ENHANCEMENT OF THE DIFFERENTIATION OF PREWEANLING RAT HEPATOCYTES IN VITRO BY RETINOIC ACID: A STUDY OF BILE CANALICULI FORMATION

by

Annie CLAUDE M.D.

A thesis submitted to the School of Graduate Studies

in partial fulfillment of the requirements

for the degree of Master in Sciences in Anatomy

Departments of Anatomy and Pathology

Faculty of Medicine, University of Ottawa

Ottawa, Canada

May 1992

ISBN 0-315-93568-5

Canada

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

To study the stimulating effects of retinoic acid on the primary culture of immature rat hepatocyte, we examined the changes in the bile canaliculi formation by observing the cytokeratins 55 kDa and 49 kDa. The formation of the bile canaliculi sheaths was our end point of differentiation. We obtained monolayer hepatocyte culture from 14-day old male Wistar Charles River rats, using the two-step collagenase perfusion method of Seglen [1976], as adapted to preweanling rats by Deschênes at al [1980]. All-trans retinoic acid 10^{-5}M dissolved in 0.1% dimethyl sulfoxide was added to the treated cultures. All cultures were maintained for 48 hours. The organization of cytokeratin was visualized by immunofluorescence using monoclonal antibodies to cytokeratins 55 kDa and 49 kDa. Under the influence of retinoic acid, the number and size of bile canaliculi formed were significantly increased. The bile canaliculi formed had a more complex architecture, such as ramification. These observations were confirmed by un-embedded whole mount electron microscopic studies on detergent extracted preparations. In retinoic acid treated colonies, the bile canaliculi appear to be functional as demonstrated by the cellular uptake followed by polarization and secretion of the fluorescein diacetate dye. Gel electrophoresis showed an apparent disparity in the relative quantity of cytokeratin 55 kDa and 49 kDa, especially in the control group. This inequality may be attributed to a selective increase in proteolysis during preparation. It is concluded that the addition of retinoic acid to the media stimulates the differentiation in hepatocyte monolayer cultures. It is possible that retinoic acid induces modification in the hepatocyte gene expression and the formation of bile canaliculi could be associated with changes in the expression of the cytokeratin genes. The two cytokeratins would be coexpressed in response to the treatment with retinoic acid.

ACKNOWLEDGEMENT

I wish to express my sincere appreciation to my thesis supervisors, Professor S. W. French, Chairman of the Department of Pathology, and Professor N. Narbaitz of the Department of Anatomy, for their guidance.

I am grateful to Dr. Fryer for providing me with the opportunity to undertake my thesis work in a stimulating environment.

I am indebted to many individuals in the Pathology Department, the Anatomy Department, and the laboratories of Dr. French and Dr. Narbaitz, for their valuable technical assistance and stimulating scientific discussions. In particular, I wish to acknowledge the guidance and assistance of Mr. R. Nicholls of Dr. Narbaitz' laboratory, in the use of electron microscopy. I would like to thank Dr. M. Cadrin of the Department of Pathology for her critical comments and challenging discussions in the course of the experimental work. Also, I wish to thank Mr. Paul Lalande of the Canada Institute of Scientific and Technical Information (CISTI), Ottawa, for his help with the bibliography.

This research was supported by a grant from the Medical Research Council of Canada to Dr. S. W. French (Grant No. IPO MNR E9S).

TABLE OF CONTENTS

ABSTRACT		i
ACKNOWLEDGMENT		ii
TABLE OF CONTENTS		iii
LIST OF TABLES		vii
LIST OF FIGURES AND ILLUSTRATIONS		viii
LIST OF ABBREVIATIONS		x
1	**INTRODUCTION**	**1**
1.1	THE LIVER	1
1.1.1	The Anatomy of the Liver	1
1.1.1a	Macroanatomy	1
1.1.1b	Microanatomy	1
1.1.2	The Bile Canaliculi System	4
1.1.3	The Functions of the Liver	7
1.1.4	The Cellular Integrity	8
1.2	THE CYTOSKELETON	9
1.2.1	The Cytoskeleton	9
1.2.2	The Intermediate Filaments	11
1.2.3	The Cytokeratins	15
1.2.4	The Cytoskeleton of Hepatocytes	17
1.2.5	The Dynamic Role of the Intermediate Filaments	17
1.3	CELLULAR DIFFERENTIATION	18
1.3.1	Cellular Differentiation *in vivo* (Embryo)	18
1.3.2	Cellular Differentiation in Regeneration	19
1.3.3	Cellular Differentiation in Culture	20
1.3.4	Differentiating Factors Used in Culture	23
1.3.4a	Dexamethasone	23
1.3.4b	Dimethyl Sulfoxide	24
1.3.4c	Retinoids	25
1.4	HEPATOCYTES IN CULTURE	27
1.4.1	The History of Hepatocyte Culture	27
1.4.2	Evolution of Methods of Maintaining Rat Hepatocytes in Monolayer Culture	29
1.4.3	The Surfaces of Attachment	30
1.4.4	The Kinetics of Hepatocyte in Culture	32
1.4.5	The Human Hepatocytes in Culture	33

1.5	RETINOIC ACID	34
1.5.1	The History of Vitamin A	34
1.5.2	The Chemical Formula and Functions of Retinoids	36
1.5.3	The Metabolism of Retinoids in the Liver	37
1.5.4	The Role of Retinoids on Epithelial Differentiation	38
1.5.5	Gene Expression and Receptors	39
1.6	EXPERIMENTAL RATIONALE	42
1.6.1	A short background review	42
1.6.2	The Experimental Design: The Hepatocyte Monolayer Culture	45
1.6.3	The Preliminary Tests	45
1.6.4	The Experiment with Retinoic Acid	47
1.6.5	The Hypothesis	48
2	**MATERIALS AND METHODS**	**49**
2.1	PRELIMINARY EXPERIMENTS	49
2.1.1	Hepatocyte Isolation Technique	49
2.1.2	Culture Media	51
2.1.2a	Serum Free Culture Medium Supplemented with Dexamethasone	51
2.1.2b	Serum Free Culture Medium Supplemented with Epidermal Growth Factor	51
2.1.2c	Culture Medium Supplemented with Dexamethasone and Calf Serum	51
2.1.3	Hepatocytes in Monolayer Cell Culture	52
2.1.3a	Surfaces of Attachment	52
2.1.3b	Seeding Density	52
2.1.3c	Attachment Time	53
2.1.3d	Incubation Time	53
2.1.4	Dimethyl Sulfoxide as a Solvent	53
2.1.5	Retinoic Acid	54
2.2	INDIRECT IMMUNOFLUORESCENCE: SINGLE AND DOUBLE STAINING	55
2.2.1	Indirect Immunofluorescence: Single Staining Technique	55
2.2.2	Indirect Immunofluorescence: Double Staining Technique	56
2.2.3	Morphometric Analysis	56
2.2.4	Immunofluorescence: Primary and Secondary Antibodies	57
2.3	FLUORESCEIN DIACETATE UPTAKE AND SECRETION	58
2.4	ELECTRON MICROSCOPY	59
2.4.1	Cellular Extraction Technique	59
2.4.2	Electron Microscopy Preparation	60
2.4.3	Immunoelectron Microscopy Preparation	61
2.4.4	Electron Microscopy: Primary and Secondary Antibodies	61
2.5	GEL ELECTROPHORESIS	62
2.5.1	SDS-Polyacrylamide Gel Electrophoresis Technique	62
2.5.2	Densitometric Analysis	64
2.5.3	Immunoblot Technique on Gels	65
2.5.4	Immunoblot Gel: Primary and Secondary Antibodies	66

3	**RESULTS**	**68**
3.1	PRELIMINARY EXPERIMENTS	68
3.1.1	Hepatocyte Isolation	68
3.1.2	Culture Media	69
3.1.2a	Serum Free Culture Medium Supplemented with Dexamethasone	69
3.1.2b	Serum Free Culture Medium Supplemented with Epidermal Growth Factor	69
3.1.2c	Culture Medium Supplemented with Dexamethasone and Calf Serum	70
3.1.3	Hepatocytes in Monolayer Cell Culture	70
3.1.3a	Surfaces of Attachment	70
3.1.3b	Seeding Density	71
3.1.3c	Attachment Time	72
3.1.3d	Incubation Time	72
3.1.3e	Description of Culture Obtained	73
3.1.4	Dimethyl Sulfoxide as a Solvent	73
3.1.5	Retinoic Acid	74
3.2	INDIRECT IMMUNOFLUORESCENCE: SINGLE AND DOUBLE STAINING	75
3.2.1	Indirect Immunofluorescence: Single Staining	75
3.2.1a	Actin	75
3.2.1b	Microtubule	76
3.2.1c	Desmoplakin	76
3.2.1d	Cytokeratin 55 kDa	76
3.2.1e	Cytokeratin 49 kDa	77
3.2.1f	Distributions of CK 55 and CK 49 kDa in Retinoic Acid Treated Colonies	77
3.2.1g	Ramification of Bile Canaliculi	78
3.2.2	Indirect Immunofluorescence: Double Staining	78
3.2.3	Morphometric Analysis of the Bile Canaliculi	79
3.3	FLUORESCEIN DIACETATE UPTAKE AND SECRETION	81
3.4	ELECTRON MICROSCOPY	82
3.4.1	Cellular Extraction Technique	82
3.4.2	Electron Microscopy	82
3.4.3	Immunoelectron Microscopy	84
3.5	GEL ELECTROPHORESIS	84
3.5.1	SDS-Polyacrylamide Gel Electrophoresis	84
3.5.2	Densitometric Analysis	85
3.5.3	Immunoblot on Gels	86
4	**DISCUSSION**	**87**
4.1	PRELIMINARY EXPERIMENTS	87
4.1.1	Hepatocyte Isolation	87
4.1.2	Culture Media	87
4.1.3	Hepatocytes in Monolayer Cell Culture	88
4.1.4	Dimethyl Sulfoxide as a Solvent	90

4.2 THE EFFECT OF RETINOIC ACID ON BILE CANALICULI FORMATION 91
4.2.1 Indirect Immunofluorescence 92
4.2.2 Fluorescein Diacetate Uptake and Secretion 94
4.2.3 Electron Microscopy 97
4.2.4 Gel Electrophoresis 101

4.3 CONCLUSION 102

Appendix A Composition of Solutions 104

A.1 Solutions for Hepatocyte Culture 104

A.2 Solutions for Culture Media 105

A.3 Solutions for Hepatocyte Extraction 107

A.4 Solutions for Gel Electrophoresis 110

A.5 Solutions for Immunoblot on Gel 112

Appendix B Statistical Equations 113

Appendix C Tables 114

Appendix D Figures and Illustrations 118

Reference 152

LIST OF TABLES

1	Types of intermediate filaments	115
2	Characteristics of the bile canaliculi observed in control and retinoic acid treated hepatocyte colonies	116
3	Ratio of the absorption peak and the integral of the CK55 absorption curve to those of the CK49 curve, in whole control and treated cultures	117

LIST OF FIGURES AND ILLUSTRATIONS

1	The dual vascular system of the liver	119
2	The three-dimensional liver structure as seen by Hans Elias	120
3	The hepatic acinus	121
4	The direction of the vascular and the bile flow in the lobule	122
5	The primary and secondary structure of intermediate filaments	123
6	Cytokeratin protofilaments and the formation of intermediate filaments	124
7	Artistic drawing of the cytoskeleton of the hepatocytes	125
8	Fibronectin	126
9	The molecular structure of retinol and retinoic acid	127
10	Procedure for indirect immunofluorescence: single and double staining	128
11	Procedure for fluorescein diacetate uptake and secretion	129
12	Procedure for hepatocyte extraction	130
13	Procedure for electron microscopy	131
14	Procedure for Electrophoresis and Electroimmunophoresis	132
15	Control hepatocyte colony seen in a phase contrast	133
16	Retinoic acid treated colony seen in a phase contrast	134
17	Immunofluorescence photograph of a control hepatocyte colony stained for actin	135
18	Immunofluorescence photographs of a control hepatocyte colony stained for tubulin	136
19	Immunofluorescence photographs comparing the distribution of cytokeratin 55 kDa in control and treated hepatocyte colonies	137

20	Immunofluorescence photographs comparing the distribution of cytokeratin 49 kDa in control and treated hepatocyte colonies	138
21	Immunofluorescence photographs comparing the distribution of cytokeratin 49 and 55 kDa in retinoic acid treated hepatocyte colonies	139
22	Immunofluorescence photograph of a retinoic acid treated hepatocyte colony showing a bile canaliculus ramification	140
23	Immunofluorescence photographs of a control hepatocyte colony comparing the distribution of cytokeratin 55 and 49 kDa	141
24	Immunofluorescence photographs comparing the distribution of cytokeratin 49 and 55 kDa in a retinoic treated hepatocyte colony	142
25	Immunofluorescence photographs of the uptake and the secretion of fluorescein diacetate in a control hepatocyte colony	143
26	Immunofluorescence photographs of the uptake and the secretion of fluorescein diacetate in a retinoic acid treated colony	144
27	Immunofluorescence photograph of the polarization phase with fluorescein diacetate in a retinoic acid treated colony	145
28	Electron microphotograph of the filament distribution in a control hepatocyte colony	146
29	Electron microphotograph of the filament distribution along the pericanalicular sheath in a retinoic acid treated hepatocyte colony	147
30	Electron microphotograph of a bile canaliculi ramification in a retinoic acid treated hepatocyte colony	148
31	SDS-PAGE of whole control and retinoic acid treated colonies	149
32	SDS-PAGE of extracted control and retinoic acid treated colonies	150
33	Example of a densitometric scanning curve of a SDS-PAGE	151

LIST OF ABBREVIATIONS

AFP	Alpha-feto-protein
Arg	Arginine
AS	Ammonium sulfate
Asp	Aspartate
BC	Bile canaliculi
C	Celcius
CK	Cytokeratin
CK49	Cytokeratin of 49 kiloDaltons
CK55	Cytokeratin of 55 kiloDaltons
CRABP	Cell retinoic acid binding protein
CRBP	Cell retinol binding protein
CSK	Cytoskeletal buffer solution
CSK-AS	Cytoskeletal buffer solution containing ammonium sulfate
DIG	Digestion, suffix reffering to solution with protein digestion properties
DMSO	Dimethyl-sulfoxide
DNA	Dexoxyribonucleic acid
DNAse	Dexoxyribonuclease
ECM	Extracellular matrix
EGF	Epidermal growth factor
EM	Electron microscopy
FD	Fluorescein diacetate
FITC	Fluoresceine iso-thiocyanate
Gly	Glycine
GFAP	Gliofilament acid protein
G1	Gap period in eukaryotic cell cycle, preceeding the S phase
G2	Gap period in eukaryotic cell cycle, following the S phase
HEPES	N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid
hRAR	Human retinoic acid receptor
IAA	Iodoacetamide
IF	Intermediate filaments
IgG	Immunoglobuline G
IgM	Immunoglobuline M
kDa	kilo-Dalton
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline solution
PIPES	1-4-piperazine-ethane sulfonic acid
PMSF	Phenyl-methyl-sulfonyl-fluoride
RNAse	Ribonuclease
S	Synthetic phase of the interphase in eukaryotic cell where replication of DNA and synthesis of histone occurs
s.d.	Standard deviation
SDS	Sodium-dodecyl sulfate
Ser	Serine
TEMED	N,N,N',N'-tetramethyl-ethylene-diamine
TRITC	Tetramethyl-rhodamine iso-thiocyanate

1. INTRODUCTION

1.1 THE LIVER

1.1.1 Anatomy of the Liver

1.1.1a Macroanatomy

In order to understand the importance of the relationship between the preservation of the liver structure and the maintenance of its functions, it is necessary to review some aspects of the liver anatomy. Despite differences in size and lobulation, the basic organization of the liver is similar in most mammals. The embryology of the liver will be briefly reviewed in Section 1.3.1. The brief review in this subsection is based on the works of Netter (1979), Delmas (1974), Fawcett (1986), and Ishak and Stromeyer (1983).

The liver, the largest organ in mammals, has many complex functions (Section 1.1.3). The key to understanding the relationship between the liver structure and its functions is its dual blood supply. The liver receives the largest amount of blood (venous) from the intestinal tract system via the valveless portal vein, and a smaller amount of blood (arterial) from the common hepatic artery, a branch of the celiac trunk. Blood from these two entry systems leaves the liver via the supra-hepatic (hepatic) vein, which empties its blood into the inferior vena cava (Figure 1).

1.1.1b Microanatomy

The anatomical unit of the liver is the classical lobule. In rats and in human beings, the lobules are not well delimited. Their boundaries are not as clear as in other species, such as pigs, in which the lobules are clearly defined by the presence of connective tissue.

Our present synopsis of the normal three-dimensional structure of the liver (Figure 2) is due to Elias (1953), who proposed the "Plate Theory" to replace the previous cord structure theory. It is interesting to note that the cord structure theory was already challenged a century ago by Hering, who described the rabbit liver as a continuous cellular mass traversed by blood capillaries.

According to the Plate Theory of Elias, the center of a lobule is occupied by a central vein, a branch of the hepatic veins. Radially, the epithelial cells of the liver are arranged in plates which are typically one cell thick. As shown by Elias (1953), the sizes and shapes of the individual liver cells vary greatly depending upon their position in the plate. The cells near a hole in the plate (*i.e.* a perforation of the lamina into the hepatic lacuna) are usually smaller than those at the corners where several cells meet. The plates are exposed to blood on both sides: the hepatic sinusoids. At the angles between the lobules, there are connective tissue spaces known as portal spaces which contain interlobular veins (branches of the portal vein), small branches of the hepatic artery, lymphatics and small branches of the bile ducts. The blood from the small branches of the hepatic artery and from the portal vein flow into the hepatic sinusoids along the liver plates through the sinusoids in a centripetal direction (towards the central vein). Therefore, there is a gradient of oxygen, nutrients and occasionally toxins along the hexagonal section of the lobules.

The functional unit of the liver is the hepatic acinus (Figure 3). As described by Rappaport et al. (1954), this unit is centered around a portal space. It contains the interlobular veins, the small branches of the hepatic artery, and the bile ducts. The blood

flows in a centrifugal direction. This concept is functionally more logical than that of the hepatic lobule, and makes it easier to explain pathological lesions of the liver (Ruebner et al., 1991).

Rappaport et al. (1954) have also divided the parenchyma around the portal space in three zones (Figure 3). The cells in zone 1 (nearest to the portal space) have first call upon the incoming oxygen and nutrients and are larger in size, while the cells in zone 2 are less favored and those in zone 3 (near the terminal hepatic venules) are the least favorably situated and the most vulnerable to ischemia, anoxia, congestion and nutritional deficiency (Rappaport, 1976). On the other hand, toxic substances such as tetrachloride, halothane and acetone, will damage cells in zone 1 first.

In the healthy liver, a blood pressure gradient exists between the incoming and the outgoing blood flow. The portal vein and the hepatic artery branches have a higher blood pressure which progressively decreases to the level of the blood pressure in the central vein branch. The existence of this pressure gradient appears only after birth when the centripetal blood flow is established in the newborn.

This architecture is not fixed, and a reversal of the lobular architecture is observed when the normal blood pressure gradient is modified, for instance by stasis in passive congestion of the liver. Thus, the liver lobules are not a static structure and their organization and function depend upon the blood flow.

A system of anastomosis exists between the portal and the systemic venous systems. This is of little consequence in the healthy body, but assumes great importance in mammals suffering from portal hypertension, a situation where the excess pressure is

rerouted into the lower systemic venous pressure system.

The hepatic cells in the liver plates are not in direct contact with the blood in the sinusoids. The Ito cells lie in the space of Disse and provide collagen to maintain the connective framework of the sinusoids. The endothelial cells rest on the tips of numerous microvilli of the hepatic cells. The hepatic cells are thus separated from the endothelial cells by a space, the space of Disse which is more like a lymphatic space. The wall of the sinusoids contains macrophages known as Kupffer cells in addition to perforated endothelial cells. They form a continuous layer but are not attached to each other by desmosomes (Ruebner et al., 1991).

The liver's lymphatic drainage system originates in the perisinusoidal space of Disse where fluid exchange takes place between the blood and the hepatocytes. These spaces drain into lymphatic vessels of gradually increasing sizes.

1.1.2 The Bile Canaliculi System

The centrifugal flow of bile toward small bile ducts is in the opposite direction of the flow of blood (Figure 4). The bile canaliculi system is an extensive network of small canals that run between hepatocytes throughout the parenchyma of the liver. The bile canaliculus is a special structure characteristic of the liver cells. Bile canaliculi do not have a wall of their own, but represent spaces between adjacent hepatocytes. The plasma membrane of these cells constitutes the wall of the canaliculi and the space is limited laterally by continuous junctions. The bile canaliculi form a three dimensional cylindrical continuous network of tight junctions and belt desmosones which joins small canals called the intermediate canals of Hering (Figure 2) at the periphery of the lobule. The

progressive anastomosis of the canals of Hering gives rise to the portal bile ducts, which flow into the intra-hepatic ducts, the extra-hepatic ducts, and finally to the lowest pressure system, the common hepatic ducts outside the liver. The common hepatic duct is joined by the cystic duct to form the common bile duct which eventually empties into the duodenum. This system carries the bile produced by the hepatocytes all the way to the digestive system.

The system of bile canaliculi is unique to the liver. Bile canaliculi vary in diameter and appear more distended when active secretion of bile is present. Experimentally, bile canaliculi may be rendered visible by using special stains such as silver impregnation techniques, or by using enzyme histochemical reactions such as those for adenosine, di- or triphosphatase, or alkaline phosphatase.

Phillips et al. (1982) and Watanabe et al. (1991) have studied the bile canalicular peristaltic movements using fluorescein diacetate in cultured hepatocytes. The functional capacity of bile canaliculi may be assessed in culture with the use of fluorescein diacetate because this chemical is captured intracellularly by the hepatocytes and later secreted into the bile canaliculi. The bile canaliculi are dynamic structures capable of distension and contraction, as demonstrated by Oshio and Phillips (1981). At that time, it was not clear whether bile canaliculi simply dilated and collapsed passively, or they actually contracted actively. Microscopic studies by French and Davies (1975) showed the presence of membrane-associated actin microfilaments along the bile canaliculi and the microvilli. These authors suggested a role for these proteins in canaliculi contraction.

Both Watanabe et al. (1983) and Phillips et al. (1983), by using phalloidin and

cytochalasins B and D, respectively, have demonstrated that one can abolish the contractile activity of the bile canaliculi by interfering with the action of actin. However, the controversy as to whether the bile canaliculi contracted or not remained unresolved until, 1985, when Smith et al. (1985), Watanabe and Phillips (1984), and Watanabe et al. (1985) further demonstrated the non-random nature of the spontaneous contraction of bile canaliculi seen *in vitro* following calcium ion microinjections. Kawahara et al. (1990) demonstrated that vasopressin regulates the contraction of the bile canaliculi in culture. Watanabe et al. (1991) demonstrated that bile canaliculi did indeed contract.

Despite the observations showing that fluorescein diacetate is a good model for studying the secretory function of hepatocytes, it is important to keep in mind that the conclusion derived from such a model in hepatocyte culture may not represent exactly what happens in the organ *in vivo*, when bile is secreted.

The hepatocyte culture model simulates a clearly abnormal phenomenon. The bile canaliculi in the intact liver are open at one end, towards which they evacuate their content. Thus, the system is an open one, with an unidirectional flow. In contrast, the bile canaliculi in culture are a closed system. The fluorescein diacetate secreted by the hepatocyte fills the bile canaliculi, which dilate under the pressure induced by continued secretion. The commonly observed leak of fluorescein diacetate into the culture media is believed to be due to the disruption (leak or rupture) of the tight junctions limiting the canaliculi. Phillips et al. (1982) have demonstrated this; they showed that the canalicular contraction, which would normally propel bile along the canalicular system, actually forces the fluorescein through the tight junctions.

The hepatocyte is a polarized cell with microvilli forming at the surface bordering Disse's spaces. Along the margin of the bile canaliculi there is close contact of the two adjacent cellular membranes forming occluding junctions comparable to the zonula occludens described in other epithelial tissues. The two bands of tight junctions are believed to seal the canaliculus and prevent leakage of bile into intercellular spaces. The cytoplasm immediately adjacent to bile canaliculi is free from organelles.

1.1.3 The Functions of the Liver

The liver is the metabolic center of the body. The synthesis, breakdown, storage, and excretion of many substances, both nutrients and toxins, occurs in the liver. There are eight major categories of functions: bile formation and excretion, fat metabolism, protein metabolism, carbohydrate metabolism, metabolism of the vitamins, blood coagulation factors, detoxification, and phagocytosis (Kupffer cells) functions.

The first category of functions - bile formation and secretion - will be discussed briefly in the following paragraphs. The other categories of functions have been summarized in the classic treatise of Lehninger (1976). The transformation and breakdown of carbohydrates, proteins and fats are referred to as intermediary metabolism.

The bile is a major product of the hepatocytes. This viscous liquid is composed of water (97.0%); bile salts (0.7%) which are sodium or potassium salts of bile acids conjugated to glycine or taurine; bile pigments (0.2%) consisting of the breakdown products of haemoglobin (biliverdin and conjugated bilirubin); inorganic salts (0.7%); fatty acids (0.15%); phospholipids - mainly lecithin (0.1%); fat (0.1%) - mainly cholesterol; and a small quantity of mucin, alkaline phosphatase and several metabolites

(from Review of Medical Physiology, Ganong, 1981). The osmolarity of the bile is 300 mOsm, similar to the plasma osmolarity.

The bile salts represent the main route for cholesterol elimination by the body. The majority of the bile acids (95%) that enter the duodenum are reabsorbed at the level of the distal ileum. This is referred to as the enterohepatic circulation of the bile salts.

The bilirubin is the main product of the haemoglobin breakdown. This breakdown is performed mainly in the reticuloendothelial system of the bone marrow, the spleen and the liver (Kupffer cells). The breakdown consists of an opening of the heme ring followed by the separation of the iron from the globin to form the biliverdin, which is then reduced into unconjugated bilirubin. The unconjugated bilirubin (hydrophobic) bound to albumin is brought to the liver where it is conjugated with glucuronic acid. The conjugated bilirubin is then secreted into the bile canaliculi and is excreted into the bile. About one third of the bilirubin will re-enter the enterohepatic circulation as the metabolite urobilinogen.

1.1.4 The Cellular Integrity

In summary, for the hepatocyte to perform its various functions, it must preserve its polarity. Through its lateral surfaces, the cell receives nutritive material, hormonal factors, oxygen, toxic substances, steroids, drugs and nonconjugated bilirubin. From the sinusoids and through the same surfaces or poles, it secretes proteins, glucose, carbon dioxide, ketone, urea, toxic substances, steroids, and drugs. Through the biliary pole, it secretes the components of the bile (including conjugated bilirubin, cholesterol, bile acids, water and electrolytes) and many of the conjugated by-products produced by the various

detoxification reactions.

Kawahara et al. (1990) showed that the intermediate filaments were part of the pericanalicular sheath and were essential to the functioning of the canaliculi. When cultures of hepatocytes were treated with nickel, the bile canaliculi were destroyed, the intermediate filaments detached from the plasma membrane, and the microfilaments became dispersed over the whole hepatocyte surface. This led to the loss of attachment between adjacent hepatocytes and the loss of the pinocytotic function, as measured by the hepatocyte uptake of horse radish peroxidase.

Thus, the function of the cells depends on the integrity of the bile canaliculi and the relationship between its lateral surfaces and the sinusoids. The cytoskeleton of hepatic cells plays an important role in maintaining the structure and function of bile canaliculi. Probably, it is also responsible for the organization of the transit of substances to and from the blood and biliary poles of the cell. On the other hand, this architecture depends on the integrity of the connective tissue, the reticular framework, and the cytoskeletal organization of the cell.

1.2 THE CYTOSKELETON

1.2.1 The Cytoskeleton

The concept of a three dimensional cytoskeleton in cells was originally advanced around 1870. In eucaryotic cells, this network of protein filaments is believed to play an important role in the preservation of cellular shape, internal organization and the spatial arrangement and movement of organelles. It is also important for cellular movements along the substrata, cell division, special functions such as protein transport, cellular

absorption and secretion, and cell-cell interactions.

The cytoskeleton is composed of three main types of filaments: thin filaments (actin), intermediate filaments, and the microtubules. Actin filaments were once thought to be present in muscular cells only, but are now known to be present in almost all cell types (Lazarides and Weber, 1974; Adelstein, 1982). They are likely responsible for the viscoelastic and contractile properties of the cell.

Actin is a thin filament of approximately 7 nm. It is found in abundance in skeletal muscle cells, and is present in almost all cell types, especially at the periphery. It is also found in special arrangements along bile canaliculi (French and Davies, 1975) and at the base of microvilli. It is an important component of the myofibril structure. At least six different types of actin are synthesized in vertebrates. Four of these types are called alpha-actins and are found in muscle cells only. The two other types are called beta- and gamma-actins, respectively, and are found in the cytoplasm of all cells. The different types of actins have slight differences in their amino acid sequences. All polymerize into a double helix. All stimulate the ATPase activity of muscle actin. G-actin is a globular subunit of 55 Å formed by a single polypeptide of 41,800 MW. It is usually found dispersed in the cytosol and its density is similar to that of water. F-actin is the polymerized form of the G-actin. It has a higher density (at least *in vitro*), and it significantly increases the viscosity of the cytosol (Pollard and Craig, 1982).

The microtubules have a complex, cylindrical architecture, with a diameter of approximately 25 nm and a wall thickness of 5 to 7 nm. The microtubule is composed of 13 protofilaments, each composed of a pair of linear polymers of tubulin of 55,000

MW each. The microtubule filament is formed by the polymerization of tubulin (the globular dimer alpha plus beta). It has a polarity and may be assembled or disassembled (Dustin, 1980, Margolis and Wilson, 1981). There appears to be a dynamic equilibrium in the cytosol between the two forms. The rate of tubulin addition and removal at the positive (+) end appears to be about twice as fast as that at the negative (-) end. The microtubule grows in length when the net addition of molecules exceeds the net loss. A steady state exists between the free tubulin subunit concentration and the microtubule assembly-disassembly reaction under certain conditions (Kirschner and Mitchison, 1986).

1.2.2 The Intermediate Filaments

The intermediate filaments are a large class of heterogeneous filaments (Table 1). The intermediate filaments were discovered by Sternlieb (1965), who proposed that they were constituents of the cytoskeleton and they played a structural role in maintaining cellular shape in vertebrate cells. Their role in the cells is still poorly understood. Their close association with the nuclear envelope and the cell membrane suggests an involvement in signal transduction. It is also believed that they play a major role in cytoplasmic organization. Metuzals et al. (1974) studied the cytoskeletal attachment to cell structures in neurons. They proposed the concept that neurofilaments fulfil a transducing function through their link between the cellular membrane and the nuclear pores. This connection of filaments was later confirmed in hepatocytes (Irie et al., 1982). Recently, Goldman et al. (1985) proposed that they may possibly serve as the connector between the cellular surface and the nucleus.

Since then, intermediate filaments have been observed to be present in most

eucaryotic cells, and to exist in a wide range from 40 to 70 kDa (Table 1). Their size is intermediate between actin and microtubules; hence their name. They measure between 8 and 10 nm in diameter. Little is known about their assembly. No evidence exists to indicate if they treadmill, or whether an unpolymerized pool exists; however, a more dynamic state is likely to exist. The regulation of intermediate filaments appears to be more complex than the equilibrium in general chemical reactions.

The assembly of the protofilaments is not yet fully understood, but the primary (polypeptide chain) and the secondary (alpha-helix) structures in bovine epidermal keratin filaments were described by Steinert et al. (1985). There are three proteolytically defined domains: the C-terminal head, the central rod, and the N-terminal tail. The rod segment is a constant alpha-helical structure of 312 amino-acids. The head and tail segments are non-alpha-helical structures, and show variability in their segments of amino acids as well as in their length. Each segment may consist of a sequence ranging from zero to 100 amino acids (Steinert et al., 1985) (Figure 5).

Models of the intermediate filament structure based on dimers of interchain alpha-helix are supported by chemical cross-linking experiments on desmin rods from chicken muscle cells (Geisler and Weber, 1982). The formation of the quadrary structure is not yet understood. A trimer model consisting of triple-stranded coils has been proposed by Steinert et al. (1980, 1985), and a tetramer model consisting of the alignment of a pair of two-chain coiled-coil molecule was proposed by Weber and Geisler (1982, 1985), Geisler et al. (1985), and Quinlan et al. (1985). (Figure 6). More recently, Potschka (1986) postulated a model for the formation of vimentin filaments containing multiples

of the previous tetramer model. These could contain as much as 28 or 32 subunits in a discal formation, and a series of such discs would form a filament. This would require further binding levels which are not yet described. Also, neither the number nor the arrangement of protofilaments is yet established.

Lazarides (1980, 1982) described these intermediate filaments as the main filaments responsible for the mechanical integration of the various structures of the cytosol space, and Goldman et al. (1985) advanced that the intermediate filaments may play an important role as a cell surface-nucleus signal transducer. The intermediate filaments are cell type and tissue specific, and may be classified into 5 types. The Type I and II intermediate filaments are the largest group; they are the cytokeratins found predominantly in epithelial cells. Type III intermediate filaments include the vimentin (in mesenchymal cells), the desmin (in muscular cells), and the glial-fibrillar filaments (in astrocytes). The neurofilaments (in neuronal cells) and the nuclear lamine are classified as Type IV and V intermediate filaments, respectively (Steinert and Roop, 1988; Table 1).

Recently, Coulombe et al. (1991) and Vassar et al. (1991) demonstrated that transgenic mice expressing a mutant keratin had abnormal bullous epidermis. The skin of the mice suffered a disease which resembled epidermolysis bullosa simplex, a group of human skin disease. Furthermore, they demonstrated a strong correlation between the severity of the disease, and the extent of disruption of the network of cytokeratin filaments in the basal layer of the epithelium.

Extensive studies are presently undertaken to identify other possible functions of

intermediate filaments, aside from their structural roles. These studies concentrate especially on the possible role of intermediate filaments in cellular differentiation. It has been postulated that the subunits of the intermediate filaments may have a function that is not necessarily related to their filamentary formation. The intermediate filaments could be post-translationally modified polypeptides in response to intra or extracellular signals. Recently, Traub et al. (1983), Traub and Vorgias (1984) and Traub (1985) conducted *in vitro* studies on vimentin, which suggested that in at least some intermediate filaments, proteins of non-epithelial origin were nucleic acid binding proteins. Traub (1985) hypothesized that these independent intermediate filaments would enter the nucleus and bind to a DNA to activate some nuclear processes such as mitosis and gene expression. The details of such a mechanism are still unclear, but evidence in support of the hypothesis of intermediate filaments binding to DNA is accumulating.

The intermediate filaments are insoluble in physiological buffers. This may lead to an erroneous impression of their being a static structure with only a cellular structural role. Since a pool of solubilized intermediate filaments subunits has not yet been identified, the only evidence for a more dynamic role is the presence of the coiled-up intermediate filaments attached to the membrane during mitosis, as observed by Lane et al. (1982) and Franke et al. (1982). Depolymerization of intermediate filaments may occur at times, but this has not been confirmed.

Traub (1985) and others have searched for an alternative function for intermediate filaments. Traub postulated that the filaments may be a form of storage and transportation for the intermediate filament subunits, in addition to their structural role. These

polypeptide subunits may play a vital role in various cellular functions, such as gene expression, and may respond to intra or extracellular signals. Nelson and Traub (1983) hydrolysed vimentin and desmin by activating specific neutral proteases with calcium. Traub (1985) attempted to ascertain his postulate by showing that intermediate filaments of non-epithelial origin were nucleic acid-binding proteins. It was speculated that the subunits would become active inside the nucleus and could control nuclear processes such as mitosis, gene expression or processing, and transport of nuclear RNA. This postulate is not inconsistent with the structural role (Lasek and Hoffmann, 1976). The works of Traub and others suggest that the intermediate filaments play a dynamic as well as a structural role.

1.2.3 The Cytokeratins

The cytokeratins are the largest group in the family of intermediate filaments (Table 1). To date, two-dimensional gel electrophoresis has identified approximately 30 different subunits of cytokeratins in human epithelium (Moll et al., 1982, Steinert and Roop, 1988). The cytokeratins are a large multigene family comprising two types of polypeptide subunits: one acidic (type I) and one basic (type II). The two types are normally synthesized in pairs. Their pattern of expression is differentially regulated and cell type specific (Moll et al., 1982). Osborn (1983) reported that these two types of subunits have different primary amino acid sequences, different isoelectric points (pH 5 to 8) and antigenic determinants. Their distribution in two-dimensional gel electrophoresis is also different. Sun et al. (1985) and Eichner et al. (1984) hypothesized that the phosphorylation on serine (a posttranscriptional modification) of the subunits of a specific

cytokeratin may be located at different sites on the molecule and therefore the isoelectric point is different on the gel electrophoresis.

The two main cytokeratins identified in rat hepatocytes are the cytokeratins of 49 and 55 kDa, corresponding to human cytokeratin number 18 and 8, respectively (Moll et al., 1982). They constitute an acid-base pair (type I-type II). Quinlan et al. (1984) isolated cytokeratin subunit complexes (CK55 and 49 kDa) in a ratio of 1:1 by solubilization of cytokeratin residues from rat liver and cultured hepatoma cells in low salt buffers containing 4M urea.

The variation in the type of cytokeratins found in various cell types has led Sun et al. (1984) to suggest an evolutional role in keratins, to explain the cellular differences between various types of epithelial tissues and those between epithelial tissues of various species. It has been repeatedly demonstrated that intermediate filaments are reliable cell markers for partly differentiated neoplasia because they often retain the characteristics of the cells of origin (Osborn, 1983). The intermediate filaments typing is therefore very useful in the pathological diagnosis of tumor tissue in addition to cellular differentiation.

During cell differentiation, the function of intermediate filaments, and especially of cytokeratins, possibly varies with changes in the expression of the different subunits of the cytokeratin. Brulet et al. (1980) and Oshima et al. (1983) have shown that certain vertebrate cells do not express intermediate filaments in early embryogenesis, and, from *in vitro* studies, that intermediate filaments are not necessary for proper cellular function. Transport may well be one of the functions of intermediate filaments, since they are deployed from the nuclear membrane to the plasma membrane. The cytokeratins consist

of a highly complex multigene family of proteins, subsets of which express differentiation of various cell types (Steinert et al., 1984).

1.2.4 The Cytoskeleton of Hepatocytes

Hepatocytes are epithelial cells and their cytoskeleton is composed of thin filaments of actin, intermediate filaments, and microtubules.

In healthy hepatocytes, the actin filaments, microtubules and cytokeratins are arranged around the bile canaliculi to form the pericanalicular sheath (Oda et al., 1974, French and Davies, 1975, French et al., 1982). The microtubules and the intermediate filaments are also found as a network in the cytoplasm. The intermediate filaments are thought to serve as a connection between the nuclear membrane and the plasma membrane (French et al., 1987, 1989) (Figure 7). The preservation of an intact cytoskeleton is crucial for maintaining cellular contact, growth and function of the hepatocytes.

1.2.5 The Dynamic Role of the Intermediate Filaments

The intermediate filaments were traditionally thought to be a static structure whose main role was to preserve the cellular shape and the relative position of cytoplasmic organelles and the nucleus. This original idea still holds, but the intermediate filaments may have many more primordial functions. The fact that the various types of intermediate filaments differ in subunits, number, molecular weight, cellular distribution and organization, immunological and biochemical properties, supports their potential role in non-static functions in various types of cells.

