CHAPTER I

INTRODUCTION

General Introduction on Isolated Hepatocytes

The isolation of hepatocytes in pure fraction are now being used in an increasing number of biochemical and pharmacological investigations, because of having several advantages as an in vitro model. They allow the study of specific cellular functions without the difficulties caused by the presence of other "unrelated" cells. It is possible to control the surrounding medium without intervening epithelial and vascular barrier. Repeating samples from a single batch of cells can be withdrawn during the course of experiments, so that both intra and extracellular biochemistry can be studied, simultaneously. The biological variability can be minimized by dividing cells prepared from a single animal and running paired control and test cultures.

A. Isolation of Hepatocytes

Before the developement of enzymatic methods for the isolation of hepatocyte preparations, nonenzymatic methods were used. Firstly, the mechanical methods which included homogenization (Palade and Claude, 1949; Harrison, 1953), and forcing of tissue through steel meshes (Schneider and Potter, 1943).
Secondly, chemical mechanical methods using chelators for removing of Ca^{2+} which play a role in cellular adhesion, prior to mechanical

treatment. The most commonly used chelators are citrate (Anderson, 1953) and EDTA (Coman, 1954). The chelation of K^+ by tetraphenylboron (TPB) has also been used (Rappapart and Howze, 1966). Finally, several enzymes, single or in combination, were tried in attempt to disperse liver slices. These enzymes include trypsin, papain, lysozyme, and pepsin (St. Aubin and Bucher, 1952 ; Hommes et al., 1970). All of the above methods resulted in a poor yield (0.4-2% hepatocytes) of relatively damage cells (Jeejeebhoy and Phillips, 1976).

A major developement was the introduction of collagenase and hyaluronidase for the dispersion of liver slices by Howard et al. (1967). This early technique resulted in a rather poor yield of intact hepatocytes (about 5% of the total). The enzymatic technique was subsequently improved by Berry and Friend (1969) who introduced a recirculating collagenase perfusion under physiological conditions ($37^{o}C$) and high yield of about 60% of the intact hepatocytes were obtained.

After the introduction of collagenase perfusion, there were the numerous modifications of this technique. Wagle and Ingebretsen (1975) simplified the procedure by using only collagenase as the digestive enzyme. Further, Seglen (1973 a,b) used a two-step procedure of perfusing the liver with Ca^{2+}-free medium, prior to the perfusion with collagenase in Ca^{2+}-contaning medium. These modifications decreased the perfusion time and increased the viable cell yields.

The successful preparation of intact liver cells by perfusion with collagenase is technically quite difficult, and still remains mostly an art. Almost every worker has incorporated his own modifications, but any successful method for obtaining well preserved hepatocytes in high yields must involve three critical steps, namely exposure of the tissue to a Ca^{2+}-free medium, digestion with collagenase, and gentle mechanical treatment (Wanson et al., 1979).

The numerous modifications introduced at various steps in the cell preparation procedure have been published. It is difficult to assess the value of these techniques, but the main features which required to yield the viable cells are considered below.

1. Preperfusion

Ca^{2+} has been found to play a role in cellular adhesion (Anderson, 1953). For the effective enzymatic dispersion, Ca^{2+} must first be removed from the tissue by a nonrecirculating preperfusion with Ca^{2+}-and enzyme-free buffer, or with Ca^{2+} chelators (Wagle and ingerbretsen, 1975; Moldeus et al., 1978). The uniform perfusion of liver as indicated by complete wash out of blood is probably the most important feature of the collagenase perfusion technique (Wagle and ingebretsen,1975; Jeejeebhoy and Phillips, 1976).

2. Collagenase Perfusion

For economic reasons, the collagenase perfusion after the preperfusion step is mostly performed in the recirculating condition using a fix volume of collagenase buffer in the concentration ranging from 0.01 to 0.08% (w/v). The optimal concentration may possibly

depend upon the total volume of recirculating perfusate in the dynamic system (Seglen, 1976 a). Hyaluronidase has traditionally been used along with collagenase for liver dispersion, but it has been found to be unneccessary by several investigators (Seglen, 1973 b; Wagle and Ingebretsen, 1975; Moldeus et al, 1978).

3. Buffer Composition, pH and Oxygenation

Since the perfused liver acidified the perfusion medium quite rapidly (Seglen, 1976 a), a strong buffering system is required for pH maintainance even during perfusion periods of 10-15 minutes. Maintainance of pH can be accomplished by using a pH-stat which infused NaOH continuously (Seglen, 1972), the CO_2/bicarbonate system equilibrated with 5% CO_2 (Berg et al., 1972), or the organic buffers like HEPES at a high concentration (Seglen, 1973 b). All the methods mentioned are fully acceptable. HEPES buffer is technically simple, the pH-stat method requires special equipment, and CO_2/ bicarbonate buffer combines perfusate oxygenation (95% O_2, 5% CO_2) with a very high buffering capacity.

