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REVIEW ARTICLE

BICONCAVE SHAPE AND ITS TRANSFORMATIONS IN HUMAN RED CELLS

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1. Discovery of the disk-sphere transformation of red cells

The shape transformation of biconcave red cells *via* crenated "echinocytes" into microsphaerocytes was first observed in washed red cell suspensions investigated between glass slide and coverslip [15, 9]. PONDER [23] demonstrated that — in addition to the glass capillary effect — haemolysins and the elevation of pH induce the same shape transformation. DEUTICKE [10] reported that many anionic or non-ionic membrane-active drugs are also echinocytogenic.

Biconcave disk, however, may be transformed into microsphaerocyte in another way, *via* cup-shaped "stomatocytes". It was JOLLY [16] who first described cup-formation of red cells. This shape change was shown to be induced also by low pH [38] and a great number of penetrating membrane-active cationic drugs [10].

Thus the microsphaerocytic transformation of red cells may follow two patterns: one is represented by the echinocytic, the other by the stomatocytic route. Both processes are characteristic of the majority of mammalian red cells the ovalocytes of the *Camelidae* (camel, llama) being an exception.

2. The steps of the microsphaerocytic transformation

Echinocytic and stomatocytic shape changes were investigated first under optical microscope. Recently scanning electron microscopy is used for resolving the finer details of the surface. Figure 1 demonstrates the characteristic transitory forms of the echinocytic, whereas Fig. 2 those of the stomatocytic, transformation. The degree of transformation in a cell suspension can be estimated by calculating morphological indices (see legends to Figs 1—2). The electron microscopy of cell sections has revealed that the spicules of echinocytes undergo fragmentation (exocytosis), whereas in stomatocytes

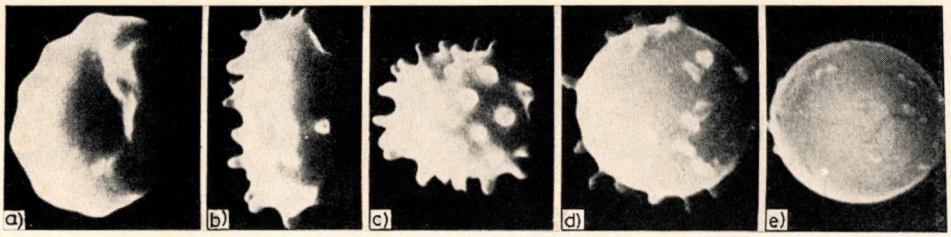

Fig. 1. Transitory forms of the sphaeroechinocytic shape changes. Scanning electron micrographs (× 4000). *a)* Crenated disk; *b)* crenated discoid; *c)* crenated spheroid; *d)* crenated sphere; *e)* smooth sphere (microsphaerocyte). The morphological index of the echinocytic shape change $(I_m)_e$ was calculated as follows: normal biconcave shape was characterized by the value 1.0, the shapes designated a → e by 0.8, 0.6, 0.4, 0.2, 0.0, resp. The percentile distribution values of the individual shapes were multiplied by these factors. $(I_m)_e$ is the sum of these products. Thus, a cell population consisting of homogeneous biconcave disks is characterized by an I_m value of 100, whereas a homogeneous microsphaerocyte population by 0.0. If transitory forms are present, the I_m value varies between 100 and 0 [12]

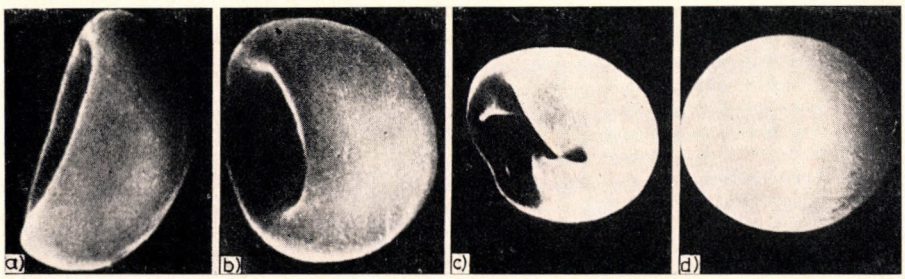

Fig. 2. Transitory forms of the stomatocytic shape changes. Scanning electron micrographs (× 4000). *a)* Monoconcave disk; *b)* stomatocyte I; *c)* stomatocyte II; *d)* microsphaerocyte. The morphological index of the stomatocytic shape changes $(I_m)_s$ was formed like the $(I_m)_e$, but the multiplying factors of the shape forms *a*, *b*, *c*, *d*, were 0.75, 0.5, 0.25, 0.0, resp.

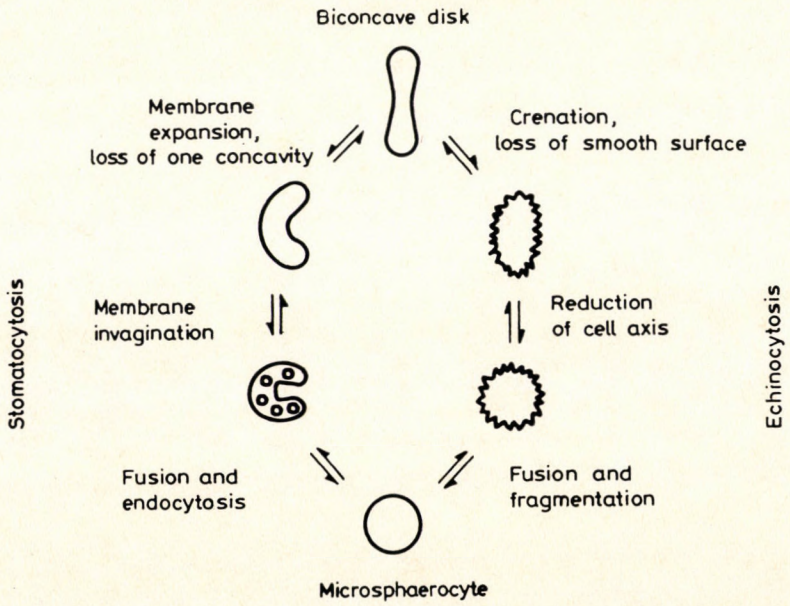

Fig. 3. The main steps of the two ways of microsphaerocytic changes of red cell shape

membrane vesicles are invaginated (endocytosis) [27]. Figure 3 schematically demonstrates the main steps of the two processes leading to microsphaerocytosis. So far it has been clarified that general membrane phenomena (fragmentation, invagination, fusion) are involved in the shape changes. The intriguing problem is the molecular mechanism and the coordination of these membrane phenomena reflected in cell morphology.

3. Biochemical changes in the background of the sphaeroechinocytic transformation

NAKAO et al. [20, 21] have revealed that the maintenance of the biconcave shape is an ATP-requiring process. In their opinion shape is determined by the ATP *level* and the echinocytic shape change starts when the physiological ATP concentration has decreased by 50%.

The role of Ca^{2+} in the sphaerocytic shape changes was stressed by WEED et al. [38, 39]. They reported that the shape transformation of glucose-depleted cells incubated in the presence of Ca^{2+} set in when the ATP concentration had decreased by 90%. At these low ATP levels Ca^{2+}-pumping becomes insufficient and thus cell Ca^{2+} level increases. WEED et al. assume that — according to the distribution of the Ca^{2+}-pump molecules — Ca^{2+}-rich and Ca^{2+}-poor regions develop within the membrane. It is this inhomogeneity in the membrane that is reflected in crenation. This explanation is fascinating but our following findings seem to contradict it: *a)* During ATP-depletion crenation develops also in Ca^{2+}-free media; *b)* it is accelerated by Ca^{2+} addition but slightly [36]; *c)* it occurs even in the presence of lanthanum, an inhibitor of the Ca^{2+}-pump (Table 1). As mentioned above, the reported data

Table 1

Comparison between Ca^{2+}-induced K^+-efflux and echinocytic shape changes

	K^+-efflux	Echinocytosis
ATP-depletion	no absolute precondition	
Mg^{2+}-depletion	no inducer	inducer
Metal ion uptake	precondition	no precondition
Inducer metal ions	Ca^{2+}, Sr^{2+}, Pb^{2+}	Ca^{2+}, Sr^{2+}, Cd^{2+}, Cu^{2+}
Inducer drugs	few cationic drugs (causing stomatocytosis)	many anionic or non-ionic drugs (causing no selective K^+-efflux)
Extracellular lanthanum	inhibitory (at low Ca_i^{2+})	not inhibitory
pH dependence	not definite	very definite

as to the extent of ATP reduction inducing shape changes are also rather contradictory.

Therefore, we investigated the connection between cellular ATP and Ca^{2+} levels and shape changes in a more detailed way.

The relationship between changes in ATP level and cell morphology

NAKAO in his experiments used either red cells treated with 20 *mM* NaF, or red cells stored in ACD. We found that both experimental conditions have direct disadvantageous effect on ATP: morphology relation [12, 32]. Hence, we used experimental systems that exerted no direct effect on the shape: for ATP-depletion: 8 *mM* NaF, 2.5 *mM* iodoacetate or iodoacetamide combined with agents modifying energy metabolism of red cells, like inosine, pyruvate, hydrogen sulphite, tetrathionate, arsenate, *etc.*, for ATP regeneration: glucose, inosine, adenine or pyruvate [12]. By introducing the morphological index (see legend for Fig. 1) shape changes could be estimated quantitatively. This way we could establish that the distribution of cell shape is not determined by the ATP level itself, but shape changes follow ATP changes with a time delay. This delay is slight in the course of the shape regeneration following the elevation of low ATP levels, but it is significant in the course of the sphaero-echinocytic changes following ATP breakdown [12]. As a consequence, in the

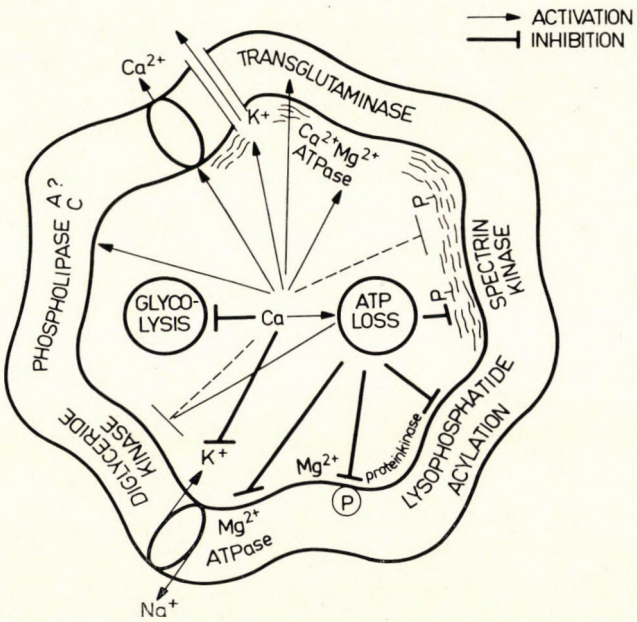

Fig. 4. Metabolic effects of ATP-depletion and intracellular Ca^{2+} in red cells

course of ATP breakdown or resynthesis, the relationship between ATP level and morphology is different even in the same experimental system.

Thus, some "transmission" is to be postulated between the changes of ATP level and shape transformation. We started to search for the "missing link" between ATP and shape changes.

Part of Fig. 4 demonstrates the effects of ATP breakdown on the membrane processes. The pumps (Ca^{2+}, $K^+—Na^+$-pump) are in the standstill, the phosphorylation of spectrin — the fibrillar protein covering the inner surface of the membrane — gradually ceases and the function of the Mg-proteinkinases (phosphorylating membrane proteins by ATP) and ATPases in general fall out. In the lipid metabolism the reacylation of lysophosphatides and the phosphorylation of the diglyceride (1,2-diacylester of glycerol) are affected.

ATP-dependent membrane-lipid changes and cell morphology

Since among the lipid breakdown products lysophosphatides and free fatty acids were found to be potent echinocytogenic agents [10], we considered at first the possible role of the accumulation of lysophosphatides (+ free fatty acids) in the membrane. SHOHET and HALEY [31] reported that in red cells incubated in autologous serum without added glucose, ATP-depletion — after a delay — is followed by lysolecithin accumulation. Our own investigations confirmed this fact: after ATP-depletion of defibrinated blood by 4—8 *mM* NaF, the lysolecithin content of red cells increased to 4—5 fold within 24 h. However, in heat-inactivated serum or crystalloid medium, ATP-depletion and shape changes developed, whereas lysolecithin accumulation could not be detected. This finding indicates that lysolecithin is not produced within the cell, but is formed by the lecithin-cholesterol acyltransferase (LCAT) enzyme of the plasma, with simultaneous cholesterol ester formation. The produced lysolecithin is incorporated into the membrane from outside and then it contributes to the sphaeroechinocytic shape changes.

The enzyme LCAT functions even in the plasma of ACD-bloods, at a lower rate corresponding to the low temperature of storage. After prolonged storage, lysophosphatide reacylation becomes deficient and lysolecithin accumulates in the membrane, although to a lesser extent than in experiments performed at 37 °C. This explains our observation that after 6—8-week storage, ATP level can be regenerated by adequate treatment (adenine + inosine + phosphate), whereas the regeneration of the biconcave shape is not complete. By adding fatty acid-poor albumin during ATP regeneration, however, the complete restitution of shape could be achieved even in these cells [33]. SATO's [25] works have confirmed that the lysolecithin accumulated in the membrane can be eliminated by albumin.

Our observation that in crystalloid medium there is no lysolecithin accumulation in red cells following ATP-depletion is in accordance with the statement that human red cells possess no active phospholipase A, cleaving off fatty acids from phosphatides [40]. However, we have to consider the role of the accumulation of diglyceride. There are two ways for the formation of diglyceride in red cells. One is the Ca^{2+}-dependent phospholipase C enzyme (Fig. 4). Since this enzyme needs very low Ca^{2+} concentrations ($K_M < 1\ \mu M$) [3] to be activated, it may become activated even by the Ca^{2+} traces liberated from the potent Ca^{2+}-chelator ATP during energy depletion. The other way of diglyceride production is the breakdown of phosphatidic acid catalyzed by phosphatidate phosphatase. The rephosphorylation of diglyceride catalyzed by the diglyceride kinase is ATP-dependent. By incubating fresh or ATP-depleted red cells by pure 1,2-distearylglycerol we could not observe any echinocytogenic effect of even high doses of exogeneous diglyceride incorporated into the membrane. ALLAN et al. [2], on the other hand, report on a stomatocytic effect of a non-purified extract containing diglyceride of mixed fatty acid composition. Thus, in itself, the accumulation of diglyceride in the membrane (in contrast to lysolecithin accumulation) is not echinocytogenic. Endogenous diglyceride formation will be discussed below in connection with the Ca^{2+}-induced shape changes.

ATP-dependent changes in membrane proteins and in cell morphology

After the investigation of the lipid breakdown products we had to focus our interest on the changes of the membrane proteins. We established that certain circumstances which are advantageous for the maintenance of the biconcave shape, *e.g.* the limited proteolysis of the cell surface, result in an increased labelling of the protein fraction in red cells containing ^{32}P-ATP, whereas disadvantageous impacts (16—32 *mM* NaF) reduce protein labelling [32]. AVRUCH and FAIRBANKS [7] demonstrated that the major phosphorylated proteins of the red cell membrane are the compounds named spectrin 2 and Band 3. BIRCHMEIER and SINGER [8] studied the characteristics of the phosphorylation of the spectrin 2 fraction catalyzed by the enzyme spectrin kinase. They established that the phosphorylation is rapid, and steady state can be reached within 15—30 min. The dephosphorylation of the prelabelled ghost, however, is inhibited by ATP concentrations much lower than the physiological level. This reaction type refers to a similarity with the ATP-dependent shape changes. Therefore, we prepared red cells containing ^{32}P-ATP and induced in them the breakdown of ATP (by iodoacetate + inosine treatment). At appropriate time intervals membranes were isolated from the cells and the phosphorylation of the protein fractions obtained with polyacrylamide gel electrophoresis was studied (see Fig. 7). The dephosphorylation of the spectrin

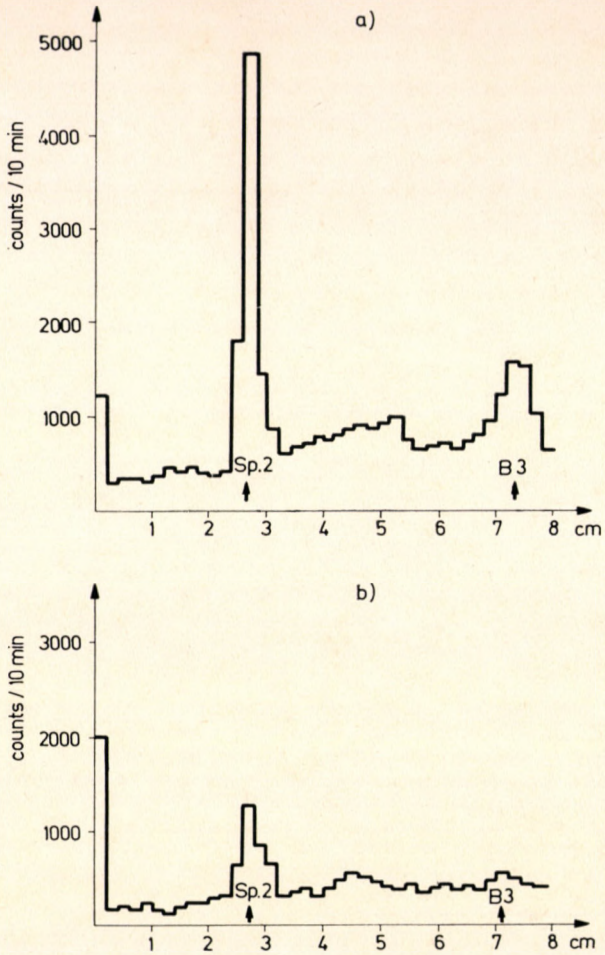

Fig. 5. Radioactivity of the polypeptide fractions of membranes prepared from cells containing ^{32}P-ATP and separated by polyacrylamide gel electrophoresis. Membranes were prepared according to WOLF as modified by SCHATZMANN [26]. Polyacrylamide gel electrophoresis was performed according to SHAPIRO et al. [28]. 100 μg of membrane protein was added to the gel. Running time was 4 h. Radioactivity of 2-mm sections of the gel was measured according to GEZELIUS [13]. *A:* Control red cells. ATP concentration: 2.1 *mM*. *B:* Red cells treated with 2.5 *mM* iodoacetamide + 10 *mM* inosine for 90 min. ATP concentration < 10 μM

2 fraction in intact cells followed the breakdown of ATP with a significant delay: after 90 min the ATP level fell from 2 *mM* to 10 μM, whereas spectrin phosphorylation decreased only to one fifth of the initial value (Fig. 5). It is the phosphospectrin that is assumed to polymerize [8, 30], according to some authors as a copolymer with actin [22, 37]. It is most probable that the intactness of this network of polymerized proteins covering the inner membrane surface is necessary for the normal maintenance of biconcave shape, and the relatively slow dephosphorylation of spectrin is accompanied by sphaero-echinocytic shape changes.

Effects of divalent cations

In contrast to the effect of ATP-depletion, rapid shape changes are induced by pH alteration or by various drugs. The shape changes induced by pH changes are further increased by Ca^{2+} in the medium (Fig. 6). The Cu^{2+}- or Cd^{2+}-induced sphaeroechinocytic shape changes described by us [32] can also be made instantaneous if the penetration of these cations is accelerated by the divalent cation ionophore A23187. In a similar manner, with A23187 + Ca^{2+} microsphaerocytes develop instantaneously through

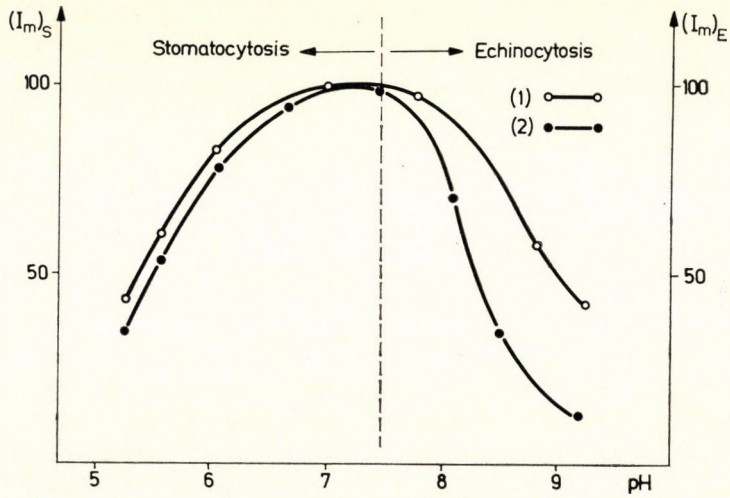

Fig. 6. Effect of pH changes on the shape of red cells. For definitions of $(I_m)_e$ and $(I_m)_s$ see the legends to Figs 1 and 2. Neutral and alkaline pH values in the red cell suspensions were adjusted by Tris-HCl, acid pH values by acetate-NaOH buffers
(1) $[Ca^{2+}]_e < 10\ \mu M$; (2) $[Ca^{2+}]_e = 5\ mM$

sphaeroechinocytic forms. It is interesting that Mg^{2+}-depletion of cells by A23187 + EDTA also induces rapid microsphaerocytosis, which persists even after the elimination of the ionophore with albumin. Certainly, there is an interaction between the divalent cations and the negatively charged phospholipids (phosphatidylserine) of the inner surface of the membrane. This charge neutralization, in turn, alters the lipid structure [40]. However, the structure of the membrane proteins is also influenced by divalent cations. HAEST et al. [14] experienced that on treatment of red cell ghosts with Cu^{2+} + *o*-phenanthroline the proportion of the aggregated fraction not penetrating into the polyacrylamide gel after SDS-solubilization increased. We observed the same phenomenon with A23187 + Cu^{2+}, Cd^{2+}, Ca^{2+} and even with La^{3+}. We demonstrated that the phosphorylation of the aggregated fraction after Ca^{2+},

or La^{3+} treatment is very definite, so that the presence of spectrin 2 in it can be postulated (Fig. 6). These deviations refer to the altered interactions of the spectrin (actin) network.

Effects of Ca^{2+}

It was WHITE [41] who first reported sphaeroechinocytosis induced by A23187 + Ca^{2+}. He, however, found these shape changes to be irreversible. We established that the shape changes were fully reversible if the inner Ca^{2+} level was not increased above 3 *mM* [24]. In order to interpret the role of intracellular Ca^{2+}, its manifold metabolic effects have to be surveyed (Fig. 4). Intracellular Ca^{2+}, by inhibiting glycolysis, reduces ATP synthesis, whereas it increases ATP consumption first of all by activating the Ca^{2+}-pump. It causes selective K^+-permeability increase. The phospholipase C enzyme of the membrane is significantly activated, which results in an increase of diglyceride content. As mentioned above, the existence of Ca^{2+}-dependent phospholipase A and B producing lysophosphatides is doubtful in human red cells. The lysolecithin content of the membrane spicules formed and fragmented under the effect of Ca^{2+} was found to be four times of that of the membrane. This fact, however, can also be explained by an altered lipid distribution within the membrane [40].

According to recent results, the transglutaminase enzyme of the membrane that builds covalent bridges between protein chains is activated by relatively high concentrations of Ca^{2+} [19]. In membrane preparations Ca^{2+}-dependent proteinase activity resulting in degradation of spectrin was also demonstrated [6, 42]. In connection with the Ca^{2+}-dependence of spectrin-kinase, different data have been published [17]. According to our own results, the spectrin kinase is Mg^{2+}-dependent and Ca^{2+} does not disturb its activity up to 100 μM concentration, higher Ca^{2+} concentrations being partially inhibitory. Inhibition of diglyceride kinase needs still higher Ca^{2+} concentrations [5]. The K^+—Na^+-pump activity is very sensitive to Ca^{2+}. The reduction of ATP level influences all the energy-requiring functions described in the above section.

Of all these effects the function of the Ca^{2+}-activated transglutaminase seems to be the most probable cause of the irreversible changes observed above 3 *mM* intracellular Ca^{2+} concentration. This enzyme, however, is inhibited by unusually low (10 μM) concentration of iodoacetamide [6]. We demonstrated that during intensive Ca^{2+} loading of red cells the presence of 10—20 μM iodoacetamide is advantageous for the cell shape, but it cannot fully eliminate irreversible shape transformation. This indicates that other events than the formation of abnormal covalent links between proteins are also involved in the irreversible shape changes. According to our experiments,

the role of Ca^{2+}-activated protease in the shape changes can be excluded, since these latter develop even in the presence of the inhibitors of the protease enzyme (hydrogensulphite, tetrathionate) [6].

This survey clearly indicates that the effects of intracellular Ca^{2+} are manifold. Quantitative relationship between cell Ca^{2+} and shape postulates that the direct interactions between Ca^{2+}-protein and Ca^{2+}-lipid play the most decisive role in the shape changes. When searching for quantitative relationships, the control of the Ca^{2+}-buffer system (ATP + 2,3DPG) of the cell is most important, since it is only free Ca^{2+} that elicits shape changes. At normal ATP and 2,3DPG levels, however, only 30—50% of the intracellular Ca^{2+} is in the ionized form [11]. Under standard conditions, there is a well-reproducible relationship between intracellular Ca^{2+} level and shape. Fresh cells

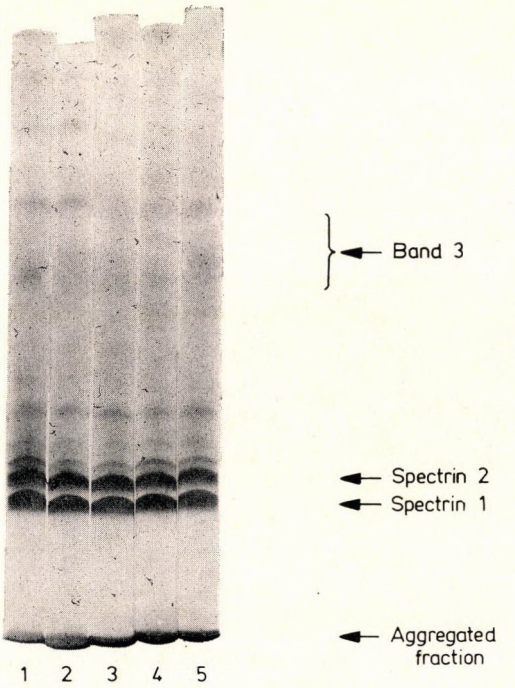

Fig. 7. Polyacrylamide gel electrophoretograms of red cell membranes treated with ^{32}P-ATP under various circumstances. Red cell membranes were prepared according to WOLF as modified by SCHATZMANN [26]. The fractions between Spectrin 2 and Band 3 are protein breakdown products. The formation of these bands can be prevented if protease activity is inhibited by 10 μM N-α-p-tosyl-l-lysine chloromethyl ketone HCl (TLCK) + 300 μM phenylmethylsulphonylfluoride (PMSF). ^{32}P-ATP (spec. act: 16 Ci/mmole) was added at a final concentration of 0.5 μM. Incubation time: 30 sec, t: 0 °C, pH: 7.0. Incubation systems: (1) No divalent cation added; (2) 500 μM EDTA; (3) 200 μM Cu^{2+}; (4) 200 μM Ca^{2+}; (5) 200 μM La^{3+}. For solubilization before gel electrophoresis 1% SDS + 1% mercaptoethanol was used with the exception of system 3, where mercaptoethanol was omitted. The radioactivity (counts 10 min) of the first 2 mm gel sections was: (1) 600, (2) 250, (3) 440, (4) 3500, (5) 4600

were loaded to 0.4—0.6 *mM* intracellular Ca^{2+} level, and in the course of Ca^{2+}-pumping at various time intervals the changes of Ca^{2+} level were stopped by lanthanum addition, and cell Ca^{2+} and the proportion of various shape forms were determined. We have calculated the cell Ca^{2+} concentration transforming normal biconcave shape into transitory shape and it was found to be 49 μM (range 23—63) on the average, whereas the concentration transforming transitory shape into microsphaerocytes was 73 μM (range 48—94) [35]. The two values are very near to each other and are in good agreement with the rapidity of Ca^{2+}-induced shape changes. The correlation between Ca^{2+} level and cell shape makes direct Ca^{2+}-membrane (protein, lipid) interactions probable. It is worth mentioning that in the whole Ca^{2+} range where the rapid shape changes develop phospholipase C (producing diglyceride) is unchanged in the maximally activated state.

We have to emphasize again the importance of considering the circumstances when searching for Ca^{2+}-shape connections. Under the above conditions at 0.4—0.6 *mM* intracellular Ca^{2+} level, the diglyceride kinase enzyme is active. This sensitizes the cells towards shape changes by producing phosphatidic acid (see Fig. 4), which we have demonstrated to be an echinocytogenic agent. Above 2 *mM* intracellular Ca^{2+} level this sensitizing effect falls out

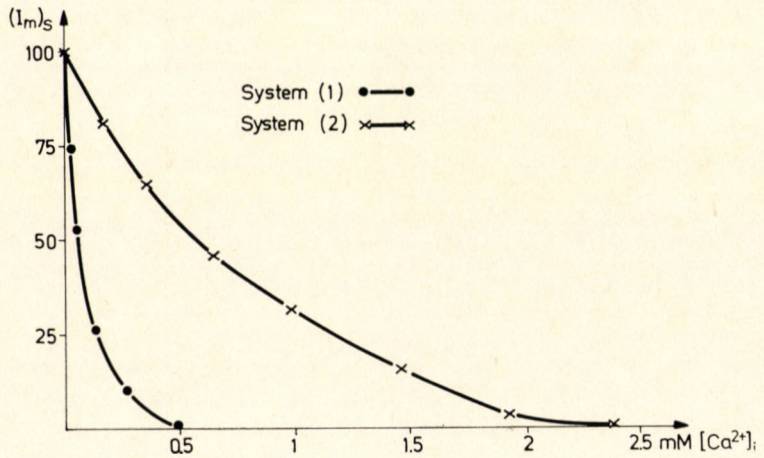

Fig. 8. The relation between cell Ca^{2+} and $(I_m)_s$ in the course of Ca^{2+}-pumping from cells loaded with Ca^{2+} to various extent. Initial cell concentrations: System (1) 0.5 *mM* calcium, 1.2 *mM* ATP, 3 *mM* 2,3-DPG; system (2) 2.4 *mM* calcium, 0.8 *mM* ATP, 3.06 *mM* 2,3-DPG. ATP consumption during the first 20 min of Ca^{2+}-pumping was 0.8 *mM* in system (1), 0.3 *mM* in system (2)

(phosphatidate formation is inhibited, ATP consumption is lower), consequently, the relationship between Ca^{2+} and cell shape changes (higher Ca^{2+} level is needed for eliciting the echinocytic shape) (Fig. 8). Within these conditions, however, the Ca^{2+}:$(I_m)_e$ ratio is also well-reproducible.

We failed to confirm the exclusive role of diglyceride in Ca^{2+}-induced sphaeroechinocytosis suggested by ALLAN et al. [1—5] *viz.*, treatment of red cells with A23187 + EGTA made diglyceride detectable among membrane lipids, most probably as a consequence of intracellular Ca^{2+} translocation; in spite of this, shape change did not occur. Conversely, diglyceride did not further accumulate on A23187 + Ca^{2+} treatment but cells were transformed into perfect microsphaerocytes (Fig. 9). In latter case phosphatidic acid accu-

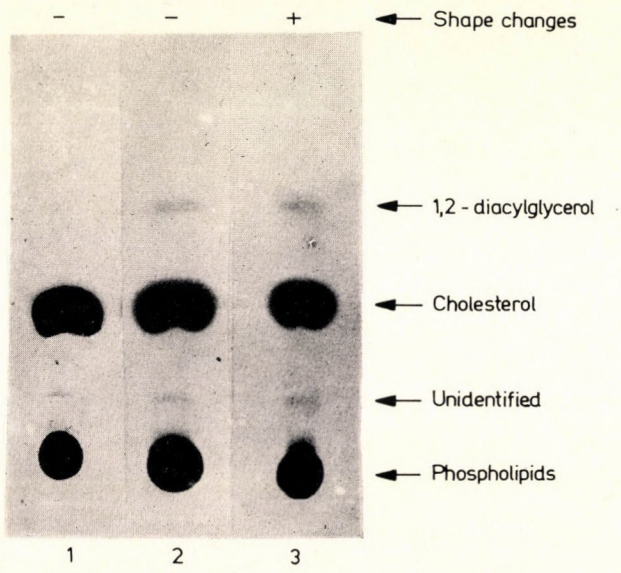

Fig. 9. Thin-layer chromatograms of neutral lipids extracted from washed red cells treated with A23187 + EGTA, or Ca^{2+}. Incubation systems: (1) No addition; (2) 10 *mM* A23187 + 500 *μM* EGTA; (3) 10 *mM* A23187 + 1.25 *mM* Ca^{2+}. Incubation time: 1 min, t: 37 °C. Lipid extracts were chromatographed on silica gel G layer. Solvent: petrol ether : acetone (80 : 25). Spots were developed with 50% H_2SO_4 (180 °C, 30 min)

mulation was well detectable (Fig. 10). In sheep red cells ALLAN and MICHELL [4] could not induce echinocytic shape change by A23187 + Ca^{2+}, and they failed to detect diglyceride formation either. We saw in these cases incomplete shape changes that could not be completed by addition of distearylglycerol. In our opinion, if diglyceride contributes to the echinocytic shape changes, this may be only the endogenously-formed diglyceride in the inner lipid region participating in special interactions. This, as a factor may play a role in shape changes, but is not their exclusive cause.

LEW and FERREIRA [18] suggest that sphaeroechinocytic shape changes and Ca^{2+}-dependent K^+-permeability increase are the consequences of the same membrane structure changes. Several of our comparative experiments

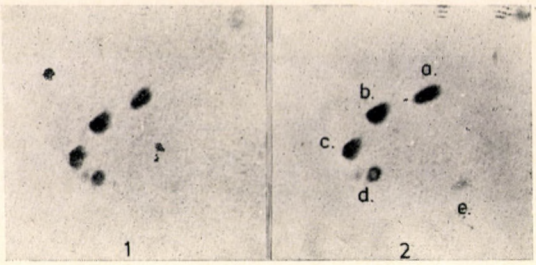

Fig. 10. Two-dimensional thin-layer chromatograms of phospholipids extracted from washed red cells treated with A23187 + EGTA, or Ca^{2+}. Incubation systems: (1) 10 μM A23187 + 500 mM EGTA; (2) 10 μM A23187 + 1.25 mM Ca^{2+}. Incubation time: 1 min, t: 37 °C. Lipid extracts were chromatographed on precoated silica gel high-performance TLC plates (Merck, Darmstadt). Solvent systems: first dimension-chloroform: methanol : conc. NH_4OH (60 : 25 : 4); second dimension-chloroform : methanol : acetone: acetic acid : water (75 : 15 : 30 : 15 : 7.5). Spots were developed by the phosphomolybdene blue reaction: lower limit of detection, $\sim$ 0.05 μg phosphorus. (a) Phosphatidylethanolamine; (b) phosphatidylserine; (c) sphingomyelin; (d) phosphatidylserine; (e) phosphatidic acid

disprove this idea (Table 1). ALLAN and MICHELL [4] in pig red cells also observed the diversity of the two phenomena, so far as rapid K^+-transport developed under the effect of A23187 + Ca^{2+}, whereas shape changes did not. Ca^{2+}-induced *K^+-permeability increase*, however, results in loss of cell K^+, Cl^- and water, and the consequent shrinking promotes the reduction of cell axis accompanying sphaeroechinocytosis.

4. Explanation of the biconcave shape and the microsphaerocytic shape changes

After the presentation of the above experimental results let us try to construct a unifying picture. From among the many theories elaborated for the explanation of shape and shape changes, we prefer the "bilayer couple" hypothesis of SHEETZ and SINGER [29]. This theory assumes that the two lipid layers of the membrane are strongly attached at certain points, but between them the individual layers may slide above each other. According to our view, indeed two half-layers can be hypothesized in the membrane, the outer with its own lipids and immersed proteins and the inner with its lipids, the immersed proteins and attached polymerized spectrin (actin) network. The borderline, however, is not between the two monomolecular lipid leaflets, there are rather two functionally separated half-layers. These two half-layers are connected at certain points strongly, however, they may be translocated above each other elsewhere. The relative expansion of the outer layer manifests itself in crenation (evagination) and in the fragmentation of the spicules (echinosphaerocytosis); the relative expansion of the inner layer in invagination and endo-

cytosis (stomatosphaerocytosis). The dynamic character of the borderline between the two halves of the membrane is indicated by the fact that the fragmented spicules are very rich in the major spanning protein of the membrane, the so-called Band 3 [1]. Since phosphatidylserine, possessing net negative charge at physiological pH, is localized in the inner region of the membrane, the permeable cationic drugs are also enriched here, whereas the anionic drugs accumulate in the outer region. The incorporated drugs induce the relative expansion of the layer in question. *The physiological expansion of the inner layer*, which *results in the maintenance of the* physiological *biconcave shape*, is regulated by an interaction with the dynamically changing polymerized spectrin (actin) network.

Inserting the above-described results into the frame of this concept, sphaeroechinocytosis induced by ATP-depletion or Ca^{2+} uptake can be interpreted in the following way:

During ATP-depletion one has to consider, on the one hand, the translocation of the intracellular Ca^{2+} and, on the other, the slow dephosphorylation of spectrin and the consequent destruction of the polymerized spectrin (actin) network. (Protein SH-group oxidation—a consequence of the failure of the ATP-dependent glutathione resynthesis—contributes to the disorganization of the protein network.) It is possible that the endogenous diglyceride formation also alters the structure of the inner lipid region and promotes shape changes. These factors in themselves are sufficient for the induction of the sphaeroechinocytic shape changes. The following factors may accelerate the process: in the presence of external Ca^{2+} the changes induced by entering Ca^{2+}, in plasma medium the accumulated echinocytogenic lysolecithin. According to our own experiments based on the time delay between ATP breakdown and shape changes, the process of spectrin dephosphorylation seems to be the most important factor.

In the course of *Ca^{2+} uptake* one has to consider, *a*, the direct effect of Ca^{2+} on membrane proteins (polymerized spectrin-actin network) and membrane lipids (phosphatidylserine, phosphatidylinositol), and *b*, the metabolic effects of Ca^{2+}, *i.e.* the formation of diglyceride and accumulation of phosphatidic acid, the inhibition of spectrin phosphorylation and the abnormal protein interactions brought about by the Ca^{2+}-activated transglutaminase enzyme. According to our own experiments, up to 3 *mM* inner Ca^{2+} level the direct interactions of the cell membrane with Ca^{2+} are decisive.

The rapid shape changes following *pH alteration* can be attributed to the charge shifts of the membrane (at low pH a positive and at high pH a negative charge excess develops). According to our investigations, mainly at alkaline pH, ATP- and Ca^{2+}-dependent processes also contribute to the rapid sphaeroechinocytic shape changes. At alkaline pH, namely, due to the activation of hexokinase a rapid ATP breakdown takes place. In addition, the Ca^{2+}-bind-

ing capacity of the membrane and the passive Ca^{2+} permeability of the cell increases to a great extent [34].

In conclusion, the maintenance of the biconcave shape and its changes in the echinocytic or stomatocytic direction result from interactions between a great number of membrane components. Our investigations — both in the field of ATP-and Ca^{2+}-dependent shape changes — refer to the paramount importance of phosphorylation and interactions of the protein network covering the inner surface of the membrane.

Note added in the proof: Recently it has been published (D. ALLAN, R. H. MICHELL 1978: *Biochim. Biophys. Acta* **508,** 277—286) that the phosphatidyl inositol phosphates are the main substrates of the Ca^{2+}-activated diglyceride production. This finding strongly supports our view delineated above. *Viz.*, the replacement of the highly charged substrate molecules with the fusogenic diglyceride moieties promotes membrane fusion—one step of the disk-sphere transformation.

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DEGRADATION OF LOW-DENSITY LIPOPROTEINS (LDL) AND LDL — PROTAMINE COMPLEXES BY LYSOSOMAL PROTEASE

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Abstract

Native LDL are degraded by the protease of the lysosomal extract but they are not sensitive to isolated cathepsin D. Protamine increases the sensitivity of LDL to the effect of lysosomal protease and makes them sensitive to the effect of cathepsin D. Degradation of LDL by lysosomal protease is most intensive between pH 4.0 and 4.5 but in case of LDL bound with protamine it is most intensive at pH 4.5—5.0.

Introduction

Changes in the sensitivity of proteins to proteolytic enzymes depend on physicochemical changes and chemical modification of the molecules and on formation of intermolecular associations.

We have shown [10, 11] that LDL form soluble and insoluble complexes with protamine and other basic proteins. But it is not known if formation of such complexes influences the rate of LDL degradation. Tracing LDL degradation in mixture with protamine is possible on account of differences in the amino acid content of these proteins, owing to the presence of aromatic amino acids in LDL and their lack in protamine.

Material and methods

LDL was isolated from human blood serum [1]. The protamine sulphate was produced by Koch Light Laboratories LTD, England. Britton and Robinson universal buffer of pH 2.5 to 6.0 was used. Lysosomal extract containing 1.6 mg protein/ml was obtained from rat liver [12]. Immediately before use, this extract was activated with an equal volume of activating mixture containing 10 *mM* NaCl, 10 *mM* EDTA and 250 *mM* DL-cysteine. Cathepsin D (0.24 mg/ml) was obtained from bovine spleen [16].

The inhibitor of cathepsin B_1 (0.3 mg/ml) was obtained from egg white [2], that of cathepsin D (0.6 mg/ml) from potatoes [14].

Degradation of LDL and LDL — protamine complexes at pH 2.5 to 6.0 was examined in the following system: 0.4 ml buffer or protamine (10 mg/ml) and 0.2 ml activated lysosomal extract or cathepsin D diluted 1 : 2 were added to 0.4 ml LDL (20 mg/ml).

The influence of inhibitors on the degradation of LDL in the presence of protamine was examined at pH 5.0. 0.1 ml cathepsin B_1 or D when lysosomal extract was used instead of the activating mixture was introduced into the above-mentioned systems, whereas in case of the degradation with cathepsin D 0.1 ml of the inhibitor was added to 0.1 ml of the undiluted enzyme. After 60 min incubation at 37 °C, the remaining protein was precipitated with 2.5 ml TCA (20%) and absorption of the clear filtrate was determined at 280 nm.

Results

Degradation of LDL by lysosomal proteolytic enzymes is most intensive between pH 4.0 and 4.5 (Fig. 1). Addition of protamine to LDL evokes a marked increase in LDL degradation. Besides, LDL in mixture with protamine is degraded most intensely at pH 4.5—5.0.

Cathepsin D does not degrade LDL (Fig. 2). Addition of protamine makes LDL sensitive to the enzyme and the degradation is most intensive at pH 4.5—5.0. The inhibitor of cathepsin B_1 from the egg white and the potato

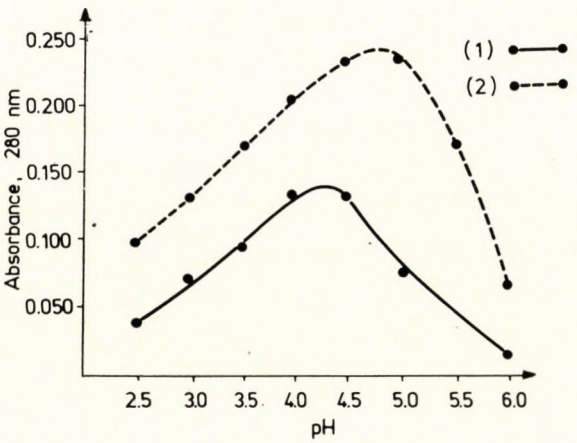

Fig. 1. Degradation of LDL by lysosomal extract at different pHs: (1) LDL (2) LDL + protamine sulphate

inhibitor of cathepsin D inhibit the degradation by lysosomal extract of the LDL present in mixture with protamine. It is shown by the decrease of extinction at 280 nm, which was 0.240 in controls and 0.035 in the system with the inhibitor of cathepsin B_1 and 0.180 in the system with the cathepsin inhibitor D. It means a decrease in the LDL degradation by 85% and 25%, respectively.

Degradation of LDL-protamine complexes by isolated cathepsin D evokes an increase of extinction decreases to 0.020 that denotes inhibition of degradation by about 70%.

In a separate experiment it was found that maximum LDL sensitivity to the effect of protease was observed at 10 mg/ml protamine concentration.

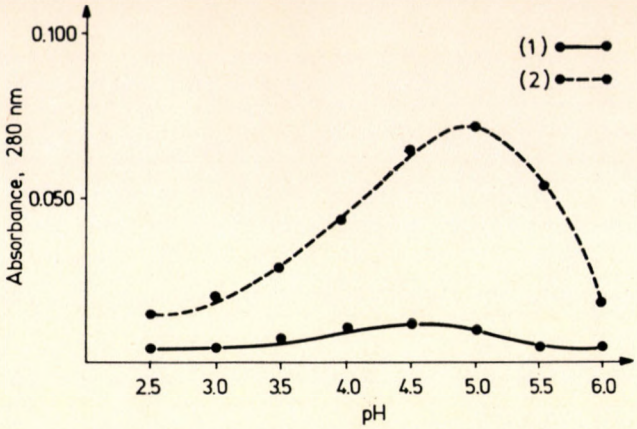

Fig. 2. Degradation of LDL by cathepsin D at different pHs: (1) LDL (2) LDL + protamine sulphate

Discussion

Low-density lipoproteins as well as high-density lipoproteins are degraded in the cell lysosomes [7, 13]. Degradation of lipoproteins by lysosomal protease is most intensive between pH 3.8 and 4.5 [6]. Cathepsin B_1 is mainly responsible for degradation of native proteins, which are resistant to cathepsin D.

Many authors attribute an essential role in the enzymatic degradation of different proteins to formation of associations with basic proteins [4, 8, 15].

The increase in the sensitivity of protamin-bound LDL to the lysosomal protease and its sensitivity to cathepsin D seem to be due to conformational changes in the LDL molecule. The changes display sensitive peptide bonds which are not present in native LDL.

Results of examinations concerning the influence of specific cathepsin B_1 and D inhibitors on degradation of LDL present in the mixture with protamine show that cathepsin B_1 is mainly responsible for this process.

The change of optimum effect of lysosomal protease from pH 4.0—4.5 for LDL alone to pH 4.5—5.0 for LDL — protamine complexes is probably evoked by the change of electrostatic charge that makes interaction between protease and LDL most intensive at higher pH. The mechanism of pH change has been described in the trypsin — haemoglobin — polyacryl acid system [5]. An increase in the sensitivity of LDL to the effect of lysosomal protease evoked by protamine and perhaps other basic cell proteins may play an essential role in degradation of LDL in the organism through the change of degradation optimum of LDL towards an indifferent pH, *i.e.* towards conditions existing in the cell. It seems that more intensive degradation of LDL may take place

in the walls of arteries changed by atherosclerosis. It may be evoked by an increased level of basic proteins in vessels changed by atherosclerosis [9] and by increased activity of lysosomal protease [3]. The lipid fraction released after degradation of the protein fraction loses its solubility and it is deposited in affected vessels.

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ISOLATION AND QUANTITATIVE DETERMINATION OF SEX HORMONES IN CARPS (*CYPRINUS CARPIO* L.)

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Abstract

Changes in the oestrogen and progesterone level in the blood and ovarian tissue of mature carps from fish farms were examined using gas chromatographic method, before and after spawning as well as under wintering. Due to the high individual variation the observations cannot be regarded as quantitative. The highest oestrogen level was found before spawning, both in the ovaries and the blood, this level was somewhat lower after spawning and did not show any further change under wintering. The resting progesterone level had a low value, both in the ovaries and the blood, but it increased after spawning. The oestrogen concentration of blood was somewhat higher in wild carps than in carps bred in fish farms, whereas that of tissues appeared to be higher in the latter. Effect of pituitary extracts on changes of the sex hormone concentration in blood and ovary of wild carps was investigated too. The results of the hormone determinations, changes in body weights and "coefficients of maturity" are presented.

Introduction

Research on the reproduction of fish began after the recognition that ovulation can be induced with exogenous gonadotrop hormones [5] in fish with mature ova. Since then methods have been developed for induction of ovulation in various fish species, and to-day an extensive literature is available on ovulation-inducing effect of hormones [9]. However, little is known about the biochemical mechanism of ovulation induction and of the site of biosynthesis of sex hormones [7, 8]. According to Beach [1], the theca layer of the follicles is responsible for oestrogen synthesis. Cedard and Nomura [2] studied oestrogens in the blood of fishes [3]. These hormones were detected in the ovaries of sea fish by Cedard et al. [3] and Gottfried [6]. Cedard et al. [3] studied the seasonal changes in hormone levels in the blood of salmons. In these early studies an increased blood oestrogen level was observed in the spawning season [3]. The paucity of data is due to the lack of biochemical methods for assaying active hormones in blood and tissues. In the present work (1) we isolated oestrogens from the blood of mature female carps, and

progesterone from the ovarian tissues, furthermore, the structure of these hormones was identified; (2) we investigated the seasonal changes in the oestrogen and progesterone concentrations in the blood and ovaries of mature carps before and after treatment with exogenous gonadotrop hormone.

Material and methods

Material

Seasonal examinations were performed on carps of large body weight, bred in fish farms. In the temperate zones, these fishes ovulate in great masses of eggs once a year (in spring). The effect of gonadotrop hormones was studied on wild-carps with scales. These are much easier to supply, and ovulate several times during the natural spawning season. Gonadosomatic ratio ("coefficient of maturity") indicates the weight of the ovary as the per cent of body weight.

Induction of ovulation with hormones

Hormone treatment was performed with carp pituitary glands previously kept in acetone for 3 × 8 h and dried at room temperature.

The pituitary glands, each averaging 3 mg, were dried, pulverized in a mortar, then suspended in 0.65% saline.

Female carps were given two injections; first a hypophysis suspension, prepared from 1 gland, was injected into the back muscles of each and after 12 h, suspension from 3 mg dried hypophysis was injected per kg body weight.

Determination of steroid hormones

The gas chromatographic method of FEHÉR et al. [4] was used with minor modifications, for the isolation and quantitative determination of steroid hormones from heparinized plasma of fishes. After deproteinization, the plasma was hydrolyzed in conc. HCl, since the hormones were generally present in both conjugated and free form. The hydrolysate was neutralized with NaOH solution and the steroids were extracted with diethyl ether. The extract was purified with 8% $NaHCO_3$, then the oestrogens were extracted by shaking the mixture with 1*N* NaOH. The remaining organic phase contained the progesterone. Hereafter, thin-layer chromatography of both extracts was performed in benzene-ethanol (95 : 5).

The oestrogens were acetylated and the oestrogen acetates rechromatographed in chloroform-heptane (80 : 20) using reference steroids. The purified extracts were gas chromatographed in a Pye Unicam series 104 gas chromatograph equipped with a flame ionization detector and a 3% SE-30 column, at 228 °C. Heptafluorobutyrate 3-enolester of progesterone was prepared and gas chromatographed on a 1% XE-60 column at 212 °C with electron-capture detector.

The plasma hormone concentration was given as μg/100 ml plasma.

Hormone determination in the ovarian tissue was performed as described by POTECZIN et al. [10]. In principle, the method is similar to that used for plasma. Before extraction, the tissues were homogenized at 20,000 r.p.m., lipids were removed by freezing the homogenate in a methanol-water mixture at —20 °C. After thin-layer chromatography of the steroids, gas chromatographic determination was performed as above. Thin-layer and gas chromatographic properties of the steroids were checked by using reference compounds. Identification of the steroids was performed by isotope dilution technique by comparing the radio-chemical properties of the tracer and of the isolated compound.

Results and discussion

Little is known of the steroid hormone production and metabolism in fish. Most of the data available are related to sea fish and are mostly qualitative [2, 3, 6]. To our knowledge, the gonadal hormone metabolism of freshwater fish has not been studied in detail. Furthermore, it is yet unkown which sex hormones are synthesized in the various phases of reproduction by the ovaries of carps. The first aim of the present work was the isolation of sex hormones and the determination of their structure. Although the isolated hormones were assayed by gas chromatography, due to the low number of specimens studied and the high individual variation, our observations cannot be regarded as quantitative.

Since observations of this kind are yet lacking in the literature, it seemed to be of interest to report them.

17 β-oestradiol (oestra — 1,3,5(10)-trien-3,17β-diol), oestrone (3-hydroxy-oestra-1,3,5,(10)-trien-17-one) and progesterone (4-pregnen-3-20-dione) were isolated from the peripheral blood and ovarian tissue of carps bred in pond and of wild carps. The structure of these hormones were determined by thin-layer and gas chromatographic techniques based on their mobility and retention properties, furthermore by isotope dilution technique, using radioactive tracers [4, 10]. 17α-oestradiol and oestriol were not detectable in the blood and ovarian tissue of the fish.

The quantities of sex hormones found in the peripheral blood and ovary of carps were in agreement with levels for sea fish as determined by hydrolysis of the esters [2, 3, 6]. The oestrogen and progesterone concentration in blood and tissue is much higher in fish than in mammals, because mammalian blood and ovarian tissues are poor in hormone esters [11, 12]. Presumably, the concen-

Table 1

Seasonal changes of sex hormone concentration in the blood and ovary of mature female carps

Time of measurements	Body weight (g)	Weight of ovary (g)	"q"	Hormone concentration			
				oestrogens		progesterone	
				mg/ovary	μg/100 ml plasma	mg/ovary	μg/100 ml plasma
Before spawning	5150	840	16.3	4.70	1.2	<0.02	3.1
	3350	750	22.3	7.50	4.8	0.08	5.9
After spawning	4100	360	8.8	1.84	2.5	0.29	6.9
	4500	370	8.2	—	0.6	—	5.9
	5000	470	9.4	2.59	0.8	0.42	4.5
Under wintering	4500	430	9.5	1.29	2.0	0.04	5 2
	3100	310	10.0	2.17	2.3	<0.02	3.2

"q" = coefficient of maturity
— = not measured

tration of biologically active (nonesterified) sex hormones in carps is also lower than our measured values.

Tables 1 and 2 summarize the results of our hormone determinations and present body weights and "coefficients of maturity". Results of both seasonal and hormone induction studies showed a high individual variation of the hormone levels, reflecting a tendency of changes only, not characteristic differences. Probably species, age, morphometric and yet unknown biochemical factors exist in the background of the great individual variation. Our observations nevertheless indicate that the oestrogen concentration of blood is somewhat higher in wild carps than in carps bred in fish farms, whereas that of tissues is higher in the latter, in spite of their lower "q" values.

Table 2

Effect of pituitary extracts on sex hormone concentration in blood and ovary of wild carps

Time of measurements	Body weight (g)	Weight of ovary (g)	"q"	Hormone concentration			
				oestrogens		progesterone	
				mg/ ovary	μg/100 ml plasma	mg/ovary	μg/100 ml plasma
Before treatment	715	92.5	12.9	1.27	17.6	0.29	5.5
	508	86	16.9	1.10	8.4	0.10	2.8
	437	65.8	15.0	0.49	3.2	0.10	3.9
5 hr after the first dose	770	66.5	8.6	0.33	5.2	0.09	5.3
	670	65.4	9.8	0.23	—	0.04	—
5 hr after the second (decisive) dose	460	78	16.9	0.76	7.5	0.02	19.5
	390	80	20.5	0.69	6.5*	0.03	10.4*
	570	88	15.4	0.89	—	0.13	—
	660	110	16.7	0.47	8.0	0.15	10.3
10 hr after the second dose	499	122	24.4	0.73	8.5	0.15	3.2
	501	94	18.8	0.72	24.2	0.34	12.0
During spawning	580	100	17.2	0.35	2.2	0.04	tr
	570	120	21.0	0.23	8.1*	0.05	tr
	540	122	22.6	0.65	—	0.06	tr
	410	88	21.5	1.00	2.7	0.02	5.2
No ovulation	680	140	20.6	0.99	6.9	0.03	7.0
	770	160	20.7	1.21	3.6	0.03	3.8
	560	145	25.9	0.67	10.3	0.12	6.2

"q" = coefficient of maturity
tr = traces
* = mutual assay of two animals
— = not measured

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HISTOLOGY AND ULTRASTRUCTURE OF THE HARDERIAN GLANDS — ACCESSORY LACRIMAL GLAND — OF THE CHICKEN

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Abstract

The Harderian gland is an exocrine gland located in the orbit of the chicken. In the stroma of the folds, an extreme amount of plasma cells can be observed. Electron-microscopic observations revealed that the surface epithelium over the margin of the folds is joined up to deeper epithelial cells with processes rich in tonofibrils and the space among them is filled with plasma cells. The basement membrane separating the surface epithelium from the underlying connective tissue is lacking in these areas, therefore, plasma cells are in direct contact with epithelial cells. A fine network of reticular fibrils can be found inside the folds and reticulum cell-like elements with long processes are intercalated among the plasma cells. This morphology resembles that of the lymphoepithelial organs. Based on this similarity the lymphoepithelial character and a possible immunological function of the Harderian gland is supposed.

Introduction

The organization of the immune system of the fowl differs from that of mammals in many aspects. It is characterized by a lower degree of evolution; *viz.*, by topographical separation of the central lymphoid tissue to the thymus and to the bursa of Fabricius, generating different lymphocyte populations displaying cellular and humoral immunological functions, respectively. The extra-thymic lymphoid organs of the chicken (bursa of Fabricius, caecal tonsils, gut-associated lymphoid tissue) play a fundamental role in local humoral immunological defense and therefore, they exhibit the ability of local antigen reception and recognition [6, 8].

The differentiation of lymphoid cells to perform their function needs a specialized tissue environment: the lymphoreticular or lymphoepithelial structure. The so-called central lymphoid organs, in which lymphoid cells gain their fundamental immunological competence, *viz.*, the thymus and the bursa of Fabricius, show a characteristic lymphoepithelial tissue architecture, *i.e.* a three-dimensional meshwork formed by epithelial cells and populated with cells of lymphoid origin [9].

Another organ of the chicken contains a considerable number of lymphoid elements: the accessory lacrimal gland, *i.e.* Harder's gland, a paired gland in the orbit. Its tubuloalveolar structure was described in detail over 100 years ago [1, 4, 5]. Investigations on the ontogeny and phylogeny of the immune system were focused on the possible immunological role of the Harderian gland in the last decade. These observations have revealed that the lymphoid cells populating the Harderian gland are predominantly plasma cells and plasma cell progenitors and the mature plasma cells produce mainly IgA which appears in secretory form in the tear. It was shown that the Harderian gland exhibit local humoral immune response. Based upon these findings, it was suggested that the Harderian gland may act as a peripheral lymphoid organ [2].

Considering that the chicken's peripheral lymphoid organs are very primitive and that the histological and immunological findings argue for the lymphoid nature of the Harderian gland, we analyzed its morphology by light and transmission electron microscopy. Special importance was attached to the identification of lymphoid cells and to the relationship between lymphoid and epithelial elements. According to the results presented in this work, there is a striking similarity between the lymphoepithelial structure of the Harderian gland and that of the central lymphoid organs of the chicken. The origin of the plasma cells in the Harderian gland remains unclear and the dependence of the function of these cells from the bursa of Fabricius needs further elucidation.

Material and methods

Harderian glands from hatching and 2- 3- 4- and 6- weeks- old chickens were investigated. The glands were reached by removing the bulbus. For light microscopy, Carnoy fixation was employed and the specimens were embedded into ester wax. The sections (3—4 μm thin) were stained with hematoxylin and eosin. For the transmission electron-microscopic studies the specimens were fixed in glutaraldehyde (3.5 mg/100 ml 0.1 *M* cacodylate buffer, pH 7.2) and a second fixation in osmic acid (1 g/100 ml) and embedding in araldite were employed. The semi-thin sections were stained with a mixture of toluidine blue and azure II, while the ultra-thin sections were contrasted by uranyl acetate and lead. A JEM 6C electron microscope was used.

Results

The Harderian gland contains a central collecting tubule along its longitudinal axis (Fig. 1). The cross-section of this tubule seems to be star-shaped, owing to several longitudinal folds bulging into the lumen (Fig. 2). The tubuloacinar units are located under the surrounding capsule. They lead into the central tubule among the folds (Fig. 2). Transmission electron-microscopic observations show that the acini have a lining of cylindrical secretory epithelium. A number of vacuoles containing a lipid-like material of low electron density can be found in the apical part of epithelial cells. The con-

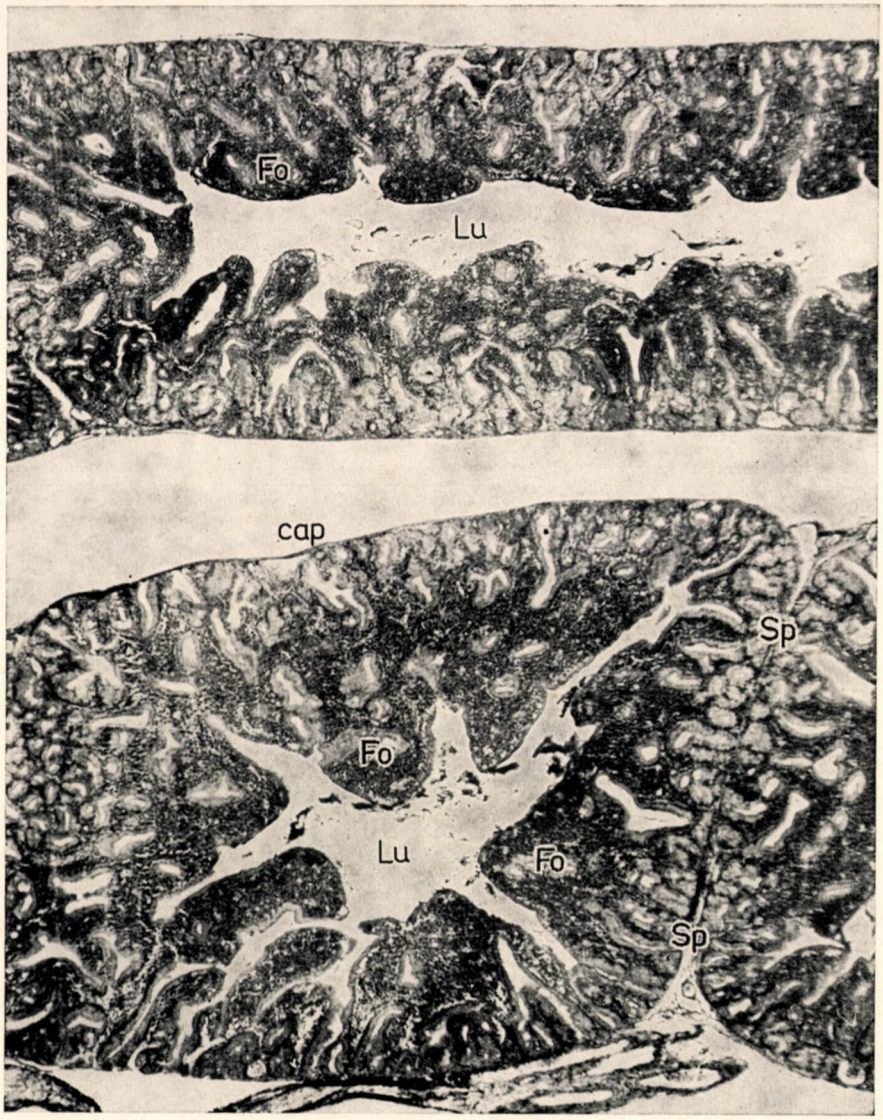

Fig. 1. Longitudinal section of the Harderian gland. The central collecting tubule (Lu)
Fig. 2. Cross-section of the Harderian gland. The folds (Fo) filled with lymphoid cells intruding the lumen (Lu). The tubuloacinar units are under the rows of connective tissue (Sp) and the capsule (Cap)



Fig. 3. Acini with cylindrical lining cells (AEC) with secretory vacuoles (Sv) over the continuous basement membrane (BM)

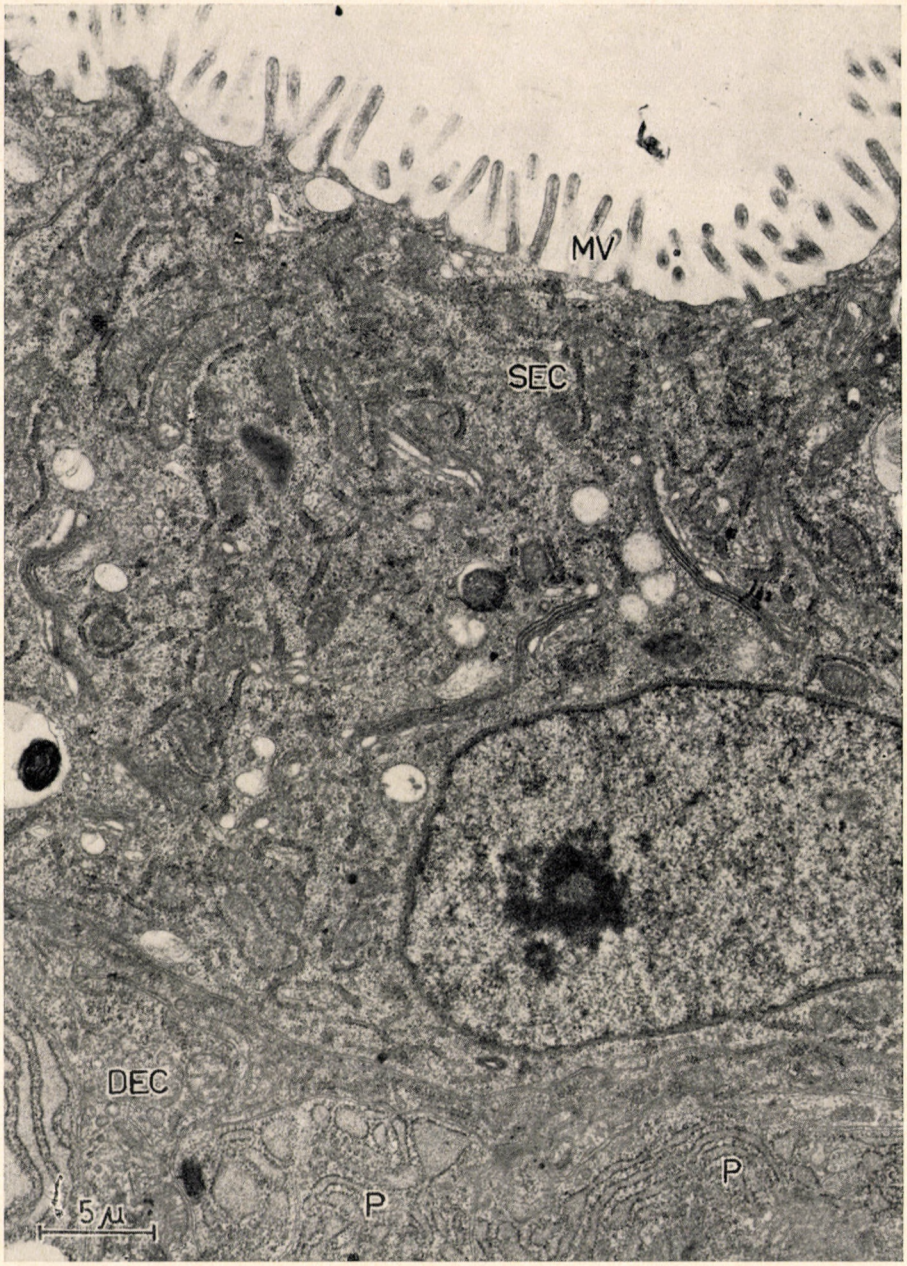

Fig. 4. Surface epithelial lining cells (SEC) over the fold. Note the microvilli (MV) on their apical surface. The basement membrane is lacking under the surface epithelial cell SEC). This cell is in connection with a process of a deep epithelial cell (DEC). Plasma cells (P) contact with the deep epithelial cell (DEC)

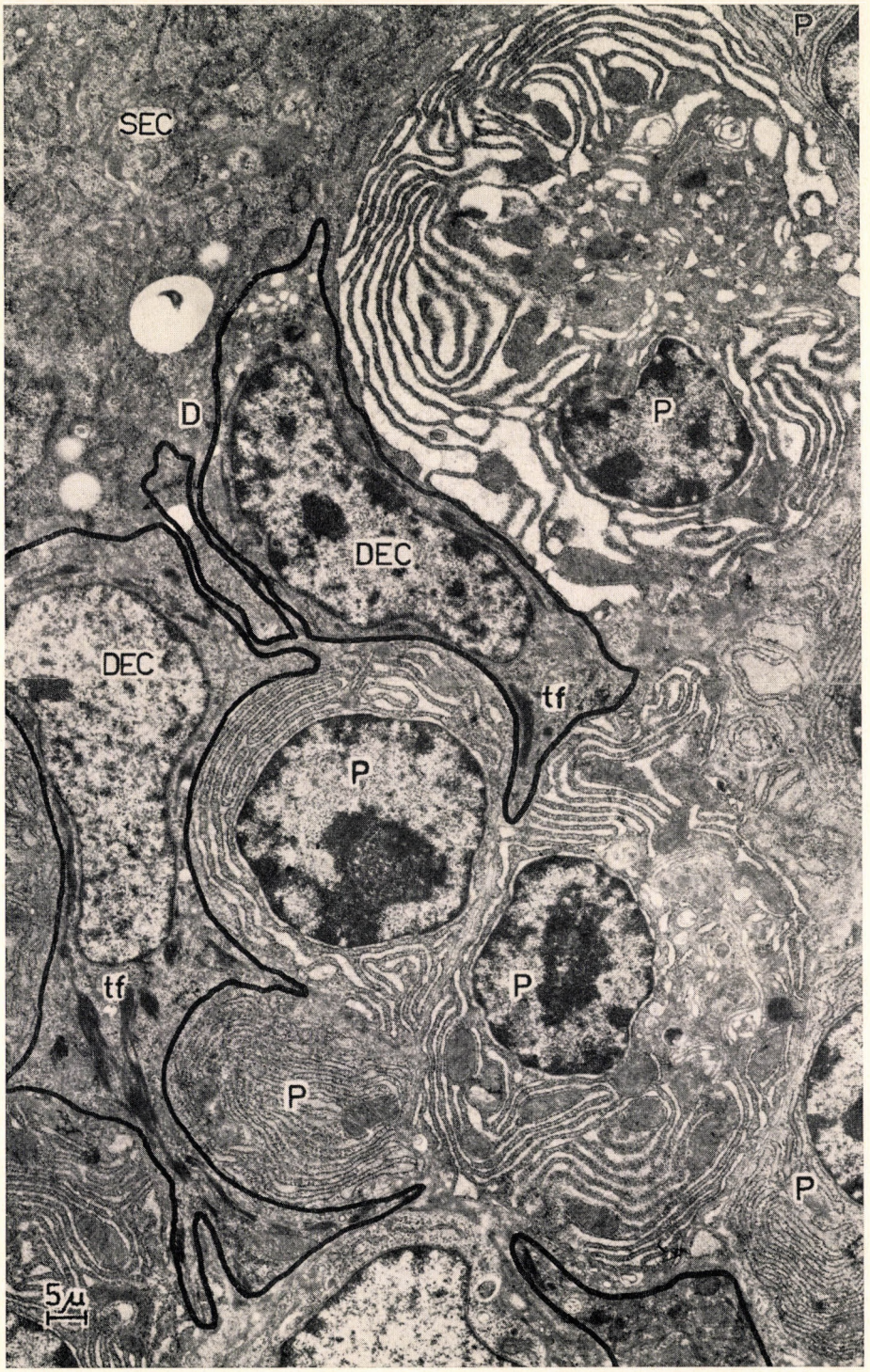

Fig. 5. Surface epithelial cell (SEC) is joined up with desmosomes (D) to the deep epithelial cell (DEC). Note the rich tonofibrillar apparatus (tf) and the different maturation forms of plasma cells (P)

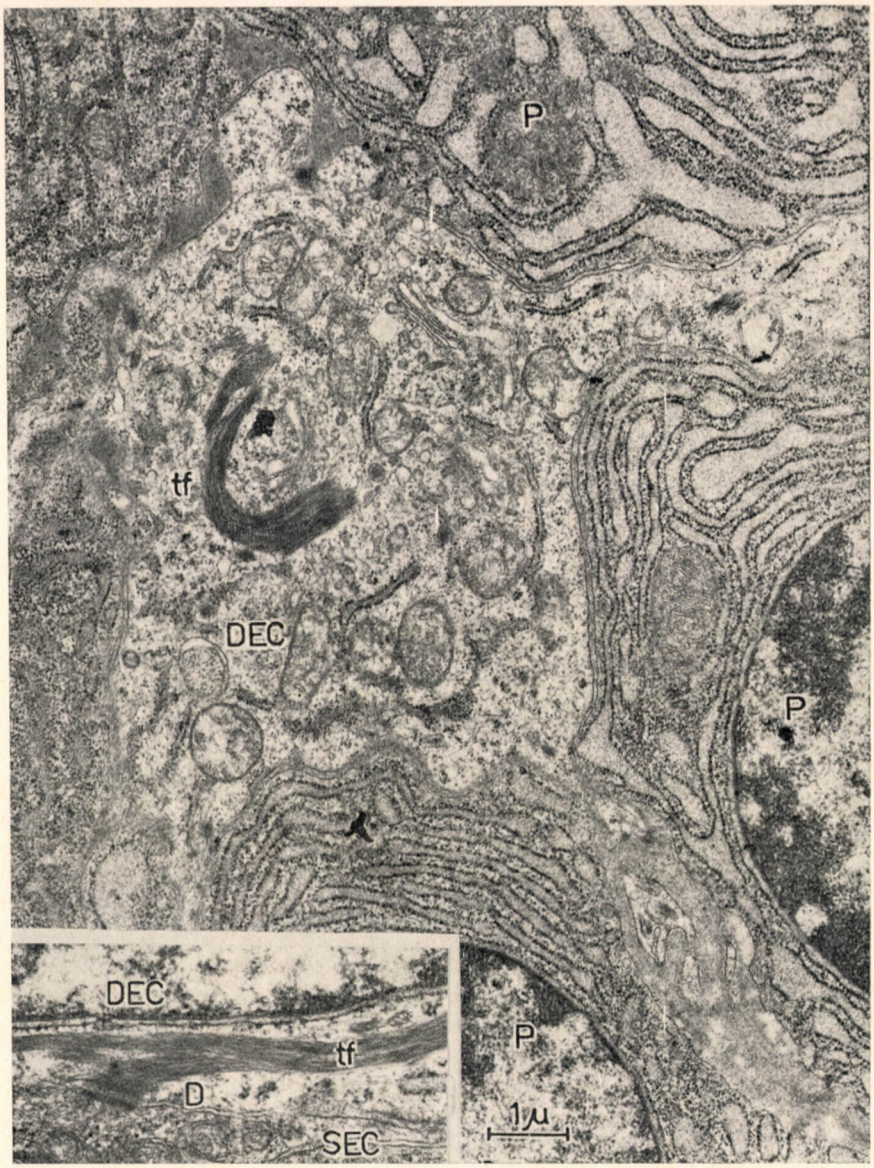

Fig. 6. Tonofibrillar apparatus of a deep epithelial cell (DEC) among plasma cells (P). Inset: Desmosome (D) joining up a surface epithelial cell (SEC) to a deep epithelial cell (DEC)

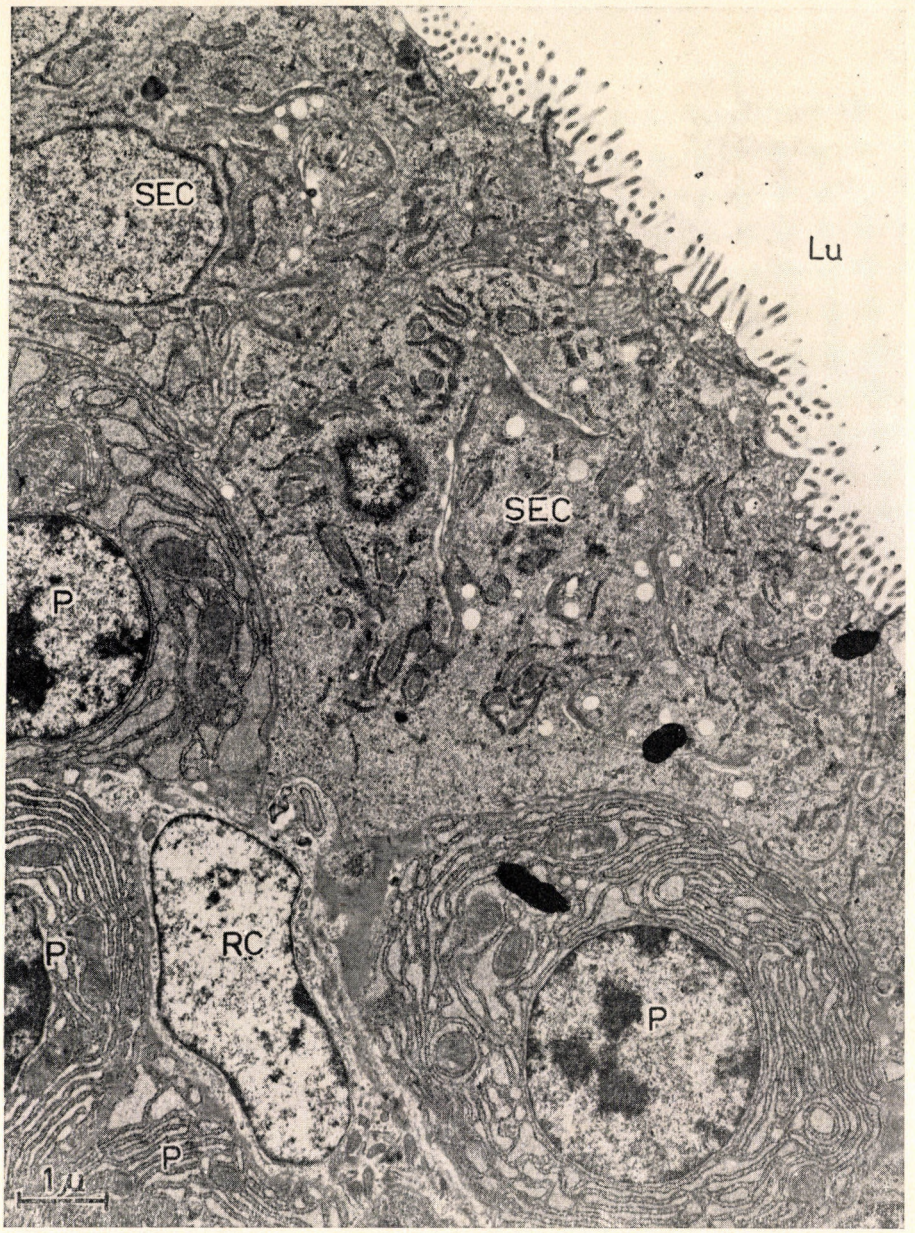

Fig. 7. Plasma cells (P) intruding among the basal parts of surface epithelial cells (SEC) are separated from the central lumen (Lu) only by the apical 1—2 μm part of the surface epithelial cells. RC: reticulum cell

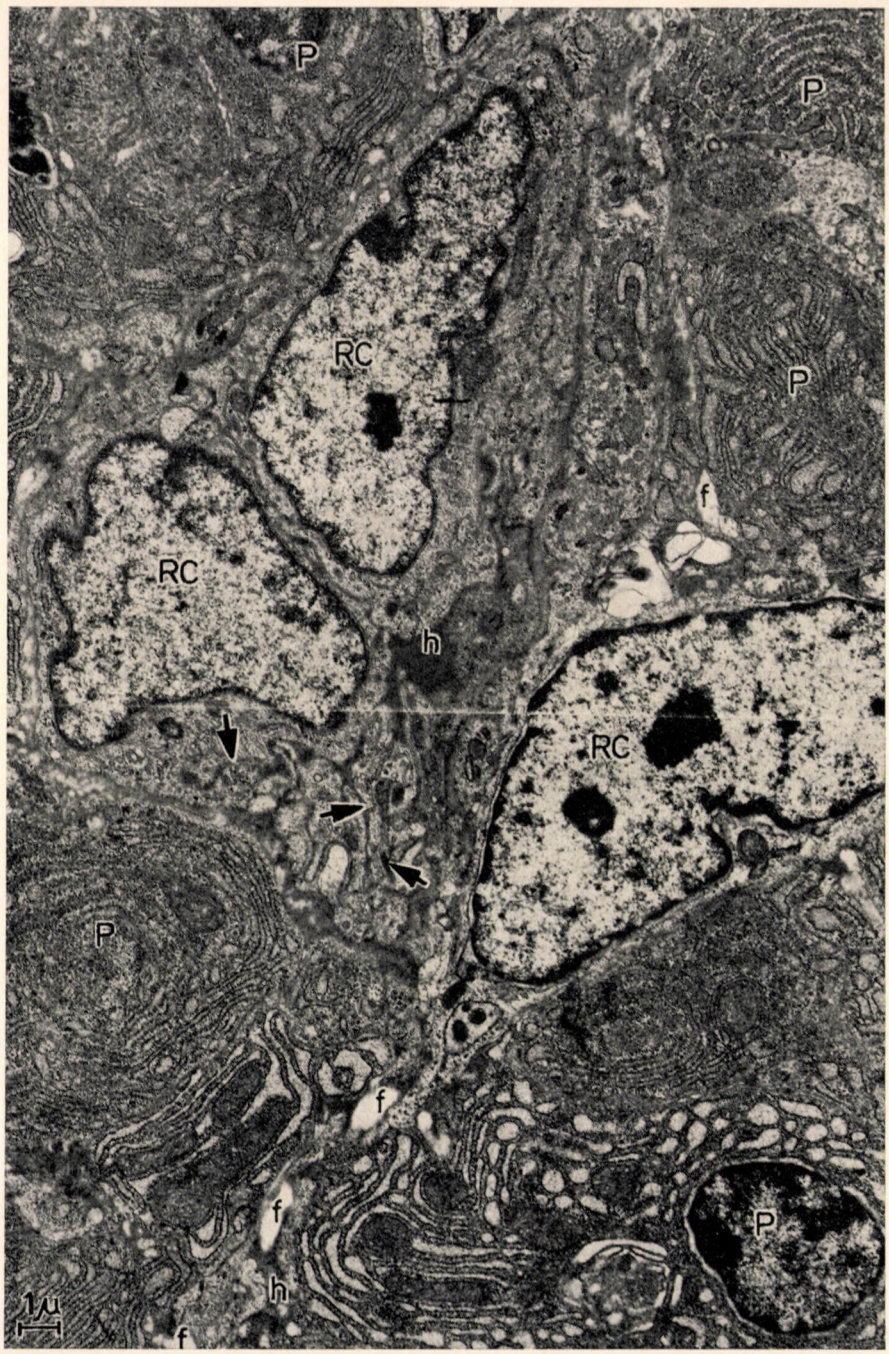

Fig. 8. The supporting apparatus of the folds is formed by reticular fibres, by homogeneous (h) and fibrillar (f) extracellular substance and by reticulum cells. Note the invaginations characteristic of the cytoplasmic processes of reticulum cells (arrow)

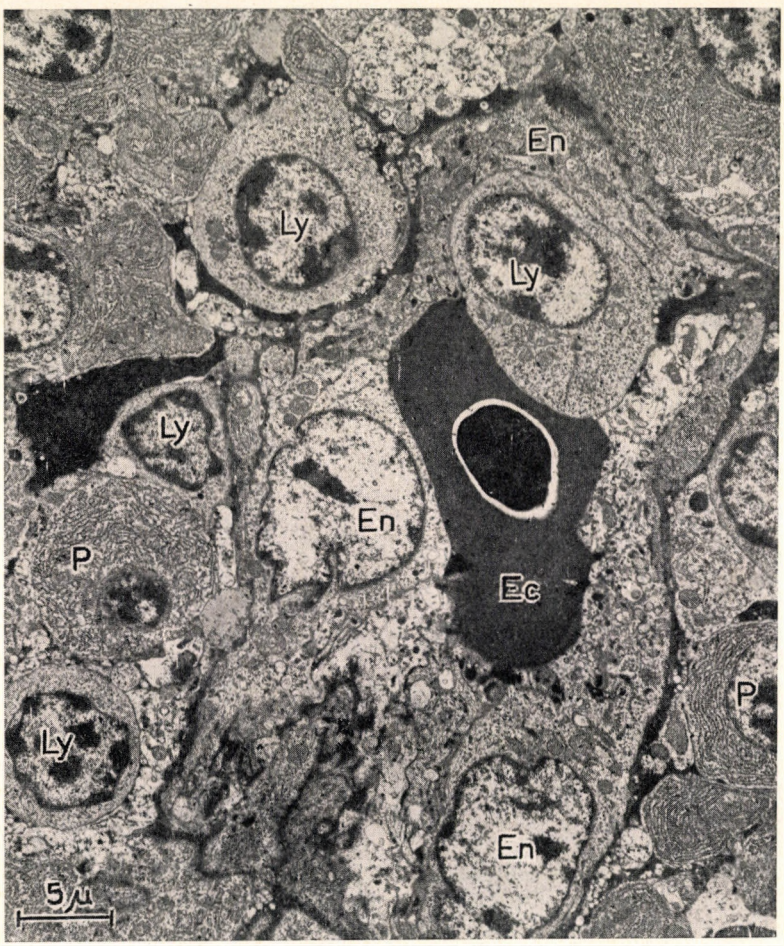

Fig. 9. Capillary lumen filled with nucleated erythrocytes (EC) and lymphocytes. Note the lymphocytes crossing the endothelial wall (En)

nective tissue elements are separated from the cells of the acini by the continuous basement membrane (Fig. 3).

The folds were investigated with particular interest because of the presence of lymphoid tissue (Fig. 2). The epithelial cells covering the folds significantly differ from the epithelial lining of the acini in the presence of microvilli on their apical surface and in lacking of secretory vacuoles (Fig. 4).

Cells with other cytological characteristics are located in a deeper layer of the epithelium and are joined up by desmosomes to the surface epithelial cells. Their processes are directed toward the inside of the folds and their cytoplasm is characterized by a well-developed tonofibrillar system. Plasma cells are interposed between them (Figs 5 and 6). Occasionally, plasma cells intrude

among the basal parts of the surface epithelial cells, too. In such a position only the luminal parts of the surface epithelial cells separate the plasma cells from the central lumen (Fig. 7).

The supporting apparatus of the fold are formed by reticular cells and by connective tissue containing small amounts of collagen fibres. The reticular cells are characterized by an endoplasmic reticulum of vesicular type and a nucleus with loose chromatin structure and a dense nucleolus. Pronounced invaginations of the cell membrane were observed. The invaginations and the intercellular space are filled with a material of medium electron density (Fig. 8).

Lymphocytes or lymphoblasts occur very rarely in the almost homogeneous population of plasma cells, most frequently in the vicinity of capillaries. All differentiating forms, from plasmoblasts to mature plasma cells, can be easily distinguished in the gland. Russell bodies produced by the concentration of the content of the ergastoplasmic cysternae occur frequently. Only a few blood vessels can be found within the folds. Lymphocyte migration across the wall of capillaries was observed in some cases. Few lymphocytes can be found in the proximity of the subendothelial connective tissue but never in further areas (Fig. 9).

Discussion

Although the plasma cell invasion characterizes only the Harderian gland of the fowl, and lymphoid elements are not abundant in the accessory lacrimal gland of mammals [3, 11], the categorization of the Harderian gland among the lymphoid organs has been proposed only recently [2, 7, 10]. The predominance of plasma cells in the Harderian gland of the chicken has been explained as a consequence of the continuous infection with leucosis virus but the characteristic plasma cell invasion develops in germ-free chickens and in other birds, too [2]. The appearance of plaque-forming cells in the Harderian gland after local or systemic immunization was examined by MUELLER et al. [7]. Antibody-producing cells were shown in the Harderian gland after an introduction of sheep erythrocytes into the eye but they were absent after systemic immunization. On the other hand, plaque-forming cells were also found after local immunization in other peripheral lymphoid organs, suggesting the migration and homing of the antigen-sensitive or antibody-producing cells. Such reception of antigen is a known function of tonsils in mammals and the same capacity is attributed to the bursa of Fabricius [6, 8, 9]. No cytotoxic cellular lymphocyte response was found against chicken chorioallantoic membrane cells and this was interpreted as complete absence of lymphoid cells of thymic origin in the Harderian gland [10]. Based on these findings the Har-

derian gland is thought to be a peripheral lymphoid organ playing a role in the local immunological defense [2, 10].

The topographical pattern of epithelial and plasma cells in the Harderian gland is identical with that of the epithelial and lymphoid elements of the thymus and the bursa of Fabricius. The close relation between these cell types is expressed by the penetration of plasma cells among the epithelial cells. The three-dimensional meshwork of the epithelial cells, which is pronounced by the surface and deep epithelial cells in the Harderian gland, is an essential characteristic of lymphoid organs playing a role in lymphoid cell differentiation. Based on these morphological similarities to the structure of thymus and bursa, the Harderian gland can be regarded as one of the lymphoepithelial organs.

It seems that there is a contradiction between the local humoral immunological function and the lymphoepithelial structure of the Harderian gland. The key of the solution might be the origin and dependence of the homogeneous plasma cell population. The presence of lymphoid follicles has been shown in the connective tissue around the lacrimal duct of the Harderian gland, and the migration of their cells into the folds, and differentiation into plasma cells were suggested [2]. The lack of lymphocytes and lymphoblasts in the folds argues against this hypothesis. The lymphocyte migration across capillary walls into the deeper layers of the folds directs attention towards another possibility. This phenomenon supports the supposition of the transport of plasma cell precursors from other lymphoid organs to the gland *via* the blood circulation. Our unpublished results seem to prove the existence of a considerable extent of cell wandering to the Harderian gland from the bursa of Fabricius, and also from the thymus. On the other hand, surgical or chemical removal of the bursa does not influence the development of the lymphoepithelial structure characterizing the Harderian gland.

These findings seem to be in accordance with the pertinent data concerning the ontogeny of the B system, namely, that the differentiation of B lymphocytes to plasma cells may happen parallelly in different places of the immune system where the following conditions are given:

(a) B committed lymphocytes,

(b) T help,

(c) epithelial or reticular framework,

(d) continuous immunogenic stimulus.

The presence of these conditions in the Harderian gland of the chicken may serve as an explanation of its dual nature but must be confirmed by further direct experimental evidence.

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EFFECT OF SOME PESTICIDES ON THE RHYTHMIC ADDUCTOR MUSCLE ACTIVITY OF FRESH-WATER MUSSEL LARVAE

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Abstract

The effect of some pesticides on the spontaneous and tryptamine (TA)-induced adductor muscle activity of glochidiae of the fresh-water mussel *Anodonta cygnea* was examined in short-term experiments. The pesticides used were: Bi-58 EC, Novenda, Malathion, Shell-DD, Dikonirt and pure 2,4-D the active ingredient of Dikonirt. High concentrations ($\geq 10^{-1}$ g/l) of the examined substances have a short, slight excitatory influence on the spontaneous rhythmic adductor activity of larvae, in addition their effect results in lasting closure of some specimens. In lower concentrations, they inhibit the TA-induced adductor activity. The threshold of inhibitory concentrations may be lower with prolonged exposure time.

Introduction

The tolerance of bivalve molluscs to different pesticides and polluting substances is variable. Furthermore, the resistance against a given substance is changing during ontogenesis, eggs and larvae being generally more resistant than adult specimens [5, 11]. For examining the influence of toxic substances on the bivalve larvae, the mortality has been considered as an indicator of the effect in short-term experiments, while the supression of larval growth in chronic experiments [1, 2, 3, 5]. In earlier investigations with pesticides and heavy metal salts, we found that only very high concentrations of the toxic substances had an excitatory effect on the spontaneous adductor activity of larvae [4, 12]. On the other hand, the spontaneous adductor activity of larvae is not suitable for testing the inhibitory influences because of its low frequency. KCl and tryptamine (TA) have a considerable excitatory effect on the rhythmic adductor activity [8, 9], thus, the inhibition of the TA-induced activity can be considered a suitable indicator of the inhibitory effect of toxic substances.

We have described the effect of some pesticides on the TA-induced activity of the larvae of fresh-water mussel *Anodonta cygnea* [12]. The aim of the present examinations was to obtain similar type of information for other pesticides which can enter streams and stagnant waters.

Material and methods

Experiments were performed on glochidiae of fresh-water mussel *Anodonta cygnea* L in the months November to March, at room temperature (20—23 °C). Before the experiments, glochidiae were divided into groups, each of them contained 25 specimens, and were allowed to stay for 30 min under binocular microscope, for assuring the adaptation to temperature and light. Following this, the spontaneous adductor contractions in the first group were counted in every minutes during 5 min, then, after an application of 100 mg/l TA solution, the TA-induced contractions were counted for 30 min in the same way. The number of larvae showing lasting closure was noted, too. In case of the other groups, the TA solution contained the given concentration of the pesticide to be tested. Thus, each concentration was tested on 200—300 larvae at least [12].

For estimation of the results the contractions of 100 larvae per minute (a/min) and the value of lasting closures in per cent of the total number of larvae (C%) were calculated and graphically presented. The number of contractions per 30 min (whole experimental period) was also calculated (Σ a), and the percentage inhibition of TA-induced activity by the pesticides was graphically presented as a function of pesticide concentration. The concentration that resulted in 50% inhibition of TA-induced activity was graphically estimated from this curve.

In case of the examination of influence of the exposure time on the TA-induced activity each group of larvae was incubated in pesticide solution of different concentration for 24 h at +4 — +5 °C, thereafter the pesticide solution was changed for 100 mg/l TA solution, and the TA-activation was measured. The data obtained were evaluated as described above.

The solutions were freshly prepared in filtered Balaton-water. The water-insoluble compounds were used as suspension or colloid. The chemicals used were, as follows:

Insecticides: Bi-58 EC (VEB Chemie-Kombinat, Bitterfeld, DDR), Novenda (Nitrochemie Industry, Fűzfő, Hungary), Malathion (Shell Chemicals Ltd., England).

Nematocide: Shell-DD (Shell-Int. Petr. Co. Ltd., England).

Herbicide: Dikonirt (Nitrochemie Industry, Fűzfő, Hungary) and its active ingredient 2,4-D.

The pH of Balaton-water was 8.45, while that of the solutions varied between 7.9 and 8.45.

Results

The own effect of the insecticide Bi-58 EC on the spontaneous adductor muscle activity and on the lasting contraction of the glochidiae required a concentration of 5×10^{-1} ml/l or more. It was a short-time, weak excitatory effect. The total contraction number evoked was a low as 1—2% of the TA-induced contractions. Thereafter a great percentage (50% at 5×10^{-1} ml/l 80—90% at 1 ml/l) of larvae showed lasting closure.

More than 10^{-1} ml/l of Bi-58 inhibited the TA-induced adductor activity of glochidiae (Fig. 1), and a concentration of 1 ml/l completely prevented it. The initial short-term, weak excitation as well as the lasting closure observed on most of the larvae seems to be the own effect of Bi-58. Figure 2 shows the inhibition of TA-activation by various concentrations of Bi-58, considering the total contraction number per 30 min. On the basis of this curve the 50% inhibition was caused by 4×10^{-2} ml/l Bi-58, *i.e.* 10^{-2} g/l active ingredient, Dimethoate.

Novenda in concentrations lower than 1 g/l did not show any own excitatory effect on the adductor muscle activity of glochidiae. Over 10^{-4} g/l concentration it inhibited the TA-induced activity (Fig. 3). The inhibition reached 50% at 2×10^{-1} g/l and was almost complete at 1 g/l, *i.e.* 5×10^{-2} g/l active ingredient DNOC (Fig. 4).

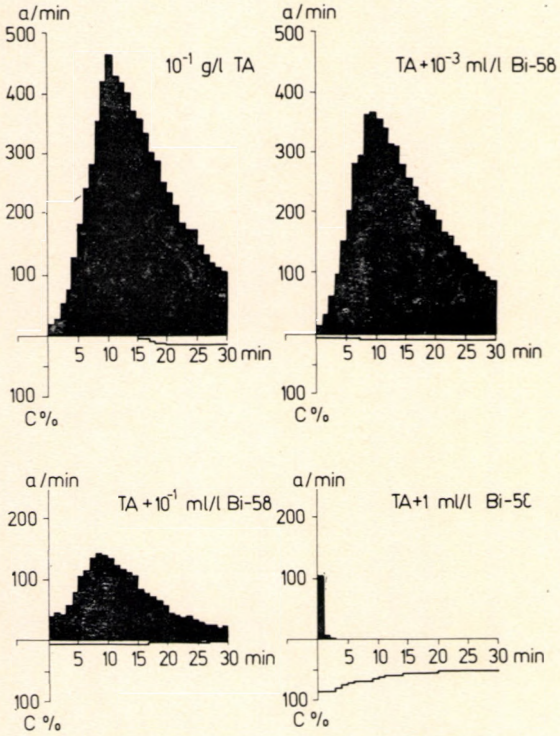

Fig. 1. Effect of different concentrations of Bi-58 on the TA-activation of glochidiae. Ordinata in the upper part: the number of contractions of 100 glochidiae per min (a/min); in the lower part: percentage of the larvae being in lasting closure (C %). Abscissa: duration of the treatment (min)

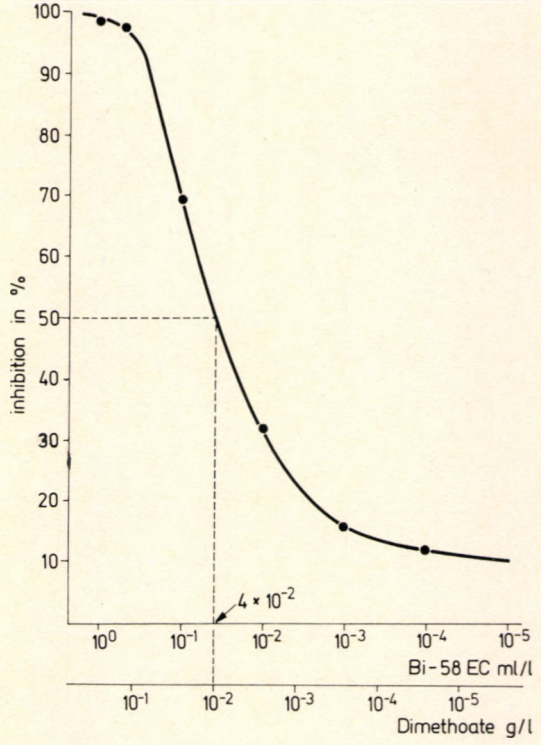

Fig. 2. Inhibition of TA-activation in per cent as a function of concentration of Bi-58 and the effective ingredient Dimethoate considering the total activity/30 min (Σ a)

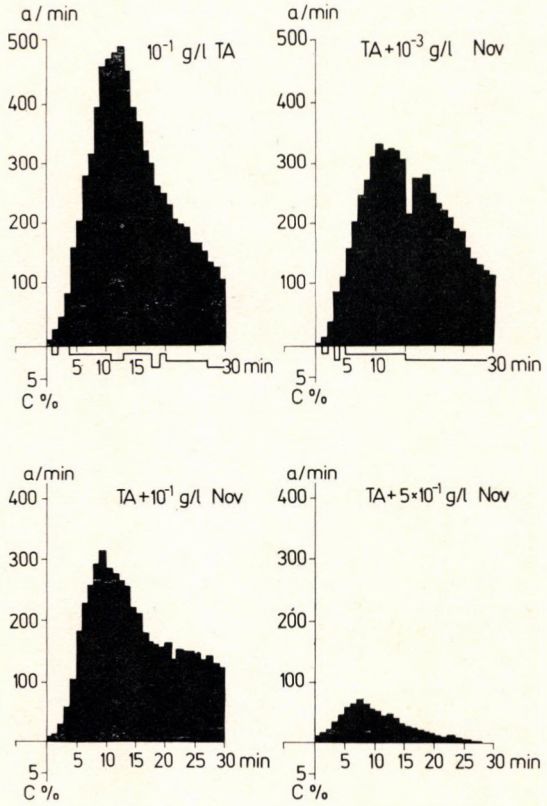

Fig. 3. Effect of different concentrations of Novenda on the TA-activation of glochidiae

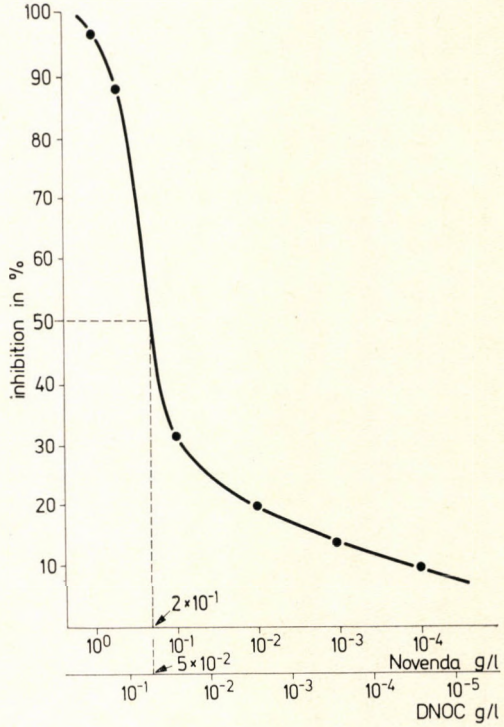

Fig. 4. Inhibition of TA-activation in per cent as a function of concentration of Novenda and the effective ingredient DNOC considering the total activity/30 min (Σ a)

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Malathion was used as emulsion in water. In concentrations 10^{-1} ml/l or higher it had a short-term, slight own excitatory effect on the spontaneous adductor muscle activity of glochidiae, and lasting closure of 20—30% of larvae occurred. A concentration as low as 10^{-5} ml/l inhibited the TA-induced adductor muscle activity considerably (Fig. 5), and the excitatory effect of TA was completely blocked by a 10^{-1} ml/l Malathion, and only the own effect of this insecticide was manifested. The 50% inhibition was reached at 1.8×10^{-3} ml/l (Fig. 6).

The nematocide Shell-DD was used as emulsion, too. In concentrations of 10^{-1} ml/l or more it had a slight own excitatory effect, and resulted in a lasting closure of 30—70% of larvae. Over 10^{-4} ml/l an inhibiton of TA-induced activity was observed. In 10^{-1} ml/l concentration the own effect of Shell-DD was realized, though this concentration did not completely prevent the TA-induced activity (Fig. 7). A complete blockade required 1 ml/l concentration. The 50% inhibition of the TA-induced adductor muscle activity was caused by a concentration of 2×10^{-2} ml/l, *i.e.* 10^{-2} ml/l active ingredient 1,3-dichlorpropene + 1,2-dichlorpropane (Fig. 8).

The herbicide Dikonirt in concentrations of 5×10^{-1} or more had an own excitatory effect on the spontaneous adductor activity of glochidiae lasting 5—7 min. On most (70—90%) of the larvae it caused immediately a lasting closure, which was quickly eliminated in the first 10 min, then the elimination process was slowed down. This latter effect was manifested even in the presence of TA (Fig. 9). Over 10^{-2} g/l concentration Dikonirt inhibited the TA-induced adductor muscle activity of glochidiae. A complete inhibition needed 5 g/l concentration, while the 50% inhibition was evoked by 3.5×10^{-1} g/l Dikonirt (Fig. 10), *i.e.* 2.5×10^{-1} g/l active ingredient 2,4-D.

The inhibitory effect of pure 2,4-D active ingredient was examined separately. It had some slight own excitatory effect on the spontaneous adductor activity of glochidiae over 5 g/l concentration. This latter effect lasted 10—15 min., and following a 5 min treatment the number of closed larvae increased up to 90%. This own effect was maintained even in the presence of TA (Fig. 11). The TA-induced adductor activity was inhibited only by concentrations as high as 1 g/l or more; 50% inhibition required 7 g/l 2,4-D (Fig. 12).

The effect of Malathion was examined also following 24 h pretreatment of glochidiae with solutions of different concentrations, thereafter the TA-induced activity was tested. The TA-induced activity of glochidiae pretreated in this way was inhibited by as a low concentration of Malathion as 10^{-8} ml/l (Fig. 13). The concentration dependence of the inhibition of TA-induced adductor activity is demonstrated in Fig. 14. The 50% inhibition was observed at a concentration of 7.5×10^{-6} ml/l.

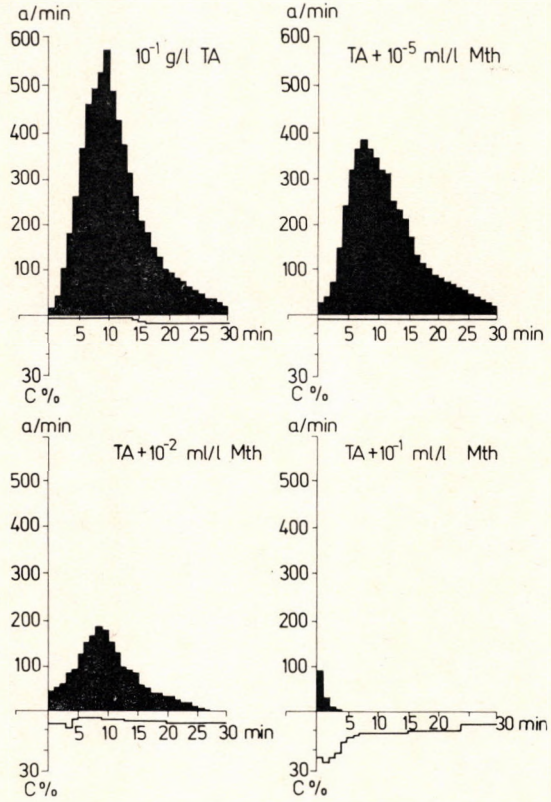

Fig. 5. Effect of different concentrations of Malathion on the TA-activation of glochidiae

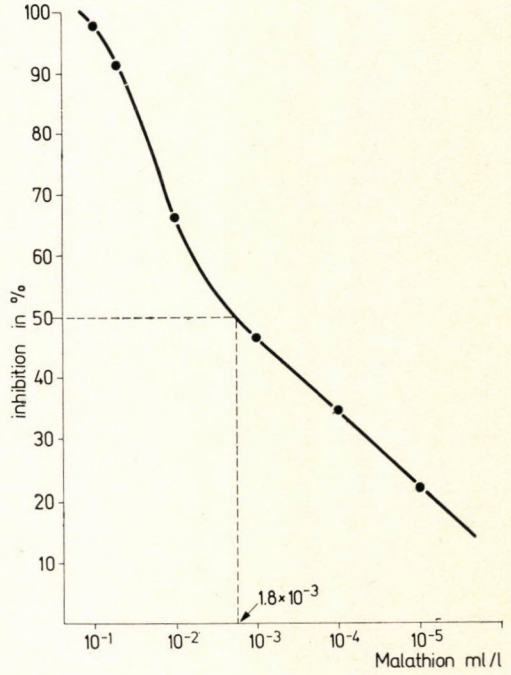

Fig. 6. Inhibition of TA-activation in per cent as a function of concentration of Malathion considering the total activity/30 min (Σ a)

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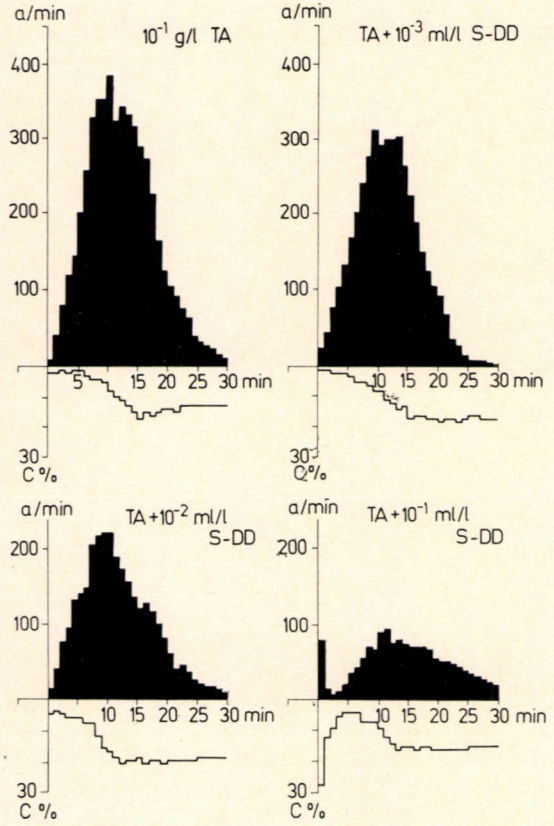

Fig. 7. Effect of different concentrations of Shell--DD on the TA-activation of glochidiae

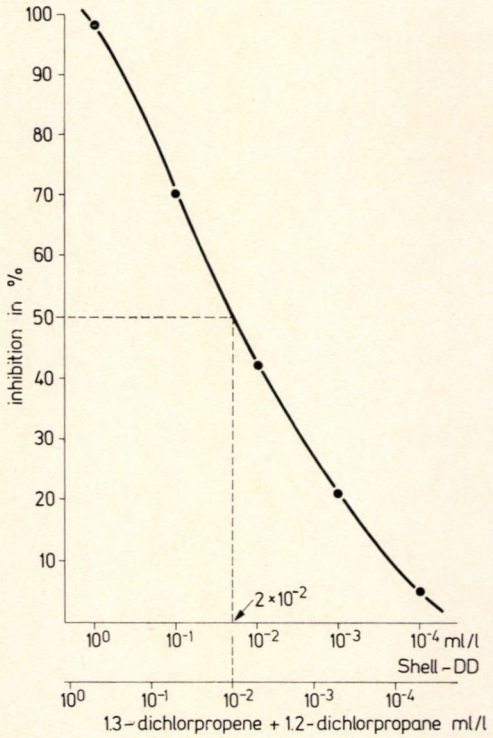

Fig. 8. Inhibition of TA-activation in per cent as a function of concentration of Shell-DD and the effective ingredient 1,3-dichlorpropene + 1,2-dichlorpropane considering the total activity/30 min (Σ a)

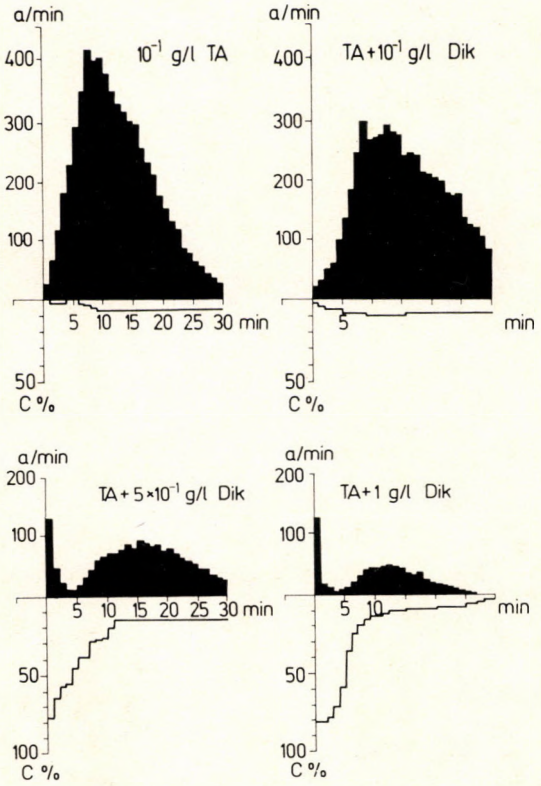

Fig. 9. Effect of different concentrations of Dikonirt on the TA-activation of glochidiae

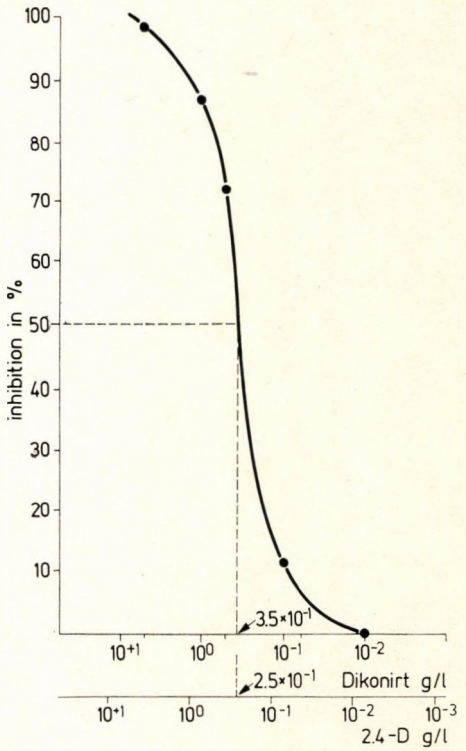

Fig. 10. Inhibition of TA-activation in per cent as a function of concentration of Dikonirt and the effective ingredient 2,4-D considering the total activity/30 min (Σ a)

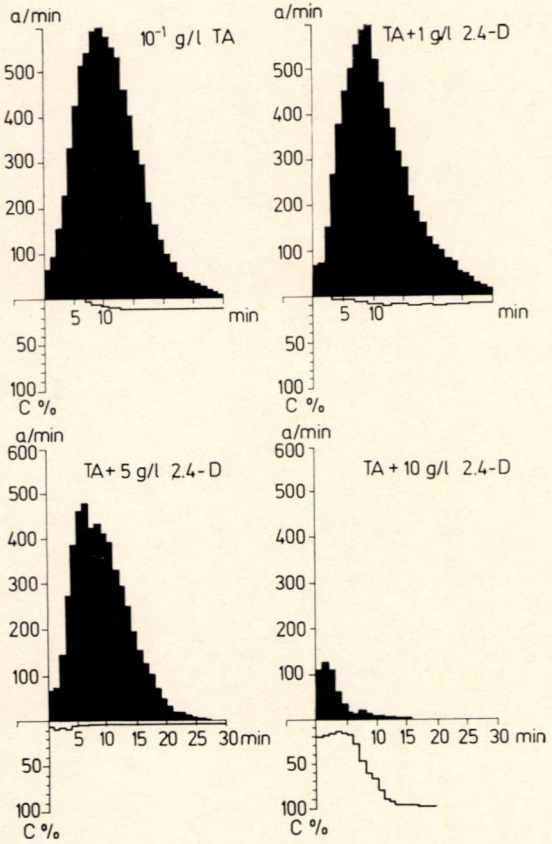

Fig. 11. Effect of different concentrations of 2,4-D on the TA-activation of glochidiae

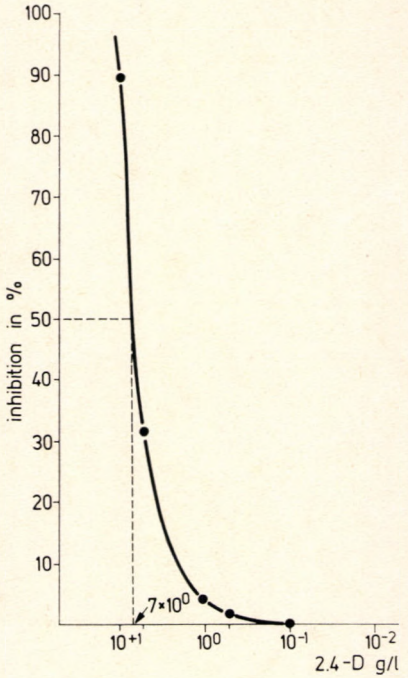

Fig. 12. Inhibition of TA-activation in per cent as a function of concentration of 2,4-D, considering the total activity/ 30 min (Σ a)

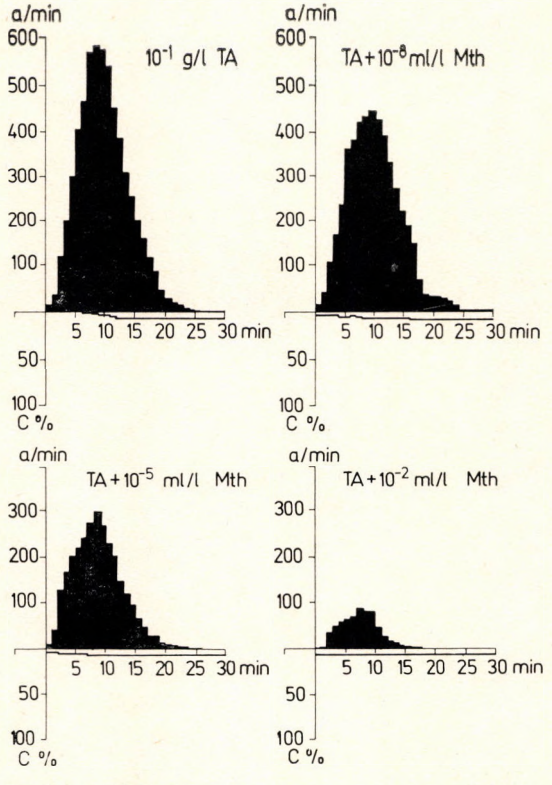

Fig. 13. Effect of different concentrations of Malathion on the TA-activation of glochidiae following a 24-h pretreatment with the drug

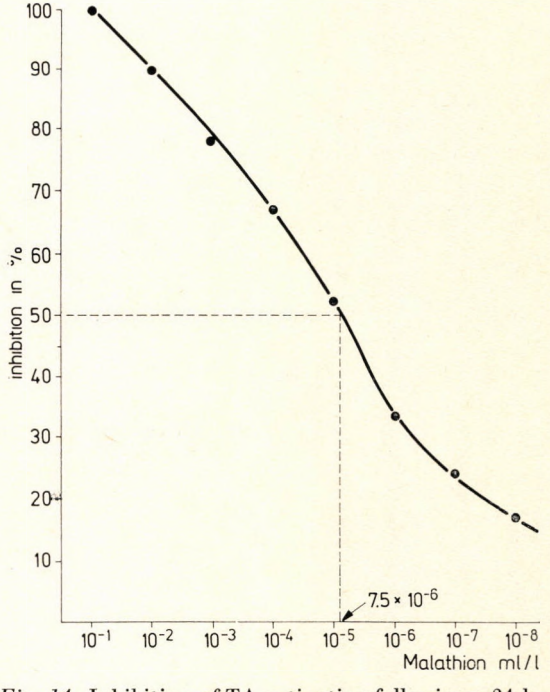

Fig. 14. Inhibition of TA-activation following a 24-h-pretreatment with Malathion, considering the total activity/30 min (Σ a)

Discussion

The glochidiae exhibit two types of spontaneous movement activity. One of these is the rhythmic adductor muscle activity, which consists of the fast, phasic contraction and relaxation of the larval adductor muscle. The other is the lasting closure, which is a result of the tonic contraction of the adductor muscle. The frequency of the rhythmic adductor muscle activity is generally very low (0—0.02 cpm), therefore, it seems only to be suitable for examining excitatory effects. The substances investigated in the present experiments caused excitatory effect only in high concentrations 10^{-1} ml/l of Malathion or Shell-DD, 5×10^{-1} ml/l of Bi-58 or Dikonirt and 10 g/l or higher of 2,4-D. Novenda had no such effect up to as high as 1 g/l concentration. This excitatory effect on the rhythmic adductor activity usually was slight and lasted only a few minutes (3—5 min), except for 2,4-D, which had an effect of 15 min duration. The number of contractions evoked by different substances was not more than 1—3% of the TA-induced ones. The slight excitatory effect of the above substances on the spontaneous activity was connected with producing lasting closure of the larvae. This latter effect was induced in about 20—30% of larvae by Malathion, 30—70% of larvae by Shell-DD, and 50—90% of larvae by Bi-58, Dikonirt and 2,4-D.