The modern view of the cytoskeleton as a non-static structure with "dynamic

reciprocity" among the various cellular components was advanced by Peters in 1956. This "dynamic reciprocity" model was later supported by Bissel et al. (1982). The latter authors argued that the extracellular matrix may send "physio-chemical" messages to the cellular membrane receptors, which would be transmitted to the nucleus via the intermediate filaments. This model would involve a network of structurally interconnected molecules that could convey messages from the surface membrane of the cell to the inside of the nucleus and ultimately alter the chromatin.

1.3 CELLULAR DIFFERENTIATION

1.3.1 Cellular Differentiation *in vivo* (Embryo)

The term cellular differentiation refers to the maturation process of an immature cell, in which the cell undergoes cellular modifications to permit it to perform specific functions that are characteristic of its cell line. Usually, during the differentiation process, the mature cell loses its versatility and some of the functions characteristic of the immature cell. The phenomenon of differentiation is therefore related to the capacity to perform specific functional activities and to lose some (or all) of its proliferative and adaptation capacities.

The embryology of the liver is very similar among mammals. The hepatic system originates from the endodermal foregut at a site close to what will become the duodenum (Moore, 1988). The liver develops from the hepatic diverticulum of the embryo. Its caudal portion rapidly gives origin to the cystic duct and, in some mammals, the gallbladder, while the cephalic portion gives origin to the liver cell plates. (The gallbladder is absent in rats.) The cell plates extend ventrally into the splanchnic mesoderm of the septum

transversum. The endodermal cells are irregularly arranged around vascular plexus, branching off from vitelline vessels, and differentiate into liver cells. The cells limiting this primitive vascular plexus will differentiate into Kupffer cells. Soon, the liver occupies the largest volume inside the abdomen of mammals.

In rats, the haematopoietic function is active around the sixth week of gestation. Bile canaliculi are present along with bile canal and intrahepatic ducts around the same time and becomes immediately functional, as bile pigments are formed and excreted into the duodenum. All these functions appear between the 13th and the 16th week of gestation in human.

Le Douarin (1975) showed that hepatoblasts do not differentiate from the endoderm unless a cell contact is established with the mesoderm, and that after weanling, the liver of the rat becomes mature and loses some of it proliferative capacity. As the hepatoblast differentiates into mature hepatocyte, it loses some of its adaptative properties.

The origin of bile ducts is still controversial, as to whether it originates from periportal hepatocytes (cephalic) or hepatic duct (caudal). Shirojiri and Katayama (1987) studied the formation of bile ducts during the hepatogenesis of mouse embryo. They observed the discontinuous lamin immunofluorescence pattern around the portal vein branch. As the bile ducts developed, they concluded that intrahepatic bile ducts originated from discrete cell population (type I hepatocytes) and later became confluent, and not from the cells of the hepatic ducts; therefore, they would have a cephalic origin.

1.3.2 Cellular Differentiation in Regeneration

Organ regeneration is a phenomenon of great biological importance. Despite its

cellular maturation, the liver, unlike various other tissues, is capable of regeneration under certain conditions. The normal adult hepatocyte has little turnover and rarely undergoes mitosis. Its life span is believed to be that of the life of the organism. However, when partial surgical hepatectomy or other types of liver destruction take place, experimentally or as a result of disease, adult hepatocytes quickly enter into mitotic activity to regenerate the mass lost. It was shown that it is the mature adult hepatocytes and not a stem cell that divide. The remaining tissues respond as if the partial hepatectomy sends a signal to the remaining healthy hepatocytes to enter into mitosis. In rats, it takes 10 to 14 days to regenerate 2/3 of the liver mass surgically excised. The rate of regeneration in human is not determined, but is known to exist (Orloff, 1981).

Some species have extraordinary regenerative capacity, not only for the tissue mass of an organ, but also for multi-tissue systems such as an entire limb (in salamander).

1.3.3 Cellular Differentiation in Culture

The characteristics of hepatocyte differentiation may be divided into morphological and functional characteristics. The main morphological characteristics are the presence of microvilli and a cellular polarity associated with the presence of bile canaliculi. An important functional characteristics of hepatocytes that is closely related to the morphological characteristics is the secretion of bile. During the differentiation process, the hepatocytes lose part of their capacity to produce alpha-feto protein, as well as their capacity to massively enter into phase S of the mitotic cycle. Albumin is secreted by mature hepatocytes in culture even in the absence of bile canaliculi formation (Marceau et al., 1985).

The cytoskeleton of rat hepatocytes in culture is largely dependent upon its environment. The shape and functional capacity of hepatocytes are largely dependent upon the cell adhesion and the cytoskeletal arrangements that take place *in vitro*.

The cell adhesion to the substrata and the cellular migration to form cell contacts are known to be influenced by the extracellular matrix composition. Fey et al. (1984) and Fey and Penmans (1985) studied the importance of the cytoskeletal structure in the three dimensional organization of the epithelial cell. In hepatocytes, one can recognize typical cellular morphology after cellular extraction, by observing the distribution of intermediate filaments along the bile canaliculi.

Baribault et al. (1985) demonstrated that in hepatocyte cultures, most of the immature hepatocytes isolated from pre-weanling rat livers are diploid. These immature hepatocytes can proliferate rapidly compared to mature hepatocytes isolated from adult rat livers, which contain a higher proportion of polyploidic cells. Brodsky and Uryvalva (1978) observed that the proliferative activity is inversely related to the degree of ploidy of the liver cell.

When hepatocytes are put in culture, they are detached from one another (Section 2.1.1), and lose at least some of their characteristics. They lose their polarity and the structure of their bile canaliculi disappears. The isolated hepatocytes can attach to the new matrix of the culture plate (polarization), reorganize and form colonies with other hepatocytes (Deschênes et al., 1980).

The cellular differentiation observed in culture can be promoted or modulated by hormonal and/or chemical factors added to the cellular environment. Baribault and

Marceau (1986) studied the effects of dexamethasone and dimethyl sulfoxide on the growth and differentiation of cultured rat hepatocytes. Some of these differentiating factors will be briefly reviewed in Section 1.3.4.

In culture, a number of morphological changes are observed during cellular differentiation in various cells, including hepatocytes. They are related to: (1) the cellular ability to attach to the substrata (fibronectin, collagen, plastic); (2) the cellular configuration change; (3) the capacity of the cells to establish intercellular contacts and junctions; (4) the capacity to perform cell-type specific functions which are characteristic of the cell line; and (5) the ability in some cell lines to perform specific functions related to the reproductive process.

In culture, the hepatocytes have some specific characteristics which are expressed in differentiated mature hepatocytes. They include the cessation of alpha-foeto protein production, a characteristic of immature hepatocytes and of certain malignant neoplasia; and the preservation and increase of albumin production. Morphologically, the formation of bile canaliculi between adjacent hepatocytes is very characteristic of mature hepatocytes, and is closely linked to the mature hepatocyte function (bile secretion). Also, bile canaliculi are not present in other cellular types and are by themselves a specific characteristic of hepatocytes. The presence of bile pigments in culture of hepatocyte is another characteristic.

Finally, because the structure of the differentiated hepatocyte is so closely linked to its function and morphology, it is important that one or more morphological parameters be used in cultures to assess differentiation. The single most important characteristic is

the hepatocyte polarity. This polarity is closely linked to the vascular supply (sinusoid) and the bile formation (bile canaliculi) *in vivo* (see Sections 1.1.1 and 1.1.2). The establishment of polarity and the formation of bile canaliculi are good end-point criteria for judging the degree of hepatocyte differentiation in culture. These were the criteria chosen in the present study.

1.3.4 Differentiating Factors Used in Culture

A number of differentiating factors have been studied on hepatocytes in culture. The variation of the cytoskeleton expression during cellular differentiation in culture has been studied by numerous authors. The following briefly outlines the effect of three of these factors (dexamethasone, dimethyl sulfoxide and retinoids) on cellular differentiation *in vitro*, and describes some of the work done on hepatocyte culture.

1.3.4a Dexamethasone

It is beyond our scope to review the extensive literature on the effects of dexamethasone on differentiation of various cells in culture. It suffices to note that dexamethasone has been shown to modulate cellular proliferation and to promote tissue differentiation in various types of cells.

In hepatoma cells, dexamethasone treatment results in loss of cell growth *in vitro*, increases albumin production, and decreases cellular division (Higgins and Borenfreund, 1980, Higgins et al., 1983). In normal hepatocytes cultured in monolayer, it suppresses alpha-foeto protein production and DNA synthesis (Belanger et al., 1981, Marceau et al., 1982, and Baribault et al., 1985). These two cellular functions disappear in hepatocytes fully differentiated *in vivo*.

Marceau et al. (1986) observed a selective dose-dependent increase in cytokeratin synthesis by hepatocytes when dexamethasone was added to a serum-free culture medium which was supplemented with insulin. Baribault et al. (1989) observed an increase in cytokeratin synthesis when epidermal growth factor and insulin were added to a similar culture medium. In the latter case, the epidermal growth factor promoted hepatocyte growth and maintained alpha-foeto protein production - both characteristics of less differentiated hepatocytes. In addition, there was a rapid re-distribution of cytokeratin filaments from the surface membrane to the cytoplasm, resulting in a disruption of the pericanalicular sheath.

1.3.4b Dimethyl Sulfoxide

The bulk of previous studies of dimethyl sulfoxide were on neoplastic cells in culture. Dimethyl sulfoxide induces differentiation in numerous cultured neoplastic cells, especially in highly undifferentiated embryogenic teratocarcinoma cells. McBurney et al. (1982) cultured totipotent cells from a embryonal carcinoma source with dimethyl sulfoxide, and found these highly undifferentiated cells to differentiate into myoblastic-like cells with actin in their cytoplasm. Dimethyl sulfoxide was also shown to promote cellular differentiation in various neoplastic cultures.

The effect of dimethyl sulfoxide on cultured rat hepatoma cell lines has been studied. Higgins et al. (1983) observed, after adding dimethyl sulfoxide to the culture medium, a suppression of uncontrolled cellular division characteristic of hepatoma cells. They also observed an increase in the secretion of albumin, and a decrease in the expression of gamma-glutamyl transpeptidase in these hepatoma cells. Baribault and

Marceau (1986) observed that the addition of dimethyl sulfoxide, at a concentration of 2% in the culture medium, inhibited hepatocyte growth, but at the same time supported the production of both alpha-foeto protein and albumin, regardless of the hormonal growth regulators used.

1.3.4c Retinoids

The importance of nutritional retinoids (vitamin A) in cellular growth and differentiation is well known. However, the effect of retinoids at the cellular level is not well understood. Until recently, retinoic acid was considered only as a toxic substance at high concentrations, and retinol was considered the only substance of the group to be of physiological value, its lack being responsible for poor night vision and various skin conditions.

Green and Watt (1982) observed that the previously normal epithelial lining of the trachea in rabbits fed with a vitamin-A deficient diet presented squamous metaplasia. However, when the squamous metaplastic epithelial cells were placed in culture and incubated with an enriched retinoic acid medium, the metaplastic cells reversed to normal differentiated epithelial cells.

Amatruda et al. (1985) observed that the presence of retinoic acid in media in which neuroblastoma cells were cultured, suppressed the expression of an oncogenic gene (named N-myc) which is normally present in these tumor cells. Sidell (1982) demonstrated a suppression of the growth curve in these same cultures when retinoic acid was added in the culture media.

Speers and Altmann (1984) transplanted a control quantity of undifferentiated

embryonal carcinoma tissue into the subcutaneous tissue of six mice. The mice were then treated with retinoic acid for 100 days. Four of the mice showed no residual embryonal carcinoma and had mainly benign teratomas; three of them had mitotically active areas. The remaining two mice showed progressively growing tumors: a pure chondrosarcoma in one and a mixture of glioma and chondrosarcoma in the other. In both mice, the tumors were potentially lethal but were more differentiated than the tumor implanted originally. Using cytogenetic and DNA analysis, Speers and Altmann (1984) demonstrated that both tumors had retained the marker chromosomes of the original tumor cell line but that they had a different karyotype. It is important to note that these observable changes in the differentiation level of cancer cell lines, after retinoic acid addition (or treatment), involve some degree of change in the intermediate filaments of those cells. Also, the level of cellular retinol (and retinoic acid) binding proteins was increased in the embryonal cells treated with retinoic acid (Eriksson et al., 1986).

Thaller and Eichele (1987) proposed a totally new concept of the potential role of retinoids in the organism. They reported that retinoic acid was endogenously present in a gradient concentration across a developing chick limb bud. It was postulated that the posterior tissue can enzymatically synthesize retinoic acid, and then secrete it to the rest of the limb, hence creating a gradient in the concentration of retinoic acid along the limb bud and directly influencing cells in this manner. In addition, it was postulated that retinoic acid in the posterior compartment has a local permissive effect that maintains posterior cells in the posterior compartment, while retinoic acid in the anterior compartment has no pattern formation function. To date, neither of these postulates has

been confirmed. More recently, Durston et al. (1989) showed a similar pattern in the nervous system. They linked an antero-posterior transformation in the developing central nervous system to the concentration of retinoic acid found in various locations.

The presence or absence of retinoic acid was known to produce dramatic effects on the pattern of regenerating amphibian limbs after amputation (Thoms and Stocum, 1984).

Our present knowledge of the effect of retinoic acid on hepatocytes is limited. Denk and Lackinger (1986) suggested a relationship between the formation of Mallory bodies and a state of vitamin A deficiency. Also, retinoic acid was shown to slow down the growth of a transplanted hepatoma (Morel-Chany et al., 1986). The toxic effect of vitamin A is better known, and is well summarized in the book by Ruebner et al. (1991). The administration of an excessive amount of vitamin A leads to focal lipid accumulation in the Ito cells, which may progress to fibrosis and even cirrhosis. No study of the effects of retinoic acid on differentiation of hepatocytes has been reported to this date.

1.4 HEPATOCYTES IN CULTURE

1.4.1 The History of Hepatocyte Culture

Human liver cell cultures were first obtained by adapting the procedures developed by Coon for culture of avian cells (Coon, 1966, 1968). His technique involved a mechanical separation of the cells from the tissue before being put in culture, followed by repeated passages of clones to obtain a pure culture.

This technique was applied to primary cultures of rat hepatocytes by Cahn et al. (1967). The liver fragments obtained from rats were washed and minced with scissors or

scalpel in 1 to 2 mm fragments. The small fragments were gently agitated in a solution containing 0.1% collagenase. The supernatant solution containing the suspended cells was then sedimented and the pellet was subsequently seeded on a culture containing a rich media. After 2 to 3 weeks, when the colonies were approximately 1 mm in diameter, they were re-seeded. This technique would lead to a plating efficiency in the order of 50%. Unfortunately, only 0.01 to 0.1% of the colonies yielded hepatocytes depending on experimental conditions and the age of the specimen used. As reported by Kaighn (1973), the technique gives similar results from fetal, infant and adult human liver specimens obtained from autopsy or after surgery. In all cases, the proportion of colonies yielding hepatocytes was small whatever the number of re-seeding or clone passage used.

Berry and Friend (1969) introduced a two-step collagenase perfusion method of the liver in the living animal, and succeeded in enhancing the viability of the isolated hepatocytes when cultured. Other perfusion methods, using collagenase alone (Laishes and Williams, 1976a, 1976b) or in association with hyaluronidase (Bonney et al., 1974), improved the viability of various types of isolated cells. However, the majority of the seeded hepatocytes died rapidly and these cultures were short-lived and usually non-proliferating (Bissel et al., 1973). Seglen (1976) modified the technique of Berry and Friend (1969) in the following manner: the perfusate was introduced via vascular pathway and the liver was digested *in situ* instead of in a glass container. It was recognized that the method of isolation of hepatocytes was crucial to the success of the culture. The suspension of hepatocytes obtained in this manner contained a much larger proportion of live and healthy hepatocytes at the time of seeding. Undoubtedly, this method enhanced

the attachment capacity of the cells to the culture surface.

Isolated hepatocytes were thus available to be used for various research experiments. Hepatocyte suspensions are used to prepare cultures on which studies can be performed, or for transplantation purposes especially in the field of tumor research (Laishes and Farber, 1978, Mito et al., 1979, Jirtle et al., 1980).

1.4.2 Evolution of Methods of Maintaining Rat Hepatocytes in Monolayer Cell Culture

The methods used in the present investigations were the result of numerous improvements suggested by a long series of increasingly successful attempts of maintaining rat hepatocytes in culture. Deschênes et al. (1980) adapted Seglen's perfusion method (Seglen, 1976) to pre-weanling rats, and obtained cultures of hepatocytes of even greater quality. Deschênes et al. (1980) maintained the concentration of collagenase and calcium ions in Seglen's perfusion solutions (Seglen, 1976), and searched for the optimum conditions for rates and durations of the two perfusates. It was determined that up to approximately 96% of the cells isolated with this method were hepatocytes. The composition of the culture medium used in newborn hepatocyte cultures was modified to eliminate the need of serum, minimizing the number of modifications to the culture medium which might affect the interpretation of experimental observations. The final medium used by Deschênes et al. (1980) was composed of commercially available William's E medium, supplemented with dexamethasone, insulin, l-ornithine, penicillin, streptomycin and fatty acid-free serum bovine albumin (Appendix A.2). The immature hepatocyte appears to have a greater capacity of surviving as isolated cells, attaching to

the fibronectin surface, and forming hepatocyte colonies with appearance characteristic of redifferentiation. The organization of the cytoskeleton of these hepatocytes was found to have some common characteristics with that observed *in situ* (Marceau et al., 1983).

Marceau et al. (1983) examined the hormonally-induced changes in the cytoskeleton and compared their organization between cultures obtained from adult and newborn animals. They concluded that dexamethasone and insulin were necessary for differentiation to take place. L-ornithine, an amino-acid present in the urea cycle but not available in the William's E medium, was added to the culture medium. Insulin was added to preserve viability (Laishes and Williams, 1976a). Penicillin and streptomycin were added to prevent bacterial contamination of the cultures. Serum bovine albumin was added as a source of protein and as a neutralizer for fatty acids released by dying cells. A fatty acid-free composition for the serum bovine albumin prevents the lytic effects of fatty acids on cellular membranes. This adaptation of the two step collagenase perfusion method of the liver *in situ* and the use of the culture media outlined earlier gave the best colonies of hepatocytes to date.

1.4.3 The Surfaces of Attachment

A surface promoting cellular attachment is crucial, since the monolayer cell culture will die quickly if the cells do not attach to the surface in an effective manner. It was observed that when serum was added to the culture medium (providing numerous growth substances) an increase in the attachment and viability of the hepatocytes in the cultures were obtained (Laishes and Williams, 1976a, Leffert et al., 1978, Savage and Bonney, 1978).

Hook et al. (1977) reported that, by precoating the surface with purified plasma fibronectin (called cold insoluble globulin at the time), the hepatocytes would attach well even in the absence of serum in the culture media. The high molecular weight glycoprotein fibronectin is composed of a sequence of 108 amino acids. Its secondary structure is a beta-fold. Close to the C-terminal end is an hydrophillic sequence composed of 4 amino acids (Arg- Gly- Asp- Ser) believed to be the site of attachment to the cellular membrane (Pierschbacher and Ruoslahti, 1984) (Figure 8).

Couchman et al. (1982) demonstrated that a surface of attachment (fibronectin) was needed for locomotion and cellular division of chicken fibroblasts. Gibson et al. (1983) suggested that fibronectin contribute to the adhesion capacity of the keratinocytes both in culture and in the formation of the skin. He demonstrated that epidermal cells could synthesize fibronectin and that its distribution was increased in areas of cell-cell and cell-substratum contact.

Collagen is an important component of the extracellular matrix. It is believed to be involved in the differentiation of the muscle cells in colonies (Hauschka and Konigsberg, 1966). Also, it influences cell adhesion (Kleinman et al., 1981), cell division (Adamson 1983, Couchman et al., 1982), cell differentiation (Couchman et al., 1979, Reddi and Anderson, 1976), and cell migration (Rovasia et al., 1983).

Early studies suggested that fibronectin binds to collagen and is responsible for cellular attachment. Recent evidence indicates that various types of cell surface components other than fibronectin, such as factor XIII of coagulation (Saito et al., 1986), collage type IV (Kurkinen et al., 1984), proteoglycan (Koda and Bernfied, 1984), may

also attach directly to collagen. Specifically, there is evidence that three polypeptides having molecular weights of 250, 70 and 30 kDa, respectively, behave as cell surface receptors for type I collagen, particularly polypeptides with the Arg-Gly-Asp sequences (Dedhar et al., 1987).

Fibronectin is a substratum that permits good hepatocyte attachment *in vitro*. Nevertheless, an ideal substratum that would perfectly mimic the *in vivo* conditions remains to be found. None of the substrata tested so far for hepatocyte culture has been demonstrated to give culture conditions of unchanged activity of the P-450 enzymes (Lilja et al., 1988). Also, it is important to bear in mind that the testing of most substrata is done *in vitro*, and therefore may not replicate what happens *in vivo*, in the interpretation of the present experiment.

1.4.4 The Kinetics of Hepatocytes in Culture

Deschênes et al. (1980) studied the kinetics of hepatocytes in culture supplemented with dexamethasone and insulin, by comparing the viability of hepatocyte suspension and its capacity to attach to fibronectin substrata and survive in a monolayer culture, between newborn and adult rat hepatocytes. They found variation in these parameters depending on the age of the rat used to produce the monolayer cultures. The newborn hepatocytes were on average smaller than the adult ones at the time of seeding, but gradually increased in size and became more polygonal within the first day in culture. Also, they attached to the fibronectin in a more efficient manner and their viability was superior one hour after seeding. Of the attached cells after one hour, about an equal fraction (80%) was still alive after one day in culture in both the newborn and the adult hepatocyte cultures.

The superior viability of the newborn hepatocyte cultures is particularly evident at the time of isolation and seeding. The hepatocyte spreading (organization in colonies) was faster in the newborn culture (2 hours) than in the adult culture (6 hours). Furthermore, the hepatocytes from both the newborn and the adult rats demonstrated increased capability to produce albumin and decreased capacity to produce alpha-foeto proteins.

Marceau et al. (1985) compared the morphology of hepatocyte monolayer culture under various conditions, and found that the use of dexamethasone and insulin in the culture medium promoted the formation of compact monolayer colonies of hepatocytes with highly ordered filaments in the cells. When the same cultures were exposed to epidermal growth factor and insulin, the colonies spread widely and the cytokeratin filaments were found to be more stretched. Thus, dexamethasone promotes hepatocyte differentiation, while epidermal growth factor promotes cellular growth.

1.4.5 The Human Hepatocytes in Culture

Human hepatocyte culture would be a great source of material for experimental research. However, the two-step collagenase perfusion method described by Seglen (1976) and adapted to pre-weanling rats by Deschênes et al. (1980) cannot be easily implemented on human hepatocytes, because of the technical difficulty associated with the large set-up required to perfuse the large quantity of perfusate that a human organ would require. In addition, ethical considerations preclude the possible use of this method under anaesthetic conditions such as those used in animals. Thus, the source of material is limited to small quantity of liver tissue obtainable from partial hepatectomy. Strom et al. (1982) scaled down the two-step perfusion method of Seglen (1976) for surgical specimen, and obtained

good quality cultures from this source. Unfortunately, this approach is not readily available for routine research. Another possible approach is to adapt a variation of the Seglen method (1976) to liver needle biopsy. However, this approach is technically very difficult considering the small size of the material present in the perfusion and the limited quantity of culture obtained from each needle biopsy.

Primary cultures of human hepatocytes of good and reproducible quality would undoubtedly be of great value for studies on hepatocytes differentiation and intermediary human hepatic metabolism; as well, they would provide a good substrate for the culture of hepatitis viruses or the study of effects of chemical carcinogens.

The cultured hepatocytes behave differently from the hepatocytes *in vivo*, since they grow in monolayers and establish only lateral contacts with other hepatocytes. The colonies in culture are also different because the orderly relationship between hepatocytes and other liver cells does not exist. Finally, the bile canaliculi formed between 2 or 3 hepatocytes in culture do not form a large network with progressively larger canals and a unidirectional flow, but rather a closed system.

These limitations notwithstanding, cultured hepatocytes constitute a relatively good model for the study of hepatocyte differentiation. Differentiation can be analyzed through the study of the organization of the cytoskeleton (especially the cytokeratins) and their response to differentiating factors that are added to their culture media.

1.5 RETINOIC ACID

1.5.1 The History of Vitamin A

The history of vitamin A is a big part of the history of biochemistry. Its link with

illness and diet has been recognized since the age of antiquity. Many diseases that are now known to be related to hypovitaminosis A were treated in ancient medicine by correction of the diet.

Beta-carotene, the precursor of retinol, is found in yellow vegetables and fruits. Carotenes have no intrinsic vitamin A activity per se and must be converted by cleavage into two identical retinol molecules by enzymatic reactions in the intestinal mucosa and in the liver. Retinoid is a generic term that includes natural molecules with vitamin A activities, and synthetic analogs of retinol with or without biological activity. The term vitamin A, strictly speaking, should be used to refer to a biological activity and not to a particular molecule.

Retinoids are stored in acid phosphatase membrane globules of the specialized Ito cells. This storage is mainly in the form of retinyl ester (the esterified retinol). The Ito cells (stellate cells) of the liver normally contain 80 to 90% and the hepatocytes contain the remaining 10 to 20% of the retinoids stored in the liver; minute amounts may be found in other cells (Modern Nutrition in Health and in Disease, Olson, 1988).

A deficit of retinoids in children produces a "dryness" of the cornea, called xerophthalmia, which causes blindness; in adult, it induces night blindness and dry skin (Section 1.5.4). On the other hand, hypervitaminosis has long been known to produce abnormalities in the foetus (Morriss, 1972); the dangers of acute intoxication from hypervitaminosis A were extensively documented among early arctic explorers who consumed large quantities of polar bear liver, where retinoids are stored.

1.5.2 The Chemical Formula and Functions of Retinoids

The Vitamin A (mainly the ester form) was one of the earliest vitamins discovered in fish liver oil (McCollum and Davis, 1915). It was first synthesized around 1937 from beta-ionone, and became commercially available in Switzerland in 1947, where it was developed by Otto Isler Company. Today, analogs of retinoids are synthetically made.

Retinol is a large molecule composed of three segments: a hydrophilic head forming a beta-ionone ring, a conjugated double bound isoprenoid chain which is responsible for the isomerization of the molecule, and a polar terminal alcohol group (Figure 9). The polar terminal group is the site of chemical modifications that form several different metabolites: esterification forms retinyl palmitate, aldehyde forms retinal, and oxidation forms retinoid acid.

The physiological functions of retinols may be divided into three groups: those related to vision, reproduction, and growth, respectively. The vision-related physiological functions are purely a photoelectric reaction, with no enzymatical involvement (Wald, 1960, Dowling and Wald, 1960, Wolf, 1980). The need of retinoids for reproduction was first noted by Evans and Bishop (1922). Later, Thompson et al. (1964) established the need of retinoids to maintain spermatocytogenesis, oogenesis, and fetal development in rats. Retinoid acid supports testosterone synthesis by the Leydig cells (Appling and Chytil, 1981). The precise mechanism is not clear; however, binding proteins have been identified on testis, ovaries, uterus and mammary glands (Mehta and Moon, 1981).

Retinoids play an important role in growth and development, including cellular differentiation. Retinol, retinyl esters, retinal and retinoic acid have all been shown to

support somatic function of growth and differentiation, especially on epithelial structures (Shapiro, 1985).

Research in the role of retinoids on cellular differentiation has so far been concentrated on immature cell lines or cancerous cells. These immature cell lines retain their ability to differentiate, often in more than one direction. Also, they usually preserve a higher turnover rate which possibly makes them more receptive to the influence of differentiating factors.

Brockes (1989) recently reviewed the profound effects of retinoic acid on vertebrate limb morphogenesis. Earlier, Thaller and Eichele (1987) reported the observation of an antero-posterior gradient of retinoic acid in chick limb bud. This led to the hypothesis that retinoic acid may provide a positional information for the growth of the limb. A similar antero-posterior gradient of retinoic acid was also found in the central nervous system by Durston et al. (1989).

1.5.3 The Metabolism of Retinoids in the Liver

The liver plays an important role in the metabolism of retinoids. It is involved at the different stages of the metabolic process, including the cellular uptake and the storage of the retinoids for further utilization. The plasma levels of retinoids remain remarkably constant, and so there must exist an equilibrium between the intake and the utilization of retinoids by other tissues (Goodman, 1980).

The precursor of retinol, beta-carotene, is converted into retinol at the level of the intestinal mucosa. First, beta-carotene is cleaved into two molecules of retinaldehyde by the enzyme beta-carotene-15-15'-dioxygenase; then the retinaldehyde molecule is reduced

to retinol by a second enzyme, the retinaldehyde reductase.

The other precursors of retinol, retinyl esters, are found in some animal tissues. They are first hydrolyzed in the intestinal lumen into retinol before being absorbed by the intestinal epithelial cells. Then, the retinol is re-esterified by the acyl-coenzyme-A-retinol acyltransferase enzyme (Helgured et al., 1983), and absorbed in association with the chylomicrons into the lymphatic system. Almost the entire component of retinyl esters of the chylomicrons is removed from the circulation by the liver, and then hydrolysed and re-esterificated into mainly retinyl palmitate for storage.

In non depleted mammals, the retinoids stored in the liver may account for up to 90% of the total reserve of active retinoids of the body (Underwood, 1984). The storage of retinoids occurs mainly in the stellate cells of the liver (Ito cells). It is possible that hypervitaminosis A toxic effects appear only after the Ito cells are saturated (McLaren, 1981), since the plasma level of retinoids remains remarkably constant over a wide range of dietary intake and liver storage.

1.5.4 The Role of Retinoids on Epithelial Differentiation

Retinoids are essential to the normal differentiation of epithelia (Wolbach, 1954, Wolf, 1980, Elias and Williams, 1981). In the normal human epidermis, the innermost epithelial cells synthesize keratin molecules of 46-59 kDa while the outermost cells synthesize larger keratins (>60 kDa) (Fuchs and Green, 1981).

Fuchs and Green (1981) studied the ability of retinoids to regulate the synthesis of epithelial keratins by controlling the retinoids in the culture of human epidermal cells. They demonstrated that the nature of the keratins synthesized was regulated by the

concentration of retinoids in the culture. In particular, a low concentration of retinoids led to the synthesis of terminally differentiated keratinized epidermal cells (67 kDa), while an excessive concentration reduced the synthesis to keratins in the 40-52 range. This result may have a bearing on xerophthalmia found in Vitamin A-deficient children, where the cornea behaves more like a keratinized epithelia than a conjunctival cell.

Brown et al. (1985) studied the effect of retinoids on cell cultures of cutaneous keratinocytes from newborn rats which were grown in a delipidized serum that was deficient in retinoids. These stratified cultures expressed the same morphological features as epidermis from intact skin. The addition of retinoic acid resulted in an enhancement of the features that are characteristic of a secretory epithelium, i.e., more extensive endoplasmic reticulum, larger Golgi zone and an increased number of vacuoles; and a reduction of features that are characteristic of a terminally differentiated and stratified squamous epithelium, i.e., keratinization. On the other hand, in the cultures maintained in medium of low retinoid concentration, the cells contained densely packed tono filaments. In addition, the cells stained positively to the monoclonal antibody AE2; the latter is specific for keratin polypeptides, and is a marker of epidermal differentiation.

Similarly, Jetten et al. (1987) demonstrated the effect of retinoids on the regulation of differentiation of rabbit tracheal epithelial cells into squamous or secretory type by controlling the concentration of retinoic acid in cell culture.

1.5.5 Gene Expression and Receptors

Retinol circulates bound to a protein, the retinol-binding protein. The human retinol-binding protein has been isolated by Kanai et al. (1968). It is synthesized by the

hepatocytes and consists of a single polypeptide of 21000 MW. Each molecule of retinol-binding protein binds one molecule of retinol to form a retinol-retinol binding protein complex. The primary structure of this retinol-binding protein was determined by Rask et al. (1979), and its three-dimensional structure by Newcomer et al. (1984).

The process by which the level of retinol regulates the synthesis of retinol-binding protein is not yet known. The small fraction of free retinol-binding protein is slowly catabolized by the kidneys, as it is small enough to be filtered by the renal glomeruli (Goodman, 1988). Therefore, it is expected that hepatic diseases would decrease the plasmatic level of retinol-binding protein, while renal disease would increase it (Goodman, 1988).

Cell surface receptors for the retinol-binding protein carrying the retinol molecule are believed to be involved in the delivery process of the retinol to various cells, such as monkey small intestine epithelial cells, bovine pigment epithelial cells, chicken testicular cell membranes and differentiated F9 embryonal carcinoma cells (Eriksson et al., 1986). After the retinol is delivered to the cell, the retinol-binding protein returns to the circulation.

Furthermore, recent studies have demonstrated the intracellular binding proteins for retinol (CRBP) and for retinoid acid (CRABP) in many types of cells. In rats, the Ito cells, the renal cortex, the Sertoli cells and certain cells of the pancreatic islets of Langerhans are particularly rich in CRBP, while the germinal cells and testis are rich in CRABP (Goodman, 1988). Li et al. (1986) reported another intracellular binding protein (CRBP II) that is similar to CRBP but is so far limited to the intestinal mucosal cells.

They postulated that the CRBP II may play a specific role in the absorption of retinol from the gastro-intestinal tract.

Since retinoids are known to play an important role in cellular differentiation and growth, the specific retinoid-binding proteins are believed to be responsible for mediating biological activities which are related to their respective ligand, retinol and retinoic acid, to the nucleus and the chromatin where receptors for retinoids have recently been reported (Petkovich et al., 1987, Crow et al., 1987).

An important advance in recent research of retinoic acid receptors was made by Giguere et al. (1987), who isolated a specific human nuclear receptor for retinoic acid, and called it retinoic acid-receptor alpha (hRAR-α) as a member of the steroid/thyroid hormone receptor family. This was followed by the discovery of a nuclear receptor for retinoic acid in human hepatoma by Brand et al. (1988), who named it hRAR-β; and of a receptor called hRAR-γ, from human breast cancer cells by Krust et al. (1989). The hRAR-γ is the predominant RAR-RNA species in the human skin. This suggests that the hRAR-γ may mediate some of the effects of retinoids observed in epithelial tissues. Another retinoic acid receptor was identified from hepatocellular carcinoma (where it surrounds sites of integration of hepatitis B virus) by Benbrook et al. (1988).

Retinol and retinoic acid may influence the gene expression of the target cells. Denning and Verma (1991) demonstrated the involvement of retinoic acid and nuclear receptors (alpha and beta) in retinoic acid-induced tissue transglutaminase gene expression in rat tracheal cells. However, the specific role of retinoids at the molecular level is not fully understood, despite the accumulation of evidence that link retinoids, intracellular

binding proteins and nuclear binding proteins to gene expression that would lead to cellular growth and differentiation (Goodman, 1988, Crow et al., 1987, Petkovich et al., 1987). For example, there is no good correlation between gene expression and the presence of cellular binding proteins for retinol or retinoic acid, and retinol and retinoic acid binding proteins have been demonstrated in cells that are apparently not targets for retinoids.

Retinoids play a complex range of roles in the cells of the body, including the areas of reproduction, embryologic and cellular growth, and the newly emerging area of cellular differentiation. Many of these functions are increasingly believed to be genetically controlled. The importance of retinol and retinoic acid to the body was recognized only recently, as studies of the effects of retinoids on differentiation in various cell types became available. Previously, such studies were limited to the epithelial cells (normal and abnormal).

In view of the important role of retinoic acid on cellular differentiation and that of the liver as the main storage for retinoids in the body, an experimental study was set up to test the hypothesis that retinoic acid may have a promoting or inducing differentiation effect on hepatocytes in culture.

1.6 EXPERIMENTAL RATIONALE

1.6.1 A Short Background Review

Eucaryotic cells have a three-dimensional cytoskeleton composed of three types of filaments: thin filaments constituted by actin, intermediate ones constituted by cytokeratins, vimentin, desmin, glial-fibrilar, neurofilament, lamins (Table 1) and

microtubules. This complex network of protein filaments plays an important role in the preservation of cellular shape, the internal organization of cellular components, as well as cellular movements, cell division, protein transport and cell-cell interaction. It is necessary for a cell to maintain an intact cytoskeleton, in order to be capable of performing its functions.

Cytokeratin expression varies as cellular differentiation occurs. A number of differentiating factors have been observed to promote or inhibit cytokeratin expression *in vitro*, and hence cellular differentiation. One of the numerous differentiating factors that have been recognized so far is retinoic acid. Since 1987, three specific human nuclear receptors for retinoic acid have been identified (Giguere et al., 1987, Brand et al., 1988, Krust et al., 1989). Retinoic acid and other retinoids are known to induce differentiation in epithelial neoplastic cells and to promote some degree of differentiation in poorly differentiated embryonal carcinoma *in vitro*. A brief review of some examples of cell differentiation obtained from retinoic acid is given below.

Jones-Villeneuve et al. (1982) observed *in vitro* the differentiation of murine embryonal carcinoma cells into a varied spectrum of cell types, such as neuro-like and fibroblast-like types. Sidell (1982) observed that the presence of retinoic acid produced a growth inhibition in human neuroblastoma cells *in vitro*. Also, it induced morphological differentiations such as the formation of long neurites, suggesting an altered expression of the malignant phenotype. Chopra (1983) observed a reversal of the metaplasia of squamous trachea cells of hamsters fed on a Vitamin-A deficient diet, by treating them with retinoic acid in culture. In malignant rat osteoblasts *in vitro* culture, a reversible,

time- and dose-dependent inhibition of the cellular growth with fewer mitotic figures was seen by Ng et al. (1985). Amatruda et al. (1985) demonstrated the suppression of the oncogenic N-myc gene expression in neuroblastoma cell cultures. Brown et al. (1985) observed a promotion of secretory characteristics of cells obtained from newborn rats when retinoic acid was added to the culture media of their cutaneous keratinocytes. The addition of retinoic acid promoted the formation of an extensive endoplasmic reticulum, the enlargement of the Golgi zone, and an increase in the vacuoles. These morphological changes were attributed to a change in the expression of the tonofilaments. Fisher et al. (1987) observed that cultured glioma cells became aligned in pallisades, as in fibroblast like cells, in the presence of retinoic acid.

Some studies of medical conditions that were treated with retinoic acid have reported clinical improvement attributed to the retinoic acid treatment. King et al. (1982) demonstrated in a double blind study of adults suffering from refractory acne that systemic intake of 13-cis-retinoic acid improved the clinical picture as well as reducing the sebum excretion rate and the free fatty acid production. Fontana et al. (1986) reported the case history of a patient, who suffered from acute promyelocytic leukemia which was resistant to conventional chemotherapy, and achieved a 1-year remission when given 13 cis-retinoic acid per os.

Retinoic acid has been shown to induce differentiation in other type of cells, especially epithelial cells. In this study, the effect of retinoic acid on hepatocyte differentiation was investigated. Primary cultures of hepatocytes were treated with retinoic acid, and the formation of bile canaliculi (a characteristic of morphological differentiation

of hepatocytes) was observed. This study focused on the cytokeratin pair 49 and 55 kDa.

1.6.2 The Experimental Design: The Hepatocyte Monolayer Culture

In order to study the effects of retinoic acid on the differentiation of hepatocytes, an *in vitro* technique to obtain monolayer hepatocyte culture was used. The method used was the two-step collagenase perfusion method developed by Seglen (1976) with the adaptation described by Deschênes et al. (1980) for pre-weanling baby rats. To facilitate further comparison with other works, monolayer hepatocyte cultures were obtained from 14 day old male Wistar Charles River rats.

