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Exploration du Parc National de la Garamba

MISSION H. DE SAEGER

en collaboration avec

P. BAERT, G. DEMOULIN, I. DENISOFF, J. MARTIN, M. MICHA, A. NOIRFALISE,
P. SCHOEMAKER, G. TROUPIN et J. VERSCHUREN (1949-1952).

FASCICULE 36

ENTERIC BACTERIA FROM REPTILES

BY

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ENTERIC BACTERIA FROM REPTILES [1]

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CHAPTER I.

Introduction.

1. REASON FOR PRESENT STUDY.

Only a limited amount of investigations have been devoted to the intestinal flora of reptiles. Most published reports reveal as their main object a search for certain restricted kinds of bacteria especially those of the *Salmonella* and *Arizona* groups. On the basis of the frequencies of the organisms belonging to these two groups and the numbers of new types encountered in the reptiles, a hypothesis was formulated : The bacterial flora of the intestinal tract of reptiles differs from the mammalian by its high frequency of bacteria belonging to the *Salmonella-Arizona* categories and the comparative scarcity of the coliform group of bacteria which predominates in the mammals.

Some detailed studies of the intestinal flora of reptiles have been performed by D. YARASHUS (1959) and D. HALKIAS (1959). In these studies

[1] A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science (June 1960).

of captive reptiles at two local zoos, differences were found between the reptilian and mammalian intestinal flora. However, there was still the question unanswered : Was the intestinal flora of these reptiles altered to any extent by their captivity

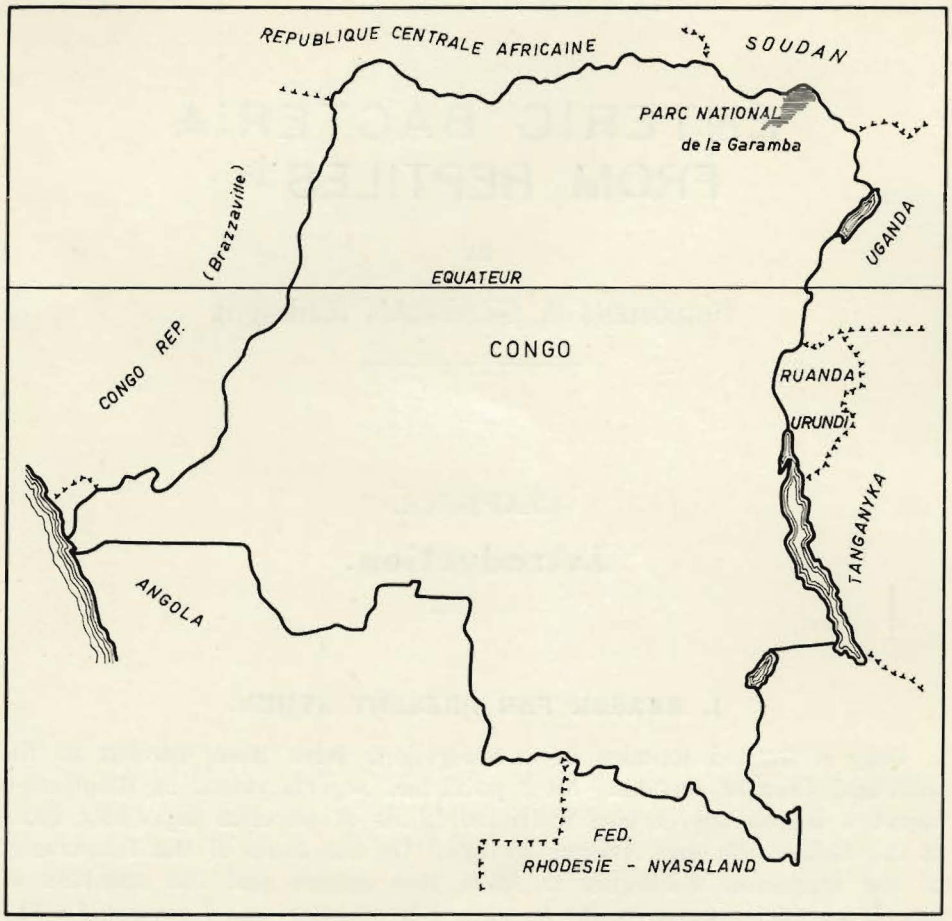

FIG. 1. — Map of the Congo depicting the location of the Parc National de la Garamba.

About this time, occasion arose for securing specimens from wild reptiles. This provided the material for the present investigation. Therefore, the objectives of this study are : 1) to identify a large collection of bacteria isolated from wild reptiles and 2) to compare these with the organisms isolated from captive reptiles in the work of YARASHUS and of HALKIAS.

2. PLAN OF THE WORK.

In the Spring of 1959, Dr. R. INGER, of the Chicago Natural History Museum, participated in an expedition to the *Parc National de la Garamba* in the Congo, to study and collect reptiles and amphibians. He agreed to dry some specimens of fresh intestinal contents into sterile filter-paper discs and forward these airmail to Chicago. The Parc authorities consented to this additional work and cooperated most fully.

As the specimens were received, they were immediately placed in brain heart infusion broth after which desoxycholate agar plates were streaked at intervals of incubation to obtain isolated colonies. These plates were refrigerated until further work could be performed. Then, about 30 colonies were picked from each plate, replated to insure purity of the cultures and stock cultures were established.Subsequently, the cultures were identified by the usual bacteriological methods. Those which possessed typical *Salmonella* characteristics were sent to the State of Illinois, Department of Healt, enteric laboratories for serotypic determinations.

ACKNOWLEDGMENTS.

Sincere thanks are extended to the following for their valuable contributions and cooperation in the many phases of this study : Dr. R. INGER, of the Chicago Natural History Museum, the Parc National authorities of Belgium, and Dr. H. J. SHAUGHNESSY and Mr. M. LESKO of the State of Illinois, Department of Healt. I am especially indebted to Dr. M. FULTON for his advice and supervision throughout the course of this research.

CHAPTER II.

Review of literature.