Considering that in the glochidiae TA has a functional significance in physiological condition [6, 9, 10], the TA-induced adductor muscle activity may be a suitable parameter for toxicological examinations. The concentration reducing the TA-induced adductor activity to 50% seems to be a good index for the toxicity. For the pesticides examined, these concentrations were as follows: Malathion: 1.8×10^{-3}, Shell-DD 20×10^{-3}, Bi-58 EC 40×10^{-3}, Novenda 200×10^{-3}, Dikonirt 350×10^{-3} g/l.

The TA-activation of glochidiae is known to be more pronounced in an alkaline medium than in a neutral one. In the latter conditions the number of contractions per min. and the percentage of larvae ensuring tonic contraction decreases, and the duration of the activation is diminished in the first few minutes following TA application [7]. In the present experiments, Bi-58 in 1 ml/l concentration was the least alkaline solution. In this solution, a short-term TA-activation was observed, which can only be attributed to the substance, not to a pH effect, because of the lasting closure of most of the larvae. The situation is the same in case of Malathion, Dikonirt and 2,4-D. Concerning Shell-DD, the considerable duration of TA-activation is inconsistent with the supposition of a pH-effect. Finally, the pH of the solutions of each concentration of Novenda was 8.45.

The above concentrations of pesticides are too high, to occur in natural waters. Nevertheless, it must be taken into consideration that we carried out mostly short-term experiments, the larvae were exposed to toxic effect of

pesticides for 30 min. Earlier we supposed that an increase in the exposure time should decrease the threshold of toxic concentrations. In the present work, this was experimentally demonstrated for Malathion. In this case an increase in the exposure time from 30 min to 24 h led to a decrease in the concentration resulting in 50% inhibition from 1.8×10^{-3} ml/l to 7.5×10^{-6} ml/l. A similar decrease in the effective concentration with increasing the exposure time is probable in the case of other pesticides, too. It may be supposed that in natural waters glochidiae may be exposed to pesticides for still longer times, and thus a further diminution of the effective inhibitory concentrations may diminish further.

2,4-D, the active ingredient of Dikonirt was examined separately. While 50% inhibition of the TA-induced adductor muscle activity required 350×10^{-3} g/l Dikonirt, the corresponding concentration for the pure 2,4-D salt was 700×10^{-3} g/l. Since pesticides often contain activators and synergists in addition to their active ingredient(s) the present experimental data suggest that in toxicological tests the pure active ingredient cannot substitute the given preparation. The additional compounds potentiating the toxic effect of the active ingredient may also enter streams and stagnant waters, nevertheless in the course of a chemical analysis of the pesticide-polluted water usually the presence and concentration of the active ingredient are only examined, though at a given concentration of the active ingredient the presence of an activator may multiply toxicity.

According to the data of DAVIS and HIDU [2], the value of 14 day's TL_m for *Crassostrea virginica* larvae is 2.66×10^{-3} ml/l, a value close to $1.8 \times \times 10^{-3}$ ml/l obtained in our short-term experiments. Using this concentration, the 50% inhibition of TA-induced adductor contractions could be observed in 30 min. The 14 day's TL_m value of 2,4-D salt on oyster larvae has been reported to be 64.29×10^{-3} g/l, while the concentration decreasing the TA-activation of *Anodonta* larvae to 50% proved to be 7000×10^{-3} g/l in our experiments.

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CHANGES IN ACTIVITY OF THE ORGANON VASCULOSUM LAMINAE TERMINALIS IN THE ANNUAL CYCLE IN *RANA TEMPORARIA*

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Abstract

In 70 sexually mature male and female *Rana temporaria* frogs captured in natural habitat, mean nuclear volumes for the cells of the pars ependymalis and pars parenchymalis of the organon vasculosum laminae terminalis (OVLT) were determined in seven characteristic stages in life. The mean nuclear volume for the cells of the pars ependymalis and pars parenchymalis of the OVLT showed distinct annual fluctuation. Maximum nuclear volume of the cells in both investigated parts of the OVLT were observed during the breeding period (Ist decade of April), and minimum volume of the nuclei of the pars ependymalis at the beginning of hibernation (IIIrd decade of October), and in the pars parenchymalis near the end of active life (Ist decade of September).

Introduction

The third and fourth cerebral ventricles, containing ependymal and subependymal areas of special cellular structure, are of special interest. These are the subfornical organ, subcommissural organ, paraventricular organ and area postrema [3, 4, 7, 19, 25, 35, 36], and the organon vasculosum laminae terminalis (OVLT) [7, 15, 22, 23].

The OVLT is commonly present in vertebrates [13]. Its morphology and structure have been investigated thoroughly in fishes, amphibians, reptiles, birds and mammals [32]. The structural model is similar in all investigated vertebrates. It consists of three parts: pars ependymalis, pars parenchymalis and pars duralis [32, 33]. The ependymal part contains typical ependymal cells and tanycytes [6, 15, 17]. The parenchymal part is built of loosely-arranged parenchymal cells, which dominate among the cells of the OVLT. The pars duralis, which is richly vascularized, is believed to supply the OVLT with blood [33]. The OVLT in *Rana temporaria* L. is interesting because of the high degree of vacuolization and presence of catecholinergic cells and nerve fibres and cytoplasmic autofluorescent granulations [13]. The function of this organ is unknown. In view of its position and rich vascularity it is hypothetically assumed to play an important role in the formation of the blood cerebro-

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spinal fluid (CSF) barrier [14, 22, 23, 32, 33]. Despite numerous data on the morphology and structure of the OVLT, little is known as yet about the seasonal changes in the activity of this organ in amphibians.

The aim of the present study was an investigation of the activity of this organ in the annual cycle in *R. temporaria* L., a species with an interesting life cycle characterized by a change from aqueous environment during the period of hibernation and breeding period to terrestrial environment during active life.

Materials and methods

Thirty-five sexually mature male and 35 female *R. temporaria* L. frogs were studied in seven stages of the annual cycle, *i.e.:* IIIrd decade of January (middle of hibernation), Ist decade of March (end of hibernation), Ist decade of April (breeding period), IIIrd decade of May (active life), IInd decade of July (middle period of active life), Ist decade of September (end of the period of active life), and IIIrd decade of October (beginning of hibernation). These periods represent the most important phases in the life of amphibians in the annual cycle, and were adopted according to JUSZCZYK [16].

Frogs from each period were captured in the vicinity of Cracow (50°04 N, 200—220 m above sea level), five males and five females directly from their natural habitat, and killed in the laboratory by decapitation, always in the afternoon hours. The brains were fixed in Bouin fluid, imbedded in paraffin, and stained with GOMORI's chrome hematoxylin and floxin as modified by BARGMANN [27]. All sections from each brain were stained and changes in nuclear volume in the pars ependymalis and pars parenchymalis were determined (Fig. 1.). Nuclear volume was calculated by the equation of PALKOVITZ [26]: $V = \frac{\pi}{6} L B^2$, where L is the long axis, and B the short axis of the nucleus. This method was chosen because, as shown, changes in nuclear volume reflect cell activity [1, 2, 5, 20, 24, 30, 31]. Arithmetic means and standard deviation of the nuclear volumes were calculated. Significance of differences in nuclear volume in cells of the OVLT between males and females in different seasons were calculated from these values. Differences were considered significant at $P \leq 0.01$. The results are assembled in Table 1 and illustrated in Fig. 2.

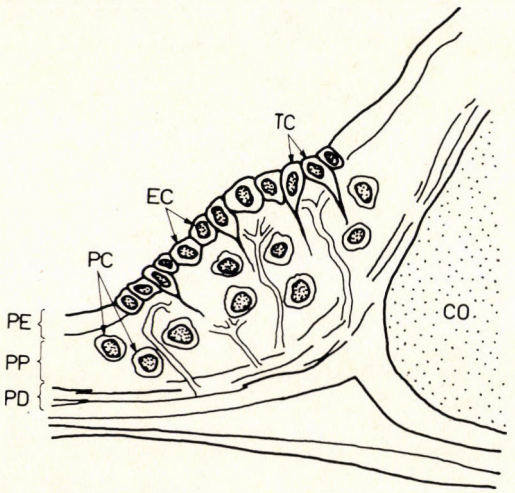

Fig. 1. Scheme of the organon vasculosum laminae terminalis in *Rana temporaria* L. according to GOOSSENS et al. (1973) and WENGER and TÖRK (1968), modified. EC = ependymal cells, TC = tanycytes, PC = parenchymal cells, PE = pars ependymalis, PD = pars duralis, PP = pars parenchymalis, CO = optic chiasma

Results

Pars ependymalis

The data pertaining to the pars ependymalis are assembled in Table 1 and illustrated in Fig. 2.

In females (136.464 μm^3) and males (136.864 μm^3) highest nuclear volume in this of the OVLT was observed in the breeding period (Ist decade of April), followed by a steady drop to minimum mean volumes in females (93.412 μm^3) and males (97.339 μm^3) at the beginning of hibernation (IIIrd decade of October), followed by a rise in nuclear volumes until the maximum mean volumes in the breeding period were reached.

Comparison of adjacent periods in females shows statistically significant differences only between the Ist decade of April and IIIrd decade of May; and

Table 1

Changes in mean nuclear volume of cells in the pars ependymalis and pars parenchymalis of the organon vasculosum laminae terminalis in the brain of Rana temporaria L. *in the annual cycle*

Group	Period investigated	Sex	Nuclear volume in cells of the pars ependymalis		Nuclear volume in cells of the pars parenchymalis	
			mean	± SD	mean	± SD
I	IIIrd decade of January	♀	104.978	± 11.37	147.006	± 6.55
		♂	100.239	± 7.11	122.634	± 10.17
II	Ist decade of March	♀	126.453	± 23.25	176.224	± 18.02
		♂	129.714	± 26.49	175.395	± 21.67
III	Ist decade of April	♀	136.464	± 28.92	189.783	± 37.57
		♂	136.864	± 11.73	200.411	± 9.00
IV	IIIrd decade of May	♀	104.902	± 16.05	172.200	± 10.63
		♂	111.513	± 9.74	165.940	± 14.09
V	IInd decade of July	♀	101.582	± 7.64	170.945	± 14.06
		♂	106.275	± 13.07	162.835	± 13.50
VI	Ist decade of September	♀	95.266	± 17.57	159.333	± 2.35
		♂	101.400	± 16.42	147.745	± 6.48*
VII	IIIrd decade of October	♀	93.412	± 15.13	175.509	± 15.76
		♂	97.339	± 7.55	175.524	± 17.79

Each group consisted of 5 males and 5 females
* $P < 0.01$ was compared with females of the same group

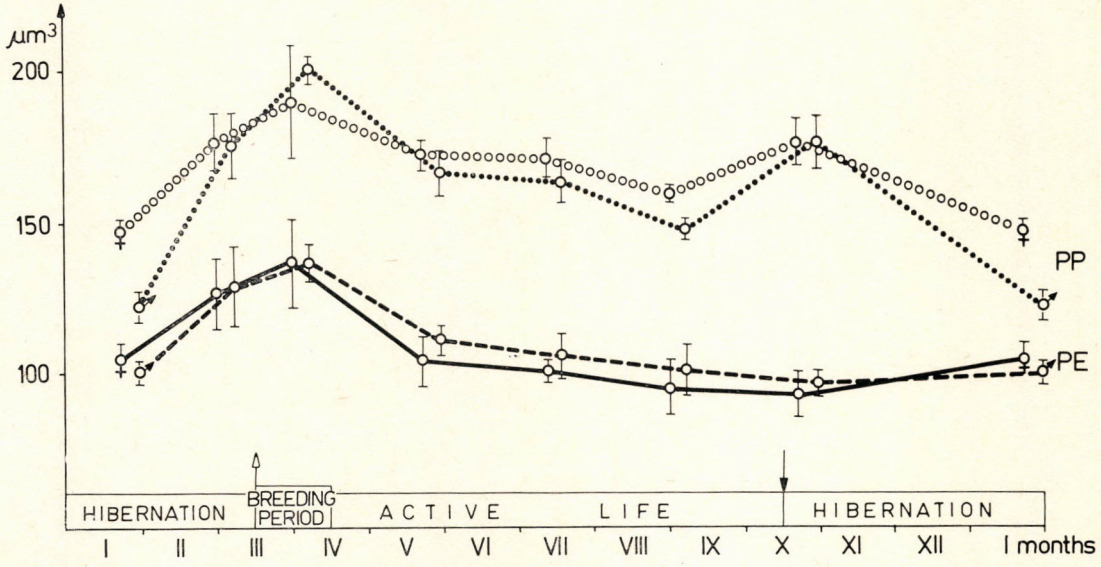

Fig. 2. Changes in nuclear volume in the cells of the pars ependymalis (PE) and pars parenchymalis (PP) of the organon vasculosum laminae terminalis in female and male *Rana temporaria* L. in the annual cycle. Arrow pointing upward — emergence from winter lairs. Arrow pointing downward — descent into winter lairs

in males between the IIIrd decade of January and Ist decade of March, and between the Ist decade of April and IIIrd decade of May. In the vast majority, nuclear volumes of ependymal cells were higher in males, than in females.

Pars parenchymalis

The data pertaining to the pars parenchymalis are assembled in Table 1 and Fig. 2.

Table 2

Statistical comparison of results (t) between different periods of investigation in the organon vasculosum laminae terminalis of Rana temporaria L. *in the annual cycle*

Period investigated	Pars ependymalis OVLT		Pars parenchymalis OVLT	
	♀	♂	♀	♂
IIIrd decade of January— Ist decade of March	4.15	5.33*	7.63*	11.04*
Ist decade of March— Ist decade of April	1.35	1.23	1.63	5.33*
Ist decade of April— IIIrd decade of May	4.78*	8.34*	2.26	9.12*
IIIrd decade of May— IInd decade of July	0.94	1.61	0.36	0.80
IInd decade of July— Ist decade of September	1.51	1.16	4.06	4.93*
Ist decade of September— IIIrd decade of October	0.37	1.12	5.05*	7.25*
IIIrd decade of October— IIIrd decade of January	3.06	1.35	8.36*	12.93*

* Statistically significant at $P < 0.01$

The highest mean nuclear volume in perenchymal cells in females (189.783 μm^3) and males (200.411 μm^3) were observed in the breeding period (Ist decade of April) and the lowest values in females (159.333 μm^3) and males (147.745 μm^3) in the last phase of active life (Ist decade of September). From then onward a slight increase in nuclear volume occurred in both sexes in the beginning of hibernation (IIIrd decade of October), followed by a drop in the middle of hibernation (IIIrd decade of January) and a renewed rise took place until the maximal mean values were attained in the breeding period (Ist decade of April).

Comparison of adjacent periods showed statistically significant differences in females between the IIIrd decade of January and Ist decade of March, between the Ist decade of September and IIIrd decade of October, and between the IIIrd decade of October and IIIrd decade of January; and in males the differences were statistically nonsignificant, except between the IIIrd decade of May and IInd decade of July.

Discussion

It is apparent from the findings that the mean nuclear volume in OVLT cells in *Rana temporaria* L. undergoes seasonal fluctuation. Changes were observed in the mean nuclear volumes of ependymal and parenchymal cells. A particularly pronounced rise of the mean nuclear volumes of the cells was observed during the breeding period (Ist decade of April). This was followed by a gradual decrease in the activity of the organ, lasting until the end of the active period of life (Ist decade of September) in case of pars parenchymalis and until the initial period of hibernation (IIIrd decade of October) in case of pars ependymalis. After beginning of hibernation, the pars ependymalis increases in activity until a maximum in the breeding period. On the other hand, the activity of the pars parenchymalis increases slightly at the beginning of hibernation, decreases again in the middle of hibernation to the minimum yearly values, and then increases suddenly until a maximum is reached in the breeding period. The course of the changes of maximum and minimum values in pars parenchymalis and in pars ependymalis are similar in both sexes.

Because of the structural system of this organ and its rich vascularity, it is supposed to play part in formation of the CSF-blood barrier [14, 22, 23, 33], and direct exchange of components between blood and the CSF is thought to take place in this organ [34], without excluding a receptor function of the organ, particularly osmo-chemo- and thermoreceptor. These assumptions, however, are difficult to prove because the data about the nervous connections of this organ with the hypothalamus and other parts of the brain are incomplete. In the light of these findings and the above-mentioned suppositions, it may be concluded that an intense exchange of components between blood and CSF in the OVLT occurs during the breeding period, and is minimal in the end of the period of active life, but the activity of pars parenchymalis increases at the beginning of hibernation (IIIrd decade of October), too, when *Rana temporaria* changes its habitat from land to water, which is connected, among others, with the change of osmotic and temperature conditions.

It is of interest that GOOSSENS et al. [13] found cells containing catecholamines and monoaminergic fibres in this organ in *Rana temporaria* L., *i.e.* fibres which play an essential role in regulation of the anterior pituitary lobe

by releasing hypothalamic hypophysiotropic hormones from the neurosecretory endings to the vascular portal system [18]. These factors are produced in the nucleus infundibularis ventralis in amphibians [9, 10] and take part in regulation of gonadotropic activity in amphibians. The work of SREBRO et al., [29] has shown that the nucleus infundibularis ventralis in *Rana esculenta* L. shows the highest activity during the breeding period, *i.e.* at the time when OVLT activity is also the highest. In rhesus monkeys ZIMMERMAN and ANTUNES [38] found OVLT Gn-RH (gonadotropic realesing hormone) as well as vasopressin and oxytocin. Recent studies by PELLETIER et al. [28] confirm the presence of LH-RH in OVLT in rats, besides earlier studies by ZIMMERMAN et al. [37], DUBE et al. [11]. If one assumes that LH-RH is produced in this organ in amphibians, then its high activity in the breeding period would indicate that it takes part in regulating ovulation. According to PELLETIER et al. [28], participation in the regulation of ovulation is based on release of LH-RH from this organ into the circulation. A further argument for participation of OVLT in reproductive processes is that in amphibians the OVLT and the subfornical organ are morphologically similar [8]. Moreover, the highest activity in the subfornical organ was shown in *Rana esculenta* L. in the same period of the year [12, 21]. According to these authors, the subfornical organ is in some way involved in reproductive processes in amphibians. In view of the morphological similarity and similar activity in the annual cycle of the OVLT and subfornical organ, it is reasonable to assume that they have a similar physiological function, namely, regulation of gonadotropic activity.

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CELL WALL CHARACTERISTICS OF *PSEUDOMONAS AERUGINOSA* AND ITS CARBENICILLIN-INDUCED L-FORM

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Abstract

L-forms of *Pseudomonas aeruginosa* were induced and cultured on a medium supplemented with carbenicillin. Morphological studies of the passaged variant revealed the presence of a triple-layered cell wall similar to that found in the parent species. Furthermore, the L-form was found to be more susceptible to gentamicin, kanamycin, tetracycline and colistin sulphate. Chemical analysis of the lipopolysaccharide fraction showed a difference in phosphorus content, and changes in cell wall envelope fatty acid content were also exhibited. It is suggested that these differences may influence the transport of certain antibiotics through the cell wall.

Introduction

Successful chemotherapeutic treatment of a number of bacteraemias associated with *Pseudomonas aeruginosa* [5, 17] have been obtained using the semi-synthetic penicillin, carbenicillin (disodium α-carboxyl benzyl penicillin). Unfortunately, such therapy has been shadowed by the emergence of carbenicillin resistant strains [16]. Moreover, morphological studies have recognised these variant species as typical L-forms [12]. L-forms of *Pseudomonas aeruginosa* have been produced *in vitro* by continued passage of the micro-organism in the presence of carbenicillin [2]. Since relatively little is known of the cell wall structure of L-forms, this study was undertaken to compare ultrastructural and certain chemical characteristics of the native bacterium with carbenicillin-induced L-forms.

Materials and methods

Pseudomonas aeruginosa, serotype 6 (NP6) was isolated at the Department of Pathology, Radcliffe Infirmary, Oxford. The organism was grown at 37 °C in a medium consisting of: 3.75% brain-heart infusion broth (Oxoid Ltd) 3.0% NaCl supplemented with 10% horse serum as an osmotic stabiliser. L-forms were induced by culturing NP6 in the above medium supplemented with carbenicillin, up to 200 μg/ml. Bacteria were harvested during the exponential phase by centrifugation at 5000 × *g*.

Antibiotic Sensitivity and Pigment Production

Inoculations from NP6 and P6 cultures were evenly spread on an agar surface and Multodisks (Oxoid Ltd) containing different combinations and concentrations of antibiotics were applied to the agar surface. Zones of inhibition were compared after a 24 h incubation period at 37 °C. Fluorescein and pyocyanin pigment production were determined as described previously [12].

Cell Wall and Lipopolysaccharide Isolation

Suspensions of washed bacteria were treated with ultrasonics and cell walls isolated by differential centrifugation [19]. The lipopolysaccharide moiety of both NP6 and P6 species was isolated by suspending samples (about 25 mg) in 46% aqueous phenol and heating to 70 °C for 15 min. The aqueous layer was removed and the lipid A fraction was removed by hydrolysis with 1% acetic acid [10].

Analytical Procedures

For hexosamine estimation, samples were hydrolyzed in constant-boiling HCl (1 mg/ml) at 100 °C under nitrogen for various time intervals and total hexosamine determined by a distillation procedure [6]. Lipopolysaccharide samples (0.2 ml) were heated with concentrated H_2SO_4 (5 ml) at 100 °C for 15 min. Phosphorus, glucose, heptose and rhamnose concentrations were determined by colorimetric procedures [1, 9, 14]. Lipids were extracted from the cell wall fraction by first hydrolyzing cell wall samples (approx. 50 mg) with 5 ml constant-boiling HCl at 100 °C for 4 h under vacuum. Lipid-containing material was removed by repeated extraction with ether. Dried ether extracts were saponified in 5% ethanolic KOH for 40 min. at 100 °C and the solution adjusted to pH 2, with HCl. Freed fatty acids were then extracted with petroleum ether and dried in a slow stream of nitrogen to prevent oxidation. Methylated fatty acid derivatives were obtained using a diazomethane reagent which was generated by mixing equal volumes of 80% KOH in ether with 1 mg/ml N-methyl-N-nitrosurea solution. The methyl esters were determined by gas liquid chromatography on a 20% diethylene glycol succinate column containing Chromosorb N HMDS (80—100 mesh, Perkin Elmer F II).

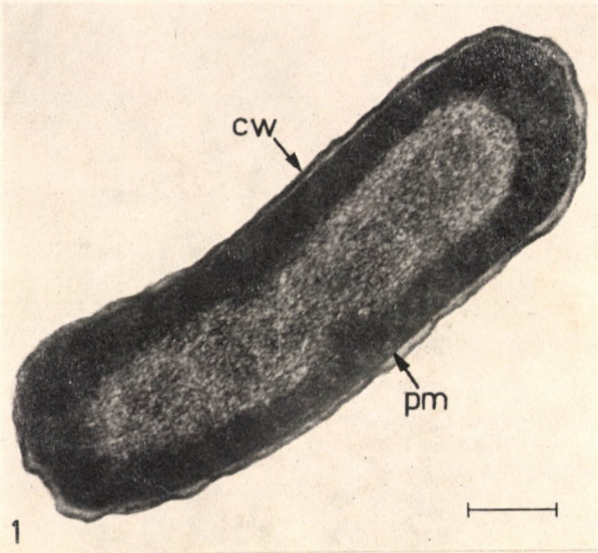
Electron Microscopy

The bacteria were processed [13] and after embedding in Araldite, ultrathin sections were cut on an LKB Ultrotome III, and collected on uncoated grids. They were stained with uranyl acetate (2% in 40% ethanol) and lead citrate [15]. Photographs were taken at set magnifications using a Phillips EM301 electron microscope.

Results

The native (NP6) form of *Pseudomonas aeruginosa* (Figs 1 and 2) shows the typical cylindrical spheroid shape, whereas the L-form (Fig. 2) prepared during this study had a larger cell volume (approximately five-fold that of the native bacterium). It is also apparent from our electron micrographs that both the cell membrane and cell wall constituents are present in the native and L-form variant of *Pseudomonas aeruginosa*.

Antibiotic sensitivity tests on a range of reagents demonstrated that the L-form was more sensitive to gentamicin, kanamycin, tetracycline and colistin sulphate (Table 1). Resistance to ampicillin, cephaloridine, nalidixic acid, nitrofurantoin and sulphafurazole was exhibited by both NP6 and P6 forms. Furthermore, inclusion of inactivated horse serum in the medium, which is essential for growth of the L-form, had no observable effect on the antibiotic sensitivity.



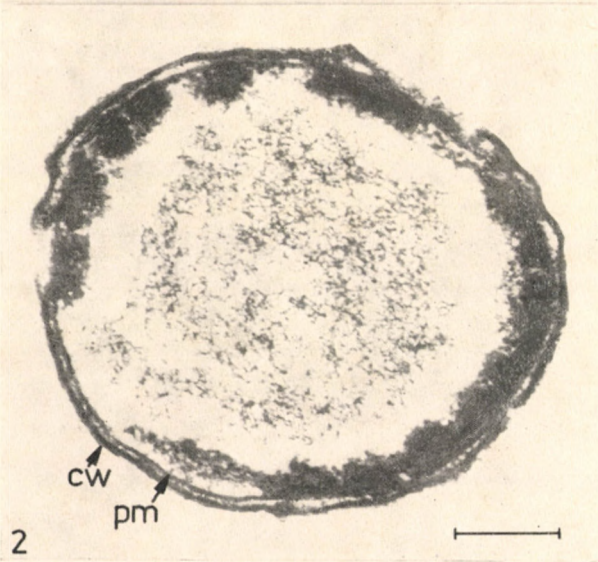


Fig. 1. Pseudomonas aeruginosa parent (NP6) form. The external triple layer of the cell wall (cw) is typical of gram-negative organisms. The inner plasma membrane (pm) can also be seen. Calibration bar = 0.2 μm

Fig. 2. Pseudomonas aeruginosa L-form. Triple-layered cell wall and plasma membrane are visible. Calibration bar = 0.2 μm

Table 1

Comparison of sensitivities of NP6 and the P6 variant form of Pseudomonas aeruginosa to a range of antibiotics

	Antibiotic				
	Carbenicillin	Colistin sulphate	Gentamicin	Kanamycin	Tetracycline
Disc. conc. (μg)	100	10	10	30	50
Diameter of Inhibition zone (mm) NP6	8 ± 1.0 **	4 ± 0.6 **	4 ± 0.5 **	0 **	3 ± 0.4 **
P6	0	9 ± 1.1	11 ± 1.4	8 ± 0.9	9 ± 1.0

* No observable differences in overall zones of inhibition were obtained after a further 24 h incubation, apart from tetracycline which showed a 10% increase in sensitivity.
The values given are the means and standard errors of the means (S.E.M.), $n = 10$.
** $P < 0.001$.

Agar cultures of the induced variant did show some colour variation for fluorescein and pyocyanin pigment formation. Absorption maxima of 475 nm for NP6 and 498 nm for P6 were observed when the organisms were grown on pyocyanin producing media. Studies with fluorescein showed that NP6 produced a pigment at 570 nm whereas the pigment produced by the variant had a maximum absorption at 580 nm.

Total lipid contents of NP6 and P6 were found to make up 25.2% and 24.8% of the dry cell wall fraction respectively. The fatty acid composition of the methyl ester using diazomethane is shown (Fig. 3). With the exception of C_{11}, C_{20} and C_{22} chain length fatty acids, both passaged and non-passaged species appeared to have similar fatty acid components. However, the relative concentrations of individual fatty acids did vary between the two bacterial forms. The majority of fatty acids (78%) associated with the native species were of the C_8, C_{10} and C_{14} type, while C_{10}, C_{14} and C_{16} fatty acids made up 84% of the passaged variant.

Table 2

Composition of the major components of lipid-free lipopolysaccharide

	Glucose	Hexosamine (Glucosamine and Galactosamine)	Rhamnose	Heptose	Phosphorus
NP6	10.2 ± 1.2	1.2 ± 0.2	5.3 ± 0.6	1.1 ± 0.1	3.7 ± 0.3
P6	10.4 ± 1.4	1.2 ± 0.1	5.1 ± 0.4	1.0 ± 0.1	6.7 ± 0.5

Results are expressed as mean percentages (± S.E.M.) of lipopolysaccharide ($n = 10$) and have been extrapolated to zero time of hydrolysis.
* Glucosamine and galactosamine were separated on an amino acid analyzer.

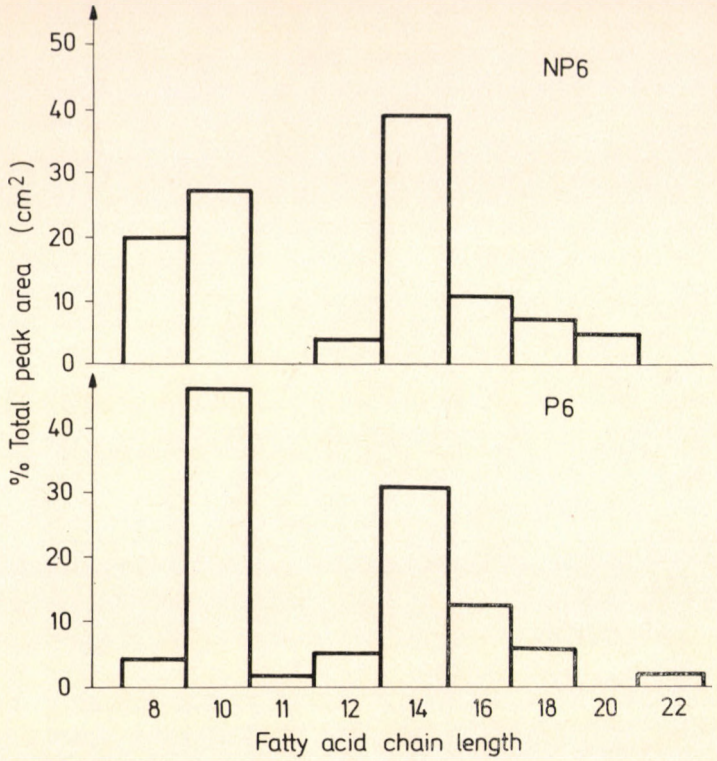


Fig. 3. Fatty acid composition of the cell wall envelope fraction of NP6 and P6

The major components of the lipopolysaccharide fraction for both bacterial types are reported (Table 2). Individual carbohydrates and amino sugars were estimated after they had been released into solution by acid hydrolysis. Optimum values for both the NP6 and P6 forms were obtained after hydrolyzing for 9 h. A 70% decrease in total carbohydrate content was observed after a 24h acid treatment. Both the passaged and non-passaged bacteria showed little variation in chemical composition apart from a higher phosphorus content exhibited by the P6 fraction.

Discussion

Gross structural changes in the morphology of bacterial L-forms have been reported. However, depending on the bacterial species and mode of induction, interpretations of such changes have been questioned [8]. A previous study on a carbenicillin-induced L-form *Pseudomonas aeruginosa* has suggested

that at least six morphological changes are evident [12]. These workers found that one parent bacterial form has both cell wall and plasma membrane triple layers, the L-form being devoid of all cell wall components. In this study both NP6 and P6 contained a triple-layered cell wall structure, differences in cell wall morphology may be attributed to variation within carbenicillin-induced L-form species. It could be postulated that, as the changes in cell wall structure occur by the continuing passaging with carbenicillin, a point is reached where the repair mechanism necessary for complete reversion back to the native species is not possible. At this stage the bacterium is thus committed to the new form which need not involve a complete loss of cell wall material.

Carbenicillin, like all other penicillins, interferes with bacterial cell wall peptidoglycan synthesis. The final stage of complete peptidoglycan synthesis involves a cross-linking reaction with the removal of a terminal D-alanine residue [20]. However, the existence of a number of penicillin-binding type proteins within the bacterium may be an alternative mechanism to that previously outlined [11].

Recent studies in our laboratory (BRANFORD WHITE, personal observations) have shown little alteration in the chemical composition of this fraction in the native and L-form species. It would appear that changes in the macromolecular organisation of this matrix could contribute to one of the factors influencing the overall changes in morphology as observed in the P6 bacterium.

Analyses of the lipid content of *Pseudomonas aeruginosa* have been previously attempted [3], After extraction with ether and acetone, the extract accounted for about 16% of the total dry weight of cell walls. Furthermore, C_{16} and C_{18} chain length fatty acids were found to be the most abundant with relatively low yields of C_{10}, C_{12} and C_{18} fatty acids. Since our analysis was done on the total cell wall envelope which would include possible contaminants from inner (cytoplasmic) and outer membranes, care must be taken in making a too exacting comparison at this time.

It has been shown that treatment of *Pseudomonas aeruginosa* with chelating agents releases lipopolysaccharides from cell walls, and this can potentiate the action of many antibacterial agents [18]. In addition, isolation of a mutant strain of *Pseudomonas aeruginosa* lacking a lipopolysaccharide polymer containing mannose, rhamose and glucosamine was found to be particularly sensitive to certain antibiotics [10].

The major components of the lipopolysaccharide moiety for the passaged and non-passaged variant are consistent to those for other serotypes of *Pseudomonas aeruginosa* [7]. *Pseudomonas* species containing relatively high concentrations of phosphorus associated with the cell wall were found to be susceptible to antibiotics [4]. In this study, the L-form variant was found to contain more phosphorus-containing material in the lipopolysaccharide fraction, which may account for an increase in antibiotic sensitivity.

In conclusion this study has shown that formation of a stable L-form species by passaging with carbenicillin not only affects the overall morphology of the organism without apparent removal of the cell wall, but also induces some changes in the chemical composition of the cell wall. Such changes in structure, either morphological or chemically induced, appear to influence certain transport phenomenon through the cell wall envelope.

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ON THE INTRACEREBRAL LOCUS FOR ANDROGENIZATION OF FEMALE RATS

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Abstract

Comparison of control and androgenized rats has revealed that many nerve terminals ending on arcuate nerve cells of rats treated neonatally with testosterone phenylpropionate show a decrease in dense-core vesicles, and an increase in the number of clear vesicles and of vesicles that exhibit various degrees of electron density. The same changes were observed in nerve terminals ending on preoptic neurons of rats androgenized neonatally. These findings are discussed in view of the intracerebral locus for androgenization of female rats.

Introduction

BARRACLOUGH and GORSKI [1] were the first to suggest that the preoptic LH trigger was deranged in the androgen-sterilized female rat. Oestrogen-sensitive preoptic and anterior hypothalamic neurons seemed to be indispensable in the neurohormonal feedback mechanisms controlling the secretion of gonadotrophic hormones (GTH) [6]. Inactivity, or at least reduced functional capacity, of these neurons was the result of the early postnatal androgen action both in male [18] and female [19] rats. Reduced oestradiol-binding capacity of the preoptic and anterior hypothalamic oestrogen-sensitive neurons following early postnatal androgen treatment was found to account for the inactivity or reduced functional capacity of these neurons [8, 9]. It was close at hand to assume, therefore, that the loss of hypothalamic neurohormonal feedback might account for the establishment of the masculine, *i.e.* non-cyclic and non-ovulatory, pattern of gonadotrophin secretion in the neonatally androgenized rat [7].

On the other hand, significant structural changes of the preoptic-anterior hypothalamic area (PAHA) were demonstrated in neonatally-castrated and androgen-treated male rats [4]. Furthermore, a relation between the action of steroid hormones and the variation of the average cell nuclear size in the medial preoptic nucleus during prepubertal life was assumed from the comparison of cell-nuclear volume in neonatally castrated males, intact females, androgen-

ized females and intact male rats [3]. MARTYN [15] used a Quantimet 720 image analyzing computer to measure the cross-sectional areas of cell nuclear profiles from selected parts of the rat brain. The frequency distribution of these areas was found to be very similar in the male and female brain regions studied. In the medial preoptic area and in the suprachiasmatic nucleus, however, the frequency distribution of the nuclear areas of the androgenized rat was markedly different. RAISMAN and FIELD [21] found sex differences in the mode of termination of the afferent fibres to the preoptic area. It is possible, therefore that the functional differences in the brain-cycle mechanism between male and female rats, especially those of the preoptic LH trigger, may be due in part to some difference in the neural connection with limbic afferent fibres. It has recently been reported by GORSKI *et al.* [10] that in rats there exists a gross sex difference in the volume of a darkly-staining cellular group within the medial preoptic area. All these suggest that the primary site of action of the neonatal testosterone would be the PAHA.

NADLER [16] found that development of the anovulatory, persistent vaginal cornification syndrome (PVCS) occurred most consistently following implantation of testosterone propionate (TP) micropellets in the ventromedial-arcuate nucleus (VM-ARC) area of the rat brain. Furthermore, implants in the same area were associated with the earliest ages at onset of PVCS. On the basis of the above findings, it has been assumed by NADLER [16] that the primary action of early androgenic stimulation in the rat, with respect to sexual differentiation of gonadotrophin regulation, occurs at the VM-ARC area of the brain rather than in the PAHA. This assumption seemed to be supported by the observation of RATNER and ADAMO [21], who found that nerve terminals ending on arcuate neurons in rats treated with TP early postnatally exhibited a decrease in dense-core vesicles (700—1200 Å), an increase in the number of clear vesicles, and of vesicles that exhibited various degrees of electron density. Since the larger clear vesicles were similar in size to the dense-core vesicles seen in control rats, it is believed that the larger clear vesicles might represent granulated vesicles that had lost their dense core. The results suggest that the nature of the vesicles in nerve terminals ending on arcuate neurons is modified after neonatal androgen treatment. The possibility exists that alteration in the vesicles of axonal endings occurs in terminals from neurons within the arcuate-ventromedial area itself. If so, the alteration of the vesicles in neonatally-androgenized rats may support the assumption of an arcuate-ventromedial locus for androgenization. Alternatively, as mentioned by RATNER and ADAMO [21], the changes in the axonal endings seen in the arcuate nucleus might be a reflexion of changes that occur in another area that has fibre connections with the arcuate nucleus, since no neuronal changes were observed by them within the arcuate nucleus itself. Such an area may be the medial preoptic area (MPA) which has been implicated as the area involved in

the triggering of ovulation [2, 5, 12, 14] and indicated to be the locus of action of early postnatal androgen treatment [1]. Since RATNER and ADAMO [22] did not investigate this area, we decided to explore the MPA of androgen-sterilized rats under the electron microscope.

Material and methods

Newborn female rats from our inbred strain, derived from a Wistar stock, received a single injection of 1250 μg of testosterone phenylpropionate (Retandrol, Gedeon Richter Chemical Works, Budapest) dissolved in 0,05 ml sesame oil two days after birth. Littermate control rats recived 0.05 ml sesame oil at the same age. All rats were maintained in a light (14 h light and 10 h dark) and temperature-controlled room, and were fed a standard pellet diet after weaning. Besides 6 control rats, having normal oestrous cycles of four days, we employed for electron microscopy only androgenized rats which revealed persistent vaginal cornification at least for a period of one month before killing, and had polyfollicular ovaries without corpus luteum at killing. Control and androgenized rats were anesthetized with hexobarbital (VEB Arzeneimittelwerk, Dresden; 100 mg/kg body weight) at 100 days of age, and perfused with a fixative containing 1% paraformaldehyde, 1% glutaraldehyde, % polyvinylpyrrolidone in 0,1 *M* phosphate buffer (pH 7.4 at 20 °C). After a perfusion of 10 min, the animals were killed by decapitation, the brains were removed, and the areas of brains to be studied (arcuate nucleus and medial preoptic area) were removed as small blocks and kept in the fixative for 2 h. Then the blocks were washed with 0.1 *M* phosphate buffer and post-fixed in 1% phosphate-buffered osmic acid solution for 2 h. After washing with 0.1 *M* phosphate buffer, the blocks were dehydrated in alcohol series and embedded in Durcupan (Fluka). Sections were cut with the LKB Ultramicrotome III. Semithick sections were stained with toluidine blue, the ultrathin sections were contrasted with uranyl acetate and lead citrate. The brain areas to be studied from six control and six androgenized rats were examined with a TESLA BS 242 electron microscope.

Results and discussion

In accordance with the findings of RATNER and ADAMO [22], comparison of control and androgenized rats revealed that many nerve terminals ending on arcuate nerve cells of rats treated neonatally with TP showed a decrease in dense-core vesicles, and an increase in the number of clear vesicles and of vesicles that exhibited various degrees of electron density (Fig. 1a and b). The same changes were observed in nerve terminals ending on preoptic neurons of rats androgenized neonatally (Fig. 1c and d). This finding indicates that alteration of the vesicles in the nerve terminals of androgenized rats described by RATNER and ADAMO [22] is not characteristic exclusively of the terminals ending on arcuate neurons. Consequently, this alteration cannot be considered a structural evidence supporting the idea that the VM-ARC area of the rat brain would be the site of the primary action of early androgenic stimulation in the rat with respect to sexual differentiation of gonadotrophin regulation. Furthermore, it is also possible that the vesicular changes in certain nerve endings of androgen-sterilized rats occur as a result of an indirect effect of persistent oestrogen action on the brain as it has already been stressed by RATNER and ADAMO [22], and not as a direct effect of the neonatal testosterone.

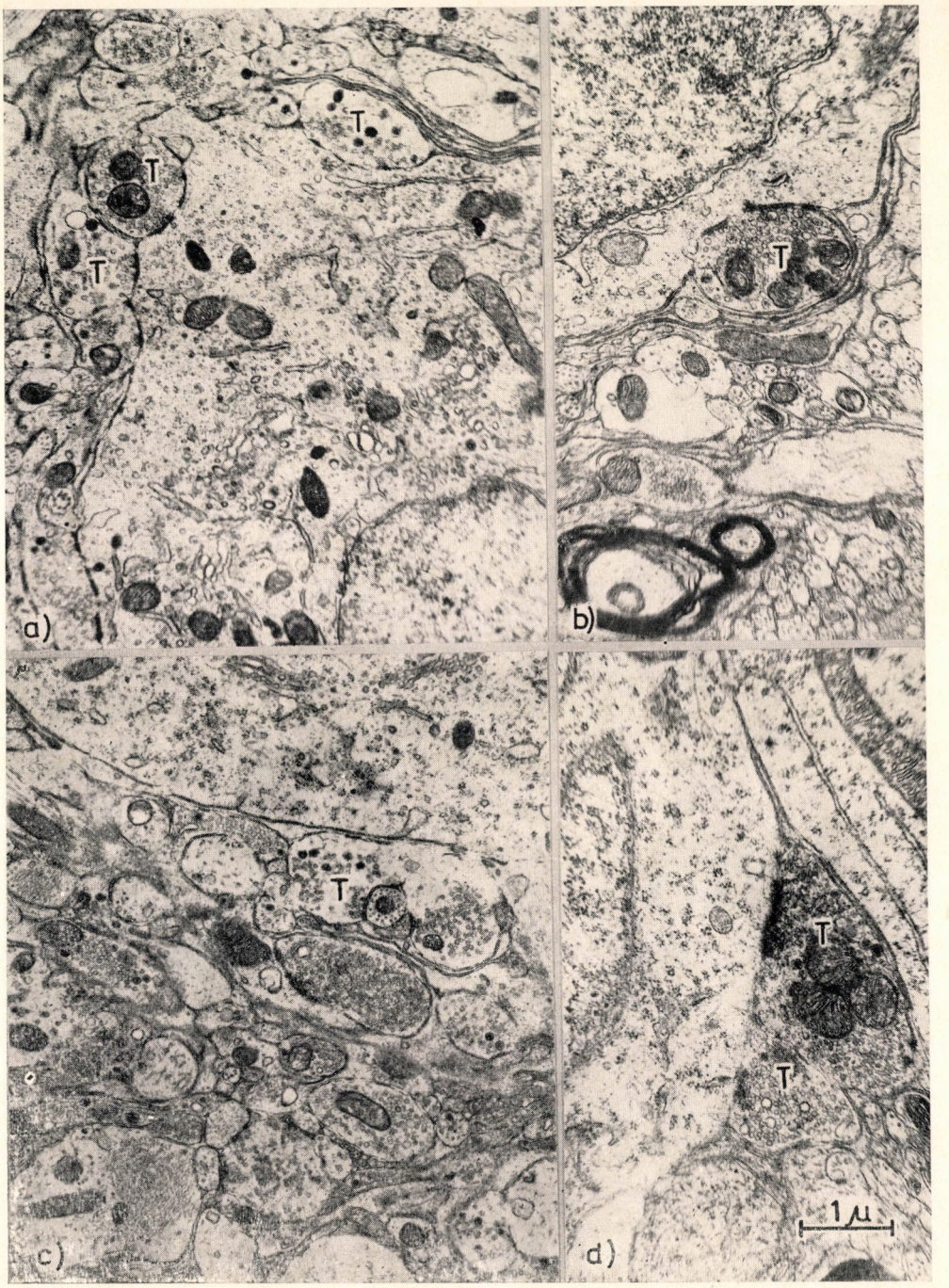

Fig. 1. Dense-core and small clear vesicles in an axon terminal (T) in the (a) arcuate and in the (c) medial preoptic nucleus of a control rat. × 12,500. Axon terminal (T) in the (b) arcuate and (d) medial preoptic nucleus of a TP-treated rat. The terminals contain small and larger clear vesicles and a few larger vesicles that exhibit various degrees of electron density. × 12,500

NADLER [17] could be correct in suggesting that it is equally plausible to describe the androgen-induced PVCS in terms of a relatively refractory condition of the luteinizing hormone-releasing hormone-(LH-RH)-producing neurons, the system that responds to the stimulation of the LH trigger. It is conceivable that, when the LH-RH synthesizing or releasing capacity of the LH-RH neuron system is reduced by the local neonatal testosterone action, the LH-trigger cannot release LH-RH and, hence, LH sufficient in amount to induce ovulation. However, it would be more difficult to accept NADLER's [17] argument that the neural locus, which is sexually differentiated in the rat with respect to the control of gonadotrophin secretion, would be in the vicinity of the arcuate nucleus rather than in that of the PAHA.

The axons of the LH-RH-producing neurons are very densely-packed in the periventricular and especially in the suprachiasmatic-arcuate region and in the median eminence [23, 24]. Hence, a considerable part of the nerve fibres of the LH-RH neuron system are exposed to the action of TP micropellets located in the VM-ARC area [16, 17]. In contrast, the oestrogen-sensitive neurons contributing to the LH-trigger mechanism are diffusely scattered in the PAHA, as revealed by radioautographic studies [20, 25]. It appears very likely, therefore, that only a very limited number of the neurons contributing to the LH-trigger were exposed to testosterone action when NADLER's TP micropellets were located in the PAHA. This may explain the failure to induce PVCS with small TP micropellets implanted into this area. However, when testosterone was infused into the PAHA [26] or testosterone micropellets larger than those used by NADLER [16] were implanted in the same region [13], testosterone reached a considerable number of the neurons of the LH-trigger and was successful in inducing PVCS by local action exerted in the PAHA. The situation appears to be analogous to that described by HILLARP [11] in connection with electrolytic lesions inducing PVCS in the rat. Whereas very small, bilateral, basal lesions between the paraventricular nuclei and the oral part of the pituitary stalk invariably induced the PVCS in rats, the same syndrome could be produced only by very large lesions in the PAHA immediately anterior and ventral to the paraventricular nuclei.

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COMPACT ANISOTROPIC BONE: ELASTIC CONSTANTS, *IN VITRO* AGING EFFECTS AND NUMERICAL RESULTS OF A MATHEMATICAL MODEL

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Abstract

This paper briefly examines the experimentally observed effects in, and symmetry arguments for, the constituents of bone which lead to a transversely isotropic configuration for the elastic behavior of bone. Based on this, a regime for computing the anisotropic elastic constants is considered in terms of ultrasonic transit time measurements. To extend the usefulness of ultrasonic techniques to an *in vivo* situation, a mathematical model for bone is developed in outline form and numerical results for the characteristic frequency equation discussed.

Introduction

The mechanical properties of bone have been a subject of scientific investigation for over a hundred and thirty years, but only in 1954 did DEMPSTER and LIDDICOAT [1] reveal that compact bone is anisotropic in nature. This aspect of bone behavior has, however, been either overlooked or ignored in most studies since then and few papers have attempted to establish an effective crystal structure for bone in order to study the nature of anisotropy in bone. To date, the study by LANG [7] on the ultrasonic determination of the anisotropic elastic constants of bone considering it as a hexagonal material, has been the only definitive study of this aspect. A few recent papers have examined piezoelectric effects in bone [5, 9, 10]. The rather extensive studies reported by EVANS [2] and YAMADA [12] are not appropriate to the work described in this paper. Since the ultimate aim of most studies on bone properties is to be able to measure such properties *in vivo*, it appears that non-destructive low-intensity ultrasound methods will continue to be a major form of testing on bone both *in vivo* and *in vitro*. A few of the benefits that might accrue from such an approach include: *a)* a fuller understanding of bone ultrastructure in terms of its individual constituents, *b)* the development of prosthetic materials with a better mechanical match to bone than those currently employed and

c) a quantification of studies on bone fractures, fracture healing and pathological bone disorders.

In the higher vertebrates, bone comprises 1) a protein framework of collagenous fibres forming the main bulk of bona, 2) the crystalline bone mineral, mostly calcium hydroxyapatite, which gives strength to the bone matrix, 3) the ground substance (cement), an apparently structureless material, which envelops and permeates the collagenous matrix and 4) the osteocytes (the bone inhabiting cells) each of which occupies its own cavity or lacuna within the matrix. Compact bone tissue encloses only sparse, small vascular channels while spongy bone contains large vascular spaces with an aggregate volume equalling or even exceeding that of the bone matrix itself. A typical long bone may be considered to be a thick-walled, two-layered cylinder made up of a thin layer of cancellous bone and a thick outer layer of compact bone. The spongy layer increases in thickness towards the flared ends of bone and is negligible toward the middle third portion (diaphysis).

Bone has been shown to be pyroelectric in nature [6]. Hydroxyapatite, which belongs to the crystallographic point group 6/m (a sixfold axis of symmetry perpendicular to a mirror plane) can, by virtue of its possessing a center of symmetry, exhibit neither the pyroelectric nor the piezoelectric effects. The mechanism producing pyroelectricity in bone might therefore originate in the organic phase of bone *i.e.* collagen. The collagen molecule displays both pyroelectric and piezoelectric effects. Pyroelectric behavior in collagen is associated with an electrical moment in the direction of the longitudinal axis of the collagen fibres which corresponds, more or less, to the long axis of bone itself. In a study on tendon, which is essentially pure collagen fibre, FUKADA and YASUDA [3] evaluated the piezoelectric matrix to be of the form

$$d_{ij} = \begin{bmatrix} 0 & 0 & 0 & d_{14} & d_{15} & 0 \\ 0 & 0 & 0 & d_{15} & -d_{14} & 0 \\ d_{31} & d_{31} & d_{33} & 0 & 0 & 0 \end{bmatrix} \qquad (1)$$

This form of the matrix is consistent with the linear texture of tendon and corresponds to the crystal class 6 — a hexagonal material. Collagen is a linear molecule and in both bone and tendon the collagen fibres lie in parallel along the long axis of bone. Since the molecules are randomly rotated about their threefold axis of symmetry, this produces a plane of transverse isotropy perpendicular to the axis. The hydroxyapatite crystals are closely packed, epitaxially overgrown formations, parallel to each other and lie along the collagen fibres. The resulting symmetry axis for bone, therefore, becomes infinitefold. The effective crystal structure of bone may thus be assigned the point group ∞/m, *i.e.* an infinitefold axis with a mirror plane normal to it. Thus, bone may be regarded as a transversely isotropic material, with the plane of isotropy perpendicular to the long axis of bone, and hence characterized be five inde-

pendent elastic constants, akin to a hexagonal material. The matrix of elastic constants of bone may be now denoted by:

$$c_{ij} = \begin{bmatrix} c_{12} & c_{11} & c_{13} & 0 & 0 & 0 \\ c_{13} & c_{13} & c_{33} & 0 & 0 & 0 \\ 0 & 0 & 0 & c_{44} & 0 & 0 \\ 0 & 0 & 0 & 0 & c_{44} & 0 \\ 0 & 0 & 0 & 0 & 0 & c_{66} \end{bmatrix} \tag{2}$$

where $c_{66} = \frac{1}{2}(c_{11} - c_{12})$.

Material and methods

Ultrasonic determination of the elastic constants: The propagation of acoustic waves entails the consideration of three distinct types of plane waves that can be transmitted in a medium. In isotropic solids these comprise a longitudinal wave with particle motion along the direction of propagation and two shear waves with particle motions perpendicular to the direction of propagation. In anisotropic media, no wave is strictly longitudinal or transverse, but, based on symmetry considerations, it is possible to extract special directions for which particle motions are in fact perpendicular to or along the direction of wave motion. Such directions form the basis for measurement of elastic constants in crystals. It must be emphasized here that (1) bone is not perfectly poly-crystalline and possesses imperfections to blur somewhat the crystallinity and (2) a definitive set of measurements of acoustic waves along different directions must ensure that an axis of symmetry is already known. These problems can be readily overcome by using diaphyses from long bones such as the tibia and femur, since these are regions of cortical bone where the structural uniformity, regularity and axial direction are well defined. Since testing must be accomplished in various directions and orientations with respect to the bone axis, rectangular parallelepipeds of bone cut along known directions are best suited for this purpose.

Knowing the measured shear and longitudinal wave velocities in the appropriate directions, the five elastic constants for bone may be evaluated through the following equations [8], where the direction (001) corresponds to the long axis of bone:

$$c_{33} = \varrho v_L^2 \tag{3}$$

where V_L is a longitudinal wave along (001)

$$c_{11} = \varrho V_T^2 \tag{4}$$

where V_T is a longitudinal wave perpendicular to the (001) axis

$$c_{44} = \varrho V_{S4}^2 \tag{5}$$

where V_{S4} is a shear wave along (001)

$$c_{12} = c_{11} - 2\varrho V_S^2 \tag{6}$$

where V_S is a shear wave perpendicular to the (001) axis

$$c_{13} = [(c_{11} + c_{44} - 2\varrho V_{qS}^2)(c_{44} + c_{33} - 2\varrho V_{qS}^2)]^{\frac{1}{2}} \tag{7}$$

where V_{qS} is a quasi-shear wave at an angle of 45° to the (001) axis. These equations indicate that longitudinal wave propagation will determine two of the elastic constants c_{33} and c_{11}, the rest being determined using shear waves. Further, the first four constants c_{33}, c_{11}, c_{44} and c_{12} may be measured on the same sample provided it is cut in the form of a rectangular parallelepiped as shown in Fig. 1. The last measurement requires a sample cut at an angle of 45° to the bone axis.

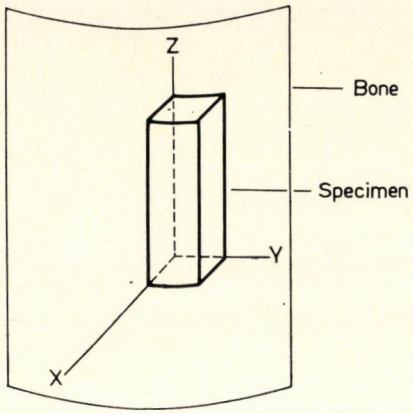

Fig. 1. Diagram showing location from which specimens were machined from bone and the coordinate system for measurement

Instrumentation: The two most commonly used techniques for the determination of the ultrasonic velocity of propagation are the transit time technique and the pulse echo method. In essence, both consist in the generation of short pulses of ultrasound in the test sample, using piezoelectric transducers, with subsequent measurement of the delay (propagation) time of these pulses through a fixed distance. The velocity of sound in the sample is obtained by dividing the measured value of the acoustic path length by the delay time through the sample.

For our measurements, we used a commercially available diagnostic unit, the Metrix Echo-Trace® II. This is an echo-ranging device which we modified so that separate transmitting and receiving transducers could be used. The Echo-Trace cathode-ray tube display was bypassed and connected, instead, to a Tektronix 564 B storage oscilloscope with expanded time base. The 564 B time base was calibrated against a time standard to insure ultrasonic pulse transit time measurements accurate to 0.1%.

Sample Preparation: Not many studies on the mechanical properties of bone have detailed the preparation methods for the samples used in such studies. To have a basis for comparison with the only previously published study by LANG [7] on anisotropic ultrasonically determined elastic constants, the samples were prepared in the manner implied by LANG.

Whole ovine and bovine tibiae and femora were obtained. Attached tissue was removed from these bones and the midshaft sawed away from the rest of the whole bone. Rectangular parallelepiped samples of the largest possible dimensions were machined from the midshaft, these dimensions being dictated by the fact that the bone had to be reasonably flat throughout this length and the thickness had to be as large and uniform as possible. Up to four samples could be obtained from one bone. The bone specimens were thoroughly degreased by setting them aside, prior to measurements, in trichloroethylene. The total time taken from the moment of obtaining the bones to that of initial testing was five to six weeks. Measurements to determine *in vitro* aging effects were made on the same samples after a period of eight to nine months. Shear wave measurements were also conducted on the aged specimens.

Method of measurement: Longitudinal wave measurements were made using Metrix UST-224 transducers. The diameter of the crystals was 1 cm and the operating frequency 2 MHz. The bone specimen was coupled to the transducer through K—Y Jelley which proved quite adequate as a coupling medium. Due to the viscous nature of K—Y Jelley it was possible, using slight pressure on either end, to have the specimen free of any lateral stresses during the course of measurement.

To make shear wave measurements, 1 cm^2 ceramic wafers, cut and polarized to emit shear waves at 2 MHz were used. Transducers were fabricated from the wafers by first coating the two surfaces with conducting silver paint to form the electrodes and then attaching the leads using conducting epoxy resin cured at room temperature. This method of fabrication was chosen because the Curie temperature of the piezoceramic was quite low and conventional soldering might have raised the temperature to a point where the polarization vanished. In the absence of coupling media commonly used for shear wave measurements such as Nonaq stopock grease, it was decided to use conducting epoxy itself as the coupling medium. This

gave good results, but due to the adhesive nature of this compound, the transducers had to be detached from the bone specimen by immersing in a solvent such as trichloroethylene, and a few transducers were damaged in the process. The number of shear wave measurements made was much less than the corresponding longitudinal wave measurements.

For each sample tested, the length or the appropriate dimension along which ultrasonic transmission took place was accurately measured. Density was determined by standard methods.

Results

Longitudinal wave measurements were made on more than a hundred samples. Of these, about half were used again after eight months and the velocities remeasured, to establish *in vitro* aging effects, if any. Table 1 shows

Table 1

Measured velocities for bone

Animal	Type	Age (mo.)	V_L(m s^{-1})	V_T(m s^{-1})
64	BF	18	3757	2610
324	BF	18	3769	2465
244	BF	18	3821	2691
H1	BF	36	3780	2467
H2	BF	36	3726	2430
H3	BF	36	3810	2428
S1	BF	36	3769	2435
S2	BF	36	3659	2534
S3	BF	36	3731	2503
S4	BF	36	3726	2531
E1	BF	36	3703	2501
E2	BF	36	3756	2593
E3	BF	36	3706	2531
64	BT	18	3580	2545
324	BT	18	3361	2908
344	BT	18	3308	2739
A1	OT	10	3251	1941
A2	OT	10	3343	1972
A3	OT	10	3322	1970
A4	OT	10	3381	1982
LANG's Values BF			4060	3420

The following abbreviations have been used:
BF: Bovine femur
OT: Ovine tibia
VT: Velocity perpendicular to axis
BT: Bovine tibia
VL: Velocity along axis

results of measurements of the velocities of longitudinal waves along the axis of bone and perpendicular to it, while Table 2 lists the computed values of the two elastic constants c_{33} and c_{11} using the measured densities of each specimen and the appropriate measured velocities. Each value is the average of up to four values recorded for up to four specimens taken from the same bone. Each set of measurements in one row corresponds to the animal whose identifying number appears in the first column.

Table 1 shows that the measured velocity in the longitudinal direction (V_L) is always greater than that measured perpendicular to it (V_T) and consequently c_{33} is always greater than c_{11} for bone. A comparison with the values obtained by LANG [7] for V_L of dried bovine femur (4,060 m sec^{-1}) indicates an average difference of about 8 per cent from those recorded in Table 1. There is, thus, fair agreement between the two, especially in view of the wide variability in results of many studies on bone properties. The difference in the average value of V_T for dried bovine femur is, however, 27 per cent and is probably significant. In this context the following facts need be mentioned. First, LANG's experiments were performed on animals of unspecified age. It is not clear if specimens of different age groups were mixed together. This factor is important because the age and diet of the animal can cause variations in the observed mechanical properties of bone. Second, LANG's measurements were made more than six months after the specimens were obtained. As he points out, during this interval, bone may have acquired a moisture content. It is questionable whether his samples of "dry" bovine femur can be regarded as perfectly dry or include a hydration content based on the environment in which the samples were stored for the six-month interval. This aspect involves two considerations. One is that moisture content can drastically alter the mechanical characteristics of bone. The other is the effect of *in vitro* aging on the properties of bone and it is shown later in this paper that aging does indeed produce a definite increase in the values of elastic constants. Another factor which must be considered where dynamic measurements are concerned is strain rate. LANG's measurements were conducted at a higher frequency than ours and consequently at a higher strain rate which would lead to increased values of both the measured velocities and the elastic constants. Even allowing for all these considerations, the difference of 27 per cent in the measured values of V_T between his results and ours is significant.

Table 2, which gives the values of c_{33} and c_{11}, shows average values for dried bovine femur as 2.94 and 1.31, respectively, all values being in 10^{10} N m^{-2}. These differ from LANG's figures by about 15 per cent and 45 per cent respectively. These percentage differences are almost twice as large as those for the velocities but this is not surprising due to the nature of the equations governing the relations between the c_{ij} and velocities which involve squares of the latter quantity.

Tables 1 and 2 also contain values of V_L, V_T, c_{33} and c_{11} for bovine and ovine tibiae. Values for these types have not been published in the literature and must therefore be considered to be the most representative to date. One published study on bovine tibia by SIMKIN and ROBIN [11] lists the YOUNG's Modulus of this type as 2.38 in tension. If this value is taken to imply the elastic modulus in the axial direction, *i.e.* c_{33}, it would differ from the average value of 2.28 of the present study by less than 5 percent. Though this percentage difference is small, it must be pointed out that such a comparison may not be valid since, to mention just one factor, SIMKIN and ROBIN used wet samples for their studies, which were conducted under static conditions.

From Table 2, it is observed that values of c_{33} for bovine tibiae are considerably lower than those for their femoral counterparts. This points to the fact that bovine tibia is mechanically weaker than bovine femur. On the other

Table 2

Densities and computed elastic constants for bone

Animal	Type	Density ($g\ cm^{-3}$)	$c_{33}(\times 10^{10} N\ m^{-2})$	$c_{11}(\times 10^{10} N\ m^{-2})$
64	BF	2.06	2.90	1.33
324	BF	2.04	2.91	1.25
344	BF	1.91	2.80	1.25
H1	BF	2.06	2.96	1.26
H2	BF	2.21	3.07	1.31
H3	BF	2.11	3.07	1.25
S1	BF	2.01	2.86	1.19
S2	BF	2.02	2.70	1.29
S3	BF	2.27	3.18	1.41
S4	BF	2.08	2.90	1.33
E1	BF	2.15	2.96	1.36
E2	BF	2.08	2.94	1.40
E3	BF	2.17	2.98	1.39
64	BT	1.95	2.51	1.41
324	BT	1.94	2.19	1.64
344	BT	2.02	2.15	1.54
A1	OT	1.91	2.04	0.72
A2	OT	1.79	1.99	0.69
A3	OT	1.88	2.03	0.73
A4	OT	2.13	2.06	0.77
LANG's values (BF)		2.02	3.34	2.38

BF, BT and OT stand for bovine femur, bovine tibia and ovine tibia respectively.

hand, values of c_{11} for bovine tibia are consistently larger compared to bovine femur. Whether this has any biological significance is not known.

One distinct fact that emerges from the results of Tables 1 and 2 is that the ratio of c_{33}/c_{11} is much greater than that for LANG's values. Since this ratio is also a measure of anisotropy, it leads to the important conclusion that bone may be much more anisotropic than previously believed.

As mentioned earlier, only a limited number of measurements were made for the determination of velocities of the shear waves in bone specimens. Results of these measurements are indicated in Table 3. The specimens used for measuring c_{13} were cut in the proper orientation from the original samples. Values of the elastic moduli c_{12}, c_{13} and c_{44} all fall within 20 per cent of LANG's values but it must be reemphasized that the measurements for shear waves here were performed on aged specimens.

Discussion

Only femoral specimens were used for measurements to study *in vitro* aging effects. Results of these measurements involving the velocities and computed elastic constants are listed in Tables 3 and 4. The results immediately reveal that there is a small but consistent increase (6.5 percent) in the values of c_{33} and a slightly greater increase (12 per cent) in those of c_{11} after aging. Since no changes in either the measurement technique or the experimental set-up were made for the aging experiments and since no other parameters *e.g.* temperature, were varied, the difference in the values of the original and aged values of the elastic constants clearly indicates that as the specimens are aged, they become mechanically stiffer. Further, the results of aging reduce the differences in c_{33} and c_{11} between the present measured values and those of LANG by nearly a factor of three and bring our values much closer to his.

It has been found that part of the bone mineral exists in amorphous form and replacement of this amorphous component with crystalline mineral takes place with age [4]. This implies that the mechanical properties of bone may

Table 3

Shear wave measurements

Sample	V_s(m s^{-1})	Elastic constant ($\times 10^{10}$N m^{-2})
F3	1773	$c_{44} = 0.66$
S4	1860	$c_{44} = 0.71$
64F	1810	$c_{12} = 0.86$
64F	1833	$c_{13} = 0.96$

change after death especially if all the amorphous phase gradually reverts to the crystalline form. This hypothesis would seem to be borne out by the present study. It also seems certain that these properties should stabilize after a long elapse of time.

Table 4

Regional variations in bone properties

Sample	Location	V_L (m s^{-1})	V_T (m s^{-1})	c_{33} ($\times 10^{10}$N m^{-2})	c_{11} ($\times 10^{10}$N m^{-2})	ϱ (g cm^{-3})
1	A	3796	2692	3.34	1.68	2.32
1	B	3596	2500	2.61	1.26	2.02
1	C	4008	2001	3.55	1.49	2.21
1	D	3684	2700	2.92	1.57	2.15
2	A	3833	2500	3.11	1.32	2.12
2	B	3889	2800	2.95	1.82	1.95
2	C	4067	2826	3.51	1.69	2.12
2	D	3903	2800	3.02	1.55	1.98
3	A	3936	2818	3.41	1.75	2.20
3	B	4014	2600	3.34	1.40	2.07
3	C	3935	2700	3.19	1.50	2.06
3	D	3894	2857	2.99	1.61	1.97
4	A	3472	2549	2.44	1.31	2.02
4	B	3462	2685	2.49	1.50	2.08
4	C	3688	2391	2.81	1.18	2.06
4	D	3416	2500	2.14	1.15	1.84

Table 5

Computed elastic constants for bone after aging

Animal	c_{33} ($\times 10^{-10}$N m^{-2})	c_{11} ($\times 10^{-10}$N m^{-2})
E1	3.20	1.49
E2	3.13	1.62
E3	3.25	1.64
H1	3.13	1.44
H2	3.29	1.45
H3	3.17	1.50
S1	2.98	1.20
S2	2.99	1.55
S3	3.34	1.63
S4	3.02	1.41

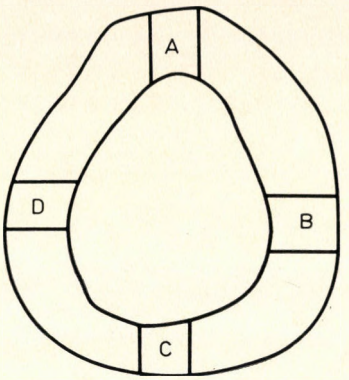

Fig. 2. Position of samples (in cross-section) in bone, used for measurements on regional variation of mechanical properties

Regional differences in the same bone: Figure 2 shows, in cross-section, the various locations from which samples were taken to conduct tests on regional differences in the elastic constants in the same bone. The results, listed in Table 6, reveal a variation in density, elastic constants and wave velocities

Table 6

Measured velocities for bone after aging

Animal	V_L (m s^{-1})	V_T (m s^{-1})
E1	3842	2628
E2	3877	2789
E3	3865	2748
H1	3893	2647
H2	3856	2594
H3	3869	2654
S1	3845	2440
S2	3853	2772
S3	3825	2684
S4	3794	2590

but do not lend themselves to any specific conclusions as to statistical or quantitative relationships between the position of the specimen in bone and its mechanical properties. In three of the four cases, however, location C is associated with the largest value of c_{33} and location A with the largest value of density. It is well known that bone deposition and absorption takes place in relation to the stresses induced. Such stresses would then contribute to denser

or stiffer portions on one face of bone and make the other face differ in its mechanical characteristics. To establish a quantitative relationship, if any, between induced stresses and regional variations, however, many more similar measurements must be performed.

To summarize, results of measured velocities of wave propagation in ovine and bovine femur and tibia and the computed values of the elastic constants from these velocities indicate that:

(1) Bone may be a much more anisotropic material than hitherto assumed.

(2) Aging *in vitro* has the effect of making bone mechanically stiffer.

(3) There are regional variations in the mechanical properties within the same bone, but no statistical relationships can be extracted.

Appendix

Numerical results of a mathematical model: If nondestructive or nontraumatic ultrasonic testing is to be applied to an *in vivo* situation it must be emphasized that such testing must be performed on whole bone and not on small samples cut in preferred orientations. A mathematical model was therefore developed to study the nature of wave propagation in *in vivo* bone considering it as an infinite two layered cylinder of transversely isotropic, piezoelectric materials. The model has been discussed elsewhere (AMBARDAR, 1976 unpublished) but some of the results of a numerical evaluation will be discussed here. It turns out that the characteristic frequency equation involves a sixteenth order determinant equalling zero. The nonzero terms of this determinant involve Bessel functions and their derivatives. The various parameters involved in the characteristic frequency equation include:

the frequency ω

the wavenumber α and hence the wave length L $(= 2\pi/\alpha)$

the dimensions of the model (outer radius r(2), inner radius r(1))

the elastic constants c_{ij}

the piezoelectric constant e_{ij}, and

the dielectric constants $\varkappa_{ij}$

The frequency equation is transcendental in nature and for each value of α there will be an infinite number of roots corresponding to the frequency ω where the determinant becomes zero. A frequency-wavelength graph can thus reveal the salient features of the frequency equation.

In the two layered model the inner layer is assumed to comprise spongy bone. The elastic constants of spongy bone have not been measured to any degree of accuracy. Futher, even though bone has been shown to be piezoelectric in nature, various investigators have arrived at different configura-

tions for the piezoelectric matrix of bone (WILLIAMS, 1974). The dielectric constants for bone are not readily available. This leads to the conclusion that a numerical study of the general model is not worth-while at present. It was decided to obtain results by numerical evaluation of the sixth order determinant obtained as the frequency equation for the single layered model in the absence of both the inner spongy layer and of the piezoelectric effect. Since the equation is transcendental in nature, it has an infinite number of solutions or modes. Results of the numerical approach are presented in Figs 3—5 which show curves of the first six modes for normalized frequency versus normalized wavenumber for various dimensions and orders of axial invariance n. The values of R are chosen so as to correspond to physically realistic values for bone and the parameters are normalized as follows:

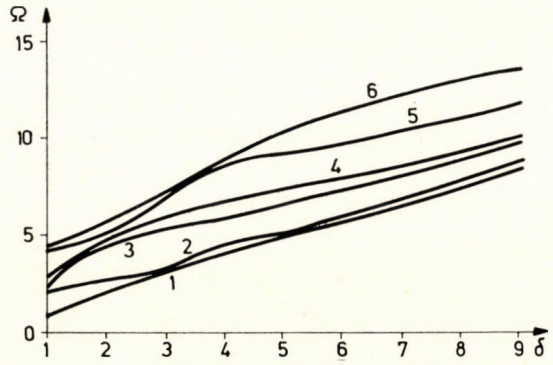

Fig. 3. Plot of frequency vs. wavelength for the first six roots, with n = 0 and R = 2

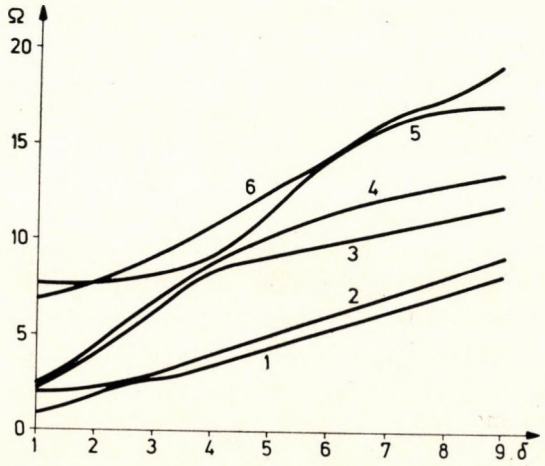

Fig. 4. Plot of frequency vs. wavelength for the first six roots, with n = 0 and R = 4

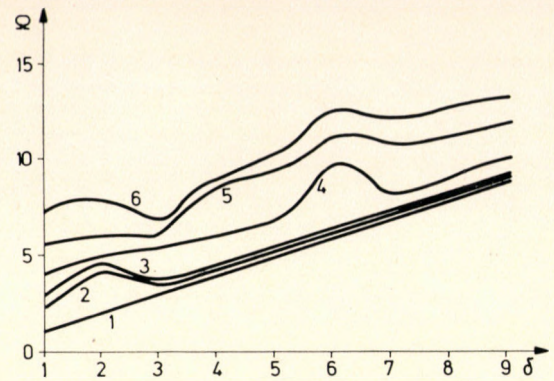

Fig. 5. Plot of frequency vs. wavelength for the first six roots, with $n = 1$ and $R = 2$

$$\Omega = \frac{\omega h}{c_s} \tag{A1}$$

where $h = r(2) - r(1) = 1$ for convenience

$$c_s = \left(\frac{c_{44}}{\varrho}\right) 1/2 \tag{A2}$$

$$\delta = \frac{2\pi h}{L} = h\alpha = \alpha \tag{A3}$$

$$R = \frac{r(1) + r(2)}{2} = \text{average radius of cylinder.} \tag{A4}$$

Though the results cannot be compared with any experimentally known data, a few remarks are in order for values of n = 0 (*i.e.* the case of axisymmetric motion). The value $\Omega = \varrho$ was found to be a simple root of the frequency equation and independent of R. The graphs also reveal the possibility of coupling between some of the modes *e.g.* 5 and 6 in Fig. 3. To ensure that such coupling does indeed occur, the result must be substituted into the solutions for the various displacements and the nature of these solutions studied in detail. With increasing R, the modes tend to drift further apart and, for the same wavelength, the frequency of propagation is seen to be higher. With increasing n, however, the first few modes are bunched closer together. For n = 0, node 2 represents the lower torsional mode and mode 1 the axi-symmetric shell flexural mode, while mode 3 corresponds to the "breathing mode". All modes higher than the third are either thickness-shear or thickness-stretch vibrations.

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RECENSIONES

Neurobiology of Invertebrates. Gastropoda Brain

Ed. SALÁNKI J., Akadémiai Kiadó, Budapest (1976), pp. 656. £ 23.

This book is the proceedings of the third International Symposium on Invertebrate Neurobiology held at the Biological Research Institute in Tihany, Hungary. The topic, Gastropoda brain, was selected because this group of *Mollusca* possesses central giant neurons suitable for *in vivo* identification, further, on the regulation of both simple and more complicated behaviour can be studied at cellular level.

The book divides into the following chapters: 1. Functional identification and mapping in the central nervous system. 2. Monoamine metabolism, uptake and localization. 3. Receptor pharmacology. 4. Electrical properties and mechanisms of excitation. 5. Sensory pathways and central projection. 6. Input-output relations and control of behaviour.

Each of the topics is presented by well-known specialists arriving from 15 countries. Altogether 41 papers are published each of which is completed by a summary and followed by the essence of the discussion conducted at the meeting. Such type of material can be interesting and informative for the specialists. Besides the new results, the volume refers to the main unsolved problems of invertebrate neurobiology. An attempt is made to generalize the results obtained at different *Gastropoda* species and to accept common principles in the identification of the cells as well as in understanding and interpreting basic neuronal functions.

The book is well-illustrated and completed with a subject index.

The volume can be recommended to those who are working in the field of biophysics, neurophysiology, neurochemistry, neuromorphology, behaviour and in experimental and theoretical neurosciences in general.

KATALIN S.-RÓZSA (Tihany)

Neuron concept today

Ed. SZENTÁGOTHAI J., HÁMORI J., and VIZI E. S. Akadémiai Kiadó, Budapest (1977).
Proceedings of a Symposium, Tihany (Hungary)
August 26—28, 1976. pp. 310. ISBN 963 05 1331 5

The discrete neuronal organization as the basic structure of the nervous system in all representatives of the animal kingdom cannot be questioned today. The title of this Volume refers mostly to the heavy discussions conducted between the supporters of the neuron and reticulum theory in the first part of this century. Nevertheless, there are numerous and extremely important problems which have arisen a consequence of the neuron concept, and have been solved only partly or have remained unsolved up to the recent years. The papers presented at the Symposium were dealing with present ideas and basic results concerning the main question of the neuron concept: what is the actual structure and mode of the functioning of the nervous system at neuronal and interneuronal level, as well as in the neuronal networks.

The book contains 20 full papers and over 50 abstracts dealing with morphology, physiology, biochemistry, pharmacology and modelling of the nervous elements, connections and circuits. A rather wide field of questions is discussed approaching the neuron concept from different aspects, including the development of nerve elements, trophical functions and

transport mechanisms, the acceptability of DALE's principle, recent problems concerning transmitter synthesis and release, significance of dendrites and in general of the connection of neurons in the determination of the neuronal functioning, elementary processes of learning and principles of neuronal organization.

The international assembling of the symposium (12 countries were represented) gave a further value to the meeting: eminent specialists of recent neurobiology gave an account on their own current research. Although for the understanding of the neural functions new and more exact discoveries and interpretations have been elaborated and are required in the future, the contributors agreed upon, as it was stressed by the chairman (J. SZENTÁGOTHAI) of the Symposium, that for the time being there is no reason to modify or reject any of the fundamental assumptions upon which the neuron concept has been based.

Altogether, the book can be warmly recommended to physiologists, morphologists and all the specialists working in neurobiological research or teaching in advanced courses at universities.

J. SALÁNKI (Tihany)

Hypothalamic Peptide Hormones and Pituitary Regulation

Ed. J. C. PORTER. In *Advances in Experimental Medicine and Biology, Vol 73*, Plenum Press, New York—London (1977), pp. 366. Figs. 139, $39.

The proceedings of a workshop held in Bethesda, Md., 1—2 November 1976 — the third in a series of symposia on neuroendocrinology sponsored by NIH.

This book concisely reviews critically analyses the current state of basic and clinical research on the hypothalamic hypophysiotrophic hormones and their influence on anterior pituitary regulation. It contains 15 papers covering a wide variety of topics, among others biochemistry, physiology, pharmacology and cellular biology of LH-RH, TRH and somatostatin, as well as agonistic and antagonistic analogues of these three peptides. It starts with "The endocrinology of the neuron and the neuronal origin of endocrine cells" (by R. GUILLEMIN). This lecture revealed the pertinence of two new concepts to neuroendocrine research (the possibility of interneuronal communication by multiple contacts of complex dendritic arborizations; all peptide-hormone-producing cells are derived from the neural ectoderm as are all neurons). S. OCHS reviews current knowledge on axoplasmic transport (mainly orthograde) in peripheral nerve and hypothalamo-neurohypophyseal systems. M. J. BROWNSTEIN deals with the distribution of biologically active peptides in the brain. A. BARNEA et al. describe studies on the subcellular compartmentalization of hypothalamic peptides. The next author (J. F. MCKELVY) summarizes current knowledge on the biosynthesis of hypothalamic peptides. A. V. SCHALLY and D. H. COY discuss the relationship between the structure of LH-RH and its biological activity and the development of a great number of stimulatory and inhibitory analogues, pointing out that the inhibitory analogues might eventually form the basis of a new birth control method. W. VALE and associates deal with the pharmacology of TRH, LH-RH, somatostatin and their synthetic analogues. F. LABRIE et al. summarize the role of adenosine 3,5 — monophosphate in the action of TRH, LH-RH and somatostatin and the modulation by peripheral hormones of the pituitary responses to the releasing hormones. J. C. PORTER et al. concentrate on the release of hypothalamic hormones under *in-vivo* and *in-vitro* conditions. Authors demonstrated, among others, the cause-effect relationship between the increased concentration of LH-RH in the portal blood and the increased release of LH from the pituitary after electrochemical stimulation of the hypothalamus in proestrus rats, and present data consistent with a retrograde transport of pituitary hormones and retrograde blood flow in the pituitary stalk. J. D. NEILL et al. reported on the LH-RH secretion in the monkey. M. SAFFRAN reviews the current status of hypothalamic hormones that control MSH and ACTH release. Recent knowledge on the factors affecting prolactin release is summarized by A. ARIMURA and A. V. SCHALLY. Data suggesting that the gonadotrophins exist in two functional pools in the pituitary gonadotrophs in women with regular cycle, one that is rapidly releasable, the other requiring prolonged stimulus input are presented (by S. S. C. YEN et al.). A further question to be discussed (by A. G. FRANTZ) was the effect of TRH in man. J. E. GERICH reports on the use of somatostatin in the study of carbohydrate homoeostasis in man. Summary and concluding comments (by R. A. GORSKI) offered a new classification of the releasing hormone neurons (classical neurons, neuroendocrine transducers and neuroendocrine integrator cells).

All papers are presented clearly. The well-produced book provides a vast amount of important new information and stimulating ideas of many of the presently hot fields of neuroendocrinology (both basic and clinical). It will be of particular interest to neuroendocrinologists, and all those interested in bioregulatory mechanisms can benefit from it.

B. HALÁSZ (Budapest)

Intercellular Communication

Ed. WALMOR C. DE MELLO. Plenum Press, New York—London (1977), pp. 251 + 3, 9 chapters, 63 figs, 5 tables, $ 30.

Living organism contain a system of communicating cells. Data accumulated in the last two decades unequivocally demonstrate the outstanding importance of intercellular communication in the functioning of different organs.

This volume provides an excellent report on the most recent findings in that field and at the same time an unbiassed critical evaluation of the subject. The high scientific level of the book can be anticipated by the first look at the list of contributors.

Although, the nine chapters in the book deal with quite divergent aspects of intercellular communication in various tissues, the editor and the contributors have succeeded in revealing ideas for a general treatment of different forms of cell-to-cell communication. Thus, they should challenge other fields of research, not mentioned in the book, by demonstrating that intercellular communication is a general phenomenon and plays a prominent role in every tissue. The leading idea is concerned to the importance or surface phenomena in forming gap junctions as morphological entities specialized for the performance of cell-to-cell communication.

However, the reader has sometimes the feeling that cell-to-cell interconnections performed by a long-distance signalization system of chemical transmitters between different cells are neglected. Also, when reading Chapter 7 on "Cell interactions in neuroblastoma" one might ask why the editor omitted dealing with problems of glial-neuronal or glia-to-glia communication.

Nevertheless, the book provides an excellent survey of the subject. Chapter 1, "Gap junctions in development", and also the last chapter, "Inter-cellular communication in the early embryo" render evidences for the significance of cell interactions in the differentiation of any tissue and in the development of any organ.

Chapter 2. "Cell contact phenomena and their implication in cell communication" and Chapter 3, "Junctional permeability and its consequences" deal with general phenomena in cell-to-cell coupling.

Chapter 4, "Intercellular communication in heart muscle" and Chapter 5, "Protein semiconduction: an alternative explanation of electrical coupling" give a fascinating discussion in findings concerned to the problem of electrical coupling of the cells.

Chapter 6, "Intercellular communication and cancer",

Chapter 7, "Cell interactions in neuroblastoma", and

Chapter 8, "Cell communication in the immune response" provide examples for the outstanding importance of cell coupling in the regulation of basic biological processes.

O. FEHÉR & L. LATZKOVITS (Szeged)

Advances in General and Cellular Pharmacology. Vol. 1.

Eds T. NARAHASHI and C. P. BIANCHI, Plenum Press New York and London (1976), pp. 252, Figs 66, Tables 16, $ 29.40.

The book is a successful attempt to summarize the present knowledge about the mechanism of action of drugs at cellular, subcellular and molecular levels with the aid to contribute to the improving of existing drugs and giving the basis of therapeutic application. A variety of concepts utilizing the methods of physics, chemistry, biophysics, biochemistry and physiology is synthetized in this book for clearing up the mechanism of action resulting in a new dimension of pharmacological research. The first volume in this series offers detailed studies in (1) cardiac automaticity, (2) opiates and their antagonists, (3) drug actions on developing cardiac tissues, (4) dose-response relation and (5) ganglionic transmission.

In the Volume the application of intracellular microelectrode technics is well presented in the cardiac pharmacology. The nature of cholinergic and adrenergic receptors in postsynaptic membranes is analyzed in detail.

Each chapter is written by outstanding experts from their own point of view, resulting in newer approaches and techniques with solid theoretical and experimental bases.

The standard of the book is excellent, and the references following each chapter will be very valuable for the readers.

The subject index gives a good basis for orientation in the topics.

The book can be recommended for biophysics, biochemists, physiologists and pharmacologists alike. The research workers and students will benefit from this volume and can use it as a manual for the today's general and cellular pharmacology.

KATALIN S.-RÓZSA (Tihany)

Comparative pathobiology, Vol. 1.

Biology of the *Microsporidia* Ed.: LEE A. BULLA, JR., THOMAS C. CHENG. Plenum Press, New York and London (1976), p. 371, 136 fig., — ISBN O-306-38121-4, $ 45.

Microsporidia constitute one of the largest group of parasitic animals. Nearly all major animal groups suffer from their parasitism. They are extremely common among *Insecta* and *Crustacea*. These facts create opportunities for detailed taxonomic studies in zoology. There are other observations evoking general interest: *e.g.* there are few reports about the occurrence of *Microsporidia* in man, infections caused by these parasites inhibit the development of various kinds of cancer in mice.

This volume presents a critical review on the structure, development, physiology and extracorporeal ecology of *Microsporidia*. The nature of host-parasite relation in Vertebrates and Invertebrates and data on their occurrence in mammalian tumours are also summarized. The book contains a short paragraph of methodology and reviews international type collections. A glossary, and indexes are attached.

For biologists of general interest the first paragraph, the structure of this smallest eukaryote cell, written by JIŘI VÁVRA is rather suggestive. Electron-microscopic studies show a Golgi-like primitive apparatus, missing mitochondria, specially-developed nucleus and modulations of the cell membrane somewhat into a cell wall structure. Similarly, the development of sporulation is quite special. There are also interesting examples of parasitic invasion and cytopathogenicity. For research workers in the field of oncology the interference between *Microsporidia* and transplanted tumours has an utmost significance.

I. KÉTYI (Pécs)

Human gene mapping 3.

BERGSMA, D. (Ed) *Baltimore Conference, 1975. Third International Workshop on Human Gene Mapping.* —. *Birth Defects: Original Article Series*, Vol. XII, No. 7. The National Foundation, New York. — S. Karger, Basel — München — Paris — London — New York — Sidney, 1976. pp. 452. SFr 110.—

This volume surveys one of the most dynamically developing fields of human genetic: human gene mapping, based on the Proceedings of the Third International Workshop, Baltimore 1975; with an Introduction by V. A. MCKUSICK. (The first of these workshops was held in 1973 at the Yale University, New Haven, USA (the second one in 1974 at the Erasmus University, Rotterdam, The Netherlands).

The principal aim of these conferences was to collect new information on the human gene map, with special interest invested in somatic cell hybridization and linkage studies in families. The summary of laboratory results obtained in different parts of the world and the comparison of the same proved more than useful.

In this volume one can find the most recent map of the human chromosomes. Definite information on specific autosomal location is available for over 70 loci and tentative information on about 40 others. (For comparison: the corresponding figures were 24 and 23 after the New Haven conference 1973, and 39 and 29 after the Rotterdam conference 1974.) A particu-

larly important result in Baltimore Conference was the ABO linkage group to chromosome 9. The mapping of chromosomes 1 and 6 provides fine examples: the Rh blood-group locus is on chromosome 1, and the major histocompatibility complex (HLA, *etc.*) is on chromosome 6. The Conference paid particular attention to the X chromosome.

The first part of the volume contains the committee reports summarizing the genetic constitution of the 1st and 2nd, the 6th, and of the further autosomal chromosomes, as well as of the X and Y chromosomes, dealing with such important problems as nomenclature, comparative mapping, *etc.*

The major part of the book comprises short reports presented at the Conference. Forty-four papers deal with the chromosome assignments and regional mapping of gene loci by utilizing somatic cell methods. Thirty studies discuss the chromosome and regional assignments of gene loci *via* linkage analysis of families or *via* studies of subjects with chromosome anomalies. Some papers investigate the localization of genes *via* hybridization *in situ*, others the new general somatic cell genetic and mapping methods, again others the comparative mapping of gene loci.

The common characteristics of these studies are concise objectivity, very informative wording and the interpretation of the most important results. The nine committee reports and the almost 100 short studies outline a very imposing picture: our recent knowledge of the constitution of human chromosomes. This is the reason why every biologist, cytologist, human genetist, physical anthropologist, clinician *etc.* will frequently consult this book.

O. G. EIBEN (Budapest)

Pollination Mechanisms, Reproduction and Plant Breeding

FRANKEL R., GALUN E.:
Springer Verlag Berlin—Heidelberg—New York (1977), pp. 281, Figs 77,

The book is the second volume of the series: Monographs on Theoretical and Applied Genetics (Edited by R. FRANKEL, BET DAGAN, G. A. E. GALL, DAVIS, M. GROSSMANN, Urbana, H. F. LINSKENS, Nijmegen, D. DE ZEEUW, Wageningen). The authors furnish a comprehensive, yet highly detailed, analytical treatment of the reproductive processes of the spermatophytes—mainly angiosperms as well as of the genetical and breeding-methodological aspects of these processes. They give an exhaustive description of pollination mechanisms and illustrate them, whenever practicable, by examples of cultivated plants. The book is based on an advanced course in plant-breeding taught at the Hebrew University of Jerusalem. The text is divided into three main parts. The first part starts with an evaluation of the pollination mechanisms significant for plant breeding and crop production. It is followed by a discussion of the reproductive processes in higher plants and the ecology and dynamics of pollination. The second part deals with crops propagated by self pollination and describes breeding procedures for such plants. The third part is devoted to allogamy. It handles the mechanisms involved in the prevention of self pollination and their utilization in plant breeding. The mechanisms considered are: sex expression, incompatibility and male sterility. The book is to be recommended to biology and agriculture students, but to specialists working in these fields as well, since it reveals highly significant relationships between botany, genetics, plant breeding and agriculture.

L. FRIDVALSZKY (Budapest)

Pesticides in Aquatic Environment

Ed. KHAN, M. A. Q. Plenum Press, New York (1977), ISBN 0-306-36310-0
Proceedings of a Symposium for the International Congress of Entomology. Washington, D. D., August 22, 1976. pp. 257, Figs 46, Tables 68. Price $ 35.40

The increasing interest in environmental pollution caused by the extensive use of pesticides in agriculture demands detailed investigations on the fate and role of these substances in the ecosystems and particularly in waters, water plants and animals. Though tendency to produce substances which are strictly specific towards target organisms (insects, fungi, weed, *etc.*) or which undergo fast degradation after use is very strong in recent years, most of the potent chemicals do not meet these requirements.

The book contains selected papers dealing with the dynamics of some pesticides starting from the place of their use up to the water organisms including their biodegradation.

The volume is divided into three sections. The first, including three papers, gives important and basic quantitative information about the origin of pesticides in waters, on their solubility, stability and persistence in sediment, water and water organisms. Data on organochlorines, organophosphates, carbamates, and also the nonpesticide polychlorinated biphenyls (PCBs) are recount in this chapter.

The four papers of the second section deal with the dynamics of pesticides in aquatic organisms. The dependence of accumulation on various factors, deposition and distribution in different organs and also the metabolism of pesticides mainly in animals are discussed in detail for several substances. The question of accumulation in fishes and studies in model ecosystems deserves special attention.

The third section, containing also four papers, is focused on the degradation of pesticides by bacteria, algae, mussels, crustaceans and fishes. Various pathways of biodegradation are described for several, widely used, substances and also the role of specific enzymes and their localization are discussed.

Each paper is followed by related literature, and subject index is added at the end of the Volume.

The book can be recommended for biologists, chemists and engineers working interested in questions of environmental protection and/or as researchers, teachers and/or experts in the practice. It can be very useful also for specialists dealing with agricultural chemistry and water management.

J. SALÁNKI (Tihany)

Second International Congress of Systematic and Evolutionary Biology (I CSEB-II)

The second international congress of systematic and evolutionary biology (I CSEB-II) will be held at The University of British Columbia, Vancouver, Canada, 17—24 July 1980.

The provisional list of symposia topics include:

1. Arctic refugia and the evolution of arctic biota
2. Origins and evolution of the north Pacific marine biota
3. Evolution of reproductive strategies
4. Evolutionary epigenetics
5. Evolution of community structure
6. Green algae and land plant origins
7. Macromolecular mechanisms in evolution
8. Allozymes and evolution
9. Coevolution and foraging strategy
10. Evolution of colonizing species
11. Rare species and the maintenance of gene pools
12. Paleobiology of the Pacific rim

Addition symposia may be included.

Sessions for contributed papers and for papers in specialized fields, taxonomic as well as methodological will also be organized.

Those interested in receiving an information circular in the spring of 1979, should write to the following:

Dr. G. G. E. Scudder
Department of Zoology
The University of British Columbia
2075 Wesbrook Mall
Vancouver, B. C. V6T 1W5
Canada.

Atlas der Biochemie

Übersichten zum Intermediärstoffwechsel

Von Dr. med. RAINER LASEK
Medizinisch-poliklinisches Institut der Karl-Marx-Universität, Leipzig
Mit einem Geleitwort von MR Prof. Dr. sc. med. WOLFGANG ROTZSCH, Leipzig
1976. 16 Seiten, 8 Tafeln (4 Tafeln 61 × 86 cm, 4 Tafeln 43 × 61 cm)
In Plastmappe (34 × 26 cm) 24,50 M · Bestell-Nr. 793 308 6

Aufgliederung des Stoffwechsels: Kohlenhydrat-, Lipid-, Aminosäurern-, Eiweiß- und Nukleinstoffwechsel, Eisen- und Kalziumstoffwechsel, biologische Oxydation und Photosynthese. Ihren Zusammenhang finden die einzelnen Gebiete in einer Gesamtübersicht. Die Stoffwechselwege sind in kurzen Anleitungen zu den einzelnen Tafeln erläutert.

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JOHANN AMBROSIUS BARTH LEIPZIG

Leopoldina-Symposium

Secondary Metabolism and Coevolution

Cellular, intercellular, and interorganismic aspects

Edited by Martin Luckner, Kurt Mothes and Lutz Nover
(Nova Acta Leopoldina. Neue Folge. Suppl. Nr. 7)

1976. 614 Seiten, zahlreiche Abbildungen und Tabellen, 35 Bildtafeln
In englischer Sprache
Kunstleder 80,— M

Das Buch behandelt Probleme der Coevolution und der koordinierten Realisierung von Prozessen des Sekundärstoffwechsels mit anderen Bereichen des Metabolismus. Besprochen werden die Koordination der Synthese sekundärer Naturstoffe mit der Ausbildung zytologischer Strukturen zu ihrer Speicherung, sowie die Coevolution von Rezeptorstrukturen mit der Bildung sekundärer Naturstoffe, die als physiologische oder ökologische Effektoren wirken. Darüber hinaus befaßt sich das Buch mit der Organisation von Genexpressionsprogrammen, die die molekulare Grundlage für die Koordinierung darstellen.

Bestellungen an den Buchhandel erbeten

JOHANN AMBROSIUS BARTH LEIPZIG

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója — Műszaki szerkesztő: Zacsik Annamária

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The manuscripts should not exceed 16 typed pages in general. The manuscripts should be typed double-spaced, on one side of the paper. In order to assure rapid publication, contributors are requested to submit two copies of the manuscript including an abstract (max. 200 words), tables and figures. Each table should be typed on a separate sheet, numbered and provided with a title. All figures, either photographs or drawings or graphs, should be numbered consecutively. Photographs should be labelled not directly, but on a transparent sheet of paper covering the photo. Figure legends should be typed in sequence on a separate sheet.

Papers should be headed with the title of the paper, the names of the authors (male authors use initials, female authors use one given name in full), department, institute and town where the work was performed.

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5. *Discussion*
6. *References.* Papers — the essential ones only — cited in the manuscript should be listed on a separate sheet in alphabetical order according to the first author's surname. The references should be numbered so that each may be referred to in the text by its number only. Examples:

1. BOAS, N. F. (1953) Method for determination of hexosamine in tissue. *J. biol. Chem.* **204,** 553—563.
2. DE DUVE, C. Lysosomes, a new group of cytoplasmic particles. HAYASHI, T. (ed.) Subcellular Particles. Ronald Press, N. Y. 1959.
3. UMBREIT, W. E., BURRIS, R. H., STAUFFER, I. F. Manometric Techniques. Burgess Publishing Co., Minneapolis 1957.

Short communication. Manuscripts, in English, should not exceed 1 000 words (4 typed pages) including references. The text of manuscripts containing tables and/or figures must be correspondingly shorter. Accepted short communications will be published within six months after submission of manuscripts. In order to speed up publication, no proof will be sent to authors.

Authors will be furnished, free of charge, with 100 reprints. Additional reprints may be obtained at cost.

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REVIEW ARTICLE

MEMBRANE FUNCTION ALTERATIONS OF RED BLOOD CELL IN NEUROPSYCHIATRIC DISORDERS

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(Received 1978—06—11)

Recent results in neurobiology suggest that changes in permeability of the cell membrane play an essential role in both physiological and pathological activity of the excitable tissues. These transport processes are of profound significance in the maintenance of resting potential and in both initiation and propagation of neuronal discharge. Moreover, numerous transport processes play a prominent role in the storage, release and re-uptake of the neurotransmitters, *i. e.* in neurochemical transmission. Membrane excitability by receptor stimulation is also combined with the optimum function of specialized membrane devices. It is evident that the brain barrier system, consisting of highly organized membrane structures, provides special membranological problems [6, 27]. Consequently, detailed studies on pathologically altered membrane transport processes have an outstanding importance in the analysis of several neuropsychiatric diseases in terms of both general pathology and neuropathology [11, 60, 121]. Recently, a great number of data have been accumulated suggesting that quite a general disturbance of transport processes should be taken into consideration when certain neuropsychiatric disorders are analysed; not only disturbances of specific neural transport mechanisms should be analysed for revealing the cellular background of some neuropsychiatric phenomena.

One of the general problems in neuropathological research is that in the case of most neuropsychiatric diseases there is no suitable experimental model at hand. There are toxic substances, drugs, which may produce experimental psychosis or degenerative neurological diseases in animals. However, these are, in spite of the fact that their symptomatology simulate the human disease, totally different regarding the pathological factors. So the possibilities to get living tissues or cells from the diseased organism which can be used for *in vitro* experiments are very limited. This is one of the reasons why human red blood cells (RBCs) and platelets, which are easy to obtain, are the preferable models for the examination of cellular membrane functions and transport processes

in neuropsychiatric diseases [18, 80, 82, 83, 110]. On the other hand, the RBC is an especially suitable model for the study of the elementary membrane functions under both normal and pathological conditions. Moreover, a lot of data suggest that factors altering membrane functions affect RBCs in the same way as they do with the other tissues in the organism [40, 13, 20, 39, 53]. The study of the change in ion transport of RBCs has contributed to the understanding of the pathomechanisms in the most different pathological states, such as uraemia, shock, myocardial infarction, collagenoses, cancer and hyperthyroidism [3, 4, 28, 89, 111, 112, 124, 127]. It is also known that the membrane transport functions and the specific features of the membrane-ATPase system in the RBC show a qualitatively significant agreement with the corresponding membrane functions of nerve cells [10, 13, 20, 39, 53, 114]. Certain drugs of membrane effect, metabolic substrates and toxic substances cause the same membrane biochemical alterations in RBCs as in the neuron [9, 36, 62].

Certain authors, assuming a "generalized membrane disturbance" in myopathia and affective psychoses, use the term "membrane disease" [45, 74, 79, 110]. For the time being, in spite of the accumulated data, it is difficult to form an opinion about the definitiveness of the new membrane theory on the pathogenesis of some neuropsychiatric diseases. But on the basis of the published data this direction of research seems to be promising and its result may attract the interest of the neuropsychiatrist and of the membranologist alike.

Disturbances in the function of RBC membrane in neuromuscular diseases

The idea that in myopathic diseases the permeability of the muscle tissue membrane is altered arose from the clinical-chemical observation that enzymes of the muscle tissue appear in the serum in great quantity [31]. CORSINI and CACCIARI [25] supposed alteration in the ion transport of the muscle membrane. The possibility that, parallel with ion transport disturbances on the plasma membrane of muscle tissue, the ion transport of the RBC membrane may be altered, arose on the basis of the observation that the Na^+-K^+-ATPase activity of the sarcoplasmatic reticulum of the heart muscle of myopathic duck and that of the plasma membrane preparation of their RBCs were not inhibited, even stimulated, by ouabain in 10^{-4} M concentration [17]. BROWN et al. [16] published that in Duchenne-type progressive muscular dystrophy, the ghost ATPase activity of the RBC significantly increased on the effect of 10^{-4} M ouabain. This observation was supported by PETER et al. [93] and ARAKI [2] and MAVATARI, while disproved by KLASSEN and BLOSTEIN [67] and PROBSFIELD et al. [94]. CHO and MELTZER [21] explained the contradictory data by assuming that ATPase activity was measured by different methods

(in presence of ouabain the increase of the ghost ATPase activity of the RBCs of dystrophic patients was observed in a medium of unusual ion composition: 1 *mM* NaCl, 2 *mM* KCl, 1*mM* $MgCl_2$). As PROBSFIELD et al. [94] pointed out, in such a medium it is not the Na^+-K^+-ATPase activity which was measured. This is supported by the examination of KLASSEN and BLOSTEIN [67], who, measuring the activity of Na^+-K^+-ATPase of the RBCs in a medium of usual ion composition, did not find any alteration in the inhibition of ouabain on the RBCs of dystrophic patients. According to the results of PROBSFIELD et al. [94], in the case of Duchenne muscular dystrophy, the $^{86}Rb^+$ uptake of the RBC shows no alteration compared to the control. According to HULL and ROSES [58] the Na^+ and K^+ transport of the RBC shows different stoichiometry in myotonic dystrophy. It is worth to underline that in this form of myopathy none of the mentioned authors observed disturbances in ATPase activity.

On the basis of all these observations, it can be stated that the data regarding the transport-ATPase activity of the RBC and the active transport of cations are contradictory. But it can be supposed that this "paradoxical effect" of ouabain has an influence on some Mg^{++}-dependent ATPase as it is underlined by CHO and MELTZER [21]. It is also unquestionable that the "paradoxical effect" of ouabain increasing the ATPase activity was unanimously observed by three research teams. Moreover, the authors who did not accept this result have never tried to reproduce it on the same experimental material. These data seem to support the opinion of ROSES and APPEL [97] that myopathic diseases mean the "diffuse defect" of the RBC membrane. This statement has been proved mainly by the data of ROSES et al. [96, 99, 100], who demonstrated the significant and important alteration of the phosphorylation of the RBC membrane in different diseases of the muscle. Accordingly, the protein-kinase activity of the RBC, being responsible for the membrane protein phosphorylation, is suitable for the identification of the carriers. According to their extensive investigations, the membrane phosphorylation is much more suitable for the clinical test of the carrier than the previously used human-genetical, clinical and other methods, including scanning electron microscopy. ROSES et al. demonstrated parallelism between the phosphorylation of certain protein fractions of the RBC membrane [96, 99] and the membrane fluidity, with spin-label technique [18, 97]. Their results show that in myotonic muscular dystrophy both the RBC and the muscle tissue plasma membrane fluidity increased. On the effect of phenytoin treatment, which is used therapeutically in this illness, the membrane fluidity of the "diseased RBCs" decreased to the normal value [98].

A number of authors [68, 73, 74] have dealt with shape alterations of the RBCs of myopathic patients. DROMGOOLE et al. [32] produced myotonic deviations by diazacholesterol in animals. Besides the shape alterations of the RBCs, development of cataract was found to simulate human myotonia.

In Duchenne muscular dystrophy, the decreased plasticity of the RBC membrane and the change in the membrane elasticity have also been demonstrated by different elastometric methods [92]. The change in shape of the RBCs reflects a characteristic membrane function [41, 42] in which the specific distribution of the membrane proteins and lipids and their interactions play the main role. The role of Ca^{++} is important in this process. APPEL and ROSES [1] reported a detailed study on the pathological membrane biochemical changes which can be observed in myotonic muscular dystrophy. We examined the Ca^{++}-dependent rapid K^+efflux [40, 115] *in vitro* on the RBCs of 9 patients with Duchenne-type progressive muscular dystrophy. It was found that the Ca^{++}-promoted K^+ efflux was significantly lower in the diseased group than in healthy control persons [119]. The significant increase of the K^+efflux was demonstrated in the muscle tissue of dystrophic mouse and chicken strains and in RBCs of patients with muscular dystrophy [1, 57]. Increased K^+ uptake was observed in the RBCs of dystrophic chickens and human patients by SA'AFI et al. [109] with an increased microviscosity of RBC plasma membrane. Structural deviations of the sarcolemma in Duchenne-type muscular dystrophy were reported by SCHOTLAND et al. [103].

The changed lipid metabolism observed in neuromuscular diseases and in experimental muscular dystrophy is suggestive of differences in the composition of certain membrane lipids of muscle tissues and other organs. The increase of the cholesterol/phospholipid ratio seems to be one of the characteristics of the dystrophic membrane disease [51, 66, 69].

Alterations in the ion transport and ATP metabolism in endogenous psychoses

There is considerable evidence that abnormalities of the electrolyte metabolism occur in affective (manic-depressive) disorders [59, 75, 81, 125, 126]. Extensive experiments have been started to elucidate the disturbances of Na^+ and K^+ ion-household. It has been stated that Na^+ retention occurs mainly in the depressive phase of psychosis while at "mood-swing" to manic phase Na^+ diuresis starts [26]. Recent examinations, modern techniques (metabolic "balance" method, isotope dilution technique) have confirmed this observation, and it is widely accepted nowadays that in endogenous depression Na^+ retention occurs which is abolished by improvement [7, 22, 23, 24, 44, 101, 106]. The pathomechanism of the mentioned electrolytic alterations is generally interpreted as a consequence of disturbed neuro-endocrine regulation [7, 75]. Studies with isotope-dilution technique suggest that the Na^+ retention, which can be demonstrated in endogenous depression, relates to the intracellular space. But the intracellular electrolyte concentrations regulated well-defined ion transport processes [41].

During clinical experiments some observations about the psychopharmacological effect of Li^+, and more recently Rb^+ ions, have called attention to the theoretical and practical importance of the examination of the transport processes taking place at cellular level. The antimanic, phase-prophylactic and antidepressant effects of Li^+ influence the outcome of affective psychoses in a favourable way [19, 43, 65, 104]. As the result of Li^+ therapy the course of the phases of affective and cycloid psychoses becomes favourable. All over the world, as in our country, the admission of patients with this illness, the number of nursing days (hospitalization time) and the frequency of relapses have significantly decreased. During Li^+ therapy the affective episodes (manic and depressive phases) appear with milder symptoms, which can be solved with ambulant treatment. The psychopharmacological effect of Rb^+ has also been known for a long time [35, 78]. MELTZER and FIEVE [76] have recently reported on the reasonability of psychiatric application of Rb^+.

As to the Li^+ therapy, investigations of the cellular ion transport regulation led to the membrane theory of affective psychoses [79, 80]. A lot of experiments were carried out parallel with the clinical course of the disease on patients with affective psychoses regarding the electrolyte concentration of RBC and plasma [36, 79], the Na^+-K^+-ATPase activity of the RBCs, the ouabain-sensitive K^+ influx and the Na^+-pump activity of RBCs in connection with Li^+ therapy. DICK et al. [29] carried out experiments for 20 weeks on a patient with manic-depressive psychosis having frequent phase changes. The Na^+-K^+-ATPase activity of the RBC membrane decreased in every case in depressive phase and change of the ouabain-sensitive K^+-influx paralleled with the changes of the mood during clinical observation. Later NAYLOR et al. [86] demonstrated in a great number of patients with endogenous depression that the Na^+-K^+-ATPase activity of their RBC membrane and their ouabain-sensitive K^+-influx were significantly diminished. NAYLOR et al. [84, 85] published also that during Li^+ therapy the Na^+K^+-ATPase activity of the RBC membrane increases significantly as compared to the placebo group. Recently HOKIN-NEAVERSON et al. [55] have observed decreased Na^+-pump activity of RBCs in depressive psychoses. They also stated that during chronic Li^+ therapy the ouabain-sensitive Na^+-efflux of the RBCs and the ouabain-sensitive ATPase activity of the RBC membrane increased [52, 54].

We demonstrated these alterations also under physiological conditions in *in vitro* experiments carried out in intact RBCs of both bipolar and unipolar patients, taking the nosological subgroups into consideration [118].

The therapeutic dose of Li^+ is close to its toxic dose (0.8—1.5, 2.0 mEq/l serum, respectively). Extensive clinical laboratory experiments have been carried out to find the optimum dosage and to prevent toxic symptoms. In animal experiments FRAZER et al. [36] found the Li^+ concentration in the brain to be in a closer correlation with the Li^+ level of RBCs than with that of

plasma. So they suggested to follow-up the Li^+ concentration of the RBCs, too, during lithium therapy. Based on an analysis of a great number of clinical data they stated that the RBC/plasma ratio of Li^+ points to the therapeutic prognosis, thus contributing to the estimation of the antidepressant effect of Li^+ [43]. Among Hungarian authors, Szücs has confirmed that the RBC/plasma Li^+ ratio does provide prognostic information on the prophylactic effectiveness of Li^+ treatment [120].

The *in vitro* and *in vivo* experiments carried out in RBCs for the genetic determination of the distribution of the Li^+ [30] support the theory of MENDELS and FRAZER [79, 80] that the disturbance of the elemental membrane ion transport processes in affective psychoses is a pathologically significant factor.

Clinical and pharmacokinetical examination have revealed correlation between the effectiveness of lithium therapy and the *in vivo* RBC/plasma Li^+ ratio. On the other hand, a close correlation reported between the value of the RBC/plasma Li^+ ratio and the change and characteristics of the clinical patterns, and between the frequency and severity of the side-effects and the effectiveness of the treatment of rare toxic symptoms [34, 87]. These studies support the practical importance of the examination of the Li^+ transport pathways responsible for the *in vivo* steady-state intracellular Li^+ levels during Li^+ therapy. The laboratory and experimental studies performed during Li^+ therapy have unanimously proved that under physiological conditions the steady-state intra- and extracellular distribution of Li^+ is unequal, the Li^+ concentration ratio of intra/extracellular-state is less than 1, not only in the blood, but also in other tissues. This fact is of great importance from membrane-biochemical aspects, *viz.* in the exact understanding of the basic physiological mechanisms concerning the *in vivo* intra- and extracellular cation distribution, and also in understanding the mechanism of action of Li^+ therapy. Furthermore, it may have a pathological importance in the research of the affective psychoses from the biological point of view.