1.6.3 The Preliminary Tests

The details of the preliminary tests are reviewed in Section 2.1, Materials and Methods. The logistics of these tests are briefly reviewed here.

After good-quality hepatocyte suspension (evaluated by the Trypan Blue dye method) was obtained using the method of Deschênes et al. (1980), preliminary tests were performed. Two seeding surfaces were tested to evaluate the cellular attachment: seeding directly on the plastic of the tissue culture dishes, and seeding on a fibronectin coated glass cover slip. The evaluation criterion was the quality of the hepatocyte colonies obtained.

Comparative studies were then performed to define the best culture medium to be used to study hepatocyte differentiation. The experiments from Marceau et al. (1983) (Experiment 1 and 2 below) were duplicated and the bile canaliculi obtained under the following conditions were evaluated: the culture medium using serum-free William's E was supplemented with (1) dexamethasone and insulin, (2) epidermal growth factor and

insulin and, (3) dexamethasone, insulin and foetal calf serum.

The optimal density of hepatocytes in the suspension at the time of seeding was determined by the quality of colonies of hepatocytes observed after 48 hours of incubation for various original concentration of hepatocytes in the suspension.

The optimal time duration for the hepatocytes to be kept in culture before observation was determined by observing the hepatocyte dynamic in forming intercellular contacts and bile canaliculi, and the point of optimal hepatocyte survival under pre-defined conditions.

A solvent was needed to dissolve the retinoic acid powder used in the experiments. This was because the culture medium is hydrophilic, while retinoic acid is a hydrophobic molecule. Dimethyl sulfoxide was tried initially because of it solvent properties and the very small amount needed. Since dimethyl sulfoxide has been reported to promote cellular differentiation in other cell types, a dose response study was first conducted. Determination of the toxic dose, the effective dose on differentiation, and the dose below which dimethyl sufoxide had no observable effects on the hepatocyte culture compared to a control hepatocyte culture devoid of the solvent was determined. A dose of dimethyl sulfoxide which could effectively dissolve retinoic acid and yet did not promote observable differentiating effects on the hepatocytes in culture was chosen.

A dose response study of the effects of retinoic acid on a series of hepatocyte cultures was performed to determine the optimal dose of retinoic acid that maximized the hepatocyte differentiation in culture. This dose should have no toxic effect on the hepatocyte, and should maximize the formation of bile canaliculi per hepatocyte under

predetermined conditions resulting from the preliminary tests described above.

1.6.4 The Experiment With Retinoic Acid

After the optimum parameters for readily reproducible, healthy hepatocyte cultures were established, and the optimal dose of retinoic acid and its solvent determined, the experiments to study the effects of retinoic acid on hepatocyte differentiation in culture were undertaken.

All experiments were repeated at least three times, unless specified otherwise. All experiments included pairs of controls and retinoic acid treated cultures. Two types of controls were used, the first one with the solvent dimethyl sulfoxide but without the retinoic acid, and the second one without the dimethyl sulfoxide and the retinoic acid. This double control was deemed necessary to ensure that the observations were not due to the effects of the solvent or a synergistic effect between the solvent and the retinoic acid.

The end-point criterion of hepatocyte differentiation in monolayer culture was chosen to be the formation of bile canaliculi, which were assessed indirectly through the study of two types of cytokeratins found in the pericanalicular sheath.

The distribution of cytokeratins 49 and 55 kDa found in the pericanalicular sheath of the bile canaliculi were studied in control and in retinoic acid treated hepatocyte cultures using the following methods: indirect immunofluorescence against cytokeratins 49 and 55 kDa, morphometric analysis of bile canaliculi formation, electron microscopy of the intermediate filaments in the extracted hepatocyte preparation, gel electrophoresis studies, and fluorescein diacetate uptake and secretion by the hepatocyte to assess the

functionality of the bile canaliculi formed *in vitro*.

1.6.5 The Hypothesis

Retinoic acid induces differentiation of various epithelial cells *in vitro* (Brown et al., 1985). In the present experiment, the hypothesis is that retinoic acid induces differentiation in hepatocytes *in vitro*. This hypothesis will be tested by examining the bile canaliculi formation.

2. MATERIALS AND METHODS

2.1 PRELIMINARY EXPERIMENTS

This section describes the preliminary experiments done on monolayer hepatocyte cultures obtained from the liver of Wistar Charles River strain rats. After the preliminary testing (Section 2.1.1) was completed, all hepatocytes were isolated for culture in the same manner, and were used to conduct the experiments described in the following sections of this chapter.

2.1.1 Hepatocyte Isolation Technique

The basic method used for hepatocyte isolation was adapted to pre-weanling rats by Deschênes et al. (1980) from the original method of Seglen (1976).

The monolayer cultures were prepared after isolation of the hepatocytes from the liver of 14 day old pre-weanling male Wistar Charles River rats (Deschênes et al., 1980). The animals were anesthesized by intra-abdominal injection of phenobarbital (0.2 mg/g of total body weight) in the left lower quadrant of the abdomen. Under general anaesthesia, the animal was tied to the support board and cleaned from the neck down using ethanol 70%.

Using straight scissors, a vertical medial incision was done from the pubis to the xiphoid cartilage. The surgical incision was then extended bilaterally by sub-thoracic horizontal incisions. Special care was exercised so as not to damage the diaphragm or open any viscera, in order to maintain the respiratory function intact and to prevent contamination.

The gastro-intestinal tract was displaced to the left to expose the portal vein. A 6-0

silk traid was passed through the epiploic foramen, and a loose knot was made over the portal vein (more precisely around the hepato-duodenal ligament). The portal vein was cannulated with a double-lumen intravenous catheter (Jelco 24G; Critikon Canada Inc. Markham, Ontario, Canada), and the cannula was secured in place by tightening the loose knot around it.

The cannula was connected to a perfusion pump set to deliver a flow of 10 to 20 ml/min. When the perfusion was established, the inferior vena cava was sectioned caudally to the right renal vein. A volume of 100 ml of the washing solution (Appendix A.1) was perfused through this system to displace the blood from the liver vascular system. This was followed by a perfusion of the liver with 100 ml of collagenase 0.05% in stock solution (Appendix A.1) to achieve a digestion of the liver structure within the Glisson's capsule.

The liver was removed by fragments, using fine straight scissors and carefully avoiding accidental contamination, and deposited in washing solution (Appendix A.1) in a sterile beaker put on ice. With blunt straight scissors, the liver fragments were minced and gently agitated into suspension.

The hepatocyte suspension was filtered through a nylon mesh. The filtrate was centrifuged at 50G for two minutes and the supernatant was decanted and discarded. Fresh washing solution (Appendix A.1) was added to the pellet and the centrifugation was repeated three times.

Finally, the pellet containing the isolated hepatocytes was suspended in culture media. The viability and the concentration of the hepatocyte suspension were determined

by adding an equal volume of 0.6% Trypan Blue dye solution (Appendix A.1) to the hepatocyte suspension, and calculating the percentage of live hepatocytes as seen under a phase contrast microscope in a hemocytometer chamber.

2.1.2 Culture Media

The effects of three different culture media on the hepatocyte culture were studied to determine the optimal composition of the culture medium to be used for the proposed work.

2.1.2a Serum Free Culture Medium Supplemented with Dexamethasone

This culture medium (Appendix A.2) is composed of the commercially available Williams' medium E (10.8 mg/ml) (Williams and Gunn, 1974) supplemented with dexamethasone (4 μg/ml), insulin (150 ng/ml), ornithine (67.5 mg/l), penicillin (200 μg/ml), streptomycin (0.1 mg/ml), and fatty acid free bovin serum albumin (5 mg/ml), following the method described by Marceau et al. (1982). The use of a serum free medium minimizes the number of parameters that may affect cellular growth and differentiation.

2.1.2b Serum Free Culture Medium Supplemented with Epidermal Growth Factor

This culture medium had the same composition as the one described above (see Section 2.1.2a), except that the dexamethasone was replaced by epidermal growth factor (10 ng/ml; Appendix A.2).

2.1.2c Culture Medium Supplemented with Dexamethasone and Calf Serum

This culture medium had the same composition as the one described above (Section 2.1.2a), except that calf serum was added to the solution (Appendix A.2).

2.1.3 Hepatocytes in Monolayer Cell Culture

2.1.3a Surfaces of Attachment

Two surfaces of attachment were tried: untreated bottom of Falcon tissue culture dishes and fibronectin coated glass cover slips. In the first case, the suspension of hepatocytes was directly seeded on sterile 35mm plastic tissue culture dishes. In the second case, the suspension was seeded on a surface of fibronectin.

To prepare the fibronectin surface, a drop of 5% fibronectin solution (Appendix A.1) in sterile water was deposited on a glass cover slip which was previously left in ethanol overnight. The fibronectin solution drop was let to dry and was sterilized under ultraviolet light for a minimum of three hours, but usually overnight. Fibronectin was used as an attachment surface for immunofluorescence observation and for gel electrophoresis studies of the hepatocytes in culture.

The tissue culture dishes to be used for electron microscopic observations were prepared in similar manner. First, gold grids (G100 and G200) had been coated with 0.5% Formvar in Ethylene Dichloride solution (Appendix A.1) and laid on a 35mm tissue culture dish. Then, a drop of 5% fibronection solution (Appendix A.1) was deposited on the prepared gold grids.

2.1.3b Seeding Density

To determine the optimum density of seeding of hepatocytes, the suspension of isolated hepatocytes was seeded on the fibronectin coated glass cover slips or the Formvar and fibronectin coated gold grids at various quantity of hepatocytes, ranging from 10^3 to 10^5 cells per 35mm tissue culture dish.

2.1.3c Attachment Time

To determine the optimum attachment time needed to produced the best monolayer culture, a known quantity of hepatocytes (ranging form 10^3 to 10^5 cells per 35mm tissue culture dish) was seeded on the fibronectin surface, and left to attach for a fixed incubation period in a humid environment with 5% carbon dioxide at 37°C. Attachment times of 1, 3 and 6 hours were chosen for test. After these periods, the floating dead cells were rinsed away with washing solution (Appendix A.1), and the remaining attached cells were maintained in culture media. The medium was changed once after the first 20 to 24 hours, and the quality of the culture obtained was evaluated after various periods of time (see Section 2.1.3d).

2.1.3d Incubation Time

To determine the optimum incubation time, the seeded and attached hepatocytes were incubated in Williams' medium E supplemented with dexamethasone (Appendix A.2), in a humid environment with 5% carbon dioxide at 37°C for various periods of time from 24 to 72 hours.

2.1.4 Dimethyl Sulfoxide as a Solvent

Other preliminary experiments were conducted to determine the optimum concentration of the solvent to be used for dissolving the retinoic acid powder. The concentration that would effect a good solubility of the hydrophobic retinoic acid powder and yet not produce any visible effect on the hepatocyte monolayer culture was deemed to be optimum.

Dimethyl sulfoxide was added to the hepatocyte culture media immediately

following the attachment period. A number of dimethyl sulfoxide concentrations in the range from 0.1 to 0.75% were maintained for the entire incubation period. The other parameters were determined by the preliminary experiments described in Section 2.1.3, and were kept fixed (see results in Section 3.1.3e). Possible toxic effects and differentiating effects on the hepatocyte itself were evaluated at 24 and 48 hours of incubation.

2.1.5 Retinoic Acid

Finally, the optimum concentration of all-trans retinoic acid needed was determined by adding retinoic acid in concentration ranging from 10^{-9} to 10^{-2}M to the culture media during the incubation period (Appendix A.1). The concentration that is non-toxic to the hepatocytes and yet produces maximum differentiation was deemed to be optimum.

After all these preliminary experiments were done and the basic parameters were defined, various techniques were used (see Sections 2.2 to 2.5) to study the differentiating effect of retinoic acid on the hepatocyte in monolayer cultures. Retinoic acid treated hepatocyte cultures were done simultaneously with control cultures. In order to ascertain that the solvent was not responsible for the observations noted, two control groups were used from identical suspension of hepatocytes cultured in identical environment at the same time. The first group of control was deprived of retinoic acid and the second group was deprived of both the retinoic acid and the dimethyl sulfoxide during the full incubation period. This latter control group was considered necessary to demonstrate that the observed differentiating effects on the hepatocyte cultures were attributable to the presence of retinoic acid in the culture media and not to the presence of the solvent in the

culture media.

2.2 INDIRECT IMMUNOFLUORESCENCE - SINGLE AND DOUBLE STAINING

2.2.1 Indirect Immunofluorescence - Single Staining Technique

The indirect immunofluorescence technique (Figure 10) was used to observe the spatial arrangements of a number of cytoskeletal elements, such as actin, microtubule, desmoplakin, and especially the cytokeratins 49 and 55 kDa.

After the 48-hour incubation period, the medium was discarded. The hepatocytes growing on fibronectin-coated cover glass slips were thoroughly rinsed with phosphate buffer saline solution (Appendix A.3) at 4°C. The hepatocytes were partially extracted by adding a cytoskeletal buffer solution (Appendix A.3) for 10 minutes at 4°C. The partially extracted hepatocytes were fixed in 70% ethanol for 5 minutes at -15°C and then thoroughly rinsed with phosphate buffer saline solution (Appendix A.3) at 4°C. The phosphate buffer saline solution was removed from the glass cover slips, and the primary antibody (see Section 2.2.4) was applied and left to incubate for a period of 30 minutes at room temperature. The excess antibody was rinsed away with phosphate buffer saline solution (Appendix A.3) at 4°C before the secondary antibody (see Section 2.2.4) was applied and left to incubate for 30 minutes at room temperature. The glass cover slips were again well rinsed with phosphate buffer saline solution (Appendix A.3) to remove all excess antibody.

The specificity of the secondary antibody to the primary antibody was determined by simultaneously making a control culture in which the primary antibody was replaced by phosphate buffer saline solution.

The cover slips were mounted with the p-phenylenediamine (Appendix A.1) mounting medium described by Johnson et al. (1981). The slide was then ready for immunofluorescence visual study.

2.2.2 Indirect Immunofluorescence - Double Staining Technique

Double immunofluorescence staining (Figure 10) was done, in the same manner as described in Section 2.2.1, by adding a second primary antibody and a second secondary antibody (see Section 2.2.4). Two different classes of Ig monoclonal antibodies were used (IgG for CK55 kDa and IgM for CK49 kDa), with corresponding secondary antibodies that fluoresce at different wavelengths.

The double immunofluorescence staining makes it possible to compare the distributions of two different types of intermediate filaments on the same preparation in the same visual field. The observation of this double staining was done by double photographic exposure and parallel photography of the same field.

All immunofluorescence staining was observed under a Zeiss immunofluorescence microscope model III-RS, and photographed with a 35 mm camera.

2.2.3 Morphometric Analysis

Representative photographs of control and retinoic acid treated hepatocyte colonies, stained for CK55 kDa, were taken and studied in detail. The criteria for selection of the photographed fields include a good viability of the colonies, a good cellular contact without crowding or death of the hepatocytes (i.e. a similar density of hepatocytes in the colonies), and the presence of at least one bile canaliculi formation.

The number of bile canaliculi in each photograph was counted and the average

number of bile canaliculi per hepatocyte was calculated for both the control and the treated colonies. Each bile canaliculus was then measured in its two axis. Finally, the eccentricity ratio of the bile canaliculus was calculated to define its configuration.

2.2.4 Immunofluorescence - Primary and Secondary Antibodies

The actin filaments were labelled using a mouse IgM monoclonal anti-actin (Amersham) diluted at 1:50 in phosphate buffer saline solution (Appendix A.1), and were coupled with a goat anti-mouse IgM conjugated with fluorescein isothiocyanate (FITC, Dimensions, Missassauga, Canada) diluted at 1:50 in phosphate buffer saline solution (Appendix A.1). The F-actin was also observed using a one step labelling with fluorescent Rhodamine Phallotoxin (Molecular Probes Inc., Eugene, Oregon, USA) isolated from the Amanita phalloides mushroom.

The microtubules were labelled using a mouse IgG monoclonal anti-alpha-tubulin (Amersham) similarly diluted in phosphate buffer saline solution and coupled with goat anti-mouse IgG conjugated with fluorescein isothiocyanate diluted in phosphate buffer saline solution (Appendix A.1).

The desmoplakin was labelled using a mouse IgG monoclonal anti-desmoplakin I-II (Boehringer, Dorval, Canada) diluted at 1:100 in phosphate buffer saline solution (Appendix A.1); and was coupled with goat anti-mouse IgG conjugated with fluorescein isothiocyanate diluted in phosphate buffer saline solution (Appendix A.1).

The cytokeratin 49 kDa was labelled using mouse IgM monoclonal anti-cytokeratin 49 kDa (Leroux-Nicollet et al., 1983), kindly provided by N. Marceau (Cancer Research Center, Hotel-Dieu, Quebec, Canada) and diluted at 1:100 in phosphate buffer saline

solution (Appendix A.1). It was coupled with a goat anti-mouse IgM conjugated with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate (TRITC, Dimensions, Missassauga, Canada) in either case diluted at 1:50 in phosphate buffer saline solution (Appendix A.1).

Likewise, the cytokeratin 55 kDa was labelled using mouse IgG monoclonal anti-cytokeratin 55 kDa (Leroux-Nicollet et al., 1983) kindly provided by N. Marceau and diluted at 1:500 in phosphate buffer saline solution, and coupled with a goat anti-mouse IgG conjugated with fluorescein isothiocyanate diluted at 1:50 in phosphate buffer saline solution. All the above monoclonal antibodies were specific to rat proteins (Leroux-Nicollet et al., 1983).

2.3 FLUORESCEIN DIACETATE UPTAKE AND SECRETION

The function of the bile canaliculi seen between adjacent hepatocytes was evaluated by studying the capacity of these hepatocytes to take up fluorescein diacetate and their potential for subsequent secretion into the bile canaliculi. After the monolayer hepatocyte cultures were incubated for 48 hours, fluorescein diacetate in 0.1% dimethyl sulfoxide (Appendix A.1) was added to the culture medium at a concentration of 25 µg/ml as described by Barth and Schwarz (1982). Serial observations of the fluorescein diacetate uptake and secretion were made at regular time intervals for up to an hour. The reaction was stopped at a predetermined incubation time (5, 10, 20, 30, 45 or 60 minutes) by thoroughly rinsing the preparation with phosphate buffer saline solution (Appendix A.1). The slides were mounted and immediately observed under a Zeiss immunofluorescence microscope (Figure 11).

2.4 ELECTRON MICROSCOPY

The slides for electron microscopy were prepared from the hepatocytes after their 48-hour incubation in Williams' medium E supplemented with dexamethasone (Appendix A.2). As for the immunofluorescence studies described above (see Section 2.2), the isolated hepatocytes were obtained by the two step collagenase perfusion method. The isolated hepatocytes were seeded on fibronectin covered gold grids (G100 or G200) coated with 0.5% Formvar in ethylene dichloride solution (Appendix A.1).

2.4.1 Cellular Extraction Technique

After the 48-hour incubation period and prior to the electron microscopy, the cellular components of the hepatocytes were extracted and a non-ionic detergent was used to remove the soluble elements from the cell (Figure 12) (Fey et al., 1984, and Fey and Penmans, 1985). The cytoskeleton was rendered more visible in the electron microscopy (Figure 13) by a carbon coating of the filaments.

After the monolayer hepatocyte cultures were incubated for 48 hours, the medium was discarded. The culture preparation on the gold grids or the glass cover slips were thoroughly rinsed with phosphate buffer saline solution (Appendix A.1) at 4°C. The monolayer hepatocyte cultures were successively extracted in cytoskeletal buffer solution (Appendix A.3) for 4 minutes at 4°C; in ammonium sulfate cytoskeletal buffer solution (Appendix A.3) for 4 minutes at 4°C; in nucleic acid digestion buffer solution containing Triton X-100 0.5% (a non-ionic detergent) RNAse (bovine pancreatic type II-A), DNAse (bovine pancreatic type IV), and protease inhibitors: iodoacetamide (IAA, Appendix A.3) and phenyl-methyl-sulfonyl-fluoride (PMSF; Appendix A.3) for 10 minutes at room

temperature; and finally in ammonium sulfate nucleic acid digestion buffer solution containing RNAse, DNAse, IAA and PMSF (Appendix A.3) for 4 minutes at room temperature, following the method described by Fey et al. (1984).

2.4.2 Electron Microscopy Preparation

The above extraction procedure (Section 2.4.1) yielded enriched intermediate filaments of the cytoskeleton which is composed mainly of intermediate filaments, actin and nuclear matrix.

The preparation was then fixed in gluteraldehyde 2.5% in cacodylate buffer 0.1M (Appendix A.3) for 30 minutes at 4°C, followed by a thorough washing of the fixative with cacodylate buffer solution 0.1M (Appendix A.3). Since the preparation for electron microscopy preparation is lengthy, the cover glasses were typically stored in the cacodylate buffer solution (Appendix A.3) at 4°C overnight.

On the next day, the extracted monolayer hepatocyte cultures were dehydrated with successive rinsings of 5 minutes duration, in ethanol solution of progressively increasing concentration (50%, 70%, 80%, 90%, 95% and finally in absolute ethanol.)

The gold grids were then transferred to a grid-holder, and dried with a critical point drying apparatus using cold running water and carbon dioxide gas. The critical point was reached at a pressure of approximately 82 Barr (1200 lb/sq in) and a temperature of 32°C.

Immediately after drying, the extracted preparation was carbon coated to increase the visibility and strength of the filaments for their observation under the electron microscope. The carbon coating was done using a Pirani Penning (Model 4, Edwards).

The preparation was studied under a Phillips 420 or a Zeiss transmission electron microscope. Regular and stereopair photographs were taken.

2.4.3 Immunoelectron Microscopy Preparation

Attempts were made to differentiate the cytokeratins 55 and 49 kDa and to compare their distributions and organizations in the preparations from the retinoic acid treated and the control cultures, respectively.

The cellular extraction was carried out in a manner identical to that described for the direct study in electron microscopy in Section 2.4.1 (Figure 12).

The enriched intermediate filament preparation was then rinsed in phosphate buffer saline solution (Appendix A.3) and incubated in the first primary antibody (see Section 2.4.4) for 30 minutes. The preparation was thoroughly rinsed in phosphate buffer saline solution (Appendix A.3), and then incubated with the first secondary antibody (see section 2.4.4) coated with colloidal gold particles for a period of 30 minutes. The excess antibody was then rinsed away with phosphate buffer saline solution (Appendix A.3), and the process was repeated for the second primary and secondary antibodies (see Section 2.4.4). The two secondary antibodies were labelled with colloidal gold particles of different sizes. The cytokeratin 49 kDa was labelled with 5nm gold particles, and the cytokeratin 55 kDa by 10 nm gold particles. The slides were then fixed, dehydrated, carbon dioxide dried at the critical point, and carbon coated (Figure 13).

2.4.4 Electron Microscopy - Primary and Secondary Antibodies

The cytokeratin 49 kDa was labelled using a mouse IgM monoclonal anti-cytokeratin 49 kDa (Leroux-Nicollet et al., 1983), kindly provided by N. Marceau (Cancer

Research Center Hotel-Dieu, Quebec, Canada) diluted at 1:100 in phosphate buffer saline solution, and was coupled with a goat anti-mouse IgM coated with colloidal gold particles of 5 nm in diameter (SPI Supplies, West Chester, Pennsylvania, USA).

The cytokeratin 55 kDa was similarly labelled, using a mouse IgG monoclonal anti-cytokeratin 55 kDa (Leroux-Nicollet et al., 1983) isolated from rat hepatocytes and kindly provided by N. Marceau, and diluted at 1:500 in phosphate buffer saline solution, and coupled with a goat anti-mouse IgG coated with 10 nm gold particles.

2.5 GEL ELECTROPHORESIS

2.5.1 SDS-Polyacrylamide Gel Electrophoresis Technique

The whole hepatocytes and the intermediate-filament enriched fractions were dissolved in sodium-dodecyl-sulfate (SDS) reducing buffer solution (Appendix A.4) and denatured by heating the solution at 95°C for 5 minutes. The proteins from retinoic acid treated and control hepatocyte cultures were run through a 10% SDS - polyacrylamide gel electrophoresis following the method described by Laemmli (1970) (Figure 14).

The SDS electrophoresis was done using a vertical slab electrophoresis instrument (Protean II - Slabgel, Biorad). The unit accommodates 2 gels of 16 cm long and up to 10 wells on each gel.

In the eve of the gel electrophoresis, the instrument was set and the separating gel (10% 0.375M tris HCl pH 8.8; Appendix A.4) was prepared. The separating gel was composed of 20 ml of demineralized water, 2.5 ml of 1.5M Tris HCl pH 8.8, 0.5 ml of 10% SDS and 16 ml of acrylamide/Bis solution (Appendix A.4). The solution was degassed for 20 to 30 minutes, and then 250 µl of freshly prepared, 10% ammonium

persulfate (Appendix A.4) and 25 µl of TEMED were added to it. The slabs were filled with the solution up to 2 cm from the top. The surface was covered with demineralized water to prevent evaporation and the 10% separating gel was left to polymerize overnight.

On the day of gel electrophoresis, the surface water was removed by absorption with a filter paper and the stacking gel (4% 0.125M Tris HCl pH 6.8) was added over the separating gel (Appendix A.4). The stacking gel was composed of 6.1 ml of demineralized water, 2.5 ml of 0.5M Tris HCl pH 6.8, 100 µl of 10% SDS and 1.3 ml of acrylamide/Bis solution (Appendix A.4). The solution was degassed for 20 to 30 minutes, and then 50 µl of freshly prepared, 10% ammonium persulfate (Appendix A.4) and 10 µl of TEMED were added to it.

The stacking gel was added to the top of the slabs, which were already containing the polymerized separating gel, and was left to polymerize for an hour with the plastic comb in place to form the wells.

While the stacking gel was polymerizing, the hepatocyte cultures were prepared. Some hepatocyte cultures were extracted and treated following the cellular extraction procedure described in Section 2.4.1 (Figure 12). Whole and extracted hepatocytes were detached from the cover glass slips using phosphate buffer saline solution (Appendix A.3) and centrifuged at 8000 G to remove the supernatant. The pellets were separated in aliquots.

To denature the proteins, the aliquots were diluted at 1:4 in SDS reducing buffer solution (Appendix A.4) and brought to 95°C for 4 to 5 minutes using a hot water bath.

The comb was removed from the polymerized gel and each well was thoroughly

washed with electrode running buffer solution. An electrode running buffer stock solution (Appendix A.4) composed of 15 g of Tris base, 72 g of glycine and 5 g of SDS (per liter of solution) diluted in water was initially prepared, and the stock solution was then diluted at 1:5 just before using it as electrode running buffer solution.

The apparatus was set and checked against leaks. Then the electrode running buffer solution (Appendix A.4) was added at the bottom and the top of the gel. A fixed quantity of the prepared samples (an average of 5 cover slips for extracted cultures and 2 cover slips for non-extracted preparations) was added into the wells, together with a fixed amount of known commercial standard from Bio Rad (5 µl of actin diluted at 1:8, and 15 µl of standard diluted at 1:10 in reducing buffer solution; Appendix A.4). The cooling system consisted of a cold tap water circuit running around the apparatus. The Protean II cell (from Biorad) was fully closed by placing the lid on its top. The cathode and anode connections were installed, and an electrical current was applied between the two poles. The current was in the order of 18 mA for the stacking gel run, and 21 mA for the separating gel run, when two 16-cm gels were run concommittantly.

Some smaller gels of 10 cm were also run in a similar apparatus. These required an increased current, in the order of 35 and 40 mA, respectively, to run the proteins through the stacking and the separating gel.

2.5.2 Densitometric Analysis

The gels for direct observation were stained with 0.1% Coomassie brilliant blue dye solution (Appendix A.4), and dried in cellophane (Cellophane #224150, Biorad) under vacuum. The dried gels could then be photographed. Densitometric scanning and

calculation were done on a densitometric analyzer (Beckman DU-88 Densitometer, USA) using a built-in computer program on 3 control and 3 retinoic acid treated gels containing whole hepatocyte colonies.

2.5.3 Immunoblot Technique on Gels

The bands corresponding to cytokeratins 55 and 49 kDa on SDS polyacrylamide gel were identified by immunoblots. After the SDS polyacrylamide gel electrophoresis was obtained, the gel was equilibrated in a transfer buffer solution (Appendix A.4) for a minimum of 30 minutes. This equilibrating solution was composed of 3.03 g of Tris base, 14.4 g of glycine, and 200 ml of methanol diluted in water, per liter of solution. The proteins were then transferred from the gel to the nitrocellulose paper (Transphor Transfer Medium, Hofer Sc. Instruments) using a trans-blot cell apparatus (Trans-blot, Biorad Labs, Richmond, California, USA). The transfer was achieved by passing a current of 80 mA through the gel overnight. A cold tap water circuit was used for cooling.

The nitrocellulose paper was rinsed in phosphate buffer saline solution (Appendix A.3). To render the protein bands visible, the paper was stained in a solution of Ponceau S 0.2% (Appendix A.5) for 5 minutes at room temperature. The protein bands of interest were identified with a pencil dot, and the nitrocellulose paper was de-stained by washing the Ponceau S away with phosphate buffer saline solution (Appendix A.3).

The paper was incubated in 0.5% skim milk in phosphate buffer saline solution (BDH Chem, Toronto, Canada) (Appendix A.5) for 2 hours at room temperature to mask the unspecific binding sites on it. It was then thoroughly washed with 0.1% v/v tween-20 in phosphate buffer saline solution (Appendix A.5). The nitrocellulose paper was

incubated with the primary antibody (see Section 2.5.4) in 0.5% skim milk in phosphate buffer saline solution (Appendix A.5) for 2 hours at 37°C. The excess antibody was then thoroughly washed away with 0.1% tween-20 in phosphate buffer saline solution (Appendix A.5). The second biotinilated antibody (see Section 2.5.4) was added to the nitrocellulose paper for 1 hour at room temperature. The excess antibody was washed away in a similar manner and the preparation was incubated with streptavidin peroxidase 2 µg/ml (Jackson Immuno Lab., Pennsylvania, USA) in 0.5% skim milk in phosphate buffer saline solution (Appendix A.5) for 20 minutes at room temperature. The paper was successively rinsed with 0.1% tween-20 in phosphate buffer saline solution (Appendix A.5) and phosphate buffer saline solution (Appendix A.3). The labelled antibody bands were revealed with freshly prepared solution of 4-chloro 1-naphtol development solution in hydrogen peroxide (Appendix A.5). The reaction was stopped at the desired exposure by rinsing the paper in demineralized water. The bands identified were then compared with known controls and bands from Coomassie Brilliant blue stained gels.

2.5.4 Immunoblot Gel - Primary and Secondary Antibodies

The cytokeratin 49 kDa was labelled using mouse IgM monoclonal anti-cytokeratin 49 kDa (Leroux-Nicollet et al., 1983), kindly provided by N. Marceau (Cancer Research Center, Hotel-Dieu, Quebec, Canada) diluted at 1:100 in phosphate buffer saline solution (Appendix A.1), and was coupled with a biotinilated goat anti-mouse IgM (Jackson Lab, Missassauga, Canada) diluted at 1:1000 in 0.5% skim milk in phosphate buffer saline solution (Appendix A.5). Likewise, the cytokeratin 55 kDa was labelled using anti-cytokeratin 55 kDa (Leroux-Nicollet et al., 1983) provided by N. Marceau and diluted at 1:500,

and coupled with a biotinilated goat anti-mouse IgG (Jackson Lab, Missassauga, Canada) diluted 1:1000 in 0.5% skim milk in phosphate buffer saline solution (Appendix A.5).

3. RESULTS

3.1 PRELIMINARY EXPERIMENTS

3.1.1 Hepatocyte Isolation

In the two-step collagenase perfusion method of Seglen (1976) as adapted to pre-weanling rats (Deschênes et al., 1980), the best results were obtained when the animal under anaesthesia was kept alive until the perfusion was well established. The physiological electrolyte balance of the washing and the collagenase solutions kept the cardiac muscle in function, and allowed the blood to be washed out from the liver. This washing discolored the liver from its usual dark brown color to a pale yellow. During the collagenase perfusion, the connective tissue of the liver was digested and the organ became progressively very soft. The texture inside the Glisson capsule became a semi-liquid.

After mincing, washing and centrifuging the digested liver, a pellet of isolated hepatocytes was obtained. Care was taken to perform these steps quickly over a cold surface to minimize cellular death. Minor disruption or interruption at this stage (between digestion and cellular seeding) had major impact on the viability of the hepatocyte monolayer culture, to the point that some could not be used for further studies.

At the time of seeding, the viability of the isolated hepatocytes was expressed as a percentage of viable hepatocytes among the total number of cells in a sample of the hepatocyte suspension. This percentage was determined by adding an equal volume of 0.6% Trypan Blue dye solution to the suspension, whereupon the dead hepatocytes were readily identified under phase contrast microscope, as the Trypan Blue dye penetrated

through their non-competent cellular membrane. The percentage of viability ranged from 60 to 95%. A viability of 80% or more at the time of seeding typically yielded colonies of good quality after 48 hours.

In summary, the two-step collagenase perfusion method developed by Seglen (1976) and adapted to pre-weanling rat liver by Deschênes et al. (1980) produces viable and reproducible hepatocyte colonies after 48 hours of incubation in a medium composed of Williams' E supplemented with dexamethasone. A viability of 80% or higher (as determined by the Trypan Blue dye method) at the time of seeding was a predictably translated into hepatocyte colonies of good quality after 48 hours of incubation.

3.1.2 Culture Media

3.1.2a Serum Free Culture Medium Supplemented with Dexamethasone

The cultures obtained using Williams' E media supplemented with dexamethasone produced good-quality hepatocyte colonies after 48 hours of incubation. The size of the colonies ranged from 20 to several hundred hepatocytes, organized in a monolayer culture with some 'channel' structures (later demonstrated to be bile canaliculi) between adjacent hepatocytes (see Sections 3.2, 3.3 and 3.4).

3.1.2b Serum Free Culture Medium Supplemented with Epidermal Growth Factor

The cultures obtained using Williams' E media supplemented with epidermal growth factor instead of dexamethasone exhibited good viabiiity after 48 hours of incubation. However, they were morphologically different from those grown with dexamethasone. The colonies were rare and usually smaller; no large colonies were seen. The hepatocytes were often found to be isolated. No bile canaliculi were seen. On the

other hand, cellular division was often seen and metaphases were quite evident in microtubule immunofluorescence stain (see section 3.2.1b). Unfortunately, these results were not readily reproducible, and some cultures showed only hepatocytes in isolation without mitotic activities. This route was not pursued further.

3.1.2c Culture Medium Supplemented with Dexamethasone and Calf Serum

The cultures using Williams' E medium supplemented with calf serum were the easiest to obtain. After the initial critical seeding time, the culture could be maintained alive for much longer than 48 hours. Unfortunately, the fibroblasts in this case invaded the hepatocyte colonies and completely took over the culture within days, even though they were difficult to identify immediately following isolation. This method was not used further in our study.

In summary, the Williams' E medium supplemented with dexamethasone was chosen as the culture medium in our study, since it produced good, reproducible and predictable hepatocyte colonies, showing signs of morphological differentiation.

3.1.3 Hepatocyte in Monolayer Cell Culture

To ensure valid comparison, monolayer hepatocyte cultures originating from the same experiment and grown under identical conditions were used to test each parameter.

3.1.3a Surfaces of Attachment

Two surfaces of attachment were compared: the fibronectin-coated glass cover slip, and the direct plastic from the tissue culture dish. The cover slips were 20 mm by 20 mm, and the tissue culture dishes were 35 mm in diameter. After 3 hours of attachment, a marked difference was seen between identical quantities of isolated hepatocytes seeded

on the two surfaces. When the hepatocytes on the plastic were rinsed gently, a large quantity of hepatocytes became detached from the surface and were lost in the rinse. The final colonies were usually found at the periphery of the dish forming a very small rim. In comparison, the attachment of hepatocytes on the fibronectin appeared stronger, and more and larger colonies were present after 48 hours of incubation.

It was found that increasing the quantity of isolated hepatocytes improved the quality of the hepatocyte colonies on the plastic dish, but that ten times as many isolated hepatocytes were needed to yield comparable quality of hepatocyte colonies: typically one liver would produce 3 to 4 preparations of colonies on the plastic dish compared with 30 to 40 on fibronectin-coated cover slips, despite the factor of 2.5 larger surface area of the tissue culture dish. Therefore, the use of the plastic dish as attachment surface was found to be wasteful and fibronectin-coated cover slips were used in our study instead.

3.1.3b Seeding Density

The isolated hepatocytes were seeded on fibronectin-coated cover slips and put in 35-mm plastic tissue culture dishes. A seeding of 10^3 hepatocytes per dish resulted in isolated and distant hepatocytes forming few rare colonies. However, this did not affect the quality of their attachment and viability, presumably because nutrients were abundant.

At a density of 10^5 hepatocytes per dish, the cells would pile over each other and form a thicker surface than the monolayer cellular cultures. The result was too dense for adequate observation; also, many dead cells seemed to be entrapped in this cellular crowd.

A density ranging between 1×10^4 to 3×10^4 hepatocytes per tissue culture dish at the time of seeding gave healthy colonies spread over a monolayer cell culture,

especially at the periphery of the fibronectin. Colonies of various sizes were separated from one another by visible space. This seeding density gave good visualization of the hepatocytes within the colonies studied.

3.1.3c Attachment time

An attachment time of 1 hour was found to be inadequate, because the majority of hepatocytes were washed away during the rinsing step. On the other hand, when the hepatocytes were left to attach for 6 hours or longer, they quickly started to die and were seen floating in the suspension even before rinsing. At the rinsing step, the numerous dead hepatocytes were simply washed away.

An attachment time of 2 to 4 hours gave the best results. Despite the fact that some floating cells were always seen at the end of the attachment time, no further effort was made to identify the optimum attachment time more precisely. Instead, the attachment time was chosen to be 3 hours post-seeding.

3.1.3d Incubation Time

With the exception of those supplemented with calf serum, which could be kept alive for many days, the culture started to show major degeneration after 72 hours of incubation: a large fraction - often almost all - of the hepatocytes detached and were found floating in the media.

After 24 hours of incubation, a significant number of colonies were formed in the hepatocyte cultures; also, there was intercellular `channel´ (bile canaliculi) formation between adjacent hepatocytes. After 48 hours of incubation, the colony and bile canaliculi formation was better established. This was also the optimal incubation time used by

others. Since bile canaliculi formation was the end-point criterion chosen for our study of retinoic acid, an incubation time of 48 hours was adopted.

3.1.3e Description of Culture Obtained

In summary, the control monolayer hepatocyte cultures were obtained under the following conditions. Isolated hepatocytes, from Wistar Charles River strain 14 day old male rat livers, were obtained using the two step collagenase perfusion method (Section 2.1.1). The culture medium was the commercially available Williams' E medium supplemented with dexamethasone (Section 2.1.2a, Appendix A.2). A volume of hepatocyte suspension containing between 1×10^4 to 3×10^4 hepatocytes (Section 2.1.3b) was seeded on fibronectin-coated cover slips (Section 2.1.3a) put in 35-mm plastic tissue culture dish, and left to attach to the fibronectin matrix for a 3-hour period (Section 2.1.3c). The hepatocyte colonies were incubated for 48 hours (Section 2.1.3d) in a humid environment of 5% carbon dioxide at 37°C. The culture medium was changed once after the first 24 hours of incubation.

