METABOLIC AND FUNCTIONAL ACTIVITIES OF PHAGOCYTIC CELLS DURING GALACTOSEMIA

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ABSTRACT

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By

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To account for enhanced susceptibility to infection among infants deficient in galactose-1-phosphate uridylyltransferase (EC 2.7.7.10), the acute effects of D-galactose on metabolic and functional activities of phagocytic cells involved in host defense were investigated. Human and guinea pig polymorphonuclear leukocytes (PMN) when incubated in medium containing 30 mM galactose displayed substantially less killing of Escherichia coli than when incubated in medium with 5 mM glucose. Impaired bactericidal activity was dependent upon galactose concentration but could be partially averted by supplementing the galactose-containing medium with 15 mM glucose. Phagocytic activities of guinea pig PMN and peritoneal macrophages were assayed by following ingestion of ^{32}P-labelled E. coli and were also depressed by elevated galactose.

The inhibitory action of galactose on phagocyte function was further investigated in normal and galactosemic chicks by monitoring the in vitro killing of E. coli by

leukocytes and the *in vivo* clearance of colloidal ^{125}I-labelled bovine serum albumin (^{125}I-BSA) from the circulation. Elevated levels of galactose (30 mM) impaired the bactericidal activities of both normal and galactosemic leukocytes. However, the latter cells were more susceptible to the galactose dependent inhibition. Phagocytic indexes, obtained from data on the clearance of ^{125}I-BSA, were calculated to be 0.0553 and 0.0297 for normal and galactosemic chicks, respectively. Chicks fed a control diet displayed a logarithmic clearance of colloid with postinjection time, whereas this relationship was not as apparent when galactosemic chicks were employed. Moreover, galactose impaired phagocytic functions of both circulating leukocytes and tissue-fixed macrophages as well as the overall development of the reticuleondothelial system.

Although galactose and glucose were transported into PMN, competition between these two hexoses for cell entry was eliminated as a mechanism of galactose toxicity. Neither galactose nor fructose competed with [G-^{3}H]2-deoxyglucose for uptake by guinea pig PMN, whereas competitive inhibition was observed when either glucose or mannose were employed. Transport of [G-^{3}H]2-deoxyglucose proceeded *in vitro* with K_m of 1.8 mM and V_{max} of 0.67 nmole min^{-1} per 10^6 cells. This uptake was temperature dependent, and phosphorylation of the 6-position was necessary for intracellular concentration. Uncouplers of oxidative phosphorylation did not alter 2-deoxyglucose uptake.

However, uptake was sensitive to inhibitors of glycolysis and could be inhibited by pre-incubating cells with 2 mM iodoacetate, 40 mM fluoride, or 30 mM galactose. The latter observation indicated that galactose could interfere with glucose entry by blocking its phosphorylation rather than competing with its uptake.

Significant decreases in intracellular levels of adenosine 5'-triphosphate and in levels of glycolytic intermediates were found to explain the galactose dependent loss of phagocytic activity. These decreases were attributed to (i) the presence of a futile adenosine triphosphatase cycle, and (ii) the accumulation of galactose intracellularly with subsequent inhibition of glycolysis.

To account for the impaired bactericidal activity of PMN during galactosemia, a number of biochemical parameters associated with the oxygen-dependent killing mechanism were investigated. Galactose did not affect either oxygen consumption or extracellular hydrogen peroxide formation. However, the ability of PMN to reduce extracellular cytochrome c via superoxide anion was significantly impaired. The latter action could not be accounted for by decreased superoxide generation but could be attributed to oxidation of ferrocytochrome c by galactose radical. Formation of this carbohydrate radical was observed in two chemical systems, and its presence in PMN was supported by data on cellular reduction of nitroblue tetrazolium, formate oxidation, and chemiluminescence. These observations suggested

that galactose could impair the killing of bacteria by reacting with hydroxyl radical and subsequently scavenging superoxide anion.

METABOLIC AND FUNCTIONAL ACTIVITIES OF PHAGOCYTIC CELLS DURING GALACTOSEMIA

By

William John Litchfield

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AMP, ADP, ATP	adenosine 5'-mono-, di-, or triphosphate
AO	acridine orange
BSA	bovine serum albumin
CGD	chronic granulomatous disease
CPM	counts per minute
EDTA	ethylenediaminetetraacetic acid
G6PDH	glucose-6-phosphate dehydrogenase
i.p.	intraperitoneal
KRPS	Krebs-Ringer phosphate solution
NADH	reduced nicotinamide adenine dinucleotide
MPO	myeloperoxidase
PMN	polymorphonuclear leukocytes
PNP-NAG	para-nitrophenyl-N'-acetylglucosamine
RES	reticuloendothelial system
SD	standard deviation
SEM	standard error of the mean
SOD	superoxide dismutase

INTRODUCTION

Organization

This dissertation is divided into four chapters, each of which is in a form acceptable for publication in a biochemical journal. Chapter I has been published in Infection and Immunity under the authorship of William J. Litchfield and William W. Wells (1976) 13, 728-734. Chapter II and III have been submitted to Infection and Immunity and Journal of Biological Chemistry, respectively. Chapter IV is in preparation to be submitted to the latter journal. The first chapter is presented with the permission of the American Society for Microbiology, copyright holder. An appendix containing supplementary data is included at the end of the text.

Rationale and Objectives

Frequent occurrences of bacterial infection among patients with poorly controlled diabetes mellitus (1,2) and of Escherichia coli septicemia leading to death among infants with galactose-1-phosphate uridylyltransferase deficiency (3,4,5) have been well documented but poorly understood. Recent reports concerning host defense among diabetics demonstrated that the ability of polymorphonuclear

leukocytes (PMN) to ingest and destroy bacteria was depressed during hyperglycemia (6,7,8). This action was primarily attributed to elevated plasma glucose and was not a result of insulin or opsonin deficiencies (9). These observations suggested that a similar type of impairment could occur during galactosemia. However, there was no pertinent information about the function of PMN during this condition, and leukocytes from galactosemic infants were not readily available.

To test this hypothesis, studies were therefore conducted on PMN isolated from the peripheral blood of normal human and guinea pig sources. Levels of galactose employed in these studies (1 to 30 mM) were comparable to those encountered during the pathological condition (10,11), and it was assumed that any deleterious effect of galactose in these cells would be more pronounced in PMN of galactosemic origin. This assumption was supported by comparing the effects of galactose upon bactericidal activity of leukocytes from normal and galactosemic chicks. All assays of bactericidal activity were performed *in vitro* using serum-treated *E. coli*, whereas phagocytic assays were performed under similar conditions using ^{32}P-labelled *E. coli* or polystyrene latex particles. Extrapolation of results from these experiments to the *in vivo* state was facilitated by observing the inhibitory action of galactose upon the *in vivo* clearance of colloidal BSA from the circulation of galactosemic chicks.

The biochemical basis of galactose toxicity in

phagocytes was explained after investigating metabolic parameters associated with the physiological functions. Moreover, effects of galactose upon phagocytic activity were correlated with cellular dependence upon glycolysis, whereas effects upon bactericidal activity were correlated with the oxygen-dependent killing mechanism.

Literature Survey

The ability of phagocytic cells such as PMN, monocytes, and macrophages to ingest and destroy a wide variety of microorganisms is of particular importance to the protection of a host against infection (12,13,14,15,16). This physiological relationship is exemplified by enhanced susceptibility to infection observed during a number of clinical disorders which predominantely affect phagocytosing cells. These disorders include leukemia (17,18,19), chronic granulomatous disease (CGD)(16,20), glucose-6-phosphate dehydrogenase (G6PDH) deficiency (21,22), myeloperoxidase (MPO) deficiency (23), and Chediak-Higashi syndrome (24,25). It is significant that three, and perhaps four, of these disorders affect the oxygen dependent killing mechanism of PMN, whereas the latter syndrome affects phagocyte degranulation. In CGD and G6PDH deficiency the ability of PMN to phagocytose microorganisms is not impaired (16,21,22). However, killing of catalase-positive bacteria such as E. coli and Staphylococcus aureus is significantly reduced (3,16,21,22). These observations indicate that phagocytosis

and intracellular killing are two distinct physiological functions in PMN and that each function may be explained in separate biochemical terms. Nevertheless, since intracellular killing depends upon previous bacterial uptake, both of these functions are impaired during disorders of phagocytosis per se. These disorders can result from opsonin difficiencies in which phagocytes do not properly recognize bacteria (14) and also from a number of nutritional states in which the phagocytic cells may be metabolically impaired (26). Enhanced susceptibility to infection, primarily by encapsulated bacteria (14), is thus associated with decreased opsonization in various abnormalities of the complement system (27) and of the properdin system among neonates (28) and patients with sickle cell anemia (29). Infection is further associated with metabolically impaired phagocytes during vitamin deficiency (26), protein-calorie kwashiorkor (30), and increased glucose or fructose ingestion (8). Moreover, the latter observations indicate that phagocytosis is a "finely tuned" process which may undergo daily fluctuations <u>in vivo</u> depending upon blood glucose and other nutrient levels. This is supported by impaired phagocytic activity during hyperglycemia (6,7,8) and during starvation (30,31).

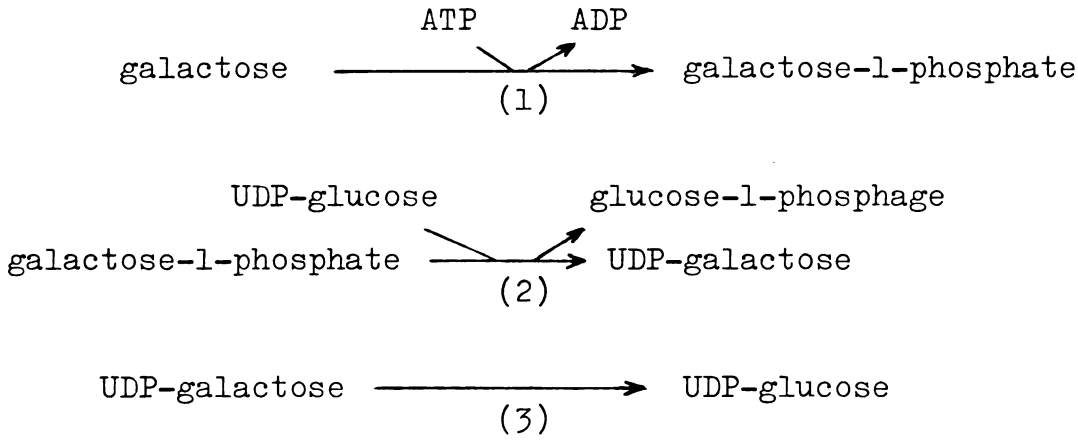
Since the initial demonstration by Sbarra and Karnovsky that phagocytosis in PMN could be inhibited by agents which antagonize glycolysis (32), a number of studies have been published in support of the hypothesis that phagocytosis

depends upon energy supplied by this metabolic pathway (33,34,35,36). This hypothesis holds for other phagocytes such as macrophage (36,37,38) and disputes the earlier view of Fenn that phagocytosis proceeds without metabolic expenditure (39). While phagocytosis in PMN is not altered by inhibitors of oxidative metabolism such as antimycin A, cyanide, and dinitrophenol, this function is very sensitive to iodoacetate and fluoride (32,33) which simultaneously inhibit cellular lactic acid production (32) and depress intracellular levels of ATP (40). In addition, phagocytosis can be impaired by inhibitors of glucose transport such as N-ethylmaleimide and cytochalasin B (41,42). The above hypothesis is further supported by observations that PMN are not gluconeogenic (43,44) and that these cells contain relatively few mitochondria (32).

Bactericidal activity, although dependent upon phagocytosis for supply of substrate, is a separate process which can be accounted for in different biochemical terms. This function is associated with a number of toxic and bacteriostatic agents which are released into the phagolysosome during degranulation. Agents such as acid, lysozyme, lactoferrin, and cationic proteins contribute synergistically to this function (45,46). However, killing of bacteria is mainly an oxidative process in PMN (45,47). This process is proceeded by dramatic increases in oxygen consumption (32,38), in hexose monophosphate shunt activity (32,48), and in enzyme activities of glutathione reductase

(49,50) and of NADH oxidase (51,52) and NADPH oxidase (53, 54). Killing is significantly reduced in the absence of catalase (59), superoxide dismutase (59,60) and benzoate (59). These studies indicate the involvement of hydrogen peroxide, superoxide anion, and hydroxyl radical, while it is further suggested that singlet oxygen may participate (61,62). PMN are known to produce measurable levels of hydrogen peroxide (63,64,65) and superoxide anion (66,67), which may be bactericidal by themselves (60,68) or in combination with myeloperoxidase plus halide (45,69). Significant decreases in killing capacity are found in phagocytes from patients with MPO dysfunction (23) and from patients with CGD or with G6PDH deficiency (16,21,22). In the latter deficiencies, NADH oxidation and hydrogen peroxide formation are not stimulated during phagocytosis.

Although glucose metabolism in normal and diabetic phagocytes has been vigorously studied (43,44,70,71), much less attention has been given to the fate of other sugars. Normal PMN can convert ^{14}C-labelled galactose, mannose, and fructose into labelled glycogen (72,73). However, when PMN are incubated with [2-^{14}C] galactose, there is less randomization of ^{14}C in the glucose molecules of glycogen than when [2-^{14}C] glucose is employed (72). This indicates that galactose follows a more direct pathway to glycogen and that the following pathway described by Lelior (74) and Kalckar et al. (75) is present:



UDP-glucose derived from ^{14}C-labelled galactose could then enter glycogen synthetase or be converted to glucose-1-phosphate via UDP-glucose pyrophosphatase. In contrast, [2-^{14}C] glucose is initially phosphorylated by hexokinase (71), and the ^{14}C is more likely to transverse the glycolytic and pentose cycle reactions before conversion to glycogen (72). Galactose-1-phosphate and UDP-galactose have been isolated from leukocyte homogenates (76), and the activities of both galactokinase (eq. 1) and galactose-1-phosphate uridylyltransferase (eq. 2) have been measured in PMN (77). The latter enzyme is not detectable in phagocytes from galactosemic patients (77,78,79), and its levels are depressed in cells from asymptomatic heterozygotes (77,80).

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CHAPTER I

INHIBITORY ACTION OF D-GALACTOSE ON PHAGOCITE METABOLISM AND FUNCTION

Abstract

To account for enhanced susceptibility to infection among galactosemics, the acute effects of D-galactose on metabolic and functional activities of phagocytic cells *in vitro* were investigated. Human and guinea pig polymorphonuclear leukocytes (PMN) when incubated in medium containing 30 mM galactose displayed substantially less killing of *Escherichia coli* than when incubated in medium with 5 mM glucose. Impaired bactericidal activity was dependent upon galactose concentration but could be partially averted by supplementing the galactose-containing medium with 15 mM glucose. Phagocytic activities of guinea pig PMN and peritoneal macrophages were assayed by following ingestion of ^{32}P-labelled *E. coli* and were also depressed by elevated galactose. Galactose was readily epimerized to glucose by resting PMN, and this conversion was stimulated by phagocytosis. Incubation of macrophages in the presence of galactose resulted in depletion of intracellular levels of adenosine 5'-triphosphate as well as other metabolites.

Introduction

Although bacterial infection (3,22) and *E. coli* septicemia (9,17) are frequently reported among infants deficient in galactose-1-phosphate uridylyltransferase (EC 2.7.7.10), little is known about the functional integrity of the host defense mechanism during galactosemia. Among patients fed diets restricted in galactose content both cellular and humoral immune systems are believed to be functional (15), but there is no pertinent information about the function of these systems in patients during galactose toxicity.

In the present communication, we suggest that increased susceptibility to infection may result from impaired phagocyte function during galactosemia. We demonstrate that elevated levels of galactose *in vitro* exert direct inhibitory effects upon the bactericidal activities of human and guinea pig polymorphonuclear leukocytes (PMN) and upon the phagocytic activities of guinea pig PMN and peritoneal macrophages. We also present data that indicate that the action of galactose on phagocytes is not primarily osmotic but may be attributed to changes in the intracellular levels of adenosine 5'-triphosphate (ATP) or other metabolites (results of preliminary studies were reported in Fed. Proc. 35:578, 1975).

Materials and Methods

Phagocyte preparations. Human blood samples were provided from 10 healthy adult volunteers through the courtesy of the American Red Cross Regional Blood Bank. Guinea pig blood was obtained from adult male guinea pigs (Connaught Laboratories, Ltd., Willowdale, Ontario) fed a commercial diet and water with 0.04% L-ascorbate ad libitum. Guinea pig serum, fetal calf serum, Medium 199, and Hanks balanced salt solution were purchased from Grand Island Biological Corp., Grand Island, N.Y. D-glucose and D-galactose were purchased from Mallinckrodt Chemical Works and Sigma Chemical Co., respectively, of St. Louis, Mo.

Human and guinea pig PMN were isolated from 10 ml samples of venous blood supplemented with 100 U of heparin (grade I, Sigma) per ml and 2 ml aliquots of 6% Dextran 250 (Pharmacia Fine Chamicals, Uppsala, Sweden) in phosphate buffered saline, pH 7.4. After one hour of sedimentation at room temperature, leukocyte-rich supernates were decanted and centrifuged at 250 xg for 5 min. Preparations were washed once with 0.87% NH_4Cl to lyse remaining erythrocytes (2), and leukocytes were suspended in Krebs-Ringer phosphate solution without calcium, pH 7.4, containing 10% autologous serum. Cell suspensions were gassed with 95:5 mixture of O_2: CO_2, adjusted to the appropriate carbohydrate concentration and incubated for 1 hour at 37°C prior to assaying for phagocytic or bactericidal activities. Amounts of D-glucose used as

supplement were calculated after determining by gas chromatography the levels of carbohydrate contributed by sera (24). Levels of glucose in sera varied from 4 to 6 mM, whereas levels of galactose were too low to be quantitated (less than 0.05 mM).

Guinea pig peritoneal macrophages were obtained 4 days after injecting, intraperitoneally, 5 ml of sterile 1% caseinate in saline. Exudates were obtained by flushing peritoneal cavities with Hanks balanced salt solution and centrifuged as described above. Macrophages suspended in Medium 199 plus 20% guinea pig serum were allowed to spread on plastic culture dishes for 2 hours at 37°C and were rinsed twice with fresh medium. Cells were cultured aerobically in this medium with appropriate concentrations of carbohydrate for 2 hours prior to use. Cell viability was determined by exclusion of 0.04% trypan blue. Differential cell counts, performed on Wright stained smears, showed homogeneities of human PMN, guinea pig PMN, and macrophage populations to be between 70 and 85%, greater than 95%, and greater than 98%, respectively.

Photomicrographs were taken of guinea pig peritoneal macrophages using a Universal model Zeiss microscope with phase optics (Figure 5a). The same microscope equipped with a BG-12 fluorescence excitation filter, as well as an OG-530 emission filter, was employed to take bright-field fluorescence photomicrographs of guinea pig exudate cells (Figure 5b). The latter cell population was stained for

30 min with 50 μM acridine orange. Both photographs are of cells in the presence of Krebs-Ringer phosphate solution, pH 7.4, with 5.0 mM glucose.

Bacteria. E. coli grown aerobically at 37°C in thioglycolate broth (Baltimore Biological Laboratory, Cockeysville, Md.) were harvested in late log phase and concentrated using a 0.45 μm membrane filter (Millipore Corp., Bedford, Mass.). Bacteria were washed extensively with Krebs-Ringer phosphate solution and suspended in autologous sera or guinea pig sera for 15 min before exposure to phagocytes. Radioactive bacteria were prepared in the same manner, except that 10 μCi of carrier-free [^{32}P] orthophosphate (New England Nuclear Corp., Boston, Mass.) was included per ml of growth medium.

Growth of E. coli in 6.0 ml suspensions supplemented with 10% fetal calf serum and carbohydrate was monitored by following absorbance at 620 nm using a Klett-Summerson colorimeter.

Phagocytic and bactericidal assays. Phagocytic activities, expressed as the average number of bacteria ingested per phagocyte, were determined by monitoring the uptake of viable ^{32}P-labelled E. coli into guinea pig PMN in suspensions (6.2×10^6 cells/ml) and into macrophages in monolayer on glass cover slips (9.1×10^5 cells/cover slip). Using a method similar to that described by Lentz and DiLuzio (11), 2.0 ml aliquots of PMN suspension were

placed in siliconized Erlenmeyer flasks (25 ml) and incubated at 37°C in a shaking Dubnoff water bath. Upon addition of E. coli (164 cpm per 10^6 bacteria), 0.2 ml volumes were removed from each flask at 20, 60, and 120 min. Prior to removal of each aliquot, cells were thoroughly resuspended by briefly shaking each flask with a Vortex mixer. Volumes were transferred to 3.0 ml siliconized centrifuge tubes, and PMN were washed three times by repeated suspension and centrifugation at 250 xg for 10 min. Pellets of washed cells were directly dispersed in 10 ml of Bray's solution and counted using a Beckman CPM-100 liquid scintillation counter.

Cover slips containing peritoneal macrophages were removed at the same time intervals and at 180 min from culture media containing labelled E. coli (338 cpm per 10^6 bacteria). Cover slips were rinsed three times with fresh media and transferred directly into scintillation vials with Bray's solution. All assays were performed in triplicate on cells pooled from three guinea pigs.

Phagocytic activities were also determined by observing the uptake of polystyrene latex particles by peritoneal macrophages. Cells were cultured for 4 hours at 37°C in the presence of Medium 199 supplemented with 20% fetal calf serum. Macrophages were allowed to adhere to cover slips, and at one hour prior to assay, cover slips were transferred to the same medium containing elevated carbohydrate. Polystyrene latex particles were then added in

a 50 fold particle to cell ratio, and uptake was observed after an additional hour using a Zeiss microscope with phase optics. Both the number of phagocytosing cells and the number of particles ingested per cell were determined.

Killing of bacteria by human and guinea pig PMN was assayed by exposing 1.0 ml cell suspensions (1.3×10^7 PMN/ml) in sterile Erlenmeyer flasks to serum-treated bacteria and incubating at 37°C in a shaking water bath. Aliquots of 0.2 ml, removed immediately and at 120 min, were serially diluted with sterile distilled water and then mixed with sterile 4% Trypticase soy agar plus 0.5% glucose in petri dishes at 45 to 50°C. After incubating each dish at 37°C for 24 hours, the number of viable bacteria per ml was determined by counting the number of colonies per dish and multiplying by the corresponding dilution factor. Each experiment was performed in triplicate using two plates per dilution with cells pooled from eight human donors or from at least three guinea pigs.

Oxidation of glucose and galactose. [1-^{14}C] glucose, [6-^{14}C] glucose, and [1-^{14}C] galactose with specific activities of 48.2, 55.8, and 45.0 mCi/mmol, respectively, were purchased from New England Nuclear Corp. Conversion of each substrate to $^{14}CO_2$ was performed in sealed Erlenmeyer flasks at 37°C. Each reaction, run in triplicate, was initiated by adding 2.4 µCi of labelled carbohydrate with the appropriate amount of non-isotopic carrier in 0.5 ml

of Krebs-Ringer phosphate solution to 0.5 ml suspensions of PMN (3.7×10^6 to 15.5×10^6 cells) with 20% guinea pig serum. After one hour of incubation, suspensions were acidified to release bound CO_2 by adding 0.2 ml of 20% H_2SO_4 and incubating at room temperature for an additional hour. Evolved CO_2 was collected from the start of each experiment on folded strips of Whatman 3MM paper (1 x 3cm) with 0.2 ml of 20% KOH in plastic centerwells. Centerwells were transferred to scintillation vials with 10 ml of Bray's solution and counted. Oxidation of carbohydrate by phagocytosing cells was determined in the same manner, except that a 20 fold (bacteria per PMN) excess of serum-treated E. coli (heat-killed for 15 min at 90°C) was included in the 0.5 ml aliquot of labelled carbohydrate.

Conversion of galactose to glucose by resting and phagocytosing PMN was determined by comparing the specific activities of [1-^{14}C] glucose remaining after incubation of cells (12.5×10^6 PMN) in media containing either 1.0 mM [1-^{14}C] glucose or [1-^{14}C] glucose with 10 mM galactose. After a 3 hr incubation at 37°C, cells were centrifuged from the medium, and neutral sugars were isolated from the supernants by adding 1.0 ml volumes of 0.3 N $Ba(OH)_2$ and 5% $SnSO_4$ (21). Specific activities were determined by converting labelled glucose to glucose-6-phosphate, separating it by paper chromatography, and dividing the counts per minute in this fraction by the number of micromoles (5).

Metabolite assays. Aliquots of peritoneal macrophages (approximately 5.0 x 10^7 cells) were cultured aerobically in plastic culture dishes at 37°C in the presence of Eagle basal medium (Grand Island Biological Co.) with 10% fetal calf serum and either 5.0 mM glucose or 5.0 mM glucose with 30 mM galactose. After 2 hr, cells in the monolayer were removed and centrifuged as described above. Pellets of cells were immediately frozen by placing centrifuge tubes in a bath of dry ice and isopropanol. Cells were stored at -80°C until homogenizing for 10 min at 4°C with 3 volumes of 3 N perchloric acid. Homogenates were centrifuged at 5000 xg for 10 min to remove precipitated protein, and supernates were neutralized with a mixture of 2 N KCH, 0.4 M imidazole, and 0.4 M KCl. Levels of protein were determined in each homogenate by the method of Lowry et al. (13), using bovine serum albumin (fraction V, Sigma) as a protein standard. Lactate was quantified spectrophotometrically using lactate dehydrogenase (EC 1.1.1.27) and by monitoring the reduction of acetylpyridine adenine dinucleotide (Boehringer Mannheim Corp., New York, N.Y.) at 366 nm (6). All other soluble metabolites were determined fluorometrically employing pyridine nucleotide-coupled enzyme reactions as previously described (12). Lactate dehydrogenase, pyruvate kinase (EC 2.7.1.40), and adenylate kinase (EC 2.7.4.3) were used sequentially to determine levels of pyruvate, adenosine 5'-diphosphate and adenosine 5'-monophosphate, whereas glucose-6-phosphate dehydrogenase

(EC 1.1.1.49) and hexokinase (EC 2.7.1.1) were used sequentially to determine glucose-6-phosphate and ATP. Glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, and fructose-1,6-bisphosphate were quantified using glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), triosephosphate isomerase (EC 5.3.1.1), and fructose-bisphosphate aldolase (EC 4.1.2.13), sequentially. All enzymes were purchased from Sigma except the latter three, which were obtained from Boehringer.

Results

The action of galactose upon bactericidal activity of phagocytes was observed with PMN from both human and guinea pig sources. When serum-treated E. coli and human PMN were incubated together in medium containing 5 mM glucose, a substantial decrease in the number of viable E. coli occurred over a 2 hr period (Table I, 0.005% survival). Incubation of bacteria without phagocytes resulted in bacterial growth (187% survival), and incubation of bacteria with PMN, but without added carbohydrate, resulted in significantly less bactericidal activity (97.4% survival). The use of 30 mM galactose as the sole carbohydrate source also failed to support normal killing of E. coli. Moreover, in the presence of 5.0 mM glucose, 30 mM galactose severly inhibited bactericidal activity (Table I, 34.2% versus 0.005% survival); however, this inhibition could be almost completely averted by increasing the glucose

concentration from 5.0 mM to 15 mM in the presence of 30 mM galactose (0.052% survival). Similarly, when bacteria and guinea pig PMN were incubated together (Table II), the inhibitory action of galactose in the presence of 5.0 mM glucose was found to be concentration dependent. This action could not be attributed to hyperosmolarity, because the impaired state was again prevented by elevating the glucose level to 15 mM, and morphological changes in PMN associated with hypertonicity (20) were not observed. Viability of PMN was not altered by incubation with galactose during these assays, and although some cell clumping did occur this was kept to a minimum by employing siliconized glassware, and using Krebs-Ringer phosphate solution without calcium.

Since the systems under study in Tables I and II were dynamic, depending not only upon phagocytic and killing processes but also upon the rates of bacteria growth, it became necessary to establish whether galactose could be affecting the growth of E. coli. For this purpose, growth curves were investigated under similar conditions but without PMN (Figure 1). High levels of galactose slightly impaired the growth of organisms, and this effect could not be prevented by increasing glucose levels. Thus, impairment of both PMN function and bacterial growth occurred in the presence of galactose.