The classical, basic, membrane transport examinations of the active ion transport and the understanding of its enzymatic mechanism and the functioning of the Na^+-K^+-ATPase have demonstrated that under physiological circumstances the transport ATPase does not take part, or its role can be neglected in the active transport of Li^+. From the theoretical point of view, all these support the importance of the Li^+ transport mechanisms. Investigations carried out in the last three years led to significant result in the cognition of the Li^+ transport mechanisms [33, 38, 48, 90, 102]. Detailed studies as to the principles of Li^+ transport of RBCs were carried out by the teams of TOSTESON; DUHM and FUNDEAR and WIETH (reviewed in [12]).

It has been demonstrated that there are 4 pathways in the lithium transport:

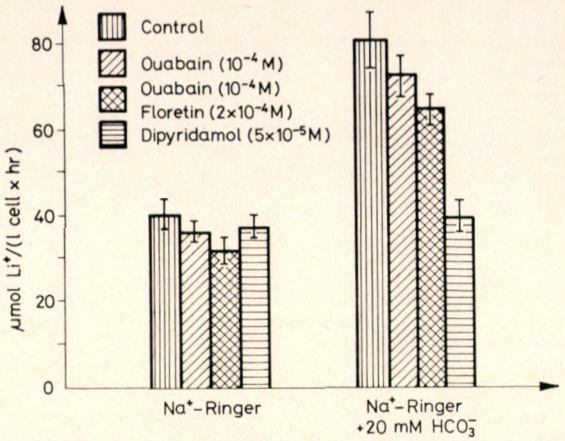


Fig. 1. Effect of 20 *mM* HCO_3^- on Li^+ uptake into the human red blood cells. The washed red blood cells were incubated in Na-Ringer or Na-Ringer + 20 *mM* HCO_3^- media supplemented with 1.5 *mM* LiCl and different transport inhibitors, pH 7.4, 37 °C. Haematocrit, 10%. Samples were taken in 20-min periods for two hours and intracellular Li^+ concentration was determined by washing the cells in ice-cold isotonic $MgCl_2$ solution and measured by atomic absorption spectrophotometry

1. bicarbonate-stimulated diffusion which can be inhibited by the transport blocker, dipyridamol;
2. Na^+-dependent floretin-sensitive Li^+ countertransport;
3. K^+- and ouabain-sensitive Li^+-uptake through the Na^+-K^+-pump;
4. passive diffusion.

We have examined the role of transport pathways taking place in the formation of the steady-state intracellular Li^+ level which can be observed while lithium is administered under physiological circumstances. In *in vitro* experiments with RBCs, we demonstrated [117] that under physiological circumstances the bicarbonate-stimulated diffusion plays a significant role in the determination of the intracellular Li^+ level at steady-state. It increases the Li^+-uptake to its double when 20 *mM* bicarbonate is applied extracellularly (Fig. 1). Besides Na^+-K^+-pump, the Na^+-dependent floretin-sensitive Li^+ transport and the passive diffusion also participate in the Li^+-uptake. In determining the RBC/plasma Li ratio at steady-state, the Na^+dependent floretin-sensitive Li^+ countertransport system has the primary role [33, 48, 117, 118].

Studies on the phosphate transport and ATP metabolism of the RBC in endogenous psychoses

There are few data in the literature regarding the ATP metabolism in manic-depressive psychosis. JENNER found [63] a close correlation between the blood ATP level and the "emotional life" of some patients with affective psychosis. The surprising findings of HANSEN [49] and HANSEN and DIMIT-

RAKOUDI [50] that in endogenous depression the ATP concentration of the blood taken from finger tip is 50% lower than the control, has not been confirmed so far [64].

As to the other form of endogenous psychoses, the schizophrenia, it was proposed a few decades ago that energy household of the organism is disturbed. According to this hypothesis, several authors and teams have examined the ATP metabolism of the RBCs *in vitro* [5, 14, 15, 46, 47, 56, 88]. The changes in phosphate incorporation and in the concentration of different intracellular phosphate fractions were studied in RBCs incubated in own plasma containing ^{32}P-labelled phosphate. GOTTLIEB et al. [47] and HONDA [56] observed that the specific activity of the ATP of RBCs taken from schizophrenic patients was 2—3 times higher than that of the control red cells. ARNOLD and HOFMANN [5] failed to observe this deviation in similar experiments. At the same time they found a significant difference in the ATP/ADP ratio, which was low in the RBCs of schizophrenic patients. Most of the mentioned authors [5, 14, 15, 46, 47, 56] observed in such experiments a similar change in the ATP level, and also in the phosphate incorporation into ATP after *in vivo* administration of insulin or succinate (insulin and succinate are mentioned as "metabolic stressors"). As the result of both effects, the specific activity of ATP in the RBCs of patients with chronic schizophrenia decreased, while an increase was observed in the controls.

The cited authors did not study the inorganic phosphate uptake of the RBCs simultaneously with the incorporation of phosphate into ATP. Such investigations were carried out by USUNOFF et al. [122, 123], who found that the RBCs of schizophrenic patients took up inorganic phosphate *in vitro* at a lower rate than the controls. However, these authors disregarded the close connection between the phosphate transport and the metabolism. None of the authors have defined the kinetic curves of phosphate incorporation: they gave the values of specific activity only for a single point of time. No valuable data can be found in literature regarding the glycolytic metabolism of RBCs of schizophrenic patients, either.

SEEMAN and O'BRIEN [107] described an increased Na^+-K^+-ATPase activity of the RBCs of schizophrenic patients. In contrast with this, PARKER and HOFFMAN [91] failed to find any difference in the Na^+-K^+-ATPase activity. CHO and MELTZER [21] observed significant increase in the ouabain-insensitive ATPase activity in the RBC membrane of schizophrenic patients.

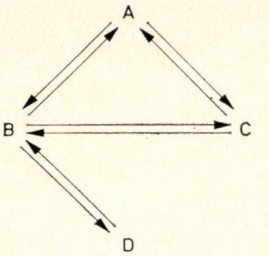
On the basis of the cited literature disturbances in the phosphate metabolism of RBCs of schizophrenic patients is presumable. But it has remained for be clarified, whether the ATP synthesis or the rate of consumption is disturbed. The literary data are also contradictory concerning the description of the alteration of the phosphate metabolism, first of all regarding the explanation of the observed deviations.

Our own experiments carried out in this field have aimed at simultaneous examination of the phosphate uptake and the ATP metabolism of the RBC. We wished to present data about the possible alterations in ATP metabolism which are in connection with the disturbances of the *in vitro* active ouabain-sensitive Na^+ and K^+ transport of RBC observed in affective psychosis; further we made efforts to show whether the phosphate transport of RBCs is altered in endogenous psychoses.

The ATP metabolism of intact RBCs cannot be examined separately from the phosphate transport on the basis of incorporation experiments with ^{32}P-labelled phosphate [61, 70]. Thus, our experiments were carried out in the following way: washed RBCs from healthy control donors kept on standard diet without medication, and those of patients with chronic schizophrenia and bipolar and unipolar depression, were incubated at 37°C in Krebs–Ringer phosphate medium of inorganic phosphate concentration 0.6—1.0 *mM*, which equals the inorganic phosphate concentration of the plasma; in simultaneous experiments, own plasma with 20% haematocrit was used as medium. The phosphate was labelled with ^{32}P in both media. These two kinds of extracellular (incubation) media were used in order to study the role of the plasma factors. Samples were taken at different intervals of incubation, the plasma and the medium, respectively, was separated and the cells were washed three times in ice-cold physiological saline. The following phosphate fractions were isolated from the samples, their concentration was determined, and the values of the specific activity were counted.

1. Inorganic phosphate in Krebs–Ringer phosphate medium and in plasma (pool A);
2. inorganic phosphate in the RBC (pool B);
3. acid-labile phosphate (pool C);
4. acid-resistant phosphate (pool D).

The materials and methods applied in these experiments were described earlier [116]. Glucose and lactic acid were determined according to SEIFERT et al. [108] and BARKER and SUMMERSON [8], respectively. The kinetic curves of the specific activity of phosphate fractions were analysed by tracer-kinetic method. We had developed an isotope-kinetic model of four compartments [71] which enables us to separate the phosphate uptake by the RBCs into two simultaneous processes, these may be characterized by transport rates. The transport rates can be determined by applying the tracer-distribution equations (Fig. 2). One of the rate values (W_{AC}) refers to the ATP synthesis taking place on the membrane, the other (W_{AB}) to the direct phosphate uptake which avoids the membrane ATP synthesis. Furthermore W_{BC} and W_{BD} are suitable for the representation of the intracellular phosphate ester synthesis. The model analysis was performed to attempt an approach to the mechanism of phosphate uptake and to decide which pathways of phosphate transport are altered.



$$\frac{dI_A}{dt} - \frac{d_A}{dt} S_A = W_{AC}(S_C - S_A) + W_{AB}(S_B - S_A) + W_{AD}(S_D - S_A)$$

$$\frac{dI_B}{dt} + \frac{d_A}{dt} S_A = W_{AB}(S_A - S_B) + W_{BC}(S_C - S_B) + W_{BD}(S_D - S_B)$$

$$\frac{dI_C}{dt} = W_{AC}(S_A - S_C) + W_{BC}(S_B - S_C) + W_{CD}(S_D - S_C)$$

$$\frac{dI_D}{dt} = W_{AD}(S_A - S_D) + W_{BD}(S_B - S_D) + W_{CD}(S_C - S_D)$$

Fig. 2. Scheme of isotope-kinetic model for the uptake and distribution of phosphate in red blood cells. Abbreviations: A, B, C and D are the amounts of phosphate of the particular pools (μ mole phosphorus per ml washed blood cell or 4 ml Krebs–Ringer solution) I_A, I_B, I_C and I_D are total activities of pools (cpm per ml washed blood cell or per 4 ml Krebs–Ringer solution) W_{XY} is the rate of exchange between the two pools denoted by the indices (μmole phosphorus per min per ml washed blood cell or 4 ml Krebs–Ringer solution)

The most important results of our experiments carried out simultaneously on the phosphate transport and ATP metabolism of the RBCs of patients with endogenous psychosis [116] are summarized as follows.

Figs 3, 4, 5 and 6 show the time curves of the total activity and specific activity of inorganic phosphate got during the incubation of the RBCs in Krebs–Ringer phosphate medium in the four groups of patients. It is shown that the total and specific activity of the medium decreases at a slower rate in the *in vitro* experiments carried out with RBCs of depressive patients. Accordingly the slope of the kinetic curves of the total and specific activity of inorganic phosphate of the RBC is of lower value. The time curves of the total and specific activity of the organic phosphate fractions of the RBC are shown in Figs 5 and 6. The kinetic curves of the total and specific activities of the acid-labile phosphate run flatter in both subgroups of affective psychosis. According to the time curves of the specific activity (Figs 5 and 6) the result of the isotope-kinetic model analysis is predictable: the W_{AB} and W_{AC} values for patients with unipolar and bipolar depression are significantly ($P < 0.01$) lower those for healthy control donors or chronic schizophrenics (Fig. 7). There is no difference in the W_{BC} and W_{BD} rate values between the four groups. Figure 8 shows that there is no difference in glucose consumption and lactic acid production among the four groups during the incubation of 120 min.

The incorporation of ^{32}P-phosphate in certain intracellular phosphate fractions was examined *in vitro* in plasma medium. Figure 9 shows that, also

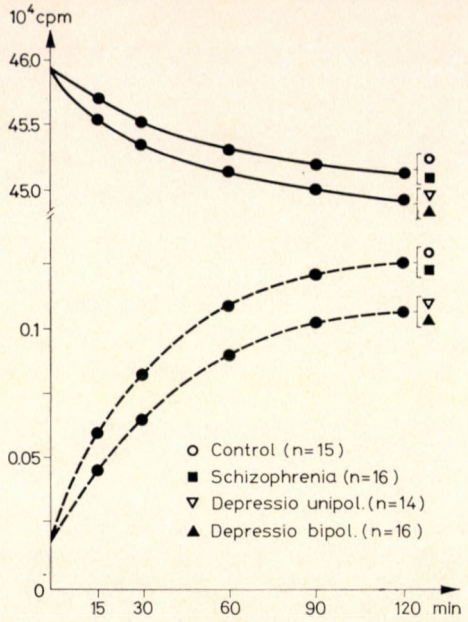


Fig. 3. Change in total activity of the inorganic phosphate pools in different diagnostic groups. Solid lines: extracellular P_i pool (cpm per 4 ml Krebs–Ringer solution). Dotted lines: intracellular P_i pools (cpm per ml red blood cell). Each point of the curves represents the mean value for the experiments in different diagnostic groups. Standard deviations are within 10%

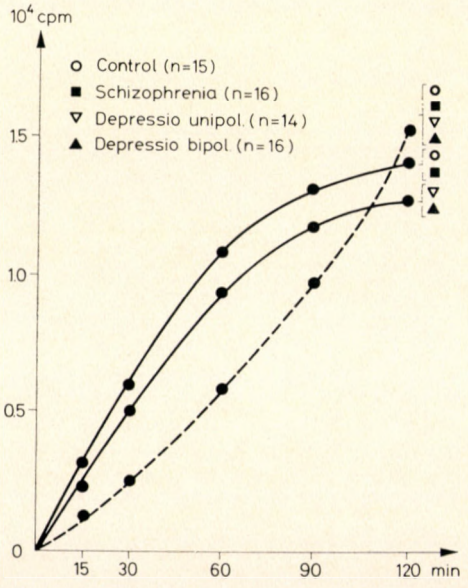


Fig. 4. Change in total activity of the organic phosphate pools in different diagnostic groups. Solid lines: acid-labile phosphate pools (cpm per ml red blood cell). Dotted lines: acid-resistant phosphate pool (cpm per ml red blood cell). Each point of the curves represents the mean value for the experiments in different diagnostic groups. Standard deviations are within 10%

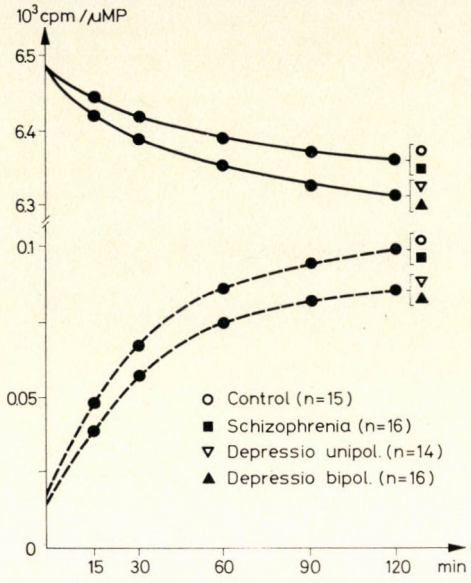


Fig. 5. Specific activity curves of the inorganic phosphate pools. Solid lines: extracellular P_i pools. Dotted lines: intracellular P_i pools. Each point of the curves represents the mean value for the experiments in different diagnostic groups. Standard deviations are within 10%

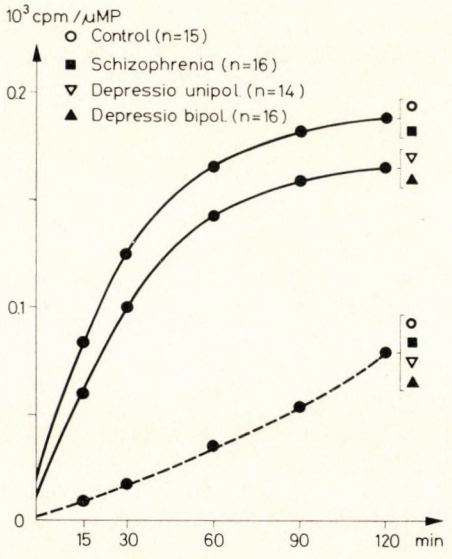


Fig. 6. Specific activity curves of the organic phosphate pools. Solid lines: acid-labile phosphate pools. Dotted lines: acid-resistant phosphate pool. Each point of the curves represents the mean value for the experiments in different diagnostic groups. Standard deviations are within 10%

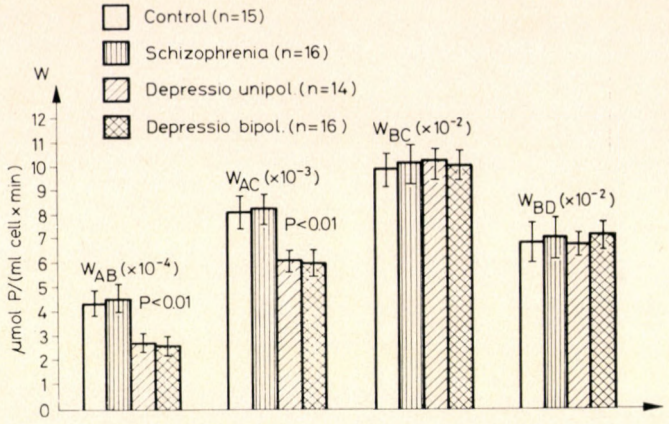


Fig. 7. Exchange rate values between the phosphate pools (shown in Fig. 2) in different diagnostic groups. Rate values were calculated from the kinetic curves in Figs 3—6

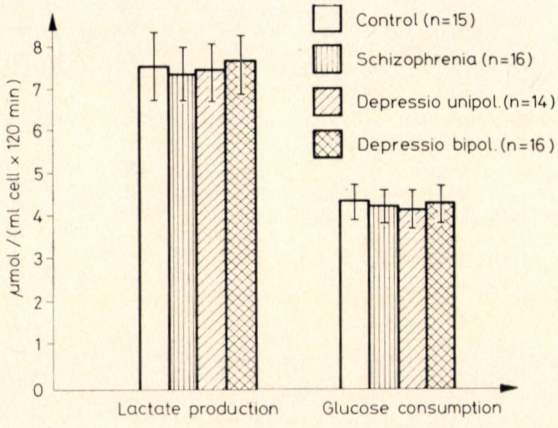


Fig. 8. Glucose consumption and lactate production during incubation in different diagnostic groups

in the case of chronic schizophrenia, a significant decrease of the passive phosphate uptake was demonstrated in plasma medium ($P < 0.01$). At the same time, there was no difference in the incorporation of ^{32}P-phosphate into the acid-labile phosphate fraction of RBCs. In the case of unipolar and bipolar depression, the values of the phosphate uptake were significantly lower in both pathways, even the experiments carried out in plasma medium.

According to our experimental results on the phosphate uptake into the RBCs of patients with endogenous depression, the decreased phosphate uptake and/or incorporation into acid-labile phosphate fractions representing the ATP, cannot be associated with the decrease of the ATP turnover. In this case the rate of glycolysis and, therefore, the intensity of lactic acid production and

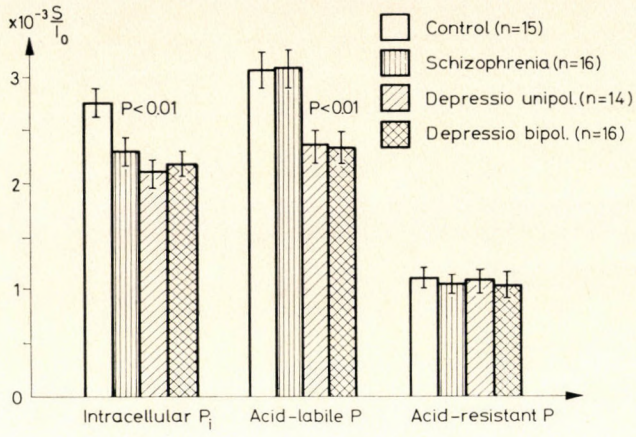


Fig. 9. Incorporation of the extracellular ^{32}P into intracellular phosphate pools during incubation in plasma medium

of the loss of glucose would have to change, unless we suppose the more or less isolated damage of a certain partial process of the glycolysis.

In connection with this problem, the compartmentalization of the enzymes within RBC arises: decrease in the quantity of the inorganic phosphate in the intramembrane compartment may result in decreased activity of the glyceraldehyde-3-phosphate-dehydrogenase phospho-glycerate-kinase enzyme system localized to the membrane [37, 72, 95, 105, 113]. This leads to slowing-down of the ATP synthesis taking place on the membrane in the RBCs of patients with endogenous depression. The results of our experiments carried out in Krebs–Ringer phosphate medium with washed RBCs of patients suffering from unipolar and bipolar affective psychoses suggest the disturbance of the membrane function affecting the anion transport mechanism. Thus, our results are in line with the literary data, *viz.*, in psychotic phases of affective psychoses disturbances can be observed in the "generalized membrane defect" [45, 79, 80].

In patients with chronic schizophrenia, decreased passive phosphate uptake could be demonstrated only in plasma medium. This is an agreement wtih the results of USUNOFF et al. [123], that the RBCs of schizophrenic patients take up iorganic phosphate at a lower rate. This deviation cannot be demonstrated in Krebs–Ringer phosphate medium, suggesting the contribution of plasma factors in the changed membrane transport processes.

Literary data and our experimental results suggest that studies on pathological aspects of membrane research may open new perspectives in the better understanding of the pathomechanism of several neuropsychiatric diseases.

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EFFECTS OF FEREBRAIN ABLATION ON GROWTH IN TELEOST FISHES

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Abstract

The effect of forebrain extirpation on food uptake and growth was studied in different teleost species. In the case of wild species such as *Carassius auratus gibelio* and *Macropodus opercularis concolor*, a prolonged enhancement of food uptake and body growth was found following forebrain ablation. In the case of domesticated species such as the common goldfish *(Carassius auratus)* and *Macropodus opercularis*, there was no effect of forebrain extirpation on either food uptake or growth.

Introduction

Ablation of the forebrain in various species of teleosts has been shown to result in marked deficits in avoidance conditioning, reproductive behaviour, parental activities, aggressive behaviour and schooling. In some cases the effects of forebrain ablation are unclear. WARREN [7] reported that fish without forebrain produce more errors than normals during reversal learning. However, INGLE [4] found just the opposite, with forebrain-ablated fish learning more quickly than normal fish in a Y-maze during successive reveals. The role of the forebrain in feeding behaviour also needs further clarifying. There was no effect of forebrain ablation on feeding in experiments made by SEEGAR [6], while during electrophysiological stimulation of certain forebrain areas, the complete feeding response was observed by BERNSTEIN [2]. PRIVAT [5] also found a change in the feeding response, depending on the light intensity after forebrain ablation.

In the present experiments, we found that the effect of forebrain extirpation on feeding was also expressed in an enhanced growth of the fish.

Materials and Methods

Animals

The following species of fish were used: common goldfish *(Carassius auratus)*, six months old from the stock of our laboratory, triploid clonal goldfish *(Carassius auratus gibelio)*, six months old. The stock was based upon the progeny of one female caught in the Danube

near Ercsi in Hungary several years ago and propagated every subsequent year by gynogenesis using carp sperm. Only one female was used as mother in every year. The individuals of this stock are genetically identical sisters.

Paradise fish (*Macropodus opercularis* Linné), 3 months old from the 12th generation of a closely inbred laboratory strain. Black paradise fish (*Macropodus opercularis concolor*), six months old. The original stock was obtained from the Far East and maintained in our laboratory by random breeding. Ten fish were used in each experimental group.

Surgical procedures

Forebrain ablation was performed in the following way. After anesthetization in 0.5% MS solution, the fish was put on a wet sponge and a 2mm diameter hole was bored in the skull by a dental drill. The fatty tissue overlaying the forebrain was sucked throught a glass tube probe. For fish in the sham operation group no additional tissue was removed, for fish in the ablation group both lobes of the forebrain were also removed. After the appropriate tissues had been removed the hole in the skull was filled with gel foam and the fish was put back in water. The whole operation was finished within 40 sec. The mortality rate was below 10% .

Upon completion of all experimental procedures, the fish was again anesthetized and then sacrificed to allow verification of the surgical procedures. The operations were very consistent, resulting in about 90% telencephalic extirpation, leaving only a small amount of tissue in the region of the preoptic nucleus. During the experimental period, no sign of regeneration of the forebrain tissue was observed macroscopically. In some cases, the removal of the olfactory epithelium was performed by burning out the nasal cavity with an electric device, under anesthesia. For visual deprivation in some animals both eyeballs were removed, under anesthesia.

Postoperative care and measurement

The operated and the control animals were maintained in groups in 40 l tanks or individually in 4 l tanks. Each tank was provided with an air stone and an inner filter. The tanks were filled with tap water, changed weekly, and maintained at room temperature, and were lit with fluorescent light for 12 h/day. The fish were fed live tubifex worms ad libitum. In some cases we collected and measured the worms that had remained in the tank after the illumination period to calculate the total amount consumed. At regular intervals, body weight was measured separately in the morning before feeding had started.

Results

The food uptake of forebrain-ablated clonal goldfish was greatly enhanced after operation (Fig. 1). Also, there was a great increase in body weight of the operated group (Fig. 2). As the food uptake of the fishes correlates with body weight, it is meaningful to compare the $\frac{\text{food uptake (g)}}{\text{body weight (g)} \cdot \text{day}}$ values, which were significantly higher for the operated group than for the control group in every day during the experimental period ($x = 0.088 \pm 0.01$ *vs.* 0.058 ± 0.02; $P < 0.05$). Experiments in prolonged periods for up to one year have shown that forebrain ablation permanently changed the growth pattern of the clonal goldfish, rate of growth being constantly about 30% higher compared to the control.

We performed experiments to find explanations for this effect. Considering that extirpation of the forebrain destroys olfaction ability, we removed the olfactory epithelium of a group of fish by burning and compared their

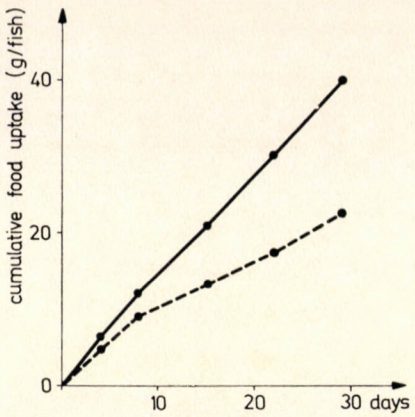

Fig. 1. The effect of forebrain extirpation of food uptake of clonal goldfish. The sum of food uptake was measured in two groups of clonal goldfish housed separately. The control group (dotted line) consisted of ten sham-operated fish (mean weight in the first day after the surgery: 13.8 g). The other group (continuous line) consisted of ten forebrain-extirpated individuals (mean weight: 12.1 g)

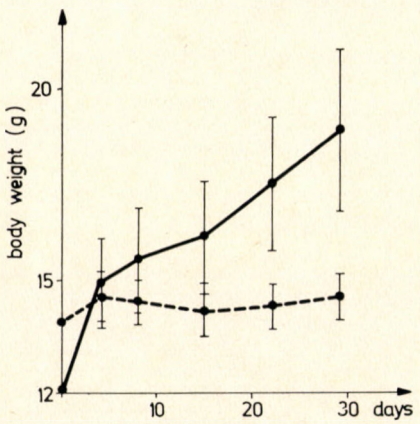

Fig. 2. The effect of forebrain extirpation on body growth of clonal goldfish. The changes in body weight/fish of the same two groups of clonal goldfish as in Fig. 1

growth to the untreated controls (Fig. 3). Growth was not altered by the cessation of olfaction in the presence of the forebrain. During the experimental period, no regeneration of the olfactory epithelium was observed.

The clonal goldfish is a very shy animal and reacts extremely fearfully to every unfamiliar visual stimulus. If the increased food uptake were a result of some damage to the optic tract having occurred during the forebrain extirpation, removal of both eyes also would enhance growth. Experiments with enucleated fishes excluded this possibility (Fig. 4).

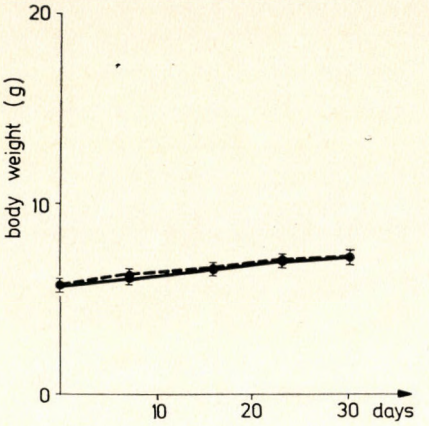

Fig. 3. The effect of the removal of the olfactory epithelium on the growth of clonal goldfish. The olfactory epithelium of ten clonal goldfish were removed (continuous line) and the group was mixed with ten untreated controls (dotted line), and the body weights were measured

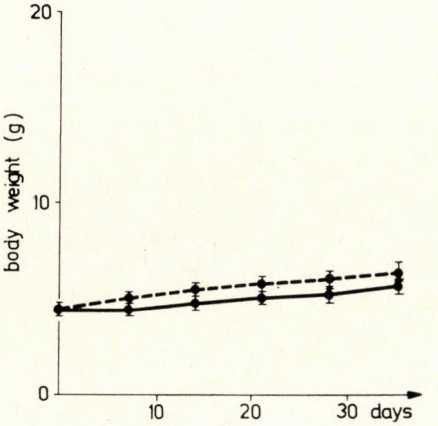

Fig. 4. The effect of enucleation on the growth of clonal goldfish. Ten clonal goldfish were enucleated (continuous line), the group was mixed with a group of ten untreated controls (dotted line) and the body weights were measured

When we wanted to check the generality of these findings on different species we found some unexpected results which may be the key to the whole problem.

Common goldfish *(Carassius auratus)* is the closest relative of the clonal goldfish, both originating from a common diploid ancestor. We tested the effect of forebrain extirpation on the growth in a stock of common goldfish. The operated animals were paired with the control ones and kept in separated 4-l tanks (Fig. 5). There was no effect following the forebrain removal on the growth of common goldfish, in contrast to the clonal goldfish.

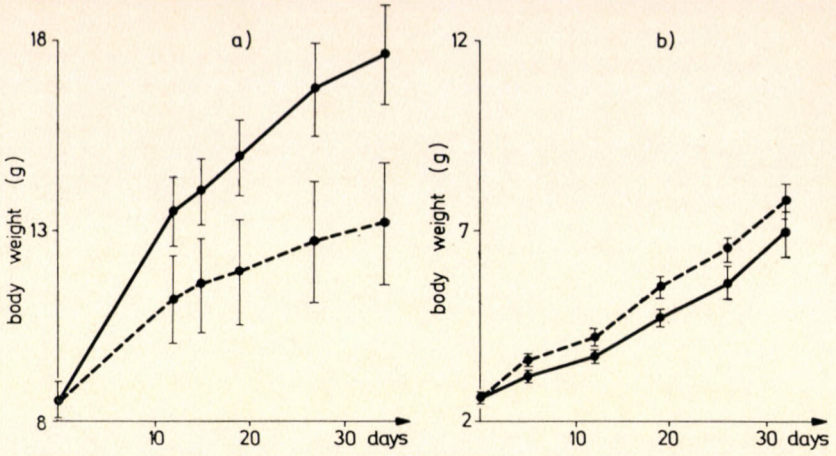

Fig. 5. Effect of forebrain extirpation on the growth of clonal goldfish and common goldfish. Pairs of forebrain-extirpated (continuous line) and shame-operated fish (dotted line) of the same weight were housed separately. Clonal goldfish (A) and common goldfish (B). The body weights were measured

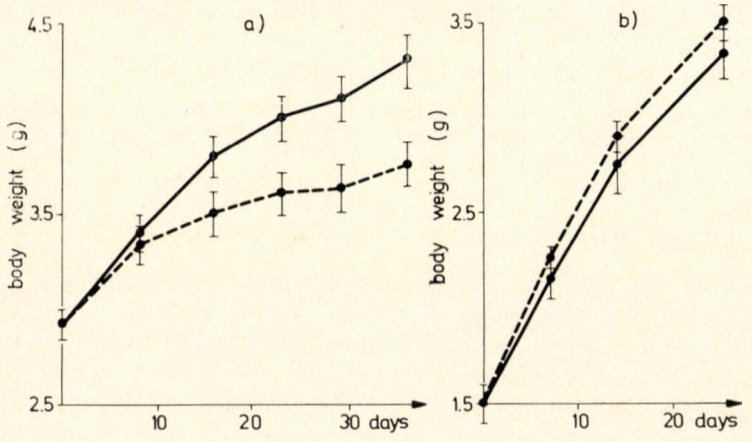

Fig. 6. Effect of forebrain extirpation on the growth of Macropodus opercularis and Macropodus opercularis concolor. Pairs of forebrain-extirpated (continuous line) and shame-operated fish (dotted line) of the same initial weight were housed separately. *Macropodus opercularis concolor* (A) and *Macropodus opercularis* (B). The body weights were measured

We looked for another pair of wild type — domesticated type of fish and choose *Macropodus opercularis* L. aquarium-bred in Europe for one and a half century; and a subspecies *Macropodus opercularis concolor*, a recent import from the Far East. Extirpation of forebrain did enhance growth in the wild form but had no effect on the domesticated one as was the case with the goldfish (Fig. 6).

Discussion

The forebrain of teleosts is a subject of intensive research. A role of integration of sensory stimuli and different motivational processes resulting in smooth tuning of overt behaviour has been suggested [1, 3]. We have found that a prolonged enhancement of food uptake followed by intensive growth are the results of the extirpation of the fish forebrain. Such a characteristic response to forebrain removal was observed only in the case of wild-type animals *(Carassius auratus gibelio, Macropodus opercularis concolor)*. In domesticated species *(Carassius auratus, Macropodus opercularis)* there was no effect following forebrain ablation either on food uptake or on growth.

It seems that the forebrain has a pronounced inhibitory function in the complex regulatory system of food uptake. If the forebrain is removed surgically, this system will be free of its inhibition and the daily food uptake will rise up by 30%. This increment in food uptake is followed by enhanced growth.

The enhancement of food uptake is not the result of some sensory deprivation, for in experiments where either olfaction or vision have been eliminated there was no change in food uptake.

The most curious result is that the inhibitory function of the forebrain seems to be non functional in the case of domesticated species. The extirpation of the forebrain caused no changes in the level of food uptake, which is normally higher than in wild type species.

Perhaps the inhibitory function of the forebrain was lost due to genetic changes occurring during domestication. Those genes or gene groups which are involved in the inhibitory function of the fish forebrain may be discovered by appropriate breeding experiments of the *Macropodus* species. This would be the way to reveal the exact physiological mechanisms of the inhibitory function, too. Our future work will follow this line of investigation.

Aknowledgement

We wish to thank Mrs Edith Somogyi and Miss Ildikó Simon for technical help.

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DETERMINATION OF DOSE AND TIME DEPENDENCE OF MUSCLE CONTRACTURES ELICITED BY CAFFEINE

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Abstract

Caffeine contractures were registered on fibre bundle preparations of frog m. semitendinosus. The drug was applied to muscles in normal Ringer for 30 min in concentrations of 3—40 *mM*. Biphasic contractures were found in all cases above mechanical threshold concentration of caffeine (2.35 *mM*. calculated value). Parameter values belonging to the two contracture phases (amplitude, Δl_1 l_2; time, t_1 t_2) were analysed by computer programmes in correlation with caffeine concentration. It was found that 1. both contracture phases have the same concentration threshold of caffeine; 2. a caffeine-binding site with $K_d = 24.6$ *mM* can be supposed in the surface membrane being responsible for the induction of the first contracture phase of caffeine in the muscle; 3. caffeine has a concentration-dependent action on the surface membrane binding site with a tendency of muscles to contract to zero length at infinite caffeine concentration; 4. the speed of caffeine action is practically equal to the solution exchange emphasizing the action site at the surface membrane of the muscle (sarcolemma and T-tubule membrane); 5. the second caffeine contracture phase develops with maximal amplitude above contracture threshold concentration, during which muscles contract to about 40% of their slack length; this corresponds to a sarcomere length less than that of the myosin filament, consequently, a shortening of this filament must also take place; 6. the concentration dependence of the second contracture phase results in a monotonic decrease of t_2 with increasing caffeine concentration.

A possibility for the mechanism of caffeine action through binding to proteins involved in the regulation of intracellular calcium level is discussed.

Introduction

The effect of caffeine on the mechanical response of skeletal muscle depends on the caffeine concentration, on the time for which caffeine is allowed to act, on the type of muscle preparation (single or multifibre), and also on the mode of registration (isometric or isotonic), *etc.*

The threshold concentration of caffeine to elicit muscle contracture proved to be 2—3 *mM* in different experiments. Above this concentration, the caffeine action is graded, the measure of tension development or the size of contracture amplitude increases with increasing caffeine concentration [2].

Caffeine contractures of muscle show a time-dependence. On single fibre preparations, the tension or the contracture amplitude has irregular

alterations [11]. On whole muscles or multifibre preparations, however, in spite of the dependence of caffeine effect on diffusion, the alteration of contracture phases with time was found to be more systematic: there has been described an initial rapid phase [4] as well as a first and a second phase [12, 14] of caffeine contractures separable by their pharmacological sensitivity and reversibility, too.

In our earlier experiments on fibre bundle preparation of frog semitendinosus muscle depolarized in potassium solution, some changes were found in the course of development of caffeine contractures compared to that of control muscle. These changes depended on the anion species in the solution, and on the time of depolarization; they were also observed for several minutes after repolarization of the muscle in normal Ringer [9].

In the experiments to be described here it was also found that caffeine contractures are always biphasic on multifibre preparations if the caffeine is left on the muscles for enough time. Concentration and time dependence of the caffeine effect on muscles were determined by mathematical analysis of values of parameters, selected for characterizing the phases of caffeine contractures. These values were measured in several experiments and the expected values of them were calculated as the function of caffeine concentration by curve-fitting method.

Materials and Methods

Fibre-bundle preparations of frog *(Rana esculenta)* muscle containing 100—200 fibres were dissected from m. semitendinosus. They were mounted in Sartorius vessels, their upper ends being fixed to a torsion lever arm. The slack length was measured on muscles suspended in solution. They were then stretched to 125—130% of their slack length by increasing torsion force. Mechanical responses were recorded on smoked drum using a kymograph. The speed of registration as well as changes in muscle length and tension were controlled repeatedly.

Resting tension of stretched muscles was about 1 g/mm^2 and did not change more than 3fold during maximal contracture (at 35—40% of slack muscle length), registration conditions were therefore regarded isotonic.

Normal Ringer was freshly prepared from substances of analytical grade (Reanal, Budapest) in the following composition: NaCl 115 *mM*, KCl 2.5 *mM*, $CaCl_2$ 1.8 *mM*, and histidine 5 *mM*, pH = 7.2. Caffeine was dissolved in Ringer in concentrations between 3—10 *mM*. Above 10 *mM* caffeine concentration osmotically equivalent amount of NaCl was omitted.

Solution exchange in the Sartorius vessel was effected by flushing solution in 5 fold volume of the vessel with a syringe from below within 5 sec. Excess of solution was drained from above. In this way, mechanical artifact of the solution exchange was effectively diminished. Caffeine-containing solutions were left on each muscle uniformly for 30 min.

To be able to compare the results gained on different muscles as well as for the sake of calculation process, we chose characteristic parameters (Fig. 1) and determined their normalized values in the individual experiments. These parameters were: the time necessary to the full development of each phase of the contracture (t_1 and t_2) and the amplitude of them (Δl_1 and l_2*. In order to find principal correlations, values of the parameters were plotted against caffeine concentrations and simple (cone section and exponential) functions in different analyt-

* Different dimensions for the amplitude of the first and second contracture phase (length change = Δl_1, or absolute length = l_2) can be reasoned by their different dependence on caffeine concentration (see below).

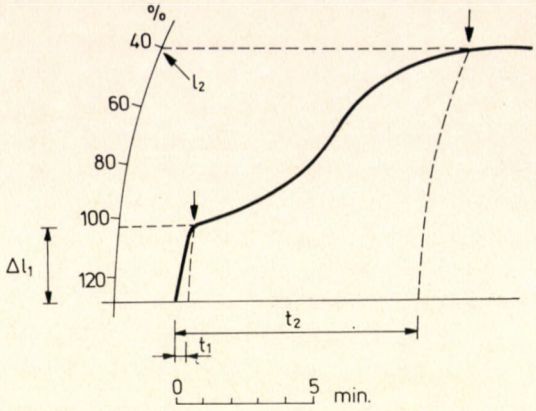

Fig. 1. Parameters of caffeine contractures of muscles indicating the amplitude (Δl_1, l_2) and time of development (t_1, t_2) of the first and second contracture phases

ical forms were fitted to them using least-square and maximum-likelihood methods. Calculations were done by computation, using fitting and error function calculating programme. Goodness of fitting was controlled by the calculated values of error function. To reduce the scattering of the measured values regarding the same parameter and caffeine concentration, their selection was carried out using another computation programme based on arithmetic mean and standard deviation. Some stages of this stepwise process are sometimes indicated in the figures.

Starting populations of parameter values were collected in experiments carried out on 113 different muscles. Caffeine concentrations applied and the number of muscles (in brackets) used at the given caffeine concentration were as follows: 3 *mM* (4), 4 *mM* (9), 5 *mM* (5), 6 *mM* (9), 8 *mM* (15), 10 *mM* (13), 13 *mM* (13), 16 *mM* (15), 20 *mM* (10), 25 *mM* (6), 30 *mM* (9), 40 *mM* (5).

Using parameter values indicated by the well-fitting functions, contracture curves modelling caffeine effect were constructed by projecting characteristic individual curves to the parameter values (Figs 1 and 2).

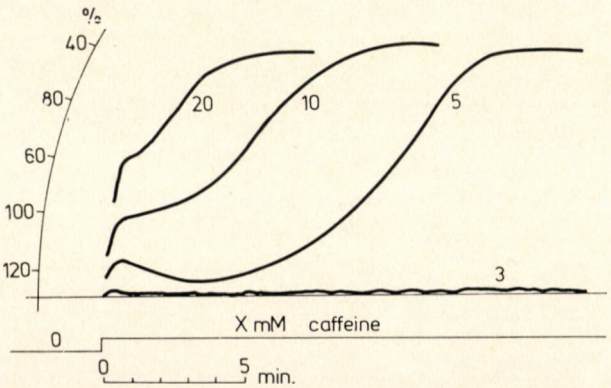

Fig. 2. Model caffeine contracture curves gained on the effect of different caffeine concentrations. Expected values (see Fig. 1) of contracture parameters were calculated by curve-fitting method. Time course of the development of curves belonging to different caffeine concentrations were redrawn by projection of characteristic individual curves to the calculated values of the parameters (not indicated in the figure)

Results

Biphasic character of caffeine contractures

On modelled curves (Fig. 2) characteristics of caffeine effect are visualized. Separation of the contracture curves into two phases is well seen on the curve belonging to 5 *mM* caffeine. The first phase develops quickly, then a tendency for relaxation can be observed. The second phase starts to develop in this case with some latency but it is completed relatively quickly afterwards. On curves belonging to 10 and 20 *mM* caffeine this latency period is shorter and the tendency for relaxation decreases. Transition between these two phases can be assigned, however, at the higher caffeine concentration as well.

Concentration dependence of t_1

The t_1 values and the fitted

$$t_1 = Ax + B \tag{1}$$

function are shown in Fig. 3, with calculated constants A = 0.329 and B = = 6.16 sec. The large scattering of t_1 values can be explained by the relatively low time-resolution of the registration applied, and by the variation in the diameter and sensitivity of the muscle preparations. In spite of this, the constant 6.16 sec indicates a fast development of the first caffeine contracture phase practically equal to the time of solution exchange in the Sartorius vessel. This fact strongly suggests a site of caffeine action in or near to the structural elements of the surface membrane.

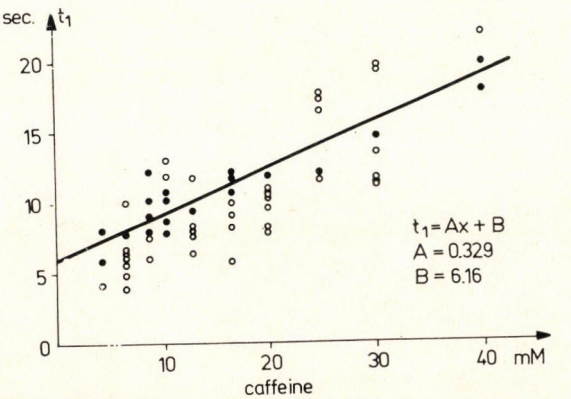

Fig. 3. Concentration dependence of t_1. Symbols indicate two different stages of data selection. The open and filled circles together indicate an early, while filled circles only the final stage of selection. Calculated values of the constants and best-fitting curve are also indicated

Concentration dependence of Δl_1

By curve-fitting calculations a hyperbolic correlation described as

$$\Delta l_1 = \frac{A}{x - B} + C \tag{2}$$

was found to fit with high probability to the values of the amplitude of the first contracture phase plotted against caffeine concentration (Fig. 4). It

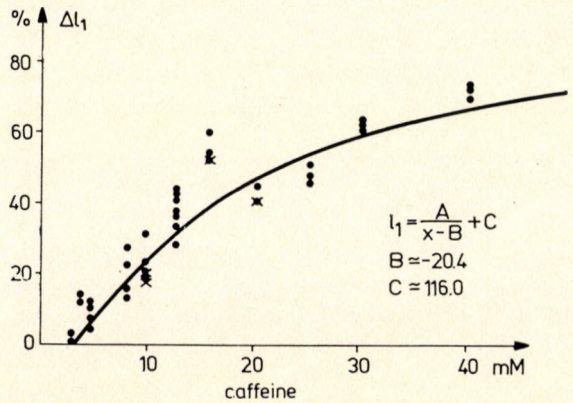

Fig. 4. Concentration dependence of Δl_1. Points represent data after selection. Crosses indicate doubled values. Constants and fitted curves are also indicated

occurred that definite physical meaning can be attributed to the different constants. B = —20.4 *mM* has an "apparent" Michaelis constant character [10], while C = 116% indicates a maximal contracture of muscle at infinite caffeine concentration. The latter value is quite close to the stretched muscle length (125—130%), therefore it shows as if muscles could contract practically to zero length at high caffeine concentrations.

Data are accumulating about the binding of caffeine to proteins [1], therefore, it seemed reasonable to suppose a binding site for caffeine in the surface membrane of the muscle, where its binding may follow the mass law. Caffeine effect is supposed to be proportional to the concentration of the binding site — caffeine complex: $\Delta l_1 \sim [PC]$ where [PC] is the concentration of protein-caffeine complex. The dissociation constant of the binding site can be expressed as

$$K_d = \frac{([P_0] - [PC])([C_0] - [PC])}{[PC]} \tag{3}$$

where $[P_0]$ and $[C_0]$ are the concentrations of the binding site and caffeine, respectively. Further,

$$[PC] = \frac{[P_0]([C_0] - [PC])}{K_d + ([C_0] - [PC])} \tag{4}$$

if $[P_0] \ll [C_0]$ and $[PC] \ll [C_0]$, then

$$\Delta l_1 \sim \frac{[P_0]\,[C_0]}{K_d + [C_0]} \tag{5}$$

appears to be a proper approximation. With appropriate normalization and taking into account a mechanical threshold concentration of caffeine C_m (subthreshold caffeine concentrations may potentiate muscle twitch on electrically stimulated muscles while they do not cause contracture themselves, see *e.g.* Ref. [11])

$$\Delta l_1 = A\,\frac{[C_0] - [C_m]}{K_d + ([C_0] - [C_m])} \tag{6}$$

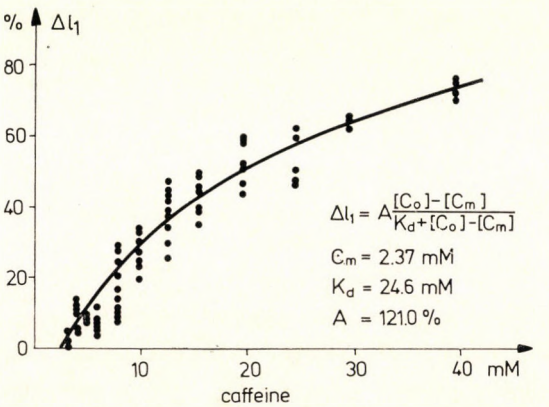

Fig. 5. Concentration dependence of Δl_1: fitting of a function which contains K_d and constant of threshold concentration C_m. Population of points were gained after moderate selection

correlation is gained. It was also successfully fitted to the Δl_1 values (Fig. 5) and constants of the function were on the one side similar to those determined by the hyperbolic correlation ($A = 121\%$ is rather close to $B = 116\%$ in eq. (2), and its meaning can be the same), on the other side their physical meaning occurs to be more clear: $K_d = 24.6\ mM$, and $C_m = 2.37\ mM$.

It can be concluded from these data that at the mechanical threshold less than 5% of the receptor sites are occupied by caffeine, while their full saturation induces theoretically a maximal muscle contracture (to zero length).

Concentration dependence of t_2

A hyperbole of (2) type fitted well to the points of t_2 values but with increasing caffeine concentrations time values decreased monotonically (Fig. 6). In this case $B = 2.35\ mM$ can be regarded another determination of the C_m, naturally for the second caffeine contracture phase. Several experiments

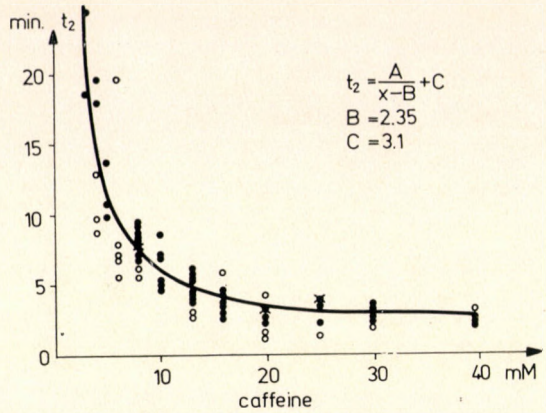

Fig. 6. Concentration dependence of t_2. Symbols as in Figs 3 and 4. Fitted curve and calculated constants are indicated

prove, however, that threshold caffeine concentration for both phases is the same. This is nicely proved by the close values of C_m and B.

As the C = 3.1 min value shows, there is a limit of time which is needed to the full development of the second contracture phase. This involves the diffusion of caffeine to the site of action, the liberation of calcium in an amount which is enough for the full activation of the contractile elements, and the development of muscle contracture itself. In the lack of detailed information, the rate-limiting step of the process can not be predicted as yet.

Concentration dependence of l_2

The second phase of caffeine contracture develops practically with maximal amplitude above threshold caffeine concentration (Fig. 7). The

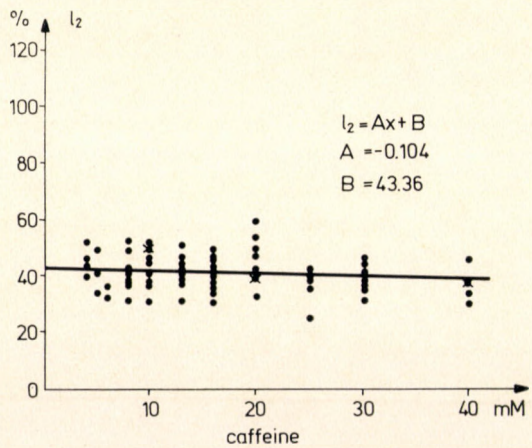

Fig. 7. Concentration dependence of l_2

length of muscle at maximally contracted state (40% of the slack length) characterizes a non-physiological mechanism for contraction at which sarcomere length can only be a fraction of that of the myosin filament, that is, myosin filament must also be shortened [8].

Discussion

Caffeine exerts its effect on muscle by activating its mechanical machinery through an elevation of intermyofibrillar calcium level. There are morphological proofs that it causes swelling of the SR[1] [7, 15]. Its effect is more pronounced on a heavy fraction of FSR[2] [6, 13] in which elements of the T—TC[3] junction are enriched.

Although caffeine is claimed to act through the sensitization of calcium-induced calcium release [5], there has not been observed any inhibition of calcium-liberating action of caffeine in the absence of calcium (buffering the FSR medium by high EGTA[4] concentration).

Similarly to BSA[5], caffeine is binding to several types of proteins and more effectively to FSR as proved by PMR[6] investigations (KÓNYA et al. unpublished results). The mechanism of its interaction with proteins, and details of its calcium-liberating effect has not been proved yet. Its combination with calcium-binding calsequestrin has been proved only indirectly (*e.g.* by the mentioned morphological observations). Its action is less clear on the surface membrane. It seems to induce a coupling mechanism without depolarization of the surface membrane [2]. Combination of caffeine with intrinsic proteins of the surface membrane of muscles with consequent calcium liberation from membrane binding sites (S and R calcium, see Ref. [3]) as well as an initiation of a calcium-induced calcium release mechanism of the junctional surface of the terminal cisternae needs further proof about the existence and characteristics of such calcium stores [9].

[1] = sarcoplasmic reticulum

[2] = biochemically prepared fragmented SR containing SR vesicles with well-preserved structure and function,

[3] = junctional structure constituted with the participation of the transversal-(T)-tubules and the terminal cisternae (TC) of the longitudinal (L) elements of the sarcotubular system. L-elements including TC compose the so-called SR.

[4] = ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid,

[5] = bovine serum albumin,

[6] = proton magnetic resonance spectroscopy.

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ULTRASTRUCTURE OF SYNAPSES IN THE CENTRAL NERVOUS SYSTEM OF LAMELLIBRANCH MOLLUSCS

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Abstract

Fine-structural characteristics of synaptic contacts were investigated in the central nervous system of different species of lamellibranch molluscs. Neuropile of the ganglia is characterized by regular occurrence of ultrastructurally well-defined polarized chemical synapses resembling those described in other invertebrate species and vertebrates. In addition to the generally observed membrane thickenings, enhanced density of synaptic membranes, cleft material and vesicle clustering on the presynaptic membrane, synapses are occasionally characterized by other pre- and postsynaptic specializations, like subsynaptic cistern, axon spine and pinocytotic invaginations. Synaptic connections were distinguished on the basis of the vesicle content of the presynaptic terminal. Different forms of synaptic configurations (divergence, convergence, presynaptic modification) were observed in the ganglia.

Introduction

Biochemical [21, 22, 23, 39, 42, 43] and fluorescence histochemical [24, 38, 40, 47] investigations have shown that biogenic monoamines occur in high concentrations and can be considered transmitter substances in the ganglia of lamellibranch molluscs. The role of biogenic monoamines in the central regulation of periodic activity [23, 35] and in the regulation of other physiological processes, like gill movement or smooth muscle contraction seems to be of great significance [1, 5, 20,].

There is little information concerning the morphological basis of chemical transmission in the lamellibranch CNS [7, 24, 48]. Several investigations on the molluscan CNS underlined the very rare occurrence, or lack, of true, ultrastructurally well-defined, polarized chemical synapses [7, 8, 12, 14, 32, 33]. It was suggested that chemical transmission in molluscan CNS is realized at axon varicosities possessing no pre- and postsynaptic membrane specializations [2].

Taking into account the above findings, we have undertaken the present work in order *(i)* to investigate the fine structural characteristics of synaptic connections in the CNS of lamellibranch molluscs; *(ii)* to provide fine struc-

tural data for transmission processes taking place in the ganglia; *(iii)* to compare the fine structural characteristics of synaptic connections found by us with findings in different phyla.

Materials and Methods

The following species were examined: *Anodonta cygnea* L., *Mactra stultorum* L., *Glycimeris pilosa* L., *Solen vagina* L., *Venerupis decussata* L. The latter four species were collected in the Bay of Naples.

Ganglia of the fresh-water bivalve *Anodonta cygnea* L. were fixed for 2h at room temperature in 3% glutaraldehyde diluted in MARCZYNSKY's [28] physiological solution, or sometimes in *Anodonta* haemolymph. After brief washing, the ganglia were postfixed for 30 min at 4 °C in 2% OsO_4 buffered with s-collidine. After dehydration in graded ethanol and propylene oxide, ganglia were embedded in Araldite (Durcupan, ACM, Fluka).

Sometimes, *Anodonta* ganglia were fixed only in 2.5% glutaraldehyde for 2 h at room temperature or overnight at 4 °C.

In the case of marine species, the ganglia were in most cases fixed in 2.5% glutaraldehyde buffered with 0.1 *M* cacodylate [29] for 2 h at room temperature or overnight at 4 °C. Then, following short washing in the same buffer, ganglia were postfixed in 2% OsO_4 buffered also with 0.1 *M* cacodylate for 30—60 min at 4 °C. Sometimes, ganglia were fixed only in 2% OsO_4 for 1—2 h at 4 °C. In the course of dehydration in graded ethanol and propylene oxide, block staining was performed with saturated uranylacetate in absolute ethanol for 30 min. After dehydration ganglia were embedded in epoxy resin [37].

Ultrathin sections, cut on LKB Ultrotome III or Reichert OMU_2 ultramicrotome and contrasted with uranylacetate and lead citrate [34] or only with lead citrate [46], were investigated in TESLA BS 413A, TESLA BS 500 and Philips 200 electron microscopes.

Results

Ultrastructural characteristics of synaptic connections

Synaptic contacts in the ganglia can well be seen at low magnification, since the enhanced density makes these sites prominent as compared to other parts of neuropile (Fig. 1). Following glutaraldehyde–osmium fixation, the synaptic connections in the neuropile of ganglia of both *Anodonta* and marine species are characterized by the enhanced density of the pre- and postsynaptic membranes (Fig. 2). In *Anodonta*, dense appositions characterizing synaptic membranes can be observed mainly on the presynaptic side (Fig. 2a, b). The presynaptic appositions may be as wide as the synaptic cleft (Fig. 2b). Postsynaptic membrane thickenings are never as pronounced as the presynaptic ones. In marine species, the synaptic membranes are, in general, similar to each other in fine-structural appearance (Fig. 2c); membrane thickenings and appositions may not be pronounced (Fig. 2d).

The 18—20-nm wide synaptic cleft is always filled with finely granulated material of intermediate density and is, in most cases, bisected by a dense line (Fig. 2a, c).

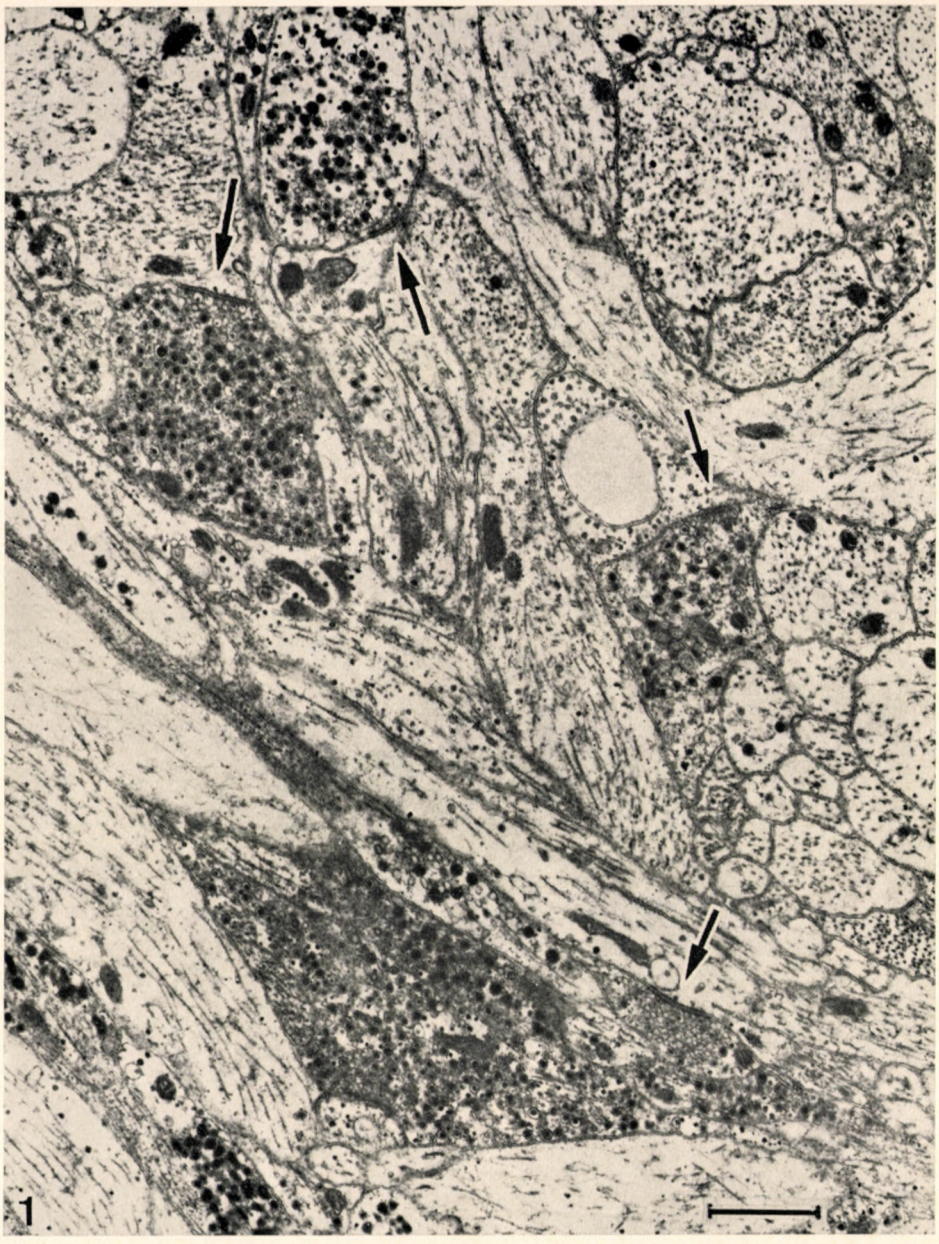


Fig. 1. Low-power electron micrograph taken from the neuropile of the pedal ganglion of *Anodonta cygnea*. Arrows indicate sites of synaptic connections. Glutaraldehyde–osmium fixation. Bar represents 1 μm

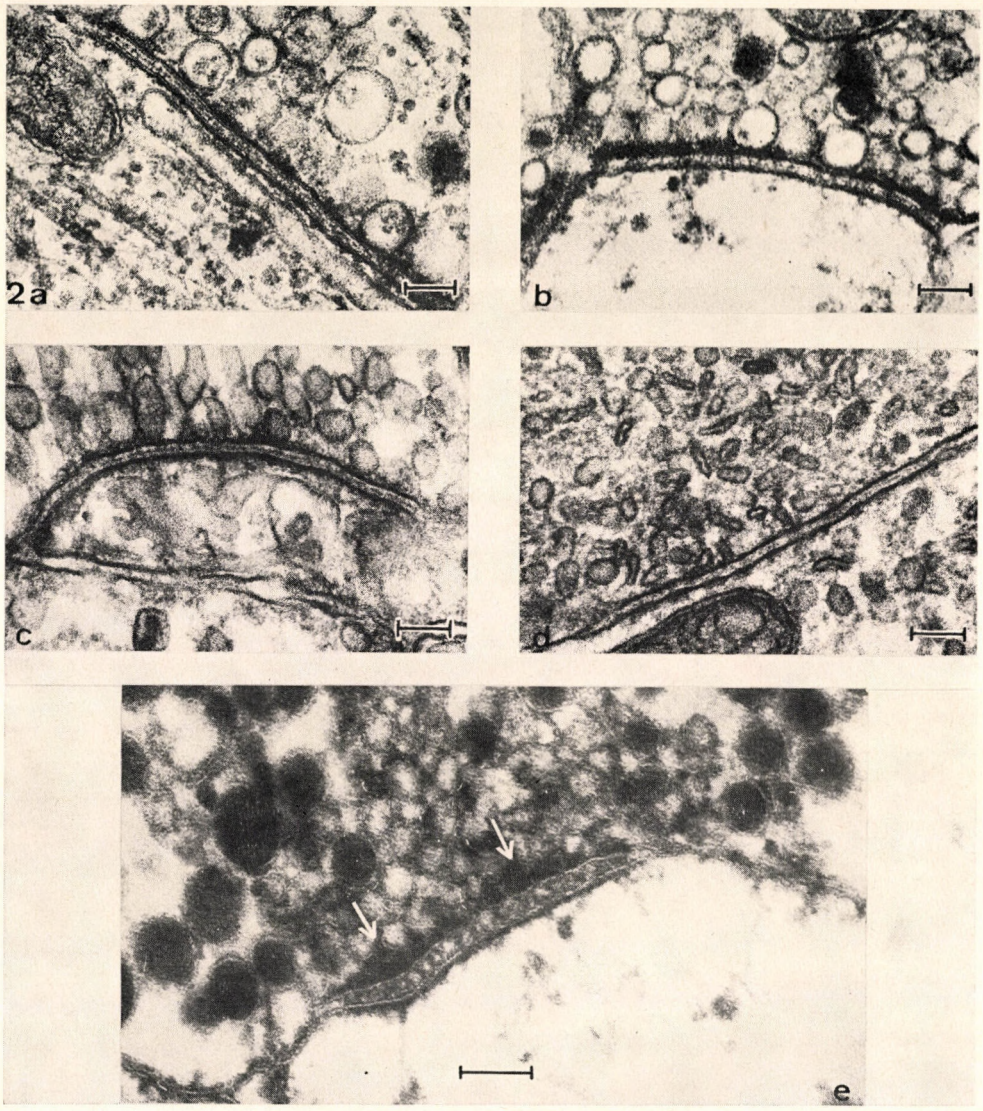
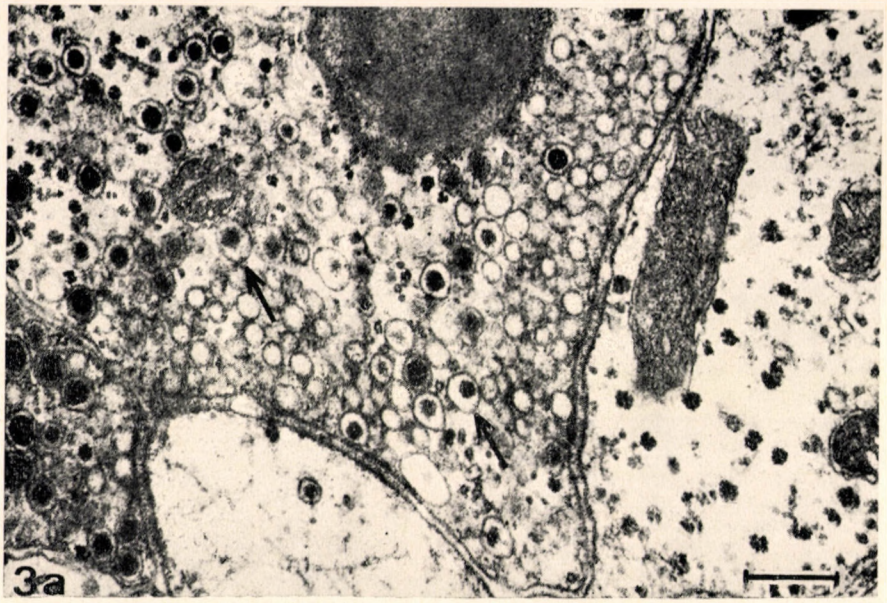


Fig. 2. Ultrastructure of synaptic contacts in the CNS of fresh-water and marine bivalves. a) *Anodonta cygnea.* Note the increased density of pre- and postsynaptic membrane and the characteristic arrangement of the intersynaptic cleft material. b) *Anodonta cygnea.* Apposition of thickened dense material along the total length of the presynaptic membrane. The width of the dense apposition is approximately equal to that of the synaptic cleft. c) *Glycimeris pilosa.* Synapse similar in ultrastructure to that in Fig. 2a. Note the dense line bisecting the cleft. d) *Solen vagina.* Synapse characterized by increased pre- and postsynaptic membrane density and finely-granulated cleft material. Some postsynaptic apposition can only be observed. e) *Anodonta cygnea.* Dense projections (arrows) are localized on the presynaptic membrane, and much thinner, continuous dense apposition is connected to the postsynaptic membrane. Note the inverse arrangement of intersynaptic cleft material compared to Figs 2a and c. Fig. 2a—d glutarladehyde–osmium fixation, 2e glutaraldehyde fixation. Bars represent 0.1 μm



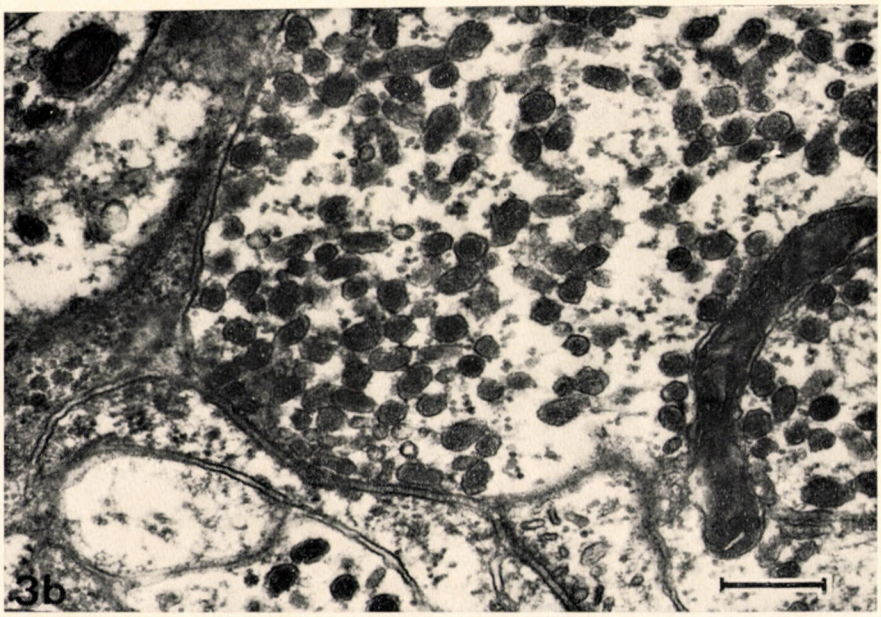


Fig. 3. Two typical presynaptic vesicle populations. a) *Anodonta cygnea.* Mixture of spheroid clear synaptic (50—70 nm), dense core (80—100 nm) and eccentric dense core (arrows, 120—140 nm) vesicles. b) *Glycimeris pilosa.* Terminal containing almost exclusively neurosecretory-like granules (100—200 nm) with variable form and finely granulated intragranular content. Note that along the presynaptic membrane only neurosecretory-like granules are situated. Glutaraldehyde–osmium fixation. Bars represent 0.25 μm

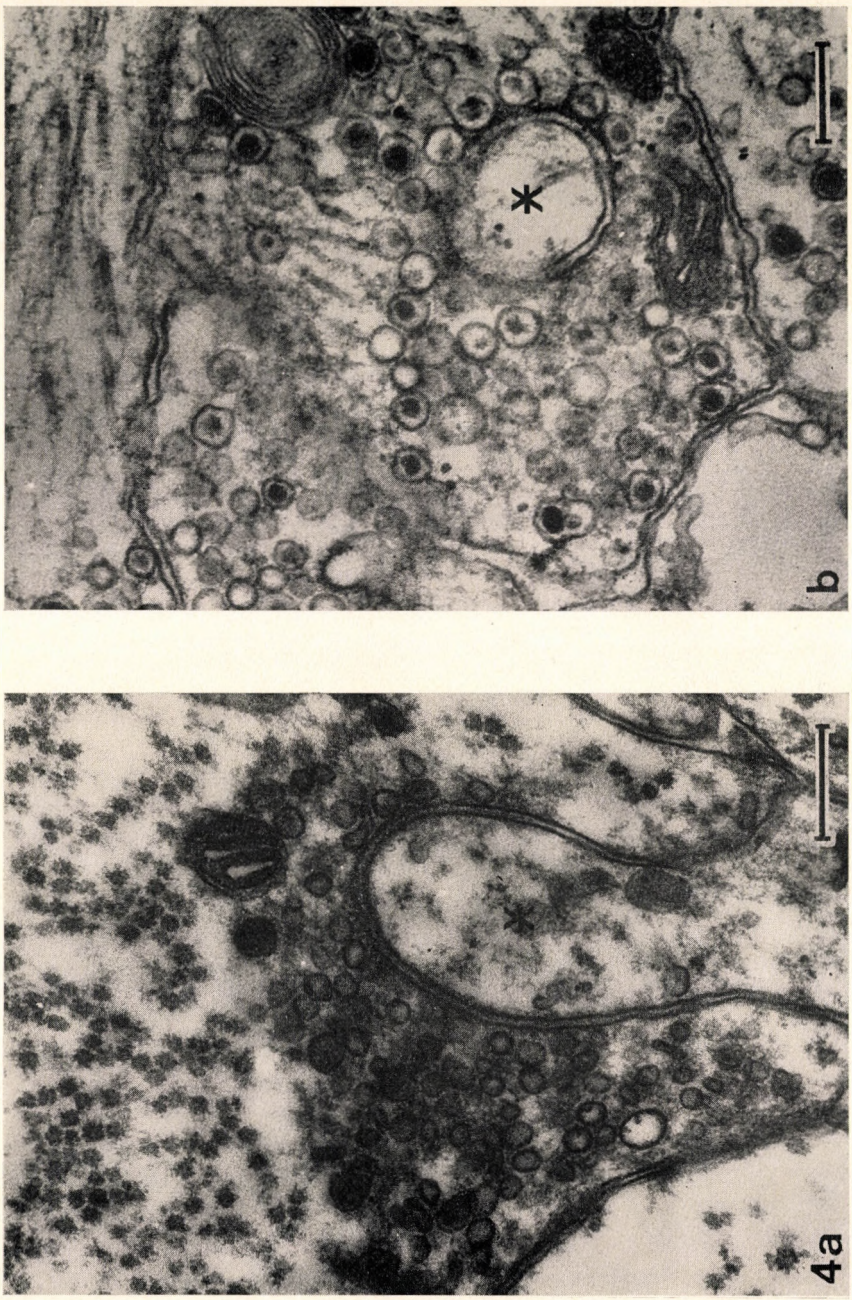


Fig. 4. a) Axon spine (asterisk) as a characteristic postsynaptic specialization. *Mactra stultorum*. b) Cross sectioned axon spine (asterisk). *Anodonta cygnea*. Glutaraldehyde–osmium fixation. Bars represent 0.25 μm

In *Anodonta* ganglia, fine-structural characteristics of synaptic contacts are even better revealed by simple glutaraldehyde fixation (Fig. 2e). Electron-dense projections of the presynaptic membrane appeared to be very conspicuous. Postsynaptic paramembranous density is present, but it is very thin. The structure of the synaptic cleft material is different from that seen after glutaraldehyde–osmium fixation. Dense material alternating with electron-transparent parts appears to be arranged transversally between pre- and postsynaptic membranes.

Two types of synapses can be distinguished according to the *composition of the presynaptic vesicle population:* 1. Synapses with homogeneous vesicle population, that is, only one type of vesicle is present in the terminal; 2. synapses with heterogeneous presynaptic vesicle population consisting of two or more different types of vesicle.

Presynaptic terminals with homogeneous vesicle population were relatively rarely seen. In *Anodonta*, so-called eccentric dense-core vesicles [9] and spheroid clear synaptic vesicles occur, while in marine species pleomorphous synaptic vesicles as well as neurosecretory-like granules (Fig. 3b) are observed as the exclusive components of presynaptic vesicle populations. However, the ganglia of the investigated lamellibranch species are generally characterized by presynaptic terminals containing two different types of vesicles. Clear synaptic vesicles are seen together with dense-core vesicles or neurosecretory-like granules (Fig. 5b). In *Anodonta*, different types of dense-core vesicles mixed with each other in the same terminal are also observed (Fig. 3a).

Among the *further fine structural specializations of the synaptic connections* observed in the neuropile, coated pits of the postsynaptic membrane are frequently encountered. However, such formations also occur outside the junctional part of the pre- and postsynaptic profiles. Smooth-surfaced invaginations of the presynaptic membrane are also seen.

A typical postsynaptic specialization is the axon spine (Fig. 4a and b), which is a deep invagination of the postsynaptic process into the presynaptic terminal. The spines are tightly surrounded by the vesicles of the presynaptic terminal.

A subsynaptic cistern is sometimes seen at synaptic contacts (Fig. 5). It is situated along the total length of the postsynaptic membrane. The fine-structural appearance of the cisterns resembles the smooth endoplasmic reticulum elements seen in other parts of the axon. Occasionally, a material similar in density and appearance to that observed in the synaptic cleft can be seen between the postsynaptic membrane and the subsynaptic cistern (Fig. 5).

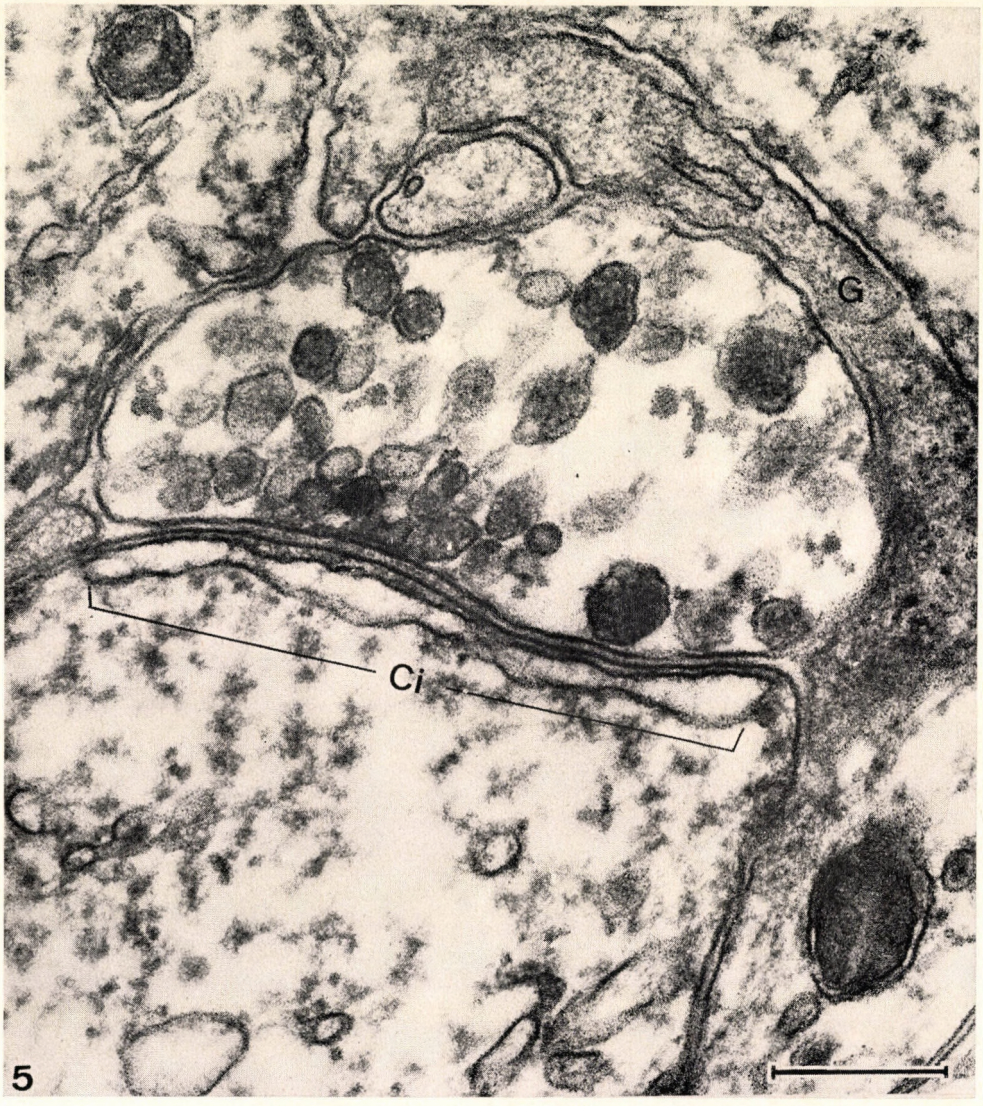


Fig. 5. Subsynaptic cistern (Ci). Note the presence of dense material between the subsynaptic cistern and postsynaptic membrane, being similar in appearance to the intersynaptic cleft material. *Glycimeris pilosa.* G — glial process. Glutaraldehyde–osmium fixation. Bar represents 0.25 μm

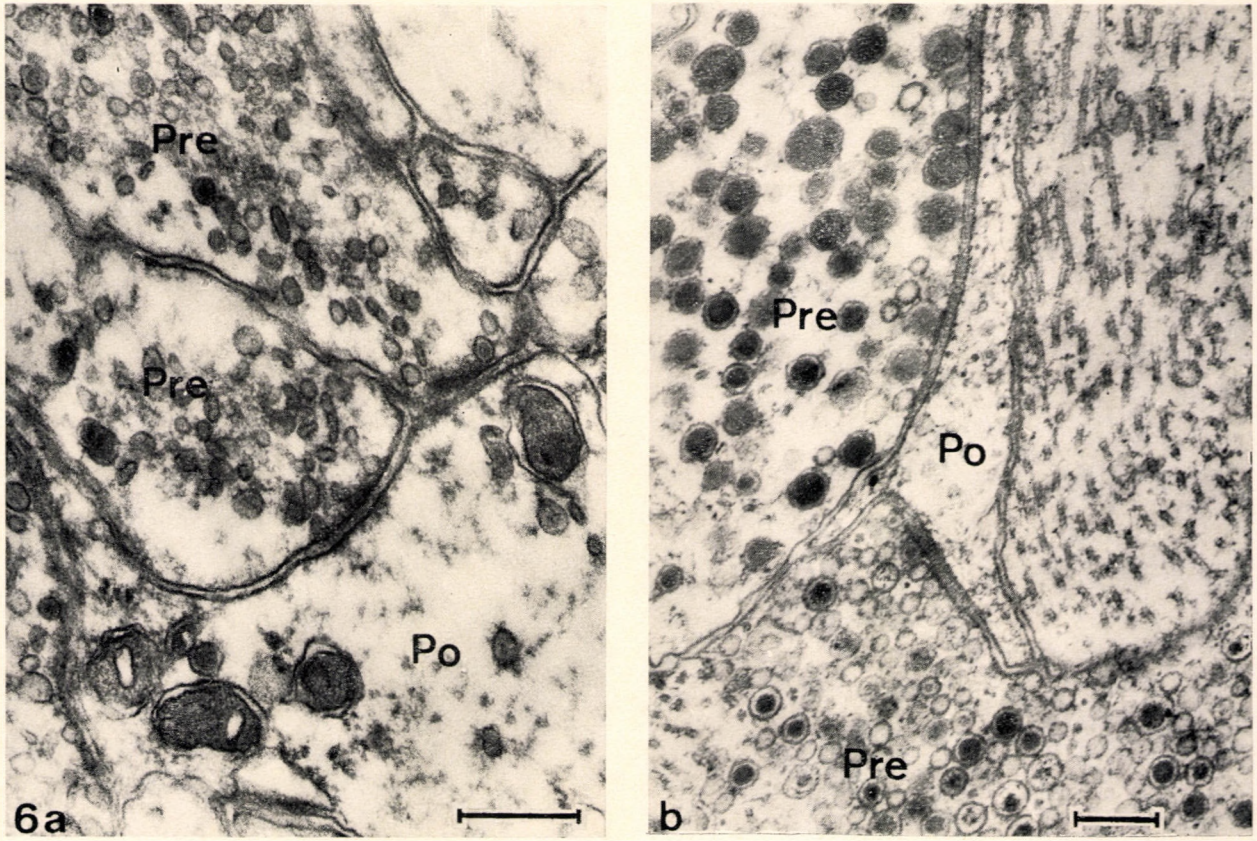


Fig. 6. Different types of synaptic convergence. a) Convergence of presynaptic terminals (Pre) containing similar vesicle population on one postsynaptic element (Po). *Mactra stultorum.* b) Convergence of terminals (Pre) containing different vesicle populations. *Anodonta cygnea.* Glutaraldehyde–osmium fixation. Bars represent 0.25 μm

Forms of synaptic configurations

In all of the ganglia investigated the main form of synaptic interaction proved to be the simple connection (*i.e.* synaptic contact between a presynaptic and a postsynaptic element). However, different forms of synaptic configurations are also found, less frequently than the simple connection. The following forms may be distinguished:

Synaptic convergence

Two or more presynaptic elements terminate on one postsynaptic profile (Figs 6 and 7), suggesting the convergence of different afferent routes. The vesicle population may be different (Fig. 6b) or similar (Fig. 6a) in the presynaptic profiles.

Synaptic divergence

One presynaptic element terminates on two or more postsynaptic profiles. In the extreme case, 6—9 synaptic junctions were observed in a single terminal and its preterminal segment (Fig. 8).

Presynaptic modification

A presynaptic terminal can be the postsynaptic element in another synaptic engagement (Fig. 9).

These three types of synaptic configurations may be observed together within a small area of the neuropile in the ganglia, suggesting the existence of a complex functional unit (Fig. 9).

Discussion

According to recent electron microscopic findings obtained from investigations on the molluscan CNS [7, 8, 12, 14, 32, 33], polarized chemical synapses of the vertebrate type are very rare and the chemical transmission is realized at axon varicosities rich in vesicles. This kind of junction resembles neuromuscular junctions of the autonomic nervous system of vertebrates [2]. However, our present findings show that in the CNS of lamellibranch molluscs structurally well-defined synaptic connections regularly occur. These are similar to those described in the CNS of other invertebrate species [6, 12, 17, 25, 27, 30], and may represent the structural basis for chemical transmission. Many of them resemble the vertebrate chemical synapse [16, 31]. However,

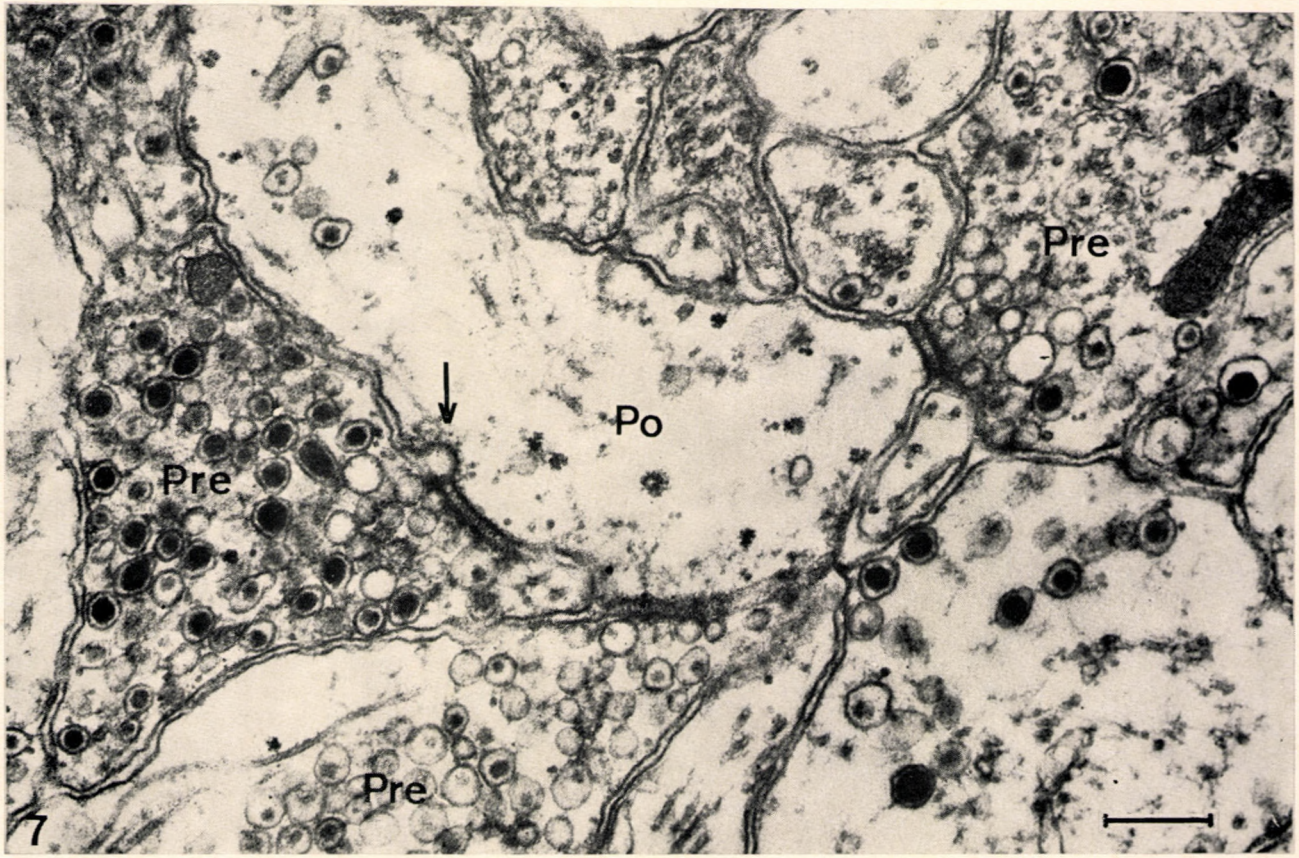


Fig. 7. Polysynaptic convergence. Arrow indicates a coated pit on the postsynaptic membrane. *Anodonta cygnea.* Pre — presynaptic terminal, Po — postsynaptic element. Glutaraldehyde–osmium fixation. Bar represents 0.25 μm

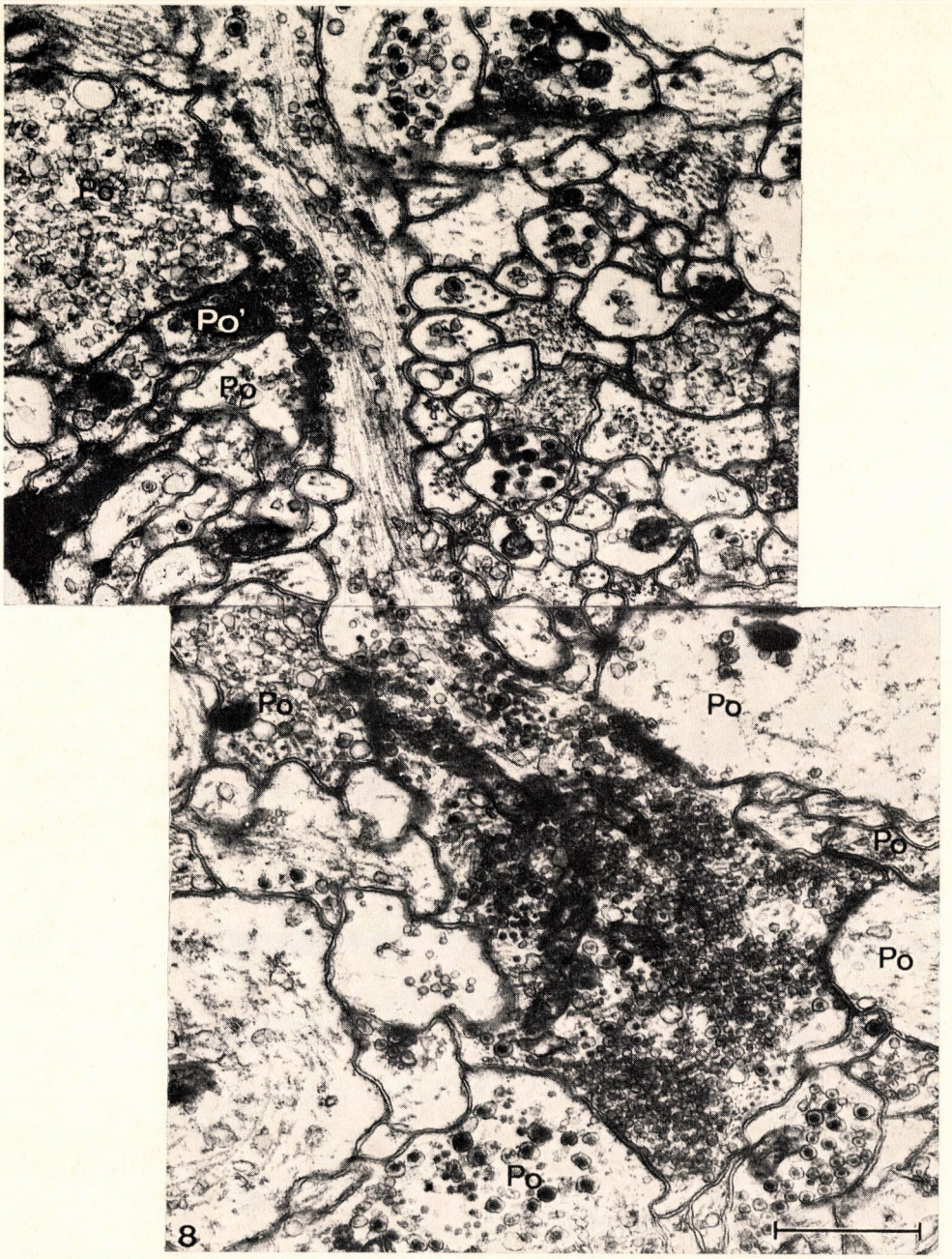


Fig. 8. Polysynaptic terminal as an extreme case of synaptic divergence seen in bivalve ganglia. *Glycimeris pilosa.* Po — postsynaptic element at the level of the presynaptic terminal, Po' — postsynaptic element at the level of the preterminal segment. Osmium fixation. Bar represents 1 μm.

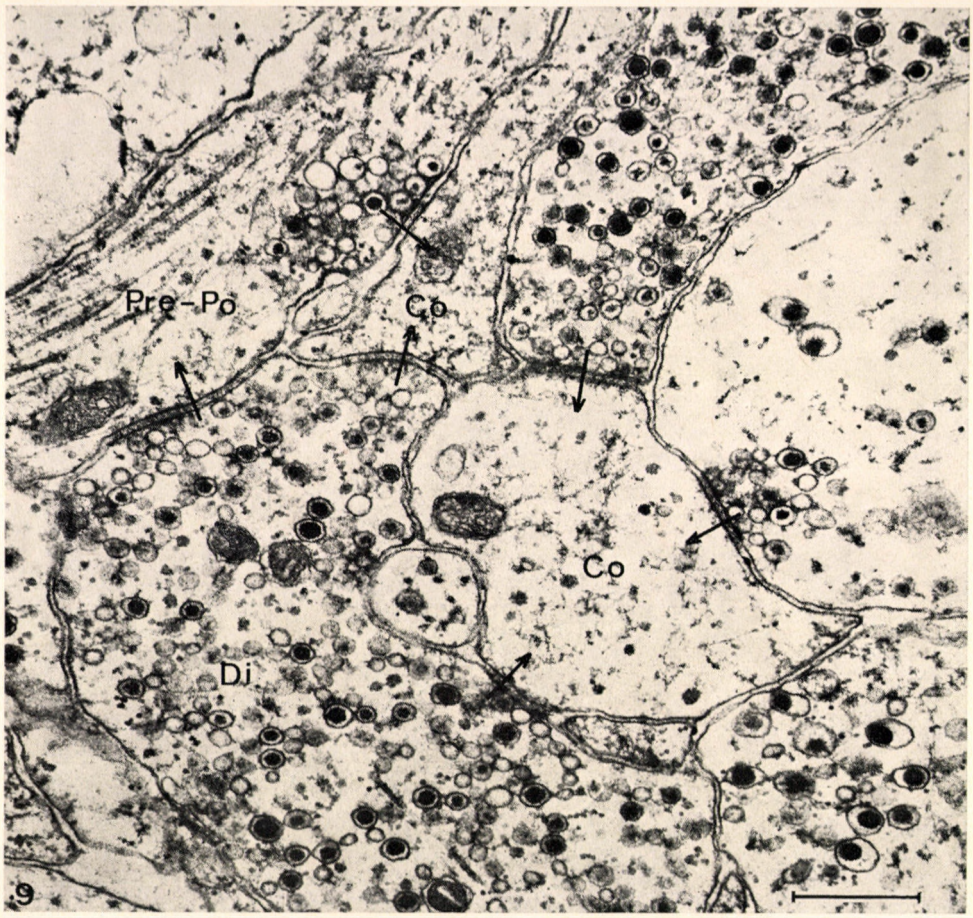


Fig. 9. Occurrence of complex synaptic configurations in a small area of the neuropile. Synaptic convergence (Co), divergence (Di) and presynaptic modification (Pre-Po) are seen together. Arrows indicate the direction of the synaptic transmission. *Anodonta cygnea*. Glutaraldehyde–osmium. Bar represents 0.5 μm

the possibility of a non-synaptic chemical transmission cannot completely be ruled out at the present level of investigations for the following reasons: (1) in the neuropile of the ganglia varicosities containing a number of vesicles can also be seen without any synaptic specialization; (2) microelectrophysiological investigations performed on gastropod CNS and concerning the synaptic transmission processes [33] are lacking in the case of lamellibranch CNS.

Postsynaptic axon spine seen in the ganglia of lamellibranch molluscs resembles the structure described as "finger-like branching" in molluscan CNS [10, 13, 25]. On the other hand, it is completely homologous with the axon spines found in the crab optic lamina [19] and, finally, it may be consid-

ered as an analogous structure with the dendritic spine of vertebrate CNS [15, 18]. Taking into consideration that bivalve central neurons are almost exclusively unipolar [4], the postsynaptic axon spine may play the same role as the dendritic spines in vertebrates.

The different forms of synaptic configurations can unequivocally be compared with structures described in other invertebrates. The occurrence of divergent and convergent connections are suggestive of integrative processes taking place in the neuropile of lamellibranch ganglia. Similar structures have already been found in lower invertebrates [3, 26]. At the same time, these complex connections may presumably be related to synaptic glomeruli described in the insect brain [11, 36, 41] and in the vertebrate CNS [44]. Finally, the presynaptic modification might play an important role in the integrative processes of the lamellibranch CNS, as it has already been supposed in gastropods on the basis of electrophysiological [45] and ultrastructural [25] data.

Part of the results was presented on the "Neuron Concept Today" Symposium held in Tihany, 1976.

Part of this work was done in Naples, 1975, within the framework of an agreement for the exchange of scientific researchers between the Hungarian and Italian Ministries of Foreign Affairs.

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CYTOCHEMICAL LOCALIZATION OF EXOGENOUS PEROXIDASE IN ADRENAL MEDULLARY CELLS OF HAMSTER

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Abstract

The endocytosis in hamster adrenal medullary cells has been studied by intravenously administered horseradish peroxidase, which was then detected with the Graham–Karnovsky method. The protein uptake was investigated 30, 60, and 120 min after the peroxidase injection. The exogenous protein was accumulated mostly in the lumen of the sinusoids, around the capillaries as well as at the apical pole of chromaffin cells 30 min after the peroxidase injection. After another 30 min, a large amount of the reaction product was detectable also in the chromaffin cells. The protein uptake was very intensive beside the plasma membrane, both apically and laterally, through coated- and smooth-surfaced vesicles. Two hours after peroxidase injection, the majority of the reaction product was present in multivesicular bodies. It is supposed that the primary endocytotic vesicles were taken up by multivesicular bodies and digested by lysosomal enzymes. The ultimate fate of the incorporated granule's membranes seems to be digestion by lysosomal enzymes, thus they are not reutilized during granulogenesis in the chromaffin cells.

Introduction

It has been generally accepted that the endocrine, as well as a great number of exocrine, cells release their secretory material by exocytosis from granules [1, 2, 3, 4, 5, 8]. However, the release of secretory material by exocytosis requires secretion product present in membrane-bounded secretory granules in the cytoplasm. It is not surprising, therefore, that exocytosis has not yet been observed in steroid-producing adrenocortical cells, where cytoplasmic storage of steroids is negligible. During exocytosis, the membrane-bounded secretory granules are closely attached to the plasma membrane, thereafter the two membranes, namely the membrane of granules and the plasma membrane, fuse. After the membrane fusion, the apical part of the cell membrane opens, and thus the secretory material can diffuse into the extracellular space [1, 3, 5, 8].

The phenomena of the exocytosis have been thoroughly studied by biochemical, physiological and morphological methods. Several questions have been clarified, however, the fate of the granule's membranes dissolved

in the plasma membrane during exocytosis has remained unknown. It is obvious that during repeated exocytosis the plasma membrane significantly increases in volume and, owing to the incorporation of excessive amounts of granule's membrane, its chemical composition, molecular organization and physicochemical character are altered. The question arises: does the plasma membrane reorganize its fine structure after exocytosis, and if it does, what kind of mechanism can provide the process of reconstruction?

Morphological studies [1, 2, 3, 5, 8] have suggested that there exists a reorganization process in the plasma membrane after exocytosis, and this takes place by endocytosis. Unfortunately, the exocytosis–endocytosis coupling has been studied mainly in rats [1, 9] where the morphological features of the exocytosis are poor.

Since exocytosis is common in the hamster adrenal medulla and its cytochemical properties have not been studied, it was reasonable to investigate the exocytosis–endocytosis coupling in this species. Horseradish peroxidase was injected into hamsters to study the endocytosis activity of the plasma membrane in adrenomedullary cells.

Materials and Methods

Adult golden hamsters (100—120 g) of both sexes were used. Before the injection of horseradish peroxidase, the animals were anaesthetized with 1 ml, 3.5% chloralhydrate by the i. p. route. Twenty mg horseradish peroxidase (Schwarz, New York) was dissolved in 0.5 m saline and was injected into the vena jugularis. Control animals were injected with 0.5 ml saline. The uptake of peroxidase was studied 30, 60 and 120 min after the injection. Before fixation, the animals were perfused with saline for 2 min to remove the red blood cells. Perfusion fluid (and later the fixative, too) was introduced via a needle into the left ventricle of the heart, and was allowed to escape through a cut in the right auricle. The fixative contained 2.5% glutaraldehyde and 4% formalin in cacodylate buffer (pH 7.4). The duration of perfusion fixation was 25 min, and 1 h more immersion in the same fixative. For cytochemical incubation 70—100 μm sections were made from the adrenal by a Smith-Farquhar tissue sectioner. These sections were thoroughly washed for 1 h in 0.05 *M* Tris-HCl buffer (pH 7.65). Cytochemical detection of peroxidase was carried out as described by GRAHAM and KARNOVSKY [7]. After incubation, the sections were washed in Tris-HCl buffer for 5 min and postfixed in OsO_4 for 1 h. Blocks were dehydrated in a series of alcohol and embedded in Durcupan ACM (Fluka). Ultrathin sections were cut with LKB Ultrotom and were stained with saturated aqueous solution of uranyl acetate for 30 min, followed by Reynold's lead citrate for 2 min. The sections were examined in a JEM 100 B electron microscope.

Results

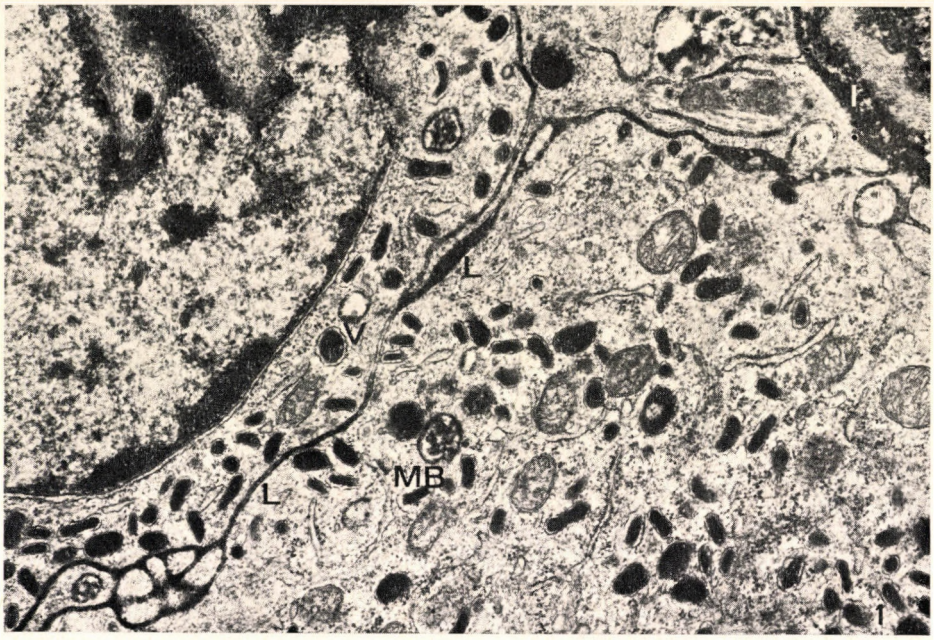
Since 30 min after peroxidase injection, we found only small amounts of the peroxidase reaction product in chromaffin cells, micrographs were taken from blocks which had been incubated for 1 and 2 h after injection. A large amount of the electron-dense precipitate (which represents the presence and roughly the amount) of the injected horseradish peroxidase was

found at the apical pole of the chromaffin cells (Figs 1, 2) around the capillaries (Fig. 2) as well as between the lateral plasma membranes (Fig. 1). Besides the extracellular horseradish peroxidase, a considerable amount of peroxidase reaction product was present intracellularly. Peroxidase activity was associated with cell organelles only in the cytoplasm of chromaffin cells (Figs 1, 3, 4, 5).

The distribution of peroxidase-containing cell organelles is rather homogeneous in the adrenomedullary cells; peroxidase reaction product appeared beside the nucleus, in the Golgi area, as well as at the peripheral part of the cytoplasm (Fig. 5). The uptake of horseradish peroxidase took place anywhere on the cell surface, most frequently at the apical poles of chromaffin cells, near or beside the capillaries (Fig. 2). A great number of deeply-invaginated plasma membrane portions were found at the apical poles of glandular cells (Fig. 2) rich in the reaction product; moreover, at the inner surface of these invaginations coated pits and vesicles with electron-dense reaction product were often seen. Exogenous peroxidase-containing coated vesicles occurred not only beside the apical plasma membrane, but also in the deeper part of the cytoplasm (Fig. 4) as well as near the lateral plasma membrane (Figs 2, 3).

Peroxidase was taken up not only by coated vesicles, but frequently by smooth-surface vesicles and vacuoles, too (Figs 2, 4). These organelles can be found near the plasma membrane, sometimes also in the Golgi apparatus (Fig. 5).

The electron-dense reaction product of peroxidase was most frequently found in the multivesicular bodies of chromaffin cells. These bodies may be present anywhere in the cytoplasm (Fig. 5), most often near the plasma membrane (Figs 2, 4). The multivesicular bodies may be filled with the electron-dense reaction product (Fig. 5), but sometimes it is possible to identify the individual peroxidase-containing vesicles in them (Fig. 1). In a few cases, smooth- and coated-surface vesicles and saccules with peroxidase reaction product in their lumina were present beside the multivesicular bodies (Fig. 5). We have never observed electron-dense reaction product in the lamellae of the Golgi apparatus (Fig. 4), in mitochondria, in secretory granules and in the rough-surfaced endoplasmic reticulum. However, Golgi vesicles (both coated and uncoated) occasionally accumulated the injected horseradish peroxidase (Fig. 5).



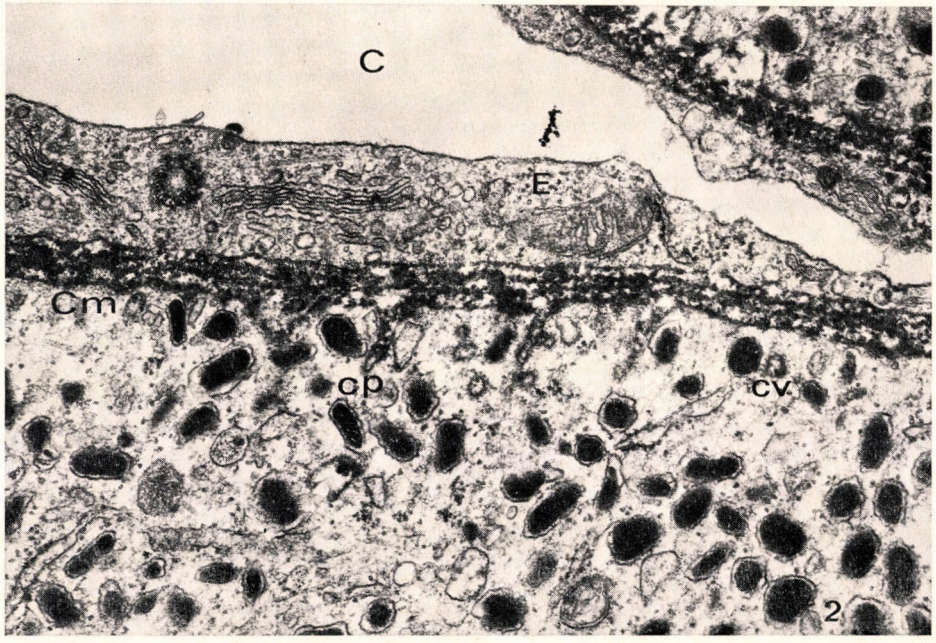


Fig. 1. Chromaffin cells, 1 h after horseradish peroxidase injection. A large amount electron-dense reaction product is seen at the cell surface both apically (I) and laterally (L). Multivesicular bodies (MB) and vacuoles (V) also contain peroxidase reaction product. In multivesicular bodies small vesicles are conspicuous. × 24,000

Fig. 2. Reaction product is detectable neither in the capillary (C) nor in the endothelial cell (E) two hours after peroxidase injection. Note the peroxidase containing coated pits (cp) and vesicles (cv) beneath the cell membrane (Cm) of chromaffin cell. × 36,000

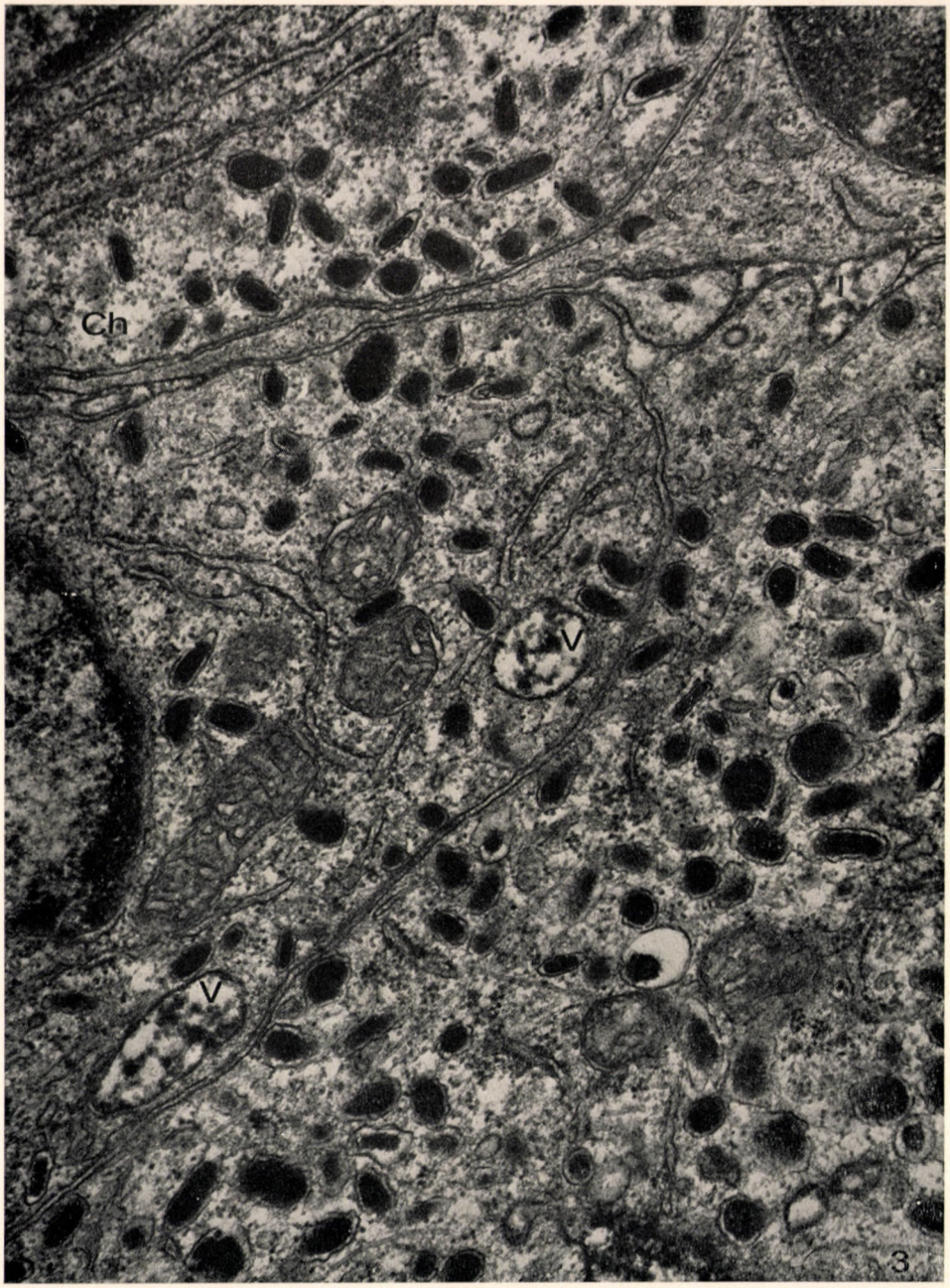


Fig. 3. Large vacuoles (V) often contain electron-dense reaction product 2 h after the peroxidase injection in chromaffin cell (Ch). I = Intercellular space. × 36,000

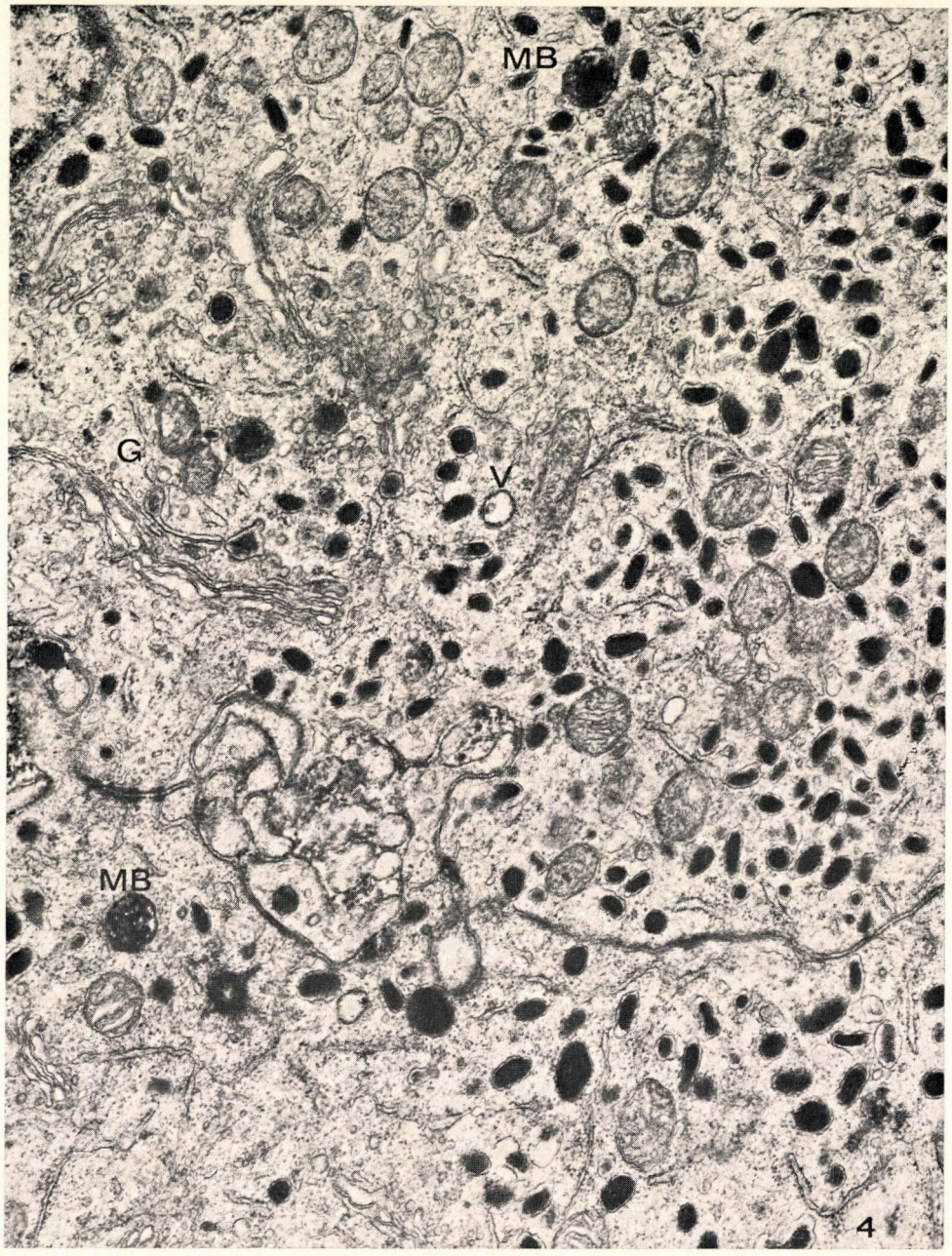


Fig. 4. Two hours after horseradish peroxidase injection, the majority of the enzyme was present in multivesicular bodies (MB) and vacuoles (V). Golgi apparatus (G) is free from reaction product. × 24,000

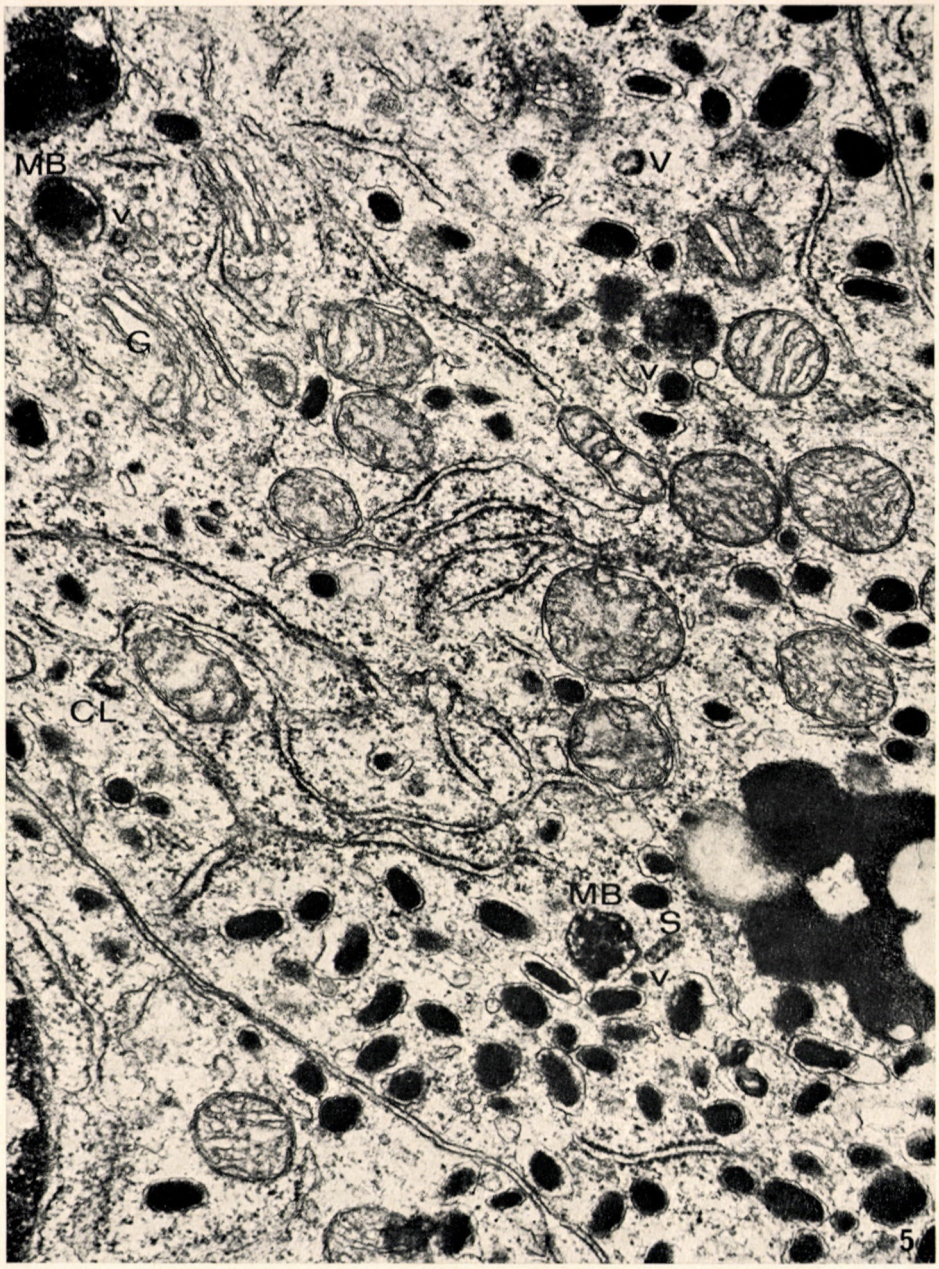


Fig. 5. The occurrence of peroxidase in cell organelles may be nearly homogeneous in the chromaffin cell. Apically, a multivesicular body (MB) can be seen, which is full with enzymes and beside it occurs a peroxidase containing vesicle (v) and a sacculus (S) in close contact with the multivesicular body. Note the "cup-like" body (CL) at the left side of the micrograph and the peroxidase-containing multivesicular body (MB) and vesicle (v) in the Golgi apparatus (G). Right, not far from the Golgi area, there are several peroxidase-containing vacuoles (V) and vesicles (v) in close morphological contact to each other. $\times 36,000$

Discussion

The endocytosis of rat adrenal medulla was studied as early as in 1968 by HOLTZMAN and DOMINITZ [9] using horseradish peroxidase as tracer molecule. These authors established that injected horseradish peroxidase was taken up first of all by vesicles, tubules and "cup-like" bodies, which immediately were transformed to multivesicular bodies. In that paper [9], HOLTZMAN and DOMINITZ did not associate the endocytotic activity of the gland with exocytosis since the existence of exocytosis was not unambiguously accepted at that time. The connection between exo- and endocytosis was re-investigated by ABRAHAMS and HOLTZMAN [1] in 1973, after insulin treatment. Although their observations concerning exocytosis are not convincing, they succeeded in corroborating their previous results, among them, the intensive peroxidase uptake by chromaffin cells after insulin treatment. According to ABRAHAMS and HOLTZMAN [1], one group of the peroxidase-containing structures proved to be lysosome, the others after the uptake fuse with lysosome, in which the exogenous protein is digested by lysosomal enzymes. It was suggested [1] that the membrane of chromaffin granules. which has been recaptured by coated vesicles from the plasma membrane, will be eliminated by a similar process, after the hormone has been released.

