glutinin by dissociation of conglutinated zymosan are in progress.

Summary. The conglutination of sensitized sheep erythrocytes or zymosan requires Ca^{++}. Mg^{++} and Ba^{++} are inactive but Sr^{++} appears to substitute to a limited extent for Ca^{++}. Zymosan inhibits conglutination of sensitized sheep cells by bovine serum. Bovine properdin and zymosan react readily in the absence of Ca^{++}, but in the presence of Mg^{++}, without any interference due to conglutination.

1. Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C., *Science*, 1954, v120, 279.

2. For a comprehensive review of conglutination see Schmidt, H., *Fortschritte der Serologie, Darmstadt, Verlag,* Dr. Dietrich Steinkopff, 1955.

3. Bukantz, S. C., Rein, C. H., and Kent, J. F., *J. Lab. and Clin. Med.,* 1946, v31, 394.

4. Leon, M. A., Plescia, O. J., and Heidelberger, M., *J. Immunol.,* 1955, v74, 313.

5. Osler, A. G., Strauss, J. H., and Mayer, M. M., *Am. J. Syph.,* 1952, v36, 140.

6. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.,* 1946, v84, 535.

7. Schwarzenbach, G., and Ackermann, H., *Helvet. Chim. Acta.,* 1948, v31, 1029.

8. Leon, M. A., *J. Exp. Med.,* 1957, v105, 403.

Received June 26, 1957. P.S.E.B.M., 1957, v96.

A Method of Securing Bacteriologically Sterile Snails (*Australorbis glabratus*).* (23433)

ELI CHERNIN (Introduced by T. H. Weller)

Department of Tropical Public Health, Harvard School of Public Health, Boston, Mass.

Recent progress in the axenic cultivation of multicellular organisms suggested that snails might also be cultivated in a bacteriologically-sterile environment. Accordingly, investigations were begun in 1954 to determine whether the eggs of *Australorbis glabratus,* the Western Hemisphere vector of schistosomiasis, could be induced to hatch under sterile conditions after being subjected to surface disinfection.

Materials and methods. 1. *Maintenance and handling of snails and snail eggs.* Eggs were derived from a Puerto Rican strain of *A. glabratus* maintained in our laboratory. The methods employed in rearing these snails have been described(1,2). Since large numbers of eggs were required, several 3-gallon aquaria were set aside for breeding purposes. Each aquarium contained 10 liters of water, a supply of water cress, and 25 snails which were replaced as they died or as fecundity diminished. Water cress was replenished at 5-7 day intervals at which time all egg-masses present on the vegetation were recovered by 'peeling' off the egg-masses using the edge of a watchmaker's forceps. The masses were then kept in a bowl of continuously aerated aquarium water at 76-78°F. Since snail embryos develop readily under these conditions, a supply was always available for experimental use (Fig. 1). Egg-masses containing immature embryos could not be employed since they commonly failed to hatch once disinfected. Consequently, when eggs were required, the stock was examined with a low-power microscope and only 'fully mature' masses selected. Such masses contained embryos which might be expected to hatch within 1-2 days; in fact, a crude guide to a 'mature' egg-mass was provided if one or more snails had already hatched at the time of examination (Fig. 2). The mature unhatched snail nearly fills its egg capsule, moves actively within its confines, and has a fully-formed shell which appears slightly yellow by indirect light. 2. *Composition and preparation of handling solution (HS).* The desirability of using a sterile solution of known composition in which disinfected snails would hatch was apparent. Such a solution was devised empirically by modifying 2 others which had been used, re-

* This investigation was supported (in part) by a Grant from the Natl. Inst. of Allergy and Infectious Diseases, from the United Fruit Co., and by a Senior Research Fellowship from the Public Health Service.

