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In the frame of the "Bioindicator" Programme of the International Union of Biological Sciences (IUBS), a Workshop was organized in November, 1986, in Cairo, Egypt. This issue contains one part of the presented reports on scientific results.

The principal aim of the Workshop was partly to review and discuss the recent results and application of biological monitoring of the state of the environment and partly to promote the exchange of views and collaboration between the scientists from Arabic countries working in the field of environmental biology.This double purpose was indicated by the fact that the environmental pollution ensuing from the speedy industrial development still continues; today not only causing problems in the highly developed industrialized countries, but also becoming an increasing hazard in the developing countries – thus in the Arabian world as well. Furthermore, it could be said that while in the highly developed countries more and more sweeping measures are taken in the interest of preventing and decreasing environmental pollution, in the developing countries the recognition of the damage is often delayed, the experiences are few in respect to the application of biological methods and only moderate sums are at disposal for the purpose of prevention.

The biological methods, biological organisms may play important role in the recognition and monitoring of the environmental hazards. The broader exposition of the achieved results, the attraction of attention, the enhancement of the prestige of such studies are important aims of the "Bioindicator" Programme of the IUBS, and this object is whished to be furthered by the present issue.

The Editor

Acta Biological Hungarica 38 (1), pp. 5–11 (1987)

SIGNALIZATION, MONITORING AND EVALUATION OF ENVIRONMENTAL POLLUTION USING BIOLOGICAL INDICATORS

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(Received: 1986–10–05)

Human activities have always had significant impact on the environment. This effect had, however, further increased in the period of the industrial revolution and has become rather hazardous during the last half century, when chemical pollution has become a constant concomitant of industrial and agricultural activities. Today there is no such branch in industry or agriculture which apart from the extreme use of raw materials, does not imply another great challenge in relation to man; namely, the hazardous effect on our environment, the unfavourable influence on global essential conditions. In the highly industrialized countries this problem presented itself much sooner than in the less developed countries; nevertheless, nowadays it has turned into an everyday problem in almost every country of the world. Today the economically less developed countries also greatly endeavour at increasing their production, which can only be attained by the introduction of more effective technologies. In most cases, the adoption of new techniques in both industry and agriculture involves the wide-spread use and/or waste of chemical substances as well, resulting in environmental pollution, the increased endangerment of the fauna and flora – and in final turn that of human life.

Naturally, the danger of environmental pollution is long not an unknown issue neither before those causing the pollution, nor before the society, and throughout the world great forces have been set in motion in

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order to estimate and decrease this danger. Numerous measures have been made which eliminate or decrease the emission of environmentally pollutant substances. The possibility for this, however, is not open in every case — even if the coverages would be at disposal.

The experiences of the recent decades have evidenced that not even the well off countries are capable of entirely avoiding the risk of environmental pollution, the damages to the ecosystem by means of preventive measures. Not even the input of large sums can eliminate the pollution of the air, waters and soil; and the less wealthy countries — due to financial causes — frequently cannot even employ the known techniques for the prevention of pollution.

Accordingly, the human race should make preparations on the long run for the detection of all the damaging effects implying human activities having unfavourable influence on our environment, and against which fight should be attempted constantly and regularly. It should also be taken into consideration that newer and newer danger sources are constantly developed mostly in the form of newer chemical agents, but also with the world-wide spread of the already present, environmental polluting technologies. This is especially true since the spread of technologies is not accompanied by the similar spread of environmental protection techniques.

Therefore, the need presents itself to be able to perceive environmental pollution throughout the world with the help of appropriate techniques; the damaging agent in question being either the appearance of a long known or new one. Attention should be focused constantly and continuously on the well known pollution sources as well as on every sign referring to pollution. Further on, the estimation of these effects is necessitated with consideration on the degree of the risk to human environment and on the way the damages could be prevented or ceased.

The constituents of the so-called "anthropogenic pollutions" arising on the effect of human activity may be of several kinds, but can be divided into two main groups. One is comprised of those occurring in smaller or greater quantities in nature itself. These are continuously released by means of the natural forces and geological processes, too, causing a background pollution of the environment. The degree of this may differ at the various regions of the earth, but due to the long-lasting "coexistence" there are no damaging effects perceivable in respect to the living organisms native there. Nevertheless, when as the consequence of human intervention the release of these substances, their entrance into

the soil, water and air greatly increases, their uptake in the living organism becomes multiplied, with the appearance of their damaging effect on the vital processes as well, and this makes them hazardous. Each toxic heavy metal is a constant component of the material forming our planet. In stronger of weaker chemical binding, in the form of minerals or ores, they represent a definite quantity similarly to the rest of the inorganic compounds, which was and remains practically the same during the course of Earth geohistory. That proportion of toxic metals, however, which becomes dissolved and is capable of entering into the living organisms is not only determined nowadays by geological disintegration as well as the natural processes, but also by human intervention. In certain cases the aim of these procedures is the extraction of these materials, while in other cases the mobilization of the toxic metals is a secondary consequence deriving from other processes. Metal mining results in the large-scale extraction of iron, copper, lead, or other metals, at the same time a smaller share becomes dispersed on the surface of the earth, considerably increasing their background level in the environment. During the course of processing, transportation and utilization, the compact and cleaned metals also become environmental pollutants in part, since – although releasing locally – they become diffused in the whole biosphere by means of the water, air and the foodchain. This is the increased level which in certain regions and organisms may reach a degree that becomes toxic to the vital functions either in the uptaking plant or animal, or in the secondary consumer.

Similarly to the heavy metals, the radioactive agents are also of natural origin. The radiation level, too, is concomitant to life on earth, without any damaging effect on the ecosystem. The artificial effectuation of great radiation sources by human activities, however, has implied the inclusion of radioactive radiation among the effects hazardous to the environment. This danger is not a theoretical possibility, but can be a very serious endanger to large populations as shown by both the atomic explosions and the accidents of nuclear plants.

The compounds releasing and getting into the air in the course of coal combusting are also to be included among the natural environmental polluting agents. From these, the most studied are the sulphur-derivatives, as these are the sources of the acidic rains which are greatly hazardous to not only the forests, but also to organisms living in waters and the soil.

The other group of environmental polluting agents is that not oc-

curring in nature. These chemical compounds have been synthesized by man himself, in order to be of help in the production of goods. The chlorinated hydrocarbons should be mentioned in the first place, such as the DDT-derivatives as well as the non-pesticide polychlorinated biphenyls (PCBs). This group comprises a number of herbicides, fungicides and insecticides used in plant protection, as well as several other synthetic materials and derivatives used in industry.

In principle, there are several possibilities for the detection, continuous follow-up and evaluation of the effect of the known environmental polluting agents. Chemical determination at the direct site of pollution – i.e. from the air, water, soil – seems the most obvious, since analytical methods are at disposal for measuring the chemical agent in question and sampling does not cause any problems either. However, studies of samples directly from air or water involve two great difficulties, furthermore, a principle obstacle is also to be taken into account. One of the difficulties derives from the fact that the polluting agent reaching the water or air becomes diluted to an extreme degree and is sometimes present in concentration of $10^{-10} - 10^{-12}$ or even lower. Exact measurements can only be made with the help of well-developed measuring techniques, or previous laboratory concentration is necessitated, encumbering the whole measuring procedure. The other difficulty is represented by the fact that the continuous measuring of every polluting agent at a large number of locations is practically unaccomplishable; samples can only be taken at determined time-points. But, as in most cases the pollution is periodical and changes are observable in regard to the level, such kind of sampling might not represent the periods when the pollution reaches the peak. Naturally, continuous measuring can be effectuated; due to the expenses, however, it is only applicable at few sites and in relation to small number of materials (e.g. radioactivity measurements).

From biological, environmental protection point of view the fundamental defectiveness of evaluating chemical determination from surrounding medium is that although everything present is measured, no knowledge is gained in respect to whether this presence has any harmful effect on the living organisms. And the question is not whether there are any pollutants present, but rather whether they have any harmful effect on the ecosystem or not. From environmental protection point of view, these substances only have significance if they get into direct contact with the living organisms. Relatively ineffective are, for example, the heavy metals bound to parti-

cles in marine sediment, and though the chemically detectable Hg and Cd are by far higher here than in the water, they prove to be much more hazardous at the latter site.

These difficulties and contradictions have resulted that the living organisms themselves have attained great significance in the estimation of the presence of polluting agents, as well as in the continuous monitoring of their level, and the living systems seem the most suitable for the evaluation of their effects too.

The biological indicators have the property of taking up, concentrating the polluting agents present in the environment in low concentrations, thus facilitating the possibility of chemical detection. Mussels, for example, may contain toxic heavy metals in 1000–100,000 fold concentration compared to their environment, especially in the gill and hepatopancreas – the organs most suitable for the binding of heavy metals. DDT and its derivatives are still demonstrable from the lipids of fish and wild animals of the forest, when in the environment they do no longer reach the level of chemical demonstration. In lichens and other plants, even those toxic agents become enriched, which occur only in traces in the air, therefore such accumulating roganisms are well applicable for the signalization of environmental pollution.

Another great advantage of biological indicators is that they do not only demonstrate the momentary state of pollution, but also give an image of a certain process. The living organisms spend several days, weeks, but in the majority of the cases, months and years in the environment where the polluting agents are present, during which interval their concentrations undergo several changes. After taking up the polluting agents, the plants and animals store these for shorter-longer period, only releasing them with delay. In consequence, they also give evidence of the shorter, transitional pollutions occurring in our environment as well as of the transitional increase or decrease in the concentration of the pollutants, respectively.

Those organisms are suitable for continuous monitoring which show good reaction to transitional pollution changes, too, i.e. which are not only capable of concentration, but also of releasing the polluting agent.

The third and greatest advantage of the biological indicators is that direct evidence is given of the substances and their concentrations harmful to the living systems, as well as of the conditions under which they are hazardous to the living world. Only the biological test gives

answer to whether a substance is harmful or not, whether we should beware of them, whether protective measures should be made. The evaluation of the effect may even go further; those targets in the living organisms can be determined, through which the life functions are damaged, in shorter-longer run the health and existence of the organism and later the individual number of the species are influenced by the polluting agent.

Both field studies and laboratory tests have role in the judgement of the damaging effects. Field studies firstly have significance in that the harmful effect exerted on the population is detectable in a striking manner and the effect manifest at an area is measurable. Observations on the ecosystem also provide information on the species and the developmental stages of the species, respectively, suffering the most, and the hazardous substance and its source can be determined relatively with ease. These studies providing us with data on the degree and cause of the damage, however, have a disadvantage; namely, they have no preventive role in respect to the given area, since the object of the observation and estimation is the ensued damaging effect itself. Nevertheless, the discovered causes can subsequently be eliminated and, on the other hand, studies of this kind provide us with experience on how this type of damage can be avoided at other endangered areas.

The significance of laboratory studies is twofold in regard to the evaluation of the effect of the environmental polluting agents. On the one hand, damages having striking symptoms are frequent in the living world, but the causes can only be revealed by means of laboratory analyses and experiments. Mostly the physiological processes, the metabolism, respiration, photosynthesis, the intake and utilization of food, the regulatory system, certain key processes of reproduction become damaged upon the effect of a polluting agent. The detection of sublethal effects is possible by means of biochemical, physiological, morphological, genetical methods, by which means conclusions can be drawn as to the harmful substance and the way it exerts its effect. On the other hand, the effect of substances discharged or to be discharged into the environment, as well as the effect of their products of decomposition can be determined well in advance with the help of laboratory studies, rendering information in regard to the permission of utilization of the new drug. In our days such preliminary investigations are performed according to a system also stipulated in international contracts; at least in respect to the widely used agents. In reality, however, the use of bioindicator organisms has not become practice

sufficiently enough despite numerous attempts, proposals and decisions, whereas their usefulness, importance and applicability are not a matter of dispute. The reason for this is partly that it is difficult to find such biological objects which would be available everywhere in the world, and which would respond to the polluting agents in the same manner besides divergent climatic conditions. The standardization of study objects and conditions would require the allocation of considerable amounts, however, the environmental protection expenditures have not reached this stage in case of most countries.

For this very reason, nowadays the only procedure seems to be the elaboration of general directives related to biological monitoring, the description and comparison of methods applied with success at various places, the elaboration of recommendations for the use of biological indication of environmental pollution by appropriate bodies, as well as the wide-spread establishment of local conditions for the adoption of the methods and for the evaluation of the results. The Bioindicator Programme of the IUBS aims at promoting the realization of this goal.

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BIOLOGICAL MONITORING OF HEAVY METAL POLLUTION IN THE REGION OF LAKE BALATON (HUNGARY)

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(Received: 1987–01–15)

The concentrations of toxic heavy metals (Hg, Cd, Cr, Cu, Zn and Pb) were measured by atomic absorption spectrophotometry in the gills of mussels (Unio pictorum L.) both living in Lake Balaton as well as transferred to various parts of tributaries of the Lake. The measurements were performed separately with two-week intervals during the course of several months.

It was found that (1) the concentration of the studied metals varied with time at each location, less variation occurred in the mussels living in the Lake itself.

(2) There were both increases and decreases in the heavy metal concentration of the gills, presumably reflecting the changing level of pollution of the water.

It is concluded that mussels can be used as biological indicators for detecting temporal variations in the degree of toxic heavy metal contamination in surface waters, and are good objects for signalizing local events of pollution.

Keywords: biomonitoring – heavy metals – freshwater mussel – Unio pictorum L. – atomic absorpiton spectrophotometry

INTRODUCTION

The monitoring of the heavy metal loading of surface waters (lakes, rivers), the confining of the site of pollution sources may be performed in three ways; namely, on the basis of studies on sediment, water and some living organisms, respectively.

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The sediment provides proof on the pollution of waters for a relatively long duration, thus the heavy metal analysis of the waters may be made use of for the identification of polluted areas /16, 26/. However, since the actual rate of sedimentation has not been revealed in the majority of the cases, not even the approximate spatial confining of the sediment-surface values can be realized without the help of further complementary (e.g. paleolimnological) methods /17/.

Depending on the rate of dilution and the actual physico-chemical conditions, there may be a change in the heavy metal concentration of the water, and in certain cases – precisely, in respect to the toxic metals – the concentration stays below the level of the detection limit values of the most wide-spread analytical methods /14, 32/. Furthermore, particularly high numbers of water samples or continuous measurements are necessary for the detection of the possible casual contamination waves /5, 8, 27/.

Most aquatic organisms are able to incorporate and accumulate heavy metals, introduced into their environment, and the change in their levels can well be followed in time and space /4, 6, 15/. Since the accumulator organisms "integrate" the pollution waves affecting them, even rarer samplings provide estimable results. In such manner a technically simple method is at disposal for the identification of polluted areas as well as for the confining of contaminating sources.

The accumulation potential of living systems can be most variable depending on the chemical nature of the various metals and the functional properties of organisms. To the group of organisms possessing a good ability to accumulate and store substances from the aquatic environment, thus usable as indicator of heavy metal contamination, belongs the Pelecypoda which lead a filter-feeding life, letting a considerable amount of water through their bodies in the active filtering period /13, 22/.

During previous investigations it has been pointed out that the distribution of toxic heavy metals in molluscs living in Lake Balaton is not uniform; Hg, Cd, Cu, Zn and Pb occur in the highest concentrations in the gills from the organs easy to dissect /23/. Concerning Cd, similar data were reported for marine molluscs /33/.

Repeated measurements conducted in different periods of the year showed that both increases and decreases occurred in the concentration of heavy metals in the mussels, crustacean plankton and fishes inhabiting Lake Balaton, suggestive of the fact that the heavy metals taken up were not bound for an unlimited period /28, 29/.

Seasonal changes were found in the concentration of heavy metals in the case of other aquatic organisms, too /3, 18, 21, 31/. Therefore, it seems obvious that the changes of the heavy metal contamination in the environment may influence the heavy metal concentration of living organisms both increasingly and decreasingly. Allowing for this, biological systems do not only indicate by means of accumulation the presence of a particular substance, but the concentration changes thereof, too, i.e. they can be the indicators of the increases and decreases of the heavy metal concentration of water.

In the present study the concentrations of toxic heavy metals were measured in the gills of mussels (Unio pictorum L.) at regular intervals for several months. The animals were transferred from Lake Balaton to three tributaries of Lake Balaton and to three, presumedly divergently contaminated reaches of the Zala river (main tributary of Lake Balaton).

The locations chosen for this purpose were near each other and the heavy metal pollution at the various sites was likely to change according to different patterns.

MATERIALS AND METHODS

The mussels Union pictorum L., (shell length 7.7 cm in average) were collected on April 14, 1982 and May 12, 1983 from the open water area of the Keszthely basin of Lake Balaton. In 1982 250, in 1983 150 individuals were placed per study area into the bed of the tributaries by means of fish-nets. The study areas (Fig. 1) were the followings: in 1982 the Tapolca brook (B), the Western Main Canal (C) and the inlet of the Zala river (D), while in 1983 downstream the Zala river (before the city Zalaegerszeg (E), after the city Zalaegerszeg (F) and at the mouth area (D). Each of the above tributaries flows into the western part of Lake Balaton. The catchment area of the Tapolca brook covers 39.5 km^2, its length is 10.2 km; that of the Western Main Canal covers 604.5 km^2, its length is 49.6 km; that of the Zala river covers 2621.8 km^2, with length of 138.8 km.

The heavy metal concentration of the mussel gills was determined at the time-point of the collections from Lake Balaton (A). The obtained values were regarded as the control, initial level. Following this, the heavy metal concentrations were measured in the transferred animals at about two-week intervals. In 1982, mussels were collected monthly for heavy metal measurements also from the control area (A). On every occasion parallel determinations were carried out from 3–3 samples.The gills of 2 animals were used for one sample.

Atomic absorption spectrophotometry was used as analytical method. The gill samples were prepared according to our previous study /23/; in case of Hg determination according to Paus /19/ setting out from wet matter, for the determination of the other metals on the basis of the method

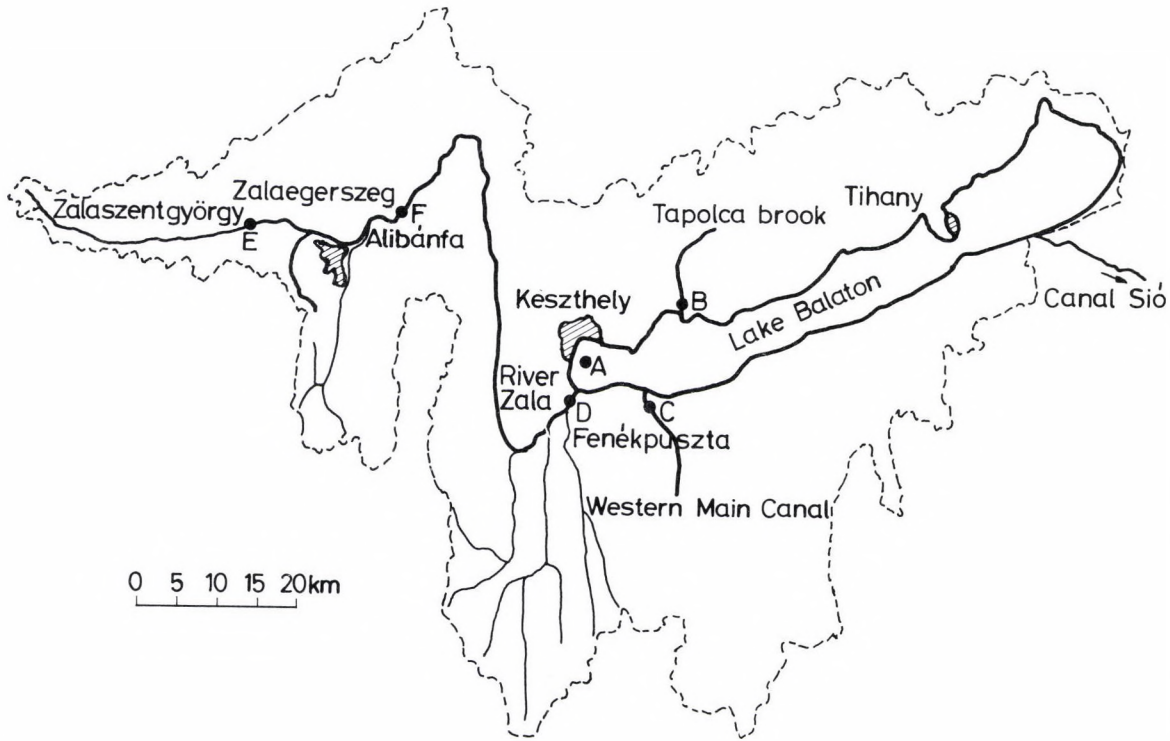

Fig. 1. Map of catchment area of Lake Balaton and sampling locations

of Krishnamurty et al. /12/ at 105°C setting out from dried matter. The Hg-concentration of the digested sample solutions was measured by cold vapour method /9/ with the intercalation of Spektromom Hg-detection unit; the concentration of the rest of the metals was measured in airacetylene flame using Zeiss AAS 1 type equipment. The data are related to dry weight (mean ±SEM). The Hg-concentration was determined in wet tissue, however, these data were also calculated to dry weight. The wet weight/dry weight ratio of the gill tissue was 7.98±1.35 (mean±SD).

RESULTS

1. Changes of toxic heavy metal concentrations in the gills of mussels exposed to the mouth of the tributaries in 1982

The exposition period lasted from 14 April till 22 September. During this time the heavy metal concentrations of the gills showed the following changes: at the control area (A – Table 1), the concentration of mercury decreased a little by June, and practically persisted at this level until September. At the same time, in the three tributaries the Hg concentration (Fig. 2) of the gills became 2–5 times higher as early as by the first sampling time. By June this value diminished somewhat below the control level, then an increase followed which, however, did not essentially exceed the double of the initial value. The highest Hg concentration (0.892 ± 0.246 mg kg^{-1}) was measured on 28 April, in the gills exposed to the Western Main Canal (location C).

The concentration of cadmium exhibited the least changes (Fig. 3) in the gills of molluscs exposed to the Western Main Canal (C), whereas at the other sites, including the control area (A – Table 1), it increased by 1.5–2 times to the end of the experimental period. The highest Cd concentration (12.7 ± 6.58 mg kg^{-1}) was measured on 14 July in the samples from the Zala river (location D).

The concentration of chrome (Fig. 4) showed only slight changes in the molluscs transferred to the Tapolca brook (B) and the Western Main Canal (C), while in the Zala river (D) the concentration of Cr increased to twice that measured at the beginning of the exposure by 2 June and to a 5 times higher level by 11 Aug. The highest Cr concentration (20.9 ± 1.5 mg kg^{-1}) was measured in the sample taken from the Zala river (D) on 14 July.

In the gills of the transferred molluscs, the concentrations of copper (Fig. 5) showed essential increases at almost every site of examination. In the three tributaries the concentration changes of the samples

Table 1

Heavy metal concentrations in the gills of the mussels (Unio pictorum L.) living in Lake Balaton (location A)

date / metal	14th April (control level)	2nd June	18th June	23rd July	13rd September
Hg	0.178±0.048	0.085±0.021	0.135±0.051	0.136±0.036	0.126±0.011
Cd	4.01 ±0.589	8.11 ±1.84	4.85 ±1.24	5.22 ±0.364	7.81 ±0.386
Cr	4.20 ±0.067	9.60 ±4.70	4.55 ±0.051	3.42 ±0.240	11.1 ±1.00
Cu	11.5 ±2.45	6.39 ±1.30	10.2 ±0.972	12.9 ±3.84	99.0 ±67.0
Zn	296 ±41.4	363 ±87.5	206 ±133	372 ±70.4	390 ±151
Pb	22.5 ±1.06	20.9 ±4.92	24.6 ±6.33	23.3 ±2.40	20.6 ±0.720

mg metal kg^{-1} dry weight
(mean ± Standard Error of Mean)

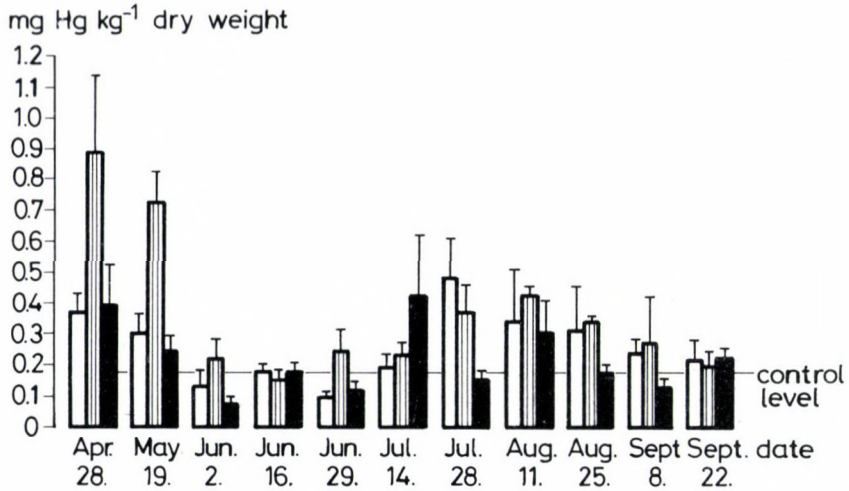

Fig. 2. Mercury concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three tributaries of Lake Balaton. (White column – location B; Striped column – location C; Black column – location D)

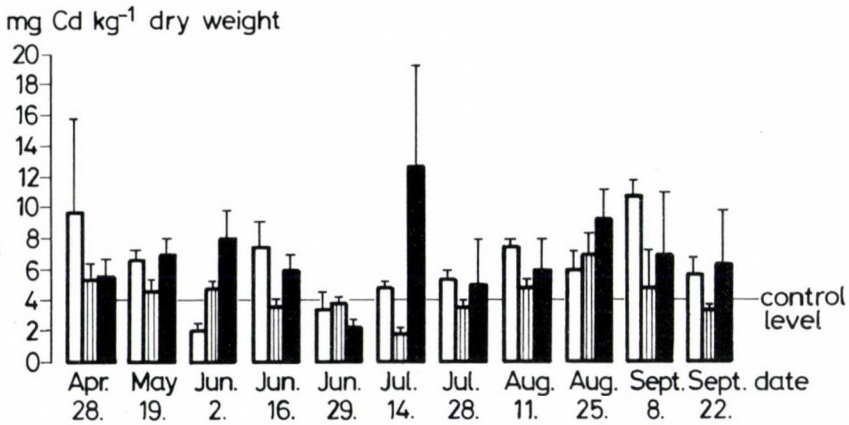

Fig. 3. Cadmium concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three tributaries of Lake Balaton. (White column – location B; Striped column – location C; Black column – location D)

did not take place according to a uniform pattern, albeit in some instances coincidences also occurred. The highest value (232 ± 41.8 mg kg^{-1}) was measured in samples from the Zala river (D) on 25 Aug. In the gills of molluscs taken from the control area (A – Table 1), the Cu concentration remained practically unchanged over the whole period of investigation, though there was an increase by approx. one order of magnitude on 13 Sept.

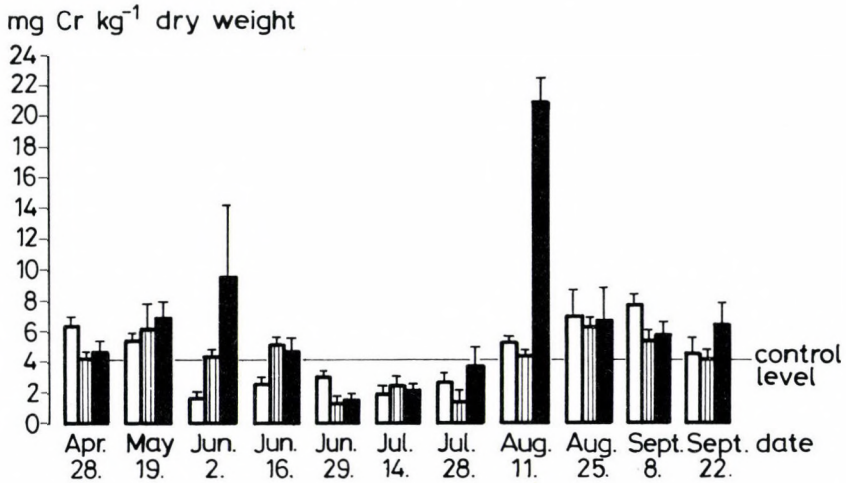

Fig. 4. Chrome concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three tributaries of Lake Balaton. (White column — location B; Striped column — location C; Black column — location D)

At the time of the first sampling the concentration of zinc (Fig.6) diminished below the initial value in the samples from each affluent, then an increase followed. Concentration values were generally never higher than 1.5 of the original value, but at the three sampling sites the increases and decreases of concentrations in the gills of molluscs did not take place at the same time, moreover, there were definite opposite tendencies, too. Zn concentrations of molluscs collected from Lake Balaton (A — Table 1) also exhibited some changes, i.e. there was a decrease in June and a slight increase in September compared to the initial value.

The concentration of lead (Fig. 7) did not show changes at any of the locations, except in the gills of molluscs exposed to the Zala river (D), where two time greater values were measured in 29 June (49.9 ± 8.04 mg kg^{-1}). This increase was followed by a diminution, then again by a gradual increase, and at the end of the experimental period the value measured was 1.5 times that of the initial one.

2. Changes of toxic heavy metal concentrations in the gills of mussels exposed to different reaches of the River Zala in 1983

The exposition period lasted from 12 May till 3 August. The mercury concentration in the mussel gill was low at every time-point at location E

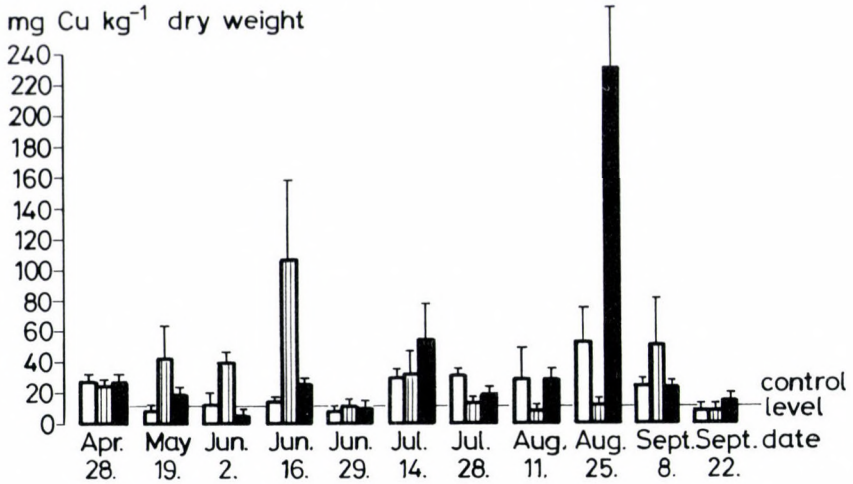

Fig. 5. Copper concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three tributaries of Lake Balaton. (White column – location B; Striped column – location C; Black column – location D)

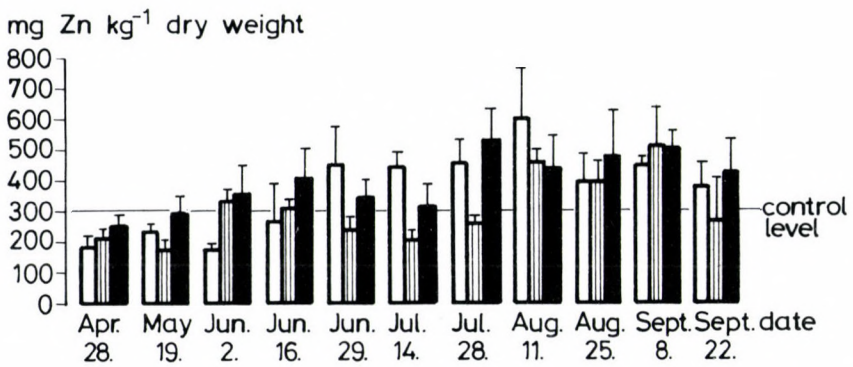

Fig. 6. Zinc concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three tributaries of Lake Balaton. (White column – location B; Striped column – location C; Black column – location D)

(Fig. 1), not surpassing the initial level (0.271 ± 0.063 mg kg^{-1}). Significantly higher ($P < 0.001$) values were detected, however, on three occasions at location D, and on one occasion at location F. The highest Hg-concentration (3.67 ± 1.59 mg kg^{-1}) was experienced at location D on 8 June (Fig.8).

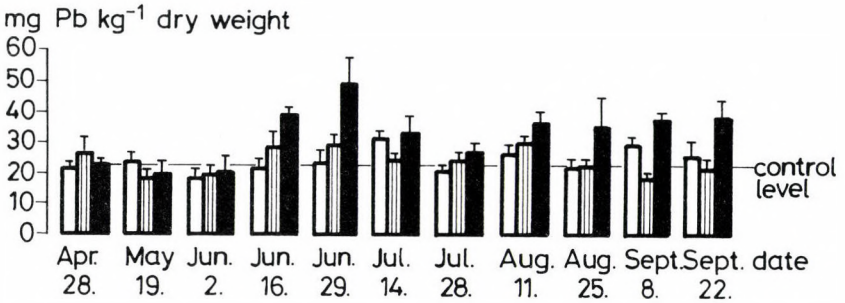

Fig. 7. Lead concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three tributaries of Lake Balaton. (White column – location B; Striped column – location C; Black column – location D)

In comparison to the control level, significantly higher ($P \leq 0.001$) cadmium concentration values were manifest on 8 June at locations F and D in the gills of the transferred mussels. Following this time-point, the level of the metal sinked below the initial value (4.38 ± 1.90 mg kg^{-1}). Although there was some increase at the later time-points, the Cd level of the gills did not surpass the control value significantly ($P \geq 0.05$) in any of the cases. The highest Cd-concentration (14.1 ± 9.10 mg kg^{-1}) was found at location D on 8 June (Fig. 9).

Compared to the level manifest at the time of transfer (10.6 ± 2.41 mg kg^{-1}), significantly higher Cu-concentrations were detected on 8 June at all three study sites, however, there was an approximately twofold increase at locations E and D ($P \leq 0.05$), while an increase higher by an order (150 ± 46.7 mg kg^{-1}) was observable at location F. Following this, the Cu-concentration in the gills was significantly higher ($P \leq 0.001$) as compared to the initial level on two occasions at location F (Fig. 10).

The Zn-concentrations of the mussel gills did not differ significantly from the initial level (325 ± 5.33 mg kg^{-1}) during the exposition period. The Zn-concentration was the highest (520 ± 197 mg kg^{-1}) in the mussels placed into the river Zala at location D on 5 July (Fig. 11).

The lead (Fig. 12) concentrations in the gills were mostly near to, or somewhat below the initial level (29.5 ± 2.27 mg kg^{-1}) during the study period at all three sites.

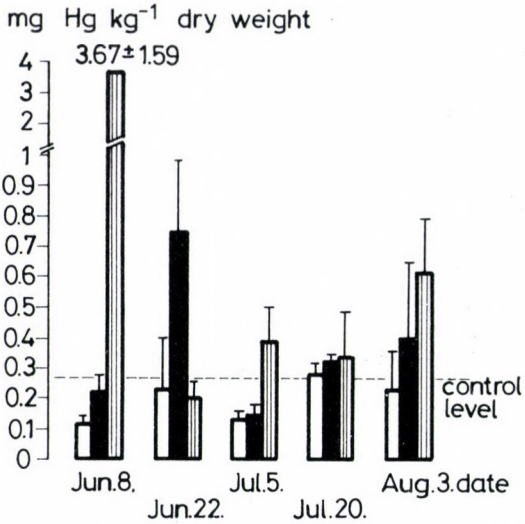

Fig. 8. Mercury concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three reaches of the Zala river. (White column – location E; Black column – location F; Striped column – location D)

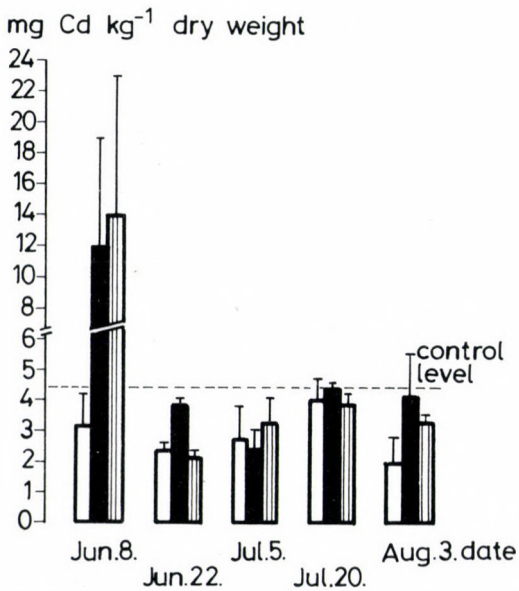

Fig. 9. Cadmium concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three reaches of the Zala river. (White column – location E; Black column – location F; Striped column – location D)

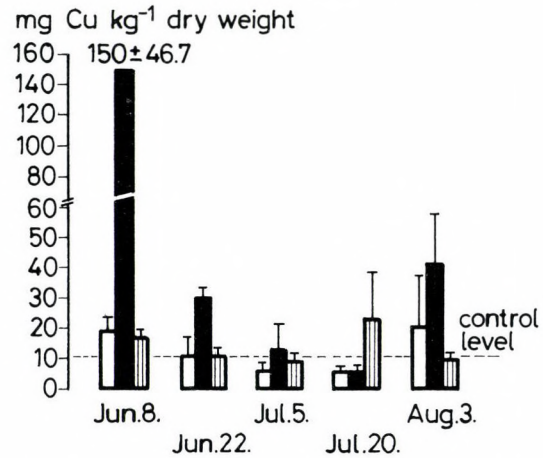

Fig. 10. Copper concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three reaches of the Zala river. (White column — location E; Black column — location F; Striped column — location D)

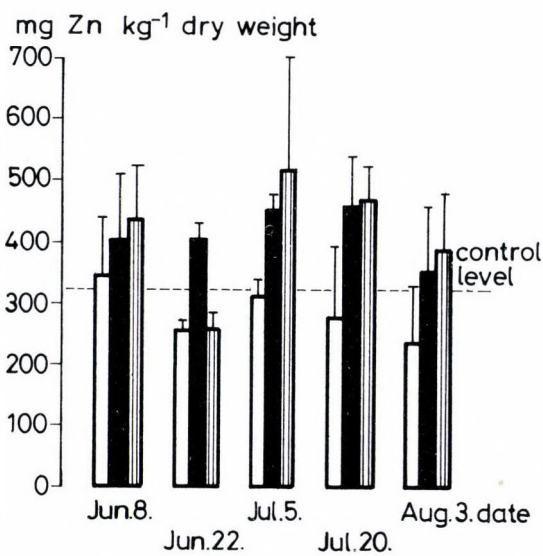

Fig. 11. Zinc concentrations in the gills of the transferred mussels (Unio pictorum L.) at the three reaches of the Zala river. (White column — location E; Black column — location F; Striped column — location D)

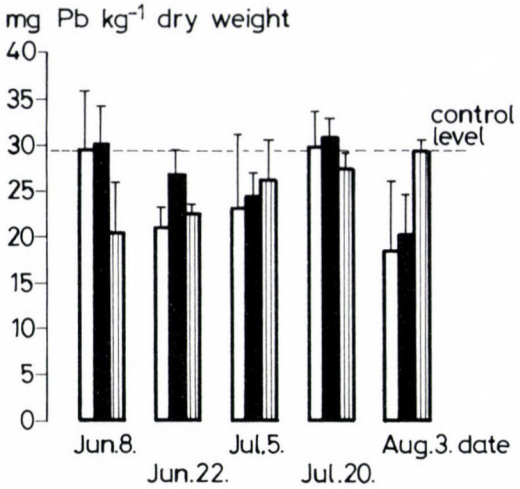

Fig. 12. Lead concentrations in the gills of the transferred mussels (*Unio pictorum* L.) at the three reaches of the Zala river. (White column – location E; Black column – location F; Striped column – location D)

DISCUSSION

The heavy metal concentrations of the tributaries investigated can depend on various factors. From these the most important is the geochemical background which determines a basic level, whereas from the variable factors, the anthropogenic sources and the precipitation falling on the catchment area play an important role /7, 10/. In the catchment areas of the three tributaries lying near one another, identical precipitation conditions prevail, as verified also by discharge measurements. This suggests that the irregularity of anthropogenic sources can be responsible for the differences in the heavy metal loading, and it seems evident that the heavy metal concentrations measured in the gills of molluscs reflect this irregularity.

The results show that the accumulation of heavy metals in the gills of molluscs significantly increased and decreased, resp. in the two-week periods. This must be due to the fact that during that time the concentration of the particular metal changed in the environment of the animals. Laboratory studies have also verified the observation that even a short (1–2 h) increase of the Cd level can change the Cd concentration of the gill

tissues, whereas an influence of longer duration can cause continuous accumulation lasting for several weeks /24, 30/.

The changes of the heavy metal concentrations in the gills of molluscs exposed to various locations of the three tributaries as well as to the control area suggest that during the experimental period increases and decreases temporarily occurred in the heavy metal contamination of these waters.

It appears that in the Tapolca brook (location B) mainly Hg and Cu, in the Western Main Canal (location C) Hg, Cd and Cu, and in the Zala river (location D) Hg, Cd, Cr, Cu and Pb occurred occasionally in higher concentrations and got into Lake Balaton.

On the basis of the heavy metal concentration values found in the gills of the transferred mussels in parallel measurements at three different locations of the Zala river (E, F, and D), it is evident that no heavy metal pollution of perceptible degree was manifest at the reach before the city Zalaegerszeg (location E). At the sampling site after Zalaegerszeg (location F) the presence of Hg, Cd, Cu and Fe in the river water can well be rendered as probable. Near the place where the river flows into Lake Balaton (location D), the frequent appearance of high Hg-concentrations is striking during the course of the study period, which, however, is with all probability for the most part independent of the contaminating effect of the city Zalaegerszeg. In respect to Cd also appearing at the lower reach, however, it cannot be decided unambiguously whether it is the result of an earlier pollution affecting the upper reach of the river, also exerting effect at this area, or whether it is due to some other source, e.g. identical with that of Hg.

In comparison with the results of the accumulation tests performed in the previous year at the upper section of the Zala river /28/, the present data are more favourable since at that time the Hg and Cd concentrations showed a 7– and 4–fold increase, resp. at the end of the investigation period, relative to the initial level.

The question arises, which contaminating sources emitting heavy metals could come into account at the catchment area?

As it is known, the contaminating sources of heavy metals can be ranked among five groups, namely: 1. geological weathering; 2. industrial processes of ores and metals; 3. the use of metals and metal components; 4. leaching of metals from garbage and solid waste dumps; and 5. animal and human excretions which contain heavy metals /7/.

The largest point source in the Balaton region is the city Zalaegerszeg, with a daily sewage discharge of about 16 000 m^3. The existing two-stage treatment plant is heavily overloaded /11/.

Although treated both mechanically and biologically, it cannot be regarded exempt from persistent substances, thus heavy metals. Since these waste substances reach the river in a direct manner, they may be significant source for the toxic heavy metals. Accordingly, the increased metal levels found directly after the city Zalaegerszeg (Hg, Cd, Cu and Fe) can unambiguously be attributed to the contaminating effect of the city. The corrosion of the urban water-supply system, for example, may contribute to the heavy metal loading of the communal sewages, which concerns Cu and Cd from the metals studied by us /20/.

The various detergents used in households may also be such sources. It had been established /1/ that the majority of detergents contain various metals (Cr, Co, Zn, etc.). The solid waste dump located near the city also carries the probability of contamination after washing out.

The phosphate rocks and phosphate chemical fertilizers frequently contain large amounts of trace metals, in particular Cd (30 mg kg^{-1} /25/ 0.1–80.8 mg kg^{-1} /2/). The fact that the level of Cd showed an increase in the monitor organisms at the same time-point far from the city as well, also renders probable the contaminating effect of the chemical fertilization performed at the region being under agricultural cultivation, since 3000 tons of P (6900 tons of P_2O_5) were applied to the Zala watershed in 1981 /11/.

The amount of Zn was found to show a slight increase downstream. Apart from the mentioned contaminating sources, the possibility arises that the great amounts of Zn-containing droppings from the livestock colony /7/ get into the water at several parts of the river.

The results seem to justify the supposition that from the three examined inflowing waters, it is the Zala river that can first come into consideration as a source of heavy metal pollution of the Lake, obviously by means of the heavy communal and certain industrial contamination originating from the city Zalaegerszeg. On the other hand, the polluting effects at the Western Main Canal and the Tapolca brook cannot be regarded negligible either, despite the fact that the size of their catchment areas remains well behind that of the Zala river.

ACKNOWLEDGEMENT

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EXCITABLE MEMBRANES – OBJECT FOR EVALUATING THE EFFECT OF HEAVY METAL POLLUTION

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Abstract: In acute experiments the interaction of heavy metals ($CdCl_2$ and $HgCl_2$) with neurotransmitters (ACh and 5HT) was studied on the excitable membrane of the identified neurons in the central nervous system of Helix pomatia L. (Gastropoda, Mollusca). It was shown that cadmium and mercury ions exert different influence on both resting and action potentials as well as on the responsiveness of the neural membranes to ACh and 5HT. The selective blocking effect of cadmium and mercury ions can be interpreted on the basis of specificity of postsynaptic receptor structures responsible for the transmitter action and of ion-channels involved in the excitatory processes. The heavy metal effect was not uniform for the different types of neurons, suggesting that pollutants can modify various functions to a different degree. The results show that testing on nerve cell membranes can serve as a useful method and model in investigating the effect of sublethal environmental contamination, as they may cause a profound modulation on the elements of the neural circuitry responsible for the regulation of the animal's behavior.

Keywords: Helix neurons – membrane effects of heavy metals – membrane effects of sublethal doses – pollution

INTRODUCTION

Heavy metal pollution constitutes a major part of the environmental problem as heavy metals have become increasingly common contaminants of sea or freshwater as well as landscape. Although the metal uptake by plants and animals has been thoroughly studied far less work has been carried out

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in respect to their effects in the excitable membrane regulating vital physiological functions of the living organisms.

The effect of heavy metals in the nervous system has been studied mainly in regard to their toxic effect on the synaptic transmission /3, 6, 20/. Most of the authors have claimed the heavy metals to act as modifiers of the transmitter liberation at the presynaptic part of synaptic connections while to have no effect at the postsynaptic receptors /2, 5, 6, 8, 9/. In the aquatic gastropod, Lymnaea stagnalis L., however, the postsynaptic site of action of heavy metals has also been demonstrated /18/. The postsynaptic effects of heavy metals have been found to block or modify cholinergic, dopaminergic or serotonergic receptor structures and also to have influence on the ionic channels located to the soma membrane in CNS of Lymnaea /18/.

As a result of the above finding the complex mode of action of heavy metals seems more probable. The manifestation of heavy metal toxicity can be a consequence of the disruption of various biochemical and physiological mechanisms related to the neurotransmission both the in pre-, and postsynaptic parts of the synapses in the nervous system. The heavy metals may have several sites of action in the peripheral and central nervous system, leading to damage of the nerve function and as a consequence, the behavior. These are:

— synthesis, release, uptake and storage of neurotransmitters /5, 6, 21/

— the postsynaptic receptor structures /1, 10, 12, 13/

— the second messenger system and phosphorylation of the membrane proteins /4, 11, 14, 19/.

The presynaptic mechanism of heavy metal toxicity may involve mainly the alterations in intracellularly free cations, including both the liberation of Ca-ions from their storage sites and the Ca-influx from the extracellular sites /3, 6/. At the same time, the postsynaptic sites of actions are directed towards the blocking of the active groups of receptor proteins, leading to the receptor desensitization or inactivation /4, 12, 18, 19/.

The synapses using various neurotransmitters offer different sites of action for heavy metals (Figs 1 and 2). For example, in cholinergic synapses where the uptake mechanism in the elimination of the ACh-response has less importance than of its enzymatic inactivation, the heavy metals are more efficient in acting on transmitter liberation, inactivation or

receptor structures /21/. On the contrary, in adrenergic or serotonergic synapses modification of the uptake mechanism by heavy metals can have far reaching consequences in changing the transmitter effects.

In an effort to understand better the effects and specific sites of action of the heavy metals as environmental toxicant, the microelectrophysiological technique was used to evaluate their membrane effects in a model animal, the Helix pomatia L. The related species, Helix aspersa, was shown to accumulate a large amount of heavy metals in the natural habitat /7/. For this reason the Helix pomatia L. living at the Tihany peninsula was chosen as a model animal to investigate the membrane effect of heavy metals in the terrestrial gastropod.

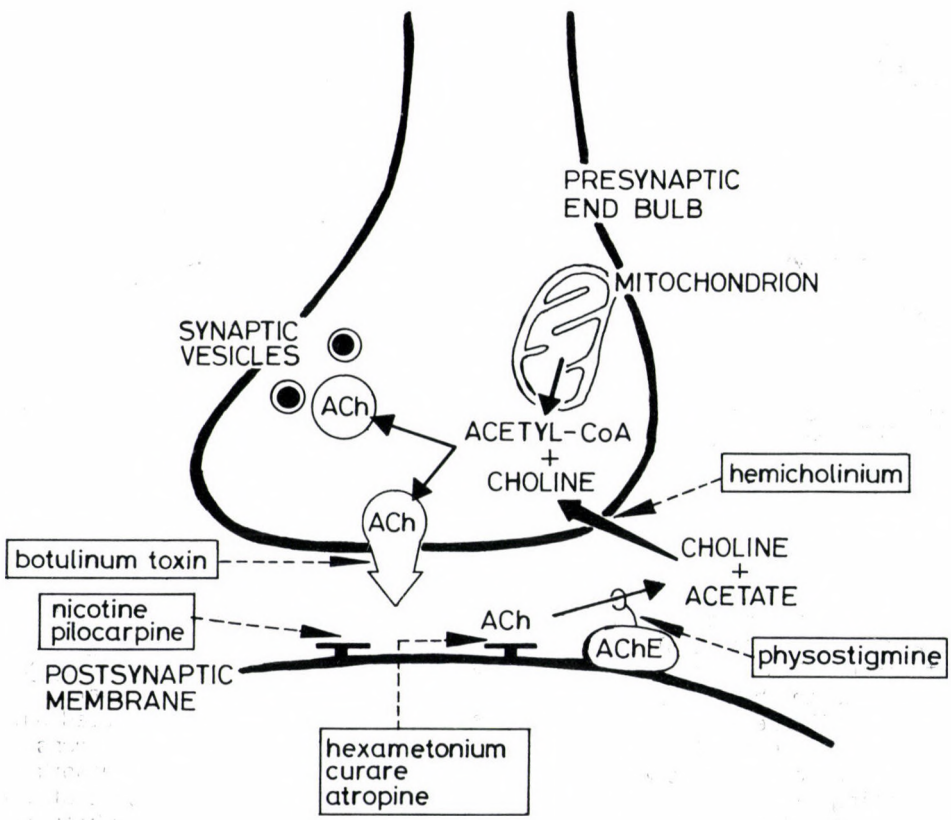

Fig. 1. Diagram of a cholinergic synapse indicating pre- and postsynaptic sites which can be effected by heavy metals. ACh – acetylcholine. Nicotine and muscarinic receptor sites. Postsynaptic hydrolysis of ACh by acetylcholinesterase (AChE)

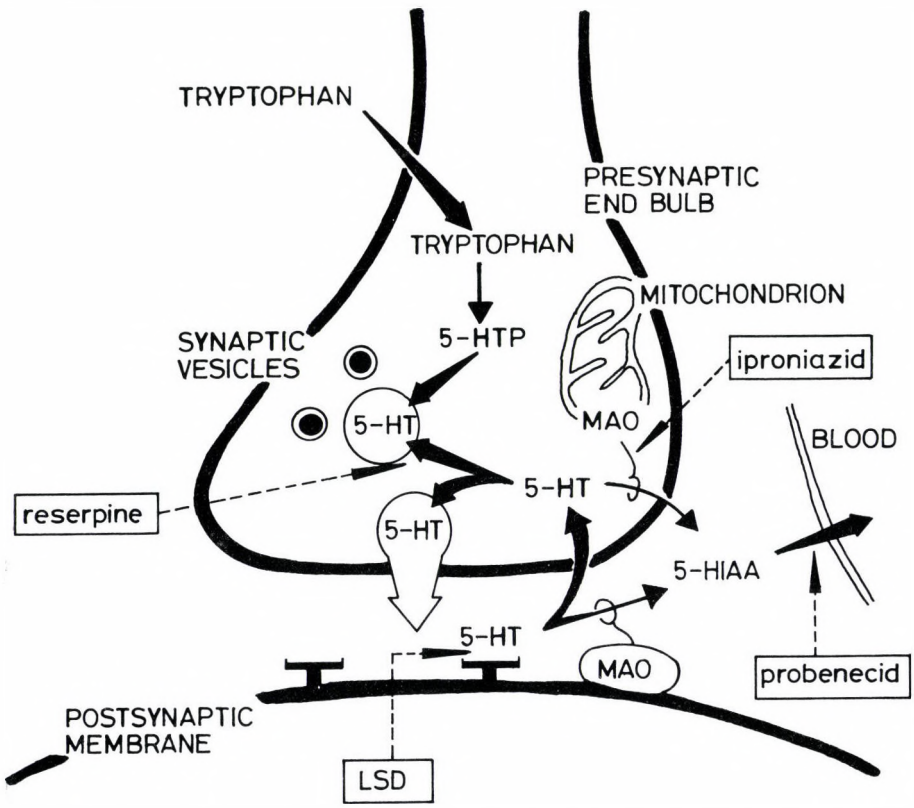

Fig. 2. Diagram of a serotonergic synapse indicating synthesis, breakdown and uptake of serotonin (5HT). MAO – monoamino oxidase, destroying 5HT

MATERIAL AND METHODS

The experiments were carried out on the identified neurons (V21, V2 and RPa2) of the isolated suboesophageal ganglionic ring of Helix pomatia L. (Gastropoda, Mollusca). The isolated ganglion complex of Helix was pinned to the Sylgard in such way that neurons of the dorsal surface became available (Fig. 3). The identification of the cells was carried out earlier using morphological and physiological criteria /16/. The neurons used in the experiments were identified as members of the neural network regulating visceral function /17/. The cell RPa2 is the largest cell of the right parietal ganglion forming an afferent pathway to the heart, while the cell V21 is located to the visceral ganglion and has been found to be a multifunctional interneuron /16/.

The electrical activity of the neurons was recorded with conventional microelectrophysiological method using KCl-filled glass-microelectrodes. The ganglia were perfused permanently during control conditions

with the saline containing heavy metals. For displaying and recording action potentials four-channel Tektronix oscilloscope (R103N) and Gould-Brush recorder were used.

The specific response of the neurons to transmitter substances was studied under control conditions and following application of heavy metal ions. From the heavy metals, $CdCl_2$ and $HgCl_2$ were used, while the sensitivity of the neurons was tested to acetylcholine (ACh) and 5-hydroxytryptamine (5HT). Transmitter substances were applied either in drop application into the bath, or in a small quantity from a micropipette directed to the soma of the investigated neuron /17/.

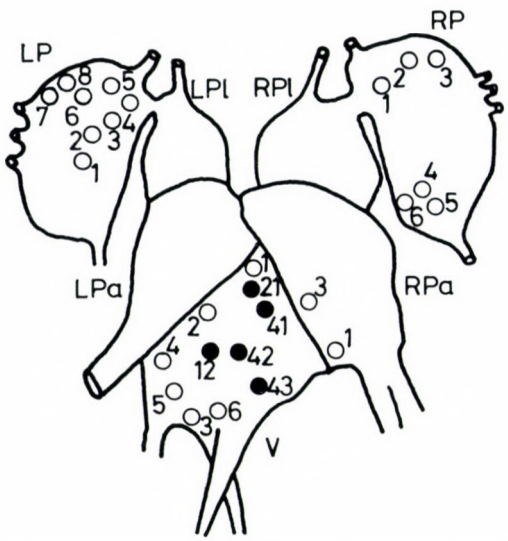

Fig. 3. Dorsal surface of the suboesophageal ganglionic ring and distribution of the identified cells regulating visceral functions in Helix pomatia L. LP and RP – left and right pedal ganglia, LPl and RPl – left and right pleural ganglia. LPa and RPa – left and right parietal ganglia. V – visceral ganglion

RESULTS

The effects of the neurotransmitters ACh and 5HT on the neurons used here have been described earlier /17/. On the interneuron V21 both acetylcholine and 5HT were shown to elicit biphasic effect (an excitation followed by inhibition of the firing), while in the cell RPa2 only the ACh caused biphasic effect, and 5HT as a rule depolarized the cell membrane leading to increased firing /17/. In case of biphasic effect to neuro-

transmitters one or another of the responses could be more expressed in different preparations. Treatment of the ganglia with $HgCl_2$ or $CdCl_2$ solution did not cause any noticeable effect on the membrane potential or spike generation within 60 min. At the same time, the transmitter response was modified following heavy metal treatment in the investigated neurons.

1. Interaction of the $CdCl_2$ with ACh and 5HT responses

On the interneuron V21 both the high frequency firing and the depolarization caused by ACh application were modified following $CdCl_2$ treatment. The interaction of $CdCl_2$ and acetylcholine was found to be dose-dependent.

The membrane of the interneuron V21 was depolarized by ACh at 10^{-4}M accompanied by high frequency firing (Fig. 4A). At the first 10 min of $CdCl_2$ (10^{-4}M) application the ACh-induced depolarization declined to 65 per cent of the control value (Fig. 4B, C) and the high frequency firing was correspondingly decreased, too. The ACh-evoked depolarization was not completely eliminated, not even by the 40 min of $CdCl_2$ treatment, on the contrary, the decrease in ACh-evoked response stabilized at about 70 per cent of its original value (Fig. 4E, F, G). In the long-lasting $CdCl_2$ treatment the ACh-elicited high frequency firing was also partially restored (Fig. 4 E, F). In case the ganglia were treated with a lower concentration of $CdCl_2$ (10^{-5}M), the ACh-elicited depolarization was only decreased by 20–25 per cent of its control value (Fig. 5) and an ACh-evoked high frequency firing remained more stable even after 90 min treatment with $CdCl_2$ (Fig. 5). However, the spontaneous firing of the cell V21 was inhibited by treatment of the ganglia for more than 80 min with $CdCl_2$ (Fig. 5D).

Not all the investigated neurons showed the same interaction between ACh and $CdCl_2$. As can be seen in Fig. 6, on the cell V2 ACh-elicited depolarization and the high-frequency firing were potentiated by $CdCl_2$ treatment. In this case the ACh-evoked high frequency firing became more pronounced, while the depolarization increased by 15–20 per cent following $CdCl_2$ treatment (Fig. 6).

The membrane of the cell RPa2 reacted to the ACh (10^{-5}M) in a very similar manner to that of the neuron V21; it caused a depolarization and a superimposed high frequency firing (Fig. 7A). The $CdCl_2$ at 10^{-3}M caused within 5 min a slight decrease in the amplitude of ACh-evoked depolarization with simultaneous prolongation of the wave of depolarization and high

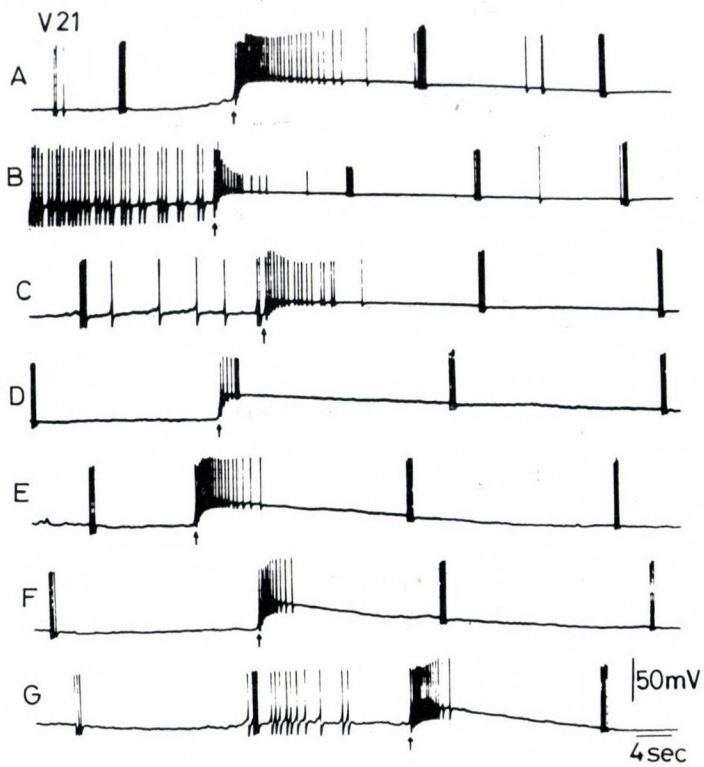

Fig. 4. Modulation of ACh-evoked depolarization and high frequency firing folloqing $CdCl_2$ treatment of the isolated ganglia of Helix pomatia L. A– control and application of ACh at 10^{-4}M to the some of the neuron V21. B,C,D,E,F and G – the effect of ACh at 10^{-4}M at 1,5,10,25,35 and 40 min of $CdCl_2$ (10^{-4}M) application – here and in the following figures, arrow indicates the moment of ACh (or other neurotransmitter) application

frequency firing (Fig. 7B, C). The washing out of $CdCl_2$ led to the partial restoration of the ACh-response (Fig. 7D). In the same experiment, the long-lasting treatment of the ganglia with a higher concentration of $CdCl_2$ (10^{-2}M) first led to the prolongation of both depolarization and high-frequency firing (Fig. 7E, F), then due to the 30 min of $CdCl_2$ treatment the high frequency firing was significantly weakened and the depolarization decreased by 60 per cent of its control value (Fig. 7G). The 60 min of $CdCl_2$ (10^{-2}M) treatment caused the complete elimination of the ACh-evoked high frequency firing, while the depolarization still survived, although

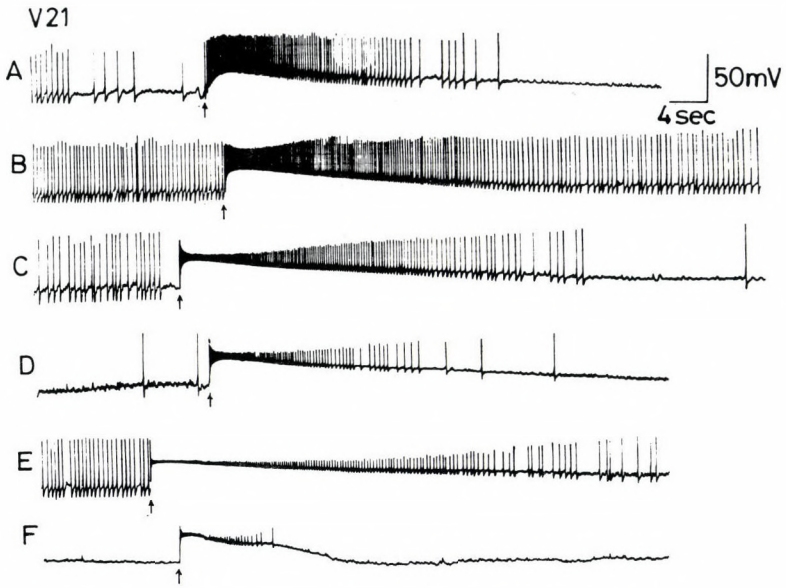

Fig. 5. Effect of $CdCl_2$ (10^{-5}M) on the ACh-elicited depolarization and high-frequency firing on the cell V21. A — control response of the neuron to ACh (10^{-4}M). B,C and D — modulation of the ACh-response at 20, 80 and 90 min of $CdCl_2$ (10^{-5}M) treatment. E and F — ACh-response following washing out of the $CdCl_2$

by a 65 per cent lower level than that of the control (Fig. 7H).

However, if the ACh concentration was elevated to 10^{-4}M the $CdCl_2$ at 10^{-2}M failed to eliminate the high frequency firing or to decrease the ACh-evoked depolarization even after 40 min treatment with $CdCl_2$ (Fig. 8D). On the contrary, in such combination the $CdCl_2$ treatment caused a potentiation by 20–60 per cnet of the ACh-evoked depolarization and the prolongation of high frequency firing (Fig. 8B, C, D). The potentiation of the ACh-evoked response was partially reversible by washing out the $CdCl_2$ (Fig. 8E).

On the neuron RPa2 the 5HT-evoked response was a slight depolarization and a firing frequency increase (Fig. 9A). As a rule, the 5HT evoked response was potentiated at the first 10 min of $CdCl_2$ treatment, then by the 20th min of $CdCl_2$ application it almost returned to the control value (Fig. 9B, C and D). High frequency firing like that evoked by ACh (Figs 7, 8 and 9E) was never observed introducing 5HT to the soma membrane of the neuron RPa2 (Fig. 9A).

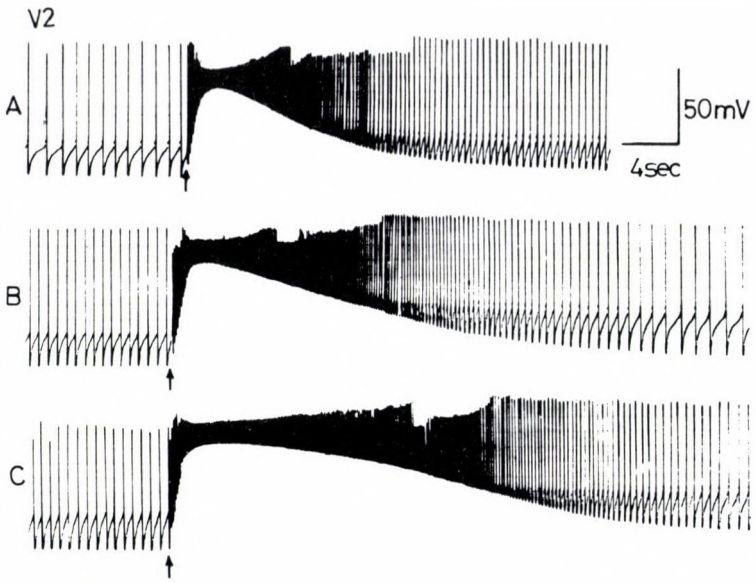

Fig. 6. Potentiation of ACh-evoked depolarization with $CdCl_2$ treatment. A – control effect of ACh (10^{-4}M). B and C – ACh effect following 10 and 15 min treatment with $CdCl_2$ (10^{-5}M)

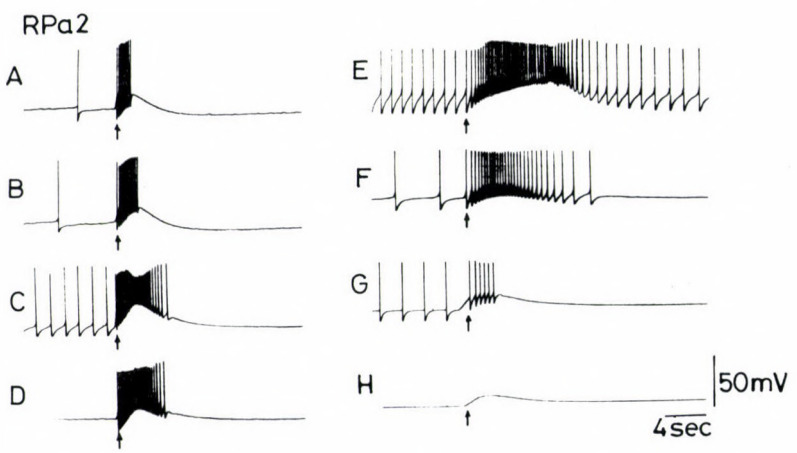

Fig. 7. Interaction of ACh and $CdCl_2$ on the membrane of the neuron RPa2. A – control effect of ACh (10^{-5}M). B and C – ACh (10^{-5}M) effect following the perfusion of the ganglia with $CdCl_2$ (10^{-3}M) for 1 and 5 min, respectively. D – ACh effect after washing out of $CdCl_2$. E,F,G,H – ACh-evoked response at 10,20,30 and 60 min of $CdCl_2$ (10^{-2}M) perfusion

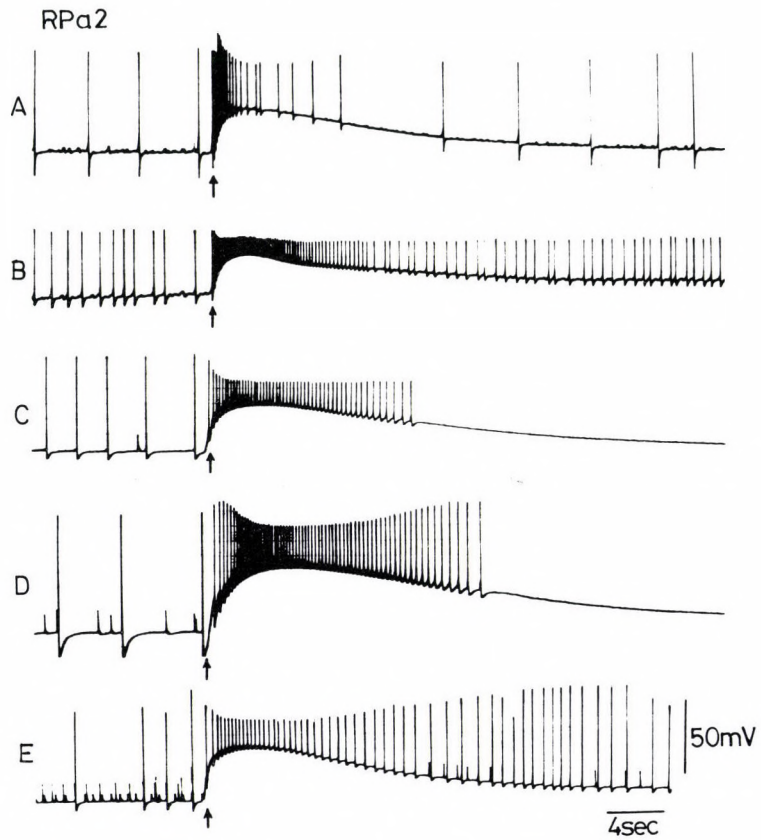

Fig. 8. Interaction of 10^{-4}M ACh and 10^{-2}M $CdCl_2$ on the cell RPa2. A — control response of ACh at 10^{-4}M. B,C,D — modulation of ACh response following, 2, 20 and 40 min $CdCl_2$ (10^{-2}M) treatment, respectively. E — ACh response after washing out of the $CdCl_2$

2. Interaction of $HgCl_2$ with neurotransmitter-evoked membrane effects

The $HgCl_2$ effectively and completely blocked the ACh-evoked depolarization and high frequency firing of the cell RPa2 (Fig. 10). The $HgCl_2$ was found to be more effective in eliminating the ACh response than that of the $CdCl_2$. As can be seen in Fig. 10, the $HgCl_2$ at 10^{-4}M gradually decreased the ACh evoked depolarization, the high frequency firing and the hyperpolarization (Fig. 10B, C). 50 min of $HgCl_2$ treatment resulted complete blocking of the ACh-evoked responses (Fig. 10E). The inhibitory effect of $HgCl_2$ on the ACh-response was found to be irreversible.

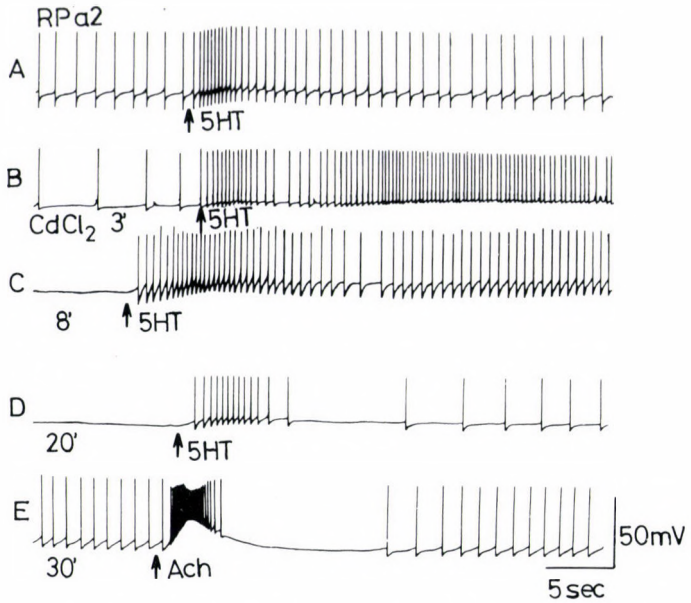

Fig. 9. Interaction of 5HT (10^{-4}M) and ACh (10^{-4}M) with $CdCl_2$ (10^{-2}M) on the cell RPa2. A – control effect of 5HT (10^{-4}M). B,C and D – modulation of 5HT-response following 3, 8 and 20 min $CdCl_2$ treatment, respectively. E – ACh response at the 20th min of $CdCl_2$ treatment

The $HgCl_2$ treatment was less effective in modifying 5HT response than that of the ACh (Fig. 11). In fact, the 5HT-evoked increase in firing was not eliminated by $HgCl_2$ treatment on the cell RPa2, however, here the depolarization evoked by 5HT was less expressed (Fig. 11).

DISCUSSION

The results showed that heavy metals selectively and significantly modified the membrane effects of neurotransmitters in the CNS of Helix pomatia L. The observed effects are connected to the modifying or blocking of cholinergic and serotonergic receptors, as well as the influencing ionic channels located at the soma membrane in a similar way as has been described for the central neurons of the pond snail, Lymnaea stagnalis L./18/.

Here, the heavy metals may have a site of action on the active groups of the receptors for neurotransmitters. It is well known that mercury ions have high affinity for sulfhydryl (-SH) and disulfide (S-S)

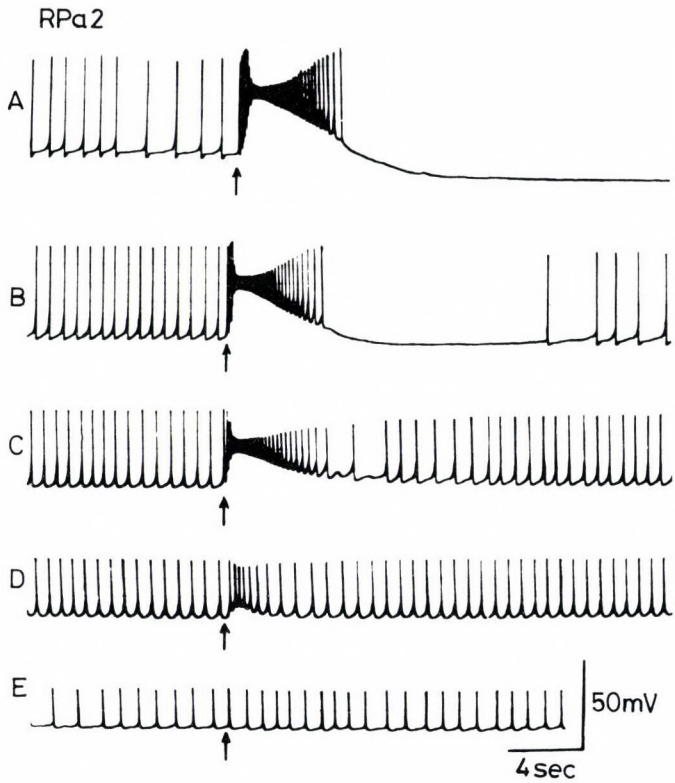

Fig. 10. Elimination of ACh-evoked responses by $HgCl_2$ in the neuron RPa2. A — control response to ACh (10^{-4}M). Here the depolarization, high frequency firing and post-hyperpolarization caused by ACh can be seen. B,C,D and E — modulation of ACh-evoked response following $HgCl_2$ (10^{-4}M) treatment of the ganglia at 6,20,30 and 50 min of its application

groups and their toxic effect is based on the mercury-sulfure interactions /12, 15, 19/. Cadmium has been shown to block the SH groups of various membrane enzymes and receptor proteins as well /10/. The muscarinic acetylcholine receptors have also been found to be modified by both mercury and cadmium ions in the vertebrate nerve system /1,21/. In addition, cadmium is known to be able to inhibit neuromuscular transmission and to interfere with the influx of calcium after binding to the SH groups in the membranes /20/. In chronical experiments the mercury and cadmium ions can also produce decrease in the ACh and 5HT level in vertebrate brain /11/.

In our experiments the effects of mercury and cadmium ions had a

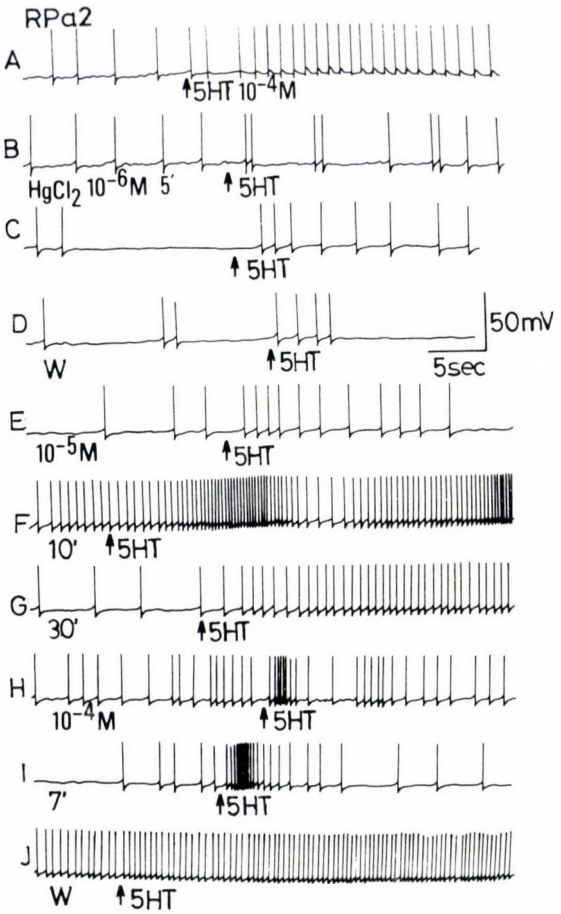

Fig. 11. Effect of $HgCl_2$ treatment on the 5HT evoked response in the cell RPa2. A – control response to 10^{-4}M 5HT. B and C – 5HT (10^{-4}M) response following $HgCl_2$ (10^{-6}M) treatment for 5 and 8 min. D – washing out of the $HgCl_2$. E,F and G – 5HT (10^{-4}M) response following $HgCl_2$ (10^{-5}M) treatment for 5,10 and 30 min respectively. H and I – effect of $HgCl_2$ (10^{-4}M) on the 5HT (10^{-4}M) response at 5 and 7 min of application. J – washing out of the $HgCl_2$

postsynaptic site of action rather than a presynaptic one. In the Helix CNS heavy metals have been shown to modulate the neurotransmitter effects likewise as in Lymnaea central neurons /18/. As the heavy metals are able to sensitize or inhibit the neurotransmitter effects, it can be suggested that under the influence of Cd or Hg ions the whole regulatory process can be modulated which has its consequences in the behavior of the animals.

The alteration of receptor affinity to neurotransmitter caused by mercury and cadmium ions simultaneously also affects the channel function of the membrane. The interaction between the heavy metals and neurotransmitters was found to be very specific. The 5HT response was less affected by heavy metals than that of the ACh. At the same time, the short-term treatment of the ganglia with $HgCl_2$ or $CdCl_2$ caused more dramatic inhibition of the transmitter effect than the long-lasting one. This means that the nerve membrane can mobilize the receptors embedded deeper into the membrane following inactivation by heavy metals of the active groups of surface receptors in the membrane. The protection of the receptors can also take place in this phenomenon /6/ underlying the tolerance of the animals to environmental pollution.

Our results showed that terrestrial snails may be a useful tool for predicting early, sublethal effects of heavy metals. The modifying of the transmitter effects on the cells regulating visceral function (e.g. reproduction) may contribute to the prediction of the conseuqnces of heavy metal pollution in the population of the ecosystem.

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THE ROLE OF AQUATIC TOXICITY TESTS IN PREDICTING AND MONITORING POLLUTION EFFECTS

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The complementary role of aquatic toxicity tests in relation to other forms of pollution assessment is discussed. These tests may be predictive and designed to estimate hazard, or used for monitoring waters to assess compliance with standards. The effects of pollutants are significantly modified by certain water quality characteristics and by a variety of biological factors. The latter, particularly those causing physiological stress, are discussed.

INTRODUCTION

Aquatic pollution assessment and control have traditionally been dependent upon physico-chemical monitoring to identify and quantify toxicants and to provide data which, for regulatory purposes, could be compared to allowable concentrations for a particular receiving water. Although great advances have been made in the design and accuracy of chemical sensors and associated analytical procedures, such monitoring techniques are inadequate on several counts: (a) unless monitoring is continuous, the results are valid only for that particular sampling time (weekly, monthly?); (b) however accurate the analytical techniques are, they can only monitor those specific pollutants which they are designed to detect and without doubt many waters, particularly those receiving complex effluents, contain

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toxicants for which there are no routine monitoring procedures; (c) the demonstration that a toxicant is present gives no indication of the biological effect, which may be influenced by the presence of other stressors including additional pollutants and a variety of water quality characteristics; (d) toxic effects may occur at concentrations below the detection limits for some pollutants.

It is clear therefore, that a realistic interpretation of chemical toxicity to biological systems can only be carried out by use of a system which actually employs living organisms. Consequently, biological monitoring programmes have developed alongside and complementary to physico-chemical monitoring. These give a more direct indication of pollutant effect and are conventionally carried out by a programme of biological surveys designed to detect changes in species distribution and community structure. Largely dependent upon benthic macroinvertebrates, several biotic and diversity indices can be calculated on the basis of key species or bio-indicators characteristic of particular water types /23/. These biomonitoring methods are normally based upon the presence, absence, abundance and spatial distribution of certain indicator species and consequently provide data which reflect water quality at the sampling site for an indeterminate period before the sampling event.

TOXICITY TESTING

An extension of this biomonitoring approach is to make use of some specific response or functional attribute of the test organism to indicate quality of the water to which it is exposed, i.e. a bioassay. The most common application of the bioassay procedure is seen in the toxicity test /10, 37, 46/. Such tests carried out for pollution regulation purposes may be of two principal types (Fig. 1) (a) predictive and (b) monitoring.

(a) Predictive Tests

These are normally laboratory based and designed to determine the likely effects of a substance if it should enter a water body, i.e. is there a hazard? Such tests, particularly those involving acute toxicity but increasingly those of chronic and sublethal nature, provide basic data required by the regulatory agencies and by those responsible for the formulation of water quality standards.

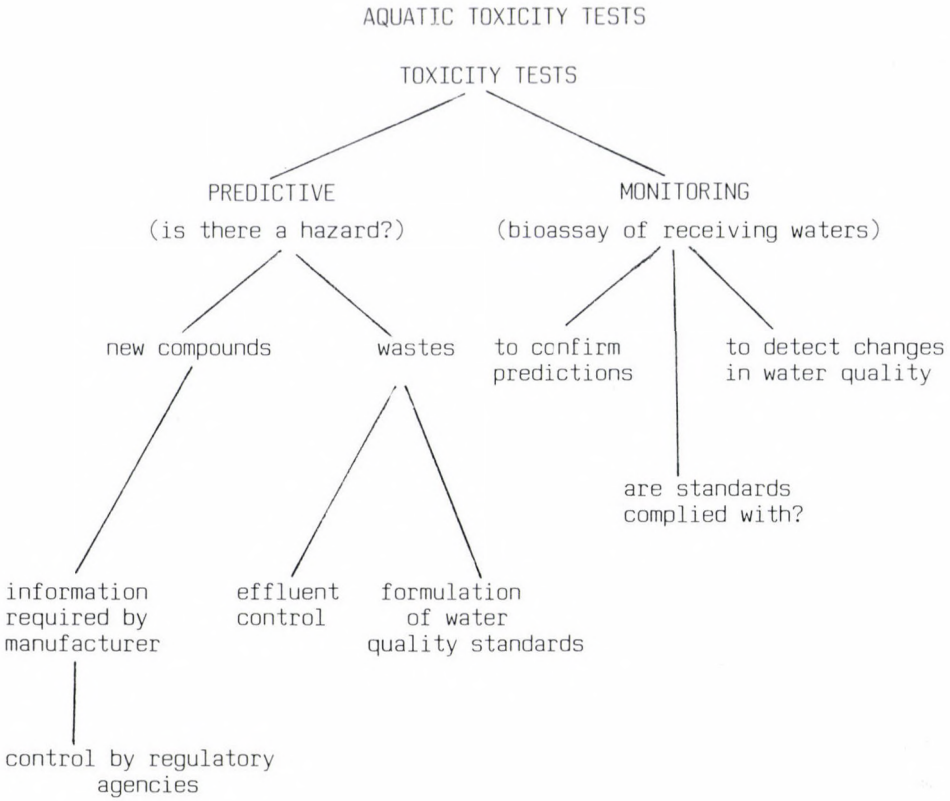

Fig. 1. The various applications of aquatic toxicity tests

(b) Monitoring Tests

These tests are carried out with samples taken from the water body into which the test substance or effluent has been discharged, in order to assess the extent to which water quality is in compliance with standards and to assist in the explanation of effects detected by routine biomonitoring procedures.

FACTORS MODIFYING TOXICITY

Within the overall scheme indicated in Fig. 1 a vast array of acute, chronic, lethal and sublethal toxicity tests at various organisational levels from the subcellular in vitro test to the artificial channel and microcosm is available /37/. Nevertheless, there remains the problem of extrapolating experimental data from such tests to the real world, where

numerous unknown and/or uncontrollable factors may contribute to the toxicity of a pollutant. These include water quality and biological factors.