1. PREVIOUS SUMMARIES.

Approximately 37 published reports concerned with the bacterial flora of reptiles were reviewed in the theses of YARASHUS (1959) and HALKIAS (1959). In reviewing this literature again, the possibility of reptiles being a source of *Salmonella* organisms was again apparent. For example : HINSHAW and MC NEIL (1947) reported the isolation of *Salmonella rubislaw* and paracolons antigenically related to *Salmonella* from Pacific fence lizards (*Sceloporus occidentalis occidentalis*) in California. In 1954, HIRSCH and SAPIRO-HIRSCH found 9 different serotypes of *Salmonella* in the feces of tortoises caught in Israel. MILLE, LEMINOR and CAPPONI (1958) isolated 248 *Salmonella* of various serotypes from autopsies of lizards (*Leiolepis belliana guttata*), in central and south Viet-nam. During a course of investigations in the Belgian Congo, VAN OYE (1952, 1953, 1955) isolated *Salmonella* from various sources including snakes. In another report from the Belgian Congo, VAN OYE, GHYSELS, and GLAUDOT (1958) describe a new type, *Salmonella kintambo*, which they isolated form the intestine of a common lizard. As indicated in the few reports summarized above, the occurrence of *Salmonella* in the intestinal tract of reptiles is definitely not confined to a specific area. Isolations of *Salmonella* organisms from reptiles were encountered in North America, Europe, Asia, the Far East, and Africa.

In most of the reviewed reports, no descriptions were included of the actual surrounding of the animals at the time of capture. The reports seem to indicate that most of the wild reptiles were captured in vicinities close to human habitations. It is very likely that these supposedly wild animals could have picked up the organisms by association with man, his sewage or his garbage. No actual evidence was offered to prove that the *Salmonella* organisms were native to the reptiles.

2. ADDITIONAL LITERATURE.

Since the work of YARASHUS (1959) and HALKIAS (1959), there have been additional publications concerned with bacteriological findings in reptiles. There are also several papers which were omitted in their reviews.

WITTIG, SULZBACHER and SEELIGER (1958) described a new *Salmonella* type, *S. halle* which they isolated from a turtle fecal specimen in a zoo in Germany. HEMMES (1958) reported the transmission of *S. newport* by tortoises (*Testudo graeca*) in the Netherlands. CLARENBURG and KAMPELMACHER (1959) isolated *Salmonella* from various sources in the Netherlands including a Boa constrictor snake, from which they isolated *S. typhi murium* and *S. potsdam*. A number of tortoises which originated from the Mediterranean region and were imported to Oslo, were examined by BOVRE and SANDBU (1959). Nineteen serological types of *Salmonella* type, *S. ramatgan* was isolated by SAPIRO-HIRSCH, ALTMAN and HIRSCH (1959) from the snake *Coluber nummifer* in Israel, which was caught by a person bitten by this snake.

More pertinent to the present study are the reports by investigators studying reptiles in Africa. LEMINOR, DARRASSE and CHARIE-MARSAINES (1959) described three new *Salmonella* serotypes isolated at Dakar, West Africa, from lizards (*Agama agama savatieri*). The three types were named : *S. oukam*, *S. camberene* and *S. yoff*. At Brazzaville, LEMINOR, RAVISSE and DREAN (1958) isolated two new *Salmonella* serotypes from snakes. The one serotype was named *S. bacongo* isolated from the snake, *Philothamnus semi-variegatus*, and the other was *S. gamaba*, isolated from the snake, *Crotaphopeltis hotambeia hotambeia*. Another new *Salmonella* serotype, *S. tanger* was isolated by LEMINOR, NEEL, DELAGE and DREAN (1959) from a tortoise (*Testudo graeca*) in Tanger, Morocco.

Besides *Salmonella*, reptiles have been found to harbour another group of organisms, the *Arizona*, which is closely related to the *Salmonella*. They were first isolated from fatal infections of certain reptiles in Arizona. Antigenically, the *Arizona* group is so similar to the *Salmonella*, that frequently it is difficult to differentiate one from the other. The evidence for pathogenicity is also similar in both the *Salmonella* and *Arizona* groups. LEMINOR, EDWARDS, FIFE, CHAMBON and RAVISSE (1959) isolated six new *Arizona* serotypes from normal reptiles in Saigon (Indochina) and Brazzaville. They described the antigenic relationships of these serotypes to *Salmonella*. Another six new *Arizona* serotypes were isolated by FIFE, EDWARDS, LEMINOR and SERIE (1959) from normal reptiles in Ethiopia. Their antigenic relationships to *Salmonella* were also described. Additional *Arizona* types of organisms were recovered from reptiles described as « normal » by EDWARDS, LEMINOR and FIFE (1958). A total of 6 new types were encountered, from snakes in the regions of Saigon, Ethiopia and Brazzaville, and from chame-

leons in Tunis. The antigenic structure of these organisms was described and their relationships to *Salmonella* indicated. SERIE and LEMINOR (1959) examined 575 snakes from Erytria and Libya, of the families *Colubridae* and *Viperidae*. No *Salmonella* were isolated from individuals of the *Nahia* genus, but *Arizona* and *Salmonella* were isolated from the snake *Dendraspis* (*Colubridae*), and from individuals of the family *Viperidae*.

3. DEDUCTIONS.

No true picture of the intestinal flora of reptiles can actually be drawn even after an exhaustive review of the pertinent literature. This is due to the investigators'failure to indicate the presence or absence of other bacterial types in their search for new serotypes of *Salmonella* and *Arizona*. Published reports give the impression that no other enteric bacteria were present in the reptiles investigated. In any event, the impression has been created, that *Salmonella* is the most common and most abundant organism found in reptiles. As the literature indicates, the origins of the reptiles studied were variable, in that some animals were imported from other countries, some were caught in the wild state on ranches, barn-yards, farms, etc., and others were children's house pets. Information which would describe the habitat of the animals is absent in most reports. It seems likely, that in most cases, the animals were in close association with human habitation prior to capture. Therefore, the study of the intestinal flora of reptiles as such, has not as yet been satisfactorily accomplished, due to the uncertain elements of human contamination.

4. FURTHER STUDY.

Review of literature shows a need for a study defining the bacterial flora of reptiles in their native state. This study should be made by identifying all the kinds of bacteria present, not by selecting only *Salmonella* or *Arizona*. For technical reasons, it would be best to limit this study to the enteric bacilli, which could be isolated and identified accordying to a selected group of well-defined procedures. The opportunity to secure dried cloacal discharges from reptiles in the Congo prompted the performance of the following study.

CHAPTER III.

Materials and methods.

