# THE INFECTION BY PLASMODIUM LOPHURAE OF DUCK ERYTHROCYTES IN THE CHICKEN EMBRYO

By R. BARCLAY McGHEE, PH.D.

*(From The Laboratories of The Rockefeller Institute for Medical Research)*

(Received for publication, December 10, 1952)

The erythrocytes of several species of mammals have been shown to be susceptible to the avian malaria parasite *Plasmodium lophurae* (1, 2). This was done by the intravenous injection of the washed erythrocytes into chick embryos infected with *P. lophurae*. Of 18 species of animals tested, the erythrocytes of the baby rat (1), pig, mouse, rabbit, and possibly man (2) were invaded by the parasite. It was apparent that in these animals the erythrocyte cannot be greatly concerned in the resistance of the intact creature, whereas in others the cell as such offered a barrier to invasion by the parasites of *P. lophurae*. Attempts to produce obvious infections in intact adult mice failed, but they were successful in infant mice (3), lending further emphasis to their differing degrees of immunity. The parasitization of certain non-nucleated erythrocytes after injection into chick embryos furnished a method for study of the inherent susceptibility of cells, but this study was brief because of various complications due to the foreign cells in the embryo host. The cells of the pig and rabbit were neither heavily parasitized nor stable in the embryo circulation and breaking down of them was quickly followed by the death of the embryo. The erythrocytes of the baby rat were quite susceptible to infection but were likewise unstable and caused death, while those of the adult mouse were stable but rather resistant to infection.

It has now been found that the erythrocytes of the adult duck can be identified with relative ease in the blood stream of infected chick embryos. They are stable and harmless to the embryo and are readily parasitized by the merozoites of *P. lophurae*.

The following studies were initiated to determine: (*a*) the amount of invasion by *P. lophurae* of uninfected duck erythrocytes injected into the chick embryo, and (*b*) the amount of secondary invasion of duck erythrocytes already infected when injected.

## *Methods and Materials*

The 12A strain of *P. lophurae* (4) which has been in continuous passage through white Leghorn chick embryos for a period of 7 months (5) was used for all embryo infections. The same strain, carried in ducklings, was used to infect adult male and female Pekin ducks. Blood was secured from infected and uninfected ducks, from either a lateral neck vein or

wing vein, into 27 mg. per cent heparin in saline, 0.1 ml. of heparin solution to 1.0 ml. of blood. The erythrocytes were washed twice in several volumes of 0.85 per cent sodium chloride solution and sufficient saline was added to make up a final volume of equal parts packed cells and saline. 0.2 ml. of this suspension was injected intravenously into 10-day-old chick embryos which had been inoculated with *P. lophurae* 2 or 3 days previously. Blood films (1) were made just following the introduction of the experimental erythrocytes and at various intervals thereafter; they were stained with Giemsa's stain. Counts of erythrocytes and parasites respectively were large enough to exclude a probable error of more than 15 per cent (6). The injection of uninfected or infected duck erythrocytes produced no apparent reaction in the embryo, and with few exceptions there were no embryo deaths.

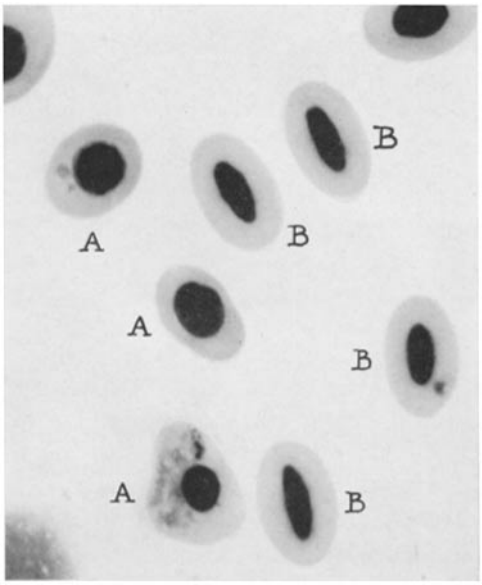


FIG. 1. Photomicrograph[1] of blood film from an infected chick embryo 4 hours after the intravenous injection of duck erythrocytes previously uninfected. *A*, chick embryo erythrocytes. *B*, duck erythrocytes, one containing a young trophozoite. × 1545.