Water Quality Characteristics

Although there have been numerous reports of water quality factors which may modify (increase or decrease) the toxicity of a pollutant, in many cases the mechanism by which this occurs, whether directed towards the test organism (e.g. affecting the physiological processes leading to detoxication), the pollutant itself (solubility, chemical speciation and availability) or both, is not clear. Temperature /11/, pH /17/, hardness /38/, and dissolved oxygen /18/ are of particular importance as is the simultaneous presence of other pollutants and the possibility therefore of interactions leading to synergistic or antagonistic effects /8, 19/.

Biological Factors

In contrast to water chemistry, the influence on toxicity of biological factors has not been so comprehensively considered. However, it must be appreciated that observed toxicity represents the overall balance between the rates of toxicant uptake, metabolism and excretion and each of these processes may be modified by biological factors such as (a) the stage in the life cycle (b) physiological stress and (c) changes resulting from prior exposure to toxicants.

(a) Stage in the life cycle

There is now ample evidence that different stages in the life cycle of aquatic animals may show differing responses to toxicants with juvenile macroinvertebrates, for example, generally being more sensitive than mature animals.This phenomenon is clearly illustrated in Tables 1 and 2 and may be explained for some pollutants /9/ at least in part by the larger surface: volume ratio of juveniles providing for greater exchange with the environment, and their high lipid content increasing the uptake of lipid-soluble pollutants.

In the case of the freshwater detritivore Chironomus riparius, larvae clearly become more tolerant of cadmium as they mature /50/, with the most resistant stage (fourth instar) having a 24 h LC50 of 2400 mg l^{-1}, about 950 times greater than the recorded value of 2.1 mg Cd l^{-1} for the most sensitive (first instar) stage. Despite widespread recognition of such

Table 1

Median lethal concentrations (96 h LC50) of cadmium for Asellus aquaticus at different stages of development (data from Green, Williams and Pascoe /21/

Development stage	96 h LC50 (µg Cd l^{-1})
Embryo (appendages beneath membrane, yolk mass visible	2000
Embryo (appendages released, yolk mass depleted)	300
1.3 mm	80
1.6 mm	170
2.3 mm	175
3.5 mm	320
5.9 mm	540
9.8 mm (adult)	1000

Table 2

Median lethal concentrations (LC50) of cadmium for Chironomus riparius at different stages of development (data from Williams, Green, Pascoe and Gower /50/

Development stage	LC50 (mg Cd l^{-1})			
	12 h	24 h	48 h	96 h
1st instar	325	2.1	–	–
2nd instar	1350	260	45	13
3rd instar	2200	500	72	22
4th instar	5100	2000	725	54
Ratio 4th: 1st instar	15.7	952	–	–

differences, toxicity tests are traditionally carried out with the most convenient, largest or most readily available stage in the life cycle rather than the most sensitive. This presents the possibility, when the

toxicity data are used in formulating water quality standards, that these may not be protective for sensitive components of the biota. It is interesting to note that while larval stages are normally sensitive, early embryos of fish /44/ and some invertebrates /21/ appear to be particularly resistant. This is probably due to the protective action of the fish egg chorion /4/ and invertebrate egg membranes /21/.

Sensitivity, however, is not restricted to the juveniles and at particular phases e.g. moulting and brood-carrying, even mature animals such as Gammarus pulex (McCahon, pers. comm.) show lowered tolerance of heavy metals.

(b) Physiological stress

Many authors /24, 28, 42, 45, 48/ have noted that animals suffering from the effects of a pollutant are more likely to succumb to disease, particularly bacterial and fungal infections than normal animals. This is possibly the result of immunosuppression due to stress-induced corticosteroids. However, it appears that the converse situation also occurs, with physiological stress such as that due to parasitic disease or dietary deficiency leading to a state of increased susceptibility to an additional stressor such as a pollutant. Paperna /34/ for example, in a comprehensive discussion of the parasites and disease of striped bass, Morone saxatilis, considered that heavily infected fish are more likely than healthy fish to succumb when water quality deteriorates and pollutants are present. Nepszy and Dechtiar /33/ studying the stress imposed by the microsporidian parasite Glugea hertwigi on rainbow smelt, Osmerus mordax, and Adelman and Smith /1/ investigating reference toxicant effects on healthy and "unhealthy" fathead minnows (Pimephales promelas) speculated similarly. Quantitative data supporting this hypothesis for fish and one invertebrate are presented in Table 3. In view of these observations it is evident that laboratory toxicity tests performed with animals cultured in the absence of parasites, or with animals collected from the field and then quarantined for identification, and treatment or removal of diseased specimens, may not always realistically simulate real pollution incidents.

Similarly, the nutritional status of test animals particularly fish, can modify their response to pollutants with dietary deficiency /2, 29/, poor diet quality /13, 30, 31, 49/ and nature of body reserves /25/ effecting sensitivity to pollutant stress. Since animals used in toxicity tests

are frequently cultured on synthetic, commercially prepared diets of variable constitution the data derived from tests may again not be directly applicable to real pollution problems. Pascoe & Woodworth /40/ investigating the effects of parasitism (due to plerocercoids of Schistocephalus solidus), dietary stress (limited availability of food) and cadmium (0.032 mg l^{-1}) reported that the combined effect of the three stressors on the three-spined stickleback, Gasterosteus aculeatus, was significantly greater than and other combination.

(c) Previous exposure to the toxicant

There is considerable evidence to show that previous exposure to a pollutant can alter an animal's response to that pollutant. In some cases, e.g. with arsenic /14/ and with metals /6, 15, 16, 35/ fish generally become more tolerant while with others, e.g. cyanide /14/ they become more sensitive. Pretreatment with the organophosphate parathion /3/, however, neither increased nor decreased the toxicity of the insecticide to rainbow trout. To add confusion to this already complex situation it now appears that for fish, as is well recognized in mammals /47/, pretreatment with one metal, e.g. zinc can also induce resistance to others such as cadmium /27/.

The significance of this pretreatment phenomenon becomes evident when the nature of aquatic pollution is considered. Many pollutants, rather than occurring at constant concentration, are found in fluctuating /12/ or isolated episodic /39/ events which may provide animals with the opportunity to develop the tolerance or increased sensitivity considered above.

There is also information to suggest that some populations have developed tolerance to pollutants by genetic adaptation following long-term exposure through many generations /7, 20/. The use of these tolerant animals in toxicity tests for hazard evaluation could be very misleading.

CONCLUSIONS

Although toxicity tests have wide application both for predicting the potential hazard of chemicals and for assessing the effects, should such a hazard become reality, many authors have drawn attention to the problems of relating laboratory toxicity data to real pollution problems. It is generally accepted, for example, that there is no single 'correct' value for the toxicity of a pollutant to a test organism but that the value de-

Table 3

Data from joint stress experiments illustrating the increased sensitivity
Toxicity in most examples is expressed by the LC50 (median lethal

Test species	Primary stressor	Toxicant
Pimephales promelas (Fathead Minnow)	Skin flukes and bacteria	Sodium chloride
Oncorhynchus kisutch fry (Coho Salmon)	Anodonta oregonensis glochidia (Mollusca)	Water soluble fraction of crude oil
"	"	Naphthalene
"	"	Toluene
Oncorhynchus kisutch juveniles (Coho Salmon)	Corynebacterium salmoninus (bacterial kidney disease)	Sodium pentachlorophenate
Oncorhynchus nerka (Sockeye Salmon)	Eubothrium salvelini (Cestoda)	Zinc (1 mg l^{-1})
Gasterosteus aculeatus (Three-spined Stickleback)	Schistocephalus solidus plerocercoids (Cestoda)	Cadmium (0.5 mg l^{-1})
"		Cadmium (0.032mg l^{-1})
Lymnaea stagnalis (Mollusca)	Schistosomatium douthitti larvae (Trematoda)	Zinc (24 mg l^{-1})
Lymnaea stagnalis	Trichobilharzia sp. larvae (Trematoda)	Zinc (24 mg l^{-1})
Carp, juveniles	Bothriocephalus gowkongensis (Cestoda)	p, p' DDT (1000 µg l^{-1})
Morone saxatilis (Striped Bass)	Anisakis sp. larvae (Nematoda)	Zinc
"	"	Benzene

*fish were additionally stressed by dietary deficiency

+data derived by extrapolation from authors' graphs

pends upon the conditions and nature of the test.

One significant factor is water quality, with (a) differences between tests and between laboratories leading to variable toxicity data and (b) the varying and variable quality of natural water preventing a direct extrapolation of laboratory results to the field. However, since the relationship between toxicity and many water quality variables is well understood, most workers do present details of test conditions and of actual, rather than nominal, toxicant concentrations so reducing, as far as possible, the margin of error in laboratory-field comparisons.

The other major factor modifying toxicity is biological variability, both of the test animals and of those animals actually exposed to pollution

to toxicants of animals already stressed by parasitism.
concentration) or LT50 (median lethal time).

Toxicity index	Toxicity (data for non-parasitized control animals in parentheses)	Reference
96 h LC50	6170 mg l^{-1} (7341 mg l^{-1})	Adelman and Smith (1976) /1/
96 h LC50	2.28 mg l^{-1} (10.38 mg l^{-1})	Moles, A. (1980) /32/
96 h LC50	0.77 mg l^{-1} (3.22 mg l^{-1})	"
96 h LC50	3.08 µl l^{-1} (9.36 µl l^{-1})	"
96 h LC50	0.039 mg l^{-1} (0.065 mg l^{-1})	Iwama and Greer (1980) /26/
LT50	3.94 h (52.8 h)	Boyce and Yamada (1977) /5/
LT50	14.6 days (35 days)	Pascoe and Cram (1977) /36/
LT50	12.8 days* (44 days*)	Pascoe and Woodworth (1980) /40/
LT50	8 h^{+} (14 h^{+})	Guth et al. (1977) /22/
LT50	4.5 h^{+} (10 h^{+})	"
%Mortality at 24 hours	54% (87.3%)	Perevozchenko and Davydov (1974) /41/
haematocrit	reduced levels in parasitized fish	Sakanari et al. (1984) /43/
"	"	"

in the natural environment. As discussed above, response to toxicants may change with the stage of the life cycle, with physiological stress and with any history of exposure to toxicants. Unfortunately, such biological conditions are considerably more difficult to quantify than water quality parameters and may well receive limited attention in toxicity investigations. Although it would clearly be impracticable and certainly of little value to attempt to eliminate all such variable factors from toxicity tests, there would seem to be a case for further studies to help clarify the complex interrelationships between biological variability and pollutant toxicity.

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PRINCIPLES OF USING WILD ANIMALS AS BIOINDICATORS OF GLOBAL RADIOACTIVE POLLUTION

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The great attention paid to the environmental conditions has aroused interest in the problems of biological indication. In industrial countries all over the world special branches of municipal economy were created to preserve an environment suitable for the life of man. The state of the environment is one of the indicators of the "life quality" of the population. In the last decade a new trend in bioindication – environmental monitoring – had been successfully developed, called forth by a sharp increase of load on living organisms deriving from new anthropogenic factors. Any site of Earth surface represents a more and more increasing value for people under conditions of depletion of biological resources with the growth of population, an increase of industrial production and the power of engineering. That is why one of the most important tasks of nature protection is to prevent the impoverishment of the biotic cover of the Earth.

The problem of bioindication covers a complex of phenomena known to everybody as the "pollution". This implies the effect of physical and chemical agents non-existent as factors of evolution in animals, plants and microorganisms. According to Ramad /17/ these factors have become important only over the last 40 years (ionizing radiation, industrial pollution, surface-active substances, mineral fertilizers, pesticides, etc.), when their distribution and action assumed a global scale.

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Radionuclides, i.e. isotopes or chemical analogues of the basic biogenic elements which readily incorporate into animal tissues and pass along food chains: ^{3}H, ^{14}C, ^{166}Ru, ^{131}I, ^{137}Cs, are of great importance in pollution studies. The following requirements are important in selectivity animals as bioindicators of industrial pollution /9, 18/: high density and metabolism level, great length of life, intensive reproduction, a small home range, sedentary way of life, permanent contact with the anthropogenic factor under study, available collection of mass samples, sensitivity to the factor concerned, fairly large size to facilitate dissection.

The ecologist's attention has always been attracted by the fission products of uranium and plutonium, particularly long-lived ^{91}Sr and ^{137}Cs (half-life period of 28.4 years and 30 years, respectively) found on the ground surface after nuclear weapon tests as well as after the processing of the uranium-containing ores and regeneration of the utilized nuclear fuel. Many radionuclides, including ^{90}Sr and ^{137}Cs, were proved to be readily involved in the biogenic turnover and can be accumulated by animals in detectable amounts. However, the radiation of these radionuclides is not so high in comparison with natural radiation background and no deleterious effects were demonstrated. Epigeous and soil-dwelling animals involved in the biogenic migration of elements, including man-made radionuclides, can serve as one of the biological indicators of environmental pollution with toxic elements or substances. At the same time, the presence of certain amounts of man-made radionuclides involved in the biological turnover in all living biospheric objects opens up new possibilities and prospects for field ecology. The use of radionuclides as tracers allows us to define the flows of a substance in ecosystems more precisely, as well as to clarify trophic chains of organisms and their productivity.

GLOBAL RADIOACTIVE FALL-OUT AND THE ANIMAL KINGDOM

Maximal global radioactive fall-out on the USSR territory was recorded in 1959–1963. After signing in 1963 the Moscow Agreement banning nuclear tests in the atmosphere and on the Earth surface, an equilibrium of ^{90}Sr and ^{137}Cs radioisotopes was reached.

In 1975 the average ^{90}Sr content in the soil was 1.5 GBq/km^2 on the USSR territory and 2.5 GBq/km^2 for ^{137}Cs /11/.

The penetration of radioisotopes into the body of terrestrial ani-

mals occurs by means of the alimentary canal – with food and water as well as via respiration of radioactive aerosols or gases. However, radioactive penetration as absorption of polluted soil particles – swallowed by animals during cleaning their integuments should also be taken into account. In addition, radionuclides may penetrate into the terresterial animal body via diffusion through integuments, particularly in case of amphibians and some soil-dwelling invertebrates. Accumulation of radioactive isotopes in animals is proportional to the pollution density of ecosystems with fission products, ^{90}Sr and ^{137}Cs in particular.

Investigating regularities of the accumulation of radioactive isotopes in animals, it is necessary to find out species differences in the concentration of radioactive substances. These differences may be connected with physiological features of the animals, their morphology or diet, behavior, peculiarities of the distribution of populations in ecosystems, etc. Epigeous animals can be subdivided into the following zoogeochemical groups according to the character of the accumulation of certain chemical elements, including radioactive ones: 1) accumulators containing the element under study in greater concentration than in trophic substrate (concentrating factor > 1); 2) diffusers, containing equal concentrations of the element as the trophic substrate (concentration factor ≈ 1). Due to the migration or burrowing activities, these animals promote the intensificationof the biogenic turnover, dispersion of the element in space; 3) decontaminators, containing the element under study in lower concentration than food substrate (concentration factor < 1) and promoting decontamination of the food chain from the element under study /9/.

The most intensive study of the ^{90}Sr and ^{137}Cs migration from the global fall-out along the food chains, involving wild and domestic animals and leading to man, was performed in regions of the extreme North of the USSR. The necessity of these studies was determined by the radionuclide concentration in the links of the chain – lichen – deer – man, exceeding by 10–100 times those in the identical links of other chains and other regions /13/. In this case the clearly defined seasonal variations of the ^{137}Cs concentration in muscles of deer were marked. In winter it was 15–18 times higher than in summer. A similar situation occurs in Alaska and Sweden /5, 12, 21/.

Seasonal variations of ^{137}Cs content were also noted in red deer (Cervus elaphus), moose (Alces alces) and roe deer (Capreolus capreolus), inhabitating the middle part of the USSR /10/.

Many studies indicate that ^{137}Cs and ^{90}Sr are accumulated more intensively by the animals inhabiting high mountain regions, which are characterized by significant precipitation with global fall-out of ^{90}Sr and ^{137}Cs.

Intensive global fall-out following nuclear weapon tests led to the pollution of animal integuments, particularly avian plumage, with radionuclides. Direct dependence was revealed between the bird size and the amount of radioisotopes on their skin and plumage, furthermore, certain differences in the pollution of plumage, being in connection with the time differences spent on search of food. The skin and plumage of birds spending much time in the air were more polluted in comparison with those spending more time in contact with vegetation /15/.

Many studies are available reporting in detail on the penetration of Cs and Sr radioactive isotopes into the body of vertebrates of various species, their distribution in the body, tissues, etc. Most of these experiments were carried out on laboratory and farm animals. The investigations on wild animals in nature show that the radionuclide penetration and distribution in them are subjected to the same rules of distribution and migration in the body as those of the element-analogues.

In California (USA) the concentrations of ^{90}Sr in the skeletons of sheep and black-tailed deer (Odocoileus nemionus) showed deviations, despite the fact that the animals used the same pastures /3/. The authors connect this with their food differences. In the muscle tissue samples obtained from red deer (Cervus elaphus), roe deer (Capreolus capreolus) and moose (Alces alces), the concentrations of ^{137}Cs were higher by 3.10 and 20-fold as compared with cows from the same region (1.10). Differences were also manifest in the ^{137}Cs concentrations in the muscles of fox (Vulpes fulva), wolf (Canis lupus) and deer (Rangifer tarandus) inhabiting the same regions. In carnivores the radionuclide content exceeded that in herbivores by 2–3-fold /4/.

Thus, radionuclide concentration icreases as it migrates in the food chain if the concentration is calculated for the organs critical for the given radionuclide. If the calculation is estimated for non-critical organs, radionuclide passage is marked by a considerable decline of concentration.

Investigations on wild terrestrial vertebrates of the USSR /6, 7/ demonstrated that the ^{137}Cs concentration factor is ≤ 1 /6, 7/ in all the links of the food chain. Thus, in insects serving as bird food this factor

in relation to plants was 1.6; in insectivorous birds in relation to insects – 0.48; and in all other trophic chains in which birds were present, the concentration factor was 0.5 /8/.

In poikilothermal organisms the level of ^{137}Cs accumulation strongly depends on temperature. Thus, for insect larvae at $5^{o}C$ it was not over 5% of the radionuclide concentration in food; at $15^{o}C$ it was up to 11%, and at $25^{o}C$ – up to 24%. The half-life period was 4–5 days; it did not exceed the usual half-life period of microelements in insects.

The biological half-life period of ^{137}Cs for mass groups of soil invertebrates (wood-lice and millipeds) was about 30 days at the normal temperatures for these animals. Evidently, this fact shows a month delay in the dynamics of radionuclide content in individual links of the trophic chain: an increase and decrease of ^{137}Cs concentration in herbivorous insects occurs one month later than in plants as food; the same process in observed in predatory insects in respect to their food /2/.

When radionuclides migrate through trophic levels, their concentration in organs and tissues is affected by isotopic and non-isotopic carriers. In chemistry the term "carrier" implies a weighty amount of the element, followed by the "weightless" amount of another element in chemical reactions. The isotopic carrier is a stable isotope of the given element; its chemical properties are identical to its radioactive isotope (e.g. ^{31}P and ^{32}P). The non-isotopic carrier is a stable isotop (or isotops) of a chemical analogue of the element identical to the radionuclide by the group chemical properties only (e.g. Ca is a carrier in respect to ^{90}Sr, or ^{90}K in respect to ^{134}Cs, ^{137}Cs).

The term "observed ratio" (OR) was introduced to establish the $^{90}Sr/Ca$ or $^{137}Cs/K$ ratio in the biological system and the ratio of the ions in the source from where these ions enter the biological system. It expresses overall discrimination characterizing the transport of these elements from the source into the biological system.

The decrease of ^{90}Sr and ^{137}Cs concentrations in relation to Ca and K is typical for the first trophic level of both invertebrate and vertebrates. It is also true for insectivorous vertebrates belonging to different classes.

The relative concentrations of ^{90}Sr, and in some cases ^{137}Cs increase in predatory invertebrates and necrophages.

The observed relations between herbivorous and insectivorous mam-

mals are close for different species despite their food both as for ^{90}Sr-Ca and ^{137}Cs-K /1, 20/.

In the highest links of the trophic chain of vertebrates (victim-predator) the ^{90}Sr relative concentration decreases in relation to Ca and the ^{137}Cs relative concentration increases in relation to K.

During the investigations of ^{90}Sr and ^{137}Cs biogenic migration originated from global fall-out, soil, vegetation and animal samples were taken from the main geographical zones. With the aim of leveling anthropogenic factor effects, the investigations were carried out in the biosphere reserves (zapovedniki) — the Berezinsky, Central Black Sea, Repeteck reserves in the USSR; Great Goby reserve in Mongolia, and besides the Pechero-Ilychsky, Pshu-Gumistinsky, and Ritsa reserves in the USSR the Moscow University White Sea biostation, as well as some other regions with minimum technogenic and agricultural impact on the environment. In all zones, animals with a skeleton containing different Ca amounts accumulated a greater part of ^{90}Sr. these are mainly all the vertebrates, and among the invertebrates — Fam. Julidae, Oniscidea and molluscs. In all regions the ^{137}Cs concentration was higher in vertebrates and insects. ^{90}Sr was accumulated in chains of vertebrates, Fam. Julidae, wood-lices (Oniscidea) and molluscs. Thus, such animals as Julidae, Oniscidea, molluscs and vertebrates can be used as bioindicators of radioactive pollution in biogeocenosis with ^{90}Sr. But it is advisable to use vertebrates as bioindicators of relative pollution in ecosystems with ^{137}Cs. The ^{90}Sr content in the animal biomass from different geographical zones changes over a wide range. However, in humid zones, animals accumulate in the biomass on an average of 0.006–0.016% and in arid zones 0.00003–0.0006% of the ^{90}Sr supply in ecosystems.

The function of animals from various groups in the biochemical turnover of ^{90}Sr is different. The presence of invertebrates such as Julidae, Oniscidea, Lumbricidae and molluscs in the zoocenosis structure defines their high share in the participation of the biogenic migration of a radioactive isotop of stroncium. On the average, 75–87% of ^{90}Sr, accumulated in the whole zoomass of these zones, account for these animals.

In mixed forests the most important role in ^{90}Sr migration is attributed to molluscs accumulating 37 cBq/m^2 of the radionuclide, when their density is 5.7 per m^2 (i.e. 55% of ^{90}Sr, contained in all animals). Taking the lengthy life-time of the animals into account, it is necessary to note their importance in ^{90}Sr biogenic migration particularly in the ecosystems, where mollusc density is very high.

In broad-leaved forests of the Caucasus the greater amount of ^{90}Sr is accumulated by the Julidae, molluscs and earthworms (75.22 and 14 cBq/m^2, respectively). Totally 80% of the radionuclide contained in the zoomass, accounts for these animals. Fam. Julidae, which are scarcely eaten by other animals, can become an effective depot of ^{90}Sr.

In the animal population of the forest-steppe oak-grove the main quantity of ^{90}Sr (about 60%) is accumulated by earthworms due to their high density and biomass, which is 20 g/m^2 here. The ^{90}Sr content in the earthworm biomass makes up 70 cBq/m^2 and on the average, considerably exceeds the amount of this radionuclide in other groups of soil-dwelling invertebrates.

The role of vertebrates in ^{90}Sr accumulation is not so great: 10 cBq/m^2 of the radionuclide in mixed forests. This makes up 16% of the total zoomass stock. In broad-leaved forests these indices are 15 cBq/m^2 or 11%, in forest-steppe oak-grove 15 cBq/m^2 or 14% of ^{90}Sr, accumulated in zoomass, respectively.

One of the important indices characterizing the participation of different organisms in the chemical turnover is the flow of elements through the population.

In the forests of Northern Caucasus the monthly litter consumption by Julidae is 250–400 kg/ga, and about 300 cBq/m^2 of ^{90}Sr pass through their population. In the forest-steppe, earthworms are one of the most numerous groups of saprophages; their population monthly passes through about 100 cBq/m^2 of radioactive strontium, when its average densitiy is 80 Ex./m^2.

Wood-lice (Trachelipes rathkei), when their average density is 16 Ex./m^2, assimilate 3–6 g/m^2 of dry litter (8–15 cBq/m^2 of ^{90}Sr) within a month. Molluscs (Bradybaena fruticum) are able to pass through 29 cBq/m^2 of ^{90}Sr, consuming 8 g of litter per individual within a day, providing their density is 50 Ex./m^2.

The ^{137}Cs content in the zoomass is 0.003–0.005% in humid zones and 0.00002–0.0004% of the total amount of radiocaesium in arid zones. Such low indices are mainly defined by high capacity of ^{137}Cs, particularly in soils rich in clay particles and containing a lot of organic material.

In ^{137}Cs migration the vertebrates are of great importance, and in less degree the earthworms and insects. On the average, there are 56% to 92% of ^{137}Cs, accumulated in the whole biomass, in the animals mentioned above. Vertebrates and insects, dominating in arid zones, contain practically all ^{137}Cs accumulated in the zoomass.

In mixed forests, in addition to the groups of animals already mentioned, containing 83% of radiocaesium, the terrestrial molluscs contain 9 cBq/m^2 or about 15% of theis radionuclide accumulated in the biomass. In broad-leaved forests the ^{137}Cs content in vertebrates, earhtworms and insects (21 cBq/m^2) is close to that in Fam. Julidae, accumulating about 37% of radiocaesium in the zoomass.

Wehn the density of earthworms and biomass is high, they can accumulate considerable amounts of ^{137}Cs as in the forest-steppe, for instance, where about 86% of radiocaesium conatined in the animal biomass are in earthworms.

The monthly ^{137}Cs flow through the saprophage population is from 20 cBq/m^2 in wood-lice (T. rathkei) to 210 cBq/m^2 in Pachyiulus foetidissiumus. The fairly small role of animals in biogenic radionuclide migration along the trophic chain is mainly compensated by the burrowing animal activities. Furthermore, radionuclide redistribution in the soil significantly affects their further life /16, 18, 22/.

In the forest-steppe, Spalax microphtalmus is one of the active soil-burrowing animals. To estimate the participation of these animals in the process of ^{90}Sr redistribution in the soil, the radionuclide dispersion in the out-burrowed soil was investigated. As a result of the burrowing activities of Spalax microphthalmus, ^{90}Sr is evenly dispersed in the soil layer in the range of 0–30 cm, while the control to 70% of radionuclide is concentrated in the upper 10 cm layer.

CONCLUSION

The use of animals as bioindicators of global fall-out, a proper estimation of the role of animals in the biological turnover and a possibility of modelling this process all greatly depend on factors influencing the accumulation of elements in the animals as well as on the element migration along the food chains in the ecosystems.

In the last few years biosphere reserves have become the main base for carrying out complex ecological investigations of global environmental changes. It is also necessary to perform radioecological experiments in biosphere reserves, since these are the only sites nowadays where it is possible to investigate the animal kingdom on the background of detailed studies of natural ecosystems.

WILD ANIMALS AND RADIOACTIVE POLLUTION

Despite the large number of literature devoted to radioecology, the migration of elements along trophic chains and the regularities of this process are not sufficiently known as yet, in order to model the migration of particular elemerts on the trophic level of biocenosis. Radioecologists consider /18/ that the construction of such models requires the understanding of: patterns of transfer of the elements one trophic level to another; factors of concentration of the elements in the body; the degree of accumulation of the elements within the body.

One of the important and not adequately studied factors of migration of mineral substances in trophic chains is the interaction of elements while they are concentrating in a body. Although ^{40}Ca serves as a non-isotopic carrier of ^{90}Sr, there are definite competative relations between them. This can be expressed in the predominant assimilation of ^{40}Ca to the detriment of ^{90}Sr and a respectively shorter life period of ^{90}Sr in the body. That is why the ratio of ^{90}Sr and ^{40}Ca differs in the animal skeleton comparison with food containing ^{90}Sr; and while transferring from food into animal body, Ca is likely to protect an organism against ^{90}Sr, i.e. the process of ^{90}Sr discrimination takes place.

However, it is necessary to use as many as possible species belonging to different trophic groups and various taxons, in order to set up general regularities of distribution. Thus, when attempting the use of wild animals as bioindicators of global fall-out of man-made radionuclides, sufficient amount of information must be obtained on the radionuclide content in the most different wild animal groups, furthermore, standard well-known indices are necessitated, objectively characterizing radionuclide migration along food chains in ecosystems.

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IS IT POSSIBLE TO USE THE HONEY BEE ADULT AS A BIOINDICATOR FOR THE DETECTION OF PESTICIDE RESIDUES IN PLANTS ?

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Pesticide residues are usually determined by physical, chemical and biological methods. The simplicity and adaptability of bioassay methods have won their acceptance in the field of residue analysis. Theoretically, any organism that is susceptible to a pesticide may be used for its bioassay in any environmental sample.This means that such organism may serve as a bioindicator for the detection of certain pollutants. The susceptibility of honey bees (Apis melifera L.) to many insecticides commonly used in crop protection led to an attempt to use it as a bioindicator for the determination of residues of some insecticides in plant materials, as well as to detect toxicity hazards to honey bees of some commonly used insecticides. Results of this work which have been recently published may suggest "Yes" to answer the question posed in the title of this subject.

INTRODUCTION

Today some 35 000 pesticides containing about 1500 active ingredients are used for the control of different agricultural and other pests. In spite of the role of pesticides to increase crop yields, several problems have arisen due to the extensive randomized application of pesticides. The majority of these problems are those concerned with: a) resistance in agricultural insect pests and disturbance of the natural biological balance; b) toxicity hazards to mammals and other beneficial organisms; c) long persistence and accumulation in human body; and d) teratogenic and carcinogenic effects of certain pesticides.

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For the former reasons, pesticides are considered at the present time as one of the most dangerous environmental pollutants.

From the viewpoint of environmental protection, the basis of choice of any pesticide should be derived from a risk/benefit evaluation programme of study. Among the studies included in such a programme are those concerned with residues in different environmental components, and toxicity hazards to mammals and other beneficial organisms.

A word on bioassay of pesticide resudes and toxicity hazards

Pesticide residues are usually determined by specific physical and chemical methods, as well as biological methods. The main disadvantage of bioassay methods is the lack of specificity. In the bioassay method of pesticide residues any chemical which is toxic to the test organism will interfere with the assay of the pesticide /7/. So, residues determined by bioassay methods may represent residues of the intact molecule of the toxicant and/or residues of other toxic metabolites or degradation products derived from the toxicant during and after its application. In certain circumstances, this property may add to the value of bioassay methods; i.e. when the aim is to assess the dangers of pesticides residues or toxicity hazards to a beneficial organism. Bioassay methods could achieve these purposes simply and in few steps, whereas much more extensive work would be required with non-biological methods. Moreover, the bioassay methods may be considered as the only available precise tools for detecting the toxic hazards of pesticides.

The use of insects in bioassay of pesticide residues

Theoretically, any insect organism that is susceptible to a pesticide may be used for its bioassay, but in practice only a few species of insects have been widely used, e.g. house flies (Musca domestica L.), pomace flies (Drosophila melanogaster Meig.) and mosquito larvae (Aedes spp.).

The susceptibility of honey bees (Apis mellifera L.) to many insecticides commonly used in crop protection has been long recognized. However, it is not recorded as a test organism for bioassaying of pesticides residues. During the last 10 years, many investigators used honey bee workers to determine the residual toxicity of insecticide deposits on plants. As examples, Korpela & Tulisalo /3/ in Finland exposed worker honey bees to the flowers of rape plants previously sprayed with some insecticides and

evaluated the residual contact toxicities. Johansen et al. /2/ in Washington State of America, used alfalfa plants and evaluated their suggested "RT 25" values which indicate the residual time required to bring bee mortality down to 25% in cage test exposures to field-weathered spray deposits. Probably at the same time /5/, in Iraq determined the residual toxicity on the flowers of clover plants and they used "t_{50}" criterion to compare persistence of the toxic action of the tested insecticides, and "t_{20}" criterion to compare the dissipation. Furthermore, by using the latter criterion they suggested a classification to compare the degree of hazard of the tested compounds.

In another work, Mansour & Al-Jalili /4/ suggested a bioassay method for the determination of residues of some insecticides in clover flowers using the honey bee workers as a test organism.

Hereinafter, a short account on the methodology and results of our work is, given.

Assessment of toxicity hazards

Flower samples from clover fields (Trifolium alexandrinum) previously sprayed with 10 different insecticides were collected 0,2,4,7 and 10 days after application. The flowers were mixed with 25% sucrose solution and blended by a high speed blender. The resultant mixture was transferred to 30x30x30 cm cages provided with the tested honey bee workers where mortalities in the bees were determined after 24 h. Results shown in Table 1 indicate that for 6 of the insecticides tested, mortality was over 90% on the day of spraying. Thereafter mortality declined with time to a negligible level at day 7 after spraying. The rate of decline varied somewhat with the insecticide.

To estimate more precisely the persistence of toxic action of the tested insecticides, the data of Table 1 were plotted on log-log scale graph paper. The mortality-time lines thus obtained were used to determine times after application for mortality for each compound to decline to levels of 50% (t_{50}) and 20% (t_{20}). These values (Table 2) indicate the degree of persistence (or dissipation) of the toxic action.

On the basis of the t_{50} values, carbaryl was the most persistent compound and fenvalerate the least persistent.

Assuming that 20% mortality in bioassay evaluation tests represents the natural rate of attrition, the t_{20} values obtained could be used to de-

Table 1

Percent mortality over 24 h in worker honey bees caged with sucrose-syrup blended with clover flowers sprayed with one of 10 insecticides

Insecticide	Rate of insecticide application ($g^*/400\ m^2$)	% mortality at day				
		0	2	4	7	10
Carbaryl, 85WP	68.0	100.0	96.8	72.9	15.3	0.0
Pirimiphos-methyl, 50EC	40.0	97.7	45.2	6.2	0.0	0.0
Fenitrothion, 50EC	40.0	100.0	90.2	32.3	5.1	0.0
Chlorpyrifos, 40.8EC	32.6	100.0	47.3	12.5	0.0	0.0
Dicofol, 18.5EC	29.6	73.0	15.0	6.6	0.0	0.0
Fenvalerate, 20EC	10.0	50.0	18.3	16.0	0.0	0.0
Propoxur, 20EC	44.8	100.0	85.0	32.0	7.0	3.0
Methomyl, 25WP	27.2	94.0	66.0	33.3	3.0	2.0
Phenthoate, 50EC	40.0	54.7	25.0	16.0	7.0	0.0
Diazinon, 60EC	57.6	85.3	43.3	25.0	13.0	5.0
Control	0	0.0	2.5	0.0	5.0	0.0

*Active ingredient

Four cages, each containing 25 bees, were used for each combination of insecticide and sampling date. (Values of mortality were adjusted by Abbott's formula)

termine the time after application required for any tested compound to cause negligible mortality to adult honey bees. From the viewpoint of honey bee protection, compounds with the lowest t_{20} values will be the most desirable. The following classification was suggested to evaluate toxic hazards of the tested compounds based on t_{20} values shown in Table 2:

1. Compounds of low hazard; t_{20} = 1–2.9 days (e.g. dicofol and fenvalerate).
2. Compounds of moderate hazard; t_{20} = 3–4.9 days (e.g. pirimiphos-methyl, chlorpyrifos and phenthoate).
3. Compounds of high hazard; t_{20} = 5–6.9 days (e.g. carbaryl, fenitrothion, propoxur, methomyl and diazinon).

The above classification was proposed as a basis for advising beekeepers in Iraq about how long bees should be kept out of areas sprayed with insecticides.

In one tries to compare the results of Johansen et al. /2/ concerning RT 25 values with our results concerning t_{20} values, he will conclude a

Table 2

Days after insecticide application required for mortality over 24 h in caged honey bees to decline to 50% (t_{50}) and 20% (t_{20}), and evaluation of insecticide hazard according to t_{20}

Insecticides	Days to reach:		Degree of hazard
	t_{50}	t_{20}	
Carbaryl	5.2	6.8	HH
Pirimiphos-methyl	1.8	3.3	MH
Fenitrothion	3.4	5.3	HH
Chlorpyrifos	1.9	3.6	MH
Dicofol	0.8	1.8	LH
Fenvalerate	0.1	1.9	LH
Propoxur	3.3	5.3	HH
Methomyl	3.0	5.4	HH
Phenthoate	0.3	3.0	MH
Diazinon	1.7	5.2	HH

HH = high hazard; MH = medium hazard; LH = low hazard

good agreement in results of some insecticides tested in both cases. However, even unagreement of results in such cases would also be quite expected for many reasons. But the most important result which could be concluded is that both RT 25 and t_{20} are considered to be safety precaution criteria for honey bee protection against hazardous pesticides. Owing to the effects of environmental conditions on the stability of pesticides, such criteria should be evaluated under the conditions of each specific environment.

Determination of residues

The flower samples collected from clover fields previously sprayed with 6 different insecticides were extracted with acetone, and extracts were applied topically to bees without need to remove co-extractive (i.e. without clean-up). This method of extraction gave good recoveries as shown in Table 3.

Dosage-mortality relationships (ld-p lines) for the tested compounds were accomplished according to the recommended methods /1, 8, 9/. Honey bees used in the bioassay tests were obtained from selected colonies

with homogeneous population. Seven days old workers were used in all tests /6/.

The obtained ld-p lines were used as "standard curves" for recovery and residue determinations.

Table 3

Slope of l-p line, LD_{50} values and recovery data for the tested insecticides

Insecticide	Mean slope of ld-p line	Mean LD_{50} ug/bee	Recovery determinations			
			Added ppm	Observed mortality %	Recovered,ppm	Recovery%
Carbaryl (100%)	2.87	0.212	70.7	40	56.6	80
Fenitrothion (96%)	7.00	0.310	103.3	55	113.6	110
Chlorpyrifos (99%)	7.71	0.115	38.3	46	35.2	92
Pirimiphos-methyl (93%)	3.63	0.066	22.0	50	22.0	100
Methomyl (90%)	9.03	0.068	22.7	52	23.6	104
Propoxur (100%)	2.14	0.112	37.3	55	41.0	110
Standard error (%)	22	27				5

All data are corrected to the purity given under the insecticide names. Slopes, LD_{50} values and recovery data are means of 3 determinations

The amounts of insecticide residues determined in the clover flowers generally decreased with time (Table 4), but detectable amounts remained up to 10 days after application. Taking into consideration that the residues determined biologically may represent residues of the intact molecule of the toxicant and/or any other toxic residues derived from this toxicant, the data presented in Table 4 may provide us with information about how much "total effective toxic residue" may be present, as a result of spraying of each of the tested compounds, in a field of clover foraged by honey bee workers. For the information to be used in this way it is essential that the assay organism is the same as the one possibly in danger.

Table 4

Residues of insecticides in clover flowers at different time intervals after application, as determined biologically using honey bee adults

Insecticide	Rate of application g(a.i.)/plot*	Mean residues (ppm in flowers, days after application				
		0	2	4	7	10
Carbaryl (85% WP)	68.0	71.3	55.7	17.7	12.3	8.3
Fenitrothion (50% EC)	40.0	36.7	33.3	30.0	13.3	10.0
Chlorpyrifos (40.8% EC)	32.6	28.0	9.9	7.9	3.3	2.0
Pirimiphosmethyl (50% EC)	40.0	16.7	11.7	11.3	6.3	5.7
Methomyl (25% WP)	27.2	9.0	8.3	8.3	6.8	4.2
Propoxur (20% EC)	44.8	12.5	7.0	5.7	2.7	1.6

*Plot area = 400 m^2

CONCLUSION

One of the studies discussed in this lecture suggests a safety precaution criterion (t_{20}) to be used for the protection of honey bees against pesticide hazards. The other study suggests a bioassay method for pesticide residue analysis in plant materials.Both study results may support the possibility to use the adult honey bees as a bioindicator for the detection of hazardous pesticides to bees and their residues contaminating the field crops.

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THE EFFECT OF AN ORGANOPHOSPHORUS PESTICIDE ON THE ENZYMES OF CARP (CYPRINUS CARPIO L.)

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1. The effect of 1.5, 2.0 and 5.0 ppm methidation was studied on the ASAT, ALAT, LDH and AChE enzymes, as well as the blood sugar and adrenaline levels in carp.

2. According to our studies methidation implies a potential hazard on the normal biochemical processes of fishes:

– causing tissue necrosis indicated by the increase ASAT, ALAT and LDH activities,
– inducing continual stress effect reflected by the increased blood sugar and adrenaline levels,
– inhibiting AChE activity in the various organs, the consequence under acute effects being fish kill.

Keywords: methidation – fish – tissue-necrosis – stress – acetylcholinesterase

INTRODUCTION

Methidation (S-2, 3-dihydro-5-methoxy-2-oxo-1,3,4-tiadiazol-3 ilmethyl-o, o-dimethyl-phosphoditioate) has been used as an insecticide since 1966. For exerting its blocking effect on acetylcholinesterase (AChE EC 3.1.1.7.), methidation – just as every other so-called thiophosphoric acid ester with P = S binding – first undergoes oxidative desulphuration, which has been proved by Chopade and Dauterman /6/.

In Hungary it is used in large quantitites firstly against the insect pests

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of orchards and the grapevine as well as against the Colorado beetle, but it can also be employed with success against scales.

During the course of complex studies, the enzyme activities of transaminase and lactic acid dehydrogenase have been measured for the detection of tissue damage in fishes /4, 15, 19, 20/. Measurements of the blood sugar, catecholamine and cortisol levels may serve for the demonstration of the stress effect exerted on the organism /17, 21, 28, 29/. Damage of the nervous system is most frequently studied by the measurement of acetylcholinesterase levels /7, 8, 22, 23/.

In consideration of these, studies were performed on the effect of methidation on tissue damage and stress situation, in addition to its expectable acetylcholinesterase blocking effect.

MATERIALS AND METHODS

Carp (Cyprinus carpio L.) of either sexes with body weights of 850–1000 g were used in the experiments. Three fishes were kept in an aquarium containing 100 l of O_2 saturated water. The temperature was maintained at 18 ± 1°C.) Methidation stock solutions were added to the water to achieve final concentrations of 1.5; 2.0; 5 ppm (mg/l), respectively. The durations of the treatment were 24 and 48 h or 2 h when the CI_{50} values (concentrations producing 50% inhibition) were determined in vitro. All experimental animals survived this exposure time.

After the treatment, blood-samples were taken from the tail vein. The blood was centrifuged at 4°C for 20 min. (6000 rpm). The ASAT (aspartate aminotransferase, EC 2.6.1.1.); ALAT (alanine aminotrasferase, EC 2.6.1.2.); LDH (lactate dehydrogenase, EC 1.1.1.27.) and AChE enzyme (acetylcholinesterase, EC 3.1.1.7.) activites as well as the blood glucose and adrenaline levels were determined from the haemolysis-free sera. The ASAT and ALAT activites were measured according to Reitman and Frankel /26/, LDH activity according to Anon /1/, AChE activity according to Ellman et al. /9/ and adrenaline levels as proposed by Anton and Sayre /2/. The blood glucose concentration was determined with the glucose-oxidase peroxidase (GOD-POD) method. Values given are the average of samples from 5–8 animals and are expressed as the percentage of control (untreated) values.

RESULTS AND DISCUSSION

The highest concentration of methidation applied in the present study caused a twofold increase of ASAT, and a fourfold increase of ALAT activities as soon as 24 h following treatment. These values remained at a similar level even after 48 h. Following an addition of 1.5 and 2 ppm amounts to the water, there were no significant changes in enzyme activities

after 24 h. Following 48 h, only the ASAT activity increased by about 2–3-fold (Figs 1, 2). On the effect of 5 ppm concentration the serum LDH activity showed a 4–5-fold increase following 24 and 48 h of treatment (Fig. 3).

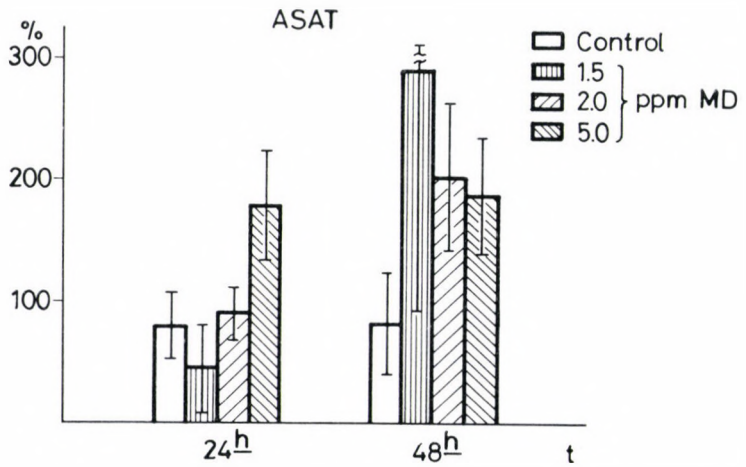

Fig. 1. The effect of different concentrations of methidation on serum ASAT activity of carp at water temperature of 18 ± 1 °C. Exposure time 24 and 48 h. The values are averages of samples from 5–8 animals and are expressed as the percentage of the control (untreated) activity

The increase in the serum transaminase enzyme activities indicates the rapid metabolism of methidation /10/ as well as its subsequent tissue damaging effect. The increase in serum transaminase activity has been reported as a consequence of liver damage in humans and rats /11/. Its measurement has also been used for diagnosing the tissue damage in fishes /5/. According to other authors the gill /25/ and kidney /27, 28/ may also become damaged on the effect of pesticides. From these, it may be assumed that presumably the liver may become damaged on the effect of methidation, however, damage of the gill, kidney or skeletal muscle cannot be excluded either. The damage of the latter organ on the methidation effect has been demonstrated in carp by Asztalos and Nemcsók /4/ by determining the LDH isoenzyme patterns.

On the effect of all insecticide concentrations, the blood sugar level increased by 50 and 100 %, respectively (Fig. 4). The serum adrenaline level showed a change of similar nature, however, the increase of

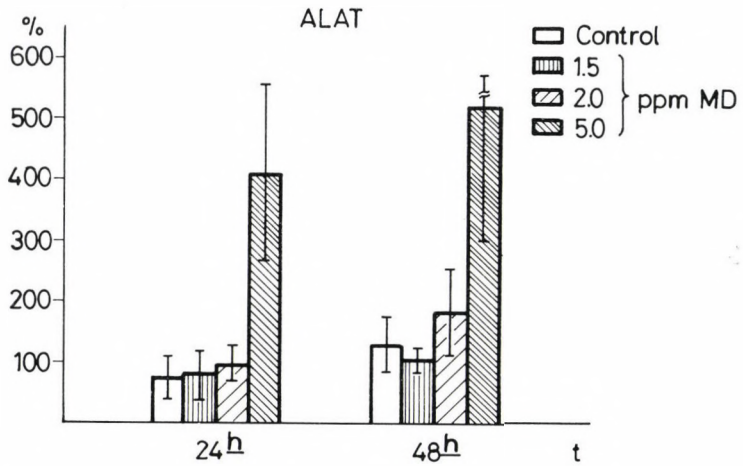

Fig. 2. The effect of different concentrations of methidation on serum ALAT activity of carp at water temperature of 18 ± 1°C. Exposure time 24 and 48 h. The values are averages of samples from 5–8 animals and are expressed as the percentage of the control (untreated) activity

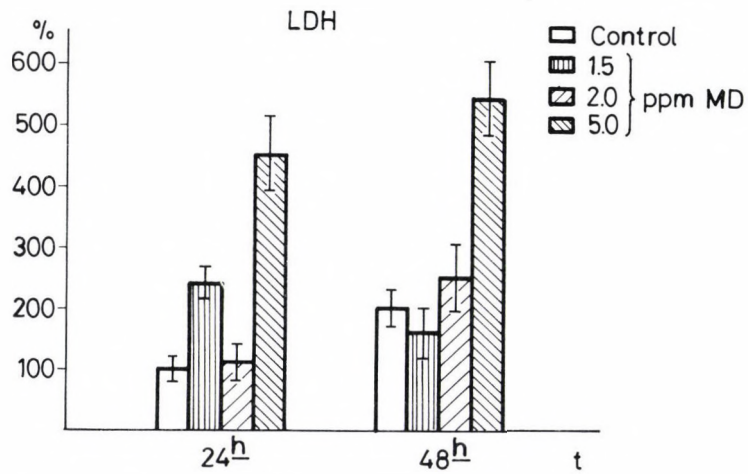

Fig. 3. The effect of different concentrations of methidation on serum LDM activity of carp at water temperature of 18 ± 1°C. Exposure time 24 and 48 h. The values are averages of samples from 5–8 animals and are expressed as the percentage of the control (untreated) activity

this level was 3–5-fold in some cases (Fig. 5). Alteration of the blood sugar and adrenaline levels reveal a stress susceptibility of fishes against methidation /18, 30/.

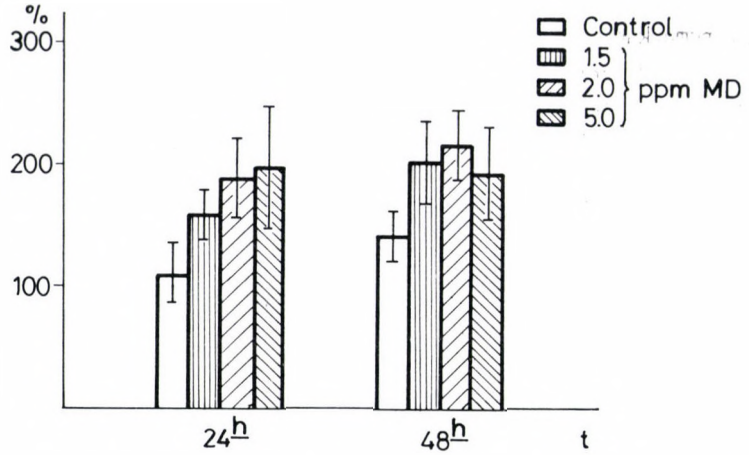

Fig. 4. The effect of different concentrations of methidation on blood sugar level of carp at water temperature of 18 ± 1°C. Exposure time 24 and 48 h. The values are averages of samples from 5–8 animals and are expressed as the percentage of the control (untreated) activity

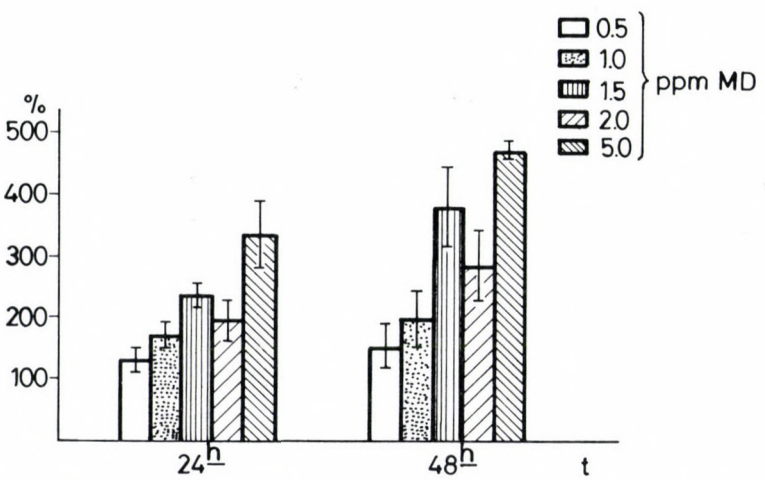

Fig. 5. The effect of different concentrations of methidation on blood adrenaline level of carp at water temperature of 18 ± 1°C. Exposure time 24 and 48 h. The values are averages of samples from 5–8 animals and are exposressed as the percentage of the control (untreated) activity

On the basis of its cholinesterase blocking effect, methidation is widely used in agriculture as an insecticide. According to our studies, similarly to other organic phosphate esters, methidation significantly in-

hibited the AChE activity in certain organs of fishes /8, 12, 13/. During our in vivo experiments the inhibition of cholinesterase activity was found to be most remarkable in the heart and brain tissues, and to be lower in the muscle. The differences of inhibition measurable in vivo in various organs may be explained by the formation of toxic metabolites of methidation, as a result of the variable rates of accumulation and decomposition of the exogenous compound, as suggested by Esser and Müller /10/ as well as Chopade and Deuterman /6/. Under in vitro circumstances it was found to exert the least inhibitory effect in the brain, while the most pronounced inhibition could be observed in the serum (Fig. 6).

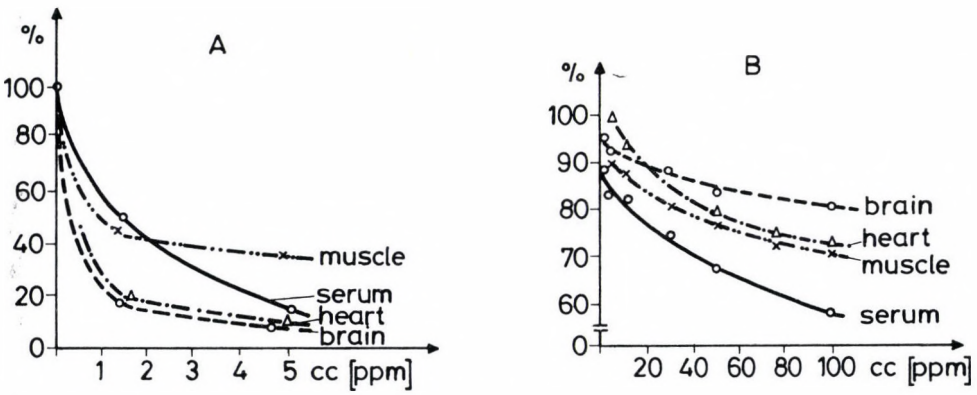

Fig. 6. Effect of MD in vivo (A, exposure time 24 hours) and in vitro (B, exposure time 2 hours) on cholinesterase activity in serum, brain, heart and skeletal muscles of common carp. The values expressed in the percentage of the control are averages of measurements form 6–10 individuals

The CI_{50} values obtained under in vivo and in vitro circumstances varied considerably. The concentrations causing a 50% inhibition were lower under in vivo circumstances by 2 orders in the serum, 3 orders in the muscle, and 4 orders in the brain and heart, as compared to the in vitro circumstances (Table 1). This effect has been found much lower than those observed previously for other pesticides /22/. This is due to the fact that methidation – similarly to the rest of the phosphoric acid esters – exerts its blocking effect only through its metabolites formed by its transformation in the organism /6/. The strong AChE blocking effect of methidation caused in fishes is in conformity with its effect observed in other animal species, such as in lobster /16/ and earthworm /14/.

Table 1

MD concentration values inducing 50% acetylcholinesterase inhibition in serum, brain, heart and skeletal muscle of common carp

Organ	CI_{50} (MD)	
	in vitro	in vivo
Serum	$(3.83\pm0.41)\cdot10^{-4}$M	$(4.13\pm0.71)\cdot10^{-6}$M
Brain	$(1.54\pm0.19)\cdot10^{-3}$M	$(6.62\pm0.50)\cdot10^{-7}$M
Heart	$(1.20\pm0.09)\cdot10^{-3}$M	$(9.93\pm0.76)\cdot10^{-7}$M
Muscle	$(1.12\pm0.10)\cdot10^{-3}$M	$(2.98\pm0.34)\cdot10^{-6}$M

As a summary of our results, methidation has been found to cause a considerable cholinesterase blocking effect. In addition, it played a potential role as inducer of tissue damage and stress situation in fishes. It is assumed that even a very low concentration in the water may cause severe damage to the fish stock. This may give rise to mass fish death by acute effects, while a subacute dose would exert deleterious influence on the locomotor activity of fishes. Thus, they get into an unfavourable situation in respect to their competition for food or their escape responses.

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EVALUATION OF OXYHEMOGLOBIN DETERMINATION ON THE BASE OF HEME-HEME INTERACTION BAND

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Abstract. Oxyhemoglobin estimation on the base of heme-heme interaction band (spin state band) according to Lambert-Beer's law on 50 normal and 300 traffic policemen subjects gave 82.2% and 72.4% of the total Hb respectively. This method excludes the other derivatives such as HbCo, Met-Hb and HbS that increased in air pollution and in many diseases as well. Moreover, this method makes it possible to determine the actual degree of anaemia, besides its high reproducibility.

Keywords: Oxyhemoglobin – cyanmethemoglobin – carboxyhemoglobin – methemoglobin – sulfohemoglobin

INTRODUCTION

The most common method for determination of hemoglobin is the cyanmethemoglobin which is recommended by the International Committee for Standardization in Haematology (ICSH). One disadvantage of the method is the use of cyanide in the reagent, which demands cautious preparation and use /3/. A second disadvantage is the conversion of different hemoglobin ligands such as HbCo HbO_2 and Met-Hb to cyanmethemoglobin. Thus, the clinical picture of oxygen transportation ability of blood sample which is related to the cyanmethemoglobin possesses some errors such as the amount of HbCo and Met-Hb /1/. In many diseases as well as in polluted areas, the above mentioned error is consequently increased /1/. Thus, the present study deals

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with the determination of HbO_2 on the base of heme-heme interaction band or spin state band of iron-heme

MATERIALS AND METHODS

Blood samples were collected from normal and traffic policemen in vacutainer tubes with EDTA as anticoagulant. The cyanmethemoglobin (HbCN) reference determinations were performed according to the method recommended by TSCH, using Drabkin's reagent /6/.

HbO_2 reagent was prepared by mixing 5 ml Nonidet-P_4O with 250 ml distilled water as a stock solution. The working solution was 1:10 (v/w) stock solution to distilled water. 0.02 ml well-mixed anticoagulated blood was pipetted into 4 ml reagent to obtain a 1:200 dilution. The mixture was mixed well by shaking for about 10 sec. The absorbance was read at 577 nm, against a blank of diluting fluid. Hb amount was carried out according to Lambert-Beer's law.

$$Hb = \frac{64458 \cdot f \cdot A577}{\varepsilon} \quad g/L$$

f = dilution factor
ε = extinction coefficient
Mol.W. of Hb = 64458

HbCo was estimated according to the modified method of Van Assendelft /2/. Met-Hb determination is based on the work of Evely and Malloy /4/. Sulfhemoglobin determination was preformed by the modified method of Van Assendelft /2/.

RESULTS

Table 1 shows the mean values of oxyhemoglobin estimated on the base of heme-heme interaction band, HbCo, Met-Hb and Hb-S of normal subjects and traffic policemen. Also the concentration of Hb of the above mentioned subjects was determined using Drabkin's reagent. It is clear that the total Hb derivatives, i.e. of different ligands, showed insignificant difference as compared to Hb of the same subject estimated by Drabkin's reagent.

HbO_2 estimated according to Lambert-Beer's law on the base of heme-heme interaction bands gave 89.19 % and 72.36 % of the total content concerning normal subject and traffic policemen, respectively. This difference was due to the elevation of HbCo, Met-Hb and Hb-S concentrations in traffic policemen.

It is obvious that the Hb concentrations of the two different sub-

Table 1

Oxyhemoglobin estimated on the base of heme-heme interaction band and other Hb-derivatives as well as Hb concentration estimated by cyanmethemoglobin method in normal and traffic policemen subjects

Subjects	HbO_2	HbCO	Met-Hb	Hb-S	Total	Hb-estimated by Drabkin's reagent
Normal	12.37 ± 0.82	1.42 ± 0.16	0.72 ± 0.1	0.54 ± 0.06	15.05 ± 1.22	14.66 ± 0.52
n = 50	(82.19%)	(9.44%)	(4.78%)	(3.59%)		
Traffic policemen	10.29 ± 0.48	2.01 ± 0.3	1.20 ± 0.2	0.72 ± 0.07	14.22 ± 0.93	13.82 ± 0.78
n = 300	(72.36%)	(14.14%)	(8.44%)	(5.06%)		

Table 2

Oxyhemoglobin estimated on the base of heme-heme band and its reproducibility

Ten replications	Reproducibility %	Ten replications	Reproducibility %
13.3 ± 0.03	99.77	12.4 ± 0.03	99.76
13.2 ± 0.02	99.85	13.4 ± 0.02	99.85
12.3 ± 0.02	99.84	10.3 ± 0.02	99.80
12.5 ± 0.03	99.76	10.5 ± 0.02	99.81
13.5 ± 0.02	99.85	10.2 ± 0.02	99.80

jects estimated by Drabkin's reagent were within normal values, i.e. without anaemia. Whereas, HbO_2 concentration according to Lambert-Beer's law showed a degree of anaemia in respect to traffic policemen.

DISCUSSION

The physiological function of Hb in blood is to transport oxygen from the lungs to the tissues and to facilitate the transportation of CO_2 from the tissues to the lungs. This function depends on the heme-heme interaction of tetramer Hb, which in its role determines Hb-O_2 affinity /1/. Once oxygen binding to hemoglobin has begun, the oxygen affinity of the remaining heme groups increases /7/. Thus, estimation of the concentration of Hb on the base of heme-heme interaction band (spin state band) at 577 nm gives the actual functional Hb.

It is well known that the above mentioned band appears at 577 and 569 nm for HbO_2 and HbCo, respectively /6, 8/. Therefore, although HbCo may not completely changed to HbO_2 in the present procedure, oxyhemoglobin has approximately more double absorbance at 577 nm. Methemoglobin is not changed to HbO_2 The former has approximately less than one-fourth the absorbance of HbO_2 at 577 nm. This is confirmed by the work of Abdel-Baset /1/ according to which the conversion of Hb to Met-Hb leads to reduction in A577/A540 ratio, i.e. increased absorbance at 540 and decreased absorbance at 577 nm. The reduction in this ratio is concomitant with the appearance of a new band at 630 nm. Thus, one can speculate that as spin state changes due to this conversion, the new band appearing at 630 nm is on the account of the absorbance at 577 nm.

The affinity of CO to Hb is 210 times that of oxygen, so that inhalation of CO-containing air readily leads to the formation of HbCO, which markedly reduces the oxygen-carrying capacity of blood. At a CO concentration of 0.1% in the inhaled air, over 5 % of the Hb is not available for oxygen transport. Also the toxic effect of certain drugs on Hb not only leads to Met-Hb formation but also to S-Hb production.

Thus, it is clear that in many cases the available Hb is greatly different from the total hemoglobin derivatives estimated by the cyanmethemoglobin method. In addition, false high results may be obtained if high serum globulin levels produce a precipitate with the cyanmethemoglobin reagent that results in turbidity of the solution /5/.

Furthermore, Hb estimation without cyanide at 540 nm according to Lambert-Beer's law /3/ possessed some disadvantages such as: 1) wavelength 540 nm is an isobestic point for HbO_2 and HbCO, 2) conversion to Met-Hb leads to an elevation in the absorbance band at 540 as mentioned above, 3) absorption band at 540 represents the bonds between the iron and nitrogen in porphyrin ring, while absorption band at 577 represents heme-heme interaction or spin state of iron-heme.

In conclusion, the estimation of HbO_2 on the base of heme-heme interaction band (spin state of iron-hem band) pronounces the availability of blood to carry oxygen, and excludes the other derivatives of Hb that increased in air pollution as well as in many diseases. Thus, using this method, it is possible to determine the actual degree of anaemia.

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PRELIMINARY STUDY OF ORGANOCHLORINE RESIDUES IN HUMAN MILK AND CORD BLOOD

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A large collection of human milk samples and cord blood from various hospitals of Tunis is studied for organochlorine residues. Some results obtained by ECD–GC are given.

INTRODUCTION

The present paper provides some preliminary results on organochlorine residues in human milk and cord blood.

To the best of our knowledge, it is the first time that such a report is given in our country.

Furthermore, apart from the well known case of use Guatemalan mother's milk /8/, few instances of such studies may be cited in the Third World countries /5, 6/.

MATERIALS AND METHODS

The human milk and cord blood samples were randomly collected from hospitals located at the Tunis area. The donors had no professional exposure to pesticides (Table 1). All samples were kept frozen until the day of analysis and volumes ranged from 50 to 150 ml. All the containers were glass tubes.

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Table 1

Sample	Reference	Age	Progeny	Location
L_1	N.F	28	4	–
L_2	N.Z	28	2	Grombalia
L_4	S.M	25	1	–
L_7	S.B	26	2	Medjez El Bab
L_8	M.B	20	1	–
L_9	M.R	30	1	Le Bardo
L_{12}	B.S	32	2	–
L_{13}	Z.D	19	3	Grombalia
L_{15}	B.A	26	2	Béja
L_{18}	B.H	34	5	Tunis
L_{21}	B.C	23	1	Mellasine
L_{22}	M.A	19	1	Ezzahra
L_{34}	Z.M	21	3	–
L_{33}	M.H	33	5	Grombalia
L_{35}	F.F	23	2	Grombalia
L_{36}	L.E	29	3	Hammamet
L_{39}	R.S	29	2	–
L_{42}	S.M	35	4	Sidi Youssef
L_{45}	A.T	27	3	B. Argoub
L_{47}	E.N	23	3	Ksar Hellal

The human milk samples were prepared for GC according to Suzuki et al. /7/ (Table 2).

The cord blood samples were treated as described by Dale /3/.

Indentification and titration of the residue were made by GC with EC detection.

The assessment of a residue is made with three different columns (Table 3).

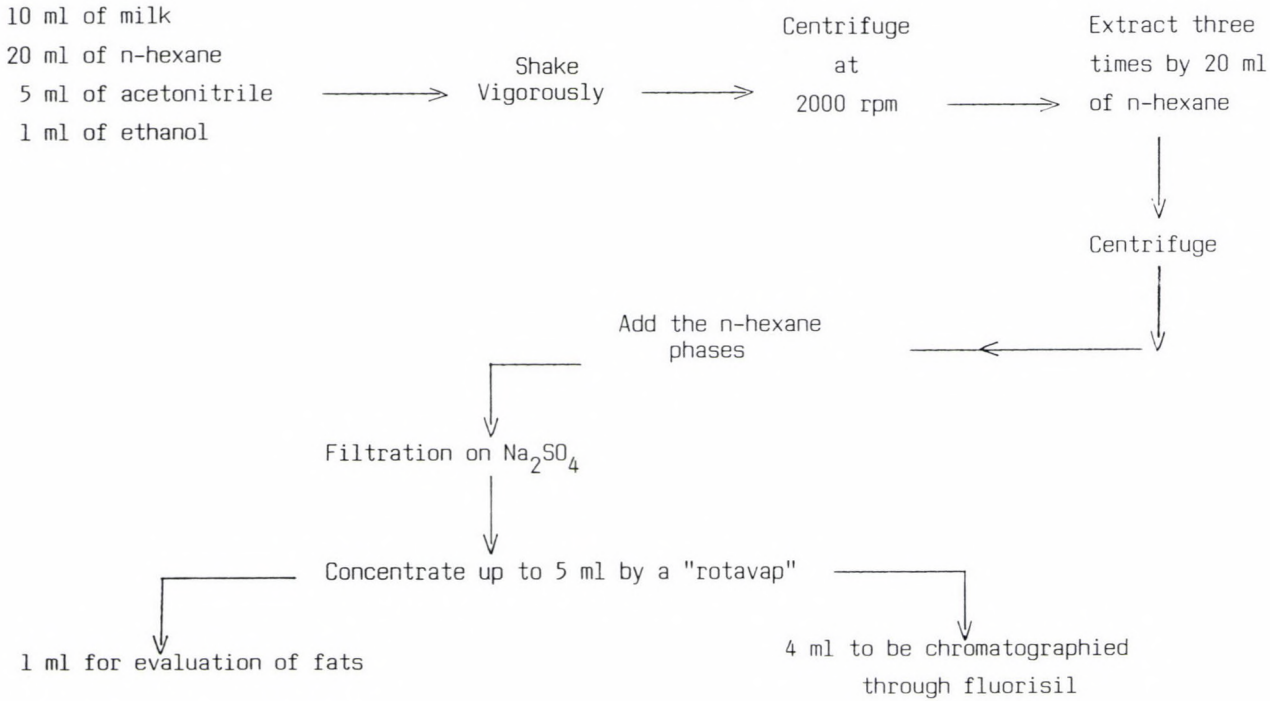
All compounds were identified by their retention times as compared to known standards. Only signals observed on three columns were reported and evaluated.

Our standard solution contains:

γ HCH (Lindane)	0.05 g/ml
pp' DDE	0.05 g/ml
pp' DDD	0.05 g/ml
pp' DDT	0.05 g/ml.

Owing to relation (1) we were able to titrate the amount of the residue

Table 2



after Suzuki et al. (J.O.A.C. 62, n° 3, 1979)

Table 3

Column	Length	Internal diameter	Phase	Support
OV 17 glass	1.80 m	1/4	3% OV 17	Chromosorb WHP 100/120 mesh
DC 200 glass	1.80 m	1/4	5% DC 200	Chromosorb WHP 100/120 mesh
OV 101 stainless steel	2 m	1/8	10% OV 101	Chromosorb WHP 80/100 mesh

VARIAN 3700 (Pulsed Feeding 0.64 μ sec)
Integrator : CDS 111
Temperature : Column 190°C Injector: 250°C Detector: 280°C
Vector gas : Nitrogen U P = 1.8 Bar Flow rate: 40 ml/mn
Detection : EC ^{63}Ni
Attenuation : 64 et 128 x 10^{-12}
Recorder : 2.5 mm/mn

detected. Results are expressed in mg/l:

$$x_{mg/l} = \frac{V_f \times S_E \times C_S \times V_{inj\ s}}{V_1 \times S_s \times V_{inj\ E}} \qquad (1)$$

where x : quantity of residue in mg/l
S_E: peak area of detected compound
S_s: peak area of the same compound in the standard solution
C_s: concentration (g/l) of the compound in the standard solution
V_f: final volume of extraction
V_1: treated sample volume
$V_{inj\ s}$:standard solution injected volume
$V_{inj\ E}$:sample injected volume
If $V_{inj\ E} = V_{inj\ s}$,(1) becomes:

$$x = \frac{V_f \times S_E \times C_s}{V_1 \times S_s} \text{ in mg/l}$$

RESULTS

a) Human milk

Out of 35 samples, 24 were positive for organochlorine compounds (68.57 %) (Table 4). All positive samples contained detectable amounts of "total DDT" as well as p,p'DDE. Only 87.5 % of the positive samples contained HCH. Our results are compared on Table 5 with those of Fournier/4/.

Table 4

Sample	Expressed in mg/l of milk				
	HCH	p,p'DDE	p,p'DDD	p,p'DDT	DDT Total
L_{22}	0.010	0.009	n.d.	n.d.	0.009
L_{34}	0.080	0.412	0.006	0.004	0.422
L_{32}	0.093	0.009	n.d.	n.d.	0.009
L_{33}	0.010	0.005	n.d.	n.d.	0.005
L_{35}	0.041	0.045	n.d.	n.d.	0.045
L_{36}	0.017	0.026	0.004	0.001	0.031
L_{39}	0.070	0.540	0.008	0.006	0.554
L_{42}	tr.	0.004	n.d.	n.d.	0.004
L_{45}	0.017	0.032	0.001	0.006	0.039
L_{47}	0.053	0.090	0.005	0.003	0.098
$\bar{L}$	0.021	0.092	0.002	0.002	0.097

Table 5

	HCH Total	p,p'DDE	p,p'DDD	DDT	
Mean Value (mg/l)	0.0399	0.028	–	0.078	Réf. 1
	0.0210	0.092	0.002	0.098	xx
Minimum (mg/l)	0.0013	0.0009	–	0.0072	Réf. 1
	0.0020	0.004	0.001	0.004	xx
Maximum (mg/l)	0.1543	0.0876	–	0.2677	Réf. 1
	0.0800	0.540	0.015	0.5540	xx

Ref. 1: F. Fournier et Cd. Intoxications par les pesticides. Prévention dans les pays en developpement OMS 1980; xx: The present work

Our mean value for DDT was found to be greater by 25.64 % and our maximum for DDT by 106.94 %, but Fournier's figures for HCH were higher than ours. It is to be noted that our poorest residue sample (L 42) belonged to our oldest donor, who was a mother of four children, and the next poorest (L 33) was a 33 years old donor with five children. Moreover, L 18 belonging to a 34 years old mother of five children account for 0.025 mg/l, but still being a small figure in our collection. However, at this stage of our work, it is too early to draw any general conclusions.

b) Cord blood

The twelve samples analyzed contained detectable quantities of HCH and p,p'DDE (Table 6). The values are scattered, however, 58.33 % of our samples were richer in HCH than in p,p'DDE.

Nevertheless, our values compared to the scarce literature /1/ seem nearer to the figures reported from Brazil than those given for USA. One again, we prove that organochlorine compounds do cross the placental barrier.

Table 6

Sample	Expressed in ng/ml	
	HCH	p,p'DDE
Sc_2	0.210	3.15
Sc_2	29.900	15.47
Sc_3	1.150	10.99
Sc_4	0.800	7.91
Sc_5	6.700	8.05
Sc_6	59.000	2.10
Sc_7	144.500	13.30
Sc_8	4.45	22.12
Sc_9	141.500	0.504
Sc_{10}	93.000	21.63
Sc_{11}	18.750	1.89
Sc_{12}	0.75	2.38
$\overline{Sc}$	41.72	9.12

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ORGANOCHLORINE RESIDUES IN THE TUNISIAN MARKET BASKET

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Abstract. This paper provides preliminary findings on organochlorine residues in Tunisian foodstuffs, i.e.: butter, milk, cheese, fish, canned tuna and sardines, beef, egg yolk, chicken and beef liver. The techniques used are HPLC, MS-GC, ECD-GC.

INTRODUCTION

Pesticide residues in eggs, dairy products, are source of human pesticide contamination. Some results are reported here on organochlorine residues in Tunisian foodstuffs. Despite the use of some organochlorine pesticides in Tunisia, no monitoring on residues has been made at the best of our knowledge. The foodstuffs studied were: butter, cheese, milk, egg yolk, beef, beef liver, chicken, canned tuna and sardines.

We focused on the following residues: αHCH, βHCH, γHCH, DDE, DDD, DDT, dieldrine, heptachlore epoxyde.

Our work was first qualitative, then after the setting up of conditions quantitative.

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MATERIALS AND METHODS

High purity analytical grade chemicals and solvents were used.

Collection of samples

All samples were randomly taken from public markets starting from the last quarter of 1978. The collected samples were placed in aluminium foil and frozen until analysis which usually took place within a week.

Methods

We used the methods already reported in the literature /1, 3–5, 7–9/.

Techniques

1) HPLC: Our high performance liquid chromatograph is a Tracor with a pump type Tracor 996 and a solvent programmator type Tracor 980 A, equipped with both variable wavelength UV detector type Tracor 970 and a differential refractometer type Tracor 910. The column used was of stainless steel (L: 25 cm, diameter: 3 mm) packed with Lichosorb SI 60, the mobile phase was n-hexane and detection was at 254 nm.

2) MS coupled to GC: The mass spectrometer was a Varian Mat 112 and the gas chromatograph a Varian 1400. The conditions were as follows:

- GC: Column 36 % SE 30, 2m x 3mm; Varaport 30 (100–120 mesh); Varian 1400 Vector gas He at 15 ml/mn; Programmed t 12°C/mn; Detection ECD; Injected vol 4 l;
- MS: Electrons energy 70 eV; Emission current 0.7 mA; Varian Mat 112 Pressure in the ionisation chamber 5×10^{-6} torr.

The so-called specific ion monitoring technique of MS was also used (SIM).

Our quantitative results were obtained solely by ECD-GC on two different columns. Only residues detected on the two columns are reported. All compounds were identified by their retention times compared to known standards (Aldrich Gold Label). The columns used are described in Table 1.

Table 1

Column	Lenght m	i.d. inch	Phase	Support
Glass	1.8	1/4	O.V.17 3%	Chromosorb W.H.P. 100/120 mesh
Glass	1.8	1/4	D.C200 5%	
Stainless steel	2	1/8	O.V.101 10%	Chromosorb W.H.P. 80/100 mesh

Detection ECD with Ni 63 source; Vector gas nitrogen U flow rate 60 ml/mn; Column temperature 190°C; Injector temperature 240°C; Detector temperature 300°C.

RESULTS

(1) Our qualitative results are shown in Tables 2 and 3 for HPLC and MS-GC, respectively.

Table 2

Sample	Residue	
Chicken	p,p'DDE	o,p'DDE
Chicken feed	p,p'DDE	
Milk	p,p'DDE	o,pDDE
Yolk	p,p'DDE	
Butter	p,p'DDE	o,pDDE

Table 3

Sample	Ion mass	Residue
Milk	246	o,p'DDE p,p'DDE o,p'DDT
	235	p,p'DDE o,p'DDT
Butter	246	o,p'DDE p,p'DDE
	235	p,p'DDE o,p'DDT
Chicken	246	o,p'DDE p,p'DDE
	235	p,p'DDE o,p'DDT p,p'DDT
Yolk	246	o,p'DDE p,p'DDE

Table 4. gives a summary of our qualitative findings:

Table 4

Sample	p,p'DDT	o,p'DDT	p,p'DDE	o,p'DDE	3 isomers DDD
Chicken	1 +	1 +	1-2-3 +	1-3 +	1-2-3 -
Chicken feed	2-3 -	2-3 -	3 +	2-3 -	2-3 -
Fish (fresh)	1-2-3 -	1-2-3 -	1-2-3 -	1-2-3 -	1-2-3 -
Cheese (imp.)	1-2-3 -	1-2-3 -	1-2-3 -	1-2-3 -	1-2-3 -
Milk	1-2-3 -	1 +	1-3 +	1-3 +	1-2-3 -
Butter	1-2-3 -	1 +	1-3 +	1-3 +	1-2-3 -
Yolk	1-2-3 -	1-2-3 -	1-3 +	1 +	1-2-3 -

1) Mass spectrometry (SIM); 2) SM-GC; 3) HPLC; +: residue detected; -: residue not detected

Except for cheese and fresh fish (two species), various isomers of DDT and its metabolite DDE were found in Tunisian foodstuffs. We were not able to detect any isomers of DDD by the three techniques. But chicken was the only foodstuff which was positive for DDT and DDE by all three techniques. It might thus be the most contaminated.

2) Quantitative results:

a- Eggs: figures are presented in Table 5.

b- Others results are shown in Table 6.

c- Table 7 gives the results obtained for canned foods.

DISCUSSION

Except for one case, namely O_2, Tunisian eggs contain amounts of HCH (total) and DDT (total) below one ppm. These amounts compared to EEC standards /6/ are good for αHCH, HCB and total DDT. Moreover, for other foodstuffs, further surveys must be aimed toward the Market Basket /2/.

Table 5

Organochlorine residues in eggs

Sample	αHCH	βHCH	γHCH	Total HCH	HCB	Diel	Hep-epox	DDE	DDD	DDT	Total DDT
O_1	N.D.	0.008	0.143	0.151	0.085	0.382	0.008	N.D.	N.D.	N.D.	N.D.
O_2^1	0.037	0.328	0.238	0.603	0.087	N.D.	0.025	12.205	2.08	0.153	14.438
O_3^2	0.014	0.242	0.029	0.285	0.159	N.D.	N.D.	0.113	0.106	0.034	0.253
O_4^3	N.D.	0.062	0.018	0.080	0.058	N.D.	N.D.	0.233	N.D.	N.D.	0.233
O_5^4	0.009	0.066	0.213	0.288	N.D.	N.D.	0.025	0.34	0.14	N.D.	0.48
O_6^5	0.015	0.015	0.017	0.047	N.D.	N.D.	N.D.	0.334	N.D.	N.D.	0.334
O_{7*}^6	0.016	0.782	0.0313	0.831	0.0046	N.D.	N.D.	0.065	N.D.	N.D.	0.065
O_{8*}^{7*}	0.005	0.240	0.016	0.261	0.042	N.D.	N.D.	0.04	N.D.	N.D.	0.04
O_{9*}^{8*}	0.005	0.152	0.009	0.166	0.025	N.D.	N.D.	0.025	N.D.	N.D.	0.025

*: Samples O_7, O_8, O_9 are pooled samples of 10 eggs originating from the same breeding; Data expressed in ppm (fat matter);
N.D.: $\prec$ 0.005 ppm

Table 6

Organochlorine residues in various samples

Sample	αHCH	βHCH	γHCH	Total HCH	HCB	Diel	Hep-epox	DDE	DDD	DDT	Total DDT
Milk	0.122	1.354	0.034	1.510	0.700	N.D.	N.D.	2.917	N.D.	N.D.	2.917
Beef	N.D.	2.291	0.149	2.440	0.292	N.D.	N.D.	0.891	4.739	0.496	6.126
Chicken	N.D.	0.241	0.112	0.353	N.D.	N.D.	N.D.	0.918	0.605	0.170	1.693
Cheese	0.086	0.525	0.115	0.726	0.101	N.D.	N.D.	0.761	0.139	1.481	2.381
Butter	0.087	0.219	0.170	0.476	0.043	N.D.	0.053	0.200	0.396	0.136	0.732
Beef liver	0.010	0.106	0.409	0.525	N.D.	N.D.	N.D.	0.211	0.345	0.064	0.620

N.D. $\prec$ 0.005 ppm; Results expressed in ppm (fat matter)

Table 7

	αHCH	βHCH	γHCH	Total HCH	DDE (ppm)
Canned tuna	0.007	0.004	0.018	0.029	0.032
Canned sardines	0.004	0.002	0.05	0.056	0.013

Table 7 (cont.)

	DDD	dieldrine	Total DDT	HE	DDT
Canned tuna	0.020	0.016	0.052		
Canned sardines	0.014	0.011	0.030	0.006	0.003

However, it must be pointed out that the amount of cyclodienes are very small in Tunisian foodstuffs.

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THE EFFECT OF ORGANIC LOADINGS ON THE PERFORMANCE OF ROTATING BIOLOGICAL CONTACTORS

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It has become a mere fact that the ever expanding growth of the population and the development of technology in our societies are causing damaging pollution problems in our natural waters.

Biological treatment processes have become a major technology for wastewater treatment whether municipal or industrial.

The Rotating Biological Contactors (RBC's) Process is a relatively new biological wastewater treatment process compared to other biological treatment processes, such as activated sludge and trickling filters.

Although the biological mechanisms for wastewater treatment for RBC's and activated sludge are the same, there are some differences relative to fixed bed growth versus fluidized bed growth systems.

The performance of RBC's is affected by a number of design parameters such as flow rate, rotational speed, detention time, temperature of wastewater, disc surface area, submerged disc depth, and organic loading.

The work presented here gives a description, analysis and discussion of the effect of organic loading which is a paramount parameter of the performance of RBC's.

The study shows the tremendous effect of organic loading on the performance of RBC's, through experimental as well as analytical techniques. It also correlates the performance at each stage of the process to the organic loading which is a major design factor for the manufacturing system of RBC's.

INTRODUCTION

Since biological treatment processes have become a major technology

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for wastewater treatment both municipal and industrial, their contribution in controlling water pollution is escalating in our societies nowadays.

The Rotating Biological Contactors (RBC's) Process is a relatively new biological wastewater treatment process if compared with other processes such as activated sludge and trickling filters.

In designing an RBC system different parameters influencing the design cirteria have to be considered, such as flow rate, rotational disc speed, detention time, surface area, wastewater temperature, submerged disc depth, number of stages and organic loading.

The objective of this work was to examine and determine the effect of organic loadings on the organic removal rates and on the overall performance of a rotating biological contactor.

Description of the RBC's Process

Rotating biological contactors are aerobic, continuous flow, fixed-film reactors.

A rotating biological contactor is constructed of bundles of plastic packing attached radially to a shaft, forming a cylinder of media. The shaft is placed over a contour-bottomed tank so that the submersion of the media is approximately 40 percent. The contactor surfaces are spaced so that during submergence wastewater can enter the voids in the packing. When rotated out of the tank, the liquid trickles out of the voids between the surfaces and is replaced by air. A fixed-film biological growth, similar to that on a trickling filter packing, adheres to the media surfaces. Alternating exposures to organics in the wastewater and oxygen in the air during rotation are similar to the dosing of a trickling filter with a rotating distributor. Excess biomass sloughs from the media and is carried out in the process effluent for gravity separation.

The microbial population in the process depends on the type of wastewater being treated and the position of each disc in the reactor. In general, the population usually consists of more filamentous and fewer slim-forming organisms. Therefore the sloughed solids are relatively dense with good settling characteristics /3/. Antonie /1/ has mentioned that due to the high sludge solid contents obtained with the rotating disc process, sludge thickening before treatment and disposal is not necessary.

One of the advantages of RBC's is that the biomass passes through the wastewater, ensuring adequate wetting of all organisms at any flow rate /4/.

RBC's do not need solid recycling /1/, they require low operating and maintenance costs along with power consumption /1/. The process is easy to operate and noiseless /2/. RBC's have a better shock load response and great ability to handle fluctuations in hydraulic and organic loadings /7/.

According to EPA, design manual for RBC, temperatures lower than $55^{o}F$ may affect the organic removal efficiency. However, in some highly loaded RBC systems, lower wastewater temperatures do not always result in decreased carbonaceus removal rates, but may enhance removals. This phenomenon may be attributed to increasing DO saturation values with decreasing temperature, which promote increased oxygen transfer and possible reduction of the sulfied oxidizing organisms /2/.

Some of these parameters control the oxygen transfer characteristics. Kima /7/ related the oxygen transfer coefficient in the RBC process to the rotational velocity, the disc size, and the space between the discs. Antonie /1/ suggested that the optimum disc velocity is 2 rpm. However, all these parameters have been standardized to optimize the process design and operation /8/. The minimum submerged disc depth is 40 percent. The typical full scale mechanical drive RBC disc diameters range from 10 to 12 feet. The typical rotation speed is 1.6 rpm or a peripheral velocity of about 60 fpm /8/. Due to the standardization of these parameters, each RBC has its own standard oxygen transfer capabilities /8/. Therefore, the flow rate and the organic concentration of the wastewater are the major factors to be controlled to meet the oxygen transfer capabilities and the required substrate removal rate and efficiency for each design. The notion of combining the two concepts of the hydraulic loading and the substrate concentration has many advantages. One of the advantages of this notion is its capability to predict the efficiency of the substrate removal at any loading condition, irrespective of zero, first or second order kinetics.

