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Insulin and Altered Reticuloendothelial System Function

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INSULIN AND ALTERED RETICULOENDOTHELIAL SYSTEM FUNCTION

by

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INTRODUCTION

The entire pancreatic output of insulin is secreted into the portal venous blood which then passes through the liver before reaching the systemic circulation. During a single transhepatic passage, 40-50% of the insulin is removed (47, 133, 202, 236, 237). Regulation of both hepatic and peripheral glucose metabolism is dependent on insulin. Therefore, hepatic extraction and degradation of insulin may be important determinants in the control of glucose homeostasis.

Past investigations of the liver's role in insulin regulation treated it as a homogeneous organ composed of only parenchymal cells (47, 57, 63, 64, 88, 116, 133, 155, 173, 194, 202, 209, 215, 219, 232, 233, 236, 237, 252, 318, 319, 322, 326, 327, 336, 337, 338, 339, 340, 361). However, the liver contains several types of cells with the following approximate percent distribution: 60.6% parenchymal cells, 33.4% RES or reticuloendothelial system cells, 2.0% bile duct cells, 1.8% endothelial cells and 2.0-5.0% connective tissue or fat (vitamin A)-storing cells (31, 122, 317). Thus, 40% of the cells are non-parenchymal and of these the Kupffer cells of the RES are in the majority, making them a numerically significant cell population.

The Kupffer cells are tissue or fixed macrophages which form part of the hepatic sinusoidal lining. Because of their location, the Kupffer cells are in intimate contact with the pancreatic insulin percolating through the liver. Depression of RES endocytic function by carbon blockade (36), lead (143, 330) or endotoxin (20, 23, 179, 192,

323) has resulted in changes in glucose regulation (52, 55, 56, 79, 84, 87, 140, 142, 144, 145, 146, 188, 225, 263, 272, 290, 291, 292, 303, 363, 364). Besides their highly active endocytic processes, the Kupffer cells, like all macrophages, also carry out numerous metabolic, degradative and secretory functions (7, 8, 73, 113, 127, 210, 239, 240, 264, 265, 268, 276, 310, 311). Therefore, the Kupffer cells may play a role in hepatic insulin regulation.

The purpose of this study was to investigate the relationship between altered RES endocytic function, especially of the hepatic Kupffer cells and insulin regulation. In order to alter RES function, two models of RES depression, acute lead treatment (143, 330) and colloidal carbon blockade (36), were utilized in male rats. Functional evidence regarding the insulin status of the animals was studied by determination of the susceptibility to insulin-induced hypoglycemic convulsive death and iv glucose tolerance testing. Finally, both basal and glucose-stimulated serum insulin and glucose levels were measured.

LITERATURE REVIEW

The Reticuloendothelial System

Several attempts have been made to classify phagocytic mononuclear cells and to define the cell system which they form. During the 1880's, Metchnikoff first demonstrated the role of certain phagocytic cells in host cellular immunity (24, 158, 276). In his studies, he emphasized that ingestion and destruction of foreign bacteria by the "macrophage system" were essential metazoan disease defense mechanisms (24, 276). Later Ribbert and Nagoa and other workers found that intravenously injected colloidal dyes or India ink were intensely localized in phagocytic cells lining blood sinuses in the liver, spleen and bone marrow (7, 196, 244, 276). Subsequently, in 1924, Aschoff introduced the term "reticuloendothelial system" (RES) to describe a body of mesenchymal-derived cells forming a diffuse system of wandering and fixed macrophages having in common the physiological function of rapid ingestion and storage of foreign colloidal or particulate matter (7, 276). Aschoff included the reticulum and endothelial cells of the lymph nodes, spleen and bone marrow, the endothelial cells lining the sinuses in the adrenal cortex and anterior pituitary, blood histiocytes or monocytes and connective tissue macrophages (7, 268). Volterra and Thomas proposed the name "reticulohistiocyte system", which included all cells that could acquire the "histiocytic state" of enhanced metabolic activity, cell proliferation and growth, as well as the powers of motility and phagocytosis (158). Their system included bone, muscle, epithelial and Schwann

cells in addition to those which Aschoff had placed in the RES (158).

Unfortunately, true endothelial and reticulum cells are not phagocytic rendering the terms RES and reticulohistiocyte system misnomers (59). In 1969, a new classification of the monocyte-macrophage system was proposed, the "mononuclear phagocyte system" or MPS (59, 158, 159, 210, 229). Cells were included in the MPS on the basis of similarities of morphology, function, origin and kinetics of phagocytosis (158). Only "professional" phagocytes having immunoglobulin and complement receptors at the cell surface, the ability to attach to glass and avid phagocytic and pinocytic processes were included (158). The MPS currently is comprised of bone marrow precursor cells, blood monocytes, microglial cells of the nervous system and connective tissue, liver, lung, lymph node, bone marrow and pleural and peritoneal macrophages (159). In this thesis the older name "reticuloendothelial system" is used since it is still the most popular, with the understanding that only the blood monocytes and macrophages are being discussed.

Monocyte-Macrophage Morphology

Mononuclear phagocytes are descendants of hemopoietic stem cells (159, 229), not of mesenchymal elements as are the fibroblasts, reticulum and endothelial cells (159). Within the bone marrow, the committed stem cell gives rise to the monoblast, which undergoes mitosis and differentiates into the promonocyte (158, 159, 210, 229). Promonocytes divide to form monocytes which are released into the blood for transportation to the tissues where further maturation and transformation takes place resulting in the formation of the tissue macrophages or histio-

cytes (158, 210, 301, 321, 345).

Monocytes leave the circulation and are transformed into macrophages via various morphological and functional changes. The circulating monocyte is a large 10-15 μm diameter cell with an indented or reniform nucleus having prominent nucleoli, numerous mitochondria and lysosomes and a well-developed Golgi apparatus and rough endoplasmic reticulum (158, 210). Monocytes can undergo mitosis (320). Like tissue macrophages, monocytes phagocytose, adhere to glass and show cytoplasmic spreading (210). Locomotion is promoted by undulating cytoplasmic veils (210). Immunoglobulin G (IgG) receptors, instrumental in phagocytosis, have been found on the cell membrane (320). The half-time of disappearance of monocytes from the circulation has been calculated to be 22 hours (268).

After arrival at the tissues, the monocytes become avidly phagocytic macrophages having increased numbers of cytoplasmic granules, vesicles and lysosomes (268, 320). These cells are larger with reported diameters of 10-40 μm with either an oval or reniform nucleus and increased endoplasmic reticulum (16, 158, 268). Mature tissue macrophages infrequently may be stimulated to undergo mitosis in situ (212, 213, 268, 358). In chronic inflammatory states, macrophages have been demonstrated to have increased lysosomal enzymes and plasma membrane with hypertrophy of the Golgi apparatus, all characteristics of "stimulation" or "activation" (2, 210). Studies have shown that macrophages have a dynamic membrane system which, as a result of repeated endocytosis, requires replenishment of plasma membrane and synthesis of new primary lysosomes (120, 354, 355). Recently Emeis has reported that liver Kupffer cells

possess a two-layered cell coat composed of an outer filamentous protein layer and an inner layer of glycoproteins or glycolipids which may be important in endocytosis (121, 123). Receptors for both the Fc portion of IgG and the third component of complement (C3) also have been found on the macrophage cell membrane (210, 246, 310). The cytoplasm contains a cytoskeletal system of microtubules and also microfilaments, reported to be composed of the contractile proteins actin and myosin, which may be instrumental for phagocytosis (73, 188, 246, 312, 313). The life span of tissue macrophages is long; it has been estimated to be from 60 days to several years (210).

Endocytosis

As discussed above, macrophages have been classified as a cell system because of their fundamental ability to engulf material from their external environment (7, 24, 59, 158, 159, 210, 229, 276). Mononuclear phagocytes are the only cells which exhibit all three types of endocytosis: phagocytosis, macropinocytosis and micropinocytosis (159). Endocytosis involves an invagination of the plasma membrane with subsequent fusion to form vacuoles (174, 242, 266) which may fuse with each other prior to transportation to the Golgi or peri-nuclear region where they fuse with either primary or pre-existing secondary lysosomes and are themselves converted into secondary lysosomes (72, 73, 351). These fusions are selective, involving only membrane derived from the plasmalemma or Golgi apparatus (73). The molecular mechanisms of membrane fusion have not been clarified but defective fusion of vacuoles and lysosomes has been shown to occur following ingestion of virulent

tubercle bacilli, viable toxoplasma, symbiotic algae (73), polymeric dextran sulfate (113) and concanavalin A (73, 110, 111), a lectin (293). Failure of degranulation or fusion of primary lysosomes with phagosomes also has been reported in human monocytes with the hereditary Chediak-Higashi syndrome (310). The three types of endocytosis, phagocytosis, macropinocytosis and micropinocytosis (2, 210, 240, 268, 275, 313) have been shown to involve different sized particles, membrane events and to have different metabolic requirements (2, 70, 113, 210, 240, 268, 275, 310, 311, 313).

It has been suggested that the term "phagocytosis" should be restricted to the uptake of particles $\geq$ 1 μm in diameter (2, 210, 313). Stossel has divided phagocytosis into 7 events: 1) recognition of the particle surface by the phagocyte; 2) the phagocyte receives the message of recognition; 3) the phagocyte relays this message to its cytoplasm; 4) effector mechanisms are aroused which cause the phagocyte to adhere to the particle; 5) pseudopods are induced to assemble; 6) and move around the particle, and; 7) the tips of pseudopods fuse at the distal side of the particle (311). How the macrophage recognizes a particle to be phagocytosed is unclear. Several theories have proposed that particles are ingested on the basis of molecular properties such as surface hydrophobicity, net surface charge or perhaps surface tension forces (8, 73, 311). In 1903, Wright and Douglas demonstrated that normal human serum contained a substance that stimulated bacterial phagocytosis, which they called an "opsonin" (276). Investigators have also found opsonic activity in other vertebrate plasma and sera (150,

153, 248, 264, 309). Some have considered the opsonins to be specific IgG or a fragment of C3 (242, 310, 311) while others have believed that opsonins may be "natural antibodies" possibly α-globulins (276, 277, 279, 280, 309). Opsonic activity has been reported to be increased by heparin (147, 148, 149, 150, 264, 276). The binding of the particle, which may be opsonized, has been shown to occur at specific receptors, including those for the Fc portion of IgG and C3 (210, 246, 310, 311). Chemical or mechanical events within the membrane have been suggested to transmit the receptor binding "message" to the effector mechanism which causes the phagocyte to adhere to the particle to be ingested (311). Stossel has proposed that the cell surface contact causes the assembly and activation of cytoplasmic actin and myosin, present in microfilaments, which may then result in movement of the pseudopods around the particle or ingestion (310, 311, 312, 313). The energy needed for phagocytosis is provided by the anaerobic glycolytic pathway with the exception of alveolar and hepatic macrophages which also depend on oxidative metabolism (2, 8, 208, 210, 264, 265).

"Pinocytosis" refers to the cellular uptake of particles < 1 μm in diameter with their fluid medium (210, 268, 313). This process has been further divided into "macropinocytosis" which produces vacuoles 0.3-2.0 μm in diameter that are visible by phase contrast microscopy and "micropinocytosis" which produces 70-100 nm diameter "microvesicles" demonstrable only by electron microscopy (2, 210, 240, 268). Pinocytic vesicles have been seen to flow centripetally in the cell, apparently guided by microtubules (73, 231). Pinocytosis is still an obscure process and involvement of specific cell membrane receptors has been

proposed but not proven (240, 275). Although a single particle can trigger phagocytosis, it is now thought that a number of particles must accumulate on a given area of cell surface for pinocytosis to occur (268). Cohn and Parks have demonstrated that adenosine and ATP as well as certain anionic proteins, carbohydrates, amino acids and nucleic acids increase macropinocytosis by mouse macrophages (71, 76, 77, 268). Serum has also been shown to induce pinosome formation in mouse macrophages (70, 78, 113, 231), particularly newborn calf serum which contains an antibody to mouse cell membranes (2, 71, 72, 78, 113). Increased pinocytic activity has been demonstrated to result in increased lysosome formation (113). Inhibitors of protein synthesis, glycolysis and oxidative phosphorylation have been shown to block macropinocytosis (2, 70, 71, 72, 113). Micropinocytosis, on the other hand, has been reported to be more resistant to metabolic inhibitors and appears to be inhibited by low temperatures when there is less membrane fluidity (2, 240). Cytochalasin B, a fungal metabolite which interferes with microfilament function, inhibits phagocytosis (312) and macropinocytosis (2) but is ineffective in blocking micropinocytosis (2, 240). It should be noted that in the early RES literature, all endocytic acts were commonly labeled phagocytosis or phagocytic clearance despite the fact that the particles were often < 1 μm in diameter, as in the case of colloidal gold, 30 nm (268, 309) or colloidal carbon 25 nm (36, 180, 309, 359). Lewis had discovered pinocytosis in mammalian cells in the 1930's but this term was considered to apply only to the cellular uptake of fluid with dissolved substances (113).

RES endocytic activity has been evaluated *in vivo* by the intravascular clearance method (20, 21, 36, 179, 276, 309). Various test particles have been utilized including colloidal carbon, colloidal gold, saccharated iron oxide, denatured serum albumin, lipid emulsions, latex beads, foreign red blood cells, silicon dioxide and chromium phosphate, as well as isotopically labeled colloids (20, 21, 36, 37, 179, 276, 309). Benacerraf *et al.* have described the characteristics of a good test particle preparation as follows: the particles should 1) be phagocytosed by RES cells in contact with the blood, 2) not cross capillary walls, 3) be homogeneous in size, 4) stable in the blood, 5) be nontoxic for the RE cells or the organism, 6) be accurately measurable in blood or tissues, and 7) be cleared only by the cells of the RES (21, 179, 276). Another criterion, added by Saba, is that the functional status of the RE cells be the rate-limiting factor, i.e., the availability of plasma opsonic factors should not be compromised by an excessive dose of test material (21, 276, 309). However, Stiffel *et al.* have emphasized that phagocytosis of larger organized particles such as bacteria or red cells is dependent on serum opsonin concentration in contrast to endocytosis of simple colloids (309). The test dose should also be large enough to not be rate-limited by hepatic blood flow (276). Kinetics studies have demonstrated that the test particle clearance is exponential in normal circumstances, allowing the calculation of a half-time of disappearance ($t_{1/2}$) by a semilog plot of concentration versus time and the equation $k = 0.639/t_{1/2}$ or by the equation $K = 0.301/t_{1/2}$ where K is the "phagocytic index" and $K = k \times 0.4343$ (20, 21, 36, 179, 276, 309). It has been demonstrated that 70-96% of a test dose is localized

in the hepatic Kupffer cells with most of the remainder found in the spleen (36, 179, 276, 309). Similar types of phagocytosis studies measuring the extraction of colloidal carbon, colloidal radiogold, radioiodinated albumin aggregates, RE test lipid emulsion and opsonized sheep red cells have also been done in vitro in both the isolated perfused rat liver and isolated rat Kupffer cells (8, 148, 150, 153, 239, 264, 279).

The Concept of RES Blockade

The classical method for evaluation of the physiological role of an organ or system is its surgical removal. However, the diffuse nature of the RES made this impossible, so "blockade" techniques were devised in which depression of RES phagocytic function was caused by injection of a large dose of foreign particulate matter such as colloidal carbon, (12, 20, 21, 22, 36, 38, 103, 179, 205, 213, 243, 259, 309), lead acetate (143, 288) and many other types of colloids, particles and emulsions (12, 20, 21, 38, 90, 179, 212, 243, 278, 309, 343, 359). In vitro blockade studies have also been done using foreign blood cells in rat and mouse Kupffer cells (239, 301). After the colloidal or particulate overload, a state of decreased endocytic activity has been observed for a variable period of time which is the blockade state (12, 20, 21, 22, 36, 38, 103, 143, 179, 205, 213, 248, 259, 276, 278, 301, 309, 359). Blockade was then followed by a recovery or even a slight increase in phagocytic function (22, 38, 90, 103, 205, 212, 213, 259, 301, 309, 343).

