dioxin analogues (Bradlaw et al., 1980) in a rat hepatoma cell culture (H-4-II-E) that is extremely sensitive to AHH induction. The EC$_{50}$ values for AHH and EROD induction in the same cell system varied over 7 orders of magnitude for 14 different CDDs, the most potent being TCDD and the least potent being 2,3,6-triCDD (Mason et al., 1986). Additional details on these and other enzyme induction dose-response characteristics and modeling are included in Chapter 8 of this report.

**Appraisal**

Based on data from Kitchin and Woods (1979), Abraham et al. (1988), Kruger et al. (1990), and Neubert (1991), a NOAEL value of 1 ng/kg bw can be calculated for enzyme induction for both rats and marmoset monkeys. At this dose, the tissue concentrations for both species were found to be 4 ppt for adipose tissue and 3 ppt for the liver. It is interesting to note that the wide range of sensitivities toward the acute toxicity of TCDD is also reflected in the wide range of sensitivities for enzyme induction both in vivo and in vitro, although the two groups of effects are not necessarily parallel. Finally, it is evident that the structure-activity relationships revealed from in vitro testing correlate fairly well with in vivo studies within a given species or strain.

**Endocrine Effects**

In many respects, TCDD toxicity mimics endocrine imbalance. Alterations in endocrine regulation have been suggested from human exposure to TCDD that resulted in hirsutism and chloracne. Chronic exposure to TCDD causes impaired reproduction in experimental animals, possibly by interfering with the estrus cycle in combination with some steroid-like activities of TCDD. This has prompted studies on the interaction of TCDD with steroid hormones and their receptors.

Evidence has been provided suggesting that chronic or subchronic exposures to TCDD impair thyroid functions. Dose-dependent reductions of plasma thyroid hormone levels have been observed in TCDD- and PCB-exposed animals (van der Kolk et al., 1992; van Birgelen et al., 1995a,b).
In a subchronic 13-week TCDD feeding study with female Sprague-Dawley (S-D) rats, a decrease in thyroid hormone (T₄) levels occurred, associated with elevation of microsomal UDPGT activity when thyroxine was used as substrate for thyroxine glucuronosyltransferase (T₄:UGT) (van Birgelen et al., 1995b). In addition, involvement of CYP1A1 and UGT1A1 by TCDD indicates that the TCDD-induced thyroid functional abnormalities are mediated though the AhR (van Birgelen et al., 1995b). 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156) has also been shown to reduce plasma total T₄ levels, and induces UDPGT by using thyroxine as substrate for T₄:UGT (van Birgelen, 1995a). Similar results have also been observed in a 30-week chronic study with female S-D rats suggesting that TCDD-induced thyroid hormone function is caused by chronic perturbation of the liver-pituitary-thyroid axis (Sewell et al., 1995).

van Birgelen et al. (1995a,b) demonstrated the effects of TCDD and coplanar PCB126 (3,3,4,4',5-PCB) on thyroid hormone metabolism in female SD-rats. Oral exposure to 0.2, 0.4, 0.7, 5, and 20 µg/kg diet of TCDD and 7 to 180 µg/kg diet of PCB 126 significantly decreased the plasma total thyroxine (TT₄) levels. An intake of 0.047 µg/kg/day was estimated to be the LOAEL for decrease in plasma thyroid hormone levels.

A dose-dependent decrease in serum T₄ levels has also been observed in male and female SD rats as a result of high-dose subchronic exposures to 1,2,3,7,8-pentaCDD (PeCDD) or 1,2,3,4,7,8-hexaCDD (HxCDD) and low-dose subchronic exposures to TCDD/kg. Serum T₄ levels in PeCDD or HxCDD-exposed males returned to close to normal levels by the end of the off-dose period (Viluksela et al., 1998).

van Birgelen et al. (1995b), in a 13-week TCDD feeding study using 7-week-old female S-D rats, found that the LOAEL for decrease in plasma T₄ was 47 ng/kg bw/day. Dose-response relationships for CYP1A1 and CYP1A2 were determined by nonlinear curve fitting. The critical values for the 95% confidence limits for CYP1A1 and CYP1A2 inductions ranged from 0.7 and 4 ngTCDD/kg bw/day.

