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ÉTABLISSEMENT DE LA VRAIE ESPÈCE *AHAVANELLA MACULATA* (MEIGEN) ET DESCRIPTION D'UNE NOUVELLE ESPÈCE PALÉARCTIQUE AFFINE (*DIPTERA, SARCOPHAGIDAE*)

ANDY Z. LEHRER

The true species *Ahavanella* (= *Sarcophaga*) *maculata* (Meigen), from Europa and Circummediterranean Africa, is rehabilitated. The new palaearctical species *Ahavanella dreyfusi* sp. n., from East-Europe and Asia, is described.

Pendant la révision du groupe *Helicophagella* ENDERLEIN (s. lat.) (Lehrer, sous presse) nous avons constaté que l'espèce *Ahavanella* (= *Bellieria*) *maculata* (Meigen) n'était pas bien connue par les spécialistes et qu'elle est en permanence confondue avec le taxon décrit par Rohdendorf (1937) sous ce nom. Pour cette raison, sa distribution géographique a été tellement élargie artificiellement (Verves, 1986), que personne ne peut plus séparer les zones où ils sont identifiés.

En comparant les armatures génitale mâles de *Sarcophaga maculata* Meigen à celles présentées par Rohdendorf et les auteurs contemporains, qui ont copié ses dessins, mais surtout avec nos préparations microscopiques, nous sommes arrivé à la conclusion que tous ces spécialistes n'ont pas reconnu la véritable espèce de Meigen et qu'ils ont toujours été en face d'une espèce nouvelle affine que nous avons nomée *Ahavanella dreyfusi* sp. n. Après nos observations et la description de cette dernière, nous avons mis au point les clés pour leur identification.

DESCRIPTION DES ESPÈCES

Ahavanella maculata (Meigen, 1835)

= *Sarcophaga maculata* sensu Böttcher 1912, Deutsch. Ent. Zeitschr., (5) : 711, fig. 5 ; — sensu Salem 1935, Egypt. Univ., Fac. Méd., Publ. nr. 5 : 37, fig. 7 ; — Salem 1936, Egypt. Univ., Fac. Méd., Publ. nr. 8 : 233 ; Séguy 1941, Encycl. Ent., A 21 : 115, fig. 138.

= *Helicophagella maculata* sensu Verves 1986 (partim), Catalogue of palaearctic Diptera, 12 : 137.

Remarques. Sur l'armature génitale mâle de cette espèce il n'y a que deux descriptions sommaires, mais très exactes et suggestives. La première est celle de Böttcher (1912 : 712) qui souligne les données suivantes :

« Genitalsegmente sehr dicht und lang behaart, auch im Profil dreieckige, an den der *S. hirticrus* Pand. erinnernde Forceps bis fast zur Spitze zottig behaart. Penis statt der kurzen, schuppenartigen prox. Fortsätze, wie wir sie bei *S. melanura* finden, mit langen, schräg seitwärtz abstehenden, an der Spitze etwas zurückgebogenen Apophysen ».

REV. ROUM. BIOL. — BIOL. ANIM., TOME 39, N° 2, P. 83—88, BUCAREST, 1994

La deuxième a été faite par Salem (1935 : 37—38), qui complète avec beaucoup d'exactitude son beau dessin sur la génitalie mâle (fig. 1). Il dit :

« *Terminalia of the male. Anal cerci* black, both margins evenly curved to tip which is blunt.

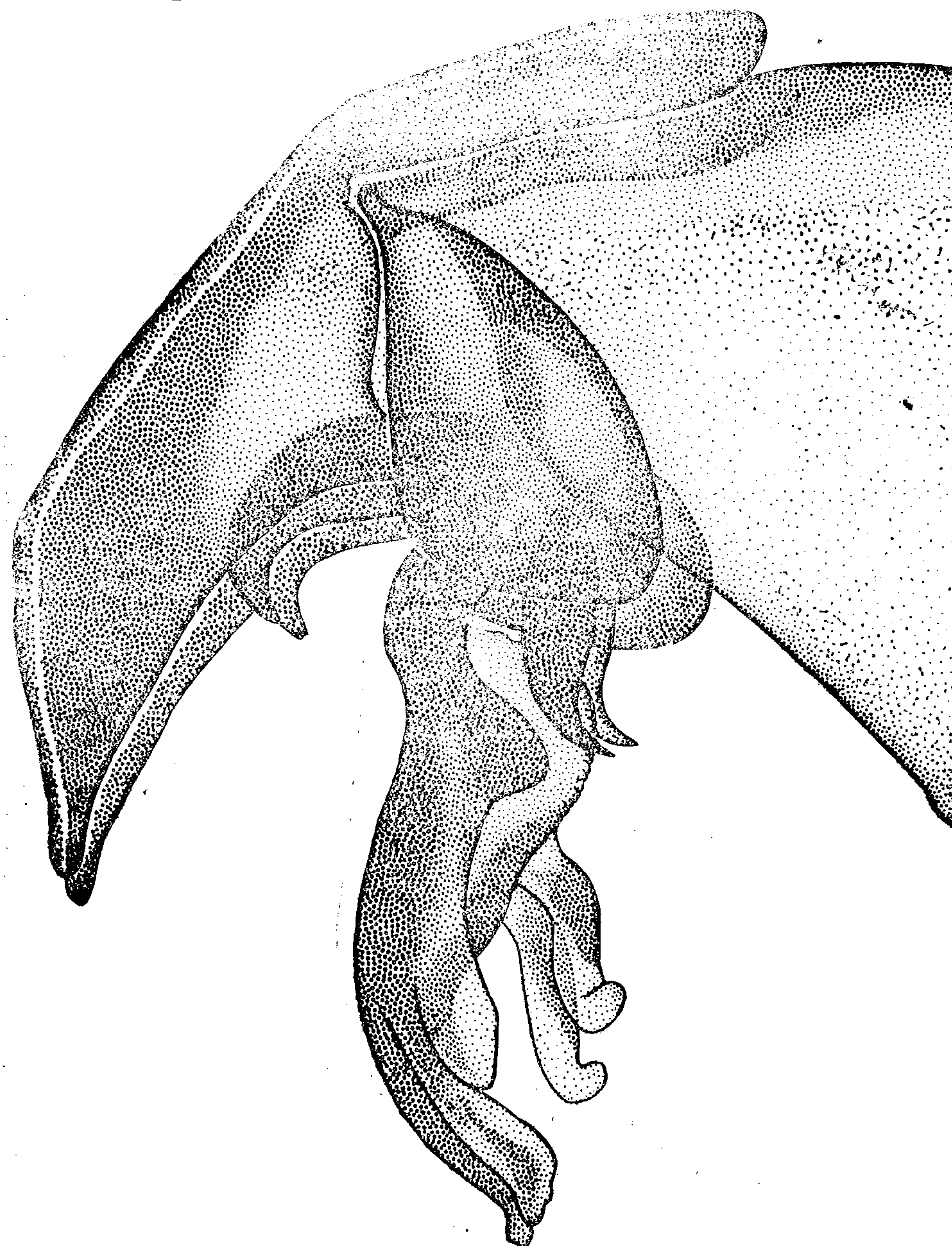

Fig. 1. — Armature génitale mâle d'*Ahavanella maculata* (Meigen). D'après Salem, 1935 (un peu modifié).

Accessory plate. Large and with very long hairs.

Parameres. Posterior parameres very slightly longer than the anterior ones, and forewardly curved apically. Both are rather thick and of very dark colour.

Phallosome. Shaft rather short. End piece elongated and broad and markedly curved forwards with its posterior surface strongly convex. One strong arm-like apophyses is found on either side of the basal part of the end piece pointing outwards, and distally curving downwards. Apical part of the end piece very blunt and without and apophyses ».

Séguy (1941 : 116, fig. 138) a reproduit schématiquement le dessin de Salem, mais en même temps il a fait la grave erreur (comme tous les spécialistes d'aujourd'hui) de synonymiser *Bellieria maculata* sensu Rohdendorf (1937 : 132), sans accorder aucune importance aux détails très différents de la structure du phallosome de cette dernière, qui sont caractéristiques à l'espèce nouvelle *Ahavanella dreyfusi* sp. n.

Distribution géographique. Europe et Afrique circum-méditerranéennes.

Ahavanella dreyfusi sp. n.

= *Bellieria (Bellieria) maculata* sensu Rohdendorf 1937, Faune de l'URSS, Insectes Diptères, 19(1) : 132, fig. 166—167.

= *Bellieria maculata* sensu Stackelberg 1956, Sinantropnye dvukrylye fauny SSSR, 60 : 85, fig. 67A ; — sensu Fan zi-De 1965, Key to the common synanthropic flies in China, 231, fig. 900—903 ; — sensu Rohdendorf 1970, Opred. nasek. evrop. časti SSSR, 5 : 654 ; — sensu Mihalyi 1972, Fauna Hung. 135, 15(16) : 115, fig. 68, D—E.

= *Helicophagella maculata* sensu Verves 1986 (partim), Catalogue of palaearctic Diptera, 12 : 137.

MÂLE

Tête. Noire et couverte d'un tomentum argenté, excepté les parafacialies et le vibrissarium, qui sont d'un brun foncé. Front, vu du dessus et au niveau le plus étroit, est presque égal à la largeur d'un œil. La bande frontale noire à nuances brunâtres et aussi large qu'une parafrontalie. Antennes noires brunâtre avec un peu de tometum ; le troisième article est presque deux fois plus long que le deuxième. Arista noire brunâtre, avec de poils moyens sur les deux parties. La trompe noire ; les palpes bruns noirâtre. Péristome mesure 1/3—2/5 du grand diamètre oculaire.

Chétotaxie de la tête. Macrochètes verticaux internes longs, forts et rétroclines ; macrochètes verticaux externes fins et 1/2 de la longuer des précédents ; les ocellaires proclines et les préverticaux rétroclines sont très bien développés ; macrochètes frontaux au nombre de 7 paires, macrochètes parafaciaux fins et courts ; les petites vibrisses montent un peu sur les bordures faciales ; il y a 1—2 postocellaires et 1 postvertical sur

chaque côté de l'occiput ; les microchètes occipitaux disposés sur deux rangs. Péristome couvert de poils noirs ; la partie postérieure de la tête pourvue de poils blancs, assez courts et rares.

Thorax. Noir, avec tomentum argenté pas très dense, trois bandes médio-dorsales longitudinales noires larges et deux bandes latérales plus étroites. Propléures glabres ; prosternum poilu. Stigmates d'un brun foncé à nuances oranges. Pattes noires à teinte brunâtre sur les fémurs et tibias.

Chétotaxie du thorax. ac = 0 + 1, dc = 4 + 3(4) (premier postsutural manque ou piliforme et petit), ia = 0 + 2, prs = 1, sa = 3, h = 3, ph = 2, n = 4, pa = 2, sc = 3 + 1, pp = 1 (plus quelques poils), pst = 1, st = 1 : 1 : 1.

Ailes. Transparentes et légèrement brunâtres. Épaulette noire ; basicosta et costagium jaunes. La nervure r_1 glabre ; la nervure r_{4+5} ciliée jusqu'à la moitié de la distance entre son origine et r-m. Cubitulus courbé en angle droit et prolongé d'un pli. Épine costalebien développée. Les écailles blanches jaunâtre ; les balanciers brunâtres.

Chétotaxie des tibias. Les tibias antérieurs ont 3 ad proximaux petits, 1 pd et 1 pv ; les tibias médians pourvus de 2—3 ad, 0—1 av, 1 pd et 1 pv ; les tibias postérieurs ont quelques ad, 2 ad forts, 2 av, quelques pd, 1 pd très fort et une longue pilosité sur les parties antéro- et postero-ventrales.

Abdomen. Noir, avec tomentum argenté et dessins caractéristiques, formés de taches longitudinales médianes sur les tergites II—III et de taches ovoïdales disposées sur la marge antérieure des tergites III—V. La formule chétotaxique : 0 + 0 + 2 + série. Tergite génital noir, long, luisant et pourvu de macrochètes marginaux piliformes. Tergit anal noir et court.

Armature génitale : fig. 2. Sternite V (A) relativement court a une base plus longue et de brosses courtes. Cerques (B) légèrement courbés, devenant graduellement étroits vers le sommet ; les paralobes plus ou moins triangulaires, avec les angles arrondis. Distiphallus (C) allongé, étroit et courbé en angle droit ou un peu aigu. Le paraphallus n'est pas divisé en deux parties, étant pourvu d'une dent antérieure petite à proximité de la base des styles ; ces derniers sont tubulaires, courts, un peu courbés et n'ont pas de dents récurrentes sur leurs marges. La partie apicale du paraphallus se termine avec quelques formations dentées à son bout. Les lobes membranaux, vus de profil, semblent avoir un aspect bidenté, mais en réalité ces dents resultent de deux apophyses sclérifiées, pigmentées et soudées sous la forme de cornes forts, courbés latéralement et en haut, très visibles en position ventrale (D). Prégonites (E) aussi longs que les postgonites (F) ; les premiers sont plus étroits, courbés et aigus à l'apex ; les seconds ont une forme de crochets, avec la base plus large et l'apex brusquement courbé.

Longueur du corps : 6—16 mm.

FEMELLE. Inconnue.

Distribution géographique. Europe de l'est (y compris la Roumanie), Asie.

Fig. 2. — Armature génitale mâle d'*Ahavanella dreyfusi* sp. n. A = sternite V ; B = cerques et paralobes ; C = distiphallus vu du profil ; D = lobes membranaux vus ventralement (détail) ; E = prégonites ; F = postgonites. Original.

CLÉS POUR L'IDENTIFICATION DES ESPÈCES

1 (2) Cerques assez larges et courbés d'une manière plus accentuée dans la partie apicale. Paraphallus courbé en forme d'arc et prolongé antérieurement avec les lobes paraphalliques longs, larges et plus ou moins arrondis aux bouts. Les lobes membranaux sont paires et, vus du profil, ont une forme de baguettes longues, larges et avec les sommets arrondis et courbés latéralement. Prégonites plus longs que les postgonites . . . *Ahavanella maculata* (Meigen).

2 (1) Cerques plus étroits, moins courbés et graduellement effilés jusqu'au sommet. Paraphallus largement courbé en angle droit. Les lobes paraphalliques sont longs, minces et presque indistincts. Les lobes membranaux sont relativement courts et forment une pièce unique avec les sommets aigus, sous la forme de cornes, courbés latéralement et en haut. Prégonites et postgontes subégaux ...... *Ahavanella dreyfusi* sp. n. . . . .

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THE ULTRASTRUCTURE OF PIGMENTED CELLS IN *TRITURUS VULGARIS* (L.) LIVER

MISCALENCU D., MAILAT FLORICA

The pigmented cells in *Triturus vulgaris* liver exhibit bodies of variable electronodensity. From large bodies of moderate electronodensity detach ovoidal bodies of maximum electrodensity which are melanin granules. The premelanosomes are absent. The hepatocytes are partially dystrophied and some of them are disintegrated following increased activity of pigmented cells.

INTRODUCTION

The variable structure of pigmented cells (melanocytes) from hepatocytes seems to depend by different liver functions in different seasons (8, 16) and also by the physiological state induced by toxins (1, 11 16).

In winter time, the animals are inactive so the liver functions are also diminished; this fact is expressed by the numerous dis aggregated hepatocytes and by the presence of some pigmented aggregates (8). The same situation was described in the case of intoxications (11).

At high vertebrates the effect of serious intoxications is expressed by the modifications of some hepatocyte organites, by the appearance of lysosomes and, in the end, by disaggregation of liver parenchyme (5, 6, 7, 14).

This paper describes the presence and the structure of pigmented cells in *Triturus vulgaris* liver.

MATERIAL AND METHODS

Small liver fragments from *Triturus vulgaris* were fixed for one and half hour in 2.5% glutaraldehyde in 0.1 M buffer cacodylate, washed in the same buffer and fixed in OsO_4. The pieces were embedded in epon, sectioned at 40 millimicrons, doubly stained with uranyl-acetate and lead-citrate. The sections were examined on a Philips electron microscope.

RESULTS

The granules in pigmented cells in *Triturus vulgaris* liver have the same electronodensity as tegumentar melanosomes, but their sizes are variables. These structures are disseminated in the whole cells cytoplasm. In some areas, there are aggregates of big bodies with variable electronodensity in which there are typical melanosomic granules inside and also among them (Figs. 1, 2). It seems that these pigmented granules are detached from the periphery of these bodies. They have a definite contour and contain granular material. Sometimes, this material is fragmented in small bodies with melanic granules inside (Fig. 2).

The pigmented cells nuclei are ovoidal, homogeneously granulated and their nucleolemma is absent on a large part of them. The nuclei look like those of neighbouring hepatocytes (Figs. 1, 3). The hepatocytes exhibit incipient disaggregation signs of the cytoplasmic organites. Their nuclei have a granular electronodense nucleoplasma (more electronodense than the cytoplasm) and from place to place they exhibit small strong electronodense granular aggregates. The nucleolemma is discontinuous on a large distance (Figs. 1, 3). The mitochondria look like spherical vesicles with an electronodense membrane which in some cases is partially disaggregated or even absent. Those mitochondria, which exhibit granules on their membrane, tend to disaggregate ; they are smaller and sometimes are melted with the cytoplasm (Figs. 1, 2, 3). The mitochondria do not exhibit cristae and their matrix has some granules which tend to agglutinate.

Among the mitochondria one can observe the vesicles which are smaller than mitochondria, and on their membrane, have small granules which look like ribosomal granules. These vesicles are not so electronodense and in fact they are active lysosomes in the primary phase.

The lipidic large vesicles are important lysis centres ; at their periphery they fuse with the mitochondria, with the vesicles and with the granules together melted in a homogeneous mass (Fig. 3).

Some hepatocytes are broken on their vascular pole so their content discharges in Disse space. In this situation, the endothelial cells are bounded by vesicles, electronodense granules and mitochondria. The disaggregated material, including the hepatocyte nuclei, are observed inside of the capillary lumen.

DISCUSSIONS

The pigmented cells (melanocytes) are characteristic structures in Amphibians' liver (1, 2, 8, 11). They are more evident by the end of the cold season, when they reveal a close relationship with the macrophages together which build up the granular aggregates (8, 16). In the case of intoxication, they are more abundant (11) and intervene in the capture of disintegrated cells.

Cicero R. et al. (2) sustain that the pigmented cells in *Rana esculenta* liver are histiocytes with melanosomes inside, which reveal the activity of tyrosinase. In *Triturus vulgaris*, the melanosomes seem to originate from an amorphous mass with moderate electronodensity, but the fragments which detach from this mass are of maximum electronodensity which is typical for melanosomes.

We do not observe the premelanosomes which always are present in the tegumentary melanocytes or in other organism structures.

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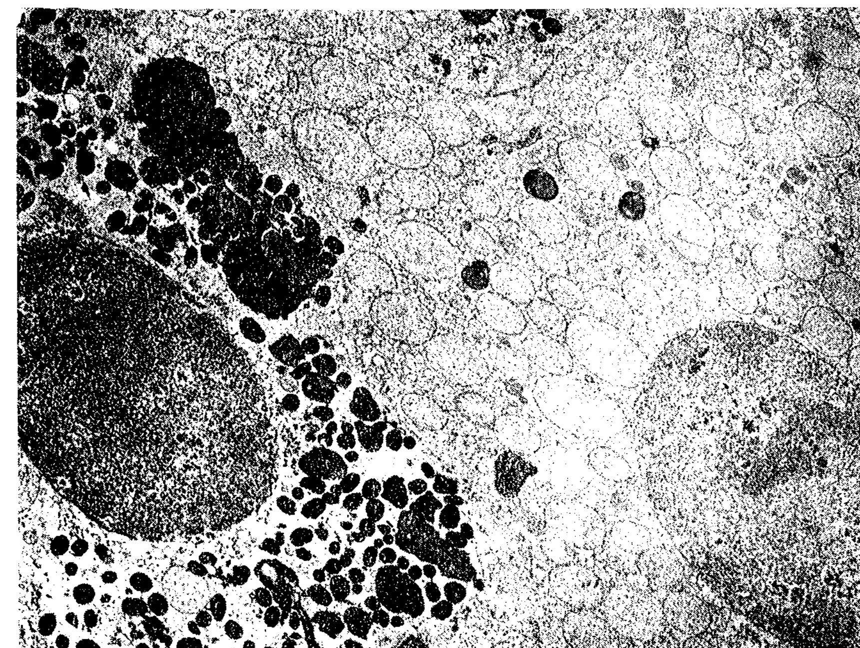

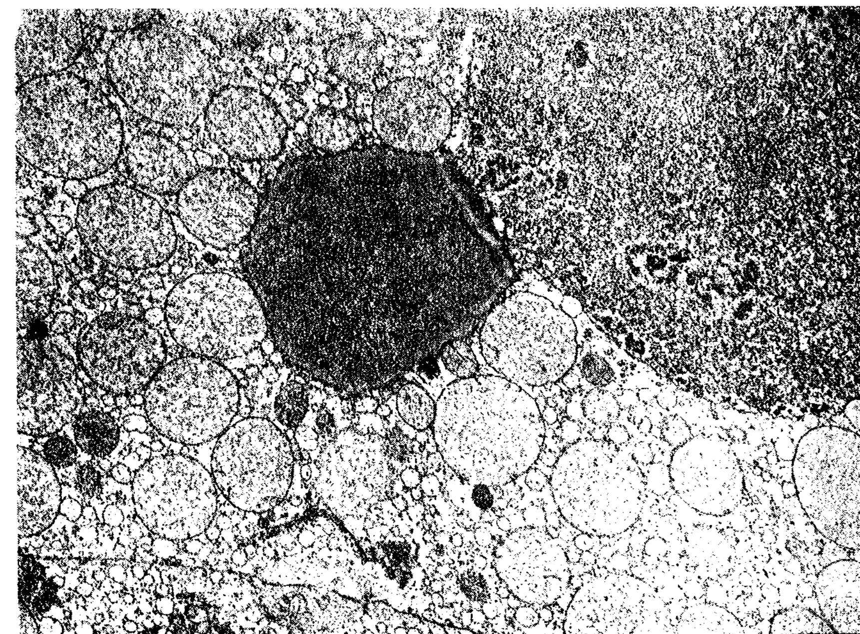

Fig. 1. — A fragment from *Triturus vulgaris* liver. a) One pigmented cell which contains black pigmented granules of different sizes is observed ; some of them are inside of the amorphous mass from which they detach. b) The hepatocytes which exhibit primary and secondary lysosomes, mitochondria and homogeneous granular nuclei can also be observed.

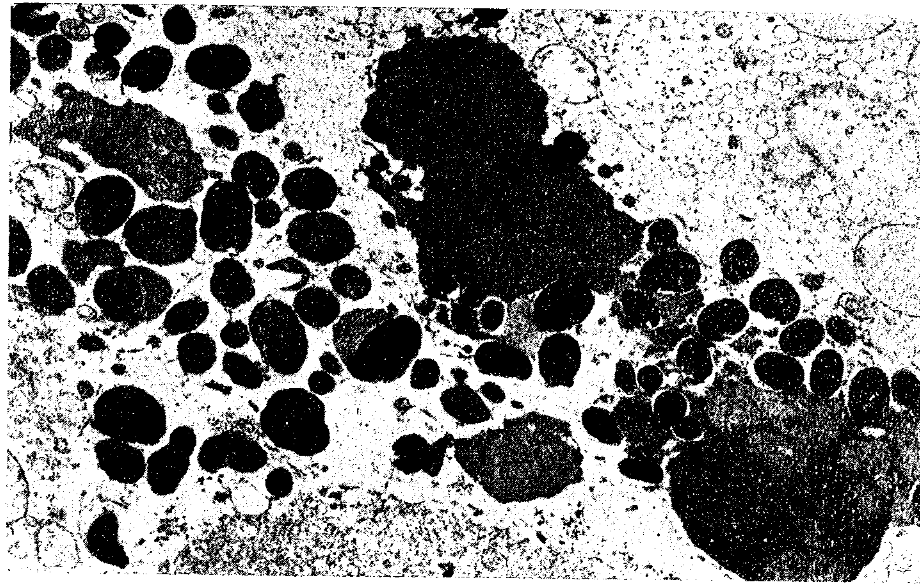

Fig. 2. — A fragment from *Triturus vulgaris* liver. One pigmented cell, which is visible, demonstrates the way in which pigmented granules aggregates are formed. These aggregates exhibit a moderate electronodensity.

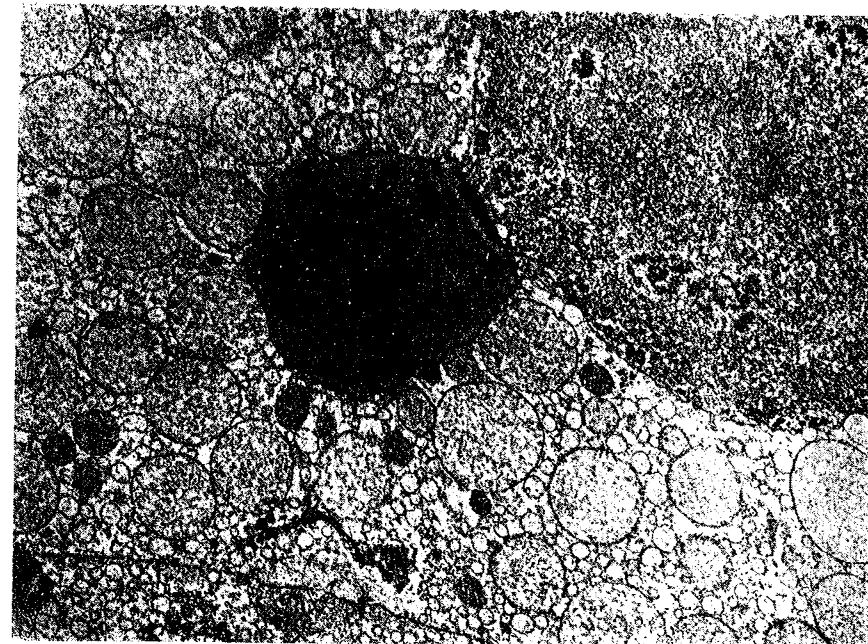

Fig. 3. — A fragment from *Triturus vulgaris* liver. It can see a hepatocyte in which are evident the structure of nucleus, of mitochondria (with no crists) and in special of lipid vesicles inside of the neighbouring organites are lysed.

