ASSESSMENT OF PHAGOCYTIC FUNCTION IN MULTIGENERATIONALLY PROTEIN-CALORIE MALNOURISHED RATS

by

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This Thesis Is Dedicated to My Parents
ASSESSMENT OF PHAGOCYTIC FUNCTION IN MULTIGENERATIONALLY PROTEIN-CALORIE MALNOURISHED RATS

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Ann Mary Hart

Submitted to the Department of Nutrition and Food Science on May 7, 1976, in partial fulfillment of the requirements for the degree of Master of Science

ABSTRACT

The possibility of an effect of malnutrition on the function of free macrophages has been largely neglected in the literature. Rats from a colony with a history of 16 to 17 generations of protein-calorie malnutrition have been found to be at higher risk for S. aureus-infected dermatitis. The purpose of this experiment was to determine whether there were defects in macrophage function in the PCM rats which might explain the higher risk. The function of elicited peritoneal macrophages was evaluated, although sufficient numbers of polymorphonuclear leukocytes were present to alter the results. Total cell yield was lower in the malnourished rats, but a correction for weight eliminated the difference. However, a significant difference in the number of cells per weight0.67 was found. Similar percentages of macrophages and PMN leukocytes were present in the exudates from control and PCM rats. However, the percentage of mature macrophages was less in the malnourished animals. Initial ingestion rates, assessed spectrophotometrically with simulated bacteria, were similar in the control and malnourished animals. Due to insufficient cell yields from the malnourished rats, results of the quantitative NBT test were inconclusive. Opsonization of test particles was less effective with serum from some of the malnourished rats, suggesting that serum opsonin levels were reduced in these animals.

It was concluded that the increased susceptibility to infection with malnutrition might be explained in part by the decreased numbers of exudative phagocytes, the relative immaturity of the macrophage population, and the reduced efficiency of opsonization. These findings were compared to results reported in the literature, and were generally in agreement with results on rats malnourished for a single generation.

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I. INTRODUCTION

The adverse effect of protein-calorie malnutrition on susceptibility to infection has been recognized for many years. However, the effect of malnutrition on each of the systems for host defense has not yet been completely determined. Polymorphonuclear leukocytes from malnourished children have been extensively studied. Macrophages are much more difficult to obtain from humans, and they have consequently received considerably less attention. In animals, a blood clearance test indicative of fixed macrophage function has frequently been used. However, the effect of malnutrition on the wandering macrophages of rats has not yet been completely established. Even where initial investigations do exist, the importance of the stage of maturation of the macrophage is sufficient to warrant further study.

Most animal models for malnutrition are designed to consider only one generation. We were fortunate to have the opportunity to study rats malnourished for 16 to 17 generations, a model more closely resembling the typical situation in humans. The purpose of this thesis was to determine the effect of multigenerational malnutrition on various functions of the wandering macrophage. Chemotaxis, initial ingestion rate, NBT reduction, and the effect of serum opsonins were evaluated. They are representative of most of the important and basic functions of the macrophage.
II. BACKGROUND

A. Malnutrition

Malnutrition is a major problem in the world today (Scrimshaw et al., 1955; Scrimshaw and Béhar, 1961; Berg, 1973). Many studies have investigated the effect of malnutrition on physical (Peckos, 1953; Gomez et al., 1957; Sabharwal et al., 1966; Jelliffe and Jelliffe, 1969) and mental (Hertzig et al., 1972; Stein et al., 1972; DeLicardie and Cravioto, 1974) capabilities. Animal models have been developed to supplement studies in humans for the elucidation of more subtle changes under more controlled conditions (Edozien, 1968; Philbrick and Hill, 1974).

Human malnutrition, unlike the malnutrition in previous animal models, is often a condition present over many generations. The lack of precise dietary information for human populations over the years imposes limitations on attempts to study intergenerational malnutrition in humans (Richardson, 1968). Consequently, about fifteen years ago, Dr. R. J. C. Stewart developed an animal model for intergenerational malnutrition (Stewart and Sheppard, 1971; Stewart, 1972, 1973).

After initial studies with dogs, a more extensive study was begun using rats. However, the heavy mortality rate in malnourished rats on a diet NDpCal% = 5.0 soon necessitated a change to NDpCal% = 6.8. Finally, litters of black and white hooded rats were divided into two groups
at weaning. One group was maintained on an adequate diet (NDpCal% = 10). The other group was provided with a marginally protein-deficient diet (NDpCal% = 6.8), which while probably adequate for nonreproducing adults, was inadequate during early growth, pregnancy and lactation. The two groups were maintained over many generations. Changes found in the malnourished group included an increased mortality rate in the young, decreased birth weights, lower organ weights, and delayed sexual development.

In 1974, rats from the high and low protein groups of the thirteenth through the fifteenth generations on these diets were transferred to MIT by Dr. Janina Galler. Here the low protein group receives a 7.5% casein diet, as opposed to a 25% casein diet for the controls. The malnourished rats are still losing weight with successive generations. Food consumption is below that in the control rats, as is typically found with a low protein diet. Lower intake may result in various vitamin, mineral, or free fatty acid deficiencies. The malnourished rats are often anemic. They have a lower basal metabolic rate and lower body temperature than the controls. Their hair is brittle and lighter, but there is no edema and there are not many fatty livers. All rats are weaned at 28 days of age.¹ Rats of the sixteenth and seventeenth generations from this colony were used in our experiments.

¹Information on the current status of the colony is from Dr. Galler, personal communication.
Malnourished rats, particularly parous females, from Dr. Galler's colony have been found to be at higher risk for *Staphylococcus aureus*-infected dermatitis (Galler et al., 1976). Malnutrition is known to increase susceptibility to infection with *S. aureus* (Kendall and Nolan, 1972) and with other bacteria and viruses (Newburne et al., 1968; Seth and Chandra, 1972; Bhuyan and Ramalingaswami, 1974). *Staphylococcus aureus* and other infections are also common in chronic granulomatous disease, where functional defects in PMN leukocytes and monocytes have been found (Douglas, 1970).

The effect of malnutrition on the PMN leukocyte has been investigated extensively in humans (Altay et al., 1972; Kendall and Nolan, 1972; Rosen et al., 1975). However, the mononuclear phagocytes are more difficult to obtain from humans. The only report we have been able to find dealing with this subject (Douglas et al., 1974), used blood monocytes, which are not mature. Even in animals, where mononuclear phagocytes are more easily studied, only one report on the effect of malnutrition on the free macrophages was found (Keusch et al., 1976). Again, the mononuclear phagocytes were primarily immature monocytes.

Rather than assess the validity of the human model in determining the effect of malnutrition on PMN leukocytes in the rat, we decided to study the effect of protein-calorie malnutrition (PCM) on macrophage function. We chose to measure chemotaxis, phagocytosis, and NBT reduction, and to
assess serum opsonin levels, which may modulate macrophage activity in vivo (Saba, 1970). Although reports in the literature are conflicting, there is a possibility that malnutrition may affect phagocyte function through the impairment of one or more of these activities in some species (Mills and Cottingham, 1943; La Via et al., 1956; Kendall and Nolan, 1972; Selvaraj and Bhat, 1972a and 1972b; Deo et al., 1973; Bhuyan and Ramalingaswami, 1974; McGhee et al., 1974; Sirisinha and Suskind, 1974; Jose et al., 1975).

B. Macrophages

The macrophage traditionally has been defined as "a mononuclear cell capable of phagocytosis" (Gottlieb and Waldman, 1972). Macrophages and PMN leukocytes are together classified as phagocytes. Macrophages are more important than PMN leukocytes in dealing with certain organisms (Keusch et al., 1976), and in fact are the only phagocytes which can effectively kill certain organisms (Furness and Axelrod, 1959; Slonecker and Omsanski, 1974). Of course, the PMN leukocytes would be more important in other infections.

Macrophages comprise the reticuloendothelial system (RES). The RES has many roles, one major role being "host defense" (Saba, 1970). The importance of the RES in host defense lies in its capacities for phagocytosis, bactericidal activity, and antigen concentration, degradation, and
processing (Gottlieb and Waldman, 1972). The cells of the RES have generally been characterized by their phagocytic behavior. The "colloid clearance" test is commonly used, where an identifiable material is injected and cells of the RES are those which clear it from the blood stream (Saba, 1970).

Cells of the RES are not all located in one organ. Rather they are found in many different parts of the body. They are generally divided into two classifications: the "fixed" and the "free" or "wandering" macrophages. Most of the "fixed" macrophages are located in the liver, spleen, lungs, bone marrow or lymph nodes (Saba, 1970). The "free" macrophages include the blood monocytes, tissue macrophages, and connective tissue histiocytes (Saba, 1970).

Different properties have been attributed to macrophages of different origins. For example, the enzymatic activities and metabolic rates of peritoneal macrophages (free macrophages) have been found to differ from those of alveolar macrophages (Solotorovsky and Soderburg, 1972). Immunogenic RNA from rat peritoneal macrophages has been found to induce antibody synthesis by rat spleen cells (Bishop et al., 1967) and to be more active than immunogenic RNA from rat Kupffer cells (Archer and Wust, 1973). Macrophages of different origins may, then, have different biochemical and functional properties.

Fixed macrophages are more difficult to obtain than the wandering variety. The Kupffer cells of the liver have
been studied in liver slices (Saba and DiLuzio, 1966) or after isolation (Pisano et al., 1968). Cell isolates have the advantage of a more uniform population of cells, but the enzymatic methods of isolation used may alter the integrity or function of the phagocytes (Pisano, 1973). The cell yield of alveolar macrophages is a limiting factor in rats (Pisano, 1973).