Janz and Bellward (1997) reported that a single intraperitoneal dose of 20 µg/kg bw of TCDD to adult great blue heron, Ardea heidias, increased plasma T₄ levels (control: 39 ± 4 ng/mL; exposed: 55 ± 5 ng/mL; p<0.05), but no effect occurred on plasma total T₃ levels or on the plasma T₃ to T₄ ratio.

Increased systemic levels of glucocorticoids may mimic some of the symptoms of TCDD toxicity (e.g., involution of lymphoid tissues, edema, and mobilization of fatty acids from adipose tissues). Thus, it has been suggested that TCDD increases glucocorticoid activity through indirect effects on glucocorticoid receptors. Poland et al. (1976) demonstrated that cortisol and synthetic glucocorticoids do not bind to the TCDD receptor.

Conflicting data have been reported on TCDD-induced levels of glucocorticoids. However, significant changes to the liver cytosolic glucocorticoid receptor were induced by
TCDD at doses 10,000-fold lower in adrenalectomized SD rats than in control rats (Sunahara et al., 1989). The data further indicate that the binding capacity of hepatic glucocorticoid receptor was altered, but not the apparent equilibrium dissociation constant (Kd). Studies in congenic strains of Ah-responsive and Ah-nonresponsive C57BL/6J female mice (Goldstein et al., 1990; Lin et al., 1991a,b) have also demonstrated that TCDD decreased the maximum binding capacity of the hepatic glucocorticoid receptor in both strains of mice by -30%.

Steroids are endogenous substrates for the hepatic MFO system. TCDD influences the activity of this enzyme system and may alter steroid metabolism in vivo and the magnitude of steroid-mediated functions.

Early studies reported contradictory data on changes in steroid levels. Umbreit and Gallo (1988) suggest that estrogen receptor modulation, and the animal's physiological response to this modulation, can explain some of the toxicity observed in TCDD-treated animals. The susceptibility of different species to TCDD correlates, to some extent, with their steroid glucuronidation capacity. For example, hamsters have low steroid UDPGT activity while guinea pigs have a corresponding high activity. Another example is given by comparing the SD and Gunn rat, the latter being defective in producing some UDPGTs. The homozygous Gunn rat is 3-10 times more resistant to the effects of TCDD than is the SD rat (Thunberg, 1984; Thunberg and Håkansson, 1983). The results of TCDD exposure in various species and strains are complex. The ability of the strain to counteract TCDD-induced modulation of the estrogen receptor depends on its ability to synthesize and excrete estrogens. Interactions of TCDD and related compounds with estrogen have been reviewed by Safe et al. (1991).

The importance of estrogens as modulators of TCDD-induced toxicity has also been demonstrated by Lucier et al. (1991), who found that the tumor-promoting effects of TCDD could be effectively prevented by removing the ovaries from female rats before exposure to TCDD. This finding agrees with the results obtained from long-term bioassays that demonstrated liver tumors only in female rats (Kociba et al., 1978; NTP, 1982).

Studies on congenic strains of Ah-responsive and Ah-nonresponsive C57BL/6J female mice found a statistically significant difference in the responsiveness of the hepatic estrogen receptor. This indicates that the Ah receptor regulates the effects of TCDD on the hepatic estrogen receptor (Goldstein et al., 1990; Lin et al., 1991a, b).

TCDD-induced changes in levels or activities of testosterone or its metabolites have been reported from several studies (Keys et al., 1985; Mittler et al., 1984; Moore and Peterson, 1985; Neal et al., 1979). A single oral dose of 50 µg TCDD/kg bw increased the plasma corticosterone level in SD rats 7 and 14 days postexposure (Neal et al., 1979). It has also been shown, however, that a single oral dose of 25 µg TCDD/kg bw decreases the plasma corticosterone in
SD rats 14 and 21 days postexposure. It is important to note that Neal et al. (1979) also observed a slight decrease in serum corticosterone during days 1-4 posttreatment.

Mittler et al. (1984) demonstrated a decreased activity of testicular 16\'-testosterone hydroxylase, 6\'-hydroxytestosterone, and 7\'-hydroxytestosterone in young SD rats 90 hours postexposure to single intraperitoneal doses of 0.2, 1, or 5 µg TCDD/kg bw.