When sulfide is present, either in the influent wastewater or by its production deep within the biofilm sulfide oxidizing organisms such as Beggiatoa can frequently grow on the biofilm surface. They compete with heterotrophic organisms for oxygen and space on the RBC media surface. They reduce sulfate to sulfide and then reoxidize it to elemental sulfur. For each mole of sulfate to be reoxidized and deposited as elemental sulfur, 1.5 moles O_2 are needed. Beggiatoa predominance can result in an increase in the concentration of biomass, while causing a substantial reduction in the organic removal. In extreme cases, a progressive Beggiatoa takeover of the entire system may cause significant deterioration of effluent quality.

EXPERIMENTAL WORK

The laboratory-scale RBC pilot employed in the study was 3.25 feet long, with 8.97 liters capacity. It consisted of a tank made of plexiglass, divided into five stages separated by baffles with holes in the bottom of each baffle to allow flow from one stage to the next. The first stage consisted of eight rotating discs and each of the other four stages consisted of four rotating discs, with a total of twenty-four discs for the whole unit. Each disc was approximately six inches in diameter and 1/8 inch thick.

The total disc surface area was 9.42 square feet for the entire unit.

Three small styrofoam paddles were placed between every two discs to create sufficient turbulence condition for complete mixing of the wastewater, and to keep the mixed liquor solids in suspense. A 1/20 horsepower electric speed reducer motor was used to rotate the discs at a speed of approximately 8 rpm.

Forty percent of the surface of the disc was submerged into the liquor.

The wastewater was placed in a tank with a capacity of approximately 25 liters. The wastewater was pumped into the first stage by using a 1/4 horsepower motor driven controlled volume pump. From the first stage the wastewater flowed through the next stage and out to the effluent inlet, where it was collected in a 25 liter capacity tank.

A hydraulic loading of 8 ml/min, which equals 0.32 gpd/ft^2, was applied to all loading conditions.

The synthetic waste used in this experiment was composed of glucose ($C_6H_{12}O_6$) as the carbon source and as growth limiting factor with nutrients and buffer. Sodium bicarbonate was used as a buffer to keep the pH constantly around 7.0. The wastewater was prepared daily by mixing the concentrated waste with a specific amount of tapwater in order to achieve the desired organic concentration. The composition of the wastewater for feeding 100 mg/l glucose is shown in Table 1.

The RBC unit was seeded with about 9 liters of effluent from the municipal sewage treatment plant, and was run as a batch process for there days until some growth started on the disc. Then, the unit was operated as a continuous flow process by pumping the wastewater to it.

The study was performed by using six different organic loadings. For each loading condition the system was run as a continuous flow unit for about six to seven days to reach equilibrium before samples were collected.

Table 1

Composition of feed for 100 mg/l glucose as substrate

Constituent	Concentration (mg/l)
glucose	100 mg/l
NH_4Cl	30 mg/l
H_3PO_4	4 mg/l
$MgSO_4.7H_2O$	10 mg/l
$FeCl_3.6H_2O$	0.1 mg/l
$CaCl_2$	1 mg/l
$MnSO_4.H_2O$	1 mg/l

Once the system reached equilibrium, samples were collected every other day.

Six samples were collected in each sampling period. The first sample was taken at the influent line. The other five samples were collected at the end of each of the following stages starting with the last stage. The samples were filtered directly after sampling procedure.

The analytical tests performed during this study were uninhibited soluble BOD and inhibited soluble BOD. The inhibited BOD tests were applied during the second and third loading condition for the samples which were taken from third, fourth and fifth stages to inhibit nitrification. HACH inhibitor consisting of 2-chloro-6 (trichloromethyl) pyridine, coated on an inert substrate, was used during the study. The BOD tests were run immediately after collecting the samples.

Biochemical oxygen demand (BOD) was used to analyze the organic concentration of the wastewater and the effluent from each stage. All the tests were run according to the procedure described in Standard Methods for the Examination of Water and Wastewater.

RESULTS AND DISCUSSION

In the study six different total organic loadings were used. A constant hydraulic loading of 11.5 L/day (0.32 gpd/ft^2) was used for the entire study. The different organic loadings were achieved by changing the concentration of the influent substrate. The organic concentrations used were 400,

800, 1200, 2000 mg/l $sBOD_5$. There organic concentrations yielded total organic loadings of 1, 2, 3, 5.4, lb $sBOD_5$/day/1000 ft^2. According to the data obtained from this study, these four loading conditions can be divided into two categories. Each category has a common performance data and operating conditions. The first category includes the first two loads (1,2 lb $sBOD_5$/day/1000 ft^2). The second category includes the third and fourth loads (3, 5.4 lb $sBOD_5$/day/1000 ft^2).

The performance of the unit during the entire study was based on the sBOD removal capability of the unit. The results of the first category are shown in Figs 1, 2, 3, 4, 5 and 6. The performance of the RBC based on BOD removal as a function of time is shown in Figs 1, 2, 4 and 5.

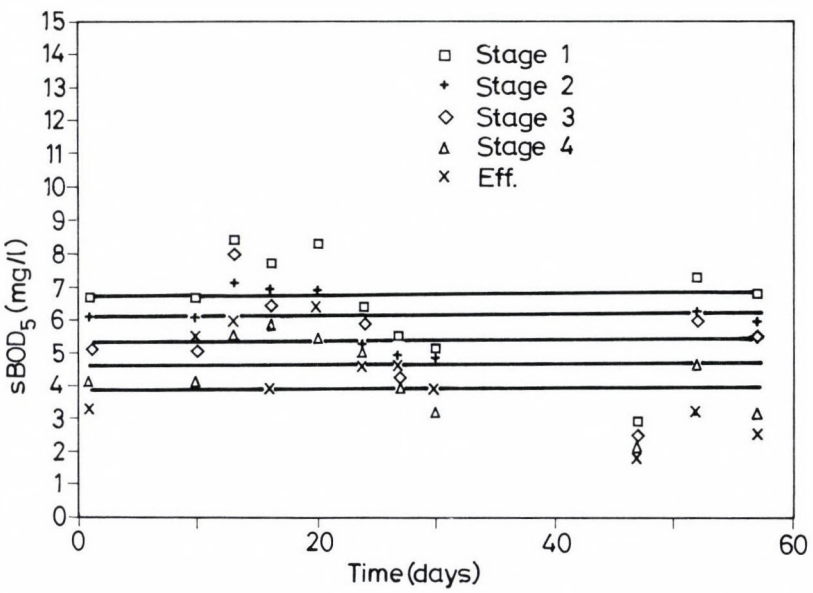

Fig. 1. $sBOD_5$ removal vs. time for the first organic loading condition (1 lb $sBOD_5$/day/1000 ft^2)

The performance of the RBC based on BOD removal as a function of stages is shown in Figs 3 and 6.

Figures 1 and 2 show that the removal efficiency for the first total organic load =1.0 lb $sBOD_5$/day/1000 ft^2) is almost constant with time. The effluent BOD ranges from 1.8 mg/l to 6.0 mg/l. The treatment efficiency ranges from 98.1 percent to 99.5 percent. Figure 3 shows that the greatest

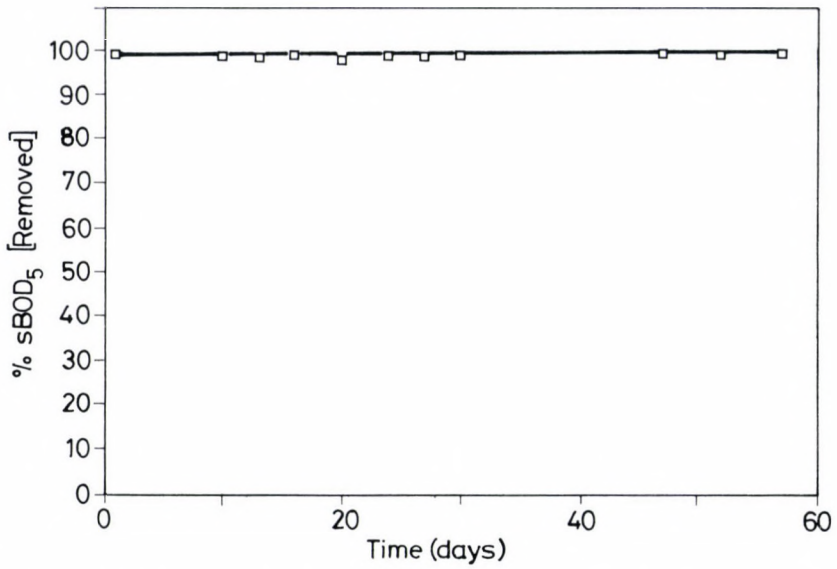

Fig. 2. Efficiency of treatment vs. time for the first organic loading condition (1 lb $sBOD_5$/day/1000 ft^2)

BOD removal was accomplished by the first stage. An average of 98 percent of the substrate was removed in the first stage (2.9 lb $sBOD_5$/day/1000 ft^2).

As shown in Figs 4 and 5, the removal efficiency for the second organic load (2.0 lb $sBOD_5$/day/1000 ft^2) is also almost constant with time. The effluent BOD ranges from 0.5 to 3.1 mg/l. The treatment efficiency ranges from 99.6 to 99.9 percent.

Figure 6 indicates that the major BOD removal occurred in the first stage (about 6 lb $sBOD_5$/day/1000 ft^2).

The operating conditions for this category were very good. The system was free of any problems or clogging. The biomass film was about 1/4 inch thick with a dark white colour in the first stage, which got thinner and darker in the following stages.

The results of the second category, which includes total organic loadings of 3 and 5.4 lb $sBID_5$/day/1000 ft^2 are shown in Figs 7–15. The performance of the RBC unit based on the BOD removal versus the time for the third organic load (3.0 lb $sBOD_5$/day/1000 ft^2) is shown in Fig 7. It indicates that the BOD removal in the first stage increased with time during the first 12 days of applying this load. The BOD leaving the first stage de-

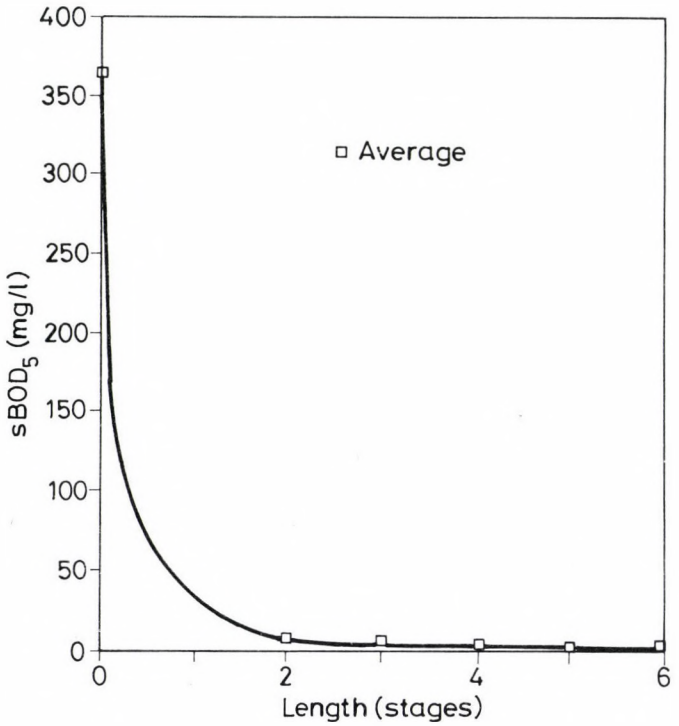

Fig. 3. $sBOD_5$ remaining vs. stages for the first organic loading condition (1 lb $sBOD_5$/day/1000 ft^2)

creased to 13.7 mg/l, then it started to increase to reach 30.2 mg/l after 17 days. While the first stage BOD started to increase, the effluent BOD started to decrease. Figure 8 represents the BOD removal efficiency versus the time. It ranges from 98 to 99.7 percent, being almost constant. Figures 9 and 10 indicate the BOD removal as a function of the stages.

They represent the average of the data collected before and after the deterioration, respectively.The figures indicate that the BOD removal of the first stage decreased after the deterioration from 98.7 percent to 97.5 percent, while the BOD removal of the last stage increased from 98.6 to 99 percent.

The two curves indicate that the greatest BOD removal occurred in the first stage. An average of 98.3 percent of the BOD was removed in the first stage (9.1 lb $sBOD_5$/day/1000 ft^2).

Figure 11 shows the BOD removal as a function of time for the

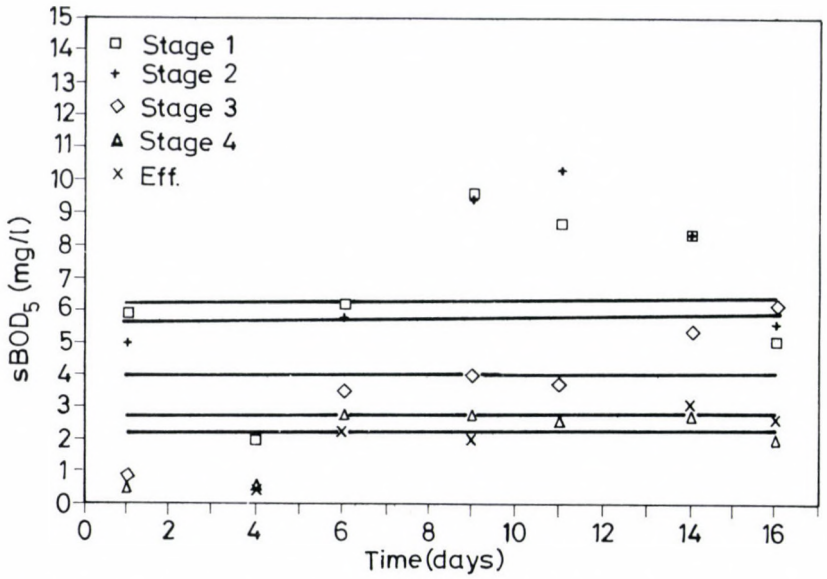

Fig. 4. $sBOD_5$ removal vs. time for the second organic loading condition (2 lbs $sBOD_5$/day/1000 ft^2)

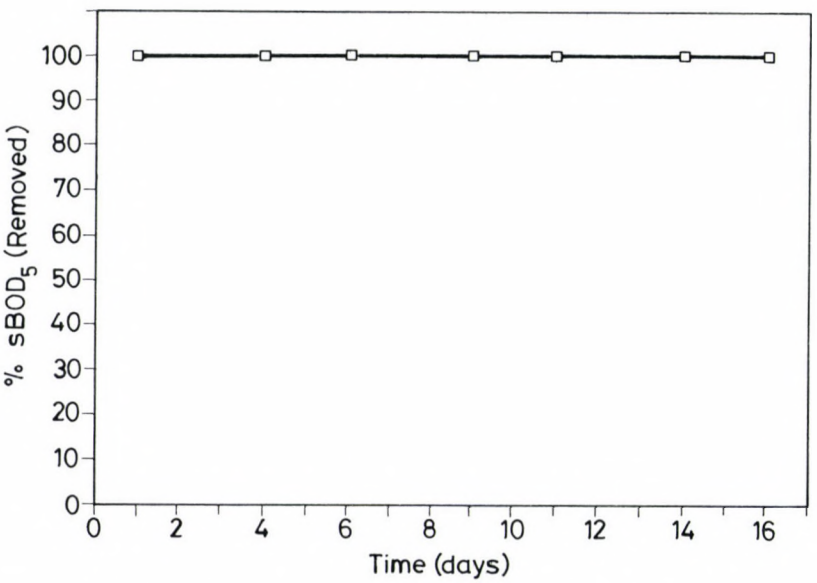

Fig. 5. Efficiency of treatment vs. time for the second organic loading condition (2 lbs $sBOD_5$/day/1000 ft^2)

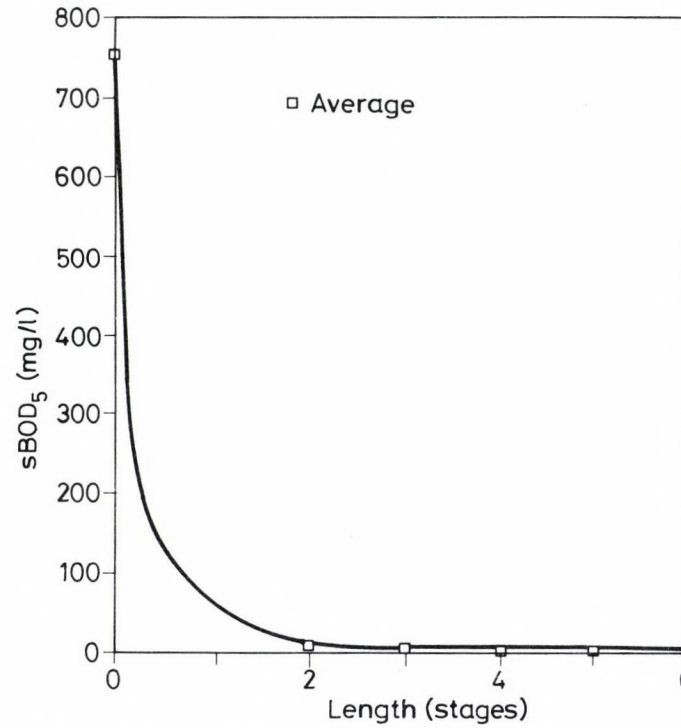

Fig. 6. $sBOD_5$ remaining vs. stages for the second organic loading condition (2 lbs $sBOD_5$/day/1000 ft^2)

fourth total organic load (5.4 lb $sBOD_5$/day/1000 ft^2). It indicates that the BOD leaving the first stage decreased from 249.0 mg/l to 140.0 mg/l within 12 days of applying this load and 5 days from starting BOD tests. Then, a sudden and significant deterioration occurred in the system.

The BOD jumped to 999.0 mg/l, then decreased to 408.0 mg/l, and increased again.

While the first stage BOD started to increase, the effluent BOD started to decrease. Figure 12 indicates that the efficiency of treatment increased from 91 to 96.4 percent, decreasing suddenly to 52.3 percent after 19 days of applying this load, then starting to increase again, to reach 83.6 percent after 24 days. Figures 13–15 represent the BOD removal as a function of stages for the data collected before, during and after deterioration. The results show that the greatest removal occurred in the first stages and a slight treatment occurred in the next stages. An average of 90

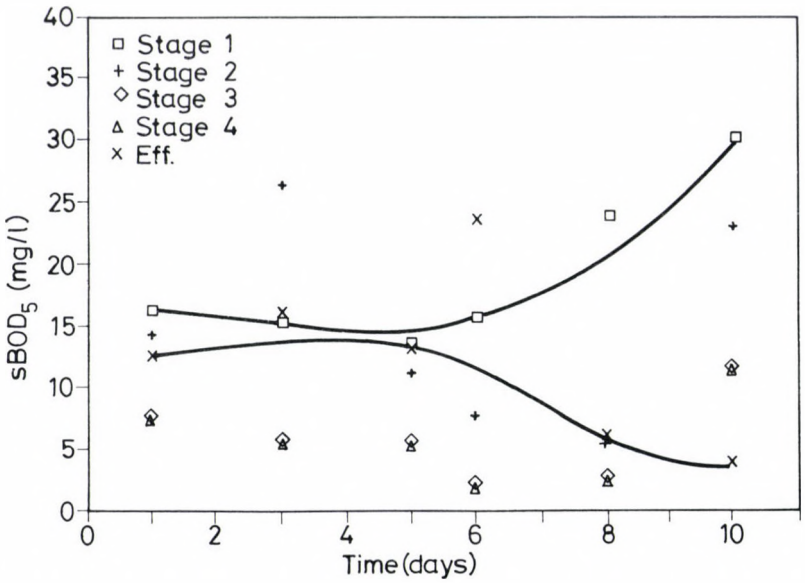

Fig. 7. $sBOD_5$ removal vs. time for the third organic loading condition (3 lbs $sBOD_5$/day/1000 ft^2)

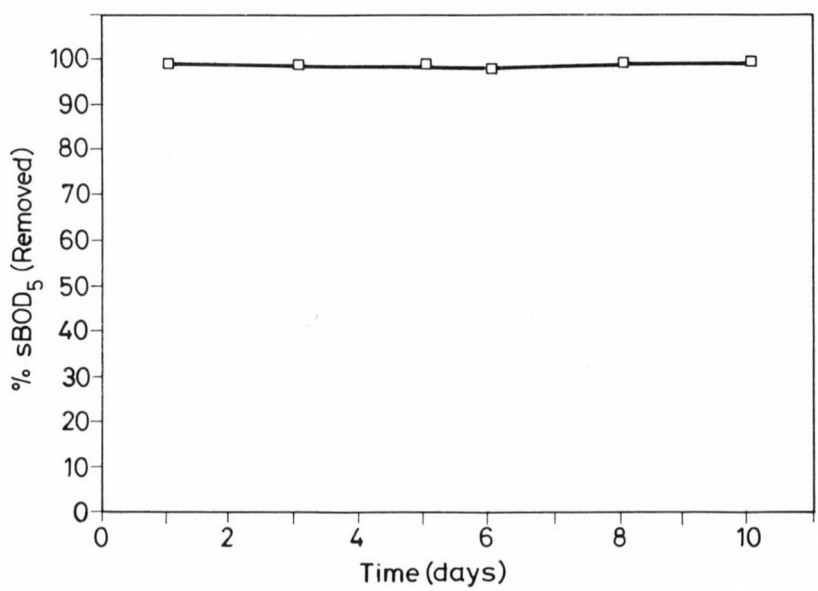

Fig. 8. Efficiency of treatment vs. time for the third organic loading condition (3 lbs $sBOD_5$/day/1000 ft^2)

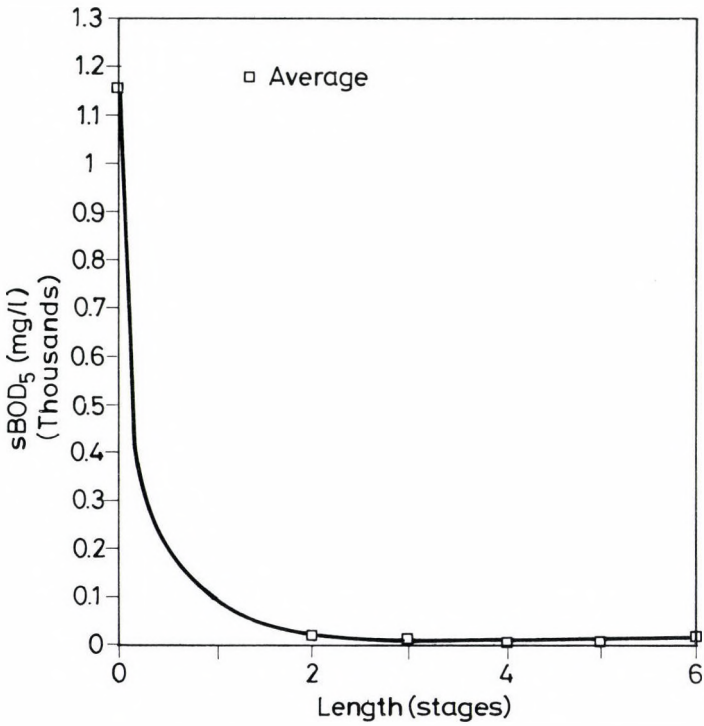

Fig. 9. $sBOD_5$ remaining vs. stages for the third organic loading condition (3 lbs $sBOD_5$/day/1000 ft^2). Before the deterioration

percent of the BOD was removed in the first stage (14.4 lb $sBOD_5$/day/1000 ft^2) and 3.8 percent in the next four stages; see Fig. 13. An average of 45.4 percent of the BOD was removed in the first stage (6.72 lb $sBOD_5$/day/1000 ft^2) and a very slight treatment, almost zero, occurred in the next three stages. Then 6.9 percent of the BOD was removed in the last stage, as shown in Fig. 14. As seen in Fig. 15, the BOD removal increased again to 74.8 percent in the first stage and a very little removal (2.4 percent) occurred in the next three stages, 4.7 percent of the BOD was removed in the last stage.

The problem in operating conditions appears in this cetagory. The odour problem started to appear during applying the first load and it became stronger during the second load. At first load, the biomass film was about 1/2 inch thick with a white colour, being firm and attached to the media in the first stage, and becoming thinner and darker in the next four

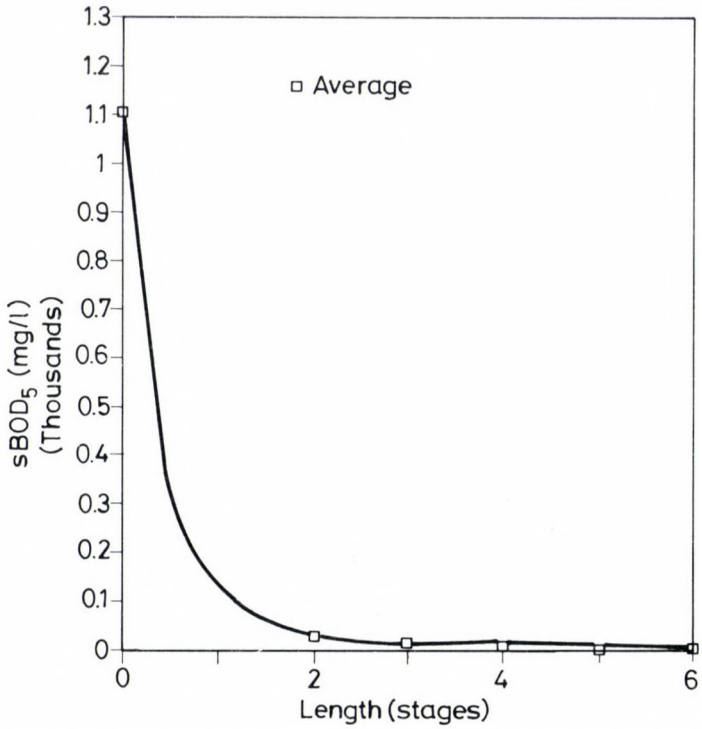

Fig. 10. $sBOD_5$ remaining vs. stages for the third organic loading condition (3 lbs $sBOD_5$/day/1000 ft^2). During the deterioration

stages. At the second load, the biomass film was thicker and darker and it was sloughing from the media and concentrating between discs causing clogging problems. A white filament film grew around the discs and the interior surface of the tank.When the failure happened in the system the biomass film turned to a slightly yellowish-white colour. Red and black worms were found in the system, too.

Kinetic analyses

The kinetic analyses in this work were based on the concept of the total organic loading. This concept was first introduced by Kincannon and Stover in the early 1970's /6, 8/. In this concept the specific substrate utilization rate is a function of the specific loading (FS_i/A) as lb $sBOD_5$ applied/day/1000 ft^2. Figure 16 represents the relationship of sol-

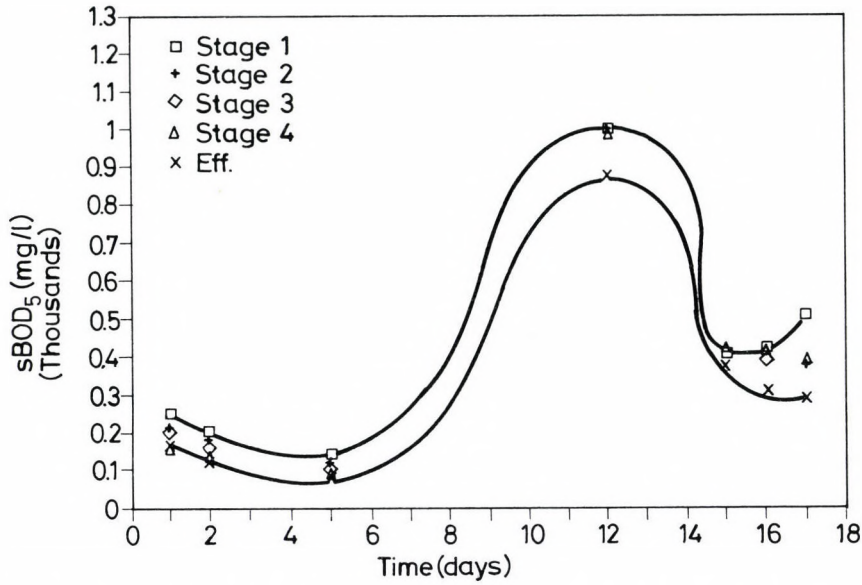

Fig. 11. $sBOD_5$ removal vs. time for the fourth organic loading condition (5.4 lbs $sBOD_5$/day/1000 ft^2)

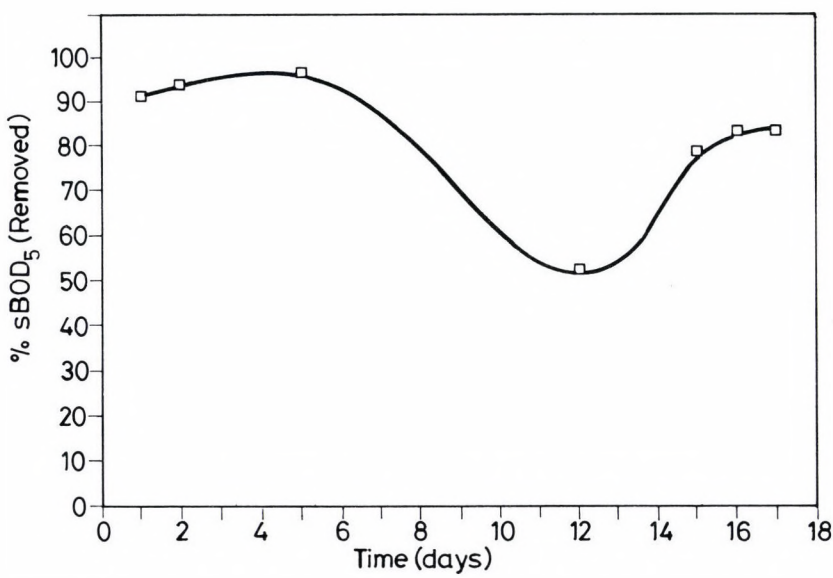

Fig. 12. Efficiency of treatment vs. time for the fourth organic loading condition (5.4 lbs $sBOD_5$/day/1000 ft^2)

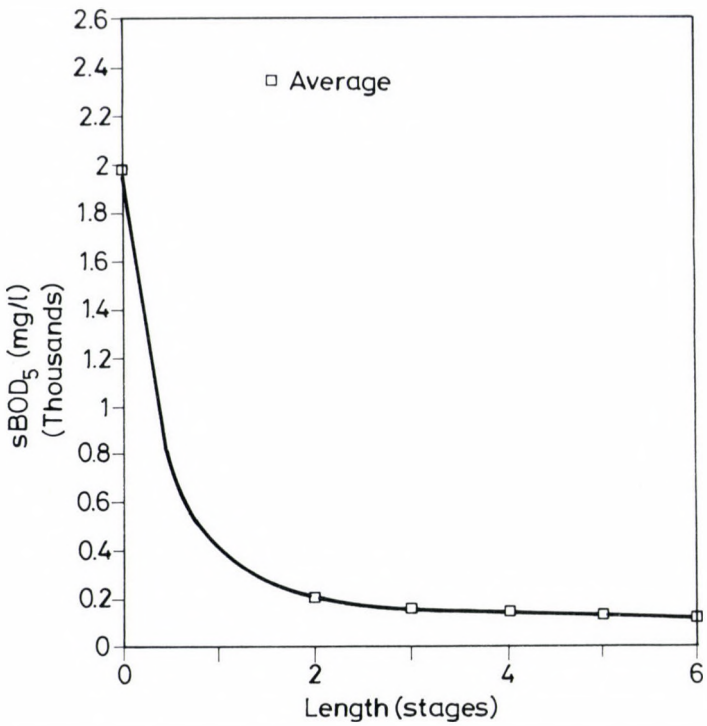

Fig. 13. $sBOD_5$ remaining vs. stages for the fourth organic loading condition (5.4 lbs $sBOD_5$/day/1000 ft^2). Before the deterioration

uble BOD removed (lbs/day/1000 ft^2) as a function of soluble BOD applied (lbs/day/1000 ft^2).

The specific loadings and specific substrate utilization rates were calculated by considering the influent substrate concentration, S_i, as the influent $sBOD_5$ to the entire stages. The effluent substrate concentration, S_e, was considered as the effluent from any stage under consideration.

Figure 16 indicates that the removal capabilities of the RBC unit at these four different organic loadings followed monomolecular kinetics and it could be fitted with a hyperbolic function similar to the "Monod equation" as described by Kincannon and Stover /6, 8/. This relationship can be shown as follows.

$$L_R = \frac{L_{R(max)} \cdot L_o}{K_s + L_o}$$

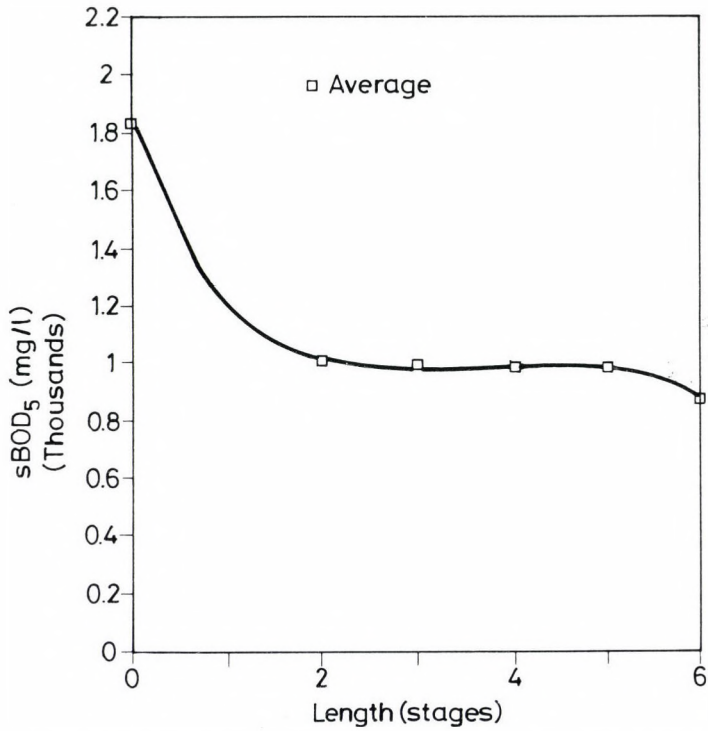

Fig. 14. $sBOD_5$ remaining vs. stages for the fourth organic loading condition (5.4 lbs $sBOD_5$/day/1000 ft^2). During the deterioration

where:

L_o = Applied BOD loading in lb BOD/day/1000 ft^2 (FS_i/A)

L_R = BOD removed in lb BOD/day/1000 ft^2 ($F(S_i - S_e)/A$)

$L_{R(max)}$ = Maximum BOD removed in lb BOD/day/1000 ft^2

K_s = Applied BOD loading rate at which the rate of BOD removal is one-half the maximum rate or the saturation constant

Figure 16 indicates that the amount of BOD removed increased as the amount of BOD applied increased until the breaking point of 6.0 to 6.5 lb BOD/day/1000 ft^2), where the amount of BOD removed per BOD applied started to decrease significantly. Beyond these loading conditions the removal capabilities of the unit decreased and the BOD removed approached a maximum value at a loading condition of 20 lb BOD/day/1000 ft^2.

Figure 17 represents the reciprocal plot of $sBOD_5$ removed versus $sBOD_5$ applied for the averages of every stage at every applied load, and

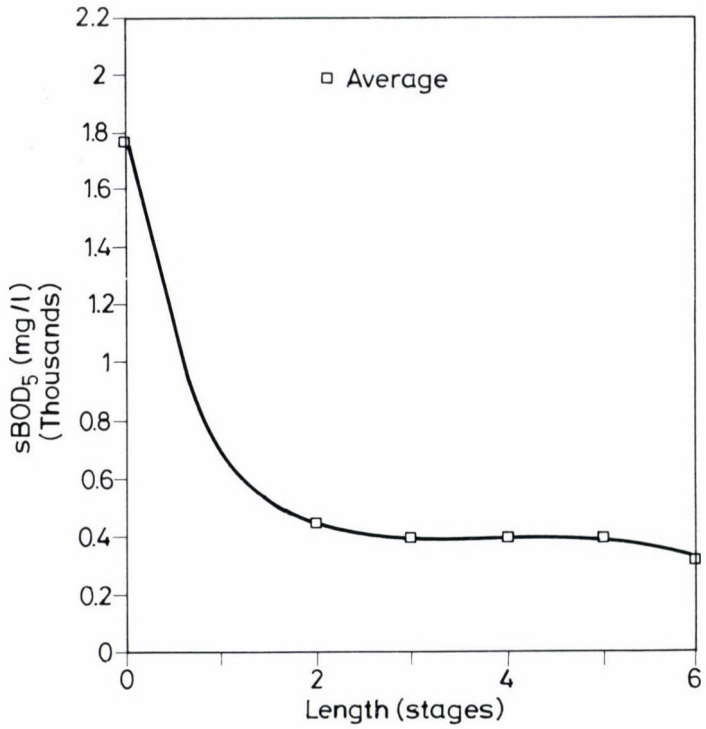

Fig. 15. $sBOD_5$ remaining vs. stages for the fourth organic loading condition (5.4 lbs $sBOD_5$/day/1000 ft^2). After the deterioration

$L_{R(max)}$ and K_s can be determined by the intercept and the slope of the curve in Fig. 17.

The maximum theoretical BOD removal rate $L_{R(max)}$ was 20 lb $sBOD_5$/day/1000 ft^2 and K_s was 19.88 lb $sBOD_5$/day/1000 ft^2.

CONCLUSIONS

The following conclusions were drawn from the results of this work.

1. The major BOD removal occurs in the first stage of the unit for this type of wastewater. Approximately 98 percent of the total BOD removal occurred in this stage for the first three total organic loads (1, 2, 3 lb $sBOD_5$/day/1000 ft^2). Ninety percent of the BOD was removed in this stage for the organic load (5.4 lb $sBOD_5$/day/1000 ft^2).

The BOD removal was very little in the other four stages for the

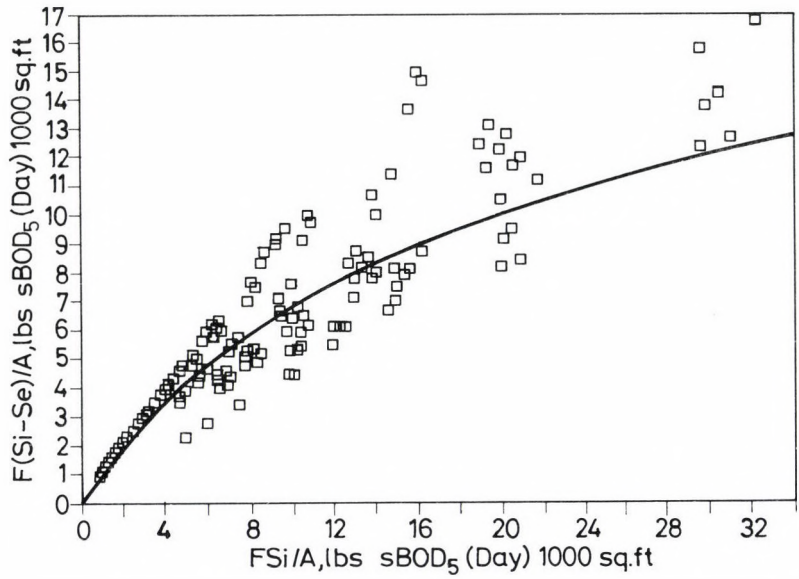

Fig. 16. Specific substrate utilization rate as a function of loading rate

four organic loads.

2. Up to 6.0 lb $sBOD_5$/day/1000 ft^2 was removed in the first stage and the unit was free from any nuisance organisms such as Beggiatoa, the operating conditions were very good and treatment efficiency was stable.

3. At organic loadings higher than that applied to the first stage (5.4 lb $sBOD_5$/day/1000 ft^2), Beggiatoa started to grow in the system, causing deterioration.

4. A maximum removal rate ($L_{R(max)}$ of 12.5 lb $sBOD_5$/day/1000 ft^2 occurred at an applied loading rate of 20.0 lb $sBOD_5$/day/1000 ft^2. Beyond this loading, the unit became oxygen transfer-limited.

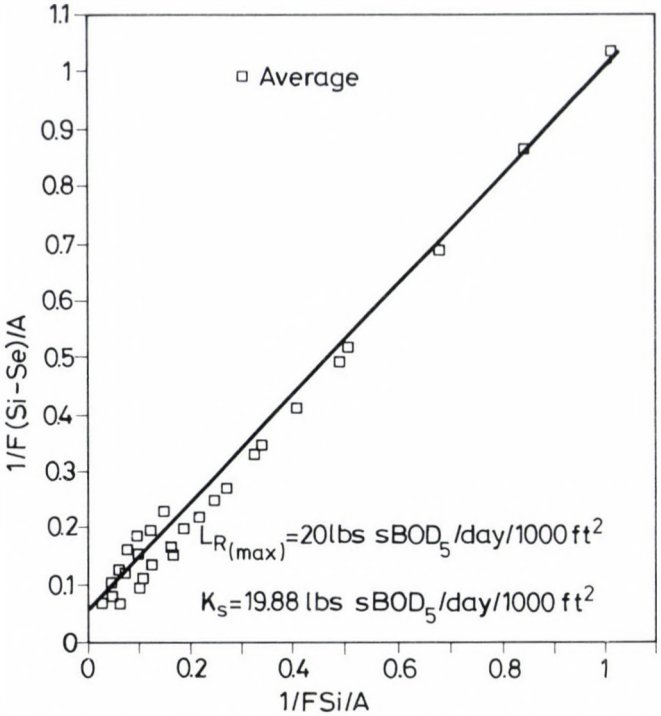

Fig. 17. Reciprocal plot of sBOD$_5$ removed (lb sBOD$_5$/day/1000 ft^2) vs. sBOD$_5$ applied (lb/day/1000 ft^2)

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EFFECTIVENESS OF MINE REHABILITATION IN RELATION TO WATER QUALITY

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When mining is completed the sites may be completely restored to the original ecosystem, rehabilitated for some desirable environmental characteristics, desirable alternative ecosystems created or just neglected. The strategy adopted will depend on the intended uses of the parts of the environment (including water) affected by the mining. An example of rehabilitation of a metal mine near the Australian Federal Capital is used to illustrate the problems that may be encountered. These include: lack of controls while mining is underway; catastrophic events, such as the collapse of a settling dam, lack of site specific understanding of pyrite oxidation processes, particularly those that are biologically enhanced; the need for adequate biological information on which to base decisions to meet biological information on which to base decisions to meet biological objectives. Experience has shown that biological collections such studies should be stored in museums where they will be valuable for comparisons of changes over long periods.

INTRODUCTION

Wind and Water

Mining will always have some environmental impact. Wind and water are often the agents for distributing the materials responsible for the otherwise local impacts of mining activities. Wind may distribute toxic gases and particulate material produced by ore treatment processes. These materials are deposited, usually with rainfall, at some distance from the

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source sometimes resulting in defoliation of large areas, consequent erosion and run-off of toxic substances into rivers.

Water is required for many mining operations from extraction to treatment of the ore. As mines are dug they may also fill will water from rainfall or from the water table and this water needs to be removed to continue mining. After mining ceases water entering the workings, and then flowing out carrying toxic effluents will be a problem leading to environmental impacts well away from the mine probably over an extended period.

Alternatives

When mining ceases four alternative courses of action may be taken (as discussed by Cairns /4/.

i) Complate restoration to the original ecosystem

ii) Rehabilitation of some desirable environmental characteristics

iii) Intervention to create an alternative ecosystem

iv) Neglect, which may result in natural rehabilitation, or no change.

Governments usually promote mining because of the political, social and economic benefits which are derived from it.At the outset these benefits are seen, but little thought is given to reclamation at the cessation of mining. Generally mining companies have not been responsible for cleaning up when they leave mines. Governments or other private individuals, will usually be left the responsibility for reclamation. Fortunately increased environmental awareness of governments and the public is leading to more controls being placed on mining operations and reclamation.

Long lasting and environmentally damaging effluents commonly result from mining of metal rich ore bodies. Sources producing the most serious metal pollution from abandoned mines are:

i) acidic water from tailings dumps or settling ponds;

ii) water from the mine which has been pumped in or from groundwater, or surface drainage;

iii) collapse or erodable particulates from tailings dams or dumps.

The availability of metals may be maintained or increased with time because of oxidation, often bacterially assisted, of the exposed ore and waste materials (principally pyrite FeS_2). Harries and Ritchie /7/ reviewed the various processes of pyritic oxidation and they pointed out that it proceeds by a number of steps. The details of the oxidation mechanisms

will depend on the detailed nature of the pyritic material, including the physical form of the pyrite and the physicochemical regime within the piles of pyrite /3, 10, 13/. The process of the oxidative decomposition of pyrite is considerably accelerated by the activity of certain microorganisms, Thiobacillus thiooxidans, T. ferrooxidans and T. denitrificans. All of the reactions, both chemical and biological require an abundant supply of both oxygen and water and mining activities usually increase the availability of both of these to the pyrite. Harries and Ritchie /7/ conclude that rehabilitation of pyrite ore bodies will require the control of these oxidation processes. They also conclude that there are few studies in which more than the first few years after rehabilitation have been studied to assess the control of the oxidation processes.

The persistence of metal pollution from mines has been well documented and many early studies, chiefly in Wales, have been reviewed by Hynes /9/ Whitton and Say /21/ and Weatherley et al. /20/. Rehabilitation of metal mines will be used as a basis of the discussion presented here, but the conclusions should be generally applicable.

The uses to which water affected by a mine will be put to, will determine the level of reclamation to be attempted. However, it should be remembered that the biota is often the most sensitive indicator of mine waste pollution and has been considered by many as the best way of measuring impact /8, 9, 21/.

Through examples, it is the intention of this paper to highlight the most important factors that need to be considered when undertaking mine rehabilitation.

REHABILITATION OF A ZINC POLLUTED RIVER

The problem

In 1913 the American architect Water Burley Griffin won a world wide competition for the design of the city of Canberra, the Australian Federal Capital. By 1963 the essentials of his plan had been carried out and a city built bissected by a lake which was subsequently named after him. The Molonglo River, which fills the lake, has been polluted by effluents from a mine about 50 km upstream (Fig. 1) ever since a slimes dump burst its banks in 1939. A further major mishap occurred in 1943 when about 30 000 m^3 of fine tailings collapsed into the town reservoir displacing an equivalent

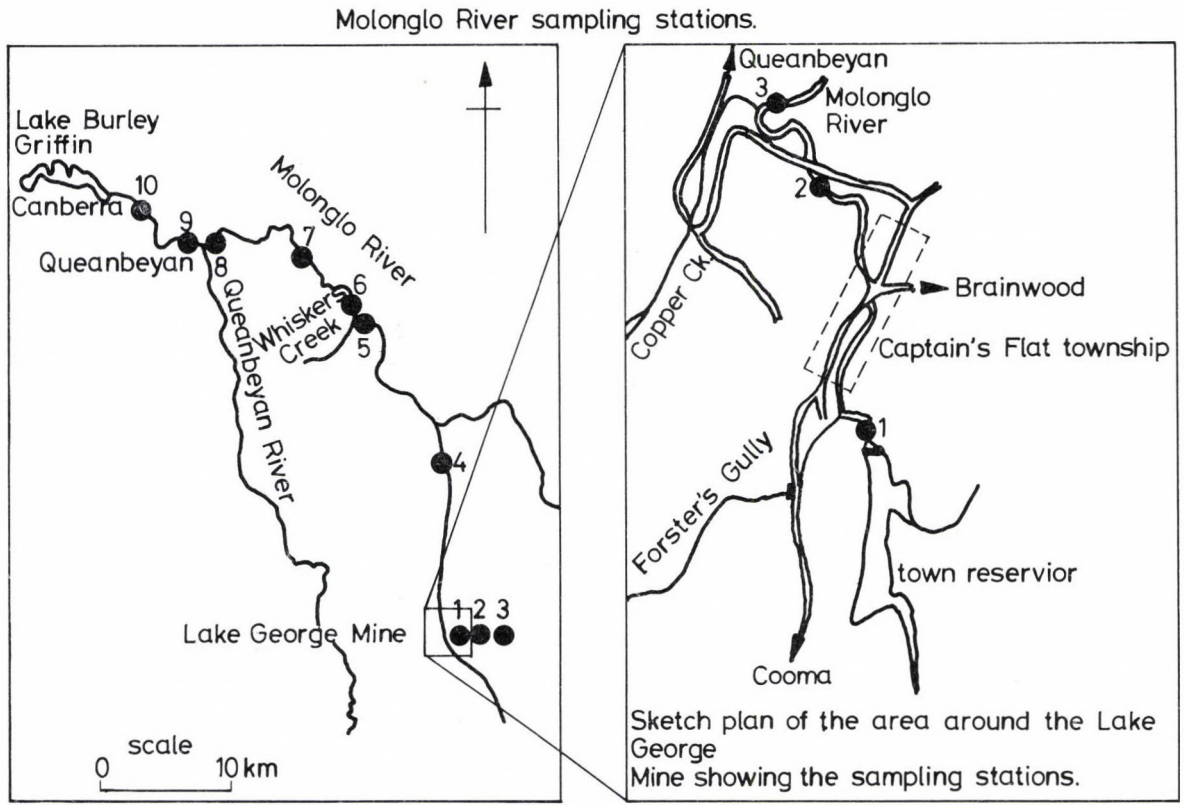

Fig. 1. Molonglo River sampling stations

volume of water over the dam wall. This water carried large quantities of tailings and the sudden rush of water caused extensive flooding along the plain between sites 4 and 5 (Fig. 1). Mine water with high levels of dissolved metals (mostly Zn, Cu, Mn and Fe) has been a continuing problem during the mines operation and since its closure in 1962.

The Molonglo River and Lake Burley Griffin are both central features of the capital. Multiple uses were planned for them, including recreational fishing, swimming and boating. The suitability of the water for these planned uses needed assessment before its filling in 1964. Weatherley et al. /18/ reported on work that was designed to assess the extent to which the pollution had affected the fauna of the river.

Weatherley et al. /18/ concluded that mine waste contamination in the Molonglo River depressed both the numbers of species and their abundance and that the absence of Crustacea, Mollusca and Oligochaeta from regions of even mild zinc contamination was likely to have been caused by lethal and sublethal concentrations of zinc. They further suggested that instability of the substratum during times of flood may produce harmful effects on the zinc-tolerant fauna by grinding and scouring. Field experiments using caged fish showed that the water of the river was also unsuitable for the survival of Salmo gairdneri both before and after the closure of the mine. All caged fish at polluted sites died between four and twelve days exposure (Fig. 1). A further biological study by Nicholas and Thomas /14/ on the Molonglo River from December 1974 to June 1977 showed that the distributions and abundances of macroinvertebrates followed the same general trends as those demonstrated by Weatherley et al. /18/. Unfortunately, their methods differed from those of Weatherley et al. /18/, thus making comparisons difficult.

An additional complicating factor was the proposed construction (completed in 1980) of a water supply reservoir on the Queanbeyan River which enters the polluted Molonglo River about 15 km from the lake (Fig. 1). Before the dam's construction the Queanbeyan River diluted the Molonglo by about three times. Thus its construction caused a relative increase in the contribution of the Molonglo River to Lake Burley Griffin through a reduction in dilution.

The solution

The objectives of remedial measures to control mine waste pollution

of the Molonglo River were as follows (Anon /1/):

i) the measures should prevent any serious deterioration in water quality that could be caused by a sudden, major collapse of mine waster damps or by increased erosion of dumps surfaces;

ii) the measures should protect and upgrade the quality of the waters of the Molonglo River as a supply of water for drinking, agricultural irrigation and stock watering;

iii) the measures should protect and upgrade the biological and aesthetic quality of the waters, and ensure maintenance of water quality in Lake Burley Griffin for present uses;

iv) the effect of the measures should be permanent.

The remedial works commenced in 1974 and were substantially completed by 1976. The works at the Captains Flat mining area involved reshaping the tailings dumps to sides with slopes of less than one in three and capping them with impervious material to prevent surface runoff and erosion. The caps to the reshaped dumps were stabilized with shallow vegetation (mainly grasses) to avoid breaking through the impervious caps thereby allowing leaching of material from the dumps /5/. Slimes dams were removed and small streams in the area were rerouted away from the dumps and other areas containing toxic materials. Some effort was also made to prevent water from entering the underground workings of the old mine. The costs of this rehabilitation were initially $ 2.5 million with $ 200 000 allocated for maintenance over the next five years. Subsequently an additional $ 500 000 was allocated for maintenance in 1984.

The result

Norris /15/ repeated the macroinvertebrate study of Weatherley et al. /18/ and concluded that there was very little difference between the two studies in the distribution and abundance of the benthic fauna, or in the chemical conditions during periods of low flow (Fig. 2). Seeps and previously deposited particulate material from the mine workings were thought to be still major sources of contamination to the Molonglo River. Graham et al. /6/ held rainbow trout in cages in the Molonglo River, repeating work also done by Weatherley et al. /18/ but with the addition of a control site upstream of Captains Flat. Rainbow trout at the control site survived for the experiments duration (12 days) while those downstream of the inflow of mine effluents survived between 4 and 12 days, similar to the findings of

Weatherley et al. /18/. Interestingly fish downstream of Carwoola Plain (between sites 5 and 6 Fig. 1) survived only 3 to 5 days compared with 7 to 12 days immediately upstream of it. A minor flood occurred at the time of the experiment and Graham et al. /6/ concluded that previously deposited metals were being released from the plain. This also concurred with the findings of Weatherley et al. /18/ 20 years earlier.

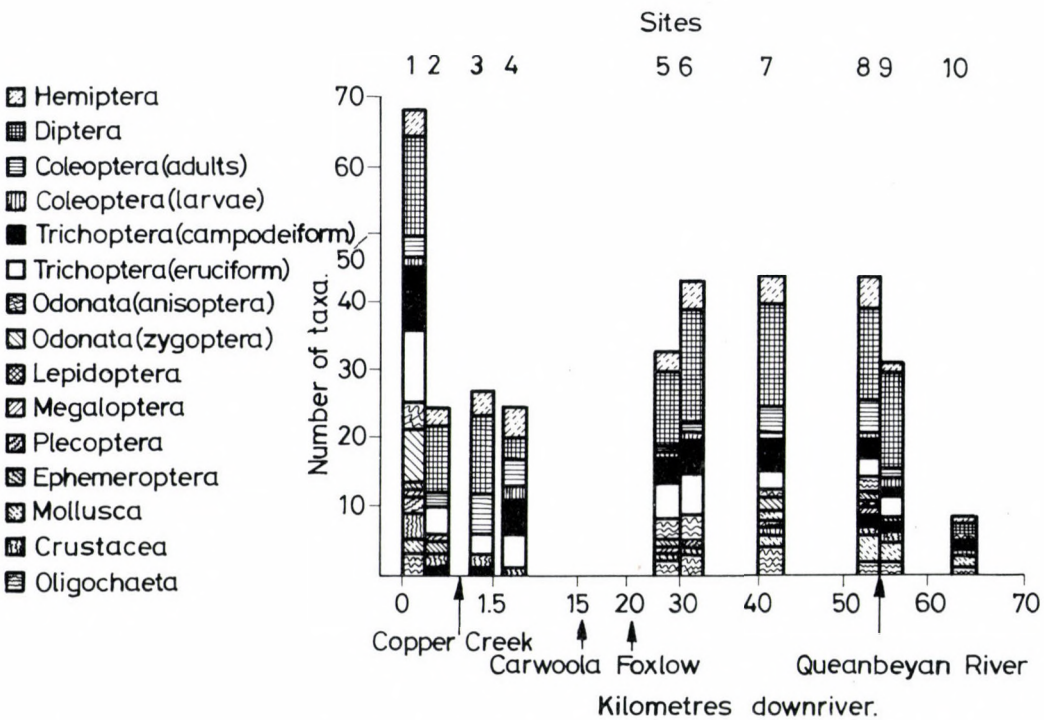

Fig. 2. Groups of taxa collected from sites in the Molonglo River 1982

Zinc concentrations in the Molonglo River may vary by a factor of 5 or 6 times over a period of a few days or less /1, 12, 18/. For this reason both loads and potentially lethal concentrations of metals may be difficult to measure if they are used alone as a measure of water quality. The studies on the river by Weatherley et al. /18/, Nicholas and Thomas /14/, Norris /15/ and Graham et al. /6/ illustrate the importance of biological data as a basis of determining impacts from mine effluents.

There are some shortcomings in the biological data that need to be

considered and these have been reviewed by Norris and Georges /16/. Species identification, or at least identification to discreet taxa, is important in most studies of water quality that include examination of the biota /2, 22/. Species from one genus may respond differently to the same environmental stress /17/. The taxonomy of most freshwater invertebrate groups in Europe and North America is quite well known. However, the taxonomy of aquatic organisms in most of the rest of the world is not as well documented. Resh and Unzicker /17/ have documented an example of the value of museum collections of caddisflies dating back to the 1930s for assessing changes in the water quality of Lake Erie and the Rock River in the United States of America. following their advice Marchant et al. /11/ placed the animals collected during their study of the La Trobe River, Victoria Australia (a river suffering from a variety of coal mining, industrial and urban impacts), in a voucher collection at the Museum of Victoria. The major benefits of this collection are as follows (from Norris and Georges /16/).

i) As the taxonomy of the groups is completed, species names could be associated with the animals collected.

ii) After naming, and along with the extensive physicochemical data collected on study areas such as the La Trobe River, much information would be available on ecological tolerances at the species level.

iii) The specimens would be available for future studies, enabling accurate assessment of temporal changes.

iv) the collection will be an aid to taxonomy in future studies.

Specimens from the Molonglo River from the study of Weatherley et al. /18/ were stored for some time in the Zoology Department of the Australian National University. Unfortunately, they were discarded before the reassessment study by Norris /15/ and several questions arising from the latter study, which might have been easily addressed by comparisons with the original collections, remain unanswered. It is not sufficient merely to make the collections; they must be housed where they will be appropriately curated in the long term /16/. The costs of future studies might be substantially reduced by adequate curation of specimens allowing the study design to be simplified and aiding species identification. Resh and Unzicker /17/ suggested that at least some of these cost savings might be passed on to cover curatorial costs.

One of the major problems with chemical data collected over long periods and throughout long-term studies is that samples cannot be stored

and methods and apparatus for analysis change with technological advances. Such changes may invalidate some statistical tests and confuse interpretation of the data. Weatherley et al. /18/, Nicholas and Thomas /14/, Millington and Walker /12/ and Norris /15/ all used different methods for measuring zinc concentrations in the Molonglo River and so the validity of comparing chemical data was limited. The biological methods of Weatherley et al. /18/ were repeated by Norris /15/ but not by Nicholas and Thomas /14/ in their studies of the Molonglo River. Therefore Norris' study could be directly compared with that of Weatherley et al., though it was unfortunate that the original collections had been discarded. The components of any long term study must always be kept constant, or at least if methods are changed any differences because should be assessed and correction factors calculated. If biological collections are stored appropriately, they will always be available for reference, whereas no such possibility exists for water samples collected for chemical analysis.

Nicholas and Thomas /14/ studied the biological release and recycling of toxic metals from lake and river sediments and they concluded that "so long as the river water contains high levels of zinc it will accumulate through biological action in the sediments and be redistributed by both plants and animals". Based on this conclusion it may be doubted whether the Molonglo River can be restored to a viable condition in terms of the benthic invertebrate and fish populations, given that much of the source of the pollution is present both within the river and the surrounding catchment /14, 15, 18, 19, 20/.

Conclusions relative to rehabilitation objectives

The remedial measures at Captains Flat have probably prevented the possibility of another major collapse of waste dumps and significant erosion of the dump surfaces.

Under low flow conditions the quality of water available for domestic supply, agricultural irrigation and stock watering has hardly been changed. Under high flows the quality may have been improved, largely because of the reduction in erosion of the mine dumps. Unfortunately remobilization of previously deposited materials within the river and its flood plain during higher flows may mean that little overall improvement in water quality is seen.

The rehabilitation measures have failed to upgrade the biological and aesthetic quality of the Molonglo River.

Break down of the dump capping, erosion, springs seeps and the continued pyrite oxidation all lead to ongoing pollution from the mine site and the need for maintenance and upgrading of the work.

REHABILITATION CONSIDERATIONS

The Captains Flat rehabilitation programme has not been successful in relation to all but the first of the stated objectives. Rehabilitation works with many similar components to Captains Flat example have been undertaken at the abandoned Rum Jungle Uranium Mine in Northern Australia. The government is undertaking these works which are costing about $ 20 million in total.

Other uranium mines in northern Australia have recently started operations. Controls over the operations of these mines have been the subject of the most detailed public environmental and social assessment ever seen in Australia. The costs and procedures for rehabilitation have been included in the agreements covering the mine operations. For the initial years of operation the Ranger Uranium Mine has been operated without any release of contaminated water. Despite all of the controls over operations the company is now arguing for the release of water contaminated at low levels by radionuclides and trace metals. One of the reasons being used in favour of the release is that it will be less harmful if it is done in a controlled manner during the wet season, rather than an uncontrolled release through the collapse of one of the tailings dams. This situation seems to have arisen because of miscalculations in the amount of water likely to come into the site in wetter years. Even under the best controlled conditions environmental problems are still possible.

Several aspects that must be considered when making decisions on mine rehabilitation need to be emphasized.

a) The problems at Captains Flat exist largely because strict environmental controls were not imposed while the mine was operating. This is true of many mines operating around the world and even where they are in place problems may still occur. The costs of rehabilitation may be a substantial cost of the operations (2% of sales are allocated to rehabilitation at Ranger Uranium Mine).

b) Catastrophic releases of mine waste effluents at Captains Flat have left considerable quantities of toxic materials spread widely in the

environment. These materials have been added to by the release and build-up of metals from the mine operations since they began in 1882. Biological studies have indicated that remobilization of these sources may be a serious problem but the conditions and mechanisms of their remobilization are poorly understood. Objectives for target concentrations of metals to be achieved do not consider the problem of long-term accumulation and possible subsequent release. Unless it can be shown that contaminants are entering a permanent sink, target concentrations should probably be divided by a time factor.

c) Pyritic oxidation is greatly enhanced by biological processes. These processes need better site specific understanding if the right rehabilitation strategies are to be implemented.

d) One aim of the rehabilitation of the Captains Flat mine was supposed to have resulted in biological improvements being achieved in the river. Apart from descriptive work, biologists were hardly consulted and they played virtually no role in the decisions on the rehabilitation strategies to be employed. Loads of contaminants to the Molonglo River probably have been reduced through the necessary first steps of control of erosion and prevention of dump collapse. However, it is clear that lethal concentrations, or unfavourable conditions related to metals, still existed at the time of the study by Norris /15/. Biological measures of the impacts of mine effluents summarize the foregoing conditions and they should be incorporated with equal weight to chemical measures into decisions on mine rehabilitation.

e) The components for long-term studies that are designed to assess the effectiveness of rehabilitation strategies should remain constant. Species identification and curation of the collections in museum collections will aid future studies directly and be valuable for comparisons for changes over long periods.

ACKNOWLEDGEMENTS

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ATTACHED ALGAL FLORA IN THE RIVERS RECEIVING EFFLUENT FROM COPPER MINES IN JAPAN

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INTRODUCTION

Attached algal flora is affected with heavy metal contamination as reported by many authors /5, 6, 7, 9, 11, 12, 13/. In most cases, the number of species is reduced with elevated concentrations of heavy metals. However, the species specific to the contamination is not necessarily consistent. Does this mean the local variation of the study sites although the algal species are cosmopolitan? It is also probable that the variation in the composition of heavy metal species in rivers causes such local variation of the flora, since mine wastes contain many kinds of metals which may interact with each other on the toxicity to aquatic organisms. Environmental conditions such as pH and temperature varying from site to site may affect the solubility and toxicity of heavy metals as well. Therefore, more information is needed even though there seems to be many reports.

There are many copper mines in Japan which have stopped mining activity for years. For example, about 80 such mines are distributed in a valley of the Kosaka district, northern Japan, only two of which are in operation now. Such closed mines are major sources of heavy metal contamination to rivers. The effluent from such mines, usually as small streams running through a slag pile or ground water, contains not only copper but also cadmium and zinc which are toxic to most aquatic organisms.

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Akadémiai Kiadó, Budapest

We have studied the flora and fauna in more than 15 rivers polluted with heavy metals in Japan. The present paper deals with a part of the studies, particularly in northern Japan.

The methods of sampling and examining attached algae were described in our previous paper /2/ and the procedure of analysis of heavy metals in water and algal cells was described in Hatakeyama, et al. /3/.

1. Community indices and specific species in the rivers polluted with heavy metals

As noted by Hynes /4/, the number of species tolerable to pollution by toxic substances decreases with the increase of the concentration. There is a clear tendency that the number of attached algal species decreases in proportion to the increase in the copper concentration in rivers (Fig. 1).

Similar tendency can be recognized in the relationship between the number of species of macrozoobenthos and the electric conductivity representing the heavy metal concentration. Since the number of species is an important parameter in determining the diversity of the community, we may find a exponential relationship between the electric conductivity and diversity index (H') for diatoms and macrozoobenthos, respectively (Fig. 2). In general, the diversity index seems to indicate the degree of the heavy metal contamination.

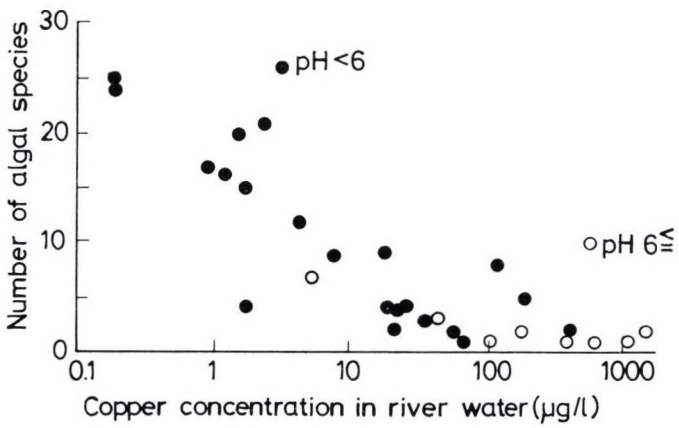

Fig. 1. Relationship between the number of algal species and copper concentrations in river water

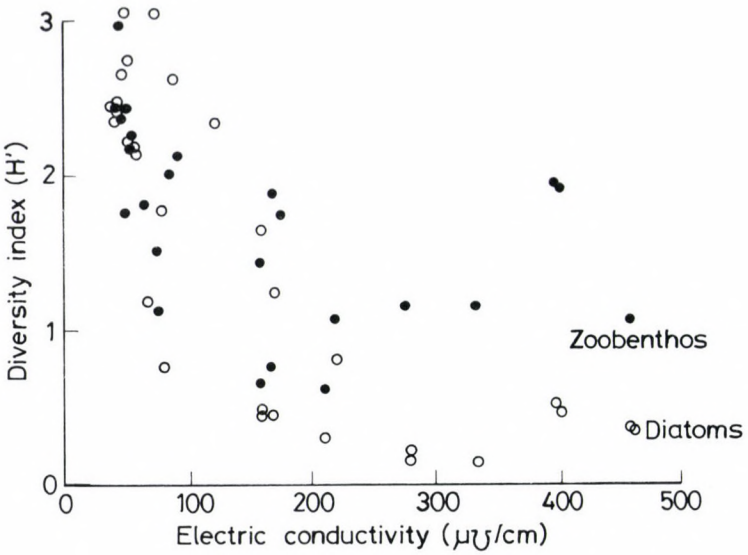

Fig. 2. Relationship between electric conductivity of water and diversity of macrozoobenthos and diatom community

In respect to individual species tolerable to heavy metals, we have found several specific species of chironomids (Diptera, Insecta) /15/. Since these species have reached unusually high density in the contaminated site, they will never be missed. Baetis thermicus (Ephemeroptera, Insecta) is also found in the polluted rivers but is not as tolerable as chironomids. This species is a peculiarly efficient accumulator of heavy metals, particularly cadmium. Its tolerance mechanisms seem to be different from that of chironomids.

As for attached algae, Achnanthes minutissima is most common in the heavy metal-polluted rivers in Japan. This species is not necessarily specific, but flourish in the polluted river and as a result, the percentage of this species of total diatoms may indicate the pollution of heavy metals (Fig. 3). Regarding other taxa, Phormidium luridum and Chamaesiphon minutus of blue-green algae appear to be tolerable to heavy metal pollution. Table 1 shows the mean and standard deviation of copper concentrations and pH in the rivers of the Wakayama District, Japan, where the respective species of attached algae were collected. The appearance of two species of green algae are characteristic to the high concentration of copper together with low pH. The acidic condition strongly influences the toxicity of heavy metals and their

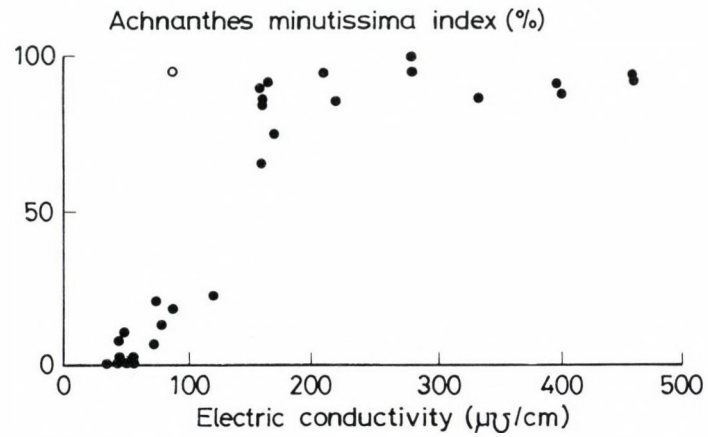

Fig. 3. Achnanthes minutissima percentage of total diatoms at various sampling sites with different conductivity values in the Kosaka River

multiple effect may form different species composition /1/. Achnanthes minutissima and Phormidium luridum are not tolerable to low pH. Most of the other species are neither tolerable to high concentration of copper nor to low pH. Table 2 is the summary list of the typical attached algae in the rivers studied. Hydrurus foetidus is also a typical tolerant species. The environmental conditions in a reservoir of the Yoshino River are extraordinarily severe, but quite different from other places since the leaf litter accumulated at the bottom of the reservoir may become a carbon source.

Table 1

Mean copper concentration and pH at the locations where the respective attached algae were collected

Species	Number of locations	Concentration of copper	pH
Chamaesiphon minutus	20	31.2±55.4	6.7±0.5
Homoeothrix janthina	11	2.2± 2.1	7.0±0.2
Achnanthes japonica	11	1.1± 1.1	7.1±0.1
Chmaesiphon polymorphus	8	6.8± 9.4	7.0±0.3
Phormidium luridum	12	91.3±74.7	6.2±0.6
Achnanthes microcephala	4	0.4±0.3	7.1±0.1
Achnanthes minutissima	13	143.3±201.3	7.4±0.1
Cymbella turgidula v. nipponica	5	0.2±0	7.1±0.1
Gomphonema tetrastigmatum	9	1.5±1.4	7.2±0
Synedra ulna	5	0.4±0.3	7.1±0.1
Hormidium sp.	4	611.0±422.8	4.0±0.4
Microsphora sp.	4	214.1±264.4	5.7±0.8

Table 2

Typical algal species in the river contaminated with copper in Japan

River	Copper conc.	pH	Typical species
Kosaka R.	under 80 ppb	above 6.0	Achnanthes minutissima, Hydrurus foetidus, Chamaesiphon minutus
Kakehashi R.	under 5300 ppb	above 6.2	Phormidium luridum, Achnanthes minutissima
Jinryuu R.	under 180 ppb	above 7.0	Achnanthes minutissima
Mazawa R.	under 190 ppb	above 6.4	Achnanthes minutissima, Phormidium luridum, Chamaesiphon minutus, Hydrurus foetidus
Senasaka R.	under 620 ppb	above 6.1	Achnanthes minutissima, Ochromonas sp.
Yoshino R.	under 640 ppb	above 6.0	Achnanthes minutissima, Ochromonas sp., Chamaesiphon minutus, Hydrurus foetidus
Yoshino R.	270 ppb	4.7	No algae
Yoshino R.*	6200 ppb	2.6	Euglena mutabilis, Pinnularia braunii, Cyanidium caldadium
Kakehashi R.	360 ppb	under 3.5	Hormidium sp.
Senasaka R.	1070 ppb	6.0	No algae

*A reservoir

2. Continuous observations on the flora in a polluted river

Most studies on flora and fauna in a river are based on seasonal, or at the most, monthly observations. However, the heavy metal concentration in a river varies day by day and the occasional high concentration which is often missed, would affect the flora. For this reason, we have monitored the heavy metal concentrations together with flora and fauna in a river on weekly basis throughout a year /2/, Hatakeyama et al.,(unpublished). The Mazawa River is situated at the Yamagata Prefecture of northern Japan. The average discharge is approximately 0.6 m^3/s, but there is a seasonal change which influences the algal standing crop; it rises in April and May due to snow melting and falls in August and September. Water temperature was 0.5–2.0°C in the winter and 15 to 22°C in the summer, and the pH remained around 6.5 except in May 1983, when it was 5. The concentration of heavy metals in the water rose in May and June when the discharge was high (Fig. 4). The cadmium concentration was 18 μg/l at the maximum and 7.6 μg/l on the average. The concentration of copper was nearly 200 μg/l at a peak, after that remaining around 50 μg/l till November when it slightly rose again. The zinc concentration was 1140 μg/l on the average and reached 2500 μg/l at the maximum. The high concentration of heavy metals in May and June might be ascribed to the flush washing the sediment.

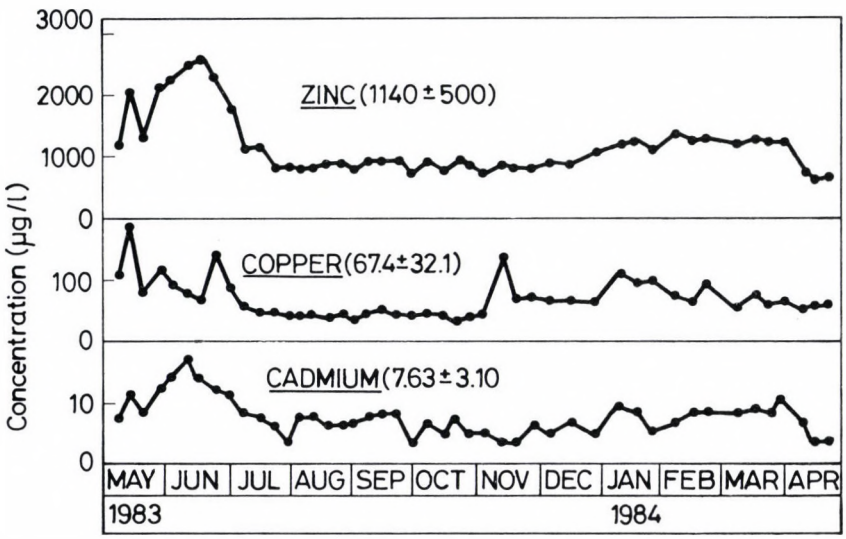

Fig. 4. Seasonal changes in the concentrations of zinc, copper and cadmium at a fixed station in the Mazawa River (mean+S.D.)

The seasonal changes of the standing crop in the main species of attached algae are shown in Fig. 5. The flora in May and June was rather poor, possibly because of the turbidity. Phormidium luridum appeared first and dominated in June and July. Achnanthes minutissima increased in July and predominated in most of the months, though fluctuating. Chamaesiphon minutus was found with low density almost every month, while Hydrurus foetidus appeared only in the winter. Ochromonas sp. Phormidium sp. and Pluerocopsa fluviatilis appeared occasionally. A total of 22 species was recorded from this particular site contaminated with heavy metals over a one-year period.

3. The succession of attached algae on the gravel exchanged between polluted and non-polluted rivers

The flora on gravel in the heavy metal-polluted river was simple. These species are certainly tolerable to pollution and are able to grow in non-polluted rivers, however, they must be less competitive in the rivers with no stress. To clarify this hypothesis, an experiment of gravel exchange between a polluted and non-polluted river was conducted.

The Mazawa River (Ma) is polluted with heavy metals (Zn: 1142 $\pm$ 502 μg/l, Cu: 67.4 $\pm$ 32.1 μg/l, and Cd: 7.63 $\pm$ 3.10 μg/l) and the adjacent Mizusawa River (Mi) is a non-polluted river (Zn: 2.2 μg/l, Cu: 5.6 μg/l, and Cd: 0.31 μg/l). the gravel collected from the riffles of each river was placed on a stainless steel tray and set in the other river. The flora on gravel in the tray was examined in the two rivers on the 7th day and then 14 days later (Table 3). The floral change could be recognized a week after. On the gravel transferred from the polluted to the non-polluted river, five species of diatom appeared on week later and 10 species 2 weeks later. The predominant species, Phormidium luridum and Achnanthes minutissima did not decrease for one week (actually increase), but decreased the next week. In the opposite transference, the number of species decreased from 16 to 13 within a week and to 9 two weeks later. Homoeothrix janthina, the predominant species in the non-polluted river disappeared soon. Another dominant species, Ochromonas sp. had persisted 2 weeks after transference to the polluted river. The appearance of Phormidium luridum and Achnanthes minutissima was fast. They became predominant species on the gravel two weeks after transference. Chamaeosiphon minutus seems to be a non-polluted river species, but seems to be able to grow in polluted rivers as well. The two species of green algae found in the non-polluted river must be quite susceptible to the heavy metal pollution.

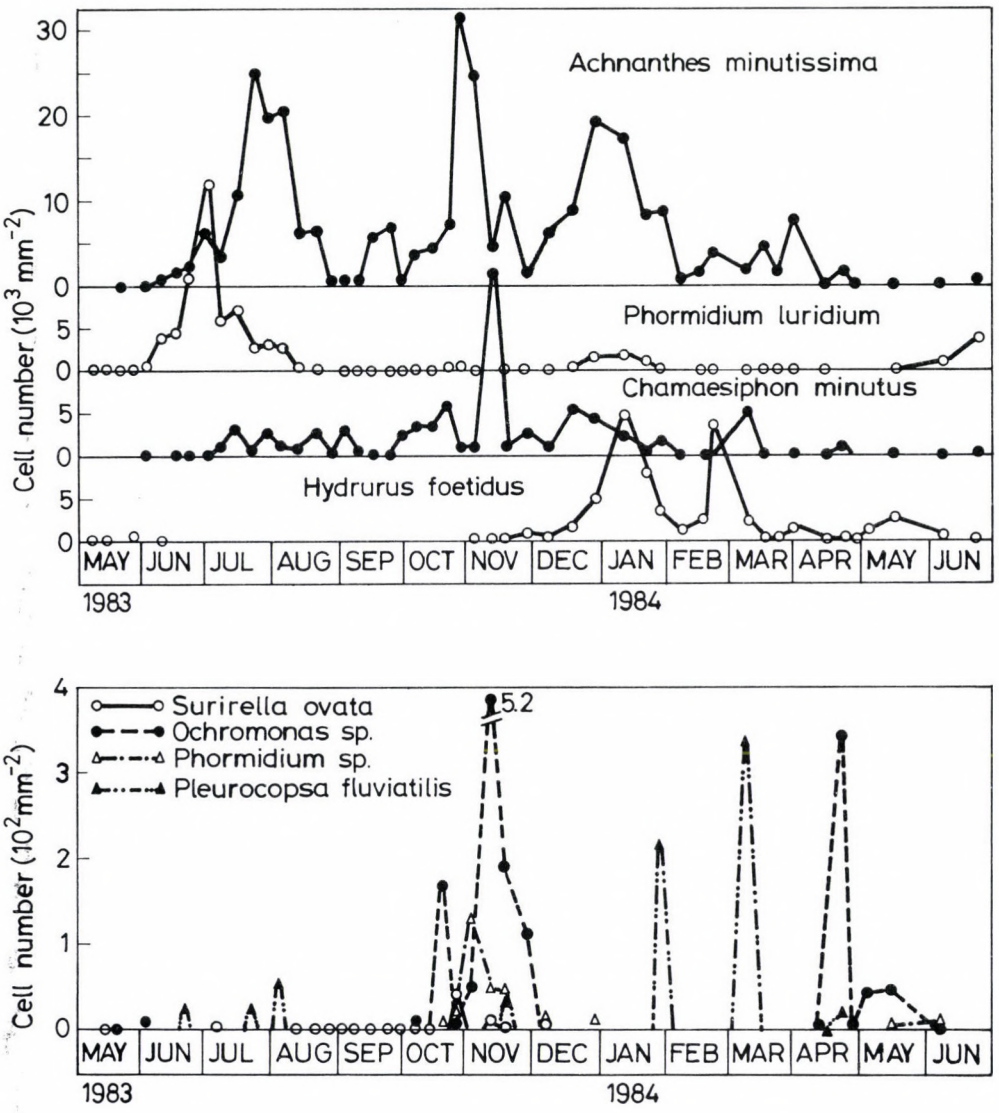

Fig. 5. Seasonal changes in the standing crop of attached algae at a fixed station in the Mazawa River

Table 3

The change in the algal species on gravel transferred from a metal-polluted river to a non-polluted river and vice versa

Algal species	Rivers	Ma ⟶ Mi			Mi ⟶ Ma		
	Week	0	1	2	0	1	2
Chamaesiphon minutus		**	*	***	***	**	*
Homoeothrix janthina					****		
Phormidium luridum		****	****	**		***	****
Hydrurus foetidus				*	***		**
Ochromonas sp.				*	****	****	***
Achnanthes lanceolata					**	*	
Achnanthes minutissima		***	***	**		**	***
Ceratoneis arcus			**	*	**		*
Cymbella sinuata			*			*	
Cymbella turgidula v. nipponica			*	*	**		**
Cymbella ventricosa				*	**	*	**
Diatoma hemale v. mesodon					**	*	
Gomphonema devei v. javanica			**	*	***	**	*
Gomphonema separatipunctatum					**	*	
Navicula cryptocephala			*	*	**	*	
Navicula sp.				*			
Nitzschia dissipata				*			
Nitzschia frustulum v. perpusilla					**	**	
Rhoicosphenia curvata					**	*	
Surirella linearis				*			
Geminella sp.					***		
Ulothrix sp.					**		

Ma: The River Mazawa, polluted with heavy metals

Mi: The River Mizusawa, adjacent non-polluted river

Number of cells/mm^2: * $\prec$ 100, ** 100 $\sim$ 1000, *** 1000 $\sim$ 10000, **** 10000 $\prec$

4. Attached algae on artificial substrates

Various types of artificial substrates have been developed to study the flora in rivers. The most common are the glass slides or plastic sub-

strates. Ungrazed tyles are also used since they are more similar to natural substrates /14/, but it is not recommendable to place glass slides or any other artificial substrates at the bottom of a river when the water level is verying or the turbidity is high. In the present study, therefore, polyethylene bottles on strings were floated in the middle of the rivers as artificial substrates. The bottles were placed at three stations from the upper to lower reaches of the Mazawa River and the Yoshino River, repsectively. Both rivers were receiving heavy metal effluent but the upper reach station in the Yoshino River had not been polluted. A total of 34 species were found on gravel at this control station and 37 species were from the artificial substrates 40 days after placing. However, the flora were quite different from each other. At the other stations, the number of species on natural substrate was limited and the flora colonized on the arificial substrate for 40 days were similar to those on gravel. There were some differences in the similarity between the substrates among the stations. The values of the similarity index (Morishita's $C\lambda$) were high at the two stations in the Mazawa River, whereas those in the Yoshino River were lower. Fig. 6 indicates the similarity between the flora on the natural substrate and the artificial one at the sites with different diversity values. The flora on the artificial substrate may represent more precisely the natural one when the flora is simple.

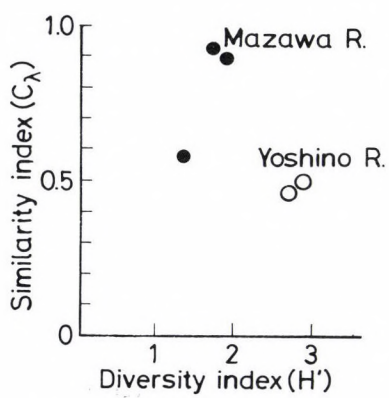

Fig. 6. Similarity between the algal flora on artificial and natural substrates in the Rivers Mazawa and Yoshino where the diversity of flora differed

The heavy metal contents of these attached algae are illustrated in Fig. 7. There is a clear correlation between the contents of the algae on

the natural substrate and those on the artificial one. In respect to the heavy metal contents, the attached algae colonized on the artificial substrate for 40 days accumulated them likewise as the natural one did. Nevertheless, further studies are necessitated to determine the appropriate exposure period. It must be noted that the concentration of heavy metals in the algal cells was considerably high in the present study. Algae might not necessarily take up such amount of heavy metals and some might remain on the surface of the cells despite careful washing with EDTA. Nevertheless, it is noteworthy that the content of heavy metals in attached algae correlated well with the concentration of those in river water /3/.