The colonies of hepatocytes obtained in this manner were reproducible. When observed in phase contrast (Figure 15), the colonies showed an agglomeration of juxtaposed hexagonal shaped cells, with a homogeneous nucleus occasionally showing a pale center. Cellular borders were well defined between adjacent cells.

3.1.4 Dimethyl Sulfoxide as a Solvent

Dimethyl sulfoxide (DMSO) was added to the basic hepatocyte culture (Section 3.1.3e) in various concentrations. When DMSO was added at a concentration of 0.1 or 0.2% to the culture medium, no difference in colony formation or in the number of bile

canaliculi (rare) was observed, compared with the basic hepatocyte culture (control culture without DMSO) at 24 and 48 hours.

At a DMSO concentration of 0.4%, the result were less predictable. In some cultures, the colonies appeared much larger when compared with the control colonies. However, no precise morphological measurements were made and therefore it was not clear if a difference did exist.

At a DMSO concentration of 0.6 to 0.75%, there were visible variations in the number of bile canaliculi formed, as well as in the morphology of the hepatocytes: the cytoplasm had a more granular appearance compared with the control cultures. However, these observations were not always reproducible from series to series.

At a DMSO concentration of 0.75 to 1.0% toxic effects were often visible at 24 hours and would result in the death of the culture at 48 hours. The cultures would present large fractions of hepatocytes floating in the medium. The remaining attached cells would be more spherical and smaller than the hepatocytes in the control culture.

The concentration of DMSO of 0.1% was chosen as it was sufficient to dissolve the retinoic acid powder, and yet well within the range where no visible changes in the hepatocyte cells were observed under light microscope, relative to the hepatocyte cells in the control cultures.

3.1.5 Retinoic Acid

All-trans retinoic acid was added to the basic hepatocyte cultures (Section 3.1.3e) in various concentrations. The results were compared to the basic hepatocyte culture, and to a second control culture composed of the basic hepatocyte culture added with 0.1% of

DMSO. This second type of control culture was used to ensure that the observations were attributable to the presence of retinoic acid in the culture medium, and not to its solvent (the DMSO), and that no synergistic effect existed between DMSO and retinoic acid.

In these preliminary experiments, no differences were apparent between the control and the retinoic acid treated cultures, at a concentration of 10^{-9}M. At a concentration of 10^{-3} to 10^{-2}M, the hepatocyte cultures simply did not survive. At a concentration of 10^{-4}M, toxic effects were seen on the hepatocyte cultures, such as poor survival, nuclear degeneration, cytoplasm clumping. Also, when their cytokeratins (49 or 55 kDa) were stained by immunofluorescence, they appeared in granular patterns instead of filamentous formation.

At a retinoic acid concentration of 10^{-5}M, the hepatocyte cultures survived well. Also, the colonies appeared more organized. Moreover, the treated hepatocyte cultures displayed a more elaborate bile canaliculi formation both in quantity and in architecture (Figure 16), compared with the control cultures. These results were reproducible. A concentration of 10^{-5}M was therefore chosen for further studies.

3.2 INDIRECT IMMUNOFLUORESCENCE: SINGLE AND DOUBLE STAINING

3.2.1 Indirect Immunofluorescence: Single Staining

3.2.1a Actin

The presence of actin was confirmed in the control hepatocyte cultures. The thin filament was more concentrated along the cellular periphery (Figure 17), including the area around the bile canaliculus structure, and formed the stress fibres at the periphery of some hepatocytes (figure not included). The negative control showed no staining.

3.2.1b Microtubule

In the control cultures, the microtubules were labelled with monoclonal anti-alpha tubulin antibodies as a general preliminary observation of the study of hepatocytes in monolayer culture.

The microtubule immunofluorescence stain (Figure 18) showed a filamentous distribution of the microtubules in the cytoplasm of the hepatocytes, and there was no or little staining seen over the nucleus. Along the cellular membrane of the hepatocytes and particularly the bile canaliculi, the tubulin stain had a tendency to be parallel to the long axis of the cells. During mitosis, the spindle, which is known to be an arrangement of polymerized microtubules, was rendered visible by the tubulin labelling. The negative control showed no staining. The microtubules in the retinoic acid treated hepatocyte cultures were not studied.

3.2.1c Desmoplakin

The desmoplakin immunofluorescence staining repeatedly failed to reveal any noticeable difference between the retinoic acid treated cultures, the control cultures, and even the negative control.

3.2.1d Cytokeratin 55 kDa

In control hepatocyte colonies, cytokeratin 55 kDa was present throughout the hepatocyte (Figure 19a), with a higher concentration of filaments in the pericanalicular sheath around the bile canaliculi and along the periphery of the hepatocytes. The cytokeratin 55 kDa filaments were visible over the hepatocyte nucleus as well as at its periphery. The negative control showed no staining.

The distribution of cytokeratin 55 kDa in retinoic acid treated colonies was similar to that of the control hepatocyte colonies, with a larger concentration of the stained filaments along the bile canaliculi (Figure 19b). The brightness of the filament obscured the cellular details of the hepatocytes on immunofluorescence photographs. The negative control showed no staining.

3.2.1e Cytokeratin 49 kDa

In the control hepatocyte colonies, cytokeratin 49 kDa was present throughout the hepatocyte (Figure 20a), with a higher concentration of filaments around the nucleus and at the periphery of the hepatocyte.

The distribution of cytokeratin 49 kDa in the retinoic acid treated hepatocyte colonies was similar to that in the control cultures, with a large concentration of the stained filaments along the bile canaliculi (Figure 20b). However, in the treated colonies, the staining of cytokeratin 49 kDa along the pericanalicular sheaths was much more prominent. The comparative quantity and size of the bile canaliculi in the treated and control colonies will be discussed in Section 3.2.3. The negative control showed no staining.

3.2.1f Distributions of Cytokeratin 55 and 49 kDa in Retinoic Acid Treated Colonies

The distributions of cytokeratin 55 and 49 kDa in the retinoic acid treated hepatocyte colonies (Figure 21) were very similar. This was demonstrated by superposed double staining (Section 3.2.2). The filaments were seen throughout the hepatocyte cytoplasm with a concentration of the filaments along the bile canaliculi.

The bile canalicular sheath showed the contours of larger-than-expected bile

canaliculi, many as large as the diameter of the hepatocyte nucleus. The very large bile canaliculi exhibited a striation along the perpendicular axis in both cytokeratin 55 and 49 kDa (Figure 21).

3.2.1g Ramification of Bile Canaliculi

In control hepatocyte colonies, the observed bile canaliculi were sparsely spaced. Their configuration was round and they measured only a small fraction of the size of the hepatocyte nucleus. They were discrete and often difficult to identify under light microcopy.

In comparison, the bile canaliculi observed in retinoic acid treated hepatocyte colonies were easy to identify. They were numerous, elongated, and often very large in size, sometimes as large as the diameter of the hepatocyte nucleus. Their pericanalicular sheath stained strongly to both cytokeratin 55 and 49 kDa immunofluorescence antibodies.

Some of the bile canaliculi formations observed in retinoic acid treated colonies would form a ramification of its structure (Figure 22). This phenomenon was studied further under electron microscopy.

3.2.2 Indirect Immunofluorescence: Double Staining

To compare the distributions of cytokeratin 55 and 49 kDa, a double staining for each cytokeratin was done on the same visual field. Identical visual fields from a control hepatocyte colony were stained with both cytokeratin 55 kDa (Figure 23a, green) and 49 kDa (Figure 23b, red) antibodies. The photographs were taken separately with the appropriate color filters. A third photograph of the superimposed exposition (Figure 23c, green + red = yellow) was also taken. These photographs (Figure 23) demonstrate the

identical distributions of cytokeratin 55 and 49 kDa, especially around the nucleus and along the filamentous cytoplasmic network.

Similar photographs were taken for the cytokeratins from a retinoic acid treated hepatocyte colony. The two cytokeratins had similar distribution in the formation of the pericanalicular sheaths and in the cytoplasm. The superimposed stains (Figure 24c) showed an identical pattern to the individual distributions of cytokeratin 55 (Figure 24a) and 49 kDa (Figure 24b), implying a co-localization of the two cytokeratins.

3.2.3 Morphometric Analysis of the Bile Canaliculi

In order to quantify the differences in the number and size of bile canaliculi between the retinoic acid treated and the control hepatocyte colonies, some quantitative measurements were carried out (Table 2). Only the fields that contained at least one bile canaliculus were included in the calculation.

A total of 95 bile canaliculi were found in 16 fields in the treated cultures. These 16 fields contained a total of 467 hepatocytes. About 24% of the bile canaliculi (23 out of 95) presented ramified structures. In the control cultures, 26 bile canaliculi were identified in 11 fields. These 11 fields contained 248 hepatocytes. No ramification was seen.

In the retinoic acid treated cultures, there was a mean of 0.203 bile canaliculi formation per hepatocyte compared to 0.105 in the control cultures. The factor of two difference is statistically significant at $p = 0.0005$ (Student's t-distribution test). The number of bile canaliculi quoted in the control cultures is likely to be higher than the actual value due to our choice of an appropriate field for morphometric calculation. The

criterion that at least one bile canaliculus be present in the field results in a large number of visual fields being discarded from the control cultures because they did not contain any visible bile canaliculi.

Morphologically, the bile canaliculi observed in the retinoic acid treated hepatocyte colonies were larger than those in the control cultures in both length and width. Their average length (the longest dimension) was 2.98±1.36 units and their average width was 1.01±0.37 units. In comparison, their control culture counterparts measured 1.52±0.65 units in length and 0.70±0.19 units in width. In other words, the average length of the bile canaliculi in the treated group was about a factor of 3 larger than that in the control group; the average width was about a factor of 2 larger. The differences in both measurements are statistically significant with $p = 0.0005$ (Student's t-distribution test).

To quantify the difference in shape between the bile canaliculi in the treated and the control cultures, the respective eccentricity index was calculated. For the bile canaliculi from the treated cultures, the index was 3.35±1.40. For those from the control cultures, the index was 2.39±0.99. The bile canaliculi from the treated and the control cultures displayed an elliptical shape of different eccentricity indices. The difference in eccentricity index was statistically significant at $p = 0.005$ (Student's t-distribution test).

This difference in the shape of the bile canaliculi persisted even when the ramified bile canaliculi were excluded from the retinoic acid treated group. The eccentricity index for the control cultures remained to be 2.39, while the index for the treated cultures changed to 2.98. The difference between the two indices was significant at $p = 0.025$ (Student's t-distribution test).

3.3 FLUORESCEIN DIACETATE UPTAKE AND SECRETION

In control hepatocyte colonies, the uptake of fluorescein diacetate molecules from the medium was relatively even in the cytosol of each hepatocyte. However, variation in the uptake intensity was seen from hepatocyte to hepatocyte within the same colony. The bile canaliculi formation often could not be identified with certainty (Figure 25a). The maximum uptake of the dye was seen after 5 to 10 minutes. No noticeable changes were seen at about 20 minutes, when the majority of hepatocytes were still evenly stained without visible pooling or secretion. Then, at about 30 minutes of incubation, the rare bile canaliculi would appear, filled with fluorescein diacetate dye (Figure 25b); the bile canaliculi observed on these occasions were larger than the ones observed with immunofluorescence staining.

In retinoic acid treated hepatocyte colonies, the uptake of the fluorescein diacetate dye from the medium took place within a similar time of about 5 minutes. The distribution of fluorescein diacetate into the hepatocyte was also evenly distributed into the cytosol, with the shadow of the nucleus often visible. Even though hepatocyte-to-hepatocyte variations in the uptake were present, they were generally less marked than in the control colonies. Also, the non-uptake regions could easily be identified. Both intercellular (bile canaliculi) and occasionally intracellular (lipid vacuoles) non-uptake regions were visible (Figure 26a). As in the control colonies, the size of intercellular bile canaliculi formation was large, often the size of the hepatocyte nucleus diameter.

After 20 minutes of incubation in fluorescein diacetate dye, a polarization of the stain was visible (Figure 27). The fluorescein diacetate appeared to have migrated to one

side of the hepatocyte and the shadow of the nucleus was again easily identified. This polarization seemed to indicate that the bile canaliculi, if present, should be immediately adjacent to the dye shifting gradient. This polarization of the dye into the hepatocyte cytosol appeared before any dye was seen secreted into the bile canaliculi.

A secretion phase of the fluorescein diacetate into bile canaliculi was observed at about 30 minutes of incubation. Bile canaliculi were numerous, large in size and very fluorescent (Figure 26b) compared with the occasional small ones seen in the control colonies. After this short period, the fluorescein diacetate re-diffused slowly into the medium, and the overall picture became more and more fuzzy.

3.4 ELECTRON MICROSCOPY

3.4.1 Cellular Extraction Technique

All electron microscopy was done on detergent extracted, unembedded whole mounts of hepatocytes. The cellular components of the hepatocytes in the monolayer culture were extracted and non-ionic detergents were used to remove the soluble elements of the hepatocytes to leave an enriched cytoskeletal preparation. This cytoskeletal structure was rendered more solid and visible by a carbon coating prior to electron microscopy observations.

3.4.2 Electron Microscopy

Figure 28 shows the electron micrograph of two adjacent hepatocytes seen in a control hepatocyte colony. The cytoskeleton consisted of interlocking filaments throughout the cell. The filaments tended to be more straight along the cellular border. The filaments had a more circumvoluted distribution around the nucleus of the cell. There were different

sizes of filaments, and some of the smaller ones appeared to "bridge" between the larger ones. The filaments were more densely distributed along the cellular border and along the nucleus border, and were less dense in the cytoplasm.

In the control hepatocyte colonies, the bile canaliculi were extremely difficult to see under both the light microscope and the electron microscope.

Numerous dark roundish components, likely nuclear debris, were visible in all our electron microscopic preparations, but they had no special spatial distributions in relation to the bile canaliculi.

As expected from the earlier observations under immunofluorescence, the pericanalicular sheaths of bile canaliculi from retinoic acid treated hepatocyte colonies were much easier to identify under electron microscopy. They had a much more complex structure (Figure 29) compared with those observed in the control colonies. Their width was a fraction of the hepatocyte nucleus diameter, in contrast with the observations made under immunofluorescence (cytokeratin 55 and 49 kDa, and fluorescein diacetate uptake and secretion), where the bile canaliculi were as large as the diameter of the hepatocyte nucleus diameter.

The pericanalicular sheaths formed by two adjacent hepatocytes consisted of a cylindrical structure (as demonstrated by stereo photographs which are not included here), formed by a concentration of semi-parallel filaments running in the longitudinal axis of the cylindrical configuration of the bile canaliculi.

These semi-parallel filaments appeared to be the points of attachment of long filaments originating from the cytoplasm at various sites. Some of these cytoplasmic

filaments appeared to stop (or start) at the points of attachment, while others appeared to be anchored there and to continue their course back into the cytoplasmic space.

Ramification of the bile canaliculi was also seen under electron microscopy (Figure 30). Again, a higher concentration of filaments were seen forming the pericanalicular sheaths, and filaments from the cytoplasm appeared to anchor onto the periphery of these pericanalicular sheaths. The observation of ramification of the bile canaliculi formation correlates well with the observations of ramification made under immunofluorescence microscopy (Figure 22).

3.4.3 Immunoelectron Microscopy

Immunogold staining was done in an attempt to identify cytokeratins 55 and 49 kDa from the actin filaments in detergent extracted, unembedded whole mounts of hepatocytes. Two different sizes of secondary gold particle antibodies were used to identify the two cytokeratins of interest.

Unfortunately, the attempt was not successful. While many of the gold particles appeared to be attached to the filaments, just as many were found in the background where there were no filaments. The majority of the filaments present were labelled by both gold particle sizes. In addition, the negative control also presented numerous non-specific labelling of both filaments and background.

3.5 GEL ELECTROPHORESIS

3.5.1 SDS-Polyacrylamide Gel Electrophoresis

The cytokeratin 55 and 49 kDa were studied by gel electrophoresis. Whole hepatocytes and extracted hepatocytes obtained from both retinoic acid treated and control

hepatocyte colonies were run through a sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). The protein bands corresponding to cytokeratin 55 and 49 kDa were identified by the immunoblot technique (Sections 2.5.3 and 3.5.3).

The SDS-PAGE of whole hepatocytes from the control and the retinoic acid treated colonies showed that cytokeratins 55 and 49 kDa are major cellular proteins in both cultures (Figure 31). The unequal amounts of proteins in the whole treated and control gels precluded a quantitative comparison between the cytokeratins in the respective gels, and only the ratios may be studied (Section 3.5.2). In the extracted hepatocyte colonies (Figure 32), the large band 66 kDa (albumin) was extracted. The protein bands from both cultures appeared to be similar in quantity.

3.5.2 Densitometric Analysis

Densitometric analysis (Figure 33) was performed to quantify the difference of cytokeratins between control and treated cultures in whole preparations. The standardized absorption peaks and integral values are summarized in Table 3, where 3 control and 3 retinoic acid gels were studied using a densitometer analyzer program (Beckman DU-88).

In the whole hepatocyte colonies, the standardized CK55/CK49 maximum absorption peak was 1.20±0.04 for the control colonies compared to 1.13±0.12 for the treated colonies. The difference of 0.07±0.16 was not significant. However, the standardized integrals of the absorption peaks were different between the treated and the control colonies. The measured CK55/CK49 integral ratio (the ratio between the integrated areas under the respective peaks) was 1.85±0.05 for the control cultures, compared to 0.95±0.05 for the treated cultures. The difference between these ratios is

significant at p = 0.0005 (Table 3).

Therefore, it appears that even though the standardized maximum absorption peak ratios of the cytokeratins 55 and 49 kDa were identical, there appears to be a difference in the relative quantity of cytokeratins 55 and 49 kDa found in the control and the treated colonies. Specifically, the quantities of the two cytokeratins were nearly identical in the retinoic acid treated colonies. In contrast, there were more CK55 kDa than CK 49 kDa in the control colonies.

The densitometric analysis of the SDS-PAGE of extracted hepatocytes from the control and retinoic acid treated colonies showed no significant difference in the CK55/CK49 maximum absorption peak ratios or the CK55/CK49 integrals of the absorption peak ratios (data not shown).

3.5.3 Immunoblot on Gels

The bands of interest on the SDS-PAGE, cytokeratins cytokeratin 55 and cytokeratin 49 kDa, were identified using the immunoblot technique described in Section 2.5.3, using primary and secondary antibodies against cytokeratin 55 and cytokeratin 49 kDa. This preparation was unstable to light and therefore no photograph was taken.

4. DISCUSSION

4.1 PRELIMINARY EXPERIMENTS

4.1.1 Hepatocyte Isolation

Suspension of hepatocytes was obtained using the two-step collagenase perfusion method of Seglen (1976) as adapted to pre-weanling rats by Deschênes et al. (1980). The suspension had a viability range from 60 to 95% at the time of seeding, as measured by the trypan blue exclusion test. A viability of 80% or higher yielded culture of good quality after 48 hours of incubation. Viability of hepatocytes in pre-weanling and adult rats up to 90-95% was previously reported by Marceau et al. (1983) and Deschênes et al. (1980). Marceau et al. (1980, 1985) identified the hepatocytes and the non-parenchymal cells in the hepatocyte culture by labelling the cytokeratins (hepatocytes) and the vimentin (endothelial and Kupffer cells), and found that at least 96% of the cells were hepatocytes. The remaining 4% were endothelial and Kupffer cells.

4.1.2 Culture Media

Marceau et al. (1980) observed that the composition of the rat liver culture varied with the isolation procedure used and the duration over which the culture was kept. The culture obtained from collagenase perfusion was of better quality than that made by enzyme digestion from the liver extracted and digested from a decapitated rat. When the culture was kept with bovine serum added to the medium, it was able to survive 1 to 2 weeks, and in some cases 2 to 3 weeks. In the latter cases, cell lines with a pale translucent cytoplasm appeared: they were identified to be epithelial cells. Cell lines with a dense cytoplasm were also seen; their identity was not known. The culture obtained

from enzyme digestion contained a higher percentage of non-hepatocyte cells, presumably because of cellular damage during the isolation procedure. The two-step collagenase perfusion method produced a higher percentage of hepatocytes. The longer the culture was kept, the more the fibroblasts proliferated, eventually invading the whole culture.

Some of these observations were also made during the preliminary experiments in the present study. The two-step collagenase method was used, and it gave cultures of good quality as long as 80% or more hepatocytes were viable at the time of seeding.

We observed that the addition of dexamethasone to the culture medium was needed to produce hepatocyte colonies with bile canaliculi after 48 hours of incubation. The addition of EGF instead of dexamethasone promoted mitosis but did not lead to the formation of colonies or bile canaliculi. Recently, Baribault et al. (1989) demonstrated that the presence of EGF in hepatocyte monolayer cultures induced a reorganization of intermediate filaments from the periphery of the cell to a more cytoplasmic localization, and the disappearance of the bile canaliculi. There was an increase in the mitosis function associated with an increase in cytokeratin synthesis and phosphorylation.

The addition of calf serum kept the colonies alive for days, but at the same time resulted in the fibroblasts proliferating and eventually invading all hepatocyte colonies in a matter of days.

4.1.3 Hepatocytes in Monolayer Culture

We have compared two surfaces of attachment: the tissue culture dish surface and the fibronectin-covered glass slips. The hepatocytes on the latter (glycoprotein) surface attached more strongly to the surface. Also, the colonies were superior in quantity after

48 hours of incubation. Whether the colonies yielded on the glycoprotein surface presented more differentiated functions was not evaluated. A density of 1×10^4 to 3×10^4 hepatocytes per glass slip (4 cm^2 in area) gave the best results.

Since the Second International Colloquium on Extracellular Matrix Influences on Gene Expression in 1974, much attention has been drawn to the importance of the surface of attachment for monolayer cultures, and to the possible influence of cell - extracellular matrix (ECM) interaction on gene expression. Such an interaction remains to be demonstrated. However, extracellular matrices used *in vitro* appeared to influence the course of epithelial differentiation in some experiments (Hay, 1980). It was recognized that the placement of cells in a culture, in itself, can lead to possibly dramatic changes in the expression of the differentiated functions, and that placing the cells on an ECM component (such as a collagen, glycoprotein, or glycosaminoglycan) may preserve some of the differentiated functions of the original cells (Bissel, 1981). The ECM also appears to play a role in growth regulation, cellular shape, and possibly the functional and biochemical interactions between the plasma membrane, the cytoskeleton and the nucleus (Bissel et al., 1982). However, the precise mechanism by which the ECM may alter gene expression is not yet understood.

The attachment time for cell cultures was previously reported to vary from 1 hour for hepatocytes (Baribault and Marceau, 1986) to up to 6 hours in epithelial cells. We found the optimum attachment time to be between 2 and 4 hours.

In previous studies, the incubation time for hepatocytes in monolayer cultures varied greatly, from 24 hours to 3 weeks. The main factor in determining the length of

incubation is the presence of serum in the medium. Linked to this factor is the invasion of the culture by the fibroblasts. Since we opted not to use serum in our culture medium, we chose a short incubation time of 48 hours. At the 48-hour point, the cultures were still very healthy and a maximum amount of bile canaliculi (our end-point criterion) was present. Thereafter, the cultures started to degenerate.

In summary, the monolayer hepatocyte cultures obtained from 14-day old Wistar strain male rats using a two-step collagenase perfusion method and the following parameters gave reproducible colonies: (1) Williams' E culture medium supplemented with dexamethasone, (2) a seeding density of 1×10^4 to 3×10^4 hepatocytes per tissue culture dish, (3) a surface of attachment of fibronectin, (4) a seeding time of 3 hours, (5) an incubation time of 48 hours in a humid, 5% carbon dioxide environment at 37°C, and (6) a change of culture medium at 24 hours. The quality of our colonies was comparable to that described previously in the literature, for cultures obtained using the same method and similar parameters.

We recognize that the hepatocyte colonies, despite their reproducibility and good quality, represent only a model of an hepatocyte functional unit. The model is a very crude reproduction of a liver, and does not mimic the functions of a liver in many respects. The observations or conclusions made from the present study provide a further understanding of the cells of the liver, but it is not valid to extrapolate them to the entire organ.

4.1.4 Dimethyl Sulfoxide as a Solvent

Dimethyl sulfoxide (DMSO) is a potent differentiating factor that is used

extensively to induce cellular differentiation in various cultured tumor cell lines (McBurney et al., 1982, Yen, 1985) and on hepatoma *in vitro* (Higgins and Borenfreund, 1980, Higgins et al. 1983). In general, it promotes cellular differentiation (to a new phenotype of a more differentiated level), and decreases the rate of mitosis of the cancerous cells. It was demonstrated that the addition of 2% of DMSO to the culture medium greatly enhanced the longevity of adult rat hepatocytes in monolayer cultures (Baribault and Marceau, 1986). As well, it maintained the albumin (Isom et al., 1985) and the alpha-foeto protein production (Higgins et al., 1983).

In our experiment, we chose a DMSO concentration of 0.1%, to dissolve the retinoic acid. At this concentration level, no visible difference in the culture was observed compared with the control culture without DMSO.

At a DMSO concentration of 1%, we found toxic effects on the cells in the colonies, in particular more granular cytoplasm and an increase in floating, dead cells. At a DMSO concentration of 2%, the colonies did not survive. We note that DMSO at 2% concentration (i.e. double our apparent toxic level) was used previously to promote cellular differentiation on cancerous cell lines (including hepatoma) and hepatocytes from adult rats. Since the hepatocytes in pre-weanling rats are relatively more differentiated than cancerous cell lines but less differentiated than mature hepatocytes, it is conceivable that they have a different level of resistance to the toxicity of DMSO. However, no attempt was made to ascertain this possibility.

4.2 THE EFFECTS OF RETINOIC ACID ON BILE CANALICULI FORMATION

The aim of our experiment was to determine the effects of retinoic acid on

hepatocyte monolayer cultures. The end-point criterion used was the presence of bile canaliculi. Specifically, we studied the presence of cytokeratin 55 and 49 kDa in the pericanalicular sheaths in retinoic acid treated and control hepatocyte colonies *in vitro*. Our observation included indirect immunofluorescence of cytokeratin 55 and 49 kDa; uptake of fluorescein diacetate compound by the hepatocytes and secretion into bile canaliculi; electron microscopy of enriched cytoskeletal preparation of hepatocyte colonies; and gel electrophoresis of both whole and extracted preparations of hepatocyte colonies.

4.2.1 Indirect Immunofluorescence

The single and double indirect immunofluorescence of cytokeratin 55 and 49 kDa showed the distributions of the two cytokeratin filaments to be almost identical under optical microscopy. Both cytokeratins were found throughout the hepatocyte, with a higher concentration of filaments in the pericanalicular sheath, along the periphery of the cell, and over the hepatocyte nucleus.

The distribution of cytokeratins was similar in both the control and the retinoic acid treated colonies, except for an increased concentration of cytokeratins - and therefore a brighter fluorescence - along the pericanalicular sheaths in the treated colonies, with little fluorescence from the cytoplasm. This may be inferred from the fact that under automatic photographic exposure, the bright, labelled canalicular sheath overshadowed the immunofluorescence labelling of the cytoplasm, which was present in both the control and the treated colonies.

In Figures 23 and 24, which show the photographs of the control and the retinoic

acid treated colonies respectively, the cytokeratin 55 kDa clearly displayed a brighter fluorescence and a more filamentous staining than did the cytokeratin 49 kDa. However, because of the slower fluorescent rate of TRITC at the longer (red) wavelength, it is not possible to unambiguously attribute the observed difference to a difference in the cytokeratins.

A similar structure of the pericanalicular sheaths was observed by Katsuma et al. (1988), in which cytokeratin 55 and 49 kDa in frozen sections of the rat liver were observed by indirect immunofluorescence. These authors described the 3-dimensional architecture of the cytokeratin filaments in the intact rat hepatocytes *in situ* using thick (10μm) frozen sections of the rat liver. The cytokeratins were present throughout the cytoplasm, with a higher concentration of filaments around the cellular border and the bile canaliculi.The cytokeratin 55 kDa had an apparent, higher staining, and a more filamentous and less punctuated staining pattern, compared with cytokeratin 49 kDa.

We have demonstrated by morphometric analysis that the bile canaliculi formed in a retinoic acid environment were larger and longer, had more complex structures (such as ramification) and a higher eccentricity index, and were more numerous than those observed in the control colonies.

In contrast to Katsuma et al. (1988), which demonstrated the presence of belt desmosome along the bile canalicular sheath in frozen sections of the rat liver, we were unable to stain the desmoplakin along the bile canalicular sheaths in our (control and treated) colonies, despite using the same monoclonal antibody under similar laboratory conditions. This once again underscores the possible difference in the quality of gene

expression between cells in a culture medium on an attachment surface and those in an organism. It is likely that the belt desmosome is present along the bile canalicular sheath in the hepatocyte culture, in view of the fact that the structure of the bile canaliculi appears to be maintained in the culture. It is of interest to note that to date, we have not been able to obtain a positive stain to desmoplakin in monolayer culture, even though the same antibody would stain the belt desmosome clearly in tissue section of the liver. Since the protein that is labelled by the desmoplakin antibody is unlikely to be absent, we postulate that perhaps the protein has a different conformation in culture.

The presence of large, ramified bile canaliculi in the retinoic acid treated hepatocyte colonies (Figure 22) is interesting. These ramifications were similar to those observed in fresh and frozen liver sections by Katsuma et al. (1988). In comparison, the control colonies (Figures 19a and 20a) displayed small, rarely visible bile canaliculi formation, without bifurcation. It appears that the addition of retinoic acid to the culture medium during the incubation period promotes the formation of bile canaliculi, and that the configuration of the bile canaliculi is closer to that observed in the intact liver. To our knowledge, these bifurcations of bile canaliculi in hepatocyte monolayer cultures originating from pre-weanling rat livers have not been reported previously. The presence of bile canaliculi is a characteristic of hepatocyte differentiation. Retinoic acid appears to promote the formation of bile canaliculi, and is therefore believed to be a differentiating factor.

4.2.2 Fluorescein Diacetate Uptake and Secretion

Hepatocytes are cells that have a secretory function. We used fluorescein diacetate

to evaluate the secretory function of bile canaliculi observed *in vitro*, after their formation in hepatocyte colonies.

Previously, it was speculated that an undamaged cytoskeletal structure was necessary to maintain the bile secretion in the liver. A number of studies were undertaken in which the integrity of the cytoskeletal structure was disrupted and the effect of the disruption on fluorescein diacetate or horse radish peroxidase secretion was then observed. In particular, Kawahara et al. (1990) demonstrated that in nickel chloride treated hepatocyte colonies ($NiCl_2$ 150μg/ml of medium), the uptake of horse radish peroxidase and the secretion of fluorescein diacetate was blocked. Also, the cytokeratin and desmoplakin became focally detached from the cell cortex, and were found to be aggregated around the nucleus. It was concluded that an intact cytokeratin intermediate filament along the bile canaliculi was needed for both the formation and the proper function of the bile canaliculi.

An intact cytokeratin arrangement along the bile canaliculi is not the only necessary element for a functional bile canaliculus; functional microtubules also need to be present. Kawahara et al. (1989) found that the addition of colchicine to the culture medium caused the disappearance of the microtubule, with the result that it completely inhibited the secretion of fluorescein diacetate into bile canaliculi *in vitro*.

In the retinoic acid treated hepatocyte colonies, we observed the uptake of fluorescein diacetate by the hepatocytes to commence within 5 to 10 minutes of its addition to the culture medium. The uptake occurred in the cytoplasm, and it varied in intensity from hepatocyte to hepatocyte. Fluorescein diacetate is known to be hydrolyzed

by esterases in hepatocytes, resulting in fluorescence. A polarization of the dye was observed (Figure 27), and after about 30 minutes of incubation, the fluorescein diacetate was secreted into the bile canaliculi, demonstrating an active process of secretion by hepatocytes.

There is a major difference in size between bile canaliculi observed in tissue and those in our hepatocyte cultures. In the slides prepared from fresh or frozen rat liver, the bile canaliculus is usually a fraction of the size of the hepatocyte nucleus. In the retinoic acid treated colonies, the size of the bile canaliculi was typically comparable to the diameter of the hepatocyte nucleus; in the control colonies, it was a fraction of the diameter. If the retinoic acid promotes the formation of bile canaliculi, and the bile canaliculi thus formed are closer in architecture to those seen in tissue than those in our control, the question then arises as to why the diameter of the bile canaliculi in the treated colonies is disproportionately larger. A possible physiological explanation is the following. In the whole organ, the bile canaliculi are small cylindrical structures which progressively anastomose with others to form larger cylindrical structures. The larger structures eventually form bile ducts which are structurally different from the bile canaliculi. A unidirectional flow of bile exists from the bile canaliculi to the larger cylindrical structures, and is eventually diverted to the bowel. Thus, unless an intrinsic or extrinsic blockage exists, the flow of bile does not result in an increase in the pressure inside the structure in any way. This distended pattern of the bile canaliculi is reminiscent of the bile canaliculi distension seen in bile duct obstructive liver disease.

In the hepatocyte monolayer culture, each of the bile canaliculi is a close structure

that does not anastomose with others into larger cylindrical structures. Therefore, secretion from the hepatocytes into the bile canaliculi can result in a buildup in pressure inside the bile canaliculi and distend it. We observed a rapid diffusion of the dye back into the culture medium immediately after the secretion of the fluorescein diacetate into the bile canaliculi. This may indicate a diffusion from the bile canaliculi once its surface pressure exceeds a certain critical threshold, artifact or the presence of another active secretion process, although the latter is believed to be less likely.

Compared with the control colonies, the retinoic acid treated colonies appeared to be more dynamic, and their uptake, polarity and secretion were more readily observed. The fluorescence observed in the control colonies was more blurry, as if the fluorescein diacetate remained at a higher concentration in the medium or diffused faster. In retrospect, if a lower concentration of fluorescein diacetate was used, the observation might be improved. It might reveal for example whether there were indeed fewer bile canaliculi in the control colonies and whether the absorption and secretory phases were less effective in the control colonies. The question remains as to whether the retinoic acid also has an effect on the fluorescein diacetate uptake capacity of the hepatocyte, whether it stimulates or facilitates fluorescein diacetate secretion, and if so, by what mechanism. Further studies are needed to address these questions.

4.2.3 Electron Microscopy

We observed the bile canaliculi from both the control and the treated colonies in enriched cytoskeletal preparations using electron microscope. The carbon coated filaments formed a circumconvoluted distribution around the nucleus of the hepatocytes, and a

larger concentration of semi-parallel filaments along the cellular border. In the cytoplasm space, larger filaments appear to be bridged by smaller ones in both the control and the treated colonies. The filaments often appeared to be anchored onto other filaments, and not to have distinct ends.

Aggregates of various sizes that absorbed the electron beam strongly were found randomly. They are believed to be fragments of DNA, which were removed from the nucleus during the extraction process and attached to the filaments. They were seen in all of our preparations.

The bile canaliculi formation was almost non-existent in the control preparations. When seen, they were small, poorly defined, and therefore difficult to observe. This is consistent with the small and rare bile canaliculi found in the immunofluorescence observation. In contrast, the bile canaliculi in the retinoic acid treated colonies were easy to identify.

The bile canaliculi observed in the retinoic acid treated colonies were a complex structure. Their cylindrical shape was demonstrated by stereo pair photographs (data not included). The cylinder was formed by the anastomosis of filaments forming the pericanalicular sheath. Filaments that run through the cytoplasm appeared to anchor on the pericanalicular sheath without being terminated. The connection of the intermediate filaments to the cytoplasmic surface was described years ago (Oda et al., 1974, French and Davis, 1975). The more recent study of intermediate filaments in hepatocytes by Katsuma et al. (1988) demonstrated that the intermediate filaments extending from the cell surface in the region of the plasma membrane or the bile canaliculi only run along the

cytoplasmic surface over a certain distance to re-enter the cytoplasma without terminating.

Bifurcation of bile canaliculi was seen under electron microscopy, demonstrating the level of complexity of bile canaliculi formed under retinoic acid treatment, *in vitro*.

The size of the bile canaliculi was a fraction of the size of the nucleus, about 1/4 to 1/3 of its diameter. This was in apparent contrast with the size of bile canaliculi seen under immunofluorescence, where it was comparable to the diameter of the nucleus. This apparent contrast may be explained as follows. While the immunofluorescence and the fluorescein diacetate studies were done on whole hepatocytes, the electron microscopy studies were done on extracted hepatocytes. In the latter case, since the membranes making up the wall of the bile canaliculi were extracted to leave an enriched filamentous preparation, the wall could well collapse due to absence of internal pressure.

However, assuming that the bile canaliculi are a dynamic structure that would normally contract to propel its contents toward large canals *in vivo*, it may be speculated that the extracted structure under electron microscopy represents the resting position of the bile canaliculi in configuration and in size. Therefore, the large bile canaliculi observed during fluorescein diacetate secretion would represent a distension of the basic structure, when pressure is applied against its walls in a closed system and fluoresceedin diacetate molecules escape through intercellular junctions. This does not represent the normal situation *in vivo* as observed by the size of bile canaliculi in tissue sections, but it is somewhat similar to the bile canaliculi distension seen in tissue section of bile duct obstructive liver disease.

Do bile canaliculi contract or collapse? We know that bile is actively secreted into

bile canaliculi (Phillips et al., 1982); that perturbations of the actin filaments impair the bile secretion (Watanabe et al., 1983, Phillips et al., 1983); that the contraction of bile canaliculi is stimulated by calcium and inhibited by inhibitors of calmodulin (Watanabe and Phillips, 1984); that it is a spontaneous coordinated phenomenon *in vitro* (Smith et al., 1985); that bile secretion is inhibited by alteration of the organization of cytokeratins along the pericanalicular sheath when nickel is added *in vitro*; and that fluorescein diacetate regurgitates back through the hepatocytes during bile canaliculi contraction in culture when exposed to vasopressin (Kawahara et al., 1990). The bile canaliculi also contracted *in vivo*, as recently demonstrated with the infusion of sodium fluorescein, without regurgitation or leakage as seen in hepatocyte cultures (Watanabe et al., 1991).

It is still uncertain as to whether canalicular motion under permissive factor is attributed to active contraction, and whether it facilitates a passive collapse of the canaliculi. There are accumulating observations in favor of an active contractile phenomenon.

A part of the difficulty in resolving this question is due to the artifacts and assumptions associated with experimental models. For examples, our model of bile canaliculi formation using retinoic acid as a promoting factor for cellular differentiation represents a very abnormal situation compared with the liver structure and functions *in vivo*. Our model of bile canaliculi is a closed system between two or three adjacent hepatocytes. Also, the fluorescein diacetate used to simulate bile secretion into bile canaliculi leaked through the intercellular junctions, instead of following a uni-directional flow towards larger canals.

The assumption that bile canaliculi collapse under low pressure implies that a regurgitation is possible. This was observed when vasopressin was added to the hepatocytes in culture, but appears illogical for a healthy *in vivo* system.

4.2.4 Gel Electrophoresis

The visual examination of the gel electrophoresis analysis confirmed that the cytokeratins 55 and 49 kDa are major cellular proteins in the whole retinoic acid treated colonies.

