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***Mémoire.***

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*Mots-clés : Trypanosoma musculi. T. crocidurae. Herpetosoma.* Cycle biologique. Infection.

# LABORATORY AND FIELD STUDIES ON *HERPETOSOMA* TRYPANOSOMES FROM PORTUGAL[1]

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SUMMARY

Several small mammals were trapped in the Arrabida region (Portugal) and checked for the presence of trypanosomes which were found in 33 of the 197 (11.1 %) *Mus spretus* and in 9 of the 29 (31 %) *Crocidura russula* observed. To our knowledge, this was the first time that trypanosomes were isolated from these mammals species. In the liver of one dead *C. russula* was observed different parasite forms. The studies of infectivity to experimental rodents, analyses of the DNA buoyant density and the isoenzymatic profils, show that trypanosomes isolates from *M. spretus* were identical to *Trypanosoma musculi* isolates from *Mus musculus*. However the isolates from *C. russula,* although related to the isolates from murine rodents, were clearely separated from these and close to *Trypanosoma microti.* These findings may allow further studies on the detection of their vectors and on the study of trypanosome reproduction.

RÉSUMÉ : **Études expérimentales de Trypanosomes *(Herpetosoma)* de petits mammifères portugais.**

Dans la région d'Arrabida (Portugal), 197 *Mus spretus* et 29 *Crocidura russula* ont été capturés. 11,1 % des *M. spretus* et 31 % des *C. russula* ont des trypanosomes qui n'avaient pas encore été signalés chez ces hôtes. Les différentes formes du parasite observées dans le foie d'une Crocidure trouvée morte sont décrites. L'infectivité chez des rongeurs expérimentaux, les gradients de densité de l'ADN et les profils enzymatiques montrent que les souches isolées de *M. spretus* sont identiques à *Trypanosoma musculi* isolé de *Mus musculus.* Les souches isolées de *C. russula,* bien qu'apparentées aux précédentes, sont plus proches de *Trypanosoma microti.* Ces données pourraient permettre d'étendre les recherches à la découverte des vecteurs et aux phénomènes de reproduction des trypanosomes.

## INTRODUCTION

Over the past decade studies have been undertaken to elucidate the animal reservoir hosts of *Leishmania* in Portugal. During this period four isolates of *Leishmania* parasites have been isolated from the fox *(Vulpes vulpes)* (Abranches *et al.,* 1983, 1984) and characterized as *L. infantum* (Abranches *et al.,* 1984). In the course of the work, various species of small mammals have been caught and examined for *Leishmania* during which time trypanosomes have been found in two species of small mammals; *Mus spretus* and *Crocidura russula.* Trypanosomes identified as *T. (Herpetosoma) musculi* and *T. (Herpetosoma) crocidurae* have been isolated in culture and studied in the laboratory. The isolate of *T. (H.) musculi* represents the first isolate of a trypanosome from *M. spretus* and provides a basis for comparison of new isolates with the only other available *T. musculi* isolate (Partinico 2) isolated by Krampitz (1969) in Sicily. The isolation of *T. (H.) crocidurae* for the first time provides an opportunity to compare an insectivore trypanosome with rodent trypanosomes of the subgenus *Herpetosoma.* Brumpt (1923) first described *T. crocidurae* from *C. russula* in France and later Rodhain (1930) in Belgium and Krampitz (1961) in Germany recorded the parasite although Rodhain (1930) described *T. crocidurae* from *Sorex aranea* (Syn. *Crocidura aranea*). The method of division of *T. crocidurae* is not known; the parasite resembles a typical *Herpetosoma* trypanosome and Krampitz (1961) considered it was transmitted by the flea *Ctenophthamus agyrtes* which he considered to be the common vector of other small mammal trypanosomes in Germany.

