

1968

Biological Studies on Various Strains of Trypanosoma Avium

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BIOLOGICAL STUDIES ON VARIOUS
STRAINS OF TRYPANOSOMA AVIUM

A Thesis
Presented to
the Graduate Faculty
Central Washington State College

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Wayne Gordon Cordell
June, 1968

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ACKNOWLEDGMENTS

The author wishes to express gratitude to Dr. Glen Clark for the selection of this problem and for his guidance and assistance throughout its duration. Thanks are extended also to Mr. Ronald Nussbaum for his help with the statistical aspect of this problem. The author also extends appreciation to his wife, Diane, for her patience and encouragement during this time.

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INTRODUCTION

Trypanosomes belong to a group of hemoflagellated blood parasites circumscribed within the following taxonomic scheme: phylum Protozoa, class Zoomastigophorea, family Trypanosomidae, and genus Trypanosoma. They are actively motile, spindle shaped bodies of protoplasm possessing a large nucleus. Division is by binary or multiple fission. Milder and Deane(10) studied the finer structure of Trypanosoma conorhini, relative to the crithidial stage. They offered an excellent report and description of the organelles of the crithidial stage of this trypanosome, which is similar to that of other trypanosomatids.

Oliver(12) stated the most probable vectors of T. avium are mosquitoes and hippoboscids; indicating that infection occurs when the vector is ingested. Bennett's(2) experiments on transmission showed positive infection via posterior station. He suggested that the natural transmission of trypanosomes is by penetration of infective flagellates from the feces through open lesions in the skin of the host.

The literature covering flagellated protozoans of the family Trypanosomidae, contains several direct

references to avian trypanosomes, however, in most instances the trypanosome under study, if associated with an avian host, is referred to as Trypanosoma avium.

The study herein, originated from a previous study with the American Robin, Turdus migratorius. This study showed a gross morphological divergence between trypanosomes of the robin and the Magpie, Pica pica.

The prime objective of this study was the speciation of the Magpie strain of trypanosomes. It involved growth rate phenomenon (increase in the number of individuals) and gross morphology. Later, trypanosome strains from the American Kestrel, Falco sparverius and Steller's Jay, Cyanocitta stelleri were incorporated into the study for statistical comparisons.

Exposure of a select strain of Robin trypanosomes to Cobalt 60 irradiation will be advanced as a possible tool in trypanosome speciation.

LITERATURE REVIEW

Incidence and Terminology

Historically the earliest descriptions of trypanosome-like parasites comes from the works of Antony van Leeuwenhoek(6). His description does not concretely confirm the presence of trypanosomes, but does present a description which might very well be a trypanosome.

Danilewsky, in 1885, was the first investigator to label an avian trypanosome as T. avium(4). In his findings, Danilewsky, recorded two distinct sizes; a smaller one, T. minus and a larger one, T. majus. The only criteria given for distinction was overall length. Laveran, in 1903, reported finding a trypanosome in an owl and labelled it T. avium(8). In 1905, Novy and MacNeal discovered two sizes of T. avium in many North American birds(11). Coatney and Roudabush's (4) study of birds from Nebraska included the following birds: baltimore orioles, dickcissel, grosbeaks, blue jays, cuckoos, purple martin, and an owl, in which, they classified all trypanosomes found as T. avium. They described both a larger and smaller form and recorded varied sizes of each. The larger form varied from 3.15μ to 60.30μ throughout the entire sample of birds. The smaller form

varied from $.90\mu$ to 41.85μ . There was no mention of modality or natural grouping of organisms of similar size. The wide range presented by these authors indicated the possibility of different species. It is assumed that these measurements were not treated statistically, since only averages were reported for each bird. Averages alone, present weak support for the validity of significant difference in a taxonomic comparison between closely related species.

Baker (1) worked with a strain of trypanosomes isolated from a rook. The strain was cultured in vitro and morphologically identified as T. avium.

Clark (3) reported the presence of T. avium in the American Robin, Turdus migratorius, located in Kittitas county. In his study, seventy robins were examined. Marrow from the tibio-tarsus was removed and examined for trypanosomes. Out of the seventy birds examined, forty three (61.4%) showed positive marrow parasitemia while only one bird had positive blood infection. These data substantiates the findings of Stabler (1966) and Diamond and Herman (1954) of higher trypanosome incidence in marrow tissue (3).

Bennett (2) stated that trypanosomes carried via their invertebrate host, through eleven families of birds

resulted in organisms which were found to be morphologically the same. He found no statistically significant data to distinguish these individuals from previously described forms of T. avium. Bennett pointed out there does exist a need for establishing new criteria for separating new species of avian trypanosomes. He suggested the use of serological test, culture media alternation, and the ability of the trypanosome to produce infective flagellates in a variety of true bloodsucking vectors.

The forementioned researchers all labelled their specific parasite as T. avium. Could all of the independent investigators have found the same parasite, T. avium, in such a wide diversity of avian species? One finds it incredulous that the many varied morphological reportings of T. avium belong to the same taxonomic category.

Irradiation

Fitzgerald (7) exposed unsporulated oocysts of Eimeria stiedae to gamma irradiation at rates ranging from 10,000 rads to 400,000 rads. He reported no "immediate change in the appearance of the oocysts after irradiation." Fitzgerald showed that the irradiation had no affect up through the 100,000 rad dosage level. Those

at 200,000 rads exposure were able to sporulate, but were unable to cause infection. Those at the 400,000 rad level were unable to sporulate.

Williams (14) reported the effects of X-irradiation on Spathidium spathula as having five general expression patterns. They were listed as follows:

- (1) death of an undivided cell (primary death);
- (2) death of a descendant of an irradiated cell (secondary death);
- (3) permanent injury.....;
- (4) temporary division retardation for 1 to 2 days followed by apparent complete recovery; and
- (5) no apparent injury.

Behavior patterns 1, 2, and 3 increased directly as the dosage level was increased from 22 to 55 krads. Patterns 4 and 5 were observed at a dosage level of 6 krads.

METHODS AND MATERIALS

Culture Techniques and Medium

Trypanosome cultures were established for three species of birds endemic to Kittitas County; the Black-billed Magpie, Pica pica; the Steller's Jay, Cyanocitta stelleri; and the Sparrowhawk or American Kestrel, Falco sparverius. Ten Magpies were taken from the nest just prior to fledging and reared in a converted sparrow trap measuring 4' x 4'. The Magpies were fed mixed mink food daily, which proved to be a very sufficient diet. As the need arose to re-establish an original culture, a Magpie was chosen at random and sacrificed. All ten of the Magpies revealed a positive trypanosomal infection.

The Steller's Jay and the Sparrowhawks were collected in the field as needed.

Trypanosomes were taken from the bone marrow of the tibio-tarsus of the respective birds. The procedures followed for invitro transmission of trypanosomes were similar to those described by Oliver. The tibio-tarsus was removed, cleaned, flamed and the proximal end removed. A sterile wire probe was inserted into the

the marrow cavity, rotated, and then transferred to the liquid phase of the invitro medium.