The peak absorption ratio of cytokeratin 55 kDa to 49 kDa (CK55/CK49) was 1.13±0.12 in the whole treated cultures, and 1.20±0.04 in the whole control cultures. The difference between the two ratios is statistically insignificant. In contrast, the integral ratio (the integrated area under the absorption peak) of cytokeratin 55 kDa to 49 kDa (CK55/CK49) was 1.85±0.05 in the control colonies, and 0.95±0.05 in the treated colonies. The statistically significant difference was surprising (Table 3). Why the ratio in the control colonies was elevated relative to that in the treated colonies remains unclear, particularly in view of the near-unity ratio for the treated colonies. It is now widely accepted that pairs of cytokeratins are found in equal quantity of molecules in the living cells. Differences may be attributed to a more selective increase of the proteolysis (degradation) of CK49 kDa in the control culture. Also, a difference in the molecular uptake of Coomassie blue stain may exist depending on the number of binding sites available on the cytokeratin molecule, but this cannot explain the difference between the control and treated cultures unless the cytokeratins were different in their specifications and not in their quantity.

In the previous studies of Marceau et al. (1985), the addition of dexamethasone to the culture media (our controls) resulted in a selective stimulation of the synthesis of cytokeratin 55 kDa and, to a lesser degree, cytokeratin 49 kDa. Our observation of the quantity of cytokeratins present was made after treatment, and therefore the synthesis was not studied.

The implications of these observations are not clear. Dynamic studies of the bile canaliculi functions in addition to simple morphological observations will possibly lead to new understanding of the roles of cytokeratins. More specifically, further comparison of the composition and arrangements of cytokeratins in the pericanalicular sheaths between our model (which uses retinoic acid as a differentiating factor) and tissues from the whole liver will be important. Also, the synthesis of cytokeratin during the exposure to retinoic acid may be assessed by radioactive methionine incorporation. Whether retinoic acid can induce a selective phosphorylation of CK55 and CK49 kDa remains to be examined.

4.3 CONCLUSION

In conclusion, we studied the effects of retinoic acid 10^{-5}M on the formation of bile canaliculi on monolayer cultures of hepatocytes obtained using the two-step collagenase perfusion method; specifically the presence of cytokeratins 55 and 49 kDa.

We observed an increase in the quantity of bile canaliculi, their size, and the complexity of their morphology. Specifically, ramifications were observed both under immunofluorescence and under electron microscopy.

We also demonstrated that the hepatocyte colonies formed in the presence of

retinoic acid were capable of uptake of fluorescein diacetate into their cytoplasm, followed by polarization of the dye at the canaliculi pole and secretion of fluorescein diacetate into the bile canaliculi.

Finally, we demonstrated in our studies on gel electrophoresis that the quantities of the two cytokeratins were identical in the treated colonies but that the two cytokeratins were present in unequal quantity in the control colonies. In our control colonies, we observed a larger integrated area of cytokeratin 55 kDa. We did not study the synthesis process of cytokeratins directly. However, we did investigate quantitatively its presence after its treatment with retinoic acid. The similarity in the quantity of the cytokeratin 55 and 49 kDa in the retinoic acid treated colonies may be due to a proportionally higher degradation of the cytokeratin 55 kDa than in the control colonies. A higher degradation of cytokeratin 49 kDa in the control colonies was also possible.

Retinoic acid acts as a differentiating factor on hepatocytes in culture by promoting the formation of bile canaliculi. The fact that it induces morphological changes that are suggestive of a higher degree of cellular differentiation may have clinical implications. Finally, retinoic acid may play a role in the maturation of the hepatocytes possibly during embryogenesis and repair of the liver.

APPENDIX A

COMPOSITION OF SOLUTIONS

A.1 SOLUTIONS FOR HEPATOCYTE CULTURE

Washing Solution

KCl	0.5 g
NaCl	8.3 g
HEPES	2.4 g (Sigma, St. Louis, Missouri)

- Adjust pH to 7.4.
- Add distilled water, to a total volume of 1 liter.
- Sterilize solution by heat, and store solution at 4°C.

Stock Solution

KCl	0.5 g
NaCl	8.3 g
HEPES	2.4 g (Sigma, St. Louis, Missouri)
$CaCl_2$	822 mg

- Adjust pH to 7.4.
- Add distilled water, to a total volume of 1 liter.
- Sterilize solution by heat, and store solution at 4°C.

Collagenase Solution 0.5% w/v

Collagenase-I	0.5 g (Sigma, St. Louis, Missouri)

- Dissolve in 100 ml of stock solution (Appendix A.1),
- Sterilize through a 0.45 micron Millipore filter (Fisher Sc., Canada), and store at -20°C.

Fibronectin 5%

Fibronectin	0.5 g (Boehringer, Dorval, Quebec)

- Dissolve in 10 ml of sterilized water, separate in 1 ml aliquot, and store at -20°C.

Trypan Blue Dye Solution 0.6%

Trypan blue	0.06 g (Sigma, St. Louis, Missouri)

- Dissolve in 10 ml of demineralized water, and keep at room temperature.

Fluorescein Diacetate

Fluorescein diacetate	25 µg (Sigma, St. Louis, Missouri)
DMSO	1 ml (Sigma, St. Louis, Missouri)

Retinoic Acid 10^{-2}M (Stock)

All-trans retinoic acid	0.3 g (Sigma, St. Louis, Missouri)
DMSO	100 ml(Sigma, St. Louis, Missouri)

Mounting Medium for Slides

p-phenylenediamine (1,4-benzene diamine)	0.1 g
demineralized water	10 ml

then add to:

Glycerol	50 ml
PBS (Appendix A.3)	50 ml

Formvar 0.5% in Ethylene Dichloride

Commercially available from J.B. EM Service, Ste-Claire, Quebec.

A.2 SOLUTIONS FOR CULTURE MEDIA

Williams Medium E

Cell culture reagent with L-glutamine and without sodium bicarbonate, commercially available from Sigma, St. Louis, Missouri.

Component	g/L
L-Alanine	0.090
L-Argine	0.050
L-Aspartic Acid	0.030
L-Asparagine.H_20	0.020
L-Cysteine	0.040
L-Cystine	0.020

L-Glutamic Acid	0.0445
L-Glutamine	0.292
Glycine	0.050
L-Histidine Free Base	0.015
L-Isoleucine	0.050
L-Leucine	0.075
L-Lysine.HCl	0.08746
L-Methionine	0.015
L-Phenylalanine	0.025
L-Proline	0.030
L-Serine	0.010
L-Threonine	0.040
L-Tryptophan	0.010
L-Tyrosine 2Na	0.05045
L-Valine	0.050
Ascorbic Acid Na	0.00227
d-Biotin	0.0005
Choline Chloride	0.0015
Ergocalciferol	0.0001
Folic Acid	0.001
Glutathione	0.00005
myo-Inositol	0.002
Menadione Sodium Bisulfite	1×10^{-5}
Linoleic Acid Methyl Ester	3×10^{-5}
Niacinamide	0.001
Pyridoxal HCl	0.001
Riboflavin	1×10^{-4}
Pyruvic Acid Na	0.025
Thiamine HCl	0.001
Tocopherol Phosphoric Acid 2Na	1×10^{-5}
Vitamin A Acetate	1×10^{-4}
Vitamin B12	2×10^{-4}
D-Pantothenic Acid Ca	0.001
Calcium Chloride $2H_2O$	0.265
$CuSO_4.5H_2O$	1×10^{-7}
$Fe[NO_3]_2.9H_2O$	1×10^{-7}
$MnCl_2.4H_2O$	1×10^{-7}
$MgSO_4$ Anhydrous	0.0977
Potassium Chloride	0.400
Sodium Chloride	6.800
NaH_2PO_4 Anhydrous	0.122
$ZnSO_4.7H_2O$	2×10^{-7}
Phenol Red Na	0.0107
Glucose	2.000

When 2.2g of sodium bicarbonate is added to one liter of the medium, the osmolarity of the solution is 290 mOs/L ±5%, and the pH is 7.5±0.3 at 25°C.

Culture Media Supplemented with Dexamethasone

William E medium	21.6 g	(Sigma, St. Louis, Missouri)
Dexamethasone	7.8 mg	(Sigma, St. Louis, Missouri)
Sodium Bicarbonate	4.4 g	
Penicillin/Streptomycin	200,000 IU	(Sigma, St. Louis, Missouri)
L-Ornithine	0.135 g	(Sigma, St. Louis, Missouri)
Insulin	0.02 ml	(Sigma, St. Louis, Missouri)

- Adjust volume to 1.8 l with distilled water and sterilize through a 0.22 Millipore filter (Fisher Sc. Ottawa, Ontario).
- Dissolve 10 g of Albumine Bovine Fatty Acid Free (Sigma, St. Louis, Missouri) into 200 ml of distilled water and add to the preparation above.
- Store at 4°C.

Culture Media Supplemented with Epidermal Growth Factor

William E medium	21.6 g	(Sigma, St. Louis, Missouri)
Epidermal Growth Factor	20 µg	(Sigma, St. Louis, Missouri)
Sodium Bicarbonate	4.4 g	
Penicillin/Streptomycin	200,000 IU	(Sigma, St. Louis, Missouri)
L-Ornithine	0.135 g	(Sigma, St. Louis, Missouri)
Insulin	0.02 ml	(Sigma, St. Louis, Missouri)

- Adjust volume to 1.8 l with distilled water and sterilize through a 0.22 Millipore filter (Fisher Sc. Ottawa,Canada).
- Dissolve 10 g of Albumine Bovine Fatty Acid Free (Sigma, St. Louis, Missouri) into 200 ml of distilled water and add to the preparation above.
- Store at 4°C.

Calf Serum Culture Media Supplemented with Dexamethasone

Calf Serum (10%) (Difco)

A.3 SOLUTIONS FOR HEPATOCYTE EXTRACTION

Phosphate Buffer Saline Solution (PBS x 10)

NaCl	79.0 g
KCl	2.0 g
KH_2PO_3	2.0 g

Na_2HPO_3	11.6 g	

- Adjust volume to 1 liter with distilled water, and adjust pH to 7.4.

Cytoskeletal Buffer Solution (CSK)

PIPES 6% (pH 6.8)	60 ml (10 mM)	(Sigma, St. Louis, Missouri)
Sucrose	61.6 g (300 mM)	
$MgCl_2.6H_2O$	0.366 g(3 mM)	
Triton X-100	3 ml	(BDH Chem. Toronto, Ontario)
NaCl	3.54 g (100 mM)	

- Adjust volume to 600 ml and store at 4°C.
- Just before using, add:

PMSF	200 µl (120 mM)	(Appendix A.3)
IAA	200 µl (10 mM)	(Appendix A.3)

to 20 ml of CSK solution.

Cytoskeletal Buffer Solution with Ammonium Sulfate (CSK-AS)

PIPES 6% (pH 6.8)	60 ml (10 mM)	(Sigma, St. Louis, Missouri)
Sucrose	61.6 g (300 mM)	
$MgCl_2.6H_2O$	0.366 g(3 mM)	
Triton X-100	3 ml	(BDH Chem. Toronto, Ontario)
NaCl	3.54 g (100 mM)	
Ammonium Sulfate	19.8 g (250 mM)	

- Adjust volume to 600 ml and store at 4°C.
- Just before using, add:

PMSF	200 µl (120mM)	(Appendix A.3)
IAA	200 µl (10 mM)	(Appendix A.3)

to 20 ml of CSK-AS solution.

Nucleic Acid Digestion Buffer (Nucleic Acid-Dig)

PIPES 6% (pH 6.8)	60 ml (10 mM)	(Sigma, St. Louis, Missouri)
Sucrose	61.6 g (300 mM)	
$MgCl_2.6H_2O$	0.366 g(3 mM)	
Triton X-100	3 ml	(BDH Chem. Toronto, Ontario)
NaCl	3.54 g (100 mM)	

- Adjust volume to 600 ml and store at 4°C.
- Just before using, add:

PMSF	200 µl (120 mM)	(Appendix A.3)
IAA	200 µl (10 mM)	(Appendix A.3)
DNAase	2 µg	(Sigma, St. Louis, Missouri)
RNAase	2 µg	(Sigma, St. Louis, Missouri)

to 20 ml of Chromatine-Dig solution.

Nucleic Acid Digestion Buffer with Ammonium Sulfate (Nucleic Acid-Dig-AS)

PIPES 6% (pH 6.8)	60 ml (10 mM)	(Sigma, St. Louis, Missouri)
Sucrose	61.6 g (300 mM)	
$MgCl_2.6H_2O$	0.366 g(3 mM)	
Triton X-100	3 ml	(BDH Chem. Toronto, Ontario)
NaCl	3.54 g (100 mM)	
Ammonium Sulfate	19.8 g (250 mM)	

- Adjust volume to 600 ml and store at 4°C.
- Just before using, add:

PMSF	200 µl (120mM)	(Appendix A.3)
IAA	200 µl (10 mM)	(Appendix A.3)
DNAase	2 µg	(Sigma, St. Louis, Missouri)
RNAase	2 µg	(Sigma, St. Louis, Missouri)

to 20 ml of Chromatin-Dig-AS solution.

Phenyl-Methyl-Sulfonyl-Fluoride (PMSF)

PMSF	0.2 g
Ethanol 70%	10 ml

Iodoacetamide (IAA)

IAA	19 g
Distilled Water	10 ml

Gluteraldehyde 2.5% in Cacodylate Buffer

Gluteraldehyde 50%	1 ml	(J.B. EM Service, Pte-Claire, Quebec)
Cacodylate Buffer 0.1M	19 ml	(J.B. EM Service, Pte-Claire, Quebec)

A.4 SOLUTIONS FOR GEL ELECTROPHORESIS

Acrylamide-BIS

Acrylamide	146 g	(29.2 % w/v)	(BioRad, Richmond,
N'N'-bis-methylene-acrylamide	4 g	(0.8 % w/v)	California)

- Adjust volume to 500 ml with distilled water, and store at 4°C, away from light for 30 days maximum.

Sodium-dodecyl-sulfate (SDS) 10%

SDS	10 g
Distilled Water	100 ml

SDS Reducing Buffer (Sample Buffer)

Distilled water	4.0 ml
Tris-HCl 0.5M, pH 6.8	1.0 ml
Glycerol	0.8 ml
SDS 10% w/v	1.6 ml (Appendix A.4)
2-beta mercaptoethanol	0.4 ml
Bromophenol blue 0.05% w/v	0.2 ml

The sample is to be diluted at least 1:4 with the SDS reducing buffer and heated at 95°C for 4 minutes.

Electrode Running Buffer (5x)

Tris base	45 g	(1.5 g%)	(BioRad, Richmond, California)
Glycine	216 g	(7.2 g%)	
SDS	15 g	(0.5 g%)	

- Adjust to 3 liters of distilled water.
- Warm to 37°C before using if precipitation occurs, and dilute 5 times with distilled water when ready to use.

Separating Gel Preparation 10%

Distilled water	20.0 ml
Tris-HCl 1.5M, pH 8.8	2.5 ml
SDS 10% w/v	0.5 ml
Acrylamide-Bis	16.0 ml

- Degas the solution under vacuum for 20 minutes, and add immediately before using:

Ammonium persulfate 10% (fresh)	250 µl	
TEMED	25 µl	(Sigma, St. Louis, Missouri)

Stacking Gel Preparation 4%

Distilled water	6.1 ml
Tris-HCl 0.5M, pH 6.8	2.5 ml
SDS 10% w/v	100 µl
Acrylamide-Bis	1.3 ml

- Degas above solution under vacuum for 20 minutes, and add immediately before using:

Ammonium persulfate 10% (fresh)	50 µl	
TEMED	10 µl	(Sigma, St. Louis, Missouri)

Transfer Buffer Solution

Tris base	9.09 g (23 mM)	(BioRad, Richmond, California)
Glycine	43.20 g(192 mM)	
Methanol	600 ml	

- Adjust volume to 3 liters with demineralized water.

Ammonium Persulfate 10%

Ammonium persulfate	0.1 g
Demineralized Water	1 ml

Coomassie Blue Dye 0.1%

Coomassie Brillant Blue Dye	0.1 g
Demineralized Water	100 ml

A.5 SOLUTIONS FOR IMMUNOBLOT ON GEL

Ponceau S Dye 0.2%

Ponceau S	1 g	
Tricholoroacetic acid 20%	75 ml	

- Adjust volume to 500 ml with demineralized water.

Skim Milk 0.5% in PBS

Skim milk	0.5 g	(BDH Chem., Toronto, Ontario)
PBS	100 ml	(Appendix A.3)

Tween-20 0.1% in PBS

Tween-20	0.1 g	(BioRad, Richmond, California)
PBS	100 ml	(Appendix A.3)

4-Chloro 1-Naphtol Development Solution

Chloronaphtol	100 mg	
Methanol (-4°C)	40 ml	
PBS	200 ml	(Appendix A.3)
Hydrogen peroxide	240 µl	

- Use immediately after preparation.

Streptavidin Peroxidase

Streptavidin peroxidase	12µg	(Jackson Immuno Research, USA: distributed by Bio-Can Scientific, Mississauga, Ontario)
Skim milk 0.5% in PBS	6 ml	(Appendix A.5)

APPENDIX B

STATISTICAL EQUATIONS

Mean of a Sample

$$\overline{x}=\frac{1}{n}\sum_{i=1}^{n} x_i$$

Standard deviation (s.d.) of the mean

$$\sigma=\sqrt{\frac{1}{n}\sum_{i=1}^{n} x_i^2-\overline{x^2}}$$

Student's t-distribution (Confidence intervals for means comparison)

$$T=\frac{\overline{x}-\overline{y}}{\sqrt{\left(\frac{ns_x^2+ms_y^2}{n+m-2}\right)\times\left(\frac{1}{n}+\frac{1}{m}\right)}}$$

APPENDIX C

TABLES

Table 1: Types of Intermediate Filaments

Type	Filament	Subunits	MW (kDa)	Occurrence
I	Acidic Keratins	~ 15	40-60	Epithelial cells carcinoma cells specialized epithelial derivatives
II	Basic Keratins	~ 15	50-70	
III	GFAP*	1	51	gial cells (astrocytes, gliomas)
III	Desmin	1	52	myogenic cells
III	Vimentin	1	53	mesenchymal cells (fibroblasts)
IV	Neurofilament	≥4 vertebrates ≥2 invertebrates	57-150 60-200	neuronal cells
V	Lamins	≥4 vertebrates	60-70	nuclear lamina of all eukaryotic cells

* GFAP = glial fibrilar acidic protein

The intermediate filaments comprise many types of filaments. The largest group is the cytokeratins found in most eucaryotic cells, except in early embryonic cells. The cytokeratins have similar properties.

Table adapted from Steinert and Roop (1988).

Table 2. Characteristics of the bile canaliculi observed in control and retinoic acid treated hepatocyte colonies

	RA Treated (16 Fields)	Control (11 Fields)	p
Number of bile canaliculi	95	26	--
Total number of hepatocytes	467	248	--
Number of BC with ramification	23 (24%)	0 (0%)	--
Average number of BC per hepatocyte	0.203	0.105	0.0005
Average length of BC	2.98±1.36★	1.52±0.65★	0.0005
Average width of BC	1.01±0.37★	0.70±0.19★	0.0005
Index of Eccentricity	3.35±1.40★	2.39±0.99★	0.0005

All observations were made from fields containing at least one visible bile canaliculi. Slides were immunostained for cytokeratin 55 kDa.

BC = bile canaliculi
★ = standard deviation

Table 3. Ratio of the absorption peak and the integral of the CK55 absorption curve to those of the CK49 curve, in whole control and treated cultures.

		CK55/CK49 Maximum Absorption Ratio	CK55/CK49 Integral Ratio
Control	#1	1.19	1.89
	#2	1.23	1.82
	#3	1.18	1.85
Mean		1.20±0.04	1.85±0.05
Retinoic Acid	#1	1.16	0.93
	#2	1.10	0.99
	#3	1.19	0.93
Mean		1.13±0.12	0.95±0.05
p		n.s.	0.0005

n.s. not significant

APPENDIX D

FIGURES AND ILLUSTRATIONS

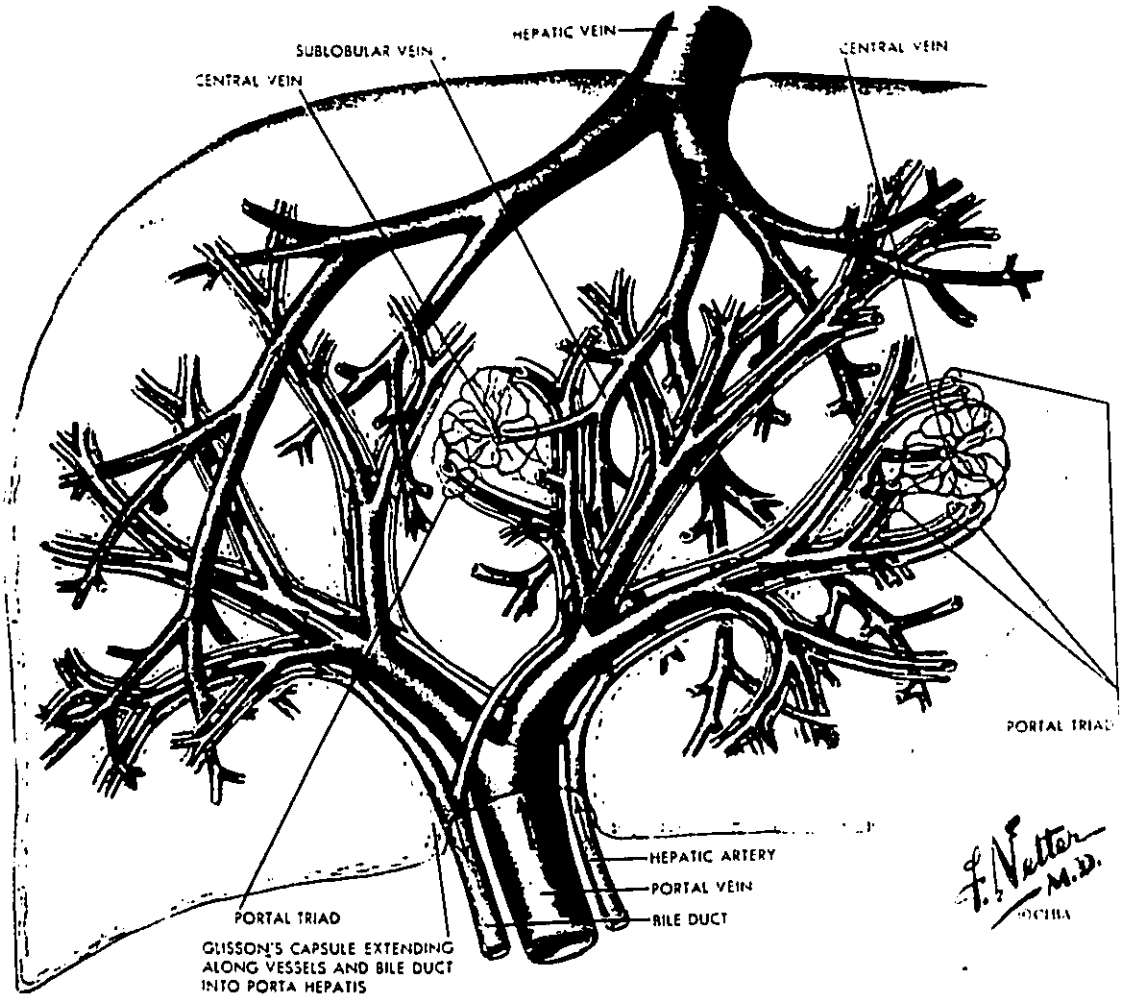


FIGURE 1. The dual vascular system of the liver.

The liver receives blood from two vascular systems. The first one is the venous blood from the intestinal tract that enters via the portal vein. This blood is rich in nutrients.

The second system is the oxygen-rich arterial blood from the hepatic artery. Circulating blood from both systems leave the liver by a common system via the hepatic vein, and finds its way back to the heart.

Netter F. H. (1979): Intrahepatic Structure. *In* Ciba-Geigy, Eds. Digestive System: Liver, Biliary Tract and Pancreas. New York, Donnelley and Sons, p. 10.

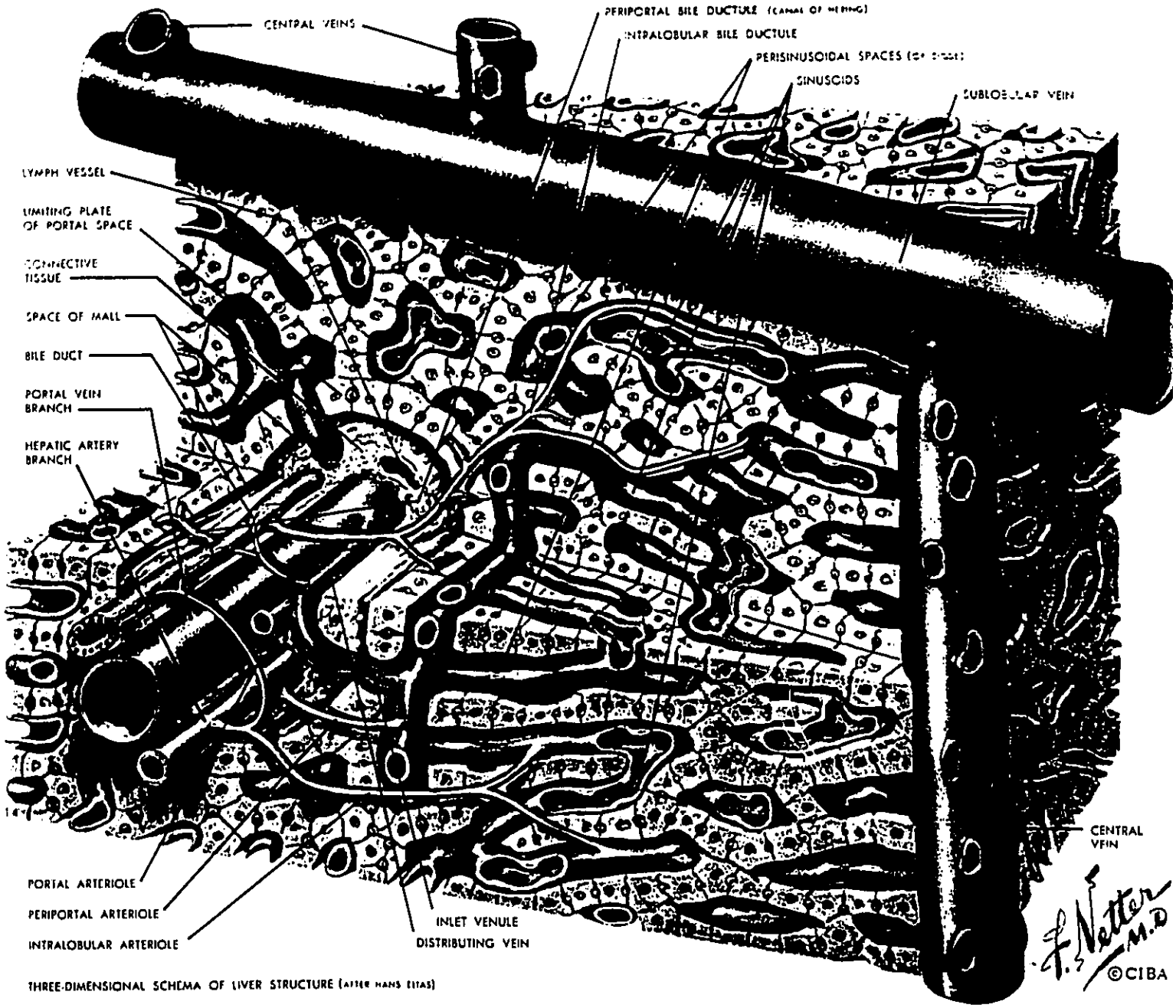


FIGURE 2. The three-dimensional liver structure as seen by Hans Elias.

Hans Elias described the "Plate Theory" of the liver to explain the liver anatomy. The center of a lobule is occupied by a branch of the hepatic vein called the central vein, and radially to it, the epithelial cells are arranged in plates of one cell thickness. He also described the presence of connective tissue in the spaces occupied at the angles between lobules.

Netter F. H. (1979): Intrahepatic Structure. *In* Ciba-Geigy, Eds. Digestive System: Liver, Biliary Tract and Pancreas. New York, Donnelley and Sons, p. 8.

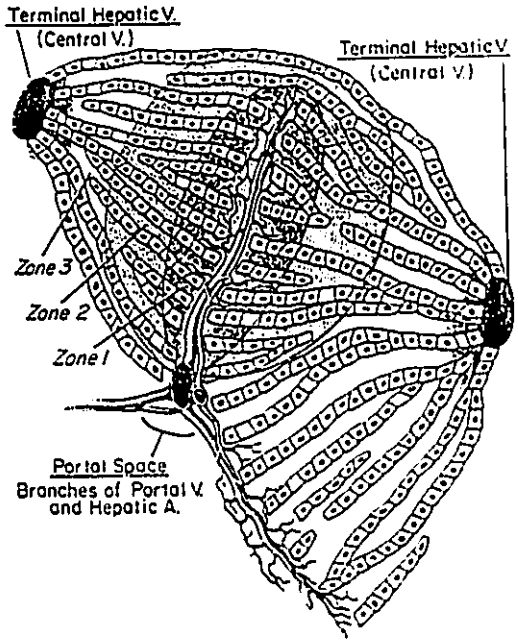


FIGURE 3. The hepatic acinus.

This diagram of the hepatic acinus, according to Rappaport et al., (1954) has the advantage over the plate theory of Elias (Fig.1) in that it takes into consideration the physiology of the liver.

The center of the hepatic acinus is occupied by the portal space which consists of parenchyma containing the small branches of the hepatic artery, the portal vein and the bile duct. The blood flow from the portal space to the central vein, such as the zone 1, nearest to the portal space, receives first call upon the incoming oxygen and nutrients. The zone 3, furthest to the portal space, is therefore most vulnerable to hypoxia while, the zone 1 is most vulnerable to intestinal toxins.

Fawcett D.W. (1986): The Liver and Gall Bladder *In* Saunders W.B., Eds. A Textbook of Histology, USA, Saunders Press, p. 679.

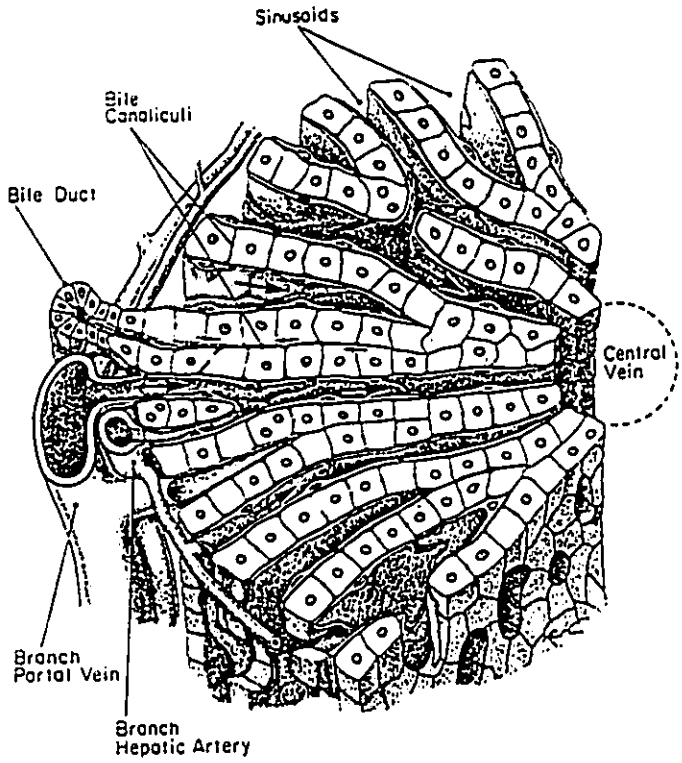
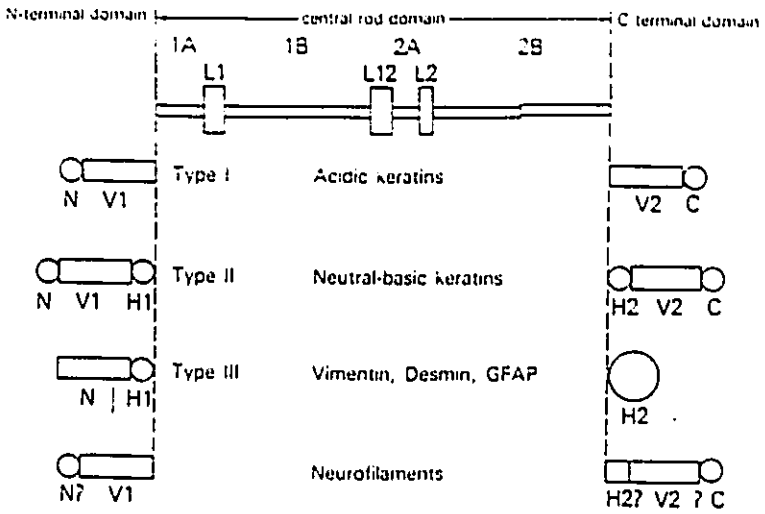


FIGURE 4. The direction of the vascular and the bile flow in the lobule.

The blood originating from the dual vascular system enters the acini via the portal space, then circulates in the sinusoids between the hepatocytes and finally reaches the central vein. The bile flow circulation is in the opposite direction. The bile is produced by the hepatocytes and secreted into the bile canaliculi, which progressively get larger in size until they form the bile duct.

Fawcett D. W. (1986): The Liver and Gall Bladder *In* Saunders W.B., Eds. A Textbook of Histology, USA, Saunders Press, p. 678.



IF Subunit Structure

All IF subunits possess a central α-helical rod domain flanked by end domains. The rod domain in all cases consists of four segments of invariant size. These are composed of a 7-residue (heptad) quasi repeat, which can form a coiled-coil structure. Segment 1A is 35 amino acids long, 1B is 101, 2A is 19, and 2B is 121. These are separated by noncoiled-coil linkers: L1 varies from 8 to 14 residues in different subunits, L12 is 16 or 17, and L2 is always 8. At the middle of segment 2B, the polarity of the progression of heptads is abruptly reversed, a feature that imposes a "stutter" in the regularity of the coiled-coil at this point. Despite these conserved features, the exact sequences of the heptads in the segments differ and permit classification into distinct sequence types. Each sequence type of rod domain is coupled with a specific set of end domains, which may be further divided into subdomains based on homologous (H) sequences, variable (V) sequences, and basic terminal (N or C) sequences. In Types I and II keratin IF subunits, the very basic N and C subdomains are 15–30 residues long, while the highly variable V1 and V2 subdomains range from 0–130 residues. The homologous sequences of the H1 and H2 subdomains of Type II keratins are 30 and 20 residues long, respectively. In Type III IF subunits, H1 and H2 are 20 and 55 residues long; V1 varies from 60 to 75 residues. Full specification of the subdomainal organization of the end domains of neurofilaments must await more complete sequence information.

FIGURE 5. The primary and secondary structure of intermediate filaments.

The primary structure of intermediate filament consists of three proteolytically defined domains: the C-terminal head, the central rod, and the n-terminal tail. The rod segment is a fixed chain of 312 amino acids. The C-terminal and the N-terminal segments are variable, and may each consist of a sequence of up to 100 amino acids.

The secondary structure of intermediate filament is an α-helix of the constant central rod segment, and does not involve the head or the tail segment.

Steinert P. M., Steven A. C., Roop D. R. (1985): The Molecular Biology of Intermediate Filaments. Cell 42: 412.

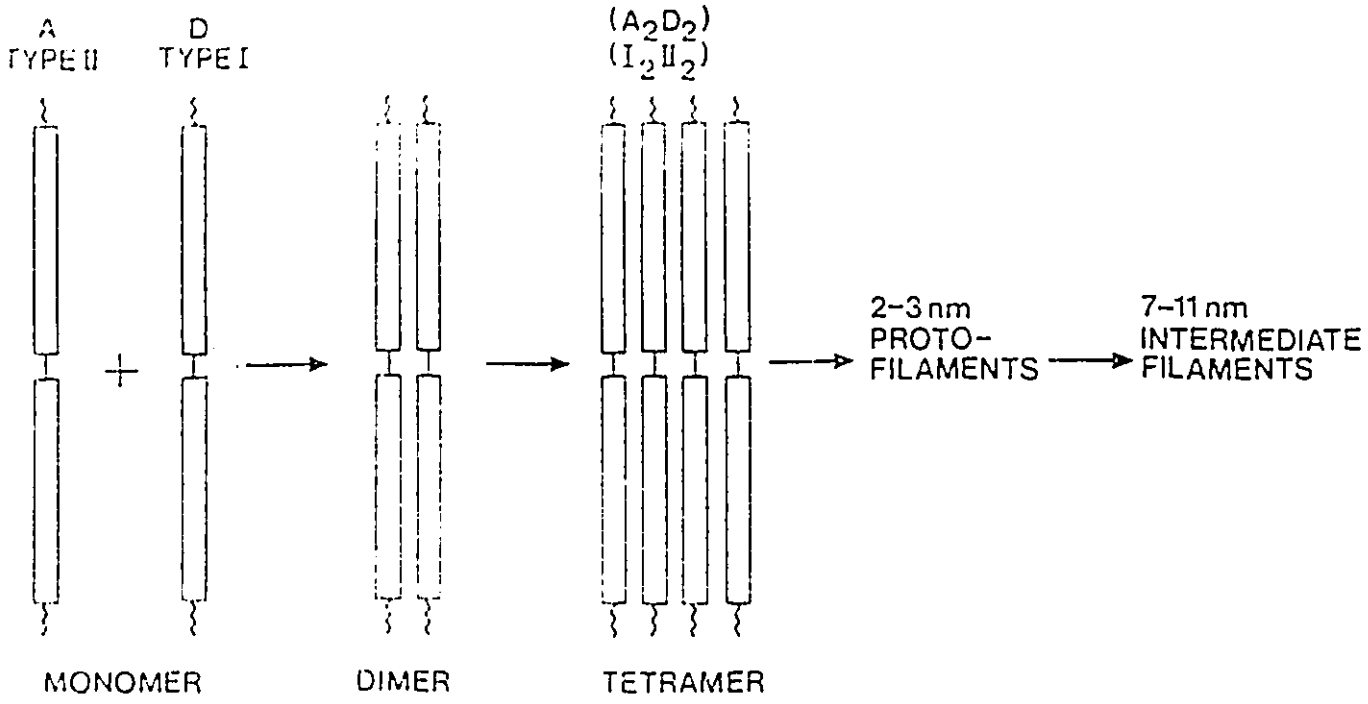


FIGURE 6. Cytokeratin protofilaments and the formation of intermediate filaments. Schematic representation of the arrangement of cytokeratin polypeptides in the formation of intermediate filaments.

The dimer is formed by a coil-coil arrangement of two types of cytokeratins. Two dimers then form a tetramer. Quinlan et al. (1985) postulated that tetramer polypeptides pack themselves into protofilaments and intermediate filaments. However, this postulate - and the orientation of the dimer in the formation of the tetramer - remain to be demonstrated.

Quinlan R.A., Schiller D.L., Achtstatter T., Moll R., Jorcano J.L., Margin T.M., Franke W.W. (1985): Patterns of expression and organization of cytoskeleton intermediate filaments. Ann NY Acad Sci 445: 303.

