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METABOLISM OF SUPPLEMENTAL IRON BY HEPATIC PARENCHYMAL AND  
SINUSOIDAL CELLS OF THE NEONATAL PIG /

by

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(ABSTRACT)

Methods were developed to isolate and culture the predominant cell types from porcine liver to investigate hepatic accumulation, distribution and intracellular metabolism of supplemental iron. Hepatocytes were prepared from collagenase perfused livers by differential centrifugation, while Kupffer cells and endothelial cells were isolated by centrifugal elutriation. One day old piglets were injected with iron-dextran (Fe-dextran) and the concentration of accumulated iron was determined in all three cell types 1, 5, and 10 days later. The concentration of iron increased markedly in all three cell types when compared to cells isolated from untreated piglets (Kupffer cells > endothelial cells >> hepatocytes). Accumulated iron was subsequently mobilized from all three cell types.

The role of ferritin in metabolism and storage of accumulated iron was investigated. An antiserum was prepared against porcine liver ferritin and the quantity of cellular ferritin was measured by

immuno-electrophoresis. The amount of cellular iron associated with ferritin was assessed by ion exchange chromatography. All three types of liver cells accumulated ferritin in response to Fe-dextran treatment. Higher concentrations of ferritin-iron and ferritin-protein were present in Kupffer and endothelial cells than in hepatocytes at all times after iron treatment. However, at 1 day after treatment 48% of the total iron within hepatocytes was associated with ferritin; ferritin-iron accounted for only 10% of total cell iron by day 10. In contrast, ferritin-iron represented only approximately 9% of the total iron in sinusoidal cells throughout the study period.

The possibility that accumulation of Fe-dextran enhanced peroxidation of membrane lipids was evaluated. Lipids extracted from heart and liver of iron-treated piglets contained increased levels of conjugated dienes. High levels of conjugated dienes were present in endothelial cells and hepatocytes 1 day after treatment and only in endothelial cells by day 5. Although Kupffer cells accumulated substantial quantities of Fe-dextran, conjugated dienes were not detectable. These studies indicate that treatment of piglets with Fe-dextran may selectively impair function of hepatic endothelial cells and perhaps hepatocytes, and define new criteria for evaluating compounds that are used for iron supplementation.

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## DEDICATION

No project such as this can be accomplished without the support and encouragement from people who really care. I would like to dedicate this work to the three women that have made it possible for me to begin these studies and who have helped me enjoy the fruits of hard work.

To my wife and best friend, who initially encouraged me to pursue a career in experimental science. She has taught me much about life, hard work and love and how to keep it all in perspective. During my studies at Virginia Tech we performed, in all probability, the most successful experiment of both of our careers: The biosynthesis of our daughter, .

To, who has shed a new light on the meaning of the word, friend. Her encouragement, ideas and ability to lighten the dark spots and brighten the highlights of the past four years will always be remembered.

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**CHAPTER 1.**

**INTRODUCTION**

During the past several years, there has been increased interest among nutritional scientists in studying metabolic regulation in large animals at cellular and molecular levels (1). Moreover, the biomedical community has developed a marked interest in utilizing the domestic pig as an animal model for investigations related to human physiology, nutrition, pathology and toxicology (2). The impetus for such enthusiasm is the anatomical and physiological similarity between several major organ systems of pigs and humans.

Mammalian liver is a complex tissue consisting of two major classes of cells, viz. parenchymal cells or hepatocytes and nonparenchymal cells. Distinct morphologic and metabolic differences exist among the various types of liver cells (3). Hepatocytes play a significant role in the synthesis of plasma proteins, energy conversion and metabolism of minerals and xenobiotics, whereas nonparenchymal cells are involved in phagocytosis and endocytosis of foreign matter and the metabolism of vitamin A, prostaglandins and lipoproteins. Kupffer cells and endothelial cells line the hepatic sinuses and constitute the major portion of the nonparenchymal cell population. Thus, the ability to isolate high yields of viable hepatocytes, Kupffer cells and endothelial cells affords the opportunity to evaluate the relative contribution of each of the three predominant liver cell types to specific biochemical processes. To facilitate the investigation of liver cell metabolism

in young pigs, my initial objective was the development of methods for the isolation and subsequent culture of liver cells from neonatal swine (Chapter 2).

Having succeeded with this objective, I proceeded to investigate the accumulation and metabolism of supplemental iron by parenchymal and sinusoidal liver cells in the neonatal pig (Chapter 3). Pigs reared in modern production facilities are administered a large dose of chelated iron shortly after birth to prevent the development of anemia. Similarly, many human infants, and particularly premature babies and infants born in underdeveloped countries receive supplemental iron either orally or parenterally (4-6). Information concerning intracellular pathways of supplemental iron metabolism in distinct populations of liver cells is largely unavailable. Studies attempting to delineate such information may be important in developing improved methods for the treatment of anemia in pigs, and perhaps, humans.

In comparison to the vast literature available on the physico-chemical characteristics of iron-containing enzymes and proteins, iron nutrition and the pathology of iron overload, there is relatively little information concerning iron metabolism by any other cell type other than the reticulocyte. Although methods for isolation of rodent hepatocytes have been available for the past two decades, techniques to isolate sinusoidal cells with mild enzyme

treatments (collagenase) have only been developed in the past four years (7,8). Detailed studies of the metabolic characteristics of sinusoidal cells have also been hindered by the limited amount of cellular material that can be obtained from rats, the most widely used animal for liver cell research. In contrast, sufficient numbers of sinusoidal cells, as well as parenchymal cells, can be isolated from the liver of a single neonatal pig to quantitatively investigate various aspects of iron (or any other nutrient) metabolism by these cells.

Hepatocytes are believed to play a central role in the storage, turnover and mobilization of iron. Intracellular storage and detoxification of iron are primarily mediated by ferritin, a 450 K dalton multimeric protein capable of binding up to 4500 g atoms of iron per molecule. The net flux of iron in anemic animals is to erythropoetic tissues for the synthesis of hemoglobin. Transport of iron throughout the body is mediated by transferrin, an 80 K dalton sero-glycoprotein that contains two high affinity binding sites for iron. Although low concentrations of a glycosylated form of ferritin are also found in plasma, the contribution of this protein to iron transport is probably negligible. Extensive reviews on the biochemistry of ferritin (9-11) and transferrin (11,12) are available.

Hepatocytes synthesize and secrete apotransferrin. Some

investigators have suggested that macrophages and perhaps other reticuloendothelial cells also synthesize transferrin, thus providing a mechanism for the mobilization of accumulated iron from cells (13). Data in support of the synthesis of transferrin by macrophages are limited and suspect. It is also believed that hepatocytes possess specific cell surface receptors for transferrin (14). Recent evidence suggests that hepatic endothelial cells also possess transferrin receptors (15). It is clear that the mechanisms involved in the regulation of iron movement into and out of liver cells are unresolved. I propose that the research described in Chapter 3 provides a basis for the use of neonatal swine liver cells in addressing such matters in the near future.

Since the accumulation of high levels of iron in cells is often associated with cytopathology (16), the possibility that cellular iron derived from treatment with Fe-dextran may be toxic to the piglet liver was also addressed (Chapter 4). The toxic effects of iron appear to be related to its ability to enhance or initiate membrane lipid peroxidation. The chemistry of iron-mediated free radical attack of lipids has been recently reviewed (17,18). Accumulation of iron has been implicated in damage of organelles in vivo (19) and in vitro (20). There are reports that parenteral iron administration to piglets may occasionally lead to death and that increased levels of peroxides in muscle are present in piglets following injection of Fe-dextran (21). The objective of experiments

described in Chapter 4 was to assess whether the administration of the recommended dosage of Fe-dextran was associated with altered membrane chemistry in tissues (liver and heart), erythrocytes and isolated liver cells. Membrane lipids were extracted by classical methods and the presence of conjugated dienes was determined by spectral analysis. Increased levels of conjugated dienes in membrane lipids is indicative of free radical attack on polyunsaturated fatty acids (20).

The findings reported in this study suggest that the metabolism of supplemental iron in baby pigs is not completely analogous to metabolism of iron in adult rodents. It is difficult to directly compare the different models because investigators have approached the question from different perspectives. The apparent discrepancies are probably due to differences in the types of iron compounds utilized, the route and frequency of injections, the dose administered and the physiological status of the animal. In the present study, I have attempted to use conditions that mimic the practices of a typical swine producer. In doing so, I believe my results related to the cellular uptake, intermediary metabolism and possible cytotoxic effects of supplemental iron are relevant to the use of this material in the swine industry. The importance of my findings to clinical nutrition requires additional investigation.

## LITERATURE CITED

1. Allen, C.E. [ed.] (1985) Current Concepts of Animal Growth I. J. Animal Sci. 61, Supplement 2, 1-184.
2. Tumbleson, M.E. [ed.] (In Press) Swine in Biomedical Research. Plenum Press, New York.
3. Popper, H. & Schaffner, F. [eds.] (1979) Progress in Liver Diseases VI. pp. 1-662. Grune & Stratton, Inc., New York.
4. Finch, C.A. (1980) Drugs effective in iron-deficiency and other hypochromic anemias. In: The Pharmacological Basis of Therapeutics. (Gilman, A.G., Goodman, L.S. & Gilman, A., eds.) pp. 1315-1330, Macmillan Publishing Co. Inc., New York.
5. Stekel, A. (1984), Prevention of iron deficiency. In: Iron Nutrition in Infancy and Childhood. (Stekel, A., ed.) pp. 179-194, Raven Press, New York.
6. Reeves, J.D., Vichinsky, E., Addiego, J. & Lubin, B.H. (1983) Iron deficiency in health and disease. Adv. Pediatrics 30, 281-320.
7. Caperna, T.J. & Garvey, J.S. (1982) Antigen handling in ageing. II. The role of Kupffer and endothelial cells in antigen processing. Mech. Ageing Develop. 20, 205-221.
8. Parise, E.R., Taylor, M.E. & Summerfield, J.A. (1982) Localization of a carbohydrate mediated glycoprotein receptor on hepatic sinusoidal cells. In: Sinusoidal Liver Cells. (Knook, D.L. & Wisse E., eds.) pp. 287-304. Elsevier Biomedical Press, Amsterdam.
9. Munro, H.N. & Linder, M.C. (1978) Ferritin: Structure, biosynthesis, and role in iron metabolism. Physiol. Rev. 58, 317-396.
10. Theil, E.C. (1985) Ferritin: Structure, function and regulation. In: Iron Binding Proteins without Cofactors or Sulfur Clusters 5. (Theil, E.C., Eichorn, G.L. & Marzilli, L.G., eds.) pp. 1-38. Elsevier Biomedical Press, New York.
11. Aisen, P. & Listowsky, I. (1980) Iron storage and transport proteins. Ann. Rev. Biochem. 49, 357-397.

12. Tavill, A.S. & Morton, A.G. (1978) Transferrin metabolism and the liver. In: *Metals and the Liver* (Powell, L.W., ed.) pp. 93-129. Marcel Dekker, Inc. New York.
13. Haurani, F.I. & Meyer, A. (1976) Iron and the reticuloendothelial system. In: *The Reticuloendothelial System in Health and Disease*. (Reichard, S.M., Escobar, M.R. & Freidman, H., eds.) pp. 171-187. Plenum Press, New York.
14. Morley, C.G.D. & Bezkorovainy, A. (1985) Cellular iron uptake: Is endocytosis the only mechanism? *Int. J. Biochem.* 17, 553-564.
15. Kishimoto, T. & Tavassoli, M. (1985) Endothelial binding of transferrin in fractionated liver cell suspensions. *Biochem. Biophys. Acta* 846, 14-20.
16. Jacobs, A. (1980) The pathology of iron overload. In: *Iron in Biochemistry and Medicine II*. (Jacobs, A. & Worwood, M., eds.) pp. 427-459. Academic Press, New York.
17. Bus, J.S. & Gibson, J.E. (1979) Lipid peroxidation and its role in toxicity. *Rev. Biochem. Toxicol.* 1, 125-149.
18. Sevanian, A. & Hochstein, (1985) Mechanisms and consequences of lipid peroxidation in biological systems. *Ann. Rev. Nutr.* 5, 365-390.
19. Bacon, B.R., Tavil, A.S., Brittenham, G.M., Park, C.H. & Recknagel, R.O. (1983) Hepatic lipid peroxidation in vivo in rats with chronic iron overload. *J. Clin. Invest.* 71, 429-439.
20. Abok, K., Hirth, T. Ericsson, J.L.E., & Brunk, U. (1983) Effect of iron on the stability of macrophage lysosomes. *Virchow Arch.[Cell Pathol.]* 43, 85-101.
21. Patterson, D.S.P., Allen, W.M., Thurley, D.C. & Done, J.T. (1976) The role of tissue peroxidation in iron-induced myodegeneration of piglets. *Biochem. J.* 104, 2p-3p.

CHAPTER 2.

ISOLATION AND CULTURE OF PARENCHYMAL AND NONPARENCHYMAL  
CELLS FROM NEONATAL SWINE LIVER

Adapted from J. Animal Science, 61, 1576-1586, 1985.

## INTRODUCTION

Development of methods for the isolation and culture of hepatocytes has greatly facilitated elucidation of the characteristics and regulation of hepatic metabolism. Maintenance of differentiated gene expression in culture, as well as the ability to alter hormonal and nutritional status of the medium, continues to contribute new information concerning lipid, carbohydrate, nitrogen, mineral and xenobiotic metabolism in the mammalian liver (1). Recently, techniques have also been developed to isolate and culture the other major cells within the liver, viz., endothelial and Kupffer cells. While these nonparenchymal cells (NPC) have long been known to possess phagocytic capacity (2), they have recently been found to play important roles in vitamin A (3), lipoprotein (4), iron (5) and heavy metal (6) metabolism. Most studies have utilized rat liver for the preparation of viable parenchymal and nonparenchymal primary cell cultures. Our objective was to develop suitable techniques for porcine liver cells. This primary cell culture model would permit the examination of specific characteristics of nutrient metabolism and the endocrine regulation of such processes.

Several groups have reported the isolation of hepatocytes from neonatal (7-9) and weaned pigs (10-12). Suspensions of freshly isolated porcine hepatocytes have been shown to synthesize glycolipids (10) and glucose (9). Porcine hepatocytes cultured for 2

to 3 d in high levels of serum (10 to 20%) synthesize and secrete plasma proteins, and also transport and catabolize lipoproteins (7, 8). There are no previous reports in the literature concerning the isolation and in vitro culture of NPC from swine liver.

Marked morphological and biochemical changes occur in the liver during the early postpartum period (13, 14). The study of nutrient metabolism in the liver and the responsiveness of this organ to various endocrine and growth factors in the newborn pig would be facilitated by the availability of primary monolayer cultures of liver cells. Here we demonstrate that high yields of viable and metabolically active hepatocytes and NPC are readily isolated from baby pigs 1 to 15 d after birth. These cells have been maintained in vitro as monolayers in medium with or without serum (hepatocytes) for at least 6 d. The incorporation of  $^3\text{H}$ -leucine and  $^3\text{H}$ -thymidine into hepatocyte protein and DNA, respectively, was influenced by the duration of culture period and type of medium utilized to maintain monolayers of hepatocytes. Incorporation of  $^3\text{H}$ -thymidine into DNA was also influenced by age of the donor pig.

## MATERIALS AND METHODS

Animals. Male crossbred piglets were used between 1 and 15 d of age. The sow and nursing piglets were housed in farrowing crates that had partially slatted floors. Piglets were neither castrated nor injected with iron. Male Sprague Dawley rats (250 g) were obtained from the Virginia Polytechnic Institute and State University Vivarium, and were maintained on a 12-h light-dark cycle, with free access to a commercial diet (Purina Rodent Chow 5012, Ralston Purina Co., St. Louis, MO) and water.

Isolation of Liver Cells. Piglets were anesthetized by intramuscular injection of sodium pentobarbital (100 mg/kg). After washing the torso and limbs in a warm soapy bath, the pig was immobilized, sternum up, on an angled surgical board. Before the removal of the skin of the abdomen and thorax, these regions were thoroughly rinsed with 70% (v/v) ethanol.

Liver cells were isolated by the two-step collagenase perfusion technique (15) with slight modifications. The peritoneal cavity was exposed by a lateral incision cranial to the umbilicus and the muscle was retracted over the sternum. The umbilical vein was ligated and cut caudal to the suture. A loose ligature was placed around the vena cava above the right renal vein. The thoracic portion of the vena cava was exposed by cutting through the ribs and excision of the diaphragm. A cannula was inserted into the thoracic vena cava approximately 1 cm above the diaphragm. Once the cannula was

secured, perfusion of the liver with 200 ml buffer A [142 mM NaCl, 6.7 mM KCl, 10 mM HEPES (N-2 hydroxyethylpiperazine- N'-2-ethane - sulfonic acid) and .5mM EGTA (ethylene glycol-bis (b-aminoethyl ether) N,N'-tetraacetic acid), pH 7.4] at 38° C was initiated at a flow rate of 25 ml/min. The ligature around the lower vena cava was tightened above the right kidney and the portal vein was severed for outflow. Buffer flow rate was increased to 35 ml/min and the liver was carefully dissected from the carcass. The excised liver was transferred to a stainless-steel screen with portal region facing up and the lobes properly positioned. Approximately 600 ml of buffer A minus EGTA was then passed through the liver. Next, 200 ml of an enzyme solution [67 mM NaCl, 6.7 mM KCl, 100 mM HEPES, 5 mM CaCl<sub>2</sub>, 20,000 units collagenase II (Cooper Biochemical, Freehold, NJ), 5 mg soy trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) and 5 mg DNAase (Sigma Chemical Co.), pH 7.6] was recirculated through the liver at a rate of 50 ml/min. Enzyme perfusion was terminated after 10 to 30 min and the liver was placed in 100 ml ice-cold medium M199 (Flow Laboratories, Dublin, VA). The gallbladder and remaining connective tissue were removed and liver cells were released by disruption of the capsule and subsequent raking of the liver stroma with a Teflon spatula. Suspended cells were separated from debris by successive filtration through two and eight layers of cotton cheesecloth and, finally, through nylon mesh (250 um, Tetko, Elmsford, NY).

Isolation and Culture of Hepatocytes. Suspended cells were centrifuged in 150 ml bottles (25 x g, 4 min, 4° C). The pellet (hepatocytes) was gently resuspended in medium M199 and was washed an additional four times by centrifugation. All supernatants were pooled for the isolation of NPC. The final suspension of hepatocytes was filtered (60 um nylon mesh) and kept on ice. Aliquots were diluted 1:1 with 0.04% (w/v) trypan blue in HEPES-buffered saline, pH 7.4, and cells were counted in a standard Neubauer hemocytometer chamber. Cells that excluded the dye were scored as viable.

