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MECHANISMS AND REGULATION OF MACROPHAGE GLYCOGEN METABOLISM

by

Paul W. Gudewicz

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

February

1975

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CHAPTER I

INTRODUCTION

The inflammatory response is a complex interdependent sequence of local vascular, cellular and tissue events that launch reparative processes at the host's damaged tissue. The acute inflammatory response is heralded by vascular dilatation and marked alterations in vascular permeability leading to the exudation of protein and cellular elements at the inflammatory site. The macrophage, i.e. the mononuclear phagocyte, and polymorphonuclear leukocyte (PMNL) constitute the two major phagocytic cell populations that accumulate at the site of tissue injury. The cellular development of the local inflammatory reaction classically features an early infiltration by the PMNL which is subsequently replaced by the inflammatory macrophage as the principal phagocytic mediator of the host's defense reactions (105). Macrophages and PMNL at the inflammatory locus perform the vital functions of clearance, degradation and detoxification of invading foreign organisms and cellular debris by employing endocytic and intracellular digestive mechanisms. However, the homeostatic control mechanisms and cellular interactions influencing the metabolic demands among inflammatory cell populations are poorly understood. The maintenance of PMNL and macrophage function is ultimately contingent upon the continual provision of substrate supply and cellular energy. The relative importance of glycolytic and oxidative pathways

as energy sources varies markedly among the different macrophage and PMNL populations (85). Early in vitro studies centering on macrophage metabolism focused attention on metabolic alterations subsequent to phagocytosis (66). Glycolysis and respiration were transiently stimulated during the phagocytic event in macrophages, however certain macrophage populations are capable of deriving their energy for phagocytosis exclusively from glycolysis under anaerobic conditions either in vitro or at the inflammatory site where low oxygen tensions prevail.

Macrophage endocytic capacities include both phagocytosis and pinocytosis - terms utilized to describe the cellular ingestion of particulate matter and soluble components of their external environment, respectively. In addition to its role in microbial host defense, endocytosis affords a mechanism whereby the inflammatory macrophage enriches its fuel supply by ingestion and degradation of macromolecules found at the inflammatory site. Glycogen, an important carbohydrate fuel reserve, is one such macromolecule which is found in abundance in the PMNL (109). Histological studies have provided evidence that the glycogen-laden PMNL sheds cytoplasmic fragments during the early course of the inflammatory response while the macrophage rapidly engulfs this potential source of fuel molecules (141).

Although glycogen content and the mechanisms responsible for glycogen synthesis and degradation have been extensively investigated in various leukocyte populations, glycogen content and its regulation within macrophages have not received similar quantitative attention. Thus the intents of this study are to evaluate in vitro the physiological responses of macrophages to exogenously added glycogen and to

assess glycogen metabolism and those control mechanisms responsible for glycogen synthesis and utilization within the macrophage derived from the inflammatory locus.

CHAPTER II

HISTORICAL REVIEW

A. The Role of the Macrophage in Inflammation

1. Historical development and knowledge of the macrophage system

Although it was known that different forms of leukocytes occurred in blood was in evidence as early as 1865 (108), the early functional history of the mononuclear phagocyte pivots around the exhaustive studies of Metchnikoff (82). In a series of comparative studies concerned with the cellular response following tissue injury, Metchnikoff investigated the phagocytic activity of inflammatory cells derived from both invertebrate and vertebrate species. He demonstrated the ultimate dominance of the phagocyte in the defense reactions of the host to inflammatory stimuli. Furthermore, he also was the first to distinguish between the macrophage, i.e., big eater, and the microphage, the smaller polymorphonuclear leukocyte. In the following decade, Goldman (49) and others employed vital staining techniques and illustrated the ubiquitous occurrence and organ localization of mononuclear phagocytes. As an outcome of these early histological studies Aschoff (3) coined the term reticuloendothelial system (RES) to describe the concept of a widely distributed collection of mononuclear phagocytes which are united by similar morphological and functional properties and potentialities. The RES includes both the fixed macrophage populations localized primarily in the blood

sinuses of the liver, lung, spleen, and bone marrow and the mobile macrophage pool found in the serous cavities of the organism. Thus following Aschoff's formulation of the RES concept as a phagocytic functional system and metabolic apparatus involved in host defense mechanisms, all subsequent investigations have resulted in determining the potential and multiple roles of the mononuclear phagocyte in physiological and pathophysiological settings.

2. Origin of the inflammatory macrophage

The origin and identity of the precursor cell which gives rise to the macrophage of the inflammatory exudate have been studied and debated for more than thirty years. Ebert and Florey (39) using rabbit ear chambers observed that monocytes infiltrated from the peripheral blood into the inflammatory site and differentiated into macrophages. However, it was argued by Rebuck and his colleagues (96, 98) that inflammatory macrophages were derived primarily from lymphocytes. Rebuck utilized the "skin window" technique in which glass coverslips were applied to areas of abraded human skin and then periodically removed and examined microscopically. It was concluded from these studies that small lymphocytes evolved through a series of intermediate cell types into inflammatory macrophages.

Recent in vivo and in vitro labelling studies have supported the conclusions that under physiological conditions and during the acute inflammatory response, the inflammatory macrophage is principally derived from peripheral blood monocytes. That mononuclear phagocytes ultimately originated from bone marrow precursors was first demonstrated by Balner (5) in radiation chimeras given allogenic bone marrow cells.

Both transfusion studies with ^3H -thymidine labelled bone marrow cells into syngenic rats (129) and labelling studies in X-irradiated animals with shielding of the bone marrow (126) have provided direct evidence not only for the existence of monocyte precursors in the bone marrow but also that the peritoneal macrophage population is derived from blood monocytes.

Induction of an inflammatory response in the peritoneal cavity of mice resulted in a two- to threefold increase in the number of peritoneal macrophages by 72 hours following the introduction of an irritant (19). In vitro incubation of these peritoneal macrophages in the presence of ^3H -thymidine indicated a labelling index comparable to peritoneal macrophages from unstimulated animals, suggesting that the increase in macrophage population as a result of the inflammatory stimuli is not the result of increased mitotic activity (126). However, when peripheral blood monocytes were labelled in vivo with repeated injections of ^3H -thymidine and a peritoneal inflammatory response was induced, these experiments resulted in a 45 to 65% increase in the labelling of inflammatory peritoneal macrophages. Thus these experiments add overwhelming evidence in favor of the blood monocyte origin of the inflammatory macrophage.

3. Morphology and maturation of the inflammatory macrophage

Maximow and Bloom (80) provided the first detailed account of macrophage morphology and distinguished macrophages from the blood lymphocyte by their "active amoeboid protoplasm" and inclusion bodies. Macrophages are large (20-40 μ) cells having an eccentrically located horseshoe-shaped nucleus with an azurophilic granular cytoplasm

containing numerous dense bodies and mitochondria (17). An extensive Golgi apparatus occupies the perinuclear area with lysosomal-like granules and mitochondria located at the periphery of the Golgi zone. At the light microscope level, the macrophage cell membrane is characterized by a ruffled appearance. When macrophages are allowed to adhere to a glass surface, the cell membrane becomes dramatically well spread out and intensely ruffled (46).

Recently, the ultrastructure of the peritoneal macrophage has been under intense investigation (13, 22, 37) and has revealed a complex cytoplasmic structure and many prominent cell membrane processes. The macrophage membrane surface is studded with spherical indentations 0.1μ in diameter suggesting ongoing active pinocytic vacuole formation. A granular endoplasmic reticulum and Golgi apparatus are well developed and have been shown to be the site of formation of the primary lysosome. In the mature macrophage many smooth surfaced vesicles (50-100 nm in diameter), the primary lysosomes, are distributed throughout the Golgi zone. The most prominent cytoplasmic inclusions are the large number of electron-dense granules demonstrated to be lysosomal in character by their positive acid phosphatase reaction (14). Such heterogeneous granules are seen to contain varying amounts of lipid or dense staining material and are clearly secondary lysosomes. Labelled marker particles have been demonstrated to be incorporated within these lysosomal structures by combination with phagocytic or pinocytic vacuoles formed at the membrane surface (16).

Peritoneal macrophages in culture have been extensively used to study the maturation process of monocyte to macrophage differentiation.

Lewis (77) first observed, by culturing mixed blood cells, that blood monocytes altered their morphological appearance and matured into large macrophages, epithelioid and giant cells. More recent studies have concluded that the induction of macrophage maturation by in vitro culturing techniques or in vivo, by lipopolysaccharide stimulated animals, resulted in similar morphological and biochemical alterations (19, 21). It was demonstrated by these authors that mouse peritoneal cells underwent a temporal sequence of morphological alterations including an increase in the number of phase-dense, acid phosphatase-positive granules and mitochondria. In addition, the size of the Golgi apparatus was markedly increased as well as the number of large lipid inclusions that were associated with the rough endoplasmic reticulum. Thus it is apparent that the arrival of the blood monocyte to the inflammatory site alters the macrophage into a large cell with more digestive capacity and phagocytic potential resulting in a dramatic stimulation in the functional properties of the inflammatory macrophage.

4. Migration behavior of the macrophage

Subsequent to all injury, alterations occur in the immediate vasculature and local tissue milieu leading to the emigration of phagocytic cells and the extravasation of plasma constituents. Although the PMNL is the preponderant cell type mobilized during the initial stage of the inflammatory reaction, within 24 hours, macrophages infiltrate the inflamed area and develop into the dominant phagocytic cell (105). The mechanisms underlying this temporal sequence and the eventual localization of the macrophage are poorly understood. Difference in PMNL and macrophage migration rates have been postulated as to why these two

cell types do not appear simultaneously during the developing inflammatory reaction (138).

Chemotaxis, the directional movement of cells toward a chemical substance, has been the in vitro approach utilized to study the mechanism of phagocytic cell migration (54, 81). The chemotactic effect of a variety of substances is thought to be due to the formation of mediators (cytotaxins) produced by the interaction of these substances with normal plasma or serum (117). Several authors have suggested that there is no striking difference in the chemotactic response in vitro of macrophages and PMNL (54, 69). However, Ward (133) has recently reported several chemotactic factors specific for mononuclear cells. One of these factors was derived from PMNL lysates indicating a functional relationship between these two cell's migratory behavior. Furthermore, the absence of circulating neutrophils has been reported to markedly reduce the appearance of mononuclear cells in experimentally induced inflammatory reactions (87).

Other chemotactic factors to which mononuclear cells respond include a factor generated in serum by interaction with antigen-antibody complex, fragmentation products of the third and fifth components of complement, and soluble bacterial factors. The interaction of lymphocytes derived from animals exhibiting delayed hypersensitivity reactions with specific antigen produces a material, migration inhibiting factor, inhibiting the random motion of normal macrophages in vitro (30).

From these studies, it is obvious that a variety of processes at the inflammatory site generate soluble chemotactic factors whose

activities can be demonstrated in vitro, however the relative importance of the factors under in vivo conditions remains to be identified. Although the cellular energetics involved in macrophage migration has not been adequately explored, recent information relative to the biochemical events of how chemotactic agents organize cell movements has assigned a major role in the mobility of phagocytes to contractile proteins and a microtubular system which possesses divalent cation-sensitive ATPase activity (119).

5. Endocytosis and digestion by the macrophage

The physiological expression and functional classification of the macrophage are ultimately dependent upon its ability to engulf material from the external environment. This endocytic process encompasses both phagocytosis, the ingestion of particulate matter, and pinocytosis, the ingestion of soluble components of the external medium. The literature pertaining to phagocytosis is enormous and has been repeatedly reviewed (9, 17, 83, 84, 119). Although pinocytosis was first described over forty years ago (77), only recently has this endocytic process been extensively examined and reviewed (17, 41).

a. Phagocytosis:

Phagocytosis is a complex cellular phenomenon requiring on the part of the macrophage the expenditure of energy and the interaction of the particulate matter with certain properties of its limiting membrane.

Investigations into the mechanism of phagocytosis have classically divided the endocytic event into the following sequence-recognition and attachment, ingestion, and digestion of foreign material.

The macrophage displays selectivity in what will be phagocytized

by recognizing certain characteristics on the surface of the material to be ingested. The importance of surface requirements for phagocytosis was first recognized by Wright and Douglas (140) who observed that bacteria had to interact with unknown serum components, which they named "opsonin", before phagocytosis would occur in vitro. Recent investigations have identified certain immunoglobulins that are able to bind selectively to the macrophage surface membrane (10). These antibodies cytophilic for macrophages have been identified as IgG and represent the heat stable portion of hyperimmune serum opsonic activity although other immunoglobulins can also bind to the macrophage surface (8). A heat labile component of serum recently demonstrated to express opsonic activity has been identified as the C3 component of the complement protein (65). Although the mechanism by which macrophages respond to an opsonic surface is not known, there is evidence that the Fc fragment of the IgG molecule attaches to the membrane surface of the macrophage and somehow the cell recognizes a conformational change in the antibody molecule when it combines with the particular antigenic determinant (91). The attachment of large particulate matter, i.e. red blood cells, to the macrophage surface has been studied (93, 127) with the result that attachment of aldehyde-treated red blood cells to the macrophage cell membrane is temperature dependent but independent of serum or cation, while the ingestion process required the presence of both serum and cations in the medium. Thus the coupling of macrophage receptor molecules with opsonized particles may not only serve a recognition role but also function to trigger the ingestion phase in some phagocytic systems. Ingestion of

particulate matter by phagocytic cells is an energy dependent process initiating a complex sequence of morphological events. Following contact with the macrophage's cell surface, the particle is surrounded by the plasma membrane by pseudopodia which fuse at the distal side of the particle to form the phagocytic vacuole or phagosome. As the phagosome moves centripetally into the cytoplasm, primary lysosomes interact and fuse with the phagosome (60), resulting in the formation of a phagolysosome. A network of microfilaments has been observed in macrophage pseudopodia during ingestion and is thought to be the intracellular contractile element involved in macrophage movement and membrane deformability (95). Cohn and Wiener (25) have investigated the intracellular events subsequent to ingestion and have demonstrated that acid hydrolyases are released from the primary lysosomes and are redistributed about the newly-formed phagocytic vacuole. Thus the degranulation of the vacuole contents involves the transfer of digestive enzymes from the lysosome converting the phagocytic vacuole into a digestive organelle.

b. Pinocytosis

Although both PMNL and macrophages actively engage in phagocytosis, of the two, only the macrophage is capable of pinocytic activity (84). Pinocytosis serves as a mechanism whereby the macrophage transports exogenous material into lysosomal granules. Small invaginations of the membrane surface form pinosomes which migrate inward and fuse with Golgi residues containing newly synthesized acid hydrolases forming secondary lysosomes. Pinocytosis in peritoneal macrophages in vitro has been demonstrated to be regulated by medium constituents (24)

and depressed by inhibitors of glycolytic or oxidative phosphorylation (16). The rate of pinocytic activity in vitro is significantly increased by a variety of molecules: 1) anionic molecules including albumin, acidic polysaccharides, RNA, and DNA and 2) nucleotides such as ATP. The presence of optimal concentrations of serum in the incubation media not only stimulated pinocytic activity but also increased the number of lysosomes as well as the levels of acid hydrolases, suggesting a direct correlation between serum concentrations, pinocytosis, and enzyme accumulation. Macrophage lysosomes contain a variety of acid hydrolases that are capable of degrading macromolecules to low molecular weight products which then are excreted or utilized by the cell. Recent observations have been made on the uptake and intracellular fate of soluble proteins by peritoneal macrophages (40). Iodinated human serum albumin was pinocytosized and transferred into lysosomes where the protein was degraded and a TCA-soluble isotope excreted. These studies point out the probable role of macrophage proteases and peptidases in digestion of foreign protein with the concomitant return of TCA-soluble peptides or amino acids to the extracellular environment. The mechanisms of uptake, storage and hydrolysis of carbohydrates were recently studied in the mouse peritoneal macrophage (20). Nonutilizable disaccharides were pinocytized and formed large acid phosphatase-positive vacuoles in the perinuclear region. Monosaccharides with molecular weights up to 220 did not produce lysosomal vacuolization. Those oligosaccharides which did produce vacuolization were shown to be resistant to the complement of macrophage acid hydrolases and are quantitatively

retained within the cell during the incubation period. Thus at the inflammatory site the macrophage is capable of responding to a variety of stimuli by displaying a state of heightened physiological activity. This maturation process is accompanied by profound alterations in function and morphology which have been referred to as differentiation. It is evident that the potential of macrophages to adapt and respond to external stimuli at the inflammatory locus by functional hypertrophy and subsequent modification of the immediate environment is of vital importance in the functioning of macrophages at sites of tissue injury and repair. The increased pinocytic activity and content of hydrolytic enzymes of the activated macrophage suggest an increased synthetic activity requiring an abundant and continuous supply of metabolic energy. Therefore, investigations into the pathways of energy supply and production would add further insight into the energetics of activation and maturation of the inflammatory macrophage.

B. Macrophage Metabolism

1. Mononuclear phagocyte vs. polymorphonuclear leukocyte

The biochemical characterization of leukocyte function has yielded valuable information concerning the metabolic consequences underlying many macrophage and PMNL functions in host defense physiology. However, a direct comparison of leukocyte metabolism is complicated by the fact that several morphologically different types of white blood cells exist and are distinctive in their complement of enzymes and other features of their metabolism. Furthermore, a homogeneous white cell population may display significant alterations in

its metabolic patterns depending upon the functional state of the cell (e.g. resting vs. phagocytizing, stage of differentiation, etc.) or whether the cells were derived from the normal or diseased state. Therefore such factors must be considered in attempting to critically review or evaluate leukocyte metabolism.

Until recently, the biochemical properties of the PMNL have received almost exclusive attention in evaluating leukocyte metabolism due to the relative ease of obtaining a homogeneous population from the peripheral blood or inflammatory exudate. The general aspects of leukocyte metabolism have consequently been thoroughly reviewed (15, 67, 124). Since the role of the PMNL in host defense reactions is intimately related to its ability to engulf and destroy a wide variety of microorganisms, the metabolic events accompanying phagocytosis by leukocytes have received considerable attention and have been extensively reviewed (66, 67). In contrast, the biochemical literature pertaining to macrophage metabolism is relatively sparse when compared to the PMNL. This is due in part to the smaller yields of a homogeneous population of macrophages from blood, tissue, or exudate preparations. Recently, however, relatively pure suspension of macrophages have been obtained from lung, peripheral blood, and peritoneal cavity (7, 85) resulting in more extensive investigations into the functional biochemistry of the macrophage (4, 68).