## EXPERIMENTAL RESULTS

### *The Morphology of the Erythrocytes of the Chick Embryo and the Adult Duck*

The erythrocytes of 10- to 13-day-old chick embryos were found to be, in general, more rounded than those of the hatched chicks. The principal difference between the chick embryo erythrocytes and those of the adult duck lay in the shape of the nuclei. The nuclei of the embryo erythrocytes were nearly spherical, with clearly distinguishable chromatin masses (Fig. 1, *A*), while those of the duck were almost spindle-shaped and stained a homo-

[1] Photograph by Mr. J. A. Carlile.

geneous color with Giemsa's stain (Fig. 1, *B*). Other differences, for example the slightly greater affinity of the cytoplasm of the duck erythrocyte for eosin and the general ellipsoidal form of the entire cell, were by no means consistent and could be used only as suggestive evidence of the species of erythrocyte. In older, uninfected chick embryos, the erythrocytes were much more difficult to distinguish from duck cells, and as a consequence only infected embryos of less than 14 day's incubation were used.

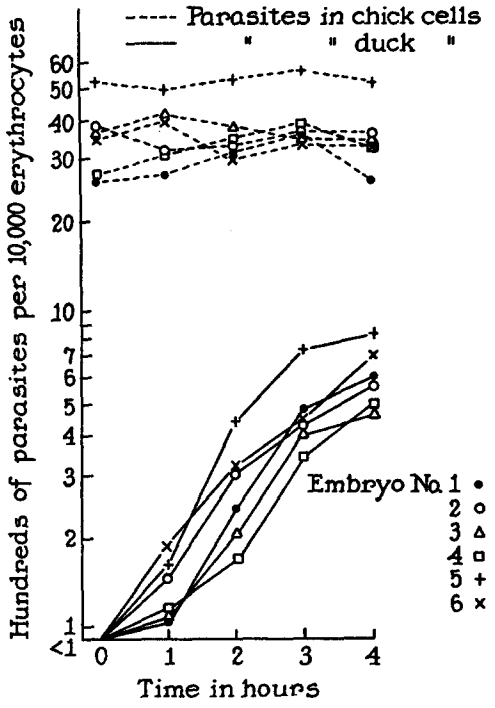


FIG. 2. Relation of infection of chick and duck erythrocytes circulating in infected chick embryos.

### *The Susceptibility of Uninfected Duck Erythrocytes*

*Experiment 1.*—0.2 ml. of washed, resuspended cells from an adult female duck was injected into an allantoic vein of 6 infected chick embryos which had received malaria parasites 2 days before. Just after injection, blood films were made and the parasitemia was found to be from 2700 to 5000 parasites per 10,000 chick erythrocytes (average 3600). From 100 to 190 parasites (average 140) per 10,000 duck erythrocytes were observed at 1 hour, 160 to 440 parasites (average 277) at 2 hours, 310 to 738 (average 460) at 3 hours, and 450 to 800 (average 600) at 4 hours after introduction of the foreign erythrocytes. At the end of 4 hours the parasitemia in chick cells had decreased in embryos 2, 3, and 6; was the same as at 0 hour in embryo 1; and was increased in embryos 4 and 5 (see Fig. 2).

Fig. 2 shows that during the period of observation in this experiment the number of parasitized duck cells steadily increased whereas the amount of invasion of the chick cells remained approximately constant. This constancy may have been due to the failure of any new chick cells to become infected, or on the other hand the amount of new infection of them may have been balanced by the amount of loss. In order to explore this possibility further, 3 more experiments were performed like Experiment 1 except that counts were made at 4 and 24 hours. The 4 hour period was selected because the parasitemia had assumed proportions which allowed for accurate counts and because it was thought that the time was short enough to minimize any changes occurring in