4. Perfusate Flow

Perfusion rates ranging from a high of 100 ml per minute to a low of 10 to 20 ml per minute have been recommended. Seglen (1976) suggested that a flow rate of 40-50 ml per minutes with oxygen saturated perfusate is necessary for maximal oxygenation of an 8 to 10 gm liver (300 gm rat). Albumin, which is routinely included in the perfusion buffer, increases the viscosity of perfusate and helps to produce the uniform penetration of the collagenase buffer (Moldeus, 1978; Autuori et al., 1979).

It must be stressed that a uniform flow collagenase buffer is extremely important for a successul hepatocyte preparation. All factors that may interupt the flow must be avoided. These factors include the clogging of hepatic venules by solid particles or microbubbles and vascular collapse that may occur if rats fall into too deep ether anesthesia, or if the portal blood flow is arrested for too long.

5. Liberation of Cells

After successful perfusion with collagenase the liver consists of cells embedded in a connective and vascular tissues. The use of minimal mechanical force to liberate cells is very important. These include gentle shaking or using a spatula, forceps (Berry and Friend, 1969), a comb with widely spaced teeth (Seglen, 1973 b), etc.

6. Purification of Parenchymal Cells

The initial cell suspension obtained after the perfusion of collagenase contains mainly the intact cells with the minor portions of variable numbers of nonparenchymal and damaged cells, some cell clumps, pieces of connective and vascular tissues, and subcellular debris. The purpose of the purification is to remove all of these contaminants. The most commonly used procedures are the combination of filtration and differential centrifugation.

Preincubation with gentle shaking of the initial cell suspension at 37^{o}C for 10 minutes (Berry and Friend, 1969) serves several purposes. Firstly, the structural damaged cell become lighter by the release of their soluble contents and can be separated

from the intact hepatocytes by differential centrifugation . The second purpose is to round up the intact cells which may reduce cellular aggregation, and small aggregates may break up into single cells. And thirdly,kupffer cells may aggregate with cell debris and can be selectively removed by filtration. However, many intact cells are also lost in such aggregations (Seglen, 1976 a).

Filtration is used to remove aggregates and tissue fragments. Both gauze and nylon cloth are suitable filter materials. Special nylon mesh offers a wide choice of exact mesh width. Two filters with decreasing mesh size are commonly used. The resulting suspension contains single cells and some aggregates of two or three cells. Doublets are relatively frequent, about 10% of the total cells (Drochman et al, 1978).

Differential centrifugation is used to separate intact parenchyma from nonparenchymal cells. The cells are sedimented at a low centrifugation force which sufficient to sediment some of the cells, but not all of them. The use of low centrifuge speed, low temperature and flat bottom centrifuge tubes are recommended, in order to prevent the damage of cells by mechanical force. Albumin or serum may help to prevent aggregation and offer protection against mechanical damage through a lubricating action (Segle, 1976 a).

7. Incubation of Cell Suspension

The final preparation of purified parenchymal cells can be incubated in several types of vessel, such as tubes, cell culture flasks, Erlenmeyer flasks,petri dishes immersed in a shaking water bath.

Various buffers, including Krebs' bicarbonate, Hanks' buffer and Eagle's medium have been used as the incubation medium. Albumin or serum is often included in incubation mixture and may protect cells against mechanical stress. Most investigators gassed the hepatocytes with 95% O_2 and 5% CO_2. The use of wide vessels with a shallow depth of incubation medium was suggested to provide the sufficient oxygenation.

B. Criterion for Viability of Isolated Hepatocytes.

Various cellular parameters are used as criterion for viability of isolated hepatocytes. These include morphological feactures, plasma membrane integrity, functional state of mitochondria, and complex synthesis and metabolic capabilities.

The most commonly employed criteria for cell viability is plasma membrane integrity. The trypan blue exclusion test is very easy to perform , less expensive and reflects a change in permeability of the plasma membrane. Intact cells exclude dyes such as trypan blue, nigrosin, and eosin, whereas damaged cells become stained, intensively in the nucleus. The membrane potential , the intracellular K^+ and Na^+ concentration have been suggested as the sensitive criterion for cell viability which detect a lessor damage of cell membrane than the trypan blue exclusion index (Baur et al., 1975). The leakage of soluble enzymes such as lactate dehydrogenase (LDH), alanine aminotransferase (ALT) are also the expression of membrane damage (Baur et al., 1975). Respiratory rates have been used for quality evaluation. A sensitive test is the stimulation of cellular respiration by substrates (e.g. succinate). Only a damaged plasma membrane

allows succinate permeation at a rate sufficient to stimulate respiration (Baur et al., 1975; Letko, 1978).