This paper presents results of comparative studies on the morphology, behaviour and biochemical characteristics of Portuguese isolates of *Herpetosoma* from *Mus* and *Crocidura.*

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## MATERIALS AND METHODS

Mammals were live trapped in the Arrabida region near Lisbon which is situated in the south of the Setúbal peninsula. The area is made up of marine deposits of the Tertiary and Quaternary period but dominated by the calcareous massif of the Arrábida. The highest point reaches 510 m above sea level. The area is designated a National Park so habitation and cultivation are limited. The climate is of Mediterranean type with an Atlantic influence (Gaspar, 1979). The vegetation is rich in shrub and pines (*Pinus pinea* and *P. pinaster*) whilst in the southern part of the area there are olive groves on the slopes.

Animals were transported to the laboratory in Lisbon, were they were anaesthetised and heart blood collected for culture into NNN medium. Blood was examined for the presence of trypanosomes by wet smear examination and impression smears made of heart, lungs, spleen, liver and kidneys. The measurements of trypanosome found on *C. russula* were performed according Ranque and coll. (1974) with a micrometric objective at a magnification of × 100. NNN cultures of trypanosomes of *M. spretus* and *C. russula* were sent to the Department of Biological Sciences, Salford University were they were maintained either in Schneider's Drosophila or Grace's medium with additional foetal calf serum of 20 % or on a feeder layer of *Microtus* embryonic fibroblasts (MAEF) or on Baltz's semi defined media (Baltz *et al.*, 1985). Further details of these media and the growth of *Herpetosoma* in them is given by Mohamed and Molyneux (1987) and Mohamed *et al.* (1987). Parasites were compared by a variety of different techniques. Morphological studies were undertaken of 100 parasites of five strains of trypanosome isolated from *M. spretus* and these were compared with the *T. musculi* (Partinico 2) strain initially isolated by Krampitz (1961) and used in immunological studies by many authors (Targett and Viens, 1976). The persistence of parasites in the kidneys of laboratory mice was examined in mice infected with trypanosomes isolated from *M. spretus* and compared with *T. musculi* (Partinico 2) in inbred C3H mice. Outbred mice were used as recipient animals of kidneys of dead mice which had been previously infected with the trypanosomes of *M. spretus*. The infectivity of strains of trypanosomes from Portuguese *M. spretus* and *C. russula* were studied in various strains in mice (C57BI/10, BALB/c and C3H inbred mice), in B.K. rats and *M. agrestis, C. glareolus* and *A. sylvaticus*.

Studies on the DNA buoyant density of trypanosome isolates were undertaken at the Institute of Molteno Institute Cambridge by Dr D. Barker. The technique is described by Newton (1971) and Godfrey (1984) and has been used extensively to compare *Leishmania parasites* (Chance *et al.*, 1974) and trypanosomes (Baker *et al.*, 1978; Gardener and Molyneux, 1988). In addition to *T. musculi* (Partinico 2), three strains of trypanosome isolated from *M. spretus* were examined by DNA buoyant density centrifugation (strains IMT 135, 136,137) and compared with *T. microti, T. grosi, T. evotomys* and an isolate of *T. acomys* from Jordan isolated from *Acomys caherinus* by Pr S. K. Abdel Hafez. The details of the hosts and localities of isolation of *T. microti, T. evotomys* and *T. grosi* are given in Mohamed *et al.* (1986). DNA buoyant density measurements were not undertaken on the trypanosomes of *C. russula*. Isoenzyme studies on three isolates from *C. russula* (IMT 155, 157, 159) and one isolate from *M. spretus* (IMT 137) were undertaken and compared to isolates of *T. microti* and *T. musculi* (Partinico 2) using cellulose acetate (CAE) and polyacrylamide gel electrophoresis (PAGE). The techniques for CAE follow those described by Lanham *et al.* (1981). The polyacrylamide gel electrophoresis was carried out by using the Phast Separation and Development System, native gels of gradient 10-15 % native buffer strips and the 8/0.5 Phast gel Applicator (all Pharmacia) were used. The separation unit was programmed to give runs 120 Avh, 350 V, 10 mA, 2.5 Watt at 15° C. Enzyme visualizations were performed as for CAE. Electrophoresis gels were placed on the agar face down and incubated until bands developed satisfactorily (20 to 50 minutes). Gels were photographed and then preserved in 10 % acetic acid in 5 % glycerol. The following enzymes were used for comparison of the different isolates: MDH, ICD, SOD, PEP 1, PEP 2, PEP D, GPI, PGM and 6 PGD.