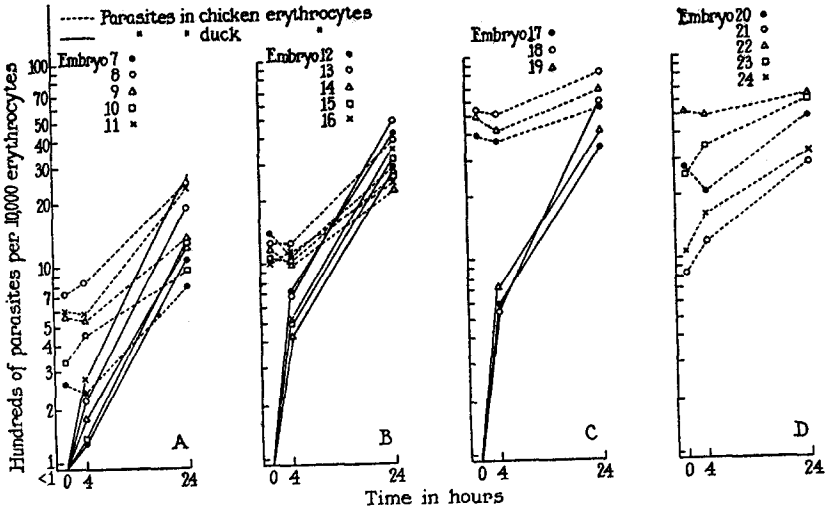


FIG. 3. The effect of the initial extent of chick cell parasitemia on the infection of duck erythrocytes at 4 and 24 hours.

the duck cells because of their presence in a foreign environment. The 24 hour period, although not considered as too accurate an indication of infectivity, was chosen to determine whether the presumably greater degree of susceptibility was still apparent after a relatively long interval of time.

Fig. 3 presents fewer instances than were studied but taken as a whole they are representative instances. Those not shown were omitted to avoid undue complication of the charts.

*Experiment 2 (Fig. 3 A).*—The average parasitemia of chick erythrocytes in the initial films was 674 with a range of from 233 to 1250 parasites per 10,000 chick erythrocytes. After 4 hours the average parasitemia in duck erythrocytes was 214. In 7 embryos out of a total of 14, the parasitemia had decreased, as figured in embryos 7, 9, and 11, while in the remaining 4 embryos there was a small rise in the numbers of infected chick cells, as shown

in embryos 8 and 10. After 24 hours, infections of the duck erythrocytes were comparable to those in the chick.

*Experiment 3* (*Fig. 3 B*).—The average chick cell infection was 1354 parasites per 10,000 chick erythrocytes. After 4 hours the parasitemia in chick cells had decreased in a manner shown in the infections in embryos 12, 14, and 15, remained approximately the same in one embryo, 13, and had increased in embryo 16. As in Experiment 2, the infection of duck cells approached that in chick cells after 24 hours.

*Experiment 4* (*Fig. 2 C*).—The average parasitemia per 10,000 chick erythrocytes was initially 4456. There was no increase in the number of parasites in chick erythrocytes in the 3 embryos at 4 hours, but the parasitemia in duck erythrocytes was somewhat higher (513) than in the preceding experiments. After 24 hours the average parasitemia in duck erythrocytes, although higher than in previous experiments, did not exceed that found in the chick red blood cells.

For comparison, the amount of infection of 20 embryos receiving no duck cells, and with initial parasitemias ranging from 500 to 6000 parasites per 10,000 erythrocytes, was determined at 4 and 24 hours. (Fig. 2 D). The parasitemias in all embryos with initial counts from 500 to 2000 parasites per 10,000 erythrocytes increased in 4 hours in a manner similar to that depicted in embryos 21 and 22. In embryos in which the initial parasitemia was between 2000 and 3500 parasites per 10,000 erythrocytes, about half (embryo 20) showed decreased parasitemias after 4 hours while in the remaining embryos the parasitemias increased (embryo 23). There was a decreased parasitemia in all embryos in which the initial parasitemia was 4500 to 6000 parasites, as shown for embryo 22. After 24 hours had elapsed, the parasitemias were not materially different from those of chick embryos injected with uninfected duck erythrocytes.

From these experiments it is evident that the introduction of duck erythrocytes into chick embryos with relatively slight infections at the time (less than 2000 parasites per 10,000 chick red cells) (Fig. 3) was followed 4 hours later by a lower rate of parasitization of chick erythrocytes than that seen in embryos infected to the same extent but not injected with duck red cells. In very heavily infected embryos (Fig. 3 C and D) the rate of infection of additional chicken erythrocytes at 4 hours after the start of the observations was low regardless of whether duck erythrocytes were injected. In all embryos which received duck red blood cells, there was a high rate of parasitization of those cells at both 4 and 24 hours after their introduction.

It seemed possible that these results might be consequent on a high death rate of merozoites, and that the introduction of uninfected duck red cells tended to decrease this death rate. In chick embryos infected with *P. lophurae* the mean number of merozoites per segmenter has been found to be 15. An approximate estimate of the number of available merozoites can therefore be obtained by multiplying by 15 the count of segmenters. It was assumed that those parasites having over 5 nuclei at the start of the experiment (blood films prepared at 0 hour) would segment and liberate merozoites during the next 4 hours, and only these were classed as segmenters. In the blood films made 4 hours after the introduction of the duck erythrocytes, the number of new parasites in chick red cells and in duck red cells was determined. Each of these

numbers was divided by the number of merozoites calculated from the initial blood film to determine the percentage of merozoites entering each type of erythrocyte. The sum of these percentages subtracted from 100 gave the merozoite death rate.