Free macrophages are easily obtained. They are isolated from the blood in humans, and often from the peritoneum in animals. Peritoneal macrophages, unlike the immature monocytes of the blood, are morphologically similar to the fixed macrophages (Gottlieb and Waldman, 1972). There are three different classifications for peritoneal macrophages. Normal or resident macrophages are those which are always present. Elicited macrophages are those obtained following the injection of an irritant, and "activated" macrophages are found after the injection of bacteria. Differences between these types of peritoneal macrophages have been observed in phagocytosis and in killing function (Drath and Karnovsky, 1975; Karnovsky et al., 1975).

The elicited macrophages were chosen for our experiments.

Elicited peritoneal macrophages, like other macrophages of acute nonbacterial inflammations, derive from the monocytes of the blood, which develop in turn from precursors in the bone marrow (Volkman and Gowans,
1965a and 1965b; Volkman, 1966). Elicited macrophages are believed to live for about one week. Macrophages are capable of division until they reach full maturity (Cline and Golde, 1973).

C. Chemotaxis

The "directional movement of phagocytes in response to chemotactic gradients" is termed chemotaxis (MacGregor et al., 1974).

Chemotaxis may be assessed either in vitro or in vivo. In vitro assessments are often done using a Boyden chamber or a modification thereof. They have the advantage of a more precisely defined system, but the disadvantage of requiring a larger number of cells than some in vivo methods. Ward (1974) has written an excellent review on in vitro methodologies and chemotactic factors. Baum and associates (1971), Cutler (1974) and Naidu and Newbould (1974) have described "simple methods" for measuring chemotaxis in vitro, and Hirsch (1975) explains some of the terminology in use today.

The measurement of chemotaxis in vivo simply involves the enumeration of the total cell yield in response to an irritant after a fixed time period. Unfortunately, the measurement of chemotaxis of macrophages in this system can be affected by the blood count of monocytes, their precursors, among other things.
Some relationship has been found between the irritant used and the cell type obtained (Cutts, 1970). Macrophages are attracted by glycogen, mineral oil, starch, saline, caseinate, broth, peptone, triolein, and thioglycollate medium (Solotorovsky and Soderburg, 1972). Of these, we chose to use caseinate. Caseinate is also chemotactic for the PMN leukocyte. Unlike some other irritants, which act by denaturing proteins found in the peritoneal cavity, caseinate is itself a chemotactic factor. It is thought that its normal random coil conformation, which other proteins acquire only after denaturation, is responsible for its chemotactic activity (Wilkinson, 1971). Attempts at biochemical characterization of the chemotactic activity have been reported (Wilkinson and McKay, 1974).

The amount of time between the injection and the peritoneal washes has a major effect on which cell type predominates in the exudate. Using sodium caseinate, PMN leukocytes should be harvested in 12-16 hours, whereas rat macrophages are usually harvested after four days (Pisano et al., 1971). A high percentage of macrophages is to be expected, but 5-25% contamination by other cell types is common (Cutts, 1970).

Besides the type of irritant and the amount of time, the weight, the maturity, and perhaps the size of the peritoneal cavity have been found to affect total cell yield in guinea pigs.

\[\text{Dr. Thomas Stossel, personal communication.}\]
D. Ingestion Rate

Ingestion rate is one way of assessing phagocytic function. It measures the rate at which macrophages and PMN leukocytes engulf particles, the rate of phagocytosis.

Phagocytosis is an energy-requiring process. Various metabolic changes are normally associated with it, including increased oxygen consumption, increased hexose monophosphate shunt (HMS) activity, and increased hydrogen peroxide production (Sbarra et al., 1974). While macrophages and PMN leukocytes are believed to respond to phagocytosis similarly in terms of metabolic changes, there is some evidence that HMS activity may be less prominent, and the citric acid cycle more active, in the macrophage (Douglas, 1970).

During phagocytosis, invagination of the membrane occurs, followed by vacuole formation. Increased lipid metabolism and increased membrane synthesis therefore should accompany phagocytosis (Douglas, 1970; Karnovsky, 1973).

Although the morphodynamics of phagocytosis are similar between macrophages and PMN leukocytes, some differences do exist between the two types of phagocytes. Macrophages are able to ingest more particles and larger particles than are the PMN leukocytes (including dead PMN). Macrophages are also capable of surviving phagocytosis, unlike most PMN leukocytes (Douglas, 1970).

Phagocytosis can be measured in a variety of ways. One of the most sophisticated techniques measures the
ingestion of radioactive particles, including $^{14}$C-labeled bacteria, by a monolayer of phagocytes (Karnovsky, 1973). Older methods still in use involve the microscopic enumeration of the number of bacteria (Mills and Cottingham, 1943; Guggenheim and Buechler, 1946), yeast (Jose et al., 1975), or other particles (Douglas and Schopfer, 1974) per phagocyte. The percentage of phagocytes containing particles is sometimes also determined (Douglas and Schopfer, 1974).

There is an elegant spectrophotometric method for measuring the ingestion rate (Stossel et al., 1973; Stossel, 1973). The particles used are simulated bacteria, droplets of oil saturated with oil red O and coated with *E. coli* lipopolysaccharide. Following ingestion of the simulated bacteria, phagocytes and extracellular particles are separated by density centrifugation. The cells are washed and lysed, and the extracted dye is measured spectrophotometrically. The ingestion rate is significantly increased by the prior opsonization of these simulated bacteria, as it is for actual bacteria (Stossel et al., 1973).

The technique of Stossel and associates (1973) measures the initial ingestion rate, over the first five minutes. Measurements which can be made with other techniques include the maximal ingestion rate and the total capacity of the phagocyte (Karnovsky, 1975).
E. Nitroblue Tetrazolium Reduction

Baehner and Nathan (1968) developed the quantitative NBT reduction test for the identification of patients with chronic granulomatous disease (CGD). Incubated with normal phagocytes, the NBT dye was found to be reduced and converted to formazan, whereas there was essentially no NBT reduction by phagocytes from patients with CGD. The dye reduction was found to depend on enzyme activity, and to increase when in cells engaged in phagocytosis.

Stossel (1973) slightly modified the NBT test of Baehner and Nathan. This modified NBT test allowed the simultaneous assessment of ingestion rate and NBT reduction, so that the NBT reduction could be corrected for the rise with phagocytosis. He expressed his results in terms of the ratio of reduced NBT to ingested particles. This modified NBT test of Stossel was used in our experiments.

The increase in NBT reduction with ingestion rate is due to the fact that the NBT must enter the cell for reduction to take place (Nathan, 1975). It has been observed that erythrocytes can interfere with the NBT test (Patriarca et al., 1974).

There is some controversy over exactly what reduces the NBT. Briggs and associates (1975) maintain that there is direct enzymatic reduction by NADH oxidase, whereas Baehner and associates (1975) claim that the majority of NBT reduction by PMN leukocytes is due to superoxide anion. Whatever the specific biochemistry, Stossel (1973)
states that the NBT test correlates with hydrogen peroxide production in PMN leukocytes. Hydrogen peroxide production increases with phagocytosis in the PMN, as does NBT reduction; different increments in hydrogen peroxide production have been observed with different particles (Root, 1975).

The NBT test is a measure of hexose monophosphate shunt activity (Douglas, 1970), or at least an indirect measure thereof (Karnovsky, 1973). Karnovsky points out that the NBT test "nonspecifically measures diaphorase activity, of which NADH oxidase constitutes a significant but variable part." Bellanti and associates (1975) found the demonstration of a correlation between NBT reduction, hexose monophosphate shunt (HMS) activity and bactericidal activity in PMN leukocytes.

There is a general consensus that the halide-hydrogen peroxide-myeloperoxidase system described by Klebanoff is the most important bactericidal system in the PMN leukocyte (Karnovsky, 1973; Sbarra et al., 1974; Klebanoff, 1975). This system is effective against "gram-positive and gram-negative bacteria, fungi and viruses" (Sbarra et al., 1974). The correlation of hydrogen peroxide production with the NBT test suggests that the NBT test is an indicator of bactericidal activity in PMN leukocytes.

The hexose monophosphate shunt provides a significant portion of the energy for phagocytosis in PMN leukocytes (Bellanti et al., 1975), and HMS activity is also, indirectly,
measured by the NBT test. However, macrophages seem to differ from PMN leukocytes. HMS activity may be less prominent (Douglas, 1970). Whereas PMN use glycolytic energy for phagocytosis, alveolar macrophages rely on oxidative energy (Sbarra et al., 1974). Superoxide release has been found to be significantly lower in elicited peritoneal macrophages than in PMN from mice (Drath and Karnovsky, 1975). Perhaps of more significance is the fact that cells of the monocytic series seem to have very little peroxidase (Drath and Karnovsky, 1975). Thus even if the macrophages had HMS activity and hydrogen peroxide production similar to that in the PMN, their potency would be, at best, seriously depressed with little peroxidase available. It is not clear that in the rat elicited macrophage NBT reduction reflects the most important bactericidal activity. However, it should give an indication of superoxide and hydrogen peroxide production, which may have some bactericidal activity even in the absence of an enzyme.

Karnovsky suggests that in the macrophage as well as in the PMN leukocyte the NBT test measures NADH oxidase activity. 3

F. Opsonins

Wright and Douglas (1903) coined the term "opsonin" for what is now best defined as a type of natural antibody,

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3Personal communication from Dr. Manfred Karnovsky, 1976.
as distinct from a specific immunoglobulin (Saba, 1970). Opsonins are found in serum or plasma, and are recognized by their ability to enhance phagocytosis of bacteria and of many inert substances (Saba, 1970). It has been postulated that opsonins are the "humoral recognition factors" (Pisano et al., 1972) which enable the phagocyte to discriminate between Burnet's "self" and "nonself" (Vaughan, 1965).