Computer analysis of the results was undertaken as described by Ward (1963) and was previously used by Gibson *et al.* (1980) and Godfrey *et al.* (1990) for *T. brucei*.

## RESULTS

266 small mammals consisting of 197 *M. spretus*, 62 *Apodemus sylvaticus*, 5 *Rattus rattus*, 3 *Pitymus lusitanicus* and 29 *C. russula* were captured. Trypanosomes were found in 33 (11.1 %) *M. spretus* and in 9 (31 %) *C. russula* *. The parasites from *M. spretus* were only found as trypomastigotes in the blood and in impression smears of wild caught infected *M. spretus*. The morphological characteristics the parasite was similar to *T. (H.) musculi* (Krampitz, 1969). Parasites were isolated in NNN cultures and later transferred to a variety of media for morphological studies and to harvest the parasites. *Table I* provides a detailed analysis of the morphology of the five strains of trypanosome isolated from *M. spretus* compared with *T. musculi* (Partinico 2) in the peripheral blood of outbred mice taken on the 11th day of infection.

In *C. russula*, the trypomastigote was the most frequent form observed on blood smears. On heart, liver, spleen and kidney impression smears the trypomastigote was also observed usually associated with blood. The morphological characteristics of trypomastigotes were identical to those described by Brumpt (1923) and Hoare (1972) for *T. (H.) crocidurae*. The trypomastigote dimensions on blood slides are given in *Table II*. The degree of parasitaemia observed in blood slides was variable since a few trypomastigotes for microscope field to one in all slide.

### Infectivity to rodents

The trypanosomes isolated from *M. spretus* in culture produced infections in outbred white mice and C57/BL/6, C57/BL/10 and BALB/c inbred mouse strains but only *T. musculi* (Partinico 2) and one isolate from *M. spretus* (IMT 137) infected C3H inbred mice. No infections of *Microtus agrestus, C. glareolus, A. sylvaticus*, or B.K. rats were observed following injections of metacyclic trypanosomes from *M. spretus* isolates from culture. It can be concluded from these studies that the trypanosome from *M. spretus* is *T. musculi*.

* The specific identification of the animals was carried out by Pr Maria da Luz Madureira, Dr Isabel Teixeira and Dr António Paulo Mira from Faculdade de Ciências da Universidade Clássica de Lisboa.

TABLE I. — *Body measurement* of* T. musculi *(Partinico 2), and newly isolated trypanosomes from* Mus spretus *in Portugal (IMT 135, 136, 137, 146 and 148).*

