PHAGOCYTOSIS OF MYCOBACTERIUM TUBERCULOSIS AND MYCOBACTERIUM SMEGMATIS STAINED WITH INDICATOR DYES

By

Marian Grace Sprick

AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

Year 1954

Approved W. N. Mack

This investigation was concerned with experiments designed to determine whether as low a degree of acidity as pH 3.0 would be produced in the mammalian neutrophil and monocyte after assimilation of indicator dyes or after phagocytosis of organisms stained with these dyes.

In the first part of the study intraperitoneal injections of azolitmin into the mouse, guinea pig, and rabbit, and intraperitoneal injections into mice of the sulfonphthalein dyes, brom phenol blue and brom cresol green, were made.

It was found that the monocytes and neutrophils in the peritoneal cavity of the mouse, guinea pig, and rabbit possessed sufficient acidity or produced the amount necessary to change the phagocytized blue particles of azolitmin to red.

The experiments with azolitmin were in agreement with the work of Rous (1925, a,b). Since the indicator is red at a pH of 6.0 (determined electrometrically), it should not necessarily be assumed that the acidity surrounding these granules in the monocyte is considerably lower because they appear to be intensely red. However, in contrast to the experiments of Rous (1925 a,b) the development of a granular acidity as low as pH 3.0 was not observed in either the monocyte or neutrophil of the adult white mouse when injected intraperitoneally with one and two per cent solutions of the indicator dyes brom phenol blue and brom cresol green.

In the second and third phases of the problem investigations of *in vitro* and *in vivo* phagocytosis by the neutrophil and monocyte of the adult white mouse, guinea pig, and rabbit of *Mycobacterium tuberculosis var hominis* strains H37Rv and H37Ra and *Mycobacterium smegmatis* stained with indicator dyes were undertaken.

M. tuberculosis var hominis strains H37Rv, H37Ra, and *M. smegmatis*, the organisms used, were stained with the sulfonphthalein dyes, brom phenol blue, brom cresol green, and brom cresol purple. Under the experimental conditions employed, it was found that sufficient acidity did not develop in either the neutrophil or the monocyte of the mouse, guinea pig, and rabbit to shift the color of the phagocytized stained organisms to the acid range of the indicator dyes.

When indicator dyes were injected intraperitoneally subsequent to injection of stained organisms, granular acidity as low as pH 3.0 in the cytoplasmic matrix surrounding the ingested bacteria was not observed.

PHAGOCYTOSIS OF MYCOBACTERIUM TUBERCULOSIS AND MYCOBACTERIUM SMEGMATIS STAINED WITH INDICATOR DYES

By

Marian Grace Sprick

A Thesis

Submitted to the School of Graduate Studies
of Michigan State College in partial
fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Bacteriology

Year 1954

ACKNOWLEDGEMENTS

The author wishes to express her sincere thanks to Dr. Walter Mack under whose helpful guidance and direction this investigation was undertaken. His valuable aid in obtaining the photomicrographs is also appreciated.

Grateful acknowledgement is also due to Dr. Russell Gottschall for his pertinent suggestions, kind guidance, and sustaining interest.

The writer deeply appreciates the two year scholarship provided by the Michigan Department of Health and extends her sincere thanks to Dr. Albert Heustis, Commissioner of Health and to Dr. G. D. Cummings, Director of Laboratories for their permission to carry on the study and to use the materials contained therein.

VITA

Marian Grace Sprick
candidate for the degree of
Doctor of Philosophy

Dissertation:

Phagocytosis of Mycobacterium tuberculosis and Mycobacterium smegmatis Stained with Indicator Dyes.

Outline of Studies:

Major- Bacteriology

Minor- Pathology and Mycology

Biographical Items:

Born- May 22, 1908

Undergraduate studies- B.S., 1931 Michigan State College

Graduate Studies- M.S.P.H., 1943 University of Michigan

Experience:

1931-1934, Bacteriologist, Butterworth Hospital Grand Rapids, Michigan
1934-1937, Bacteriologist, Pertussis Immunization Project, Grand Rapids Branch Laboratory, Michigan Dept. of Health
1937-1943, Bacteriologist, Powers Branch Laboratory, Michigan Dept. of Health.
1943- Assistant Director, Powers Branch Laboratory, Michigan Dept. of Health

Member:

Phi Kappa Phi
Society of American Bacteriologists
American Public Health Association
Registry of Medical Technologists

TABLE OF CONTENTS

INTRODUCTION

Many interesting studies have been conducted, the majority in the past thirty-five years, using indicator dyes as a means of determining the relative hydrogen-ion concentration of the granules, cytoplasm, and nucleus of both plant and mammalian cells.

In recent years the internal environment of the cell, especially in regard to the acidity produced, has been of considerable interest. The work of Rous (1925 c), in which he concluded that the granular acidity of the macrophage became as low as pH 3.0 or lower when the cell was subjected to injury has been frequently referred to in the current literature (Cameron, 1951; Rich, 1951; Grogg and Pearse, 1952; Dubos, 1953).

In considering the bacteriostatic action of streptomycin on tubercle bacilli the thought occurred that one of the reasons why streptomycin may not kill or inhibit the intracellular phagocytized bacteria (Machaness, 1952; Suter, 1952 b), might be the fact that streptomycin is most effective in a pH range of 8.0 to 9.0. If the granular acidity of the neutrophil and macrophage is as low as pH 3.0 as indicated by Rous' experiments, and since the tubercle bacillus can survive in an environment of this low pH, then it might be assumed that the effectiveness of streptomycin against the organisms in the intracellular fluids is limited and cannot be exerted on the

phagocytized tubercle bacilli existing in such an acid environment.

The fact that streptomycin is not effective against phagocytized intracellular organisms of other genera has been shown recently by Shaffer et al. (1953) who demonstrated that when brucellae were phagocytized by the neutrophils and monocytes of the adult male white rat, the bacilli were protected to a remarkable degree against the action of streptomycin.

Since in a review of the literature no other reference stating that the macrophages developed a granular acidity as low as pH 3.0 was found, it was thought that it would be of interest to repeat the work with litmus and the phthalein dyes which are the indicators Rous used. Rous used the term, "granular acidity" to denote the small non refractive granules in which the acid vital stains are stored.

It was thought it also would be of interest to investigate whether the phagocytic cells of the mouse which has more natural resistance to tuberculosis (Rich, 1951) would show a more marked granular acidity when subjected to injury induced by phagocytized bacteria than the cells of the guinea pig and rabbit. The latter animals have much less natural resistance to tuberculosis.

Thus, in vitro and in vivo phagocytosis experiments with Mycobacterium tuberculosis and Mycobacterium smegmatis stained with indicator dyes were made.

This study is divided into three parts (1) experiments designed to repeat the work of Rous- the intraperitoneal injection of azolitmin into the mouse, guinea pig, and rabbit, and the injection of sulfon-

phthalein dyes into mice: (2) experiments to observe *in vitro* phagocytosis by the neutrophil and monocyte of the mouse, guinea pig, and rabbit of organisms stained with indicator dyes; (3) investigation of *in vivo* phagocytosis of stained organisms by the neutrophil and monocyte of the mouse and guinea pig.

HISTORICAL REVIEW

Before the beginning of the century leDantec (1890) conducted experiments in which he observed the ingestion of litmus particles by protozoa. He found that the ingested particles of litmus became red in the body of the Stentor polymorphus and remained red for some time, as long as 12-13 hours. He concluded that the acid surrounding the particles was due to a secretion which was progressive and that it was a strong acid. Metchnikoff (1905) also found that protozoa could change the blue litmus particles to red after phagocytizing them but did not find the same reaction developing in the phagocytes of mammals and birds. He developed a law according to which the degree of acidity about phagocytized material is less the higher up the organism is in the animal kingdom. At that time the general concensus was that there was insufficient acidity developed in the granules of mammalian cells to turn blue litmus to red.

Rhode (1917) used indicator dyes to study changes in the inner reaction of plant cells and the infusoria.

Sabin (1923) used neutral red in a study of vital staining of human blood cells. She found that the granules took up the dye more slowly than the vacuoles, and that the dye could be used to ascertain how much phagocytic activity goes on in the leucocyte. In rabbits with pneumonia large vacuoles showed a brilliant scarlet color, acid reaction of neutral red whereas smaller vacuoles were an orange

color - neutral reaction of neutral red.

Rous (1925 a, b, c, d,) conducted an extensive series of investigations concerned with vital staining of mammalian tissues. He used purified litmus to stain living mammals such as rats and mice and observed that the animals became colored blue a few hours after the litmus was injected by the peritoneal route. The color gradually changed to pink-violet and persisted as long as 10 days after some injections. He also found that many body cells both in fixed tissues and in the exudate were capable of developing a rather higher degree of granular acidity. The work with mice included indicators of the phthalein series, brom thymol blue, brom phenol blue, brom cresol green, and brom cresol purple. The ranges of the phthalein indicators were all more to the acid side than litmus. These solutions also indicated that the acidity of the non refractive granules of the macrophage may approach pH 3.0 or less.

He also found that when the cells of the peritoneal exudate which had been stained with litmus were crushed, the acid reaction prevailed and that the acid was not derived from the dye but was inherent in the living elements. When the cells died, there was a shift from the acid to the basic stain. The shift from the acid to the alkaline reaction after death was thought to be due to the absorption of intracellular fluid rather than the protoplasm itself becoming alkaline. One important conclusion was that by means of vital staining it was shown that the intracellular reaction during life was independent of that of the body fluids.

Irving (1926) found in the study of living starfish that the reaction of the cellular fluids may be independent of the surrounding fluid. The normal reaction in the caeca was pH 6.7, that of the coelomic fluid which surrounded them was pH 7.6 and of sea water pH 8.3.

Experiments on living human neutrophils were performed by Ishikawa (1935) who used a micromanipulator and indicator dyes. He found that the pH of the cytoplasm of the neutrophil was 6.6, the pH of the nucleus 7.0, and of the granules 7.1. No definite figure was given but the statement was made that the granules of the monocyte inclined more to the acid side.