2. Macrophage carbohydrate metabolism and energy supply

The relative contribution of either glycolysis or oxidative respiration as the principal energy source is significantly different among the various macrophage populations examined. The primary energy

source for the blood monocyte and peritoneal macrophage is glycolysis; the alveolar macrophage however appears to be more dependent on oxidative mechanisms (7, 85). Harris and Barclay (55) observed that most of the glucose utilized by peritoneal macrophages was converted to lactate, even in the presence of adequate oxygen, and that under anaerobic conditions glycolysis supplied cellular energy requirements. Thus, they concluded that peritoneal macrophages are facultative anaerobes which utilize glycolysis as their principal energy pathway. Recently, West et al. (135) confirmed the high glucose utilization ($31-37 \mu\text{moles/hr}/10^8$ cells) displayed by peritoneal macrophages and also demonstrated a high aerobic lactate production. In addition, a comparison of the rates of glucose utilization by PMN leukocytes, lymphocytes and macrophages indicated that the macrophage is the most active of the three cell types studied.

Glucose oxidation via the hexose monophosphate shunt is present in the non-particulate fraction of PMN leukocytes and macrophages. The alveolar macrophage of the guinea pig is far more active in converting glucose-1- ^{14}C and glucose-6- ^{14}C to $^{14}\text{CO}_2$ than the peritoneal macrophage (85). The ratio of $^{14}\text{CO}_2$ production from glucose-1- ^{14}C to that from glucose-6- ^{14}C is about 6:1 for the resting alveolar macrophage and 20:1 for the peritoneal macrophage. The oxidation of glucose-6- ^{14}C to $^{14}\text{CO}_2$ is extremely low in rat exudate PMNL (99), demonstrating a glucose-1- $^{14}\text{CO}_2$ /glucose-6- $^{14}\text{CO}_2$ ratio of 92 in the resting state. Rat PMNL are also characterized by a low rate of oxygen uptake, a high aerobic glycolytic rate, and an active direct oxidative pathway, thus the resting metabolism of peritoneal PMNL appears to be similar to that of the

peritoneal macrophage.

3. Macrophage lipid metabolism

Studies in macrophage lipid metabolism have addressed themselves to two important unsolved problems in macrophage physiology: 1) the tremendous lipid turnover of the cell membrane subsequent to endocytosis and 2) the function of macrophages at the site of fatty plaque formation and their role in the clearance of blood cholesterol.

Day (32) and his colleagues have investigated the uptake and metabolism of ^{14}C -labelled cholesterol either in aqueous solution or as a component of chylomicra and determined that exogenous cholesterol was incorporated by macrophages and rapidly hydrolyzed. The in vitro synthesis of cholesterol from ^{14}C -acetate was demonstrated by peritoneal macrophages (31), along with cholesterol esterifying activity (33). The principal fatty acids synthesized by peritoneal macrophages from ^{14}C -acetate were palmitic, oleic and linoleic acids.

The uptake of ^{14}C -labelled fatty acids has been investigated in the alveolar macrophage. ^{14}C -linoleic and ^{14}C -palmitic acid were readily taken up by the macrophage, esterified and incorporated into the triglyceride and phospholipid pool (42). Inhibitors of glycolysis effectively eliminated esterification, whereas inhibition of oxidative metabolism was without effect. Furthermore, triglyceride incorporation was investigated by the use of labelled tripalmitate, free or chylomicra-bound, and the cellular incorporation of labelled palmitate was examined following 60 minutes of incubation. Approximately 60% of the labelled palmitate was found in the triglyceride fraction, 35% in phospholipids, and 5% as free fatty acids.

A prominent feature in atherosclerotic plaque formation is the presence of a large number of lipid-laden macrophages infiltrating the vascular endothelium. Duff et al. (36) concluded that these lipid-laden macrophages, called foam cells, were derived from the blood monocyte population and were incorporated into the vessel wall while the endothelium grew over the macrophages. Recently, Cookson (27) has proposed a dual origin for foam cells in atherosclerosis. One cell type contained numerous cytoplasmic fibrils suggestive of a smooth muscle cell origin. The other cell type manifested itself as a macrophage, exhibiting many cytoplasmic processes, inclusions, and positive acid phosphatase reactive granules. Cholesterol and its esters have been shown to be fibrogenic and produce large granuloma when injected into connective tissue (1). These authors suggest that phospholipid injection somehow stimulated macrophage metabolism of cholesterol and thereby removes the cholesterol before it can exert a fibrogenic effect on the vascular wall.

Thus the macrophage's role in the metabolism of a variety of lipids establishes an important trophic function by the RES and clearly implicates the macrophage as an important site of metabolic fuel uptake and processing in health and disease states.

4. Macrophage protein synthesis

The fact that macrophages are quite active in the synthesis and subsequent release of active molecules necessary to host defense mechanisms suggests, an active protein forming machinery. On morphological evidence alone, macrophages and monocytes are quite active in protein synthesis as shown by the presence of abundant endoplasmic reticulum and

ribosomes. Cohn et al. (21) have demonstrated in macrophages cultured in vitro that, following a pulse of leucine- ^3H , a flow of newly formed protein occurs from the site of protein synthesis in the endoplasmic reticulum through the Golgi apparatus and finally to the primary lysosome. These authors concluded that the majority of the newly synthesized protein accumulated in Golgi vesicles prior to their fusion with endocytic vacuoles.

Interferon represents a heterogeneous class of antiviral proteins produced in many tissues in response to viral infection. Several investigators have now clearly established that macrophages can produce and secrete large amounts of interferon. Acton and Myrvik (2) demonstrated that alveolar macrophages can produce interferon following the in vitro inoculation of parainfluenza-3 virus. Incubation of normal alveolar macrophages with this substance before challenging the macrophages with pox virus protected the cells against destruction. Peritoneal macrophages are also capable of producing interferon which is detectable in the culture medium two hours following the exposure of macrophages to viral agents (116).

Stecher and Thorbecke (118) have studied the in vitro incorporation of ^{14}C -labelled amino acids into serum proteins by macrophages. By utilizing autoradiographic and immunoelectrophoretic techniques, they found that peritoneal macrophages synthesize in vitro B $_{1c}$ globulin (C'3) and transferrin far more actively than either thoracic duct lymphocytes or blood leukocytes. Although the macrophage is important for complement component synthesis and probably the major site of complement formation in lymphoid tissue, it must be realized that it is

probably not the only cell capable of producing these proteins. In all likelihood the parenchymal cells of the liver are also capable of synthesizing serum complement proteins.

Blood monocytes and peritoneal macrophages do not normally synthesize DNA under culture conditions (126), although following stimulation by an adjuvant, macrophages are able to divide and incorporate thymidine into DNA. Active RNA synthesis has been demonstrated in macrophages (17). Utilizing ^3H -uridine and radioautographic techniques, macrophages incorporate uridine initially into the nucleus and nucleoli followed by migration of the label into regions of the cytoplasm containing ribosomes.

5. Phagocytosis associated metabolism in PMNL and macrophages

Phagocytosis is associated with profound alterations in the metabolism of phagocytic cells. The nature and magnitude of these metabolic perturbations vary and are ultimately dependent upon a host of factors including: 1) the cell type (PMNL vs. macrophage), 2) the origin of the phagocytic cell (blood, lung, or peritoneal cavity), 3) the type of particle ingested (bacteria vs. synthetic), and 4) the incubation conditions (serum concentration, gas phase, cell suspension or monolayer, etc.).

a. PMN Leukocytes

Until recently, the PMNL has been the phagocytic cell population which has been most studied in an effort to understand the metabolic events accompanying phagocytosis (66). For example, it has been demonstrated that the uptake of particulate matter by the PMNL is accompanied by a stimulation of oxygen consumption, as well as by increases in aerobic

and anaerobic glycolysis, glycogenolysis, hexose monophosphate shunt activity, lipid turnover, and formate oxidation (23, 63, 106, 107). Glycolytic inhibitors (e.g. sodium fluoride and iodoacetate) have a profound inhibitory effect on phagocytosis, whereas the respiratory inhibitors (e.g. sodium cyanide and antimycin A) have little effect. It is clear that phagocytosis by PMNL is an active process requiring a net expenditure of energy derived primarily from glycolysis.

The dramatic respiratory burst that is associated with phagocytosis has been linked functionally to a bactericidal mechanism in the PMNL. Klebanoff (70) has postulated an effective antimicrobial scheme consisting of a halide, myeloperoxidase, and the production of hydrogen peroxide. The increment in oxygen uptake subsequent to phagocytosis appears to be largely insensitive to inhibitors of cytochrome-linked respiration and is accompanied by the direct oxidation of glucose via the hexose monophosphate shunt. Two oxidases have been demonstrated in the PMNL capable of producing hydrogen peroxide (12, 145). It has been suggested that the activation of one of these two oxidases during phagocytosis accounts for the increased oxygen utilization and direct oxidation of glucose-1-¹⁴C due to the generation of NADP either by indirect coupling with an NADPH lactic dehydrogenase or by direct NADPH oxidation.

b. Macrophages

The recent advent of improved techniques for separating mononuclear cells from the peritoneal cavity, lung, or peripheral blood has elicited quantitative information concerning the metabolic events of macrophages during phagocytosis. Oren et al. (85) compared the metabolic characterizations of peritoneal macrophages and PMNL along with

alveolar macrophages harvested from the guinea pig. Following the ingestion of starch particles, these authors observed that both respiration and glycolysis were stimulated in peritoneal macrophages as evidenced by a three-fold increase in oxygen uptake concomitant with a ten-fold increase in the recovery of $^{14}\text{CO}_2$ from glucose-6- ^{14}C . Phagocytosis by peritoneal macrophages was not impaired by anaerobiosis, inhibitors of respiration (10^{-3}M cyanide) or by uncouplers of oxidative phosphorylation (10^{-4}M 2,4-dinitrophenol). However, the glycolytic inhibitors, sodium fluoride (10^{-2}M) and iodoacetate (10^{-4}M), exerted a dramatic inhibitory effect on phagocytosis. Thus it was concluded that the peritoneal macrophage derived its energy needs for phagocytosis from glycolysis.

The alveolar macrophage, with a resting respiratory rate three times that of the peritoneal macrophage, demonstrated only a slight increase in oxygen uptake or $^{14}\text{CO}_2$ production from either glucose-1- ^{14}C or glucose-6- ^{14}C following phagocytosis. Furthermore, phagocytosis by alveolar macrophages was markedly depressed in the presence of inhibitors of both oxidative and glycolytic metabolism.

Cytochemical investigations involved with the metabolism of alveolar and peritoneal macrophages basically concur with the biochemical evidence that alveolar macrophages require oxidative metabolism to meet energy demands while peritoneal macrophages derive their energy needs predominantly from glycolytic metabolism. Portugalov et al. (92) demonstrated that peritoneal macrophages displayed high activity of such glycolytic enzymes as phosphorylase and lactic dehydrogenase, whereas the alveolar macrophage demonstrated higher levels of tricarboxylic

acid cycle enzymes than did the peritoneal macrophage.

In contrast to the PMNL where phagocytosis was associated with a three-fold increase in the ratio of $^{14}\text{CO}_2$ from glucose-1- ^{14}C to glucose-6- ^{14}C , Oren et al. (85) could not demonstrate any significant increase in hexose monophosphate shunt activity in either the alveolar or peritoneal macrophage subsequent to phagocytosis. However, West et al. (135) calculated hexose monophosphate shunt activity in guinea pig peritoneal macrophages and were able to demonstrate a small but significant increase during phagocytosis.

Recently Romeo et al. (101) have analyzed the link between the stimulated respiration and HMP shunt activity in the macrophage. Their results show a stimulation of both processes within seconds after the addition of bacteria. Furthermore, NADPH oxidase activity has been demonstrated in macrophage 20,000 x g subcellular fraction suggesting that the activation of this oxidase, producing hydrogen peroxide, may be the event linking the stimulation of macrophage respiration to that of HMP shunt activity, a mechanism similar to that found in the PMNL.

Stimulation of macrophage metabolic pathways is not only dependent upon particle ingestion but also on the state of maturation of the mononuclear cell population. Nonstimulated macrophages washed from unstimulated mouse peritoneum displayed no respiratory burst during phagocytosis (68). However, peritoneal macrophages harvested five days following the intraperitoneal injection of caseinate, demonstrated the typical respiratory response subsequent to the ingestion of particles.

The metabolic requirements for macrophage pinocytic activity

have been investigated in the mouse peritoneal macrophage (16) and are clearly distinctive from those required for phagocytosis. Pinocytosis by the peritoneal macrophage was depressed by inhibitors of respiration ($10^{-5}M$ cyanide, $10^{-7}M$ antimycin A), by anaerobiosis, and by inhibitors of oxidative phosphorylation (0.6 g/ml oligomycin, $10^{-6}M$ 2,4-dinitrophenol). Pinocytosis can also be arrested by inhibitors of protein synthesis suggesting that the synthesis of new plasma membrane is probably required for continuing membrane interiorization processes.

6. Glycogen metabolism in the PMNL

The preceding summary of selected aspects of the metabolism of PMNL and macrophages have emphasized the major role performed by aerobic and anaerobic glycolysis in meeting the energy demands of phagocytic cells. The high rate of glucose utilization in the macrophage and the accelerated rates of glycolysis demonstrated by PMNL and macrophages during the endocytic event necessitate a ready supply of glucose in order to maintain functional integrity. Furthermore, at the inflammatory site, where low oxygen and substrate supply may prevail, it would be advantageous if exudate cells were able to store glucose in a reserve form to meet continual energy demands. Glycogen is the principal macromolecular storage form of carbohydrate in animal cells and, in concert with glycogen synthetic and degradative enzymes, functions as a dynamic ready source of glucose.

Investigations approaching the study of glycogen metabolism and its regulation in white blood cells have primarily focused attention on the leukocyte phagocytic cell population. Early studies using histological

techniques demonstrated that most of the glycogen in circulating white blood cells is found in neutrophils and platelets, while both lymphocytes and monocytes contained relatively small amounts of stainable glycogen. Wachstein (130) demonstrated that eosinophils and basophils contained glycogen but in much smaller amounts than in the neutrophil. This histochemical data in conjunction with the relative ease of procuring a homogeneous population of blood leukocytes stimulated the appearance of quantitative estimates of leukocyte glycogen.

Wagner (131) estimated leukocyte glycogen, utilizing the micro-method of Pfluger (90), to be 4.2 micrograms per 10^6 granulocytic leukocytes and calculated that the glycogen content of leukocytes was in the same order of magnitude as striated muscle (i.e. 0.5-1.0%). Valentine et al. (125) investigated the glycogen content of human leukocytes in health and in various disease states by the anthrone technique (113). The mean leukocyte glycogen per 10^{10} granulocytic leukocytes was 75.1 mg. Leukocyte glycogen remained relatively unchanged during fasting and the postprandial rise in blood glucose. Leukocytes obtained from chronic myelocytic leukemic patients displayed a glycogen content approximately one half that of normal subjects. In contrast, leukocyte glycogen in polycythemia vera was elevated to a mean value per 10^{10} leukocytes of 116.2 mg.

Recently, an elegant study on leukocyte glycogen turnover and the macromolecular state of leukocyte glycogen was performed by Scott et al. (112). The glycogen content of human leukocytes averaged 7.36 ± 2.05 mg glycogen per 10^9 neutrophils and, when placed in a glucose-free medium, decreased 38% following a two hour incubation. Leukocyte glycogen

synthesis was investigated in vitro by determining the glucose level at which intracellular glycogen was not consumed and the glucose level which provided maximal glycogen resynthesis. The glucose load which gave no net change in leukocyte glycogen for 60 minutes of incubation was found to average 17.6 mg %, while the maximal change in glycogen content was obtained at a glucose load of 200 mg %. After preincubation of leukocytes in glucose-free medium, glycogen resynthesis with glucose- ^{14}C was estimated and revealed that nearly 90% of the intracellular radioactivity was found in the isolated glycogen fraction. An electron micrograph study of leukocyte glycogen revealed a rather uniform particle size of about 20-30 $\text{m}\mu$ in diameter, unlike liver glycogen particles which appear in a spectrum of sizes ranging from 40 to 200 $\text{m}\mu$ in diameter (112).

In view of the observations that leukocyte glycogen levels appeared to be markedly labile in various physiological and disease states, several investigators have attempted to elucidate those factors controlling glycogenolysis and glycogenesis in the leukocyte. Williams and Field (137) measured leukocyte phosphorylase activity in normal humans and in two patients with glycogen storage disease with low liver phosphorylase activity. The mean level of leukocyte phosphorylase was 29.6 micrograms of inorganic phosphorus per 10^7 leukocytes in normal patients and an abnormally low mean value of 6.3 micrograms of inorganic phosphorus per 10^7 in those patients manifesting glycogen storage disease. The authors concluded that the determination of leukocyte phosphorylase activity derived from a blood sample may assist in diagnosing certain glycogen storage diseases, ideally eliminating the need

for a liver biopsy. Glycogen phosphorylase was assayed in normal leukocytes and those derived from patients with chronic granulocytic or lymphocytic leukemia (144). Their results indicated that normal leukocyte phosphorylase existed predominantly in the active form (i.e. active in the absence of 5'-AMP) suggesting that this leukocyte enzyme is closely related to liver phosphorylase. Phosphorylase levels in leukemic leukocytes did not differ significantly from the normals.

The properties of leukocyte glycogen phosphorylase were further studied in rats with chloroma, a tumor composed entirely of immature granulocytes (143). Two forms of the enzyme were demonstrated to exist, an active form exhibiting 65-80% of its activity in the absence of 5'-AMP and an inactive form that was not significantly active even in the presence of the nucleotide. The authors noticed striking similarities in the properties of leukocyte glycogen phosphorylase when compared to liver phosphorylase.

Sbarra and Karnovsky (106) observed that, when leukocytes are ingesting latex particles in a glucose-free medium, glycogen breakdown was accelerated during the first fifteen minutes of incubation. However, these same authors found that in the presence of glucose no change in glycogen content occurred during phagocytosis. Recently, Stossel et al. (120) incubated leukocytes with latex particles in the absence of glucose and demonstrated that during the first fifteen minutes the rate of glycogenolysis was accelerated while glycogen phosphorylase or synthetase activity did not significantly differ from leukocytes incubated without particles. Epinephrine and glucagon, hormones which increase phosphorylase activity in liver preparations, had no effect in leukocyte

phosphorylase activity, although Williams and Field (137) did report that glucagon increased phosphorylase activity.