Since the rate of phagocytosis can be the rate-limiting step in bacterial destruction (Jenkin and Rowley, 1961), opsonins are very important. Virulent bacteria are cleared more rapidly when the serum has a high opsonic titer (Saba, 1970). Depressed opsonin levels have been implicated in carcinogenesis (Pisano et al., 1972). Current evidence even suggests that there may be a requirement for opsonins for phagocytosis to take place (Saba, 1970). Saba also identifies plasma opsonin levels, which apparently are physiologically regulated, as a determinant of the functional behavior of the reticuloendothelial system.

Many different factors have been implicated in the opsonization process. Complement components have been found to be particularly important (Douglas, 1970), and will be discussed in some detail below. Specific antibodies, while not always considered to be opsonins since they are absent in nonimmune animals, are known to be active in opsonization. Properdin (Slopek et al., 1965), noncomplement thermolabile factors and possibly basic polypeptides, including lysozyme...
and basic polyamino acids (Douglas, 1970) have also been considered to be opsonins. An opsonin for inert particles ingested by the fixed macrophages of the RES was found in the α-2 globulin fraction of serum by Saba and associates (1966). It has since been purified (Pisano and DiLuzio, 1970; Saba et al., 1973), and represents the first opsonic protein(s) to reach this stage of identification.

The existence of a "multiple opsonin system" was proposed by Saba and DiLuzio (1966), based on evidence that depletion of opsonins for one type of particle does not interfere with opsonization of a different type of particle (Saba and DiLuzio, 1969). The opsonin specificity might depend on various physical and chemical characteristics of the particle. Different opsonins are now known to exist not only for various types of particles but also for different bacterial species (Douglas, 1970).

Complement is part of the complement system. Its presence may be essential for adherence and ingestion during phagocytosis (Douglas, 1970). More specifically, certain one or ones of the many components of complement are important for opsonization. The most recent review (Alper et al., 1975) concludes that opsonic activity is generated by the alternate pathway. C3 fixation by the alternate pathway alone is necessary for the opsonization of the simulated bacteria used in our experiments. There is a C3 receptor on PMN leukocytes, monocytes (Müller-Eberhard, 1975), and macrophages (Unanue, 1975).
Most complement protein is synthesized by macrophages, many of which are located in the liver and spleen (Ruddy et al., 1972). Almost all of the C3 is produced in the liver, under normal conditions (Alper et al., 1975). C3 can be activated by either the classical or the alternate, otherwise known as the properdin, pathway. The alternate pathway, which is activated by lipopolysaccharides including those of *E. coli* and staphylococcus (Müller-Eberhard, 1975), is thought to be especially important in the initial stages of host defense (Alper et al., 1975).

Complement is involved not only in opsonization but also in chemotaxis. One or more C3 fragments are known to be chemotactic for PMN leukocytes and for monocytes (Ward, 1974).
III. MATERIALS AND METHODS

A. Animal Model System

Black and white hooded rats from an inbred strain were generously provided by Dr. Janina Galler. The experimental and control groups were developed from littermates by Dr. R. J. C. Stewart. The experimental group has been maintained for multiple generations on a low protein diet (7.5% casein), whereas the control group has been maintained on an optimal protein diet (25% casein). For additional information regarding the history and current status of Dr. Galler's colony, refer to the Background section.

A total of 34 rats were used, 17 from each group. Of the 17 rats, 10 were male and 7 were female. The rats had histories of 16 to 17 generations on the low protein or high protein diets. The average age at the time of sacrifice was 12 weeks, with a range from 10.4 to 13.3 weeks. All of the experimental animals (protein-calorie malnourished or PCM) and 8 out of 17 of the control animals were from litters of 8 pups; the remaining control animals were from litters of 6 pups. Litter size was limited in these cases by still births and cannibalistic behavior on the part of these mothers. The rats used were selected

\[ \text{Litter size in the PCM group was 8 until 14 days of age, when the litter size was reduced to 7 in half of the litters.} \]
TABLE I
Composition of Experimental Diets

<table>
<thead>
<tr>
<th></th>
<th>7.5% Casein g/kg</th>
<th>25% Casein g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>75</td>
<td>249</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Dextrose</td>
<td>217</td>
<td>173</td>
</tr>
<tr>
<td>Sucrose</td>
<td>208</td>
<td>151</td>
</tr>
<tr>
<td>Dextrin</td>
<td>235</td>
<td>178</td>
</tr>
<tr>
<td>Celluloflour</td>
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<td>50</td>
</tr>
<tr>
<td>Rogers-Harpers Salt Mix</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vitamin Mix&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Chloride&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>

<sup>a</sup>Contributed in milligrams per kilogram diet: thiamine hydrochloride, 20.0; riboflavin, 40.0; pyridoxine hydrochloride, 20.0; niacin, 100.0; calcium pantothenate, 100.0; cyanocobalamin (.1% solution), 100.0; para-aminobenzoic acid, 200.0; biotin, 1.0; folic acid, 4.0; inositol, 400.0; α-tocopherol, .8; vitamins A and D, 18.4; vitamin K, 10.0; ascorbic acid, 400.0

<sup>b</sup>40% solution
from 5 PCM litters and 5 control litters, taking 2 to 4 rats from each litter.

Rats were housed in suspension galvanized wire cages, with one to five rats per cage. They were on a reversed lighting schedule, with white light from 8:00 pm to 8:00 am. There was additionally continuous red fluorescent lighting.

In treatment for the endemic chronic respiratory disease, the rats were maintained throughout their lives on tetracycline (1 mg/ml) in their drinking water. The water was given ad libitum.

B. Elicitation of Phagocytes

15% sodium caseinate (Nutrose) (Difco, Detroit, Mich.) in isotonic saline was prepared by the slow addition of the caseinate to warm (about 60°C) isotonic saline with vigorous stirring. After the caseinate was added, stirring continued at 60°C for an additional 15 to 30 min. After cooling, the solution was divided into 100 ml aliquots and placed in 500 ml bottles. It was autoclaved (30 min. 15 lbs - liquid setting), at which time the color changed to red or pink. It was refrigerated until needed.

Rats were weighed, and the volume of sodium caseinate to be injected was calculated as 5 ml per 100 g body weight. With the rats under light ether anesthesia, the site for the injection was washed with an alcohol wipe. The sodium caseinate, now at room temperature, was injected slowly
through a #21 needle into the peritoneal cavity. The rats were allowed to fully recover from the anesthesia before being returned to their cages.

C. Sacrifice and Wash Procedure

Four days later, each rat was given a physical examination. The eyes, nose, and inside of the front paws were inspected for discharge. The condition of the coat was noted, where a smooth, sleek, glossy coat indicates good nutrition. Breathing was observed for labored abdominal breathing or sneezing. Where possible, the feces were checked for diarrhea. Finally, the animal was weighed.

With the rat under ether anesthesia, a cardiac puncture was done. The death of the animal ensued. The blood obtained was left at room temperature for about 20 min, then placed on ice.

The rat was stretched out rather tautly on its back and its legs were pinned down. The entire abdomen was washed with 70% ethanol. A midline incision was made through the skin only, from the lower abdomen up over part of the chest. Two cuts were made to the left, at the top and bottom of the midline incision. The skin on this side was partly pulled back, then pinned down, creating a space in the peritoneum where the wash fluid might accumulate. A #19 1-1/2 inch needle was used for the washes. It was inserted at the midline. With the hole facing up it was moved along close to the peritoneum until the space to
the left was reached. The buffer was then injected, the abdomen was palpated gently, and the wash was drawn up once again into the plastic syringe.

Four washes were done per rat, with cold Phosphate Buffered Saline with Glucose (PBSG, pH 7.5 ± .1) with 10 units of heparin per ml. The experimental rats were washed with 10 ml per wash. The control females had 25 ml per wash, and the males had 30 ml per wash. Washes were collected in 50 ml plastic conical centrifuge tubes, and kept on ice.

After the completion of the four washes, the skin was similarly pulled back from the right side and any fluid which had accumulated there was collected. Usually almost all of the fluid injected was recovered.

Finally, the animal was opened up and examined for gross abnormalities.

D. Chemotaxis

The washes were centrifuged for 10 min at 1000 rpm. Pellets were resuspended in 3 ml and 6 ml for PCM and control rats, respectively. A chilled siliconized pasteur pipette was used to resuspend them in the cold PBSG.

Duplicate aliquots of the cell suspension were diluted 1:20 with 2% acetic acid and counted on a hemocytometer. The total yield, a measure of in vivo chemotaxis to sodium caseinate, was calculated from the cell count and the volume of the cell suspension. Smears were made, after the addition
of serum to a small aliquot of the cell suspension to prevent excessive lysis during the process of smearing.

E. Phagocytic Ingestion Rate

First an ammonium chloride lysis was done to eliminate erythrocyte contamination. The volume of the cell suspension was brought to 9 ml with the heparinized PBSG. 1 ml of 0.25 M NaHCO$_3$ in isotonic saline was added, resulting in a final concentration of NaHCO$_3$ in the cell suspension of 0.025 M, the mean concentration of human venous serum (Henry, 1964). The NaHCO$_3$ is necessary for a successful ammonium chloride lysis. 20 ml of 0.87% ammonium chloride were added. The tubes were covered with parafilm, gently inverted three times, and spun immediately at 1000 rpm for 10 min. This and all other centrifugations were done in a refrigerated centrifuge.

The supernatant was removed immediately and the cell pellet was resuspended in 10 ml of PBSG with 10 units of heparin per ml. The tubes were spun again.