Hepatocytes were added to collagen-coated, 35 mm multiwell plates (Costar, Cambridge, MA) and 60 mm dishes (Nunc, Denmark) at inoculum densities of  $1.5 \times 10^6$  and  $3.5 \times 10^6$  viable cells, respectively. Collagen was prepared by dissolving acid-soluble calf skin collagen (Sigma Chemical Co.) in 0.5 M acetic acid, followed by dialysis vs 5 mM sodium acetate in 0.1 M NaCl, pH 4.2. Twenty and 40 ug of collagen were evenly spread over 35 and 60 mm plates, respectively, and dried at 40° C. Before addition of cells, plates were washed with 2 ml 10 mM HEPES-buffered saline, pH 7.4. Cells were incubated in arginine- and hypoxanthine-free Waymouth's MB 752/1 containing 5.5 mM glucose and 0.15 mM L-ornithine (hereafter referred to as MB 752/1 M) at 37° C in 95% air:5% CO<sub>2</sub> (16). The basal medium was supplemented with insulin (0.5 ug/ml) and 10% (v/v) fetal calf serum (FCS). After 1 h, attached cells were washed twice with 10 mM HEPES-buffered saline, pH 7.4. Hepatocytes were maintained in MB

752/1 M supplemented with gentamycin (50 ug/ml), insulin (0.05 ug/ml) and either 5% (v/v) FCS or 0.2% (w/v) fatty acid-free bovine serum albumin (Sigma Chemical Co.) and 10 uM oleic acid. The latter is referred to as serum-free medium. Monolayers were washed twice with HEPES-buffered saline and fresh medium was added daily.

Isolation and Culture of Kupffer and Endothelial Cells. The supernatants collected from washings of hepatocytes obtained during the initial 25 x g centrifugations were recentrifuged (350 x g, 10 min, 4° C) to collect NPC, residual hepatocytes and blood cells. The cell pellet was resuspended in Gey's balanced salt solution (GBSS: NaCl, 137 mM; KCl, 5 mM; CaCl<sub>2</sub>.2H<sub>2</sub>O, 1.6 mM; MgCl<sub>2</sub>.6H<sub>2</sub>O, 9mM; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.3mM; Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 1.7 mM; KH<sub>2</sub>PO<sub>4</sub>, 0.2 mM; NaHCO<sub>3</sub>, 2.7 mM; glucose, 5.5 mM; HEPES, 5 mM; pH 7.2) and centrifuged. Cells were resuspended in 8 ml GBSS and mixed with 7 ml of a 30% Metrizamide (Accurate Chemical Scientific Corp., Westbury, NY) solution prepared in GBSS without NaCl (17). One milliliter GBSS was layered on top of the Metrizamide-cell solution before centrifugation (1,400 x g, 15 min, 20° C) in a swinging bucket rotor. Erythrocytes, hepatocytes and cell debris were pelleted, while NPC formed a band at the gradient interface. The NPC-rich fraction also contained leucocytes and residual hepatocytes. Endothelial and Kupffer cells were further purified by centrifugal elutriation (JE 6B elutriator rotor, Beckman Instruments, Palo Alto, CA), essentially as described by Caperna and Garvey (18).

The elutriation buffer consisted of GBSS without  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{NaHCO}_3$ , but containing 1.0% (w/v) bovine serum albumin, 0.05 mM EDTA (ethylenediaminetetraacetic acid) and 10 mM HEPES, pH 7.2. Due to the high numbers of cells, the suspension was introduced into the elutriator at 7 ml/min. The flow rate was gradually raised to 10 ml/min and the cells were washed with a total volume of 220 ml buffer. Endothelial cells were then collected at 16 ml/min. An intermediate fraction was collected at 19.5 ml/min before Kupffer cells were collected at 31 ml/min. Each cell fraction was collected in 220 ml buffer.

Cells were pelleted by centrifugation, washed and cultured as described below. Identification of Kupffer and endothelial cells was primarily based on size, morphology and ability of Kupffer cells to phagocytize latex particles (19). Kupffer cells ( $3 \times 10^6$  viable cells) were added directly to 35 mm dishes. Endothelial cells were added to 35 mm plates coated with collagen. Both cell types were maintained in MB 752/1 M supplemented with 0.36 mM L-arginine, gentamycin (59 ug/ml) and 20% FCS in 95% air:5%  $\text{CO}_2$  environment.

Protein and DNA Synthesis in Cultured Hepatocytes. The uptake and incorporation of  $^3\text{H}$ -leucine and  $^3\text{H}$ -thymidine into acid insoluble macromolecules by monolayers of hepatocytes maintained in either serum-free or serum-containing medium was assessed over a 3-d period. At 24, 48 and 72 h after cells were seeded, monolayers were washed twice with 10 mM HEPES-buffered saline at  $37^\circ\text{C}$ . Monolayers

were incubated in 1.5 ml serum-free MB 752/1 M medium containing 1.6 uCi  $^3\text{H}$ -thymidine (Thymidine, [Methyl- $^3\text{H}$ ], 2.0 Ci/mmol., New England Nuclear, Boston, MA) for 3 h. Replicate monolayers received 1.5 ml leucine and serum-free MB 752/1 M medium containing 0.8 uCi  $^3\text{H}$ -leucine (L-Leucine, [4,5- $^3\text{H}$ ], 54 Ci/mmol., I.C.N., Irvine, CA), and were incubated for 1 h. All metabolic studies were performed at 37° C in 95% air:5% CO<sub>2</sub>. Incubations were terminated by removal of labeled media and by washing monolayers twice with ice-cold, 10 mM HEPES-buffered saline. Cells were removed from each well in 1 ml sodium dodecyl sulfate (0.2% w/v) solution. Trichloroacetic acid (TCA) was added to a final concentration of 7.5% (w/v). Acid-insoluble material was collected by centrifugation (2,000 x g, 30 min, 4° C) and the pellets were washed with 10% (w/v) TCA. The final precipitate was dissolved in 1.0 ml of 1.0 N NaOH. The quantity of  $^3\text{H}$  in aliquots was determined and dpm were calculated by the external standard method. Cell protein was determined by assaying an aliquot of the dissolved precipitate by the modified Lowry method (20), using bovine serum albumin as the standard. All assays were performed in triplicate culture wells and the data are presented as dpm/ug cell protein. Individual values were within 10% of the triplicate mean.

Statistical Analysis. Results were expressed as means  $\pm$  SE. Data were evaluated for significant differences ( $P < .05$ ) by analysis of variance with animal age, media type and time in culture considered in the model. All statistical procedures were accomplished with the general linear models routine of the statistical analysis system (21). To rank the sources of variation, a priority rating was obtained by dividing the treatment mean squares by the residual error mean squares in the model.

## RESULTS AND DISCUSSION

Yields of hepatocytes, Kupffer cells and endothelial cells isolated from the neonatal pigs between one and 15 d of age are shown in Table 1. Although livers increased in size with age, similar numbers of cells were isolated from younger (less than 1 wk) and older (1 to 2 wk) pigs. This is due in part to the lack of any attempt to isolate as many cells as possible by more rigorous raking of the stroma, or by increasing the duration of perfusion with collagenase. When the outlined procedure was used with pigs as old as 22 d of age, yields of viable liver cells were similar to those from younger animals when the time of perfusion was increased to 40 or 50 min. Viability was consistently greater than 85% for hepatocytes and greater than 90% for NPC, as determined by trypan blue exclusion. The ability to isolate high numbers of viable NPC from baby pigs makes this animal a useful model for the investigation of NPC metabolism during the neonatal period.

Porcine hepatocytes rapidly attached to the collagen-coated surface of the culture dish. After 3 h in culture, cells were flattened and had the polygonal morphology characteristic of primary monolayer cultures of hepatocytes isolated from adult rat liver (22). Cells retained their appearance for at least 6 d. Hepatocytes maintained either in the presence or absence of serum appeared to be identical (Figure 1). The use of arginine-free media was essential to prevent fibroblast growth when monolayers of hepatocytes were

TABLE 1. YIELD OF LIVER CELLS ISOLATED FROM BABY PIGS 1 to 15 DAYS OLD BY THE COLLAGENASE PERFUSION TECHNIQUE.

Cell type	Viable <sup>a</sup> cells $\times 10^{-8}$ /liver
Parenchymal cells (hepatocytes)	21 $\pm$ 2.0 (20) <sup>b</sup>
Kupffer cells	1.2 $\pm$ 0.1 (17)
Endothelial cells	3.0 $\pm$ 0.2 (17)

<sup>a</sup>Viability as determined by trypan blue exclusion was greater than 85% for all cell types.

<sup>b</sup>Values are means  $\pm$  SE. The numbers in parentheses indicate number of donor pigs from which the liver cell types were isolated.

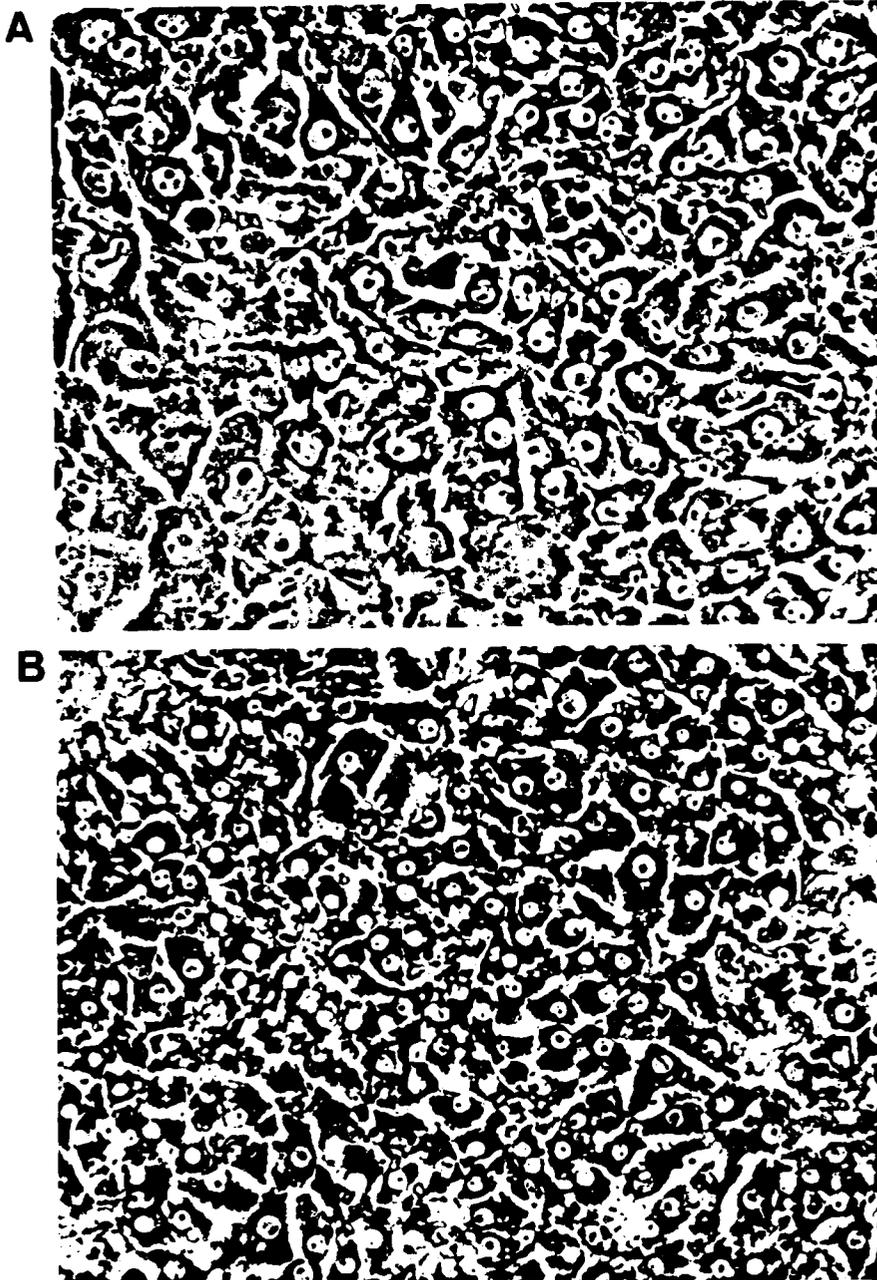


Figure 1. Morphology of porcine hepatocytes in monolayer culture. At 24 and 72 h after the initiation of culture period, monolayers were washed and unfixed cells were photographed at a magnification of 100X. (Enlarged magnification is 700X.) A) Medium + 5% FCS at 24 h; B) Serum-free medium at 24 h;

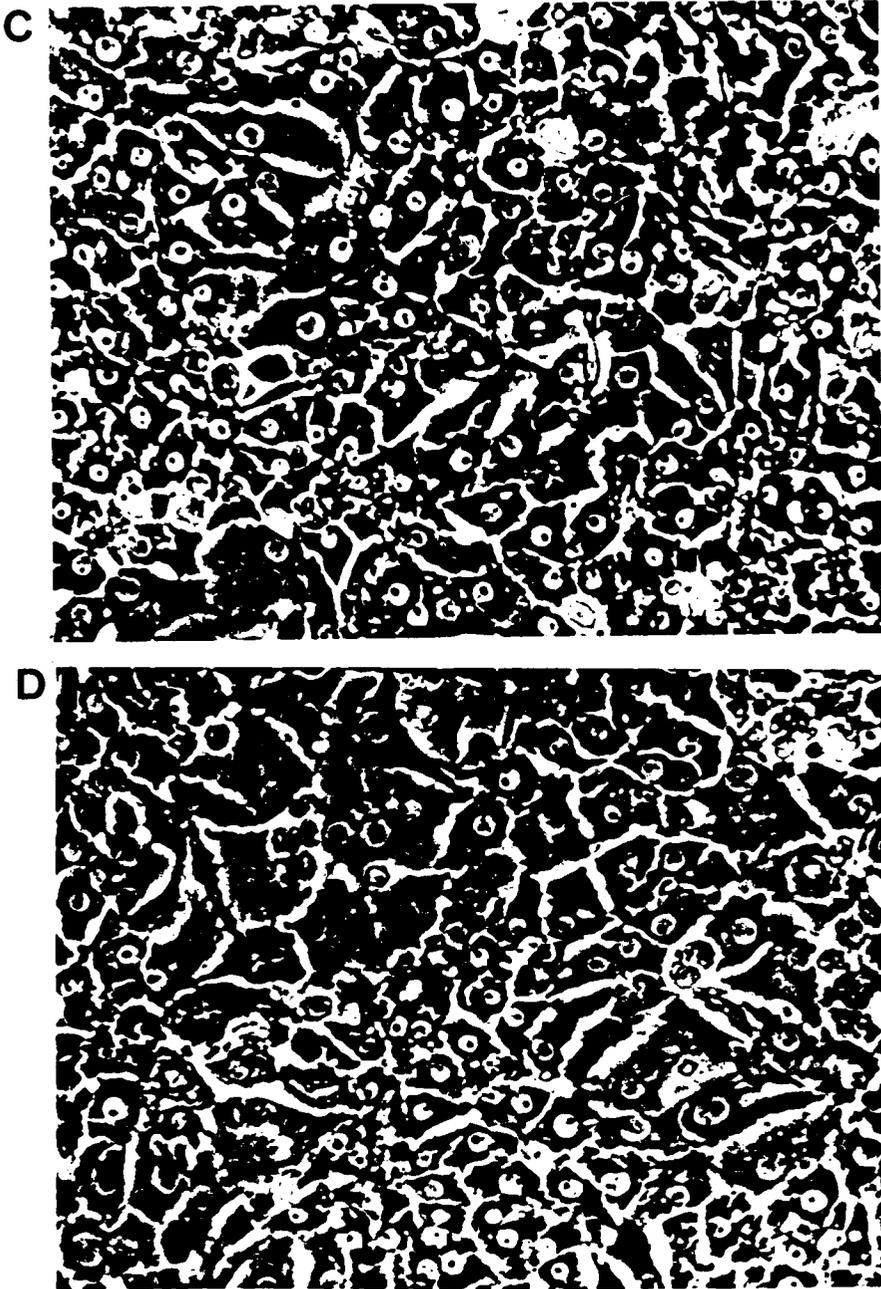


Figure 1, continued. C) Medium + 5% FCS at 72 h; D) Serum-free medium at 72 h.

maintained for extended periods, i.e., greater than 4 d.

Cultured swine endothelial and Kupffer cells exhibited morphologies similar to those described for such cells isolated from rat liver (18, 23, 24). Kupffer cells phagocytized 5  $\mu$ m latex particles, while endothelial cells did not exhibit this capability (Figure 2).

The metabolic integrity of cultured hepatocytes prepared from neonatal pigs varying in age (1 to 8 d) was assessed by incorporation of  $^3\text{H}$ -leucine and  $^3\text{H}$ -thymidine into acid-insoluble macromolecules. Pigs were separated into three age groups, viz., 1 d, 3 to 5 d and 7,8 d of age. Henceforth, these donor groups will be referred to as being 1-d, 4-d and 7-d-old pigs, respectively, to simplify presentation of data. Monolayers were maintained in serum-free and serum-containing media, and precursor incorporation was assayed at various times during the 3-d culture period.