1. COLLECTION OF SPECIMENS.

Before Dr. INGER departed for his field trip to the Congo, he was given a supply of sterile filter paper discs (SCHLEICHER and SCHULL, No. 740 E) enclosed in cellophane coin envelopes. Specimens from the wild reptiles were collected by immersing filter paper discs in fresh cloacal deposit of captured animals, after which the discs were placed in the cellophane envelopes and sealed. Each envelope was labeled with the collection number that was assigned to reptile specimen itself. The specimens were sent to Chicago by Air Mail. The suitability of the dried disc method for transporting specimens has been reviewed by YARASHUS (1959) and was supported by his experiments.

2. PLATING OF SPECIMENS.

Upon receipt, each specimen disc was immediately placed in 10 ml of Brain Heart Infusion broth (Difco) contained in a 16 by 100 mm tube. The tubes were incubated at 30 °C for various amounts of time. Samples from each tube which showed growth were then plated on large (150 mm diameter) Petri plates containing Desoxycholate agar (Difco). Of the 18 specimens received, 11 proved to be sterile : these were specimen numbers, 2369, 2370, 2400, 2406, 2457, 3353, 3384, 3385, 3386, 3448 and 3598. Others, however, grew rapidly and heavily; they were plated at various times as follows : numbers 2465, 2466 and 3316 were plated after 4 and 6 days incubation; number 3677 was plated after ½, 1, 2, 3, 5, and 8 days; specimen numbers 3819, 3883, and 4136 were plated after 1, 2, 3, 5, and 8 days incubation. A total of 27 plates accumulated from the 7 specimens which showed growth in the broth. The plates were stored in the refrigerator until work on them could be continued.

3. ISOLATION OF COLONIES.

After storage of 1-3 months in the cold, isolation of colonies on the large Desoxycholate plates began. There were approximately 100-300 colonies on each plate. It was decided, that a representative sample of the bacteria on each plate could be obtained by picking 30 colonies from each plate. The 30 colonies were picked at random into Brain Heart Infusion broth, trying to avoid partiality to colonies which possibly had growth characteristics of *Salmonella*. This was not difficult to accomplish, since most of the colonies were altered in a way that made them all similar in appearance, because of the prolonged storage in the refrigerator. As each was picked, the general growth characteristics of the colonies were recorded on the reverse sides of special cards used in this laboratory for the identification of organisms by biochemical methods. A number was assigned to each isolate, which was also entered on the special card bearing its colony growth characteristics and date of isolation. In all, 810 bacterial colonies were fished from the 7 specimens. All the tubes were incubated overnight at 30 °C. Each was re-streaked on a standard Desoxycholate agar Petri plate and after 24 hours incubation at 30 °C, the purity of the culture was evident. Stock cultures were established of each isolate by stabbing duplicate tubes of semisolid heart infusion agar. The permanent stock number was placed on each tube, and the tubes were stored in the refrigerator.

4. METHODS OF IDENTIFICATION.

After completing the isolation of the colonies from each of the 27 plates, the growths from the brain heart infusion broths were inoculated into various media for biochemical identification. Details for each of the media and the general procedures were essentially those presented by SIGTENHORST (1954) with slight modifications. SIGTENHORST employed the following 17 tests for the identification of enteric microorganisms :

adonitol, glucose for acid and gas, lactose, maltose, mannitol, sucrose, xylose, urea, indol, methyl red, Voges-Proskauer, citrate, gelatin, motility and sulfide.

All the carbohydrates were added in 0,5 % amounts to Purple Broth Base (Difco). Fermentation of lactose was tested in both 0,5 % and 10 % concentrations of lactose; the 10 % concentration was added to Purple Agar Base. In addition to these tests, 8 more were employed in the present study, which are the following :

aesculin, dulcitol, salicin, phenyl pyruvic acid production, production of nitrite and gas in Nitrate broth (Difco), and the oxidative-fermentative property.

Aesculin was added in 0,5 % amounts with the addition of 5 ml of 5,0 % Ferric Ammonium Sulfate per 100 ml of broth. Salicin was used in a 1,0 % concentration. Production of phenyl pyrovic acid was determined in phenylalanine agar (Difco). The oxidative-fermentative properties were studied in Hugh-Leifson semisolid agar (Difco). The motility semisolid medium was modified by the addition of the indicator, triphenyltetrazolium. All inoculated media were incubated at 37 °C for 24-48 hours. The media which necessitated the addition of reagents for development of the tests were incubated for 48 hours. The biochemical reactions, positive or negative, of each organism in each medium, were recorded in the proper special identification card. These cards constitute the permanent record of the results obtained. All the procedures thus far followed were essentially the same as those employed by YARASHUS and by HALKIAS. A sample of the special cards used in this laboratory for the biochemical identification of enteric bacteria can be found in either thesis.

Classification of each organism from the biochemical reactions consisted of assigning a group name to each organism. The basis for the group names was found largely in the manuals of the *Enterobacteriaceae* by KAUFFMANN (1951) and EDWARDS and EWING (1955). Organisms with characteristics typical of *Salmonella* were studied in greater detail by submitting them to the enteric laboratory of the State of Illinois, Department of Health, 1800 W. Fillmore St., Chicago, Illinois. Complete serological analysis of each possible *Salmonella* was performed by Mr. M. LESKO, the head enteric bacteriologist. The type names of each *Salmonella* were then sent to our laboratory. This valuable contribution was possible through the kind cooperation of Dr. H. J. SHAUGHNESSY, Chief, Division of Laboratories.

At the completion of the experimental work, identification cards bearing the results of all the tests of each organism isolated had accumulated in one file. The file consisted of cards labeled with stock numbers 4458 to 5285. A total of 827 cards were accumulated. Each card represented a pure culture derived from an isolated colony picked from the original platings of the specimens. However, 240 colonies out of the 827 were dead upon being picked, leaving a total of 587 which were viable. Therefore the 25 biochemical reactions employed were tested on each of the 587 isolates and each culture was accordingly identified. Since each culture was examined for a minimum of 25 characteristics, each of which required the preparation, inoculation and testing of 1 tube of culture medium, more than 14.675 tubes were used in the study. This effort provided the information concerning the nature of the intestinal flora of wild reptiles which is analyzed and discussed in the succeeding chapters of this thesis.

CHAPTER IV.

Results.

Tables I through XV present the results obtained in this investigation.