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OBSERVATIONS ON THE MALE GENITAL APPARATUS AT *ANODONTA CYGNEA PISCINALIS* IN NORMAL AND INTOXICATION CONDITIONS WITH MERCURY CHLORIDE

MANOLACHE VIORICA, NĂSTĂSESCU MARIA, ZĂRNESCU OTILIA, TESIO CĂLIN

The male genital apparatus was investigated in optic microscopy at *Anodonta cygnea piscinalis*, both in normal and intoxication conditions with mercury chloride. The intoxication doses used were: 0.1 μg and 1 μg/l $HgCl_2$/4 months; 5 μg/l $HgCl_2$/4 days; 5 μg and 10 μg/l $HgCl_2$ 1 month.
The modifications both of male sexual cells and of the other associated cells from the male gonade are presented.

Male genital apparatus at Lamellibranchiata (= Bivalvia) was intensely investigated both in optic and electron microscopy in normal and experimental conditions.

In this direction genus Anodonta was little researched especially by electron-microscopy.

In this paper there are presented the histological observations on male genital apparatus in normal conditions. After that we had proposed the investigation on the structural modification appeared in the mentioned system by intoxication with different quantities of mercury chloride.

MATERIAL AND METHODS

Many specimens of *Anodonta cygnea piscinalis* were disecated and the male genital apparatus was used as control. Other male exemplars were under the influence of mercury chloride intoxications. The pollutant was used in different concentrations and sacrifications were made at various times: 0.1 μg/l and 1 μg/l at four months; 5 μg/l at four days; 5 μg/l and 10 μg/l at one month. The gonads were fixed in Bouin and calcic formol. The sections were stained with histological and histochemical current technics (Hemalaun-eosine; alcian-blue; PAS).

The species of Anodonta genus have a great captation pollutant capacity. For this reason they are used as biological indications and for this purpose we appreciated the remained mercury quantity in the water.

RESULTS

The genital male apparatus of *Anodonta cygnea piscinalis* is composed of testis, gonoduct and annexed glands. Sometimes, hermephrodit species also appeared and in this case we observed female gonads too.

The gonads are situated on the dorso-lateral and postero-dorsal sides of the visceral mass. At the maturity, the gonads are more developed and form prolongations penetrated between hepatopancreas diverticles.

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The male gonad is surrounded by the vacuolar connective tissue. The testis is formed by seminiferous tubules with chists. In the last there are the sexual cells in the same development stage (Fig. 1). At the periphery seminiferous tubules are big little chromatic nuclei cells, with 1—2 nucleoli. At some specimens we observed the tubules with sexual cells in all spermatogenesis phases. The spermatogonia, as spherical cells with little chromatic nucleus and visible nucleolus have an abundant cytoplasm. The spermatogonia present some usual mitotic divisions and transform into the spermatocyte I. These have a little evident growth period, in this way the size of cells is not so big. The I spermatocytes undergo the first maturation division, which is meiosis as other animal species. At the end of this process the little-sized spermatocytes II appear. For this reason, the chists are very compact with numerous sexual cells. The spherical nucleus is more chromatic. The II spermatocytes undergo the second mitotic division and transform in spermatides.

The last spherical cells have a visible nucleus with little cytoplasm. In their evolution, the spermatid changes the form, becomes more elongate with an ovale nucleus which gradually becomes chromatic. In the evoluated spermatid we observed the presence of a short flagellum. These cells transform in spermatozoa, so in the distal end of the seminiferous tubules, there are numerous chists with sperms. These tubules continued with the gonoduct where penetrate the sperms. The gonoduct is lined with an unistratified ciliated epithelium (Fig. 2). The spermatozoa are very little with a very chromatic nucleus. At the apical end one can observe the acrosome in certain colorations : P.A.S., alcian-blue. The axial filaments of the sperms towards in the lumen chists of the gonoduct.

In some disections effectuated at different time intervals we remark in the male gonad the frequent existence of chists with spermatids and spermatozoa. These observations were made especially in long time experiments.

In the testis there are nutritive cells together with sexual cells. The last have numerous prolongations in the direction of evoluated stages of sexual cells and the nucleus remains at the periphery of tubules. The nutritive cells have a role and in the phagocitosis of relict cytoplasm of spermatide.

At the periphery of the tubules and chists there are the conjunctive tissue, with numerous nuclei and collagen fibers. Also, in these tissue in the proximity of tubules, we observe the presence of sanguine cells, especially amaoebocytes. Also we remark the amoebocytes in the seminiferous tubules with a phagocitose role.

In the intoxicated specimens with mercury, in the testis there are presented different histological modifications in correlation with various utilised doses of pollutant.

These histological aspects are very significant at the same dose and time of experiment.

At specimens treated with 1 μg $HgCl_2$/l, for a long time — four months — we observed the very pronounced destructions. So the many chists were disintegrated, the sexual nutritive and conjunctive cells are scattered. In this case it is very hard to identify these cells. Sometimes we

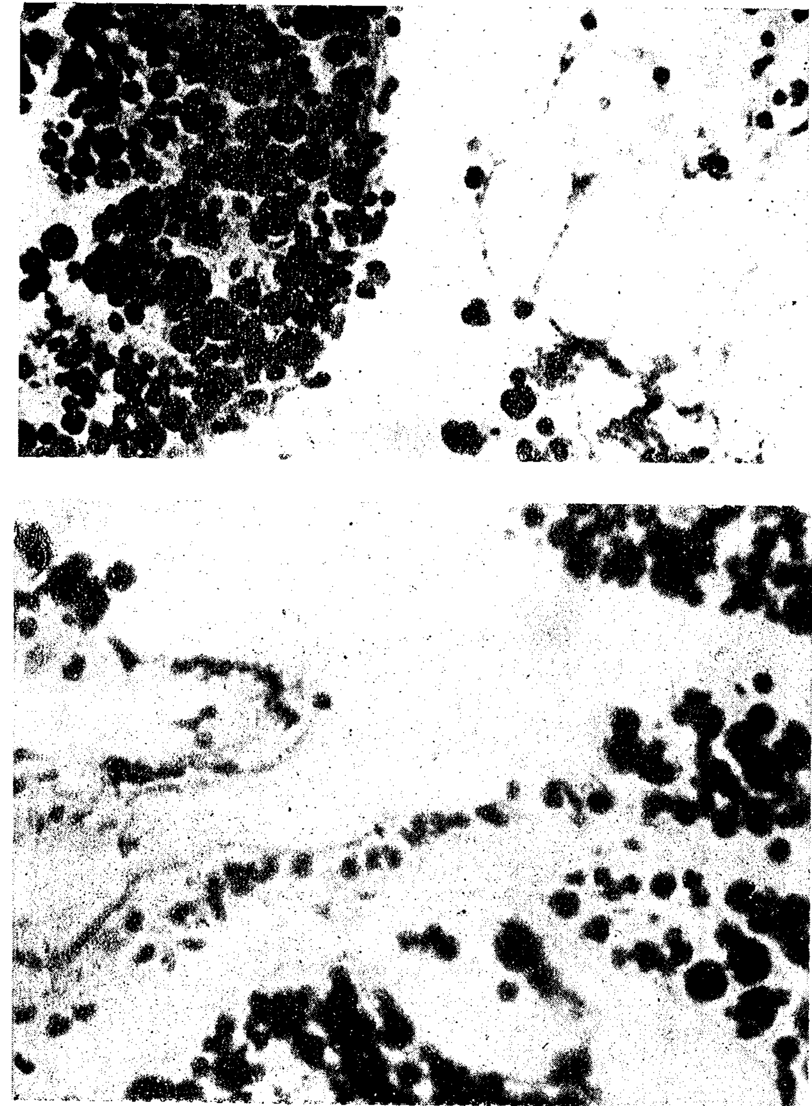

PLATE I — *Anodonta cygnea piscinalis* — male control.
Fig. 1. — Normal aspect of the testis.
Fig. 2. — The gonoduct with an unistratified epithelium.

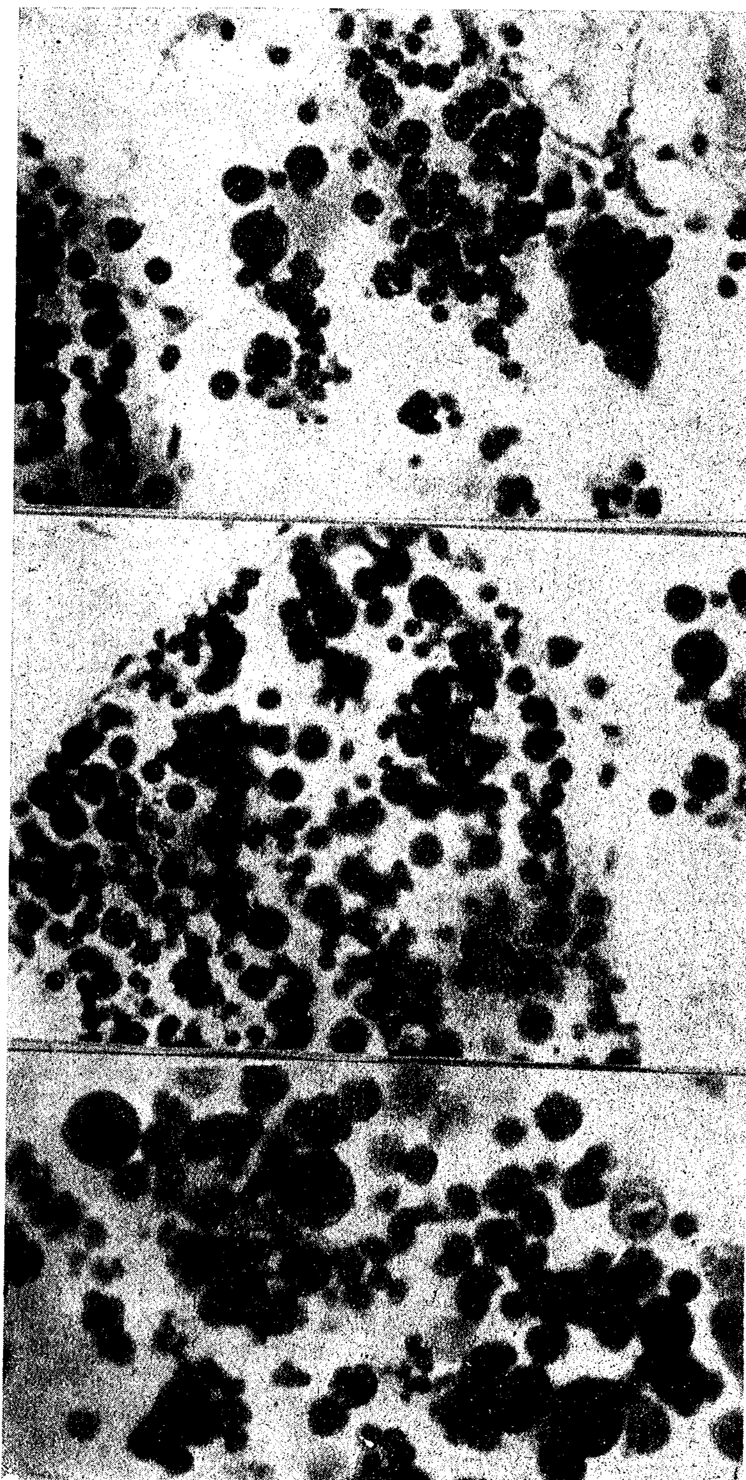

PLATE II — *Anodonta cygnea piscinalis* — male gonad under the action of different doses of mercury chloride.

Fig. 3. — 4 months after treatment with 1 μg $HgCl_2$/l.
Fig. 4. — 4 days after treatment with 5 μg $HgCl_2$/l.
Fig. 5. — 1 month after treatment with 5 μg $HgCl_2$/l.

remark a rarefaction of chists (Fig. 3). Amoebocytes invade the seminal tissue with a phagocitose role.

Striking is the fact that the nuclei of nutritive cells are normal. Sometimes numerous mercury granulations are evident both inside of tubules or in the adjacent conjunctive tissue.

At 5 μg $HgCl_2$/l-four days there are present the chists with normal aspect and completely partial destroyed cells. The nuclei of disintegrated cells are in the main pycnotic. The membrane of nealtered chists is sometimes evident.

At 5 μg $HgCl_2$/l-one month the modifications of the chists are not more pronounced than it is observed at the specimens exposed to the same dose for four days. Probably, it was the result of the medium adaptation of these animals which accumulate the pollutant.

In the microscopic sections we observe many normal chists with spermatogonia, spermatids and sperms. The many destructions appear especially between spermatids and sperms (Fig. 5). Sometimes, the membrane seminiferous chists are disintegrate. Also, the nuclei became pycnotic in the remain cells. In the gonoduct cells the nuclei are pycnotic.

At the moment of sacrification, we appreciated the quantity of $HgCl_2$ remained in the water. This was: 1.72 μg/l at a dose of 5 μg $HgCl_2$/l — 1 month; 1.63 μg $HgCl_2$/l at a dose of 10 μg $HgCl_2$/l — 1 month and 1.09—1.12 μg $HgCl_2$/l at 1 μg $HgCl_2$/l — 4 months.

DISCUSSION

The normal testicle of *Anodonta cygnes piscinalis* L. has the same structure with those met in other moluscs and anthropods (6, 5, 10, 7, 4, 2). As *Anodonte cygnea* species, at our species, we observe the presence of all stages of spermatogenesis inside the seminiferous tubules. Sometimes at *Anodonta piscinalis*, the distal end of tubules points only to small-sized spermatids and sperms. This fact confirms Kelley's (1982) observations on *Mytillus californianus* Conrad. This author remarks that in certain periods of development the spermatogenesis is not detected.

In electron microscopic researches, other authors (11) at *Cipangopaludina malleata* Reeve recognize the presence in the testis of both the sexual and nutritive cells. We observe the same aspects in optic microscopy at *Anodonta cygnea piscinalis*. In addition, in the central part of the chists, we describe the antholysis figures. These observations demonstrate the role of nutritive cells in the phagocitosis of cytoplasmic relict of spermatide.

The investigations concerning the influence of mercury of spermatogenesis in moluscs are scarce. In this direction, we mention the electron microscopic researches on *Crassostrea gigas*. Durfort M. 1991 (3) remark the spermatozoa alterations in the presence of internal parasites. Bozzo G. M., 1993 (1) observe the same alterations due to environment modifications.

We describe the pronounced alterations of the sexual cells at a 1 μg $HgCl_2$/l—4 months dose. Nevertheless, at the same dose, the nuclei nutritive cells keep their normal structure. Also, we remark the presence

of amoebocytes inside the seminal tissue, with a phagocitosis role. At 5 μg $HgCl_2$/l-1 month, we observe especially the advanced alteration stages of spermatogenesis.

The researches of reproduction on *Anodonta cygnea piscinalis* as other animal species have a great value in the ecological investigations. These offer important data in connection with the distribution and structure of populations.

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IN VITRO AND *IN VIVO* CYTOSTATIC ACTION OF SOME VEGETABLE POLYPHENOLIC PREPARATIONS

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In vitro investigation of the different doses effect of PA_2 III and PA_3 on HeLa cell cultures development has confirmed the high inhibitory potential of these polyphenolic preparations, obtained from *Asclepias syriaca* plant leaves. *In vivo* testing of their action on the evolution of the Guérin T–8 lymphotropic epithelioma and of the Walker 256 carcinosarcoma was also performed. The comparative analysis of our values of the pharmacodynamics effect evaluation indices with those imposed by the reference preclinical screening programs revealed their compatibility. Thus, the antitumoral pharmacotherapeutic effect of the PA_2 III and PA_3 polyphenolic preparations has been h ghlighted.

The investigation of the effect of a total crude alkaline vegetal extract, obtained from Asclepias syriaca plant, on HeLa cell cultures evolution and on the tumoral development process pointed out its cytostatic action both *in vitro* and *in vivo* [20].

In order to make a final selection of an active cancerostatic agent with therapeutic significance, some total polyphenolic preparations have been separated from different morphological components in various stages of vegetation [21] and certain phenolic fractions from the October harvested lea ves were obtained by the successive extraction with different solvents — [22]. Their *in vitro* preliminary screening revealed that the various samples were characterized by different degrees of cytostatic effectiveness, the most active being the PA_2 III and PA_3 polyphenolic preparations [21], [22].

In the present paper are exposed the testing results of the *in vitro* and *in vivo* cytostatic and antitumoral actions of the PA_2 III and PA_3 polyphenolic preparations on HeLa cell cultures and on some experimental tumor systems, respectively.

MATERIALS AND METHODS

The PA_2 III and PA_3 polyphenolic preparations have been separated and purified from the crude alkaline vegetal extracts, obtained from *Asclepias syriaca* leaves harvested in October 1991, after the chemical removal of hemicellulosic compounds. The chemical composition of both preparations is similar, PA_3 being distinguished from PA_2 III by the absence of latex, wax, fatty alcohols, fatty acids as well as of terpenoids, which were excluded by an extraction with cyclohexane.

The *in vitro* cytostatic action was assessed by a comparative follow-up of the total protein dynamics during the evolution of the treated and control HeLa cell cultures, of human neoplastic origin, as an expression of the cell proteinosynthesis level.

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The test tubes were inoculated with 1×10^5 cells in a IC—65 growth medium with 10% calf serum. After 24 hours, when the monolayer stage was attained and the cultures were in the logarithmic phase of development, the initial medium was replaced with a medium containing different doses (0.5, 1.0 and 1.5 mg/ml) of polyphenolic preparations.

At 24, 48 and 72 hours of culture development the medium was discarded from the control and treated test tubes. The cell layer was washed with TFS and subjected to total protein determination [14], [18].

For each culture type and time interval five culture tubes were used and the statistic analysis of the results was performed using Student's "t" test [28].

In vivo testing of the polyphenolic preparations antitumoral activity was made on Wistar white female rats of 125—150 g, bearing either T—8 Guérin lymphotropic epithelioma or Walker 256 carcinosarcoma, both experimental tumoral lines being of solid type.

24 hours after the tumoral transplant the antitumoral treatment started and lasted for 16 days, in the case of Guérin T—8 tumor and for 19 days, in the case of Walker 256 tumor. It was applied by intraperitoneal (ip) daily injection of the polyphenolic preparations in different doses (mg/kg body weight), which are presented in the tables. An equivalent volume of physiological serum was administered to the control animals.

The estimation of the antitumoral activity was based on the comparative follow-up of the mean tumor weight (M.T.W.) at sacrifice in the treated and control groups.

The evaluation of antineoplastic action was realized by the percentage determination of mean tumor regression (% M.T.R.) and by the calculation of the T/C value (where T = MTW for the treated group and C = M.T.W. for the control) and of the statistic significance using the Student's "t" test.

The appreciation of the *in vitro* cytostatic and *in vivo* cancerostatic effects was performed by a comparative analysis of our values of the evaluation indices with those imposed by the selection criteria of antitumoral substances established by the preclinical screening programs of the National Institute for Chemotherapy of Cancer from the U.S.A. [13] and of the Institute of Microbiology and Experimental Therapy from Germany [11] for these preliminary steps.

RESULTS

The *in vitro* investigations followed the effect of the PA_2 III and PA_3 different doses on HeLa cell culture development, the experimental results being included in Fig. 1 and Fig. 2, respectively.

It can be observed from Fig. 1 that the total protein dynamics, which characterizes the evolution of HeLa cell cultures incubated with 0.5 mg/ml, presents — as compared to the control one — slight fluctuations due to the decreases of protein concentrations induced by PA_2 III polyphenolic preparation. These allow the estimation of some culture development inhibitions of 28.9% at 48 hours and of 37.5% at 72 hours. More profound alterations of the cellular proteinosynthesis

have appeared to an PA_2 III increased dose (1.0 mg/ml). The significant cytostatic effect (of 42.8% and 54.0%, respectively) is illustreated by the diminished protein values registered during the treated culture evolution as compared to the control. The maximum inhibitory action of the PA_2 III preparation was induced at a 1.5 mg/ml dose, the protein

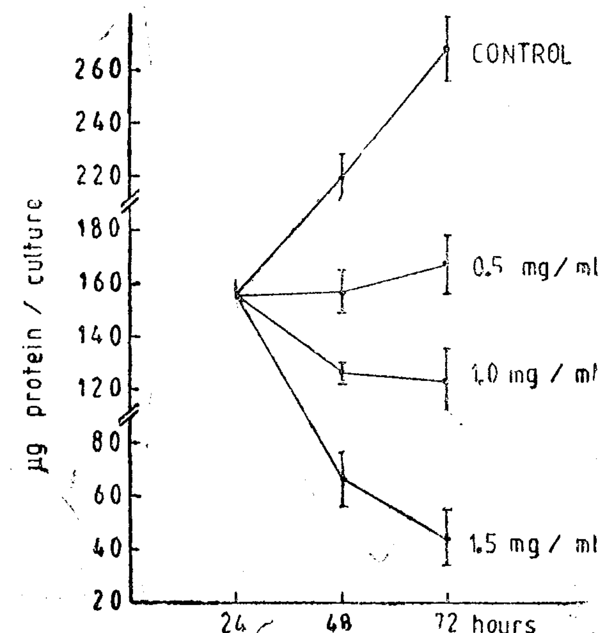

Fig. 1. — Protein dynamics of HeLa cell cultures incubated with different doses of PA_2 III polyphenolic preparation.

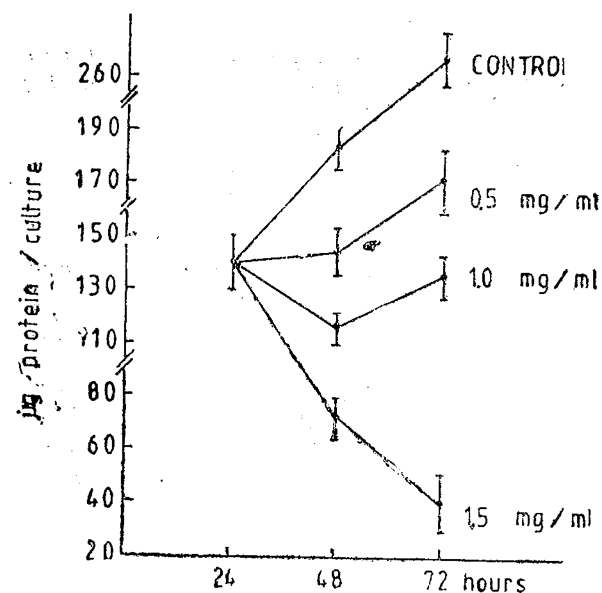

Fig. 2. — Protein content of HeLa cell cultures treated with different doses of PA_3 polyphenolic preparation.

level decreases reaching 70.2% and 83.6%, respectively, from the normal values of the controls at both intervals analysed.

In another experiment, illustrated by Fig. 2, the effect of PA_3 different doses on the HeLa cell culture development was tested. It is observed that the 0.5 mg/ml dose induces a small modification of the cellular protein biosynthesis. This is expressed by decreases of only 21.1% and 33.6% of the protein concentrations comparatively with the corresponding control values. The increase of the incubating dose to 1.0 mg/ml was correlated with an intensification of the cytostatic effect. It is argued by the protein level diminutions at 48 hours and 72 hours, which allowed the assessment of some culture development inhibitions of 36.8% and 47.4%, respectively. Finally, the augmentation of a dose to 1.5 mg/ml was accompanied by a very significant potentiation of the culture development inhibitory effect. In this case, the total protein concentrations were lower with 60.8% and 84.2%, respectively, than the corresponding protein values of the control cultures.

It can be concluded from these experiments that the *in vitro* cytostatic effect of the PA_2 III and PA_3 polyphenolic preparations is reproducible and that the inhibitory potential is dependent on the dose used at incubation of the HeLa cell cultures. The bulk of the experimental results presented above imposes including of these products in a primary step of the *in vivo* preclinical screening on rats bearing of different experimental tumoral systems in order to evidence their antitumoral pharmacodynamic effect.

The *in vivo* effect of the PA_2 III and PA_3 polyphenolic preparations on Guérin T–8 lymphotropic epithelioma development can be analysed from the experimental data presented in Table 1. In comparison with the control group, it is observed that the i.p. daily treatment with PA_2 III,

Table 1

Antitumoral therapeutic effect of the PA_2 III and PA_3 polyphenolic preparations, administered daily i.p. in specific doses to the rats bearing of Guérin *T–8* lymphotropic epithelioma. Figures in brackets indicate the number of experimental animals

Group/Treatment	M.T.W. (g)	% M.T.R.	T/C value	Statistical significance
CONTROL	12.4 ± 1.8 (15)	–	–	–
PA_2 III (5 mg/kg. b.w.)	6.1 ± 1.6 (10)	50.8	0.49	$p<0.01$
CONTROL	15.8 ± 1.6 (15)	–	–	–
PA_3 (45 mg/kg. b.w.)	7.1 ± 1.3 (10)	55.1	0.45	$p<0.001$

in a dose of 5 mg/kg.b.w., has induced a significant decrease of MTW ($p<0.01$). This allows the estimation of a MTR of 50.8% and of a T/C value of 0.49. It can be also seen that the i.p. daily therapeutical administration of PA_3 (45 mg/kg.b.w.) has inhibited the Guérin T–8 development comparatively to the control group, the MTR and T/C value being of 55.1 % and 0.45, respectively.

The antitumoral activity investigation of the PA_2 III and PA_3 polyphenolic preparations was extended on Walker 256 carcinosarcoma, the experimental results being included in Table 2. The i.p. daily therapy has induced a significant antitumoral effect ($p<0.01$ and $p<0.001$) as compared to the normal tumoral development of the controls. The values of the evaluation indices are in the case of :

– PA_2 III preparation : MTR of 50.0% and T/C of 0.50 ;
– PA_3 preparation : MTR of 45.4% and T/C of 0.54.