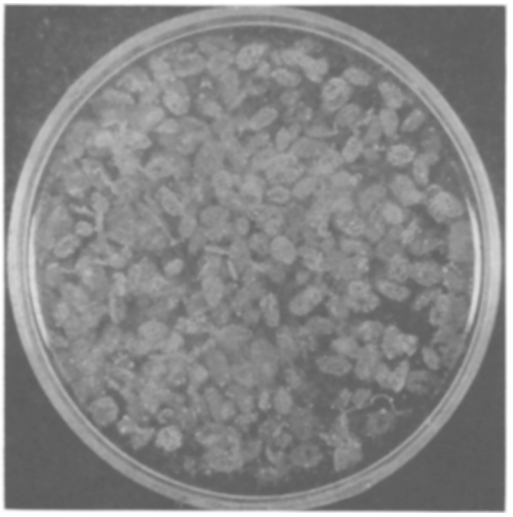

FIG. 1. Stock of *A. glabratus* egg-masses in various stages of development. Shown in an 8 cm Petri dish.

spectively, for snail tissues(3,4) and for physiologic studies on mussels(5). The composition of HS is shown in Table I. Salts #1-4 are dissolved in 800 ml water. Salt #5 is dissolved in 300 ml water and then combined with the first solution. Phenol red is added and the mixture is autoclaved for 10-15 minutes at 10-15 lb pressure. The volume of fluid allows for a 10% loss in autoclaving. The sterile solution is stored at 5°C until needed. The pH of the solution should be 7.2-7.4 after autoclaving; however, after several days the pH drops to about 7.0. If desired, a 4-fold concentrated solution of salts #1-5 may be prepared and later diluted and sterilized. For certain purposes, sterile HS is modified by the addition of: a) $NaHCO_3$ (1.4% aqueous stock sterilized by filtration; use 1.0-2.0 ml stock/100 ml HS); and b) penicillin G (cryst.-pot.) and streptomycin sulfate (100 units and μg respectively per ml HS). HS thus modified is designated HS(M). Glucose (20% aqueous stock; autoclaved) was used only as noted in a few experiments as a further addition to HS(M). 3. *Disinfecting solution.* The use of sodium hypochlorite solutions for disinfecting certain nematode eggs and larvae (see (6) for review) suggested that it might also serve for external disinfection of *A. glabratus* eggs. The solution was prepared by dilution of Clorox,† a commercial liquid bleach which contains 5.25% sodium hypochlorite by weight. The physical properties and mode of disinfection of NaOCl have been reviewed(7). 4. *Glassware.* Glassware and rubber stoppers were washed and otherwise processed as for purposes of tissue culture(8). 5. *Sterility tests.* Experiments, except as otherwise indicated, were controlled by inoculation of aliquots of HS(M), snail eggs, or newly-hatched snails into tubes of thioglycollate broth. These controls were maintained at 28-32° C for 7 days and at room temperature for at least 7 days more before being read and discarded. Supplementary bacteriologic tests were utilized

TABLE I. Composition of Handling Solution (HS).

	Constituents	g/liter H_2O†
1.	NaCl*	2.0
2.	KCl	.1
3.	Na_2HPO_4 (anh.)	.05
4.	$MgSO_4 \cdot 7H_2O$	.2
5.	$CaCl_2 \cdot 2H_2O$	.1
6.	Phenol red (0.4% aqueous stock)	.5 ml/100 ml HS

* Merck, sodium chloride for biological work. Other salts reagent grade.

† Double (glass) distilled H_2O used in preparing all salt and stock solutions.

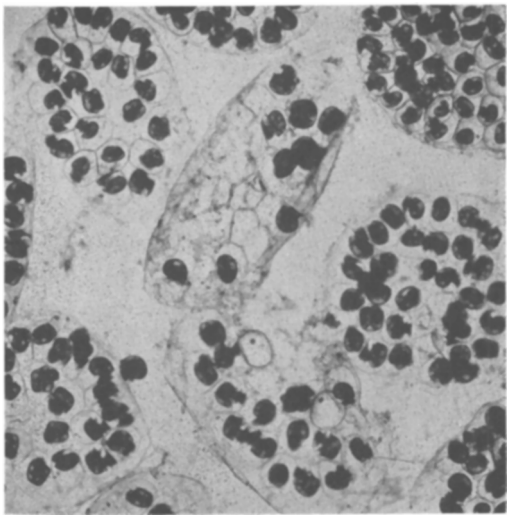

FIG. 2. Well-developed egg-masses of *A. glabratus* (×5). Many snails already hatched from clutches in center.