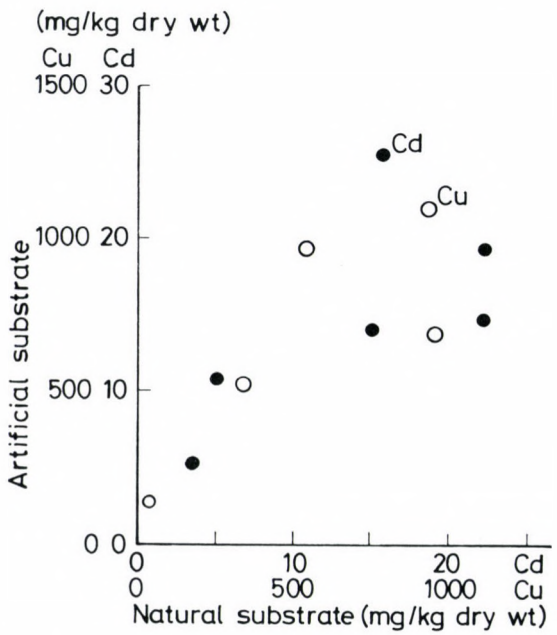

Fig. 7. Comparison of heavy metal contents of algal cells on natural and artificial substrates

DISCUSSION

Most studies so far have shown no specific species of algae adapting heavy metal contamination as found in chironomids of insect. Achnanthes minutissima is a common species in non-polluted streams both in Europe and North America /8, 9, 11/. This species can also be found in non-polluted rivers in Japan, but it has never reached high density. Therefore it is

noteworthy that the marked increase of this species in the heavy metal-contaminated rivers is a common phenomenon in Japan as reported in the present study. The increase of Achnanthes minutissima contrasted with the reduction of the number of diatom species. Say /10/ reported the predominance of Gomphonema and this species in the river contaminated with zinc and cadmium. Leland and Carter /5/ reported that only Achnanthes minutissima responded to the Cu-enrichment in an experimental study in a creek. In the River Sasu, Tsushima, Japan, where the zinc concentration in the water was high, but the copper was negligible, Chamaesiphon minutus and Stigeoclonium sp. were dominant and Achnanthes minutissima was rare (Fukushima, unpublished data). Thus, we may assume that Achnanthes minutissima is especially tolerable to copper and possibly cadmium compared with other species (Hormidium luridum is also a tolerant species). Zinc is not so toxic to most species as copper, therefore other species may overcome Achnanthes minutissima in competition in rivers contaminated with zinc alone, or in non-polluted rivers. In fact, the growth of Achnanthes minutissima was not inhibited by up to 10 μM of copper if EDTA was present (Takamura personal communication). This concentration is about 10 time the average concentration observed in our study sites.

The heavy metal content of attached algae on artificial substrates could be used for monitoring the contamination level /12/. The present study also indicates that the algae on natural as well as artificial substrates take up copper and cadmium in proportion to the concentration of the metals in the water.

The present study also points to the seasonal variation in the discharge of heavy metals in the river water, resulting in the changes in flora. The low water temperature also affects the species composition. Namely, Hydrurus foetidus, known as a species adapted to low temperature, appeared in the water of the polluted river. It is also conceivable that the toxicity of copper has been reduced with low temperature /7/.

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DISTRIBUTION OF LEAD AND CADMIUM IN TROPHIC LEVELS OF SOME MARINE ORGANISMS

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The concentrations of lead and cadmium in two species of algae, two species of invertebrates, and one species of fish (from Fehmern Baltic Sea) were determined using atomic absorption spectrophotometry. The concentrations of these metals at the producer level (algae) were always higher than at the consumer level. Also, among the consumers there was an obvious difference according to feeding habits, in such a way that they could be ranked according to their high content of lead and cadmium as follows: filter feeder (detritous feeder), plankton feeder. In addition, distribution of lead and cadmium varied within the individual producer (Fucus vesiculosus) in such a way that the holdfast exhibited the highest concentration followed by the apcial tip and the branches of the first dichotomy was the lowest. This alga reflects clear selectivity in absorbing more lead than cadmium.

INTRODUCTION

There has been increasing concern recently about the rising quantities of heavy metals such as cadmium, lead, zinc and copper in river waters of the North Sea /12, 20, 26, 29/. More attention has been paid to heavy metal concentrations in the Baltic Sea /17, 26, 28/ and to those in the North Sea or seawaters near the British islands and the German coasts /1–3, 10–13, 22–26/.

Various investigators have reported about concentrations and determinations of heavy metals in marine macro-algae /4–9, 14–16, 18–21, 27, 29/

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which were mostly collected from the British coasts. However, none of these investigations deal with concentrations of heavy metals in various trophic levels at any of the above localities.

In the present study data are presented on lead and cadmium concentrations which were found in some organisms at various trophic levels collected from Fehmern (Baltic sea proper) and Helgoland (North Sea). The data shed some light on the level of heavy metals at various trophic levels.

MATERIALS AND METHODS

Materials

Samples of the 3 species of algae: a) Fucus vesiculosus (from Helgoland), b) Enteromorpha intestinales and c) Elachista fucicola; the mollusc Mytilus edulis, the shrimp Gammarus locusta and the fish Clupea harengus were collected from Fehmern island in the Baltic sea. The samples were transported on ice to the laboratory and studied as described under methods. All chemicals used were of reagent quality.

Methods

Whole animal samples and pieces of the algae were lyophylized separately using a Christ lyophylizer (Model Beta I). 30 mg of the lyophylized algae samples and the whole lyophylized animal samples were digested in perchloric acid for 10 h at gradual increments in temperature from 70 to 180°C. After digestion the material was diluted with glass distilled water (4 x – distillation). Then the lead and cadmium contents were determined by the standard addition method using a Hitachi – atomic absorption spectrophotometer (model –180 – 70) fitted with a Hitachi – hollow cathode lead or cadmium lamp. Results are expressed in ppm on dry weight basis.

RESULTS AND DISCUSSION

The concentrations of lead and cadmium in two types of algae, two invertebrates and one species of fish are given in Table 1. Three different levels of lead concentrations are shown in this table: a) highest level is exhibited among algae; b) intermediate level is exhibited among the species Mytilus edulis and Gammarus locusta, and c) the lowest level is seen among the fry (young fish). The concentrations of cadmium also reflect three different levels similar to that for lead except in the mollusc Mytilus edulis which contains higher cadmium concentration than the alga Elachista fucicola. The lead concentration always exceeded the cadmium concentration in the same species of algae or animal.

Table 1

Concentrations of lead and cadmium in algae and animals collected from Fehmern

Organism	Lead (ppm) dry weight of tissue	Cadmium (ppm) dry weight of tissue
CHLOROPHYCEAE		
Enteromorpha intestinalis	7.547 ± 0.072	0.947 ± 0.024
PHAEOPHYCEAE		
Elachista fucicola	8.1024 ± 0.036	0.2256 ± 0.017
*MOLLUSCS		
Mytilus edulis L. b	2.017 ± 0.086	0.368 ± 0.057
*CRUSTACEAE		
Gammarus locusta b	1.816 ± 0.34	0.0915 ± 0.027
**PISCES		
Clupea harengus c	0.5413 ± 0.027	0.0736 ± 0.0134

*values are mean ± S.D of three animals

**values are mean ± S.D of four animals

Table 2

Distribution of lead and cadmium in Fucus vesiculosus

Material assayed	Lead (ppm) dry weight of tissue	Cadmium (ppm) dry weight of tissue
*Holdfast	1.294 ± 0.22	0.47 ± 0.34
*Branches of the first dichotomy	0.869 ± 0.074	0.469 ± 0.27
*Receptacles (apical tip)	1.096 ± 0.017	0.675 ± 0.142

*each value is the mean of six plants ± S.D.

These results indicate that the producer trophic level always contains the highest concentration of lead and cadmium. Although the consumer animal (Primary) contains lower concentrations of lead and cadmium, there

is an obvious difference according to the feeding habit. Mytilus edulis, a filter feeder, has higher concentrations of lead and cadmium than the detritus feeder crustacean Gammarus locusta, which in turn contains more lead and cadmium than the plankton feeder fish Clupea harengus.

The distribution of lead and cadmium within the individual organism was studied using Fucus vesiculosus from Helgoland. The results in Table 2 show that the highest concentration of both metals occurred in the holdfast (rhizoid) and the apical tip (receptacle). Branches of the first dichotomy (middle part of the thallus) contain lowest level of cadmium which is intermediate between the holdfast and the apical tip. Although the branches of the first dichotomy (BFD) contain intermediate amounts of lead and cadmium compared to the holdfast and apical tip, the ratio of heavy metals in this region compared to the other two regions is quite different. While the BFD: holdfast ratio for the lead is 67%, the same ratio for cadmium is 32%. On the other hand, the BFD: apical tip ratio for cadmium and lead is almost identical.

The results indicate that the holdfast of Fucus vesiculosus tends to accumulate more lead and cadmium than the rest of the thallus. This could be due to the contact of the holdfast to the bottom sediments which usually contain more heavy metals than the water. A similar result was reported by James et al. /18/ for distribution of mercury in this alga. In addition, the results also indicate selectivity of this alga in absorbing more lead than cadmium. Thorell /28/ has also reported a higher affinity of Fucus alginate to lead than to cadmium.

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THE CONCURRENT GROWTH OF PLANTS AND CHEMICAL PURIFICATION OF WASTEWATER USED AS A HYDROPONIC UNIT

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In this study the seedling of a variety of plants were successfully grown hydroponically on raw wastewater obtained from one of the main sewer outfalls in Beirut.

In the first phase, a series of experiments was run on a batch system in glass or plastic containers provided with aeration facilities. A continuous-flow system with recirculation was adopted in the second phase. Iron supplementation was applied in all cases to compensate for its deficiency in the raw wastewater used.

The immediate and ultimate objectives of the project were threefold: (a) to demonstrate the feasibility of utilizing as a hydroponic medium untreated municipal wastewater having relatively high mean values for BOD and mineral content; (b) to achieve the growth of useful plants on such readily available hydroponic media, thereby saving on fertilizers and scarce water resources; and (c) reclamation of the wastewater through biological purification leading to the gradual depletion of the nutritive constituents.

Experimental conditions are described, and the data presented leads to the conclusion that the system is practicable on a laboratory scale. It has great potential for trial on a pilot scale prior to field applications in developing countries suffering from water shortage and hard currency expended on imported fertilizers and wastewater purification facilities.

Keywords: Wastewater purification – hydroponic system – plant growing

INTRODUCTION

Previous hydroponic studies with secondary treated wastewater centered on demonstrating the feasibility of growing commercial crops in

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green-house experiments using secondary wastewater effluent. These showed that the growth medium was a satisfactory source of nutrients except iron /1/. Iron deficiency could be corrected by the addition of insoluble Fe-Fe oxide, or soluble Fe chelate, applied to plant roots /2/. Commercial scale production of cucumbers, tomatoes, and chrysanthemum was achieved in (9.2 x 9.2 m) green-houses /1, 3/.

Studies based on continuous flow of the growth medium were also conducted to determine the optimum flow rates required for maximal yield of both plant materials and fruits /4, 5/.

Plants grown hydroponically in secondary treated effluent with added iron accumulated Ca, Mg, K, Na and B in the fruits. The trace elements contained in the effluent were found to accumulate outside and/or inside the roots, with little translocation to arial portions, and much less to fruits /4, 6/.

The present study aims at determining the feasibility of utilizing raw wastewater as a hydroponic medium for its reclamation and reuse, as well as for growing useful plants.

MATERIALS AND METHODS

Preparatory Work

The initial study was devoted to the assessment of the physico-chemical quality of raw wastewater collected for experimentation. Accordingly, hourly, daily, and monthly fluctuations were recorded, and 9:30 a.m. was selected as an appropriate time for the collection of the samples with average physico-chemical composition for experimentation.

Phase One – Hydroponic Batch System

Plant growth in untreated wastewater was then studied. Laboratory experiments designed to assess the feasibility of attaining the crop production stage were conducted. The wastewater medium used throughout the experiments was fortified by the addition of 2.5 mg/l Fe chelate as Fe EDDHA* (ethylene diamine di-o-hydroxy phenylacetic acid). The experimental plant species grown hydroponically included the following:

- cucumbers (Cucumis sativis cv. "Toska hybrid" and Asgraw Beit Alpha)
- tomatoes (Lycopersicon esculentum cv. "Tropic" and "Local")
- beans (Phaseolus vulgaris cv. "Improved Tender Green"),
- celery (Apium graveolens cv. "Rapaceum")
- pepper (Capsicum trutescens cv. "Floral Gen")

*Ciba-Geigy, Basel, Switzerland

- lettuce (Lactuca sativa L. cv. "Local")
- carnations (Dianthus caryophyllus cv. "Lener", "Scania", "Tangerine", "Arthur", "Majestic", "Flame".

The experimental plant seedling swere transplanted in 20 l capacity glass or plastic containers for periods ranging from 4 to 8 weeks. The wastewater media used in fruiting experiments were changed at average intervals of 3 to 5 days. Changes in certain physico-chemical parameters were monitored at intervals of 3 days. Controls utilizing Hoaglands nutrient solution /7/ were conducted simultaneously.

The effect of plant growth on changes in the physico-chemical quality of untreated wastewater used as a hydroponic medium was studied next. Toska hybrid cucumbers and tropic tomatoes grown hydroponically in 3.7 l capacity glass containers were utilized. As in the previous experiments the wastewater medium was fortified by the addition of 2.5 mg/l Fe chelate.

A few experiments were conducted in an attempt to identify the following factors that could influence the rate of reduction in the chemical constituents of the wastewater medium; plant species and variety, number and growth stage of seedlings, volume and aeration of wastewater medium.

Several macro-elements (NH_3, NO_3, Cl, Na, K, Mg, SO_4 and PO_4), and some micro-elements were monitored throughout these experiments. The physical parameters monitored included pH specific conductance, biological oxygen (BOD) and dissolved oxygen (DO).

Phase Two – Continuous Flow System

Continuous flow experiments were conducted to determine the relationship between the flow rates of the wastewater hydroponic media applied, and the reduction in its mineral content by the plants grown.

The continuous flow system used consisted of two units, each divided into two subunits with six compartments of 3.7 l capacity per compartment. Figure 1 shows the set up for the continuous flow system and the recirculation pattern for the wastewater hydroponic medium. Flow rates of 10, 20 and 30 ml/min were selected for experimentation on the basis of previously performed trials. These correspond to detention periods of 24, 26 and 72 h respectively. The values for pH, specific conductance, NO_3, K, Na and Ca were determined at 3 days interval.

Tomato plants (Lycopersicon esculentum cv. "Tropic") and cucumber plants (Cucumis sativis cv. "Toska hybrid" were used in these experiments. Either one type of plant was solely grown in both subunits of a hydroponic unit or the two kinds of plants were grown in series within the subunits indicated. An average of two seedlings were grown in each compartment of the continuous flow system.

RESULTS AND DISCUSSION

Composition of Raw Wastewater

The initial part of the study was devoted to the assessment of the physico-chemical quality of the raw wastewater collected for experimentation. Daily fluctuations in the specific conductance of wastewater collected between 6:00 a.m. and 6:00 p.m., resulted in a morning peak between

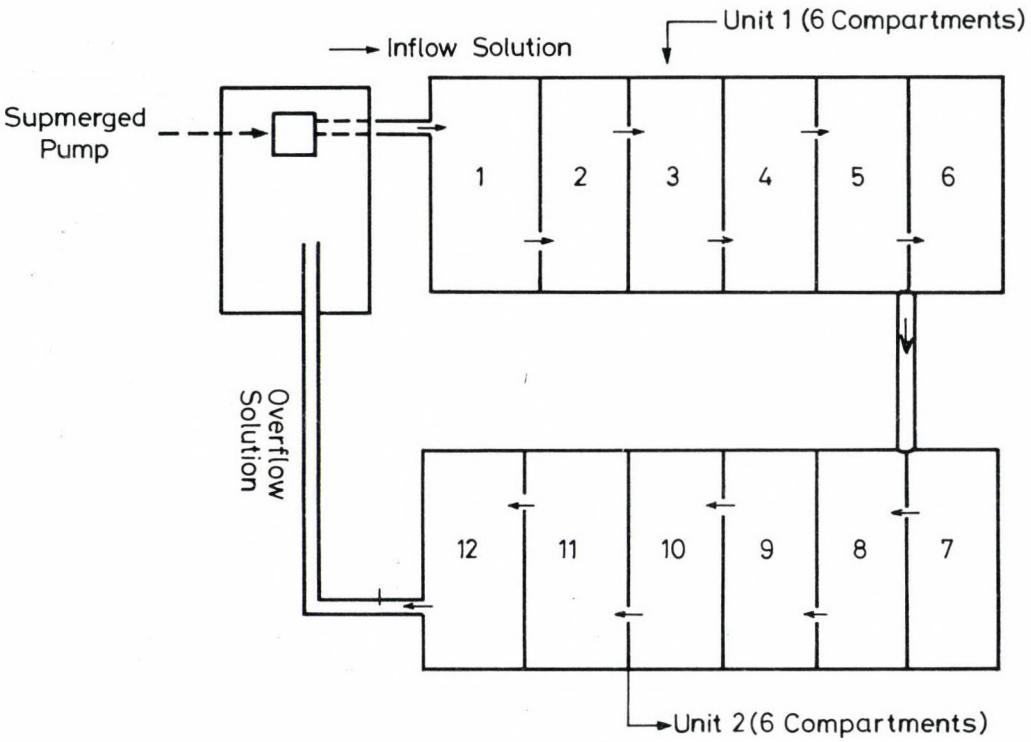

Fig. 1. Schematic diagram of a continuous flow unit

10:00 a.m. and 12:00 noon. Two smaller peaks also appeared around 2:00 p.m. and 5:00 p.m., respectively (Fig. 2). Accordingly, 9:30 was selected as an appropriate time for collection of wastewater samples with average composition for experimentation.

The specific conductance of the raw wastewater collected regularly for experimentation was about 1400 mhos/cm at 25°C. The pH values ranged between 7.50 and 8.60.

Chemical analysis of the raw wastewater used in this study as a hydroponic medium for plant growth are shown in (Table 1). The data showed only traces of Fe, low concentrations of heavy metals (Ar, Cd, Cr, Pb, Hg, Ni, Ag, Se), and relatively high concentrations of PO_4 and NO_3 (two major chemicals associated with eutrophication of surface water supplies).

The overall chemical composition except for (NH_4–N), of the wastewater used for experimentation is comparable to that of the secondary treated effluent used by Wallace et al. /3/ as a hydroponic medium for the growth of a variety of crops and ornamentals.

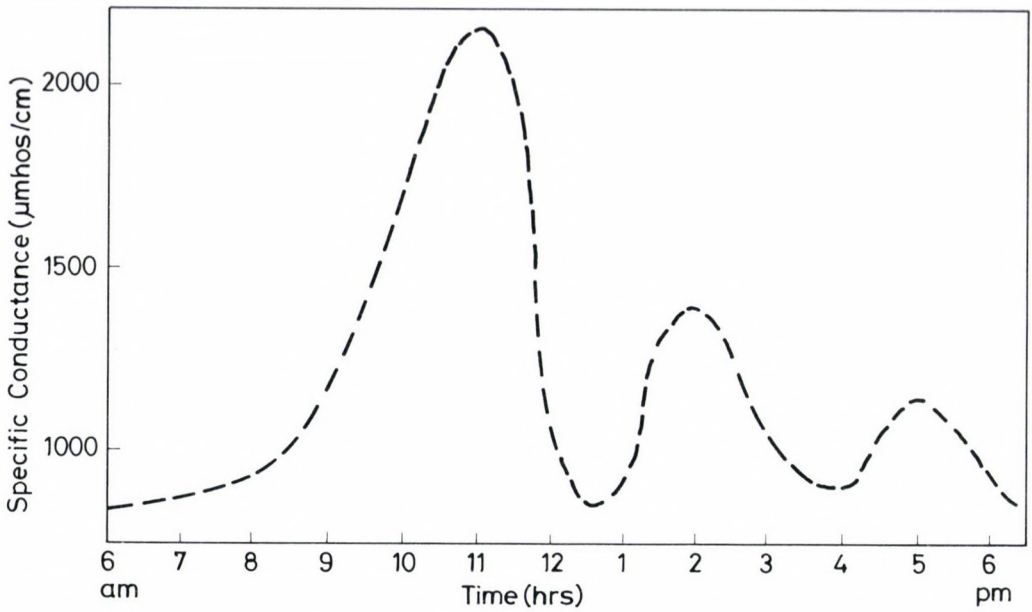

Fig. 2. Variation of the specific conductance of wastewater collected between 6:00 a.m. and 6:00 p.m. daily at different time intervals

Table 1

Chemical composition of raw wastewater collected daily between 6:00 a.m. and 6:00 p.m. at different time intervals[ab]

Chemical element	Concentration range mg/l	Chemical element	Concentration range mg/l
Boron	0.5–0.8	Arsenic	0.001–0.002
Calcium	60–80	Cadmium	0.002–0.005
Chloride	150–180	Chromium	0.006–0.009
Copper	0.06–0.08	Lead	0.02–0.04
Iron	traces	Mercury	0.0003–0.0005
Magnesium	25–35	Nickel	0.02–0.03
Manganese	0.15–0.17	Silver	0.0005–0.001
Nitrate-nitrogen	20–40	Selenium	0.015–0.025
Phosphorus	8–12		
Potassium	15–30		
Sulfur	60–90		
Zinc	0.80–0.90		

[a] pH ranged between 7.50 and 8.60
[b] Specific conductance ranged between 700 and 3200 µmhos/cm at 25°C

The overcome the deficiency in iron concentration, a vital parameter for plant growth, the raw wastewater was fortified with 2.5 mg/l of iron prior to use as a hydroponic medium for experimentation.

Growing Plants on Wastewater

The experimental plants were grown successfully on raw wastewater to various stages of their life cycles.

They were successfully grown to fruiting, flowering, or ultimately to the vegetative stage of their life cycle. This is in accordance with the findings of Berry et al. /8/, who were able to grow fruits and ornamentals on secondary treated wastewater effluents.

Local and tropic tomato varieties, Toska hybrid and Asgraw Beit Alpha cucumber varieties, Improved Tender Green beans, and local pepper were grown to fruiting on raw wastewater for period of 6–8 weeks. A yield of 11.5 kg/plant was reported for Toska hybrid cucumbers, and a crop yield of 5.50 kg/plant for Asgraw Beit Alpha cucumbers. Comparable yields for Toska hybrid cucumbers grown on secondary wastewater effluents were reported by Wallace et al. /3/.

Quality, as indicated by the texture of the crop and uniformity in size, was competitive. Results in this study also agree with those reported by Wallace et al /3/ who grew Marglobe tomatoes, Japanese F, eggplants, and California Wonder bell pepper, on secondary wastewater effluent.

Plants grown for the purpose of flower production were maintained on wastewater media for shorter periods of 5 weeks. Six varieties of carnations (Lener, Scania, Tangerine, Arthur, Majestic, Flame) were grown successfully. Similarly, Bright Golden Anne Chrysanthemum has been reported by Wallace et al. /5/ to be grown successfully on secondary wastewater effluent.

For the purpose of vegetable production lettuce and celery were grown on wastewater for periods of 3–4 weeks.Celery grew better on raw wastewater, while lettuce did poorly due to the high room temperature of around 25^{o}C. Lettuce is known to do well at lower temperatures ranging between 10–15^{o}C, while celery does well at higher temperature of 24–28^{o}C. Fardhook lettuce has been reported by Wallace et al. /9/ to be grown well in glass houses under controlled environmental conditions.

In depletion experiments with tropic tomato plants grown ultimately to the vegetative stage of their life cycle. Experimental results showed

that the vegetative growth of Tropic tomato plants on wastewater was about double that for control plants grown on Hoaglands nutrient solution. The yield, reported in grams dry weight supporting this observation is shown in (Table 2).

Table 2

Comparison of wastewater and nutrient solution as hydroponic media on the vegetative growth of tropic tomato plants

Plant parts	Nutrient sol. yield[a] gm. dry wt.	%	Wastewater yield[a] gm. dry. wt.	%
Roots	0.718	15	0.717	7
Stems	3.124	65	5.280	54
Leaves	0.910	20	3.867	39
Whole plants	4.752	100	9.864	100

[a]Average values of four sets of experiments (8 replicates)

Chemical analysis of plant samples grown on raw wastewater showed that the experimental plants taken as a whole, were able to accumulate the mineral nutrients from the wastewater. Trace elements (Cd, Zn, Cu, Ni) were mainly concentrated in, or at the root surfaces. Stems mainly concentrated P, while Na, K, Ca and Mg were slightly concentrated in the leaves. In addition, Na, K, Mg and B were concentrated in the fruits. However, B concentrations were not at levels toxic to man. The results thus obtained agree with previously reported findings /8, 10/ where a similar pattern of distribution of the chemical elements within a variety of crops and ornamentals grown on secondary wastewater effluent was observed.

Quality Changes in the Wastewater Hydroponic Medium

Statistical analysis of experimental data showed that the reduction in the mineral content of wastewater medium by roots of Toska hybrid in cucumber plants correlates exponentially with time. Reduction in the mineral content to tap water levels as indicated by a specific conductance of

about 300 µmhos/cm at 25°C was achieved in 18 days. However, reduction in the concentrations to distilled water levels (specific conductance 10 µmhos/cm at 25°C) were achieved in 28 days (Fig. 3).

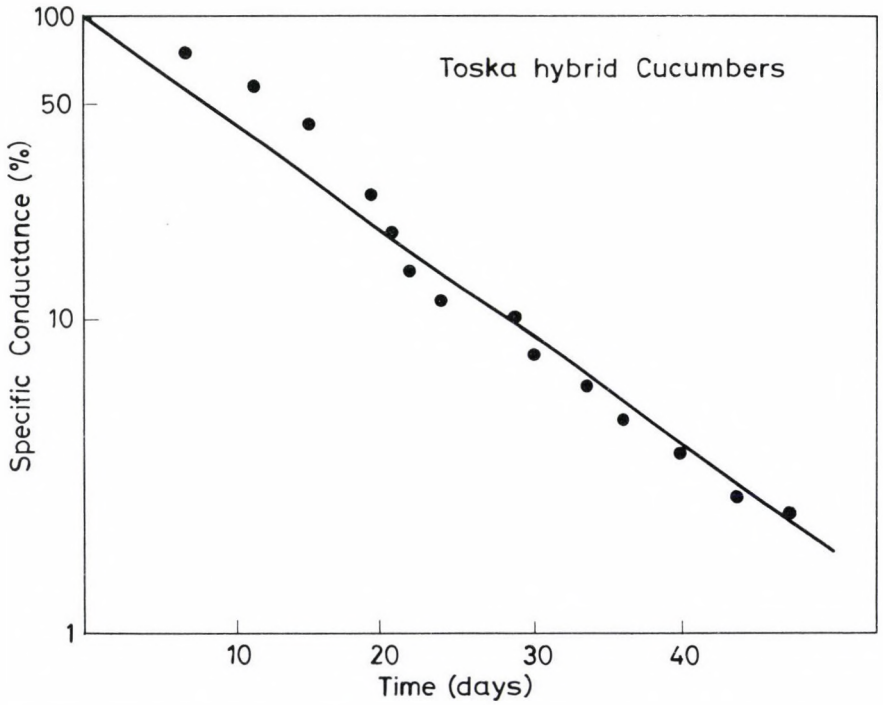

Fig. 3. Changes in the specific conductance of wastewater within the hydroponic system

A period of 4 to 12 days was needed to attain a decrease of 50% in the concentrations of some macro elements for plant growth, such as (NO_3–N, Cl, Na, PO_4, Mg, Ca, SO_2). Changes in the levels of the various parameters correlate exponentially with time as shown in (Fig. 4). As expected, a decrease in nitrate concentrations to low levels (< 0.1 mg/l) was found to be the limiting factor for vegetative plant growth. In contrast, a decrease of 50% in the concentrations of micro-elements (Zn, Ca, Mn and B) required 25 days (Fig. 5).

Reduction in the mineral content of wastewater by tropic tomatoes was also found to correlate exponentially with time. The different patterns of change in the various parameters are shown in (Figs 6–8). The time

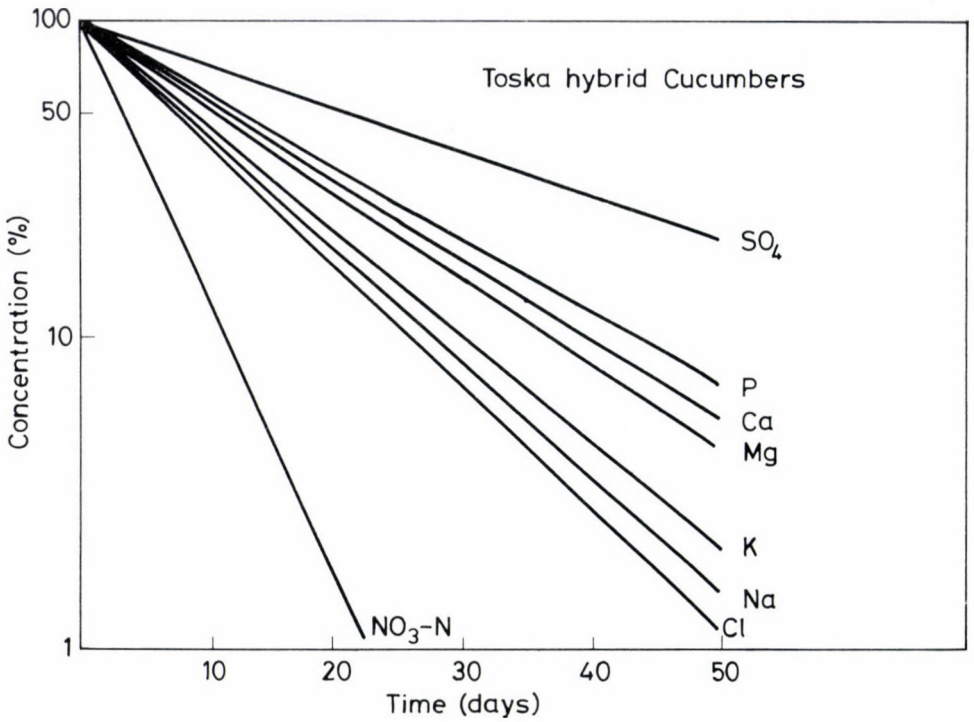

Fig. 4. Changes in the concentrations of certain macroelements in wastewater within the hydroponic system

needed for depletion of the mineral content to tapwater levels was comparable to that required by Toska hybrid cucumber plants.

A number of factors were observed to have an effect on the reduction in the mineral content of wastewater. These include plant species and variety, number and growth stage of the seedlings, as well as the volume and aeration of wastewater.

On the whole, both cucumber and tomato plants were good depleters of the macroelements and poor depleters of the microelements monitored. However, the rate of reduction in the specific conductance, on the whole, was higher for cucumber plants at all time intervals. These findings are in accordance with the results obtained by Ward /11/ who reported that cucumber plants remove nutrient elements from the soil at a rate 1.5 times faster than that of tomato plants.

Varying the number of seedlings per fixed volume of wastewater used

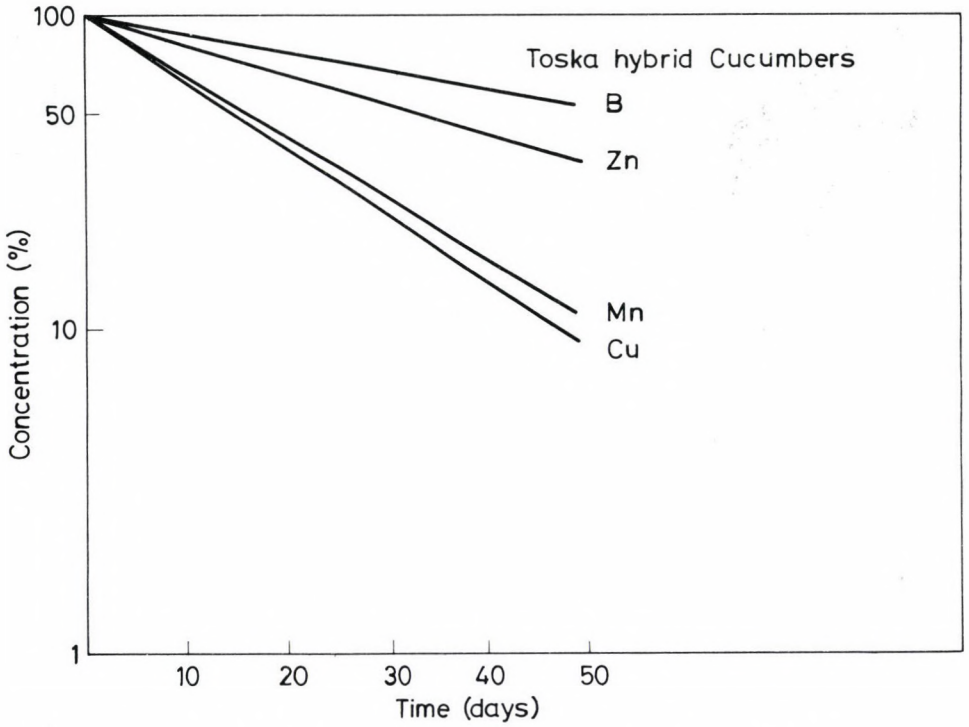

Fig. 5. Changes in the concentrations of some microelements in wastewater within the hydroponic system

had a variable effect on the reduction in the specific conductance. Experimenting with Toska hybrid cucumber plants, for example, higher reduction rates were observed in the hydroponic units utilizing two seedlings per fixed volume of 3.7 l.

The increase in the rate of reduction tapered off at the level of three to four seedlings (Fig. 9). Accordingly, Toska hybrid cucumber plants seedlings grown in each hydroponic unit. This is indicated by the weak correlation coefficient of 0.524, which is not significant at the 0.05 level (Table 3). In contrast, a significant correlation at the 0.05 level ($r = 0.974$) was found between the extent of depletion in the mineral content of wastewater and the final dry weight of the plant seedlings grown in each hydroponic unit.

It was also observed that the smaller the volume of wastewater used per fixed number of plant seedling the higher is the reduction in the mineral content of wastewater (Fig. 10).

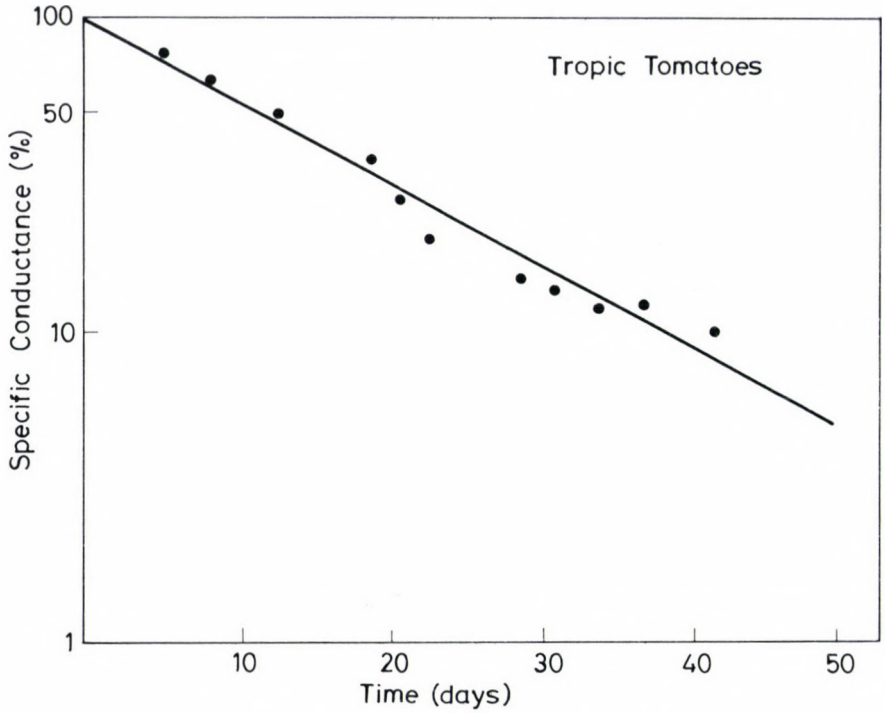

Fig. 6. Changes in the specific conductance of wastewater within the hydroponic system

Transplanting 5-week-old Toska hybrid cucumber seedlings in wastewater lessened the period required to achieve 90% reduction in the mineral of wastewater from 17 to 9 days (Fig. 11).The reduction correlated significantly with the final dry weights of the plants at the 0.05 level (r=0.974).

Applying the wastewater medium at flow rates of 10, 20 and 30 ml/min influenced the percentage reduction in the mineral content of wastewater.

(A steady state in the reduction of the mineral content by Toska hybrid cucumber plants was attained at a flow rate of about 20 ml/min (Fig. 12). However, in the case of tropic tomato plants, it was observed that an increase in the flow rate resulted in a significant lowering in the mineral content (Fig. 13). Reduction in P concentration to low levels ($\prec$ 0.1 mg/l) was the limiting factor for plants growth at the higher flow rates of 20 and 30 ml/min.

Experimenting with Toska hybrid cucumber plants in sequence with

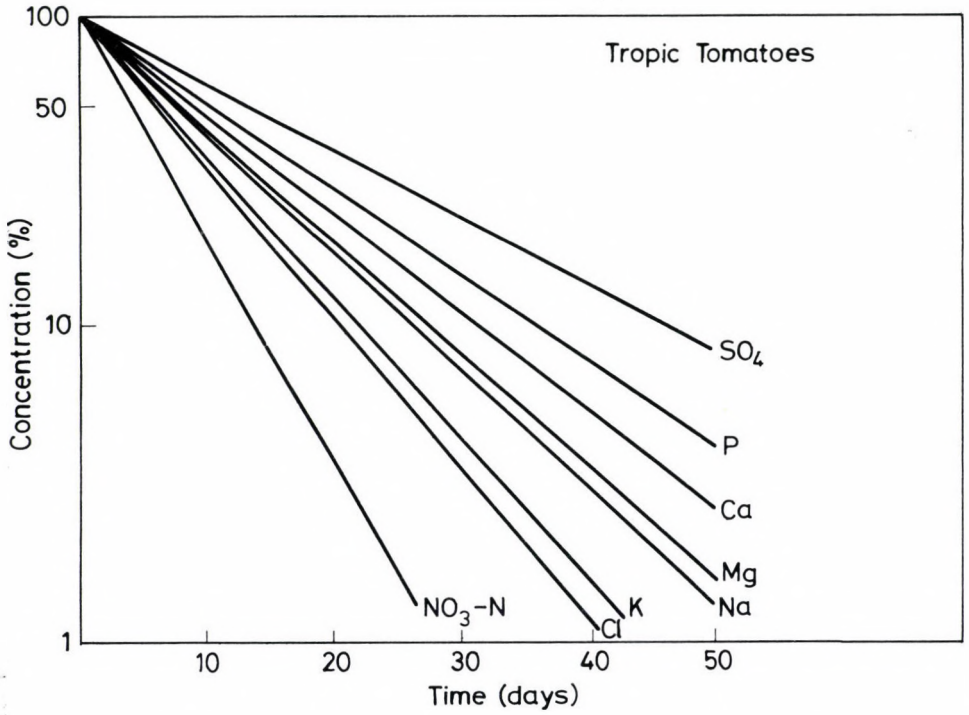

Fig. 7. Changes in the concentrations of some macroelements in wastewater within the hydroponic system

tropic tomato plants within the continuous flow system. Increased the potential to utilize higher flow rates with maximal reduction in the mineral content of the wastewater hydroponic medium. Thus reduction in the mineral content of wastewater within a continuous flow system utilizing more than one plant species is governed by the presence of the limiting depleter, in this case, the tomato plants (Figs 14–16).

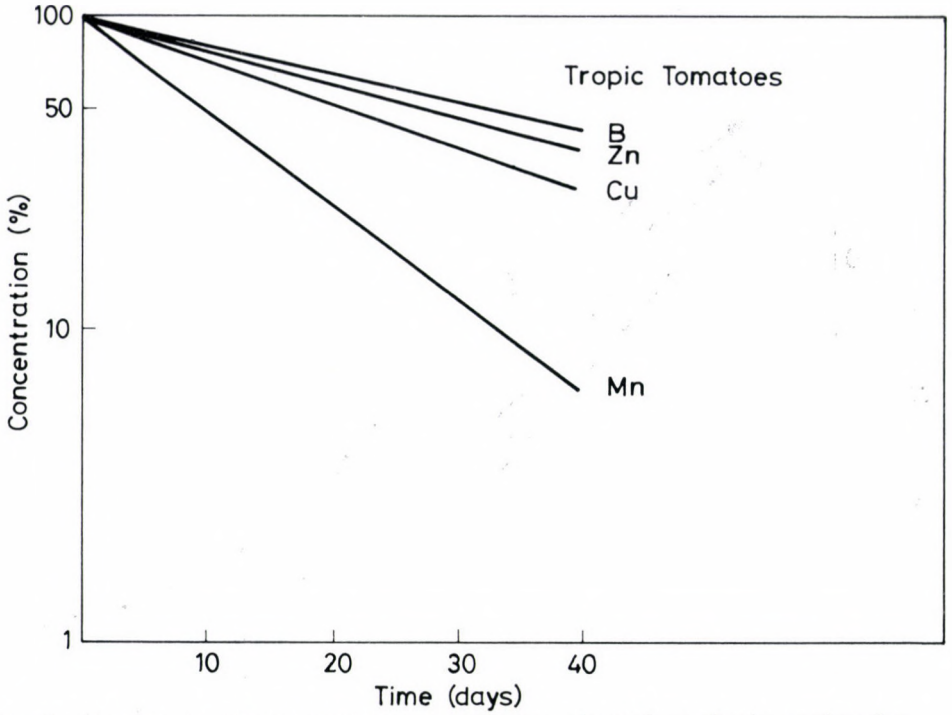

Fig.8. Changes in the concentrations of some microelements in wastewater within the hydroponic system

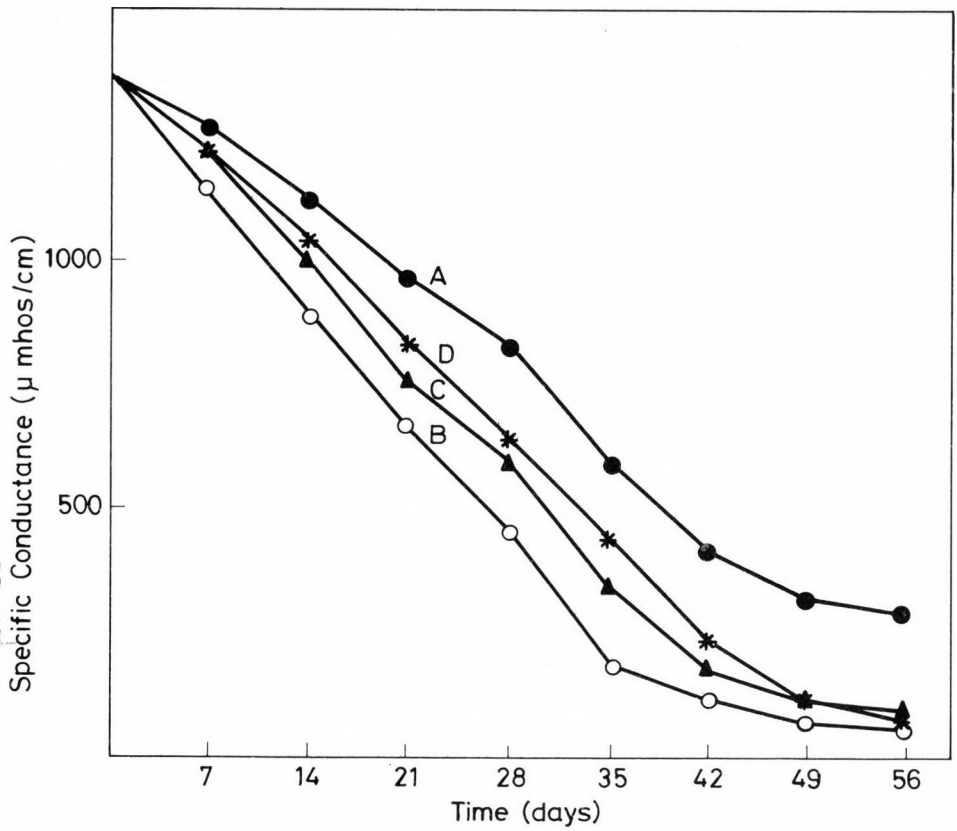

Fig. 9. Effect of varying the number of seedlings per fixed volume of wastewater on the reduction of the specific conductance in wastewater within the hydroponic system. Number of Toska hybrid cucumber seedlings per fixed volume (A) one, (B) two, (C) three, (D) four

Table 3

Effect of varying the number of seedlings per fixed volume on the yield and reduction of the specific conductance in wastewater within the hydroponic system[a]

No. of seedling/ Fixed volume	Yield[b] gm. dry wt.	Specific conductance % reduction
1	4.013	79
2	9.464	97
3	7.894	94
4	7.322	93

[a]Plant species used: Toska hybrid cucumber plants

[b]Average values of 8 sets of experiments (40 replicates)

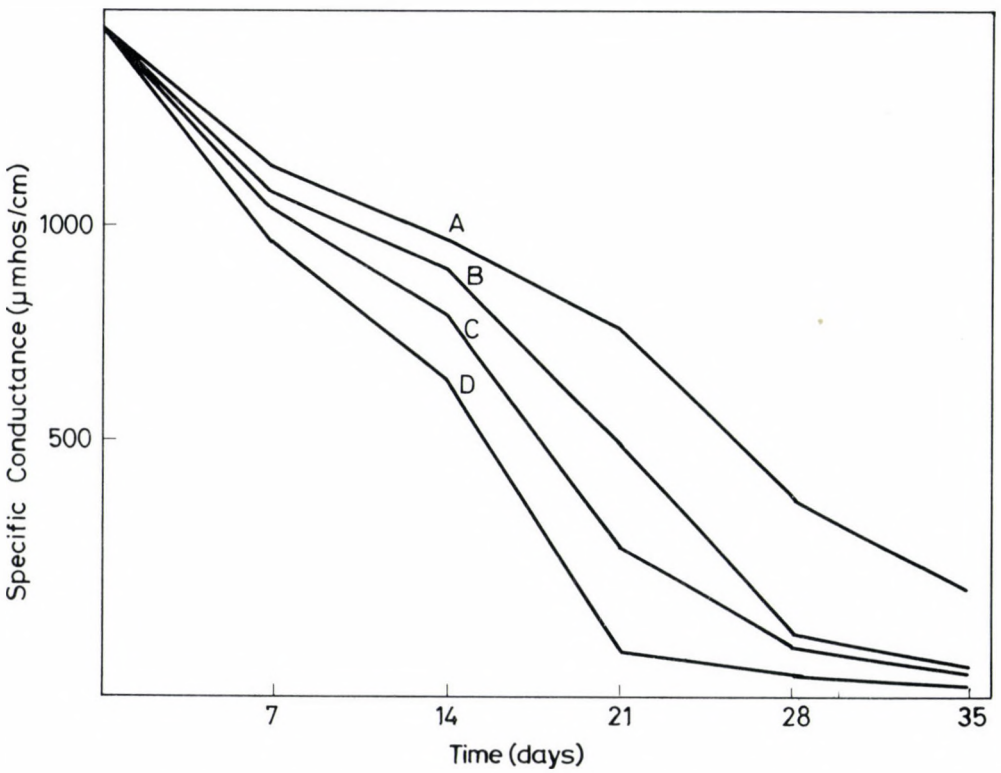

Fig. 10. Effect of varying the volume of wastewater per fixed number of seedlings within the hydroponic system on the reduction of the specific conductance in wastewater. Volume per fixed number of tropic tomato seedlings: (A) 1 l, (B) 2 l, (C) 3 l, (D) 4 l

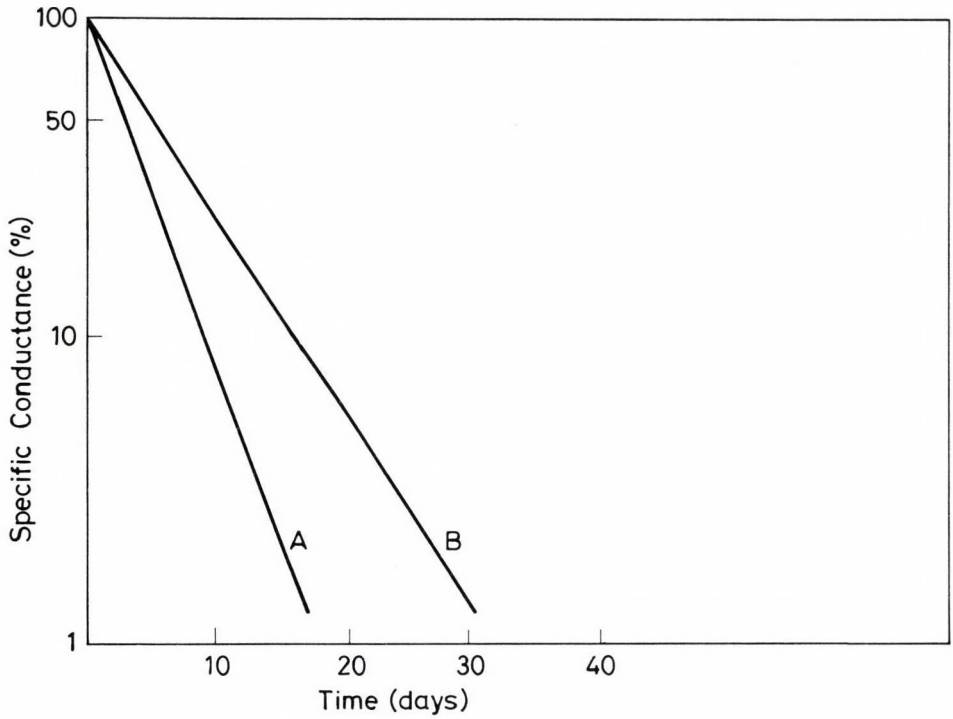

Fig. 11. Effect of the growth stage of the seedlings on the reduction of the specific conductance in wastewater within the hydroponic system.
(A) 5-week-old Toska hybrid cucumber seedling
(B) 3-week-old Toska hybrid cucumber seedling

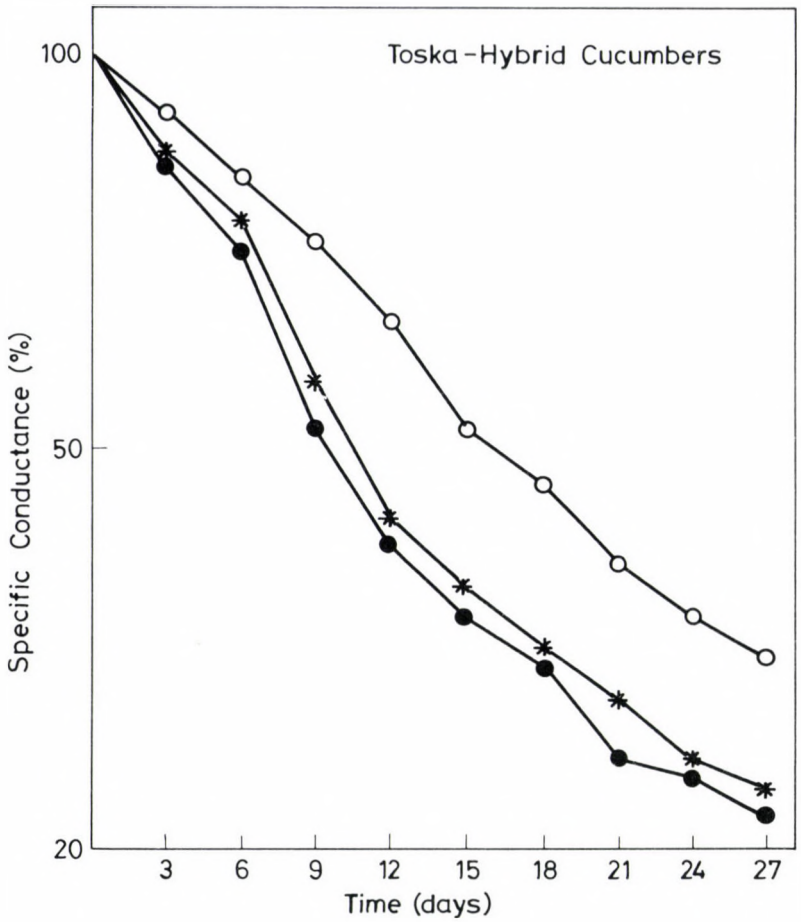

Fig. 12. Effect of different flow rates of wastewater within the hydroponic system on the reduction of the specific conductance in wastewater

Flow rate: ○○○ 10 ml/min
*** 20 ml/min
●●● 30 ml/min

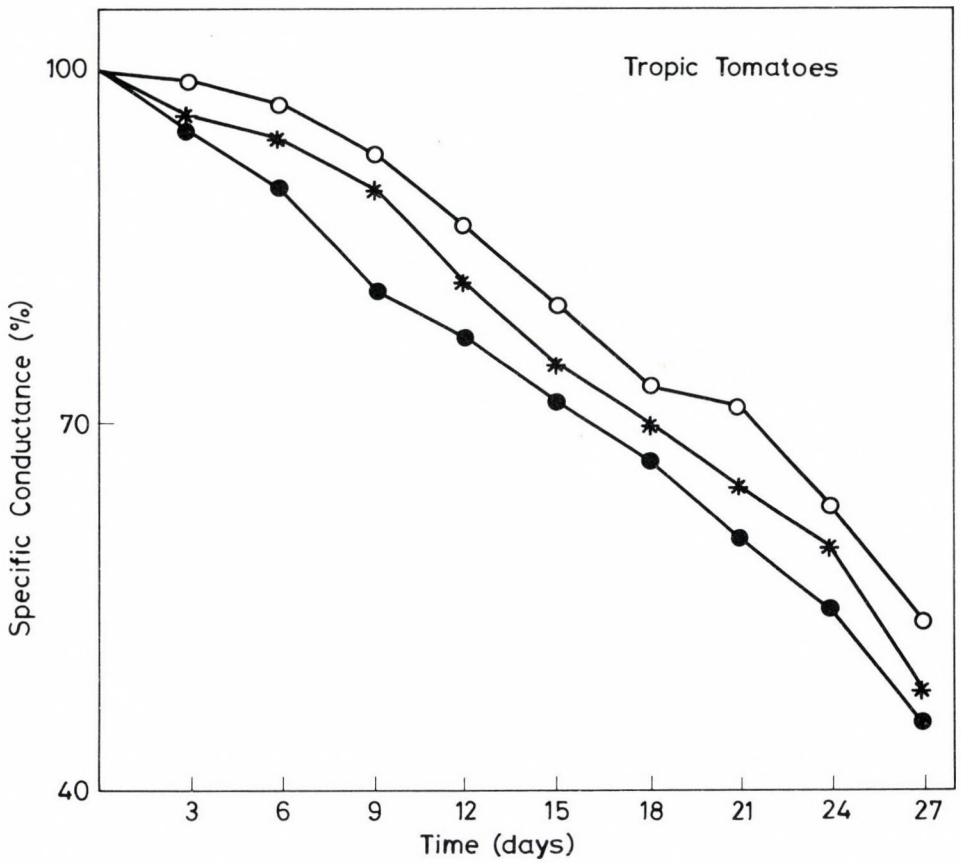

Fig. 13. Effect of different flow rates of wastewater within the hydroponic system on the reduction of the specific conductance in wastewater.

Flow rate: ○○○ 10 ml/min
✳✳✳ 20 ml/min
●●● 30 ml/min

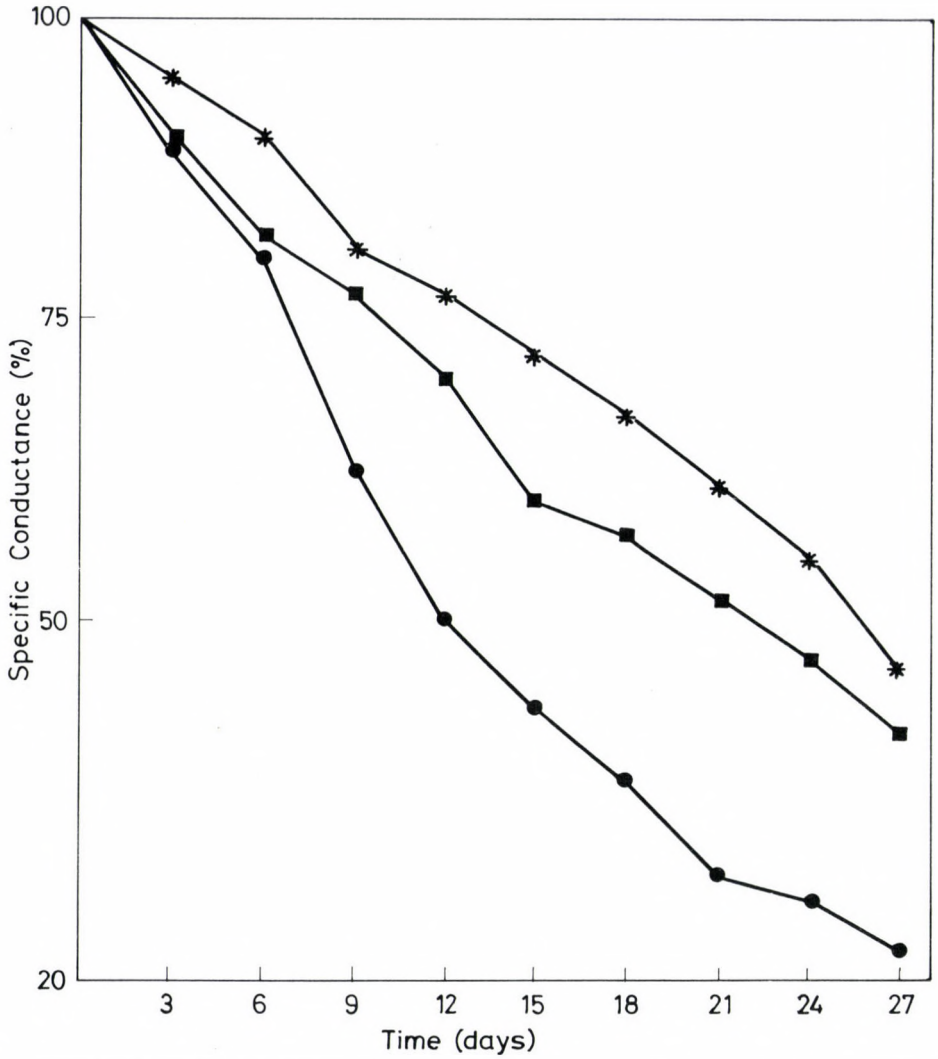

Fig. 14. Effect of plant species on the reduction of the specific conductance in wastewater within the flow hydroponic system.
Plant species ●●● Toska hybrid cucumber plants
✱✱✱ Tropic tomato plants
■■■ Tropic tomato and Toska hybrid cucumber plants

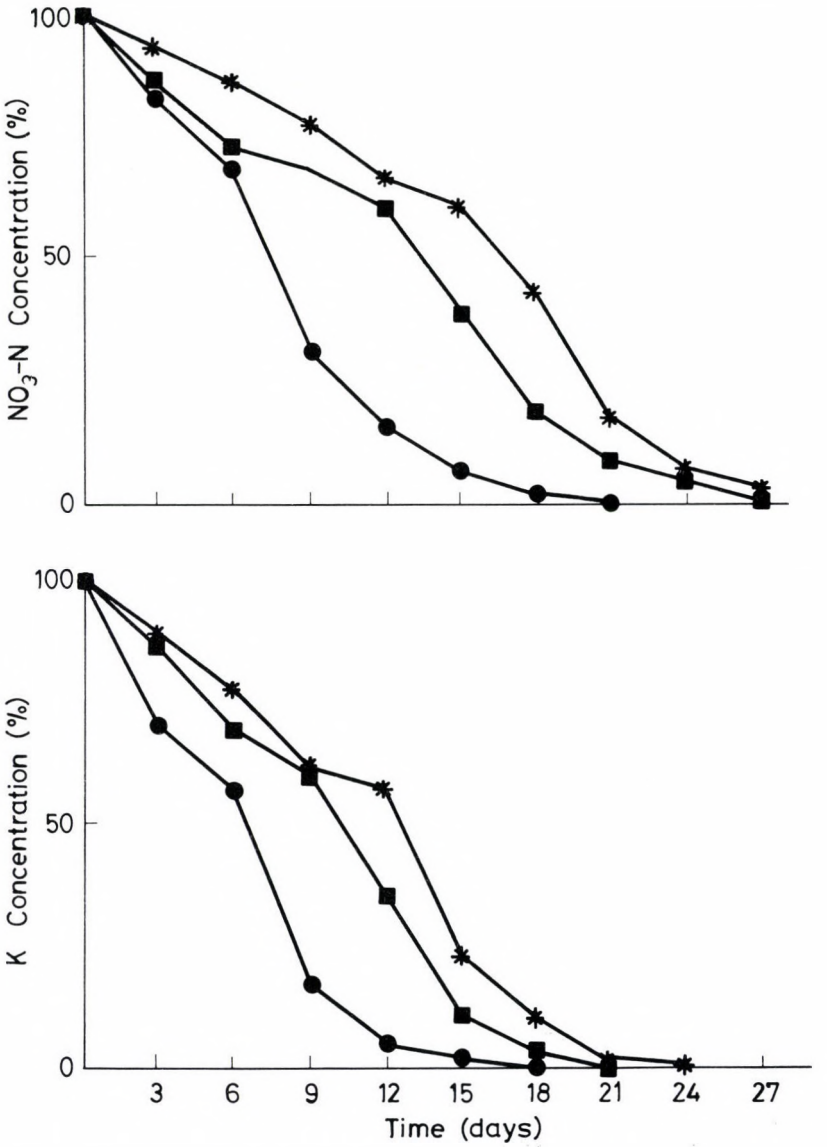

Fig. 15. Effect of plant species on the reduction in the concentrations of nitrate-nitrogen and potassium in wastewater within the flow hydroponic system.

Plant species ●●● Toska hybrid cucumber plants
■■■ Tropic tomato plants
✱✱✱ Tropic tomato and Toska hybrid cucumber plants

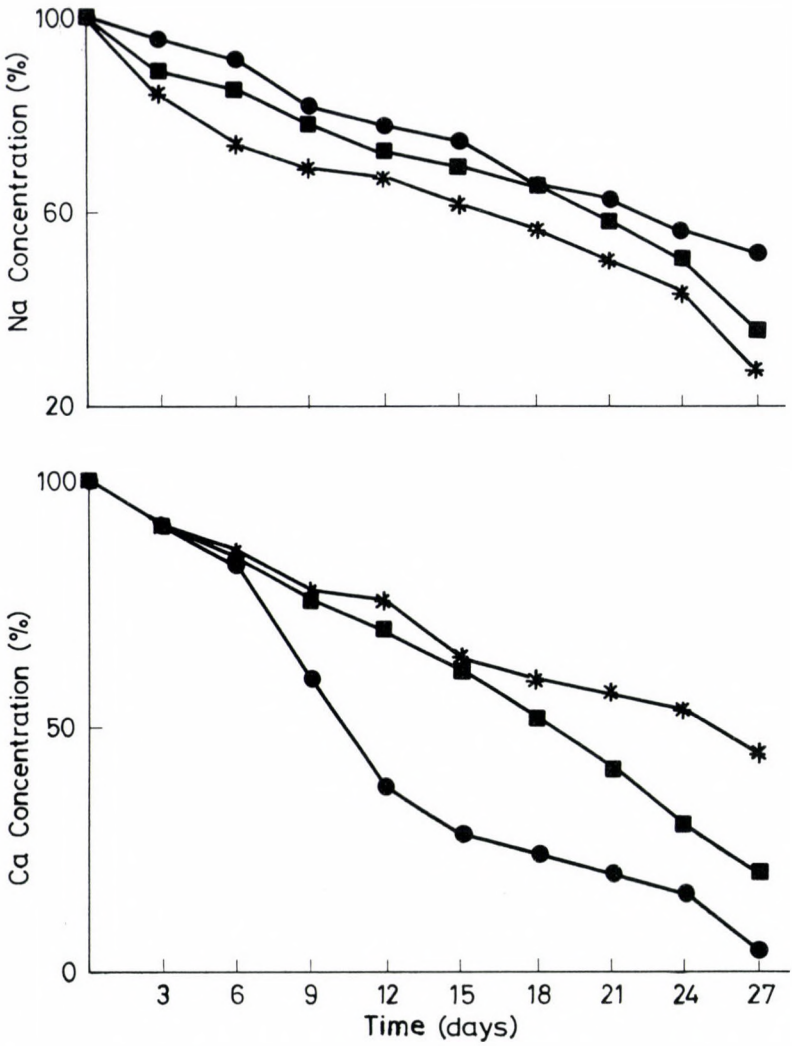

Fig. 16. Effect of plant species on the reduction in the concentrations of sodium and calcium in wastewater within the hydroponic system.
Plant species ●●● Toska hybrid cucumber plants
■■■ Tropic tomato plants
*** Tropic tomato and Toska hybrid cucumber plants

CONCLUSION

The presented experimental data demonstrates the feasibility of utilizing raw wastewater as a hydroponic medium for its reclamation and reuse, as well as for growing useful crops. However, pilot-scale applicator is essential prior to field application. A diversity of advantages can result from such applications:

1. Removal of the chemical constituents of raw wastewater by the roots of plants grown hydroponically.
2. Water recycling for specific uses in water-short areas.
3. Protection of water resources from contamination by discharged wastewater.
4. Production of high prices, out of season crops.
5. Benefits in terms of reclaimed wastewater, and reduction in fertilizers used.

ACKNOWLEDGEMENT

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INFLUENCE OF ETHANOL ON GLUTATHIONE LEVEL IN THE BLOOD, LIVER AND KIDNEYS OF RANA TEMPORARIA L. IN THE ANNUAL CYCLE[+]

H. Lach, J. Surowiak[++], K. Dziubek, S. Krawczyk,
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(Received 1986–08–06, revised 1986–10–25)

The effects of single doses (3 g/kg and 9 g/kg) of 35% ethanol, on the glutathione (GSH) contents of the blood, liver and kidneys of Rana temporaria L. were studied in the annual cycle. It was found that the single doses of ethanol generally caused a significant increase of GSH in the blood and liver of males and females of Rana temporaria L. in each period of the annual cycle as compared with the control values. In time, it was found that the same doses of ethanol caused a significant decrease in the GSH content of the kidneys of the male and female Rana temporaria L. during their active land life and a strong increase of this tripeptide during hibernation.

Keywords: Reduced glutathione (GSH) – blood – liver – kidney – Rana temporaria L. – ethanol – annual cycle

INTRODUCTION

GSH (gamma-glutamylcysteinyl-glicine) plays a key role in the liver in several detoxification reactions and in the reduction of lipid peroxides, and appears also to play an important role in a variety of cell functions, including amino acid transport and the storage of thiol moieties /25/.

Studies on chronic ethanol consumption in rats have provided

[+]Supported by the scientific program R-III-14 coordinated by the Jagiellonian University, Kraków, Poland

Send offprint requests to: Prof. H. Lach, Dept. of Animal Physiol., Teacher Training College, Podbrzezie 3, 31-054 Kraków, Poland

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evidence in favour of /20, 21, 23, 28, 29, 31/ and against /22, 30, 36/ the involvement of lipid peroxidation.

Likewise, it has been reported that chronic ethanol feeding results in increased /2, 15, 17, 39/, decreased /12, 14, 32, 37, 38/ or unchanged /19, 30/ hepatic GSH levels in rats. The mechanism by which this effect is exerted is, however, not clear. Three general mechanisms have been suggested, namely (i) binding of GSH to acetaldehyde produced in the metabolism of ethanol /38/, (ii) oxidation of GSH by lipid peroxides produced by ethanol /37/, and (iii) binding of acetaldehyde to cysteine, a precursor of GSH /32/.

Under normal physiological conditions the GSH level in tissues shows evident diurnal /3, 5, 6, 7, 11, 13, 35/, as well as cyclic seasonal changes /9/. The presently available investigations concerning ethanol effects on the circadian changes of behaviour and various physiological processes are concentrated mainly on mammals /4, 8, 16, 27, 34/. On the other hand, as far as the amphibians are concerned, no studies have been performed as yet on the effects of ethanol on GSH metabolism in the blood and tissues in the annual cycle.

Therefore, the present paper aimed at determining whether — and if so — how the GSH level changes in the blood, liver and kidneys of Rana temporaria L. in the annual cycle upon the administration of single doses of ethanol.

MATERIALS AND METHODS

420, sexually mature individuals of Rana temporaria L. (210 males and 210 females) were used in the studies, coming directly from their natural habitat, captured in 6 characteristic periods of their life, namely: 3rd half of January (middle of hibernation), 3rd half of March (breeding period), 3rd half of May (active life), 2nd half of July (middle period of active life), 1st half of September (end of active life), and 3rd half of October (beginning of hibernation). The adopted annual phases were taken from the work of Juszczyk /18/. The frogs were captured in the environs of Kraków (50° 04' N, 200–220 m above sea level), 10 males and females from each period.

The animals were divided into three groups, namely, one control and two experimental groups. The frogs of the first group received a single, intragastric dose of 35% ethanol, 3 g/kg, in each of the studied periods, whereas the frogs of the second experimental group received a dose of 9 g/kg of 35% ethanol.

The frogs of the first experimental group were killed by decapitation after 3 h, whereas the frogs of the second group 6 h after the administration of ethanol was ended. Then, the blood, liver and kidneys were collected in order to determine the GSH content. In the blood, liver and kidney homogenates, the GSH was determined by the method of Ellman /10/. The obtained results were statistically analyzed by the "t" and F tests.

RESULTS

Analysis of the obtained results revealed that the GSH content in the blood, liver and kidneys of the males and females of the control group of Rana temporaria L. underwent cyclic changes throughout a year.

The GSH content in the blood of the control male and female Rana temporaria L. showed a maximum in the initial period of their active life on land, i.e. in the 3rd half of May, throughout a year, whereas the minimum was observed in the final period of hibernation (1st half of March) (Figs 1 and 2). The curves illustrating the changes in GSH level in the blood of control males and females showed a similar run throughout a year.

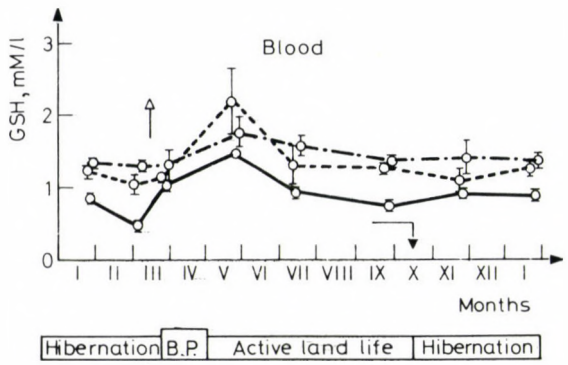

Fig. 1. Effects of ethanol on GSH content in the blood of males of Rana temporaria L. in the annual cycle. Continuous line – control broken line – experimental group I, broken-spotted line – experimental group II, B.P. – breeding period, vertical lines – standard deviations, upward arrows – frogs' exit from their hibernation half, downward arrows – frogs' entering the hibernation half

In the first and second experimental groups in case of both sexes, the single dose of ethanol induced an increase of the GSH content in the blood in each experimental period throughout a whole year as compared with the control values (Figs 1 and 2). Analysis of variance revealed that the obtained changes of the GSH content in the blood of the control and experimental males and females were statistically significant throughout a year.

In the liver of the control males and females, a maximal GSH content was found in the 2nd half of July, i.e. in the middle period of their active land life, whereas the minimum fell to the 1st half of March (Figs 3 and 4). Ethanol administration caused an increase of the GSH content

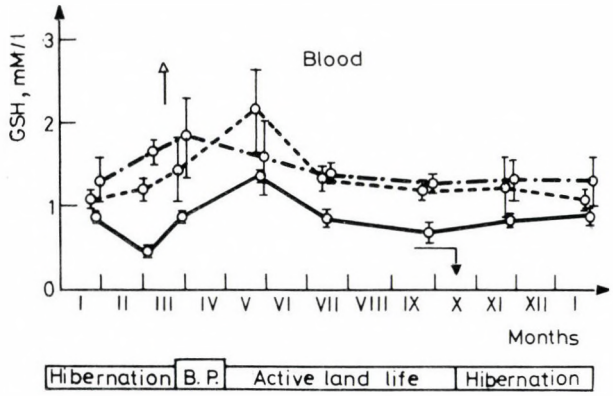

Fig. 2. Effects of ethanol on GSH content in the blood of females of Rana temporaria L. in the annual cycle. Designations as in Figure 1

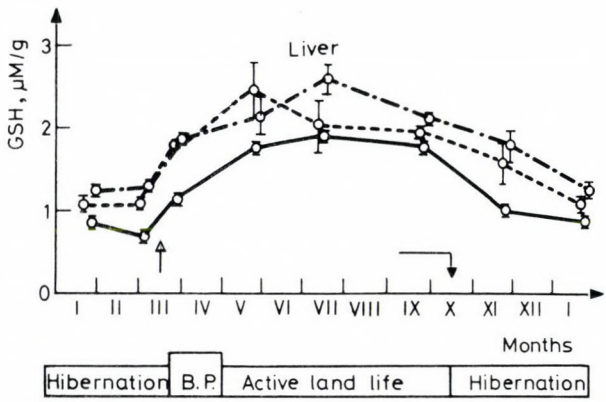

Fig. 3. Effects of ethanol on GSH content in the liver of males of Rana temporaria L. in the annual cycle. Designations as in Figure 1

in the liver of both males and females of the first and second experimental group within the whole annual cycle as compared with the control group (Figs 3 and 4). In the first experimental group in both males and females, the GSH maximum was shifted from the 2nd half of July to the 3rd half of May. Like in the blood, statistical analysis revealed the obtained changes in GSH level in the liver of males and females of Rana temporaria L. to be statistically significant throughout a year.

During the hibernation period, GSH in the kidneys of control males and females was not found. As late as during the breeding period, a small amount of GSH was noted, reaching its maximum value by the 3rd half of May, i.e. in

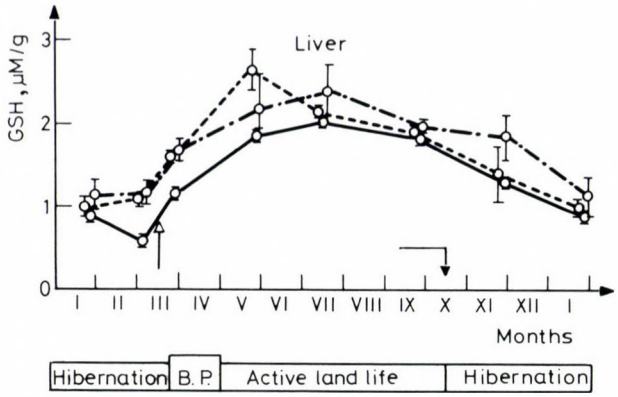

Fig. 4. Effects of ethanol on GSH content in the liver of females of Rana temporaria L. in the annual cycle. Designations as in Figure 1

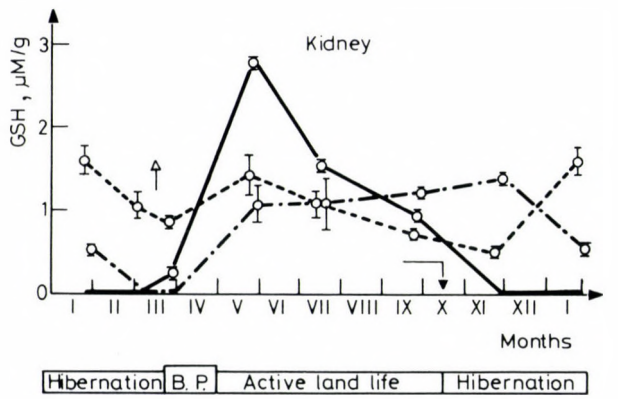

Fig. 5. Effects of ethanol on GSH content in the kidney of males of Rana temporaria L. in the annual cycle. Designations as in Figure 1

the initial period of their active land life (Figs 5 and 6). In contrast to this, ethanol administration caused a considerable amount of GSH in the kidneys of males and females of both experimental groups during the whole period of hibernation. In spite of this, ethanol caused a total drop of the GSH content in the kidneys of the males and females of both experimental groups during their active land life. The administration of ethanol in case of the females of the first and second experimental groups did not change the maximum GSH level throughout a year as compared with the control group. On the other hand, in the males of the first experimental group, the maximum GSH content was shifted from the 3rd half of May to the 3rd half of January.

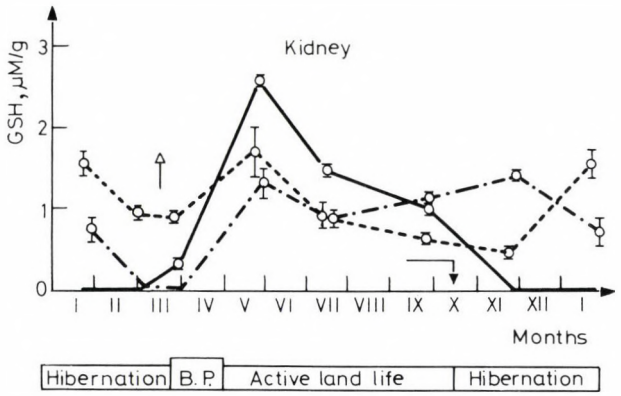

Fig. 6. Effects of ethanol on GSH content in the kidney of females of Rana temporaria L. in the annual cycle. Designations as in Figure 1

Similarly in the males of the second experimental group the maximum GSH content was shifted from the 3rd half of May to the 1st half of November, i.e. to the initial period of hibernation (Figs 5 and 6). Analysis of the obtained results with the "t" and F tests showed statistically significant changes in the GSH content in the kidneys of Rana temporaria L. in the annual cycle.

DISCUSSION

The results obtained in the control group entirely confirm earlier studied performed by Dziubek /9/, who evidenced identical changes in GSH content in the blood, liver and kidneys of males and females of Rana temporaria L. in the annual cycle. On the other hand, administration of single doses of ethanol caused a significant increase of the GSH content in the blood and liver of males and females of Rana temporaria L. during the entire annual cycle as compared with the control material.

The results obtained are in confirmity with those of Yalcin et al. /39/ who found an increase of the GSH content as well as the GSH-transferase activity in the blood of rats after administration of a single dose of ethanol. The increase of the GSH content in the liver after chronic ethanol doses was observed by Hetu et al. /17/, Hassing et al. /15/. Also Aykac et al. /2/ found that chronic ethanol administration induces statistically significant GSH increases as well as the presence of GSH-peroxidase and

GSH-transferase in the rat liver. Chronic ethanol doses also result increased activities of numerous enzymes, including the oxidase of mixed function, the cytochrome P-450 and NADPH-cytochrome C reductase /33/.

At the same time, it was found that the same doses of ethanol caused a significant drop of the GSH content in the kidneys of males and females of Rana temporaria L. during their active land life and a strong increase of this tripeptide during hibernation. The increase in the level of GSH upon the administration of single doses of ethanol in the kidneys of Rana temporaria L. during hibernation can be brought into connection with the immediate balancing response of the organism to a poorer delivery of oxygen to the tissues, moreover, with the fact that frogs stay in water of $4^{o}C$. According to Molenda et al. /26/, after an intravenous administration of 20 ml of 20% ethanol, oxygen consumption in the tissues decreases on the average by 13% and returns to a normal level very slowly. In the process of ethanol oxidation by alcohol dehydrogenase, the level of NADH increases and is accumulated in the cytoplasm, limiting the process of ethanol oxidation as well as it affects the oxidation-reduction balance in a cell /33/. Thus, breaking the oxidation-reduction balance in cells caused by ethanol administration, can be a factor inducing an increased GSH synthesis. On the other hand, during the whole active land life of the Rana temporaria L. the same ethanol doses caused a reduction of this tripeptide amount in the kidneys in comparison with the control values.

A main function of GSH in the process of aminoacids transport consists in the delivery of the gamma-glutamyl groups in a so-called gamma-glutamyl cycle occurring mainly in kidenyes /1, 24/. It seems that an acceleration of the gamma-glutamyl cycle in the kidneys under the effect of ethanol during amphibian's active land life, i.e. a high level of kidney metabolism, in consequence, it may lead to an increased GSH demand in the process of amino acid transport, and at the same time, to a decrease of the amount of this tripeptide. One cannot exclude that lipid peroxides formed on the effect of ethanol cause GSH oxidation or a decrease of the cysteine level acting as a GSH precursor /32/. In conclusion, the presented studies suggest that ethanol administration induces increased GSH levels which reflects ad adaptive change against ethanol-induced lipid peroxide toxicity.

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ALTERATIONS OF THE SEROTONIN LEVEL IN THE CENTRAL NERVOUS SYSTEM OF *LOCUSTA MIGRATORIA* DURING LARVAL-ADULT TRANSFORMATION

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Serotonin level was measured in the cerebral, suboesophageal, thoracic and abdominal ganglia of *Locusta migratoria* during larval-adult transformation. It has been established that the serotonin content shows a progressive increase in the period between the last larval stage and the first 7 days of adult life, being of the highest value in the abdominal ganglia (146%), followed by the thoracic (110%), and then by the cerebral and suboesophageal (75%) ganglia. No significant changes are manifested in the serotonin level in the course of moulting. Considerable increase is also detectable in the protein content of the ganglia during the studied development phase. No correlation is present between the protein and serotonin contents of the ganglia, the changes of the protein content was found to be faster as compared to that of serotonin.

Keywords: Serotonin – *Locusta migratoria* – CNS – larval – adult transformation

INTRODUCTION

The presence of biogenic monoamines has been proved in the nervous system of many insects /6, 14, 29/. Using biochemical methods, serotonin has been demonstrated in the CNS of *Schistocerca gregaria* /16/, *Locusta migratoria* /12/, *Periplaneta americana* (18, 31/, *Formica rufa* /17/ and *Calliphora erythrocephala* /26/.

Immunocytochemical method has exhibited 5-hydroxytryptamine in the

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brain of Schistocerca, Periplaneta and Calliphora /2, 25, 27, 34/, occurring in largest amounts in the optic lobes of the brain. The synthesis of serotonin in the brain /4, 10, 22, 30/ as well as its cellular localization in the nervous system /9, 14, 19, 27, 28, 29, 34/ serve as further proof for its role as transmitter or modulator.

The function of serotonin in insects has also been studied. According to Hinks /11/ and Fowler et al. /8/, serotonin may play a role in the regulation of the daily rhythm and the correlating locomotor activity, respectively. Muszynska-Pytel and Cymborowski /23, 24/ reported on the circadian alterations of the serotonin level in the brain, this rhythm of serotonin, however, shows no correlation with the daily rhythm of the locomotor activity. The functional role of serotonin has been revealed in visceral organs and the glandular function /1, 21, 13, 33/, too.

5HT was found to stimulate the secretion of the Malphighian tubules in Carausius morosus /21/ and the salivary gland function in Calliphora erythrocephala /1/. The contractions of the fore- and hindguts of Locusta became more frequent by 5HT treatment /13/. The stimulatory effect of 5HT has been reported on the heart muscle fibers of the Locusta and Periplaneta /5, 33/, as well as on the extensor-tibiae muscle of the hopping leg /7/.

The aim of our present investigation was to obtain quantitative measurements of serotonin level in different regions of the adult and larval CNS of Locusta migratoria migratorioides using high performance liquid chromatography (HPLC) and to connect it to the regulation of larval-adult transformation.

MATERIALS AND METHODS

Specimens of the migratory locust, Locusta migratoria migratorioides R.F. were reared in our Institute. The animals were kept at 32 ^{o}C during the 12 h light period and at 28^{o}C during the subsequent 12 h dark period. The animals were fed on wheat seedlings grass and bran. Synchronized populations were used for our studies in case of both the 5th larval instar and the adult insects. The synchronization means that at time of the 4th and last moulting, respectively, the individuals from a population made up of 300-400 animals moulting within 3 h were collected and regarded as synchronized population.

The lifespan of such populations in the 5th larval instar was 9 days.

The cerebral, suboesophageal, thoracic and abdominal ganglia were used separately for the serotonin estimations /32/. The tissue were homogenized in 100 µl 0.1 M $HClO_4$ containing 0.2 mM ascorbic acid. The homogenates were centrifuged for 15 min at 30 000 g and an aliquot of the supernatant was injected into the HPLC. The identification and the quanti-

tative estimation of serotonin were performed, using a Model 6000A solvent delivery system with a Model U6K sample injector, uBondapak C-18 reverse phase column (Water Associates) glassy carbon electrode and amperiometric detector (Bioanalytical System). The mobile phase consisted of a sodium acetate buffer (0.1 M pH 4.7) containing 6% methanol. The flow rate was 1.5 ml/min and the column was operated at 40 °C. The electrochemical detector was set at a potential of 0.7 Volt.

The pellet was dissolved in n NaOH and the protein was estimated by the method of Lowry et al. /20/.

RESULTS AND DISCUSSION

A significant increase was observable in the serotonin content of ganglia starting from the first day of the 5th larval instar (Fig. 1). This increase was found to be gradual throughout the development. The increase of serotonin content was found to be 75% in the cerebral and suboesophageal

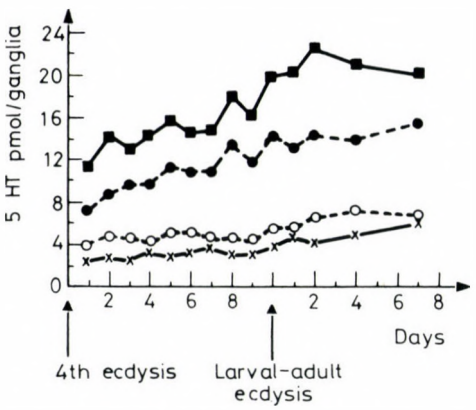

Fig. 1. Changes of serotonin content of the locust ganglia during the larval-adult transformation

■ – cerebral ganglia, ● – thoracic ganglia,
○ – suboesophageal ganglia, x – abdominal ganglia

ganglia, while it increased by 110% in the thoracic, and by 146% in the abdominal ganglia. The elevation of the serotonin level was uniform in the cerebral and thoracic ganglia, the increase took place in 50% in the larval form and in 50% in the adult stage. In the larval stage the serotonin level showed a slighter degree of increase in the suboesophageal and abdominal ganglia (15 and 35%) as compared to the adult form (59 and 111%). No significant changes were manifested in respect to the serotonin content of the ganglia at the time of moulting. It is true in case of each ganglion,

however, than the increase ensuring in the larval form mostly fell to the first 5 days of the larval state.