Incorporation of  $^3\text{H}$ -leucine into acid-insoluble components of porcine hepatocytes is shown in Table 2. The extent of  $^3\text{H}$ -leucine incorporation was markedly influenced ( $P < .0001$ ) by the length of time the monolayers were cultured before the addition of labeled substrate. There was a general trend towards increased  $^3\text{H}$ -leucine incorporation with increased time in culture. This phenomenon was observed in all groups, except for liver cells that were prepared from 7-d-old animals and that had been maintained in serum-free medium. Incorporation of  $^3\text{H}$ -leucine into protein was also affected

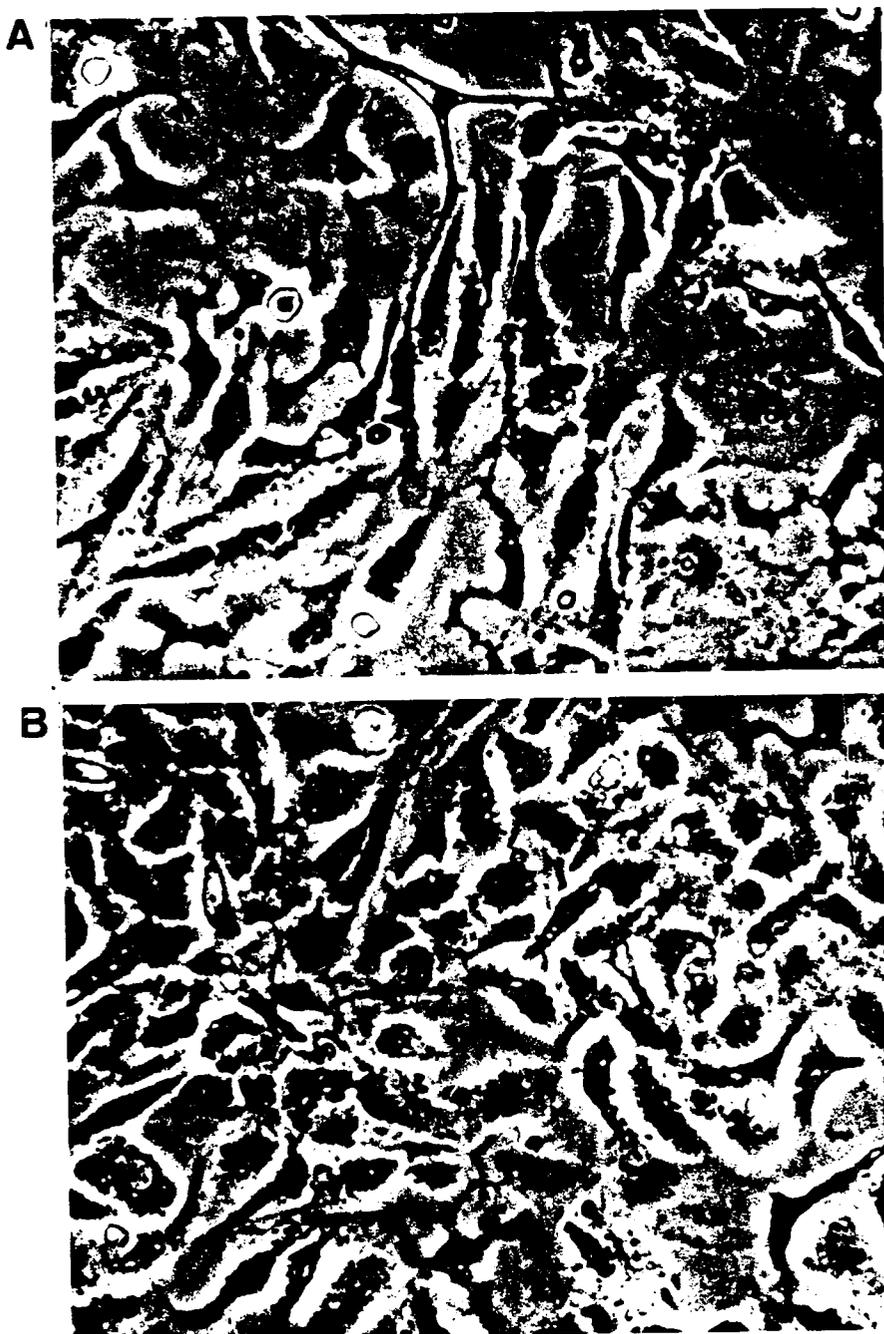


Figure 2. Morphology of porcine liver nonparenchymal cells in monolayer cultures. Unfixed cells were photographed at a magnification of 100X. (Enlarged magnification is 750X.) A) Endothelial cells; B) Kupffer cells;

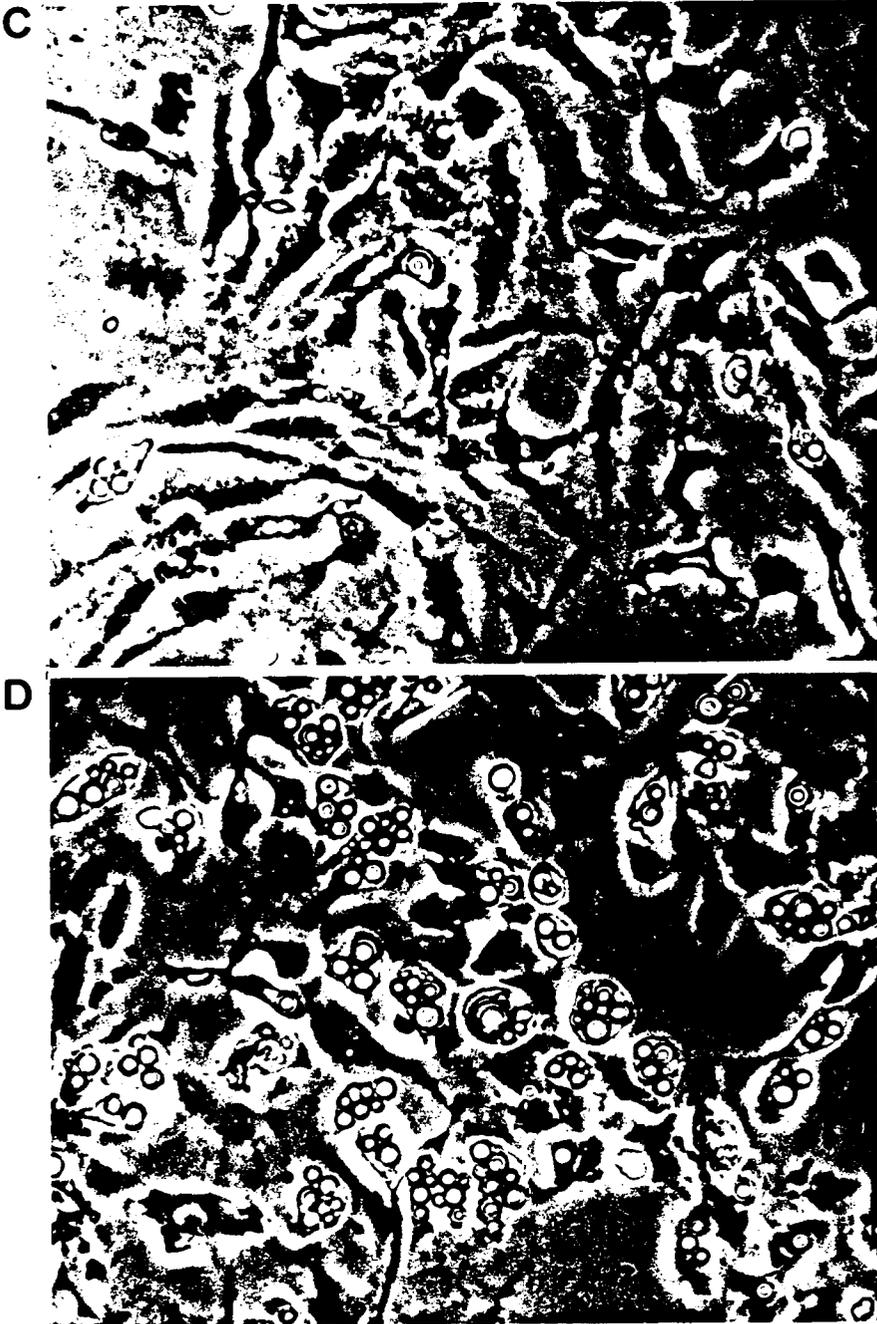


Figure 2, continued. C) Endothelial cells incubated with 5-um latex particles; D) Kupffer cells incubated with 5-um latex particles. NPC were cultured for 24 h in 20% FCS. After exposure to latex particles for 2 h, monolayers were washed extensively to remove particles that had not been phagocytized.

TABLE 2. INFLUENCE OF ANIMAL AGE, TIME IN CULTURE AND MEDIA CONDITIONS ON  $^3\text{H}$ -LEUCINE INCORPORATION INTO PROTEIN<sup>a</sup> BY PRIMARY MONOLAYERS OF PORCINE HEPATOCYTES<sup>b</sup>

Age of pig <sup>cd,d</sup>	N	Days in culture <sup>e</sup>		
		1	2	3
I. Medium + 5% FCS <sup>f</sup>		dpm/ug cell protein		
1	4	178 ± 3	277 ± 7	352 ± 79
4	5	315 ± 32	377 ± 21	506 ± 58
7	5	259 ± 14	380 ± 51	568 ± 123
II. Serum-free medium <sup>df</sup>				
1	4	158 ± 2	239 ± 17	343 ± 97
4	5	260 ± 33	296 ± 23	392 ± 59
7	5	211 ± 16	194 ± 22	220 ± 30

<sup>a</sup>Values are means ± SE.

<sup>b</sup>Hepatocytes were isolated from neonatal pigs (1 to 7 d old) and cultured as described in Materials and Methods. N represents the number of donor baby pigs from which hepatocytes were isolated and cultured for each group. At 1, 2 and 3 d after initiation of cultures, maintenance media was replaced with fresh serum-free medium containing  $^3\text{H}$ -leucine. After 1 h, cells were washed, harvested and radioactivity incorporated into acid-insoluble material determined. All assays were performed in triplicate for cells from each pig.

<sup>c</sup>Age of pigs ( $P > .19$ ).

<sup>d</sup>Age X medium ( $P < .002$ )

<sup>e</sup>Time in culture ( $P < .0001$ ).

<sup>f</sup>Medium ( $P < .0001$ ).

( $P < .0001$ ) by the type of culture medium. The rate of  $^3\text{H}$ -leucine incorporation was higher in hepatocytes that had been maintained in serum-containing medium than serum-free medium for cells isolated from donors more than 1 d old. This serum effect was particularly evident in cells isolated from 7-d-old pigs. In contrast, medium type did not influence incorporation of radiolabeled precursor in cells isolated from 1-d-old pigs ( $P > .4$ ). The extent of  $^3\text{H}$ -leucine incorporation was not influenced by age of the donor ( $P > .19$ ), although there was a significant interaction between donor age and type of medium ( $P < .002$ ). In an attempt to rank the sources of variation involved in assessing  $^3\text{H}$ -leucine incorporation, the following pattern was observed: time in culture > media supplement > age of donor animal.

The ability of cycloheximide, an inhibitor of translation by 80S ribosomes, to inhibit  $^3\text{H}$ -leucine incorporation by cultured hepatocytes was examined. When cells were exposed to cycloheximide (1.7  $\mu\text{M}$ ), beginning 30 min before the addition of  $^3\text{H}$ -leucine, incorporation of the label into acid-insoluble material was inhibited by greater than 80% (data not shown). However, cycloheximide also decreased  $^3\text{H}$ -leucine uptake from the medium by 40 to 60%. Risser and Gelehrter (25) have also reported that exposure of rat hepatoma cells to cycloheximide (100  $\mu\text{M}$ ) inhibited  $\alpha$ -amino isobutyric acid transport by 25%. While the biochemical basis for the cyclohexamide-mediated reduction in  $^3\text{H}$ -leucine accumulation from

The medium by porcine hepatocytes is unknown, the results suggest that uptake is dependent in part on the size of the intracellular pool of free amino acids.

Incorporation of  $^3\text{H}$ -thymidine into DNA by monolayers of porcine hepatocytes is shown in Table 3. The rate of  $^3\text{H}$ -thymidine incorporation was influenced ( $P < .0001$ ) by the length of time in culture and by the type of medium used to maintain the monolayers. All hepatocytes maintained in medium containing FCS demonstrated marked increases in  $^3\text{H}$ -thymidine incorporation by the second and third day in culture. This trend was less pronounced when cells were maintained in serum-free medium and was not observed in the 7-d-old group. Thus, in contrast to  $^3\text{H}$ -leucine incorporation, incorporation of  $^3\text{H}$ -thymidine into DNA was markedly influenced ( $P < .017$ ) by the age of the pig at the time of cell isolation. Hepatocytes prepared from 4-d-old pigs demonstrated the highest rates of  $^3\text{H}$ -thymidine incorporation, particularly during the first 2 d in culture. Higher rates of  $^3\text{H}$ -leucine incorporation were also observed in the 4-d-old pigs. A priority rating for the sources of variation involved in assessing  $^3\text{H}$ -thymidine incorporation indicates the following hierarchy: time in culture > media supplement > age of donor.

The incorporation of  $^3\text{H}$ -leucine and  $^3\text{H}$ -thymidine into protein and DNA, respectively, by monolayers of adult rat hepatocytes was determined to provide a direct comparison with porcine hepatocytes

TABLE 3. INFLUENCE OF ANIMAL AGE, TIME IN CULTURE AND MEDIA CONDITIONS ON  $^3\text{H}$ -THYMIDINE INCORPORATION INTO DNA<sup>a</sup> BY PRIMARY MONOLAYERS OF PORCINE HEPATOCYTES<sup>b</sup>

Age of pig <sup>cde, d</sup>	N	Days in culture <sup>dfg</sup>		
		1	2	3
I. Medium + 5% FCS <sup>egh</sup>		dpm/ug cell protein		
1	4	46 ± 6	131 ± 13	158 ± 47
4	5	157 ± 24	419 ± 45	612 ± 145
7	5	55 ± 11	284 ± 58	729 ± 171
II. Serum-free medium <sup>egh</sup>				
1	4	40 ± 6	110 ± 28	118 ± 43
4	5	93 ± 23	208 ± 40	339 ± 89
7	5	37 ± 4	29 ± 11	41 ± 11

<sup>a</sup>Values are means ± SE.

<sup>b</sup>Hepatocytes were isolated from neonatal pigs (1 to 7 d old) and cultured as described in Materials and Methods. N represents the number of donor baby pigs from which hepatocytes were isolated and cultured for each age group. At 1, 2 and 3 d after initiation of cultures, maintenance media was replaced with fresh serum-free medium containing  $^3\text{H}$ -thymidine. After 3 h, cells were washed, harvested and radioactivity incorporated into acid-insoluble material determined. All assays were performed in triplicate for cells from each pig.

<sup>c</sup>Age of pig (P<.017)

<sup>f</sup>Time in culture (P<.0001)

<sup>d</sup>Age X time (P<.02)

<sup>g</sup>Time X medium (P<.0002)

<sup>e</sup>Age X medium (P<.0004)

<sup>h</sup>Medium (P<.0001)

(Table 4). Similar overall rates of incorporation of  $^3\text{H}$ -leucine were observed when rat and porcine hepatocytes were compared (Tables 2 and 4). However, rat cells did not incorporate more  $^3\text{H}$ -leucine when cultured in serum-containing medium for longer periods. Unlike pig hepatocytes (Table 3), the incorporation of  $^3\text{H}$ -thymidine into DNA was minimal in primary monolayer cultures of adult rat hepatocytes, regardless of type of medium and duration in culture. Only porcine hepatocytes from the 7-d-old donors maintained in serum-free medium exhibited minimal  $^3\text{H}$ -thymidine incorporation similar to the low levels observed with adult rat cells. These results suggest that hepatocytes isolated from younger pigs (1 to 4 d of age) retained their ability to synthesize DNA in the absence of serum-containing growth factors. In contrast, cells prepared from the older pigs apparently require these factors to enhance the rate of incorporation of exogenous thymidine into DNA.

Marked increases in incorporation of  $^3\text{H}$ -thymidine and, to a lesser extent,  $^3\text{H}$ -leucine by 72-h cultures of hepatocytes maintained in medium supplemented with FCS (Tables 2 and 3) suggested that cells were dividing in vitro. Consequently, DNA and protein content per dish were assayed for replicate cultures of hepatocytes at 1 and 3 d after initiation of the cell incubation. The amounts of DNA and protein per dish did not increase with time in culture (Table 5). Moreover, the protein to DNA ratio did not change during the 3-d culture period in representative samples of hepatocytes isolated from

TABLE 4. METABOLIC ACTIVITY OF CULTURED ADULT RAT HEPATOCYTES<sup>a</sup>

Item	Days in culture		
	1	2	3
dpm/ug cell protein			
A. <sup>3</sup> H-leucine incorporation			
I. Medium + 5% FCS			
Rat 1	320	225	160
Rat 2	299	242	187
II. Serum-free medium			
Rat 1	299	228	217
Rat 2	317	221	210
B. <sup>3</sup> H-thymidine incorporation			
I. Medium + 5% FCS			
Rat 1	15	19	20
Rat 2	18	19	25
II. Serum-free medium			
Rat 1	16	18	20
Rat 2	17	18	17

<sup>a</sup>Hepatocytes were isolated from male Sprague-Dawley rats (250 g). Culture conditions and experimental protocol were identical to those described for swine hepatocytes.

TABLE 5. INFLUENCE OF PRESENCE OF SERUM AND DURATION OF CULTURE PERIOD ON THE DNA AND PROTEIN CONTENT OF MONOLAYER CULTURES OF PORCINE HEPATOCYTES<sup>a</sup>

Days in culture	DNA, ug/plate	Protein, mg/plate	$\frac{\text{DNA (ug)}}{\text{Protein (mg)}}$
I. Medium + 5% FCS			
1	16.7 $\pm$ 1.3	1.26 $\pm$ 0.2	13.3
3	12.8 $\pm$ 1.8	1.06 $\pm$ 0.2	12.1
II. Serum-free medium			
1	16.1 $\pm$ 1.1	1.15 $\pm$ 0.2	14.0
3	11.0 $\pm$ 1.4	0.92 $\pm$ 0.2	12.0

<sup>a</sup>Viable hepatocytes ( $3.5 \times 10^6$  cells) were seeded onto 60-mm, collagen-coated plates. At 1 and 3 d after initiation of culture, triplicate plates were washed and cells were scraped from plates in buffered saline. The DNA was quantitated fluorometrically by the method of Fiszler-Szafarz et al. (30), and protein was quantitated as described in Materials and Methods. Each value represents the mean  $\pm$  SE of cells isolated from six pigs (two representative pigs from each of three age groups).

pigs 1 to 7 d old. The majority of hepatocytes isolated from neonatal pigs (1 to 7 d) are mononucleate, and nuclear division was not observed over 3 d in culture (Figure 1). The likelihood of cell replication is further diminished in this system because hepatocytes were maintained at or near confluency. Recent evidence suggests that hepatocyte growth and replication is dependent on low plating density and the presence of specific polypeptide growth factors in cultured neonatal (26) and adult (27) rat hepatocytes. Thus, the enhanced incorporation of  $^3\text{H}$ -thymidine does not appear to represent significant increases in total DNA or cell replication. Apparently, monolayers of porcine hepatocytes were not dividing in medium with or without serum. Pangburn et al. (7) also reported that monolayers of neonatal pig hepatocytes maintained in serum containing medium exhibited enhanced rates of  $^3\text{H}$ -thymidine incorporation into DNA without evidence of cell proliferation.

Initial attempts to investigate RNA synthesis in cultured porcine hepatocytes by addition of  $^3\text{H}$ -orotic acid to the medium were unsuccessful, because this precursor was not accumulated by the cells. Since growing rats (28) and neonatal pigs (29) excrete high levels of orotic acid when fed an arginine-free diet, we examined the possibility that the lack of  $^3\text{H}$ -orotic acid incorporation was due to the absence of arginine in the medium. It is believed that increased urea-cycle activity increases the pool of carbamoyl phosphate, thereby stimulating de novo pyrimidine synthesis (28).

When hepatocytes were incubated overnight in medium containing arginine,  $^3\text{H}$ -orotic acid was accumulated and incorporated into acid-insoluble material. Moreover, porcine and rat hepatocytes maintained in low-phosphate medium incorporated similar amounts of  $^{32}\text{P}$ -phosphate into 28S, 18S, 5.8S, 5S and 4S (data not shown).

Our experiments show that metabolically active, nonreplicating porcine hepatocytes can be maintained as monolayers in either serum-free or serum-containing medium for at least 3 d. The ability to culture cells in serum-free medium should facilitate studies aimed at elucidating the influence of specific hormones on the metabolism of specific nutrients by hepatocytes during the neonatal period. The data also indicate the need to compare cells that have been isolated from similarly aged pigs and maintained in culture for similar times in investigations of nutrient transport and metabolism by neonatal swine hepatocytes. The aforementioned techniques may be of value for the study of hormone-receptor interactions and avoid difficult physiological interpretations arising from studies with broken cell preparations (31).