It should be noted that the assay systems under study in Tables I and II were of similar design but differed

with respect to the species of PMN and the type of serum employed. The number of bacteria added to each system also varied, and this probably accounts for observed discrepancies between the percentage of survival of E. coli in these tables at comparable carbohydrate levels (34.2 versus 112% survival in the presence of 5 mM glucose plus 30 mM galactose with human and guinea pig PMN, respectively).

The extent to which galactose affects phagocytosis was determined by monitoring the uptake of ^{32}P-labelled E. coli into guinea pig PMN and peritoneal macrophages. PMN incubated in medium containing 5 mM glucose displayed most phagocytosis, whereas cells incubated in similar media with 15 to 30 mM galactose displayed 87 or 76% of this activity, respectively (Figure 2). Effects of galactose on phagocytic activity were more pronounced when employing macrophages (Figure 3), where the same relative amounts of galactose decreased uptake to 67 and 47%, respectively. Although inhibition of phagocytosis contributes to the loss of bactericidal activity, the extent of this inhibition did not appear sufficient to account for all the impairment in killing capacity.

Visual observation of phagocytosing macrophages showed that both the number of polystyrene latex particles ingested per phagocytosing cell and the number of phagocytosing cells were lower in the presence of 30 mM galactose (Table III). Addition of 10 mM glucose to the medium containing 30 mM galactose appeared to partially relieve this

depression. However, higher levels of glucose without galactose were also inhibitory. Moreover, the number of ingested particles varied greatly (from 1 to 30) between phagocytosing cells.

To further investigate losses of phagocytic and bactericidal function, the action of galactose upon metabolic processes associated with these functions were studied. Effects of galactose upon the oxidation of [1-^{14}C] glucose and [6-^{14}C] glucose by guinea pig PMN are presented in Figure 4. Although 10 mM galactose reduced the amount of $^{14}CO_2$ evolved from [1-^{14}C] glucose by both resting and phagocytosing cells, a 2.5 fold increase in glucose oxidation associated with phagocytosis was relatively unaffected. Evolution of $^{14}CO_2$ from [6-^{14}C] glucose by resting cells was 50 fold less than that evolved from [1-^{14}C] glucose and was impaired by galactose to a greater extent. Thus, either galactose inhibited glucose transport, and/or its oxidation, or galactose was converted to glucose in sufficient amounts to lower its specific radioactivity.

The latter explanation appears correct (Table IV). When resting or phagocytosing PMN were incubated for 3 hours in media with 1.0 mM labelled glucose, the specific activities of residual glucose were essentially equal. Addition of 10 mM galactose to either medium, however, resulted in decreased specific activities. That phagocytosing cells converted more galactose to glucose than resting cells was

suggested by these data and further verified by monitoring the evolution of $^{14}CO_2$ from [1-^{14}C] galactose. Resting and phagocytosing cells incubated in media with 1.0 mM labelled galactose oxidized 0.59 and 1.70 nmole of galactose per hour per 10^6 cells, respectively. These oxidation rates were approximately 10 fold less than those for glucose (data not shown) and appeared to be insufficient for normal bactericidal action of PMN when 30 mM galactose was provided (Table I).

Peritoneal macrophages cultured for 2 hr in basal medium (Eagle) containing 5 mM glucose, 10% fetal calf serum, and 30 mM galactose showed significant decreases in intracellular ATP ($P < 0.005$) as well as elevations in levels of adenosine 5'-diphosphate and adenosine 5'-monophosphate (Table V). Cell viability was lower under these conditions, and levels of all glycolytic intermediates, with the exception of pyruvate, were depressed.

Discussion

The present results demonstrate that elevated levels of D-galactose inhibit the phagocytic activities of guinea pig PMN and peritoneal macrophages as well as the bactericidal activities of guinea pig and human PMN. Since levels of galactose employed in this study (5 to 30 mM) are observed in infants deficient in galactose-1-phosphate uridylyltransferase (14,16), it is reasonable to assume that phagocyte function is also impaired in these patients when

blood galactose levels remain elevated. Such impairment of phagocyte function could severely compromise the host defense system and may represent an underlying cause for increased susceptibility to E. coli infection observed among galactosemics.

The effects of galactose upon phagocytes are probably very similar to the effects of other carbohydrates previously implicated in impairing phagocyte function. Sanchez et al. (18) reported that elevated levels of blood glucose depressed phagocytic activity of human PMN toward Staphylococcus epidermidis, and Bagdade and co-workers (1) found that the ability of human PMN to ingest and destroy type 25 pneumococcus was impaired by increasing glucose levels to 55 mM. In studying the correlation between susceptibility to infection and poorly controlled diabetes mellitus, Drachman et al. (4) followed the ingestion of type 25 pneumococcus by rat PMN and found that phagocytosis was depressed both in vivo by hyperglycemia and in vitro in crowded cell suspensions by 43 mM concentrations of glucose, fructose, sucrose, mannose, xylose, and arabinose. Noting previous reports that solute concentrations above 40 milliosmolal inhibited phagocyte function (20), Drachman concluded that the action of elevated carbohydrate was primarily osmotic.

Our results, however, indicated that the action of galactose upon phagocytes was not related to osmolality, since the inhibitory effect of 30 mM galactose upon

bactericidal activity could be averted by elevating glucose levels to 15 mM, and since the morphological changes in PMN and macrophage associated with hypertonicity were not seen. Correlations between impaired activity and PMN viability, as well as between activity and cell clumping, were also not observed.

Since phagocytosis by PMN (19), as well as by macrophage (8), is known to depend largely upon energy supplied by glycolysis, suppression of this metabolic pathway may represent a primary action of galactose. Depressed levels of intracellular ATP and glycolytic intermediates were found in guinea pig peritoneal macrophages when incubated with elevated levels of galactose (Table V), and similar results were reported to occur in other tissues during galactose toxicity (5). Suppression of glycolysis could arise from either of the following mechanisms: (i) an inhibition of glucose transport into the phagocyte, or (ii) an accumulation of galactose, or one of its metabolites, intracellularly with subsequent inhibition of one or more glycolytic enzymes. However, both galactose and galactose-1-phosphate are concentrated by leukocytes (7,23), and we have recently found that glucose, but not galactose, competitively inhibits the uptake of[G-^{3}H]2-deoxyglucose into guinea pig PMN (see Chapter III). Thus, competition for transport is an unlikely explanation for galactose action in these cells.

The rapid conversion of galactose to glucose in

phagocytes (Table IV) can be accounted for by the combined action of the Lelior pathway enzymes and either a specific or nonspecific phosphatase. This pathway, while functioning to dilute the glucose label in our oxidation experiments, was stimulated by phagocytosis and may have contributed to depletion of intracellular ATP via a futile adenosine triphosphatase action. Similar cycles operating during galactose neurotoxicity have been previously reported (10).

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TABLE I. Effect of galactose on bactericidal activity of human polymorphonuclear leukocytes[a]

Carbohydrate Content of Medium (mM)			Average Viable E. coli/ml		Percent
Glucose	Galactose	PMN	0 Min.	120 Min.	Survival
5.0	0	+	3.50×10^8	1.81×10^4	0.005
5.0	0	-	1.22×10^8	2.29×10^8	187.0
0	0	+	4.81×10^8	4.68×10^8	97.4
0	30	+	3.39×10^8	3.25×10^8	96.0
5.0	30	+	3.63×10^8	1.24×10^8	34.2
15.0	30	+	3.87×10^8	1.95×10^5	0.052

[a]Human PMN (1.3×10^7 cells) were incubated at 37°C in 1.0 ml of media containing 10% autologous sera and the indicated levels of carbohydrate. Serum-treated bacteria were added after one hour of preincubation. Each experiment was performed in triplicate.

TABLE II. Effect of galactose on bactericidal activity of guinea pig polymorphonuclear leukocytes[a]

Carbohydrate Content of Medium (mM)			Average Viable E coli/ml		Percent
Glucose	Galactose	PMN	0 Min.	120 Min.	Survival
5.0	0	+	2.02×10^6	1.72×10^4	0.571
5.0	0	-	2.93×10^6	4.06×10^6	139.0
5.0	7.5	+	3.24×10^6	1.32×10^6	4.07
5.0	15.0	+	2.42×10^6	9.70×10^6	40.1
5.0	30.0	+	2.49×10^6	2.80×10^6	112.0
15.0	30.0	+	2.62×10^6	4.13×10^4	1.58

[a]Guinea pig PMN (1.3×10^7 cells) were incubated at 37°C in 1.0 ml of media containing 10% guinea pig sera and the indicated levels of carbohydrate. Serum-treated bacteria were added after one hour of preincubation. Each experiment was performed in triplicate.

TABLE III. Influence of carbohydrate on phagocytosis of latex particles by guinea pig peritoneal macrophages[a]

Carbohydrate Content of Medium (mM)		% Phagocytosing Cells[b]	Particles Ingested Per Phagocytosing Cell[c]
Glucose	Galactose		
5.0	0	90.9	8.0 ± 0.8 (37)
10.0	0	88.5	4.8 ± 0.4 (46)
20.0	0	85.7	4.3 ± 0.4 (47)
30.0	0	80.9	4.5 ± 0.4 (47)
5.0	10.0	76.3	4.3 ± 0.4 (95)
5.0	30.0	74.8	3.3 ± 0.3 (50)
10.0	30.0	89.0	5.4 ± 0.7 (34)

[a]Peritoneal macrophages were cultured aerobically for 4 hr at 37°C in the presence of Medium 199 supplemented with 20% fetal calf serum. One hr prior to assay dishes were adjusted to the appropriate carbohydrate concentration. To commence assay, a 50 fold particle to cell excess of polystyrene latex particles were added. Counting was performed by phase contrast microscopy at 800x, one hr after particle addition.

[b]Percent of macrophages that have ingested at least one particle.

[c]Average number of particles ingested per phagocytosing cell ± SEM. Brackets indicate the number of cells observed.

TABLE IV. Conversion of galactose to glucose by guinea pig PMN as indicated by the specific activity of [1-^{14}C]D-glucose[a]

Carbohydrate Content of Medium (mM)		Cell State	Glucose Specific Activity After 3 Hours with PMN (cpm x 10^{-5}) ± SEM
Glucose	Galactose		
1.0	0	Resting	3.03 ± 0.35
1.0	0	Phagocytosing	3.08 ± 0.35
1.0	10	Resting	2.07 ± 0.16
1.0	10	Phagocytosing	1.43 ± 0.29

[a]PMN (12.5 x 10^6 cells) were incubated at 37°C in 1.0 ml of media containing 10% guinea pig sera, 2.0 µCi of [1-^{14}C] D-glucose, and the indicated amounts of carbohydrate. After 3 hours, specific activities of extracellular glucose were determined in triplicate.

TABLE V. Effect of galactose on levels of adenine nucleotides and glycolytic intermediates in guinea pig peritoneal macrophages[a]

Metabolite	Intracellular Levels (nmoles/mg protein ± SEM) After Cells were Incubated With: 5 mM Glucose	5 mM Glucose + 30 mM Galactose	P
ATP	6.88 ± 0.21	2.16 ± 0.12	0.005
ADP[b]	0.73	2.80	--
AMP[b]	0.80	4.47	--
Glucose-6-phosphate	0.93 ± 0.02	0.70 ± 0.01	0.01
Fructose-1,6-diphosphate	2.69 ± 0.17	2.34 ± 0.06	NS
Dihydroxyacetone phosphate[b]	0.48	Below 0.05	--
Glyceraldehyde-3-phosphate[b]	0.43	Below 0.05	--
Pyruvate[b]	1.76	2.06	--
Lactate	545 ± 12.2	406 ± 4.9	0.01
Decrease in Cell Viability during Incubation	2.8%	16.0%	

[a]Macrophages (5.0×10^7 cells) were incubated at 37°C for 2 hours in basal medium (Eagle) with 10% fetal calf serum and the indicated levels of carbohydrate. Levels of significance (P) were calculated by the Student's two-tailed t-test and are given for values determined in triplicate for three separate experiments; NS refers to means not significantly different.

[b]Values are the means of triplicate determinations from one study.

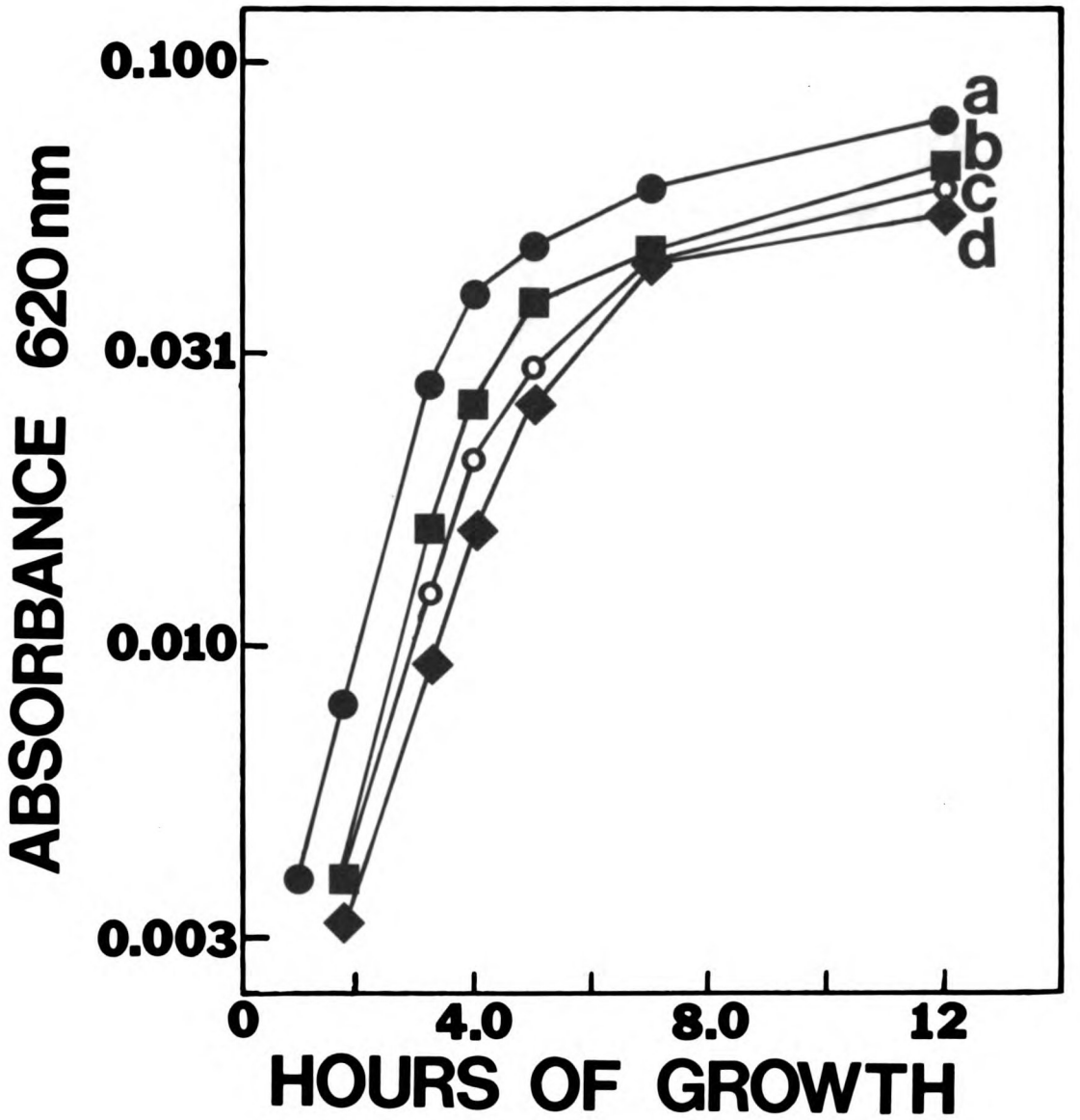

Figure 1. Semilogarithmic plot of E. coli growth in high galactose media.

Bacteria were grown at 37°C in 6.0 ml of KRPS with 10% fetal calf serum and a) 5 mM glucose, b) 5 mM glucose and 15 mM galactose, c) 5 mM glucose and 30 mM galactose, or d) 15 mM glucose and 30 mM galactose.

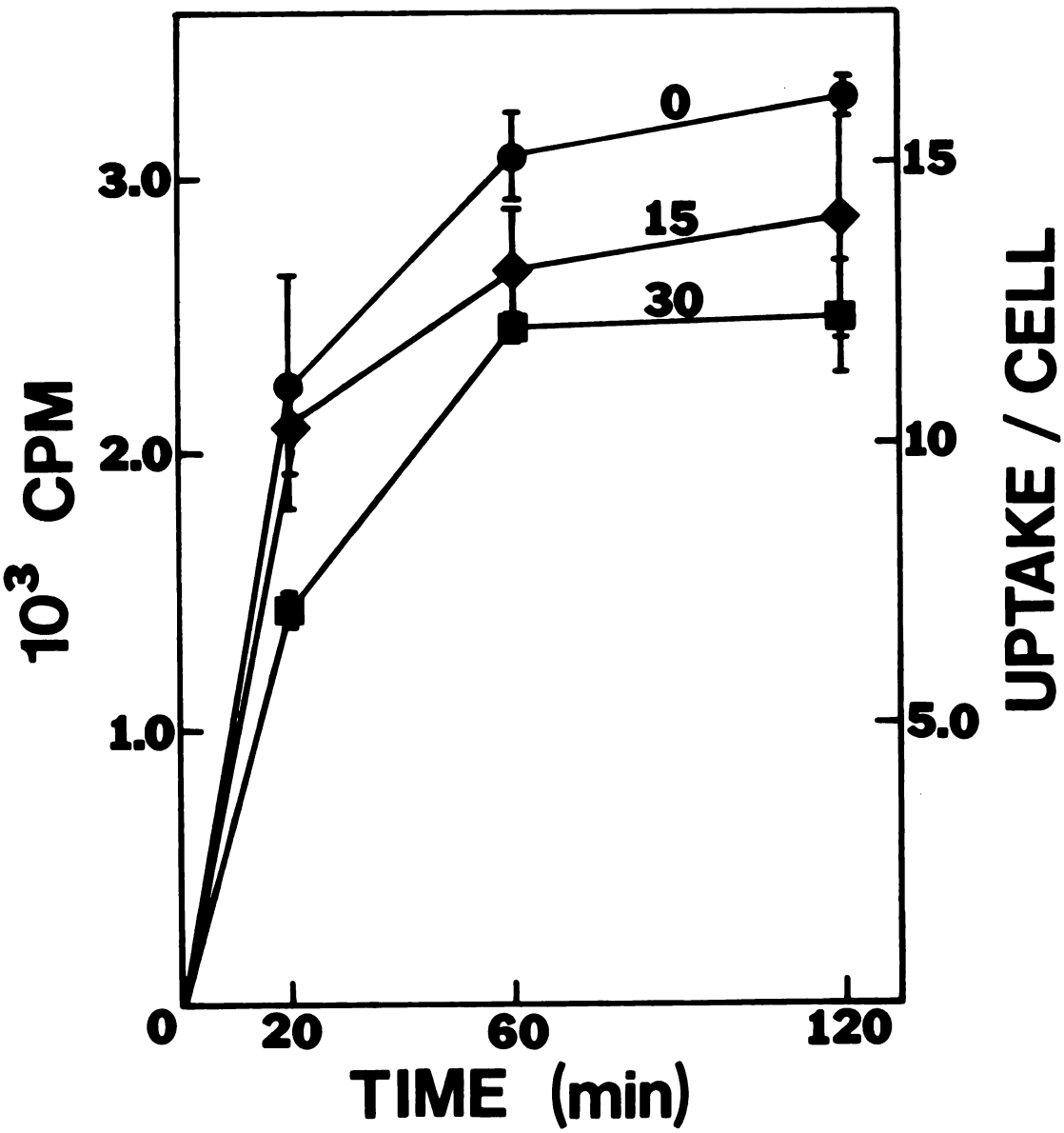

Figure 2. Inhibitory effect of galactose on phagocytosis of ^{32}P-labelled E. coli by guinea pig PMN.

Labelled bacteria were added at zero time to PMN suspensions in media with 10% guinea pig sera, and 0, 15, or 30 mM galactose. Uptake/cell represents the average number of bacteria ingested per phagocyte. Each point is the mean of triplicate determinations and bar line indicate S.E.M.

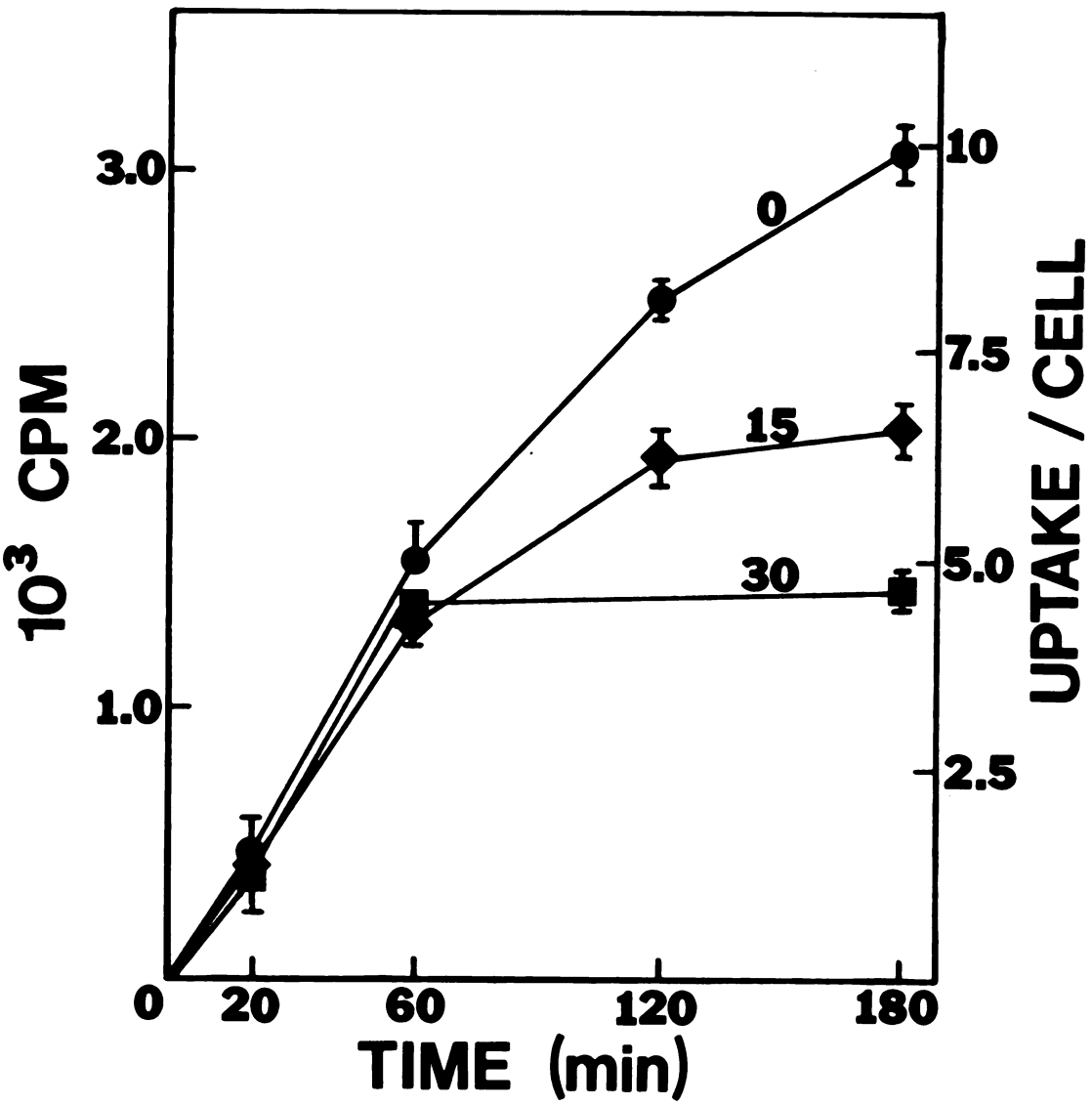

Figure 3. Inhibitory effect of galactose on phagocytosis of ^{32}P-labelled E. coli by guinea pig peritoneal macrophages.

Labelled bacteria were added at zero time to macrophage monolayers in media with 10% guinea pig sera and 5.0 mM glucose with 0, 15, or 30 mM galactose. Uptake/cell represents the average number of bacteria ingested per phagocyte. Each point is the mean of triplicate determinations and line bars indicate S.E.M.

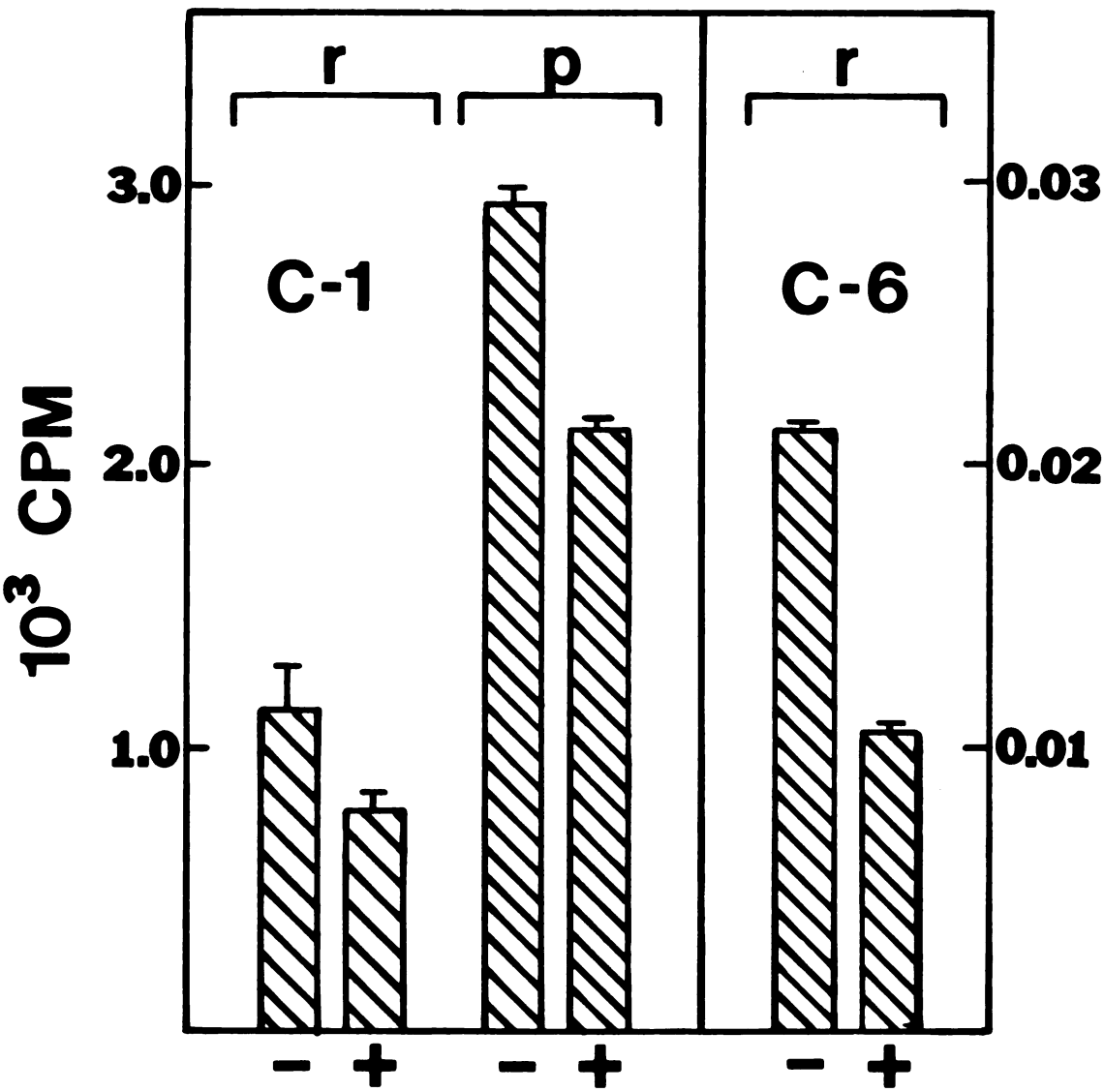

Figure 4. Effect of galactose on oxidation of [1-^{14}C]glucose and [6-^{14}C] glucose by guinea pig PMN.