The reaction of the protoplasm of amoeba and echinoderm eggs has been extensively studied by the microinjection of indicator dyes. Kite (1913) was probably the first to use this technique. He injected azolitmin, sodium alizarin sulphonate, methyl orange, and congo red into Amoeba proteus. A neutral to slightly alkaline reaction was found by all the indicators. Needham & Needham (1925) using neutral red vital staining technique found the cytoplasm of Amoeba proteus to have an internal reaction of pH 7.6 which is more alkaline than Pollack (1928) found. He obtained pH values of not less than 6.6 and not more than 7.2 for the cytoplasms of Amoeba proteus and Amoeba dubia. Chambers and Pollack (1926-27) found the normal cytoplasmic pH of starfish eggs to be 6.7 ± 0.1.

Studies have been carried out also with respect to the degree of acidity produced when the cell was subjected to injury. Chambers and Pollack (1926-27) found that when the cytoplasm of a starfish egg

was injured by a slow tear, very little acid was produced and that it was quickly neutralized by the buffering action of the cytoplasm. Only when cytolysis occurred did the pH drop to 5.5 ± 1.

Pollack (1928) introduced buffers of a pH of 5.6, 5.8 and 6.0 with Amoeba proteus and Amoeba dubia previously injected with brom cresol purple - a momentary shift in the blue color to a distinct yellow occurred but the reversal to the blue color was rapid and constant.

Reznihoff and Pollack (1928) injected solutions of hydrochloric acid (pH 2.0) into amoeba colored orange-yellow by previous injection with phenol red. Usually the cytoplasm reverted within a few seconds to the color of the normal cytoplasm. However, if there was considerable damage to the cytoplasm, the injured portion of the amoeba, which had a bright yellow color (acid), was pinched off indicating the amoeba could not tolerate such a low pH and live. Spek and Chambers (1933) found no recognizable change of the plasma components when the amoeba were subjected to injury, and that even when they were pulped into a brei, no acid was produced. Various concentrations of lactic, butyric, and hydrochloric acid were injected into the cells but no change in the reaction of cytoplasm occurred unless an irreversible coagulation of the protoplasm took place, the coagulated portion staining acid. They also noted that the amoeba injected with indicator dyes did not show a uniform reaction but that the granuloplasma showed a more acid reaction (highest pH 6.0 possibly lower) than the hyaloplasm which had a more alkaline reaction pH - 7.3.

Marshak (1944) attempted to change the cytoplasmic hydrogen ion concentration of Amoeba dubia colored with phenol red by injection of egg albumin. He noted that the color of the cytoplasm indicated a pH of 6.9 and when albumin was placed in the surrounding medium the pH was raised to 8.3 and that the nucleus was extruded. However, if the nucleus remained in the cell the reaction returned to the original pH 6.9. Experiments carried out by first injecting phenol red and then the albumin, showed that the dye and albmin permeated the cytoplasm but were distributed to different phases of it. The existence of two phases in the cytoplasm also occurred when albumin entered through the cell membrane. This agreed with the results of Spek and Chambers.

The isoelectric point of the cytoplasm and nucleus has also been determined by means of staining fixed films with certain acid and basic dyes in a series of buffered solutions. The intensity of staining with an acid and basic dye is then plotted against the pH. The isoelectric point is theoretically the pH value where the two curves cross.

Mommisen (1927) using cytochemical technique and air dried blood smears found the isoelectric point of neutrophils to be a pH of 6.5 and of the neutrophilic granules to be approximately the same value.

Schartz-Karsten (1927) used air dried blood films which gave a relative value of 4.5 - 5.0 for the isoelectric point of the monocyte granules.

More recently Weiss (1953) using light green, an acid dye, and methylene blue, a basic dye, determined the pH range of blood cells stained with these dyes but no estimation of the approximate isoelectric point of the cells was given. However, Levine (1940) and Lamanna and Mallette (1953) do not think that the procedure of determining the isoelectric point by staining at controlled pH values is reliable. They think that the protoplasm is complex and that the proteins composing it would not all react or absorb the various cations and anions added to the system in the same way and thus would have a broad isoelectric range rather than a true isoelectric point.

MATERIAL AND METHODS

I. In Vivo Injection of Indicator Dyes

Indicators. Azolitmin, National Aniline and Chemical Company Lot 8834, was used since Kahlbaum cube litmus which Rous (1925 a) used was not available and attempts at purifying ordinary cube litmus (Sutton, 1911) were not successful. The azolitmin was sterilized by allowing it to remain in ether for several days. The ether was then decanted and 1 g of azolitmin was suspended in 15 ml of 1.5 per cent agar made up in 0.9 per cent saline solution. The color of the azolitmin at various pH concentrations was checked on a Beckman pH Meter and gave the following results; pH 5.5, red; pH 6.0, red; pH 6.5, magenta; pH 7.0, magenta; pH 7.7, blue or purple.

Sulfonphthalein dyes used in the experiments were National Aniline and Chemical Company products. These dyes are very stable chemically and are less affected by proteins and the presence of neutral salts (Conn, 1953) than are a large number of other dyes. Also, they are not subject to loss of color on reduction. Hundred mg of the dye was placed in a sterile mortar and the amount of sterile N/20 sodium hydroxide as indicated by Clark (1921), added to the indicator. The solution was made up to volume, 5 or 10 ml with 0.9 per cent saline solution. In some experiments 2 per cent solutions were too toxic for the mice so 1 per cent solutions were used and more injections were given.

Locke's solution. The solution used in experiments contained sodium chloride, 0.2 g; calcium chloride, 0.2 g; potassium chloride, 0.4 g; sodium bicarbonate, 0.2 g; Bacto-dextrose, 2.5 g; distilled water, 1 liter. All chemicals were reagent grade.

Experimental animals. Adult male white mice weighing 27-30 g were used for experiments with azolitmin and the sulfonphthalein dyes. Male guinea pigs weighing 300-350 g and white rabbits weighing 2 kg were used for experiments with azolitmin. All animals were from stock bred at the Michigan Department of Health Laboratories, Lansing, Michigan.

Intraperitoneal injections. Before injection, the litmus-agar was heated slightly so the litmus could be suspended evenly in the agar before being drawn up in the syringe. The material was allowed to form a soft jel in the syringe before intraperitoneal injections into the animals. Two tenths ml was injected into the mouse, 1.0 ml into the guinea pig, and 15 ml into the rabbit.

II. *In Vitro* Phagocytosis Experiments with Stained Organisms.

Preparation of bacterial suspensions. *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis var hominis*, strains H37Rv and H37Ra were obtained from the stock cultures of the Michigan Department of Health Laboratories. Organisms were grown in Dubos' liquid medium for 1 week at 37 C. The organisms were killed by placing cultures, contained in flasks, in flowing stream for 1 hour. The medium was centrifuged and sedimented organisms were washed once with 0.9 per cent sterile saline solution. The organisms were resuspended in a small amount of saline solution.

Preparation of indicator stains. In the preliminary work on this study, many methods were tried in an attempt to stain the organisms intensely. Dyes were made up in 50 per cent alcoholic solutions, addition of mordants such as aluminum amonium sulfate, surface depressants such as propylene glycol, and oxidation of the proteins of killed organisms with chromic acid to enhance penetration of the stain were attempted. None of these procedures were effective in producing intensely stained organisms.

Rawlins and Schmidt (1930) found that the more acid the solution of dye was the more dye was absorbed by gelatin granules. However, the dye concentration and the length of time particles were in contact with the dye solution were also factors in the amount of dye absorbed.

Harris (1951) found that the absorption of acid dyes, such as brom thymol blue, brom cresol purple, and brom cresol green, by

Bacillus subtilis, Bacillus megatherium, Escherichia coli, Sarcina lutea, and other bacteria, was increased and that the negative charge on the cell decreased as the pH of the suspending medium was lowered. Therefore, the method of Mallmann et al. (1942) which consists of using 1 per cent solutions of the indicator made up in 95 per cent alcohol with the addition of 0.5 per cent concentrated hydrochloric acid was finally adopted as the method of choice and gave uniformly successful results.

Procedure for staining organisms. Two tenths ml of packed organisms suspended in 1 ml of 0.9 per cent saline solution was placed in a 15 ml centrifuge tube. Four ml of the indicator was added, the tube was stoppered, and then inverted several times to mix the organisms with the dye. The dye suspension was then stored at least a week, more frequently 2 weeks, at room temperature before using in experiments. The tube was then centrifuged until the stained bacteria were well packed, the excess dye was decanted and 10 ml of buffered distilled water, pH 7.0 was added. Washing was repeated with 5 ml of distilled water and the sediment resuspended in 0.5 ml of buffered distilled water. The bacteria stained intensely in the acid-alcoholic solutions and were the color of the acid range of the indicator. When washed twice with the buffered distilled water, the color of the organisms shifted to the basic side of the indicator.

Indicator Dyes Used.

TABLE 1

Indicator Dyes

Dye	Color		pH interval	pK value
	Acid	Basic		
Tetrabromo phenol sufon - phthalein (Brom phenol blue)	Yellow	Blue violet	3.0 - 4.6	4.0
Tetrabromo-m-cresol sulfon-phthalein (Brom cresol green)	Yellow	Blue	3.8 - 5.4	4.7
Dibromo-o-cresol sulfon phthalein (Brom cresol purple)	Yellow	Purple	5.2 - 6.8	6.3
Dimethyldiamino phenezine chloride (Neutral Red)	Red	Yellow orange	6.8 - 8.0	

Color of stained organisms in buffer solutions. To ascertain whether the organisms stained with the sulfonphthalein indicator dyes would change color in the designated pH range buffer solutions according to Mac Ilvaine (1921) were prepared varying from pH 2.8 to 6.8. After the bacteria had been washed with buffered distilled water (pH 7.0) several times, the organisms were placed in the buffer

solutions of known pH value and the color change in the stained bacilli, both macroscopic, and microscopic was observed. The results are shown in table 2.

TABLE 2

Color of stained organisms in Mac Ilvaine's buffer solutions

pH of Buffer Solution	Color of organisms		
	Brom phenol blue	Brom Cresol green	Brom cresol purple
2.8	green-yellow	yellow	
3.0	green-yellow	yellow	
3.4	green-blue	yellow	
4.0	blue-green	green-yellow	
4.4	blue	green-yellow	
4.8	blue	green	yellow
5.2		green-blue	yellow
5.6		blue	yellow-green
6.0		blue	green-yellow
6.4		blue	green-yellow
6.6			blue
6.8			blue

Both brom phenol blue and brom cresol purple have a certain disadvantage in that they both manifest "dichromism" which is the

quality or condition of presenting one color in reflected and another in transmitted light. Thus, the color of the indicator, depending on the depth of the liquid observed and on the concentration of the indicator, may appear blue in a thin layer of a solution and purple when viewed through a greater depth. This condition, however, did not cause any difficulties in the interpretation of the experimental results as either the blue or purple color is associated with alkalinity and both change to yellow in the acid range of the indicator.