*Experiment 5.*—Five sets of embryos were used in which the mean parasitemia ranged from 700 to 5800 parasites per 10,000 chick erythrocytes in initial blood films (Table I). When the initial infection in the chick embryo was slight (700 to 1300 parasites) the death rate of merozoites was approximately the same in both uninjected embryos and those receiving duck cells. When the initial infection of the embryo reached 3500 parasites the death rate of merozoites in uninjected embryos was 96 per cent, while in injected embryos it remained at 80 per cent with only 1 per cent of the merozoites entering chicken erythrocytes. In 2 other groups in which the mean initial parasitemias were 4500 and 5800 parasites per 10,000 erythrocytes, 100 per cent of the merozoites died in control embryos whereas in the injected groups the death rates were 82 and 80 per cent, respectively. In neither group was there invasion of chick erythrocytes.

TABLE I

*The Effect of the Introduction of Uninfected Duck Erythrocytes on the Merozoite Death Rate in Infected Chicken Embryos*

| Experiment No. | Initial blood film | | 4 hr. blood film | | | | |
|---|---|---|---|---|---|---|---|
| | | | Percentage of merozoites | | | | |
| | Parasites per 100 chicken erythrocytes | Merozoites per 100 chicken erythrocytes | Entering duck erythrocytes in injected embryos | Entering chick cells in | | Dying in | |
| | | | | Injected embryos | Uninjected embryos | Uninjected embryos | Injected embryos |
| 5 A | 7 | 19 | 16 | 0 | 28 | 72 | 84 |
| 5 B | 13 | 40 | 11 | 0 | 13 | 87 | 85 |
| 5 C | 35 | 34 | 19 | 1 | 4 | 96 | 80 |
| 5 D | 45 | 33 | 18 | 0 | 0 | 100 | 82 |
| 5 E | 58 | 68 | 20 | 0 | 0 | 100 | 80 |

The experiments indicated that the merozoite death rate was not materially altered when duck erythrocytes were introduced during early stages of the chick embryo infection, but later, when the parasitemia had reached a higher level, the introduction of duck cells was followed by a significant lowering of the death rate of merozoites below that found in comparable infections in embryos not injected with duck erythrocytes.

### *The Effect of Parasitization on the Susceptibility of Duck Erythrocytes to Infection with P. lophurae*

The high merozoite death rate in uninjected embryos which were undergoing heavy infections, as contrasted with the continued low death rate in embryos receiving duck erythrocytes, indicated that some mechanism might be oper-

ative in the latter which rendered infected chick erythrocytes less susceptible to infection. If this hypothesis were correct, the introduction of markedly infected duck erythrocytes into embryos undergoing relatively slight infection of their cells would result in a diminution of the numbers of merozoites entering duck erythrocytes and a return, in the chick embryo, to the death rate usually associated with the stage of infection initially present. To test the hypothesis, the red cells in samples of blood representing various degrees of duck cell infection were washed and injected into infected chick embryos.

It was essential that the introduced duck erythrocytes should not contain excessive numbers of segmenting parasites, since the liberation of merozoites from these cells would complicate any counts made in relation to the disposition of merozoites. Fortunately the re-

TABLE II

*The Effect of Parasitization on the Susceptibility of Duck Erythrocytes*

| Experiment No. | Parasites per 100 duck cells at 0 hr. | Per cent merozoites invading duck cells at 4 hrs. | Paraistes per 100 chick cells at 0 hr. | Per cent merozoites entering chick cells at 4 hrs. | Per cent merozoites not invading cells at 4 hrs. |
|---|---|---|---|---|---|
| 6 A | 2 | 20 | 33 | 1 | 79 |
| 6 B | 3 | 24 | 26 | 0 | 66 |
| 6 C | 13 | 12 | 38 | 0 | 88 |
| 6 D | 45 | 23 | 49 | 0 | 77 |
| 6 E | 84 | 0 | 26 | 5 | 95 |
| 6 F | 89 | 0 | 34 | 4 | 96 |
| 6 G | 95 | 0 | 5 | 17 | 83 |
| 6 H | 170 | 0 | 39 | 7 | 93 |

production of *P. lophurae* is more nearly synchronous in the duck than in the chick embryo and samples of duck blood could be obtained with negligible numbers of segmenters. In all experiments the percentage of segmenters in the duck erythrocytes was determined, the numbers of merozoites calculated, and the combined merozoite count used as a base for figuring the death rates of merozoites. No less than 6 embryos were used to an experiment.