| | DISTANCE BETWEEN** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| STRAIN | P-K | K-MN | P-MN | MN-A | P-A | FF | BW | L | B |
| *T. musculi* | 5.11 | 6.63 | 11.85 | 6.12 | 18.88 | 8.28 | 1.93 | 2.56 | 1.52 |
| | 1.06 | 1.20 | 1.46 | 1.29 | 2.11 | 1.59 | 0.52 | 0.57 | 0.44 |
| Partinico 2 | (3.33-7.77) | (4.44-8.88) | (8.88-15.54) | (4.44-9.99) | (12.21-23.31) | (5.55-11.10) | (1.11-3.33) | (1.33-3.88) | (0.88-2.77) |
| | 6.08 | 6.31 | 12.40 | 7.18 | 19.67 | 7.51 | 1.42 | 2.36 | 1.52 |
| IMT 135 | 1.38 | 0.72 | 1.64 | 1.56 | 2.51 | 1.18 | 0.25 | 0.50 | 0.20 |
| | (3.88-9.99) | (5.55-7.77) | (8.88-17.76) | (3.33-9.99) | (12.21-24.42) | (5.55-11.10) | (1.11-2.22) | (1.33-3.33) | (0.88-1.88) |
| | 5.57 | 6.76 | 12.31 | 7.49 | 19.99 | 7.89 | 1.67 | 2.52 | 1.36 |
| IMT 136 | 0.98 | 0.89 | 1.49 | 1.47 | 2.42 | 1.28 | 0.40 | 0.50 | 0.35 |
| | (2.22-8.32) | (5.55-8.08) | (9.99-16.09) | (4.44-9.99) | (13.87-25.53) | (6.66-11.10) | (1.11-2.22) | (1.65-4.44) | (0.93-1.99) |
| | 5.61 | 6.33 | 11.96 | 7.45 | 19.42 | 7.43 | 1.52 | 2.65 | 1.28 |
| IMT 137 | 0.95 | 0.95 | 1.50 | 1.34 | 2.38 | 1.12 | 0.22 | 0.53 | 0.21 |
| | (3.88-7.77) | (4.44-7.77) | (8.88-15.43) | (5.55-11.10) | (14.43-23.31) | (5.55-9.99) | (1.11-2.22) | (1.65-4.44) | (1.11-1.99) |
| | 5.54 | 7.17 | 12.71 | 7.43 | 20.15 | 9.60 | 1.69 | 2.90 | 1.30 |
| IMT 146 | 0.95 | 0.97 | 1.48 | 1.19 | 1.65 | 1.56 | 0.37 | 0.55 | 0.24 |
| | (3.33-3.66) | (4.44-8.88) | (10.54-15.54) | (5.55-9.43) | (13.33-22.75) | (6.10-13.32) | (1.11-2.44) | (1.65-4.44) | (1.11-1.99) |
| | 5.61 | 7.02 | 12.71 | 7.43 | 20.45 | 9.06 | 1.72 | 2.94 | 1.27 |
| IMT 148 | 1.07 | 0.82 | 1.33 | 1.45 | 2.50 | 1.41 | 0.32 | 0.59 | 0.23 |
| | (3.33-6.66) | (5.55-8.88) | (10.54-15.54) | (5.55-9.99) | (16.65-25.53) | (5.66-11.10) | (1.11-2.77) | (1.65-3.88) | (0.88-1.65) |

* Mean followed by standard deviation and range (in microns).
**P -Posterior end
K -Kinetoplast
MN - Middle of nucleus
A -Anterior end
FF - Free flagellum
BW - Body with across the nucleus without undulating membrane
L - Lenght of nucleus
B - Breadth of nucleus

TABLE II. — *Trypomastigote dimensions of* T. crocidurae *in blood slides.*

| | Range ($\mu$) | Mean ($\mu$) |
|---|---|---|
| Total lenght | 25.2-36.4 | 25.5 |
| Width | 1.4- 2.8 | 2.3 |
| d(N-AE) | 15.4-21.0 | 16.7 |
| Nucleus lenght | 1.5- 5.6 | 3.2 |
| d(K-N) | 5.6- 9.8 | 8.0 |

N - Nucleus; AE - Anterior end; K - kinetoplast

*T. crocidurae* from cultures was inoculated into mice, rats, *C. glareolus* and *A. sylvaticus. M. agrestis* was not available for inoculation at the time when *T. crocidurae* was obtained. No infections were produced in any of the above recipient animals.

*T. musculi*

The course of infection of *T. musculi* in suceptible mice was similar to that of the Partinico 2 strain (Krampitz 1961; Targett and Viens, 1976); trypanosomes were detected between days 4-5 postinfection and parasites were eliminated from the blood 22-26 days postinfection.