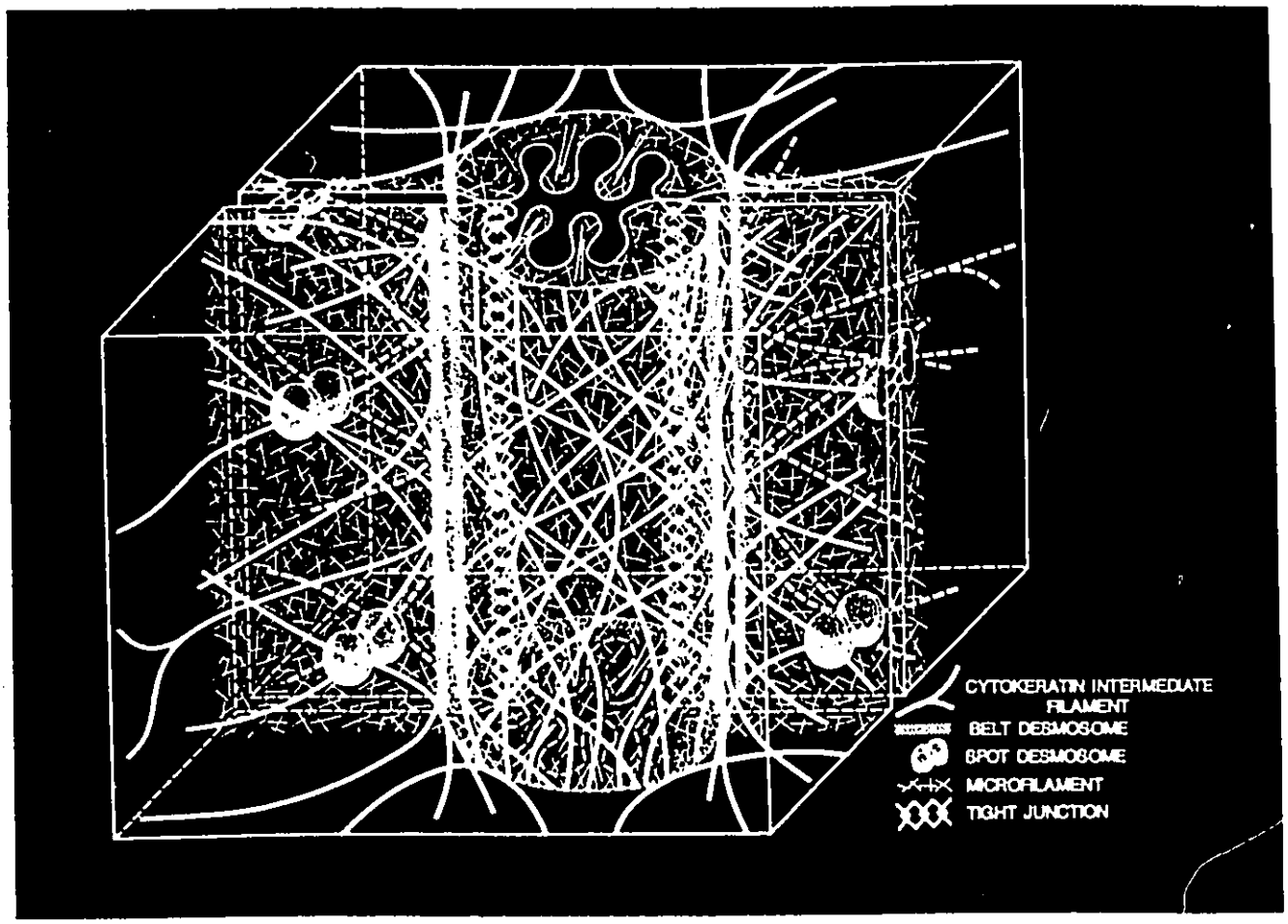


FIGURE 7. Artistic drawing of the cytoskeleton of the hepatocytes: The model of a bile canaliculi formation and its relation to the cytokeratin in the cytoplasm.

This diagram shows the connections of the intermediate filaments (IF) lattice work with the pericanalicular sheath (PS), and the belt and spot desmosomes which bind adjacent heaptocytes.

French S.W., Kawahara H., Katsuma Y., Ohta M., Swierenga S.H.H. (1989): Interaction of intermediate filaments with nuclear lamina and cell periphery. Electron Microsc Rev 2(1): 17-52.

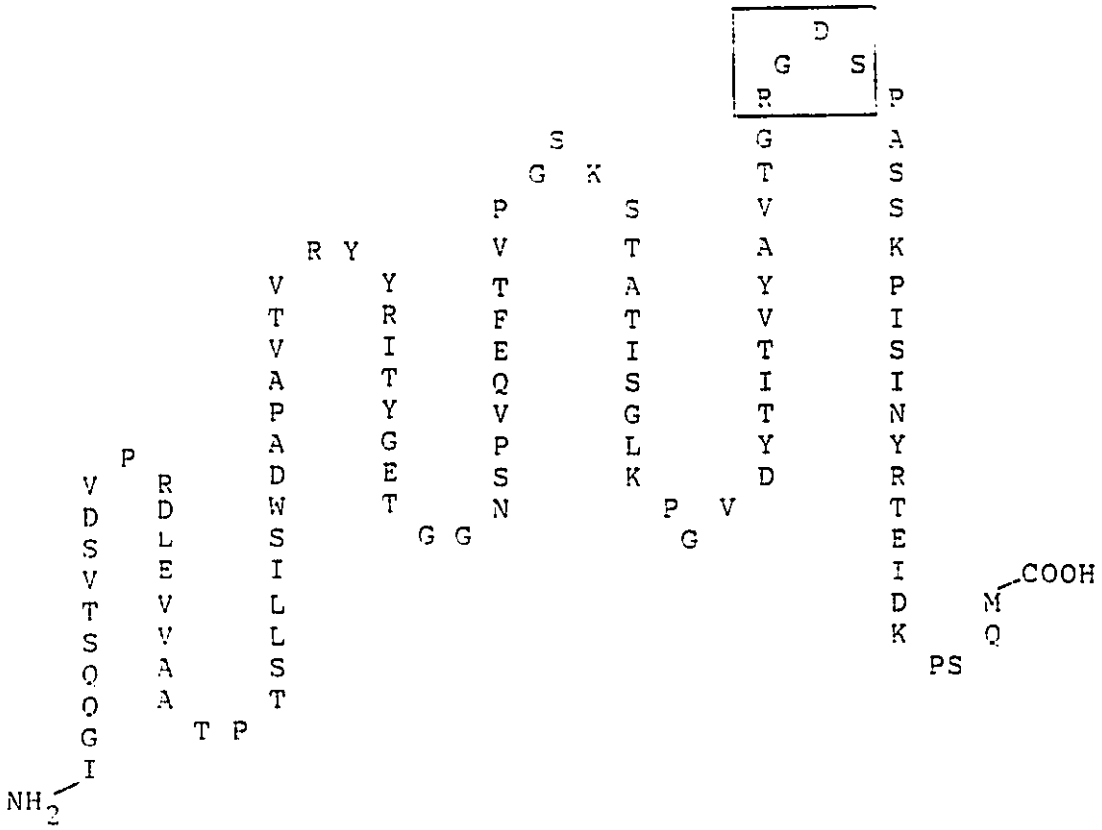
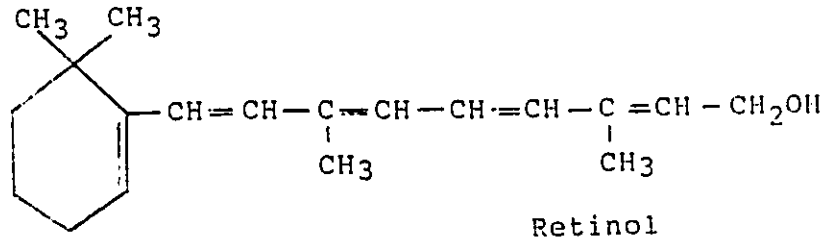


FIGURE 8. Fibronectin

Fibronectin is a glycoprotein of molecular weight of 11,500 with a primary structure of a sequence of 108 amino acids. Its secondary structure is a series of β-turns, with the most hydrophillic of it (in the box) containing the 4-amino-acid sequence (Arg-Gly-Asp-Ser or R-G-D-S) where the binding capacity resides.

Pierschbacher M., Ruoslahti E. (1984): Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature, 309: 30.



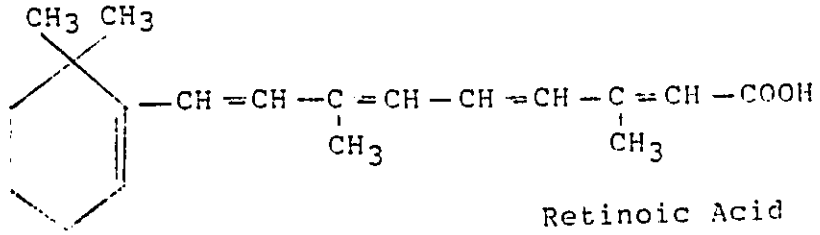


FIGURE 9. The molecular structure of retinol and retinoic acid.

The retinol is a large molecule of 20 carbon atoms composed of three segments: a hydrophillic head forming a β-ionone ring, a conjugated double bound isoprenoid chain responsible for isomerization, and a polar terminal alcohol group. This polar group is the site of chemical reactions that produce the vitamin A metabolism. The retinoic acid is the product of oxidation of the retinol molecule.

Modern Nutrition in Health and Disease (1988): 7th ed. Shils & Young, p. 294.

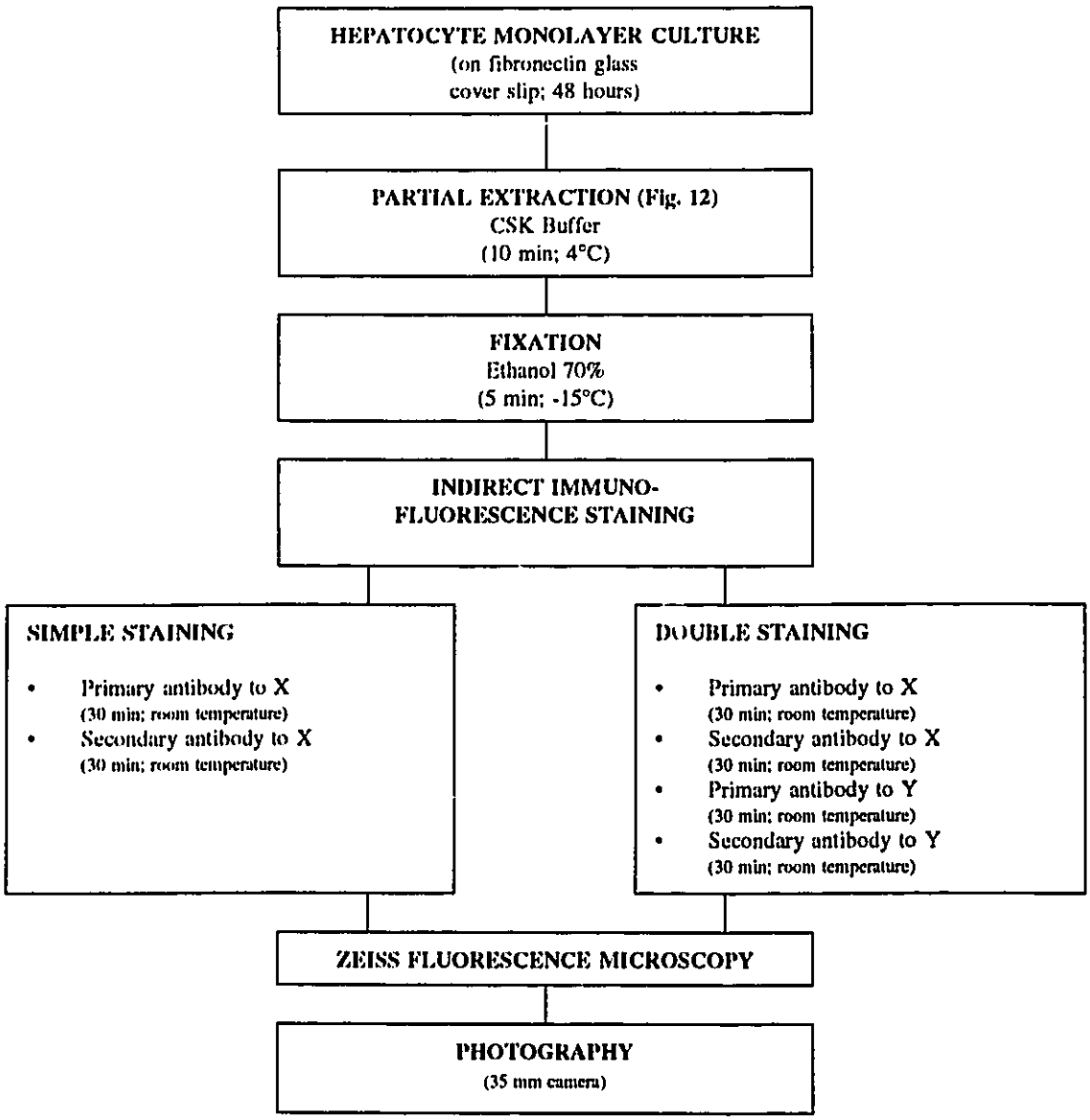


FIGURE 10. Procedure for Indirect Immunofluorescence: Single and Double Staining

Flow chart summarizing the technical steps for indirect single and double immunofluorescence staining of cytokeratins 49 and 55 kDa present in the hepatocytes obtained from monolayer cell cultures.

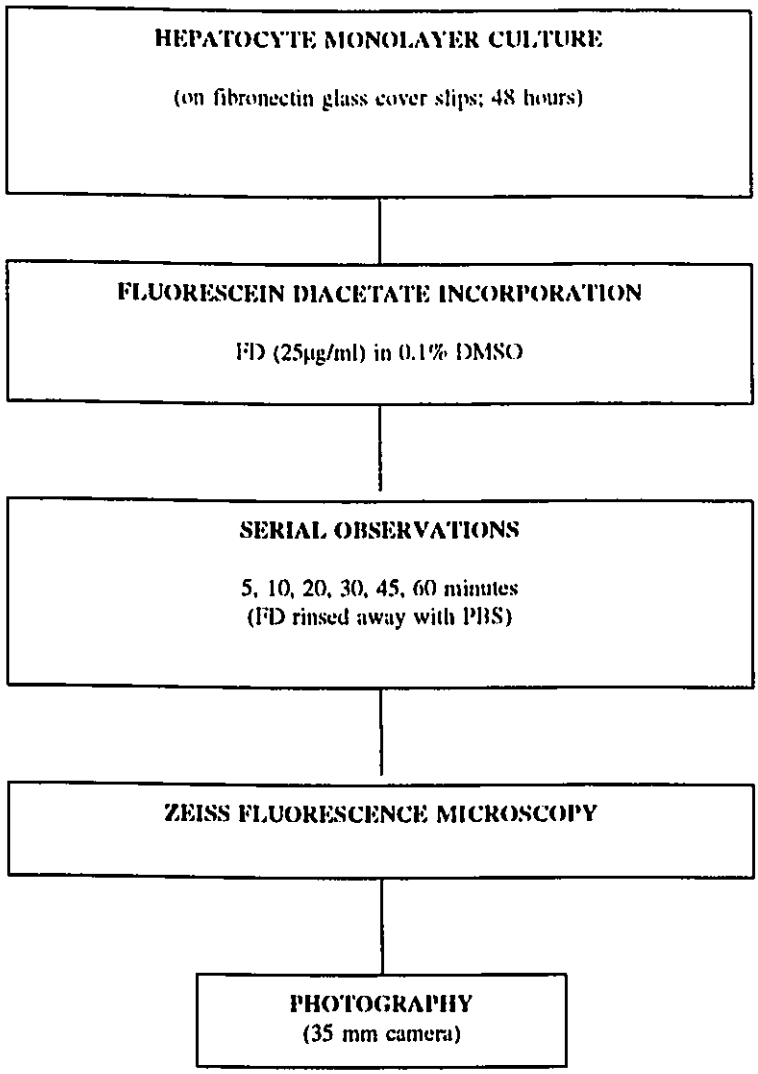


FIGURE 11. Procedure for Fluorescein Diacetate Uptake and Secretion

Flow chart summarizing the technical steps leading to the observation of the uptake by hepatocytes and the secretion into bile canaliculi of fluorescein diacetate in the hepatocytes in monolayer cell cultures. The study of fluorescein diacetate migration is a model to observe the function of the bile canaliculi in hepatocyte culture.

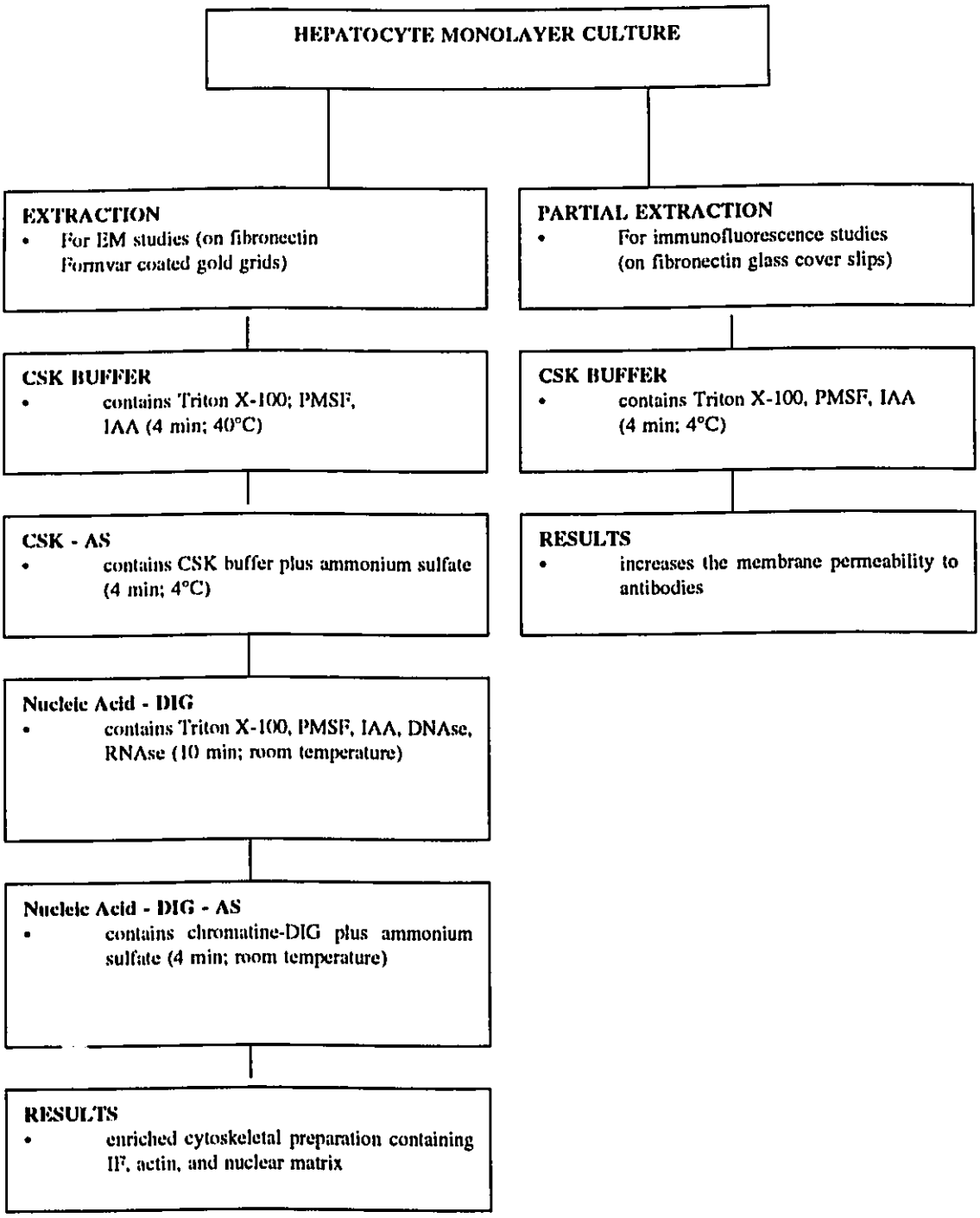


FIGURE 12. Procedure for Hepatocyte Extraction

Flow chart summarizing the technical steps involved in the extraction of the soluble cellular components of the hepatocytes. The extraction procedure follows the method of Fey et al. (1984) and Fey and Penmans (1985).

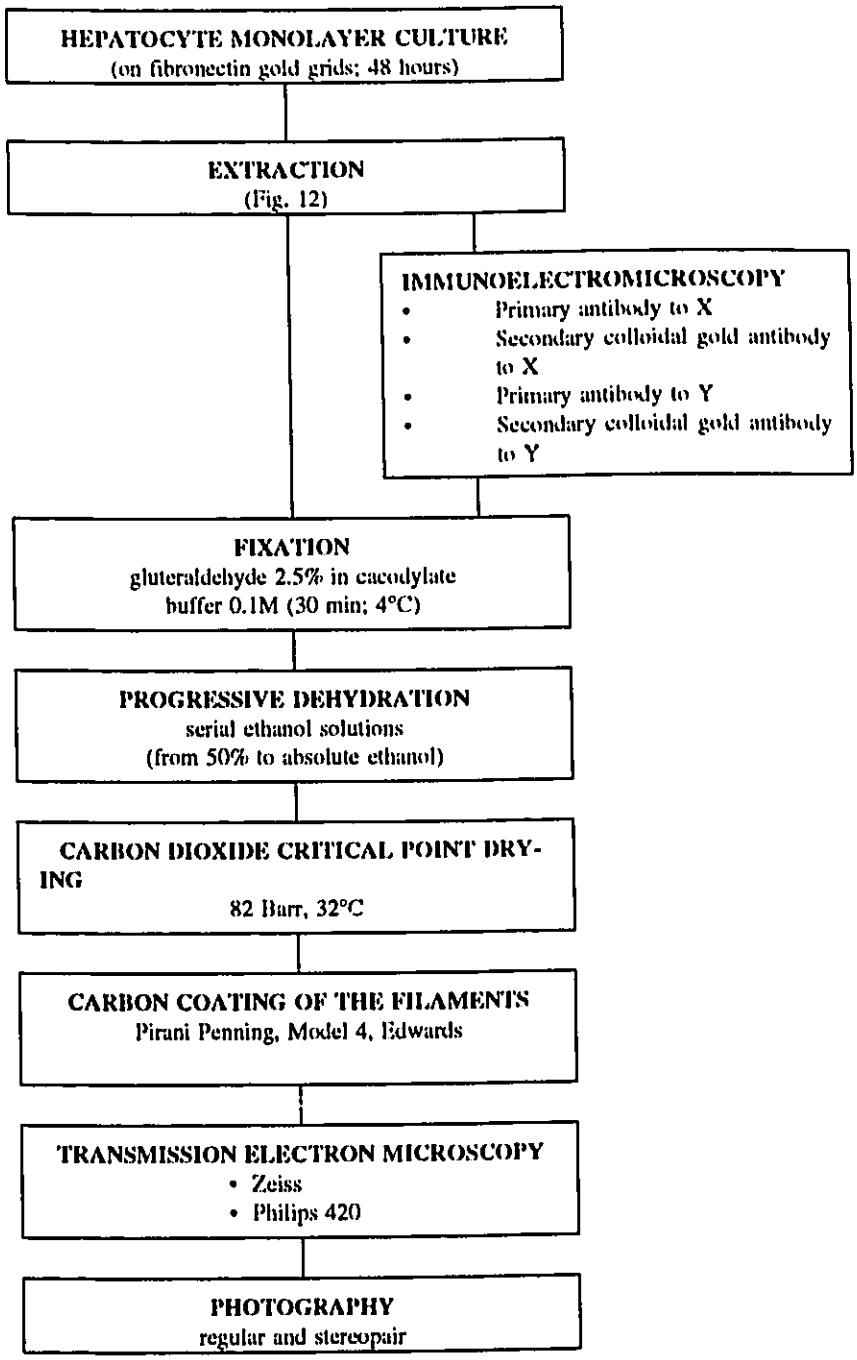


FIGURE 13. Procedure for Electron Microscopy

Flow chart summarizing the technical steps involved in the preparation of the hepatocyte monolayer cell culture, after extraction (see Fig. 12), for further electron microscopic observations of their intermediate filament structure.

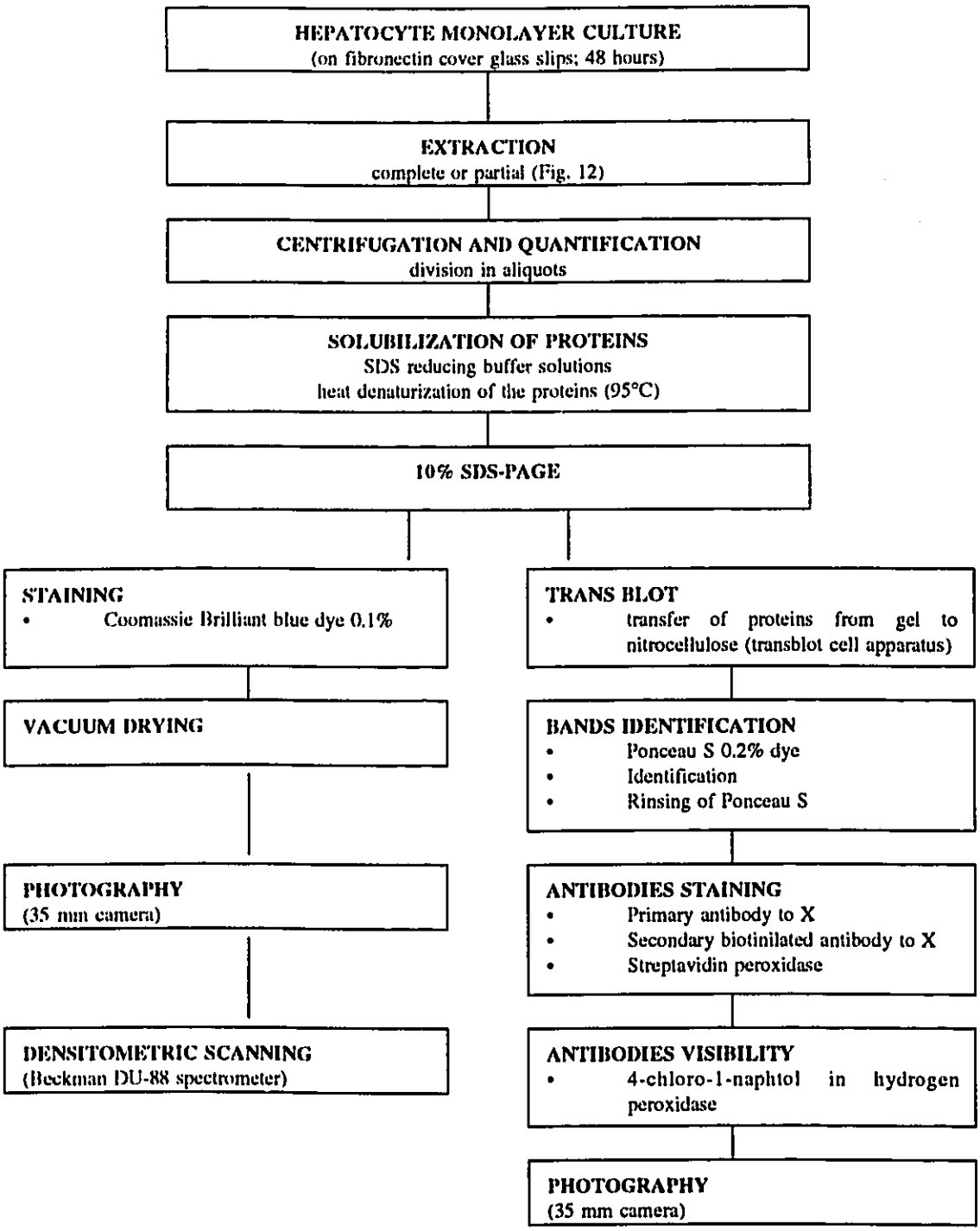


FIGURE 14. Procedure for Electrophoresis and Electroimmunophoresis

Flow chart summarizing the technical steps involved in the electrophoresis studies of proteins CK 49 and CK 55 kDa from hepatocytes monolayer cell cultures.

FIGURE 15. Control hepatocyte colony seen in phase contrast

Photograph of a control hepatocyte colony seen in phase contrast from a culture of isolated hepatocytes after 48-hour incubation in a William's E medium supplemented with dexamethasone. The hepatocytes have an hexagonal shape and are found in a juxtaposed position. The nuclei are visible, but bile canaliculi formation are not easily identified.

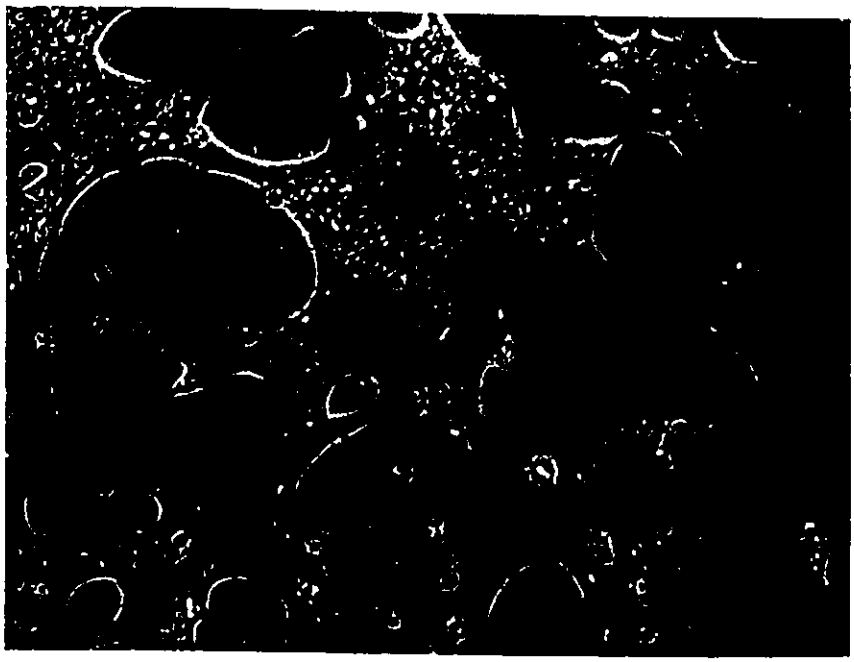


FIGURE 16. Retinoic acid treated colony seen in phase contrast

Photograph of a retinoic acid treated hepatocyte colony seen in phase contrast from a culture of isolated hepatocytes after 48-hour incubation in a William's E medium supplemented with dexamethasone and containing retinoic acid 10^{-5}M.

The culture is composed almost exclusively of hepatocytes. The hepatocyte nucleus appears as a white circle in the center of the cell (←), and numerous bile canaliculi formation may be seen between two or three adjacent hepatocytes as a small white thickening of the cellular membrane (→).

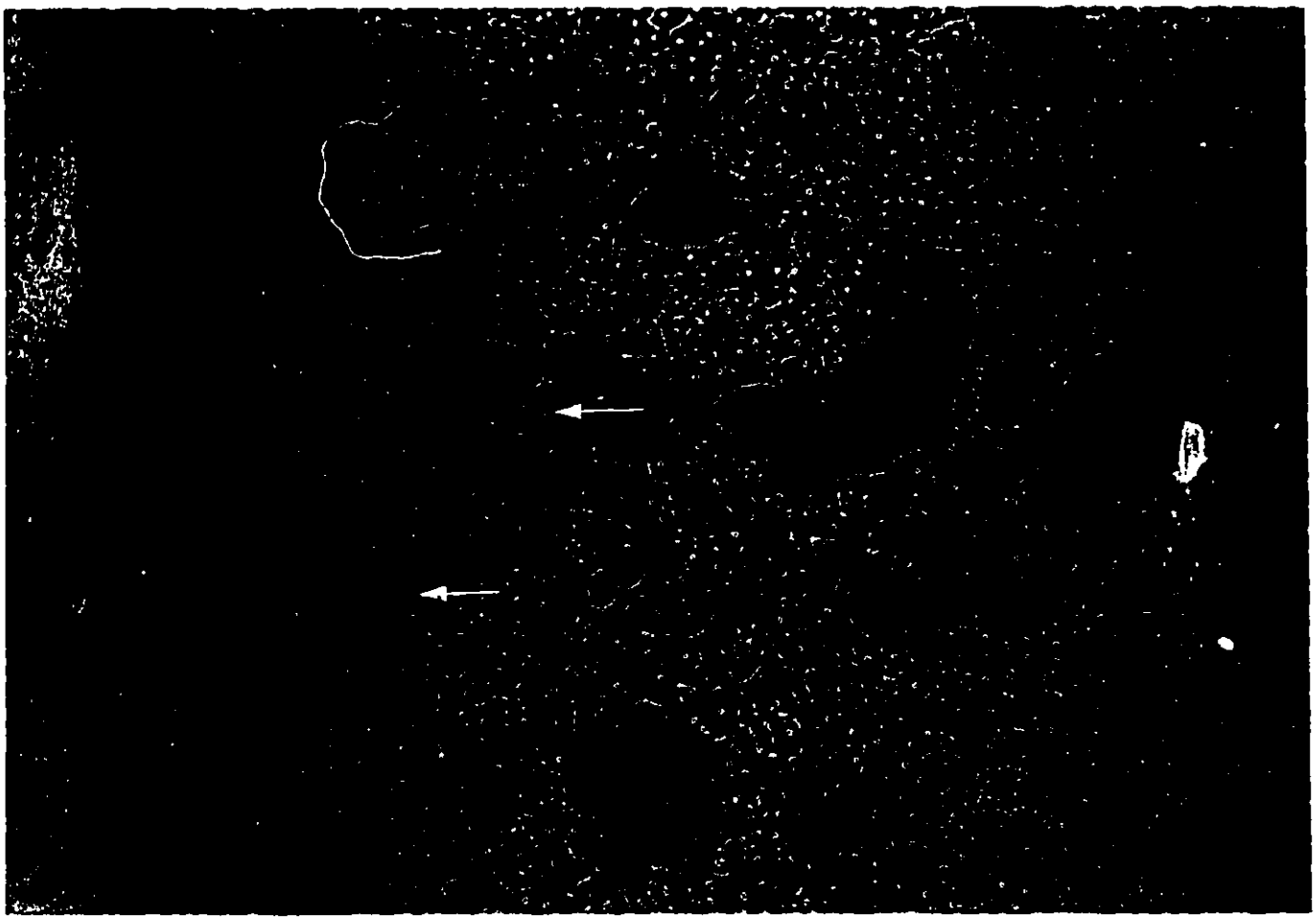


FIGURE 17. Immunofluorescence photograph of a control hepatocyte colony stained for actin.

The actin filament is more concentrated at the periphery of the hepatocyte and around small bile canaliculi (⇒). (× 400)

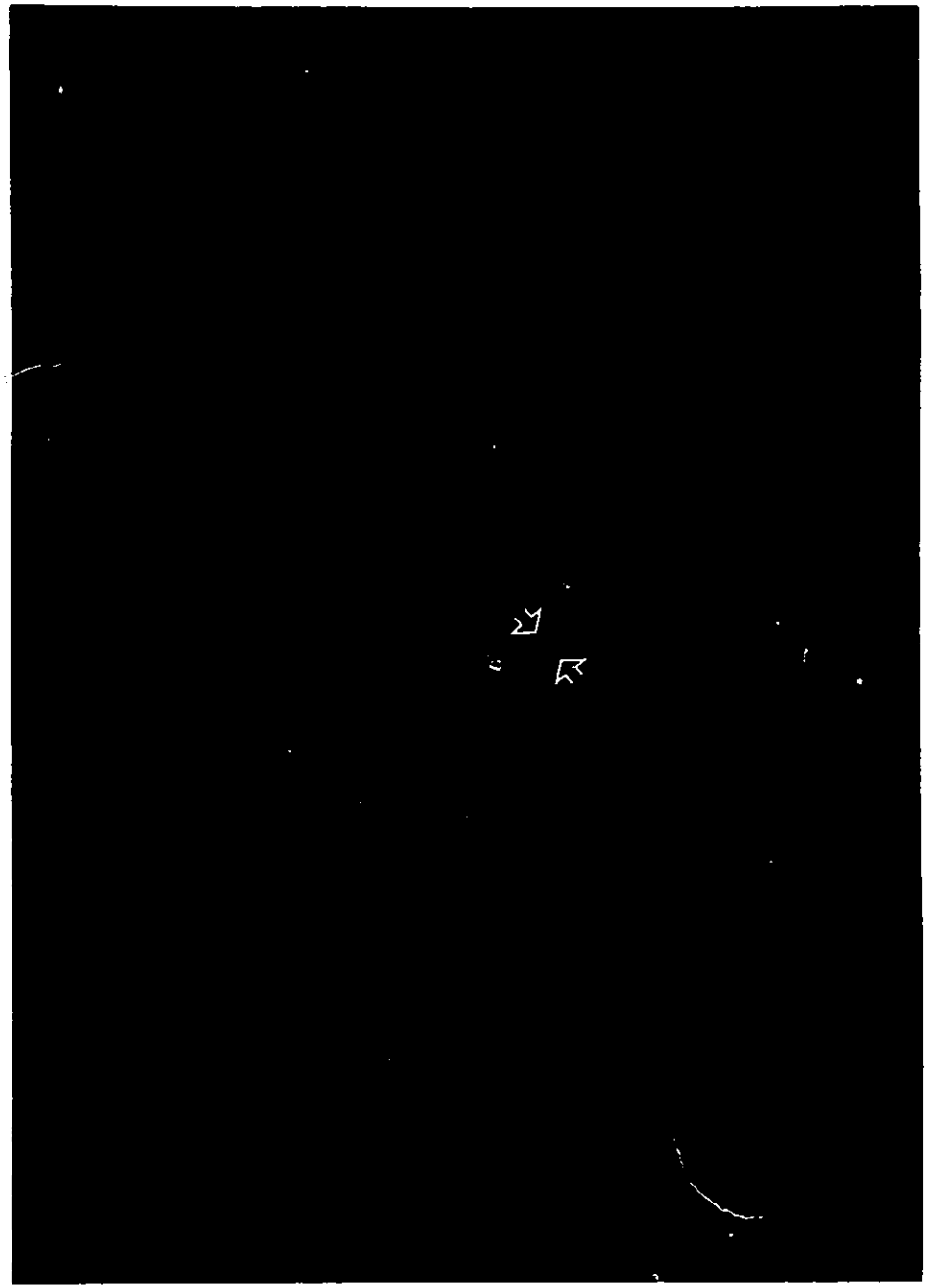
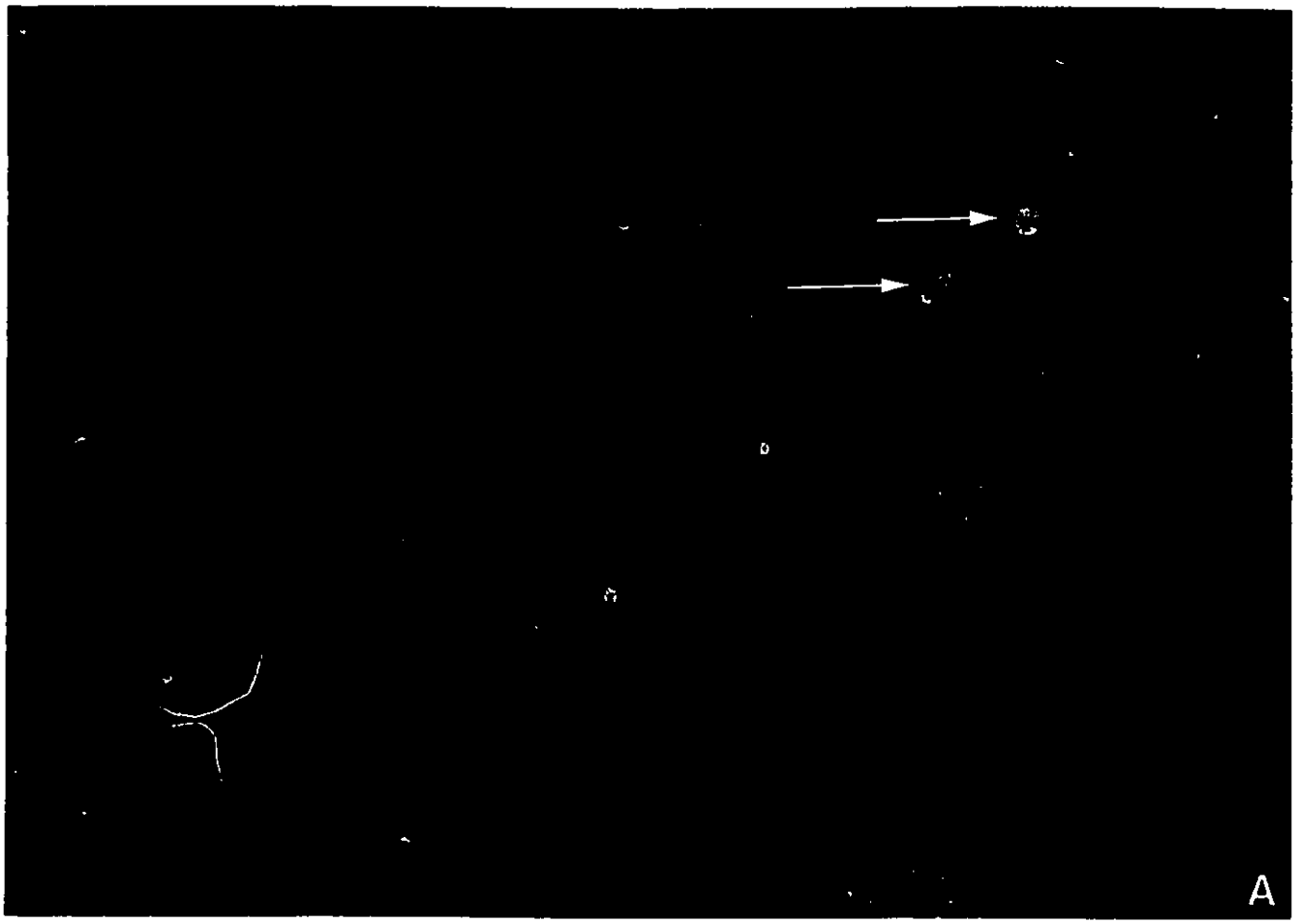


FIGURE 18. Immunofluorescence photographs of a control hepatocyte colony stained for tubulin.

