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SÉRIE DE BIOLOGIE ANIMALE

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SOMMAIRE

PECTINAPSEUDES CAROLINENSIS, new. gen., n. sp. *(TANAIDACEA)* DISCOVERED IN CONTINENTAL SLOPE WATERS OF NORTH AND SOUTH CAROLINA (USA)

MIHAI BĂCESCU [1] and ISABELLE WILLIAMS [2]

A new Tanaid — *Pectinapseudes carolinensis*, new. gen., n. sp. — discovered in the deep waters (800 m) off North and South Carolina (USA) is described in this paper. Ecological and catching data are also provided.

Pectinapseudes gen. new.

Diagnosis. Apseudes-like tanaidacean, with corpus of mandible anteriorly provided with a series of 5—8 strong spines ; optic lobes, spiniform, downwards and frontally curved ; 5 pairs of couplers in maxillipedes.

Pectinapseudes carolinensis new species
Fig. 1. (A—D)

Diagnosis. In addition to the diagnosis of the genus, we add that the basis of cheliped shows 2 spines on the lower edge and the bases of peraeopods III—VII are swollen and have a carpus longer than merus. Abdomen strongly curved downwards from its middle (pleonites IV—V).

Material, origin : 9 specimens originating as follows : 1 ♂ without abdomen from cruise SA 6, St. 11, Rep. 2, 11/22/1985 ; 804 m. 33°04.94′N ; 76°25.17′ W and 4 adults ♂♂, 4 ♀♀ with oostegites rudimentary, partly broken and 1 manca from SA 4, St. 11, Rep. 2 ; 5/22/85 ; 802 m.33°04.92′N 76°24.97′ W. There also were some manca specimens in the replicate stations.

This material was collected in 2 repeated, very close, benthic stations, one off North Carolina and the other off South Carolina.

These stations were made during a 3-year field programme designed to characterize the biological, chemical and sedimentray processes on the slope and rise off Georges Bank, New Jersey and Northland, South Carolina. This new genus and sp. was found in only two of the 44 stations. The samples were collected using a Hessler-Sandia MK III box core (0.25 m²).

Description (♂ ♀) Small-sized Apseudid (6—7 mm ♂, ♀) from Apseudidae Family. Tegument glabrous, pereonites twice wider than high, with short hairs only on the corners. Carapace with slight dorsal folds with a wide rounded rostrum ending in a short spiniform prolongation (Fig. 1 A). The sides of the carapace, strongly excavated, leave the mandible completely visible (Fig. 1 J). The optic lobes practically cannot be seen from above as they abruptly disappear under the basis of antennule, and appear laterally like some long anteriorly curved spines. A strong epistomal spine,

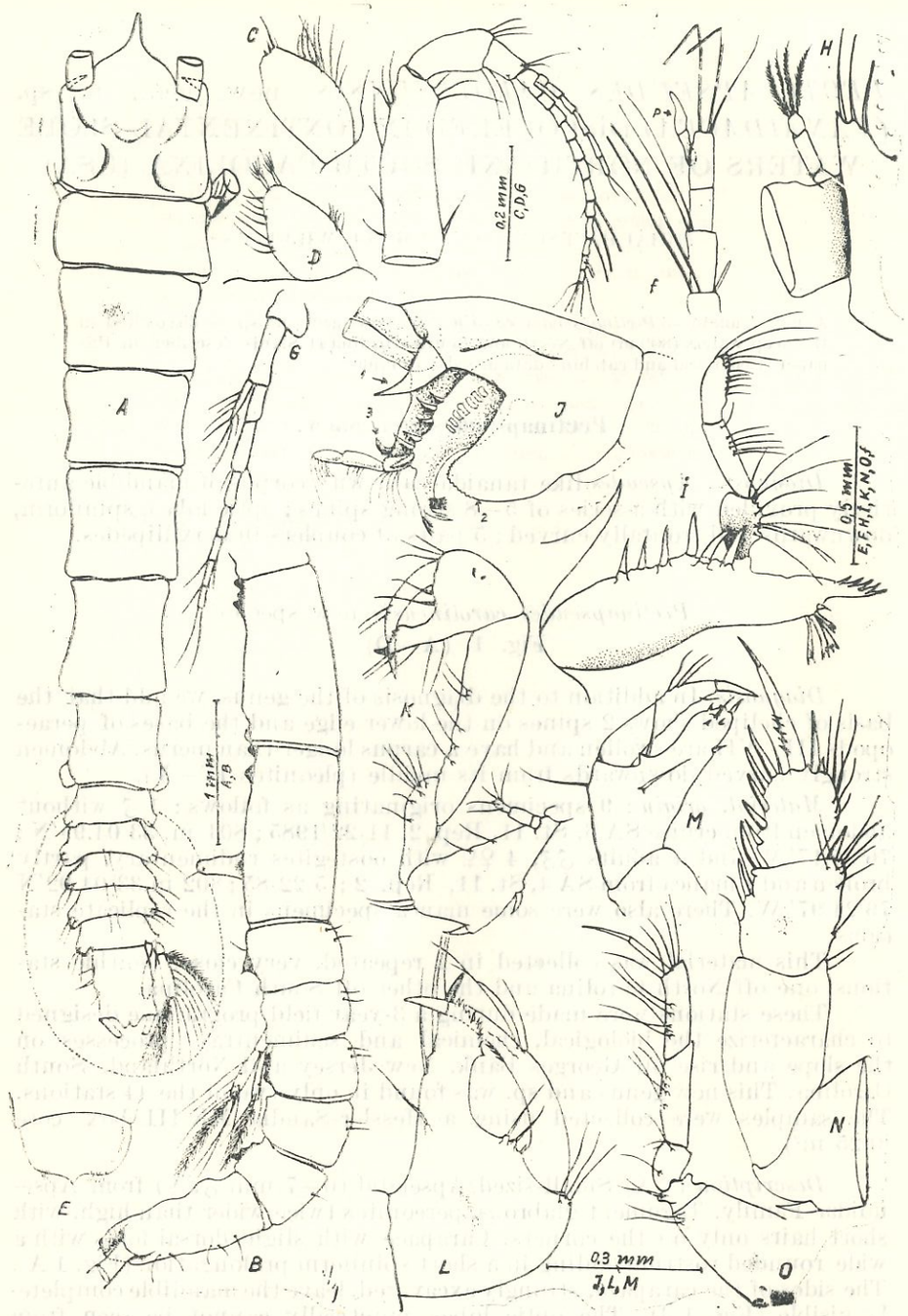

Fig. 1. — A, ♂ = 6.5 mm seen from above (dotted, the area covered by the setae of pleopods); B, ditto, the posterior half in lateral view; C, abnormal endopodal lobe; D, normal endopodal lobe; E, labrum; F, antennule, f, its distal part, magnified, with the last aesthetascs and with a parasite (p); G, antenna; H, exopodite and epimere of peraepod II; J, cephalic side in lateral view; 1 = optic lobe; 3 = epistomal spine; 2 = lower side of cephalotoracic shield; I, mandible with 6 spines, magnified: K, peraepod I; L, its chela, magnified; M, chela of another specimen (anomaly?); N, distal side of peraeopod II; O, pereaeopod V.

curved upwards. Penis, an aplically rounded cone with a posterior hair, gradually narrower to wards the abdomen; strong hyposphenial spines between the peraeopods II and under the pereonites III and IV, also present in females and broad. The buds of oostegites decrease caudally.

Pleon (Fig. 1 B), slightly shorter than the last 3 thoracomeres, provided with short segments, with epimeral prolongation obliquely truncated and showing lots of short setae. Pleotelson only a little longer than broad, with prominent anal lobes and terminal insertion of uropods. Hyposphenia lacking.

Appendages. Labrum with a short median prominence and with two groups of lateral short spinules (Fig. 1 E). The labial lobes may have 1 or 2 apical spines, with or without other short ones between them (Fig. 1 C, D); their palp with 10 setae.

Antennule with the basis without spines, shows 12—13 articles in long flagellum and 5 articles in the short one (Fig. 1 F) (without counting the basal article common to both flagelli). In the ♂, 2 aesthetascs on each of the articles 6, 7, 8, 10, 11 and 12, in the ♀, only one on articles 8, 9 and 11. The last two apical articles narrower than the others.

Antenna (Fig. 1 G) with 7 articles in flagellum and exopodite (scaly) elongate, with 6 setae.

Mandible (Fig. 1 I) shows a unique characteristic among the Tanaidaceans; its corpus is provided with 5—8 strong perpendicular spines anteriorly directed; the number of these spines varies according to the size of the crustacean.

Palp, with 3 articles, provided with 5—7 strong external setae on its basal article; a bunch of 4—5 bifid phanera form *lacinia mobilis* and pars *masticatoria* shows 5 chitinous denticules.

Maxilla and maxillipede common to the family as far as shape is concerned; the latter shows 5 retinacles and its epignath ends in a strong spine.

Cheliped (Fig. 1 K), with exopodite, provided with 2 spines on the curvature of the basis — the anterior one being twice thicker. A short chela with a wide palm; the fixed finger with a thick denticulation (Fig. 1 L). Peraeopod II (Fig. 1 N) twice stronger than chela, provided with only 5 spines on the lower edge of propod (a manca has only 4 spines); with exopodite and a strong spine on ischium, anteriorly a short epimeral ± triangular apophysis, with 3 long setae (Fig. 1 H). The other peraeopods with a very developed basis (Fig. 1 O), get easily detached during handling; in some specimens they were completely missing; dactyloclaws, very fine, as long as propod or longer in Pr 2—5. Marsupial buds decrease in size from peraeopod II to V.

Pleopods (5 pairs) foliaceous in both sexes, with a short basis, with 3 external feathered setae and a strong basal feathered seta.

Exopodite 1/3 shorter than endopodite, but both rami show long feathered setae whose joining forms a broad oval texture around pleon (Fig. 1 A, dotted).

Uropods have a short basis with terminal insertion; flagellum short (exopodite) provided with 5 articles; the long one is absent in all specimens.

REMARKS

As a general appearance, *Pectinapseudes* resembles a small Apseudes (of *A. robustus* type.); a mere view of the large excavation of the lateral shield of the cephalothorax is sufficient for seeing the 6—8 thick spines of the body of the mandible stretching up to the basis of palp. This is a unique type of mandible within the Tanaidacea group. The number of spines varies according to age; from 1—2 in manca stage up to 6—8 in adults.

Other characteristics drawing one's attention as soon as one sees the crustacean laterally are the strong bent of the abdomen (Fig. 1 B) and the curvature of the optic lobes.

Three specimens have a kind of worm-shaped parasite thrust into one of the distal articles of the big flagellum of A_1 (Fig. 1 f).

Some anomalies are also remarked; in a ♂ a fixed finger of the chela appears blunt with tiny spines (Fig. 1 M); in another ♂, a whole cheliped is reduced, in process of regeneration.

The palp of maxilla is not always provided with the group of phanera (3 as a rule), but only with some tiny spinules.

Derivatio nominis. The Crustacean was baptized after the comb of mandibular spines and after the place where it was collected.

Both upper slope stations 11 and 14 are highly diverse with approximately 400 species found at each station (Blake et al. 1987). A small orbiniid polychaete was the dominant species in both stations, counting for less than 10 % of the total fauna. St. 11 had 17 other species of Tanaidaceans; prevailing were a Leptichelid (n = 484) and many species of *Cumella*.

The more diverse St. 14 had 23 species of Tanaidaceans, the most abundant (n = 41) was a sp.n. of *Leptognathiella*, which was the second dominant at St. 11. Our new genus is one of the 7 Tanaidacean species that had only 1 individual present, while at St. 11, it represented the third most common species.

The field programme was performed by personnel from Battelle Ocean Sciences, Woods Hole Oceanographic Institution and Lamont Doherty Geological Observatory. This is Woods Hole Oceanographic Institution's Contribution Number 6693.

Location of material. The type of the species (1 ♂) USNM 236900 and 2 paratypes (♂ and ♀) USNM 236901 are deposited at the Smithsonian Institution, USA; likewise, some unstudied specimens.

The allotype (♀ with marsupial sheets) and 2 paratypes (♂ and ♀) parly dissected, are deposited at the "Grigore Antipa" Natural History Museum of Bucharest, under No 702, and 703, respectively.

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APAUSTIS RUPICOLA DEN. et SCHIFF. *(LEPIDOPTERA NOCTUIDAE)* IN ROMANIA

LÁSZLÓ RÁKOSY* and VASILE CRIȘAN **

The paper presents the second record (after 81 years) of *Apaustis rupicola* Den. et Schiff. in the Romanian fauna. The description of its biotope and distribution is accompanied by a drawing of the male and female genitalia. Considering the morphological characteristics (head, labial palpus, venation and genitalia), the authors place the genus *Apaustis* after the genus *Panemeria* Hb. within the classification of European Noctuidae.

The collecting of lepidoptera from Dobrudja, mostly from its southern part, has revealed over the last 10—15 years the presence of many rare or even new species for the Romanian fauna. Some of them reach here their northern distribution limit.

Continuing researches in this geographical area, one of the authors (V. Crișan) collected in 1984 and 1986 some samples of heliophilous lepidoptera, which proved to belong to *Apaustis rupicola* Den. et Schiff: (Fig. 1). The species was recorded only once in this country (81 years ago) by A. Caradja [3] after the specimens collected by A. L. Montandon at Iglitza near Măcin (Tulcea county).

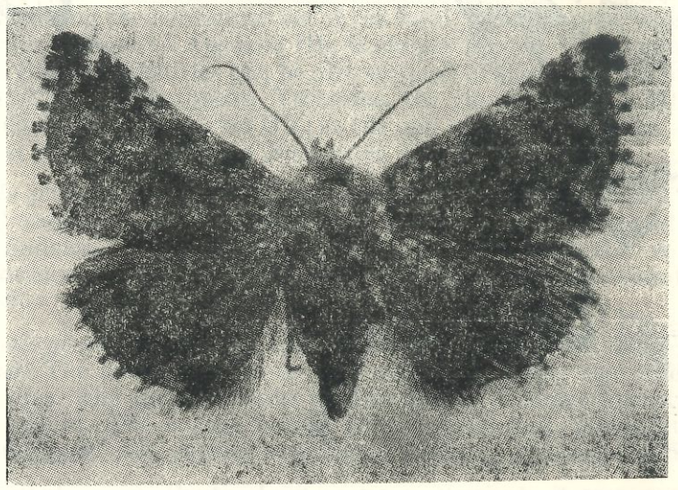

Fig. 1. — *Apaustis rupicola* Den. et Schiff. ♀ Canaraua Fetti 31.V.1984,

Material: 4 ♂♂ Canaraua Fetti 2—5.V.1986.
14 ♀♀ Iortmac Valley (S-Dobrudja) 25.V.1984; Canaraua Fetti 31.V.1984, 2—5.V.1986.
Wingspan: 15—17 mm ♂♂; 17—18 mm ♀♀

The habitat : From a geographical point of view [9], Canaraua Fetii and the Iortmac Valley with Iortmac forest belong to the Danubian subprovince, being characterized by a plain relief, with an altitude varying between 100 and 200 m, and a dry pre-Mediterranean climate. Because of

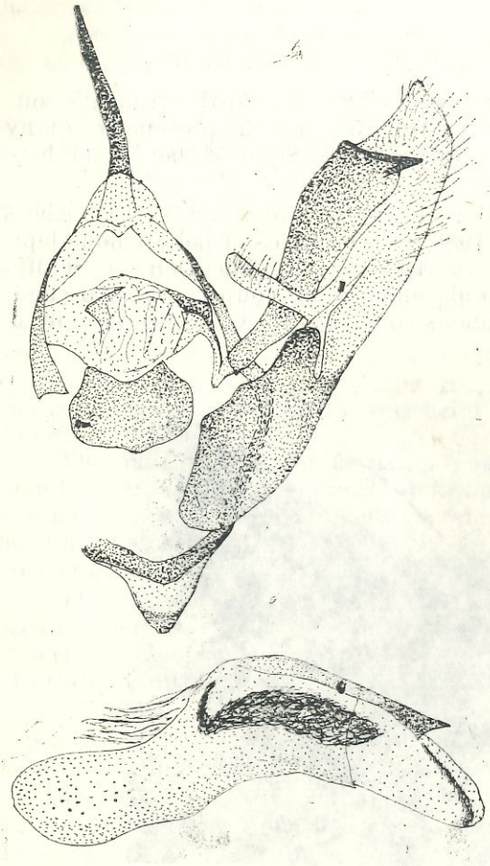

Fig. 2. — Male genitalia of *Apaustis rupicola* Den. et Schiff.

Fig. 3. — Female genitalia of *Apaustis rupicola* Den. et Schiff.

the gritstone and limestone substratum, the waters have cut deep canyon-like valleys. The influence of the Mediterranean climate brought about the formation of microclimates favouring the development of some Mediterranean elements. The vegetation is characteristic for the steppe and

forest steppe. Of the evident steppe species, one can find here *Stipa lessingiana*, *S. capilliata*, *Adonis vernalis*, *Gagea pusilla*, *Euphorbia dobrogensis*, *E. stepossa*, *Poa bulbosa*, *Bromus tectorium*, *Thymus zygioides* [9]. The forest steppe vegetation, housing several species of *Quercus* (*Q. pubescens*, *Q. robur*, *Q. frainetto*), *Carpinus orientalis*, *Fraxinus excelsior*.

Apaustis rupicola Den. et Schiff. was collected on limestone slopes of S and S—V aspect, with a xerothermal vegetation, at the entrance of Canaraua Fetii and the Iortmac Valley.

Biology : The early stages have not been described and the food of the larvae is not yet known.

The imagos are heliophilous and, on sunny days feed on the nectar of different *Thymus* species. All the samples from Dobrudja were collected around noon (till 3 p.m.) on Thymus *Thymus zygioides* Gris.

According to Gozmány [6], the imagos are not attracted either by light or sugaring. They fly single brooded in May-June.

Taxonomy : The genus *Apaustis* Hb., with its two species *A. rupicola* Den. et Schiff. and *A. theophila* Stgr. known in Europe, has a changing taxonomic position, depending on the authors. With Warren [12] it is placed between *Pyrocleptria* Hamps. and *Panemeria* Hb. ; with Hruby [8] between *Aedophron* Led. and *Oxytripia* Stgr. ; Forster and Wohlfahrt [4] places it between *Pyrrhia* Hb. and *Panemeria* Hb. ; Hartig and Heinicke [7] between *Aedophron* Led. and *Janthinea* Gn. ; while with Ganew [5] we find it between *Axylia* Hb. and *Janthinea* Gn.

Considering the configuration of the head, labial palpus, venation and the genitalia, the genus *Apaustis* Hb. is closely related to the genus *Panemeria* Hb. Similar affinities can be also noticed in the behaviour of the imagos. On the List of European *Noctuidae*, the genus *Apaustis* Hb. should have its place after *Panemeria* Hb., at the end of Trifinae, as the subfamily *Heliothinae* is placed between Noctuinae and Hadeninae [2].

Male gentalia (Fig. 2). The valva presents a slightly sclerotised well-developed ampulla, and a strongly sclerotised pointed dorsal process, Uncus cylindrical, elongated. Aedeagus longer than half the valva, with vesica provided with a cluster of very thin cornuti and a sclerotised dorsal process.

Female genitalia (Fig. 3). The papillae anales are well developed and prolongued. The apophyses posteriores are much longer than the apophyses anteriores. The membraneous ductus bursae becomes sclerotised towards the collum. The corpus bursae is well developed, presenting on its median and basal side various ornaments made up of lines and dots.

Distribution : A Ponto-Mediterranean element, it was recorded in Austria [4], Hungary [6], Czechoslovakia [[8], [Bulgaria [1, 5], Jugoslavia [11], S—E S.U., N—W Kurdistan and the Altai Mountains (12).

This report of *Apaustis rupicola* from South Dobrudja confirms once again the presence of this species in Romania, urging Lepidopterists to new researches in the South-East of this country.