A single dose of 0.06 µmol TCDD/kg bw decreased levels of 3\'-, 6\'-, and 16β-hydroxytestosterone and an increase of 7\'-hydroxytestosterone has also been observed in young male Wistar rats (Keys et al., 1985). Moore et al. (1985) noted decreases in serum testosterone and dihydrotestosterone levels in 15 µg TCDD/kg bw-dosed male SD rats. The data do not, however, allow for any conclusions with regard to the possible relationship to receptor-mediated toxicity. TCDD induces several enzymes related to testosterone metabolism, which suggests that the changes observed may be secondary to the induction of various enzymes. Serum testosterone and dihydrotestosterone were found to be dose-dependently depressed by TCDD treatment in male SD rats, when compared with pair-fed and ad libitum-fed controls. The ED₅₀ for this effect was -15 µg/kg (Moore et al., 1985). It was further shown that testosterone synthesis was decreased in the animals due to depressed production of pregnenolone by the testis (Kleeman et al., 1990). In the same strain of rats, a single 100 µg/kg oral dose of TCDD was found to cause a 55 percent decrease in testicular cytochrome P-450sc activity and to inhibit the mobilization of cholesterol to cytochrome P-450sc. The authors concluded that the latter effect probably was responsible for the inhibition of testicular steroidogenesis (Moore et al., 1991). Maternal exposure to TCDD has been shown to affect the male reproductive system at low doses; the lowest dose tested was 64 ng/kg (Mably et al., 1991, 1992a,b,c). This is discussed in Chapter 5.

In ovo exposure of white Leghorn chickens to TCDD, in the dose range of 1-10,000 pmol/egg, increased the cardiac release of prostaglandins (Quilley and Rifkind, 1986). Studies on chick embryos have indicated that the induction of cytochrome P-450 by TCDD results in a major increase in the NADPH-dependent metabolism of arachidonic acid (Rifkind et al., 1990). These effects are clearly related to receptor-mediated enzyme induction.

**Vitamin A Storage**

Decreased hepatic vitamin A storage has been reported in animals exposed to various chlorinated aromatic compounds. Because only minute quantities are needed to produce ill effects, and because of its persistence in nature, TCDD is unique in its capacity to reduce the vitamin A content of the liver. A single oral dose of 10 µg TCDD/kg bw decreased both the total amount and the concentration of vitamin A in the liver of adult male SD rats (Thunberg et al., 1979). The decrease was evident 4 days after dosing and progressed with time. After 8 weeks, the treated animals had a total liver vitamin A content corresponding to 33% of that of controls.
Decreased dietary intake of vitamin A could not account for this difference. A significant increase in the UDPGT activity was observed, suggesting an increased excretion of glucuronide-conjugated vitamin A. No correlation between the UDPGT activity and the hepatic vitamin A reduction was seen, however, when homozygous Gunn rats lacking inducible UDPGT (Aitio et al., 1979) and heterozygous Gunn rats with inducible UDPGT were treated with a single oral dose of 10 µg TCDD/kg bw (Thunberg and Håkansson, 1983).

A study combining pair-feed restriction and a single TCDD treatment found that decreases in liver reserves of vitamin A were not related to a decreased intake of vitamin A via the diet (Håkansson et al., 1989b).

Puhvel et al. (1991) reported a comparative study in which congenic haired (+/+ ) and hairless (hr/hr) HRS/J mice were fed a vitamin A-deficient diet and treated topically with TCDD. The sensitivity to TCDD-induced cutaneous changes was essentially 100 times higher in hairless mice than in haired mice (0.01 and 1.0 µg 3 times/week for 3 and 2 weeks, respectively). In the haired phenotype, the effects of vitamin A depletion by itself were not seen by cutaneous histology, nor were any changes observed in cutaneous morphology attributable to TCDD. In the hairless mice, however, vitamin A deficiency increased the keratinization of dermal epithelial cysts and increased the sensitivity of these cysts to TCDD-induced keratinization. Analysis of vitamin A demonstrated that TCDD exposure did not affect cutaneous levels of the vitamin but did significantly lower levels of vitamin A in the liver. TCDD-induced body weight loss and atrophy of the thymus glands were not affected by the vitamin A status in either strain.