As regards the connection between exo- and endocytosis several authors supposed a close coupling between them. First, PALADE [10] supposed that the intact membrane segments of the extruded zymogen granules can be reutilized in the course of granulogenesis in the Golgi apparatus. In contrast to this, FAWCETT [6] stated that the membrane of the secretory granules degrades after the secretory material has been released. The hormone liberation process of adrenal medulla had been explained similarly to other endocrine glands until 1967, when DINER [4] proposed a new hormone extrusion process, called exocytosis. On the omega-shaped membrane invaginations of the exocytosis DINER [4] often observed coated pits and vesicles, but then, she did not explain their role in the secretion. On the basis of our electron microscopic studies [2, 3] we supposed that the coated vesicles might play a role in the retrieval of the granule's membranes, which dissolved in the plasma membrane during exocytosis. The thorotrast uptake, which reflects the endocytotic activity of coated vesicles, was proved by DOUGLAS et al. [5] both in the hypophysis and in the rat adrenal medulla. Not much later, ABRAHAMS and HOLTZMAN [1] published cytochemical observations concerning the peroxidase uptake by rat adrenal medulla, however, the endocytotic activity of the hamster adrenal medulla remained still obscure. Since the adrenal medulla of the hamster has been accepted as the classical test object of exocytosis, we supposed it is basically important to study the morphological response of the chromaffin cells on the injected horseradish

peroxidase. We found that both coated- and smooth-surfaced vesicles are able to take up and store the exogenous protein. Since the number of peroxidase-containing cell organelles was rather high in the adrenomedullary cell of the hamster, it can be stated that the endocytotic capacity of the chromaffin cells is rather high. In the chromaffin cells not only the endocytotic capacity is high, but the protein transport, too; 1 h after the peroxidase injection, the protein was present everywhere in the cytoplasm. The fact on the other hand, that 2 h after peroxidase injection, the majority of the protein was found in the multivesicular bodies suggests that the ultimate fate of exogenous protein is lysosomal digestion in chromaffin cells. Based on this experiment we suppose that the fate of granule's membrane is similar during catecholamine secretion, namely it will be digested by lysosomal enzymes. This is supported by our negative cytochemical results. We have never found electron-dense reaction product in the Golgi's lamellae; this means that the elements of the degraded granule's membranes are not reutilized during de novo granulogenesis.

Since the results of our experiment are consistent with those of ABRAHAMS and HOLTZMAN [1], we can state that though exocytosis is different in many aspects in the adrenal medulla of hamster and rat, the morphological features of endocytosis are practically the same in the two species.

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GENETIC RELATEDNESS BETWEEN STREPTOMYCIN-PRODUCING AND NON-PRODUCING STRAINS OF *STREPTOMYCES GRISEUS*, STUDIED BY MEANS OF DNA–DNA HYBRIDIZATION

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Abstract

DNA–DNA hybridization was studied in order to determine the genetic relatedness between a streptomycin-producing and a non-producing mutant of *Streptomyces griseus*. The latter strain of short vegetative life cycle had been developed from the streptomycin-producing strain by means of nitrogen mustard treatment. Since the two strains differ in several features, we were prompted to examine the sequence homology between their DNAs. Hybridization experiments carried out with the membrane filter method yielded 73.8% and 67.3% DNA homology values, respectively. The results indicate that there is a significant, but not very close, relatedness between the DNA sequences that corresponds to the similarities and differences observed between the phenotypes of the two strains. Further aspects of established homology are also discussed.

Introduction

In order to understand their biological significance and the function antibiotics have in the life of the producing strain, two mutants of *Streptomyces griseus* have been investigated in our Institute. *S. griseus* No. 52-1, a streptomycin-producing (SM-producing) mutant, which is closely related to industrial strains, does not sporulate in submerged culture, *i. e.* its development is blocked. *S. griseus* No. 45-H, a streptomycin-non-producing (SM-non-producing) mutant, has complete life cycle; it sporulates well in submerged fermentation [20]. The latter strain has been obtained from the SM-producing one by nitrogen mustard treatment [16].

Comparison of the two strains reveals that they differ significantly in their life cycle [22], the pentose–hexose ratio in the cell wall [1, 3], the quantity of the cell wall in the vegetative mycelia [3], the amino acid composition of their mucopeptide [4], the heat resistance of their spores [7, 23] and their capability to germinate on and utilize different carbon sources [16]. At the same time the fact that streptidine, a component of the SM molecule, can be isolated from the cell wall of the SM-non-producing strain indicates certain relatedness between the two strains [2, 3].

A further indication of some relatedness is assumed because the fermentation liquid of strain No. 45-H contains a substance which, if added to the culture medium of strain No. 52-1, causes the latter strain to produce conidia [17, 18, 21].

The existing differences between the two strains, however, raised the question whether strain No. 45-H could originate from the SM-producing one by a single mutational event or it is a result of a laboratory contamination. Our studies were based on the comparison of the DNAs isolated from the two strains.

Earlier VALU and SZABÓ [19] studied the base composition of DNA in the two strains by means of chemical hydrolysis and thermal denaturation. The results indicated that the two strains were identical in this respect.

The genome size estimated from the DNA content of single spores was also found to be identical in the two strains, *i. e.* 1.32×10^{-14} g DNA per spore [15]. This value is in good agreement with the results described for *S. coelicolor* and *S. rimosus* spores [5].

However, the degree of genetic homology between the two strains can be best evaluated by means of DNA–DNA hybridization experiments. Applying this technique we attempted to measure the percentage of sequences identical in the two genomes. This value was considered to represent the common fraction of the gene sets of the two strains.

Materials and Methods

Strains and their cultivation. The SM-producing strain No. 52-1 and its non-producing mutant No. 45-H were cultivated in liquid, filtered soy-bean medium. Incubation was performed in a rotatory shaker at 27 °C. Vegetative mycelia were harvested in the exponential phase of growth. The strains and their cultivation were described earlier [16, 20].

For DNA isolation [11, 12, 14] 20—25 g of wet mycelia was harvested, washed three times with 0.15 *M* NaCl, 0.01 *M* EDTA (pH 8.0) solution, then suspended in 0.033 *M* Tris-HCl (pH 8.0), 0.01 *M* EDTA buffer. Afterwards lysozyme (crystalline, from egg white, Reanal, Hungary) was added in 500 μg/ml final concentration and the mixture was incubated at 37 °C for 30—60 min. After adding 0.5 volume of 0.1 *M* Tris-HCl (pH 9.0), 0.1 *M* NaCl buffer the SDS concentration was adjusted to 1% and the mixture was incubated at 60 °C for 10 min, then cooled quickly. Deproteinization was achieved by several treatments with an equal volume of freshly distilled phenol saturated with Tris-SDS buffer (0.1 *M* Tris-HCl, pH 9.0; 0.1 *M* NaCl, 1% SDS) at 4 °C. The pH of the saturated phenol was adjusted to 9.0 with 10 *N* NaOH. The emulsion thus obtained was separated by centrifugation, and the upper aqueous phase was carefully pipetted. After the last phenol extraction DNA was precipitated from the aqueous fraction with 2 volumes of cold (−20 °C) ethanol. The precipitated DNA was collected on a glass rod and dissolved in 0.1 ×SSC (1 ×SSC = 0.15 *M* NaCl + 0.015 *M* sodium citrate, pH 7.0). RNase treatment was carried out with 50 μg/ml pancreatic RNase (crystalline, from bovine pancreas, Reanal, Hungary) and 100 units per ml T_1 RNase (from *Aspergillus orizae*, Serva, FRG) at 37 °C for 1 h. Before its application, the pancreatic RNase was deprived of DNase activity by heating at 100 °C in a water bath for 10 min. Afterwards, Proteinase K (fungal, Merck, FRG) was added in 50 μg/ml final concentration and incubated at 37 °C for 60 min in the presence of 0.5% SDS [8]. Then the salt concentration was raised to 1 ×SSC adding 0.1 volume of 10 ×SSC. This was followed by several phenol treatments and precipitation with ethanol. The precipitate was dissolved in 0.1 ×SSC, and 0.1 volume of 3 *M* Na-ace-

tate, 0.001 *M* EDTA, pH 7.0 solution was added. The DNA was precipitated with 0.54 volume of isopropanol, and dissolved in 0.1 × SSC. This step was repeated once or twice as required. The DNA was stored either as precipitate on a glass rod in ethanol at −20 °C, or in concentrated solution with several drops of chloroform at 4 °C.

The purity of DNA preparations was characterized by UV absorbance ratios. The A_{260}/A_{280} and the A_{255}/A_{230} ratios were above 1.9 and 2.1, respectively.

Labelled DNA was prepared by adding 5—10 μCi/ml ^{32}P-labelled Na-orthophosphate (carrier-free, Isotope Institute of the Hungarian Academy of Sciences) to the culture medium after inoculation. The ^{32}P-labelled DNA had a specific activity of $5-7 \times 10^3$ cpm/μg.

For *DNA–DNA hybridization experiments* we used a membrane filter technique described by LEGAULT-DÉMARE and modified by DE LEY and TIJTGAT [6, 10]. Solutions containing 150 μg of DNA per ml in 0.1 × SSC, were denatured for 15 min at 100 °C, then chilled quickly in an ice-bath, and adjusted to 6 × SSC concentration. 8.3 ml of DNA solution ($A_{260} = 1.0$) was passed through a membrane filter (Selectron Filter, type: BA 83/6, diameter: 50 mm, pore size: 0.2 μ, Schleicher and Schuell, Dassel, FRG) presoaked and washed with 6 × SSC. The filters were washed again with 100 ml of 6 × SSC, dried at room temperature overnight, then discs of about 10 mm diameter, each containing about 25 μg of DNA, were punched from it. Drying was continued for 2 h at 80 °C in a vacuum oven. The amount of DNA fixed was determined spectrophotometrically both before and after filtration.

Hybridizations were carried out at 65 °C for 18 h in 1.0 ml of 2 × SSC solution containing 30% dimethylsulphoxide (2 × SSC-DMSO). 1 μg (5-7000 cpm) of denatured homologous ^{32}P-labelled DNA was hybridized to filter-fixed DNA (1), in the presence of homologous competitive DNA, (2), in the presence of heterologous competitive DNA and (3) in the absence of competitive DNA.

Both the labelled and the unlabelled competitive DNAs were degraded by ultrasonic treatment in an ultrasonic disintegrator (MSE Ltd., Power Unit No. 3000) at 1.4—1.5 A Ammeter position for six times 15 sec with 45 sec intervals in ice-bath. Before their application the DNA samples were denatured at 100 °C for 15 min and cooled quickly. After hybridization the filters were washed three times with 10 ml of 2 × SSC-DMSO by shaking for 5 min, then once with 10 ml of 2 × SSC. The filters were dried and their radioactivity was measured in a Nuclear Chicago Isocap Liquid Scintillation System. The scintillation fluid contained 5 g of PPO, 100 mg of POPOP in 1000 ml of toluene.

Results

In a part of our experiments 1 μg of ^{32}P-labelled DNA from *S. griseus* No. 52-1 was hybridized to filter-fixed unlabelled homologous DNA, in the presence of 250 μg of homologous and 250 μg of heterologous DNA (from *S. gri-*

Table 1

Hybridization of ^{32}P-DNA from S. griseus *52-1 to filter-fixed homologous DNA in the presence of homologous and heterologous competitive DNAs*

Filter-fixed DNA	Relative amount of ^{32}P-DNA from *S. griseus* 52—1 hybridized		
	without competitive DNA (A)	in the presence of 250 μg of homologous competitive DNA (B)	in the presence of 250 μg of heterologous competitive DNA (C)
S. griseus 52—1	100.0 (1444)*	15.0	37.3
Blank filter	(22)		
Total ^{32}P-DNA	(7000)		

* cpm values are in parentheses

Table 2

Hybridization of ^{32}P-DNA from S. griseus *45-H to filter-fixed homologous DNA in the presence of homologous and heterologous* (S. griseus *52-1) DNAs*

Filter-fixed DNA	Relative amount of ^{32}P-DNA from *S. griseus* 45-H hybridized		
	without competitive DNA (A)	in the presence of 250 μg of homologous competitive DNA (B)	in the presence of 250 μg of heterologous competitive DNA (C)
S. griseus 45-H	100.0 (752)*	16.2	43.6
Blank filter	(19)		
Total ^{32}P-DNA	(4711)		

* cpm values are in parentheses

seus No. 45-H), respectively. The results we obtained are shown in Table 1. The percentage of DNA relatedness between the two organisms was calculated as follows:

$$\text{Homology index } (\%) = 100 \times \frac{A - C}{A - B}$$

In this experiment the degree of homology between the two DNAs was 73.8%.

In the reciprocal experiment 1 μg of ^{32}P-labelled DNA from *S. griseus* No. 45-H was hybridized to filter-fixed unlabelled homologous DNA in the presence of 250 μg of homologous and 250 μg of heterologous DNA (from *S. griseus* No. 52-1), respectively. Results obtained are presented in Table 2.

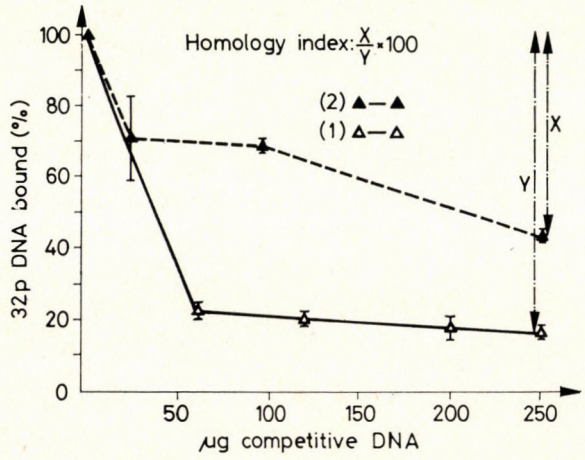

Fig. 1. Effect of the concentration of competitive DNA on the extent of hybridization: continuous line: homologous competition; dotted line: heterologous competition. Each point represents the average of two parallels. For details, see the text

In the reciprocal experiment the homology index was 67.3%. In the latter type of experiments we studied hybridization at various concentrations of competitive DNAs (Fig. 1). 1.0 μg of ^{32}P-labelled DNA from *S. griseus* No. 52-1 was hybridized to 25 μg of filter-fixed homologous DNA in the presence of increasing amounts of homologous and heterologous DNA's. The heterologous DNA originated from *S. griseus* No. 45-H.

Discussion

The values of genetic homology indicate considerable similarity between the two *S. griseus* strains regarding their base sequences, though this relatedness is not extremely close. The results of the reciprocal experiments do not differ significantly, thus supporting the reliability of the method.

A similar degree of homology (75%) was measured by KOIKE et al. [9] between two tetracycline producing *S. aureofaciens* strains of identical morphology, *i. e.* such a degree of relatedness does not exclude belonging to the same species.

OKANISHI et al. [13] found 66—71% homology between the SM-producing *S. griseus* strains and the grisein-producing *S. griseinus* strains, which was considered high enough to classify the latter strains together with the SM-producing ones to the *griseus* group.

On the basis of our experiments concerning genetic homology, we cannot as yet give a definite answer to the question whether strain No. 45-H had originated from the SM-producing one as a result of mutagenic treatment. It is unlikely that a single mutation may alter the structure of the genome to such an extent. Therefore, the possibility arises that the SM-non-producing strain was selected from a genetically inhomogenous strain by nitrogen mustard treatment.

This well-sporulating *Streptomyces* of complete life cycle is more similar to commonly occurring (wild-type) strains than the SM-producing one. Therefore, the possibility that strain No. 45-H originates from a laboratory contamination cannot be excluded.

The values of DNA homology determined in our experiments indicate that the two genomes contain considerable amounts of common sequences. However, the genetic differences existing between the two strains may well be responsible for the stable differences found in their phenotypes, *i. e.* the carbohydrate and amino acid compositions of the cell wall, the heat resistance of the spores, the carbon source utilization, *etc.*

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EFFECT OF SOME INSECTICIDES ON THE PERIODIC ACTIVITY OF THE FRESH-WATER MUSSEL (*ANODONTA CYGNEA* L.)

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(Recived 1978—04—10)

Abstract

The effect of the insecticides Hungaria L-7, Dimecron-50, Thimet-10 G and Satox 20 WSC containing lindane, phosphamidon, phorate and trichlorfon, respectively, as active ingredient was investigated on the survival and pumping behaviour of the fresh-water mussel *Anodonta cygnea* L. The shortening of active periods was used as indicator of the sublethal effects. Hungaria L-7, Thimet-10 G and Satox 20 WSC caused significant inhibition of the filtering activity, while Dimecron-50 was less effective. Fifty per cent shortening of active periods required 6×10^{-3} g/l Hungaria L-7, 8×10^{-3} g/l Thimet-10 G and 2×10^{-2} ml/l Satox 20 WSC. Thimet and Satox were lethal at 10^{-1} g/l and 1 ml/l concentrations, respectively. Although the mechanism of the effect can be different with various substances, the results have shown, that monitoring of the activity of mussels can be a suitable test for the biological indication of water pollution.

Introduction

The general spread and growing use of specific plant protecting substances has called attention to their influence on water organisms both in order to clear up the direct toxic effect and for determining the possible chronic damage they can cause when reaching surface waters [3, 7, 12]. According to previous investigations, mussels, which are filter-feeding animals, react very sensitively to lowering of the O_2 content of waters [14] and to some chemicals [8, 18]. The activity of mussels alternates periodically with rest, both lasting for several hours. Active periods are characterized by fast pumping movements of the shells, which are closed during rest [1]. The reaction of these animals to unfavourable substances is the change of active filtering behaviour, which can be monitored by recording the position and movement of the valves [16]. Besides the filtering activity, other physiological processes, like growth of animals [4, 10], activity of gonads [11], and formation of byssal threads [12], have been used as markers of sublethal effects caused by environmental factors. These approaches have led to definite results as concerned the inhibitory effect of various pollutants. At the same time, it was found that, considering the lethal doses, mussels show a rather great tolerance and a high accumulation capacity to a number of toxic agents [2, 19].

In the present experiments, we analysed the survival and the motoric activity of mussels in order to (a) estimate the lethal concentrations of some insecticides containing lindane, phosphamidon, phorate or trichlorfon as active component and, (b) using sublethal concentrations, we wished to determine the degree of their influence on the filtering behaviour, connected both with feeding and with the water-cleaning function of mussels.

Materials and Methods

Experiments were carried out on 10—12-cm-long specimens of *Anodonta cygnea* L. in spring, summer and autumn. Animals were placed separately in 3 litres of water taken directly from Lake Balaton. Activity was permanently recorded by a mussel actograph [15]. The water was changed every day, in the morning. Water temperature varied during the experimental period between 17 and 27 °C.

Before starting with the application of substances, the normal activity of mussels was recorded for a week. After this period, the desired amount of insecticide was added to the water. The exposure lasted for a week. During this period the insecticide concentration was kept constant by renewing daily, when the water was changed. Parallel experiments were carried out on 6—10 animals.

Evaluation of the results was made separately for each animal. The duration of active periods was measured and mean values for the control period and for the period following treatment were determined. The activity in the presence of insecticide was expressed as per cent of the control activity. The mean of these percentual values obtained for each concentration at least in 6 animals were plotted against the insecticide concentration. The concentration necessary for a 50% inhibition of activity was determined from the curve by interpolation. Besides the concentration of the complete insecticide the concentration of its active compound is also given in the graphs.

The following substances were used in the present experiments: Hungaria L-7, containing 7.8% lindane as active component, Dimecron-50 (phosphamidon, 50%), Satox 20 WSC (trichlorfon, 20%) and Thimet-10 G (phorate, 10%). The substances were dissolved or suspended in Balaton water; pH values varied between 8.0 and 8.4 The pH of Balaton water is normally at 8.4.

Results

The effect of Hungaria L-7 was investigated within the concentration range 10^{-6} to 10^{-1} g/l. Shortening of active periods occurred at 10^{-4} g/l and higher concentrations (Fig. 1). Plotting of mean duration of activity expressed

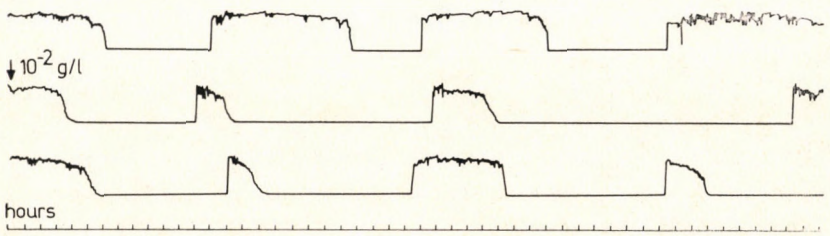

Fig. 1. Effect of 10^{-2} g/l Hungaria L-7 on the activity of *Anodonta*. Continuous recording. Arrow marks the adding of the chemical. During active period when the shells are open, rhythmic valve movements are present. Closed position of the shells (rest) is represented by the straight lines of the record close to the time scale

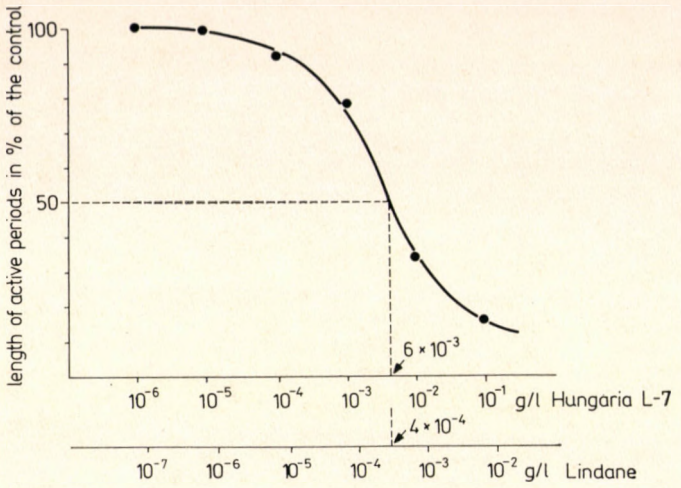

Fig. 2. Dependence of the duration of active periods on the concentration of Hungaria L-7 and its active ingredient, expressed in per cent of the control duration

in per cent of the control against the drug concentration resulted in an S-shaped curve (Fig. 2). At 10^{-1} g/l, the mean duration of active periods was as short as about 15% of the control. From the curve, the concentration necessary for 50% inhibition was estimated. It was 6×10^{-3} g/l Hungaria L-7, which is equivalent to 4×10^{-4} g/l lindane. All the mussels survived the one-week experiment. Recovery of control activity occurred in 2—10 days after removing of the drug from the water.

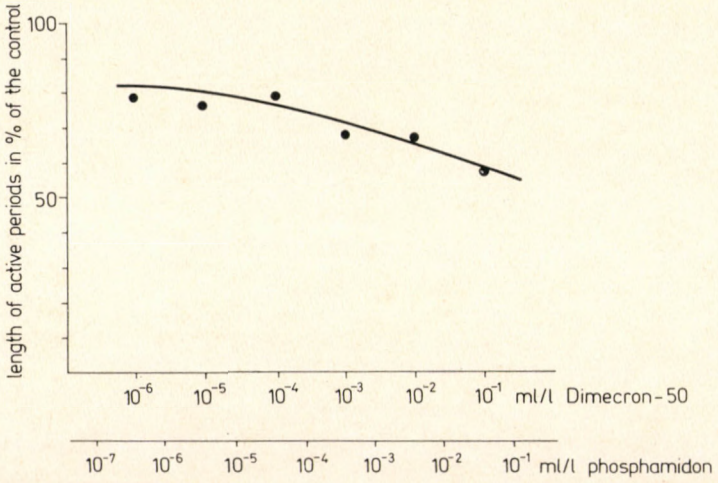

Fig. 3. Dependence of the duration of active periods on the concentration of Dimecron-50 and its active ingredient

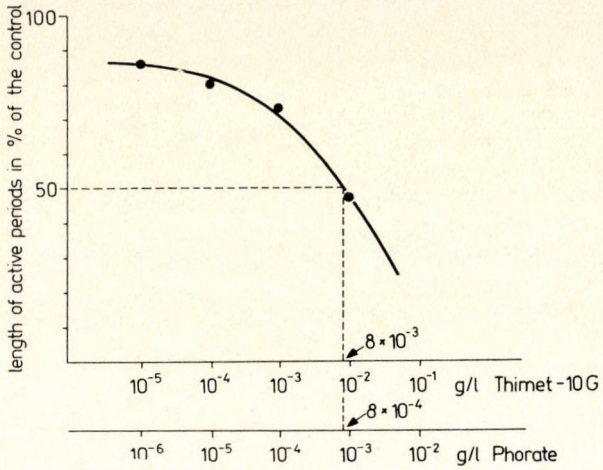

Fig. 4. Dependence of the duration of active periods on the concentration of Thimet-10 G and its active ingredient

The effect of Dimecron-50 was investigated in the same range of concentrations. At 10^{-6} ml/l, the mean duration of active periods was 78% of the control, while at 10^{-1} ml/l it was 57%. So the curve (Fig. 3) is rather flat, and the inhibition does not reach 50% within the investigated concentration range. No perishing occurred.

Thimet-10 G was applied in concentrations from 10^{-5} to 10^{-1} g/l. The mean length of activity was 86% of the control at 10^{-5} g/l and 47% at 10^{-2} g/l (Fig. 4). Fifty per cent inhibition required 8×10^{-3} g/l Thimet-10 G, *i. e.* 8×10^{-4} g/l phorate. Thimet-10 G proved to be lethal for mussels in 10^{-1} g/l, the mean survival time being 113 h. Before death the rhythmic activity of animals increased, a long active period occurred and, as a result of the decay of muscle tone, the shells opened. In cases, when the mussel had not died by

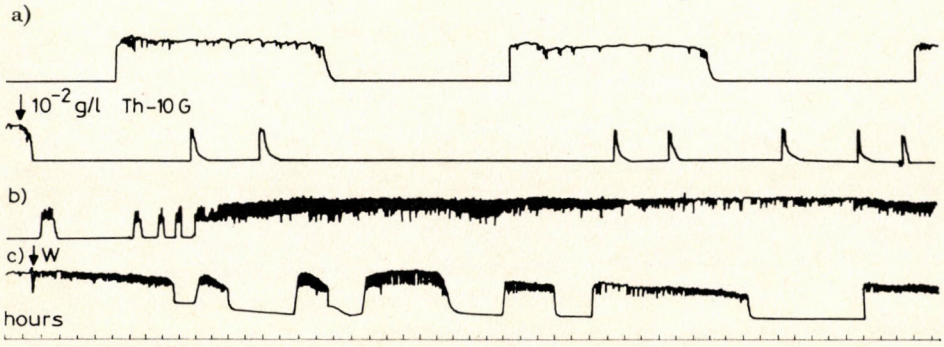

Fig. 5. Effect of 10^{-1} g/l Thimet-10 G on the activity of *Anodonta*. a — Control and adding of the insecticide (at arrow); b — after 18 h; c — restoration of activity after washing out

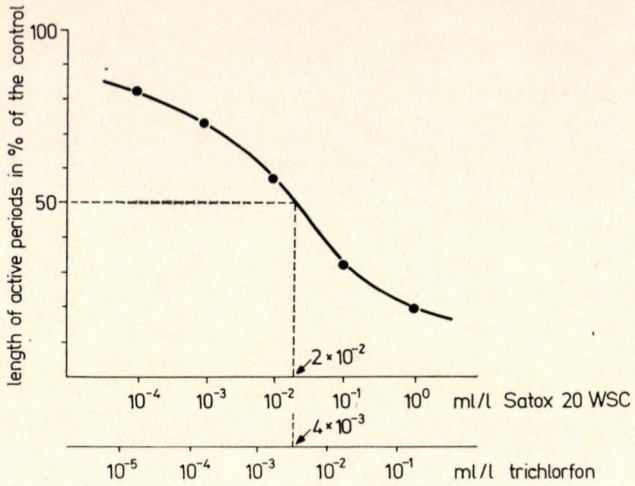

Fig. 6. Dependence of the duration of active periods on the concentration of Satox 20 WSC and its active ingredient

the end of the first week, the control activity returned within a few days after the insecticide had been washed off (Fig. 5).

The effect of Satox 20 WSC was tested at 10^{-4}—10^{0} ml/l concentrations. 1 ml/l proved to be lethal, the mean survival time being 209 h. The strong effect of this concentration could not be eliminated by washing even after a 120-h application time. The animals perished as a rule after a long active period, in closed state. As a result of the treatment the shortening of active periods is characteristic, being dependent on the drug concentration (Fig. 6). Fifty per cent inhibition of the duration of active periods requires 2×10^{-2} ml/l Satox, *i. e.* 4×10^{-3} ml/l trichlorfon.

Elimination of the effect of 10^{-1} ml/l Satox lasted rather long, it required 8—10 days. Lower concentrations required 4—5 days to be eliminated.

Satox 20 WSC over 10^{-3} ml/l concentration decreased the ability of the adductors for tonic contraction, thus preventing the total closure of the shells during rest. In the presence of 10^{-1} ml/l concentration of the drug, the shell margins were 8—15 mm apart from each other, the foot was out of the shell cavity, but no rhythmic activity was present.

Discussion

The results of the presented experiments show that, depending on concentration, Hungaria L-7, Thimet-10 G and Satox WSC significantly influence the activity of the fresh-water mussel. Thimet and Satox even can kill the animals. Dimecron-50, on the other hand, exerts a slight effect on the activity of

Anodonta. The inhibitory effect is manifested first of all in the shortening of active periods, while the frequency of rhythmic pumping activity shows no characteristic change. The concentrations of the active components necessary for 50% inhibition are considerably high, far higher than the concentrations possibly reached in highly polluted natural waters. However, these values should be evaluated otherwise if taking into consideration the slow decomposition rate of these substances and, also, their presence in the sediment and in lower organisms from where mussels can accumulate them further.

There are a lot of data about accumulation of substances in mussels [12]; VALIELA et al. [20] reported on the accumulation of heavy metals, while BEDFORD et al. [2] found the accumulation of DDT and its metabolites to be time-dependent in *Anodonta* up to a given concentration gradient. This possibility cannot be excluded in the case of the substances used in our experiments. Restoration of the animals' activity in fresh water required several days, suggesting that the accumulation of drugs was significant and their washing out demands a considerable time.

During the experimental period marked perishing of animals was observed at 10^{-1} g/l concentration of Thimet-10 G and 1 ml/l Satox. Animals died usually without preceding reduction of active periods; on the contrary, at the terminal stage of life the adductor muscles relaxed and frequent pumping rhythmic activity of valves occurred for several hours.

It is known that the motor activity of mussels may be influenced by a variety of external factors, like oxygen content of the water [14] or temperature [9] while in the internal regulation of activity the monoamines of the nervous system play the main role [17]. By influencing the metabolism of serotonin and catecholamines with specific pharmacons the activity of *Anodonta* can be modified [6], however, little is known about the effect of external factors on the monoamine metabolism in mussels. It is very probable that heavy metals (Hg, Cs, Cu) which also inhibit the activity of mussels [13, 18] evoke their effect by blocking enzymes important in cell respiration.

The insecticides we investigated recently must influence also the internal regulatory system. Phorate, phosphamidon and trichlorfon are cholinesterase inhibitors [5], but the great difference between the effect of Thimet, Satox and Dimecron makes questionable whether their effect realizes through the cholinesterase system. Nevertheless, they can influence in some way the neural regulation or can cause the inhibition of activity by disturbing the respiratory system either at the level of the external mechanism or in the tissues. Similar mechanism can be supposed for lindane.

The change of pumping activity under the influence of pesticides have been described previously by BUTLER et al. [4], however, these authors concluded that, because of the slight effect and the great variation of results, this method cannot be used in short-time experiments for testing of the biological

effect of pesticides. Our result show that the effect of the used chemicals can be well detected in several days long experiments if the shortening of active periods occurring after pesticide application is taken into consideration. As investigation of growth of animals, of gonad activity and of byssal attachment require much longer experimental period, it can be stated that the monitoring and analysis of the length of active periods in mussels can be used as a good test for detecting the harmful effect of various pollutants both in short-term and in chronic experiments.

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EFFECT OF HERMAPHRODITIC GLAND HOMOGENATE ON THE REGULATION OF THE GAMETOGENESIS IN *HELIX POMATIA* (GASTROPODA, PULMONATA)

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Abstract

On the effect of ovotestis (glandula hermaphroditica) homogenate the number of the ovocytes of 1st and 2nd order and of the eggs increased very significantly, while spermiocytes of 1st order and sperms significantly decreased in number. When snails with extirpated tentacles were injected with ovotestis homogenate the ovogenesis was activated which was proved by a significant increase in the number of ova.

Introduction

The number of cells belonging to cell types characteristic of ovogenesis was found by us highly elevated in edible snails 1—2 weeks after extirpation of the optic tentacles during hibernation or during the period of awakening in spring, or before egg-laying. At the same time, injection of optic tentacle homogenate into intact animals had a very strong effect — already after a week — on the spermiogenesis having increased the number of its cell types. The cotrolling role of the optic tentacles in the spermiogenesis has been proved by our earlier examinations, too [1—9, 22]. Furthermore, we have proved the role of the cerebral ganglion in the control of gametogenesis [7]. In our present work we have attempted to clear up the role of the hermaphroditic gland (glandula hermaphroditica *seu* ovotestis) in the control of gametogenesis.

Materials and Methods

Our experiments were performed on edible snails *(Helix pomatia)* being before the period of the egg-laying. In the first group, 40 intact control snails and 40 experimental snails injected with hermaphroditic gland homogenate were investigated.

In the second group, 40 snails with extirpated tentacles served as controls and 40 experimental snails with extirpated tentacles were injected i. m. with hermaphroditic gland homogenate.

Hermaphroditic glands were homogenized in glass Potter homogenizator, then centrifuged at 4 °C. The homogenate separated from the supernatant was weighed in fresh state and

dispersed, thereafter, in bidistilled water. The solution got by this way contained 0.5 mg substance per ml and each animal was injected with one ml of this solution.

After a week, the animals were decapitated, and the hermaphroditic glands were prepared and fixed in Bouin solution. The materials were serially cut, stained with hematoxylin and eosin and, finally, analysed and evaluated regarding the different developmental forms of gametocytes. In this way, the following phases of gametogenesis were quantitatively evaluated in individual follicles: ovocytes of 1st and 2nd order, ova, furthermore, spermiocytes of 1st and 2nd order and sperms, and finally if they were at all, the "many sperms" per follicle. ("Many sperms" means that the number of sperms per follicle was six times or more than the control value). On the basis of the absolute numerical values obtained by microscopic observation percentage values were calculated which show that in how many per cent of the follicles counted was the cell type in question found. The cell types mentioned were investigated at least in 1000—1500 follicles in every experimental group. The mathematical analysis of the numerical values was performed by the Department of Numerical Method and Computer Science (Eötvös Loránd University, Budapest) and by the Computing Group of the Semmelweis Medical University.

Results

In the first experimental group, snails with intact tentacles were injected with hermaphroditic gland homogenate. Compared to the normal control group, the number of the ova and ovocytes of 1st and 2nd order very significantly increased in the follicles of the hermaphroditic gland upon the effect of the homogenate (Fig. 1).

In this experimental group, the number of spermiocytes of 1st order as well as the number of sperms significantly decreased, compared to the control (Fig. 2).

In the second group snails without tentacles were injected with hermaphroditic gland homogenate. As a result, ovocytes of 1st order significantly

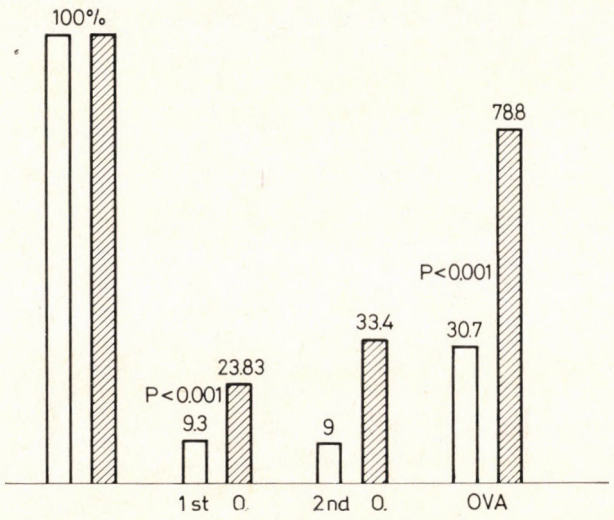

Fig. 1. Ovogenesis. 8—15 June, 1975. White columns = intact, untreated snails not injected with ovotestis homogenate. Hatched column = intact experimental group injected with ovotestis homogenate. 1st O = ovocyte of 1st order; 2nd O = ovocyte of 2nd order

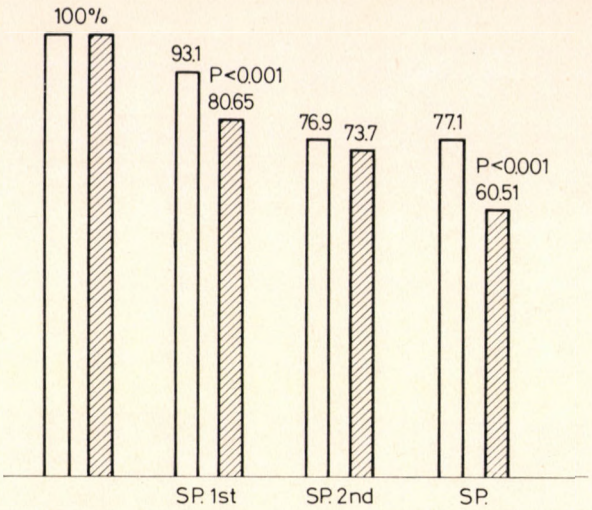

Fig. 2. Spermiogenesis 8—15 June, 1975. White columns = group of intact snails not injected with ovotestis homogenate. Hatched columns = experimental group injected with ovotestis homogenate. 1st SP = spermiocyte of 1st order; 2nd SP = spermiocyte of 2nd order; SP = = sperm

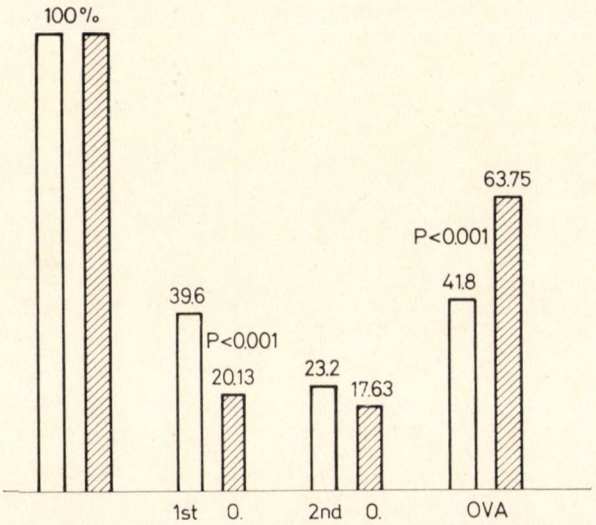

Fig. 3. Ovogenesis. 8—15 June, 1975. White columns = group of control snails, after extirpation of the optic tentacle but without injecting ovotestis homogenate. Hatched columns = = experimental group injected with ovotestis homogenate following extirpation of the optic tentacle. 1st O = ovocyte of 1st order; 2nd O = ovocyte of 2nd order

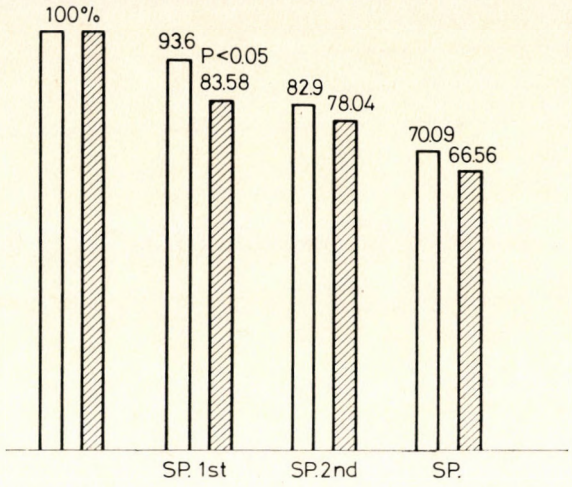

Fig. 4. Spermiogenesis. 8—15 June 1975. White columns = control group, ovotestis homogenate was not injected following the extirpation of the optic tentacle. Hatched columns = experimental group injected with ovotestis homogenate following the extirpation of the optic tentacle. 1st SP = spermiocyte of 1st order; 2nd SP = spermiocyte of 2nd order

increased in number, compared to the corresponding control group (Fig. 3). The number of the ova also significantly increased (Fig. 3). In this experimental group, too, the spermiocytes of 1st order significantly decreased in number, compared to the control. The mean number/follicle of spermiocytes of 2nd order and that of sperms did not show any significant alteration (Fig. 4).

Other part of the results is shown in the Tables 1, 2, 3 and 4.

Table 1

Absolute number and per cent of different cells found in the follicles

Total number of follicles		Number of the follicles per group					
		Ovocyte of 1st order		Ovocyte of 2nd order		Ovum	
E	C	E	C	E	C	E	C
1137	1596	271	149	380	144	896	480
		Comparative mathematical method					
100%	100%	181.88%	100%	263.88%	100%	186.66%	100%

C = Control group: animals with intact optic tentacle injected with ovotestis (glandula hermaphroditica) homogenate

E = Experimental group: animals with intact optic tentacle injected with ovotestis homogenate

Table 2

Absolute number and per cent of cells found in the follicles

Total number of follicles		Number of the follicles per group							
		Spermiocyte of 2nd order		Spermiocyte of 2nd order		Sperm		Many sperm	
E	C	E	C	E	C	E	C	E	C
1137	1596	917	1490	838	1230	688	1248	0	0
Comparative mathematical method									
100%	100%	61.54%	100%	68.13%	100%	55%	100%		

C = Control group: animals with intact optic tentacles and not injected with ovotestis homogenate

E = Experimental group: animals with intact optic tentacles and injected with ovotestis homogenate

Table 3

Absolute number and per cent of cells found in the follicles

Total number of follicles		Number of follicles per group					
		Ovocyte of 1st order		Ovocyte of 2nd order		Ovium	
E	C	E	C	E	C	E	C
1316	1585	265	629	232	368	839	663
Comparative mathematical method							
100%	100%	42.13%	100%	63.04%	100%	126.55%	100%

C = Control group: animals not injected with ovotestis homogenate following the extirpation of the optic tentacle

E = Experimental group: animals injected with ovotestis homogenate following the extirpation of the optic tentacle

Table 4

Absolute number and per cent of cells found in the follicles

Total number of follicles		Number of follicles per group							
		Spermiocyte of 1st order		Spermiocyte of 2nd order		Sperm		Many sperm	
E	C	E	C	E	C	E	C	E	C
1316	1585	1100	1485	1027	1315	876	1110	0	0
Comparative mathematical method									
100%	100%	74.1%	100%	78.1%	100%	78.92%	100%		

C = Control group: animals not injected with ovotestis homogenate following the extirpation of the optic tentacle

E = Experimental group: animals injected with ovotestis homogenate following the extirpation of the optic tentacle

Discussion

Many papers have dealt with the neurosecretion of Molluscs [10, 11, 18, 19, 20, 21] and also with the problem of gametogenesis [12, 13, 14, 15, 16, 17, 21].

In earlier experiments we found ova and ovocytes of 1st and 2nd order in highly elevated numbers in follicles of *Helix pomatia* after extirpation of the optic tentacles during hibernation, being the main life period of pulmonate snails, or in the period of awaking in spring or later, before egg-laying [5, 6, 8]. Optic tentacle homogenate administered to snails having intact tentacles activated the spermiogenesis. According to our present investigation, ova and ovocytes of 1st and 2nd order per follicle very significantly increased in the hermaphroditic gland on the effect of hermaphroditic gland homogenate. The number of the spermiocytes of 1st order and of the sperms significantly decreased, compared to the control. The spermiogenesis was inhibited to some extent. In snails without tentacles injection of hermaphroditic gland homogenate was followed by a significant increase in the number of the ova, while the spermiocytes of 1st order significantly decreased in number.

It may be concluded that the ovotestis possesses effective substances playing an important role in the control of gametogenesis.

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METAPHASE ARREST IN THE BONE MARROW CELLS OF *RATTUS NORVEGICUS* BY THE β-EXOTOXIN OF *BACILLUS THURINGIENSIS*

SHORT COMMUNICATION

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(Received 1977—09—23; revised 1978—04—30)

Abstract

The β-exotoxin, thuringiensin A, from *Bacillus thuringiensis* var. *thuringiensis* has been found to be C-mitotic in the bone marrow cells of *Rattus norvegicus*. This exotoxin therefore appears to be a potential tool for metaphase arrest in karyological studies. The δ-endotoxin protein has no similar activity.

Bacillus thuringiensis var. *thuringiensis* produces an endotoxin [1] the protein subunit of which is known to be insecticidal and antineoplastic in some systems [6]. Some strains of this bacterium produce a β-exotoxin, *i. e.* thuringiensin A, which is an adenosine monophosphate derivative and toxic to insect, nematode and mammalian systems [1, 4, 5]. The exotoxin has been recently reported to be C-mitotic in the *Allium* test in comparatively low concentrations [9]. Herewith we record a similar activity for exotoxin, and a negative response for endotoxin protein, in the bone marrow of *Rattus norvegicus*.

The endotoxin protein was extracted from the cultures of *Bacillus thuringiensis* var. *thuringiensis* according to an established method [5]. It is of about 90% purity. The exotoxin had been supplied by the Sittingbourne Research Centre, Kent, U. K. Aqueous solutions of 100—1000 μg/ml endotoxin protein and of 5—50 μg/ml exotoxin were used. The different concentrations were i.p. administered for 1—3 in separate experiments to albino rats, *Rattus norvegicus*, following the customary dosage pattern *i.e.* 1 ml/100 g body weight. Rats administered with 100 μg/ml colchicine served as positive controls. In normal controls, no chemical is administered. The air-dried preparations from the bone marrow, stained in Giemsa, were made following the conventional methods [2]. Detailed experiments could not be conducted owing to difficulties in extracting or obtaining more exotoxin.

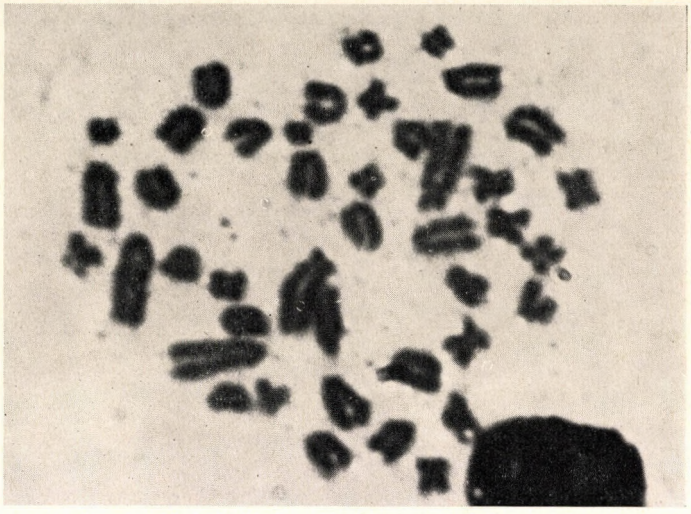

Fig. 1

The observations are presented in Fig. 1 and Tables 1 and 2. The endotoxin protein is found to be ineffective, in accordance with its known toxicity data [1]. In the exotoxin-treated rats, metaphases accumulate with concentration similar to colchicine; though not to the same extent. However, the strength of exotoxin needed to arrest metaphases in the bone marrow is less than that required in plant systems [9] and it appears that very few other chemicals cause metaphase arrest at such low concentrations [3, 8]. However, the dynamics of exotoxin action on dividing cells cannot be understood from a pilot study like this and needs much detailed investigation.

Table 1

Metaphase indices for the bone marrow of Rattus norvegicus *treated with exotoxin for two hours*

Treatment, μg/ml	Total dividing cells	Total metaphases	Metaphase cells, per cent
Control	6400	89	1.38 ± 0.09
Colchicine 100	6400	800	12.53 ± 0.36
β-exotoxin			
10	6400	582	9.10 ± 0.21
25	6400	589	9.24 ± 0.23
50	6400	602	9.40 ± 0.20

Data are from the randomized slides from the bone marrow of five rats

Table 2

Ranges of C-mitotic concentrations of β-exotoxin (in μg/ml)

System	β-exotoxin	δ-endotoxin	colchicine*
Allium cepa root meristems	25—100 and more	—	250—1000
Rattus norvegicus bone marrow	10—50 and more	—	100—500

* Laboratory practice

Surprisingly, the results from mammalian cultures are conflicting. While 10 μg/ml exotoxin brought about metaphase arrest in VERO cell lines of the African Green Monkey and human lymphocyte cultures, no such effect has been noticed in He-La cells even at higher concentrations [7].

The C-mitotic activity, which is in confirmity with our earlier report on plant systems [9] has probably resulted from an interference with the protein synthesis in the spindle microtubular systems. The present study indicates that the exotoxin is yet another C-mitotic agent comparing well with colchicine in mammalian chromosome preparations. However, it may constitute an environmental hazard only at the unlikely events of nontarget systems getting exposed to it.

Acknowledgements

We thank the authorities of the Berhampur University for facilities, Dr. S. S. V. Prasad, Indian Institute of Science, Bangalore for help in extracting the endotoxin and Dr. M. E. Shilling of Sittingbourne Research Centre, England for the gift of exotoxin.

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RECENSIONES

Relations between Structure and Function in the Prokaryotic Cell. Ed. STANIER, R. Y., ROGERS, H. J., WARD, J. B.: Cambridge University Press, Cambridge—London—New York—Melbourne (1978), pp. 369, Figs 75, £ 17.50

This is the 28th member of the series of *Symposia of the Society for General Microbiology*, Great Britain. The annually published books of the Society are almost always the most enjoyable and useful readings for microbiologists of any interest (unless they limit themselves to too narrow topics). The contributions and problems of the Symposia are very well-selected and the distinguished authors, who belong to excellent laboratories and who themselves are actively pursuing research in the field they are writing about, summarize the state of affairs of their problems clearly and authentically.

The problems dealt with are very diverse in their nature, but all of them are about life processes in prokaryotes (no viruses, no eukaryotic microorganisms!). Although the analysis, the description of certain structures and processes are at the molecular level, they are about relatively complex structures and functions, like: bacterial ribosome; the translation machinery; Gram-negative bacterial cell envelope; structure and growth of the walls of Gram-positive bacteria; structure and immunostimulant properties of mycobacterial cell walls; plasma membranes; the purple membrane of halobacteria; phospholipoproteins in enzyme excretion by bacteria; the reversion of bacterial protoplasts and L forms; bacterial flagella; leptospiral mobility; spore-specific structures and their function and the gas vesicles of aquatic prokaryotes.

Reading, *e. g.*, the fundamental progress about the ribosomes or the elegant and many-sided approach in flagella research one surely feels the truth in what the editors say: "... our colleagues who have deserted the microbes to study higher things ... may see the error of their ways and realize that for many relevant problems bacteria may still be the necessary and most expeditious model organisms to use."

Nevertheless, I am sure that some are also happy with eukaryotes.

G. SZABÓ (Debrecen)

D'AMATO, F.: **Nuclear Cytology in Relation to Development.** Cambridge University Press, Cambridge (1977), pp. 283, Figs 83, Tables 40, £ 15.00

The original purpose of this book is to give a summary on the role of the nucleus in development. The selected topics (life cycle, cell cycle, meiosis, mosaics, chromosome complement, differential DNA replication, gene expression during development, regulation of gene activity, regeneration and totipotency) are carefully chosen and the data on plant systems well presented.

The author is a well-known representative of plant cytology, having interest in fields of molecular biology. In spite of this, the interpretation of certain topics (meiosis, cell cycle, gene expression) is lost in descriptive details and not concentrated on theoretical approaches of development. There are many comments on different types of meiosis and peculiarities about the fertilization process, but the biochemistry and genetics of these events are very superficially touched, using old references.

It is very difficult to summarize the role of differential gene activity in development from the point of view of a cytologist. However, it is nearly impossible not to mention the findings of R. KORNBERG on histones and DNA structure, these findings have up a large part of contradictions caused by "chromatin research". It would be also extremely important to take into account the recent results of J. GURDON and his co-workers with the injection of purified mRNAs and the theoretical consequences of these experiments. The most serious setback of the book is the total lack of genetical data on the well-known developmental systems *(Drosophila*, axolotl, *Xenopus)* where at least there are examples about the importance of the "maternal effect" genes in regulating eukaryotic development.

Perhaps the best part of the work is the chapter on differential DNA replication where all the data on ribosomal genes in different systems are carefully collected and critically analysed.

J. GAUSZ (Szeged)

MARTIN, S. J.: **The Biochemistry of Viruses** *(Cambridge Texts in Chemistry and Biochemistry)* Cambridge University Press, Cambridge, London, New York and Melbourne, (1978), pp. 145, Figs 78, Tables 6, Appendix for Virus classification.

According to the author, the book is "designed to provide a rapid overall picture of virology at the molecular level".

It contains seven chapters. In the first chapter a brief history of virology is given. The second chapter deals with the principles of virus classification, describes the cryptograms summarizing the characteristics of the different viruses. This chapter is completed by an Appendix for virus classification at the end of the book. In Chapter 3 the author outlines the methods of assaying viruses, while in Chapter 4 he summarizes the principles of virus-purification. Chapters 4 and 6 contain the essence of the book describing the architecture of different viruses and the "strategy of virus infection" in lytic, transforming and persistent infections. Finally, in Chapter 7 the relationships of viruses and biosphere, including the role of viruses in evolution, the fight against virus diseases with vaccines and chemotherapeutic agents, are dealt with.

This booklet does not give detailed information for the virologist dealing with special problems of virology, even author did not intend to do this. As the book is written in a most ingenious style and fulfilled its original intention, *i. e.* to give an up-to-date picture of virology at the molecular level, it can be recommended to medical and veterinary students, to students of biochemistry and biology and to all wishing to get a survey of molecular virology without considerable efforts.

I. FÖLDES (Budapest)

Integration of Activity in the Higher Plant *Symp. Soc. Exp. Biol. No. 31.* Ed. JENNINGS, D. H. Cambridge University Press, Cambridge, (1977), pp. 539, Figs 118, Tables 18, £ 20

The volume contains the lectures delivered at the *31st Symposium of the Society for Exerpimental Botany*, devoted to the problem how a higher plant is able to function in a coordinated manner. Clearly, but a few aspects of this up-to-date and difficult topic could be covered and the selection might seem somewhat arbitrary. However, it is most welcome that in addition to the "fashionable" regulatory problems, *e. g.* "Regulation of carbon dioxide fixation in plants" (M. KLUGE) and "Growth substances and integration in the whole plant" (P. F. WAREING) some areas which usually receive less attention have also been discussed like "Gravity and light-sensing guidance systems in primary roots and shoots" (M. B. WILKINS), "Stomatal function in relation to leaf metabolism and environment (I. E. COVAN and G. D. FARQUHAR) and a mathematical approach to "Root: shoot interactions" J. H. M. THORNLEY). Great emphasis has been laid on the regulation of transport processes and on their role in "whole-plant" integration: "Regulation of solute transport at the cell level" (J. A. RAVEN), "Cell turgor pressure regulation and turgor pressure-mediated transport processes" (V. ZIMMERMANN), "The role of nutrition in apical dominance" (G. I. MCINTYRE), "Regulation of ion content in whole plants" (M. G. PITTMANN and W. J. CRAM), "Integration and regulation of translocation within the whole plant" (J. MOORBY), "Regulation of water movement in whole plants" (J. S. BOYER). Some selected, typical areas of biochemical regulation have also been covered: "Conservation of carbohydrate by the non-photosynthetic cells of higher plants" (T. REES), "Factors determining nucleic acid and protein synthesis in the early hours of

germination" (D. J. OSBORNE, M. DOBRZANKA and S. SEN), "The greening of leaves" (J. W. BRADBEER, G. P. ARRON, A. HERRERA, R. J. KEMBLE, G. MONTES, D. SHERRATT and O. WARA-ASWAPATI). The old problems of the "Molecular basis of physiological rhythms" (E. WAGNER), "The control of cell enlargement" (R. E. CLELAND), and the "Interacting processes in vegetative development and in the transition to flowering at the shoot apex" (R. F. LYNDON) are described in new perspectives.

The volume can be recommended to all plant physiologists, irrespective of their focus of interest (molecular or whole-plant) because the treatment of the subject is typically interdisciplinary in nature.

G. L. FARKAS (Szeged)

Printed in Hungary

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REVIEW ARTICLE

THE MOLECULAR STRUCTURE OF THE INTERFIBRILLAR MATRIX IN CONNECTIVE TISSUE

L. Módis

INSTITUTE OF ANATOMY, HISTOLOGY AND EMBRYOLOGY
MEDICAL UNIVERSITY,
DEBRECEN

Introduction

The biology of the matrix deals with the structure and function of the material composed of organic and inorganic components occupying the extravasal and extracellular spaces in multicellular organisms. The extracellular matrix is made up of the glycocalyx (cell coat) connected closely with the cell membrane, and of connective tissue matrix filling the intercellular spaces.

The organic structure of the intercellular matrix consists of fibres and of the interfibrillar matrix (ground substance). The fibrous component is represented by highly-organized collagen and, in certain sites, by elastic and reticular fibres. The interfibrillar matrix is composed of different complexes of non-collagenous structural proteins and carbohydrates. The carbohydrate components of proteoglycans are polyanionic glycosaminoglycan (mucopolysaccharide) molecules of high functional importance, while the carbohydrate components of glycoproteins are oligosaccharides composed of neutral sugars and neuraminic acid. The physicochemical state and biological function of the matrix are determined by the quantity, the primary structure and the conformation of its organic components, and by the interaction of these components.

The intercellular matrix constitutes a significant part of the human organism. Its bulk corresponds roughly to the extracellular-extravasal water content of the body. More important is the fact that the matrix is in contact with the large bordering surface of the totality of cells. Cell surfaces contact one another and the capillary walls through an interposed thinner of thicker matrix space. Thus, in all multicellular animal organisms supracellular biological units (tissues, organs, organ systems) are connected by the matrix, which, in this way, provides the morphological integrity of the organism, the appropriate conditions for specific activities of different tissues and organs, and the functional integrity of the organism.

In multicellular animals, life processes are bound to continuous interactions between cells and the surrounding matrix.

This is illustrated in Fig. 1, which is a schematic representation of some salient features of the matrix function. A blood capillary is surrounded by connective tissue cells of different degrees of differentiation. These cells synthesize and secrete the organic components of the matrix in compliance with their degree of differentiation (arrows with circles). Spaces of varying extent between the capillary and the cells are occupied by the matrix. The distance between capillaries and cells may vary from a few nanometers to hundreds of micrometers, and in some connective tissues the gap may attain even the magnitude of a few centimeters. Within the same animal species the quantity, composition, and structure of the matrix depend on the type and degree of differentiation of the connective tissue, on the equilibrium between the synthesis and degradation of the matrix by the cells (short, dense arrows), on the humoral factors regulating cellular activity (dotted arrows), on mechanical factors, and on various pathological circumstances. The quantity, composition, and structure of the matrix influence a series of metabolic processes including the anabolic transport between capillaries and cells, the diffusion of nutritive materials and oxygen (empty arrows), and the catabolic transport between cells and capillaries (long, dense arrows). These effects on cell metabolism are exerted by the matrix in conformity with the quantity, with the primary structure, and with the conformation of its macromolecular components. The components released by cells into the extracellular space interact and build up the structure of the organic matrix; at the same time, the components affect the synthesizing activity of the cells. This feedback known only in outlines, may be positive or negative (arrows with triangles). Figure 1. shows the effect of inductive factors (hatched arrows) involved in differentiation. The matrix has, of course, important mechanical functions which ensures the shape, the elasticity, and the frame of varying stability of tissues and organs.

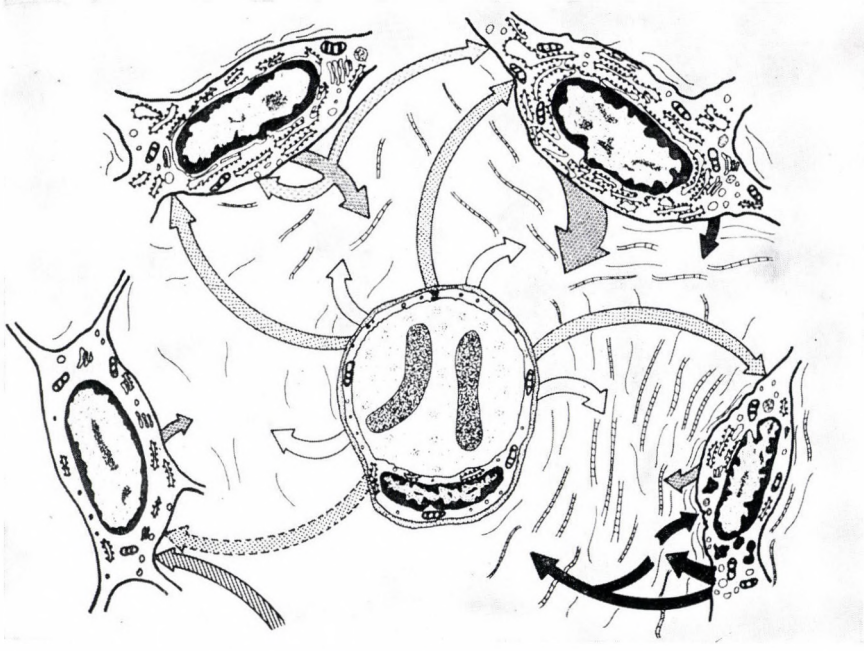
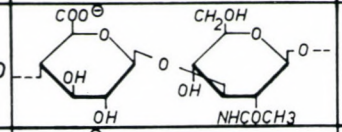
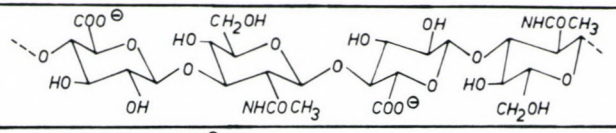
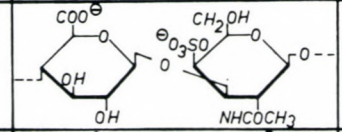
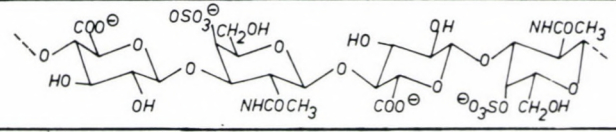
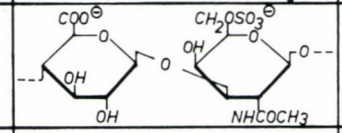
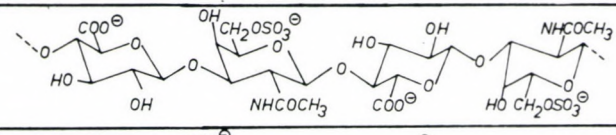
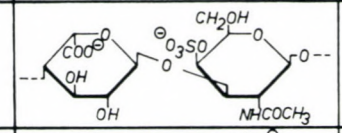
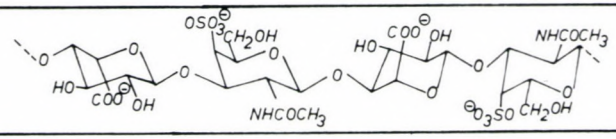
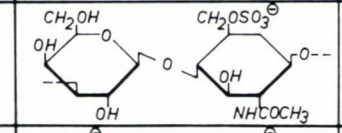
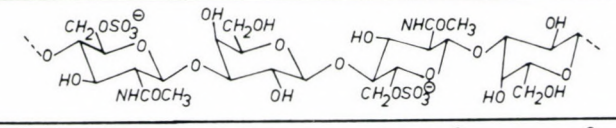
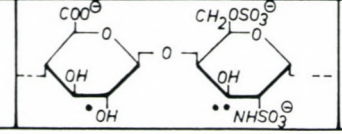
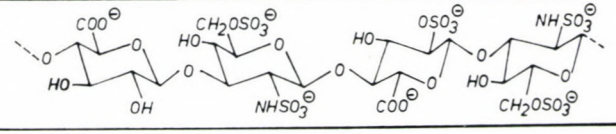


Fig. 1. Schematic diagram illustrating some selected functions of the connective tissue matrix. In the centre, a blood capillary can be seen. Around the capillary, connective tissue cells of varying degree of differentiation are shown (from left to right: undifferentiated, differentiating, mature and ageing cells). The space between the cells and the capillary is occupied by the matrix (the fibrillar components are marked by a few fine lines). Empty arrows = anabolic transport; dotted arrows = humoral regulating factors; arrows filled with circles = activity of cells synthesizing and secreting matrix components; arrows filled with triangles = feedback between matrix and cell; dense arrows = matrix degradation and catabolic transport

Table 1

Summary of repeating units of glycosaminoglycans present in vertebrates

Right part of Table is based on data published by NIEDUSZYNSKI and ATKINS [75], PHELPS and ATKINS [79], WOOD-HEAD-GALLOWAY and HUKINS [117].

GAG	IDEALIZED CHEMICAL FORMULAS OF REPEATING PERIODS	
	IN HAWORTH-FORM	IN CHAIR-FORM
HYALURONIC ACID		
CHONDROITIN 4-SULFATE		
CHONDROITIN 6-SULFATE		
DERMATAN SULFATE		
KERATAN SULFATE		
HEPARIN		

* = —OH groups may be sulphated if the uronic acid component is L-iduronic acid [51].
** = here the "heparan sulphate" contains no sulphate.

The appreciation of the vital importance of the connective tissue matrix has given inspiration to many investigators to explore finer and finer structural details. In the last 20 years the increasing interest in matrix biology and the dynamic growth of this field led to the publication of more than two hundred textbooks and innumerable papers on this subject. It is now a challenging task for the investigators to question KARL MAYER's statement which the great pioneer of the molecular biology of the matrix advanced in 1970: "Compared to protein or nucleic acid biochemistry, the glycosaminoglycans and related compounds are still very much the poor relatives." It is hoped that the present review can convincingly show that the matrix components are, in their biological significance, equal counterparts of proteins and nucleic acids.

1. Glycosaminoglycans (mucopolysaccharides)

Primary structure

The following glycosaminoglycans (GAGs) are encountered in vertebrates: hyaluronic acid (HA), chondroitin (Ch), chondroitin 4-sulphate (Ch4S), chondroitin 6-sulphate (Ch6S), dermatan sulphate (DS), corneal keratan sulphate (KS I), skeletal keratan sulphate (KS II), heparan sulphate (HS) and heparin (H). All of them are linear polyelectrolytes of varying anionic character. Their molecular weights are in the range of 4×10^3—15×10^6 daltons, KS I, KS II and H having the smallest molecular weights. Molecular weight may vary even with the same GAG, as it depends on the technique of preparation and method of determination on the species, tissue, and age. It may even change within the same tissue.

The basic GAG structure has not changed, or has changed very little, during the evolution of the animal world. Most GAGs consist of repeating disaccharide units, except HS and H, which are composed of tetrasaccharide units (Table 1). The repeating units are composed of hexosamine and hexuronic acid. The two types of KS contain no hexuronic acid; D-galactose and D-glucosamine constitute their repeating units. As chain terminals, or side chains, N-acetylneuraminic acid and neutral monosaccharides (fucose, galactose, mannose) can be found in both types of KS [15, 43, 61]. KS II has a branched structure containing more neutral monosaccharides than KS I. Therefore, the structural features of KS II resemble glycoproteins more than GAGs [60]. In the HA, Ch, Ch4S and Ch6S molecules hexuronic acid is present in the form of D-glucuronic acid. In DS, the hexuronic acid component is L-iduronic acid, while the HS and H molecules contain both glucuronic acid and iduronic acid in varying proportions. In H preparations the major uronic acid component is L-iduronic acid [51]. With the exception of HA, the hexosamine component is sulphated in all GAGs. The degree of sulphation and the position of the

sulpho groups vary according to the type of GAG. Heparin with its 5—6 sulpho groups bound to each tetrasaccharide unit, has the highest anionic charge density. The lowest degree of sulphation is shown by chondroitin, which is the non-sulphated, or low-sulphated precursor of Ch4S and Ch6S. The average degree of sulphation in Ch4S, Ch6S, DS, KS I, and KS II is similar: each disaccharide has about one ester sulphate group. KS II is over-sulphated in the aging hyaline cartilage. The structure of HS is less known, its degree of sulphation is lower than that of heparin.

The most important common feature of GAG molecules is their anionic character, which is due to the carboxyl groups of hexuronic acid and to the sulpho groups of hexosamine. The weakest polyanion is the polycarboxylated HA, while the strongest is the polysulphated-polycarboxylated H. The anionic charges show dense and regular localizations on the surface of the GAG molecule. The greatest distance that can be observed between the anionic groups (about 1.0 nm), is on the surface of HA [6, 7, 8], and the distribution of charges is the most dense on the surface of H. In this molecule, the sulpho groups are spaced at a distance of about 0.3 nm from each other [6, 73, 75].

Owing to their polyanionic character, the GAG molecules are capable of performing many physiological and *in vitro* interactions. GAG molecules with different polyanionic character differ also in their interactions. The formulae shown in Table 1 are only of approximative value in estimating the polyanionic character of GAGs. Due to their heterogeneity, variability and polydispersity, the stucture of GAG molecules is more complicated [61].

By heterogeneity, or hybridization, we mean that within a GAG chain the monosaccharide sequence is not entirely regular as one or more monosaccharide species are replaced by others. Variability results from differences in the number and position of exo-groups (neuraminic acid, fucose, sulphate). Variability can be demonstrated between different chains of the same GAG type, and even between different segments of the same chain. For example, in the cartilage matrix the degree of sulphation of the chondroitin sulphate and of the keratan sulphate II changes with age [61]. It has also been shown that the distribution of ester sulphate groups is not uniform within chondroitin sulphate and heparin chains. As it will be shown below, GAG chains are linked to protein core through the so-called linkage region. The degree of sulphation of chondroitin sulphate increases with the distance from the linkage region [114]. Heterogeneity and variability are characteristic properties of all GAGs except hyaluronic acid.

Polydispersity means the dispersion of the molecular weight. This is an inherent property of all GAG types. It is clear that we have to reckon with a multitude of finer structural differences within the different GAG molecules built on the same basic structural principle. So far we do not know the exact chemical background of GAG heterogeneity, variability, and polydispersity

dependent on species, tissue, and age. This is the main reason why we try to explain most GAG functions on the basis of an idealized formula of the covalent structure. While this is helpful in explaining some basic interactions (*e.g.* ion binding), it deprives us of the possibility of interpreting finer details of the extremely variable matrix functions. This situation underlines the importance of investigations of GAG primary structures.

As to the localization of GAG molecules, heparin occurs intracellularly (in the granules of mast cells and blood basophils). Extracellular occurrence of heparin can be noted only when these cells degranulate, but even so heparin does not contribute to the matrix structure. The rest of GAGs are encountered both intra-, and extracellularly: they are formed in cells and released into the intercellular space where they become incorporated in the hierarchic structure of the matrix. GAG molecules are differently distributed in different tissues. In mammals, a typical occurrence of GAG molecules involved in matrix composition is as follows:

GAG	Tissue
HA	synovia, vitreous body, umbilical cord, embryonic tissues, skin, arterial wall, cartilage
Ch4S	cartilage, cornea, bone, arterial wall, skin
Ch6S	cartilage, skin, arterial wall, heart valve
DS	skin, tendon, arterial wall, sclera, heart valve
KS I	cornea
KS II	cartilage

(For details see [1, 14, 17, 50, 61, 68, 107]).

The composition and contents of GAGs in the various connective tissue matrices differ considerably according to species and age. GAG composition and content may vary even within the same tissue. Thus, the matrix structure, merely on the basis of GAG distribution, shows an incredible diversity, which becomes apparent in the multifariousness of the matrix. In view of our limited knowledge of the species, tissue- and age-dependent differences of GAG structures, we understand very little of the matrix function.

Functional and methodological implications of the primary structure of GAGs

The physiological functions of GAGs in the matrix can be explained by their interactions. The following types of interactions are sufficiently understood: electrostatic interactions with cations; non-electrostatic interactions with other macromolecules (steric exclusion); covalent interactions with proteins resulting in proteoglycans.

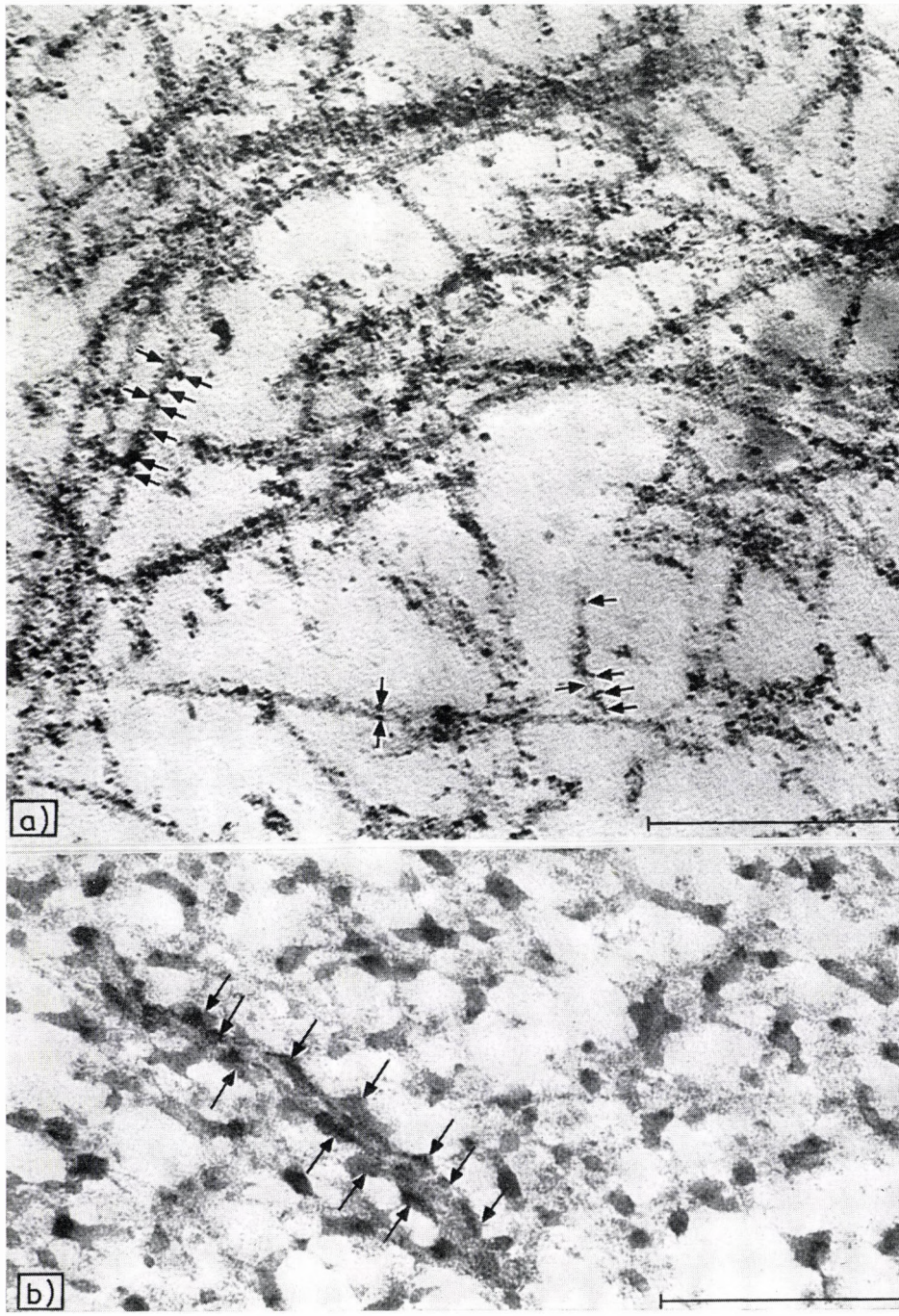
(a) Electrostatic interactions of different GAGs

Under physiological conditions, interactions occur between anionic groups of GAG molecules on the one hand and micro- and polycations on the other. The interactions with polycations are discussed in Part 4.

Microcation binding is dependent on the charge intensity of the anionic groups of GAGs, and on microcation affinity. The whole electrostatic field of the GAG molecule takes part in the interaction, which is, therefore, a non-specific binding [19, 49, 94]. The cation affinity of different GAG molecules was established by DUNSTONE [19] in model experiments. The order of cation affinity of chondroitin 4-sulphate is: $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} > Na^{+} > > K^{+}$; of dermatan sulphate: $Ba^{2+} \simeq Sr^{2+} \simeq Ca^{2+} \simeq Mg^{2+} > Na^{+} > K^{+}$; of heparin: $Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+} > K^{+} > Na^{+}$.

Model experiments are also adequate to determine the order of affinity of microcations to different GAG molecules. The order of affinity of Ca^{2+} is: H > DS > Ch4S > HA [55]. In these model experiments, however, the physiological conditions were poorly maintained, since no attention was paid to interactions between polyanions and other macromolecules. Microheterogeneity, polydispersity and conformation of polyanions were also disregarded. Moreover, as orders of affinity indicate, cations either absent, or present only in traces (Ba^{2+}, Sr^{2+}) under physiological conditions, have also been considered in these experiments. It is supposed that the physiological counterion is Ca^{2+}, then the Ca^{2+} may connect two or more adjacent GAG chains [61]. By their differential ion-binding capacity, GAG molecules have a cation-exchanging effect and, in this way, they are involved in the extracellular ion transport influencing the homoeostasis of different microcations. The electrolyte concentration, on the other hand, influences the configuration of GAG molecules: increased microcation concentration reduces the extended shape of GAG molecules and promotes their coiling [55, 57].

Trivalent metal cations show a great affinity to GAGs. We know the biological importance of this finding since the publication of SOKOLOFF's [103] results. In his *in vitro* experiments SOKOLOFF observed that in the presence of trivalent cations (Al^{3+}, La^{3+}, Fe^{3+}, $Co(NH_3)_6^{3+}$) cartilage deformation occurred more markedly than in the presence of mono-, or divalent cations. MATHEWS [55] was able to demonstrate the strong binding of $Co(NH_3)_6^{3+}$ to GAG molecules in the following order of affinity: H > DS > Ch4S > Ch6S > KS. In recent electron-microscopic investigations we have verified the binding of $Co(NH_3)_6^{3+}$ in the cartilage tissue (Fig. 2). It is known that GAG molecules are precipitated by La^{3+} [54]. From these observations, it may be supposed that trivalent cations by their strong electrovalent interactions are capable of modifying or abolishing the extended configuration of GAG molecules (this is seen in Fig. 2), and make these molecules collapse. This reduces the elasticity of the GAG-containing matrix, changes its permeability to different solutes, resulting in the degeneration of the matrix. A similar phenomenon can be noted in haemophiliacs suffering from haemarthrosis and from subsequent cartilage degeneration. The cartilage degeneration is caused by the precipitation of GAGs by Fe^{3+}, which gets into the articular cartilage from the deterio-


a)
b)

rating haemoglobin. The synovial fluid contains increased amounts of Fe^{3+} and Al^{3+} in rheumatoid arthritis [74], and this may enhance the impairment of cartilage by the former mechanism.

Water binding of GAGs is greatly influenced by the electric field generated by the anionic groups of GAG molecules. The GAG molecules are present in a hydrated state in tissues. They are able to bind water 10^3—10^4 times their own volumes [48]. The GAG chains are surrounded by cylinders of water 1.6 nm in diameter [47]. A good correlation exists between GAG content, water binding and elasticity of connective tissues. These properties decrease with age [16], because the biosynthesis of GAGs, which are the major water binding components, becomes reduced in the aging connective tissue.

(b) Steric exclusion

In solutions, large GAG molecules are able to interact with other large molecules, mainly with proteins. The basis of this interaction is the fact that two or more molecules cannot occupy the same position at the same time and they therefore mutually exclude each other from the domains they occupy. Steric exclusion is directly related to the size of the GAG molecule, to the degree of asymmetry in its shape, and to its concentration [48, 49]. Thus, the result of steric exclusion is precipitation of macromolecules penetrating the GAG interphase. This phenomenon has important physiological implications. For the sake of simplicity, the matrix may be considered as a three-component system: a GAG phase of gel state in which GAG molecules are kept in place by linkages to proteins, a solvent phase, and a mobile macromolecular phase. The GAG phase reduces the solubility of mobile macromolecules in the function of the size and shape of GAG molecules, and of the GAG concentration, and leads to precipitation of these macromolecules. Thus, steric exclusion may be involved in the formation of collagen fibres (tropocollagen molecules are released by the cell precipitate and form fibrils in the extracellular GAG phase), in immune precipitation processes (antigen-antibody complexes are precipitated in the GAG phase) which may explain the connective tissue symptoms of different immunological diseases, and in the formation of pathologic deposits (*e.g.* lipoprotein deposition in the arterial wall).

Fig. 2. Electron micrograph taken from the femoral articular cartilage of a newborn rat. Fixation in 5% glutaraldehyde (0.2 *M* cacodylate buffer, pH 7.4), counterstaining in block with cobalt hexamine (a) and ruthenium red (b), and embedded in Durcupan. No contrasting with other heavy metals was made. Scale: 0.5 μm. (MÓDIS and LÉVAI, unpublished observation.) *(a)* Cartilage slices were incubated in 3% $[Co(NH_3)_6]Cl_3$ solution in 0.2 *M* cacodylate buffer, pH 7.4. After rinsing in buffer, the bound cobalt was precipitated with H_2S dissolved in buffer. The material was then embedded and sectioned, and the thin sections were treated with a physical developer to intensify the cobaltous sulphide precipitate [108]. On the surface of non-contrasted collagen fibrils small matrix granules are seen. The granules represent collapsed and precipitated glycosaminoglycans. Some granules are marked with arrows. *(b)* After counterstaining with ruthenium red, larger matrix granules are seen. This method also serves for the visualization of the close connection between granules and collagen fibrils

(c) Exploitation of the polyanionic character of GAGs in morphological methodology

Numerous morphological methods make use of the cationbinding capacity of GAGs. The intense negative field of these polyanions attracts positively charged cationic dyes (thiazines, acridines, phthalocyanins, *etc*), metal ions, and colloids. With the aid of appropriate selection of cationic reagents and staining conditions, the different GAG components of the matrix can be studied *in situ*. Especially, cationic dyes are used in large number for these "GAG-specific" histochemistry, and the stainings are very often regarded as "specific" reactions. It must be, however, kept in mind that there are no specific GAG stains. Specific GAG staining depends entirely on staining conditions including pH, "critical electrolyte concentration" [62, 95, 97], *etc*. The failure in recognizing this fact may have resulted in the countless methods of "specific" GAG stainings based on empirical grounds, and turned GAG histochemistry into a vast irrational jungle.

With the aid of crystalloid and colloid solutions of metals of higher atomic number, or using cationic dyes containing these metals, the localization of GAGs in the tissues can be studied with the electron microscope [24, 38, 63, 89, 90, 95, 96, 99, 118]. The specificity of these methods is also contestable without the use of proper control. The contradiction in the increasing striving for ultrastructural localization without a theoretical background in the methods, is present in GAG electron histochemistry as well. For the illustration of this opinion, we refer to two allegedly specific methods by SHEPARD and MITCHELL [101, 102]. These authors used two dyes: toluidine blue (in which sulphur has the highest atomic number) and safranin 0 (in which nitrogen has the highest atomic number), for the study of electron-microscopic localization of GAG-containing proteoglycans. Obviously, both sulphur and nitrogen are too light to be shown with the electron microscope, and the electron density seen in their photographs must have been caused by heavy-metal contamination present in the dyes.

Conformation of glycosaminoglycans

The exploration of the specific conformation of GAG molecules by means of different physical methods (CD, ORD, X-ray diffraction, NMR) opened a new era in matrix biology. These investigations were carried out on pure systems (isolated GAG preparations); they still contribute to the understanding of macromolecular interactions in the connective tissue. At this critical point of polysaccharide chemistry REES [83] rightly wrote: "The ugly ducklings have begun to look a little more like swans. In this sense, polysaccharides begin to appear attractive molecules, shapely molecules."

The first examinations were performed on GAG solutions to which cationic dyes of planar character (methylene blue, or acridine orange) were added. The helical structure of GAG molecules was deduced from the chiroptical spectroscopic parameters (CD, ORD) of the GAG — dye complex [104, 105, 106]. By means of this method, NÉMETH—CSÓKA [70, 71] and NÉMETH—CSÓKA et al. [72, 73] established several structural preconditions of the helical conformation of GAG molecules and clarified the role played by the helical structure in fibrillogenesis. The chemical preconditions of GAG conformation are as follows:

(a) The glycopyranose rings of GAG monosaccharides are not flat, but are in chair form, most of them showing C-1 conformation (Table 1). The side groups occupy equatorial positions in this energetically advantageous confor-

mation. The chair conformation renders possible the formation of H-bonds stabilizing the secondary structure.

(b) Periodicity of the primary structure (repeating mono-, di-, and tetrasaccharide units).

(c) The minimum length of the GAG chain should be at least composed of 4—6 disaccharides [72, 73].

(d) Appropriate degree of sulphation of the GAG chain. At least one sulpho group is required for each disaccharide unit in the case of chondroitin sulphate [72].

(e) Appropriate humidity, ionic strength, pH and ionic quality of the environment.

By means of X-ray diffraction method, it was demonstrated that the polysaccharides of seaweeds (carrageenan, agarose) have a double-helical structure [2, 4, 83]. The experiments have shown that a correlation exists between the conformation of polysaccharides and their colloidal state. When seaweed polysaccharides assume a double-helical structure, the polysaccharide solution turns into gel. The gel structure develops only at a certain length of helix segments. At this critical length double helices also may assume the gel form, provided they interact with another type of polysaccharides and form quaternary structures with them. It is not clear what changes in conformation may cause the animal polysaccharides transform into gel state. A similar mechanism may be assumed in this case as well.

X-ray diffraction examinations have indicated that GAG molecules extracted from animal organisms have also helical conformations. The chemical similarities among the GAGs results in conformational similarities: an elementary "cell", that is, one turn of the helix, is built up of two or three repeating disaccharide units. Thus, most GAG molecules are twofold or threefold helices. GAG helices are almost entirely extended, as shown by the relatively long axial translation of one disaccharide unit (= h = 0.93 to 0.97 nm). Within the individual GAG types the helical parameters are influenced by the relative humidity of the GAG preparation, by its ion content, and by the quality of the counter ions.

Salts of the *hyaluronic acid* formed with bivalent cations (*e.g.* Ca^{2+}) show a left-handed threefold single-helical character. The pitch (= a rise per turn = axial period) of this extended helix is 2.8 nm, and the axial periodicity per disaccharide (h) is 0.95 nm [6, 7]. Adjacent antiparallel chains running in the elementary cells are linked together by $COO^- \ldots Ca^{2+} \ldots {}^-OOC$ bridges [6]. Twofold single helices were also observed under acid conditions [7]. In the presence of monovalent cations, compressed fourfold single helices with h = 0.85 nm were obtained [28]. This h value of the fourfold helix is similar to the low values of h measured in double helices of polysaccharides extracted from seaweeds (agarose, carrageenan) [2, 4]. For this reason, a coaxial double-helical structure has been proposed for all systems with low values of h [2, 3, 4, 18, 83].

The double helix models proposed earlier for hyaluronic acid [3, 18] are not generally accepted any more [28, 100, 117]. Recently SHEEHAN et al. [100] proposed an antiparallel double-helical structure for hyaluronic acid in the presence of cations K^+, NH_4^+, Rb^+ and

Cs^+. In their model, each chain forms a contracted left-handed fourfold helix (h = 0.82 nm), and the tetragonal elementary cell is passed by two double helices. The double-helical state is presumably metastable [100]. A single to double transition of the helical structure may occur dynamically under the influence of salt and water content, and of the environmental pH [8].

Chondroitin-4-sulphate is a simple two-, or threefold helix, the length of its axial period is 2.0—2.9 nm, and the axial translation of one disaccharide is 0.93—0.98 nm [6, 11, 45, 79].

Chondroitin-6-sulphate can form twofold (h=0.93 nm), threefold (h=0.95 nm) and eightfold (h=0.98 nm) helixes. The twofold helixes have been obtained only under acidic conditions [6]. The average axial periodicities for the three- and eightfold helixes are 2.86 and 7.8 nm, respectively [3, 6, 79]. The strutures of chondroitin-4-sulphate and -6-sulphate differ most noticeably in the disposition of their sulphate groups, those in the -6-sulphate being at a greater radical distance (0.8 nm) from the fibre axis than the -4-isomer (0.6 nm) [73, 79].

Dermatan sulphate has a left-handed single helical structure [9, 10, 79], and its iduronate residue is in C-1 conformation [6, 9]. Three different conformations for dermatan sulphate have been described: eightfold helix (h = 0.93 nm), threefold helix (h = 0.95 nm), and twofold helix with h = 0.97 nm [9]. Most probably, its conformation is an 8/—3 helix, that is, 8 repeating disaccharide units constitute 3 complete turns, and the axial period is 7.44 nm [9, 10].

Keratan sulphate is a twofold helix, its elementary cell is 1.89 nm high, the axial period per disaccharide is 0.94 nm [5, 6].

One turn of the *heparin* twofold helix contains a tetrasaccharide unit, the height of the elementary cell is 1.59—1.65 nm, and five sulphate groups on the average are situated in the cell [6, 75]. It means that in this molecule the sulpho groups repeat themselves in the direction of the helical axis at 0.34 nm distance. In chondroitin sulphate, on the other hand, there is a distance of about 1.0 nm between these groups.

We have no direct evidence as to what extent the *in vitro* observation on conformation are applicable to *in situ* physiological circumstances. According to ATKINS et al. [11] it is very likely that in matrix with high GAG content the GAGs are present in a crystalline form. X-ray diffraction studies of proteoglycans and proteoglycan aggregates have proven that the GAG conformation is similar both in isolated forms and in complexes existing *in vivo* [6, 11, 79]. From abnormal dispersion of induced birefringence observed in tissue section stained with cationic dye, VIDAL [111, 112] came to the conclusion that GAGs have helical conformation also when built up in the connective tissue matrix.

We know only some aspects of the *functional implications* of GAG conformation. As already mentioned, the helix content increases in the gel state. The gel-forming polysaccharides exist under biological conditions in the form of a swollen three-dimensional network of hydrated chains. The interstices of the network are occupied by water and other small molecules and ions. Some properties including the great capacity in holding its own shape the elastic response to applied stress, derive from the helical structure of the gel [1, 83, 100].

There is a spatial change in the distribution of charges on the surface of the GAG when a helical conformation is assumed. The interactions of GAGs with micro- and macromolecules are determined by this specific charge distribution, which may play an important role in collagen-GAG interaction, that is, in fibrillogenesis. It is known that dermatan sulphate binds more strongly to collagen and to other polycations than do other GAGs [22, 110]. It is also known that in dermatan sulphate the carboxyl group of iduronic acid residue

has a C-1 conformation, and thus it is axially-positioned. On the other hand, in chondroitin sulphate and hyaluronic acid the carboxyl group of D-glucuronic acid residue, though having a C-1 conformation, is in an equatorial position [19]. This stereo-chemical difference may explain the strong polycation affinity of dermatan sulphate in comparison with chondroitin sulphate [9].

NÉMETH—CSÓKA et al. [72, 73] called attention to a correlation existing between the helical structure of GAG and its effect on fibrillogenesis. Heparin, with its high charge density and low value of axial periodicity, inhibits collagen fibre formation, while chondroitin sulphate with sparser distribution of sulphates, and with longer elementary cell of its helix, stimulates fibrillogenesis.

2. Proteoglycans

Structural principles of proteoglycans

Under physiological conditions, the great majority of GAG molecules are bound to polypeptide chains in connective tissues. The linkage is of the covalent connective tissues. The linkage is of the covalent type and it is formed intracellularly. This protein-polysaccharide complex with a molecular weight of 10^5—10^6 dalton range is termed proteoglycan (PG). The PG molecular complex consist of GAG molecules varying in number and quality, of a non-collagenous polypeptide backbone (core protein), and of the so-called linkage region connecting the GAG molecules with the core protein (Fig. 3). The number of GAG molecules varies by species and by the type of the tissue and it changes with age. Thus, PG molecules vary in size, that is, they are polydisperse. Moreover, PG molecules show a high degree of heterogeneity due to the variations in the primary structure of the PG components, mainly of GAGs. PG molecules may contain one or two types of GAG. Those containing two types are the so-called hybrid PGs. Hybrid PGs are, for example, proteokeratan chondroitin-4-sulphate and proteochondroitin dermatan sulphate. The first is present in the aging hyaline cartilage, and the second can be found in the aorta wall. Most PG molecules in the hyaline cartilage form aggregates (see Part 3). These types of PGs are very often termed proteoglycan subunits (PGs).

The structure of core protein

Core protein is a 100—400 nm long, a presumably linear polypeptide composed of several hundreds, or a few thousands, amino acid residues. It is suggested that in certain PG molecules the core protein is a double-strand polypeptide [36, 61]. The double-strand structure seems to be due to the linear aggregation of polypeptide subunits composing the core protein [98].

The amino acid sequence of the core protein differs by species and tissue. Most of the information available relates to the core protein structure of the PGs of the cartilage matrix. Approximately 30% of the amino acids building up the core are charged. The ratio of acidic to basic groups is about 2:1 [58]. On the basis of periodical binding of GAG chains to the core, the latter has been

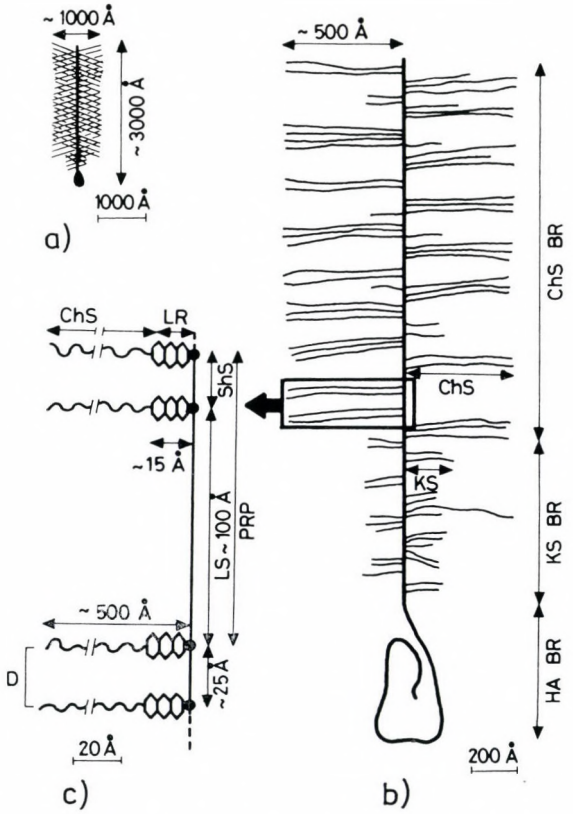


Fig. 3. Schematic representation of the shape and size of proteoglycan subunit (PGS) present in cartilage tissue (ref.: [29, 39, 40, 42, 52, 58]). *A.* Drawing showing the steric structure of PGS. The long line in the middle is the core protein which has a globular configuration at one end (dense oval). Core protein binds the longer chondroitin sulphate chains and the shorter keratan sulfate chains. The latters bind preferentially to the core portion next to the globular region. Chondroitin sulphate and keratan sulphate molecules are arranged perpendicularly to the core protein. The spatial structure of PGS reminds a bottle brush. *B.* Scheme illustrating the different parts of PGS. In extreme right, the parts of the core protein are shown globular hyaluronic acid binding region (HA BR), linear keratan sulphate binding region (KS BR), and linear chondroitin sulphate binding region (ChS BR). Chondroitin sulphate (ChS) and keratan sulphate (KS) chains are bound periodically to core protein. The length of a ChS chain indicates 50 nm. *C.* The enframed portion of *B* at higher magnification showing a possible repeating period (= PRP) of the core protein and the linkage region (= LR). LS = the long sequence of core protein ShS: the short sequence of core protein D = chondroitin sulphate (ChS) doublet bound to ShS. Dots on the ShS portion represent binding amino acids to which GAG chains are bound through trisaccharides marked with hexagons. The length of a GAG chain indicates 50 nm

divided into repeating units. According to MATHEWS [58], each period consists of a long and a short sequence (Fig. 3). The long sequence is usually composed of 35, and the short sequence of 10, or less, amino acids. The long sequence is sensitive to proteolytic enzymes, whereas the short one is resistant to enzymes except papain and pronase. In the so-called doublets, two GAG chains are bound to the short sequences. Core periods containing more GAG chains may also exist [13].

On the basis of their amino acid composition and participation in interactions, three different segments can be distinguished in the core protein of PG (PGs) extracted from differentiated hyaline cartilage [29, 39, 40, 42] (Fig. 3). The longest part of the core is the *chondroitin sulphate-binding region* (Fig. 3 = ChS BR). This part of polypeptide is of various lengths, has an extended configuration and is rich in serine, threonine and glycine. The serine residues of this region bind the ChS chains, a total of about 100. The threonine residues of the region bind about 20% of all KS chains to be found in PGS. ChS BR is the most variable part of the core, being liable to change with species, tissue and age.

The second, shorter part of the core is the so-called *keratan sulphate linkage region* (Fig. 3 = KS BR). This part of polypeptide has an extended configuration and is rich in glutamic acid and proline. It binds about 50% of the KS chains present in PG. The KS chains are linked by alkali-stable bonds to the glutamic acid or glutamine present in the core. The KS chains are shorter and have lower molecular weights than the ChS chains. ChS BR and KS BR contain different antigenic determinants [46]. The antigenic determinants of the core protein located in the vicinity of the link present between GAGs and core protein (= linkage region) represent the so-called species-common antigenic determinants of the connective tissues [91]. A structural similarity in the short sequence of the core protein of all animal species studied may account for the species-common antigenicity of this region [58, 60]. The antigenicity of core protein made possible to perform immune histochemical investigations on non-collagenous proteins. Recently, core protein filaments have been demonstrated in the cartilage matrix by means of immune electron histochemical methods in our Institute [26] (Fig. 4). This method, or its advanced form by using more specific antibodies, offers new possibilities in the investigation of the matrix structure. The method seems to be promising in the finer localization of the core protein relative to other components of the matrix.

The third part of the core protein is synthesized only by cartilage cells. This is a region with a molecular weight of 9×10^4 daltons, and it is rich in aspartic acid, methionine and cysteine, and has a globular configuration. No chondroitin sulphate and only a small amount of keratan sulphate is bound to this part of the polypeptide backbone. The most important property of this region is its high affinity and non-covalent linkage to a decasaccharide unit of

the filamentous hyaluronic acid present in cartilage matrix [31, 33, 34]. The term *hyaluronic acid-binding region* refers to this property (Fig. 3 = HA BR). The linkage to hyaluronic acid is higly specific. No binding to other GAG molecules takes place even if these are very similar to HE in structure. In specific binding, when the corresponding segment of HA is recognized, arginine, tryptophan, tyrosine residues, and lysine ε-amino groups are involved. For this "recognizing" and binding function an intact tertiary structure of this region is also required. This structure is stabilized by 5 to 7 disulphide bridges.

There are few data on the *conformation of PG core protein*. EYRING and YANG [20] suggested that the polypeptide core has no secondary structure. On the basis of X-ray diffraction pictures of PG molecules [6, 11, 79], the only conclusions that can be drawn is that the helical conformation of GAG chains in PG is identical with the conformation observed in pure GAG preparations. On the basis of CD measurements, GELMAN et al. [23] have found that in the case of PG interaction with poly(L-arginine), or poly(L-lysine) alpha-helical conformation is induced in about 40% of the core protein. These investigations are of particular interest because the interaction between PG and these basic homopolypeptides is very similar to the PG-collagen interaction occurring in the matrix under physiological conditions.

Structure of the linkage region

GAG chains form covalent bonds with preferential amino acid residues (binding amino acids) of the core protein through an oligosaccharide structure. The amino acid residue and the oligosaccharide together constitute the linkage region (Fig. 3 = LR). The ordered sequence of this region in the case of Ch4S, Ch6S, DS and H molecules is: serine-D-xylose-D-galactose-D-galactose [52, 84]. Some authors suggest that in the case of GAG molecules containing D-glucuronic acid, the first glucuronic acid residue of the GAG is also involved in the structure of the linkage region. This first uronic acid residue is attached to D-galactose by a glucosyl uronic transferase that differs from the one responsible for the addition of the subsequent glucuronic acid residue in the GAG chain. On account of this biosynthetic distinction, the first uronic acid residue is taken together with the neutral sugar sequence to comprise the linkage region of the proteoglycan [52, 60].

Other structures of linkage region are also known. In the case of hyaluronic acid the following sequence of the linkage region was supposed: serine or threonine-arabinose-glucose-glucose [113]. It is an unsettled question, however, whether hyaluronic acid does or does not exist in proteoglycan form. The linkage region of corneal keratan sulphate is composed of asparagine or glutamate-N-acetylglucosamine-mannose-mannose [12]. The skeletal keratan sulphate is bound to the core protein through a linkage region with branched structure. Available data suggest the following sequence: serine or threonine or glutamate-N-acetylgalactosaminemannose. As a side chain, a galactose-N-acetylneuraminic acid sequence is linked to the N-acetylgalactosamine [43].

The linkage region and its immediate environment have three biological functions. First, it connects the parts of the PG molecule. The second function is the role in GAG biosynthesis. While the biosynthesis of core protein is genetically coded, the biosynthesis of polysaccharide chains is not. Assembly of the monosaccharide residues of the linkage region and attachment of the GAG chain requires the sequential action of different glycosyl transferases. In the case of ChS, for example, the immediate vicinity of the binding amino acid (*e.g.* serine) on the core protein is "recognized" by xylosyl transferase, which binds the first monosaccharide (xylose) to the serine. It is followed by the addition of subsequent sugar residues in proper sequence. The acceptor specificity of the growing polysaccharide chain for the next glycosyl transferase is determined by the nature of the terminal sugar residue. Thus, the linkage region serves a site of initiation for GAG biosynthesis [58, 60, 84]. The third function of the linkage region is that the core sequence in its immediate vicinity, which is a phylogenetically ancient portion, represents the species-common antigenic determinant of connective tissues.

Size and shape of proteoglycans

The dimensions of proteoglycans have been deduced from biochemical assays of proteoglycan components (the average dimensions are shown in Fig. 3). On the basis of these data, several hypotheses have been built. They obtained support from physical and electron-microscopic investigations carried out on isolated proteoglycan preparations. The proteoglycan molecule has a bottle-brush like form (Fig. 3, part a). The bristles of the brush are the GAG chains with an extended configuration and helical conformation arranged perpendicularly to the axis of the brush, which is the core protein. We do not know how this molecular shape can be modified in the matrix by various interactions. By determination of electric birefringence (KERR-effect) of proteoglycan solutions, FOWERAKER et al. [21] concluded that the hyaline cartilage PG molecules are prolate ellipsoids of 380 ($\pm$20) nm length and of 40 nm semiminor axes. The length of the ellipsoid is consistent with other data on the length of the core protein, and the half of the shorter axis corresponds to the average length of a ChS chain. With electron-microscopic investigations of a monomolecular layer made from a mixture of isolated PG molecules and cytochrome-C-oxydase, the shape and size of PG molecules could be directly studied [88]. The most important data are summarized in Table 2. The bottle-brush-like shape supposed on the basis of biochemical and physical data, has been confirmed by electron microscopic investigations. The electron micrographs showed cluster formation of GAG chains, each cluster consisting of four GAG chains [41, 109]. This phenomenon is illustrated in Fig. 3, part *b*. It is clear that the number of GAG side chains in Table 2 refers to the number

Table 2

Dimensions of isolated proteoglycan molecules on the basis of electron microscopic investigations

Tissue	Core length (nm)	GAG side chains		References
		number	length (nm)	
Bovine nasal cartilage (BNC)	203—340	19—30	48—54	ROSENBERG et al. [88]
BNC	170—340	20—24	52—54	WELLAUER et al. [115]
BNC	240—360	25	45	THYBERG [109]
Bovine tracheal cartilage Guinea pig costal cartilage				
BNC	170—370	25	39—46	LOHMANDER [53]

of clusters rather than the real quantity of GAGs. The occurrence of clusters seems to support the concept that the core protein may be divided into periods according to the periodic GAG binding (Fig. 3, part *c*).

The electron micrographs may also be used for the tentative estimation of the molecular weight of the whole PG molecule and that of its components. In the calculation, an amino acid residue was considered to be 0.25 nm long with a molecular weight of 100 daltons, and a disaccharide was taken to be 1.0 nm long having a molecular weight of 500 daltons [53]. Starting from these data, a 300 nm long PG molecule with 100 GAG chains each 45 nm long, has a molecular weight in the order of 2.4×10^6 daltons [53]. This is in good correlation with the biochemical molecular weight range: $0.7—3.8 \times 10^6$ daltons [37]. On the basis of this principle, the number of anionic charges of a PG molecule was calculated to be 9×10^3.

PG molecules, owing to their heterogeneity and polydispersity, show great variations in shape and size, even within the same tissue. The structural

Table 3

In the course of evolution of vertebrates the following principles can be recognized in the structure of ChS—PG.

Constant structural features	Variant structural features
1. Molecular architecture (bottle-brush-like attachment of GAG to a core)	1. The length of ChS binding region in the core protein
2. The length of the HA- and KS-binding regions in the core protein	2. The amino acid sequence of the long sequence
3. The "doublet" chain substructure	3. The number of GAG chains bound to the core
4. The amino acid sequence and the length of the short sequence	4. The length of GAG chain
5. The length of the long sequence	5. Degree of sulphation of GAGs
6. The binding amino acid residue and neutral sugar sequence on the linkage region	6. Microheterogeneity of GAG chains
	7. Substitution of other glycosaminoglycans (*e.g.* KS) for polypeptide

principles have hardly changed during evolution, but numerous variants on the composition and size can be found. When finer data will be obtained, this structural variability may help to explain certain functional differences of connective tissue matrices. For illustration of this concept, we have summarized the structural principles, which are constant or changing during evolution, in proteochondroitin sulphate, which is the most frequent proteoglycan molecule in vertebrates [29, 59] (Table 3).