Table 2

Cancerostatic activity of the PA_2 III and PA_3 i.p. daily therapies on rats bearing of Walker 256 carcinosarcoma. Figures in brackets indicate the number of experimental animals

Group/Treatment	M.T.W. (g)	% M.T.R.	T/C value	Statistical significance
CONTROL	12.2 ± 1.5 (15)	–	–	–
PA_2 III (5 mg/kg. b.w.)	6.1 ± 1.0 (10)	50.0	0.50	$p<0.01$
CONTROL	15.2 ± 1.0 (15)	–	–	–
PA_3 (45 mg/kg. b.w.)	8.3 ± 1.3 (10)	45.4	0.54	$p<0.001$

DISCUSSION

A maximum significance desideratum of contemporary pathology is represented by the effectiveness augmentation of the antineoplastic chemotherapy. The discovery of some new cancerostatics represents a very important concern at present, when chemotherapy holds pride of place. The identification of an antitumoral agent – the main purpose of the multistage screening chemotherapeutic programs – is the consequence of some numerous and complex preclinical and clinical pharmacological researches on adequate experimental models using different biological systems of testing [10], [29], [30], [32].

The great number, the various nature (biosynthesis, semisynthesis and synthesis) and the structural diversity of the active biological substances with supposed antitumoral action haves conditioned the introduction of an *in vitro* preliminary testing phase on cancerous cell cultures [1], [4], [6], [7], [10], [13], [23 – 26], [31]. This assures, by the imposed appreciation criteria, the selection only of the active cytostatic and/or cytotoxic agents. They will be included subsequently ulteriorly in the successive and interdependent steps of the *in vivo* preclinical screening on animals bearing of diverse experimental tumoral lines. The qualitative and quantitative evaluations of their specific action of tumoral development inhibition will assure or not the final preclinical pharmacological characterization of these as active cancerostatic agents [5], [7], [10–13], [24–26].

The *in vitro* testing of the PA_2 III and PA_3 polyphenolic preparations highlighted their inhibitory effect on HeLa cell culture develop-

ment, expressed by a profound and significant perturbation of the cell total protein dynamics.

The progressive increase of the incubating doses was correlated with a corresponding intensification of the *in vitro* cytostatic efficiency, demonstrating the existence of a dose-effect relationship. Thus, the dose increase from 0.5 mg/ml to 1.0 mg/ml amplified the final inhibitory effect with 44.0%, in the case of PA_2 III preparate and with 41.0%, in the case of PA_3. Once again, the utilization of the maximum dose (1.5 mg/ml) was accompanied by a final augmentation of cytostatic action with 55% and 77% for PA_2 III and PA_3, respectively.

The high intensity of the specific action, its rapidity and reproducibility, the dependence of the inhibitory potential of the dose used at cell culture incubation – the appreciation criteria imposed of the American screening program for this preliminary stage [13] – allow the characterization of the polyphenolic preparations PA_2 III and PA_3 as *in vitro* cytostatic and/or cytotoxic agents. At the same time, the *in vitro* effect imposes their passage in the circuit of the *in vivo* preclinical screening.

Consequently, in a first step, we followed, on rats bearing different experimental solid tumoral systems, the influence of the PA_2 III and PA_3 therapy on the development of the Guérin T–8 lymphotropic epithelioma and Walker 256 carcinosarcoma in order to evidence their antitumoral pharmacodynamic effect.

The tests, performed on both experimental models, revealed the antitumoral activity of the polyphenolic preparations, it being illustrated in the case of:

– PA_2 III, by MTR of 50.8% and T/C of 0.49 as well as by MTR of 50.0% and T/C of 0.50, corresponding to Guérin T–8 tumor and Walker 256 tumor, respectively;

– PA_3, by MTR of 55.1% and T/C of 0.45 as well as MTR of 45.4% and T/C of 0.54, estimated on Guérin T–8 tumor and Walker 256 tumor, respectively.

The appreciation of the antitumoral action significance of the studied products involves the comparative analysis of our values of the evaluation indices with those stipulated by the selection criteria of the active cancerostatic agents. The standard values were established for this primary step of multistage preclinical screening programs elaborated by the Institute of Microbiology and Experimental Therapy from Germany [11] and by the National Institute for Cancer Chemotherapy from the U.S.A. [13].

According to these reference programs, in a first step, the anticancerous experimental therapy – on at least on tumoral system from three tested ones – must induce a MTR of at least 35% in the treated groups comparatively to the controls [11] or must allow the assessment of a T/C ratio of 0.54 – 0.64, for the solid tumor lines.

The comparative analysis of the evaluation indices values of the induced antitumoral action with those standard – by their compatibility – revealed the cancerostatic pharmacotherapeutic effect of the PA_2 III and PA_3 polyphenolic preparations.

Data from the oncobiological bibliography pointed out the *in vitro* and *in vivo* activity of some phenolic structures on the malignant cells, too [2], [3], [8], [9], [15], [16], [17], [19], [27].

Finally, our results – giving a positive answer to the first question of the qualitative evaluation of the specific pharmacological effect – have assured the extension of the researches in order to demonstrate the reproducibility of the PA_2 III and PA_3 antitumoral action – the second problem of the preclinical qualitative evaluation.

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INFLUENCE OF THE PARAVENTRICULAR NUCLEUS UPON THE CELLULARY – MEDIATED DEFENCE FUNCTION OF THE ORGANISM

V. P. HEFCO and I. NEACŞU

Experiments were performed on two groups (an operated and a sham-operated one, respectively) of Wistar male, mature rats. Mechanical lesion of the hypothalamic paraventricular nucleus (PVN) induces a decrease in the number of total leukocytes and of the leukocytary groups, as well. One week after the surgical intervention, the most ample decreases are to be observed with eosinophils (84 %) and monocytes (47 %), the other indices decreasing with about 30 %, as compared with the sham – operated group. The variations recorded are maintained along the whole duration of the experiment (3 weeks). Modifications in the leukocytary formula are explained through the functional modifications of the endocrine and nervous vegetative systems, respectively, and of the CNS connections, induced by PVN lesion, which has consequences upon both leukopoiesis and the organism's defence function.

Paraventricular nucleus (PVN) presents a series of neurons known as inducing various neurosecretions, such as thyroliberin (2), corticoliberin (26), (36), PHI (23), a neuropeptide belonging to the family of secretin (20), VIP (vaso-active intestinal peptide), encephaline (17) etc.

With all vertebrates taken into study, an interaction between the nervous, endocrine and immune systems, respectively, known as neuroimmunomodulation (NIM) (39), was observed. Among the endocrine substances influencing directly the immune function, there are worth mentioning : corticoadrenal and sexual steroids, thymozin, prolactin, the growth hormone, melatonin, catecholamines, serotonin and several other neurotransmitting substances and peptides acting upon the nervous system (40), (41). PVN has numerous nervous connections with the neural lobe and with the cerebral cortex, brain stem and spinal cord (3), (11), (35), as well. Through these connections, several vegetative and endocrine functions, the defence function of the organism included, may be influenced (38), (41).

The present study analyses the influence of the paraventricular nucleus upon the cellulary-mediated defence function of the organism, as evidenced by the dynamics of the number of leukocytes and of the leukocytary formula, as well, under conditions of mechanical lesion of this nucleus.

MATERIAL AND METHODS

Experiments were made on two groups of Wistar male rats (sham-operated = SO ; and operated = OP), weighing around 220 g, at the beginning of the experiments.

The PVN has been injured stereotaxically, the animal's head being placed so that the plane crossing the auditory conduct and behind the

incisors should form an 11° angle (nose downwards) with the horizontal plane. The lesion was made with a rotating knife designed to make an inverted cone-shaped lesion centered on PVN, with a radius of 1.8 mm and 2 mm in height. To this end, the knife, directed caudally, was introduced downward through the sagittal sinus to the brain's base, 1.8 mm caudally to the bregma, followed by its 360° rotation to the right and to the left, as well.

In sham-operated rats, the knife was lowered 5 mm beneath the skull's surface, without any subsequent rotation. After the intervention, the skull's surface was powdered with streptomycine. At the end of the experiment, a histological control of the intervention performed was made, according to the method of Guzman-Flores and coworkers (15).

The total number of leukocytes was established by direct counting, on a microscope, by means of a hemocytometer, while the various categories of leukocytes were determined by the classical staining method of May Grünwald-Giemsa. Determinations were made on the 7th, 14th and 21st day, respectively, after the surgical intervention, with blood collected from tail vessels. Statistic evaluation employed Student's "t" test.

RESULTS

The PVN lesion induces a decrease in both the total number of leukocytes (leukopenia) and in each leukocytary group forming the leukocytary formula (Table 1).

Table 1

Variations of leukopoiesis after PVN lesion. The values represent the Mean ± SE, while the numbers given in parenthesis — the number of animals. SO = sham-operated ; OP = operated ; W = weeks postoperatively.

Group	Weeks (W)	Groups of leukocytes (number and % of total)					
		Total leukocytes	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
SO (8)	1 W	16345±700 100%	6937±350 42.44%	78±20 0.47%	106±11 0.64%	8798±300 53.82%	245±20 1.49%
	2 W	12465±366 100%	4377±408 35.11%	16±1.5 0.13%	72±3 0.58%	7819±390 62.65%	185±15 1.48%
	3 W	17600±1306 100%	6629±449 37.66%	25±1 0.14%	78±8 0.44%	9065±780 51.50%	204±16 1.15%
OP (6)	1 W	12512±1100 100%	5694±400 45.49%	13±2 0.10%	69±12 0.55%	6586±500 52.62%	131±18 1.04%
	2 W	9080±1013 100%	3127±396 34.43%	11±2 0.15%	62±2 0.70%	5737±591 63.18%	134±12 1.47%
	3 W	12430±998 100%	5117±427 41.16%	19±1 0.15%	56±6 0.45%	6961±238 56.0%	155±17 1.24%

The most pronounced per cent variations are observed with eosinophils and monocytes which, a week after the operation, decrease to 84% and 47%, respectively, the other indices decreasing with about 30%. Within each group, the variations appearing one week after the intervention do not suffer significant modifications along the three weeks of the experiment, which indicated the persistency of the above-mentioned modifications.

DISCUSSIONS AND CONCLUSIONS

The obtained data have evidenced that lesion of PVN induces a significant decrease in both the total number of leukocytes and in each group of leukocytes, separately, the decrease being maintained along the whole 3 week-period of the experiment; in this way, the cellulary-mediated defence capacity of the organism is affected.

Recently, at the level of PVN, about 10 subnuclei — differing in their cellular composition, neuropeptides and neuromediators content, and in their cell composition, and afferent and efferent connections (3), (4), (19), (24), (27), (32), (34), as well — have been identified. The existence of an immense number of afferent and efferent connections of PVN attests the major role played by this nucleus in maintaining homeostasis.

Application of the method of lesion or of PVN stimulations evidenced that this nucleus has a significant role in controlling the adenohypophysis secretion of corticotropin and of other related peptides (30). Certain PVN neurons contain corticoliberin and arginin-vasopressin (17). PVN lesion reduces a decrease of the ACTH and plasma corticosterone concentration which usually appears through the action of the stressing agents or hypothalamus' stimulation (5), (9), (21). The lymphopenia evoked by the action of the stressing agents has been explained through the hyperfunction of the corticoadrenal and involution of the thymus and spleen (16), (22), (31). Decrease of the number of leukocytes, generally, and of eosinophiles and mastocytes, especially, cannot be attributed, in our experimental conditions to the hypersecretion of glucocorticoids, as lesion of PVN provokes a decrease in the activity of the hypothalamo-pituitary-adrenal cortex system.

The thyroid, too, intervenes in the regulation of leukopoiesis, hyperthyroidism being accompanied by eosinophilia and lymphocytosis, while the hyperthyroidia is characterized by leukopenia. The leukopenia observed in our experiments may be partially attributed to the reductions of the thyroid activity, as PVN secretes also thyroliberin (2).

A stimulating effect upon leukopoiesis is exercised, too, by the androgens, growth hormone and prolactin. Although the structure of prolactoliberin has not been established up to now, a prolactoliberin-like action was attributed to certain neuropeptides, such as VIP (1), oxytocin (29), thyroliberin (28) and PHI—27 peptide (44), that may be identified in the porthypophyseal blood vessels. The neurons from PVN secrete most of these neuropeptides, that arrive in the porthypophyseal blood vessels (8), (18), (33), (42). Kiss and coworkers (25) have observed that PVN lesion does not affect the basal concentration of prolactin from the plasma, yet it reduces the hypersecretion of prolactin, induced by some

external factors, such as the stimuli starting from the mammary region, produced by suckling.

It is therefore possible that, in our experiments, too, the effect of the exogenous and/or endogenous stimuli, acting continuously upon the immune system by means of CNS, should have diminished prolactin secretion, which might determine, partially, a decrease of leukopoiesis.

The morphine-like substances, too, exert immunosuppressing effects, along with those inducing intensification of the cancerous tumour development (37). Nevertheless, in our experimental conditions, the decreases of global leukopoiesis could not be attributed to the hypersecretion of morphine-like substances, as, after lesion of PVN, known as secreting encephalins (17), too, the concentration of morphine-like substances may be expected to decrease. Recently issuing of numerous data may be mentioned, evidencing that the organism's defence function may be regulated through CNS and through mechanisms others than the humoral ones. For example, it has been demonstrated that lesion of the anterior hypothalamus induces an increase of the number of T lymphocytes in the case of hypophys ectomized rats (13).

The direct link between CNS and leukopoietic tissues could be manifested by direct nervous ways, as well. The sympathetic and parasympathetic nervous terminations have been identified at the level of the thymus, spleen and lymphatic ganglions (10), (12), (14), (45). The parasympathetic enervation of the thymus comes from the ambiguous nucleus, the neurons of which produce, too, some parasympathetic fibers, attached to the vagus nerve (10).

PVN may influence the sympathetic and parasympathetic function by means of its connections with the vagus nerve and the sympathetic pre ganglionary neurons from the thoracic intermediolateral column of the spinal cord (43). PVN lesion intensifies the activity of the vagus nerve and diminished the activity of the splanchnic nerve (43). According to the ideas of Benetato et al. (6), stimulation of the sympathetic hypothalamic region with UV rays induces an increase in the number of circulating leukocytes.

The leukopenia observed in our experiments as a result of PVN lesion might be therefore mainly induced by the diminishing of the sympathetic tonus. Without denying the role played by the hypothalamus upon the organism's defence function through the endocrine system, our data suggest that the hypothalamus may modulate the immune function on a direct nervous way, as well, if considering its regulating role upon the vegetative nervous system. This might explain the prolongued maintenance of leukopenia as a result of the PVN lesion, in the absence of corticoadrenal hyperfunction. Besides these data, the identification of the presence of adrenergic and cholinergic receptors on the lymphocytes' surface (7), (36) represents another argument supporting the direct connection between the central nervous system and the organism's defence function.

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THE ROLE OF THE PVH NUCLEUS IN MODULATING NEUTROPHILS' PHAGOCYTOSIS OF RATS

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Lesion or isolation of the hypothalamic paraventricular nucleus (PVH), alone or together with the hypophysotropic region, induces a significant decrease of the phagocytosis index in neutrophils. Along the whole period of the experiment, i.e. one month – the lowest values of the phagocytosis index have been recorded following complete isolation. In our opinion, the PVH nucleus belongs to a more extended hypothalamic region, stimulating phagocytosis to neutrophils. This role is exerted by modulating the presence of some essential humoral agents in the phagocytic process on an endocrine and a nervous way, especially through the efferent pathways localised posterior to the median hypothalamus.

Key words : PVH nucleus, phagocytosis, circulating neutrophils, humoral factors.

By means of neuromediators and neurohormones, central nervous system (CNS) modulates several and various functions of specific immunity (3, 4, 8, 9). Less known is the role of CNS in modulating the organism's natural resistance and especially in the phagocytic activity of infection agents. The response of phagocytes is very complex, including various vegetative reactions, aimed at annihilating the stimuli and also at regulation the homeostatic mechanisms. Among the first studies, in this way, are those of Baciu et al., whose idea is that the hypothalamus should play a modulating role in the phagocytic function, thus intervening in the regulation of immune homeostasis (2).

On the other side, the PVH nucleus, best individualized among the hypothalamic structures, plays a very important role in controlling the vegetative functions, through integration of the nervous and endocrine signals (11).

Because of the importance of phagocytosis in the organism's natural resistance, as a first active barrier against infection stimuli, as well as the significant number of information the PVH nucleus may receive from somato-, visceroreceptors and some CNS regions (1,10), along with its efferents with the vegetative nervous system (1, 10, 13), the present study analyzes the role of PVH in modulation of neutrophils' phagocytosis.

To this end, the PVH nucleus has been lesioned stereotaxically, then isolated, alone or together with the hypophysotropic region, prior to the in vitro determination of the phagocytosis index of the circulating neutrophils.

MATERIALS AND METHODS

Experiments were performed on Wistar male rats, weighing around 200–300 g in the beginning of the experiment; they have been fed according to the Mc Collum's standard diet, and water ad libitum.

By the stereotaxic method, the PVH nucleus has been injured mechanically (PVHL), isolated it alone (PVHI), or we have isolated frontal

and lateral (FLI) the median region of the hypothalamus, which includes the PVH nucleus, too, or we have isolated completely (CI) the hypophysotropic region. With sham-operated (SO) animals, the stereotaxic knife has been lowered 5 mm under the skull's surface.

Histologic control of the interventions performed was made according to the method of Guzman Flores et al., (6). Blood samples have been collected every week, for one month, from the venous retroorbital plexus after ether anaesthesia.

Investigation of the phagocytic capacity of the circulating neutrophils was performed in vitro in the whole blood with a culture of *Staphylococcus albus*. Consequently, 100 μl heparinized blood were mixed with 100 μl Staphylococus suspension (4.10^9 bact./ml), and incubated at 37°C for 30 minutes on stirring from 10 to 10 minutes (2—3 rpm), thus favourising the neutrophils' contact with the phagocytated material. Smears have been prepared from the suspension thus obtained; they were May Grünwald-Giemsa coloured. The phagocytated particles of one hundred neutrophils have been counted and the phagocytosis index hoto-been obtained.

The results were analysed statistically using Student's "t" test.

RESULTS

The significant decrease of the phagocytic activity is maintained weekly, for one month, with all studied groups, comparatively with SO. After the first week (Fig. 1) PVHI and FLI induce more pronounced decreases of the phagocytosis index than PVHL, while in the case of CI, the lowest values are obtained. After 2 (Fig. 2), 3 (Fig. 3) and 4 weeks (Fig. 4), respectively, the phagocytosis index evidences approximately the same values for the PVHL, PVHI and FLI groups, while after CI, the lowest activity is maintained.

DISCUSSION

Mechanical lesion and isolation of the PVH nucleus, along with the fronto-lateral isolation, induce significant decreases of the phagocytosis index, in the case of circulating neutrophils. A significant, even more ample decrease was recorded after CI of the hypophysotropic area.

The role played by "professional" phagocytes, i.e. macrophages, monocytes, neutrophils is of assuring the organism's natural resistance to both infectious and neoplasic affections. This function is achieved together with other compounds of the immune system. On the other side, receptors for various hormones have been discovered on the neutrophils' surface, which might affect their phagocytic function. Thus, there are receptors for insulin, glucocorticoids, β-endorphin, the P substance, sexual hormones (8).

Insulin has a positive effect upon phagocytosis, the deficiencies of insulin being associated with the depressed movement, phagocytosis and bactericidal activity of phagocytes, which explains the increased susceptibility of diabetics to infection diseases (8). Lesion of the PVH nucleus reduces the sympathetic activity and increases the activity of the vagal nerve. Radioimmunological assay of insulin after PVHL indicates a significant increase (personal data), so that diminishing of the phagocytic activity would not be caused by insulin's low level.

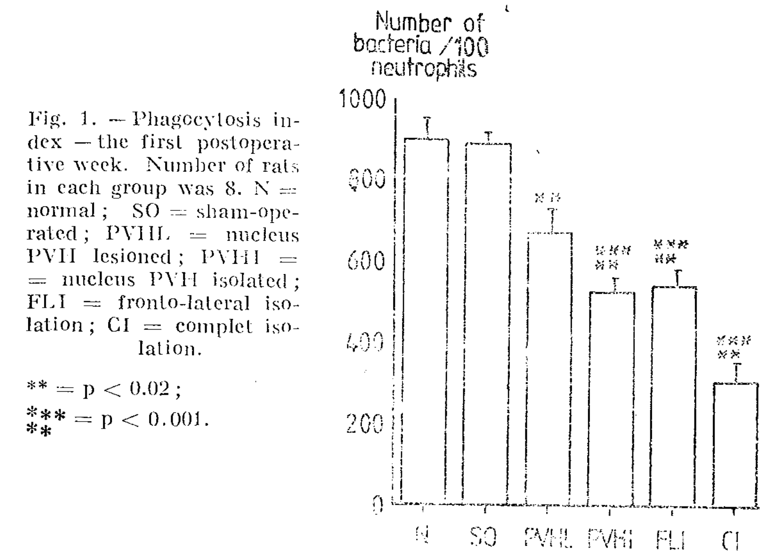

Fig. 1. — Phagocytosis index — the first postoperative week. Number of rats in each group was 8. N = normal; SO = sham-operated; PVHL = nucleus PVH lesioned; PVHI = = nucleus PVH isolated; FLI = fronto-lateral isolation; CI = complet isolation.

** = $p < 0.02$;
*** ** = $p < 0.001$.

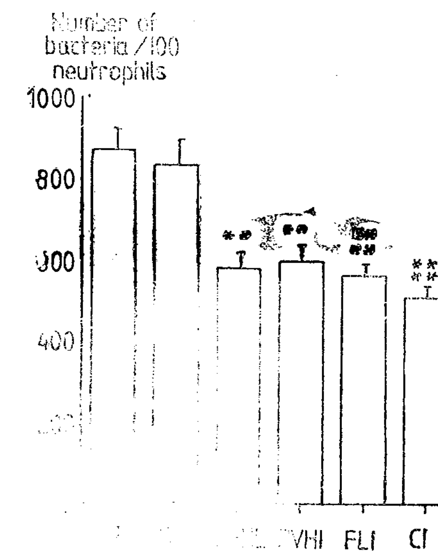

Fig. 2. — Phagocytosis index — the second postoperative week. The legend as in Fig. 1.

** = $p < 0.02$;
** ** = $p < 0.002$.

High doses of glucocorticoids induce phagocytic depression, either by the reduction of the number of active cells or by the decrease of their phagocytic efficiency, both *in vivo* and *in vitro* (8). Radioimmunological

assay of corticosterone indicates an insignificant increase with all studied groups (personal data). As the small doses of corticosterone have no effect or may even stimulate the activity of the phagocytic system (8), the assertion may be made that the considerable reduction of the phagocytic activity—as observed by us—may not be explained by the action of glucocorticoids.

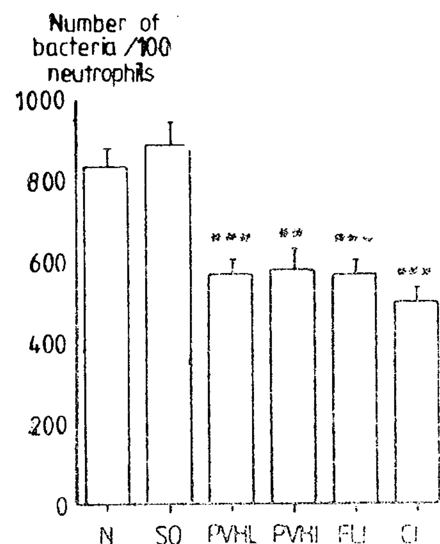

Fig. 3. — Phagocytosis index — the third postoperative week. The legend as in Fig. 1. ** = $p < 0.02$; *** = $p = 0.01$.

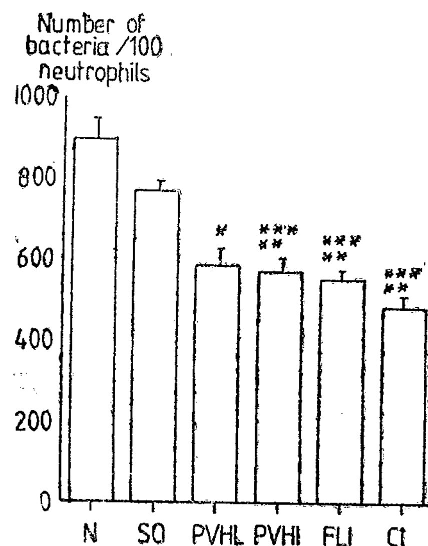

Fig. 4. — Phagocytosis index — the fourth postoperative week. The legend as in Fig. 1. * = $p < 0.05$; *** ** = $p < 0.001$.

β-endorphin stimulates chemotaxis of both monocytes and neutrophils (4). Lesion of the PVH nucleus induces a decrease of the β-endorphin-IR level in the hypothalamus. According to Wiegant, this might be

due to the presence of certain vasopressinergic nervous terminations starting from the PVH nucleus and reaching the arcuate nucleus (12), being thus responsible, at least partially, for the secretion of POMC, from which the β-endorphin-IR is derived. The lack of β-endorphin seems to affect negligibly the phagocytic activity, because low—although quite close—values of the phagocytosis index have been recorded both for animals with lesioned PVH nucleus and for those whose PVH nucleus, or frontolateral region of the hypothalamus have been isolated.

The lowest values of the phagocytosis index are recorded with animals with CI of the hypophysotropic area. With this section, the hypothalamus region starting in the anterior part, immediately behind the optic chiasma, laterally up to the fornix and posterior up to the mammilary bodies, is isolated.