Metabolic capabilities may give an overall impression of preparation quality, but they show great biological variability (Seglen, 1976 a). The choice of suitable metabolic tests to assess the competence of the cells depend on the experimental conditions used of normal functions such as gluconeogenesis and protein synthesis which should be functionally demonstrated.

In morphological terms, the cells in a wet mount should have well defined outline, yellow color, and refractile appearance. Morphology at light microscopic level is inadequate in assessing functional activity. On electron microscopy, the cell membrane should show microvilli and organelles with their normal structure and distribution. Nuclear and nucleolar detail should be well preserved.

It has been pointed out that no single test supplies full information about the metabolic competence of isolated hepatocytes. One should use criterion questioning at least the integrity and function of plasma membrane and/or the metabolic performances of the cells.

C. Properties of Isolted Hepatocytes

Much work on the properties of isolated parenchymal cells have been published. Freshly intact isolated hepatocytes retain many of the essential properties of the intact tissue, including complete normal internal ultrastructure (Seglen, 1976 a; Berry and Friend,

1969; Wanson et al., 1979), similar characteristics of permeability (Moldeus, 1978). Variety of metabolic properties are well observed, Carbohydrate metabolism : gluconeogenesis (Van De Werve, 1980; Cornell and Filkins, 1974; Seglen, 1976), glycogen synthesis from high concentration of glucose (Wanson et al., 1979; Drochmans et al., 1978), glycogenolysis (Wagle et al., 1973; Seglen, 1974), Lipid metabolism : fatty acid ester and ketone synthesis (Jeejeebhoy and Phillips, 1976; Wagle et al., 1973), protein and nucleic acid metabolism : protein and RNA synthesis (Seglen, 1976 b; Drochmans, 1978), protein degradation (Seglen, 1977), DNA synthesis (Hersiger et al., 1972). The respiration (oxygen uptake) of isolated hepatocytes has also been measured (Letko, 1978; Berry and Friend, 1969). Drug metabolizing enzymes are presented in isolated hepatocytes which can metabolize a variety of drugs (Holtzman et al, 1972; Moldeus et al., 1973; Ronald et al., 1980).

D. Uses of Isolated Hepatocytes

Suspensions of isolated hepatocytes are suitable for biochemical studies. They contribute the understanding of cell function and regulation. This experimental tool has, for example, been successfully employed in studies on gluconeogenesis, glycolysis, lipid, fatty acid, and urea synthesis, ketone body production, protein metabolism, ethanol oxidation, membrane transport, and response to hormones (Tager et al., 1976; Cain and Skilleter, 1980; Stacey and Klaassen, 1980).

Isolated hepatocytes have also been used in studies on cytochrome P-450 linked drug metabolism and drug- induced toxicity, and have

been proved to be valuable for further elucidation of many aspects of this process. (McMahon, 1980; Lowery et al., 1978; Moldeus et al., 1974; Grundin, 1975;Yasuhara et al., 1979; Mitchell and Jollow, 1973).

Recently, hepatocyte suspensions have been employed effectively as a test system by means of in vitro sereening for various hepatotoxic agents and drugs (Zimmerman et al., 1974; Sear and MeGivan, 1979; Stacey et al, 1980; Cantilena et al., 1983; Tsao et al., 1981; Muller and Ohnesorge, 1982).

Isolated hepatocytes have relatively short duration of viability in typical incubation experiments using relatively simple media. In order to extend the experimental period, primary monolayer hepatocytes cultures have been used (Seglen, 1979; Inmon et al., 1981; Poole et al., 1981).

General Introduction on Cadmium

Cadmium (Cd) is a newcomer, having been identified as a distinct element in 1817 (Hammond and Beliles, 1980). It occurs in nature in association with zinc and lead. After isolation, it is quickly found application as an alloy, in electroplating of other metals, and as pigments in paints. Later it is used extensively in the manufacture of alkali storage batteries and plastics (Winter, 1982).

Cadmium ranks close to lead and mercury in toxicological importance due to increasing levels in the environment as a result of past and present industrial practices. The acute toxic effect of cadmium was first discovered as local effects on the gastrointestinal tract (after ingestion) and lung (after inhalation) (Sovet,

1858; Wheeler, 1876). The specific systemic effects of this heavy metal remained virtually unknown until recent years. With increasing production and utilization, certain industrial workers became acutely exposed to high concentrations of cadmium oxide fumes and developed pulmonary edema. However, the possible long-term effects on the industrial workers received no attention. It was not until 1948, when Friberg first reported the coexistence of emphysema and renal damage characterized by proteinurea and glycosurea in men working in an alkali storage battery plant (Friberg, 1948). The specific feature of chronic cadmium poisoning in the industrial workers gradually became recognized. Much of the attention in chronic cadmium poisoning was triggered by the report of cadmium-induced "Itai-Itai" disease in Japan among general population of the Jintsu River Valley who consumed cadmium-contaminated rice as a result of contamination of river water by a mine discharging cadmium-raden wastewater. Middle aged to elderly multiparous women were mainly affected. The disease features included osteomalacia with spontaneous multiple bone fracture, proteinurea and glycosuria (Kazantizis, 1973; Tsuchiya, 1969). Interest in cadmium was further stimulated when Schroeder published a provocative epidemiologic study in 1965 linking dietary cadmium to hypertension in general population (Schroeder, 1965). Later, Corrol (1966) reported that there was a positive correlation between cardiovascular disease and cadmium in the air of 28 American cities studies (Carrol, 1966).