## LITERATURE CITED

1. Borek, C. & Williams, G.M. [eds.] (1980) Differentiation and carcinogenesis in liver cell cultures. *Ann. New York Acad. Sci.* 349, 1-429.
2. Bjorneboe, M. & Prytz, H. (1976) The mononuclear phagocytic functions of the liver. In: *Immunological Aspects of the Liver and Gastrointestinal Tract* (Ferguson, A. & MacSween, R.N.M., eds.), pp. 251-289, University Park Press, Baltimore.
3. Berg, T., Blomhoff, R. & Norum, K.R. (1982) Transfer of retinol between parenchymal and non-parenchymal liver cells. In: *Sinusoidal Liver Cells* (Knook, D.L. & Wisse, E., eds.), pp. 37-44, Elsevier Biomedical Press, Amsterdam.
4. Van Berkel, T.J.C., Nagelkerke, J.F. & Harkes, L. (1982) Liver sinusoidal cells and lipoprotein metabolism. In: *Sinusoidal Liver Cells* (Knook, D.L. & Wisse, E., eds.), pp. 305-318, Elsevier Biomedical Press, Amsterdam.
5. Hulcrantz, R., Hogberg, J. & Glauman, H. (1983) Studies on the rat liver following iron overload: An analysis of iron and lysosomal enzymes in isolated parenchymal and nonparenchymal cells. *Virchows Arch. [Cell Pathol.]* 43, 67-74.
6. Caperna, T.J. & Failla, M.L. (1984) Cadmium metabolism by rat liver endothelial and Kupffer cells. *Biochem. J.* 221, 631-636.
7. Pangburn, S.H., Newton, R.S., Chang, C.-M., Weinstein, D.B. & Steinberg, D. (1981) Receptor-mediated catabolism of homologous low-density lipoproteins in cultured pig hepatocytes. *J. Biol. Chem.* 256, 3340-3347.
8. Bachorik, P.S., Franklin, F.A., Virgil, D.G. & Kwiterovich, P.O., Jr. (1982) High-affinity uptake and degradation of apolipoprotein E-free high-density lipoprotein and low-density lipoprotein in cultured porcine hepatocytes. *Biochemistry* 21, 5675-5684.
9. Pegorier, J.P., Duee, P.H., Girard, J. & Peret, J. (1982) Development of gluconeogenesis in isolated hepatocytes from fasting or suckling newborn pigs. *J. Nutr.* 112, 1038-1046.
10. Belfrage, P., Borjesson, B., Hagerstrand, I., Nilsson, A., Olsson, A., Wiebe, T. & Akesson, B. (1975) Dispersion of viable pig liver cells with collagenase. *Life Sci.* 17, 1219-1225.

11. Makowka, L., Rotstein, L.E., Falk, R.E., Falk, J.A., Langer, B., Nossal, N.A., Blendis, L.M. & Phillips, M.J. (1980) Reversal of toxic and anoxic induced hepatic failure by syngeneic, allogeneic and xenogeneic hepatocyte transplantation. *Surgery* 88, 244-253.
12. Gammeltoft, S. (1981) Receptor binding of biosynthetic human insulin on isolated pig hepatocytes. *Diabetes Care* 4, 235-237.
13. Bischoff, M.B., Richter, W.R. & Stein, R.J. (1969) Ultrastructural changes in pig hepatocytes during the transitional period from late foetal to early neonatal life. *J. Cell Sci.* 4, 381.
14. Mersmann, H.J. (1974) Metabolic patterns in the neonatal swine. *J. Anim. Sci.* 38, 1022-1030.
15. Seglen, P.O. (1973) Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. *Exp. Cell Res.* 82, 391-398.
16. Waymouth, C. (1959) Rapid proliferation of sublines of NCTC clone 929 (strain L) mouse cells in a simple chemically defined medium (MB752/1). *J. Natl. Cancer Inst.* 22, 1003-1017.
17. Knook, D.L. & Sleyster, E. Ch. (1976) Separation of Kupffer and endothelial cells by centrifugal elutriation. *Exp. Cell Res.* 99, 444-449.
18. Caperna, T.J. & Garvey, J.S. (1982) Antigen handling in aging. II. The role of Kupffer and endothelial cells in antigen processing in Fischer 344 rats. *Mech. Age. Dev.* 20: 205-221.
19. Sleyster, E.Ch., Westerhuis, F.G. & Knook, D.L. (1977) The purification of nonparenchymal cell classes by centrifugal elutriation. In: *Kupffer Cells and Other Liver Sinusoidal Cells* (Knook, D.L. & Wisse, E., eds.), pp. 289-298, Elsevier Biomedical Press, Amsterdam.
20. Nerurkar, L.S., Marino, P.A. & Adams, D.O. (1981) Quantification of selected intracellular and secreted hydrolases of macrophages. In: *Manual of Macrophage Methodology* (Herscowitz, H.B., Holden, H.T., Bellanti, J.A. & Ghaffer, A., eds.), pp. 229-247, Marcel Dekker, Inc., New York.
21. SAS (1979) SAS User's Guide. Statistical Analysis System Institute, Inc., Cary.

22. Laishes, B.A. & Williams, G.M. (1976) Conditions affecting primary cell cultures of functional adult rat hepatocytes. 1. The effect of insulin. *In Vitro* 12, 521-532.
23. De Leeuw, A.M., Barelds, R.J., de Zanger, R. & Knook, D.L. (1982) Primary cultures of endothelial cells of the rat liver. A model for ultrastructural and functional studies. *Cell Tissue Res.* 223, 201-215.
24. Smedsrod, B., Eriksson, S., Fraser, J.R.E., Laurent, T.C. & Pertoft, H. (1982) Properties of liver endothelial cells in primary monolayer cultures. In: *Sinusoidal Liver Cells* (Knook, D.L. & Wisse, E., eds.), pp. 263-270, Elsevier Biomedical Press, Amsterdam.
25. Risser, W.L. & Gelehrter, T.D. (1973) Hormonal modulation of amino acid transport in rat hepatoma cells. *J. Biol. Chem.* 248, 1248.
26. 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.
27. Nakamura, T., Yoshimoto, K., Nakayama, Y., Tomita, Y. & Ichihara, A. (1983) Reciprocal modulation of growth and differentiated functions of mature rat hepatocytes in primary culture by cell-cell contact and cell membranes. *Proc. Natl. Acad. Sci.* 80, 7229-7233.
28. Milner, J.A. & Visek, W.J. (1973) Orotic aciduria and arginine deficiency. *Nature* 245, 211-213.
29. Southern, L.L. & Baker, D.H. (1983) Arginine requirement of the young pig. *J. Anim. Sci.* 57, 402-412.
30. Fiszer-Szafarz, B., Szafarz, D. & deMurillo, A.G. (1981) A general, fast, and sensitive micromethod for DNA determination: Application to rat and mouse liver, rat hepatoma, human leukocytes, chicken fibroblasts and yeast cells. *Anal. Biochem.* 110, 165-170.
31. Etherton, T.D. & Kensinger, R.S. (1984) Endocrine regulation of fetal and postnatal meat animal growth. *J. Anim. Sci.* 59, 511-527.

CHAPTER 3.

ACCUMULATION AND METABOLISM OF IRON-DEXTRAN BY HEPATOCYTES,  
KUPFFER CELLS AND ENDOTHELIAL CELLS IN THE NEONATAL PIG LIVER

Submitted to J. Nutrition, 1986.

## INTRODUCTION

Confinement reared pigs develop microcytic hypochromic anemia shortly after birth (1). The onset of anemia occurs in part because tissue stores of iron at birth and the quantity of iron received from sow's milk fail to provide the necessary amount of the micronutrient for the growing animal. This problem has been exacerbated in modern production facilities by denying young pigs access to exogenous sources of iron, such as soil and feces. To prevent development of anemia, current management practices mandate the administration of a single intramuscular injection of a large amount of colloidal iron during the first week after birth. Iron-carbohydrate complexes, and particularly iron-dextran (Fe-dextran), are widely used for the prevention of anemia in swine (1) and humans (2).

The hematologic profile of anemic and iron-treated piglets has been thoroughly investigated (1). More recently, the effects of an injection of Fe-dextran on the concentrations of ferritin (3,4), and trace elements (5) in the serum of neonatal pigs have been reported. Earlier studies established the central role of the liver in metabolism and storage of supplemental iron in pigs (6,7). However, the partitioning and subsequent metabolism of iron by the various types of liver cells in the neonatal pig are unknown and have been only partially characterized in laboratory rats (8-11).

The aim of the present investigation was to study accumulation and metabolism of Fe-dextran by hepatocytes and sinusoidal cells (Kupffer and endothelial cells), the predominant cell types present in the neonatal pig liver. In addition, the influence of Fe-dextran administration on the concentrations of zinc and copper in porcine liver cells was examined.

## MATERIALS AND METHODS

Animals. Male and female crossbred piglets between 2 and 4 d of age were used. Sows and nursing piglets were housed in farrowing crates that had partially slatted floors. Fe-dextran (Anchor Laboratories, St. Joseph, MO, 100 mg Fe/ml) was administered to piglets at a dose of 50 mg Fe per kg body weight at 20 to 30 h after birth. One-half of the dose was administered into the muscle of each thigh to insure complete retention of injected material. Control piglets were not injected. Piglets were anesthetized with sodium pentobarbital prior to laparotomy.

Histology of Liver Tissue. Whole tissue was prepared for histochemical analysis by initially perfusing the liver in situ with 20 mM HEPES (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid)-buffered saline, pH 7.4, at 4° C. The liver was next perfused with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 at 4° C. Fixed liver slices were dehydrated in ethanol, embedded in paraffin, sectioned and stained for the detection of non-heme Fe<sup>3+</sup> and Fe-dextran by Perl's Prussian Blue reaction (12).

Isolation of Liver Cells. Methods for the isolation of hepatocytes, Kupffer cells and endothelial cells from porcine liver are described in detail elsewhere (13). Briefly, cells were obtained by perfusing the liver with collagenase (Cooper Biochemical, Freehold, NJ). Hepatocytes were subsequently isolated by differential centrifugation. After removal of the hepatocytes from

the cell suspension, Kupffer cells and endothelial cells were purified by metrizamide (Accurate Chemical, Westbury, NY) density gradient centrifugation followed by centrifugal elutriation. Yields of isolated cells were similar to those previously reported (13) and viability exceeded 80 and 90 % for hepatocytes and sinusoidal cells, respectively, as assessed by trypan blue exclusion.

Analysis of Iron, Copper and Zinc in Cell Isolates. After purification, cell types were collected by centrifugation, frozen in liquid N<sub>2</sub> and stored at -70° C until analyzed. Cell pellets were resuspended in 3 ml distilled, deionized water (Nanopure System, Barnstead, Boston, MA) and disrupted by either homogenization (hepatocytes; Tissuemizer, Tekmar Corp., Cincinnati, OH) or exposure to ultrasound (sinusoidal cells; Fisher Model 300 Sonicator at 30 % maximum power for 1 min with a titanium microprobe). After removal of aliquots for assay of protein concentration, the remaining material was transferred to porcelain crucibles and dried at 90° C for 12 h. The temperature was elevated to 440° C over a 6 h period and the samples were maintained at this temperature for 24 h. Ashed samples were solubilized and diluted in 0.12N HCl (Ultrex, J.T. Baker Chemical Co., Phillipsburg, NJ) and analyzed by atomic absorption spectroscopy (Model 560, Perkin-Elmer Corp., Norwalk, CT) with an air:acetylene flame. Recovery of iron, copper and zinc exceeded 95% as determined with Standard Reference Material #1577a (bovine liver) from the National Bureau of Standards that was prepared and analyzed

in an identical manner for each set of cell samples. Protein was determined by a modified Lowry procedure (14) with bovine serum albumin as the standard.

Ferritin Purification and Antibody Production. Ferritin was purified from livers of fetal (90 d), neonatal (2-6 d following Fe-dextran treatment) and adult pigs by the method of May and Fish (15) with the following modifications described by Arosio et al. (16). The redissolved, dialyzed ammonium sulfate precipitate was chromatographed on Sepharose 6B and final purification to homogeneity was attained by preparative gradient polyacrylamide gel electrophoresis (PAGE). The purity of ferritin was confirmed by SDS-PAGE and size exclusion gel-permeation high-performance liquid chromatography.

Antiserum to Fe-dextran-induced neonatal ferritin was raised by immunizing each of several rabbits with 1 mg ferritin in Freund's complete adjuvant followed by three biweekly injections of 1 mg ferritin in incomplete adjuvant. Rabbits were exsanguinated 1 wk after the final injection. The antiserum gave strong precipitation lines in Ouchterlony double diffusion plates at a 1:150 dilution. Ferritin purified from fetal, neonatal and adult pig liver crossreacted with complete identity in double diffusion plates (Figure 1).

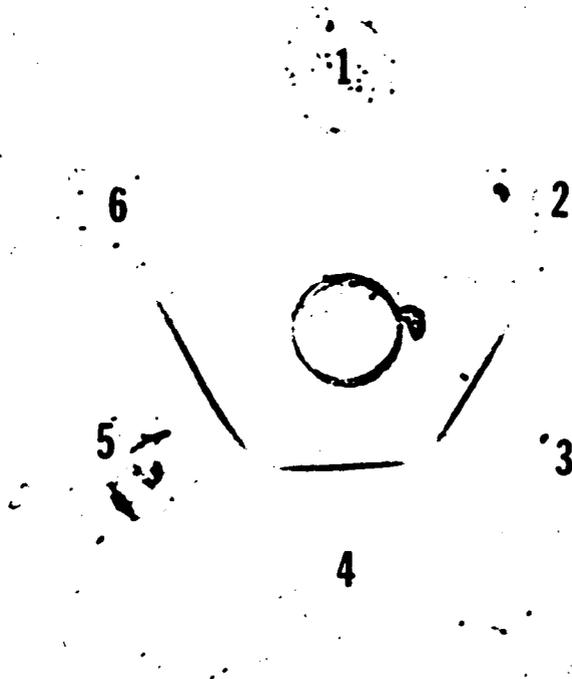


Figure 1. Double diffusion analysis of anti-porcine liver ferritin immune serum. The center well contained 10 ul of a 1:150 dilution of immune serum. Peripheral wells contained (1) buffer; (2) 0.5 ug horse spleen ferritin; (3) 0.5 ug fetal porcine liver ferritin; (4) 0.5 ug neonatal porcine liver ferritin; (5) 0.5 ug adult porcine liver ferritin; (6) 10 ug horse spleen ferritin. All solutions were added in 10 ul aliquots.

Quantification of Ferritin. Ferritin was measured by rocket immunoelectrophoresis, essentially as described by Garvey and associates (17). Antiserum (4 ul) was incorporated into 10 ml agarose gel prepared in 25 mM barbital buffer, pH 8.6, and poured onto 50 x 75 mm prewarmed glass plates. Three millimeter wells were punched into the gel and ferritin standards (25-600 ng) were added. Isolated cells were sonicated in 5 mM Tris-HCl, pH 7.8, containing 5 mM 2-mercaptoethanol and 0.1% Triton X-100 at a concentration of 2 to 6 mg of cell protein per ml. Aliquots (5-20 ul) of each sonicate were analyzed in duplicate. Gels were electrophoresed for 12 h in 25 mM barbital buffer, pH 8.6. Ferritin levels were determined from rocket peak area compared to porcine ferritin standards. The limit of detection was approximately 0.2 ug ferritin per mg cell protein.

Determination of Ferritin-Iron. Whole cell sonicates prepared for immunoelectrophoresis were also chromatographed on DEAE cellulose (DE-53, Whatman, Ltd., England) to separate the various classes of iron-binding species. Aliquots of cell protein (5-15 mg) in a total volume of 5 ml were added to columns packed with 2 ml ion exchange resin and equilibrated with 10 mM Tris-HCl in 10 mM 2-mercaptoethanol, pH 7.8 (Buffer A). The concentration of iron in fractions was determined by atomic absorption spectroscopy. Fraction 1 contained materials that eluted during sample application and a 7 ml wash with Buffer A. Fraction 2 was obtained by washing with 15 ml of Buffer A containing 85 mM NaCl. Fraction 3 was collected during a

wash with 15 ml high salt buffer (350 mM NaCl, 100 mM Tris-HCl, and 10 mM 2-mercaptoethanol, pH 7.8). Greater than 90% of purified neonatal pig liver ferritin eluted from the column in Fraction 3 when applied either directly to the packed resin or after addition to an aliquot of liver cell sonicate. To elute metabolized derivatives of Fe-dextran and other iron containing materials that remained bound to the column after the high salt wash, the resin was washed successively with 15 ml of 0.5 M NaOH (Fraction 4) and 0.5 M HCl (Fraction 5). Following Fe-dextran treatment, the majority of cellular iron was either eluted in Fractions 4 and 5 or remained bound to the ion exchange resin. This was particularly evident in homogenates from sinusoidal cells where the majority of iron appears to be associated with dextran metabolites (see Discussion). In a preliminary study, the contents in each eluted fraction were concentrated by membrane centrifugation (Centricon 10, Amicon Corp., Danvers, MA) and analyzed by nondenaturing electrophoresis using 7.5% polyacrylamide gels. Replicate gels were stained with potassium ferrocyanide to detect  $\text{Fe}^{3+}$  (18) and Coomassie Blue to detect protein. All detectable iron that had the same electrophoretic mobility as purified porcine liver ferritin was present in Fraction 3. Moreover, the only band in this fraction which was iron-positive had the same relative mobility as ferritin. Consequently, the amount of iron that eluted in Fraction 3 was used to estimate the quantity of iron bound to ferritin in each cell sample.

Statistical Analysis. All data are presented as the mean  $\pm$  standard error of the mean (SE). Where appropriate, statistical significance of the differences between control and Fe-dextran treated animals were determined at each age by a two-tailed t-test. P values for unequal variances were utilized where necessary.

## RESULTS

Hepatic distribution of iron following treatment with Fe-dextran was initially investigated by histochemical methods. Positive staining with Prussian Blue was primarily associated with sinusoidal cells at 24 h after injection (Figure 2). Positive staining was not observed in age-matched control liver tissue or in tissue prepared 10 d after treatment with Fe-dextran.