No kidney stages were observed in smears prepared from the kidneys of outbred mice after the parasitaemia was lost when outbred mice had been inoculated with *T. musculi* (Partinico 2) or strains IMT 135, 136, 137, 146, 147, 148. Infection however developed in recipient mice inoculated with kidney homogenates from mice killed 240 days postinfection. Trypanosomes were however found in kidney impression smears of C3H mice which had been infected with *T. musculi* (Partinico 2) 240 days previously. Inoculation of kidney homogenates produced a patent infection in recipient inbred (C3H) and outbred mice when donor mice were killed 240 days postinfection 200 days after

patency had disappeared. Strain IMT 147 in C3H mice whose kidney homogenates were inoculated into recipient mice, produced an infection of *T. musculi* but the strains IMT 146 and 148 did not.

No parasitaemias developed in outbred mice challenged with homologous strains IMT 135, 136, 137, 147 and 148 when inoculated into recipient mice which had been first inoculated 240 days earlier.

### *T. crocidurae*

In a single dead *C. russula* typical *(Fig. 1)* and atypical trypomastigote forms were observed in heart and spleen impression smears. An atypical trypomastigote had lost the lanceolate shape and cell was enlarged.

In the liver impression smears amastigotes and sphaeromastigotes of *T. crocidurae* were found. The amastigote forms were round and oval (4.2-5.6 μ). The sphaeromastigotes (8.4 × 4.2 μ) had a long flagellum and no undulanting membrane. Some of these forms appeared to be in division.

The inoculation in NNN medium of blood or spleen homogenate was performed in 9 animals. Four cultures became positive after 8 days.

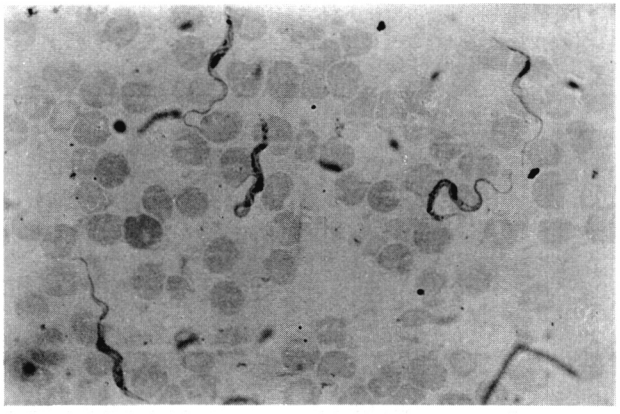

FIG. 1. — Trypomastigote form in blood smear of *C. russula*. Giemsa stained (× 1 000).

## CULTIVATION

### *T. musculi*

The growth and the behaviour of the 9 strains of trypanosomes from *Mus spretus* were similar to *T. musculi* (Partinico 2) in the various media used. All strains grew in both Schneider's Drosophila medium and Grace's medium attaching to the culture flasks (Mohamed and Molyneux, 1987) and produced both attached and floating rosettes with epimastigote and trypomastigote forms in the supernatant. Trypanosomes in the MAEF feeder layers moved between cells and divided in the intracellular spaces. Similary there was rapid growth in the medium of Baltz (Baltz *et al.*, 1985) with trypomastigotes morphologically similar to bloodstream forms in the supernatant 2-3 days after initiation of the culture. Three strains from *C. russula* (IMT 155, 157, 159) also grew well in Schneider's Drosophila medium, Grace's medium and in the medium with the MAEF cell lines.

## DNA BUOYANT DENSITY

The buoyant densities of the nuclear and kinetoplast DNA of *T. microti, T. evotomys, T. grosi, T. acomys, T. musculi* (Partinico 2) and IMT 135, 136 and 137 are summarized in *Table III.*