PMN were incubated for 1 hr in media with 10% guinea pig sera and either 1.0 mM glucose (-) or 1.0 mM glucose with 10 mM galactose (+). Evolved $^{14}CO_2$, expressed as 10^3 CPM per 10^6 cells, was determined for both resting (r) and phagocytosing (p) PMN. Each experiment was performed in triplicate on cells from each of 3 guinea pigs. Bar lines equal S.E.M.

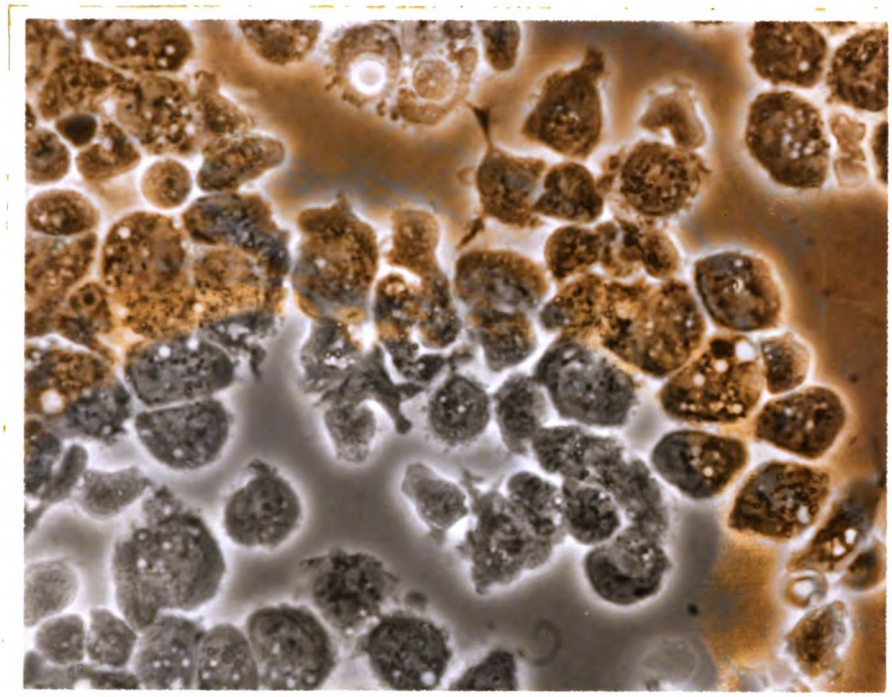

Figure 5a. Phase contrast photomicrograph of guinea pig macrophages.

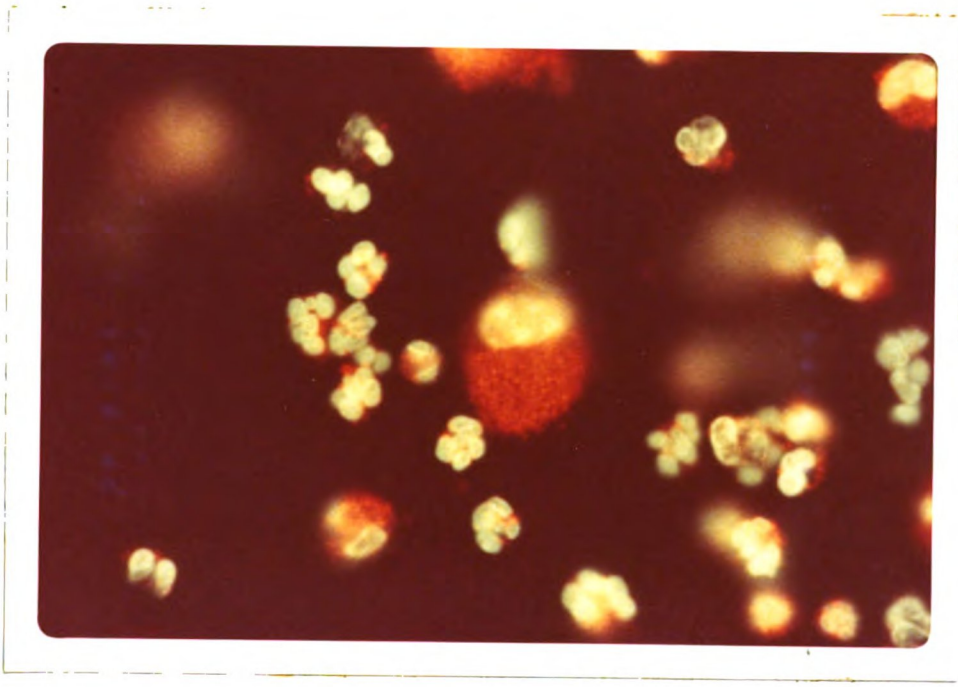

Figure 5b. Bright-field fluorescence photomicrograph of guinea pig exudate cells stained with acridine orange.

CHAPTER II

INHIBITORY ACTION OF GALACTOSE ON PHAGOCYTES FROM NORMAL AND GALACTOSEMIC CHICKS

Abstract

The inhibitory effect of galactose on phagocyte function was investigated in normal and galactosemic chicks by monitoring the *in vitro* killing of *Escherichia coli* by leukocytes and the *in vivo* clearance of colloidal ^{125}I-labelled bovine serum albumin (^{125}I-BSA) from the circulation. Elevated levels of galactose (30 mM) impaired the bactericidal activities of both control and galactosemic leukocytes. However, the latter cells were more susceptible to the galactose dependent inhibition. Galactosemic leukocytes displayed near normal bactericidal activity when assayed *in vitro* under simulated normal conditions.

Phagocytic indexes, obtained from data on the clearance of colloidal ^{125}I-BSA, were calculated to be 0.0553 and 0.0297 for control and galactosemic chicks, respectively. Chicks fed a control diet displayed a logarithmic clearance of colloid with postinjection time, whereas this relationship was not as apparent when galactosemic chicks were employed. Moreover, galactose impaired phagocytic functions

of both circulating leukocytes and tissue-filled macrophages as well as the overall development of the reticuloendothelial system.

Introduction

Previous studies have demonstrated that elevated levels of galactose, as encountered during galactosemia, are inhibitory to phagocyte function *in vitro* (Chapter I). These studies were performed with elicited guinea pig macrophages and with polymorphonuclear leukocytes isolated from the peripheral blood of normal guinea pigs or human donors. These studies were not performed with phagocytes from galactosemic sources. Therefore, data regarding galactose action in galactosemic cells and *in vivo* were not obtained. To acquire this information, experiments were designed using leukocytes from galactosemic chicks and using a standard test of reticuloendothelial system (RES) function in these animals.

Major objectives of the present research are threefold: 1) to determine whether the killing of *E. coli* by leukocytes of normal and galactosemic animals is impaired to similar extents by elevated levels of galactose, 2) to test whether galactosemia impairs *in vivo* phagocytic activity of tissue-fixed macrophages, as judged by the intravascular clearance of colloidal ^{125}I-labelled bovine serum albumin (^{125}I-BSA), and 3) to ascertain whether experimentally induced galactosemia in developing Leghorn cockerels is a good model

system for studying the effects of galactose upon human phagocytes.

Materials and Methods

Animals and materials. Day-old Leghorn cockerels (Gallus domesticus) were obtained through the generosity of MacPherson Hatchery and Rainbow Trail Hatchery of Ionia and St. Louis, Michigan, respectively. The basal 2 diet of Rutter et al. (1) was fed ad libitum to both control and galactosemic chicks. However, in the latter case, this diet contained either 30% or 50% (w/w) D-galactose substituted for an equal amount of cerelose (D-glucose monohydrate). Chicks were kept in brooders at 32°C.

Adult chicken serum, trypan blue, and Hank's balanced salt solution were purchased from Grand Island Biological Co., Grand Island, N.Y., whereas all other reagents were obtained from Sigma Chemical Co., St. Louis, Mo. Radioactive iodine ($Na^{125}I$), as well as other materials necessary for protein labelling, were kindly provided by Dr. Pamela Fraker of Michigan State University.

Phagocyte preparations. Leukocytes were isolated from 10 ml samples of whole blood obtained by cardiac puncture of ether anestetized chicks. Samples were pooled from either six control chicks or eight galactosemic chicks that had been fed the 50% galactose diet for 48 hours. Blood was supplemented with heparin (100 U/ml) and with dextran

(1.2% w/v). After 1 hour of sedimentation at room temperature, leukocyte-rich supernatants were decanted and centrifuged at 250 xg for 5 min. Preparations were washed once with 0.87% NH_4Cl to lyse remaining erythocytes, and leukocytes were suspended in Hank's balanced salt solution containing 10% adult chicken serum. Cell suspensions were gassed with a 95:5 mixture of O_2: CO_2, adjusted to the appropriate carbohydrate concentration and incubated for 1 hour at 37°C prior to assaying for bactericidal activity. Amounts of glucose and galactose used as supplements were calculated after determining by gas chromatography the levels of carbohydrate contributed by adult chicken sera (see Appendix). Levels of galactose were too low to be quantitated (less than 0.05 mM).

Bacteria. A phage resistant strain of E. coli, designated E. coli B/r (ara^-), was kindly provided by Dr. R. Anderson of Michigan State University. Bacteria were grown aerobically at 37°C in thioglycolate broth and were harvested in late log phase using a 0.45 μm membrane filter. Bacteria were washed extensively with Krebs-Ringer phosphate solution and were suspended in adult chicken serum for 15 min before exposure to leukocytes.

Bactericidal assays. Killing of bacteria by chick leukocytes was assayed by exposing 1.0 ml cell suspensions in sterile Erlenmeyer flasks to 10^8 serum-treated bacteria and incubating at 38°C in a shaking water bath. Aliquots

of 0.2 ml, removed immediately and at 120 min, were serially diluted with sterile water and then mixed with sterile 4% Trypticase soy agar plus 0.5% glucose in petri dishes at 45 to 50°C. After incubating each dish at 37°C for 24 hours, the number of viable bacteria per ml was determined by counting the number of colonies per dish and multiplying by the corresponding dilution factor. Each assay was performed in triplicate using two plates per bacterial dilution and using between 4.9×10^7 and 6.4×10^7 leukocytes per ml. Quantities of leukocytes were determined by counting cells in a hemocytometer using phase optics. Leukocyte viability was measured by the exclusion of 0.04% trypan blue (2).

Intravascular clearance of colloidal ^{125}I-BSA. Radioactive BSA (1.88×10^5 cpm/μg) was prepared according to the method of Fraker and Speck (3) by exposing 100 μg of BSA in 100 μl of 6.7×10^{-10} M potassium iodide to 15.5 μCi of $Na^{125}I$ in the presence of 0.4 μg tetrachloroglycoluril. Iodination was performed for 5 min at 4°C and was terminated by decanting the reaction solution into test tubes without tetrachloroglycoluril. Solutions were extensively dialyzed against borate-saline buffer, pH 8.2, and finally against Krebs-Henseleit bicarbonate solution, pH 7.4. Aliquots of protein precipitated with 15% (w/v) trichloroacetic acid prior to and after dialysis contained 54.7% and 99.2% of the soluble radioactivity, respectively.

Colloidal ^{125}I-BSA was prepared by heat denaturing 3.0 ml of a 1.0% BSA solution (4) containing the above labelled material. The solution was adjusted to pH 10.0 by adding 0.2 N NaOH, heated to 79°C for 20 min, and cooled on ice. The pH was further reduced to the isoelectric point of BSA by adding 0.2 N HCl, and the colloidal suspension was centrifuged at 22,000 xg for 15 min at 4°C. The resulting pellet was washed twice and resuspended in 1.5 ml of Krebs-Henseleit bicarbonate solution containing 150 U of heparin. Particle diameters ranged from 0.1 to 5 μm as judged by visual observation with a Zeiss microscope.

Intravascular clearance of colloidal ^{125}I-BSA was determined using 10 day old chicks fed either a control diet or a 50% galactose diet from 4 days of age. Each chick was anestetized with diethyl ether, and the thoracic cavity was surgically opened to expose the heart. An injection of colloidal ^{125}I-BSA (50 μl/100 gm chick weight) was immediately given via a 25 gauge needle into the left ventricle of the heart, and 0.1 ml aliquots of whole blood were removed at 1.0 min intervals from the right ventricle. Aliquots of blood were placed directly into vials and counted for 10 min with a Beckman Biogamma scintillation spectrometer set for ^{125}I counting. Levels of ^{125}I-BSA remaining in the blood at each time were determined for 10 control chicks and 10 galactosemic chicks. Whole body, spleen and liver weights were noted for each animal, and subsequently, the distribution of counts in each liver and

spleen was determined.

Control chicks used in this experiment displayed blood glucose levels of 12.5 ± 1.2 mM, whereas galactosemic chicks showed blood glucose and galactose levels of 14.9 ± 2.6 mM and 18.4 ± 2.6 mM, respectively. The latter chicks were demonstrating galactose neurotoxic behavior.

RES development. Spleens and livers were surgically removed from control chicks and galactosemic chicks fed a 30% galactose diet from 7 days of age. Organs were washed once with sterile saline, blotted dry, and immediately weighed using an analytical balance. In a separate experiment, chicks were placed on each diet at 4 days of age and immunologically stressed by injecting, i.p., 10^7 viable E. coli. Organ weights were determined at 12 days of age and compared to the weights of organs from unstressed animals. At least six chicks were employed in each group.

Results

Effect of galactose on bactericidal activity. The inhibitory action of galactose upon the bactericidal activity of chick leukocytes in vitro is illustrated in Figure 1. Leukocytes from 9 day old chicks, fed a control diet, killed the most serum treated E. coli during a two hour incubation (8.5% bacterial survival), while leukocytes from chicks, fed a 50% galactose diet, displayed near normal bactericidal activity in the absence of galactose (13.4% bacterial

survival). Substantially lower activities, however, were found in both groups when cells were incubated and assayed with 30 mM galactose. Killing of E. coli by galactosemic cells was greatly impaired in this case (83.5% bacterial survival), whereas killing by control cells was impaired to a lesser extent (56.2% bacterial survival). The presence of 30 mM galactose did not affect the growth of E. coli nor the viability of chick leukocytes under these conditions.

Effect of galactose on clearance of colloid. The effect of galactose in vivo upon intravascular clearance of colloidal ^{125}I-BSA is shown in Figure 2. Ten day old chicks fed a control diet displayed a logarithmic clearance of colloidal protein with postinjection time. However, this relationship was not as apparent from the data on galactosemic chicks. The former data was easily fit to the following empirical formula for RES clearance (5):

$$C = C_o \, 10^{-kt}$$

where C equalled CPM/ml blood postinjection, C_o equalled CPM/ml blood at zero time, t represented postinjection time, and k was the global phagocytic index, a measure of intravascular clearance. Thus, values of k were equivalent to the opposite of the slope of the logarithmic form of this equation ($\log C = -kt + \log C_o$) and were directly obtained from the lines in Figure 2. Both lines in this

figure were obtained by the method of least squares. Control chicks and galactosemic chicks displayed global phagocytic indexes (k) of 0.0553 and 0.0297, respectively.

Whole body weights of galactosemic chicks (57.8 ± 1.9 gm) were significantly lower than the weights of control chicks (87.3 ± 2.37 gm) during this experiment. Similarly, the weights of galactosemic livers (2.02 ± 0.06 gm) and spleens (48.6 ± 0.4 mg) were substantially lower than the weights of control livers (5.45 ± 0.13 gm) and spleens (99.0 ± 0.6 mg). These data in combination with values for k permitted calculation of corrected phagocytic indexes (α) by the following equation (5):

$$\alpha = k^{1/3} \frac{W}{W + L + S}$$

where W, L, and S corresponded to whole body, liver, and spleen weights, respectively. Corrected phagocytic indexes for control chicks and galactosemic chicks were 0.358 and 0.299, respectively.

By the termination of each intravascular clearance study (approximately 6 min postinjection), livers and spleens of control chicks acquired 28.2% and 1.83%, respectively, of the total injected colloidal ^{125}I-BSA. On the other hand, galactosemic livers and spleens sequesterred 23.4% and 1.83% of the dose, respectively.

RES development. The effect of galactose diet on development of liver and spleen is illustrated in Figure 3. Chicks on control diet showed normal increases in spleen and liver

weights, whereas chicks on 30% galactose diet displayed abnormal weight increases. Significant differences between these groups were apparent by 14 days of age. Whole body weight was also lower in galactosemic chicks (data not shown). However, liver and spleen weights were depressed to a greater extent.

Table I shows that spleen weight of control chicks was responsive to E. coli injection. Increases in spleen weight were not found, however, with galactosemic chicks, and increases in liver weights of either group were not observed following the bacterial stress.

Discussion

Although bactericidal activities of leukocytes from both control and galactosemic chicks were inhibited by 30 mM galactose in vitro, activities of leukocytes from the latter source were affected to a greater extent (Figure 1). These observations demonstrated that the inhibitory actions of galactose were not limited to phagocytes of mammalian origin (Chapter I) and that the bactericidal functions of galactosemic cells were previously compromised in vivo. The latter finding was not, however, a result of permanent cellular damage since near normal bactericidal activity was observed by galactosemic cells in the absence of galactose.

These observations strongly suggested that galactosemic cells contained elevated levels of galactose and/or its metabolites which as glycolytic antagonists could inhibit

phagocytosis (6) and which as free radical scavengers could impair oxidative bactericidal activity (7). Moreover, both galactose and galactitol were observed in galactosemic leukocytes (data not shown), and the diffusion of these agents from galactosemic cells could be a mechanism for the restoration of bactericidal activity.

Since levels of galactose-1-phosphate uridylyltransferase (EC 2.7.7.10) are low in both control and galactosemic chick leukocytes (8), the existence of a futile ATPase cycle converting galactose to glucose (Chapter I) is unlikely in these cells. Nevertheless, a similar ATPase cycle, involving the cyclic phosphorylation and dephosphorylation of galactose, is found in galactosemic chick brain (9) and could be present in galactosemic chick phagocytes. Such a cycle might occur in phagocytes by the action of either a specific or non-specific phosphatase upon galactose-1-phosphate.

Clearance of colloidal ^{125}I-BSA from the circulation of control chicks (Figure 2) obeyed an exponential function commonly employed to define RES activity in other species (5,10). Intravascular clearance by galactosemic chicks, on the other hand, did not follow such a clear relationship, and this was probably a result of experimental error rather than a delay in the onset of phagocytosis. Global phagocytic indexes for RES function of control and galactosemic chicks were calculated to be 0.0553 and 0.0297, respectively. However, since these animals were substantially different in

in both size and RES organ weight, the corrected phagocytic indexes of 0.358 and 0.299, respectively, were better measures of *in vivo* phagocytic activity by tissue-fixed macrophages. These experiments were limited by three major assumptions: 1) that colloid was not entrapped by capillaries, 2) that opsonins were not necessary or limiting for ^{125}I-BSA phagocytosis, and 3) that anesthesia and anoxia affected both control and galactosemic chicks equally.

Although spleens of control and galactosemic chicks accumulated the same levels of ^{125}I-BSA (1.83% of the dose), livers of these animals acquired 28.2% and 23.4% of the dose, respectively. These observations indicate that at 10 days of age chick RES relies upon macrophages of liver rather than of spleen and that the action of galactose regarding the RES is predominantly upon cells of the former organ. Table I demonstrates, however, that spleen weight in control chicks is sensitive to injection of *E. coli*. This response is not a measure of RES function *per se* but is related to an influx or proliferation of immunocompetent cells (11). Thus, inhibition of this response during galactosemia suggests that cellular immunity may also be depressed *in vivo*.

Moreover, the present results indicate that experimentally induced galactosemia in chicks may be a good model system for studying the effects of galactose on human phagocytes. Chick leukocytes, like those from galactosemic infants, are deficient in galactose-1-phosphate

uridylytransferase (12) and, like normal human leukocytes, are functionally impaired by elevated levels of galactose. The impaired activity of RES cells in galactosemic chicks, as well as the retarded RES organ development, further suggest that these chicks, like galactosemic infants (13), are more susceptible to bacterial infection. On the other hand, human leukocytes are morphologically quite different from chick leukocytes (14), and human RES function depends to a greater extent upon the macrophages of liver and spleen (5). In addition, galactosemic infants commonly display enlarged livers and spleens, whereas these symptoms were not observed in galactosemic chicks.

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Table I. Response of RES to injection of *E. coli*.[a]

Diet	Injection	Spleen Weight mg ± SEM	Liver Weight gm ± SEM
Control	-	117 ± 22.6	5.30 ± 0.43
Control	+	182 ± 22.8	5.79 ± 0.84
Galactose	-	46.7 ± 13.6	1.89 ± 0.34
Galactose	+	49.4 ± 15.6	1.79 ± 0.34

[a]Six chicks were placed on each diet at 4 days of age and sacrificed eight days later. Each injected chick received 10^7 viable *E. coli*. Galactose diet was 30% (w/w) galactose.

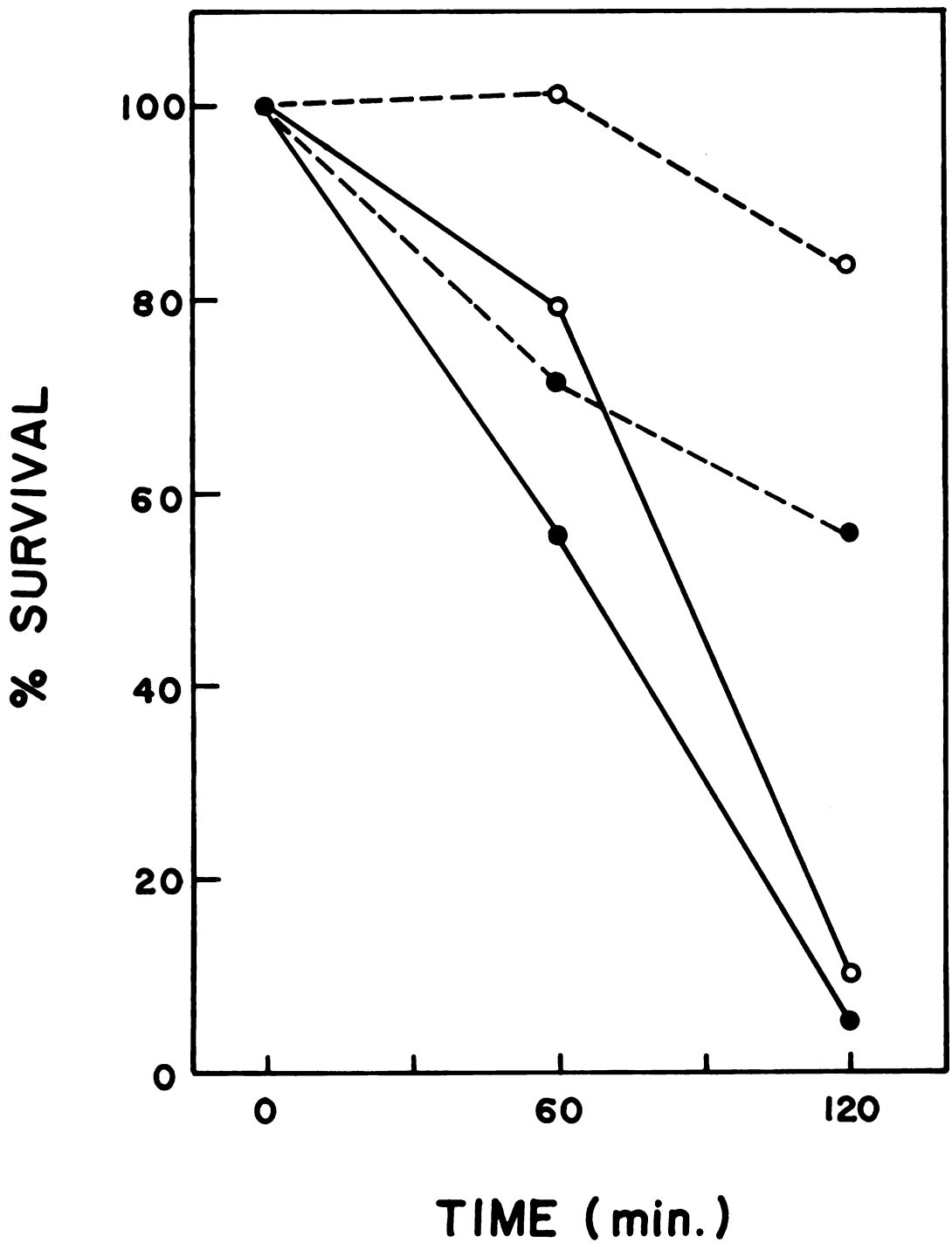

Figure 1. Effect of galactose on bactericidal activity of normal (●) and galactosemic (o) chick leukocytes in vitro.

E. coli were added at zero time to cell suspensions incubated with 5.0 mM glucose (———) or 5.0 mM glucose and 30 mM galactose (------).

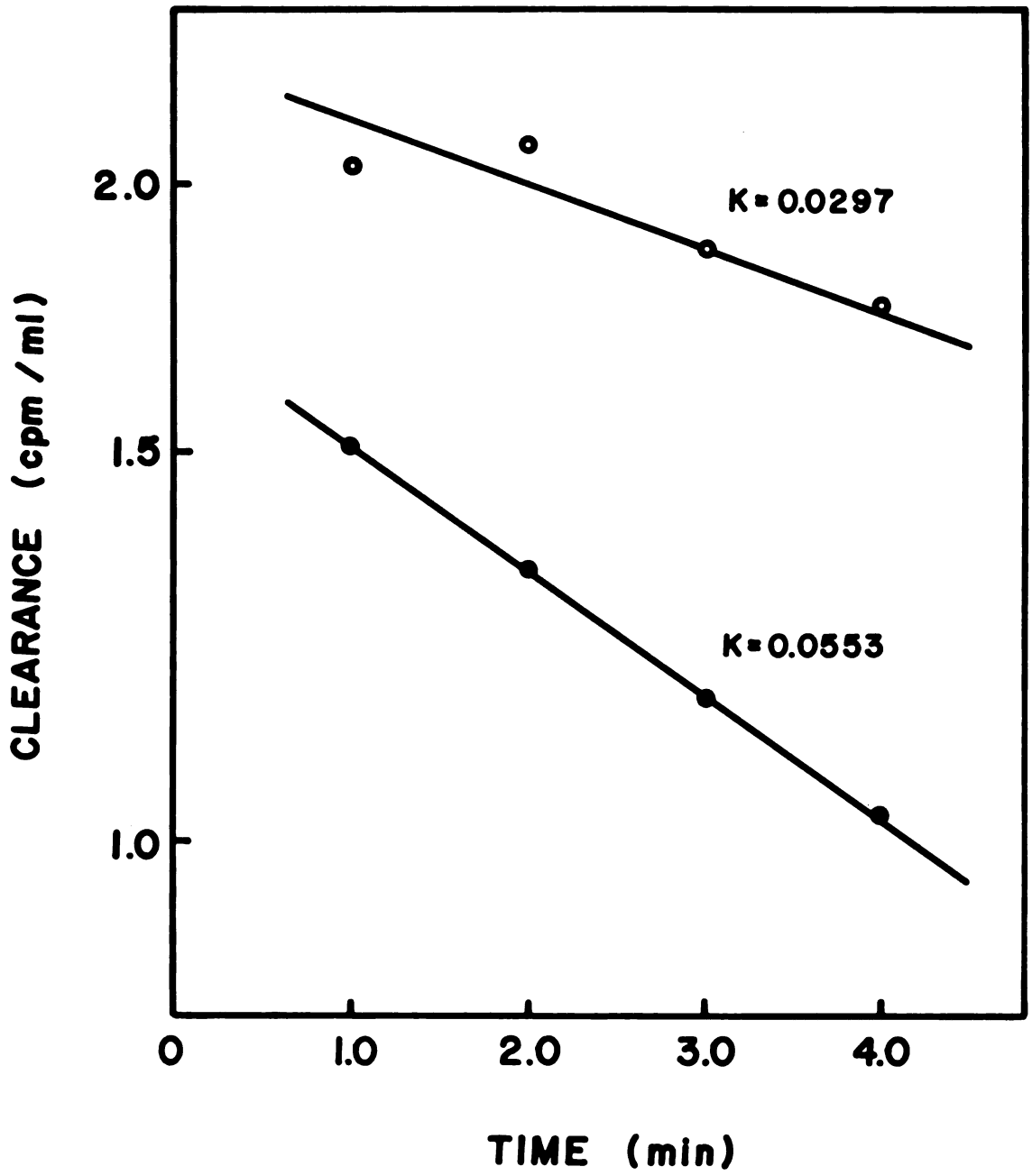

Figure 2. Intravascular clearance of colloidal ^{125}I-BSA in normal and galactosemic chicks.