To investigate the accumulation and metabolism of supplemental iron by the liver during early neonatal life, the three predominant liver cell types, *viz.* hepatocytes, Kupffer cells and endothelial cells, were isolated from Fe-dextran treated piglets 1, 5 and 10 d after injection. For comparison, liver cells were similarly isolated from age-matched piglets not injected with supplemental iron. The concentration of iron ( $\mu\text{g Fe/mg cell protein}$ ) was  $0.4 \pm 0.04$ ,  $0.4 \pm 0.1$  and  $0.5 \pm 0.1$  in hepatocytes, endothelial cells and Kupffer cells, respectively, isolated from control piglets at 2 d after birth. Minimum levels of iron were present by 6 d after birth (Figures 3 and 4). Administration of supplemental iron resulted in a 5-, 54- and 62-fold increase in the concentration of iron in hepatocytes, endothelial cells and Kupffer cells, respectively, by 1 d after injection. The concentration of iron in hepatocytes at 5 d after treatment was only 25% that at 1 d, whereas sinusoidal cells retained 80% of their complement of accumulated iron during the same period of time. Although the majority of iron was mobilized from

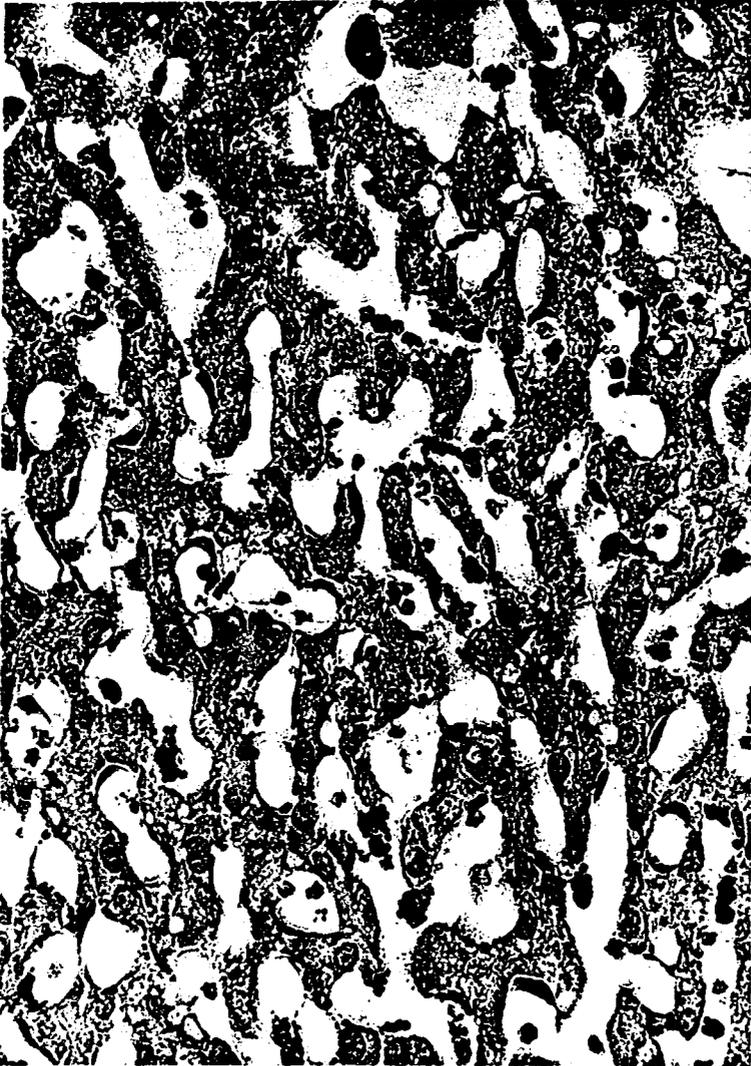


Figure 2. Liver section obtained from piglet 1 d after Fe-dextran treatment was stained with Prussian blue. Note intense staining of cells lining the sinuses.

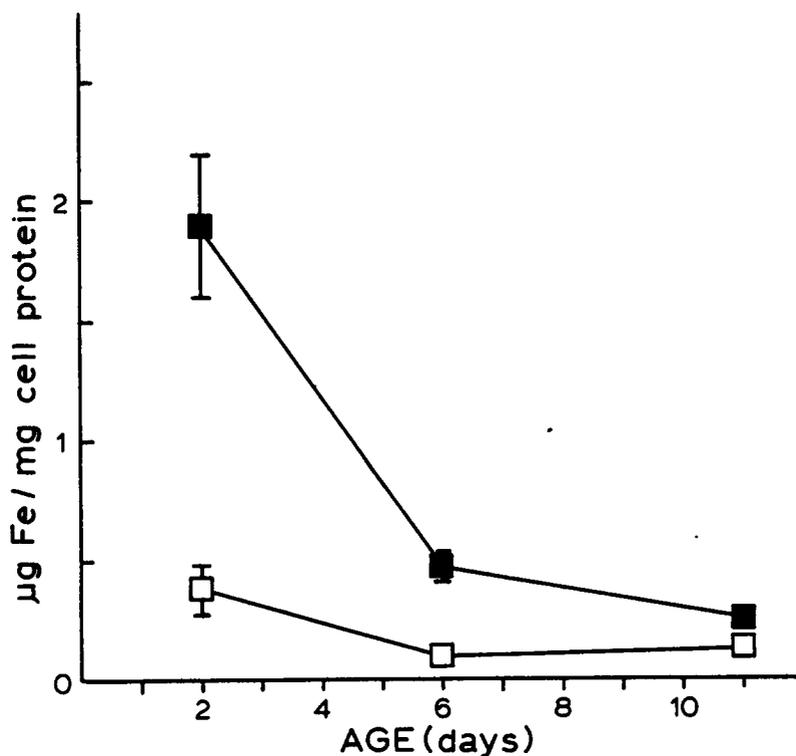


Figure 3. Concentrations of iron in hepatocytes isolated from control and Fe-dextran treated piglets. Hepatocytes were isolated from control pigs (□) at 2, 6 and 11 d of age. Another group of pigs was injected with Fe-dextran (50 mg/kg body weight) 1 d after birth. Hepatocytes were isolated from iron treated pigs (■) 1, 5 and 10 d following injection. Values are means  $\pm$  SE; n = 4-6 pigs per group at each time.

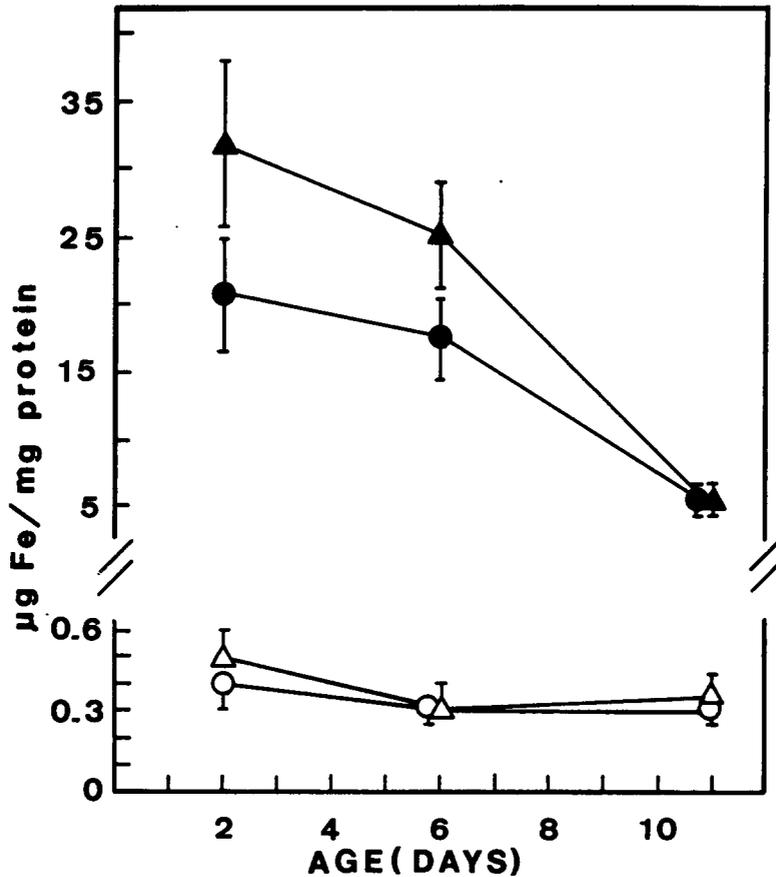


Figure 4. Concentrations of iron in sinusoidal cells isolated from control and Fe-dextran treated pigs. Kupffer cells and endothelial cells were isolated from control pigs at 2, 6 and 11 d of age. Another group of pigs was injected with Fe-dextran (50 mg/kg body weight) 1 d after birth, and Kupffer cells and endothelial cells were isolated 1, 5 and 10 d later. Values are means  $\pm$  SE;  $n = 4$  pigs per group at each time. Control endothelial cells, (○); control Kupffer cells, (△); endothelial cells from iron-treated pigs, (●); Kupffer cells from iron-treated pigs, (▲).

sinusoidal cells by 10 d post-treatment, the concentration of iron in these cells was 20-fold higher than in sinusoidal cells isolated from age-matched control pig liver.

The influence of iron administration on the zinc and copper status of the different liver cell types also was evaluated. At early times after injection (1 and 5 d), cellular concentrations of zinc and copper were similar in iron-treated and control pigs (Table 1). However, the concentration of zinc was 43% higher in hepatocytes and approximately 30% lower in sinusoidal cells at 10 d after treatment than that in the respective cell types isolated from control livers. The concentration of copper in endothelial and Kupffer cells isolated 10 d after Fe-dextran treatment was 50 ( $P < .05$ ) and 62 ( $P < 0.1$ ) percent, respectively, of that in sinusoidal cells isolated from age-matched control piglets.

At birth, the ferritin concentration in whole liver was approximately 3 ug/mg tissue protein. Ferritin concentrations ranged from undetectable ( $< 0.2$  ug/mg cell protein) to approximately 1 ug/mg protein in cells isolated from control piglets at 2 d after birth. By 6 d, the cellular concentration of ferritin was below the limit of detection. The concentration of ferritin increased from 5- to 75-fold in each liver cell type by 1 d after the administration of Fe-dextran (Figure 5). For the three time points investigated, maximum concentrations of ferritin were observed on d 1 in hepatocytes and Kupffer cells. In contrast, peak ferritin levels

TABLE 1. THE CONCENTRATIONS OF ZINC AND COPPER IN LIVER CELLS  
ISOLATED FROM CONTROL AND IRON-DEXTRAN TREATED PIGLETS<sup>1,2</sup>

Cell type	Treatment	ug metal/mg cell protein		
		Day 2	Day 6	Day 11
A. Zinc				
Hepatocytes	Control	0.47 ± 0.04	0.65 ± 0.0	0.76 ± 0.1
	Fe-dextran	0.54 ± 0.11	0.76 ± 0.11	1.09 ± 0.02*
Endothelial	Control	0.27 ± 0.01	0.55 ± 0.12	0.35 ± 0.01
	Fe-dextran	0.37 ± 0.10	0.44 ± 0.11	0.26 ± 0.01*
Kupffer	Control	0.40 ± 0.09	0.86 ± 0.28	0.40 ± 0.03
	Fe-dextran	0.47 ± 0.09	0.63 ± 0.17	0.27 ± 0.03*
B. Copper				
Hepatocytes	Control	0.33 ± 0.05	0.32 ± 0.03	0.54 ± 0.04
	Fe-dextran	0.40 ± 0.01	0.50 ± 0.11	0.55 ± 0.10
Endothelial	Control	0.14 ± 0.02	0.26 ± 0.07	0.20 ± 0.02
	Fe-dextran	0.26 ± 0.07	0.15 ± 0.04	0.10 ± 0.01*
Kupffer	Control	0.32 ± 0.06	0.48 ± 0.17	0.29 ± 0.03
	Fe-dextran	0.36 ± 0.11	0.41 ± 0.14	0.18 ± 0.03

<sup>1</sup>Values are means ± SE; n = 4 to 8 pigs in each group.

<sup>2</sup>Significant difference by t-test analyses between control and Fe-dextran treated pigs in each age group: \*P<0.05.

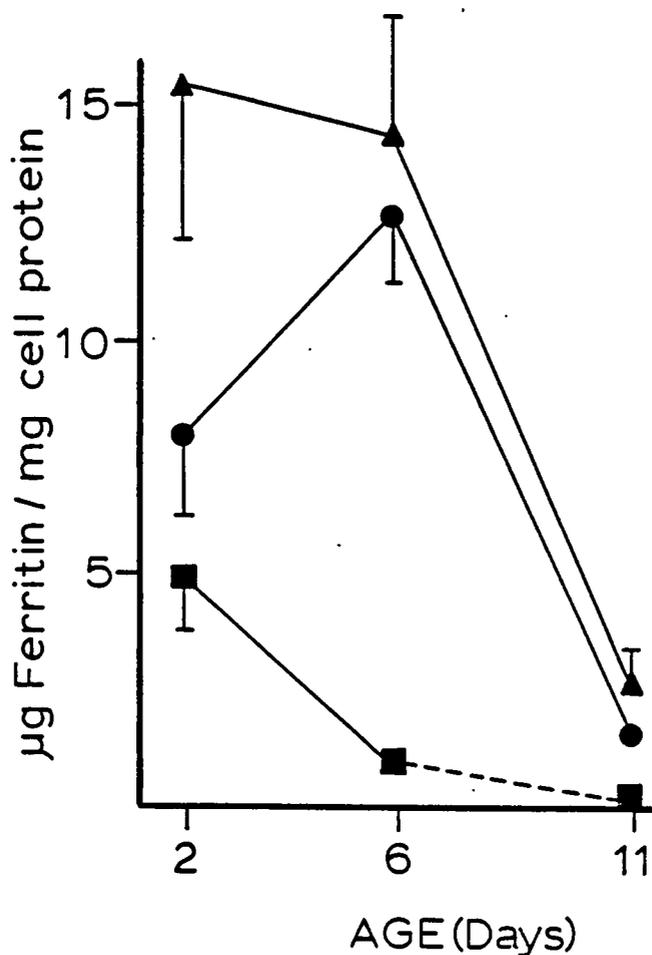


Figure 5. Concentrations of immunoreactive ferritin in hepatocytes, Kupffer cells and endothelial cells isolated from pigs treated with Fe-dextran. Fe-dextran was injected into piglets 1 d after birth and liver cells were isolated at 1, 5 and 10 d later. Concentrations of ferritin were determined by rocket immunoelectrophoresis as described in Materials and Methods. Values are means  $\pm$  SE; n = 4 pigs per group at each time. Immunoreactive ferritin levels were below limits of accurate quantitation in hepatocytes on d 11. Hepatocytes, (■); endothelial cells, (●); Kupffer cells, (▲).

were not attained until 5 d post injection in endothelial cells. After reaching maximal levels, immunoreactive ferritin decreased in concert with the concentrations of iron in each cell type.

The amount of iron associated with ferritin in each cell type was determined by quantitating the concentration of iron that eluted in the high salt fraction from DEAE-cellulose (Table 2). Ferritin-iron concentrations attained maximum levels in each cell type by 1 d after treatment. Thereafter, the amount of iron associated with ferritin decreased in each cell type in a manner similar to that observed for the decrease in immunoreactive ferritin-protein and total cellular iron. The decreases in concentrations of total iron and ferritin-iron in liver cells were probably due to mobilization of the micronutrient to extrahepatic compartments.

The degree of iron saturation of the ferritin pools ( $\mu\text{g Fe}/\mu\text{g ferritin-protein}$ ) was calculated for each cell type and was determined to be relatively constant in hepatocytes and Kupffer cells (Table 2). However, the saturation of endothelial cell ferritin varied markedly, ranging from 0.13 to 0.65  $\mu\text{g Fe}/\mu\text{g ferritin}$ .

The percentage of total cellular iron that was associated with ferritin in each cell type following iron treatment was also determined (Table 2). Approximately one-half of the iron present in hepatocytes was associated with ferritin at 1 d after treatment with Fe-dextran. The decrease in the percentage of cellular iron

TABLE 2. ANALYSIS OF IRON ASSOCIATED WITH FERRITIN IN LIVER  
CELLS ISOLATED FROM IRON-DEXTRAN TREATED PIGLETS<sup>1,2</sup>

Parameter	Cell type	Age		
		Day 2	Day 6	Day 11
Ferritin-iron (ug Fe/mg cell protein)	Hepatocytes	0.77 ± 0.20	0.18 ± 0.03	0.03 ± 0.01
	Endothelial	1.94 ± 0.23	1.86 ± 0.23	0.46 ± 0.03
	Kupffer	2.39 ± 0.39	2.09 ± 0.40	0.42 ± 0.07
Degree of iron saturation (ug Fe/ug ferritin)	Hepatocytes	0.15 ± 0.01	0.23 ± 0.06	N.D. <sup>3</sup>
	Endothelial	0.29 ± 0.06	0.15 ± 0.01	0.38 ± 0.11
	Kupffer	0.16 ± 0.01	0.14 ± 0.01	0.21 ± 0.05
Cellular iron as ferritin (%)	Hepatocytes	48.0 ± 11.0	38.0 ± 6.7	10.0 ± 1.6
	Endothelial	8.1 ± 1.8	11.0 ± 1.4	8.1 ± 0.6
	Kupffer	9.7 ± 1.9	8.5 ± 1.1	7.4 ± 0.3

<sup>1</sup>Values are means ± SE; n = 4 pigs in each group.

<sup>2</sup>Piglets were injected 1 d after birth and liver cells were isolated 1, 5 and 10 d later.

<sup>3</sup>N.D., immunoreactive ferritin levels were below limits of detection.

associated with ferritin in hepatocytes paralleled the decrease in total level of iron in these cells. This phenomenon was not observed in sinusoidal cells, where only approximately 7 to 11% of the total iron content at any time after injection was associated with ferritin.

## DISCUSSION

The mammalian liver is a histologically complex organ and is composed of at least five types of resident cells: Hepatocytes, Kupffer cells, endothelial cells, pit cells and stellate cells, that are morphologically and functionally distinct (19). Identification of which hepatic cell types participate in the accumulation, metabolism and storage of exogenous and endogenous iron has been limited primarily to histochemical (20-22) and ultrastructural (12, 23-25) analyses. Development of methods for the isolation of hepatocytes, Kupffer cells and endothelial cells provided an opportunity to investigate the cellular uptake and metabolism of supplemental iron by the neonatal pig liver in a quantitative manner. The finding that sinusoidal cells accumulated much higher concentrations of iron than hepatocytes after a single injection of Fe-dextran confirms and extends results obtained by histochemical studies in the baby pig (Figure 2, and ref 20) and neonatal rat (Caperna and Failla, unpublished observation). However, hepatocytes account for approximately 90% of the total cellular mass of the liver (assuming that morphometry of rodent liver is similar to porcine liver). About 40 and 30% of the total amount of iron in liver was contained within hepatocytes at 1 and 5 d, respectively, after treatment of the pigs with Fe-dextran. Thus, all three predominant liver cell types actively participate in the metabolism of Fe-dextran in the neonatal pig.

The concentration of iron in sinusoidal cells at 1 d after the administration of Fe-dextran was 12-15 times higher than in hepatocytes (Figures 3 and 4). These data are in agreement with our previous finding that primary monolayer cultures of Kupffer cells and endothelial cells from livers of control piglets accumulated markedly more iron than monolayers of porcine hepatocytes on a protein basis, during a 24 h exposure to  $^{55}\text{Fe}$ -dextran (26). This ability of sinusoidal cells to accumulate more colloidal iron than hepatocytes in vivo and in vitro is related in part to their greater relative surface area (27). Moreover, the unique architecture of the hepatic sinus provides sieving properties that impede effective diffusion of some high molecular weight complexes to the surface of hepatocytes (28). Thus, high molecular weight colloidal (29) and azo-derivatized (30) albumin and polyvinyl pyrrolidone (29) were preferentially accumulated by sinusoidal cells in the rat liver, while low molecular weight materials such as iron chelates (31) and small lipid vesicles (32) were concentrated in hepatocytes. To determine whether this sieving effect contributed to enhanced uptake of Fe-dextran by sinusoidal cells in baby pig liver, levels of iron in liver cells were determined 1 d after treatment with equivalent doses (50 mg Fe/kg body weight) of Fe-dextran and Jectofer (iron-polysorbitol-citrate), a low molecular weight iron complex. Sinusoidal cells accumulated 12- and 4-fold more iron than

hepatocytes in livers from pigs treated with Fe-dextran and Jectofer, respectively (26). This observation supports the conclusion that differential cellular accumulation of supplemental iron in neonatal pig liver is influenced in part by the form of iron administered. In contrast to these findings, Hulcrantz et al. (9) have reported that hepatocytes accumulated higher concentrations of iron on a protein basis than sinusoidal cells following the administration of Jectofer to rats. In their study, however, normal rats received a total of 500 mg Fe/kg body weight in a series of multiple injections over a 3 wk period. Thus, in addition to the form of iron, the cellular accumulation and subsequent metabolism of supplemental iron in the liver is probably influenced by dosage, regimen of treatment and the physiological status (e.g., age) of the animal.