Several explanations for the phenomena of blockade and recovery have been put forth. Classically, blockade was thought to be due to a "physical saturation" of the RE cells which resulted in reduced cellular clearance of particles with similar surface characteristics (20, 21,

36, 179, 243, 247, 309, 359). More recently the idea that the increased endocytic activity produces a depletion of opsonins has also been presented since addition of opsonic substances reversed a state of phagocytic depression (248, 277, 278, 309). Although the depletion of opsonins could be instrumental in causing RES depression, other investigators have reported that the limiting factors for endocytic activity seem to be the amount of plasma membrane internalized and the cytoplasmic volume of the cell, with recovery of endocytosis occurring only after synthesis of new membrane (73, 239, 301, 354, 355). Recovery from blockade has also been demonstrated to occur by local cellular proliferation and hypertrophy (90, 196, 212, 213, 243) or by replenishment of cells from a bone marrow source (301).

Monocyte-Macrophage Substrate Metabolism

Mononuclear phagocytes have a dynamic intracellular metabolism which must provide substrates and energy for both ongoing cellular activities and sudden bursts of endocytic activity. The intracellular metabolic activity is dependent on several factors including stage of cellular maturation, specific tissue location, "activation" and endocytosis (210). The bone marrow monocyte precursor cells have an active glycolytic pathway (210). Maturation into blood monocytes and tissue macrophages is accompanied by augmentation of glucose consumption and lactate production (210). Hexose monophosphate shunt, aerobic metabolism and cellular respiration are present but less prominent (210, 214). With increased development of intracellular organelles, there is an increase in protein synthesis and a corresponding elevation of lysosomal enzyme activities (210). Specific details regarding macrophage glucose,

lipid and amino acid-protein metabolism and their relationship to the specific cell type and functional status will be discussed below.

The macrophage possesses an active glucose metabolism which has been the subject of numerous studies. Several investigators have reported that both alveolar (165) and peritoneal macrophages (45, 238) accumulate glucose by a facilitated diffusion transport system. In the alveolar macrophage, this system was inhibited by theophylline and was insulin independent (165). This is in contrast to early studies on mixed leukocyte populations which reported both an *in vitro* and *in vivo* insulin stimulation of glucose uptake and lactate production (108, 126). Phagocytosis augmented glucose transport in peritoneal macrophages by lowering the Km value (45).

Kupffer cells, peritoneal and inflammatory exudate macrophages have been shown to contain glycogen stores (131, 175, 176, 269). Interestingly, the wandering macrophage appeared to maintain these stores in two ways: 1) via a glycogen synthetase which was found to exist in 2 forms, either independent of or dependent on glucose-6-phosphate for activity (176) and 2) by uptake of extracellular or exogenous glycogen from incubation media (175, 176), or from glycogen shed from neutrophils into exudative fluids (269) or by direct transfer by mononuclear neutrophil cellular apposition (269). Although *in vivo* insulin treatment increased glycogen synthesis in human leukocytes (126), it had no effect when added to peritoneal macrophages *in vitro* (174). The glycogen pool of peritoneal macrophages *in vitro* was observed to be very labile and readily depleted by incubation in glucose-free media and by phagocytic activity (174, 176). Two glycogen degradative systems are also present in the

peritoneal macrophage: 1) a glycogen phosphorylase system and 2) an α-1,4 glucosidase of probable lysosomal origin (176).

Glycolysis seems to be a universal method of energy production in mononuclear phagocytes (8, 70, 208, 210, 214, 264); however, the reliance on this pathway may be affected by the microenvironment of the cell (210). The alveolar macrophage, which is chronically exposed to high oxygen levels, has been demonstrated to utilize oxidative phosphorylation as its primary energy source in contrast to the peritoneal macrophage (208, 210). Several workers have emphasized that both pinocytosis and phagocytosis are dependent on glycolysis in monocytes and peritoneal, hepatic and alveolar macrophages since they were inhibited by glycolytic inhibitors (2, 8, 70, 208, 210, 264). During phagocytosis, peritoneal macrophages have also demonstrated great increases in Krebs cycle activity and smaller increases in hexose monophosphate shunt activity (2, 208). Alveolar and hepatic macrophages which normally utilize oxidation of glucose and the hexose monophosphate pathway (8, 210, 264, 265, 268) did not show such a marked elevation in oxidative metabolism during phagocytosis (8, 208, 210, 264, 265, 268). Glucose oxidation in isolated Kupffer cells has also been found to be non-responsive to *in vitro* addition of insulin (264). "Activation" is a phenomenon which results in increased cell size and enhanced macrophage phagocytic digestive and microbicidal activity (210). Activated or "elicited" macrophages have been found to possess increased size and number of lysosomes, elevation in acid hydrolases, an enlarged Golgi zone and increased plasma membrane ruffling as well as an increased glucose oxidation (208, 210). Activation has been induced by exposure to various agents which include caseinate (208) thioglycollate, bacterial lipopolysaccharide, immune

complexes and facultative intracellular bacteria (210).

In addition to their continuous utilization of glucose, macrophages also carry on a dynamic lipid metabolism. This area of investigation has been divided into 3 research topics: 1) cellular synthesis and oxidation of fatty acids and triglyceride synthesis and degradation, 2) the relationship between macrophages and atherosclerosis including cholesterol and lipoprotein metabolism and 3) the tremendous lipid turnover of the cell membrane following endocytosis. Two studies by Pisano et al. demonstrated that isolated rat Kupffer cells oxidize exogenous acetate to CO_2 (264, 265) and that this oxidation decreased during phagocytosis (265), suggesting that marcophages might possess a significant fatty acid metabolism. Both hepatic and peritoneal macrophages have been shown to oxidize 1-^{14}C-palmitate (91, 264). Rabbit peritoneal and alveolar macrophages also oxidized ^{14}C-labeled triglycerides (91, 118) implying the existence of a lipolytic system. Elsbach found a high lipase activity in rabbit alveolar macrophages and obtained evidence that lipolysis preceded uptake of glycerides which were then esterified in the cell (118). Other histochemical and biochemical studies have indicated that macrophages possess both cytoplasmic esterase and lipase activity (92). Alveolar macrophages also incorporated free fatty acids complexed to albumin or in chylomicrons into cellular lipids; this incorporation was suppressed by the addition of glycolytic inhibitors (118). Macrophages have also been shown to synthesis fatty acids and mono-, di- and triglycerides from acetate (93, 210). Recently, Wagle et al. have reported that epinephrine, but not glucagon, stimulated lipolysis of triolein and tripalmitin by isolated rat Kupffer cells (348).

A number of early observations implicated a role for the RES in the uptake of triglycerides, cholesterol, phospholipids and lipoproteins. Many of those experiments were done using artificial triglyceride emulsions which were readily ingested by Kupffer and other RE cells and suggested, therefore, that the Kupffer cells might remove lipid from the circulation and transfer it to the liver parenchymal cells (92, 304). However, as mentioned above, macrophages do metabolize the lipid they take up (91, 92, 118). Additionally it was found that Kupffer cells handled artificial emulsions differently than biological lipoproteins (92), possibly because the emulsions were recognized as foreign and because lipids from chylomicrons were taken up directly by the parenchymal cells (92).

Cholesterol, a component of lipoproteins, has been shown to accumulate in RE cells after oral, ip or iv administration (1, 33, 92, 94, 107) and in Tangier Disease, a genetic deficiency of heavy-density lipoprotein or HDL (195). Macrophages also internalized cholesterol from in vitro incubation media (33, 91, 92, 355). Despite one isolated report of macrophage synthesis of cholesterol from acetate (93), it is generally thought that macrophages do not synthesize it and rely on the external medium as their sole source of cholesterol (210, 354, 355). Rabbit peritoneal macrophages could not oxidize cholesterol (91), supporting other studies which demonstrated active macrophage cholesterol esterification (92, 93, 94, 95). Normally cholesterol esters are not further metabolized, but are stored in the cell (83, 92, 94, 195) and excluded from incorporation into cellular membranes (83). A recent study by Drevon et al., however identified the presence of a lysosomal cholesterol

esterase in hepatic macrophages which hydrolyzed cholesterol esters contained in HDL (106). In vivo, cholesterol, free or esterified, is normally transported within plasma lipoproteins because sterols are virtually insoluble in aqueous media (83). It has been hypothesized that free cholesterol is then incorporated into cell membranes by exchange diffusion from the lipoproteins (83). Once in the cell, the free cholesterol equilibrates between the plasma and organelle membranes (83). There may also be an endocytic uptake of lipoproteins by macrophages as has been implicated in fibroblasts and other cells (48, 49). Brown and Goldstein have proposed that low-density lipoproteins (LDL) first bind to a cell surface receptor, leading to endocytosis of the LDL with lysosomal protease hydrolysis of the protein component to amino acids, released from the cell, and hydrolysis of the cholesterol ester component by a lysosomal lipase, with the resulting unesterified cholesterol being incorporated into cell membranes (48, 49). They have also suggested that the level of LDL presented to the cell has a negative regulatory influence on the synthesis of LDL receptors (48, 49). Kupffer cells have been suggested to be a site of LDL catabolism (245); uptake of HDL has been demonstrated in hepatic macrophages (106). Concanavalin A inhibited cholesterol ester uptake by hepatic nonparenchymal cells, suggesting the presence of a carbohydrate-containing lipoprotein binding site (106).

Knowing about the macrophage's propensity for lipid uptake, it should be no surprise that many investigators found lipid-laden macrophages or "foam cells" at the sites of atherosclerotic plaque formation (1, 82, 92, 107, 183). Histological studies verified that these foam

cells were of two types, lipid-containing macrophages and smooth muscle cells, in the intimal layer of arterial blood vessels (1, 82, 92, 107, 183). Adams et al., demonstrated the sclerosing effect of cholesterol on connective tissue and that phospholipid promoted macrophage uptake of cholesterol (1). They interpreted this as evidence of a protective role of macrophages in removing tissue-irritating cholesterol aggregates (1). As was mentioned above, macrophages tended to accumulate cholesterol esters (91, 92, 94, 106) which seemed in turn to promote deposition of other plasma or newly synthesized lipids in macrophages lining the arterial wall (92). These cells are then tought to die, leaving a sequestered pool of interstitial lipids, forming the gruel-like core of the plaque (82, 183). When exposed to an increased cholesterol-phospholipid ratio, such as occurs with ingestion of a cholesterol-rich, atherogenic diet, mammalian cells tend to accumulate cholesterol in their membranes (83). Cooper has hypothesized that the formation and storage of cholesterol esters occurring in atherosclerosis may be an adaptive process designed to divert the cholesterol to a form excluded from cell membrane structure, preventing deleterious changes in fluidity (83). In fact a decrease in membrane fluidity due to an increase in the membrane ratio of cholesterol to phospholipid content has been associated with a decreased endocytic ability in macrophages (83).

During a bout of endocytic activity, a macrophage may internalize 50-60% of its plasma membrane (73, 354), resulting in an increased lipid turnover (268). Surface IgG receptors and 5'-nucleotidase present on the plasma membrane also have been found to disappear after phagocytosis (239, 285, 354, 355). After such maximal endocytosis, cells rounded up

and ceased both pinocytic and phagocytic activity for 5-6 hours (73, 355). Over this period of time, biochemical changes suggesting synthesis of new membrane occurred including increased RNA, protein and phospholipid synthesis and a net increase in membrane RNA, protein and phospholipid synthesis and a net increase in membrane cholesterol (73, 119, 120, 210, 354, 355). Plasma membrane synthesis required the presence of exogenous cholesterol and was blocked by inhibition of protein and RNA synthesis (354, 355). The increased membrane synthesis does not occur at the site of vacuole formation but is only evident 6 hours after endocytosis (210, 354). Macrophages have been shown to synthesize phospholipids from labeled acetate (93), choline (354) and lysolecithin (120). Phospholipids containing labeled choline have been localized in plasma membrane and secondary lysosomes; they have also been shown to be exchanged between cytomembranes via a specific cytosolic protein (210, 354). The amount of new membrane synthesized has been reported to be linearly related to the amount of membrane initially internalized (73) with quantitative restoration of its original composition (354).

After formation of an endocytic vacuole, there is fusion of the membrane resulting in a pinosome or phagosome lined with the plasma membrane constituents which were originally on the exterior of the cell (73). Subsequent fusion of the vacuole with lysosomes leads to discharge of acid hydrolases and digestion of endocytized macromolecules, including membrane polypeptides, to small molecular weight products (73, 113). Thus plasma membrane molecules such as 5'-nucleotidase, phosphodiesterase and IgG receptors have been observed to disappear following endocytosis,

only to be replaced later (73, 239, 285, 354, 355). Macrophages are capable of nucleic acid synthesis, both RNA and DNA, (210, 212, 213, 268) and possess a well-developed endoplasmic reticulum for protein synthesis (210, 268). The replacement of membrane components has been shown to occur after RNA and protein synthesis (73, 354, 355). Induction of lysosomal enzymes and increased formation of lysosomes has also been noted in response to endocytic activity (113, 354). Cohn et al., followed the intracellular flow of leucine-H^3 in mouse peritoneal macrophages (75). They found that there is initial labeling of the rough endoplasmic reticulum, followed by appearance of the label in the Golgi complex and finally the dense granules contained the majority of the isotope and suggested that lysosomal hydrolytic encymes are synthesized in the endoplasmic reticulum and then transferred to the Golgi apparatus where the enzymes are packaged into small Golgi vesicles resulting in formation of primary lysosomes (75).

Alveolar macrophages have been shown to possess carrier-mediated transport systems for adenosine and lysine (30, 332, 354). These transport sites were apparently preserved after 35-50% of the membrane was internalized since constancy of transport activity did not depend on production of new transport sites (332). A role for microtubules in maintaining a separation of the transport and phagocytic sites has been suggested (30, 246, 332, 354). This preservation of amino acid and nucleotide transport during endocytosis seems appropriate for the succeeding bout of protein synthesis. However, a recent report presented evidence that protein synthesis and leucine transport were inhibited in suspended alveolar macrophages after repeated washing, perhaps by the

removal of a necessary surface membrane RNA (334).

Besides synthesizing proteins to maintain their cellular integrity, macrophages synthesize and secrete many other proteins. Macrophages, as discussed previously, actively synthesize lysosomal enzymes which have been reported to be released outside the cell during endocytosis (2, 73, 89, 96, 127, 210, 224, 230, 299, 310,352). Only about 10-15% of the total cellular lysosomal acid hydrolase activity appeared extracellularly (73). In contrast, 85-90% of the enzyme lysozyme was secreted independent of cellular activity (73, 127) and may be a major macrophage secretory product (73). Another enzyme, plasminogen activator, which is involved in lysis of fibrin and cleavage of complement proteins and Hageman factor via conversion of plasminogen to plasmin, was secreted primarily by activated macrophages (73, 127). Synthesis of collagenase and elastase for export has been observed (127, 230, 349). Macrophages have been shown to synthesize serum complement proteins Clq, C2, C3, C4, and C5 (71, 115, 127, 210, 230, 305, 306) as well as transferrin (125, 210, 305, 306). Production of a variety of proteins related to immunological functions by macrophages such as colony-stimulating factor, interferon and lytic, stimulatory or inhibitory factors has also been demonstrated (89, 127, 210, 230, 249, 275, 320). After phagocytosis, macrophages, especially Kupffer cells have been reported to produce the protein endogenous pyrogen (9, 19, 42, 43, 102, 127, 171, 178, 210, 351) which may or may not be related to leukocytic endogenous mediator, a protein synthesized and released by macrophages and blood leukocytes (19, 206, 260). Two recent reports have even implicated macrophages in regulation of the renin-angiotensin system by production of angiotensin-

converting enzyme (156, 222). Thus, the secretory function of macrophages gives the RES the potential to exert a regulatory influence on its environment within the organism and to function almost as an endocrine organ.

Metabolic Role of the RES in the Organism

Aschoff in his studies on the RES was obviously aware of the important role of macrophage metabolism in the organism when he proposed the name "histiocytic metabolic apparatus" as an alternate title for the RES (7). The macrophages have been implicated in many catabolic and transformation reactions such as drug metabolism and detoxification (100, 276) and, as discussed above, in lipoprotein and cholesterol transformation and metabolism (1, 7, 20, 33, 91, 92, 93, 94, 95, 106, 107, 118, 245, 276, 354, 355). Berliner demonstrated that adrenal cortical RE cells are involved in cortisol production from progesterone while the hepatic RE cells clear steroids and cause steroid ring A reduction preparatory to excretion (31, 32). Thyroxine degradation during bacterial phagocytosis has also been reported (98). A relationship between RE cell activity and transcobalamin II metabolism has been suggested (167). Macrophages seem to regulate the clotting system by several routes since they produce plasminogen activator (73, 127) and have been demonstrated to clear both fibrin aggregates (220) and thromboplastin (302).