Table I, page 20, describes in general all the specimens received from the Congo. A total of 18 specimens was received. The specimen numbers which correspond to numbers of the reptile specimens themselves, are arranged in the numerical order which also is the order in which they were received. A variety of groups of reptiles were involved in the specimen collection. Of the total 18, 9 specimens were obtained from snakes, 7 from lizards, 1 from a toad (*Bufo*) and 1 from a frog (*Rana*). The snake *Crotaphopeltis hotamboeia*, is represented by specimens from 5 individual snakes, and the genera *Philothamnus*, *Scaphiophis*, *Psammophis*, and *Natriciteres* are each represented by a specimen from 1 individual. Of the specimens collected from lizards, 5 were collected from *Mabuya sudanensis*, and 1 was obtained from a true chameleon, *Chameleo senegalensis laevigatus*. A general description of the locale where the animals were captured is also included in Table I. All of the animals were captured in the vicinity of a guard post with 10-15 residents. Six specimens were collected from lizards and snakes which are captured in a spot completely isolated from apparent human habitation.

Unfortunately, not all the specimens which were received could be studied. This was due to the sterile condition of many, which was evident from their failure to grow in culture media. Table II, page 21, lists the 11 specimens which proved to be sterile and the remaining 7 which grew and subsequently were studied in detail. Dates of collection, first plating, and approximate dates of colonies picked are also indicated in Table II. The specimens were collected in March, April, and May of 1959 and arrived in Chicago in the latter parts of these 3 months. On the average, 26 days elapsed between the time the specimens were collected and the time they were received at our laboratory and initially plated. Approximately 45 days elapsed between the plating of the specimens and fishing of the colonies. Therefore, the average time spent between the collection and shipment of specimens and the actual isolation of bacterial colonies was 2 ½ months.

Tables III to IX enumerate all the bacterial groups isolated from each of the specimens and the number of strains of each group encountered from platings after various incubation times. Each of these 7 tables is devoted

to one of the 7 specimens. The bacterial groups are listed alphabetically, with the exception of *Salmonella* which was placed first in the lists.

The kinds of bacteria isolated from 2 plates of specimen No. 2465 are shown in Table III, page 22. A total of groups as follows : 40 strains of *Salmonella*, 4 strains of Atypical *Coli*, 2 strains of *Citrobacter*, 1 strain of *Cloaca* and 9 strains of *Escherichia*. The *Salmonella* proved to be type *tel-aviv*.

Table IV, page 22, shows the 6 different groups of bacteria isolated from 2 plates of specimen No. 2466. A total of 46 strains were identified as follows: 18 strains of *Hafnia*, 5 strains of *Paracolon aerobacter*, and 13 strains of *Paracolon intermedium*. Two strains of *Cloaca*, 1 of *Oxytocum* and 4 of *Pseudomonas*, were isolated only after 6 days incubation and not after 4 days.

Table V, page 23, shows the bacterial groups isolated from 2 plates of specimen No. 3316. Fifty-three strains were identified, 25 of which *Salmonella*, 1 strain of *Bethesda*, 1 strain of *Citrobacter*, 7 strains of *Escherichia*, 4 strains of *Paracolon citrobacter*, 7 strains of *Paracolobactrum intermedium* and 8 strains of *Hafnia*. The single strains of *Bethesda* and *Citrobacter* were isolated after 6 days of incubation and were not encountered after 4 days incubation. *Hafnia* was not found after 6 days incubation but only after 4 days. Three *Salmonella* types were found in this specimen, which were : *S. champaign*, *S. plymouth*, and *S. ramat-gan*.

Table VI, page 23, shows the identification of 128 strains of bacteria from 6 plates of specimen No. 3677. Two bacterial groups were found, the *Salmonella* (115 strains), and the *Hafnia* group (13 strains). *Salmonella* was relatively just as abundant for the whole 8 days. The *Hafnia* group, however, was not encountered during the first 24 hours of incubation but appeared only after 2 days and persisted through the fifth day, disappearing by the eighth day. The *Salmonella* type proved to be *S. plymouth*.

Table VII, page 24, shows the identification of 109 strains of bacteria from 5 plates of specimen No. 3819. Three bacterial groups were found, the *Salmonella* (87 strains), *Bethesda* (4 strains), and *Pseudomonas* (18 strains). Again, the incidence of *Salmonella* remained approximately the same throughout the 8 days of incubation. The *Bethesda* group was encountered after 1 and 2 days of incubation, but was not isolated after subsequent incubations. *Pseudomonas* was isolated after 2 days incubation and persisted after 5 days, but it was not found after 8 days of incubation. The *Salmonella* isolated from this specimen probed to be type, *S. gatow*.

Table VIII, page 24, shows the 7 groups of bacteria isolated among the 116 strains from 5 plates of specimen No. 3883. The 7 groups were *Citrobacter* (1 strain), *Cloaca* (1 strain), *Escherichia* (16 strains), *Hafnia* (46 strains), *Oxytocum* (26 strains), *Paracolon citrobacter* (18 strains) and *Pseudomonas* (8 strains). Only *Hafnia* was isolated after 1 day of incubation and the remaining groups were observed after at least 2, 3, or 5 days of incubation.

Table IX, page 24, shows the identification of 80 strains of bacteria from 5 plates of specimen No. 4136. Three groups were identified which are the following : *Cloaca* (1 strain), *Hafnia* (66 strains), and *Paracolon citrobacter* (13 strains). The *Hafnia* group persisted throughout all the times of incubation. *Paracolon citrobacter* appeared first after 2 days and was isolated after all the incubation times thereafter. *Cloaca* was observed only after 3 days of incubation and not at any other time.

A summary of all the kinds of bacteria found in each of the specimen studied is presented in Table X, page 25. The most frequent kind of bacteria encountered in each specimen is indicated by the underlined group. This was *Salmonella*, which was most frequent in 4 of the specimens, namely, 3 snakes and 1 lizard, and *Hafnia* which was most frequent in the 3 remaining snake specimens. At least 2 different groups of bacteria were found in each specimen, and in most, 3 or more bacterial groups were found.

Table XI, page 26, outlines the enteric flora of the snake, *Crotaphopeltis hotamboeia* as obtained from specimens from 3 individuals. Five or more different bacterial groups were found in each specimen, the *Citrobacter* and *Escherichia* groups being present in all 3 individuals. *Salmonella* was the most frequent group in 2 of the individuals and *Hafnia* was the most frequent in the third. *Hafnia*, *Paracolon Citrobacter*, and *Oxytocum* groups were present together in 2 of the 3 individuals. The enteric flora of 2 other kinds of snakes, *Philothamnus heterolepidotus* and *Natriciteres olivacea* is outlined in Table XII, page 26. Both snakes desplay similarities in their enteric flora in that *Hafnia* was the most frequent group found in both and both snakes contained bacteria of the *Cloaca* group.

