THE «PARANUCLEAR CORPUSCLES» IN POIKILOTHERMIC VERTEBRATES: DESCRIPTION OF A NEW SPECIES OF *PIRHEMOCYTON* IN *IGUANA IGUANA* OF VENEZUELA, WITH REMARKS ON THE NATURE OF THESE ORGANISMS AND THEIR RELATION TO ALLIED PARASITES[1*]

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(With 1 plate)

The presence of RNA in the paranuclear corpuscles in *Pirhemocyton* surrounded by a halo of DNA is reported and the significance of this evidence is discussed on the light of the identification of the genus as a virus on grounds of the morphology of the ultrastructure by Stebbens & Johnstone 1965.

The oxidative enzimatic reaction of succinic dehydrogenase, used by Dodin and Brygoo in 1960, was repeated, demonstrating enzimatic respiratory activity in this organism, activity which does not exist among the viruses.

MATERIAL AND METHODS

Blood smears from *I. iguana* of the region of Marapa (Dto. Federal of Venezuela) were stained first with Giemsa.

The following cytochemical reactions were carried out on *Toddia lacertiliarum* Arcay, Nasir & Diaz 1968, and on *Pirhemocyton iguanae* sp. nov.:

Giemsa. — Smears fixed with methanol and stained with Giemsa were used for diagnosis, and in all the subsequent technics, smears made at the same time were stained with Giemsa to control the behavior of the organism under the distinct reactions practiced. Sorensen phospfate buffer at a pH of 7.2 was used.

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Nile blue. — Freshly drawn blood from the iguanas was examined on a slide under a coverslip with phase contrast, in 2% Nile blue chloride in physiologycal saline, in order to observe these organisms under the action of a vital stain.

Feulgen. — The reaction of Feulgen and Rossenbeck 1924 was applied to fresh blood smears, fixed with methanol, 10% formol, Bouin and 0l-formol for 10 minutes.

Methyl green-pyronine. — This reaction was utilized to differentiate the granular content of RNA in the paranuclear corpuscles of *T. lacertiliarus* and *P. iguanae,* from the outer parts of the same. The modification of Taft 1951, was used, and the fixatives were the same as in the Feulgen reaction.

Periodic acid-Schiff (PAS reaction). — According to Hotchkiss, 1948. Fixation: 10 minutes in alcohol-formol.

Gallegos trichromic stain. — Used to differentiate certain types of proteins. Fixed for 5 minutes with Cytofixemart spray.

Succinic dehydrogenase activity. — The technic described in 1960 by Dodin & Brygoo was used, in which blood was taken from iguanas parasitized by *P. iguanae* by cardiopuncture. The erythrocytes were washed three times in a centrifuge at 2.000 RPM, and incubated 48 hours at 37°C in Farber's medium. The blood was examined under phase contrast at two-hour intervals, beginning at 4 hours incubation time, for a total of 10 hours. It was examined again at 24 and 48 hours.

Acid phosphatase activity. — The technic perfected by Gomori in 1950 was used to detect this enzime, in which blood was taken from the parasitized animal by cardiopuncture and fixed with cold acetone.

Experimental infections. — Healthy lizards of various genera were inoculated intraperitoneally with blood extracted by cardiopuncture from iguanas parasitized with *P. iguanae,* in order to demonstrate the specificity of the parasite.

Four specimens of *Iguana iguana* (from a non-endemic zone), three specimens of *Ameiva ameiva,* fous *Cnemidophorus l. lemniscatus,* two *Tropidurus torquatus* and three *Leposoma percarinatum* were inoculated. Blood smears of all these lizards had been previously examined to make sure that they were not parasitized with any organism related to *Pirhemocyton.* All lizards were checked daily for 18 days, and later, twice a week for 60 days.

Cross infections. — Iguanas parasitized with *P. iguanae* and others infected with a species of *Pirhemocyton* not identified (from different locality), were inoculated with blood containing a heterologous species of *Pirhemocyton.*

RESULTS

The citochemical reactions observed in *Toddia lacertiliarum* and in *Pirhemocyton iguanae* are given in Table Nº 1.

The results of these reactions permit us to show the affinity between these parasites, as well as the differences between them. On the other hand, it appears that the Feulgen reaction, so long used to investigate the nature of these paranuclear corpuscles, is unsatisfactory, as it shows only in the composition of the nucleoprotein, the presence of DNA, without differentiating between the content of the nucleic acids. For the latter purpose, we have used methyl green pyronin, which stains the paranuclear corpuscles bright red, showing them to be composed of one or more granules of RNA. The granules are surround by a greenish halo, the result of the action of the methyl green on the DNA. (fig. 6). The same green color is seen in the nuclei of the parasitized erythrocytes. In the "hernias" observed in these nuclei, the evaginations contain a granule, in the apical extremity, which is stained with the pyronine.