The health hazards of cadmium have attracted increased attention since it was realized that apart from the occasional acute intoxication, usually related to occupational hazards of metallurgical

processes, long-term exposure to low concentrations is cumulative. Severe effects of cadmium to health may be fatal, and that increasing industrial pollution may endanger the general population (Flick et al, 1971).

A. Cadmium in Man and Environment

The relationship between geographic distribution, tissue levels of cadmium, deposition and clinical evidence of cadmium toxicity is unresolved. Based on current knowledge, it can be said that there is a positive correlation between tissue cadmium content and signs of cadmiosis (Flick et al, 1971).

Schroeder and Balassa (1961) reported that cadmium deposition in man was cumulative, with kidney and liver having the highest levels. Renal cadmium (mean level) among Japanese was 6,030 ± 800 ppm ash, Hong Kong 5,620 ± 950 ppm ash, Bangkok Thai 4,910 ± 1,470 ppm ash, Taiwan Chinese 4,150 ± 1,160, United States 2,910 ± 110, England over 1,000 ppm, Bern Swiss, 2,260 ± 480 ppm, Delhe-Lucknow Indian 2,120 ± 310 ppm, and Nigerian Negro 1,700 ± 150 ppm. Liver levels of cadmium, about one-tenth the respective renal levels, showed a similar pattern (Schroeder, 1960; Schroeder and Balassa, 1961)

Cadmium has always been leaching from rocks into soil. In addition, man has contributed in a minor way of burning the fossil fuels in the production of iron, steel and other metals. Due to this normal background, biological materials, as well as airborne dust, always contaminated with unavailable traces of cadmium (0.001 - 1 μg/g). Open seawater contains small amount of

cadmium, about 0.02-0.3 µg/l (Chester and Stoner, 1974).

With increasing industrialization, man is increasingly adding cadmium to the environment. A most important source of pollution is mining and smelting (Buchauer, 1973). Motor fuels are a main reason for high cadmium levels in the air of large cities. In Los Angeles and Tokyo, levels of 0.004 $\mu g/m^3$ and 0.01 $\mu g/m^3$ have been measured (Yagamata, 1973). The other important source of pollution are incineration of refuse and use of sewage sludge as fertilizer. These are the sigificant sources of atmospheric pollution of cadmium that settles on plants and soil (Winter, 1982).

B. Cadmium : Absorption, Distribution and Excretion

Metabolism of cadmium in the mammalion system have been examined extensively in recent years. This briefly review summarized a portion of the experimental datas which have contributed to our current understanding of cadmium toxicity. It may be pointed out that once adsorbed, its excretion from the body is very slow resulting in its accumulation which increase with age. This property of cadmium to be retained in mammalion cells might be an important factor in its potential for toxicity.

1. Absorption

In human, the primary routes of cadmium intoxication are through the respiratory and gastrointestinal tracts by contaminated cadmium in air, food and water.

Studies on absorption of inhaled cadmium in various species of animals, suggested an efficient absorption of cadmium from

respiratory tract. Initial experiments by Harrison et al (1947) showed a rather sufficient absorption of inhaled cadmium as evidence by rapid accumulation in lungs, liver, and kidneys of dogs exposed to cadmium chloride aerosols. Using the data of Harrison et al (1947), Friberg et al (1974) have calculated an absorption efficiency of 40% via the lungs of dogs. Moore et al. (1973) have suggested a retention of greater than 40% of a inhaled cadmium dose in rat 30 days following exposure.

Absorption of cadmium from the gastrointestinal tract is relatively minor, ranging from 0.5 to 8% in various species of animals (Decker et al,1957; Cotzias et al, 1961; Norberg et al, 1971).

Following parenteral administration, cadmium is efficiently retained in animal tissues independent of the injection route. Datas indicated that approximately 80% of an injected cadmium is retained in animals (Decker et al., 1957, Moore et al., 1973; Shaikh and Lucis, 1972).

Absorption of cadmium through intact skin has been investigated. Up to 4% of cadmium is absorbed in 5 hours through the skin of guinea pig (Hammond and Beliles, 1980).