† Made by the Clorox Chemical Co., Oakland, Calif.

when required.

Summary of preliminary experiments. Attempts were first made to surface-disinfect entire egg-masses of *A. glabratus* and permit the snails to hatch under controlled conditions. Groups of mature egg-masses were exposed for 10-15 minutes to Clorox diluted 1:100 with HS(M), washed free of disinfectant, and isolated in separate tubes each containing 5 ml of HS(M). Over 50% of snails in egg-masses so treated hatched within 3-5 days (28°C). but a variable proportion of tubes proved to be contaminated. Further studies revealed that the presence of antibiotics in the HS(M) diluent interfered with the disinfecting activity of the hypochlorite. It was found that the omission of antibiotics during disinfection resulted in an increased proportion of egg-masses rendered sterile, but also in a decrease in rate of hatch; converse results were produced by increasing the concentration of antibiotics present during disinfection. A further complication arose from the frequent detection, by direct microscopic examination of the tubes, of protozoan contaminants. Microscopic examination of mature egg-masses from stock then revealed the occasional presence of ciliates *within* apparently intact, and otherwise normal, eggs. These protozoa superficially resembled forms commonly seen in aquarium water or in egg capsules from which snails had already hatched. The presence of these organisms within snail eggs cannot now be explained, but this observation made it apparent that whole egg-masses could not be used successfully.

Outline of technic. It appeared that the problem might be resolved by disinfecting *individual* eggs of *A. glabratus*. Preliminary experiments to separate eggs from the mass by a variety of enzymatic means were unsuccessful. Attempts were made to free eggs from the mass and from each other by manual means. This ultimately proved feasible and permitted development of the following technic.

1. Stocks of egg-masses are maintained and mature clutches are selected just prior to use. One or 2 mature egg-masses at a time are placed in half a Petri dish containing HS to which 0.2% Triton N-100‡ has been added. The detergent facilitates manipulation since particles do not tend to stick to the dissecting needles. It also rapidly kills bristle worms, protozoa, and perhaps other external contaminants. While snails within intact capsules are not affected by the detergent after 1-2 hours of immersion, those within perforated eggs die quickly. A simple means is thus afforded for distinguishing usable eggs.

2. Dissection of masses is done under a stereo-microscope with 20-30x magnification and transmitted light. Dissecting needles are prepared by breaking the hollow shaft of a 19-22 gauge hypodermic needle from its hub and inserting a very small insect pin ("Minutien", size 0.15) at one end of the cannula; this is crimped firmly into place and the opposite end of the needle is secured in the pin vise of a needle holder. Eggs are separated using 2 such needles. No effort is made to separate the closely adjacent eggs characteristic of most masses; rather, eggs surrounding one to be isolated are punctured and the selected egg freed by a 'scissoring' action of the 2 needles. However, in some clutches eggs do not lie immediately adjacent to one another and it is possible to separate them rapidly. Attached debris is trimmed away as each egg is separated from the mass and the eggs are then transferred by pipette to a dish containing HS. When the desired number of eggs has been accumulated, they are washed several times with HS (by decantation) and are checked microscopically for viability. A normal snail may be recognized by gross movement and by its characteristically rapid heartbeat. Eggs in which the snail is withdrawn, in which the capsule appears to be collapsed or punctured, or in which protozoa are observed, are discarded. The process of securing individual eggs becomes rapid with practice. An experienced person can free 50-100 viable eggs (Fig. 3) in about an hour, the variation depending on the nature of available masses.