Table XIII, page 26, shows the enteric flora of the lizard, *Mabuya*. Only 2 groups were present, the *Salmonella*, which was the most frequent, and the *Hafnia* group.

The frequency of groups of enteric bacilli in wild and captive reptiles is compared in Table XIV, page 27. A total of 17 bacterial groups are compared which are arranged in the order of decreasing frequency as found in the wild reptiles. The number of strains of each group was calculated as per cent of the total number strains isolated. The figures on the captive reptiles were extracted from the theses of YARASHUS and of HALKIAS. As Table XIV indicates, the most frequent group of bacteria found in wild reptiles was *Salmonella* which comprised 45,4 % of the total bacteria isolated, as compared to the 10,2 % or 5,0 % *Salmonella* found in the two series of captive reptiles. The most frequent group of bacteria found in captive turtles and lizards was *Citrobacter* (28,0 %) as compared with only 0,6 % *Citrobacter* found in wild reptiles. The most frequent group observed in captive snakes was the *Proteus* group which comprised 40,8 % of the total bacteria isolated. In comparison, the *Proteus* group was completely absent in the specimens from wild

reptiles. Other groups, besides *Proteus*, present in captive reptiles and not observed in wild reptiles were : *Alkaligenes*, *Arizona*, *Lophomonas*, *Klebsiella*, *Anitratum*, and *Serratia*. The *Cloaca* and *Bethesda* groups present in small numbers in both wild reptiles and captive turtles and lizards, were not observed in captive snakes.

The serotyping of the *Salmonella* strains is summarized in Table XV, page 28. Five types were identified among the 257 strains isolated, which are the following : *S. champaign*, *S. plymouth*, *S. ramat-gan*, *S. tel-aviv* and *S. gatow*. The antigenic formulas of each type are indicated in the table and also the specimen numbers and the scientific names of the animals from which the types were isolated. As the second column of Table XV shows, all the *Salmonella* types identified bear high numbered somatic antigens with the exception of the type *gatow*, which belongs to *Salmonella* group C_1, possessing somatic antigens 6 and 7. All 5 types were diphasic. Two similar types were identified, *S. champaign* and *S. ramat-gan* both of which contain flagellar antigens k : 1,5. *S. plymouth* shows a similarity to the typhoid bacillus, in that it possesses phase 1 flagellar antigen, d, which is also present in *S. typhi*. It is evident from the column listing the names of specimens from which the *Salmonella* were isolated, that the snake genus *Crotaphopeltis* proved to be a rich reservoir for *Salmonella*. Another snake, *Psammophis*, and a lizard, *Mabuya*, were also *Salmonella* sources.

The frequency of each type in a specimen is indicated in the fourth column of the table. Only 1 type of *Salmonella* was found in specimen numbers, 3677, 2465, and 3819. In specimen No. 3316, 3 types of *Salmonella* were found : *S. champaign*, *S. plymouth*, and *S. ramat-gan*. *S. plymouth* also occurred in specimen No. 3677. The most frequent type isolated in any one specimen was *S. plymouth* of which 112 strains were isolated from specimen No. 3677 among a total 128 bacteria examined. The rarest type isolated was *S. ramat-gan* of which only 2 strains were isolated from specimen No. 3316. *S. gatow* was also isolated in quantity, 83 strains being isolated from specimen No. 3819.

CHAPTER V.

Discussion.

Many phases of this investigation could be discussed at length. Arbitrarily, 4 have been chosen which are the following : 1) Collection of Specimens; 2) Plating Specimens and Picking Colonies; 3) Frequency of *Salmonella*, and 4) Intestinal Flora of Wild and Captive Reptiles.

1. COLLECTION OF SPECIMENS.

It would be most advantageous to be present at the collection of the specimens and to examine and study them as soon as possible. This was not possible in the present study. Provisions had to be established, whereby the specimens which were to be collected by a second person during an expedition in the Congo, could be received in our laboratory. It was learned from similar investigations that the dried disc method would be of value for this purpose. Varela's study of fecal specimens in Mexico in 1955 employed the dried disc method with much success, which was a method originally proposed by lie KIAN JOE in 1950. *Salmonella* and *Shigella* were the groups of main concern to these investigators. A study of this method was performed in our laboratory prior to this investigation. Unpublished work by FORNEY and by YARASHUS indicated the suitability of the dried disc method. YARASHUS (1959) compared the swab method and dried disc method for collecting specimens and found no essential differences. The present study added to the evidence that the dried disc method could be employed for transporting the whole content of fecal specimens; not only do *Salmonella* and *Shigella* survive but also many other intestinal bacteria. Although approximately 2 months elapsed between collection and plating of the specimens, the dried discs preserved various groups of bacteria without altering their physiological characteristics. However, heavily inoculated discs are necessary for good recovery. As observed in this study, clean-looking discs arrived sterile, whereas the darkly soiled discs yielded growth. A possible reason for this outcome may be that in the heavily inoculated discs, more liquid containing possible nutriments together with the bacteria was introduced, which helped to maintain the viability of the organisms over a longer period of time. The opposite would be true of the scantily inoculated discs.

2. PLATING OF SPECIMENS AND PICKING COLONIES.

The several platings performed on each specimen after various incubation times proved to be of value in detecting both the fast-growing and slow-growing varieties of bacteria. Some groups of bacteria were not encountered after the first 1 or 2 days of incubation, but only after the third, fourth, or fifth day. For example, in the study of specimen No. 3883, strains of the *Pseudomonas* and *Paracolon citrobacter* groups were not detected until after 5 to 8 days incubation. They were not encountered on plates of the specimens after 1 to 3 days incubation. Therefore, some groups of bacteria would be missed entirely if plates were streaked after 1, 2, or 3 days only.