2. Distribution

Animals exposed either acutely or chronically to cadmium accumulated this metal in nearly every tissue. In general, the liver, kidneys, testis, pancrease, heart, lungs and spleen contain the highest cadmium concentrations (Kotsonis and Klaassen, 1977). Undergoing temporal redistribution liver and kidneys become the

major sites of accumulation, about 50% of the total body burden is found in these two organs.

a) Cadmium in Blood

Cadmium is rapidly accumulated and cleared from blood. Maximal concentrations were reached 5-10 minutes after injection, and 90% clearance occurred after several hours (Gunn et al., 1968; Nordberg, 1972).

b) Hepatic Cadmium

Following either acute or chronic exposure irrespective of the route of administration, liver becomes a primary site of accumulation. About 60-70% of a single injected cadmium dose were rapidly found in the liver (Decker et al., 1957; Cotzias et al., 1961; Shaikh and Lucis, 1972, Kotsonis and Klaassen, 1977). However orally administered cadmium appeared in the liver at a much slower rate (Decker et al., 1957).

In addition to rapid accumulation, cadmium levels are maintained in the liver for extended periods of time although a time-dependent decrease of hepatic cadmium coupled with a continual increase in renal cadmium has been observed. According to Kotsonis and Klaassen (1977), the tissue concentrations of cadmium after 2 days following single oral dose of ^{109}Cd was highest in the liver. After 2 weeks, most tissue concentrations decreased by 50%, except liver (unchanged level at high doses) and the kidney (a three to four-folds increase). Similar hepatic-renal cadmium redistribution have been reported by others (Decker et al., 1957; Cotzias et al, 1961).

The mechanism of this redistribution is not clearly understood, although it has been suggested that cadmium binding protein metallothionein may play a role in the distribution and retention of cadmium.

The kinetics of hepatic cadmium transport have been studied and suggested that cadmium uptake into the liver occurred by a combination of simple diffusion and carrier mediated process which involved zinc transport (Frazier and Kingsley, 1976; Frazier and Puglese, 1978; Stacy and Klaassen, 1980). By using a cell separation technique, Cain and Skilleter (1980) showed that the uptake of cadmium was more selective to the liver parenchymal cells than the non-parenchymal cells.

c) Renal Cadmium

Unlike liver, the kidneys accumulated cadmium more slowly, where it was preferentially concentrated in the cortex (Gunn and Gould, 1957). According to Decker et al (1957), renal ^{115}Cd levels in rats showed a gradual increase from 1.6% at 4 hours to 5.1% after 5 weeks following a single intravenous dose. Similar results were also observed by Kotsonis and Klaassen (1977).

d) Cadmium in Other Tissues

Kinetics of cadmium uptake in pancreas paralleled to hepatic retention in that there were the rapid accumulation and retention for at least 25 days. Pancreatic cadmium concentrations, were found at least four folds lower than hepatic levels, and two folds higher than testicular levels with a gradual decline as a function of time

(Shaikh and Lucis, 1972). In addition to the major sites of cadmium accumulation, cadmium was found in a wide variety of other tissues, though to a much lesser extent. These tissues included intestine, spleen, heart, blood vessels, lungs, muscle, brain, bone, uterus and placenta (Kotsonis and Klaassen,1977; Berlin and Ullberg, 1963).

3. Excretion

The primary routes of cadmium excretion are through the kidney and the gastrointestinal tract.

Urinary excretion studied in normal renal function aminals showed that less than 0.5% of a single parenteral cadmium was found in urine within one week (Miller et al., 1968; Klaassen and Kotsonis, 1977; Shaikh and Lucis, 1972, Moore, 1973). Chronic subcutaneous administration resulted in urinary excretion of about 1% of the injected dose per day. After several months of exposure the 20 to 100 folds increase were observed, accompanied with renal dysfunction as manifested by the onset of proteinuria (Axelsson and Piscator, 1966; Nordberg, 1972).

Fecal excretion was dependent on the route of cadmium administration. About 85% of orally administered cadmium to mice, was excreted in the feces within 3 days, this reflected limited absorption of cadmium through the gastrointestinal tract (Decker et al, 1957). After a single parenteral dose, about 1-2% was excreted in feces per day (Axelsson and Piscator, 1966; Miller et al., 1968). In chronic studies , 1.6-2.8% of the daily dose was excreted in feces (Axelssen ans Piscator, 1966).

In addition to urinary and fecal excretion,it has been suggested that cadmium may also be secreted in bile (Friberg et al, 1974; Frazier and Kingsley, 1976; Klaassen and Kotsonis, 1977), in milk (Lucis et al., 1972; Miller et al., 1967), and through hair follicles and hair (Miller et al., 1968). These auxillary secretory pathways accounted for the clearance of only a limited portion of the total body burden of cadmium. In summary, with the exception of proteinurea enhanced urinary excretion, limited excretion of absorbed cadmium occurred following either acute or chronic exposure.