Production of neutrophils and monocytes - mice. Mice were inoculated intraperitoneally with 2.0 ml of 7.2 per cent sodium caseinate (Martin et al., 1950) 8 hours before sacrificing the animal and withdrawing peritoneal fluid. Two injections were often necessary as less exudate was found in the mouse than in the guinea pig. Usually it was necessary to rinse out the peritoneal cavity with Locke's solution in order to obtain sufficient cells. Cells were washed with Locke's solution and then resuspended in a small amount of the same solution. A cell count of the fluid showed 90 per cent neutrophils and 10 per cent monocytes and lymphocytes.

Monocytes were obtained by inoculating mice intraperitoneally for 7 days with 2 ml glycogen saline suspension containing 0.01 mg glycogen per ml. On the 8th day the animal was sacrificed and peritoneal fluid withdrawn and placed in Locke's solution. A pool of the peritoneal fluid from several animals was necessary to secure a heavy suspension of cells. A cell count of the fluid showed 40 per cent monocytes and 60 per cent neutrophils.

Production of neutrophils and monocytes - guinea pig. Normal guinea pigs weighing 300-350 g were used. Ten ml of a 10 per cent solution of sodium caseinate (Difco brand) in saline solution, pH 7.0, was injected into the peritoneal cavity of the guinea pig. After 20-24 hours the animal was sacrificed and the peritoneal cavity was opened immediately aseptically and the fluid withdrawn with a sterile syringe and placed in a 15 ml centrifuge tube. Four tenths of a ml of a 1-100 dilution of heparin per 10 ml of fluid was used as an anticoagulant. The cells were placed immediately in the incubator (37 C). The pH of the fluid was 7.0.

Stained slide preparations were made of the peritoneal exudate and the differential count showed 88-90 per cent neutrophils and 10 per cent monocytes and lymphocytes.

The method of Suter et al. (1952 a) was used to produce monocytes in the guinea pig. Ten ml of sterile saline solution, pH 7.0, containing 0.01 mg glycogen per ml was injected into the peritoneal cavity for a period of 7 days. On the 8th day the animal was sacrificed and the peritoneal fluid withdrawn aseptically. Four tenths ml of a 1-100 dilution of heparin was added to 10 ml of the fluid. A good yield of monocytes, 50 per cent, was obtained by this method, although not as high as reported by Suter.

Production of neutrophils and monocytes - rabbit. White rabbits weighing 2 to 2.5 kg. were used. Animals were anesthetized with ether and 75 ml of 7.2 per cent sodium caseinate was injected intraperitoneally

on two successive days. On the 3rd day, in the morning, 75-100 ml was injected intraperitoneally, and 6 hours later the animal was anesthetized and the fluid aspirated from the peritoneal cavity. Heparin (0.5 ml of 1-100 dilution) was added to the fluid to prevent coagulation. The percentage of neutrophils and monocytes was 56 and 44 per cent respectively.

Sera. Mice, guinea pigs and rabbits were bled from the heart, the blood allowed to clot and the serum decanted aseptically.

Technique for in vitro phagocytic tests. Preliminary experiments were carried out with slight modification according to the method of Mudd et al. (1929) - Cells, serum, and stained organisms were placed in shell vials with paraffin coated stoppers in a rollator machine which rotated 4-6 revolutions per min for 30 min in the incubator (37 C). Phagocytosis of stained bacteria was not marked when this method was used. Tullis and Kochow (1952) recommended coating the surface of vials with liquid silicone to promote phagocytosis, but since silicone was not available, the centrifuge method described by Mackanness (1952) was tried and found to be much more satisfactory than the rollator method.

The procedure was as follows: five tenths ml of cells suspended in sodium caseinate, 0.5 ml of homologous serum and 0.1 ml stained organisms were placed in a sterile centrifuge tube. The tube was stoppered and centrifuged at 850 G for five minutes. The supernatant fluid was decanted. The bottom of the tube was tapped gently and a

small amount of the mixture was placed on a plastic cover slip and the cover slip inverted on a well slide ringed with vaseline. The slides were placed on a warm stage - 37 C. Slides were examined under the oil immersion lens.

III. *In Vivo* Phagocytosis Experiments with Stained Organisms.

Mice. White mice weighing from 25 to 28 g were injected intraperitoneally with 2.0 ml of 7.2 per cent sodium caseinate on 2 successive days. Six hours following the 2nd injection, 0.1 ml of the packed stained organisms which had been washed and resuspended in buffered distilled water pH 7.0 was inoculated into the peritoneal cavity. At intervals of 2, 24, 48, 72, and 96 hours a small amount of the peritoneal fluid was withdrawn with a sterile syringe. For most of the preparations no anesthesia was used so that cells aspirated from the peritoneal cavities would not be subjected to the effects of any narcotizing substances. Ether anesthesia was used, however, when the abdomen was incised, occasionally at 48 hours, more often at the end of 96 hours or a longer interval after injection of organisms. A small drop of the fluid was placed on plastic cover slip and inverted on a well slide which had been ringed with vaseline to exclude air. Preparations were examined immediately under the oil immersion lens. A warm stage was used to keep preparations at 37 C.

Guinea pigs. Animals weighing 300-350 g were used. Animals were usually injected intraperitoneally with 10 ml of 7.2 per cent of sodium caseinate on two successive days, although in some experiments only one injection was given. Approximately 6 hours

after the last injection, 0.5 ml of the packed stained organisms, suspended in 10 ml buffered distilled water pH 7.0, was inoculated into the peritoneal cavity. Material from the peritoneal cavity was aspirated with a sterile syringe at 6, 24, 48, 72, and in some instances 96 hour intervals. No anesthesia was used except when the abdomen was incised. Hanging drops prepared as already described were examined immediately.

Some experiments using organisms which were washed with and resuspended in distilled water of pH 3.0 were undertaken.

Preparation of syringes. Since it was found that the moisture left in syringes after boiling produced an alkaline reaction in the aspirated fluid from the peritoneal cavity, the syringes were placed in cleaning solution for 12 hours. They were then rinsed thoroughly with tap water and placed in distilled water for 24 hours. Syringes and needles were sterilized with dry heat.

Organisms - amount of stained organisms injected. The preparation of stained organisms, M. smegmatis, M. tuberculosis H37Rv, and H37Ra, for the in vivo experiments was the same as that described in in vitro procedures. After stained organisms had been washed twice with sterile buffered distilled water pH 7.0, the organisms were centrifuged, and for mouse inoculations 0.1 ml of the packed organisms was suspended in 0.2 ml buffered distilled water pH 7.0, whereas for the guinea pig 0.5 ml of packed organisms was suspended in 10 ml of buffered distilled water pH 7.0.

RESULTS

I. Experiments with azolitmin and phthalein dyes.

Experiments with azolitmin. In all of the experiments described, the phagocytic cells of the peritoneal fluid will be characterized as neutrophils and monocytes rather than polymorphonuclear cells and macrophages, terms used by Rous. Protocols of the intraperitoneal injection of azolitmin into the mouse, guinea pig, and rabbit were as follows:

Mouse. Two ml of 7.2 per cent sodium caseinate was injected intraperitoneally which was followed in 6 hours with 0.2 ml of azolitmin suspended in 1.5 per cent agar. Twelve hours after the dye was injected fluid was aspirated from the peritoneal cavity, a dry sterile 1 ml tuberculin syringe being used. The fluid was placed on a slide, covered with a cover slip both of which had been cleaned with cleaning solution and thoroughly washed with distilled water. Many monocytes filled with red granules of azolitmin were observed. One cell contained both red and blue granules. The agar litmus particles on the outside of the cells suspended in the peritoneal fluid were all blue.

In the experiments Rous (1925) conducted with litmus, he found that the litmus was concentrated around the refractive granules in both the polymorphonuclear cell and macrophage. He also observed

that the litmus did not penetrate these granules but that the refractive granules surrounded by the litmus coalesced to form large globules which contained the indicator. Similar findings were observed in the present experiments. However, in some instances, it appeared as though intact particles of the azolitmin had been engulfed by the neutrophils and monocytes.

The animal was again aspirated after 36 hours. At this time many of the refractive granules around which the azolitmin had clustered, had coalesced and large red globules were observed. The background cytoplasm of the monocytes was a pinkish red. Only one cell was observed with a blue particle of litmus. After five days, aspiration of the peritoneal cavity showed many large monocytes containing bright red particles. In a few cells the cytoplasm appeared a magenta color. Some of the extracellular litmin was apparently reduced to the yellow form although there were a few blue particles. Nineteen days after injection of the azolitmin the animal was sacrificed. There were still many monocytes with intense red particles. At this time there were still a few blue extracellular particles of azolitmin.

Guinea pig - intraperitoneal injection. The same procedure was used with the guinea pig only larger amounts of the materials were injected. Ten ml of 7.2 per cent sodium caseinate was injected intraperitoneally followed in 24 hours by 1.0 ml of the azolitmin. Fluid from the peritoneal cavity was aspirated 12 hours after the dye was inoculated. The color of the fluid was a bluish red. A count of the cells showed 65 per cent neutrophils and 35 per cent

monocytes. The background cytoplasm of many monocytes was pink and some contained red granules. No cells were observed with blue granules. After 36 hours 0.2 ml of blue fluid was withdrawn. The pH of the fluid tested with universal indicator paper was 7.0. Many monocytes, 15-17 cells per oil immersion field, contained red granules of litmus and the cytoplasm was tinted pinkish red. At 48 hours many of the smaller granules in the cells had coalesced into large globules which appeared a vivid red. Some of the fluid was placed in Mac Ilvaine's buffer solution (pH 7.4) and the pinkish red background slowly turned to a magenta color and the granules appeared violet. A portion of the fluid was added to the Locke's solution and placed in the incubator (37 C) for 76 hours. The cells still showed the red coloration and as they were motile they were apparently living. Phagocytic activity and amoeboid motility are usually considered to be indicative of functional viability (Tullis, 1953). After 5 days there were many red particles of azolitmin in the cells. The extracellular azolitmin was blue although some had been reduced to the yellow form.