The survey of the mixed population recovered on the plates present some problems, one of which still remains unsolved at the conclusion of this study. Random picking of colonies is sometimes difficult since one is tempted to pick white *Salmonella*-like colonies and descriminate against all others. This difficulty did not occurr in the present study since all the colonies appeared alike due to their prolonged storage in the refrigerator. Another problem, which is the one that still remains unsolved, is the number of colonies necessary to be picked to constitute a representative sample. Since there were 100 to 300 colonies on each plate, not all could be identified. Consequently, the number 30 was arbitrarly chosen. This resulted in picking approximately 10 % of the total number of colonies present on each plate. wheter every kind of bacteria recovered on the plate would be encountered among the 30 colonies picked involves a change problem that still remains to be solved. The problem is further complicated by the unequal amounts of each of the bacterial groups present. A bacterium, present as only 1 or 2 colonies among 300, would be likely missed no matter how large a fraction of the colonies was picked. The whole question is really one concerning the theory of sampling and probably needs to be approached first by way of theoretical statistics.

3. FREQUENCY OF SALMONELLA.

The high frequency of *Salmonella* encountered in the specimens studied was remarkable and could not have been predicted. The actual number found was 267 out of a total of 587 strains of bacteria isolated or 45,4 %. By the Chi Square test, this was found not significantly different from 50 %. Therefore, in this specimen collection from 6 snakes and 1 lizard, the Gram negative bacteria in the cloacal dejecta were about ½ *Salmonella* and ½ other kinds of bacteria. The figure, 50 %, cannot be compared to the results of other similar investigations. No one else has reported in a way so as to present the frequency of *Salmonella* among the total strains of

bacteria isolated. Other workers have examined a larger number of specimens and found a high percentage of the individuals harboring *Salmonella*. For example, HINSHAW and MCNEIL (1945) found that 26,8 % of the 41 snakes which they examined yielded *Salmonella*. MILLE, LEMINOR and CAPPONI (1958) in examining 609 lizards from the region of Viet-Nam, found that 40 % of the individuals harbored *Salmonella*. Although, the frequencies of the *Salmonella* in the positive specimens were not reported, there is reason to suspect that the frequency was high. If the *Salmonella* were present in only small numbers, many would tend to be lost during the isolating procedures and the reported high percentages of *Salmonella*-yielding individuals would not be observed. Judging from the literature, it is not difficult to isolate *Salmonella* from reptiles; rather the opposite is true, that *Salmonella* are readily encountered in reptiles. Furthermore, many individuals appear to be heavily infected with *Salmonella*.

The *Salmonella* types found, tend to be rare, belonging to groups consisting of organisms with high-numbered somatic antigens. This means that the antigens were only recently discovered. *S. gatow* belongs to *Salmonella* group C, a « lower » group. This is a group common in human and animal salmonellosis. Since there are many organisms closely related to *S. gatow*, which are known to be pathogenic for man, *S. gatow* can also be presumed to be a human pathogen, given the opportunity. In an answer to a letter requesting information about the isolation of *S. gatow*, KAUFFMAN (1960) replied : « I received *S. gatow* in April, 1959 from Dr. S. HOFMANN, Robert Koch Institute, Berlin, for confirmation. The culture was isolated from sewage in Berlin-Gatow. The type is not published as yet. » Each of the other types isolated has been found in human infections except *S. ramatgan*, which has been reported only once before, from a snake in Israel.

4. INTESTINAL FLORA OF WILD AND CAPTIVE REPTILES.

In comparing the kinds and frequencies of bacteria found in this study with those obtained in the study of captive reptiles by YARASHUS (1959) and HALKIAS (1959), 2 major differences immediately stand out : 1) the high frequency of *Proteus* in captive reptiles and its complete absence in wild reptiles, and 2) the high frequency of *Salmonella* in wild reptiles and its relatively low frequency in captive reptiles. These differences would seem to imply that a major change took place in the reptilian intestinal flora during captivity, i.e., it changed from the wild type with *Salmonella* predominating, to the captive type in which *Proteus* was predominant. It is therefore tempting to formulate the hypothesis that the *Salmonella* present in high frequency in wild reptiles are replaced during captivity by other bacteria such as *Proteus*. However, there is still insufficient evidence in

support of such a hypothesis. For one thing, the comparison of wild and captive reptiles should be made with the same or very similar kinds of animals, with similar living and feeding habits. The isolating and test procedures should be identical for both studies. This was not so for the 2 studies compared here, since the work on captive reptiles proceeded from swab specimens, while that on wild reptiles was done from discs. Furthermore, it should be remembered that a considerable number of colonies on the isolation plates were nonviable by the time the cultural identifications were begun. There is no evidence to suggest what these nonviable organisms were. It is possible, although not probable, that they might have been a *Proteus* component of the wild reptile flora. If this were true, the argument that *Proteus* is characteristic of captive as opposed to wild reptiles would not be valid. It would still be true, however, that *Salmonella* were a major component of the wild reptile flora.

The present state of knowledge concerning the intestinal flora of reptiles amounts to the following :

1) There is sufficient evidence that *Salmonella* can be isolated from reptiles, especially snakes, without difficulty.

2) The present study has contributed to this, the knowledge, that in wild reptiles which shun association with man, a large proportion of the enteric bacteria of the intestine in a considerable number of individuals consists of *Salmonella.*

3) On the other hand, there is a relative absence of *Salmonella* in captive reptiles, and an increased proportion of *Proteus*, as established by the work of HALKIAS and of YARASHUS.

What is not known is, do the above observations represent replacement of a wild type flora by a captive type flora ? A further study is needed, taking into account the various factors mentioned. Most of the problems could be solved by departing on a well-equipped safari to the Congo.

TABLE I. — **Description of Specimens received from the Congo.**

Specimen No.	Scientific name	Kind	General description of locale where animal lived
2369	*Bufo*	Toad	Nagero. A settlement
2370	*Rana mascareniensis*	Frog	Nagero. A settlement
2400	*Crotaphopeltis hotamboeia*	Snake	Nagero. A settlement
2406	*Crotaphopeltis hotamboeia*	Snake	Nagero. A settlement
2457	*Mabuya sudanesis*	Lizard	Nagero. A settlement
2465	*Crotaphopeltis hotamboeia*	Snake	Nagero. A settlement
2466	*Philothamnus heterolepiootus*	Snake	Nagero. A settlement
3316	*Crotaphopeltis hotamboeia*	Snake	Ndelele. A permanent guard post with 10-15 residents
3353	*Scaphiophis albopunctatus*	Snake	Ndelele. A permanent guard post with 10-15 residents
3384	*Mabuya sudanensis*	Lizard	Away form human habitation in park
3385	*Mabuya sudanensis*	Lizard	Away from human habitation in park
3386	*Mabuya sudanensis*	Lizard	Away from human habitation in park
3448	*Mabuya quinquetaeniata*	Lizard	Ndelele. A permanent guard post with 10-15 residents
3598	*Chameleo senegalensis laevigatus*	Lizard	Savanna. Not a settlement, but passed by people every 3-5 days
3677	*Mabuya*	Lizard	Away from human habitation in park
3819	*Psammophis subtaeniatus sudanensis*	Snake	Away from human habitation in park
3883	*Crotaphopeltis hotamboeia*	Snake	Away from human habitation in park
4136	*Natriciteres olivacea*	Snake	Away form human habitation in park