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INTRASPECIFIC VARIATION IN *CRICONEMOIDES ANNULATUS* TAYLOR, 1936 *(NEMATODA: CRICONEMATIDAE)* FROM ROMANIA

IULIANA POPOVICI

A population of *Criconemoides annulatus* Taylor, 1936 was recorded from an acid brown soil of a spruce forest in the Apuseni Mountains (Romania). Its identification widens the species distribution as well as the range of intraspecific variation in this species.

Many new data on the taxonomy of *Criconematidae (Nematoda)* and its species distribution have been published in the last two decades. The diversity of taxa, great variations between species belonging to the same genus brought about critical studies on these nematodes. They consisted in taxonomic revisions (1, 2), estimations on the value of some characters used in taxonomy (6) as well as studies on the interrelationships of the genera (5).

The great variability of morphological structures, often not known well enough, renders more difficult the interpretation of the diagnostic characters of some species, especially in the absence of an abundant biological material for the taxonomic study of some structures. The critical study of intraspecific variation of morphological and numerical characters in some *Criconematidae* (3) showed a clear-cut correlation between some characters (spermatheca, annule number, spear length) as well as the constant value of others (i.e. vulva shape).

Among the species of the genus *Criconemoides, C. annulatus* Taylor, 1936 presents specific characteristics. However, the wide range of spear size, an important indicator characteristic of speciation, "leads to the conclusion that *annulatus* is so variable a species" (6).

This species is known in USA and Canada. It has never been found in Europe, except for the island of Spitzbergen (4).

The present paper gives new data on intraspecific variation in *Criconemoides annulatus* and on its geographical distribution. A population belonging to this species was recorded during some ecological researches on the pedofauna of montane ecosystems in Romania.

The material (22 females and several juveniles) was collected from an acid brown soil of a spruce forest situated at 1 100 m altitude in the upper basin of the Someşul Cald river, the Apuseni Mountains (Western Carpathians). The species, distributed in clusters, was found in deeper soil layers (from 10 to 60 cm depth). The study is based on permanent glycerine mounted specimens, fixed in TAF solution.

Figure 1 illustrates some morphological details of *C. annulatus* collected from the Apuseni Mountains.

Measurements (based on adults) are : (n = 22) ; L = 0.69 mm (0.53 — 0.89) ; a = 12.4 (9.7 — 14.8) ; b = 4.2 (3.4 — 5.2) ; c = 32.7 (26.7—49.6) ; vulva = 94.7 % (92.1—96.1) ; spear = 106.5 μm (96—114) ; prorhabdion = 84.5 μm (75—90) ; total body annules = 159 (145—170).

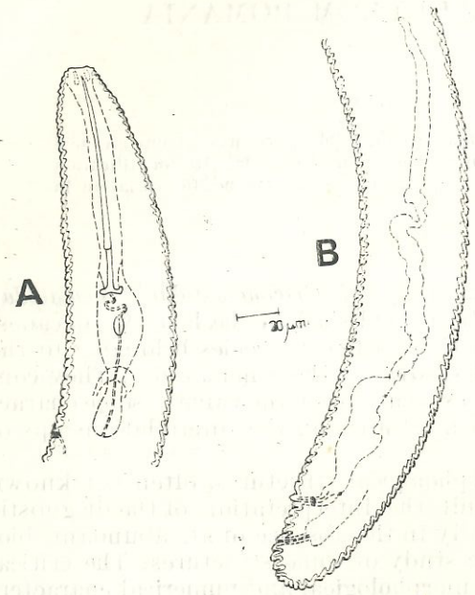

Fig. 1. — *Criconemoides annulatus* Taylor, 1936 A = oesophagus region; B = reproductive system and tail.

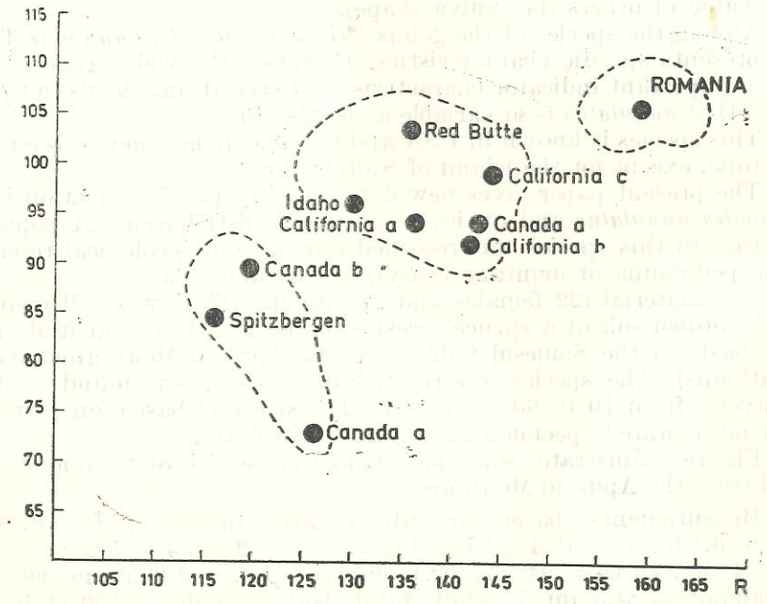

Fig. 2. — *Criconemoides annulatus*, females. Variation in spear length and annule number (R). Each circle represents the mean of a population with localities.

Figure 2 shows distribution of mean values of spear length and annule number comparatively for all populations (ten) known until now. The ranges are given in Table 1.

Table 1

Variations in *Criconemoides annulatus* Taylor, 1396
A= our own data; B = literature data

Population	n	Spear length (µm)	R	Rex	RV
A. *Romania*					
Apuseni Mountains	22	96—114	145—170	40—51	6—9
B. Spitzbergen Island	1	84	116	36	10
(4)					
Canada					
a) Alberta (8)	35	65—81	114—137	34—40	7—12
b) Hazen Lake (8)	6	86—92	113—125	31—38	7—12
c) Alberta, Vancouver	15	84.5—102.5	132—153	39—42	9—11
(8)					
USA					
Red Butte, Montana (6)	3	99—106	133—139	3	10
California a (Placer County) (6)	9	83—108	123—149	34—43	7—10
California b (Pyramid Ranger Station) (6)	1	92	102		
California c (Pyramid Ranger Stn.) (6)	3	97—101	139—149		
Idaho, Kanisko National Park (6)	2	90—96	129—130		

Note: n = specimen number; R = total body annule number; Rex = annule number from the anterior end to the excretory pore; RV = annule number from posterior end to vulva opening.

There are differences among these populations. Mean annule number ranges from 116 (Spitzbergen) to 159 (Romania). Only two populations, Red Butte (USA) and Apuseni Mountains (Romania), have the mean spear length over 100 µm (103 and 106.5 µm, respectively).

The species characteristics for *C. annulatus* are present in all populations: great body length, spear length, head and tail shape.

In the populations from Romania the variation coefficient is quite normal (between 4.6—4.8), both for spear length and for number of annules from the anterior end to the excretory pore (7). Hence, these are good diagnostic characters. On the other hand, the great value (11.6) of the va-

riation coefficient for the position of the vulva (RV) indicates that there are specimens of different ages in this population.

The variation of some characters is given in Table 1 for all the known populations (under A our own observations are listed, under B data taken from literature). Generally, the populations with a small annule number (R = 125 or less) have shorter spear, under 90 μm. The population from Romania shows a greater spear length and a more posterior position of the excretory pore (Rex = 45), as compared to all the other populations.

The identification of *Criconemoides annulatus* from the Apuseni Mountains (Romania) widens the species distribution as well as the range of intraspecific variation in this species.

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A POX VIRUS IN THE SALIVARY GLANDS OF *CHIRONOMUS PLUMOSUS*

GR. MIHĂESCU, L. GAVRILĂ and D. MIȘCALENCU

In the salivary gland cells of *Chironomus plumosus* larvae a virus from the pox group is replicating. Immature viral particles which correspond to the early steps in the pox group viral morphogenesis, are freely disseminated in the cytoplasm. The morphogenesis climax with its tardy stages takes place in the proximity of inclusion dense bodies, in which the virions remain aggregated. These dense bodies from *Chironomus* salivary gland cells, are similar to those resulted through vaccinal virus replication in mammalian cells and are homologous structures to spheroidal crystalline inclusions formed in the fat body cells of insects, after infection with a pox virus. Spindle-shaped inclusions may be a general feature of cytopathic effects of pox viruses infection in insects.

Some viruses from the pox group (Entomopoxviruses) are infective agents for some representative of *Coleoptera*, *Diptera* and *Lepidoptera* orders. In *Melolontha melolontha*, a pox virus is the causative agent of an infeetion which is characterized by the presence of spindle inclusions and spherules in the host cell cytoplasm (2). Pox viruses in Insects are usually infecting and they are replicated in fat body cells. Their replication cycle records the same essential steps and the same cytoplasmic location as pox viruses in Vertebrates. In insects, virions resulted after a replication cycle are included in a crystalline spheroidal proteinaceous matrix (3, 8) and exhibit the fine structure pecularities of pox virus (5,6).

In *Chironomus*, a virus from the iridocytoplasmic group (ICDV) is known, which infects the larval fat body cells (9). Gland localization of viral infection in insects is seldom encountered. The paper signals the replication of pox virus in the salivary glands of *Ch. plumosus*. A knowledge of its infectious cycle is important both theoretically and practically, because it suggests new ways of fighting against deleterious insects.

MATERIALS AND METHODS

Larval salivary glands 4th stadium of *Ch. plumosus* were collected and fixed in 2% glutaraldehyde solution, phosphate buffered, pH = 7.4. After washing, the pieces were postfixed in 2% osmic teteraoxide and included in Epon. The fine sections were contrasted with uranyl acetate and lead cytrate. The examination was done under a Phillips 201 Electron Microscope.

RESULTS

Pox virus, which infects salivary gland cells in *Ch. plumosus* larvae in the replication cycle, goes through intermediary steps, with the formation of immature particles (130 nm in diameter), like pox viruses in ver-

tebrates. In the cytoplasm of infected cells, granular, relatively dense areas appear. In these areas the viral replication takes place. They represent the viroplasm. Inside these areas, incomplete membraneous calots (Fig. 6) gradually enclosing electrondense core material are "de novo" built. In the viroplasm, immature viral particles, with uncondensed core appear (Fig. 3, 4). These mark the ending of the early step in pox virus morphogenesis (7). Then, gradually, condensation of the viral core begins and this marks the beginning of the late steps in viral morphogenesis (Fig. 5). The final steps in viral particle maturation are proceeding in intimate contact with the proteinaceous matrix of dense inclusion bodies. These are disseminated in the whole cytoplasm (Fig. 1), especially in the close vicinity of the nucleus (Fig. 3, 4). The origin of these inclusions must be sought in the precursor materials of the viroplasm, which being in excess, are condensed. Immature viral particles with uncondensed or partially condensed core, are comming in contact with the electrondense inclusion periphery. The contour of inclusion bodies is irregular and exhibits depths in which peripheral virions are located. These depths are not primary because their appearance does not precede immature virus adherence. They are formed as a result of the new materials apposition which includes peripheral virions (Fig. 7, 8). The peripheral virions of inclusion have a morphology characteristic for mature virions (Fig. 8), with all the pox virus envelopes. Viral maturation outside inclusion bodies (Fig. 7) has rarely been encountered.

In the cytoplasm of infected cells, spindle inclusions appear (Fig. 1, 2). They are the result of viral replication but are not directly related to virion morphogenesis. Spindle inclusions are characteristic for "spindle disease", due to the replication of a pox virus in fat body cells in *M. melolontha* (2).

DISCUSSION

The pox virus replication in the larval salivary glands in *Ch. plumosus* is not a surprising phenomenon. Viral tropism for cells with a high metabolic activity is well known. In *Chironomus* at the beginning of larval evolution, mitoses take place ; very frequently and after a while, they are stopped, and endoreduplication processes of genetic material take place. As a consequence, polytenic chromosomes appear, exhibiting a high transcription activity. The salivary gland cells in this stadium exhibit a high metabolic activity.

The steps of pox virus morphogenesis in the salivary glands of *Ch. plumosus* are the same as those of vaccina virus which replicates in mammalian cells. In both situations, the early stages of morphogenesis take place in the viroplasm. In insects the late stages of pox virus morphogenesis are preponderently proceeding in dense inclusion bodies or in crystalline spheroidal inclusion in which the virions remain aggregated (1). In mammalian cells, inclusion bodies are relatively rare and the virions can be released (5, 6).

The final steps of pox virus morphogenesis in the salivary gland cells of *Ch. plumosus* are proceeding at the periphery of inclusion dense bodies.

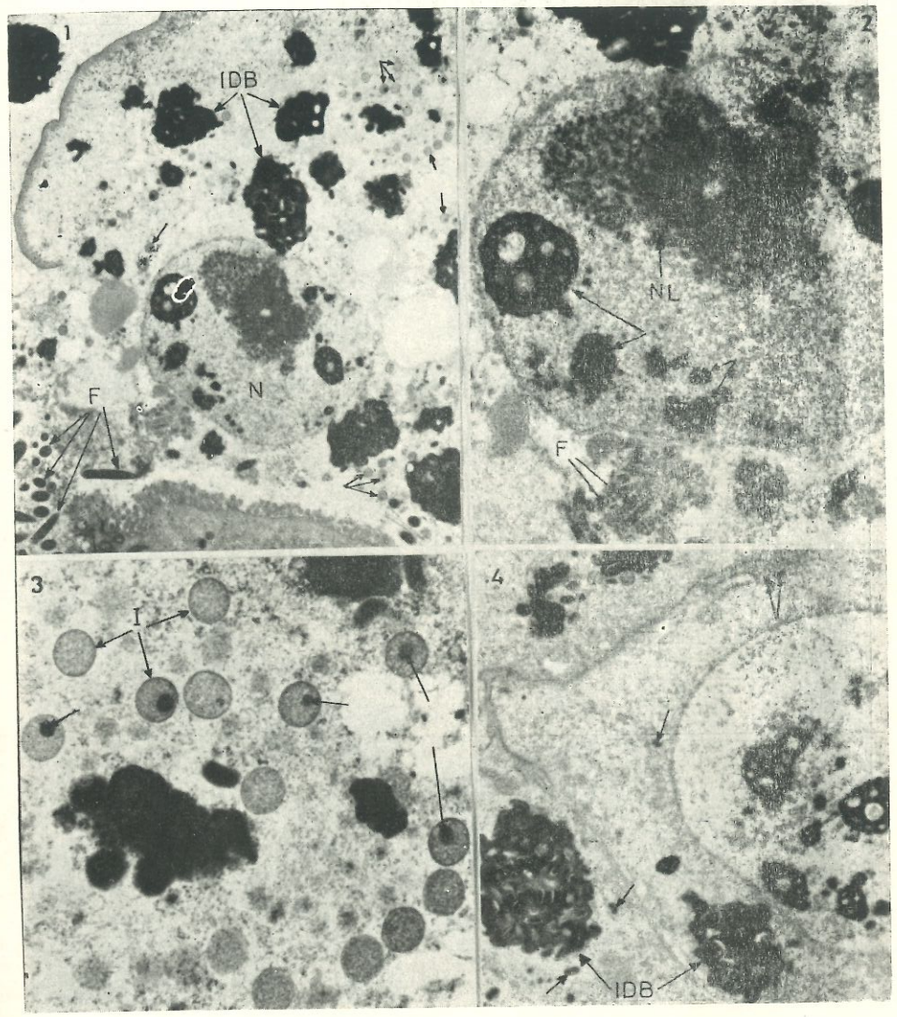

Fig. 1. — General view of an infected cell from the salivary glands of *Ch. plumosus*. Immature viral particles are disseminated in the viroplasm (arrows). Inclusion dense bodies (IDB) in which mature aggregated virions are seen, have a preponderently paranuclear disposition (F = spindle-shaped inclusions ; N = nucleus), × 8 400.

Fig. 2. — A detail of fig. 1. Nucleolus (NL) subunits have segregated. Intranuclear dense structures are nonfunctioning nucleoli (simple arrows). Normal cells exhibit ribosomal gene amplification phenomenon, having multiple extrachromosomal nucleoli, × 18 900.

Fig. 3. — Immature stadium of pox group viruses (I) is represented by ovoid bodies with dense granular content, delimited by a membrane. The formation of virion core, by condensation, begins outside the inclusion dense body (arrows), × 38 700.

Fig. 4. — The final step in morphogenesis takes place usually inside inclusion dense bodies. Rarely the virions are freely assembled in the viroplasm (arrows), × 18 900.

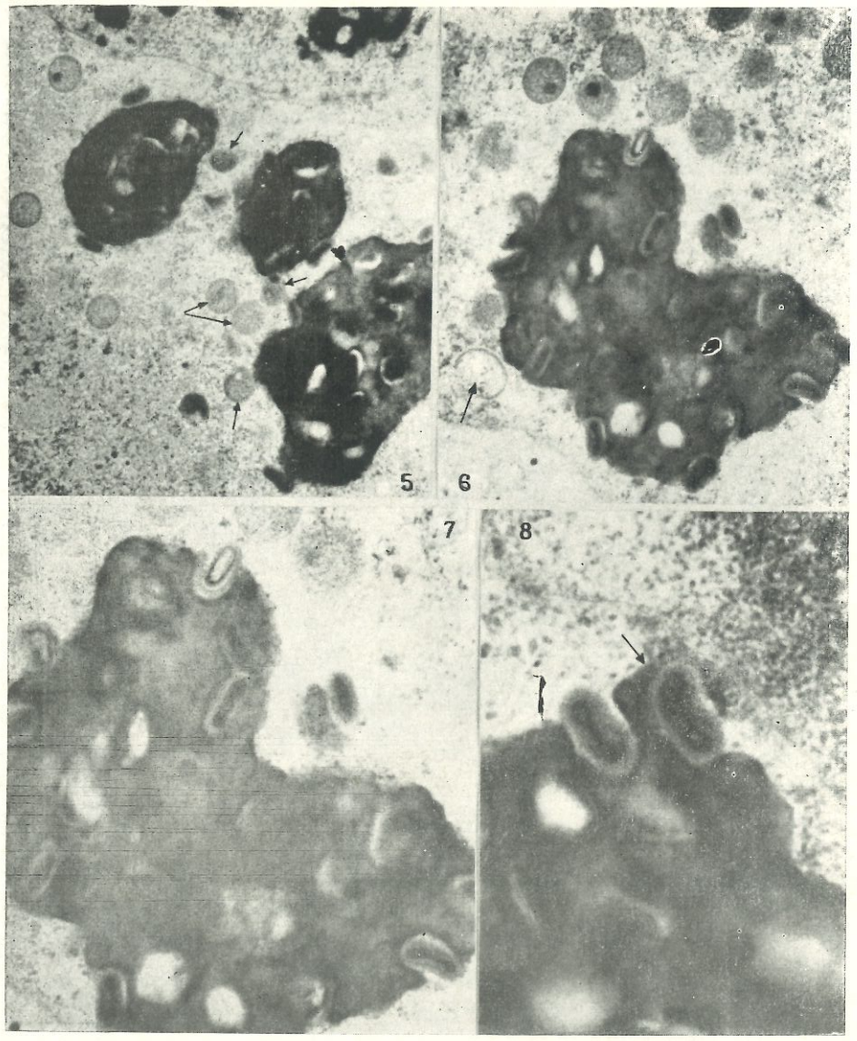

Fig. 5. — Immature forms of pox virus are comming in contact with the periphery of inclusion dense bodies, where the late morphogenesis stages are proceeding (arrows), × 29 400.
Fig. 6, 7. — The arrow indicates an incomplete membraneous calote. The virions are assembled at the periphery of the inclusion dense body, and are gradually incorporated into the inclusion, × 38 700 and 58 800, respectively.
Fig. 8. — A fragment from an inclusion dense body. The virions are located in secondary excavations. The space between virions is occupied by the inclusion material which is directly continued with viroplasm granulations (arrow), × 88 800.

The profound zone of inclusion has a high electrondensity and perhaps does not allow the viral assembly. Our images suggest that simultaneously with the apposition of new viral particles at the periphery, virional degrading processes take place inside the inclusion. This later process is illustrated by the presence of electronoclear spaces (Fig. 6—8).