In the reactions of methyl green pyronine, we have observed that the crystals of *Toddia* are stained bright pink, without ever being stained red. This appears to indicate that the crystals are some metaproteic product of the nucleoproteins; this the first report of any cytochemical staining behavior for the crystals of *Toddia*. The globoids of *Pirhemocyton* never react with methyl green pyronine, always remaining transparent.

PAS reaction. — The granules of the paranuclear corpuscles are stained purplish-blue for *P. iguanae* and reddish-blue for *T. lacertiliarum,* the granules being surrounded by a refringent halo. This indicates that the granules are readting only with the hematoxylin content of this stain, only the nucleoprotein giving a reaction.

Gallegos's trichrome. — With this stain, only the reaction of the nucleoprotein to the hematoxylin was observed, indicating that the proteins of the paranuclear corpuscles are all nucleoproteins.

Succinic dehydrogenase activity. — Observation by phase contrast of blood parasitized with both organisms, after 6 hours incubation, showed the presence of a granule of formazán near the paranuclear corpuscle of both forms, and 2 to 3 granules around the nucleus of the erythrocyte. The presence of these dark formazán granules near the paranuclear corpuscles indicates the activity of reduction in respiration. Although the respiratory activity of the nucleus of the erythrocyte itself must not be left of account, we consider that, between 6 and 8 hours of incubation, there is a critical period in which the formazán granules produced by the paranuclear corpuscles may be observed. The granule is seen to be well separated from the granules produced by the nucleus, which adhere to it.

Acid phosphatase activity. — In this reaction no vestige of the enzime was detected.

Result of experimental infections. — Of the 16 lizards inoculated with *P. iguanae* taken from *I. iguana,* only the specimens of *Iguana* were positive for the organism, so that the course of the parasitemia is described only in this species.

TABLE I

COMPARATIVE CYTOCHEMICAL REACTIONS BETWEEN *PIRHEMOCYTON IGUANAE* AND *TODDIA LACERTILIARUM*

		Nile Blue	Giemsa	Feulgen	Methyl-green Pyronine	PAS Reaction	Gallegos trichromic stain	Succinic dehydrogenase activity	Acid Phosphatase activity
PIRHEMOCYTON	Corpuscle	granule pink-bluish halo blue pale	red granules blue halo	doubtful reaction	red granules pale greenish halo	purplish blue granules refringent halo	fuchsia	1 granule of formazán in periphery	—
	Globoids	—	Soluble in methanol	—	—	—	—	—	No Reaction
TODDIA	Corpuscle	compact brilliant blue	red granule blue halo	positive reaction	red granule greenish halo	blue granule refringent pink halo	bright red granule refringent halo	1 granule of formazán in periphery	—
	Crystal	—	Insoluble in methanol	—	Pink	—	—	—	—

Paranuclear corpuscles appeared on the fifth day after infection, possessing only one granule in the interior; later, 2 to 3 granules surrounded by a delicate covering were observed.

On the tenth day, vacuolization was seen. Between 10 and 12 days, the rectangular globoids characteristic of *P. iguanae* appeared, and corpuscles were seen outside the erythrocytes.

It was observed that the number of globoids bore an inverse relation to the number of paranuclear corpuscles, the globoids increasing as the corpuscles decreased and viceversa. Such fluctuations might alternate in chronic infections, although no chronic infections were observed by us in experimental infections, all lizards dying from 10 to 25 days after infections. The young iguanas being less resistant and showing acute symptoms. In the state when the globoids decreased, the hernial deformations of the nuclei increased.

Result of cross-infections. — The iguanas with *P. iguanae* from Marapa, were inoculated with *Pirhemocyton* sp. from Maracay, and were examined daily for a month; they were always negative to *Pirhemocyton* sp.. On the other hand, the iguanas from Maracay parasitized con *Pirhemocyton* sp. and inoculated with *P. iguanae* were negative for *P. iguanae.* In both cases, the animals retained the original infections.

Description of Pirhemocyton iguanae sp. nov.