Supernatants were drawn off, and the pellets were resuspended in Hanks balanced salt solution (Hanks BSS), pH 7.4 ± .1. The volume necessary for a count of 200 WBCs/mm$^3$ was estimated from the earlier count, assuming some cell loss during the lysis and wash. One cell count was then done per cell suspension, and the volume was adjusted to lower the count to 200 WBCs/mm$^3$, if this was necessary.
Using 1 ml plastic pipettes, 0.4 ml of the cell suspension were added to siliconized 15 ml glass conical centrifuge tubes. 0.4 ml of the Hanks BSS were added to each tube. Duplicates were run where possible.

Phagocytic ingestion rate was measured using a slight modification of the procedure of Dr. Thomas Stossel (1973).

Simulated bacteria, the 'particles' of Dr. Stossel (1973), had been prepared previously. Briefly, approximately 2 g of oil red O (Sigma, St. Louis, MO) were ground into 50 ml of diisodecyl phthalate (ICN Pharmaceuticals, Inc., Plainview, N.Y.) (density 0.94) with a large mortar and pestle for about 20 min. The saturated suspension was then centrifuged at top speed in an International Centrifuge for one hour. The solution was poured off into a dark glass bottle for storage. 10 microliters were added to 10 ml of dioxane (Fisher Scientific, Fair Lawn, N.J.), and the optical density at 525 nm was used to determine the conversion factor for the calculations. The conversion factor is equal to the density of the oil divided by the OD\textsubscript{525}. In these experiments, the conversion factor was 0.8138 mg/OD.

30 mg of \textit{E. coli} lipopolysaccharide 026:B6 (Boivin preparation) (Difco Laboratories, Detroit, Mich.) were dispersed in 3 ml of the Hanks BSS by brief sonication.

\textsuperscript{5}A different type of oil was used, and a different procedure for dissolving the oil red O, both at Dr. Stossel's suggestion (personal communication).
1 ml of the oil red O in diisodecyl phthalate was layered on top, and sonication was continued, with brief stops when the tube became warm, until the color was homogeneous and no layering was observed with time. The particles could be frozen between experiments (Stossel et al., 1973). They were briefly sonicated just prior to their use.

An equal volume of fresh control serum--pooled serum unless contraindicated--and simulated bacteria were combined, mixed well, and preincubated in a 37°C water bath. After 10 min, the tubes containing the cell suspension were added at 30 sec intervals. Five minutes later, 0.2 ml of the preincubated opsonized simulated bacteria were added to the tubes sequentially, at 30 sec intervals. After a 5 min incubation of the cells with the opsonized simulated bacteria, ingestion was stopped by the addition of 6 ml of 1 mM N-ethyl maleimide (NEM) (Sigma, St. Louis, MO) in isotonic saline, followed by vortexing for 5 sec. The total opsonization time was never allowed to exceed 23 min.

The tubes were spun at 1000 rpm for 10 min. They were gently shaken horizontally to free the oil layer from the side, then inverted. One wash was done, with 6 ml of NEM. Then the tubes were inverted as before and allowed to drain.

Cell counts were done in duplicate on the remaining cell suspension. Smears were made when possible, following the addition of serum. Viability was assessed with trypan blue (see below).
The sides of the tubes from the incubation were wiped very carefully to remove any excess oil and dye, which would adversely affect the results. 1.5 ml of dioxane were added to each tube. Tubes were vortexed and allowed to sit briefly while the cells lysed and the oil dissolved in the dioxane. They were then centrifuged for 15 min at 2000 rpm to remove cellular debris. Supernatants were read in microcuvettes at 525 nm in a Bausch and Lomb Precision Spectrophotometer against a dioxane blank.

Ingestion rate (mg diisodecyl phthalate/10^7 phagocytes/min) was calculated from

\[
\text{ingestion rate} = \frac{\text{OD}_{525} \times 0.8138 \times 2.5 \times 1.5}{5 \times (\text{no. WBCs/mm}^3) \times 10^{-4} \times \% \text{ phagocytes}}
\]

where 0.8138 is the conversion factor calculated from the OD_{525} of the oil red O in diisodecyl phthalate (see above), 2.5 is a correction factor for the volume of cell suspension used, 1.5 is a correction for the volume of dioxane used, 5 is the length of the ingestion period in minutes, and phagocytes include all macrophages and monocytes, histiocytes, PMN leukocytes and bands.

F. Nitroblue Tetrazolium Reduction

To assess nitroblue tetrazolium (NBT) reduction, the procedure for ingestion rate described above was followed, with the following modifications. The 0.4 ml of Hanks BSS were not added to the 0.4 ml of cell suspension in the siliconized centrifuge tubes. During the
incubation, 0.4 ml of NBT (Sigma, St. Louis, MO) were added to the tubes just prior to the addition of the opsonized simulated bacteria. The NBT was prepared by saturating Hanks BSS (2 mg NBT/ml Hanks BSS), followed by filtration through a 0.3 μ Millipore filter and storage in a dark bottle.

Ingestion rate was always assessed simultaneously with NBT reduction, because the rate of NBT reduction is dependent upon the ingestion rate. Where possible, duplicate incubations were run for the determination of each of the rates.

Following the addition of dioxane to the tubes and vortexing, the NBT tubes were corked and heated in an approximately 83°C water bath for 30 min, to extract the NBT-formazan. The NBT tubes were read at 580 nm. The ingestion rate (ORO) tubes were also read at 580 nm, so the NBT reading could be corrected for ORO absorbance.

The NBT reduction rate (μg formazan reduced/10^7 phagocytes/min) was calculated from:

\[
\text{NBT reduction rate} = \frac{[\text{OD}_{580} \text{(NBT tube)} - \text{OD}_{580} \text{(ORO tube)}] \times 14.14 \times 2.5 \times 1.5}{5 \times (\text{no. WBCs/mm}^3) \times 10^{-4} \times \% \text{phagocytes}}
\]

For an explanation of the various numbers, refer to the discussion of ingestion rate calculations above.

Since the rate of NBT reduction is dependent upon the ingestion rate, the NBT:ORO ratio (μg formazan/mg
diisodecyl phthalate) is the form in which the NBT reduction rate is most meaningfully expressed.

G. Serum Opsonization Factors

The ingestion rate was measured as described above, using serum from the malnourished rats with cells from the control rats. The serum was pooled unless contraindicated. Duplicate incubations were run where possible. Calculations were done as described under ingestion rate.

H. Trypan Blue Staining

The procedure followed was basically that of Boyum (1968). One drop of cell suspension and two drops of Hanks BSS were placed in a small plastic tube—more cells and less buffer were used if the cell count was below 200 WBCs/mm$^3$. One drop of 1% trypan blue (Direct Blue 14) (Matheson, Coleman & Bell, Norwood, Ohio) in isotonic saline was added and mixed. After 5 min, the number of stained cells out of 200 cells was determined. Viability was then expressed as a percent.

I. Cell Type Classification

Smears were stained with Diff-Quik (Harleco, American Hospital Supply Corp., Gibbstown, N.J.), which gives similar results to a Wright-Giemsa stain.

Duplicate differentials of 200 cells each were done, classifying the cells as one of ten types: monocytes, immature macrophages, mature macrophages, histiocytes,
polymorphonuclear (PMN) leukocytes, lymphocytes, mast cells, eosinophils, mesothelial cells, and dead cells.

Criteria for the classification of cells were based on a composite of information from many sources. Bennett (1967) has pictures of rat peritoneal macrophages. Sandoz (1973) contains useful information regarding stains and, more importantly, artefactual differences in the appearance of cells due to smearing technique. With information on morphology from Diggs et al. (1954) and the descriptions of the various stages of differentiation of mononuclear phagocytes in guinea pigs (Adams, 1974, 1975)—the latter were particularly useful—various criteria were chosen for the differentiation.

Monocytes were small cells with relatively large nuclei. The nuclei were central for classification as a monocyte unless the nucleus was so large and the cell so small that the nucleus took up most of the cell and could not really be central. Mature macrophages were large cells with eccentric nuclei. The nucleus:cytoplasm ratio was small—that is, the nucleus was relatively smaller. Immature macrophages fell between the other two categories, where the nucleus was too eccentric or too small or the cell too large for classification as a monocyte. Alternately the nucleus might be too central or too large or the cell too small for classification as a mature macrophage, so the classification would be immature macrophage.
Histiocytes were identified by their large pinkish nuclei and cytoplasm, and were often found in clumps. PMN leukocytes were identified by their typical multilobular nucleus and neutral staining cytoplasm. Bands were counted as PMN leukocytes. Lymphocytes were recognized by their round nuclei and their extremely sparse cytoplasm (even less than a monocyte). Mast cells and eosinophils were identified on the basis of their purple and red granules, respectively. Mesothelial cells had rather large nuclei. The cytoplasm stained blue in a blur around the nucleus. Due to the difficulty of distinguishing mesothelial cells from lysed or broken cells, they were counted only where an outline of the cell membrane was visible beyond the stained cytoplasm. Finally, the dead cells were identified by pyknosis and other standard characteristics (La Via and Hill, 1971).

The duplicate differentials were averaged and the result expressed as a percent of the total cells.

J. Total Protein Determination

A modified Biuret reagent test was done in duplicate on all rat sera. The amount of hemolysis of the serum was noted, as it seemed to affect the color of the sample in the Biuret reagent. The test was done within one week of the experiment. Serum was frozen in the interim.
K. Serum Albumin Determination

Serum albumin levels were measured with Albustrate serum albumin reagent (General Diagnostics, Warner-Lambert Co., Morris Plains, N.J.). The readings were done almost immediately. Again, the test was done in duplicate within one week of the experiment, on serum frozen in the interim.