Guinea pig - subcutaneous injection. Eight tenths ml of azolitmin suspended in agar was inoculated subcutaneously. At the end of 7 days the subcutaneous nodule was the size of a nickel. The center of the lesion contained azolitmin and agar, and the edges were red and indurated. The material at the edge of the lesion was scraped and placed in a drop of saline solution. There were many large monocytes, some with blue granules and others red. Some cells had both red and blue particles.

Rabbit. The rabbit was inoculated with 50 ml sodium caseinate on two successive days followed by a 100 ml injection on the 3rd day.

A few hours after the last injection 1 g of azolitmin, suspended in 15 ml of 1.5 per cent agar, was injected into the peritoneal cavity. Twenty six hours later a small amount of blue colored fluid was aspirated from the peritoneal cavity. The pH of the fluid was 7.0-universal indicator paper. A count revealed 50 per cent monocytes. These cells, 7-8 per oil immersion field, contained bright red globules of azolitmin. The azolitmin outside the cells was blue. An occasional cell showed blue granules and in nearly all cases the cells containing the blue azolitmin granules were neutrophils. LeDantec (1890) was of the opinion that cells with blue granules had phagocytized the litmus more recently and that due to the short time within the cells, they had not had time to turn red.

It is of interest that after 72 hours one large monocyte was observed which had phagocytized two neutrophils. The particles in the monocyte were bright red whereas they were blue in the neutrophil. One would think that the particles of azolitmin would have had to be in the neutrophil even longer than in the monocyte as they no doubt had already been ingested by the neutrophil when it was phagocytized by the monocyte. When N/20 sodium hydroxide was added under the cover slip the red azolitmin in the cells became blue, and when N/20 hydrochloric acid was added the colors were reversed, the blue particles becoming red again.

After 96 hours only an occasional neutrophil was seen but monocytes still contained red particles. One cell was observed with blue ones. Some blue azolitmin was not phagocytized and some had been reduced to the yellow form.

Intraperitoneal injections with the phthalein dyes. Solutions of brom phenol blue and brom cresol green were injected into white mice weighing 27-30 g in order to attempt to ascertain whether results found by Rous could be repeated.

Dyes were prepared according to the methods outlined under experimental methods. The experiments were repeated a number of times. The procedure followed that of Rous as closely as possible. Rous (1925 a) described two types of granules in the macrophage. One type was the highly refractive granule like those found in the polymorphonuclear, and the other type was a non-refractile relatively small granule which at times attained the diameter of an erythrocyte. He found that it was in the latter type of granule the acid vital dyes became stored. In the following experiments the term granule will refer to the non refractive particles in the cytoplasm which take the dye in a uniform manner and do not increase in size during the functional activity of the cell.

A specimen protocol was as follows:

Brom phenol blue (acid-yellow, basic-blue). Rous injected mice weighing 30 g with 1 ml of a 1 or 2 per cent solution of brom phenol blue in 0.9 per cent saline for three successive days and aspirated peritoneal fluid 24 hours after the last injection.

It was found that a 1 ml dose was too toxic for the mice so 0.5 ml was injected on six successive days, a total of 3 ml being given. Within 3 or 4 minutes after the dye was injected, the tail became tinted blue and gradually the eyes, ears, and skin all became

a vivid blue. The blue color was present for 8-12 hours but had disappeared in 24 hours, or by the time the next injection was given. The urine and feces were blue.

Since the phthalein dyes are so easily assimilated by the animal, it was thought that if the fluid was aspirated from the peritoneal cavity at intervals less than 24 hours after the dye was administered, it might be possible to detect the dye in the cells. Aspiration of fluid from the peritoneal cavity was made 1 hour after the injection of 0.5 ml of the dye. There did not appear to be any increase in the normal cell granules in either the neutrophil or monocyte. However, clustered about the normal refractive granules were very fine, minute particles which were a definite blue. No fine lemon-yellow granules were observed. Six hours after the 4th injection a very small amount of peritoneal fluid was withdrawn. The ears, eyes and tail of the animal was still blue. There were now many large refractive granules in the cells which had a greenish blue cast. No fine blue particles were observed nor were any bright yellow ones found. In some monocytes there were small globules of blue dye, possibly coalescing fine particles.

Brom phenol blue suspended in 7.2% Na caseinate. In order to evoke more fluid in the peritoneal cavity, a 2 per cent solution of brom phenol blue was suspended in 7.2 per cent sodium caseinate. Six hours after the 2nd injection of the dye a small amount of peritoneal fluid was aspirated. A number of cells were observed with opaque blue masses inside the cell. The granules did not appear to have taken up any of the dye. No lemon-yellow granules were observed. In some cells there was fine blue granular

material interspersed with the refractile granules. One monocyte was observed with a large blue mass and another inclusion inside of which were many fine blue granules. This appeared to be a phagocytized neutrophil although due to the granular appearance one could not be certain.

Brom cresol green (acid-yellow, basic-blue). A 27 g mouse was injected with 0.4 ml of a 1 per cent solution of brom cresol green for six successive days. After each injection of the dye the animal became a bright green within 5 minutes after the dye was administered. The skin, tail, ears, eyes and feet all became a vivid green. The color persisted for 6-8 hours but had entirely disappeared in 24 hours or by the time the next injection was given. Two hours after the second injection a very minute amount of pale green fluid was aspirated from the peritoneal cavity. Cells were nearly all neutrophils, an occasional monocyte was observed. There were fine blue-green granules of dye in the cells. The normal refractive granules of the cytoplasm appeared to be a greenish yellow. Some of the epithelial cells appeared blue-green.

Six hours after the 4th injection the animal was still green. A very minute drop of bright green fluid was aspirated. There were now more monocytes which had bright blue-green masses of dye in the cell. Twenty-four hours after the 4th injection a very small amount of fluid was withdrawn. The normal refractive granules in the monocytes and neutrophils appeared greenish yellow. There were no bright lemon colored non refractive granules observed like those Rous describes.

Twenty-four hours after the 6th injection of dye, the mouse was placed under ether anesthesia and the abdomen incised. There was only a minute amount of fluid in the peritoneal cavity. There were no colored masses nor any fine bright lemon-yellow non refractive granules found in the monocytes. The normal refractive granules were greenish yellow.

The results of the experiments with azolitmin are in agreement with those of Rous. The azolitmin used in the above investigations was checked electro-metrically and gave the following results: pH 5.5, red; 6.0, red; 6.5, magenta; 7.0, magenta; 7.7, blue. Since the indicator is a bright red at a pH of 5.5, it therefore does not necessarily follow that the acidity surrounding these ingested granules in the monocyte is considerably lower because the granules appear very red.

The granular acidity in the macrophage of the mouse which was evoked by repeated intraperitoneal injections of brom phenol blue and brom cresol green, and described by Rous (1925 a) as "non refractive translucent yellow granules" was not observed in experiments described above which followed the original work as closely as possible.

Based on the observation of these "yellow translucent granules" it was concluded that the acidity of the macrophage granule may be as low as pH 3.0 and that the reaction about phagocytized bacteria may be very acid and that this acid material is held in the cytoplasm in granular or globular form.

It was thought that possibly one reason for failing to conform the work of Rous was that he may have used German manufactured dyes.

However, Clark and Lubs (1917) described an improved method for the preparation of the sulfonphthalein indicators in 1917 and the dye can be purchased in the United States. Furthermore, it was not until 1923 that Cohen (1923, 1926) introduced brom cresol green as a new synthesized sulfonphthalein, so it is probable that the indicators Rous used were manufactured in this country.

Since his observation of this "granular acidity" could not be confirmed, it was thought that it would be of interest to ascertain whether organisms stained with indicator dyes would change color when phagocytized by the neutrophil and monocyte.

II. *In Vitro* Results

The animals used in these *in vitro* experiments were the guinea pig, mouse, and rabbit. The guinea pig and rabbit have less natural resistance to tuberculosis than the mouse (Rich, 1951). Moreover, Gross and Pearse (1952), using histochemical techniques, found that the mononuclear phagocytes of the mouse contain a strong acid phosphatase, and that in the susceptible guinea pig no acid phosphatase could be demonstrated. The mononuclear cells of the rabbit showed a moderately strong acid phosphatase and the blood monocytes gave a strong esterase reaction. They postulated that the tuberculo-phosphatide may be the substrate for acid phosphatase. They suggested that the cells of a more resistant animal like the mouse might be able to metabolize the tuberculo-phosphatide to acid phosphatase, whereas the cells of the susceptible guinea pig would not have this ability. Therefore, tubercle formation would be induced more readily in the guinea pig due to the larger amount of phosphatide available.

It was therefore thought it would be of interest to attempt to determine whether a difference in the acidity of the neutrophil and monocyte of the mouse, guinea pig, and rabbit could be demonstrated by *in vitro* phagocytosis of organisms such as *M. smegmatis* and *M. tuberculosis var hominis* H37Rv and H37Ra stained with indicator dyes.

A protocol on the phagocytosis of M. smegmatis by the neutrophils and monocytes of the guinea pig was as follows:

A 300 g guinea pig was inoculated intraperitoneally for 7 days with 10 ml of saline solution containing 0.1 mg glycogen. On the last day the animal was inoculated in the morning and sacrificed 8½ hours later. The abdomen was quickly incised and the peritoneal exudate withdrawn with a sterile pipette. The fluid was purulent and free from any visible erythrocytes. Approximately 5-6 ml of fluid was obtained and 0.2 ml of 1-100 solution of heparin was added as an anticoagulant. The pH of the exudate was 6.9 - 7.0 using universal indicator paper. A cell count was made and it was found that there were 50 per cent neutrophils and 50 per cent monocytes. This was not as high a percentage of monocytes as Suter et al. (1925 a) found, but they were sufficiently numerous for the phagocytosis experiments.

The centrifugation method was used as outlined under procedures. M. smegmatis was used. These bacilli stained well and were readily phagocytized. The organisms were stained with brom phenol blue, brom cresol green, and neutral red.