Some of the earliest known RES functions were the sequestration and destruction of aged, foreign or damaged red blood cells and circulating hemoglobin with subsequent bile pigment or bilirubin formation and conservation of the heme iron in ferritin (7, 20, 72, 125, 159, 160, 239, 268, 276). The globin moiety of hemoglobin is degraded by proteases and

the heme by heme oxygenase (320). Iron stored as ferritin is transported back to erythroid precursors via the protein transferrin, secreted by the macrophage (102, 125, 210, 306). Transferrin molecules have been proposed to deliver the iron to the cell surface of the erythroid precursors and return empty to the RE cells to pick up another load (125, 320). Others have suggested that macrophages in the bone marrow serve as "nurse" cells in an island of developing cells, supplying iron by transferrin or by direct cell-to-cell delivery (125, 320).

RE cells are very active in removal and degradation of effete, autologous tissue, antigens and proteins by means of their extensive array of lysosomal enzymes (2, 25, 74, 89, 111, 113, 210, 224, 241, 276, 352). Macrophages have been shown to arrive at the site of inflammation later than polymorphonulcear leukocytes (89, 137, 224). The macrophages appear to function as "scavenger" cells, phagocytizing fibrin and cellular debris in preparation for repair (224). Several in vitro studies have demonstrated macrophage segregation and digestion of radiolabeled serum albumin, bovine gamma globulin, hemoglobin, horse radish peroxidase and polypeptides in secondary lysosomes (20, 71, 72, 111, 112, 113). Apparently the macrophages digested the proteins down to the level of amino acids (71, 72, 113). Chronic inflammatory conditions can be perpetuated by macrophage release of lysosomal enzymes, collagenase, elastase, cytotoxic activities, prostaglandin release and activation of osteoclasts (127, 168, 224, 230, 349, 351, 352). Macrophages may also help in elimination of unwanted protein by secreting proteolytic enzymes during absorption of the tadpole tail during metamorphosis and the period of collagen resorption in the post-partum uterus (96, 127). Experi-

mental absence of macrophages led to an increased amount of fibrin debris and a slowing of fibrinogenesis in wounds (127). Rebuck et al., also discussed the phenomenon of leukocytic glycogen transfer to macrophages at inflammatory sites (269). Both Cohn (74) and Gudewicz (175, 176) found that macrophages possess enzymes of probable lysosomal origin capable of hydrolyzing α-lined polysaccharides. Macrophage α-1,4 glucosidase activity may provide an additional source of glucose via endocytosed glycogen not only to the inflammatory macrophage but to other cells at the site since a portion of this glucose is released to the outside (175, 176).

The cells of the RES tend to accumulate phagocytizable organic material when it is present in the body in amounts that cannot be rapidly disposed of by normal catabolic or excretory processes such as in storage diseases or conditions with excessive cell turnover and death (74, 96, 159, 320, 351). Hemosiderosis, asbestosis, silicosis and xanthomas are examples of storage diseases caused by macrophage endocytosis of nondigestible materials (159). Inborn genetic enzyme deficiencies can result in macrophage lysosomal enzyme deficiency storage diseases characterized by the presence of undigested material in the lysosomes (74, 159, 320, 351). Cohn was able to induce a similar disorder in vitro by allowing mouse peritoneal macrophages to pinocytose sucrose or Ficoll, a polysucrose (74). Interestingly, antigenic substances, for example, α-globulins and keyhole limpet hemocyanin, may bypass extensive catabolism by either remaining bound to the plasma membrane or undergoing only partial degradation and processing for release from the cell to stimulate lymphocyte function (85, 210).

Since the time of Metchnikoff, the role played by the RES in host cellular immunity by clearance of potentially harmful bacteria and viruses from the body has been emphasized (14, 20, 38, 58, 89, 179, 196, 263, 276, 287). The RES has also been considered to be instrumental in the clearance of endotoxin, the lipopolysaccharide component of gram negative bacterial cell walls having a molecular weight of about 1 million, normally preventing a fatal shock episode (18, 20, 23, 41, 44, 53, 101, 129, 171, 179, 192, 196, 274, 276, 323, 350, 353, 370). Bona found that macrophages appeared to take up endotoxin by pinocytosis (44). Macrophages have been shown to detoxify endotoxin via lysosomal activity or perhaps by elaboration of a blood antiendotoxin system (135, 136, 137, 138, 139, 141, 329, 353). It has long been known that the acute administration of endotoxin to an animal results in depression of RES function (20, 23, 179, 192, 323). This RES depression may be the result of the profound alterations in glucose metabolism caused by endotoxemia (52, 54, 55, 56, 79, 87, 134, 140, 145, 146, 188, 225, 263, 272, 290, 291, 292, 303, 363, 364).

RES - Hormone Interactions

In prior sections of this review, several instances of RES interaction with various segments of the endocrine system were mentioned. Although the RES does participate in the metabolism and synthesis of hormones and in turn RES functions are affected by certain hormones, the elucidation of this area of RES physiology has only begun. Some of the earliest observations concerned the effects of estrogens on the endocytic function of the RES. It is generally believed that the female mammal is

more resistant to stress than the male. This belief has not been systematically investigated; however, it has been suggested that the female hormones, especially the estrogens, may stimulate RES function and thus increase cellular immunity (4). In 1935, Nicol found that estrogenic hormones seemed to induce the formation and stimulate the activity of guinea pig uterine macrophages (316). Several investigators reported that phagocytic activity increased during the two peak levels of estrogen present during the human menstrual cycle and in the estrous cycle of lower mammals (316, 342). Ovariectomized rodents showed decreased phagocytic activity with a decline in the number of uterine macrophages (342). Estrogen replacement increased the number of macrophages in spayed animals (342). During the early implantation and late stages of pregnancy, RES endocytic activity is elevated (316, 342). Ovariectomy reduced survival against highly virulent infections (342). In the rat, females were reported to have greater ability to clear colloidal carbon and greater resistance to lethal circulatory trauma (4). In male rats, estrogen treatment conferred trauma resistance and caused hyperactivity of the RES (4). Thus, in general, estrogenic steroids have an unequivocal stimulatory effect on RES endocytic activity (4, 179, 316, 342). Several mechanisms for this stimulation have been presented including an increased migration of mononuclear cells from the bone marrow (331, 342), local cellular proliferation, especially in the liver (342), higher phagocytic activity of the available macrophages (342) and an elevated rate of energy metabolism, especially of hexose monophosphate shunt activity (214, 342).

Another group of steroids, the adrenal corticosteroids also have profound effects on the RES. Patients with Cushing's syndrome, characterized by an excess of corticosteroids or with Addison's disease and a lack of corticosteroids both showed increased susceptibility to infection (316). Pharmacological doses of cortisone also were known to reduce the resistance of man and animals to bacteria, viruses, fungi and bacterial toxins (22, 38, 316, 342). Therefore, experiments were done to determine the reasons for the impairment of immunity, particularly of the phagocytic function of the macrophages. Heller reported that cortisone administration depressed RES colloid clearance in both rats and mice (186, 316). Others reported no effect of cortisone on the basic RES endocytic activity (21, 22). Cortisone administration did delay the recovery phase following colloidal blockade (22, 179, 316) and suppressed the normal stimulatory reaction of the RES to bacterial products (38). Insight into the mechanism by which corticosteroids influence RES kinetics was provided by studies on inflammation (130, 324). In normal mice, subcutaneous injection of corticosteroids resulted in a rapid drop in the number of blood monocytes released from the bone marrow (324) and inhibition of macrophage accumulation in sites of inflammation (130). It is likely then that corticosteroids do not modify the phagocytic properties of macrophages per se (130). In contrast to the above findings, smaller doses of cortisone reportedly stimulated RES phagocytic activity (316, 342). This is consistent with the report that cultured peritoneal macrophages required cortisol in the culture medium to maintain normal functions such as serum protein synthesis (156, 306). Altura has also found that glucocorticoids prevented the early RES depression seen in circula-

tory shock (3). Yet the same small doses of cortisone which enhanced RES endocytic activity inhibited macrophage digestive and bactericidal capacities (130, 342). This last effect may be the result of corticosteroid inhibition of degranulation (130, 310) and stabilization of lysosomes (224, 351). It is now accepted that small doses of corticosteroids maintain or enhance RES function while large doses depress it (99, 342). It should be reiterated that adrenal cortical macrophages produce cortisol from progesterone while hepatic Kupffer cells clear steroids and cause ring A reduction preparatory to conjugation and excretion (31, 32). Thus macrophages also have a role in regulation of steroids which in turn influence their activity.

Because of the known interactions of the RES and adrenal corticosteroids, studies were done to ascertain the effects of hypophysectomy on RES function (35, 99, 211). DiCarlo et al. found that hypophysectomy did not affect the rate of phagocytosis in mice (99). However, others found that long term effects of this operation included a progressive fall in RES phagocytic capacity (35, 211) possibly due to a decrease in RES mass and blood volume (211). Although Keefe et al. found that zymosan-induced stimulation was still possible after pituitary removal in rats (211), DiCarlo et al. could not cause RES stimulation in hypophysectomized mice unless they were treated with ACTH (adrenocorticotropin), pituitary suspension or corticosterone (99). The latter group hypothesized that ACTH may be stimulatory to the RES via increased adrenal cortical secretion (99).

Bilder noted both thyroidectomy and hypophysectomy decreased the RES maximum phagocytic rate or V_{max} (35). She attributed this effect to

the resulting decrease in thyroid hormone since thyroxine treatment restored V_{max} to intact levels (35). The thyroid hormones may maintain RES function by regulating whole body metabolism (35) rather than by a direct effect, as proposed by Stambul (204). Recently DeRubertis and Woeber demonstrated the uptake and metabolism of thyroxine by hepatic macrophages during Samonella typhimurium sepsis in monkeys (98). They proposed that thyroxine could influence the host phagocytic cell function (98).

In addition to metabolizing hormones or being affected by them, macrophages also produce hormone-like substances which influence the secretion of endocrine glands. Besides adrenal cortical macrophage participation in glucocorticosteroid production (32), macrophages have been shown to release large quantities of prostaglandins (168, 230). Rabbit alveolar macrophages, exposed to physiological concentrations of corticosteroids, contained increased levels of angiotensin converting enzyme (156), suggesting that the RES may influence the regulation of blood pressure. In macrophage related diseases, such as Gaucher's disease, increased macrophage production of serum angiotensin converting enzyme has also been observed (156, 222). After in vivo stimulation with bacteria, macrophages have been shown to produce leukocytic endogenous mediator, a protein produced by leukocytes, which causes a shift of amino acids from muscle to liver, increased hepatic amino acid uptake, cAMP levels and glycogenolysis as well as elevation of plasma insulin and glucagon levels with host hypoglycemia (19, 166, 206, 260). Therefore, the macrophages may regulate pancreatic insulin and glucagon levels and the host's metabolic responses to infection (166). Moore et al. have likewise demonstrated that macro-

phages exposed to endotoxin produced an anti-gluconeogenic substance which inhibited corticosteroid induction of phosphenolpyruvate carboxykinase (235), an insulin-like effect.

Classically the effects of hormones affecting hepatic metabolism were thought to occur only at the parenchymal cells. This generalization is no longer valid since the identification of a hormone sensitive adenylate cyclase in Kupffer cells (270, 347, 348, 362). Despite one report of glucagon-sensitive adenyl cyclase being located in the RE cells (270), studies on isolated cells demonstrated that epinephrine sensitive adenylate cyclase predominated (347, 348, 362). Wincek and coworkers found that Kupffer cell adenylate cyclase was stimulated by fluoride ion, GTP and catecholamines as monitored by cAMP production (362). Wagle et al. demonstrated that epinephrine, but not glucagon, stimulated hepatic macrophage lipolysis (348). The latter group also claimed that both epinephrine and, to a lesser extent glucagon stimulated gluconeogenesis in Kupffer cells (347, 348); however, these results could be due to parenchymal cell contamination.

Because Kupffer cells are responsive to catecholamines and glucagon, and by inference possess "receptor sites" for these hormones, it seems reasonable to investigate the effect of insulin on Kupffer and other RE cells. Initially, the increased susceptibility to infection seen in diabetics (10) sparked interest in this topic. Several immunodeficiencies have been identified in human diabetics including decreased or missing antibody formation to bacterial antigens (223), possibly due to inhibition of lymphocyte blastogenesis, as was reported in diabetic rats (256), decreased numbers of peripheral T-lymphocytes (60) and impaired

leukocyte migration (161). Several workers also noted depressed phagocytosis by granulocytes and macrophages in the diabetic state (10, 11, 105, 269). Yet Berken and Sherman found that RES phagocytosis was normal in diabetes (27) and Gabrieli and associates found it to be abnormally accelerated (161). The discrepancies in these results have been explained by the direct correlation between successful blood glucose management with insulin and the return to normal phagocytic function (10, 11, 27). Apparently the elevated blood glucose levels and hypertonicity depressed phagocytosis (67, 105, 283, 310). Rebuck et al. using his "skin window" technique demonstrated that diabetics did show a defective inflammatory response which he attributed to a failure of leukocytic glycogen transfer to mononuclear phagocytes (269). Neutrophils from severely diabetic patients, in that study, showed abnormally high glycogen concentrations accompanied by decreased neutrophil migration and phagocytosis in contrast to cells from well-controlled diabetics and controls which shed their glycogen to lymphocytes, monocytes and macrophages (269).

Still other investigators sought to further characterize the effect of insulin directly on white blood cells and macrophages. Dumm studied the effect of insulin on glucose utilization by mixed white blood cells and reported increases in cells from both normal and diabetic subjects after both in vivo insulin administration and in vitro using 0.1-0.5 U/ml of insulin (108). Esmann, also saw a slight in vitro insulin effect on glucose uptake by human leukocytes with 1 U/ml and a 4 hour incubation; no effect was observed on glycogen synthesis (126). Studies specifically on macrophage glucose metabolism yielded similar results since insulin

had no effect on glucose transport in alveolar macrophages (165) or on peritoneal macrophage glycogen synthesis (174). Kupffer cell glucose oxidation was also unaffected by insulin (264). Generally speaking, insulin seemed to have no effect on macrophage glucose metabolism.

The apparent leukocyte insensitivity to insulin puzzled investigators since mixed leukocytes had been reported to bind large amounts of insulin compared to other tissues (184) and binding was similar in normal and diabetic cells (296). Gavin, Archer and their coworkers subsequently demonstrated that circulating and cultured B-lymphocytes contained specific insulin binding sites (163, 164). However, a study by Krug et al. detected no such receptors on resting lymphocytes; it is very likely that they had a T-lymphocyte cell population (217). Insulin receptors appeared to be present on the T-cell only after in vitro transformation with concanavalin A (217) or after immune activation (185, 315). Insulin caused an augmentation of T-lymphocyte cytotoxicity (185, 315) and in another study, it increased both glucose transport and membrane ATPase activity in human lymphocytes (177). Thus insulin binding had functional consequences in lymphocytes. Gavin and coworkers also reported that chronic (5-16 hr) exposure of cultured human lymphocytes to insulin resulted in a reduction of insulin receptor concentrations, suggesting a reciprocal relationship between the amount of insulin in extracellular fluid and the number of insulin receptors per cell which directly affects target-cell hormone sensitivity (164). Lymphocytes were then utilized in several studies for investigation of possible insulin receptor binding defects to explain the conditions of insulin resistance (5), diabetes (253) and obesity (6).

In a study on insulin receptors on human mononuclear leukocytes, Swartz and associates demonstrated that although lymphocytes constituted 80% of these cells, the amount of ^{125}I-labeled insulin specifically bound correlated significantly only with the number of monocytes (286). Autoradiograms of blood smears also showed that only monocytes bound the labeled insulin (286). Apparently many of the experimental "lymphocyte" preparations were contaminated with monocytes and thus showed insulin binding and likewise no binding if measures were taken to insure depletion of monocytes (185). Monocytes were then immediately used to further investigate the disorders of obesity (251) and diabetes (254, 255). Diabetes mellitus and obesity were both accompanied by decreases in insulin receptor number (250, 254). The use of circulating monocytes was instrumental in identifying a cause for the diabetic syndrome of acanthosis nigricans in which patients show a marked resistance to insulin probably due to the presence of insulin receptor antibodies (154, 203, 204) or a decrease in binding affinity (204). Recently, Thorsson and Hintz reported that monocytes also possess specific receptor sites for the insulin-like substance somatomedin (325).