The decrease of the phagocytosis index, after interruption of all afferent and efferent connections to this region assumes intervention, in the neutrophil's phagocytosis, of a nervous way, as well, an idea supported by neuroimmunological data. Thus, Baciu et coll. in 1988 (2) have observed that, after electrical bilateral and symmetric lesions in some nuclei of the anterior hypothalamus or the tubero-mammilary region, or sectioning of the spinal cord in the C_6 region, decreases the phagocytic activity. After that, chlorpromazine, administered to guinea pigs in narcotic doses, has a very pronounced central sympathicolitic effect, inducing a decrease of about 50% of the phagocytic activity (5). According to the other data, the peripheral sympathetic system is not involved in the activation of phagocytosis by means of epinephrine and of the B-adrenergic receptors, even if agonists of norepinephrine in vitro at macrophages, decrease phagocytosis of the immune complexes (7).

During our experiments, by means of CI those have been interrupted efferent nervous ways through which the signals stimulating the phagocytic activity are probably transmitted. Phagocytosis's inhibition effect, as observed in our experiments, might be due—on the one side—to the lack of some stimulating humoral factors in the process of non-specific opsonisation (characteristic to non-immunized animals, as it is our case) or in the activation of the complement for the generation of the C_3b fragment. On the other side, it is possible that sectioning of the posterior efferents might lead to the appearance factors inhibiting opsonisation and activation of the complement.

The general conclusion of the study is that the PVH nucleus belongs to a more extended hypothalamic region that stimulates neutrophils' phagocytic activity. The stimulating role is played both on an endocrine and nervous way, by modulation of the presence of certain humoral factors, playing an essential role in activation neutrophils' phagocytosis.

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IMMUNOMODULATING EFFECTS OF PAGOSTEN-1

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The effect of the acetylated product Pagosten-1 (pentaacetylglucose) upon total lymphocytes and leukocytary groups was followed on Chinchilla rabbits, on employing three different doses, for six weeks, as compared with the effects of the immunostimulating agent Rodilemid. Pagosten-1 influences the dynamics of total leukocytes and the leukocytary groups, as well, manifesting immunostimulating effects, as depending on dose, duration of treatment and type of leukocytes. The most pronounced immunostimulating effects, similar to those of Rodilemid, were observed especially with lower doses (2.5 mg/kg body/day) and for shorter treatment periods (2–4 weeks).

The study of the organism defence mechanisms, alongwith that of the possibilities of influencing the immune response, constitutes a problem of increased interest (2), (11).

Generally, it is considered that the immune response involves a series of specific, cellular and humoral reactions, of an organism having contacted bacteria, fungi, viruses, some foreign cells (such as antigene ones) or different high molecular weight substances (2), (11), (15). There exist, nevertheless, a series of low molecular weight substances (such as antibiotics, alkaloides, hormones, alkylating substances etc.) (7), (11), (16), (18), that may influence the immune response (immunomodulators), inducing either its stimulation (immunostimulators) or inhibition (immunosuppressors) (5), (9), (11), (17). The action mechanism of such low weight immunomodulators is still far from being wholly elucidated.

A previous paper of ours (1) has been devoted to the immunomodulating action of some acetylated polyolic derivatives, evidencing their preponderance by immunostimulating effects upon the cellularly mediated immune response.

The present paper analyzes the immunomodulating effects of an acetylated glucidic derivative, Pagosten-1 (pentaacetylglucose), possessing important pharmacological properties, and a chemical structure similar to that of the previously-investigated products (1), (12–14). The effects of this agent upon the cellularly-mediated immune response have been thus studied, in parallel with the dynamics of the leukocytary formula during the treatment with various doses of agent, as compared to the effects of Rodilemid – a known immunostimulating agent (4).

MATERIAL AND METHODS

The experiments were made on laboratory Chinchilla rabbits, grouped in five batches of seven individuals each, subjected to various treatments, as follows : group I – untreated reference batch, II – treated with Pagosten-1 (Pag-1), in doses of 2.5 mg/kg body/day (dose 1), III – treated with Pag-1 in doses of 5 mg/kg body/day (dose 2), IV – with

Pag-1 in doses of 7.5 mg/kg body/day (dose 3) and V — with 10 mg/kg body/day Rodilemid (a dose similar to the therapeutical one applied to humans). All treatments have been applied for six weeks, as intramuscular injections, at intervals of two days, the products being administered in 0.4 mL physiological salt solution, to the reference group being injected only a physiological salt solution. All batches have been analysed before initiation of the treatments, for the establishment of the initial (normal) values and then at two week intervals, along the whole duration of the treatment.

The total number of leukocytes was determined by the hemocytometric method, as well as the leukocytary formula, on integral blood with 1% EDTA as anticoagulant, by microscopical numbering of the leukocyte type, on glass blades with blood smears coloured by the May-Grümwald-Giemsa method (10), followed by calculation of the percent ratio of each leukocytary group, as compared with total leukocytes.

RESULTS

The numerical values of the various groups of leukocytes, as well as the percent ones, compared to the total leukocytes of the control group of rabbits, range within the normal limits recorded for this species (8). The three administered doses of Pag-1 induce specific modifications both in the total number of leukocytes and in that of the leukocytary groups, as compared with the reference-untreated group (Table 1). Thus, at a dose 1 total leukocytes (TL) increase significantly in the first four weeks, then the process slows down, the final value recorded being of 114.08%, against the initial value of 100 %, which is nevertheless higher than that of the reference group, characterized by minor oscillations. At a dose 2, LT records a slow but continuous increase the final value being of 109.93%, comparatively with the initial one. The dose 3 induces a more pronounced increase of LT, up to a final value of 119.67% as compared with the initial one. At the control batch, treated with Rodilemid, a continuous increase of LT up to a final value of 123.50 %, compared with the initial value, has been recorded.

With dose 1, neutrophils (N) also evidence a pronounced increase in the first four weeks, followed by a slower one, the final value recorded being of 107.73 %, as compared with the initial one (108.40 % with the non-treated, reference batch). With dose 2, nevertheless, a significant decrease of N occurs in the first two weeks (76.56 %), followed by a slight decrease, up to six weeks (87.75 %). A similar dynamics of N was recorded with a dose 3 of Pag-1, too, the final value being nevertheless slightly lower (85.19 %) as compared with dose 2. Rodilemid induced a constant increase of N, up to a final value of 114. 80 %, as compared with the initial one.

Eosinophils (E) are generally decreasing during the treatment with all three doses of Pag-1 the final value being of 71.22 % against the initial one—dose 1 ; 61.08 % — dose 2 ; and 70.69 % — dose 3 (the reference final value —106.50 %). An E decrease up to a final value of 70.98 %,

Table 1

Effect of different doses of Pag-1 (Pag-D_1, D_2, D_3) on the leukocytary formula, comparatively with the effect of Rodilemid (Rod.). 1 = % of normal, initial values (100 %), 2 = % of Rodilemid effect (100 %), TL = total leukocytes, N = neutrophils, E = eosinophils, B = basophils, L = lymphocytes, M = monocytes

Leukocytes	Treatment	2 weeks		4 weeks		6 weeks	
		1	2	1	2	1	2
TL	Pag-D_1	124.25	122.32	134.40	112.34	114.08	92.37
	Pag-D_2	102.54	100.94	109.24	91.31	109.93	89.01
	Pag-D_3	111.95	110.21	124.25	103.86	119.67	96.90
	Rod.	101.58	100.00	119.63	100.00	123.50	100.00
	Control	99.77	98.22	104.39	87.26	109.00	88.26
N	Pag-D_1	105.46	98.63	125.95	112.98	107.73	93.84
	Pag-D_2	76.56	71.60	89.86	80.61	87.75	76.44
	Pag-D_3	74.96	70.11	87.41	78.41	85.19	74.21
	Rod.	106.92	100.00	111.48	100.00	114.80	100.00
	Control	93.94	87.86	103.38	92.73	108.40	94.42
E	Pag-D_1	120.14	141.01	100.71	133.48	71.22	100.63
	Pag-D_2	71.26	83.64	58.08	76.98	61.08	86.05
	Pag-D_3	88.79	104.21	81.90	108.55	70.69	99.59
	Rod.	85.20	100.00	75.45	100.00	70.98	100.00
	Control	104.15	122.24	101.04	133.92	106.50	150.04
B	Pag-D_1	327.52	377.98	204.58	343.25	117.43	294.60
	Pag-D_2	95.07	109.71	86.62	145.33	143.66	360.41
	Pag-D_3	102.52	118.31	59.75	100.25	84.90	212.99
	Rod.	86.65	100.00	59.60	100.00	39.86	100.00
	Control	106.13	122.48	94.85	159.14	116.59	292.50
L	Pag-D_1	144.75	137.75	144.32	113.30	121.00	91.39
	Pag-D_2	137.49	130.84	136.82	107.41	141.16	106.62
	Pag-D_3	165.43	157.43	178.61	140.22	171.72	129.70
	Rod.	105.08	100.00	127.38	100.00	132.40	100.00
	Control	108.31	103.07	105.76	83.03	108.56	81.99
M	Pag-D_1	118.63	54.05	127.03	52.45	113.67	40.99
	Pag-D_2	147.10	67.02	96.91	40.01	89.38	32.23
	Pag-D_3	267.30	121.79	246.68	101.73	125.12	45.12
	Rod.	219.47	100.00	242.19	100.00	277.28	100.00
	Control	97.24	44.30	103.32	42.66	109.28	39.41

compared with the initial one, was also recorded during treatments with Rodilemid.

Basophils (B) show a different dynamics for the three doses of Pag-1. Thus, with a dose 1, a very pronounced increase occurs in the first two weeks, followed by a weaker one, up to a final value of 117.43 % *versus* the initial one ; with dose 2 an initial decrease (86.62 %) in the first four weeks is recorded, followed by a significant (143.66 %) final increase, while, in the case of dose 3, a slight increase (102.52 %) in the first two weeks is recorded, followed by a considerable decrease after four weeks (59.75 %) and a weaker one (84.90 %) after six weeks. With the reference batch, slight fluctuations occur, followed by a final increase (116.59 %).

With the Rodilemid treatment group, a continuous drecrease is recorded, up to a final value of 39.86 %, comparatively with the initial one.

Lymphocytes (L) are seen as increasing with all doses of Pag-1, the final value being of 121.00 % for dose 1 ; 141.16 % for dose 2 and 171.72 % for dose 3, respectively, as compared with the initial value. With the reference sample, the final value is of 108.56 %, and of 132.40 % for Rodilemid, comparatively with the initial value.

Monocytes (M) record increases in the treatment with doses 1 and 3, in parallel with a decrease for dose 2. Thus, in the case of dose 1, an initially more pronounced increase occurs, followed by a more reduced one, up to a final value of 113.67 %. With dose 2, an initial increase of 147.10 % (after two weeks) is recorded, followed by a decrease up to a final value of 89.38 %, comparatively with the initial one. Dose 3 induces a considerable increase (267.63 %) after two weeks, and a weaker one (125.12 %, compared with the initial value) in the end. With the reference sample, the final value recorded is of 109.28 %, while the group treated with Rodilemid recorded a very pronounced increase, up to a final value of 277.28 % compared with the initial one.

DISCUSSIONS AND CONCLUSIONS

The experimental data obtained show that the agents considered in the study influence the cellularly-mediated immune response, by modifying the numerical dynamics of total leukocytes and of the various leukocytary groups, as well. The effects of Pag-1 depend both on the doses employed and on the treatment duration, being characteristic to each group of leukocytes (Table 1).

Thus, the treatment with Pag-1 determines a general tendency of increasing leukocytary values ; nevertheless, while dose 1 induces increases of all leukocytary groups, doses 2 and 3 have stimulating values only upon LT, L and M, their effects an N, E and B being generally depressing and less constant, as depending on both the dose and treatment duration. Actually, the duration of treatment influences specifically the peculiar effects of Pag-1 upon the leukocytary image, with all doses employed (Table 1). The observation is therefore made that both the stimulating effect upon some groups of leukocytes and the depressing ones, manifested upon other groups, are generally more pronounced in the first two or four weeks of treatment, and weaker towards the end of the experiment. It is also to be observed that the effects of dose 1 are more constant than those of doses 2 and 3, a case in which the depressing effects are, usually, less stable along the duration of treatment.

All the data gathered evidence too, that the effects of Pag-1 upon the leukocytes' dynamics are generally of the same type with those of the immunostimulating agent Rodilemid (4) the effects upon N excepted, a case in which only dose 1 is stimulating along the whole duration of the treatment, as it is the case of Rodilemid, doses 2 and 3 manifesting adverse effects (Table 1). Worth mentioning is also the fact that the effects of Pag-1 are generally weaker then those of Rodilemid (% as compared with Rodilemid) towards the end of the treatment. The effects

manifested upon B and L are nevertheless stronger along the whole duration of the experiment, which is to be observed, too, with TL and E, in the first two or four weeks of treatment, especially with doses 1 and 3.

If considering the general effects of Pag-1 upon the dynamics of leukocytary groups, this product may be considered as an immunomodulating agent with immunostimulating action, similar to that manifested by Rodilemid (4) taken as reference and also to that of Pag-2 (hexaacetylmanitol) and Pag-3 (hexaacetylsorbitol), discussed in a previous paper (1), both acetylated derivatives of the same type as Pag-1 (pentaacetylglycose). As a matter of fact, it is known that acetylation leads to the obtainment of pharmacological products with improved bioavailability and significant biochemical and pharmacological effects (3), (5), (6), (12–14), (18). Consequently, there becomes possible that the immunostimulating effects of Pag-1, manifested upon the leukocytes' dynamics, should result not only from its direct action upon the mechanism of the cellularly-mediated immune response or upon leukopoiesis, but also from its involvement in various metabolic processes (1), (8).

For a superior application of the immunostimulating action of Pag-1, the dose employed and the treatment duration should be nevertheless considered lower doses and shorter duration, repeated at certain time intervals.

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INTER-INDIVIDUAL VARIATIONS OF THE GAMMA GTP VALUES WITH ALCOHOLICS AS A RESULT OF ETHANOLIC STIMULATION

ELISABETA NAUM and ION NEACŞU *

Individual variations of the gamma GTP values from blood have been followed on a group of 25 volunteers, formed of 8 women (4 of them non-alcoholics) and 14 men, 9 of them non-alcoholics, after ingestion of 0 5 mL absolute ethanol/kg body. Determinations were made initially (control values) and after 30, 90 and 150 minutes respectively, after the alcohol's ingestion. A great variability of values was recorded, which attests the individual character of the gamma GTP activity. At the same time, an initial increase (after 30 minutes) of the values was recorded with all subjects, yet ampler with alcoholics, followed by a slight decrease or plateau-levelling. The data obtained demonstrate the importance of gamma GTP as a marker in stating the various stages of alcoholism, alongwith its treatment to be applied.

Gamma glutamyl transpeptidase (gamma GTP) evidences a marked specificity in chronic alcoholism, being considered the most susceptible enzyme in liver's chronic affection (10–14).

The mechanism inducing increase of the seric values of gamma GTP in chronic alcoholism is still discussed. Several clinical and experimental studies have been devoted upon to this aspect, most authors explaining the increase in the gamma GTP values through the activation of the proteic synthesis at microsomal level, as due to the inducing effect of alcohol (6), (11).

Teschke et al. (11) have dosed the enzyme activity both in the serum and in the liver, on patients suffering from alcoholic hepatic steatoze, as well as on normal individuals, considerably increased values being recorded with alcoholics, which suggests the major part played in such an increase by the microsomal enzymatic induction provoked by alcohol. Such a theory is also supported by the studies of Ebihara et al. (3), who administered phenobarbital to rats for three weeks, which resulted in a considerable increase of cytochrome P_{450} and of the gamma GTP activity as well. Other studies (7) demonstrated that the seric activity of gamma GTP with alcoholics is only partially caused by hepatic microsomes, an important part being due to enzyme intestinal production, which has been evidenced histochemically at the level of the brush border of the intestine.

If considering enzyme great susceptibility to alcoholic ingestion, the present study has aimed at evidencing both individual and group variations, on applying the same method to both alcoholic and non-alcoholic subjects.

MATERIALS AND METHODS

25 volunteers, between 30 and 60 years, hospitalized in the clinics of the "Socola" Psychiatry Hospital of Jassy, have been studied in various phases of chronic alcoholism. The group was formed of 8 women, 4 of them non-addicted to alcohol, and 17 men, 9 of which nonalcoholics. The experiment started a week after hospitalization, any possible ingestion of alcoholic beverages being thus excluded along this whole period. The data in the observation sheets show the absence of other clinical signs of the liver's chronic affection, the subjects selected having either normal or insignificantly increased levels of the seric transaminazes and/or of the hepatic parameters.

Analysis of the enzymatic activity of gamma GTP employed the reactive pouches produced by the Institute of Chemico-Pharmaceutical Researches in Bucharest, by application of a clinical investigation technique perfected in the Laboratory of Clinical Biochemistry within the University of Medicine and Pharmacy in Cluj-Napoca.

Blood samples have been taken over "á jeun", successively at preestablished time intervals, after individual ingestion of 0.5 mL absolute ethanol/kg body. The alcohol was ingested in about 10 minutes. The initial (zero) sample was made of blood taken over before the alcohol administration, representing the individual reference moment, while the subsequent samples were taken 30 min (sample 1), 90 (sample 2) and 150 min (sample 3) respectively later, thus reflecting the dynamics of enzymatic values.

RESULTS

The individual values obtained from gamma GTP determinations for the subjects taken into study, are plotted graphically in Figures 1 to 4. The observation is made that the values obtained vary within large limits, which attests the individual, varying character of the enzyme's activity. In spite of this inter-individual variability of the enzyme's values, mention has to be made, nevertheless, of a common characteristic of all variation curves, namely their ascending character up to minute 30 with all subjects (100%). This increase may be observed with 45% of subjects up to minute 90, 17% remaining as plateau values, for the rest of 38% decreases being observed. Starting with minute 90, up to minute 150, with 34% of patients the curves' values remain on the plateau, as compared with minute 90, various decreases being observed for the remaining 66%.

Analysis of the values recorded by alcoholics draws the attention upon curves 6 (Fig. 2) 20 and 21 (Fig. 4), characterized by a pronounced amplitude and various dynamics of values, as follows; all the three curves increase spectacularly up to minute 30, followed by a rapid decrease of curve 6 up to minute 90, and then, by a slight decrease up to minute 150, while curves 20 and 21 differ both from curve 6 and between them as to their values' dynamics. Curve 21 increases up to minute 90, remaining then as a plateau up to minute 150, while curve 20 evidences a pronounced increase up to minute 30, decreasing then rapidly up to minute 150. As the the final values (minute 150) of gamma GTP, compared with the initial (zero) moment, it is only 20 % that return to the zero level, the remaining ones being maintained at higher values.

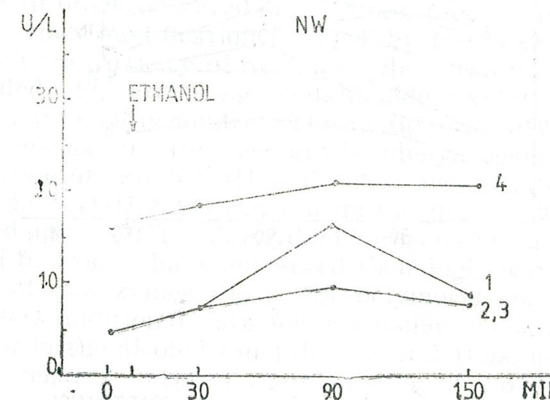

Fig. 1. — Dynamics of the individual values of gamma GTP (U/L) in the case of non-alcoholic women (NW) patients (curves 1 to 4).

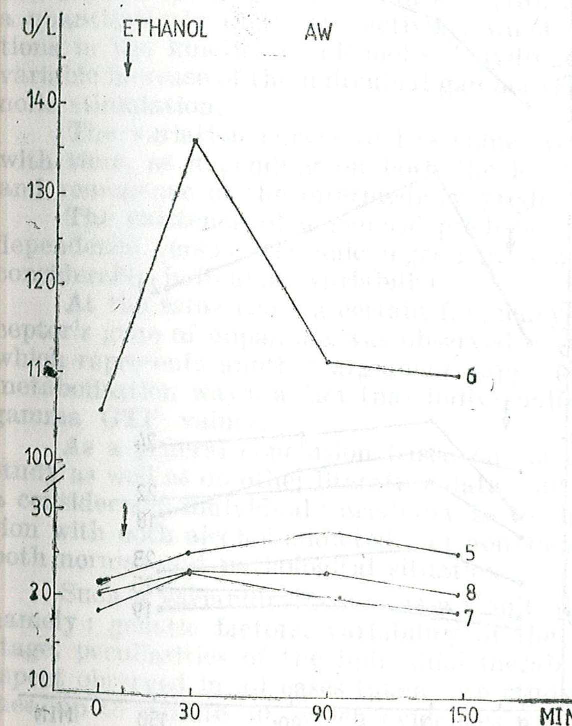

Fig. 2. — Dynamics of the individual values of gamma GTP (U/L) in the case of alcoholic women (AW) patients (curves 5 to 8).

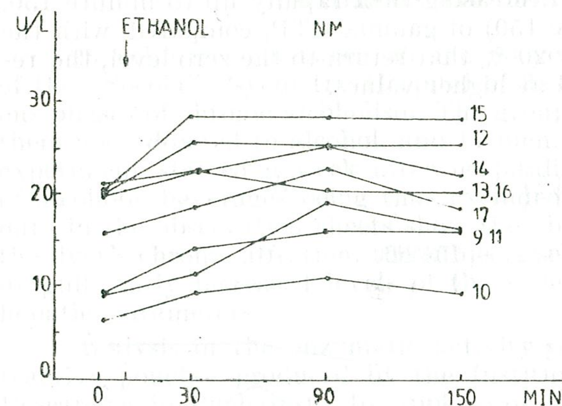

Fig. 3. — Dynamics of the individual values of gamma GTP (U/L) in the case of non-alcoholic men (NM) patients (curves 9 to 17).

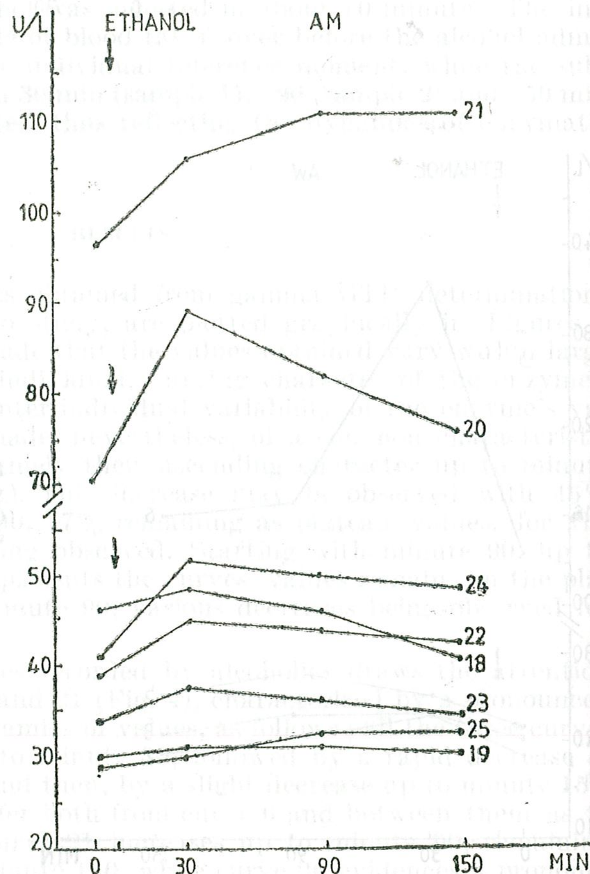

Fig. 4. — Dynamics of the individual values of gamma GTP (U/L) in the case of alcoholic men (AM) patients (curves 18 to 25).

DISCUSSIONS AND CONCLUSIONS

It is generally known that the toxic effects of ethanol are closely related to its metabolism and therefore, to the enzymes involved in its oxidative removal.

Many of the metabolic disordes observed as a result of alcoholic intoxication results from the redox modification induced by the noxious formation of NADH, to which the toxicity of acetaldehyde — the intermediate metabolite — is added (8). Following acetaldehide oxidation, most of the acetate is shifted into mitochondria. The metabolism of an ethanol molecule leads, by means of the acetaldehyddehydrogenase (ALDH) system, to the formation of two NADH molecules, accumulated in the liver, which modifies the NADH/NAD ratio in its favour.

Recent studies devoted to ADH (aldehyddehydrogenase) and ALDH, to their structure and functions, have evidenced their high degree of complexity. The existence of izoenzymes and of the polymorphism of some "gene loci" variants offers additional data for a possible explanation of the differences recorded in the alcohol's metabolism and thus, implicitly, in acetaldehyde. The relation between the character of various enzymes and the individual and rasial differences as to the susceptibility to alcohol, as well as the various affections they cause have been discussed in several studies (4). The number and electrophoretic mobility of the molecular formes of individual ADH vary from one individual to another, and depend on the genetic conditions (13). The ADH isoenzymes evidence a quantitatively different activity, which explains the individual variations in the kinetics of alcohol's dehydrogenation, thus influencing the variable increase of the individual gamma GTP values, as a result of ethanolic stimulation.

The variation curves of the values resulted from the study vary with time, as depending on both the level of alcohol's metabolization and remanence of the intermediary products.

The existence of a genetic predisposition, regarding to a possible dependence versus ethanolic ingestion, is assumed, which might induce considerable individual variability.

At the same time, a certain frequency of the A allela of the D_2 receptor's gene of dopamine was observed with alcoholics (1), (2), (5), (12), which represents another argument supporting the diversity of alcohol's metabolization ways, a fact that individualizes, as well, variations of the gamma GTP values.

As a general conclusion based on the information provided in this study as well as on other literature data, one may observe the existence of a considerable individual variability as to the response to ethanol ingestion with both alcohol-addicted and non-alcoholics, within limits defining both normal and pathological situations.