TABLE II. — **Dates of collection and study of Specimens received from the Congo.**

Specimen No.	Date collected	Date of first plating	Date colonies picked
2369	March 9, 1959	March 25, 1959	Specimen sterile
2370	March 9, 1959	March 25, 1959	Specimen sterile
2400	March 10, 1959	March 25, 1959	Specimen sterile
2406	March 14, 1959	March 25, 1959	Specimen sterile
2457	March 19, 1959	April 30, 1959	Specimen sterile
2465	March 19, 1959	April 30, 1959	Early June 1959
2466	March 20, 1959	April 30, 1959	Mid June 1959
3316	April 2, 1959	April 30, 1959	Late June 1959
3353	April 7, 1959	April 30, 1959	Specimen sterile
3384	April 9, 1959	April 30, 1959	Specimen sterile
3385	April 9, 1959	April 30, 1959	Specimen sterile
3386	April 10, 1959	April 30, 1959	Specimen sterile
3448	April 11, 1959	April 30, 1959	Specimen sterile
3598	April 21, 1959	May 31, 1959	Specimen sterile
3677	April 23, 1959	May 31, 1959	Mid July 1959
3819	May 2, 1959	May 31, 1959	Early July 1959
3883	May 2, 1959	May 31, 1959	Mid July 1959
4136	May 8, 1959	May 31, 1959	Late July 1959

TABLE III. — **Identification of 56 strains from Specimen No. 2465.**

Groups	Broth plated after incubating :		Total
	4 days	6 days	
Salmonella	27	13	40
Atypical coli	0	4	4
Citrobacter	1	1	2
Cloaca	1	0	1
Escherichia	2	7	9

TABLE IV. — **Identification of 46 strains from Specimen No. 2466.**

Groups	Broth plated after incubating :		Total
	4 days	6 days	
Cloaca	0	2	2
Hafnia	15	3	18
Oxytocum	0	1	1
Paracolon aerobacter	4	1	5
Paracolobactrum intermedium	7	6	13
Pseudomonas	0	4	4

TABLE V. — **Identification of 53 strains from Specimen No. 3316.**

Groups	Broth plated after incubating :		Total
	4 days	6 days	
Salmonella	15	10	25
Bethesda	0	1	1
Citrobacter	0	1	1
Escherichia	3	4	7
Hafnia	8	0	8
Paracolon citrobacter	3	1	4
Paracolobactrum intermedium	2	5	7

TABLE VI. — **Identification of 128 strains from Specimen No. 3677.**

Groups	Broth plated after incubating :						Total
	½ day	1 day	2 days	3 days	5 days	8 days	
Salmonella	25	16	22	18	12	22	115
Hafnia	0	0	1	7	5	0	13

TABLE VII. — **Identification of 109 strains from Specimen No. 3819.**

Groups	Broth plated after incubating :					Total
	1 day	2 days	3 days	5 days	8 days	
Salmonella	19	12	12	18	26	87
Bethesda	2	2	0	0	0	4
Pseudomonas	0	5	10	3	0	18

TABLE VIII. — **Identification of 116 strains from Specimen No. 3883.**

Groups	Broth plated after incubating :					Total
	1 day	2 days	3 days	5 days	8 days	
Citrobacter	0	0	1	0	0	1
Cloaca	0	0	0	1	0	1
Escherichia	0	1	0	5	10	16
Hafnia	20	7	15	4	0	46
Oxytocum	0	9	6	8	3	26
Paracolon citrobacter	0	0	0	8	10	18
Pseudomonas	0	0	0	1	7	8

TABLE IX. — **Identification of 80 strains from Specimen No. 4136.**

Groups	Broth plated after incubating :					Total
	1 day	2 days	3 days	5 days	8 days	
Claca	0	0	1	0	0	1
Hafnia	4	20	11	16	15	66
Paracolon citrobacter	0	3	3	1	6	13

TABLE X. — **Summary of the kinds of bacteria found in each Specimen studied.**
(Most Frequent Group is Underlined.)

Specimen No.	Scientific name of animal	Bacterial groups isolated
2465	*Crotaphopeltis hotamboeia* (Snake)	*Salmonella* *Atypical coli* *Citrobacter* *Cloaca* *Escherichia*
2466	*Philothamnus heterolepidotus* (Snake)	*Cloaca* *Hafnia* *Oxytocum* *Paracolon aerobacter* *Paracolobactrum intermedium* *Pseudomonas*
3316	*Crotaphopeltis hotamboeia* (Snake)	*Salmonella* *Bethesda* *Citrobacter* *Escherichia* *Hafnia* *Oxytocum* *Paracolon citrobacter* *Paracolobactrum intermedium*
3677	*Mabuya* (Lizard)	*Salmonella* *Hafnia*
3819	*Psammophis subtaeniatus sudanensis* (Snake)	*Salmonella* *Bethesda* *Pseudomonas*
3833	*Crotaphopeltis hotamboeia* (Snake)	*Citrobacter* *Cloaca* *Escherichia* *Hafnia* *Oxytocum* *Paracolon citrobacter* *Pseudomonas*
4136	*Natriciteres olivacea* (Snake)	*Cloaca* *Hafnia* *Paracolon citrobacter*

TABLE XI. — **Enteric flora of the snake, « Crotaphopeltis hotamboeia ».**
(Most Frequent Group is Underlined.)

Specimens from three individuals		
No. 2465	No. 3316	No. 3883
Salmonella	*Salmonella*	*Citrobacter*
Atypical coli	*Bethesda*	*Cloaca*
Citrobacter	*Escherichia*	*Escherichia*
Cloaca	*Hafnia*	*Hafnia*
Escherichia	*Oxytocum*	*Oxytocum*
—	*Paracolon citrobacter*	*Paracolon citrobacter*
—	*Paracolobactrum intermedium*	*Pseudomonas*

TABLE XII. — **Enteric flora of two kinds of snakes.**
(Most Frequent Group is Underlined.)