The biosynthesis of glycogen via glycogen synthetase or transferase has been demonstrated in human PMNL (103) and in lymphocytes (57). In normal human PMNL, synthetase activity existed only in the glucose-6-phosphate dependent D-form, whereas lymphocytes possessed the system for interconversion between the D and I-form (i.e. independent of glucose-6-phosphate). However, in leukocytes from diabetic patients, glycogen synthetase was found in both the I and D-form (45) and the D to I transformation was greatly increased by insulin treatment. Leukocytes from rat peritoneal exudates differed from human PMNL in that incubation greatly stimulated the D to I transformation in both normal and diabetic leukocytes (102). Recently Wang et al. (132) incubated human PMNL in a glucose-free buffer for two hours and observed a large decrease in glycogen content and phosphorylase activity. The subsequent addition of a 2 mg/ml glucose load resulted in a three-fold increase in the I activity, thus demonstrating that a D to I interconversion system, similar to those found in liver, muscle and rat PMNL preparations, is present in normal human PMNL. Insulin had no demonstrable effect on either the D to I conversion of glycogen synthetase or on the glycogen content of human PMNL.

7. Glycogen metabolism in inflammatory cells

Information regarding the role of glycogen in inflammatory exudate cells is meager and consists primarily of histological studies demonstrating the presence of stainable glycogen deposits. Page and Good (87), utilizing the skin window technique of Rebuck followed the

progression of phagocytic cells infiltrating into the inflammatory site in neutropenic patients. They observed a marked depletion of mononuclear cells at the inflammatory site in the absence of circulating neutrophils and concluded that viable leukocytes somehow contributed to the subsequent appearance of macrophages in the inflammatory response. Wulff (141), utilizing the periodic acid-Schiff (PAS) technique to stain for glycogen, investigated the glycogen content of leukocytes and macrophages infiltrating into the inflammatory locus. Initially, neutrophils at the exudate site were observed to contain variable amounts of PAS positive reaction product and as the inflammatory reaction proceeded, the neutrophils stained more intensely. With the appearance of macrophages, neutrophil cytoplasmic fragments containing PAS positive reaction product were eventually observed to be amassed in membranous aggregations around the macrophage population. Rebuck et al. (97) also demonstrated a direct transfer of neutrophil cytoplasmic glycogen to monocytes during the course of the inflammatory response in humans. Furthermore, they observed a failure to transfer glycogen to mononuclear cells in severely acidotic patients and suggested that the absence of membranous-glycogen in the mononuclear cells accounted for the depressed phagocytic ability in diabetic phagocytes and offered a mechanism for the increased susceptibility of diabetics to infection.

Recently, Scott and Cooper (111) studied the leukocyte glycogen responses in guinea pig inflammatory exudates and demonstrated a dramatic rise in exudate leukocyte glycogen content as compared to blood leukocyte glycogen levels. Glycogen synthetase activity was found to be significantly elevated in inflammatory leukocytes when compared to

peripheral blood leukocytes whereas, no significant change in glycogen phosphorylase activity was detected in blood or exudate leukocytes. Fasting the animals for up to three days appeared to have no effect on the glycogen accumulating ability of inflammatory leukocytes. Thus the authors concluded that the dramatic rise in leukocyte glycogen levels at the inflammatory site is a significant event in the course of the inflammatory response and can be accounted for by the relative increase in the D-form of glycogen synthetase activity in the exudate leukocyte.

C. Statement of the problem

In contrast to the abundant data which exists relevant to glycogen metabolism and its regulatory mechanisms in blood and exudate leukocytes, no quantitative information is available that has explored the role of glycogen or its metabolism within the inflammatory macrophage nor the mechanisms by which macrophages acquire glycogen at the inflammatory site.

The overall purpose of this study is to evaluate in vitro the physiological responses of macrophages derived from the inflammatory site to exogenous glycogen. Studies were undertaken to demonstrate the mechanisms by which inflammatory macrophages acquire a large intracellular glycogen level from the inflammatory environment. Additional studies were also performed to examine those mechanisms employed by inflammatory macrophages to maintain and utilize this carbohydrate reserve for continual macrophage function.

Furthermore, in the present study in vitro methods were developed and employed to demonstrate macrophage glycogen synthesis and degradation by determining the activity of glycogen phosphorylase and synthetase

and also by identifying an α -glucosidase of probable lysosomal origin.

In view of the observation that macrophages perform trophic functions by ingestion of particulate material and the subsequent release of digested material into the extracellular environment, studies were employed to examine a new trophic function of glucose export and the relevant contribution of extracellular glycogen ingestion to this glucogenic mechanism.

CHAPTER III

EXPERIMENTAL METHODS

A. Experimental Animals

Male rats of the Sprague Dawley strain weighing between 250 and 350 grams were obtained from the Holtzman Co., Madison, Wisconsin. The rats were housed at a room temperature of 74-75°F and maintained on Purina Laboratory Chow and tap water ad libitum.

B. Preparation of Inflammatory Cell Populations

1. Exudate production

Inflammatory peritoneal exudates containing either PMNL or macrophages were induced by a modification of the procedure described by Reed and Tepperman (99). Rats were anesthetized with ether and injected intraperitoneally (16 ml/100 gm body wt) with a 1% solution of sodium caseinate (Eastman Kodak Co., Rochester, N.Y.) in 0.02M phosphate-buffered saline (PBS) at pH 7.4. Non-inflammatory mononuclear cells were obtained by lavage of normal rat peritoneum without prior stimulation with caseinate. Peritoneal exudates containing PMNL were collected three hours following the introduction of caseinate while inflammatory macrophages were harvested 96 hours after the injection of the irritant.

2. Harvesting procedures

Following decapitation, 20 ml of cold PBS containing 20 units/ml of heparin were injected into the peritoneal cavity, mixed briefly, and

the exudate contents withdrawn with a large syringe and needle and delivered into 50 ml Nalgene centrifuge tubes at 4°C. Cells were sedimented by centrifugation at 150-200 x g for 10 minutes at 4°C and erythrocytes were effectively removed from the resulting cell pellets by a 10 second exposure to 5.0 ml of distilled water followed immediately by the addition of 15.0 ml of 1.2% saline to adjust isotonicity. Following recentrifugation at 200 x g for 10 minutes at 4°C, cells were resuspended in cold glucose-free Hanks solution (GFH),* pH 7.4, and washed twice in GFH prior to resuspension to their final volume.

3. Cell counting, differential and protein determination

Duplicate cell counts were obtained from 0.1 ml sample of the final cell suspension diluted into 10 ml of GFH by routine hemocytometry and expressed as 10^6 cells per ml of cell suspension. Cell differentials of PMNL, macrophages and lymphocytes were performed from Wright-stained smears. Cell protein was determined from a 1:100 dilution of cell suspension according to the method of Lowry et al. (78) utilizing the Oyama and Eagle modification (86) with bovine albumin as a reference standard.

C. Glycogen Determination in Inflammatory Leukocytes and Macrophages

1. Chemical determination of glycogen

Glycogen was isolated from inflammatory macrophages and PMNL by a modification of the procedure originally described by Good, Kramer, and Somogyi (50). Glycogen was extracted by adding 2.0 ml of 30% KOH to cell pellets ($80-120 \times 10^6$ cells) in 12 ml glass centrifuge tubes and

*Glucose-free Hank's balanced salt solution (pH=7.4), concentrations in grams/L: NaCl 8.0, KCl 0.40, Na₂ HPO₄ 0.09, KH₂ PO₄ 0.06, Mg SO₄ 0.1, CaCl₂ 0.14, Mg CL₂ 0.1, NaHCO₃ 0.35.

heating in a boiling water bath for 15 minutes until the pellet dissolved. Following hydrolysis, tubes were cooled to room temperature and 2.0 ml of 95% ethanol (ETOH) was added, mixed, reheated to near boil, and stored at 4°C overnight to precipitate glycogen. Glycogen was sedimented by centrifugation at 1000 x g for 10 minutes at 4°C and the resulting supernatant discarded. The glycogen pellet was washed twice in 60% ETOH and the final pellet drained briefly to remove the alcohol. 1.0 ml of distilled water and 1.0 ml of 2.0 N H₂SO₄ were added to the dried glycogen pellets and heated in a boiling water bath for 3 hours. Following acid hydrolysis of the glycogen, the tubes were cooled to room temperature and neutralized to pH 7.0 by titrating with 1.0 N NaOH and the final volume brought to 5.0 ml with distilled water. Duplicate 1.0 ml aliquots were added to 4.0 ml of glucose oxidase reagent (Worthington Biochemical Co., Freehold, N.J.) and color development read after 60 minutes of incubation at 37°C using a Klett-Summerson colorimeter with blue filter (400-450 mμ). Results are expressed as either μg of glycogen per mg of protein or μg glycogen per 10⁶ cells.

2. Incorporation of ¹⁴C-U-glucose into macrophage glycogen

4.0 ml of 96 hour inflammatory macrophages (30 to 40 x 10⁶/ml) in GFH, pH 7.4, were incubated at either 37° or 4°C in 25 ml Erlenmeyer flasks containing ¹⁴C-U-glucose at a final concentration of 10 mM. ¹⁴C-U-glucose (specific activity 285 mCi/mM) was diluted with non-radioactive glucose in GFH to a specific activity of either 0.1 or 1.0 μCi/10 mM glucose. Following the incubation interval, the contents of the flasks were transferred to 12 ml glass centrifuge tubes at 4°C and the macrophages washed twice in cold GFH to remove any extracellular

^{14}C -glucose. 2.0 ml of 30% KOH was added to the final macrophage pellet and glycogen precipitated by the method of Good, Kramer, and Somogyi (50) as described above. Following two washings in 60% ETOH, the final glycogen pellet was dissolved in 1.5 ml of distilled water and duplicate 0.5 ml aliquots of the glycogen solution were added to 10 ml of PCS and counted in the Isocap 300 (Searle Analytic Inc., Des Plaines, Illinois) for 10 minutes. Activity was expressed as the dpms incorporated into macrophage glycogen per mg protein per incubation time.

3. ^{14}C -glycogen isolation from rat liver

Rat liver ^{14}C -glycogen was prepared by the incorporation of ^{14}C -U-glucose into liver glycogen as described by Brown (11). Male rats weighing 300-350 gms were fasted overnight and anesthetized with sodium pentobarbital (12 mg/300 gm). 2-deoxyglucose (40 mg/100 gm body weight) was injected i.v. into the dorsal vein of the penis followed after 30 minutes with an intraperitoneal injection of D-glucose (150 mg/100 gm body weight) containing ^{14}C -U-glucose at a specific activity of $6.0 \mu\text{Ci}/\text{mmole}$. Following three hours of light anesthesia maintained by subcutaneous injection of pentobarbital, the rats were decapitated, the livers quickly removed, weighed, cut into small pieces, and placed immediately into 250 ml beakers containing hot 30% KOH and heated to near boil with stirring for 15 minutes. After tissue digestion, beakers were cooled to room temperature and 50 ml of 95% ETOH added, mixed and the contents poured into centrifuge tubes and the glycogen allowed to precipitate overnight at 4°C . Glycogen pellets were centrifuged for 10 minutes at $200 \times g$ and washed twice in three volumes of 60% ETOH. The final glycogen pellets were dissolved in distilled water and dialyzed (mol.

weight cut off 12,000-14,000) against water overnight at room temperature. Following dialysis, glycogen was reprecipitated by adding 1 volume of 95% ETOH, washed in 60% ETOH and dried in a vacuum desiccator. The resulting glycogen was weighed and diluted in GFH and activity expressed as cpm per mg of glycogen by adding 1.0 ml of a known concentration of ^{14}C -glycogen in GFH to 10.0 ml of Phase Combination System (PCS) Solubilizer and counted for 10 minutes in Isocap 300 Liquid Scintillation System, using efficiency calculations based on quench correction factors determined by the sample channels ratio method.

4. Uptake of ^{14}C -glycogen by inflammatory macrophages

4.0 ml of inflammatory macrophages ($30\text{-}40 \times 10^6$ cells/ml) in GFH, pH 7.4, were incubated in 25 ml Erlenmeyer flasks containing 10 mg/ml of ^{14}C -glycogen, specific activity ($2\text{-}3 \times 10^3$ cpm/mg glycogen), at either 37°C or 4°C with shaking (60/min). Following the incubation period, contents of the flasks were transferred to 12 ml glass centrifuge tubes at 4°C and the flasks rinsed with cold GFH. Cells were washed twice in cold GFH to remove extracellular ^{14}C -glycogen and 2.0 ml of 30% KOH was added to the cell pellets and macrophage glycogen isolated by the method of Good, Kramer, and Somogyi (50) as described above. The final glycogen pellet was dissolved in 1.5 ml of distilled water and duplicate 0.5 ml aliquots were added to 10 ml of PCS and counted in the Isocap 300 for 10 minutes. Activity is expressed as the μg of ^{14}C -glycogen incorporated into macrophage glycogen per mg protein.

D. Glycogen Regulatory Enzyme Determinations in Inflammatory Macrophage

1. Macrophage phosphorylase activity

Macrophage phosphorylase activity was determined in the direction

of polysaccharide synthesis by measuring the rate of liberation of inorganic phosphorus from glucose-1-phosphate in the presence of glycogen by a modification of the method of Sutherland (121). 3.0 ml of 96 hour inflammatory macrophages ($20-40 \times 10^6$ cells/ml) in GFH, pH 7.4, were incubated in 25 ml Erlenmeyer flasks containing 1.0 ml of additions at 37°C in a Dubnoff metabolic shaking bath (60/min) with air as the gas phase. Incubations were terminated by the rapid transfer of the flask contents into 12 ml glass centrifuge tubes at 4°C, the flasks rinsed with cold GFH, and the cell suspensions centrifuged at 200 x g for 10 minutes at 4°C. Following centrifugation, the supernatant was discarded and the macrophage pellets were resuspended in 2.0 ml of 0.1 M NaF at 4°C. Macrophages were effectively disrupted by a 2 minute sonication at 4°C using a Biosonik IV Sonifer (Brownwill Scientific Inc., Rochester, N.Y.) with microprobe and centrifuged for 10 minutes at 3000 x g to remove cellular debris. 0.4 ml of the resulting sonicate supernatant was added to duplicate 12 ml glass centrifuge tubes at 4°C containing 1.0 ml of 0.05 M glucose-1-phosphate (pH 6.1) containing 5.7 mg/ml of glycogen and 0.05 M NaF and either 0.1 ml of 0.02 M adenosine-5'-phosphate or 0.1 ml of distilled water. Reagent blank tubes without sonicate were included in each experiment. Reaction mixtures were incubated for 15 minutes at 37°C and terminated by the addition of 2.0 ml of cold 5% TCA and centrifuged for 10 minutes at 500 x g at 4°C. Following centrifugation, duplicate 0.2 ml aliquots of deproteinized supernatants were added to cuvettes containing 0.8 ml of distilled H₂O and inorganic phosphorus was determined by the method of Fiske and Subbarow (47). 3.0 ml of molybdc acid reagent were added to each cuvette and, at 10 second

intervals, 1.0 ml of elon reagent was added to each tube and incubated for 15 minutes at 37°C. Color development was read immediately in a Klett-Summerson colorimeter using a red filter. Results are expressed as micrograms of inorganic phosphorus liberated per mg protein per 15 minutes of incubation.

2. Macrophage α -glucosidase activity

Macrophage α -glucosidase activity was measured by the formation of glucose from maltose, isomaltose, and glycogen as described by Hers (58). A 96 hour inflammatory macrophage cell suspension ($1-10 \times 10^6$ cells/ml) in saline was sonicated for 2 minutes at 4°C. 0.5 ml duplicate aliquots of macrophage sonicate were added to 12 ml glass centrifuge tubes containing either (1) 0.5 ml of 8 mg/ml of glycogen in 0.1 M acetate buffer, pH 4.0; (2) 4 mg/ml of maltose in 0.1 M acetate buffer, pH 4.0; or (3) 4 mg/ml of isomaltose in 0.1 M acetate buffer, pH 4.0 and incubated for 60 minutes at either 4°C or 37°C. Protein was determined in the macrophage sonicate according to the method of Lowry (78). A substrate blank containing 0.5 ml of substrate in 0.1 M buffer and 0.5 ml of saline was incubated with each experimental run. 4 mg/ml of maltose substrate was also prepared in 0.1 M sodium cacodylate-HCl, pH 6.0; 0.1 M imidazole-HCl, pH 7.0-8.0; and 0.1 M glycine -NaOH, pH 9.0 to obtain a pH profile of macrophage α -1,4 glucosidase activity. Following incubation, samples were deproteinized with 1 ml of 1.8% BaOH and 1 ml of 2% ZnSO₄ and centrifuged for 10 minutes at 200 x g. Duplicate 1.0 ml aliquots of deproteinized supernatant were added to Hycell cuvette tubes and glucose concentration determined by the glucose oxidase method with the glucose oxidase reagent dissolved in .025 M tris-HCl buffer to

inhibit the maltase present in commercial preparations of glucose oxidase (28). The α -glucosidase activity was measured by the difference between the amounts of glucose present in samples incubated at 40 or 37°C and expressed as μ g glucose per mg of protein per 60 minutes of incubation.