L. Statistical Analysis

Results are expressed as the mean plus or minus the standard error of the mean. Possible differences between populations were evaluated with the Student's t-test. Correlation coefficients were used to compare different variables within a population.
IV. RESULTS

A. Health and Nutritional Status

The findings of the physical examinations are shown in Table II. As can be seen, nasal discharge was found only in the PCM rats whereas diarrhea was found only in the controls. Sneezing and labored breathing were found with essentially equal frequency in the controls and the PCM rats. Dried discharge was never observed on the front paws, so this category was omitted from the table. For lack of another means of assessment, the existence of any one or a combination of these signs was taken as the criterion for an unhealthy condition.

The condition of the coat probably more nearly reflects nutritional status. The coat was described as 'healthy,' 'satisfactory,' 'poor' meaning rumpled and dull but fairly complete, 'thin' indicating a poor coat with hair loss at the neck and/or shoulders, and 'very poor' when there was more extensive hair loss, often resulting in patches of denudation. As was anticipated, the control coats were generally in fine condition whereas the coats of PCM rats fell almost exclusively into the range indicating poor nutrition.

Two PCM rats originally included in this study were injected with the sodium caseinate. By the end of the four day waiting period, however, their three to four cagemates had turned cannibalistic, a finding apparently
### TABLE II

Scores on the Physical Examination

<table>
<thead>
<tr>
<th>Nasal Discharge</th>
<th>Sneezing</th>
<th>Labored Breathing</th>
<th>Diarrhea</th>
<th>Unhealthy by one or more criteria</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Malnourished</td>
<td>8</td>
<td>5</td>
<td>2</td>
<td>0</td>
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</table>

<table>
<thead>
<tr>
<th>Coat</th>
<th>Healthy</th>
<th>Satisfactory</th>
<th>Poor</th>
<th>Thin</th>
<th>Very Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malnourished</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>7</td>
</tr>
</tbody>
</table>

*a Number afflicted out of 17 per group*

### TABLE III

Biochemical Data Relating to Nutritional Status

<table>
<thead>
<tr>
<th>Serum Protein (g/100 ml)</th>
<th>Serum Albumin (g/100 ml)</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.27 ± .10</td>
<td>3.40 ± .05</td>
</tr>
<tr>
<td>Malnourished</td>
<td>5.18 ± .04</td>
<td>2.67 ± .03</td>
</tr>
<tr>
<td>t</td>
<td>9.86</td>
<td>12.77</td>
</tr>
<tr>
<td>p</td>
<td>&lt;.001</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*a Mean ± SEM*
not uncommon in protein-malnourished rats. Evidence for this interpretation in one of the two cases included the skeleton, and the elevated serum protein and albumin levels of one of its cagemates which was sacrificed soon thereafter.

Nearly half of the PCM rats were observed eating either feces or paper towels, when these were accessible. Presumably this reflects their malnourished condition. One of the PCM females had a large ovarian cyst. However, this should not have affected the results. The majority of the control rats (13/17) were observed to have fatty infiltration of the liver, but this was never observed in the PCM rats. Finally, it has been suggested that many rats from this colony, regardless of diet, suffer from a chronic enteritis, which could affect the nutritional status, in addition to chronic respiratory diseases such as the chronic pneumonia which is endemic in the colony.

Serum protein and albumin levels are given in Table III. A very significant difference between the control and PCM rats was found for both protein and albumin levels.

Despite the good duplication and consistency of the protein and albumin data within each group, no correlation between protein and albumin levels was observed for either the control (\( r = .386 \)) or the PCM (\( r = .271 \)) rats. A possible explanation for this may lie in the pronounced effect serum hemolysis was found to have on the determined total protein level but not on the albumin level. Serums
were rated for hemolysis on a scale of 1 to 5 (no hemolysis to extreme hemolysis) and the protein and albumin levels of those rating 5 (7/17 controls and 3/16 PCM rats) were compared to all other rats within their groups. A very significant difference ($p < .001$) was found with hemolysis in the control group, and a difference ($p < .05$) was found in the PCM group. Hemolyzed serum resulted in an apparently higher protein level. No difference was found in albumin levels with hemolysis in either group.

It was not always possible to evaluate each rat on the entire battery of tests. This was particularly a problem in the PCM rats (see Table IV), where cell yields were low. Duplicate incubations for each test were given priority over single incubations for more tests. The priorities were set such that ingestion rate with control serum was determined first, followed by the NBT reduction rate, with ingestion rate with malnourished serum receiving the lowest priority.

### TABLE IV

The Number of Data Points Available for a Given Variable

<table>
<thead>
<tr>
<th></th>
<th>Total Proteins</th>
<th>Serum Albumins</th>
<th>Chemotaxis</th>
<th>ORO</th>
<th>NBT: ORO</th>
<th>Opsonization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>17</td>
<td>16</td>
<td>17</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Malnourished</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>16</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>
B. Cell Type

The effect of diet on the kind of phagocyte found in the peritoneal cavity four days after sodium caseinate injection is tabulated in Table V. Although 5 other cell types were differentiated, numbers were too low and too variable for any except the dead cells (refer to the trypan blue section below) to be tabulated or discussed.

The results of the differential may be presented in a variety of ways. When macrophages are expressed as the percent of all cells obtained, the average is about 55% and there is no significant difference with diet. When PMN are similarly expressed, the average is 20%, with no significant difference between the control and PCM groups. The proportion of mature macrophages, expressed either as the percentage of all cells obtained or the percentage of all non-histiocyte macrophages, was significantly higher \((p < .05)\) in the control than in the PCM rats. Relative monocyte levels in the control and malnourished animals, expressed similarly as either the percentage of total cells or the percentage of macrophages, were not significantly different.

In terms of cell maturation, there was a significant negative correlation \((r = -.65, p < .01)\) between mature macrophages and monocytes, expressed as a percentage of total cells, for both the control and the PCM rats. Thus, as more macrophages reach the mature stage, apparently
<table>
<thead>
<tr>
<th></th>
<th>Macrophages</th>
<th>Mature Macrophages</th>
<th>Monocytes</th>
<th>PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Total Cells</td>
<td>% Phagocytes</td>
<td>% Total Cells</td>
<td>% Macrophages</td>
</tr>
<tr>
<td>Control</td>
<td>53 ± 2</td>
<td>70 ± 2</td>
<td>8 ± 2</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>Malnourished</td>
<td>56 ± 2</td>
<td>76 ± 2</td>
<td>4 ± 1</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

\[ t \] = -1.107, -1.880, 2.156, 2.298, .697, .892, 2.119

\[ p \] = >.05, >.05, <.05, <.05, >.05, >.05, <.05

\(^a\)Mean ± SEM
fewer are still in the initial stage identifiable in the peritoneal cavity as the monocyte (see Figure 1).

C. Chemotaxis

A significantly higher total cell yield and higher total numbers of macrophages and PMN were found in the control rats over the PCM rats (see Table VI).

Possible reasons for the differences were also tabulated in Table VI. A very significant difference ($p < .001$) in body weights exists between males and females of the control and PCM groups, a difference which has been noted for many generations in this colony. Also evident is a significant sex difference in control weights but not in malnourished weights. Total cell number correlates with body weight both in the control ($p < .02$) and the PCM rats ($p < .05$). When cell yields are therefore corrected for body weight, there are no longer significant differences between the groups.

The possibility that health was also playing a role was investigated. The information in Table II was used to group the rats. No significant difference in the number of cells per gram between the healthy and the unhealthy rats was found in either the controls or the malnourished group.

One other correlation was found in the control rats. When total cell yield was compared to the percent of mature macrophages (of all cells), a significant
Figure 1

Comparison of the Percentage of Mature Macrophages and Monocytes for Control and PCM Rats.

The solid lines are the least squares lines. The arrow heads represent data points, which are the mean percentages (percent cells of all types) from duplicate differential counts of 200 cells each. Control data is black, whereas data from the malnourished rats is represented in red.
### TABLE VI

The Effect of PCM on Cell Yield, Macrophage and PMN Yields, and Weight<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>Total Cell Yield x 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Total Macrophages x 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Total PMN x 10&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Cells/g body wt. x 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Control</td>
<td>52.783±9.608</td>
<td>28.391±5.785</td>
<td>11.815±2.359</td>
<td>258±38</td>
<td>231±4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malnourished</td>
<td>13.685±1.679</td>
<td>7.762±1.089</td>
<td>2.308±0.315</td>
<td>214±23</td>
<td>65±3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

|                  |                                  |                                  |                             |                                 | Female     |
|                  |                                  |                                  |                             |                                 |            |
|                  | t                                | 4.128                            | 3.608                       | 4.118                           | 36.534     |
|                  | p                                | <.001                            | <.01                        | <.001                           | <.001      |

<sup>a</sup>Mean ± SEM

<sup>b</sup>Control Male vs. Female wts., t = 15.629, p < .001

<sup>c</sup>Malnourished Male vs. Female wts., t = 1.555, p > .05
correlation \( (r = .683, p < .01) \) was found in the control rats, especially in the males \( (r = .793) \). This correlation was not found in the PCM rats as a whole \( (r = .413) \) or in the males alone \( (r = -.454) \).

D. Ingestion Rate

The ingestion rate results are presented in Table VII. No significant difference was found in the ingestion rate of control versus PCM phagocytes. There was no correlation between the percent of the phagocytes which were macrophages and the ingestion rate in either control \( (r = .133) \) or PCM \( (r = .230) \) rats. The possible effect of health on ingestion rate was investigated as above, but no significant difference was found.

