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Enteric Bacteria from Reptiles in the Belgian Congo

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ENTERIC BACTERIA FROM REPTILES IN THE BELGIAN CONGO

By

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LIFE

Philomena A. Szafran was born in Chicago, Illinois, September 16, 1933. She was graduated from Lourdes High School, Chicago, Illinois, June, 1951.

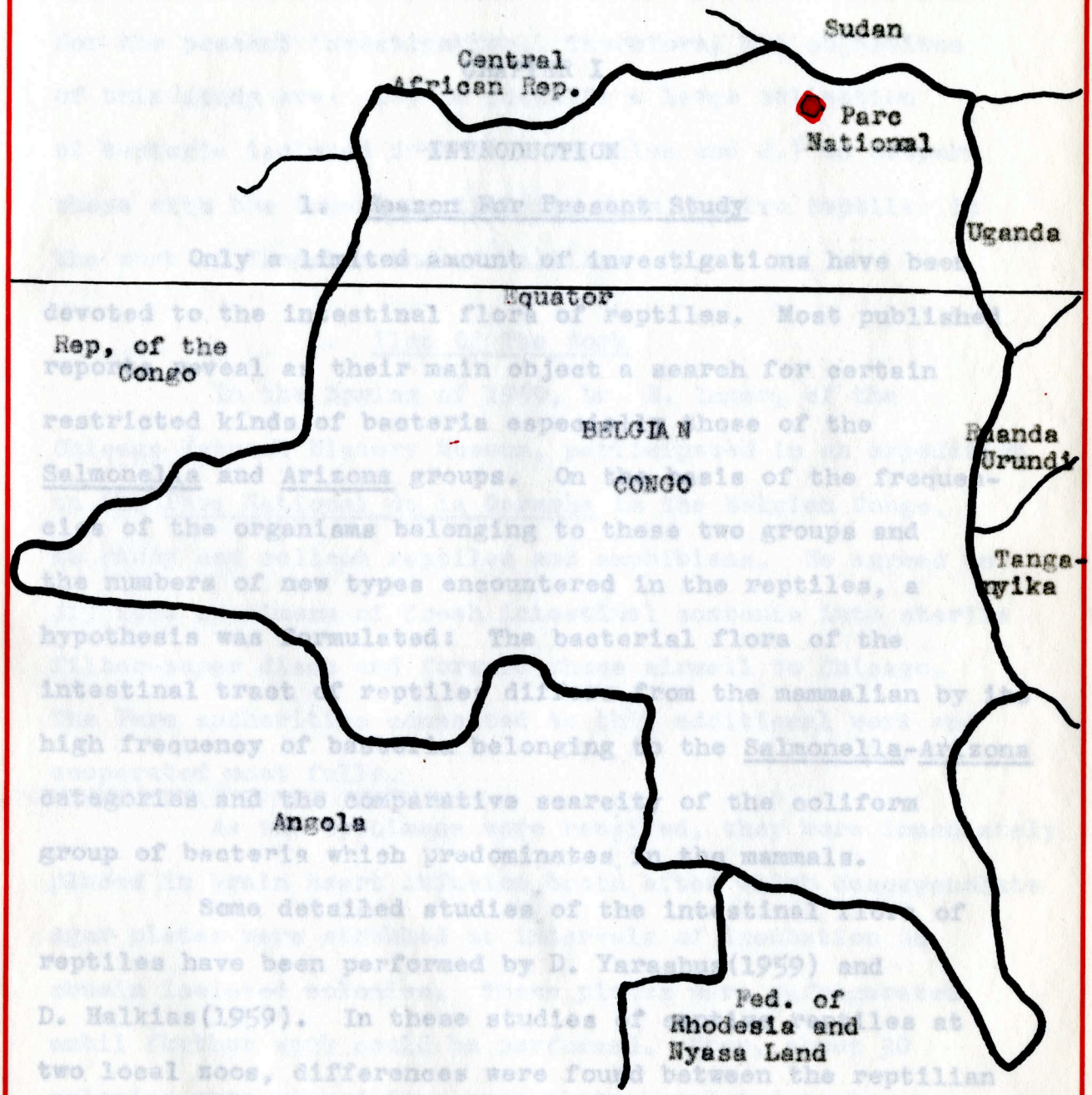
She began attending Loyola University in Chicago, Illinois, September, 1951, from which she graduated in June, 1955, with the Degree of Bachelor of Science. She received a Master of Science Degree in the Chemistry Department of the Graduate School of Loyola University in January, 1958. She began work for her second Master of Science Degree in the Department of Microbiology in September, 1958.