In a study on tumor promotion by TCDD, in which enzyme-altered hepatic foci were induced in the livers of female SD rats, Flodström et al. (1991) found that vitamin A deficiency by itself enhanced foci development. The effects of TCDD treatment were also markedly enhanced, including TCDD-induced thymus atrophy.

Several studies have been performed to elucidate the mechanism of TCDD-vitamin A interaction. Håkansson et al. (1989c) and Håkansson and Hanberg (1989) have demonstrated that TCDD specifically inhibits the storage of vitamin A in liver stellate cells. Brouwer et al. (1989) demonstrated that a single dose of TCDD (10 µg/kg) to female SD rats reduced vitamin A in the liver, lungs, intestines, and adrenal glands, while increasing its concentration in serum, kidneys, and urine. They also found a 150% increase in the free fraction of serum retinol binding protein. Taken together, all of these data in the rat indicate that TCDD induces an increased mobilization of vitamin A from hepatic and extrahepatic storage sites into the serum, accompanied by an enhanced elimination of the vitamin via the kidney into the urine.

In a comparative study of TCDD toxicity in male SD rats and Hartley guinea pigs (Håkansson et al., 1989a), the animals were given single intraperitoneal doses of 40 and 0.5 µg/kg bw, respectively (i.e., comparable fractions of their respective LD₅₀). Similar
reductions in hepatic vitamin A were observed for both species, while serum and renal vitamin A concentrations were increased in the rat but unaffected in the guinea pig. Hepatic EROD activity was markedly increased in the rat but unchanged in the guinea pig. Furthermore, rats seemed to recover from the wasting, thymic atrophy, and liver enlargement and resumed their ability to store vitamin A in the liver at 4-8 weeks after exposure. No such trends for wasting and vitamin A storage were observed in guinea pigs, even 16 weeks after exposure. A complementary study also included C57BL/6 mice, DBA/2 mice, and Syrian Golden hamsters (Håkansson et al., 1991). The effects on TCDD-induced decrease of vitamin A in the liver and lung correlated reasonably well with other toxic symptoms observed in the animals. On the other hand, studies of two strains of rats, L-E and H/W (the H/W being >300 times more resistant to TCDD toxicity), could not demonstrate significant differences in the TCDD-induced changes in vitamin A in the liver, kidney, testicles, or serum after a sublethal dose of 4 µg/kg (Pohjanvirta et al., 1990). These findings show that the correlation between TCDD-induced lethality and changes in vitamin A status found among other species also apply to these strains of rats.

The interaction of 3,4,3′,4′-TCB with vitamin A has been studied by Brouwer and van den Berg (1983, 1984, 1986), Brouwer et al. (1985, 1986), and Brouwer (1987). The effects of TCB on vitamin A differ in many respects from those of TCDD. TCB is rapidly converted in vivo into a polar 5-OH-TCB metabolite, which binds with a relatively high affinity to transthyretin (TTR). As a consequence of this interaction, the physiological functions of TTR in retinoid and thyroid hormone transport are severely affected in TCB-exposed animals. The model proposed by Brouwer (1987) may explain some of the characteristic toxicological lesions related to exposure to this PCB. This mechanism of action seems to be clearly separated from the Ah receptor-mediated toxicity of CDDs and CDFs. Hydroxylated metabolites of TCDD have also been demonstrated to bind in a similar manner to TTR (Lans et al., 1993). Due to the very slow metabolism of TCDD (or other 2,3,7,8-substituted CDDs/CDFs), however, this mechanism probably plays a very minor role in toxicity.

Taken together, these data indicate that TCDD interferes with the metabolism and storage mechanisms for vitamin A (Kelley et al., 1998). Because supplementation of dietary vitamin A seems unable to counteract all of the observed toxic effects, this would imply either that the effect on vitamin A storage is secondary to TCDD toxicity or that the cellular utilization of vitamin A is affected by TCDD.

**Lipid Peroxidation**

Lipid peroxidation and oxidative stress have been indicated as factors that affect the acute toxicity of TCDD (WHO/IPCS, 1989; Wahba et al., 1989a,b, 1990a,b; Pohjanvirta et al., 1989; Alsharif et al., 1990; Stohs et al., 1990). Among the effects noted have been membrane
lipid peroxidation, decreased membrane fluidity, and increased incidence of single-strand breaks in DNA. No studies relating these observations to the Ah receptor have been performed. When considering the available data on TCDD and lipid peroxidation, it is not possible to define a relationship between lipid peroxidation and TCDD-induced lethality. However, oxidative stress is observed only at high doses of TCDD following acute exposure. Acute TCDD exposure at high doses has been shown to produce reactive oxygen species (Alsharif et al., 1994 a,b), lipid peroxidation (Alsharif et al., 1994b), and decreased membrane fluidity (Alsharif et al., 1990) in the mouse and rat.