The culture strains were isolated in NNN medium at room temperature (24-28°C). Preparation of the media followed a traditional recipe given by Baker (1). The ingredients and proportions used were as follows:

Preparation of NNN media with liquid overlay

NaCl, 6g.; agar, 16g.; distilled water, 950ml. This solution was transferred in 8ml. quantities to 22ml screw-cap tubes and autoclaved at 15lbs. pressure for 15 minutes. After removal from the autoclave and cooling, 1ml of sterile, fresh rabbit blood was added to each tube. This mixture constituted the base of the media and was allowed to solidify on a slant. After solidification, 1ml of the liquid overlay was added. The overlay was a Locke's solution containing 2.86mg of the antibiotic streptomycin sulfate. The Locke's solution consisted of the following: NaCl, 8g; KCL, 0.2g; KH_2PO_4 , 0.3g; glucose, 2.5g; water, 1 liter. The tubes were then incubated at 37°C for twenty four hours. After incubation they were stored in refrigeration at 5°C until needed.

Subinoculations were made of all original cultures. These were made by aseptically passing a platinum loopful of the liquid overlay to the new tube. Cultures of the Magpie, Sparrowhawk, and Steller's Jay were maintained at 15°C, 20°C, and room temperature. All cultures were counted daily with the aid of a hemo-

cytometer. In order to insure uniform distribution, each tube was shaken twenty five times, after which a loopful of the liquid phase was aseptically transferred to the hemocytometer. Only those trypanosomes found in 1 sq. mm., which corresponds to 0.1 cu. mm. of fluid, were counted. Counting included only active motile forms.

Irradiation

The accessibility of a Cobalt 60 irradiating source, located at Battelle Northwest in Richland Washington, enabled this investigator to indulge in a relatively new area of trypanosome investigation. The author was interested mainly in seeing what effects, if any, irradiation had upon the growth patterns of well known and previously investigated trypanosome strains and to initiate basic research in this area. For these experiments, trypanosomes from the American Robin, Turdus migratorius, was chosen, due to its proven hardiness and the fast growth rate which it exhibits. The irradiated cultures were maintained at a constant temperature of 25°C.

Robin trypanosomes were inoculated into the defined NNN media and exposed to the respective dosages on the same day. Five tubes were exposed at each of the

following dosage levels: 5 krads, 15 krads, 50 krads, 75 krads, 100 krads, and 300 krads, while five tubes were maintained as a control, void of radiation exposure. The series of tubes were placed on a 4'x8' table equal in height to that of the radiating source. To obtain the correct dosages, a tube rack containing five tubes of inoculum was placed at the proper footage according to the inverse square law. The dosage and footage was as follows:

300 krad	1'
100 krad	1'9"
75 krad	2'
50 krad	2'5"
15 krad	4'6"
5 krad	7'9"

The dosage period was three hours. Dosimeters of lithium fluoride were placed, as a check of correct dosage, at the 5, 50, and 300 krad levels. The dosimeter readings were checked later by Battelle Memorial Institute and forwarded to the author.

Morphology and statistics

Morphological studies entailed the taking of culture smears. These were taken consecutively from the inoculation date up to and past the peak of growth for a given series. A smear was made by transferring a loopful of the liquid overlay to a glass slide where it was

allowed to dry. After drying, they were fixed in Ponselle's fixative (methyl alcohol, 5 parts; tincture of iodine, 1 part), for five minutes and then rinsed in methyl alcohol. Again, they were air dried. Smears were then hydrolyzed in a 1 N solution of HCL at 60°C for two minutes, rinsed with buffered water, stained with Giemsa for twenty minutes, and dried. A cover slip was applied, preparing the material for observation and measurement under oil immersion.

Measurements were taken by superimposing a micron scale, located in the right ocular of a phase contrast microscope, upon the stained crithidial forms. Measurements taken included overall length, maximum width, and distance from the anterior end of the nucleus to the anterior end of the organism. One hundred twenty individuals were measured for each of the three strains.

The specimens of each strain were arranged into an array from the shortest to the longest individual. From the many accounts found in the literature attesting to the fact that trypanosomes change shape with increased age or size, it was decided to arbitrarily divide these arrays into three size classes consisting of forty specimens each. This procedure would naturally group individual specimens exhibiting similar characteristics,

thereby eliminating the need to differentiate qualitatively between various forms. Comparison, then, was made between equivalent size classes of the three strains of trypanosomes.

From the measurements taken the following ratios were calculated: (R_1) the width at the widest point (W) divided by the total length (L) giving the ratio W/L and (R_2) the distance from the anterior end of the nucleus to the anterior end of the individual ($N-A$) divided by the total length (L) giving the ratio $N-A/L$. By establishing such ratios, the author has defined a definite body proportion or character and has thus eliminated the need of discussing morphology in terms of pyriform, bulbous, and slender forms.

The following is an outline of the procedure used to obtain an average ratio for one of the size classes with reference to ratio $R_1(W/L)$. This procedure was used to obtain average ratios for each of the three size classes in each strain of trypanosomes for both ratios R_1 and R_2 .

- Step I Sum the numerator and denominator of the expression W/L of all individuals of that size class.
- Step II Divide the summed numerator by the summed denominator obtaining the decimal equivalent.
- Step III Record the decimal equivalent as the average ratio of that size class

with reference to that particular ratio, in this case $R_1(W/L)$.

The foregoing ratios were treated with standard statistical tests. Variances, standard deviations, and 95 per cent confidence intervals were calculated for each ratio of each size class in each of the three strains of trypanosomes.

Simpson, Roe, and Lewontin (13) pointed out that the largest confidence interval within a given size class must be assigned to all strains in that size class in order to obtain valid significant difference. Then, a non-overlap of confidence intervals indicates a valid significant difference at the 95 per cent confident level. Comparative analysis of these data, consisting of intra and inter-size class comparison, will follow later.

RESULTS

The individual readings represent the mean trypanosome population in a given series of cultures and the average number of organisms per 0.1 cu. mm. of fluid. Since some problem existed in obtaining precise measurements, the ranges and averages referred to will be in terms of length only.

Effect of Temperature on the Trypanosome strains

Trypanosomes from the Magpie inoculated at 15°C failed to show any appreciable growth. Nine cultures were sustained for a period of twenty days. One tube showed a population of eleven individuals on the twenty-first day. Over a fifty-four day period, this culture recorded an average reading of four. These results do not concur with those found by Oliver (12) for T. avium from the American Robin at the same temperature. At 20°C the growth of the Magpie strain reached a peak of 1,575 individuals. A five day lag preceded any significant growth. This was then followed by rapid growth until peak population was reached (Fig. 1). The readings for

Fig. 1. Growth of the Magpie strain at 20°C in NNN medium.