Histochemical examination of liver sections at 10 d after administration of Fe-dextran failed to reveal the presence of high concentrations of iron in either sinusoidal or parenchymal regions. Direct analysis of iron levels in isolated cells showed that concentrations of the metal in hepatocytes, Kupffer and endothelial cells at 10 d post-injection were markedly lower than those at 1 and 5 d after treatment (Figures 3 and 4). The reductions in cellular concentrations of iron exceeded those which would be expected due to doubling of the mass of liver during the 2 wk period after birth. Our results suggest that the amount of supplemental iron initially

accumulated by the liver was processed (see below) and subsequently transferred from sinusoidal cells and hepatocytes to extrahepatic sites. This finding is similar to the report by Hulcrantz et al. (9) that phlebotomy induced the mobilization of stored iron from hepatocytes and sinusoidal cells in adult rats. Kuchta et al. (23) have also reported that iron accumulated in sinusoidal cells after treatment of anemic rats with Fe-dextran was subsequently mobilized from Kupffer cells, and to a lesser degree, from endothelial cells. Together, these findings demonstrate that metabolism and transport of iron in liver sinusoidal cells as well as in hepatocytes are affected by extrahepatic factors, such as enhanced rate of erythropoiesis. Transferrin, ceruloplasmin and hepatic xanthine oxidase are proteins that are directly involved in the transfer of iron from liver to other tissues. Since the levels of these proteins are very low in piglets during the first week of life (33) the mechanism(s) responsible for the mobilization of iron from liver cells is an enigma.

Ferritin serves a primary role in the storage and detoxification of intracellular iron (34,35). Although the rate of incorporation of  $^{14}\text{C}$ -labeled amino acids into immunoreactive ferritin was increased by exposure to high levels of inorganic iron (36) and ferritin protein was identified in Kupffer cells isolated from livers of adult rats treated with Fe-dextran (8), it is generally believed that

ferritin levels in Kupffer cells are not responsive to changes in iron status (37). Our results demonstrate that the concentration of immunoreactive ferritin markedly increased in hepatocytes, Kupffer cells and endothelial cells following administration of Fe-dextran to neonatal pigs (Figure 5). The higher concentrations ( $\mu\text{g}/\text{mg}$  cell protein) of ferritin-protein in the sinusoidal cells compared to hepatocytes were correlated with the higher concentrations of iron in the former (Figures 3 and 4). However, because of the greater mass of hepatocyte protein, 79% of the total liver ferritin was present in hepatocytes 1 d after Fe-dextran administration. In contrast, 62% and virtually all of the liver ferritin protein was present in sinusoidal cells at 5 and 10 d, respectively, after treatment.

The percentage of cellular iron associated with ferritin in hepatocytes was 4.9 and 5.9 times greater than in Kupffer cells and endothelial cells, respectively, 1 d after the injection of Fe-dextran. Van Wyk et al. (8) also found the cellular complement of iron that was associated with ferritin in Kupffer cells to be very low (<6%), in cells isolated from adult rats that had been treated with Fe-dextran. Ultrastructural analysis (transmission electron microscopy) of cells isolated from baby pigs 1 d after treatment showed that the majority of electron dense material (presumably iron) within sinusoidal cells was amorphous and localized within organelles that were limited by a single membrane with an appearance similar to secondary lysosomes (Caperna and Failla, unpublished observations).

Such deposits were not evident in hepatocytes. Therefore, the degradation of endocytosed Fe-dextran derivatives within lysosomes appear to be rate limiting in sinusoidal cells and responsible in part for the lower percentage of cellular iron associated with ferritin in sinusoidal cells.

The influence of Fe-dextran treatment on the concentrations of zinc and copper in liver cells was investigated because of the known interactions between these essential elements (38-40). Our data show that intramuscular administration of Fe-dextran did not affect concentrations of zinc and copper in liver cells 1 and 5 d after treatment, while slight alterations were noted at 10 d (Table 1). Neither the basis for these differences nor the significance, if any, are known. Recent studies have shown that parenteral administration of Fe-dextran to piglets (5) and oral iron supplements to children (41) were not accompanied by alterations in zinc and copper levels in plasma.

Iron deficiency anemia continues to be a major problem for rapidly growing newborn pigs and human infants. Although treatment with parenteral iron is widely accepted and believed to be safe, there is increasing evidence that parenteral iron treatment compromises nutritional immunity (42,43). Moreover, the presence of excess iron in cells is also associated with peroxidative damage to cell membranes (44,45). It has been recently reported that a single injection of Fe-dextran to adult rats was associated with enhanced

membrane lipid peroxidation (46,47). Since sinusoidal cells accumulate high levels of iron after treatment of neonatal pigs with a single injection of the recommended dose of Fe-dextran, we are currently assessing whether the treatment adversely effects cellular function.

## LITERATURE CITED

1. Zimmerman, D.R. (1980) Iron in swine nutrition. In: NFIA Literature Review on Iron in Animals and Poultry Nutrition (Conrad, C.R., Zimmerman, D.R. & Combs, G.F., eds.), pp. 1-56, NFIA, West Des Moines, Iowa.
2. Finch, C.A. (1980) Drugs effective in iron-deficiency and other hypochromic anemias. In: The Pharmacological Basis of Therapeutics (Gilman, A.G., Goodman, L.S. & Gilman, A., eds.), pp. 1315-1330, Macmillan Publishing Co., Inc., New York.
3. Furugouri, K., Miyata, Y. & Narasaki, N. (1983) Developmental changes in serum ferritin in piglets. *J. Anim. Sci.* 57, 960-965.
4. Smith, J.E., Moore, K., Boyington, D., Pollman, D.S. & Schoneweis, D. (1984) Serum ferritin and total iron-binding capacity to estimate iron storage in pigs. *Vet. Pathol.* 21, 597-600.
5. Ku, P.K., Miller, E.R. & Ullrey, D.E. (1983) Effect of parenteral iron on serum electrolytes of the baby pig. *J. Anim. Sci.* 57, 638-644.
6. Braude, R., Chamberlain, A.G., Kotarbinska, M. & Mitchell, K.G. (1962) The metabolism of iron in piglets given labelled iron either orally or by injection. *Brit. J. Nutr.* 16, 427-449.
7. Furugouri, K. (1973) Developmental changes in the nonheme iron composition of the liver and spleen in piglets. *J. Anim. Sci.* 36, 265-270.
8. van Wyk, C.P., Linder-Horowitz, M. & Munro, H.N. (1971) Effect of iron loading on non-heme iron compounds in different liver cell populations. *J. Biol. Chem.* 246, 1025-1031.
9. Hulcrantz, R., Hogberg, J. & Glauman, H. (1983) Studies on the rat liver following iron overload: An analysis of iron and lysosomal enzymes in isolated parenchymal and non-parenchymal cells. *Virchows Arch. (Cell Pathol.)* 43, 67-74.
10. Tyson, C.A., Green, C.E., LeValley, S.E. & Stevens, R.J. (1982) Characterization of isolated Fe-loaded rat hepatocytes prepared by collagenase perfusion. *In Vitro* 18, 945-951.

11. Lake-Bakaar, D.M. (1980) Relative contribution of parenchymal and non-parenchymal cells to the hepatic distribution and metabolism of iron-poly (sorbitol-gluconic acid) complex in the rat. *Acta Pharmacol. et Toxicol.* 46, 345-352.
12. Parmley, R.T., May, M.E., Spicer, S.S., Buse, M.G. & Alvarez, C.J. (1981) Ultrastructural distribution of inorganic iron in normal and iron-loaded hepatic cells. *Lab. Invest.* 44, 475-485.
13. Caperna, T.J., Failla, M.L., Kornegay, E.T., Richards, M.P. & Steele, N.C. (1985) Isolation and culture of parenchymal and nonparenchymal cells from neonatal swine liver. *J. Anim. Sci.* 61, 1576-1586.
14. Nerurkar, L.S., Marino, P.A. & Adams, D.O. (1981) Quantification of selected intracellular and secreted hydrolases of macrophages. In: *Manual of Macrophage Methodology* (Herscovitz, H.B., Holden, H.T., Bellanti, J.A. & Ghaffer, A., eds.), pp.229-247, Marcel Dekker, Inc., New York.
15. May, M.E. & Fish, W.W. (1977) The isolation and properties of porcine ferritin and apoferritin. *Arch. Biochem. Biophys.* 182, 396-403.
16. Arosio, P., Adelman, T.G. & Drysdale, J.W. (1978) On ferritin heterogeneity. Further evidence for heteropolymers. *J. Biol. Chem.* 253, 4451-4458.
17. Garvey, J.S., Cremer, N.E. & Sussdorf, D.H. (1977) *Methods in Immunology*. pp. 340-346, W. Benjamin, Inc., Reading, MA.
18. Drysdale, J.W. (1974) Heterogeneity in tissue ferritins displayed by gel electrofocusing. *Biochem. J.* 141, 627-632.
19. Wisse, E. & Knook, D.L. (1979) The investigation of sinusoidal cells: A new approach to the study of liver function. In: *Progress in Liver Diseases*. Vol. VI, (Popper, H. & Schafner, F., eds.), pp. 153-171, Grune and Stratton, New York.
20. Thoren-Tolling, K. & Jonsson, L. (1977) Cellular distribution of orally and intramuscularly administered iron dextran in newborn piglets. *Can. J. Comp. Med.* 41, 318-325.
21. Goldberg, L., Smith, J.P. & Martin, L.E. (1957) The effects of intensive and prolonged administration of iron parenterally in animals. *Br. J. Exp. Pathol.* 38, 297-311.

22. Shoden, A., Ing, K. & Sturgeon, P. (1962) Iron storage IV. Cellular distribution of excess liver iron. *Amer. J. Pathol.* 40, 671-683.
23. Kuchta, B., Wulfhekel, U. & Dullman, J. (1982) Experimental siderosis of liver sinusoidal cells in the rat: The role of Kupffer cells for transformation and utilization of colloidal iron. In: *Sinusoidal Liver Cells* (Knook, D.L. & Wisse, E., eds.), pp. 223-230, Elsevier Biomedical Press, Amsterdam.
24. Iancu, T.C., Lichterman, L. & Neustein, H.B. (1978) Hepatic sinusoidal cells in iron overload. Ultrastructural observations. *Israel J. Med. Sci.* 14, 1191-1201.
25. Doolittle, R.L. & Richter, G.W. (1981) Isolation and culture of Kupffer cells and hepatocytes from single rat livers with observations on iron-loaded Kupffer cells. *Lab. Invest.* 45, 558-566.
26. Caperna, T.J., Failla, M.L., Richards, M.P. & Steele, N.C. (In Press) Uptake and distribution of iron-dextran by neonatal swine liver cells. In: *Symposium on Swine in Biomedical Research*, (Tumbleson, M.E., ed.). Plenum Press, New York.
27. Blouin, A., Bolender, R.P. & Weibel, E.R. (1977) Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. *J. Cell Biol.* 72, 441-455.
28. Wisse, E., DeZanger, R.B., Charels, K., Van Der Smissen, P. & McCuskey, R.S. (1985) The liver seive: considerations concerning the structure and function of endothelial fenestrae, the sinusoidal wall and the space of Disse. *Hepatology* 5, 683-692.
29. Praaning-van Dalen, D.P., Brouwer, A. & Knook, D.L. (1981) Clearance capacity of rat liver Kupffer, endothelial and parenchymal cells. *Gastroenterology* 81, 1036-1044.
30. Garvey, J.S. & Caperna, T.J. (1982) The role of endothelial cells and Kupffer cells in antigen processing by Fischer-344 rats. In: *Sinusoidal Liver Cells* (Knook, D.L. & Wisse, E., eds.), pp. 421-428, Elsevier Biomedical Press, Amsterdam.
31. Brissot, P., Wright, T.L., Ma, W.L. & Weisiger, R.A. (1985) Efficient clearance of non-transferrin-bound iron by rat liver. Implications for hepatic iron loading in iron overload states. *J. Clin. Invest.* 76, 1463-1470.

32. Rahman, Y.E., Cerny, E.A., Patel, K.R., Lau, E.H. & Wright, B.J. (1982) Differential uptake of liposomes varying in size and lipid composition by parenchymal and Kupffer cells of mouse liver. *Life Sci.* 31, 2061-2071.
33. Furugouri, K. (1975) Characteristic aspects of iron metabolism in piglets. *Jap. Agri. Res. Quart.* 9, 171-176.
34. Munro, H.N. & Linder, M.C. (1978) Ferritin: Structure, biosynthesis and role in iron metabolism. *Physiol. Rev.* 58, 317-396.
35. Aisen, P. & Listowsky, I. (1980) Iron storage and transport proteins. *Ann. Rev. Biochem.* 49, 357-393.
36. Doolittle, R.L. & Richter, G.W. (1981) Isoferritins in rat Kupffer cells, hepatocytes, and extrahepatic macrophages. Biosynthesis in cell suspensions and cultures in response to iron. *Lab. Invest.* 45, 567-574.
37. Young, S.P. & Aisen, P. (1982) The Liver and Iron. In: *The Liver: Biology and Pathobiology* (Arias, I., Popper, H., Schachter, D. & Shafritz, D.A., eds.), pp. 393-404, Raven Press, New York.
38. Hill, G.M., Miller, E.R., Whetter, P.A. & Ullrey, D.E. (1983) Concentration of minerals in tissues of pigs from dams fed different levels of dietary zinc. *J. Anim. Sci.* 57, 130-138.
39. McCormick, C.C. (1984) The tissue-specific accumulation of hepatic zinc metallothionein following parenteral iron loading. *Proc. Soc. Exp. Biol. Med.* 176, 392-402.
40. Price, D. & Joshi, J.G. (1982) Ferritin: A zinc detoxicant and a zinc ion donor. *Proc. Natl. Acad. Sci. USA.* 79, 3116-3119.
41. Yip, R., Reeves, J.D., Lonnerdal, B., Keen, C.L. & Dallman, P.R. (1985) Does iron supplementation compromise zinc nutrition in healthy infants? *Amer. J. Clin. Nutr.* 42, 683-687.
42. Weinberg, E.D. (1984) Iron withholding: A defense against infection and neoplasia. *Physiol. Rev.* 64, 65-102.
43. Knight, C.D., Klasing, K.C. & Forsyth, D.M. (1984) The effects of intestinal Escherichia coli 263, intravenous infusion of Escherichia coli 263 culture filtrate and iron dextran supplementation on iron metabolism in the young pig. *J. Anim. Sci.* 59, 1519-1528.

44. Jacobs, A. (1980) The pathology of iron overload. In: Iron in Biochemistry and Medicine II (Jacobs, A & Worwood, M., eds.), pp. 427-459, Academic Press, New York.
45. Seymour, C.A. & Peters, T.J. (1978) Organelle pathology in primary and secondary haemochromatosis with special reference to lysosomal changes. Brit. J. Haematol. 40, 239-253.
46. Valenzuela, A., Fernandez, V. & Videla, L.A. (1983) Hepatic and biliary levels of glutathione and peroxides following iron overload in the rat: Effect of simultaneous ethanol administration. Toxicol. Appl. Pharmacol. 70, 87-95.
47. Gavino, V.C., Dillard, C.J. & Tappel, A.L. (1984) Release of ethane and pentane from rat tissue slices: effect of vitamin E, halogenated hydrocarbons and iron overload. Arch. Biochem. Biophys. 233, 741-747.

CHAPTER 4.

IRON-DEXTRAN MEDIATED PEROXIDATION OF LIPIDS IN  
TISSUES OF BABY PIGS: A BEGINNING

## INTRODUCTION

Iron-dextran (Fe-dextran) is considered to be a relatively non-toxic form of supplemental iron in baby pigs and is routinely used by many swine producers to prevent the development of anemia. However, recent studies have demonstrated that a single injection of Fe-dextran was associated with cytotoxicity in rats (1-3). The toxic effects appear to be related to the ability of iron to enhance or initiate membrane lipid peroxidation (4,5). Mitochondrial (6) and lysosomal (7) membrane functions in liver have also been shown to be adversely affected by chronic iron overload.

We have shown in a previous study (Chapter 3) that the concentration of iron in hepatocytes, Kupffer cells and endothelial cells was markedly increased following administration of Fe-dextran to neonatal pigs. The purpose of this study was to determine whether accumulation and metabolism of Fe-dextran results in enhanced lipid peroxidation within liver cells of the neonatal pig. The levels of conjugated dienes (stable intermediates resulting from peroxidation of polyunsaturated fatty acids) were measured in tissue (heart and liver), isolated liver cells and erythrocytes from control and Fe-dextran treated piglets. The presence of conjugated dienes was chosen as an indicator of lipid peroxidation because this method has been widely used to evaluate the extent of membrane lipid damage resulting from metabolism of toxic compounds in vivo (8,9).

## MATERIALS AND METHODS

Animals. Male and female crossbred piglets between 1 and 6 d of age were used. Sows and nursing piglets were housed in farrowing crates that had plastic coated wire mesh flooring. Fe-dextran (Tech America Group, Inc., Elwood, KS, 100 mg Fe/ml.) was administered to piglets at a dose of 50 mg Fe/kg body weight at approximately 1 d after birth. One half of the dose was administered into the muscle of each thigh to insure complete retention of the injected material. Control piglets were not injected. Piglets were anaesthetized with sodium pentobarbital prior to sacrifice.

Collection of Blood, Tissue Samples and Liver Cells. Blood was withdrawn into syringes containing lithium heparin (Sigma Chemical Co., St. Louis, MO) via cardiac puncture. Following centrifugation and removal of plasma and the buffy coat, erythrocytes were washed once by centrifugation in 1.15% KCl containing 10 mM Tris-HCl, pH 7.4 (Buffer A). Erythrocytes were lysed in 0.1x Buffer A and membranes were centrifuged at 37,000 x g, for 20 min and 4° C. The membrane preparation was resuspended in buffer A and centrifuged as above. Approximately 10 volumes Buffer A were added to the erythrocyte membrane pellet and the suspension was sonicated for 1 min at 60% maximum power with a Titanium microprobe (Model W-225, Heat Systems-Ultrasonics Inc., Farmingdale, NY).

Whole liver was perfused in situ with 20 mM HEPES (N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid )-buffered saline, pH 7.4 (HEPES-saline), at 4° C to remove blood cells from the sinuses. A small piece of the medial lobe was removed and placed in HEPES-saline at 4° C until homogenized. A section from the apical portion of the heart was also removed and placed in HEPES-saline at 4° C. Tissue samples were homogenized (Polytron, Brinkman Instruments, Westbury, NY) in 9 volumes of Buffer A. Homogenates were subjected to ultrasound as described above.