A considerable increase (125–150%) was found in respect to the protein content of the ganglia, too (Fig. 2). Fifty per cent of this increase took place during the larval state and 50% in the course of the adult stage. The rate of the increase was lower in the larval stage than adult one.

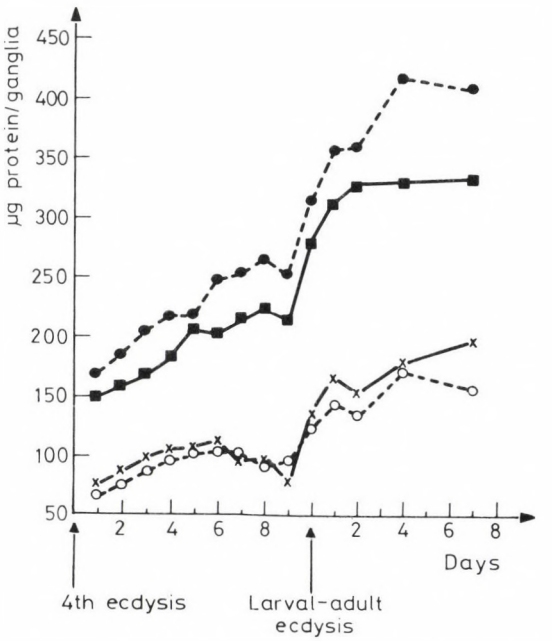

Fig. 2. Alteration of the protein content of the locust ganglia during the larval-adult transformation

■ – cerebral ganglia, ● – thoracic ganglia,
○ – suboesophageal ganglia, × – abdominal ganglia

Similarly to the change in serotonin content here, too, the majority of the increase in protein content took place during the first 5 days of the larval state. In the cerebral and thoracic ganglia the increase continued until the 9th day of the larval state, but the degree of the increase was slighter in this period. On the 6th day there was a transient decrease in the protein content of the suboesophageal and abdominal ganglia, taking place till the time-point of moulting. The change in protein content was considerable and rapid in the nervous system of the moulting and 1–2 days old adult animals, respectively.

Though both the serotonin- and protein contents showed gradual increase in the ganglia, no tight correlation can be detected between the changes in the protein- and serotonin contents of the ganglia. The changes in the protein content of the ganglia were faster than those in the serotonin content. This means that if expressing the ganglional serotonin content in pmol/mg protein concentration, from the beginning of the 5th larval instar until the 7th day of the adult stage, the serotonin concentration shows a decrease from 80 to 60 pmol/mg protein in the cerebral ganglia, from 65 to 45 pmol/mg protein in the suboesophageal ganglia, from 48 to 38 pmol/mg protein in the thoracic ganglia, and from 35 to 28 pmol/mg protein in the abdominal ganglia (Fig. 3). However, the protein content of the

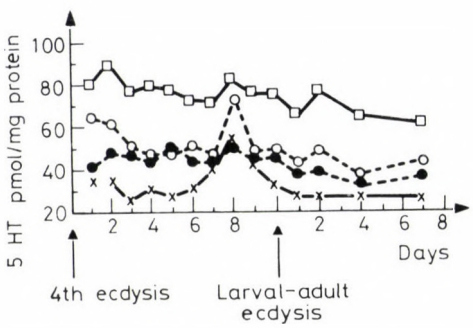

Fig. 3. Alteration of serotonin/protein ratio in the locust ganglia during the larval-adult transformation

■ – cerebral ganglia, ● – thoracic ganglia,
○ – suboesophageal ganglia, X – abdominal ganglia

suboesophageal and abdominal ganglia showed a transitional decrease between the 6th and 9th day of the 5th instar. This decrease in protein content issues in the considerable and significant increase manifested for serotonin concentration related to mg protein in these ganglia at these time-points. This increase is unambiguously the consequence of the decrease in protein content and has no physiological significance.

The importance of monoamines in cytodifferentiation during development has been stated in the dipterous flies /26, 28, 29/. The maximal value in octopamine content was found in the tenth day of adult life of Mamestra, too /3/. The same was proved for serotonin level in Locusta in our present studies. Taking together these data it can be concluded that final level of neurotransmitters are formed to the 9–10th days of adult life in insects.

However, further work is required before the functional role of serotonin can be established in the regulation of development or aging in the insects.

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CATECHOLAMINE-CONTAINING NEURONS IN THE PERIPHERAL NERVOUS SYSTEM OF APLYSIA

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Numerous green-fluorescent neurons have been revealed by means of the glyoxylic acid histochemical method in cryostat sections of several organs of two Adriatic aplysiid gastropods, Aplysia depilans and A. fasciata. Catecholamine-containing perikarya and their processes have been found to be especially abundant in the vaginal portion of the large hermaphrodite duct, in the penis and its sheath, and in the gill. In the reproductive organs, two subpopulations of catecholamine-containing neurons could be distinguished according to their size and location. Axons of larger neurons formed bundles which seemed to project ot the CNS.

Keywords: Catecholaminergic neurons – peripheral nervous system – Aplysia

INTRODUCTION

Catecholamine (CA)-containing neurons have been found in all gastropod molluscs investigated so far, and dopamine seems to be the main transmitter substance of these neurons /14/. The distribution of CA neurons considerably varies, however, within the class. A comparative analysis of available data has enabled us to propose that the evolution of pulmonate gastropods was accompanied by inclusion of the initially peripheral CA neurons into the central ganglia, while in opisthobranchs, CAergic neurons mainly retained their original peripheral positions /11/.

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In a previous study, we have found that the number of CA-containing neurons is small in the CNS of a "model" opisthobranch, Aplysia (13; in the paper, the species was erroneously referred to as A. depilans while it was A. fasciata). It appeared from this finding that peripheral neurons might be the source of the well developed CA-containing neuropile of central ganglia. Little is known, however, on monoaminergic neurons of the peripheral nervous system of Aplysia. CA-derived fluorescence was found in nerve fibers in several organs of A. californica, namely in the eye, mantle, siphon and gill /6, 16/, but their cellular origin remained unclear. In the case of the gill, the origin was attributed to neurons located in the abdominal ganglion /16/, but this suggestion could not be confirmed by the results of histochemical investigation of the ganglion /13/.

The aim of the present study was to search CA in the peripheral nervous system of two species of Aplysia. Not all potential sources of CA-containing neurons were investigated, including the naterior part of the alimentary tract which is very rich in CA-containing nerve cells in another opisthobranch, Clione /5/. Special attention was paid to the reproductive system, also known to be rich in CAs in many gastropods /2, 5, 11/. We report here that CA-containing neurons of the peripheral nervous system are, in fact, much more numerous and even larger than neurons of this chemical type located in the CNS.

MATERIALS AND METHODS

Adult specimens of Aplysia depilans and A. fasciata weighing 400–800 g were used. The animals were collected in Budva, Adriatic Sea, in May – June. They were kept in aerated aquaria at 22–24 ^{o}C before use, and exhibited sexual behaviour. Prior to dissection, the animals were anaesthetized by immersing them in I:I mixture of the sea water and isotonic $MgCl_2$ solution. The following organs were investigated by fluorescence histochemistry: the large hermaphrodite duct (its vaginal portion, 10–15 mm long, including the common genital aperture), the penis, its sheath, retractor muscles of the penis, the tentacles, the gill, and the apical part of parapodia. The glyoxylic acid condensation method /18/ modified for cryostat sections of marine animal tissues was used as described elsewhere /13/. The sections were investigated under a LUMAM I-1 fluorescence microscope (LOMO, Leningrad) equipped with FS-1 and SZS-24-2 primary filters providing a green fluorescence of CA fluorophores and a yellow one of indole reaction products.

RESULTS

1. Vaginal portion of the large hermaphrodite duct

Aplysia, like many gastropods, is hermaphoridte and possesses a common genital aperture /3, 16=. In the investigated species, the vaginal portion of the large hermaphoridte duct is an orange-yellow tube. The internal epithelium of the duct is folded and underlied by circular and longitudinal muscles, the musculature is covered by external epithelium (Fig. 2). We have found that this portion of the large hermaphrodite duct located near the common genital aperture is very rich in CA-containing neurons.

Two subpopulations of green fluorescing neurons differing in their size and location were demonstrated in the wall of the vagina. One was represented by large (100–140 μm in diameter) uni-, bi- or multipolar cells located close to the genital aperture in both A. depilans and A. fasciata. In transverse sections of the duct, these cells were seen lying between the circular and longitudinal muscle layers (Figs 1A, C, E; Fig. 2). These larger neurons were clustered in the vagina of A. depilans, whereas only single cells of this type were revealed in the corresponding area in A. fasciata. Axons of these neurons formed bundles running in the longitudinal muscle layer (Fig. 1B). The bundles were seen to contribute to small nerves believed to be branches of principal nerves projecting to the CNS. Approximately 20 such neurons could be counted along 1 mm of the wall in the vagina of A. depilans.

Another subpopulation was represented by abundant smaller multipolar nerve cells ranging from 30–80 um. They were located within the circular muscle layer and near the internal epithelium (Fig. 1D).

The circular muscle layer is densely innervated by varicose fibers, containing CA. Numerous fine green fibers run to the internal subepithelial region, forming here a fine plexus of varicose fibers (Fig. 1A).

No serotoninergic nerve elements were found in this region.

2. Penis, its sheath and retractor muscles

A male copulatory organ, or penis, is a pedal structure and situated at the base of the right anterior tentacle. The penis is completely retractile within its sheath. In A. depilans the penis is a thick-walled, short dark-brown organ, while in A. fasciata it is white, long and filiform. The sheath is a muscular cul-de-sac, the blind end of which the penis is

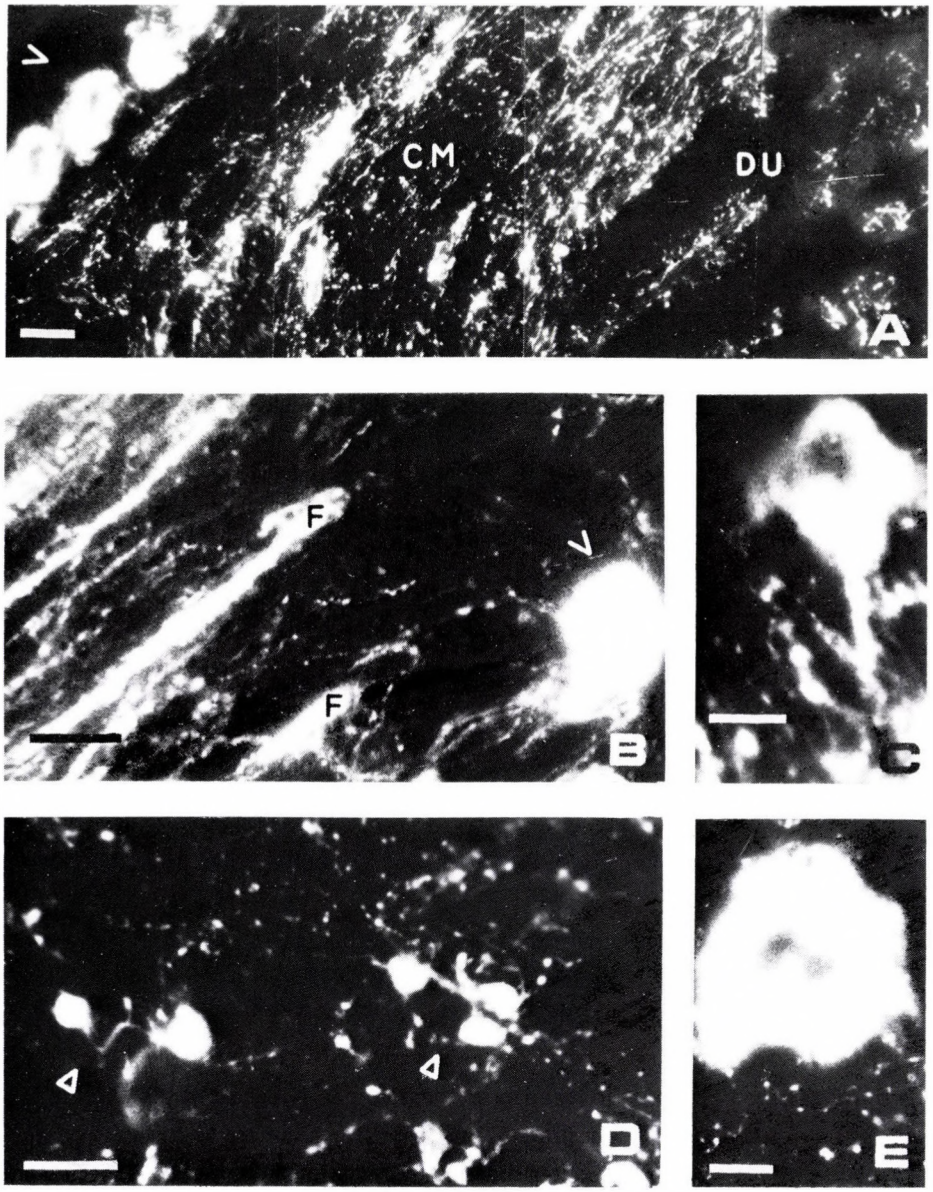

Fig. 1. CA-containing nerve elements in the wall of vaginal portion of large hermaphrodite duct in A. depilans (A,B,D,E) and A. fasciata (C). A: transverse section of the duct wall. A cluster of four large neurons (angle mark) is situated near the circular muscle layer (CM) which is densely innervated by CA-containing fibers. B: a single large nerve cell body (angle mark) and thick processes (F) of neighbouring large cells which form bundles of CA-containing fibers. C, E: Large neurons and varicosities in the longitudinal muscle layer. D: small nerve cells (triangles) in the circular muscle layer. DU – the duct. Bar in (A,B) = 100 μm; Bar in (C,D,E) = 50 μm

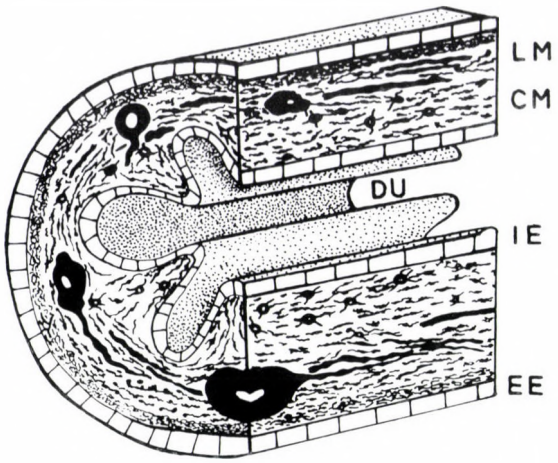

Fig. 2. Scheme summing up the location of CA-containing nerve elements in the wall of vaginal portion of the large hermaphrodite duct near the common genital aperture in the two Aplysia species. DU – the duct; EE – external epithelium; IE – internal epithelium; LM – longitudinal muscle layer; CM – circular muscle layer

attached where is posterior. Numerous muscles are attached to the walls of the sheath. From the genital orifice a shallow ciliated groove, the seminal groove, runs forward to the penis. Along the groove the sperm passes from the common genital aperture to the penis. The seminal groove terminates close to the tip of the penis /3, 4/.

Nerve elements with bright green fluorescence characteristic of CA densely innervate the muscle wall and subepithelial region of the penis and its sheath in both species. Two types of CA-containing nerve cells have been found here. The neurons differ in size and location: there are relatively large multipolar neurons 40–80 μm in diameter, and small 5–20 μm bi- or multipolar cells (Figs 3A, B, D; 4). In A. depilans the former are rather rare and localized in the penis muscle wall and its sheath. They do not form clusters. The latter are numerous in the penis muscle wall and practically absent in the sheath of the penis (Fig. 4A). In A. fasciata, unlike A. depilans, a cluster of large CA-containing neurons (10–15 cells) can be seen in the site where the penis is attached to its sheath. Besides this cluster, single neurons of this chemical type were found along the sheath spermatic groove (Fig. 3B; 4B). Small nerve cells, containing CA, are revealed in this species only in the penis sheath and are absent in the penis (Fig. 4B). The processes of large neurons form dense bundles of

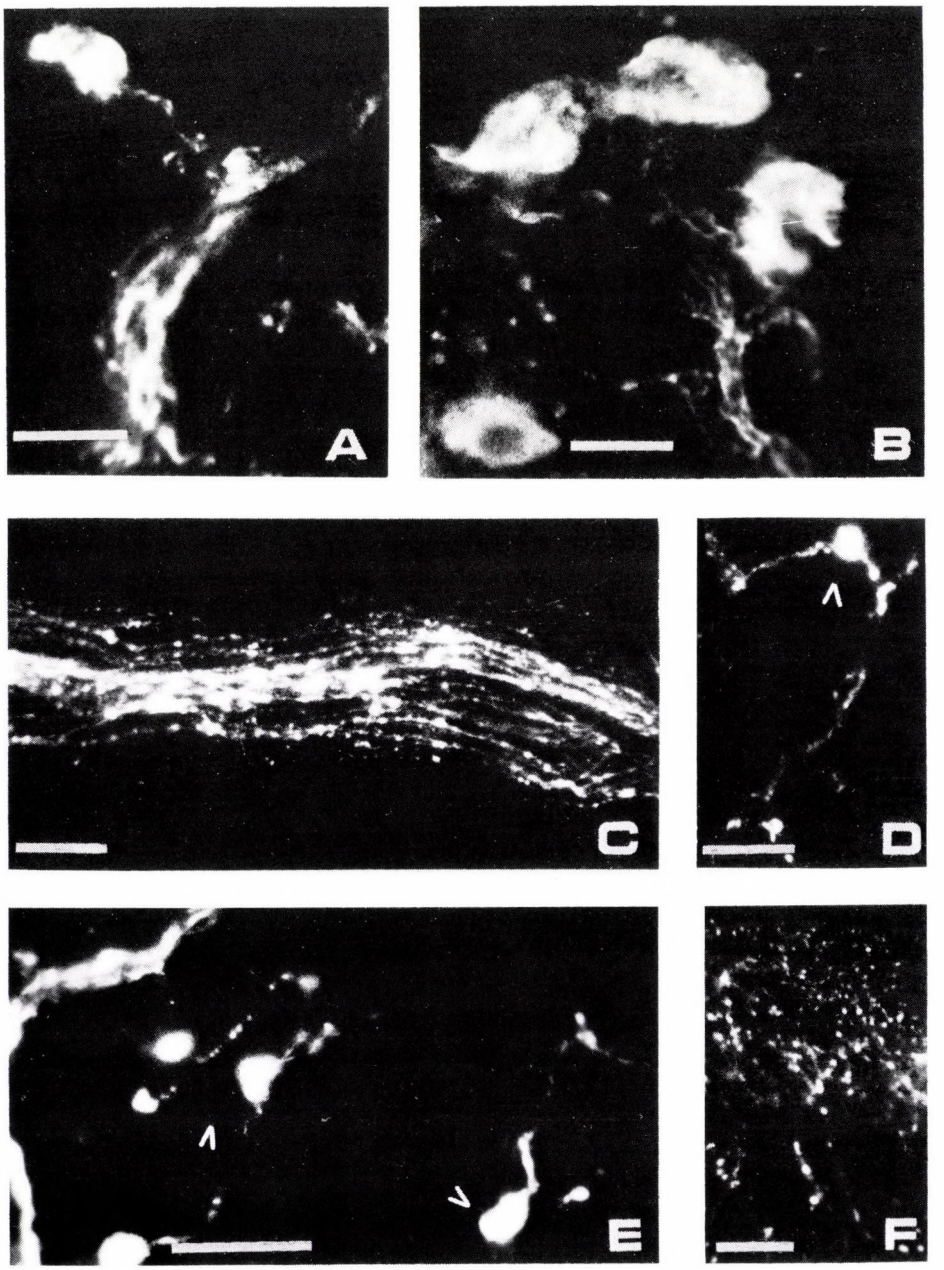

Fig. 3. CA-containing neurons and fibers in the penis (A,C,D), its sheath (B,F) and the gill (E) of A. depilans (A,C,D) and A. fasciata (B,E, F). A: large nerve cell sending its process to the thick bundle of axons. B: a cluster of four large neurons. C: thick bundle of varicose fibers. D: small multipolar nerve cell (angle mark) with varicose processes. E: small neuron (angle marks) within the gill musculature. F: dense subepithelial plexus composed of fine varicose fibers. Bar = 50 µm

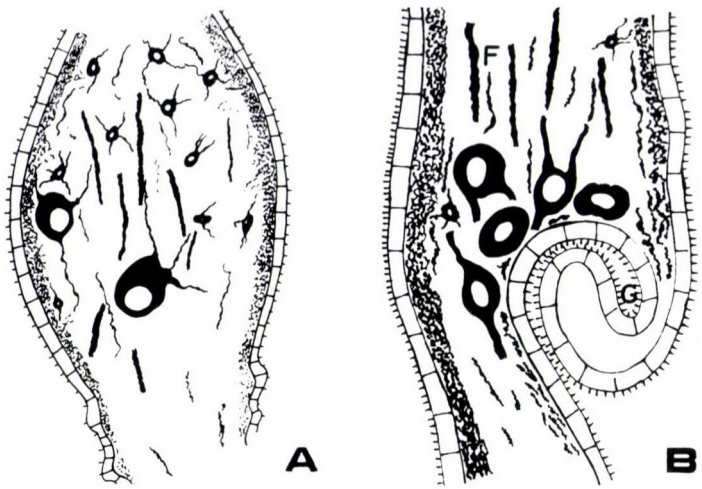

Fig. 4. Schemes of the location of CA-containing nerve elements in the penis of A. depilans (A) and in the penis sheath of A. fasciata (B). Schemes are based on the longitudinal sections of the organs. Cluster of large neurons has been located near spermatic groove. F – nerve fibers; G – spermatic groove

varicose axons in the internal muscle layer (Figs 3A,C; 4) and seem to run to the CNS. Single varicose fibers run from these bundles to the external epithelium and form a dense subepithelial plexus (Fig. 3F).

The penis retractor muscles are abundatly innervated by varicose CA-containing fibers. Numerous yellow fluorescent axons were seen in the nerves running in the direction of the retractors and in the adjacent connective tissue.

3. Gill, tentacles and parapodia

In the gill musculature, numerous green fluorescent fibers were revealed in both A. depilans and A. fasciata. This corresponds to what Swann et al. /16/ had previously found in the gill of A. californica. A striking difference is, however, that in our material numerous green fluorescing neuron perikarya, 5–20 µm in diameter, were also seen in the gill muscle (Fig. 3E).

In the tentacles and parapodia, CA-containing nerve fibers were comparatively rare. No fluorescent cell bodies could be found in the tissue samples of these organs.

DISCUSSION

Our results show that some of the organs of both Aplysia depilans and A. fasciata are densely innervated with CA-containing nerve fibers which originate from local cell bodies. This is characteristic to the investigated parts of the reproductive system, which are especially rich in CA-containing nerve elements. CAergic innervation of the musculature is also dense in the gill of the two species. In A. californica, dopamine is assumed to be the excitatory transmitter in the gill musculature, and the abdominal ganglion has been claimed to be the site of origin of dopaminergic fibers supplying the gill /16/. This interpretation is in obvious disagreement with the absence of revealable CA fluorescence in the gill influencing neurons of the abdominal ganglion /13, 16/. The finding of numerous green fluorescing neuron perikarya within the gill in A. depilans and A. fasciata indicates that the situation might be similar in A. californica.

The penis and the vaginal portion of the large hermaphrodite duct are believed to have a pedal origin in many gastropods /3, 8/. In a primitive prosobranch, Acmaea, in which the pedal ganglia are not yet developed, small CA-containing neurons have been found in the foot and in the muscular wall of the penis which is also a pedal derivative /11/. Similarly, numerous green fluorescing nerve cells and fibers have been found in the foot and penis of an advanced opisthobranch, Dendronotus /11/ whilst no CA cells have been demonstrated in the pedal ganglia of this nudibranch /11/ as well as in that of a related species, Tritonia diomedea /7/. The pteropod mollusc Clione limacina is another opisthobranch which has just a few central and numerous peripheral CA neurons including a considerable cluster at the base of the penis /5/. On the contrary, in pulmonate gastropods the CAergic innervation of the foot and the penis seems to be of a central origin and, correspondingly, numerous green fluorescing perikarya have been found in the pedal ganglia of pulmonates /1, 9, 10, 11/. Aplysia may represent a somewhat intermediate pattern. It contains clustered green fluorescent neurons in the pedal part of its CNS /13/ and, at the same time, CA neurons are abundant in a peripheral pedal derivation, as can be seen from the present study.

The branching pattern of CA neurons in the subepithelial layer in the genital organs of Aplysia indicates a sensory function. This suggestion is in accordance with previous neurophysiological and pharmacological evidence of the mechanosensory function of CAergic neurons in gastropod

molluscs /5, 12, 15/. At the same time, there appears to be a considerable involvement of CA-containing axons in muscle innervation, at least in the gill and penis wall of Aplysia. Experimental evidence of dopaminergic innervation of the gill musculature in A. californica /16/ has been mentioned already. We observed that dopaminergic drugs have a strong action on penis muscles in both A. depilans and A. fasciata. There seems little doubt that dopamine is a neuromuscular transmitter in various muscles of Aplysia, and that peripheral green fluorescing neurons are the main source of this muscle innervation. It remains unclear, however, if sensory and motor functions are combined in the same neurons or there exists a functional specialization of CAergic neurons. Large CAergic neurons of the reproductive system described in this paper seem to represent a convenient "model" cell to elucidate this problem.

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CALCIUM IN HIPPOCAMPUS FOLLOWING LIDOCAINE INDUCED SEIZURES: AN ELECTRON CYTOCHEMICAL STUDY

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The effect of lidocaine seizures on cellular accumulation of calcium was studied in hippocampal subfields CA1 and CA3 and the dentate gyrus of rats, using the combined oxalate-pyroantimonate method. The specificity of the reaction was ascertained by EGTA treatment and X-ray microanalysis. In control rats, calcium was visualized between myelin lamellae of axons, in synaptic vesicles and in some lysosomes.Two hours after onset of lidocaine seizures selective neuronal degenerations appeared in hippocampal subfields CA1 and CA3 but not in the dentate gyrus. Calcium deposists were present in numerous mitochondria of pyramidal cells and, occasionally, also of neuroglial cells. Many of these mitochondria exhibited ultrastructural alterations. Calcium uptake was most prominent in the CA3 sector but was also present in the CA1 subfield as well as the dentate gyrus. Intracellular calcium uptake, in consequence, is not the unique attribute of selectively vulnerable hippocampal neurons.

Keywords: Calcium – hippocampus – lidocaine – electron microscopy – cytochemistry

INTRODUCTION

Lidocaine, a local anaesthetic and anti-convulsant drug /1/, is known to produce seizure activity at high dose /11, 13, 14, 18/. Previous electrophysiological studies with ion-sensitive electrodes have shown that extracellular calcium activity significantly decreases during paroxysmal

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activity /10, 17/ and it has been suggested that the resulting increase in intracellular calcium activity is responsible for inducing irreversible cell damage /8/. This is supported by the observation that so-called selectively vulnerable neurons of hippocampus exhibit pronounced mitochondrial sequestration of calcium after allylglycine or bicuculline-induced status epilepticus /8, 16/. It cannot be excluded, however, that intracellular calcium accumulation is an epiphenomenon of increased neuronal activity /10/ and is not the reason for cell death after status epilepticus.

In the present study this question was dealt within the lidocaine-induced seizure model /11, 13, 14, 18/. The electron microscopical oxalate-pyroantimonate technique /2, 22/ was used for visualizing intracellular calcium accumulation in hippocampal subfields CA1 and CA3 which are known to exhibit selective vulnerability to epilepsy /8, 16/. The same technique was also applied to the dentate gyrus which is resistent. The results obtained indicate that intracellular calcium accumulation is, in fact, a generalized phenomenon which is not restricted to selectively vulnerable neurons.

MATERIALS AND METHODS

Adult female BD IX rats weighing 200–250 g were used. Three animals served as controls and 5 were subjected to lidocaine-induced seizures. The animals were lightly anaesthetized with a gas mixture containing 1.5% halothane and 70% nitrogen, the rest being oxygen. Body temperature was maintained at 37 ^{o}C with a feedback-controlled infrared heating lamp. Following tracheotomy, the animals were immobilized with d-tubocurarine (Pancuronium, 1.5 mg/kg) and mechanically ventilated. Tidal volume was adjusted to keep a PCO_2 close to 40 mmHg, and a PO_2 above 100 mmHg. Arterial blood pressure and the electrocardiogram (EEG) were continuously monitored. EEG activity was recorded from the frontal-parietal region with silverball electrodes mounted on the exposed calvarium.

Fifteen minutes before administration of lidocaine, halothane concentration was diminished to 0.4% and maintained at this level for the rest of the experiment. Seizures were induced by intravenous injection of an aqueous solution of 15 mg/kg lidocaine (20 mg/ml H_2O) and maintained for a duration of 2 h with additional doses of 5 mg/kg when necessary. Control animals received the same amount of Ringer's solution.

For electron-cytochemical visualization of calcium the combined oxalate-pyroantimonate precipitation method was used /2, 22/. Subfields CA1 and CA3 of the hippocampus, and the dentate gyrus were dissected from araldite-embedded vibratome sections; ultra-thin sections were prepared with a Reichert OMU 2 microtome, and stained with 0.5% uranyl acetate and 0.4% lead citrate. Semi-thin sections were stained with methylene blue. The specificity of the cytochemical reaction was tested by treating specimens from each tissue block with 5mM EGTA for 1 h at 60 ^{o}C /5/; EGTA treatment,

in turn, was controlled by incubation of sections for 1 h at 60 ^{o}C in distilled water. A standard electron microscope (Zeiss EM 9) was used for examination of specimens.

X-ray microprobe analysis of ultra-thin sections was carried out using a JEOL ASID-I scanning unit and an EDAX 183B Si(Li) detector attached to a JEOL JEM 100B electron microscope, as described in detail earlier /20, 21/.

RESULTS

Intravenous injection of lidocaine, after a delay of a few seconds, caused cardiac arrhythmia, decrease of arterial blood pressure by about 50 mmHg, and the appearance of high voltage slow wave EEG activity. About 25 min. later, a burst suppression pattern of EEG activity appeared which persisted throughout the two hours' observation period (Fig. 1).

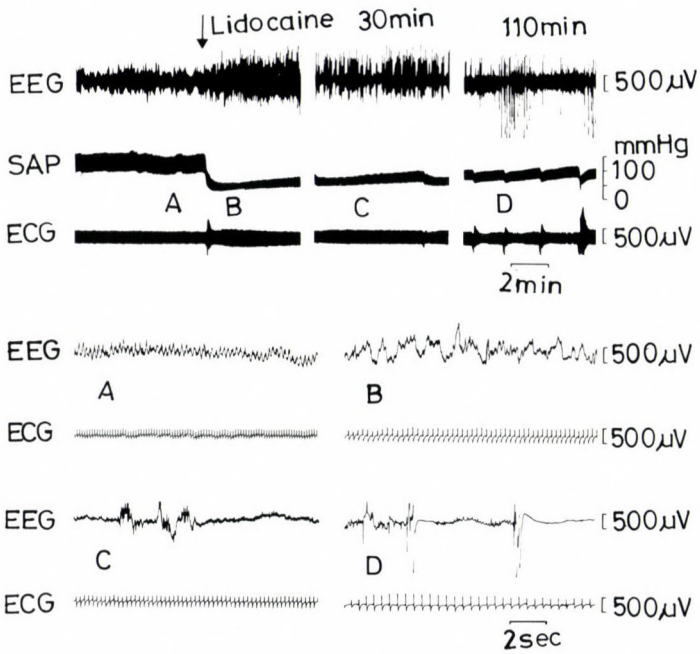
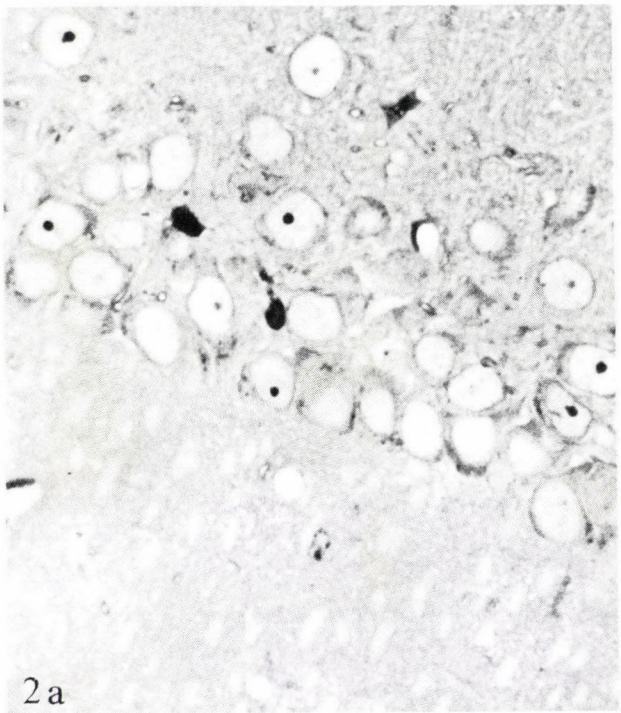


Fig. 1. Lodicaine-induced seizure of rat. Recording of electroencephalogram (EEG), systemic arterial pressure (SAP) and electrocardiogram (ECG) before (A) and at various time-points (B-D) after intravenous injection of lidocaine (marked by arrow). Note initial slowing (B), followed by burst-suppression pattern of EEG (C and D)

Light microscopy of semi-thin sections of the hippocampus revealed circumscribed foci with degenerative changes of pyramical cells in the CA1



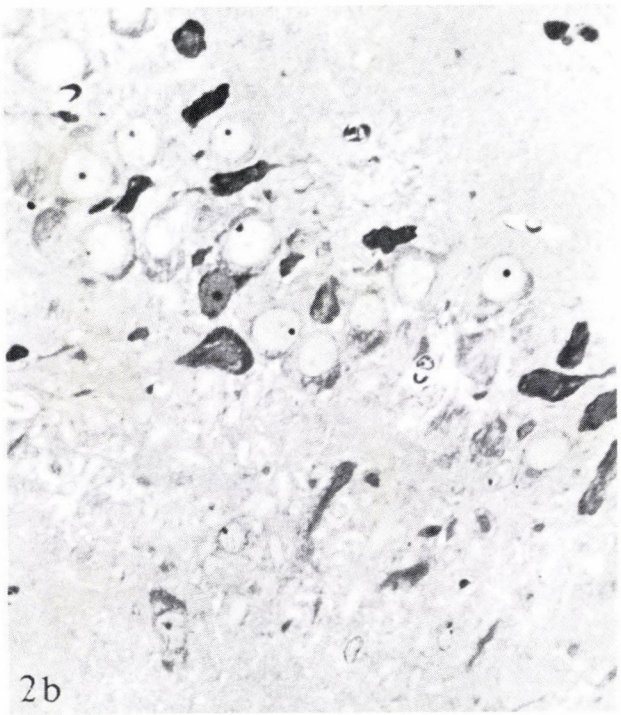


Fig. 2. Semi-thin sections of hippocampal subfield Cal. a) Control rat, b(after 2 h lidocaine seizures. Note pyramidal dark neurons. Methylene blue staining x520

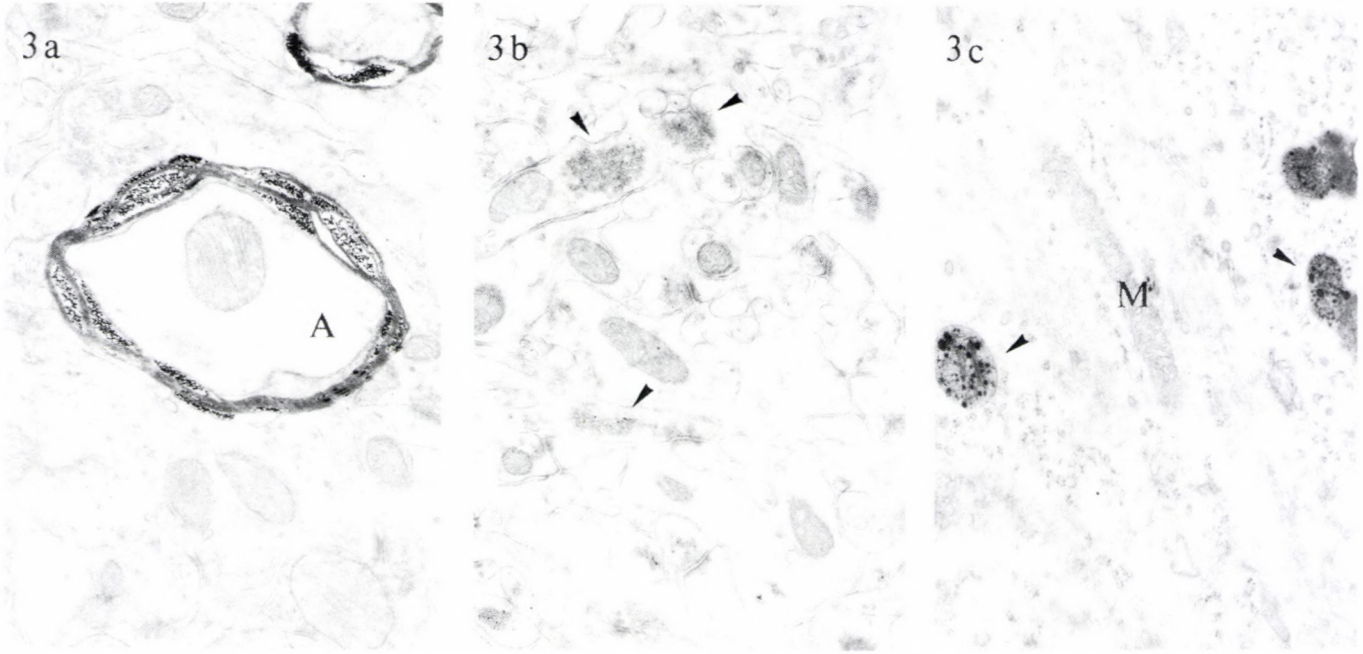


Fig. 3. Electron cytochemical localization of calcium in pyramidal cells of hippocampal subfield CA1 of control rat (oxalate-pyroantimonate technique; calcium appears as black precipitates). a) Accumulation between myelin-lamellae; b) localization in the center of synaptic vesicles (arrowheads); c) accumulation in lysosomes (arrowheads). A: axon, M: mitochondrium. x23 000

and CA3 subfields (Fig. 2). These changes consisted of shrinkage and darkening of the cytoplasm, cork-screw alterations of proximal axons, and appearance of perinucleolar vacuoles. In control rats and in the dentate gyrus of both control and epileptic rats such changes were absent.

Intracellular calcium was visualized by electron microscopy, using the oxalate-pyroantimonate precipitation method. Specificity of the reaction was controlled by X-ray microanalysis which confirmed the presence of calcium in the precipitates (Fig. 7). Further evidence for specificity was provided by treating tissue sections with EGTA which, in contrast to distilled water, led to the disappearance of precipitates.

In control animals, small amounts of calcium were detected between myelin lamellae of axons, in synaptic vesicles and, occasionally, in lysosomes (Fig. 3). After 2 h of lidocaine seizures precipitation of calcium was much more pronounced (Figs 4–6). Precipitates were regularly seen in mitochondria, particularly in dendrites, where they were frequently associated with ballooning of cristae (Fig. 4). Other locations were the cytoplasm of astroglial cells and lipofuscin granules of microglial cells (Fig. 5). Occasionally, free cytoplasmic calcium deposits were seen in dendrites (Fig. 6); these precipitates were also associated with ultrastructural alterations. Changes were most pronounced in the CA1 and CA3 subfields of the hippocampus, but they were also present in the dentate gyrus (Fig. 6). Calcium precipitates, in consequence, were much more widespread than the light microscopical changes which were confined to subfields CA1 and CA3 of the hippocampus.

DISCUSSION

Calcium-mediated processes have been considered by several investigators as a common denominator of irreversible cell damage under various pathological conditions /3, 7, 12, 19/. In the healthy cell, cytosolic calcium activity is extremely low, and any increase is immediately reversed by sodium/calcium exchange, activation of ATP-dependent calcium pump, or by sequestration in synaptic vesicles, endoplasmic reticulum and mitochondria /4, 15/. These mechanisms, however, operate only as long as intracellular calcium influx remains within certain limits. In situations such as ischemia or epilepsy calcium fluxes across voltage-dependent channels dramatically increase as evidenced by the sudden fall of extracellular calcium activity

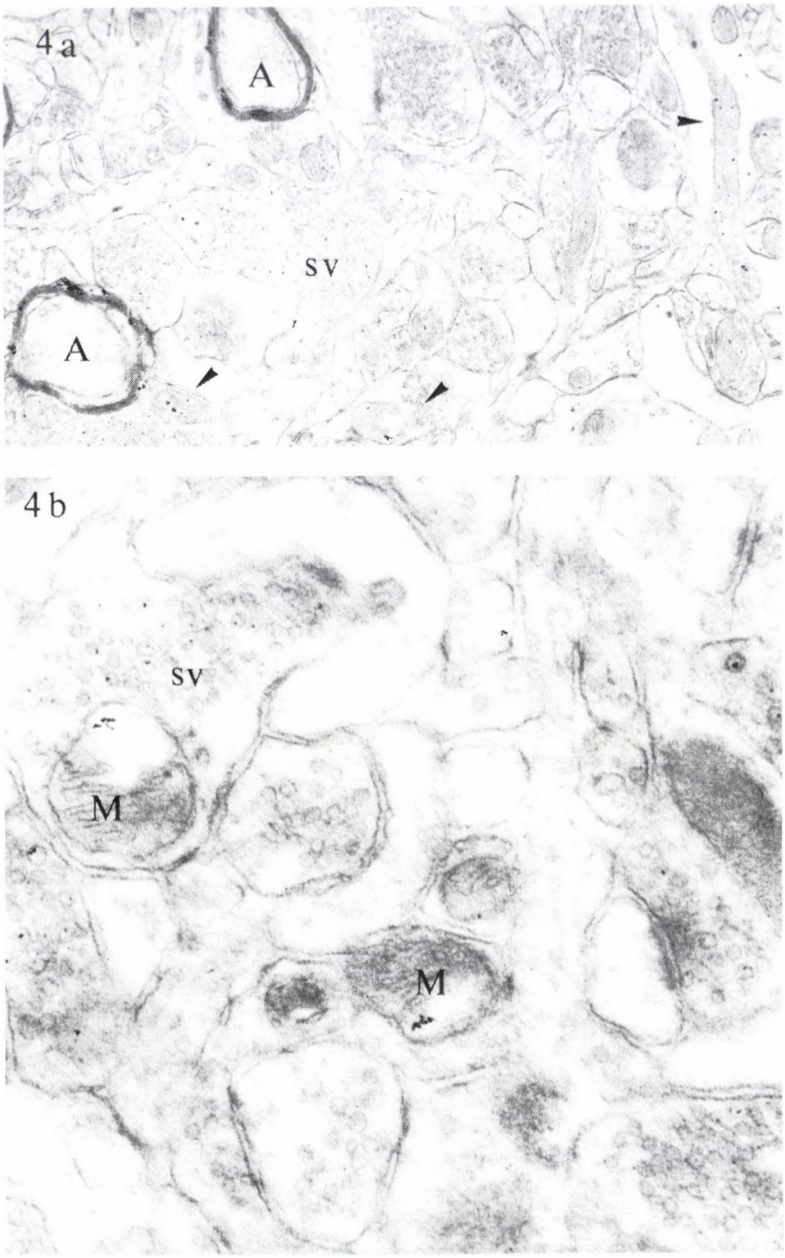


Fig. 4. Calcium accumulation after 2 h lidocaine seizures of rat. a) Hippocampal subfield CA3 Intramitochondrial deposits are marked by the arrows x23 000; b) hippocampal subfield CA1: note ballooning of mitochondria at the site of calcium sequestration. A: axon, M: mitochondria, SV: synaptic vesicles x62 000

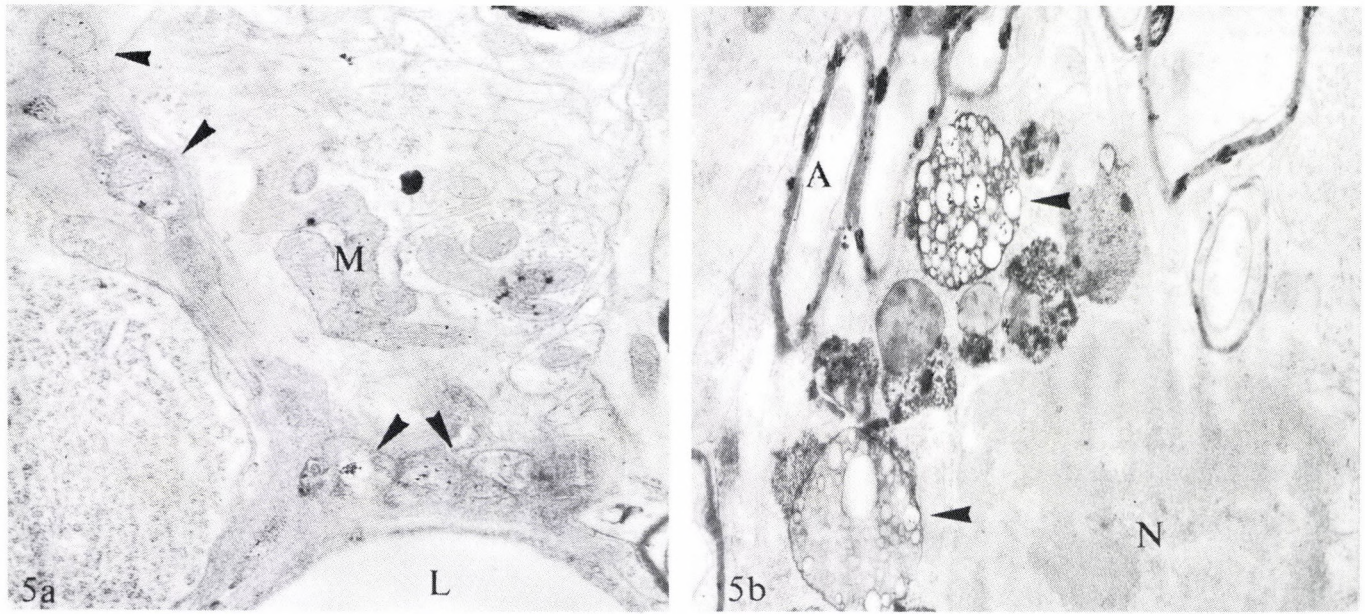


Fig. 5. Calcium accumulation in neuroglia after 2 h lidocaine seizures of rat. a) Perivascular process of astrocyte (arrowheads); b) lipofuscin granules in microglial cell (arrowheads). A: axon, L: vessel lumen, M: mitochondrium, N: nucleus x23 000

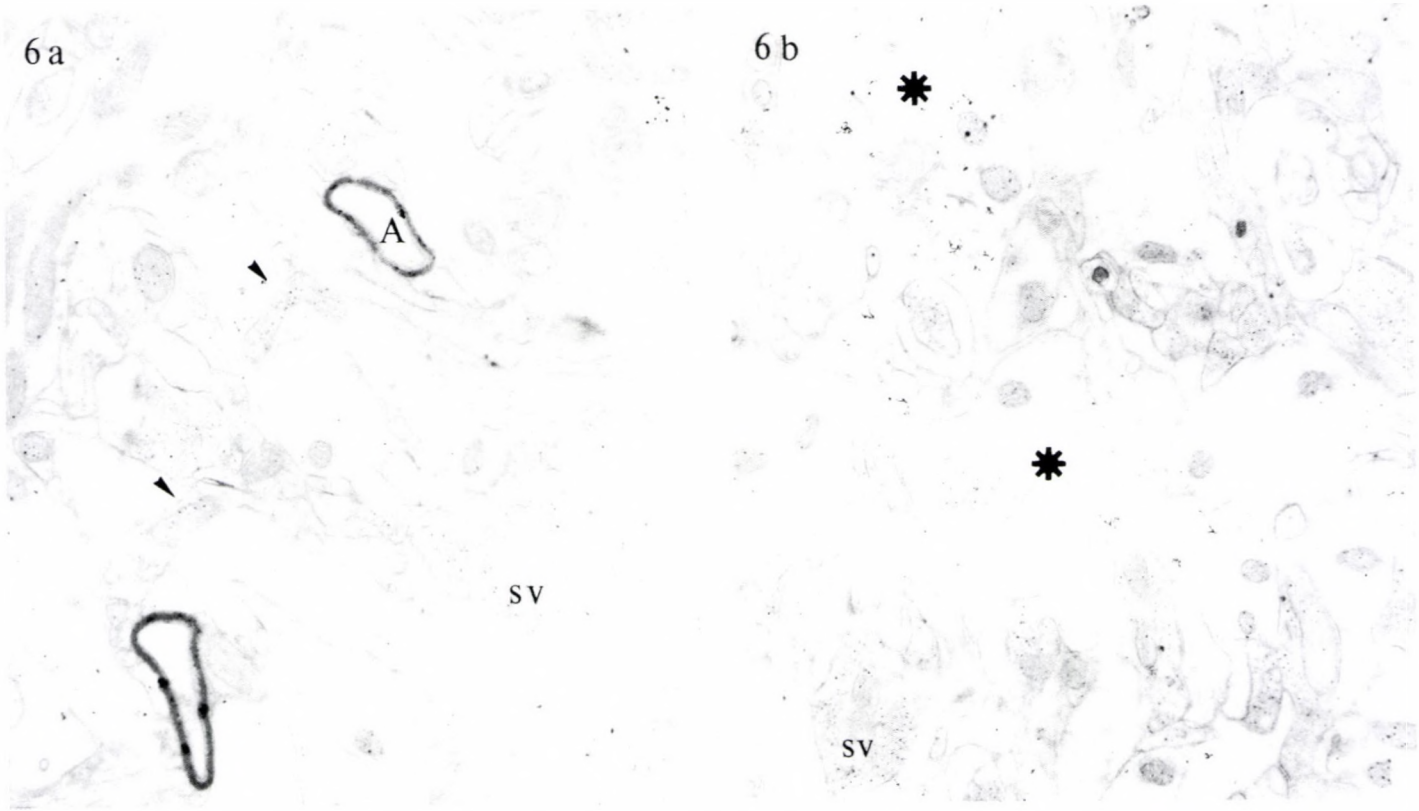


Fig. 6. Calcium accumulation in dentate gyrus after 2 h lidocaine seizures of rat. a) Mitochondrial calcium sequestration (arrowhead); b) free cytosolic calcium in dendrite (asterix). A: axon, SV: synaptic vesicles x23 000

/9, 10/. The resulting increase in intracellular calcium is thought to overload the ion-regulating homeostatic mechanisms, leading to irreversible damage of calcium-sensitive cell structures, such as the mitochondria /3, 8, 15/.

Evidence for this pathomechanism derives mainly from in vitro experiments in which extracellular calcium, and in consequence intracellular calcium influx, can be manipulated /7/. A direct cytochemical evaluation of intracellular calcium accumulation in vivo has become possible only recently /2, 22/. This method relies on the precipitation of an electron-dense reaction product which appears when calcium is exposed to oxalate and pyroantimonate, and which can be detected by conventional electron microscopy. Application of this technique to heart or brain ischemia /3, 6/, and to experimental epilepsy induced by bicuculline or allylglycine /8/ revealed accumulation of calcium precipitates in the mitochondria, thus supporting the calcium hypothesis.

The present study provides evidence of intracellular calcium accumulation in another epilepsy model, i.e. lidocaine seizures. This model has been previously used for the investigation of hemodynamic and metabolic alterations /1, 14, 18/. One of the main findings was a dissociation between reduced blood flow and increased glucose utilization in the hippocampus /14/. Uncoupling was most prominent in hippocampal subfields CA1 and CA3, but was absent in the dentate gyrus. The light microscopical changes observed in the present study follow this pattern: neuronal lesions appeared in

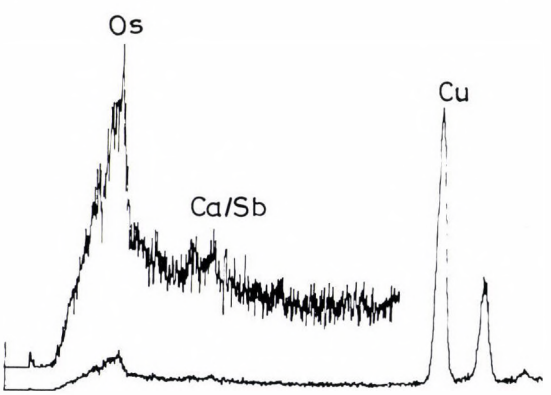


Fig. 7. X-ray microanalysis of electron-dense precipitate in mitochondrium after 2 h lidocaine seizures of rat (same spectrum shown at two different sensitivities). Os: Osmium peak (acquired during fixation); Ca/Sb: calcium/antimony complex; Cu: copper (from grid)

subfields CA1 and CA3 but not in the dentate gyrus. It is, therefore, reasonable to assume that uncoupling between blood flow and metabolic activity is one of the factors responsible for structural damage.

The role of calcium in this process remains unclear. In the present study mitochondrial calcium uptake could be detected in the regions where morphological changes occurred, but there was also distinct accumulation of calcium in the dentate gyrus where these changes were absent. Mitochondrial calcium sequestration, in consequence, seems to be a generalized effect induced by epileptic seizures, but does not produce irreversible cell damage per se. It cannot be exluded that calcium-mediated structural changes occur only if a certain threshold of ion activity is exceeded, but it is difficult to understand why this threshold should be lower in the CA1 and CA3 subfields than in the dentate gyrus. It is, therefore, more likely that intracellular calcium uptake is only one among other still unknown factors responsible for the phenomenon of selective vulnerability.

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THE BEHAVIOUR OF THE PARADISE FISH (MACROPODUS OPERCULARIS) IN TWO DIFFERENT OPEN-FIELDS. A CORRELATION STUDY

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The behaviour of the paradise fish in a traditional "closed" and in a new "transparent" open-field was investigated. The traditional way of measuring ambulation scores was extended by recording ethologically defined behaviour units. The correlations found between the scores measured in the "closed" field and those measured in the "transparent" field are discussed in this paper.

Keywords: Paradise fish — open-field — behaviour elements

INTRODUCTION

Ethology has provided several methods of observing animal behaviour /3/, but studies by comparative animal psychologists are generally done on a few species, usually laboratory rodents only /2/. It is obvious that s ides of species living in entirely different environments will enhance the validity of theoretical generalizations. Beside studying a new vertebrate species, we also aimed at including ethologically defined behavioural units so as to investigate their correlations with the traditionally used open-field scores.

The paradise fish is a relatively unknown vertebrate species in animal behaviour studies, but it has lots of advantages over other traditionally used animals, e.g., rodents. It is a diurnal animal with a well developed communication system, therefore the changes in its inner state are strongly reflected in its observable behaviour, and are clearly visible. Being a simpler vertebrate its behaviour is less complex, thus it

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can be described precisely by using ethologically defined behaviour units. Finally, one of the most important advantages of the paradise fish is that its laboratory environment, the aquarium, can be maintained and controlled in complete isolation from the observer. An ethological code system for recording the behaviour elements of the paradise fish in seminatural and laboratory environment was defined and the various relationships between these elements were studied earlier /8, 9, 16/. Using this ethological code an analysis of predator avoidance /4, 5, 7/, effects of drugs /11/, an ethological analysis of the effect of novel environment /6/, and an etho-genetic analysis of strain differences /13, 10/ were performed.

In the present experiment we studied the paradise fish in two open-fields, and described their behaviour by traditionally used open-field ambulation scores and at the same time by a sequence of spontaneously emitted ethologically defined behaviour units. To be able to record these behaviour units, the animals were observed from the side. To do so we had to modify the traditionally used /13/ open-field tank, i.e., we had to leave one side of the open-field tank transparent. The behavioural differences of the paradise fish caused by the difference between the two types of fields, the closed and the transparent tank, were investigated. The results of the present study are to be exploited in a subsequent etho-genetic analysis of a classical cross system consisting of two highly inbred strains of paradise fish and their several crosses.

MATERIAL AND METHODS

Animals and housing

An outbred population of paradise fish bred in our laboratory was tested. (The origin and maintenance of this stock were described earlier /13/.)

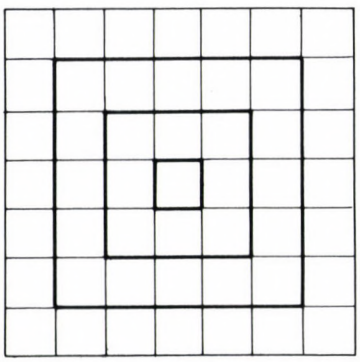
The fish were raised in groups of thirty in (60x40x35 cm), 80 l glass aquaria in well filtered water. Each unit contained water plants (Hygrophyla polysperma), planted in clay pots. The temperature (28 ^{0}C) was held constant, and a 14/10 h light-dark cycle was maintained. A radio program was transmitted as a constant noise to reduce the accidental disturbances of fish. The animals were fed daily on laboratory made fish food, consisting of beef liver, hake, eggs, wheat bran and vitamins. The 120–180 days old, fully mature fish were taken to the recording room where every condition was identical with that of rearing, except that they were housed individually in (30x15x15 cm) 6 l aquaria for three days. After the three-day habituation period every fish was recorded in the "closed" open-field for 15 min, and after

another three days the recording session was repeated in the "transparent" open-field. Between the two tests all the fish were housed in their individual aquaria. In separate preliminary experiments no detectable signs of habituation could be found owing to the first test.

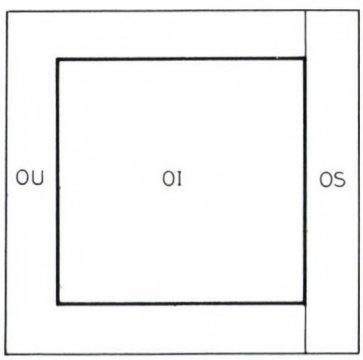
Apparatus

The "closed" open-field tank was a 70x70x20 cm glass aquarium painted white. The "transparent" tank was of the same size and painted on all but one side. The same network of squares (10x10 cm) was painted on the bottom of the two types of fields (Fig. 1). In the case of the "closed"

The bottom pattern of the open-field



The measures (ambulation scores) of the open-field



OO=OU+OI

Fig. 1.

Experimental arrangement

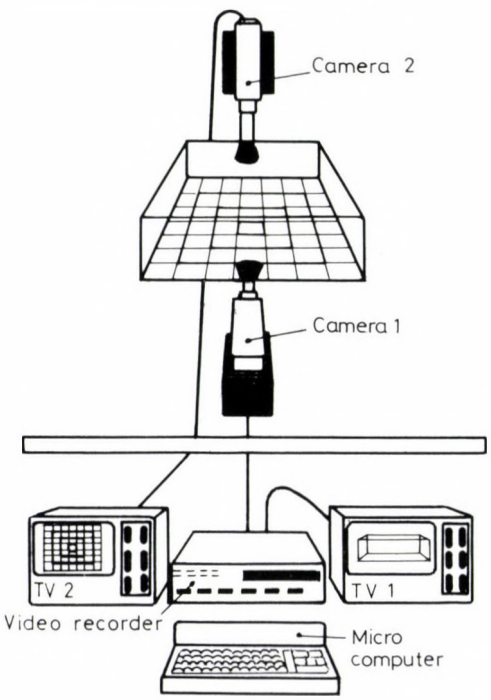


Fig. 2.

field one TV camera was placed above, and in the case of the "transparent" field, another camera was aimed at the transparent side (Fig. 2). The tanks were illuminated from above by white phototubes and filled with water of temperature 28 ^{0}C. The fish were netted individually into a clay pot and were placed into the center of the open-fields.

Behavioural measures

Latency time to emerge (LATC and LATT for the "closed" and "transparent" field, respectively) from the clay pot was recorded and the animal's movements were monitored for 15 min. The number of squares entered (ambulation score) in the various parts of the open-fields were recorded. Designations are as follows: OIC is the ambulation score in the inner part of the "closed" tank. OIT is the ambulation score in the inner part of the "transparent" tank. OOC is the ambulation score in the outer part of the "closed" tank. OOT is the ambulation score in the outer part of the "transparent" tank. The OOT score was further divided and assigned as OUT and OST. OUT is the ambulation score in the "U" segment of the outer part of the "transparent" tank (Fig. 2). OST is the ambulation score in the segment of the outer part of the "transparent" tank located next to the transparent glass side (Fig. 2).

For the behavioural units, the relative duration (a) or frequency (b) were measured as follows:

[a]Move MOV: slow, even locomotion without using caudal fin.

[a]Swim SWI: fast locomotion.

[a]Escape ESC: rapid to and fro movement, with forceful swimming perpendicular to the transparent glass side.

[a]Staccato STA: a series of quick starts and sudden stops during locomotion.

[a]Creeping CRE: very slow swimming, the fish is propelled forward only by pectoral fin fanning, all other fins are closed.

[b]Leaping LEP: an extremely quick move and a sudden halt without any specific orientation.

[a]Erratic movement ERA: extremely quick zig-zag-like locomotion performed on the bottom.

[b]Pick PIC: oriented movement with the jaw streched out to get some small pieces of food or visible spots in the environment.

[b]Air gulp A-G: being an anabantoid fish, the M.opercularis swims from time to time to the surface and gulps air to take in oxygen.

[a]Floating FLO: the immobile animal is floating under the surface by fanning its pectoral fins; the animal stays in position.

[a]Hanging in midwater HIM: as FLO, but in the middle range of the water.

[a]Resting RES: as FLO and HIM, but staying on the ground. The anal or caudal fin touches the bottom of the tank.

[a]Oblique plan position OBQ: the body axis is inclined approximately 20-30 degrees from the horizontal plane. The fish stays near the wall, often in a corner. Dorsal, caudal and anal fins are closed, the pectoral fins are fanning.

[a]Freezing FRZ: the fish is motionless, only the gills and occasionally the eyes move.

RESULTS

Means and standard deviations of the behaviour measures are shown in Table I. Correlations among open-field parameters and behavioural units were calculated by Spearman Rank Correlation Test, and are shown in Table II. The correlations can be interpreted in two different contexts: first, correlations between the scores of the first and second recording session; second, correlations between the scores of the second recording session. The latter interpretation will be discussed in another paper in the frame of the subsequent etho-genetic analysis.

Table I

	Measure	Mean	SD
Measured in the "transparent" open-field	ESC	30.1	14.3
	SWI	26.1	8.8
	MOV	20.5	10.6
	STA	10.5	11.1
	ERA	0.01	0.05
	CRE	4.5	4.8
	PIC	4	8
	A-G	29	9
	LEP	1	1
	FLO	3.8	2.6
	HIM	1.1	0.9
	RES	0.2	0.2
	OBQ	2.4	3.1
	FRZ	0.1	0.2
	LAT-T	26.1	24.2
	OIT	71	20
	OOT	353	147
	OUT	134	51
	OST	219	132
	OIT+OOT	424	149
Measured in the "closed" open-field	LAT-C	17.8	11.8
	OIC	102	44
	OOC	310	201
	OIC+OOC	412	224

Sample size=19

Table II

	ESC	SWI	MOV	STA	ERA	CRE	PIC	A-G	LEP	FLO	HIM	RES	OBQ	FRZ	LATT	OIT	OOT	OUT	OST	OIT+OOT	LATC	OIC	OOC	OIC+OOC
OIC+OOC	0.70		0.67			0.48				0.66							0.74		0.68	0.72				
OOC	0.74		0.61			0.49				0.67							0.77		0.75	0.75		0.60		
OIC			0.56							0.50	0.57													
LATC									0.53						0.65									
OIT+OOT	0.83	0.57	0.48	0.49		0.55																		
OST	0.92		0.48			0.53				0.47														
OUT		0.82																						
OOT	0.81	0.56	0.48			0.55																		
OIT																								
LATT								0.57	0.56															
FRZ																								
OBQ	0.49	0.51				0.70	0.55																	
RES								0.49																
HIM				0.56						0.57														
FLO	0.53		0.47																					
LEP																								
A-G																								
PIC						0.62																		
CRE	0.72																							
ERA																								
STA		0.54																						
MOV																								
SWI																								
ESC																								

$r_s > 0.693$ $p < 0.01$

$r_s > 0.575$ $p < 0.1$

$r_s > 0.455$ $p < 0.05$

The correlation between the total sum of the ambulations measured in the "closed" tank (OIC+OOC) and the "transparent" tank (OIT+OOT) was significant. Significant correlation was found between the OOC score and the OOT score as well. These findings seem to support the assumption that the two apparatuses are similar in measuring the overall activity, as well as the acitivity performed in the outer part of the fields. But the non-significant correlation between OIC and OIT shows that the two apparatuses are not identical.

In the case of the traditional "closed" open-field, two uncorrelated parts of the field were differentiated /13/, an inner and an outer area. In the case of the "transparent" field, three different parts were found to be uncorrelated, OIT, OUT and OST. We can suppose that the high correlation between the ambulation score recorded in the outer part of the "closed" field (OOC) and the ambulation score recorded at the transparent side of the "transparent" field (OST) means that they measure similar behavioural character of the fish. The strong correlation found between OST and the ESC unit and between OOC and the ESC unit means that the fish performs the escape behaviour mostly at the transparent glass wall and in the case of the "closed" field at the outer area. The OUT and OIT ambulation scores do not correlate with any ambulation scores of the "closed" field, thus they can be considered to measure new behavioural character of the fish. The OIT score does not correlate with any behaviour units either. This means that the animals do not show any particular behaviour more frequently or for a longer period when in the inner part of the field. The strong correlation between the OUT score and the SWI unit supports the idea of fish swimming mostly in the "U" segment of the "transparent" field, and escaping only at the transparent side, while in the case of the "closed" field the fish show the escape behaviour in the whole outer part. The MOV unit is connected with slow locomotion, thus it is not surprising that its correlation with ambulation scores is negative. The situation is quite the same in the case of the CRE unit. The STA, PIC, A-G and LEP units do not correlate with the ambulation scores. The correlations of the FLO unit with all the ambulation scores of the "closed" field are negative, and this is also the case in respect to the OST score. The HIM unit correlates with the OIC score only. The other behaviour units do not show any significant correlation with the ambulation scores.

DISCUSSION

The use of the traditional open-field test is widespread in the field of animal psychology /3/ and there are some preliminary works done by ethologists on chickens /12, 14, 15, 19, 20/, ducks /17/, mice and rats /18, 22/, guinea pigs /21/ and other non-domesticated rodents /23/.

Open-field as an "environment" can ethologically be conceptualized as a dangerous place from where the animals try to escape in order to reinstate social contact with conspecifics and to evade predation /12, 17, 20, 21/.

In the present study we compared well defined ethological units and ambulation scores of the paradise fish. In several cases significant correlations were found between behaviour units and ambulation scores. Previous studies, which permitted the functional interpretation of the ethological units /4, 8, 9/, have also helped in constructing a natural description of the paradise fish's behaviour in the open-field. In the ethological analysis /8, 9/ we have found that in the presence of a perceived danger the behaviour of the paradise fish is characterized by two sets of behaviour elements, namely by the active defense complex (ESC, ERA, LEP) and by the passive defense complex (RES, FRZ, OBQ, FLO). These complexes represent alternatives in defense, depending on particular stimulus-settings. There is also a territorial complex (SWI, MOV, HIM, PIC, A-G), which characterizes the peaceful home-living animal.

In the open-field study presented here, the high positive correlation found between OOC and ESC shows that ambulation in the outer part of the open-field is a tendency to leave the unfamiliar environment.

Territorial activity, which is characterized by MOV, HIM and PIC, was also observed occasionally. The negative correlations found between OIC and MOV and HIM mean that "home-behaviour" is connected with a motionless state in the inner part of the open-field.

Passive strategy was used as an alternative to the active defense complex that was reflected in significant negative correlation between OOC and FLO.

Our present results are in close agreement with the ethological interpretation of animal behaviour in open-field worked out for other animals /19, 20/.

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CHEMOATTRACTION OF INFECTIVE LARVAE OF ANCYLOSTOMA BRAZILIENSE TO RODENT PLASMAS AND TO SALTS*

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Infective larvae of Anyclostoma braziliense were tested for orientational response to rat plasma, to mouse plasma, to rat plasma fractions, and to salts. A high percentage of larvae accumulated at sources of rat plasma, mouse plasma, rat plasma diffusate, concentrated rat plasma dialysate, and some salts, notably sodium chloride. Because sodium chloride is present at an effective concentration in mammalian plasma, and because this salt may form a gradient between the blood and the skin surface, sodium chloride from the blood may direct the penetrating larvae through host's skin. Preliminary tracking of the larvae in gradients of rat plasma and of sodium chloride suggests that orientation to sources of these attractants was via a taxis, possibly a klinotaxis, whereas accumulation at these sources was via a klinokinesis.

Keywords: Chemotaxis – hookworm – Ancylostoma braziliense – infective larvae – behaviour

INTRODUCTION

The third-stage hookworm larvae may infect the host by active skin penetration /17, 18, 19/ followed by vascular and lymphatic transport to the lungs /12/. They then migrate up the respiratory tree and are swallowed, and develop to the adults in the small intestine /14/. The dog and cat hookworm

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Ancylostoma braziliense is medically important because the infective larvae can penetrate human skin, resulting in a transient dermatitis known as "creeping eruption" /2/, and an understanding of the invasive behaviour of the infective larvae is therefore of vital concern. Infective larvae of Ancylostoma caninum showed, to a varying degree, an affinity for sera from a variety of mammalian species, and such an affinity may direct the penetrating larvae through host's skin /11, 22, 24, 25/. One chemoattractant has been identified as a polypeptide with a M.W. of 480 /20/. Certain inorganic salts are present at significant concentrations in mammalian plasmas, and these have not previously been tested for chemotactic activity. Here is a first report of chemoattraction of the infective hookworm larvae to salts.

MATERIALS AND METHODS

Ancylostoma braziliense was originally obtained from a naturally infected cat in Jacksonville, Florida. Infection was maintained in cats and dogs. Fresh faeces were mixed with a large quantity of granulated charcoal, and a minimum amount of water to uniformly moisten the mixture. This culture was maintained at 25 ^{o}C in the dark (Model 806 Incubator, Precision Scientific Co., Chicago 60647) and was moistened periodically. Infective larvae began to appear 5 days later. Larvae were collected using the "pad technic" /1/, washed in d. water, and examined. They were collected just prior to the experiment to ensure that only living larvae were tested.

Rats (SD or LE) and a mouse (Swiss) were lightly anaesthetized with ether, then killed by cervical dislocation. Whole blood was obtained by cardiac puncture, using a heparinized syringe and needle. Plasma was isolated by centrifugation (International Clinical Centrifuge, Model CL), and was used immediately, or used within the next 3 days following storage at 5 ^{o}C.

Whole rat plasma and mouse plasma. A 9 cm Petri disch was filled with 25 ml of 1% Bacto agar (Difco). Four test points, each 35 mm from the center of the dish and at an angular displacement of 90^{o} from adjacent test points, were marked on the bottom of the dish (Fig. 1). Plasma (0.1 ml) was added to one of the test points, whereas d. water (0.1 ml) was added to each of the other three test points. Two hours elapsed to allow a chemical gradient to form between the source of the plasma and the center of the dish. About 500 or 1000 larvae in 0.05 ml d. water were added to the center of the agar surface. The dish was left uncovered (to facilitate dissipation of water from the larval suspension, eventually allowing the larvae to freely disperse) at 25 ^{o}C in the dark in an environmental chamber. For the sake of uniformity, the four test points were aligned in a north, east, south, and west direction. Twelve or 14 h later, larvae found within a circle of 15 mm diameter at each test point were counted.

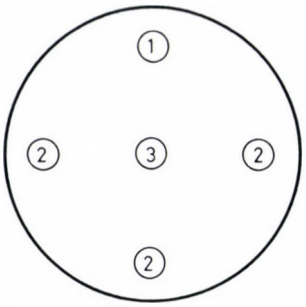


Fig. 1. Agar plate showing the positions of (1) the test point to which a test substance had been added, (2) the test points to which d. water had been added, and (3) the point to which the larvae had been added

Rat plasma diffusate and concentrated dialysate. The same method was used, except that a 2x2 cm double layer of cellulose material (Dialysis sack, 250-9U, Sigma, with cut-off molecular weight of about 12 000), was positioned between the agar surface and the added plasma or d. water. About 500 larvae in 0.05 ml d. water were added to the center of the agar surface. Larvae were counted 12 h later. Alternatively, plasma was dialyzed (Dialysis sack, 250-9U, Sigma) in 4 volumes of d. water at 5 °C for 48 h. The dialysate was heated in a water bath at 50 °C until it was reduced to the original volume of the plasma. In one experiment, 2000 larvae in 0.05 ml d. water were added, and the duration of the experiment was 6 h. In the other, 5000 larvae in 0.05 ml d. water were added, and the duration of the experiment was 3 h.

Dialyzed rat plasma. One ml of rat plasma was dialyzed (Dialysis sack, 250-9Z, Sigma) in two changes of 1000 ml d. water at 5 °C over a 48 h period. The dialyzed plasma was tested using the original method. Two experiments were conducted using this method. In one, 2000 larvae were used; in the other, 1000 larvae were used. The duration of both experiments was 6 h.

Organic and inorganic salts. Each salt was standardized at a concentration of 150 meq/l, the total ionic concentration of human plasma /23/. The original method was used. One thousand larvae in 0.05 ml distilled water were used, and the duration of the experiment was 3 h.

Direct observation

Orientation of the infective larvae to a source of rat plasma and to a source of sodium chloride was observed under a dissecting microscope, using a reflected incandescent light beam.

Table 1

Osmolality of 16 selected salt solutions at a concentration of 150 meq/l

Salt solution	Osmolality (mOs/kg)
1. potassium chloride	224
2. sodium chloride	286
3. sodium carbonate	199
4. sodium citrate	129
5. sodium fluoride	185
6. sodium acetate	365
7. sodium tartrate	160
8. lithium carbonate	403
9. sodium sulfide	220
10. sodium oxalate	228
11. magnesium chloride	199
12. sodium sulfate	205
13. sodium nitrite	351
14. ammonium chloride	249
15. manganous sulfate	176
16. magnesium sulfate	102

RESULTS

A consistently high percentage of larvae (mean, 85.8 to 95.0%) accumulated at the test points to which rat plasma, mouse plasma, rat plasma dialysate, and concentrated rat plasma dialysate had been added. A relatively low percentage of larvae (mean, 35.5%, pooled results from two independent experiments) accumulated at the test points to which dialyzed rat plasma had been added (Fig. 2).

The percentages of larvae, that accumulated at sources of 41 salts are given, in decreasing order, in Figures 3 and 4. Thirty-five salts (85%) showed mean percentages of accumulation greater than 25%. Three salts (magnesium bromide, ammonium nitrate, and magnesium nitrate) showed a consistently low accumulation of 5.8 to 11.4%.

Figure 5 shows the paths of two larvae in response to a source of rat plasma. After the water margin of the larval suspension had receded sufficiently for the larvae to disperse, ten larvae were found within the

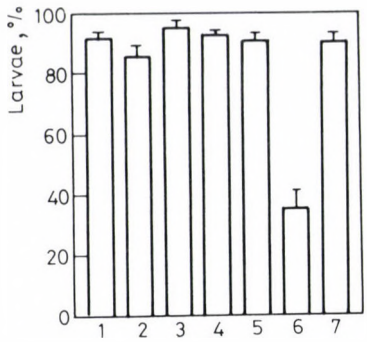
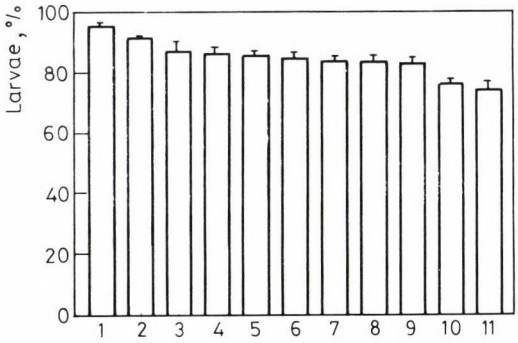


Fig. 2. Percentage of larvae that accumulated at the test point to which plasma or plasma fraction had been added. Bars represent group mean (±SE). 1. rat plasma: 12 h duration, 12 replicates
2. rat plasma: 14 h, n = 8
3. rat plasma dialysate concentrate: 6 h, n = 9
4. rat plasma dialysate concentrate: 3 h, n = 7
5. rat plasma diffusate: 12 h, n = 9
6. rat plasma (dialyzed): 6 h, n = 18
7. mouse plasma: 12 h, n = 2

plasma area in 3 min whereas none were found at comparable test points to which d. water had been added. Many larvae moved directly to the source of plasma (Fig. 5a), while others, on approaching the plasma source, moved along the observable outline of the plasma, a few made turns and loops before entering the plasma area (Fig. 5b). The orientational response to a source of sodium chloride was similar, except that larvae arriving at the plasma source slowed and eventually stopped moving, whereas those arriving at a source of sodium chloride continued to locomote. On the other hand, locomotory response to a control point where d. water had been added appeared to be undirected. Most larvae stopped before they came near to this point, the few that came near to this point did not accumulate.

DISCUSSION

Results suggest possible chemotactic response to diffusable substances in the plasma. Na^+ (at 132 to 150 meq/l) and Cl^- (at 100 to 110 meq/l) are the predominant ions in human plasma /23/; comparable concentrations of these ions were found in new-born puppies /8/, and in rats /13/. Therefore, sodium chloride could have been the principal chemoattractant in



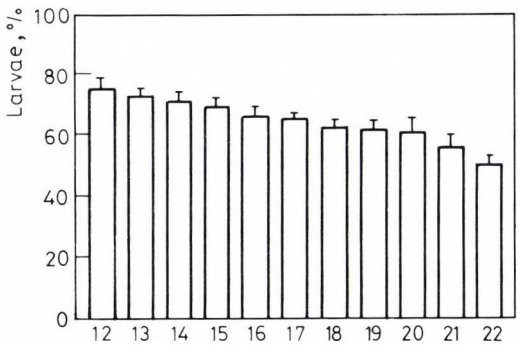
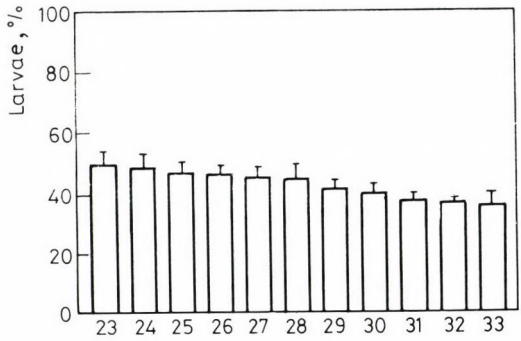


Fig. 3. Percentage of larvae that accumulated at the test point to which a salt had been added. Bars represent group mean (±SE)

1. potassium chloride
2. sodium chloride
3. sodium carbonate
4. sodium citrate
5. sodium fluoride
6. sodium bicarbonate
7. sodium bromide
8. sodium acetate
9. sodium tartrate
10. lithium carbonate
11. sodium sulfite
12. potassium acetate
13. cupric chloride
14. potassium carbonate
15. lithium chloride
16. sodium dihydrogen phosphate
17. potassium bromide
18. sodium oxalate
19. magnesium chloride
20. sodium iodide
21. potassium monohydrogen phosphate
22. copper sulfate



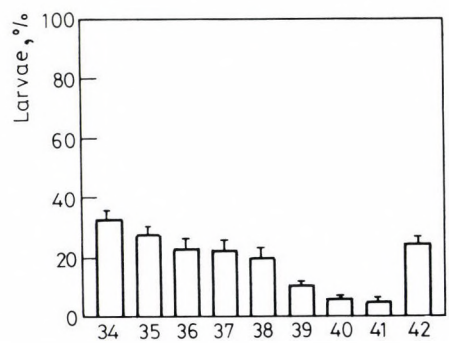


Fig. 4. Percentage of larvae that accumulated at the test point to which a salt had been added. Bars represent group mean (±SE)

23. sodium sulfate
24. sodium monohydrogen phosphate
25. sodium nitrite
26. strontium chloride
27. manganous chloride
28. ammonium chloride
29. zinc sulfate
30. aluminum chloride
31. manganous sulfate
32. potassium dihydrogen phosphate
33. potassium sulfate
34. magnesium sulfate
35. ferric chloride
36. calcium chloride
37. silver nitrate
38. potassium iodide
39. magnesium bromide
40. ammonium nitrate
41. magnesium nitrate
42. d. water

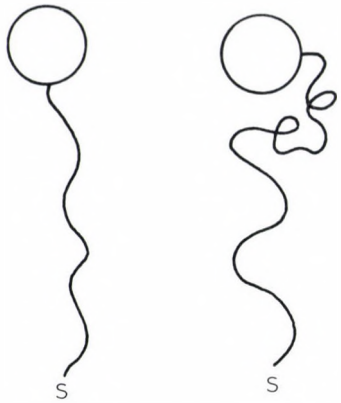


Fig. 5. The paths (wavy pattern not shown) of two larvae on agar in response to a source of rat plasma (circle) s = start

the plasma of dog (definitive host for A. braziliense), and in the plasma of man and rodents (abnormal hosts for A. braziliense). A high percentage of larvae also accumulated at sources of potassium chloride and sodium bicarbonate. The plasma concentrations of bicarbonate ions (at 24 to 30 meq/l in man, and 23 meq/l in dog) and potassium ions (at 3.8 to 5.4 meq/l in man and 5.8 meq/l in dog) are low relative to the concentration of sodium chloride, but may augment the effect of sodium chloride. The percentage of larvae that accumulated at sources of other salts containing sodium, potassium, and chloride ions varied from low (mean of 19.8% for potassium iodide) to high (mean of 88.0% for sodium carbonate), suggesting that accumulation of larvae at sources of these salts may have been the combined effects of both the cations and the anions in a salt.

Infective larvae of Ancylostoma caninum showed a greater affinity for dog serum than for sera from the horse, rat, sheep, mouse, cat, guinea-pig, hamster, pigeon, rabbit, or chicken, and such a preference might explain how the infective larva could orient much better in dog, the normal host, but that creeping eruption could otherwise develop in the abnormal host /25/. It is possible that /1/ more than one chemotactic factors existed, and dog serum contained more of these factors than did sera from other animal species, or /2/ only one factor was responsible for such chemoattraction, but dog serum contained a higher concentration of this factor than did sera from the other animal species /25/. One such chemotactic factor has been identified as a polypeptide with a molecular weight of 480 /20, 22/. The present study identified sodium chloride (M.W., 58.5) as a second chemoattractant present in mammalian plasma.

Direct observation was undertaken to determine the mechanism involved in chemoattraction of the larvae. The direction of the observation beam was perpendicular to the horizontal movement of the larva, and therefore would not have influenced, tactically, larval orientation in a horizontal plane. The direct approach of the larvae towards a source of plasma or sodium chloride suggests a taxis. Side-to-side movements of the anterior end during up-gradient orientation suggest possible sampling of the chemical gradient at successive points in time /7/, and therefore a klinotaxis /21/. However, the mechanism may not have been a klinotaxis because infective hookworm larvae naturally move with the anterior end swinging from side to side, and orientation might have been mediated via some other mechanism such as a tropotaxis /7/, a mechanism in which simultaneous comparison of inputs from all the receptors resulted in orientation. The concepts of the various taxes proposed thus far (e.g. tropotaxis, klinotaxis, telotaxis) have been based on the movement patterns of specific animals and may not fit into the movement patterns of other animals /7, 16/. The continually locomoting larvae turned more frequently at a source of plasma and at a source of sodium chloride, suggesting that accumulation was via a direct klinokinesis /16/.

Dog serum or serum fractions and phosphate buffered saline (PBS) both attracted infective larvae of A. caninum; however, larvae that arrived at sources of PBS (which contains sodium, chloride, potassium, and phosphate ions) continued to "migrate" /22/. Infective larvae of A. braziliense reported here slowed and eventually stopped moving at a source of rat plasma, but continued to locomote at a source of sodium chloride. The larvae might have slowed and eventually stopped moving due to drying and hardening of the plasma, or in response to certain factor in the plasma.

Coiling and cessation of movement occurred at sources of copper sulfate and at sources of cupric chloride, and might have resulted in a moderately high accumulation of larvae at sources of these salts. On the other hand, the consistently low accumulation of larvae at sources of magnesium bromide, ammonium nitrate, and magnesium nitrate, suggests a possible avoidance response to these salts.

Attraction to the ions Na^+ and Cl^- has been identified in larvae and adults of Caenorhabditis elegans /4, 21/, a free-living nematode, and in second-stage larvae of Rotylenchulus reniformis /15/, a plant parasitic nematode, and is probably quite common. Penetrating infective larvae of Ancylostoma caninum resulted in acute inflammation of the skin of dog, which

is often accompanied by serous exudate, a serum gradient may then develop and direct the larvae to the blood vessel /25/. Salts from human perspiration may likewise act as an attractant.