At least 70 per cent of the cells showed phagocytosis. The organisms stained with the sulfonphthalein dyes did not shift to the acid range of the indicator but retained the colors of the alkaline range when phagocytized by the neutrophil and monocyte. The bacteria stained with brom phenol blue were blue, and those stained with brom cresol

green were blue-green. The color of the organisms appeared to be the same in the monocytes and neutrophils.

Cells showing phagocytized M. smegmatis stained with neutral red were striking. The bacteria inside the cells were red and orange-yellow in the surrounding fluid. However, Conn (1953) stated, "neutral red is red in weak acids and the reaction of ordinary tap water is sufficient to bring out the acid color". Neutral red, like litmus, is not a good indicator to determine a low degree of acidity but it was of interest that the indicator stain on the organisms was capable of shifting to the acid side when phagocytized by the guinea pig neutrophil and monocyte.

Mouse and rabbit. In vitro experiments were also carried out with neutrophils and monocytes of the mouse and the rabbit. The organisms and dyes used were the same as in the guinea pig experiments.

The results obtained with the cells of the mouse and rabbit were similar to those found in the guinea pig. The ingested organisms stained with brom phenol blue appeared blue, and those stained with brom cresol green appeared green-blue. Organisms in the cells stained with neutral red were red whereas the extracellular bacilli were orange-yellow.

There was some clumping of M. tuberculosis H37Rv and H37Ra, and the cells surrounded the clumps of organisms. Few cells revealed phagocytized bacteria. The neutrophil and the monocyte of the mouse did not differ from those of the guinea pig and rabbit in that a low degree of granular acidity was not detected in these cells after they

had ingested phagocytized bacteria stained with indicator dyes. This is in contrast to the findings of Grogg and Pearse (1952) who found that after inoculation with the Vallée strain of bovine Mycobacterium tuberculosis the monocyte of the mouse differed from the monocytes of the guinea pig and rabbit with respect to the type of enzyme present.

III. *In Vivo* Results

The conditions under which *in vitro* phagocytosis takes place are not exactly comparable to those within the body of the animal, and the time that the ingested organisms are in contact with the fluids of the cell is limited. Therefore, *in vivo* tests were made using the adult white mouse and the guinea pig. The procedures used for these tests were described under Methods.

Adult white mice. Many experiments have been performed. Observations on a typical one undertaken on a mouse weighing 28 g and injected with *M. tuberculosis var hominis* H37Rv stained with brom phenol blue (acid-yellow, basic-blue) were as follows:

Two hours after injection a small amount of peritoneal fluid was aspirated. There were 4-5 cells per oil immersion field with intensely blue organisms in the cells. The cells at this time were nearly all neutrophils. The extracellular organisms were all blue. The cell refractive granules did not appear to be colored. At 24 hours there were more monocytes. Some neutrophils with ingested brilliant blue organisms had been engulfed by monocytes. Other monocytes and neutrophils also showed phagocytized blue stained organisms. No color in the normal cell granules was noted. At 48 hours the exudate was very scanty but the same observations were made. At 72 hours many monocytes had ingested organisms which were vivid blue.

Few neutrophils and no extracellular organisms now were observed. Under the conditions of the experiment there is no way of knowing just how long the ingested bacteria had been in any particular cell. However, it would seem that since no extracellular organisms were found after 48 or 72 hours following injection, the ingested organisms had probably been phagocytized for some hours.

Five days after inoculation the animal was anesthetized with ether. Since on exposure to air the tissues of animals injected with vital stains may take on a more intense hue as Rous (1925 c) noted, care was taken to make a very small incision in the peritoneal lining. Sometimes, before the incision was made, the small blue nodules could be observed underneath the peritoneum. Many intensely blue nodules on the intestine, the omentum, and an occasional one on the liver and the spleen were observed. A nodule was quickly excised, gently crushed under a plastic cover slip, and examined microscopically. Many monocytes and neutrophils exhibited organisms stained blue in the cells. Refractive granules in the cells did not appear to be colored.

In a repeat experiment a photomicrograph was taken 5½ hours after intraperitoneal injection of M. tuberculosis H37Rv stained with brom phenol blue. This is illustrated in figure 1. It will be noted that there is a clump of five or six organisms which has been ingested by a large monocyte, and that the bacilli have retained the blue color of the basic range of the indicator. A suggestion of a vacuole can be observed about the phagocytized bacteria. This is

EXPLANATION OF PLATE I

Figure 1. Phagocytosis by monocytes of mouse of M. tuberculosis H37Rv stained with brom phenol blue. Photomicrograph taken 5½ hours after intraperitoneal injection. Bacilli are blue - color of basic range of indicator.

Figure 2. Phagocytosis by monocytes of mouse of M. smegmatis stained with brom phenol blue. Cells shown from nodule on large intestine. Organisms are blue 48 hours after intraperitoneal injection.

Figures 3 and 4. Monocytes of peritoneal fluid of a mouse 18 hours after intraperitoneal injection of M. tuberculosis H37Rv stained with brom cresol green. Organisms in cells are green with tinge of yellow. Large refractive granules appear green.

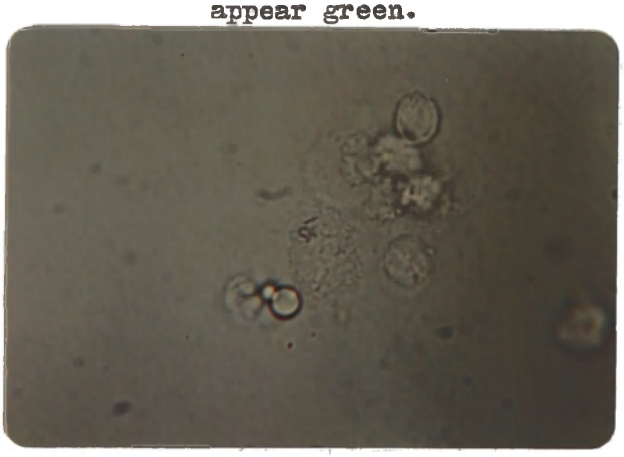


FIG. 1.

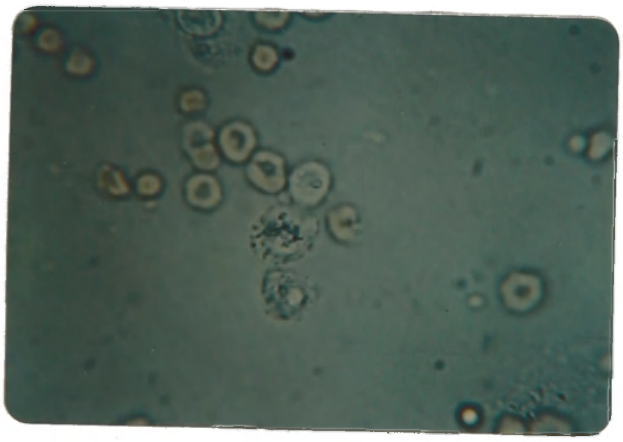


FIG. 2.

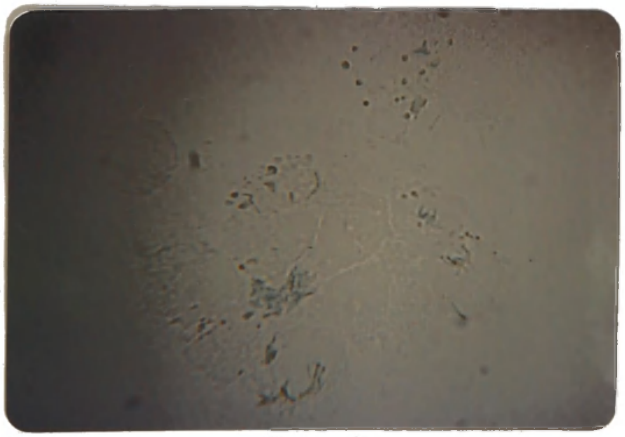


FIG. 3.

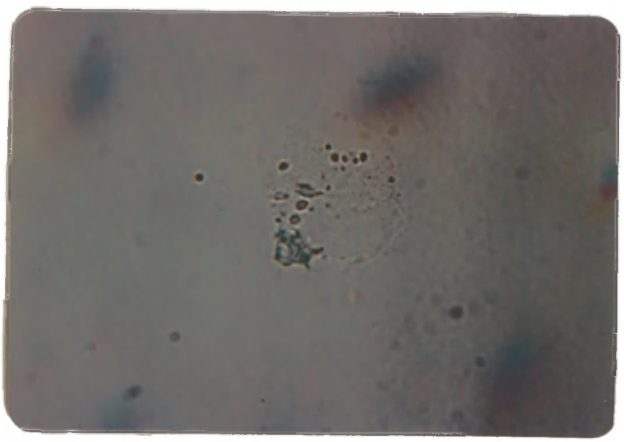


FIG. 4.

clearer in the photomicrograph than when the preparation was viewed under the oil immersion lens of the microscope. The argument may be advanced that the organisms had not been in the cell long enough for sufficient acidity to develop to change the color of the organisms. However, in figure 2 a cell is shown which was taken from a nodule on the large intestine 48 hours after the stained bacteria, in this case M. smegmatis had been phagocytized. The bacteria in the monocyte were definitely blue after this period of time.

Brom cresol green (acid-yellow, basic-blue). The following observations were made on a mouse injected intraperitoneally with M. tuberculosis H37Ra stained with brom cresol green. Two hours after injection a very small amount of fluid was aspirated from the peritoneal cavity. A few cells exhibited ingested blue-green organisms. Extracellular organisms were blue-green. The cells and organisms appeared more clumped than when M. smegmatis was used. At 24 hours the organisms appeared blue-green in the cells. At 48 hours there were many monocytes with vivid blue-green stained organisms in the cells. There were also many refractive granules which had a faint yellow-green appearance. These granules were all of the refractive type and not the non refractive small granules which may appear when solutions of the sulfonphthalein dyes are injected. However, care should be taken in describing relative shades of cell granules as even in cells from animals which have not been injected with dyes, the refractive granules appear to have a yellow-green tinge, sometimes yellow. Claude (1947-48) stated that the large

granules of the cytoplasm are yellow-brown, sometimes a lemon color. Intraperitoneal injections of brom cresol green and of organisms stained with this dye, elicited a greater number of refractive granules in the cells than did brom phenol blue. At 72 hours the organisms in the monocyte appeared to be slightly more greenish yellow than previously observed. Ninety-six hours after injection the mouse was subjected to ether anesthesia. The abdomen was incised while the animal was under the anesthetic. Tiny blue-green nodules were found on the intestine. A small retroperitoneal nodule was crushed gently on a plastic cover slip. There were many more monocytes observed in the tissue than occurred after injection with M. smegmatis. The organisms in the monocytes appeared to be greener with a very slight yellow tinge whereas in the neutrophils the bacteria were bluish green. This may possibly indicate a slightly lower pH about the engulfed bacteria in the monocyte than in the neutrophil. The extracellular organisms were blue.