E. Nitroblue Tetrazolium Reduction

Since the NBT enters the cell before being reduced, the rate of NBT reduction depends upon the ingestion rate (Stossel, 1973), as it does not enter the cell by diffusion or active transport across a membrane. Therefore, NBT reduction rates are expressed as the ratio of NBT:ORO. As a check on the procedure, a test for correlation between ingestion rate and NBT reduction itself was done, but no significant correlation was found for the control group \( (r = .446) \) or the PCM group \( (r = .557) \). However, when control and PCM rats are grouped together, there is a significant although weak correlation \( (r = .453, p < .05) \). It is believed that a few divergent controls and the small
Table VII
Phagocyte Function and Serum Factors in PCM

<table>
<thead>
<tr>
<th>Phagocytes</th>
<th>Ingestion Rate (mg diisodecyl phthalate/10⁷ phagocytes/min.)</th>
<th>NBT (µg formazan/10⁷ phagocytes/min.)</th>
<th>NBT:ORO (µg formazan/mg diisodecyl phthalate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Serum (ORO)</td>
<td>Malnourished Serum</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>.1515 ± .0089</td>
<td>.1289 ± .0083b</td>
<td>1.493 ± .115</td>
</tr>
<tr>
<td>Malnourished</td>
<td>.1688 ± .0073</td>
<td>--</td>
<td>1.471 ± .184</td>
</tr>
</tbody>
</table>

p > .05 > .05 > .05

a Mean ± SEM
b Ingestion Route, Control vs. Malnourished Serum; Paired one tailed t test, t = 1.913, p < .05
c NBT:ORO vs. ORO - Control r = -.611, p < .02
 Malnourished r = -.471, p > .05
number (4) of PCM rats prevented a correlation within the individual groups. The results have been expressed in terms of the ratio because the data supporting a dependence of NBT reduction on ingestion rate seems convincing.

There was no significant difference in the mean NBT:ORO ratio of the control versus the PCM rats (see Table VII). There was no correlation \( r = -0.004 \) between the NBT:ORO ratios and serum albumins when control and PCM rats were grouped together.

The ratio of NBT:ORO is supposed to be directly proportional to the ingestion rate. However, when a correlation was done, there was instead a negative correlation \( r = -0.611 \), \( p < 0.02 \) in the controls. In the PCM rats, with only four NBT:ORO ratios, \( r = -0.471 \) and was not significant.

F. Opsonization

When a one-tailed paired t test was used to analyze the effect of control versus malnourished serum on ingestion rate, a significant difference \( (p < 0.05) \) was found. As anticipated, the ingestion rate was significantly lower when malnourished serum was used for opsonization.

When two control rats were studied on a given day, for each rat two incubations were done using malnourished serum and two using control serum. The same malnourished serum and control serum were used for the two rats.
Therefore the ratio of the mean ingestion rate with malnourished serum to the mean ingestion rate with control serum should be identical for each rat. In fact, the ratios were very similar the last three times two control rats were used: 68.0% and 68.1%, 100.8% and 97.8%, 101.9% and 97.0%. This suggests that rather than a consistent decrease in serum opsonins in the PCM rats, there is probably a range from quite low to actually normal levels. The reduced opsonization in the one example above was associated with the lowest mean serum albumin level (\( \bar{X} = 2.51 \text{ g/100 ml} \)) and both PCM rats were sick, which was not the case on the other two occasions. Individual rat sera must be used for the opsonization before meaningful correlations can be run with respect to health or serum albumin levels.

No comment can be made concerning the effect of malnourished serum as compared with control serum on PCM cells, because only one data point is available. The very fact that only one rat had a high enough cell yield for this test suggests that the result was not representative.

G. Trypan Blue

The results of the trypan blue test are given in Table VIII. There was no significant difference in viability as assessed by trypan blue between the malnourished and control rats. The percentage of cells not classified as dead from the differentials gives another
indication of viability. The results here are also shown in Table VIII. No correlation was found between the two tests. This may indicate that different cell types were being assessed. The trypan blue results were not used in the calculations.

TABLE VIII

Comparison of Two Assessments of Cell Viability

<table>
<thead>
<tr>
<th>% Viable</th>
<th>Trypan Blue</th>
<th>Differential</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>81 ± 1</td>
<td>85 ± 1</td>
<td>.034</td>
</tr>
<tr>
<td>Malnourished</td>
<td>78 ± 2</td>
<td>84 ± 2</td>
<td>-.069</td>
</tr>
<tr>
<td>t</td>
<td>&gt;.05</td>
<td>&gt;.05</td>
<td></td>
</tr>
</tbody>
</table>

aMean ± SEM
V. DISCUSSION

A. Health and Nutritional Status

The various coat changes observed were those characteristic of PCM, and were found in the PCM rats. Signs of infection, including nasal discharge, sneezing, labored breathing, and diarrhea, were found in both the control and PCM rats. Should it have been true that rats from both groups suffered from chronic enteritis, this would have compromised the nutrition model.

The existence of infection could have affected almost all of our tests, although reports in the literature are not always in agreement as to whether infection would enhance or depress the particular test. Infection has been reported to affect chemotaxis, at least when measured in vitro (Mowat and Baum, 1971; Anderson et al., 1974; Meltzer et al., 1975), ingestion rates and NBT:ORO ratios (Stossel, 1973), and complement levels (Rose and Molotchnikoff, 1973; Chandra, 1975). We found no statistical effect of infection on any of these tests. However, infection probably contributed to the scatter in our results.

Our control serum protein and albumin levels were in the general range reported by others for control rats (Edozien, 1968; Keusch et al., 1976). Our PCM albumin levels were slightly higher on the average than those reported by Edozien (1968) for marasmic rats. However, our serum proteins were considerably higher than those he
reported for the same rats. It is suggested that the apparent increase in protein level caused by serum hemolysis may be responsible for this discrepancy.

On the basis of coat changes and serum protein and albumin levels, our PCM rats may be classified as marasmic-kwashiorkor in the spectrum of protein-calorie malnutrition.

B. Chemotaxis

Chemotaxis is the "directional movement of phagocytes in response to chemotactic gradients" (Mac Gregor et al., 1974). When chemotaxis was assessed in vivo by the determination of the total cell yield four days after i.p. sodium caseinate injection, an almost fourfold difference (p < .001) was found in total cell yield between the control and PCM rats. However, this difference could be explained by the dissimilarity in weights between the two groups. The number of cells per gram body weight was not found to be significantly different (p > .05) in the PCM versus the control rats.

Gray (1964) studied the effect of protein malnutrition on chemotaxis. His malnourished rats were females of the Sprague-Dawley strain which were tested 30 days after being weaned onto a 20% gluten diet, where the controls had a 26% casein diet. He measured chemotaxis both with an adaptation of the Rebuck "skin window" and additionally at various times after i.p. sodium caseinate injection. The latter method, which we used, proved superior in
distinguishing between the control and malnourished rats. Gray found less rapid leukocyte mobilization in the malnourished rats, and a lower peak response. However, if one corrects his cell yields at 50 hours after the injection for reported differences in body weight between the two groups, one finds an equal to slightly increased number of cells per gram body weight for the malnourished rats. Thus, his difference in cell yield can, like ours, be explained by differences in body weight.

The curves drawn by Gray to demonstrate the change in cell yield with time had not plateaued by 50 hours post-injection. However, the data points between 20 and 40 hours fell well below the curve in each group. Perhaps this inaccuracy, then, explains the apparent difference between these curves and those of Slonecker and Osmanski (1974), where plateauing was observed at 8 hours post-injection and the decline had begun by 48 hours. The total number of cells was drastically reduced by 72 hours, a full 24 hours before we harvested our peritoneal exudates.

Slonecker and Osmanski studied male Wistar rats, 30 days after weaning onto a 0.5% protein or an 18.0% protein diet for the malnourished and control rats, respectively. They assessed chemotaxis in response to the intraperitoneal injection of latex particles, at various times up to 72 hours. The malnourished rats were found to have approximately one-half the number of cells of the control rats at the various times. However, weight differences
were not considered, nor was information on body weight given. Judging from the results of Gray (1964), who used a similar nutrition model but had the malnourished rats on a diet somewhat higher in protein, one would estimate that the malnourished rats weighed approximately one-half as much as the controls. If this was the case, the difference in cell yield was once again associated with a parallel and compensatory difference in body weight.

The apparently declining total cell yield found by Slonecker and Osmanski (1974) at 72 hours was significantly lower than the total cell yield we found at 96 hours in both control and PCM rats. However, this difference may be related to a difference in irritant (latex versus caseinate). Although our control rats at 3 months of age were quite possibly heavier than their controls at 2 months of age, our 16th and 17th generation malnourished rats were most likely not significantly heavier than their experimental group. Consequently the difference in cell yield cannot be totally explained by reference to body weights.

The results of Aschkenasy (1972) reveal further problems with the weight correction for cell yield. Using male Sherman rats, Aschkenasy had malnourished rats which were deprived of protein for two months starting at 56 days of age. The renourished rats were then fed an 18% casein diet for 15 days. The controls were fed a normal diet throughout. Cell yields were enumerated 5 hours after the intraperitoneal injection of glycogen. The total cell
yield was very depressed in the malnourished rats, as expected. However, the renourished rats, after only 15 days of rehabilitation, had cell yields approximately double those of the malnourished rats and already around 75% of those of the controls. Although weights of the various groups of rats were not given, it seems quite unlikely that the weights paralleled the cell yields in this case.