This organism is seen in two forms that are considered characteristic of the genus *Pirhemocyton* Chatton & Blanc, 1914, the paranuclear corpuscles and the globoids. The paranuclear corpuscles appear as circular rings with 1, 2 or 3 granules in the interior, at times 4; the granules have a characteristic marginal position within the corpuscle, in which they shown great similarity to *Anaplasma.* The granules are surrounded by a halo which shows a circular, ring-like aspect. The paranuclear corpuscles of *Pirhemocyton iguanae* measure 1 to 4 micra in diameter, according to the number of marginal granules they contain (figs. 1, 2, 3, 4).

It should be noted that the "paranuclear corpuscles" are found not only in the cytoplasm of the erythrocytes, but also outside, these cells (fig. 2).

We have observed the corpuscles, both within and outside the erythrocytes, undergoing binary division and schizogony, in the latter case forming groups of 8 to 16 merozoites.

The globoids have the appearance of empty vacuoles, not taking any of the stains used in our investigation. The globoids of *P. iguanae* generally appear as elongated rectangular forms, with rounded borders at the ends. The most characteristic globoids of *P. iguanae* are long, 1. 5 by 5 micra, but others are shorter, sometimes seen joined together (fig. 1).

The paranuclear corpuscles with marginal granules, associated with the globoids, are the two structures most characteristic of the genus *Pirhemocyton,* as established by Chatton & Blanc in 1914. However, there exist other structures, such as fusiform bodies, chains of red gra-

nules, and the nuclear evaginations also observed in other species such as *P. eremiasi* Blanc & Ascione, 1959.

In *P. iguanae* we have also been able to observe these above mentioned structures which has been associated to the genus: a) irregular nucleated fusiform bodies; b) fusiform bodies with granules, one behind the other; c) chains of red granules in the cytoplasm of the erythrocytes, similar to those seen in *P. eremiasi;* d) small red granules, separated but connected by a fine hilium which is stained the same as the granules; e) abundant rounded vacuoles in the cytoplasm of the erythrocytes, not having the refringent appearance of the globoids. These vacuoles give the cytoplasm a characteristic cribose aspect; they measure 1.5 to 2 micra (fig. 3).

The nuclei of the erythrocytes are often seen to have an irregular outline, and to have evaginations of a hernia type (fig. 5). These evaginations have a granule within the distal extremity, a granule which is stained intensely red with pyronine.

DISCUSSION

Since Chatton and Blanc in 1914, described *Pirhemocyton tarentolae* from the lizard *Terentola mauritanica,* other species have been described, always from lizards or chamaleons. Chatton & Blanc 1914, present an incomplete parasite, describing two types of structures: an inert nucleated corpuscle of 1 micron, which becomes pyriform, reaching a size of 3-4 by 1.5-2 micra, and a colorless spherical, refringent globule, which they represent as a product elaborated by the cytoplasmic changes caused by the parasite.

In 1916, the same authors gave a more complete description, recognizing three forms: a) spherical, with diffuse chromatin (1-4 micra); b) ameboid, with massive chromatin (2-4 micra); c) spherical, with peripheral granular chromatin (3-5 micra). They did not observe multiplication nor extraerythrocytic forms.

Brumpt & Lavier 1935, observed in *Lacerta viridis* a hematozoon presenting the same forms described for *P. tarentolae,* which they put forward as a new species, *P. lacertae.* They did not observe globoids, but reported the presence of grouped forms that they regarded as a result of shizogony; they also reported a leishmanoid-like form. Like Chatton & Blanc 1916, Brumpt & Lavier 1935, recalled *Toddia buffonis* França 1911, related these two organisms, establishing that the crystalloids of *Toddia* could be compared to the globoids of *Pirhemocyton* spp., and that the said globoids were no more than a product of the parasitized blood cells. He also put forward that the schizogonic forms were comparable to these of *Dactylosoma* sp. Brumpt & Lavier, stating that *P. lacertae* can be inoculated into animals of the same species.

Dodin & Brygoo 1956, described *P. chamaleonis* in *Chamaleo lateralis,* describing five forms obtained from blood smears and organ appositions, in which the chromatin could be either central or clumped in marginal

packets which gave a granular appearance to the form. They also described globoids attached to the relatively dense chromatin mass.

They also mentioned deformations in the erythrocytes, the ends being sharp so that the whole cell was of a spindle shape. The illustrations show anisocytosis, there being both atrophied and hypertrophied cells, in addition to the normocytes.