TABLE OF CONTENTS

LIST OF TABLES

Location of the Parc National de la Garamba,

Belgian Congo, Central Africa



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 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?

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 Belgian 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 Health, enteric laboratories for serotypic determinations.

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 McNeil (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 from the intestine of a common lizard. As indicated in the few reports summarized above, the occurrance 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 were found in the excreta of 27 tortoises. A new Salmonella type, S. ramat-gan was isolated by Sapiro-Hirsch, Altmann, 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, French West Africa, from lizards (Agama agama savatieri). The three types were named: S. oukam, S. camberene and S. yoff. At Brazzaville, French Camerons, 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 th 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, French 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, French Indochina, and Brazzaville. They described the antigenic relationships of thse 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 chameleons 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 according to a selected group of well-defined procedures. The opportunity to secure dried cloacal discharges from reptiles in the Belgian 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 Belgian Congo, he was given a supply of sterile filter paper discs (Schleicher and Schull, No. 740E) 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 colony 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 pyruvic 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, triphenyl-tetrazolium. 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 on 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 Yarmoshus 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 33, describes in general all the specimens received from the Belgian 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 ere 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 34, 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 isolatiion of bacterial colonies was 2½ 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 #2465 are shown in Table III, page 35. A total of 56 strains were identified representing 5 different bacteria 1 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 36, shows the 6 different groups of bacteria isolated from 2 plates of specimen #2466. A total of 46 strains were identified as follows: 18 strains of Hafnia, 5 strains of Paracolon Aerobacter, and 13 strains of Pen. 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 37, shows the 7 bacterial groups isolated from 2 plates of specimen #3316. Fifty-three strains were identified, 25 of which were Salmonella, 1 strain

of Bethesda, 1 strain of Citrobacter, 7 strains of Escherichia, 4 strains of Pcn. 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 38, shows the identification of 128 strains of bacteria from 6 plates of specimen #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 39, shows the identification of 109 strains of bacteria from 5 plates of specimen #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 proved to be type, S. gatow.

Table VIII, page 40, shows the 7 groups of bacteria isolated among the 116 strains from 5 plates of specimen #3883. The 7 groups were Citrobacter (1 strain), Cloaca (1 strain), Escherichia (16 strains), Hafnia (46 strains), Oxytocum (26 strains), Pen. 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 41, shows the identification of 80 strains of bacteria from 5 plates of specimen #4136. Three groups were identified which are the following: Cloaca (1 strain), Hafnia (66 strains), and Pen. Citrobacter (13 strains). The Hafnia group persisted throughout all the times of incubation. Pen. 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 specimens studied is presented in Table X, page 42. 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. A least 2 different groups of bacteria were found in each specimen, and in most, 3 or more bacterial groups were found.

Table XI, page 43, 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, Pcn. 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 Natrid teres olivacea is outlined in Table XII, page 44. Both snakes display 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 44, 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 45.

A total of 17 bacterial groups are compared which are arranged in the order of decreasing frquency 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 Malkias. As Table XIV indicates, the most frquent 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 frquent 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 46. 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, Psamrophis, 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 #3316, 3 types of Salmonella were found: S. champaign, S. plymouth, and S. ramat-gan. S. plymouth also occurred in specimen #3677. The most frequent type isolated in any one specimen was S. plymouth of which

112 strains were isolated from specimen #3677 among a total 128 bacteria examined. The rarest type isolated was S. ramatgan of which only 2 strains were isolated from specimen #3316. S. gatow was also isolated in quantity, 83 strains being isolated from specimen #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) Freqency 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 detecing 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 #3883, strains of the Pseudomonas and Pcn. 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 presents 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 arbitrarily chosen. This

resulted in picking approximately 10% of the total number of colonies present on each plate. Whether every kind of bacteria recovered on the plate would be encountered among the 30 colonies picked involves a chance 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, Kauffmann (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. Howevever, 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 proceded 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 Belgian 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 heterolepidotus	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 from 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	Chamaleo 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 from 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 #2465

GROUPS	BROTH PLATED AFTER INCUBATING: 4 days	6 days	TOTAL
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 #2466

GROUPS	BROTH PLATED AFTER INCUBATING: 4 days	6 days	TOTAL
Cloaca	0	2	2
Hafnia	15	3	18
Oxytocum	0	1	1
Pcn. Aerobacter	4	1	5
Pcn. Intermedium	7	6	13
Pseudomonas	0	4	4

TABLE V

IDENTIFICATION OF 53 STRAINS FROM SPECIMEN #3316

GROUPS	BROTH PLATED AFTER INCUBATING: 4 days	6 days	TOTAL
Salmonella	15	10	25
Bethesda	0	1	1
Citrobacter	0	1	1
Escherichia	3	4	7
Hafnia	8	0	8
Pcn. Citrobacter	3	1	4
Paracolobactrum intermedium	2	5	7