Immunofluorescence microphotographs of a control hepatocyte colony showing the staining of tubulin. The staining shows a filamentous presence of tubulins in the cytoplasm (the non polymerized form), and the arrangement of polymerized microtubules forming the spindle during the mitosis process (→).

(a) × 250
(b) × 400


A


B

FIGURE 19 Immunofluorescence photographs comparing the distribution of cytokeratin CK55 kDa in control and treated hepatocyte colonies.

(a) control hepatocyte culture
(b) retinoic acid 10^{-5}M treated hepatocyte culture

The staining shows a filamentous cytokeratin 55 kDa network in the cytoplasm, and its concentration around the bile canaliculi. In the retinoic acid treated colonies (b), the pericanalicular sheath appears thicker and larger, and more numerous bile canaliculi (→) are seen. The intensity of the stain obscured the cellular details. (× 400)

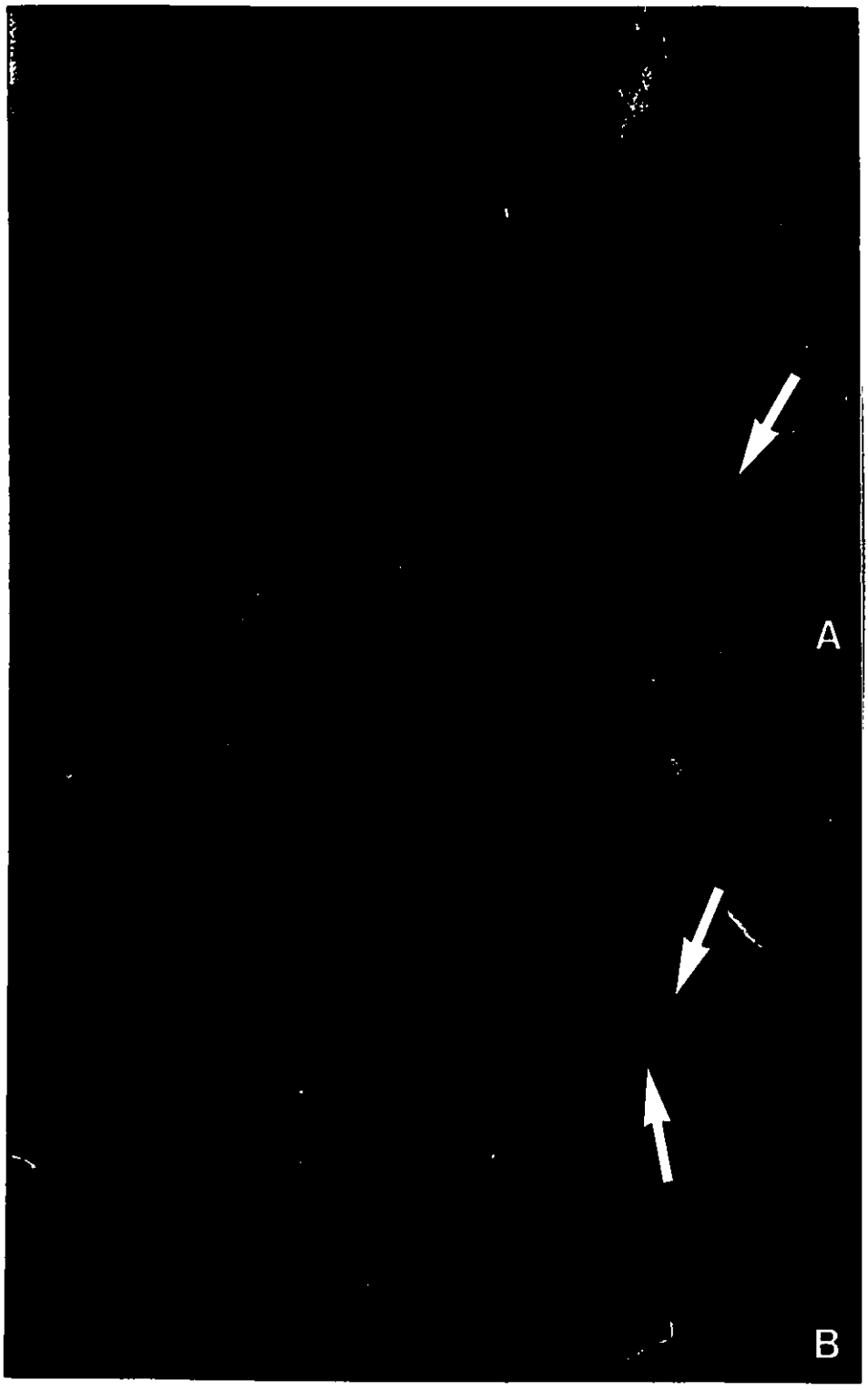

A
B

FIGURE 20 Immunofluorescence photographs comparing the distribution of cytokeratin 49 kDa in control and treated hepatocyte colonies.

(a) control hepatocyte culture
(b) retinoic acid 10^{-5} M treated hepatocyte culture

The staining shows a filamentous cytokeratin 49 kDa network in the cytoplasm, and its concentration around the bile canaliculi. In the retinoic acid treated colonies (b), the pericanalicular sheath appears thicker and larger, and more numerous bile canaliculi (→) are seen. (× 400)


A


B

FIGURE 21. Immunofluorescence photographs comparing the distribution of cytokeratin 49 and 55 kDa in retinoic acid treated hepatocyte colonies.

(a) cytokeratin 49 kDa
(b) cytokeratin 55 kDa

The distribution of the two cytokeratins forming the pericanalicular sheaths is similar. The pericanalicular sheaths are large and thick compared with the cellular size, and striations are present along the perpendicular axis of the bile canaliculi (→). (× 400)


A

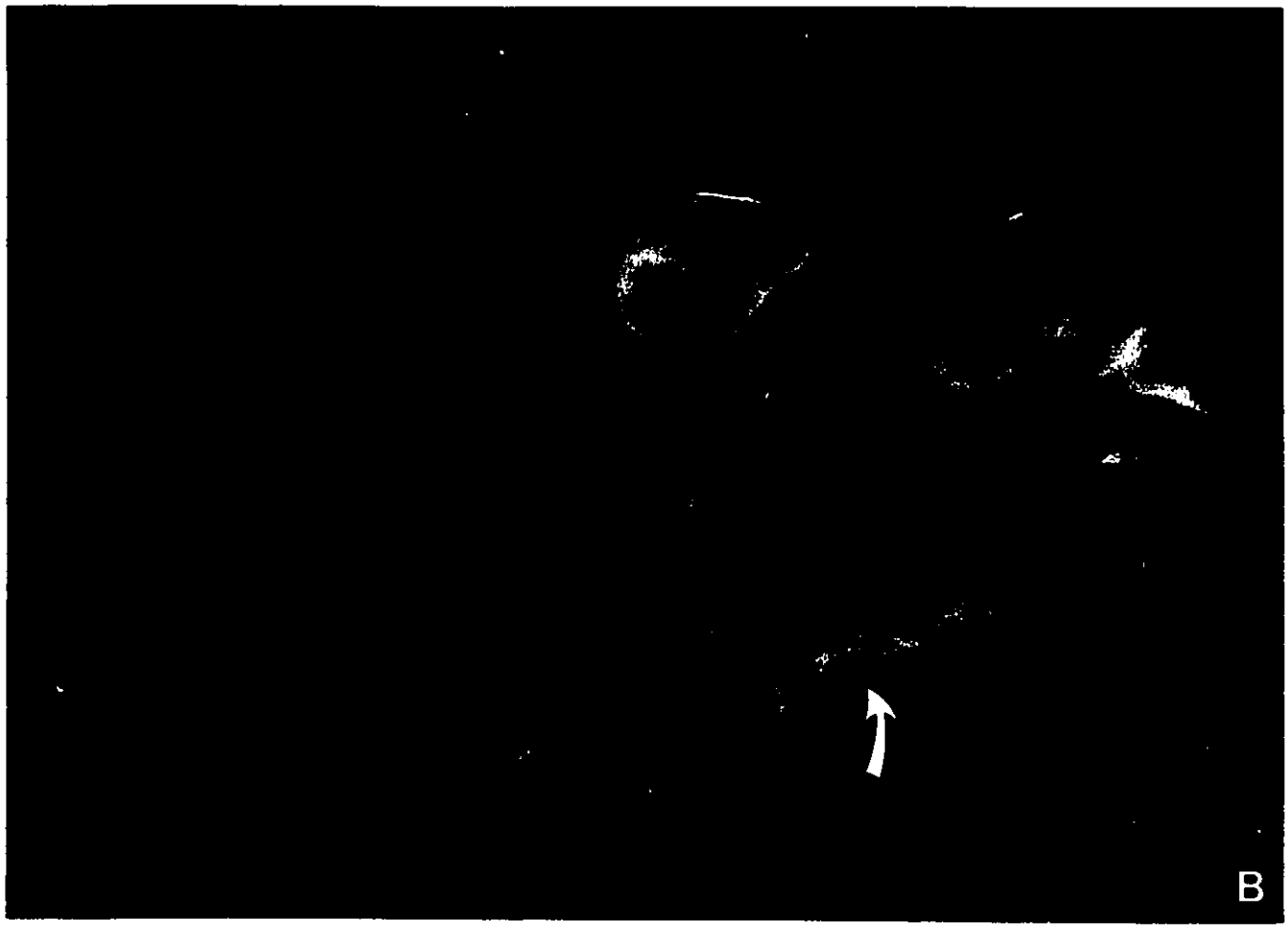

B

FIGURE 22. Immunofluorescence photograph of a retinoic acid treated hepatocyte colony showing a bile canaliculus ramification.

A bile canaliculi ramification (→) is demonstrated by the immunofluorescence staining of the pericanalicular sheath with cytokeratin 55 kDa. (× 400)

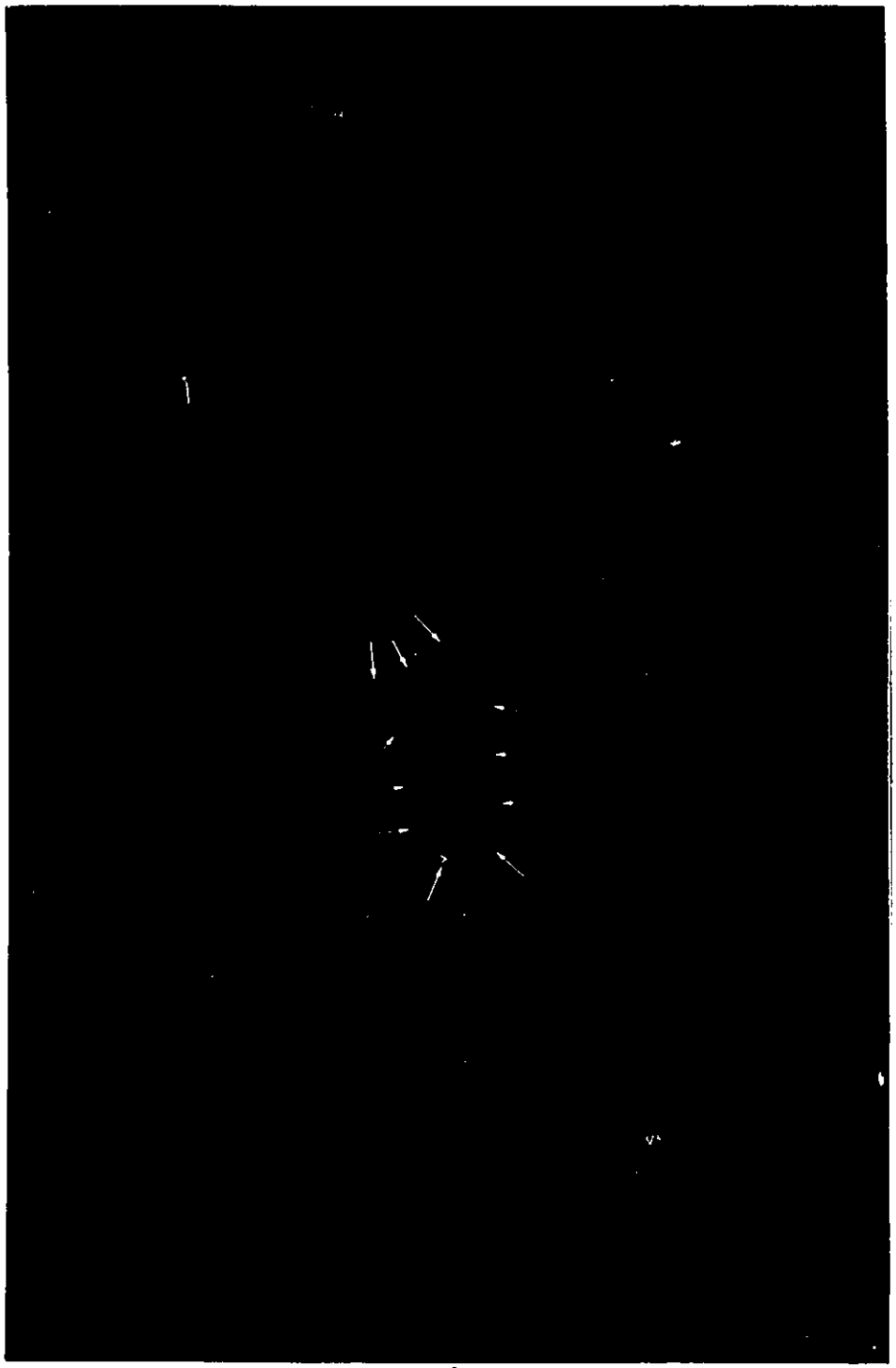


FIGURE 23. Immunofluorescence photographs of a control hepatocyte colony comparing the distribution of cytokeratin 55 and 49 kDa.

(a) cytokeratin 55 kDa (FITC)
(b) cytokeratin 49 kDa (TRITC)
(c) cytokeratin 55 and 49 kDa superposed

The cytokeratin 55 and 49 kDa are seen arranged around the hepatocyte nucleus (→) and in the cytoplasm. The cytokeratins are more concentrated at the hepatocyte periphery. The microphotograph (c) showing the superposition of the filaments labelled with the immunofluorescence stained to cytokeratin 55 and 49 kDa appears to demonstrate that these two filaments present a co-localization in the cell. (× 400)


A


B

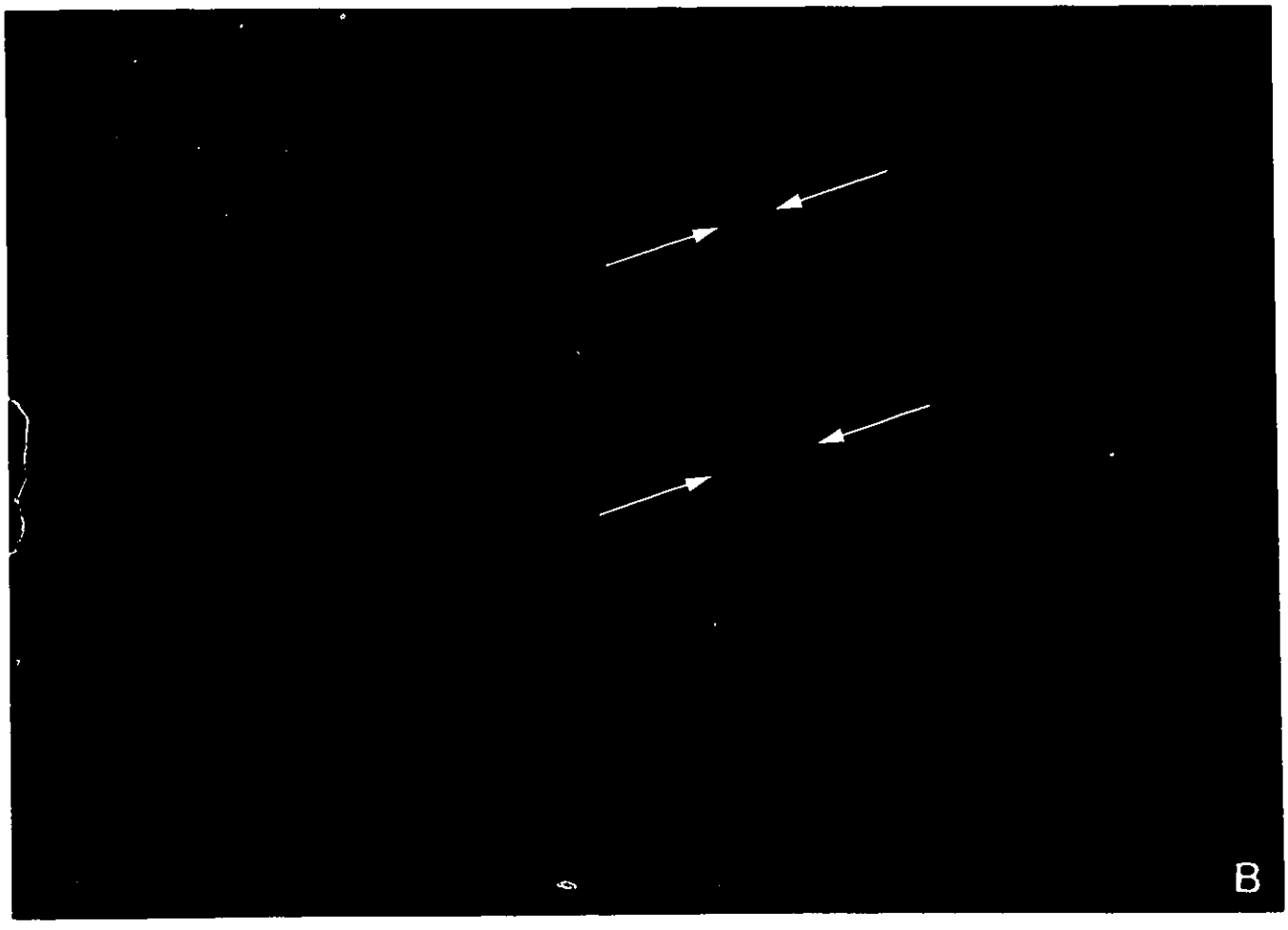

C

FIGURE 24. Immunofluorescence photographs comparing the distribution of cytokeratin 49 and 55 kDa in a retinoic acid treated hepatocyte colony.

(a) cytokeratin 55 kDa (FITC)
(b) cytokeratin 49 kDa (TRITC)
(c) cytokeratin 55 and 49 kDa superposed

The two cytokeratins have a similar distribution in the formation of the pericanalicular sheaths of the bile canaliculi and in the cytoplasm. The superposition photograph shows a golden coloration of the pericanalicular sheaths (→) implying a co-localization of the two cytokeratins. (× 400)


A


B

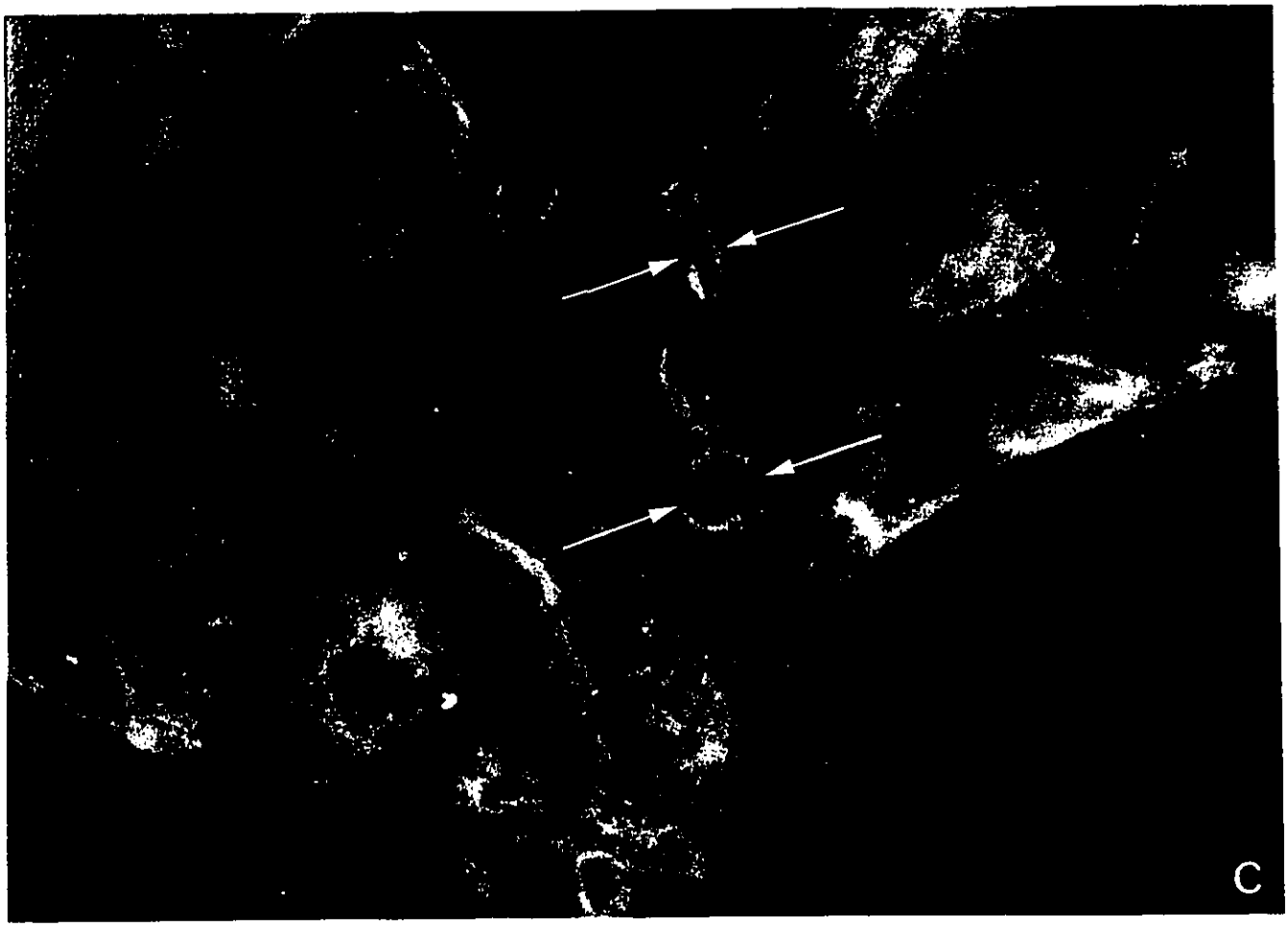

C

FIGURE 25. Immunofluorescence photographs of the uptake and the secretion of fluorescein diacetate in a control hepatocyte colony.

(a) uptake phase: control colony at 5 minutes (× 400)
(b) secretion phase: control colony at 30 minutes (⇒) (× 1000)


a

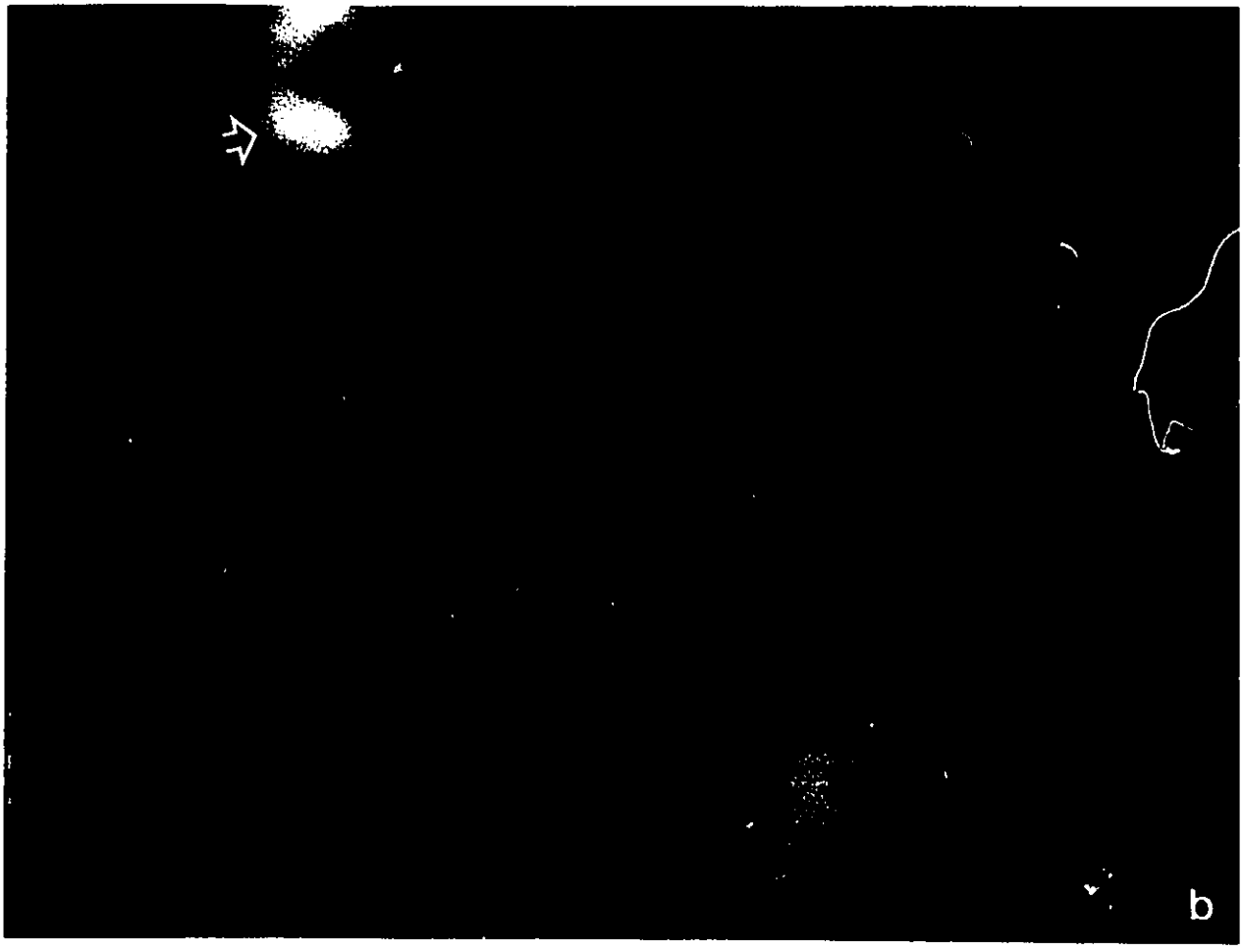

b

FIGURE 26. Immunofluorescence photographs of the uptake and the secretion of fluorescein diacetate in a retinoic acid treated colony.

(a) uptake phase: treated colony at 5 minutes (× 250)
(b) secretion phase: treated colony at 30 minutes (⇒) (× 400)


a

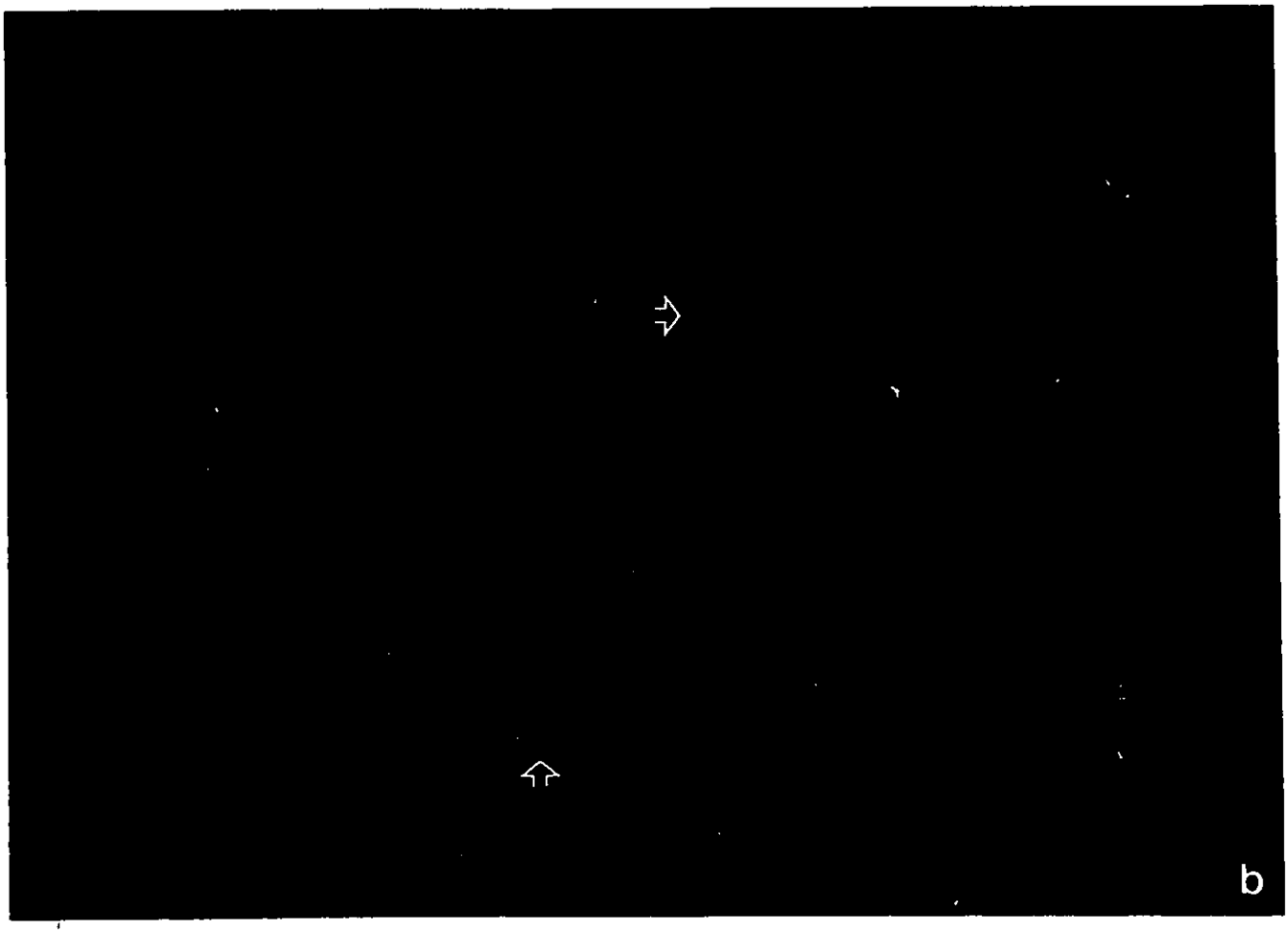

b

FIGURE 27. Immunofluorescence photograph of the polarization phase with fluorescein diacetate in a retinoic acid treated colony.

This microphotograph demonstrates the polarization phase of the fluorescein diacetate in the cytoplasm, immediately preceding the secretion phase (→). This photograph was taken after 20 minutes of incubation. (× 400)

FIGURE 28. Electron microphotograph of the filament distribution in a control hepatocyte colony.

The photograph shows the distribution of cytoskeletal filaments after digestion and extraction of its cellular components.

The border between two adjacent hepatocytes (→) and the remaining of the nucleus is identified (▸) by the higher density of filaments composing these structures. The dark agglomerates (⇒) correspond to nuclear debris that were attached to the filaments during the extraction process. (× 3800)

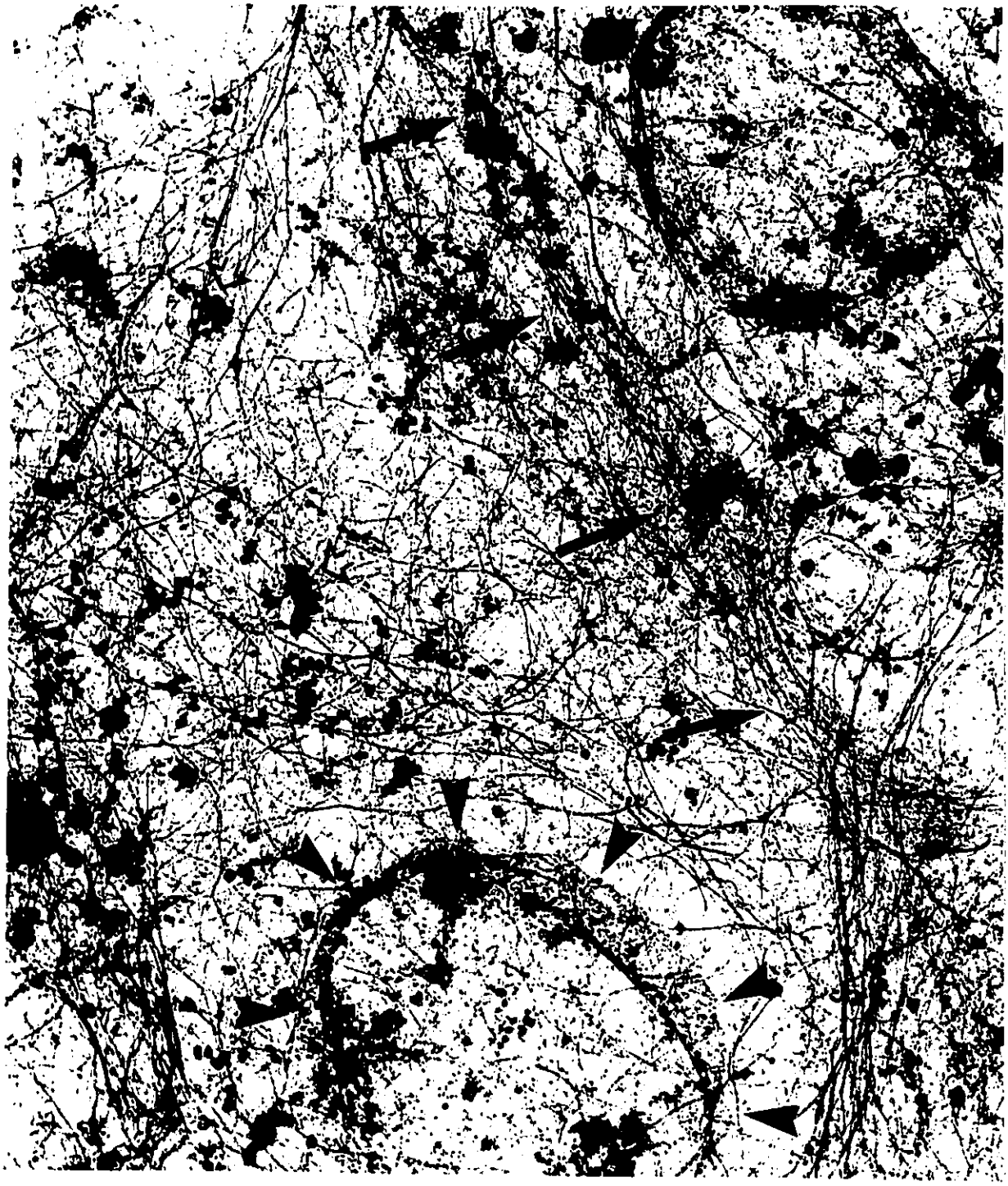


FIGURE 29. Electron microphotograph of the filament distribution along the pericanalicular sheath in a retinoic acid treated hepatocyte colony.