The large number of salts shown to attract the infective hookworm larvae suggest that the larvae could have responded to an osmotic gradient (osmotaxis), osmolality being a physical property of any soluble compound. Caenorhabditis elegans wild type, for example, showed chemotaxis to low concentrations of sodium chloride /4, 21/, but is strongly repelled by high osmotic strength solutions of salts and sugars /3/. Chemotaxis to sodium chloride and osmotic avoidance of sodium chloride (or fructose) shared some common gene functions /5/. Osmotic avoidance appears to be mediated by a receptor distinct from that mediating chemotactic behaviours /6/. Infective larvae of Ancylostoma braziliense reported here apparently showed a greater affinity for some salts but not for others. Measurement of 16 salt solutions at a concentration of 150 meq/l showed that their osmotic strengths (Table 1) were of the same order of magnitude (range = 102 to 403 mOs/kg). Two of the salts tested, sodium chloride and ammonium chloride, had similar molecular weights (58.5 and 54.5 respectively), and were therefore expected to diffuse in agar at similar rates; at a concentration of 150 meq/l, they also had comparable osmolalities (286 and 249 mOs/kg respectively), the osmotic gradients produced by the two salts would be expected to be similar. However, sodium chloride consistently attracted a far higher percentage of larvae than did ammonium chloride. This suggests that osmotaxis may not have been a deciding mechanism resulting in larval accumulation. In one experiment, larvae accumulated at sources of concentrated rat plasma dialysate at 6 h, but such an accumulation pattern disappeared 11 h later, and at this time the larvae appeared to be evenly distributed over the agar surface. This suggests that accumulation at, and orientation to a source of attractant depended on the concentration of the attractant at its source.

Successful inhibition of chemotactic behavior in C. elegans using enzymes /9/ and lectins /10/ opens new possibilities in the study of the invasive behaviour of infective hookworm larvae. Similar inhibition in infective hookworm larvae has practical application in that it may prevent invasion of the larvae via the skin route, and deserves further study.

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ISOLATION OF PATHOGEN-SYNTHESIZED FRACTION THAT ELICITS PHYTOALEXINS IN LUCERNE (MEDICAGO SATIVA L.)

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The production of phytoalexins by lucerne cultivars Dupuits (susceptible to Verticillium wilt) and Maris Kabul (resistant to Verticillum wilt) in response to substances (= elicitors) produced by pathogenic and non-pathogenic isolates of Verticillium albo-atrum was investigated. The elicitors are produced in culture filtrates from log-phase and past log-phase stages. The elicitors were isolated by gel-filtration and found to contain both protein and carbohydrate. They were found to be thermostable and non-dialyzable and proved to be sensitive to protease and sodium hydroxide treatment which suggests that they are glycoproteins. There was no evidence of specificity of the elicitors.

Keywords: Phytoalexins — lucerne — Medicago sativa L.

INTRODUCTION

Lucerne plants produce phytoalexins in response to challenge with drops of spore suspensions in their leaves /15, 19/. This response is postulated to be a factor in the resistance of lucerne plants as in many other plants such as cotton /2, 3/ and tomato /25/ in which these antifungal substances have been produced more in reaction to the presence of incompatible isolates of the pathogen. In addition to spores, other compounds (biotic and abiotic) have been reported to elicit the production of these phytoalexins in plants /1, 5, 10, 11, 12, 18, 20, 27/. In the present study, the objective was to determine if spore-free extracts of isolates of Verticillium are capable of inducing phytoalexin production in lucerne plants.

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MATERIALS AND METHODS

Isolates of Verticillium used were obtained from the culture collection maintained at the University College of Swansea, U.K. These isolates are for convenience designated V_1 and V_2 as follows:

V_1 = Verticillium albo-atrum ex lucerne
V_2 = Verticillium albo-atrum ex tomato

The cultures were grown in liquid Czapek-Dox's medium at 23 °C in a shake-incubator at 100 r.p.m. Each liquid medium culture was inoculated with 10^7 spores (conidia) of the isolate. Conidial suspension was obtained from 2--3-week-old cultures on PDA or prune lactose agar.

Culture filtrates from the isolates were obtained by removal of mycelial fragments and spores through filtration. Filtrate was centrifuged at 7000 r.p.m. at 4 °C in an MSE centrifuge for 20 min, then filtered twice through glass microfibre and finally through 0.45 µm Millipore filters. The spore-free culture filtrate was dialysed for 48 h against six changes of distilled water at 4 °C at the end of which the filtrate was again filtered through 0.45 µm Millipore filters. 200 ml of the dialyzed spore-free culture filtrate was freeze-dried in a Leybold Haraeus GT 2 freeze-drier and stored at 4 °C until required.

For gel-filtration, 8.0 g of Sephadex G-200 (Superfine) was swollen in 400 ml of phosphate D buffer, pH 6.8. The gel was degassed and packed in a Pharmacia K 26/40 column. Flow of buffer was initiated with a Peristaltic pump p-3 and adjusted to 7.14 ml cm^{-2} h^{-1} and maintained at room temperature for 12 h to equilibrate. The bed was checked for uniformity using Blue Dextran 2000.

To fractionate the culture filtrate, the freeze-dried residue was suspended in 5 ml of 25 mM phosphate D buffer, pH 6.8. The resulting suspension was centrifuged at 7000 r.p.m. for 10 min. 3 ml of the clear supernatant was withdrawn and applied to the column of Sephadex G-200 and eluted with the same buffer. 5 ml fractions were collected and assayed for elicitor activity.

Bioassay of elicitor activity

Bioassays were conducted by placing detached shoots of cv. Dupuits or cv. Maris Kabul standing in the test solution for 48 h at 25 °C in growth rooms with 16 h light (4200 Lux) and 8 h darkness. At the end of this period, elicitor activity was present if the plant extract exhibited the characteristic ultraviolet absorption spectrum of lucerne phytoalexins as shown by Khan and Milton (1975).

Extraction of phytoalexins from leaf tissue

The method of Higgins /15/ was used. Leaf tissue (0.8--2.0 g fresh weight) was homogenized in 20 ml 95% (v/v) ethanol and the homogenate filtered through Whatman No. 1 filter paper. The ethanolic filtrate was evaporated to dryness in a stream of air; alternatively, the ethanolic solution was dried under vacuum in a rotary evaporator at 30 °C.

The dried residue was washed three times with 7 ml of carbon tetrachloride and the washings partitioned three times with one volume of

0.2 NaOH. The sodium hydroxide fraction was centrifuged at 6000 r.p.m. for 20 min to remove an emulsion that formed. The extract was acidified to pH 3.0 with 6N HCl, and then partitioned three times with one half volume of carbon tetrachloride. The carbon tetrachloride fraction was dried under a stream of air and the residue dissolved in 5 ml of 95% (v/v) ethanol, or an appropriate dilution of this and scanned on a recording spectrophotometer. Following the establishment of phytoalexins in the extract, the ethanolic solution was dried under an air stream and the phytoalexins separated by thin layer chromatography.

RESULTS

Lucerne phytoalexins — medicarpin and sativan — were obtained from the leaves of shoots inoculated with culture filtrates of both isolates, V_1 and V_2, after 24 h of incubation with the levels of phytoalexin increasing up to 48 h. The stems of inoculated plants did not yield any phytoalexins. In all the experiments, a large portion of the leaves from shoots treated with dialyzed culture filtrates did not show wilt symptoms before 48 h. Up to 72 h, a visual estimate showed an increase in foliar symptoms up to 60% in the shoots treated with culture filtrate V_1.

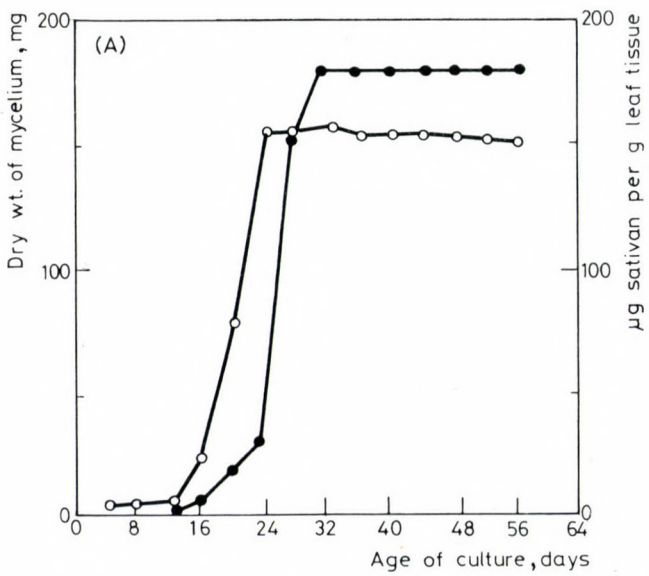

Fig. 1.

It is readily seen from Fig. 1 that elicitor activity was obtained

from older cultures. This production of elicitors lagged behind growth of the fungus and reached a peak level after growth of the fungus had ceased.

Table 1

Effect of heat on culture filtrate elicitor

Source of culture filtrate	Treatment of culture filtrate	Phytoalexin production (sativan) as a % control
V_1	25 ^{o}C for 24 h	100($\pm$ 3.0)
V_1	Boiling at 100 ^{o}C for 10 min	100(+ 5.3)
V_1	Autoclaving at 121 ^{o}C for 15 min	92($\pm$ 2.6)
V_1	Cooling to 4 ^{o}C for 24 h	102($\pm$ 4.6)
V_2	25 ^{o}C for 24 h	100($\pm$ 6.6)
V_2	Boiling at 100 ^{o}C for 10 min	98($\pm$ 4.3)
V_2	Autoclaving at 121 ^{o}C for 15 min	95($\pm$ 3.0)
V_2	Cooling to 4 ^{o}C for 24 h	100($\pm$ 1.6)

Figures in parentheses represent standard deviation. Results are the mean of six replicates

Table 1 shows the effect of heat treatment on the elicitor. Neither cold treatment nor boiling at 100 ^{o}C for 10 min had any significant effect on the elicitor activity. The stability of the culture elicitor to pH was tested using standard solutions of the culture filtrate adjusted to 60 ug protein equivalents ml^{-1}. The results are presented in Table 2. The elicitors proved more stable at low than high pH. After only one hour, the effect of exposure of the culture filtrates of both isolates to pH 12.0 resulted in declining activity. There was an appreciable loss of elicitor activity of both isolates with more prolonged exposure.

Sensitivity of the culture filtrate elicitors to enzymatic breakdown was examined using protease, ribonuclease-A, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase and α-mannosidase

Table 2

Effect of pH on culture filtrate elicitors

Isolate	Treatment of culture filtrate	Phytoalexin production as a % of control
V_1	pH = 12.0; 0.5N NaOH for 1 h	80(± 2.2)
V_1	pH = 12.0; 0.5N NaOH for 24 h	55(± 5.4)
V_1	pH = 3.0; 0.5N HCl for 1 h	100(± 1.6)
V_1	pH = 3.0; 0.5N HCl for 24 h	102(± 4.2)
V_1	Control	100(± 2.2)
V_2	pH = 12.0; 0.5N NaOH for 1 h	86(± 2.6)
V_2	pH = 12.0; 0.5N NaOH for 24 h	52(± 4.3)
V_2	pH = 3.0; 0.5N HCl for 1 h	98(± 1.2)
V_2	pH = 3.0; 1.5N HCl for 24 h	100(± 1.0)
V_2	Control	100(± 3.6)

Figures in parentheses represent standard deviation. Results are the mean of six replicates

Table 3

Effect of enzyme action on culture filtrate elicitor activity

Treatment of culture filtrate	Phytoalexin production as a % of control	
	Isolate V_1 culture filtrate	Isolate V_2 culture filtrate
Control	100(± 2.2)	100(± 3.6)
Boiled at 100 °C for 10 min	100(± 5.3)	98(± 1.3)
Protease	12(± 4.2)	28(± 5.6)
RNA-ase	103(± 0.9)	100(± 2.3)
α-glucosidase	97(± 3.3)	95(± 4.2)
β-glucosidase	100(± 2.0)	100(± 1.6)
α-galactosidase	96(± 1.5)	98(± 3.3)
β-galactosidase	98(± 4.3)	96(± 1.8)
α-mannosidase	98(± 2.8)	100(± 3.6)

Figures in parentheses represent standard deviation. Results are the mean of six replicates

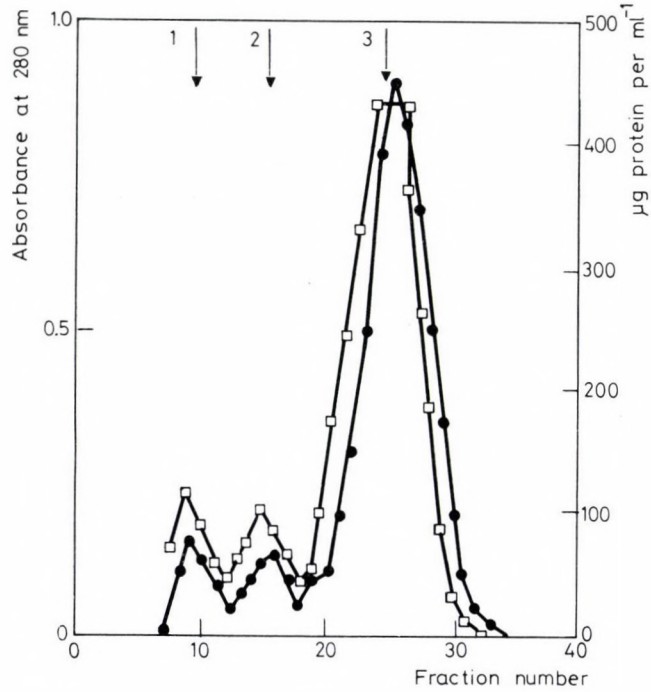
Absorbance at 280 nm
1.0
0.5
0
1
2
3
500
400
300
200
100
0
µg protein per ml^{-1}
0
10
20
30
40
Fraction number

Fig. 2.

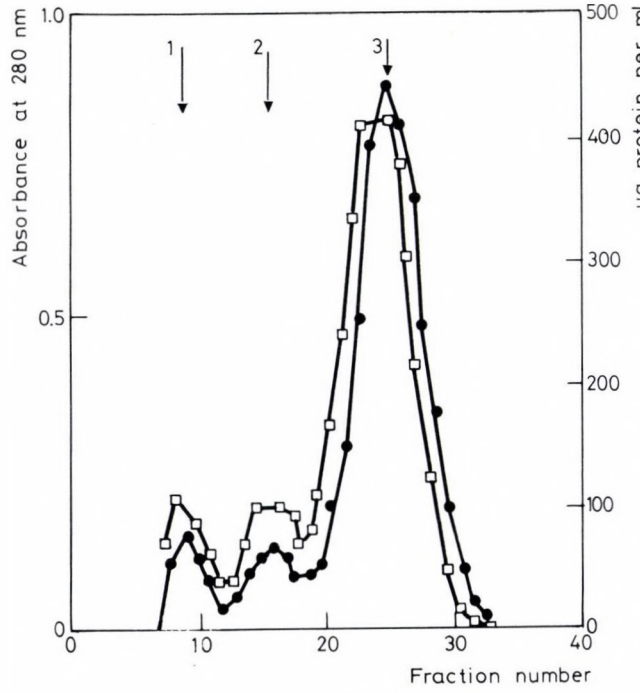
Absorbance at 280 nm
1.0
0.5
0
1
2
3
500
400
300
200
100
0
µg protein per ml
0
10
20
30
40
Fraction number

Fig. 3.

respectively. The results which are contained in Table 3 show that protease destroyed much of the elicitor activity. RNA-ase, α- and β-glucosidase, α- and β-galactosidase, and α-mannosidase did not destroy elicitor activity.

The elution profile of the culture filtrate from isolate V_1 is presented in Fig. 2. Three peaks were obtained: fractions 8 to 11 eluted in peak 1; fractions 13 to 17 in peak 2; and fractions 20 to 29 eluted in peak 3. All pooled peak fractions contained both proteins and carbohydrates (proteins were detected by the Lowry method as modified by Hartree while carbohydrates were determined by the anthrone procedure). Elicitor activity was detected in peak 3 but not in peaks 1 and 2. The eluting profile of V_2 culture filtrate is shown in Fig. 3. The sample also eluted in three peaks as V_1. Both proteins and carbohydrates were detected in the three peaks but elicitor activity was detected only in peak 3 but not in peaks 1 and 2. Attempts to precipitate the active components responsible for the elicitation of the phytoalexins from the dialyzed culture filtrates with ammonium sulfate were unsuccessful.

DISCUSSION

Although Khan and Milton /19/ had used the drop diffusate technique to distinguish between pathogenic and non-pathogenic isolates of Verticillium albo-atrum, Walsh /26/, Flood /9/ and Onuorah /22/ failed to make such a distinction using spore-free culture filtrates. Consequently, the drop diffusion method has not been employed in the present study.

Detached shoots were treated with culture filtrates by standing them in the filtrate under conditions of moderate transpiration. The culture filtrate was passively absorbed into the shoots and transported via the transpiration stream into the stems and leaves. Using this experimental system, elicitor activity was detected in culture filtrates from log-phase, and past log-phase cultures. The technique was considered suitable since such extracellular elicitors are likely to be released in the vascular tissues by Verticillium growing in the plant. Blackhurst and Wook /4/ and Sinha and Wook /24/ have used similar methods with spore suspensions in investigating the nature of resistance of tomato plants to V. albo-atrum.

Culture filtrates from the pathogenic (V_1) and non-pathogenic isolate (V_2) induced similar levels of phytoalexin in the leaves of shoots treated with the culture filtrates. These results represent a major dif-

ference from the interaction between the surface of lucerne leaves and spores in that the elicitors failed to exhibit specificity. This specificity shown by the spores on the leaf surface involves the recognition of the different isolates within 12 h leading to the differential response by the leaf cells. In the present study, phytoalexins were not detected from the leaves of treated shoots earlier than 24 h of the treatment with the culture filtrate.

Brian /6/ emphasized that specificity is exhibited early in the infection process, the latest and frequently the most critical point being the moment of contact between the pathogen cell wall and the plasmalemma of a penetrated cell. This is true also of the hypersensitive response which is the most highly developed form of specificity. In accounting for the necessary recognitional events between plants and potential pathogens in specific interactions, many workers /14, 16, 17, 23/ postulated that incompatible pathogens form constituents termed elicitors which interact, with specific plant sites termed receptors in a highly specific complementary manner to initiate events leading to incompatibility — phytoalexins and necrotrophy. From the present study, lucerne phytoalexin elicitors were obtained from both compatible and incompatible isolates of V. albo-atrum. A comparison of the activity of the elicitors from the two isolates showed little difference between them which suggests that these culture filtrate elicitors are non-specific.

The elicitors were found to be heat-stable and non-dialyzable. The instability of the elicitor at pH 12.0 suggested that proteins are involved. The loss of elicitor activity following treatment with protease further emphasized the involvement of proteins and the action of the high pH seems to result from the loss of serine and threonine components of glycopeptides as pointed out by Neuberger et al. /21/. Evidence for the presence of a molecule containing both protein and carbohydrate in the elicitor is overwhelming. Other aspects of this large molecule are currently being further studied in this laboratory.

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CHARACTERIZATION OF FS(2)1, A GERM-LINE DEPENDENT DOMINANT FEMALE STERILE MUTATION OF DROSOPHILA

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Fs(2)1 is a germ-line dependent dominant female sterile mutation of Drosophila melanogaster. Fs(2)1 heterozygous females deposit very few abnormal eggs (collapsed, with malformed chorion). The degeneration of egg primorida starts around the end of egg maturation. Mitotic recombination mapping locates Fs(2)1 in a distal region of the left arm of the 2nd chromosome. Fs(2)1 is a good tool for studying germ-line functions (by the dominant female sterile technique) because the frequency of germ-line mosaicism exceeds 20% upon irradiation of adult females. Salivary gland polytene chromosomes of Fs(2)1 and the revertant heterozygous larvae appear normal.

Keywords: Dominant-female-sterile technique — germ-line mosaics — Drosophila

INTRODUCTION

A possible approach to the understanding of the genetic and developmental regulation of oogenesis, maternal effects and embryogenesis is to analyze the effects of mutations on germ-line cells. Mosaic egg primordia are usually created, in which mutant germ-line cells (the oocyte and 15 nurse cells) are surrounded by phenotypically wild-type follicular cells. When the development of the mosaic egg primordia or the resultant embryo is affected, the mutation is considered to be germ-line dependent and it can be deduced that the gene identified by the mutation has germ-line functions.

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However, if the mosaic egg primordium exhibits normal development, the mutation is generally considered to be somatic (follicular) dependent.

Mosaic egg primordia are generated either by the transplantation of pole cells (ancestors of germ-line cells, /8/ or by the dominant female sterile technique, /9/). The dominant female sterile technique makes use of germ-line dependent dominant female sterile mutations (Fs). Mitotic recombination is induced in oogonial cells heterozygous for Fs and the m mutation to be studied. (The mutations Fs and m are located in trans on the same arm of the homologous chromosomes.) The Fs-free daughter cell may continue development (unless the m mutation interferes with it) and give rise to mosaic egg primordia in which the m homozygous germ-line cells are surrounded by m heterozygous follicular cells. (Although the follicular cells are Fs heterozygous, they develop normally because Fs does not disturb the functions of these cells.)

Two Fs mutations have been reported and used in the dominant female sterile technique: Fs(2)D /10, 12/ and Fs(1)K1237 /2, 4/ for a review see /5/. Fs(1)K1237 is X-linked, while Fs(2)D is located on distal 2R.

We have recently carried out a systematic screen for the isolation of EMS-induced autosomal Fs mutations. From over 70 Fs mutations induced only one — Fs(2)1 — emerged that can be used for the construction of germ-line mosaics /3/. This paper describes the characteristics of Fs(2)1.

MATERIALS AND METHODS

The analysis of Fs(2)1/al dp b prcc px sp females is described. They were derived from a cross between al dp b pr c px sp homozygous females and Fs(2)1/Cy Roi or Fs(2)1/Bc Gla males. al, dp, b, pr, c, px and sp are recessive, while Cy, Roi, Bc and Gla are dominant marker mutations located on balancer chromosomes. /For a description see 6./ The Fs(2)1 males were derived from a stock in which Bc Gla/Cy Roi females were mated with Fs(2)1/Bc Gla or Fs(2)1/Cy Roi males, and the fertile Bc Gla/Cy Roi males were discarded in every generation.

For the study of egg morphology, eggs deposited by the Fs(2)1 heterozygous females were fixed in an acetic acid: glycerol (1:1) mixture at 60 ^{o}C for one hour, mounted in Hoyer's medium and cleared for 1-2 days at 60 ^{o}C /11/. Ovaries were analyzed on Feulgen-stained whole mounts. For the induction of germ-line mosaicism, late second to early third instar larvae (46-50 h after oviposition) or 3-5-day-old adult females were irradiated with 1500 R of X-rays (150 kV, 0.5 mm Al filter, 1000 R/min). In the case of adult irradiations, females were mated with al dp b pr c px sp homozygous males prior to X-raying. Lots of five females plus 8-10 males were transferred into vials and screened for mosaicism throughout 10-14 days. This period is necessary to identify over 95% of the mosaics /10/. Upon the detection of mosaicism, females were screened individually for several more days.

RESULTS AND DISCUSSION

Fs(2)1 is an EMS-induced dominant female sterile mutation. The mutant phenotype is fully penetrant and expressive: Fs(2)1 females with 10 different genetic backgrounds gave the same phenotype. Fs(2)1 has no influence on the rate of development or on male fertility. The viability of the Fs(2)1 females is excellent. They each produce 0-4 eggs per day (40-70 in the control). The eggs are rudimentary. The dorsal appendages are missing and the anterior part of the eggs is often not covered with chorion (Fig. 1). The eggs usually collapse shortly after deposition.

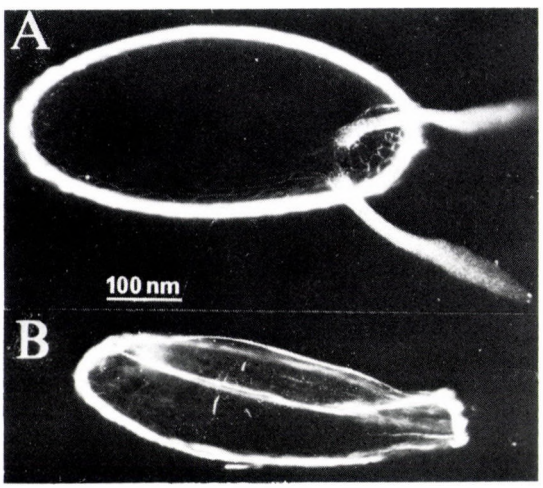

Fig. 1. Eggs deposited by wild-type (A) and Fs(2)1 (B) females

Indications of egg degeneration can be visualized on Feulgen-stained whole mounts of ovaries towards the end of egg maturation. Egg primordia develop normally up to stages 8-10, when pyknosis of nurse cell nuclei begins, chorion defects develop and the resorption of 90-95% of the egg primordia takes place (Fig. 2).

The results of larval ovary transplantation show that the mutant phenotype is ovary autonomous: Fs(2)1/+ovaries displayed a mutant type of development in normal hosts (5 chimeras), while the Fs(2)1/+ hosts provided excellent conditions for the development of normal ovaries (5 chimeras).

Whether the Fs(2)1 phenotype is germ-line or follicular dependent was studied through the induction of Fs-free germ-line cells (by the induction of mitotic recombination) in Fs(2)1/al dp b pr c px sp larvae and

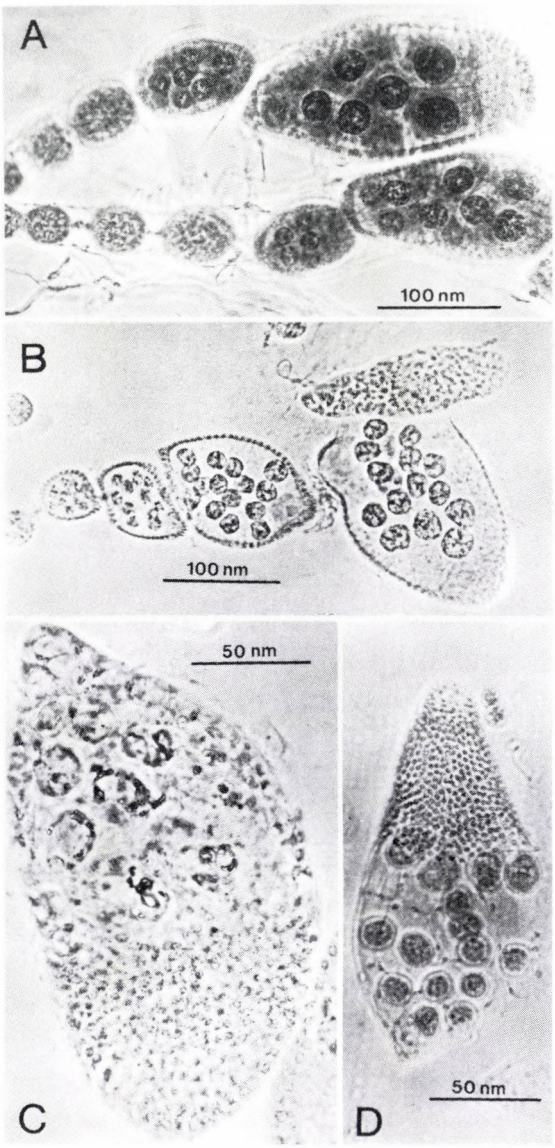

Fig. 2. Feulgen-stained egg chambers of wild-type (A) and Fs(2)1 (B) females. Pyknosis of nurse-cell nuclei is a general feature of the Fs(2)1 egg primordia (C). Although follicular cells migrate over the oocyte (D), it remains rudimentary because the nurse cells fail to supply material to its cytoplasm. The Fs(2)1 egg primordia degenerate in about 95% of the cases, and when deposited, they are flaccid and rudimentary

females. The results of the germ-line clonal analyses are listed in Table 1. Only two of the 384 control females were mosaic, indicating a low (0.5%) frequency of mosaicism. As a consequence of X-irradiations, offspring developed from 27 and 68% of the lots. There were 18 and 23 mosaic females identified among 261 and 132 females irradiated as larvae or adults, respectively. The actual numbers of germ-line clones were estimated on the basis of a Poisson distribution: $P(i) = (v^i/i!)e^{-v}$, considering that $e^{-v} = N_0/N$ and $N(i) = N\,P(i)$, where N and N_0 stand for the total and non-offspring-yielding numbers of lots. Thus, mosaicism frequencies of 7.7 and 22.7% emerged for larval and adult irradiations.

There may be a number of reasons why the Fs(2)1 heterozygous females give rise to offspring following X-irradiations. Reversion of the dominant allele or clones of Fs(2)1-free cells (that come about following mitotic recombinations) may be the sources of offspring. However, the phenotypes of the progeny and the pattern of offspring-production provide clues to distinguish among sources of mosaicism. In the case of reversions and Fs-free follicular clones the germ-line cells remain heterozygous for all the marker mutations and thus their wild-type alleles can be recognized in the subsequent generation. When, however, the offspring is generated from Fs-free germ-line cells, wild-type alleles of certain marker mutations will be absent in the descendants. This principle can be used for mapping Fs mutations by mitotic recombination. A similar approach was described by Busson et al. /2/.

When mitotic recombination takes place in an Fs heterozygous cell, one of the daughter cells will be Fs-free and can be the source of egg and offspring (Fig. 3). As reported earlier /1/, a large proportion of the mitotic recombinations takes place in the heterochromatin adjacent to the centromere (Fig. 3A). In this case, the Fs-free daughter cell is homozygous for all the marker mutations located on the same arm of the 2nd chromosome as Fs(2)1, while those located on the other arm will be heterozygous. In a test cross, therefore, wild-type alleles of the former marker mutations will not appear, while mutant and wild-type alleles of the latter will be present in a 1:1 ratio. Indeed, of the 41 mosaic females identified (following irradiations), 23 were of this type. They gave rise to 95 offsprings. All these were homozygous for al, dp, b, and pr, while mutant and wild-type alleles of c, px and sp developed in a 1:1 ration (46/49, 44/51 and 44/51). These results show that in these females the progeny derived form Fs-free germ-line cells and consequently Fs(2)1 is germ-line dependent. Besides, it

Table 1

Germ-line mosaicism of the Fs(2)1 heterozygous females

Stage at irradiation	Lots							Clones	Females screened	Frequency of mosaicism (%)	Identified mosaics
	Total	"Empty"	With 1-5 clones[a]								
	N	N_0	N_1	N_2	N_3	N_4	N_5				
Control	80	78	2	-	-	-	-	2	384	0.5	2
Larval	62	45	14.4	2.3	0.2	-	-	20	261	7.7	18
Adult	28	9	10.2	5.8	2.2	0.6	0.1	30	132	22.7	23

a = determined on the basis of a Poisson distribution (for details, see text)

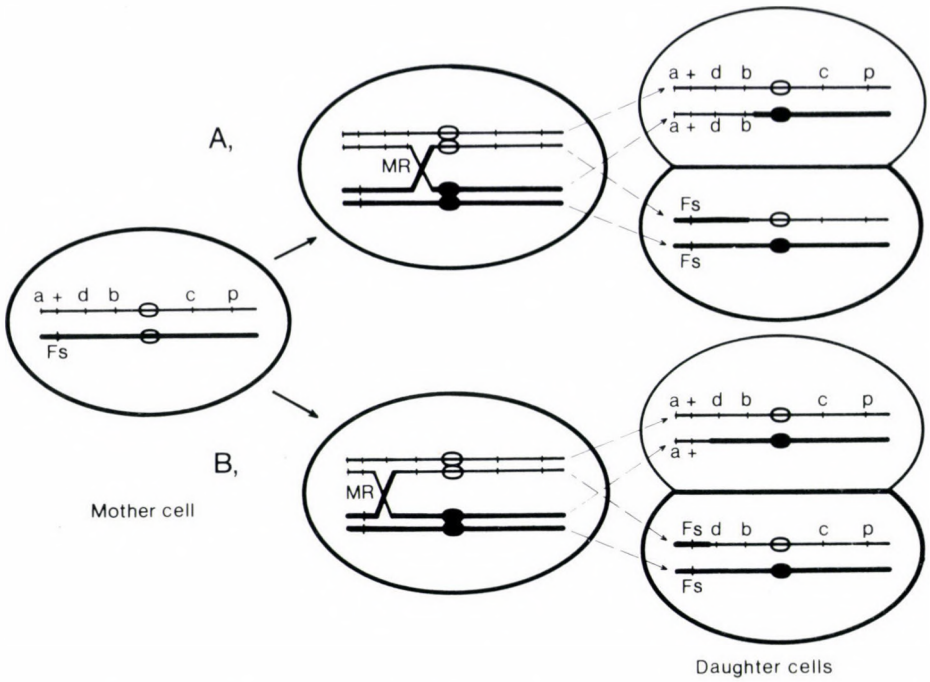

Fig. 3. Schematic representation of mitotic recombination (MR) and the consequences in case of proximal (A) and distal (B) recombinations. Symbols a, d, b, c and p stand for recessive marker mutations. Their wild-type alleles (that are located on the paternally derived thick chromosome) are not shown. For details, see text

can also be deduced that Fs(2)1 is located on the left arm of the 2nd chromosome.

Mitotic recombinations that take place in distal regions of 2L can also result in the formation of Fs-free daughter cells. However, they will be heterozygous for the marker mutations proximal to the site of mitotic recombination (Fig. 3B). Wild-type alleles of these mutations will appear in the subsequent generation, while wild-type alleles of the marker mutations located distally from Fs will be absent. Thus, Fs(2)1 can be located between two marker mutations: the wild-type allele of the distal will not be observed, while that of the proximal will appear among the progeny of the mosaic females.

Of the 41 mosaic females, 16 yielded offspring (167 altogether) with dp^+, b^+ or pr^+, but al^+ was missing. Mutant and wild-type alleles of al, dp, b and pr appeared with the following frequencies: 0/42; 33/34; 36/31; 39/28. These results locate Fs(2)1 between the marker mutations al

and dp, and are in agreement with the germ-line dependent nature of Fs(2)1. (It is worth mentioning that meiotic recombinations took place in the developing Fs-free oocytes, and as expected, mutant and wild-type alleles of the marker mutations appeared in different combinations.)

Two of the mosaic females gave rise to progeny that, besides the wild-type alleles mentioned, carried the al^+ allele. Both of them developed following adult irradiations. Progeny analysis of these females revealed that they were heterozygous for all the marker mutations. These females might have contained Fs-free follicular clones, which would imply that (i) Fs(2)1 is both germ-line and follicular dependent (and by rendering either components of the egg primordia Fs-free, a condition is achieved sufficient for egg development) and (ii) the follicular clones are very seldom.

It is, in principle, also possible that the location of Fs(2)1 is distal to al and mitotic recombination between Fs and al can lead to Fs-free germ-line clone (similarly as shown in Fig. 3B). However, this possibility is very unlikely, because al is located at the very tip of the left arm of the 2nd chromosome (at a position of 0.01 on the meiotic map /6/, and the chance for a mitotic recombination in such short distal regions is very small.

Double mitotic recombinations (one between the centromere and al and the other between al and Fs) will also result in al/al^+ condition (as seen in the two mosaics), however, in the case of occasional double mitotic recombinations the Fs-free daughter cell will generally be homozygous for dp b and pr.

By considering the above possibilities we believe that the two al and al^+-producing females originated as a consequence of reversion of Fs(2)1. That this hypothesis is likely was shown in another experiment, in which Fs(2)1/Cy Roi adult males were irradiated (by 4000 R of X-rays), mated with Bc Gla/Cy Roi females, and the descending females screened for fertility. From the 2009 females screened, two Fs(2)1 revertants were recovered. The revertants are not homozygous viable (probably due to second site lethal mutations). There are no visible rearrangements on the polytenic chromosomes of the Fs(2)1/+ or the revertant heterozygous larvae.

In addition to the difference in the phenotypes of offspring deriving from Fs-free germ-line or follicular clones, the pattern of offspring-production is also expected to differ in the two types of clones. In the case of adult irradiations, the Fs-free germ-line cell remains an undifferentiated stem line cell in 50% of the cases (and will be the source of

several offspring), while in the other 50% it will differentiate to an egg (and only progeny fly /10/). When, however, Fs-free follicular clones are the sources of offspring, all the clones are expected to yield several progenies. (There have been no cases of this latter type reported so far.)

Results of the adult irradiations approve the germ-line dependent nature of Fs(2)1. Among the 19 lots of 5 females 4, 2 and 13 yielded 1, 2 and ≥ 3 offspring, respectively. Since induction of mitotic recombinations is a random process, it was estimated that 10.2, 5.8 and 2.9 of the lots contained 1, 2 and ≥ 3 clones, respectively (Table 1). Based on the unequeal type of stem-cell divisions /10/, half of the one-clone-lots (about 5) is expected to yield only 1 offspring (the other 5 will yield several). There were 4 such lots observed. One fourth of the 5.8 two-clone-containing lots is expected to give rise to only two offsprings, and there were two such lots seen. In about 13 of the lots, production of several (≥ 3) offsprings is to be expected, and indeed, there were 13 such lots identified. These results clearly show that the progeny of the Fs(2)1 heterozygous irradiated adult females derives from Fs-free germ-line cells and thus Fs(2)1 is a germ-line dependent dominant female sterile mutation of Drosophila.

The relatively high frequency of germ-line clones renders Fs(2)1 a suitable tool for the dominant female sterile technique, as was actually found /7/.

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INHIBITION OF IN VITRO SPLICING OF A MOUSE INSULIN PRE-mRNA BY COVALENT CROSS-LINKS IN THE INTRON REGION

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Recent studies have indicated that in vitro splicing of a mouse insulin pre-mRNA by a HeLa cell nuclear extract is accompanied by the unwinding of substrate RNA. The present experiments were performed to determine whether this melting of the secondary structure of the precursor RNA is essential for the splicing reaction. ^{32}P-labelled mouse insulin pre-mRNA synthesized in vitro in a SP6 transcription system was cross-linked with aminomethyltrimethyl psoralen and fractionated by polyacrylamide gel electrophoresis. RNA species containing different intramolecular cross-links were eluted from the gel and the sites of cross-links were mapped by primer extension analysis using synthetic oligonucleotide primers. Under conditions that allow accurate in vitro splicing of intact pre-mRNA, precursor molecules with psoralen cross-links within their intron region were not spliced by a HeLa cell nuclear extract. This observation strongly supports the assumption that unwinding of precursor RNA molecules is necessary for the splicing reaction.

Keywords: Mouse insulin pre-mRNA — psoralen cross-linking — in vitro splicing — unwinding of RNA

INTRODUCTION

Many eukaryotic protein-coding genes contain intron sequences that are transcribed into pre-mRNA molecules but are subsequently removed from the primary transcripts by splicing /1/. The excision of intervening sequences proceeds in two steps /11, 16/. First, an endonucleolytic cleavage is introduced at the 5' (or left) splice junction followed by the esterfica-

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tion of the 5' end of intron to the 2'-OH group of an A nucleotide upstream from the 3' (or right) splice junction resulting in an intermediate that contains a covalently closed circular structure (lariat). In the second step, the intron-lariat is released by a cleavage at the right splice junction and the two exons are ligated to form the functional mRNA molecule. These reactions require accurate juxtaposition of the cleavage sites that is achieved by the assembly of a large, multicomponent complex /4, 6, 7, 10, 13, 17/. This spliceosome contains the precursor RNA, snRNPs, and hnRNP proteins.

The mechanism of recognition of the splicing sites is only partly understood. Consensus sequences at the 5' and 3' splice junctions are important for efficient splicing, but, especially in the case of large introns, may not be sufficient for splice site selection. Recently, Solnick /18/ presented strong evidence for the involvement of the secondary structure of precursor RNA in splice site accessibility. He found that splice sites sequestered in stable hairpin structures became optional both *in vitro* and *in vivo*. The role of the conformation of pre-mRNA was further supported by experiments showing that an unwinding of substrate RNA takes place in the early phase of *in vitro* splicing reaction /21/.

The experiments described in this paper were conducted to obtain further information concerning the role of secondary-tertiary structure of pre-mRNA in the splicing reaction. Precursor RNA species covalently cross-linked with the bifunctional photoreagent aminomethyltrimethyl (AMT) psoralen /3/ were used as substrates for *in vitro* splicing. Our results suggest that melting of the secondary structure of pre-mRNA is a precondition for splicing.

MATERIALS AND METHODS

In vitro transcription of pSDP64/MINS

The plasmid pSP64/MINS containing a mouse insulin (MINS) gene ligated to the SP6 promoter was linearized by cleavage with EcoRI and transcribed *in vitro* by SP6 RNA polymerase /5, 8/. The reaction mixture (25 µl) contained 1 µg of linearized DNA template, 40 mM of Tris-HCl (pH 7.4), 6 mM of $MgCl_2$, 10 mM of dithiothreitol, 2 mM of spermidine, 2.5 µg of albumin, 25 units of RNasin, 1.76 mM of cap, 500 µM of ATP, CTP and UTP, 50 µM of GTP, 50 µCi of α-^{32}P-GTP, and 30 units of SP6 RNA polymerase. The mixture was incubated at 37 °C for 60 min, followed by digestion with DNase I (8 µg per mixture) at 37 °C for 10 min. Labelled RNA was extracted with phenol-chloroform-isoamyl alcohol and ethanol-precipitated.

Psoralen cross-linking

Small scale cross-linking with AMT psoralen was performed in a mixture (25 µl) containing 400 000 cpm (about 10 ng of RNA) ^{32}P-labelled MINS-transcript, 12 mM Hepes (pH 7.9), 60 mM KCl, 3.2 mM $MgCl_2$, 1 mM dithiothreitol, 12% glycerol, 2.1% (w/v) polyvinyl alcohol, 25 units of RNasin. Reactions were incubated for 10 min on ice, followed by the addition of AMT psoralen (20 ng per 12 ng of MINS pre-mRNA) and irradiation at 365 nm for 7 min in a high intensity mercury light irradiator as described by Isaacs et al. /9/. The samples were then digested at 37 °C for 30 min with Proteinase K (1 mg/ml) in the presence of 0.5% SDS and 20 mM EDTA. RNA was extracted with phenolchloroform-isoamyl alcohol and ethanol-precipitated. The pellets were resuspended in loading buffer (80% formamide, 0.025% xylene cyanol, and 0.025% bromphenol blue) and electrophoresed in 5% polyacrylamide gels containing 8M urea at 500 V for 12—14 h. For preparative psoralen cross-linking the same procedure was followed, except that 1.25 ml mixtures were used. The fractionated cross-linked RNAs were eluted from the gel and subjected to further analysis.

Primer extension analysis

Psoralen monoadducts and cross-links were mapped by using individual MINS pre-mRNA fractions as templates for reverse transcription /14/. 5' end labelled synthetic oligonucleotides were hybridized to the RNA species and reverse transcribed by avian myeloblastosis virus reverse transcriptase. cDNAs were purified and electrophoresed in 8% denaturing polyacrylamide gels at 1900 V for 2 h.

In vitro splicing reaction

In vitro splicing of MINS transcripts was carried out with a crude nuclear extract isolated from HeLa cells /14/. Reaction mixtures (25 µl) were the same as those described for psoralen cross-linking (see above), but ATP (0.5 mM final concentration), creatine phosphate (20 mM) and HeLa nuclear extract (30—60 µg protein) were also added. Reactions were incubated at 30 °C for 90 min, terminated, extracted with phenol-chloroform-isoamyl alcohol, precipitated with ethanol and subjected to polyacrylamide gel electrophoresis in 5% gels containing 8M urea.

RESULTS

The substrate RNA used for the present experiments was a run-off transcript of the linearized pSP63/MINS plasmid /10/. The transcripts (412 nucleotides) were initiated from the SP6 promoter of pSP64, and consisted of a part of the plasmids polylinker region and sequences from a mouse insulin gene containing two exons (E1 and E2) and an intron (I). ^{32}P-labelled MINS transcripts were cross-linked with a psoralen derivative (AMT psoralen) *in vitro*. Psoralens are bifunctional heterocyclic compounds that

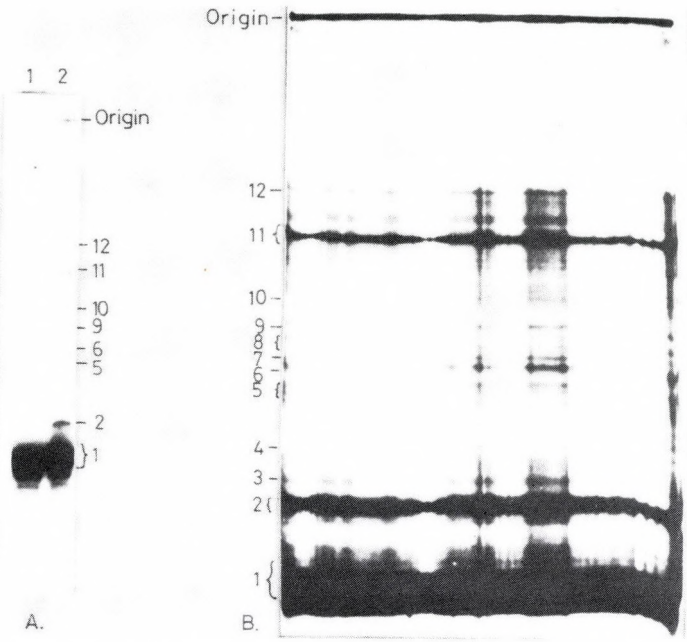

Fig. 1. Analytical (A) and preparative scale (B) fractionation of AMT psoralen cross-linked MINS pre-mRNA. ^{32}P-labelled MINS pre-mRNA was incubated without lane 1, (A) or with AMT psoralen lane 2, (A) and (B) irradiated and fractionated by polyacrylamide gel electrophoresis as described in Materials and Methods

are able to intercalate into double stranded regions of nucleic acids. Following UV irradiation photocycloaddition takes place between bound psoralen molecules and the bases of the polynucleotide chain (in the case of RNA usually U is the target of the photoreaction). As a result, psoralen monoadducts and cross-links are formed /3/.

RNA species containing covalently cross-linked loops have altered electrophoretic mobility in denaturing polyacrylamide gels (Fig. 1): cross-linked MINS transcripts (species 2 to 12 in Figs 1A and 1B, were easily separated from monoadducts and intact RNA molecules (species 1). (The covalent binding of AMT psoralen to the MINS pre-mRNA species was substantiated by the use of ^{3}H-labelled psoralen (data not shown).) Fractionation of AMT psoralen treated precursor RNA yielded multiple RNA species: beside the consistently reproducible strong bands (e.g. fractions 1, 2, 6, 9, 11 in Fig. 1) numerous minor bands appeared on our autoradiograms showing some variation from experiment to experiment; only the major RNA species were used for further analysis.

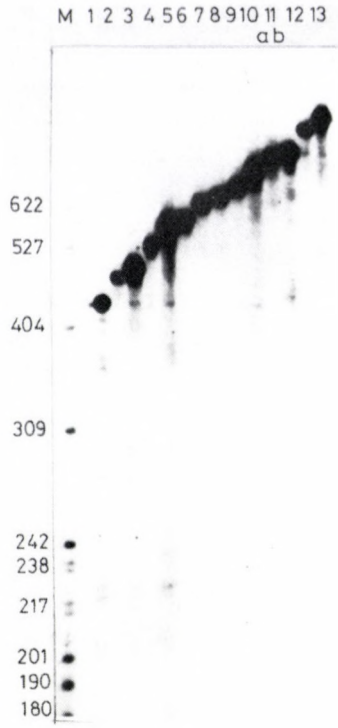

Fig. 2. Polyacrylamide gel electrophoresis of isolated cross-linked MINS pre-mRNA species. Individual RNA fractions of a large scale AMT psoralen cross-linking experiment similar to that presented in Fig. 1B were eluted from the gel and reelectrophoresed in a 5% denaturing polyacrylamide gel. M, 5' end labelled marker DNA prepared from HpaII digested pBR322

AMT psoralen treated RNA species were eluted from preparative polyacrylamide gels and their purity was checked by reelectrophoresis. As visible in Fig. 2 the RNA species were fairly pure and did not show extensive degradation or photoreversion. (Psoralen adducts are sensitive to UV light of short wavelength (see Ref. 3). Note that the amount of material comigrating with the intact transcript (lane 1) is small in all RNA species (lanes 2 to 13) showing that the extent of photoreversion was negligible during manipulation of the eluted RNA samples.) It should be mentioned that in the gel from which the RNA species of Fig. 2 were eluted, species 11 RNA appeared as a doublet (a and b) and an additional major band (species 13) was also visible (for comparison see Fig. 1).

The sites of AMT psoralen adducts of different MINS pre-mRNA species were mapped by primer extension analysis. 5' end labelled synthetic

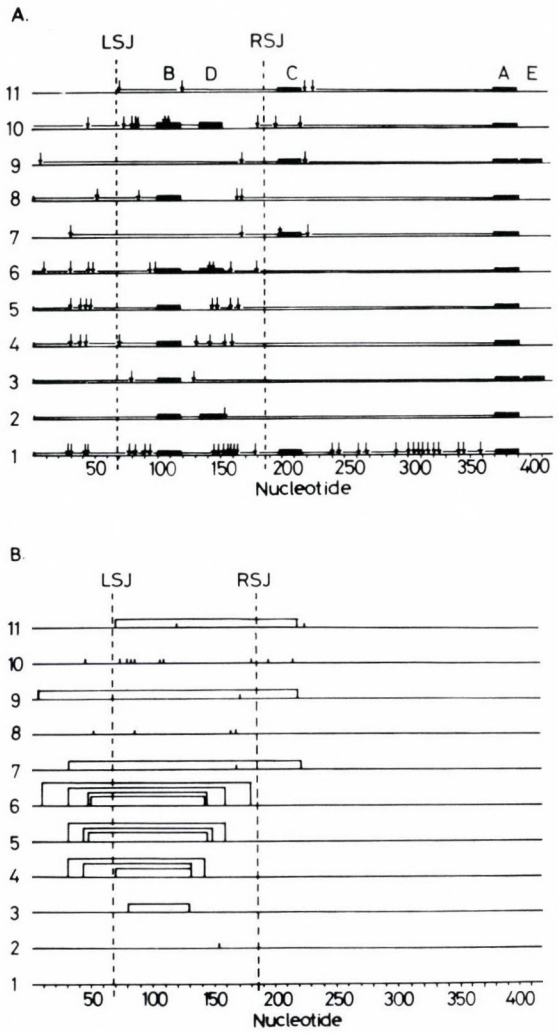

Fig. 3. Mapping of AMT psoralen monoadducts and cross-links on MINS pre-mRNA species. A: Individual RNA species eluted from a gel similar to that of Fig. 1B were subjected to primer extension analysis using synthetic oligonucleotides (A to E) as primers for reverse transcription. The arrows indicate stop sites for reverse transcriptase. B: Possible cross-links and monoadducts present in AMT psoralen treated RNA species as deduced from the results of primer extension analysis and the sequence of MINS transcript. Left (LSJ) and right splice junctions (RSJ) are located at positions 68/69 and 186/187, respectively

oligonucleotide primers complementary to different regions of the MINS transcripts (see oligonucleotides A to E in Fig. 3A) were hybridized to

the isolated AMT psoralen treated RNA species and these template-primer complexes were used for in vitro DNA synthesis by avian myeloblastosis virus reverse transcriptase. The products of primer extensions were purified and fractionated in sequencing gels (not shown). Bands of such autoradiograms correspond to prematurely terminated products of reverse transcription due to the covalent binding of AMT psoralen molecules. The stop-sites can be mapped by determining the size of primer extension products (see the arrows in Fig. 3A). The sites of possible cross-links can be deduced by conferring the results of reverse transcription experiments with the sequence of MINS precursor RNA (Fig. 3B). Some of the RNA bands (fractions 4, 5 and 6) contain more than one AMT psoralen cross-links and/or consist of RNA species with different cross-links. In species 2 RNA only a single reverse transcription stop-site (position 154) was found. However, since this RNA migrates much slower than intact MINS transcripts or molecules with mono-adducts, we assume that it should contain a cross-link forming a rather small loop. We could not find any obvious complementarity within the psoralen binding regions of species 8 and 10 RNAs. Since the electrophoretic mobility of these RNA species is low, they should contain cross-links that are formed between adjacent sites of tertiary rather than secondary structures.

In order to study the effect of cross-links on the in vitro splicing of MINS pre-mRNA, individual RNA species were incubated with a HeLa nuclear extract in a cell-free splicing mixture, and the products were fractionated in polyacrylamide gels (Fig. 4). Untreated transcripts (T in Fig. 4) were efficiently spliced: both the final product (E1E2) and one of the splicing intermediates (intron-lariat-E2) were detected in the gel. (Under our electrophoresis conditions the other intermediates (intron-lariat, E1) ran out of the gel and do not appear on the autoradiogram of Fig. 4). Species 1 RNA that consists of intact molecules and precursors with AMT psoralen mono-adducts was likewise a good substrate for the splicing extract. On the other hand, incubation of psecies 2 to 12 RNAs under splicing conditions resulted only in the conversion of substrate molecules to a slightly faster migrating heterogeneous population of molecules.These broad bands might contain (I) degradation products due to non-specific nucleases in the HeLa extracts, or (II) authentic splicing products and/or intermediates that have unusual electrophoretic properties caused by intra and/or intermolecular AMT psoralen cross-links. To decide between these two possibilities, a UV photoreversion experiment was performed. Species 2 RNA incubated with

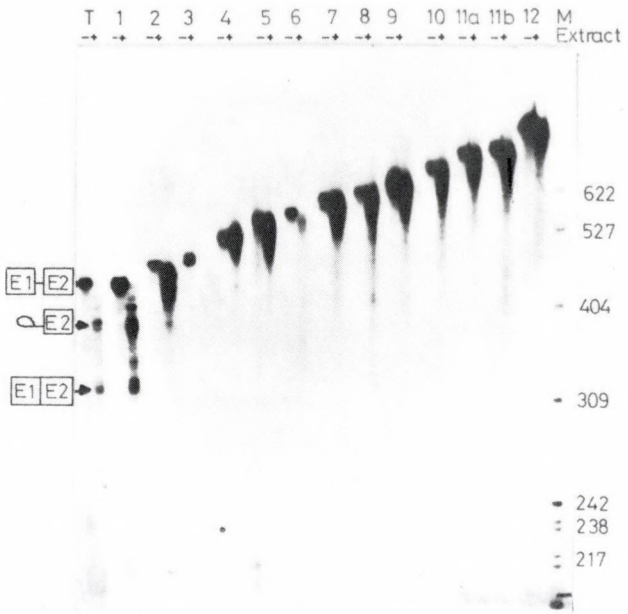

Fig. 4. In vitro splicing of AMT psoralen cross-linked MINS pre-mRNA species with HeLa cell nuclear extract. Intact transcripts (T) and individual AMT psoralen treated pre-mRNA species (1 to 12) were incubated under standard splicing conditions (see Materials and Methods) without (—) or with HeLa nuclear extract (+). Reaction products were fractionated in denaturing 5% polyac rylamide gels. M, 5' end labelled marker DNA prepared from HpaII digested pBR322

HeLa nuclear extract under splicing conditions was irradiated with UV light (254 nm) and subjected to electrophoresis in a denaturing gel. Bands that may correspond to either the precursor or to splicing products or intermediates did not appear on this autoradiogram (not shown). This observation indicates that the splicing, but not the degradation of band 2 RNA was inhibited by cross-linking. In fact, some of the cross-linked RNAs (especially species 3, see Fig. 4) are extremely sensitive to degradation. This can be due to the fixing of the precursor molecule by cross-linking in a conformation that renders the RNA accessible to non-specific nucleases.

DISCUSSION

Using the bifunctional photoreagent AMT psoralen, we prepared precursor mRNA species that contained covalent cross-links within or around

their intron region. These cross-linked pre-mRNA molecules were no longer substrates for the splicing machinery of a HeLa cell nuclear extract in vitro.

Fractionation of AMT psoralen cross-linked MINS pre-mRNA in denaturing polyacrylamide gels revealed multiple RNA species of different electrophoretic mobility (see Figs 1 and 2). Beside the major RNA species (bands 1 to 12 in Fig. 1) numerous minor bands were observed on the autoradiograms after longer exposure. Although only the most prominent RNA fractions were subjected to primer extension analysis (see Fig. 3) it is obvious that many different cross-linked products can be formed from MINS pre-mRNA molecules incubated in an in vitro reaction mixture optimal for splicing. This observation reflects a conformational heterogeneity among precursor RNA molecules: several regions of the transcripts may be involved in the formation of double stranded structures, some of them are even able to base-pair with more than one sequence. (See for example bands 4, 5 and 7: they contain RNA species in which nucleotide 30 is cross-linked with nucleotides 142, 161 and 222, respectively (see Fig. 3B).) It is also worth mentioning that all the cross-links mapped in AMT psoralen treated MINS pre-mRNA are within the intron or in its flanking sequences. It is conceivable that this compact conformation of the intron region may play a role in the earliest phase of splicing, but is destroyed thereafter (see below).

MINS pre-mRNA species containing AMT psoralen cross-links are not spliced in vitro (see Fig. 4). There are two possible explanations for this phenomenon: (I) the presence of AMT psoralen molecules in the precursor RNA per se interferes with the reaction, or (II) the splicing is hampered by stable intramolecular cross-links. Since band 1 RNA, that represents precursor molecules with psoralen monoadducts at different positions (see Fig. 3), but without cross-links, is efficiently spliced by the HeLa nuclear extract, we conclude that the presence of cross-links rather than that of psoralen is inhibitory for the splicing reaction.

Recent observations support the assumption that the secondary-tertiary structure of the substrate may have a significant impact on the splicing reaction. Solnick /18/ constructed substrate RNA molecules in which one or both splice sites were located in loops of stable double stranded stems. He found that splice sites sequestered in the hairpin structures were less efficiently used for splicing both in vivo and in vitro. It was suggested by this author that unwinding of the substrate RNA may be necessary for the splicing. Further evidence supporting this

assumption was obtained from our experiments /21/. Using the MINS pre-mRNA/HeLa extract system we found that an ATP-dependent unwinding of the substrate takes place during the early phase of in vitro splicing.

The factors involved in the melting of the secondary structure of precursor RNA are not known, but the most likely candidates may be among the hnRNP proteins /12, 16/. Szer and his co-workers /19, 20/ described a helix destabilizing protein (HD40) in hnRNP particles of Artemia salina. Circumstantial evidence suggests that a similar protein may be associated with hnRNP particles of rat liver /15/. Monoclonal antibodies against C proteins (components of hnRNP) inhibit the in vitro splicing reaction /2/ and the melting of the secondary structure of substrate RNA as well /21/. These data are consistent with a mechanism in which splicing is preceeded by the unwinding of substrate by specific hnRNP proteins, but further experiments are necessary to substantiate this model.

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HEAT AND pH DEPENDENCE OF CATALASE. A COMPARATIVE STUDY

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Effects of pH and heat were examined on the activity of enzyme catalase from human sources (normal and pathological sera, tissue homogenates, purified catalases).

The pH optimum, temperature optimum and T50 values of purified catalases were lower than those of normal, or pathological sera and tissue homogenates. On contrast, the activation energy showed its highest value in purified catalase. These findings might be explained by the post-translational modification of enzyme catalase.

The obtained results failed to enhance the diagnostic role of serum catalase determination, nevertheless, gave the optimal values of pH and temperature for catalase assay.

Keywords: Catalase — serum — tissues — pH — heat — comparison — diagnosis

INTRODUCTION

The enzyme catalase (EC 1.11.1.6) catalyses the decomposition of hydrogen peroxide into oxygen and water. It can be found in human tissues with its highest activity in the erythrocytes, liver and kidney /2, 8/, while its activity is very low in human serum /8/. In tissues catalase is localized in the mitochondria /2/ and peroxisomes /5/, while in erythrocytes it can be found in a soluble form /2/.

Catalase is a well known enzyme but its physiological function is still obscure /2/, although its role is underlined by newer findings on the

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protective mechanism of different organs against free radicals. These new results gave a further impetus to the catalase determinations.

We have developed a simple, fast and cost saving polarographic method /12/ for the determination of serum catalase activity. This assay can be used for pathological sera with icterus, lipemia and hemolysis and tissue homogenates as well. Neither the colour nor the turbidity of the sample influences this polarographic determination of hydrogen peroxide substrate.

The present paper reports the effect of pH and heat on catalase activity of pathological sera and tissue homogenates, using purified catalases for comparison. Our first aim was to determine the pH as well as temperature optima. These values can be used for the determination of enzyme activities from different sources, applying the same method. The references in the literature for these values are either very poor or show a wide range /2/ due to the different methods of determination. Furthermore, in case of some serum enzymes the variation of the enzyme activity with pH and temperature (alkaline phosphatase) and the values /22/ of activation energy (macro CK) can be used for diagnostic purposes. We have detected increased serum catalase activities in acute pancreatitis /9/, hemolytic diseases /10/, liver and heart diseases /12/. Our previous studies on electrophoretic mobility /11/ and substrate dependence /13/ of catalase in sera and tissue homogenates yielded no valuable results which could be used for differential diagnosis between diseases with elevated serum catalase activity. Therefore, our second goal was a diagnostic one, when we tried to detect organ specific catalase forms in serum.

MATERIALS AND METHODS

Human sera with normal (40—90 U/mL) and elevated catalase activities (200—700 U/mL) were taken from patients at the Department of Internal Medicine. Patients with pathological serum catalase activity had liver /10/, heart /6/ and pancreas /9/ diseases, while 7 patients had hematological /erythrocyte) problems.

Tissue samples from different human organs (liver, heart, pancreas) were taken by autopsy. The samples were washed with ice-cold phosphate buffer (pH 7.4, 0.06 mol/L) and then homogenized with a glass-teflon homogenizer. This homogenate was centrifued at 2000 rpm for 20 min and the supernatant was used for determination.

Whole blood was taken with anticoagulant EDTA. After centrifugation plasma was removed and red blood cells were washed three times with NaCl solution (0.9 g/L). The packed cells were hemolyzed with distilled water (1:2) at room temperature for one hour then the hemolysate was centrifugated at 2000 rpm for 20 min and the supernatant was used.

HEAT AND pH DEPENDENCE OF CATALASE

Purified beef liver catalase was purchased from Boehringer Mannheim (FRG), Sigma (USA) and Reanal (Hungary). Erythrocyte catalase was separated from human red cells by the method of Mörikofer-Zwez and co-workers /19/.

Samples were diluted with phosphate buffer (0.06 mol/L, pH 7.4) for heat dependence and with distilled water for pH dependence studies, resp. This phosphate buffer was used to adjust the samples to the required pH.

The catalase activity was determined by our polarographic method /12/ based on the change in the concentration of hydrogen peroxide substrate (65 μmol/mL) at 37 °C, except for the heat dependence measurements.

Samples were incubated either at the required temperate or pH for 20 min before starting the determination.

Values of pH optimum, T (temperature) optimum, T 50 (heat stability, i.e. temperature with half max imal activity) were determined graphically. The activation energy was calculated according to Arrhenius, based on determination of enzyme activities at four temperatures between 20 and 33 °C.

The Student's t test was used for calculation of significance.

RESULTS AND DISCUSSION

The effects of pH and temperature on catalase activity are shown in Table 1 and Figs 1, 2.

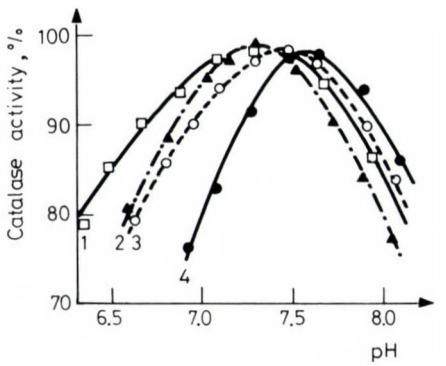

Fig. 1. Variation of catalase activity with pH: 1. purified catalase (SIGMA); 2. tissues homogenate (liver); 3. pathological sera (liver diseases); 4. normal sera

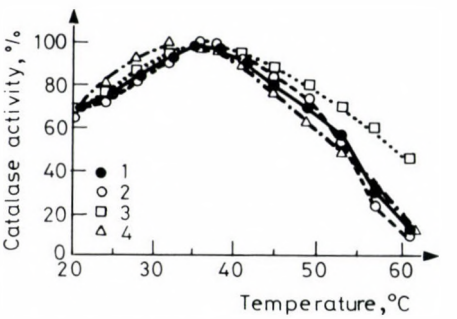

Fig. 2. Variation of catalase activity with temperature: 1. normal sera; 2. pathological sera (liver diseases); 3. tissue homogenate (liver); 4. purified catalase (SIGMA)

The lowest pH otpimum was detected in purified catalase samples ranging from pH 7.19 to 7.25. It was higher in tissue homogenates (7.23—7.28) and the highest ($0.001 < p < 0.01$) in pathological (7.42—7.47) and normal sera (7.54). The range of pH optimum for purified catalases and

Table 1

pH optima, activation energy (E), T optima and T_{50} values of different catalase samples

	pH optima			E Kcal/mol			T optima °C			T_{50} °C		
	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n	$\bar{x}$	SD	n
Normal sera	7.54	0.07	14	1.38	0.20	14	36.3	0.26	14	53.8	2.0	14
Pathological sera in												
disease of pancreas	7.47	0.08	6	1.58	0.18	5	37.3	0.30	5	51.8	1.8	5
liver	7.42	0.07	7	1.88	0.21	7	37.0	0.27	7	54.8	1.6	7
heart	7.43	0.07	7	1.64	0.26	6	37.5	0.28	6	52.3	1.9	6
erythrocyte	7.45	0.06	5	1.85	0.19	7	37.2	0.32	7	54.4	2.2	7
Tissue homogenizates												
of pancreas	7.27	0.06	6	1.46	0.21	5	37.0	0.19	5	61.5	2.1	5
liver	7.28	0.05	7	1.82	0.23	6	37.3	0.20	6	60.6	2.4	6
heart	7.24	0.07	6	1.28	0.18	5	37.1	0.18	5	58.8	2.1	5
erythrocyte	7.23	0.08	8	1.69	0.21	11	37.1	0.15	11	56.4	1.9	11
Purified catalses from												
Sigma	7.25	0.04	7	1.96	0.19	6	34.0	0.31	6	50.8	1.1	6
Boehringer	7.24	0.06	6	2.37	0.22	6	34.1	0.28	6	52.2	1.3	6
Reanal	7.19	0.07	7	2.28	0.21	9	34.9	0.24	9	51.2	0.9	9
Erythrocyte	7.22	0.04	7	2.24	0.20	6	34.9	0.29	6	52.4	1.2	6

tissue homogenates, resp. is 6.8—7.5 /2/ and this is in accordance with our findings. At pH 7.0, recommended by manufacturers (Sigma, Merck and Serva), we found 96.8 per cent of the maximal activity in purified catalase samples.

The lowest activation energy was found in normal sera (1.38 kcal/mol). It was slightly higher (1.46—1.69 kcal/mol) in the homogenates of pancreas, liver and erythrocyte hemolysate, as well as in pathological sera (1.58—1.88 kcal/mol). The highest values (p 0.001) were calculated for the purified catalases (1.96—2.37 kcal/mol), while the lowest one in heart tissue (1.28 kcal/mol). Our findings are in agreement with the results of Aebi /2/ (0.6—1.7 kcal/mol), except for the purified catalase samples.

The highest T optimum value was detected in the pathological sera (37.0—37.5 ^{o}C) and tissue homogenates (37.0—37.3 ^{o}C). It was lower ($p < 0.001$) in normal sera (36.3 ^{o}C), while the lowest values were found in purified catalase (34.0—34.9 ^{o}C) samples. These results are in agreement with those of other authors /16, 20/ who have found it to be between 30 and 40 ^{o}C. The moderate change in heat dependence of catalase activity is in accordance with its low activation energy.

The value of T_{50}, representing the heat stability, was the lowest in purified catalases (50.8—52.4 ^{o}C). It showed no significant rise ($0.05 < p$) in pathological (51.8—54.8 ^{o}C) and normal sera (53.8 ^{o}C). The T_{50} was the highest (p 0.001) in tissue homogenates (56.4—61.5 ^{o}C). These results agree with the findings of other authors /6, 14, 15, 25/ who have found wide ranges (46—68 ^{o}C) for the T_{50} values.

The values of activation energy and pH optima were different ($p < 0.05$) for normal sera as well as pathological sera obtained from patients with liver, heart and hemolytic diseases. In case of T optima, significant difference could be found between the normal and all types of pathological sera. These findings could be explained by the ageing of the enzyme and/or by other changes which might occur in the serum /17, 18, 24/.

We were only able to find a slight correlation ($r = 0.677$) between the activation energies of catalase of pathological sera in patients with liver diseases and those of homogenizates of liver tissues in one case. However, this correlation cannot be used for the differential diagnosis of liver diseases due to the wide ranges of activation energies in tissue homogenates and pathological sera.

The different changes in catalase activity on the effect of pH and heat in sera, tissue homogenates as well as purified catalases might be explained by post-translational modifications as complex formations

/1, 3, 4, 7, 20, 21, 23, 24/, and effects of proteolysis /17, 18/, which may take place in tissues, sera and during purification.

The pH and T optima of catalase activity presented here may be helpful in respect to the more exact determinations of catalase activities in tissue homogenates and that of purified catalase samples.

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SERUM CATALASE ENZYME ACTIVITY IN LIVER DISEASES

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Serum catalase activity was moderately increased in fatty liver, acute alcoholic hepatitis and in the decompensated form of cardiac circulatory failure. It showed significant increase in acute yellow atrophy and in toxic hepatitis while no changes were detected in liver cirrhosis and viral hepatitis.

Serum catalase activity showed a good correlation ($r = 0.820$) with the serum glutamate dehydrogenase activity.

In accordance with our results, the inexpensive assay of serum catalase activity is suggested for the detection of severe liver cell damage.

Keywords: Serum catalase — liver diseases — diagnosis

INTRODUCTION

Enzyme catalase (EC 1.11.1.6) decomposes hydrogen peroxide into oxygen and water. This enzyme has an important role, in the defensive mechanism of the different organs, against the free radicals. The highest catalase activity can be found in the liver, kidney and erythrocytes /1—3/, being very low in the blood serum /3/.

Earlier we found increased serum catalase activity in hemolytic disease /4/ and in acute pancreatitis /5/ which can be used as a diagnostic tool for the detection of these diseases.

In this paper we report on an investigation of one serum catalase activity in liver diseases, since an increased serum catalase activity might be expected in some diseases of the liver rich in catalase.

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METHODS AND PATIENTS

Serum catalase activity was measured by our fast and cost saving polarographic method /6/. Its normal value (mean and SD) is 56.7 ± 21.3 kU/L, n = 111.

For the determination of other serum enzyme activities, optimized monotests (German Society for Clinical Chemistry) were used at 25 °C obtained from Boehringer Mannheim (FRG). These enzymes and their normal values were GOT: 0—18 U/L, GPT: 0—22 U/L, LHD: 120—240 U/L, GLDH activated: 0—4 U/L, gamma—GT: 4—28 U/L.

The serum catalase activity was determined and compared with the GOT, GPT, GLDH and gamma—GT enzyme activities in the following liver diseases: cirrhosis (52), viral hepatitis (315) and its final stage (acute yellow atrophy 18), acute alcoholic hepatitis (42), different types of toxic hepatitis due to toxic liver damage (toadstool toxin 4, drugs 4, pesticides 7), as well as in the decompensated form of cardiac circulatory failure (221).

RESULTS

Serum catalase, GOT, GPT, GLDH and gamma—GT enzyme activites and the pathological frequency of serum catalase activity are shown in Table 1.

No changes ($p > 0.05$) of catalase activity were found in liver cirrhosis and acute viral hepatitis, while the GOT, GPT activities showed slightly and highly increased values, resp.

In fatty liver and acute alcoholic hepatitis slightly elevated catalase (1.68—1.85-fold), GOT, GPT and GLDH activities were detected.

The highest serum catalase activity (4.79—4.84-fold) was manifest in acute yellow atrophy of the liver and in toxic hepatitis with very high GOT, GPT, GLDH and gamma—GT activities. The gamma—GT activity was the highest in acute alcoholic hepatitis. On average a 1.96-fold elevation of serum catalase activity was detected in the decompensated form of cardiac circulatory failure. These increased serum enzyme activities may be the results of liver congestion which is frequent in this disease.

The glutamate dehydrogenase enzyme is localized in the liver mitochondria, and its increased serum activity is known as a good indicator of severe liver cell damage.

Good correlation ($r = 0.820$, intercept = -14.99, slope = 0.340, $n = 326$) was found between the activities of serum catalase and GLDH. Normal GLDH activity (4.1 ± 1.2 U/L) with elevated serum catalase activity (337.5 ± 289.6 U/mL) was detectable in 34 cases (10.4 per cent), while normal serum catalase (77.4 ± 15.1 U/mL) with increased GLDH activity (36.2 ± 23.7 U/L) was measured only in 17 cases (5.2 per cent).

Table 1

Serum catalase, GOT, GPT, gamma-GT, GLDH activities and pathological frequences of serum catalase in liver diseases

	Cirrhosis n=52	Viral hepatitis n=315	Fatty liver n=21	Acute alcoholic hepatitis n=42	Acute yellow atrophy n=18	Toxic hepatitis n=19	Circulatory failure n=221	Normal value/ range
Catalase U/mL								
$\bar{x}$	55.9	59.1	90.8	105.1	211.8	216.8	117.4	56.7
SD	24.1	24.8	31.9	48.2	a	a	a	21.3
n	91	675	31	95	83	54	221	111
GOT U/L								
$\bar{x}$	48.5	486	42.7	35.3	389.6	240.9	32.1	0—18
SD	15.4	249	22.3	20.4	146.1	92.4	14.2	
n	86	610	31	95	83	54	221	
GPT U/L								
$\bar{x}$	32.6	542	36.7	28.1	450.8	290.6	26.3	0—22
SD	8.7	238	10.5	9.5	158.7	100.8	7.1	
n	86	610	31	95	83	54	211	
gamma—GT U/L								
$\bar{x}$	—	—	30.6	284	86.2	143.8	—	4—28
SD			16.2	110.3	39.4	90.5		
n			24	86	40	44		
GLDH U/L								
$\bar{x}$	—	—	7.6	9.8	210.3	180.7	51.6	0—4
SD			4.1	2.9	a	a	a	
n			35	54	52	45	140	
Catalase pathological frequency %	6.6	19.7	38.6	48.4	79.5	87.1	49.1	

a=log/normal distribution

DISCUSSION

Catalase-rich liver contains this enzyme in the mitochondira /1/ and peroxisomes /2/.

The serum catalase activity was not increased in liver cirrhosis and viral hepatitis. On the contrary significantly increased serum catalase activity was found in acute yellow atrophy and toxic hepatitis, diseases which are characterized by the damage of liver cell particles.

The serum glutamate dehydrogenase activity is a good indicator of the damage of liver mitochondria, and a good correlation was found between the activities of the serum catalase and GLDH enzymes. The pathological frequency of serum catalase was high in acute yellow atrophy and toxic hepatitis, furthermore its sensitivity was higher than that of serum glutamate dehydrogenase.

In agreement with these facts, the inexpensive serum catalase determination can be proposed in the diagnostics of acute yellow atrophy as well as in toxic hepatitis. Furthermore, the increased serum catalase activity might indicate a severe form of fatty liver, acute alcoholic hepatitis and the decompensated form of cardiac circulatory failure.

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Acta Biologica Hungarica 38(2), pp. 291—295 (1987)

BOOK REVIEWS

Bestimmung wirbelloser Tiere im Gelände

(Determination of Invertebrates in Country)

H.J. MÜLLER Ed.

VEB Gustav Fischer Verl., Jena (1985) pp. 280.

Together with eight co-workers, the publisher has provided those interested with a very clear-cut taxonomic book of animals.

The taxonomic book satisfying requirements of higher standard is not an easy literary form. The often extremely similar species can only be differentiated even by the line-specialists on the basis of great practice, and the studies necessitated for the identification can frequently only be carried out in laboratory. This, however, leads to the detachment of the object from its environment, which makes difficult the improvement of the so diesirable ecological approach. In the knowledge of these facts such taxonomic books, merely providing fundamentals, are being prepared which can also be used without more thorough grounding and are suitable for familiarizing those interested with the autotypes of the fauna of various areas.

This book belongs to the latter group. It has firstly been prepared for biology students, future teachers and those showing interest towards the fauna in general. A further purpose of the book is to train for the perception of the manifoldness and beauty of nature. Its first and foremost aim is to be descriptive. It is easily usable, in the interest of which it does not discuss the groups of microscopic order and the representative of the endoparasites. In the case of the more difficult taxons the genus or family are described, otherwise the demonstration of the frequent or easily identifiable species is given.

Illustrative taxonomy. Line diagrams or part-drawings of animals are the base of this work. These are grouped in a taxonomic key-like manner, in a dichotomic system, with one-two words beside the figure calling attention to the distinguishing characters also visible to the naked eye, or observable by a simple magnifying glass.

The book which includes an introduction of a few pages as well as an index of the discussed genera and species comprises 147 tables, demonstrating 2097 invertebrates from which 1609 are species and 488 are genera.

Akadémiai Kiadó, Budapest

The book can also well be made use of by the interested public of the countries neighbouring the German Democratic Republic.

G. Gere (Budapest)

Heavy Metals in Water Organisms

J. SALÁNKI Ed.

Symposia Biologica Hungarica, Vol. 29

Akadémiai Kiadó, Budapest (1985) pp 441, 96 tables, 115 figures

The volume contains 32 contributions in the form of full papers, presented at a symposium held at the Balaton Limnological Research Institute of the Hungarian Academy of Sciences, Tihany, Hungary in September, 1984. The papers, each followed by a discussion with the participants, are arranged around the following four main topics: "Accumulation of heavy metals", "Ecological monitoring of heavy metal pollution", "Monitoring heavy metals at the individual level", and "Functional aspects of heavy metal pollution". Thus — about one third of the papers being a review of the works done by the contributors in recent years — this volume presents complex information on both observations and an experimental approach to ecotoxicology and the bioindication of heavy metal pollution of natural waters. Another advantage of this collection is the special attention paid — due to the location of the Symposium — to the heavy metal status of Lake Balaton, this well-investigated and largest lake of Central Europe serving as an extremely important recreation, bath, and sport area of international significance.

Therefore, this book should attract the interest of ecologists, environmental scientists, toxicologists, limnologists, and experts of regional planning.

G. Réz (Budapest)

The Possibilities on Increasing Genetic Variability in the Plant Kingdom

GY. PÁL Ed.

Proc. of the Hungarian-Italian Plant Genetic Conference held in Martonvásár from 26 to 30 June, 1984

Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, pp 208.

Several methods are known for increasing genetic variation such as hybridization, mutation, poliploidy, chromosome manipulation, etc. In addition to these, nowadays plant cell and tissue cultures, protoplast fusion are being used more and more widely. It is an important task to determine how these methods contribute or are able to contribute to the increase of genetic variation and how these can be used in the development of new varieties.

The book contains the 15 papers read at the Hungarian—Italian

joint conference on the above subject. The papers cover the main aspects of increasing genetic variability as follows: induced mutation, polyploidy, intervarietal and distant hybridization, chromosome manipulation in cereals, somaclonal variation. Some papers deal with the utilization of increased variation in plant breeding.

The book is a useful summary for university professors, students, as well as for plant breeders.

J. Sutka (Martonvásár)

Color Atlas of Physiology

A. DESPOPOULOS and S. SILBERNAGL Ed.

(3rd revised and enlarged edition) Georg Thieme Verlag, Stuttgart, New York (1986) pp 356, 154 color plates, Price: DM 33.-

The book is translated from a German original first published in 1979 and re-edited in a revised form in 1983. Two main advantages are attributable to the book, (1) it provides brief information about all questions and recent knowledge in human (and animal) physiology, (2) a large number of color illustrations help to understand the rather complex and dynamic processes in a very didactical way. Nevertheless, this is not the book which would be a primary source for university students for studying physiology, but is very good for summarizing and overviewing what has been learnt from textbooks. Both the short, concise definitions and the careful, demonstrative illustrations oriented to visual perception may be extremely useful for readers in physiology and may also be made use of in popular lectures for non-specialists in helping to make a number of physiological processes understandable.

As usual in such types of pocket-books, there are numerous simplifications both in the text and the figures, but it is quite obvious that such a limited volume cannot comprise the detailed description and explanation of all the varieties of functions occurring in the animal kingdom.

The book is a useful and enjoyable information source for both students and teachers in physiology.

J. Salánki (Tihany)

The Protean Gate. Structure and Plasticity of the Primary Nociceptive Analyzer

B. CSILLIK and E. KNYIHÁR—CSILLIK

Akadémiai Kiadó, Budapest (1986) pp 294, 151 figs, 8 tables

The Protean Gate is a metaphoric label to denote the plasticity of sensory function in the spinal cord. The primary nociceptive analyzer in the upper dorsal horn is a readily changing form, appearance and function, like Proteus, the famous sea-god of the Greek. The authors have provided major contribution to our knowledge on the plasticity of the gate for sensory information, particularly in relation to pain. They present their

work in this book with many schematic diagrams and original histochemical and electron microscopic pictures of high quality. There are detailed descriptions about the applied techniques, too.

The most important topics are the followings. Structure of the primary nociceptive analyzer, immunocytochemical localization of substance P and somatostatin, analysis of axon terminals in the glomeruli of the Rolando substance, features of the transganglionic degenerative atrophy induced by transection or crushing and ligating the peripheral nerves, transneuronal degeneration and restoration of the primary nociceptive analyzer; and electrophysiological correlates and therapeutic considerations are also included.

The book is recommended to those interested in the theoretical and clinical aspects of the gating function of the spinal cord, which plays an important role in the processing of sensory information.

L. Kovács (Debrecen)

Multidomain Proteins

Proceedings of the Unesco Workshop on Structure and Function of Proteins

Budapest, September 13—15, 1984

L. PATTHY and P. FRIEDRICH Eds.

Akadémiai Kiadó, Budapest (1986)

This book provides an opportunity for researchers to read 16 interesting papers about the structure and function of multidomain proteins. The lectures had been delivered at the "Workshop on Structure on Multi-domain Proteins" held in Budapest, September 13—15, 1984. The general discussion by the participants and a list of contributors are presented at the end of the book.

The discussed proteins include fibronectin, fibrinogen, serine proteases, plasmin, plasminogen activators and immunoglobulins.

O.B. Ptitsin et al. showed both the perspectives and the limitations of diffuse X—ray scattering for the study of domain displacements in proteins. Fibronectin was the subject of several lessons, existing in two forms, a soluble monomer present in plasma and a fibrillar variant predominantly found on the surface of adherent cells. J.E.Schwarzbauer et al. described how the analysis of cDNA and genomic clones encoding fibronectin has helped to explain some of the structural and functional similarities and differences of the two forms. Their investigation indicated that the sequence of the region of the fibronectin gene encoding the difference segment clearly shows the pattern of RNA processing which gives rise to the three forms of mRNA.

In the case of immunoglobulins which are the very representative members of multidomain proteins, the structural and fucntional folding and genetic autonomy of the "domains" frequently make demarcation of the units self-evident. P. Zavodszky et al. discussed the domain structure and signal transduction within the antibody molecule.They concluded that the IgG molecule is not just the sum of its structural domains and ligand binding increases the conformational stability of the molecule, which effect extends to domains distant to the binding site.

At the closing roundtable discussion of the meeting general, philosophical questions were disputed such as: What is the definition of

domain? What kind of structural elements are coded by exons? What is the role of exons and introns in evolution?

The book is highly recommended to those interested in the structure and evolution of multidomain proteins.

Margit Balázs (Debrecen)

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The *full paper* should be divided into the following parts in the order indicated:

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3. *Materials and methods*
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3. Umbreit, W. E., Burris, R. H., Stauffer, I. F. (1957) *Manometric Techniques.* Burgess Publishing Co., Minneapolis.

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CONTENTS

TWENTIETH ANNIVERSARY OF A SUCCESSFUL VENTURE

In the early sixties it became clear in our Country that biological teaching and research needs urgently new, up-to-date chairs and laboratories in the framework of our Universities. Namely neither the traditional departments of the non-medical section of the Universities, nor the Medical Schools could fulfil the requirements of educating well trained high- and primary-school teachers as well as research workers in biology. This evidence was the primary motivation for the foundation of a series of new chairs in biological sciences in Budapest and Szeged.

Among the first departments established in those years — shortly after the foundation of its homologue in the Capital — has been the Department of Comparative Physiology of the József Attila University in Szeged in 1967, precisely twenty years ago.

Its founder, Professor Ottó Fehér arrived from another University, namely from the Medical School of Debrecen where he taught for many years Physiology as a leading pupil of the late Professor István Went. He was full of enthusiasm, new ideas and energy coupled with a supreme knowledge of human and animal Physiology, research strategies and new techniques.

The new Department showed quickly marked signs of high quality and consolidation. It attracted a constellation of talented young scientists and students, it inspired first class publications in neurophysiology, it drew the attention of the international scientific community.

Nowadays — as demonstrated by the excellent publications of the present volume — the scientific achievements of the Department, celebrating its jubilee, deserves acknowledgement and appreciative attention.

The Hungarian and the international scientific communities congratulate our colleagues in Szeged on the occasion of their bidecennial anniversary whishing them further creative enthusiasm and much success.