Using a different experimental design, Bhuyan and Ramalingaswami (1974) studied adult male rabbits which had been maintained on 20% casein or no casein diets for 8 weeks. The rabbits were then immunized with BCG. A poorly developed primary complex was observed in the malnourished rabbits, with only focal, ill-formed granulomas, in contrast to a normal, healthy response in the control animals. Thus, in the final analysis, the phagocytic response in a test case in protein malnourished animals was inadequate.

Our findings of a difference in total cell yield between control and PCM rats associated with a parallel difference in weight is in agreement with the results of others (Gray, 1964; Slonecker and Osmanski, 1974). It should be noted that in this test system a defect in chemotaxis could not be readily distinguished from reduced production or release of precursor cells from the bone marrow, or from faulty diapedesis from the blood vessels (MacGregor et al., 1974). In fact, the apparent correlation of cell yield with body weight suggests that reduced production of
phagocytes in a smaller animal compared to a larger animal may be the best explanation. The decreased blood white cell count in malnourished animals reported by Gray (1964) also supports the idea of reduced production with malnutrition.

While differences in total cell yield may often correlate with differences in body weight, it is not clear that this is always the case (Aschkenasy, 1972) nor that this implies corresponding adequacy of the response (Bhuyan and Ramalingaswami, 1974). There is no reason to assume that the number of phagocytes necessary for adequate host defense is related directly to body weight. Since pathogens come from the environment, the area of interaction with the environment--best approximated by the surface area of the body--is more likely to indicate the amount of protection required.

The relative surface areas of the control and the PCM rats can be approximated from body weight^{0.67} (Guyton, 1971). When the total cells per gram body weight^{0.67} are calculated, the control rats average 1485 ± 235 compared to an average of 842 ± 93 for the PCM rats. The major difference noted here suggests an impaired capacity to mobilize enough phagocytes for adequate host defense in our PCM rats, compared to the control rats of the same age. Reduced phagocyte mobilization may be one explanation for the increased susceptibility to infection found in our 16 and 17 generation PCM rats.
C. Cell Type

Of all the peritoneal exudate cells, 55% were macrophages and 20% were PMN leukocytes, with no significant difference between the control and the PCM rats. One might have expected a lower percentage of macrophages with malnutrition based on reports in the literature (see discussion below). However, if one observes the trends reported by Slonecker and Osmanski (1974), it may be that a long period of depression in macrophage mobilization had previously occurred. However, at 72 hours post-injection they show steady rates of decline for PMN leukocytes in both control and PCM rats, and for macrophages in the controls. The macrophages for the PCM rats, on the other hand, seem to be almost at a plateau level. The coincidence of these four trends at 96 hours, might result in a similar percentage of macrophages and PMN leukocytes in the two groups despite actual marked differences in the rates of mobilization of phagocytes. Proportionately decreased rates of macrophage mobilization in response to Salmonella typhimurium have been reported in protein-malnourished rats (Newburne et al., 1968). Bhuyan and Ramalingaswami (1974) observed a delayed and deficient mobilization of macrophages in protein-deficient rabbits, and Dobsin and Bawden (1974) found fewer macrophages in response to parasite infestation in protein-malnourished sheep. However, although differences in the rates of mobilization may have been masked by examining only one
time point 96 hours after the injection, the fact remains that we found no difference in the percent of macrophages between our two groups at this point.

Of course there were significant differences in the total numbers of macrophages and PMN leukocytes from the control versus the PCM rats, due to the significant difference in total cell yield. The possible significance of these differences is reflected in the discussion of the significance of differences in total cell yield in the section on chemotaxis.

The average proportion of macrophages we found in the exudates was about 55%, which compares favorably to the "more than 50%" macrophages at the time macrophage yield was maximal in the results of Slonecker and Osmanski (1974). On the other hand, one might have hoped that macrophages would constitute a higher proportion of all phagocytes than approximately three out of four. However, even this proportion appears to exceed that found in the controls of Slonecker and Osmanski (1974) at 72 hours post-injection. Although macrophages constituted the majority of the phagocytes in our rats, the PMN leukocytes were present in sufficient numbers that the effect of their presence cannot be overlooked in the interpretation of the data regarding phagocyte function.

In both control and PCM rats, the percentage of mature macrophages was found to increase as the percentage of monocytes decreased. Monocytes develop to become immature
macrophages and then mature macrophages, and the inverse relationship found follows logically from this sequence. The apparent difference in the rate of increase in the percentage of mature macrophages as the percentage of monocytes decreases, between the control and PCM rats may suggest a slower rate of transition between immature macrophages and mature macrophages in the malnourished rats.

In fact, the percentage of mature macrophages was found to be higher in the control rats than in the PCM rats. Slonecker and Osmanski (1974) also found differences in the subclasses of macrophages in the peritoneal exudates from their malnourished rats in comparison with their controls. Bhuyan and Ramalingaswami (1974) observed deficient activation of macrophages in protein-deficient rabbits.

The meaning of these results depends upon the significance of cell maturation in the macrophage. Changes associated with maturation in the macrophage include increased phagocytic ability, increased numbers of lysosomes with changes in lysosomal enzymes, increased numbers of Ig G receptors, and increased antigen processing and interaction with lymphocytes (Cline and Gold, 1973). Applying our terminology to Cline and Gold's information (1973) (i.e., reclassifying his cell types using our criteria), it is clear that only in our mature macrophages had full functional potential been attained. Adams (1974)
states that the maturation or lack thereof of the macrophages may determine the outcome of a pathogenetic attack on the host.

Thus, despite the relative similarity in the proportions of macrophages and PMN leukocytes between the control and the PCM rats, the relative deficiency of mature macrophages in the PCM rats may help explain the lower resistance of their host defense systems.

D. Ingestion Rate

No significant difference was found between the ingestion rates of the control and the PCM rats.

The fact that the rats had been maintained on 1 mg of tetracycline per ml of drinking water for their entire lives is unfortunate. A single dose of tetracycline (100 mg/kg) has been found to depress phagocytic activity in rats for at least 48 hours (Lemperle et al., 1971). Information from the animal caretakers indicates that rats drink less per gram body weight as they get heavier. However, from their experienced estimations, all of our rats were receiving each day at least 3 times the dose of tetracycline found to depress phagocytosis. Thus, the rates determined may have been depressed in comparison with the true rate, in the absence of the antibiotic. Since the malnourished rats were probably drinking a larger volume of water per gram body weight than the controls, the effect on ingestion rate may have been more pronounced in the PCM rats.
To the best of our knowledge, only one study has been done previously on the effect of protein-calorie malnutrition on peritoneal macrophages. Keusch and coworkers (1976) compared infected marasmic-kwashiorkor Sprague-Dawley rats which had been maintained on a 0.5% lactalbumin diet for 4 weeks, since a week after weaning, to controls which were on an 18% lactalbumin diet. The peritoneal exudates were harvested 24 hours after the i.p. injection of 1% glycogen, and macrophages present were observed to be "relatively immature monocytes." No difference was found in the rate of ingestion of latex particles between the control and the PCM rats. Thus despite differences in the nutritional model, and in the maturity and perhaps the relative proportion of macrophages at 96 versus 24 hours post-injection, our results brought us to the same conclusion as did those of Keusch and associates.

In humans, blood monocytes have been studied for changes in kwashiorkor (Douglas and Schopfer, 1974). The monocytes from children ages 10 to 30 months were isolated by adherence to glass. Ingestion of polystyrene particles and of antibody-coated erythrocytes was examined. There was no significant difference with kwashiorkor in either the percentage of monocytes involved in phagocytosis or the number of particles per monocyte. Again, despite experimental differences, our work supports this finding in humans.
In contrast to these results in monocytes and peritoneal macrophages, a variety of studies on the effect of protein malnutrition on the fixed macrophages of the reticuloendothelial system have all found a decrease in ingestion rate in the malnourished animals (La Via et al., 1956; Ratnakar et al., 1972; Deo et al., 1973; Passwell et al., 1974). The studies were done primarily in males, using mice, rats, or rhesus monkeys. Clearance of carbon, radio-labelled bacteria, or erythrocyte stroma was measured. The nutritional models varied, although all began deprivation in post-weaning or adult animals. The reason for the different responses to protein malnutrition of free and fixed macrophages is unclear.

The effect of PCM on PMN leukocytes was studied in male rats by Mills and Cottingham (1943) and by Guggenheim and Buechler (1946). Their results were contradictory. Mills and Cottingham found a decrease in the number of bacteria per blood phagocyte for a 6% versus an 18% casein diet. Interestingly, they also observed a decrease in ingestion rate (not statistically significant) as the protein concentration increased to 24% and again to 36% casein. Guggenheim and Buechler (1946) found instead an increase in phagocytic activity when caloric intake was restricted.

More recent work on the effect of PCM, or a history of PCM, on the ingestion rate of PMN leukocytes has been done in rabbits (Bhuyan amd Ramalingaswami, 1974) and in children (Selvaraj and Bhat, 1972; Seth and Chandra, 1972;
Schopfer and Muralt, 1974; Jose et al., 1975). These studies have uniformly found no effect of PCM on ingestion rate in PMN leukocytes.

On the basis of these studies, we expected no difference in ingestion rate in the PMN leukocyte in our malnourished rats. The fact that no difference was to be expected in the ingestion rate of either macrophages or PMN leukocytes with protein-calorie malnutrition explains the lack of correlation of the type of phagocyte with the level of the ingestion rate.

E. Nitroblue Tetrazolium Reduction

No difference was found in the rate of NBT reduction between the 16 control and the 4 PCM rats in which NBT reduction was measured. Neither was a difference found when the ratio of NBT reduction to ORO (ingestion rate) was compared. However, due to the small number of PCM rats studied, these findings cannot be considered conclusive.