Normal chicks and galactosemic chicks are represented by (●) and (o), respectively.

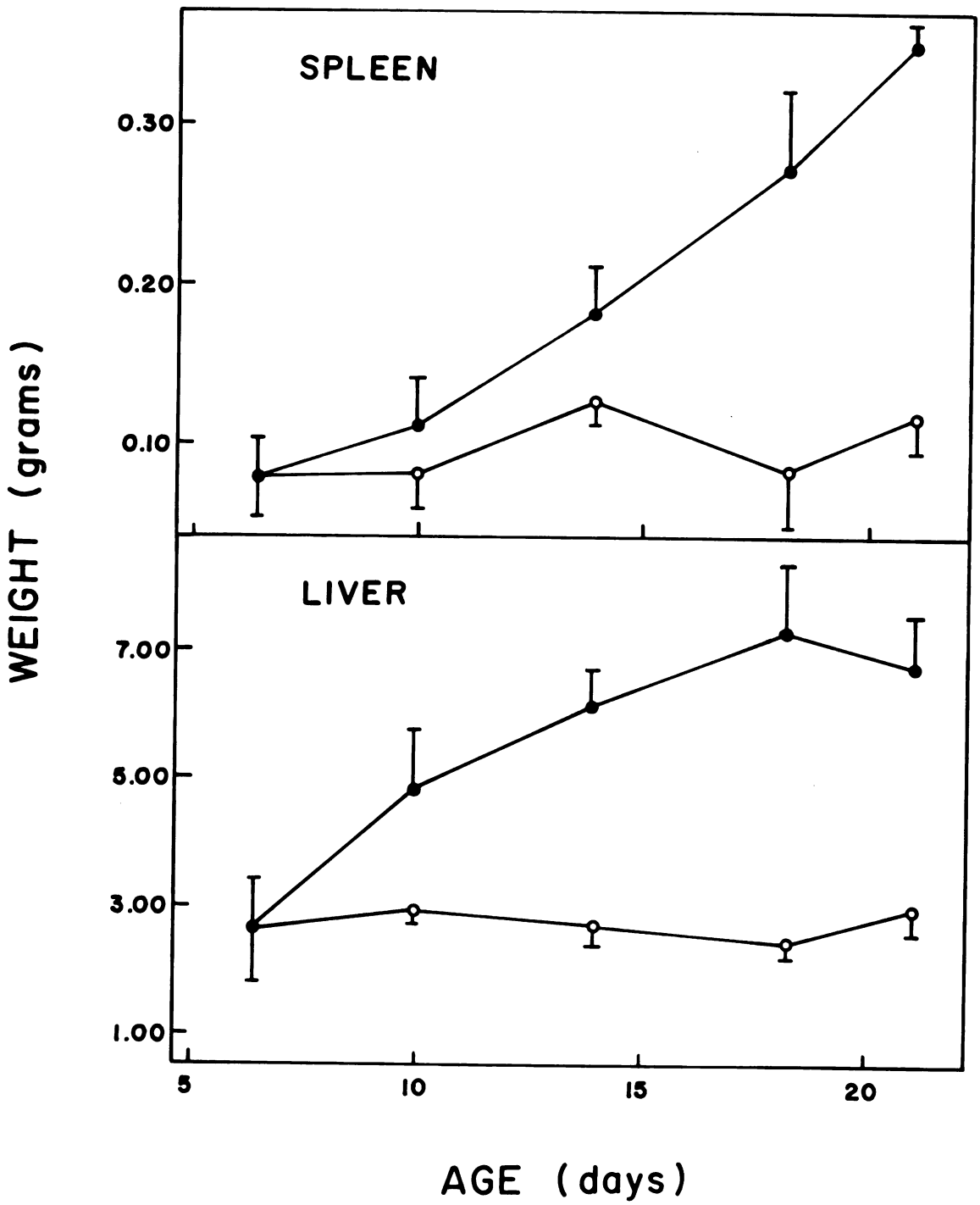

Figure 3. Effect of galactose diet on spleen and liver development in chicks.

Normal chicks and galactosemic chicks are represented by (●) and (o), respectively.

CHAPTER III

HEXOSE TRANSPORT IN HUMAN AND GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES

Abstract

The properties of 2-deoxyglucose and 3-O-methylglucose transport in human and guinea pig polymorphonuclear leukocytes (PMN) were investigated. Uptake of [G-^{3}H] 2-deoxyglucose by guinea pig PMN proceeded *in vitro* with a K_m and V_{max} of 1.8 mM and 0.67 nmoles min^{-1} per 10^6 cells, respectively. This system was competitively inhibited by glucose and mannose but was not significantly affected by galactose, fructose or 3-O-methylglucose. Maximal uptake of 2-deoxyglucose occurred at 41°, and phosphorylation of the 6-position was necessary for its intracellular concentration. This process, while not altered by uncouplers of oxidative phosphorylation, was sensitive to inhibitors of glycolysis. Preincubation of cells with 2 mM iodoacetate for 30 min significantly reduced the uptake of 2-deoxyglucose and the intracellular levels of adenosine-5'-triphosphate without decreasing cell viability.

[Methyl-^{3}H]3-O-methylglucose, on the other hand, was not phosphorylated after entry, and uptake was insensitive

to the presence of either heterologous hexoses or metabolic inhibitors. These and kinetic results indicated that uptake of 3-O-methylglucose by guinea pig PMN occurred by simple diffusion, whereas uptake of 2-deoxyglucose occurred by facilitated diffusion with subsequent phosphorylation. Similar transport systems were found in PMN isolated from human peripheral blood.

Introduction

Phagocytosis and intracellular killing of microorganisms are two primary functions of neutrophilic polymorphonuclear leukocytes (PMN). These activities are of particular importance to the protection of a host against infection. However, in certain disorders, such as diabetes mellitus (1,2) and galactosemia (3), the capacity of PMN to ingest and destroy bacteria is impaired. Previous studies indicate that this impairment directly results from elevated levels of plasma carbohydrate (1-3) and is not an effect of insulin or opsonin deficiencies (1).

Since phagocytosis is sensitive to inhibitors of glycolysis (4) and glucose transport (5), the deleterious effect of galactose on PMN function could largely result from a competitive inhibition of glucose transport. To test this suggestion, we undertook a detailed study of sugar transport in human and guinea pig PMN.

Materials and Methods

Materials. All radioisotopes were purchased from New England Nuclear Corp. with the exception of [1-^{14}C]fructose which was obtained from Amersham Searle Corp. All carbohydrates were of the D-configuration and were purchased from Sigma Chemical Co., Mallinckrodt Chemical Works, and Nutritional Biochemical Co. Other reagents were primarily from Sigma with the following exceptions: iodoacetic acid, Matheson Coleman and Bell Manufacturing Chemists; potassium cyanide, Baker Chemical Co.; p-chloromercuribenzoate and cytochalasin B, Calbiochem; phlorizin, Nutritional Biochemical Co.; N-ethylmaleimide, Aldrich Chemical Co.; U-80 regular Iletin insulin, Eli Lilly and Co.; dextran 250, Pharmacia Fine Chemicals, Inc.; guinea pig serum and Hank's balanced salt solution, Grand Island Biological Co.; and polystyrene latex particles (1.1 μ dia.), Dow Diagnostics. All glassware was siliconized with a 1% solution of Siliclad, Clay Adams.

Cell preparations. PMN was isolated from adult male guinea pigs (Connaught Laboratories, Ltd.) fed a commercial diet and water with 0.04% L-ascorbic acid, ad libitum. Guinea pigs were injected i.p with 5.0 ml of sterile 1% caseinate in saline, and exudates were removed 15 to 20 hours later by flushing peritoneal cavities with Hank's balanced salt solution. Exudate cells composed of greater than 95% PMN were pooled and centrifuged at 250 xg at room temperature

for 5 minutes. Cells were washed and resuspended in Krebs-Ringer phosphate solution without calcium, pH 7.4.

Human PMN were prepared from 10 ml samples of venous blood taken from 15 healthy adult volunteers. Samples were pooled, supplemented with 1.2% dextran 250, and allowed to sediment for one hour at room temperature. Leukocyte-rich supernatants were removed and centrifuged as described above. To lyse remaining erythrocytes, pellets of leukocytes were twice suspended and incubated for one hour at 37° in 200 ml of 0.015 M Tris, pH 7.2, containing 0.75% NH_4Cl. After the second incubation, cells were again centrifuged and were suspended in 5.0 ml of fresh autologous plasma. Cells in plasma were then applied to 20 ml of siliconized glass beads (0.3 mm dia.), and PMN were isolated as described by Rabinowitz (6). Human PMN of at least 90% purity as judged by visual count were washed and resuspended in Krebs-Ringer phosphate solution without calcium, pH 7.4. Cell viability was determined by exclusion of 0.04% Trypan blue (7).

Oxidation of carbohydrate. Conversion of ^{14}C-labelled carbohydrate to $^{14}CO_2$ by guinea pig PMN was performed in sealed 25 ml Erlenmeyer flasks as previously described (4). Cell suspensions (7×10^6 PMN) were incubated for 1 hour at 37° in 1.0 ml aliquots of Krebs-Ringer phosphate solution containing 10% guinea pig serum and 1.0 mM labelled carbohydrate, 0.25 μCi. Reactions were stopped by adding 0.2 ml

of 7.3N H_2SO_4 to the cells, and evolved CO_2 was collected on 1 x 3 cm folded strips of Whatman 3MM paper with 0.2 ml of 3.75N KOH in plastic centerwells. Labelled CO_2 was absorbed from the start of each experiment and for 1 hour after acidifying the medium. Centerwells were transferred directly to scintillation vials with 10 ml of Bray's solution and counted. Oxidation of carbohydrate by phagocytosing cells was determined in the same manner except that a 20-fold (particle/PMN) excess of polystyrene latex particles was included in each flask.

Purity of each ^{14}C-labelled carbohydrate was determined to be greater than 99% by ascending paper chromatography with 7:3 (v/v) ethanol: 1M ammonium acetate. Chromatograms were subsequently monitored with a Packard model 7201 chromatogram scanner.

Transport of carbohydrate. Uptake of ^{3}H-labelled carbohydrate was determined by monitoring the incorporation of both tritium counts and [U-^{14}C]sucrose counts into pellets of cells. Sucrose, which was shown to be excluded from entering PMN, was employed in each assay to correct for trapped ^{3}H-carbohydrate present in the extracellular space. Such corrections were made by multiplying [U-^{14}C]sucrose counts in the pellets by the ratio of tritium to [U-^{14}C] sucrose counts in the supernatants (8). Counts attributable to intracellular ^{3}H-carbohydrate were then calculated by subtracting extracellular tritium counts from total tritium

counts in each pellet.

Unless otherwise stated, reactions employed approximately 4 x 10^6 PMN suspended in 0.5 ml of Krebs-Ringer phosphate solution, pH 7.4. Suspensions were incubated at 37° for 30 min prior to adding 0.5 ml volumes of buffer containing 0.5 μCi of [G-^{3}H]2-deoxyglucose, 0.05 μCi of [U-^{14}C]sucrose, and various amounts of nonisotopic 2-deoxyglucose or inhibitors. After addition of labelled carbohydrate, cells were further incubated for 5 min at the reaction temperature. Since uptake as well as loss of labelled 2-deoxyglucose was found to be negligible at temperatures below 4°, reactions were stopped by placing tubes with cells on ice and centrifuging at 3000 xg at 0° for 5 min. Supernatants were immediately aspirated, and pellets were transferred directly into scintillation vials with 10 ml of Bray's solution. Pellets of cells and supernatant fractions were counted with a Beckman CPM 100 liquid scintillation counter set for double label counting. Uptake of [methyl-^{3}H]3-O-methylglucose was determined by the same procedure.

Quantities of PMN in each assay were determined by counting cells in a hemocytometer. Cell counts were performed at least in quadruplicate with a Universal model Zeiss microscope using phase optics.

Intracellular levels of phosphorylated 2-deoxyglucose were measured after the uptake experiments were performed. Scintillation fluid from each vial was dried, and

carbohydrate was removed from each napthalene residue by extracting with 20 ml of distilled water. Extracts were concentrated under a stream of nitrogen and were spotted on Whatman 3MM paper for ascending chromatography. After development for 15 hours with 7:3 (v/v) ethanol: 1M ammonium acetate, chromatograms were cut into segments corresponding to fast and slow migrating bands of radioactivity. Phosphorylated 2-deoxyglucose associated with the latter segments and free 2-deoxyglucose plus sucrose present in the former segments were eluted with buffer into separate scintillation vials, dried, and counted as described above. In some experiments, phosphorylated 2-deoxyglucose was precipitated by Somogyi's method (9), and levels were determined by calculating the difference between counts attributable to total and free 2-deoxyglucose. Results of both procedures were comparable.

Levels of ATP. PMN (15.2×10^6 cells/assay) were incubated either with or without inhibitors at 37° in 1.0 ml aliquots of Krebs-Ringer phosphate solution, pH 7.4. After 30 min of incubation, cells were centrifuged for 1 min at 3700 xg. Pellets were immediately frozen by immersing tubes in liquid nitrogen, and cells were stored at -80° until homogenizing with 0.21 ml of 3N perchloric acid. Homogenates were centrifuged at 5000 xg for 10 min to remove precipitated protein, and supernates were neutralized with 0.18 ml of 2N KOH: 0.4M imidazole: 0.4M KCl. Levels of ATP were

determined in each supernate by monitoring the reduction of NADP at 340 nm in the presence of excess glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and hexokinase (EC 2.7.1.1) (10).

Results

Oxidation of carbohydrates to $^{14}CO_2$. Table I shows the levels of $^{14}CO_2$ produced by guinea pig PMN during a 1 hour incubation at 37° with various ^{14}C-labelled carbohydrates. Values obtained with [1-^{14}C]glucose were higher than those obtained with other carbohydrates, and participation of the hexose monophosphate shunt in this process is indicated by comparing values for the oxidation of [1-^{14}C]glucose to those of [6-^{14}C]glucose. Addition of latex particles, which stimulates hexose monophosphate shunt activity (11), enhanced $^{14}CO_2$ production by the following extents: with glucose, 2.6 fold; mannose, 2.6 fold; galactose, 2.8 fold; and fructose, 1.3 fold. Increases in $^{14}CO_2$ evolution during phagocytosis were not observed when ^{14}C-labelled 2-deoxyglucose, 3-O-methylglucose, or sucrose were employed. The latter carbohydrates were not readily converted to $^{14}CO_2$ by PMN, and the values obtained in each of these cases could be attributed to traces (less than 1%) of labelled contamination as judged by paper chromatography.

Exclusion of sucrose from PMN. That [U-^{14}C]sucrose was not taken up by PMN is illustrated in Table II. Radioactivity associated with pellets of cells did not change significantly

over the course of a two hour incubation. Similar values for pellet-associated radioactivity were also found when cells were preincubated for 10 min with 5.0 mM nonisotopic sucrose before the addition of equal amounts of labelled sucrose. This eliminated the possibility that sucrose rapidly entered PMN before the initial measurement and supported our contention that pellet-associated radioactivity indicated the amount of trapped extracellular medium.

Time course for 2-deoxyglucose uptake. Data in Figure 1 indicate the time course for uptake of 5.0 mM [G-^{3}H]2-deoxyglucose into guinea pig PMN. Uptake was essentially linear with time for the first ten minutes, after which a maximal value of 4.8 nmoles/10^6 cells was reached. In order to determine whether this value represented a concentration of sugar in either its free or phosphorylated form, intracellular levels of 2-deoxyglucose were calculated upon dividing uptake by the intracellular water space of 0.42 µl/10^6 PMN (12). An approximate 2-fold concentration of sugar then became apparent (Figure 1, right ordinate). Intracellular 2-deoxyglucose was 69.1-84.9% phosphorylated.

Uptake of 2-deoxyglucose versus cell concentration. Before data from separate experiments could be pooled, we determined that uptake was linear with cell concentration and that "crowding effects," as discussed later, were not present. Figure 2 indicates the former relationship for cell concentrations up to 3.6 x 10^6 PMN/ml when 0.2 mM [G-^{3}H]2-

deoxyglucose was employed. Subsequent experiments using this and other levels of 2-deoxyglucose showed linearity up to approximately 8 x 10^6 PMN/ml.

Kinetics of uptake. Values illustrated in Figure 3 were determined by stopping cell incubations after 5 min and measuring the penetration of labelled 2-deoxyglucose or 3-O-methylglucose under conditions of varying substrate concentrations. Initial velocities for uptake were therefore not directly measured but were calculated by dividing the average nmoles of sugar/10^6 cells by the 5 min time interval. Unlike the uptake of 3-O-methylglucose, penetration of 2-deoxyglucose clearly followed saturation type kinetics and yielded a K_m and V_{max} of 1.8 mM and 0.67 nmoles $min^{-1}/10^6$ cells, respectively, when analyzed by the method of Lineweaver and Burk (13). Data obtained with 3-O-methylglucose gave an apparent K_m and V_{max} of 68 mM and 21 nmoles $min^{-1}/10^6$ cells, respectively. However, the latter values could not be verified using a Hofstee type analysis (14).

Effects of heterologous carbohydrates on uptake. When PMN suspensions were incubated with either 4.0 mM glucose or mannose together with 0.2 mM [G-^{3}H]2-deoxyglucose, (Table III), the uptake of labelled sugar was significantly impaired. However, inhibition by similar levels of galactose and fructose was not observed. Addition of 3-O-methylglucose or insulin had no significant effects on 2-

deoxyglucose uptake, and the presence of insulin did not increase the inhibition by glucose. In similar experiments where 3-O-methylglucose uptake was monitored, no inhibitors of uptake were found, and insulin was also without effect (data not shown).

Experiments were then conducted using various 2-deoxyglucose levels at fixed concentrations of glucose or mannose (Figures 4a and 4b, respectively). Both glucose and mannose appeared to be competitive inhibitors of 2-deoxyglucose uptake; the former giving a K_i of 2.67 $\pm$ 0.32 mM and the latter of 2.28 $\pm$ 0.33 mM. All lines in Figure 4 were fit to the data by the method of least squares using a weighted computor program as previously described (15).

Effects of temperature and metabolic inhibitors. As seen in Figure 5, the velocity of 0.2 mM [G-^{3}H]2-deoxyglucose penetration rose significantly with increasing temperature until 41°. Beyond this point velocity decreased and was particularly sensitive to temperatures between 48° and 53°. In contrast, the rate of 0.1 mM [methyl-^{3}H]3-O-methylglucose uptake did not change significantly during similar experiments.

The dependence of 2-deoxyglucose uptake upon temperature suggested that this process relied, in part, upon metabolic activity. To test this suggestion, PMN were preincubated for 30 min at 37° with various metabolic inhibitors, and the rates of [G-^{3}H]2-deoxyglucose uptake,

as well as phosphorylation, were measured (Table IV). In the presence of 2.0 mM iodoacetate rates of both uptake and phosphorylation were significantly reduced to 33% and 11% of the control values, respectively. Preincubation of cells with either 40 mM sodium fluoride or 0.2 mM iodoacetate resulted in similar degrees of inhibition in both uptake and phosphorylation. However, uncouplers of oxidative phosphorylation, such as antimycin A, potassium cyanide, and dinitrophenol, did not show significant effects. Phlorizin, N-ethylmaleimide, and p-chloromercuribenzoate produced slight reductions of 2-deoxyglucose uptake, but these were only significant when the former two agents were employed. In subsequent experiments, cytochalasin B at 0.5 μg/ml and 1.0 ug/ml also inhibited uptake by 30.3% and 35.7%, respectively. These differences were significant at the $P < 0.01$ level. Uptake of [methyl-^{3}H]3-O-methylglucose was not affected by any of the above metabolic inhibitors (data not shown).

A number of other agents were preincubated with cells for 30 min but were without significant effects on [G-^{3}H] 2-deoxyglucose uptake. These included 10 mM sodium barbital, 3 mM theophylline, 3 mM caffeine, 4 mM myo-inositol, 5 mM colchicine, 1 mM cAMP, 1 mM dibutryl cAMP, 1 mM cGMP, and 1 mM dibutryl cGMP. Preincubation with polystyrene latex particles (200:1 particles: cell) and 0.5 mU of insulin, resulted in higher (127% and 143% of control, respectively) but not significantly different, uptake rates. Effects of

insulin were not altered by substituting Krebs-Henseleit bicarbonate solution for Krebs-Ringer phosphate solution, nor did this substitution affect [methyl-^{3}H]3-O-methylglucose uptake. However, rates for [G-^{3}H]2-deoxyglucose uptake were 30% lower when the former medium was employed. Dialyzed 10% guinea pig serum did not affect the uptake of either glucose analog.

ATP levels. Intracellular levels of ATP in guinea pig PMN were significantly lowered by the presence of 2.0 mM iodoacetate (Table V). Conditions during the incubation of cells were identical with those employed during the inhibitor experiments (Table IV).

Effects of heterologous carbohydrates on uptake by human PMN. The uptake of 0.2 mM [G-^{3}H]2-deoxyglucose by human PMN was approximately 2 fold greater than the uptake by guinea pig PMN (0.227 vs 0.100 nmoles $min^{-1}/10^{6}$ cells). Despite this difference, inhibitors of uptake for human PMN (Table VI) were very similar with those for guinea pig PMN (Table III). Galactose, fructose, and 3-O-methylglucose did not significantly impair 2-deoxyglucose uptake when present at a 20:1 inhibitor to substrate ratio, whereas glucose and mannose resulted in significant inhibition. Mannose in this case was a slightly better inhibitor than glucose, and some inhibition did occur in the presence of 30 mM galactose, but the latter was not significant.

Discussion

Although the conversion of [1-^{14}C] glucose to $^{14}CO_2$ by PMN has been frequently reported (16,17), studies on the oxidation of other carbohydrates to CO_2 have not been previously made. Our observations (Table I) demonstrate that [1-^{14}C] mannose, [1-^{14}C] galactose, and [1-^{14}C] fructose are also metabolized to $^{14}CO_2$ and that this oxidative metabolism is stimulated by phagocytosis. The degree of stimulation of [1-^{14}C] glucose oxidation is comparable to values previously reported (16), and the rate of oxidation by phagocytosing cells agrees with the 20 nmoles $hr^{-1}/10^6$ cells observed by Stjernholm et al. (18). That a constant degree of stimulation (2.6-2.8 fold) occurred, when either labelled glucose, mannose or galactose (but not fructose) were employed, strongly suggests that the former three hexoses are converted into a common intermediate, i,e., glucose-6-phosphate, prior to oxidation by the hexose monophosphate shunt. Support for this suggestion is threefold: 1) PMN contain a complete Leloir pathway and are capable of converting galactose to glucose-6-phosphate (3,19), 2) ^{14}C-labelled mannose is converted to lactate, via mannose-6-phosphate and fructose-6-phosphate, with the same labelling pattern as lactate derived from ^{14}C-labelled glucose (20), and 3) PMN are not gluconeogenic and are not able to convert fructose-1-phosphate or fructose-1, 6-bisphosphate to glucose-6-phosphate (21).

The inability of guinea pig PMN to transport or oxidize ^{14}C-sucrose (Table II) is in agreement with results on human PMN described by Englhardt and Metz (12) and with observations on rabbit alveolar macrophages described by Gee et al. (5). Both of these studies similarly employed the exclusion of sucrose to correct for extracellular space during hexose transport experiments. The use of this technique also corrects for a non-specific type of uptake encountered during phagocytosis. Esman refers to this as "piggy-back" phagocytosis or the "concomitant engulfment of extracellular medium with the phagocytosis of particles" (22). Such non-specific uptake increases the insulin space in pellets of leukocytes by 4% to 6% (23) and, thus, could result in elevated values for 2-deoxyglucose uptake if ^{14}C-sucrose was not also employed.

When 5 mM [G-^{3}H]2-deoxyglucose is presented to guinea pig PMN, maximal intracellular levels of label approach 11.4 mM after 40 min of incubation (Figure 1). Since 69% to 85% of this label is phosphorylated, the maximal intracellular levels of free 2-deoxyglucose can be calculated to range from 1.7 mM to 3.5 mM. These values suggest that entry of 2-deoxyglucose occurs in the free form and that phosphorylation is necessary for 2-deoxyglucose concentration. These results are in accord with those of Esman (22), who found free intracellular glucose in PMN at low external glucose concentrations, and also with those of Luzzatto and Leoncini (24), who concluded that the rate of carbohydrate

utilization was slower than the rate of carbohydrate entry.

While data in Figure 1 eliminate an active transport system for 2-deoxyglucose uptake, those from Figure 3 and Figure 4 rule out simple diffusion and indicate a facilitated transport mechanism. Uptake of 2-deoxyglucose displayed saturation-type kinetics and was competitively inhibited by glucose and mannose which is suggestive of a common carrier mediated transport system. Similar inhibition of 2-deoxyglucose uptake by glucose occurs in rabbit alveolar macrophages (5), and inhibition by glucose and mannose, but not galactose and fructose, is reported for rat diaphram muscle (25). Studies on guinea pig lymph node cells by Helmreich and Eisen (26) also imply a common carrier mechanism for hexose transport; however, this mechanism appears to be more selective in PMN, since our results did not show competitive inhibition by fructose or 3-O-methylglucose.

Further evidence for facilitated diffusion of 2-deoxyglucose arises from the temperature dependence of [G-^{3}H] 2-deoxyglucose uptake (Figure 5) and from the effects of metabolic inhibitors upon this process (Table IV). Since hexose phosphorylation is required for sequestering of label, inhibition of phosphorylation by depleting intracellular ATP would be expected to lower the diffusion gradient for free 2-deoxyglucose and, thus, impair the rate of 2-deoxyglucose entry. We observed significantly lower rates of entry when guinea pig PMN were incubated at temperatures below 37°,

when an isotonic bicarbonate solution was substituted for Krebs-Ringer phosphate solution, and when cells were pre-incubated with 40 mM sodium fluoride or 2.0 mM iodoacetate. Pre-incubation with the latter inhibitor not only impaired uptake and phosphorylation to the greatest extents but also significantly decreased the intracellular levels of ATP (Table V). These results, together with the lack of significant inhibition by potassium cyanide, antimycin A, and dinitrophenol, further demonstrate that active glycolysis, and not oxidative phosphorylation, is the primary source of ATP in PMN. This agrees with previous observations that PMN contain few mitochondria and that inhibitors of glycolysis can depress phagocytic function (4).

The lack of an in vitro insulin effect on either 2-deoxyglucose uptake or an inhibition of uptake by glucose (Table III) is consistent with our findings that the rate of carbohydrate entry was always greater than the rate of phosphorylation. However, this also implies that phosphorylation of 2-deoxyglucose is not affected by the presence of insulin. Similar results were obtained by Beck (27), who could not find insulin sensitivity in leukocyte hexokinase, by Englhardt and Metz (12), who did not observe an insulin effect upon glucose transport into human PMN, and by Helmreich and Eisen (26), who were unable to demonstrate an insulin effect upon glucose uptake into guinea pig lymph node cells. On the other hand, Luzzatto (28) observed an increase in xylose penetration into leukocytes at a

non-physiological level of insulin (1 U/ml), and Klant and Schucher (29) found an increased disappearance of glucose when leukocytes were incubated with 0.3 U/ml of insulin. Levels of insulin employed in our studies (0.5 mU/ml) were slightly higher than physiological and did not affect either 2-deoxyglucose or 3-O-methyglucose uptake.

The uptake of [methyl-^{3}H]3-O-methylglucose in our experiments was remarkably different from the uptake of [G-^{3}H] 2-deoxyglucose in three respects: 1) 3-O-methylglucose was not concentrated nor phosphorylated after entry, 2) uptake was insensitive to the presence of heterologous carbohydrate, metabolic inhibitors, and to varying temperature, and 3) uptake failed to follow saturation-type kinetics. Whereas kinetic parameters were determined for this glucose analog using a Lineweaver-Burk type analysis (Figure 3b), these values were substantially different from those for 2-deoxyglucose and could not be verified by the method of Hofstee (14). Since both K_m and V_{max} for 3-O-methylglucose uptake tend towards infinity, it is apparent that transport of this analog in PMN occurs by simple rather than facilitated diffusion.