Dodin & Brygoo in 1956 described the development and transmission of *P. chamaleonis* and discussed its position in relation to *Dactylosoma* sp., and to the other species of *Pirhemocyton* already described. Brygoo in 1957 rectified the name of the species to *P. chamaleonis* Rousselot 1953, as Rousselot had described the same species from *Chamaleo basiliscus* in 1953.

Blanc & Ascione 1959, presented *P. eremiasi* as a new species, found in the lizard *Eremias guttulatus olivieri,* differing from the three species already described in the polymorphism of the chromatic elements, all of which might be found within one erythrocyte. They also reported extremely small forms, appearing as chains of colored granules, and also elongated forms which gave the appearance of being in division. They also reported, together with the large globoids, small bodies with a vacuolar aspect.

Mackerras 1961, in Australia, found *Pirhemocyton* in three different lizards species, but considered that they were all one species, refering them to *P. tarentolae.*

Pessoa and Souza Lopes 1963, described a species of *Pirhemocyton* from *Cnemidophorus l. lemniscatus,* which they provisionally identified as *P. tarentolae,* although it appeared to be more similar to *P. eremiasi,* since the globoids were generally smaller and more numerous, measuring 2-3 micra, very rarely 8-10 micra, as in *P. tarentolae.* In the same way, the corpuscles vary between 1-3 micra, and very rarely, 4-5 micra, being in the latter case very close to the nucleus of the blood cell and not showing differentiated cytoplasm. Deformations of the blood cells were not reported.

Pirhemocyton iguanae sp. nov., differs from *P. tarentolae* in that the structure we have defined as the paranuclear corpuscle is always spherical, never pyriform. Also the form of the globoids of *F. iguanae* is always rectangular with rounded edges.

P. iguanae shows strong dimorphism, similar to that of *P. eremiasi,* in the chains of small granules. However the two forms are never found within the same erythrocyte. There are also similarities in the vacuolar formations, which can be distinguished from the globoids by the more strongly refringent appearance of the latter, and also by the appearance in different stages of development of the infection in *Iguana iguana.*

The specificity of the infection to *I. iguana* has been demonstrated by attemts to infect other species of lizards, in the same way that Dodin & Brygoo in 1956 proved the specificity of *P. chamaleonis* for its respective host. Also we have demonstrated specificity by the negative result of the cross infection of *P. iguanae* from Marapa (Dto. Federal)

with *Pirhemocyton* sp. (species not identified) of iguanas from Maracay (Edo. Aragua). Like these authors, we have also been able to follow the sucession of forms in the development on parasitemia on *Iguana iguana.*

We consider that one of the most striking morphological characteristics of the species is the form and size of the globoids, the rectangular form of *P. iguanae* not having been described in any other species. Also the parasitic specificity of this organism permits us to diagnose it as a new species, proposing it as *P. iguanae,* in order to mantain the taxonomic status of the genus.

The nature of P. iguanae and its relation to Toddia lacertiliarum.

The fundamental object of this investigation has been to relate the two species, at the same time differentiating between them by cytochemical reactions, with the end of clarifying their position as parasitic or viral organisms.

There are currently two views of the nature of *Pirhemocyton*:

(A). Dodin & Brygoo in 1960 showed the parasitic nature of this organism, by its enzymatic activity, by experimental infection with the filtrate of the organism, and by demonstrating its specificity to its natural host.

(B). Stebbens and Johnston in 1965, in their work on the ultrastrusture of *P. chamaleonis* maintain that it is... "an icosohedral virus of DNA type"...

The use of the electron microscope shows details of the ultrastructure, showing... "numerous polygonal bodies resembling virus particles"... They describe an area of more luminous density than that of the cytoplasm of the affected erythrocyte, areas of 3.5 micra diameter which they consider to be... "a factory of virus"... They observed two types of vacuoles, of which one would corresponding to the globoids, called "albuminoid body", and the other corresponding to a granular material of little electronic density.

They speak of ..."fibrils of similar dimensions to nucleic acids"... and mantain that as they are Feulgen-positive, they must be DNA.

However, we have demonstrated that the Feulgen reaction is incomplete and unsatisfactory for determining the composition of the paranuclear corpuscles, since it only indicates the presence of DNA, which is scarce in *Pirhemocyton.* For this reason, we have employed the reaction of methyl green pyronin, which differentiates between RNA and DNA. Thus we have demonstrate a higs content of RNA, since the granules in the interior of the paranuclear corpuscles are stained intensely red with pyronine, showing that they contain RNA, while the periphery of the paranuclear corpuscle has a pale green halo, showing the presence of DNA.