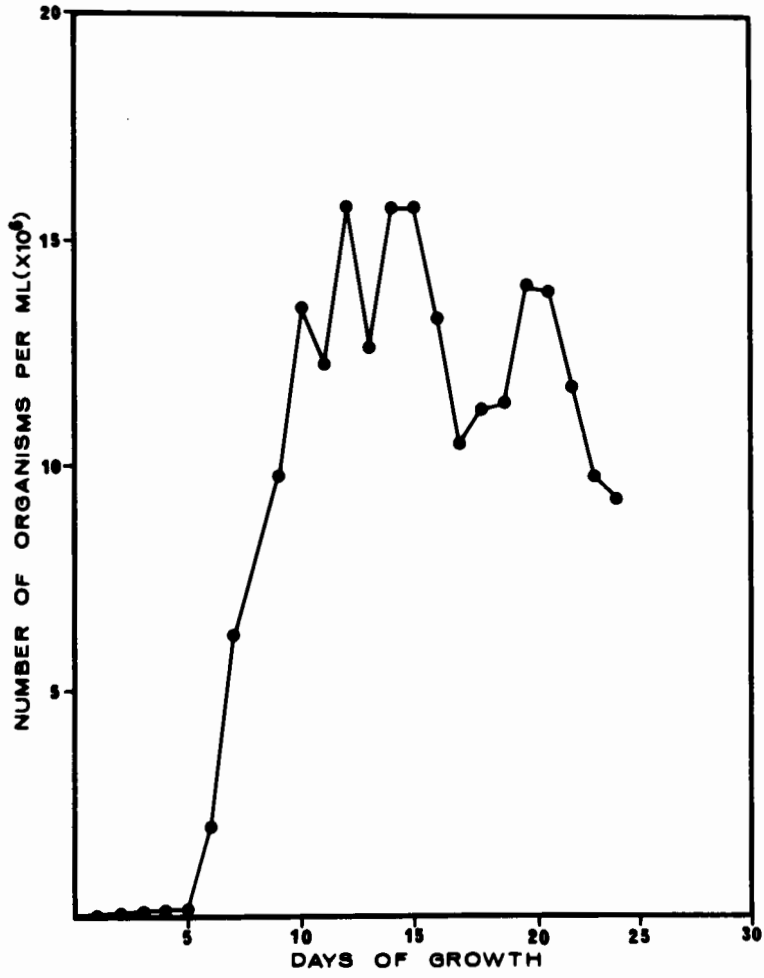


Figure 1

Magpie growth at room temperature were the mean population of seventeen cultures. Growth at room temperature was more responsive than at 20°C but the peak was considerable lower (Fig. 2). The range in length for this strain was 6.6-56.1 with an average of 20.33 .

The trypanosome strain taken from the Steller's Jay exhibited growth phenomenon somewhat similar to the findings of Oliver for T. avium from the Robin at 15°C. The overall growth was slow, peaking on the thirty-first day. The peak population was 1,225 individuals (Fig. 3). The growth lag for this strain, before significant growth occurred, was ten days. Growth of this strain at 20°C and room temperature closely parallel one another; both exhibited immediate growth response and peaked on the seventh and eighth day respectively with peak populations of 1,350 and 1,472 individuals (Fig. 4,5). The 20°C growth pattern of the Jay strain is the mean of nine cultures while the room temperature curve is the mean total of ten cultures. Size range of this strain was 7.7-29.7 , with the average organism being 15.59 long.

The Sparrowhawk trypanosomes cultured at 15°C exhibited a very slow rate of growth (Fig. 6). The peak of 1,245 was not reached until the thirty-second day. The growth lag for this particular strain was approximately sixteen days. At 20°C, the peak was not as high

Fig. 2. Growth of the Magpie strain at room temperature
in NNN medium.

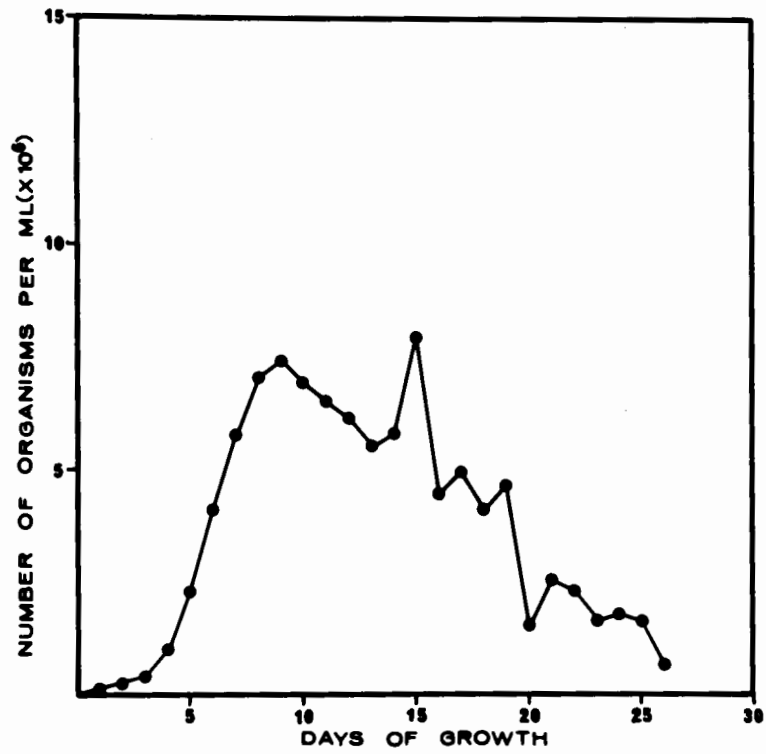


Figure 2

Fig. 3. Growth of the Jay strain at 15^oC in NNN medium.