Hepatocytes, Kupffer cells and endothelial cells were isolated by collagenase perfusion as previously described (10). Hepatocytes were isolated by differential centrifugation. Kupffer cells and endothelial cells were purified by centrifugal elutriation. Isolated cells were washed twice in HEPES-saline prior to preparation of 10% suspensions in Buffer A. Cell suspensions were also subjected to ultrasonic disruption as described above.

Determination of Conjugated Dienes. The presence of conjugated dienes in tissue and cell samples were determined essentially by the method of Buege and Aust (11). Three ml of methanol was added to 1 ml samples of sonicated material in 15 ml screw cap tubes. Tubes were vortexed and incubated on ice for 10 min. Chloroform (6 ml) was added, tubes were vortexed, and reincubated on ice. One ml 0.5x Buffer A was added and tubes inverted several times. Tubes were centrifuged at 1000 x g for 15 min at 4° C in a swinging bucket

rotor. The methanol-aqueous phase was removed and 5 ml of the lower chloroform phase was transferred into a glass vial. Chloroform was evaporated under a stream of  $N_2$  at  $30^{\circ} C$ . A 0.5 ml aliquot of each chloroform extract was also saved for the determination of phosphorus (12). Each lipid extraction was performed in duplicate on samples that were freshly isolated from an equivalent number of control and iron-treated pigs.

The dried lipid fraction was solublized in cyclohexane and absorbance was monitored at various wavelengths between 220 nm and 300 nm. Absorbance at each wavelength was expressed per mg P and differences between samples from iron-treated and control pigs were calculated.

## RESULTS

Lipids were extracted from liver, heart and erythrocytes obtained from piglets 5 d after treatment with Fe-dextran and from age-matched controls. Absorbance spectra of liver samples are shown in Figure 1. Average UV (220-300 nm) absorbance per mg P was greater in tissues from iron-treated piglets than in controls. Difference spectra [average  $A_{220 \text{ to } 300 \text{ nm}}$  (iron-treated) - average  $A_{220 \text{ to } 300 \text{ nm}}$  (control)] for both liver and heart tissue demonstrate the presence of two distinct peaks, one in the region of 230 to 240 nm and a second minor peak near 270 nm (Figure 1, inset). These findings indicate that the administration of supplemental iron was associated with enhanced lipid peroxidation as assessed by the presence of conjugated dienes. For comparative purposes, absorbance values in the major peak region (233 nm) for lipids extracted from liver, heart and erythrocytes of control and iron-treated piglets are provided in Table 1. In contrast to liver and heart, membrane lipids from iron-treated erythrocytes had reduced absorbance in the regions of the spectrum characteristic for conjugated dienes. The difference spectra for erythrocytes between 225 and 300 nm was relatively constant, suggesting the possibility that iron-treatment resulted in a change in fatty acid composition rather the presence of conjugated dienes in the control erythrocytes (data not shown).

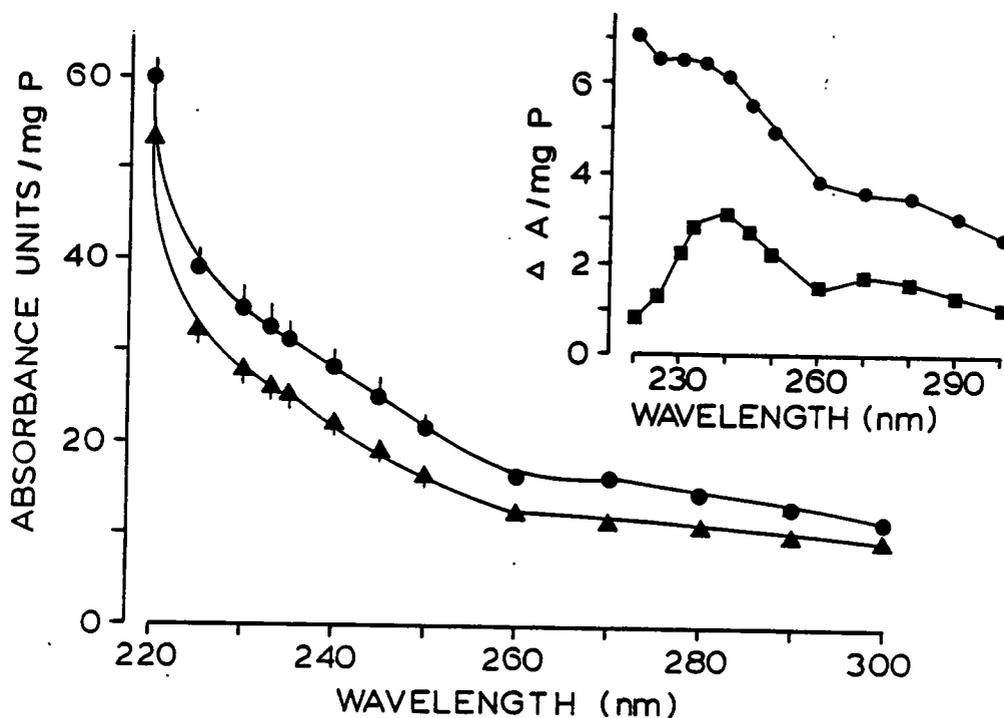


Figure 1. Iron-dextran induced formation of conjugated dienes in liver and heart. UV absorption spectra are shown for lipids extracted from livers of control (▲) and Fe-dextran treated (●) piglets. Each point is the mean absorbance per mg P; bars represent SE. N = 6 pigs per group (2 controls and 2 Fe-treated piglets from each of three litters).

Inset: The difference spectra for liver (●) and heart (■) were calculated by subtracting the mean absorbance of lipids at each wavelength of controls from that of Fe-treated piglets.

TABLE 1. LEVELS OF CONJUGATED DIENES IN LIPIDS EXTRACTED FROM LIVER, HEART AND ERYTHROCYTES FROM CONTROL AND FE-DEXTRAN TREATED PIGLETS

Tissue	(N)	Absorbance 233 nm/mg P <sup>a</sup> Control	Absorbance 233 nm/mg P <sup>a</sup> Fe-dextran treated	A <sub>233 nm</sub> <sup>b</sup>
Liver	(6)	26.0 ± 1.6	32.5 ± 2.4 <sup>c</sup>	6.5
Heart	(6)	23.6 ± 1.3	26.4 ± 4.5	2.8
Erythrocytes	(3)	123.0 ± 12.4	114.0 ± 3.0	-9.0

<sup>a</sup>Total lipids were extracted from liver, heart and erythrocytes of either 6 d old control piglets or from 6 d old piglets that had been injected with Fe-dextran 1 d after birth. Data are presented as mean ± SE.

<sup>b</sup>The arithmetic difference between the mean absorbances/mg P for Fe-dextran treated minus controls.

<sup>c</sup>Significantly different (P < 0.05) from control value when data were compared by Student's t-test

Freshly isolated hepatocytes, Kupffer cells and endothelial cells were similarly analyzed for the presence of conjugated dienes (Figure 2A). At 5 d after treatment with Fe-dextran, endothelial cells contained high levels of conjugated dienes. In contrast, levels of conjugated dienes in Kupffer cells and hepatocytes were similar to levels observed in whole liver preparations. Although, definitive peaks were not observed at 230 to 240 nm and at 270 nm in hepatocytes and Kupffer cells, the increased absorbances in samples from iron treated piglets are probably due to peroxidative damage. Difference spectra for samples prepared 1 d after injection of Fe-dextran also revealed that this treatment was associated with the presence of conjugated dienes in endothelial cells and hepatocytes, but not Kupffer cells (Figure 2B).

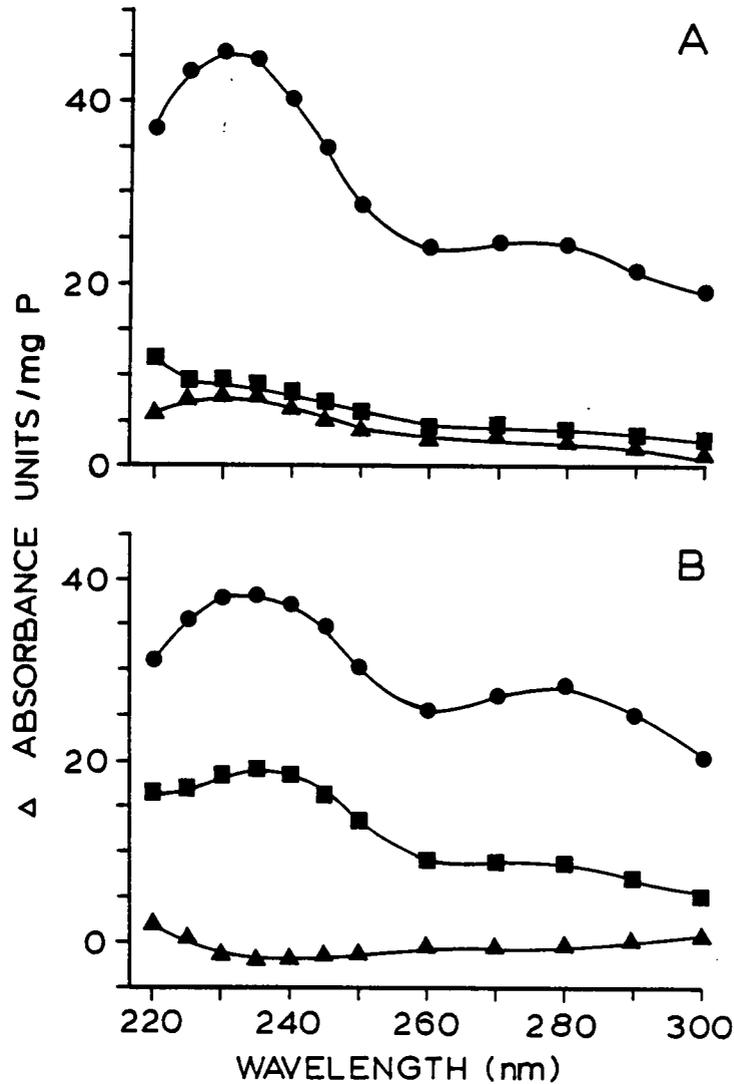


Figure 2. Difference spectra of lipids prepared from liver cells isolated from Fe-dextran treated and control piglets. A. Hepatocytes (■), Kupffer cells (▲) and endothelial cells (●) were isolated from pigs 5 d after treatment with Fe-dextran and from age-matched controls. Lipids were extracted and the difference spectra were obtained from UV absorption curves as described in Figure 1 (N = 2 pigs per group). B. Hepatocytes (■), Kupffer cells (▲) and endothelial cells (●) were isolated from a pig 1 d after treatment with Fe-dextran and from an age matched control pig. Lipids were extracted and the difference spectra were obtained from UV absorption curves as described in Figure 1.

### Discussion

Difference spectra reported here for liver and heart tissue and isolated liver cells demonstrate the presence of elevated levels of conjugated dienes after the administration of recommended levels of Fe-dextran to baby pigs. These spectra are similar to those obtained with pure standard lipid peroxides (5,13) and peroxidized mitochondrial membrane lipids (8,13). Absorbances per mg P at wavelengths between 220 and 300 nm for samples from liver and heart tissue were generally greater in iron-treated pigs than in controls. However, it is important to note that not every iron-treated pig in this study had elevated concentrations of conjugated dienes in liver and heart when directly compared to an age-matched littermate. For example, absorbance at 233 nm for liver tissue was greater in one control animal than in a treated littermate in a particular litter. However, this litter contained only five piglets. Additional research is required to determine whether factors such as litter size and growth rate may alter the pattern and extent of iron-mediated lipid peroxidation.

The relative absorbances at 233 nm of lipid extracts from liver and heart of iron-treated piglets were 25 and 12% higher, respectively, than that in control tissues after 5 d. These levels of conjugated dienes are comparable to data presented by other investigators. In vitro exposure of hepatocytes to a lethal concentration of bromobenzene resulted in a 120% increase in

absorbance at 233 nm over control levels (14). Bacon et al. (15) reported a 67% increase in peak absorbance values in mitochondrial lipids from adult rats fed carbonyl iron for 7 weeks. The concentration of non-heme iron in livers of rats fed carbonyl iron was 50-fold higher than in controls.

Lipid samples from endothelial cells isolated from iron-treated pig liver were markedly altered. We believe that the observed levels of conjugated dienes in endothelial cells indicate selective peroxidative damage of these cells in vivo. In contrast, the concentration of conjugated dienes in Kupffer cells from iron-treated pigs were only slightly higher than that in cells from control pigs, despite the presence of 25- to 50-fold higher levels of iron in the former. However, additional experiments are required to validate this differential cellular response to iron loading. First, antioxidants and free radical scavengers, such as BHT (butylated-hydroxy toluene), catalase, superoxide dismutase and mannitol, should be added to buffers used to isolate, incubate and homogenize isolated sinusoidal cells. These species will protect cells from peroxidation catalyzed by endogenous or exogenous iron during isolation and subsequent manipulations. Second, liver cells that have been freshly isolated or cultured from control piglets will be exposed to iron salts (or Fe-dextran) and EDTA at 37° C to determine relative susceptibility of each cell type to iron-mediated peroxidative damage of lipids in vitro. In a preliminary study, these conditions were

effective in stimulating conjugated diene production in hepatocytes isolated from control pig liver (data not shown).

Recent reports have indicated that vascular endothelial cells are also sensitive to peroxidative damage (16,17). Kupffer cells may be less susceptible to oxygen-mediated damage since they, like most macrophages, utilize oxygen metabolites to kill bacteria and probably possess an adequate defense system that affords protection against oxygen metabolites. Whether these enzyme systems are operative in Kupffer cells of neonatal pig liver is unknown. Although hepatocytes have been shown to be sensitive to peroxidative membrane lipid degeneration (14), the concentration of iron is only marginally and transiently increased after injection with Fe-dextran (Chapter 3). Thus, increased lipid peroxidation may be prevalent only at times soon after treatment (< 5d). The need for further studies involving determination of the activities of enzymes that participate in the metabolism of oxygen and its reactive species in the various populations of liver cells are discussed in Chapter 5.

In conclusion, we have demonstrated that parenteral administration of a recommended dose of Fe-dextran in newborn piglets results in enhanced lipid peroxidation in liver and heart tissue and isolated liver cells. To our knowledge this is the first report of identification of conjugated dienes in hepatic sinusoidal endothelial cells. Whether these findings are indicative of impaired cellular function remains to be determined.

## LITERATURE CITED

1. Valenzuela, A., Fernandez, V., & Videla, L.A. (1983) Hepatic and biliary levels of glutathione and peroxides following iron overload in the rat: Effect of simultaneous ethanol administration. *Toxicol. Appl. Pharmacol.* 70, 87-95.
2. Dougherty, J.J., Croft, W.A., & Hoekstra, W.G. (1981) Effects of ferrous chloride and iron-dextran on lipid peroxidation in vivo in vitamin E and selenium adequate and deficient rats. *J. Nutr.* 111, 1784-1796.
3. Gavino, V.C., Dillard, C.J. & Tappel, A.L. (1984) Release of ethane and pentane from rat tissue slices: Effect of vitamin E, halogenated hydrocarbons, and iron overload. *Arch. Biochem. Biophys.* 233, 741-747.
4. Jacobs, A. (1980) The pathology of iron overload. In: *Iron in Biochemistry and Medicine II.* (Jacobs, A. & Worwood, M., eds.) pp. 427-459. Academic Press, New York.
5. Bus, J.S. & Gibson, J.E. (1979). Lipid peroxidation and its role in toxicity. *Rev. Biochem. Toxicol.* 1, 125-149.
6. Masini, A. Trenti, T., Ventura, E. Ceccarelli- Stanzani, D. & Muscatello, U. (1984) Functional efficiency of mitochondrial membrane of rats with chronic iron overload. *Biochem. Biophys. Res. Commun.* 124, 426-469.
7. Seymour, C.A. & Peters, T.J. (1978) Organelle pathology in primary and secondary haemochromatosis with special reference to lysosomal changes. *Br.J. Haematol.* 40, 239-253.
8. Bacon, B.R., Tavill, A.S., Brittenham, G.M., Park, C.H. & Recknagel, R.O. (1983) Hepatic lipid peroxidation in vivo in rats with chronic iron overload. *J.Clin. Invest.* 71, 429-439.
9. Casini, A.F., Pompella, A. & Comporti, M. (1985) Liver glutathione depletion induced by bromobenzene, iodobenzene, and diethylmaleate poisoning and its relation to lipid peroxidation. *Am. J. Pathol.* 118, 225-237.
10. Caperna, T.J., Failla, M.L., Kornegay, E.T., Richards, M.P. & Steele, N.C. (1985) Isolation and culture of parenchymal and nonparenchymal cells from neonatal swine liver. *J. Animal Sci.* 61, 1576-1586.

11. Buege, J.A. & Aust, S.D. (1978) Microsomal lipid peroxidation. *Methods Enzymol.* 52, 302-310.
12. Bartlett, G.R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem.* 234, 466-468.
13. Recknagel, R.O. & Glende, E.A. (1984) Spectrophotometric detection of conjugated dienes. *Methods Enzymol.* 105, 331- 337.
14. Casini, A., Giorli, M., Hyland, R.J., Serroni, A. Gilfor, D. & Farber, J.L. (1982) Mechanisms of cell injury in the killing of cultured hepatocytes by bromobenzene. *J. Biol. Chem.* 257, 6721-6728.
15. Bacon, B.R., Chanho, H.P., Brittenham, G.M., O'Neil, R. & Tavill, A.S. (1985) Hepatic mitochondrial oxidative metabolism in rats with chronic dietary iron overload. *Hepatology* 5, 789-797.
16. Andreoli, S.P., Bahner, R.L. & Bergstein, J.M. (1985) In vitro detection of endothelial cell damage using 2-deoxy-D-<sup>3</sup>H-glucose: Comparison with chromium 51, <sup>3</sup>H-leucine, <sup>3</sup>H-adenine, and lactate dehydrogenase. *J. Lab. Clin. Med.* 106, 253-261.
17. Harlan, J.M., Levine, J.D., Callahan, K.S. & Harker, L.A. (1984) Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. *J. Clin. Invest.* 73, 706-713.

**CHAPTER 5 .**

**EPILOGUE**

## EPILOGUE

The main focus of research in our laboratory is the elucidation of regulatory mechanisms of trace mineral metabolism in animals. My primary interest during the past three years has been the hepatic metabolism of supplemental iron in baby pigs. Since I wanted to investigate uptake, distribution and metabolism of supplemental iron at the cellular level, the first part of my research effort involved development of methods to isolate the predominant cell types from the neonatal pig liver.

Procedures for the isolation and monolayer culture of hepatocytes and sinusoidal (Kupffer and endothelial) cells from livers of neonatal pigs (1 to 15-d-of age) are described in Chapter 2. Cell suspensions were obtained by a modification of the two step collagenase perfusion technique. Hepatocytes were collected by low-speed centrifugation and sinusoidal cell populations were purified by centrifugal elutriation.