The pox virus replication in salivary gland cells is accompanied by formation of spindle inclusions. These express the existence of evident similarities of molecular mechanisms of cytopathic effects induction of this virus and of "spindle disease" virus in *M. melolontha*.

The dense inclusion bodies from salivary gland cells and crystalline spheroidal inclusions from fat body cells (1, 7, 8) due to virus replication process, are homologous structures. The inclusions from the first category are similar to the dense bodies encountered in the vaccina virus infected mammalian cells The spheroidal inclusions which are formed in the fat body cells represent not only a particular manifestation of the cytopathic effect, but also, a synthesis of materials for virion morphogenesis.

Obvious similarity between pox virus from *Chironomus* and vaccina virus from mammalian cells, which is suggested by the structural similarity of maturation inclusions, is important for the possible isolation and testing "in vitro" replication of pox virus from *Chironomus*, in mammalian cells. This attempt is justified by the difficulties in insect cells and tissues cultivation.

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AGE-RELATED INSULIN EFFECT ON GLUCOSE UPTAKE BY ISOLATED AORTA OF NORMAL AND HYDROCORTISONE-INJECTED WHITE RATS

IOSIF MADAR, NINA ŞILDAN and ANA ILONCA

The *in vitro* insulin-sensitivity of rat thoracic aorta was evaluated by calculating the insulin-stimulated net glucose uptake. In normal rats an age-dependent polyphasic insulin-sensitivity of aorta wall was established. Against the background of hydrocortisone hemisuccinate injection an obvious reduction of the polyphasic character of aorta insulin-responsiveness was pointed out.

It has been established that under *in vivo* conditions glucose is a major insulin-dependent metabolic substrate of aorta wall (1), (2), (4), (13), and that hormonal status and age of white rats influence the glucose utilization of isolated aorta (3), (14). However, the role of age in the sensitivity of the aorta to insulin is yet unknown. Starting from these considerations, and from the conditioning role of age in rapid antiinsulinic effect of glucocorticoids upon the tissular metabolism of glucose (9), (10), (11), (12), we proposed to study the ontogenetic dynamics of *in vitro* insulin-sensitivity of the aorta of normal, as well as of hydrocortisone-injected rats, by evaluating the insulin-stimulated net glucose uptake.

MATERIALS AND METHODS

Male albino Wistar rats from the stockfarm of our laboratory as donors of thoracic aorta were used, their age being 35, 60, 95, 120 and more than 365 days. The animals were bred under standardized laboratory condition and maintained on a dry Larsen diet, drinking water being provided *ad libitum*.

Hydrocortisone hemisuccinate (U.M.B., Bucharest) was administered intraperitoneally in a single dose of 2.5 mg/100 g b.w., 60 minutes prior to isolation of the aorta.

After fasting for 18 hours, the animals were sacrificed by cervical dislocation and exsanguination. Thoracic aorta was excised and rinsed for 20 minutes in ice-cooled Krebs-Henseleit bicarbonate buffer, without glucose. Each aorta was sectioned in approximately equal hemipieces and incubated for 2 hours at 37.6°C under basal conditions (BAS; without insulin) and in the presence of insulin (INS), by using an original device (8). Incubation medium for each hemiorgan was 0.5 ml Krebs-Henseleit solution (pH = 7.4), containing 16.7 micromoles glucose (p.a. Merck) plus 2 mg gelatine (p.a. Merck) per ml, and 10^{-3} I.U. recrystallized bovine insulin (Organon) per ml, respectively. The gaseous phase of the system was 95% O_2 + 5% CO_2.

The initial and final glucose content of the medium was determined enzymatically, according to the glucose oxidase-peroxidase method of Hugget and Nixon (7).

REV. ROUM. BIOL. – BIOL. ANIM., TOME 33, Nº 1, P. 19–23, BUCAREST, 1988

The basal and global glucose consumption of the aorta in the presence of insulin was expressed in micromoles/100 mg tissue for 2 hours. The sensitivity of the aorta to insulin was estimated by calculating the insulin-stimulated net glucose uptake, i.e. by Δ(INS—BAS).

Statistical calculations were done as usual. The statistic significance of differences was evaluated by Student's *t* test, the limit of significance being accepted at $P = 0.05$.

RESULTS

Insulin *in vitro* significantly stimulated glucose consumption by the aorta as compared to the corresponding basal values (Fig. 1), excepting in more than 365-day-old normal rats and in 35-day-old hydrocortisone-injected ones.

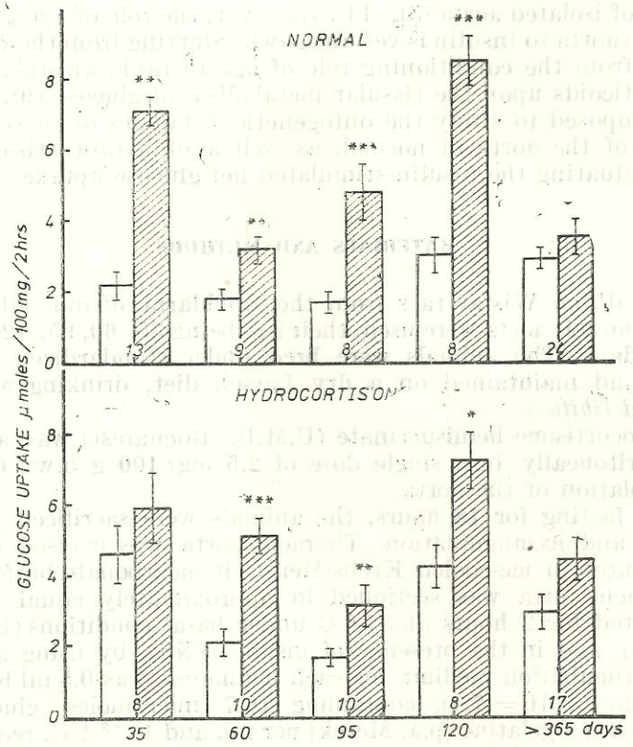

Fig. 1. — *In vitro* glucose uptake by normal and hydrocortisone-injected rats, as depending on the age of individuals. □ = basal glucose uptake; ▨ = global glucose uptake in the presence of insulin. The values represent means ± S.E. The number of experiments is given in the columns. Asterisks show statistically significant differences between basal and global glucose consumption.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

In normal rats the insulin sensitivity of the aorta (Fig. 2) changed in a polyphasic manner during postnatal ontogenesis. In 35-day-old group Δ(INS—BAS) was 4.89 ± 0.67 micromoles glucose uptake per

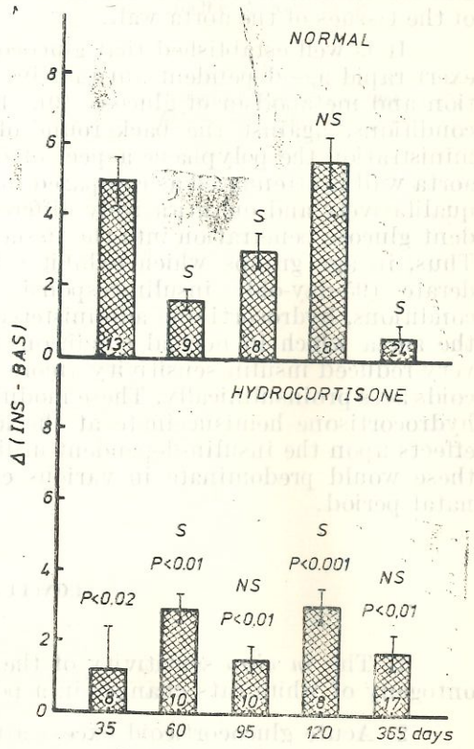

Fig. 2. — The sensitivity to insulin of normal and hydrocortisone-injected rats, as depending on the age of animals. Δ(INS — BAS) = insulin-stimulated net glucose uptake/100 mg tissue per 2 hours. Values are means ± S.E. The number of experiments is indicated in the columns. S and NS show the significant or nonsignificant differences vs. the 35-day-old group. P show the significance of modifications vs. corresponding normal groups.

100 mg tissue/ 2 hours. In comparison with this reference value, the sensitivity to hormone of the aorta isolated from 60- and 95-day-old rats significantly decreased, in 120-day-old animals it remained unchanged, while in more than 365-day-old ones it reached a minimal level (0.06 ± ±0.36 micromoles).

Acute excess of glucocorticoid, induced by intraperitoneal administration of hydrocortisone hemisuccinate, attenuated the polyphasic character of aorta insulin-sensitivity during postnatal ontogenesis. Thus, in the age group of 35, 95 and 120 days Δ(INS—BAS) decreased appreciably as compared to normal, while in those of 60- and more than 365-day-old it increased.

DISCUSSION

Corresponding to the present conception, the *in vivo* or *in vitro* tissular sensitivity to insulin depends on the interaction of insulin with its receptors (5), (6), (15), (16). Our data plead for a polyphasic character of

sensitivity to insulin of the aorta wall during postnatal ontogenesis of white rats. In this context it seems plausible that the age of individuals influences characteristically both the insulin-binding capacity of insulin receptors and the insulin-dependent transmembranal glucose transport at the level of the tissues of the aorta wall.

It is well established that glucocorticoids administered in white rats exert rapid age-dependent antiinsulinic effects upon the cellular penetration and metabolism of glucose (9), (10), (11), (12). In our experimental conditions, against the background of hydrocortisone hemisuccinate administration the polyphasic aspect of *in vitro* sensitivity to insulin of the aorta wall is attenuated as compared to normal, this glucocorticoid having qualitatively and quantitatively different actions upon the insulin-dependent glucose penetration into the tissues of the aorta as depending on age. Thus, in age groups which exhibit a high (35- and 120-day-old) or moderate (95-day-old) insulin-responsiveness of the aorta under normal conditions, hydrocortisone administered acts antiinsulinically, while upon the aorta which in normal conditions shows a reduced (60-day-old) or very reduced insulin sensitivity (more than 365-day-old) the glucocorticoids acts proinsulinically. These modifications suggest the possibility that hydrocortisone hemisuccinate at the level of aortic tissues has multiple effects upon the insulin-dependent utilization of glucose and that some of these would predominate in various critical stages of the studied postnatal period.

CONCLUSIONS

1. The *in vitro* sensitivity of the aorta to insulin during postnatal ontogeny of white rats changes in a polyphasic manner.

2. Acute glucocorticoid excess attenuates the polyphasic character of the aorta insulin-sensitivity, exerting qantitatively and qualitatively different actions, as depending on the age of individuals.

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THE EFFECT OF PROCAINE-BASED DRUGS ON PHOSPHATE-ESTER HYDROLASE AND ATP-ASE ACTIVITIES OF RAT BRAIN AND LIVER

MARIA BORŞA, VICTORIA DOINA SANDU, A. D. ABRAHAM and C. PUICA-DAT

Histological examinations of the brain and liver of Wistar rats treated with Procaine. HCl (0.1 g/kg) and procaine-based drugs (Gerovital H_3 and Aslavital) revealed no obvious changes in comparison with control. Biochemical determinations showed an increase of alkaline para-nitrophenylphosphate-ester hydrolase, while histoenzymatical examinations an enhancement of both acid- and alkaline β-glycerophosphate hydrolases, and Mg^{2+}-ATPase activities, especially at the level of the endothelium of blood vessels in the cerebral cortex and liver. This suggests a stimulatory effect of the drugs on membrane transport processes.

Despite the large number of papers dealing with the various effects of procaine-based drugs on biological membranes, there are only few data concerning their effect on certain cellular metabolic activities. Nevertheless, some enzymes might supply information on the changes occurring at the level of different biological membranes or subcellular organelles (e.g. : lysosomes, mitochondria, a.s.o.). It was suggested that procaine exerts a protective effect on lysosomal membrane (1), (8), (13), (14), and influences membrane transport involving the function of some ATPases (3), (9).

In this paper, data concerning the effect of Procaine, HCl (PRC), Gerovital H_3 (GH_3) and Aslavital (ASL) on some acid- and alkaline phosphatases and Mg^{2+}-ATPase activities in rat brain and liver are presented.

MATERIAL AND METHODS

Adult male albino Wistar rats from our stockfarm (inbred strain), weighing 140 to 160 g, were kept under standard conditions with free access to food and water.

Sixty rats were randomly divided into four groups : the 1st group served as control, the 2nd group received intraperitoneally 100 mg/kg PRC in 0.9 % NaCl solute, the 3rd group received GH_3 and the 4th group ASL containing the same amount of PRC. After 24 hours the rats were killed by decapitation. The brain and liver were quickly removed. The cerebral hemispheres (200 mg) and the liver central lobe (200 mg) were homogenized in cooled 0.14 M KCl and centrifuged at 3 000 RPM and 4°C for 20 minutes. Supernatants were used for the determination of acid and alkaline para-nitrophenyl-phosphate ester hydrolases (E.C.3.1.3.2) (PNPH) according to Bergmeyer (5). Protein content was determined by Lowry's technique (12).

The regional distribution of some enzyme activities was estimated histoenzymatically as follows : the brain and liver were frozen in liquid

nitrogen and 10 μm slices were prepared by sectioning with a SLEE (London) cryotom. In the case of brain, the slices were obtained by transverse sectioning of the cerebral cortex and diencephalon at frontal planes A 5.2 to 5.6 according to Albe-Fessard et al. (2). Acid and alkaline β-glycero-phosphate ester hydrolases (E.C.3.1.3.3) (BGPH) and Mg^{2+}-ATPase (E.C.3.6.1.3) activities were estimated as described in (6), (10).

Enzyme activities were expressed depending on reaction intensities according to Barka et al. (4) (0 = no detectable activity; +2 = slight activity; + 4 = mean activity; +6 = strong activity).

The results were statistically treated according to Chauvenet's test. $\overline{X}$ = arithmetical mean, SE = standard error of the mean; D% = per cent differences versus control, N = number of determinations, t and P values were calculated according to Student's test.

RESULTS AND DISCUSSION

Histological examinations of the brain and liver of Wistar rats treated with PRC, GH_3 and ASL revealed no obvious changes versus control. The acid PNPH activity under the influence of drugs tended to decrease, but the changes were not statistically significant (Table 1). Histoenzymological determinations of the acid BGPH activity showed a heterogeneous distribution in the different regions of the brain. The activity was intensely positive in the plexus choroideus, vascular endothelium of the blood vessels from the cerebral cortex and in the hippocampus, as proved by the occurrence of granular black course precipitants (Table 2). Administration of PRC, GH_3 and ASL caused a marked increase of acid BGPH activity

Table 1

The change of acid PNPH activity in the brain and liver of PRC, GH_3 and ASL treated rats versus control (the results are expressed in $mIU.min^{-1}.mg^{-1}$ proteins)

GROUPS	$\overline{X} \pm SE$	N	D%	t	P
BRAIN					
CONTROL	26.07±3.55	6	100	—	—
PRC	19.46±3.17	6	74.65	1.39	<0.1
GH_3	21.83±3.17	6	83.74	0.91	>0.3
ASL	23.54±2.16	6	90.30	0.61	>0.5
LIVER					
CONTROL	133.3±7.59	6	100	—	—
PRC	135.3±6.12	6	101.50	0.64	>0.5
GH_3	140.4±6.57	6	105.33	0.70	>0.5
ASL	143.2±4.25	6	107.43	1.14	<0.2

in the hippocampus and vascular endothelium of the cortex. Slight enzyme reaction was characterized by the appearence of fine granular precipitants in the cerebral cortex cells, thalamic nuclei, hypothalamus, fascia dentata, amygdala and putamen. No activity in the corpus callosum was detected. Administration of PRC and GH_3 caused slight enhancement of acid BGPH activity in the thalamic nuclei, and that of ASL in the fascia dentata (Table 2). The acid BGPH activity was intensely positive in liver extra-

Table 2

Reaction intensity of acid- and alkaline BGPH, and Mg^{+2}-ATPase in some brain regions of control, PRC-, GH_3- and ASL-treated rats

	Acid BGPH				Alkaline BGPH				Mg^{+2}-ATPase			
	C	PRC	GH_3	ASL	C	PRC	GH_3	ASL	C	PRC	GH_3	ASL
Cortex	+2	+3	+3	+3	+1	+2	+2	+2	+2	+3	+3	+3
Hippocampus	+3	+5	+5	+5	0	0	0	0	+1	+1	+1	+1
Thalamus	+1	+2	+2	+1	+1	+1	+1	+1	+2	+2	+2	+2
Hypothalamus	+1	+1	+1	+1	+1	+1	+1	+1	+2	+2	+2	+2
Fascia dentata	+1	+1	+1	+2	+2	+2	+2	+2	+1	+1	+1	+1
Amygdala	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
Putamen	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1	+1
Plexus chor.	+3	+3	+3	+3	+3	+4	+4	+4	+4	+6	+6	+6
Corpus call.	0	0	0	0	0	0	0	0	0	0	0	0

periportal parenchyma and in the Kupffer cells. Under the influence of the drugs an increase of the enzyme activity, only at the level of the vascular endothelium of the blood vessels, was noticed.

These effects of procaine-based drugs could be correlated rather with their stimulating effect on membrane transport processes at the level of the blood-brain barrier and of the vascular endothelium of liver blood vessels, than with a damaging effect on lysosomal membrane, as neither cell lysis nor membrane ruptures were found.

Both alkaline PNPH and BGPH activities were slightly stimulated by the administration of PRC, GH_3 and ASL. A more significant increase of PNPH activity in the brain of ASL treated rats was noticed (Table 3), while administration of PRC caused the most significant increase at liver level. An enhancement of the alkaline BGPH activity in the plexus choroideus and vascular endothelium of the brain cortex, and that of liver blood vesels, was noticed after the administration of PRC, GH_3 and ASL.

The stimulatory effect of procaine-based drugs on alkaline phosphate ester hydrolase isoenzymes in rat brain and liver, detected especially at the level of the complex biological membranes and barriers of these organs could be the result of an activatory effect on transport channels (1), (14), which is due mainly, but not entirely, to their procaine content. This hypothesis is also underlain by our data concerning the stimulatory effect of PRC, GH_3 and ASL on Mg^{2+}-ATPase activity localized especially in the cell membrane. An enhancement of this enzyme activity was noticed at the level of the plexus choroideus and vascular endothelium of the brain cortex, and that of the liver blood vessels (Table 2). These data are in agreement with the stimulatory effect of some anesthetic drugs on Mg^{2+}-ATPase

Table 3

The change of alkaline PNPH activity in the brain and liver of PRC, GH_3 and ASL treated rats versus control (the results are expressed in $mIU.min^{-1}.mg^{-1}$ proteins)

GROUPS	$\bar{X}\pm SE$	N	D%	t	P
		BRAIN			
CONTROL	40.86±3.56	6	100	—	—
PRC	47.14±4.40	6	115.40	1.10	<0.2
GH_3	45.32±1.29	6	110.92	1.18	<0.2
ASL	51.29±2.62	6	126.99	2.49	<0.05
		LIVER			
CONTROL	23.89±1.67	6	100	—	—
PRC	33.60±2.88	6	140.64	2.92	<0.02
GH_3	27.46±3.92	6	114.94	0.83	>0.4
ASL	24.80±2.39	6	103.81	0.31	>0.7

reported by other authors (14), who explained this effect as a result of the change of membrane fluidity. The stimulation of the alkaline phosphate ester hydrolase activity might be correlated with the effect of procaine-based drugs on the release of the enzyme from its inactive membrane bound form (11).

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EFFECTS OF FEEDING OF *SPIRULINA* TO HENS UPON SOME PARAMETERS OF OVIDUCT PROTEIN METABOLISM

RODICA GIURGEA, C. WITTENBERGER, D. COPREAN and T. BUHĂȚEL

Roso type laying hens were fed during 50 days with 5% or 10% Spirulina biomass in their standard concentrated fodder. The smaller dose elicited decreases of the content and synthesis rate of proteins, mainly in the magnum and isthmus. The larger one generally determined increases in the nucleic acids contents.

Many investigations have been aimed at checking the nutritional values of the alga Spirulina for various animal species (1), (2), (3), (4), (8). Blum et al. (3) showed a dose-dependent influence of Spirulina in hens upon the number of eggs and the colour of their yolk. Based on this observation, we studied the effects of Spirulina administration in the fodder upon the oviductal segment of laying hens.