Such a variability occurs as a result of a complex series of factors, namely: genetic factors, variability of the alcoholics' hepatic affection stage, peculiarities of the individual metabolism etc. The characteristic aspect observed in all cases taken into study is the initial increase of values, up to minute 30, which evidences the ethanolic stimulation of the

gamma GTP activity. The values of both initial and maximum stimulation do not exceed — in the case of non-alcoholics — the normal values, with the exception of curve 4 (Fig. 1) situated at the high limit of the normal values, the curve's maximum exceeding this line, which might suggest a possible evolution towards alcoholism. Evidencing of such a moment is of special interest for the recovery phase of the medical assistance.

The study demonstrates that the GTP activity represents quite a reliable marker in stating the exact stage of the alcoholic-type hepatic affections. Also, the results obtained, correlated with clinical data, (9), may efficiently contribute to a timely tracing of alcoholic hepatopathies.

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IDENTIFICATION AND CHARACTERIZATION OF A PROTEIN PHOSPHATASE IN SPERMATOZOA OF NEWTS

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Electron microscopic study identified a serine/threonine protein phosphatase associated with microtubules in spermatozoa of newts. The protein phosphatase activities in extracts of newt testis, catalyses dephosphorylation of phosphocasein and a synthetic substrate, p-nitrophenyl phosphate. Protein phosphatase activity is inhibited by adenine nucleotides, fluoride and Zn^{2+} and stimulated by Mn^{2+}. The enzyme was also stimulated by calmodulin. The results suggest that a calmodulin-dependent protein phosphatase is one of the phosphatases that interacts with microtubules in spermatozoa. The identification of a calmodulin-dependent protein phosphatase in spermatozoa suggests a role for Ca^{2+} — dependent dephosphorylation in flagellar movement.

The reversible phosphorylation of protein is a major mechanism for the control of many intracellular functions in eukaryotic cells, and plays a dominant role in controlling the activities of proteins involved in signaling. The activities of protein phosphatases are key components of cellular signaling transduction pathways. Protein serine/threonine phosphatases have been found in many eukaryotic cells and were divided in several interrelated families (3), (4), (5), (10). The sperm flagellum represents an organelle that contains a number of specialized signaling elements which are important for the control of the movement of the cell (2), (8), (14), (18).

Several protein phosphatases activities have been demonstrated in the testis of some species. In the rat testis, protein phosphatase 1 was identified in the particulate fraction, while protein phosphatase 2A is mainly present in the soluble fraction (13), (11). A cDNA of the testis-specific serine/threonine proteinphosphatase 2B has been isolated and its mRNA seems to be involved in sperm motility (14). Thus, the testis contains several protein serin/threonine phosphatases, suggesting differential regulatory properties. Brokaw (2) used partially purified muscle protein phosphatase to dephosphorylate sperm cAMP-dependent phosphoproteins. Rephosphorylation of the dephosphorylated sperm proteins reversed the reduction in reactivation produced by phosphatase treatment. A calmodulin-dependent protein phosphatase was identified in dog sperm (16), which suggests a role for Ca^{2+} — dependent dephosphorylation in flagellar function.

The results presented in this article show that the flagellum of newt sperm contains a serin threonine protein phosphatase associated with microtubules. The sperm phosphatase is stimulated by calmodulin, suggesting that it is a calmodulin-dependent protein phosphatase.

REV. ROUM. BIOL. — BIOL. ANIM., TOME 39, Nº 2, P. 129—133, BUCAREST, 1994

MATERIAL AND METHODS

Animals. In our experiments we used specimens of *Triturus cristatu* captured in May from Bacău. The animals were anaesthetized and th testis were dissected.

Cytochemical localization. For electron microscopic study, smal pieces of about 1 mm from the testis were fixed in 2% glutaraldehyd prepared in cacodylate buffer 50 mM, pH 7.0, containing 3% formalde hyde and 6% sucrose, for two hours. After fixation, the pieces were was hed several times in cacodylate buffer 50 mM, pH 7.0. The ultrastructur localization of phosphatase activity was accomplished by incubation o pieces in a medium consisting of Tris-HCl 50 mM, pH 7.2, 2 mg/ml phos phocasein, 1 mM lead nitrate, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.1% mercap toethanol. Incubation was carried for 60 min at 37°C. After incubatio the pieces were washed with Tris-HCl buffer 50 mM, pH 7.0 and wer postfixed in 1% osmium tetraoxide prepared in the same buffer, for tw hours at cold. The pieces were dehydrated and embedded in Epon 81

Ultrathin sections were examined either unstained or stained wit uranyl acetate and lead citrate in a Philips EM 201 electron microscop

Phosphatase assay. Phosphatase activity was measured by the re lease of orthophosphate from phosphocasein. The enzyme was assaye in a reaction mixture (50 µl) containing 50 mM Tris-HCl pH 7.0, 0.1% mercaptoethanol, 1 mg/ml bovine serum albumin, 1 mg/ml phosphopro tein, 0.3 mM $CaCl_2$ and an appropriate amount of enzyme. Assays wer carried out for 5 min at 37°C and were initiated with the enzyme. The re leased orthophosphate was measured by a malachite green procedure (1 Phosphatase specific activity is expressed as nmol of phosphate release per mg of protein/min, the background having been subtracted. Calmo dulin-dependent phosphatase activity was measured as the differenc between total phosphatase activity and calmodulin-independent phos phatase activity defined as activity in the presence of EGTA or trifluo perazine, both of which inhibit the biological activity of calmodulin (17 Calmodulin was removed from the phosphatase by a batch DEAE-cellu lose fractionation, as described by Wang and Desai (19).

Protein determination. Protein was determined according to Lowr et al. (9), using bovine serum albumin as a substrate.

Preparation of tissue extract. Newt testis was homogenized in 3 vo lumes of ice-cold 50 mM Tris-HCl pH 7.0, containing 0.6 M NaCl, 0.1% mercaptoethanol, 2 mM EDTA and 0.1 mM phenylmethylsulphonylfluo ride using a Potter-Elvehjem homogenizer. The extract was centrifuge at 20.000 × g for 20 min, and the resulting supernatant was used in dete mination, or was used for further purification. The supernatant was ad sorbed on a column of DEAE-Sephadex A−50 and the enzyme was elu ted with a NaCl gradient. The fractions containing the phosphatase acti vity were pooled, concentrated and made 10% in glycerol, and stored a −20°C.

RESULTS AND DISCUSSION

Ultrastructural localization. In figure 1, several spermatozoa are seen showing the localization of the protein phosphatase in the cells. The reaction product was clearly detected in the flagellum of spermatozoa along the microtubules (arrow). Apart from this localization, a moderate reaction is also observed in some mitochondria (arrow head). Studies of the subcellular localization of protein phosphatases in rat testis with antibodies developed against protein phosphatase 1 showed the presence of enzyme in the nuclei of late spermatocytes (11), (12). Moreover, an isoform of protein phosphatase 2A is also expressed in rat testis (13).

Biochemical characterization. Knowing that a role for protein phosphatase 2B in regulation of the sperm flagellum has been implied by the presence of this enzyme associated with the flagellum (16), we examined the activity of the enzyme in the newt testis homogenate. To determine if the testis contains a calmodulin-dependent protein phosphatase we assayed the activity of the enzyme in the extract by the release of orthophosphate from phosphocasein and the calmodulin-dependent activity was measured as the difference between total phosphatase activity and calmodulin-independent phosphatase activity in the presence of EGTA. Using EGTA to inhibit calmodulin, we found that calmodulin-dependent phosphatase activity in the sperm supernatant was approximately 0.18 nmol/mg/min, which accounted 20% of the total phosphatase activity (Fig. 2). In the presence of 10 mM $MgCl_2$ (to activate protein phosphatase 2C) a slight increase in the rate of dephosphorylation of casein in the sperm extract was observed, indicating that protein phosphatase 2C makes a contribution to the dephosphorylation of this substrate. This suggests that in the newt testis homogenate the protein phosphatases 2B and 2C are functionally active. It is not excluded also the presence of protein phosphatase 2A, but we have not tested the sensitivity of phosphatase to okadaic acid (which inhibits this type of phosphatase).

The effect of various modulators on the protein phosphatase activity is shown in Table 1. Adenine nucleotides significantly decreased the specific activity of both basal (in the presence of EGTA) and calmodulin-dependent protein phosphatase, with the following order of effectiveness: ATP, ADP, 5-AMP. It was suggested that an inhibition of protein phosphatase activity by ATP, which is independent of substrate effects,

Table 1

The effect of some modulators on protein phosphatase activity from newt testis

Addition		Phosphatase activity (% of control) −CaM	+CaM
5−AMP	1 mM	75	86
ADP	1 mM	62	70
ATP	1 mM	40	53
$MnCl_2$	1 mM	130	162
NaF	5 mM	54	60
$ZnCl_2$	1 mM	36	30

can be due to the removal of metal ion from the enzyme, since the effe of ATP is partially reversed by preincubation with magnesium.

Several serine/threonine protein phosphatases have been identifie which are involved in the dephosphorylation of microtubule-associated pro teins (6), (7), (20), but a role for Ca^{2+}—dependent dephosphorylation i flagellar movement was restricted to calmodulin-dependent phosphatas (14), (16). This phosphatase is the only one whose activity is controlle directly by a second messenger and exhibits a high degree of substrat specificity. The enzyme was identified in many cells and is likely to pla a central role in cellular communication (5), (10).

The calmodulin-dependent protein phosphatase identified in th newt testis dephosphorylated casein, phosvitin and the synthetic subs trate p-nitrophenylphosphate, but the phosphoproteins used in this stud are not likely to be physiological substrates for the enzyme. An additiona insight into the function of the enzyme is the identification of multipl phosphoprotein substrates in the sperm that are specific to the motilit apparatus. Tash et al. (15), (16) show the presence of a calmodulin-de pendent protein phosphatase in the dog sperm and have identified 1 substrates for this phosphatase in the whole sperm.

The relationships between changes in the protein substrates fo cAMP dependent phosphorylation and changes in axonemal function ar still unknown. However, the Ca^{2+} sensitivity of a calmodulin-dependen protein phosphatase in spermatozoa indicates that it is likely to be invol ved in Ca^{2+}-dependent signaling pathway. Moreover, the reponse to che moattractants involves changes in the asymmetry of flagellar bendin waves, which can be produced in vitro by increases in calcium (2). It i likely that this enzyme may be needed for the modulation of the dynamics o microtubule sleeding, that is a key to flagellar movement in sperm cells Given the evidence that the enzyme is closely associated with microtu bules, it was suggested that the enzyme could by optimally positione for this function by virtue of its association with the cytoskeleton.

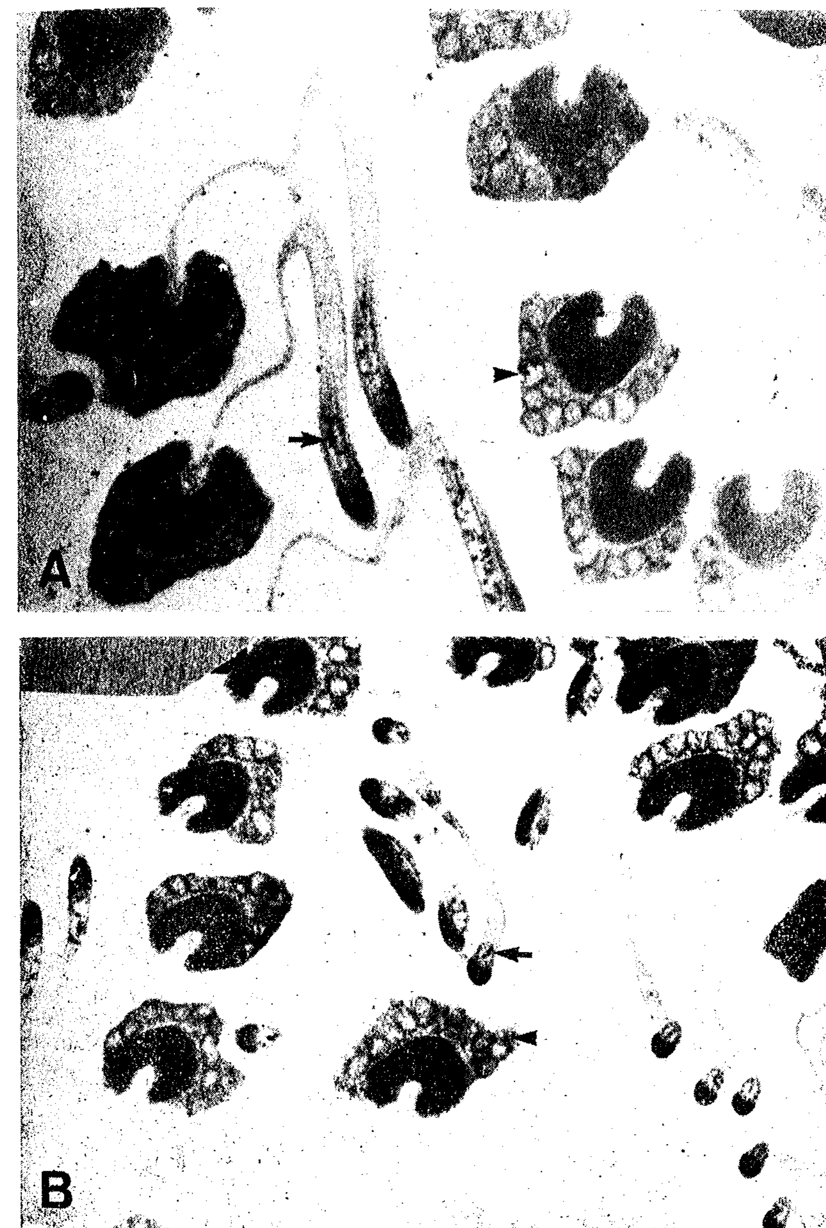

Fig. 1. — Electron microscopic localization of protein phosphatase in spermatozoa of newts.

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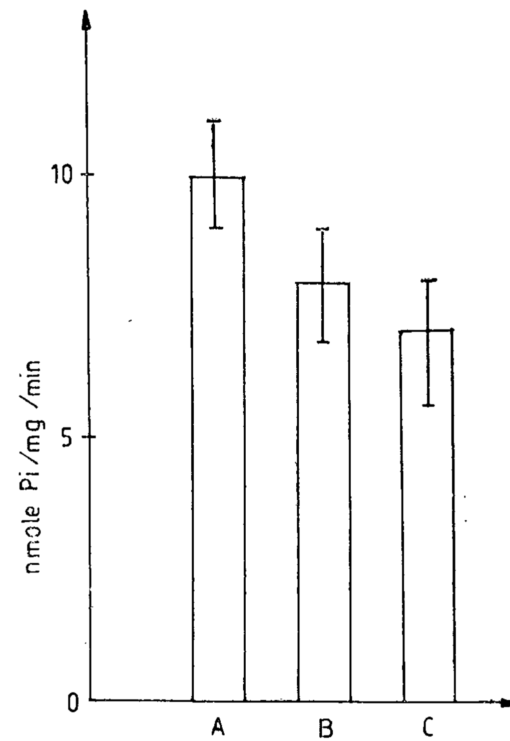

Fig. 2. — Protein phosphatase activity in the extract of newt testis. Phosphatase activity was measured either in the presence of 1 μM calmodulin and 0.3 μM Ca^{2+} or in the presence of 0.3 μM Ca^{2+} and 1 mM EGTA, using phosphocasein as a substrate. A-phosphatase activity with Ca^{2+} and calmodulin; B — phosphatase activity with EGTA and calmodulin; C — phosphatase activity in the presence of 10 mM $MgCl_2$.

THE EFFECT OF GLYCOSAMINOGLYCANS ON ACROSOME REACTION IN BOAR SPERMATOZOA "IN VITRO"

ANCA OANCEA *, LUCIA MOLDOVAN * and OTILIA ZĂRNESCU **

Boar spermatozoa were incubated for 9h in the presence of 0, 10, 50, 100 μg/ml of chondroitin sulfate (CS) and heparin (Hep) to induce acrosome reaction "in vitro". Acrosome reactions were evaluated by light and electron microscopy. The addition of glycosaminoglycans (GAG_s) in medium significantly increased the incidence of acrosome reaction. When observed by electron microscopy, acrosome-reacted sperm had undergone vesiculation of the plasma membrane. These results suggest that glycosaminoglycans in the female reproductive tract may be responsible for some of the biochemical changes associated with fertilization.

The capacitation and the subsequent acrosome reaction in spermatozoa are prerequisites for mammalian fertilization "in vivo" and "in vitro" (1).

Mammalian spermatozoa must reside for a time in female reproductive tract before they acquire the capacity to fertilize ova (2). After capacitation has occurred the sperm are able to undergo an acrosome reaction. The acrosome reaction releases enzymes which digest the matrix of the cumulus cells surrounding the ovum and permits the zona pellucida to be penetrated by spermatozoa there by allowing the sperm to make its way toward the ovum (3).

It was reported that the follicular fluid (4) and flushings from female reproductive tracts (5) are able to promote capacitation and acrosome reaction.

It was also reported that classes of carbohydrates known as glycosaminoglycans, which have been identified in reproductive tracts of swine (6) and in follicular fluids of cows (7), enhance the occurrence of acrosome reaction in sperm "in vitro" (8).

The present study was designed to evaluate the effects of two glycosaminoglycans (chondroitin sulfate and heparin) on faciliting acrosome reaction in boar sperm. Light and electron microscopy were used to demonstrate that a normal morphological acrosome reaction had occurred.

MATERIALS AND METHODS

Boar sperm was obtained from Romsuin-Test Peris. The sample with a percent motility 75 was evaluated for spermatozoa concentration with a hemocytometer. The concentration of spermatozoa was $4 \cdot 10^5$ sperm/ml.

Sperm was removed from seminal plasma by centrifugation at 500 rpm for 10 min at room temperature and resuspended in a modified Tyrode's buffer (containing 162.83 mM NaCl and 2.25 mM $CaCl_2$).

In experiment one, spermatozoa were incubated in the presence o 0, 10, 50, 100 μg/ml of CS from bovine trachea and in the presence o some quantities of heparin. The samples were examined with light mi croscopy to determine the frequency of sperm which excluded acrosoma stain and were considered to have undergone an acrosome reaction.

In a second experiment the staining method used to assess the acro some reaction by light microscopy was validated by electron microscopy Spermatozoa before and after exposure to GAG_s were examined for vesi culation of the outer acrosomal and plasma membrane.

Acrosomal stain procedure: staining of boar spermatozoa was a we have previously described (9).

Electron microscopy: spermatozoa were fixed for electron micros copy after 18h, according to the procedure described by Jones (10). Fo initial fixation, spermatozoa were concentrated by centrifugation fo 10 min at 200 × g. The sperm pellet was diluted with picric acid, formal dehyde, glutaraldehyde and sodium cacodylate. Spermatozoa were post fixed for 30 min in osmium tetroxide and placed in agar after osmifica tion. The samples were dehydrated, embedded, sectioned and staine with uranyl acetate and lead citrate. A JEOL 7C electron microscope wa used to assess vesiculation of the acrosome.

RESULTS AND DISCUSSIONS

The percentage of sperm exhibiting an acrosome reaction in th presence of 0, 10, 50, 100 μg/ml CS, after 9h of incubation at 37°C is shown in Fig. 1. In the presence of 100 μgCS/ml, 68% of spermatozoa un derwent an acrosome reaction.

Viability of sperm did not differ significantly among controls or th various doses of CS, in agreement with previous work (Lenz) (11).

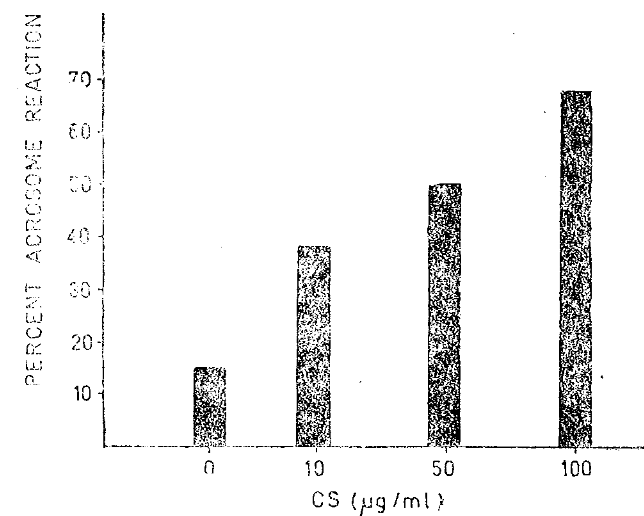

Fig. 1. — Percent acrosome reaction of sperm after 9h of incubation in the absence or presence of CS.

When the swine sperm was incubated in the presence of glycosaminoglycan heparin (0, 10, 50, 100 μg/ml), 70% of spermatozoa underwent an acrosome reaction after 9h of incubation at 37°C (Fig. 2).

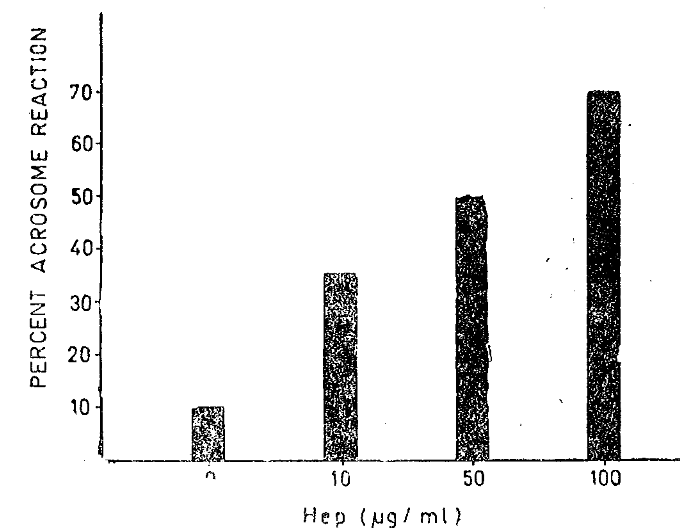

Fig. 2. — Percent acrosome reaction of sperm after 9h of incubation in the absence or presence of Heparin.

Light microscopy showed that induction of the acrosome reaction (in the presence of both GAG_s) was apparent as a lack of uptake of the dye by the acrosome (Fig. 3 — A, B). Sperm that are acrosome reacted (AR) are apparent as lighter uptake of stain as compared to sperm with intact acrosome (IA). Our data were comparable with those reported by Lenz (12) for bovine epididymal spermatozoa.

Electron microscopy showed that induction of acrosome reaction by both CS and Hep was morphologically normal (Fig. 5). The criteria by which an acrosome reaction can be noticed as normal include the occurrence of regular vesiculation of the outer acrosomal membrane and plasma membrane (13, 14). By these criteria, the acrosome reaction induced by the two studied GAG_s appeared normal (Fig. 4 — A, B).

The transmission electron micrographs showed that the sperm in the presence of GAG_s had undergone vesiculation. The sperm in the absence of GAG_s (control) did not demonstrate a true vesiculation characteristic of the acrosome reaction (15).

These experiments demonstrated that CS and Hep were able to stimulate an acrosome reaction in boar spermatozoa. The staining procedure that we have used for light microscopy permitted to evaluate the incidence of acrosome reaction.

It is known that a GAG in porcine uterine fluid activated the zymogen proacrosin to acrosin (16) and this GAG lost its effect after exposure to testicular hyaluronidase or chondroitin ABC lyase. Parish et al (17) also reported that commercially available GAG_s enhanced the conversion of proacrosin into acrosin in bovine spermatozoa.

Our conclusion was that GAG_s CS and Hep interact with sperm to cause membrane perturbations associated with a normal acrosome reaction remarked by light and electron microscopy.

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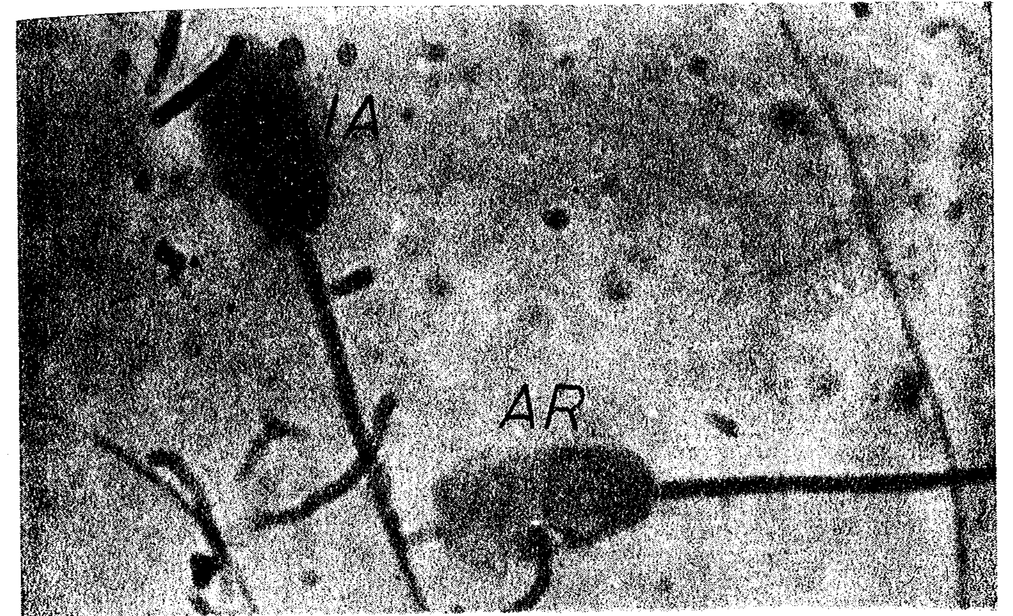

Fig. 3. — Photomicrographs of swine sperm using light microscopy. Sperm were stained for acrosome reaction with naphthol yellow and erythrosin B. Sperm that are acrosome reacted (AR) are apparent as lighter uptake of stain as compared to sperm with intact acrosomes (IA) (×400).
A — in the presence of CS
B — in the presence of Hep.