The scarce content of DNA explain why many of the Feulgen reactions were doubtful for *Pirhemocyton,* although *Toddia* gave more

positive reactions. In *Pirhemocyton* and *Toddia,* the granules of the paranuclear corpuscles are of RNA and the envelope of DNA (Fig. 6).

Until a short time ago, one could speak of a "mixo-virus" containing both types of nucleic acids, but today it is accepted that there can be only one type of nucleic acid in a virus (Lwoff 1957, Schaffer & Schwerdt 1965). For this reason, the organism which cause the diseases of psittacosis and lymphogranuloma venerea, have been separated from the viruses proper, as these former contain both RNA and DNA. They now form a group of small bacteria, the Bedsoniae (Meyer 1964).

Dodin & Brygoo in 1960, demonstrated the experimental infection by the unfiltrable portion of a suspension of erythrocyte infected with *P. chamaleonis.* Stebbens & Johnston 1965, refuted this experiment, stating that the filter used by Dodin & Brygoo ... "is not in current use because of its tendency to adsorb the virus particles to its surface" ... However, the work od Dodin & Brygoo does not specify the type of filter used. It would be worth while to repeat this experiment with an acceptable filter.

We have repeated, in our species, the reaction of succinic dehydrogenase carried out by Dodin & Brygoo on *Pirhemocyton chamaleonis,* with the object of demonstrating the respiratory enzimatic activity of our organism. We have observed, clearly and precisely, the formation of a granule of formazán attached to the paranuclear corpuscle in *Pirhemocyton* and *Toddia,* indicating a respiratory activity which cannot occur in a virus, according to present criteria. The reactions of succinic dehydrogenase and of cytochrome oxidase are evidence that the organism is a parasite. Although these reactions can only be considered as really effective in the parasites separated from the lysed erythrocytes, since the blood cells themselves are nucleated, and their nuclei show enzimatic activity, as can be seen from the formazán granules that form around them, our observations at the critical moment show the formazán granule of the parasite well separated from the nucleus of the erythrocyte.

Neither should we leave out the possibility that some of the structures are mature and degenerating forms in the nucleated blood cells of vertebrates, as Billet envisaged in 1904, and Bremer 1895 stated that the phenomenon might be interpreted as a degenerative process in the nucleus, leading to the expulsion of a nucleolus surrounded by chromatin material. However, *Toddia* has a single granule of RNA surrounded by DNA, while *Pirhemocyton* shows single, double or triple granules, as has been emphasized by varios authors already cited.

With respect to the globoids, these were described by Chatton and Blanc, for *Pirhemocyton tarentolae,* and were said to be reactions of the parasitized cytoplasm.

Having in mind that the parasite, in this case represented especially by the paranuclear corpuscles, on consuming the hemoglobin of the erythrocytes would stimulate the formation of vacuoles filled with acid phosphatase, we have employed the technique perfected by Gomori in 1950 to detect this enzime and to test whether the globoids contain it.

In this reaction, the globoids remained intact, and no vestige of a precipitation of lead sulfide appeared.

Conclusions: Toddia and *Pirhemocyton* appear as intracytoplasmic corpuscles in the erythrocytes of poikilothermic vertebrates, being comparable to the Piroplasmida of homeothermic animals, and having similarities to *Dactylosoma* and other parasites of cold-blooded animals, such as *Babesiosoma, Tunetella,* etc.

Toddia and *Pirhemocyton* cause alterations in the parasitized erythrocytes, which lead, in the invaded cells, to a process of degeneration marked by the appearance of vacuolization. They cause great alterations in the nucleus of the parasitized erythrocytes, causing herniated degenerations in both cases, and total destruction of the hemoglobin with *Toddia,* which culminates with the formation of the characteristic crystal. The alterations of the parasitized erythrocytes lead also to anisocytosis and changes in the form of the cells.

We have demonstrated that the paranuclear corpuscles of both organisms are composed of one or more granules of RNA, surrounded by an envelope of DNA.

It may be regarded as fixed that both genera have characteristic structures associated with the paranuclear corpuscle, the crystal in *Toddia,* and the globoids in *Pirhemocyton.* The crystal of *Toddia* is insoluble in methanol, and is stained pale pink in pyronine, indicating to us that it is derived from nucleoprotein. The globoids of *Pirhemocyton* dissolve in methanol, and to date we have been unable to determine the nature of their content, as they remain intact under all the cytochemical and biochemical reactions we have employed.