3. The remaining procedures are done macroscopically, preferably in a hood, with sterile equipment and precautions. The eggs are transferred in groups of 10-20 to screw-cap

‡ Made by Rohm and Haas, Philadelphia. Other detergents were not investigated.

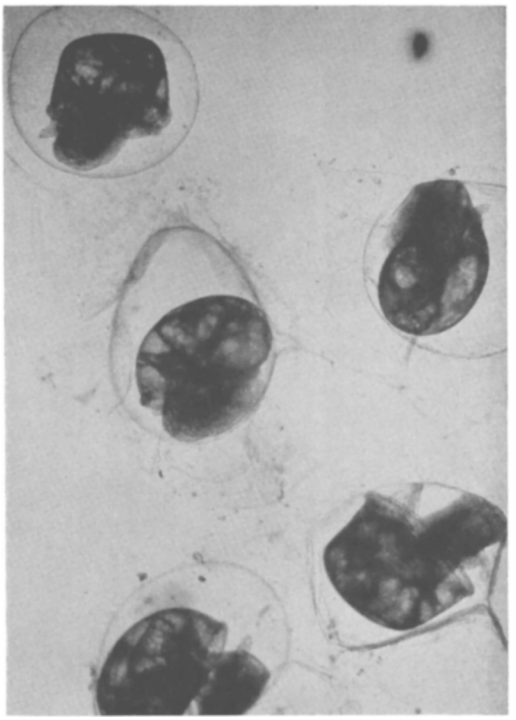

FIG. 3. Characteristic appearance of separated eggs of *A. glabratus* containing fully-developed snail embryos (×28).

vials (20x75 mm) using a Pasteur pipette. A minimum of fluid should be carried over, and fluid and eggs should be deposited on the bottom of the vial without contaminating its sides. All but a small amount of the transfer fluid is removed using the same pipette, and the cap is tightened to prevent drying.

4. Disinfecting fluid is prepared as follows: Place 19.95 ml HS (room temperature) in a sterile container. Add 0.05 ml (approximately one drop) of Clorox using a 1.0 ml serological pipette; this represents a 1:400 dilution of Clorox stock. Upon addition of Clorox the HS will turn deep purple, and it should be used within a few minutes.

5. Transfer 5 ml of the disinfecting solution to a vial and flood the eggs with it, tapping gently to swirl them about. Agitate the contents once or twice during the next 10 minutes. After 10 minutes the solution is removed by aspiration, using a narrow-bore Pasteur pipette. The eggs are washed free of the disinfectant with three 5 ml changes of HS(M), the wash fluids being removed by aspiration. About 2 ml of the last wash are left as transfer fluid.

6. A separate Pasteur pipette is used for the contents of each vial. Eggs are taken up in small groups and transferred to previously prepared tubes (*i.e.* 20x150 mm culture tubes containing 5 ml, or Wasserman tubes (10x100 mm) containing 2 ml of HS(M)), and one egg is deposited per tube; these are closed with rubber stoppers or screw caps. After completion of the procedure tubes are examined (using a stereo-microscope) in the same manner as roller tube tissue cultures. Snails which may accidentally have been killed during treatment are discarded. Tubes are maintained in an upright position in an incubator at 28°C.

Summary of results. 1. The concentration of disinfectant and the treatment period indicated above were selected after experiments in which the 2 factors were varied. In each such experiment, snail eggs were disinfected in groups of about 10 (using one of the schedules shown in Table II), washed and isolated in tubes containing 2 ml HS(M). The tubes were examined daily and hatch records compiled. On the 7th day each snail (or unhatched egg) was transferred by Pasteur pipette to a tube of thioglycollate for sterility tests. It is apparent from the summarized data (Table II) that there is some freedom in the choice of treatment schedule, and that the selection of Clorox 1:400 for 10 minutes seemed to strike a good balance between % hatch and % rendered sterile. Further experience with this schedule has shown that hatch and sterility rates of 90-100% can usually be obtained.