TABLE III. — *DNA buoyant densities of* T. microti, T. evotomys, T. grosi, T. acomys, *IMT 135, IMT 136, IMT 137 and* T. musculi *(Partinico 2).*

| | 18h | | 20h | |
|---|---|---|---|---|
| Species | kDNA | nDNA | kDNA | nDNA |
| *T. microti* | 1.6952 | 1.7055 | 1.6953 | 1.7054 |
| *T. evotomys* | 1.6951 | 1.7056 | 1.6952 | 1.7054 |
| *T. grosi* | 1.6981 | 1.7060 | 1.6977 | 1.7056 |
| *T. acomys* | 1.6978 | 1.7062 | 1.6975 | 1.7063 |
| IMT 135 | 1.6953 | 1.7044 | ND | ND |
| IMT 136 | 1.6968 | 1.7067 | ND | ND |
| IMT 137 | 1.6986 | 1.7083 | ND | ND |
| *T. musculi* (Partinico2) | 1.6985 | 1.7070 | ND | ND |

ND - Not done

## ISOENZYME RESULTS

All 9 enzymes tested gave a clear resolution for most lysates tested *(Fig. 2)*. The three isolates from *C. russula* (IMT 155, 157, 159) were similar in profiles of 7 of the enzymes (GPI, PGM, SOD, PEP D, ICD, PEP 1, MDH) and they were similar to *T. microti* in the profiles of 5 enzymes (GPI, ICD, PGM, PEP D, MDH). *T. musculi* (Partinico 2) and isolate IMT 137 from *M. spretus* had identical profiles in PGM and PEP D and had one or more common bands in all the other 7 enzymes. The profiles of the 9 enzymes from all isolates were subjected to computer analysis wich divided the parasites into two distinct groups, the first comprising *T. musculi,* IMT 137 from *M. spretus* and *T. acomys* and the second group which was there isolates from the *C. russula* and *T. microti* as shown in the dendrogram *(Fig. 3)*.

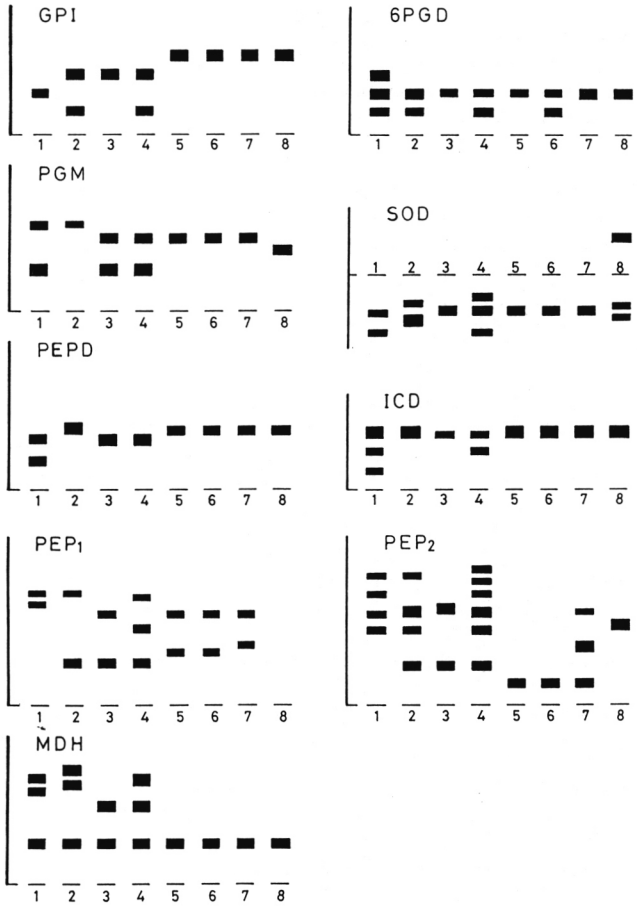


FIG. 2. — Diagrams of the electrophoretic pattern for 9 enzymes of trypanosomes isolated from *C. russula* (IMT 155, 157, 159) and from *M. spretus* (IMT 137). The trypanosomes in all enzymes are: (1) NLB-759 *T. acomys* (Kenya), (2) *T. acomys,* (3) IMT 137, (4) *T. musculi,* (5) *T. microti,* (6) IMT 155, (7) IMT 157, (8) IMT 159.