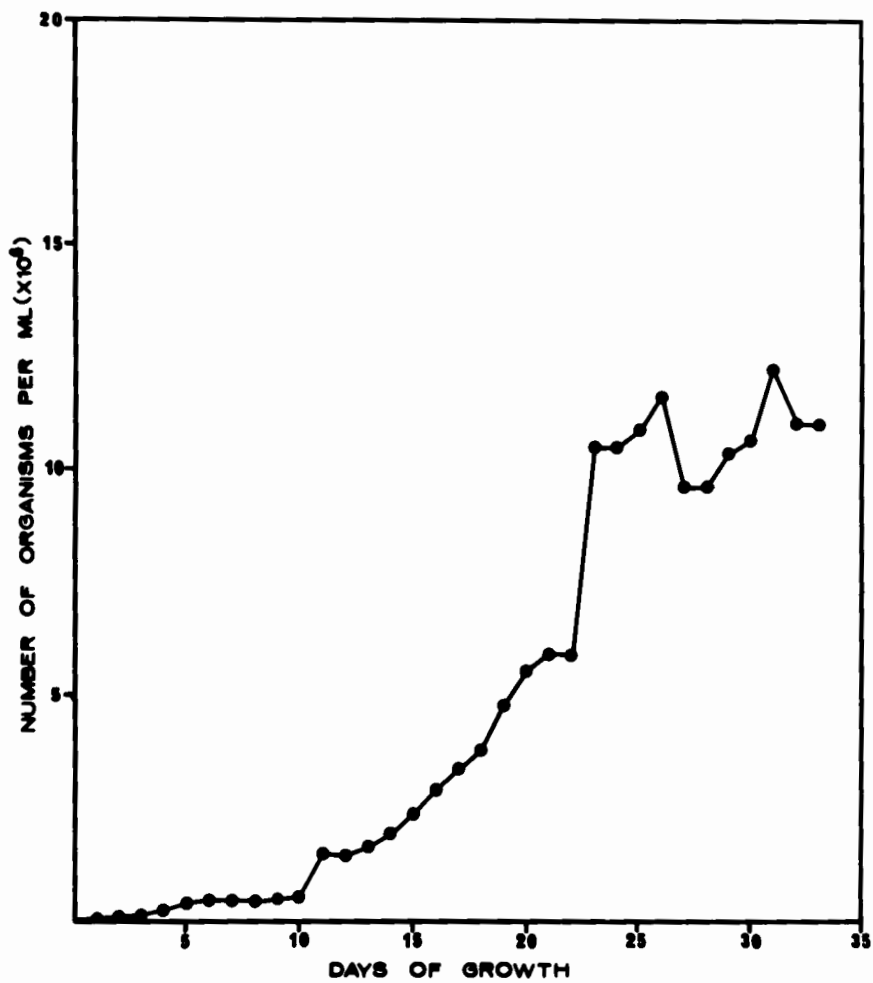


Figure 3

Fig. 4. Growth of the Jay strain at 20°C in NNN medium.

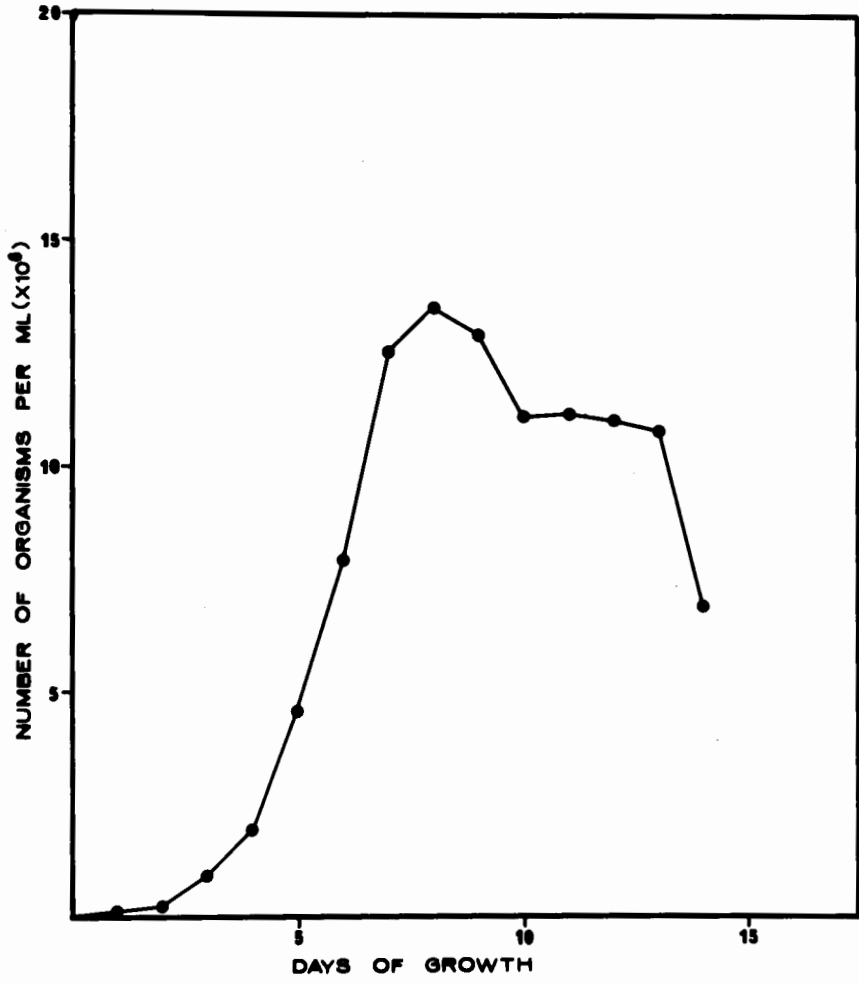


Figure 4

Fig. 5. Growth of the Jay strain at room temperature in
NNN medium.

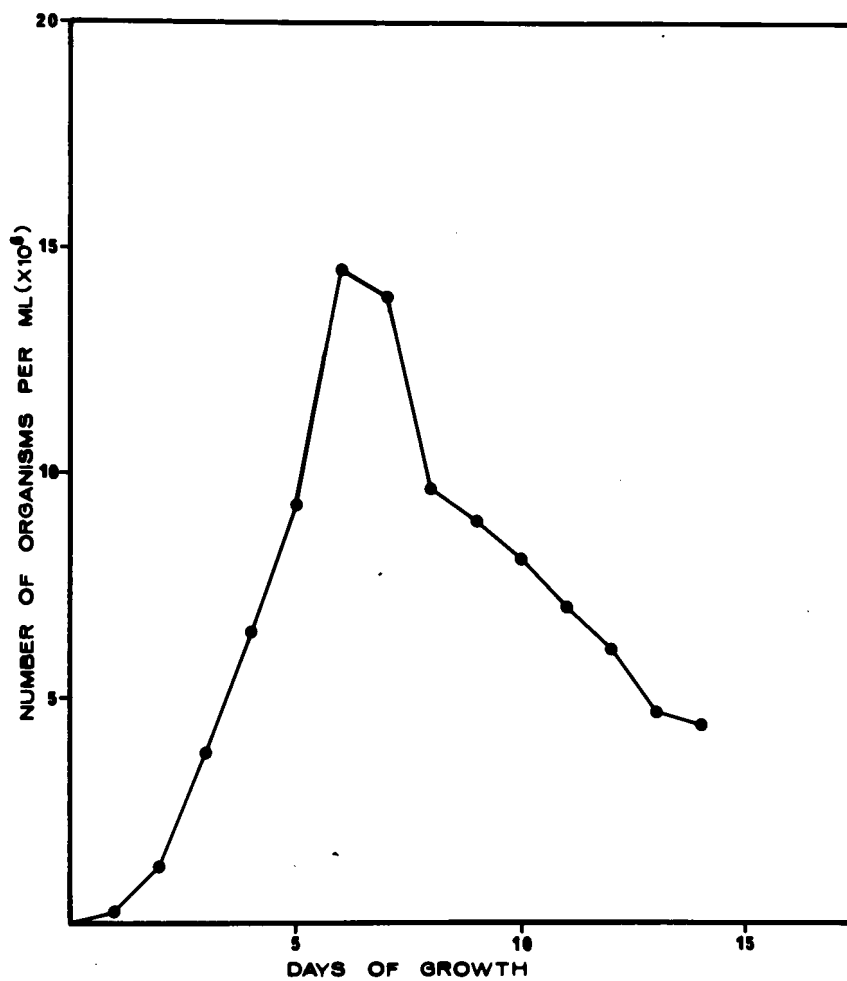


Figure 5

Fig. 6. Growth of the Sparrowhawk strain at 15°C in
NNN medium.