3. Macrophage glycogen synthetase activity

Uridine diphosphate glucose: α -1,4-glucan α -4-glucosyl transferase (E.C. 2.4.1.11), the enzyme responsible for the elongation of the outer chains of glycogen, was determined by measuring the amount of 14 C-glucose activity incorporated into macrophage glycogen from UDP-U- 14 C glucose according to a modification of the method described by Gold and Segal (48). 1.0 to 2.0 ml of packed 96 hour inflammatory macrophages were sonicated in two volumes of 0.1 M glycylglycine buffer (pH 7.4) at 40°C for 2 minutes and the sonicate centrifuged for 10 minutes at 3000 x g at 40°C. The assay mixture contained 1μ mol of UDP-glucose, 0.2 μ Ci of UDP-glucose -U- 14 C, 264 μ mol of glycylglycine, pH 7.4, and 20 mg of glycogen in a total volume of 3.8 ml. When the glucose-6-phosphate dependent or D form of the enzyme was assayed, 0.01 M glucose-6-phosphate was also present. The reaction was initiated by adding 0.2 ml of macrophage 3000 x g sonicate supernatant to the assay mixture in 25 ml Erlenmeyer flasks and incubated at 37°C for 10 minutes with shaking (60/min). The reaction was stopped by the addition of 6.0 ml of hot 30% KOH to each flask and the contents of the flasks transferred to 40 ml centrifuge tubes and heated for 15 minutes in a boiling water bath. Following heating, 0.5 ml of saturated NaSO₄ was added to each centrifuge tube and glycogen precipitated with 14.0 ml of 95% ETOH. The mixture was again heated to boil,

cooled for 30 minutes at 4°C and centrifuged for 10 minutes at 200 x g. The glycogen- Na_2SO_4 pellets were dissolved in 5.0 ml of distilled water and reprecipitated by adding 6.0 ml of 95% ETOH. The tubes were heated, cooled and centrifuged as above. The glycogen pellet was dissolved in distilled water to a final volume of 3.0 ml and duplicate 1.0 ml samples of the glycogen solutions were added to 10-15 ml of Phase Combining System (PCS) Solubilizer, (Amersham/Searle Corp., Arlington Heights, Illinois,) and counted in the Isocap 300 for 10 minutes. Background incorporation was determined in samples to which the enzyme was added after the addition of KOH to the flask. Enzyme activities are expressed as nanomoles of glucose transferred from ^{14}C -uridine diphosphate glucose (UDPG) to glycogen per 10 minutes per mg of protein.

4. Macrophage glucose-6-phosphatase activity

Macrophage glucose-6-phosphatase activity was measured by the liberation of inorganic phosphorus from glucose-6-phosphate as described by DeDuve (34). Inflammatory macrophages ($40\text{-}50 \times 10^6$ ml) were suspended in 0.25 M sucrose and sonicated for 2 minutes at 4°C using a Biosonik IV (Brownwill Scientific Inc., Rochester, N.Y.) with microprobe. Protein was determined in the macrophage sonicate according to the method of Lowry (78). Duplicate 0.5 ml aliquots of macrophage sonicate were added to 12 ml centrifuge tubes containing 0.5 ml of .04 M glucose-6-phosphate, .001 M EDTA, and .007 M histidine monohydrate, pH 6.5, and incubated for 10 minutes at 37°C. Enzyme blanks were determined in tubes to which the macrophage sonicate was added after the addition of 10% TCA and incubated for 10 minutes at 37°C. Reagent blanks were prepared by incubating 0.5 ml of the glucose-6-phosphate substrate with

0.5 ml of 0.25 M sucrose. Following incubation, 5.0 ml of 10% TCA were added to assay and reagent blank tubes and all samples were centrifuged for 15 minutes at 200 x g at 4°C. 1.0 ml of deproteinized supernatant was added to cuvettes and inorganic phosphorus was determined by the method of Fiske and Subbarow (47). Activity was expressed as mg phosphorus liberated per 10 minutes per mg protein.

E. Glucogenesis in Inflammatory Macrophages and Polymorpho-nuclear Leukocytes

Two ml of cells ($20-50 \times 10^6/\text{ml}$) or derived sonicate were suspended in GFH and added to 25 ml Erlenmeyer flasks at 4°C. One ml of additions was added and all incubations were conducted at 37°C with air as the gas phase and the flasks were shaken at 60/minute in a Dubnoff metabolic shaking bath. Incubations were terminated by rapidly freezing all samples along with appropriate blanks at -20°C. After thawing, samples were deproteinized with 1 ml of 1.8% BaOH and 1 ml of 2.0% ZnSO₄ and the gluco-stat method (Worthington Biochemical Co., Freehold, N.J.) was used to determine glucose concentrations from deproteinized supernatants. Glucose concentrations are expressed as μg glucose per mg of protein per hour of incubation.

F. Statistics and Data Analysis

Throughout the entire study, all results are expressed as the mean \pm the standard error of the mean with the number of independent in vitro observations described as "n". Non-paired analysis were employed, placing the confidence limit at 95% for all experiments.

CHAPTER IV

RESULTS

A. Factors Regulating the Glycogen Content of Inflammatory Macrophages

1. Inflammatory cellular yields from the rat peritoneal cavity

Lavage of normal, unstimulated rat peritoneal cavities yielded an average $18-20 \times 10^6$ mononuclear cells per rat with small macrophages and lymphocytes constituting 90% of the cell population recovered. Caseinate induction resulted in consistently high yields of inflammatory exudate cells from the rat peritoneum; from the 3 hour exudate, $6-7 \times 10^7$ cells were collected from each rat with greater than 90% of the cell population examined being PMNL. The 96 hour inflammatory exudate produced $2-3 \times 10^8$ cells per rat with inflammatory macrophages accounting for 85-90% of the cell population; PMNL and lymphocytes comprised the remainder of the exudative population. Approximately 90% of the 96 hour inflammatory cell population was observed to adhere to a glass surface when incubated for 60 minutes in Leighton tubes; furthermore, the cells avidly phagocytized latex beads, 0.8μ in diameter, and carbon particles as determined by light microscopy.

2. Glycogen content of inflammatory PMNL and macrophages

The glycogen contents of caseinate-induced inflammatory PMNL and macrophages harvested from the rat peritoneum are presented in Table 1. The 96 hour inflammatory macrophage was observed to contain a large intracellular glycogen reserve. When the glycogen level of inflammatory

TABLE 1
GLYCOGEN CONTENT AND DEPLETION IN INFLAMMATORY PMNL AND MACROPHAGES a.)

Cell Type	Number of Incubation Samples	Glycogen Content	
		μg glycogen/ 10^6 cells	% of control
<u>Inflammatory</u>			
PMNL			
<u>Control - 60 min at 4°C</u>	4	9.55 \pm 0.44	100
<u>60 min in GFH at 37°C</u>	4	5.53 \pm 0.36	58
<u>Inflammatory</u>			
Macrophages			
<u>Control - 60 min at 4°C</u>	7	11.79 \pm 1.59	100
<u>60 min in GFH at 37°C</u>	6	5.78 \pm 0.58	49

a.) 4.0 ml ($20-40 \times 10^6/\text{ml}$) of inflammatory macrophages were incubated in GFH for 60 minutes at either 4° or 37°C. Glycogen content is expressed as μg glycogen per 10^6 cells.

macrophages was compared with the 3 hour exudate PMNL, there was no significant difference in the glycogen levels of these two inflammatory cell populations. Furthermore, when either inflammatory PMNL or macrophages were incubated in glucose-free Hanks (GFH) media for 60 minutes, both cell populations exhibited a labile intracellular glycogen pool as demonstrated by an approximately 50% reduction in either inflammatory cell's carbohydrate reserve.

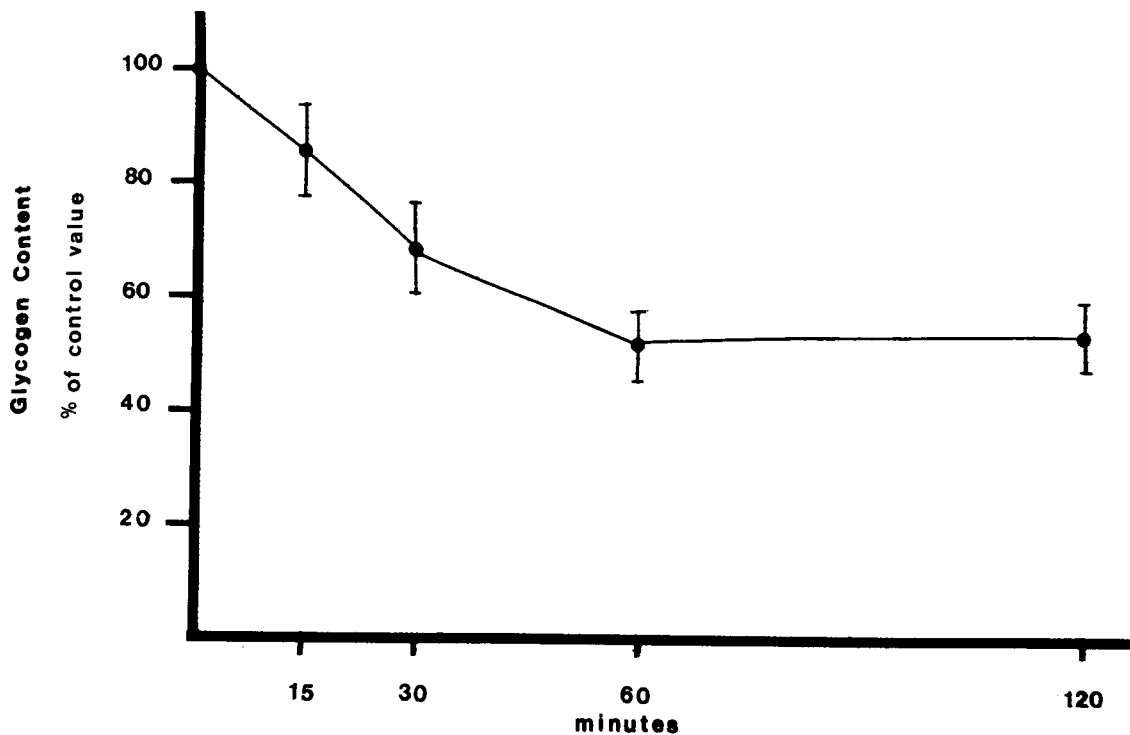
Figure 1 depicts the effect of incubation in GFH on the glycogen content in inflammatory macrophages. Increasing the length of incubation in a glucose-free media to 120 minutes did not further reduce the glycogen levels in inflammatory macrophages. This inability to further deplete the intracellular glycogen store suggests that only a certain fraction of macrophage glycogen is available for rapid mobilization as a fuel reserve.

3. Effect of anaerobiosis and phagocytosis on glycogen content in inflammatory macrophages

Since the peritoneal macrophage has been demonstrated to ultimately rely on glycolytic mechanisms to meet energy demands under anaerobic conditions (85), it was of interest to determine whether in vitro incubations of inflammatory macrophages in the presence of a nitrogen atmosphere would markedly affect macrophage glycogen stores (Table 2). During 60 minutes of incubation under anaerobic conditions in a glucose-free media, inflammatory macrophages did not demonstrate a further reduction in their glycogen content when compared to macrophages incubated similarly with air as the gas phase.

Furthermore, since leukocyte glycogen breakdown has been shown

FIGURE 1
EFFECT OF IN VITRO INCUBATION IN GFH
ON MACROPHAGE GLYCOGEN CONTENT



4.0 ml of macrophages ($20-40 \times 10^6/\text{ml}$) were incubated at 37°C in GFH shaking (60/min) for the various incubation intervals. Glycogen content is compared with control value at 4° and expressed as per cent of control. Each point represents the mean \pm standard error of at least four experiments.

TABLE 2
EFFECT OF ANAEROBIOSIS AND PHAGOCYTOSIS ON THE GLYCOGEN CONTENT
IN INFLAMMATORY MACROPHAGES

Incubation a.) Conditions	Number of Incubation Samples	Glycogen Content <u>μg glycogen/mg Protein</u>
A. Control - 60 min at 4°C	8	110.3 ± 12.8
B. 60 min in GFH	8	58.5 ± 6.12
C. 60 min + N ₂ Atmosphere	4	56.0 ± 11.8
D. 60 min + Latex Particles b.)	4	40.0 ± 3.40 c.)

a.) 4.0 ml of inflammatory macrophages (20-40 x 10⁶ cells/ml) were incubated at 37°C with shaking (60/min) for 60 min. Glycogen content is expressed as μg glycogen/mg protein.

b.) Latex particles (0.8 μdia) were added at a particle to cell ratio of 50:1 in a total volume of 0.1 ml in GFH.

c.) p < .05 as compared to group B

to be accelerated during phagocytosis in the absence of adequate glucose in the media (23, 107), experiments were undertaken to determine if macrophage glycogen provided additional fuel molecules during the course of the phagocytic event. When inflammatory macrophages were incubated in the presence of latex particles (0.8μ in dia) in a glucose-free media, the glycogen content of phagocytizing macrophages was significantly diminished when compared to non-phagocytizing macrophages (Table 2).

4. Effect of glucose and exogenous glycogen on inflammatory macrophage glycogen content

The maintenance of intracellular macrophage glycogen content by the addition of either glucose or exogenous glycogen is presented in Table 3. Inflammatory macrophages were incubated in the presence of 10mM glucose or 10 mg/ml of glycogen (Rabbit Liver Glycogen, Type III, Sigma Co., St. Louis, Mo.) for 60 minutes and macrophage glycogen content determined. The presence of 10mM glucose in the incubation media adequately maintained macrophage glycogen content during the 60 minute incubation interval. Moreover, the addition of 10 mg/ml of exogenous glycogen to the glucose-free medium also enabled macrophages to maintain their intracellular glycogen pool. In the presence of exogenous glycogen, the glycogen content of inflammatory macrophages following 60 minutes of incubation did not differ significantly from those samples incubated in the presence of a high glucose load. In addition, incubation of exogenous glycogen with inflammatory macrophages in an anaerobic atmosphere did not significantly diminish the macrophage's ability to maintain glycogen levels when compared to samples incubated under air atmosphere.

TABLE 3
EFFECT OF GLUCOSE AND EXOGENOUS GLYCOGEN ON INFLAMMATORY MACROPHAGE GLYCOGEN CONTENT

Incubation a.) Conditions	Number of Incubation Samples	Glycogen Content	
		$\mu\text{g Glycogen/mg Protein}$	% of Control
A. Control - 60 min at 4°C	8	110.3 \pm 12.8 c.)	100
B. 60 min in GFH	8	58.5 \pm 6.12	53
C. <u>60 min</u> + 10mM Glucose	6	95.8 \pm 4.20	87
D. <u>60 min</u> + 10mg/ml Glycogen b.)	6	85.4 \pm 9.08	75
E. <u>60 min</u> + 10mg/ml Glycogen + N ₂ Atmosphere	3	76.6 \pm 3.65	70

a.) 4.0 ml of macrophages ($20-40 \times 10^6/\text{ml}$) were incubated for 60 min at 37°C with shaking (60/min)

b.) Rabbit liver glycogen (Sigma Co., St. Louis, Mo.) was dissolved in GFH to a final incubation concentration of 10 mg/ml.

c.) P values in determining significant differences between groups

gp A vs B	$p < .01$
gp A vs C,D	not significantly different
gp B vs E	$p < .05$

5. Effect of glucose or glycogen on the glycogen content
in glucose-depleted inflammatory macrophages

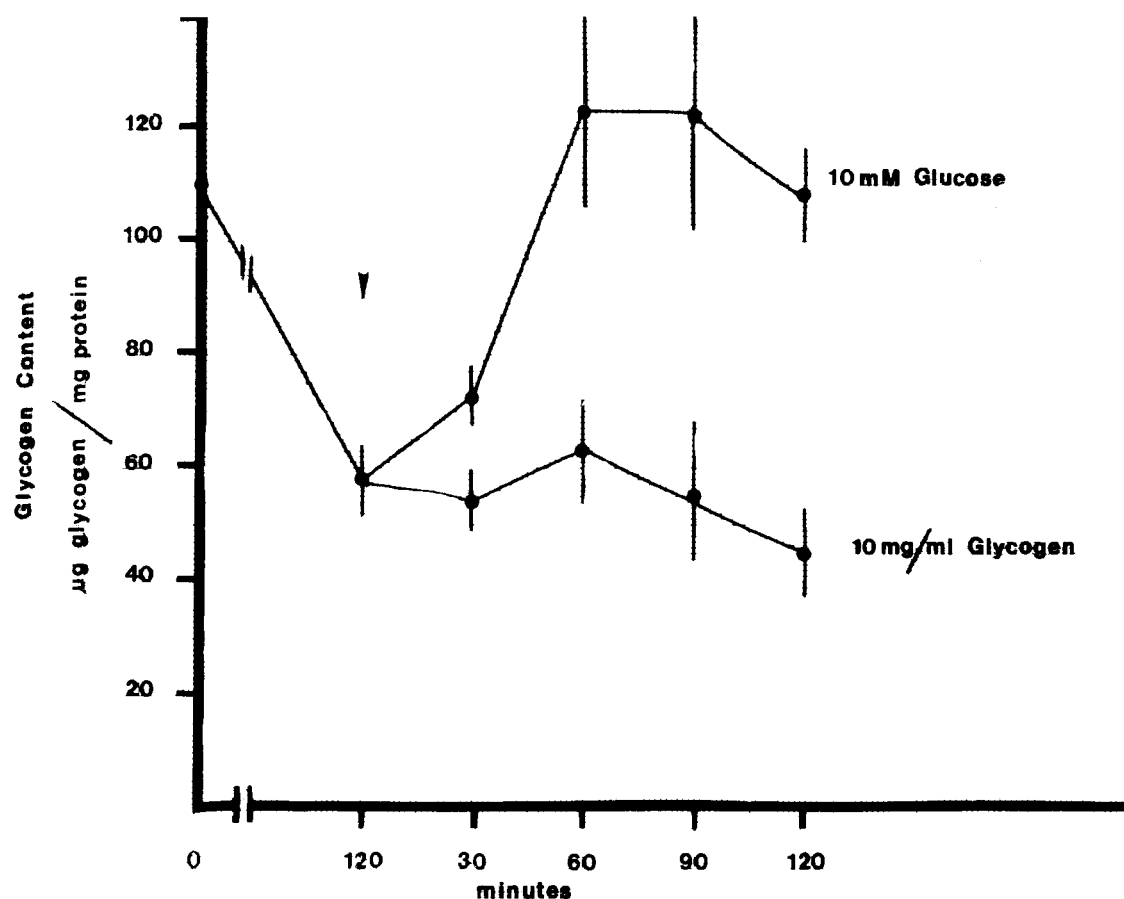
One approach to determine by what mechanism exogenous glycogen is able to maintain macrophage glycogen content is shown in figure 2. Incorporation of exogenous glycogen into the inflammatory macrophage glycogen pool could occur by two possible mechanisms, i.e., phagocytosis of macromolecular glycogen particles or by the extracellular hydrolysis of glycogen to glucose with the subsequent transport of glucose into the macrophage directed toward glycogen resynthesis. Since an adequate glucose supply to maintain glycolytic mechanisms is one necessary requirement for particle ingestion by phagocytic cells (23), incubation of inflammatory macrophages in a glucose deficient environment would therefore suppress phagocytosis of glycogen particles. Macrophage glycogen resynthesis from glucose can be adequately stimulated by preincubating macrophage cell suspensions for two hours in a glucose-free medium. The addition of 10mM glucose to glycogen-depleted inflammatory macrophages initiated a dramatic increase in glycogen resynthesis by inflammatory macrophages, reaching control levels by 60 minutes of incubation. In contrast, the addition of 10 mg/ml of glycogen to macrophages with a low energy reserve in a glucose-free medium did not result in any significant changes in macrophage intracellular glycogen content.