C. Biological Half-life

As described previously covering absorption, distribution, and excretion indicates that cadmium is effectively accumulated and retained in soft tissues. These observations suggest a long biological half-life for obsorbed cadmium. The biological half-life of cadmium has been estimated to be between 50 and 250 days for rodents (Moore et al,1973; Cotzias et al, 1961), more than 700 days for monkeys, and between 9 and 30 years for man (Friberg et al, 1974).

D. Cadmium Intoxication

It is realized that apart from the occasional acute intoxication, small doses over long periods are cumulative and may have severe toxic effects. Intoxication occurs mainly by ingestion and inhalation (Flick et al., 1971; Winter, 1982).

Numerous cases of acute cadmium poisoning have been documented. Most of them inhaled the very high concentrations of cadmium (Mac Farland, 1979). Others resulted from consumption of cadmium contaminated beverages and foods (Frant and Kleeman, 1941).

Acute lethal doses by inhalation in man can only be estimiated rather crudely. They are considerably depended on the chemical form, particle size, and period of time over which inhalation occurred. The lethal dose of cadmium fumes has been estimated to be 2,600 mg/M^3 for one minute inhalation and the minimum toxic dose for an eight-hour inhalation is probably 1 to 3 mg/M^3 depending on the particle size (Commission of the Europeon Communities,1978). The principal toxic effects of cadmium inhalation are attributable to local irritation of the respiratory tract. Death is usually due to massive pulmonary edema. Signs and symptoms are delayed (few hours) and consist mainly of irritation of upper respiratory tract, chest pains, nausea, and dizziness. Gastrointestinal effects, e.g., nausea and diarrhea, may also occur. Long-term or permanent lung damage may occur as emphysema and peribronchial and perivascular fibrosis (Casarett and Doull's, 1980).

Oral lethal doses in man are also difficult to estimate and very considerably depended on the chemical forms. In experimental animals the acute oral LD_{50} varies from approximately 100 mg/kg for soluble salts of cadmium to several thousand mg/kg for metallic cadmium powder or the insoluble salts. Estimated lethal doses in man range from 350 to 8,900 mg. The minimum acute toxic dose is probably less than 10 mg. As with inhalation of cadmium, major toxic effects are referable to local irritant effects. In cases of oral intake the manifestations are nausea, vomiting, salivation, diarrhea, and abdominal cramps. Death may occur within 24 hours due to shock and dehydration or may be delayed one or two weeks following onset of various systemic effects, notably renal and cardiopulmonary failure.

Extensive damage to the liver may also occur (Casarett and Doull's, 1980).

Long-term exposure to low concentrations of cadmium is cumulative, may have severe systemic effects and may be fatal. Cadmium is toxic to virtually every system of the body, but the critical target organ in chronic cadmium exposure is generally the kidneys.

The toxic effects caused by cadmium to various systems described follow are those recognized so far in man and experimental animals. In animals, a surprising number of pathological conditions have been produced. Their implications in man are not yet fully known.

1. Renal Effects.

Early investigations in experimental animals found that cadmium administration produced a diffuse inflammation of kidneys in addition to infarction of the lungs, fatty degeneration of liver and heart (Marme, 1867), necrosis of the convoluted tubules and tubular casts (Severi, 1896). Several subsequent reports confirmed and elaborated on the ability of cadmium to produce the morphological damage of renal tissue. However, it was not until 1950 that the effects of cadmium on human renal function began to recognize. Workers exposed chronically to cadmium oxide dust produced renal damage reflected by low molecular weight proteinurea and diuresis. (Friberg, 1950). Further evidence of renal dysfunction was glycosuria and aminoacidurea, hypercalciurea and renal stone formation (Kazantzis et al., 1963). A cadmium-induced syndrome similar to that described above also found in the nonindustrially exposed population, the Itai-Itai patients characteristically displayed proteinurea and

glycosurea. Other common symptoms included aminoacidurea and hypercalciurea (Kazantzis et al, 1963).

Studies on renal damage in man and animals following cadmium exposure suggested that renal damage were the tubular (Nomiyama et al, 1973; Murakami and Webb, 1981) and glomerular changes (Bonnell et al, 1960; Itokawa et al, 1974). However, the morphological details of kidney damage, like damage of other organs was so far not fully described in man. It was estimated that under normal condition in an unpolluted environment, the human kidney should accumulate 50 µg/g wet weight by the age of 50 years. The critical concentration appeared to be 200 µg/g when kidney failure setted in (Friberg, 1974). Friberg et al. also suggested that after 10 years of occupatical exposure to 25 $\mu g/M^3$, the kidney cortex may accumulate the critical concentration of 200 µg/g (Friberg et al., 1974).