The filaments are seen dispersed in the hepatocyte cytoplasm of two adjacent hepatocytes and become denser when forming the pericanalicular sheath (⇒) of the bile canaliculi between the two adjacent hepatocytes.
The filaments are arranged parallel to the longitudinal dimension of the bile canaliculi. It appears that some filaments (→) from the cytoplasm are somewhat anchored to the pericanalicular sheaths. (× 13500)

FIGURE 30. Electron microphotograph of a bile canaliculi ramification in a retinoic acid treated hepatocyte colony

This photograph of the distribution of the filaments shows the detailed architecture of a pericanalicular sheath ramification (→) forming a complex bile canaliculi structure between three adjacent hepatocytes. The presence of bile canaliculi ramification as demonstrated by the visualization of the pericanalicular sheaths in extracted preparation corresponds to the ramification observed under immunofluorescence staining (see Figure 22). (× 6350)

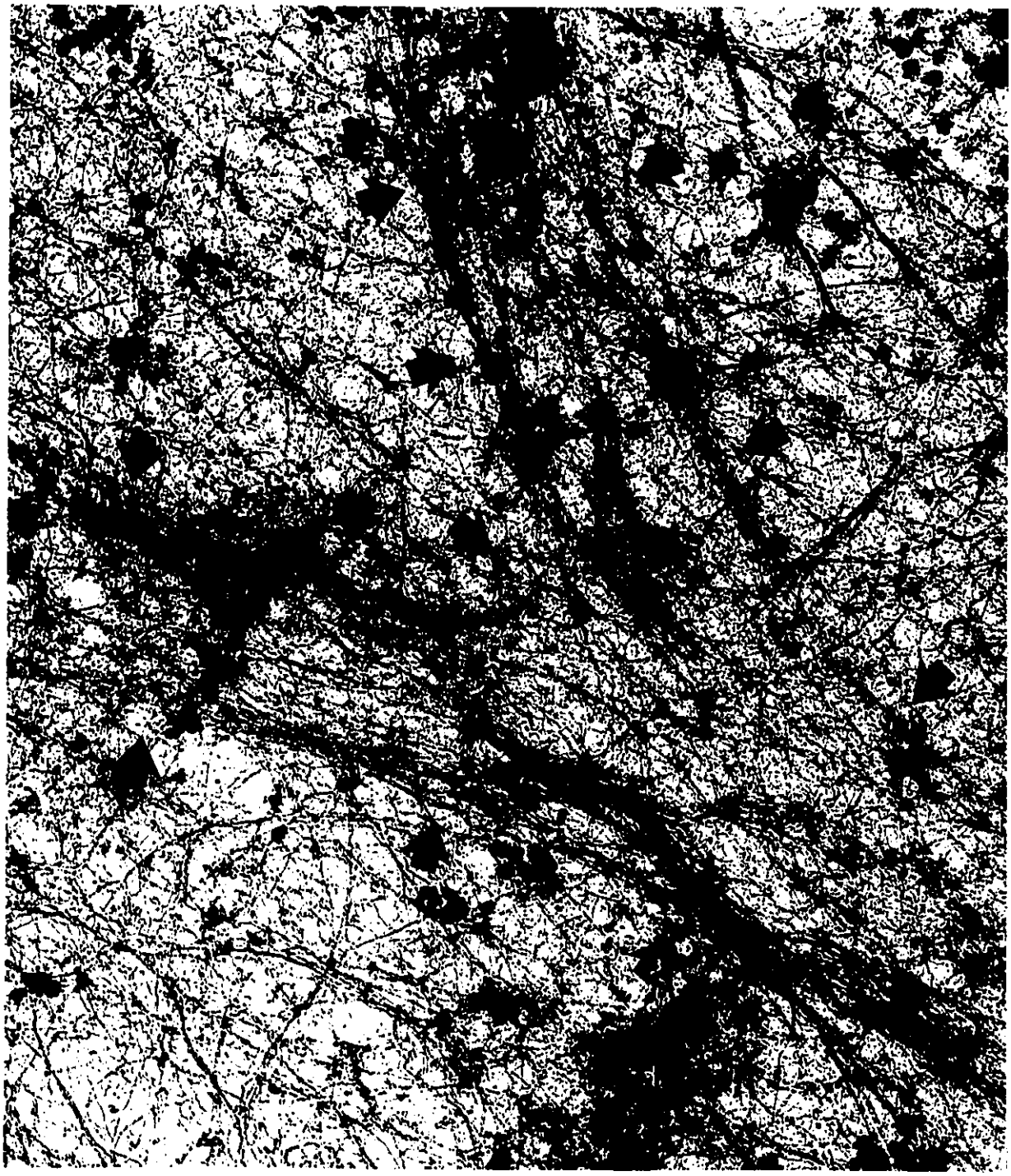


FIGURE 31. SDS-PAGE of whole control and retinoic acid treated colonies

This SDS-polyacrylamide gel electrophoresis used non-extracted hepatocyte proteins from an identical quantity of petri from both control hepatocyte colonies and retinoic acid 10^{-5} M treated hepatocyte colonies.

C: proteins obtained from the control hepatocyte colonies
T: proteins obtained from the retinoic acid treated colonies
S: low molecular weight standard (Bio-Rad):

97 kDa = Rabbit muscle phosphorylase b
66 kDa = Bovin serum albumin
42 kDa = Hen egg white ovalbumin
21 kDa = Soybean trypsin inhibitor

A: actin

55: cytokeratin of 55 kDa
49: cytokeratin of 49 kDa

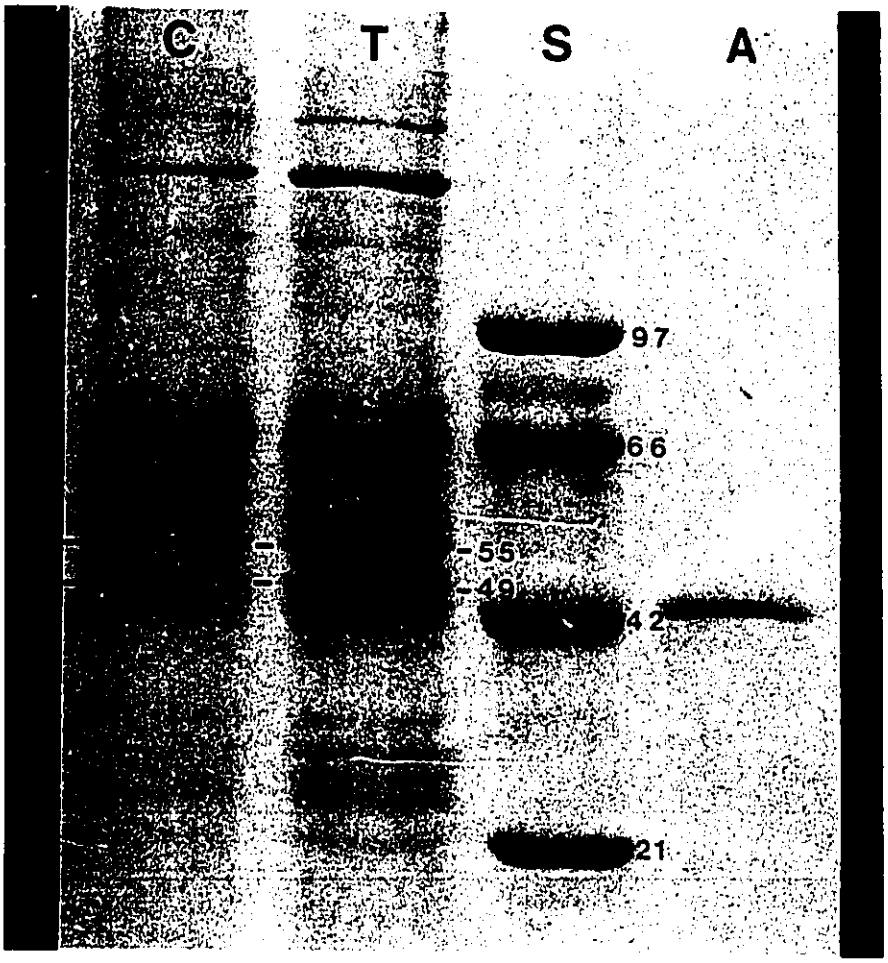

C
T
S
A
97
66
55
49
42
21

FIGURE 32. SDS-PAGE of extracted control and retinoic acid treated colonies

This SDS-polyacrylamide gel electrophoresis used hepatocyte proteins extracted from an identical quantity of proteins from both control and retinoic acid 10^{-5} M treated hepatocyte colonies (see Section 2.4.1).

C: proteins obtained from the control hepatocyte colonies
T: proteins obtained from the retinoic acid treated colonies
S: low molecular weight standard (Bio-Rad):

97 kDa = Rabbit muscle phosphorylase b
66 kDa = Bovin serum albumin
42 kDa = Hen egg white ovalbumin
21 kDa = Soybean trypsin inhibitor

A: actin

55: cytokeratin of 55 kDa
49: cytokeratin of 49 kDa

FIGURE 33. Example of densitometric scanning curve of a SDS-PAGE

The densitometric scanning curve was obtained from the SDS-polyacrylamide gel electrophoresis of the extracted hepatocyte proteins from a retinoic acid 10^{-5} M treated hepatocyte colony.

The densitometry was done using the densitometric analyzer program Beckman DU-88.


SENSITIVITY = 1
0.0000
0.3000
40.00
90.00
45.0 MM 0.344
51.6 MM 0.191
61.2 MM 0.436
66.6 MM 0.200
70.8 MM 0.196
73.7 MM 0.245
77.1 MM 0.190
84.6 MM 0.261
88.3 MM 0.288
92.5 MM 0.239
95.4 MM 0.291
98.3 MM 0.318
100.4 MM 0.350
103.7 MM 0.607
110.8 MM 0.416
117.5 MM 0.198
120.4 MM 0.263
123.3 MM 0.211
126.6 MM 0.309
128.5 MM 0.336
133.3 MM 0.371
137.5 MM 0.275

REFERENCE

Adamson E.D. (1983): The effect of collagen on cell division, cellular differentiation and embryonic development. *In:* Jayson, Weiss, eds. Collagen in Health and Disease. London, Churchill-Livingston, 218-243.

Adelstein R.S. (1982): Calmodulin and the regulation of the actin-myosin interaction in smooth muscle and nonmuscle cells. Cell 30: 349-350.

Amatruda T.T., III, Sidell N., Ranyard J., Koeffler P. (1985): Retinoic acid treatment of human neuroblastoma cells is associated with decreased N-Myc expression. Biochem Biophys Res Commun 126: 1189-1195.

Appling D.R., Chytil F. (1981): Evidence of a role for retinoic acid (vitamin A-acid) in the maintenance of testosterone production in male rats. Endocrinology 108: 2120-2123.

Baribault H., Blouin R., Bourgon L., Marceau N. (1989): Epidermal growth factor-induced selective phosphorylation of cultured rat hepatocyte 55 kD cytokeratin before filament reorganization and DNA synthesis. J Cell Physio 109: 1665-1676.

Baribault H., Leroux-Nicollet I., Marceau N. (1985): Differential responsiveness of cultured suckling and adult rat hepatocytes to growth-promoting factors: entry into S phase and mitosis. J Cell Physiol 122: 105-112.

Baribault H., Marceau N. (1986): Dexamethasone and Dimethylfulfoxide as distinct regulators of growth and differentiation of cultured suckling rat hepatocytes. J Cell Physiol 129: 77-84.

Barth C.A., Schwarz L.R. (1982): Transcellular transport of fluorescein in hepatocyte monolayers: Evidence for functional polarity of cells in culture. Proc Natl Acad Sci (USA) 79: 4985-4987.

Belanger L., Frain M., Baril P., Gingras M.C., Bartkowiak J., Sala-Trepat T.M. (1981): Glucocorticoid suppression of alpha-1-fetoprotein synthesis in developing rat liver. Biochemistry 20: 6665-6672.

Benbrook D., Lernhardt E., Pfahl M. (1988): A new retinoic acid receptor identified from a hepatocellular carcinoma. Nature 333: 669-672.

Berry M.N., Friend D.S. (1969): High yield preparation of isolated rat liver parenchymal cells. J Cell Biol 43: 506-520.

Bissel D.M., Hammaker L., Meyer U.A. (1973): Parenchymal cells from adult rat liver

in non-proliferating monolayer culture. J Cell Biol 59: 722-734.

Bissel M.J. (1981): The differentiated state of normal and malignant cells or how to define a "normal" cell in culture. Int Rev Cytol, 70: 27-100.

Bissel M.J., Hall H.G., Parry G (1982): How does the extracellular matrix direct gene expression? J Theor Biol 99: 31-68.

Bonney R.J., Becker J.E., Walker P.R., Potter V.R. (1974): Primary monolayer cultures of adult rat liver parenchymal cells suitable for study of regulation of enzyme synthesis. In Vitro 9: 399-413.

Brand N., Petkovich M., Krust A., Chambon P., De The H., Marchio A., Tiollais P., Dejean A. (1988): Identification of a second human retinoic acid receptor. Nature 332: 850-853.

Brockes J.P. (1989): Retinoids, homeobox genes, and limb morphogenesis. Neuron 2: 1285-1294.

Brodsky W.Y., Uryvalva I.V. (1978): Cell polyploidy: Its relation to tissue growth and function. Int Rev Cytol 50: 275-332.

Brown R., Gray R.H., Bernstein I.A. (1985): Retinoids alter the direction of differentiation in primary cultures of cutaneous keratinocytes. Differentiation 28: 268-278.

Brulet P., Babinet C., Kemler R., Jacob F. (1980): Monoclonal antibodies against trophectoderm-specific markers during mouse blastocyst formation. Proc Natl Acad Sci (USA) 77: 4113-4117.

Cahn R.D., Coon H.G., Cahn M.B. (1967): *In:* Methods in developmental biology. Eds. Wilt and Wessells: 493-530.

Chopra D.P. (1983): Retinoid reversal of squamous metaplasia in organ cultures of tracheas derived from hamsters fed on vitamin A-deficient diet. Eur J Cancer Clin Oncol 19: 847-857.

Coon H.G. (1966): Clonal stability and phenotypic expression of chick cartilage cells *in vitro*. Proc Natl Acad Sci (USA) 55: 66-73.

Coon H.G. (1968). Clonal culture of differentiated rat liver cell. J Cell Biol 39: 29a.

Couchman J.R., Gibson W.T., Thom D., Weaver A.C., Rees D.A., Parish W.E. (1979): Fibronectin distribution in epithelial and associated tissues of the rat. Arch Dermatol Res 266: 295-310.

Couchman J.R., Rees D.A., Green M.R., Smith C.G. (1982): Fibronectin has a dual role in locomotion and anchorage of primary chick fibroblasts and can promote entry in the division cycle. J Cell Biol 93: 402-410.

Coulombe P.A., Hutton M.E., Vassar R., Fuchs E. (1991): A function for keratins and a common thread among different types of epidermolysis Bullosa Simplex Diseases. J Cell Biol 115(6): 1661-1674.

Crow J.A., Ong D.E., Chytil F. (1987): Specificity of cellular retinol-binding protein in the transfer of retinol to nuclei and chromatin. Arch Biochem Biophys 254: 372-375.

Dedhar S., Ruoslahti E., Pierschbacher M.D. (1987): A cell surface receptor complex for collagen type 1 recognizes the Arg-Gly-Asp sequence. J Cell Biol 104: 585-593.

Delmas A. (1974): Le foie. *In:* Le Rouviere: Anatomie Humaine, Descriptive, Topographique et Fonctionnelle. tome 2, (11th ed.) Masson Press. pp. 432-458.

Denk H., Lackinger E. (1986): Cytoskeleton in liver diseases. Seminars in Liver Disease 6(3): 199-211.

Denning H.F., Verma A.K. (1991): Involvement of retinoic acid nuclear receptors in retinoic acid-induced tissue transglutaminase gene expression in rat tracheal 2C5 tissue. Biochem Biophys Res Commun 175(1): 344-350.

Deschênes J., Valet J.P., Marceau N. (1980): Hepatocytes from newborn and weanling rats in monolayer culture: isolation by perfusion, fibronectin-mediated adhesion, spreading, and functional activities. In Vitro 16(8): 722-730.

Dowling J.E., Wald G. (1960): The biological function of vitamin A acid. Proc Natl Acad Sci (USA) 46: 587-608.

Durston A.J., Timmermans J.P.M., Hage W.T., Hendriks H.F.J., de Vries N.J., Heideveld M., Nieuwkoop D.P. (1989): Retinoic acid causes an anterioposterior transformation in the developing central nervous system. Nature 340: 140-144.

Dustin P. (1980): Microtubules. Sci Am 243(2): 66-76.

Eichner R., Bonitz P., Sun T.T. (1984): Classification of epidermal keratins according to their immunoreactivity, isoelectric point, and mode of expression. J Cell Biol 98: 1388-1396.

Elias H. (1953): *In:* Research in the service of medicine. 37.

Elias P.M., Williams M.L. (1981): Retinoids, cancer and the skin. Arch Dermatol 117:

160-180.

Eriksson U., Hansson E., Nilsson M., Jonsson K.H., Sundelin J., Peterson P.H. (1986): Increased levels of several retinoid binding proteins resulting from retionic acid-induced differentiation of F9 cells. Cancer Res 46: 717-722.

Evans H.M., Bishop K.S. (1922): On an invariable and characteristic disturbance of reproduction function in animals reared on a diet poor in fat soluble vitamin A. Anat Rec 23: 17.

Fawcett D.W. (1986): The liver and gall bladder. *In:* Saunders W.B., eds. A Textbook of Histology. (11th ed.) Saunders Press, USA pp. 679-708.

Fey E.G., Penmans S. (1985): New views of cell and tissue cytoarchitecture: Embedment-free electron microscopy and biochemical analysis. Hepatology 5: 152-158.

Fey E.G., Won K.M., Penman S. (1984): Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: Three-dimensional organization and protein composition. J Cell Biol 98: 1973.

Fisher I., Nolan C.E., Shea T.B. (1987): Effects of Retinoic Acid on expression of the transformed phenotype in C6 glioma cells. Life Sc 41: 463-470.

Fontana J.A., Rogers J.S., II., Durham J.P. (1986): The role of 13 cis-retinoic acid in the remission induction of a patient with acute promyelocytic leukemia. Cancer 57(2): 209-217.

Franke W., Schmid W.E., Grund C., Geiger B. (1982): Intermediate filament proteins in non-filamentous structures: transient disintegration and inclusion of subunit proteins in granular aggregates. Cell 30: 103-113.

French S.W., Davies P.L. (1975): Ultrastructural localization of actin-like filaments in rat hepatocytes. Gastroenterology 68: 765-774.

French S.W., Kawahara H., Katsuma Y., Ohta M., Swierenga S.H.H. (1989): Interaction of intermediate filaments with nuclear lamina and cell periphery. Electron Microsc Rev 2(1): 17-52.

French S.W., Kondo I., Irie T., Ihrig T.J., Benson N., Munn R. (1982): Morphologic study of intermediate filaments in rat hepatocytes. Hepatology 2: 29-38.

French S.W., Okanoue T., Swierenga S.H.H., Marceau N. (1987): The cytoskeleton of hepatocytes in health and disease. *In:* Williams and Wilkins, eds. Pathogenesis of Liver Diseases: IAP mono 28, Baltimore, MD, USA, pp. 95-111.

Fuchs E., Green H. (1981): Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. Cell 25: 617-625.

Ganong W.F. (1981): Liver and biliary system. *In:* Review of Medical Physiology. (10th ed.) Lange, ed. Los Altos, California, pp. 393-397.

Geisler N., Kaufman E., Weber K. (1985): Antiparallel orientation of the two double stranded coiled coils in the tetrameric protofilament unit of intermediate filaments. J Mol Biol 182: 173-177.

Geisler N., Weber K. (1982): The amino acid sequence of chicken muscle desmin provides a common structural model for intermediate filament proteins. J Euro Mol Biol Org 1: 1649-1656.

Gibson W.T., Couchman J.R., Badley R.A., Saunders H.J., Smith C.G. (1983): Fibronectin in cultured rat keratinocytes: distribution, synthesis and relationship to cytoskeletal proteins. Eur J Cell Biol 30: 205-213.

Giguere V., Ong E.S., Sequi P., Evans R.M. (1987): Identification of a receptor for the morphogen retinoic acid. Nature 333: 624-629.

Goldman R., Goldman A., Green K., Jones J., Lieska N., Yang H.Y. (1985): Intermediate filaments: Possible functions as cytoskeletal connecting links between the nucleus and the cell surface. Ann NY Acad Sci 445: 1-17.

Goodman D.S. (1980): Plasma retinol-binding protein. Ann NY Acad Sci 348: 378-390.

Goodman D.S. (1988): Vitamin A metabolism and the liver. *In:* The Liver, Biology and Pathobiology. New York, Raven Press.

Green H., Watt F.M. (1982): Regulation by vitamin A of envelope cross-linking in cultured keratinocytes derived from different human epithelia. Mol Cell Biol 2(9): 1115-1117.

Hauschka D.S., Konigsberg I.R. (1966): The influence of collagen on the development of muscle colonies. Proc Natl Acad Sci (USA) 55: 119-126.

Hay E.D., (1980): Development of the vertebrate cornea. Int Rev Cytol 63: 263-322.

Helgerud P., Peterson L.B., Norum K.R. (1983): Retinol esterification by microsomes from the mucosa of human small intestine. J Clin Invest 71: 747-753.

Higgins P.J., Borenfreund E. (1980): Enhanced albumin production by malignantly

transformed hepatocytes during *in vitro* exposure to dimethylsulfoxide. Biochem Biophys Acta 610: 174-180.

Higgins P.J., Dazynkiewicz Z., Melamed M.R. (1983): Secretion of albumin and alpha-feto protein by dimethylsulfoxide stimulated hepatocellular carcinoma cells. Br J Cancer 48: 485-493.

Hook M., Rubin K., Olberg A., Obrink B., Vaheri A. (1977): Cold-insoluble globulin mediates the adhesion of rat liver cells to plastic petri dishes. Biochem Biophys Res Commun 79: 726-733.

Ishak K.G., Stromeyer F.W. (1983): Medical diseases of the liver. *In:* Silverberg ed. Principles and Practice of Surgical Pathology. 2, pp. 937-941.

Irie T., Benson N.C., French S.W. (1982): Relationship of Mallory bodies to the cytoskeleton of hepatocytes in griseofulvin-treated mice. Lab Invest 47: 336-345.

Isom H.C., Secott T., Georgoff I., Woodworth C., Mummaw J. (1985): Maintenance of differentiated rat hepatocytes in primary culture. Proc Natl Acad Sci (USA) 82: 3252-3256.

Jetten A.M., Brody A.R., Deas M.A., Hook G.E.R., Rearick J.I. (1987): Retionic acid and substratum regulate the differentiation of rabbit tracheal epithelial cells into squamous and secretory phenotype. Lab Invest 56: 654-664.

Jirtle R.L., Biles C., Michalopoulos G. (1980): Morphologic and histochemical analysis of hepatocytes transplanted into syngeneic hosts. Am J Pathol 101: 115-123.

Johnson G.D., Nogueira Araujo G.M. (1981): A simple method of reducing the fading of immunofluorescence during microscopy. J Immunological Methods 43: 349-350.

Jones-Villeneuve E.M.V., McBurney M.W., Rogers K.A., Kalnins V.I. (1982): Retinoic acid induces embryonal carcimona cells to differentiate into neurons and glial cells. J Cell Biol 94: 253-262.

Kaighn M.E. (1973): Human liver cells. *In:* Kruse and Patterson eds. Liver Tissue Culture, Methods and Applications. pp. 54-58.

Kanai M., Raz A., Goodman D.S. (1968): Retinol-binding protein: The transport protein for vitamin A in human plasma. J Clin Invest 47: 2025-2044.

Katsuma Y., Marceau N., Ohta M., French S.W. (1988): Cytokeratin intermediate filaments of rat hepatocytes: different cytoskeletal domains and their three-dimensional structure. Hepatology 8(3): 559-568.

Kawahara H., Cadrin M., Perry G., Autilio-Gambetti L., Swierenga S.H., Metuzals, J., Marceau N., French S.W. (1990): Role of cytokeratin intermediate filaments in transhepatic transport and canalicular secretion. Hepatology 11(3): 435-448.

Kawahara H., Marceau N., French S.W. (1989): Effect of agents which rearrange the cytoskeleton *in vitro* on the structure and function of hepatocytic canaliculi. Lab Invest 60(5): 692-704.

King K., Jones D.H., Daltrey D.C., Cunliffe W.J. (1982): A double-blind study of the effects of 13-cis-retinoic acid on acne, sebum excretion rate and microbial population. Brit J Dermatol 107: 583-590.

Kirschner M., Mitchison T. (1986): Beyond self-assembly: from microtubules to morphogenesis. Cell 45: 329-342.

Kleinman H.K., Klebe R.J., Martin G.R. (1981): Role of collagenous matrices in the adhesion and growth of cells. J Cell Biol 88: 473-485.

Koda J.E., Bernfield M. (1984): Heparan sulfate proteoglycans from mouse mammary epithelial cells. Basal extracellular proteoglycan binds specifically to native type I collagen fibrils. J Biol Chem 259: 11763-11770.

Krust A., Kastner P.H., Petkovich M., Zelent A., Chambon P. (1989). A third human retinoic acid receptor, hRAR-γ. Proc Natl Acad Sci (USA) 86: 5310-5314.

Kurkinen M., Taylor A., Garrels J.I., Hogan B.L.M. (1984): Cell surface-associated proteins which bind native type IV collagen or gelatin. J Biol Chem 259: 5915-5922.

Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227: 680-685.

Laishes B.A., Farber E. (1978): Transfer of viable putative preneoplastic hepatocytes to the livers of syngeneic host rats. J Natl Cancer Inst 61: 507-512.

Laishes B.A., Williams G.M. (1976a): Conditions affecting primary cell cultures of functional adult rat hepatocytes. I. The effect of insulin. In Vitro 12: 521-532.

Laishes B.A., Williams G.M. (1976b): Conditions affecting primary cell cultures of functional adult rat hepatocytes. II. Dexamethasone enhances longevity and maintenance of morphology. In Vitro 12: 821-832.

Lane E.B., Goodman S.L., Trejdosiewicz L.K. (1982): Disruption of keratin filament network during epithelial cell division. J Euro Mol Bio Org 1: 1365-1372.

Lasek R.J., Hoffmann P.N. (1976): *In:* Goldmann, Pollard and Rosenblum eds. Cell Mobility. 3: pp. 1021-1049.

Lazarides E. (1980): Intermediate filaments as mechanical integrators of cellular space. Nature 283: 249-256.

Lazarides E. (1982): Intermediate filaments: A chemically heterogeneous developmentally regulated class of proteins. Ann Rev Biochem 511: 219-250.

Lazarides E., Weber K. (1974): Actin antibody: the specific visualization of actin filaments in non-muscle cells. Proc Natl Acad Sci (USA) 71: 2268-2272.

Le Douarin N.M. (1975): An experimental analysis of liver development. Med Biol 53: 427-455.

Leffert H.L., Moran T., Skelly S.H., Sell S., Ibsen K., Mueller M., Arias I. (1978): Growth state-dependent phenotypes of adult hepatocytes in primary monolayer culture. Proc Natl Acad Sci (USA) 75: 1834-1838.

Lehninger A.L. (1976): Vitamins and coenzymes. *In:* Biochemistry. (2nd ed.) Worth Publishers, pp. 350-362.

Leroux-Nicollet I., Noel M., Baribault H., Goyette R., Marceau N. (1983): Selective increase in cytokeratin synthesis in cultured rat hepatocytes in response to hormonal stimulation. Biochem Biophys Res Commun 114: 556-563.

Li E., Demmer L.A., Sweetser D.A., Ong D.E., Gordon J.I. (1986): Rat cellular retinol-binding protein II: Use of a cloned cDNA to define its primary structure, tissue-specific expression, and developmental regulation. Proc Natl Acad Sci 83: 5779-5783.

Lilja S., Merker H.J., Ghaida J. (1988): The influence of matrix components on the morphological differentiation of a proliferative hepatocyte line from liver of newborn mice. Histopathology 3: 249-263.

Marceau N., Baribault H., Germain L., Noel M. (1986): The cytoskeleton of cultured hepatocyte. *In:* Guillouzo and Guguen-Guillouzo eds. Isolated and Cultured Hepatocytes. John Libbey Eurotext. pp. 39-61.

Marceau N., Baribault H., Leroux-Nicollet I. (1985): Dexamethasone can modulate the synthesis and organization of cytokeratins in cultured differentiating rat hepatocytes. Can J Biochem Cell Biol 63(6): 448-457.

Marceau N., Goyette R., Deschênes J., Valet J.P. (1980): Morphological differences

between epithelial and fibroblast cells in rat liver cultures, and the roles of cell surface fibronectin and cytoskeletal element organization in cell shape. Ann NY Acad of Sci 349: 138-152.

Marceau N., Goyette R., Pelletier G., Antakly J. (1983): Hormonally induced changes in the cytoskeleton organization of adult and newborn rat hepatocyte cultured on fibronectin precoated substratum: effect of dexamethasone and insuline. Cell Mol Biol: 29(5): 421-435.

Marceau N., Noel M., Deschênes J. (1982): Growth and functional activities of neonatal and adult rat hepatocytes on fibronectin coated substratum in serum-free medium. In Vitro 18: 1-11.

Margolis R.L., Wilson L. (1981): Microtubule treadmills: possible molecular machinery. Nature 293: 705-711.

McBurney M.W., Jones-Villeneuve E.M.V., Edwards M.K.S., Anderson P.J. (1982): Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature 299: 165-169.

McCollum E.V., Davis M. (1915): The nature of dietary deficiencies of rice. J Biol Chem 23: 181.

McLaren D.S. (1981): The luxus vitamins: A and B12. Am J Clin Nutr 34: 1611-1616.

Mehta R.G., Moon R.C. (1981): Hormonal regulation of retinoic acid binding proteins in the mammary gland. Biochem J 200: 591-595.

Metuzals J., Mushynski W.E. (1974): Electron microscope and experimental investigations of the neurofilamentous network in Deiter's neurons. Relationship with the cell surface and nuclear pores. J Cell Biol 61: 701-722.

Mito M., Ebata H., Kusano M., Onishi T., Saito T., Sakamoto S. (1979): Morphology and function of isolated hepatocytes transplanted into rat spleen. Transplantation 28: 499-505.

Moll R., Franke W.W., Schiller D.L., Geiger B., Krepler R. (1982): The catalog of human cytokeratins: Patterns of expression of normal epithelia, tumors and cultured celles. Cell 31: 11-24.

Moore K.L. (1988): *In:* The Developing Human (4th ed.) Saunders, pp. 217-243.

Morel-Chany E., Aujard C., Lafarge-Frayssinet C. (1986): Effects of retinoic acid on the transformation and metabolic cooperation of rat liver cells *in vitro*, and on the growth

of hepatoma cells *in vivo*. Carcinogenesis 7(4): 653-658.

Morriss G.M. (1972): Morphogenesis of the malformation induced in rat embryo by maternal hypervitaminosis A. J Anat 113: 241-250.

Nelson W.J., Traub P. (1983): Proteolysis of vimentin and desmin by the calcium ions activated protease specific for these intermediate filament proteins. Mol Cell Biol 3: 1146-1156.

Netter F.H. (1979): Liver, Biliary Tract and Pancreas. *In:* Digestive System (2nd ed.) New York, Donnelley and Son Press. pp. 2-12.

Newcomer M.E., Jones T.A., Aqvist J., Sundelin J., Eriksson U., Rask L., Peterson P.A. (1984): The three-dimensional structure of retinol-binding protein. J Euro Mol Bio Org 3: 1451-1454.

Ng K.W., Livesey S.A., Collier F., Gummer P.R., Martin T.J. (1985): Effect of retinoids on the growth, ultrastructure and cytoskeletal structures of malignant rat osteoblasts. Cancer Res 45: 5106-5113.

Oda M., Price V., Fisher M.M., Phillips M.J. (1974): Ultrastructure of bile canaliculi, with special reference to the surface coat and the pericanalicular web. Lab Invest 31: 314-323.

Olson, J.A. (1988): *In:* Shils M.E., Young V.R. eds. Modern Nutrition in Health and Disease. pp. 292-312.

Orloff M.J. (1981): *In:* Sabiston ed. Textbook of Surgery. p. 1131.

Osborn M. (1983): Intermediate filaments as histologic markers: an overview. J Invest Derm 81: 104s-107s.

Oshima R.G., Howe H.E., Klier F.G., Adamson E.D., Shevinsky L.H. (1983): Intermediate filament protein synthesis in preimplantation murine embryos. Dev Biol 99: 447-455.

Oshio C., Phillips M.J. (1981): Contractility of the bile canaliculi: implications for liver function. Science 212: 1041-1042.

Peters R.A. (1956): Hormones and the cytoskeleton. Nature 177: 426.

Petkovich M., Brand N.J., Krust A., Champon P. (1987): A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330: 444-450.

Phillips M.J., Oshio C., Miyairi M., Katz H., Smith C.R. (1982): A study of bile canalicular contractions in isolated hepatocytes. Hepatology 2: 763-768.

Phillips M.J., Oshio C., Miyairi M., Smith C.R. (1983): Intrahepatic cholestasis as a canalicular motility disorder: evidence using cytochalasin. Lab Invest 48: 205-211.

Pierschbacher M., Ruoslahti E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecules. Nature 309: 30-33.

Pollard TD, Craig SW (1982): Mechanism of actin polymerization. Trends Biochem. Sci 7: 55-58.

Potschka M. (1986): Structure of intermediate filaments. Biophys J 49: 129-130.

Quinlan R.A., Cohlberg J.A., Schiller D.L., Hatzfeld M., Franke W.W. (1984): Heterotypic tetramer (A_2D_2) complexes of non-epidermal keratins isolated from cytoskeleton of rat hepatocytes and hepatoma cells. J Mol Biol 178: 365-388.

Quinlan R.A., Schiller D.L., Achtstatter T,, Moll R,, Jorcano J.L., Margin T.M., Franke W.W. (1985): Patterns of expression and organization of cytoskeleton intermediate filaments. Ann NY Acad Sci 455: 282-306.

Rappaport A.M. (1976): The microcirculatory acinar concept of normal and pathological hepatic structure. Beitr Pathol 157: 215-243.

Rappaport A.M., Borowy Z.J., Lougheed W.M., Lotto W.N. (1954): Subdivision of hexagonal liver lobules into a structural and functional unit. Role in hepatic physiology and pathology. Anat Rec 119: 11-27.

Rask L., Anundi H., Peterson P.A. (1979): The primary structure of the human retinol-binding protein. FEBS Lett 104: 55-58.

Reddi A.H., Anderson W.A. (1976): Collagenous bound matrix-induced endochondrial ossification and hemopoiesis. J Cell Biol 69: 557-572.

Rovasio R.A., Delouvee K., Yamada K.M., Timpl R., Thiery J.P. (1983): Neural crest cell migration: requirements for exogenous fibronectin and high cell density. J Cell Biol 96: 462-473.

Ruebner B.H., Montgomery C.K., French S.W. (1991): The acinus *In:* Diagnostic pathology of the liver and biliary tract (2nd ed.), Hemisphere Publ. 492 pp.

Saito Y., Imada T., Takagi J., Kikuchi T., Inada Y. (1986): Platelet factor XIII. The collagen receptor? J Biol Chem 261: 1355-1358.

Savage C.R. Jr, Bonney R.J. (1978): Extended expression of differentiated function in primary cultures of adult liver parenchymal cells maintained on nitrocellulose filters. Exp Cell Res 114: 307-315.

Seglen P.O. (1976): Preparation of isolated rat liver cells. *In:* D. Prescott ed. Methods Cell Biol 13. New York, Academic Press, pp. 29-83.

Shapiro S.S. (1985): *In:* Sherman ed. Retinoids and Epithelial Differentiation. CRC Press, USA, pp. 30-35.

Shiojiri N., Katayama H. (1987): Secondary joining of the bile ducts during the hepatogenesis of the mouse embryo. Anat Embryol 177: 153-163.

Sidell N. (1982): Retinoic acid-induced growth inhibition and morphologic differentiation of human neuroblastoma cells *in Vitro*. J Natl Cancer Inst 68(4): 589-596.

Smith C.R., Oshio C., Miyairi M., Katz H., Phillips M.J. (1985): Coordination of the contractile activity of bile canaliculi. Evidence from spontaneous contractions *in vitro*. Lab Invest 53: 270-274.

Speers W.C., Altmann M. (1984): Malignant Neoplasms of differentiated cells occurring after retinoic acid treatment of murine embryonal carcinomas *in Vivo*. Cancer Res 44: 2136-2143.

Steinert P.M., Idler W.W., Goldman R.D. (1980): Intermediate filaments of baby hamster kidney cells and bovine epidermal keratinocytes have similar ultrastructure and subunit domain structures. Proc Natl Acad Sci (USA) 77: 4534-4538.

Steinert P.M., Parry D.A.D., Racoosin E.L., Idler W.W., Steven A.C., Trus B.L., Roop D.R. (1984): The complete cDNA and deduced amino acid sequence of a type II mouse epidermal keratin of 60,000 Da: Analysis of sequence differences between type I and type II keratins. Proc Natl Acad Sci (USA) 81: 5709-5713.

Steinert P.M., Roop D.R. (1988): Molecular and cellular biology of intermediate filaments. Ann Rev Biochem 57: 593-625.

Steinert P.M., Steven A.C., Roop D.R. (1985): The molecular biology of intermediate filaments. Cell 42: 411-419.

Sternlieb I. (1965): Perinuclear filaments and microtubules in human hepatocytes and biliary epithelial cells. J Micros 4: 551-558.

Strom S.C., Jirtle R.L., Jones R.S., Novicki D.L., Rosenberg M.R., Novotny A., Irons G., McLain J.R., Michalopoulos G. (1982): Isolation, Culture, and Transplantation of

Human Hepatocytes. J Natl Cancer Inst 68(5): 771-778.

Sun T.T., Eichner R., Schermer A., Copper D., Nelson W.G., Weiss R.A. (1984): Classification, expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. *In:* Cancer Cells 1, The Transformed Phenotype, Cold Spring Harbor Laboratory, pp. 169-176.

Sun T.T., Scheffer C., Tseng G., Huang A.J., Cooper D., Schermer A., Lynch M.H., Weiss R., Eichner R. (1985): Monoclonal antibody studies of mammalian epithelial keratins: A review. Ann NY Acad Sci 445: 307-329.

Thaller C., Eichele G. (1987): Identification and spatial distribution of retinoids in the developing chick limb bud. Nature 327, 625-628.

Thoms S.D., Stocum D.L. (1984): Retinoic acid-induced pattern duplication in regenerating Urodele limbs. Dev Biol 103: 319-328.

Thompson J.N., Howell J.M.C., Pitt G.A.J. (1964): Vitamin A and reproduction in rats. Proc Roy Soc (London) 159: 510-535.

Traub P. (1985): Are intermediate filament proteins involved in gene expression? Ann NY Acad Sci 455: 68-78, 1985.

Traub P., Nelson W.J., Kuehn S., Vorgias C.E. (1983): The interaction *in vitro* of the intermediate filament protein vimentin with naturally occurring RNAs and DNAs. J Biochem 258: 1456-1466.

Traub P., Vorgias C.E. (1984): Differential effect of Arginine modification with 1,2-Cyclohexanedione on the capacity of vimentin and desmin to assemble into intermediate filaments and to bind to nucleic acids. J Cell Sci 65: 1-20.

Underwood B.A. (1984): Vitamin A in animal and human nutrition. *In:* The retinoids. New York, Academic Press.

Vassar R., Coulombe P.A., Degenstein L., Albers K., Fuchs E. (1991): Mutant keratin expression in transgenic mice causes marked abnormalities resembling a human genetic skin disease. Cell 64: 365-380.

Wald G. (1960): The visual function of vitamin A. *In:* Vitamins and Hormones 18: New York, Academic Press, pp. 417-430.

Watanabe N., Tsukada N., Smith C.R., Phillips M.J. (1991): Mobility of bile canaliculi in the living animal: Implications for bile flow. J Cell Bio 113: 1069-1080.

Watanabe S., Miyairi M., Oshio C., Smith C.R., Phillips M.J. (1983): Phalloidin alters bile canalicular contractility in primary monolayer cultures of rat liver. Gastroenterology 85: 245-253.

Watanabe S., Phillips M.J. (1984): Calcium causes active contraction of bile canaliculi: direct evidence from microinjections studies. Proc Natl Acad Sci (USA) 81: 6164-6168.

Watanabe S., Smith C.R., Phillips M.J. (1985): Coordination of the contractile activity of bile canaliculi. Evidence from calcium microinjection of triplet hepatocytes. Lab Invest 53: 275-279.

Weber K., Geisler N. (1982): The structural relationship between intermediate filament proteins in living cells and the α-keratins of sheep wool. J Euro Mol Biol Org 1: 1155-1160.

Weber K., Geisler N. (1985): Intermediate filaments from wool α-keratin to neurofilaments: a structural overview. *In:* Cancer cells 1, the transformed phenotype. Cold Spring Harbor, pp. 153-159.

Williams G.M., Gunn J.M. (1974): Long-term cell culture of adult rat liver epithelial cells. Exp Cell Res 89: 139-142.

Wolbach S.B. (1954): Effects of vitamin A deficiency and hypervitaminosis A in animals. *In:* The vitamins 1: New York, Academic Press, 106-137.

Wolf G. (1980): Vitamin A. *In:* Human Nutrition. Plenum Press, pp. 97-203.

Yen A. (1985): Control of HL-60 myeloid differentiation. Exp Cell Res 156: 198-212.