6. Incorporation of ^{14}C -U-glucose into inflammatory
macrophage glycogen

^{14}C -U-glucose was utilized to compare the rates of glycogen synthesis in glycogen-laden inflammatory macrophages and glycogen

FIGURE 2

EFFECT OF GLUCOSE (10mM) OR GLYCOGEN (10mg/ml) ON GLYCOGEN
CONTENT IN GLYCOGEN-DEPLETED MACROPHAGES



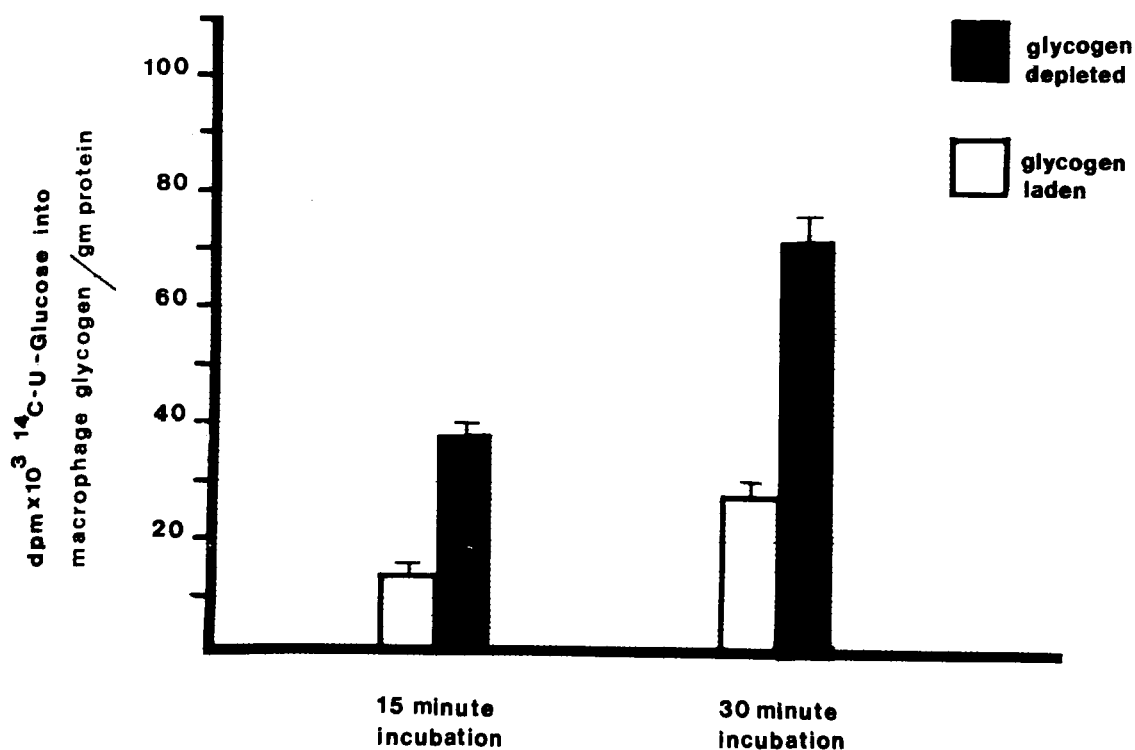
4.0 ml of macrophages ($20-40 \times 10^6/\text{ml}$) were preincubated for 2 hours at 37°C with shaking (60/min) in GFH. 10mM glucose or 10 mg/ml of glycogen were then added and glycogen content determined at 30 minute intervals. Each point represents the mean \pm standard error of at least three experiments.

resynthesis in macrophage depleted of their glycogen pools (Figure 3). Inflammatory macrophage cell suspension were preincubated in a glucose-free media at either 4° or 37°C. Following the preincubation period, 10mM glucose containing 0.1 μ C of 14 C-U-glucose was added to each flask and, following various incubation intervals, macrophage glycogen was isolated and radioactivity counted from aliquots of dissolved glycogen. Glycogen-laden inflammatory macrophages incorporated 14 C-U-glucose into intracellular glycogen at a rate of $27.2 \pm 3.8 \times 10^3$ dpm of 14 C-glucose per 30 minutes per gram of cell protein. Glycogen resynthesis in glycogen-depleted macrophages was determined to be $71.5 \pm 4.4 \times 10^3$ dpm of 14 C-U-glucose incorporated into macrophage glycogen per 30 minutes per gram of cell protein. The rate of 14 C-glucose incorporation into macrophage glycogen was directly related to the length of incubation in both macrophage populations (Figure 3). Thus glycogen depleted macrophages demonstrated an accelerated rate of glycogen resynthesis and suggests a probable inverse relationship between macrophage glycogen concentration and the percent of glycogen synthetase in the active form as has been observed in such tissues as skeletal muscle (29), cardiac muscle, (62) and liver (35).

7. The influence of a pinocytic activator and insulin in stimulating 14 C-U-glucose incorporation into macrophage glycogen

Pinocytosis affords the macrophage another mechanism of transporting extracellular solutes by membrane vesicle formation and invagination into the macrophage interior. Numerous factors stimulate macrophage pinocytosis including anionic proteins, serum factors, ATP,

FIGURE 3

INCORPORATION OF ^{14}C -U-GLUCOSE INTO INFLAMMATORY MACROPHAGE GLYCOGENEFFECT OF GLYCOGEN DEPLETION ON ^{14}C -GLUCOSE INCORPORATION

4.0 ml of macrophages ($30\text{--}40 \times 10^6/\text{ml}$) were incubated in the presence of 10mM glucose containing $0.1\mu\text{Ci}$ of ^{14}C -U-glucose per flask and glycogen isolated from cell pellet.

Glycogen depleted macrophages were preincubated for 120 minutes at 37°C in GFH prior to the introduction of ^{14}C -glucose.

Radioactivity was counted from duplicate 0.5 ml samples of isolated glycogen dissolved in 1.5 ml of distilled H_2O .

Each point represents the mean \pm standard error of at least four experiments.

and mucopolysaccharides (24). Since albumin is one such activator of macrophage pinocytic activity, experiments were undertaken to determine whether activation of pinocytosis increased the incorporation of extracellular ^{14}C -U-glucose into macrophage glycogen (Table 4). Inflammatory macrophages incubated in the presence of 1% albumin doubled their incorporation of ^{14}C -U-glucose into macrophage glycogen as compared to cells incubated in a glucose-free media alone.

Insulin has been shown to be without effect in PMN leukocytes incubated in vitro in either augmenting glycogen synthetase activity or increasing leukocyte glycogen levels (44). Inflammatory macrophages incubated in the presence of insulin ($200\mu\text{U/ml}$) did not demonstrate any increased incorporation of ^{14}C -U-glucose into macrophage glycogen. However, the addition of insulin to the incubation media containing 1% albumin significantly decreased ^{14}C -U-glucose incorporation into macrophage glycogen when compared to macrophages incubated in the presence of 1% albumin alone (Table 4).

8. ^{14}C -glycogen uptake by inflammatory macrophages

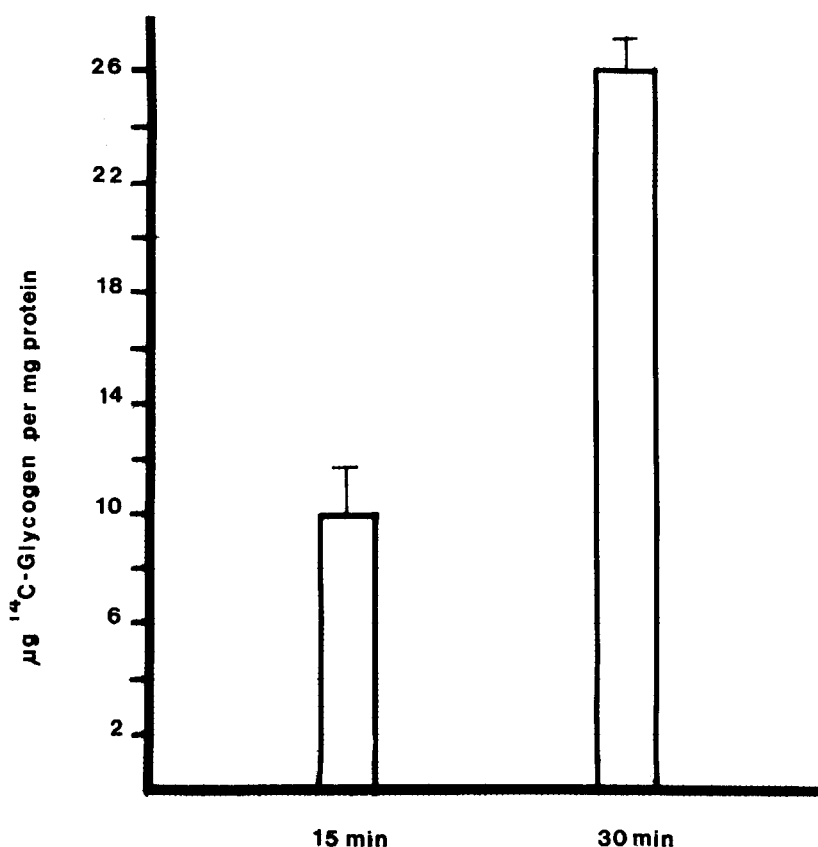
In view of the apparent significance that exogenous glycogen serves as a source of energy reserve to the inflammatory macrophage and the lack of any quantitative information concerning the ingestion of this important fuel macromolecule, preliminary experiments were undertaken to determine whether ^{14}C -labelled glycogen could be ingested in vitro by inflammatory macrophages. Figure 4 presents a plot of the in vitro uptake of ^{14}C -labelled glycogen by inflammatory macrophages in a glucose-free media. ^{14}C -glycogen was prepared from rat liver as described in Methods and dissolved in GFH containing unlabelled glycogen to a final incubation concentration of 10 mg/ml. Using a medium glycogen

TABLE 4
INCORPORATION OF ^{14}C -U-GLUCOSE INTO MACROPHAGE GLYCOGEN
EFFECT OF INSULIN AND ALBUMIN PINOCYTIC ACTIVATION

Addition to a.) Incubation Medium	Number of Incubation Samples	dpm of ^{14}C -U-glucose b.) incorporation into glycogen/mg protein/60 min
10mM ^{14}C -U-Glucose	8	71.9 \pm 2.23
10mM ^{14}C -U-Glucose + 1% Albumin	12	146.9 \pm 3.42
10mM ^{14}C -U-Glucose + 1% Albumin + 200 $\mu\text{U/ml}$ Insulin	13	122.8 \pm 3.15

- a.) 4.0 ml of macrophages ($30-40 \times 10^6$ cells/ml) were incubated with 1.0 ml of additions at 37°C with shaking (60/min) for 60 minutes.
- b.) Radioactivity was counted from duplicate 0.5 ml samples of isolated macrophage glycogen dissolved in 1.5 ml of distilled H_2O .

FIGURE 4

 ^{14}C -GLYCOGEN UPTAKE BY INFLAMMATORY MACROPHAGES

4.0 ml of macrophages ($30\text{--}40 \times 10^6$ cells/ml) were incubated at 37°C and 4°C with 0.5 ml of ^{14}C -glycogen in a total volume of 5.0 ml.

Radioactivity was counted from duplicate 0.5 ml samples of isolated macrophage glycogen dissolved in 1.5 ml of distilled H_2O .

0.5 ml of a 100 mg/ml glycogen in GFH contained 90 mg/ml of unlabeled glycogen + 10 mg/ml of ^{14}C -glycogen. Specific activity of final ^{14}C -glycogen solution was approximately 1600 cpm/mg of glycogen.

concentration of 10 mg/ml containing 16×10^3 cpm/ml of ^{14}C -glycogen, the amount of isotope incorporated into inflammatory macrophage glycogen by 15 minutes of incubation corresponded to $10\mu\text{g}$ of glycogen per mg of cell protein. Samples containing ^{14}C -glycogen were incubated at 4°C with each experimental run and any activity in the isolated glycogen was subtracted from those samples incubated at 37°C . The incorporation of ^{14}C -glycogen was linear over a 30 minute incubation period utilized in these experiments. Assuming no change in the total macrophage glycogen level of $110\mu\text{g}$ of glycogen/mg protein over the 15 minute incubation period, the incorporation of exogenous glycogen amounted to approximately 10% of the total macrophage intracellular pool.

9. ^{14}C -glycogen uptake in glycogen-depleted inflammatory macrophages

Since glycogen-depleted macrophages manifested a marked inability to increase their glycogen content in the presence of exogenous glycogen in a glucose-free media, it was of interest to compare the rate of incorporation of ^{14}C -glycogen by glycogen-laden and glycogen-depleted inflammatory macrophages. In Table 5 is presented the in vitro incorporation of ^{14}C -labelled glycogen by glycogen laden macrophages and by macrophages depleted of their glycogen reserve by a 2 hour preincubation in a glucose-free media. Incorporation of ^{14}C -glycogen by glycogen-depleted macrophages was markedly depressed in the presence of 10 mg/ml of ^{14}C -labelled glycogen at either 15 or 30 minutes of incubation at 37°C . When compared to inflammatory macrophages containing their full complement of glycogen reserve, the ingestion of exogenous glycogen was depressed nearly 50% in glycogen-depleted inflammatory macrophages,

TABLE 5
 ^{14}C -GLYCOGEN UPTAKE BY INFLAMMATORY MACROPHAGES
 EFFECT OF GLYCOGEN DEPLETION ON ^{14}C -GLYCOGEN UPTAKE

Macrophage a.) Population	Incubation Interval	Number of Incubation Samples	^{14}C -Glycogen Content c.)	
			$\mu\text{g } ^{14}\text{-C Glycogen/}$ mg Protein b.)	% of Control
Glycogen-Laden	15 min	4	29.69 ± 0.75	100
Glycogen-Depleted	15 min	4	12.56 ± 0.63	56
Glycogen-Laden	30 min	4	36.26 ± 1.60	100
Glycogen-Depleted	30 min	4	21.38 ± 1.92	56

- a.) 4.0 ml of macrophages ($30\text{-}40 \times 10^6/\text{ml}$) were incubated at 37°C with 0.5 ml of 10 mg/ml of ^{14}C -glycogen in a total volume of 5.0 ml.
- b.) Radioactivity was counted from duplicate 0.5 ml samples of isolated macrophage glycogen dissolved in 1.5 ml of distilled H_2O .
- c.) ^{14}C -glycogen specific activity was 2100 cpm/mg of glycogen.

suggesting a reduced functional endocytic capacity in inflammatory macrophages whose carbohydrate reserve has been severely diminished.

B. Enzymes Regulating Macrophage Glycogen Metabolism

1. Macrophage phosphorylase activity

a. Effect of glycogenolysis on macrophage total
phosphorylase activity

Table 6 demonstrates that the initial total phosphorylase activity, derived from whole sonicates of 96 hr inflammatory macrophages, was $37.9 \pm 1.56 \mu\text{g}$ inorganic phosphorus (P_i) liberated per 15 minutes per mg of protein. Total macrophage phosphorylase activity was determined in the presence of $5'$ -AMP in the reaction mixture. Since it was of interest to determine the relative states of macrophage phosphorylase activation prior to and during active glycogenolysis, experiments were performed to determine if any change occurred in macrophage total phosphorylase activity during 60 minutes of active glycogenolysis in a glucose-free media. In Table 6 it is observed that inflammatory macrophages incubated in GFH demonstrated no significant change in total phosphorylase during the 60 minutes of active glycogenolysis.

b. Effect of exogenous glycogen on macrophage total
phosphorylase activity

The activation of macrophage $5'$ -AMP dependent phosphorylase activity by exogenous glycogen is presented in Table 7. Inflammatory macrophages were incubated in GFH for 15 minutes in the presence or absence of 10 mg/ml of exogenous glycogen in the incubation media. Following the 15 minute incubation interval, total phosphorylase

TABLE 6
EFFECT OF GLYCOGENOLYSIS ON MACROPHAGE TOTAL PHOSPHORYLASE ACTIVITY a.)

Incubation Intervals	Number of Incubation Samples	Total Phosphorylase Activity ^{b.)}
		$\mu\text{g Pi}/15 \text{ min}/\text{mg protein}$
Control	10	37.9 ± 1.56
15 min	10	42.3 ± 4.31
30 min	5	42.3 ± 5.68
60 min	5	39.9 ± 4.50

a.) 3.0 ml of macrophages were incubated at 37°C with shaking (60/min) in GFH

b.) Total phosphorylase activity measured in the presence of 5'-AMP was determined on whole sonicates in 0.1 M NaF.

TABLE 7
EFFECT OF EXOGENOUS GLYCOGEN ON TOTAL MACROPHAGE PHOSPHORYLASE ACTIVITY

Additions to a.) Incubation Medium	Number of Incubation Samples	Total Phosphorylase Activity b.)
		$\mu\text{g P}_i/15 \text{ min/mg protein}$
Control	10	37.9 ± 1.56
15 min in GFH	10	42.3 ± 4.31
15 min + 10 mg/ml Glycogen	8	48.4 ± 3.37

a.) 3.0 ml of macrophages ($20-40 \times 10^6$ cells/ml) in GFH were incubated with 1.0 ml of additions at 37°C with shaking 60/min.

b.) Total phosphorylase activity was measured in the presence of $5'$ -AMP and determined from whole sonicates in 0.1 M NaF.

activity was immediately determined on macrophage sonicates. Inflammatory macrophages incubated in the presence of 10 mg/ml of glycogen significantly increased phosphorylase activity by nearly 30%, whereas macrophages incubated in the absence of glycogen did not demonstrate any further activation of 5'-AMP dependent phosphorylase activity.

c. The active form of macrophage phosphorylase

An active or a form of the phosphorylase enzyme, whose activity is measured in the absence of 5'-AMP, has been demonstrated in such tissues as skeletal muscle (26), liver (139) and leukocytes (143, 144). Since the form independent of 5'-AMP has been shown to be the fraction of phosphorylase activity responsible for initiating glycogenolysis, studies were attempted to determine what fraction of macrophage phosphorylase activity is in the active form. The active and total phosphorylase activities derived from 3000 x g sonicate supernatant is presented in Table 8. Total macrophage phosphorylase activity measured in the presence of 5'-AMP was 41.2 ± 1.82 g Pi/15 min/mg protein. Macrophage active phosphorylase activity measured in the absence of 5'-AMP was 30.4 ± 1.86 μ g Pi/15 min/mg protein. Thus approximately 73% of macrophage phosphorylase activity was in the active form. The macrophage phosphorylase activity ratio (active phosphorylase activity/total phosphorylase activity) of 0.73 was similar to the 0.75 activity ratio of liver phosphorylase (139) and the 0.80 activity ratio of leukocyte phosphorylase (143).

d. Effect of Mg^{++} -ATP on macrophage phosphorylase activity

The conversion of inactive to active phosphorylase has been shown to occur in crude liver extracts (94) and, more recently, in leukocyte

TABLE 8
ACTIVE AND TOTAL PHOSPHORYLASE ACTIVITY IN INFLAMMATORY MACROPHAGES

	Number of Incubation Samples	Macrophage Phosphorylase Activity a.) μg Pi/15 min/mg protein
Total Phosphorylase Activity (+ 0.02 M 5'-AMP)	9	41.2 ± 1.82
Phosphorylase a Activity (- 0.02 M 5'-AMP)	9	30.4 ± 1.86 b.)
Per Cent Activity in the Active Form	9	73.1

a.) Macrophage phosphorylase activity was determined on 3000 x g sonicate supernatants in 0.1 M NaF.

b.) Significantly different from total phosphorylase activity ($p < .001$).