2. A subsequent experiment was aimed at more rigorous bacteriologic screening for contaminants. Four groups of mature eggs (totalling 56) were disinfected and tubed, and during the succeeding 7 days 44 (79%) hatched. On day 7 each snail or unhatched egg, together with some HS(M) from its tube, was transferred to thioglycollate medium. Aliquots of the remaining HS(M) from each tube were inoculated into nutrient broth, a blood agar slant, and a slant of Sabouraud's dextrose agar. Tubes containing solid media

TABLE II. Effects of Time and Concentration of Clorox Disinfectant on Hatch and Sterility Rates of Eggs of *Australorbis glabratus*.

			Separated eggs of *A. glabratus*				
Clorox in HS	Min. treatment	No. exp.	No. treated	No. hatched*	% hatched	Sterile snails or eggs† No.	%
1:400	2	6	85	52	61	74	87
"	5	7	77	63	82	63	82
"	10	5	50	39	78	47	94
"	15	4	37	25	68	37	100
1:200	2	5	45	29	64	44	98
"	5	5	48	28	58	47	98
"	8	3	28	15	54	28	100
1:100	2	3	30	15	50	30	100
Totals		38	400	266	67	370	93

* By day 7 after treatment.
† "Eggs" refers to those which did not hatch by day 7.

were sealed with Parafilm to prevent drying during the incubation period. All tests were held at 28-32° C for 10 days and then at room temperature for an additional 21 days. No contaminants were detected in this series.

3. Experiments were done to determine ability to hatch and length of survival under different sets of sterile conditions, eggs having first been disinfected in groups and tubed individually using the routine procedure. Maintenance conditions were as follows (all at 28° C): a) In cotton-stoppered Wasserman tubes containing 2 ml HS(M), (9 experiments). The initial pH was 7.6-7.8 but rose rapidly to 8.5-8.7 or higher. In no case was the original HS(M) replenished or changed, although considerable loss through evaporation occurred during the observation period of several weeks. b) As in 'a' but with 500 mg% glucose added (6 experiments). c) As in 'a' but tubes closed with rubber stoppers (3 experiments). The initial pH was 7.6-7.8 and no changes in pH were observed during several weeks of observation. d) As in 'c' above but bicarbonate omitted (3 experiments). The initial pH of 6.8-7.0 remained during the period of observation. In these experiments a total of 340 mature eggs were treated and tubed, 292 (86%) hatched, and only 9 (3%) of the hatched snails or unhatched eggs proved to be contaminated. Variations in hatch or sterility rates among the several experiments were negligible. All tubes were examined daily and a snail was recorded as having died only when heartbeat could not be detected. Using 50% survival, in terms of days after hatch, as an index, there were no significant variations in length of survival, although snails in HS(M) plus glucose tended to live slightly longer. Thus, all the 50% end-points fell between 11 and 20 days with the majority of them falling between 15 and 20 days. Deaths were rare in the first 5-day period after hatch, a few deaths occurred in the next 5-day interval, with a sharp increase during the succeeding 10 days. Maximum survival in the various maintenance conditions ranged from 21-29 days except in one instance (in group 'd') in which a single snail survived for 52 days. Similar observations were made when treated eggs were isolated in 5 ml HS(M) (500 mg% glucose in one group) in rubber-stoppered 20x150 mm culture tubes. The results paralleled experiments noted above in which smaller fluid volumes in smaller tubes were used.