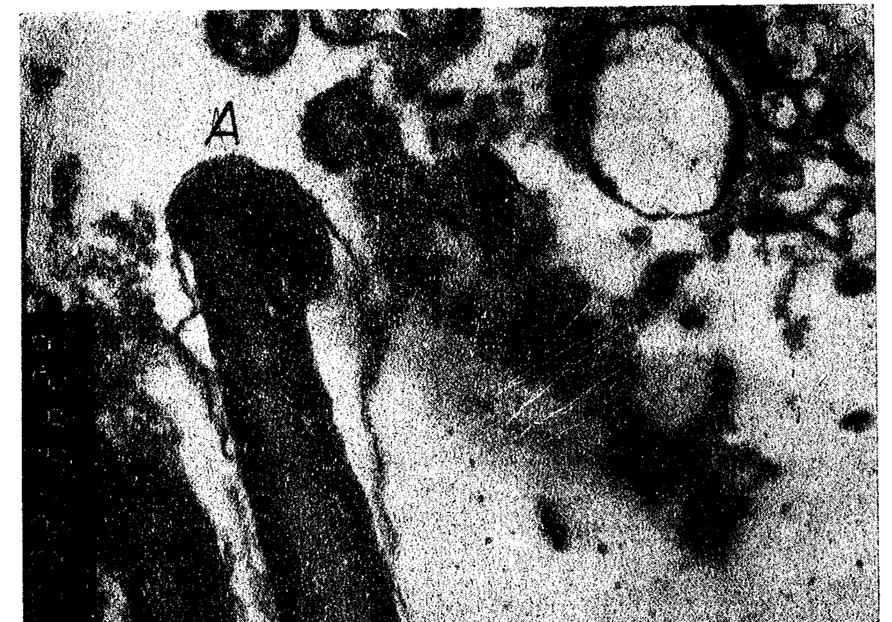

Fig. 4A

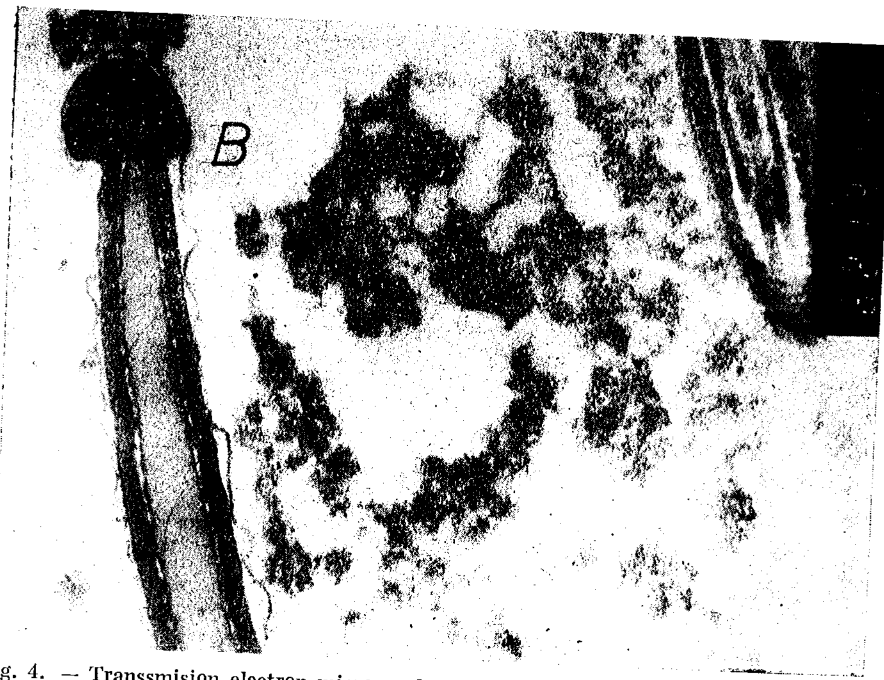

Fig. 4. — Transsmision electron micrographs of swine spermatozoa after CS induction (A) and after Hep induction (B) of the acrosome reaction. Vesiculation of the plasma membrane and outer acrosome membrane had occurred in the acrosome region overlying the inner acrosomal membrane (× 24.000).

Fig. 5. — Spermatozoa which exhibit the vesiculation of the plasma membrane (arrow) (× 48.000).

CELL AND TISSUE CHANGES INDUCED IN CARP YOUNG FISHES BY THE COMPONENTS FROM WATERS DERIVED FROM BREEDING FARMS

MESTER LOTUS*, TESIO CALIN*, POPESCU-MARINESCU VIRGINIA**

In the present work we have pointed out the structural changes at the cell and tissue level in *Cyprinus carpio* young fish generated by the components' actions from residual waters derived from swine breeding Farms. We have to mention strong and irreversible actions at the level of gills, intestine and liver, then, following long term action (even in very dilute waters) at the other organs' level as well: tegument, spleen, brain, muscles, pancreas.

Intense breeding of fishes in conditioned basins is strongly influenced by the quality of used waters. Residual waters from animal breeding farms along with the organic substances charge contain also a high concentration of ammonium (NH_4^+), nitrates (NO_3^-) and nitrites (NO_2^-). Among these, ammonium has the highest concentration and plays a particular role in animals toxicity. Although early investigations pointed out that only nonionised ammonium is toxic for fishes, more recent studies have shown the noxious effects of ionized ammonium form (4), (13), (15), (16). Moreover, the ammonium is the major excretion nitrated product of teleost fishes, eliminated through gills into external environment. The ionized form (NH_4^+) may be excreted in exchange of natrium ions, suggesting several cells regulating mechanisms (8), (10), (17), (18). Besides the lethal effect of ammonium in increased concentrations in water, the sublethal doses have pleiothropic effects on fishes due both to alteration in ionic exchanges at gills level and to its concentration increase in the body liquids, affecting systemic functions and the metabolism of numerous tissues (3), (5), (9), (11). Paley and co-workers (12) have proved that by exposing the hatched young's for 24 h to increased ammonium concentrations, this ion level increases by about 6 folds in the body liquids.

Due to the toxic effects of ammonium one must consider the influence of the pH and of the temperature too. An increase of one unit in the medium pH will cause an increase in nonionized ammonium level by a 10 factor (12).

In the present paper we intended to observe the cell and tissue changes induced in fishes by the components from residual waters derived from breeding farms, in various dilutions.

MATERIALS AND METHODS

Carp young fish, 2 month-old, from the Nucet Fish Research Station, was acclimated to laboratory conditions, in an aquarium of 30 l capacity. On this material ecotoxicologic tests have been carried out using the utilized water in proportions of 5%, 10%, 25%, and 50% mixed with

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tap water. A fish batch was kept as control. In waters with high concentrations, the carp young fish rapidly died (after 20 minutes at 50% concentration and after 60 minutes at 25% concentration). At lower concentrations (10% and 5%) some fishes survived for a longer period and they were killed 7 days after the starting of the experiments.

From the intoxicated material some samples have been taken of gills, lateral muscle, tegument, liver, kidney, intestine, brain, heart. The material was fixed in buffered formalin and in Bouin for 1—2 days, and afterwards it was dehydrated and included into paraffin. 7 μ sections were colored by Mayer hemalaun — eosin and P.A.S.

RESULTS

All four variants of concentrations of the components from the used waters have caused a series of structural changes in the analyzed material, as follows:

At 50% concentration

The gills are strongly affected, the respiratory epithelium being thinned and detached from the skeletal support. In certain areas, epithelial cells are flattened or detached and the capillary endothelium appears discontinued. One can notice extravasated blood between the skeletal support and the gill epithelium. At the tip of certain gill lamellae' one can see capillaries without contents (Fig. 1 C, D).

The *liver, pancreas and intestine* appear histologically modified in some sections. In certain zones at hepatocyte level the intracellular organelles are altered: endoplasmic reticulum and mitochondria disappear, nuclei disintegrate. In certain hepatocytes, nuclei appear as pycnotic, suggesting a disorganization of nuclear chromatin, finally leading to the disappearance of nuclei in the affected cells. The other zones have greatly dilated sinusoids, being noticed extravasation of figured elements.

In *intestine* there are partially affected muscles, submucosa and the basis of some intestinal folds that appear as discolored, with extra cellular matrix injured by the action of toxic substances present in water (Fig. 1 A, B, E).

At 25% concentration:

The *striated muscles* appear partly affected, with myofibril packages losing their affinity with dyestuff. The connective cell nuclei appear here and there hypertrophied or fusioned (Fig. 2 A).

The *gills* appear again with detached zones, suggesting the effect on the support of gill epithelial cells, with white cells, hyperemia and extravasation. Cartilage cells are hypertrophied with nodule aspect. In some areas of gills lamellae one can notice infiltration of white cells (Fig. 2 B).

The *liver* has hepatocytes with modified aspect. On the zones there appear lysed hepatocytes with chromatin network poorly delineated and pycnotic nuclei. The sinusoids appear greatly dilated in some areas, with zones of extravasation (Fig. 1 F).

At the *intestine* level there appear either disaggregations at the end of some folds, or a vacuolising of enterocyte cytoplasm, which lose the dye affinity. The number of goblet cells increases very much. In submucosa one notices the blood vessels dilating and the penetration of some white cells at the basis of intestinal folds (Fig. 2 C).

At 10% concentration:

The *striated muscles* exhibit some myofibrils' packages with poor affinity with dyes and crevice zones. Sarcolemas appear discontinued at certain levels, the muscle's consistency being weak (Fig. 3 A). The *gills* appear with hyperemia, hyperplasia and epithelium detachment from the sustain skeletal support. The gill lamellae appear shortened, fusioned, clavated or flattened. On the gill lamellae a high amount of mucus is deposited, preventing the performing of respiratory exchanges in good conditions (Fig. 2 E, F).

The *liver* is affected by the action of toxic substances present in waters, the display in plates of hepatocytes being altered. The cytoplasm appears vacuolary and toward the liver periphery the cell organelles are damaged and the nuclei appear point form. The dilated sinusoids can be noticed throughout the hepatic parenchyma and among hepatocytes there appear extravasated figured elements (Fig. 3 B, C).

The *intestine* has enterocytes with vacuolary cytoplasm and hypochromatic nuclei or with pycnotic nuclei, infiltration of leukocytes and disintegrated intestine folds at the apical pole (Fig. 2 D).

At 5% concentration:

The *striated muscles* exhibit myofibril packages with gaps between them, with interpose and affinity alterations to dyestuff.

The *tegument* is intensely pigmented at epidermal level and with hypertrophied derma, with loose display connective tissue fibers (Fig. 3 D).

The *gills* are characterized by clavated lamellae and by gill epithelium detachment from the skeletal support. At the basis of certain gill lamellae appear zones with necrosis, certain lamellae are flattened or clavated and the lamellar blood sinusoid is heavily dilated, with extravasation of white cells (Fig. 3 E; 4 A, B).

The *brain* is congested, has a vacuolised aspect of the basic substance and neurons with lysed cytoplasm. Neuron nuclei have a fine granular chromatin or they are pycnotic. Around certain dilated blood vessels some empty spaces appear (Fig. 4 C).

The *liver* appears with partially affected parenchyma basic structure, with hepatocytes with vacuolised cytoplasm and nuclei with granular chromatin. One notices zones with sinusoids extremely dilated, with numerous white cells (Fig. 4 D).

The *intestine* has either enterocytes with vacuolised cytoplasm, or here and there all the structures affected (mucosa, submucosa, the muscularis) appearing discolored, with cells lacking cytoplasmic organelles and nuclei (Fig. 3 F).

The *pancreas* exhibits a strong dilatation of blood vessels here and there, with extravasated leukocytes (Fig. 4 E).

The *spleen* appears with an intense hematopoietic activity, but the normal cell structure is altered. One notices groups of histiocytes and dilated blood vessels (Fig. 4 F).

DISCUSSIONS

Our histologic investigations correlated with numerous previous studies (1), (2), (6), (7), (11), (14) point out the fact that industrial waters and natural waters secondarily used cause various morphological alterations of different tissues of fishes. The most obvious effects are noticed at gills and liver levels manifested by the gill epithelium hypertrophy, gill epithelium and liver tissue necrosis. Cell disorganisation and cell death can be explained by particular mechanisms : direct lithic action of toxic substances or by alteration of lysosomal membrane permeability causing the cell autolysis.

The toxic substances present in aquatic medium can affect aquatic organisms in different ways. First, they can affect permeability and functions of the cells with which they come into direct contact. Once entered in organism in all tissues they can be accumulated in different degrees in a series of structures and they exert various physiologic effects. Our observations concerning the structures point out the noxious effects of used waters on the capillaries in various tissues, suggesting morphofunctional alterations at the whole organism level. In many tissues the capillaries appear dilated, showing alterations of their permeability, favoring the efflux of some molecules into the interstitial space. It is also affected the connective tissue nearby capillaries, very often altered and infiltrated by leukocytes.

A comparative study on fish gills' structural changes induced by various toxic substances in sublethal acute and chronic conditions has revealed that histopathologic injuries (necrosis, hyperplasia, vascular system alterations, etc..) are non-specific and are due to numerous factors' interference (9). Our observations, at histologic level, suggest that toxic substances from industrial waters deeply affect cell functions and organization. On photonic microscopy there were observed cytoplasmic vacuolising, alterations of cytomembrane organization and especially, nuclear alterations (pycnotic nuclei or completely disorganized). These changes indicate effects on cell organization that can lead to cell death (in the liver). It is quite probable that the toxic substances from polluted waters have particular effects on various tissues, variably expressed. The degree of structural and ultrastructural changes of various cell types must be systematically considered in different fish species.

The correct evaluation of aquatic medium quality, especially of some waters from polluted sources for fish breeding should be made with great discernment. Industrial waters used in our investigations contain a mixture of toxic substances. It appears as very important the direct evaluation of the toxicity of each component in different species, but the correlation with other environmental factors as well, to appreciate more accurately the quality of natural and industrial waters used in pisciculture.

CONCLUSIONS

1. Waters derived from animal breeding farms, concentrated (25% and 50%) have a toxic synergetic action, determining the rapid death of young fish (probably due to very high ammonium contents). The action preponderantly occurs on major ways of penetration into organism of the

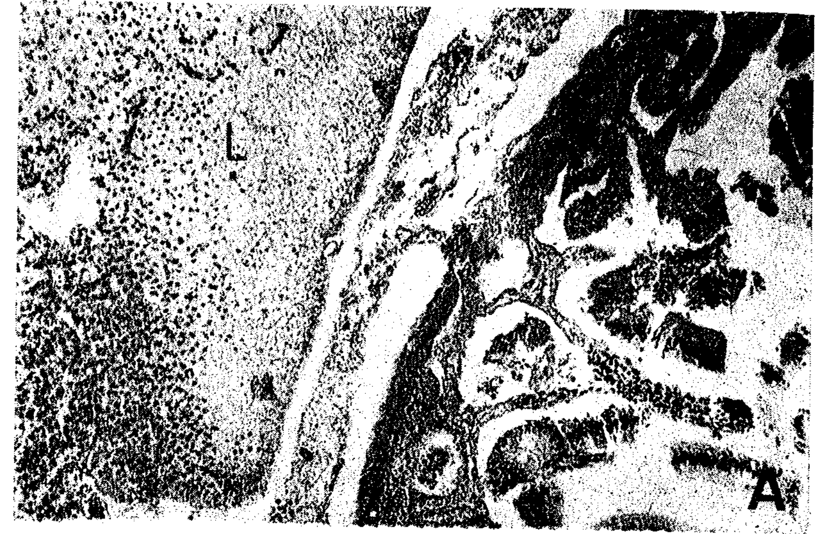

Fig. 1. — A. Carp digestive system and liver sections, kept for 20 minutes in 50% used water. 10 × 0.25.

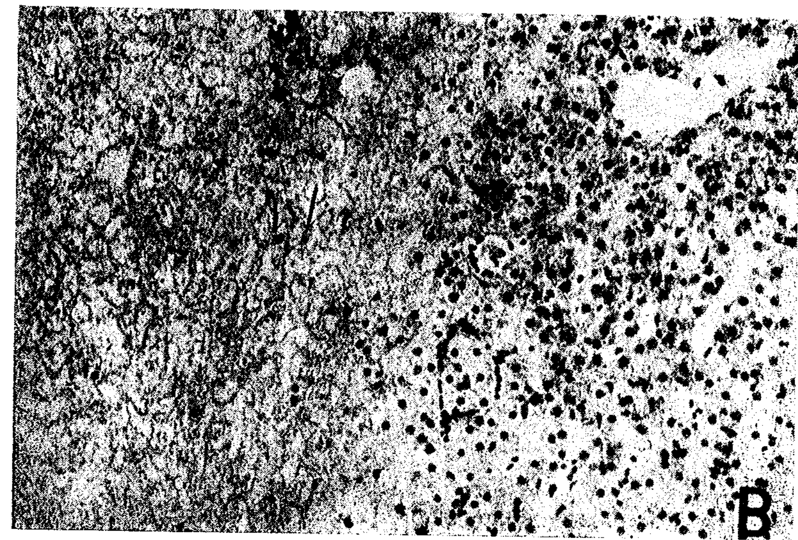

Fig. 1. — B. Carp liver, kept for 20 minutes in 50% used water. One can observe the area of hepatocytes completely lacking cell organelles and nuclei. 20 × 0.40.

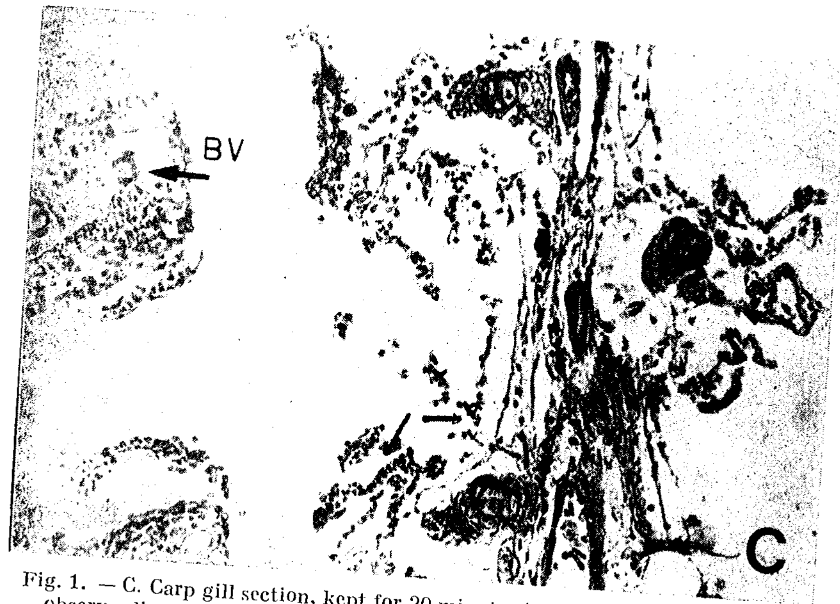

Fig. 1. — C. Carp gill section, kept for 20 minutes in 50% used water. One can observe disorganized gill lamellae (arrows) and cartilaginous nodule. 40 × 0.65.

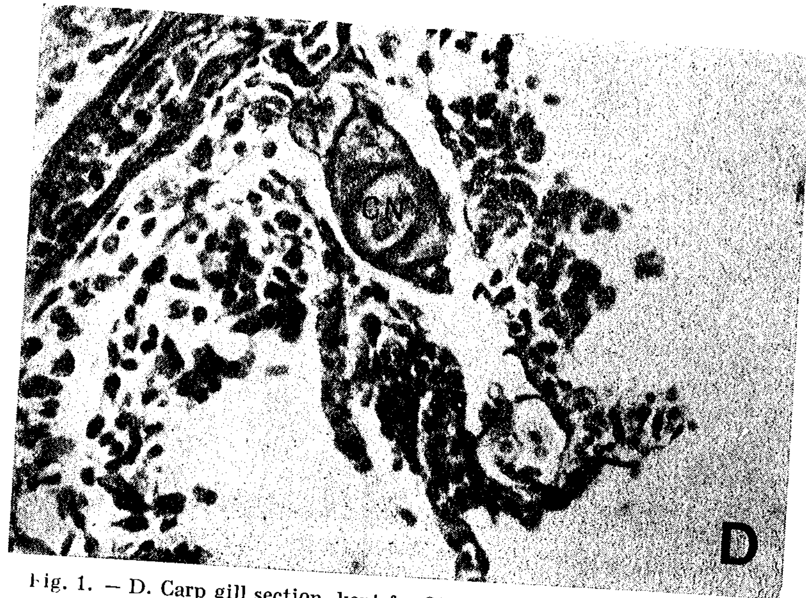

Fig. 1. — D. Carp gill section, kept for 20 minutes in 50% used water. 40 × 0.65.

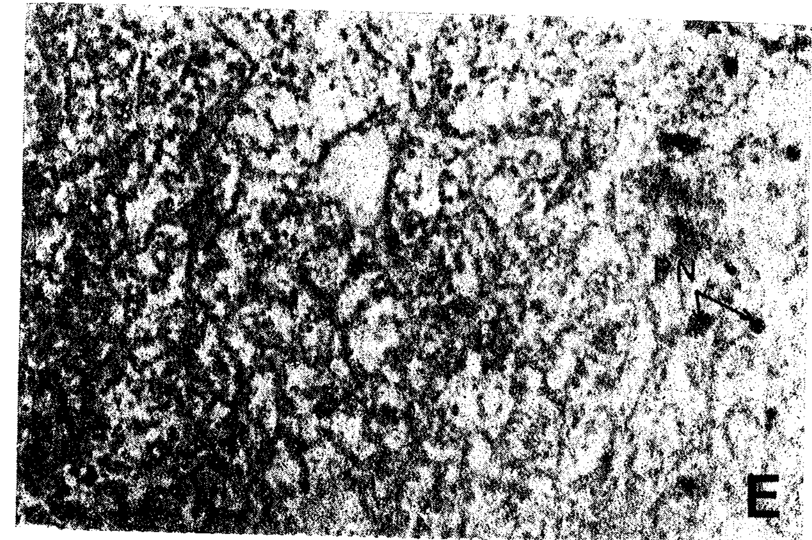

Fig. 1. — E. Carp liver deeply affected by the compounds from 50% used water. 40 × 0.65.

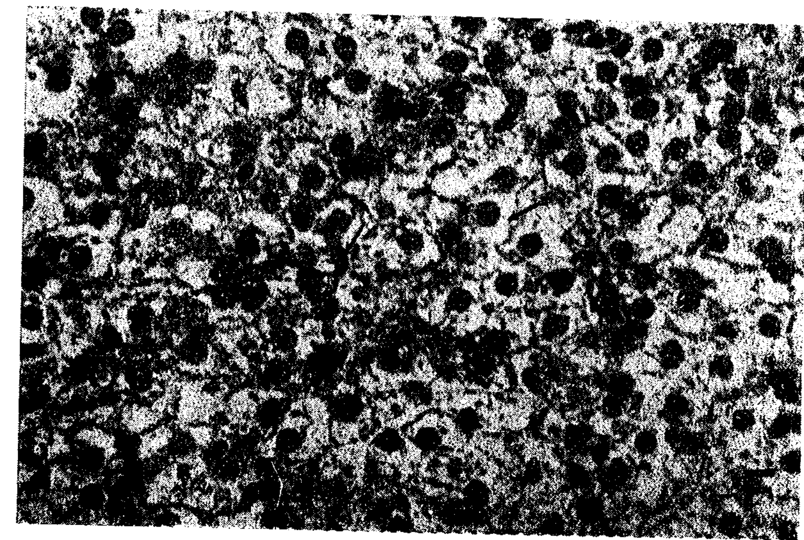

Fig. 1. — F. Carp liver section, kept 1 h in 25% used water. One can notice hepatocytes with vacuolised cytoplasm and some pycnotic nuclei (arrows). 40 × 0.65. Abbreviations : CN — cartilaginous nodule ; PN — pycnotic nuclei ; BV — blood vessel ; L — liver ; IN — intestine.

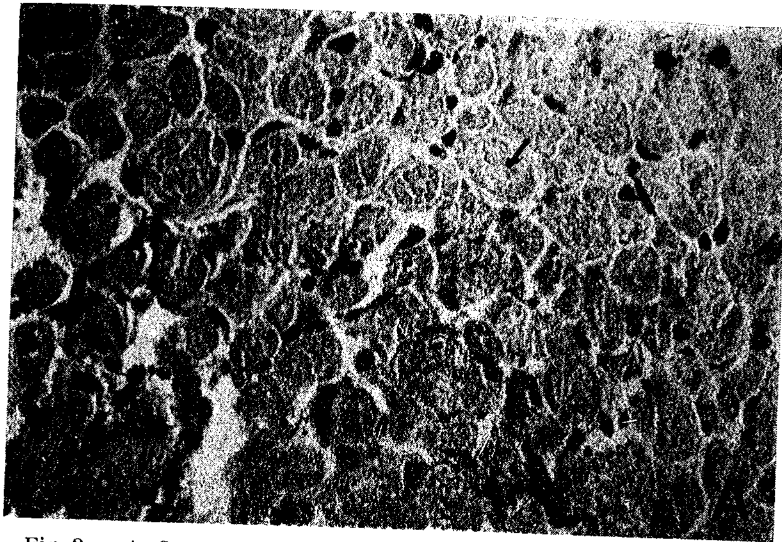

Fig. 2. — A. Carp lateral muscle section, kept for 1 h in 25% used water. One can notice myofibril packages that loose their affinity to dyestuff (arrow). 40 × 0.65.

Fig. 2. — B. Carp gill section, kept for 1 h in 25% used water. The arrows indicate the affected zones. 20 × 0.40.