Two methods of calculating the disposition of merozoites were used. The first was similar to that described in previous experiments: that of estimating the percentages of merozoites entering chick or duck cells and those dying. The second was devised to determine whether it required the presence of 1, 2, 3, or 4 parasites in a single erythrocyte to assure insusceptibility. This was done by making counts of 100 infected duck erythrocytes and obtaining the percentage of cells with 1 to 4 parasites in a given infection, both at the time of introduction of the foreign cells and after 4 hours.

The results of these tests (Table II) indicated an over-all decrease in the rate of penetration by parasites into infected erythrocytes. When the number of parasites was small the rate of penetration into duck cells was practically the same as when uninfected duck cells were present. As the parasitemia in duck erythrocytes increased, the rate of merozoite penetration decreased, until at

the time when the parasitemia reached 84 parasites per 100 duck erythrocytes, there was no longer any invasion of duck cells and the merozoite death rate was similar to that in uninjected embryos. In series 6 G the initial chick cell infection was only 465 per 10,000 red cells and after 4 hours it had increased markedly with only 83 per cent of the merozoites dying. A similar infection with approximately equal death rate can be seen in Table I, Experiment 5 A.

Differential counts of parasitized erythrocytes were uniform in all instances, in that there were no shifts in the direction of triply or quadruply infected duck cells after 4 hours (Table III). There was some increase in the number of cells having 2 parasites, indicating that the presence of a single parasite did not

TABLE III

*The Percentages of Duck Erythrocytes Showing Multiple Infection at Time of Injection and 4 Hours Later*

| Experiment No. | Initial blood film | | | | 4 hour blood film | | | |
|---|---|---|---|---|---|---|---|---|
| | Cells with 1, | 2, | 3, | 4 parasites | Cells with 1, | 2, | 3, | 4 parasites |
| | *per cent* | | | | *per cent* | | | |
| 6 A | 98 | 2 | 0 | 0 | 95 | 5 | 0 | 0 |
| 6 B | 99 | 1 | 0 | 0 | 98 | 2 | 0 | 0 |
| 6 C | 96 | 4 | 0 | 0 | 93 | 7 | 0 | 0 |
| 6 D | 79 | 19 | 2 | 0 | 82 | 15 | 3 | 0 |
| 6 E | 72 | 24 | 4 | 0 | 73 | 23 | 3 | 1 |
| 6 F | 66 | 29 | 5 | 0 | 65 | 30 | 5 | 0 |
| 6 G | 69 | 26 | 5 | 0 | 66 | 29 | 5 | 0 |
| 6 H | 36 | 50 | 12 | 2 | 46 | 41 | 12 | 1 |

reduce the cell susceptibility. In heavily infected erythrocytes the differential counts were essentially unchanged after 4 hours, with the exception of series H. In this group of embryos there was a rather decided shift toward an increase in numbers of singly infected erythrocytes.

## DISCUSSION

When duck erythrocytes were introduced into chick embryos infected with *P. lophurae*, the number of parasites which had entered the foreign cells by the end of a 4 hour period was always greater than the number which had newly entered chick embryo cells. If the infection of the chick embryo was slight (between 700 and 1300 parasites per 10,000 erythrocytes), the death rate of the merozites was unchanged by the introduction of duck erythrocytes. With heavier infections the higher death rate of the merozoites ordinarily seen in embryos receiving no duck cells was not in evidence if these latter had been introduced. Instead the death rate remained similar to that in embryos during infections of less intensity. The introduction of duck cells, therefore, provided

erythrocytes which were not only uninfected but more susceptible to infection than chick embryo cells. The combination of these two conditions reduced the death rate of the merozoites by affording hosts for them when in the free state.

The results presented in Table I suggest at first glance that when duck erythrocytes were present there was no invasion of fowl cells by merozoites. However, the observed maintenance of the level of parasitemia or even a slight decrease in this would indicate that the rate of invasion was sufficient to balance the reduction in infected chick cells as a result of destruction of these latter by segmenting parasites.