Since the discovery of monocyte insulin receptor binding, investigators have endeavored to find some associated biological functions in RES cells. One of the earliest observations on a possible effect of insulin was in a study by Stossel et al. on phagocytosis (314). Although that study utilized polymorphonuclear leukocytes, it is significant to note that in vitro addition of insulin at a physiological concentration of 2 mU/ml slightly stimulated the phagocytic rate (314). In a later report, Rhodes demonstrated that guinea pig peritoneal macrophage Fc

receptor expression is modulated by insulin and cyclic nucleotides (271). In both human monocytes and mouse spleen macrophages, insulin inhibited antibody-dependent cytotoxicity (13) possibly by decreasing the number of Fc receptors (271). Therefore, macrophages appear to possess functionally significant specific insulin receptors. Several groups, working independently, demonstrated that peripherally injected ^{131}I or ^{125}I-insulin tended to be localized in hepatic Kupffer cells (17, 307, 366), as did injected insulin-antibody complexes (157). Chandler and Varandani, working with a mixed blood leukocyte population, have identified the insulin-degrading activity present as the enzyme glutathione-insulin transhydrogenase (65). Additionally, both glutathione-insulin transhydrogenase and insulin specific protease were found in the lysosomal fraction of rat liver (173). Because Kupffer cells are rich in lysosomes and lysosomal enzymes (25, 241, 299) and are in intimate contact with pancreatic insulin secreted into the hepatic portal circulation and possess active secretory, metabolic and degradative processes, it is possible that Kupffer cells may participate in the modulation of insulin activity in the organism.

The Effect of RES Depression on Glucoregulation and Shock Susceptibility.

Since it was known that the RES played a significant role in the removal of pathogenic organisms and endotoxin, which if left unchecked could produce a shock type of death, studies were conducted to determine if blockade of the RES by other substances could increase shock susceptibility. Depression of the RES by saccharated iron oxide, thorotrast, large doses of cortisone or whole body irradiation increased the inci-

dence of death from traumatic or endotoxin shock (142, 152, 227, 369). Similarly lead acetate treatment also sensitized rats to death by endotoxin shock (79, 80, 81, 135, 143, 288, 329). Interestingly, stimulation of RES function by zymosan, endotoxin, estrogens or induction of trauma resistance decreased susceptibility to shock death (4, 151, 192, 369, 371).

RES depression by colloidal carbon blockade has also been demonstrated to cause alterations in glucose regulation as judged by 1) the enhanced hypoglycemic response to insulin, 2) increased insulin-induced hypoglycemic lethality, 3) decreased gluconeogenesis and 4) increased total body glucose oxidation (142, 144). Lead administration has also been shown to cause decreased gluconeogenesis and increased hypoglycemic response to insulin (52, 84, 140). Endotoxemia and gram negative sepsis also depress the RES (20, 23, 179, 192, 323) producing similar but more profound changes in glucose homeostasis along with changes in insulin activity (26, 55, 56, 79, 86, 87, 142, 145, 146, 172, 188, 189, 225, 263, 272, 290, 291, 292, 303, 363, 364) suggesting that the RES governs the host metabolic response to endotoxin not only by clearance of bacteria and endotoxin but by modulating insulin levels. Consideration of the above date indicates a probable relationship between RES functional status and insulin regulation.

Statement of Purpose

The purpose of this study was to investigate the relationship between altered RES endocytic function and insulin regulation. In specific, experiments were performed:

1. to investigate the effect of RES depression, using acute lead acetate administration, on susceptibility to insulin-induced hypoglycemic convulsive death;

2. to evaluate the influence of RES depression on iv glucose tolerance and;

3. to ascertain the effect of RES depression by either colloidal carbon blockade or acute lead treatment on actual basal and glucose-stimulated serum insulin levels as measured by the Phadebas radioimmunoassay.

METHODS

A. Animals

Adult male albino Holtzman rats (Madison, Wisconsin) were used in all experiments. The rats were housed 3-5 per cage and allowed to acclimate to our animal quarters which were kept at 26-28°C with a 12 hour light-dark cycle--lights on at 7 a.m. and off at 7 p.m. (Central Standard Time) for 7-15 days prior to use. Rats were given Purina Laboratory Rat Chow (Ralston Purina Co., St. Louis, Missouri) and tap water ad libitum until approximately 4 p.m. the afternoon preceding experiments when only the food was removed to allow for an overnight fast of about 18 hours. The rats weighed 302 $\pm$ 25 grams (mean $\pm$ standard deviation, N = 353) on the day of the experiments.

B. Acute Lead Treatment

In order to alter RES function, acute administration of lead acetate (PbAc) 5 mg/300 g rat was utilized since this dose has been shown to depress RES phagocytic function (143,330). Powdered lead acetate (Code 5684, Mallinckrodt Chemical Works, St. Louis, Missouri) was prepared in deionized distilled water to a concentration of 5 mg/ml on the day of the experiment. Depending on the experiment protocol, control treatment consisted of 1 ml of either 0.9% pyrogen-free saline (Travenol Laboratories, Inc., Deerfield, Illinois) or a 5 mg/ml solution of sodium acetate (NaAc) (Code 7372, Mallinckrodt) in deionized distilled water. Rats received the 1 ml injection iv via the dorsal vein of the penis

under light ether anesthesia (Anesthesia Grade, Mallinckrodt) induced in a glass ether jar.

C. Colloidal Carbon Blockade

As a second method of RES depression classic colloidal carbon blockade (36) was induced by iv injection of 32 mg/100 g body weight of colloidal carbon. An inert biological ink was used--Pelikan Special Ink, C11/143la (Gunther-Wagner, West Germany, Koh-I-Noor Radiograph, Inc., Bloomsbury, New Jersey) which is a colloidal suspension of homogeneous 25 nm carbon particles (36, 180, 359) stabilized in a 4-5% fish glue gelatin solution (147, 247) with 1% phenol as a preservative (36, 152). To standardize the carbon concentration, the ink stock was diluted 1:10,000 with deionized distilled water or 0.9% saline to permit densimetric determination of 3 ml aliquots using 1 x 1 cm cuvettes in a Beckman Model DU Spectrophotometer (Beckman Instruments, Inc., Fullerton, California) at a wavelength of 675 nm. The conversion factor of 0.04 OD U/mg carbon per liter (147) allowed calculation of the carbon concentration which was 83.5 mg/ml which is in agreement with the reported concentration of approximately 100 mg/ml (36).

For blockade induction, the ink was diluted 1 part ink to 1.6 parts 0.9% non-pyrogenic saline (Travenol) to achieve a concentration of 32 mg/ml, pH adjusted to 7.4 and kept refrigerated until shortly before use. A sham blockade vehicle control for the ink diluent was obtained by ultracentrifugation of the ink at 25,000 x g in an International Preparatory Ultracentrifuge Model B-35 (International Equipment Co., Needham Heights, Massachusetts) which produced a supernatant that was

diluted as the carbon, pH = 7.4 and refrigerated. Sham and carbon solutions were prepared the evening before or day of the experiment. In carbon blockade experiments, the rats were separated into 3 groups. One group received a slow 3 ml iv injection of the 32 mg/ml colloidal carbon or 32 mg/100 g under light ether anesthesia. The 2 control groups were a sham vehicle group that was iv injected with 3 ml of the sham and an uninjected control group.

D. Insulin Lethality Experiments

Overnight fasted rats were divided into two groups, one received the non-lethal dose of 5 mg PbAc iv, the other pyrogen-free isotonic saline under light ether anesthesia. Immediately following the first injection, a second 1 ml subcutaneous injection of Lilly U-40 Iletin® regular insulin (Eli Lilly and Co., Indianapolis, Indiana) diluted to one of several concentrations with isotonic pyrogen-free saline was given in the subscapular region. Preparation of insulin solutions was done with non-glass labware to avoid losses due to non-specific binding. Dilutions ranging from 0.5 U/ml to 1.5 U/ml were utilized. The rats were then weighed, marked for identification and returned to their cages with only tap water provided. The number of hypoglycemic convulsive deaths which occured in the 24 hours after injection were tabulated. Significance of the difference in lethality between groups was assessed by the chi square test with Yates correction factor run on a PDP-12 Digital Computer (Digital Equipment Co., Maynard, Massachusetts). A "p" value less than 0.05 for chi square (104) was considered statistically significant.

E. Intravenous Glucose Tolerance Testing

Fasted rats were given either 5 mg PbAc or 5 mg NaAc 4 hours before the tolerance test. At t = -15 minutes, the animals were anesthetized with 32.5 mg/kg Pentobarbital Sodium (Holmes Serum Co., Inc., Springfield, Illinois) administered as a 1 ml ip injection of a fresh pre-diluted solution of the 65 mg/ml sodium pentobarbital in isotonic nonpyrogenic saline. The level of anesthesia was considered adequate when the tail was limp and breathing was regular. Control (C_i) and succeeding blood samples were taken from a tail snip. Using a sharp pair of scissors, just the tip of the tail was cut off and then the tail was milked to promote blood flow. Samples were collected directly into 250 µl heparinized, fluoride-treated microfuge tubes (Beckman). At time zero, after the control sampling, 200 mg of D-glucose (Sigma Laboratories, St. Louis, Missouri) as 1 ml of 20% glucose in isotonic saline was administered iv. The glucose solution was prepared the night before to allow for mutarotation and kept refrigerated. Subsequent blood samples of about 0.2 ml were taken at 5 minute intervals from 5-45 minutes. After the last sample, the blood was rapidly separated into plasma and cells in a Beckman Microfuge Model 152 and then frozen at 0°C until glucose analysis.

Plasma glucose concentrations were determined using the Model 23A Yellow Springs Glucose Analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). This instrument measures glucose concentration in a 25 µl sample by the amount of hydrogen peroxide produced in the glucose oxidase reaction (367). After thawing on ice and mixing of the

plasma layer, samples were done in triplicate except when the first 2 readings differed by 2 mg/dl or less and then the mean calculated for each sample. To check for linearity of response, the analyzer was standardized with 200 and 500 mg/dl glucose solutions (Yellow Springs).

In order to analyze the data, the $\log_{10}$ of the plasma glucose concentrations was plotted against time in minutes for each rat. Then the best straight line through the 5-30 minute points was drawn and extrapolated to the ordinate so that the theoretical concentration at time zero (C_0) could be determined. The time at which the glucose concentration was one half of the C_0 was considered the half-time ($t_{\frac{1}{2}}$) of glucose disappearance from the vascular compartment (298, 360). The k value or % glucose disappearance/min can be obtained by the formula $k = 0.693/t_{\frac{1}{2}} \times 100\%$, since during the initial 30 min measured, the change in plasma glucose with time appeared to be a single exponential function. The mean, standard deviation and standard error were calculated for C_i, C_0 and $t_{\frac{1}{2}}$. The significance of the difference between the means was determined using Student's unpaired "t" test with $p < 0.05$ considered statistically significant done on the PDP-12 computer.

F. Determination of Basal and Glucose-Stimulated Serum Insulin Levels in RES Depressed Rats

Both basal and glucose-stimulated serum immunoreactive insulin levels (IRI) were measured in rats at various times after administration of an RES depressant and in controls. Sodium pentobarbital at a dose of 32.5 or 43.3 mg/kg as a 1 ml ip injection of a freshly prepared dilution in isotonic nonpyrogenic saline was used. For basal samples, a cardiac

puncture of at least 1-3 ml was taken with a 5 ml syringe with a 20 gauge needle at 15 min after induction of anesthesia or time zero. Glucose-stimulated serum samples were obtained 5 min after a 1 ml iv injection of 400 mg of D-glucose (Sigma) at time zero as 40% glucose in isotonic saline prepared the previous night and refrigerated. It has been shown that the peak insulin response to a glucose pulse occurs between 2-5 min following the stimulus (39, 267, 289) and 5 min also allowed for adequate mixing in the vascular compartment. After the cardiac puncture, the needle was removed from the syringe and blood was carefully transferred to glass tubes on ice to prevent hemolysis and allowed to clot. Clots were rung and the samples centrifuged, the serum removed and frozen at 0°C in plastic vials. Serum glucose was measured using the YSI Glucose Analyzer as described previously. Serum IRI was determined on 4 replicates of the serum samples using the Phadebas Insulin Test radioimmunoassay (RIA) kit (Pharmacia Laboratories, Inc., Diagnostics, Piscataway, New Jersey) as described below. Data were compared using Student's unpaired "t" test with $p < 0.05$ as the level of statistical significance.

Serum samples were taken at 4 hours after 5 mg PbAc iv in overnight fasted rats because the RES is depressed (143) and metabolic changes have been noted at 4-6 hours after lead (140). For the carbon blockade studies 3 time points were chosen for measurement of basal serum glucose and insulin. Fasted rats were subjected to either a 4 or 8 carbon blockade since there is decreased RES function at 4-8 hours (12, 22, 142, 144, 152, 205, 213, 259, 301) and altered glucoregulation has been reported (144). An 18 hour carbon blockade was also used with a slight

difference in protocol in that rats were injected with carbon or vehicle and then fasted overnight for a total of 18 hours since both carbon and lead treatment before an overnight fast resulted in depressed hepatic gluconeogenesis (84, 144) and a trend toward recovery of phagocytic function after carbon blockad has been demonstrated within one day (22, 38, 103, 205).

G. Immunoreactive Insulin Assay

The Phadebas Insulin Test RIA kit (Pharmacia) was used to assay serum insulin (IRI) levels in rat serum samples. This RIA utilizes guinea pig anti-porcine insulin antibodies covalently bound to Sephadex particles as the solid phase which has several advantages such as ease of separation of bound and unbound insulin, high stability, high antigen binding capacity and little tendency for non-specific adsorption of proteins and polypeptides (356, 357). The concentration of insulin in an unknown sample was evaluated by its capacity to compete with a fixed amount of labeled I-125 insulin for insulin antibody binding sites compared to known concentration insulin standards. Since rat insulin is not readily available, a porcine insulin standard was used as a relative reference.

Seven insulin standards of 3.2, 8, 16, 32, 80, 160 and 320 μU/ml were prepared by both single and serial dilution steps (261). At the time of assay, serum was thawed and 250 μl of each well-mixed sample was mixed with 250 μl of the RIA kit buffer solution resulting in 500 μl half-dilutions of the samples which was done for the following reasons: 1) only a small quantity of serum was required for assay, 2) samples with high insulin levels were assayed at a more readable portion of the

standard curve and 3) dilution of high insulin samples made dilution of all samples obligatory because IRI measured by RIA is augmented by dilution (284). Four 100 µl replicates of each sample were assayed following the manufacturer's procedure (261). Each time the RIA was done, triplicates of the buffer and duplicates of the standards, Pharmacia human reference serum and pooled fed rat serum were run preceding and following the unknowns to check the reproducibility of the RIA. The coefficient of variation for samples was $\leq$ 15%. Detailed information on the precision and accuracy of this RIA method is available (51, 341). Oxford Samplers (Oxford Laboratories, Inc., Foster City, California) were used for pipetting except for the Sephadex anti-insulin complex which was pipetted with a 1 ml Cornwall Syringe (Type BD, Becton, Dickinson, Rutherford, New Jersey) specially adapted with narrow polyethylene tubing to prevent sedimentation. Assay mixtures containing I-125 insulin, sample and sephadex antibody complex in buffer were incubated 24-36 hours in Pharmacia plastic 12 x 55 mm stoppered RIA tubes at room temperature on a horizontal RIA Shaker (Pharmacia). Incubation was terminated by centrifugation for 2 min in a 96 position IEC swinging bucket head at 1,500 x g in an IEC Model PR-J refrigerated centrifuge. After the stoppers were removed, the centrifugation was repeated at 1,500 x g for 4 min, after which an adjustable collared pipette coupled to a water vacuum pump was used to aspirate the supernatant to approximately 5 mm from the bottom of the tubes. Three washes of the Sephadex particles were done with 2 ml of pH 7.0-7.5 isotonic saline kept on ice using a 5 ml Cornwall syringe followed by a 4 min centrifugation. The tubes containing the sediment were gently placed into uncapped 16 x 25

mm polystyrene gamma counting vials (Amersham Corp., Arlington Heights, Illinois) and counted for 10 or 20 min in a Searle/Analytic Model 1185 Gamma Counter (Amersham/Searle, Arlington Heights, Illinois).

In order to calculate the sample insulin concentrations, the mean count rate for each of the standards in cpm minus background was expressed as a percentage of the background corrected count rate of the zero samples which contained only buffer plus Sephadex antibody complex and I-125 insulin. A linear transform of the plot of the percent activity bound versus the $\log_{10}$ of the insulin concentration was calculated with a regression program run on the computer. Another program calculated the mean insulin concentration, standard deviation, standard error and coefficient of variation for each sample (51). The resulting concentrations were multiplied by 2 to correct for dilution of the samples.