MATERIAL AND METHODS

Roso type laying hens were divided into three groups of five animals each: *control group* fed with standard, age-fitted, concentrated fodder; *experimental groups*, fed the same fodder, to which 5% (S_5) or 10% (S_{10}) biomass of Spirulina had been added. The treatment lasted 50 days. No attempt was made to estimate the metabolizable energy content of the alga. Life conditions of the hens were similar to those in fowl farms; fodder and water were given *ad libitum*.

The hens were killed by decapitation, after 16 hours of starvation. The oviduct was immediately sampled, its five segments (infundibulum, magnum, isthmus, uterus and vagina) separated and used to determine nucleic acids (RNA and DNA) (7) and protein (5) contents, and the maximal capacity of protein synthesis. For the last determination, tissue slices were incubated during one hour, at 40°C, in a shaker water-bath, in 1 cm^3 heparinized blood plasma obtained from the hens of the same group; glucose was added to the incubating medium, to reach a final concentration of 10 mM; the medium was labelled by adding 2.5×10^6 dpm (disintegrations per minute) (1—^{14}C)leucine (a product of the Institute for Physics and Nuclear Engineering, Bucharest) per vial. After incubation, the slices were rinsed, blotted, homogenized, and the proteins were precipitated with trichloroacetic acid, washed with 80% formic acid, dehydrated with acetone, dried, weighed, and dissolved in 1 N NaOH. Protein radioactivity was determined in a Betaszint BF 5 000/300 liquid scintillation spectrometer (Berthold, F.R. Germany), using Bray's solution as scintillator.

The results were statistically processed by Student's "t" test, after eliminating the aberrant values according to Chauvenet's criterion. The significance limit was considered at $p = 0.05$. Percentage differences of experimental values against those of control group are given.

RESULTS AND DISCUSSIONS

The biochemical modifications in the oviductal segments (Table 1) showed, on the one hand, the importance of the dose, and on the other hand, the marked differences between the reactions of various segments to Spirulina. Most intense modifications were observed on the magnum,

Table 1

Total protein (TP), RNA and DNA contents, and protein synthesis rate (PS) in the oviductal segments (Infundibulum — IN, Magnum — M, Isthmus — IS, Uterus—U, Vagina—V) of 5% or 10% Spirulina biomass fed laying hens. For the control group mean values ± standard errors are given in mg/g for TP, RNA and DNA, and dpm/mg protein/h for PS; percentage differences against control in S_5 and S_{10} groups. Number of individual results = 8, except PS value in IN where n = 3

GROUPS		Control	S_5	S_{10}
IN	TP	64.36±1.95	+25 (p=NS)	− 1.23 (p=NS)
	RNA	1.86±0.06	− 3.77 (p=NS)	+33.33(p<0.001)
	DNA	1.50±0.15	−17.34(p=NS)	− 8 (p=NS)
	PS	737	−14.50(p=NS)	+65.1 (p=NS)
M	TP	136.08±7.59	−31.71(p<0.001)	−28.01(p=NS)
	RNA	2.34±0.06	− 4.71(p=NS)	+48.71(p<0.001)
	DNA	0.74±0.01	+24.32(p=NS)	+36.48(p<0.05)
	PS	1189.00±184	−47.10(p<0.01)	+77.00(p=NS)
IS	TP	113.66±6.77	−25.39(p<0.01)	−43.85(p<0.001)
	RNA	2.32±0.39	+ 6.03 (p=NS)	− 3.45 (p=NS)
	DNA	0.66±0.02	−59.09(p<0.05)	+92.42(p<0.001)
	PS	1659.00±600	−70.40(p<0.001)	−60.30(p=NS)
U	TP	82.00±7.07	+21.03(p=NS)	− 8.49(p=NS)
	RNA	3.21±0.80	− 36.45(p=NS)	−32.04(p=NS)
	DNA	1.37±0.31	−23.36(p=NS)	−32.85(p=NS)
	PS	758.00±154	−48.2 (p<0.01)	−48.20(p=NS)
V	TP	78.22±5.35	+74.44(p<0.001)	+28.18(p=NS)
	RNA	2.55±0.07	−40.40(p<0.001)	−27.85(p<0.001)
	DNA	1.44±0.06	− 3.48 (p=NS)	−11.81(p=NS)
	PS	1289.00±314	−8.30(p=NS)	− 6.5(p=NS)

isthmus and vagina. The uterus showed only a decrease in protein synthesis, and the infundibulum an increase of RNA content in S_{10}. While in S_5 mainly the proteins were affected (their amount decreased in the magnum and isthmus, and increased in the vagina), as well as their synthesis, in S_{10} the nucleic acid content was more affected, this being generally increased.

A correlation of the decrease in protein content and protein synthesis rate was clearly found in the magnum and isthmus, but not in the uterus. In the latter, the protein synthesis was slowed down, but the total protein content was not affected.

If the protein synthesis rate is calculated globally for the whole oviduct, the value obtained is by 40.6% lower in S_5 (665±79 dpm/mg) than in the control group (1120 ± 171), while in S_{10} no significant difference appears (1116±317).

The modifications noticed in the protein metabolism may be accounted for by the high amino-acid content of the alga (8). But the high oligo-elements content of the latter (6) should also be taken into consideration as a factor contributing to determine the rate of egg formation and its composition.

There are data in the literature (2) showing that the efficiency of Spirulina fodder additive may be compared with that of traditional protein sources (fish meal, soybean groat, etc.), but only if a limit of 10% in the fodder is not overpassed. Above this limit Spirulina has negative effects.

In conclusion, Spirulina biomass addition to the fodder of laying hens affects the protein metabolism of their oviduct in a manner which depends on the dose and on the segment of the organ.

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POTENTIATION OF THE ANTITUMORAL THERAPEUTIC EFFECTIVENESS OF SOME CYTOSTATICS BY CHEMICALLY MODIFIED NYSTATIN (NsMC)

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Therapeutic association of Antipholan, Levopholan and Cyclophosphamid — cancerostatic agents for clinical use — with chemically modified nystatin (NsMC) induced a significant increased antitumoral effect on rats bearing solid or ascitic Guérin T-8 tumors. The comparative analysis of the evaluation index values of antitumor activities induced by the combined treatments with those obtained by separate administrations of the drugs allows us to appreciate this intensified response as a potentiation of the antitumor activities of cytostatics by NsMC.
The augmented effectiveness of these preclinical associated therapies imposes the use of NsMC in human antineoplastic plychemotherapy beside other cytostatics in order to potentiate their cancerostatic actions.

Although there is a continuous progress in cancer diagnosis and treatment, the antineoplastic chemotherapy is still characterized by a low efficiency. This imposed extended and intense investigations for the identification of new cancerostatic pharmacological agents as well as the discovery of new ways of action on the malignant process (15).

One way of increasing the efficiency of human anticancerous chemotherapy is provided by identification of some substances which intensify the cancerostatic action of the antitumoral agents for clinical use (5), (8) (9), (15), (16).

In a previous paper (14), we reported the "in vivo" potentiation of the antitumor activity of Girostan IOB in rats bearing various tumoral lines by its therapeutic association with NsMC.

The "in vivo" effectiveness of the association of some cytostatics for clinical use with NsMC on the tumoral development process was investigated in the present study.

MATERIAL AND METHODS

White Wistar female rats, weighing 130 g, bearing Guérin T-8 lymphotropic epithelioma, in solid subcutaneous form and its intraperitoneal ascitic variant (10), were used as experimental animals.

24 hours after the transplant the treatment started and lasted for 16 days in the case of the solid tumor or until the death of the last control animal for the ascitic tumor.

The treatment was applied either alone, by daily intraperitoneal administration of the drugs, NsMC (in a dose of 50 mg/Kg.b.w.), Antipholan (in a dose of 0.075 mg/Kg.b.w.), Levopholan (in a dose of 0.15 mg/Kg.b.w.) and Cyclophosphamid (in a dose of 0.8 mg /Kg.b.w.) or combined, by daily i.p. injection of each cytostatic in association with

NsMC in the same doses. It should be mentioned that in the case of the associated treatments the injected solutions were prepared extemporaneously.

The estimation of the antitumor activity was based on the follow up of the mean tumor weight (MTW) at sacrifice, in the case of solid tumor, or of the mean survival time (MST), in the case of ascitic tumor, in the treated groups comparatively with the controls.

The evaluation of antitumor activity was made by establishment of mean tumor regression (MTR) for solid tumor, by determination of the increase of the MST for ascitic tumor and by calculation of the statistic significance and the T/C value (where T = mean tumor weight or mean survival time for the treated groups and C = mean tumor weight or mean survival time for the control group).

The appreciation of induced antitumoral activity was performed by comparative analysis of the evaluation index values obtained by us with those imposed by the selection criteria of anticancerous substances established by the screening programs of the Institute of Microbiology and Experimental Therapy in G.D.R. (6) and of the Cancer Chemotherapy National Service Center in U.S.A. (7) (a MTR of at least 35% for solid tumors or an increase of the MST of 30—50% for ascitic tumors, in the treated groups compared to the controls, or a T/C value of 0.54 for solid tumors and 1.25 for ascitic tumors).

RESULTS

The results of the experiment testing the antitumoral efficiency of the combined treatments, comparatively with that of the singular treatments, on rats bearing solid Guérin T-8 tumors, are included in Table 1.

Table 1

Antitumor activity of the associated treatments with Antipholan, Levopholan, Cyclophosphamid and NsMC on solid Guérin T-8 tumor. The therapeutical doses are given in *Material and methods*. Figures in brackets indicate the number of animals

Group/ Treatment	Mean tumor weight (g)	% tumor regression	Statistic significance	T/C value
CONTROL	13.5±1.7(13)	—	—	—
NsMC	11.8±1.7(9)	12.6	N.S.	0.87
Antipholan	10.9±2.4(9)	19.3	N.S.	0.80
Antipholan + NsMC	8.1±2.1(8)	40.0	$p<0.05$	0.60
Levopholan	11.1±2.7(9)	17.8	N.S.	0.82
Levopholan + NsMC	13.2±2.5(9)	2.3	N.S.	0.98
Cyclophosphamid	11.6±2.2(9)	14.1	N.S.	0.86
Cyclophosphamid + NsMC	5.8±1.6(9)	57.1	$p<0.001$	0.42

In comparison to the control group, it is observed that NsMC administration induced a nonsignificant MTR of 12.6%, correlated with a T/C value of 0.87.

The daily treatment with Antipholan determined a nonsignificant antitumoral effect, the MTR registered and the T/C value being of 19.3% and 0.80, respectively.

On the contrary, comparatively with the control group and also with those treated only with NsMC or Antipholan, in the case of the animals submitted to the combined therapy with Antipholan and NsMC the MTW showed a significant decrease ($p<0.05$). This reveals a MTR of 40% with a corresponding T/C value of 0.60.

The daily administration of Levopholan induced a nonsignificant antitumoral effect, the MTR and the correlated T/C value being of 17.8% and 0.82, respectively.

On the other hand, in the group treated with Levopholan associated with NsMC, the MTR (2.3%) showed a very slight antitumoral effect comparatively with the control groups (untreated rats, and those injected with NsMC or Levopholan).

Finally, in the case of the daily injections of Cyclophosphamid, a nonsignificant decrease of MTW (14.1%) was observed, as compared to the control group.

On the contrary, comparatively with the control group and also with those treated only with NsMC or Cyclophosphamid, the associated therapy with these two drugs induced a significant ($p<0.01$) antitumoral action. The increased effectiveness of the combined administration of Cyclophosphamid and NsMC is argued by a MTR of 57.1 and a T/C value of 0.42.

The investigation of the antitumor activity induced by the therapeutic association of the cytostatics with NsMC was extended to the Guérin T-8 ascitic tumor line, the registered results being reported in Table 2.

Table 2

Antitumor activity of the associated treatments with Antipholan, Levopholan, Cyclophosphamid and NsMC in ascitic Guérin T-8 tumor. The therapeutical doses are given in *Material and methods*. Figures in brackets indicate the number of animals

Group/ Treatment	MST (days)	%increase MST	Significance	T/C value	% tumor undevelopment
CONTROL	16.8±0.8(12)	—	—	—	—
NsMC	19.6±1.3(10)	16.6	N.S.	1.16	—
Antipholan	36.8±4.7(6)	119.0	$p<0.001$	2.19	40.0
Antipholan + NsMC	42.8±1.3(5)	154.7	$p<0.001$	2.55	50.0
Levopholan	43.4±1.7(10)	158.3	$p<0.001$	2.58	—
Levopholan + NsMC	43.5±2.2(4)	158.9	$p<0.001$	2.59	60.0
Cyclophosphamid	13.3±0.6(10)	−20.8	$p<0.01$	0.79	—
Cyclophosphamid +NsMC	25.7±2.5(10)	53.0	$p<0.01$	1.53	—

Compared to the control group, the daily NsMC treatment induced a slight and nonsignificant antitumoral effect, illustrated by a MST of 12.6 only and a T/C value of 1.16.

It is observed that the daily Antipholan therapy was correlated with a strong antitumor activity, characterized by a significant ($p<0.001$) increase in MST of 119.0 %, by a T/C value of 2.19 and by four cases of tumor undevelopment.

This cancerostatic effect of Antipholan is intensified by its association with NsMC. Thus, the Antipholan + NsMC combined therapy, comparatively with the control group and also with the group treated only with NsMC or Antipholan, induced a MST increase of 154.7 %, correlated with a T/C value of 2.55 and five cases of tumor undevelopment.

Another cytostatic tested under the same experimental conditions was Levopholan. The animals treated only with Levopholan showed a significant ($p<0.001$) increase of MST (158.3 %) with a corresponding T/C value of 2.58. It can be emphasized that the Levopholan + NsMC associated therapy in the rats bearing ascitic tumor induces an augmented antitumoral response. This strong cancerostatic activity was suggested by the similar increase (158.9%) of MST and by a high precentage of tumoral undevelopments (60 %), compared to the animals treated only with Levopholan.

Very interesting results were obtained in testing the antitumoral activity of Cyclophosphamid, administered, either alone or associated with NsMC, to the rats bearing ascitic Guérin T-8 tumor.

The treatment with Cyclophosphamid alone, in doses of 2.5 and 1.6 mg/Kg.b.w./i.p./daily failed to demonstrate its antitumcr activity in this tumor line (unpublished data). The same thing was observed in the present testing which used the cytostatic in a dose of 0.8 mg/Kg b.w. In this case, the treated group was characterized by a significant ($p<0.01$) decrease of MST (20.8 %) in comparison to the control group.

On the contrary, comparatively with the untreated animals and also with those treated only with NsMC or Cyclophosphamid, their therapeutical association induced a significant ($p<0.01$) MST increase of 53 %, correlated with a positive T/C value of 1.53.

DISCUSSIONS

The results of these investigations clearly show that the therapeutic associations of Antipholan, Levopholan and Cyclophosphamid with NsMC induce a significant increase of the antitumoral activity of these cytostatics.

This appreciation is based on the comparative analysis of the evaluation index values of the cancerostatic activities induced by the combined treatments with those obtained by singular administrations of drugs.

Thus, the MTR, MST, and T/C values and the percentage of tumoral undevelopments, registered in the two tumor lines subjected to the associated therapies are better than those corresponding to the separate administrations of the drugs.

The tumor regression induced by the associated treatment with Antipholan and NsMC was 3.2 time higher (with 27.4 %) than that obtained by NsMC administration and twice (with 20.7 %) than that registered after Antipholan therapy. Also, the prolongation of the MST observed after Antipholan+NsMC treatment was 1.3 time higher (with

35.7 %) than that obtained by Antipholan administration. The number of tumor undevelopments registered after combined therapy was greater (by 10 %), too.

The antitumcral activity of the associated therapy with Levopholan and NsMC, registered in rats bearing solid Guérin T-8 tumor, was lower than that induced by the separate administrations of the two drugs. On the contrary, in the case of ascitic tumor line, the associated treatment with Levopholan and NsMC was correlated with an increased antitumoral effectiveness illustrated by a high tumoral undevelopment percentage of 60 %. It is difficult to explain at present the reason of the different antitumoral effect of this combined therapy on the two tumoral systems.

At last, the simultaneous administration of the Cyclophosphamid and NsMC in the rats bearing solid Guérin T-8 tumor induced an increased antitumoral activity, comparatively to the control groups. Thus, the tumor regression registered in this case was 4.5 time higher (by 44.5 %) than that obtained by NsMC injection and 4-time higher (by 43 %) than that registered after Cyclophcsphamid therapy. Very interesting results in testing the antitumoral activity of Cyclophosphamid, administered either alone or associated with NsMC to the rats bearing ascitic Guérin T-8 tumor, were obtained. The treatment with Cyclophosphamid alone failed to demonstrate its antitumor activity in this tumor line. On the contrary, therapeutical association of Cyclophosphamid with NsMC was correlated with a significant cancerostatic effect. Although the administration of the Cyclophosphamid was not compatible with the ascitic animals' life, its association with NsMC conditioned the manifestation of its antitumoral activity in this tumor line, too. This fact suggests a bioprotective effect and/or an increase of the drug bioavailability induced by NsMC.

The increased cancerostatic activity induced by the therapeutic association of cytostatics with NsMC did not represent only the sum of their effects because the intensity of the obtained action was higher than the sum of the antitumoral effects registered after the separate administration of the agents.

Therefore, it can be appreciated that the augmented effectiveness of the combined therapies on the two tumor lines is the result of a potentiation of the antitumoral activity of Antipholan, Levopholan and Cyclophosphamid by NsMC.

The potentiation effect of the antitumoral activities of the cytostatics taken in study is probably due to intracellular concentration increases of these drugs, as a result of the increase in membrane permeability induced by the concomitant specific action of NsMC polyene antibiotic (1), (2), (3), (4), (14).

Implication of the membrane properties modifications in the initiation and development of the malignant process justifies this preferential mode of action of NsMC membranotropic agent especially on the cancerous cells (1), (2), (3), (4), (15).

A bioprotective action, an increase of the cytostatic bioavailability or an activation of the immunological system could be also implicated in the potentiation of antitumoral activities of Antipholan, Levopholan and Cyclophosphamid by NsMC.

The potentiation of the antitumoral activity of some cytostatics used in human antineoplastic chemotherapy by NsMC, probably reflects a similar effect with that described for Amphotericin B, a polyene antibiotic which enhances the cancerostatic effect of some antitumoral agents (8), (15), (16).

The results of the present investigation have important practical implications by the identification of a potentiation agent, NsMC, and also by confirmation of its possible bioprotective effect (11), (12), (13), imposing the use of NsMC in human antineoplastic polychemotherapy beside other cytostatics.

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THE EFFECT OF SOME NEW HYPOLIPEMIC PRODUCTS UPON SERUM TOTAL AND NEUTRAL LIPIDS

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The new biosynthesis antibiotic, A.12.3, administered orally as powder in Chinchilla rabbits subjected to an atherogenic regimen, in doses of 400 u/kg body/ /day, associated with ergosterol (0.5 mg/kg body/day) or with the original product, rutacyl (4.5 mg/kg body/day), possessing vasoactive properties, reduces serum total lipids and triglycerides, which in atherogenic regimen have high values. Generally, this effect is more pronounced than that of the chlofibrate drug (25 mg/kg body/day), evidencing significant hypolipemic properties of such products and thus, the possibility of their application in therapeutics.

Several investigations evidenced the specific interaction of some polyene antibiotics with sterols from cell membranes (9), (11), (20). There has been thus proved that the fungicide effect of polyenes occurring through their interaction with the ergosterol from cell membranes (7), (10). The penetration of these antibiotics in animal cells is conditioned by the presence of cholesterol in the cell membrane (12), (15), being also influenced by the cholesterol/phospholipid ratio in the membrane structure (7) and by the free cholesterol, as well (13).

Starting from these observations, the idea has been advanced on the hypocholesterolemic action of polyenes (16), which has been also proved, for nystatin and A.20.5, by some of our previous investigations (1), (2), (3), (4).