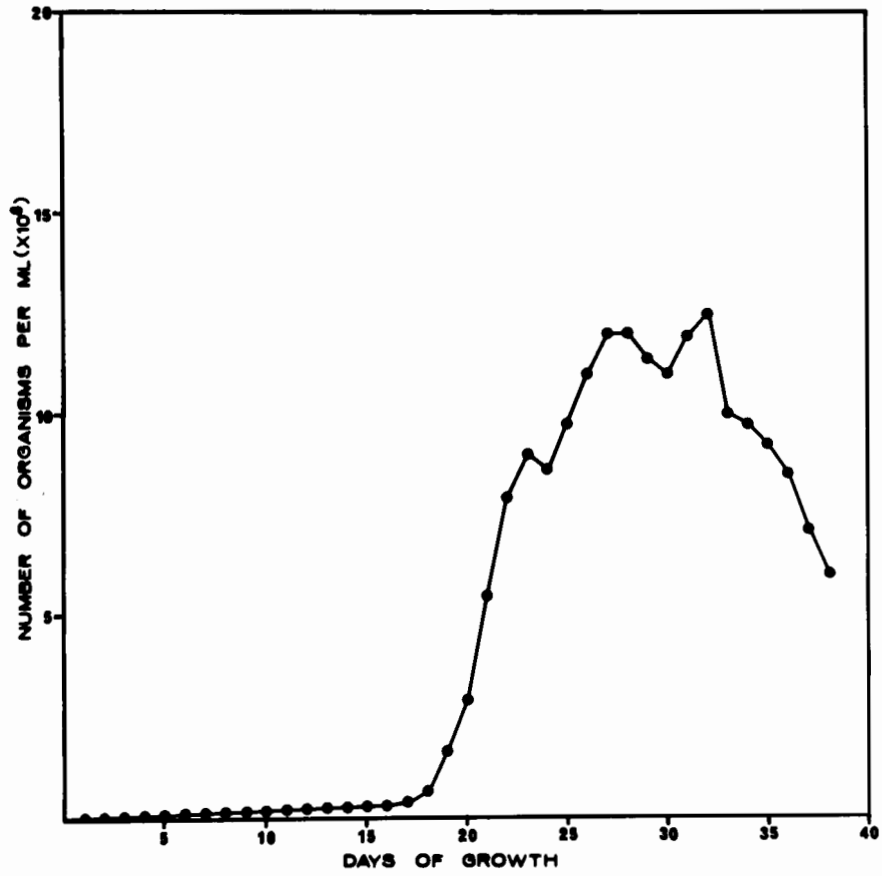


Figure 6

as at 15°C, however, the growth response was much quicker and peak population was attained in about one-third the time of that at 15°C (Fig. 7). The number of cultures examined at 20°C was eight. The peak growth of fifteen inoculated cultures grown at room temperature was attained in six days and maintained at that level for a period of four days (Fig. 8). The average length for the Sparrowhawk strain was 15.86 μ , with the range being 5.5-31.9 μ .

Cultures were incubated for all three strains at 37°C, however, the cultures exhibited good growth for only the first four days, after which, they began a gradual decline, terminating on the seventh day.

Irradiation results

The dosimeters of lithium fluoride powder, were analyzed by Battelle Northwest and found to be in the general range of our anticipated dosages of radiation. The dosimeter at the 5 krad level revealed the correct dosage to be 4.6 krads. The dose we calculated as 50 krads, was in fact between 75 and 80 krads. Due to the magnitude of the 300 krad exposure, the correct dosage at this particular level was not discernible on the dosimeter powder. Based on the two dosimeter readings obtained for

Fig. 7. Growth of the Sparrowhawk strain at 20°C in
NNN medium.

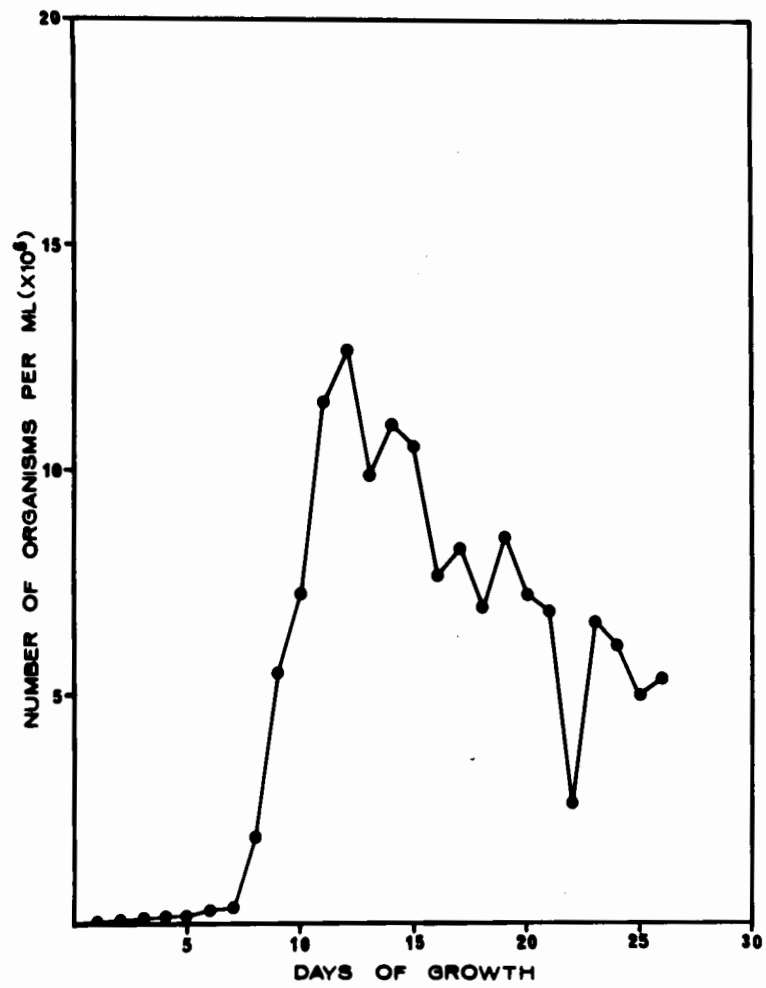


Figure 7

Fig. 8. Growth of the Sparrowhawk strain at room temperature in NNN medium.