RESULTS

Insulin Induced Hypoglycemic Lethality with Acute Lead Administration

A series of experiments was done to determine whether RES depression by acute lead acetate administration would affect the incidence of insulin-induced lethal hypoglycemia. These data are displayed in figure 1 and table 1. Figure 1 shows that 5 mg lead acetate (PbAc) iv elevated the incidence of lethal hypoglycemic convulsions at the 4 insulin dosages used. Both figure 1 and table indicate that the lower doses of insulin, 0.5 and 0.75 U, though not lethal to saline controls did cause some deaths in lead-treated animals. However, statistically significant enhancement of lethality occurred with 1.0 U insulin (70%) and 1.5 U insulin (95%) with lead administration. Comparison of the overall % lethality in the saline and lead-treated groups regardless of the insulin dosage also reveals an increased sensitivity to insulin-induced hypoglycemic death. Lead acetate given alone is non-lethal at a dose of 5 mg/300 g rat (135).

Intravenous Glucose Tolerance After Acute Lead Administration

In order to further evaluate the altered insulin sensitivity accompanying acute lead administration, iv glucose tolerance testing was done 4 hours after 5 mg of lead acetate (PbAc) or sodium acetate (NaAc), using 200 mg of glucose. Figure 2 demonstrates a 50% reduction in half-time ($T_{½}$) of glucose disappearance, i.e. increased glucose tolerance in the lead treated rats. The basal or initial plasma glucose levels (C_i) of the 2 experimental groups were not significantly different (table 2). The

TABLE 1

INSULIN HYPOGLYCEMIC LETHALITY WITH ACUTE LEAD ADMINISTRATION[a]

Experimental Group	Number of Rats	Insulin Dose (Units/Rat)	Lethality
Control, 0.9% Saline	20	0.50	0%
	10	0.75	0%
	20	1.00	5.0%
	20	1.50	40.0%
	70[b]		12.8%
Lead Acetate, 5 mg	30	0.50	16.7%
	10	0.75	30.0%
	30	1.00	70.0%[c]
	20	1.50	95.0%[c]
	90[b]		53.3%[c]

[a] Overnight fasted rats received a 1 ml iv injection of either 5 mg/ml lead acetate or 0.9% saline immediately followed by a 1 ml injection of insulin sc. Lethality was recorded at 24 hours.

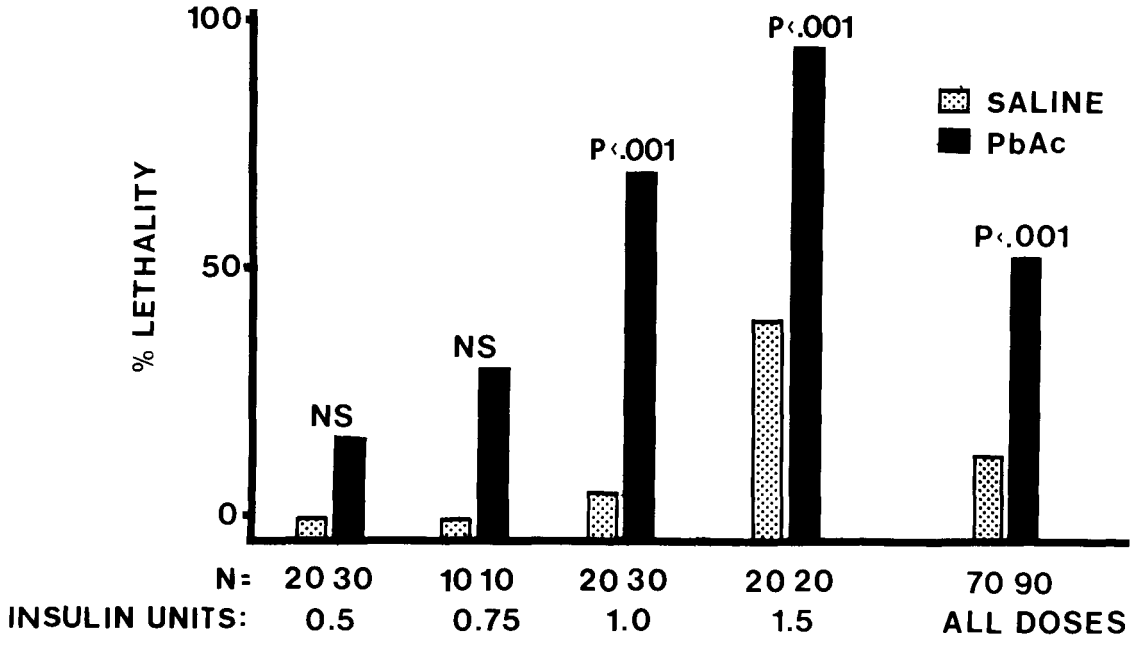
[b] Total lethality of all rats in the experimental group regardless of insulin dose.

[c] $p<0.001$ as compared to control groups using the Chi Square test with Yates correction factor.

FIGURE 1

EFFECT OF ACUTE LEAD ADMINISTRATION ON

INSULIN HYPOGLYCEMIC LETHALITY



Overnight fasted rats received a 1 ml iv injection of either 5 mg lead acetate or 0.9% saline immediately followed by a 1 ml insulin injection sc of one of 4 dosage levels. Lethality was recorded at 24 hours. N = the number of rats in the group. The 2 bars on the far right show % lethality of all rats in the experimental group regardless of insulin dose (all doses). Statistical significance of the data was evaluated using the Chi Square test with Yates correction factor. N.S. = not significant.

TABLE 2

GLUCOSE TOLERANCE (200 mg iv) AFTER ACUTE LEAD ADMINISTRATION[a]

Experimental Group	Number of Rats	Plasma Glucose Basal (C_i)[b] (mg/dl)	Plasma Glucose At Time Zero (C_o)[c] (mg/dl)	Half-Time ($T_{½}$) Glucose Disappearance (min)
Control, Sodium Acetate 5 mg	10	84 ± 3[d]	305 ± 10	49 ± 6
Lead Acetate 5 mg	10	75 ± 4	359 ± 21	24 ± 3
P-Value[e]		N.S.	0.032	<0.001

[a] Overnight fasted rats received a 1 ml iv injection of either lead acetate or sodium acetate (5 mg/ml) 4 hours before iv glucose tolerance testing.

[b] Plasma glucose immediately before injection of 200 mg glucose iv at 0 min.

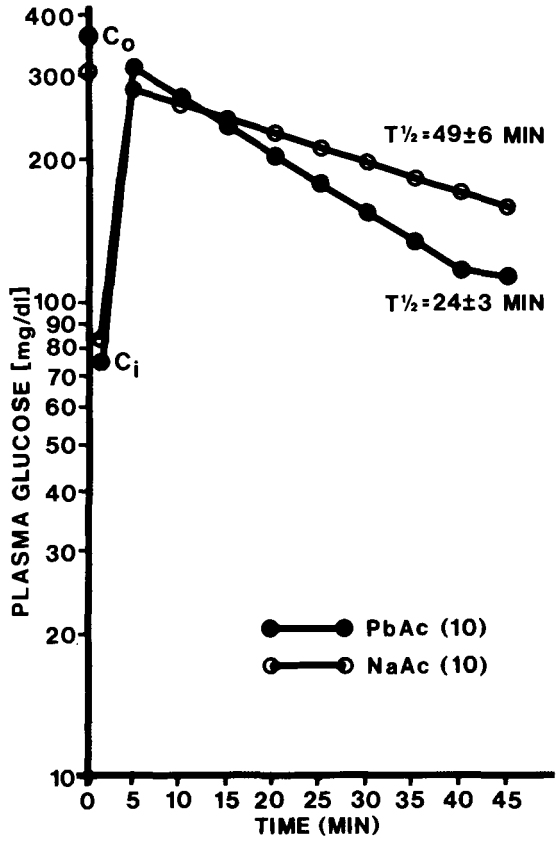
[c] Value of plasma glucose at time = 0 min obtained by extrapolation of the line -- $\log_{10}$ plasma glucose vs. time -- to 0 min.

[d] Mean ± standard error.

[e] Group data were compared using the unpaired Student's "t" test. P = <0.05 was considered statistically significant. N.S. = not significant.

FIGURE 2

EFFECT OF ACUTE LEAD ADMINISTRATION ON THE HALF-TIME OF GLUCOSE DISAPPEARANCE [200 mg iv]



Overnight fasted rats received a 1 ml iv injection of either 5 mg lead acetate or sodium acetate 4 hours before iv glucose tolerance testing. C_i is the mean basal plasma glucose of the experimental groups immediately before iv injection of 200 mg glucose at 0 min. Note that the lines are not drawn through the mean plasma glucose concentration at each time point but were drawn utilizing the mean theoretical plasma glucose concentration at time zero (C_0) and the mean half-time of glucose disappearance ($T_{½}$) of the 10 rats in each group.

group mean C_0, the glucose level at time zero, was significantly higher in the PbAc group compared to the NaAc group (table 2).

Basal and Glucose-Stimulated Serum Glucose and Insulin Levels 4 Hours After Acute Lead Administration

Since the preceding experiments suggested a role for the RES in modulating insulin activity, actual measurements of serum glucose and insulin (IRI) levels were done both in the basal fasting state and 5 min after 400 mg glucose iv. As shown in table 3 and figure 3, RES depression by administration of PbAc 4 hours prior raised basal serum insulin levels ($p = 0.018$) although serum glucose was unchanged, resulting in the elevation of the I/G (insulin to glucose) ratio. At 5 min after 400 mg glucose iv (table 4 and figure 4), there was a 6-fold increment in serum insulin levels ($p < 0.001$). Though serum glucose was higher in the PbAc group, the I/G ratio still showed a 6-fold increase compared to that for NaAc controls. This suggests an exaggerated insulin response to the prevailing glucose levels.

Basal and Glucose-Stimulated Serum Glucose and Insulin Levels After Colloidal Carbon Blockade

Further investigation of the effect of altered RES function on insulin regulation was carried out using inert non-toxic colloidal carbon (180) to induce a state of blockade or decreased phagocytic function (36). At 4 hours after injection of 32 mg/100 g of colloidal carbon, there was a decrease in basal serum glucose levels compared to the uninjected controls, ($p = 0.016$) but not the sham vehicle controls (table 5). The 4 hr carbon

TABLE 3

SERUM GLUCOSE AND INSULIN LEVELS 4 HOURS AFTER ACUTE LEAD ADMINISTRATION[a]

Experimental Group	Number of Rats	Serum Glucose (mg/dl)	Serum IRI[b] (μU/ml)	I/G[c]
Control, Sodium Acetate 5 mg	8	116 ± 2[d]	9.4 ± 0.5	0.082 ± 0.005
Lead Acetate 5 mg	8	110 ± 3	15.2 ± 2.1	0.139 ± 0.022
P-Value[e]		N.S.	0.018	0.024

[a] A 1 ml iv injection of 5 mg/ml lead acetate or sodium acetate was administered to fasted rats 4 hours before blood sampling.

[b] Immunoreactive insulin (IRI) was measured by the Phadebas method.

[c] Ratio of the serum IRI in μU/ml to serum glucose in mg/dl.

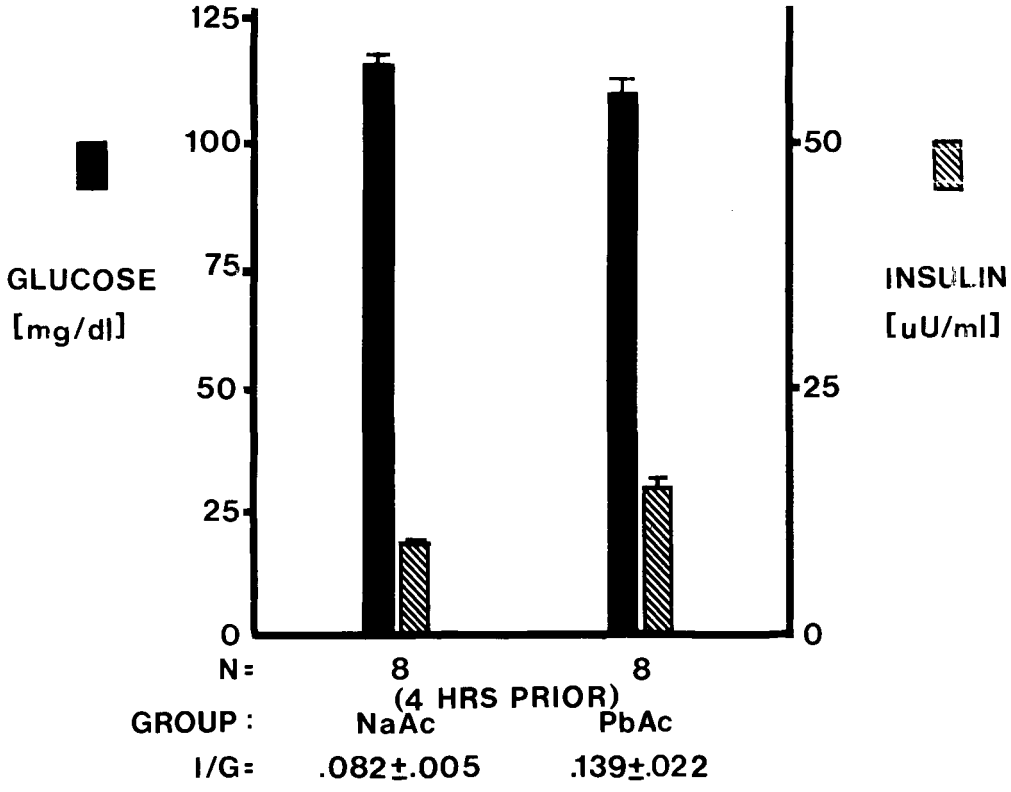
[d] Mean ± standard error.

[e] Group data were compared using the unpaired Student's "t" test. $P < 0.05$ was considered statistically significant. N.S. = not significant.

FIGURE 3

EFFECT OF ACUTE LEAD ADMINISTRATION ON

SERUM GLUCOSE AND INSULIN LEVELS



A 1 ml iv injection of either 5 mg lead acetate or sodium acetate was administered to fasted rats 4 hours before blood sampling. Basal serum glucose and insulin (IRI) levels are shown. I/G is the ratio of the serum IRI in μU/ml to serum glucose in mg/dl. N = number of rats per group.

TABLE 4

EFFECT OF ACUTE LEAD ADMINISTRATION[a] ON SERUM GLUCOSE AND INSULIN LEVELS 5 MIN AFTER 400 mg GLUCOSE iv

Experimental Group	Number of rats	Serum Glucose (mg/dl)	Serum IRI[b] (μU/ml)	I/G[c]
Control, Sodium Acetate 5 mg	8	569 ± 9[d]	34.6 ± 4.7	0.062 ± 0.009
Lead Acetate 5 mg	8	652 ± 27	235.5 ± 17.4	0.362 ± 0.024
P-value		0.011	< 0.001	< 0.001

[a] A 1 ml iv injection of 5 mg/ml lead acetate or sodium acetate was administered to fasted rats 4 hours before the time of glucose injection. Blood samples were taken at 5 min after 400 mg glucose iv.

[b] Immunoreactive insulin (IRI) was measured by the Phadebas method.

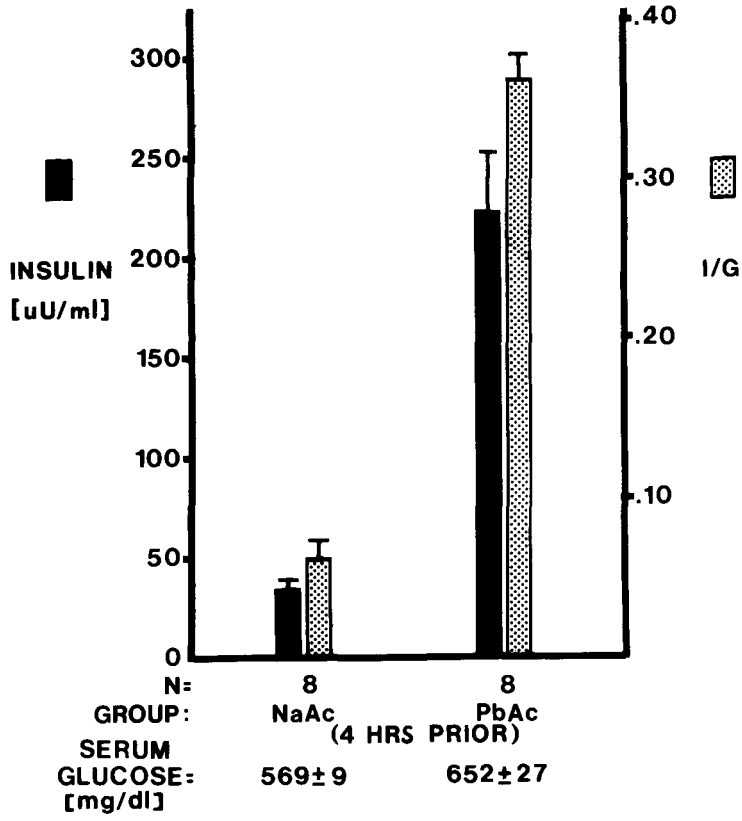
[c] Ratio of the serum IRI in μU/ml to serum glucose in mg/dl.