It has also been found that yeast shows positive effects in atherosclerosis (17), while germinated wheat oil administered in the food of animals subjected to an atherogenic regimen reduces the action of cholesterol (8), due to their rich content in ergosterol (5). As a matter of fact, in a previous paper we have evidenced that ergosterol opposes itself to the atherogenic action of cholesterol intensifying the hypolipemic and hypocholesterolemic effect of nystatin (2).

Certain investigations into the original product rutacyl, a diacetylated derivative of the rutosid (rutin) flavonoid, drew the attention upon its vasoactive improving properties and vascular trophicity (14).

All these observations determined us to follow the hypolipemic action of a new biosynthesis antibiotic, A.12.3, associated either with ergosterol or with rutacyl.

MATERIAL AND METHODS

Experiments were performed on Chinchilla rabbits having a body weight about 2.0 kg. They were divided into 4 groups of 10 individuals each, and subjected to a uniform atherogenic regimen, each animal being orally

administered a cholesterol dose of 0.125 g/kg body/day, on the whole duration of the treatment.

In the first stage of the experiment (from T_0 to T_1) all groups were given only cholesterol, without any other treatment.

In the following stages (T_1—T_3), the four groups were treated differently : group I (control) was given no treatment at all ; group II was orally treated with a dose of 400 u/kg body/day powder of A.12.3, associated with 0.5 mg/kg body/day ergosterol ; group III was treated, again orally, with a dose of 400 u/kg body/day A.12.3 associated with a dose of 4.5 mg/kg body/day rutacyl, while group IV was also orally treated with chlofibrate, as reference drug (6), in a dose of 25 mg/kg body/day.

The effect of the atherogenic regimen and of the treatments applied upon serum total lipids (TL) and triglycerides (TGL) was followed by analyses performed at the beginning of the experiments (T_0) after a fortnight of atherogenic regimen (T_1), a fortnight (T_2) and four weeks (T_3) of treatment with hypolipemic agents, all determinations being carried out by spectrophotometric methods (18), (19).

RESULTS

Serum total lipids had in all animal groups normal initial values, ranging between 337.50—472.50 mg/100 ml serum. After a fortnight of atherogenic regimen, serum total lipids increased in all groups, up to values of 450.00—654.50 mg/100 ml serum.

In the following stage, the control group — atherogenic regimen, no treatment — recorded a ceaseless increase in total lipids, reaching in the end of the experiment a value of 174.92 % compared with the initial normal value, and 126.27 % compared with that recorded in the beginning of the treatment in the other groups (Fig. 1). In the groups treated with different products, total lipids registered, nevertheless, a decrease, depending on the treatment applied, the T_3 values being — as compared to the T_1 ones, of 90.02 % in group II, 80.00 % in group III and 98.41 % in group IV.

Serum triglycerides had initial normal values of 94.23—107.48 mg/100 ml serum, the values recorded after a fortnight of atherogenic regimen being of 75.89—117.03 mg/100 ml serum. The final value of triglycerides in the control group increased up to 136.35 %, as compared with the initial one, and up to 176.54 % against the T_1 one (Fig. 2). In the treated groups, the T_3 values compared with those at T_1 were of 77.92 % in group II, 133.29 % in group III and 120.92 % in group IV.

DISCUSSIONS AND CONCLUSIONS

In the case of pathological hyperlipemia, both serum total lipids and lipid compounds, among which triglycerides (neutral fats), constituents of the VLDL fraction (6), are markedly increasing.

Our results show that, in an atherogenic regimen, a significant increase of serum total lipids and triglycerides occurs, which is nevertheless

much reduced by the treatment with the A.12.3 antibiotic, associated with ergosterol or rutacyl. In order to get a more accurate evaluation of the hypolipemic effects of the treatments applied on serum total lipids

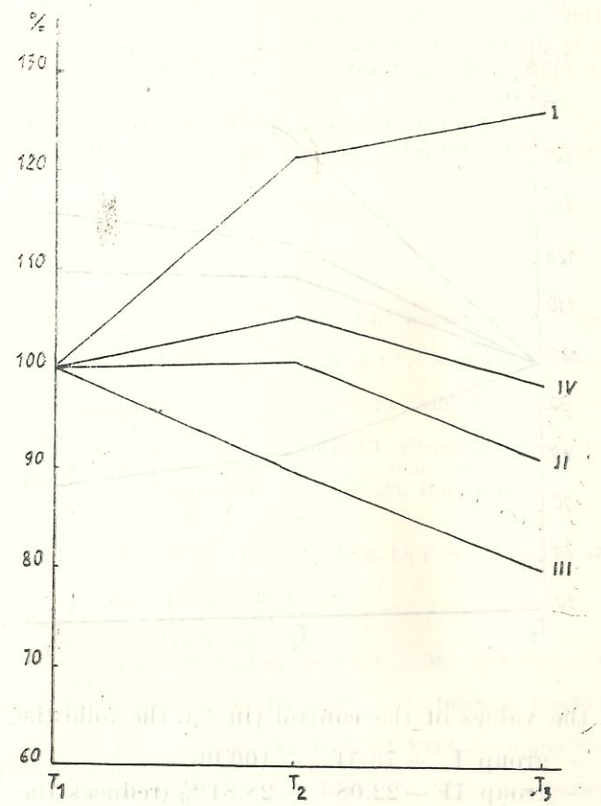

Fig. 1. — Variation of serum total lipids in rabbits subjected to an atherogenic regimen : untreated (I), treated with A.12.3 and ergosterol (II), A. 12.3 and rutacyl (III), or with chlofibrate (IV) — expressed in %, as compared with the value recorded in the beginning of the treatment.
T_1, T_2, T_3 — stages of treatment.

and triglycerides, which have high values in the atherogenic regimen, the final increase of the value of each group was compared with the final increase of the value in the control group, considered as 100 %.

At the end of the treatment (T_3), the variation of serum total lipids as compared with the initial moment of this period (T_1), expressed as %, and the hypolipemic effect (again in %) of the treatments may be expressed in the following form :

— group I + 26.27 + 100.00 %,
— group II — 8.18 — 34.18 % (reduces the TL level with 134.18 %),
— group III — 20.00 — 76.13 % (reduces the TL level with 176.13 %),
— group IV — 1.59 — 6.05 % (reduces the TL level with 106.05 %).

As regards the variation of serum triglycerides, at T_3 as compared with T_1 (in %), and the effect of the applied treatments, compared with

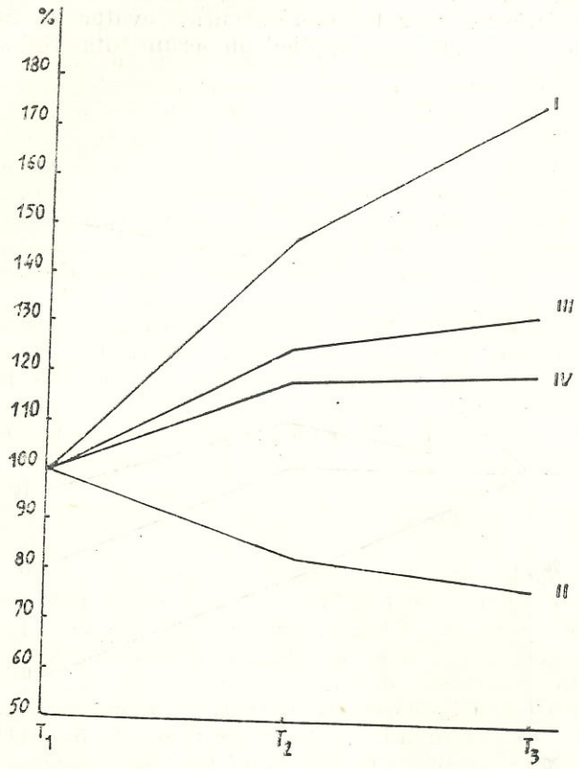

Fig. 2 — Variation of serum triglycerides in rabbits subjected to an atherogenic regimen: untreated (I), treated with A.12.3 and ergosterol (II), A.12.3 and rutacyl (III) or with chlofibrate. See also explanations to Fig. 1.

the values of the control (in %), the following situation is encountered:

— group I + 76.54 + 100.00 %
— group II — 22.08 — 28.84 % (reduces the TGL level with 128.84 %)
— group III + 33.29 + 43.49 % (reduces the TGL level with 56.51 %)
— group IV + 20.92 + 27.33 % (reduces the TGL level with 72.67 %).

Thus, it can be observed that all treatments applied are efficient in reducing the level of serum total lipids and triglycerides, which is high in conditions of atherogenic regimen. The strongest hypolipemic effect is induced by the treatment with A.12.3, associated with rutacyl, which determines the lowering of the level of serum total lipids, almost down to the initial normal value (106.66 %, as against it). The effect of the treatment with A.12.3 associated with ergosterol is also remarkable, leading to the decrease of the level of serum lipids below that recorded in the beginning of the treatment (with 34.18 %, as compared with the control). Both treatments are more effective than the one with chlofibrate, which reduces the level of serum lipids by only 6.05 %, as compared with that at the beginning of the treatment, and with the control.

The effect of the A.12.3 and rutacyl and A.12.3 and ergosterol treatments is 1.66 times and 1.26 times, respectively stronger than that of chlofibrate.

As for serum triglycerides, the strongest effect recorded in reducing their level (below the initial one) is evidenced by the treatment with A.12.3, associated with ergosterol, which is thus 1.77 times stronger than that of chlofibrate. The treatment with A.12.3 and rutacyl determines a significant decrease of the level of serum triglycerides, yet its effect is lower than that of chlofibrate (77.76 % as compared with the latter).

Thus, the product A.12.3, associated with ergosterol or with rutacyl evidences significant hypolipemic properties, that should be further used in therapy.

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THE EFFECT OF NITROGEN LASER ON THE SUPEROXIDE DISMUTASE ACTIVITY IN *E. COLI*

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The superoxide dismutase activity in *E. coli* cultures irradiated with a pulse nitrogen laser (3371 Å) has been investigated. Following the irradiation of the *E. coli* culture the superoxide dismutase activity increased 2.2 fold indicating the presence of the superoxide radicals that trigger the enzyme biosynthesis. Changes of the temperature optimum, pH optimum and the riboflavin concentration at which the enzyme exhibits the highest activity were recorded. Electrophoretic studies showed quantitative and qualitative changes of the superoxide dismutase isoenzymes ; the biosynthesis of a new molecular form and an increased activity of the isoenzyme 1.

Superoxide dismutase (EC 1.15.1.1), a rather recently discovered enzyme (10)is, together with the catalase and the peroxidase, involved in the hydrogen peroxide metabolism :

$$O_2 \xrightarrow[\text{superoxide dismutase}]{+e^-} O_2^- \cdot \xrightarrow[\text{catalase}]{+e^-+2H^+} H_2O_2 \xrightarrow[\text{peroxidase}]{+e^-+H^+} HO^- \xrightarrow{+e^-+H^+} H_2O$$

and catalyses the reaction :

$$O_2^- \cdot + O_2^- \cdot + 2H^+ \rightarrow O_2 + H_2O_2$$

Fridovich et al. (5, 10, 13, 14) characterized the superoxide dismutase of bovine erythrocytes and *E. coli*. The erythrocyte enzyme is identical to the copper proteins — hemocuprein (8) and erythrocuprein (6, 9) isolated without knowledge of their function from the bovine erythrocytes and human erythrocytes, respectively. Various tissues from a large number of mammals have been found to contain a large amount of superoxide dismutase. The mammalian enzyme is blue and contains copper and zinc, whereas the microbial superoxide dismutase is purple-red and contains manganese.

Since the superoxide dismutase is involved in the protection of the cell against the toxic effects of the superoxide radicals one should expect an increase in the enzyme activity when the concentration of the free radicals in the medium is higher. As a result of laser irradation, when $O_2^- \cdot$ radicals are produced, there might be an increased biosynthesis of superoxide dismutase in the cells of the irradiated organisms.

We have used *E. coli* as enzyme source because the superoxide dismutase from this organism is well characterized and the enzyme induction is fast in *E. coli*.

Here we report on the superoxide dismutase activity in *E. coli* cul tures irradiated with nitrogen laser as well as in cultures not subjected to the laser beams.

REV. ROUM. BIOL.—BIOL. ANIM., TOME 33, Nº 1, P. 45—51, BUCAREST, 1988

MATERIALS AND METHODS

E. coli 029 was obtained from the "Dr. I. Cantacuzino" Institute of Microbiology. The cells were harvested by centrifugation at 10, 000 r.p.m. for 20 min, then washed three times with 0.05 M potasium phosphate buffer pH 7.8, 10^{-4} M EDTA. The pellet was taken up in a small volume of the same buffer and sonicated in a Sonomatic 150 apparatus for 15 min. The homogenate was centrifuged at 10,000 r.p.m. for 30 min and the clear supernatant was referred to as a crude protein fraction.

The *E. coli* was irradiated with nitrogen pulse laser at 3371Å, power 30 KW/pulse ; time pulse 10 ns, frequency 15 Hz for 10 min.

Protein concentration was determined according to Lowry et al. (7), using bovine serum albumin as standard.

The assay of superoxide dismutase activity was performed as described by Beauchamp and Fridovich (1), modified by Winterbourn et al. (15). The superoxide radicals generated by the illumination of riboflavin reduce the tetrazolium salt (nitro BT) to formazan. The enzyme activity is assayed by the inhibition of the photoreduction of the tetrazolium salt, the superoxide radicals being decomposed by the superoxide dismutase. The assay mixture contained 0.15 μmoles nitro BT, 6 nmoles riboflavin, 20μmoles EDTA, 0.1 ml enzyme solution (25—44 μg/ml) in a final volume of 3 ml buffered with 0.036 M potassium phosphate buffer pH 7.8. The assay and the control — without the enzyme, were illuminated for 5 min, then the absorbance at 560 nm was read against distilled water. In the presence of light, the riboflavin is oxidized and superoxide radicals are formed. The extent of inhibition produced by the control was considered as 0 % and the percentage inhibition achieved by the sample is calculated by the following equation :

$$\% \text{ Inhibition} = 100 - \frac{A_{560nm\ (sample)}}{A_{560nm\ (control)}} \times 100$$

One superoxide dismutase unit is defined as the amount of enzyme that causes a 50 % inhibition in the reduction of the tetrazolium salt. Superoxide dismutase activity is expressed in units/mg protein.

The superoxide dismutase isoenzymes were separated by polyacrylamide gel electrophoresis in 7.8 % acrylamide gels, pH 8.9 using the Tris-glycine buffer pH 8.3 in the electrode vessels. 500μg protein were applied per gel and the electrophoresis was carried out at 2 mA/gel for 5—6 hours (3). After electrophoresis the protein bands were detected by staining the gels with 1 % Amido Black 10 B in acetic acid : methanol : water (1 :5 :5) for 20 min and destained with 7 % acetic acid solution according to Pastewka et al. (11).

The superoxide dismutase isoenzymes were detected by incubating the gels in a solution containing 0.075 mM Nitro BT, 5 mM EDTA, 10 μM riboflavin, 0.036 M potassium phosphate buffer pH 7.8 for 20 min in the dark followed by illumination with UV light for 5—10 min. The enzyme bands appear colourless against a dark-blue background.

RESULTS AND DISCUSSION

Figure 1 shows the superoxide dismutase activity in crude extracts from control *E. coli* cultures and cultures irradiated with nitrogen laser — as a function of the protein concentration from the reaction mixture. In

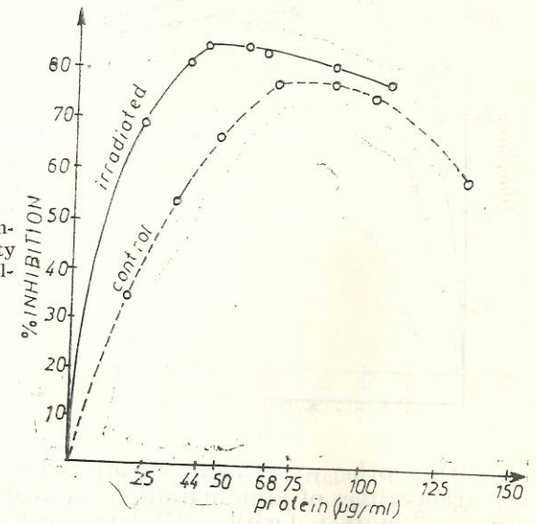

Fig. 1. — The effect of protein concentration on superoxide dismutase activity in the control and laser irradiated cultures.

the irradiated cultures the enzyme activity increases linearly with the protein concentration in the range 0—44 μg/ml, whereas in the control cultures the linear range is 0—68 μg/ml. The specific activity of the superoxide dismutase was 41.2 units/mg/min. for the control culture and 90.9 units/ /mg/min. for the irradiated culture. Following the laser irradiation there was an increase in the superoxide dismutase activity by 2.2 fold. The laser irradiation causes the appearance in the cells of $O_2^{-}\cdot$ radicals that may induce an increased biosynthesis of the enzyme or its activation. The protein concentration in the control cultures was 3.4 mg/ml, whereas in the irradiated culture it was 2.2 mg/ml. In the irradiated culture one could even observe with the naked eye some protein coagulation demonstrating the degrading effect of the laser radiation.

Riboflavin may be looked upon as a true substrate for this enzyme since it generates superoxide radicals and between the riboflavin concentration in the reaction mixture and the amount of $O_2^{-}\cdot$ there is a direct relationship. Figure 2 shows the saturation curves of the superoxide dismutase preparations from the two *E. coli* cultures. The enzyme kinetics follows the Michaelis-Menten pattern ; in the control culture the enzyme has a V_{max} at 3.6 nmoles riboflavin/assay mixture, whereas in the irradiated culture it exhibits a V_{max} at 1.8 nmoles riboflavin. In the irradiated cultures the enzyme is saturated at a lower riboflavin concentration and this may be due to some conformational changes in the enzyme molecule that has a higher affinity for its substrate. The increased affinity of the enzyme for the substrate may also be due to an increased amount of $O_2^{-}\cdot$ as

a result of the laser irradiation and these radiacals add up to those produced by the riboflavin. In this way, the actual substrate concentration in the reaction mixture is higher.

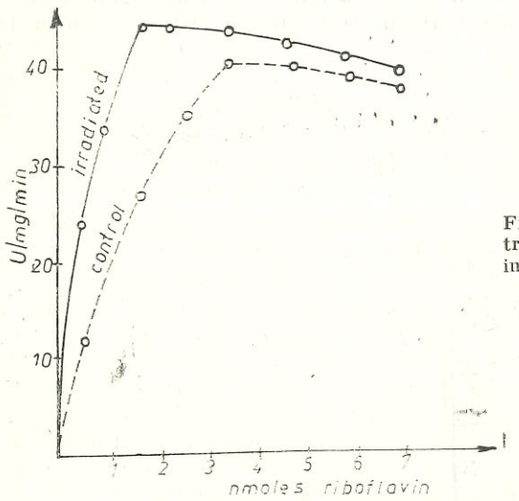

Fig. 2. — The effect of riboflavin concentration on superoxide dismutase activity in the control and laser irradiated cultures

In another experiment we tested the superoxide dismutase activity at various times of illumination of the reaction mixture. The assay mixture was illuminated with an UV lamp for 1—6 min (Fig. 3). The enzyme

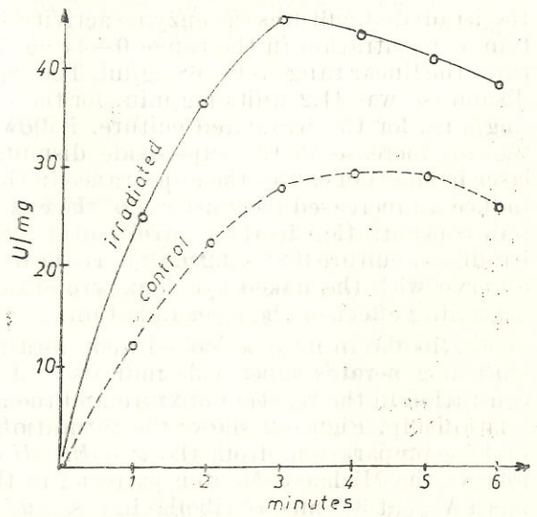

Fig. 3. — The effect of the illumination time on the superoxide dismutase activity in laser irradiated and control *E. coli* cultures.

activity in the control culture increases linearly in the first two minutes of illumination, the highest reaction rate being reached after 5 min. In the laser irradiated culture the highest reaction rate is reached after 3 min of UV illumination. These findings strongly suggest that the laser irradiation yields superoxide radicals inside the bacterial cell.