SUMMARY

This paper discusses the relations between the genera *Toddia* and *Pirhemocyton,* describing certain cytochemical reactions that clarify their nature, and discussing the position of these organisms as being of a parasitic or viral nature.

A new species of *Pirhemocyton* is described from *Iguana iguana* from Mamo, Marapa (Dto. Federal) of Venezuela; characterized by rectangular globoids with rounded borders. Attempts at experimental infections of other genera of lizards indicate that the new species, *Pirhemocyton iguanae,* is specific to the natural host, *Iguana iguana.*

The course of the parasitemia in the lizard is described.

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PLATE I

Pirhemocyton iguanae sp. nov.

Fig. 1 — Paranuclear corpuscle with single granule. Characteristic rectangular globoids. Vacuolization.

Fig. 2 — Corpuscle in the plasma, showing two marginal granules.

Fig. 3 — Paranuclear corpuscle with three granules.

Fig. 4 — Paranuclear corpuscle with four granules.

Fig. 5 — Fusiform structure composed of granules in a chain. Stained red with Giemsa.

Fig. 6 — Reaction with methyl-green pyronine. Paranuclear corpuscle with granule stained intense red with pyronine, surrounded by a greenish halo. Globoids colorless.

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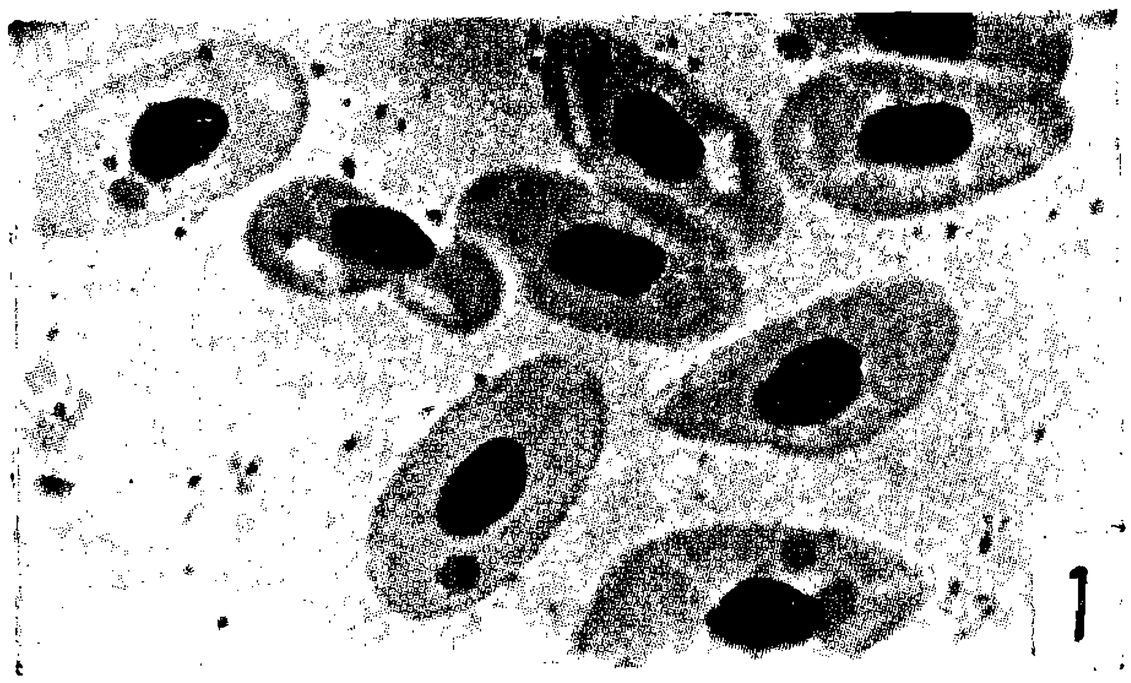
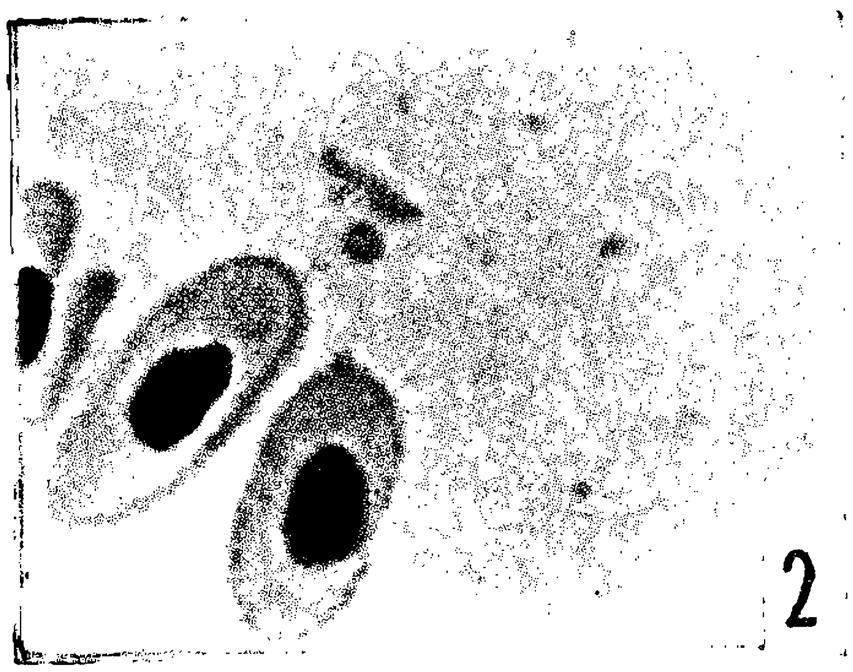
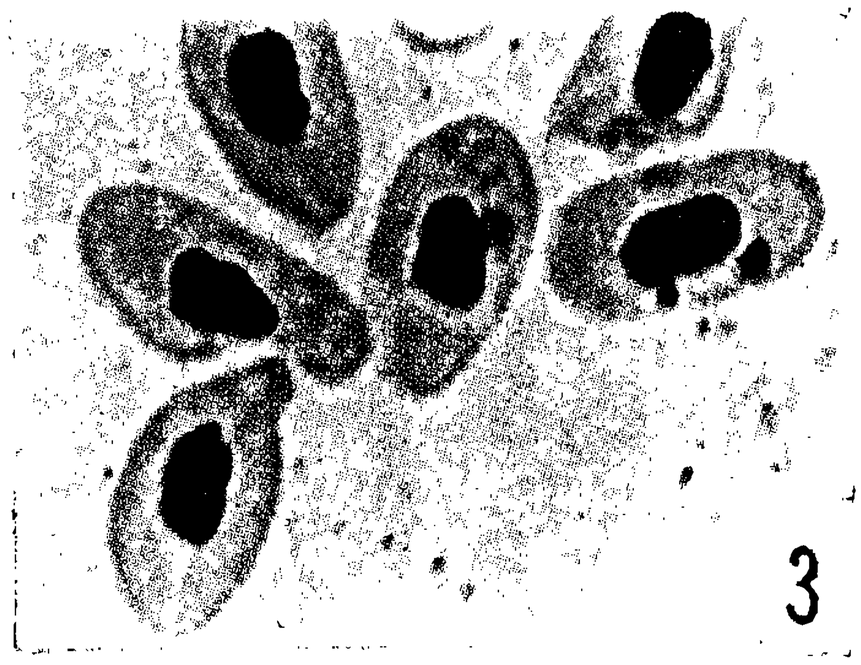
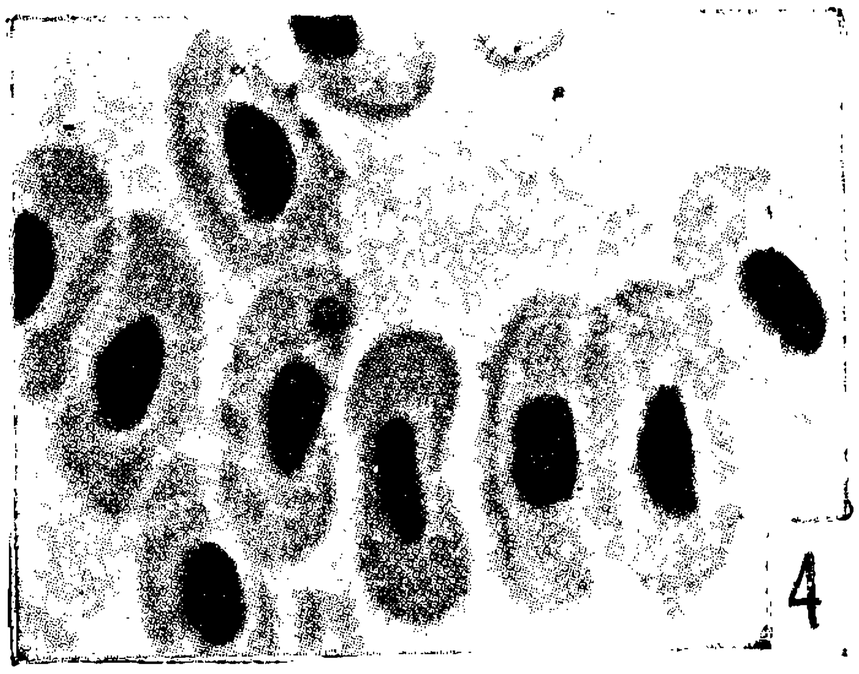
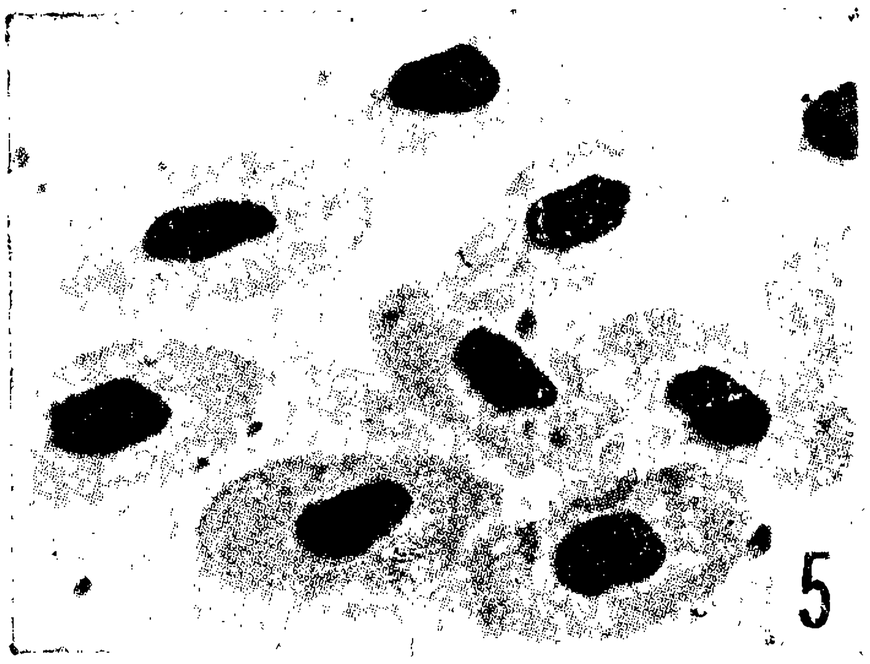
PLATE I