[d] Mean ± standard error.

[e] Group data were compared using the unpaired Student's "t" test. $P < 0.05$ was considered statistically significant.

FIGURE 4

EFFECT OF ACUTE LEAD ADMINISTRATION ON SERUM INSULIN LEVELS 5 MIN AFTER GLUCOSE [400 mg iv]



A 1 ml iv injection of either 5 mg lead acetate or sodium acetate was administered to fasted rats 4 hours before the time of glucose injection. Blood samples were taken 5 min after 400 mg glucose iv. Serum insulin (IRI) levels and the ratios (I/G) of the serum IRI (μU/ml) to serum glucose (mg/dl) are depicted. N = number of rats per group.

TABLE 5

SERUM GLUCOSE AND INSULIN LEVELS AFTER COLLOIDAL CARBON BLOCKADE[a]

Experimental Group	Serum Glucose (mg/dl)	Serum IRI[b] (μU/ml)	I/G[c]
A) Uninjected Control			
1) 4 hr prior	87 ± 4 (8)[d]	32.4 ± 4.0 (8)	0.385 ± 0.064 (8)
2) 8 hr prior	103 ± 4 (15)	20.9 ± 1.5 (14)	0.213 ± 0.019 (14)
3) 18 hr prior	68 ± 3 (6)	26.8 ± 2.6 (4)	0.384 ± 0.033 (4)
B) Sham Vehicle Control			
1) 4 hr prior	80 ± 5 (8)	20.9 ± 2.9 (8)	0.277 ± 0.049 (8)
2) 8 hr prior	106 ± 4 (14)	20.7 ± 2.0 (15)	0.193 ± 0.021 (14)
3) 18 hr prior	77 ± 2 (16)	27.0 ± 2.3 (6)	0.350 ± 0.028 (6)
C) Carbon Blockade			
1) 4 hr prior	65 ± 7 (8)	29.8 ± 0.8 (7)	0.460 ± 0.048 (7)
2) 8 hr prior	108 ± 4 (17)	39.8 ± 2.8 (17)	0.378 ± 0.033 (17)
3) 18 hr prior	67 ± 3 (8)	27.9 ± 2.7 (8)	0.427 ± 0.050 (8)

[a] Overnight fasted rats received 32 mg/100 g of colloidal carbon (3 ml of 32 mg/ml) or equivalent volume of vehicle iv or no treatment either 4 or 8 hr prior to blood sampling. For 18 hr blockade, fed rats were injected and then fasted 18 hr prior to blood sampling.

[b] Immunoreactive insulin (IRI) was measured by the Phadebas method.

[c] Ratio of the serum IRI in μU/ml to serum glucose in mg/dl.

[d] Mean ± standard error (number of animals).

TABLE 5 (cont.)

SERUM GLUCOSE AND INSULIN LEVELS AFTER COLLOIDAL CARBON BLOCKADE[a]

	Critical Comparisons[e]	P-values
Serum Glucose	A-1 vs. C-1	0.016
	A-3 vs. B-3	0.032
	B-3 vs. C-3	0.016
Serum Insulin	A-1 vs. B-1	0.035
	B-1 vs. C-1	0.011
	A-2 vs. C-2	< 0.001
	B-2 vs. C-2	< 0.001
I/G	B-1 vs. C-1	0.019
	A-2 vs. C-2	< 0.001
	B-2 vs. C-2	< 0.001

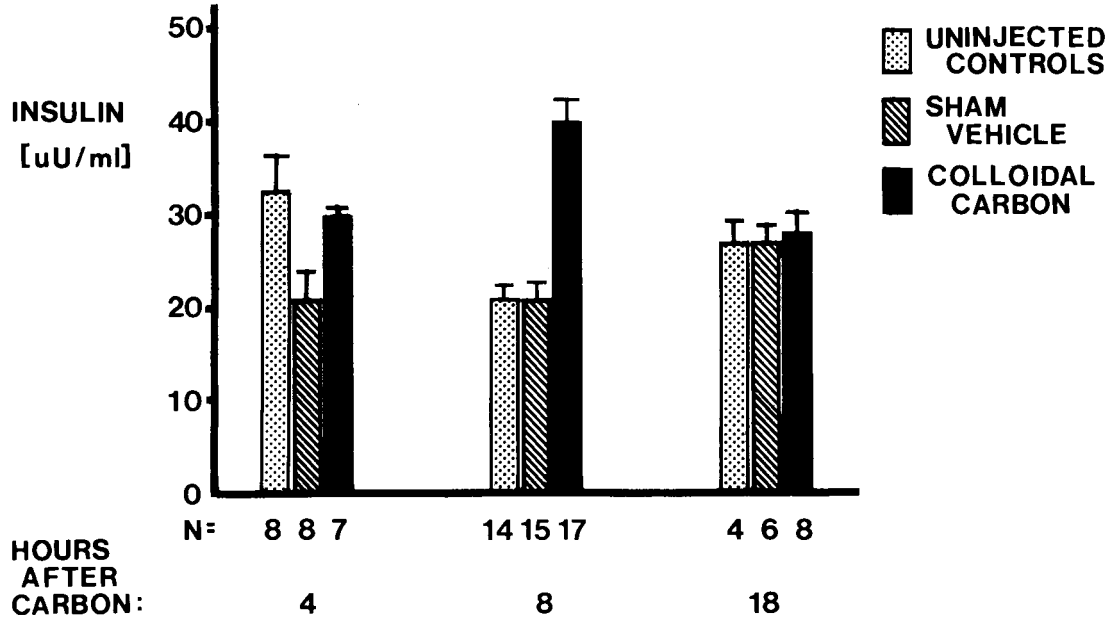
[e] Group data were compared using the unpaired Student's "t" test. Only statistically significant comparisons with p-value < 0.05 are given.

rats also showed an elevation in basal serum insulin ($p = 0.011$) in comparison to the sham vehicle controls resulting in an elevated I/G ratio (figure 5, table 5). But uninjected control basal serum insulin levels were higher than those of the sham vehicle group ($p = 0.035$) and not statistically different from those of the carbon-treated rats (table 5, figure 5). In contrast to the 4 hr carbon blockade study, 8 hr carbon blockade resulted in a highly significant doubling of basal serum insulin concentration compared to both control groups ($p < 0.001$) although the serum glucose concentration remained the same in all 3 groups (table 5, figures 5 and 6) resulting in a doubling of the I/G ratio. Table 6 and figure 7 display the effect of an iv 400 mg glucose stimulus on serum insulin levels 5 min after injection in the 8 hr carbon blockade group. An even more exaggerated 3-fold serum insulin increase occurred compared to both sham vehicle and uninjected controls ($p < 0.001$). Serum glucose levels were similar in all 3 groups, however, resulting in a corresponding rise in the I/G ratio (table 6, figure 7) in the 8 hr carbon blockade animals after glucose. In the fed rats which received carbon and were then fasted 18 hr (18 hr carbon blockade), basal serum glucose, insulin and the I/G ratios were not significantly different from the controls as seen in figure 5 and table 5.

FIGURE 5

EFFECT OF ALTERED RES FUNCTION

ON SERUM INSULIN LEVELS

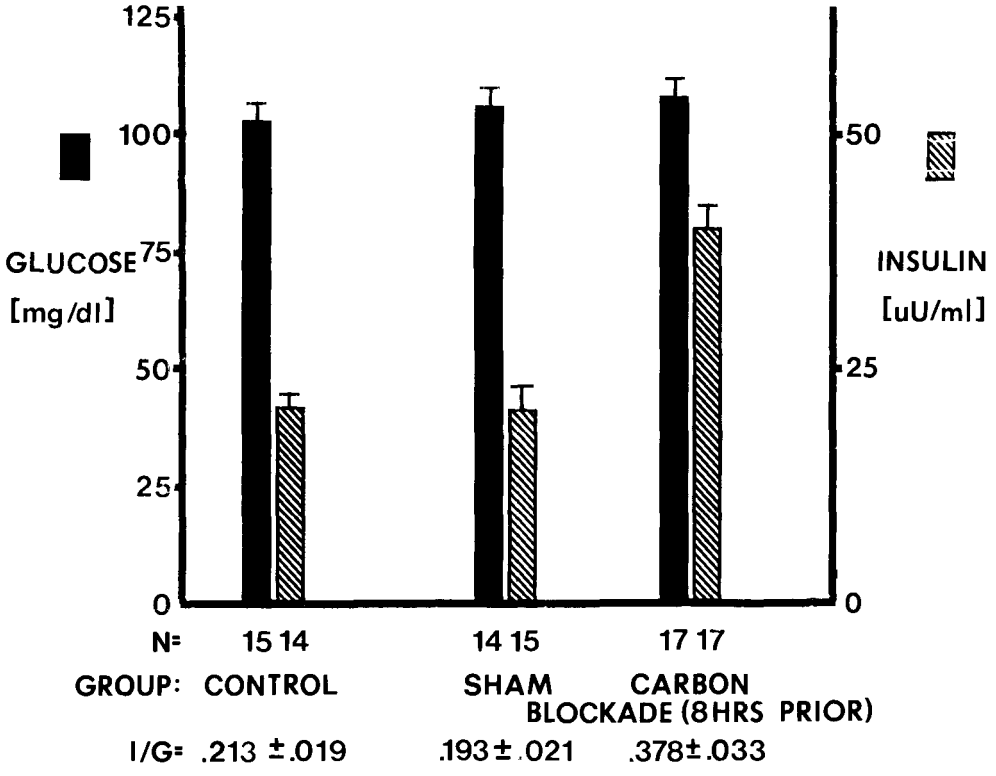


Overnight fasted rats received colloidal carbon (32 mg/100 g) iv, an equivalent volume of vehicle iv or no treatment either 4 or 8 hours prior to blood sampling. For the 18 hour group, fed rats were injected and then fasted 18 hours prior to blood sampling. Basal serum insulin (IRI) levels are depicted. N = number of rats yielding samples in each group.

FIGURE 6

EFFECT OF CARBON BLOCKADE ON SERUM

GLUCOSE AND INSULIN LEVELS



Data from the 8 hr colloidal carbon injected (carbon blockade), sham vehicle controls (sham blockade) and uninjected controls (Figure 5 and Table 5) are shown in detail. Serum glucose and insulin (IRI) levels are depicted. I/G is the ratio of the serum IRI in μU/ml to serum glucose in mg/dl. N = number of rats yielding samples in each group.

TABLE 6

EFFECT OF 8 HOUR CARBON BLOCKADE[a] ON SERUM GLUCOSE AND INSULIN LEVELS 5 MIN AFTER 400 mg GLUCOSE iv

Experimental Group	Serum Glucose (mg/dl)	Serum IRI[b] (μU/ml)	I/G[c]
Uninjected control	544 ± 21 (16)[d]	38.2 ± 2.2 (14)	0.077 ± 0.005 (14)
Sham Vehicle Control	531 ± 17 (17)	34.6 ± 2.1 (17)	0.065 ± 0.004 (17)
Carbon Blockade	539 ± 28 (17)	116.6 ± 9.7 (17)[e]	0.226 ± 0.022 (17)[e]

[a] Overnight fasted rats received 32 mg/100 g of colloidal carbon (3 ml of 32 mg/ml) or equivalent volume of vehicle iv or no treatment 8 hr before the time of glucose injection. Blood samples were taken at 5 min after 400 mg glucose iv.

[b] Immunoreactive insulin (IRI) was measured by the Phadebas method.

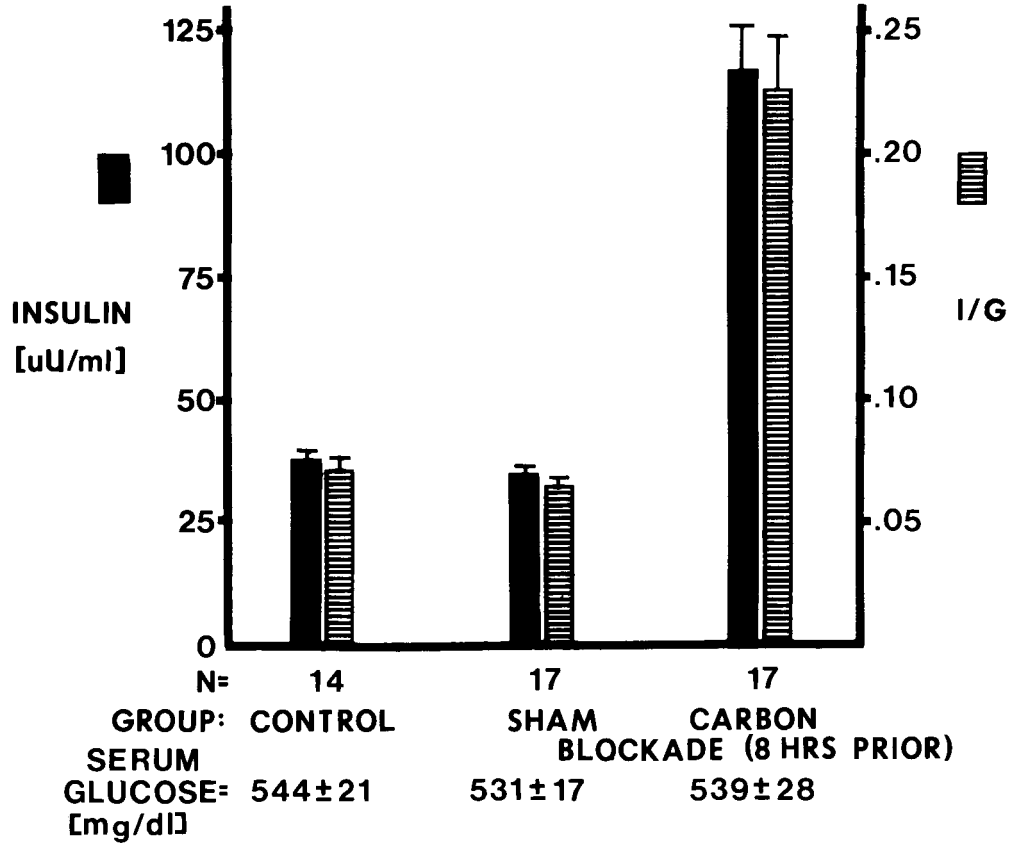
[c] Ratio of the serum IRI in μU/ml to serum glucose in mg/dl.

[d] Mean ± standard error (number of animals).

[e] $P < 0.001$ as compared to either the uninjected control or sham vehicle control groups using the unpaired Student's "t" test. $P < 0.05$ considered statistically significant.

FIGURE 7

EFFECT OF CARBON BLOCKADE ON SERUM INSULIN LEVELS 5 MIN AFTER GLUCOSE [400 mg iv]



Overnight fasted rats received colloidal carbon (32 mg/100 g -- carbon blockade) iv, an equivalent volume of vehicle (sham blockade) iv or no treatment 8 hr before the time of glucose injection. Blood samples were taken 5 min after 400 mg glucose iv. Serum insulin (IRI) levels and the ratios (I/G) of the serum IRI (μU/ml) to serum glucose (mg/dl) are depicted. N = number of rats yielding serum insulin samples in each group.

DISCUSSION

Effect of Acute Lead Administration on Insulin Hypoglycemic Lethality.

The toxic effects of lead were recognized since antiquity and descriptions of acute lead poisoning date back to Hippocrates and Nicander (69, 182). Starting from the seventeenth century the more subtle aspects of lead poisoning have been investigated, especially because lead is an ubiquitous environmental contaminant which primarily affects children with pica causing a constellation of hematological, neuromuscular, gastrointestinal and central nervous system disorders (69, 182, 234). One of the more ominous of the reported consequences of lead ingestion in man is a fatal shock episode (182).