3. Proteoglycan aggregates

In cartilage tissue matrix, the majority of proteoglycans are present in the form of multimolecular aggregates. Previously it was supposed that the aggregates arise from the attachment of PG molecules and glycoprotein [35]

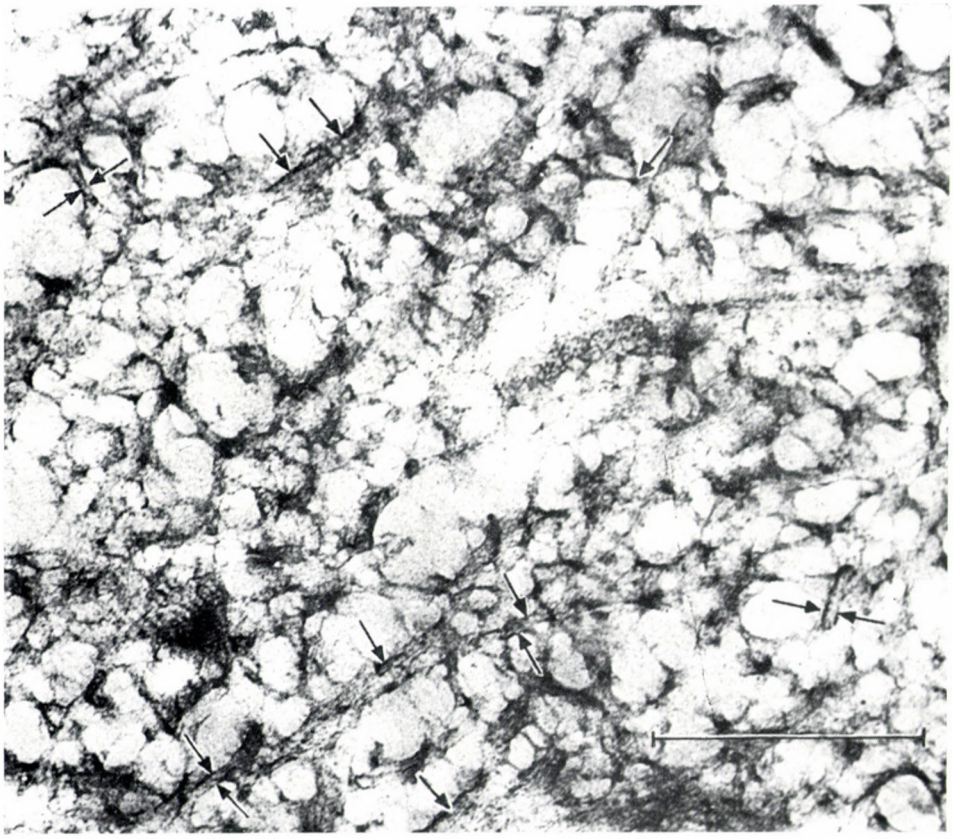


Fig. 4. Immune electron histochemical reaction of non collagenous proteins in bovine nasal cartilage matrix, modified from GLANT et al. [26]. Immune peroxidase reaction was carried out after hyaluronidase digestion, no counterstaining with heavy metal salts was applied. Non collagenous proteins appear as fine granules and filaments (arrows) on the collagen fibrils and in their vicinity. Scale: 0.5 μm

HARDINGHAM and MUIR [30] have demonstrated that the aggregate results from interaction of PG molecules and filamentous hyaluronic acid molecules. Using equilibrium density-gradient ultracentrifugation under associative and dissociative conditions, it was demonstrated that the aggreates consist of three parts: PG (= PGS), HA, and the so-called protein link [30, 31, 32, 33, 34, 40, 67]. As described in Part 2, a region of the core protein with a special

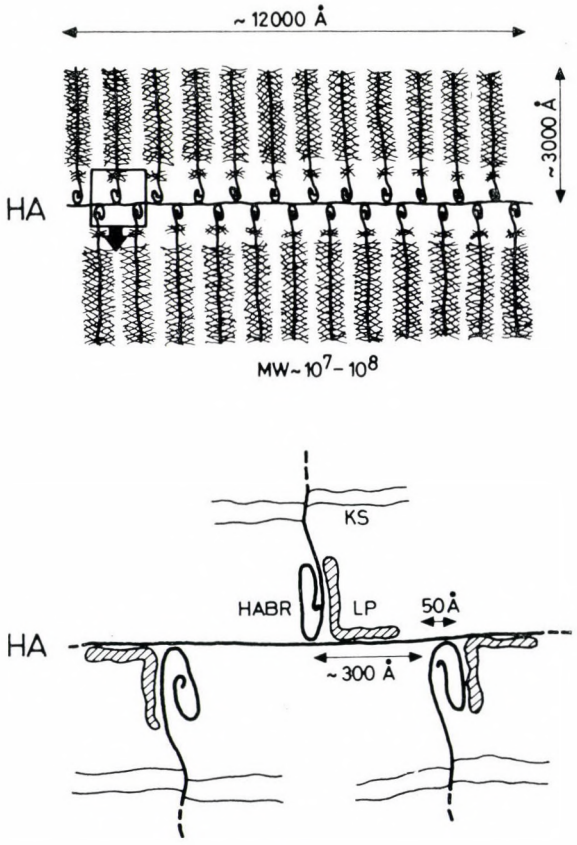


Fig. 5. Two-dimensional scheme of proteoglycan aggregate present in cartilage matrix composed on the basis of references [29, 30, 31, 33, 34, 67]. In the upper part of the figure, a filamentous hyaluronic acid (HA) molecule, about 1200 nm long, with 24 proteoglycans, each about 300 nm long, are delineated. The core proteins of proteoglycan molecules are bound to HA by their ends of globular configuration (hyaluronic acid-binding region = HA BR) at a distance of about 30 nm from each other. The molecular weight of the aggregate is in the order of $10^7 - 10^8$. The framed portion of the aggregate is shown with higher magnification in the lower part of the figure. Here, we can see a portion of HA with 3 proteoglycans attached by means of HA BR with globular configuration. The globular region passes to the linear part of the core protein which binds keratan sulphate (KS) molecules. The interaction between hyaluronic acid and HA BR region of core protein occupies an area about 5 nm long on the HA chain. The linkage between HA and HA BR is stabilized by link protein (LP)

structure is responsible for HA binding in the PG molecule. Within the hyaluronic acid molecule, having a molecular weight of 5×10^5 daltons and a length of 1200 nm (400—4000 nm), a decasaccharide part is the minimum length (4—5 nm) which can bind PGS (Fig 5, lower part). This binding is highly specific, it does not occur if HA is replaced by chondroitin in the system, though these two GAGs differ only in the conformation of hexosamine C4—OH. The interaction is not covalent but partly H-bond, and partly ionic binding. One HA molecule can bind 30 to 40 PGS and the distance between the PGS molecules bound is approximately 30 nm (Fig. 5). The bond is stabilized by one or two proteins presumably of glycoprotein in nature (protein links) [29, 33, 34, 67]. The molecular weights of link proteins are: 45×10^3 and 65×10^3 daltons, respectively [33, 67]. One part of these proteins binds to the globular region of PGS, the other part to a portion of the HA molecule (Fig. 5, lower part). The nature of this linkage is not known.

From the interactions mentioned above, a giant supramolecular complex arises with a molecular weight of the order 10^7—10^8 daltons. Its steric structure may be imagined as a long string (HA) to which 30—40 bottlebrushes (PGS) are attached at regular distance from each other, and in alternating direction. This structure is illustrated in a two-dimensional scheme in Fig. 5. Electron-microscopic investigations of isolated PG-aggregates have supported this assumption [87]. Morphological evidence has been obtained that hyaluronic acid-proteoglycan interaction represents a phenomenon occurring physiologically in the cartilage both intra- and extracellularly [82].

The function of aggregates is not fully understood. Presumably, the hierarchic structure of the aggregate has an immobilizing effect on PG molecules in the cartilage matrix, and reduces the sensitivity of PG molecules to proteolytic enzymes [67]. Elasticity, one of the essential mechanical properties of the cartilage matrix is due to this highly-oriented structure. In normal cartilage matrix, only a minor part of PGs is non-aggregated. The proportion between the aggregated and non-aggregated forms changes with age. Under pathological conditions, *e.g.* in osteoarthritis, the quantity of PG-aggregates diminishes [67], and this may explain the severe dysfunctions observed in this disease. It may be noted that mineralization of the cartilage matrix is preceded by a marked reduction in aggregated PG [44]. It is supposed that aggregated PG inhibits calcification, while non-aggregated PG may form the organic matrix required for the deposition of calcium crystals.

It may be also supposed that the aggregates play a role in the formation of collagen structure characteristic of hyaline cartilage. It remains to be investigated whether this cartilage-specific supramolecular structure has any specific function.

4. Proteoglycan-collagen interaction

The *in vitro* and *in vivo* interactions of collagen and glycosaminoglycans are known since long. The interaction depends on the pH, ionic composition and ionic strength of the environment, and it is thought to be electrostatic in nature [56, 60, 77, 78]. Excluded volume effects and hydrogen bonding are also important and operate simultaneously with electrostatic factors in GAG-collagen interactions [49, 60].

The electrostatic interaction occurs between the anionic groups of GAG chains and the protonated nitrogen of lysine, hydroxylysine and arginine residues of the collagen. Due to this interaction, both the nucleation and the growth phases of fibrillogenesis are influenced by GAGs [77, 78]. The retarding or accelerating influence of diffent GAGs on fibrillogenesis is dependent upon the type, the charge density, the length, and the conformation of the GAG molecule. As compared to other GAGs, dermatan sulphate and its proteoglycans interact more effectively with collagen [22, 110]. This strong reaction is probably due to the special conformation of DS (see Part 1). According to recent studies, the interacting capacity of GAGs with collagen is determined by the degree of sulphation of the GAG molecule [70, 71, 72, 73]. As a consequence of the similar direction of rotation of their helices, GAGs of high sulphate content are linked strongly to collagen [73]. In view of their high charge density, these complexes repel each other and inhibit fibrillogenesis. Chondroitin sulphate with more extended helical structure and lower degree of sulphation, on the other hand, plays a decisive role in fibrillogenesis. A minimal length of 15—20 nm in the chondroitin sulphate chain is required to bind tropocollagen molecules [73].

Proteoglycans interact with collagen stronger than their isolated glycosaminoglycans [60]. According to model experiments, the GAG side chains of the PG can bind 25—30 tropocollagen molecules [76]. It was supposed that linkages develop also between core protein and collagen during collagen-PG interaction [76]. Electron micrographs prepared in the course of immunohistochemical assays of non-collagenous protein also show close interconnections between these proteins and collagen fibrils (Fig. 4). This type of interaction, however, remains to be verified.

The predominant ionic character of GAG-collagen interaction was proven by the competing effects of micro-ions. Increasing the ionic strength diminishes or blocks the GAG-collagen interaction in model experiments [1, 56, 57, 77, 78, 92]. Probably, the *in vivo* GAG-collagen interaction is also dependent on the ionic strength and composition of the extracellular environment.

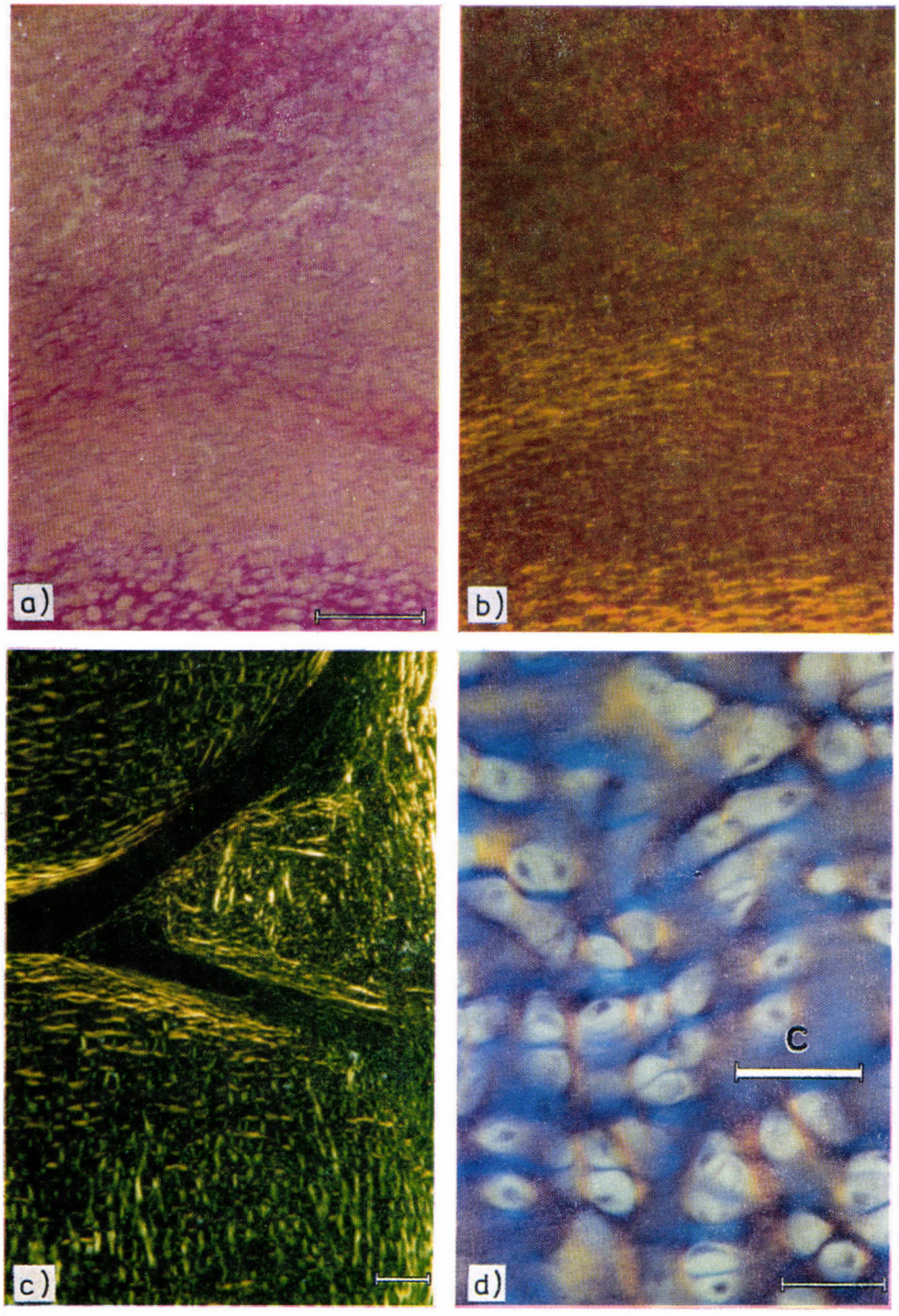
Accepting this supposition, we may speculate on the problem of ageing of the matrix structure. With the aid of X-ray microanalysis PIERI et al. [80, 81], Zs.-NAGY et al. [119] have demonstrated a markedly increased microcation concentration in nuclei of ageing cells.

According to these authors, this process inhibits DNA-protein interaction and results in an alteration of protein synthesis of the cell. A similar process operating in the matrix may reduce the GAG-collagen interaction. Such a mechanism may explain the phenomenon that a structure composed of coarse aggregated fibrous collagen develops in the extracellular matrix of certain types of connective tissue with advancing age. It should not be forgotten that a possible increase in the extracellular electrolyte concentration may be only one aspect in the interpretation of changes in extracellular macromolecular interactions associated with age; the biosynthetic and degradation activities of cells change also in the ageing connective tissue.

As a result of GAG-collagen interaction, the two components become spatially oriented in relation to one another. Biochemists have surmised for a long time that collagen fibrils and GAG chains show a parallel orientation [56, 60, 93]. The supposition has been verified by morphologists with polarization-microscopic methods [64, 65, 66, 69, 85, 86]. According to many observations, the GAG molecules are not amorphous components of the matrix, but they form a micellar structure along the collagen fibres. The physiological importance of the organized GAG structure is not fully understood. It may be supposed that the oriented GAG structure plays a role in the formation of the regular collagen structure. This possibility has been raised by several authors [27 61, 116]. In connection with this, WEISS [116] expressed the following opinion: ". . . hyaline ground substance in reality possesses definite structural order analogous to crystallinity". MEYER [61] has suggested that the GAG molecules "are a code of extracellular information for the arrangement of organization of the fibrillar proteins, chiefly collagen fibrils". This "code function" presupposes the existence of organized GAG molecules. By means of the topo-optical method, we succeeded in verifying these assumptions. We have also demonstrated that the organized GAG structure was the first to appear in the course of embryonic differentiation of the cartilage matrix [62, 65] (Fig. 6a, b). This is followed by the appearance of oriented collagen structure. As a result of GAG-collagen interaction, the degree of GAG orientation increases at this time (Fig. 6 c,d).

The oriented GAG structure is probably involved in anabolic and catabolic processes of the cartilage matrix. We have demonstrated that the so-called osteocyte capsule surrounding the bone cell and its processes has a submicroscopically-oriented GAG structure, and the GAG chains are arranged parallel to the cell membrane [62, 66] (Fig. 7a). The transport of materials between the perivascular spaces and cells takes place in the osteocyte capsule. Immunohistochemical investigations carried out in this Institute indicate that this oriented GAG structure is bound to the core protein that carries the antigenic determinants of bone tissue [25] (Fig. 7b). Experimentally induced injuries slacken or abolish the oriented structure of the osteocyte capsule [66].

The interaction between proteoglycans and collagen has, therefore, three consequences: it induces formation of fibrillar collagen, stabilizes the oriented collagen structure, and increases the degree of orientation of GAG chains.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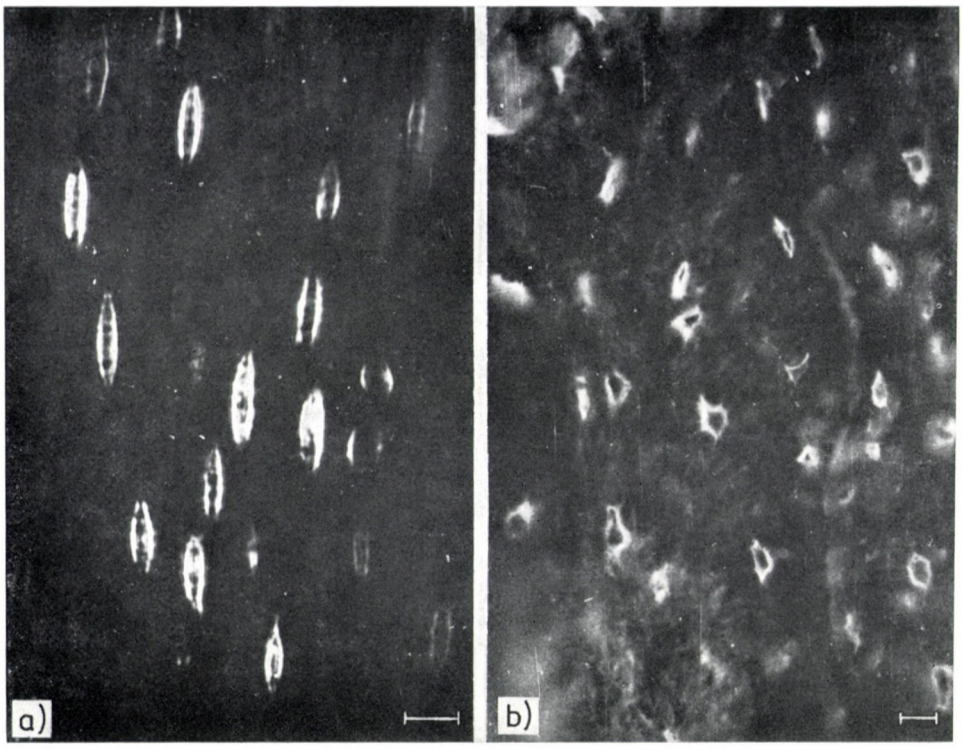

Fig. 7. Photomicrographs showing the structure of osteocyte capsule (OC). *(a)* Portion of cortical bone from adult rat photographed in polarized light after fixation, decalcification, and staining with N,N-diethylpseudoisocyanine. Strong induced birefringence of OC shows the orientation of the stained GAG molecules. Scale: 10 μm. *b)* Portion of bovine cortical bone. Fluorescence-microscopic picture demonstrating indirect immunohistochemical reaction performed after fixation of the material in cold acetone, EDTA decalcification and hyaluronidase digestion (modified from GLANT et al. [25]). Induced fluorescence verifies the presence of non-collagenous proteins. Scale: 20 μm

Fig. 6. Demonstration of oriented glycosaminoglycan structure in human embryonic cartilage matrix by topo-optical reactions. *(a)* Cartilaginous blastema of the posterior limb anlage from 4-week embryo after N,N-diethylpseudoisocyanine staining for glycosaminoglycans. On common light photomicrograph the glycosaminoglycans are marked by violet metachromasia. *(b)* The same visual field photographed in polarized light. Induced birefringence indicates the orientation of glycosaminoglycan molecules. In this area, no oriented collagen structure could be demonstrated. Scale: 50 μm. *(c)* Section from a knee-joint of an 8-week ebryo after a selective topo-optical reaction for collagen photographed in polarized light. The strong induced birefringence indicates the oriented collagen structure of the cartilage matrix. Scale: 50 μm. *(d)* Articular cartilage (deep zone) of a 6-month-old human embryonic femur photographed in polarized light after staining with toluidine blue at pH 3.5. The induced optical activity of glycosaminoglycans is compensated by a 13 nm retardation value (the slow axis of the compensator plate is marked = C). Parallel to the slow axis of the plate extinction of the birefringence (= blue subtractive anomalous colour), in perpendicular direction intensification of the birefringence (= orange additive anomalous colour) is seen. The direction-dependent distribution of the anomalous colours suggests an ordered network of GAGs in the cartilage matrix. The same orientation pattern of collagen suggests the parallel orientation of these two components. Scale: 50 μm

This interaction is the final step in the development of the hierarchic structure of the matrix. The assumption that ordered GAG structure is the precondition of the formation of oriented collagen structure has been verified in cartilage tissue. The general validity of this statement remains to be investigated.

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BEHAVIOUR OF AMINOHEXOSES AND SIALIC ACIDS IN SUBACUTE INTOXICATION OF GUINEA PIGS WITH "ELOCRON 50 WP"

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Abstract

The behaviour of sialic acids and aminohexoses was examined in subacute intoxication of 60 guinea pigs with "Elocron 50 WP". Elocron 50 WP was administered in soy-bean oil emulsion by means of a stomach tube for 28 and 85 days. The daily dose was 1/3 LD50. Further animals were kept for 28 days on food sprayed with "Elocron 50 WP". These animals were kept in quarantine during the period of treatment. The sialic acid level was found to be increased in the liver and in the other examined organs (heart, brain, kidney, lung) and slightly decreased in the serum. The aminohexose level was increased in the heart, kidney and brain, and decreased in the liver and lungs, in the early stage of intoxication (28 days) and in the group of the so-called "fodder" animals. In the late stage of the intoxication (85 days) an increased content of aminohexoses was observed in the brain and in the kidney, the increase was very slight in the heart and brain, whereas in the lung tissue the aminohexose level was subnormal.

Introduction

Owing to their short persistence in the environment and their low toxicity for homoiotherms carbamates have been used widely. However, carbamates are not quite safe because they are strong inhibitors of enzymes, evoke changes in the protein biosynthesis and impair the liver [1, 3].

This paper presents results of examinations on the influence of "Elocron 50 WP", a purified carbamate preparation on the turnover of glycoproteins.

Materials and Methods

Experiments were carried out on 60 male guinea pigs weighing 350—400 g, aged 6—8 months, kept in the same conditions and on the same diet. The animals were divided into 5 groups:

Group 1 included 20 animals which were given about 1/3 LD50/day of "Elocron 50 WP" in soy-bean oil suspension by means of a tube for 28 days.

Group 2 included 20 guinea pigs which were given "Elocron 50 WP" for 85 days. The daily dose was as in group 1.

Group 3 included 10 guinea pigs fed for 28 days during four morning hours on food sprayed with "Elocron 50 WP". The animals were kept in quarantine in the period of treatment.

Group 4 included 5 guinea pigs which were given only soy-bean oil by means of a stomach tube.

The animals in group 5 were given neither soy-bean oil nor "Elocron 50 WP".

Groups 4 and 5 were considered as control groups.

A hundred grams of "Elocron 50 WP" was diluted in 150 ml of ethyl ether and left for 15h at room temperature. Then the ether was evaporated at 40 °C, and the rest was again rinsed twice with ether. 40 g active substance — dioxacarb (2-(1,3-dioxolate-2-yl)-phenyl-methylcarbamate) — was obtained from 100 g of the commercial praparation. 600 mg dioxacarb was suspended in 10 ml soy-bean oil prewarmed to 30 °C and given to the animals of groups 1 and 2, 30 mg/kg body weight/day. Blood was taken from the heart of intoxicated and control guinea pigs after 28 days (groups 1 and 3) and 85 days (group 2) of the administration of dioxacarb. Then the animals were decapitated and their internal organs and tissues were taken for biochemical examinations.

Aminohexoses were determined in the liver, heart, brain, lung and kidney tissues. For this purpose, the organs were hydrolysed in hydrochloric acid, and then aminohexoses were determined by means of the ELSEN—MORGAN method after an initial purification of the hydrolysates according to SPIRO on columns with Dovex 50×4 (200×400 mesh) in the hydrogen from [12, 13].

The extinction was read on spectrophotometer "Specol" at 535 nm. The result was read from the calibration curve and calculated per one gram wet tissue.

Sialic acids were determined according to the method of WARREN [15] in the same tissues as aminohexoses and in the serum; tissue hydrolysis was carried out in 0.2 *N* sulphuric acid. The coefficient of extinction was read immediately after decantation in spectrophotometer "Specol" at 549 nm against the reagent test including 0.2 ml distilled water instead of hydrolysate. The result was read from a standard curve constructed from synthetic N-acetylneuraminic acid and calculated for the number of μg/g wet tissue or mg/100 ml. Serum aminohexose was not determined because of technical reasons.

The results were evaluated statistically [4, 6, 11].

Table 1

Sialic acids and aminohexoses in the kidney, lung and brain tissues of guinea pigs intoxicated with "Elocron 50 WP"

Group of animals	Kidney		Lung		Brain	
	S.A.	H.A.	S.A.	H.A.	S.A.	H.A.
Controls	669±245	532± 65	1251±276	762±162	1640±665	403±102
Intoxicated for 28 days	1180±368	950±119	1690±555	745±124	2520±840	735±265
t	5.2	14.0	3.16	0.52	4.16	4.8
P	$\leqslant$0.001	$\leqslant$0.001	0.01$\geqslant$0.001	0.70$\geqslant$0.60	$\leqslant$0.001	$\leqslant$0.001
Intoxicated for 85 days	1040±324	670± 85	1690±540	664±110	1750±718	606±170
t	4.84	7.04	3.56	3.52	0.66	5.27
P	$\leqslant$0.001	$\leqslant$0.001	0.01$\geqslant$0.001	0.01$\geqslant$0.001	0.6$\geqslant$0.5	$\leqslant$0.001
So-called "fodder" animals intoxicated for 28 days	1208±370	816±103	1714±560	680±105	2614±850	580±155
t	6.16	11.88	3.60	3.38	4.84	4.84
P	$\leqslant$0.001	$\leqslant$0.001	0.01$\geqslant$0.001	0.01$\geqslant$0.001	$\leqslant$0.001	$\leqslant$0.001

S. A. = sialic acids
H. A. = aminohexoses
} μg/g

Results

Tables 1 and 2 present the sialic acid and aminohexose concentrations in μg/g wet tissue in particular tissues and in serum of guinea pigs intoxicated with "Elocron 50 WP" for different periods.

Table 2

Sialic acids and aminohexoses in the heart and liver tissues and sialic acids in serum of guinea pigs intoxicated with "Elocron 50 WP"

Group of animals	Heart		Liver		Serum
	S.A.	H.A.	S.A.	H.A.	S.A. mg/100 ml
Controls	328 ± 43	477 ± 113	435 ± 175	814 ± 260	87 ± 7.9
Intoxicated for 28 days	1400 ± 464	960 ± 320	690 ± 168	585 ± 146	82 ± 9.0
t	9.2	6.0	6.0	6.28	1.98
P	$\ll 0.001$	$\ll 0.001$	$\ll 0.001$	$\ll 0.001$	$0.10 \gg 0.05$
Intoxicated for 85 days	670 ± 92	591 ± 243	455 ± 120	880 ± 285	82 ± 8.2
t	11.44	1.76	0.70	1.012	2.64
P	$\ll 0.001$	$0.10 \gg 0.05$	$0.5 \gg 0.4$	$0.4 \gg 0.3$	$0.02 \gg 0.01$
So called "fodder" animals intoxicated for 28 days	832 ± 112	680 ± 260	582 ± 135	548 ± 152	68 ± 8.5
t	19.80	3.08	4.75	6.60	9.68
P	$\ll 0.001$	$0.01 \gg 0.001$	$\ll 0.001$	$\ll 0.001$	$\ll 0.001$

S. A. = sialic acids
H. A. = aminohexoses } μg/g

In group 1, the highest sialic acid concentration was found in the brain, and an elevated concentration was found in each of the heart, kidney and liver. A small increase was demonstrated in the lungs, but in the serum the sialic acid concentration was not increased. The aminohexose concentrations were increased in the kidney, brain and heart, being the highest in the kidney and heart. The liver showed a significant decrease in aminohexoses, while in the lungs the decrease was small.

Group 2 showed increased sialic acid level in the kidney, lung and heart. A distinct decrease was observed in the brain and liver, but the serum did not show any changes. The aminohexose concentration was increased in the kidney and brain. An increasing tendency, less evident than in group 1, was shown by the heart and liver. A distinct decrease in aminohexoses content was observed in the lungs.

In the so-called "fodder" group (group 3), the sialic acid level was remarkably increased in all the examined tissues, while it was decreased in the

serum; a phenomenon difficult to explain. Perhaps it was caused by absorption of the preparation through routes other than the alimentary tract. Probably, the dose of the preparation was not under control, and the decrease might be due to contaminations in the preparation. The aminohexose levels were nearly identical to those in group 1 (i.e. animals intoxicated for 28 days). Increase in aminohexoses was observed in the kidney, brain and heart tissues, and a decrease was observed in lungs and liver.

Discussion

Both the sialic acid and aminohexose levels were significantly increased in the majority of the examined organs of intoxicated guinea pigs. The level of sialic acids remained unchanged. This may suggest that dioxacarb accelerated the biosynthesis of the carbohydrate part of glycoproteins. The increased glycoprotein production may be explained by the hypothesis of Koj et al. [7, 8] suggesting that release of lysosomal enzymes results in an increased formation of specific glycoproteins which inhibit the acid hydrolysis of lysosomes, thus preventing further impairment of the cell. The increase in the glycoprotein level in the course of dioxacarb intoxication might be attributed to an immunological reaction against toxic impairment of organs, a phenomenon, similar to that shown in connection with DNOC [10] intoxication.

Some authors [2, 9] regard the increase of the level of sialic acids as a nonspecific reaction of the organism against the effect of the pathological factor.

Kossman [9], examining the behaviour of glycoproteins in the course of experimental mercurialism, found significantly increased aminohexose and sialic acid level, in the serum and liver of animals. The aminohexose levels tended to decrease after a prolonged period of intoxication and the level of sialic acids showed a tendency to increase during the whole period of the experiment. In our experiments both levels showed a tendency to normalize. The changes observed in the levels of sialic acids and aminohexoses may be explained by a direct effect of dioxacarb on cell.

There are no reports in the literature on the behaviour of phosphoglucomutase and phosphohexose-aminotransferase under the influence of carbamates. If we assume that the enzyme activity is not inhibited, then glucose-6-P is isomerized to fructose-6-P. Fructose-6-P is transaminated to glucosamine-6-P in the presence of phosphohexose aminotransferase, thus evoking an increase in aminohexoses [5, 14].

The present results suggest that this mechanism may also be valid for carbamates because the level of aminohexoses in the liver was significantly lower then in the control group. Yet, on account of the marked chemical

plasticity of aminohexoses, interpretation of their level changes is difficult, the more so in that different pathological factors such as liver anoxia, X-ray irradiation, intoxication with carbon tetrachloride [12] *etc.* evoke more or less regular changes in the level and activity of phosphohexose-aminotransferase that undoubtedly could influence the level of aminohexose.

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LIGHT AND ELECTRON MICROSCOPIC INVESTIGATION OF THE SENSORY DENDRITES IN THE EPIDERMIS OF THE FOOT OF THE SNAIL, *HELIX POMATIA* L.

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Abstract

The fine structure of epithelial cells, supporting cells and the sensory dendrites running toward the surface between the former two was investigated in the epidermis of the foot of *Helix pomatia*. Cilia orienting in regular lines and possessing 9 + 2 tubular structure occur on the surface of the cylindrical epithelial cells lying on the basal lamina. The cilia terminate in basal bodies. The epithelial cells can first of all be characterized by a well-developed Golgi-apparatus and endoplasmic reticulum system as well as a developed tonofibrillar system. The supporting cells differ from the epithelial cells mainly in the poorly-developed tonofibrillar system and small number of cilia. Septate desmosome and zonula adherens are the common connecting structures between the cells composing the epithelium. According to the surface specialization, two types of sensory dendrites running toward the surface were distinguished: dendrites with and dendrites without cilia. On the surface of the former cilia with 9 + 2 tubular structure are randomly distributed and are present in varying number. In their cytoplasm there are many mitochondria, few clear vesicles and multivesicular bodies. From the surface of the dendrites without cilia, microvilli of different diameter originate and sometimes a centriole was observed below the distal membrane. Their cytoplasm contains many clear vesicles, a well developed smooth endoplasmic reticulum system and few mitochondria. Comparing our results with literary data, we suppose that the differences between the two dendrite types in the presence and number of cilia as well as in the diameter of the microvilli show different forms of appearance of one type of dendritic terminal.

Introduction

Investigation of the peripheral nervous system of snails started in the last century. Using the Golgi technique, receptor cells in the epidermis and a subepithelial net of nerve fibres were shown by Retzius [27], Veratti [39], Schmidt [38] and Schultz [35]. Four types of receptor cells were distinguished by Schultz [35] in the epidermis of *Helix pomatia*. On the basis of electron microscopic investigations two types of sensory dendrites running toward the surface of the epidermis between the epithelial cells have been described: dendrites with and denrites without cilia [12, 17, 43, 45, 47]. Within these two types further differentiation was made on the basis of different fine-structural characteristics (number and diameter of cilia and microvilli, lenght, striating and branching of the roots, surface of the dendritic terminals). In

snails, a considerable part of the epidermis is constituted by the foot, which in the case of an active animal contacts with the largest surface with the surroundings rich in stimuli. Among the cellular elements composing the foot, only epithelial, supporting and glandular cells have been examined for their fine-structural and endocytotic features [12, 32, 33, 47]. As a part of the ultrastructural analysis of the entire epidermis, the neural elements of the foot were investigated by CRISP [12] and ZYLSTRA [47], while earlier KOSHTOYANTZ and RÓZSA [22] performed electrophysiological experiments in connection with the osmoregulation of the *Helix* foot. Apart from a work concerning the surface specializations of the epithelial cells [29], electron microscopic investigations have not been done on *Helix* species. Therefore, we analysed the ultrastructure of the distal processes of the sensory cells situated in the foot of *Helix pomatia*, and our findings were compared with data obtained on other species.

Materials and methods

Adult specimens of *H. pomatia* collected in spring and autumn were used. The foot of the active animal was cut and samples were taken by using a blade from the different regions of the foot. The samples were immediatly placed into fixative.

For light microscopy rapid Golgi-method [30] was employed. Materials were maintained in the fixative for 7—10 days at 20 °C, then after a brief washing in distilled water they were placed into 0.75% $AgNO_3$ solution for 24 h at 20 °C. Finally, materials were dehydrated in graded ethanol and after methylbenzoate treatment embedded in paraffin wax. In the light microscope 30 μm thick sections were investigated.

For the electron microscopy two methods of fixation were employed:

a) Fixation in 1.5% OsO_4 (0.2 *M* phosphate buffer, pH 7.4) at 4 °C for 3 h.

b) Fixation in a mixture of 2% OsO_4 and 4% glutaraldehyde (0.1 *M* s-collidine buffer, pH 7.2), followed by a brief rinsing in the buffer, and postfixation in 1.5% OsO_4 (01. *M* s-collidine, pH 7.2) at 4 °C for 2 h.

The fixed preparations were dehydrated in graded ethanol, block staining was done in 75% ethanol with saturated uranyl acetate. Materials were embedded in Durcupan ACM. Ultrathin sections cut on an LKB Ultratome and stained with lead citrate [28] were examined in a TESLA BS 413A electron microscope.

Results

On the basis of the light-microscopic investigations, it can be stated that the receptor cells belong to the primary type of the sensory cells on the basis of their morphological characteristics (Fig. 1a, b, c). Their dendrites run to the surface and before reaching it they are widened (Fig. 1a). Their axon goes to the centre and makes connections with the subepithelial nerve net (Fig. 1c).

According to the electron microscopic observations, the epithelium consists of one layer of cylindric epithelial cells, supporting cells, glandular cells and sensory dendrites running toward the surface, all lying on the basal lamina (Fig. 2).

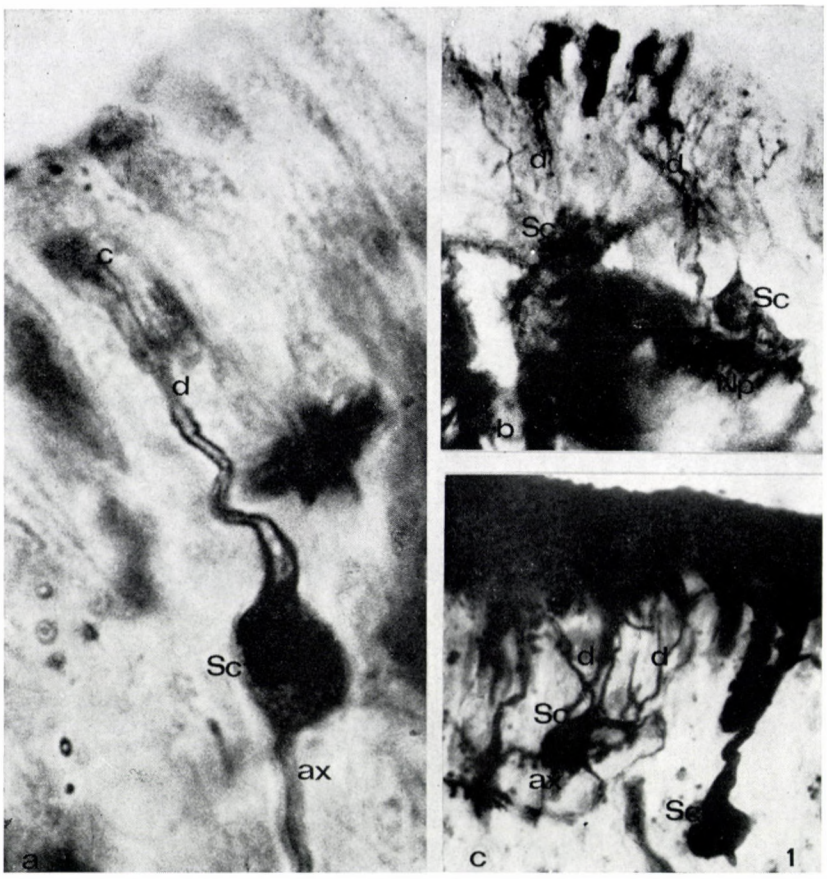
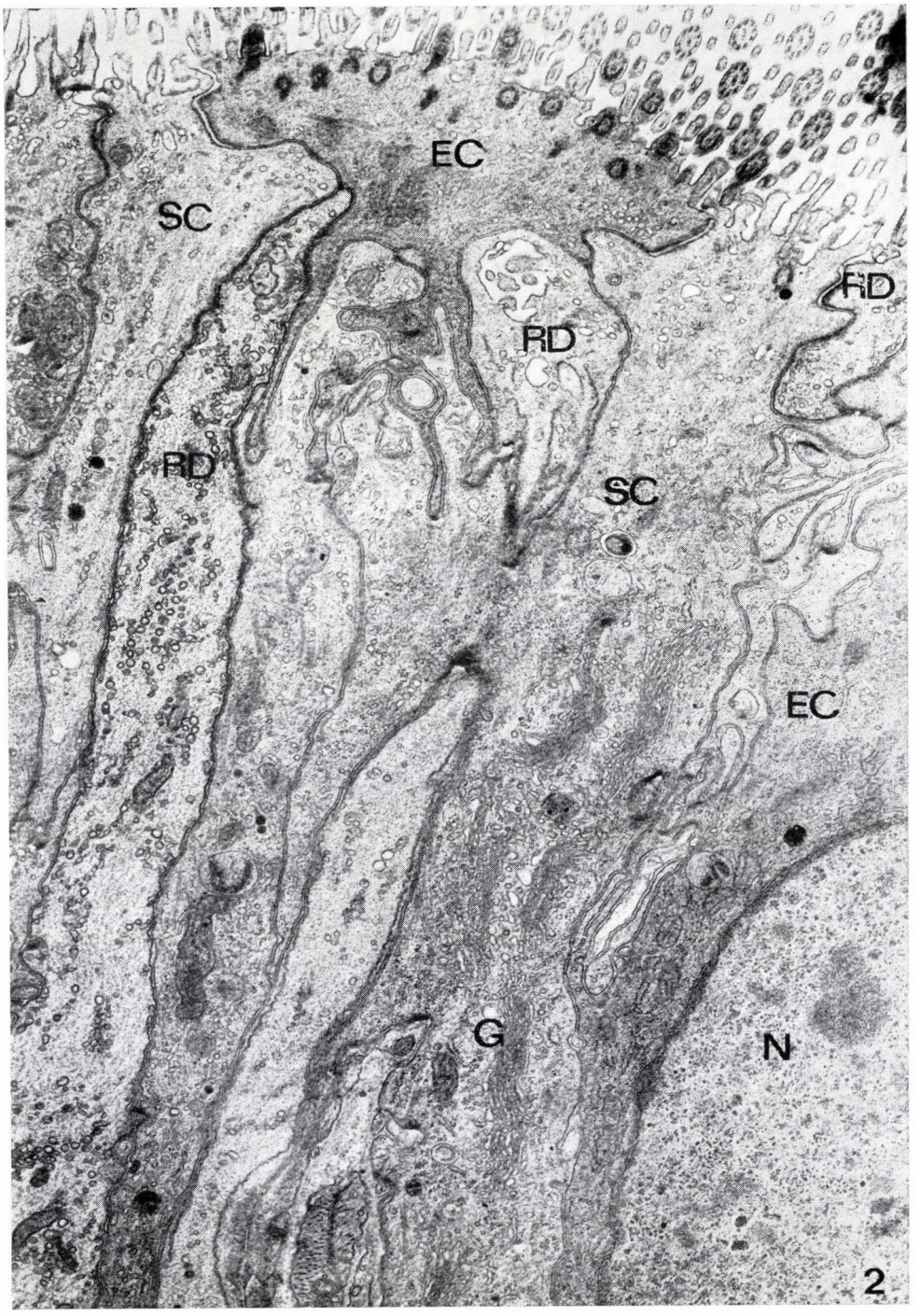


Fig. 1. Groups of primary receptor cells (Sc) and their processes. Rapid Golgi technique. a) The dendrites (d) running toward the surface are widened in a cone-like manner (c) before reaching the surface. b) High number of dendrites (d) and two groups of sensory cells (Sc). The axons of the sensory cells are connected to the subepithelial plexus (Np). c) Sensory cells (Sc) localizing in different depth. Numerous processes (d, ax) originate in the cell group on the left side
a — ×1500, b — ×380, c — ×430

On the surface of the epithelial cells cilia, and microvilli embedded in a fine filamentous layer, can be observed (Fig. 3). The microvilli are either ramifying or non-ramifying and a dense bundle of filaments can be found in their interior, which can be followed to different depth of the epithelial cells, connecting it to the tonofibrillar system (Figs 3 and 7). This well-developed tonofibrillar system sometimes reached even the basis of the epithelial cell. The cilia oriented in regular lines showing 9+2 tubular structure terminate in basal body and possess a periodically striated root (Figs 5 and 7). With the fixatives employed, the epithelial cells display higher density then the other cell types. Their cytoplasm is rich in cellular organelles. The well-developed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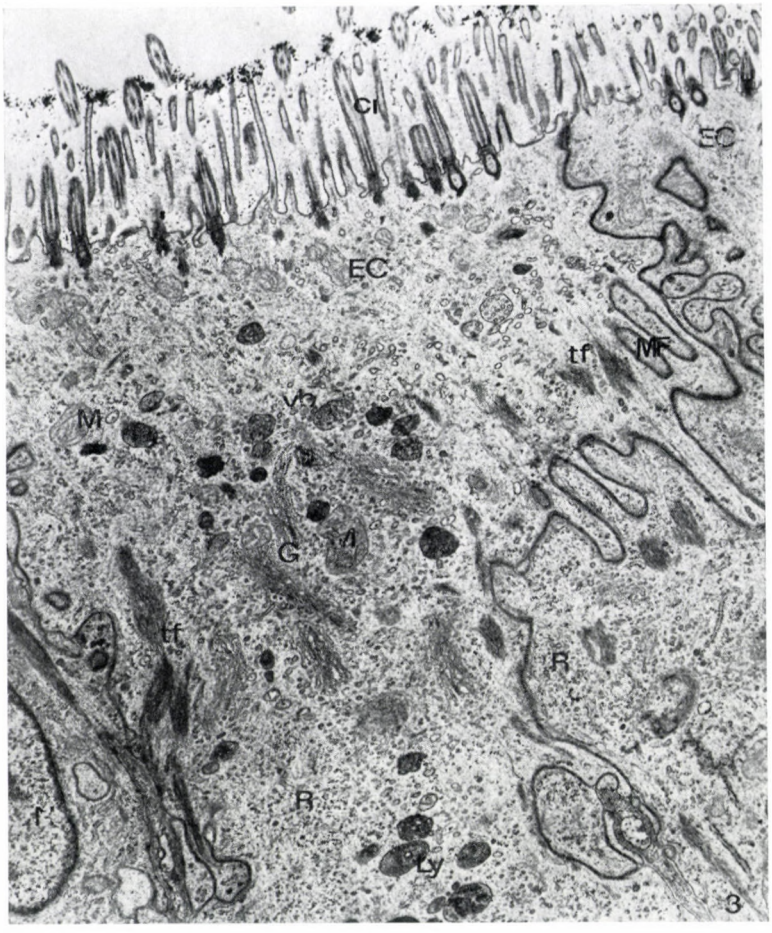

Fig. 3. Details of ciliated epithelial cells (EC) from the epidermis of the snail foot. On the lateral surface of the epithelial cells strong membrane digitations (MF) occur frequently. Note the well-developed Golgi-apparatus (G), many ribosomes (R) and a considerable amount of tonofibril bundles (tf) in the cytoplasm. N — nucleus, vb — multivesicular body, Ly — lysosome, M — mitochondrion. ×9000

Golgi-apparatus, rough endoplasmic reticulum system and the high number of free ribosomes are characteristic (Figs 2 and 3). Mitochondria, multivesicular bodies, secretory granules, pigment granules and lysosomes are variable in number (Fig. 3). The basal part of the epithelial cells leans with hemidesmosomes against the basal lamina. The basal lamina is sometimes connected with collagen fibres (Fig. 4).

Fig. 2. Low-power electron micrograph of the sensory epithelium with sensory dendrites (RD), ciliated epithelial cells (EC) and supporting cells (SC). G — Golgi-area, N — nucleus. ×13 000

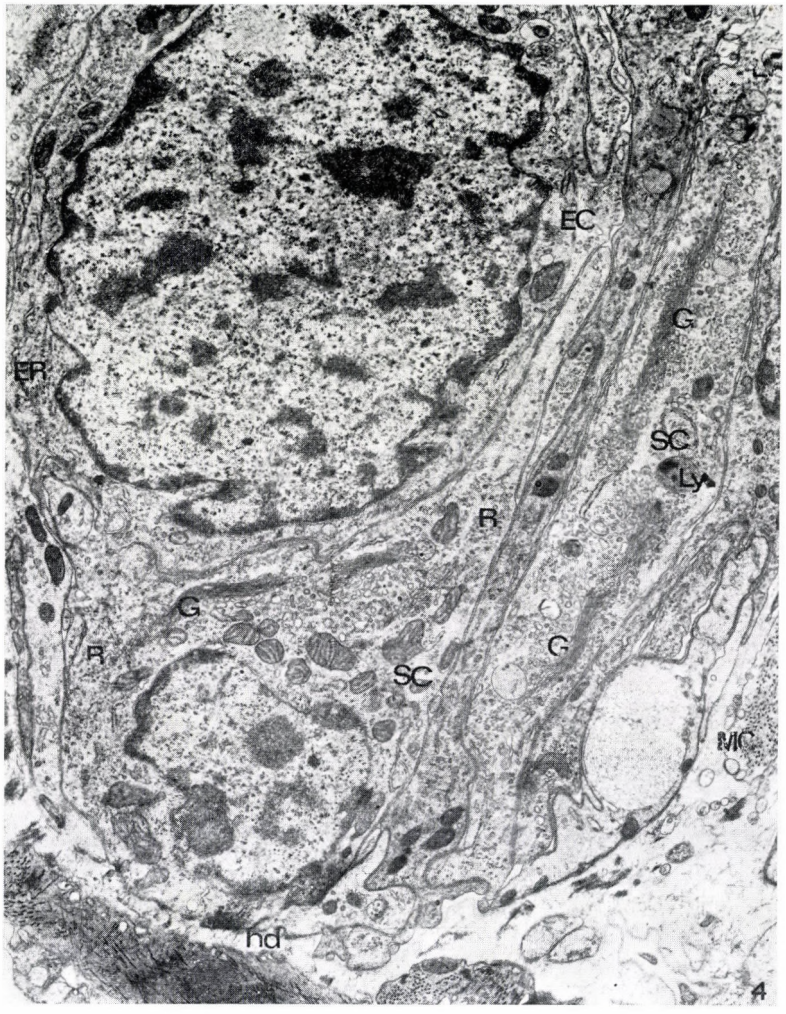

Fig. 4. General view of epithelial (EC) and supporting (SC) cells on the basal lamina. In the cytoplasm of the latter type, extensive Golgi apparatus (G) and many ribosomes (R) occur. hd — hemidesmosome, ER — rough endoplasmic reticulum, Ly — lysosome, MC — muscle cell. ×9000

The supporting cells are similar to the epithelial cells in shape, inner density and the presence of well-developed Golgi apparatus. However, they differ in their low number of tonofibrils and cilia on their apical pole and in the rare occurrence of pigment granules, multivesicular bodies and lysosomes.

Breaking through the basal lamina, the sensory dendrites of the primary receptor cells run toward the surface between the epithelial and supporting cells. The dendrites are connected by zonula adherens and septate desmosomes with the neighbouring cells. The sensory dendrites of the receptor cells could

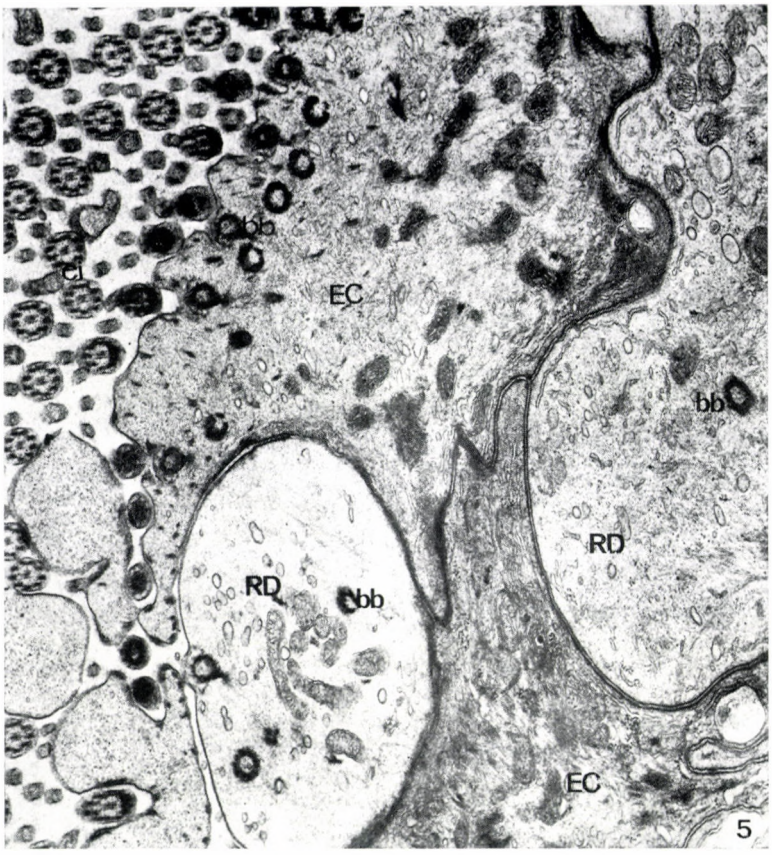

Fig. 5. Oblique section of dendrites (RD) running to the surface between epithelial cells (EC). In the epithelial cells the cilia (ci) and the basal bodies (bb) are oriented in regular lines, while in the sensory dendrites they show an irregular distribution. ×17 600

easily be identified, since they represent the most electron-transparent elements of the epithelium (Fig. 2). According to their surface specializations, *i.e.* the presence of cilia and microvilli, two main dendrite types can be distinguished, *viz.* ciliated and non-ciliated dendrites.

1. Dendrites possessing both cilia and microvilli were found in the greatest number. On these, the cilia are distributed randomly (Figs 5 and 7) and in varying number (1—14). At their origin the surface is generally indented (Figs 6 and 7). Similarly to those observed on the epithelial cells, the cilia possess 9+2 tubular structure (Figs 6 and 7) which terminates in basal body, and striated roots originate from them (Fig. 8). The neuroplasm of the ciliated dendrites is rich in cellular organelles. Generally, many mitochondria and also dense granules can be found in the distal part of the dendrites (Figs 6 and 7). Apart from the mitochondria, multivesicular bodies, clear vesicles in varying

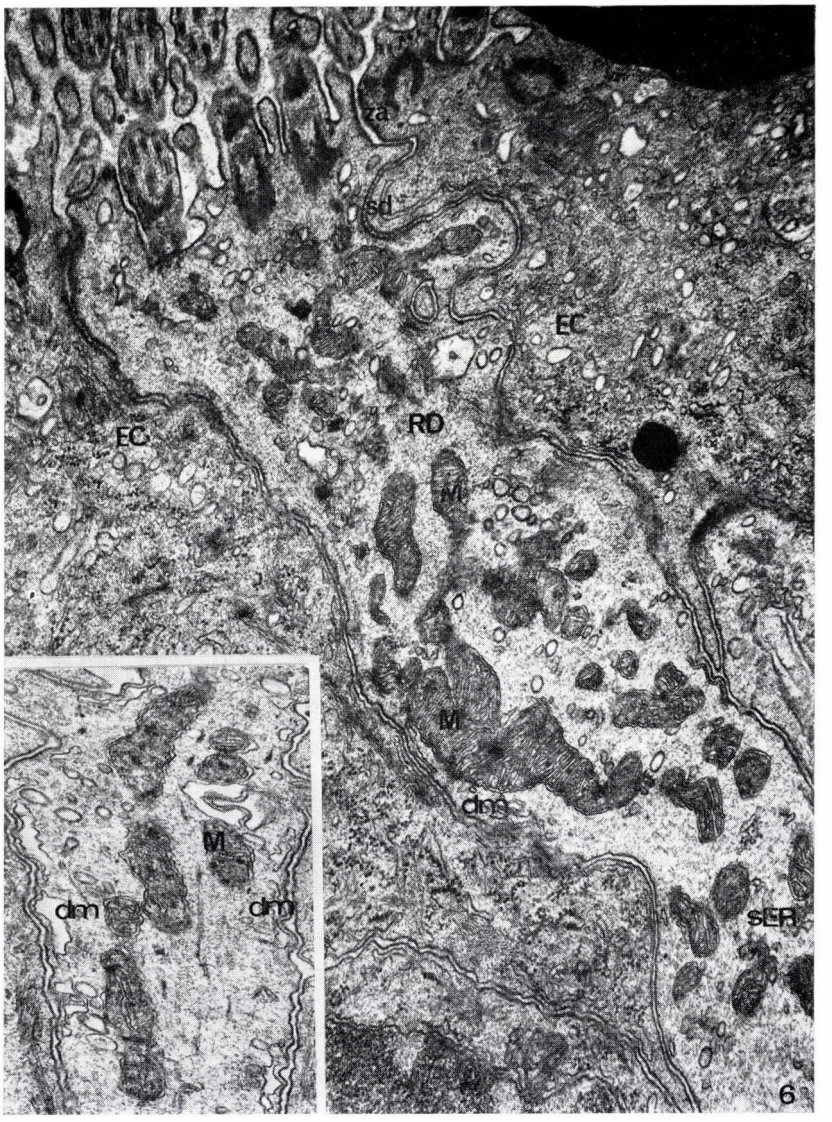

Fig. 6. Longitudinal section of a ciliated sensory dendrite (RD). The dendrite is connected with the epithelial cells (EC) by zonula adherens (za) and septate desmosomes (sd). In the cytoplasm of low electron density, many mitochondria (M) and a few elements of the smooth endoplasmic reticulum system (sER) can be seen. Near the cell membrane, membrane fragments (dm) are present. ×17 600. Insert: Detail of a dendrite in which the intramitochondrial dense particles as well as the cisterns (dm) situating below the lateral cell surface and contacting morphologically with the mitochondria (M) can well be seen. ×27 000

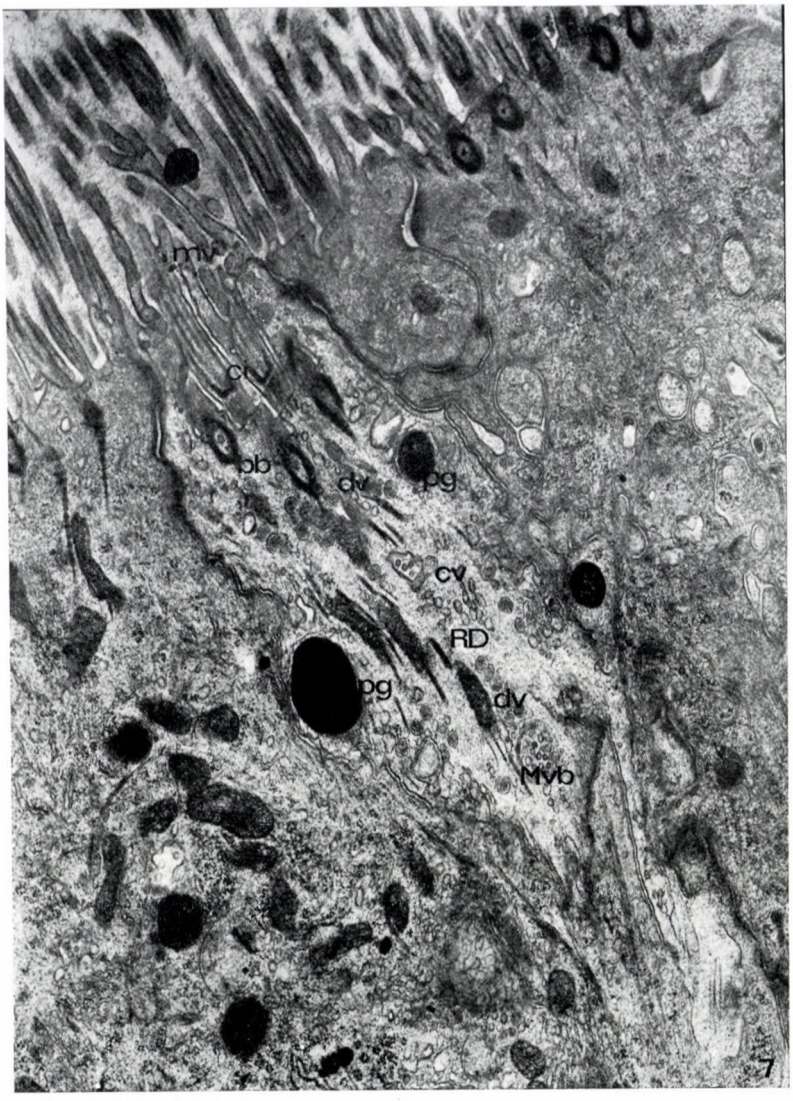

Fig. 7. Dendrite (RD) with pigment granules (pg) and with lines of dense-core vesicles (dv). Mvb — multivesicular body, cv — clear vesicles, bb — basal body, ci — cilium, mv — microvilli, r — root. ×17 600

number and relatively few elements of the smooth endoplasmic reticulum system occur in the distal part of the dendrites (Figs 7 and 8). Below the lateral cell surface a membrane system (subsurface cistern) is seen, which follows the surface at shorter or longer distance and may also be connected to mitochondria (Figs 6 and 8). In some cases, dense material was seen in their irregular, vesicle-like enlargements (Figs 6 and 8). In one case, a sensory dendrite was found in which lines of dense-core-like vesicles and pigment gran-

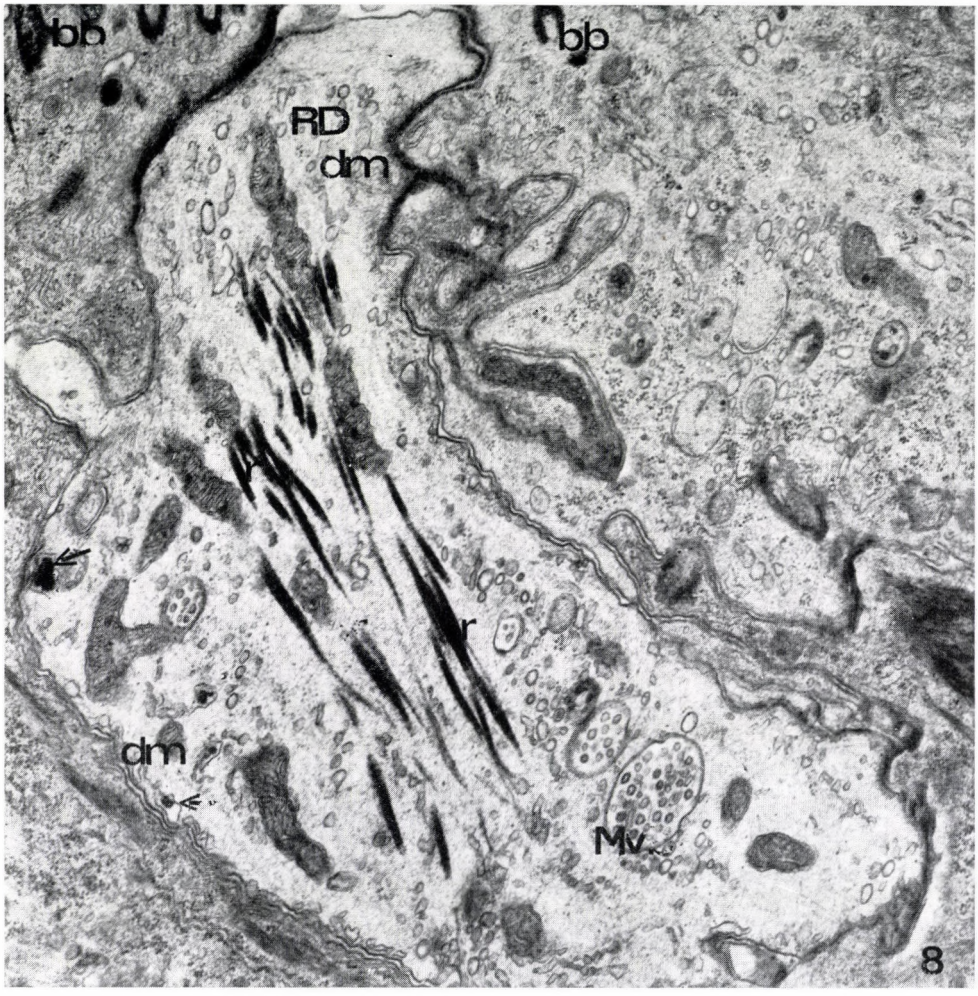

Fig. 8. Electron micrograph of a dendrite with roots (r) reaching deeply. Note the discontinuous membranes (dm) below the lateral cell surface and the dense material occurring occasionally in them (arrow). Mvb — multivesicular body, bb — basal body. ×17 600

ules were present (Fig. 7). The size of the pigment granules (3—6 μm) and their limiting membrane appear to be very similar to those described in the photoreceptor cells of the eye [17]. The proximal part of the dendrites is less abundant in cell organelles. In addition to the microvilli, few mitochondria, clear vesicles and ribosomes can be seen (Fig. 2).

2. Free nerve terminals possessing only microvilli represent the other type of the sensory dendrites (Figs 2 and 9.) From the surface of the dendrites non-ramifying microvilli originate, in which bundle of dense filaments cannot be detected. In certain cases, the microvilli show a spheroid-like swelling after

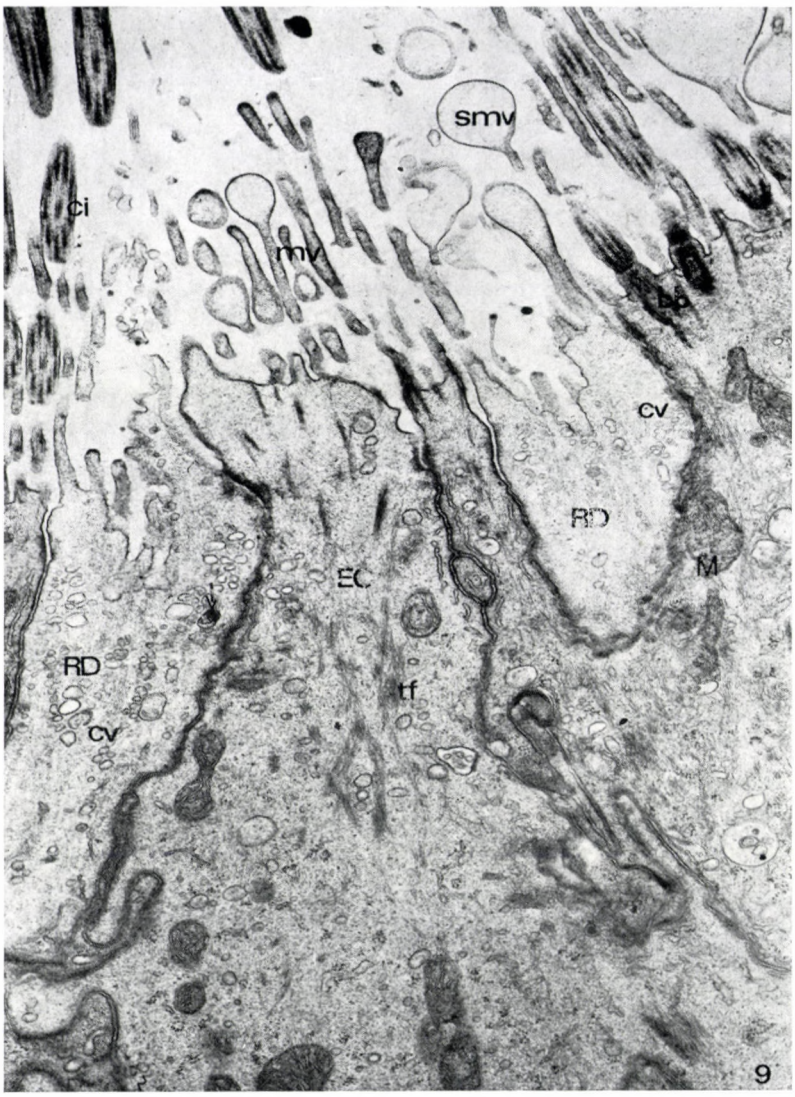

Fig. 9. Non-ciliated dendrites (RD) in the epidermis of the snail foot. Some microvilli appear to be finger-like (mv), others are spherically swollen (smv). Rows of clear vesicles (cv) within the dendrites. Note the dense material in some of the vesicles (arrow). sd — septate desmosome, tf — tonofilament bundle, EC — epithelial cell, bb — basal body, M — mitochondrion, ci — cilium. ×17 600

their origin (Fig. 9). In contrast to the ciliated dendrites, the non-ciliated ones are rich in clear vesicles and smooth endoplasmic reticulum elements and poor in mitochondria (Fig. 9 . The membrane system below the lateral cell surface is also present in this dendrite type and its elements may contain dense material (Fig. 9). Sometimes, a centriole occurs below the distal membrane.

Discussion

Electron microscopic analysis on the snail foot has shown that the ultrastructural characteristics of the epithelial and supporting cells as well as of the structures establishing the cellular connections (zonula adherens, septate desmosome) are in agreement with other results obtained on other parts of the snail epidermis [4, 9, 12, 17, 26, 29, 36, 37, 44, 46].

Light-microscopic findings concerning the sensory elements running toward the surface in the epidermis layer correspond to the results of classical investigations obtained on the different parts of the epidermis [7, 27, 35, 38, 39].

On the basis of their surface specializations, two types of sensory dendrites running toward the surface between the epithelial and supporting cells have been distinguished: ciliated and non-ciliated types. Apart from the different surface specializations (cilia and microvilli), there are also differences in respect of the cellular organelles found in the dendritic terminals. The ciliated type is characterized by a high number of mitochondria and multivesicular bodies, while in the non-ciliated type many smooth endoplasmic reticulum elements and clear vesicles, originating presumably from the former structures, are characteristic. It should be noted that the rate of the tubular and vesicular form of the smooth endoplasmic reticulum system strongly depends on the fixatives employed. Fixatives containing OsO_4 are favourable to the vesicular form [2, 3]. We failed to find either in the epithelial cells or in the ciliated dendrites a morphological connection between cilia and the smooth endoplasmic reticulum system described by CORDIER [11] in the covering epithelium of the thymus cysta. The fact that smooth endoplasmic reticulum elements and clear vesicles originating presumably from the former ones as well as membrane system situating below the lateral cell surface are present in both dendrite types suggests their role in the general process of the reception. In secondary sensory cells (outerand inner hair cells) discontinuous membranes and lamellated bodies similar to the former structures have also been described [20, 14] and they were in connection with each other and also with mitochondria.

The two types of sensory terminals described at the ultrastructural level can mostly be compared to the two terminal types found in the lip of *H. pomatia* [4].

After classifying the sensory nerve terminals the question has arisen whether unequivocal sensory functions can be bound to them. Electronmicroscopic and electrophysiological investigations by authors dealing with the reception function of invertebrate animals were generally performed on that parts of the epidermis to which some presumed receptor function has been attributed on the basis of classical investigations: on the tentacles [1, 8, 12, 26, 42, 43, 45, 47], on the osphradium [5, 6], on the lip [4, 19, 24, 34, 41],

and on the eye of the optical tentacle [10, 16, 25, 31]. As to the receptor functions of the different terminal types of the invertebrate epidermis, contradictions have emerged, since some authors attribute mechano-, chemo- and photoreception to ciliated terminals, while others attribute chemo- and photoreception to non-ciliated dendrites. It is generally accepted, on the other hand, that mechanoreception is bound to ciliated terminals. Two types of terminals, namely ciliated and non-ciliated dendrites, were also found in the photoreceptor cells of the eye, situated on the optical tentacle. To these cells a special receptor function can be attributed. Within the class *Gastropoda*, the species possessing non-ciliated photoreceptors belong to the phylogenetic line represented by the pulmonate snails, while the species with ciliated ones belong to the line of *Opistobranchiata*. Both phylogenetic lines originate in the *Prosobranchiata* [7, 23]. One of the terminals found by us, which can be considered as a photoreceptor on the basis of the size and the limiting membrane of the pigment granules and according to literary data [16], possesses cilia. At the same time, the photoreceptors of the *Helix* eye are non-ciliated, though cilia can also be observed on them in the early stage of their development [13]. These rudimentary cilia speak in favour of the common origin of the photoreceptors of the eye and of the foot.

The conclusion, a certain function cannot be connected to a certain receptor group desribed only on the basis of morphological characteristics, since the number of the characteristics (number and size of cilia and microvilli; lenght, ramification and striating of the roots; surface of the terminals) used for distinguishing the different groups is arbitrary. So, the setting-up of unfounded high number of groups is also possible, to all of which proper receptor function cannot be bound. Since, in the course of our investigations, ciliated terminals have been found in the highest number and non-ciliated occurred only rarely, we suppose that the dendritic terminals appearing to be non-ciliated can also contain cilium or centriole or basal body which could not be identified for the lack of serial sectioning. On the basis of the ectodermal origin of the primary receptor cells, it can also be supposed that all of the sensory dendrites possess cilia or a structure (centriole, basal body) from which cilia can be developed if necessary [21]. According to VINNIKOV [40], every receptor (primary, secondary) cell possesses cilia. We suppose that the non-ciliated terminals can also be regarded as the developmental and/or functional forms of the ciliated terminal. This was shown by WRIGHT's [45] results obtained after serial sectioning on the non-optical tentacle of *Arion*. This author found non-ciliated sensory terminals exhibiting centriole in their distal and in the highest number. In our experimental material non-ciliated dendrites with a centriole-like structure in their distal end near the surface were frequently found. Like the vertebrate olfactory epithelium, the centriole can develop cilia [15, 18]. Taking into consideration the terminal types described by

ZYLSTRA [47], WRIGHT [45] and WONDRAK [43] in the *Helix* epidermis on the basis of different morphological characteristics, we supposed that these types are continuous transitional forms of each other from the types with few microvilli to the types exhibiting a high number of cilia, and that the differences between the dendrite types in the presence and number of the cilia as well as in the width of the microvilli represent different forms of appearance of one sensory terminal type.

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THE EFFECT OF LESIONS OF THE EPITHALAMUS ON PLASMA ALDOSTERONE AND CORTICOSTERONE LEVELS IN JAPANESE QUAIL

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Abstract

The peripheral plasma aldosterone and corticosterone concentrations were measured by CPBA and RIA method in Japanese quails in which the subcommissural organ (SCO) or the habenula had been destroyed. Lesion of the SCO reduced the plasma aldosterone level by 50%. The lesion of n. habenularis medialis and lateralis on the other hand did not affect the plasma corticosteroid level. It is suggested that in birds the SCO takes part in a multifactorial regulation of aldosterone secretion.

Introduction

A large number of data are available about the role of mammalian epithalamus in regulation of aldosterone secretion. PALKOVITS et al. [10—15] and VAN DER WAL et al. [18] reported the SCO playing an important role in the maintenance of zona glomerulosa normofunction and aldosterone secretion by the adrenal cortex. On contrary, BROWN and AFFIFI [3] and LENYS [7] failed to find any functional correlation among the SCO, zona glomerulosa and the aldosterone production. There are publications indicating some connection of the habenula and adrenal cortex function with the equilibrium in salt and water metabolism, data suggestive of an importance of this epithalamic area in the regulation of corticosteroid secretion (MARTINI et al. [8]; LENGVÁRY et al. [6]).

Our previous histological experiments indicated that in domestic pigeon a specific morphological response of the SCO can be induced by influencing salt and water metabolism. Furthermore, a close morphological and functional correlation was found between the peripheral zone of adrenal interrenal tissue and the SCO (PÉCZELY and MURAY [16, 17]).

The aim of the present experiments was to investigate by lesion technique the hypothetical epithalamic glomerulotrop effect by studying the changes in plasma corticosteroid level.

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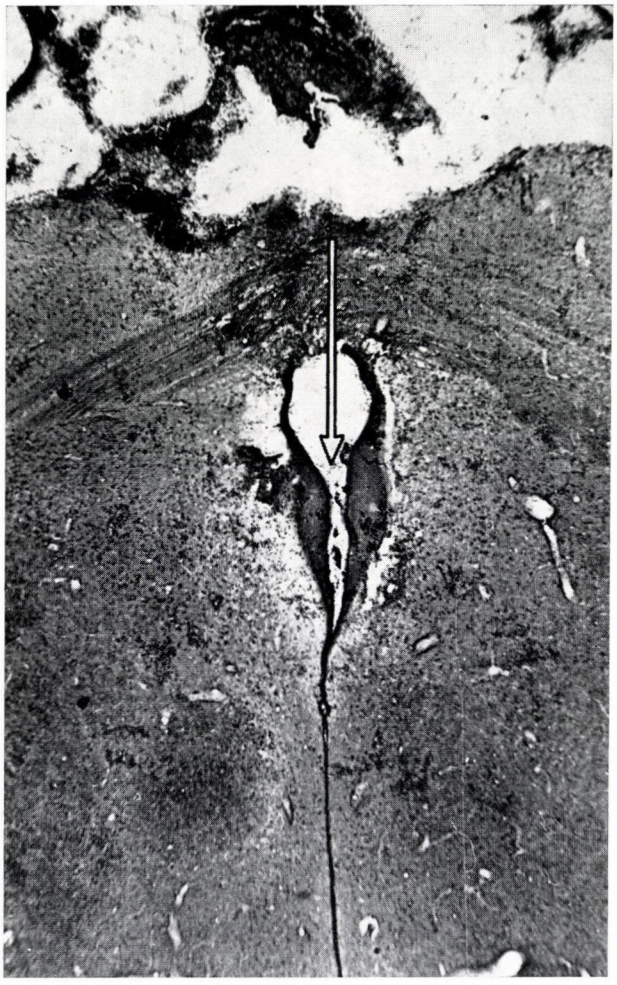

Fig. 1. Histological localization of the SCO lesion (Frontal plane: A +3.1)

Materials and methods

Thirty-one Japanese quails kept in standardized environments were divided into five groups: intact control (6 birds), SCO sham-operated (6) SCO—lesioned (5), habenula sham-operated (6) and habenula lesioned (8). Temperature was set to 26 °C and artificial illumination was used (70 lux; 18 L—6 D; light on: at 9.00 a.m., light off: at 3.00 a.m.).

Lesions were made in Equi-Thesin (Jensen—Salsbery) anaesthetized animals. The SCO and the habenular nuclei were localized according to the stereotaxis atlas of BAYLÉ et al. [1]. Destruction of the SCO was performed by electric current of 250 μA applied for 40 sec.

Lesion coordinates: ant.-post.: +3.1, lat.: 0, vert.: +3.2. (Fig. 1.) Two lesions were needed for destroying both medial and lateral habenular nuclei. Amperage used: 250 μA for 25 sec. Lesion coordinates:

1. ant.-post.: +4.0, lat.: ±0.5, vert.: +3.6;

2. ant.-post.: +3.3, lat.: ±0.6, vert.: +3.7. A platinum electrode 0.14 mm of diameter were applied.

Four weeks after operation, the birds aged 10 weeks were decapitated between 8.00 and 9.00 a.m. The heparinized blood obtained by exsanguination was centrifuged at 3000 rpm, the plasma samples were frozen and stored at −30 °C until analised. After chromatographic separation (Sephadex LH 20) of corticosteroids the aldosterone was assayed by the RIA method of DANIEL et al. [4], and the corticosterone by the CPBA method of MURPHY [9].

Results and discussions

The peripheral plasma corticosterone and aldosterone level in Japanese quail is very low (Table 1). Plasma corticosteroid concentrations in the intact and sham-operated control group were essentially agreed. BOISSIN [2] found the peripheral plasma corticosterone level of Japanese quail reaching its maximum in the morning hours and the end of dark period. By fluorometric method the maximum proved to be 2.8 μg /100 ml. According to our unpublished data, this maximum can fluctuate between 0.28 and 1.00 μg/100 ml as measured by CPBA method. The peripheral aldosterone concentration of the quail has not been published yet. DANIEL [5] found it to be 12 ng/100 ml in the duck (measured by RIA method) *i.e.* 7 times as high as the concentration found by us in the quail. It is wellknown that the plasma corticoid level in aquatic birds is relatively high.

Our results suggest that destruction of the SCO reduced the peripheral plasma aldosterone by 50% but did not reduce plasma corticosterone. Furthermore it was ascertained that lesion of the habenula affects neither plasma aldosterone nor plasma corticosterone (Table 1).

Table 1

Effects of lesions of the epithalamus on plasma corticosteroid level

Treatment	Aldosterone ng/100 ml	Corticosterone μg/100 ml
Intact control	1.60 ± 0.21 (6)	0.89 ± 0.08 (6)
SCO sham-operated	1.64 ± 0.28 (6)	1.10 ± 0.20 (6)
SCO lesioned	0.71 ± 0.16* (5)	0.70 ± 0.11 (5)
Habenula sham-operated	1.40 ± 0.33 (6)	1.07 ± 0.15 (6)
Habenula lesioned	1.49 ± 0.24 (8)	0.81 ± 0.03 (8)

* $P < 0.001$ *vs* intact or sham-operated control.

The present results seem to verify our previous hypothesis, based upon indirect results suggesting that the bird SCO would influence the aldosterone production in the adrenal gland. Simultaneously, our results are covering those of PALKOVITS et al. [14] and VAN DER WAL et al. [18], who found, in rat, a reduction of *in vitro* aldosterone production after SCO lesion. It is most likely that in birds, as in mammals, the SCO takes part in a multifactorial regulation of the aldosterone secretion. Destruction of this epithalamic ependyma organ the aldosterone secretion remarkably decreases through there are other evident regulating factors (presumably the renin-angiotensin system and ACTH) which can maintain its production at a subnormal level.

Our observation according to which in quail the habenula lesion does not affect either plasma aldosterone or corticosterone concentration can indicate a very important difference compared to mammals, in which the whole epithalamic complex seems to take part in the regulation of aldosterone production.

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HYPOSTASE, EMBRYONIC SAC AND ENDOSPERM IN *ANETHUM GRAVEOLENS* L., AND IN VARIOUS FAMILIES

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Abstract

The presence of pyriform hypostase tissue was investigated before double fertilization till the fusion of free endosperm nuclei in the embryonic sac of *A. graveolens*. Hypostase a glandular tissue, can secrete its cell contents into the embryonic sac during the development of free endosperm nuclei. Of 78 randomly selected families, 19 display different numbers of species with hypostase. In 15 of these families the hypostase is present together with the Polygonum type of embryonic sac and nuclear type of endosperm.

Introduction

Hypostase was first reported by JURICA [9] in some species of *Umbelliferae*. He and HÅKANSSON [5] described the hypostase as a "nutritive apparatus". PALIVAL [12] and ADATIA SHAH [1] termed and interpreted the hypostase as "nutritiv tissues". MAHESHWARI [11] pointed out the function of the hypostase as follows. "By the evident position* of these tissues, especially of the hypostase, it would seem natural to suggest their similarity to glandular tissues connected with secretion of enzymes or growth hormones."

The nucellar origin of hypostase was clarified by SCHULZ and JENSEN [15] (*i.e.* later than this tissue was discovered).

GUPTA [4] summarized also the problems connected with the hypostase in different species: "(*i*) inconsistency in the thickness of the cell walls constituting this tissue, and (*ii*) its varying life span".

In the literature the hypostase is sometimes confused with the postament tissue (BROWN and MOGENSEN [2], COE [3], RAGHAVAN [14]. The latter, like the hypostase tissues, is situated among the integument cells below the chalazal end of the embryonic sac, *i.e.* under antipodal cells.

According to the SCHULZ and JENSEN [15] the hypostase develops from the proliferation of some remnant nucellus cells, whereas the postament tissue

* RAGHAVAN [14] summarized the position of hypostase in the ovules as follows: "proliferation of the nucellus at the chalazal and micropylar ends of the ovules of some species designated as hypostase and epistase respectively".

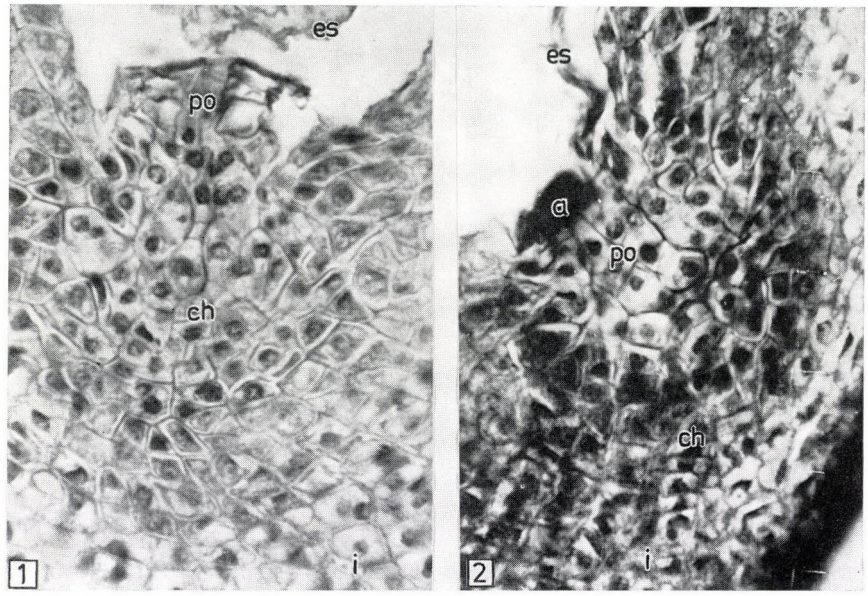


Fig. 1. Chalazal basal cells in the ovary of *Pimpinella anisum* L. ×200
Fig. 2. Chalazal basal cells and the postament cells in *P. anisum.* ×200

differentiates from the chalazal basal cells (SCHÜRHOF [16]). I have succeeded in verifying this latter statement for *Pimpinella anisum* L. (Figs 1, 2). These two tissues, however do not occur together in the same species.

Confusion of these two different tissues have led to misleading and insufficient results also from physiological points of view [4]. COE [3] studied the "hypostase" cells with $^{14}CO_2$ in *Zephyranthes drummondii* at the 8-nucleate stage of the embryonic sac; in fact, these were postament cells and showed lower activity, than the egg and synergid cells.

Anethum graveolens, together with *Foeniculum* and *Trachyspenum* was studied in detail by JOHRI and SEHGAL [8]. The hypostase of *Anethum* was mentioned first by SZUJKÓ-LACZA [19], when the hypostase in the 8-nucleate developmental phase of the embryonic sac was identified as a pyriform hole placing under the antipodal cells.

The only presence of the true hypostase was reported in many other species of *Umbelliferae*, and also in other families, by JOHRI et al. [6].

Therefore, the aim of this paper was to investigate the development and changing of the hypostase in the integument of ovule (Fig. 3) and its life span in *Anethum graveolens*, as well as, to find out the connection between the behaviour of styles and s.l. ovule. JOHRI and SEGHAL [7] noted in *Anethum* that "the stigma became receptive 5—6 days after the dehiscence of anthers. At first the two styles are very close to each other but they diverge after pollination."

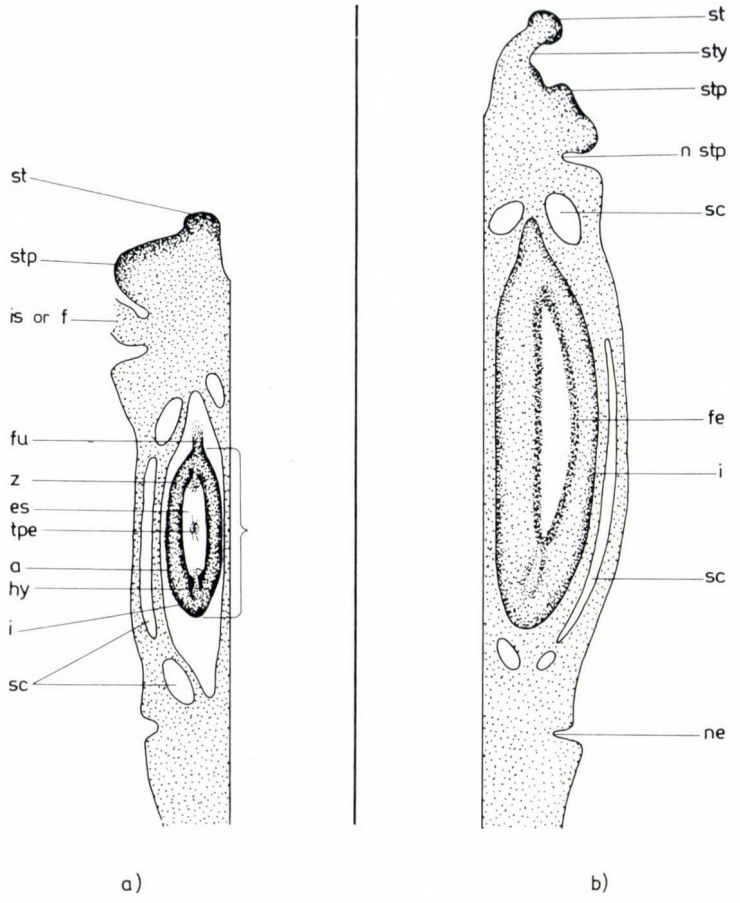


*Fig. 3*a. Phenophase 32.1 Longitudinal section from the carpel of *Anethum graveolens*
Fig. 3b. The same is in phenophase 32.3.5—32.3.6

Key to labels: a = antipode cells; ch = chalasal basal cells; cw = cell wall; di = disorganized integument cells; dw = double wall; e = epidermis; em = embryo; es = embryonic sac; en = secondary endosperm nuclei; hy = hypostase; i = integument; il = inner layer of pericarp; ini = inner rows of integument cells; is or f = insertion of sepal or filament; fe = = free endosperm nuclei; ffe = fusiform free endosperm nuclei; fu = funicle; fufe = fused free endosperm nuclei; funo = fused nucleoli; m = mesocarp; mp = micro pollen tube; n = nuclei; ne = nectar-secreting cells; no = nucleoli; oi = outer rows of integument cells; p = papillose cells; pa = parenchyma; pg = pollen grain; po = postament cells; py = pyriform hypostase; re = receptacle; rfe = rounded free endosperm nuclei; ri = rudimentary integument cells; sc = secretory canal; se = secretion cells; st = stigma; sty = style; stp = = stylopodium; tpe = triploid primary endosperm; vb = vascular bundle; z = zygote

According to literary data [6] the frequency of occurence of hypostase in different families has been reported. The possible connections between the free endosperm nuclei, the hypostase and the behaviour of some reproductive organs have been identified here. These latter results were evaluated by the SHANNON [18] model.

Materials and methods

The outermost flowers of the compound umbel were collected during the full blooming period from a garden at Dömsöd. The flowers were selected in the phenophases 31.4.2; 32.1—32.1.2; 32.3.3; 32.3.5; 32.3.6; and 33.1.1; 33.2.1 (cf. [13]), and fixed in NAWASHIN solution, embedded in paraffin and stained with toluidine blue. Sections of 10 μm thickness were prepared. The diversity values were calculated according to SHANNON [18], equitability according to LLOYD and GHELARDI [10], and the evenness according to PIELOU [13].

Results and discussion

The pyriform hypostase in *A. graveolens* appears to be sunk in the integument of the ovule at the chalazal end of the embryonic sac prior to and following the double fertilization. At the time of double fertilization — in the phenophases of 31.4.2 or 32.1* — the hypostase was 69 μm long and 64 μm wide. Following the double fertilization in the above-mentioned phenophase (s) the hypostase was 150 μm long and 82 μm wide.

According to the cytological picture the hypostase may be in a very active stage in the subsequent phases of development. Therefore, I shall attempt to give a correct description of the flower of *A. graveolens* together with the morphological and anatomical features.

In the phenophase 32.3.3 the colour of petals is yellow, the filaments are discoloured, the anther is withering and falling off. The styles and stigmas are also yellow and the styles are slightly elongated and separated from each other. The stylopodium is glistening. The nectar-secreting cells are arranged in three rows deep in the disc. At this stage, the zygote is in its resting period and there are 16—24 free endosperm nuclei in the embryonic sac.

In phenophases 32.3.5—32.3.6, the petals are gradually drying, the anthers are dehiscent and the styles set apart and moving in opposite directions (Fig. 4). Many pollen grains were found to be adhered to the surface of the stigma (Fig. 5). The zygote is large and oblonged (Fig. 6) with a bilayered wall (Fig. 7), but still in the resting stage. A number of free endosperm nuclei are visible below the zygote. The separation of cytoplasm around the nuclei now begins, but only below the zygote (Fig. 7). The endosperm nuclei are, however, variable in the middle part. They are rounded or fusiform, with one, or sometimes two nucleoli (Fig. 8). The fusion of two or three endosperm nuclei and micro pollen tube may be seen (Fig. 9) towards the base of the embryonic sac, *i.e.* above the hypostase, of 280 μ m length and 139 μ m width. Following the fusion of nucleoli, the nucleus enlarges and becomes strongly associated with the micro pollen tube (Fig. 10). (The stage of development is the same as in *Pim-*

* The interpretation of the code, with reference to the outer morphological features of flowers, was given by SZUJKÓ—LACZA and FEKETE (1973) and in detail by SZUJKÓ—LACZA (1975).

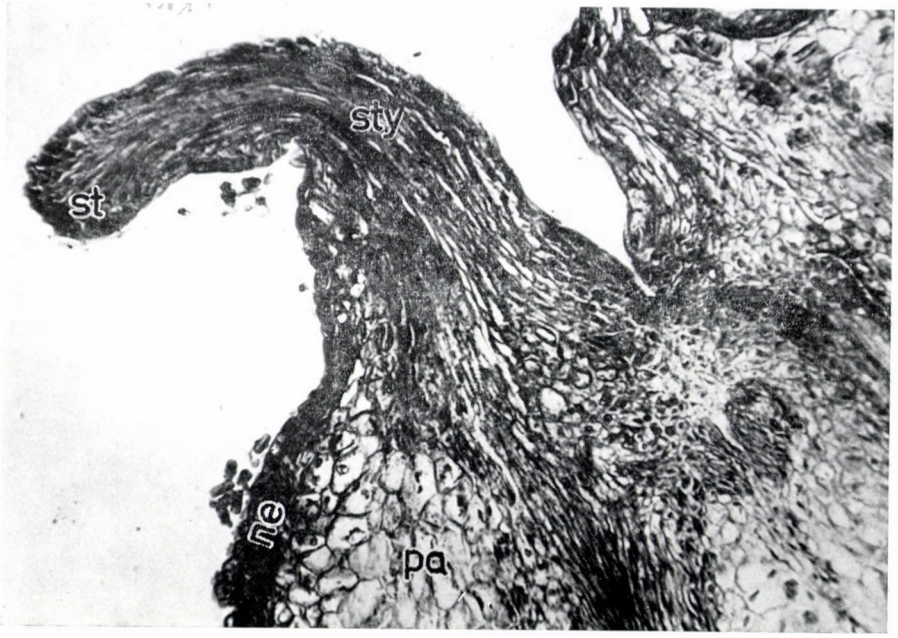


Fig. 4. Phenophase 32.3.5—32.3.6 Curved style in *A. graveolens* ×120

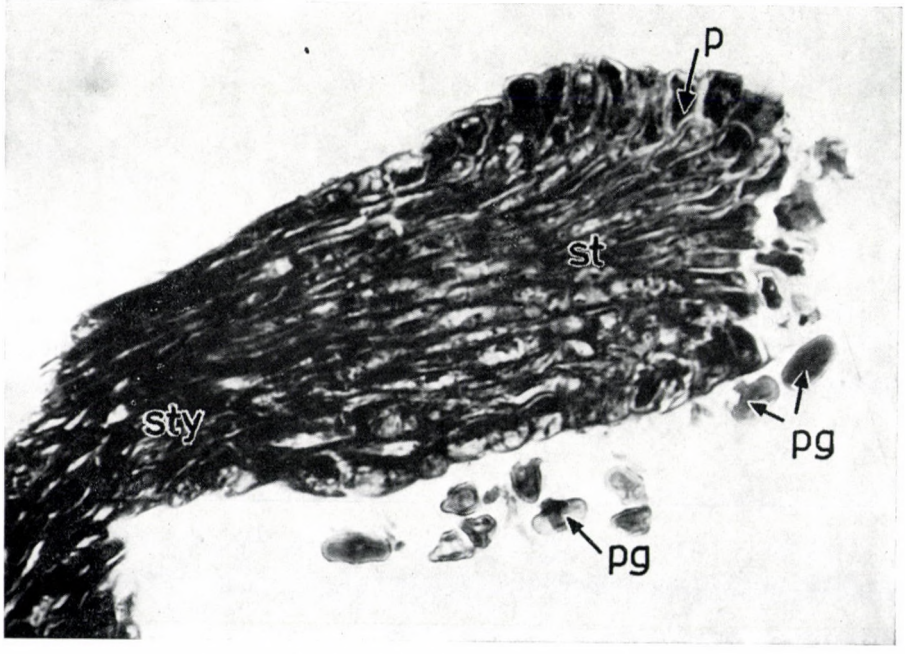


Fig. 5. Phenophase 32.3.5—32.3.6 Pollen grains on the surface of stigma. ×300

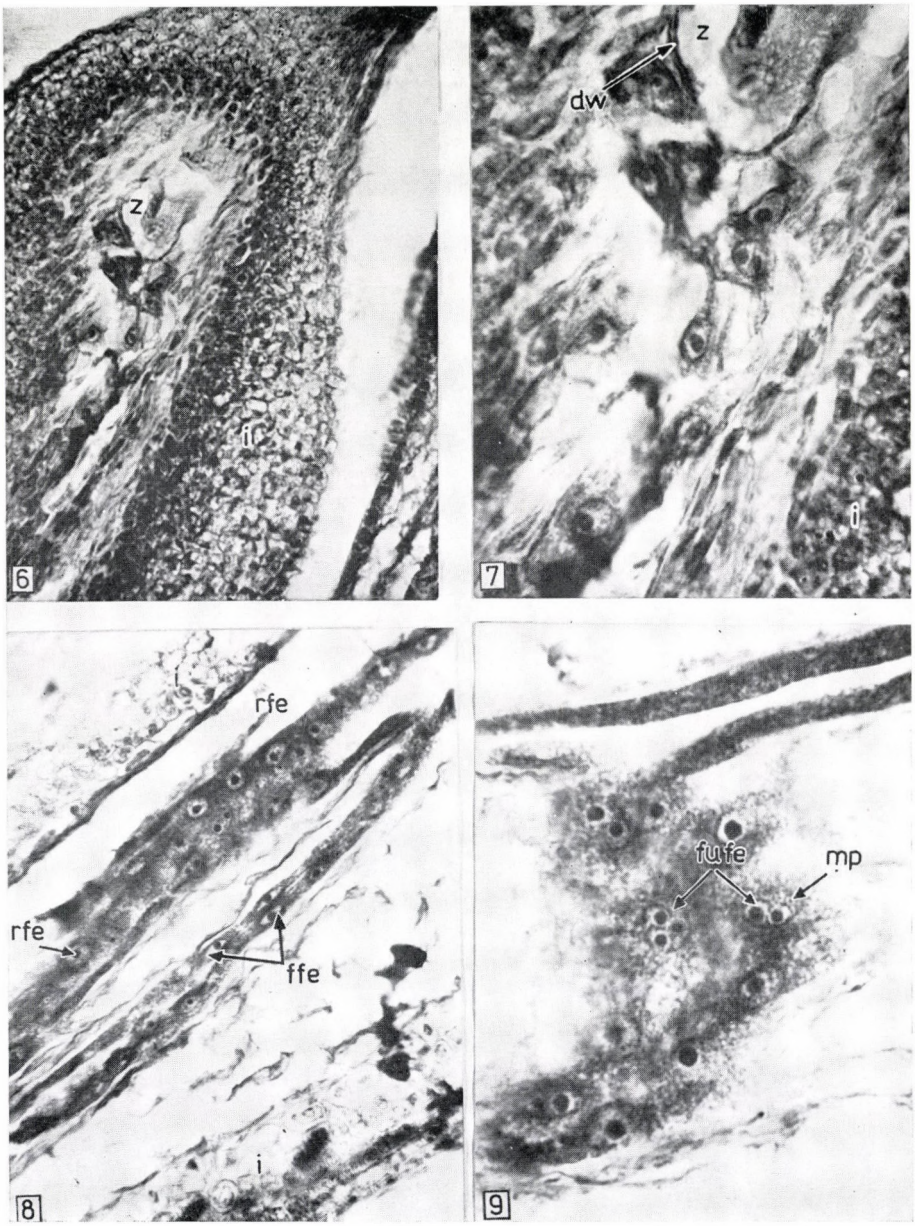


Fig. 6. Phenophase 32.3.5—32.3.6 Zygote in resting stage. ×200
Fig. 7. Phenophase 32.3.5—32.3.6 Zygote with double walls. ×500
Fig. 8. Phenophase 32.3.5—32.3.6 Free endosperm nuclei, different in size and shape. ×200
Fig. 9. Phenophase 32.3.5—32.3.6 Double and triple fused free endosperm nuclei. ×500

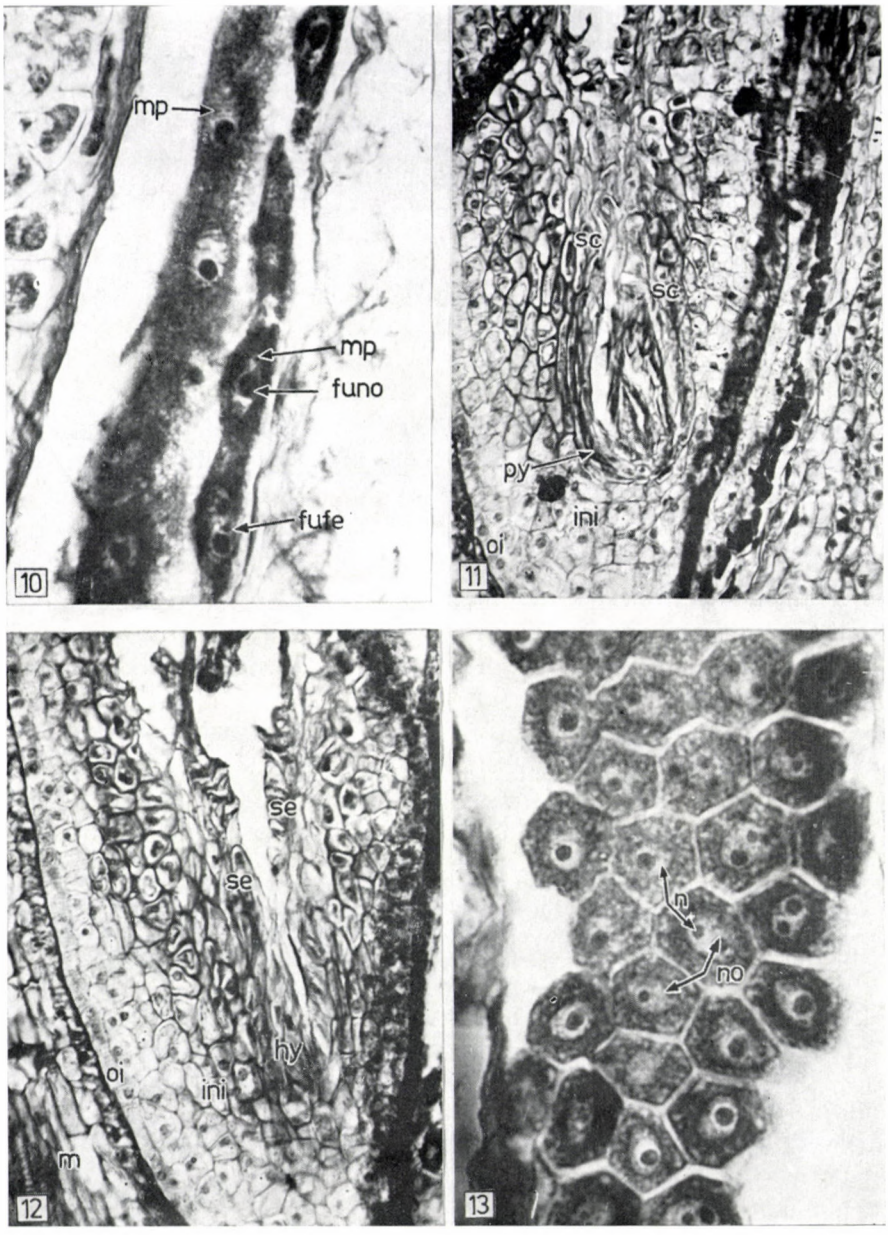


Fig. 10. Phenophase 32.3.5—32.3.6 Free endosperm nuclei with fused nucleoli and micro-pollen tubes. ×500

Fig. 11. Phenophase 32.3.5—32.3.6 The pyriform hypostase. ×200

Fig. 12. Phenophase 32.3.5—32.3.6 Connection between the embryonic sac and hypostase cells. ×200

Fig. 13. Phenophase 32.3.7—33.1.1 Hexagonal cells at the micropilar end. ×500

pinella anisum in this phenosphase.) The hypostase cells are elongated and curved (Fig. 11). They differ from the cell population of the integument in these two characters (Figs 11, 12). In the middle line of the hypostase cells, some of these have membranous walls. These cells may function only as secretory canal cells (Fig. 12). The other peripheral cells appear to be secretory cells. The secretory cells of the hypostase having large nuclei are in contrast to the canal cells.

In phenophase 32.3.7—33.1.1, the pollen grains are still present on the surface of the stigma and the walls of the nectar-secreting cells are membranous in the stylopodium. The cells seem like exhausted active cells which have ceased functioning. The zygote still remains in a resting condition. The free endosperm nuclei are of different phases, depending on their location. Below the zygote at the micropylar end, the endosperm nuclei are surrounded by a hexagonal layer of cytoplasm (Fig. 13). A centripetal wall formation is initiated at the micropylar end of the endosperm, similar to that reported in *Coriandrum* by SEGHAL [17].

The remaining cells of the integument, and the free endosperm nuclei, as well as the cytoplasm are arranged in columns. The separation of cytoplasm and wall formation in the centre of the cytoplasmic mass is becoming visible at the end of this phenophase step by step (Fig. 14). During the next stage,

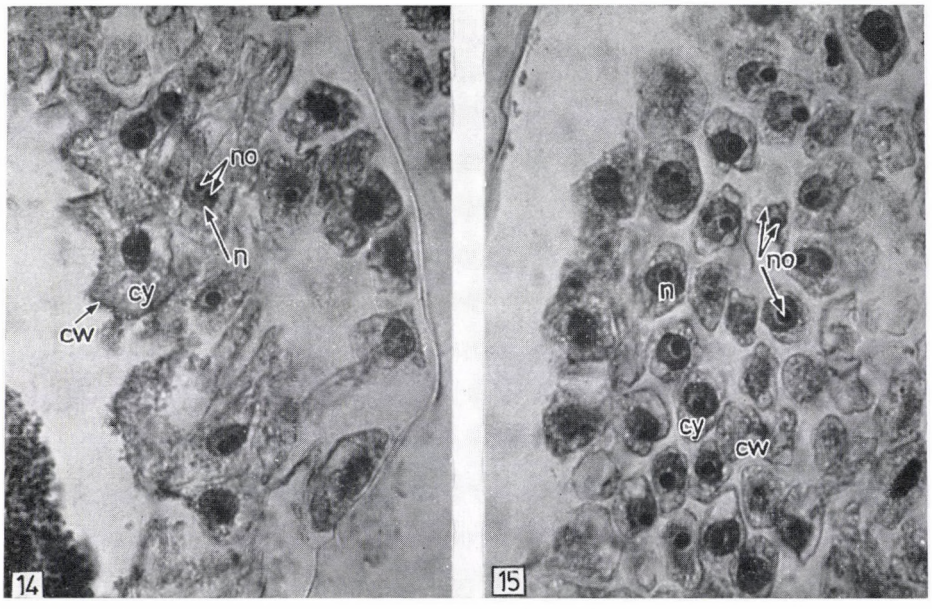


Fig. 14. Phenophase 32.3.7—33.1.1 The 2nd stage of separation of middle layer in cytoplasm ×500

Fig. 15. Phenophase 33.1.1—33.2.1 Well separated cells with nucleus, and nucleolus with prominent boundary. ×500

in the third or the fourth layer of the endosperm, the boundary of the cytoplasm, the nucleus and nucleoli become more prominent (Fig. 15). Elsewhere, the separated endosperm cells are in close contact with the disorganized integument cells (Fig. 16). The cells contain nucleus without nucleoli. The nuclei are not sharply demarcated. They are in a transitional stage. This is the last

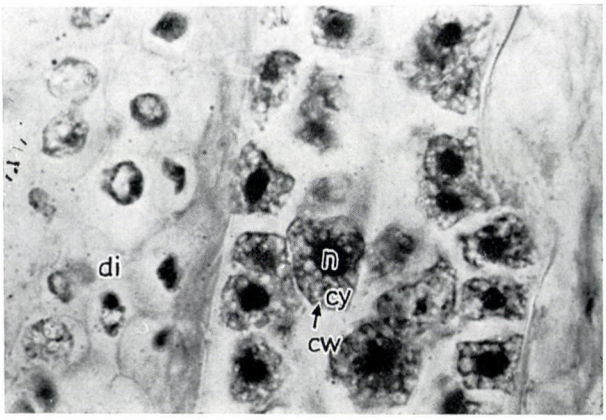
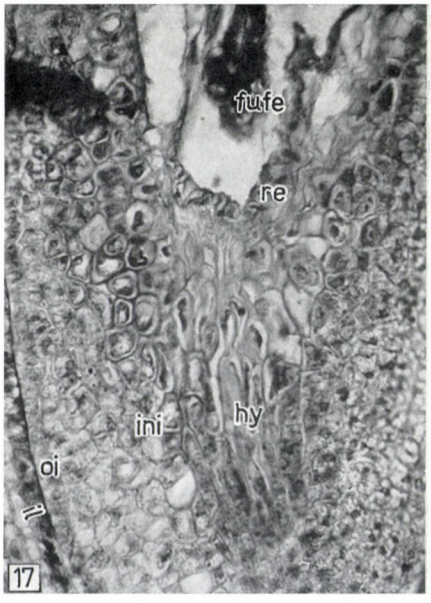


Fig. 16. Phenophase 33.1.1—33.2.1 The outer cell layer in the transitional stage; the nucleus has lost its sharp boundary; absence of nucleoli. ×500



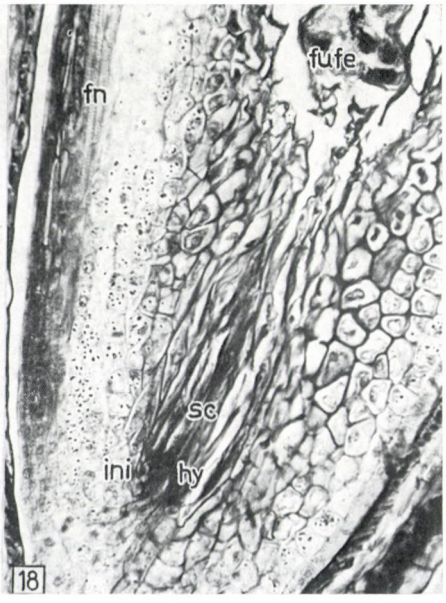


Fig. 17. Phenophase 33.1.1—33.2.1 The outer layer of hypostase cells. ×200
Fig. 18. Phenophase 33.1.1—33.2.1 The middle layer of hypostase cells without any cytoplasm. ×200

phase of the free endosperm nuclei or the first phase of the secondary endosperm cell. In the next stage, they are ready for cell division, like normal meristematic cells.

At the same time, cell division starts in the zygote and by repeated division and redivision, at different active points, it forms a Solanoid type of embryo. The hypostase cells in this phenophase are also well distinguishable. Figures 17 and 18 exhibit the hypostase cells with variable number of layers. Until it reached the maximum length (615 μm), and width (102 μm), and its cells soon become functionally exhausted. It is shown by large number of cells which are found crushed in the centre (Fig. 18). Now the inner cell rows of the integument are becoming disorganized and possibly the substance of these cells has transformed into secondary endosperm, except in the two outer rows and in the vascular bundle of the funicle (Fig. 19). The two outer cell rows ultimately form the testa of the seed, a common phenomenon in *Umbelliferae* [5].

In phenophase 33.1.1—33.2.1, the style and stigma wither and stop the secreting of nectar in the stylopodium; the gland cells are slightly compressed. In this particular stage, zygotic cell division continues simultaneously with that of the secondary endosperm cells.

In phenophase 33.2.1, the embryo develops and the endosperm cells are totally absent around the embryo (Fig. 19). Below the embryo, the endosperm cells are quite different in size and shape. This is perhaps due to the unequal



Fig. 19. Phenophase 33.2.1 The endosperm cells are absent around the embryo. ×500



Fig. 20. Phenophase 33.2.1 Secondary endosperm cells different in sizes and shape. ×650

number of division and position, or unequal fusion of the earlier free endosperm nuclei, or of both (Fig. 20). In the cytoplasm of the secondary endosperm cells, small vacuoles and small rudimentary nuclei are visible. At this stage, most of the integument cells seem disorganized and are present only as a membrane, except in the two outer cell rows (Fig. 20). The inner cell rows of the pericarps are in a very active stage: they appear as secretory cells (Fig. 20). These cells supply food to the endosperm and, at the later stage, they are transformed into endocarp cells.

It is well-known that different cell populations of a particular organ, like ovary or ovule, are interdependent (see RAGHAVAN [14]). Morphological, anatomical and occasionally functional relationships between the organs are also very clear. Connections between the functional details of style, stylopodium, cells of the micropyle, fertilization and fusion of free endosperm nuclei have been reported in *Pimpinella anisum* [21]. The same is true for Anethum graveolens. Investigations of the activities and the role of style and stigma are therefore reasonable. The styles get apart from each other before the free endosperm nuclei are pollinated and fertilized. The epidermal and hypodermal cells of stigma do not function as papillose cells before this process (Fig. 5). The styles become dried soon after the fertilization or induction for the fusion of free endosperm nuclei. The duration and functional activity of the hypostase may also be related with the development of the free endosperm nuclei. The hypostase cells as a glandular tissue [11] can secrete their content directly into the free endosperm nuclei and gradually become exhausted and form a membrane-like wall. The inner carpellary cells seem to be in an active stage. They resemble secretory cells (Fig. 20). Later these cells form the endocarp; at this stage (33.2.1) they probably supply food to the developing endosperm cells.

The function of hypostase and its connection with the free endosperm nuclei are clear in *Anethum graveolens*. This fact promted us to determine:

a) the frequency distribution of the hypostase within the families;

b) the probability of joint presence of the free endosperm nuclei, hypostase and embryonic sac of Polygonum-type

c) the probability of the other possible combinations of this triplet, that is the type of endosperm, embryonic sac and presence of hypostase.

On the basis of the embryological data of 78 families compiled in the "*Symposium on Comparative Embryology of Angiosperm*" ed. by JOHRI et al. (1970) [6]. I have reported forthcoming the coexistence of the hypostase and different types of endosperm, embryonic sac and embryos.

According to Table 1, the family *Solanaceae* is more variable because there are three types of endosperm present (as also in *Saxifragaceae*). *Solanaceae* also have different types of embryonic sacs and embryos. The next in order in the number of combinations is another large family *Compositae*. Cellular

Table 1

Distribution of type of endosperm, embryonic sacs, embryos and the hypostase in different families

Family name	Type of endosperm			Hypostase	Type of embryo sac		Type of embryo		Sum
	cell.	helob.	nucl.	present	Polygo-num	other	Solanoid	other	
Moraceae			+		+				2
Oleaceae	+	+			+	+			4
Opiliaceae	+				+				2
Santalaceae	+	+			+	+			4
Myzodendraceae	+				+				2
Loranthaceae	+			+	+			+	4
Cactaceae			+		+				2
Degeneriaceae	+				+				2
Annonaceae	+				+				2
Schisandraceae	+				+				2
Ranunculaceae			+		+	+			3
Peoniaceae			+		+				2
Nymphaeaceae	+		+		+				3
Crossomataceae			+	+	+				3
Actinidaceae	+			+	+		+		4
Theaceae			+	+		+	+		4
Capparaceae			+		+		+	+	4
Moringaceae			+	+	+				3
Crassulaceae	+				+				2
Saxifragaceae	+	+	+		+				4
Leguminosae			+		+				2
Limnanthaceae			+			+			2
Oxalidaceae			+		+				2
Geraniaceae			+	+	+		+		4
Tropaeolaceae			+		+				2
Zygophyllaceae			+		+		+		3
Linaceae			+	+	+		+		4
Erythroxylaceae			+		+		+	+	4
Euphorbiaceae			+	+	+				3
Simaroubaceae			+	+					2
Burseraceae			+		+				2
Meliaceae			+		+				2
Coriariaceae			+		+				2
Balsaminaceae	+				+	+			2

Table 1 (continued)

Family name	Type of endosperm			Hypostase	Type of embryo sac		Type of embryo		Sum
	cell.	helob.	nucl.	present	Polygo-num	other	Solanoid	other	
Cyrillaceae	+				+		+	+	4
Rhamnaceae			+	+	+	+	+	+	6
Vitaceae			+		+				2
Leeaceae			+		+				2
Eleagnaceae			+	+	+				3
Violaceae			+	+ double	+				3
Turneraceae			+	+	+				3
Passifloraceae			+		+				2
Bixaceae			+		+				2
Caricaceae			+		+				2
Cucurbitaceae			+		+	+	+	+	5
Onagraceae			+	+		+			3
Haloragaceae	+		+	+	+				4
Cornaceae	+				+				2
Umbelliferae			+	+	+	+	+		5
Clethraceae	+				+				2
Polemoniaceae			+		+			+	3
Fouqueriaceae	+				+				2
Convolvulaceae			+		+	+	+	+	5
Verbenaceae	+				+		+		3
Solanaceae	+	+	+		+	+	+	+	7
Scrophulariaceae	+				+	+			3
Acanthaceae	+		(+)		+				3
Pedaliaceae	+			+	+				3
Martyniaceae	+				+				2
Orobanchaceae	+				+				2
Lentibulariaceae	+					+			2
Plantaginaceae	+					+		+	3
Campanulaceae	+				+				3
Spenocleaceae	+				+			+	3
Pentaphragmataceae	+				+		+		3
Stylidiaceae	+				+		+		3
Compositae	+		+		+	+	+		5
Hydrocharitaceae		+			+		+		3
Sheuchzeriaceae		+			+			+	3
Juncaginaceae			+		+			+	3

Table 1 (continued)

Family name	Type of endosperm			Hypostase	Type of embryo sac		Type of embryo		Sum
	cell.	helob.	nucl.	present	Polygonum	other	Solanoid	other	
Potamogetonaceae		+	+		+			+	4
Zannichelliaceae		+			+			+	3
Najadaceae			+		+			+	3
Haemodoraceae		+			+			+	3
Amaryllidaceae		+	+		+			+	4
Hypoxidaceae		+	+		+			+	4
Burmanniaceae		+			+			+	3
Zingiberaceae		+		+	+			+	4
Sum of column	32	13	45	19	72	21	17	66	

and helobial types of endosperm were found in the *Oleaceae* and *Santalaceaea*. Cellular and nuclear endosperms are present in *Nymphaeaceae*, *Haloragaceae*, *Acanthaceae* and *Compositae*. Helobial nuclear endosperms have been found in *Potamogetonaceae*, *Amaryllidaceae* and *Hypoxidaceae*. In the rest of 36 families, only the nuclear type of endosperm was investigated. In eleven families, the variability appears in the types of embryonic sac, in six families the embryo and in four families both are visible. These differences, shown in the variance between genera and between species, whithin the same family, may be interesting from evolutionary point of view.