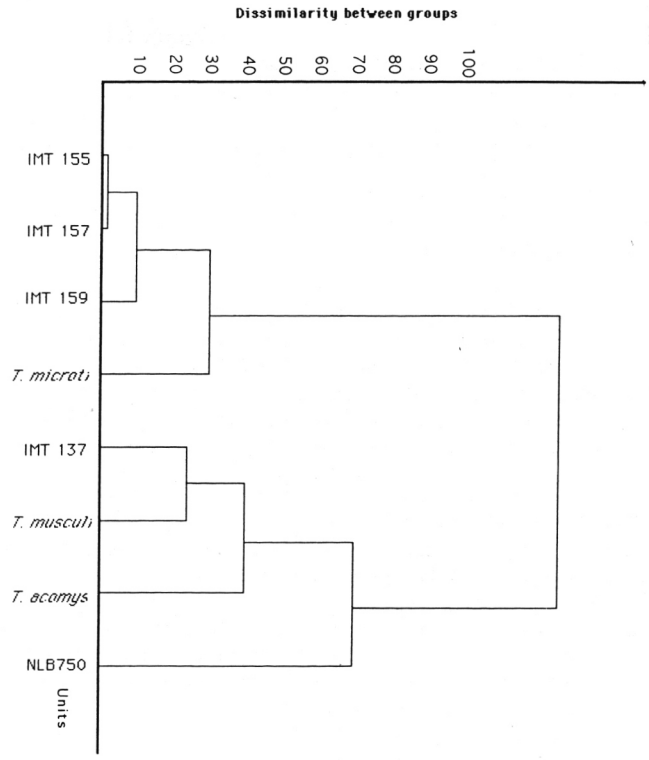


FIG. 3. — Dendogram constructed from the profiles of 9 enzymes (MDH, ICD, SOD, PEP 1, PEP 2, PEP D, GPI, PGM, 6 PGD) of *T. musculi, T. microti, T. acomys,* IMT 155, IMT 157, IMT 159, IMT 137 and NLB7 50.

## DISCUSSION

This paper describes the first isolation of trypanosomes from *Mus spretus* and *Crocidura russula.* 62 *Apodemus sylvaticus* studied no trypanosome was found although this species had been found infected with trypanosomes elsewhere in Europe (Hoare, 1972). In addition *A. sylvaticus* was resistant to the experimental inoculation with *T. musculi* and *T. crocidurae* isolated during this study, confirming the host restriction of these parasites (Hoare, 1972).

With the exception of the forms found in a single *C. russula* trypomastigotes were the only form observed in animals either in blood or liver, spleen, heart and kidney impression smear.

In the liver of one heavily parasitised *C. russula* examined *post mortem* typical trypomastigotes as well as atypical trypomastigotes, amastigotes and sphaeromastigotes were observed. If the reproductive phase is of limited duration (Molyneux, 1976) as with others *Herpetosoma* then the chance of finding tissue forms in the mammals is much reduced. Interpretation of this finding require further work.

The application of isoenzyme and DNA buoyant density techniques have shown that trypanosome isolates from *M. spretus* resembled most closely the earlier isolate from *M. musculus* by Krampitz (1961) from Sicily. Trypanosome isolates from *M. spretus* were also similarly morphologically to *T. musculi* and grew easily in culture. Isoenzyme analyses confirmed the similarity of *Mus* isolates and it can be concluded that the trypanosome from *Mus spretus* is *T. musculi.*

The isolation of *T. crocidurae* from *C. russula* into culture and its discription is an important new finding. This provides a new source of insectivore trypanosomes which because of the difficulties of maintenance of their hosts in the laboratory remain to be studied in the host animals. The isoenzyme results demonstrate *T. crocidurae* isolates were closely related but clearly separable from *Herpetosoma* isolates from murine rodents being closest to *T. microti* from *Microtus* of the parasites studied.

The availability of culture of these parasites will provide reagents for further work. It will be important to attempt to establish the method of reproduction of *T. crocidurae* as well as the vectores of both *T. musculi* of *Mus spretus* and *T. crocidurae.*

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