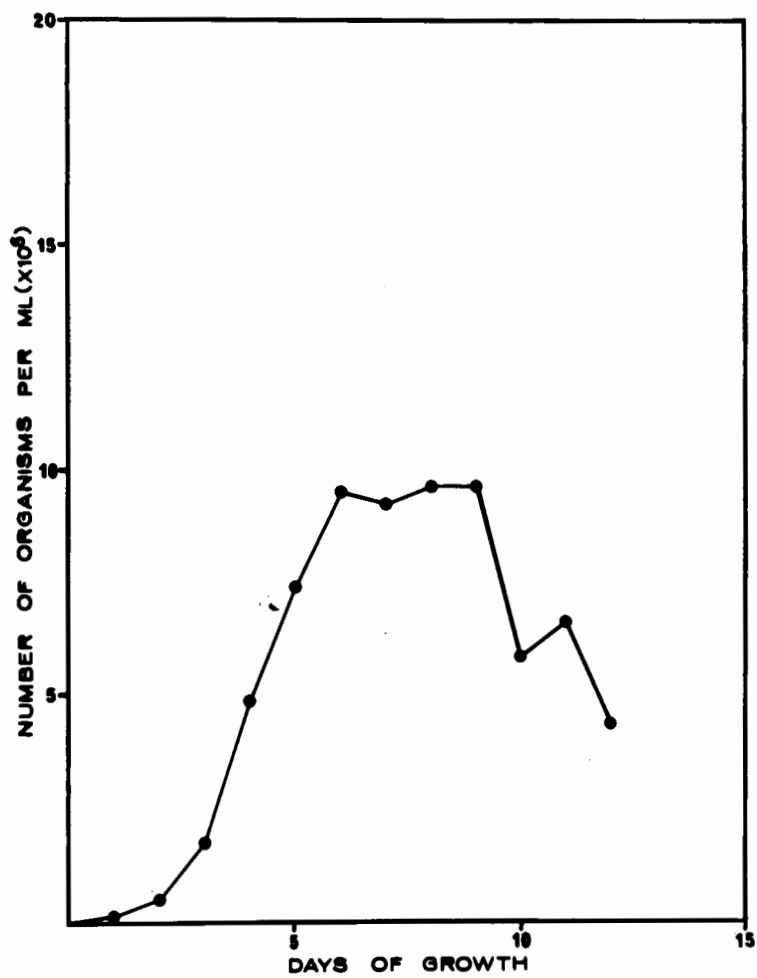


Figure 8

the lower dosages, it was recommended that we assume this exposure to be in excess of its calculated value. Figure 9 shows the growth patterns of the original inoculum upon exposure to radiation. Good growth was recorded for all dosage levels with the exception of the cultures at the 300 krad level. All five cultures at this level were void of viable forms by the third day of incubation. By comparing the exposed group to the control group, the effects of radiation upon growth is readily apparent (Fig. 9).

In the lower levels of exposure (5-50 krads), peak growth was achieved roughly the same day as in the control cultures, however, the peak itself was approximately 50 per cent of that seen for the control group. Peak growth was prolonged in the higher dosage ranges of 75 and 100 krads (14-15 days).

Three tubes of the NNN media were aseptically inoculated as subcultures from all five exposure levels plus the control group. The resultant growth rates were relatively comparable (Fig. 10).

Morphology and Statistics

Results of the morphological data from the statis-

Fig. 9. Irradiated growth of the Robin strain at 25°C
in NNN medium.

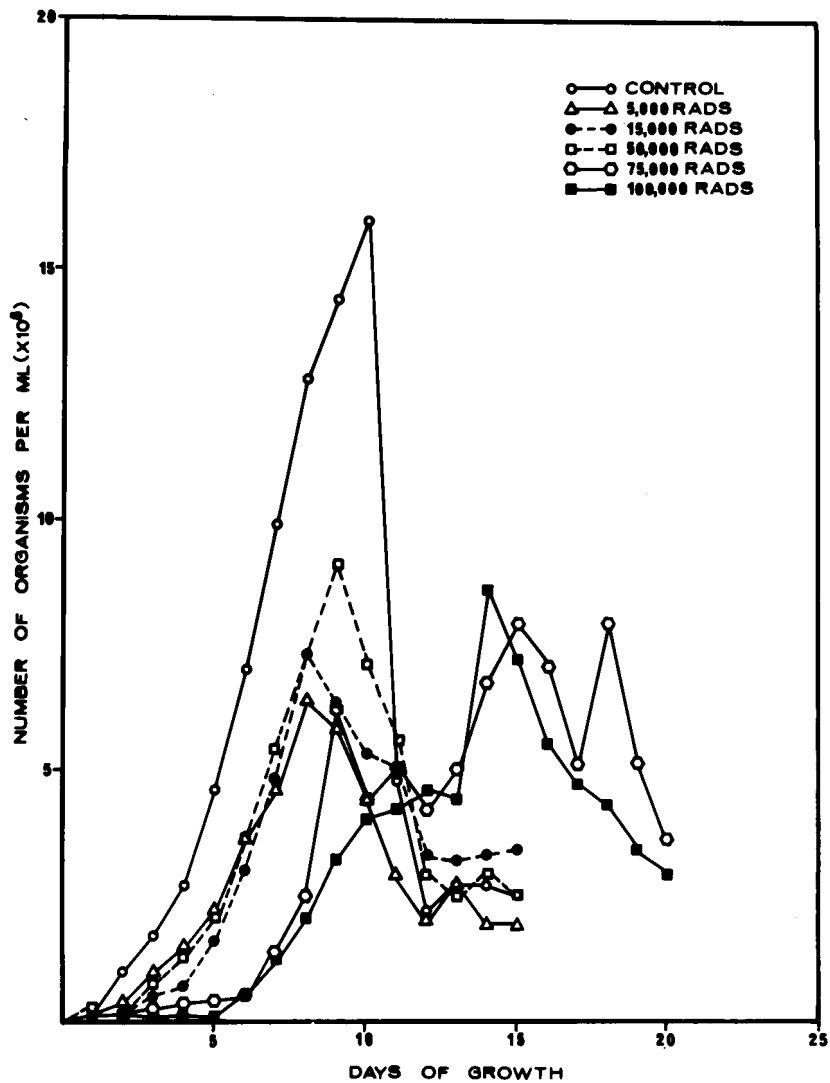


Figure 9

Fig. 10. Subculture growth of the irradiated robin strain at 25°C in NNN medium.