4. The heartbeat rate of newly-hatched (less than 24-hour old) *A. glabratus* maintained unfed in HS(M) at 28°C under sterile conditions usually averaged 100-120 per minute. This rate declined steadily during the ensuing 5 days to about 60-80 beats per minute. No effects on rate of heartbeat were observed due to the presence of bicarbonate or glucose during this 5-day interval. Similarly, no differences were noted in comparing snails kept in cotton-stoppered tubes or in tubes sealed with rubber stoppers. However, temperature did affect rate of heartbeat. Three groups of 20 eggs each were disinfected and

the eggs isolated in 2 ml of HS(M) (plus 500 mg% glucose) in rubber-stoppered Wasserman tubes maintained at 24, 28, and 32°C, respectively. The proportion of hatch was high in all 3 groups (18-19/20). However, on day 5 (calculated from day of hatch for individual snails) the mean rate of heartbeat for the group at 24° was 73 per minute, at 28°, 82 per minute, and at 32°, 97 per minute. Rate of heartbeat beyond the 5th day post-hatch varied greatly, presumably reflecting individual differences in response to inanition.

5. A variable degree of success was achieved in attempts to secure viable, bacteriologically-sterile snails other than *A. glabratus*. The outlined procedures were applied to mature eggs of *Biomphalaria pfeifferi* and results comparable to those with *A. glabratus* were obtained in regard to rates of hatch, sterility, etc. However, preliminary experiments with mature eggs of *Lymnaea* sp. and of *Marisa cornuarietis* were not similarly successful.

Discussion. Some interesting aspects of the biology of *A. glabratus* emerge from the foregoing studies. It is apparent that microbial action is not essential to the hatching process, that snails will hatch in a simple solution of salts very different from the complex composition of pond or aquarium water, and, that once hatched, they may live for long periods of time without food. The behavior of the newly-hatched isolated snail appears unaffected, except as manifestations of inanition develop, by the absence of living micro-organisms, by differences in pH in the range indicated, by the presence of antibiotics or phenol red, or by being confined to small volumes of fluid in sealed tubes.

The behavior of the normal snail *in vitro* before eclosion is rather characteristic. The snail's foot is almost always maintained in contact with the inner wall of the egg capsule even when the snail is not moving about actively. Heartbeat is distinct. Although the cilia coating the tentacles and other exposed surfaces cannot be seen under the low-power microscope, the currents they produce are evidenced by movement of the particulate matter usually present in the fluid of the egg. The egg capsule itself is slightly yellow in color and usually retains its firm-looking, elliptical outline until the process of eclosion begins. This is initiated by a 'stretching' action, in which the snail appears to brace its shell against one side of the egg capsule and extend its foot against the opposite side, thus tending to distort the outlines of the egg. As the snail moves along the inner side of the egg repeating this movement, the egg capsule eventually takes on a crinkled or partially-collapsed appearance. The snail's mouth parts are in frequent motion during this process, and eventually a small rent appears in the capsule through which the snail ultimately escapes. The snail may thereafter be seen crawling on its empty egg capsule but no effort to eat it has been observed.

Once free of the egg capsule, the newly-hatched snail (which averages 0.7 mm in diameter) moves about actively with the foot in contact with the wall of the culture tube. However, after about 10 days some snails may be found detached from the walls. The foot, while partly withdrawn, may still be observed to move. Heartbeat, although slow and irregular, may be observed for several days to several weeks after other objective signs of life have ceased. By the time heartbeat has nearly stopped, the animal's soft parts appear translucent.

The accumulated experience of rendering several thousand eggs bacteriologically sterile indicates that 90% or more hatch during the first 3-4 days after treatment and only a negligible proportion thereafter. Nothing has been noted to explain why some apparently normal mature eggs fail to hatch, even though many have been observed to live for as long as 2 weeks. In a different category are eggs which contain immature embryos at time of treatment, since a very high proportion of such eggs regularly fail to hatch. There are at least 2 other circumstances in which rate of hatch may be materially reduced: a) If mature eggs are subjected to too high a concentration of disinfectant, or if they are exposed for too long a period of time, the unhatched snails usually die within 1-2 days. The survivors rarely hatch; the foot is usually retracted and may appear partially disintegrated and bordered by loose cellular elements. b) Hatching is also inhibited by the antifungal

antibiotic Mycostatin§ at certain concentrations. This substance was added to a few tubes in some of the preliminary experiments in an effort to suppress mold contaminants. In concentrations of 12.5 or 25 units per ml molds were suppressed but few or no snails hatched; at 5 units per ml a small proportion of snails hatched but molds were not uniformly suppressed. It is of interest that a very low concentration of Mycostatin is rapidly lethal to adult *A. glabratus*(9).