Oxidative stress has been proposed as one of the reasons for increased susceptibility of female mice to TCDD-induced toxicity. In female C57BL/6J mice, intraperitoneal exposure to 5 µg/kg of TCDD for 3 consecutive days results in a long-term increase in hepatic oxidized glutathione and 8-hydroxydeoxyguanosine levels. Levels of 8-hydroxydeoxyguanosine, a product of DNA base oxidation and subsequent excision repair, remain elevated about 20-fold 8 weeks after treatment. This suggests a sustained TCDD-induced oxidative stress resulting in potentially promutagenic DNA base damage (Shertzer et al., 1998). Induction of CYP1A1 by TCDD has also been suggested to cause an increased excretion rate of 8-oxoguanine, a biomarker of oxidative DNA damage (Park et al., 1996).

Oxidative brain tissue damage may play a role in TCDD-induced central nervous system abnormalities. Hassoun et al. (1998) reported that subchronic oral exposure of B6C3F1 mice to TCDD for 13 weeks can result in a dose-dependent increase in superoxide anions (indicated by reduction in cytochrome c), lipid production, and DNA single-strand breaks in brain tissues. The authors posited involvement of the cytochrome P-450 system in TCDD-induced oxidative stress. Slezak et al. (1999), using CYP1A2 knockout (CYP1A2−/−) mice, demonstrated that TCDD-induced oxidative stress (indicated by production of thiobarbituric acid-reactive substances as a measure of lipid peroxidation, production of reactive oxygen species via in vitro reduction of CYC, and changes in glutathione) is not mediated through the cytochrome P-450 type 1A2 isozyme (CYP1A2). Hassoun et al. (1997) also posited that TCDD-induced fetal death and fetal and placental weight reductions in C57BL/6J mice may be caused by oxidative damage induced by TCDD. Ellagic acid at 6 mg/kg/day on days 10, 11, and 12 of gestation and 3 mg/kg on day 13 protected against TCDD administration on day 12 at 30 µg/kg bw. Vitamin E succinate administered at 100 mg/kg/day through gestation days 10, 11, and 12 and at 40 mg/kg on day 13, instead of ellagic acid, was a less effective protective agent.

Iron administered before TCDD administration (75 µg/kg bw) to AhR-responsive AhRb-1 C57BL/6J mice potentiated hepatic porphyria, hepatocellular damage, and plasma hepatic enzyme markers (Smith et al., 1998). The mechanism was oxidative because hydroxylated and peroxyxylated derivatives of the uroporphyrins formed from uroporphyrinogen,
and μ-glutathione transferase were also induced. Iron overcame the weak porphyria and toxicity responses of TCDD in AhRb-2 BALB/c and AhRd SWR mice, but not in DBA/2 mice, which remained TCDD resistant. Thus, metabolic factors may play a part in the responses of some mice strains to TCDD through an oxidative process that disturbs iron regulatory protein capacity.