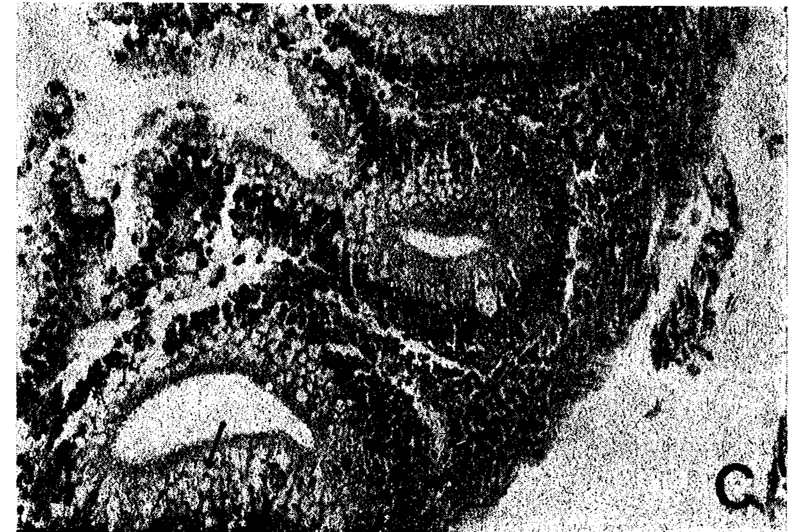

Fig. 2. — C. Carp intestine section, kept for 1 h in 25% used water. One can observe the particular increase in goblet cell number (arrow). 20 × 0.40.

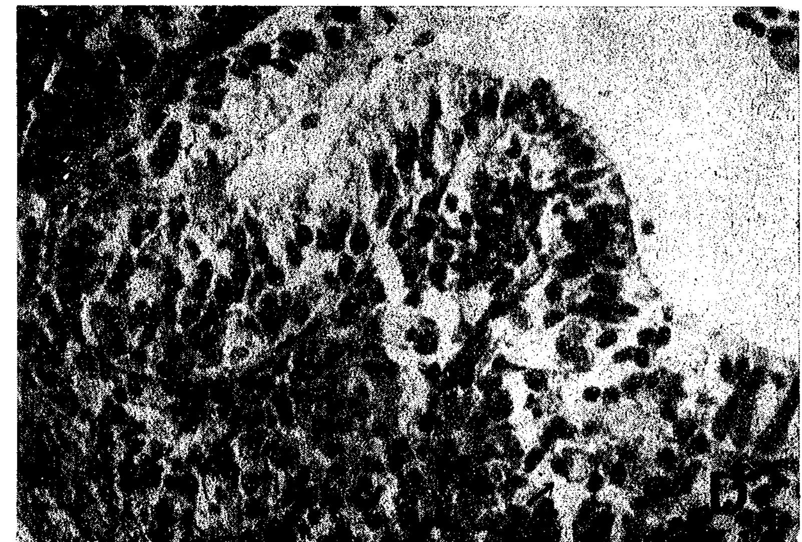

Fig. 2. — D. Carp intestinal folds, kept for 7 days in 10% used water. 40 × 0.65.

Fig. 2. — E. Carp gill section, kept for 7 days in 10% used water. Gill lamellae are flattened, shortened and with detached epithelium (arrows). 20 × 0.40.

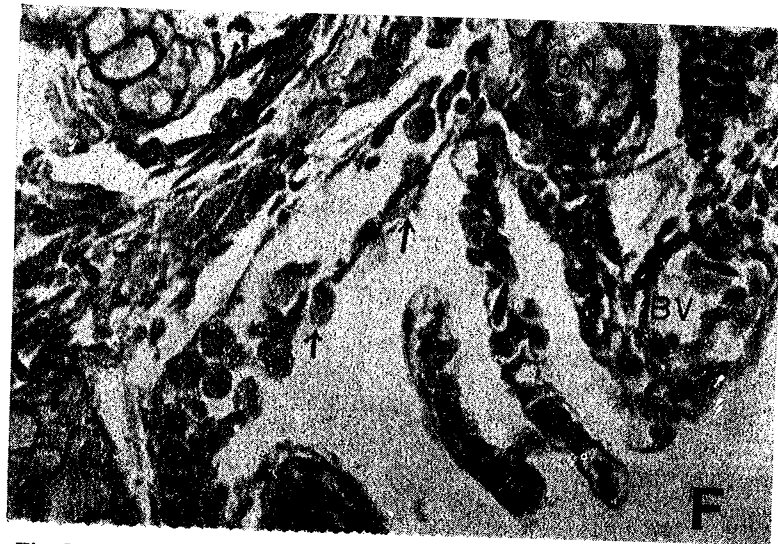

Fig. 2. — F. Carp gill section, kept for 7 days in 10% used water. One can observe the disorganized gill lamellae, dilated blood vessels, cartilaginous nodule. 40 × 0.65.

Abbreviations : CN — cartilaginous nodule ; BV — blood vessel.

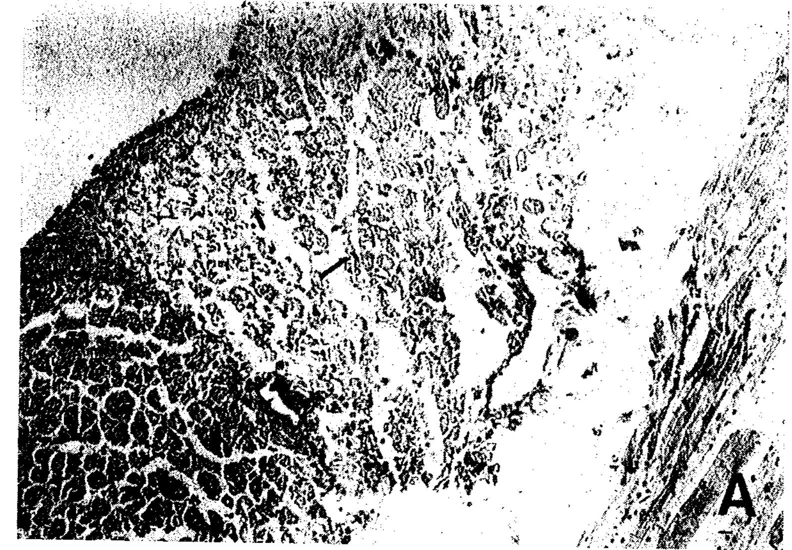

Fig. 3. — A. Carp striated muscles, kept for 7 days in 10% used water. 10 × 0.25.

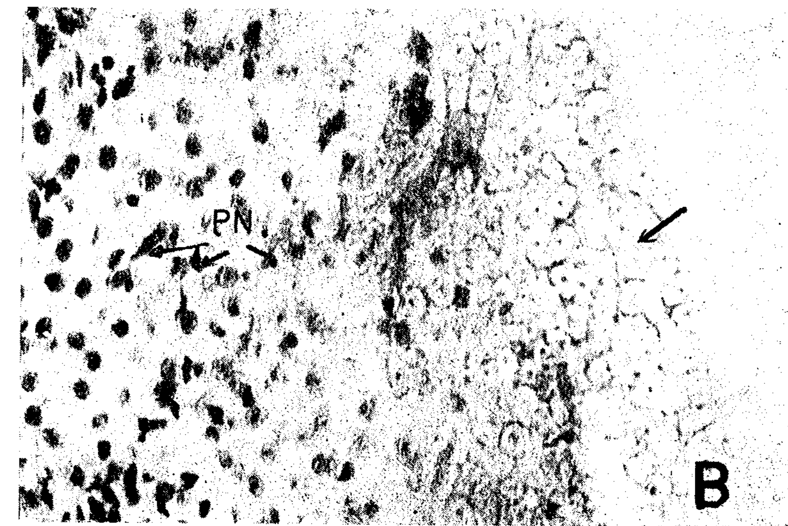

Fig. 3. — B. Carp liver, kept for 7 days in 10% used water. To the right one can notice unfunctional hepatocytes. 40 × 0.65.

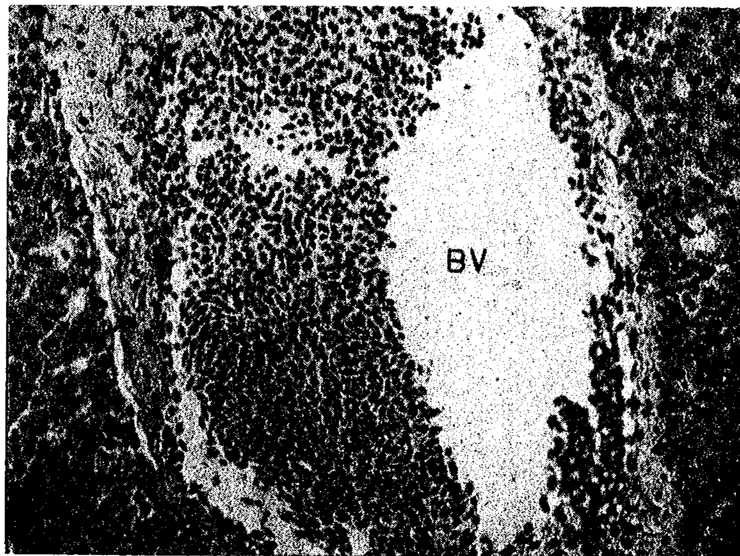

Fig. 3. — C. Carp liver, kept for 7 days in 10% used water. In the center there is a blood vessel very dilated, surrounded by degenerated hepatocytes. 40 × 0.65.

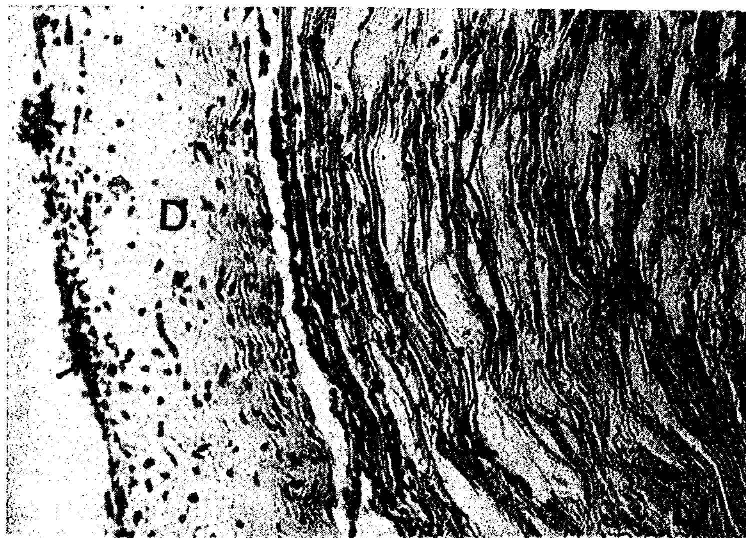

Fig. 3. — D. Carp tegument and striated muscle sections, kept for 7 days in 5% used water. 20 × 0.40.

Fig. 3. — E. Carp gill section, kept for 7 days in 5% used water. One can observe the gill epithelium detached from the skeletal support and zones with necrosis at the gill lamellae basis. 20 × 0.40.

Fig. 3. — F. Carp intestine section, kept for 7 days in 5% used water. Some enterocytes with vacuolised cytoplasm (arrow). 20×0.40. Abbreviations : CLM — clavate lamellae ; PN — pycnotic nuclei ; BV — blood vessel ; ZN — zone with necrosis.

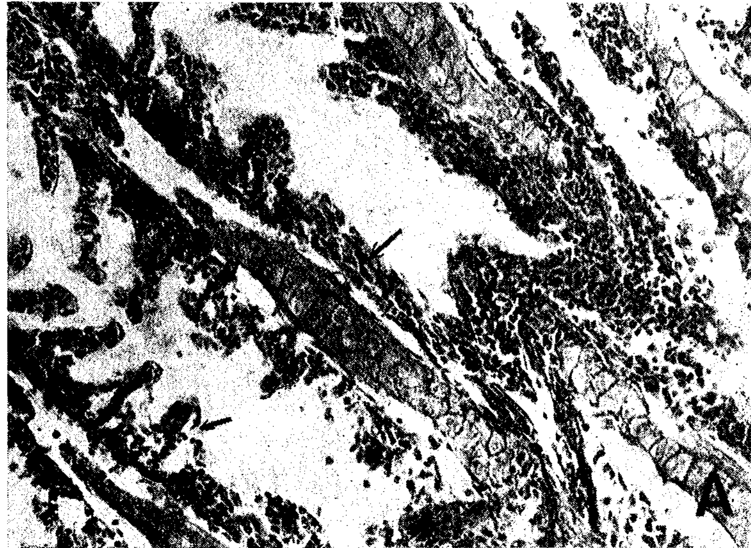

Fig. 4. — A. Carp gill, kept for 7 days in 5% used water. Some lamellae appear flattened, other with detached epithelium (arrows). 20 × 0.40.

Fig. 4. — B. Carp gill affected by the compounds of 5% used water. In lamella axis one can observe a dilated blood vessel, one side boarded by flattened lamellae. 40 × 0.65.

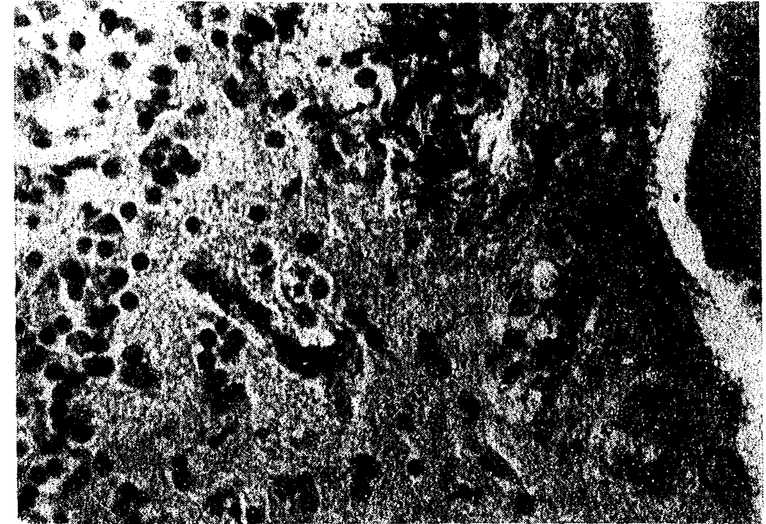

Fig. 4. — C. Carp brain section, kept for 7 days in 5% used water. One can observe vacuolised zones (arrows). 40 × 0.65.

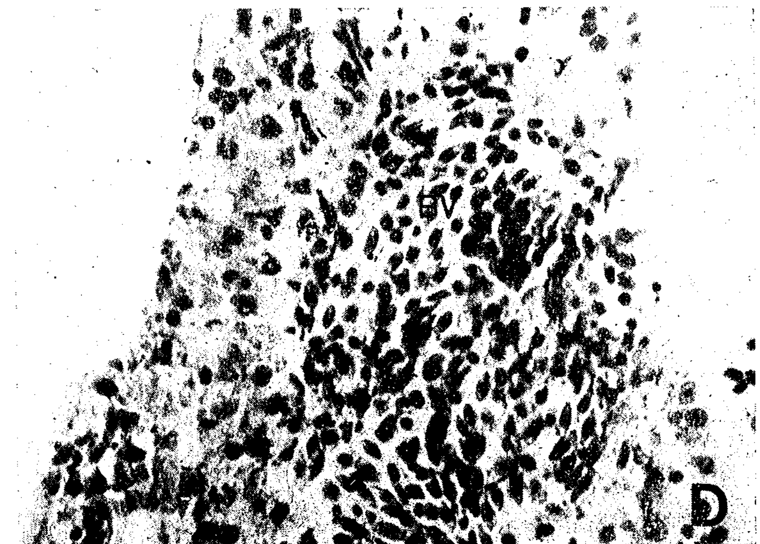

Fig. 4. — D. Carp liver, kept for 7 days in 5% used water. In the center there is a high dilated blood vessel, boarded by nonfunctional hepatocytes. 40 × 0.65.

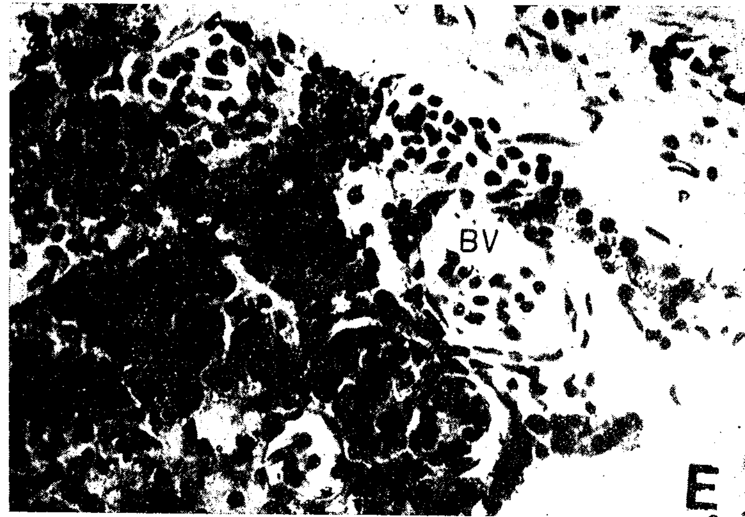

Fig. 4. — E. Carp pancreas section, kept for 7 days in 5% used water. 40 × 0.65.

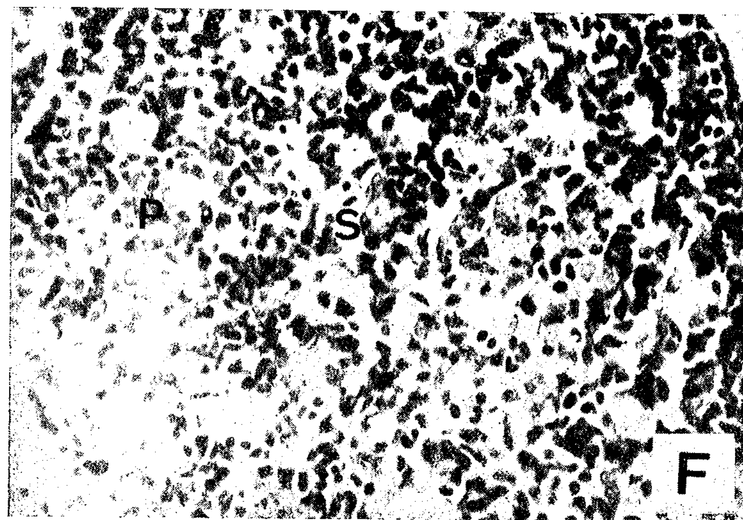

Fig. 4. — F. Carp spleen section, kept for 7 days in 5% used water. Affected parenchyma, dilated sinusoids. 40 × 0.65.
Abbreviations: P — parenchyma; S — sinusoid; BV — blood vessel; ZN — zone with necrosis.

toxic substances (gills and intestine) and strongly affects the liver, which cannot perform the detoxification action. As far as other organs are concerned the very short span of fish life (from experiments) does not allow the induction of some visible alterations.
2. Lower concentrations of these waters (10% and 5%) cause changes of irreversible character at various organs' levels. The tegument is also affected, it changes the color, the derma is hypertrophied and the muscles appear of tender consistency.
3. In all concentrations first the liver is affected; thus its mechanisms of toxic substance elimination are disturbed.
4. Respiratory exchanges are slowed down, a great quantity of mucus is accumulating on gills' lamellae and the toxic substances present in waters cause a deterioration of gill epithelium and blood vessels (even at 5% dilution, the ammonium concentration in residual waters is very high).
5. Toxic substance action also affects the brain, which appears congested, the increase in ammonium concentration causing a decrease in hemoglobin affinity for oxygen.
6. The waters from animal breeding farms, in case they are flown into the rivers without a prior well purification can lead to a massive destruction of aquatic fauna, a fact already noticed on various river sections.

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EXPERIMENTAL HYBRIDIZATION WITHIN THE *TRITURUS VULGARIS* SPECIES-GROUP (AMPHIBIA, CAUDATA)

DAN COGĂLNICEANU

I investigated the relations between taxa of the *Triturus vulgaris* species-group by performing several hybridization experiments. The following natural crosses were done: *T. helveticus* × *T. montandoni*, *T. vulgaris ampelensis* × *T. helveticus*, *T. v. ampelensis* × *T. montandoni*, and *T. v. vulgaris* × *T. helveticus*. I compared the secondary sexual characters of the parental species and F_1 hybrid males to identify the pattern of dominance. This decreases in the order *T. vulgaris* > *T. helveticus* > *T. montandoni*. I suggest that morphologically *T. montandoni* is similar to the ancestor of this group. The evolution among taxa of the *T. vulgaris* species-group was probably caused by an increase in sexual dimorphism. The evolution of belly pattern supports this hypothesis. *T. montandoni* has an unspotted belly, in *T. helveticus* numerous small spots are present in both sexes, and in *T. vulgaris* the pattern of spots is different in the two sexes.

INTRODUCTION

The genus *Triturus* is one of the most studied groups of vertebrates (15), still the relationships between species remain still uncertain. The *Triturus vulgaris* species-group includes three species of small bodied newts: *T. vulgaris*, *T. helveticus* and *T. montandoni*. Biochemical and cytogenetic data suggest that *T. montandoni* and *T. vulgaris* are the more closely related species (4, 13, 17, 22, 23), though *T. montandoni* is more similar to *T. helveticus* both in morphology and in courtship behavior (21). *T. helveticus* is distributed in Western Europe and is allopatric with *T. montandoni* that inhabits only a restricted area in the Carpathian Mountains, between Romania and Poland. Ten subspecies are currently recognized in *T. vulgaris* (7, 24), that differ mainly in male secondary sexual characters. The process of subspeciation in *T. vulgaris* probably occurred during the Pleistocene glaciations (7). Eight of *T. vulgaris* subspecies are distributed at the periphery of the areal of the nominal form *T. v. vulgaris*, except *T. v. ampelensis*, which is restricted to an area almost in the center.

Five of these subspecies *(graecus, dalmaticus, meridionalis, borealis,* and *ampelensis)* share several characters in common: low and smooth edged dorsal crests, dorso-lateral ridges and tail filaments or tips. These characters are shared with *T. helveticus* and *T. montandoni* but lack in the nominal subspecies. *T. v. vulgaris*, which is the most widely distributed, is sympatric over quite a large area with *T. helveticus* and on a narrow contact zone with *T. montandoni*. In these areas, the morphological differences between species are greater and represent a good example of character displacement.

Hybridological studies allow to evaluate the relatedness between species and subspecies, their level of differentiation and the efficiency of the isolation barriers. Newts are useful in these studies since they hybridize spontaneously in nature and in captivity, and artificial fertilization can be easily induced (17, 18). The importance of hybridization in evolu-

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tion has been repeatedly emphasized (2, 16), since it also represents a means of speciation (5). Several methods were proposed for phylogenetic analysis that consider hybrids, Dubois's (8) "hybrid distance" and Anderson's (1) hybrid index.

In an attempt to get a better understanding of the evolution in the *T. vulgaris* species-group I performed several hybridization experiments and I compared the secondary sexual characters of the F_1 hybrid males to ascertain the pattern of dominance and to infer the morphology of the ancestral form.

METHODS

The animals used in the hybridization experiments were caught from natural populations in Romania from Predeal, (*T. montandoni*), Zlatna (*T. v. ampelensis*) and Bucharest (*T. v. vulgaris*). *T. helveticus* came from a captive bred colony originating from Basel, Switzerland. All females used hibernated in captivity so were not previously inseminated. The males were freshly caught in spring.

The animals were kept in large aquariums or aquaterrariums and were allowed to hibernate about five months each winter. They were fed a mix diet of *Tubifex*, earthworms, white-worms, and *Drosophila*. The sexes were separated, except during reproduction when they were put together in 10 l aquariums. No hormonal treatment was needed. The hatching larvae were transferred to aquariums where a culture of *Daphnia* sp. was previously started. A supplement of chopped *Tubifex* and white-worms was added 2–3 times a week. The larval mortality was not recorded, only the number of metamorphosing animals was considered. The only F_1 hybrids that could be reproduced were from the *vulgaris* ♀ × *helveticus* ♂ cross, although the resulting offspring suffered high post-metamorphic mortality.

The true hybrid nature of the offspring of *helveticus* ♀ × *montandoni* ♂ and *ampelensis* ♀ × *helveticus* ♂ crosses was proved electrophoretically on two pairs of animals by Dr. Jan Rafinski.

Nine male secondary sexual characters were scored with presence (1) or absence (0) in the parental species and F_1 hybrids, considered each as an Operational Taxonomic Unit (OTU): dorsal crest (A), dorsolateral ridges (B), unspotted belly (C), unspotted throat (D), gular fold (E), smooth skin (F), tail filament (G), webbed hind feet (H), and lower side of the tail colored (I). *T. cristatus*, a large bodied newt species, was introduced as outgroup, with another character, body size (J), scored with large (1) or small (0). For the *vulgaris* ♀ × *helveticus* ♂ cross the data was completed with the one presented by Griffiths, Roberts & Sims (14). Data from literature (11, 12) and from personal observations in the field was introduced for the *vulgaris* × *montandoni* cross.

To identify the taxonomic position of the hybrids relative to the parental species the data was subjected to parsimony analysis. The most parsimonious Wagner trees were calculated using the Penny program and a strict consensus tree was found using the Consense program (PHYLIP 3.4) (9).

RESULTS

The following natural crosses were done:
- *T. helveticus* (4 females) × *T. montandoni* (2 males) in 1988, 110 juveniles metamorphosing;
- *T. v. ampelensis* (1 female) × *T. helveticus* (1 male) in 1989, 12 juveniles metamorphosing;
- *T. v. ampelensis* (1 female) × *T. montandoni* (1 male) in 1990, 50 juveniles metamorphosing;
- *T. v. vulgaris* (4 females) × *T. helveticus* (4 males) in 1990, 11 juveniles metamorphosing.

Morphologically the hybrid males show intermediate characters or a mixture of characters of the parental species (Table 1).

Table 1

Male secondary sexual characters scored for presence or absence (see text for details).

Species and hybrid combinations	Characters									
	A	B	C	D	E	F	G	H	I	J
T. v. vulgaris	1	0	0	0	0	1	0	0	1	0
T. montandoni	0	1	1	1	1	0	1	0	0	0
T. helveticus	0	1	0	1	1	0	1	1	1	0
T. v. ampelensis	1	1	0	0	0	1	1	0	1	0
T. helveticus × *montandoni*	0	1	0	1	1	0	1	0	0	0
T. ampelensis × *helveticus*	0	1	0	0	1	1	1	0	1	0
T. ampelensis × *montandoni*	0	1	0	1	1	0	1	0	1	0
T. vulgaris × *helveticus*	1	1	0	0	0	1	1	1	1	0
T. vulgaris × *montandoni*	1	1	0	0	1	0	1	0	1	0
T. cristatus	1	0	0	0	1	0	0	0	0	1

The morphological analysis of the parental species produced a parsimony tree that does not agree with the one revealed by biochemical and cytogenetical data (Figure 1). The joint analysis with the hybrids does not add more information nor do they introduce any distortions. The hybrids are distributed between two "extremes": *vulgaris* and *montandoni*. Each hybrid is nonetheless positioned between its two parental species. Reticulated trees could represent a good solution and might prove useful in case only one hybrid combination is introduced at a time (19, 25).