Although decreases in cell metabolism, as well as glucose uptake, have been noted in concentrated PMN suspensions, we did not encounter this during our experiments. This so-called "crowding effect" has been attributed to changes in extracellular pH as a result of lactate accumulation and to the subsequent inhibition of phosphofructokinase

activity (22). Although Englhardt and Metz (12) observed this in their experiments with 7.6 x 10^7 PMN/ml during a 1 hour incubation, our experiments avoided this effect by utilizing fewer cells and shorter (5 min) incubation times. Therefore, lactic acid was not allowed to build up, and linearity between 2-deoxyglucose uptake and cell concentration was established (Figure 2). This method also circumvented any problems associated with excessive 2-deoxyglucose-6-phosphate accumulation which could non-competitively inhibit the hexokinase reaction (24).

Similarities between the uptake of 2-deoxyglucose by guinea pig PMN and the uptake by human PMN isolated from peripheral blood are apparent from comparing values in Table III to those in Table VI. Although the rate of uptake with human cells was approximately 2 fold greater than the rate with guinea pig cells at 37°, uptake in both cases was substantially inhibited by the presence of glucose or mannose. Galactose, fructose, and 3-O-methylglucose at 4 mM did not significantly impair 0.2 mM 2-deoxyglucose entry into either cell type. However, uptake was slightly decreased when human PMN were incubated with 30 mM galactose. This latter effect was not observed with guinea pig PMN under similar conditions but was found, together with lower ATP levels (Table V), after a 30 min pre-incubation with elevated galactose. It therefore appears that galactose may impair 2-deoxyglucose uptake by blocking its phosphorylation rather than by competing with its uptake. Although

competition in this case with human PMN cannot be eliminated *per se*, it seems unlikely that elevated levels of galactose, as encountered during galactosemia, could impair phagocyte function by competing with glucose uptake. Thus, impaired phagocytic activity in the presence of galactose may be primarily attributed to other factors, such as i) the presence of a futile adenosine triphosphatase cycle, or ii) the intracellular accumulation of galactose or one of its metabolites with subsequent inhibition of glycolysis (3).

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Table I. Conversion of ^{14}C-labelled carbohydrate to $^{14}CO_2$ by guinea pig PMN[a]

Carbohydrate	$^{14}CO_2$ produced		P
	Resting	Phagocytosing	
	nmoles/hour/10^6 cells ± S.D.		
[1-^{14}C]Glucose	7.16 ± 1.57	18.5 ± 0.4	0.01
[1-^{14}C]Mannose	2.06 ± 0.16	5.42 ± 0.55	0.01
[1-^{14}C]Galactose	0.60 ± 0.14	1.70 ± 0.10	0.01
[1-^{14}C]Fructose	0.26 ± 0.02	0.34 ± 0.01	0.02
[6-^{14}C]Glucose	0.14 ± 0.02		
[U-^{14}C]2-deoxyglucose	0.11 ± 0.02	0.11 ± 0.01	ns
[U-^{14}C]3-O-methyl-glucose	0.01	0.01	ns
[U-^{14}C]sucrose	0.03	0.04	ns

[a]Cells (7×10^6 PMN) were incubated for 1 hour at 37° in 1.0 ml aliquots of buffer containing 10% guinea pig serum and 1.0 mM labelled carbohydrate, 0.25 μCi. CO_2 was collected from the start of each experiment on strips of Whatman 3mm paper saturated with KOH and for 1 hour after acidifying medium. Each experiment was performed in triplicate. S.D. refers to standard deviation. Significance levels, P, are given for comparison of values from resting cells to values from cells incubated with polystyrene latex particles; ns indicates no significance.

Table II. Exclusion of [U-^{14}C]Sucrose from guinea pig PMN[a]

Incubation time	[U-^{14}C]Sucrose in pellet	P
	counts/min ± S. D.	
0	914 ± 114	
2	879 ± 84	ns
30	854 ± 84	ns
60	1002 ± 251	ns
120	914 ± 181	ns

[a]PMN (1.18×10^6 cells/assay) were incubated at 37° with buffer containing 10% autologous serum and 1.0 mM [U-^{14}C] sucrose, 0.25 µCi. After each time point tubes were placed in ice and immediately centrifuged for 5 min at 3000 xg at 4°. Pellets were rinsed with cold buffer and suspended in scintillation fluid. Each time point was performed in triplicate: ns indicates no significance between values at a given time and values at 0 time.

Table III. Effect of carbohydrate and insulin on uptake [G-^{3}H]2-deoxyglucose by guinea pig PMN[a]

Additions	% Uptake	P
None	100 ± 13.1	
Insulin	95.7 ± 4.9	ns
Glucose	42.5 ± 10.1	0.001
Glucose + insulin	44.5 ± 3.6	0.001[c]
Mannose	46.9 ± 4.2	0.001
Galactose	95.8 ± 7.5	ns
Fructose	95.8 ± 6.8	ns
3-O-methylglucose	87.0 ± 7.1	ns

[a]PMN were incubated at 37° in 1.0 ml of buffer. All additions were made at 0 time. 2-deoxyglucose was 0.2 mM, whereas levels of carbohydrate and insulin were 4.0 mM and 0.5 mU/ml, respectively. P indicates levels of significance between values with additions and values without additions: ns indicates no significance.

[b]Uptake rate without additions was 0.142 ± 0.019 nmoles/min/10^{6} cells: N=6.

[c]No significance between values obtained with glucose and values obtained with both glucose and insulin.

Table IV. Effect of metabolic inhibitors on rates of [G-^{3}H] 2-deoxyglucose uptake and phosphorylation by guinea pig PMN[a]

Additions	Uptake	Phosphorylation
	nmoles $min^{-1}/10^6$ PMN	
None	0.100	0.064
Iodoacetate 0.2 mM	0.057[c]	0.018
Iodoacetate 2.0 mM	0.033[b]	0.007
Fluoride 40 mM	0.064[c]	0.017
Antimycin A 1.0 µg/ml	0.086[ns]	
Cyanide 6.0 mM	0.086[ns]	0.042
Dinitrophenol 1.0 mM	0.096[ns]	0.058
Phlorizin 10.0 mM	0.069[e]	
N-ethylmaleimide 0.2 mM	0.070[d]	
Chloromercuribenzoate 0.2 mM	0.081[ns]	

[a]PMN (2.81 x 10^6 cells/assay) were preincubated with or without additions for 30 min at 37°. After this period [G-^{3}H]2-deoxyglucose was added to a final concentration of 0.2 mM, and uptake rates were measured. Each assay was performed 5 times, and levels of significance between values with additions and values without additions are indicated by: b) 0.005, c) 0.01, d) 0.02, e) 0.05, and ns (no significance).

Table V. Effect of iodoacetate on ATP levels in guinea pig PMN[a]

Additions	ATP levels	P
	nmoles/10^6 cells ± S.D.	
None	1.32 ± 0.46	
Iodoacetate 2.0 mM	0.41 ± 0.33	0.05
Galactose 30.0 mM	0.78 ± 0.27	ns

[a]PMN (15.2 x 10^6 cells) were incubated with or without additions for 30 min at 37°. After incubation cells were immediately centrifuged at 37° for 1 min at 3700 xg. Pellets were quickly forzen by immersing tubes in liquid nitrogen. Cells were stored at -80° until assay; see "Experimental Procedures" for assay conditions. P refers to levels of significance between values with additions and values without additions; ns indicates no significance.

Table VI. Effect of carbohydrate on uptake of [G-^{3}H]2-deoxyglucose by human PMN[a]

Additions	% Uptake ± S.D.	P
None	100 ± 20.1	
Mannose 4.0 mM	39.1 ± 7.8	0.02
Glucose 4.0 mM	50.8 ± 4.6	0.02
Fructose 4.0 mM	78.2 ± 9.9	ns
Galactose 4.0 mM	78.2 ± 6.6	ns
Galactose 30.0 mM	64.3 ± 11.9	0.05
3-O-methylglucose 4.0 mM	98.8 ± 8.8	ns

[a]PMN (0.848 x 10^6 cells/ml) were incubated in 1.0 ml buffer at 37°. All additions were made at 0 time. [G-^{3}H]2-deoxyglucose was 0.2 mM, and uptake rate without additions was 0.227 ± 0.046 nmoles/min/10^6 cells: N=5. P refers to levels of significance between values with additions and values without additions; ns indicates no significance.

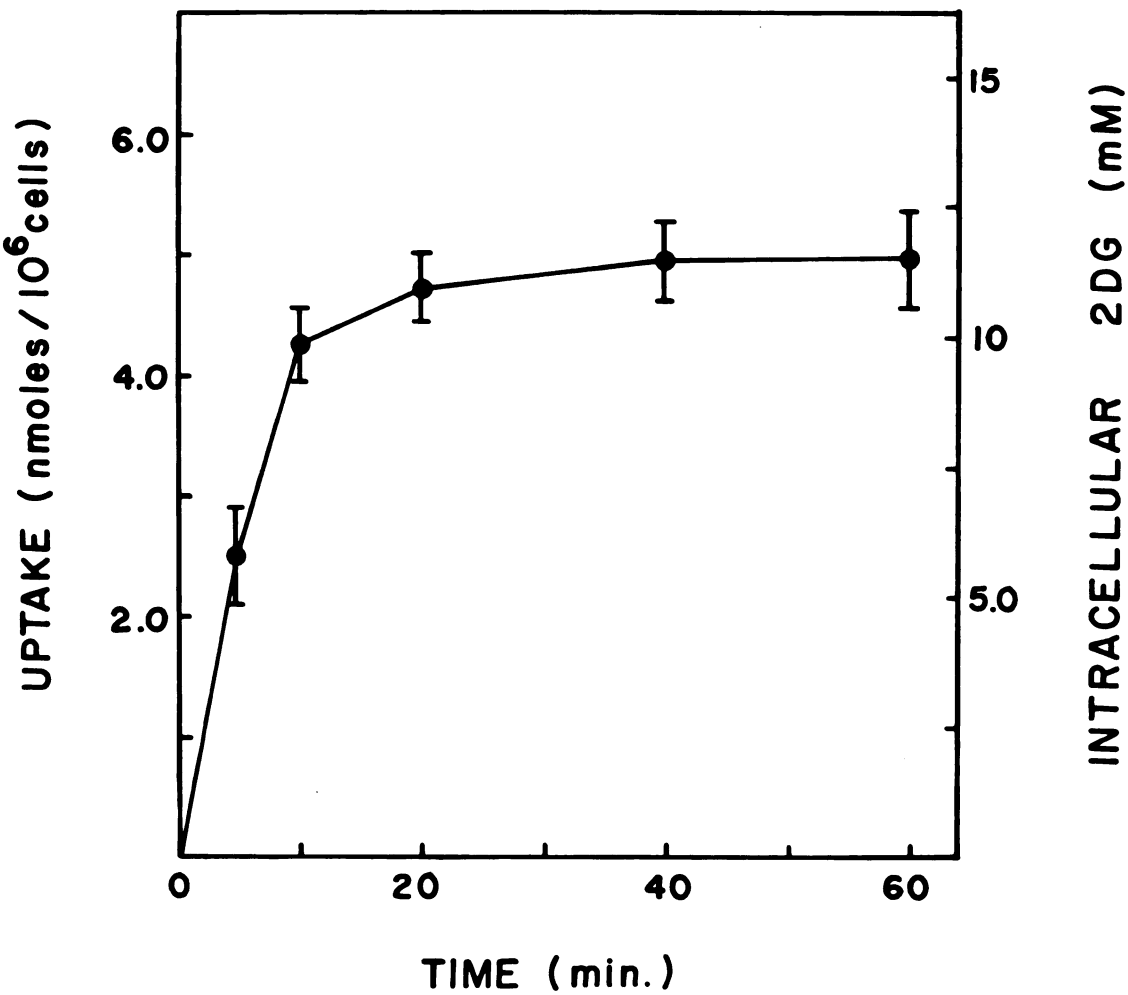


Figure 1. Time-course for uptake of 5.0 mM 2-deoxyglucose by guinea pig PMN.

Assays were performed in quadruplicate using 7 x 10^6 PMN/experiment at 37°C. Line bars indicate S.D. Intracellular concentrations (right ordinate) are derived from uptake values (left ordinate), see text.

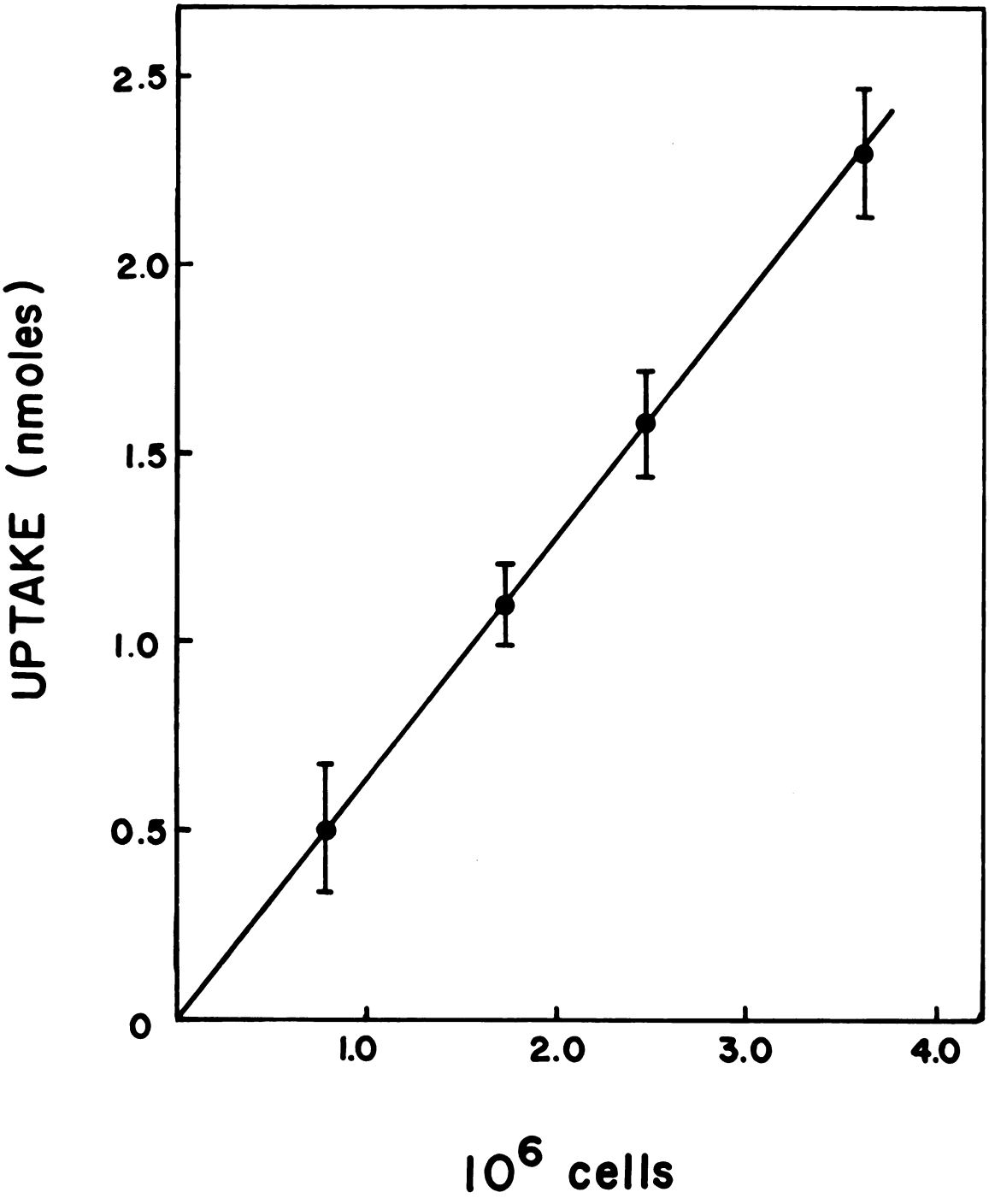


Figure 2. Uptake of 2-deoxyglucose versus cell concentration.

Uptake was measured 5 min after adding 0.2 mM labelled 2-deoxyglucose. Line bars indicate S.D. of three experiments.

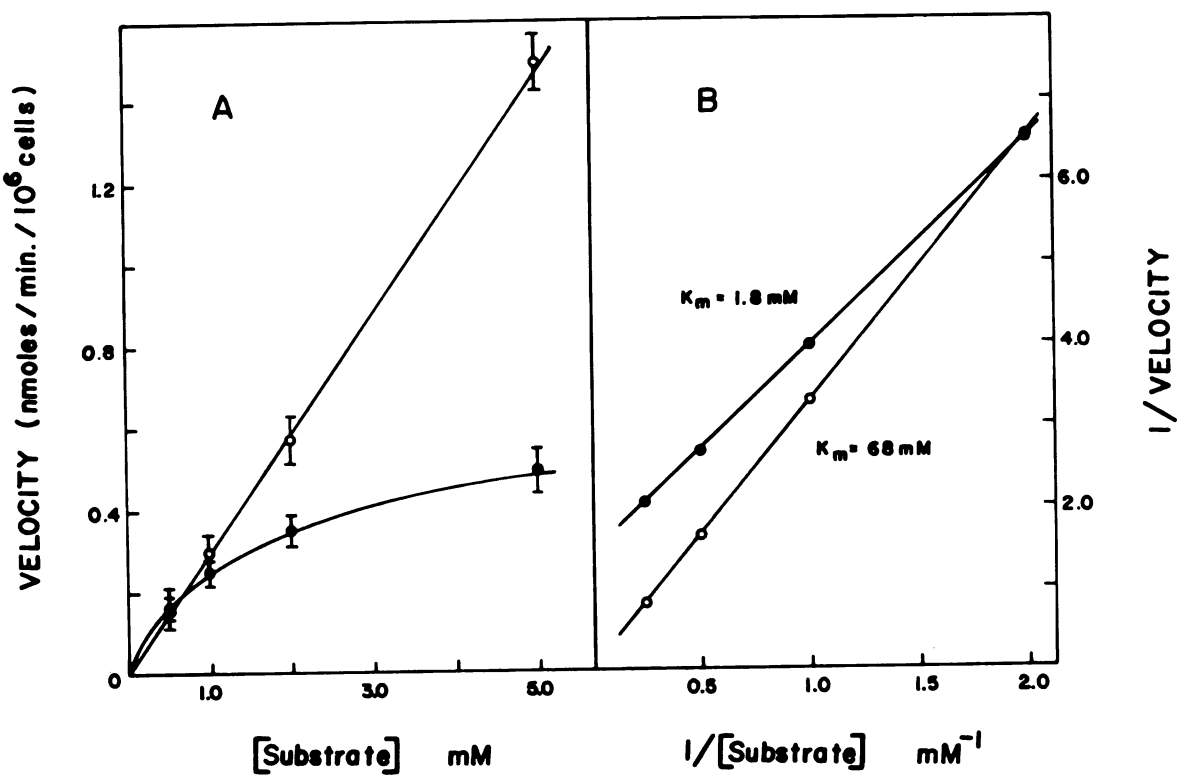


Figure 3. Dependence of 2-deoxyglucose uptake (●) and 3-O-methylglucose uptake (o) on levels of external homologous carbohydrate.

Velocity is plotted (A) against external substrate concentration, and these values are replotted (B) according to Lineweaver and Burk. Each point is a mean of values from 3 experiments that were performed in triplicate. Line bars indicate S.D.

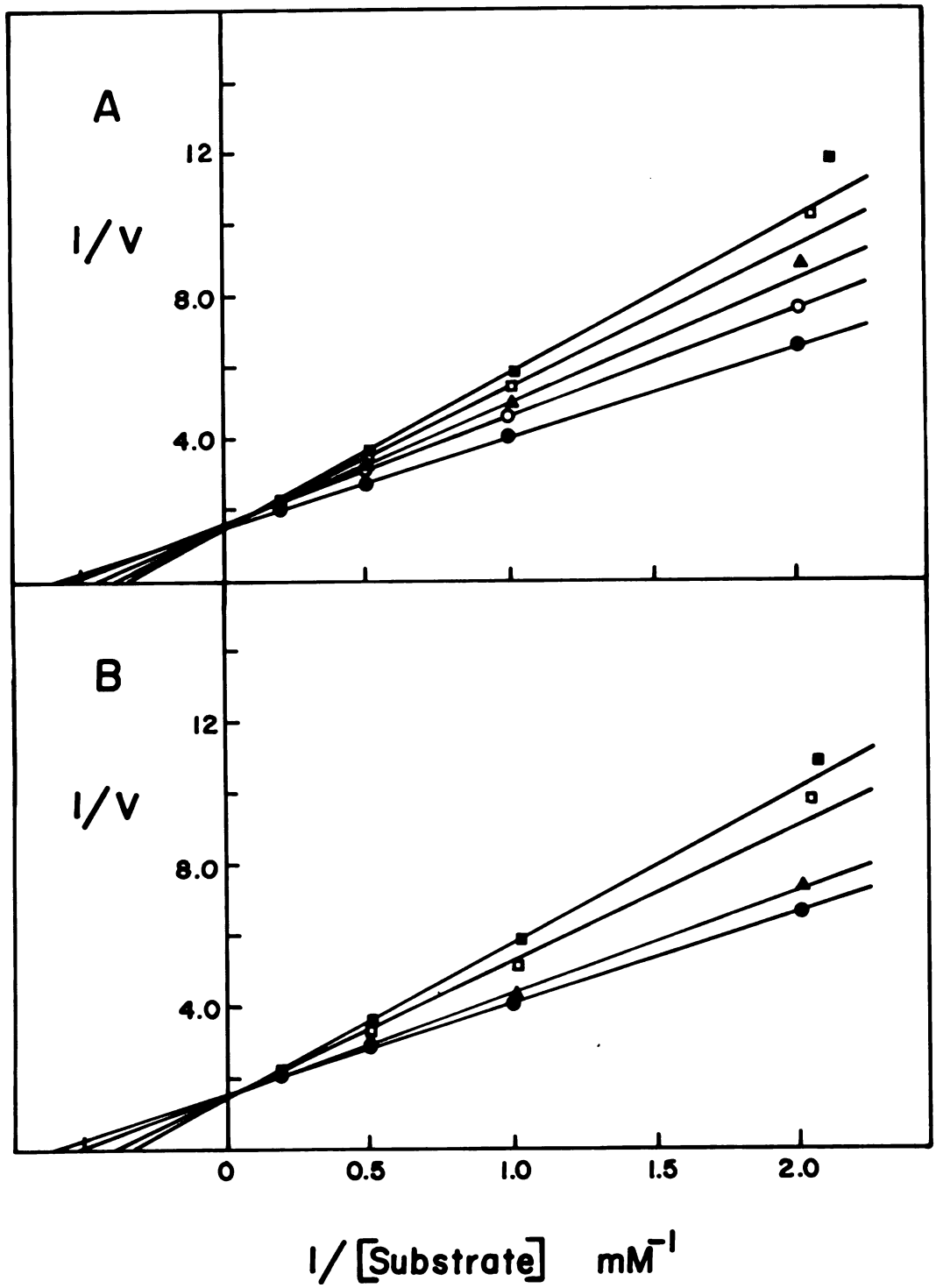


Figure 4. Inhibition of 2-deoxyglucose transport by glucose and mannose.

Lineweaver-Burk plots for inhibition by glucose (A) and mannose (B) were generated from data obtained in 4 and 3 experiments, respectively; each of which were performed in triplicate. Levels of inhibitor are (●) none, (o) 0.25 mM, (▲) 0.49 mM, (□) 0.98 mM, and (■) 1.96 mM.

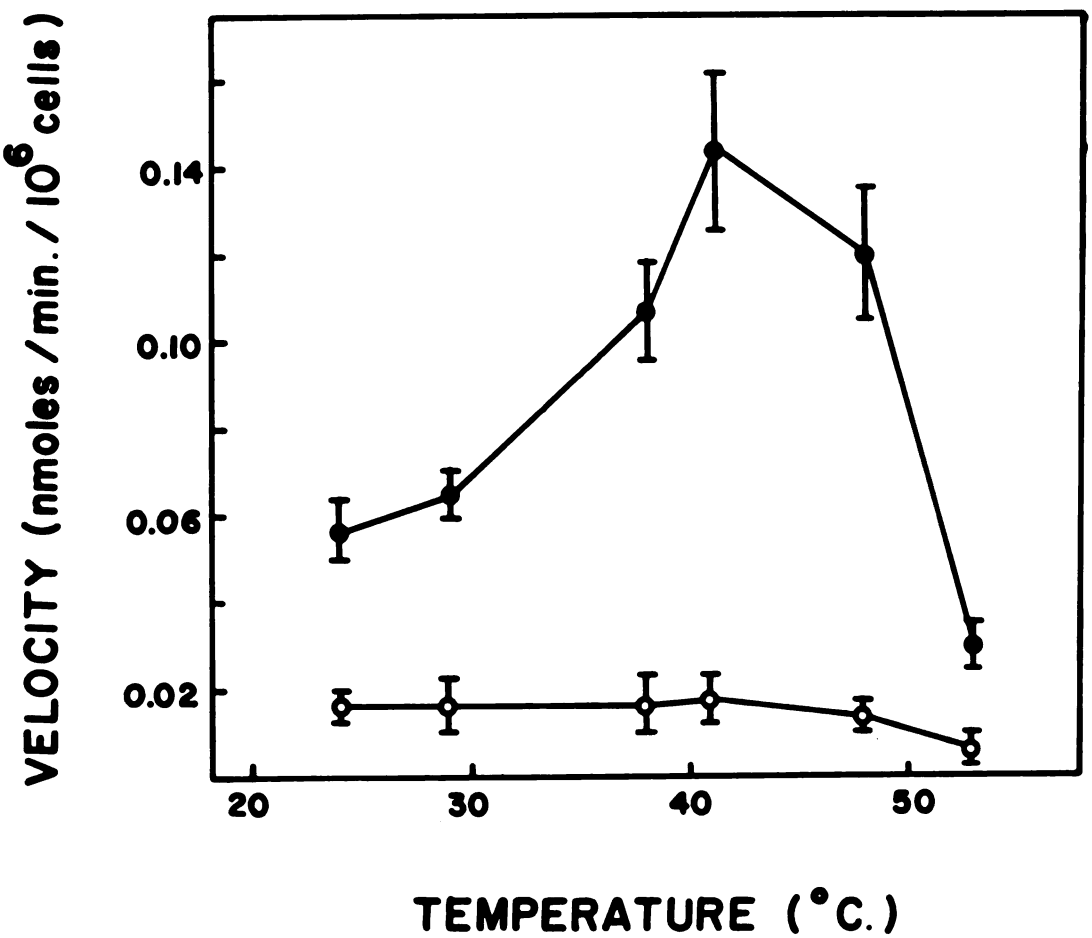


Figure 5. Temperature dependence of 0.2 mM 2-deoxyglucose uptake (●) and 0.1 mM 3-O-methylglucose uptake (o).

Line bars indicate S.D. from 5 experiments.

CHAPTER IV

EFFECT OF CARBOHYDRATE ON THE OXYGEN-DEPENDENT KILLING MECHANISM OF POLYMORPHONUCLEAR LEUKOCYTES

Abstract

To account for the impaired bactericidal activity of polymorphonuclear leukocytes (PMN) during galactosemia and hyperglycemia, a number of biochemical parameters associated with the oxygen-dependent killing mechanism of guinea pig PMN were investigated. These included the formation of superoxide anion, hydrogen peroxide and hydroxyl radical, as well as the consumption of oxygen and chemiluminescence.

Incubation of PMN in medium containing 30 mM galactose substantially impaired the extracellular reduction of cytochrome c. This action was prevented by the addition of catalase. However, this impairment could not be adequately explained on the basis of either decreased superoxide anion production or increased hydrogen peroxide generation. Further experiments demonstrated 1) that hexoses and polyols, which are accumulated by PMN, effectively competed with methional for reaction with hydroxyl radical, and 2) that hydroxyl radical oxidized hexose to hexonic acid. These

results indicated the participation of carbohydrate radicals and suggested that such radicals were formed by PMN. The formation of carbohydrate radicals by PMN could account for oxidation of extracellular cytochrome c and could be deleterious to normal bactericidal activity.