Since *in vitro* studies (7) have shown that merozoites have no power of independent movement, the possibility of their seeking out the erythrocytes of the duck in preference to those of the chick can be excluded. The initial contact between the merozoite and the cell must, therefore, be brought about as a result of contact of the erythrocytes and the parasites in the circulating blood stream. Inasmuch as the proportion of duck cells to total blood cells never exceeded 30 per cent (average 20 per cent) the rate of contact of the merozoite with chick erythrocytes would be from 3 to 5 times as great as it would be with the duck red cells. The effect of parasitization of the chick erythrocyte on susceptibility to its further parasitization is not known. If, however, it be assumed that the presence of a single parasite decreases the susceptibility of fowl cells, a recalculation of the numbers of duck to chick cells counting only the uninfected erythrocytes, would, in embryos with relatively slight infections, still show considerably more fowl than duck cells. Thus it is apparent that in spite of the inability of the merozoite to move independently, and in spite of the greater numbers of chick cells, the rate of penetration of the duck cells by the parasites was greater than that of the chick embryo cells.

One possible explanation of the selective invasion of the duck cell is that there are, in susceptible cells, only certain areas which are receptive to penetration. The fewer the areas the greater the likelihood of the parasite failing to become associated with the cell membrane. If, in the duck erythrocyte, these presumptive areas are a great deal more numerous than in the fowl cell, the possibility of parasitization by the merozoites will be more considerable.

It is plain from the findings of Table III that when the duck erythrocyte already contains 2 or more parasites at the time of its introduction into the infected embryo it has been altered in such a way that subsequent penetration by merozoites becomes more difficult. The resulting barrier is not completely impenetrable, however, since as many as 4 parasites per erythrocyte are sometimes present in ducks undergoing heavy infection (see Table III, 6 H). The experiments of Rigdon and Varnadoe (8) and Becker *et al.* (9) suggest that conditions somewhat similar to those observed in these studies obtain in intact animals. Following repeated transfusions of infected ducks with uninfected duck erythrocytes, they observed, instead of crisis or death of the animal, the main-

tenance of the parasitemia at a high level. Although they offered no explanation for this phenomenon it seems to the author that, as in the chick embryo, the transfusion of non-infected erythrocytes afforded the merozoites greater opportunities of penetrating new cells and thereby saved them from wholesale destruction because of an inability to penetrate multiply infected erythrocytes.

## SUMMARY

Certain morphological differences render it possible to recognize duck erythrocytes after introduction into the circulating blood of chick embryos infected with *P. lophurae*. 4 hours afterward, considerable numbers of merozoites have entered duck erythrocytes, while the parasitemia of the chick itself remains essentially unchanged in degree. By estimating the numbers of potentially invading merozoites from blood films made at the time of introduction of duck cells, it was learned that a relatively constant rate of invasion into duck cells by merozoites was maintained. In counter-distinction there was an ever increasing merozoite death rate in embryos not receiving duck cells concurrent with the increase in numbers of parasites. After the injection into parasitized embryos of duck erythrocytes showing but few parasites, no difference was apparent in the rate of merozoite invasion into the introduced cells and the host cells, respectively; but when the percentage of duck cells was greater, the rate of merozoite penetration diminished to zero. The selective penetration of duck erythrocytes which, under the experimental conditions obtaining, constituted only 30 per cent of the total cell numbers of most, and the inability of the merozoites to move independently, taken together, suggest that the greater susceptibility of the duck erythrocyte may be due to greater numbers of accessible areas on its surface. The decreased susceptibility following parasitization indicates that the presence of the parasite alters the cell in such wise that entry of additional parasites is rendered more difficult.

## BIBLIOGRAPHY

1. McGhee, R. B., *J. Infect. Dis.*, 1953 **92,** 4.
2. McGhee, R. B., *Am. J. Hyg.*, 1950, **52,** 42.
3. McGhee, R. B., *J. Infect. Dis.*, 1951, **88,** 86.
4. Coggeshall, L. T., *Am. J. Hyg.*, 1938, **27,** 615.
5. McGhee, R. B., *J. Parasitol.*, 1949, **35,** 411.
6. Gingrich, W., *J. Prev. Med.*, 1932, **6,** 197.
7. Trager, W., and McGhee, R. B., unpublished data.
8. Rigdon, R. H., and Varnadoe, N. B., *Am. J. Trop. Med.*, 1947, **27,** 673.
9. Becker, E. R., Marousek, A. A., and Brodine, C. E., *J. Nat. Malaria Soc.*, 1949, **8,** 290.