Selye et al., while investigating the pharmacological actions of heavy metals observed that iv administration of a non-lethal 5 mg quantity of lead acetate to rats resulted in a 100,000-fold increase in endotoxin susceptibility (288). The lead enhancement of endotoxin shock lethality was maximal when both agents were administered simultaneously (80, 288). Filkins and Buchanan utilized this synergism to develop an endotoxin bioassay (53, 135, 143). Selye's hypothesis for the lead effect was that it produced a blockade of the RES (288). Subsequent studies substantiated that hypothesis since a non-lethal dose of lead acetate--5 mg/300 g rat--did depress the RES clearance of colloidal carbon (143) and ^{131}I-labeled lipid emulsion (330). Kupffer cell phagosomes containing electron-dense material (lead?) have been identified in both hepatic and splenic macrophages along with later hepatic ultra-

structural changes (80, 190) after lead administration. It has also been reported that parenterally administered lead salts attached primarily to erythrocyte membranes with the remainder rapidly precipitating to form colloidal particles which were distributed in the liver, spleen and bone marrow (84).

The mechanism of the lead sensitization to endotoxin remained a mystery. The degenerative morphological changes noted in hepatic Kupffer and parenchymal cells did not correlate with the time response of lead-induced endotoxin hypersensitivity (80, 190). Furthermore, Filkins and Buchanan demonstrated that lead administration altered neither the in vivo vascular clearance nor the in vitro hepatic detoxification of endotoxin (143). These investigators interpreted the data as indicating that a "metabolic lesion" was the basis of lead's action (143).

The combination of lead and endotoxin produced hypoglycemia, glycogen depletion and depression of gluconeogenesis in baboons (80), mice (272) and rats (140) all characteristic of endotoxin shock (26, 52, 54, 55, 56, 79, 87, 134, 140, 145, 146, 172, 188, 189, 225, 263, 272, 290, 291, 292, 303, 363, 364). Further experimentation revealed that lead acetate alone depressed gluconeogenesis from lactate, pyruvate and alanine both in vivo and in vitro using isolated hepatocytes (84, 140). Lead also produced glycogen depletion (140, 272). In vitro addition of lead acetate to isolated hepatocytes had no effect on gluconeogenesis (84), suggesting that the lead-induced alterations in glucose metabolism must be mediated by another substance.

With the knowledge that insulin is the dominant physiologic regulator of carbohydrate metabolism (97, 132), a lethality study was done to

determine whether the metabolic changes noted after RES depression by lead acetate might be due to alterations in insulin responsivity or regulation. A series of lethality experiments was done to evaluate the susceptibility to insulin-induced hypoglycemic convulsive death in lead-treated rats versus saline controls. Four different dosages of insulin were utilized randomly in the 2 groups of fasted rats. As depicted in both table 1 and figure 1, at every dose level, insulin administration resulted in hypoglycemic convulsions followed by death in a portion of the lead-treated rats. In comparison, note that both the 0.5 and 0.75 U doses of insulin were non-lethal in the saline treated control animals. Statistically significant elevations in lethality did occur with lead treatment at the higher dosages of 1.0 and 1.5 U of insulin. Comparison of the results in the 2 experimental groups regardless of insulin dose also revealed an increase in lethality in the lead acetate group. All deaths occurred 4 to 12 hours following insulin injection and were preceded by the signs and symptoms of hypoglycemia i.e. loss of consciousness, convulsive spasms and coma (124).

These results confirmed and expanded upon an earlier study by Buchanan and Filkins, in which leat-treated rats displayed increased sensitivity to one dose level of exogenous insulin and to endogenous insulin release induced by tolazamide as manifested by an elevation of the incidence of lethal hypoglycemia (52). Recently Filkins demonstrated that RES depression by colloidal carbon blockade markedly enhanced sensitivity to lethal insulin hypoglycemia in rats (142, 144). Since acute endotoxin administration produces an RES depressed state (20, 23, 179, 192, 323), the reports of Pieroni and Levine (263) and Buchanan and

Filkins (55) that endotoxin shock lethality was increased by administration of insulin or beta cell stimulants provide additional support for the notion that RES depression results in altered responsivity to insulin. Shands et al. also reported endotoxin-induced insulin sensitivity in mice; however, their results are difficult to interpret because the mice were infected with Mycobacterium bovis BCG prior to the experiment (292). Although insulin tolerance testing in rats given the RES depressants colloidal carbon (142), lead (52) or endotoxin (142) demonstrated an enhanced hypoglycemic response to exogenous insulin as shown by blood or plasma glucose measurements, the mechanism of this insulin sensitization remained unexplained.

One potential explanation for this phenomenon is an alteration of tissue responsiveness to insulin. This possibility has not been explored in depth. A second explanation could be a failure or diminished effect of insulin counter-regulatory systems such as glucagon, growth hormone, corticosteroids and the sympathetic nervous system (97, 124). Glucagon insufficiency has been proposed as a cause for hypoglycemia but it is a rare occurrence (124). The role of glucagon in glucose regulation has recently been minimized since glucagon has an evanescent effect on hepatic glucose production and no known effect on peripheral glucose disposal (68, 132). Saccá et al. reported that even though glucagon was involved in the metabolic reaction to insulin-induced hypoglycemia in the rat, its role was not essential because rats rendered glucagon-deficient tended to recover normally (281). Growth hormone is normally released during hypoglycemia (97) and a deficit in its secretion has been demonstrated only in ateliotic dwarfs (124). A deficiency

in glucocorticoids can exacerbate or even casue hypoglycemia but adrenal or pituitary hypofunction would have to be demonstrated (124). Finally the activity of the sympathetic nervous system normally is increased in a stressful state such as hypoglycemia (97) and its dysfunction is more likely the result of rather than the cause of a hypoglycemic reaction (124). A third possibility for the sensitization to insulin manifested after RES depression could be the production of additional non-suppressible insulin-like activity or NSILA; however, Eigenmann and associates reported that NSILA levels did not change following manipulations of blood sugar in the dog and they concluded that in physiological terms NSILA appears to be only an insulin-like growth factor (114). The fourth and most obvious explanation for the increased insulin lethality with decreased RES function is a defect in insulin regulation. The hypoglycemia resulting from the insulin increase in the animal would suppress pancreatic insulin secretion (97, 124) pointing to a potential defect in insulin removal or degradation. A decrease in insulin extraction by the key insulin degrading organs, especially the liver (133), could promote an elevation of circulating insulin in the RES depressed animal as has been demonstrated in the obese-hyperglycemic mouse (207). Before the advent of modern radioimmunoassay methods, insulin was assayed by noting the incidence of severe hypoglycemic reactions in mice (368), lending further support to the notion of a defect in insulin extraction and catabolism in the RES depressed state.

Intravenous Glucose Tolerance After Acute Lead Administration.

In order to further evaluate the altered insulin status accompany-

ing RES depression, iv glucose tolerance was measured in rats 4 hours after either lead acetate or sodium acetate treatment. As displayed in table 2 and figure 2, the lead-treated group demonstrated an increase in glucose tolerance with a reduction of the half-time of glucose disappearance from 49 min in the sodium control group to 24 min in the lead group. The basal fasting plasma glucose values in the two groups were not significantly different (table 2) and were in the appropriate range for fasting (360), indicating that the rats were in a normoglycemic state immediately before tolerance testing.

The iv glucose tolerance test was used because, even though it is a relatively non-specific test, the results are readily interpreted and the decrease in plasma glucose is significantly influenced by the effect of insulin on hepatic glucose production and peripheral tissue glucose uptake (132, 298, 360). Therefore, changes in glucose tolerance can be directly related to insulin activity and barring any evidence of insulin resistance, to the insulin levels present after the glucose stimulus (298). Normally an iv infusion of glucose results in two phases of pancreatic insulin secretion, a rapid phase beginning 1 min after the start of the infusion and lasting about 3 to 5 min followed by a slower phase beginning approximately 10 min after the start of the infusion (39, 267, 289). Stress is a significant cause of glucose intolerance and therefore false tolerance test results (360, 365). Increased sympathetic activity and catecholamine release have been shown to cause inhibition of pancreatic insulin output, retardation of glucose utilization and accelerated appearance of endogenous glucose (289, 295, 365). In order to eliminate this variable, the rats were anesthetized with sodium

pentobarbital, allowing a 15 min waiting period before the start of the test to insure that they were fully relaxed and asleep with a regular breathing pattern. The dose of glucose used was 200 mg/300 g or 0.666 g/kg which is within the accepted range of glucose dosages used for glucose tolerance testing (56, 87, 144, 295, 360).

In this study, RES depression by acute lead treatment increased glucose tolerance as shown by a 50% reduction in the half-time of glucose disappearance from the vascular compartment. This data agreed with the results of another study by Filkins and Buchanan in which they demonstrated that decreasing RES function by colloidal carbon blockade caused a 3-fold reduction in the glucose disappearance half-time (145). Thus in 2 different models of RES depression there was physiological evidence of an increase in insulin activity. In interpreting these data, it should be considered that there is no evidence at present for an altered response to insulin at the tissue level with RES depression alone. It is probable that the increased glucose tolerance resulted from elevated circulating insulin levels.

Several other alterations in glucose metabolism that hint of a functional hyperinsulinemic state have been reported with reduced RES function. Depression of hepatic gluconeogenesis was observed after acute lead administration (84, 140), colloidal carbon blockade (144) and endotoxin administration (56, 145, 146, 188, 225, 291). The occurrence of increased disappearance or utilization of glucose in endotoxin shock is well known (56, 188, 363). Measurements of _in vivo_ whole body glucose oxidation after lead (Filkins, unpublished results), carbon blockade (144) and endotoxin (56, 134) demonstrated increased

insulin activity at the tissue level. The endotoxin-induced hypercatabolism of glucose could be eliminated by the use of dexamethasone (134) or by rendering the rats insulinopenic by either mannoheptulose or streptozotocin treatment (56). Hypercatabolism of glucose by various tissues taken from endotoxic rats was also diminished by anti-insulin agents (134). The combination of decreased gluconeogenesis and increased glucose utilization in endotoxemia ultimately results in hypoglycemia (26, 55, 56, 134, 145, 146, 172, 188, 189, 290, 303, 363). Buchanan, in his investigation of the metabolic alterations of endotoxemia in the rat, performed ip glucose tolerance tests with insulin measurements in groups of rats; he noted no alterations of glucose tolerance despite an exaggerated insulin response (56). Endotoxic rats receiving saline also displayed an elevation of serum insulin levels (56), strengthening the notion that RES depression may foster a hyperinsulinemic state.

Basal and Glucose-Stimulated Serum Glucose and Insulin Levels After RES Depression.

With the knowledge that both hyperinsulinemia and hypoglycemia were present in the endotoxin-treated rat (56) and since acute endotoxin administration causes RES depression (20, 23, 179, 192, 323), which is characterized by altered glucose regulation suggestive of a hyperinsulinemic state (table 2) (56, 84, 134, 140, 144, 145, 146, 188, 225, 291, 363), it seemed logical to investigate the effect of RES depression on serum glucose and insulin levels. Two models of RES depression were investigated: acute lead treatment and colloidal carbon blockade. These models were utilized because of the previous data on altered glucoregulation and because they are non-lethal (135, 180) in comparison to

endotoxin which can cause a fatal shock episode. The effect of colloidal carbon would be especially significant since this is a classic, inert, non-toxic RES test and blockade substance (20, 21, 36, 179, 180, 309) which should not produce any deleterious effects in addition to RES depression. In contrast, acute treatment of 300 g rats with 5 mg lead acetate, though non-lethal (135), might have some toxic effects since heavy metals can combine with sulfhydryl and other groups to inhibit various enzyme systems and perhaps altering membrane permeability (182, 333). Both serum glucose and insulin were measured in the basal fasting state and 5 min after 400 mg of glucose iv, which allowed for adequate mixing and equilibration time in the vascular compartment, and should reflect the rapid phase of pancreatic insulin secretion (39, 267, 289). Since blood sampling was by cardiac puncture, the rats were anesthetized to minimize any influence of stress on insulin and glucose levels (289, 295, 365).

Table 3 and figure 3 present the basal fasting serum glucose and insulin levels 4 hours after acute lead administration. Filkins and Buchanan verified that RES function is decreased at that time (143). Serum glucose levels were unchanged and normal [60-120 mg/dl] (360) yet serum immunoreactive insulin or IRI showed a statistically significant elevation in the lead group. This resulted in an elevation of the insulin to glucose ratio (I/G) which indicates that there was an abnormally large amount of circulating insulin in the lead-treated rats relative to the pancreatic glucose stimulus. This ratio has been shown by Merimee and Tyson to be superior in the diagnosis and differentiation of hypoglycemia and hyperinsulinemia due to an insulinoma (228). Both the

sodium and lead-treated rats had insulin levels within the range of reported fasting plasma or serum IRI levels of nearly 0 μU/ml to 66 μU/ml (51, 162, 289).

Since basal insulin levels were higher in the RES depressed lead group, the effect of a 5 min glucose stimulation on serum IRI and glucose was investigated. As shown in table 4 and figure 4 serum insulin levels were incremented 6-fold in the 4 hr lead treatment group compared to the sodium control group. Serum glucose levels were elevated in the lead-treated rats; however, there was still a 6-fold elevation in the I/G ratio. Therefore, the lead-treated rats had markedly elevated serum insulin levels in relation to the serum glucose concentration present after glucose administration supporting the data on basal insulin and glucose in table 3 and figure 3.

Rather than choose only one time point in the study on insulin and glucose levels after colloidal carbon blockade, an attempt was made to find some correlation between RES function and insulin status. Basal serum glucose and insulin levels were measured at 4, 8 and 18 hours after colloidal carbon blockade and are given in table 5. Three experimental groups were used in this study: uninjected control rats, sham vehicle-injected rats which received a 3 ml iv injection of the vehicle from the colloidal carbon suspension and the carbon blockade group which received 32 mg/100 g body weight of colloidal carbon as a 3 ml iv injection. Although it might be argued that the sham and carbon groups alone would have been sufficient, the uninjected groups was added to check for any influences of the injection procedure e.g. stress or possible volume expansion and to provide normal values.

At 4 hours after carbon administration, the carbon-group had a decrease in serum glucose in comparison to the uninjected controls but not the sham vehicle-injected control group. There are 2 possible explanations for these data. Four hours after a blocking dose of carbon, the dose is still being cleared by the RES (227, 370) and the increased glucose utilization by the actively endocytizing macrophages (2, 8, 45, 70, 208, 210, 264, 265) may cause a lowering of the serum glucose concentration. The other factor in the sham vehicle-injected rats could be the 3 ml iv injection of vehicle which contains a fish glue gelatin (147, 247) protein solution that may remain in the vascular compartment for several hours. Dilution may have reduced the 4 hour serum glucose slightly in the sham group, making the carbon group appear hypoglycemic compared to only the uninjected rats and not the shams. The hormonal systems which maintain blood glucose (124, 281, 360) could also be acting to counter the lowering of serum glucose by dilution, preventing an even larger decrease. Serum insulin levels and the I/G ratio were elevated in the 4 hr carbon rats compared to the sham rats but not the uninjected rats (table 5 and figure 5). In fact the serum insulin levels in the uninjected rats were higher than those of the sham control rats. Again the most likely explanation for the diminished serum insulin levels in the sham vehicle-injected rats is possible dilution of the circulating insulin. The albino rat has an estimated blood volume of about 5 ml/100 g body weight (28, 193, 258). Since the rats weighed about 300 g, the blood volume should be approximately 15 ml and, assuming a hematocrit of 45 (28), the plasma or serum volume should be about 8.25 ml. Therefore, a 3 ml injection of a protein solution could enlarge

the plasma volume by up to one-third. For example, the insulin levels in the sham group, which were 20.9 ± 2.9 μU/ml, might have been approximately 28 μU/ml without the injection of vehicle and thus similar to the uninjected control insulin levels of 32.4 ± 4.0. This dilution effect may even have lowered the serum IRI in the 4 hr carbon group. The results of the 4 hour blockade study did not permit any firm conclusions to be made.

Because the 4 hour study hinted of a possible increase in serum insulin levels in the carbon injected rats, which may have been entering a state of RES blockade (12, 152, 205, 259, 369), the next study investigated the effect of an 8 hour carbon blockade on serum glucose and insulin levels. Several workers have found that at 6 to 12 hours after a blocking dose of colloidal carbon, RES endocytic activity is depressed in several species (12, 152, 213, 259, 369, 370). The results of the 8 hour carbon blockade are displayed in table 5 and figure 6. In contrast to the 4 hr study, 8 hr carbon blockade resulted in a doubling of basal fasting serum insulin levels compared to both the sham and uninjected control groups. The serum glucose levels were unchanged causing a doubling of the I/G ratio reminiscent of the results in the lead study.