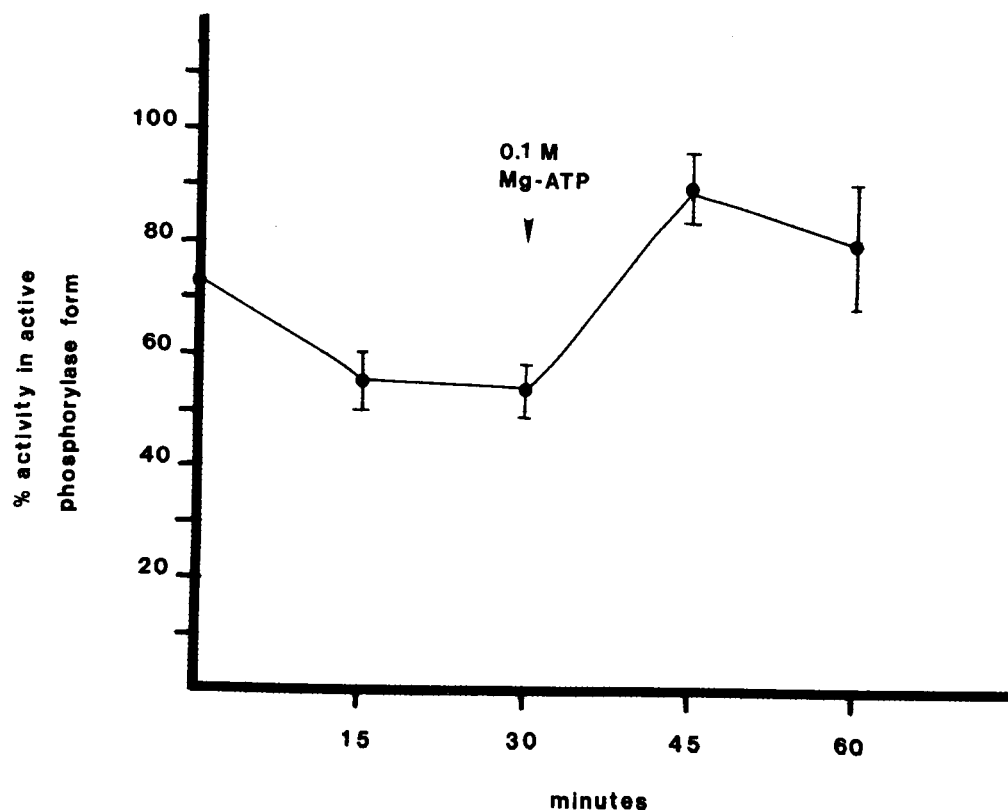
homogenates (143) by a mechanism involving a specific protein kinase requiring Mg^{++} and ATP. In order to determine whether macrophage phosphorylase was also capable of interconversion of the inactive to active form, macrophage 3000 x g sonicate supernatants were incubated with 0.1M Mg-ATP and the inactive and active phosphorylase activities determined (Figure 5). The preincubation of macrophage 3000 x g sonicate in 0.1M NaF for 30 minutes decreased the percent of macrophage phosphorylase in the active form from 73% to 55%. Following this preincubation interval, the introduction of 0.1M Mg^{++} -ATP to the samples resulted in a nearly complete conversion of phosphorylase activity to the active form by 15 minutes (90% in the active form). Total phosphorylase activity measured in the presence of 5'-AMP did not significantly change throughout the incubation interval.

2. Macrophage glucosidase activity

a. α -1,4 and α -1,6 glucosidase activity in inflammatory macrophage

Since acid α -1,4 glucosidase is capable of hydrolyzing maltose, linear oligosaccharides, and the outer branches of glycogen to glucose and of catalysing the transglucosylation from maltose to glycogen in liver, skeletal muscle (58) and in human leukocytes (136), it was of interest to determine whether this mechanism of glycogen hydrolysis was present in inflammatory macrophages. Table 9 contains the results of determinations of α -glucosidase activities measured at pH 4.0 in whole sonicates of 96 hour inflammatory macrophages. Macrophages exhibited a large α -1,4-glucosidase activity of $201.1 \pm 1.07 \mu g$ of glucose hydrolyzed per hour per mg of protein utilizing maltose as the enzyme

FIGURE 5
EFFECT OF Mg-ATP ON
INFLAMMATORY MACROPHAGE PHOSPHORYLASE ACTIVITY



- a) Macrophage 3000 xg sonicate supernatants were preincubated at 37°C in 0.1M NaF for 30 minutes prior to the addition of 0.1M Mg²⁺-ATP.
b) Per, cent in active form was determined by the phosphorylase activity ratio (activity - AMP/activity + AMP).
c) Mean \pm standard error of four experiments.

TABLE 9
 α -GLUCOSIDASE ACTIVITY IN INFLAMMATORY MACROPHAGES a.)

α -1,4-Glucosidase b.)		α -1,6-Glucosidase b.)
4 mg/ml Maltose	8 mg/ml Glycogen	4 mg/ml Isomaltose
201 \pm 1.07	27.0 \pm 2.0	4.43 \pm 1.08
(6)	(6)	(6)

a.) α -glucosidase was determined at pH 4.0 from macrophage whole sonicates and samples incubated for 60 min at 4° and 37°C.

b.) α -glucosidase activity is expressed as μ g of glucose hydrolyzed per hr per mg protein.

substrate and $27.0 \pm 2.0 \mu\text{g}$ of glucose hydrolyzed per hour per mg of protein using glycogen as substrate. α -1,6-glucosidase activity resulted in a very low activity of $4.43 \pm 1.08 \mu\text{g}$ of glucose hydrolyzed per hour per mg of protein when isomaltose, a disaccharide comprised entirely of α -1,6 glucosidic bonds was added as substrate for glucosidase activity.

b. Effect of pH on inflammatory macrophage α -1,4-glucosidase activity

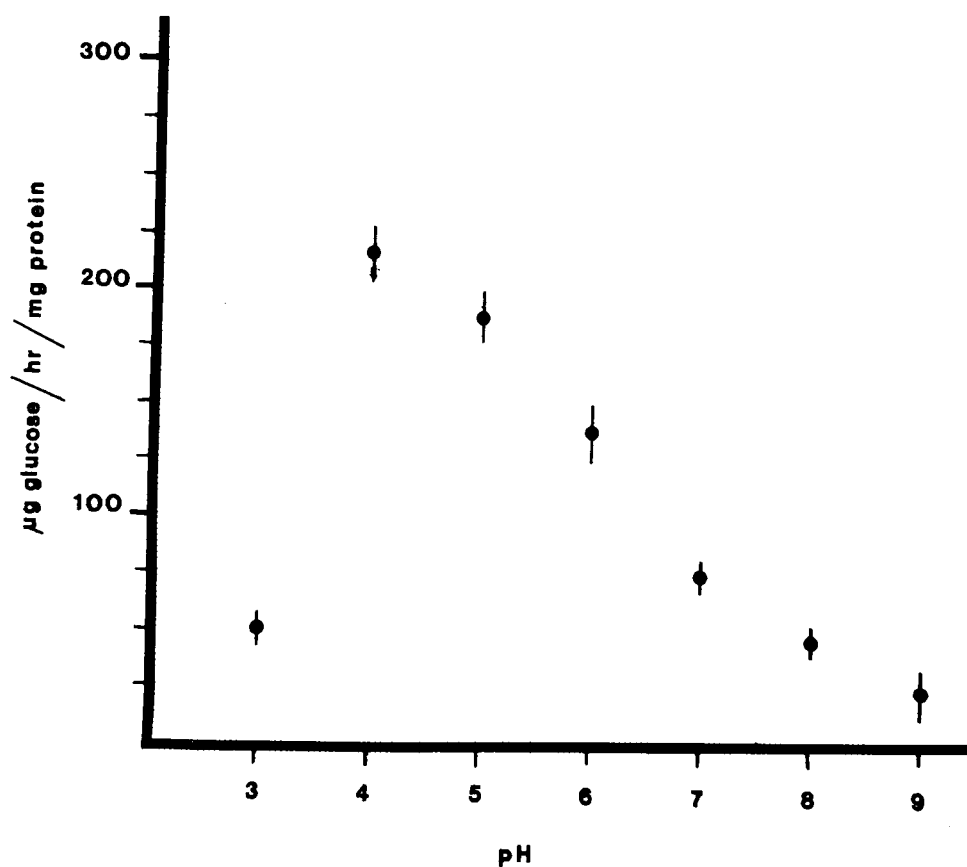
Lejeune et al. (76) reported that liver α -glucosidase had its maximum activity at pH 4.0 and was present in the lysosomal fraction of rat-liver homogenates. Figure 6 presents the results of incubating macrophage sonicates with maltose substrate at pH values ranging from 3.0 to 9.0. Macrophage α -1,4 glucosidase activity manifested its greatest activity at pH 4.0, suggesting that acid pH optimum of macrophage glucosidase activity favors a lysosomal environment.

3. Macrophage glycogen synthetase activity

a. I and D forms of macrophage glycogen synthetase

Table 10 presents the glycogen synthetase or transferase activity of inflammatory macrophage 3000 x g sonicate supernatants. Macrophage glycogen synthetase was found to exist in two forms, a dependent or D form, requiring the presence of glucose-6-phosphate and an independent or I form, manifesting activity in the absence of glucose-6-phosphate. Macrophage dependent or total glycogen synthetase activity measured in the presence of 10mM glucose-6-phosphate was $14.3 \pm 2.2 \mu\text{m}$ of UDP glucose incorporated into macrophage glycogen per 10 minutes per mg of protein, while the independent or I form of macrophage glycogen synthetase was $0.84 \mu\text{m}$ of UDP glucose incorporated into glycogen

FIGURE 6

EFFECT OF pH ON MACROPHAGE α -1,4 GLUCOSIDASE ACTIVITY

a) 0.5 ml of macrophage sonicate was incubated with 4 mg/ml of maltose in a total volume of 1.0 ml for 60 minutes at 4° and 37°C.

b) Mean \pm standard error of nine experiments.

TABLE 10
INFLAMMATORY MACROPHAGE GLYCOGEN SYNTHETASE ACTIVITY a.)

	Number of Incubated Samples	mmoles of UDP glucose incorporated into glycogen/ 10 min/mg protein
Total Synthetase Activity + 10mM Glucose-6-Phosphate	8	14.3 \pm 2.23
Independent Synthetase Activity - 10mM Glucose-6-Phosphate	8	0.84 \pm 0.08
Per Cent Activity in the Independent Form	8	5.9%

a.) Glycogen synthetase activity was determined on macrophage 3000 x g sonicate supernatants in 0.1M glycylglycine buffer.

per 10 minutes per mg protein and represented approximately 6% of the total activity.

b. Effect of glycogen depletion on macrophage glycogen synthetase activity

In view of the observation that preincubation of macrophages in a glucose-free media accelerated the rate of ^{14}C -glucose incorporation into glycogen, it was of interest to investigate whether an increase in the activity of glycogen synthetase was the underlying mechanism for the acceleration of glycogen resynthesis in glycogen-depleted macrophages. In Table 11 is shown the results of a comparison of macrophage glycogen synthetase activity, both in the glucose-6-phosphate independent and dependent forms, from glycogen-laden macrophages and macrophages depleted of their glycogen reserve by a 2 hour preincubation in a glucose-free media. Subsequent to glycogen depletion macrophages exhibited a dramatic 267% increase in the glucose-6-phosphate dependent form of the enzyme to a value of $38.2 \pm 2.1 \mu\text{m}$ of UDP glucose incorporated into glycogen per 10 minutes per mg of protein. The glucose-6-phosphate independent form of glycogen synthetase was observed to increase over five fold to a value of $4.69 \pm 0.73 \mu\text{m}$ of UDP glucose incorporated into glycogen per 10 minutes per mg of protein. As a consequence of this dramatic rise in the independent form of macrophage glycogen synthetase in glycogen depleted macrophages, glycogen synthetase activity in the independent form increased 83% as compared to glycogen laden macrophages.

4. Macrophage glucose-6-phosphatase activity

Glucose-6-phosphatase catalyzes the hydrolysis of glucose-6-phosphate to inorganic phosphate and glucose and its activity has been

TABLE 11
EFFECT OF GLYCOGEN DEPLETION ON MACROPHAGE GLYCOGEN SYNTHETASE ACTIVITY a.)

Incubation Conditions	Number of Incubation Samples	Glycogen Synthetase Activity	μ M UDPG Glucose Incorporated into glycogen/ 10 min/mg protein	% of Control
Glycogen-Laden	8	D-form	14.3 ± 2.23	100
Glycogen-Depleted b.)	4	D-form	38.2 ± 2.15	267
Glycogen-Laden	8	I-form c.)	0.84 ± 0.08	100
Glycogen-Depleted	4	I-form	4.69 ± 0.73	558

- a.) Glycogen synthetase activity was determined on macrophage 3000 x g sonicate supernatant in 0.1M glycylglycine.
- b.) Glycogen-depleted macrophages were preincubated for 2 hours at 37°C in GFH.
- c.) Percent activity in I-form in glycogen-laden macrophages = 5.9%.
Percent activity in I-form in glycogen-depleted macrophages = 10.9%.

reported in liver, kidney and in small intestine (59). Since glucose-6-phosphatase represents the final key regulatory step in glycogen metabolism in terms of glucose output, it was of interest to determine whether inflammatory macrophages could demonstrate hydrolytic activity towards glucose-6-phosphate. Table 12 presents the glucose-6-phosphatase activity of inflammatory macrophage sonicates in 0.25M sucrose. Macrophages displayed a significant glucose-6-phosphatase activity of 1.52 ± 0.11 mg Pi/mg protein/10 min, suggesting the enzymatic capability to liberate free glucose from intracellular glycogenolytic mechanisms.

C. Glucogenesis in Inflammatory Macrophages

Since the macrophage is known to perform trophic functions by the ingestion of soluble macromolecules and the subsequent transport of digested products back into the immediate extracellular environment, it was of interest to determine whether the inflammatory macrophage responded trophically to the uptake and intracellular digestion of exogenous glycogen by the production and subsequent transport of glucose, i.e. glucogenesis, back into the extracellular milieu.

1. Glucose production in normal and inflammatory exudate cell populations

In Table 13, the glucogenic capability of unstimulated peritoneal mononuclear cells, inflammatory PMNL and inflammatory macrophages is evaluated by measuring the glucose liberated by these cell populations into a glucose-free media. In the absence of exogenous glycogen, there was no significant amount of glucose liberated during a 3 hour incubation period by the three peritoneal cell populations. The addition of 50 mg of glycogen to the incubation flasks significantly

TABLE 12
GLUCOSE-6-PHOSPHATASE ACTIVITY IN INFLAMMATORY MACROPHAGES

Cell Population a.)	Number of Incubation Samples	Glucose-6-Phosphatase b.) Activity
Inflammatory Macrophage Sonicate	12	1.52 \pm 0.11

a.) Inflammatory macrophages were sonicated in 0.25M sucrose at 4°C.
Duplicate samples were incubated at 4° or 37°C for 30 min.

b.) Glucose-6-phosphatase activity is expressed as mg Pi/mg protein/10 min.

TABLE 13
GLUCOSE PRODUCTION IN NORMAL AND INFLAMMATORY EXUDATE CELL POPULATIONS a.)

Cell Population	Additions to incubation media		Glycogen (10 mg/ml) + NaF (3 mM)
	No Additions	Glycogen (10 mg/ml)	
Unstimulated Peritoneal Cells	0 (3) ^{b.)}	2.26 ± 0.78 (4)	1.63 ± 0.98 (4)
Inflammatory Leukocytes	0.63 ± 0.31 (3)	1.42 ± 0.13 (3)	1.80 ± 1.06 (4)
Inflammatory Macrophages	0.23 ± 0.13 (3)	2.68 ± 0.35 (4)	$12.30 \pm 1.75^{\text{c.)}}$ (5)

a.) Two ml of cells ($20-50 \times 10^6/\text{ml}$) were suspended in GFH in a total incubation volume of 3 ml. Incubations were for 180 min. at 37°C . Glucose production is expressed as μg of glucose per mg of protein per hour. All data represent the differences as compared to control samples incubated at 4°C .

b.) Number of experiments

c.) Significantly different from the unstimulated and leukocyte populations ($p < .001$)

augmented the glucogenic response of all three cell types examined. While the addition of 3 mM NaF, a potent glycolytic inhibitor, to the incubation media containing exogenous glycogen did not significantly alter glucose liberation in either the unstimulated peritoneal mononuclear cells or in the inflammatory PMNL, glucose production by the inflammatory macrophage was augmented from $2.68 \pm 0.35 \mu\text{g}$ glucose per mg protein per hour to $12.30 \pm 1.75 \mu\text{g}$ glucose per mg protein per hour, nearly a six fold increase in the macrophage glucogenic capacity. In contrast, macrophages did not manifest a glucogenic response to 3 mM NaF when exogenous glycogen was deleted from the incubation media. Furthermore, incubation of 50 mg of glycogen in GFH for 3 hours at 37°C did not result in any measurable glucose liberation into the media.