DISCUSSIONS

The degree of compatibility and the relations between the different taxa of the *T. vulgaris* species-group through hybridization have been intensively documented (17, 18). The present state of knowledge is synthesized in Table 2.

T. v. ampelensis is genetically different from *T. v. vulgaris* ($D_H = 0.17$; Rafinski and Cogălniceanu, unpubl. data) and intergrading with *T. v. vulgaris* is frequent (10). It is not yet known if it is parapatric or sym-

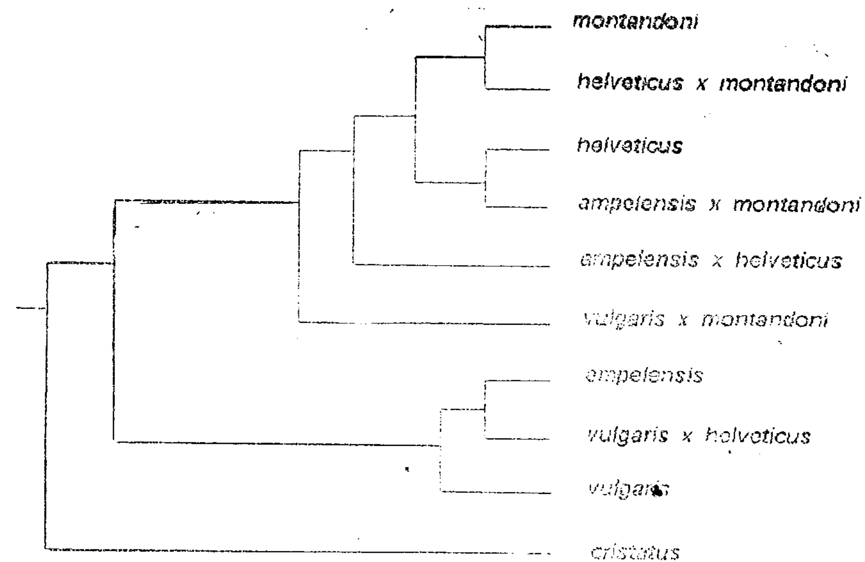

Fig. 1. — Dendrogram of the most parsimonious Wagner tree (see text for details)

patric with *T. montandoni*, so no definite conclusions can be drawn from the easiness they hybridized.

It is difficult to identify a real F_1 phenotype among the *T. vulgaris* × *montandoni* hybrids in nature. In syntopic localities hybrids represent up to 60% of the population with high introgression (21). In natural hybridizing populations animals with intermediate characters can be frequently found ranging all the way between the pure parental morphs (Cogălniceanu, unpubl. data).

Table 2

Hybridization among *Triturus vulgaris* species-group

♀ \ ♂	*T. v. vulgaris*	*T. v. ampelensis*	*T. montandoni*	*T. helveticus*
T. v. vulgaris	—	○	○ ●	○ ○ ■
T. v. ampelensis	○	—	■	■
T. montandoni	○ ●		—	
T. helveticus	○ ●		■	—

Legend : ○ — natural hybridization
● — experimental hybridization (in literature)
■ — experimental hybridization (present paper)

As previously suggested (6), the parental influences differ strongly, decreasing from *vulgaris* to *helveticus* and then *montandoni*. The male characters of *montandoni* (unspotted belly, tail filament, lack of dorsal crest and of vivid color) are lacking in hybrids and tend to be replaced with

vulgaris characters (spotted belly, dorsal crest and vivid coloration). *T. montandoni* shares the same primitive characters with two other species, *T. boscai* and *T. italicus*, that have also a restricted distribution and no subspeciation. This supports the hypothesis that *T. v. vulgaris* is a young subspecies and that *T. montandoni* is a primitive species, similar to the ancestor of this group (7).

I suggest that the evolution among members of *T. vulgaris* species-group was through an increase in sexual dimorphism, possibly involving stronger sexual selection pressures. For example, in *T. montandoni* both males and females have unspotted belly. In *T. helveticus* small spots are present in both sexes. In *T. v. vulgaris* males have larger spots than females. In some subspecies, including *T. v. ampelensis*, many individuals have unspotted bellies, thus ressembling *T. montandoni*. The possible role of the larger spots in *T. v. vulgaris* males is to present a stronger contrast and, with the vivid colors displayed during breeding season, have a higher visual impact on females.

Isolating reproductive mechanisms develop only between sympatric or parapatric species. No effective reproductive mechanisms were observed in captivity between the allopatric *T. helveticus* and *T. montandoni*, which could be easily crossed. It is possible that these two species are the result of the splitting of an ancestral form by one or more Pleistocene glaciations, similar to the case of *Bombina bombina* and *B. variegata* (3). Then *T. vulgaris* would have separated from the common lineage before the Pleistocene glaciacions. This hypothesis is not supported by the biochemical and cytogenetical studies (20), although the accuracy of the timing of the splitting events is low. One explanation is that since *T. vulgaris* and *T. montandoni* hybridize easily and there is a quite important transfer of genes, the results might be biased. Animals far from the hybridizing areas should be used in these studies.

Although the use of hybrids in phylogenetic analysis is not recommended (Arntzen, pers. commun.) further crosses and backcrosses between members of the *T. vulgaris* species-group, and a detailed study of the intergrading areas between *T. vulgaris* subspecies and between *T. vulgaris* and *T. montandoni* might offer a better understanding of the phylogenetic relations and of the evolution and speciation of this group.

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CONTRIBUTIONS OF PARASITOID HYMENOPTERA TO LIMITING THE OUTBREAK OF SOME DEFOLIATOR LEPIDOPTERA POPULATIONS IN THE OAK WOODS

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32 species of primary parasitoid Hymenoptera were recovered from three defoliator moths : *Lymantria dispar* L., *Tortrix viridana* L. and *Malacosoma neustria* L. 5. species are oophagous parasitoids of *Lymantria dispar*, 16 species are larval and pupal parasitoids of the same host. 7 species are pupal parasitoids of *Tortrix viridana* and 10 species of *Malacosoma neustria*, respectively.
Lymantria dispar is a new host in science for *Euceros serricornis* Hal. and *Casinaria punctiventris* Woldst.

In Romania, especially in the Southern part, the majority of oak woods, particularly those which contain *Quercus cerris* L., *Q. frainetto* Ten. and *Q. pubescens* Wild., are often heavily infested by the defoliator Lepidoptera.

The many negative effects, which occurred because of the intensive treatment system with pesticides for the control of forest insect pests, led to the intensive researches about the limitative action of the biotic factors of natural mortality.

In this paper we present the results of our researches on the role of the parasitoid Hymenoptera for limiting the outbreak of the following defoliators of oak woods : *Lymantria dispar* L. (gypsy moth), *Tortrix viridana* L. (green oak leaf roller) and *Malacosoma neustria* L. (lackey moth).

MATERIALS AND METHODS

Between 1977—1990 we carried out studies in 37 oak woods in the Southern Romania. In these woods our observations about the three defoliator species were made in the field stations, where we collected the necessary biological material.

In order to establish the parasitoid Hymenoptera species and their parasitization degrees achieved by them we collected *Lymantria dispar* egg masses, larvae and pupae, pupae of *Tortrix viridana* L. and *Malacosoma neustria* L. from each field station. These were isolated and kept in laboratory conditions. The parasitoid hatching was watched daily.

RESULTS AND DISCUSSION

We recovered 32 parasitoid Hymenoptera species of which five species are oophagous parasitoids of *Lymantria dispar*, 16 species are both larval and pupal parasitoids of *Lymantria dispar*, 7 species are pupal parasitoids of *Tortrix viridana* and 10 species of *Malacosoma neustria*.

The oophagous parasitoids of *Lymantria dispar* were recovered from all researched woods. The intensity of their activity varied much from year to year (0.1 % — 22.7 %) and we identified 5 oophagous parasitoid species (Table 1). *Anastatus disparis* and *Ooencyrtus kuwanai* are the most efficient parasitoids, the former is a dominant species in the oak woods of the Romanian Plain, while the latter is a dominant species in those of Oltenia. The scelionids are accidental species in the parasite complex of *Lymantria dispar* but baving faunal importance.

Table 1

Parasitoid Hymenoptera recovered from defoliator Lepidoptera:

Lymantria dispar L., *Tortrix viridana* L. and *Malacosoma neustria* L.

Host	Immature stage of host	Parasitoid species	Observations
1	2	3	4
Lymantria dispar	egg	Fam. *Eupelmidae* *Anastatus disparis* Ruschka Fam. *Encyrtidae* *Ooencyrtus kuwanai* (How.) Fam. *Scelionidae* *Gryon howardi* Mokr. et Ogl. *Telenomus laeviusculus* Thoms. *Telenomus lymantriae* Thoms.	
Lymantria dispar	larvae	Fam. *Braconidae* *Apanteles melanoscelus* Ratz. *Apanteles porthetriae* Mus. Fam. *Ichneumonidae* *Euceros superbus* Kriechb. *Euceros serricornis* Hal. *Phobocampe unicincta* (Grav). *Hyposoter tricoloripes* Grav. *Casinaria punctiventris* Woldst. *Theronia atalantae* Poda Fam. *Eulophidae* *Eulophus larvarum* L.	*L. dispar* is a new host in science for *Euceros serricornis* and *Casinaria puncti-ventris*
	pupae	Fam. *Ichneumonidae* *Euceros superbus* Kriechb. *Theronia atalantae* Poda *Pimpla instigator* F. *Itoplectis enslini* Ulbr. *Apechtis compunctor* (L.) Fam. *Chalcididae* *Brachymeria intermedia* (Nees) Fam. *Torymidae* *Monodontomerus aereus* Walk.	
Tortrix viridana	pupae	Fam. *Ichneumonidae* *Theronia atalantae* Poda *Apechtis rufata* (Gmel.) *Apechtis resinator* (Thunb.) *Apechtis compunctor* (L.) *Phaeogenes invisor* (Thunb.) *Itoplectis maculator* (F.) *Itoplectis alternans* (Grav.)	

1	2	3	4
Malacosoma neustria	pupae	Fam. *Ichneumonidae* *Theronia atalantae* (Poda) *Pimpla instigator* F. *Pimpla turionellae* L. *Gregopimpla malacosomae* Seyr. *Itoplectis kolthoffi* (Auriv.) *Scambus foliae* (Cushm.) *Scambus boulianae* (Hart.) *Euceros superbus* Kriechb. *Acropimpla didyma* Grav. *Trichoma occisor* Seyr.	

We remarked in our studies that Hymenoptera represent a less efficient group for the parasitization of larvae and pupae of defoliator moths as compared with the parasitic Diptera. Thus, in the parasite complex of larvae and pupae *Lymantria dispar* we identified 14 parasitoid species of Hymenoptera of which 7 species are only larval parasitoids, 5 species are only pupal parasitoids and *Theronia atalantae* and *Euceros superbus* are both larval and pupal.

The parasitization of the gypsy moth larvae by Hymenoptera was of 13.2 % and for the pupae was of 18.8 % (Fig. 1).

Generally, braconids and ichneumonids parasitize larvae of gypsy moth in their second to the fourth stages. For example, *Apanteles melanoscelus*, which achieved low parasitization degrees between 0.3 % (Frasinu woods, 1978) and 8.8 % (Blaj woods, 1984) and *Apanteles porthetriae* achieved an unimportant parasitization (it was present only in Frasinu woods in 1978).

Occasionally, there could be seen a multiparasitism of gypsy moth larvae, when the same larva is simultaneously parasitized by two gypsy moth parasitoids, the braconid *Apanteles melanoscelus* and the tachinid *Compsilura concinnata* Meig.

The tolerance between *Apanteles melanoscelus* and *Compsilura concinnata* probably occurs because the parasitoids fill different niches and thus they do not encounter with each other within the host. *Apanteles melanoscelus* is found in the host haemolymph, while *Compsilura concinnata* resides in the host alimentary canal. *Compsilura concinnata* develops slowly enough to permit *Apanteles melanoscelus* to emerge before *Compsilura concinnata* does.

The cocoons of *Apanteles melanoscelus* can be parasitized by hyperparasitoids. Thus, in the lot of larvae collected on May, 24, 1977, in Frasinu woods, we recovered two specimens of *Apanteles melanoscelus*. One of them had been parasitized by an *Eurytoma* sp. male and the other one by a female and 4 males of *Pediobius* sp.

Euceros superbus, *Euceros serricornis*, *Phobocampe unicincta*, *Hyposoter tricoloripes*, *Casinaria punctiventris* and *Theronia atalantae* are pupal parasitoids.

The eulophid, *Eulophus larvarum*, was recovered from the larva of gypsy moth, which achieved some important parasitization degrees, being found only in the Frasinu woods and only once. Sometimes, the

efficiency of this primary parasitoid can be limited by the secondary parasitoid, *Pediobius* sp.. The same happens with *Apanteles melanoscelus*. Thus, in the same larvae lot collected in Frasinu woods in 1977, we noticed that *Pediobius* sp. achieved a parasitization of 56.9 % for *Eulophus larvarum*.

We noticed that in the Islaz woods in 1983, where we collected 1273 host larvae, 161 of them were parasitized (12.8%), of which 35% only by Hymenoptera. In these woods only microbiological treatments with NPV (nucleopolyhedrosis virus) were applied, but they have not affected Hymenoptera populations because they constitute an entomophagous group which is very sensitive to chemical treatments.

Lymantria dispar is a new host in science for *Euceros serricornis* and *Casinaria punctiventris*.

The parasitoid Hymenoptera of the gypsy moth, green oak leaf roller and lackey moth pupae were investigated during the period when the hosts were present and we could collect many pupae. 19 Hymenoptera species were recovered from the pupae of these three defoliator species (Table 1).

Hymenoptera achieved a much smaller degree of parasitization of the *Lymantria dispar* pupae than the parasitic Diptera (Fig. 1).

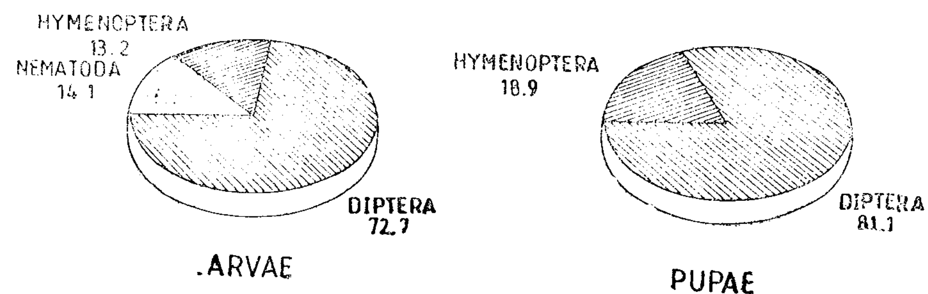

Fig. 1. — Total parasitization of gypsy moth larvae and pupae.

From the *Lymantria dispar* pupae we recovered two primary parasitoids: *Brachymeria intermedia* and *Monodontomerus aereus*, the latter as primary and secondary parasitoid. From among Hymenoptera, *Brachymeria intermedia* achieved the greatest parasitization degrees with a peak parasitism of 12.6 % in 1977 for the host pupae in Frasinu woods, but *Monodontomerus aereus* had much lower parasitization degrees with a peak parasitism of 0.4 % in 1982, in Bălășcuța woods.

We recovered *Monodontomerus aereus* as primary parasitoid from 16 oak woods and as secondary parasitoid it was recovered from *Compsilura concinnata* puparia in the lot of pupae collected in August, 5, 1977 in Rușii lui Asan woods.

Theronia atalantae is a common species of the three parasite complexes (Fig. 2), with the greatest efficiency. For example, in *Lymantria dispar* pupae this species achieved the greatest parasitization degrees, between 0.1 % — 4 %. It was present in 18 oak woods of the 32 ones from which we collected host pupae and it parasitized the pupae in almost each generation of host.

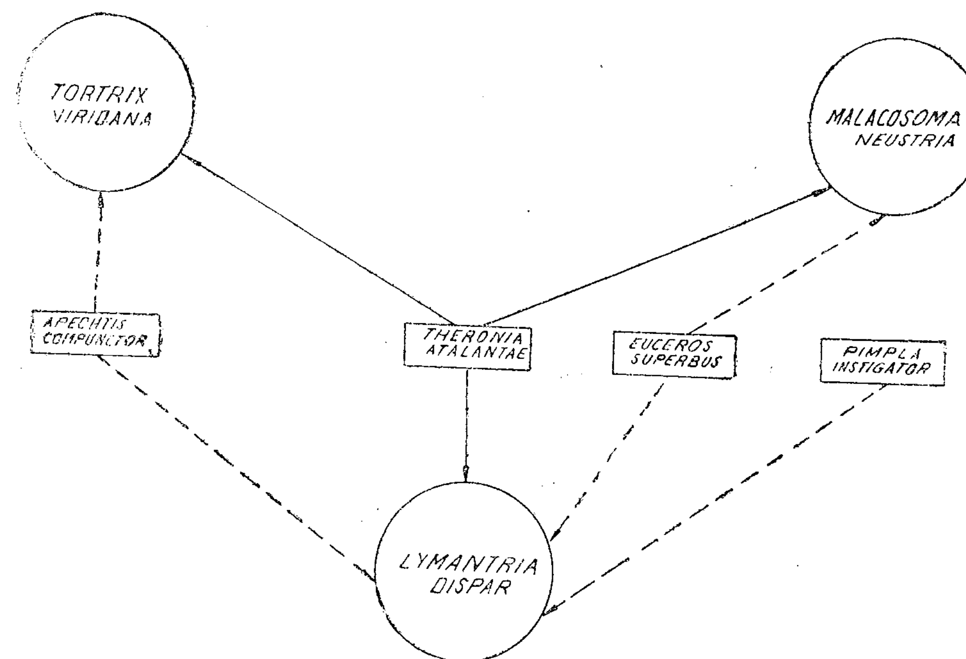

Fig. 2. — Common parasitic Hymenoptera of the parasitic defoliator complexes.

Although the parasitization percentage of the collected pupae was reduced, we noticed a great frequency of *Theronia atalantae* specimens. This fact led us to the idea of the role of this entomophagous in limiting the host pupae.

We noticed that many *Theronia atalantae* females sting the host pupae, feeding on the body fluids oazed out from the wound produced by the ovipositor.

We took to the laboratory a part of the pupae stung by *Theronia atalantae* isolated them and the other part were marked and left in the field, for 2 weeks.

We noticed that from the pupae taken to the laboratory, only a few ichneumonids emerged. Periodically, we checked on the marked pupae left in the field and after some time we noticed that a part of them were parasitized by tachinids.

These observations led us to the idea that tachinids penetrate the pupa of *Lymantria dispar* only after the latter was previosly stung by ichneumonids.

Both laboratory and field observations reveal that although parasitization of the pupae by ichneumonids is very small as compared with that by tachinids, nevertheless ichneumonids play a special part as limiting biotic factors of defoliator populations.

Euceros superbus and *Pimpla instigator* are common parasitoids of the *Lymantria dispar* and *Malacosoma neustria* pupae, while *Apechtis compunctor* parasitize the pupae of *Lymantria dispar* and *Tortrix viridana* (Fig. 2).

Important observations also result from the analysis of parasitization of pupae on sex ratio. In all field stations female pupae of *Lymantria dispar* are parasitized in a greater ratio than the male ones.

From a practical point of view, this observation has a great importance especially in prognosis. A most accurate prognosis implies the collecting of pupae from the woods towards the end of pupation, when both male as well as female pupae can be found.

If we established the parasitization degree at the beginning pupation when male pupae prevail would lead to improper results which would directly influence the protection measures for future years.

CONCLUSIONS

1. In this paper we present 32 species of primary parasitoid Hymenoptera which contributed to the limiting outbreak of defoliators: *Lymantria dispar*, *Tortrix viridana* and *Malacosoma neustria;*

2. 5 species are oophagous parasitoids of *Lymantria dispar*, 16 species are larval and pupal parasitoids of the same host, 7 species are pupal parasitoids of *Tortrix viridana* and 10 species of *Malacosoma neustria*, respectively;

3. The most efficient oophagous parasitoids proved to be *Anastatus disparis* and *Ooencyrtus kuwanai*, the former being dominant species in the central area of the *Romanian Plain*, while the latter is dominant in the oak woods in Oltenia. The scelionids are accidental species in the parasite complex of *Lymantria dispar*, but being faunally important;

4. *Theronia atalantae* is a common parasitoid species of the parasite complexes of the three Lepidoptera species, while *Euceros superbus* and *Pimpla instigator* are common of the gypsy moth and lackey moth pupae, but *Apechtis compunctor* parasitizes the *Lymantria dispar* and *Tortrix viridana* pupae;

5. *Lymantria dispar* is a new host in science for *Euceros serricornis* and *Casinaria punctiventris;*

6. Although Hymenoptera have a smaller contribution in parasitizing the larvae and pupae of defoliator Lepidoptera some species of ichneumonids by their activity favour the parasitization of these host by Diptera.

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Prof. Dr. Doc. M. AL. IENIȘTEA

(1910–1994)

The disappearance of Prof. Dr. Doc. Mircea Alexandru Ieniștea (Bucharest, 1 May 1994) stopped a carrier which has deeply influenced the Romanian entomology.

A noteworthy personality of the Romanian zoology, Prof. Dr. Doc. Mircea Ieniștea was born in Bucharest, on the 11th October of 1910. His Career of active zoologist started as early as the school time, when as a schoolboy at "Gh. Lazăr" secondary school, he got the well-knownReitter's "Fauna Germanica". He determined beetles collected not only during the excursions from country, but also during the short breaks between lessons, from the Cișmigiu gardens, several of which (ex. *Calosoma dentiolle*) being now extinct in this site.

Already endoweed with many zoological and botanical knowledge, he attended the courses of the Faculty of Biology in Bucharest and the University Pedagogic Seminar. During the university years he published the papers in the "Bulletin of the Natural History Students Society" (1930–1933), representing the results of collecting excursions in the country and took part actively in the scientic sessions of students. His conferences from this period impress through the richness of knowledge in botany but also in onithology, herpetology and entomology.

Concomitantly with the collecting programe, the young student M. Ieniștea started to develop an impressive correspondence with famous entomologists from Romania and later from abroad. He worked untiringly to realise equally a representative scientific collection and a great scientific library. The young coleopterologist M. Ieniștea contacted the Deubel's kin, after the death of this famous fan coleopterologist, and taking his collection, bibliography and optic apparatus, succeeded to save from loss, which seemed to be inherent, the valuable scientific material, which he know subsequently to conserve and utilise with ability in the profit of science.

At the beginning he worked as an assistant and after this as a chief of department at the Natural History Museum in Chișinău. In 1949 he sustained the thesis for a doctor degree, with the subject "Contributions to the Knowledge of the Coleopterofauna of the Bucegi Mountains and of the Regions near to Prahova Valley", whose very interesting contents, wasn't unfortunately, published until now. Then he worked at zoology the chairs at the Bucharest University, at Hygiene Institute in Bucharest – malaria and protozoology section, at the Research Stations at Sinaia (as a manager) and at Pantelimon. He finished his university career in Craiova and then in Constanța wherefrom he retired as a professor.

Owing to his enormous interest for zoology, especially for entomology, with the all apparent diverse tasks which he had to discharge during his

career, professor Ieniştea remain ed loyal to the greatest group of insects : Coleoptera. The inventory of the Romanian Coleopter-fauna, which he started since 1936 and continued for during 55 years of activity, until his old age, contained approximately 6900 species, many of them recorded or described by himself.

He contributed to the knowledge, from the entomofaunistical point of view, of some important areas of Romania (the Retezat, the Bucegi and the Parâng mountains, the Danube Delta). He has enriched with new species the faunal inventory from Switzerland, France, Bulgaria, Spain, also described new species and genera.

His correspondence with over 70 speciality institutes from 25 countries showed not only his international acknowiedgement as a distinguished coleopterologist, but it was one of the few contacts with world science, in a period when the Romanian entomology was isolated by the results of similar research from abroad.

Through his own efforts and titles owned thanks to his personal merits (member in the international Permanent Committee for Entomo-faunal Symposium of Central Europe, founding member of the Austrian Entomological Society, member of the Royal Society of Entomology from England, honorary co-worker of natural history museums in Budapest, Vienna, Linz, Stuttgart, Frankfurt on pe Main, Copenhagen, Basel, Zurich, Geneva, Lugano), Professor Ieniştea has Striven for the assertion of Romanian entomology merits. In this respect, his very rich personal library, kept through his own money effrots, was used sometimes as a unique source of knowledge for many Romanian zoologists.

Thanks to his pedagogic talent, professor Ieniştea has guided and formed as specialists numerous young fun-entomologists. In their scientific instruction, he has invested hundreds of hours of consultations, in his modest laboratory from the Faculty of Biology in Bucharest.

Professor Ieniştea has worked with zeal and competence oin the "Nature's Monuments Commission" and in guiding the natural history museums in Romania.

For friends, he will remain in memory not only as en eminent zoologist, but also as a man with an impressive culture and a high ethics.

During his activity, Professor Ieniştea has described 8 genera, 4 subgenera, 44 species and recorded 5 genera, 4 subgenera, 105 species new for the Romanian fauna ; 4 species for Swiss fauna and 2 for Spanish fauna.

He has published 58 papers, among which the chapter on *Hydradephaga & Palpicomia*, in illies (ed.), Limnofauna Europaea, 2nd edition, 1978.

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AVIS AUX AUTEURS

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