Figures 3 and 4 show cells of the peritoneal fluid of a mouse 18 hours after injection with M. tuberculosis H37Rv stained with brom cresol green. It will be noted in figure 3 that the extracellular organisms clumped in the center are blue-green whereas some of the bacteria ingested by the large monocyte directly above the clumped extracellular bacteria and also those in the cell farthest to the left are green with a tinge of yellow. The pK value which is the point of 50 per cent dissociation of the indicator is 4.7 for brom cresol green. This would suggest that the ground matrix surrounding

the engulfed bacteria in the monocyte may possibly be as low as 4.7. However, no cells were observed with lemon-yellow organisms which would indicate a pH of 3.8 or lower. Figure 4 shows a large clump of organisms in the monocyte that are definitely green whereas the smaller clump seems to have a greenish yellow appearance. The large refractive granules have a green tinge.

Figure 5 shows a group of cells taken from a small mass on the intestine 48 hours after intraperitoneal injection of M. tuberculosis H37Rv stained with brom cresol green. The extracellular and intracellular organisms are blue.

Guinea pigs. Animals received two intraperitoneal injections of 7.2 per cent sodium caseinate to stimulate migration of neutrophils and monocytes, followed by intraperitoneal injection of stained organisms as outlined under procedure. The results with the three organisms used were similar except that M. smegmatis and M. tuberculosis H37Rv appeared to be more readily phagocytized than M. tuberculosis H37Ra. Similar results were found in the mouse. Bloch (1948) found that after 96 hours only 1 per cent of the leucocytes in the peritoneal exudates of mice engulfed avirulent tubercle bacilli whereas 15 per cent phagocytized virulent organisms, H37Rv. Furthermore, the number of monocytes was increased earlier when M. tuberculosis H37Rv was injected than when M. smegmatis and M. tuberculosis H37Ra were used.

The following is a typical protocol on a 250 g guinea pig inoculated intraperitoneally with 10 ml of 7.2 per cent sodium caseinate on two successive days followed by 10 ml distilled water pH 7.0

containing 0.5 ml of M. tuberculosis H37Rv stained with brom phenol blue 24 hours after the last injection of sodium caseinate. Six hours after the stained organisms were injected a small amount of colorless opaque fluid, which gave a pH of 6.9 - 7.0 with universal indicator paper, was aspirated from the peritoneal cavity. A drop of the peritoneal fluid was placed on a plastic cover slip which was inverted on a well slide ringed with vaseline to exclude air. Preparations were examined immediately under the oil immersion lens. The organisms were blue in both the neutrophils and monocytes. Many monocytes had already phagocytized neutrophils containing organisms stained blue. The normal cell granules did not appear to be colored. At 30 hours a small amount of exudate was aspirated. At least 5 cells per oil immersion field contained intensely stained blue bacteria. These were observed as soon as one focused the microscope. Many large monocytes with as many as 3 or 4 phagocytized neutrophils were noted. Organisms in the monocytes and also in the phagocytized neutrophils were very blue. After 30 hours a few extracellular blue stained bacteria were found. At 50 hours the same observations were made. Three cells per oil immersion field showed phagocytized blue bacilli. After 76 hours a very minute amount of peritoneal fluid was withdrawn. Brilliantly blue stained bacilli were observed in the monocytes and neutrophils although there were fewer monocytes with phagocytized neutrophils than at 30 and 50 hours.

In a repeat experiment, similar to the one just described, photomicrographs were taken 26 hours after 0.5 ml of M. tuberculosis

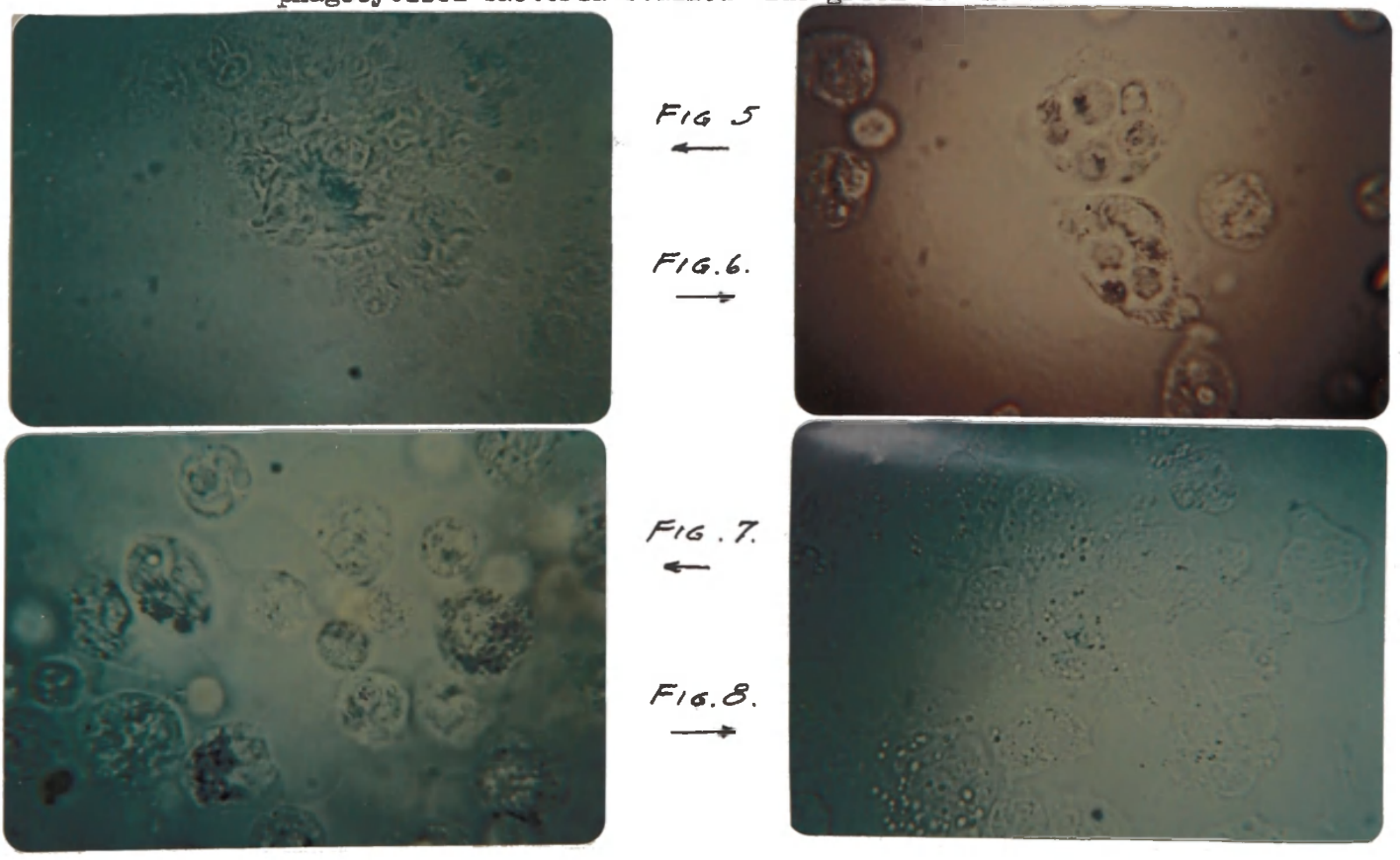
EXPLANATION OF PLATE II

Figure 5. Cells from mass on large intestine of mouse 48 hours after intraperitoneal injection of M. tuberculosis H37Rv stained with brom cresol green. The extracellular and intracellular organisms are blue - basic range of the indicator.

Figure 6. Cells of peritoneal fluid of guinea pig 26 hours after intraperitoneal injection of M. tuberculosis H37Rv stained with brom phenol blue. Neutrophils containing organisms stained blue are shown engulfed by monocytes.

Figure 7. Same as Figure 6 - 48 hours after intraperitoneal injection of organisms. Monocytes with numerous phagocytized bacilli stained blue are shown.

Figure 8. Cells of peritoneal fluid of guinea pig 12 hours after intraperitoneal injection of M. tuberculosis H37Rv stained with brom cresol green. In center of field a monocyte with phagocytized bacteria stained blue-green is shown.



H37Rv, suspended in 10 ml of buffered distilled water was injected into the peritoneal cavity of a male guinea pig. The tubercle bacilli were stained with brom phenol blue. In figure 6, guinea pig Lq478, the intensely blue stained bacteria are seen engulfed in the neutrophils which had been phagocytized by the monocytes. The blue bacilli can also be observed in the monocyte. In figure 7, guinea pig Lq478, taken 48 hours after the organisms had been injected, the bacteria still colored blue, can be observed in the monocytes and neutrophils.

Brom cresol green. The same procedure was followed except that M. tuberculosis H37Rv was stained with brom cresol green. Six hours after injection of the bacilli, the peritoneal fluid of the guinea pig was aspirated. A slightly bloody fluid was obtained. The material was placed on a plastic cover slip inverted on a well slide ringed with vaseline and viewed immediately under an oil immersion lens. Many monocytes and neutrophils contained green-blue organisms. At this time, 30 hours after injection, there appeared to be even more monocytes with phagocytized neutrophils containing stained bacteria than when brom phenol blue was employed as the stain. The same microscopic picture prevailed at 48 and 76 hours. At the end of 2 weeks the animal was anesthetized with ether. The abdomen was opened while the animal was living. Before the peritoneal lining was incised, a blue-green mass measuring 0.5 mm was visible on the peritoneal wall showing that even before exposure to air the mass was bluish green. Several smaller masses of 0.1 and 0.2 mm were observed on the omentum

and a large adhesion to the liver contained several blue-green masses. Rous (1925 a) found that 24 days after subcutaneous injection of litmus and agar, the center of the agar appeared blue but the zone around it for a distance of 2 cm was a brilliant pink which was due to numerous red granules in the macrophages and what appeared to be fibroblasts. However, with the sulfonphthalein dyes there was no evidence of any yellow coloration at the periphery of the tissue which would indicate a high degree of acidity.