The effect of the temperature on the superoxide dismutase activity has been investigated in another experiment. This type of experiment could give some information on the presence of multiple molecular forms,

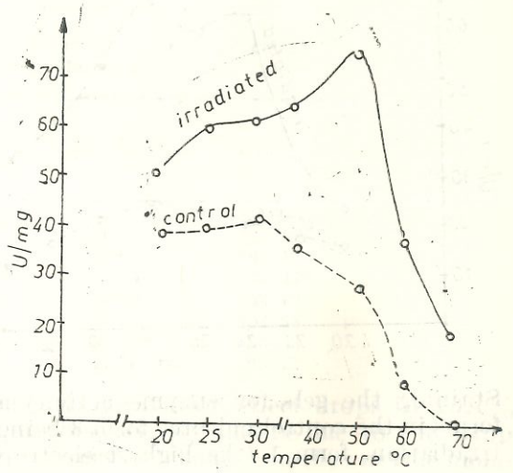

Fig. 4. — The effect of temperature on the superoxide dismutase activity.

the activation energy and enzyme thermal stability. The reaction mixture was incubated for 5 min at various temperatures. As seen in Fig. 4, in the control culture the highest enzyme activity was recorded at 30°C, after which inhibition took place : 28.7 % at 50°C, 83.2 % at 60°C and 100 % at 70°C. The superoxide dismutase from the irradiated *E. coli* culture exhibits a different behaviour. Between 20° and 30°C the activity of the control enzyme increases by 3.4 %, whereas in the irradiated culture the increase was by 20.85 %. It is likely that following the laser irradiation some conformational changes might occur in the enzyme molecule leading to increased activity or the superoxide radicals resulted from irradiation may protect the enzyme against thermal degradation. The maximum temperature for the irradiated culture was found to be 50°C.

The investigation of the effect of pH on the enzyme activity could give information on the changes in the enzyme structure and function. This experiment was realized with Tris-HCl 0.2 M buffer solutions. The superoxide dismutase from the control culture exhibits the highest activity at pH 7.8, whereas the pH-activity curve from the irradiated culture shows two maxima, at pH 7.4 and pH 7.8 (Fig. 5). This finding may be explained by some changes occurring on the catalytic site of the enzyme or/ and the biosynthesis of a new molecular form of the enzyme as a result of increasing the superoxide radicals concentration in the medium.

To see the causes of these kinetic changes, a comparative study of the multiple molecular forms of the enzyme from *E. coli* cultures — control and irradiated, separated by disk electrophoresis in polyacrylamide gel, was carried out (Fig. 6). By developing the total proteins with Amido

Black, the protein pattern from the irradiated culture showed a smaller number of bands than the control one (10 as opposed to 12). This is in agreement with the lower protein concentration in the irradiated culture.

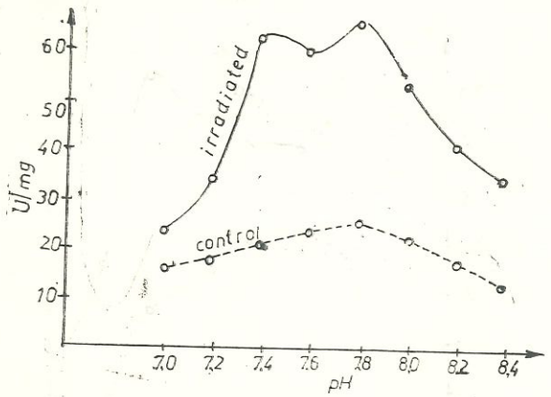

Fig. 5. — The effect of pH on the superoxide dismutase activity.

Staining the gels for enzyme activity showed three multiple molecular forms in the control culture, form 3 being the most active. Following laser irradiation, form 1 (the highest electrophoretic mobility) becomes more

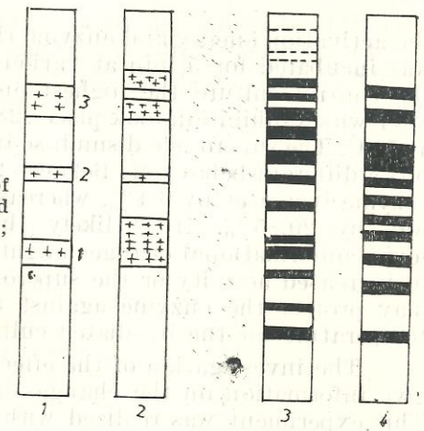

Fig. 6. — Polyacrylamide gel electrophoresis of proteins from nitrogen laser irradiated (2,4) and control (1, 3) *E. coli* cultures (1,2 activity staining; 3, 4 -protein staining).

active, the activity of form 2 decreases markedly and in the cathode area two enzyme bands are detected which were labelled 3_a and 3_b. These results indicate that the laser irradiation induces the biosynthesis of a new molecular form of superoxide dismutase, positively charged and an increased amount of form 1. It is likely that the laser irradiation may induce only conformational changes in form 1 enzyme, leading to increased activity.

There is still very little information on the effect of laser on enzyme activity and when such studies were made it was suggested that the observed changes were variable (2, 4, 12). In order to get some insight into

this phenomenon time course experiments were performed on the some *E. coli* culture (Table 1). The activatory effect of the laser irradiation is rapidly apparent and is rather constant in the first 2 hours from the

Table 1

Superoxide dismutase activity in *E. coli* cultures after nitrogen laser irradiation

Time elapsed since the irradiation moment	Inhibition (%)	U/ml
20 min	71.43	142.86
30 min	72.09	144.19
45 min	73.64	147.27
60 min	72.09	144.19
120 min	77.27	154.54
24 h	66.67	133.33
48 h	52.56	105.12

irradiation moment. After 2 hours the activation effect drops by 26.4% in 48 hours. This decrease in the enzyme activity may be due to the drop in the $O_2^{-\cdot}$ concentration or to the reversible changes in the enzyme molecule induced by the laser irradiation.

Taking into account the above, all superoxide dismutase assays from irradiated cultures were carried out on the day of irradiation.

In order to distinguish between the effects of the laser beams on the superoxide dismutase as well as on the intracellular milieu as a whole, studies on the effects of the laser irradiation on the purified enzyme are now in progress in this laboratory.

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AN ELECTRON MICROSCOPIC STUDY OF THE CHROMAFFIN AND INTERRENAL CELLS OF THE EMBRYOS OF *XIPHOPHORUS HELLERI* *(POECILIIDAE, TELEOSTEI)*

D. SCRIPCARIU, LOTUS MEȘTER, A. POPESCU and R. MEȘTER

Electron microscopic study of the head kidney embryos of *Xiphophorus helleri* has shown that the adrenal gland is composed of interrenal and chromaffin cells closely associated with the renal blood vessels. Interrenal cells contain abundant rough endoplasmic reticulum, mitochondria and Golgi complex and are intimately associated with the chromaffin cells. Two types of chromaffin cells were identified, based on the predominance and density of the chromaffin secretion. In both types of cells there are numerous mitochondria which are surrounded by rough endoplasmic reticulum and the secretion product is not aggregate in granules. It seems that these embryonic cells belong to a younger developmental stage with increased biosynthetic activity.

The results obtained by light and electron microscopy on the adrenal medulla of vertebrates have shown that adrenaline and noradrenaline cells present distinct differences in granulation. Two types of chromaffin cells were identified. Adrenaline cells contain small granules, have slightly electron-dense cytoplasm and were named "light cells". Cells having dense and larger granules were designated as "dark cells" and were appreciated as noradrenaline-storing granules. These two types of cells have been demonstrated in all classes of vertebrates, including mammalians (1), (2), (3), (8), (9), (11), (19).

In fish, the adrenal gland is composed of islets of cells lying on the walls of veins in the head kidney. Histological and histochemical studies have shown that the adrenal gland in Teleostei is formed by interrenal cells comparable with the adrenal cortical cells of higher vertebrates, and chromaffin cells, which are homologous with the mammalian cells of the adrenal medulla gland (4), (5), (7), (12), (16) (18), (21), (23). These cells had most of the histochemical reactions characteristic of the mammalian adrenal gland.

Ultrastructural studies concerning the interrenal cells and chromaffin cells of teleosts are very few (14), (15), (20), (22). Yoakim and Grizzle (22) have shown that the interrenal and chromaffin cells of the fathead minnow are intermingled in the head kidneys and are closely associated with the cardinal veins. They identified one interrenal cell type and two types of chromaffin cells with differences in cytoplasmic density and in kinds of granules. However, to our knowledge, ultrastructural studies on the adrenal gland in fish embryos are completely missing.

The aim of the present work is to elucidate the ultrastructure of the interrenal and chromaffin cells in fish embryos and to provide evidence concerning the function of the larval adrenal gland.

REV. ROUM. BIOL.—BIOL. ANIM., TOME 33, Nº1, P. 53—57, BUCAREST, 1988

MATERIAL AND METHODS

In our experiments we used specimens of the viviparous fish *Xiphophorus helleri*. The female fish were anaesthetized and from pre-hatching embryos the head kidneys were dissected under microscope.

For electron microscopic study, small pieces of about 1 mm from the head kidney were fixed in 2 % glutaraldehyde in cacodylate buffer 50 mM pH 7.4, containing 3 % formaldehyde and 6 % succrose, for two hours. After fixation, the pieces were washed several times in cacodylate buffer 50 mM, pH 7.4 with succrose 6 %. Subsequently, the pieces were treated with a methenamine silver solution, according to Lillie (13) and De Martino and Zamboni (10), with some modifications. The pieces were exposed to methenamine silver solution for 3 hours at room temperature in the dark. After incubation, the pieces were washed several times with Trismaleate buffer 50 mM pH 7.6, and were postfixed with 1 % osmium tetroxide, for 3 hours. Thereafter, the pieces were dehydrated and embedded in Epon. Ultrathin sections were cut on a LKB ultratome. The sections were stained with uranyl acetate followed by lead citrate and were examined under a Phillips EM 201 electron microscope.

RESULTS

The adrenal tissue of *Xiphophorus helleri* embryos was localized in the lymphoid head kidney and was closely associated with renal blood vessels. The interrenal and chromaffin cells were disposed in the form of small groups of islets arround the blood vessels. The cells are intermingled with one another and are in close contact (Pl.1, A, B).

The interrenal cells had a rounded nucleus and slightly dense chromatin. The cytoplasm of interrenal cells contained a large quantity of rough endoplasmic reticulum, evidentiated either as tubules or vesicles of varying size (Pl.1, B and Pl. 2, A). The mitochondria of the interrenal cells were less numerous than in the chromaffin cells and revealed a marked size variation. The mitochondrial matrix had a moderate electron opacity and contained few osmiophilic granules (Pl. 2, A). In the Golgi complex, numerous vesicles of varying size are seen, filled with electron dense material (Pl. 1, B). In general, lipid droplets are missing in the interrenal cells. However, few lipid droplets were identified in the cytoplasm or linked to the rough endoplasmic reticulum. The cell membrane of the interrenal cells appears relatively smooth in the zone in close apposition with the chromaffin cells (Pl.2, A arrow head). In the cytoplasm, some lysosome-like structures were identified (Pl. 1, B).

Two types of chromaffin cells were identified in the head kidney of fish embryos. Cells having a slightly electron-dense cytoplasm were named light cells (adrenaline cells), and the cells with a high density of secretory granules were designated as dark cells (noradrenaline cells). In both types of chromaffin cells, the secretory granules are scattered throughout the cytoplasm (Pl.1, A and B, Pl. 2, A and B). The secretory granules are variable in size and appeared without a limiting membrane.

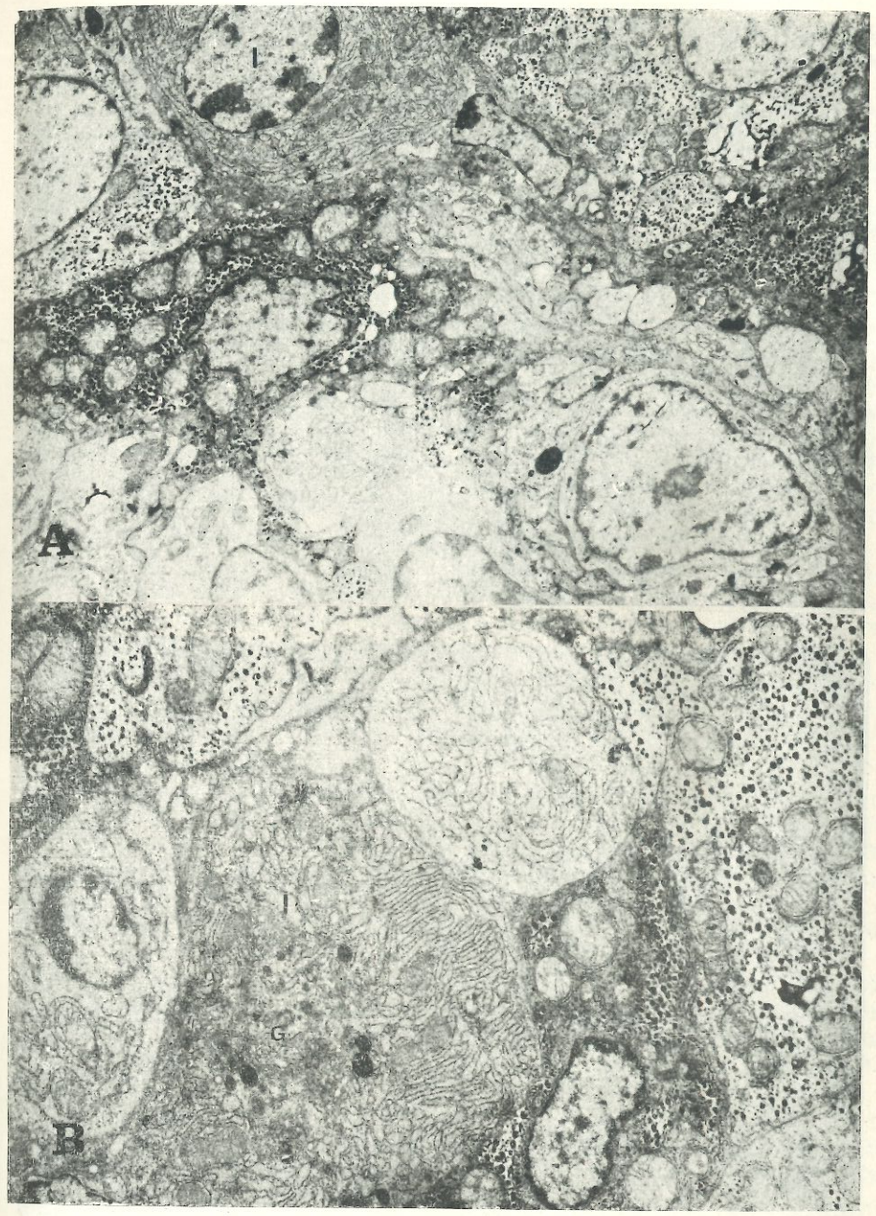

Plate 1. A — A comparison of light (adrenaline) and dark (noradrenaline) chromaffin cells and interrenal cell (I) intermingled and closely associated with the blood vessel. Endothelial cell and two smooth muscle cells are also seen. × 6020 ; B — An interrenal cell (I) surrounded by dark and light chromaffin cells. Golgi complex (G) surrounded by numerous vesicles, mitochondria and a well-developed rough endoplasmic reticulum can be seen in the interrenal cell. × 7350.

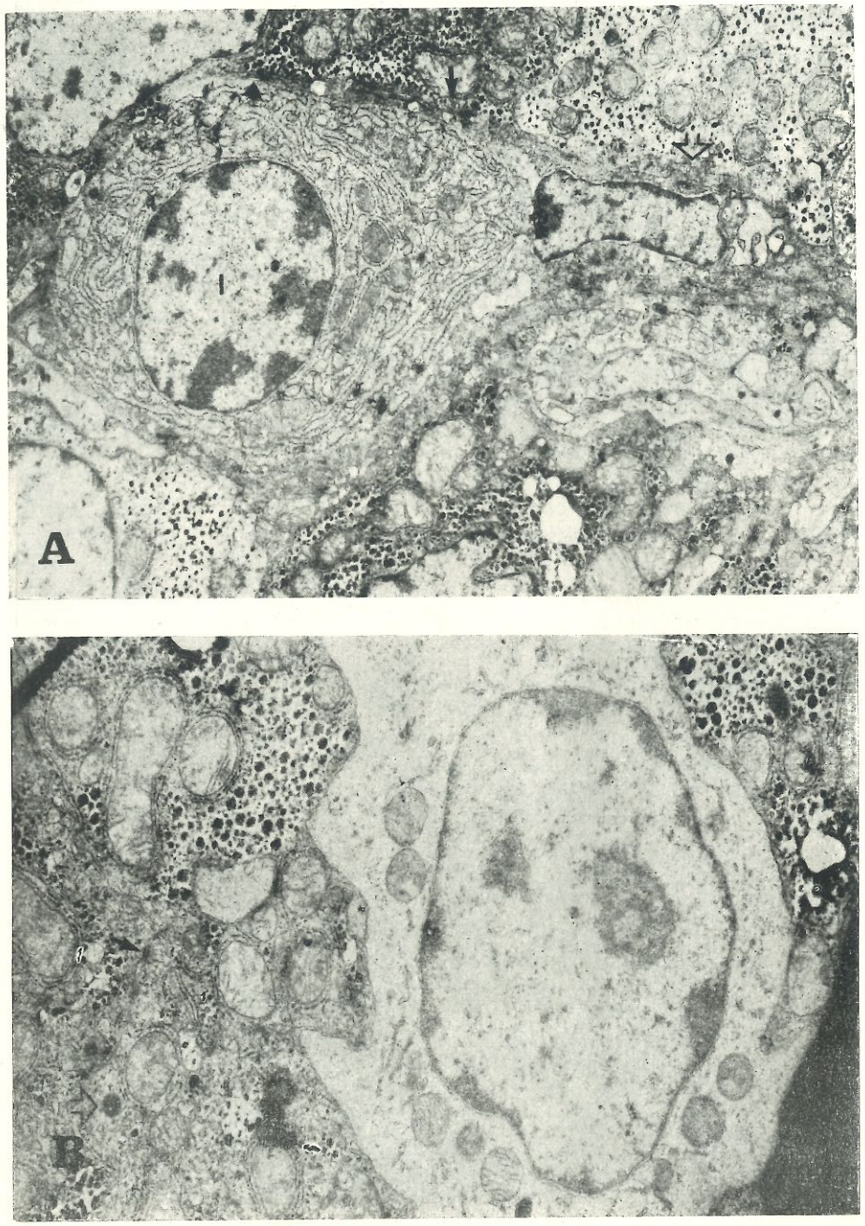

Plate 2. A — Interrenal cell (I) with developed rough endoplasmic reticulum in close association with light and dark chromaffin cells. Note the numerous mitochondria surrounded by rough endoplasmic reticulum in the chromaffin cells. The secretion product in the chromaffin cells appears freely dispersed in the cytoplasm. 12 920 ; B — A smooth muscle cell in close association with adrenaline (light) cells. Note the cell membrane between adjacent light cells and the presence of desmosome (arrow). In the cytoplasm, many secretory granules, heterogeneous in size and without a limiting membrane, are seen. × 21 350.