2. Reproductive Effects

The testes seem to be a susceptible organ to cadmium toxicity, although their cadmium concentrations are relatively low. Alsberg and Schwartze (1919) first noted that administration of cadmium in animals caused "bluis discoloration of testicle ". Later in 1957, Parizek observed that following parenteral administration of cadmium, there was a very rapid progressive changes in the testes included haemorrhagic necrosis, atrophy and permanent sterility 24 hours after injection (Parijek, 1957). Recently, Lee and Dixon reported that single injection of cadmium dose did not cause morphological testicular changes but produced a significant decrease in firtility for a period of 55 days. Histological testicular changes

in rat as a result of long term administration of cadmium in food was reported (Ribelin, 1963). Although the mechanisms of testicular lesions are not fully clarified, they appear to be primarily damaged to the testicular endothelium and circulation (Chiquoine, 1964).

So far testicular necrosis as a result of cadmium exposure has not been reported in man. However, industrially exposured workers seem to accumulate considerable amounts of cadmium in testes (Smith et al, 1960).

Ovarian changes have been produced by cadmium in prepubertal rats (Kar et al, 1959). It caused hyperaemia of the ovaries, atresia of follicles and haemorrhage into the follicular cavity, followed by recovery after about one week.

Investigations in laboratory animals showed the teratogenic capabilities of cadmium. A variety of abnormalities produced could result from a direct action of cadmium on the maternal or placental tissues, or a direct effect of cadmium on the fetal tissues (Rohrer et al., 1979).

3. Cardiovascular Effects

The ability of cadmium to produce hypertension has received considerable attention during recent years. Schroeder (1965) reported that cadmium played a significant role in the etiology of hypertension in general population. Chronic arterial hypertension has been induced in rats exposed to small amount of cadmium for a period of few months (Schroeder et al., 1970). Cadmium has been shown to depress atrioventricular (A-V) conduction, both in isolated heart

preparation and in chronically exposed rats (Kopp and Howley, 1976). Evidence for depressed conduction consisted of dose dependent lengthening of the P-R interval (Kopp, S.J., 1980). Cadmium had also been shown to induce morphologic changes in myocardium. Dotta and Fruscella (1963) observed myocardial hypertrophy. Ultrastructural changes in heart after chronic exposure to cadmium consisted of A-V nodul degeneration and the presence of electron densities in membranes associated with intercalated disces (Kopp et al., 1978). However, the role of cadmium in the etiology of hypertension is still not satisfactorily resolved.

4. CNS Effects

Histological evidence indicated that administration of cadmium caused hemorrhagic lesions in brain of new born rat (Gabbiani et al, 1967). In older animals, the lesions were produced in spinal sensory ganglia of rats, rabbits, guinea pigs and hamsters, indicating the importance of age with respect to the CNS site of cadmium toxicity (Gabbiani, 1966; 1967). Furthermore, Lucis et al (1972) showed that cadmium penetrated the blood brain barrier with more ease in fetal rats. However, cadmium has also been demonstrated to accumulate in adult brain (Stowe et al, 1972).

Cearly and Coleman (1974) observed "abnormal behavioral patterns" in fish intoxicated with cadmium a few weeks after exposure. The bass and bluegill exhibited erractic, uncoordinated swimming movements , muscle spasms, convulsion and paralysis, indicating that the nervous system was the site of damage.

5. Hepatic Effects

Several investigators have shown that liver is one of the major sites of cadminm localization following acute exposure to the metal. Cadmium has been reported to induce hepatic functional and structural alterations.

Cadmium produced fatty infiltration and lesions in liver of experimental animals following inhalation or single injection dose (Marme, 1867; Meek 1957). Evidence from histological studies by Hoffmann(1975) showed that a single injection of cadmium dose to rats produced at 10 hours, liver damage but no observable kidney changes. Ultrastructure of the liver revealed more profound changes in parenchymal cell than non parenchymal cells. The most prominent changes were single parenchymal cell necrosis, deterioration of rough endoplasmic reticulum,proliferation of smoth endoplasmic reticulum,autophagocytosis, and mitochondrial degenerative changes(Hoffman, 1975). Similar results were seen by Meiss (1982). Elevation of serum enzymes which indicated hepatic dysfunction after acute cadmium administration have also been demonstrated (Dudley et al., 1980, Cook et al, 1974). Thus, liver (mainly the parenchymal cells) is a target organ for acute cadmium toxicity. Using a cell separation technique also showed that liver parenchymal cells selectively took up more cadmium than the non-parenchymal cells (Cain and Skilleter, 1980).