Professor György Ádám

Budapest, October 1987

Acta Biologica Hungarica, 38(3–4), pp. 299–314 (1987)

EFFECTS OF EXTRACELLULAR Ca AND Ca-CHANNEL BLOCKERS ON A-CURRENTS IN SNAIL BRAIN NEURONS

L. Erdélyi

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(Received 1987—10—02)

A study was made to characterize the extracellular Ca sensitivity of the A-currents in identified neurons (LPa2, 3) of the snail, Helix pomatia L. The characteristics and voltage-dependence of activation and inactivation of isolated A-currents were examined under voltage clamp in normal, low Ca and high Ca solutions. Modification of the extracellular Ca concentration between 0 and 28 mM caused a dose-dependent attenuation of the amplitude of A-currents and alteration of the current kinetics. Anorganic Ca-channel blockers (Ni, Cd, La) also decreased the amplitude of A-currents in a dose-dependent reversible manner, but the organic Ca-channel blocker verapamil did not influence the A-current amplitude. An analysis of the Hill plots of Ca and Ni dose-inhibition data showed similar values for the Hill constants (n_H) with 1.3 and 1.2 respectively.

Lowering the potassium concentration in the saline increased the amplitude of A-currents, while Na-deficiency slightly decreased the amplitude of A-currents.

Tetraethylammonium (TEA) modulated the amplitude and kinetics of A-currents and 4-aminopyridine (4-AP) blocked all components of the A-currents.

Keywords: Snail neuron — extracellular Ca — Ca-channel blockers — A-current

INTRODUCTION

It is well-documented and generally accepted that most of the molluscan neurons have a significant fast outward current component, known as

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Akadémiai Kiadó, Budapest

the A-current or I_A /for review see, 2/. This current is reported to be responsible for some elementary and integrative neuronal functions such as control of the cell excitability in the subthreshold range, synaptic modulation, regulation of the interspike interval and it is markedly modulated during learning /3, 9, 10, 32/. The widespread appearance of the A-current in the animal kingdom and early functional existence during ontogenesis can also emphasize its importance /17, 27, 32, 33/. Recently, a calcium-dependent transient outward current was isolated in some vertebrate and few invertebrate preparations /7, 23, 28, 34, 36, 42/. Calcium- and voltage-dependent activation of an outward current (I_C) is a known component of the delayed outward current in molluscan neurons, too, but an involvement of calcium in activation of the transient outward current is still not clear /25, 26/. However, some Ca-channel blockers such as verapamil have been reported to influence the time course of A-currents /21/. TEA was reported as a non-specific blocker of the A-current or it was found practically ineffective on A-currents in other laboratory /16, 20, 30, 38/. 4-aminopyridine (4-AP) is a widely accepted but non-specific blocker of the A-currents /6, 16/.

The aim of the experiments was to assess the extracellular Ca sensitivity of A-currents.

MATERIALS AND METHODS

The experiments were carried out on identified and some unidentified neurons of the snail Helix pomatia L. The studied identified cells as identical with the left parietal 2, 3 neurons (LPa2, 3).

The preparation, the physiological solution and the recording procedure were the same as described earlier /11, 12/. The Ca concentration varied between 0 and 28 mM by an appropriate increase or decrease of the Na or Tris content in the saline. Solutions with Ca-channel blockers were prepared freshly.

A-currents were isolated under spike threshold voltages by the use of conditioning hyperpolarization protocol. The potential and current signals were recorded on a Tektronix storage oscilloscope under voltage-clamp circumstances and photographed from the screen. The recorded currents were corrected for leakage and other small contaminating currents before plots were made.

All experiments were made with active snails in each season of the year, at room temperature (22—25 ^{o}C).

RESULTS

Characteristics of A-currents in LPa2, 3 neurons

LPa2, 3 neurons are silent cells with relatively high resting mambrane potential (-55 $\pm$ 4.5 mV, n=25; mean $\pm$ S.D.) and spike threshold potential.

As Figs 1Aa and Ba show in 4K and 1K solutions when the membrane voltage is stepped from a holding potential level (V_h = -60 mV) to a hyperpolarized conditioning level (V_c = -110 mV, 500 ms) and back to near the resting membrane potential by the use of a test voltage step (V_t), the transient outward current that rapidly builds up and more slowly declines to zero, is almost entirely A-current. However, a precise isolation of A-currents needs corrections. Firstly, the leakage currents (I_L), activated by a similar size of voltage pulses as V_t, but apposite in polarity have to be withdrawn. Secondly, current traces activated by test voltages but without conditioning hyperpolarization will give another correction factor (Figs 1A, Bb). Thus the corrected A-currents are the difference of currents shown in Figs 1A-B, a and b.

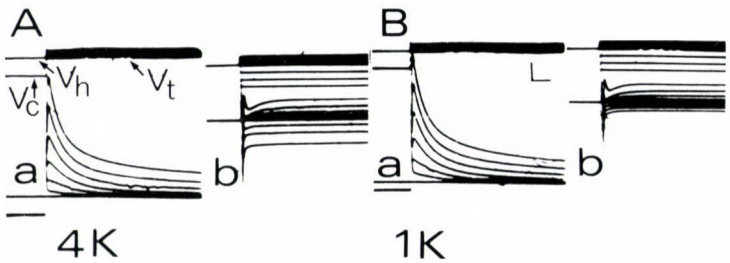

Fig. 1. Membrane outward currents in 4 and 1 mM K-containing solutions (A and B) activated with (a) and without (b) conditioning hyperpolarizations (V_c) at 25 °C. V_h = holding potential; V_t = test voltage steps. A-current is the difference of currents recorded in a and b. Leakage current (I_L) is also shwon in the b panels of the figure activated by negative V_t steps in the LPa2 neuron. Calibration: 25 nA, 100 ms, 50 mV

To determine the inactivation of A-currents the following procedure was used. Membrane voltage was stepped up from a conditioning level to a given test voltage. The A-current which caused activation during the test voltage was measured. This was repeated for a family of condition clamp voltages from -50 to -100 mV. Fig. 2 shows the activation and normalized steady-state inactivation of A-currents vs. membrane potential plots for

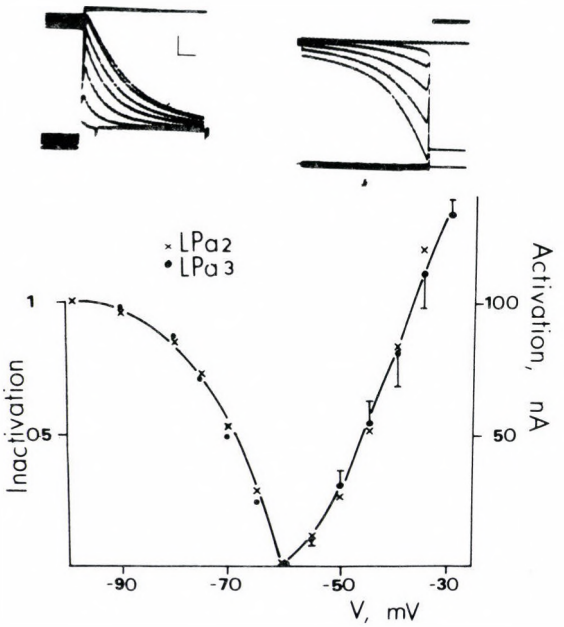

Fig. 2. Voltage-dependence of activation and inactivation of A-currents in normal solution at 22 °C. Insets show the recorded currents in a LPa3 neuron. Calibration: 20 nA, 100 ms and 50 mV. (Values: mean±S.D., n = 3)

LPa2, 3 neurons in normal solution. As can be seen in the figure the mid-inactivation point occurs at -68 mV in both neurons.

Fast outward and delayed outward currents activate at different membrane voltage. Fig. 3 shows the current-voltage (I-V) relations of separated A- (I_A), delayed outward (I_K+I_C) and leakage currents (I_L) in the LPa2 neuron respectively. Thus, for weak depolarizations, the fast outward current caused activation. Therefore, at these levels of membrane potential the fast outward current can be estimated by subtracting the extrapolated leakage current from the total peak outward current. The fast outward current has a threshold near -45 mV well below the level of significant activation of other currents.

As Figs 1—3 show A-current amplitude reach a peak at about ten ms after activation and the time course of decline after the peak can be characterized by a single exponential. Fig. 4A shows the semilogarithmic plot of normalized relative amplitudes of A-currents vs. time recorded in normal and low potassium solutions. As can be seen in the Figure, the time constants of the decline of A-currents are 163 ms at -35 mV membrane potential in the LPa2 neuron. The time course of the removal of A-current inactivation is shown in Fig. 4B. Conditioning prepulses of variable duration were applied from a given conditioning level to a test voltage to

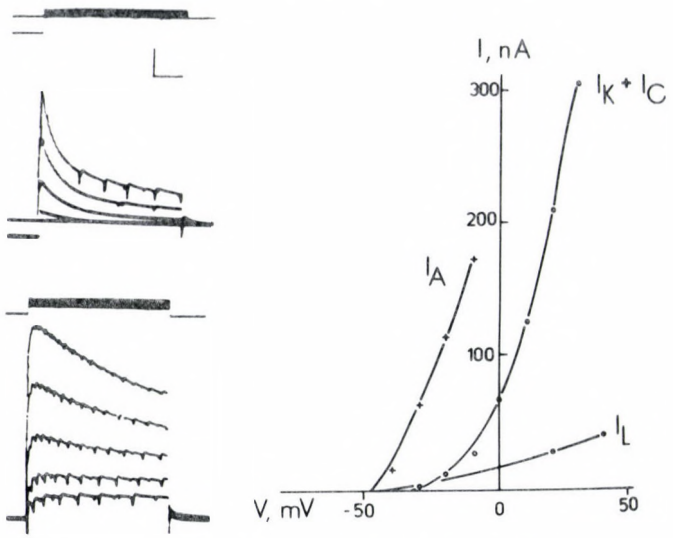

Fig. 3. Current-voltage (I-V) relations of separated fast outward (I_A), delayed outward ($I_K + I_C$) and leakage currents (I_L) in normal solution at 35 °C. Left side records show families of fast and delayed outward currents in the LPa3 neuron. Membrane currents are contaminated by axon spike artefacts. Calibration: 50 nA, 200 ms and 100 mV

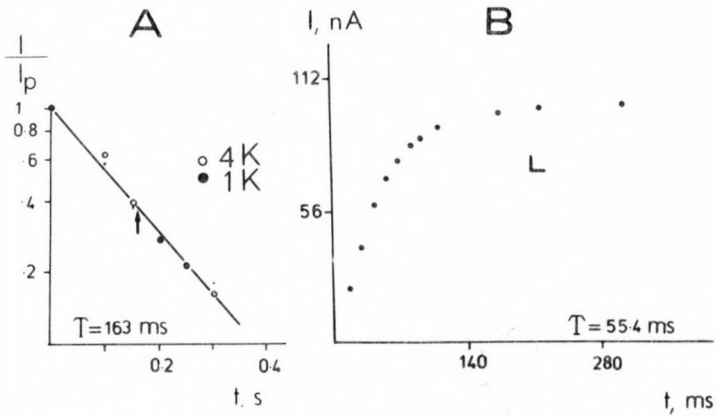

Fig. 4. A) Semilogarithmic plots of normalized relative amplitudes of A-currents-declines after peak vs. time in 4 and 1 mM K-containing normal solution at 25 °C. B) Time course of removal of inactivation. Peak A-currents during the test level are plotted against the conditioning pulse duration. Inset shows the recorded currents. V_h = -45 mV; V_c = -90 mV; V_t = -35 mV. Calibration: 20 nA, 100 ms and 50 mV

characterize the time course of the removal of inactivation. As can be seen in Fig. 4B the time constant is estimated at 55.4 ms during a protocol.

A-current is a potassium current as indicated by reversal potential measurement. In the experiment illustrated in Fig. 5A the A-current is activated, after the removal of inactivation at -70 mV, by a depolarizing step to -30 mV. At 30 ms after the current peak the membrane is repolarized to potentials ranging from -40 to -90 mV. The reversal potential of the tail currents is close to -75 mV in this experiment. Tail current amplitude vs. membrane potential plot gives the instantaneous current-voltage relations of the A-currents. The I-V relation appears to be linear over the voltage range between -40 to -70 mV. The current reversal potential (E_A) suggests that the A-current is essentially a K^+ current. E_A values in five other cells, measured as the voltage at which reversal of the tail current occurs, ranging between -70 and -80 mV.

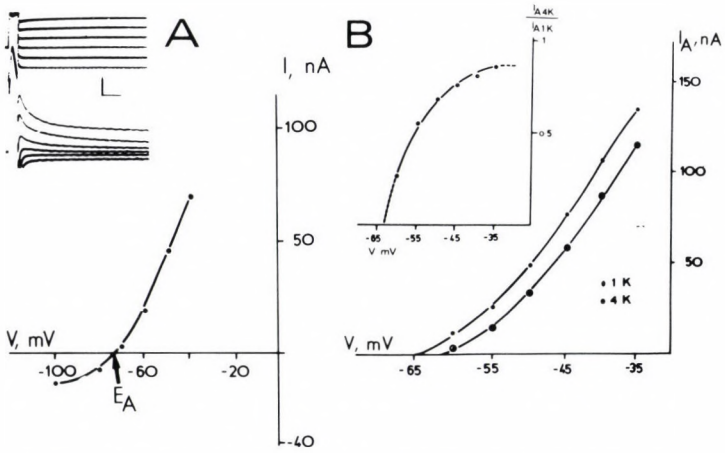

Fig. 5. A) Instantaneous I-V relations of A-current. Tail currents shown in the inset were plotted against the membrane potential to estimate the reversal potential (E_A) of A-currents. V_h = -70 mV; V_t = -30 mV. Calibration: 25 nA; 50 ms and 20 mV. B) A-current amplitude vs. membrane potential curves recorded in normal and low potassium solutions. Inset shows the potential dependence of relative amplitude of currents recorded in 4K and 1K solutions

Decreasing the potassium concentration in the bathing solution from 4 mM to 1 mM increased the amplitude of the A-currents and decreased the leakage conductance. The I-V relations of A-currents in 1 mM and 4 mM K-containing normal solution can be seen in Fig. 5B. The effect of extracel-

lular K on A-current amplitude is voltage-dependent. This is shown in the inset of Fig. 5B, where the relative amplitude of A-currents is plotted against membrane potential. An enhancement of the A-current amplitude evoked by lowering the potassium concentration from 4 mM to 1 mM decreases as the membrane potential becomes more positive. However, the time-to-peak or the time constant of decline of A-currents was not significantly modulated in the low potassium solution (Figs 1A, B and 4A).

Lowering the extracellular Na concentration in the saline from 80 mM to 0 mM (Tris substitution) had a very moderate effect on A-currents by causing a maximum 15% decrease of the amplitudes at -35 mV.

Effects of extracellular Ca on A-currents

Elevation of the Ca_o from 0 to 28 mM in the normal or Na-free saline decreased the amplitude of A-currents in a dose-dependent and reversible manner. Figure 6A shows the effects of Ca-free, 3.5, 7, 14 and 28 mM Ca_o on the I-V relations of A-currents. The effects of Ca_o on A-current amplitudes seem to be a potential-dependent event at each Ca_o concentration used. This is shown in the right side plot of the Fig. 6A where the relative amplitudes of A-currents are plotted against membrane potential. As the Figure shows Ca_o induced suppression of A-current amplitudes decreases as the membrane potential becomes more positive.The dose-response curves of Ca_o action on relative amplitude of A-currents recorded at -45, -40, -35 and -30 mV membrane potentials are shown in Fig. 6B. The half-blocking dose ($K_{1/2}$) of Ca_o increased from 5.7 to 9.4 mM at -45 and -30 mV membrane potential, respectively, by showing a decrease in the affinity with increasing membrane potential. An average value for $K_{1/2}$ varied between 7.5 and 12 mM in the different experiments. Average values of the dose-inhibition data of Ca_o on A-currents calculated in two different experiments were used to estimate the Hill constant (n_H). Depression of A-currents vs. Ca_o concentration is plotted on double logarithmic scales in Fig. 7A. The slope of the dose-inhibition curve was measured at the straight section which resulted 1.3 for n_H by showing a one-to-one binding stoicheiometry for Ca to the recognition site.

Elevation of the Ca_o concentration in the bathing solution modulates the kinetics of the A-currents, too. The time-to-peak and time constant of decay of A-currents increased with the increasing Ca_o concentration in the saline. In a typical experiment, the time-to-peak of the A-current was 10 ms at -30 mV in the Ca-free solution which increased to 15 and 20 ms in 7 and

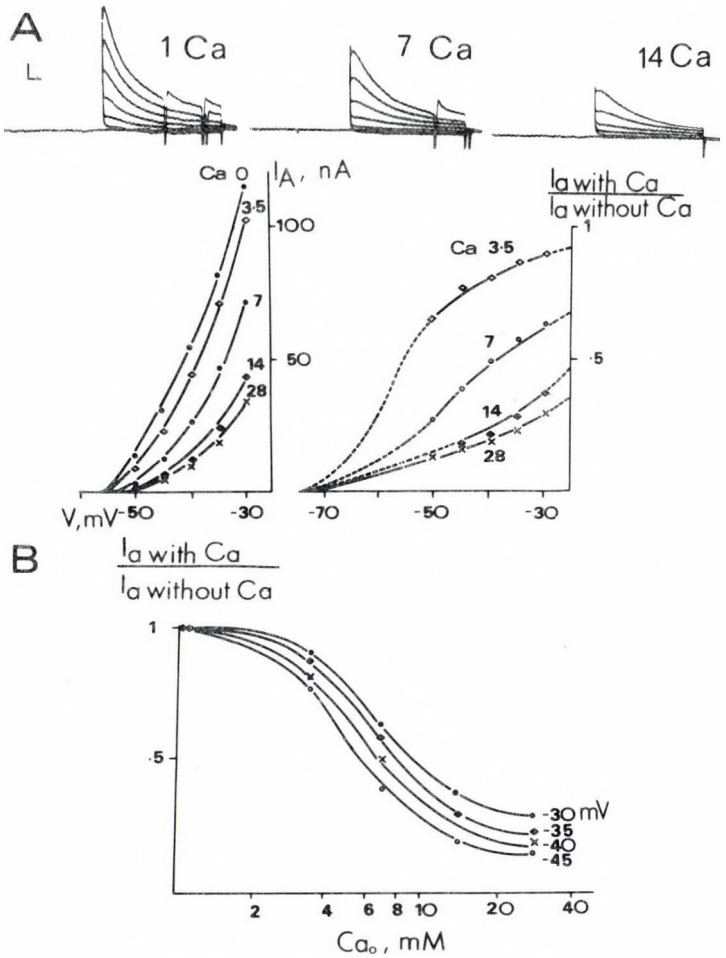

Fig. 6. A) Dose- and voltage-dependent suppression of A-current amplitudes in various Ca_o solutions. Top records show families of A-currents recorded in 1, 7 and 14 mM Ca_o solutions, respectively. V_h = -50 mV; V_c = -100 mV for 500 ms; V_t = -5 — +25 mV in 5 mV increments. Calibration: 20 nA, 100 ms. B) Dose-inhibition curve of extracellular Ca on relative amplitude of A-currents

14 mM Ca-containing solutions respectively. In the same experiment the inactivation time constant of the A-current was 145 ms in the Ca-free solution but it increased to 170 and 200 ms in the 7 and 14 mM Ca_o solution, respectively. However, the effect of Ca_o does not influence the single exponential character of the A-current decline (Fig. 7B).

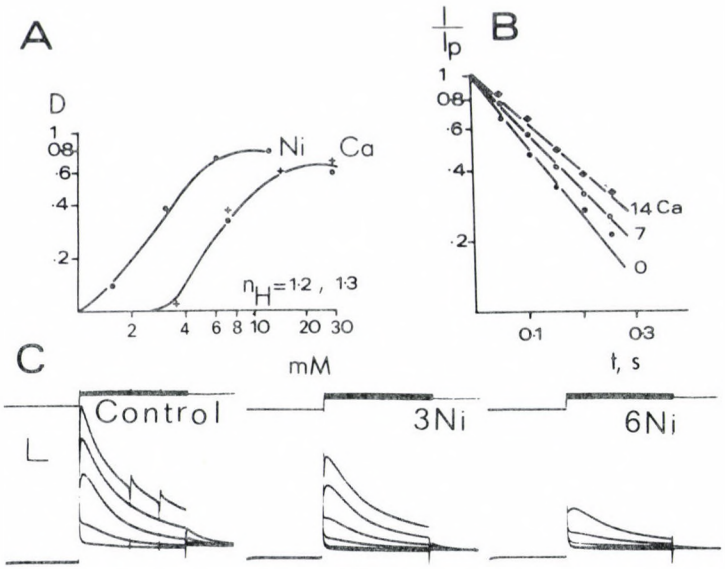

Fig. 7. A) Hill plots of dose-inhibition data of Ca_o and Ni on A-currents ($D = 1-I_{A\ test}/I_{A\ control}$). B) Semilogarithmic plots of normalized relative amplitudes of A-currents after peak vs time in various Ca_o. C) Families of A-currents recorded in control, 3 and 6 mM Ni-containing solutions, respectively. V_h = -55 mV; V_c = -110 mV for 500 ms; V_t = -5 — +15 mV in 5 mV increments. Calibration: 50 nA, 200 ms

Effects of Ca-channel blockers on A-currents

The effects of anorganic Ca-channel blockers Ni, Cd and La and the organic Ca antagonist verapamil were also studied. Similarly to Ca_o, Ni (0.5—12 mM) also decreased the amplitude of A-currents in a dose- and voltage-dependent manner (Fig. 7C). The dose-response curve of Ni on the relative amplitude of A-currents at -40, -45, and -50 mV membrane potentials is shown in Fig. 8A. The half-blocking dose for Ni was 1.5, 2.3 and 3.6 mM at -50, -45 and -40 mV membrane potential, respectively. The average value of the dose-inhibition data of Ni on A-currents was used to estimate the Hill constant, which proved to be comparable with that of Ca being 1.2 to 1.3 (Fig. 7A). Ni as Ca increased the time-to-peak and the time constant of the decline of A-currents. The effects of La and Cd on A-currents were equipotential with 1—1.5 mM for the $K_{1/2}$ values.

The organic Ca channel-blocker verapamil (5 mM) does not attenuate the A-current amplitude and in contrast to Ca or Ni it decreased the time course of A-currents (Fig. 8B). As Fig. 8B shows similarly to Ca or Ni verapamil also decreased the leakage current.

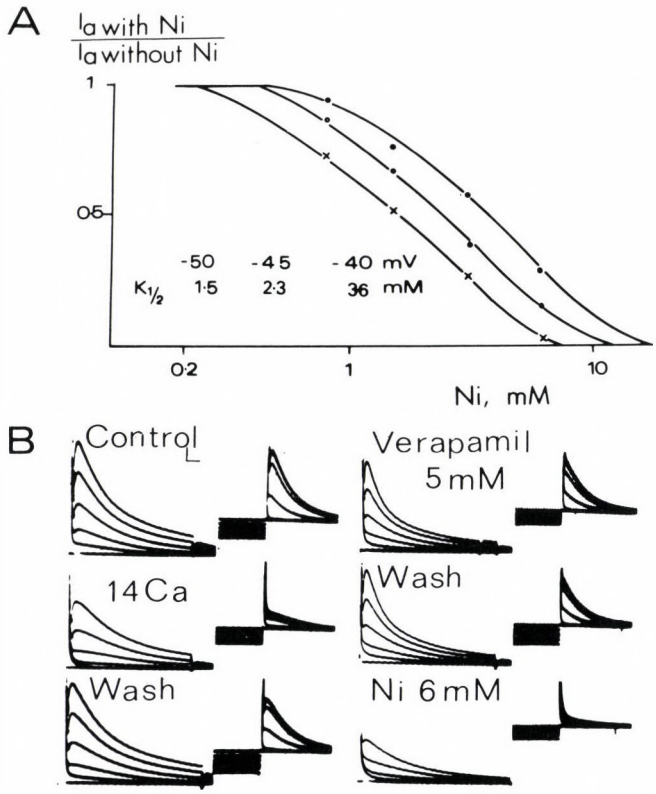

Fig. 8. A) Dose-inhibition curves of Ni on A-currents activated at different membrane potentials. B) Effects of Ca_o, verapapmil and Ni on A-currents recorded in an unidentified neuron. V_h = -50 mV; V_c = -110 mV for 800 ms (left side records), -50 — -100 mV (right side records); V_t = -5 — +15 mV (left side records) -50 mV (right side records). Calibration: 12.5 nA (left side records), 5 nA (right side records) and 50 ms

Pharmacology of A-currents in LPa2, 3 neurons

In Na-free (TEA) saline the amplitude of A-currents decreased by about 30—35% showing TEA-sensitive and TEA-insensitive components of the A-currents. The kinetics of the TEA-insensitive A-current differed markedly from that of the total- A-current. As Fig. 9 shows the TEA-insensitive A-current inactivates faster than that of the total A-current recorded in TEA-free solution. The time constant of decay of A-currents decreased by about 65—70% in the 70 mM TEA solution but it remained the single exponential in nature.

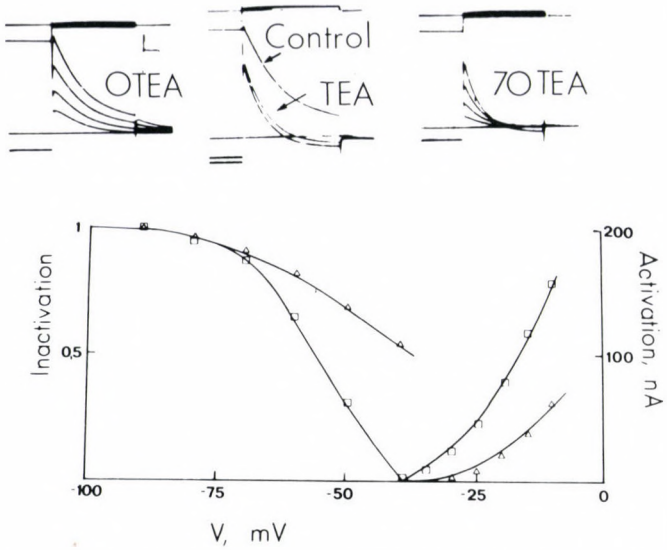

Fig. 9. Voltage-dependence of activation and inactivation of TEA-resistant A-current in 7 mM Ca solution (squares) and in the presence of 12 mM Ni (triangles). Top records show the effects of TEA on amplitudes and kinetics of A-currents. V_h = -50 mV; V_c = -100 mV for 500 ms; V_t = -5 — +10 mV in 5 mV increments. Calibration: 25 nA, 100 ms and 50 mV

Increasing the Ca_o concentration or the presence of Ni and other anorganic Ca channel-blockers decreased the amplitude of the TEA-insensitive A-currents as that of the total A-currents presented before. Fig. 9 shows the potential-dependence of activation and inactivation of TEA-insensitive A-currents (squares) and the effect of 12 mM Ni on the activation and inactivation of the currents (triangles). As can be seen in the figure Ni decreased the A-current activation and shifted the inactivation curve to a more positive voltage.

Finally, a study was made with 4-AP which showed that the drug (5 mM) decreased the amplitude of the TEA-insensitive A-currents by 75% in 15 min after application. 10 mM 4-AP totally blocked the A-current within 5 min exposure time both in the TEA-containing and the TEA-free solutions.

DISCUSSION

A-currents in LPa2, 3 neurons

A study made on identified Helix neurons show that an operationally distinct potassium current, the A-current or I_A, is present in the LPa2, 3

neurons which is separable from other outward currents on the basis of its kinetics and time and voltage dependence. The voltage dependence of activation and inactivation, the time course, the reversal potential and kinetics of A-currents are qualitatively similar to some Helix and to other molluscan neurons /8, 15, 19, 29/. The amplitude of A-currents increased in a voltage-dependent manner when the extracellular potassium ion concentration was decreased from 4 mM to 1 mM in the saline. However, the kinetics of the A-currents were not significantly altered. In contrast to potassium ion a 100% decrease of the Na^+ ion content (Tris substitution) in the solution only slightly decreased the amplitude of the A-currents by about 15%.

Effects of extracellular Ca and Ca-channel blockers

The experimental approach provided evidence that the A-currents in LPa2, 3 neurons and in some unidentified cells were markedly sensitive to modification of the extracellular Ca concentration or to application of anorganic Ca channel blockers such as Ni, Cd, and La.

A ten-fold Ca change from 0 to 10 mM decreased the amplitude of A-currents by about 50% in a dose- and voltage-dependent manner. The kinetics of A-currents were concomitantly altered by extracellular Ca as well. Raising the Ca_o slowed the rise of I_A and increased the time constant of the decline of A-currents. Anorganic Ca channel blockers acted similarly to Ca_o. However, the organic Ca-channel blocker verapamil (5 mM) did not attenuate the amplitude of A-currents. An analysis of the Hill plots of the dose-inhibition data of Ca_o and Ni showed that the recognition site can bind one Ca or Ni in turn of a decrease of the conductance and a change in the kinetics of the A-channel.

With regard to the K channel, the literature suggests that divalent cations act by two separate mechanisms, a gating action and a blocking action. An alteration of the fixed membrane surface charge, e.g., on phospholipids can cause a modification of the gating behaviour of the channel. It has also been postulated that Ca binds to, or electrostatically screens, negative charges on the membrane's external surface, thereby altering the surface potential /13, 24/. This in turn alters the energy profile in the membrane and the voltage-sensing apparatus in the channel. External Ca was reported to slow down K channel opening in squid giant axon /5/. Blocking has been clearly demonstrated for Ca ions acting on Na channels /37, 40/. Divalent cations were found to decrease the K conductance of both the Torpedo and bovine acetylcholine receptor channels /18/. Furthermore, a

non-selective cation conductance was blocked by Ca and other divalent cations in frog muscle as the light-sensitive Na channels in vertebrate photoreceptors /4, 41/.

As regards A-currents, calcium activated transient outward current was reported in Drosophila flight muscle /34/, calf cardiac Purkinje cells /36/, bullfrog sympathetic neurons /1/ and guinea-pig hippocampal pyramidal neurons /42/. Precedents for the co-existence of Ca_i-activated and voltage-sensitive outward currents can be found in nerve cells from Helix /26/, Tritonia /38/ and Limulus /22/. In all of these neurons the transient outward current appeares to be activated by depolarization but not by Ca_i. In contrast, Alkon has shown that a long-lasting reduction in the amplitude of I_A and an increase in its rate of inactivation developed during associative learning in B-type photoreceptor cells in a mollusc Hermissenda /3/. Intracellular iontophoresis of Ca, calmodulin-dependent protein kinase, phorbol esters can mimic the effect of training, suggesting that phosphorilation of the channel may ultimately be responsible for the reduction in the current. I found and described here that extracellular Ca also regulates A-currents independently of external monovalent cations but similarly to some other currents mentioned above. The binding site for Ca and for anorganic Ca-channel blocker Ni or Cd and La may be common in or around the A-channel. The affinity of the cations to the recognition site, however, decreased in the order of Cd = La > Ni > Ca. The organic Ca channel-blocker verapamil may act in another way because it was only effective on the rate of inactivation of A-currents in close correlation with previous findings /21/.

I postulate that A-currents are suppressed by about 40—50% in normal solution with 7 mM Ca_o but it may be markedly modulated when the hemolymph becomes diluted or concentrated in various life periods of the animals. It may seem strange that a possible fundamental action of Ca_o on A-currents, that is supposed here, can have escaped attention so long.

Pharmacology of A-currents

Experimental evidence reported from various laboratories can show that A-current recorded in various molluscan neurons is to a greater or lesser extent sensitive to TEA /16, 20, 30, 35, 38/. LPa2, 3 neurons have A-currents which are composed of a smaller TEA-sensitive and a larger TEA-insensitive component. However, the rate of inactivation of A-currents was markedly modulated in a high dose of TEA solution. 4-AP is also reported as

a specific and non-specific blocker of A-currents from different laboratories /6, 14, 16, 31, 39/, but it totally blocked all components of the A-currents in LPa2, 3 neurons.

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PHORBOL ESTERS THAT ACTIVATE PROTEIN KINASE C INDUCE LONG-TERM CHANGES OF MEMBRANE EXCITABILITY AND POSTSYNAPTIC CURRENTS IN NEOCORTICAL NEURONS

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Intracellularly injected tumor promoter phorbol esters (PhEs) that activate protein kinase C (PKC) increased the excitability and altered the postsynaptic responses of neurons of the motor cortex of awake cats. PhEs increased the amplitude and duration of EPSPs and decreased the amplitude and durations of IPSPs. No consistent changes in resting membrane parameters that would account for these modifications were found. Corresponding changes in peak excitatory and inhibitory postsynaptic currents (EPSCs, IPSCs) were measured directly with the single electrode voltage clamp technique. The changes lasted for 50 min or longer. Quantitative analysis of EPSCs in response to ventrolateral thalamic stimulation and IPSCs in response to pyramidal tract stimulation made in a subgroup of fast PT cells suggested that PhE acted within the injected neuron rather than presynaptically to alter the synaptic currents. PhE also reduced a voltage-dependent, 3-aminopyridine sensitive fast outward current (I_A) and an apamin and EGTA sensitive slow outward current ($I_K(Ca)$). Control injections of a phorbol ester that did not activate PKC failed to induce changes in synaptic responses or resting membrane properties. These observations provide the first evidence that activation of PKC, in vivo, can induce long-lasting changes in synaptic responses of neocortical neurons by direct modification of postsynaptic ion channel conductivities.

Keywords: Phorbol ester — protein kinase C — postsynaptic currents — motor cortex — voltage clamp

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INTRODUCTION

Calcium, phospholipid and diacylglycerol-dependent protein kinase C (PKC) is present in high concentrations in the neocortex being localized in presynaptic terminals, dendrites and cytosol of neurons /20, 36/. Through phosphorylations PKC regulates membrane conductances /3, 17, 23, 33/, synaptic transmission /24, 25, 31/ and neuronal plsticity /1, 2, 9, 18, 26/. Tumor promoter phorbol esters (PhEs) such as phorbol 12,13-dibutyrate (PdiB) and phorbol 12 myristate 13-acetate (PMA) have structure similar to diacylglycerol and selectively activate PKC /1, 13, 27/. There is no evidence as yet how PhEs influence neuronal excitability, membrane currents and postsynaptic responses of neocortical neurons, in vivo. In earlier studies of electrophysiological effects of PhEs on mammalian central neurons, phorbol esters were applied extracellularly, in vitro. Thus, some of their effects could have been produced indirectly from actions on neighbouring cells as well as directly in the recorded neurons. In order to study the direct local actions, we injected PhEs intracellularly into neurons in which electrophysiological measurements were made.

Here we summarize our observations on the postsynaptic effects of PhEs on neuronal excitability, action potentials, excitatory/inhibitory postsynaptic potentials (EPSPs/IPSPs), currents (EPSCs/IPSCs), and membrane currents in neurons of the motor cortex of awake cats. This study provides the first description of membrane currents of identified EPSCs, LPSCs, in vivo, elicited by stimulation of ventrolateral (VL) thalamus and pyramidal tract (PT). Single electrode voltage clamp (SEVC) techniques were used to study synaptic and membrane currents.

MATERIALS AND METHODS

Adult cats were prepared initially under sodium pentobarbital anaesthesia (35 mg/kg, i.p.) by implanting four bolts into the skull so that the head might be secured during subsequent recording sessions in which the animals were awake. During recording, the body of animal was comfortably restrained in a cloth sleeve while the head was fixed. Complete details of these techniques can be found elsewhere /35/. Synaptic potentials were evoked by stimulation of VL and the PT. Stimuli (4—7 V, 0.1 ms, 0.5 Hz for VL and 2—4 V, 0.1 ms, 0.5 Hz for PT) were delivered through implanted electrodes as in earlier studies /4, 5/. Micropipettes were pulled from 1.5 mm (O.D.) theta capillaries and filled with: (i) 1—5 μM PdiB, 10 μM PMA or 10 μM 4 alpha-phorbol 12,13-didecanoate (PdiD) mixed in 1 M potassium citrate containing 50 μM/ml phosphatidylserine. The aqueous solubility levels of PdiB and PMA were sufficient to activate PKC in nanomolar con-

centration /11, 13/; (ii) 50 mM 3-aminopyridine (Ap); (iii) 50 mM 3-Ap+20 mM QX 314, (iv) 100 mM apamin or (v) 200 mM EGTA dissolved in 1 M potassium citrate. The microelectrodes were bumped under oil to permit intracellular pressure injection /30/. Electrodes were connected through Ag/AgCl wire to a Dagan 8100 SEVC amplifier. Electrode rectification was tested in the cortex with current of $\pm$2—5 nA and tip polarization was kept within $\pm$1 mV to avoid errors from drift. Care was taken to minimize input capacitance and to use microelectrodes of suitable resistances (15—30 MOhm). Capacitance compensation and switching frequency (3—6 kHz, using a duty cycle of 50%) were adjusted to allow the charging transients of electrode voltage to settle completely between oscillations. These were achieved by (i) monitoring the headstage output as reasonable square /19/, (ii) the amount of current flow was identical under current-clamp and voltage-clamp mode as checked with rectangular pulses of $\pm$2 nA /15/, and (iii) by increasing the gain together with adjustment of the phase until the desired holding potential was reached /21/. The apparent input resistance (Rm) and membrane time constant (Tm) were measured by the intracellular voltage drop from the resting potential (Vr) produced by 50—100 rectangular hyperpolarizing current pulses (0.5—2.0 nA, 1—5 Hz, 40—100 ms) at 0.5—1.0 min intervals. An estimate of spike threshold (T50) was assessed by injecting 0.5—1.5 nA, 40—100 ms depolarizing pulses in the balanced bridge mode and measuring voltage deflection from the reference potential at the point where the first spike discharge was initiated. Current and voltage records were displayed on oscilloscope and stored on FM tape (DC-5 KHz band pass). Averages were made by computer by digitizing the voltage and current outputs at a rate of 10 KHz during each trial and averaging across trials.

RESULTS

Effects of phorbol ester on neuronal excitability

Baseline values of electrophysiological membrane parameters (Vr, Rm, T50), the amplitude and duration of action potentials (AP), the amplitudes of slow after-hyperpolarizations (AHPs) following APs and current-induced depolarizations, and the rate of spontaneous (background) and depolarizing pulse-induced firing activity were measured in bridge mode in 80 cells. Injections of both PdiB (in 37 cells) and PMA (in 28 cells) led to rapid transient increases in Vr and decreases in Rm and in both spontaneous and current-induced firing activities within one min after injection (Fig. 2C). Signs of increased neuronal excitability were then observed in each cell within 2—3 min which reached a maximal level 8—10 min after injection (Table 1) and were sustained throughout the time of recordings in all but four cells. Partial recovery was observed in those four cells 50 min after injection. When activity in response to constant depolarizing current pulses was measured the number of spikes gradually increased as well as the spontaneous firing activity (Fig. 1). The latency of the first AP produced

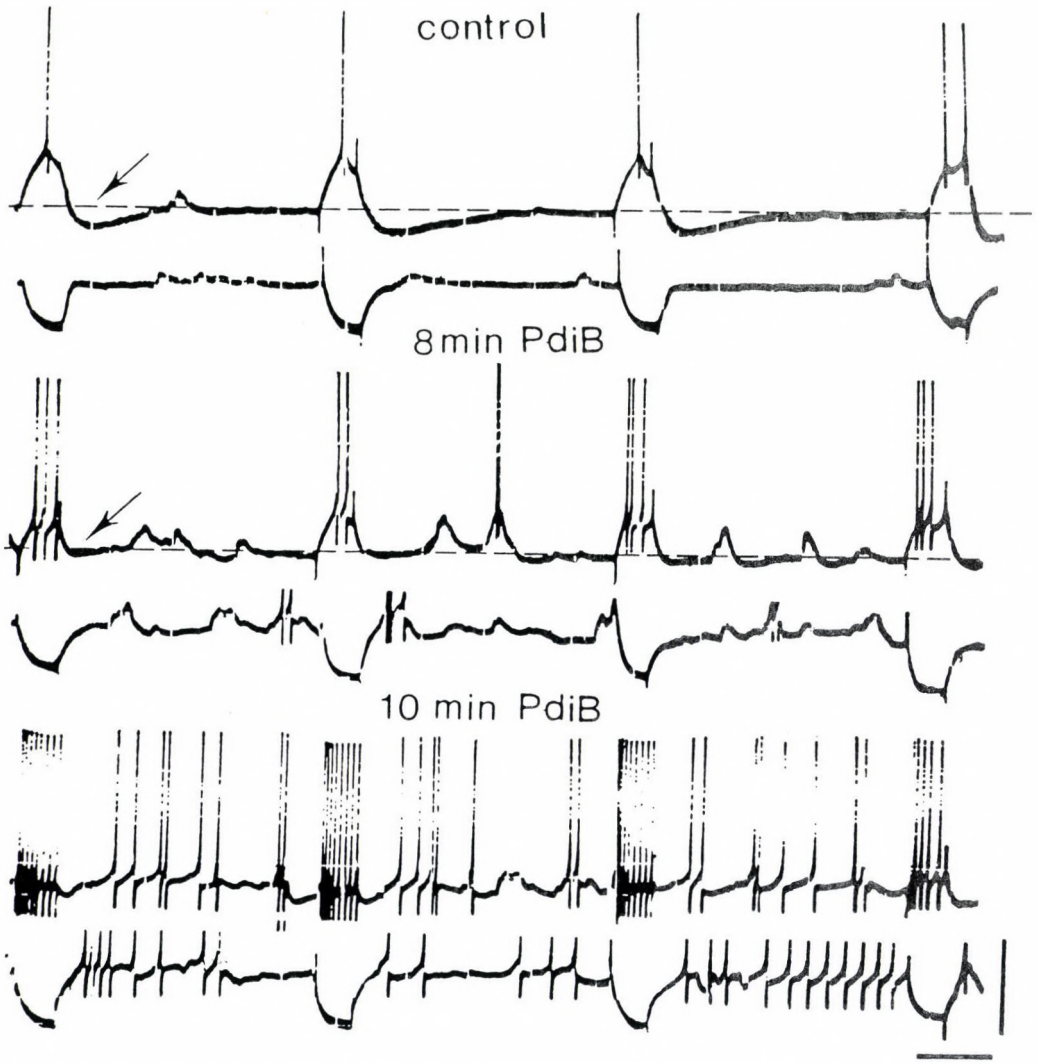

Fig. 1. Changes in neuronal excitability measured by current (+1.3 nA, 40 ms, 5 Hz)-induced responses after intracellular injection of PdiB into a PT neuron. Upper traces: PdiB gradually facilitated (8 and 20 min) the spontaneous and current-induced firing and increased the amplitude of EPSPs in background activity. The action potentials developed prominent undershooting after potentials. Slow afterhyperpolarizations (indicated by oblique arrows) following depolarizing pulses were abolished when compared with (control) preinjection values. Dashed lines indicate resting membrane potential (-67 mV). Lower traces: proportionate changes were not found in input resistance (14.8 MOhm) measured by hyperpolarizing current pulses (-1.3 nA, 40 ms, 5 Hz). Action potentials are truncated. Calibration bars: 80 ms, 40 mV

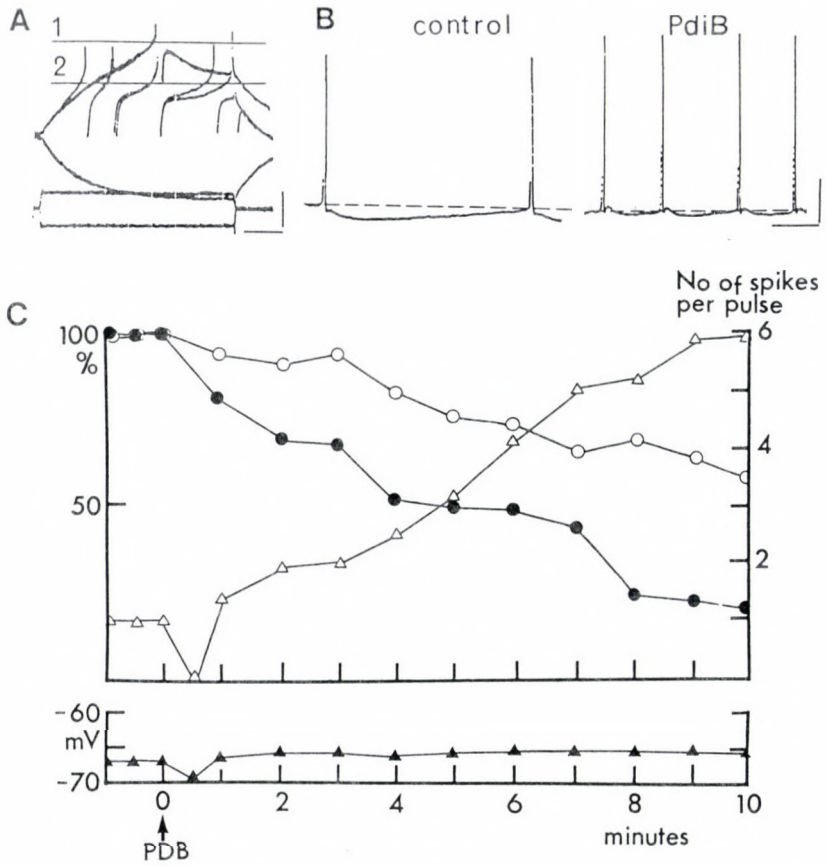

Fig. 2. A: Superimposed records at higher magnification (before and 8 min after PdiB injection) showing a reduction in latency of action potential elicited by the depolarizing pulses and a decrease in depolarization necessary to reach the threshold for spike discharge (from 1 to 2) after injection. The Rm did not change. The magnitude of the fast AHP component (asterisk) increased after injection. Calibration bars: 7.5 ms, 10 mV and 6 nA. B: The slow afterhyperpolarization following action potentials were reduced, while the amplitude of action potentials were increased by PdiB. Dashed lines indicate resting membrane potential (-69 mV). Calibration bars: 10 ms, 20 mV. C: Plots illustrating effects of PdiB on the number of spikes elicited per depolarizing current pulse (open triangles), membrane potential (full triangles below), as well as the percentage changes in latency (full circles), and threshold potential of the first spike (empty circles). Each value represents the average of ten individual data point

Table 1

Values of resting membrane potential (Vr), input resistance (Rm), firing threshold to depolarizing current pulses (T50), amplitude of action potential (AP), slow afterhyperpolarization following action potentials (AHP), and spontaneous firing activity (SF) of neocortical neurons before and 10 min after phorbol ester injection. Averaged values were determined from 10 individual data

	VR(mV)	Rm(MOhm)	T50(mV)	AP(mV)	AHP(mV)	SF(Hz)
All cells before injection (n=80)						
	64.1±5.9	8.2±3.1	53.8±1.9	66.0±9.2	7.1±2.0	7.3±8.1
Active phorbol ester-injected cells (n=50)						
Pre-injection	64.9±4.4	8.2±3.0	53.6±1.9	66.0±8.7	7.2±1.8	6.9±7.7
Post-injection	62.1±9.4	7.9±5.2	57.1±2.0*	74.0±9.6*	1.0±2.4	24.0±6.6
Inactive phorbol ester-injected cells (n=15)						
Pre-injection	64.2±5.6	8.6±3.4	55.0±4.1	65.0±9.9	7.0±1.5	8.8±6.1
Post-injection	64.0±6.2	8.5±4.7	54.0±2.3	66.0±9.8	7.3±2.3	9.7±7.7

*$p < 0.005$ Student's two-tailed t-test; All numbers are means ±S.D.

by constant depolarizing pulses decreased (Figs 2A, C) and the apparent level of membrane potential at which spikes were initiated (Fig. 2A) changed from -53.6±1.9 mV to -57.1±2.0 mV (Table 1). The amplitude of action potentials increased, without changes in duration of action potentials (Fig. 2B). The slow AHPs following APs and current-induced depolarizations decreased (Figs 1 and 2B) while the fast AHPs following APs frequently increased (Fig. 2A). An increase of background firing with progressive burst generation was found in 36% of the cells.

Neither increases in Rm nor depolarization of the resting potential sufficient to account for these excitability changes were found (Fig. 2C). Since no differences occurred between effects of PdiB and PMA, their results were combined and expressed as results of injecting PhEs (Table 1).

Effects of phorbol ester on postsynaptic responses

The synaptic actions of PhEs were studied in other 56 cells. Intracellular application of PdiB induced selective modifications of synaptic

responses in 56 cells. The effects began 2—3 min, reached their maximal level 6—10 min after injections and were long-lasting. In 14 of 17 cells no recovery of PSPs was observed after 50 min of recording. The amplitude and duration of EPSPs evoked by either VL or PT stimulation increased after injection of PdiB, resulting in multiple spike discharges (Fig. 3, Table 2). The slope of rising phases of augmented EPSPs did not change, however, their decay time constant increased (Table 3). For E-IPSPs, when the cell membrane was hyperpolarized to a level at which currents attributable to concurrent IPSP components were reduced or abolished, parameters of the EPSP component could be measured separately. Contrary to its effect on EPSPs, PdiB reduced the amplitude, duration, and decay time constant of IPSPs evoked by VL or PT stimulation (Tables 2,3). These opposite actions on EPSPs and IPSPs were found in cells with separate EPSPs or IPSPs as well as in single cells with composite E-IPSPs (Fig. 3, n=27 cells). When amplitude-voltage relationships were investigated by intracellular current injections in regions close to the Vr, neither EPSP nor IPSP showed changes in their reversal potential after injection of PdiB (Fig. 4, Table 3).

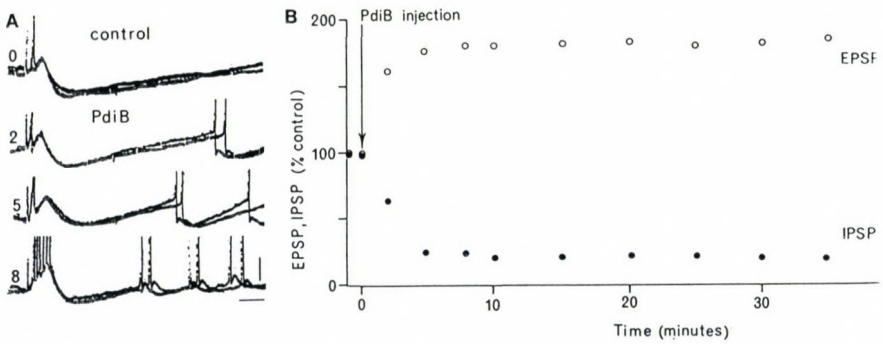

Fig. 3. A: Effects if PdiB on composite E-IPSPs evoked by stimulation of thalamic VL in a PT neuron. Two superimposed records taken before (0) and 2, 5, 8 min after PdiB injection illustrate opposite effects on EPSPs and IPSPs. The increased EPSPs resulted in a burst of action potentials. Spikes are truncated. Resting potential (-62 mV) did not change after injection. Calibration bars: 10 mV, 20 ms. B: The graph illustrates the peak amplitude of EPSPs and the amplitude of IPSPs measured 50 ms after stimuli onset, as a function of time before and after injection of PdiB. 100% represents the control values of E-IPSPs. Each point is the average of three successive responses

Table 2

Effects of phorbol 12,13-dibutyrate (PdiB) on excitatory (EPSP) and inhibitory (IPSP) postsynaptic responses elicited by VL and PT stimulation in pyramidal tract (PT) and non-pyramidal tract (nPT) cells of the motor cortex of awake cats. Averaged values were determined from twenty individual data measured before and 10 min after injection of PdiB. Values of amplitude were measured at the peak of EPSPs and IPSPs except for E-IPSPs where the amplitudes of IPSPs were measured 50 ms after stimulus onset

Synaptic response		Control		PdiB	
		PT cells	nPT cells	PT cells	mPT cells
VL EPSP					
amplitude	(mV)	5.2 ± 1.1	5.5 ± 1.7	$14.6 \pm 3.1^{*}$	$12.9 \pm 3.6^{*}$
T_{EPSP}	(ms)	14.2 ± 6.2	18.3 ± 4.6	$19.2 \pm 5.7^{*}$	$26.4 \pm 9.1^{*}$
		(n=22)	(n=8)	(n=17)	(n=8)
VL IPSP					
amplitude	(mV)	7.0 ± 2.2	8.9 ± 1.2	$2.3 \pm 0.9^{*}$	$1.5 \pm 0.6^{*}$
T_{IPSP}	(ms)	63.0 ± 19.4	72.2 ± 16.0	$22.2 \pm 5.6^{*}$	$16.4 \pm 3.6^{*}$
		(n=11)	(n=14)	(n=10)	(n=13)
PT EPSP					
amplitude	(mV)	4.1 ± 0.6	5.9 ± 2.1	7.3 ± 1.3	8.2 ± 2.2
T_{EPSP}	(ms)	20.2 ± 3.7	34.6 ± 6.1	24.2 ± 4.8	38.4 ± 5.3
		(n=9)	(n=13)	(n=7)	(n=10)
PT IPSP					
amplitude	(mV)	6.9 ± 1.4	-	$2.1 \pm 1.2^{*}$	-
T_{IPSP}	(ms)	71.6 ± 16.0	-	$30.1 \pm 6.1^{*}$	-
		(n=18)		(n=18)	

$^{*}p < 0.005$; Student's two-tailed t-test. Values are means $\pm$S.D.; measured only in fast PT cells. T_{EPSP} and T_{IPSP} are decay time constants

The modification of synaptic responses were not accompanied by consistent changes in Vr, Rm or Tm (Table 3), a finding similar to that reported after extracellular applications of phorbol esters in other systems.

Sixteen identified fast PT neurons were selected for detailed analysis of the action of PdiB on excitatory synaptic responses evoked by VL stimulation and on inhibitory synaptic responses evoked by PT stimulation (Table 3). Fast PT neurons are advantageous for studying PSPs and PSCs since the VL monosynaptic excitatory input to fast PT neurons is located electrotonically close to the usual somatic sites of recording, permitting

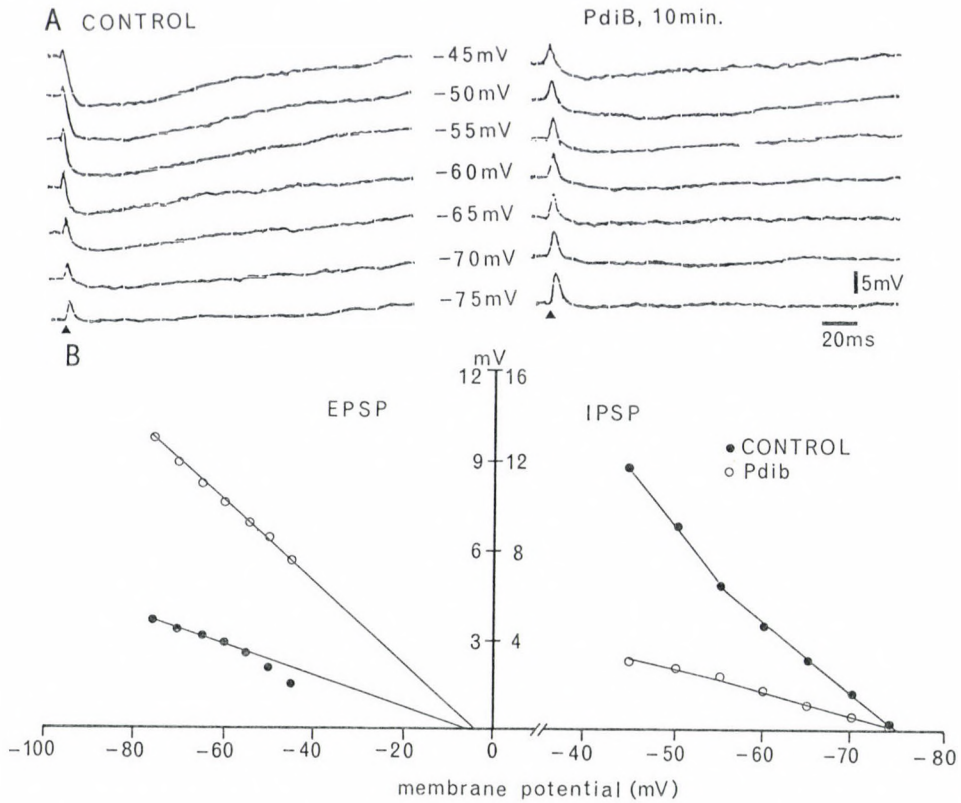

Fig. 4. Changes in current-voltage (I-V) curves of the VL EPSP and IPSP in a single nPT neuron following PdiB injection. A: The Vr (-60 mV) was shifted in 5 mV steps by intracellular current injections via the recording electrode. Upward arrows indicate VL stimulation. B: Both the EPSP and IPSP showed small non-linearities at depolarized levels of Vr above -55 mV. Increases in the amplitude of EPSPs and decreases in the amplitude of IPSPs were found on all voltage levels 10 min after PdiB injection, depicting linearly appearing I-V curves for both PSPs. The extrapolated synaptic reversal potentials did not change significantly

reasonable accurate analysis of synaptic events /32/. Similarly, most of the disynaptic, feed-forward (from VL stimulation) and recurrent (from PT stimulation) IPSPs of PT neurons are generated by axosomatic synapses /10/.

When studied in the voltage clamp mode, VL EPSCs appeared as inward currents that reached an average peak amplitude of 0.86 nA at a holding potential of -60 mV with in 3—5 ms of stimulus onset and then decayed exponentially (Fig. 5, Table 3). The decay time constant of these EPSCs averaged 9.2 ± 0.6 ms. The amplitude of EPSCs exhibited a linear appearing

Table 3

Electrophysiological membrane and synaptic response parameters of 16 fast PT neurons measured before and after PdiB injections. Excitatory post-synaptic responses (EPSP/EPSC) were elicited by VL and inhibitory post-synaptic responses (IPSP/IPSC) were elicited by PT stimulation. Averaged values were determined from twenty individual data measured before and 10 min after injection of PdiB. The synaptic response parameters were measured at resting membrane potentials in bridge mode and at -60 mV holding potential in voltage-clamp mode. The synaptic conductance increase (G) was calculated according to the equation:

$$G = I_p(V_H - V_R)$$

where V_H is the holding potential, V_R is the synaptic reversal potential, and I_p is the peak synaptic current /see Ref. 7/

Parameters	Control	PdiB
Membrane potential, V_I (mV)	63.1±3.9	62.3±5.2
Input resistance, Rm (MOhm)	8.0±2.1	8.8±3.0
Membrane time constant, T_m (ms)	11.1±1.2	11.8±1.4
Spike threshold, T50 (mV)	54.3±2.0	58.0±3.1
EPSP peak amplitude, (mV)	5.8±1.3	13.9±2.4*
IPSP peak amplitude, (mV)	7.4±1.9	2.5±1.1*
EPSP decay time constant, T_{EPSP} (ms)	9.2±0.6	13.1±1.1*
IPSP decay time constant, T_{IPSP} (ms)	74.0±1.6	24.0±6.2*
peak EPSC, I_p (nA)	0.86±0.11	1.49±0.36*
peak IPSC, I_p (nA)	1.01±0.20	0.34±0.18*
EPSC conductance increase, / G (nS)	15.8±2.9	26.1±3.3*
IPSC conductance increase, / G (nS)	37.2±4.0	13.6±2.2*
EPSC reversal potential, V_R (mV)	- 6.6±3.2	-4.9±2.9
IPSC reversal potential, V_R (mV)	-75.0±4.9	-74.3±3.6
EPSC decay time constant, T_{EPSC} (ms)	5.6±0.3	5.7±0.5
IPSC decay time constant, T_{IPSC} (ms)	52.0±3.6	51.0±2.6

*$p < 0.005$; Student's two-tailed t-test. Values are means ±S.D.

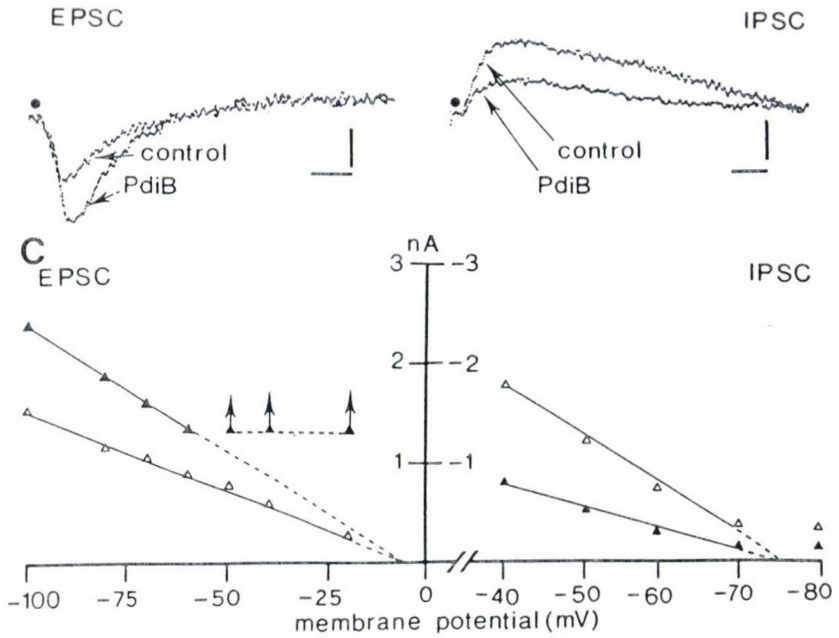

Fig. 5. Effect of PdiB on VL (EPSC) and PT (IPSC) stimulation-evoked postsynaptic currents. Dots indicate stimulation. Averaged curves of 10 individual measurements of synaptic currents (illustrated in A) at -60 mV holding potential, before (control) and 20 min after PdiB injection. Action potentials were eliminated by computer if they appeared. Prior to voltage-clamping the resting potentials were -63 mV (EPSC) and -60 mV (IPSC). Calibration bars: 0.5 nA, 5 ms for EPSC; 0.5 nA, 7.5 ms for IPSC. C: current-voltage (I-V) relationships depicting peak synaptic currents at different voltage steps before (open triangles) and 20 min after (black triangles) PdiB injection. Upward arrows symbolize action currents triggered by enhanced EPSCs after PdiB. Each point represents the mean of 15 peak synaptic currents. The standard errors of the means were smaller than the symbols used to plot each point, and thus are not included in the graphs

voltage relationship when evoked 30 ms after the onset of command steps from the holding potential to potentials varying from -110 mV to -10 mV (Fig 5C). The average slope conductance was 16 nS, and the reversal potential determined by extrapolation was -6.6 ± 3.2 mV (Table 3). Similar baseline parameters of EPSCs have been measured in hippocampal neurons, in vitro, following stimulation of mossy fiber inputs /7/.

PT IPSCs occurred as outward currents with a mean peak amplitude of 1.01 ± 0.20 nA at the holding potential of -60 mV (Table 3), and were highly sensitive to voltage steps in regions close to the resting potential

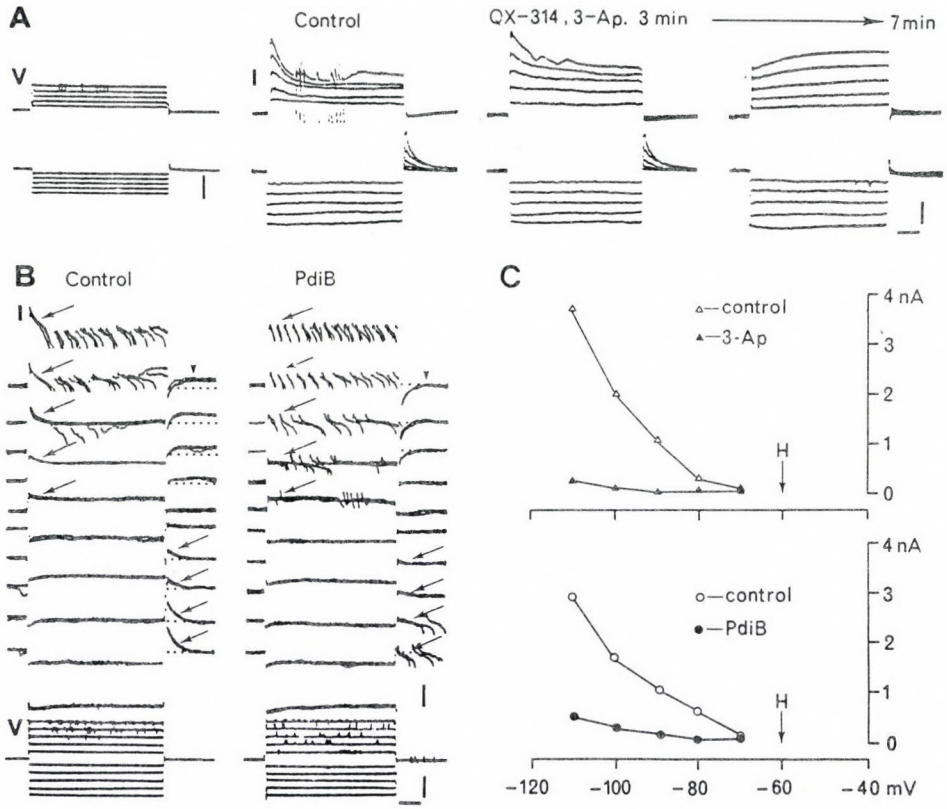

Fig. 6. A: Composite current responses (I) in a fast PT cell penetrated with microelectrode containing QX-314 and 3-Ap. Voltage steps were made (V) in 10 mV increments to +10 mV and -110 mV from -60 mV holding potential. Step depolarizations elicited fast outward currents which peaked approximately 4 ms after the onset of depolarizing steps and a slow inward current activated by steps above +10 mV (Control). The inward currents were estimated by substraction of instantaneous currents with opposite polarity. Hyperpolarizing steps removed the inactivation gating of the transient outward current which occurred as a tail current at the offset of the pulses (Control). After impalement, QX-314 quickly leaked into the cell and abolished the depolarizing command-induced fast sodium spike currents and reduced a slow inward current within 3 min. Pressure injection of 3-aminopyridine (3-Ap) with QX-314 completely blocked fast outward currents within 4 min (7 min after penetration). Note further reduction of QX-314 sensitive inward currents which revealed a voltage-dependent slow outward current to depolarizing steps. Resting potential before voltage-clamping was -63 mV. Calibration bars: 50 mV, 2 nA and 20 ms. B: Composite current traces (I) in a fast PT neuron before (Control) and after injection of PdiB. Oblique arrows indicate fast outward currents activated by voltage steps (V) from -60 mV holding potential. Slow outward currents following depolarizing pulses is indicated by downward arrowheads. Dotted lines indicate the baseline for current records. Superimposed traces taken

(Fig. 5). The average extrapolated reversal potential of -75 mV for IPSCs suggested involvement of both chloride and potassium conductances.

Both EPSCs and IPSCs were studied at command potentials in the range of -110 to -10 mV from a holding potential of -60 mV before and after injection of PdiB. PdiB induced persistent increases of peak currents and slope conductances of EPSCs and decreases of currents and conductances of IPSCs (Fig. 5, Table 3). These changes in synaptic currents were observed without signs of recovery in 5 cells which were recorded for 50 min or more. The changes in the magnitudes of peak synaptic currents reflected increases in slope conductances of EPSCs and decreases in slope conductances of IPSCs. The increased EPSCs frequently activated fast currents associated with action potentials. When estimating synaptic reversal potentials, EPSCs evoking action currents were excluded. The slope of decay time constants and reversal potentials of the synaptic currents were not significantly affected by PdiB (Table 3).

Effects of phorbol ester on membrane currents

In the next series of experiments we analyzed outward membrane currents which may support PdiB-induced changes of neuronal excitability and postsynaptic responses. At first we observed that a voltage-dependent fast outward current was consistently depressed by PdiB concomitant with the facilitation of EPSCs (Fig. 6). This fast outward current could be evoked either by depolarization or occurred as tail current at the offset of hyperpolarizing commands. It peaked within 2—4 ms and inactivated 40—80 ms after its onset and gradually increased with command pulses between 10—50 mV from the holding potential of -60 to +60 mV reaching a peak amplitude of up to 3 nA. The activation/inactivation was voltage-dependent and indicated a

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10 min after injection (PdiB) show the strong reduction of transient outward current and slow outward current. In spite of presence of sodium spikes the main current components are idential to those seen in A. Calibration bars: 25 mV, 2 nA and 20 ms. C: De-inactivation curves for the fast outward tail current evoked at the offset of hyperpolarizing commands as illustrated in A and B before and after QX-314, 3-Ap and PdiB. Note that 3-Ap and PdiB blocked the same current similarly. Arrows indicate holding potentials (H).

reversal potential of approximately -65 mV (Fig. 6C). Intracellular application of 3-aminopyridine (Ap) reduced the fast outward current within 1-4 min (Fig. 6, n=16 cells). The time course, voltage-dependence and 3-Ap sensitivity of this fast outward current resembled the I_A current as described originally in molluscan /14, 16/ and subsequently in vertebrate neurons /8, 12, 15, 29/. The peak amplitude of this current measured 3 ms after the offset of -30 mV hyperpolarizing commands was reduced from 1.32 ± 0.26 nA to 0.21 ± 0.11 nA 10 min after PdiB injection (Fig. 6B). In addition, PdiB reduced a slow, voltage-dependent outward tail current following depolarizing commands (Fig. 6B). This current had a time course similar to slow AHPs (Figs 1, 2B). Since intracellular injection of EGTA (in 5 of 6 cells) and apamin (in 13 of 15 cells) a Ca-dependent potassium current ($I_K(Ca)$/ blocker /28/ reduced both AHPs and related slow outward tail currents (not shown) we considered this component as Ca-dependent potassium current $I_K(Ca)$.

Control injections of 4 alpha-phorbol 12, 13-didecanoate (PdiD), which does not activate PKC /11, 13/, did not induce changes in resting membrane properties and neuronal excitability (Table 1, n=15), in synaptic potentials (measured in bridge mode, n=26 cells), or in membrane and synaptic currents (measured in voltage-clamp mode, n=18 cells). Thus, it seems likely that changes in membrane excitability and synaptic responses resulted from a direct activation of PKC.

DISCUSSION

When applied extracellularly, in vitro, PhEs facilitate neuronal excitability and synaptic transmission at invertebrate neurons /17, 18, 33/, at the neuromuscular junction /31/ and in hippocampal neurons /3, 24—26/. Most of the synaptic effects of PhEs have been interpreted as resulting from presynaptic actions, via an increased transmitter release.

Intracellular injection of PhEs in our experiment provided an opportunity to asses the consequences of activation of PKC postsynatpcially. Although presynaptic activation of PKC cannot be completely excluded, our results favour a direct action of postsynaptically activated PKC on local conductances; (i) Since PKC is present in the dendrites and cytosol of neocortical neurons /20, 36/, it is reasonable to assume that intracellularly injected PdiB, with high affinity for PKC /13/, activated the enzyme

locally. (ii) PdiB differentially influenced EPSCs and IPSCs in single cells. With an increased transmitter release attributable to a presynaptic facilitatory action, one would expect increases in amplitudes of both excitatory and inhibitory synaptic currents /22/. (iii) Parallel with the effects of PdiB on synaptic responses, we found other postsynaptic effects including reductions of slow AHPs, threshold of spike discharges to depolarizing currents and alterations of other membrane currents. To confirm the postsynaptic origin of the changes of synaptic responses induced by intracellular injection of PdiB, quantal analysis would be required.

Postsynaptic modulations of neuronal excitability induced by PdiB could arise directly from changes in voltage-dependent membrane currents. A voltage dependent reduction of fast outward potassium conductance (I_A) could have accounted for the decreased latency and threshold of spiking without Vr and Rm changes /29/ as could a facilitation of Na^+ or Ca^{2+}-mediated inward currents potentiated by PhE /1, 9, 17, 25, 33/. Inhibition of $I_K(Ca)$ current of the postsynaptic membrane could have caused the increase in background firing and elevated activity to depolarizing current pulses as well as the reduction of slow AHPs and burst generation, as in hippocampal neurons, in vitro /3, 25/. The generation of bursting activity could also have resulted from facilitation of calcium currents by PhEs. Our results demonstrate that postsynaptic activation of PKC induces long-lasting potentiation of excitatory and reduction of inhibitory synaptic responses. Postsynaptic modulation of synaptic responses could arise directly from changes in the availability or opening probability of transmitter-activated ion channels or indirectly through changes in other voltage-dependent membrane currents. The duration of individual channel opening is closely correlated with the decay time of PSCs /6/. Since in our experiments PdiB influenced peak synaptic current amplitudes and conductances without changing decay time constants or reversal potentials, it is reasonable to assume that PdiB increased EPSCs by opening new (and decreased IPSCs by closing already functioning) transmitter-activated channels having the same ion specificity /33/.

These changes could be supported by reductions of at least two outward potassium currents (I_A and $I_K(Ca)$) vs increases in inward (Na^+ and/or Ca^{2+}) conductances. Although the exact contribution of PdiB-induced reduction of I_A to the EPSC potentiation is difficult to asses, evidence suggest this possibility. It was shown that I_A reduces both the amplitude and duration of EPSCs in molluscan and vertebrate ganglion cells /8, 12, 16/. Accordingly, all of those EPSCs and depolarizing membrane action whose time

course and amplitude are comparable with the activation and inactivation gating of this current will increase in amplitude and duration when the I_A is reduced.The reduction of slow AHP and $I_K(Ca)$ by PhEs has been reported also in other systems /3, 18, 25/. Beside the inhibitory action of PhE on chloride currents /23/ the reduction of $I_K(Ca)$ probably contributed to the PdiB-induced decreases of IPSCs.

In conclusion our results provide the first direct measurements of identified EPSCs and IPSCs in PT and nPT cells, in vivo, and the first demonstration of a postsynaptic as opposed to presynaptic actions of phorbol ester-activated PKC on neuronal excitability and synaptic currents. In our earlier studies sustained associative facilitation of EPSPs occurred in the cat motor cortex following paired activation of convergent excitatory pathways. No significant changes in Rm were measured in of the conditioned cells vs others where increases in Rm accompained synaptic facilitations /4, 5/. Induction of LTP in the hippocampus is not associated with changes in Rm /7/. In Pavlovian conditioning experiments, increases of Rm were demonstrated in cat motor cortical cells, Hermissenda B photoreceptors and locust motoneurons /1, 18, 34/. It remains to be determined whether these long-lasting changes in postsynaptic responses found here and induced by activation of PKC are involved in long-term neuronal alterations which support learning in the neocortex.

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INTERACTIONS OF ACOUSTIC AND SOMATOSENSORY EVOKED RESPONSES IN A POLYSENSORY CORTEX OF THE CAT

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Interactions of acoustic and somatosensory evoked potentials were studied in the anterior suprasylvian gyrus of the cat. The interactions showed dynamic changes and were susceptible to different kinds of influences. The interactions could be influenced by synchronous activation of the acoustic and somatosensory inputs with 2 Hz frequency, or by elevating the stimulus frequency.

Interactions could be influenced by amphetamine and γ-glutamyl-taurine, drugs known as capable of influencing the arousal level of the brain. The antagonists of amphetamine prevented this effect. Durgs acting on the cortical GABA-ergic system proved also to be decisive in the interactions of evoked potentials of different origins.

In some experiments unit activity was recorded parallel with evoked potentials.

Keywords: Evoked potential — occlusion — facilitation — physiological plasticity

INTRODUCTION

Most animals possess multiple sensory systems with which they can simultaneously sample a variety of physical and chemical changes in their environment. Practically, the multitude of stimuli impinge on freely moving animals. It is likely that multisensory integration is a critical and continuous determinant of behaviour. Therefore, it is surprising that in

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contrast to the tremendous literature about the electrophysiological properties of the individual sensory systems, relatively few studies have been published about multisensory systems only and we are rather ignorant of how the nervous system deals with simultaneous inputs from different modalities. The study of these complicated intergrative processes is not easy. The first step in this direction was the study of interaction of heterosensory evoked potentials in the early sixties /1, 12, 13/.

Later, this method proved to be fruitful in the studies on cortical and subcortical associative systems /2, 3, 4, 5, 10, 11/.

There were only a few interaction studies in which microelectrode recording was used /6, 7, 8, 9/.

In the past ten years we have studied the interactions of acoustic (Ac) and somatosensory (Ss) evoked responses in the anterior suprasylvian gyrus of the cat. We have developed an unusual paradigm which is suitable not only for the detection of the interactions between Ac and SS evoked responses but also modulate them in order to produce plastic changes in the level of interactions and to follow these changes /16/.

In some of the experiments unit responses were also recorded in the course of procedures, parallel with the evoked potentials (EPs).

MATERIALS AND METHODS

The experiments were performed on cats anaesthetized with sodium pentobarbital (40 mg/kg i.p.). Additional doses were injected intravenously in the course of experiments to maintain the level of anaesthesia. After venous and tracheal cannulation the animals were fixed in a stereotaxic frame (type Szilas—Grastyán). The left cerebral cortex was exposed above the anterior suprasylvian gyrus (ASG) and the dura mater was removed. Then the cortical surface was covered with warm liquid paraffin and saturated with physiological saline. After surgery the animals were kept at rest for 1—1.5 h. Body temperature was maintained at 37 ^{o}C.

Acoustic stimuli (clicks) were applied to the right ear by way of a small loudspeaker. The stimuli were supramaximal. Weak electric shocks to the right fore-paw served as somatosensory stimuli, with a 5—9 V intensity and 0.3 ms duration. Both stimuli were supramaximal as measured on the evoked potentials of the anterior suprasylvian gyrus. Their repetition rate was 1 Hz.

The evoked potentials were led off with a ball-tipped silver-wire electrode from two loci at the same time, amplified by a two-channel amplifier and visualized on the screen of an oscilloscope. Blocks of 20 potentials were averaged by a MOTOROLA MC 6800 computer and drawn by an X—Y plotter. The interactions were analyzed using the method published previously /14, 15, 16/. One block of acoustic and somatosensory potentials was recorded separately, then one block was evoked and recorded with synchronous stimulation giving summated amplitudes of the two evoked

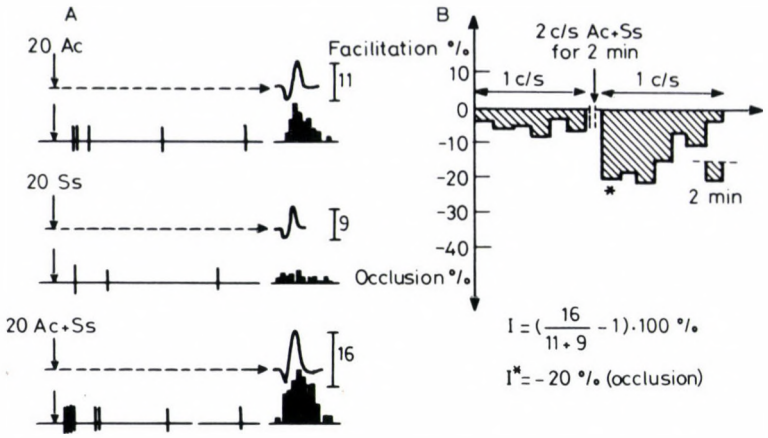

Fig. 1. A scheme to illustrate the paradigm used for the study of interaction of Ac and Ss evoked responses. A: data processing. 20 acoustic (Ac), somatosensory (Ss) and compound (Ac+Ss) potentials were recorded respectively. Evoked potentials (EPs) were averaged, while unit responses delineated by poststimulus time histograms (PSTHs). B: an example for conditioning procedure with 2 c/s. The interaction values were calculated according to the formula presented below

potential. The numerical value of interaction (I) was calculated according to the formula presented in Figs 1A, B. Negative values corresponded to occlusion, positive values to facilitation. If "I" was equal to zero, no interaction was supposed to be present.

Single unit responses were recorded extracellularly making use of Corning glass micropipettes filled with 2.5 M KCl. Electrode impedances were in the range from 5 to 15 mΩ. The electric signals were amplified and filtered using a two-cahnnel system (DISA Electromyograph) and stored on magnetic tape. Off-line analyses were carried out with a MOTOROLA MC 6800 computer. The temporal pattern of firing was delineated by poststimulus time histograms (PSTHs). All PSTHs represent averages of 20 response patterns (see Fig. 1A). In several cases unit responses were photographed from the screen of a Tektronix storage oscilloscope.

Drugs were obtained from Hungarian and foreing pharmaceutical compnaies: amphetamine, γ-glutamyltaurine and diazepam from Chinoin Ltd., haloperidol and reserpine from the Kőbányai Gyógyszerárúgyár, propranolol from Koch-Light, GABA (Reanal), baclofen (p-chlorophenyl-GABA) from Polfa, picrotoxin, bicuculline from Sigma.

RESULTS

Types of interactions of acoustic and somatosensory evoked potentials

The parameters of Ac and Ss evoked potentials of the ASG were described earlier /16/. Here we pay attention only to their interactions. In

the experiments three types of interactions occurred: (1) algebraic summation, which meant a real or apparent lack of interaction, with I=0, (2) occlusion with I 0 and facilitation at I 0.

First we examined whether the basic levels of interactions show any correlation with one another in different polysensory areas. One example of a comparison between the posterior middle suprasylvian association area (PMSA) and the anterior lateral area (ALA) is presented in Fig. 2. The records taken for as long as 30 min show that the level of interaction varied around zero with low occlusion and facilitation values in the anterior lateral association area. In the posterior middle suprasylvian area a rather high level of occlusion was present at around 30%. Beyond minute variations of occlusion no correlation could be found between the two recorded sites (r=0.18).

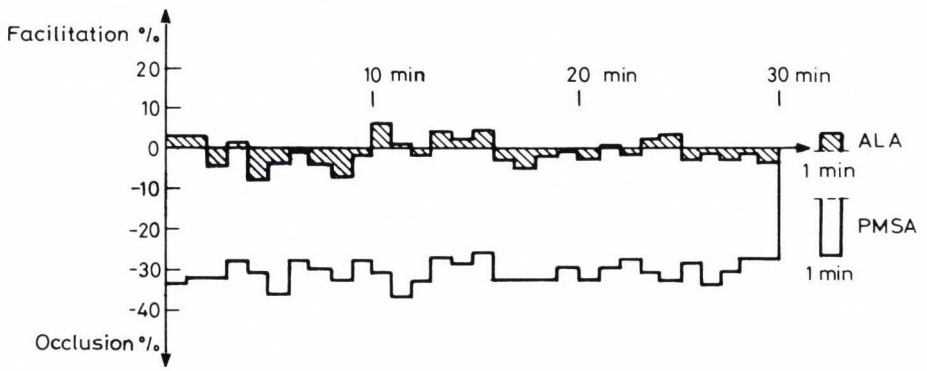

Fig. 2. Interaction of acoustic and somatosensory evoked potentials in the anterior lateral association area (ALA) and posterior middle suprasylvian area (PMSA) as recorded simultaneously over a 30 min period. In ALA the interactions were very weak, while in PMSA occlusion was dominant

The same holds for another pair of loci in the ASG 1.5 mm apart. The level of correlation of interactions between them remained under r=0.075% /16/.

After having established the level of interaction (in most cases occlusion) at a specific point of ASG, the question arose whether it is possible to influence it by shifting the actual level of interaction in any direction.

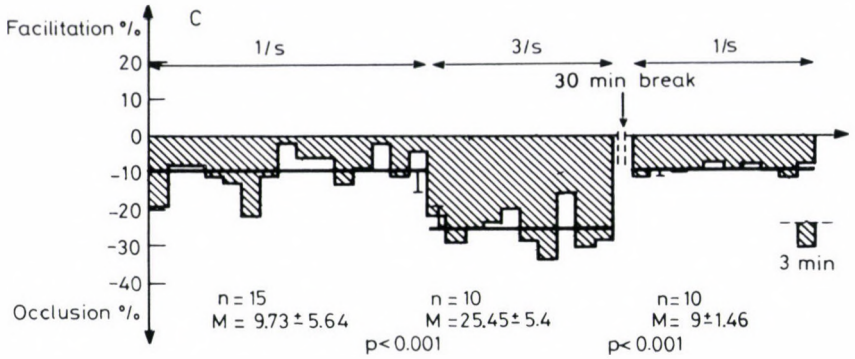

Fig. 3. Effect of increasing the stimulus frequency on the level of occlusion. During 3 Hz stimulation the mean level rose more than twice that of the control period (C) and returned to the initial one after 30 min rest. All changes are statistically significant. M, mean

MODIFICATION OF INTERACTIONS

Dependence of interaction on the stimulation frequency

The first question was: how changes of the stimulation frequency influence the interaction? The experiments showed that the elevation of frequency caused deepening of occlusion. This is exemplified in Fig. 3. After having tested the level of occlusive interaction with a 1 Hz repetition frequency, over to blocks 3 Hz were applied.The occlusion value increased from -9.73 to -25.45%. After 30 min rest the return to 1 Hz brought occlusion at -9% again. The synchronous stimulation of Ac and Ss inputs with 2 Hz exerted a peculiar effect on the interaction of evoked potentials (see below).

Modification of interactions following stimulus trains

After having established the level of interaction (mostly occlusion) at a particular point of ASG, 2 Hz compound stimulation was given for 2 min (Figs 1 and 4). The changes subsequent to it were characterized by a temporary elevation of occlusion as measured during the next 1 Hz stimulation period. After the 2 Hz stimulus process a 2 min rest was interposed. The maximal duration of such reversible changes was 16 min. The course of experiments of this type is illustrated and explained in detail in Fig. 4.