A small amount of tissue was excised and placed on a slide and pressed down gently with a plastic cover slip. Numerous blue-green organisms were in the cells 2 weeks after injection. The pH of the center of the blue-green mass was approximately 7.0 - universal indicator paper. A drop of the very slight amount of peritoneal fluid showed monocytes; only one cell was observed with blue-green organisms, and there was only a rare monocyte with engulfed neutrophils. The color of the refractive granules appeared normal.

In a repeat experiment photomicrographs were taken 12 hours after intraperitoneal inoculation with the organism, M. tuberculosis H37Rv stained with brom cresol green. A monocyte with blue-green engulfed bacteria is shown in the center of the field, figure 8.

Since the pK values of brom phenol blue and brom cresol green are 4.0 and 4.7 respectively, it was thought it would be of interest to use an indicator such as brom cresol purple which is yellow at a pH of 5.2 and purple or blue at a pH of 6.8. The pK value of this

indicator is 6.3 which is the point where there is maximum intensity of both yellow and purple or a greenish yellow color.

A protocol using M. tuberculosis H37Rv stained with brom cresol purple was as follows: A male guinea pig weighing 300 g was inoculated intraperitoneally with 10 ml of 7.2 per cent sodium caseinate on 2 successive days followed by 10 ml of buffered distilled water containing 0.5 ml of organisms stained with brom cresol purple on the third day. Six hours after the organisms were injected, fluid was aspirated from the peritoneal cavity. A very small amount of colorless fluid was obtained. Many monocytes showed engulfed bacteria which were yellow-green. Many monocytes with ingested neutrophils containing yellow-green organisms were found. Granules possessed the normal color. At 24 hours a small amount of colorless fluid was withdrawn which had a pH of 7.0. The color of the phagocytized organisms inside the monocytes was yellowish green. A small amount of N/20 sodium hydroxide was run under the cover slip and the bacilli in the cells after a period of 10-15 minutes became pale blue. The same results were found at 48 and 96 hours although there were not as many monocytes showing engulfed neutrophils at this time as were observed earlier. Six days after injection of stained organisms the animal was placed under ether anesthesia and the abdomen incised. There was a scant amount of peritoneal fluid which contained monocytes with bacteria stained yellow-green. There were also many small green-yellow nodules on the omentum and spleen. Organisms in cells of the nodules were of the same intense color as those found in the free fluid. Sodium hydroxide

N/20 was added under the cover slip and organisms became blue-green in the cells.

Indicator dyes injected subsequent to the injection of stained organisms. When 1 and 2 per cent solutions of brom phenol blue and brom cresol green were injected intraperitoneally into the mouse, no fine lemon-yellow translucent granules such as Rous described were observed. It was thought that it would be of interest to inject the dyes subsequent to inoculation of the stained organisms in order to see whether any "granular acidity" could be observed surrounding the ingested stained bacteria. Four tenths ml of a 2 per cent solution of brom phenol blue was injected into a 30 g mouse 18 hours after M. smegmatis stained with brom phenol blue had been inoculated intraperitoneally. Three injections of the dye were given and the peritoneal fluid aspirated 24 hours after each injection. Phagocytized organisms were blue in the monocytes and neutrophils, but no yellow granules surrounding the phagocytized bacilli were observed. Negative results were also obtained when 2 per cent brom cresol green was injected into a 30 g mouse after inoculation of organisms stained with the same dye.

Injection of stained organisms in the acid range of the indicator. In order to ascertain whether stained organisms would shift to the alkaline side when placed in the peritoneal cavity, organisms stained with brom phenol blue and brom cresol green were washed several times with distilled water of pH 3.0 and 0.2 ml injected into the peritoneal

cavity of a mouse 6 hours after injection of 2.0 ml of 7.2 per cent sodium caseinate. Twelve hours after organisms were inoculated, fluid was aspirated from the peritoneal cavity. The organisms ingested by the neutrophils and monocytes were a bright blue as were the majority extracellular organisms. However, one clump of extracellular bacilli was bright yellow; these evidently had not yet shifted to the basic range of the indicator. After 48 hours the animal was placed under ether anesthesia, and the abdomen incised. There was no visible peritoneal exudate, and small, bluish purple nodules were observed on the omentum. Neutrophils and macrophages showed many phagocytized bacteria, all definitely blue. At this time no intracellular or extracellular yellow stained bacteria were found.

When organisms stained with brom cresol green were suspended in distilled water, pH 3.0, and injected, the peritoneal fluid withdrawn after 12 hours showed blue-green organisms in the cells. Some extracellular organisms were blue-green but some clumps of bacteria appeared to be yellow-green and had not turned to the blue-green of the alkaline range. Small bright green nodules were observed on the omentum at 48 hours. Both the monocytes and neutrophils revealed phagocytized organisms which were greenish blue.

It is probable that the organisms shifted color before phagocytosis occurred as when sodium caseinate was added to the bacilli in the test tube, they became blue (brom phenol blue) and blue-green

(brom cresol green). Some organisms undoubtedly retained the color of the acid range 12 hours after injection, because the organisms were clumped and the fluid did not penetrate sufficiently to change the color. At 48 hours no intracellular or extracellular bacteria the color of the acid range (yellow) were observed.

DISCUSSION

The experiments described and illustrated in figures 1-8 were made in an attempt to determine whether as low a degree of acidity as pH 3.0 would be produced in the mammalian neutrophil and monocyte after phagocytosis of organisms stained with indicator dyes.

The approximate pH of the peritoneal fluid in which the neutrophils and monocytes phagocytized the stained bacteria was 7.0. Thus, the pH of the medium was slightly greater than the pH of the cytoplasm. As noted previously, Ishikawa (1935) stated that the pH of the cytoplasm of the neutrophil was 6.6. Frey-Wyserling (1948) asserted that the acid and basic groups of the molecular frame work which are screened off in the cytoplasm at the isoelectric point must first be liberated by slight hydrolysis in order to be capable of reacting with the dyestuffs. If the stained organisms are in the cytoplasm, one might postulate that there were not sufficient acid or basic groups liberated by hydrolysis or that they were neutralized immediately so they could not react with the dyestuff. Reznikoff (1926-27) suggested that the buffers in the cytoplasm of Amoeba proteus could effectively neutralize any acid formed by hydrolysis.

It does not appear probable, however, that the cytoplasmic matrix of either the neutrophil or monocyte would be changed to as low as pH 3.0 even if injured by ingested particles. Pollack (1928) found that

when buffer solutions were injected into Amoeba proteus and Amoeba dubia previously injected with indicator dyes, regardless of the pH of the buffer, the return of the color of the indicator to the usual one was quite rapid and constant. When sufficient buffer was added to change the pH of the cell, the cell died. He concluded that the cytoplasm of the amoeba has considerable buffering power and when the pH of the cytoplasm is changed, the cell dies. Chambers and Pollack (1926-27) also found that localized increase in the protoplasmic acidity in the amoeba caused by mechanical injury was immediately neutralized.

However, it is generally believed that phagocytized material ingested by neutrophils and monocytes is aggregated in a vacuole, the reaction of which is not the same as the cytoplasm (Metschnikoff, 1889; Evans and Scott, 1921; Sabin, 1923). Metschnikoff (1889) thought that the digestion of the phagocytized bacteria was aided by digestive ferments and that it took place in a vacuole, the fluid of which was weakly acid.

No vacuoles were observed around the phagocytized bacteria stained with indicator dyes when observed under the microscope. In figures 1, 3 and 4 there is a suggestion, however, that there is a clear area perhaps a vacuole, surrounding the engulfed organisms. If a phase microscope had been used, vacuoles might have been observed as Wilson (1954) noted formation of a vacuole around streptococci shortly after they were ingested by the neutrophil.

Lewis (1923) on the other hand conducted experiments with connective tissue cells of the chick embryo and found that in tissue culture these cells engulfed Bacillus radicicola, an organism isolated from soy bean and red clover, and destroyed them within a short period of time. The bacilli were not taken into vacuoles, neither did a vacuole form around them during the course of their destruction.

The environment surrounding the stained phagocytized organisms may be slightly different from the cytoplasm as the color of the organisms became more intense when slides were not ringed with vaseline and exposed to air due to the permeability of the tissue cells to ammonia. Chambers (1928) found that the color of intracellular inclusions of amoebae could be shifted by a change in the environment, either exposure to carbon dioxide or ammonia, but that the pH of the cytoplasmic matrix remained constant.

Neutral red has been used as a vital dye to stain the granules and vacuoles by Sabin (1923), Strugger (1935), and Chambers (1943) to demonstrate that they have a reaction different from that of the protoplasmic matrix. Frey Wyserling (1948) has noted that the end groups of organic compounds which are constituents of the vacuolar colloids are not screened off as in the cytoplasm and are therefore reactive, and the acid groups are free to react with the basic dyes. This would be an explanation of why the granules and vacuoles were stained, but the cytoplasm remained unchanged. Since neutral red turns red at a pH of 6.8 it cannot be used to determine whether the acidity

surrounding the phagocytized material is as low as pH 3.0.

Although there may be acid groups present in the colloid material around the ingested bacilli, there does not appear to be a sufficient number to react with the indicator adsorbed to the organisms. The organisms stained with brom phenol blue are definitely blue after being phagocytized by the neutrophil and monocytes shown in figures 1-4. The color of the organisms was not even slightly changed to green or a greenish yellow. According to Conn (1953), "these color changes in the sulfonphthalein indicators are generally assumed to be due to alterations in the structure of the molecule such as the disappearance and reappearance of the quinoid ring, but the relation of structure to color is complicated and has not yet been worked out to general satisfaction". Therefore, no theory as to what groups present in the material surrounding the stained organisms that might react with the indicator molecule has been advanced.