To recognize the early cell injury produced by cadmium, the cytotoxic actions of cadmium have recently been investigated using primary culture of rat hepatocytes (Santone and Acosta, 1982). Evidence of cytotoxicity after exposed to cadmium chloride (50-4000 μM) for 1 hour was shown by increase leakage of lactate dehydrogenase

from the hepatocytes into the culture medium, increase in lactate-to-pyruvate (L/P) ratios, and decrease in urea content in a dose-dependent manner.

Aims of the Thesis

As it is pointed out that, irrespective of the route of administration, liver (mainly parenchymal cells) initially accumulated most of cadmium (Cain and Skilleter, 1980). Previous in vivo laboratory experiments have demonstrated hepatic morphological, functional and metabolic alterations following cadmium exposure. Recognition of early cell injury induced by several chemicals is often difficult in the intact animal. Various in vitro liver preparations have been used to evaluate the potential injurous effects of xenobiotic at the cellular level. Primary culture of rat hepatocytes have been used to investigate the cytotoxic action of cadmium (Santone and Acosta, 1982). However the culture system is time consuming. Since freshly isolated hepatocytes have increasing been used for biochemical, drug metabolism and toxicity studies. A major advantage of this system is that it is possible to observe the reaction of hepatocytes to the direct action of the factors under study. Recently, the use of liver cells in suspension has been advocated as an in vitro system for the assessment of the hepatotoxic potential of drugs and toxicants (Sear and McGivan, 1979; Stacey and Klaassen, 1979; Tsao et al, 1981). Therefore, the present study was undertaken to assess the utility of isolated hepatocytes as the in vitro model to evaluate the hepatotoxicity of cadmium.

Experimental Protocol

Study 1

The purpose of the first series of experiments is to determine the viability of freshly isolated rat hepatocyte suspensions using the following criterion for cell viability.

1. Morphological features

Experiment : Hepatocytes were isolated from male rats (200-250 g.) by the method described in chapter II.

Aliqouts of rat hepatocyte suspensions were prepared for light and electron microscopic examinations (methods described in chapter II).

2. Plasma membrane integrity

Experiment : Aliqouts 3.0 ml of rat hepatocyte suspensions (10×10^6 cells/ml) were incubated at $37^{\circ}C$ in metabolic shaker bath for 30 to 60 minutes, triplicate flasks were used for each time point. Three viability tests were done before and after 30, and 60 minutes of incubation. For analyzation, every sample was divided as : 50 µl for TB exclusion test and 1.0 ml for examination of the released ALT activity and intracellular K^+ (methods decribed in chapter II).

3. Metabolic capability

Experiment 3.1 : To determine the aminopyrine demethylase activity, triplicate samples of 3.0 ml cell suspensions (10×10^6 cells/ml) were incubated with 5 µM aminopyrine at $37^{\circ}C$ in metabolic shaker bath for 30 min. The formaldehyde formed which was trapped as semicarbazone

was determined by the method of Nash (methods described in Chapter II).

Experiment 3.2 : To determine the gluconeogenic capability, triplicate samples of 3.0 ml 24 hr fasted cell suspensions (10×10^6 cells/ml) were incubated with 10 mM gluconeogenic substrates (L-alanine, β-D-fructose, L(+)-lactic acid, and sodium pyruvate, for 1 hour at $37^{\circ}C$ in metabolic shaker bath. Aliqouts 1 ml samples were taken for quantitative assay of glucose formed using the O-toluidine method (methods described in chapter II).

Study 2

The purpose of the second series of experiments is to investigate the acute hepatotoxicity of cadmium chloride on freshly isolated rat hepatocyte suspensions.

Experiment 1. To determine the acute effects of cadmium chloride on cell membrane integrity of isolated hepatocytes.

Triplicate samples of 3.0 ml cell suspensions were incubated with 50 µl cadmium chloride (1-100 µM final concentration of cadmium) or saline for 30 minutes at $37^{\circ}C$ in metabolic shaker bath. Aliqouts of the cell suspensions were removed for assay of cell injury by using : TB exclusion test, intracellular K^+ content, and the released ALT (methods described in chapter II).

Experiment 2. To determine the acute effects of cadmium chloride on gluconeogenic capability from lactate of isolated hepatocytes.

Triplicate samples of 3.0 ml of 24 hr-fasted cell suspensions were incubated with 50 µl cadmium chloride (1-100 µM final concentration of cadmium) or saline for 30 minutes prior to incubation with 10 mM final concentration of sodium lactate for 1 hour at 37°C in metabolic shaker bath. The amounts of glucose formed from lactate were determined by using the O-toluidine method (methods deseribed in chapter II).

Experiment 3. To determine the acute effects of cadmium chloride on the morphological features of isolated hepatocytes.

Triplicate samples of 3.0 ml hepatocyte suspension were incubated with 5 mM cadmium for 30 minutes at 37°C in metabolic shaker bath. At the end of 30 minutes exposure, hepatocyte suspensions were prepared for electron microscopic examination (methods described in chapter II).