In our hands, the NBT test was found to be rather insensitive. NBT reduction depends upon the ingestion rate, since the NBT must enter the cell. Additionally, hydrogen peroxide production increases with increasing ingestion rate (Root, 1975), so a resultant increase in NBT reduction would be expected as the ingestion rate increased. However, we found only a weak correlation between NBT and ORO, and the line calculated from the least squares did not have the steep slope which would have been
expected from the anticipated dual effects on NBT reduction associated with increasing ingestion rate.

With a slope of less than one for the least squares line of NBT versus ORO, the ratio of NBT:ORO when plotted against ORO must necessarily show an inverse proportionality. Only had the expected steep slope ($m > 1$) been observed would the ratio of NBT:ORO have been positively correlated with ORO. The negative correlation between the NBT:ORO ratio and ingestion rate, then, simply reflects the insensitivity of our NBT test to increasing ingestion rate. However, Shousha and Kamel (1972) found only a slight increase in NBT reduction when comparing resting cells to phagocytizing cells, with considerable overlap in the values between the two. Therefore, the lack of sensitivity of the NBT test to ingestion rate within our range of ORO values apparently reflects the limitations of the NBT test.

Others have done the NBT test on PMN leukocytes from malnourished children, with conflicting reported results: no difference from controls (Altay et al., 1972), occasional increase with kwashiorkor (Rosen et al., 1975), and a significant decrease with malnutrition (Shousha and Kamel, 1972), the decrease sometimes being noted in only some of the children (Kendall and Nolan, 1972). A decrease with malnutrition was found in the NBT test in rabbits (Bhuyan and Ramalingaswami, 1974). Clearly, considerable confusion exists in the literature regarding
the effect of malnutrition on NBT reduction in the PMN leukocyte.

No report of the effect of malnutrition on the NBT test in peritoneal macrophages was found in the literature. Keusch et al. (1976) did find diminished hexose monophosphate shunt (HMS) activity in peritoneal macrophages from rats with marasmic-kwashiorkor. Since the NBT test is believed to reflect HMS activity, one might have expected decreased NBT reduction in the PCM rats. However, infection is known to result in increased NBT reduction (Karnovsky, 1973) so perhaps the effects would neutralize each other, resulting in no difference with diet as we found.

While our results do not clarify the conflicting results of others concerning the effect of malnutrition on the NBT test, neither do they seriously differ with the reports in the literature.

F. Opsonization

When opsonization with serum from the PCM rats was compared to opsonization with control serum, a statistically significant decrease in the ingestion rate was found with PCM serum. However, upon closer examination, it was apparent that serum opsonins in some PCM rats were at approximately normal levels, whereas in other PCM rats they were quite depressed. Both infection and low serum albumin levels were associated with the lowest opsonin levels.
Keusch et al. (1976) found decreased opsonization of *S. aureus*, *E. coli* and *S. enteriditis* with serum from marasmic-kwashiorkor rats, when ingestion by "relatively immature peritoneal exudate monocytes" was measured.

Serum haemolytic complement and serum C3 were found to be significantly decreased in children with primary PCM (Chandra, 1975). Chandra also observed that infection was associated with an even more pronounced depression of serum C3. Thus the association of infection with our lowest observed opsonin levels may not be coincidental. Sirisinha and Suskind (1974) measured individual components of complement and found all but C4 to be depressed in malnourished children. They also noted that depression was greater in children with kwashiorkor, and that protein was more important than calories for rehabilitation. Again, the association we found of the lowest serum albumin levels with the lowest opsonin levels may have been more than coincidental.

Complement levels as measured by a sensitive radial hemolysis in gel have also been found to be depressed in protein-malnourished rats (McGhee et al., 1974). They placed their five-day pregnant females on 8% versus 25% casein diets. All resultant pups were found to have very low serum complement levels during the first 10 days of life. However, in the control pups complement levels increased following weaning and a low adult level was approached by day 35, whereas serum complement in the PCM
rats lagged further behind the controls with increasing age after weaning. The authors note that even by 90 days of age, when control rats had attained the adult level, the complement titer was still lower in the PCM rats. The rats in our experiment averaged 84 days of age at the time of sacrifice, and our multigenerational model of malnutrition would be expected to have, if anything, a more pronounced effect, so decreased complement levels were to be expected.

However, prolonged protein malnutrition is not necessary for an effect on serum opsonin levels in rats. When adult male rats were provided with water but no food for 24, 48, or 72 hours, the ingestion rate of control Kupffer cells was decreased 45% when starved serum rather than control serum was used for opsonization (Saba and DiLuzio, 1968). Five of a total of 10 of our adult male control rats were without food either on the day of sodium caseinate injection or on the day of sacrifice. (There was no overlap.) It is possible that one or more of these rats may have been without food for 24 hours at the time of sacrifice and blood collection. Although peritoneal exudate cells rather than liver macrophages were used in our experiments, it is possible that on some days our ingestion rates were inadvertently decreased due to opsonization with starved serum. The fact that serum from the control rats, usually a male and a female, was generally pooled may hopefully have averted any adverse effect of control serum on the ingestion rate.
G. Higher Risk of Parous Females for *S. aureus*-Infected Dermatitis

We were asked to determine the reason for the greater susceptibility of parous females, most especially those in the PCM group, to *S. aureus*-infected dermatitis. On the basis of our results and further information from the literature, we would like to suggest a possible etiology.

The opsonization of *S. aureus* has been studied using rat serum (Slopek et al., 1965). Complement and another factor, possibly properdine, were found to be necessary and sufficient for the opsonization of *S. aureus*. Our ORO test provides a "quantitative functional assay for C3 and the properdin pathway" of complement activation (Stossel et al., 1973). The lipopolysaccharide of our simulated bacteria activates this alternate pathway.

We found depressed opsonin levels in some of our PCM rats. Where complement itself was measured, depressed complement levels have been found in rats of approximately the same age which were protein malnourished during and since gestation (McGhee et al., 1974). Depressed complement levels may be found, then, in adult protein-malnourished rats.

It has also been found that pregnancy and lactation can more than halve the complement levels of normal, well-nourished female rats (mcGhee et al., 1974). The complement levels of pregnant and lactating protein-calorie malnourished females would, then, be expected to be particularly depressed.
It is proposed that parous PCM females are at higher risk for *S. aureus*-infected dermatitis because complement, which is essential for the opsonization of *S. aureus*, is particularly depressed during the pregnancy and lactation of female PCM rats. The fact that the only control rat in which *S. aureus*-infected dermatitis did not resolve spontaneously was a parous female (Galler et al., 1976) is in agreement with the concept that the depletion of complement by pregnancy and lactation is a key factor in the etiology of *S. aureus*-infected dermatitis.
VI. SUMMARY AND CONCLUSION

1. There were significantly fewer cells in the peritoneal exudates of malnourished rats, corresponding to their lower weights. The number of cells per weight$^{0.67}$, which approximates surface area, was significantly reduced.

2. Similar percentages of macrophages and polymorphonuclear leukocytes were present in exudates from the malnourished and control rats. However, the percentage of mature macrophages was lower in the malnourished animals.

3. There was no difference in the initial ingestion rate between the two groups, when measured using simulated bacteria.

4. Due to insufficient cell yields from the malnourished rats, results concerning NBT reduction were inconclusive.

5. Opsonization of test particles was less effective with serum from some of the malnourished rats, suggesting that serum opsonin levels were reduced in these animals.

6. It is concluded that the decreased numbers of exudative phagocytes, the relative immaturity of the macrophage population, and the reduced efficiency of opsonization may help explain the increased susceptibility to infection found with malnutrition.
VII. SUGGESTIONS FOR FUTURE RESEARCH

1. Similar proportions of macrophages and PMN leukocytes were found in exudates from the control and malnourished rats. In order to determine whether this similarity was only coincidental, as suggested by reports in the literature, one might determine the time course of exudation of the two types of phagocytes in the multigenerationally malnourished rats. The time course may vary with the irritant, which would explain the differing results in the literature. Blood counts and hematocrits done simultaneously with the exudate determinations might provide further insight into the primary defect.

2. Significant numbers of polymorphonuclear leukocytes were present in our exudates. The use of purified populations of macrophages might uncover certain defects masked due to the contamination. Of particular interest would be a comparison of NBT reduction in purified populations of macrophages and PMN leukocytes, as preliminary results suggested that in rats the two types of phagocytes respond differently to this test.

3. Serum opsonin levels were decreased in some of our malnourished rats. Serum complement levels might be compared. Age should be carefully controlled, and different age groups evaluated, as reports in the
literature suggest changes with age, at least to 90 days, and decreases with pregnancy and lactation. Our preliminary results on serum protein and albumin levels of older rats suggest that the malnourished rats reach normal levels eventually, presumably as the diet becomes sufficient to meet their needs. Serum protein and albumin levels should be determined concurrently with complement levels to clarify any changes in nutritional status with age.

4. Elicited macrophages were tested in our experiments. Numbers of resident macrophages in rats are too limited for extensive testing. However, activated macrophages might well be studied. In light of our results showing a decreased proportion of mature macrophages in malnourished rats, the activation of macrophages in malnourished rats might be monitored.
REFERENCES


Ann Hart was born in Chicago, Illinois. Her family soon moved to the state of New York, where she spent most of her childhood and adolescence.

She attended Wellesley College, graduating in June, 1974 with a major in molecular biology. As a cross-registrant from Wellesley, she took several courses in MIT's Department of Nutrition and Food Science, from which her interest in nutrition evolved. She has been a graduate student in the department since September, 1974. Following graduation, she will be married to Timothy Ramish. They will live in New York City, where she hopes to find a position in nutrition.