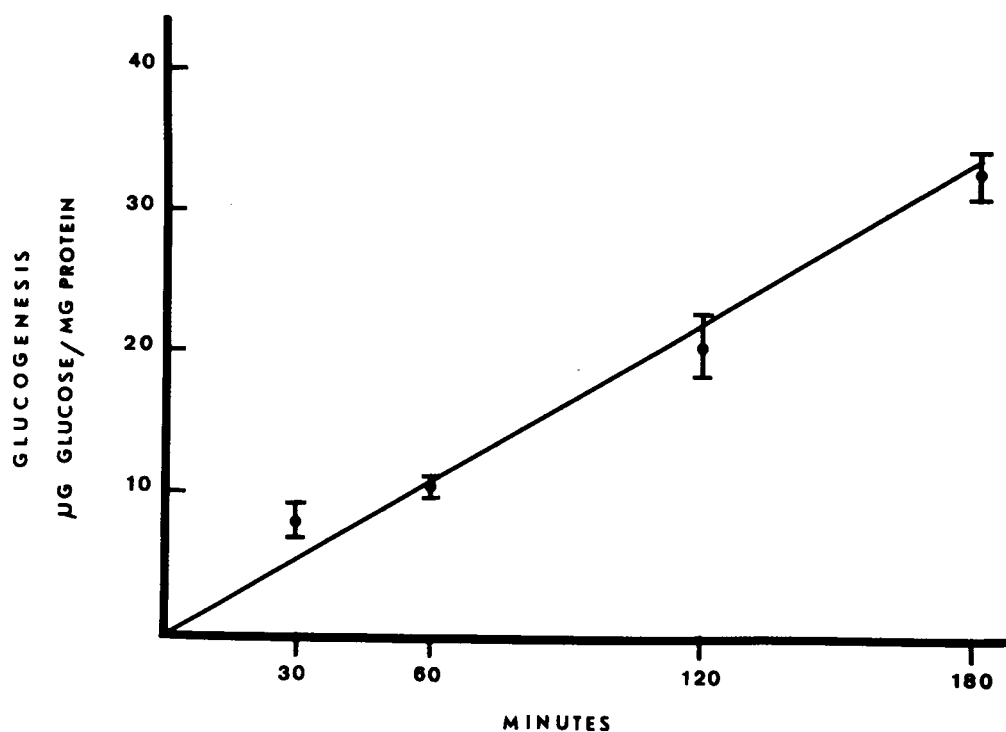
Glucogenesis by inflammatory macrophages as related to length of incubation is represented in Figure 7. The rate of macrophage glucogenesis was directly related to the 3 hour incubation interval utilized in these experiments. Additional studies have also demonstrated that the glucogenic rate was independent of exogenous glycogen concentration above 8 mg/ml. These results indicate not only that the rates of reactions involved in glycogenolysis and glucogenesis were dependent on length of incubation and substrate concentration but also that these mechanisms were maintained over the course of incubation time chosen in these experiments.

2. Glucogenesis by inflammatory macrophage cell and sonicate preparations

Table 14 presents the glucogenic response of macrophage whole cells and sonicate preparations. Macrophage sonicate preparations when compared to intact cells demonstrated a significant increase in the rate

FIGURE 7

GLUCOGENESIS BY INFLAMMATORY MACROPHAGES



Macrophages were incubated in the presence of 50 mg of glycogen and 3mM NaF. Each point represents the mean \pm S.E. of at least four experiments.

TABLE 14
GLUCOSE PRODUCTION BY INFLAMMATORY MACROPHAGE CELL AND SONICATE PREPARATIONS

	Glucogenesis - μ g glucose/mg protein/hr		
	No Additions	Glycogen (10 mg/ml)	Glycogen (10 mg/ml) + NaF (3 mM)
Macrophage Whole Cells	0.23 \pm 0.13 (3)	2.68 \pm 0.35 (4)	12.30 \pm 1.75 (5)
Macrophage a.) Sonicate	3.13 \pm 0.97 (3)	15.56 \pm 2.49 (3)	16.71 \pm 2.44 (3)

a.) Macrophages ($20-50 \times 10^6$ /ml) were sonicated for two minutes at 4°C in GFH, pH 7.4.

of glucose formation either in the absence or presence of exogenous glycogen. However, the addition of 50 mg of glycogen and 3mM NaF to macrophage sonicate preparations resulted in a rate of glucose liberated of $16.71 \pm 2.41 \mu\text{g}$ of glucose per mg protein per hour comparable to that found in the intact cell preparation, i.e. $12.3 \pm 1.75 \mu\text{g}$ glucose per mg protein per hour. Furthermore, the glucogenic response of $15.56 \pm 2.49 \mu\text{g}$ glucose per mg protein per hour demonstrated by the addition of exogenous glycogen alone to macrophage sonicates illustrates that the rate of glucose formation from sonicate glycogenolysis in the absence of any metabolic inhibitor is similar to the rate of glucogenesis by intact macrophages when substrate utilization is effectively inhibited by NaF.

3. Effect of acid pH conditions on macrophage glucogenesis

In order to evaluate the possible role of macrophage acid-hydrolytic mechanisms on glycogenolysis and subsequent glucose formation, exogenous glycogen was added to macrophage sonicate preparations under neutral and acid pH conditions (Table 15). Macrophage sonicates were incubated for 180 minutes in a citrate phosphate buffer at either pH 7.0 or pH 5.0. Glucose production by macrophage sonicates without the addition of exogenous glycogen demonstrated a probable endogenous glycogenolysis at both neutral and acid pH conditions. The addition of exogenous glycogen to macrophage sonicates resulted in an augmentation of macrophage glycogenolysis. Moreover, macrophage sonicate glycogenolysis and glucose formation in the presence of exogenous glycogen was further augmented at pH 5.0 when compared to neutral pH conditions.

4. Substrate and glycoside bond specificity of macrophage glucogenesis

TABLE 15
THE EFFECT OF pH ON GLUCOSE PRODUCTION BY MACROPHAGE SONICATES

Macrophage Sonicate a.)	Glucogenesis - μ g glucose/mg protein/hour	
	pH 7.0	pH 5.0
+ 10 mg/ml Glycogen	21.63 \pm 3.70 b.)	32.72 \pm 5.02
- 10 mg/ml Glycogen	8.50 \pm 1.29	9.02 \pm 1.97

a.) Three ml of macrophage sonicates were incubated in citrate phosphate buffer for 180 min.

b.) Each value represents the mean \pm standard error of the mean of 3 experiments.

In view of the fact that α -1,4 glucosidase predominates as the principal acid glucosidase present within the inflammatory macrophage, substrate and glycoside bond specificity of macrophage glucogenesis was investigated in intact macrophage preparations (Table 16). Glycogen and soluble starch contain α -1,4 and α -1,6 glycoside bonds linking glucose residues and both substrates can vary enormously in their particle size. Dextrans are polysaccharides produced by the action of certain bacteria (e.g. Leuconostoc mesenteroides) on sucrose and are composed almost entirely of glucose units joined by α -1,6 glycosidic linkages. Incubation of intact inflammatory macrophages with either glycogen or starch resulted in comparable rate of glucogenesis, $12.3 \pm 1.75 \mu\text{g}$ of glucose per mg protein per hour and $11.00 \pm 0.31 \mu\text{g}$ of glucose per mg protein per hour, respectively, while the addition of two different molecular weight dextrans failed to liberate any significant amount of glucose.

5. Effect of cyclic AMP on macrophage glucogenesis

In order to evaluate the potential role of a phosphorylytic mechanism mediated by glycogen phosphorylase on the glucogenic mechanism, experiments were performed to determine whether dibutyryl cyclic AMP could influence macrophage glucose formation from exogenous glycogen. Cyclic AMP and, in the presence of intact cells, the dibutyryl derivative are nucleotides known to activate phosphorylase kinase and glycogen phosphorylase (56). Macrophages and PMNL were incubated under identical conditions in the presence of 50 mg of glycogen, 3mM NaF and 1mM dibutyryl cyclic AMP for 180 minutes (Table 17). The results indicate that in the inflammatory macrophage, the addition of dbc AMP produced a 63% increase in the rate of glucose formation in the presence

TABLE 16
SUBSTRATE AND GLYCOSIDE BOND SPECIFICITY OF MACROPHAGE GLUCOGENESIS a.)

Addition to Incubation Medium a.)	Number of Incubation Samples	<u>Glucogenesis</u>
		<u>μg glucose/mg protein/hour</u>
Glycogen	5	12.30 ± 1.75
Starch	3	11.00 ± 0.31
Dextran mw= 2 x 10 ⁶	3	0.28 ± 0.23
Dextran mw= 2 x 10 ⁵	3	0.42 ± 0.31

a.) Two ml of macrophages were incubated in the presence of NaF (3 mM) and 50 mg of carbohydrate in GFH for 180 min at 37°C.

TABLE 17
EFFECT OF CYCLIC AMP ON MACROPHAGE AND LEUKOCYTE GLUCOGENESIS a.)

Cell Population	<u>Glucogenesis - μg glucose/mg protein/hour</u>	
	<u>-dbc AMP</u>	<u>+dbc AMP (1 mM)</u>
Inflammatory Leukocyte	1.80 \pm 1.06 (4)	1.20 \pm 0.31 (3)
Inflammatory Macrophage	12.20 \pm 1.75 (5)	19.92 \pm 0.90 (3) ^{b.)}

a.) Two ml of cells were incubated with 10 mg/ml of glycogen and 3 mM of NaF in a total volume of 3.0 ml for 180 min. at 37°C with or without 1 mM dbc AMP.

b.) Significantly different from macrophages incubated without dbc AMP ($p < .01$).

of exogenous glycogen. In contrast, the inflammatory PMNL did not exhibit any demonstrable change in their ability to liberate glucose from a glycogen source in the whole cell system.

CHAPTER V

DISCUSSION

The results of this study demonstrate a prominent glycogen content in inflammatory macrophages originating from a 96 hour peritoneal exudate. The inflammatory macrophage glycogen content of $9.55 \pm 0.44 \mu\text{g}$ glycogen per 10^6 cells is not significantly different from the glycogen content of 3 hour inflammatory leukocytes utilized in this study and is also comparable to glycogen values reported for normal PMN leukocytes (43, 112). Although a quantitative analysis of macrophage glycogen content has not been previously reported, histological studies have suggested glycogen to be a significant cytoplasmic constituent of mononuclear phagocytes. Weiss and Fawcett (134) cultured chicken buffy coat cells in autologous serum, allowing granulocytes and platelets to disintegrate with continuing culture and leaving a uniform population of adherent macrophages. During the in vitro culture incubations, macrophages were observed to markedly increase cytoplasmic inclusions exhibiting a positive periodic acid-Schiff (PAS) reaction by the ingestion of effete cells. Wulff (142) investigated the glycogen metabolism of developing macrophages and observed that, parallel to maturation, an increase in PAS-positivity occurred. Recently, Dvorak et al. (38) demonstrated dense aggregations of glycogen within the peripheral pseudopodia of migrating macrophages in capillary tube cultures. Furthermore, in cultures where macrophage migration was

inhibited by the presence of sensitized lymphocytes, glycogen-packed pseudopodia were less prominent. Interestingly, in the invertebrate chiton Liolophura where the phagocytic cell represents the only known host defense mechanism to foreign body invasion or tissue injury, wandering macrophages have been collected from the hemolymph and have been observed to contain large aggregations of glycogen particles, at times filling more than half of the cell's cytoplasmic space (71). Therefore, the present quantitative data discounts the previous notion that the PMN leukocyte is the only white blood cell population to contain significant stores of glycogen (109, 125) and supports the histological evidence that macrophages can accumulate a large glycogen reserve.

The acquisition of glycogen by the macrophage at the inflammatory locus appears to occur by the active ingestion of cellular debris containing large deposits of glycogen. Quantitative studies on inflammatory leukocyte glycogen content have recently been conducted on guinea pig blood and exudate leukocytes (111). The glycogen content of leukocytes entering the inflammatory site increased dramatically when compared to control peripheral blood leukocytes. The mean glycogen content of peripheral blood leukocytes of 6.5 ± 1.3 mg of glycogen per 10^9 cells was elevated to 28.7 ± 3.1 mg of glycogen per 10^9 cells in 24 hour exudate leukocytes. Indeed, the glycogen content of inflammatory leukocytes was already significantly elevated after 4 hours following the induction of the inflammatory response. Examination of histological evidence has repeatedly suggested that exudate macrophages acquire glycogen by the ingestion of leukocyte debris. Wulff (141) demonstrated that inflammatory macrophages progressively increased their glycogen

content by the ingestion of neutrophil cytoplasmic fragments. Rebuck et al. (97) have further suggested that the acquisition of PAS- positive neutrophilic debris by inflammatory macrophages was apparently dependent upon a functional macrophage carbohydrate metabolism. The authors proposed the concept that the depressed phagocytic capability of macrophages originating from insulin-deficient diabetics was due, in part, to a failure of macrophages to increase their glycogen reserve.

The results of in vitro experiments in this study demonstrating the uptake by inflammatory macrophages of exogenous glycogen clearly are in agreement with the histological evidence that macrophages are capable of ingesting glycogen at the inflammatory site. Macrophages were able to incorporate ^{14}C -labelled glycogen into intracellular glycogen stores, amounting to nearly 10% of the total glycogen content in a 15 minute incubation period. Furthermore, inflammatory macrophages demonstrated a profound increase in ^{14}C -glycogen uptake at 37°C as compared to incubation at 4°C , suggesting that the labelled glycogen was not merely being adsorbed to the macrophage membrane during the incubation interval. Moreover, macrophages were able to maintain their glycogen stores not only by the provision of glucose to the incubation media but also by the ingestion of exogenous glycogen in a glucose-free environment. These results suggest that the macrophage at the inflammatory site is able to maintain functional integrity by the ingestion of glycogen-containing cellular debris even though adequate substrate supply may be interrupted.

A functional carbohydrate metabolism is mandatory for the provision of cellular energy for the active endocytic process in phagocytic cells.

The findings that, in peritoneal PMN leukocytes and macrophages, glycolytic inhibitors prevent phagocytosis, whereas inhibitors of respiration do not indicate that the energy requirement for particle uptake is ultimately derived from glycolysis (85, 106). Pinocytosis by peritoneal macrophages, in contrast, has been shown to be severely depressed by inhibitors of oxidative phosphorylation and respiration (16). In vitro incubation under N₂ atmospheric conditions did not interfere with glycogen uptake by macrophages in a glucose-free media when compared to macrophages incubated under air as the gas phase, suggesting that anaerobic glycolysis provided the necessary energy for macrophage glycogen uptake. Whether in vitro glycogen uptake was performed by pinocytic or phagocytic mechanisms was not determined in this study, although under in vivo conditions at the inflammatory site, both endocytic mechanisms would be operative in the ingestion of soluble glycogen or aggregates of the glycogen macromolecule.

The investigation into the alteration of macrophage glycogen content during phagocytosis was an attempt to gain further insight into the functional significance of the macrophage glycogen reserve. Studies examining the metabolic perturbations during phagocytosis by either leukocytes or macrophages have demonstrated a significant stimulation in glucose utilization during the phagocytic event (23, 135). The role of leukocyte glycogen as an energy store to be utilized during phagocytosis has been proposed by Scott (109). Indeed, several studies have reported leukocyte glycogen levels to be depleted in vitro during phagocytosis (6, 23, 120). Inflammatory macrophages incubated in the presence of latex particles were observed to significantly accelerate glycogenolysis

in the absence of glucose when compared to non-phagocytizing controls. Thus the evidence suggests that the inflammatory macrophage glycogen reserve can also be called upon to provide for the augmented glucose demands during phagocytosis.

Incubation for 60 minutes in a glucose-free media mobilized half of the macrophage glycogen supply, however, incubation for longer intervals did not further deplete endogenous glycogen stores. The inability to mobilize the entire glycogen reserve in macrophages has not been determined, although studies involved in the molecular structure of glycogen have revealed that apparently only 60% of the total glucose residues forming the outer linear chains of the glycogen molecule are readily accessible to the action of phosphorylase (115). Additional support for the role of macrophage glycogen in maintaining active phagocytic mechanisms was provided utilizing glycogen-depleted macrophages. The results in this in vitro study demonstrate that the addition of exogenous glycogen in a glucose-free media to macrophages whose energy stores have been depleted did not increase macrophage glycogen levels to initial control values, although glycogen-depleted macrophages readily synthesized glycogen when extracellular glucose was available. The depression of macrophage phagocytic ability in glycogen-depleted cells was further supported by the fact that the uptake of ^{14}C -labelled glycogen by glycogen-depleted macrophages in a glucose-free media was depressed nearly 50% when compared to glycogen-laden macrophages. These results suggest that the depletion of macrophage carbohydrate reserve may significantly reduce the capacity of inflammatory macrophages to participate in the inflammatory reaction by depressing macrophage function

when glucose is not readily available.

The in vitro incorporation of ^{14}C -glucose into macrophage glycogen offers some insight into the changing balance of synthesis at different glycogen loads. The rate of glycogen synthesis in inflammatory macrophages partially depleted of their glycogen reserve was accelerated more than three-fold when compared to glycogen-laden cells when incubated in the presence of ^{14}C -U-glucose. These results were consistent with similar observations made in the PMN leukocyte (109, 111) and in glycogen-depleted human cytotrophoblastic cells -- a cell line that relies heavily on glycogen as an energy source (89). The in vitro results in the present study raise the possibility that a relationship exists between the initial glycogen content of macrophages and the rate of incorporation of glucose into glycogen. In skeletal muscle (75) and liver (35), glycogen decreases its own synthesis non-hormonally by inhibition of glycogen synthetase D phosphatase, the enzyme that converts synthetase D to synthetase I. It was suggested by Wang et al. (132) that glycogen repletion of PMN leukocytes is associated with a rapid change from the D-form of the enzyme to the I-form with a small change in total enzyme activity. More recently, Scott and Cooper (111) observed a significant elevation in glycogen synthetase activity in exudate leukocytes when compared to blood leukocytes and attributed the change in enzyme activity to an increase in the glucose-6-phosphate dependent (D) form of the enzyme.