The development of a technic for securing newly-hatched, bacteriologically sterile, *A. glabratus* should permit a variety of additional studies. For example, the metabolic characteristics and nutritional requirements of the young snails can be investigated in the absence of micro-organisms or in the presence only of selected micro-organisms. Furthermore, snail tissues free of contamination can now be secured for *in vitro* cultivation. Finally, 'germ-free' snails maintained under sterile conditions should provide an ideal tool for screening and studying microbial or viral agents which might be of value in biological control. Aspects of these problems are now under investigation.

Summary. Details of a technic are presented by means of which newly-hatched, bacteriologically sterile snails (*Australorbis glabratus*) can be secured for study. Snail eggs containing mature embryos are separated manually from the egg-mass, disinfected externally with dilute sodium hypochlorite, and isolated in sealed tubes containing a suitable salt solution and antibiotics. Using this procedure, 90-100% hatch occurs within 3-4 days of treatment, and tests have shown that only a negligible proportion are bacteriologically contaminated. Details of maintenance, behavior, and longevity of these snails are discussed.

I am indebted to Dr. E. H. Michelson for maintaining the colony of *A. glabratus* from which the eggs used in these studies were derived; to Dr. John H. Hanks, Harvard Medical School, for suggestions regarding the composition of HS; and to Miss Sarah E. Dwight for technical assistance.

§ Squibb brand of Nystatin. Kindly supplied by E. R. Squibb and Sons, New Brunswick, N. J.

1. Chernin, E., Michelson, E. H., and Augustine, D. L., *Am. J. Trop. Med., and Hyg.*, 1956, v5, 297.
2. Chernin, E., and Michelson, E. H., *Am. J. Hyg.*, 1957, v65, 57.
3. Carriker, M. R., *Biol. Bull.*, 1946, v91, 88.
4. Fraser, L. A., *Trans. Am. Micr. Soc.*, 1946, v65, 279.
5. Ellis, M. M., Merrick, A. D., and Ellis, M. D., *Bull. Bur. Fisheries*, 1931, v46, 509 (Document No. 1097).
6. Weinstein, P. P., and Jones, M. F., *J. Parasit.*, 1956, v42, 215.
7. Hadfield, W. A., Ch. 22 in *Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization*, 1954, E. F. Reddish, Ed., Lea and Febiger, Phila., 841.
8. Weller, T. H., Enders, J. F., Robbins, F. C., and Stoddard, M. B., *J. Immunol.*, 1952, v69, 645.
9. Seneca, H., and Bergendahl, E., *Antib. and Chemo.*, 1955, v5, 737.

Received June 27, 1957. P.S.E.B.M., 1957, v96.

Observations on *in vitro* Behavior of Altered Mononuclear Phagocytes Following Exposure to Tubercle Bacilli.* (23434)

JOSEPH W. JOYNER AND CALVIN L. CALHOUN (Introduced by Harold D. West)
Departments of Microbiology and Anatomy, Meharry Medical College, Nashville, Tenn.

Although cellular response to infection with the tubercle bacillus has been extensively investigated, there is considerable difference of opinion as to the role of phagocytes in tubercular infections(1,2,3). Mononuclear phagocytes have been proposed as the main factors of resistance to tuberculosis infection(4,5). Vorwald and co-workers(6) indicate that BCG will produce fatal cavitary disease in silicotic guinea pigs. Dubos(3) pointed out

* This investigation was supported in part by grant from Public Health Service.