Increased accumulation of lipofuscin pigments, which are by-products of lipid peroxidation, in heart muscles of TCDD-exposed rats (Albro et al., 1978) and iron deficiency in animals resulting in in vitro inhibition of lipid peroxidation and reduced TCDD-induced hepatotoxicity (Sweeney et al., 1979) suggested that oxidative stress may play a role in TCDD-induced acute toxicity. Subsequently, Stohs et al. (1983) demonstrated that lipid peroxidation is increased in isolated liver microsomes from TCDD-exposed rats. Further observations suggest a possible role of reactive oxygen species in TCDD acute toxicity. Alsharif et al. (1994a,b) observed that a maximum increase in superoxide anion production occurs on day 1 of posttreatment in female SD rats treated with 50 and 125 µg/kg bw of TCDD, and that TCDD-induced oxidative stress is mediated through the Ah receptor in mice. TCDD-induced superoxide anion production by peritoneal lavage primary macrophages and its mediation through the Ah receptor suggests involvement of reactive oxygen species in a broad spectrum of TCDD-induced toxicity. Bagchi et al. (1993) found that products from altered lipid peroxidation and increased oxidative stress result in elevated serum and urinary levels of certain lipid metabolic products, such as malondialdehyde, formaldehyde, acetaldehyde, and acetone, following a single oral exposure to 50 µg/kg bw of TCDD in female SD rats. Vos et al. (1978) suggested that endotoxin shock may be the cause of TCDD-induced lethality, and that the tumor necrosis factor-" (TNF-"") may be a contributing factor, even though it has not been detected in the serum of TCDD-treated mice without exposure to endotoxin. Alsharif et al. (1994c) demonstrated that anti-TNF-" antibody can decrease phagocytic cell activity following TCDD treatment. This suggests that TNF-" release, a possible activator of TCDD-induced oxidative stress, may have some role in TCDD-induced activation of phagocytic cells.

**Neurotoxicity**

Exposure to dioxin-like coplanar PCBs may result in neurotoxicity. Eriksson et al. (1991) suggested that dioxin-like coplanar PCBs have neurologic activities that affect the cholinergic receptors in the hippocampus. Seegal (1996) provided evidence that perinatal exposure to coplanar 3,3',4,4'-PCB (PCB 77) results in significant elevation of dopamine in the frontal cortex. Dopamine is an important neurotransmitter, dependent on tyrosine, that is associated with initiation and control of motor behavior, learning, and memory functions. The neurons and astroglia of rat hippocampal neural cells are responsive to relatively low levels of TCDD through mechanisms that are probably not associated with altered gene transcription and
that may involve other cellular targets (Hanneman et al., 1996). TCDD induces phosphorylation and other responses within minutes of treatment, probably through a nonnuclear role of the Ah receptor.

Eriksson and Fredriksson (1998) demonstrated that a single oral exposure of NMRI mice to either 0.51 or 51 mg/kg bw of 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) on postnatal day 10 can result in derangement of spontaneous motor behavior. In addition, permanent impairment of learning and working memory was revealed when these animals reached adulthood. This study suggests that exposure to PCB 169 during the neonatal period, at a time when there is incomplete development of the infant’s blood-brain barrier and during rapid brain development, can result in vulnerability of the brain to neurologic effects, which in many cases can only be manifested during adulthood. In utero and lactational exposure to TCDD (100 ng/kg/d) or coplanar PCBs resulted in reduction of errors on a radial arm maze working memory task in grown up S-D rats exposed through their mothers (Seo et al., 1999). The effect was more pronounced in males than females. There was no difference in performance of the Morris water maze task or the spatial discrimination-reversal learning task for exposed males and females or unexposed rats. Both adult male and female S-D rats exposed maternally to TCDD showed a deficit in learning a visual discrimination-reversal learning task, a finding also observed in monkeys.

Postnatal oral exposure of primates to PCBs can result in long-term behavioral dysfunctions. In monkeys, oral exposure from birth to 20 weeks of age to 7.5 µg/kg/day of a PCB mixture, representative of the PCB residues generally found in human breast milk samples, also results in significant impairment in discrimination-reversal learning activities (Rice, 1997).

**MECHANISMS OF TOXICITY**

The most reliable and consistent symptom of TCDD toxicity among all experimental animals is weight loss. The cause of the body weight loss seems to be reduced food intake, apparently occurring secondarily to a physiological adjustment that reduces the body weight to a maintenance level that is lower than normal. The physiological trigger for this body weight set-point might be a target for TCDD.

Delayed expression of TCDD-induced toxic responses, including lethality, suggests that these toxic responses may not be the result of a direct insult by the parent compound (Mukerjee, 1998; Rozman, 1999). Progressive hypoglycemia from feed refusal and reduced gluconeogenesis seems to be the ultimate cause of TCDD-induced lethality (Gorski et al., 1990). In the L-E strain rat, reduced gluconeogenesis, indicated by decreased PEPCK activity, has been suggested to contribute to the acute toxicity of TCDD (Fan and Rozman, 1994; Viluksela et al., 1999). One of the major causes of TCDD-induced lethality also is dose-dependent reduction of tryptophan 2,3-dioxygenase (TDO) activity (Fan and Rozman, 1994; Stall et al., 1993), indicating
that subtle differences in the regulation of intermediary metabolism may be responsible for strain differences in the susceptibility of rats to TCDD (Fan and Rozman, 1994).