Introduction

Phagocytosis and microbicidal activity are two distinct functions of polymorphonuclear leukocytes (PMN). The former is an endergonic process relying upon energy supplied from glycolysis (1), whereas the latter is mainly oxidative in character depending upon the production of reduced oxygen intermediates. Bactericidal activity is impaired under anaerobic conditions (2,3) and can be partially inhibited by incubating leukocytes with catalase (4), superoxide dismutase (4,5) or benzoate (4). Hydrogen peroxide (6,7,8) and superoxide anion (9,10) are formed by PMN, and production of hydroxyl radical (4,11), as well as singlet oxygen (4,12, 13), has been proposed. Considerable evidence suggests that the generation of these agents is linked to the univalent reduction of oxygen by a cyanide-insensitive NADH oxidase (14,15) and that this system is related to the activity of the hexose monophosphate shunt (16,17). In this regard, absence of extracellular glucose has been shown to decrease formate oxidation, and thus hydrogen peroxide production, in PMN (8). However, effects of elevated levels of hexose upon the formation of hydrogen peroxide and other reduced

forms of oxygen have not been previously determined. These effects could account for the impaired bactericidal activity of PMN during galactosemia and hyperglycemia and are described herein.

Materials and Methods

Animals and materials. Adult male guinea pigs were obtained from Elm Hill Laboratories, Cambridge, Mass., and fed a commercial diet and water with 0.04% ascorbic acid, ad libitum. Xanthine, scopoletin, phenazine methosulfate, nitroblue tetrazolium, methional, heparin (grade I), NADH (grade III), xanthine oxidase (EC 1.2.3.2), horse radish peroxidase (EC 1.11.1.7), and cytochrome c (type III) were purchased from Sigma Chemical Co. Hydrogen peroxide, catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), and potassium cyanide were purchased from Mallinckrodt Chemical Works, Worthington Biochemical Co., Miles Laboratories, Ltd., and J.T. Baker Chemical Co., respectively. All carbohydrates and polyols were purchased from Sigma, Mallinckrodt, or Nutritional Biochemical Co. Guinea pig serum and ^{14}C-labelled formate were purchased from Grand Island Biological Co. and New England Nuclear Corp., respectively. Dichlorodiacetylfluorescein and triethylene diamine were products of Eastman Organic Chemicals.

Xanthine oxidase and catalase were routinely passed through 1 x 30 cm columns of Sephadex G-10 to remove preservatives, and gel filtration was similarly used to free

ferrocytochrome c from excess ascorbate. Stock solutions of hydrogen peroxide were prepared immediately before use by diluting 30% H_2O_2 2000 fold with distilled water. Concentrations were then determined by recording the 230 nm absorbance and employing the molar extinction coefficient of 81 M^{-1} cm^{-1} (6).

Cell preparations. Guinea pig PMN and macrophages were isolated from casein injected peritonea as described in Chapter III and Chapter I, respectively. Concentrations of cells in suspension were determined using a hemocytometer under phase optics. Each cell count was performed in quadruplicate.

Extracellular reduction of cytochrome c. Reactions involving the reduction of ferricytochrome c by superoxide anions were investigated by using a method similar to that reported by Babior et al. (9). Both resting and phagocytosing PMN (0.88×10^7 to 1.06×10^7/assay) were preincubated at 37°C for 2 hr in 1.0 ml of KRPS containing 10% guinea pig serum. Cell suspensions were then diluted with one volume of ferricytochrome c solution (132 uM) and divided into two 1.0 ml aliquots. One aliquot was placed on ice to serve as a reaction blank, while the other was incubated for an additional hour at 37°C. Cells along with polystyrene latex particles were removed by centrifuging each aliquot at 3600 xg for 5 min, and levels of reduced cytochrome c were determined in each cell supernate by

monitoring the absorbance at 550 nm. Various levels of carbohydrate were added prior to the pre-incubation period, while other agents including superoxide dismutase (0.2 mg/ml) and catalase (0.3 mg/ml) were added along with cytochrome c. All assays were performed in quadruplicate on cells pooled from at least two guinea pigs. Phagocytosing cells received a 50 fold (particle to cell) excess of polystyrene latex particles.

Effects of carbohydrate upon the reduction of cytochrome c by superoxide anion were also studied in separate experiments without cells. Superoxide was generated enzymatically in 1.1 ml of 0.05 M sodium phosphate buffer, pH 7.8, containing: 50 μM xanthine, 100 μM EDTA, 10 μM ferricytochrome c, 27 mM carbohydrate, and 3.2 mU of xanthine oxidase. Reactions were performed 6 to 8 times, and rates of cytochrome c reduction at 550 nm were determined using a Gilford model 3500 spectrophotometer in the general kinetic-1 program mode.

Cellular reduction of nitroblue tetrazolium. Reduction of nitroblue tetrazolium to blue formazan was studied in suspensions of resting and phagocytosing PMN. Cells (4.9 x 10^6/assay) were pooled from at least two guinea pigs and were incubated at 37°C in KRPS containing 10% autologous guinea pig serum. In one experiment the length of pre-incubation time was varied prior to stimulating cells with polystyrene latex particles. Whereas, in a second experiment

pre-incubation time was kept constant for 1 hr, while carbohydrate concentrations were varied. In both cases stimulated cells were incubated at 37°C for an additional 30 min in the presence of nitroblue tetrazolium (140 μM), and reactions were stopped by placing cell suspensions on ice. Cells were centrifuged at 3000 xg for 5 min, supernates were decanted, and formazan was extracted from pellets of cells with 6.0 ml of pyridine. Formazan was quantitated by its absorption at 515 nm.

Effects of carbohydrate upon the reduction of nitroblue tetrazolium by superoxide anion were also determined in separate experiments without cells. Superoxide was generated non-enzymatically in 1.1 ml of KRPS containing: 78 μM NADH, 23 μM phenazine methosulfate, and 50 μM nitroblue tetrazolium. Reactions were performed 6 to 8 times at room temperature using a Gilford model 3500 spectrophotometer in the general kinetic-1 program mode.

Cellular release of hydrogen peroxide. Rates of hydrogen peroxide release from PMN were quantitated by monitoring the decrease in scopoletin fluorescence upon oxidation of scopoletin by peroxide and horse radish peroxidase (7). PMN (0.50×10^7 to 1.23×10^7/assay) were incubated for 3 hr at 37°C in KRPS, pH 7.4. Cells were contained in 0.5 ml dialysis bags and were dialyzed against 2.5 ml of the same buffer during incubation and for one hour at 4°C following incubation. Peroxide levels were determined by adding 0.1

ml of each dialysate to 1.9 ml of KRPS containing 2 μM scopoletin and 1 μg/ml horse radish peroxidase. Reactions were completed after 45 min at room temperature, and fluorescence emission was then measured between 430 nm and 470 nm upon excitation with 313 nm plus 366 nm light. Since this system was standardized in the absence of cells by adding known amounts of hydrogen peroxide (Figure 3), decreases in scopoletin fluorescence were directly related to increases in peroxide generation. When phagocytosing PMN were employed, a 200 fold (particle: cell) excess of polystyrene latex particles was included in each dialysis bag.

In similar experiments, peroxide levels were determined by monitoring the fluorescence of diacetylfluorescein after its oxidation by peroxide and horse radish peroxidase (18). This method required one-fifth the level of hydrogen peroxide, as well as 0.5 μM leuko-diacetylfluorescein, prepared by exposing its dichloro-derivative to 0.01 N NaOH for 30 min (19). Although linear standard curves were found with both hydrogen donors, variations in fluorescence were substantially less when scopoletin was employed.

PMN in all assays were pooled from at least four guinea pigs and were washed extensively with isotonic buffer to remove contaminating ascorbate and glutathione. Preliminary experiments performed on cells without washing, as well as cells without dialysis, gave inconsistent results.

Cellular formate oxidation. Conversion of ^{14}C-labelled formate to $^{14}CO_2$ by PMN was performed in 25 ml siliconized Erlenmeyer flasks fitted with gas-tight stoppers and plastic centerwells. Cells (2.07 x 10^7/assay) were incubated for one hour at 37°C in 1.0 ml of KRPS, pH 7.4, containing 1.0 μCi of ^{14}C-labelled formate, diluted with non-isotopic carrier to 2 mM. Glucose was present in all assays at a level of 5 mM, whereas galactose or potassium cyanide were included where appropriate at 30 mM or 5 mM levels, respectively. Reactions were stopped by introducing 0.2 ml of 7.3 N H_2SO_4 to each cell suspension, and evolved CO_2 was collected in plastic centerwells on 1 x 3 cm folded strips of KOH-saturated Whatman 3MM paper. Liberated CO_2 was absorbed from the start of each experiment and for one hour after acidifying the medium. Centerwells were directly transferred to scintillation vials containing 10 ml of Bray's solution and counted. Each assay was performed in triplicate on cells pooled from at least two guinea pigs.

Cellular oxygen consumption. Rates of oxygen consumption by resting and phagocytosing PMN (0.50 x 10^7 to 0.97 x 10^7/assay) were measured using a Yellow Springs Institute model 53 biological oxygen monitor with a Clark oxygen electrode. PMN were placed in siliconized glass chambers containing 3 ml of KRPS plus 10% guinea pig serum, and cells were preincubated with carbohydrate for 2 to 3 hrs at 37°C. Cell suspensions were then agitated with magnetic stirrers to

insure a continuous supply of dissolved oxygen, and the rate of consumption was recorded over a 20 min period. Monitoring was briefly interrupted for the introduction of polystyrene latex particles (200 particles/cell) and recording was resumed for another 20 min. Each experiment employed cells from one guinea pig and was repeated 3 to 4 times on subsequent days. Since rates of consumption were recorded in µl/hr/cell number, the general gas law was applied to calculate the actual concentrations of oxygen consumed under these conditions.

Methional assay for hydroxyl radicals. Interactions between carbohydrates and hydroxyl radicals were determined by monitoring the inhibition of ethylene production from the hydroxyl radical dependent degradation of methional (20). Hydroxyl radicals were generated either enzymatically as a result of the xanthine oxidase reaction or non-enzymatically by a Fenton-type hydroxylating system without ascorbic acid. The former system included 1.0 mM methional, 0.2 mM xanthine, 0.1 mM EDTA, and 150 mU xanthine oxidase in 1.0 ml of 50 mM potassium phosphate buffer, pH 7.8. Whereas, the latter system contained 1.0 mM methional and 1.0 mM H_2O_2 in 1.0 ml of the same buffer. Addition of ferrous iron was not necessary in the latter system since iron levels in phosphate buffer were sufficient to promote univalent peroxide reduction in the presence of dissolved oxygen. All reactions were performed at room temperature in 25 ml

siliconized Erlenmeyer flasks fitted with gas-tight stoppers. Gas above each reaction mixture was sampled with a gas-tight syringe and was analyzed for ethylene by injecting 1.0 ml aliquots into a Hewlett Packard model 440 gas chromatograph equipped with a 6 foot glass column of Chromosorb 102. Column temperature and flame ionization detector temperature were maintained at 90°C and 220°C, respectively. Nanomoles of ethylene present in a flask at any given time (t) were calculated using a computer program based upon the following equation:

$$\text{nmoles}_t = (R_s/R_{std}) \times (V_2/V_1) + \text{nmoles}_{t-1}$$

where R_s = detector response with sample (cm)

R_{std} = detector response with ethylene standard (cm/nmole)

V_2 = volume of flask (ml)

V_1 = volume of injection (ml)

In separate experiments, ethylene production from 1.0 mM methional was also investigated using cell suspensions of PMN (2.43×10^7/assay) and of macrophage (1.41×10^7/assay) in 1.0 ml volumes of KRPS. These reactions were performed at 37°C, and a number of controls were run simultaneously. Controls included: buffer alone, buffer plus polystyrene latex particles, and buffer plus killed cells. Cells were killed both by heating to 90°C for 15 min and by a repetitive freeze-thaw procedure.

Oxidation of hexoses by hydroxyl radicals. Products of the reaction between carbohydrates and hydroxyl radicals were generated by incubating various levels of glucose or galactose in a Fenton-type hydroxylating system without ascorbate. Reaction solutions of 1.0 ml contained: 5 mM $FeSO_4$, 15 mM EDTA, 5 mM H_2O_2, and 1 to 30 mM carbohydrate in 40 mM potassium phosphate buffer, pH 7.4. Reactions were initiated by adding hydrogen peroxide and were judged as complete when similar solutions without carobhydrate but containing 5 mM aniline gave an opaque dark-brown appearance. All solutions were incubated at room temperature and after completion were dried under N_2 gas.

Products were analyzed by gas chromatography and by ascending paper chromatography on Whatman 3MM paper using a 7:3 (v/v) ethanol: 1 M ammonium acetate solution as developer. Gas chromatography was performed on the trimethylsilyl derivatives of products using a Hewlett Packard model 5830A gas chromatograph equipped with OV-1 and XE-60 columns. Standards included the trimethylsilyl derivatives of each hexose, each corresponding hexonic acid (in both open and closed ring forms), each hexuronic acid, and a variety of pentoses and disaccharides. Derivatives were prepared as previously described (21).

Hexonic acid production by PMN was similarly investigated by incubating cells pooled from 5 guinea pigs (3.84×10^8/assay) in 1.11 ml of KRPS containing either 5.0 mM glucose or 5.0 mM glucose with 30 mM galactose.

Incubations were run for 6 hr at 37°C and were terminated by adding one volume of 30% trichloroacetic acid while vortexing. Protein was removed by centrifugation at 20,000 xg for 15 min, and supernates were extracted 4 times with 3 volumes of diethyl ether. Neutralized supernates were then dried under N_2 and derivatized for gas chromatography. Standards were similarly treated with trichloroacetic acid.

In order to determine whether hydroxyl radicals could contribute to the marked increase in glucose C-1 oxidation observed during phagocytosis, glucose and galactose oxidation experiments as described in Chapter III were repeated but in the presence of free radical scavengers. These included 0.2 mg/ml superoxide dismutase, 0.3 mg/ml cytochrome c, 0.3 mg/ml catalase, and 30 mM sucrose.

Polyol formation by PMN. Production of sorbitol and galactitol by PMN was investigated by incubating cells pooled from 3 guinea pigs in 1 ml aliquots of KRPS containing 30 mM glucose or 30 mM galactose. After incubating cells (1.52×10^7/assay) for varying lengths of time at 37°C, cell suspensions were centrifuged at 3000 xg for 1 min, and the extracellular media was discarded. Pellets of cells were immediately washed with fresh medium in the absence of carbohydrate and frozen with liquid N_2. PMN were stored at -80°C until homogenizing in the presence of 3 N perchloric acid (see Chapter I, Metabolite assays).

Quantitation of polyol levels in cell homogenates was performed by gas chromatography of the trimethylsilyl derivatives. Each determination was run at least in duplicate, and similar experiments were performed on cells incubated without carbohydrate, with 5 mM glucose, or with 30 mM galactose plus polystyrene latex particles.

PMN chemiluminescence. Chemiluminescence was monitored with a Beckman CPM-100 liquid scintillation spectrometer operated in the out-of-coincidence summation mode. The instrument was used at room temperature with an open window setting and at 100% gain.

PMN (3.98×10^7/assay) were pooled from four guinea pigs and were incubated in siliconized glass scintillation vials containing 6.0 ml of KRPS. Cells were kept in the dark for eight hours at 37°C, and were stimulated by adding a 20 fold (bacteria: PMN) excess of heat-killed E. coli in 0.4 ml of autologous guinea pig serum. Chemiluminescence was monitored every 12 seconds for 2 min before and 4 min after stimulation of cells. Bacteria were grown and heat-killed as described in Chapter I. In separate experiments, superoxide dismutase and triethylenediamine were included at 0.2 mg/ml and 0.1 mM, respectively.

Results

Extracellular reduction of cytochrome c. Effects of carbohydrate, superoxide dismutase and catalase upon the reduction of cytochrome c by PMN (Table I) can be enumerated as follows: 1) reduction was more pronounced when phagocytosing rather than resting cells were employed, 2) reduction was enhanced by pre-incubating cells with 5.0 mM glucose, 3) reduction was sensitive to superoxide dismutase but not to catalase, 4) reduction was impaired by pre-incubating cells with 30 mM glucose or 30 mM galactose, 5) reduction was also impaired by galactose when added after the pre-incubation period, and 6) the inhibitory action of galactose did not occur in the presence of catalase. The former three effects indicated that cytochrome reduction was dependent upon superoxide anion release from PMN, whereas the latter three effects suggested that a catalase-sensitive inhibition of cytochrome reduction occurred in the presence of 30 mM carbohydrate. This catalase-sensitive impairment could be explained on the basis of extracellular cytochrome re-oxidation 1) if production of an oxidizing agent, such as hydrogen peroxide, was enhanced by elevated carbohydrate or 2) if other oxidizing agents, such as hydroxyl radical or a longer-lived carbohydrate radical, were formed. To test these suggestions, we undertook further experiments to determine whether elevated levels of galactose affected hydrogen peroxide generation or oxygen consumption by PMN.

Cellular reduction of nitroblue tetrazolium. Further evidence that elevated levels of carbohydrate affected free-radical type reactions in PMN was obtained by monitoring the reduction of nitroblue tetrazolium. A time-course study (Figure 1) showed that dye reduction was independent of pre-incubation time when phagocytosing cells were kept in KRPS with 5.0 mM glucose. However, when these cells were exposed to either 30 mM glucose or 5.0 mM glucose plus 30 mM galactose, the reduction of dye was impaired after 1 hr of pre-incubation. Data in Figure 2 demonstrated that nitroblue tetrazolium reduction was stimulated during phagocytosis and that impairment of this reduction was dependent upon the extracellular galactose concentration.

In separate experiments without cells (Table II), neither the superoxide-dependent reduction of cytochrome c nor the reduction of nitroblue tetrazolium were impaired by elevated levels of carbohydrate. These observations indicated that carbohydrate could not directly interact with cellular superoxide. However, it was possible that carbohydrate could lower cellular superoxide production or that other free radicals, if formed, could interact with superoxide anion.

Cellular release of hydrogen peroxide. As measured by the oxidation of scopoletin, levels of peroxide released from phagocytosing PMN were approximately 2 fold greater than those released from resting PMN (Table III). Catalase,

when added to these cell suspensions, substantially lowered the oxidation of scopoletin by 82% and 66%, respectively. Release of hydrogen peroxide from PMN was not significantly affected by external carbohydrate. However, slight increases in peroxide formation (up to 137% of the control) were found when resting PMN were incubated with 30 mM glucose or 30 mM galactose.

Cellular formate oxidation. Intracellular steady-state levels of hydrogen peroxide were estimated by observing the oxidation of ^{14}C-labelled formate by endogenous catalase operating in the peroxidatic mode. Potassium cyanide, which should have completely inhibited this reaction, was found to decrease the evolution of radioactivity by only 46%. This suggested that labelled formate was evaporating during the experiment and that values obtained in the presence of cyanide should be considered as background. After correcting for this background, levels of $^{14}CO_2$ produced by PMN incubated in 5.0 mM glucose were 1.9 fold greater than those produced by PMN incubated in 5.0 mM glucose plus 30 mM galactose. The difference between these values, however, was not statistically significant (Table IV).

Cellular oxygen consumption. As indicated in Table V, rates of oxygen consumption by PMN were stimulated 2 to 11 fold during phagocytosis of polystyrene latex particles. Significant differences in these rates were not found,

however, to result from the incubation of either resting or phagocytosing cells in medium containing elevated levels of carbohydrate.

Methional assay for hydroxyl radicals. The production of ethylene from methional during the enzymatic conversion of xanthine to urate (Figure 4) indicated that hydroxyl radical was produced. Since both hydrogen peroxide and superoxide arise from the xanthine oxidase reaction (22), hydroxyl radical was probably formed secondarily by a Haber Weiss reaction as follows:

$$O_2^{\cdot} + H_2O_2 \longrightarrow OH^- + OH^{\cdot} + {}^1O_2 \qquad \text{(eq. 1)}$$

Hydroxyl radical then interacted with methional to produce ethylene. However, in the presence of polyol the formation of ethylene was impaired. Since polyol did not interfere with the xanthine oxidase reaction (Table II), these data indicated that galactitol, mannitol and sorbitol were effectively scavenging hydroxyl radical. Data in Figure 4 were obtained from one representative experiment in which all four reactions were run simultaneously. Subsequent experiments gave similar results for polyol inhibition, but the overall rates of ethylene production were lower. This resulted from the rapid decay in xanthine oxidase activity with time.

To avoid such variations in the rate of hydroxyl radical generation, a simple non-enzymatic system was

constructed by adding 1.0 mM H_2O_2 and 1.0 mM methional to 50 mM potassium phosphate buffer. This cyclic system (Figure 5) depended upon the following three reactions:

$$H_2O_2 + Fe^{+2} \longrightarrow OH^- + OH\cdot + Fe^{+3} \qquad \text{(eq. 2)}$$

$$H_2O_2 + OH\cdot \longrightarrow OH^- + O_2^{\cdot} + 2H \qquad \text{(eq. 3)}$$

$$O_2^{\cdot} + Fe^{+3} \longrightarrow {}^1O_2 + Fe^{+2} \qquad \text{(eq. 4)}$$

Ethylene production from the hydroxyl radical dependent degradation of methional was thus stimulated by adding more H_2O_2 or ferrous iron and was inhibited by adding superoxide dismutase or benzoate. Ethylene formation was also impaired by including 40 mM levels of polyol, hexose or myo-inositol. Each point in Figure 5 was obtained by averaging values from 4 to 6 determinations, and all differences between average control values and values obtained with inhibitors were significant at the $P < 0.01$ level after 10 min of reaction.

In separate experiments, ethylene formation from methional was also monitored when suspensions of PMN or macrophage were incubated with 1.0 ml of KRPS. Although ethylene was produced from these cell suspensions, rates ofethylene production were lower than those obtained with the controls (data not shown). Controls included: buffer alone, buffer plus polystyrene latex particles, and buffer plus heat-killed or freeze-thaw-killed cells. These results suggested either that hydroxyl radical was not

cellularly produced or that cellular components scavenged this radical, along with exogenous radical, before its interaction with methional. Moreover, additions of 10% guinea pig serum or 0.2 mg/ml hemoglobin were found to stimulate ethylene formation. These observations indicated that heme-iron contributed by these agents, and perhaps by killed cells, was promoting the formation of hydroxyl radical as in equation 2.

Oxidation of hexoses by hydroxyl radicals. Hydroxyl radicals were generated by a Fenton-type hydroxylating system without ascorbate but in the presence of 1.0 mM glucose or galactose. Products were separated from free hexose by paper chromatography and were trimethylsilylated after elution from paper. Gas-chromatographic analyses of these derivatized products are illustrated in Figures 6A and 6C, respectively, whereas analyses of derivatized standards of gluconate and galactonate are shown in Figures 6B and 6D, respectively. No other major reaction products were found, and hexonic acids were only formed when both hydrogen peroxide and ferrous sulfate were included in the reaction solution. Similar results were later found by substituting oxy-hemoglobin for ferrous sulfate and by extracting the reaction solutions with 15% trichloracetic acid prior to trimethylsilylation.

Hexonic acid production by PMN was similarly investigated when concentrated cell suspensions (3.84×10^8/assay)

were incubated for 6 hr with KRPS containing either 5.0 mM glucose or 5.0 mM glucose plus 30 mM galactose. As with previous experiments employing resting and phagocytosing cells (1.0 x 10^7/assay), hexonic acid was not detected. This observation indicated either that production of hexonate by PMN was extremely low (< 0.1 nmole/hr/10^7 cells) or that hexonate was metabolized rapidly after its formation.

In order to determine whether hydroxyl radicals contributed to the marked increase in hexose C-1 oxidation observed during phagocytosis (Chapter III), phagocytosing PMN were incubated in KRPS containing free-radical scavengers and either [1-^{14}C]glucose or [1-^{14}C]galactose. Oxidation of these labelled carbohydrates to $^{14}CO_2$ was not affected by the presence of superoxide dismutase, catalase, cytochrome c or sucrose (data not shown).

Polyol formation in PMN. The presence of galactitol in extracts of PMN, after 1 hr of incubation with 30 mM galactose, was demonstrated by gas chromatography. An analysis of derivatized sugars from these PMN (Figure 7B) showed the presence of galactitol, galactose and glucose (peaks 2, 3 and 4, respectively). Methylmannoside (peak 1) was added to this sample and to a standard galactitol solution (Figure 7A) to facilitate quantitation. Under galactosemic conditions, the intracellular levels of galactitol accumulated rapidly to 3 mM (Figure 8), whereas under

hyperglycemic conditions, the levels of sorbitol rose gradually to 1 mM. These levels were calculated by dividing nmoles polyol per 10^6 cells by the intracellular water space of PMN used in Chapter III. Levels of galactitol were increased at least 2 fold during phagocytosis.

PMN Chemiluminescence. After addition of heat-killed E. coli to PMN suspensions, chemiluminescence reached a maximum by 30 to 45 seconds (Figure 9). The amplitude of this response was enhanced by pre-incubating cells with 20 mM or 30 mM galactose. However, such pre-incubation did not appear to affect the rate of decay in light emission. In other experiments, superoxide dismutase was found to inhibit PMN chemiluminescence by as much as 50%, whereas the singlet oxygen trapper, triethylenediamine, was without effect.

Discussion

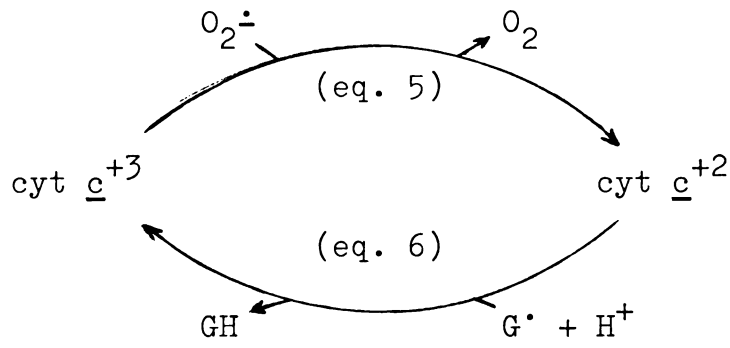
Although production of superoxide anion by PMN has been demonstrated by Babior et al. (9) and Drath et al. (10), these studies were performed under different conditions without regard to the extracellular levels of carbohydrate. The former investigators measured cytochrome c reduction by superoxide anion when cells were incubated in Hank's balanced salt solution containing 10 mM glucose, whereas the latter investigators utilized the same isotonic buffer with 5 mM glucose and 10% serum. Our system (Table

I) employed KRPS containing various levels of carbohydrate and 10% guinea pig serum, and the values obtained from this system, when PMN were incubated with 5 mM glucose (3.41 ± 0.31 nmole $O_2^{\bullet}$/hr/10^7 cells), agreed with those found by the previous investigators. Our results similarly demonstrated that cytochrome reduction was stimulated during phagocytosis and that reduction was inhibited by superoxide dismutase. We also concurred with the observation of Drath et al.(10) that the presence of serum substantially increased apparent superoxide anion production (data not shown). This increase did not result from the 0.6 mM glucose contributed by serum (see Appendix).

Our results in Table I further demonstrated that cytochrome reduction was lower when PMN were pre-incubated in the absence of glucose or in the presence of excess glucose or galactose (> 15 mM). The former observation indicated that some glucose may be necessary for maintenance of intracellular reducing equivalents through the hexose-monophosphate shunt and substantiated the results of Iyer et al. (8) that intracellular peroxide production was decreased under these conditions. The latter observation, however, could not be accounted for by decreased hexose-monophosphate shunt activity since elevated levels of carbohydrate did not significantly affect glucose C-1 oxidation (Chapter I).

Moreover, the inhibitory effect of galactose upon cytochrome c reduction did not require pre-incubation of cells

with hexose, and this impairment was not observed in the presence of catalase. Thus, galactose acted predominantely upon the cytochrome c assay system, and its reaction depended upon the presence of extracellular hydrogen peroxide. This impairment could be explained on the basis of the following model in which cytochrome c was reduced by superoxide anion and reoxidized by carbohydrate radical (G·):



Since PMN are known to produce $O_2^{\cdot-}$ and H_2O_2, it has been proposed (4,11) that hydroxyl radical is formed via the Haber Weiss reaction (eq. 1). Carbohydrates, such as glucose or galactose, could then interact with hydroxyl radical to form carbohydrate radical as follows:

$$GH + OH^{\cdot} \longrightarrow G^{\cdot} + H_2O \qquad \text{(eq. 7)}$$

This reaction has been observed with glucose (23), mannitol (20,24) and sucrose (25), and our results in Figures 4 and 5 demonstrate that a variety of hexoses and polyols are capable of this reaction. Data from pulse radiolysis studies have shown that complete scavenging of hydroxyl radical can occur with 5 mM glucose (26), and second-order

rate constants for reaction of glucose and sucrose with $OH^{\cdot}$ have been calculated to be 1×10^{10} M^{-1} sec^{-1} and 2.5×10^{9} M^{-1} sec^{-1}, respectively (23,25).