Thirty-eight families have only one type of endosperm, embryonic sac and embryo. These families seem to be homogeneous in the investigated characters.

The nuclear type of endosperm and the Polygonum type of embryonic sac occur together in 40 families. Thus, there seems to be existing an important interdependence between these two characters. The other type of embryonic sac and nuclear endosperm are co-existing only in five families.

The coexistence of hypostase and Solanoid embryogenesis was found also only in five out of seventy-eight families. Therefore, these two characters do not appear to be greatly interrelated. (The function of hypostase terminates before the embryo begins to develop.)

Taking into consideration the distribution of the data (Table 2), the number of published *joint properties* referring to the type of endosperm, embryonic sacs and embryo, we consider that, our knowledge is insufficient for 28 families (1st column). The high diversity value ($H' = 1.9110$) shows that our data have some uncertainty in sense of the information theory.

Table 2

The frequency distribution of the combination of the types of endosperm embryonic sacs and embryos (that is the triplet)

Number of any combinations of triplet (except the 1st column)	2	3	4	5	6	7
Frequency of combinations	29	28	16	4	1	1

Diversity H' = 1.9110; Evenness = J = 0.7392.

Note: each family has different numbers of examined genera and species, and the presence of the triplet was reported at the family level. It is also true to the hypostase.

Table 3

The coexistence of the hypostase, different types of endosperm and embryonic sacs

Type of endosperm	Type of embryonic sac		Hypostase present in the number of families
	Polygonum	other	
Nuclear	+		12
Nuclear	+	+	3
Cellular	+		3
Helobial	+		1

Diversity H' = 1.4832; Evenness J = 0.7416.

Out of the 78 families, there are 17 (21.7%) in which the hypostase is present. In table 3 the number of co-existence of the triplet are presented. Accordingly, the probability of co-existence of the hypostase, free endosperm nuclei and Polygonum type of embryonic sac is high.

This result supports the possible functional connection between the hypostase and free endosperm nuclei in Anethum graveolens. The other combinations are of less importance. Though the co-existence of hypostase and nuclear or helobial type of endosperm is not frequent, so the above results suggest that the life-span of the hypostase may be different in various species. On the one hand, the cellular endosperm develops at once after the double fertilization, suggesting that here the life-time of the hypostase may be short. On the other hand, the helobial type of endosperm is also nuclear substantially. The time of development of the free endosperm nuclei including helobial ones is also differing from species to species, and this fact determines the life-span of the hypostase cells.

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MONOLAYER CULTURES OF RAT PITUITARY INTERMEDIATE LOBE: GROWTH AND FINE STRUCTURE OF EPITHELIAL CELLS

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Abstract

Rat hypophyseal intermediate lobe cells, partially dispersed by trypsin, were cultivated on an inert surface. The attaching capacity, the growth of the cultures and the fine structure of the epithelial cells developed during cultivation were studied. The majority of the cell groups attached to the inert surface within 72 h after explantation. Epithelial islands developed, with a layer consisting of fibroblast-like cells beginning to form at their periphery. By the end of the second week of cultivation the epithelial islets and fibroblast-like cells formed a monolayer. The epithelial cells survived for five weeks. The granular cytoplasm of the epithelial island cells could be well identified on the phase-contrast microcinematographic frames of the young cultures; both epithelial and fibroblast-like cells divided. The mitotic ability of both cell types could be confirmed by their tritiated-thymidine incorporation, too. In young cultures, the fine-structural features of epithelial cells were practically identical with those of the glandular cells in intact glands. In older cultures, the secretory granules in the epithelial cells were lower in number; the localization of the granules at the periphery was predominating.

Introduction

It is generally accepted that an appreciable amount of adrenocorticotrophic hormone (ACTH) is present in the intermediate lobe of the rat hypophysis. When investigating the biological role of this ACTH, some researchers [14, 15, 19] demonstrated that neural stress caused ACTH depletion in the intermediate lobe, while others [6] reported that functionally significant quantities of ACTH were hardly secreted by the intermediate lobe or by the neurohypophysis. Mainly for anatomical reasons, this discrepancy is rather difficult to explain. This is why *in vitro* methods were resorted to. KRAICER and MORRIS [10, 11] extracted biologically active ACTH from mechanically dispersed intermediate lobe cells. Moreover, in short-term incubation experiments they demonstrated that both a hypothalamic extract and serotonin stimulate ACTH secretion. Later, based on short-term incubation experiments on neuro-intermediate lobe fragments, FISCHER and MORIARTY [4] stated that the hypophyseal stalk-median eminence extract, acetylcholine and serotonin stimulate, other substances either inhibit (dopamine) or do not affect (norepinephrine) the release of biologically active ACTH.

The *in vitro* systems applied by the two research teams have their incontestable advantages. However, one must not neglect their common drawback that in such incubation experiments the cells remain viable for a rather brief time and so do not permit repeated application of the same cells. The cell cultivation method eliminates these drawbacks.

Only a few cases of *in vitro* intermediate lobe cell cultivation have been reported. CHATTERJEE [2] prepared organ cultures from the intermediate lobe of differentiated rabbit hypophysis; the differentiated state of glandular cells observed *in vivo* was maintained for as long as five weeks. However, CHATTERJEE did not examine hormone production in the cultures. TIXIER—VIDAL et al. [22] reported at a conference on rat hypophyseal intermediate lobe cell cultures prepared from mechanically dispersed cells.

We have developed a simple cultivation procedure: the intermediate lobe of the hypophysis from adult rats was dispersed by trypsin and the cell suspension was explanted onto an inert surface. The present paper reports on the cultivation technique, life phenomena and fine structure of surviving and proliferating glandular cells. Cultures prepared by this method appear to be suitable for long-term and repeated study of hormone production by intermediate lobe cells.

Materials and methods

Dispersion and explantation of intermediate lobe cells.

The hypophyses from decapitated male and female CFY rats weighing 200 g were removed under sterile conditions and collected into Tissue Culture Medium 199 (Parker 199). Twenty-five to 30 animals of the same sex were used in each experimental series. The posterior and intermediate lobes were separated from the anterior lobe under the stereomicroscope without hardly any cell loss. The neuro-intermediate lobes were collected in centrifuge tubes containing 0.25% trypsin (DIFCO) solution and were mechanically agitated by a pipette for 10 min. Macroscopically, the neurohypophysis appeared intact. The intermediate lobe dispersed into individual cells or cell groups consisting of 2 to 30 cells each. After the sedimentation of

Table 1

Composition of culture media

1	80% Parker 199 + 20% fetal calf serum* (FCS) After attachment of the cells: 90% Parker 199 + 10% FCS
2	80% F-10 (HAM F-10 1× with L-Glutamine)* + 20% FCS After attachment of the cells: 90% F-10 + 10% FCS
3	82.5% Parker 199 + 15% horse serum* + 2.5% FCS
4	82.5% F-10 + 15% horse serum + 2.5% FCS

* GIBCO BIO-CULT Lab.
No antibiotic was used in any of the media.

the neurohypophyses the suspension of the intermediate lobe cells was decanted. Next, it was diluted with the same volume of a 4:1 mixture of Parker 199 and fetal calf serum (GIBCO—BIOCULT LAB.), and centrifuged at 1000 rpm at 4 °C for 10 min. The cell sediment was resuspended in various media (Table 1).

In certain experiments, 1 ml samples of the cell suspension were explanted in a multidish tray (LIMBRO disposo-tray FC 16/24): generally 12 parallel cultures were used per experiment. Since the cell suspension contained cell groups besides individual cells, no cell counting could be done.

In other experiments, 2 ml samples of the cell suspension were explanted in plastic petri-dishes (FALCON plastic, 3.5 cm) with round coverslips of 25 mm at their bottom.

The cultures were incubated at 37 °C in 5% CO_2 atmosphere. The medium was first changed after 72 h, thereafter every second day.

Phase-contrast cinematography of the coverslip cultures placed in perfusion chambers was performed as described earlier [3]. To ensure constant and uniform exchange of the medium, the chamber was connected with an infusion pump.

For autoradiographic examination, the cells were labelled with 1 μCi/ml tritiated thymidine (6-^{3}H-thymidine, 23 Ci/mM spec. act., The Radiochemical Centre, Amersham), dissolved in protein-free Eagle medium (DIFCO) for 1 h. Next, they were washed with Parker 199 five times. The cultures were fixed for 10 min in methanol, washed and covered with KODAK AR 10 stripping film; they were then exposed at 4 °C for 15 days. After development the autoradiograms were stained according to Giemsa. The labeling index was established by counting about 1000 cells per culture.

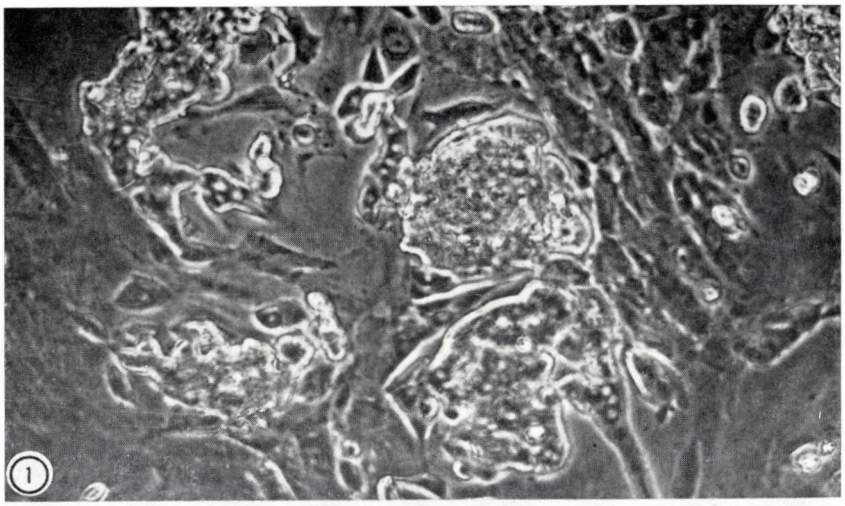
Cultures growing in multidish trays were used for *electron microscopic* study. The cells were prefixed at room temperature in 2.5% purified glutaraldehyde, dissolved in 0.1 *M*, pH 7.4 cacodylate buffer for 2 h. They were then washed in the same buffer overnight, postfixed in 1% OsO_4 and embedded in Epon. The thin sections were cut in a Reichert OmU2 microtome, stained with uranyl acetate and lead citrate, and examined in the JEOL 100 C electron microscope with 80 kV accelerating voltage.

Results

Attachment of the cells and growth of the cultures

The individual cells of the cell suspension displayed but a low tendency to attach, the majority of the cell groups attached slowly to the inert surface. The attachment was not influenced by the serum composition of the medium (Table 1). For not disturbing the attaching of the cells, the medium was not changed for 72 h. The attached cell groups mostly consisted of epithelial cells forming islands. On their periphery, a fibroblast layer started to grow, which formed a continuous monolayer in the second week after explantation. The initially dense epithelial islets grew and became less compact, while their individual cells remained closely attached to one another (Fig. 1). Neither the quality nor the quantity of the serum component in the medium influenced either the development or the growth of the epithelial islands or of the fibloblast monolayer. In three-week or older cultures the fibloblast layer ceased to be continuous, its cells detached from the surface, while the cells of the epithelial islands invariably survived even as long as for 5 weeks.

The cells in the epithelial islands were examined in 5 to 12 days old cultures by phase-contrast microcinematography. The epithelial cells were closely attached to each other and had no long processes (Fig. 2). Their cyto-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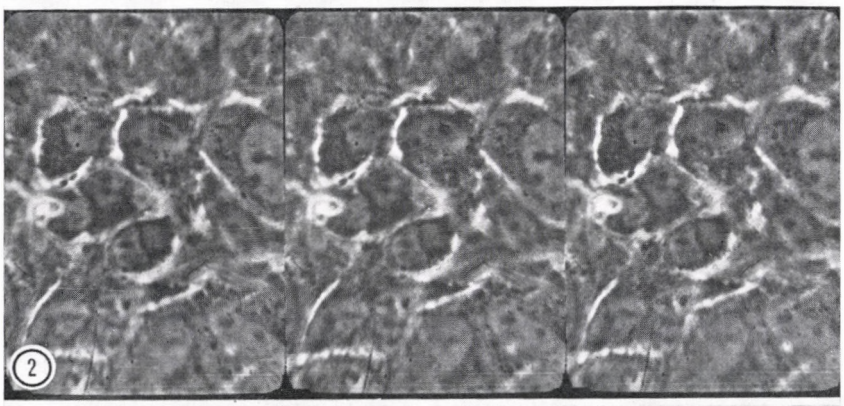
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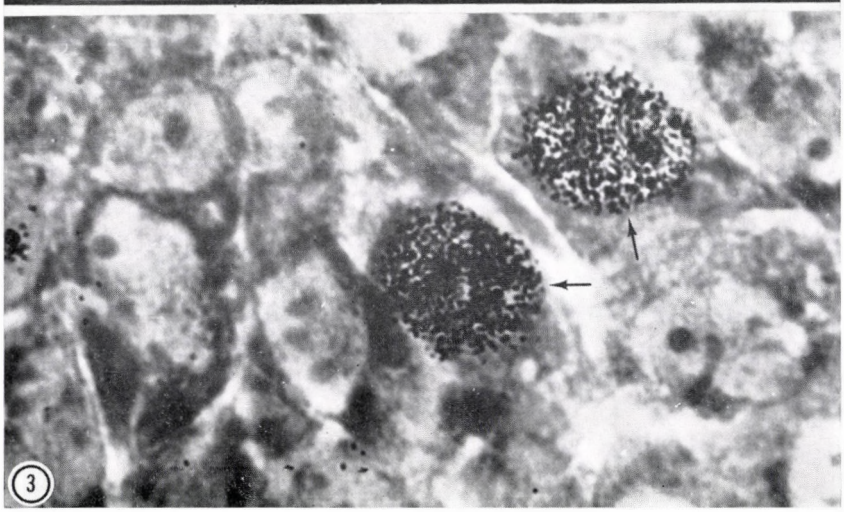
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plasmic membrane was vividly moving, but the cells did not migrate. Motile phase-dark granules — either mitochondria or aggregated secretory granules —, occurred in their cytoplasm. The epithelial cells were generally mononuclear. The nucleus was often rotating. No differences were seen in the features of the epithelial cells in the cultures of various ages. Dividing epithelial cells and fibroblast often occurred.

The dividing capacity of the epithelial cells was confirmed by their DNA precursor incorporation, too (Fig. 3). In the epithelial islands of 7-day cultures the proportion of cells incorporating tritiated thymidine run to 9.7%, in those of 10 and 12-day cultures to 11.0 and 10.2%, respectively (Table 2). The percentage of labelled cells in the fibroblast monolayer amounted to 16.5% in the 12-day cultures, a sign of the higher proliferation rate of these cells. In cultures older than 17 days, as soon as the continuous monolayers developed, the cells incorporated but a minimum of the precursor. Accordingly, no labelling index could be determined.

Table 2

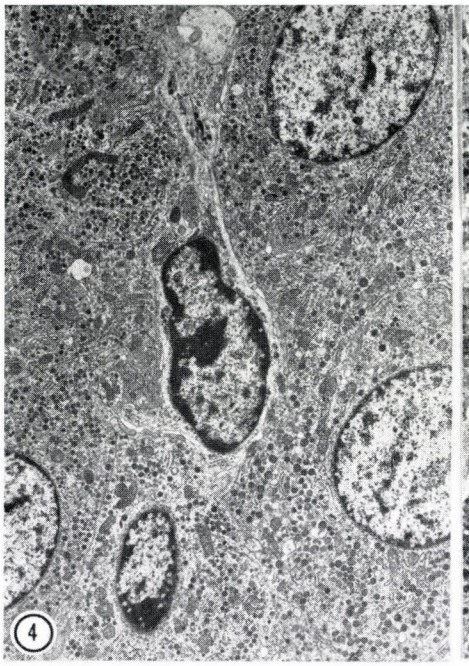
^{3}H-thymidine incorporation into cultured cells

Age of the cultures	Morphology of cells counted	Total number of counted cells	Number of labelled cells	Labeling index in %
7 days	epithelial islands	1000	73	
		1000	114	
		1000	105	
		total: 3000	292	9.7
10 days	epithelial islands	1000	111	
		1000	111	
		1000	110	11.0
		total: 3000	332	
12 days	epithelial islands	1000	102	10.2
12 days	fibroblast layer	1000	148	
		1000	183	
		total: 2000	331	16.5

Fig. 1. 8-day intermediate lobe cell culture. Phase-contrast photomicrograph. Closely fitting epithelial cells forming epithelial islands with fibroblast-like cells at their periphery (Magnification: ×250)

Fig. 2. Portion of an epithelial island from an 8-day intermediate lobe cell culture. Phase-contrast microcinematographic frames. Many phase-dark granules are seen in the cytoplasm of closely packed polygonal cells

Fig. 3. Autoradiograph of a 7-day intermediate lobe cell culture. Portion of an epithelial island. Many silver grains over cell nuclei (arrows) indicate an evidence for the incorporation of ^{3}H-thymidine. Giemsa staining. ×1350



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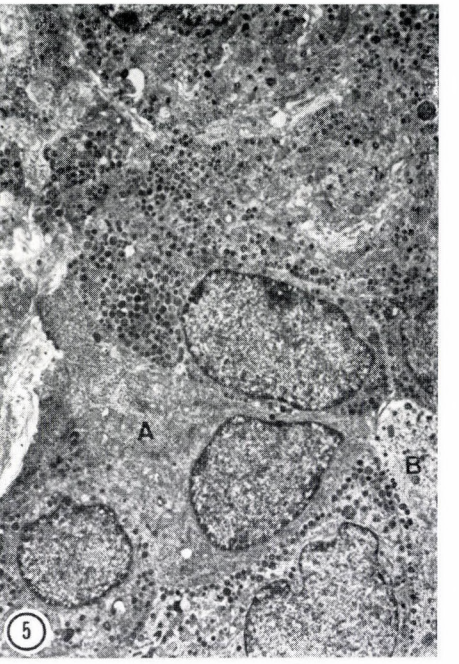

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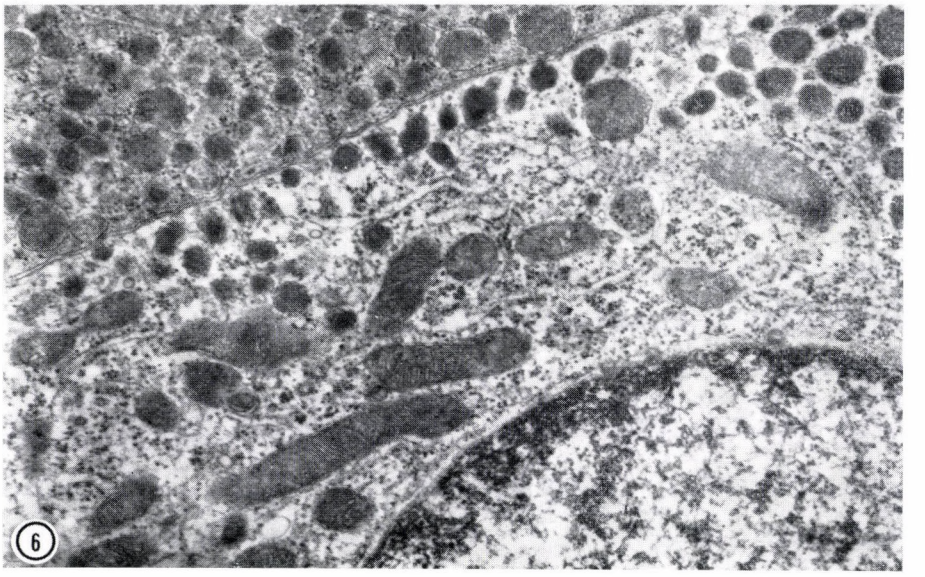


6

Fine structure of cultivated epithelial cells

The majority of the glandular cells from the intact, non-cultivated intermediate lobe contained many pleomorphic secretory granules (Fig. 4). Such mature secretory granules occurred everywhere in the cytoplasm. Signs of secretory granule formation could be observed in the Golgi region. The cells were rich in mitochondria with a dark matrix. Such parenchymal cells also occurred in certain regions of the intact lobe in which the secretory granules were aligned along the cytoplasmic membrane.

In 8-day cultures the majority of the cells in the epithelial islands contained such secretory granules and displayed the same morphological signs as those in the intact gland. Cells without cytoplasmic secretory granules were rarely seen. According to their shape and fitting to the other epithelial cells we believe these few cells to be epithelial cells, too (Fig. 5). A higher magnification permitted the differentiation also of that secretory glandular cell type wherein the granules were arranged mainly along the cytoplasmic membrane (Fig. 6).

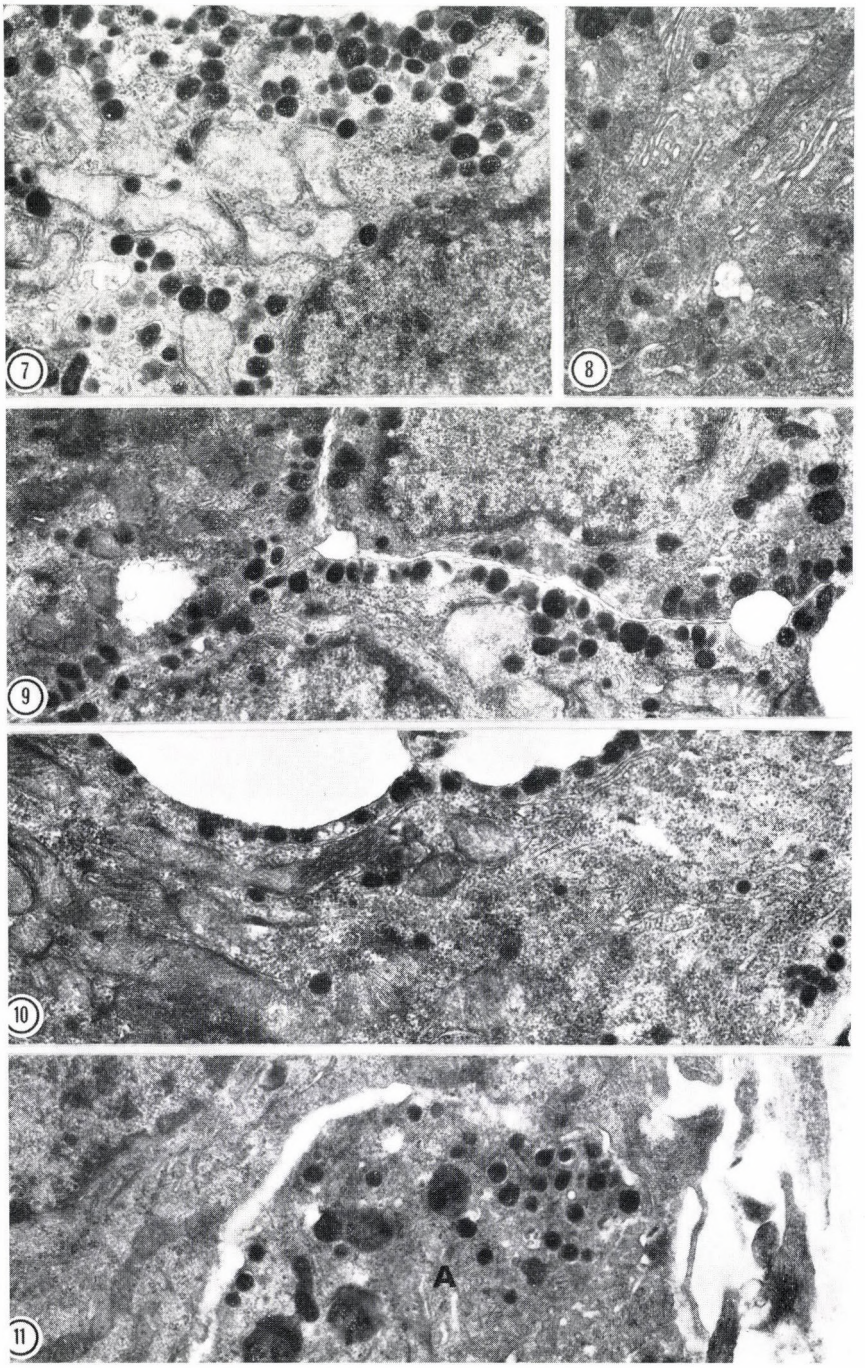
In 27-day cultures, the epithelial cells with the characteristic secretory granules could still be differentiated, though the number of the granules was lower than in the 8-day ones (Figs 7—10). The location of the granules in the cytoplasm was varied from the random distribution (Fig. 7) to localization mainly along the membrane (Figs 9 and 10). The signs of secretory granule formation in the Golgi region of epithelial cells were still present (Fig. 8). However, no sign of exocytosis of secretory granules could be observed. Both the cytoplasm and the matrix of the mitochondria were dark in the majority of the epithelial cells. Sporadically, microtubule fascicles accumulated at the periphery of the cytoplasm (Fig. 10).

In 35-day cultures, the ratio of epithelial cells with few granules was increased. As opposed to younger cultures, dense bodies more frequently occurred in the granular cells (Fig. 11) suggesting the possibility of intracellular digestion of secretory granules.

Fig. 4. Low-power (×3000) electron micrograph showing a portion of an intact intermediate lobe embedded after perfusion fixation. The majority of the cells contain many pleomorphic secretory granules. The cytoplasm is rich in endoplasmic reticulum and mitochondria

Fig. 5. Low-power (×3000) electron micrograph of an 8-day intermediate lobe cell culture. The majority of the cells displays the characteristic morphological features of intact intermediate lobe cells. While the majority of the cells contains many secretory granules in a random arrangement, a cell (A) containing hardly any granule also occurs. In other cells (B) the secretory granules are localized to the periphery of the cytoplasm

Fig. 6. High-power (×19 700) electron micrograph of an epithelial island from a 8-day intermediate lobe culture. Portions of two cells laying close to one another are shown. The secretory granules lay scattered in the cytoplasm portion seen in the upper corner, left. In the other cell the secretory granules are located along the cytoplasmic membrane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The close contact of the glandular cells, observed first under the light microscope, was confirmed by electron microscopic examination at any culture time.

Discussion

As confirmed by our results, the intermediate lobe of the rat hypophysis permits the preparation, with a relatively good yield, of cell cultures surviving for a long time. Thus, we have confirmed the findings of TIXIER—VIDAL et al. [22]. The intravital observation of the cells, their DNA precursor incorporation and the morphological features of the cultivated cells enable us to draw the conclusion that the glandular cells survive, proliferate, and display the morphological signs of intracellular hormone formation for at least 5 weeks. The qualitative composition of the various media we used practically did not influence the cellularity of the cultures.

According to light-microscopic criteria, the cells of the rat hypophyseal intermediate lobe can be categorized into three types [7]: in two cell types the cytoplasm is PAS-positive and contains granules. These cell types can be detected in the epithelial islands of our cultures, more precisely, they might be identified as epithelial cells rich in phase-dark granules. The third cell type present in the intact intermediate lobe does not display any sign that would suggest secretory activity. This cell type might be the source of the fibroblast-like cells found in the cultures.

According to LEBLOND and WALKER [12] and to HUNT and HUNT [9], the number of dividing cells is rather low in the intact hypophysis of adults. However, after adrenalectomy the ratio of glandular cells entering the cell cycle will increase both in the anterior [20] and the intermediate lobe [5]. Also, it has been shown that should the hypophyseal stalk be transected, the intermediate lobe becomes hypertrophied [13]. All these findings suggest that under certain conditions the glandular cells in the intermediate lobe are capable of proliferating. Mitotic glandular epithelial cells can be seen on the micro-cinematographic frames; in our younger cultures the proportion of epithelial cells incorporating the DNA precursor was about 10%. Accordingly, the glandular cells are capable of dividing in cell cultures, too. This is confirmed also by an earlier finding of ours [21], *viz.* immunoreactive glandular cells were capable

Figs 7—10. Electron micrographs of epithelial cells from 27-day intermediate lobe cell cultures. The incidence of secretory granules is lower than in the 8-day cultures

Fig. 7. The pleomorphic secretory granules are located scattered throughout the cytoplasm. ×14 600

Fig. 8. Well-developed Golgi region with signs of granule formation. ×15 300

Figs 9—10. The secretory granules are located along the cytoplasmic membrane. 9: ×14 700, 10: ×19 400

Fig. 11. Electron micrograph of two epithelial cells from a 35-days old intermediate lobe cell culture. Besides the secretory granules, several dense bodies may be seen in the cytoplasm of the "A" cell. ×13 370

of synthesizing DNA in our cultures. CHATTERJEE [2] established by the autoradiographic study of organ cultures from rabbit intermediate lobe that cell proliferation goes on for at least two weeks in cultures.

Quite recently, in electron microscopic studies, HOWE and MAXWELL [8] and lately MORIARTY [16] have demonstrated that melanotropic cells constitute the majority of glandular cells in the intact rat intermediate lobe. In the cytoplasm of their two types, phase-light and phase-dark cells, pleomorphic secretory granules are abundant and randomly distributed. A lower number of stellate parenchymal cells occur sporadically. In these cells secretory granules are less in number than in the above-mentioned cell types and are aligned along the cell membrane. Several authors, as *e.g.* NAIK [17, 18] and CHATTERJEE [1], consider these cells analogous to the ACTH-producing cells in the anterior lobe of the hypophysis, others [16] contest this analogy.

In our young cultures, the majority of the individual cells in the epithelial islands displayed all those fine-structural features (presence, density and localization of secretory granules and their formation in the Golgi apparatus) that had earlier been described to characterize the melanotropic cells in the intact intermediate lobe. Sporadically, also such epithelial cells occurred that failed to contain any secretory granules. The signs of the "corticotrophic" cell occurred but rarely in the epithelial islands. In the four and five weeks old cultures the amount of secretory granules decreased in the cytoplasm of most of the glandular cells and the localization of the granules at the periphery became more frequent. The signs of granule formation were invariably found in the Golgi region.

The survival of parenchymal cells in our cultures and the cytological signs of intracellular hormone production suggest that like monolayer cultures from other endocrine organs, these cultures are suitable for studying production and regulation of the hormones known to be produced in the intermediate lobe. Namely, in addition to ACTH, a fair number of hormones have been detected in the intermediate lobe of the hypophysis. These hormones are split products partly of the ACTH, partly of the lipotropin molecules [16]. Thus, the applicability of a well-functioning *in vitro* model is, we feel, more than timely in the study of these problems.

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ONTOGENESIS OF THE LH—RH-CONTAINING NEURONAL ELEMENTS IN THE HYPOTHALAMUS OF THE RAT[1]

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Abstract

LH—RH-containing neuronal elements were detected earliest in the median eminence and in the vascular organ of the lamina terminalis of newborn rats. The peroxidase-labeled antibody method was used. Since the synthesis of the gonadotrophic hormones starts already on the 18th day of intrauterine life in the rat, this finding does not support the view that LH—RH could have inductive effect on the differentiation of the gonadotropic cells of the anterior pituitary gland.

Introduction

Both morphological and functional data indicate that the neurovascular mechanism regulating the hormone synthesis and/or release of the anterior pituitary gland starts to function in the rat around birth.

SÉTÁLÓ and NAKANE [17] and SÉTÁLÓ et al. [18], using the peroxidase-labeled antibody method, have shown that cytodifferentiation of the troph-hormone-synthesizing cells of the fetal rat pituitary gland is completed before birth. Although immunoreactivity does not necessarily reflect the biological activity of these hormones, other experiments have shown that the embryonic pituitary releases at least some of the troph hormones as biologically active substances [4, 9, 11].

It has also been documented that both the nerve terminals discharging the hypophysiotrophic hormones and the adjacent capillary system are present in the hypothalamus of the rat before birth. HALÁSZ et al. [7] demonstrated the presence of such specific nerve terminals in the median eminence (ME) of 16-day-old rat fetuses. GLYDON [6] showed that in the albino rat, the supratuberal capillary plexus already existed in 18-day-old fetuses and this plexus had connecting vessels to the capillary system of the adenohypophysis. This finding was substantiated by RINNE and KIVALO [16] and CAMPBELL [2].

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These authors, however, observed capillary loops penetrating the ME earliest on the 5th postnatal day. Since in the adult rat the nerve terminals of the parvicellular neurosecretory system end preferentially along the contact zone of the ME, HALÁSZ et al. [7] assumed the significance of these capillary loops to be rather quantitative than qualitative.

The data of the above investigations strongly suggested that hypothalamic control of some anterior pituitary function through a neurovascular link might have been established several days before birth. This possibility was suggested earlier also by DAIKOKU et al. [3] and KOBAYASHI et al. [10]. Other, more functional, experiments gave further support to this assumption. JOST [9] and FUJITA et al. [5] have demonstrated that the release of ACTH and TSH in the rat is really under the control of the hypothalamus already in the embryonic life. Although the fetal pituitary gland of the rat synthesizes also gonadotroph hormones [17, 18],no data are available indicating whether this synthesis is under hypothalamic control.

Hopefully, the knowledge of the timetables of the functional differentiation of both the neuron systems elaborating the different hypophysiotrophic hormones and the cells synthesizing the troph hormones would help the better understanding of the onset of hypothalamic control on the anterior pituitary. Our knowledge on the ultrastructural characteristics of the nerve terminals of the median eminence, however, does not enable us to distinguish these terminals according to their hypophysiotrophic hormone content. Recently, specific antibodies against some hypophysiotrophic hormones have become available. Therefore, as a first step to determine the onset of the synthesis of the hypophysiotrophic hormones, we used an immunohistological approach to detect the earliest appearance of LH—RH in the hypothalamus of the rat.

Materials and methods

Hypothalami were collected from rats (originally Wistar strain) ranging in age between the 16th day of intrauterine life and the 28th postnatal day. The presence of sperms in the vaginal smears of the mothers was considered to be indicative of the first gestational day. The embryos were removed by Cesarian section under pentobarbital anesthesia. The heads were cut off, the skulls opened and, with the brains in place, they were immersed in cold picric acid-formaldehyde solution [19] for 24h. For the brains of the postnatal rats, fixation was the same, but the brains were removed from the skull immediately after decapitation. Sex of the embryos was determined by inspection of their gonads. The fixative was washed out with cold 0.1 *M* PBS of pH 7.6. The fixed tissues were dehydrated in graded alcohols and embedded in polywachs 1000 (Chemische Werke, Hüls). 5—7 μm thick sections were floated on PBS, mounted on glass slides and processed for immunohistological detection of LH—RH. At least 4 animals representing both sexes were investigated on each developmental day. LH—RH was localized on coronal or sagittal sections of the brains using an indirect immunohistological method [13]. The first antibody used was generated in rabbits against synthetic LH—RH (a gift from DR. ARIMURA; anti LH—RH No. 422; dilution 1:1000). Peroxidase-labeled anti-rabbit gamma globulin prepared according to the method of NAKANE and KAWOI [12] was used as the second antibody (dilution 1:50). The reacted sections were developed for peroxidase using hydrogen peroxide and 3,3'-diaminobenzidine (Sigma). For control staining,

either normal rabbit or anti-LH—RH serum absorbed with LH—RH were used instead of the specific serum. Neither these sera nor the peroxidase-labeled antibody itself gave positive reaction. Endogenous perixidase could only be seen over red blood cells.

Results

Deposition of DAB reaction product over some neuronal elements of the median eminence was detectable earliest in newborn rats and indicated the presence of LH—RH in these structures. This reaction was, however, very week and was not present in all investigated animals. If present, the hormone was localized in neuronal profiles located in the most lateral region of the median eminence, very close to the surface.

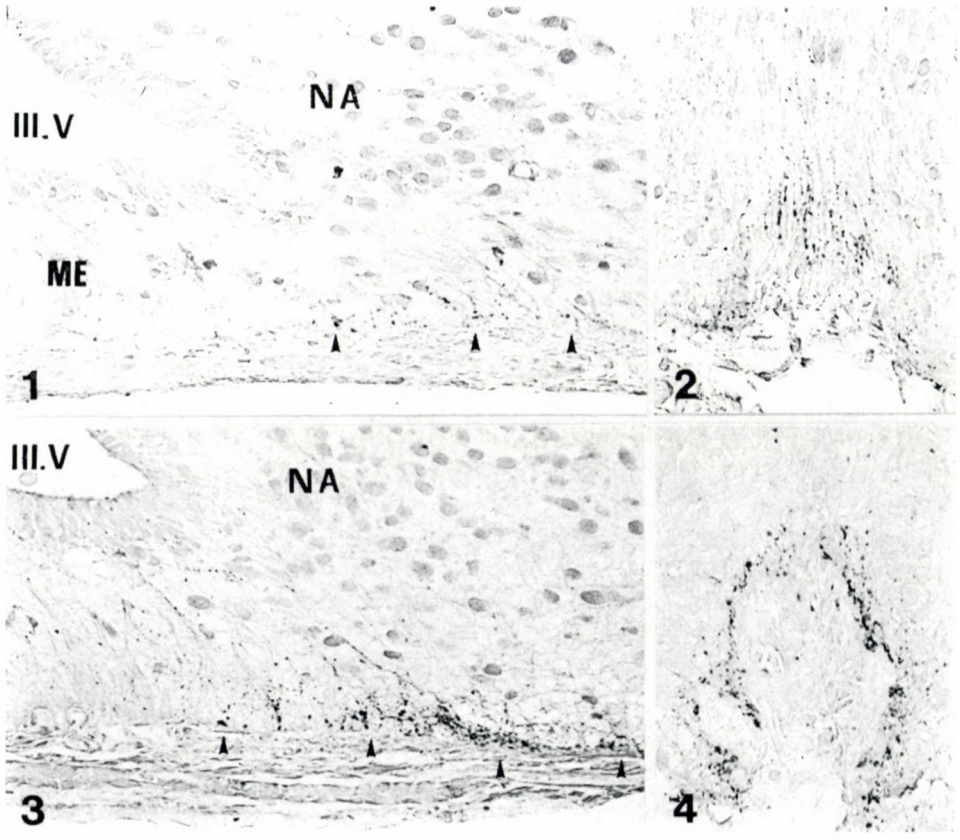

Fig. 1. Half median eminence (ME) of a rat from the second postnatal day. Arrowheads indicate axonal profiles containing immunoreactive LH—RH. III. V = 3rd ventricle; NA = arcuate nucleus. ×500

Fig. 2. LH—RH-containing axonal profiles in the vascular organ of the lamina terminalis of a two days old rat. ×500

Fig. 3. Half ME of a rat from the 5th postnatal day. Arrowheads indicate axonal profiles containing immunoreactive LH—RH. III. V = 3rd ventricle; NA = arcuate nucleus. × = 500

Fig. 4. LH—RH-containing axonal profiles in the OVLT of a five days old rat. × = 500

On the second day following birth, LH—RH-containing neuronal elements were present in all of the brain specimens. Again, the profiles containing LH—RH were predominantly found in the lateral third of the median eminence (Fig. 1). They were increased in number and reactivity compared to those found on the previous day. Drum-stick-like profiles reacting with anti-LH—RH indicated nerve terminals and occupied mainly the contact zone of the median eminence. On this day, LH—RH-containing nerve fibres and terminals were found also in the vascular organ of the lamina terminalis (OVLT). The density of LH—RH axons in this region was surprisingly high with respect to the small size of OVLT at this age (Fig. 2).

On later days, both the density and reactivity of the neuronal profiles containing LH—RH increased gradually (Figs 3 and 4) and became similar to the picture one can see in an adult animal by the end of the second postnatal week.

Differences related to sexes were not noticed in the materials investigated.

Discussion

Because of the limited access to specific antisera to the different hypophysiotrophic hormones, our knowledge on the functional differentiation of the neurones elaborating these hormones is almost restricted to the results classic light and electron microscopy have obtained. From such studies we have learned that both the specific nerve terminals devoted to the storage and discharge of the hypophysiotrophic hormones and a certain primitive portal circulation do exist in the median eminence of the rat already around the 16—17th day of intrauterine life [2, 6, 7, 16]. On the other hand, immunohistological investigations showed that the onset of hormone synthesis in the anterior pituitary cells could also be dated on the 16th day of intrauterine life [17, 18]. From the temporal coincidence of the morphological differentiation of the median eminence and the functional differentiation of the anterior pituitary, one could suppose that direct causal relationship does exist between these two events.

Halász et al. [7] found that morphological differentiation of the median eminence somewhat preceded the onset of hormone synthesis in the anterior pituitary of the rat. They found nerve terminals containing both synaptic and densecore vesicles in the zona palisadica of the median eminence in 16-day-old rat embryos, and observed that pituitary cells became granulated later, on the 18th gestational day. Although smaller differences can be found among different strains of the same species, we suppose that this late dating of the onset of granulogenesis in the anterior pituitary of their rat strain was caused by inadequate sampling. As mentioned in the introduction, immunohistological

data indicate that the hormone synthesis starts on the 16th gestational day in the pituitary gland of the rat. Therefore, immunohistological findings do not substantiate the idea that differentiation of the cells in the pars distalis would be preceded by the differentiation of the neural elements elaborating the hypophysiotrophic hormones. Furthermore, we do not think that the supposition of a causal relationship between the onset of the synthesis of the hypophysiotrophic hormones and the cytodifferentiation of the pars distalis in a general sense would have any justification. To study this relationship in connection with one of the pituitary hormones and its related hypophysiotrophic hormone seems to be more plausible. Therefore, our present observation on the ontogenesis of the LH—RH system of the rat, together with our earlier findings regarding the onset of gonadotroph hormone synthesis offers a possibility to discuss the question whether LH—RH has inductive influence on the differentiation of the gonadotroph cells of the pituitary gland or not. The results we have obtained do not support such a relationship. LH cells appear in the pars distalis on the 18th gestational day [17] and even FSH cells are present in the gland before birth [18]. On the other hand, LH—RH could be detected earliest in the hypothalamus of newborn rats. Of course, the possibility that LH—RH was present in the median eminence a few days earlier but in an amount below the threshold of the sensitivity of our immunohistological method cannot be excluded. Recently, however, ARAKI et al. [1], using a radioimmunoassay approach, found detectable gonadotrophic hormone releasing hormone first also in newborn rats.

The observation of NEMESKÉRY et al. [15] indicated that anterior pituitary cells have the capacity of self-differentiation. In embryonic pituitaries removed on the 13th day of gestation and cultured in a synthetic medium, ACTH cells could be detected by immunohistological method at the end of a 7-day culturing period. When pituitaries from 14-day-old embryos were cultured under the same conditions, besides ACTH cells, prolactin, LH and TSH cells were detectable. NEMESKÉRY et al. [14] have also demonstrated that when releasing hormones were added to the culture medium, or if the pituitaries were confronted with hypothalamic tissue, differentiation of the related pituitary cells, or of the pars distalis in general, did not speed up. The number of detectable differentiated cells, however, increased in these cases compared to the explants cultured only in synthetic medium. These observations would indicate that while hypophysiotrophic hormones have, among others, trophic effect on the anterior pituitary gland they have no inductive effect of the functional differentiation of the gland. Recent *in vitro* studies of ISCHIKAWA and SHIINO [8] on embryonic pituitary primordia led to the conclusion that while an extract of embryonic brain was obligatory to achieve differentiation of the troph-hormone-synthesizing cells, extracts from the median eminence of adult rats did not have such effect.

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SIMULTANEOUS LOCALIZATION OF LUTEINIZING HORMONE-RELEASING HORMONE (LH—RH) AND SOMATOSTATIN-CONTAINING NERVE FIBRES IN THE BRAIN OF THE RAT

SHORT COMMUNICATION

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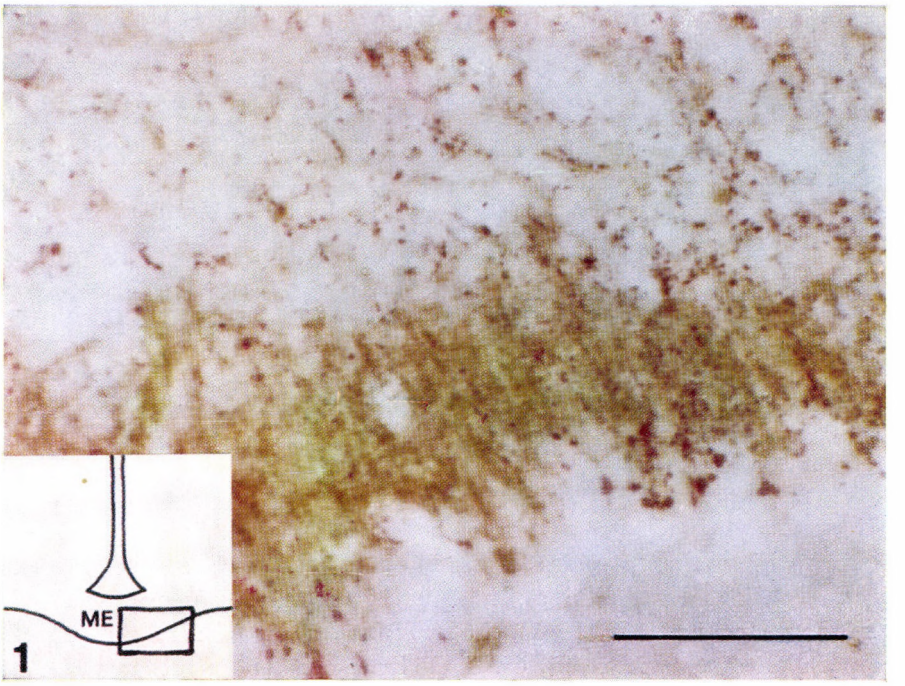
Two arrays of delicate nerve fibres containing immunoreactive LH—RH and growth hormone-release inhibiting hormone (GH—RIH or somatostatin), respectively, have been distinguished in the median eminence (ME) of the rat. In the rostral part of the ME, the two fibre systems form a flat superficial layer parallel to the surface [5, 6]. Caudally, where the pituitary stalk starts to emerge from the ME proper, somatostatin fibres appear in the middle portion of the ME, while LH—RH containing fibres occupy mainly, but not exclusively, the lateral parts of the ME [3]. Since the areas where the nerve fibres of the two systems are located partly overlap each other, and these hormones have only been detected in consecutive sections, there was no direct evidence that the two neurohormones are consequently located in separate axon profiles. Simultaneous localization of the two hormones in a single section, by the method described by NAKANE [4], offered a possibility to test this question directly. Theoretical possibility of cross-reaction between the antibodies to different hypophysiotrophic hormones gives further actuality to such a study.

It was found in an earlier study [7] that reserpine treatment, or the administration of the antiovulatory sulpiride or pentobarbital, greatly increased the amount of immunodetectable LH—RH in the hypothalamus of the rat. Therefore, we decided to use such treated animals in the present study. Female rats from our own stock (originally Wistar), ranging between 150—200 g in body weight were injected either *i.p.* with 1 mg/kg b.w. reserpine (Rausedyl, G. Richter, Budapest, Hungary) daily for 10 days, or *i.p.* with 100 mg/kg b.w. sulpiride (Dogmatil, Delagrange, France) daily for 4 days. This latter group of animals was also treated with pentobarbital (50 mg/kg b.w.) on the 3rd day of treatment with sulpiride. Six hours after the last injection, the animals were anaesthesized with sodium pentobarbital, and their head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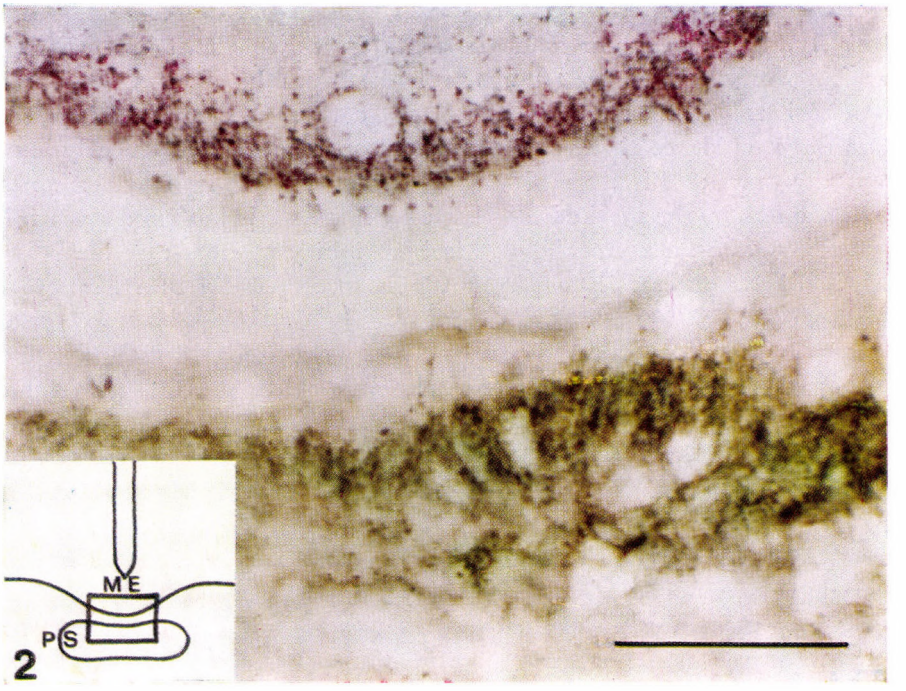
1


ME
PS
2

was perfused through the ascending aorta with 100 ml 0.1 *M* phosphate buffered 0.9% saline (PBS), pH 7.4. The perfusion was then continued with 150 ml of ZAMBONI's fixative [8]. Following perfusion, the head was cut off, the skull opened and placed for an additional 24h period in the same fixative at +4 °C. The fixative was washed from the brain with cold PBS, then the brains were dehydrated in graded alcohol and were embedded in polyethylene glycol under vacuum (Polywachs 1000, Chemische Werke, Hüls). To obtain fast penetration of the different materials used during fixation and dehydration, the brains were continuously moved with an orbital shaker at +4 °C.

For staining, 10-μm-thick sections were cut with the use of a rotary microtome. To remove the embedding medium, the sections were floated on PBS overnight. Then the sections were floated on a few drops of the LH—RH and somatostatin antibodies placed on glass slides, and were incubated in a wet chamber at +4 °C for 24—28 h. The tissue was incubated with goat anti-rabbit γ-globulin bridging antibody at a 1:50 dilution for 1 h (Antibodies Incorporated, Davis, California). Peroxidase-anti-peroxidase complex (PAP; Dako immunoglobulins) used in 1:50 dilution for 1 h served as the enzyme label in the staining procedure.

The specific antiserum to LH—RH, No 422, was raised in a rabbit using synthetic LH—RH absorbed on polyvinylpyrrholidone as antigen [1]. The antiserum was diluted 1:1000 with PBS containing 1% lamb serum (Grand Island Biological Company) and 0.1% sodium azide (Merck). The specific anti-GH—RIH, serum No. 103, was generated against human $\alpha\beta$ globulin-conjugated somatostatin in rabbit [2], and diluted 1:500 with the same diluent.

The specifically-bound enzyme was detected histochemically with 0.005% hydrogen peroxide as substrate, and α-naphthol-pyronine for the first, and 3,3'-diaminobenzidine for the second antigen to be localized, as capturing agents. Following the detection of the first hypophysiotrophic hormone, the antibody complexes bound to the antigen determinant sites of the tissue during the preceding incubations were dissociated and washed out by floating the sections on distilled water adjusted to pH 2.0 with HCl, for 2 h. The best staining was obtained if LH—RH-containing structures were detected first with α-naphthol-pyronine, and somatostatin-containing nerve fibres were localized

Fig. 1. Portion of the anterior median eminence (ME) of a female rat treated with reserpine and stained simultaneously for LH—RH (pink colour) and somatostatin (brown colour). Note the purity of colours over individual fibres. The enframed area in the schematic drawing is shown in the coloured photomicrograph. Scale: 50 μm

Fig. 2. Portion of the posterior median eminence (ME) and the pituitary stalk (PS) already detached from the base of the hypothalamus. Note that the posterior part of the ME contains almost exclusively LH—RH fibres, while the stalk is virtually lacking this hormone. The enframed area in the schematic drawing is shown in the coloured photomicrograph. Scale: 50 μm

later with 3,3'-diaminobenzidine. This way, LH—RH fibres appeared in pink, somatostatin containing structures in brown colour.

The results of our attempt to localize LH—RH and somatostatin simultaneously are demonstrated in Figs 1 and 2. Distribution of these hypophysiotrophic hormones in the double-stained sections was the same as in sections stained only for one of the hormones. LH—RH fibres appeared in clear pink-purple colour, while somatostatin-containing neuronal elements exhibited pure brown colour. Not a single fibre was spotted suggesting mixed colours as a consequence of binding both anti-LH—RH and anti-somatostatin. Even in areas where the two fibre systems are extensively intermingled, each individual fibre profile showed either clear pink or clear brown colour (Fig. 1).

Our study clearly demonstrates that LH—RH and somatostatin are present in two anatomically distinct neuronal systems in the rat brain, and no fibres containing both neurohormones do exist. The finding that all neuronal profiles stained during the two consecutive staining procedures showed clearly the colour of one of the reaction products also demonstrates the specificity of the antibodies used in the present study and in many of our earlier studies.

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GENETICAL ANALYSIS OF TONIC IMMOBILITY IN TWO SUBSPECIES OF *MACROPODUS OPERCULARIS*

SHORT COMMUNICATION

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(Received 1978—05—22)

Tonic immobility (TI) has recently been discussed as a species-specific antipredator behaviour [5]. Its biological function and possible survival value is now being investigated by some authors [2, 6]. Physiological and biochemical processes involved in this behavioural phenomenon are scarcely known (for review see [1]). Few attempts have been made to study genetical influence on TI [3, 4, 7], though data provided by genetical analysis would help to understand better the underlying mechanism of escape behaviour. This paper presents a preliminary experiment on the genetical differences in TI between two subspecies of the paradise fish *(M. o. opercularis* and *M. o. concolor).*

Subjects

Our two laboratory strains: "Y" *(M. o. opercularis)* and "F" *(M. o. concolor)* were chosen as parent populations (P_1 and P_2, respectively). The strains (both originated from a dealer in Florida, Gossington, USA) were bred in our laboratory by sibmating: at the time of the experiment strain "Y" (P_1) was at the 8th, and strain "F" (P_2) at the 6th generation of inbreeding. The two parent populations ($N_{P_1} = 21$ and $N_{P_2} = 28$ fish), their hybrids ($N_{F_1} = 27$ fish) and the two back-cross populations ($N_{B_1} = 26$ and $N_{B_2} = 39$ fish) were used for the experiment. The fish were kept in 60 l homing tanks, then moved into 4 l individual tanks one day before measurement.

Apparatus and Procedure

A simple glass cylinder, 50 cm high (d = 6.5 cm), was filled with day-old tap water, which was changed before each measurement. The outer part of the glass was marked by blue circles at heights of 12.5, 25, and 37.5 cm. The apparatus was illuminated by diffuse light. When the fish were put into the water one by one, they sink within 3—10 seconds to the bottom, where they generally stayed immobile in unusual postures (body parallel with the

bottom, and fins fully erected). Motion of the eyes could be observed during TI. TI was often interrupted for a few seconds by short intensive tail beats, followed by another period of immobility. This sequence was repeated several times until there was a return to normal motions, defined in this experiment as rising to 15 cm in height. The duration of TI here refers to the whole period until normal swimming was resumed. During swimming locomotor activity was measured by the number of circles the fish passed. Number of air gulps was also recorded for the entire 10 min of the test.

Results and Discussion

Mean values of locomotor activity and number of air gulps for each population are shown in Table 1. The hybrids exceeded the parents in both

Table 1

Mean values of locomotor activity and number of air gulp

Locomotor activity		Number of air gulp	
P_1 $\bar{x} = 220$	P_2 $\bar{x} = 103$	P_1 $\bar{x} = 19$	P_2 $\bar{x} = 5$
$s = 64$	$s = 84$	$s = 5$	$s = 4.5$
F_1 $\bar{x} = 273$		F_1 $\bar{x} = 27$	
$s = 86$		$s = 7$	
B_1 $\bar{x} = 195$	B_2 $\bar{x} = 142$	B_1 $\bar{x} = 13$	B_2 $\bar{x} = 10$
$s = 85$	$s = 80$	$s = 7$	$s = 7$

of these two parameters. Heritability and number of allelic differences could not be estimated for locomotor activity and air gulping frequency because transformations of the scale failed to bring about the means and variances fulfilling Mather's two criteria.

Distribution of data of TI is shown in Fig. 1. Applying a logarithmic scale transformation, Mather's first criterion was fulfilled. Variances of the data of the parent populations remained still significantly different ($F = 6.48$, $P < 0.001$). *Macropodus o. opercularis* (P_1) had a high dominance ($D = 0.70$) in TI over *M. o. concolor* (P_2). Heritabilities (H^2) of TI in this experiment were 31% and 33% calculated from the variances of the transformed data of the first and second back-cross populations, respectively. The minimal number of gene differences (k) between the two strains was estimated as 0.06 from the data of the first back-cross (toward the dominant parent) population, and as

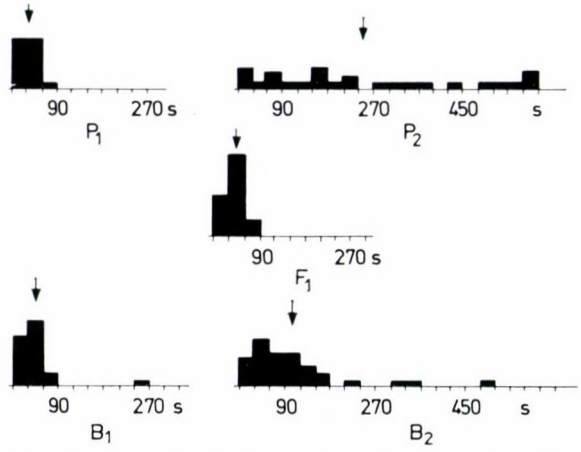

Fig. 1. Distributions of tonic immobility durations of each population

1.78 from the second back-cross (toward the recessive parent) population. The average number of k being $0.91 \approx 1$ is a rough estimation of the number of major genes responsible for the differences in this behavioural phenomenon between the two subspecies. Our results suggest that the genetical factors influencing the duration of TI in our test situation are not very complex.

Table 2

Duration of tonic immobility

Original data (sec)		Transformed data (ln sec)	
P_1 $\bar{x} = 33$	P_2 $\bar{x} = 251$	P_1 $\bar{x} = 3.4$	P_2 $x = 5.1$
$s = 13$	$s = 199$	$s^2 = 0.20$	$s^2 = 1.35$
$N = 21$	$N = 28$		
F_1 $\bar{x} = 41$		F_1 $\bar{x} = 3.65$	
$s = 15$		$s^2 = 0.14$	
$N = 27$			
B_1 $\bar{x} = 43$	B_2 $\bar{x} = 101$	B_1 $\bar{x} = 3.4$	B_2 $x = 4.2$
$s = 46$	$s = 99$	$s^2 = 0.81$	$s^2 = 0.85$
$N = 26$	$N = 39$		

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STEP-OUT LATENCY MEASUREMENTS USING CLONAL GOLDFISH

SHORT COMMUNICATION

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(Received 1978–05–22)

This communication presents preliminary experiments on triploid *Carassius auratus gibelio* clones to demonstrate the advantages of using these isogenic populations in physiological and behavioural studies. *C. auratus gibelio* occurs in nature in two forms: the usual bisexual diploid form (wild type of the common goldfish), and the gynogenetic unisexual triploid form. Both forms occur from the Asian part of the USSR to countries of Eastern Europe, the diploid form being rarer in the west.

While diploid *C. auratus gibelio* propagates by normal heterosexual reproduction, the way of reproduction of the triploid form is natural gynogenesis [1], that is, the penetration of the sperm is necessary for normal embryogenesis, but the head of the sperm does not transform into male pronucleus [3], therefore the genetical information of the male does not participate in the development of the embryo [2]. Gynogenetic offspring of triploid *C. auratus gibelio* are all females and identical to the mother, constituting a clone.

The employment of genetically identical individuals in behavioural studies allows to estimate the environmental and genetic influence on behaviour. As an example step-out latency was measured.

One pair diploid *C. auratus gibelio* (originated from a river in the Amur basin)* and one female of the triploid form (originated from river Danube at Ercsi, Hungary) were artifically propagated in our laboratory. The eggs of the triploid female were inseminated by carp *(Cyprinus carpio)* sperm. Progenies were raised up in our laboratory and individuals reaching 10 cm in length were used for the experiments.

The test apparatus consisted of a 15×15 cm plexiglass box with one guillotine door controlled by a photocell and an automatic counter, and was situated in a 40×40 cm tank (water level: 10 cm; temperature: 24 ± 1 °C; permanent filtration). The experimental set-up was placed in a diffusely-illuminated room. The animals were put in the box one by one, and after a

* Kindly provided by N. D. TCHERFAS.

5-min adaptation period the gate was automatically opened, and recovery time in seconds was measured until the fish passed by the photocell (step-out latency: SL).

The fish were maintained differently prior to the three experiments.

Exp. 1. Fish were kept in complete isolation in 4-l tanks for one week prior to the measurement.

Exp. 2. Two groups of 30 fish were kept in 60-l tanks for one week before measurement.

Exp. 3. Fish kept as in exp. 2, but were disturbed by intensive netting 2 h before measurement, *viz* removed from their home tanks and put into similar ones. (During exp. 2 and 3 only two fish were removed for the test in each day session.)

Environmental conditions have a great influence on SL. There is a relationship between the degree of environmental disturbances prior to the test and SL values. A relatively undisturbed environment was provided in the first experiment by one week isolation. The highest values of SL were measured here (Table 1). Grouping of the fishes in exp. 2 resulted in a smaller SL. The lowest mean values of step-out latency were measured in exp. 3, where the intensive netting prior to the test caused the most severe disturbances.

Table 1

	Diploid groups			Triploid groups		
	N	mean	sd	N	mean	sd
Exp. 1	17	1392	2698	10	374	232
Exp. 2	30	338	476	31	177	130
Exp. 3	10	316	540	18	128	76

Standard deviations (sd) of SL of the diploid groups (Table 1) throughout the experiments are relatively small, due to the genetic identity of the gynogenetic *C. auratus gibelio* clones. The variances here may be equated with the environmental source of variance. The higher variances of the heterogeneous diploid population reflect the genetical and environmental sources of variances and the interaction between the two.

The great difference between the variances of SL of the diploid and triploid populations allow us to assume that SL is considerably influenced by genetical factors.

On the basis of our preliminary experiments we think that the use of triploid *Carassius auratus gibelio* clones would be very advantageous in physiological and behavioural studies. Triploid goldfish can be easily propagated

and maintained. Triploidity inhibits any kind of selection, and uniformity of these populations would promote to make the experiments of different laboratories more comparable.

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RECENSIONES

NORMAN MACLEAN: **Haemoglobin**

Edward Arnold (Publishers) Ltd., London (1978), pp. 68, 30 figures, 1 table. Price: £ 1.60.

The booklet has been recently issued as the 93rd volume of the series "Studies in Biology" sponsored by the Institute of Biology, London. The series has efficiently advanced the knowledge of biology and promoted the professional usefulness of biologists. This volume is up to this tradition.

The author compares the importance of haemoglobin to the students of molecular biology and biochemistry with that of the rat to zoologists. This statement is then convincingly justified in the six chapters of the booklet. The pioneer research revealing structure, function, synthesis (and its control), variations, genetics and evolution of haemoglobin is described and its significance in modern biochemistry underlined. One chapter is devoted to the red cell, the vehicle of haemoglobin. Practical understanding and insight is greatly facilitated by the last chapter describing techniques routinely used in haemoglobin studies and three important and most informative experiments from the field of haemoglobin synthesis research. The scope and size of the booklet did not allow to treat abnormal haemoglobins in full detail.

The comments of the two referees: a layman and a professor of haematology may have contributed to the double merit of the volume: it comprises correct relevant up-to-date information and makes it easily understandable and readable.

ILMA SZÁSZ (Budapest)

LURIYA, E. A.: **Hematopoietic and Lymphoid Tissue in Culture**

Studies in Soviet Science. Consultants Bureau, New York and London, 1977. Division of Plenum Publishing Corporation. Translated from Russian by BASIL HAIGH. XIII + 181 pages, 47 figures, 14 tables. $ 42.

This Monograph, originally published in Russian by the Meditsina Press, Moscow, in 1972 gives a comprehensive survey on research carried out on *in-vitro* culture of haematopoietic and lymphoid tissue in the Soviet Union. In the Introduction the author presents a historical review on the topics and on the development of tissue culture methods. Chapter I deals with different culture methods of haematopoietic tissue and with their values for studying differentiation and gives account of factors influencing differentiation. In Chapter II, the ability of cells from haematopoietic tissue cultures to perform their specific functions after regrafting *in vivo* is discussed. Chapters III and IV are devoted to cultures and immunological functions of lymphoid tissue, including the process of antibody formation and the delayed hypersensitivity reaction *in vitro*. A short chapter deals with cell lines in lymphoid and haematopoietic tissue. A great value of the book is the description of methods completed with schematic drawings. The recent techniques used, and the results obtained by the author and her co-workers are summarized in an Appendix. A list of references, concluded with the year 1976, makes this excellent book more complete and valuable.

I. PÁLYI

A dictionary of microbial taxonomy

S. T. COWAN (Edited by L. R. HILL), Cambridge University Press, Cambridge—London—New York—Melbourne, pp. 285, price £ 12.50. ISBN: 0 521 21890.

Introduced by short chapters on methodological questions 250 pages of the book are devoted to the real dictionary. The words, expressions and idioms included are in the sphere of interest not only of microbial taxonomists, but of a much wider circle of biologists who are not very familiar with the special language of this field. The volume does not contain any taxonomic nomenclature and so the title seems to be somewhat misleading. The selection of entries is in some respect rather unexpected and curious. There are included words which do not bear any microbiological or taxonomical character, like *reprint*, *xerox*, *typography* etc. On the other hand there are missing such words as *alga*, *antisera*, *toxin* and so on.

The great advantage of the dictionary that words and terms which are used in the general literature in different meanings are defined as used in the terminology of microbial taxonomy. This is extremely useful for specialists and nonspecialists as well, and can help very much both in writing reports and understanding special texts.

The book can be recommended for students, teachers, botanists, zoologists and microbiologists and also for those who are not experts in English terms in the field of microbiology.

J. SALÁNKI (Tihany)

MCGARRITY, G. J., MURPHY, D. G. and NICHOLS, W. W.: **Mycoplasma Infection of Cell Cultures.** Plenum Press. New York and London (1978) *(Proceedings of the Institute for Medical Research Workshop on Mycoplasma Infection of Cell Cultures held in Camden, N. J., USA, March 22—23, 1977)*, pp. 342, 62 tables, 28 figures.

Mycoplasma infection of cell cultures represents a great problem in the biological research using cell culture systems. The present book, consisting of 6 chapters, discusses various important aspects of this topic. The contributors are well-known mycoplasmologists and experts of cell-culture systems. Based on their experiences, they give a unique and recent review on basic properties of mycoplasmas, incidence and sources of mycoplasma contamination (Chapter 1), cultural, microscopic, fluorescence, morphological and histochemical, autoradiographic, biochemical (enzyme assay, identification of mycoplasmal RNAs, assay of uptake of nucleic acid precursors) and electron microscopic detection of mycoplasmas in cell cultures (Chapters 2 and 3). Summaries are given on the effect of mycoplasmas on the growth, metabolism, protein synthesis, chromosomes and genes of cell, virus synthesis, virus-interferon induction, on the role of mycoplasmas in relation to aminocentesis, lymphocyte cell cultures as well as phenotypic alteration of cell lines (Chapter 4). The book presents several useful recommendations for effective prevention, control and elimination of mycoplasma infection of cells (Chapter 5). A special bibliography is attached (Chapter 6, on 89 pages) containing all available recent references on mycoplasma infection of cell cultures.

L. STIPKOVICS

Vertebrate Limb and Somite Morphogenesis.

Eds. EDE, D. A., HINCHLIFFE, J. R. and BALLS, M.

The Third Symposium of the British Society for Developmental Biology. Cambridge University Press, Cambridge—London—New York—Melbourne (1977), pp. 498 with several text figures (halftone and drawings) and tables.

The book contains 29 papers from the Society meeting held at the University of Glasgow, Sep. 1—4, 1976. As the editors note in the Preface, the organization of the symposium reflects the rapid growth of interest in limb and somite development. The many good results which have appeared since the Grenoble conference in 1972, seems to fully support the notion of the editors.

The first paper by SAUNDERS sets, as it were, the programme of the symposium. A fine review is given on the role of the apical ectodermal ridge (AER) and of the zone of polarizing activity (ZPA). It is most amazing to read the recent finding that ZPA activity is not confined to postaxial limb mesoderm, but can be obtained from non-limb chick embryo mesoderm grafted

pre-axially. Discrediting his own great experimental discovery made some 10 years ago DR. SAUNDER writes: ". . . it now seems to me that there is no ZPA in the sense that such a zone is a factor in normal limb morphogenesis, . . ." It is almost heartening to read further: "With respect to the AER . . . we can now accept that it has a role in limb morphogenesis. . ." The next six papers give account of experiments in which the effect of ZPA on limb development is studied. The experiments include transplantation (also amniote ZPA into avian hosts), tissue culture and mutant studies; and in the results the specific — non-specific controversies with respect to a morphogenetic signal are reminiscent of the old story on the primary embryonic induction. A considerable number of papers are devoted to tissue differentiation in the developing limb bud. In a formal model relying on morphogen gradients and short-range cell interaction, the simulation of normal and mutant development is attempted. Five papers deal with reptilian and amphibian limb morphogenesis, and the last 5 papers are on somite formation.

The book is a very good collection of the various strands of current limb morphogenesis research. It certainly deserves the interest of biologists and must not be missing from the shelves of embryologists.

G. SZÉKELY (Debrecen)

BRIAN K. HALL: **Chondrogenesis of the somitic mesoderm**

Springer Verlag. Berlin—Heidelberg—New York (1977). *No. 4 of Series 53 of the Advances in Anatomy, Embryology and Cell Biology*, pp. 50, 5 figures, 10 tables. DM 26.

The monograph undertakes the task of reviewing all the aspects of the question of somite-chondrogenesis. First of all, the known details of the development and division of the somite are placed in the frame of recent literature. Then, the results obtained with the usual methods (grafts to the avian chorioallantois membrane, *in vivo* transplantation and extirpation, *in vitro* chondrogenesis) are described and, on this basis, the problem of selfdifferentiation or induction is discussed. The author points out that it is evident that notochord and spinal cord do provide developmental information to the somites *in vivo*. *In vitro*, we are able to bypass the induced, but *in vivo* these tissues are prerequisites for somatic chondrogenesis. Then follows a summary of what is known of the notochord, spinal cord, as well as ectopic (ectoderm, optic epithelium, cartilage) inducers and their mechanism of action on the somites. A great number of data are presented to verify the concept that the inductive activity of the notochord and spinal cord is mediated by products of their extracellular matrices: collagen and glycosaminoglycans. The monograph which is completed by a substantial discussion and detailed bibliography, is the work of an autheútic researcher. It deserves the interest of investigators dealing with the problems of chondrogenesis.

Cs. HADHÁZY (Debrecen)

Stem Cells and Tissue Homeostasis. *British Society for Cell Biology. Symposium 2.*

Ed. LORD, B. I., POTTEN, C. S. and COLE, R. J. Cambridge University Press, Cambridge (1978), pp. 368, 73 figures, 20 tables. £ 18.50.

This book summarizes reviews presented on the Symposium held at the University of Manchaster in April 1977. The 15 papers cover a wide field of cell biology including plant growth, insect development, early mammalian development and problems of human pathology. Nearly one half of the presentations deal with different questions of the physiology and pathology of haemopoiesis. These seemingly divergent topics are uniformly treated from the aspect of cell proliferation and cell differentiation. This trace leads to the problems of genetic diseases and oncogenesis, also briefly touched in some of the reviews. The main aim of this volume is to identify the common properties of the stem cells in all tissues and to consider how these cells behave in the differentiated and developing tissues.

The illustration of this work is outstanding. Most of the figures are clearly constructed diagrams or schematic drawings, however, high quality micrographs are also available. Among these photographs usual light-and phase micrographs, as well as transmission and scanning electron micrographs are to be found.

This volume might be useful for biologists, physiologists, histologists and embryologist, as well as for pathologists and internists especially those, interested in haematology or genetics.

B. MESS (Pécs)

Growth and development . Physique *Symposia Biologica Hungarica 20*

Ed. EIBEN, O. G. Publishing House of the Hungarian Academy of Sciences Budapest, (1977) In English, pp. 498, 128 figures, 96 tables.

The Symposium, organized in Balatonfüred by the Department of Anthropology, Eötvös Loránd University, Budapest, dealt with the problems of growth and development and with the variations of physique. The present volume contains the papers of research workers from 19 countries. The book is divided into two sections. The first section discusses the genetic and environmental factors (climatic, socio-economical, nutritional factors *etc.*) influencing several biological phenomena (intrauterine growth and development, age at menarche, as well ae the so-called "acceleration" of growth and the phenomenon of secular trend). Some papers give a view about growth studies made in different countries, others deal with the clinical and/or physical educational aspects of these investigations (35 papers).

The second section presents 15 papers analysing the variations of physique. Two main methods: somatotyping and factor analysis and their results in clinical and physical educational applications are demonstrated.

The book gives a good summary on modern results concerning these two fields of human biology.

L. HORVÁTH

Isolation of Plant Growth Substances

Ed. J. R. HILLMAN. Cambridge University Press, Cambridge—London—New York—Melbourne (1978), pp. 1—157, figs. 50, £ 4.50.

The articles published in the present volume are based on lectures delivered at a Symposium held in Birmingham in 1977. The Authors of the book were carefully selected from among scientists working in laboratories actively engaged in plant hormone research and applying up-to-date techniques for the isolation, purification, identification and assay of plant growth substances. J. MCDOUGALL and J. R. HILLMAN wrote a chapter on the analysis of indole-3-acetic acid by gas chromatography and mass spectrometry. The spectrophotofluorimetric methods used in the determination of indole-3-acetic acid were described by D. M. A. MOUSDALE, D. N. BUTCHER and R. G. POWELL. The analysis of gibberellins by liquid chromatography was described by D. R. REEVE and A. GOZIER. GASKIN P. and J. MACMILLAN discussed the application of gas chromatography and mass spectrometry in the identification of gibberellins. R. HOGAN presented a survey of the analytical methods used in the detection of cytokinins. A chapter on the identification and quantitative determination of abscisic acid was written by P. F. SAUNDERS. WARD, T. M. M., WRIGHT, J. A. ROBERTS, R. SELF and D. J. OSBORNE described the analytical procedures for the assay and identification of ethylene. The articles reflect the major progress we have been witnessing during the past decade in the development of analytical techniques in plant hormone research. It is evident that the physical-chemical methods are increasingly gaining ground. An extremely valuable aspect of the description of the methods is the critical attitude of the authors and their assistance in interpreting results. This was possible because the book is not a simple "review" of methods described in the literature but rather a laboratory manual based on personal research experience and deep theoretical knowledge of the subject. Scientist both in the basic and applied field, as well as students specializing in plant biochemistry, will greatly benefit from the volume.

G. L. FARKAS (Szeged)

Major Patterns in Vertebrate Evolution

Ed. HECHT, M. K., GOODY, P. C., HECHT, B. M.
Plenum Press, New York—London (1976). *NATO Advanced Study Institutes, Series A (Life Sciences)*. Vol. 14, pp. IX + 908, tabl. 37, figs. 166.

The papers included were delivered at a symposium held at the Royal Holloway College, London, under the sponsorship of the NATO Advanced Study Institutes, July 1976. The purpose of this comprehensive volume is to review some significant advances and relevant theories made in understanding of vertebrate evolution during the last two decades.

The program was divided into three major areas: "(1) the theory and methodology of phyletic inference and approaches to the analysis of macroevolutionary trends as applied to vertebrates; (2) the application of these methodological principles and analytical processes to different groups and structures, particularly in anatomy and paleontology; (3) the application of these results to classification."

The contributions demonstrate a rather wide apply of "non-traditional" methods in character analysis and in reconstructing of phyletic ramifications, *e.g.* macromolecular sequence information (contributions of FITCH, BEINTEMA), karyological evidence (MORESCALCHI), bionic modelling (TEMPLIN, CLARK), clustering methods (LOVTRUP, FERRIS), *etc.* The time-honoured methods of comparative anatomy and embryology (BOCK, DULLEMEYER and BAREL, DELSOL, LEWIS and HOLDER, LUCKETT, XAVIER) and paleontology (SZALAY, SZARSKY, SCHULTZE, PATTERSON, GUTTMANN) are represented by numerous, partly very substantial and well-illustrated, papers.

The theoretical contributions, dealing with general and philosophical aspects of evolutionary theory and cladistic classification (HECHT and EDWARDS, BOCK, KEAST, BONDE *etc.*) lend a particular value to the volume.

There is, therefore, reason to believe that this collection of papers, covering a rather wide subject matter and complementing successfully the — not very numerous ! — recent text-books about transspecific evolution, will be of much value for a considerable range of biologists, interested in evolutionary theory, taxonomy, comparative morphology and embryology, paleontology, biogeography and ecology, *etc.*

Z. VARGA (Debrecen)

Advances in Human Genetics, Vol. 8

Ed. HARRIS, K., HIRSCHHORN, K. Plenum Press, New York—London (1977), pp. 445, figs 75, $ 42.

This new volume of the previously often commended series contains excellent critical review articles which summarize the positions reached in five different areas of human genetics.

Three chapters deal with clinically utilizable subjects. These are comprehensive papers on genetics and aetiology of human cancer (A. G. KNUDSON, Jr.), the human α-amylases (A. D. MERRITT and R. C. KARN) and the genetic aspects of facial abnormalities (R. J. GORLIN and W. S. BOGGS). Figures and text of the latter are in part identical with those of the senior author's wellknown atlas of facial dysmorphology. Two other chapters are concerned with rather theoretical problems, entitled "Population genetics theory in relation to the neutralist-selectionist controversy" (W. J. EWENS) and "Some facts and fancies relating to chromosome structure in man" (H. J. EVANS). Nevertheless, even these theoretical contributions may promote the everyday practice in medical genetics.

Each part is written clearly, illustrated with suggestive pictures and diagrams, and supplemented by a high number of relevant, up-to-date references.

This book will be a real success of contributors, editors and the publisher. It can be recommended for everybody who is interested in the theory and applied practice of the speedy advancing human genetics.

K. MÉHES (Győr)

Limitations and Potentials for Biological Nitrogen Fixation in the Tropics

Ed. DÖBEREINER, J., BURRIS, R. H., HOLLANDER, A. Plenum Publ. Co., New York (1978) *Proceedings of a Conference on Limitations and Potentials for Biological Nitrogen Fixation in the Tropics, Brasilia, Brazil, 1977.*
Basic Life Sciences, **10**, (1978), pp. 413, figs 84, tables 59.

The needs for increasing world food production and the rapid rise in costs of nitrogenous fertilizer have produced a tremendous interest in research to enhance biological nitrogen fixation. This volume, together with the previous one appeared in this series (Genetic Engineering for Nitrogen Fixation, *Basic Life Sci.*, 9, 1977) gives a picture on the present status of various areas in nitrogen fixation research. While in the previous monograph special emphasis was placed on the potentials of genetic engineering in this field, this book deals with many other possibilities to enhance biological nitrogen fixation, especially under tropical climatic and soil conditions.

Essentially four topics are reviewed in detail, namely, nitrogen fixation systems and their contribution to nitrogen balance in different ecosystems, basic metabolic properties of N_2-fixing microorganisms, physiology of these systems, and environmental requirements for free-living and symbiotic nitrogen fixation. These problems are discussed from the point of view of the potentials and limitations for nitrogen fixation in tropical legumes and grasses since the enhancement of biological nitrogen fixation is most needed in the tropical agriculture.

Research data on the application of principles of plant physiology, plant breeding and soil microbiology are also presented. Furthermore, many aspects of the symbiotic association between legumes and *Rhizobium* are reviewed. The book briefly summarizes our current knowledge on the genetics and biochemistry of nitrogen fixation.

The abstracts of short papers and posters presented at the meeting are also published.

This book will undoubtedly be useful for biologists and agricultural specialists and it certainly will help research and development of agriculture not only in the tropical countries.

A. KONDOROSI (Szeged)

SINGH, P.: **Artificial diets for insects, mites, and spiders**
IFI/Plenum, New York (1977), p. XI + 594, 2 figs, 4 tables, $ 90.

Although the first attempts to rear insects on artificial diets go back to the beginning of this century, a real breakthrough occurred in that domain only recently. The fast increasing demand for great numbers of laboratory-reared insects both for basic research (physiology, genetics, ecology) and practical purposes (pesticide screening, biological control, production of pheromones and pathogens, *etc.*) resulted in an explosion of publications dealing with artificial diets during the last two decades. Thus, the author's undertaking has been well-timed.

The nearly 2000 publications abstracted or referred to in the book include all important references. The introductory chapters deal briefly with the historical background, the terminology, the principial factors to be considered in formulating diets, the methods of evaluating diets as well as with some questions of various types of rearings. Five-sixth of the book contains abstracts of publications dealing with specific diets arranged alphabetically within the taxonomic categories. Most abstracts give the composition and the preparation of the diet, the techniques of rearing as well as the rates of growth and development of the arthropod species concerned.

The clear representation of the huge amount of data makes this handbook a most valuable source of information for everybody who is interested in the fast-developing methodology of arthropod rearing.

T. JERMY (Budapest)

HASLAM, SYLVIA M.: **River plants. The macrophytic vegetation of watercourses**

(Illustrated by P. A. WOLSELEY).
Cambridge University Press, Cambridge (1978), I—XII + 1—396 p., 35 tables, 164 figures in the text and 27 photographic plates. £ 27.50.

Running waters provide a fascinating and complex environment for plants and important water resources for man. This book gives the first full-length description of the vegetation of rivers, streams, brooks and artificial canals.

The book opens with an Introduction (Chapter 1), in which some important and fundamental definitions are outlined on the types of river habitats and of types of its inhabiting plants. To help the reader, there are numerous excellent figures including the useful innovation of symbols for the different aquatic plant species.

Chapters 2—6 deal with the physical factors that affect the distribution of plant communities and individual plants species in rivers, streams and other watercourses. The ecology and ecological physiology of river plants and their distribution are considered in the following Chapters 7—10. The vegetation of different water-course types are presented in Chapters 11—14; in the three next Chapters (15—17) the flora and vegetation of North American streams, ditches and canals is demonstrated. In Chapters 18—21 the human use and benefits of river vegetation and the hazards that these plants may present with respect to flooding and

water flow and the ways in which these may be controlled are treated in succession. The last chapter (22) is devoted to pollution in rivers and its effect on plant communities.

There is a glossary and bibliography.

The volume is attractively printed and it is illustrated with eminent, clear and beautiful figures.

In conclusion it may be stated that though the book deals with the flora and vegetation of Britain and North America, it is of more universal interest because of its general ecological principles, methods and practical aspects.

According to Editor's statement, this comprehensive account on river plants has been written for amateur naturalists, amateur and professional botanists, plant ecologists, students taking courses in limnology and hydrobiology, and planners and managers of water resources. The aquatic and shore vegetation plays an important role in works of environmental protection, too.

L. FELFÖLDY (Budapest)

PROCEEDINGS OF THE 4TH INTERNATIONAL CONGRESS OF ACAROLOGY

Ed. by E. Piffl

The volume is divided into twelve sections, each dealing with topics of current interest: General and experimental taxonomy; Physiology and genetics; Soil Acari; Marine and freshwater Acari; Mites associated with plants; Mites of stored products; Parasitic mites; Ticks; Acari and diseases; Biological control, Chemical control and Techniques. In addition, there are four further sections discussed under the following titles: Form and function of acarine mouth parts; Nutrition; Nidicolous mites; Fine structure.

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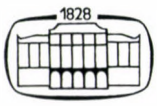

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Lili Fülöp-Aszódi:

ALLOANTIGEN SISTEMS OF HUMAN LEUCOCYTES AND PLATELETS

The monograph deals with the HLA system which is the most polymorphous human genetic system known today. Apart from surveying the specific antigens of leucocytes and platelets, the HLA system is presented in the light of recent results. Moreover, the practical importance of the HLA system in the various fields of medicine is discussed. A review is given of the most important serological and cellular techniques, their performance, value and the possible errors inherent in them. The HLA system has great genetic, immunological and theoretical importance, with an increasing practical significance in a number of fields of medicine.

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IMMUNOLOGY 1978

Proceedings of the Fourth European Immunology Meeting Budapest, Hungary, 12—14 April 1978

Ed by J. GERGELY, G. A. MEDGYESI, S. R. HOLLÁN

This is the first time that the proceedings of a European Immunology Meeting are published. The volume comprises the lectures delivered at the symposia together with the invited talks of the Fourth European Immunology Meeting in Budapest, 12—14 April 1978. Up-to-date information is to be found on the most formidable problems of immunology as well as views of leading authorities on these.

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CONTROL OF CELL PROLIFERATION BY ENDOGENOUS INHIBITORS

In the past fifteen years considerable progress has been made in the research of normal and pathological cell proliferation. Based on an extensive survey of the literature, current information concerning this important field of biology has been systematized, confronted and reconciled by the authors. The results of their own research are also presented. The authors offer a new theory on the control of cell proliferation and oncogenesis.

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NEW TRENDS IN THE DESCRIPTION OF THE GENERAL MECHANISM AND REGULATION OF ENZYMES

Symposium on Enzyme Action, 9-12 July 1978, Debrecen, Hungary

Ed. by S. Damjanovich, P. Előd i and B. Somogyi

(Symposia Biologica Hungarica 21)

The purpose of this volume, based on papers presented at a joint Summer Symposium of the Medical University School of Debrecen and the Biological Division of the Hungarian Academy of Sciences, is to give a representative survey of current research activities in the field of enzyme energetics and regulation. The range of topics covered are as follows:

Subunit catalytic cooperativity; mathematical analysis of heterotropic and homotropic interaction; thermodynamic approach to enzyme action, regulation and evolution; role of thermodanamic fluctuation in enzyme action microenvironment and organized multienzyme systems; evolutionary adaption; contribution of non-active site regions; electronic theory of enzyme action; secondary interactions; adsorptive enzyme systems; role of hydrogen ions.

The contributing authors have all adopted a true multidisciplinary approach to the problems of enzyme catalysis and the participation of the whole enzyme in catalysis.

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Acta biol. Acad. Sci. hung., **29** (4), 311—365 (1978)

REVIEW ARTICLE

ULTRASTRUCTURE OF BIOMEMBRANES AS SHOWN BY TOPO-OPTICAL REACTIONS

Gy. Romhányi

MEDICAL UNIVERSITY, PÉCS

(Received 1978—11—28)

Introduction

In view of the great significance and wide range of functional characteristics of biomembranes in regulating cell functions, there is growing interest in their ultrastructural organization. Several review articles have summarized the knowledge of various structural and functional aspects of biomembranes (Danielli and Dawson, [19], Hendler, [43], Singer [122], Bretscher [10, 11], Bretscher and Raff [12], Robertson [91], Danielli [18], Gulik-Krzywicki [40], Tanford and Reynold [129]). A good overview of the whole field with basic references is provided by the articles in [139].

Among the methods used in studying the ultrastructure of biomembranes, polarization optics, the technique that started submicroscopic morphology through Nageli's micellar theory, are referred to cursorily if at all (Wilkie, [142]). The lack of interest might be attributed to two factors.

First, it has been only recently, after the fundamental contribution of W. J. Schmidt [111, 112, 113, 115], that polarization microscopy attracts an increasing attention in biomorphological studies (Mitchison [77], Inoué [48], Inoué and Hyde [49], Wolman [144], Scheuner and Hutschenreiter [110], Módis [78], Taubert [130], Sato et al. [109]). This is a surprise in view of the theoretically well-founded possibility to detect by polarization optics structural orders at the micellar, molecular or submolecular levels, when the ordered areas span the resolution power of the light microscope. Recently, polarization microscopy has gained also importance as a modern method of quality control of the molecular structural order of polymer industrial products (Hamza and Sikorski, [41]).

Second, biomembranes reveal, as a rule, only weak intrinsic and extrinsic(-form) birefringences, owing to their lipid bilayer and their protein framework, respectively. For this reason, polarization microscopy appeared to provide no practical facilities for the study of the ultrastructure of biomembranes. Nevertheless, some fundamental data about biomembrane ultrastructure have emerged from polarization light analyses, for instance, the order of the mole-

cular chains of the photosensitive pigment retinal, parallel with the plane of the rod outer segment disc-membranes of retina (as shown by the negative dichroism of the outer segments of the frog retina [112]); or the radially-ordered lipid layers in the myelin sheath with concentrically-arranged protein layers [113]. The latter features were recognized in the electron microscope and characterized as the unit membrane structure of biomembranes (ROBERTSON [90], [91]) and the lipid layers of myelin were detected by X-ray diffraction studies (FINEAN [29], BLAUROCK and WORTHINGTON [6]), but the molecular order of the retinal in the disc membranes in the rod outer segments was detected only by polarization optics (SCHMIDT [112], DENTON [20], LIEBMAN [68]).

In most cases, however, biomembranes are not developed to such multilayered patterns as in the rod outer segment or in myelin sheath and therefore their birefringence effects are originally weak and in many cases, for instance in red cell membranes, practically undetectable in their original native state (SCHMITT et al. [116], MITCHISON [77], PONDER and BARRETO [86]).

Nevertheless, there exists a theoretical basis for making the anisotropy of biomembranes — the structural basis of their polarized vectorial functional characteristics [107] — detectable by polarization optics, namely, by oriented binding of dye molecules (or colourless anisodiametric molecules), on the membrane structure. In fact, even weak or highly-masked complex anisotropic structures have been detected in this way, and, by correlating the conformation of the dye molecules with their reacting structural components in biomembranes, a new insight into their structural order has been obtained at the molecular level by polarization-optical methods.

We have been working for several years along these lines and have succeeded in developing such types of oriented binding reactions with some dyes and colourless drug substances, manifested as strong birefringence effects of biomembranes and other biological structures.

We have introduced the term: *topo-optical (staining) reactions* [98, 97] for these optical reactions as the earlier term "*topo-chemical*" (SCHMIDT [115]) has lost its clear meaning after it had become widely applied to all kinds of histochemical reactions without any optical anisotropy effects. The term introduced by us (and taken over in the literature, MÓDIS [78]), however, stresses the topological ("*in situ*") nature as well as the optical anisotropy effects of these reactions.

Topo-optical (staining) reactions thus considerably increase (with or without inversion of the optical character) the originally weak, or in complex structures strongly-masked and often undetectable, birefringences of biological structures caused by oriented binding of dye (or colourless) molecules.

We intend to survey the topo-optical reactions of biomembranes, which may well be of great interest in view of their wide range of applicability in biology, their relatively simple methodology and the new way of ultrastructural

information they can provide. The topo-optical reactions of biomembranes depend either on the presence of the *structural lipids* or react with *RNA* components of the protein framework of the membranes, or interact with *specific groups* induced by chemical reactions in the membrane structure (for instance, carbohydrate- or *RNA*-derived aldehyde groups induced by oxidative pretreatments). Thus, in some cases these reactions may be highly specific with respect to both the underlying texture and the reacting components. The early state of knowledge of "birefringence of cytoplasm and cell membrane" has been reviewed by SWAN and MITCHISON [127], and the progress in this field, achieved mainly by the development and use of topo-optical staining reactions, can be clearly recognized (ROMHÁNYI and DEÁK [100]).

We should like to point out in advance that the introduction of *post-staining stabilization* of ordered dye binding on micellar textures (achieved by post-staining treatment with potassium ferricyanide) has proved to be the decisive step in our approach in developing practicable topo-optical staining reactions of biomembranes and other biological structures.

For lack of space, we do not describe technical details or the theoretical backgrounds of polarization-optical analyses of such reactions. The reader should consult basic works on polarization microscopy (SCHMIDT [111], [114], [115], BENNET [5], SCHEUNER and HUTSCHENREITER [110], MÓDIS [78]) and our papers on these subjects. Basic data on staining methods are included in the *legends*.

Topo-optical staining reactions

I. A. *Topo-optical staining reaction of endoplasmic reticulum (ER) membranes of pancreas, liver and plasma cells with Rivanol-potassium ferricyanide*

Among the *RNA*-containing cytomembranes, it was the endoplasmic reticulum membranes *(ER)* of the pancreatic exocrine cells which, because of their highly-developed multilayered appearance, attracted our attention. We started experiments by using *Rivanol*, a planar diacridine dyestoff (2 : 5-diamino-7-ethoxyacridine, ALBERT, [1]) because in earlier investigations we had found [39] it to precipitate *DNA* from its solutions in the form of strongly-negative birefringent filaments and sheaths (negative with respect to their lenght) indicating, beside the high affinity of these dye molecules to nucleic acids (DE BRUYN et al., [21]), their capacity to react with *DNA* molecules in a highly-ordered structural system, a property not shared by other acridine dyes.

The strong negative birefringence of the Rivanol-precipitated *DNA* filaments suggested that the dye molecules were associated transversally with

their light-absorbing bonds (the planes of the dye molecules) to the nucleic acid chains, possibly parallel to the planes of the nucleotide bases. This suggestion agreed well with the later finding of LERMAN [66] that acridine dye molecules become intercalated between the nucleotide bases, thus causing elongation of the nucleic acid chains in solutions. We made attempts, on the basis of the oriented interaction between Rivanol and *DNA*, to achieve oriented staining reaction of the *ER* membranes of pancreatic exocrine cells with Rivanol; we wished to test whether *RNA* is present in an anisotropic molecular order with respect to the membrane system.

However, in formalin-fixed paraffin sections of dog pancreas stained with Rivanol and sealed in gum arabic or in Canada balsam, we found no signs of topo-optical staining reactivity, *i.e.*, no birefringence effects could be detected on *ER* membranes. When, however, we applied a post-staining treatment of the Rivanol-stained sections with 2% potassium ferricyanide solution, the result was striking, *viz.*, most selective and strong anisotropic effects — pointing to ordered dye binding on the *ER* membranes — were achieved (Fig. 1/a and b).

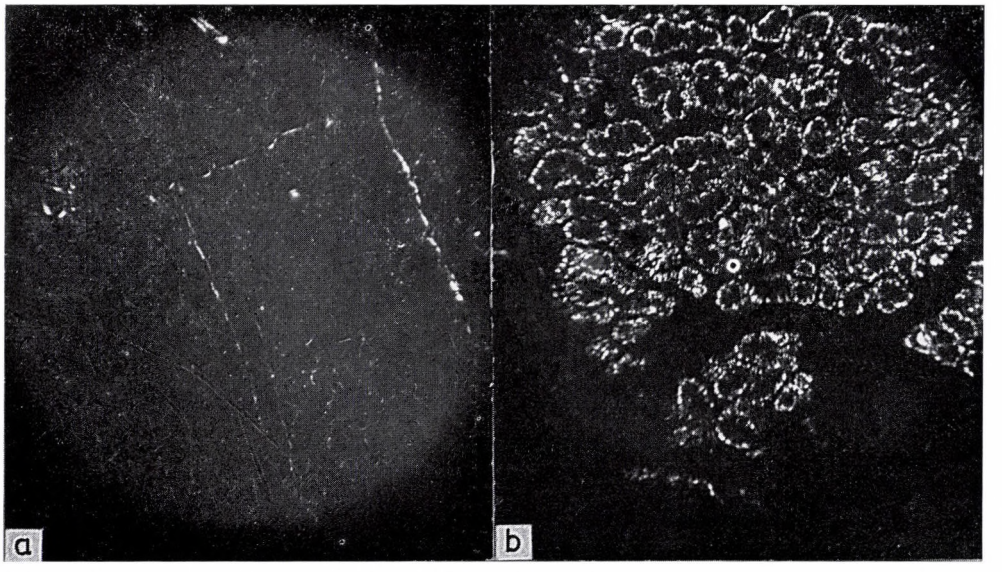

Fig. 1. Dog pancreas. Formol-fixed paraffin section. *(a)* stained with Rivanol, without post-staining stabilization; sealed in gum arabic; *(b)* stained with Rivanol followed by post-staining stabilization with potassium ferricyanide (2%), sealed in gum arabic. *(a)* and *(b)* with crossed polars. ×40. In *(a)*, there is no sign of any Rivanol-induced birefringence; in *(b)*, the acinar structure of the gland is clearly delineated by strong birefringence of the cytoplasm of the acinar cells, induced by oriented dye binding. (Rivanol staining was made for 5 min with 0.1 *M* Rivanol solution being dropped on the slide. After staining, the dye solution was rinsed off shortly with 1—2 ml tap water and the potassium ferricyanide (2%) was dropped on the slide. This stabilizes the oriented dye binding. Then without rinsing, the slide was sealed in gum arabic containing 2% potassium ferricyanide, which was allowed to dry. In this form the slide can be kept permanently or it can be sealed in Canada balsam with cover glass)

The idea to use potassium ferricyanide for post-staining stabilization of the dye molecules on Rivanol-stained structures was taken from the findings published by ALBERT [1], *viz.*, diamino-acridines can be determined quantitatively by precipitating them with potassium ferricyanide or potassium bichromate from their solutions and titrating the residual reagent. The effect of post-staining stabilization with potassium ferricyanide was remarkable and had been fruitfully used as a basic methodological principle in further topo-optical staining reactions developed later in our laboratory.

As shown in Fig. 2/a, the topo-optical staining reaction of pancreatic *ER* membranes with the Rivanol-ferricyanide method is of high selectivity and of considerable intensity. Even the cell nuclei, the *DNA* of which binds Rivanol strongly, appear isotropic, indicating random dye binding (see below). The optical character of the Rivanol-induced birefringence of *ER* membranes was negative with respect to the lenght (the plane) of the membranes, indicating orientation of dye molecules perpendicular with their planes (their light-retarding bonds) to the membranes. Since this staining effect was achieved in paraffin sections, the membrane lipids could not be implicated in this staining

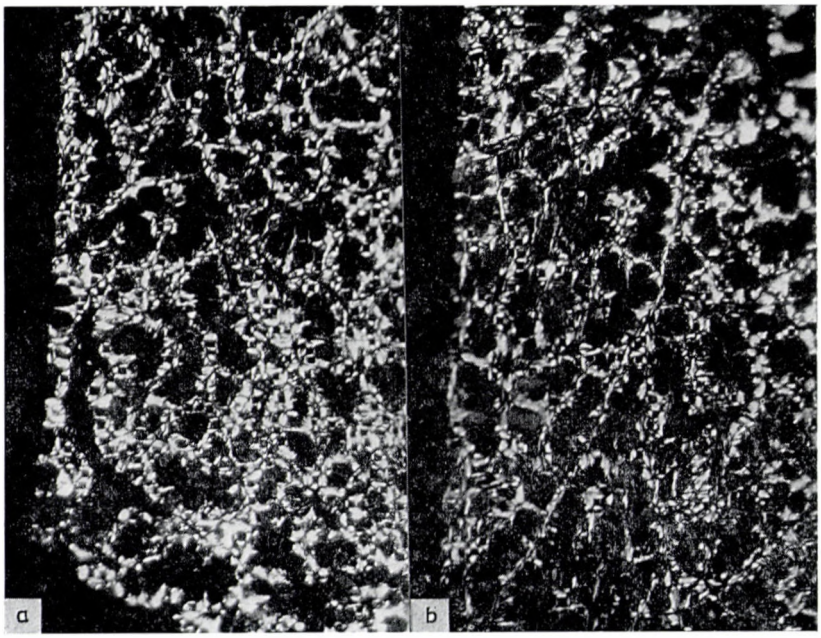

Fig. 2a, b. Dog pancreas. Formol-fixed frozen section. Rivanol-ferricyanide. *(a)* control, *(b)* ethanol-extracted. In *(a)*, there is a strong Rivanol-induced birefringence of the cytoplasmic *ER* membranes with negative character in respect to the length *(i.e.*, the planes) of the *ER* membranes, indicating oriented binding of the dye molecules perpendicular to the membrane plane. Cell nuclei are isotropic and appear as small black areas. Lipid extraction in *(b)* caused only small decrease in the anisotropic staining of the *ER* membranes. (Compare this Fig. with Fig. 9)

reaction (in contrast to the lipid-dependency of the topo-optical staining reaction of the pancreatic *ER* membranes and other biomembranes with the toluidine blue-ferricyanide method, see below). After lipid extraction in frozen sections only a weak decrease was observed in the intensity of the Rivanol reaction of pancreatic *ER* membranes (Fig. 2/b).

RNase pretreatment of frozen sections of pancreas, however, strongly decreased Rivanol-induced birefringence of *ER* membranes, leaving back only the weak intrinsic birefringence of the structural lipids (Fig. 3.). These findings indicated that *RNA* is the substrate of the oriented dye binding in the *ER* membranes and furthermore, that in formol-fixed sections the ribosomal *RNA* unlike the *DNA* of the cell nuclei in formol-fixed tissues, is freely accessible for binding of cationic dyes, in agreement with similar observations on native ribosomes (MIALL and WALKER, [74]). The orientating forces on the

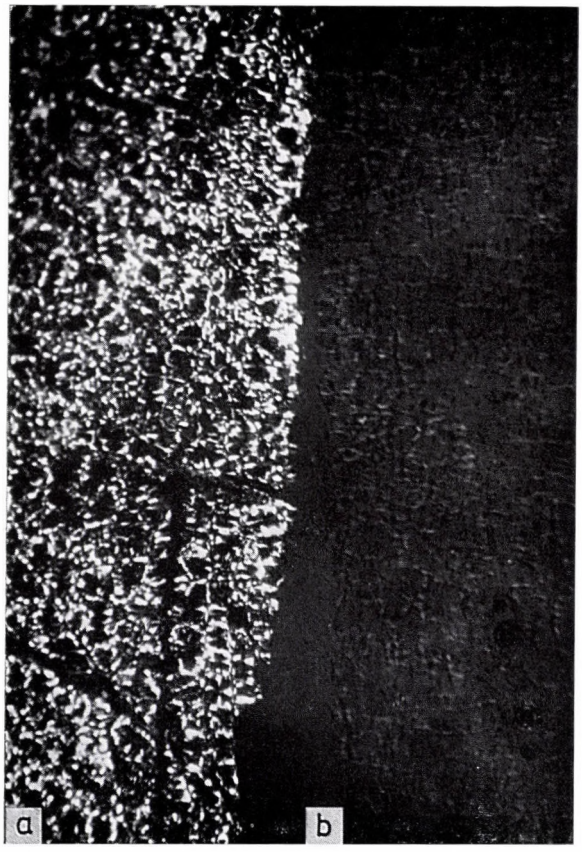

Fig. 3. Dog pancreas. Frozen section. Rivanol-ferricyanide. *(a)* control, *(b)* pretreated with RNase. In *(b)*, Rivanol-induced birefringence is abolished. The residual weak birefringence is due to the intrinsic birefringence of the membrane lipids (negative with respect to the planes of the *ER* membranes)

RNA structure, however, are weak and the dye molecules can be held in an oriented pattern only by the stabilizing effect of potassium ferricyanide. This differs clearly from the staining reactivity of the nuclear *DNA*, which in formol-fixed tissues is inaccessible to oriented binding of Rivanol, since in the chromatin the *DNA* is complexed with histone and for sterical reasons, there is no possibility for oriented, only for random, dye binding. Accordingly, the intrinsic birefringence of chromatin is appreciably less pronounced than that of free *DNA* (HOUSSIER et al., [46]). However, after removal of the histone component by trypsin treatment, we found that nuclear *DNA* was freely accessible for oriented dye binding as shown by the strong Rivanol-induced birefringence of the cell nuclei (ROMHÁNYI, [95]). This indicates that the dye binding of *DNA* is now oriented similarly as in the birefringent filaments precipitated from solutions of *DNA* with Rivanol (GUBA et al. [39]).

Similar results were obtained with the Rivanol-ferricyanide method on the *ER* membranes of liver cells (Fig. 4) and plasma cells in human material,

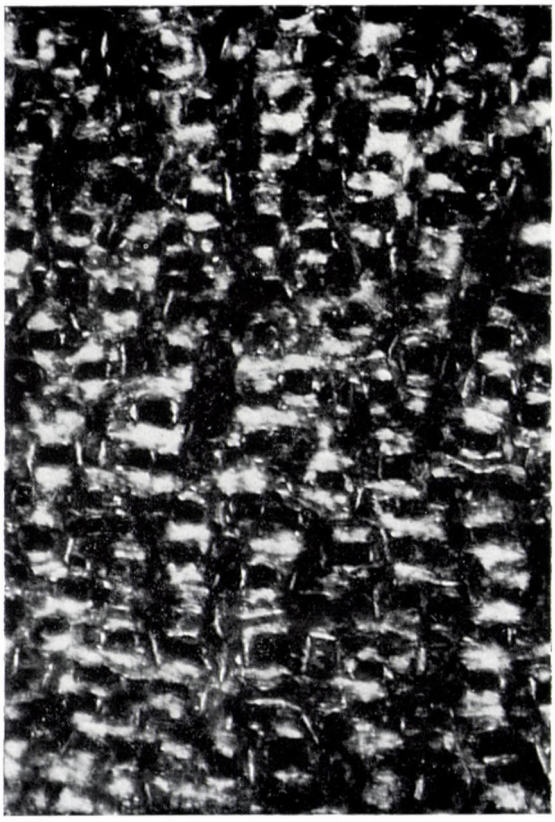

Fig. 4. Rat liver, formol-fixed frozen section. Rivanol ferricyanide. Cytoplasmic *ER* membranes reveal strong negative birefringence negative with respect to their length. Cell nuclei are isotropic. ×360

the latter being known to possess highly-developed *RNA*-containing membranes involved in immunoglobulin synthesis and secretion.

As a principle, oriented dye binding can arise only on substrates with definite underlying molecular order or micellar texture, provided they are accessible *in situ* for oriented dye binding (see the random dye binding of *DNA* in chromatin and oriented dye binding of the free *DNA* of cell nuclei). Consequently, our optical findings on the pancreatic *ER* membranes as well as those on hepatic and plasma cells led us to the conclusion that ribosomal *RNA* is present in a *definite anisotropic molecular order* with respect to the membrane structure serving as the underlying molecular pattern for the oriented dye binding shown by the staining-induced birefringence.

This observation appears of particular interest, since, in spite of intensive studies (KURLAND [63], SHORE and TATA [120]), no definite data are available so far about the molecular order of either ribosomal *RNA in situ* in the *ER* membranes or *in vitro*. In fact, recently a number of studies have been concerned with the molecular structure of ribosome-membrane interaction and of ribosome architecture (BORGESE et al. [8], BLOBEL and DOBBERSTEIN [7], KREIBICH et al. [59]). More recently, it has been reported (KREIBICH et al. [60], [61]) that two specific integral membrane proteins were demonstrated to be present only in the *rough-surfaced ER* membranes (absent in the *smooth microsomal membranes* in the liver cells of the rat) and definitely related to the binding of ribosomes to the membranes, providing specific binding structure for them ("ribosome receptors"). The two proteins, designated as ribophorin I and II, have molecular weights of 65 000 and 63 000, respectively.

It has also been suggested (KREIBICH et al. [61]) that ribophorin I and II are interconnected in the plane of the membrane forming a network in it to serve as a definite underlying pattern organizing the regular attachment and topo-graphical segregation of ribosomes to the membranes in the cell.

Thus, it appears likely that our findings suggesting definite molecular order of ribosomal *RNA* in the *ER* membranes are consistent with those results of molecular biological studies indicating highly-specialized structural conditions for the attachment and topological arrangement of ribosomes on the membranes.

Theoretically, an interpretation of our optical findings on the Rivanol-stained *ER* membranes need two assumptions, namely

(1) linear anchoring of ribosomes to the membrane plane and

(2) anisotropic molecular order of the *RNA* complex in individual ribosomes.

Ad (1) The significance of the linear anchoring of ribosomes to the membranes is evident from the finding that, in cells lacking *ER* membranes and possessing only freely-dispersed ribosomes (ganglion cells), using the same method, we have never obtained any staining-induced birefringence. This find-

ing is understandable since randomly-distributed submicroscopic particles, like ribosomes, cannot induce birefringence effects, not even with a definite internal molecular order.

Ad (2) The second assumption, *viz.*, the presence of a definite anisotropic molecular order of the *RNA* in the individual ribosomes is obvious, since even linear ordering of isotropic submicroscopic particles lacking definite internal molecular array cannot induce ordered dye binding resulting in birefringence.

Our finding suggestive of a definite anisotropic molecular order of the ribosomal *RNA* attached to the *ER* membranes is interesting in view of the very fragmentary knowledge of the architecture of animal and bacterial ribosomes (KURLAND, [63], SHORE and TATA [120]), although some data seem to speak against a complete randomness of ribosomal *RNA* or ribonucleoproteins (COX and BONANOU [17]), and are suggestive of some regular folding of *RNA* namely *(i)* ribosomes from three different origins showed similar X-ray diffraction patterns (LANGRIDGE, [65]); *(ii)* the same temperature absorbance profile of the ribosomal *RNA* and the free *RNA* suggests that the helical segments of *RNA* are not complexed with protein and probably protrude free out of the surface (COTTER et al. [16]); *(iii)*, a part of ribosomal *RNA* was found to be freely accessible to cationic dyes (MIALL and WALKER, [74]); and *(iiii)* mapping of surface ribosomal proteins by immunoelectron microscopy resulted in demonstration of anisodiametric elongated ribosomal protein fractions (TISCHENDORFF et al. [132]).

The anisotropic structure of ribosomal *RNA* in *ER* membranes *in situ*, as detected by the Rivanol reaction, is further supported by the results obtained with the toluidine blue-ferricyanide reaction (see below) and by a newly-developed selective topo-optical staining reaction of *RNA* chains based on oriented dye binding of pyrimidine-derived aldehyde groups of *RNA* (FISCHER, [32]).

I. B. *Specific topo-optical staining reaction of ER membranes based on oriented dye binding of the pyrimidine-derived aldehyde groups of RNA, as shown by permanganate-bisulphite-toluidine blue reaction*

The definite molecular order of the RNA complex *in situ* in the *ER* membranes as shown by the Rivanol-ferricyanide reaction is supported, apart from the similar results obtained by the topo-optical staining reaction with toluidine blue (see below), by the results of the newly-developed topo-optical staining reaction of the *RNA* molecules themselves on the *ER* membranes by the permanganate-bisulphite-toluidine blue-ferricyanide reaction (FISCHER, [32]).

This reaction is based on the strong ER membrane birefringence caused by oriented binding of toluidine blue to aldehyde groups induced by cleavage

of the pyrimidine rings of *RNA* chains. The cleavage is induced by pretreatment with potassium permanganate followed by sodium bisulphite and toluidine blue at pH 1.0 (corresponding to the principle of the periodate-bisulphite-toluidine blue-ferricyanide reaction, the *ABT* reaction, for the detection of carbohydrate chains in tissues, see below).

The birefringence induced by this reaction is negative with respect to the length (the plane) of the *ER* membrane, suggesting membrane-parallel orientation of the reacting *RNA* chains, similarly as with the Rivanol reaction, in which the negative phosphate groups of the *RNA* chains are involved in the dye binding. The Rivanol-ferricyanide reaction, the toluidine blue-ferricyanide reaction, (see below) as well as the permanganate-bisulphite-toluidine blue reaction provide convincing evidence for the anisotropic linear molecular order of the *RNA* complex in the *ER* membranes *in situ*.

Theoretically, some definite molecular order of the ribosomal nucleoproteins would appear in the translational process as a reasonable structural requirement for the regular interactions between *tRNA*, *mRNA* and ribosomal *RNA* on the one hand and the underlying membrane structure on the other. Such a structurally complex process could hardly be imagined to proceed without a well-defined anisotropic order of the reacting components involved, for instance, if the ribosomal *RNA* were buried haphazardly within the depth of a random ribosomal protein framework. We can therefore reasonably consider the molecular order of the *RNA* complex of *ER* membranes *in situ* — shown by topo-optical staining reactions in pancreas, liver and plasma cells, but so far not detected definitely by any other method either *in situ* or *in vitro* — as a significant structural condition for the template-guided synthetic function of the *ER* system.

In the light of these data it would appear of interest to test whether drugs like puromycin known to selectively inhibit the translation process would cause detachment of ribosomes from *ER* membranes and induce ultrastructural changes demonstrable by topo-optical staining reactions in the *ER* membrane system.

I. C. *Topo-optical staining reaction of biomembranes with Rivanol-ferricyanide, not related to their RNA content*

Rivanol, as a cationic dye with two amino groups (Fig. 19) has a great binding capacity to negatively-charged structures, such as *DNA*, *RNA* and *AMP*. It has also affinity, to a lesser degree, to proteins with negatively-charged side groups of carboxylic nature. Above pH 4.0 Rivanol reacts with a wide range of the protein framework in biomembranes, even after structural lipids were extracted. In frozen sections with the membrane lipids present, the Rivanol-ferricyanide reaction provides excellent topo-optical staining reactions

of cell membranes, peritubular and capillary basement membranes, rendering them strongly negative birefringent with respect to their length. After extraction of lipids the reactivity with Rivanol is decreased, but it is not completely abolished (Fig. 2) unlike the toluidine blue reaction of biomembranes (see below). Against this advantage of Rivanol, it has a disadvantage, namely, it lacks absorption in the visible range of the spectrum and therefore Rivanol-stained sections cannot be used for investigation of dichroism (absorption of light polarized parallel or perpendicular with respect to a given micellar texture, as an optical sign of oriented dye binding and, in turn, of the presence of an oriented underlying molecular texture) and, furthermore, Rivanol-stained sections are not suitable for comparison of the same structure of a slide in the bright-field microscope and polarization microscope.