The present results clearly demonstrate the activity of UDP glucose-glucan glucosyl transferase (i.e. glycogen synthetase) in macrophage sonicates. Macrophage glycogen synthetase activity independent of

glucose-6-phosphate (I-form) was equal to 5.9% of the total (I and D) activity. Furthermore, when macrophages were incubated for 2 hours in a glucose-free media, the I activity increased to nearly 11%. This two fold increase in I activity suggests a decreased inhibitory effect of glycogen on the synthetase phosphatase. A similar glycogen synthetase D to I conversion has recently been reported in the human PMN leukocyte (132) and also in human lymphocytes (57). Depletion of macrophage glycogen content for 2 hours significantly increased glycogen synthetase activity not only in the glucose-6-phosphate independent or I form but also in the dependent or D form. In liver, the activation of glycogen synthetase (D to I conversion) involves a dephosphorylation catalyzed by a specific phosphatase, thus the activity of glycogen synthetase, like that of phosphorylase, appears to depend on a finely tuned regulatory mechanism between the activities of kinases and phosphatases. The present evidence suggests such a regulatory mechanism for glycogen synthesis is operational within the inflammatory macrophage and furthermore, that alterations in glycogen levels within the cell will exert a profound influence on the macrophage glycogen synthetic machinery.

Insulin, a potent stimulator of glycogen synthetic mechanisms in adipose tissue and skeletal muscle, failed to increase ^{14}C -glucose incorporation into macrophage glycogen at an insulin concentration of $200\mu\text{U/ml}$. These results support the conclusions of Esmann (44) who also failed to find any in vitro effect of insulin at physiological concentrations on glucose transport or glycogen synthesis in the PMNL, although prolonged incubation and high insulin concentration (1U/ml) did result in a non-specific increase in glucose transport. However, the addition of 1%

albumin to the incubation media significantly doubled the in vitro incorporation of ^{14}C -glucose into macrophage glycogen in a 60 minute incubation period. Cohn and Parks (24) have investigated the pinocytosis-inducing effect of a number of molecular species and have found certain proteins, in particular plasma albumin, to be potent stimulators of vesicle formation in macrophages. The addition of insulin to the media containing albumin did not further enhance ^{14}C -glucose incorporation into macrophage glycogen. Thus the present results suggest that stimulation of macrophage pinocytosis results in an increased transport of extracellular glucose, offering endocytosis as a mechanism facilitating glycogen synthesis by increasing the incorporation of available extracellular glucose.

The demonstration of macrophage phosphorylase activity indicates that, in view of the broad variation in macrophage energy demands at the inflammatory site, glycogen mobilization is controlled by a finely balanced regulatory mechanism. Rapid glycogenolysis is accomplished by the activation of glycogen phosphorylase and is controlled by a complex cascade of phosphorylation-dephosphorylation reactions. Green and Cori (51) were the first to isolate rabbit skeletal muscle phosphorylase and found readily interconvertible active and inactive forms. Sutherland et al. (123) characterized the two forms of phosphorylase in liver homogenates and demonstrated that the conversion of the inactive to active form required the presence of ATP, Mg^{++} and the enzyme phosphorylase b kinase. Distinct differences exist in the phosphorylase activity from these two tissues. The inactive skeletal muscle enzyme becomes active in the presence of AMP, while the active form has an activity independent of AMP.

In contrast, the inactive liver enzyme demonstrates little activity even in the presence of the nucleotide, whereas the active form is stimulated only 40% by AMP. Detailed studies on the protein kinases responsible for the phosphorylation of muscle phosphorylase b has revealed that they also occur as active and inactive forms. Studies by Krebs et al. (74) have shown that the conversion of the inactive to active form of phosphorylase b kinase takes place on incubation with ATP and Mg^{++} , resulting in the phosphorylation of the inactive form. Maddaiah and Madsen (79) have demonstrated that 60% of resting liver phosphorylase activity is in the active form while resting skeletal muscle phosphorylase activity is predominantly in the b or inactive form (72). The levels of cofactors and substrates in the cell have been shown to influence the interconversion reactions of phosphorylase and synthetase. Krebs et al. (74) have demonstrated that elevated glycogen levels stimulate the phosphorylase b to a conversion and also the activation of phosphorylase b kinase whereas, increased glycogen levels inhibited the synthetase D to I conversion (128).

Determination of leukocyte phosphorylase activity demonstrated a behavior similar to that found in liver in that it was possible to activate leukocyte phosphorylase activity in vitro by glucagon (137). Recently, Yunis and Arimura (143) examined the properties of glycogen phosphorylase in rat chloroma cells, a tumor composed entirely of immature granulocytes, and noticed that leukocyte phosphorylase exhibited considerable activity in the absence of AMP and thus resembled liver phosphorylase. The present results of phosphorylase assays in inflammatory macrophage extracts indicate that a significantly large fraction

of the total phosphorylase activity exists in the active form (73%), thus, in this one respect, macrophage phosphorylase activity resembled leukocyte and liver phosphorylase activity. Total phosphorylase activity did not significantly change during a 1 hour period of glycogenolysis in a glucose-free media, however, phosphorylase activity increased significantly 15 minutes following the addition of exogenous glycogen when compared to initial control values, again comparable to liver phosphorylase. Conversion of inactive to active liver phosphorylase has been shown to occur readily in the presence of Mg^{++} and ATP (94). Macrophage active phosphorylase activity was markedly enhanced in macrophage sonicates incubated in the presence of 0.1M Mg^{++} -ATP following the partial inactivation of the active form by a 30 minute pre-incubation. The results suggest that the Mg^{++} -ATP stimulation of macrophage active phosphorylase activity is similar to those mechanisms described in liver and muscle. The activation of this protein kinase in these tissues by Mg^{++} -ATP is strongly stimulated by cyclic AMP. The now classic studies by Sutherland and Rall (122) on the physiology of cyclic AMP have demonstrated that phosphorylase activation via the adenyl cyclase system provides rapid glycogenolysis and increased glucose output by liver preparations. In support of this concept, Reik et al. (100) have localized a hormone-sensitive adenyl cyclase activity in reticuloendothelial cells of the liver utilizing both cytochemical and biochemical techniques. In addition, alveolar macrophages have been found to exhibit a significant increase in the intracellular level of cyclic AMP and thus suggesting a link between particle uptake and the initiation of metabolic events requiring the accelerated expenditure

of energy in the form of glycogenolysis (114). Thus the glycogen mobilization via phosphorylase by the inflammatory macrophage may also respond in a similar fashion via a hormone-sensitive adenylyl cyclase mechanism that would function in rapidly providing glucose to meet the substrate demand of the macrophage at the inflammatory locus.

The presence of an acid α -glucosidase in inflammatory macrophages provides an alternate mechanism for the degradation of the glycogen molecule. This is the only known lysosomal enzyme capable of degrading glycogen. Jeffrey et al. (64) have recently purified an α -glucosidase from the lysosomal fraction of rat liver homogenates and have demonstrated that the enzyme had both α -1,4 and α -1,6 glucosidase activity. Hers (58) initially demonstrated the deficiency of a lysosomal acid maltase, α -1,4 glucosidase, in the livers of patients with Pompe's disease (Type II glycogenosis), a fatal disorder characterized by the accumulation of glycogen in the heart, skeletal muscle, brain, liver and kidney. Williams (136) measured α -glucosidase activity in human PMN leukocytes from normal subjects and from patients with Type II glycogenosis and demonstrated that leukocytes from patients with Pompe's disease display approximately one half the activity of the normal controls.

Hsia et al. (61) have characterized an α -glucosidase in human PMN leukocytes and were able to show activity with maltose as a substrate; however, no α -1,6 glucosidase activity was observed utilizing isomaltose as substrate. The results of the present study utilizing the macrophage indicate a prominent α -1,4 glucosidase activity measured at pH 4.0 with maltose as substrate. Furthermore, that only α -1,4 glucosidase activity

was manifest in macrophage sonicates and negligible α -1,6 glucosidase activity was observed is comparable to the results found in the PMN leukocyte. The determination of a 4.0 pH optimum for macrophage α -1,4 glucosidase activity is in agreement with results described for liver α -1,4 glucosidase (58) and in PMN leukocytes (136) and strongly suggests a probable lysosomal origin within the macrophage for the acid glucosidase activity. Therefore, the identification within macrophages of a hydrolytic degradative mechanism for glycogen indicates that subsequent to the discharge of lysosomal hydrolases into a glycogen-containing phagocytic vacuole, lysosomal glucosidase could readily attack the outer linear branches of the glycogen molecule providing available free glucose for macrophage functional energy demands.

In view of the fact that glucose-6-phosphatase provides the final enzymatic link in the provision of free extracellular glucose from the glycogenolytic sequence, particular interest is raised in this study when glucose-6-phosphatase activity was observed in macrophage sonicates. It is generally accepted that glucose-6-phosphatase activity is manifested predominantly in those organs which function in the regulation of blood glucose. Thus, Hers and DeDuve (59) found glucose-6-phosphatase activity greatest in liver, kidney and in small intestine. Although no glucose-6-phosphatase activity has been reported biochemically in other white blood cells (61, 109), the blood platelet however, does contain measurable amounts of glucose-6-phosphatase (52). Recent histochemical evidence revealed that glucose-6-phosphatase activity was localized in rat and mouse RES cells, in particular, alveolar macrophages and Kupffer cells of the liver (104). The present evidence

supplies the quantitative data in support of the histochemical demonstration of glucose-6-phosphatase activity in macrophages.

The functional significance of glucose-6-phosphatase in RES cells has not been previously investigated. The active phosphorylytic attack of macrophage glycogen by phosphorylase would result in the formation of glucose-6-phosphate which predominantly would be further metabolized via glycolytic or hexose monophosphate shunt pathways to meet macrophage energy demands. However, the presence of glucose-6-phosphatase in inflammatory macrophages provides a third alternative to the metabolism of glucose-6-phosphate, whereby the hydrolytic attack on the molecule would result in the formation of free glucose. Therefore, the macrophage at the inflammatory site is equipped with the full complement of glucogenic enzymes and is capable enzymatically of supplying glucose to its extracellular environment. Thus the present characterization of glycogenolytic and glucogenic enzymes within the inflammatory macrophage reveals an additional trophic functional role for the large carbohydrate supply ingested and stored by the inflammatory macrophages.

The process of glucogenesis, i.e. the liberation of glucose, was demonstrated in vitro in the peritoneal exudate cells and significant increases were observed in macrophages in the presence of glycogen and NaF, a glycolytic inhibitor. In contrast, neither inflammatory PMN leukocytes nor unstimulated peritoneal mononuclear cells responded by augmented glucose liberation when exogenous glycogen and the glycolytic inhibitor were added. The use of a glycolytic inhibitor in vitro to exaggerate the glucogenic response by inflammatory macrophages may mimic the in vivo situation whereby the glycolytic pathway is depressed by a

decrease in intracellular pH initiated by the release of acid hydrolases into the cytosol. In support of this concept, Halpern et al. (53) recently compared the rate of glycolysis in leukocytes incubated in media of low pH values and found that the inhibition of glycolysis was due to an inhibition of the activity of phosphofructokinase, a key regulatory enzyme of the glycolytic pathway.

Recent studies by Cohn and Ehrenreich (20) relevant to the mechanisms involved in the uptake of digestible and nondigestible carbohydrates by cultured macrophages have demonstrated that large carbohydrate molecules are pinocytized and stored within lysosomal associated vacuoles. Intralysosomal hydrolysis of carbohydrates then takes place with the formation of utilizable monosaccharides which escape from the vacuole. It appears reasonable to speculate that such a mechanism is employed following the ingestion of glycogen-containing debris from the inflammatory environment. The resulting release of glucose from the phagocytic vacuole would then be either further metabolized or liberated from the macrophage and utilized by ongoing reparative processes at the inflammatory site.

In an attempt to extend these observations, macrophage sonicate preparations were employed to investigate the hydrolytic degradation of glycogen by inflammatory macrophages. The rate of glucogenesis in macrophage whole cells was found to be comparable to the rate of glycogenolysis in macrophage sonicates indicating that macrophage lysosomal glucosidase released upon sonication could account for the observed rate of glucose liberation in the intact macrophage when glucose utilization was depressed. Glycogen and starch are composed of D-glucose units linked by

α -1,4 and α -1,6 glycoside bonds whereas dextrans are polysaccharides composed entirely of glucose units joined by α -1,6 glycoside bonds. A comparison of the hydrolytic action by macrophage sonicates on large molecular weight polysaccharides revealed that glucogenic activity was manifest only in the presence of carbohydrates where α -1,4 glycoside bonds are prominent. Thus these present studies concur with the enzymatic demonstration that α -1,6 glucosidase is not active within the macrophage lysosome. Although glucosidase activity in macrophage subcellular compartments by fractionation techniques was not determined in this study, the probable lysosomal origin of the hydrolytic activity on the glycogen molecule was inferred when exogenously added glycogen was more rapidly hydrolyzed to glucose by macrophage sonicates under acid pH conditions favored by lysosomal hydrolases.

An important functional role for phosphorylase as a degradative process involved in macrophage glycogen breakdown was illustrated by the in vitro addition of dibutyryl cyclic AMP to macrophages ingesting exogenous glycogen. Macrophage glycogenolytic and glucogenic mechanisms in intact cells responded to the addition of dibutyryl cyclic AMP by a notable increase in the glucose liberated from intact inflammatory macrophages. It is reasonable to assume that dibutyryl cyclic AMP accelerated intracellular glycogenolysis by stimulating the activation of macrophage phosphorylase and, coupled with the hydrolytic action of glucose-6-phosphatase, augmented the liberation of glucose supplied by the acid hydrolytic mechanism. Park et al. (88) postulated that adenyl cyclase activation during phagocytosis may be the initiating event triggering subsequent metabolic alterations during phagocytosis, thus emphasizing a

role for phosphorylase activation and the ensuing glycogenolysis in the energetics of particle engulfment within phagocytic cells. The present study suggests that the degradative pathways involved in glycogenolysis within the macrophage include both hydrolytic and phosphorylytic mechanisms and furthermore, that both mechanisms may be operative in supporting macrophage glucogenesis.

The present studies have demonstrated that the inflammatory macrophage is equipped to harvest a prominent carbohydrate reservoir by the ingestion of exogenous glycogen as well as by glycogen synthetic enzymes. Furthermore, the dynamic state of macrophage glycogen metabolism has been evaluated by the demonstration of a complex glycogen degradative apparatus, capable of responding to regulatory control mechanisms, to provide additional glucose to meet a wide spectrum of macrophage energy demands. The studies illustrating the effect of glycogen depletion on the phagocytic ability of the macrophage emphasizes that further investigation is needed to adequately clarify the physiological role of glycogen metabolism within the phagocytic cell. The demonstration of a glucogenic capacity within the inflammatory macrophage adds a new dimension to macrophage function suggesting that an important nutrient role may be served by the macrophage at the inflammatory site in the form of glucose provision to assist the reparative processes of tissue healing mechanisms.

CHAPTER VI

SUMMARY

1. Caseinate-induced 3 hour exudates yielded 6 to 7 x 10⁶ cells with greater than 90% of the cell population examined being PMNL. The 96 hour inflammatory exudate produced 2 to 3 x 10⁸ cells per rat with macrophages comprising 85 to 90% of the cell population from the peritoneum.
2. The 96 hour inflammatory macrophage demonstrated a prominent glycogen content of 11.79 ± 1.59 ~~µg~~ glycogen per 10⁶ cells or 110.3 ± 12.8 ~~µg~~ glycogen per mg of cell protein that was comparable to the glycogen content of the inflammatory PMNL.
3. Both PMNL and macrophages exhibited a labile intracellular glycogen pool that was decreased to approximately 50% of control values by incubation in glucose-free Hank's for 60 minutes. Furthermore, when macrophages were incubated in the presence of latex particles in a glucose-free medium, the glycogen content of phagocytizing macrophages was further diminished when compared to non-phagocytizing macrophages.
4. Inflammatory macrophages incubated in the presence of either 10 mM glucose or 10 mg/ml of exogenous glycogen maintained their large glycogen reserve during a 60 minute incubation interval.

5. Macrophage glycogen resynthesis from glucose was stimulated by preincubation for 2 hours in a glucose-free medium. However, the addition of exogenous glycogen to glycogen depleted macrophages did not result in an elevation of macrophage glycogen content in glycogen depleted cells.

6. Glycogen synthesis in glycogen depleted macrophages from ^{14}C -U-glucose was accelerated three-fold when compared to ^{14}C -U-glucose incorporation into glycogen from glycogen laden macrophages.

7. Inflammatory macrophages incubated in the presence of 1% albumin doubled their incorporation of ^{14}C -U-glucose into macrophage glycogen when compared to cells incubated without the pinocytic activator. 200 $\mu\text{U/ml}$ of insulin did not enhance the incorporation of ^{14}C -U-glucose into macrophage glycogen.

8. Exogenous ^{14}C -labelled glycogen was readily incorporated into the macrophage glycogen pool. In contrast, incorporation of ^{14}C -glycogen by glycogen depleted macrophages was markedly depressed.

9. Inflammatory macrophages exhibited glycogen phosphorylase activity that was significantly increased in the presence of exogenous glycogen. Macrophage active phosphorylase activity amounted to 73% of the total phosphorylase activity and the introduction of 0.1 M Mg^{++} -ATP to macrophage sonicates resulted in a nearly complete conversion of macrophage phosphorylase activity to the active form.

10. Inflammatory macrophages demonstrated considerable α -1,4

glucosidase activity that manifested its greatest activity at pH 4.0. In contrast, macrophages displayed no α -1,6 glucosidase activity.

11. Macrophage glycogen synthetase was found to exist in two forms, a glucose-6-phosphate dependent or D-form and a glucose-6-phosphate independent or I-form. The I-form amounted to approximately 6% of the total glycogen synthetase activity.

12. Glycogen depletion in inflammatory macrophages resulted in a dramatic increase in both the I and D-forms of glycogen synthetase. Glycogen depletion produced an 83% increase in the I-form as compared to glycogen laden macrophages.

13. Inflammatory macrophages displayed a significant glucose-6-phosphatase activity suggesting the enzymatic ability to liberate free glucose from intracellular glycogenolytic mechanisms.

14. Glucogenesis, i.e., glucose liberation into the incubation medium was demonstrated by 96 hour inflammatory macrophages in the presence of NaF and exogenous glycogen. Macrophage glucogenesis was greater at pH 5.0 than at pH 7.0.

15. Macrophage glucogenesis was marked in the presence of either exogenous glycogen or soluble starch while the addition of various molecular weight dextrans was ineffective in liberating glucose by macrophages.

16. Glucogenesis in both intact macrophages and sonicate preparations was augmented by dbc AMP while PMN leukocyte glucogenesis

was not affected by dbc AMP.

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Jan 2, 1975
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