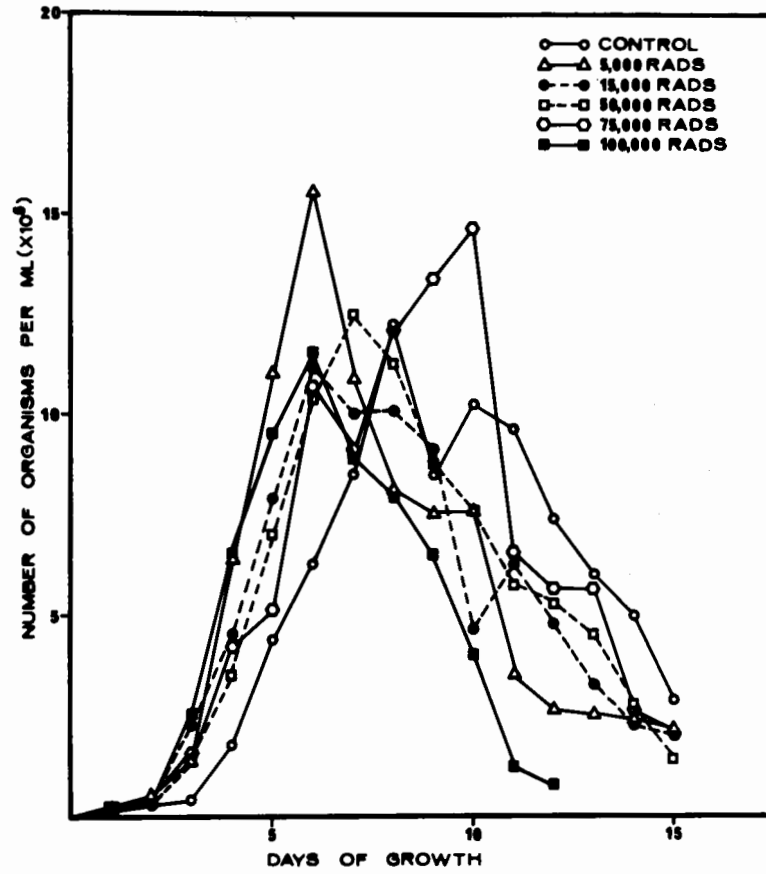


Figure 10

tically treated ratios for the three strains of trypanosomes are presented in Table I, and are to be referred to later.

TABLE I

Statistical data on Trypanosomes from the various birds

Magpie					Jay				
Size Class	Range in μ	No.	W/L	N-A/L	Range in μ	No.	W/L	N-A/L	
I	6.6-13.2	40	.227 \pm .022	.689 \pm .040	7.7-13.2	40	.290 \pm .033	.653 \pm .040	
II	13.2-25.3	40	.091 \pm .016	.684 \pm .044	13.2-16.5	40	.169 \pm .025	.645 \pm .040	
III	25.3-56.1	40	.038 \pm .002	.621 \pm .036	16.5-29.7	39	.108 \pm .001	.656 \pm .037	

Hawk				
Size Class	Range in μ	No.	W/L	N-A/L
I	5.5-11.0	40	.225 \pm .028	.587 \pm .010
II	11.0-17.6	40	.119 \pm .020	.630 \pm .030
III	17.6-31.9	43	.055 \pm .005	.647 \pm .025

DISCUSSION

Effects of Temperature

The purpose of this study was to further investigate avian trypanosomids, relative to the Magpie, Steller's Jay, and Sparrowhawk, and to initiate work for a newer aspect of trypanosome study; the effects of radiation upon trypanosome growth in vitro.

The success of the in vitro culturing of these three strains of trypanosomes offered excellent testimony to the adequacy of the NNN media described by Baker (1). Growth phenomena under specific temperatures failed to show any significant differences between the three strains of trypanosomes. The failure of the Magpie strain to grow at 15°C may be of some significance, however, based on the other results of this study, it was unexpected and unexplainable.

Growth composites at 15°C, 20°C, and room temperature showed all three strains to be relatively equal in terms of population size increase (Figs. 11, 12, 13, in the appendix). The rate of size increase was also comparable. The Jay and Sparrowhawk strains showed a comparable growth lag and peak achievement at 15°C, while all three showed a

reduced lag at 20°C. This increase in growth delay as the temperature decreased definitely indicated the need of an adjustment period. This quiescent period seemed to be advantageous as shown by the greater population attainment following the delay. The growth phenomenon of these organisms during this delay period was not fully understood and presents new avenues of investigation.

There was no growth observed for any of the three strains at 37°C. Many theories have been advanced indicating a trypanocidal effect being illicited from the serum at this temperature. In the course of this study, five tubes of the defined media were placed in a 37°C incubator for a period of five days. The tubes were free of inoculum. After an incubation period of five days, the tubes were withdrawn and inoculated. The resultant growth was normal. It is suggested, therefore, that the trypanocidal effect is not illicited from the serum, but is incorporated by the presence of the trypanosomes. In this regard, we must agree with Deane and Kirchner (5) and say, that the trypanocidal effect is probably due to the buildup of metabolic waste material. This buildup is undoubtedly due to increased metabolism imposed by the higher temperatures.

Irradiation

The data collected on the irradiation experiments indicated a definite trypanocidal effect on those individuals that underwent exposure to the Cobalt 60 source (Fig. 9). The subculturing in this experiment has stimulated continued interest in radiation exposure. Collectively, the subcultures presented a normal growth curve for this strain of trypanosomes (Fig. 10). A second subculture was run as a check on the unexpected normal curve recorded for the first subculturing. This second subculturing substantiated the first by also showing the return to a normal growth curve. This lack of residual effects of irradiation is a bit confusing. In as much as trypanosomes under go asexual reproduction, one would expect morphological and physiological changes to be evident in the following generations of individuals. It may be that the effects, if any, may not show in the first few generations, but could become evident several generations from the original inoculum. With an exposure time of three hours, it would be almost impossible for any one trypanosome to escape exposure. Since inoculation and exposure were only three hours apart, the magnitude of individuals per tube was such that it would not hinder

exposure to each specimen.

The lack of bizarre forms is good evidence that the irradiation had no morphological distorting effects, at least on the first few cultures or generations, but merely slowed basic metabolism and hence growth rate. Here again, it would be interesting to see if monster trypanosomes appeared in later generations.

Another possibility might be that the irradiation caused a breakdown in some constituent of the media in such a way that it would support only a reduced population.

Statistics

When considering ratio R_1 (W/L), there was significant change in body shape for each strain between all size classes (Table I). This difference suggested a cyclic development. Cyclic development for T. ranarum (9) has been reported in terms of "initial pear-shaped reproductive bodies, followed ... by 'sustaining' slender flagellates." This change in body shape coincides with the quantitative expression of the character W/L, herein.

There was no significant difference within size classes between the Magpie and Sparrowhawk strain. The

Jay strain, however, was significantly different from the two former strains in terms of this character (W/L, Table I).

With reference to the N-A/L character, after assigning the largest confidence interval, there was no significant difference noted within any of the three size classes. After interpreting these data, it is suggested that this character (N-A/L) remains fairly stable throughout the bodily changes observed in trypanosome development. The failure to obtain any significant difference designates this character as having no significant value in this case.

From the foregoing study, in terms of growth, the three strains of trypanosomes apparently belong to the same species. However, statistics have shown that in terms of morphology, the Steller's Jay strain of trypanosome is significantly different from the Magpie and Sparrowhawk strains with reference to the statistical character W/L. It is felt however, that the data herein is not extensive enough to warrant the establishment of a new species to house this strain of trypanosome. It is hoped that these results will initiate further investigation toward the development of competent techniques for trypanosome speciation.