Hepatocytes were readily maintained in arginine-free medium containing insulin and fortified with either fetal calf serum or bovine serum albumin and oleate for periods as long as 6 d. The ability of cultured hepatocytes to incorporate  $^3\text{H}$ -leucine and  $^3\text{H}$ -thymidine into protein and DNA, respectively, demonstrated that cells were metabolically active for at least 3 d in culture.  $^3\text{H}$ -leucine incorporation into total cell protein was constant regardless of animal age at the time of cell isolation, while

incorporation of  $^3\text{H}$ -thymidine was influenced by animal age. Incorporation of both precursors was dependent upon duration of culture period and type of medium (serum-free vs serum-containing) in which the cells were maintained. Morphological observation and analysis of the DNA and protein levels of hepatocyte monolayers suggest that cells did not replicate during the three day incubation period.

Endothelial cells readily attached to collagen-coated plates in the presence of fetal calf serum (20%) and Kupffer cells adhered to untreated polystyrene plates under the same culture conditions. Kupffer cells in monolayer cultures demonstrated the ability to phagocytize 5  $\mu\text{m}$  latex particles, whereas endothelial cells were not phagocytic. Thus, porcine sinusoidal liver cells demonstrated morphologies and phagocytic properties similar to sinusoidal cells similarly isolated from other species.

After developing methods to isolate viable liver cells, I next turned my attention to characterizing cellular accumulation of iron following treatment of piglets with supplemental iron (Chapter 3). Hepatic distribution and intracellular metabolism of Fe-dextran, a commonly used colloidal iron-carbohydrate complex, was investigated. Newborn piglets were injected intramuscularly with Fe-dextran (50mg Fe/kg body weight) and liver cells were isolated from iron-treated and age-matched control pigs 1, 5 and 10 d later. Tissue sections

were prepared 1 and 10 d after iron treatment. Cytochemical staining of tissue sections for  $\text{Fe}^{3+}$  indicated that substantial concentrations of iron were within sinusoidal regions at 1 d after treatment, but were undetectable by 10 d. Direct analysis of iron in isolated liver cells by atomic absorption spectroscopy indicated that by 1 d after treatment the concentration ( $\mu\text{g}/\text{mg}$  cell protein) of iron was 62-, 54- and 5-fold higher in Kupffer cells, endothelial cells and hepatocytes, respectively, than in cells isolated from the livers of age-matched control pigs. By 10 d, the concentration of iron in hepatocytes was similar in iron-treated and control pig liver. Although the concentrations of iron in sinusoidal cells from iron-treated pigs were markedly higher than those from control pigs after 10 d, there was also substantial mobilization of iron from these cells to extrahepatic sites. The quantities of ferritin-protein and ferritin-iron were measured by immunoelectrophoresis and ion exchange chromatography, respectively. All three types of liver cells accumulated ferritin in response to treatment with Fe-dextran. For the time periods investigated, maximal concentrations of ferritin-protein were present in hepatocytes and Kupffer cells 1 d after treatment and in endothelial cells 5 d after treatment. The concentration of ferritin-iron attained maximal levels in the three types of liver cells 1 d after treatment. Thereafter, the quantity of iron associated with ferritin decreased in concert with the decrease in total cellular iron. Higher concentrations of

ferritin-protein and ferritin-iron were present in Kupffer cells and endothelial cells than in hepatocytes at all times after iron treatment. At 1 d after treatment, 48% of the total iron within hepatocytes was associated with ferritin and the level declined to 38% and 10% on 5 and 10 d, respectively. In contrast, ferritin-iron represented 7 to 11% of the total quantity of iron in Kupffer and endothelial cells throughout the study.

In Figure 1, I have attempted to integrate the findings from Chapter 3 and preliminary results of ultrastructural studies with information from the literature to develop a schematic view of the metabolism of supplemental iron in the neonatal porcine liver. At this time it is apparent that the general pathways of iron metabolism within the various cell types are similar, e.g. all three cell types contained elevated levels of ferritin protein after iron loading. However, marked differences were evident in levels of ferritin protein, ferritin-iron and particulate (lysosomal)-iron in parenchymal and sinusoidal cells. Experiments designed to elucidate the characteristics and regulation of subcellular compartmentalization and transfer of iron to the extracellular milieu are discussed below.

The possibility that accumulation of Fe-dextran enhanced peroxidation of membrane lipids of neonatal porcine liver was evaluated (Chapter 4). Tissues and cells were collected 5 d after piglets were injected with Fe-dextran. Sonicates were prepared from

fresh samples of tissues (heart and liver), isolated liver cells (hepatocytes, endothelial cells and Kupffer cells) and erythrocytes. Total lipids were extracted to measure levels of conjugated dienes (intermediate metabolites of peroxidized polyunsaturated fatty acids). Difference spectra revealed the presence of low levels of conjugated dienes in whole liver and heart tissue from iron-treated pigs. Iron-loaded endothelial cells contained greater levels of conjugated dienes than hepatocytes or Kupffer cells. In contrast, treatment of piglets with Fe-dextran did not enhance lipid peroxidation in erythrocytes. Lipid peroxidation was also evaluated in liver cells isolated 1 d after iron treatment. High levels of conjugated dienes were present in hepatocytes and endothelial cells, but not in Kupffer cells, isolated from iron treated piglets. Whether the observed alterations in lipid chemistry of iron-treated pigs are indeed associated with altered cellular functions is a matter that requires further investigation.

My research has attempted to address some of the pertinent questions regarding hepatic metabolism of supplemental iron in baby pigs. In doing so, the need for further research has become apparent. Moreover, the usefulness of the iron-treated baby pig as a model for investigating several general problems of iron metabolism has become evident. Below, I outline several experiments concerning cellular iron metabolism and toxicity that merit consideration in the near future. Additionally, animal studies to determine whether

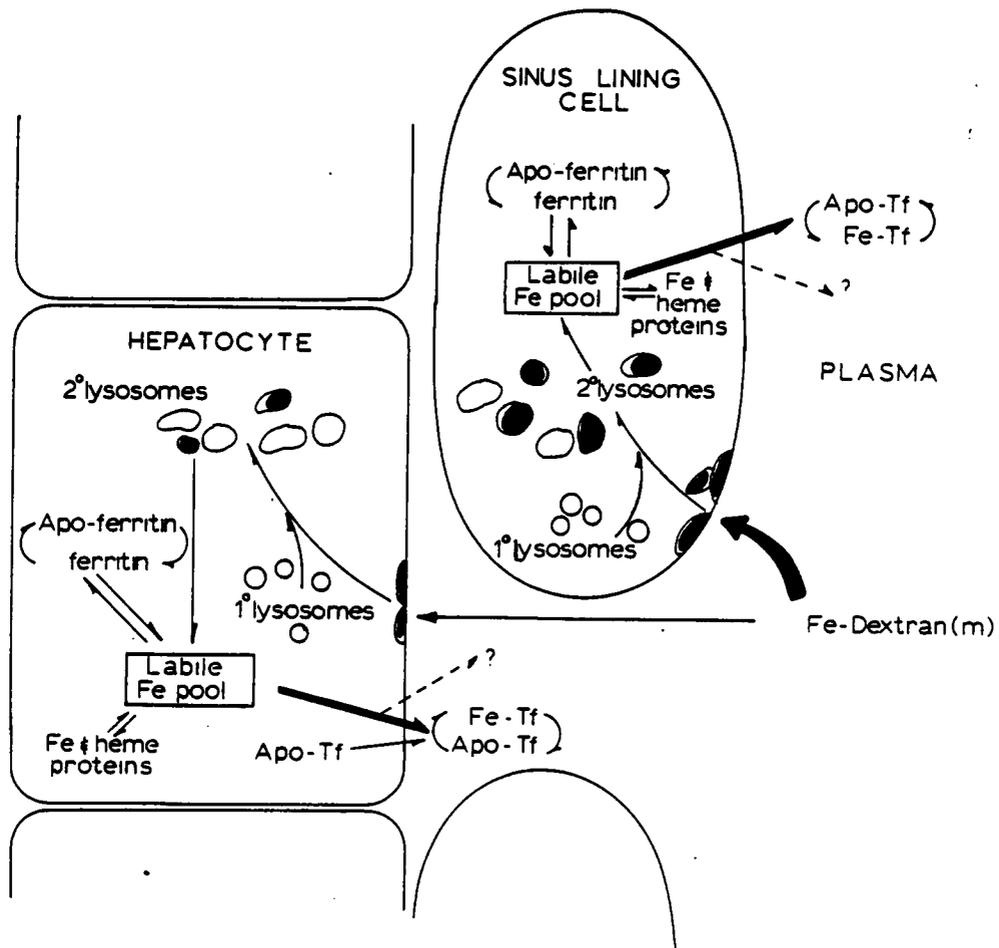


Figure 1. Schematic summary of accumulation and metabolism of Fe-dextran by liver cells in the neonatal pig.

Tf; transferrin: Fe-dextran (m); circulating form of Fe-dextran; Metabolized at the site of injection.

observed cellular peroxidative damage has any impact on health of newborn pigs are outlined.

1. Cellular metabolism of iron. Our studies on ferritin content within hepatocytes and sinusoidal cells merely suggest that ferritin is synthesized within these cells. To demonstrate that accumulation represents actual de novo synthesis of ferritin, mRNA will be isolated from each cell type, translated in a cell free system and ferritin-protein identified by immunoprecipitation. Assuming that iron accumulation is correlated with increased synthesis of ferritin-protein in a cell-free translation system, additional techniques will be employed to study the regulation of ferritin synthesis in each of the three liver cell types. It is widely accepted that hepatic synthesis of ferritin subunits is subject to translational control. It is unknown whether this phenomenon actually reflects the situation in the various populations of liver cells or if ferritin biosynthesis in neonates is subject to different regulatory mechanisms. A cDNA probe will be utilized to directly determine levels of mRNA in different liver cell types from control and iron-treated pigs.

I found my results concerning the relationship between the concentration of total cellular iron and the amount of ferritin-iron in sinusoidal cells surprising. Since sinusoidal cells contained higher levels of iron than hepatocytes why was there not a proportionately greater amount of ferritin-iron in Kupffer cells and

endothelial cells as compared to hepatocytes? One possible explanation is that the intralysosomal degradation of Fe-dextran metabolites and subsequent release into cytoplasm are rate limiting steps in the formation of mature ferritin polymers. To test this possibility I would compare levels of ferritin in monolayers of sinusoidal cells isolated from iron-treated pigs and incubated with lysosomotropic agents in vitro. These agents alter hydrolytic activity by raising intralysosomal pH, and would probably decrease the rate of release of iron from dextran complexes in lysosomes. Additionally, ferritin will be quantitated in sinusoidal cells isolated from piglets following administration of different forms of supplemental iron viz., Jectofer, Gleptoferrin, and Glussoferrin. These low molecular weight iron carbohydrate complexes may be degraded more rapidly than Fe-dextran in lysosomes. An attempt will then be made to correlate ferritin levels at various times after treatment with the rate of mobilization of iron from lysosomes.

Since levels of transferrin are particularly low in the plasma of neonatal pigs, mechanism(s) responsible for the transfer of iron to and from hepatocytes, Kupffer cells and endothelial cells are of particular interest. An initial direct approach would be to determine which cells (if any) possess transferrin receptors. Purified porcine transferrin would be labeled with  $^{55}\text{Fe}$  and  $^{125}\text{I}$  to perform binding studies. Alternatively, the receptor could be identified microscopically by covalently linking transferrin to inert

beads or sheep erythrocytes and observed directly as rosettes. Assuming the transferrin receptor plays a role in the uptake of iron by liver cells, the effect of cellular iron status on the number and affinity of these receptors will be evaluated.

Another area requiring investigation concerns which, if any, iron-binding proteins are secreted by Kupffer cells and endothelial cells. At present, lactoferrin and transferrin are two primary candidates for this function. Numerous studies demonstrating that hepatocytes synthesize and secrete transferrin in vitro are available. While it is generally believed that reticuloendothelial cells secrete lactoferrin, some investigators have suggested that they synthesize and secrete transferrin. Although Kupffer cells represent the largest single population of reticuloendothelial cells in the body, their relationship to other populations of macrophages (peritoneal exudate cells, pulmonary macrophages, circulating monocytes, etc.) remains obscure, particularly with regard to iron metabolism. Kupffer cells and endothelial cells will be isolated and maintained in serum-free media. Specific antisera to lactoferrin and transferrin will be utilized to determine whether these proteins are synthesized and secreted by sinusoidal liver cells. In addition, the effect of iron loading on synthesis and secretion of iron binding proteins by sinusoidal cells, as well as hepatocytes, will also be investigated. The question of whether enhanced secretion or mobilization of iron from cells requires increased synthesis or

secretion of specific iron-binding proteins will be addressed.

Direct examination of the mechanism(s) by which iron is mobilized out of liver cells is also needed. To do so I would pre-load cells with a radio-labeled iron compound and examine the extent and rate of iron efflux in the presence of apo-, mono- and diferric transferrin in vitro. Evaluation of the influence of other substances, viz., lactoferrin, albumin, amino acids, chelating agents and interleukin-1 (IL-1) on the characteristics of iron efflux are also of interest. It is believed that IL-1 is involved in the development of hypoferremia during infection. There is also speculation in the literature that IL-1 may also be involved in the regulation of ferritin synthesis in reticuloendothelial cells. The characteristics of iron efflux from liver cells that have been loaded with various forms of iron complexes may provide useful information for evaluating the efficiency of novel compounds as iron supplements.

2. Cellular toxicity. My preliminary results suggest that membrane alterations are present in cells isolated from pig livers 1 and 5 d after treatment with Fe-dextran. Additional, studies assessing levels of conjugated dienes in iron-treated pigs are needed. I will then investigate whether peroxidative damage adversely affects cellular function. For example, are the rates of plasma protein synthesis and secretion by hepatocytes or uptake and degradation of colloidal albumin by sinusoidal cells compromised by the presence of elevated levels of iron.

Further studies are required to evaluate the apparently greater susceptibility of endothelial cells to peroxidative damage than hepatocytes and Kupffer cells. If such is the case, then it would be important to compare levels of enzymes that participate in the metabolism of oxygen species, viz., superoxide dismutase, catalase and nonspecific peroxidases, as well as glutathione, glutathione peroxidase and xanthine oxidase, in hepatocytes, Kupffer cells and endothelial cells. If xanthine oxidase activity is localized within endothelial cells, might the observed levels of conjugated dienes in the liver be correlated with the presence of this enzymatic activity?

3. Animal studies. The emphasis in sections 1 and 2 concerned the potential usefulness of liver cells from iron-treated piglets as models for elucidating unresolved problems in iron metabolism at the cellular and molecular level. Below, I have outlined studies that may be important in the development of alternative strategies to prevent the development of anemia in neonatal swine. I found that sinusoidal cells accumulated lower quantities of the low molecular weight iron complex, Jectofer (iron poly-sorbitol-citrate) than Fe-dextran (Figure 2). The presence of lower concentrations of iron in endothelial cells following the administration of Jectofer may diminish the extent of peroxidative damage in these cells. In the past, efficacy of novel forms of supplemental iron has been primarily evaluated by following hematological parameters and the

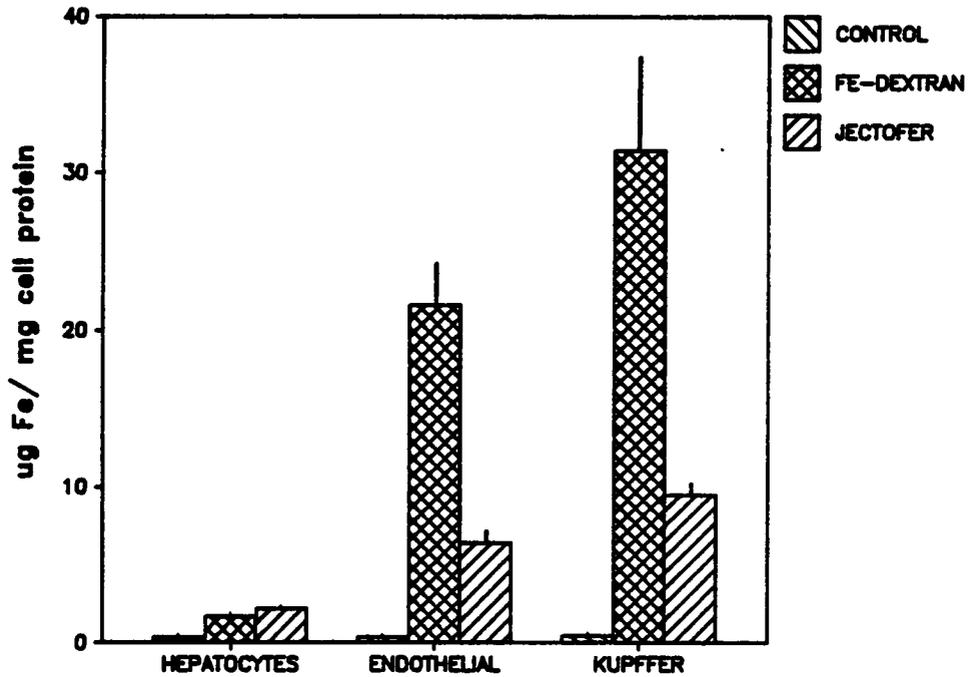


Figure 2. Effect of treatment with Fe-dextran and Jectofer on the concentration of iron in porcine hepatocytes and sinusoidal liver cells. At 1 d-of-age piglets received either Fe-dextran or Jectofer at a dose of 50 mg Fe/kg body weight. Hepatocytes, Kupffer cells and endothelial cells were isolated from livers 24 h after injection and the concentration of iron in each cell type determined. Liver cells were also isolated from control (non-injected) piglets at 2 d-of-age. Values represent means  $\pm$  SE; n = 4 for each group.

activity of a variety of iron dependent enzymes. My data suggest that an evaluation of cellular toxicity also merits consideration when testing new iron chelates.

The time course of formation of lipid peroxides warrants further investigation to determine whether the initial insult of iron influx or the sustained metabolism of iron is responsible for increased conjugated diene content. Another matter of interest concerns whether iron-mediated formation of conjugated dienes would be decreased by postponing administration of supplemental iron until 1 week after birth. Additionally, trials in which antioxidants, such as vitamin E and Se, are given to piglets prior to iron administration as an attempt to offset peroxidative damage, will be conducted. Finally, it remains to be determined whether lipid peroxidation caused by iron treatment is enhanced by general stresses, such as marked fluctuations in environmental temperature, infections and litter size.

It is apparent that many questions have been raised as a result of the data presented in this thesis. Obviously, I cannot provide answers to the economically important matter of whether to continue treating piglets with supplemental iron. However, I have provided novel information about the metabolism of supplemental iron by liver cells in the neonatal pig and have defined a need for future

investigation concerning toxicological consequences of current management practices. I believe that I have also established the usefulness of the neonatal pig as a model for investigating many of the basic questions remaining regarding the mechanisms of cellular iron metabolism. Finally, combined use of the tools I have developed and available molecular probes will facilitate my addressing several of the basic and applied problems outlined above in the near future.

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