In both types of chromaffin cells there are numerous mitochondria of varying size and with a mitochondrial matrix less abundant than that of the interrenal cells. The most prominent feature of these chromaffin cells from fish embryos was the presence of the rough endoplasmic reticulum, which surrounded almost all mitochondria (Pl. 1, A and B, Pl. 2,A and B). The cytoplasm of both types of chromaffin cells appears very poor in other organelles. Some vesicles with an electron-dense content are seen in the light cells (Pl.2, B light arrow). Occasionally, some vacuoles were identified in both chromaffin cells (Pl. 2, A and B). The cell membrane of the chromaffin cells appears rather smooth in the zone of contact with other chromaffin cells or with smooth muscle cells. Occasionally, desmosomes between two adjacent light cells were encountered (Pl 2, B arrow). There were also identified small vesicles with very small amounts of electron-dense material, located in the vicinity of the plasma membrane (Pl 2, A light arrow). Few endocytotic vesicles were encountered adjacent to the interrenal cells (Pl. 2, A arrow). The cell membrane of the chromaffin cells lying adjacent to the veins had microvillosities extending through the sub-endothelial space (Pl. 1, A).

The nucleus of the dark chromaffin cells is irregular in shape and chromatin is more deeply stained and distributed along the nuclear envelope. The nucleus of light chromaffin cells is larger, oval in shape, with distinct nuclear envelope and faintly stained chromatin (Pl. 1, A).

DISCUSSION

The morphological organization of the adrenal gland in *Xiphophorus helleri* embryos appears to be similar to that observed in other teleostei. Moreover, the ultrastructure of the interrenal and chromaffin cells from the head kidney of embryos shows many similarities with those of other species of fish (14), (15), (20), (22). In *Xiphophorus helleri* embryos one type of interrenal cell and two types of chromaffin cells were identified ultrastructurally. Both types of chromaffin cells are distinguishable on the density of chromaffin secretion product. Previous investigations had shown that the two chromaffin cells types, the adrenaline and noradrenaline secretory cells, are the common cells in the adrenal medulla of mammalian species and in many vertebrates including amphibians, reptiles and birds (1—3), (7), (9), (19), (20).

Although in the head kidney embryos of *Xiphophorus helleri* two types of chromaffin cells, similar with the adrenal medulla cells of other species were identified, embryonic cells presented some peculiar ultrastructural characteristics : many mitochondria surrounded by rough endoplasmic reticulum and secretory products of varying size without limiting membrane. Our observations indicate that these cells are in a younger development stage in comparison with the differentiated chromaffin cells of the adult, and this is reflected in an increased biosynthetic activity. Moreover, it seems that the chromaffin cells in embryos had not yet reached their full endocrine development and the catecholamines synthetized actively are not aggregated in granules before birth.

There is no indication on the relations between the differentiation of adrenal gland cells and its secretory capacity. Moreover, information is scanty concerning the function and the ultrastructure of the larval adrenals in other species of vertebrates. Rapola (17), studying the development of the adrenal cortex in *Xenopus laevis* during different stages of metamorphosis, indicated that it is activated during the metamorphic climax. The author showed a variation of lipid contents during the differentiation of adrenal cortical cells and suggested that those adrenals which exhibit large amounts of coarsely granular lipids are inactive in regard to secretion. *Chester Jones* (7) discusses the problem of the varying association of adrenocortical homologues with chromaffin tissue. He indicated that some neural crest cells give rise to chromaffin cells and their final characteristics are determined by association with adrenocortical cells. Moreover, catecholamines may be necessary for the full secretion of corticoids, and this is one consequence of the intimate association of cortical and chromaffin tissue.

Another unsolved problem is the difference between the two types of chromaffin granules and the identity of adrenaline and noradrealine cells, based only on distinct differences in granulation. Some authors suggested that adrenaline and noradrenaline storing granules are present in both light and dark chromaffin cells (22). However, considering that structurally the two hormones are very similar and that up to methylation their synthesis proceeds in the same way, the presence of methylating enzyme may decide whether a particular cell will produce adrenaline. It is also worthwhile noting, that a selective secretion of adrenaline and noradrenaline was demonstrated in some physiological conditions (2). Our ultrastructural observations indicated that the two types of chromaffin cells are functionally active in fish embryos and the differentiation of the cells occurs early during development.

Our ultrastructural observation has demonstrated the presence in the head kidney of embryos of *Xiphophorus helleri* of only one type of interrenal cell. Although there are many histochemical studies on the interrenal cells in fishes and other vertebrates (4), (7), (12), (21),ultrastructural data are very scanty (14), (15), (20), (22). Previous investigations have shown that in the teleostean interrenal there appears only one type of interrenal cell.

In the present investigation, the interrenal cells of *X. helleri* embryos revealed some ultrastructural pecularities mainly related to the large quantity of rough endoplasmic reticulum and the structure of mitochondria. It has been shown that the interrenal cells of fish and mammalian adrenocortex contain smooth endoplasmic reticulum and mitochondria of tubulo-vesicular type (14), (20), (22). The functional status of these embryonic cells is not known. On the ultrastructural basis, there is good reason to suppose that the embryonic interrenal cells of fish are active with increased biosynthetic activity, but it is impossible to say whether the activation of adrenocortical cells is correlated with a high level of steroid hormone biosynthesis.

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A RAPID METHOD FOR THE PURIFICATION OF SEMINOLIPID (SULPHOGLYCEROGALACTOLIPID)

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Sulphoglycerogalactolipid was isolated from boar spermatozoa. Purification stages were : extraction with organic solvents, partition with a saline solution in order to remove water soluble compounds. Florisil chromatography in order to remove phospholipids and preparative thin layer chromatography. Purity of the product was checked by IR spectra, sulfate and galactose determination as well as in comparison with a sulfatid sample.

Seminolipid 1-0-alkyl-2-0-acyl-3-[β-3-sulfogalactosyl)-glycerol) was isolated firstly from boar testis and spermatozoa (2) (3) and almost simultaneously from rat testis (5). This compound, named also sulfoglycerogalactolipid, is the major glycolipid from the testis in the following species : rat, mouse, guinea-pig, rabbit and man (6). Biosynthesis of this compound was also elucidated (4). Seminolipid is substrate for sulfatase A (1) (9).

The type and relative ratio of seminolipid fatty acids should have taxonomic value.

This paper presents a rapid method for purification of this compound in order to be used in characterization of sulfatases.

MATERIALS AND METHODS

Obtainment of spermatozoa. Boar sperm was centrifuged at 900 g for 10 min at room temperature in order to sediment spermatozoa. Seminal plasma was decanted and the pellet was washed three times by gently suspending in 5 volumes of NaCl 0.9 % followed each time by centrifugation at 900 g.

Extraction and partition. The pellet was suspended in 20 volumes of chloroform-methanol 2 :1 and left for 24—48 hrs at room temperature with occasional stirring. The extract was separated from residue by filtration. The residue was similarly extracted with 10 volumes of chloroformethanol 2 :1. The second filtrate was mixed with the first one and then the mixture was submitted to partition with 0.2 volumes of NaCl 0.1 M and, after separation, the chloroformic phase was concentrated to dryness in vacuum (rotavapor) at 50°C; the residue was then dried in vacuum on P_2O_5 at room temperature.

Florisil chromatography. The residue was solved in a minimum volume of chloroform-methanol 19 :1 and applied on a Florisil column at a ratio of 25 mg total lipids per gram of Florisil (Florisil had been activated for 5 hr at 120°C). The column was washed with 6 volumes of chloroform in order to remove neutral lipids after which elution was

REV. ROUM. BIOL.—BIOL. ANIM., TOME 33, Nº1, P. 59—61, BUCAREST, 1988

made with a mixture of cloroform-methanol 2 :1. Fractions of 18 ml were collected at a rate of 2 ml/min.

Thin layer chromatography. Florisil fractions were submitted to thin layer chromatography on preparative silicagel plates in the chloroform-methanol-acetone-acetic acid-water (10 :2 :4 :2 :1) (A I) solvent system. After migration, the plates were dried at room temperature and placed in a tank with vapor of iodine. Iodine spots were marked with a sharp spatula and the plates left at room temperature till iodine disappeared (48—72 hrs). The spots were scraped and washed for 1—2 hrs in 10 volumes of chloroform-methanol in order to elute seminolipid. After filtration the solvent from the filtrate was removed in vacuum and the residue was analysed for sulfate, galactose, IR spectra and analytical thin layer chromatography. The A I solvent system and another two solvent systems — chloroform-methanol-ammonia (13 :5 :1) (A II) and chloroform-methanol-water (14 :6 :1) (A III) — were used for analytical thin layer chromatography. The spots were visualised by spraying with anthrone sulfuric acid, orcinol, phosphomolybdenic acid or iodine vapor. The chromatographic behaviour of the seminolipid was compared with that of a sulfatid sample (1).

Other analytical methods. Galactose was determined after hydrolysis and removal of lipids by the method indicated by Roe (7). Sulfate was determined according to Terho and Hartiala (8) with potassium rhodizonate. Infrared spectra were obtained in KCl pellets.

RESULTS AND DISCUSSION

From 15 g spermatozoa 0.415 g of total lipids were obtained. By thin layer chromatography in solvent system A I nine spots could be seen at this stage (Fig. 1).

After Florisil chromatography thin layer chromatography revealed a major spot and three minor ones. Identification in this spot, after hydrolysis, of sulfate and galactose indicated that this was seminolipid. The concentration of the seminolipid in the total lipids, estimated from galactose determination, was found to be 1.3% (5.5 mg seminolipid from 415 mg total lipids).

After preparative thin layer chromatography the product gave a single spot at analytical thin layer chromatography (coated with a 250μ silica layer) in solvent systems A I, A II and A III. Visualization, for the same solvent system, gave positive reaction with anthrone, orcinol, phosphomolybdenic acid and iodine. The product had an R_f value greater than that of the sulfatid, in solvent system A I (Fig. 2) (1).

A galactose-to-sulfate molar ratio of about 1 :1 was found. Infrared spectra revealed absorption maxima at 1240 cm^{-1} (sulfate ester), at 820 cm^{-1} (sulfate ester bound at an equatorial hydroxyl group) and at 875 cm^{-1} (β-anomeric configuration of hexapiranoside) (3).

Acknowledgement. Thanks are due to prof. H. Kihara for the sulfatid sample sent to us.

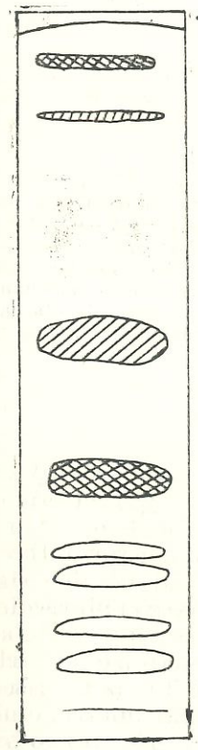

Fig. 1. — Thin layer chromatography of total lipids from boar sperm atozoa (Solvent system A I; Sprying with phosphomolybdenic acid followed by heating for 15 min at 130°C)

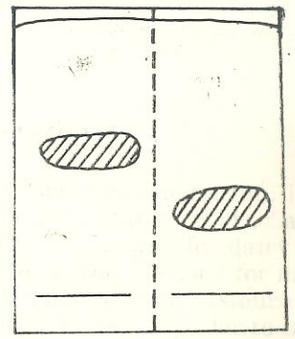

Fig. 2. — Comparative thin layer chromatography of seminolipid (left) and sulfatid (right). (Solvent system A I; Phosphomolybdenic acid).

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STOCHASTIC MODELING OF *MICROCOCCUS LYSODEIKTICUS* GROWTH ON A COMPLEX SUBSTRATE

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In order to investigate the evolution of the number of living cells, experiments were carried out on *Micrococcus lysodeikticus* NCTC— 2665 on a complex substrate containing mainly yeast extract, molasses and inorganic salts. A Herbert kinetic model describes approximately this system. A stochastic approach, which considers the kinetic constants subjected to perturbations, seems better suited to describe the experimental runs. Predicting not only the expected values of the number of living cells, but also their scattering, this second model presents interest for the engineering of the microbial catalase manufacturing process.

INTRODUCTION

Micrococcus lysodeikticus, a Gram-positive bacterium, has been the first microbial source for crystalline catalase synthesis (1). Catalase preparations are used in the food industry (2), (3), mainly for dairy products (4). Milk treatment with hydrogen peroxide-catalase is used for milk preservation under special conditions (5). It is often used over pasteurization for the manufacturing of certain types of cheese (6), (7), in order to improve body characteristics and to reduce the presence of undesirable microorganisms (8). Catalase preparations from the liver (9) and bacterial source (*Micrococcus lysodeikticus*) (10) have been approved for cheese manufacturing. Taking into account that beef liver is a limited source, the microbial manufacture of catalase seems to be an economic alternative. The purpose of this paper is to investigate the evolution of the *Micrococcus lysodeikticus* culture on a complex substrate and to provide a kinetic model of the process.

MATERIALS AND METHODS

The experiments were carried out on a submerse culture of *Micrococcus lysodeikticus* NCTC 2665 stimulated for catalase biosynthesis (11). The medium for biosynthesis was essentially that proposed by Beers (12) : yeast extract (20 g/L), molasses (20 g/L), $NaHCO_3$ (8.5 g/L), K_2HPO_4 (1 g/L) and microelements. The stock culture, obtained by freeze-drying a suspension of *Micrococcus lysodeikticus* cells in protective liquid with a Usifroid apparatus, was used for subcultivation. The inoculum was obtained by growth of the subculture on agar slant at 37°C. The submerse culture was carried out in 750 mL conical flasks, shaken at 280 rpm, containing 100 mL of medium at pH = 8 and at the temperature of 30°C (13). The number of living cells was determined by serial dilution after plating on Petri dishes (14). In the experimental runs we used different quantities of inoculum, the initial number of living cells varying between 10^3 and

REV. ROUM. BIOL.—BIOL. ANIM., TOME 33, Nº1, P. 63—69, BUCAREST, 1988

10^5 cells/mL. The lag time was up to 10 h being influenced by the inoculum incubation period which varied between 24 and 72 h. In this paper the time is recorded after the lag time.

DETERMINISTIC MODEL

Generally, kinetic models have been developed for a single microorganism fed on a pure substrate under aseptic conditions. Recently, some models have been developed for the complex cases (15), (16), but they are too complicated for engineering applications. Therefore we have adopted for our case a simple kinetic model of the Herbert type (17). The kinetic parameters have been estimated from the experimental data of living cells. The identification method used was Marquardt (18) modified by Fletcher (19) and implemented by us on a FELIX C-256 computer. This method seems to be better suited for our case than the Lineweaver-Burk plot or the direct linear methods (20). The estimated kinetic parameters, together with their standard errors, are listed in Table 1. The deterministic model is presented below :

Table 1

The kinetic parameters

Kinetic parameter	$\beta(h^{-1})$	σ
$K_s = 1 \pm 0.2$ g/L	1	0.894
$\mu_m = 1.13 \pm 0.1\ h^{-1}$	1	0.447
$\mu_d = 0.033 \pm 0.013\ h^{-1}$	1	0.058
$Y = (5 \pm 1.5) \cdot 10^{11} \frac{\text{cells}}{\text{g substrate}}$	1	$6.7 \cdot 10^{10}$

$$\frac{dX}{dt} = \frac{\mu_m \cdot X \cdot S}{K_s + S} - \mu_d \cdot X \tag{1}$$

$$\frac{dS}{dt} = -\frac{1}{Y} \frac{\mu_m \cdot X \cdot S}{K_s + S} \tag{2}$$

For computations, the substrate was considered as yeast extract, because previous experiments indicated that the other components of the substrate have a reduced influence for our case.

STOCHASTIC MODEL

The kinetic parameters are considered to be subjected to perturbations :

$$K_s = \overline{K}_s + K'_s$$

$$\mu_m = \overline{\mu}_m + \mu'_m \tag{3}$$

$$\mu_d = \overline{\mu}_d + \mu'_d$$

$$Y = \overline{Y} + Y'$$

where $(^-)$ denotes the nominal value, identified above, and $(\ )'$ its perturbation. In many cases the perturbations can be approximated with a Gaussian white noise stochastic process, that is with zero expectation and the stationary autocorrelation function given by the "Dirac delta function" multiplied by a constant. This implies that the perturbations can change infinitely fast. White noise is not physically realisable, because no process in nature can change infinitely fast. However, it is often employed as a model of random physical systems, generally for linear systems when analytical solutions are available for the expected value and the moments of higher order. For nonlinear systems, as in our case, we cannot obtain analytical solutions. The computational effort is near the same if, instead of white noise, we use a more realistically correlated noise model for the perturbations. If k is a correlated Gaussian noise process with zero expectation and the stationary autocorrelation function :

$$R(\tau) = 0.5 \cdot \sigma^2 \cdot \beta \cdot D \cdot \exp(-\beta \cdot \tau) \tag{4}$$

then it is generated by the following stochastic differential equation (21) :

$$dk = -\beta \cdot k \cdot dt + \sigma \cdot \beta \cdot dW \tag{5}$$

Here W is the Wiener process, a continuous parameter Gaussian process with zero expectation and stationary independent increments. D is its variance parameter. Although the Wiener process is not differentiable, it can be shown that formally its derivative is the white noise process (21). The stochastic differential equation (5) can be integrated using the following approximate formula (22) :

$$k_{i+1} = k_i + \sigma \cdot \beta \cdot \Delta w_{i+1} - \beta \cdot k_i \cdot \Delta t - 0.5 \cdot \beta^2 \cdot \sigma \cdot \Delta t \cdot \Delta w_{i+1} + 0.5 \cdot \beta^2 \cdot k_i \cdot \Delta t^2 \tag{6}$$

where Δt is the time step of integration, Δw is a Gaussian random number with zero expectation and variance Δt, and the subscripts i and $i+1$ denote the quantities evaluated at the step i and $i+1$, respectively.

The stochastic model presented in Table 2 consists of six equations. The first two equations are the same as in the deterministic case, but they include the perturbations. The last four equations generate the perturbations. The stochastic equations have been integrated using the Talay method (22). Simultaneously, the first two equations were integrated with a Runge-Kutta method. The integration was done for 100 systems, and, because of the stochastic process, we get different results. For each kinetic parameter, σ and β are listed in Table 1. Using these values, the standard errors of the perturbations, i.e. $(\sigma^2 \cdot \beta \cdot D/2)^{0.5}$ (see equation (4)), are equal

with those reported for the kinetic parameters, and the scattering of the simulated and experimental data is comparable.