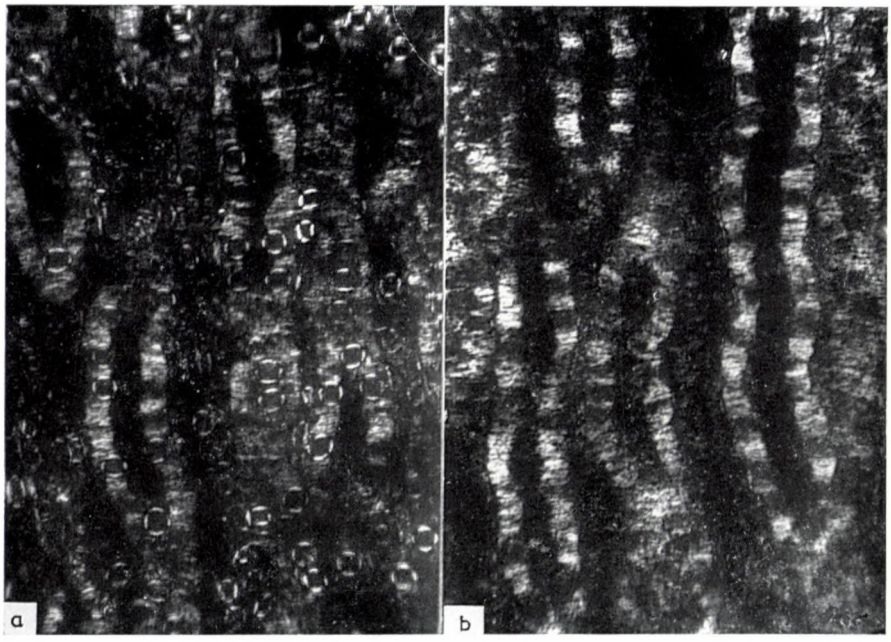

Fig. 5. Paraffin section of human kidney in the region of the thick segments of the Henle loops. Rivanol-ferricyanide; *(a)* control, *(b)* pretreated with RNase. In *(a)*, the cytoplasmic striations, corresponding to the cytomembrane invaginations seen in the electron micrographs, show negative birefringence. In addition, nuclear membranes are rendered birefringent: in the form of radially-positive spherites with radially-oriented slow axis of transmission, indicating Rivanol molecules oriented radially with their planes to the nuclear membrane. (Compare this Fig. with Fig. 16). In *(b)*, the optical reaction of the nuclear membranes is abolished but the birefringence of the cytoplasmic membranes is unchanged. This finding suggests oriented RNA association to the external surface of nuclear membranes, in agreement with electron-microscopic findings (FACETT, [27]) suggesting that the outer leaflet of the nuclear membrane is part of the *ER* membrane system

The negative Rivanol-induced birefringence of the cytomembranes in *(b)* was resistant to RNase treatment. It indicates oriented dye binding perpendicular to the protein framework of these membranes

For this reason, we turned over to the use of toluidine blue, which we found precipitable in the form of negatively-birefringent filaments and sheaths with potassium ferricyanide, and in this way very useful for achieving topo-optical staining reactions by post-staining stabilization, similarly as with Rivanol. Some differences, however, emerged between the staining reactivity of the two dyes due to their differences in molecular conformation (see below).

II. Topo-optical staining reactions of biomembranes with toluidine blue

II. A. *General remarks* (ROMHÁNYI and DEÁK [99])

Toluidine blue is a planar cationic thiazine dyestoff which we found precipitable from its solutions by potassium ferricyanide in the form of birefringent sheaths and negatively-birefringent filaments. Toluidine blue proved therefore useful in achieving, by post-staining stabilization, oriented anisotropic staining reactions of several biological structures characterized by micellar textures of a molecular order possessing negatively-charged dye-binding sites. The characteristics of the topo-optical staining reactions of biomembranes with toluidine blue differed from those with Rivanol in several aspects.

(1) Oriented staining reactions of biomembranes with toluidine blue are completely dependent on *the presence of the membrane lipids*, although the latter are not involved in the dye binding. After lipid extraction, the staining intensity did not decrease but the staining became isotropic, suggesting that the lipid bilayer of the biomembranes plays an indirect key role in the orientation of the dye molecules bound by non-lipid negative side groups of the membrane.

(2) The oriented staining reaction of *RNA*-containing *ER* membranes with toluidine blue is dependent, apart from *RNA* and structural lipids, *on the ionic strenght* of the dye solution, 0.4—0.5 *M* ionic strengths giving the best results. Without adding salts in the needed concentrations, only isotropic overstaining results (Fig 12/a-b).

(3) *ER* membranes of hepatic cells were found to be able to bind toluidine blue in an ordered pattern only in their physiological *reduced functional state*, *i.e.*, *with electrons present* in their electron-transporting chains during their metabolic activities.

(4) The best post-staining stabilization of some biomembranes (renal membranes, capillary basement membranes, red cell membranes) were achieved after toluidine blue staining with a mixture of 2% solutions of potassium ferricyanide and potassium iodide.

II. B. *Topo-optical staining reaction of the pancreatic ER membranes with toluidine blue. Dependency on the presence of the membrane lipids and the ionic strength of the dye solution* (ROMHÁNYI and DEÁK, [97])

Figure 6 shows the overall reaction of the pancreatic *ER* membranes with toluidine blue-ferricyanide in formalin-fixed frozen section at the ionic strength of 0.4 *M* of sodium chloride solution confronted with the unstained control section. The selectivity of the reaction for the *ER* membranes is apparent. Their weak intrinsic birefringence in the unstained section, due to their mem-

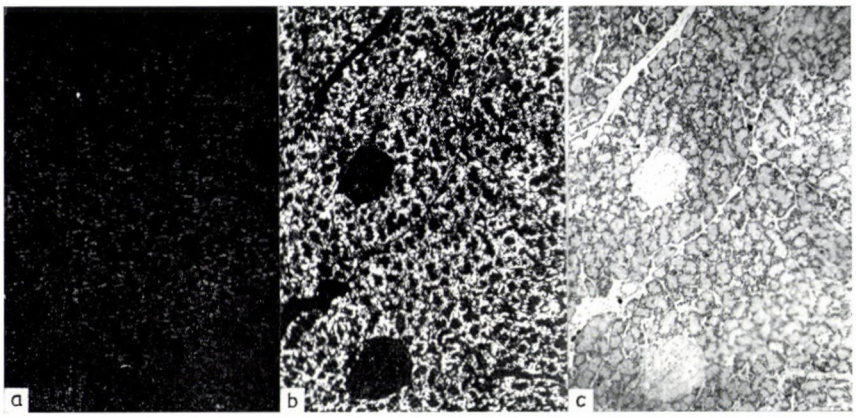

Fig. 6. Rat pancreas. Formol-fixed frozen section. *(a)* unstained control sealed in gum arabic; *(b)* and *(c)* are stained with toluidine blue-ferricyanide, pH 4.0 and 0.4 *M* ionic strength of NaCl of the dye solution for 10 min. In *(a)*, the weak intrinsic birefringence of the *ER* membranes, negative with respect to the length of the membranes, is seen. In *(c)*, there is a selective and intensive basophilic staining of the cytoplasmic *ER* membranes; cell nuclei remain unstained at this ionic strength. In *(b)*, the same field is shown as in *(c)* with crossed polars. There is strong staining-induced birefringence of the cytoplasm of the acinar cells (negative with respect to the plane of the *ER* striations). Islands of Langerhans do not participate in this reaction, ×70. (The staining was made with 0.1% buffered toluidine blue solution with NaCl added to an ionic strength of 0.4—0.5. The dye solution was dropped on the slide. After staining the dye solution was blotted off with filter paper without rinsing. Then potassium ferricyanide solution was dropped on the slide. This was followed, without rinsing, by sealing in gum arabic which was allowed to dry.)

brane-perpendicularly oriented lipid hydrocarbon chains, is strongly increased by the oriented dye binding. The birefringence of the *ER* membranes after toluidine blue staining is again negative with respect to the plane of the membranes, indicating membrane-perpendicular orientation of the dye molecules (their light-absorbing bonds) parallel to the hydrocarbon chains of the membrane lipids. The same conclusion has been reached by investigating the toluidine blue-stained *ER* membranes for dichroism (Fig. 7, 8). Short lipid extraction with ethanol completely abolished oriented dye binding without causing

any decrease in dye binding (Fig. 9). RNase pretreatment (Fig. 10) abolished the topo-optical staining reaction of the *ER* membrane system of the pancreatic acinar tissue. Figure 11 shows the selective topo-optical staining reaction of a plasma cell group in a peripancreatic lymph node centrum. The reaction is due to the strongly-developed *ER* membrane system of plasma cells.

The effect of the *ionic strength of the dye solution* on the topo-optical staining reaction of *RNA*-containing *ER* membranes is remarkable. High ionic strength caused intensive staining of *RNA* in the *ER* membranes, but at the

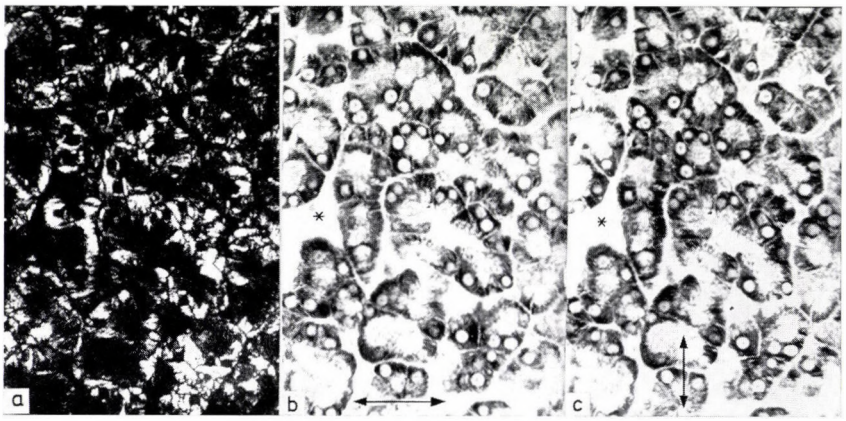

Fig. 7. Rat pancreas. Formol-fixed frozen section. Toluidine blue pH 4.0 at 0.4 ionic strength of NaCl. Stabilization with ferricyanide. *(a)*, *(b)* and *(c)* show the same fields; in *(a)* with crossed polars, in *(b)* and *(c)* in the light microscope. In *(b)* and *(c)*, the pictures were taken with incident light polarized. (The plane of polarization is marked by arrows.) The selective basophilia of *ER* membranes (striations) is apparent. Cell nuclei are unstained. Signs of linear dichroism are clearly seen (asterisks) even at this magnification. In *(a)*, the same field reveals strong staining-induced birefringence of the *ER* membranes

same time completely repressed the staining of nuclear *DNA* (Fig. 12). Initially, we assumed that the salt cations compete with the negative binding sites of *RNA*, preventing thus the overcrowding of the structure and the consequent repression of birefringence. In this connection, however, we have considered the findings of Le Pecqu and Paoletti [67] regarding the special binding characteristics of ethidium bromide to nucleic acids, depending on the salt concentration of the medium. Ethidium bromide, a cationic dye with high affinity for nucleic acids, binds at high (2%) salt concentration exclusively to double-stranded and H-bonded polynucleotide segments of *RNA* and *DNA* in an ordered pattern similar to the intercalated type as found for acridines by Lerman [66]. The difference between the staining reactivity of *RNA* and nuclear *DNA*, depending on the salt concentration *in situ* (Fig 12), may, in our opinion, be related to the fact that nuclear *DNA* is complexed *in situ* in

the chromatin with histone to form *DNP;* the helical segments of ribosomal *RNA*, however, are apparently not complexed with protein and protrude freely out of the ribosomal surface (COTTER et al. [16]).

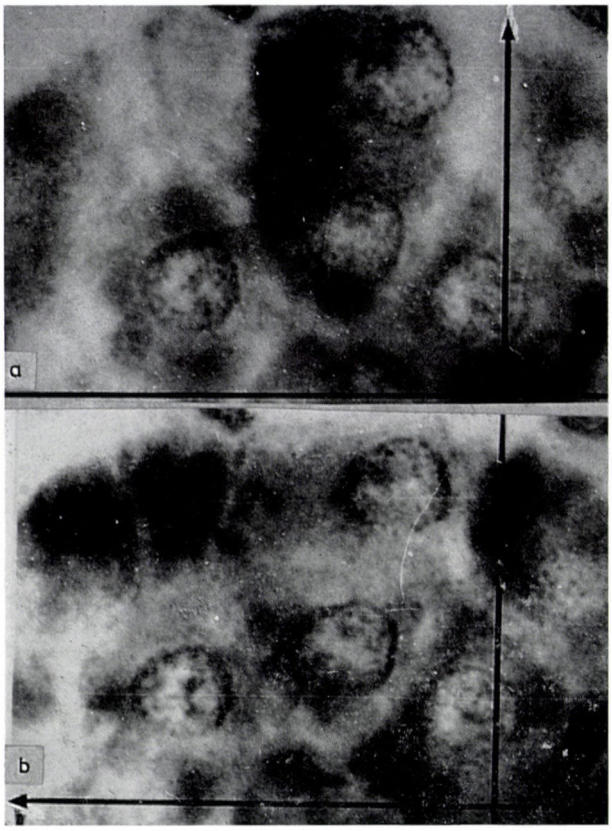

Fig. 8. Rat pancreas. Formol-fixed frozen section. Stained as in *Fig. 7*, demonstrating linear dichroism of the toluidine blue-stained *ER* membranes, ×1200. *(a)* and *(b)* represent the same acinar cell group. The incident light was polarized in two directions (arrows) to detect linear dichroism. (Planar dye molecules absorb only light polarized parallel with their light-absorbing bonds *i.e.* their planes. Therefore, the maximum absorption of polarized light indicates the orientation of dye molecules on structures with oriented dye binding.) In *(a)*, the plane of the polarization of the incident light is perpendicular and *ER* membranes with horizontal striations show maximum absorption. Note that the same area in *(b)* remained unstained. In *(b)*, the incident light is polarized horizontally and the vertical striations of the *ER* membranes show maximum absorption. This same area appears in *(a)* unstained, with minimum absorption. The strong negative linear dichroism of toluidine blue-stained *ER* membranes indicates that the dye molecules are oriented parallel with their planes to the plane of the polarization of the incident light maximally absorbed, *i.e.*, perpendicular to the *ER* membrane lipid bilayers. The same conclusion is reached by the analysis of the toluidine blue-induced birefringence of the *ER* membranes. The negative birefringence with respect to their planes (striations) indicates toluidine blue molecules oriented perpendicularly to the membranes and parallel with the hydrocarbon chains of the lipid layer. (We point to the very simple way of polarization-optical analysis providing information about dye molecule orientation in biological structures being stained and, in an indirect way, about the molecular order of the underlying biological structures.)

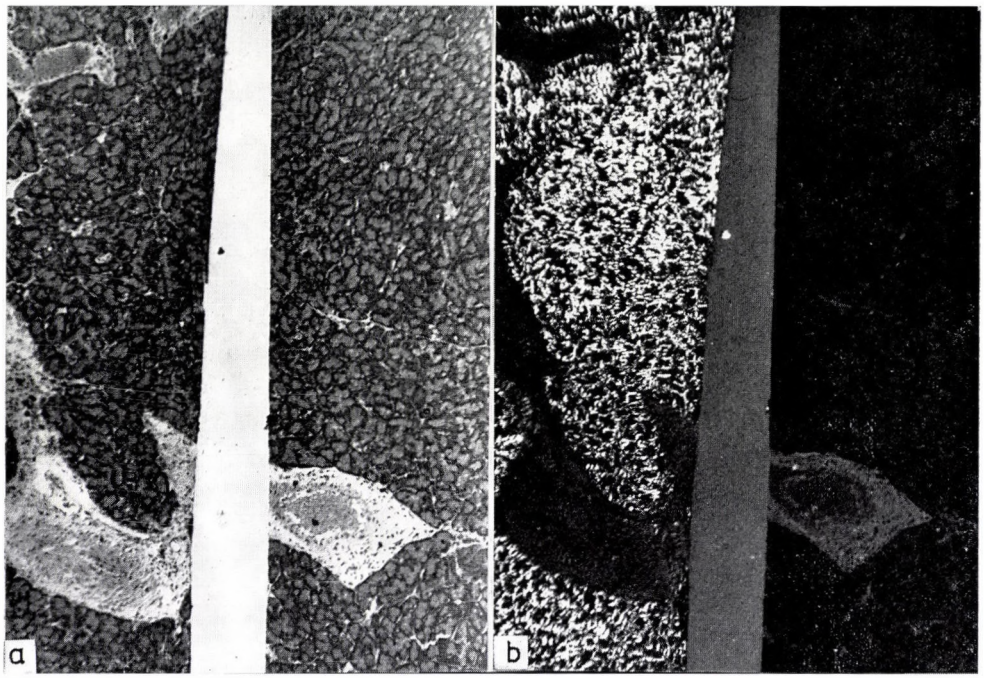

Fig. 9. Rat pancreas frozen section had been dissected into two halves. One (on the left) served as control, the other half was treated with ethanol. Both halves were then stained together on the same slide. Toluidine blue-ferricyanide *(a)* in the light microscope, *(b)* with crossed polars. In *(a)*, after lipid extraction the basophilia of the acinar cell cytoplasm is unaffected but, in spite of this intensive staining, birefringence effect in *(b)* is completely abolished, indicating randomly-distributed dye binding on the lipid-extracted *ER* membranes. This finding points to an essential role of the membrane lipids in the orientation of the dye molecules bound by other, non-lipid, membrane components. In the control half, there is strong basophilia and staining-induced birefringence of the *ER* membranes

Thus, the topo-optical staining reactions of pancreatic *ER* membranes with toluidine blue suggest — as the results obtained with the Rivanol-ferricyanide reaction — a definite anisotropic molecular structure of the ribosomal *RNA*, possibly with membrane-parallel orientation of the helical hydrogen-bonded loops of *RNA*, in near neighbourhood to the membrane surface. Such a structural order would correspond to the finding that membrane lipide played the key role in the orientation of the toluidine blue molecules bound by *RNA* side groups. This appears to need a close neighbourhood-topological correlation between the dye-binding sites and the dye-orientating lipids in the membrane.

The possible functional significance of an anisotropic molecular order of the ribosomal *RNA* complex was discussed above, in connection with the Rivanol reaction.

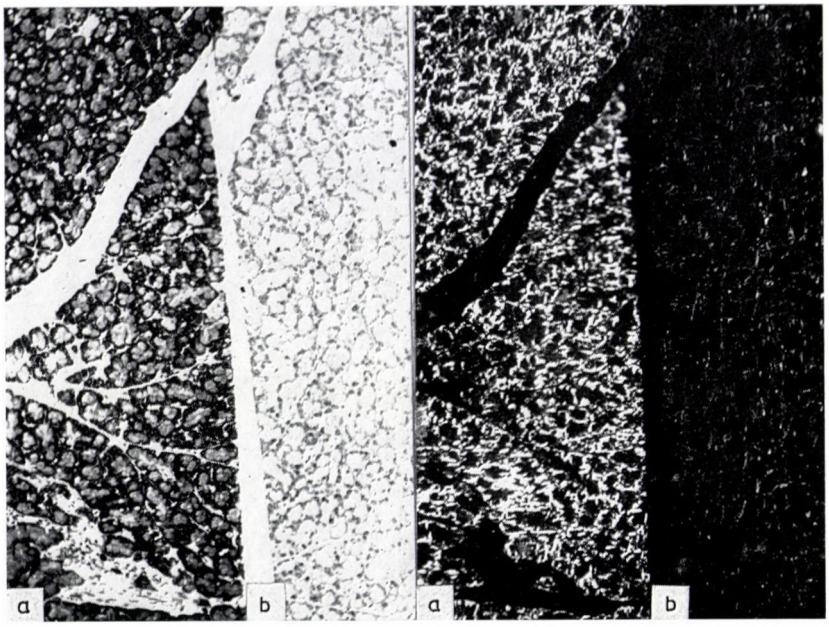

Fig. 10. Rat pancreas. Frozen section dissected into two halves. That on the left served as control, the other half had been treated with RNase. Toluidine blue-ferricyanide. In the control half there is a strong basophilia with strong staining-induced birefringence of the *ER* membranes. In the RNase-treated *(b)* half the basophilia of the acinar tissue is much weaker and there is only weak residual birefringence due to the intrinsic birefringence of the membrane lipids

II. C. *Topo-optical staining reaction of the hepatic ER membranes with toluidine blue. Dependency on the reduced state*

Figure 12 shows the effect of the ionic strength of the dye solution on the staining reaction of the *ER* membranes of rat liver cells. It took a long time until we recognized that the capacity of the *ER* membranes of the liver cells to bind toluidine blue in an ordered pattern depends not only on the ionic strength of the dye solution (as with the *ER* membranes of the pancreatic acinar cells) but also on the physiologically reduced state of the membranes, *i.e.*, the presence of electrons being carried through the electron-transporting chains during metabolic processes in these highly-active integrated enzyme systems of hepatic *ER* membranes. In toluidine blue-ferricyanide stained section of rat liver (Fig. 13) a number of rounded isotropic areas are seen among birefringent areas. This appeared a very uncommon finding, since in the light microscope there was no detectable staining difference between these areas (Fig. 13b).

Only after exclusion of many suspected causes of an artificial change, were we able to clarify that in the isotropic areas with random dye binding

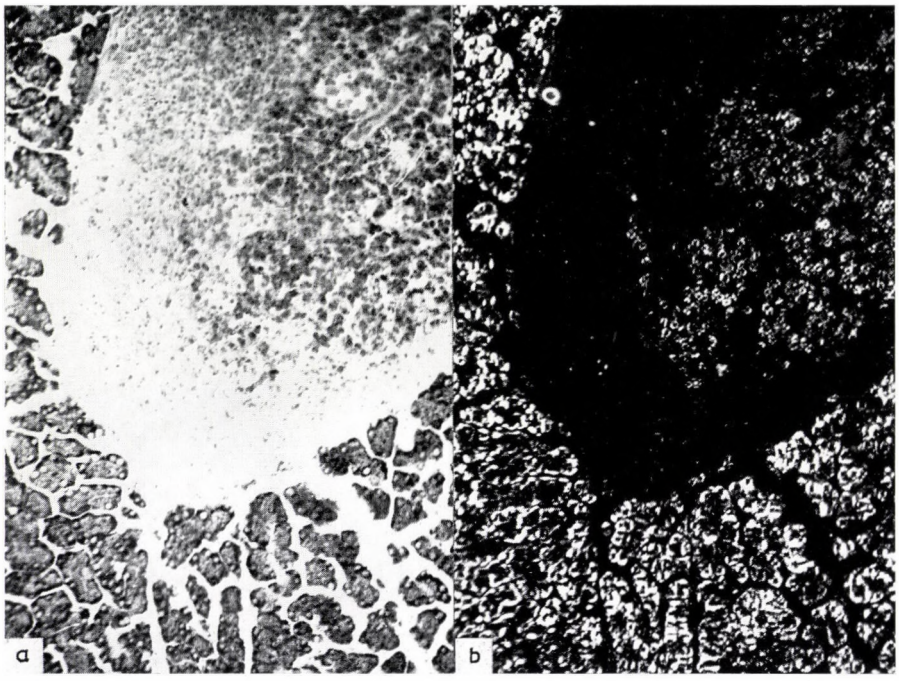

Fig. 11. Rat pancreas. Formol-fixed frozen section. Toluidine blue-ferricyanide. A part of a lymph node is surrounded by pancreatic acinar tissue with intensive birefringence of the *ER* membrane system. In the center of the lymph node a number of plasma cells are seen with intensively basophilic cytoplasm *(a)*, with crossed polars *(b)* the cytoplasm of the plasma cells appears strongly birefringent. This staining reaction is due to the strongly developed *RNA*-containing and protein-synthesizing *ER* membranes in the secretion process of immunoglobulins. In view of the great selectivity of this topo-optical staining reaction for *RNA*-containing membranes, it can be applied to the study of plasmacytic transformations of lymphatic tissues during inflammatory or antigenic stimulations. ×70.

the *ER* membranes have undergone photooxidation during the handling of the specimens and slides at day-light conditions in the laboratory (ROMHÁNYI and DEÁK, [100]). The interpretation of these uncommon findings was reached when we succeeded in restoring the anisotropic staining of the isotropic areas by reduction with sodium dithionite or by treatment with the electron donor chlorpromazine (KARREMAN, ISENBERG and SZENT-GYÖRGYI, [55]).

The photo-oxidative damage was reproduced experimentally in slices irradiated for a short time (5 min) with a small laboratory UV lamp. Irradiated areas showed complete loss of oriented dye binding and appeared isotropic, whereas the protected areas revealed strong toluidine blue-induced birefringence indicating oriented dye binding in these areas. In the bright-field microscope, however, there was no detectable staining difference between the isotropic and birefringent areas. The experimentally-induced photo-oxidation damage could be restored by dithionite or chlorpromazine (Fig. 14). In animals

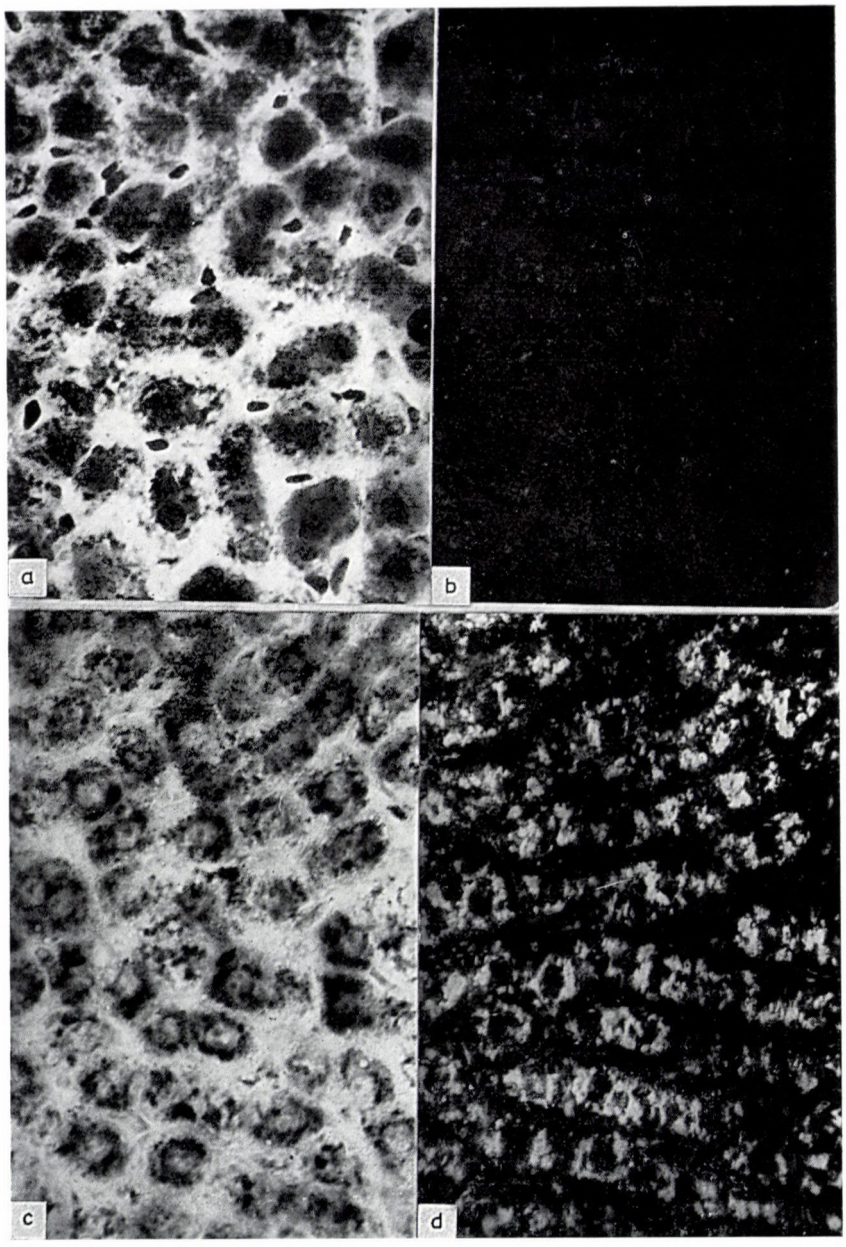

Fig. 12. The effect of the ionic strength of the dye solution on the staining effect of toluidine blue. Rat liver. Formol-fixed frozen section. In *(a)* and *(b)*, staining was made with toluidine blue at low ionic strength (0.01% NaCl) and pH 4.5 In *(c)* and *(d)*, the staining was made with toluidine blue at high ionic strength (0.5 *M* NaCl). There is a great difference between the staining results and birefringence effects of the two dye solutions. At low ionic strength in *(a)*, cell nuclei and cytoplasmic basophilic granules *(ER* membranes) are stained intensively, but with crossed polars *(b)* there is a complete isotropy. At high ionic strength *(c)*, the staining of the nuclei is repressed and in contrast there is an intensive selective staining of the cytoplasmic granules *(ER)* membranes, which with crossed polars (in *d*) reveal strong birefringence negative with respect to the membrane planes, indicating oriented dye binding perpendicular to the plane of the membranes

exhausted by swimming, the hepatic *ER* membranes showed about 50% decrease in their reactivity as calculated from the strength of their toluidine blue-induced birefringence measured electrophotometrically.

An explanation of the molecular mechanism of this dependency of oriented staining reaction of hepatic *ER* membranes on the presence of electrons was only found when we realized that ferricyanide — being used as a post-staining stabilizer in our staining reactions — is itself an electron acceptor and can very likely be assumed to interact with electrons present in the electron-transporting chains of *ER* membranes. This appears to be in accord with biochemical

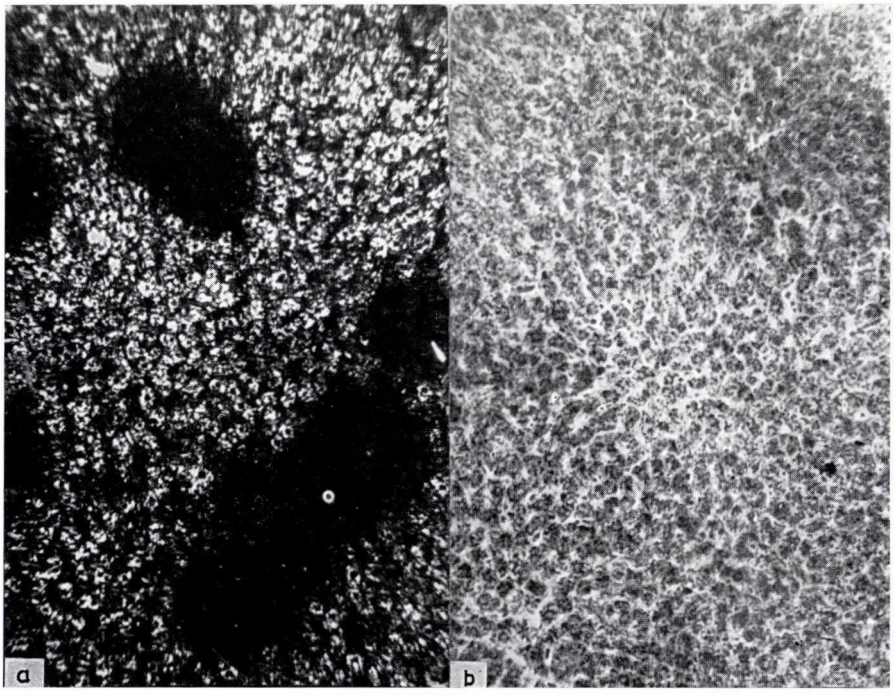

Fig. 13. Rat liver. Formol-fixed frozen section stained with toluidine blue at pH 4.5 and at ionic strength of 0.5 NaCl. Post-staining stabilization was carried out using 1 : 7 mixture of 2% solutions of potassium ferricyanide and potassium iodide; *(a)* with crossed polars, *(b)* the same field in the bright-field microscope. In *(a)*, black rounded isotropic areas are seen between anisotropic areas in which the cytoplasmic *ER* membranes show strong anisotropic staining with strong birefringence. In the isotropic areas the dye molecules, being bound randomly, do not induce birefringence, while in the birefringent areas dye binding is oriented perpendicular to the *ER* membranes, resulting in a strong negative birefringence with respect to the plane(s) of the membranes. In *(b)*, there is however no visible difference in the staining behaviour of the isotropic areas. Later, during our studies, it turned out that in the isotropic areas with random dye binding the *ER* membranes had undergone photo-oxidative damage during handling of the specimens and slides. This was clarified later, when we found that the anisotropic staining could be restored in the isotropic areas by short treatment of the slices with 2% sodium dithionite, ($Na_2S_2O_4$) a reducing agent or by treatment with chlorpromazine which has electron donor capacity (KARREMAN, ISENBERG and SZENT-GYÖRGYI [55]). On the basis of these experiments, we began to use formalin solutions with 0.5% or 1% sodium dithionite addition. This preserved well the physiologically reduced state of the *ER* membranes of the liver

data indicating that at least two electron-transporting chains are located in the hepatic *ER* membranes (DE PIERRE and ERNSTER, [22]), *viz.*, the cytochrome P450 and the cytochrome b5 reductase systems, using electrons from NADH and NADPH, respectively, as electron donors, and being able to reduce ferricyanide. Ferricyanide is often applied as an electron acceptor in biochemical and histochemical systems (SHNITHA and TALABI [121], KERPEL-FRONIUS and HAJÓS [57]).

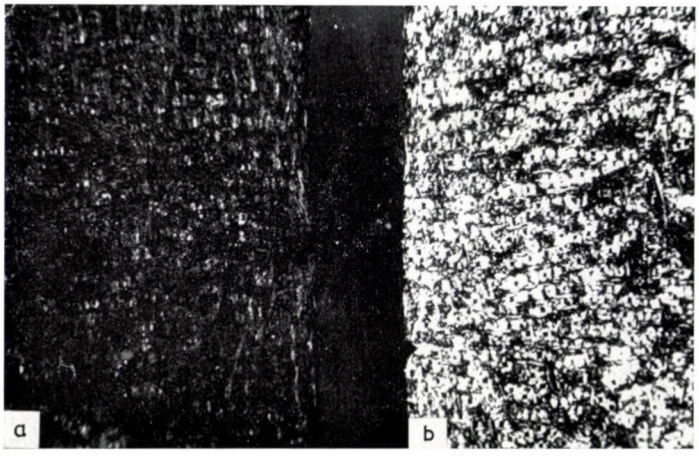

Fig. 14. Rat liver. Frozen section dissected into two halves. Both were photo-inactivated by irradiation with a small laboratory UV lamp for 5 min. *(a)* is the photo-inactivated control, *(b)* had been treated after photo-oxidation with 1% sodium dithionite for 5 min. Both halves were stained simultaneously on the same slide for 5 min with toluidine blue pH 4.5 at the ionic strength of 0.5 NaCl. In *(a)*, there is complete lack of anisotropic staining of the *ER* membranes. In *(b)*, the anisotropic topo-optical staining reaction of the cytoplasmic *ER* membranes is completely restored. The cell nuclei are isotropic and visible as small black rounded holes

Thus, oriented toluidine blue binding of *ER* membranes in liver cells needs fecricyanide molecules interacting with electrons present in the structure, being carried through the electron-transporting chains of *ER* membranes. When, however, ferricyanide molecules, owing to photo-oxidation or metabolic exhaustion, do not find electrons to interact with: then toluidine blue molecules cannot be stabilized in an ordered pattern on the *ER* membrane structure and random distribution of the dye molecules results in isotropy.

The decrease to about 50% of the topo-optical reactivity of the hepatic *ER* membranes in animals exhausted to maximal fatigue can be explained as resulting from transient insufficiency of the electron-transport processes due to metabolic overload.

In any case, the finding that the topo-optical reactivity of hepatic *ER* membranes with toluidine blue depends not only on the presence of membrane lipids and a definite ionic strength of the dye solution but even on the presence

of electrons being transported during metabolic processes, can be regarded as a convincing evidence of the unparalleled capacity of topo-optical staining reactions in providing information about the functional state of biomembranes, not provided by any other morphological methods.

I I. D. *Topo-optical staining reactions of renal membrane systems with toluidin blue-ferricyanide (-potassium iodide)*

Membrane systems in the kidney (brush border, cytomembranes, glomerular and peritubular basement membranes) play a fundamental role in the integrated function of this organ. Ultrastructural studies of the renal membrane systems have therefore gained great significance in histophysiology and histopathology as well. The optical characteristics of renal membrane systems had been studied in unstained sections by ROMHÁNYI [93] and ROLHÄUSER [92].

Topo-optical staining reactions of renal membranes are useful not only because they can detect quantitative changes in some components or their structural disorganization but offer insight not provided by other methods. Therefore, these topo-optical staining reactions are suitable for routine investigations as well.

Since the topo-optical staining reactions of biomembranes with toluidine blue are dependent on the presence of the membrane lipids, only frozen sections can be used for such investigations. Results are shown in Figs 15a—d and 16. Beside the cytomembranes and brush border, the peritubular and glomerular basement membranes are rendered birefringent (negative in character to the length; the brush border being negative with respect to the axis of the microvilli). Lipid extraction rapidly abolished the oriented staining reaction, indicating again the role of the structural lipids in the oriented binding of toluidine blue molecules in biomembranes. Figure 16 shows the result of an incomplete lipid extraction (5 min with ethanol). The nuclear membranes of the thick segments of the Henle loops are more resistant to lipid extraction than other cytomembranes and brush border. (Compare this Figure with Fig. 5.)

Analysis for linear dichroism

When toluidine blue-stained sections are investigated by light microscopy, using polarized incident light, then strong (negative linear) dichroism is observed with greater absorption for light polarized perpendicularly to membrane structures. Figure 17 shows linear dichroism of toluidine blue-stained brush border (being negative with respect to the individual microvilli), indicating toluidine blue binding perpendicular to the axis of the microvilli *i.e.*, parallel to the hydrocarbon chains of the lipids in their surface membrane. Figure

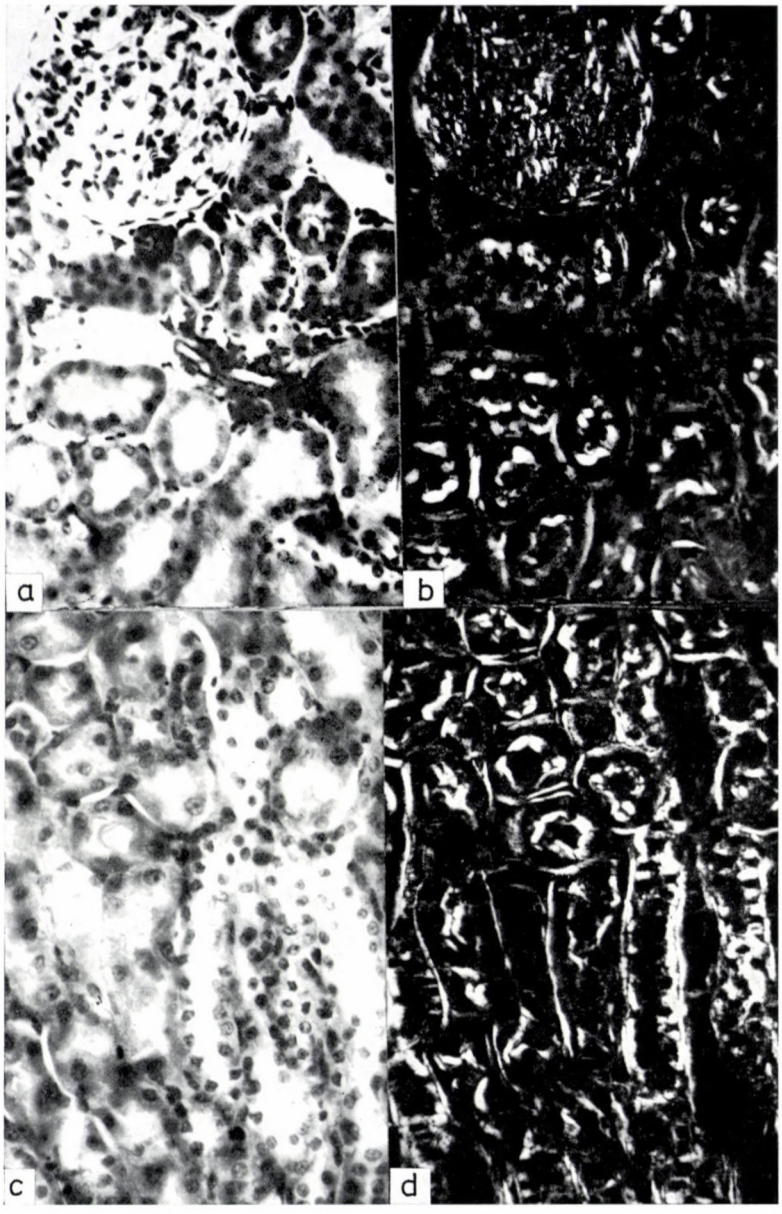

Fig. 15. Rat kidney. Formol-fixed frozen section. Toluidine blue pH 4.5, low ionic strength. Post-staining fixation was made with the potassium ferricyanide-potassium iodide mixture (1 : 7); *(a)* and *(b)* cortical region, *(c)* and *(d)* deeper region of the cortex with thick Henle loop segments. With crossed polars *(b, d)*, unexpected details are made recognizable: the strongly birefringent brush border (invisible in the light microscope), the birefringent cytoplasmic striations in the Henle loops and the birefringent peritubular and glomerular basement membranes. Cell nuclei appear isotropic. This anistotropic staining reaction of the renal membranes was dependent on the presence of the membrane lipids. Lipid extraction with chloroform-methanol, completely abolishing all membrane reactions, did not reduce the staining intensity

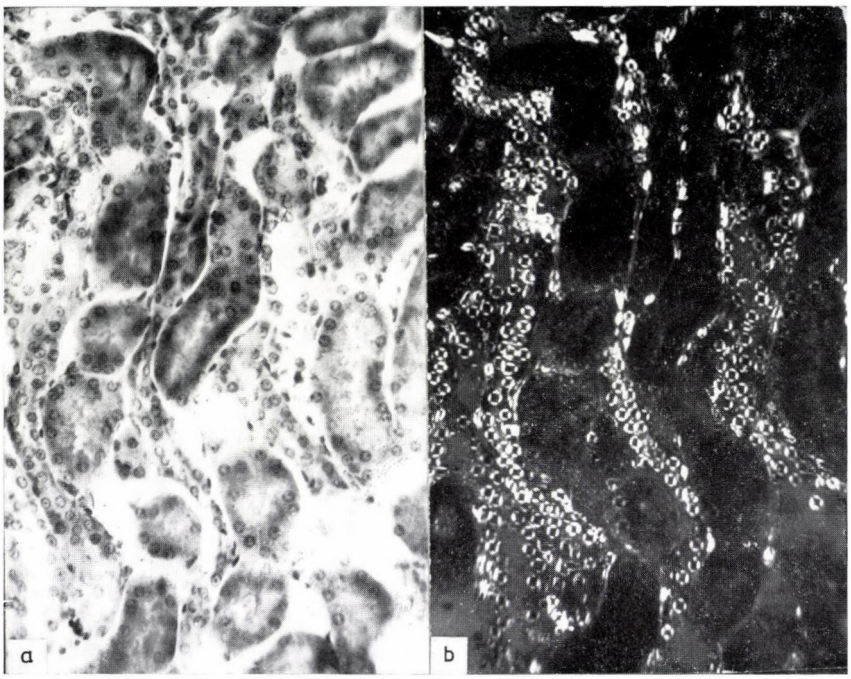

Fig. 16. Rat kidney. Stained as in Fig. 15. Prior to the staining the slide was treated for 5 min with ethanol, which caused partial extraction of the membrane lipids. Only the nuclear membranes of the Henle loops retained their topo-optical reactivity. Birefringence of other cytomembranes, brush border and basement membranes was abolished by this incomplete lipid extraction (Compare this Fig. with Fig. 5 with respect to the *RNA*-coated nuclear membranes of the Henle loops

18 demonstrates the negative linear dichroism with greater absorption for light polarized perpendicularly to the cytomembrane striations, indicating toluidine blue association perpendicular to the cytomembrane infoldings characteristic of the epithelial cells of the Henle loops, seen in the electron microscope.

II. E. *Differences in the capacity for oriented binding of Rivanol and toluidine blue*

We have seen considerable differences in the staining capacity of toluidine blue and Rivanol. Toluidine blue was able to bind in an oriented pattern to biomembranes only when the membrane lipids were present, but Rivanol, although to a lesser degree, induced oriented staining reactions even on lipid-extracted membranes. These differences can be explained in terms of differences in the molecular conformations of the dyes (Fig. 19). Both dye molecules have a planar conformation and are approximately equal in size. Rivanol, as a diamino-acridine dye, however, has two obliquely-situated positive amino-binding groups, whereas the toluidine blue molecule possesses only one amino

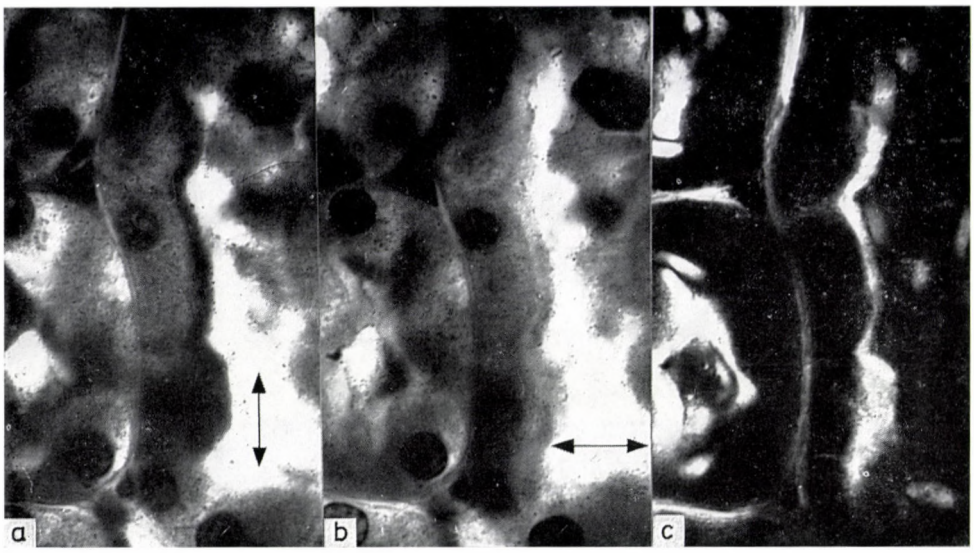

Fig. 17. Rat kidney. Frozen section. Stained as in *Fig.* 13. In order to demonstrate linear dichroism (oriented dye absorption of polarized light due to oriented dye binding) we illuminated the slide in the bright-field microscope with linearly-polarized light. The plane of the polarization is marked by arrows. ×1200.*(a)* and *(b)* in the bright-field microscope, *(c)* the same field with crossed polars. In *(a)* the brush border appears dark, in *(b)*, illuminated with horizontally polarized light, it reveals a considerably lower absorption than in *(a)*. The plane of polarization of the incident light with maximum absorption indicates the orientation of the planes of dye molecules (the planes of their light-absorbing bonds). Thus, we can state that the toluidine blue molecules are oriented perpendicularly to the axis of the microvilli, *i.e.*, they are associated parallel with the membrane-lipid hydrocarbon chains in the membrane. In *(c)*, the brush border appears strongly birefringent (*i.e.* negative with respect to the axis of the microvilli, indicating toluidine blue molecules bound transversally to the axis of the microvilli)

group for binding to negative side groups on substrates. Therefore, Rivanol has a greater binding capacity and a greater stability on substrates than toluidine blue molecules with their single binding groups which require the additional ordering interaction of membrane lipids (beside the stabilization with ferricyanide). This, of course, implies a very close topological interaction between the apolar ring system of toluidine blue and the hydrocarbon chains of the membrane lipids, the orientation being probably brought about by an intercalation of the apolar rings of dye molecules between the fatty acid chains of the membrane lipids in the interior of the membrane. In view of this interaction between toluidine blue and membrane lipids, in turn, one can conclude from the orientation of the toluidine blue molecules, as detected by polarization optics, on the orientation of the hydrocarbon chains of membrane lipids. Such an indirect conclusion is valuable in cases where the intrinsic birefringence of the membrane lipid bilayer is undetectable without topo-optical staining reaction of the membrane, for instance, in the red cell membrane.

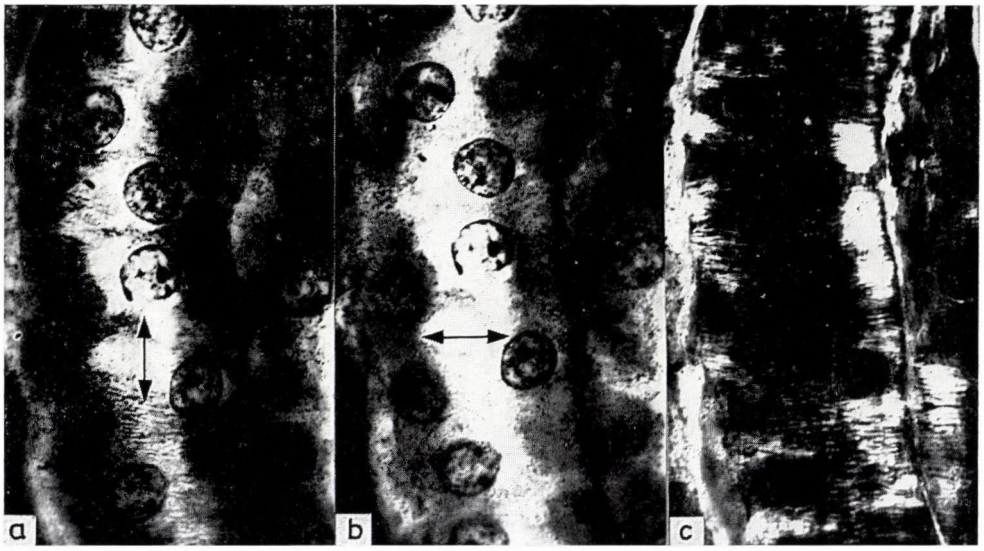

Fig. 18. Rat kidney. Thick segment of Henle loops ×1200. In *(a)* and *(b)*, illuminated in the bright-field microscope with polarized light to detect dichroism as a sign of oriented binding of toluidine blue to the cytomembranes. The planes of polarization of the light are marked by arrows; *(c)* the same field with crossed polars. In *(a)* and *(b)*, the membrane striations of the epithelial cells corresponding to the membrane invaginations in the electron microscope show negative dichroism, *i.e.* greater absorption for light polarized perpendicularly to their length. Correspondingly, in *(a)* the horizontal striations are black and reveal greater absorption as the same striations in *(b)*; clearly seen in the lower part of the figure. In *(c)*, the membrane striations are strongly birefringent (negative with respect to their length). Even the nuclear membranes show a weak birefringence. Both the negative birefringence and the negative dichroism of the cytoplasmic membrane striations indicate that the toluidine blue molecules are arranged transversally in the membrane structure

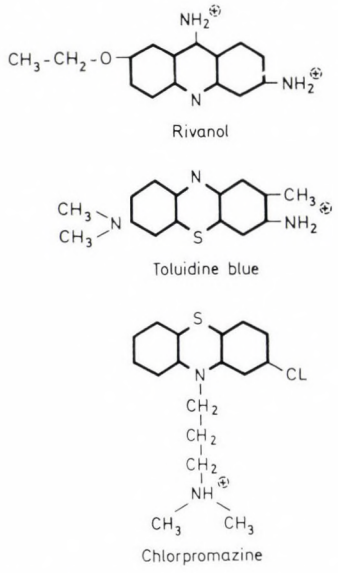

Fig. 19. Differences in the molecular conformation of Rivanol and toluidine blue as the basis of their different capacities to induce topo-optical staining reactions on membranes. Rivanol possesses two obliquely situated positively-charged binding groups, whereas toluidine blue has only one such group

The significance of the localization and number of the positive binding groups of dye molecules for topo-optical reactivity in biomembranes is shown by our failure to induce any practicable topo-optical staining reaction with 5-amino-acridine, a compound structurally very closely related to Rivanol (Fig. 20).

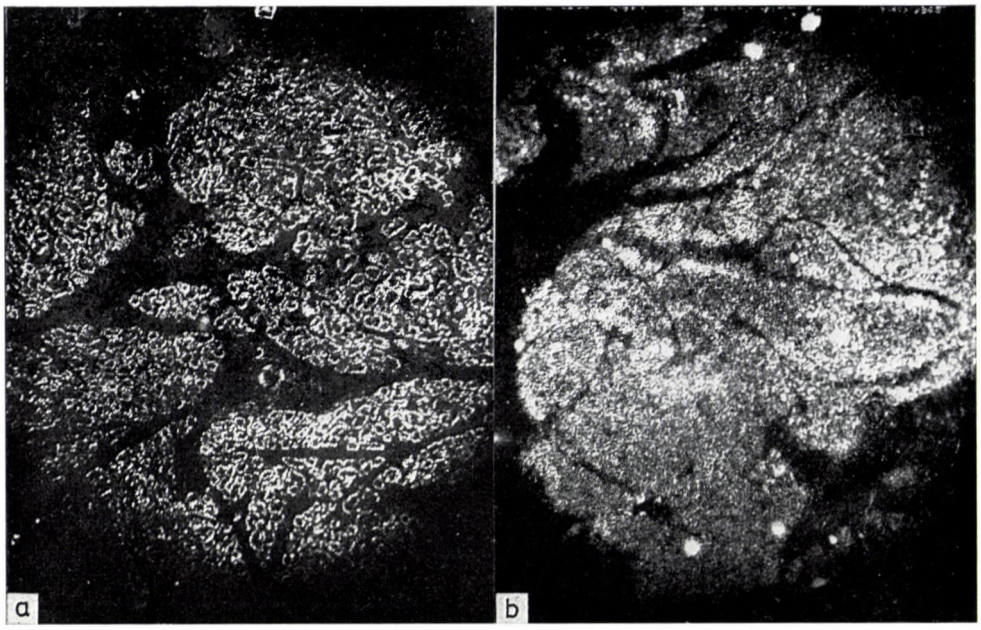

Fig. 20. Rat pancreas. Paraffin section. In *(a)*, stained with Rivanol-ferricyanide, in *(b)*, stained with 5-amino acridine-ferricyanide. In *(a)*, there is a clear topo-optical staining reaction of the *ER* membranes delineating the acinar structure of the gland; in *(b)*, only granular, amorphous staining effect, indicating the role of the dye molecular conformation in topo-optical staining reactivity

III.. A. *Topo-optical staining reactions of blood-cell elements with toluidine blue-ferricyanide-potassium iodide (1 : 7)*

The red blood cell membrane is one of the most intensely investigated biomembranes, since it is easily available in haemoglobin-free form for chemical, physical, functional and structural studies. The interest in red cell membrane biochemistry and ultrastructure (Bretscher [10], Fowler and Branton [35], Jacob [51], Hollán et al. [44], Steck [126]) has ever been stimulated by its possible relation to red cell pathology (Jacob [51], Jamieson [53], Jensen and Lessis [52], Whittman [141] and Ellory and Lev [25], with references of earlier significant publications). These studies have led to suggestions about molecular membrane models of red cells, but details of these models are not reflected in the trilaminar unit membrane structure of the erythrocyte seen in the electron microscope.

Recently, interest has increased particularly in the ultrastructural organization of lymphoid cell membranes and other types of cell membranes in view of their involvement in widespread functional activities, *viz.*, membrane changes related to cell recognition and growth regulation (EDELMAN [24]), cell stimulation or cellular contact (REES et al. [89]), reassociation (TURNER and BURGER, [134]), immunological response and graft rejection (UHR, [135], lectin and toxin binding (NICOLSON, [81]) and changing in ultrastructural organization with malignant transformation (NICOLSON [82], NICOLSON and POSTE, [84], WARREN et al. [136], ZATZ et al. [145]). Biochemical investigations of membrane proteins of lymphoid cells also revealed some differences in their glycoprotein components (TROWBRIDGE et al. [133], HUNT and BROWN [45], ZATZ et al. [145]) in various conditions. However, efforts to detect any ultrastructural difference between cell membranes of these cell types and those of the red cell membrane have failed.

So far, even polarization-optical analyses have failed to demonstrate definite differences in the ultrastructural organization of the haematological cell membranes, probably due to their very weak intrinsic birefringence (MITCHISON [77], PONDER and BARRETO [86], SCHMITT et al. [116]). We therefore were surprised by the strong selective topo-optical staining reaction of the red cell membranes with toluidine blue-ferricyanide-potassium iodide in glutaraldehyde-fixed smears and by the striking difference in the topo-optical reactivity of red cell membranes from those of myeloid, lymphoid and macrophage cell membranes [102] (Fig. 21, 22, 23).

Although we cannot present definite explanation of the molecular-structural basis of these ultrastructural differences between the membrane organization of red cell membranes and that of lympoid myeloid cell elements, these findings justify the use of topo-optical staining reactions in further comparative studies on the ultrastructural organization of different blood cell elements in physiological and pathological research. Figure 21 demonstrates the selective topo-optical staining reaction of red cell membranes in glutaraldehyde-fixed blood smear. The selectivity in the topo-optical reactivity of red cell membranes, differing sharply from that of leucocytes and lymphocytes (Figs 22, 23), is remarkable. With crossed polars, the red cell membranes appear in the form of radially-positive spherites (with radially-oriented slow axis of transmission, *i.e.*, radially-oriented greater refractive index) indicating orientation of dye molecules radially with their light-absorbing bonds *i.e.* their planes. In agreement with this finding, the red cell membranes reveal a strong radially-positive linear dichroism (Fig. 21a) with the greater absorption for light polarized radially. Therefore, two opposite sectors of the red cell membranes show greater absorption while the other two opposite sectors are invisible. This phenomenon is moving with rotating the polarizer used for illumination in the bright-field microscope with polarized light.

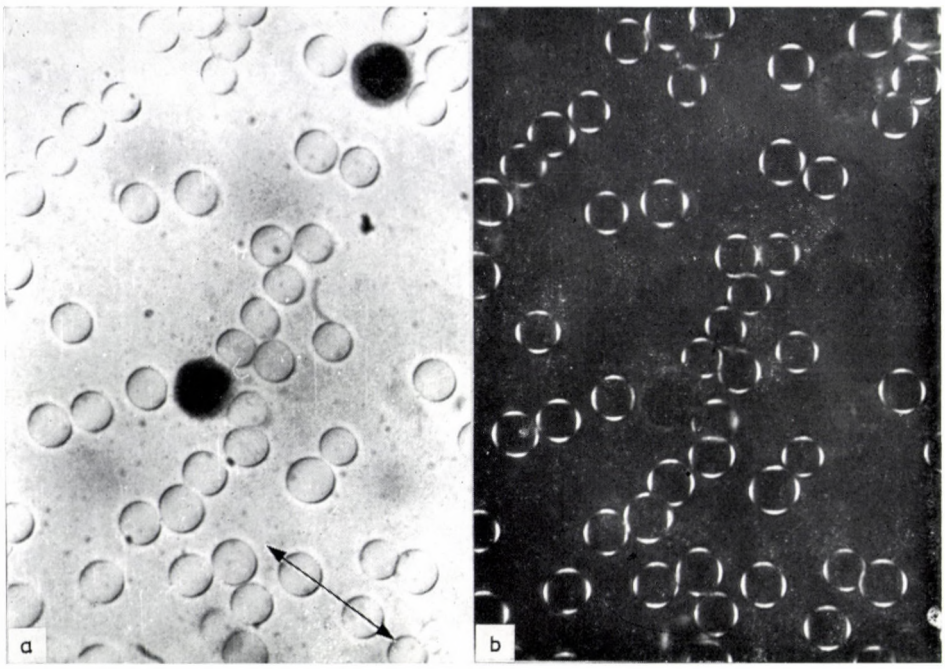

Fig. 21. Blood smear, fixed in isotonic phosphate-sucrose buffer, pH 7.5, for 5 min. Stained with toluidine blue 0.1%, at pH 6.0. Stabilization with post-staining treatment with a mixture of potassium ferricyanide and potassium iodide (1 : 7). Sealed in gumi arabic. (Blood was diluted and washed three times with isotonic sucrose-phosphate buffer of pH 7.5, then after adequate dilution with isotonic sucrose-phosphate buffer smears were made. Smears were fixed after very short drying with 2.5% glutaraldehyde in isotonic sucrose-phosphate buffer. After staining for 5—10 min the dye solution was washed off with the stabilizing mixture and the slide was sealed, without rinsing in water, in gum arabic, which was allowed to dry.) *(a)* bright-field microscope picture made with polarized incident light (marked by the arrow); *(b)* the same field with crossed polars. In *(b)*, the red cell membranes reveal selective strong anisotropy indicating oriented dye binding on the membrane. The birefringence induced by toluidine blue staining was positive with respect to the radius (corresponding to radially positive spherite). The membranes of the two leucocytes in the field reveal very weak mosaic-like birefringence, so that in the polarization-optical picture they can hardly be detected (as compared with their intensive staining seen in *a*). Thus, the leucocyte membranes, unlike erythrocyte membranes, do not bind toluidine blue in an ordered pattern. In *(a)*, linear dichroism of the toluidine blue-stained red blood cell membranes can be clearly recognized. Both sectors of the cell membranes oriented perpendicularly to the plane of the incident polarized light are dark and show greater absorption than the other two sectors, lying parallel with the plane of the polarized incident light which are only faintly visible. Toluidine blue molecules are thus oriented parallel with their planes to the plane of the polarization of the incident light *i.e.*, they are arranged radially in the membrane. Thus, both the radial positive birefringence of the red cell membrane and the radially positive linear dichroism indicate radial orientation of the dye molecules with their planes (their light absorbing bonds) in the membrane structure

Thus, the optical analyses have shown that both the radially-positive birefringence and the radially-positive linear dichroism indicate radial orientation of the dye molecules with their planes in the red cell membrane. The strength of the birefringence of the red cell membranes, with retardations of

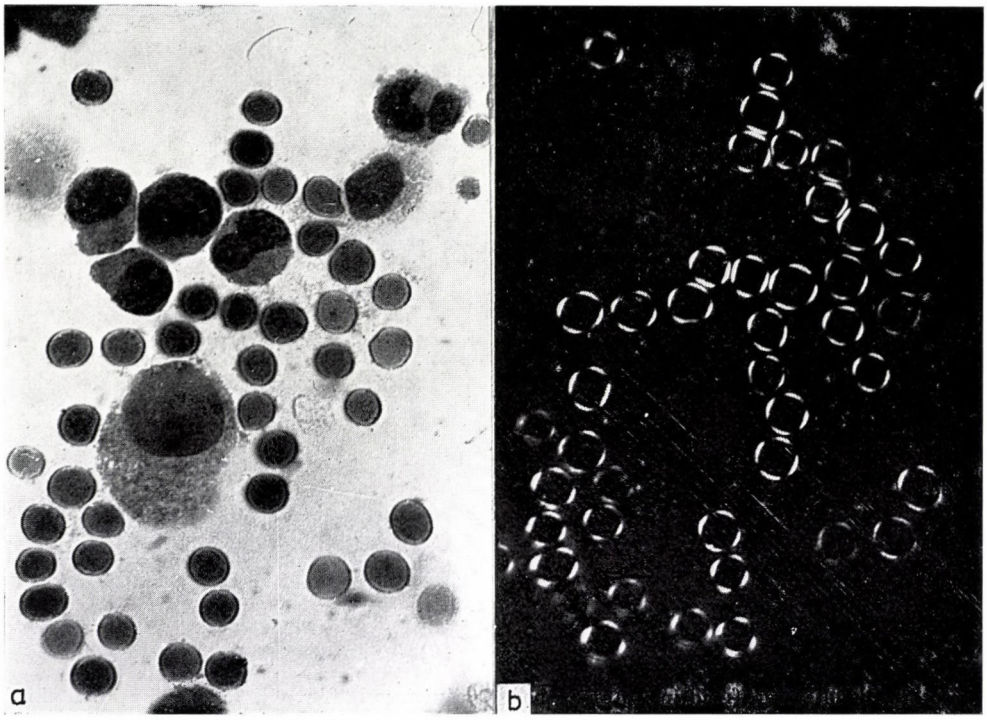

Fig. 22. Bone marrow smear stained as in Fig. 21. Erythrocyte membranes appear strongly birefringent as optically positive spherites. Even the erythroblastic cell elements reveal membrane effects similar to those of mature erythrocytes (here not shown). The membranes of the myeloid cell elements appear quite isotropic. Their membranes suggest themselves only as very faintly lighting granules or lines. ×1200

35—40 nm, is remarkable in view of the originally extremely weak or invisible birefringence of the red cell membrane (MITCHISON [77], PONDER and BARRETO [86]). This suggests a high degree of orientation and density of the coplanarly-oriented toluidine blue molecules within the membrane structure and, in turn, a high order of the hydrocarbon chains of the membrane lipids between which the dye molecules are intercalated and oriented.

This topo-optical staining reaction of the red cell membrane with toluidine blue is similarly dependent on the presence of membrane lipids as found for other biomembranes. Even short extraction of glutaraldehyde-fixed smears with ethanol completely abolished this reaction. Thus, the membrane lipids play a key role in the orientation of the toluidine blue molecules within the red cell membrane. Therefore, the view (MAKOVITZKY and GEYER [69]) that the orientation of the toluidine blue molecules in the red cell membrane depends on surface glycoproteins and not on membrane lipids cannot be accepted as valid; it apparently contradicts all observations on other biomembranes with

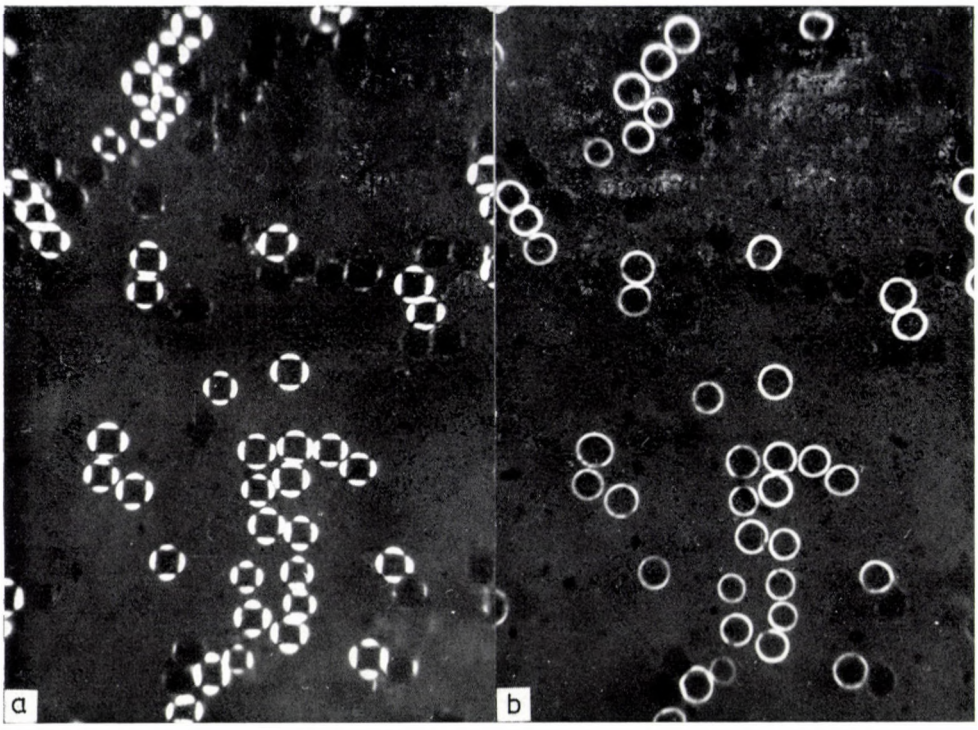

Fig. 23. Human erythrocytes mixed with rat thymus lymphocytes fixed and stained as in Fig. 21. In *(a)*, with crossed polars for linear birefringence and in *(b)* with circular polarization (obtained by interposition of correctly crossed $\lambda/4$ plates between the crossed polars, see SCHNABEL [117]). The red cell membranes appear in *(b)* continuously birefringent and sharply delineated both on their outer and inner surface, indicating that inframembraneous cytoplasmic structures do not contribute to the topo-optical staining reaction of the erythrocyte membrane. The membranes of the lymphocytes reveal only very faint anisotropic effects, invisible with circular polarization

toluidine blue and may be related to methodological problems. Since lipids are not involved in the binding of the dye molecules, non-lipid negative-dye-binding groups must be taken into consideration. These may be either carboxyl groups of the membrane proteins or similar groups of the sialic acid residues of the neuraminic acid of surface glycoproteins, or both. In either case, however, the binding sites must be located adjacent to the orientating lipid layer of the membrane, since at a greater distance between dye-binding sites and membrane lipids no orientating effect would be possible. If, as most probable, the sialic acid groups of surface glycoproteins of red cell membrane are involved with their negative charge (EYLAR et al. [26]) in the dye binding, then these groups must lie in a very near neighbourhood to the membrane lipids. Such a suggestion, however, is not consistent with the molecular structural models (NICOLSON [81], [82], NICOLSON and POSTE, [84], MAKOVITZKY and GEYER

[69]) assuming glycoprotein chains protruding far out over the membrane surface. In such a structural pattern the membrane lipids would not be able to interact with the dye molecules and orient them. The lipid dependency of the toluidine blue staining reaction of red blood cell membranes (and other biomembranes) is rather consistent with the structural model proposed by MARCHESI [71], and SHARON and GRANT [119], suggesting that the carbohydrate units of surface glycoproteins lie flat along the membrane surface, interacting partly with the hydrophilic heads of the membrane lipids and partly being interconnected and stabilized by hydrogen bonds and water structures. In such a structure, the repulsive forces between the negative sialic acid residues could act in the plane of the membranes and contribute to the maintenance of a specific pattern of the surface glycoprotein network as a cell-specific ultrastructural feature.

It appears that the sharp differences in the topo-optical staining reactions between red blood cell membranes and myeloid-lymphoid cell elements are closely related to differences in their surface glycoprotein pattern. This question of surface-membrane glycoproteins of blood cell elements will concern us later again in connection with the specific topo-optical staining reaction of periodate-reactive carbohydrate chains of biomembrane polysaccharides.

III. B. *Chlorpromazine-eosin topo-optical staining reaction demonstrating densely-ordered negative side groups on the lipid-extracted red cell membrane*

The lipid-dependent anisotropic staining of red cell membrane with toluidine blue-ferricyanide suggested the presence on the membrane surface of negatively-charged dye binding groups of non-lipid nature in high density and definite order. This suggestion can be definitely proved by the chlorpromazine-eosin topo-optical staining reaction of red cell membranes.

In formol-fixed paraffin sections, or glutaraldehyde-fixed lipid-extracted smears, erythrocyte membranes reveal a strong birefringence but only when the staining with eosin followed the chlorpromazine pretreatment (Fig. 24a). The birefringence was radially negative with tangentially-oriented slow axis of transmission: with tangential orientation of the greater refractive index (*i.e.*, with an opposite optical character to the birefringence of the toluidine blue-stained red cell membranes).

The molecular mechanism of this oriented staining reaction is based on charge-transfer reaction between chlorpromazine and eosin, as discovered *in vitro* by LÁBAS [64]. Since such an oriented charge-transfer reaction of chlorpromazine-eosin on red cell membranes could be achieved only when chlorpromazine had preceeded eosin staining negative side groups must play a primary role in the binding of chlorpromazine, which is then aggregated by the subsequent treatment with the anionic dyestoff eosin. The radially-negative

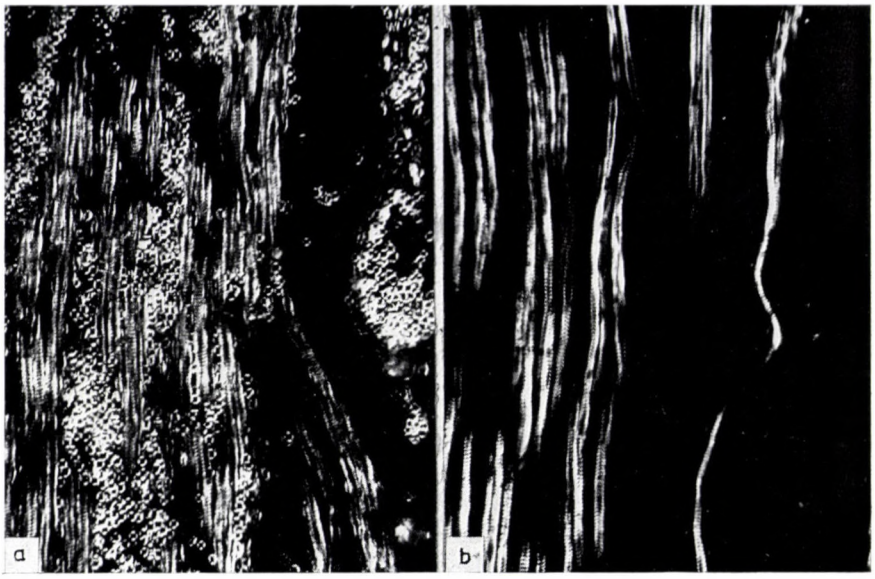

Fig. 24. Human embryonic muscle fibres with interstitial haemorrhages. *(a)* stained with haematoxylin-chlorpromazine-eosin. Between the strongly birefringent muscle fibres, erythrocytes appear in great number with strongly birefringent membranes. The birefringence was negative with respect to the radius, *i.e.*, with tangentially oriented slow axis of transmission. *(b)* After 24 h methylation the birefringence effects of the erythrocyte membranes disappeared, although the erythrocytes were strongly stained with eosin. Methylation resulted in blocking the negative side groups for binding chlorpromazine

character of the birefringence of the erythrocyte membranes caused by chlorpromazine-eosin aggregates is suggestive of linearly-ordered negative binding groups for chlorpromazine in high density on the membrane structure; similarly as concluded on the basis of the optical analysis of the toluidine blue-ferricyanide-induced birefringence.

The role of the negative side groups on the erythrocyte membrane surface — possibly the sialic acid residues of the surface glycoproteins (EYLAR et al. [26]), or the negatively-charged acidic peripheral membrane proteins (GULIK—KRZYWICKI [40) in this reaction — is evident from the finding that histochemical blocking of the negative side groups by methylation completely abolished the chlorpromazine-eosin reaction of the red cell membrane. (Fig. 24b) This reaction is independent of membrane lipids and is related to non-lipid negative binding groups on the membrane structure, which must have a definite array to provide appropriate underlying molecular pattern for the ordered association of the charge-transfer complex. The method appears also useful to study other biomembranes in paraffin tissue sections with thickness under 5 nm.

The topo-optical staining of red cell membranes with toluidine blue-ferricyanide and with chlorpromazine-eosin reaction allow the following conclusions.

(1) The red cell membrane possesses densely-situated linearly-ordered negative dye-binding groups in a definite structural array, serving as the basis of the topo-optical staining reactions. The toluidine-blue binding sites must be assumed to be localized in high density since, as we have shown in a recent study on carbohydrate chains that in agreement with the considerations of SYLVÉN [128], no ordered coplanar (birefringent) toluidine blue binding is possible when the dye-binding sites are at a distance over 6 Å, not even with ferricyanide stabilization (FISCHER and ROMHÁNYI [34]).

(2) Oriented toluidine blue binding on erythrocyte membrane is *dependent on the presence of the membrane lipids.* The radial orientation of the toluidine blue molecules in the erythrocyte membrane is probably brought about by intercalation of the apolar ring system of the dye molecules between the hydrocarbon chains of the lipid bilayer. This suggests a very near neighbourhood between dye-binding groups and the membrane bilayer and indirectly a planarly-extended network of glycoproteins on the membrane surface (as suggested by MARCHESI [7], and SHARON and GRANT [119]). Lipid extraction resulted in a disoriented random dye binding of the toluidine blue molecules, manifested in isotropy, similarly as with other biomembranes.

(3) The striking differences in ultrastructure between red cell membranes and other blood cell membranes was found already demonstrable in the early phases of erytrhoblastic differentiation and maturation process, indicating that the cell-specific membrane organization is an early event in cell differentiation, agreeing with results of biochemical analytical studies (INOUÉ et al. [50], KEHRY et al. [56], WILEY and SHATTER [140]).

III. C. *Topo-optical reaction of chlorpromazine with biomembranes*

So far we have been concerned only with staining-induced topo-optical reactions of biomembranes due to oriented intercalation of appropriate dye molecules. Much less is known about interactions of colourless substances, for instance drugs, with biomembranes.

As a matter of fact, there was early interest in the structural interaction between drugs and biomembranes (SEEMAN [118]), the interest being particularly directed to the possible molecular mechanism of activity of local and general anaesthetics. It was postulated (BANDHAM [4]) that anaesthetics are intercalated between membrane lipids (or proteins) and achieve their anaesthetic effects by membrane expansion and structural perturbation. Interesting findings of BANGHAM [4] showed that under high hydrostatic pressure the narcotic effect could be reversed.

The molecular mechanism of the general membrane activity of the historically first tranquilizer the thiazine relative chlorpromazine, has been most extensively studied (KLEINSORGE and KÖSNER [58], KARREMAN, ISENBERG

and SZENT-GYÖRGYI [55], FEINBERG and SNYDER [28], SEEMAN [118], WOLLEMAN and KELETI [143]). However, no information on a possible structural interaction of chlorpromazine and biomembranes was obtained.

More recently, TENFORDE et al. [131] have studied the influence of chlorpromazine on the electrophoretic mobility of erythrocytes. At 23 *mM* concentration of chlorpromazine, the electrophoretic mobility of the erythrocytes decreased by 10%, an effect completely reversible by washing out the chlorpromazine. The reduction of the electrophoretic mobility was attributed to a decrease in surface charge caused by binding of chlorpromazine molecules to negative binding sites by their tertiary amino groups. Glutaraldehyde-fixed erythrocytes revealed the same electrophoretic behaviour as native red cells. These findings indicate that chlorpromazine molecules bind to the cell membrane structure. About 10^6 chlorpromazine molecules are estimated to be bound in these experiments to each erythrocyte.

We based our investigations with chlorpromazine on the following considerations. *(i)* As a thiazine derivative chlorpromazine is structurally related to toluidine blue (Fig. 19), which we have used extensively in topo-optical staining reactions; *(ii)* accidentally, we observed chlorpromazine acting in liver slices as a powerful electron donor in restoring the anisotropic staining of photo-oxidized *ER* membranes; *(iii)* we found that it can bind in an oriented pattern to negative side groups on lipid-extracted red cell membranes where it could be demonstrated by the oriented charge-transfer complex reaction with eosin, as described in the preceding section.

We have carried out our experiments *(i)* in formol-fixed frozen sections of peripheral and central nerves, *(ii)* on membranes in frozen sections of kidney, *(iii)* on myelin figures formed from lecithin in water and with addition of chlorpromazine to water, and *(iv)* on the negative rod outer segments of frog retina.

Figure 25 shows the considerable decrease (near to isotropy) of the intrinsic birefringence of the myelin sheaths of the peripheral nerve as caused by chlorpromazine. In consequence of this depression of the intrinsic birefringence of the myelin sheaths the π-granules became visible, which in the control section were invisible because their birefringence was overshadowed by the strong birefringence of the myelin sheaths. The curve in Fig. 26 shows that this effect of chlorpromazine was reversible and could be eliminated by washing out the reagent from the tissues. Therefore, the effect is not attributable to a structural damage of the myelin sheath, but is brought about by overcompensation of the optical effect of the membrane lipids by chlorpromazine molecules intercalated in an ordered arrangement in the membrane structure.

The results obtained on the renal membranes, on the rod outer segments and on myelin figures were similar. Myelin figures formed in water appear in the form of optically positive spherites, however, in the presence of chlorpromazine

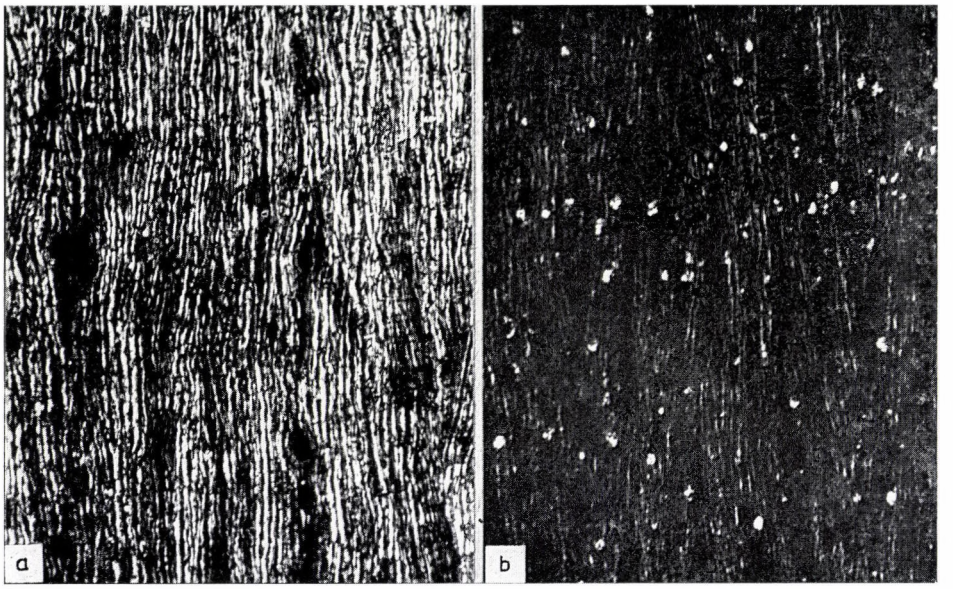

Fig. 25. Formol-fixed frozen section of peripheral nerve with crossed polars: *(a)* in water, *(b)* in 1% chlorpromazine-water; In *(a)* the myelin sheaths show strong birefringence negative with respect to their length, in *(b)* the intrinsic birefringence of the myelin sheaths is depressed near to isotropy and therefore the birefringent π-granules become visible between the myelin sheaths. Otherwise, they are overshadowed by the strong birefringence of the myelin sheaths. The chlorpromazine-induced reaction on the myelin sheath is reversible: the original birefringence could be restored by washing out chlorpromazine in water. Chlorpromazine thus reacts with the myelin sheath (and other biomembranes) in the form of *inversive* topo-optical reaction by intercalating with its ring system transversally between the hydrocarbon chains of the lipid bilayer, causing structural membrane perturbations

(1%) the spherites were optically negative, with tangentially-oriented slow axis of transmission.

Thus, chlorpromazine produces an inversive topo-optical reaction with biomembranes, an optical effect resulting from over-compensation of the optical effect of birefringence of the membrane lipid layer. This suggests that the chlorpromazine molecules are intercalated in the lipid layer oriented transversally with their ring system (their greater refractive index) to the hydrocarbon chains of the lipid layer. The chlorpromazine molecules are bound by their tertiary amino groups to negative surface side groups of the membrane structure and the ring systems penetrate between the lipid molecules and are floating between the hydrocarbon chains of the membrane lipid layer, thus producing reversible perturbation in the membrane structure. The structural perturbation may be partly responsible for the manifold membrane effects of chlorpromazine and possibly for its damaging effects at protracted dosage (*e.g.* chlorpromazine-induced retinopathia, MEYER-RUGE [73]). In frog retina, we have seen disc-like segregation of the outer segments of the retina at protracted

dosage of chlorpromazine and an increased detachment of rhodopsin-containing membranes in the form of spherulites into the pigment cells (ROMHÁNYI et al. [105]).

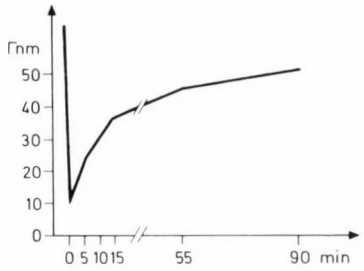

Fig. 26. The curve of the change in birefringence of the myelin sheath caused by chlorpromazine (2%). There is a rapid decrease of the birefringence, already within the first minutes. The effect is reversed by washing out with water

IV. Molecular orientation of periodate-reactive carbohydrate components in biomembranes as shown by the topo-optical staining reaction of the periodate-aldehyde-bisulphite-toluidine blue-ferricyanide reaction, the ABT reaction

IV. A. *General remarks*

Carbohydrates are widespread components of many biological structures and biomembranes in animal cells as well as in bacterial and plant cell walls in the form of polysaccharides, glycoproteins and glycolipids: partly demonstrable by electron microscopy as a carbohydrate-rich cell "coat"at the cell surface (RAMBOURG and LEBLOND [88]). Their biochemistry and biological significance have been reviewed by HUGHES [47]. The periodate-reactive carbohydrate components are demonstrable *in situ* by the *PAS* reaction. This, however, produces isotropic staining effect and so it does not provide any insight into the molecular order of the reactive components in tissues. The same applies to the demonstration of periodate-reactive carbohydrate components at the ultrastructural level, based on the silver reduction capacity of periodate-induced aldehyde groups (AINSWORTH et al. [2], BRADBURY and STOWAR [9], RAMBOURG [87], MOVAT [79], NEVELAINEN and KLEMI [80]) or to binding of labeled lectins on cell-surface carbohydrates (NICOLSON and SINGER [85], RÖHLICH [108], GROS et al. [38]). All these reactions result in formation of an amorphous aggregate on the reductive or binding sites and give information only on the presence and localization of carbohydrate components at the ultrastructural level, but not about the molecular order and orientation of the chains of the reacting carbohydrate components.

Because of these methodological inadequacies, there is no definite knowledge about the molecular order of the complex periodate-reactive carbohydrate components or their structural disorders in tissues.

Therefore, the development of theoretically well-founded and technically simple topo-optical staining reaction, capable of demonstrating the molecular order of periodate-reactive complex carbohydrate components in tissues, appears of great value in biomorphological studies in view of the widespread presence of these components in biological structures with great functional significance. We therefore may consider the development of a selective topo-optical staining reaction of periodate-reactive carbohydrate components in biological structures (ROMHÁNYI et al. [104]) a methodological breakthrough of great significance.

IV. B. *Aldehyde-bisulphite-toluidine blue (ABT) reaction as a topo-optical staining reaction of periodate-reactive carbohydrate chains in tissues*

This method is based on the oriented binding of toluidine blue to periodate-induced carbohydrate-derived aldehyde groups at pH 1.0 (stabilized by post- staining treatment with ferricyanide or ferricyanide-potassium iodide mixture, 1 : 7). Figure 27 shows the molecular mechanism of the reaction. The aldehyde groups induced by the periodate oxidation of vicinal OH groups of carbohydrates are transformed into negatively-charged dye-binding groups by addition of bisulphite, as shown initially by MALININ [70], for light microscopic glycogen demonstration. In this way strongly negative dye-binding groups are induced on the carbohydrate chains being able to bind toluidine blue at pH 1.0 and being stabilized in an ordered pattern by ferricyanide [ROMHÁNYI et al. [104]). The staining reaction thus results in strong negative birefringence with respect to the polysaccharide chain (Fig. 27).

IV. C. *Widespread applicability of the ABT reaction for structural studies of carbohydrate components in tissues*

In order to show the reliability of the *ABT* reaction in ultrastructural studies, we first demonstrate its effects on some examples of complex carbohydrates with well-known molecular order and chemical composition. In this way we can correlate the optical effects of the *ABT* reaction obtained on biological structures with those obtained on the model substances and we can conclude on the molecular order of the reacting carbohydrate chains in biological substrates.

Figure 28a, b shows the strong increase (and optical inversion) of the cellulose walls in the tip of an onion root as induced by the *ABT* reaction.

The originally weak positive birefringence of the cellulose walls (FREY-WISSLING [36]) is inverted into strong negative birefringence with respect to

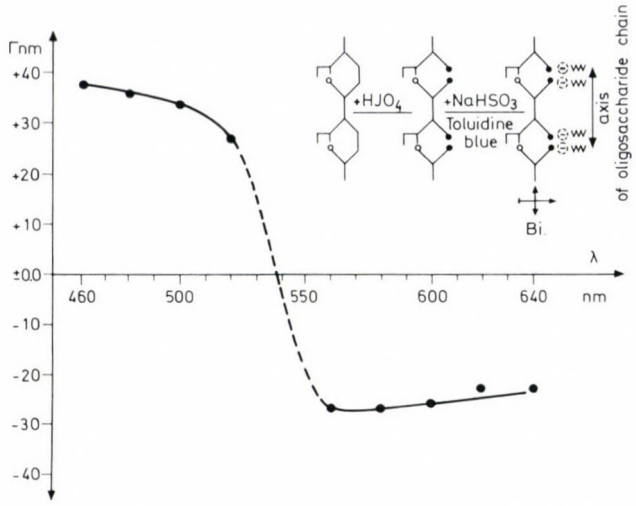

Fig. 27. Demonstrates the molecular mechanism underlying the topo-optical staining reaction of periodate reactive carbohydrate chains by the periodate-bisulphite-toluidine blue-ferricyanide reaction (*ABT* reaction). Periodate oxidizes the vicinal OH groups to dialdehyde groups by splitting the bonds between the carbon atoms in the carbohydrate ring carrying vicinal OH groups. The dialdehyde groups are then rendered strongly negative by addition of bisulphite (by treatment of the slides with saturated sodium bisulphite solution for 10 min) and capable to bind toluidine blue at pH 1.0. The post-staining treatment with potassium ferricyanide stabilizes the oriented dye binding on the carbohydrate chains inducing birefringence with negative character with respect to the length of the carbohydrate chain. The curve of the anomalous dispersion of the toluidine blue-induced birefringence with respect to the orientation of the fibre is included in this figure. The birefringence induced by the oriented binding of toluidine blue on the polysaccharide chains, for instance cellulose fibres, is negative with respect to the fibre *length for light of the long-wave side of the spectrum*, but is inverted *to positive* birefringence below 500 mm. In all our observations, the birefringences we have referred to, were measured at the long-wave side of the spectrum. For details on theoretical basis of anomalous dispersion of birefringence see: [98, 111, 145].

the length of the cell walls. The strong inversive type of the topo-optical staining reaction of cellulose indicates dye molecules being attached in high density perpendicularly to the molecular texture of cellulose.

The orientation of the toluidine blue molecules on the cellulose texture is reflected also by the strong negative dichroism seen when investigated by bright-field microscopy in polarized incident light.

As another example, we have chosen pneumococcal capsular polysaccharide, a linear polysaccharide built up of cellobiuronic acid disaccharide units (Westphal and Lüderitz [137]). The difficulty of demonstration by selective staining of this polysaccharide is well-known. With the *ABT* reaction we have obtained excellent results in the lung tissue as well as in smears of cultured microbes (Fig. 28c, d). The optical character was radially positive, indicating radially-oriented dye molecules and circularly-oriented polysaccharide chains in the capsular polysaccharide.

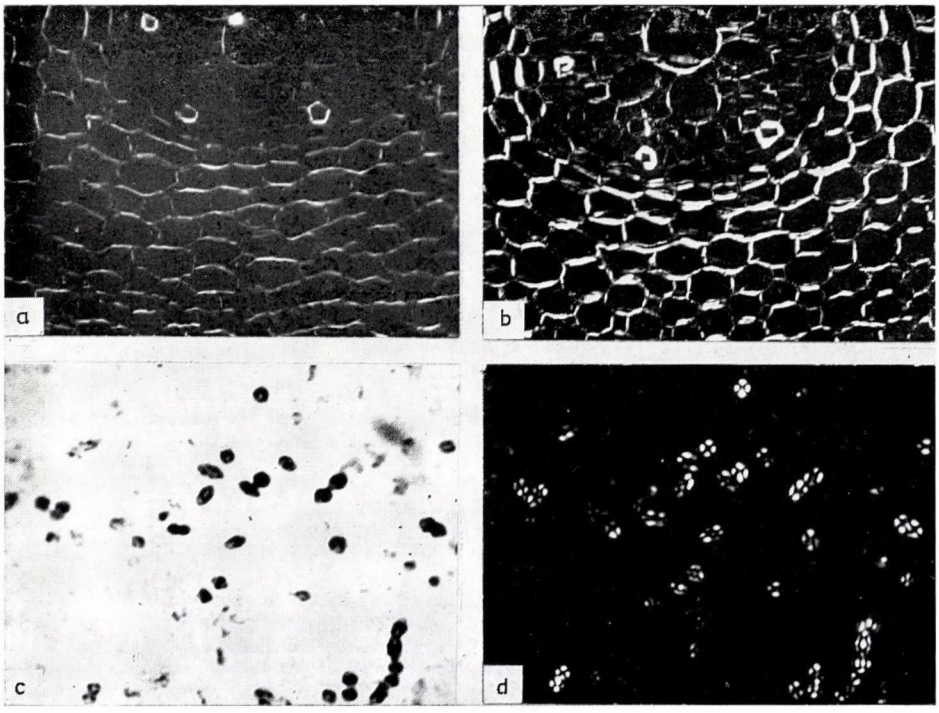

Fig. 28. (a) and *(b)* show a pectinase-treated section of the tip of onion root. *(a)* unstained *(b)* the same field after *ABT* reaction; both with crossed polars. *(a)* shows the weakly birefringent cellulose walls; *positive:* with respect to the lengths and with 8—10 retardations (FREY-WISSLING [36]) In *(b)* there is a strong toluidine blue-induced birefringence of the cell walls, *negative* with respect to their lengths and with about 40 nm retardations (ROMHÁNYI et al. [103].) *(c)* and *(d)* show glutaraldehyde-fixed smear of freshly cultured pneumococcus with *ABT* reaction, in the light microscope *(c)* and with crossed polars *(d)*. Pneumococcal capsular polysaccharide reveals strong basophilia. With crossed polars, a strong birefringence is seen of the capsular polysaccharide (with 30—35 nm retardations) in the form of radially positive spherites indicating radial orientation of the toluidine blue molecules and in turn circular orientation of the capsular polysaccharide texture ×1200

Figure 29 intends to demonstrate the practical usefulness of the *ABT* reaction in demonstrating microbes in tissues, based on the topo-optical staining reaction of the oriented carbohydrate components in microbial cell walls or membranes.

The *ABT* reaction for microbes in tissues is highly selective and very useful, since after bisulphite treatment the periodate-induced aldehyde groups can take up toluidine blue at pH 1.0, and the ordered dye binding results in an easily recognizable strong birefringence. No other tissue components except mast cell granules take up toluidine blue at pH 1. FISCHER [30, 31) and FISCHER and EMŐDY [33] have obtained interesting results about the complex structural organization of carbohydrate components in microbial cell walls.

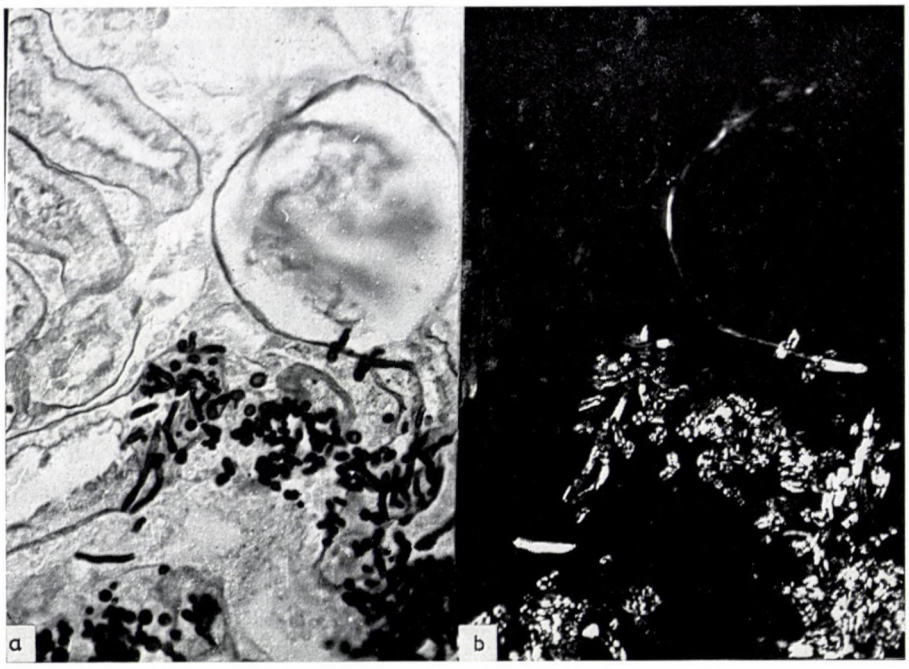

Fig. 29. Kidney of a rat infected with *Candida albicans*. *ABT* reaction *(a)* in the light microscope, *(b)* with crossed polars. In *(a)* basophilic fungus masses are seen which in *(b)* are strongly birefringent and in cross sections they appear in the form of optically negative spherites. (With 30—35 nm retardations) with tangentially oriented slow axis of transmission, indicating tangentially (circularly) oriented dye molecules bound on the polysaccharide texture of the cell wall

IV. D. *Specialized membrane structures rich in carbohydrate components demonstrated by the ABT reaction*

In this section we will demonstrate the oriented periodate-reactive carbohydrate components of the intestinal mucous membranes, the orientation of the disaccharide chain units in basement membranes, the orientation of the oligosaccharide chain of rhodopsin in the rod outer segment disc membranes and the periodate-reactive structures of blood cell membranes.

Microvilli of the epithelial surface layer of the intestinal mucous membrane

They are known from electron microscopy as made up of slender elongated processes of the cellular surface membrane, with actin-like filaments in their axis serving contractility. The microvillous membrane layer shows strong *PAS* reaction and is rich in glycosylated enzymes serving the resorptive processes. Fig. 30 demonstrates the optical finding of the microvillous layer with the *ABT* reaction, showing a strongly basophilic and highly birefringent microvillous layer. The birefringence induced by the oriented toluidine blue binding

is negative with respect to the axis of the individual microvilli, indicating carbohydrate components parallel with the membrane surface as a longitudinal framework of the membrane structure. The glycogen nature of the carbohydrate was excluded by our failure to eliminate the reaction by diastase pretreatment. Optical analyses indicate that the dye molecules were bound perpendicularly on the membranes of the individual microvilli. The method appears useful in studying structural conditions in human biopsy material.

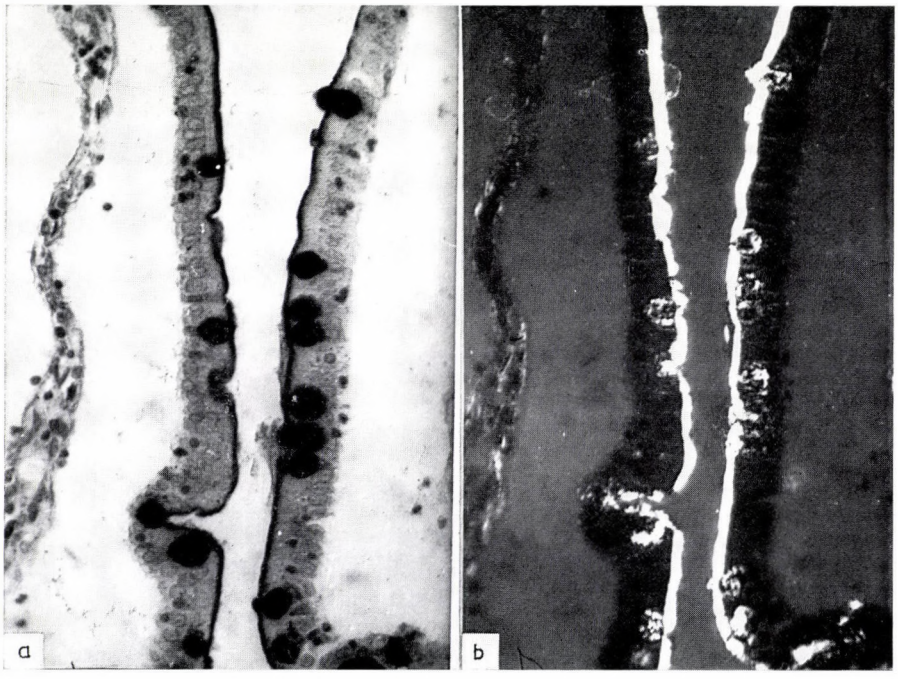

Fig. 30. Microvillous surface layer with goblet cells of the epthelial cells in human intestinal biopsy material. *ABT* reaction stabilized with a 1 : 7 mixture of potassium ferricyanide and potassium iodide *(a)* in the bright-field microscope, *(b)* the same area with crossed polars. In *(b)* the microvillous surface layer shows a very intensive birefringence (negative in character with respect to the axis of the individual microvilli) indicating that the dye molecules were bound perpendicularly to the axis of the individual microvilli. The perpendicular binding is indicative of a carbohydrate texture parallel with their membrane surface. Goblet cells' content shows similar reactivity, corresponding to its glycoprotein nature. This picture demonstrates how useful the *ABT* topo-optical staining reaction can be applied for routine investigations of biopsy materials

Topo-optical staining reaction of the carbohydrate disaccharide units of basement membranes

Basement membranes are specially-textured complex membrane structures of collagen, carbohydrate and lipid. They react with the inversive topo-optical staining reaction specific for collagen (phenol reaction, EBNER [23])

and show selective topo-optical staining reaction for collagen with toluidine blue when sulphated after blocking of the vicinal OH groups (ROMHÁNYI et al. [101]), indicating axiparallel arrangement of the collagen filaments in their texture.

Their intrinsic negative birefringence which is eliminated by lipid extraction, indicates transversally-oriented lipid structures in them (MISSMAHL [76]) similarly as in the cell membranes.

On the other hand, the structural order of the high carbohydrate content (12%, SPIRO [124, 125]) of basement membranes present in disaccharide units is unknown. Epithelial alveoli, capillary walls and glomerular tufts are surrounded by basement membranes; which in general, play an important role in several transport processes, for instance in the kidney, and may therefore rightly be included, in a broader sense, into the group of biomembranes.

We do not discuss in detail the complex structure of basement membranes in this review; we consider them here only in view of the so far unknown molecular order of their carbohydrate content, which is reflected in their strong *PAS* reaction. However, the *PAS* reaction gives no information on the molecular order of the reactive carbohydrate component. Figure 31 shows the strong topo-optical reaction of the renal basement membranes with the *ABT* reaction. The basement membranes are rendered strongly basophilic, and birefringent with retardations of 30—35 nm. The birefringence was negative with respect to their length, indicating that the disaccharide units are oriented parallel to the framework of the collagen-filamentous texture interposed between the filaments. We found considerable differences in the optical reactivity of basement membranes in different organs with *ABT*, corresponding to the varying intensities of their *PAS* reaction. For instance, there was a great difference between the optical reactivity of the renal peritubular and glomerular basement membranes, reflecting probably differences in their structurally different functional significance. The *ABT* reaction can be applied usefully in studying the common pathological changes of basement membranes, *viz.*, hyalin degeneration at the ultrastructural level.

The molecular orientation of the hexosaccharide chains of rhodopsin in the visual receptor membranes of the rod outer segment of frog as revealed by the ABT reaction

The early polarization-optical findings of SCHMIDT [112] indicated that the retinal molecules are oriented parallel to the disc membranes in the rod outer segment. This observation was based on the negative dichroism of the rod outer segments with respect to their length, *i.e.*, with maximum absorption for light polarized perpendicularly to the rod axis. It is evident that this membrane-parallel orientation of retinal may provide the maximal absorption capacity for the incident light propagating along the rod axis. Retinal has

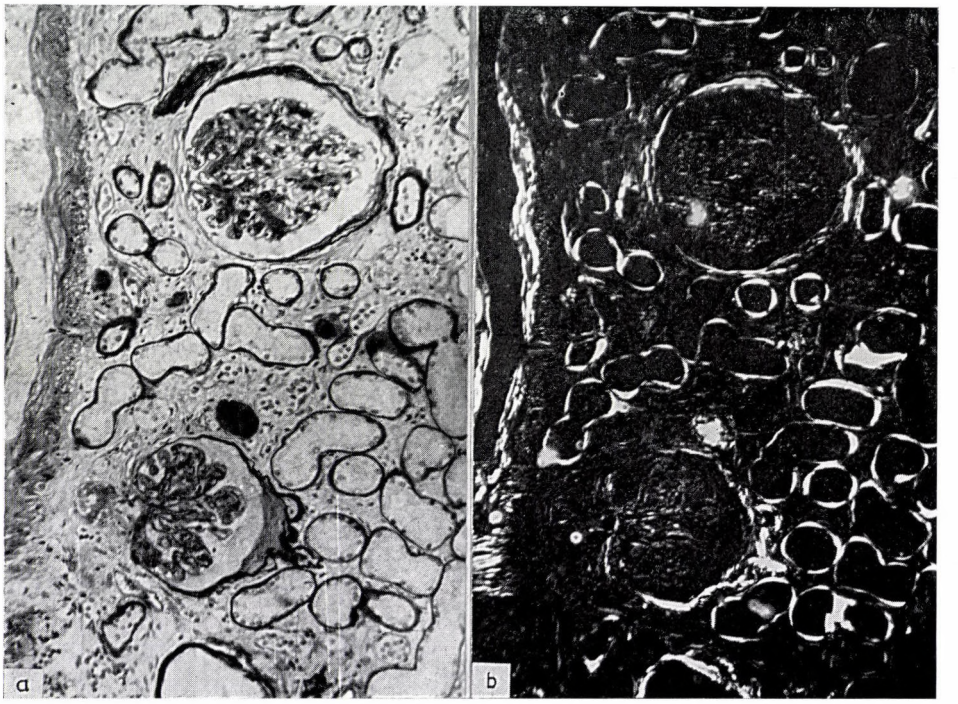

Fig. 31. Reaction of the basement membranes of human kidney. *(a)* in the light microscope, *(b)* with crossed polars. Basement membranes reveal strong reaction for glycol groups of carbohydrate components (as indicated also by their intensive *PAS* reaction). The birefringence of the basement membranes induced by the *ABT* reaction was negative with respect to the length, indicating toluidine blue binding transversally to the membranes, suggesting in turn that the disaccharide units of the basement membranes (SPIRO [124, 125]) are oriented in longitudinal direction in *between* the underlying filamentous collagen framework. Peritubular basement membranes show stronger birefringence with *ABT* reaction than the glomerular basement membranes, indicating textural differences in their ultrastructure. Peritubular basement membranes also revealed intensive negative linear dichroism after *ABT* reaction (ROMHÁNYI et al. [104]), suggesting transversally bound dye molecules on the linear texture of the basement membranes

therefore, a fixed molecular orientation with respect to the disc membrane plane. However, according to flash-light polarization-optical studies (BROWN [13], CONE [15]) rhodopsin with the retinal rotates freely in the membrane plane.

What may be the molecular structural organization which allows such a free rotational movement of rhodopsin with maintaining the plane of retinal in the disc membrane? What can serve as a potential rotational axis for this limited Brownian movement in the membrane? Rhodopsin, the main protein (about 80%) of the disc membrane contains, apart from retinal, a hexosaccharide chain (HELLER and LAWRENCE [42]). It was suggested that the hexosaccharide chain serving as a rotational axis, may play a role in maintaining

rhodopsin in its definite situation in the membrane. Thus, the question arose whether rhodopsin hexosaccharide chains have a well-defined orientation with respect to the membrane and in what direction.

We [102, 105] removed acid polysaccharides by hyaluronidase and blocked the participation of the free aldehyde groups present in formol-fixed rods (free retinal aldehyde?) by treatment with borohydride, which reduces aldehyde groups to nonreactive OH groups in the *ABT* reaction, and found a strong reaction of the rod outer segments for vicinal hydroxyl groups (Fig. 32a, b). The birefringence was negative with respect to the axis of the rods, indicating that the carbohydrate chains were oriented perpendicularly to the disc membranes. In this way the highly-ordered hexosaccharide chains may allow the suggestion that their definite orientation has a functional significance in maintaining rhodopsin molecules in a definite molecular order in the disc membrane. This order, as mentioned above, has a great functional significance for the optimum absorption of light passing along the rod axis. The nature of the so-called "free" aldehyde groups in the rod outer segment (Fig. 32c, d), revealing a high degree of molecular order in the disc membranes, needs further investigations.

Topo-optical staining reactions of carbohydrate components of blood cell elements

Recently, there is growing interest in membrane surface glycoproteins (WINZLER [138], WARREN et al. [136], TURNER and BURGER [134], MARCHESI et al. [72], ROSEMAN [106], REES [83], NICOLSON [81], EDELMAN [24], EYLAR et al. [26], GLOSSMAN and NEVILLE [37]) in view of their possible functional signicifance in determining the cell-specific character of different cell elements and functional characteristics. In the red cell membrane, MARCHESI et al. [72] have demonstrated a cell-specific glycoprotein with about 60% carbohydrate content ("glycophorin"). Glycophorin spans the whole thickness of the cell membrane. Its carbohydrate units are concentrated on the outer surface of the membrane. Glycophorin plays a definite role in determining membrane specificity and carries blood-group antigens (MARCHESI [71]).

Recently, biochemical studies have been concerned intensively with glycoprotein components of nucleated blood cell elements, especially of lymphocytes (GLOSSMANN and NEVILLE [37], HUNT and BROWN [45], JULIANO and LI [54], MISRA et al. [75]). Here a great number of major glycoprotein fractions have been detected with their changes under pathological conditions, but no components resembling glycophorin have been detected.

In view of these data we were greatly interested in the topo-optical reactivity for structural carbohydrate components of different blood cell elements as revealed by the *ABT* reaction. The results were surprising: with the *ABT* reaction the red cell membrane birefringence was strongly decreased in

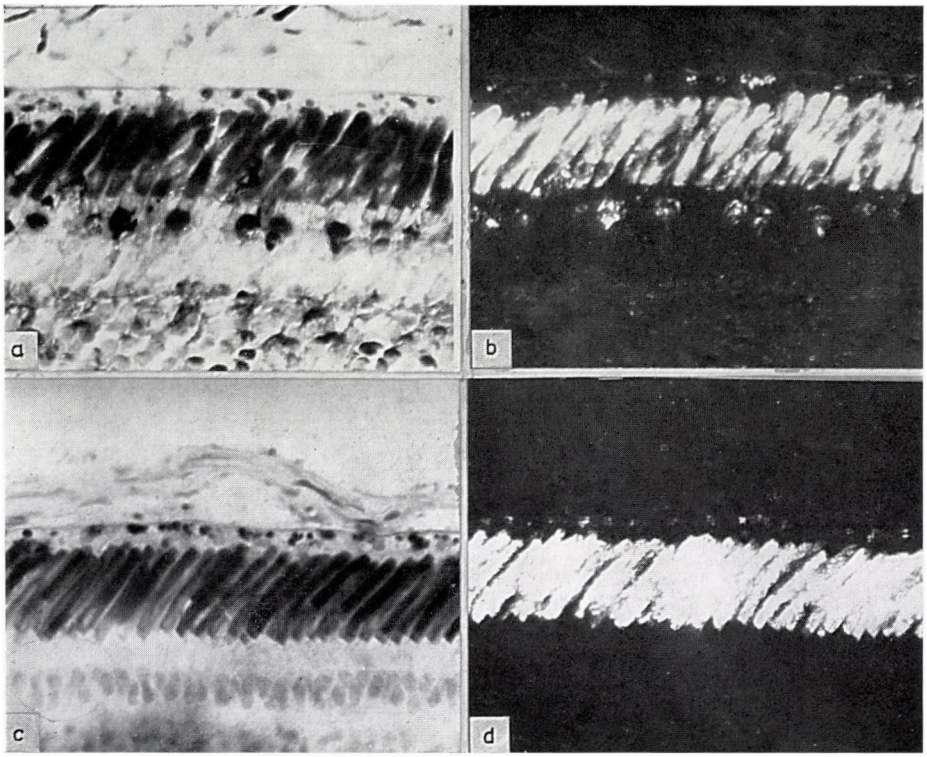

Fig. 32. (a), (b), (c), (d). Frog retina. Fixed in formalin. Paraffin section. In *(a)* and *(b)*, periodate-aldehyde-bisulphite-toluidine blue *(ABT)* reaction selective for the oligosaccharide chains of rhodopsin was made after removal of the acid polysaccharide component by hyaluronidase pretreatment and blocking with 5% borohydride the free aldehyde groups present in the visual receptor membranes possibly induced by the cleavage of the binding of retinal to rhodopsin by formalin. In *(a)*, strong basophilic staining reaction of oligosaccharide chains can be seen in the rod outer segments. In the inner segments, the coarse basophilic granules proved to be glycogen granules. In *(b)*, rod outer segments show strong staining induced birefringence, negative with respect to the rod axis, indicating transversally bound dye molecules with respect to the rod axis, and axially oriented oligosaccharide chains of rhodopsin in the visual receptor membrane. The strong birefringence effect suggests high order of the oligosaccharide chains of rhodopsin, perpendicular to the disc membranes. Above the outer end of the rod outer segments birefringent spherulites can be seen with tangentially oriented slow axis of transmission. These droplets correspond to detached and circularly wound up disc membranes with radially oriented oligosaccharide chains of rhodopsin (ROMHÁNYI et al. [105]). In *(c)* and *(d)*, the bisulphite-toluidine blue reaction was made by omitting the periodate pretreatment. Thus this reaction detects free aldehyde groups already present in the rod outer segments. There is a strong basophilic staining reaction for free aldehyde groups in the rod outer segments, with basophilic droplets included in the pigment epithelial zone. With crossed polars: the rod outer segments and the droplets in the pigment epithelial zone show strong birefringence, negative with respect to the rod axis. The nature of these freely-reacting aldehyde groups in the visual receptor membrane has not been definitely clarified; it seems likely that they correspond to retinaldehyde groups liberated from their binding with rhodopsin *in situ* by the influence of formalin fixation. The optical effects suggest a high order of the free aldehyde groups in the visual receptor membrane

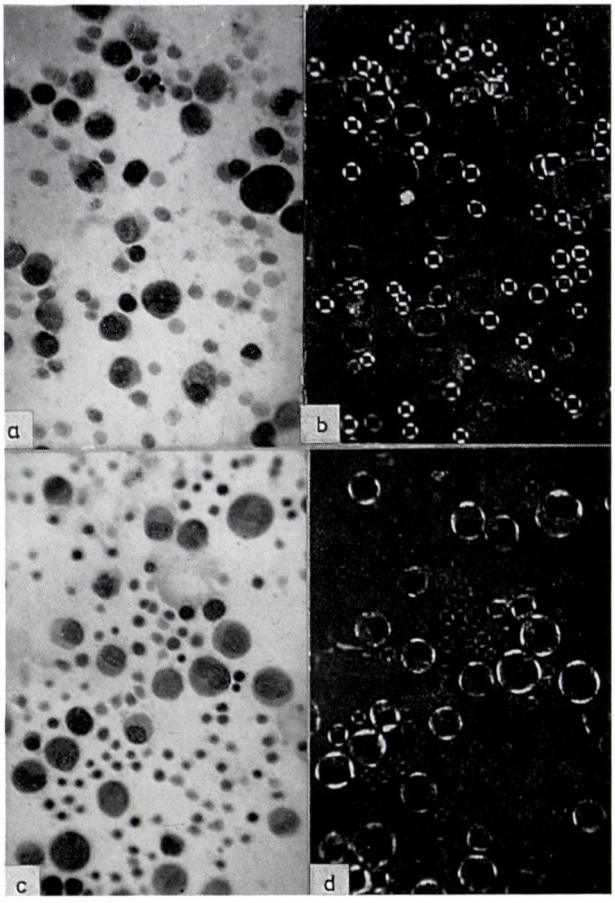

Fig. 33. (a), (b), (c), (d) Human bone marrow smear fixed in glutaraldehyde for 5 min. *(a)* and *(b)* represent the same cell group stained with toluidine blue at pH 5.0 and stabilized with potassium ferricyanide-potassium iodide mixture: sealed in gum arabic. In *(c)* and *(d)* *ABT* reaction was made., *(c)* and *(d)* represent the same cell groups. In *(b)*, the red blood cell membranes appear strongly birefringent. The membranes of the myeloid cell elements show very weak anisotropic staining. In *(c)*, the red blood cells show an increased basophilia but with crossed polars *(d)* their birefringence is markedly weaker; in contrast, the membranes of the myeloid cells appear strongly birefringent *(d)*. The birefringence was radially positive, indicating radially-oriented toluidine blue molecules on the membrane and, in turn, planarly (tangentially) extended glycoprotein network on the membrane surface

spite of the enhanced basophilia of the erythrocytes (Fig. 33). On the contrary, the membranes of myeloid and lymphoid cell elements revealed intensive topo-optical staining reaction for carbohydrate chains. The radially positive character of the induced birefringence of these cells indicated radially-oriented dye molecules and, in turn, planarly extended carbohydrate network on the cell surface. The *ABT* reaction of myeloid and lymphoid cell membranes was lipid-dependent. Initially, of course, we expected rather a strong reaction of

the red cell membranes with the *ABT* reaction, in view of their high glycoprotein surface component, and a relatively weak reaction of the myeloid-lymphoid cell elements. This, apparently paradox finding may probably be explained be suggesting that in the planarly extended glycoprotein network of the red cell membrane the periodate oxidation induces a great number of cleavages of the bonds between vicinal OH groups, resulting in a high number of densely-situated aldehyde groups which in the *ABT* reaction lead to overcrowding of dye molecules on the surface, repressing the possibility for the dye molecules to arrange themselves in an oriented pattern (the repression of birefringence in topo-optical staining reactions caused by over-staining is well-known). This suggests that on the surface of myeloid-lymphoid cell elements glycoproteins form a looser network, whence no overcrowding of dye molecules occurs in the *ABT* reaction and regular birefringence effects can arise on the surface of these cells.