There have been significant advances in understanding the cause of TCDD-induced voluntary feed refusal. The neurotransmitter 5-hydroxytryptamine (5-HT), or serotonin, controlled by the availability of the amino acid tryptophan (Carlsson and Lindquist, 1978), suppresses feed intake behavior (Leibowitz, 1993). TCDD increases the plasma level of free tryptophan in L-E rats but not H/W rats (Unkila et al., 1994a). Increase in brain tryptophan levels (Rozman et al., 1991; Unkila et al., 1994b) and 5-HT turnover are closely connected with changes in plasma tryptophan (Unkila et al., 1994b). In L-E and H/W rats, the potencies of dioxin congeners highly correlate with their ability to disrupt tryptophan homeostasis. The order of potency is: TCDD > 1,2,3,7,8-PeCDD > 1,2,3,4,7,8-HxCDD > 1,2,3,4,6,7,8-HpCD (Unkila et al., 1998). TCDD lethal dose exposure results in increased brain 5-HT synthesis in L-E rats (Unkila et al., 1993), whereas in resistant H/W rats no such increase of 5-HT occurs. The dose-related changes in plasma free tryptophan are closely associated with the severity of the wasting syndrome observed in L-E rats (Unkila et al., 1994b). Increased circulating tryptophan and rapid turnovers of tryptophan and 5-HT in the brain are associated with TCDD-induced reduced feed intake, wasting, and lethality (Rozman et al., 1991; Unkila et al., 1994b). However, tryptophan metabolism or carbohydrate homeostasis does not explain the wide interspecies differences in susceptibility to acute lethality encountered between guinea pigs (the most acutely susceptible species) and hamsters (the most resistant species) (Unkila et al., 1995).

Despite extensive research to elucidate the ultimate events underlying the toxic action of TCDD, definitive answers are not yet available. The toxicity of TCDD apparently depends on the fact that the four lateral positions of the molecule are occupied by chlorine, resulting in high-affinity binding to the AhR. Mechanisms of toxicity are discussed in detail in Chapter 2. TCDD toxicity involves many different types of symptoms, which vary from species to species and from tissue to tissue, both quantitatively and qualitatively. Age- and sex-related differences in sensitivity have also been reported. Another characteristic of TCDD toxicity is the delay before all the endpoints of toxicity are manifested (from 2 weeks to 2 months), which is seen in all species.

Polymorphism in the Ah locus, which has been shown to be the structural gene for the cytosolic receptor, seems to determine the sensitivity of genetically different strains of mice to TCDD and congeners. Ah-responsive strains of mice (e.g., C57BL/6) are characterized by high hepatic levels of a high-affinity TCDD-receptor protein, highly elevated levels of hepatic cytochrome P-4501A1 and associated enzyme activities in response to treatment with 3-MC (3-methylcholanthrene), and sensitivity to the ulcerative action of DMBA (7,12-
Based on these findings, several genetic studies have been performed to elucidate the role of the receptor in TCDD toxicity. In contrast to 3-MC, TCDD induces AHH activity and several toxic effects both in Ah-responsive and Ah-nonresponsive strains of mice. The dose required to produce the effect in an Ah-nonresponsive strain, however, is approximately 10-fold greater than that needed in an Ah-responsive strain. This indicates that the Ah-nonresponsive strain also contains the TCDD receptor, but the receptor is defective (Okey and Vella, 1982). Data from studies of DBA/2 mice given either single or multiple doses of TCDD (Jones and Sweeney, 1980; Smith et al., 1981) suggest that the LD$_{50}$ in this strain of mice is at least fivefold greater than the values recorded for the C57BL/6 and C57BL/10 strains (Jones and Greig, 1975; Smith et al., 1981; Vos et al., 1974). TCDD-induced hepatic porphyria has also been shown to segregate with the Ah locus in mice (Jones and Sweeney, 1980). The correlative differences between the C57Bl/6 and DBA/2 strains of mice, in terms of altered specific binding of TCDD and sensitivity to this compound, may not be applicable to other species (Gasiewicz and Rucci, 1984).