Table 2

The stochastic model

$$\frac{dX}{dt} = \frac{(\bar{\mu}_m + \mu'_m) \cdot S \cdot X}{(\bar{K}_s + K'_s) + S} - (\bar{\mu}_d + \mu'_d) \cdot X$$

$$\frac{dS}{dt} = - \frac{1}{(\bar{Y} + Y')} \cdot \frac{(\bar{\mu}_m + \mu'_m) \cdot S \cdot X}{(\bar{K}_s + K'_s) + S}$$

$$dK'_s = - \beta_{K'_s} \cdot K'_s \cdot dt + \sigma_{K'_s} \cdot \beta_{K'_s} \cdot dW$$

$$d\mu'_m = - \beta_{\mu'_m} \cdot \mu'_m \cdot dt + \sigma_{\mu'_m} \cdot \beta_{\mu'_m} \cdot dW$$

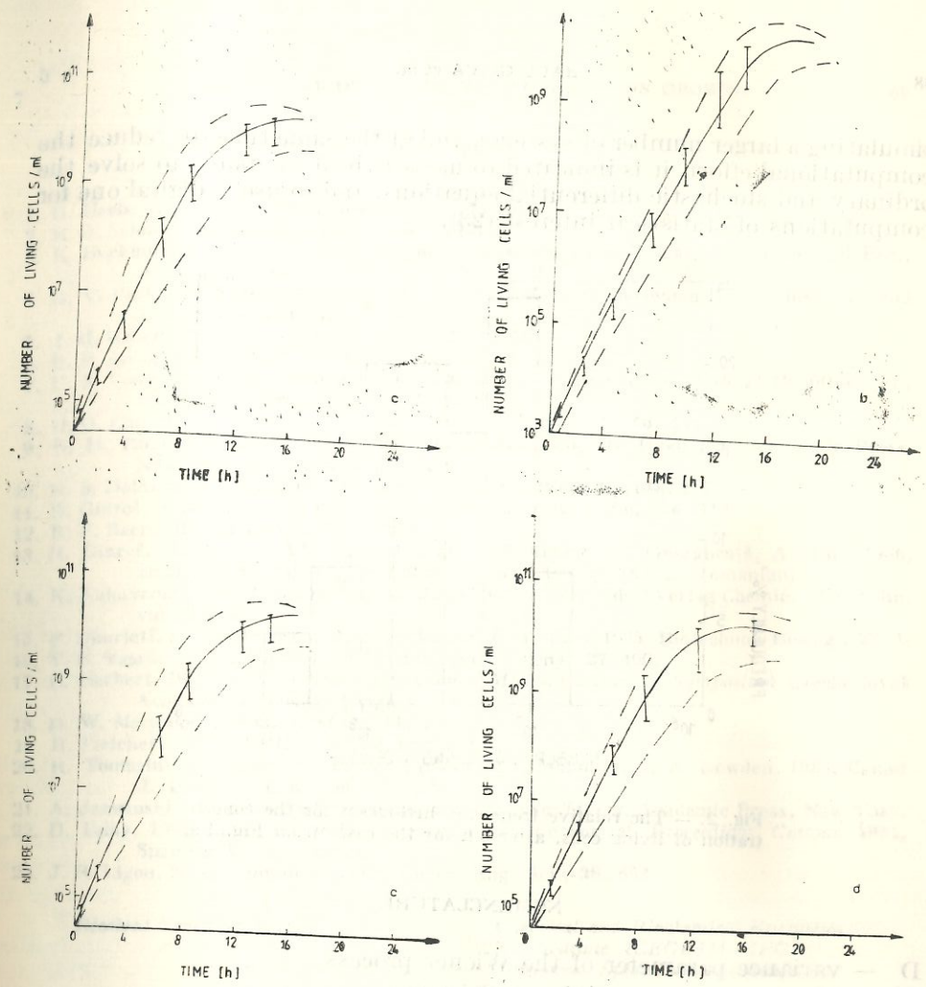
$$d\mu'_d = - \beta_{\mu'_d} \cdot \mu'_d \cdot dt + \sigma_{\mu'_d} \cdot \beta_{\mu'_d} \cdot dW$$

$$dY' = - \beta_{Y'} \cdot Y' \cdot dt + \sigma_{Y'} \cdot \beta_{Y'} \cdot dW$$

RESULTS AND DISCUSSIONS

The expected values of the concentration of living cells have been computed, at each integration step, as the means of the respective concentrations obtained for the 100 systems simulated. The distribution of the concentrations of living cells, at each moment, is strongly different from the Gaussian distribution, so the standard error does not give a significant information. That is why, instead of the standard error we give the maximum and minimum values resulted from simulations. In Fig. 1 we present with a continuous line the expected values predicted by the stochastic model, and with dashed lines the maximum and minimum values. We observe that the region between the two dashed lines covers the field of experimental values, indicating a fair agreement between our model and the experiments. Although, in general, the presence of perturbations produces a shift of the expected values [23], for our case the difference is negligible. It results that the continuous line represents the prediction of the deterministic model, too. We have to point out that the stochastic model provides informations concerning not only the expected values, such as the deterministic model, but it gives also an account of the data scattering. In order to better illustrate the simulated data scattering we present in Fig. 2 the relative frequency histogram for the concentration of the living cells, after 8 h, for the case from Fig. 1 a. We observe that the distribution is rather log-normal than Gaussian. Although there are two maxima, because the number of investigated systems is relatively small, i.e. 100, we cannot conclude that the distribution is multi-modal.

This stochastic model, tested on laboratory data, presents interest for the process scale up and engineering. In order to increase its accuracy,



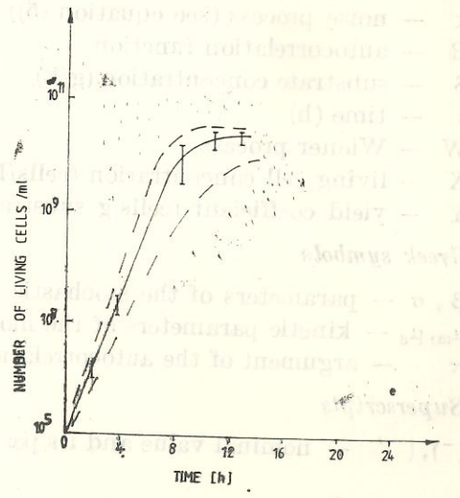


Fig. 1. — a, b, c, d and e. The concentration of living cells as a function of time, for different starting conditions.
— the field of experimental data obtained from different experimental runs (with the same starting conditions);
— the expected values predicted by the stochastic model;
– – – – the maximum and minimum values predicted by the stochastic model.

simulating a larger number of systems, and at the same time to reduce the computational effort, it is indicated to use a hybrid computer to solve the ordinary and stochastic differential equations, and to use a digital one for computations of statistical interest (23).

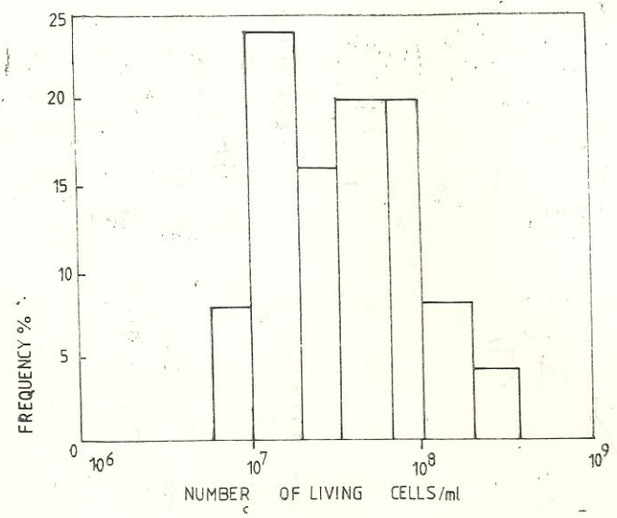


Fig. 2. — The relative frequency histogram for the concentration of living cells, after 8 h for the case from Fig. 1 a.

NOMENCLATURE

D — variance parameter of the Wiener process
K — kinetic parameter of the model (g/L)
k — noise process (see equation (5))
R — autocorrelation function
S — substrate concentration (g/L)
t — time (h)
W — Wiener process
X — living cell concentration (cells/L)
Y — yield coefficient (cells/g substrate)

Greek symbols

β, σ — parameters of the stochastic differential equation (5)
μ_m, μ_d — kinetic parameters of the model (h^{-1})
τ — argument of the autocorrelation function

Superscripts

$(^-)$, $(\ )'$ — nominal value and its perturbation

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CHROMOSOMAL ABERRATIONS IN RAT MEIOSIS INDUCED BY INTERNAL IRRADIATION WITH LOW-LEVEL TRITIATED WATER

FLORICA TRIPĂDUȘ and ILEANA PETCU

The present study represents an evaluation of chromosomal damage in rat germ cells, after continuous ingestion of HTO, in low doses range.
Male rats from Wistar-London strain were exposed to internal contamination with tritiated water in concentrations around the maximum permissible one, ranging from 0.01 μCi ^{3}H/ml to 0.3 μCi ^{3}H/ml.
After a 90-day treatment, meiotic preparations from the testes were made, using the air-drying technique.
Different types of chromosomal aberrations were found such as : breaks, fragments, multivalent configurations, aneuploidy and polyploidy cells in a rather reduced number of cells, with a gradually increasing frequency of HTO administered activity.

INTRODUCTION

Researches regarding internal irradiation with low-level doses HTO, include both results of the experiments performed on somatic cells (1, 2) and those regarding the developing female germ cells in the mouse (3), the cytological damage of mouse testes (4) and the reduction of embryos viability (5).

The studies regarding the effects of irradiation in mammal meiosis are still relatively few and the information about the action of internal irradiation with HTO on meiotic chromosomes are also incomplete.

Most of the data were obtained from the cytological analysis of primary spermatocytes after the external irradiation of spermatogonia in mice (6, 7, 8) and some studies dealing with the effects of low-doses released by uptaken radionuclides : ^{14}C and ^{239}Pu (9, 10), on meiosis in mice.

The aim of this paper is to find out the chromosomal changes in germ cells of male rat in a low concentration of HTO experiment, ranging over 0.3. MPC* and 10. MPC.

MATERIAL AND METHOD

The investigation covered 29 male rats from the Wistar-London strain selected in point of weight and with a standard nutrition. HTO was administered in drinking water "ad libitum", for 90 days. Experiments were made in 5 groups of 6—5 animals, and out of which 4 groups received tritiated water with different activities :

— group A — 0.01 μCi ^{3}H/ml (0.3.MPC) — 6 animals ;
— group B — 0.03 μCi ^{3}H/ml (1 .MPC) — 6 animals ;
— group C — 0.1 μCi ^{3}H/ml (3 .MPC) — 6 animals ;
— group D — 0.3 μCi ^{3}H/ml (10.MPC) — 5 animals ;
— control group — on tap water — 6 animals.

* MPC—maximum permissible concentration

REV. ROUM. BIOL.—BIOL. ANIM., TOME 33, Nº 1, P. 71—75, BUCAREST, 1988

The spermatocytic test was used to analyse chromosomal aberrations in meiosis.

The observations were made during the diakinese-metaphase I stage from the first spermatocytes and from a reduced number of second spermatocytes in Metaphase II.

Using the air-drying technique (Evans et al. (11)) modified by Raicu et al. (12), we obtained the meiotic chromosome preparations, examining a total number 1662 meiotic cells, out of which 1404 were in metaphases I and 258 in metaphases II.

The frequency of chromosomal aberrations was expressed in percentages. The meaning of the difference between the groups was also calculated and each of the experimental groups was compared with the control group.

RESULTS AND DISCUSSION

It has been generally accepted that germ cells have a high radiosensitivity and the changes caused in the genetic material can be transmitted to descendents. The types of chromosomal aberrations and their frequency observed in the 4 groups of male rats exposed to HTO internal irradiation and also in the control group, are shown in Table 1.

Most of the primary and secondary spermatocytes contained a normal complement of 21 bivalents, as can be seen therein.

The following types of chromosomal aberrations were observed: breaks, fragments, extrachromosomes, multivalent configurations, aneuploidy, and polyploidy cells. A gradual increase of the total frequency of chromosomal aberrations can be noticed (Table 1) together with the increasing activity of administered HTO. However, the total frequency of the chromosomal aberrations induced in A and B groups, to which a 0.01 μCi/ml — 0.03 μCi/ml activity was administered, presents no high differences compared with the control group, while in the C and D groups, which are subjected to greater activities: 0.1 μCi/ml — 0.3 μCi/ml respectively, the total frequency of the chromosomal aberrations is significantly increased, as against all the other batches.

Breaks and fragments were noticed in a rather reduced number of cells so that we considered their total frequency, that ranged from 1.42 % in A group to 3.17 % in D group in metaphase I, and from 2.08 % — 4.28 % in metaphase II. We noticed their total absence in the control group.

Although these percents have low values, they cannot be entirely neglected. However, there are some authors (13) who do not exclude that only 1 % spermatocytes with the same aberrations, can produce a very small proportion of detrimental effects.

Extrachromosomes or unidentified chromosomes were observed both in the control group and in the irradiated groups, with a frequency which does not seem to be dose dependent.

Some other authors (8.14) have also noticed their presence. They can appear also in mitosis. Their signification is not yet entirely elucidated.

Chromosomal aberrations induced by HTO internal irradiation in rat meiosis

Groups	Activity in μCi	Metaphase I (spermatocytes I)								Metaphase II (spermatocytes II)				
		Total of cells analysed	Number of metaphase I analysed	Normal metaphase I %	Cells with multivalents %		Cells with breaks fragments univalents %		Apparently polyploid cells %	Number of metaphase II analysed	Normal metaphase II %	Breaks %	Apparently polyploid cells %	Total abnormal metaphases II %
					1	2								
control 6 animals		362	322	96.28	—	—	—	1.55	2.17	40	97.95	—	2.05	2.05
A 6 animals	0.3 CMA 0.01 μCi ^{3}H/ml	328	280	94.65	—	—	1.42	1.78	2.15	48	95.84	2.08	2.08	4.16
B 6 animals	1 CMA 0.03 μCi ^{3}H/ml	350	300	94.34	—	—	1.50	2	2.16	50	94	2	4	6
C 6 animals	3 CMA 0.1 μCi ^{3}H/ml	300	250	82	0.4	0.4	2.8	2	12.4	50	92	4	4	8
D 5 animals	10 CMA 0.3 μCi ^{3}H/ml	322	252	75	5.15	0.39	3.17	1.98	14.28	70	88.6	4.28	7.14	11.4
					0.8	5.54								

Multivalent configurations were identified as quadrivalents and hexavalents, only in groups C and D that received activities higher than 1. MPC.

Yet, the number of spermatocytes with such configurations in not too big : 0.8 % in group C and 5.54 % in group D.

However, cells with 1 and 2 translocated configurations were also observed. In group C, a cell with 2 quadrivalents has been detected and in group D two cells, each of them presenting two quadrivalents. The appearance of this aberration type is very important, indicating the existence of reciprocal translocations induced in spermatogonia, which is entirely considered to be the most radiosensitive cell type of the cell series involved in the spermatogenesis process. The presence of reciprocal translocations in low dose irradiation has been reported both in external irradiation experiments with ^{60}Co (6, 7) or with neutrons of relatively low energy (8), and in experiments using low doses released by uptaken radionuclids such as ^{239}Pu and ^{14}C (9, 10).

The two activities of HTO : 0.1 μCi/ml and 0.3 μCi/ml (in groups C and D), in which we had identified translocated configurations, correspond to cumulated doses of 3.86 rad and 12.87 rad respectively, being smaller than acute doses used in the above mentioned experiments.

The frequency of germ cells in primary metaphase is low in groups A and B around the value of the control group and it is higher in groups C and D : 12.4 % and 14.28 %, respectively.

The increased frequency of polyploidy noticed in these 2 groups, may reflect, indeed, an effect of the administered HTO doses, but a certain subjectivity in their recognition cannot be excluded, either.

Thus the experimental data obtained in this study show that HTO administered for 90 days in drinking water corresponding to concentrations of 0.3. MPC, 1.MPC, 3. MPC and 10. MPC, produced chromosomal abberrations in the male rat germ cells, in a rather reduced number of cells, but increasing gradually with the ^{3}H activity.

It is likely that all these chromosomal changes noticed in meiosis, are compatible or not with the maturity process up to the sperm stage.

Also, there is a relatively small probability of the genesis of abnormal sperms or of the disturbance of the normal stages of spermatogenesis.

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P. JITARIU (Editor), *Acțiunea cîmpului magnetic și electromagnetic asupra organismelor* (The action of the magnetic and electromagnetic field on animal organisms), Ed. Academiei, București, 1987, 235 p., 134 figs, 46 tables.

The present work represents the first monographic book in Romania concerning the action of the magnetic (MF) and electromagnetic field (EMF) on the animal organisms. It was written by a famous group of specialists in the biomagnetic field, on the basis of a very rich and actual literature, and of a remarkable scientific experience of the authors. Due to technological developments, the effects of MF and EMF are continuously increasing must be known, because all the living organisms do not support such changes without to react.

In the chapter on "The electric, magnetic and electromagnetic field" (E. Luca) some physical notions, necessary for the understanding of the nature of these fields are presented. In the chapter on "Biostructures and electromagnetic biofield" (P. Jitariu), the author expands his theory on the biofield, generated by biostructures in normal and in pathological conditions. The main generating factor, playing a major role in biofield genesis, is represented by the protein macromolecule. The exogenous fields, in interaction with the biofields, induce effects, which are dependent on their intensity, on the time of action of the applied EMF, as well as on the pattern of its application. In te case of static MF, the biological effects are not related to their intensities, but they depend on the gradient amplitude of the unhomogeneous MF, as demonstrated by the studies performed on Tubifex eggs division (R. Brandsch, P. Jitariu). The effects induced by MF in egg-cell are longacting, they being manifest in many cellular cycles.

The chapter "Biochemical and physiological modifications induced by the magnetic and electromagnetic field" (M. Lazăr) reveals modifications in plasma proteins, lipoproteins and tissue nuclei acids and on some transaminases, changes which depend on MF type, its intensity, time of treatment, animal species and the age of animals. The results obtained on these experimental models, evidence a MF action especially through the neuroendocrine system.

The histological researches performed by N. Neaga, have shown that MF and EMF bring about histological and histochemical modifications in lymphoid organs, endocrine glands and other tissues, depending on the type of the applied field.

In the chapter "The magnetic field and the hypothalamo-hypophysis-adrenal system" (G. Dimitriu), the author indicates some aspects of the functional correlation of this system, mainly important in adaptation, studying the way of EMF action. The experimental results, obtained on numerous experimental patterns, lead to a predominant CNS action of the MF, especially on the hypothalamus. The secondary modifications are not greater than the capacity of cell reaction, suggesting that the MF acts as a weak, corrective, nonspecific stimulus, its action being felt against the background of a specific reaction of the system.

Due to the major role of the immunological reaction in the general homeostasis, this type of reaction was also investigated under the influence of different kinds and intensities of the EMF. The author found an evident action of EMF on the immune reactivity, which depends on the daily intensity and time of treatment. The EMF action on antibody genesis is exerted either by suppressing the inhibitory role of the reticular formation or directly on the hypothalamus, though a direct action on the reticuloendothelial system can not be ruled out.

The chapter "Zootechnical applications of the magnetic and electromagnetic field" (N. Neaga, M. Lazăr) indicates their usefulness in bird and silkworm growth. The authors concluded that interrupted EMF has a stimulatory action on the metabolic processes, while a continuous EMF has an inhibitory effect.

Utilisation of low-frequency electromagnetic fields in human therapy is described in chapter 9 (M. Zirra). There are indicated the effects of EMF in the treatment of neurotic and rheumatismal deseases, gynecopathies, postviral hepatites.

The monograph, coordinated by academician P. Jitariu, represents a model of a complex interdisciplinary research, for the clearing up of the effects and action mechanisms of these ecological factors. The presentation of and the approach to facts as well as the actuality of the problemes raised, into discussion will be of interest not only to the people concerned with theoretical and practical biological problemes, but also to readers keen on knowing these factors which are always present in our life.

Vasile P. Hefco

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AVIS AUX AUTEURS

La « Revue roumaine de biologie — Série de biologie animale » publie des articles originaux d'un haut nuveau scientifique de tous les domaines de la biologie animale : taxonomie, morphologie, physiologie, génétique, écologie, etc. Les sommaires des revues sont complétés par d'autres rubriques, comme : 1. La vie scientifique, qui traite des manifestations scientifiques du domaine de la biologie : symposiums, conférences, etc. ; 2. Compte rendus des livres de spécialité.

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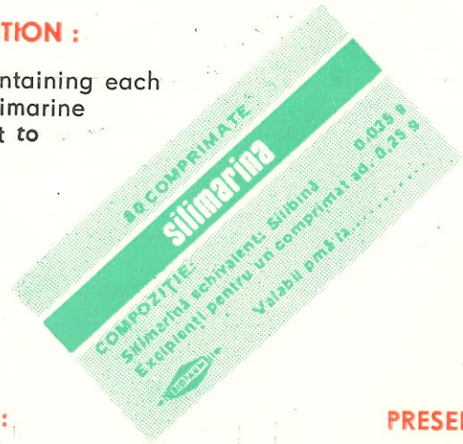

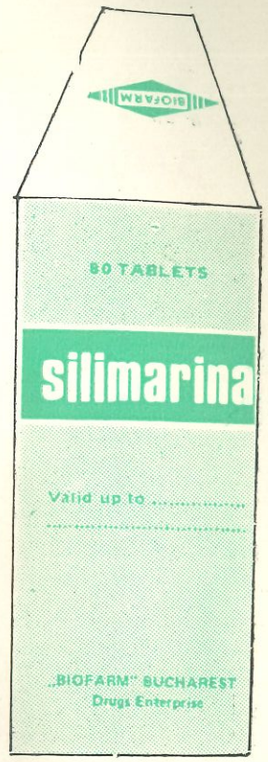

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43 489

Lei 30