Like hydroxyl radical and mannitol radical (24), carbohydrate radicals could oxidize ferrocytochrome c (eq. 6) or react with superoxide anion via the following reaction:

$$G^{\cdot} + O_2^{\dot{-}} + H^{+} \longrightarrow GH + O_2 \qquad \text{(eq. 8)}$$

However, mannitol radical does not compete well with excess ferricytochrome c for reaction with superoxide anion (24), and thus in our system, carbohydrate radicals probably do not affect the reduction of cytochrome per se. Carbohydrate radicals could, however, as in the phagolysosome scavenge both hydroxyl radical (eq. 7) and superoxide anion (eq. 8).

A major point in this discussion is that catalase did not affect the cytochrome c reduction assay when PMN were incubated with 5 mM glucose. This observation has been previously observed (9) and can be explained on the basis of ferricytochrome c and carbohydrate concentrations. As mentioned earlier, leukocyte H_2O_2 may be formed by the non-enzymatic dismutation of superoxide anion (4,27):

$$2O_2^{\dot{-}} + 2H^{+} \longrightarrow H_2O_2 + {}^{1}O_2 \qquad \text{(eq. 9)}$$

However, at high concentrations of ferricytochrome c, reduction of extracellular cytochrome occurs (eq. 5), and

the above reaction (eq. 9) is competitively inhibited. As superoxide anion is formed, levels of ferricytochrome c decrease, and the production of hydrogen peroxide becomes favored. Although peroxide concentrations may then be adequate to promote hydroxyl radical formation (eq. 1), 5 mM levels of carbohydrate may be insufficient to compete with other agents for reaction with $OH^{\cdot}$. Such agents could be contributed by serum, and this competition could be overcome by increasing the carbohydrate concentration.

That the above model (eq. 5 and eq. 6) operated extracellularly was supported by the failure of 30 mM galactose to significantly affect either hydrogen peroxide generation (Table III) or oxygen consumption (Table V). These observations suggested that extracellular levels of superoxide anion were not impaired and that the action of carbohydrate upon this system must be through cytochrome c reoxidation. These results also showed that increases in H_2O_2 generation could not account for this effect. Rates of extracellular H_2O_2 production (Table III) were in the range of previously reported values (6,7), and rates of oxygen consumption (Table V) were comparable to those reported by Root et al. (7). Both parameters were similarly stimulated by phagocytosis.

Significant decreases in nitroblue tetrazolium reduction, when phagocytosing PMN were incubated with 30 mM glucose or galactose (Figure 1), suggested that hexose or polyol accumulated intracellularly and that this accumulation

promoted carbohydrate radical formation (eq. 7). Carbohydrate radical could have then competed (eq. 8) with nitroblue tetrazolium for reduction by superoxide anion. These suggestions were supported by 1) the effect of increasing galactose concentrations upon intracellular nitroblue tetrazolium reduction (Figure 2), 2) the detection of polyol accumulation within PMN (Figures 7 and 8), and 3) the failure of carbohydrate alone to react with superoxide anion (Table II).

Nevertheless, a certain amount of caution must be expressed regarding this interpretation since the results in Figures 1 and 2 could also be explained by decreased cell permeability to nitroblue tetrazolium and since it is not clear whether all nitroblue tetrazolium reduction by PMN is superoxide-dependent. Although formazin formation can be inhibited by superoxide dismutase in strictly chemical (28) and enzymatic systems (29), some evidence has been presented that superoxide dismutase does not completely inhibit cellular dye reduction (4,30) and that this reduction is also inhibited by a variety of other proteins (31). However, formazan accumulation is stimulated by phagocytosis (32) and is impaired in leukocytes from patients with chronic granulomatous disease (4), a dysfunction of NADH-oxidase activity (33,34).

Our results obtained from studies on cellular formate oxidation (Table IV) and PMN chemiluminescence (Figure 9) were also consistent with the above model. Although rates

of ^{14}C-labelled formate oxidation were not significantly decreased by incubating cells in 30 mM galactose, the lack of significance could largely be attributed to variations resulting from labelled formate evaporation. Similar problems were encountered by Iyer et al. (8). Average rates of formate oxidation, which depended upon the reaction of H_2O_2 with catalase operating in the peroxidatic mode (35), were lower in the presence of 30 mM galactose. Thus, steady-state levels of H_2O_2 were probably decreased intracellularly. This could be accounted for by carbohydrate radical formation within the phagolysosome (eq. 7), and its subsequent reaction with superoxide anion (eq. 8) to compete with further H_2O_2 production (eq. 9). Briggs et al. have recently shown, using a histochemical method for H_2O_2 localization, that H_2O_2 was formed via NADH-oxidase at the external plasma membrane and that this activity was partially internalized during phagocytosis (30). Therefore, the intercellular reaction between carbohydrate radical and superoxide anion (eq. 8) would not necessarily affect extracellular superoxide or peroxide levels. This agreed with our data on extracellular peroxide formation (Table III) and indicated that internal hydrogen peroxide was not transferred across the plasma membrane. Such a transfer would not be probable because of the high cellular content of catalase and myeloperoxidase (36).

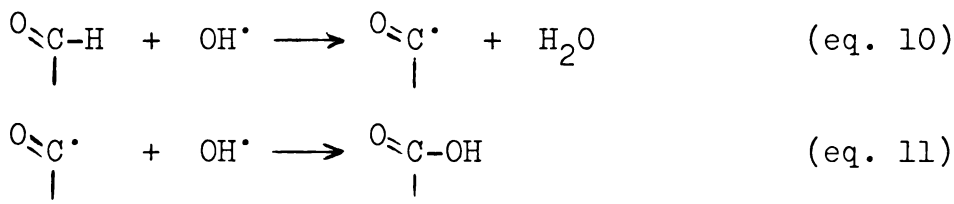
Chemiluminescence of phagocytosing PMN (Figure 9) was enhanced by pre-incubating cells with 20 mM or 30 mM

galactose. This effect indicated an interaction between galactose and free radicals which resulted in light emission from either excited carbohydrate molecules or some other meta-stable intermediate. Chemiluminescence of PMN has been associated with the production of singlet oxygen by a number of reactions (4,12,13). These have included the Haber Weiss reaction (eq. 1), the non-enzymatic dismutation of superoxide anion (eq. 9), the reduction of H_2O_2 by hypochlorite and myeloperoxidase (12,27), and the reaction between hydroxyl radical and superoxide anion (37). Since the latter reaction is analogous to (eq. 8), increased chemiluminescence could be interpreted as indicating the occurrence of this reaction when cells are incubated with galactose. Benzoate, which scavenges hydroxyl radical, as well as superoxide dismutase and catalase have been shown to partially inhibit PMN chemiluminescence (37). Our results also demonstrated this inhibition with superoxide dismutase; however, the singlet oxygen trapper, triethylenediamine (1,4-diazobicyclo-2,2,2-octane)(38), was without effect. This observation did not support the above interpretation in regard to the source of chemiluminescence with galactose, and this suggested that excited carbohydrate molecules may be the light emitting species. Allen et al. have also suggested that excited carbonyls promote this reaction (39).

Excess levels of glucose did not enhance chemiluminescence by PMN in our studies. This could be a result of the high rate of glycolysis (1) and thus low levels of glucose

accumulation (40). In addition, reduction of glucose to sorbitol was at least 3 fold less than the reduction of galactose to galactitol when comparable levels of galactose were present (Figure 8).

A variety of hexoses and polyols competed with methional for reaction with hydroxyl radical (Figures 4 and 5). These observations indicated that the resulting carbohydrate radicals were not as powerful in oxidizing potential as the hydroxyl radical which they replaced. Nevertheless, these radicals were capable of oxidizing ferrocytochrome c and superoxide anion, as previously discussed, and could undergo further oxidation by reacting with a second hydroxyl radical. The latter reaction was observed by incubating glucose and galactose in a Fenton-type hydroxylating system (Figure 6). Since the products of this reaction were gluconic and galactonic acids, respectively, the hydroxyl radical must have abstracted a hydrogen from the hexose C-1 position (eq. 10) and secondarily combined with the carbohydrate radical (eq. 11):



(eq. 10)

(eq. 11)

The formation of gluconic acid in this manner has been previously observed upon pulse radiolysis and γ-irradiation of glucose solutions (26). However, to our knowledge, this was the first observation of hexonic acid production

from a Fenton-type hydroxylating system.

As evident from our 20% yield of hexonic acid from 1 mM hexose, levels of hydroxyl radical in this system approached 10^{-4}M. However, steady-state levels of hydroxyl radical in PMN are probably at least 2 orders of magnitude less at any given period of time. Thus, even though carbohydrate radicals may be formed in PMN, the further oxidation of these radicals to hexonic acid is less likely to occur. Side reactions as proposed by McCord et al. for the dismutation of mannitol radical (24) may also be favored, and in this case only minute quantities of hexonic acid would be formed. These levels of hexonic acid may evade detection by gas chromatography.

In conclusion, carbohydrate radicals have been demonstrated in two hydroxyl radical generating systems. One of these systems produced non-biological levels of this radical, while the other produced low steady-state levels of hydroxyl radical comparable to levels associated with phagocytosing PMN. Hexonic acid formation from hexose was found in the former system, whereas carbohydrate radical formation was followed in the latter system by monitoring inhibition of the hydroxyl radical-dependent degradation of methional. These results suggested that carbohydrate radicals were formed by PMN, and indeed, most of the effects of 30 mM galactose upon parameters of the oxygen-dependent killing mechanism could be explained on this basis.

As demonstrated in Chapter I, 30 mM galactose impaired

phagocytosis of ^{32}P-labelled E. coli by 24% in guinea pig PMN. However, the killing of E. coli was almost completely inhibited (< 1% activity) under these conditions. Since this bacterium is greatly affected by the oxygen-dependent killing mechanism of PMN (27), the impaired bactericidal activity could be accounted for by the reaction of carbohydrate radicals with superoxide anion (eq. 8) within the phagolysosome. This suggestion is supported by our data on cellular nitroblue tetrazolium reduction, formate oxidation, and chemiluminescence.

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Table I. Carbohydrate effect on cytochrome c reduction by PMN[a]

Additions prior to pre-incubation		Additions with cytochrome c[b]	nmoles cytochrome c reduced/ hour/10^7 cells ± S.D.	
Glucose (mM)	Galactose (mM)		Resting	Phagocytosing[c]
0	0		2.21 ± 0.49	3.55 ± 1.50
5.0	0		3.41 ± 0.31	10.0 ± 2.22
15.0	0			8.03 ± 0.29
30.0	0		0.69 ± 0.46[d]	6.55 ± 4.81
5.0	15.0			2.95 ± 1.06[d]
5.0	30.0		0.15 ± 0.29[d]	1.22 ± 1.46[d]
5.0	0	SOD	1.48 ± 0.49[d]	3.88 ± 2.92[d]
5.0	0	Catalase	4.53 ± 0.95	10.1 ± 2.13
5.0	30.0	Catalase	4.76 ± 1.38[d]	
5.0	0	Galactose	0.08 ± 0.54[d]	2.03 ± 2.20[d]

[a]PMN (0.88 x 10^7 to 1.06 x 10^7/assay) where pre-incubated at 37°C for 2 hr in 1.0 ml of Krebs-Ringer phosphate solution, pH 7.4, with 10% guinea pig serum. Cell suspensions were then diluted with 1 volume of ferricytochrome c (132 µM) and divided into two 1.0 ml aliquots. One aliquot was placed on ice to serve as a reaction blank, while the other was incubated for an additional hour at 37°C. Reduced cytochrome in cell supernates was monitored at 550 nm. All assays were performed in quadruplicate on cells pooled from at least two guinea pigs. S.D. indicates standard deviation.

Table I (Continued)

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[b]Superoxide dismutase (SOD), catalase, and galactose were present at 0.2 mg/ml, 0.3 mg/ml, and 30 mM, respectively.

[c]Phagocytosing cells received a 50 fold excess of polystyrene latex particles.

[d]Significantly different ($P < 0.05$) from values with cells in 5mM glucose.

Table II. Effect of carbohydrate on reactions involving superoxide anion radical[a]

Addition	Cytochrome c reduced[b] nmoles/min	Nitroblue tetrazolium reduction[c] $A_{517\ nm}$/min x 10^2
None	1.49 ± 0.07	0.95 ± 0.05
Galactose	1.61 ± 0.21	1.08 ± 0.05
Galactitol	1.52 ± 0.15	0.96 ± 0.06
Sorbitol	1.55 ± 0.18	1.05 ± 0.06
Mannitol	1.55 ± 0.18	0.99 ± 0.06
Xylitol	1.57 ± 0.14	

[a]Each reaction was performed 6 to 8 times using a Gilford model 3500 spectrophotometer in the general kinetic-1 program mode. All values represent average slopes ± standard deviation. All carbohydrate levels were 27 nM.

[b]Superoxide was generated enzymatically in 1.1 ml of 0.05 M sodium phosphate buffer, pH 7.8, containing: 50 μM xanthine, 100 μM EDTA, 10 μM ferricytochrome c, and 3.2 mU xanthine oxidase.

[c]Superoxide was generated non-enzymatically in 1.1 ml of Krebs-Ringer phosphate solution, pH 7.4, containing: 78 μM NADH, 23 μM phenazine methosulfate, and 50 μM nitroblue tetrazolium.

Table III. Effect of carbohydrate on hydrogen peroxide formation by PMN[a]

Additions			nmoles H_2O_2 produced/hour/10^7 cells ± S.D.	
Glucose (mM)	Galactose (mM)	Other	Resting	Phagocytosing[b]
0	0		4.32 ± 1.16	
5.0	0		4.61 ± 0.88	9.21 ± 0.19
30.0	0		6.22 ± 1.17	9.53 ± 0.54
5.0	30.0		5.35 ± 1.39	9.01 ± 0.75
5.0	0	Catalase[c]	1.54 ± 1.09	1.61 ± 1.36

[a]PMN (0.50 x 10^7 to 1.23 x 10^7/assay) were incubated for 3 hr in Krebs-Ringer phosphate solution, pH 7.4, at 37°C. Cells were contained in 0.5 ml dialysis bags and dialyzed against 2.5 ml of the same buffer during incubation. Peroxide levels were determined in dialysates by measuring the oxidation of scopoletin by peroxidase. Each value is a mean of quadruplicate determinations performed on cells from four guinea pigs. S.D. indicates standard deviation.

[b]Phagocytosing cells were exposed to a 200 fold (particle: cell) excess of polystyrene latex particles.

[c]Catalase was present at 0.3 mg/ml.

Table IV. Carbohydrate effect on ^{14}C-formate oxidation by PMN[a]

Additions		cpm released ± S.D.	nmoles $^{14}CO_2$ $hr^{-1}/10^7$ cells
Glucose (mM)	Galactose (mM)		
5.0	0	18.2 ± 1.47	7.6
5.0	30.0	14.4 ± 1.57	4.1
5.0[b]	0	9.86 ± 1.11[c]	0.0

[a]PMN (2.07×10^7/assay) were incubated for 1 hr at 37°C in 1.0 ml of KRPS, pH 7,4, containing 1.0 μCi ^{14}C-formate. Each assay was performed in triplicate on cells pooled from two guinea pigs. S.D. refers to standard deviation.

[b]KCN was present at 5 mM.

[c]Significantly different ($P < 0.005$) from values obtained with cells incubated with 5 mM glucose.

Table V. Effect of carbohydrate on oxygen consumption by PMN[a]

Carbohydrate (mM)		nmoles O_2 consumed/hour/10^7 cells ± S.D.	
Glucose	Galactose	Resting	Phagocytosing[b]
5.0	0	127 ± 45.2	701 ± 453
5.0	10.0	80.4± 24.5	901 ± 454
5.0	20.0	136 ± 56.3	863 ± 379
5.0	30.0	114 ± 34.8	521 ± 268
30.0	0	156 ± 49.1	334[c]

[a]PMN were incubated from 2 to 3 hours at 37°C in Krebs-Ringer phosphate solution, pH 7.4, with 10% guinea pig serum. Oxygen consumption was measured using a Yellow Springs Institute model 53 biological oxygen monitor with a Clark oxygen electrode. Each experiment employed cells from one guinea pig, and each value is a mean of triplicate or quadruplicate experiments. S.D. refers to standard deviation.

[b]Phagocytosing cells were exposed to a 200 fold (particle: cell) excess of polystyrene latex particles.

[c]Single determination.

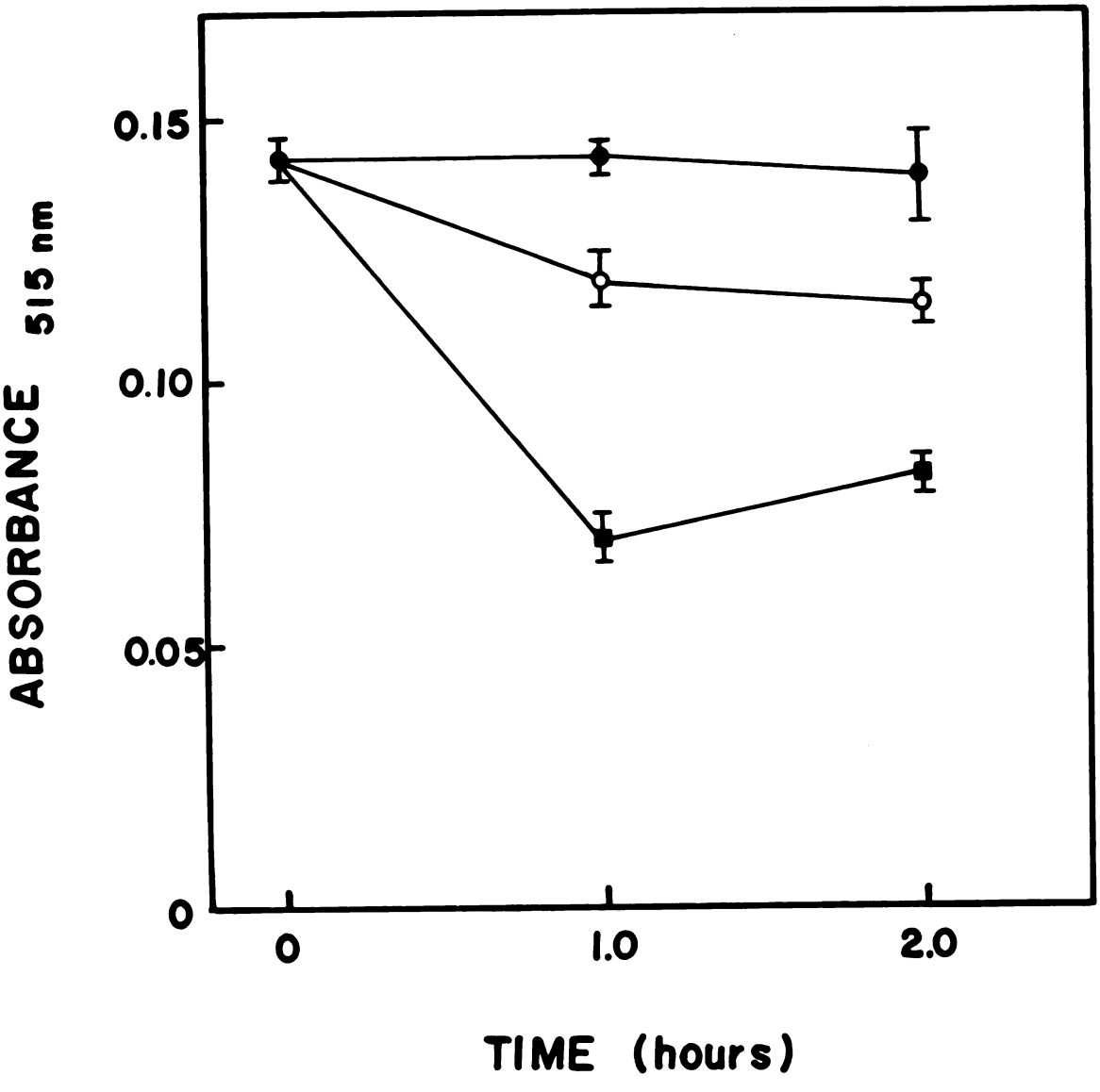

Figure 1. Effect of carbohydrate and pre-incubation time on nitroblue tetrazolium reduction by PMN.

PMN were incubated with 5.0 mM glucose (●), 30 mM glucose (o), or 30 mM galactose (■) for the indicated times, prior to assay for NBT reduction. All three groups were assayed at zero time.

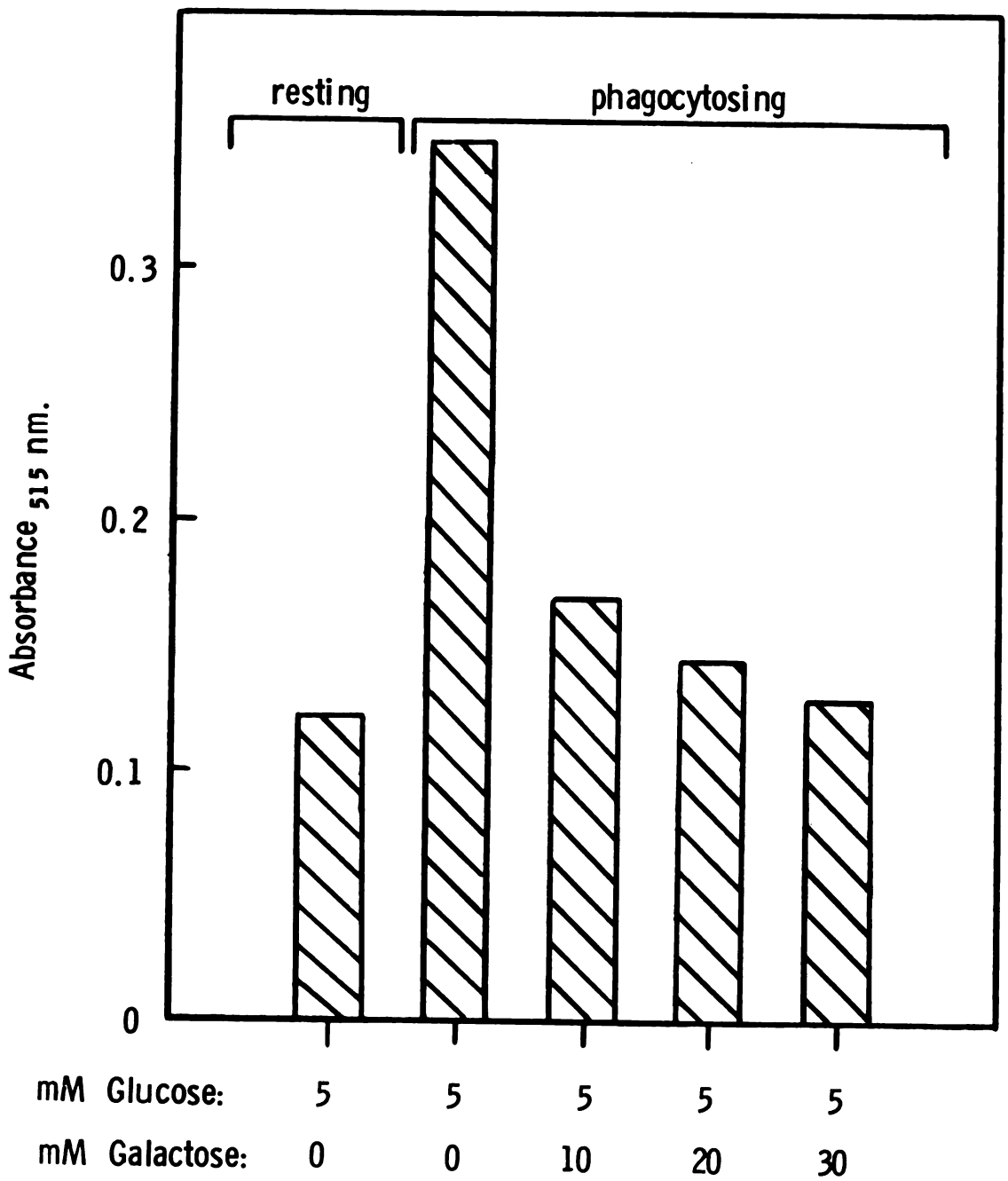

Figure 2. Effect of galactose concentration on nitroblue tetrazolium reduction by PMN.

Both resting and phagocytosing cells were incubated for 1 hr in the appropriate media prior to assay. Reduction is indicated by increased absorbance at 515 nm.

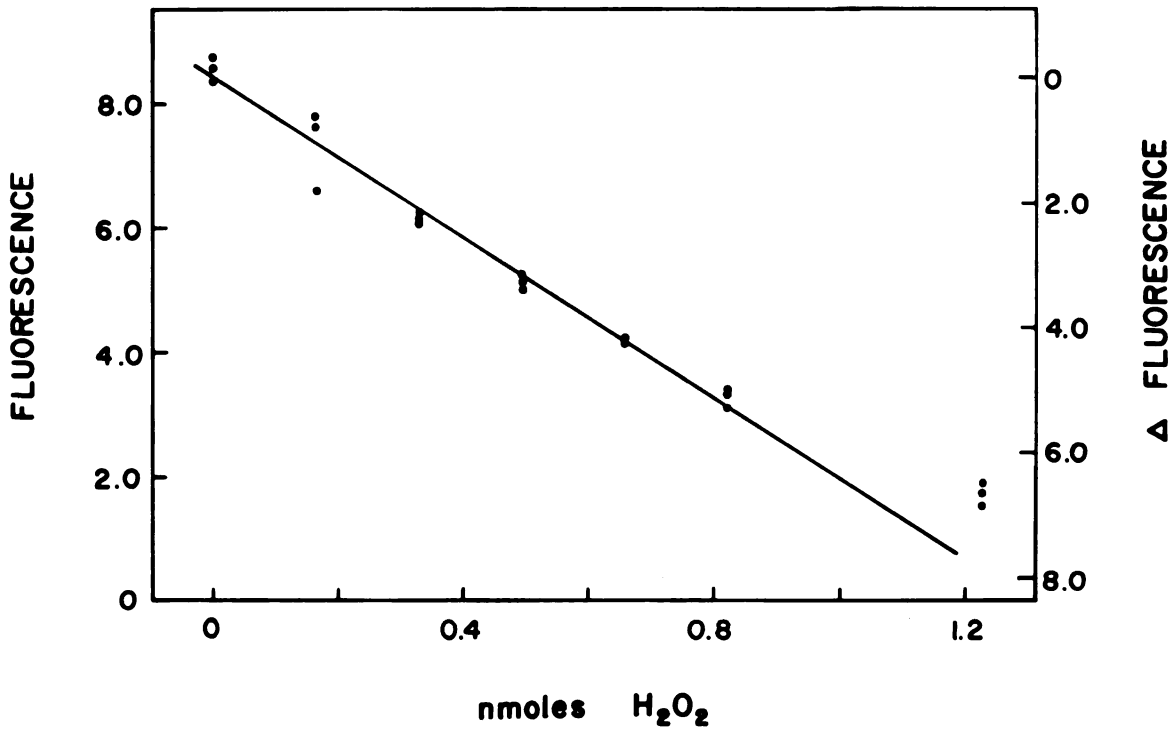

Figure 3. Relationship between scopoletin fluorescence and nanomoles of hydrogen peroxide.