SUMMARY

1. Trypanosomes from the bone marrow of the Magpie, Steller's Jay, and Sparrowhawk were cultured at 15°C, 20°C, and room temperature in NNN medium with rabbit serum. Comparable growth (increase in number of individuals) was observed for all three strains. Based on growth phenomenon, all three strains are considered as belonging to the same species. A temperature of 37°C inhibits growth of all three strains.
2. Culture smears were made of all three strains and stained in Giemsa for morphological and statistical studies. Standard deviations, variances, and confident intervals at the 95 per cent level were calculated using standard test. Comparisons were between equivalent size classes arbitrarily assigned to each strain. There was no significant difference between the Magpie and Sparrowhawk strains. The Jay strain was shown to be significantly different with reference to the ratio W/L (maximum width/total length). There was no significant difference between the three strains in terms of the ratio $N-A/L$ (distance from the anterior end of the nucleus to the anterior end of the organism/total length).

3. Trypanosomes from the American Robin were subjected to radiation exposure of a Cobalt 60 source. The dosage levels were as follows: 5 krad, 15 krad, 50 krad, 75 krad, 100 krad, and 300 krads plus a control group. Reduced growth was observed at all levels with the exception of the 300 krad level, which was void of viable forms by the third day of incubation. The upper critical dosage limit appears to be between 100 and 300 krads. A subcultured group of the original inoculum returned to a normal growth curve for this particular strain of trypanosome. There were no residual effects observed nor were there any bizarre forms present.

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APPENDIX

Fig. 11. Composite of the growth rates of the Jay and Hawk strains at 15°C.

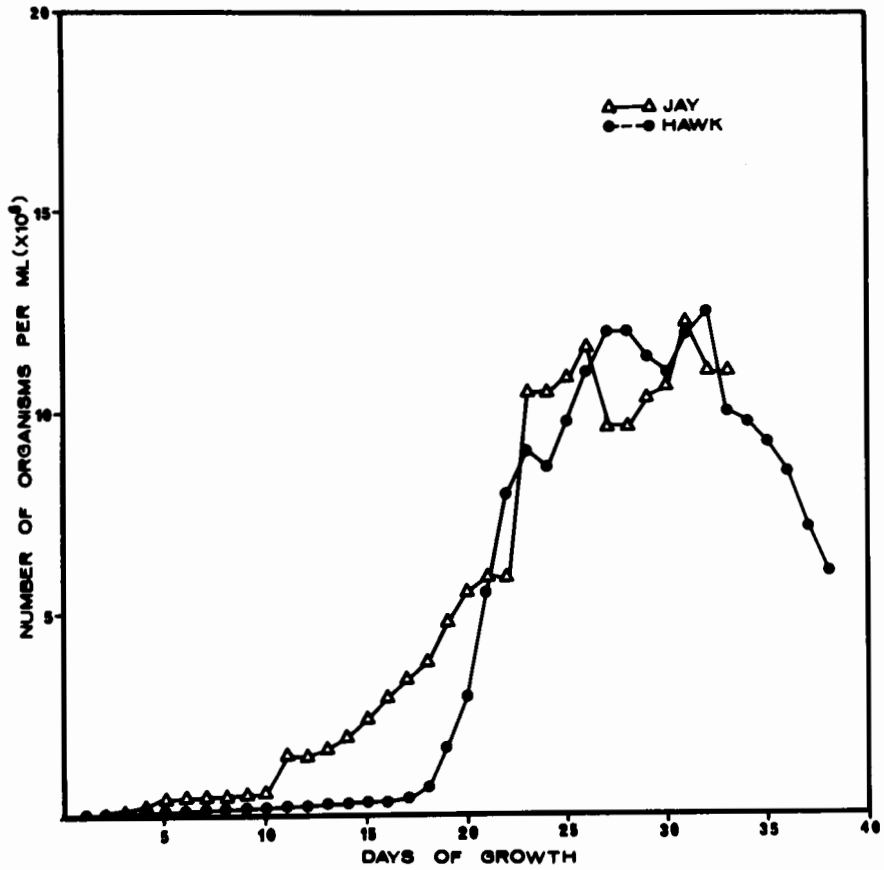


Fig. 12. Composite of the growth rates of the Magpie, Jay, and Hawk strains at 20°C.

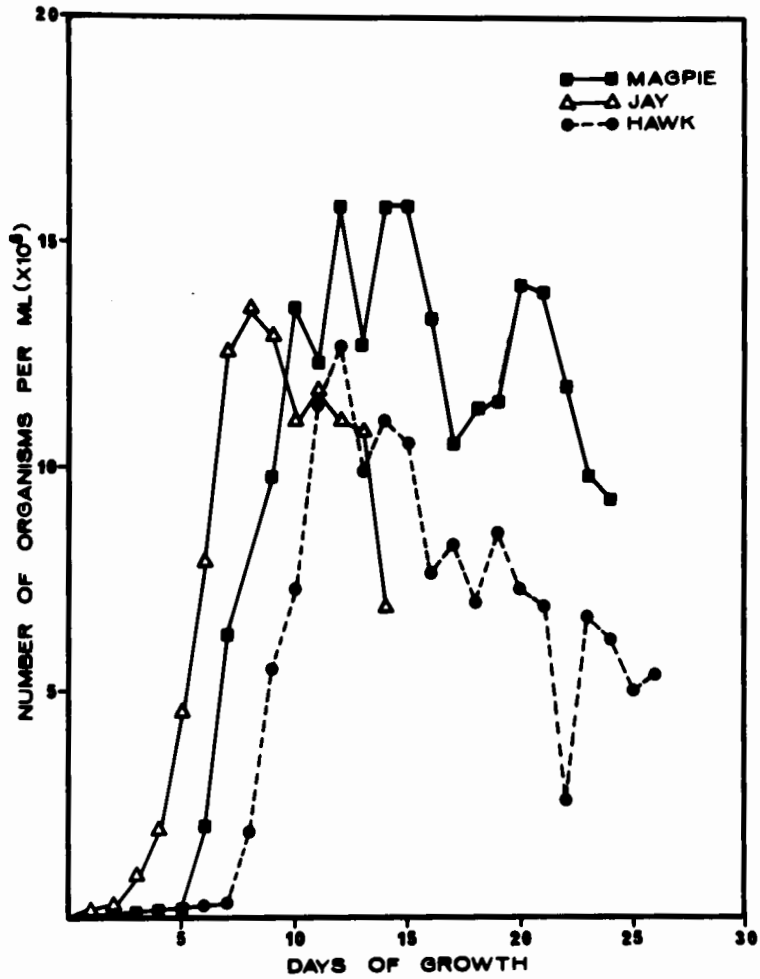


Fig. 13. Composite of the growth rates of the Magpie, Jay, and Hawk strains at room temperature.