The fact that the organisms stained with the indicator dyes do change color can be demonstrated <u>in vitro</u> by placing the stained bacteria in buffer solutions of appropriate pH values. It should be noted that in the case of brom phenol blue, when the organisms are placed in Mac Ilvaine's buffer solutions of pH 2.8 and 3.0, the color of the bacilli only changed to greenish yellow and not yellow. If, however, N/20 hydrochloric acid was added, the bacteria became intensely yellow. To test the indicator the dye was changed to the sodium salt with the proper amount of N/20 sodium hydroxide and made up to 0.04 per cent

solution. A few drops of the indicator was added to the correct amount of buffer solution and it was found that the buffer solutions of pH 2.8 and 3.0 also appeared greenish yellow rather than intensely yellow. In all the experiments conducted, however, the organisms stained with brom phenol blue were definitely blue both in the monocyte and neutrophil and were never observed to have the slightest tinge of green or yellow.

The color changes in organisms stained with brom cresol green were quite sharp and corresponded to the color of the buffer solutions when a few drops of 0.04 per cent indicator solution were added to them.

SUMMARY

Mice, guinea pigs, and rabbits were injected intraperitoneally with azolitmin suspended in 1.5 per cent agar. It was found that the monocytes and neutrophils of these animals possessed sufficient acidity or produced the amount necessary to change the phagocytized blue particles of azolitmin to red. Azolitmin was magenta colored at pH 6.5 and red at pH 6.0.

The development of granular acidity as low as pH 3.0 was not observed in either the monocyte or neutrophil of the adult white mouse when injected intraperitoneally with one and two per cent solutions of the indicator dyes, brom phenol blue, and brom cresol green.

M. tuberculosis var hominis H37Rv, H37Ra, and M. smegmatis were stained with the sulfonphthalein dyes, brom phenol blue, brom cresol green, and brom cresol purple. Under the experimental conditions employed, it was found that sufficient acidity did not develop in either the neutrophil or the monocyte of the mouse, guinea pig and rabbit to shift the color of the phagocytized stained organisms to the acid range of the indicator dyes.

When indicator dyes were injected intraperitoneally subsequent to injection of stained organisms, granular acidity as low as pH 3.0 in the cytoplasmic matrix surrounding the ingested bacteria was not observed.

References

Bloch, H. 1948 The relationship between phagocytic cells and human tubercle bacilli. Am. Rev. Tuberc., 58, 662-670.

Cameron, G. R. 1951 Pathology of the Cell. C. C. Thomas, Springfield, Illinois.

Chambers, R. 1928 Intracellular hydrion concentration studies -a. Biol. Bull. of Marine Biol. Lab., 55, 369-375.

Chambers, R., and Pollack H. 1926-27 Micrurgical studies in cell physiology. J. Gen. Physiol., 10, 739-741, 750-753.

Chambers, R. 1943 Electrolytic solutions compatible with the maintenance of protoplasmic structures. Biol. Symposia-Frontiers in Cytochemistry., 10, 91.

Clark, W. M., and Lubs, H. 1921 Determination of Hydrogen Ions 3rd ed., 581.

Clark, W. M., and Lubs, H. 1917 The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. J. Bact., 2, 1-34, 109-136, 191-236.

Claude, A. 1947-48 The Harvey lectures, Studies of cells-Morphology, chemical constitution and distribution of biochemical functions. C. C. Thomas, Springfield, Illinois.

Cohen, B. 1923 Some new sulfonphthalein indicators. A preliminary note. Pub. Health Rep., 38, 199.

Cohen, B. 1926 Indicator properties of some new sulfonphthaleins. Pub. Health Rep., 41, 3051-3074.

Conn, H. J. 1953 Biological Stains, 6th ed. Biotech. Publications, Geneva, New York.

Dubos, R. J. 1953 Effect of ketone bodies and other metabolites on the survival and multiplication of staphylococci and tubercle bacilli. J. Exper., Med., 98, 145-155.

Evans, H. M., and Scott, K. J. 1921 On the differential reaction to vital dyes exhibited by the two great groups of connective tissue cells. Contr. Embryol, Carnegie Instit. Washington, 10, 1-55.

Frey-Wyssling, A. 1948 Submicroscopic Morphology of Protoplasm and its Derivaties. New York, Amsterdam, London and Brussels.

Grogg, E., and Pearse, A. G. E., 1952 The enzymic and lipid histochemistry of experimental tuberculosis. Brit. Exper. Path., 33, 567-576.

Harris, J. O. 1951 A study of the relationship between the surface charge and the absorption of acid dyes by bacterial cells. J. Bact., 61, 649-652.

Irving, L. 1926-1927 Regulation of the hydrogen ion concentration and its relation to metabolism and respiration in the starfish. J. Gen. Physiol., 10, 345-357.

Ishikawa, A. 1935 Die Wasserstoffionen Konzentration der Leukozyten (bestimmt unter Verwendung des Mikromanipulators). (I. med. Klim. der med. Fakultat Nagoya) Z. f. Kl. Path. u. Hamatol., 4, 305.

Kite, G. L. 1913 Studies on the physical properties of protoplasm. Am. J. Physiol., 32, 146.

Lamanna, C., and Mallette, M. F. 1953 Basic Bacteriology. Williams and Wilkins Co., Baltimore, Maryland.

leDantec, F. 1890 Recherches sur la digestion intracellulaire chez les Protozoaires, Ann. Inst. Pasteur, 4, 776-791.

Levine, M. D. 1940 The determination of apparent isoelectric points of cell structures by staining at controlled reactions. Stain Technol., 15, 91-112.

Lewis, M. R. 1923 The destruction of Bacillus radicicola by the connective tissue cells of the chick embryo in vitro. Bull, Johns Hopkins Hosp., 34, 223.

MacIlvaine, T. C. 1921 A buffer solution for colorimetric comparison. J. Biol. Chem., 49, 183.

Machaness, G. B. 1952 The action of drugs on intracellular tubercle bacilli. J. Path. and Bact., 64, 429-445.

Mallmann, W. L., and Churchill, E. 1942 The detection of bacteria in liquid egg-meats during processing. Ice and Refrigeration.

Marshak, A. 1944 Changes in the apparent cytoplasmic hydrogen ion concentration of Ameba Dubia on injection of egg albumin. J. Gen. Physiol., 28, 95-102.

Martin, S. P., Pierce, C. H., Middlebrook, G., and Dubos, R. J. 1950. The effect of tubercle bacilli on the polymorphonuclear leucocytes of normal animals. J. Exper. Med., 91, 381-391.

Metchnikoff, E. 1889 Recherches sur la digestion intracellulaire. Ann. Inst. Pasteur, 3, 25-29.

Metchnikoff, E. 1905 Immunity in infective diseases, Cambridge.

Mammesn, H. 1927 Uber elektrostatische Lagung Von Zellen des menschlichen Blutes, ein Beitrag ur Frage der Azido und Basophilie. Folia Hamat., 34, 50.

Mudd, S., Lucke, B., Mc Cutcheon, M., Strumia, M. 1929 Correlation between changes in bacterial surface properties and in phagocytosis caused by sera of animals under immunization. J. Exper. Med., 49, 779-795.

Needham, J., and Needham, D. M. 1926 The hydrogen - ion concentration and oxidation reduction potential of the cell-interior before and after fertilization and cleavage. A micro-injection study on marine eggs. Proc. Roy. Soc. London Series B., 99, 173-199.

Pollack, H. 1928 Intracellular hydrion concentration studies. Biol. Bull. Marine Biol. Lab., 55, 383.

Rawlins, L. M. C., and Schmidt, C. L. A. 1930 The mode of combination between certain dyes and gelatin granules. J. Biol. Chem., 88, 271-284.

Reznikoff, P. 1926-1927 Micrurgical studies in cell physiology. J. Gen. Physiol., 10, 9-21.

Reznikoff, P., and Pollack, H. 1928 The effect of injection of acids and salts on the cytoplasmic pH of Amoeba dubia. Biol. Bull., 55, 383.

Rich, A. R. 1951 The Pathogenesis of Tuberculosis, 2nd ed., C. C. Thomas, Springfield, Illinois.

Rhode, K. 1917 Untersuchungen uber den Einfluz der frien H-Ionen im Inneren lebender Zellen auf den Vorgang der vitalen Farbung. Archiv. fur die ges Physiologie, 168, 411.

Rous, P. 1925a The relative reaction within living mammalian tissues. I. General features of vital staining with litmus. J. Exper. Med. 41, 379-398.

Rous, P. 1925b The relative reaction within living mammalian tissues. II. On the mobilization of acid material within cells and the reaction as influenced by the cell state. J. Exper. Med., 41, 399-411.

Rous, P. 1925c ibid III. Indicated differences in the reaction of the blood and tissues on vital staining with phthaleins. J. Exper. Med., 41, 451-470.

Rous, P. 1925d IV. Indicated differences in the reaction of the organs on vital staining with phthaleins. J. Exper. Med., 41, 739-759.

Sabin, F. R. 1923 Studies of living human blood cells. Bull. Johns Hopkins Hosp., 34, 373.

Shaffer, J. M., Kucera, C. J., and Spink, W. W. 1953 The protection of intracellular brucella against therapeutic agents and the bactericidal action of serum. J. Exper. Med., 97, 77-90.

Schwartz-Karsten, H. 1927 Ueber blut farburg mit gepufferten Farblo Sungen. Deutsche med. Wehnsehr, 53, 1820.

Spek, von Josef, and Chambers, R. 1933 Das Problem der Reaktion des Protoplasmas. Protoplasma, 20, 376.

Strugger, S. 1935 Beitrage zur Geweb-physiologie der Wurzel zur analyse und Methodik der Vitalfarbung Pflanzlicher Zellen mit Neutral Rot. Protoplasma, 24, 108-127.

Suter, E. 1952a The multiplication of tubercle bacilli within normal phagocytes in tissue culture. J. Exper. Med., 96, 137-150.

Suter, E. 1952b Action of streptomycin and isonicotinic acid hydrazide on M. tuberculosis within phagocytes. Am. Rev. Tuberc., 65, 775-776

Sutton, 1911 Volumetric Analysis, 10th ed.

Tullis, J. L., and Kochow, E. G. 1952 Non wettable surfaces. Blood, J. Hemat., 7, 850-853.

Tullis, J. L. 1953 Preservation of leukocytes. Blood, J. Hemat., 8, 563-575.

Weiss, L. P. 1953 Binding of acid and basic dye at varied pH by blood and bone marrow cells of man. Blood, J. Hemat., 8, 249-261.

Wilson, A. 1953 The egestion of phagocytized particles by leukocytes. J. Exper. Med., 98, 305-310.