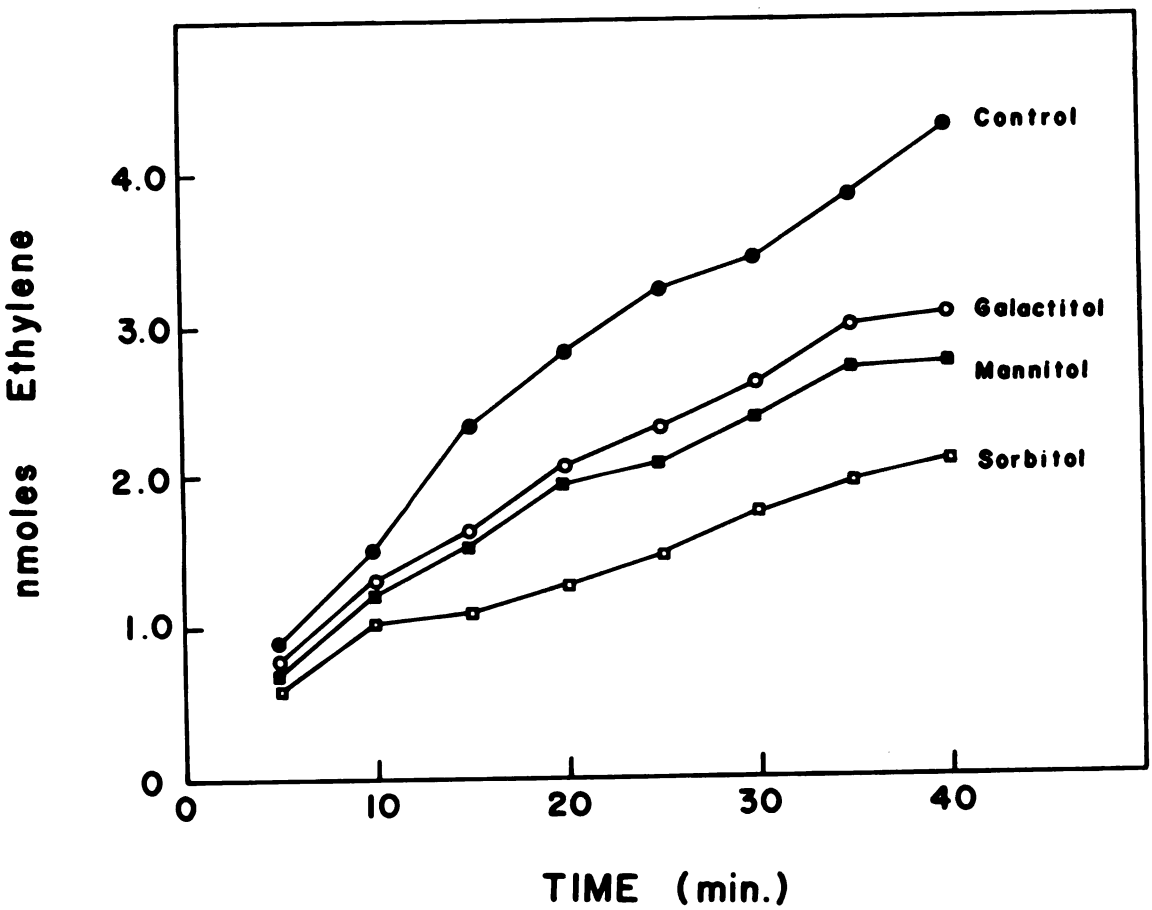

Figure 4. Effect of polyols on hydroxyl radical dependent ethylene formation from methional. Enzymatic generation of hydroxyl radicals.

All polyol levels were 40 mM.

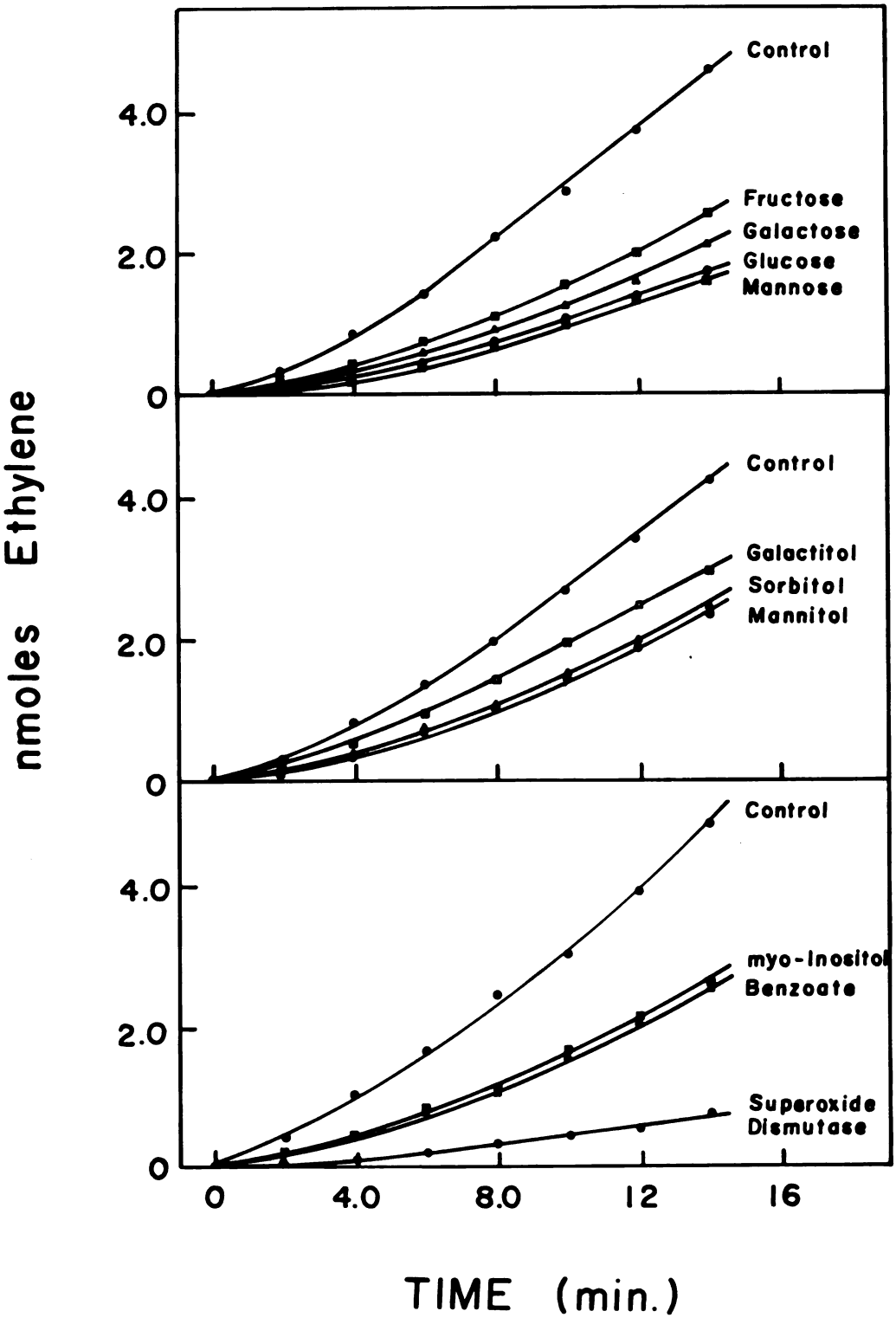

Figure 5. Effect of carbohydrates and polyols on the hydroxyl radical dependent formation of ethylene from methional. Non-enzymatic generation of hydroxyl radicals.

All carbohydrate and polyol levels were 40 mM. Benzoate and superoxide dismutase were 5 mM and 0.2 mg/ml, respectively.

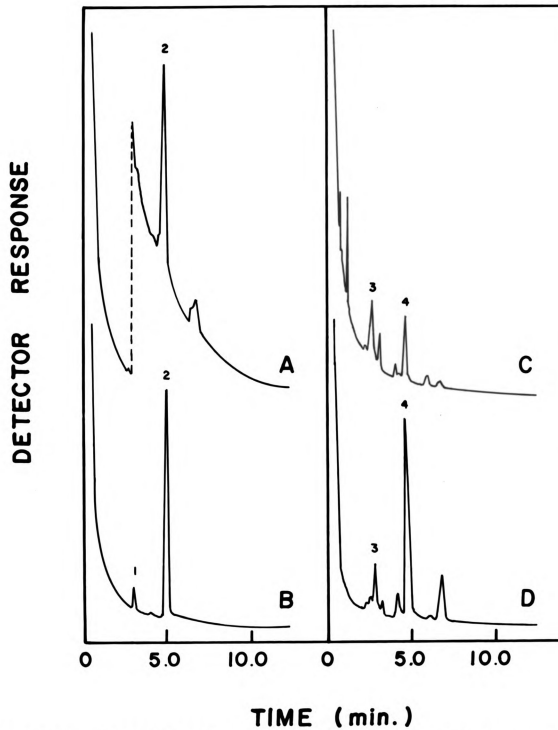

Figure 6. Identification of hexonic acids as products of the reaction between carbohydrates and hydroxyl radicals in vitro.

Products are represented in (A) and (C), while standards of gluconic acid and galactonic acid are represented in (B) and (D), respectively. Peaks are (1) glucono-δ-lactone, (2) gluconic acid (open form), (3) galactono-γ- lactone, and (4) galactonic acid (open form).

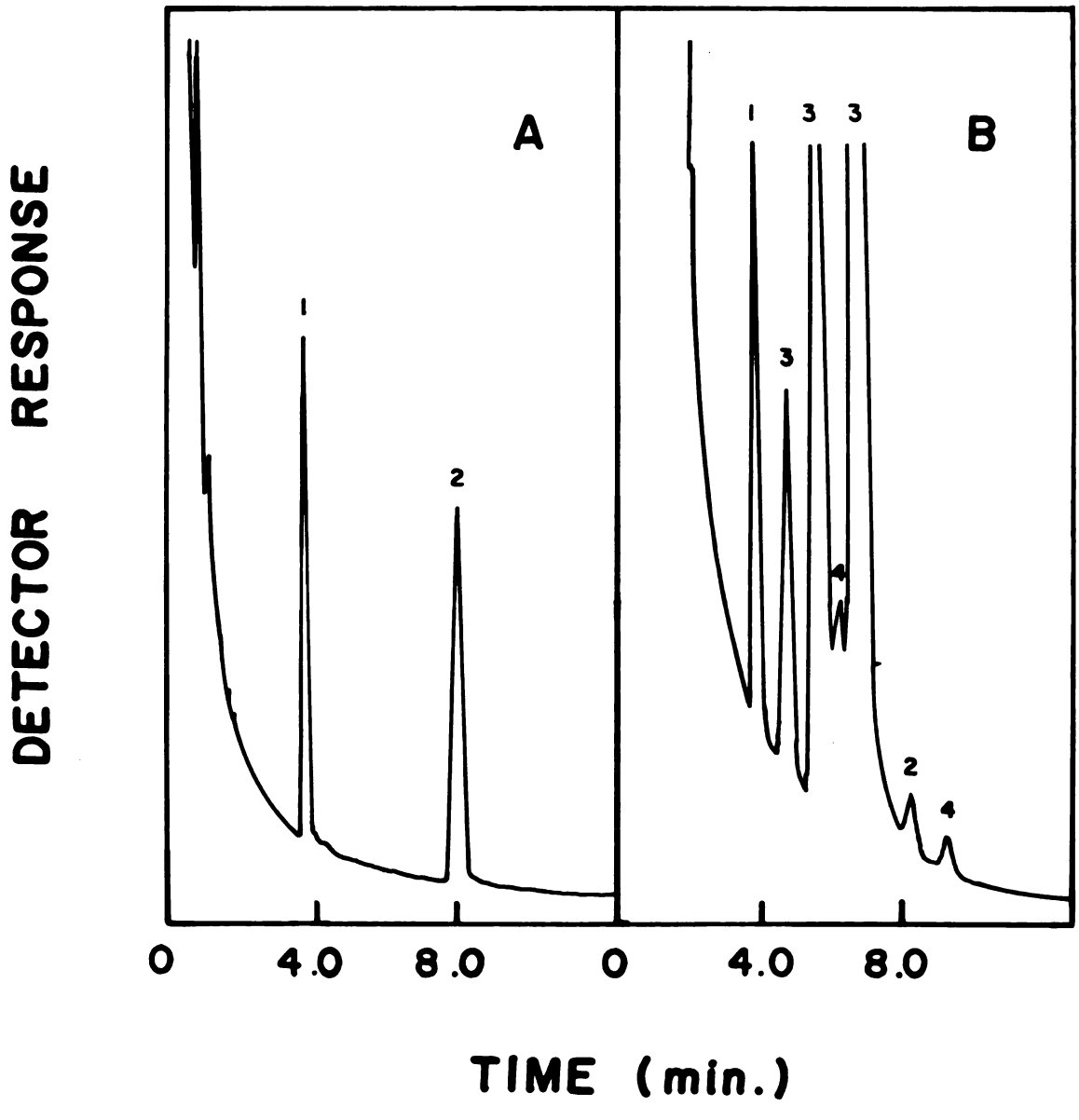

Figure 7. Identification of galactitol in homogenates of PMN after incubation with 30 mM galactose.

Standards are in (A), and cell homogenate in (B). Peaks are (1)∝-methylmannoside, (2) galactitol, (3) galactose, and (4) glucose.

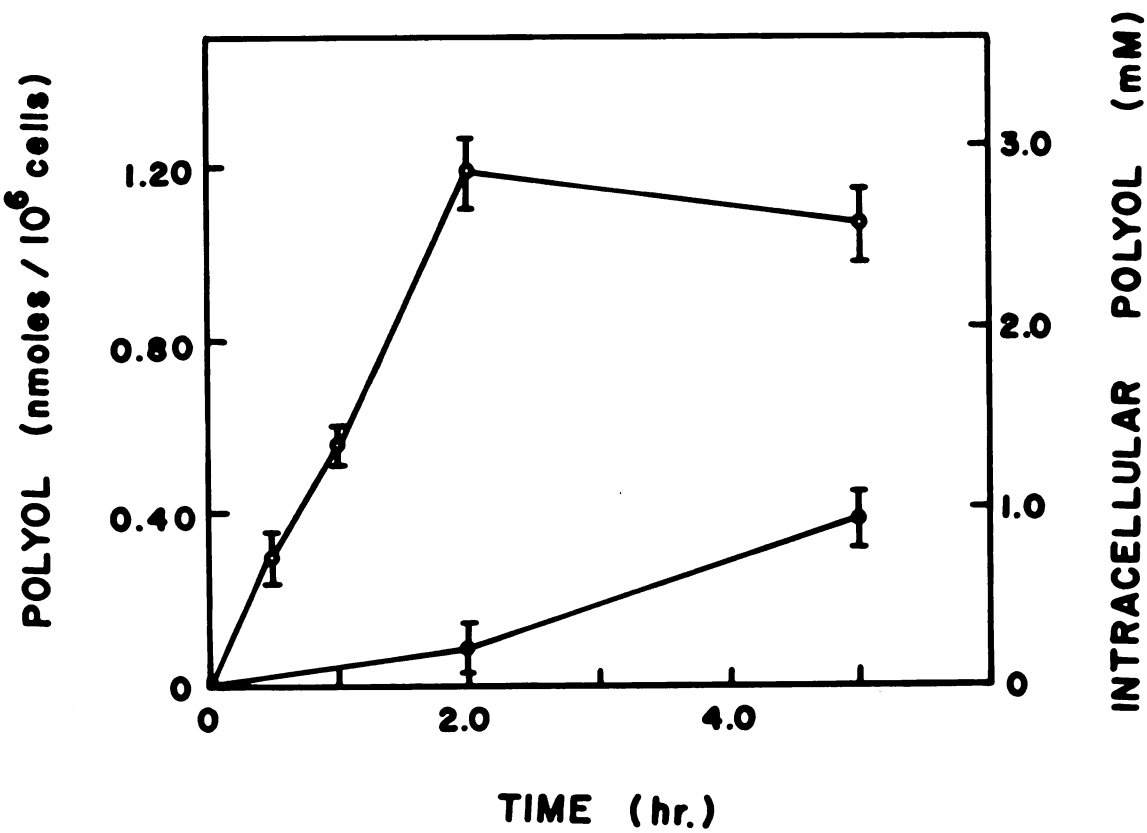

Figure 8. Accumulation of intracellular polyol during incubation of PMN with elevated levels of carbohydrate.

PMN were incubated in 30 mM galactose (o) or 30 mM glucose (●) media.

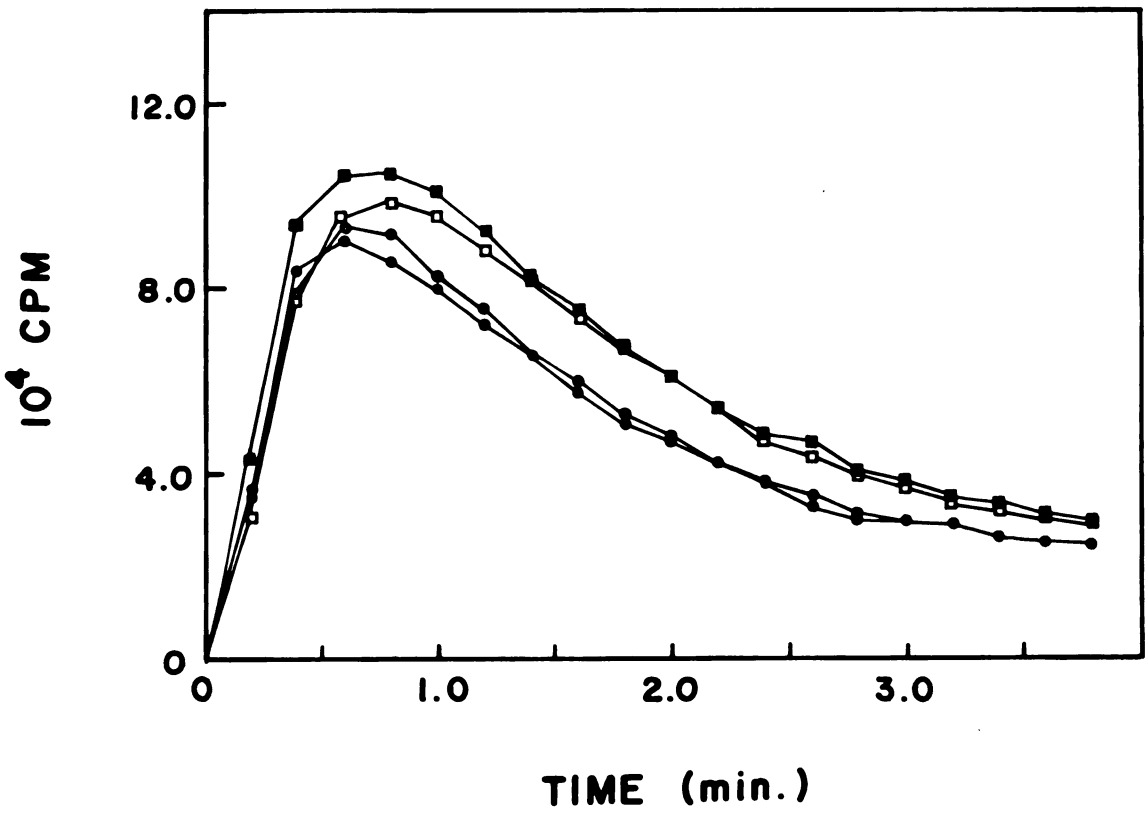

Figure 9. Effect of carbohydrate on PMN chemiluminescence. Heat-killed bacteria were added at zero time to cell suspensions pre-incubated in media containing (●) 5 mM glucose, (o) 30 mM glucose, (▫) 5 mM glucose plus 20 mM galactose, or (■) 5 mM glucose plus 30 mM galactose.

SUMMARY

Although phagocytic cells from galactosemic patients were not employed in the present research, the preceding results indicate that these cells would exhibit depressed phagocytic and bactericidal activities in the presence of galactose. Decreased killing of E. coli was observed under galactosemic conditions in vitro using normal leukocytes from peripheral blood of human (p. 32), guinea pig (p. 33), and chick (p. 57) sources. This response was impaired to a greater extent when leukocytes from galactosemic rather than normal chicks were employed. In addition, galactose was found to impair phagocytic activities of normal guinea pig PMN (p. 38) and peritoneal macrophages (p. 39) as well as the intravascular clearance of colloidal ^{125}I-BSA by chicks (p. 58). These results strongly suggest that impaired phagocyte function may be the underlying cause of susceptibility to E. coli infection among galactosemic infants. There are, however, many aspects of host defense which were not investigated and which could also contribute to increased infection. These include (i) the permeability of the intestinal lining to E. coli, (ii) the capacities of the complement, properdin, and humoral antibody systems, (iii) the capacity of the cellular immune system, and (iv) the susceptibility of E. coli to antibiotics in the presence

of elevated blood galactose.

The inhibitory action of galactose upon phagocytosis per se can be accounted for by decreased intracellular ATP. Levels of ATP were found to be lower in guinea pig PMN (p. 86) and peritoneal macrophages (p. 36) during culture of cells with 30 mM galactose. These decreases could be attributed to two basic inhibitory mechanisms. The first, and most likely in galactosemic phagocytes, is the accumulation of galactose or one of its metabolites intracellularly which could subsequently impair glycolysis by inhibiting one of the glycolytic enzymes. It is known that both galactose and galactose-1-phosphate accumulate in galactosemic PMN (1), and data with macrophages (p. 36) suggest that fructose-1,6-bisphosphate aldolase may be inhibited. Decreases in ATP could further result in lower levels of glucose uptake (p. 85) and additionally impair glycolysis.

The second mechanism by which galactose could lower ATP is present in guinea pig PMN (p. 35) and depends upon the conversion of galactose to free glucose. This pathway requires ATP (2,3) and could complete with the energy requiring steps leading to phagocytosis. That this conversion is stimulated by 2 fold during phagocytosis (p. 35) supports this suggestion. However, stimulation could also indicate an increase in intracytoplasmic phosphatase activity. Since PMN do not contain glucose-6-phosphatase (4,5), the presence of another enzyme either of lysosomal or cytoplasmic origin

is indicated. Cohn and Hirsch have shown a 2 to 3 fold increase in both soluble acid phosphatase and alkaline phosphatase activities during phagocytosis (6). Since these enzymes could retain some activity in the pH range of the cytoplasm, one or both of these could be responsible for the dephosphorylation of glucose-1-phosphate or glucose-6-phosphate in PMN during galactose loading. This suggestion is further supported by evidence indicating that lysosomal fragility is increased during galactose toxicity in other tissues (7,8). However, data on the redistribution of acid phosphatase in PMN (Appendix, Table II) do not demonstrate any significant increase in free activity, or extracellular activity, as a result of incubation with galactose. Therefore, dephosphorylation of hexose-phosphates in PMN may occur as a normal process, although apparently wasteful in terms of energy conservation. Cyclic phosphorylation and dephosphorylation of triose-phosphates in PMN (9) concurs with this suggestion.

A third mechanism for depleting intracellular ATP by competition between glucose and galactose for cell entry does not occur (Chapter III).

Effects of galactose upon bactericidal activity (p. 33) were much more pronounced than effects upon phagocytic activity (p. 38) in guinea pig PMN. These observations indicate that galactose predominately affects the former physiological function in these cells which is mainly oxidative in character (10,11). An inhibitory action of

galactose upon the oxygen-dependent killing mechanism is therefore suggested, and this is supported by data on PMN reduction of nitroblue tetrazolium, formate oxidation, and chemiluminescence as discussed in Chapter IV. Moreover, intracellular galactose and galactitol did not impair oxygen consumption (p. 132), hexose monophosphate shunt activity (pp. 35 & 40), or extracellular peroxide formation (p. 130) in these cells; even though levels of ATP were depressed (p. 86). These apparently conflicting results can be reconciled by considering the versatility of the hexose monophosphate shunt. Less than 2% of glucose carbon transverses this pathway in resting PMN, whereas up to 17% enters the shunt during phagocytosis (5). In the presence of galactose, the proportion of glucose-6-phosphate entering this pathway could increase to compensate for lower substrate levels.

Production of galactose radical or galactitol radical by PMN can explain most of the effects of galactose observed upon the oxygen-dependent killing mechanism. These radicals are probably very similar to mannitol radical, which has also been proposed in PMN to account for the protection of leukocytes against suicide during phagocytosis (12). Since this deleterious effect was also prevented by superoxide dismutase and catalase the scavenge of hydroxyl radical by mannitol was indicated (12).

Photographs of the decay of acridine orange fluorescence, induced by blue light and oxygen (Appendix, Figure 1),

demonstrate that superoxide anion is involved in this process. Pre-incubation of cells with superoxide dismutase prevented loss of some red, lysosome-associated (13) fluorescence. However, pre-incubation of cells with 30 mM galactose facilitated this decay (data not shown). Since this process likely involves the photochemical production of high superoxide anion fluxes, peroxidation of lysosomal membranes may occur (14) and result in leakage of acridine orange from lysosomes. That galactose enhances this process suggests involvement of galactose radical in promoting lipid peroxidation, perhaps through reacting with superoxide (eq. 8, Chapter IV) to form singlet oxygen. Alternatively, this could be explained by increased lysosomal fragility, but data in Tables I and II of the Appendix do not agree.

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APPENDIX

Table I. Analysis of carbohydrates in sera[a]

Carbohydrates	Concentration (mM)			
	Fetal Calf[b]	Human[c]	Guinea Pig[b]	Chicken[b]
Glucose	7.83	2.55	6.00	12.4
Fructose	5.48	-	-	-
Sorbitol	1.42	-	-	-
myo-Inositol	0.72	-	-	-
Galactose	-	-	-	-

[a]Aliquots of sera (0.2 ml) were diluted with 5 volumes of triple distilled water and treated with 0.4 ml of 0.3 N $Ba(OH)_2$ and of 0.5% $ZnSO_4$. Supernates were then taken to dryness under N_2 in the presence of 40 μg α-methyl-mannoside and later treated with 0.1 ml of TMS reagent. Each derivatized sample was then analyzed 3 times by gas chromatography, and the values represent means of these determinations. (-) indicates levels less than 0.01 mM.

[b]Obtained from Grand Island Biological Company.

[c]Obtained from North American Biologicals, Inc.

Table II. Acid phosphatase activity associated with guinea pig PMN[a]

Additions		Extracellular release[b]	Intracellular distribution[c]
Glucose (mM)	Galactose (mM)	mU/hr/10^6 cells	% Free
5.0	0	0.70 ± 0.22	14.0 ± 1.90
5.0	30.0	0.72 ± 0.11	17.8 ± 3.60

[a]PMN (2.79 x 10^6/assay) were incubated at 37°C for 8 hr in Krebs-Henseleit bicarbonate solution without phosphate, pH 7.4. Acid phosphatase was determined in 0.1 M acetate buffer pH 5.0, containing 0.2% (w/v) Triton-X-100 and 0.05 M β-glycerol phosphate. Each determination was performed 3 to 5 times.

[b]Enzyme activity released into cell supernate/hr/10^6 cells. 1mU = 1 μmole PO_4^{-3}/min.

[c]Ratio of enzyme activities in 22,000 xg supernate to that in 400 xg supernate of cell homogenates.

Table III. Distribution of hexosaminidase and peroxidase activity in guinea pig PMN[a]

Carbohydrate (mM)		Hexosaminidase[b] % Free ± S.D.	Peroxidase[c] % Free ± S.D.
Glucose	Galactose		
5.0	0	42.0 ± 7.3	22.1 ± 4.0
5.0	30.0	46.3 ± 2.2	19.8 ± 13.6
30.0	0	37.7 ± 5.9	23.9 ± 10.8

[a]PMN (1.7×10^7/assay) were incubated for 2 hours at 37°C in Krebs-Ringer phosphate solution, pH 7.4. Distribution of enzyme activity (% Free) was determined by dividing enzyme activity in the 22,000 xg supernates by that in the 400 xg supernates of cell homogenates. Each value was determined 5 times on cells pooled from 4 guinea pigs. S.D. refers to standard deviation.

[b]Hexosaminidase activity was determined in 50 mM citrate buffer, pH 4.3, with 0.1% Triton X-100 and with 5 mM PNP-NAG.

[c]Peroxidase activity was determined in 0.1 M phosphate buffer, pH 7.0, with 0.1% Triton X-100 by monitoring the initial velocity of guaiacol (0.31 mM) oxidation in the presence of 0.11 mM hydrogen peroxide.

Figure 1. Effect of superoxide dismutase on the decay of acridine orange fluorescence in phagocytes.

Cells were incubated with 5 mM glucose before exposure to blue light for 1 min (A) and 13 min (B). Cells were incubated with 5 mM glucose plus 0.2 mg/ml superoxide dismutase before exposure to light for 1 min (C) and 13 min (D).

Total incubation time prior to staining was 5 hr at 37°C. Staining was for 30 min at the same temperature with 50 μM acridine orange in the dark.