TABLE VI

IDENTIFICATION OF 128 STRAINS FROM SPECIMEN #3677

GROUPS	BROTH PLATED AFTER INCUBATING: 1/2 day	1 day	2 days	3 days	5 days	8 days	TOTAL
Salmonella	25	16	22	18	12	22	115
Hafnia	0	0	1	7	5	0	13

TABLE VII

IDENTIFICATION OF 109 STRAINS FROM SPECIMEN #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 #3803

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
Pcn. Citrobacter	0	0	0	8	10	18
Pseudomonas	0	0	0	1	7	8

TABLE IX

IDENTIFICATION OF 80 STRAINS FROM SPECIMEN #4136

GROUPS	BROTH PLATED AFTER INCUBATING:					TOTAL
	1 day	2 days	3 days	5 days	8 days	
Cloaca	0	0	1	0	0	1
Hafnia	4	20	11	16	15	66
Pcn.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)	<u>Salmonella</u> Atypical coli Citrobacter Cloaca Escherichia
2466	Philothamnus heterolepidotus (Snake)	Cloaca <u>Hafnia</u> Oxytocum Pcn. Aerobacter Pcn. Intermedium Pseudomonas
3316	Crotaphopeltis hotamboeia (Snake)	<u>Salmonella</u> Bethesda Citrobacter Escherichia Hafnia Oxytocum Pcn. Citrobacter Pcn. Intermedium
3677	Mabuya (Lizard)	<u>Salmonella</u> Hafnia
3819	Psammophis subtaeniatus sudanensis (Snake)	<u>Salmonella</u> Bethesda Pseudomonas
3833	Crotaphopeltis hotamboeia (Snake)	Citrobacter Cloaca Escherichia <u>Hafnia</u> Oxytocum Pcn. Citrobacter Pseudomonas
4136	Natriciteres olivacea (Snake)	Cloaca <u>Hafnia</u> Pcn. Citrobacter

TABLE XI

ENTERIC FLORA OF THE SNAKE, CROTAPHOPELTIS HOTAMBOEIA

(Most Frequent Group is Underlined)

SPECIMENS FROM THREE INDIVIDUALS		
#2465	#3316	#3883
Salmonella	**Salmonella**	Citrobacter
Atypical coli	Bethesda	Cloaca
Citrobacter	Escherichia	Escherichia
Cloaca	Hafnia	**Hafnia**
Escherichia	Oxytocum	Oxytocum
	Pcn. Citrobacter	Pcn. Citrobacter
	Pcn. intermedium	Pseudomonas

TABLE XII

ENTERIC FLORA OF TWO KINDS OF SNAKES
(Most Frequent Group is Underlined)

SPECIMEN #2466 Philothamnus Heterolepidotus	SPECIMEN #4136 Natriciteres Olivacea
Cloaca	Cloaca
<u>Hafnia</u>	<u>Hafnia</u>
Oxytocum	Pcn. Citrobacter
Pcn. aerobacter	
Pcn. intermedium	
Pseudomonas	

TABLE XIII

ENTERIC FLORA OF THE LIZARD, MABUYA
(Most Frequent Group is Underlined)

SPECIMEN #3677
<u>Salmonella</u>
Hafnia

TABLE XIV

COMPARISON OF FREQUENCY OF GROUPS OF ENTERIC BACILLI IN WILD AND CAPTIVE REPTILES

GROUPS	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. & NAME FROM WHERE ISOLATED	FRE- * QUENCY
S. champaign	39: k: 1,5	#3316 Crotaphopeltis hotamboeia (Snake)	15/53
S. plymouth	46: d: z_6	#3316 Crotaphopeltis hotamboeia	6/53
		#3677 Mabuya (Lizard)	112/128
S. ramat-gan	30: k: 1,5	#3316 Crotaphopeltis hotamboeia	2/53
S. tel-aviv	28: y: e,$\overset{n}{\phi}$,z_{15}	#2465 Crotaphopeltis hotamboeia	39/56
S. gatow	6,7: y: 1,7	#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 Belgian 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 wi th 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 accpted 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 occasionally 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 intstinal flora; for example, a parallel investigation could be carried on by deliberately choosing some animals in contact with human habitations and some from man-free 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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APPROVAL SHEET

The thesis submitted by Philomena A. Szafran has been read and approved by three members of the Department of Microbiology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 23 1960
Date

MacDonald Fulton
Signature of Advisor