In any case, the difference in ultrastructure between red blood cell membrane and myeloid and lymphoid cell membranes, as revealed by the *ABT* reaction, can be considered as a morphologically significant finding.

It remains also to be seen whether by means of other modified topo-optical test reactions, perhaps in connection with enzymatic or biochemical alterations of the cell membrane surfaces, further cell-specific differences could be established between different haematological cell elements according to their functional state, differentiation, or pathological transformation (KEHRY et al. [56], INOUÉ et al. [50], WILEY and SHATTER [140]).

Concluding remarks

The topo-optical reactions of biomembranes were reviewed. These reactions are brought about by oriented binding of dye molecules or colourless substances on the underlying molecular texture of biomembranes; they often provide information about their ultrastructural organization not obtainable by other ultrastructural methods.

Topo-optical staining reactions are manifested by strong birefringence effects caused by oriented binding of dye molecules on the underlying texture of biomembranes. Emphasis was made on topo-optical staining reactions obtained with two cationic dyes with planar conformation (Rivanol and toluidine blue); included was also the topo-optical reactivity of chlorpromazine with biomembranes, as a possible mechanism of its general membrane effect.

An essential point in achieving topo-optical staining reactions of biomembranes (and other biological substrates) proved the introduction of post-staining stabilization of the ordered dye binding on the substrates (using potassium ferricyanide alone or mixed with potassium iodide). This opened a

new area in polarization optics for biological structures in general and for biomembranes in particular.

An essential conclusion has emerged from the depencdeny of the toluidine blue-induced topo-optical reactions of biomembranes on the presence of membrane lipids. Although non-lipid negative side groups of the membrane structure were involved in the dye binding, the membrane lipids played a key role in the orientation of the toluidine blue molecules, probably by intercalating their apolar ring system between the hydrocarbon chains of the lipid bilayer. Lipid extraction abolished the topo-optical staining reactions with toluidine blue of all biomembranes investigated, but did not decrease the quantitative dye binding.

In view of the role of membrane lipids in the orientation of toluidine blue molecules in the topo-optical staining reactions, a very close neighbourhood between the dye-binding sites in the membranes and the orientating lipid layer must be assumed. The dye-binding groups in the cell membranes were assumed to be sialic acid residues of a planarly-extended glycoprotein network on the membrane surface, and membrane-near situated negative side groups of *RNA* in the *ER* membranes.

The newly-developed topo-optical staining reaction, a reaction capable of revealing the molecular order of complex carbohydrate chains in biological structures (the aldehyde-bisulfite-toluidine blue reaction, the *ABT* reaction) opened a new area for morphological studies of molecular structural order of periodate-reactive carbohydrate components in biomembranes and biological substrates. Similar significance can be attributed to the more recently developed selective topo-optical staining reaction of *RNA* in biomembranes.

A high sensitivity is often characteristic of the topo-optical reactions of biomembranes: detecting ultrastructural differences, for instance, between the membrane of red bood cells and myeloid-lymphoid cell elements, not amenable to other ultrastructural morphological methods.

Furthermore, the topo-optical staining reactions are highly sensitive, *i.e.* they can have strong birefringence effects even when the staining intensity in the light microscope is very weak or no staining is visible.

In view of the high sensitivity of the topo-optical staining reactions of biomembranes, it can be hoped that after covalent labelling of biomembranes (Carraway [14]) or by introducing specific chemical or enzyme-induced changes in biomembrane structures, topo-optical staining reactions can provide further possibilities for the study of biomembrane ultrastructures.

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SEASONAL VARIATIONS OF RAT THYROID C CELL POPULATION

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Abstract

With the aid of argyrophilic and metachromatic reactions male rats' thyroid C cells were investigated in spring and autumn. Autumn C cells were found to be more swollen and richer in granules and showed a more pronounced metachromasia and argyrophilia than spring C cells. The area occupied by C cells in the central part of the thyroid lobe was large in autumn and reduced in spring. A study into the proportions of C cell: follicular cell: colloid: stroma indicated a significantly higher number of C cells in autumn than in spring.

Introduction

The cells termed "parafollicular" by NONIDEZ [14] and "C cells" by PEARSE [18] produce the calcitonin [3, 8, 11] hormone which regulates the calcium level in the blood. These cells are diffusely scattered in the rat thyroid gland, showing a denser localization in the centre of the lobes and a relative sparsity at their periphery [7, 29]. The central part of the lobe containing the C cells is bordered by a zone free from C cells. The C cells are somewhat larger than the follicular cells, from which they can be well distinguished on the basis of their argyrophilia and masked metachromasia. The cytological and ultrastructural description of these cells [2, 15, 19, 27], as well as their role in maintaining calcium equilibrium [4, 5, 10, 11], are well known. STOECKEL and PORTE [28] and WELSCH [31] have found that the ultrastructural differentiation and the development of the rough-surfaced endoplasmic reticulum of C cells takes place in the last period of the embryonic life in the rat. WELSCH [31] described the ultrastructural, and STUX et al. [29] the quantitative changes of C cells in postnatal ontogenesis.

The C cell population of the thyroid sensitively reacts to any effect influencing calcium metabolism.

As indicated by our earlier investigations [21], the C cell population shows marked quantitative changes in the postnatal development. C cells increase in number until about 50 days of age, and a slight decrease can be recorded after this time. The numerical increase in C cells is usually associated

with the enhanced mineralization and growth of bones. From the 70th day on, the number of C cells attends a steady state. Recent investigations have shown that the number of C cells decreases in hypocalcaemia induced by hypovitaminosis D, whereas in hypervitaminosis D a marked rise in the number of C cells can be noted [22]. A histological picture indicative of C cells' hypofunction is observed in hibernating animals, which are known for their low serum calcium level [23]. These considerations, especially the latter finding, promted us to investigate whether there exist seasonal changes in the morphological characters of C cells in nonhibernating animals.

Material and methods

The experimental material consisted of 6-month-old male Wistar rats weighing about 240 g. The animals were fed on standard compressed rat food (LATI, Gödöllő, Hungary) and tap water. The experiments were carried out on 7 spring animals (processed in the first half of April), and on 5 autumn rats (processed in mid-October). Under Nembutal anesthesia (Serva, Heidelberg) both lobes of the thyroid gland were removed and fixed in glutaraldehyde — picric acid — acetic acid (GPA) solution at 4 °C for 24 hours. Tissue blocks were embedded in paraffin and serially sectioned at 7 μm. For the demonstration of the C cells, we made use of their argyrophilia and masked metachromasia. Alternating series of the sections were impregnated with $AgNO_3$ according to De Grandi [6], and stained with toluidine blue (0.01%) for metachromasia. The staining was made at pH 5.4, after hydrolysis in warm hydrochloric acid [26], and the dye was precipitated with a mixture of aqueous solution of 2% KI and 2%$K_3[Fe(CN)_6]$ (7 : 1) [25].

Sections impregnated with silver nitrate were used for planimetric measurements [17, 30] in which the rations of C cells: follicular cells: colloid (acinar lumen): stroma were determined in the thyroid gland. The central part of the lobe of each thyroid was projected from 50 sections on to a Zeiss Visopan (Jena) microprojector with a magnification of 400 : 1. The screen of the Visopan was covered with a 10×10 square grid. A "point count" was performed, which involved the recording of the four thyroid components seen at each of the intersection points of the grid. The count made over 100 points expressed the proportion of C cells: follicular cells: colloid: stroma in per cent. Mean values and standard deviations were calculated in the two groups. For the calculation of significance in the difference between the spring and autumn groups Student's t test was performed.

Results

In autumn animals, the swollen C cells, rich in granules, are prominent with their strong argyrophilia and metachromasia. In the centre of the lobe, the follicles are usually bordered by 6—8, or often by more, C cells (Figs 1, 3, 7). Sometimes groups of C cells can be seen to occupy an apparently interfollicular localization (Fig. 5). C cells extend over a large area in the central part of the lobe, while the marginal zone is void of them (Fig. 3). The measurements of the ratios C cell : follicular cell : colloid : stroma indicated a C cells ratio of $15.36 \pm 2.07\%$ in relation to the other components of the thyroid (Table 1).

In spring animals, the C cells are relatively small, displaying moderate or slight granulation. The granules show weak argyrophilia and metachromasia.

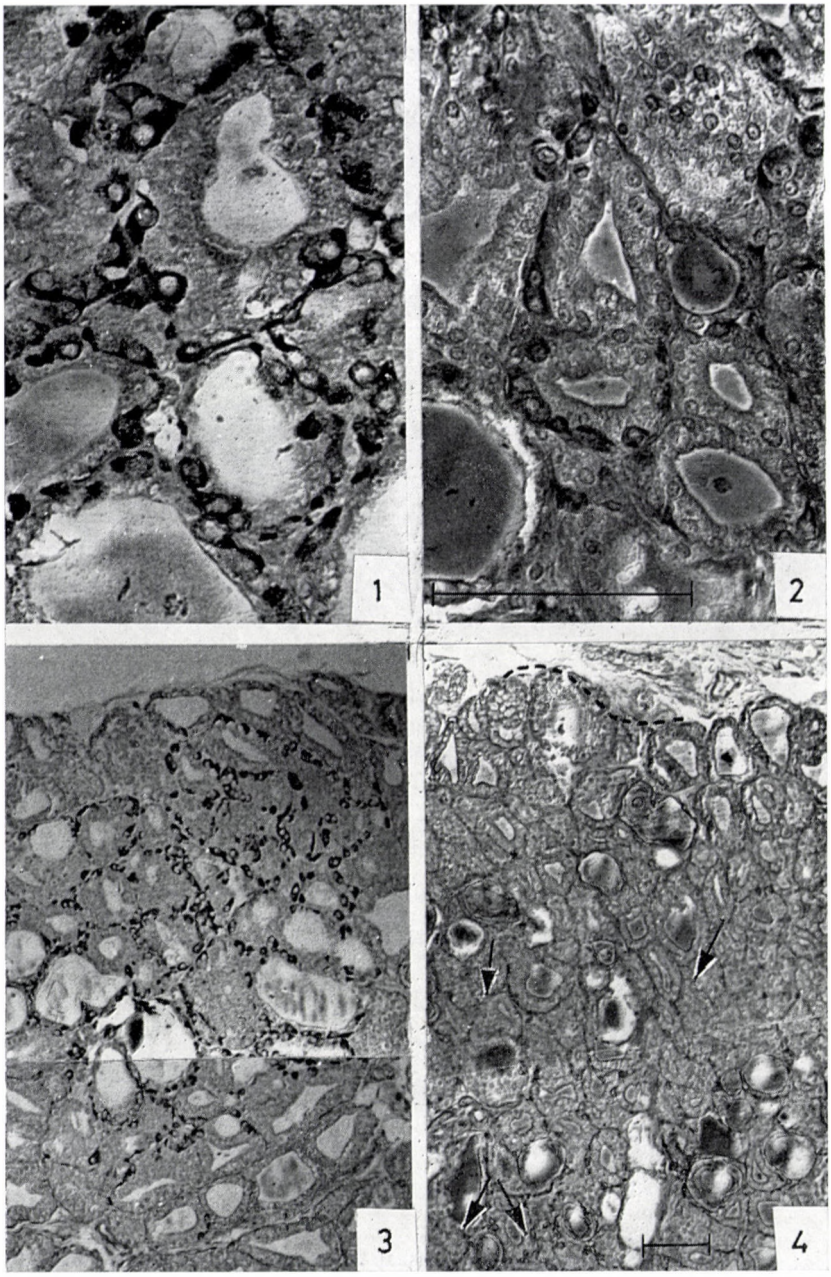

Fig. 1. Rat thyroid. Silver impregnation according to DE GRANDI. In autumn the follicles are bordered by swollen C cells displaying strong argyrophilia

Fig. 2. Rat thyroid. In spring, the follicles are bordered by a zone containing few C cells showing weak argyrophilia

Fig. 3. Same as Fig. 1 with lower magnification. In autumn there is a large area occupied by C cells in the central part of the lobe. In the peripheral narrow zone no C cells are present

Fig. 4. Same as Fig. 2 with lower magnification. In spring, C cells (arrow) occupy a small area in the middle of the lobe, bordered by a broad zone containing no C cells (broken line = margin of the thyroid lobe). Line of calibration = 100 μm

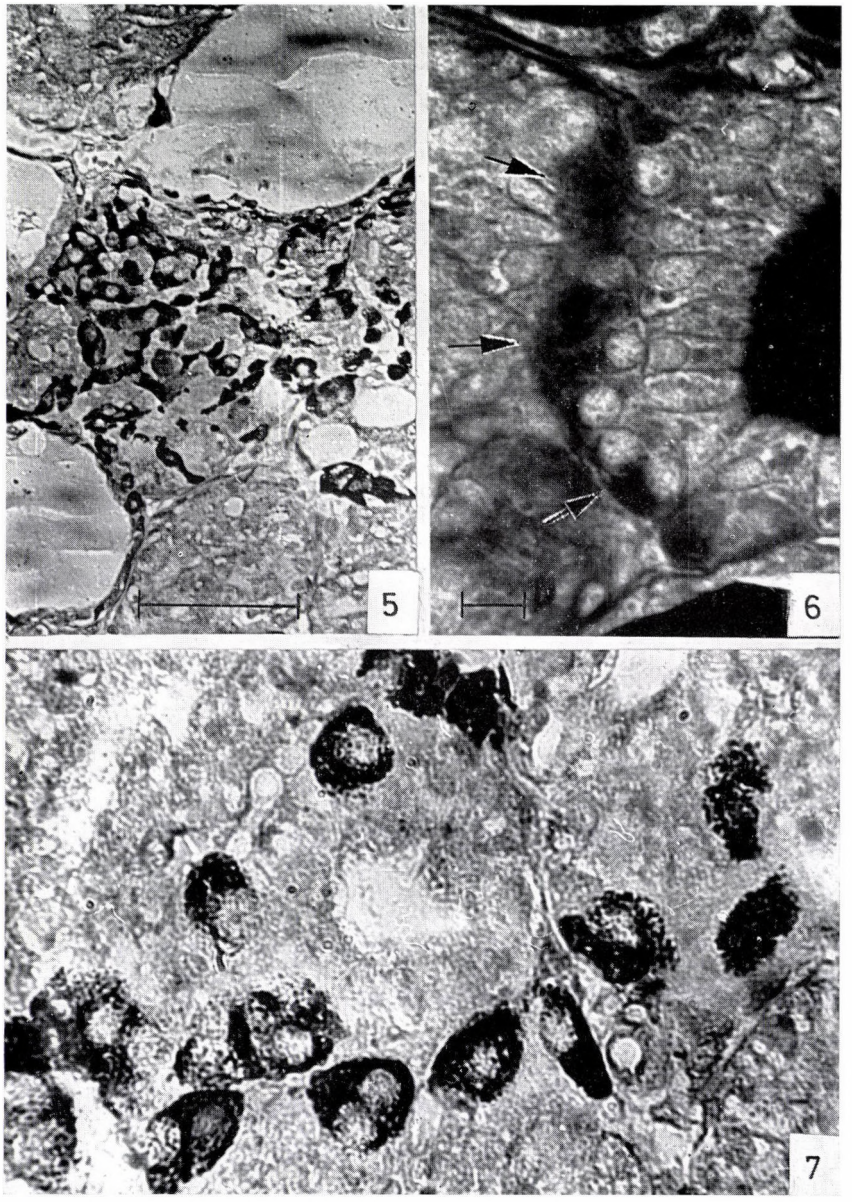

Fig. 5. In autumn, the argyrophilic C cells occasionally form groups which apparently show interfollicular localization

Fig. 6. With toluidine blue staining a pronounced metachromasia is displayed by autumn C cells (arrow)

Fig. 7. In autumn, the C cells are rich in argyrophylic granules. Line of calibration = 100 μm. (Fig. 5). Line of calibration = 10 μm (Figs. 6, 7)

Table 1

C cell : follicular cell : colloid : stroma ratios in autumn and in spring rats

	No. of rats	C cell	F cell	Colloid	Stroma
Autumn animals*	5	15.36±2.07	60.65±3.74	18.03±3.47	5.91±0.95
Spring animals*	7	7.24±1.07 P<0.001	71.79±4.19	13.92±3.47	7.03±0.67

* mean value±SD (%)

In the centre of the lobe the follicles are usually bordered by 1, 2, or at most by 3 or 4 cells (Fig. 2). C cells occupied only a small central area, which were surrounded by large zones void of C cells (Fig. 4). The C cell: follicular cell: colloid: stroma ratio showed that C cells represented 7.24 ± 1.07% of the components (Table 1), and their ratio was significantly smaller ($P < 0.001$) than in the autumn group.

Discussion

The results presented indicate that the thyroid C cells show definite seasonal changes in the rat, that is, their number increases in autumn and decreases in spring. Previous investigations on hibernating animals have revealed cytological differences between winter and summer C cells. Ultrastructural investigations by PEARSE and WELSCH [20] on the hedgehog demonstrated that C cells contain well-developed rough endoplasmic reticulum, large number of free ribosomes, and dense granules in summer. In autumn, the granules increased in number and density. In contrast to this, the rough endoplasmic reticulum was less pronounced and the number of granules decreased in winter. In the bat the largest and most numerous granules were found in August and September (NUNEZ et al. [16]). From October, granules decreased in amount until February, when usually empty vacuoles were seen in the C cells. Recently, FRINK et al. [9] have reported similar seasonal changes in the C cells of the marmot. It was generally found that C cells were highly active in summer and autumn, and almost inactive in winter in hibernating animals. We have recorded similar phenomena in the non-hibernating rat, that is, C cells. showed hyperfunction in autumn and hypofunction in spring. It is, therefore, probable that the phenomenon is not restricted to hibernating animals.

Some clinical observations made on human patients seem to be consistent with the findings described above. The original observation of McCance and Widdowson [13] that the calcium level in the urine is much higher in summer than in winter was corroborated by Robertson et al. [24] on a large number of healthy subjects. The latter authors were of the opinion that a slight increase in calcium intake cannot account for the high calcium excretion in summer. The role of vitamin D may also be questioned because the highest 25-OH-cholecalciferol level of the serum is measured 1—2 months later than the maximum amount of calcium excreted in the urine, that is, in September and October. The seasonal changes in the calcium content of the urine seem to be related to the monthly distribution to sunny hours. Aaron et al. [1] have called attention to an interesting seasonal phenomenon, namely that in patients with femoral-neck fractures the occurrence of osteomalacia is high between February and May, and low between September and November.

Our results supplement literary data in that, in addition to the calcium metabolism and to the levels of vitamin D and the parathormone, calcitonin shows seasonal changes in the rat. This seasonality should be taken into account in experiments dealing with the regulation of calcification.

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A COMPARATIVE STUDY OF DNA-INDUCED TRANSFORMANTS AND SPONTANEOUS REVERTANTS OF INOSITOLLESS *NEUROSPORA CRASSA*

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Abstract

Inositolless *(inl⁻) Neurospora crassa* strains were treated with DNA (allo-DNA) of wild type *N. crassa*. Hyphal fragments of a mycelial suspension of the *N. crassa* ragged *inl⁻* strain used as recipient in our transformation experiments were found to consist of units containing 100—1000 nuclei. In this strain the inositol-independent *(inl⁺)* nuclei appearing after DNA treatment or by spontaneous reversion are present in the cytoplasm together with a large number of *inl⁻* nuclei. Thus, both transformation and reversion initially must result in heterokaryosis. Under appropriate conditions the *inl⁻* nuclei can be detected in the transformed and spontaneous *inl⁺* phenotype revertant strains. Spontaneous revertants are usually characterized by the loss of their *inl⁺* nuclei after transfers on inositol-supplemented medium. On minimal medium, the growth rate of transformed strains is significantly lower than that of spontaneous revertants. The *inl⁺* gene appearing after DNA treatment or by spontaneous reversion is inherited as a trait bound to chromosomes. In crosses with the transformed strains, there is a significant increase in the number of non-Mendelian (6 : 2 and 5 : 3) tetrads of the *inl* locus.

Introduction

The results of DNA-mediated transformation in eukaryotes are difficult to reproduce. Even in those experiments in which an increase in the reversion of some biochemically defective mutants can be detected after treatment with wild-type DNA, the possibility of spontaneous or induced mutations (reversions) cannot be excluded.

During the past few years, considerable data have been published concerning the genetic transformation of N. *crassa* [4, 9, 10, 12, 13, 14].

It has been reported [9] that transformed and spontaneous revertant strains of *N. crassa* behave differently in crosses. It was claimed that the transformed strains often transmit newly-acquired characters to their sexual progeny in a non-Mendelian way. They assumed that it is a donor DNA fragment (or "exosome") which is responsible for the non-Mendelian transmission.

Since the efficiency of the transformation experiments with *N. crassa* is low, a comparative study of DNA-treated (transformed) and spontaneous revertant strains seems to be justified.

On the basis of our recent work, we have concluded that 1. in the transformed strains new features appear that are not characteristic of the spontaneous revertants and 2. the heterokaryotic state which develops during reversion is responsible for the genetic irregularities ("non-Mendelian ratios") of the transformed strains.

Material and methods

Strains. The following strains were obtained from the collection of the Rockefeller University: R 2506-5-101 *(inl⁻, rg⁻, mt^a)*, 89601 *(inl⁻, mt^A)* and RL-3-8 *(mt^A)*. The transformed and the spontaneous revertant strains were obtained from separate experiments carried out in our Institute.

Maintenance and growth of strains. The strains were cultured either on solid or liquid Vogel's minimal medium [16] or supplemented with inositol (100 μg/ml) when required. Crosses were carried out on Westergaard's medium [17].

Designation of DNA preparation. DNA preparation from the donor strain (RL-3-8) carrying the wild-type allele *(inl⁺)* has been designated as allo-DNA.

Genetic analysis. Random spore analysis of the progeny obtained from crosses was performed as follows: an ascospore suspension was heat-shocked at 60 °C for 30 min, then spread on solid minimal medium containing 4% sorbose, and also on inositol-supplemented plates [5]. The number of colonies which appeared on agar plates was counted after incubation in 27 °C for 72 h. For tetrad analysis, 21-day-old perithecea were dissected and ascospores were isolated from complete asci. The morphology and inositol requirement of the colonies grown from the tetrads were studied.

Detection of heterokaryosis by hypha-fragmentation. The spontaneous revertant and the transformed strains obtained from previous experiments were maintained through six transfers both on minimal and on inositol-supplemented media. After the last transfer the entire mycelial content of one slant was cut into fragments in a Waring blendor. The phenotypes and number of the multinuclear fragments were determined both on minimal and on inositol-supplemented agar media after 72 h of incubation.

Determination of the average number of nuclei in the hypha fragments. The DNA content of the different hypha fragment suspensions was determined [6]. The number of the hypha fragments was counted both in Buerker chamber and by viable count. With these data it was possible to calculate the average number of cell nuclei in the hypha fragments.

Determination of the growth-rate of the strains. 8-ml samples (each containing 4×10^4 hypha fragments per ml) were taken from the suspensions of both the transformed and the spontaneous revertant strains and they were grown in 25-ml Erlenmeyer flasks at 27 °C for 72 h at 240 rev/min in a New Brunswick rotatory shaker. After appropriate incubation periods (0 h, 24 h, 48 h, 72 h) the mycelia were washed with distilled water on filter paper and dried at 105 °C for 24 h. The growth rates were estimated as dry weight at 48 h after inoculation per dry weight at 24 h after inoculation.

Results

Ten spontaneous and 15 allo-DNA-induced inositol-independent *(inl⁺)* revertants were crossed with inositol-requiring *(inl⁻)* strains, and the frequency of the *inl⁺* and *inl⁻* ascospores was determined. As shown in Table 1, in the case of the spontaneous revertants the transmission of the revertant *(inl⁺)* character to the sexual progeny differed from the expected Mendelian ratio. The data presented in Table 2 show that in the case of the transformed strains the ratio of *inl⁺* to *inl⁻* ascospores differed from the Mendelian way of inheritance similarly to what we had observed with the spontaneous revertants.

Table 1

Genetic analysis of inl+ revertants obtained without DNA treatment

Strain	No. of progeny		Percent inl+
	inl+	inl−	
1	620	1,180	34.44
2	1,737	15,928	10.80
3	3,275	34,055	8.80
4	2,687	31,643	7.83
5	1,637	25,500	6.00
6	130	2,453	5.00
7	95	1,805	4.10
8	186	7,479	2.43
9	600	26,056	2.25
10	0	915	0.00

inl+ rev. × inl− (89601)

Table 2

Genetic analysis of inl+ revertants obtained with allo-DNA treatment

Strain	No. of progeny		Percent inl+
	inl+	inl−	
1	1,033	2,000	34.30
2	5,533	14,967	26.99
3	340	1,210	21.93
4	3,950	16,550	19.27
5	3,650	19,350	15.87
6	165	1,085	13.20
7	3,100	27,730	10.05
8	175	1,825	8.75
9	3,087	63,743	4.62
10	450	11,550	3.75
11	5,533	14,967	2.00
12	165	11,665	1.39
13	800	68,400	1.10
14	1	1,599	0.062
15	2	5,496	0.036

inl+ rev. × inl− (89601)

From the above data we concluded that in both kinds of revertant strains, in addition to the *inl*$^+$ nuclei, *inl*$^-$ nuclei are present in the same cytoplasm, thus bringing about heterokaryosis.

Since the *inl*$^+$ revertants had been arisen from hyphal fragments of the ragged strain, we wanted to know how many nuclei the fragments contained. By determining the DNA content and the number of the fragments, we found that one colony-forming unit (*i.e.* one fragment) contained 100—1000 nuclei on the average. It was an obvious consequence of the high number of nuclei present in the colony-forming units that every newly-arising *inl*$^+$ nucleus was accompanied by several *inl*$^-$ nuclei, independently of the character of reversion (*i.e.* induced or spontaneous). The initial hypha fragments had to be heterokaryotes.

In the following experiments we wanted to study how the presence or absence of inositol in the culture medium affects the ratio of *inl*$^+$ to *inl*$^-$ nuclei.

Four spontaneous revertants and six transformed strains (both isolated on minimal medium) were grown on minimal and on inositol-supplemented media. After six transfers, the strains were examined for the occurrence of heterokaryosis by establishing the phenotypes of the hypha fragments of the respective colonies. Table 3 shows that the colonies grown from spontaneous

Table 3

Examination of the heterokaryotic state. Inositol requirement of mycelial fragments of revertant strains after six transfers on minimal or inositol-containing media

Revertants	Strain	Colony count			
		Minimal subline		Inositol subline	
		*inl+	*inl+ + inl−	*inl+	*inl+ + inl−
Spontaneous	1	800	800	0	1700
	2	300	300	750	750
	3	400	400	0	450
	4	110	115	0	450
Allo-DNA induced	5	140	210	500	550
	6	65	115	200	540
	7	65	130	9	550
	8	295	340	105	200
	9	270	460	0	5100
	10	230	365	360	1650

* Phenotype

revertants on minimal medium contained only inositol-independent fragments, since an equal number of colonies could be counted on inositol-supplemented medium and on minimal medium.

We found both inositol-requiring and inositol-independent fragments among the transformed strains. This indicated that these originally heterokaryotic strains remained so in spite of the selective pressure; thus, on cutting up the mycelium, the probability of finding fragments containing only *inl*$^-$ nuclei increased. *Inl*$^+$ nuclei might occur but in such a little number that they are not sufficient to support growth on minimal medium. Table 3 also shows that with 3 out of 4 spontaneous revertants grown on inositol-supplemented medium the *inl*$^+$ nuclei disappeared in the course of six transfers, while the transformed strains except strain no. 9, which may be regarded as a spontaneous revertant, preserved their heterokaryotic state even if they were maintained on inositol-supplemented medium.

These results point to some differences between the transformants and the spontaneous revertants which are revealed if we cultivate them in the presence or absence of inositol.

The ratio of *inl*$^+$ to *inl*$^-$ nuclei in the revertants was also determined after the 1st and 6th transfers by crosses.

The data presented in Table 4 reveal that on minimal medium the proportion of *inl*$^+$ nuclei (or ascospores) increases in both revertant groups.

Table 4

Genetic analysis of inl$^+$ *revertants after six transfers on minimal medium*

Revertants	Strain	Percent of inl+ progeny after transfers	
		1	6
Spontaneous	1	16.86	36.03
	2	18.18	53.24
	3	8.48	9.92
	4	7.05	11.95
Allo-DNA induced	5	32.56	51.25
	6	46.89	47.34
	7	8.74	18.50
	8	15.67	36.48
	9	10.54	33.24
	10	10.91	14.19

inl$^+$ rev. $\times$ inl$^-$ (89601)

The data in Table 5 show that the proportion of *inl*$^+$ nuclei is low in both strains on inositol-supplemented medium. Comparing the progeny of these strains obtained after the 1st and 6th transfers, we have concluded that the *inl*$^+$ nuclei are more likely to disappear from the spontaneous revertants than from the transformed strains. (The above experiments with hyphal fragments indicated the same.)

The growth rate of the spontaneous revertant and transformed strains was also studied. The growth rate of the strain used as recipient in the transformation experiments was found to be 4.68 on inositol-supplemented medium (100 μg per ml). Table 6 summarizes the growth rates of 5 spontaneous revertant and 7 transformed strains on minimal and on inositol-supplemented media.

Table 5

Genetic analysis of inl$^+$ revertant strains maintained in the presence of inositol

Revertants	Strain	Percent of inl+ progeny after transfers	
		1	6
Spontaneous	1	2.270	0.000
	2	8.470	39.470
	3	0.530	0.000
	4	0.008	0.000
Allo-DNA induced	5	25.000	37.040
	6	8.670	0.989
	7	1.140	0.058
	8	4.290	15.340
	9	0.400	0.009
	10	6.230	14.670

inl$^+$ rev. $\times$ inl$^-$ (89601)

The growth rate of the spontaneous revertants on minimal medium usually approximates that of the original (recipient) strain, if the latter growth rate was measured in the presence of inositol. The growth rate of the transformed strains on minimal medium is significantly lower than that of the spontaneous revertants ($0.01 > P > 0.05$).

The fact that the strains (both the spontaneous revertants and the transformants) were heterokaryotic, leads us to suppose that it is the heterokaryotic state and not an exosome that is responsible for the non-Mendelian ratio of *inl*$^+$ and *inl*$^-$ ascospores in the sexual progeny.

Table 6

Growth rate of inl⁺ strains*

Strains	On minimal media	On inositol supplemented media
Spontaneous revertants (5 strains)	average: 4.36±1.28 S. E. M. 0.45	average: 4.52±1.76 S. E. M. 0.62
Transformants (7 strains)	average: 3.08±0.71 S. E. M. 0.27 t: 2.4445 0.05>P>0.01	average: 3.14±0.91 S. E. M. 0.35 t: 1.9403 0.1>P>0.05

* dry weight at 48 hr/at 24 hr

The assumed presence of allo-DNA in the recipient chromosome or as an exosome was also studied by tetrad analysis. Two transformants, one spontaneous revertant and the RL-3-8 *inl*⁺ strain, were crossed with the 89601 *inl*⁻ strain. The results in Table 7 show that in the transformed strains no. 5 and no. 6 a large number of inositol-requiring asci were found, due to heterokaryosis. Beside the regular Mendelian *(inl*⁺ and *inl*⁻*)* tetrads, some non-Mendelian tetrads were detected. The latter might have arisen as a result of gene con-

Table 7

Tetrad analysis

Cross	Ascus types inl⁺ : inl⁻					Total
	4:4	6:2	2:6	5:3	0:8	
No. 5 transformant (inl⁺) × Standard strain (inl⁻)	17	1	2	2	32	70
No. 6 transformant (inl⁺) × Standard strain (inl⁻)	19	1	—	—	14	34
No. $T_{14}K_2$ Spontaneous revertant (inl⁺) × Standard strain (inl⁻)	29	—	—	—	—	29
Control RL-3-8A (inl⁺) × R 2506-5-101 (inl⁻)	29	—	—	—	—	29

Standard strain: 89601-5-5A (inl⁻)

version. Non-Mendelian tetrads could not be detected in the crosses of the spontaneous revertant and of the standard strains.

The data obtained by the tetrad analysis led us to the conclusion that the transmission of the *inl*$^+$ character to the sexual progeny was normal both in the transformed and in the spontaneous revertant strains. The frequency of the recombinant (2 : 2 : 2 : 2) tetrads suggest that both in the transformants and in the spontaneous revertants the *inl*$^+$ genes are at the original *inl* locus (Table 8). The increase of "gene conversion" of transformants significantly exceeds the reported value [15].

Table 8

Centromer—gene distance in crosses of inl$^+$ × inl$^-$ strains

Cross	Recombinants** / No. tested	Map units***
No. 5 transformant (inl$^+$) × Standard strain (inl$^-$) *	16/38 (asci)	21.0
No. 6 transformant (inl$^+$) × Standard strain (inl$^-$) *	7/20 (asci)	17.5
$T_{14}K_2$ spontaneous rev. × Standard strain (inl$^-$) *	14/29 (asci)	24.1
RL-3-8 (inl$^+$) × R 2506-5-101 (inl$^-$)	14/29 (asci)	24.1

* Standard strain: 89601-5-5 (inl$^-$, mt^A)

** Recombinants = ascus type 2:2:2:2 (inl$^+$:inl$^-$:inl$^+$:inl$^-$)

*** Map units = $\frac{\text{Number of recombinants}}{\text{Total number of asci tested} \times 2}$

Discussion

Various experiments have been proposed [8] to show whether eukaryotic cells can be transformed by DNA; *e.g.*, to trace the intracellular fate of the donor DNA, or to detect recombinant DNA. Many research-workers claim, however, that the efficacy of the transforming DNA can be evaluated only through the manifestation of the character coded [2].

Eukaryotic transformation is difficult to evaluate because of the low frequency of transformants and because of the possibility of spontaneous reversions. Spontaneous reversion may be eliminated by the use of recipient

strains carrying large deletions of the appropriate gene. But in such experiments, even in the case of bacteria, the frequency of transformants falls by 2—3 orders of magnitude [1].

In the present transformation experiment, the *inl*$^+$ revertants induced by DNA treatment were found to possess features distinguishing them from spontaneous revertants.

The transformed and the spontaneous revertant heterokaryotes, when maintained on selective or non-selective medium, differ from each other. Six transfers on minimal medium do not eliminate *inl*$^-$ nuclei from the transformant strains to the extent as in the case of spontaneous revertants. On inositol-supplemented media *inl*$^+$ nuclei of transformed strains, less frequently, disappear.

Studying the growth of the two strains we found that the spontaneous revertants grow similarly to the recipient strain, but their rate of growth on minimal medium is significantly higher than that of the transformed strains. The low growth rate of the transformed strains might be explained by the fact that allo-DNA may integrate into different genome parts, thus exerting a non-specific mutagenic effect as well [11].

Non-Mendelian ratios observed in crosses of transformant strains were explained by MISHRA and TATUM [9] with FOX's [3] exosome model. They have assumed that there is a close association between the donor DNA and the genome of the recipient but during meiosis the non-integrated exosomes are often eliminated by nucleases.

We think that before applying the exosome model one should try to explain the results by assuming the heterokaryotic state because it follows from the multinuclear state and the coenocytic nature of *N. crassa* that it becomes heterokaryotic when the hyphal fragments are used in transformation experiments irrespective of the nature of reversion. In the heterokaryotic cell, nuclei carrying dominant as well as nuclei carrying recessive alleles are present in the same cytoplasm.

Several transfers on selective and non-selective media show that transformed heterokaryotic mycelia remain heterokaryotic even under selective pressure.

Our results also suggest that the *inl*$^+$ genes of both the transformant and revertant strains are located at the original inositol locus.

In the case of the transformed strains, the increasing number of tetrads exhibiting gene conversion suggests that during meiosis gene conversion is probably promoted by the new structure of DNA that developed from the recipient genome and the transforming DNA. This supposition is supported by the fact that the number of tetrads exhibiting gene conversion increases neither in the crosses of the spontaneous revertants nor in those of the standard laboratory strains.

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HEAVY METAL BINDING PROPERTIES OF EARTHWORM CHLORAGOSOMES

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Abstract

The highest concentration of metal in *Lumbricus terrestris* and *Lumbricus rubellus* chloragosomes was Ca^{++} followed by Fe^{+++}, Zn^{++} and Mg^{++}. Chloragosomes and body wall extracts bound Pb^{++} and to a lesser extent Cu^{++} but not Zn^{++}. Binding of Pb^{++} and Cu^{++} was greater in chloragosomes than body wall homogenate. Maximum binding capacity for Pb^{++} was higher in *Lumbricus terrestris* than *Lumbricus rubellus*. The binding of Pb^{++} to chloragosomes appears to be unrelated to phospholipid content. The release of most Zn^{++} and Ca^{++} in chloragosomes by 0.01 *M* HCl but release of Fe^{+++} requiring 5 *M* HCl suggests that the latter may be in the form of an organometallic complex. Release of Cu^{++} from chloragosomes by 0.001 *M* HCl but only 20% of the bound Pb^{++} indicates that Pb^{++} is more firmly bound to chloragosomes than Cu^{++}. Bound Pb^{++} in body wall extracts is released by 0.001 *M* HCl. The addition of Pb^{++} to a chloragosomal suspension caused a drop in pH and release of mainly Ca^{++} with smaller amounts of Zn^{++} and Mg^{++} indicating a cation exchange was taking place. It is concluded that earthworm chloragosomes bind heavy metal by a cation-exchange system which may be related to the chemical constituents of chloragosomes being similar to ion exchange compounds.

Introduction

Earthworms have been shown to take up and accumulate heavy metal [12, 14, 29]. A more detailed study of the earthworms *Dendrobaena rubida* and *Lumbricus rubellus* indicates that Pb^{++} is deposited in the chloragogenous tissue around the intestine and associated with electron dense cell organelles called chloragosomes and debris vesicles [15, 17].

The chloragosomes contain carbohydrates [3], proteins [4], lipids and phospholipids [7, 26, 28]. They also contain some redox pigments such as flavins, thiamine, carotenoids and metalloporphyrins [7, 21, 26, 28]. Chloragosomes have been shown to display cation-exchange properties and redox activity [6, 8].

The purpose of the present study was to investigate the inorganic nature of isolated earthworm chloragosomes, their affinity for heavy metals and cation-exchange properties.

* Part of the work was done while the author was on an Academic Interchange Grant from the British Council to visit the Department of Zoology, Teachers' Training College, Pécs.

Material and methods

The earthworms *Lumbricus terrestris* were collected at Aberystwyth and *L. rubellus* from sewage beds on Keele University campus. All earthworms were kept in their native soil at 15 °C for at least a week before the commencement of an experiment.

Preparation of chloragosomes and body wall extract. Chloragosomes were prepared by isolating the gut, brushing off the chloragocytes into isotonic saline and washing three times in distilled water [24]. The final insoluble residue was made up in distilled water to give a concentration of 10 mg, 20 mg or 40 mg wet weight/ml.

Fresh body wall was washed in isotonic saline, homogenised in distilled water and made up to give a final concentration of 10 mg wet weight/ml.

Binding of Pb^{++}, Cu^{++} and Zn^{++} to chloragosomes and body wall. Freshly prepared chloragosomes and body wall homogenates *of L. rubellus* and *L. terrestris* were made up to give a final concentration of 10 mg wet weight/ml. An aliquot of 0.2 ml was made up to 2 ml with distilled water to which was added an equal volume of $PbCl_2$. The final concentration of Pb^{++} in *L. terrestris* extracts was 10, 12.5, 25, 50, 75 μg/ml and the final concentration of Pb^{++}, Cu^{++} and Zn^{++} in the chloride salts used in *L. rubellus* extracts was 2.5, 5, 12.5, 25, 37.5 and 50 μg/ml. All preparations were shaken at 75 rev/min for 10 min at 15 °C, centrifuged and metals estimated in the supernatant.

Release of metals by HCl. Increasing concentrations of HCl (4 ml) were added to a freshly prepared aqueous suspension of *L. terrestris* chloragosomes (10 mg wet weight). The preparations were shaken for 10 min at 15 °C and centrifuged. This procedure was repeated for each concentration of HCl studied. Body wall homogenates and the chloragosomal fractions used to study the binding capacity of Pb^{++}, Zn^{++} and Cu^{++} were pooled for each metal (4 mg wet weight), washed three times in distilled water and the release of the metals from the residue by increasing concentrations of HCl studied. Metals were estimated in the supernatant fractions.

Release of metals and pH change after the addition of Pb^{++}. An equal volume of $PbCl_2$ was added to a fresh aqueous suspension of chloragosomes and stirred at 75 rev/min for 10 min at 15 °C, centrifuged and the metals estimated in the supernatant fraction. In separate experiments the pH was monitored continuously using the same experimental procedure.

Preparation of phospholipid-depleted chloragosomes. Phospholipid depleted chloragosomes were prepared according to the method described by Fleischer, Fleischer & Stoeckenius [10] using 4.9 ml of a mixture of acetone (Aristar) 10% distilled water and ammonia and 0.1 ml of chloragosome suspension containing 20 mg wet weight/ml. Control chloragosomes were treated with the same volume of distilled water. All preparations were left for 5 min, with intermittant shaking and centrifuged. Residues were dried in air for 10 min and resuspended in 2 ml distilled water to which was added an equal volume of $PbCl_2$ to give a final concentration of 50 μg/ml Pb^{++}. The chloragosomal suspensions were shaken for 10 min at 15 °C, centrifuged and the Pb^{++} estimated in the supernatant.

Chemical determinations. Metals were estimated using an EEL Atomic Absorption Spectrophotometer with standards made up to HNO_3, HCl or as the chloride salts where appropriate. Samples used to estimate Ca^{++} contained 0.65% $LaCl_3$ to suppress Fe^{+++} and PO_4^{---} interference. Total metal in the chloragosomes was estimated after drying at 105 °C for 48 h and extracting in concentrated HNO_3 (Aristar). Phosphate was estimated by the colorimetric method of Rockstein & Herron [23].

Protein was estimated in chloragosomal suspensions and body wall homogenates using the method of Lowry et al. [19] with bovine serum as standard.

Results

Metal content of earthworm chloragosomes

Statistical analyses of metals between species was not considered meaningful since they were collected at different sites and diet/soil metal content could affect the tissue metal concentrations. All data presented, except for

binding capacity, are the results of duplicate analyses on separate specimens.

As shown in Table 1, the main divalent cationic constituent of the chloragosomes of *L. terrestris* and *L. rubellus* is Ca^{++} followed by Fe^{+++}. Of the metals analysed Mg^{++} is the lowest. No Pb^{++} or Cu^{++} could be detected in the chloragosomes of either species before experimentation.

Table 1

Cation content of isolated earthworm chloragosomes

Metal	*L. terrestris* (mg/g protein)	*L. rubellus* (mg/g protein)
Ca^{++}	131.3	199.6
Fe^{+++}	87.9	90.3
Zn^{++}	25.1	83.0
Mg^{++}	18.4	13.5

Cation binding to chloragosomes and body wall

When Cu^{++}, Pb^{++} or Zn^{++} are incubated in the presence of a chloragosomal suspension or body wall homogenate for 10 min at 15 °C, except for Zn^{++}, different amounts of metal are retained at different concentrations of the metal in the incubation medium. The amounts of metal bound to 2mg wet weight of *L. rubellus* material is presented in Figs 1A, B. Lead and Cu^{++} results represent a single experiment using the same material, therefore a direct comparison can be made. The amount of Pb^{++} bound to both chloragosomes and body wall, when the concentration was similar in the external medium (μg/g protein), was greater than Cu^{++}. Except when 221μg Pb^{++} was present in *L. rubellus* body wall homogenate there was less Pb^{++} and Cu^{++} bound than to chloragosomes, despite the fact that 2mg wet weight of body wall homogenate contained 226.4μg protein and 2mg wet weight of chloragosomal suspension contained 48.8μg protein. The anomalous result with 221μg Pb^{++}/g protein and the high binding to body wall homogenate was not repeated for *L. terrestris* body wall homogenate where only 26μg Pb^{++} was bound and at all concentrations of Pb^{++} studied in *L. terrestris* (μg/g protein) less Pb^{++} was bound to body wall homogenates than to chloragosomes.

Zinc binding results of *L. rubellus* were the product of another experiment where 2mg wet weight of chloragosomes contained 76μg protein and 2mg wet weight of body wall extract contained 140μg protein. Zinc did not appear to bind to either chloragosomes or body wall.

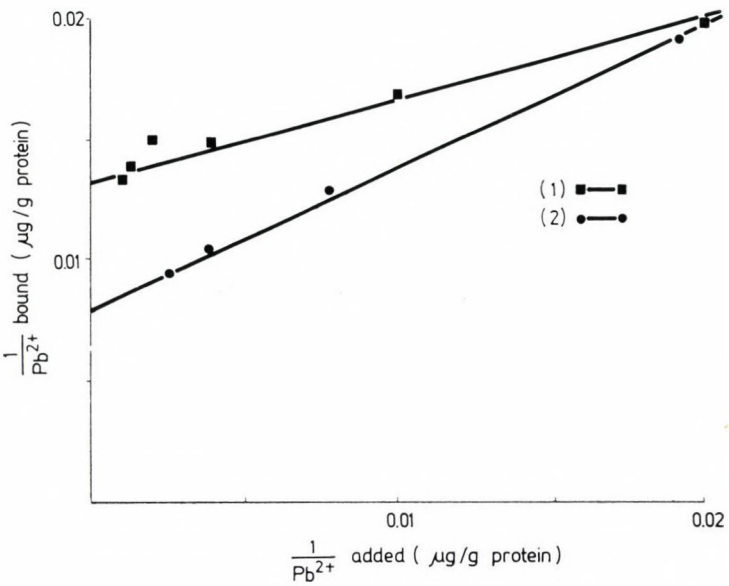

Fig. 1. The binding of Pb^{++} (1) and Cu^{++} (2) to 2 mg wet weight of *L. rubellus* chloragosomal suspension (A) and body wall homogenate (B). Shaken at 75 rev/min for 10 min at 15 °C in the presence of 51, 102, 256, 512, 768 and 1025 μg Pb^{++} and Cu^{++}/g protein in the chloragosomal fractions and 11, 22, 55, 110, 165 and 221 μg Pb^{++} and Cu^{++}/g protein for body wall homogenates

Binding capacity of Pb^{++} in L. terrestris and L. rubellus

Because of the high affinity earthworm chloragosomes show for Pb^{++} it was decided to estimate the maximum binding capacity. A double-reciprocal plot of the data for Pb^{++} added (μg/g protein) against Pb^{++} bound (μg/g protein) results in a straight line. The intercept on the ordinate is the maximum binding capacity. Under the conditions reported in Methods, the calculated maximum binding capacity for isolated *L. terrestris* chloragosomes was 120μg Pb^{++}/g protein and for *L. rubellus* was 75μg Pb^{++}/g protein (Fig. 2).

Cation binding and phospholipids

After depletion of the phospholipids from *L. terrestris* and *L. rubellus* chloragosomes the degree of Pb^{++} binding was in no way altered. Lipid "free" and normal chloragosomes isolated from *L. terrestris* bound over 91% of the Pb^{++} added, while the treated and untreated chloragosomes of *L. rubellus* bound over 86% of the Pb^{++} added.

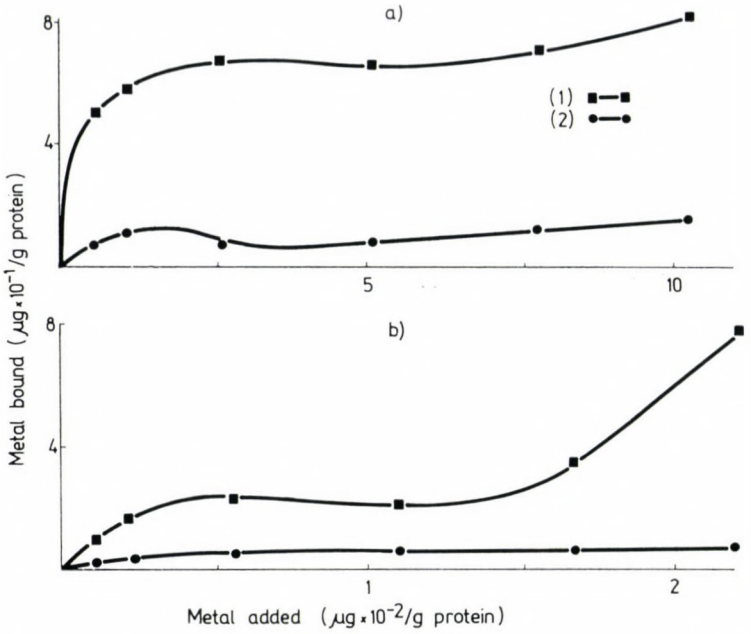

Fig. 2. A double-reciprocal plot to give the maximum binding capacity of Pb^{++} to 2 mg wet weight of a chloragosomal suspension from *L. terrestris* (1) and *L. rubellus* (2). Shaken at 75 rev/min for 10 min at 15 °C in the presence of 52, 65, 130, 260 and 391 μg Pb^{++}/g protein for *L. terrestris* and 51, 102, 256, 512, 768 and 1025 μg Pb^{++}/g protein for *L. rubellus*

Release of metals by HCl

The results are summarised in Table 2.

Table 2

Effects of HCl on the release of metals from isolated earthworm chloragosomes and body wall homogenates

L. terrestris

Eluant	Normal chloragosomes			Pb++ loaded chloragosomes (%)
	Zn++ %	Fe+++ %	Ca++ (%)	
H_2O	0.4	0.0	3.5	0.0
0.01 *M* HCl	96.6	0.0	95.5	93.6
0.1 *M* HCl	2.0	0.0	1.0	6.4
1 *M* HCl	1.0	25.0	0.0	0.0
5 *M* HCl	0.0	75.0	0.0	0.0

L. rubellus

Eluant	Pb^{++} loaded chloragosomes (%)	Cu^{++} loaded chloragosomes (%)	Body wall homogenate (Pb^{++} loaded) (%)
H_2O	0.0	0.0	0.0
0.001 *M* HCl	21.1	100.0	78.4
0.01 *M* HCl	56.1	0.0	21.6
0.1 *M* HCl	22.8	0.0	0.0

The ability of the chloragosomes to retain metals appears to depend on the nature of the metal under investigation. Only a small percentage of the Zn^{++} and Ca^{++} was present in *L. terrestris* chloragosome supernatant fraction after washing with distilled water but the effect of increasing concentrations of HCl on normal chloragosomes showed Zn^{++} and Ca^{++} to be solubilised in 0.01 *M* HCl while Fe^{+++} is not fully released until a concentration of 5 *M* HCl is used on *L. terrestris* chloragosomes.

Chloragosomes used to investigate the ability of these organelles to bind Pb^{++} and Cu^{++}, which would therefore be loaded with the respective metal, released all the Cu^{++} in 0.001 *M* HCl. Lead loaded chloragosomes appear to hold the metal more firmly since only about 21% of the bound Pb^{++} is released in 0.001 *M* HCl for *L. rubellus*. *L. terrestris* shows a similar result for Pb^{++} loaded chloragosomes.

Body wall homogenate of *L. rubellus* binds Pb^{++} much less firmly than chloragosomes and over 78% of the bound Pb^{++} is released in 0.001 *M* HCl.

Metal release from chloragosomes by Pb^{++}

The addition of $PbCl_3$ to give a concentration of 40μg Pb^{++} to 40mg wet weight of *L. terrestris* chloragosomes or 2mg protein caused a drop in pH. There was also a release of 9mg Ca^{++}/g protein, 0.15mg and 0.18mg/g protein of Zn^{++} and Mg^{++}, respectively. A similar experiment with *L. rubellus* chloragosomes showed that on addition of $PbCl_2$ (100 μg Pb^{++}, pH 5.39) to give a concentration of 50 μg Pb^{++} to 20mg wet weight or 440 μg protein caused a drop in pH from 6.82—5.21 in 10 min. As in the case of *L. terrestris*, there was more Ca^{++} released (11.4 mg/g protein) than Zn^{++} and Mg^{++} which were 2.7 mg and 0.38 mg/g protein, respectively. No metal was detected in the final distilled water wash when preparing the chloragosomal fraction. The percentage distribution of metals in the chloragosomal fraction and the supernatant are summarised in Table 3.

Table 3

Effects of added Pb^{++} on the release of metals from isolated chloragosomes

Material	Zn^{++} (%)	Mg^{++} (%)	Ca^{++} (%)	Pb^{++} (%)
	L. terrestris			
Chloragosome fraction	98.6	90.0	90.8	100.0
Supernatant	1.4	10.0	9.2	0.0
	L. rubellus			
Chloragosome fraction	88.8	88.9	69.1	58.7
Supernatant	11.2	11.2	30.9	41.3

Release of PO_4^{---} by added metal or HCl

The total amount of HNO_3 extractable PO_4^{---} in body wall is 5.3 mg/g protein compared with 94.8 mg/g protein in *L. terrestris* chloragosomes and 11.1 mg/g protein in *L. rubellus* body wall compared with 188 mg/g protein in isolated chloragosomes. Since high concentrations of PO_4^{---} were present in chloragosomes and if they were released they would form insoluble compounds with Pb^{++} and Cu^{++}, it was decided to investigate this aspect separately.

The addition of $PbCl_2$ to give a final concentration of 50 μg Pb^{++} to 10 mg wet weight of *L. terrestris* chloragosomes or 840 μg protein was ineffective in causing the release of PO_4^{---} into the supernatant fraction after stirring at 75 rev/min for 10 min and centrifuging. Similar results were found using 50 μg Cu^{++}. To verify that the negative results were not simply the result of spinning out the insoluble PO_4^{---} on centrifugation, Pb^{++} and Cu^{++} treated chloragosomes were exposed to 0.01 *M* HCl and the PO_4^{---} content of the supernatant was the same as the PO_4^{---} content of a sample of the same batch of chloragosomes not treated with Pb^{++} or Cu^{++} but exposed to 0.01 *M* HCl. The amount of extractable PO_4^{---} was 43.7 mg PO_4^{---}/g protein.

Attempts to do similar Pb^{++} and Cu^{++} experiments on body wall homogenates were impaired by precipitation during the estimation of PO_4^{---} when high concentrations of tissue extract were used (50 mg wet weight or 6.45 mg protein). It can be concluded from these experiments that a certain amount of soluble PO_4^{---} is present in the fresh body wall homogenate supernatant after centrifugation. At the concentration of body wall homogenate used in this study (10 mg wet weight) no PO_4^{---} could be detected.

Discussion

In the present investigation buffering agents were not used because the anions of most buffer solutions act as competitors for the metal ions. CLARKSON & KENCH [1], investigating the uptake of Pb^{++} by human erythrocytes, showed 84% of the added metal to be precipitated in Krebs-Ringer bicarbonate buffer. Unpublished observations have shown SORENSON's phosphate buffer to precipitate 75% of the Pb^{++} in a homogenate of *D. rubida* containing high levels of tissue Pb^{++} [16]. PIPES (piperazine-N N'-bis-2-ethanesulphonic acid) has a pK value of 6.8 and does not bind Mg^{++}, Ca^{++}, Mn^{++} or Cu^{++} [13] but it was not considered because the metal-buffer binding constants of Pb^{++} and Zn^{++} require further investigation. It was decided to rely solely on the fact that a freshly prepared aqueous suspension of the chloragosomes of *L. terrestris* and *L. rubellus* was pH 6.8.

It is interesting that the major cation species in earthworm chloragosomes is Ca^{++}. WALKER et al. [32] and WALKER [31] studying the deposition of Zn^{++} and Cu^{++} in *Balanus balanoides* found that the "zinc" and "copper" granules also contained Ca^{++}. It would be informative to know whether the Zn^{++} and Cu^{++} substitute for Ca^{++} in this system.

Lead appears to bind to chloragosomes and body wall homogenates in much higher concentrations than Cu^{++} while Zn^{++}, in the concentration used, does not appear to be bound. A similar situation is found in plasma where the plasma proteins have a greater affinity for Pb^{++} and Cu^{++} than for Zn^{++} [22], and WALDREN & SOFTEN [30] have shown Pb^{++} to have a greater affinity for binding with the SH group of the enzyme delta-aminolaevulinic acid dehydratase (Ec 4.2.1.24.) than Zn^{++}. The apparent nonbinding of Zn^{++} to body wall homogenate and chloragosome suspension may simply be a case of Zn^{++} substitution. FUCHS [11], in his study of the ion exchange properties of Ca^{++} bound to the protein troponin, has shown that protein bound $^{45}Ca^{++}$ can be completely exchanged with non-radioactive Ca^{++}. In the present study, Zn^{++} is found in both extracts. (*L. rubellus* body wall, 0.3 mg/g protein) and if Zn^{++} in the material exchanged with the Zn^{++} added there would be no appreciable change in the concentration of this metal in the supernant fractions. Since body wall homogenates contain proteins with SH groups which bind Pb^{++} and Cu^{++} [22] it is surprising that body wall extracts do not bind more Pb^{++} and Cu^{++} than chloragosomes. On the other hand, FISCHER [6] has stated that earthworm chloragosomes contain phosphoric acid, carboxyl, phenolic hydroxyl and sulphonic acid groups, all of which are constituents of ion exchange compounds [18].

According to WATANABE & YANA [33], red blood cells have an affinity for Pb^{++} which probably binds phospholipids and lipoproteins of the cell surface. Earthworm chloragosomes contain phospholipids [7, 25, 26], and

in the present study phospholipid depletion of the chloragosomes does not affect Pb^{++} binding. Chloragosomes contain histidine, alanine, glutamic acid and aspartic acid [4], but their involvement in the binding of Pb^{++} is probably minimal since all these compounds show the binding constant for Pb^{++} to be less stable than for Cu^{++} or Zn^{++} [27].

As pointed out by FISCHER [5, 6], one of the functions of earthworm chloragosomes is the cation-binding of toxic ions such as atebrin and trypaflavine in exchange for H^+ in H^+-chloragosomes. Calcium will also release H^+ from chloragosomes. He states that the binding of these acridine derivatives to body wall homogenates is not so strong as the binding to chloragosomes and he assumes that trypaflavine is bound in body wall homogenates mainly by VAN DER WALLS' forces but the binding mechanism of chloragosomes is based on cation exchange. In the presented results, a similar conclusion can be drawn from the release of Pb^{++} but not Cu^{++} from body wall homogenates and chloragosomes by HCl. Iron, on the other hand, has been reported to be concentrated in chloragosomes [2] and the fact that high concentrations of HCl are required to release it supports the suggestion of FISCHER [7] that it may be in the form of a metalloporphyrin. The addition of Pb^{++} to a fresh aqueous suspenion of earthworm chloragosomes causes a drop in pH which lends itself to a cationic exchange [20] but this further supported by the release of Ca^{++} together with small amounts of Zn^{++} and Mg^{++}. Phosphate ions do not appear to be involved in this reaction.

Earthworm chloragosomes therefore appear to function as a cation-exchange system capable of taking up and retaining heavy metals and particularly Pb^{++}, thus reducing their toxic effects on the animals' metabolism. These metals may be subsequently excreted by fractionation of the chloragosome containing cells, the chloragocytes, as suggested by FISCHER[9] for the excretion of carbamate pesticides in earthworms.

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EFFECT OF Pb^{++} ON Fe^{+++} TISSUE CONCENTRATIONS AND DELTA-AMINOLAEVULINIC ACID DEHYDRATASE ACTIVITY IN *LUMBRICUS TERRESTRIS*

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Abstract

Iron was found at the highest concentration in the chloragogenous tissue with smaller amounts in the intestine and the lowest concentration in the body wall. Earthworms treated with Pb^{++} for 20 days showed a decrease in the concentration of Fe^{+++} in the intestinal tissue but no significant changes in the other tissues. The enzyme, delta-aminolaevulinic acid dehydratase (ALAD) was not detected in isolated chloragosomes but it was found in the chloragocyte cytosol, body wall and intestine. *L. terrestris* treated with Pb^{++} showed a decrease in the activity of ALAD in all tissues where it was located and particularly in the chloragocyte cytosol.

Introduction

Lumbricidae contain haemoglobin as their respiratory pigment, and according to DELKESKAMP [2], the chloragogenous tissue of oligochaetes is the site of haem biosynthesis. The annelid haemoglobin biosynthetic pathway has been extensively studied in the polychaetes [15, 20] and the enzyme delta-aminolaevulinic acid dehydratase (ALAD), which is involved in the conversion of delta-aminolaevulinic acid (ALA) to porphobilinogen, has been reported in various tissues of the polychaete, *Neoamphitrite figulus* [1]. MACRAE and BOGORAD [22] have demonstrated a similar conversion by whole body homogenates of the oligochaete, *Allolobophora foetida* after addition of ALA. DELKESKAMP [3] has shown homogenates of *Lumbricus terrestris* body wall, gonads, intestine and chloragogenous tissue to synthetise porphyrins after the addition of porphobilinogen and the rate of synthesis was highest in the chloragogenous tissue.

Lead in humans suffering from lead-poisoning has an inhibitory effect on the enzyme ALAD, and this was first reported by LICHTMAN and FELDMAN [17]. It has more recently been shown that Pb^{++} can inhibit this enzyme in amphibia and fish [11, 14].

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The purpose of the present investigation was to examine the distribution of Fe^{+++} and ALAD in the earthworm *L. terrestris* and evaluate the effects of induced Pb^{++}.

Material and methods

L. terrestris, 2—3 animals were kept in 1 litre jars containing filter paper discs (Whatman No. 1, 24 cm) for 20 days at 15 °C in the dark. The filter paper was saturated with 1.0 μg Pb^{++}/100 ml as the chloride salt made up in tap water (20 ml). Tap water was used for controlling earthworms. Since the Pb^{++} precipitated on contact with the tap water, the solution and the filter paper were changed daily. At the concentration of Pb^{++} selected the earthworms continued to feed since on filter paper faecal deposits were observed. Higher concentratios of Pb^{++} inhibited feeding.

Metal analysis of the medium after leaving overnight gave the following composition:

Metal	mg/100 ml
Na^{+}	255.80
K^{+}	26.40
Ca^{++}	0.79
Pb^{++}	0.12

The addition of Pb^{++} to the medium had no effect on the levels of Na^{+}, K^{+}, Ca^{++}.

Preparation of earthworm extracts. Earthworms were anaesthetised in 15% ethanol and dissected to expose the gut. Chloragocytes were isolated by brushing the surface of the intestine into isotonic saline, centrifuged twice, and the supernatant discharded. The intestinal tissue was repeatedly brushed with isotonic saline to remove any adhering blood vessels and nephridia and finally rinsed in saline. Chloragosomes were prepared by adding distilled water to the chloragocyte fraction, to rupture the cells, and homogenised. After centrifugation, the supernatant was retained as the chloragocyte cytosol fraction and the chlroagosome fraction washed a further two times.

Preparations used to estimate Fe^{+++} were homogenised in 6.0 ml distilled water, an aliquot taken for the estimation of protein, the rest dried at 105 °C for 48 h and the metal extracted with concentrated nitric acid. For the estimation of ALAD, body wall and intestine were homogenised in 6.0 ml phosphate buffer pH 7.0. The chloragosomes were washed twice in phosphate buffer and made up to 6.0 ml with buffer and the cytosol fraction made up with an equal volume of phosphate buffer to 6.0 ml. All preparations were kept at 4 °C.

Assay of ALAD. After removal of an aliquot for protein estimation, 2.5 ml of fresh homogenate was incubated at 37 °C for 2 h in the presence of 0.5 ml buffered ALA (2 mg/ml). The reaction was stopped by adding an equal volume of 10% trichloracetic acid containing 0.1 M mercuric chloride, to suppress interference from SH compounds [27]. After centrifugation, 3.0 ml of supernatant were added to 3.0 ml Ehrlich reagent (1 g para-dimethylaminobenzaldehyde in 34 ml glacial acetic acid and 16 ml 70% perchloric acid) and the absorbance read at 555 nm within 5 min to give the amount of porphobilinogen formed. All samples were read against homogenate samples treated in the same way but without added ALA. The amount of material present was calculated from the molar extinction coefficient [25].

Chemical estimations. Iron, Ca^{++} and Pb^{++} were estimated by atomic absorption analysis after extraction in concentrated nicric acid. Sodium and K^{+} were measured by flame photometry. Protein was estimated by the method of LOWRY et al. [19].

Results

Tissue distribution of Fe^{+++}

As shown in Table 1, the highest concentration of Fe^{+++} was found in the chloragosomes together with high amounts in the chloragocyte cytosol.

The combined values would give the total amount of Fe^{+++} present in the chloragogenous tissue. The lowest value for Fe^{+++} was found in the body wall extracts but the values were not significantly different after the earthworms had been treated with Pb^{++}. The amount of Fe^{+++} in the intestinal fraction was lower than in chloragogenous tissue and the concentration of Fe^{+++} in the intestine of *L. terrestris* treated with Pb^{++} was significantly lower than in control earthworms.

Table 1

Distribution of Fe^{+++} in earthworm tissue homogenates after treatment with 1.0μg Pb^{+++}/100 ml for 20 days (mg/g protein, n=3)

Tissue	Treatment		
	Pb^{++}	control	P
Chloragocyte cytosol	5.40±0.79	4.89±0.49	N. S.*
Chloragosomes	5.69±1.69	5.74±0.57	N. S.
Intestine	1.44±0.08	2.30±0.19	<0.02
Body wall	0.22±0.07	0.18±0.07	N. S.

* N. S.=not significant

Table 2

Concentration of ALAD in earthworm tissue homogenates after treatment with 1.0 μg Pb^{++}/100 ml for 20 days (n moles porphobilinogen/g protein/h, n=3)

Tissue	Treatment		
	Pb^{++}	control	P
Chloragocyte cytosol	304.6±18.6	762.3±104.9	<0.01
Chloragosomes	none detected	none detected	
Intestine	127.0±6.1	182.7±17.4	<0.01
Body wall	25.4±8.6	51.8±15.6	<0.01

Tissue distribution of the enzyme ALAD

No ALAD could be detected in the chloragosomes but the amount of enzyme in the chloragocyte cytosol was the highest value recorded. Body wall homogenate extracts contained the lowest concentration of synthesised porphobilinogen while the presence of ALAD in intestinal tissue gave intermediate values.

There was a substantial drop of over 50% in the activity of ALAD in chloragocyte cytosol and body wall extracts after treating earthworms with Pb^{++} for 20 days. A small drop in ALAD activity was also observed in the intestinal fraction (Table 2).

Discussion

The presence of Fe^{+++} in the chloragogenous tissue of oligochaetes has been well established histochemically [6, 18, 23], while direct chemical analysis of Fe^{+++} has shown most of the metal found in the chloragogenous tissue of *L. terrestris* to be deposited in the chloragosomes [2]. High concentrations of Fe^{+++} in the chloragosomes of *L. terrestris* and *L. rubellus* have more recently been established [12].

In man, Fe^{+++} exists as a protein complex in the water-souble, heat-stable form, ferritin and as a water-insoluble form, haemosiderin [10]. According to LINDNER [18] and based on histological studies, Fe^{+++} is present in the chloragogenous tissue as haemoglobin vacoules. Haemoglobin together with ferritin is also found free in the cytosol. Iron in the cytosol of the chloragocytes, in the present study, could represent ferritin and "free" haemoglobin. LINDER [18] could not detect ferritin in the chloragosomes and it is probably not haemosiderin since the cationic composition of isolated chloragosomes is different from isolated haemosiderin granules though the latter are larger [12, 13, 24]. According to FISCHER [4, 5], Fe^{+++} in the chloragosomes of *L. terrestris* is in the form of a metalloporphyrin. Whatever the state of the Fe^{+++} in both the chloragogenous tissue and the body wall, it appears that induced Pb^{++} does not affect the Fe^{+++} concentrations. Contrary to this result, Pb^{++} treated earthworms have a reduced level of Fe^{+++} in the intestinal tissue. In a study of Pb^{++} uptake by isolated rat intestine, it has been reported, that over 55% of the metal is deposited in the intestinal cells [3]. Studies in the earthworm *Dendrobaena rubida* have shown that deposition of Pb^{++} in the intestine is of the same magnitude as Pb^{++} deposited in the chloragocytes [13]. Intestinal uptake of Fe^{+++} is mediated by iron-binding proteins [26] and, as pointed out by VALLEE and ULMER [29], Pb^{++} has a strong affinity for SH groups on proteins and amino acids. Such a response would result in a general upset of Fe^{+++} metabolism.

The results of lead-poisoning in mammals on haem biosynthesis is the excretion of various precursors of the haem biosynthetic pathway such as ALA, coproporphyrin III and uroporphyrin I and an increase in these compounds in the blood together with increased amounts of protoporphyrin in the erythrocytes. The resultant abnormalities in porphyrin metabolism induce symtoms similar to the diseases known as poryhyrias [8]. In the annelids, "free" porphyrins appear to be a natural part of the biosynthetic pathway of haem compounds [2,15, 20].

Body wall extracts of *L. terrestris* have been found to contain protoporphyrins [3, 16], while "free" porphyrins have been reported in the body wall and skin of marine annelids [15, 20]. The presence of ALAD in the body wall of *L. terrestris* together with these previous findings suggests that, at

least, part of the pathway of prophyrin biosynthesis [20] occurs in the body wall of *L. terrestris* since porphobilinogen is the pyrrole intermediate in porphyrin synthesis [27].

The presence of ALAD in the intestine and the chloragocyte cytosol poses an interesting problem. DELKESKAMP [3] could not detect porphobilinogen in any of the organs of *L. terrestris* analysed but reported the highest rate of synthesis of porphyrins, after the addition of porphobilinogen, in the chloragogenous tissue. Based on this evidence and the fact that chloragogenous tissue contains high concentrations of Fe^{++}, it has been suggested that this tissue may be the site of haematopoesis in oligochaetes [3]. This is supported by the present results and it is further suggested that the stage before the synthesis of porphibilinogen is also present. Since it is practically very difficult to remove all the chloragocytes from intestinal tissue, the ALAD activity of this tissue may indicate contamination from chloragocytes.

The sensitivity of ALAD to Pb^{++} has been shown in vertebrates [7, 11, 14, 28], and ALAD does exist in the invertebrates [1, 21, 22] but there are no reports on the effects of Pb^{++} on this enzyme in invertebrate tissue. In the present study, Pb^{++} has been demonstrated to effect ALAD at all sites where it is found and particularly in the chloragocyte cytosol.

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HOW PARADISE FISH (*MACROPODUS OPERCULARIS*, L.) EXPLORES A CHESSBOARD

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Abstract

We investigated the exploratory movements of Anabantid fishes in a chessboard-like aquarium (divided into 25 communicating compartments) as a new environment.The analysis was based on the observation of the route of two inbred strains of paradise fish *(Macropodus opercularis; Teleostei, Anabantoidae)*. We recorded their movement within the blocks until 100 steps, and compared the obtained process with hypothetical random walks. It turns out within the same period of time the fish meets significantly more new compartments than in the simulated process. The discrepancy between the two processes can be interpreted as the result of a successful exploratory strategy of the fish. In this exploratory movement the fish prefers the outer compartments to the inner ones, a fact that explains the striking difference between the actual behaviour and the "uniform simulation". This is obviously so, since the empirical data show that the transition probabilities from the actual compartment to the possible neighbouring compartments differ greatly. However, a model taking this inhomogeneity into account still does not fit sufficiently, indicating that this exploratory strategy is more complicated than our memoryless construction. Moreover, there are differences between the two strains investigated, which might reflect some role of genetic factors.

Introduction

The genetic aspects of exploratory behaviour have been examined mostly in mice, *e. g.* De Fries [3] and Parsons [7] demonstrated such differences among inbred strains in open field tests and van Abeelen [1] in novelty box experiments. Oliverio [5, 6] found that the differences between the exploratory activities of the strains C57BL/6By and BALB/cBy are caused by single gene effects: a single major gene is responsible for the short-term (10 min) activity, and another for that of the longer session (25 min).

The purpose of our experiments is to identify the exploratory strategies, and see to what an extent can these strategies be simulated by (essentially) memoryless random models.

Paradise fishes are very suitable for such an experiment for they live on strongly compartmentalized locations.

Material and method

Our test field was a $75 \times 75 \times 15$ cm aquarium, divided into 25 communicating compartments (Fig. 1). The walls (including the inner ones) were non-transparent, and so we followed the fish in the tank by a mirror placed over the aquarium. To facilitate the exploration, each compartment contained different objects (plastic plants and cane, pieces of foam, *etc.*). The subjects (Ss) came from two strains of paradise fish bred in our laboratory in closed inbreeding, sibmating for 4 generations. The animals were exposed to a 12 + 12 h light/dark cycle (16 + 8 h in winter months) and fed daily (after the test) approximately 0.1 g of living Tubifex. Eleven adult males and 4 females from both strains (named "L" and "S") were tested at the age of 8—9 months. The subjects (divided into two sets) were kept in tanks, 35 l in volume, before tested. The test started by placing the fish to the central compartment in a green plastic cylinder (10 cm in diameter). After an adaptation period of 5 min the cylinder was taken out, and the movement of the fish was followed for 30 min. To eliminate occasional effects of feromons [2], we changed the water in the aquarium each time. The temperature of the water was 25 ± 2 °C in both the home and the test tank.

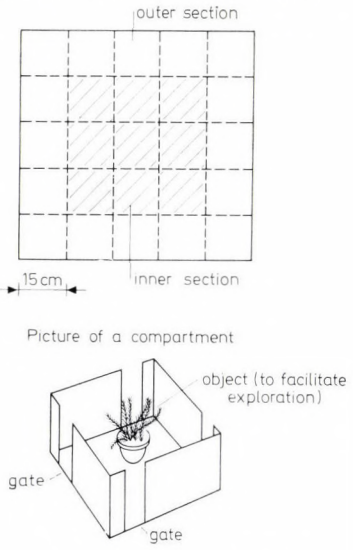

Fig. 1. The 'chessboard'

Results

The obtained data were compared to two different hypothetical random walks. In the "uniform model" it was assumed that the fish moves into one of the neighbouring compartments at random with equal probabilities, and independently of the former movements. In the second (non-uniform) random walk the above condition of equal transition probabilities was dropped, and we used the observed empirical relative frequencies .

We measured the effectiveness of the exploratory strategy in two ways: by the number of explored compartments within a given number (25) of steps, and also the inverse set-up,the number of steps needed for exploring a certain number (7, 13 or 19) of new compartments (Table 1). The first row of Table 1

shows that the uniform simulation performs poorly compared to the fish (only 11 new compartments explored, on the average). Another parameter (ratio of outher to inner compartments visited) indicates that this uniform model is a bad approximation anyway; the fish visits the outer compartments more often than suggested by the uniform model. (Such preference of the boundaries by mice and rats in open-field tests was reported by WARREN [8]).

A similar deviation can be found in the average lengths of runs, *i. e.* the number of consecutive steps on the boundary before going inside and *vice versa* (Table 2). The performance of outer compartments is automatically present in the second model, but still the effectiveness of fish is significantly better. This can also be seen in Table 1: the average number of new compartments is only 13.3 in this model, and the discrepancy also increases with the increase of the number of steps. Thus, the preference of the outer compartments does not offer an explanation for the better performance of fish (actually the fish's ratio 3.88 is too large compared to the optimal ratio $1.77 = 16/9$ of the "visit each compartment once" strategy.) Therefore, it is reasonable to assume that the exploration follows some strategy; in other words, one might get a much better mathematical model using some random process with memory (*e. g.* a higher-order Markovian chain).

Discussion

Both of the investigated strains show deviations from the random walks. However, there are also differences between the strains in the frequencies of going straight, backwards, and also in the average lengths of runs. But these differences do not seem to be significant except the greater number of crossings for the strain "S" (Table 1), and the shorter lengths of runs in the outer and inner sections for the strain "L" (Table 2). These might indicate a genetic difference, but not necessarily in the structure of strategy; perhaps only for the transition probabilities, and the speed of movement.

Thus, our investigations can be summarized as follows:

1. The exploratory behaviour of fish is not a completely random movement (comparison with a Markovian chain of order 1). The fish has preferences in choosing exits in different directions, on the one hand, and in different compartments, on the other (see also KLEEREKOPER's experiments with goldfish [4]).

2. Our second model (Markovian chain of order 2) takes these preferences into account, and we get a better fit here for the different parameters. However, the performance of the fish (Table 1) is still much better. This indicates that beyond these preferences the fish must employ a more sophisticated exploratory strategy.

Table 1

Effectiveness and speed of exploratory movement

	Strain "S"		Strain "L"		Random walk 1	Random walk 2
	M	SE	M	SE	M	M
Average number of explored new compartments (in the lst 25 crossings)	16.8	0.85	16.2	0.71	11.0*	13.3*
Number of steps needed for exploring						
7	7.1		8.0		12.6*	10.8*
13	18.9		18.7		35.3*	30.2*
19 new compartments	33.2		36.6		61.2*	55.5*
Ratio of inner and outer compartments	3.88	0.46	4.53	0.60	1.22	4.21
Crossings per min	9.1	0.86	4.6	0.65	—	—

* Data coming from simulation

Table 2

Transition frequencies and lengths of runs

	Strain "S"		Strain "L"		Random walk 1	Random walk 2
	M	SE	M	SE	M	M
2. A) *Frequencies in the inner section*						
Straight ahead	0.27	0.02	0.29	0.03	0.25	0.28
Turning right and left	0.71	0.03	0.70	0.03	0.50	0.70
Turning back	0.02	0.01	0.02	0.01	0.25	0.02
Average length of runs in the inner area	3.0	0.17	2.3	0.14	3.5*	2.71*
2. B) *Frequencies in the outer section: edges*						
Straight ahead	0.77	0.20	0.68	0.02	0.33	0.73
Turning right and left	0.12	0.01	0.16	0.02	0.33	0.14
Turning back	0.11	0.02	0.16	0.02	0.33	0.13
corners						
Proceeding	0.65	0.03	0.61	0.03	0.5	0.63
Turning back	0.35	0.03	0.39	0.03	0.5	0.37
Average length of runs in the outer area	12.5	1.25	10.0	0.96	3.75**	8.35**

* starting from the centre
** starting from a corner

Appendix

Our first model is a homogeneous Markovian chain of order 1, which is clearly ergodic. Thus, we can easily compute the (existing) limiting probabilities of the states (*i.e.* of the compartments). Because of the symmetry of the chessboard we have a system of equations with only six parameters:

$$\begin{array}{ccccc} p_1 & p_2 & p_3 & p_2 & p_1 \\ p_2 & p_4 & p_5 & p_4 & p_2 \\ p_3 & p_5 & p_6 & p_5 & p_3 \\ p_2 & p_4 & p_5 & p_4 & p_2 \\ p_1 & p_2 & p_3 & p_2 & p_1 \end{array}$$

$$\begin{aligned} p_1 &= 2/3p_2 \\ p_2 &= 1/2p_1 + 1/3p_3 + 1/4p_4 \\ p_3 &= 2/3p_2 + 1/4p_5 \\ p_4 &= 2/3p_2 + 1/2p_5 \\ p_5 &= 1/3p_3 + 1/2p_4 + 1/4p_5 \\ p_6 &= p_5 \end{aligned}$$

and solving this system, we get the following matrix of probabilities:

$$\begin{array}{ccccc} 1/40 & 3/80 & 3/80 & 3/80 & 1/40 \\ 3/80 & 1/20 & 1/20 & 1/20 & 3/80 \\ 3/80 & 1/20 & 1/20 & 1/20 & 3/80 \\ 3/80 & 1/20 & 1/20 & 1/20 & 3/80 \\ 1/40 & 3/80 & 3/80 & 3/80 & 1/40 \end{array}$$

Comparing the outer areas to the inner ones we get the value 1.22 in the 5th row of Table 1.

$$\begin{array}{ccccc} 1 & 2 & 3 & 2 & 1 \\ 2 & 4 & 5 & 4 & 2 \\ 3 & 5 & 6 & 5 & 3 \\ 2 & 4 & 5 & 4 & 2 \\ 1 & 2 & 3 & 2 & 1 \end{array} \qquad [X]$$

To compute the average length of runs, let E_4, E_5 and E_6 be the following quantities:

E_4 : the expected number of consecutive steps in the inner section, if starting from a compartment labelled "4" (see [X]);
E_5 : the same expected number starting from "5";
E_6 : the same expected number starting from "6".

Then our system of equations is as follows:

$$\begin{aligned} E_4 &= 1/2(E_5 + 1) \\ E_5 &= 1/2(E_4 + 1) + 1/4(E_6 + 1) \\ E_6 &= E_5 + 1 \quad . \end{aligned}$$

Solving this for E_6 we get $3.5 = 7/2$ (Table 2.A); this is the average number of inner steps starting from the centre. The average length of runs in the outer section can be computed similarly; its value is $3.75 = 15/4$ (starting from a corner; see Table 2.B).

To compute the (outer) average length of runs for the non-uniform model, let E_{ij} be the following:

E_{ij} : the expected number of consecutive steps if starting from compartment "j", provided that the preceding compartment has been "i".

Then, again according to [X], we get:

$$\begin{aligned} E_{21} &= E_{12} + 1 \\ E_{12} &= 0.73(E_{23} + 1) + 0.13(E_{21} + 1) \\ E_{23} &= 0.86(E_{32} + 1) \\ E_{32} &= 0.13(E_{23} + 1) + 0.73(E_{21} + 1) \end{aligned}$$

(see also the last column of Table 2.B)

This yields the value 8.35 for E_{21} (Table 2.B), which is, in fact, the expected length E_1 of runs starting from a corner, since the preceding compartment to "1" must have been "2".

In essentially the same way we get that, starting from the centre, this conditional expectation in 2.71 (Table 2.A), which is a better fit to the corresponding parameter of the fish than that in the first model. Some of the remaining data of our tables came from the observations, and the rest can easily be computed.

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IDENTIFICATION OF EPITHELIAL CELLS AND FIBROBLASTS IN HYPOPHYSIS INTERMEDIATE LOBE CULTURES BY SCANNING ELECTRON MICROSCOPY

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Abstract

Cell cultures of the rat pituitary intermediate lobe grown for eight days were studied in the scanning electron microscope. The epithelial cells and fibroblasts could be differentiated by the characteristic structures of the cell surface and the cell association features. The epithelial cells were characterized by blebs and rugae, microvilli, and occasionally by some cilia. The surface of the fibroblasts was smooth or bore microvilli. Scanning electron microscopy may provide special information for the characterization of endocrine cell cultures.

Introduction

The role of the intermediate lobe of the hypophysis has come anew into the limelight in the past few years. It has, namely, been proven that this lobe is the site of the synthesis and conversion of a peptide hormone family (corticotropin, melanotropin, lipotropin, corticotropin-like intermediate lobe peptide and incidentally, endorphins). Accordingly, beside the melanotropic effect, the intermediate lobe might also be the carrier of essential functions in mammals. Since the isolated, *in situ* physiological study of the intermediate lobe function meets with difficulties for anatomical reasons, it has seemed necessary to develop some suitable *in vitro* model for examining this function. Primary monolayer cultures of the rat intermediate lobe proved to be an appropriate model [4]. In the present paper certain surface and organizational features of such cultures were studied by scanning electron microscopy.

Materials and methods

For each experiment the hypophyseal intermediate lobes from CFY rats (180—200 g) of the same sex were dissociated by trypsin as described earlier [4]. Two ml aliquots of the cell suspension, in a 8 : 2 mixture of TCM 199 and foetal calf serum, were explanted into plastic Petri-dishes (Falcon plastic ∅ 3.5 cm), with glass coverslips (Bellco Glass Inc.) in them. The cultures were incubated at 37 °C in an atmosphere of 5% CO_2, 95% air for 8 days, changing the medium every second day. After 8 days of cultivation the cells that adhered to the coverslips were washed several times with TCM 199 at room temperature, fixed in 2,5 % glutaraldehyde buffered with 0.1 M cacodylate (pH 7.4) at room temperature for 60 min., and washed in the same buffer in the refrigerator overnight. The cultures were postfixed in 1% OsO_4 for

60 min, dehydrated in ethanol and amyl acetate and dried at the critical point from carbon dioxide. Finally, the coverslips with the cells were coated with gold (200 Å) for examination with a JEM 100 C ASID 4 D microscope.

Results

Individual cells as well as cell groups consisting of 2—30 cells, adhere to the intert surface in about three days. Cell aggregates are formed, with fibroblasts growing out from their periphery. Accordingly, epithelial cell stacks (Fig. 4), epithelial islands (Figs 2 and 5), individual flattened epithelial cells (Fig. 1) and fibroblasts (Figs 1 and 2) occur in the 8-day cultures.

Morphological identification of epithelial cells and fibroblasts

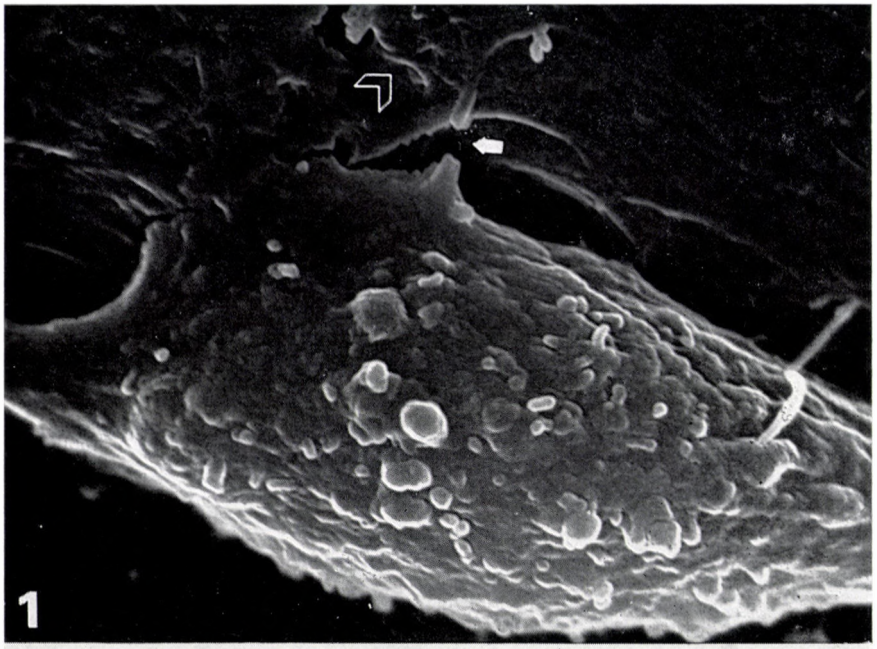
The fibroblasts form a monolayer, consisting of well spread cells. The cells are tightly packed (Figs 9, 10). The continuity of the fibroblast monolayer is interrupted either by individual (Fig. 1) or variously associated (Fig. 2) epithelial cells. These cells are less flattened, stocky; they are spherical, polygonal or spindle-shaped. In some places, the round or polygonal epithelial cells are tightly packed. Occasionally, they form cords which can associate into bundles (Figs 3 and 6). Cell aggregates consisting of 6—9 cells may also occur, with the cells superimposed and thus forming several layers (Fig. 4). In such stacks the lowest layer is formed by some flat, polygonal cells adhering to the glass by their cytoplasmic processes. Epithelial islands consisting of fully spread cells are not infrequent, either (Fig. 5).

Surface features of the epithelial cells

It is striking even in low-magnification scanning electron micrographs that the surface of epithelial cells is uneven, with many protrusions which may be classified into two groups. In high-power micrographs, solitary or multiple blebs and rugae are seen in varied arrangements, more or less densely in different areas of the cell surface (Figs 6, 7). Shorter and longer microvilli form the other group of surface structures (Fig. 8). Less microvilli are found on the surface of superimposed cells than on the surface of cells forming epithelial cords or bundles.

Narrow, longitudinal extensions, running parallel to the axis of the epithelial cords are frequent (Fig. 6). Solitary, long and bulky villi also occur on the surface of many epithelial cells. Owing to their size, they might qualify even as cilia (Figs 4, 5).

Dividing epithelial cells also occur sporadically (Fig. 5). Beside the numerous rugae and several blebs only a few microvilli are found on the surface of such cells.



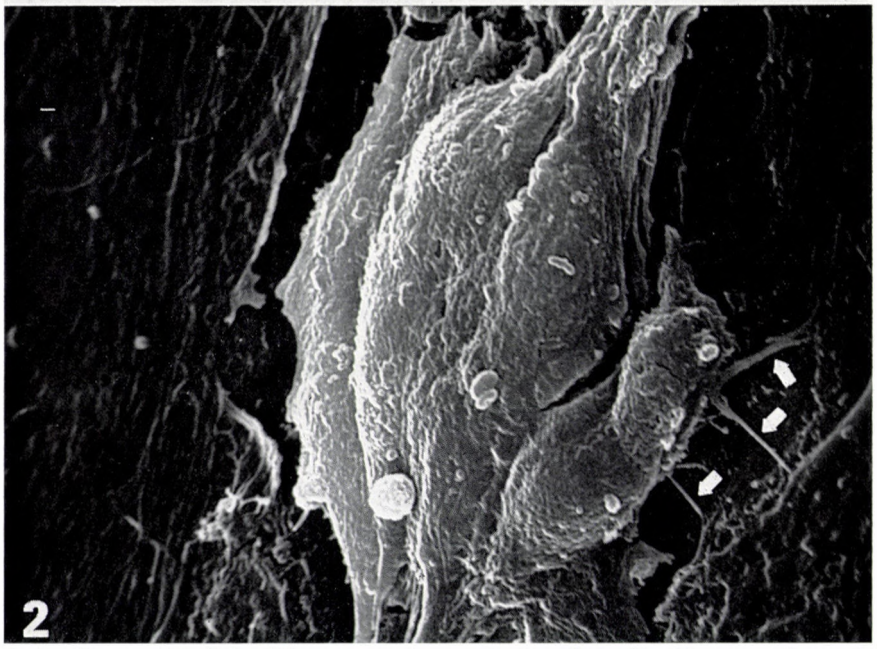


Fig. 1. A solitary epithelial cell interrupts the continuity of the fibroblast monolayer. Blebs, rugae and microvilli can be differentiated on the epithelial cell surface. The arrowhead shows the ruffled free edge of a fibroblast. The extension anchoring the epithelial cell to the fibroblast monolayer (arrow) has broken during freezing. ×8600. Scanning electron micrograph of an 8-day culture

Fig. 2. Island consisting of tightly packed elongated epithelial cells attached at its periphery to the fibroblast monolayer by extensions (arrows). Low rugae following the course of fibres and a few microvilli are seen on the fibroblasts. ×2400

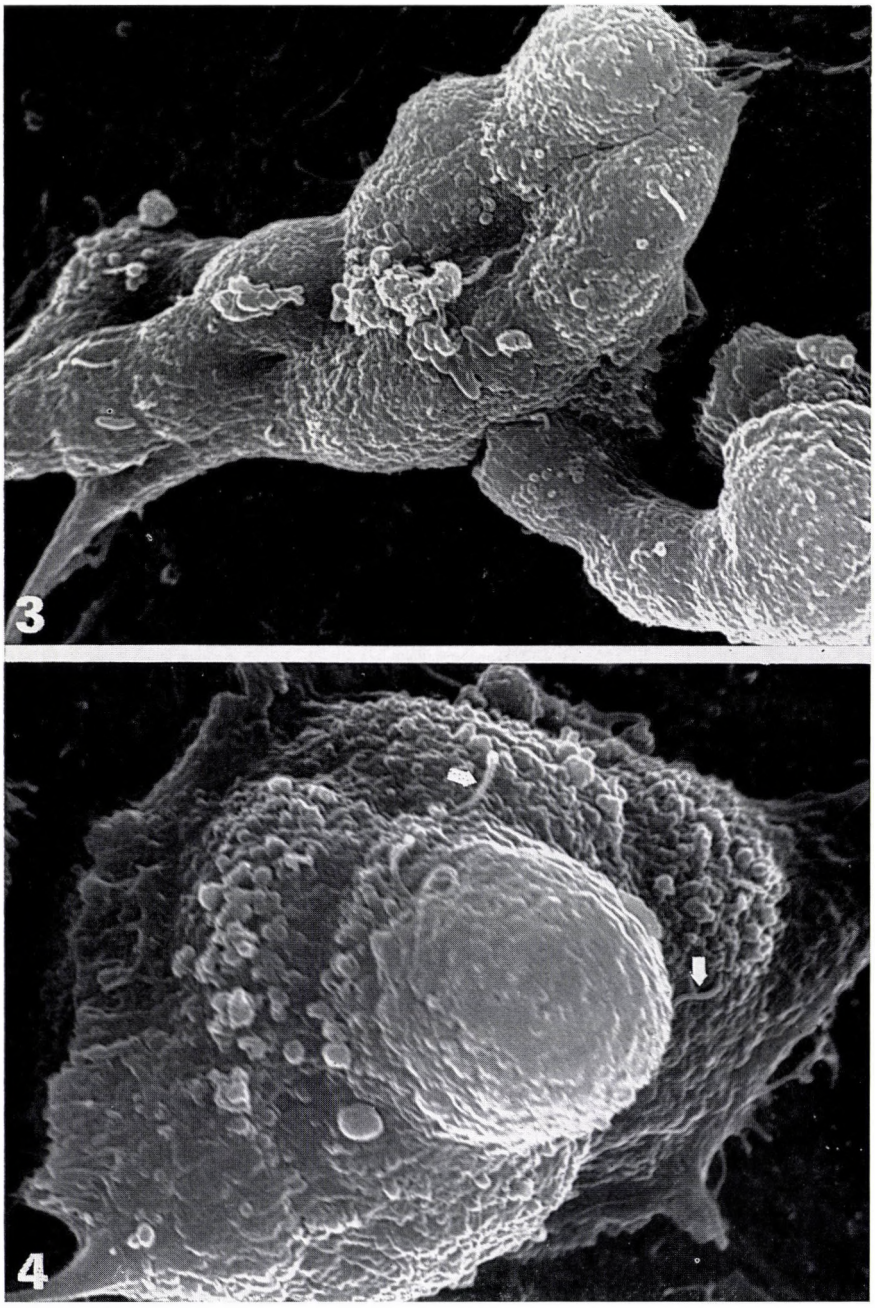
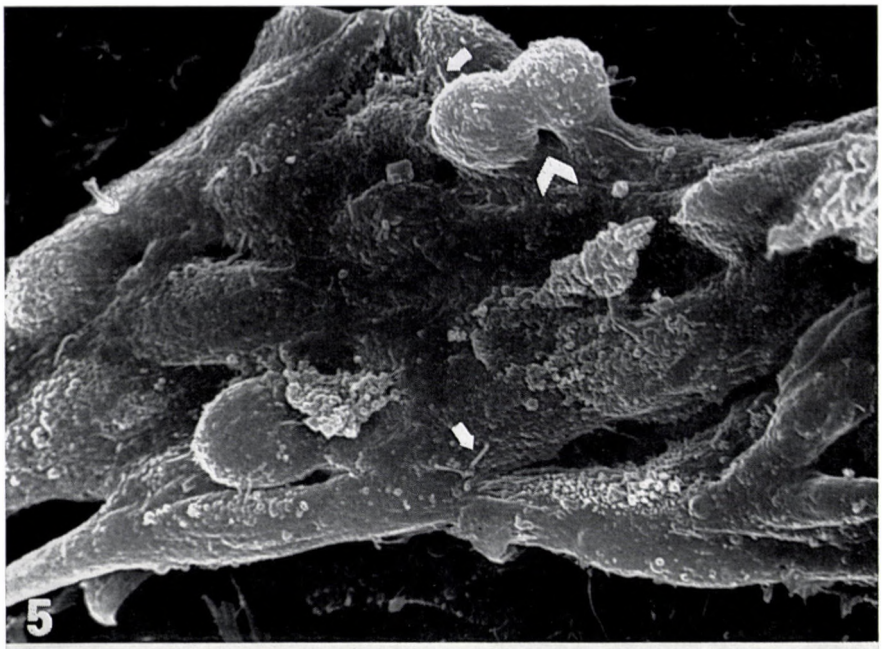


Fig. 3. Epithelial cells organized into a cord and a bundle. ×2400
Fig. 4. Cell stack consisting of several layers. The spread cells of the lowest layer attach to the fibroblasts and to the glass surface by extensions. Blebs and rugae predominate the surface of cells in the upper layer, with occasional cilia (arrows). ×4800



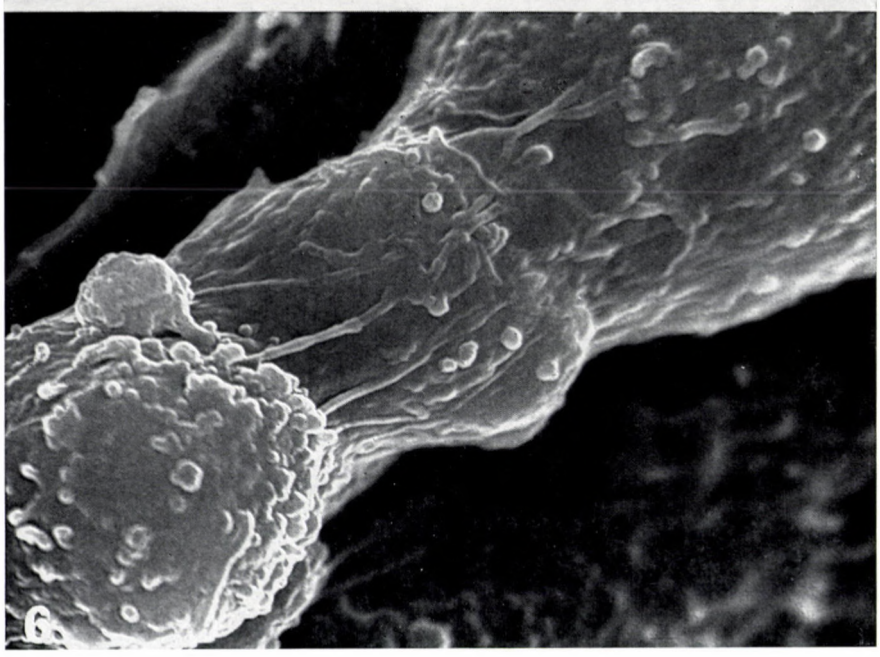


Fig. 5. Epithelial island consisting of many spread cells. Arrows: solitary cilia. An epithelia cell in the late telophase (arrow-head) is seen on the marginal part of the island. ×1800
Fig. 6. Cord consisting of cubic epithelial cells. Small individual blebs and long, narrow processes running parallel to the axis of the epithelial cord are characteristic of the surface. ×9600

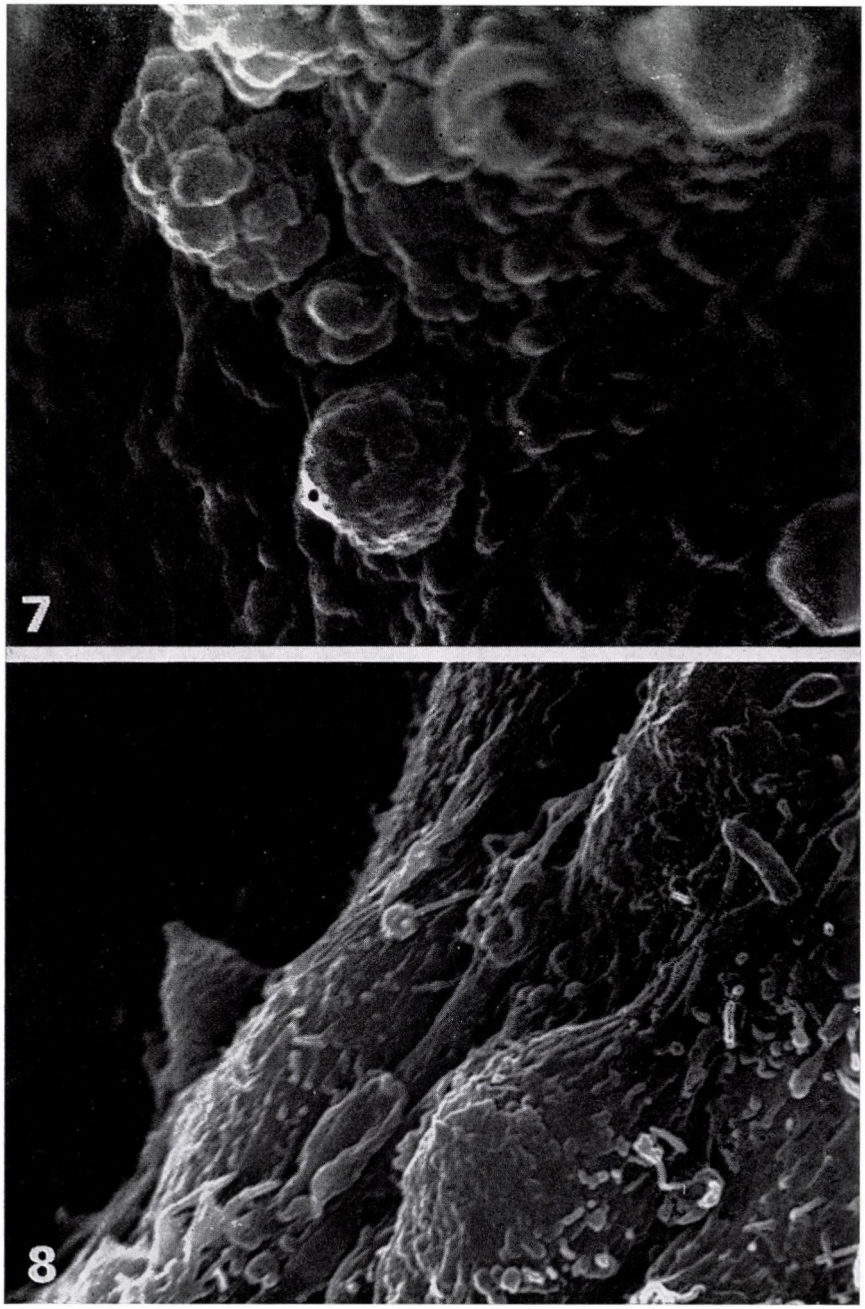


Fig. 7. Solitary and grouped blebs on the surface of an epithelial cell. ×24000
Fig. 8. Microvilli predominating the surface of some epithelial cells. ×6000

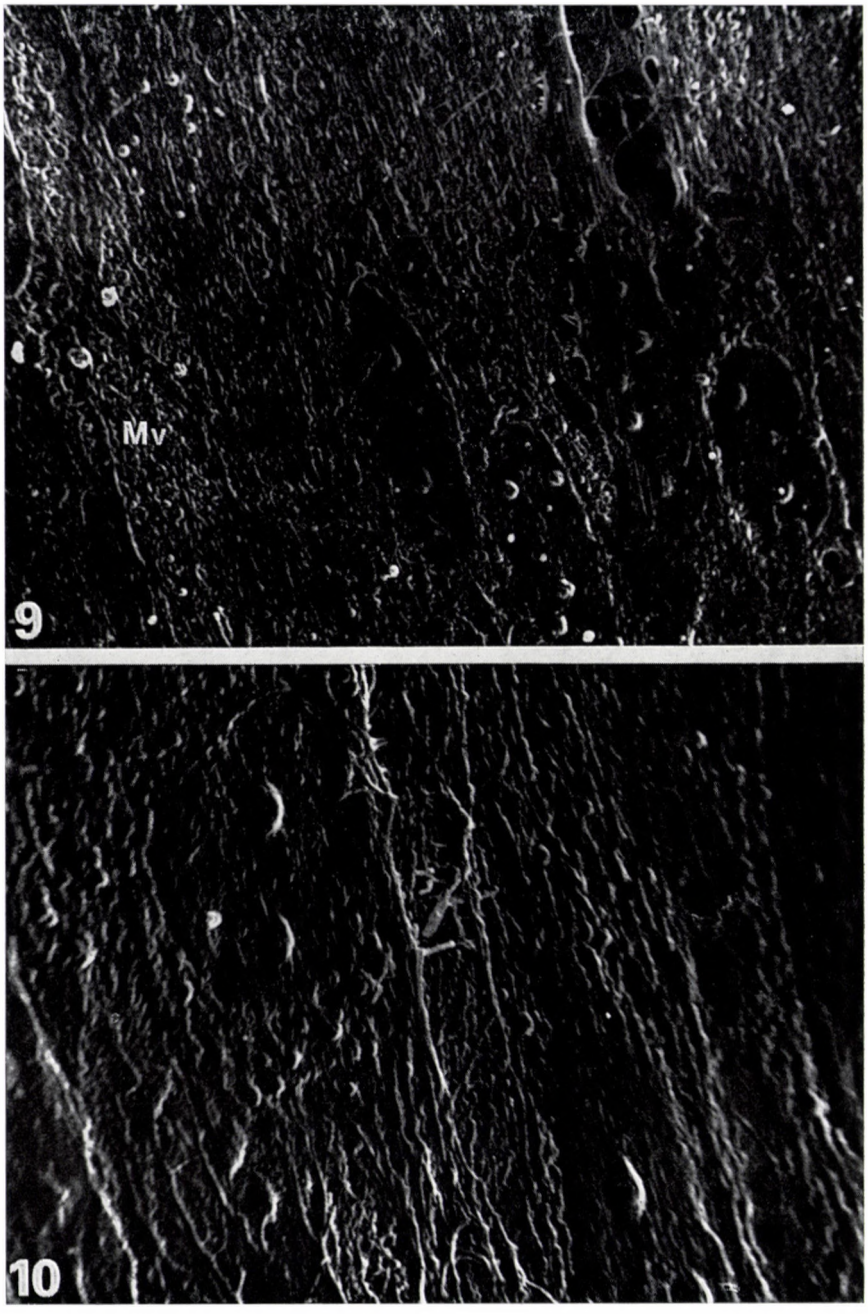


Fig. 9. Part of a fibroblast monolayer. The cells are in contact with each other; the density of microvilli (Mv) is varying on their surface. ×1800

Fig. 10. Fibroblasts poor in microvilli. Low rugation organized along the course of intracellular fibres. ×4100

Surface features of the fibroblasts

In contrast to the surface of epithelial cells, that of fibroblasts is essentially smoother and more monotonous. Microvilli may arise, less or more densely, in different areas of the cell surface (Figs 1 and 9). Low, longitudinal wrinkles also occur on the surface of certain cells following the course of intracellular fibres (Figs 2 and 10). The free edge of the fibroblasts, not contacting other cells, is ruffled (Fig. 1).

Discussion

As shown by the present studies, epithelial cells and fibroblasts can easily be differentiated in cell cultures of the rat intermediate lobe. The shape of the cells, their interrelationships, the qualitative and quantitative differences in their surface structures permit this differentiation. We have demonstrated that epithelial cells are less spread than fibroblasts; that microvilli arise in varying numbers from the monotonous surface of fibroblasts; that most frequently blebs and rugae, less frequently microvilli occur on the surface of epithelial cells, either solitary or associated.

The appearance of blebs and rugae on the cell surface is likely to be a characteristic feature of the epithelium. Similar structures were described *e. g.* by MIETTINEN et al. [5] on hepatocytes from adult rats and by BERKY and ZOLOTOR [1] on the surface of an epithelial cell line from the mouse bladder.

The abundance in surface structures of epithelial cells is a function also of the extent of their spreading and of their relationship to the neighbouring cells: the more spread, adhered cells of the epithelial islands are less rich in surface structures than the cells organized into cords or bundles. As found by ERICKSON and TRINKAUS [3] for the surface organization of cells of the BHK 21 cell line, the surface is rich in structures shortly after passage but becomes smooth on complete adherence. Equally, for the BHK 21 cell line, O'NEILL and FOLLETT [7] found an inverse relation between cell density and the number of microvilli: the surface of the cells forming a continuous monolayer was smooth.

MORIARTY and GARNER [6] identified dividing glandular cells in the pituitary intermediate lobe of intact rat by immunoelectron microscopy. Dividing epithelial cells could be detected in the microcinematographs of our cultures [4] and combined immunocytochemical and autoradiographic studies permitted to describe glandular cells in the S-phase [8]. Some mitotic epithelial cells with a few microvilli, blebs and rugae could be seen in our

scanning micrographs, too. However, the mitotic index being low, we were unable to give a detailed description of the surface morphology of cells in the various phases of the cell cycle.

The importance of the abundance of cell surface structures and their relationship to intracellular processes are not sufficiently known as yet. O'NEILL and FOLLETT [7] as well as ERICKSON and TRINKAUS [3] believe the various surface structures to be the reserves of the cell membrane. ENLANDER et al. [2] contest that the microvilli would represent some membrane reserve. The studies of VAN DER SCHUEREN et al. [10] with colchicine and cytochalasin-B suggest that the abundance of cell surface structures is connected with the intracellular microfilamentar-microtubular system.

TASHIJAN and HOYT [9] established that the cells of the hormone-producing GH_3 cell line, relatively rich in surface structures, spread out and became smooth after thyrotropin-releasing hormone administration. This finding demonstrates that the surface morphology of endocrine cells may change during hormone release. At the same time, it suggests that scanning electron microscopy might prove an auxiliary tool in the study of hormone-producing *in vitro* systems.

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Mass Spectrometry in Drug Metabolism

Eds: A. FRIGERIO and E. L. GHISALBERTI, Plenum Press, New York and London (1977), 532 pp.; 226 figs.; 63 tables, $ 51.

The book contains the papers presented at the International Symposium on Mass Spectrometry in Drug Metabolism held at the "Mario Negri" Institute for Pharmacological Research (Milan) in June 1976. Main topics covered by the volume are identification and quantitation of drugs and drug metabolites by mass spectrometry, development in methodology and drug abuse. As mass spectrometry due to its high sensitivity and high resolution is an ideal tool for characterization of compounds, it is frequently used for identification and quantitation of foreign compounds and their metabolites in biological fluids and for studying metabolic pathways. The book gives a rather large overview of these problems and the possibilities of application of new techniques, like gas-chromatography, mass spectrometry with computer systems, stable isotope labelling, twin ion technique, chemical ionization and field desorption mass spectrometry.

A list of participants and subject index complete the volume.

The book will be of interest to anyone working in pharmacology, toxicology and in the biomedical field of science.

L. VERECZKEY (Budapest)

ULRICH SUCKER: **Philosophische Probleme der Artheorie.** VEB, Gustav Fischer Verlag, Jena (1978), pp. 120, M 18.50.

After the summary of the history of the debate on the concept of species and the reconsideration of the present philosophical problems related to it, the author deals with the latest issues of epistemology and methodology, especially in the last two chapters of the book. The reference to the general epistemological development of concept theory, and the complex, interdisciplinary character of the issue of the species is justified. The systems-theory approach of species and its examination as an objective-real system, as well as the analysis of the relation between species and taxon, species and population and the relations within the population are remarkable. In this analysis the differentiation between chronological descendance or the intensive and spatial, reproductive or extensive structures plays an important role. These structures together make up the species and the population as well, but, according to the author, species is a descendance community, whereas population is rather the unity of reproductive, actual systems, *i.e.* the species system is an internally-structured developing totality, a dialectical unity of spatial and chronological, extensive and intensive structures. The species, as the system of real-objective relations, the various levels of direct interactions, represent the various degrees of materiality.

ULRICH SUCKER examines the relationship between reality of species (the species as concrete general) and its concept (the abstract general) according to the above principles.

L. KOVÁCS (Debrecen)

Subcellular Biochemistry Vol. 5.

Ed.: DONALD B. ROODYN, Plenum Press, New York and London, pp. 389, figs. 17.

Subcellular Biochemistry previously published as a quarterly journal, this continuing series has been expanded into an annual volume.
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J. MOLNÁR (Szeged)

Arthropod Venoms

(Handbook of Experimental Pharmacology: New Series: V 48)
Ed. S. BETTINI Springer-Verlag, Berlin—Heidelberg—New York, 1978, ISBN 3-540-08228-x, pp 977, figs. 293

Written by 44 specialists and divided into 26 chapters, the book is an excellent source and useful handbook for specialists working or interested in venomous arthropodes. Each chapter deals with a taxonomical group of arthropodes, summarizing ecological, morphological, physiological, pharmacological and toxicological knowledge of both old and newest investigations.

Due to the different levels of knowledge, the amount and quality of information is different for the various systematical groups, nevertheless, all of them are characterized by modern and valuable aspects and results. In the description of the venomous apparatus scanning microscopical and electronmicroscopical demonstration is often added to classical anatomical pictures, while in explaining the mechanism of action a wide scale of electrophysiological results are included, obtained not only from poisoned animals but also in isolated muscle and nerve cells. The data related to the chemistry of toxic substances include in most cases both separation and identification as well as formulae of the components, data that can be useful for pharmacologists and physiologists interested not in venoms but is specific inhibitors of physiological processes.

For some arthropodes, detailed description of the behaviour connected with venom release, and method of obtaining the venom in laboratory is given.

The toxic substances in various animal groups are of different origin. There are defensive substances, offensive weapons and also poisonous toxins which are not used by the animal itself. Such a classification as well as the mapping of the geographical distribution of animal species are important for preventive and therapeutic reasons in the field of human implications.

Each chapter is followed by related references and the volume is completed by author and subject indexes. The printing and publication of the book is excellent.

J. SALÁNKI (Tihany)

HUMAN IMPACTS ON LIFE IN FRESH WATERS

Ed. by J. Salánki and P. Biró

(Symposia Biologica Hungarica 19)

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