Specimen No. 2466 *Philothamnus heterolepidotus*	Specimen No. 4136 *Natriciteres olivacea*
Cloaca	*Cloaca*
Hafnia	*Hafnia*
Oxytocum	*Paracolon citrobacter*
Paracolon aerobacter	—
Paracolobactrum intermedium	—
Pseudomonas	—

TABLE XIII. — **Enteric flora of the Lizard, Mabuya.**
(Most Frequent Group is Underlined.)

Specimen No. 3677
Salmonella
Hafnia

TABLE XIV. — **Comparison of frequency of groups of enteric bacilli in wild and captive reptiles.**

Groupes	Wild reptiles		Captive reptiles			
			Turtles and Lizards (*)		Snakes (**)	
	No. Strains	%	No. Strains	%	No. Strains	%
Salmonella	267	45,4	11	10,2	6	5,0
Hafnia	151	25,7	3	2,8	2	1,6
Paracolon	40	6,8	8	7,4	1	0,8
Escherichia	36	6,1	5	4,6	7	5,8
Pseudomonas	30	5,1	1	0,9	3	2,5
Oxytocum	29	4,9	3	2,8	1	0,8
Intermediate	20	3,4	2	1,8	4	3,3
Cloaca	5	0,8	3	2,8	0	—
Bethesda	5	0,8	2	1,8	0	—
Citrobacter	4	0,6	31	28,0	26	21,6
Proteus	0	—	24	22,4	49	40,8
Alcaligenes	0	—	6	5,6	14	11,6
Arizona	0	—	6	5,6	3	2,5
Lophomonas	0	—	0	—	2	1,6
Klebsiella	0	—	1	0,9	1	0,8
Anitratum	0	—	0	—	1	0,8
Serratia	0	—	1	0,9	0	—
Total	587		107		120	

(*) Taken from D. YARASHUS, 1959, M. S. Thesis.
(**) Taken from D. HALKIUS, 1959, M. S. Thesis.

TABLE XV. — **Serotypic identification of 257 strains of « Salmonella » isolated from wild reptiles.**

Species identification	Antigenic formula	Specimen No. and name from where isolated	Frequency (*)
S. champaign	39 : k : 1,5	No. 3316 *Crotaphopeltis hotamboeia* (Snake)	15/53
S. plymouth	46 : d : Z_6	No. 3316 *Crotaphopeltis hotamboeia*	6/53
		No. 3677 *Mabuya* (Lizard)	112/128
S. ramat-gan	30 : k : 1,5	No. 3316 *Crotaphopeltis hotemboeia*	2/53
S. tel-aviv	28 : y : e, m, Z_{15}	No. 2465 *Crotaphopeltis hotamboeia*	39/56
S. gatow	6,7 : y : 1,7	No. 3819 *Psammophis subtaeniatus sudanensis* (Snake)	83/109

(*) Numerator : Number of *Salmonella* strains.
Denominator : Total number of strains.

CHAPTER VI.

Summary and conclusions.

The essential findings in the research were :

1) Eighteen specimens were collected from wild reptiles at locations in the Congo, judged to be remote from human habitations. The specimens were of cloacal contents which were dried on filter paper discs, sealed in cellophane envelopes and sent by Air Mail to our laboratory. The zoologist who identified the reptiles, stated that the animals chosen were kinds which are known not to associate with man. These animals also tend to feed on insects and plants and are not known to feed on rodents. This is important, because it supports the idea, that the bacteria, subsequently isolated, were native to the reptiles and not acquired by association with man.

2) Of the 18 specimens, 11 were inoculated scantily and were sterile on arrival, leaving 7 for further study, 6 from snakes, and 1 from a lizard.

3) The dried disc method of transporting specimens was effective in preserving the mixed bacterial flora, providing that the disc was heavily inoculated at the onset.

4) Streaking plates more than once from the enrichment broth was of value in revealing components of the flora that would otherwise have been missed.

5) A total of 587 cultures isolated from these specimens were examined biochemically and classified as to bacterial groups, either genus, species or type, according to the accepted methods of classification.

6) The most frequent group encountered was the *Salmonella* of which 267 strains were isolated or approximately 50 % of the total. These were identified as the types, *champaign*, *plymouth*, *ramat-gan*, *tel-aviv*, and *gatow*.

7) Strains of nine other major groups of bacteria were isolated. These were 151 *Hafnia*, 40 *Paracolon*, 36 *Escherichia*, 30 *Pseudomonas*, 29 *Oxytocum*, 20 *Intermediate*, 5 *Cloaca*, 5 *Bethesda*, and 4 *Citrobacter*.

From these experimental results, certain deductions and interpretations arise :

1) In comparing the types and frequencies of bacteria isolated from wild reptiles with those isolated from captive reptiles, some differences were noted. Whereas *Proteus* was most frequent in captive animals, not a single strain was isolated from wild reptiles. *Salmonella* was the most frequent group isolated from wild reptiles, but it was only occasionnally isolated from captive reptiles. Both, the wild and the captive reptiles showed a surprisingly low frequency of the coliform group.

2) Many wild reptiles are apparently healthy carriers of *Salmonella.* They may therefore, constitute an hitherto unsuspected reservoir of salmonellosis.

Among the many additional problems and projects suggested by this study, the following may be mentioned :

1) This limited investigation does not of course, exhaust all the information which could be obtained about the wild reptilian intestinal flora. A more complete picture of the flora could be drawn if a larger number of specimens were examined and studied in greater detail. It would be most advantageous to carry out this type of study in a laboratory where wild reptiles were conveniently available.

2) Since repeated platings of the specimens became of value in detecting growth of late growing bacteria, otherwise absent in the earlier hours of incubation, it would be beneficial to know exactly how many platings are necessary to recover every possible organism. Of even greater value would be a method whereby the dried disc could be suspended and plated in such a way as to recover the entire contents of the disc on 1 or more plates.

3) Further study is necessary on the effect, if any, of association with man on the reptilian intestinal flora; for example, a parallel investigation could be carried on by deliberately choosing some animals in contact with human habitations and some from manfree sources.

4) A considerable proportion of colonies present on the old desoxycholate plates proved to be dead. It would be of interest to determine if these represent any definite groups of bacteria e.g., *Escherichia* and *Citrobacter.* The question could easily be approached experimentally as part of a study of the viability of pure cultures on various media at different temperatures.

CHAPTER VII.

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