In a genetic-crossing experiment between L-E and H/W rats (Pohjanvirta, 1990), it was demonstrated that the F$_1$ offspring were as resistant to TCDD toxicity as the H/W rats (LD$_{50}$ >3,000 µg/kg). Further studies on the F$_2$ generation indicated that the distribution of resistant and susceptible phenotypes was consistent with inheritance regulated by 2 (possibly 3) autosomal genes displaying complete dominance, independent segregation, and an additive coeffect.

Despite enormous variability in the recorded LD$_{50}$ values for the guinea pig, rat, mouse, rabbit, and hamster, the amount and physical properties of the hepatic and extrahepatic receptors are comparable in these species (Gasiewicz and Rucci, 1984; Poland and Knutson, 1982). Furthermore, although the recorded LD$_{50}$ values for TCDD vary >100 times among the chick embryo, the C3H/HeN mouse, and the SD rat, the ED$_{50}$ doses for AHH induction in these species are comparable (Poland and Glover, 1974). Even between strains of rats with a difference of >300 times in LD$_{50}$, no differences in enzyme induction could be demonstrated (Pohjanvirta et al., 1988). In the guinea pig, the most TCDD-susceptible species, AHH induction is not a prominent symptom, even at lethal doses of TCDD. A number of cell types, including primary cultures and established and transformed cell lines from several species and tissues, are inducible for AHH activity, indicating the presence of the receptor. Yet toxicity is not expressed in these systems (Knutson and Poland, 1980a). The available data thus suggest that the receptor for TCDD may be a prerequisite but is not sufficient in itself for the mediation of toxicity.
Recent observations suggest that some of the TCDD-induced toxicity in mice require other modes of action, beyond AhR-mediated DNA transcription. For example, wasting syndrome, thymus involution, and loss of adipose tissue in c-src\(^{+/+}\) mice are correlated to c-src kinase activation, which is physically linked to AhR. These TCDD-induced toxic effects are not induced in src\(^{-/-}\) mice and are marginal in c-src\(^{+/+}\) mice (Matsumura et al., 1997a,b). These toxic effects can also be prevented in c-src\(^{+/+}\) mice pretreated with geldanamycin, a c-src kinase inhibitor (Enan et al., 1998a; Dunlap et al., 1999). Based on c-src deficiency not affecting TCDD induction of the cytochrome P-450 type 1A1 isozyme (CYP1A1), the gene activation pathway of TCDD’s action through the AhR nuclear translocator (ARNT) gene appears to be independent of the phosphorylation pathway of TCDD toxic activities modulated through the c-src gene. Involvement of c-src kinase activation in TCDD-induced toxicity has also been observed in the guinea pig. Enan et al. (1998b) showed that male guinea pigs pretreated with the src-kinase inhibitor geldanamycin did not suffer TCDD wasting. These investigators obtained similar results with src-deficient mice. Treatment with estradiol also protected male guinea pigs from TCDD-induced wasting. Furthermore, a nuclear AhR complex is not required for one of the signal transduction pathways associated with TCDD-induced early response of the c-fos and junB genes (Puga et al., 1992; Hoffer et al., 1996).

A strong correlation between lack of AhR affinity and lack of acute TCDD toxicity has been demonstrated in the knockout AhR\(^{-/-}\) mouse. No significant difference in short-term toxicity was observed between the vehicle control group and knockout homozygous AhR\(^{-/-}\) mice receiving TCDD at 2,000 \(\mu\)g/kg bw. Postexposure effects at day 28 were limited to vascularities of the lung and scattered necrosis of hepatocytes in AhR\(^{-/-}\)-resistant mice. In contrast, lipid accumulation and inflammatory cell infiltration of the liver were seen in heterozygous AhR\(^{+/+}\)-susceptible mice at the much lower dose of 200 \(\mu\)g/kg TCDD (Fernandez-Salguero et al., 1996). Although some of the TCDD-induced toxicity of the liver and thymus are mediated by the AhR, the mechanism for vascularities of the lung and the scattered necrosis of the lung and liver in AhR knockout mice may involve alternative pathways. As proposed by Matsumura et al. (1997a, b), these toxicity pathways still require the AhR and associated cytosolic proteins (Enan and Matsumura, 1996), but not nuclear AhR and DNA transcription.