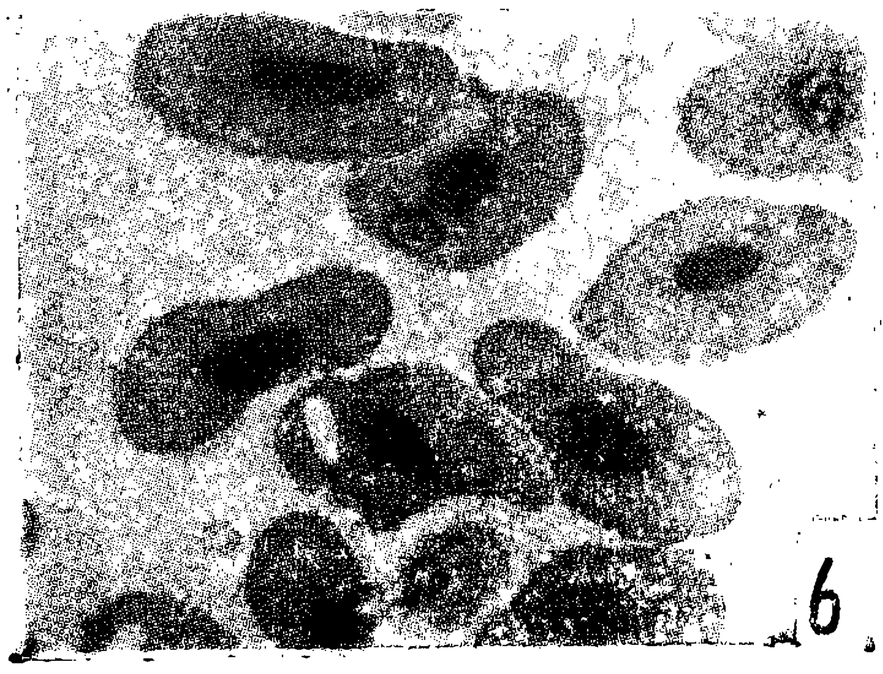












Peraza & Roca: The Paranuclear corpuscles