After 400 mg of glucose iv, the rats subjected to an 8 hr carbon blockade had a 3-fold elevation in serum IRI levels. As in the basal state, the serum glucose values were similar in all 3 experimental groups as seen in table 6 and figure 7. Consequently, the I/G ratios in the carbon blockade group also showed a 3-fold elevation. Since colloidal carbon blockade is the best model for a "pure" RES depressed state, the results of the basal and glucose stimulation studies clearly

demonstrated that during RES depression there is an increase in the circulating serum IRI. The fact that normal serum glucose levels were maintained despite the hyperinsulinemic state is not surprising if one considers that the various components of the glucoregulatory system (124, 360) may be capable of counteracting the insulin increase. In fact these data may indicate that those systems are functional in the RES depressed animal. An elevation of glucagon levels could stimulate pancreatic insulin secretion (360); however, glucagon levels were not measured in this study preventing any conclusions regarding glucagon's role in the hyperinsulinemia observed. Insulin resistance is not a likely explanation for these data in view of the results of the earlier studies of RES depression which demonstrated: 1) a decreased resistance to lethal insulin-induced hypoglycemia (table 1) (52, 55, 142, 144, 263), 2) an enhanced hypoglycemic response to exogenous insulin (52, 142), 3) increased glucose tolerance (table 2) (144), 4) increased glucose oxidation (56, 134, 144) and utilization (188, 363, 364) and 5) decreased gluconeogenesis (56, 84, 140, 144, 145, 146, 188, 225, 291). Since the RES, especially the Kupffer cells, normally clear the blood of endotoxin (18, 20, 23, 41, 44, 53, 101, 129, 171, 179, 192, 196, 257, 274, 323, 350, 353, 370), RES hypofunction may foster an endotoxemia (41, 101, 257) which would produce the alterations in glucose metabolism and insulin regulation seen with RES depression. Unfortunately this possibility cannot be tested as there is currently no satisfactory method of assaying minute quantities of endotoxin in biological fluids. The older bioassay method to detect serum endotoxin was done using RES depressed animals (53, 135) and the limulus lysate test was reported to have no

clinical usefulness for detection of endotoxemia (117).

In order to verify that the hyperinsulinemia was a concomitant of RES depression, blood samples were taken at 18 hours after administration of a blocking dose of colloidal carbon. Several workers have noted that from about 18 hours through 4 days after carbon blockade, there was a recovery of RES function to either a normal or heightened level of endocytic activity (22, 38, 103, 205, 259, 301, 309, 369). Table 5 and figure 5 show that at 18 hours after carbon blockade, serum insulin levels were not significantly different from control levels. The serum glucose levels and I/G ratios were also similar in the 3 groups. The fact that serum insulin levels were normal when the RES function should have been returning to normal along with the preceding demonstration of hyperinsulinemia during RES depression divorced from a shock episode support the existence of a relationship between altered reticuloendotheilial system function and insulin regulation.

Proposed Role of the Reticuloendothelial System in Insulin Regulation.

This study was performed to investigate a relationship between altered RES function and insulin regulation. In the two models of RES depression that were utilized, colloidal carbon blockade and acute lead treatment, both a functional and an actual hyperinsulemia were demonstrated. These results suggested a possible role for the RES, particularly the Kupffer cells of the liver, in the modulation of insulin levels.

The hepatic macrophages normally are the principal endocytic cells of the body as evidenced by clearance studies (36, 179, 276, 309) and may also be equally important in the metabolism of the body. Some of the many metabolic, degradative and secretory functions of macrophages

were discussed in the literature review. What follows is a proposition that the hepatic macrophages may participate in the control of glucose homeostasis via regulation of peripheral insulin levels by the pinocytic uptake and degradation of insulin.

All the insulin-rich pancreatic venous effluent enters the portal vein and then passes through the liver before reaching the other tissues. During one trans-hepatic passage, Field, Mortimore and others have reported that 40 to 50% of portal venous insulin is removed (47, 133, 202, 236, 237), making the liver the chief site of insulin extraction in the body (47). In support of this statement, hyperinsulinemia has been detected in cirrhotic patients with portal-systemic shunting of blood and after portocaval anastomosis (197, 294, 297). Johnston et al. have presented data suggesting that cirrhosis of the liver causes a peripheral hyperinsulinism by decreased hepatic insulin degradation and not increased insulin secretion (197). Both hyperinsulinemia or elevation of the I/G ratio have also been noted in cases of fulminating hepatitis (131, 282). However, despite the numerous investigations of hepatic insulin handling, the Kupffer cells have not been considered although they comprise about 33% of liver cells (31). Past investigators of insulin extraction and degradation tended to regard the liver as a homogeneous organ composed of only parenchymal cells since they were the largest cell population in the liver (31) which was studied in toto.

Early experiments tested the ability of the liver and other tissue homogenates to degrade insulin by rendering isotopically labeled insulin trichloroacetic acid-soluble (63, 116, 215, 322, 327, 361). These studies resulted in the recognition of two types of insulin-degrading

enzymes. One type is glutathione-insulin transhydrogenase (GIT) (63, 209, 232, 322, 326, 327, 335, 336, 340), which requires reduced glutathione or other thiol compounds (64, 335) to reduce the disulfide bonds of insulin, resulting in the separation of the A and B chains. The other type of enzyme includes the insulin proteases, which proteolytically degrade the polypeptide chains (50, 57, 215, 322, 337). Two studies on the tissue distribution of insulin degrading enzymes demonstrated that the liver possessed a high content of both GIT and insulin protease (63, 215). Generally there was an inverse relation between tissue insulin sensitivity and tissue insulin-degrading activity (63).

The exact location of the insulin degradation process remains controversial. Plasma membrane-bound insulin-degrading activity has been reported (155, 181, 252, 338) but may be an artifact of cell fractionation (338). However the insulin-degrading enzymes are primarily intracellular (50, 57, 173, 181, 215, 322, 337). This results in a paradox in that the standard model for insulin action at the cellular level entails reversible binding to an external membrane receptor protein resulting in intracellular changes via "second messengers" (88, 169, 204, 218) with the bound insulin remaining undegraded (155). However Cuatrecasas' clever study to prove that insulin interaction with superficial cellular receptors initiated insulin's metabolic effects (88) has been disproven. The insulin-sepharose complex that he utilized has since been shown to dissociate into a super-active free form of insulin when in contact with biological materials (169, 328). Isolated intact cells have also been shown to degrade insulin (62, 181, 219, 252, 318, 339), the amount of degradation being directly related to the

amount bound to the receptor (318). Thus the receptor may function in bringing insulin into contact with the degrading systems which then terminate the hormonal signal at the cell membrane (308, 318).

Misbin et al. found that insulin extraction by the isolated perfused rat liver cannot be explained by the properties of the previously described insulin-degrading enzymes (233). Others have noticed that there is a lag between the initial removal and subsequent degradation of insulin by both isolated perfused rat liver (194, 236, 319) and rat hepatocytes (318) that was not seen in the studies using broken-cell or isolated enzyme preparations (50, 57, 63, 64, 88, 155, 173, 209, 215, 232, 252, 322, 326, 327, 335, 336, 337, 338, 340, 361). Also factors which are thought to decrease the mobility of cell membrane components, such as decreased temperature (2, 83, 240, 273) and concanavalin A (29), have been shown to depress insulin degradation (236, 318, 319), with little interference with insulin extraction (236), binding (319) or degradative enzyme reactions (236). Numerous reports of intracellular localization of labeled insulin in intact cells or tissues, after iv injection or in vitro addition, exist (17, 46, 109, 116, 157, 169, 194, 221, 232, 252, 307, 322, 366). Recent studies have uncovered intracellular insulin receptors (169, 191), implying that insulin may in fact enter cells and bind to subcellular organelles. Several investigators have therefore proposed that the missing link between extracellular binding and intracellular degradation could be the entrace of insulin into the cell (194, 221, 307, 322), perhaps by pinocytosis (15, 169, 170, 232, 308).

The preceding review represents the state of the art as far as published research papers on insulin extraction and degradation are con-

cerned. It may be that Kupffer cells are the missing link in the explanation of hepatic insulin extraction and degradation. As was mentioned before, these cells are the first hepatic cells to be in contact with insulin secreted by the pancreas. Kupffer cells can carry out all the various endocytic processes i.e. phagocytosis, macropinocytosis and micropinocytosis (239, 240, 242) which formed the basis for their classification into the mononuclear phagocyte system with other endocytic RES cells (158). It seems reasonable to infer that the Kupffer cells may participate in hepatic insulin metabolism via a pinocytic mechanism. Although insulin receptors have not been identified on hepatic macrophages as of yet, they have been found on both monocytes (154, 203, 204, 250, 254, 255, 286) and splenic macrophages (13, 271). Since macrophage glucose metabolism appeared to be insulin independent, (165, 174, 264), one is left with the paradox of avid insulin binding without the familiar metabolic consequences. Insulin had been shown to cause some less orthodox effects in lymphocytes such as increased ATPase activity (177) and augmentation of T-lymphocyte-mediated cytotoxicity (315). In fact T-lymphocyte insulin receptors appear on the cell concommitant with cellular transformation and immune activation (217, 315). Insulin would thus seem to be involved in regulation of immunological functions. Bar and associates have demonstrated that insulin caused inhibition of antibody dependent cytotoxicity in spleen macrophages (13). This effect may be mediated by the insulin modulation of macrophage Fc receptor expression (271).

The reports of Rhodes (271) and Bar et al. (13) both point indirectly to a possible insulin induction of endocytosis which could be instru-

mental to the subsequent degradation of insulin by macrophages, especially the Kupffer cells. Stossel et al. did report that insulin slightly stimulated the phagocytic rate of polymorphonuclear leukocytes (314). Insulin appeared to enter adipose tissue cells (15) and renal proximal tubule cells by endocytosis (46). Incubation of fibroblasts with insulin resulted in the formation of numerous microvilli on the cell surface (128, 198). It could be argued that insulin was merely promoting the growth of the fibroblasts and causing a pleiotypic response (187) but insulin has been shown to promote microtubule assembly in cells (262, 300) without significant effects on protein synthesis (262). Microtubules and microfilaments probably mediate changes in cell membrane conformation, especially those associated with endocytosis (246, 310, 311, 312, 313). The insulin molecule has minimum solubility in body fluids at pH = 5.0, suggesting that it exists as an anionic molecule at physiological pH (162). Cohn has mentioned in several papers that anionic molecules tended to stimulate pinocytic activity in macrophages (71, 72, 76, 113). Thus insulin may induce endocytosis, probably pinocytosis in macrophages.

Heightened endocytic activity has been shown to result in a depletion of macrophage cellular membrane components including receptors (73, 239, 266, 269, 354, 355). It is then logical to suggest that insulin receptors may also be internalized during the endocytosis of a colloidal overload such as a blockade-inducing dose of carbon. The down-regulation of insulin receptors, characterized by a reciprocal relationship between insulin concentration in the extracellular fluid and the concentration of cellular insulin receptors (164), may involve

internalization of the insulin-receptor complex (204, 308). Kahn et al. have discussed the possibility of a pinocytic uptake of the receptor with its subsequent enzymatic degradation inside the cell (204). Inhibitors of protein synthesis prevented the receptor degradation (204). It seems probable that these enzymes may be contained in lysosomes which are abundant in Kupffer cells (2, 25, 241, 299). Steiner has proposed that insulin enters intact cells perhaps by endocytosis of an insulin-receptor complex which could then dissociate, allowing the hormone or its fragments to act directly on cellular components (308). Grisolia and Wallace found hepatic lysosomes to be rich in both GIT and "insulin specific protease" (173) lending support to a lysosomal theory of degradation of insulin. Human leukocytes were shown to contain GIT (65), so monocytes and other RES cells may possess insulin degrading activity. During the induction of a diabetic state with streptozotocin, wandering macrophages disposed of the remaining insulin granules within the pancreas (200, 201). Kupffer cells have also been reported to take up and degrade insulin antibody complexes (157, 347) and to concentrate labeled insulin (17, 307, 366). A preliminary report by Wagle (347) that "much higher degradation of insulin was found with Kupffer cells than with hepatocytes," which appeared after this study was completed, may confirm the participation of Kupffer cells in the hepatic degradation and thus modulation of insulin levels.

Even if Kupffer cells are proved to degrade insulin in vitro further experimentation will be needed to elucidate the exact mechanisms of the cellular uptake and enzymatic activites involved. In order to determine whether and what type of endocytosis is involved, studies using

isolated Kupffer cells and various inhibitors of endocytosis should be fruitful. Since insulin is a relatively small molecule, and in solution, the most likely mechanisms for its uptake by macrophages would be micropinocytosis, which is inhibited by low temperature or decreased membrane fluidity, or perhaps macropinocytosis, which is inhibited by cytochalasin B and agents interfering with metabolism or protein synthesis (2, 70, 72, 113, 240). Since Terris and Steiner have reported that concanavalin A depressed insulin degration in the isolated perfused rat liver (319), these results may be reinterpreted to support a pinocytic entrance of insulin into the Kupffer cell with secondary lysosome formation and degradation. Edelson and Cohn reported that concanavalin A, rather than inhibit endocytosis (29), stimulated pinosome formation but prevented fusion of the vesicles with lysosomes (110) rather than decreasing membrane fluidity (29, 319).

Studies on isolated Kupffer cells should also be done to confirm whether a GIT-like enzyme for degrading is present. Glutathione, which is utilized in the GIT reduction of insulin, is ubiquitous in the body tissues and is found in high concentration in insulin-degrading tissues (346). Glutathione has been shown to be essential to alveolar macrophage metabolism (344). It is probable that macrophages, including Kupffer cells, do produce glutathione and other thiol containing compounds (66) which could be used to degrade insulin (64, 335). Since lead inhibits enzyme systems via combination with sulfhydryl groups (182) it may interfere with the activity of GIT and insulin degradation at the molecular level in addition to causing decreased RES endocytic function. Cysteine and other sulfhydryl compounds protected rats from

lead acetate sensitization to endotoxin (34, 79, 80), perhaps by maintaining the normal function of the insulin degradative system.

To verify that RES depression is causing a diminished hepatic extraction and degradation of insulin, studies should be performed using the isolated perfused liver. In shock, which is accompanied by RES depression, in vivo measurements of insulin clearance showed a prolongation of the half-time of insulin disappearance (61, 87, 226); however, these results are complicated by a possible decrease in blood flow to the liver (40). The in vivo cardiovascular variable would be eliminated in the isolated organ preparation which would also maintain the Kupffer cells in their normal condition lining the sinusoids. There should also be studies done to determine whether macrophages, especially the hepatic Kupffer cells, may have a role in insulin regulation by influencing pancreatic insulin secretion. Leukocytic endogenous mediator is a protein reported to be produced during infection by macrophages, as well as leukocytes (19, 166, 206, 260). This substance has been shown to cause elevations in pancreatic insulin via a central nervous system-mediated response (166). Much clarification of the nature and effects of this substance is still needed.

The results of the present study and all the above evidence do suggest a potential role for the hepatic Kupffer cells in insulin regulation. Although not definitively proven, the macrophages of the RES may interact with insulin not only in terms of regulation of the immune response (13, 271) but by regulating peripheral insulin levels. The Kupffer cells seem especially well-suited for a role in modulating the amount of insulin presented not only to the peripheral tissues but to

the parenchymal cells of the liver which control blood glucose levels. By their maintenance of circulating insulin concentrations which promote a normoglycemic state in the organism, the Kupffer cells would also insure their own substrate supply. Consequently, the Kupffer and parenchymal cells of the liver may function together to insure glucose homeostasis.

Conclusions

In summary, the present study demonstrated that:

1. depression of RES function by acute lead treatment sensitized rats to lethal insulin-induced hypoglycemia;
2. RES depression by acute lead administration increased glucose tolerance;
3. RES depression by either colloidal carbon blockade or acute lead administration produced an elevation of both basal and glucose-stimulated serum insulin levels.

Therefore it is concluded that there is a relationship between altered RES function and insulin regulation since RES depression resulted in a functional and actual hyperinsulinemia. These data also suggest a novel role for the RES in the modulation of insulin levels, perhaps by participation in the hepatic extraction and degradation of insulin.

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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