Based on this type of experiments one is led to conclude that the

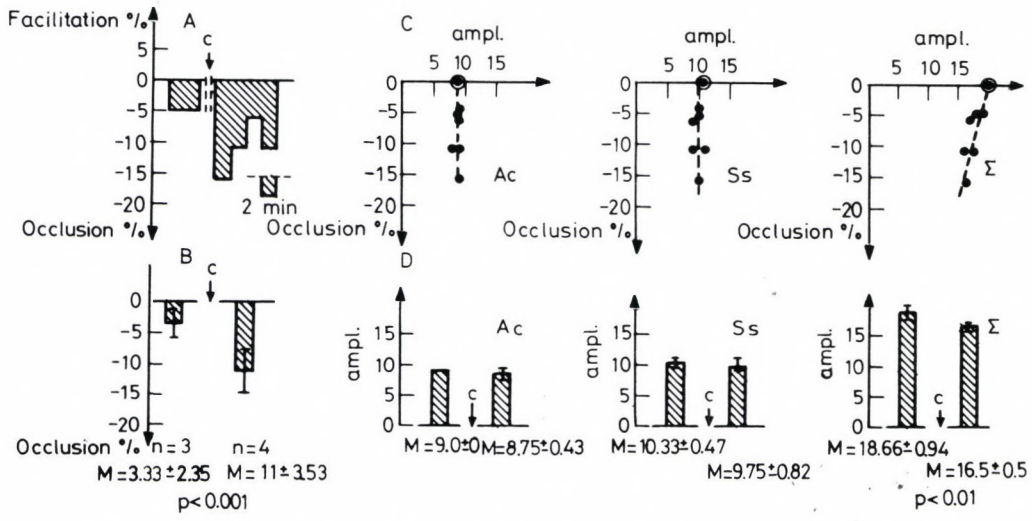

Fig. 4. The effect of 2 Hz conditioning (C) stimulation on the interactions of evoked potentials. A: Occlusion levels before and after C as measured in 2-min periods. B: Mean occlusion level with SD before and after C. The difference is highly significant ($p < 0.001$). C: Diagrams show the relation between evoked responses amplitudes (abscissa) and occlusion levels (ordinates) in all 2-min periods presented in A. D: Acoustic (Ac) and somatosensory (Ss) potentials remained practically unchanged through the recording period, while occlusion levels showed a rather wide variance. The compoun d responses decreased significantly after C and this led to an elevation of the occlusion level, presented in B. M, mean

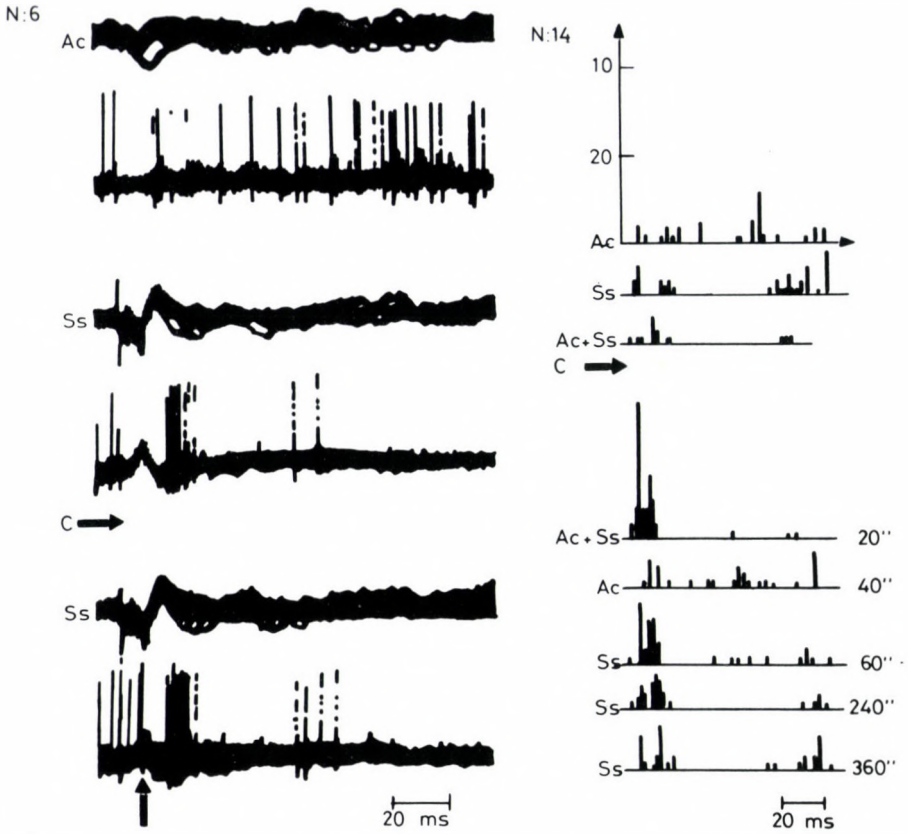

Fig. 5. Conditioned change of firing pattern of responses. N:6 neuron responded to acoustic (Ac) and somatosensory (Ss) stimuli, respectively. After conditioning (C) a new component of Ss response appeared (arrow). Upper lines: evoked potentials, Lower lines: unit responses. 5 traces were superimposed. N:14 neuron, did not respond either to Ac or Ss stimuli. A compound stimulation (Ac+Ss) also did not result in facilitation of the unit responses (3rd trace). After conditioning (C) the neuron's responses were facilitated both to compound (Ac+Ss) and somatosensory (Ss) stimulation, while Ac response did not change

amplitude of evoked potentials and the extent of their occlusion are determined by different factors and show independence from each other within wide limits.

Interactions of unit activities

After having observed the reversible changes of interactions of Ac and Ss evoked potentials subsequent to 2 Hz compound stimulation (as shown

above) we studied the changes of the evoked unit responses in the course of the conditioning described above (see paradigm in Fig. 1A). Here we deal only with the changes in evoked unit activity having a latency comparable with that of EPs (between 12 and 20 ms). After 2 Hz compound stimulation two kinds of changes could be observed: i) the evoked responses of only one of the two modalities (Ac or Ss) changed following 2 Hz compound stimulation (conditioning: c). In most cases the response pattern changed after conditioning reversibly for some minutes. Two examples for this kind of change are represented in Fig. 5. N:6 neuron was a bimodal cell which responded both to Ac and Ss stimulation (1st and 2nd rows in Fig. 5). After 2 Hz compound stimulation (c), a brief burst of spikes (arrow) appeared in the Ss response in correspondance with the deep negative phase of the Ss evoked potential. This persisted for 4 min (Fig. 5). A similar change was seen at neuron N:14 whose PSTHs are presented in Fig. 5. During the control period the evoked discharge rate did not rise over the spontaneous activity (neither at Ac nor at Ss stimulation, 1st and 2nd rows in Fig. 5) (N:14), just as at the compound stimulation (3rd row). After conditioning (C) the responses to compound (4th row) and Ss stimulation (6th, 7th and 8th rows) became enhanced, while the Ac response did not change (5th row). These change persisted for about 6 min (Fig. 5). ii) Another kind of change occurred more frequently. In these cases only the compound response changed following conditioning (Fig. 6) (N:12, N:13 and N:16 neurones).

Generally, these neurons exhibited a weak facilitation in the control period on compound stimulation (3rd row in Fig. 6) and this increased transiently in the course of conditioning. The conditioned increase of facilitation was very weak in neuron N:12 and lasted for only 3—4 min, while at neurons N:13 and N:16 it was longer (Fig. 6).

It is worth noting that there was no identifiable response to separate Ac and Ss stimulation before conditioning (C). Only neuron N:13 showed enhancement of firing upon compound stimulation before (C). However, after conditioning (C) a marked enhancement of firing ensued at all of these neurons and lasted for minutes (Fig. 6).

Modifications of interactions by drugs

The experimental data and theoretical considerations suggested that inhibitory and excitatory mechanisms play an outstanding role in these interactions. The actual level of interaction seemed to be determined by an equilibrium of inhibitory and excitatory neuronal activity. If so, this

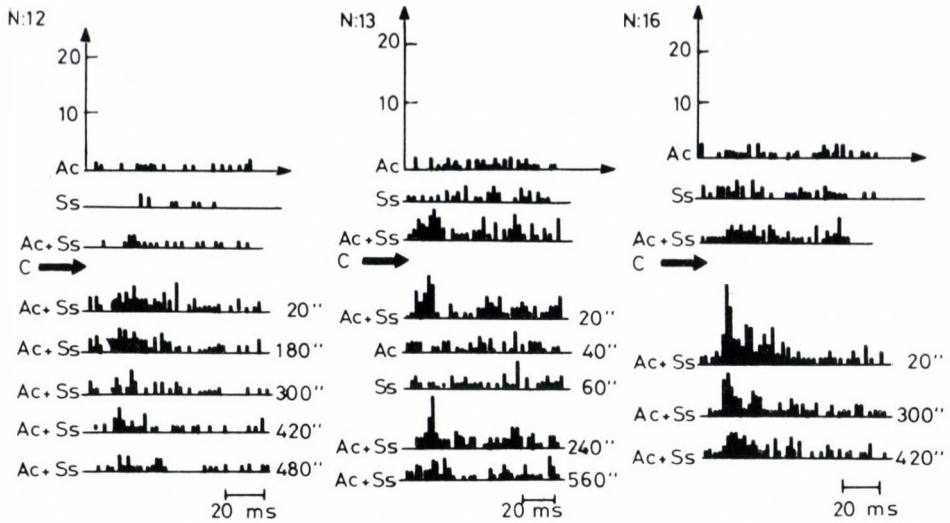

Fig. 6. Response patterns of N:12, N:13 and N:16 neurons in the course of conditioning. In this group of neurons conditioned changes could be seen only in compound responses. A weak facilitation was present before conditioning (C) (3rd row) which was enhanced after 2 Hz compound stimulation (C), while the separately evoked responses did not change (N:13, 5th and 6th rows)

equilibrium might be shifted in an occlusive or facilitatory direction, making use of different drugs influencing these systems. A series of experiments was designed in an attempt to throw light on the importance of the GABAergic synapses in the cortical occlusive and facilitatory processes.

These results of such experiments are collected in Fig. 7.

GABA was applied locally in a 1% solution to the anterior suprasylvian gyrus or administered intravenously, in a dose of 4 mg/kg. After 5 min of i.v. application, GABA enhanced the occlusion from a control value of -14.2 ± 3.1 to $-25.9\pm4.5\%$ (Fig. 7A).

Two GABA antagonists ($\pm$Baclofen and Diazepam) had a similar effect on the interaction of Ac and Ss evoked potentials (Figs 7B, C).

The effect of these durgs can be seen on the interactions of Ac and Ss evoked potentials in Figs. 7D, E.

After Baclofen treatment, the acoustic evoked potential sometimes diminished or disappeared completely, but the amplitude obtained through synchronous stimulation was lower than the somatosensory potential alone, indicating that some kind of inhibition was activated by the synchronous stimulation (Fig. 7E).

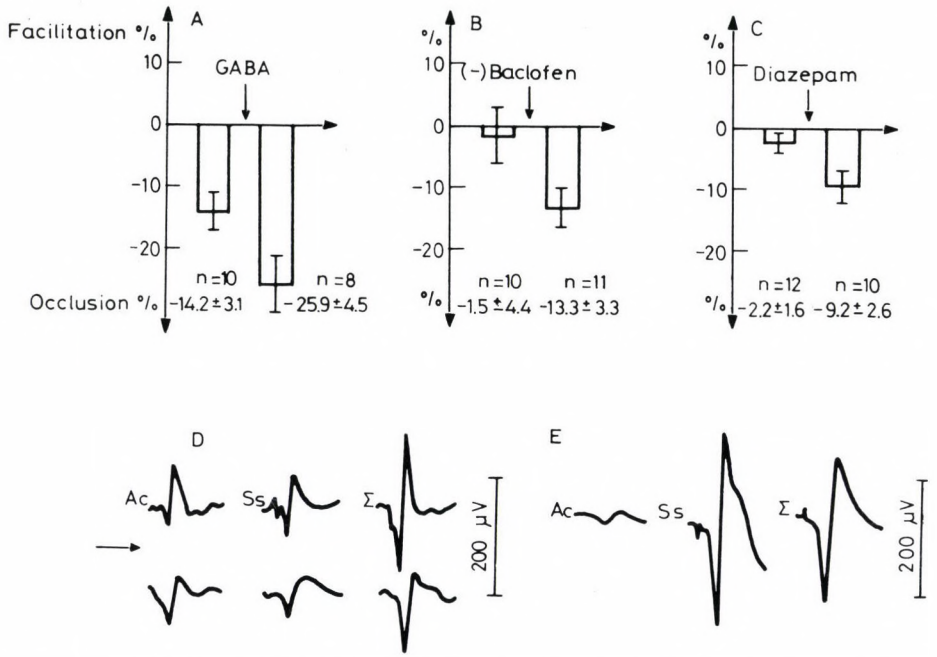

Fig. 7. The effect of GABA A:, baclofen B: and diazepam C: on the interactions of acoustic and somatosensory evoked potentials. Under the diagrams the mean values and number of blocks are denoted. All differences are highly significant as calculated with Student's double t-test. D: upper row: averaged blocks of separately recorded acoustic (Ac), somatosensory (Ss) and summated ( ) potentials before GABA. At arrow 2 mg/kg GABA was administered i.v. lower row: evoked potentials 10 min after GABA injection. E: averaged blocks of separately recorded acoustic, somatosensory and summated potentials 10 min after baclofen injection (1 mg/kg). The summated potential is lower than the somatosensory alone

We have also tested the effect of the GABA antagonists on the interaction. The GABA antagonists bicuculline and picrotoxin are known also as convulsants at higher concentrations. In our experiments they were administered only in subconvulsive doses (Fig. 8 P_1). Picrotoxin in 1 mg/kg doses increased the amplitude of the separately evoked potentials but enhanced the amplitude of synchronously evoked responses much more and this resulted in high values of facilitations (Figs 8A, C).

Similar results were obtained in case of bicuculline application (Fig. 8B).

In later stages of their action or at higher doses both GABA antagonists caused convulsive potentials, appearing with longer latency on the

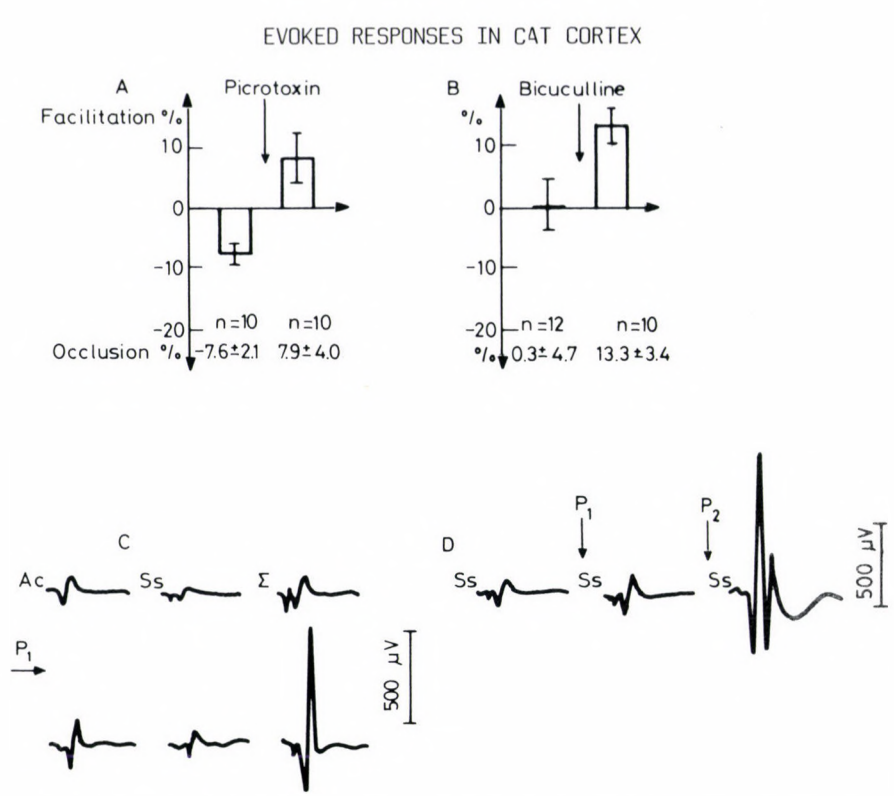

Fig. 8. The action of picrotoxin /A/ and bicuculline /B/ on the interaction of acoustic (Ac) and somatosensory (Ss) potentials. The numbers below denoted the same as in Fig. 7. C: Upper row: control series, lower row: 10 min after 1 mg/kg picrotoxin P_1 (at arrow). D: The evolvement of the facilitatory effect of picrotoxin upon somatosensory potentials. P_1: 5 min after a 1 mg/kg i.v. dose of picrotoxin, P_2: 5 min after a 2.5 mg/kg i.v. dose of picrotoxin

decaying limb of the surface negative phase of the sensory evoked potentials (Fig. 8D P_2). Stimulation series in which seizure potentials appeared, were not evaluated in interaction studies.

In other experiments we analyzed the effect of drugs capable of influencing the arousal level of the brain. One of them was amphetamine which applied in four different doses, from 0.13 to 0.5 mg/kg (Table 1). All doses caused a lowering of occlusion, i.e. a shift towards facilitation. The decrease of occlusion, as expressed as a percentage, correlated well with doses of amphetamine (r=0.93), indicating a causal relationhsip between them.

A newly discovered peptide (γ-glutamyltaurine) proved to have effects on central nervous functions analogous with those of amphetamine.

Table 1

Effect of amphetamine on the occlusion of acoustic and somatosensory evoked potentials

Amphetamine (mg/kg, i.v.)	Decrease of occlusion (%)	Correlation coefficient: r
0.13	3.1	-
0.36	6.3	-
0.42	6.2	-
0.50	9.8	0.93

In the present experiments it was tested whether this parallel also holds for the interactions of evoked potentials (Fig. 9). On administering γ-glutamyltaurine in a wide range of doses, a dose-response relationship could be observed if not as strict as in the case of amphetamine (Table 2) with a correlation coefficient of 0.53.

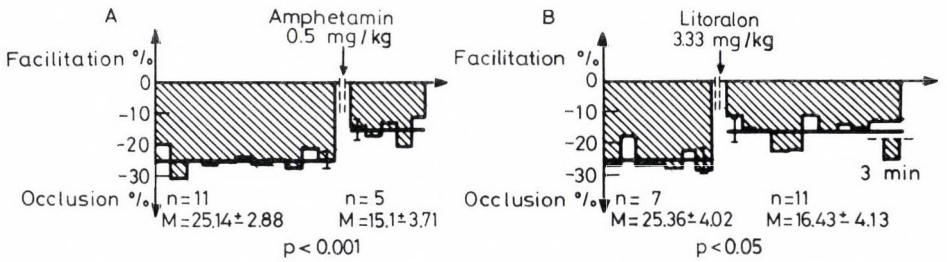

Fig. 9. The effect of A amphetamine (0.5 mg/kg) and B γ-glutamyltaurine (Litoralon: 3.33 mg/kg) on the occlusion level. All changes are significant. M, mean

The effects of haloperidol and reserpine (antagonists of amphetamine) were then studied. Haloperidol was administered i.v. in a dose of 0.5 mg/kg, 3.5 h prior to the recording, and prevented the decrease in occlusion by amphetamine (Fig. 10).

Reserpine (0.42 mg/kg) behaved similarly. None of these drugs seemed to influence the level of occlusion by itself, but blocked changes of occlusion induced by amphetamine and Litoralon.

Propranolol, a well-known blocker of adrenergic beta receptors, not only antagonized the facilitatory action of amphetamine and γ-glutamyl-

Table 2

Effect of γ-glutamyltaurine on the occlusion of acoustic and somatosensory evoked potentials

γ-glutamyltaurine (mg/kg, i.v.)	Decrease of occlusion (%)	Correlation coefficient: r
1.81	5.00	-
2.10	7.40	-
3.17	4.04	-
3.17	3.37	-
3.60	8.50	-
3.77	3.37	-
3.61	6.20	-
5.00	14.60	0.53

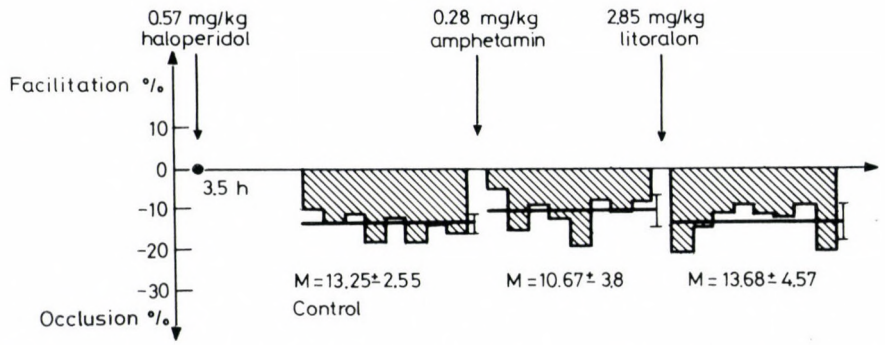

Fig. 10. The effect of haloperidol (0.5 mg/kg) on the facilitatory action of amphetamine and γ-glutamyltaurine (Litoralon). M, mean

taurine, but also strongly enhanced the occlusive interaction of acoustic and somatosensory potentials (Fig. 11).

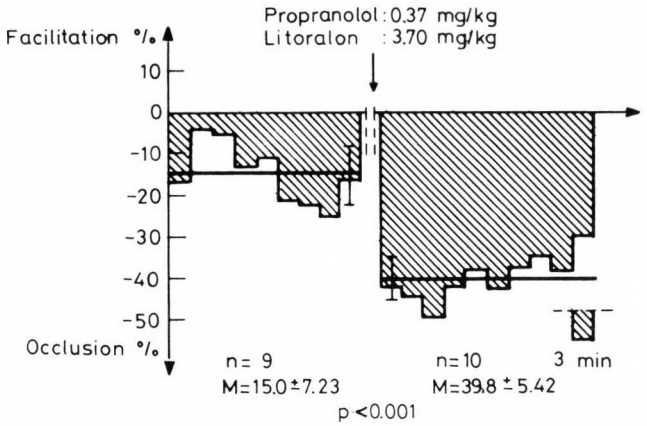

Fig. 11. The effect of propranolol (0.37 mg/kg) on the occlusion level. This drug does not only antagonize γ-glutamyltaurine (Litoralon), but cause a great enhancement of occlusion. M, mean

DISCUSSION

In this paper we have tried to summarize the main results of the interaction studies made in the past 10 years. Most of these results have been published and suggestions for explanations are discussed in detail /14, 15, 16/.

Here we only try to summarize the main results and suggestions for the mechanisms of these phenomena. It seems likely that the two kinds of stimuli excite separate populations of neurons (according to the literature, which shows that the ASG is not a usual associative cortex) /12, 13, 17/. Our experiments indicate that these, more or less separate neuron populations are bound up with each other by dynamic interactions. The following observations provide information as to the nature of the interaction: 1) the most dominant form of interactions is occlusion, which might be the result of occlusive and facilitatory mechanisms. Thus, changes in the level of interaction may be due to changes in equilibrium of facilitatory and inhibitory mechanisms. 2) Elevation of the occlusion level can be attained in more than one way: a) by 2 Hz conditioning, b) by a higher stimulation frequency at both separate and compound stimulations, c) by using GABA or GABAergic drugs, or d) by blocking the central adrenergic beta receptors with propranolol.

On the other hand, the fact seems to be important that modifi-

cations of the interaction level are largely independent of the actual amplitude of separately evoked potential, indicating that subsystems are responsible for interaction, for its level and these subsystems are not identical with those generating sensory evoked potentials.

This was supported also by the results of unit recording which showed that plastic changes were found — in most cases — in neurones which did not respond significantly either to Ss or Ac stimuli before conditioning (Fig. 6 N:12, N:13, N:16 neurons). In the case of compound stimulation in the control period a weak, if any, facilitation could be observed (Fig. 6 N:13, N:16). These results suggest that the cortical interneuronal systems play a decisive role in the integrative processes. This was supported by the experiments in which drugs (acting on GABAergic system: Figs 7, 8) or on catecholaminergic system: (Figs 9, 10, 11) influenced always prominently the interactions of evoked potentials of different origin. They did so — in most cases — without bringing about changes in their amplitudes.

However, any more progress in this field needs further pharmacological and electrophysiological investigations.

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THE IONIC MECHANISM OF THE PENTYLENETETRAZOL CONVULSIONS

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The ionic dependence and the nature of conductance was examined at slowly inactivating inward current in metacerebral giant cells of Helix pomatia, induced by 50 mM pentylenetetrazol. Ramp and square wave depolarizations in voltage clamp mode revealed, that withdrawal of sodium ions prevented this current to flow. While TTX was ineffective, Mn, Co and Ni-ions and verapamil blocked the current. It is concluded that PTZ, especially in presence of TEA impairs calcium channels, which loose their specifity and transmit sodium ions, with very slow kinetics.

Keywords: Snail neuron — pentylenetetrazol — ionic currents — ion channel blockers

INTRODUCTION

In a previous paper the effects of pentylenetetrazol (PTZ) were analyzed on the electric membrane parameters, action potentials, inward and outward currents on the metacerebral giant cell (MCC) of Helix pomatia /3/. At the same time paroxysmal depolarization shifts (PDSs) elicited by the drug were also recorded. The most obvious effect of PTZ, the depression of the I_A and I_K currents, although proved to be a corollary of the convulsive actions, failed to provide an explanation for the PDSs, all the more, because it did not modify substantially the inward Na- and Ca-currents. Our attention was therefore directed to the origin of those long-lasting depolarization plateaus, which underlie to the PDSs and bear on the crest

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normal or partially inactivated spike potentials. Our aim was to clear up i) what kind(s) of channels are used by this slow, scarcely inactivating current, and ii) what kind(s) of ions are carrying it.

MATERIALS AND METHODS

The experiments were carried out on the metacerebral giant cell (MCC) of the snail, Helix pomatia L. To prepare the identified cells, the ganglionic mass was dissected from the animal and the cerebral ganglion with its ventral surface upwards was fixed to the bottom of an organ bath, covered with Sylgard. After peeling off the connective tissue sheats the MCC was sought under binocular magnification (x40). For penetration of the neurons with a microelectrode a standard mechanical micromanipulator was used.

The preparation was continuously superfused with normal and modified Helix-physiological solution. The normal Helix-physiological solution contained (in millimoles) NaCl 80, KCl 4, $CaCl_2$ 7, $MgCl_2$ 5, Tris Cl 5, (pH 7.4). Sodium-free solution was prepared with aequimolar substitution for NaCl with Tris-HCl or choline-Cl. In Ca-free solutions $CaCl_2$ was replaced by $MgCl_2$ aequimolar. In some experiments 10 mmol/l $NiCl_2$ was used to block Ca-channels, 30—50 mmol/l tetraethyl-ammonium chloride (TEA) to block potassium channels. PTZ was dissolved in 20 to 50 mmol/l concentrations without osmotic balance.All experiments were performed at room temperature (22—25 ^{0}C).

Current-clamp and voltage clamp recordings were made by use of a single-channel voltage clamp amplifier built according to the design of Wilson and Goldner /10/ and Merickel /6/. Glass microelectrodes were filled with one molar potassium citrate; their resistance ranged from 2 to 7 MOhms. Potential and current records were visualized and photographed from the screen of a Tektronix storage oscilloscope. Occasionally, current-voltage curves were recorded with an X—Y plotter. A second oscilloscope was used for monitoring the sampling process.The duty cycle of the sample-and-hold amplifier was 50% and all current values were corrected according to this proportion.

RESULTS

Since PDSs are generally introduced by a spike potential (at least the spontaneous ones) it was supposed, that the slow inward current underlying to them is mediated by voltage dependent ionic channels. This was demonstrated previously by Gola /4/. Therefore cells operating in voltage clamp mode were depolarized in two ways: slow voltage ramps and square wave pulses were applied through the microelectrode.

The steepness of the ramps (dV/dt) in both directions ranged from 20 to 25 mV/s; the maximal depolarization extended from the holding potential (typically -40 -45 mV) to +30 mV.The I-V characteristics of a non-treated

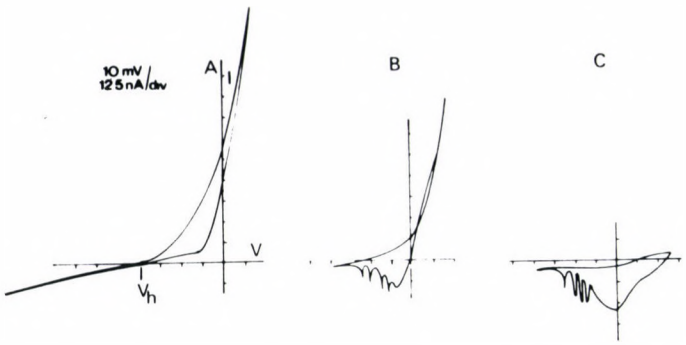

Fig. 1. A: Current-voltage characteristic of an untreated metacerebral giant cell (MCC). Left top: voltage and current calibration. B: The same under the effect of 50 mM PTZ, after 20 min application. C: The effect of 50 mM PTZ and 30 mM TEA

MCC is presented in Fig. 1A as recorded during a voltage ramp under voltage clamp conditions. The hysteresis on the descending limb can be ascribed to a slowly activating outward current. On the hyperpolarizing limb only some leakage current is present. The superfusion of 50 mM PTZ brought about considerable modifications at this I-V characteristic (Fig. 1B). With the decrease of the membrane potential pari passu an inward current appeared, which attained an apparent maximum at -10 mV; then this went over into an outward current with decreased final amplitude and without hysteresis. At the beginning of the depolarization spike artifacts appeared from poorly clamped regions of the cell.

Since it was probable, that the slow inward current induced by the PTZ might be partially masked by outward currents, being activated simultaneously, 30 mM TEABr was added to the PTZ, for depressing most part of the outward potassium currents. As it is demonstrated in Fig. 1C, under these conditions during depolarizing ramp no outward crrent appeared, and the negative resistance region of the I-V characteristic contained the slow inward current at its total amplitude. Its maximum was at 0 mV membrane potential and its reversal point was at +25 mV. At the start of the depolarization also spike artifacts could be seen. This state of the cell, when recorded in current clamp mode, was already strongly convulsive: PDSs follow each other with irregular intervals. The spike potentials,

which initiate PDSs or ride on their crest were widened, due to the presence of TEA and/or PTZ.

In further experiments the slow inward current, isolated in the aforementioned way, was analyzed in view of the ions carrying it and the kind of channels used by them.

The ion dependence was examined by use of ion-deficient solutions. In presence of 50 mM PTZ and 30 mM TEA the substitution of the sodium ions with Tris modified the I-V characteristic in a direction qualitatively well defined, but at different extents. In some cases (Fig. 2) omission of Na-ions resulted in complete disappearance of the slow inward current, indicating that it was mediated exclusively by sodium ions. In this same experiment, however, 11 min application of 15 mM $CoCl_2$ led to the same result. Application of 15 mM $NiCl_2$ had the same effect (Fig. 2B).

Since participation of sodium ions in the slow inward current was indisputable, it could not be excluded that part of them is conveyed through tetrodotoxin (TTX) sensitive "fast" sodium channels. Therefore the effect of 10 μM TTX was examined on the slow inward current induced by PTZ and TEA, on several cells. As it can be seen in Fig. 3, this concentration of TTX failed to modify the I-V characteristics also after 28 min of application. At the same cell, withdrawal of sodium ions depressed the slow inward current to a small fraction. Although there are observations /5/ that sodium channels in the Helix neuron membrane are TTX resistant, it is worth of mention that after application of TTX no spontaneous or evoked spike potentials were encountered.

The conductance, transmitting the slow inward current could be measured quantitatively by use of square voltage pulses of 4 s duration in voltage clamp mode. The current amplitude was measured at different command potentials at 1000 ms after onset of the pulse, because by this time any contamination from fast inward and outward currents (eventually not blocked by TEA) could be excluded.The voltage dependence of the slow inward current on four cells, treated with 50 mM PTZ and 30 mM TEA is demonstrated in Fig. 4. The corresponding conductance values are represented in Fig. 5. The conductances are uniformly and monotonically voltage dependent and along with the applied voltage they increase practically linearly.The current, however, diminishes over some level of depolarization because the membrane potential gets near to the reversal potential of the ion carrying it. Although in transmission of the slow inward current both sodium and calcium

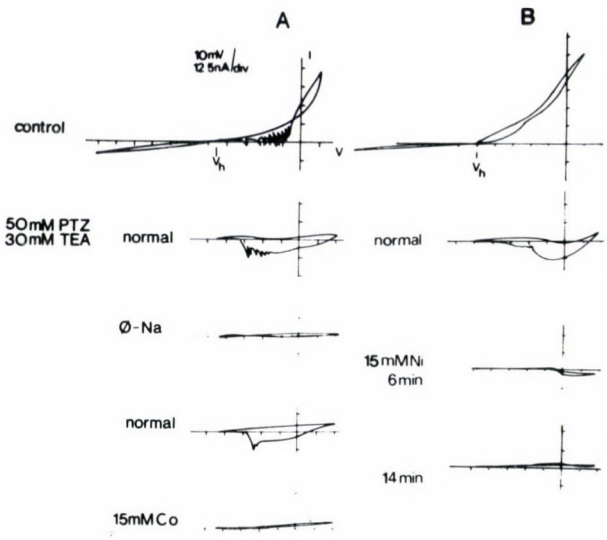

Fig. 2. A: Current-voltage characteristic of a MCC under control conditions (1st row), after application of 50 mM PTZ and 30 mM TEA (2nd row), after withdrawal of sodium ions (3rd row), after returning to PTZ+ TEA superfusion (4th row), and under the effect of 15 mM $MnCl_2$ (5th row). B: Another MCC under the same conditions but after 6 min application of 15 mM $NiCl_2$ (3rd row) and after 14 min (4th row)

ions take part, as reversal potential for computation of conductances +30 mV was chosen.

In some part of the experiments the slow inward current, elicited with ramp voltage pulses, showed not only one maximum (as in Fig. 1) but two. During superfusion of PTZ and TEA (at the usual concentrations) the slow inward current had two maxima: one at -13 mV and one at +8 mV membrane potential (Fig. 6). 15 mM $MnCl_2$ depressed this current strongly. The first maximum disappeared and the second maximum seemed to be shifted to higher potential values. There appeared, that the membrane potential shift, provided by the ramp was not enough to reach the voltage at which this

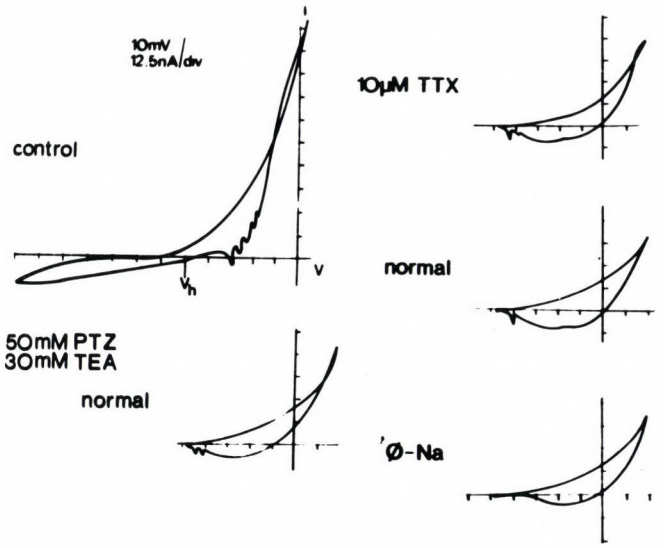

Fig. 3. The effect of 10 μM tetrodotoxin (TTX) on the current-voltage characteristic of a MCC, during application of 50 mM PTZ and 30 mM TEA, and during superfusion of Na-deficient solution, containing the same drugs. The notation "normal" refers to the sodium concentration

current could attain its maximum. This might lie over +30 mV. After washing out the manganese, practically the initial situation was restored, but with the two maxima better separated. Replacement of the sodium with Tris destroyed the first maximum almost completely, keeping the second one intact. Application of $MnCl_2$ in this situation led to complete removal of any conductance.

A similar sequence of events could be reproduced with Ni- and Co-ions with interpolated withdrawal of sodium ions, not presented here.

The time relations of activation and inactivation of the slow inward current is rather difficult to analyze. The process of activation may suffer interferences from activation of fast sodium and calcium currents. Use of TTX is not of great use, because calcium channels, taking part in generation

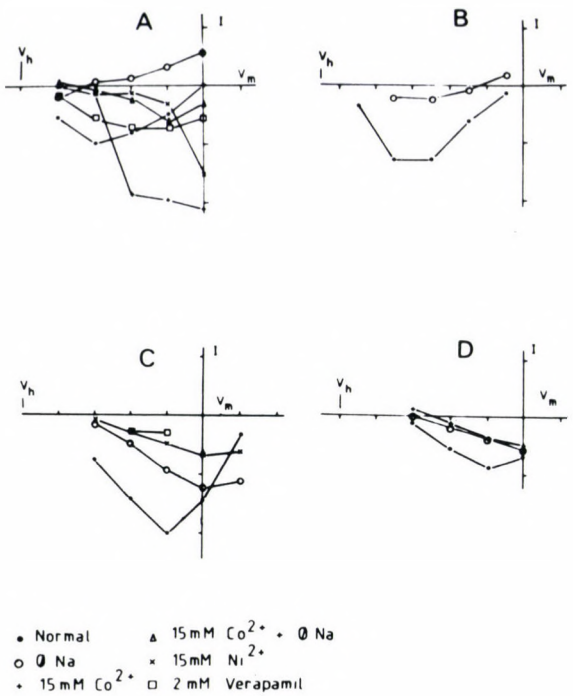

Fig. 4. Current-voltage relations of MCCs constructed from records obtained with depolarizing pulses of 4 s duration. Current values were measured and plotted at 1 s after pulse onset. From A to D four different neurons are presented, which were superfused with 50 mM PTZ and 30 mM TEA and are denoted as "normal". Sodium deficiency and application of Ca-channel blockers are denoted as shown bottom right

of spike potentials are not blocked by the drug. The process of inactivation was examined by computation of the time constants. The descending phase of the currents, elicited by square wave voltage pulses in voltage clamp mode seem to decline along two time constants: an initial shorter and a late longer one (Fig. 7). Finally the current has a time independent plateau, without any sign of inactivation. The logarithmic plots of the descending phases and time constants calculated form them are comprised in Fig. 8.

The slow inward current proved to be largely sodium dependent also in this experimental paradigm: on withdrawing the Na-ions from the superfusing fluid PTZ failed to evoke any slow inward current also in presence to TEA (Fig. 7). With omission of calcium ions no experiment was made, because lack of calcium might damage the membrane seriously.

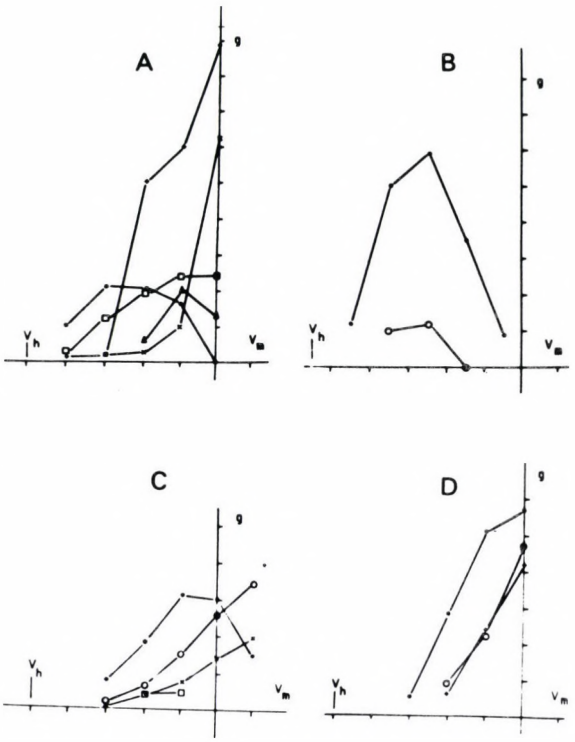

Fig. 5. Plot of conductances (g) as a function of membrane potential (V) at the same MCCs which were presented in Fig. 4. Notations are the same. Voltage calibration: 10 mV. Calibration for conductance: 100 nS for A and B, 200 nS for C and D

Blockers of the calcium channels proved to be effective also in inward current, elicited with square wave voltage pulses: Co-, Ni- ions and verapamil decreased the slow inward current largely or completely (Fig. 9).

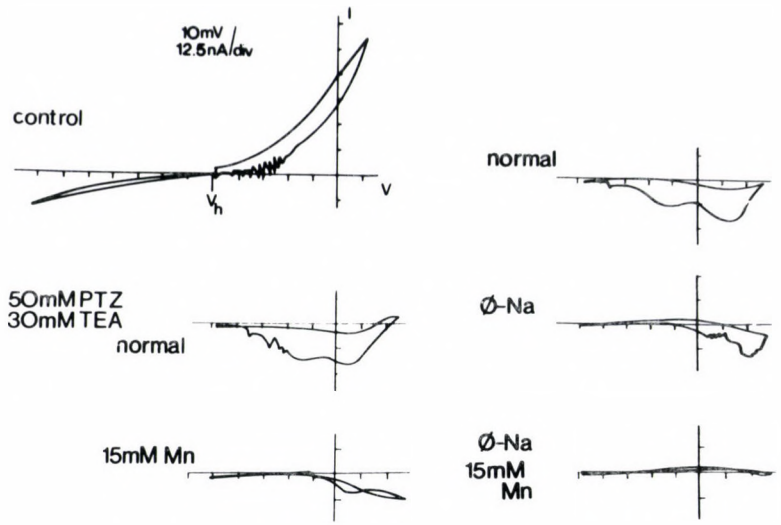

Fig. 6. Current voltage characteristic of a MCC under control conditions, under the effect of 50 mM PTZ and 30 mM TEA, then treated with 15 mM $MnCl_2$ and exposed to sodium deficient solution. The inward current had two maxima, which responded to Mn ions in different manner

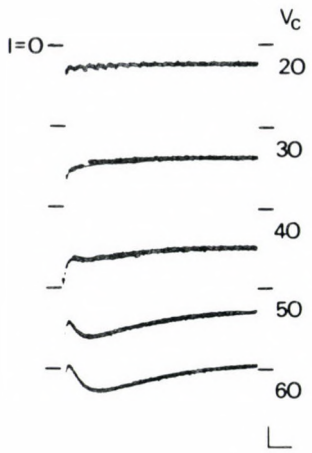

Fig. 7. Records of the slow inward current obtained with depolarizing steps of +20 to +60 mV from the holding potential (-45 mV), and with 4.5 s duration. The neuron was exposed to 50 mM PTZ and 30 mM TEA, over the whole recording period. At low depolarizations the current reached its maximum rapidly, decayed slowly and had a considerable steady state value. At depolarizations to 0 mV or more positive potentials the current showed a second maximum and faster inactivation. Pulse amplitudes are shown at right. Calibration for current: 12.5 nA, for time: 0.5 s

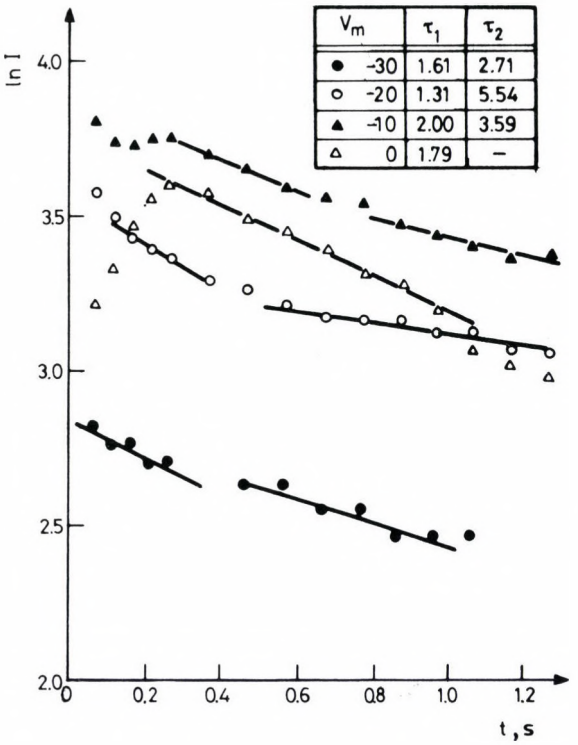

Fig. 8. Logarithmic plots of the current curves presented in Fig. 7, and the time constants calculated from them. Numerical values of time constants are tabulated at top right

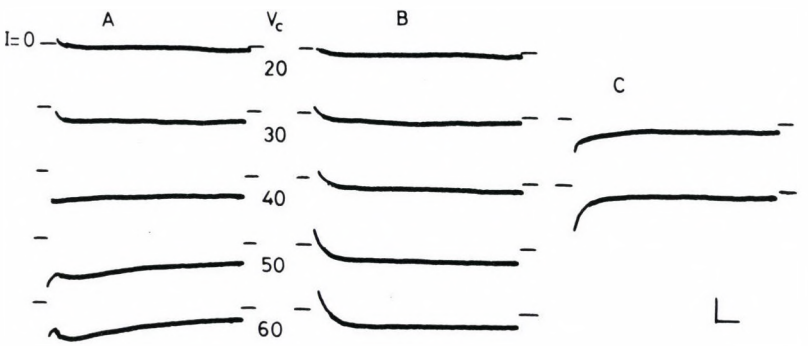

Fig. 9. Effect of sodium withdrawal (A), $NiCl_2$ (B) and verapamil (C) on the slow inward current of the MCC demonstrated in Fig. 6. The neuron was treated with 50 mM PTZ and 30 mM TEA over the whole recording time. Calibrations are the same as in Fig. 6.

DISCUSSION

It has been widely accepted that spontaneous burtsing behaviour, at least in Molluscs, finds its basis in two special modifications of membrane conductance: (i) a slow, scarcely inactivating inward current and (ii) depression of potassium conductance. Although the same has been established for mammalian neural substrates, we shall deal here only with findings obtained on Gastropoda. Smith and coll. /7/ observed in neurons of Otala and Aplysia that diminution of the sodium concentration in the superfusing fluid from 100 mM to 25 mM destroyed bursting pacemaker activity. The authors presume that bursting pacemaker activity relies upon a slowly inactivating (τ = 16 s) sodium conductance, which together with a cyclically changing potassium conductance, becomes enabled to generate a bursting pacemaker activity. Swandulla and Lux /8/ report, that such a non-specific cationic conductance can be activated by elevation of the intracellular calcium concentration. TTX was found ineffective on these slow inward conductances. Inorganic and organic Ca-channel blockers, on the other hand, were highly effective in inactivating these channels (Walden and coll. /9/). In bursting pacemaker neurons this current is permanently present in the pacemaker range of membrane potential and at moderate depolarizations it causes a negative slope resistance, as revealed in voltage clamp situation. The carriers of this current may be Ca-ions /2/ or Na-ions /1/.

The MCC neuron is not a bursting pacemaker and under natural conditions neither slow inward current, nor cyclically decreasing potassium conductance is present in it. This is indicated by I-V characteristics taken by use of ramp- or square wave voltage steps. On effect of PTZ, however, they begin to operate which results in a membrane behaviour, comparable in all respects to that of the bursting pacemaker neurons, as it was characterized by Swandulla and Lux /8/. In view of its ionic dependence, it is not surprising that it is carried mainly by sodium ions, although in some cases also Ca-ions may take a considerable part in it. This supposition seems to be justified in cases, when the slow inward current exhibits two maxima, as it was described by us in this paper. The first of them vanishes on withdrawal of sodium ions, while the other one, at more positive potentials becomes depressed by calcium channel blockers. There appears, that although sodium ions use most part calcium channels also in these cases, another part is penetrated by Ca-ions.

The present experiments have succeeded in clearing up several other

properties of this conductance. Thus, its voltage dependence proved to be almost linearly proportional to the depolarization, without attaining any maximum, as it is usual at conductances participating in the normal neuronal activity.This points to a basically different channel kinetics and cannot be dealt with as the "normal" ones. The inactivation properties are much more similar to those e.g. of the potassium, although its final stage with the time independent inward conductance appears to be unique. It is probable, that the final, time independent conductance causing long-lasting moderate depolarization gives opportunity for the gradually activating potassium currents to finish the paroxysmal depolarizations. At least at PDSs, which have an after-hyperpolarization this seems to be the case.

The "abnormality" of the conductance invoked by PTZ is indicated also by its poor specifity: this channel, which has retained its vulnerability by calcium channel blockers, has lost its specifity to calcium: it transmits sodium ions with unusual intensity. These distortions seem to be the central moments of the membrane behaviour, characteristic to the neurons, made convulsive by chemical, physical or metabolic factors.

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FINE STRUCTURE OF THE NEUROMUSCULAR JUNCTIONS IN THE ALIMENTARY TRACT OF PHYLOGENETICALLY DIFFERENT ANIMAL SPECIES

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Authors studied the fine structural characteristics of the neuromuscular junctions in the alimentary tract of phylogenetically different animal species. Nearly in each studied species the so-called close contacts were observable, where the sarcolemma and axolemma establish a junction; the gap of this contact is 10—100 nm wide, and the neurotransmitters can affect the muscles through non-synaptic release (exocytosis). The junctional gap is widest in the gut wall of earthworm: 100—200 nm. Only close contacts are to be found in the alimentary canal of snail, and here, the junctional gap is as narrow as 10—15 nm. Beside close contacts synaptic neuromuscular junctions also occur in the locust gut. Their fine structural organization refer to that of chemical synapses. As in the tench intestine both striated and smooth muscular elements can be found, motor endplates as well as close contacts take part in their innervation. Only close contacts are in the smooth muscular layer of domestic fowl. Authors emphasize the role of close contacts in the regulation of gut peristalsis and only secondary importance is attached to other different junctions.

Keywords: Gut — comparative ultrastructure — neuromuscular junction

INTRODUCTION

Electron microscopic studies /10, 19, 20, 25/ revealed that a peculiar form of nerve-muscle junctions: the so-called close contact takes part in the innervation of various visceral muscles. This type of contacts

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is basically different from motor endplates innervating skeletal muscles /10/, namely the "classical" synaptic organization: the pre- and post-synaptic membrane thickenings, the infoldings of sarcolemma, etc. are absent. Close contacts have been observed in the gut musculature of both mammals /9, 11, 18, 24/ and a few species of lower order /1, 2, 21, 22, 23/. The basic fine structural features of this sort of nerve-muscle junctions are similar in the case of every single neuroeffector. However there are differences in the distance between the terminal membranes (axolemma and sarcolemma), moreover in the vesicle population of axonterminals. Cyto-chemical studies /8/ proved the probable neurotransmitter (or modulator) role of more than a dozen candidates. The fine structural characteristics of close contacts in the mammalian gut are almost entirely clarified. Some studies have been carried out on lower vertebrates /22, 23, 27/ and on some representatives of invertebrates /4, 13, 21/ as well. Systematic examinations have not been carried out until now from phylogenetic point of view, in order to reveal the neuromuscular junctions of the alimentary tract. This field is very interesting because the structural and chemical features of muscular and neural elements composing the enteric neuromuscular junctions are very variable in the intestine of different animal species. For example, a so-called obliquely striated musculature can be found in the alimentary tract of earthworm /14/. The gut-wall of snail is built up by smooth muscle cells /21/, in contrary, a cross-striated musculature is present along the entire alimentary canal of insects /2/. Cross-striated muscle fibres occur in the oesophagus, stomach and small intestine of some fish /23/ (for example tench), but exclusively smooth muscle cells can be found in the oesophagus of pigeon.

Possessing all this knowledge our aim was to analyze the ultra-structural features of the neuromuscular junctions in the gut-musculature of different animals, and to draw conclusions about their physiological role. The present paper deals with the fine structure of neuromuscular junctions in the alimentary tract of earthworm, snail, locust, tench and domestic fowl.

MATERIALS AND METHODS

Adult individuals of Lumbricus terrestis, Helix pomatia, Locusta migratoria, Tinca tinca and Gallus domesticus were used in our experiment. Animals were killed, the alimentary tract was immediately removed and cut

into cubed blocks in ice-cold 2.5% glutaraldehyde, diluted in 0.1 M phosphate buffer. The materials were immersed into the same fixative for overnight then were washed for 5 min in 0.1 M phsophate buffer (pH 7.4) containing sucrose (7.5% wt/vol). After washing blocks were immersed in osmium tetroxid (2% wt/vol) for 2 h at 4°C. After OsO_4 fixation materials were dehydrated in ascending series of alcohol, then embedded in Durcupan. Contrasting was performed in blocks with uranyl-acetate and on section with lead citrate. Electron micrographs were taken by TESLA BS 500 and JEOL 100 B electron microscopes.

RESULTS

Earthworm (Lumbricus terrestris)

Nerve cells have not been detected in the earthworm gut until now. Nerve bundles are situated between the blood sinuses and muscle fibres (Fig. 1). The nerve fibres are surrounded by glial processes. At places the glial cover is absent, and neuromuscular junctions are established at these sites. A very characteristic feature of the neuromuscular contacts in the earthworm is the wide junctional gap, where the distance between the sarcolemma and axolemma is about 100—200 nm (Fig. 2b). A well visible basal lamina can be found along both axo- and sarcolemma. The vesicle-population of axonprofiles is very varied. Axon profiles containing agranular vesciles only, are relatively rare (Fig. 2a insert), while the occurrence of agranular (average diameter: 40—50 nm) and granular (peptidergic) vesicles of different density (average diameter: 100—120 nm) together in the same axon terminal, is more frequent. (Figs 2a, b). Pre- and postsynaptic membrane thickenings are usually not observable, but, at places the sarcolemma forms finger-like processes, with mitochondria in them (Fig. 2b).

Snail (Helix pomatia)

A well-developed smooth musculature are to be found along the entire length of snail alimentary tract. A wide extracellular space rich in collagen fibrils and glial processes is situated among the muscle cells (Fig. 3). Thinner and thicker nerve bundles are also frequent in this extracellular space. Their structure is very varied depending on the plane of section. Beside varicose axonprofiles, both thin and thick nerve fibres are common in the loose neuropil (Fig. 3). The majority of varicosities contain highly electron dense, so-called peptidergic neurosecretory granules. The shape and size of these granules are very variable. A smaller proportion of

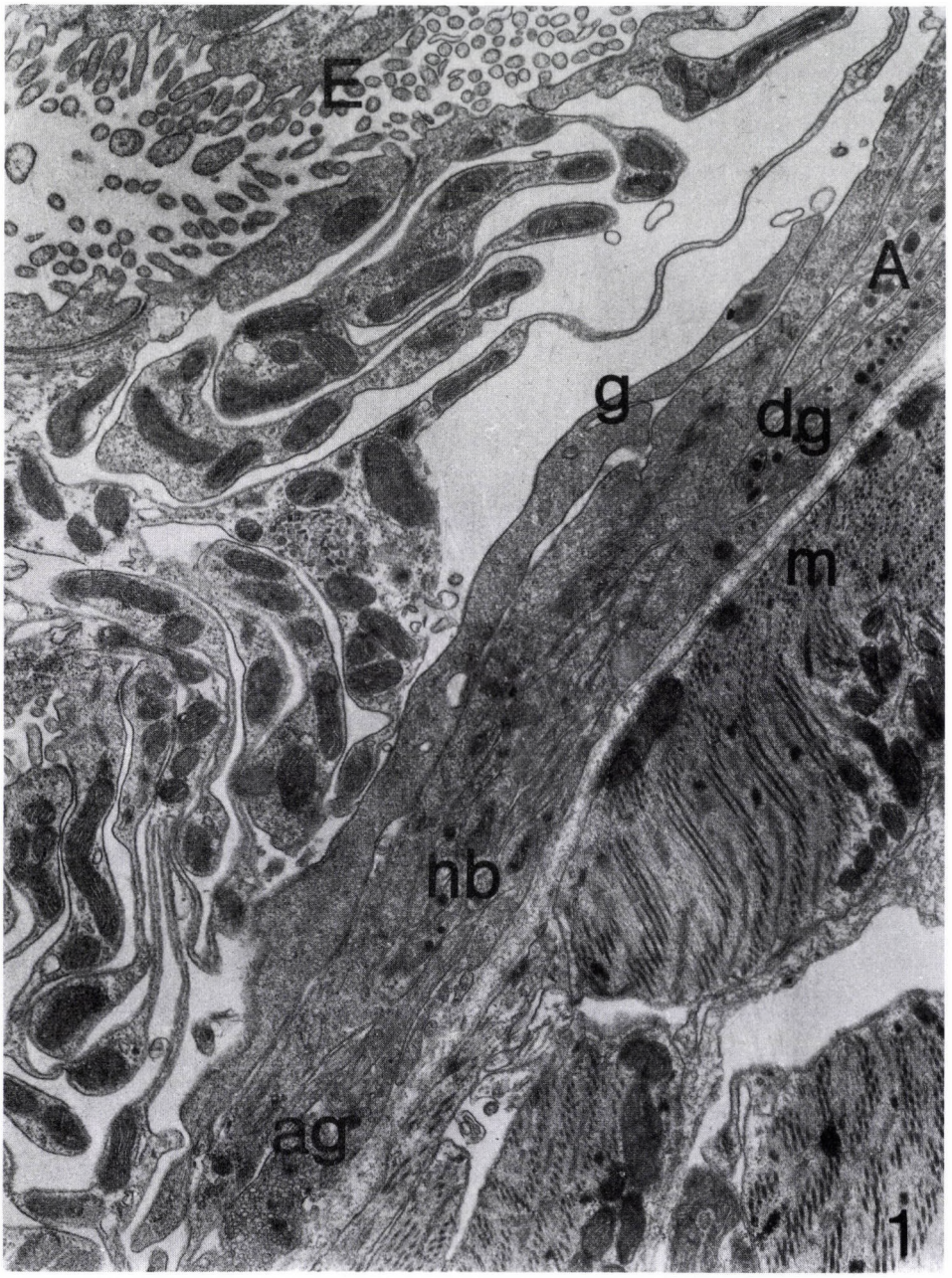

Fig. 1. Nerve bundle (nb) is situated between epithelial (E) and muscular (m) layer in the gut of Lumbricus terrestris. ag = agranular vesicles; dg (dense granular vesicles; g = glial process; A = axon x15 000

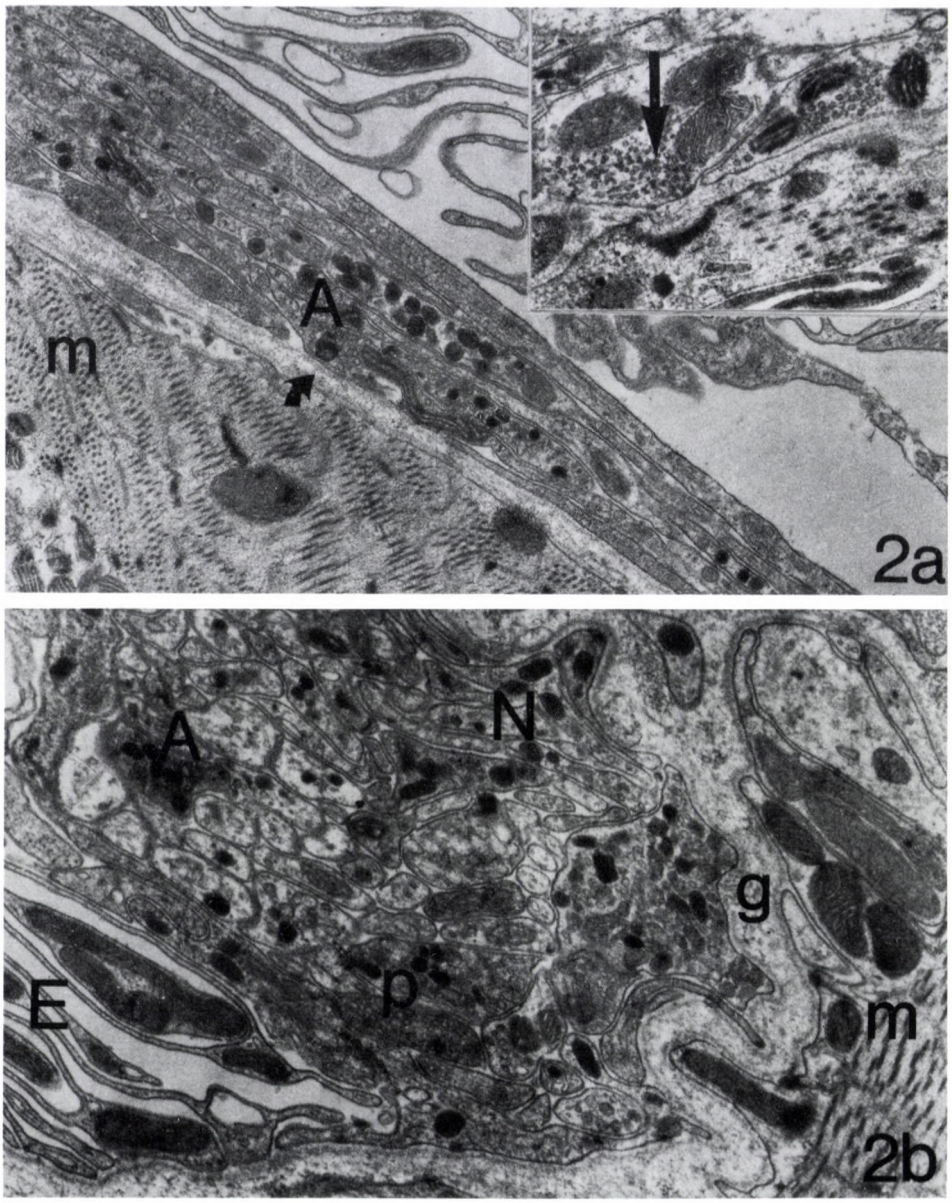

Fig. 2. A: Lumbricus terrestris. Axon (A) profile forming neuromuscular contact (arrow) with the muscle cell (m). Insert: Agranular clear vesicles (arrow) in the axon terminal. m = muscle x15 000 B: Loose neuropil (N) in the wall of the gut of earthworm. Note axons (A) with large opaque granules (p). E = epithel cell; m = muscle; g = gap between axolemma and sarcolemma x15 000

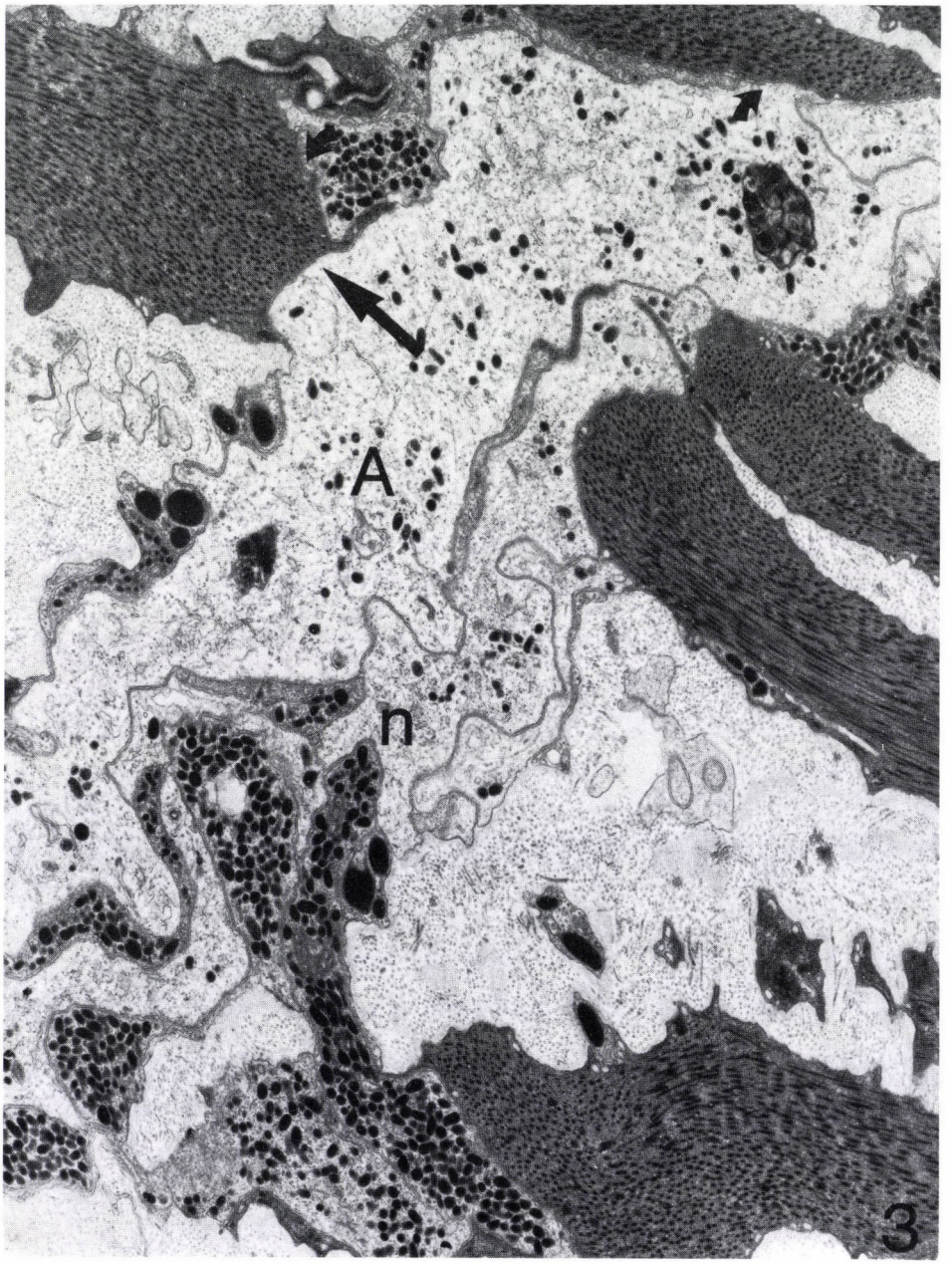

Fig. 3. Smooth muscle is characteristic for the gut of Helix pomatia. Axons (A) form close contacts (arrow) with the sarcolemma. n = neuropil x10 000

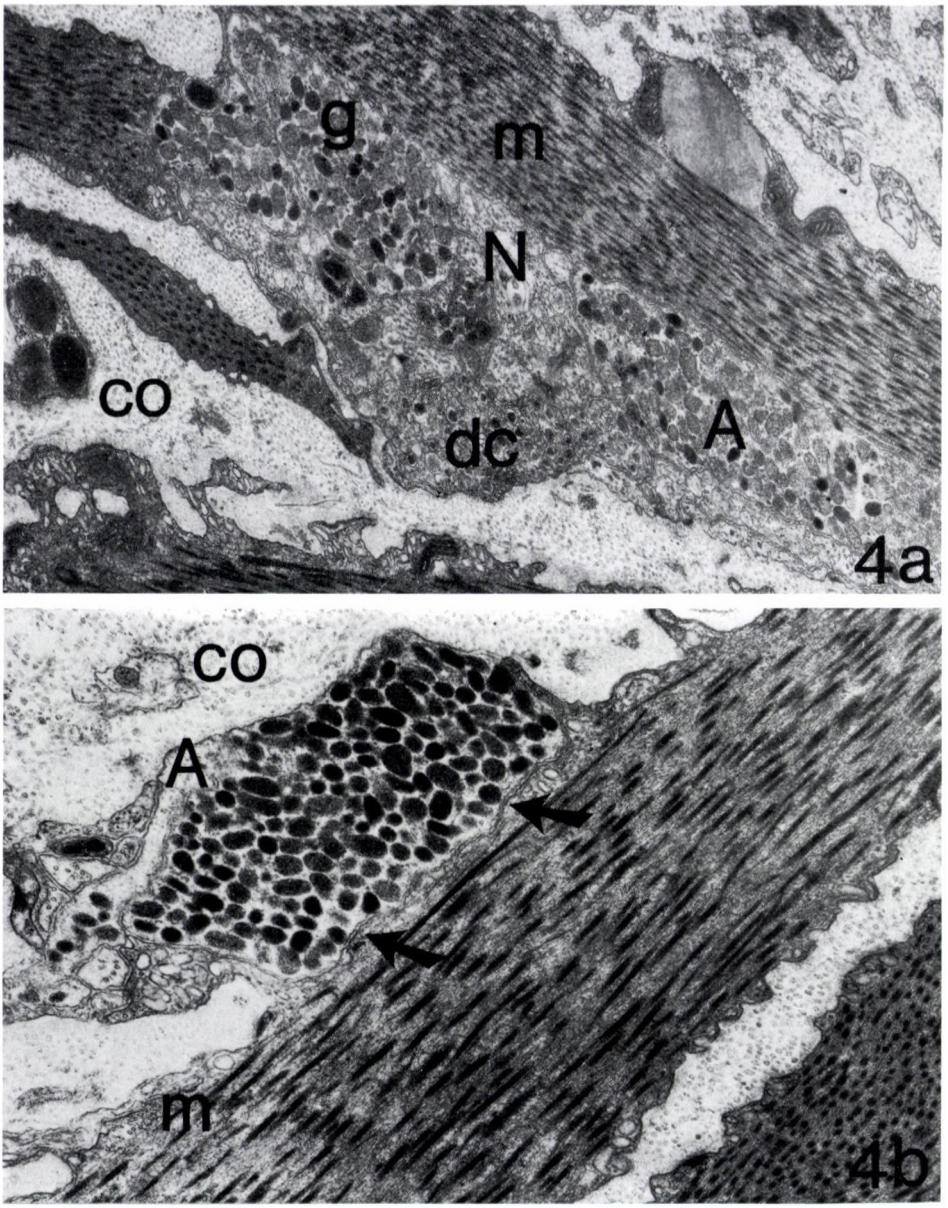

Fig. 4. A: Among muscle cells (m) of the gut of Helix pomatia axons (A) form a loose neuropil (N). Moderately dense, large granules (g) and dense-cored vesicles (dc) can be seen in axon terminals co = collagen. x15 000 B: Close contact (arrow) is the typical neuromuscular junction in the gut wall of Helix pomatia. The axolemma lies closely to the sarcolemma. A = axon; m = muscle; co = collagen. x12 000

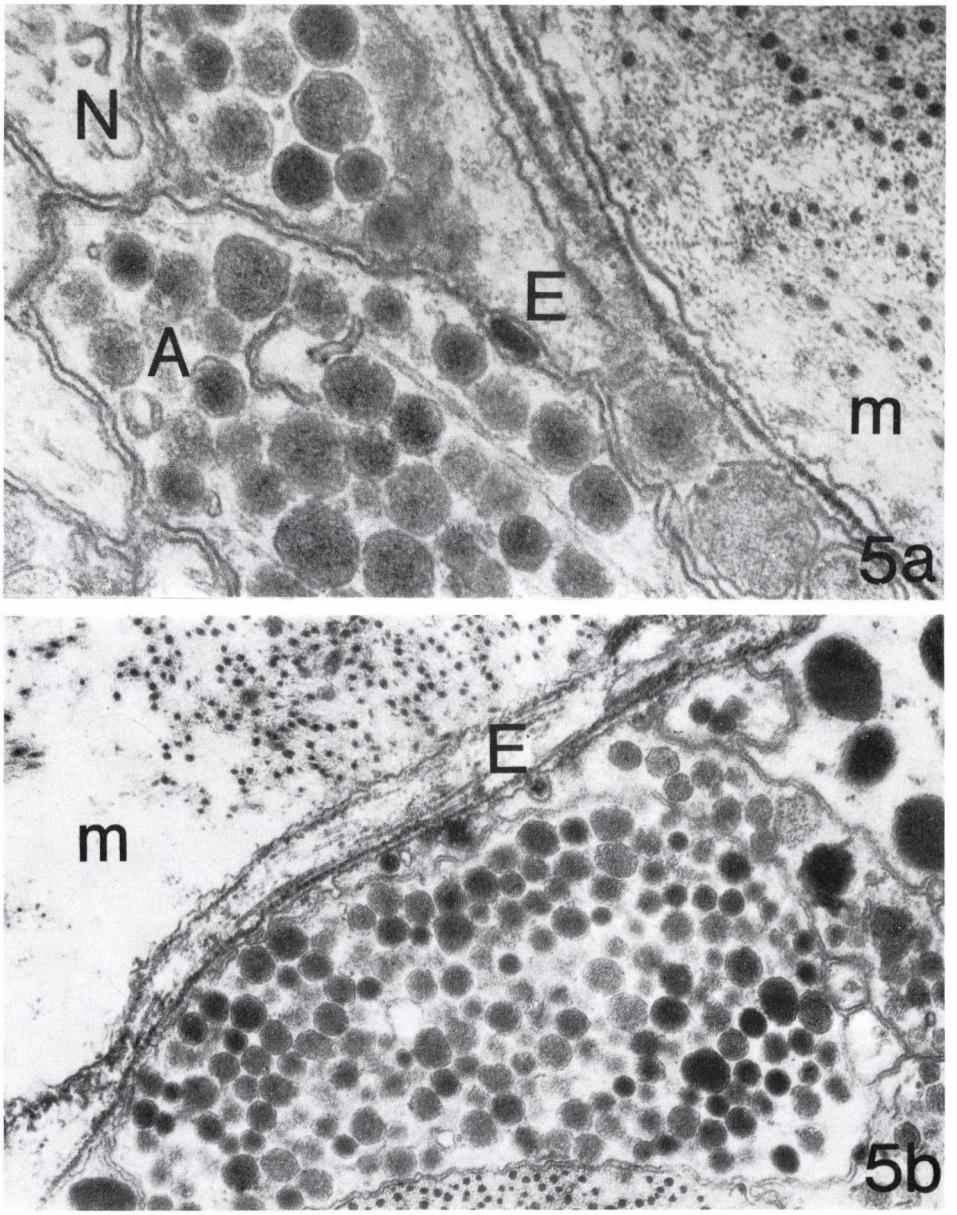

Fig. 5. A: Exocytosis (E) of transmitter containing granules in the neuropil (N). m = muscle; A = axon; Gut wall of Helix pomatia. x60 000
B: Exocytoses profiles (E) on the surface of an axon terminal near the muscle (m) in the gut wall of Helix pomatia. x40 000

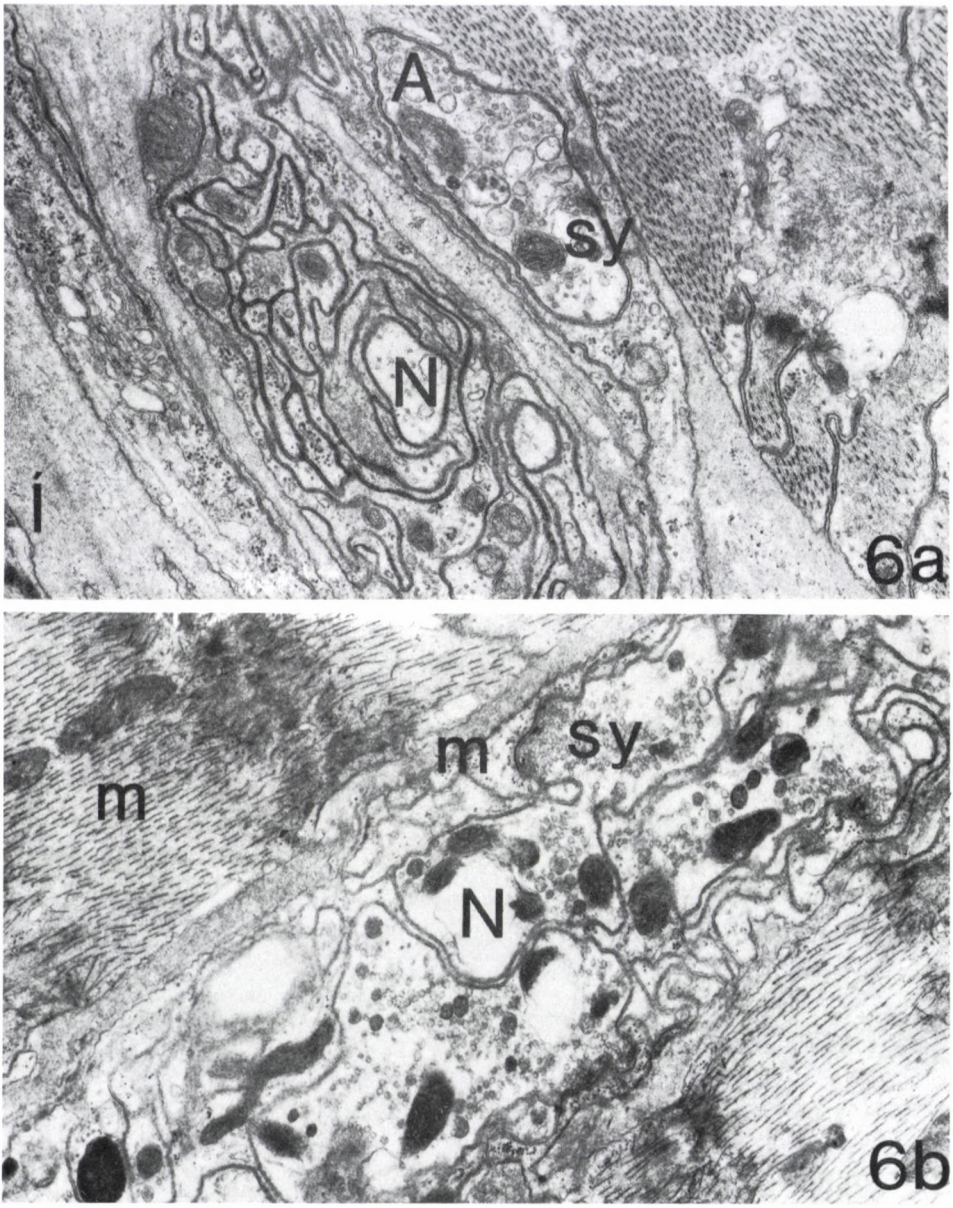

Fig. 6. A: Gut wall of the locust. Extensive neuropil (N) and axon terminal (A) can be seen on the picture. Axon forms synapse (sy) on the muscle. I = interstitium. x12 000. B: Synaptic specializations (sy) often can be seen in the neuropil (N) of the gut wall of locust. Muscle process (m) is invaginated into the neuropil. m = muscle. x15 000

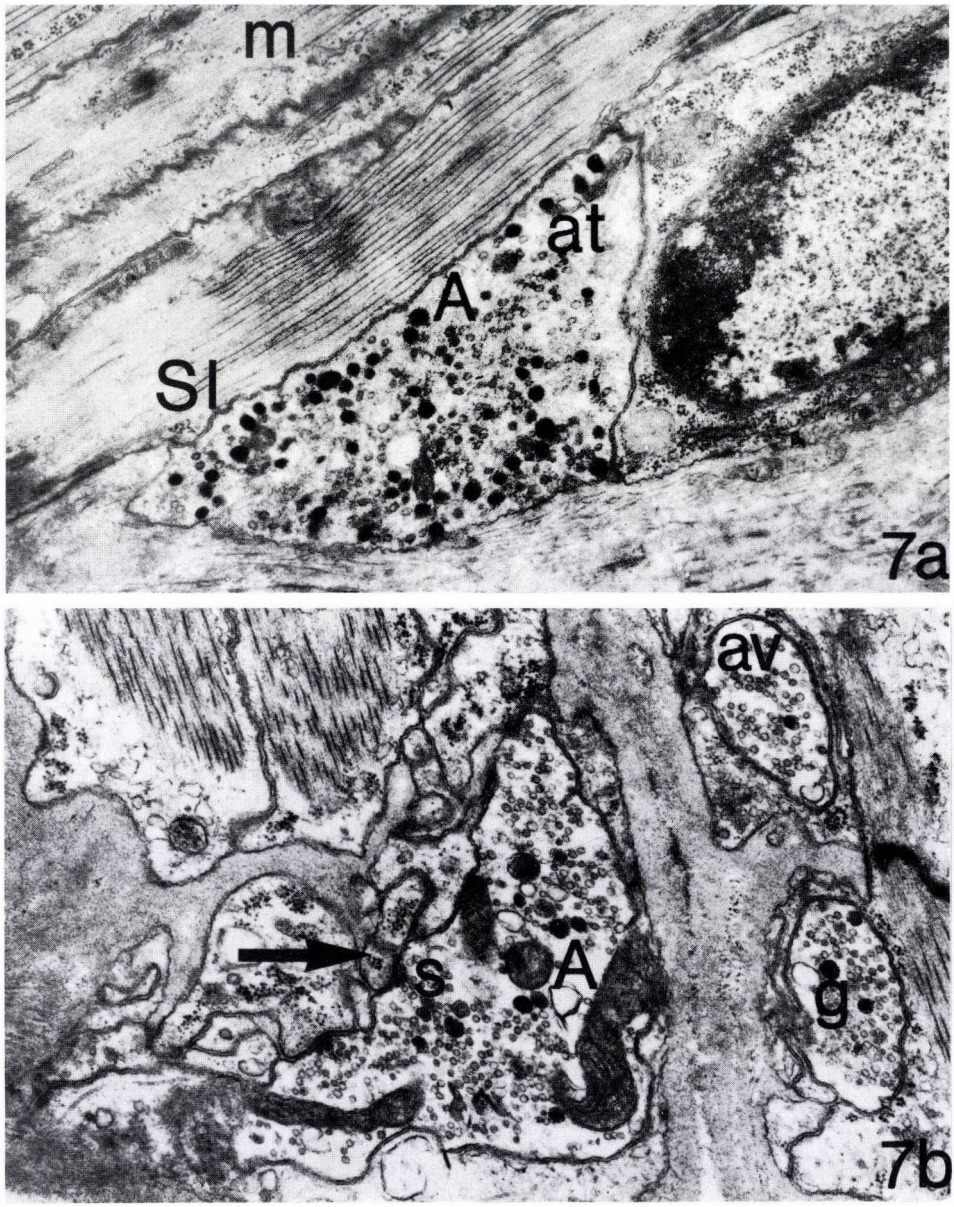

Fig. 7. A: "Close contact" in the hindgut of locust. Axolemma (A) runs parallelly with the sarcolemma (Sl). m = muscle; at = axon terminal. x22 000. B: Muscle process (arrow) form conventional chemical synapse (s) with an axon terminal (A). Both agranular vesicles (av) and large opaque granules (g) are present in the axomplasm. x30 000

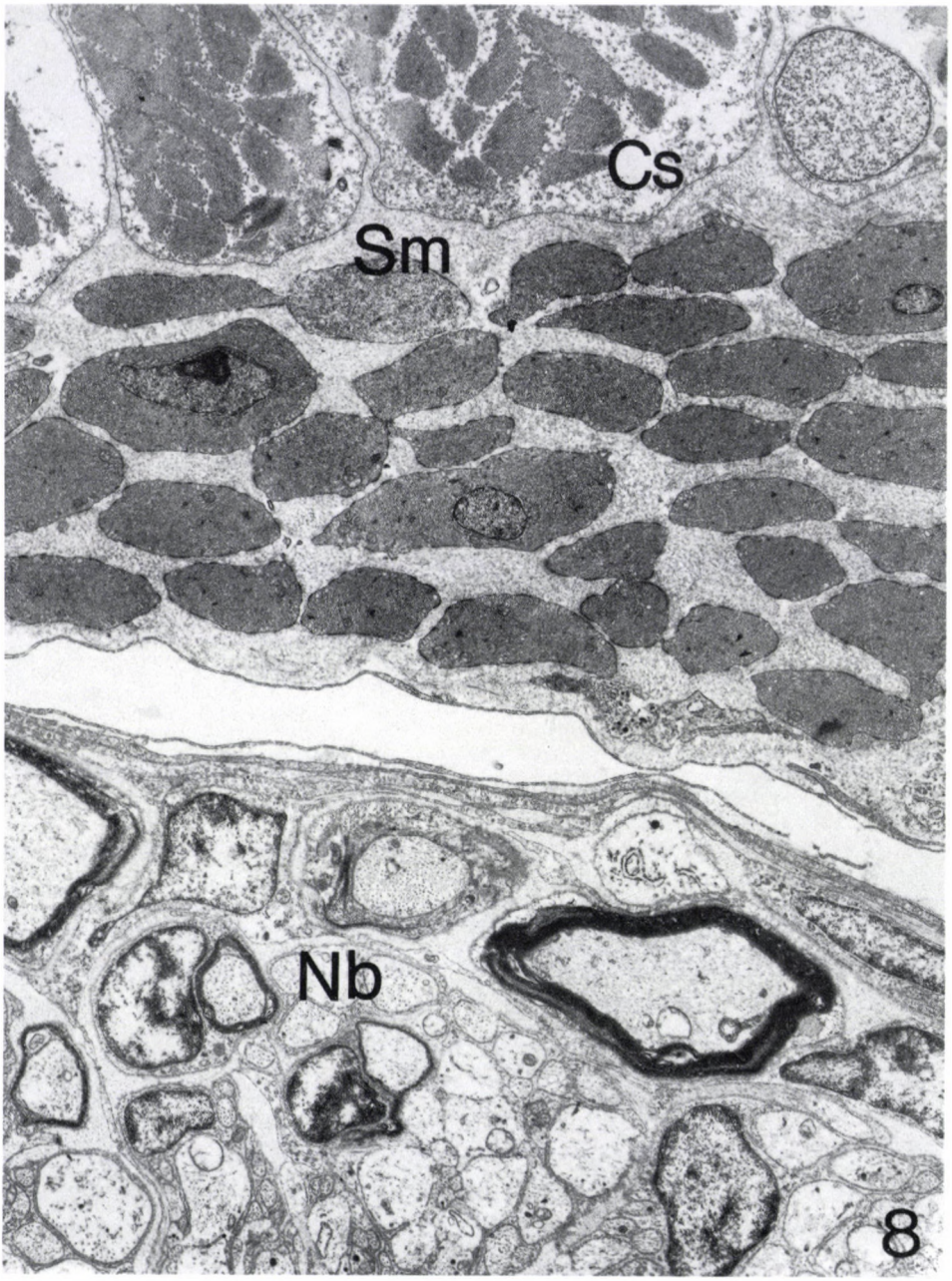

Fig. 8. Low power electron micrograph on the gut of tench. Cross striated (Cs) and smooth muscle layers (Sm) can be found side by side also in the small intestine. Besides the smooth muscle layer a large nerve bundle (Nb) can be seen. x7000

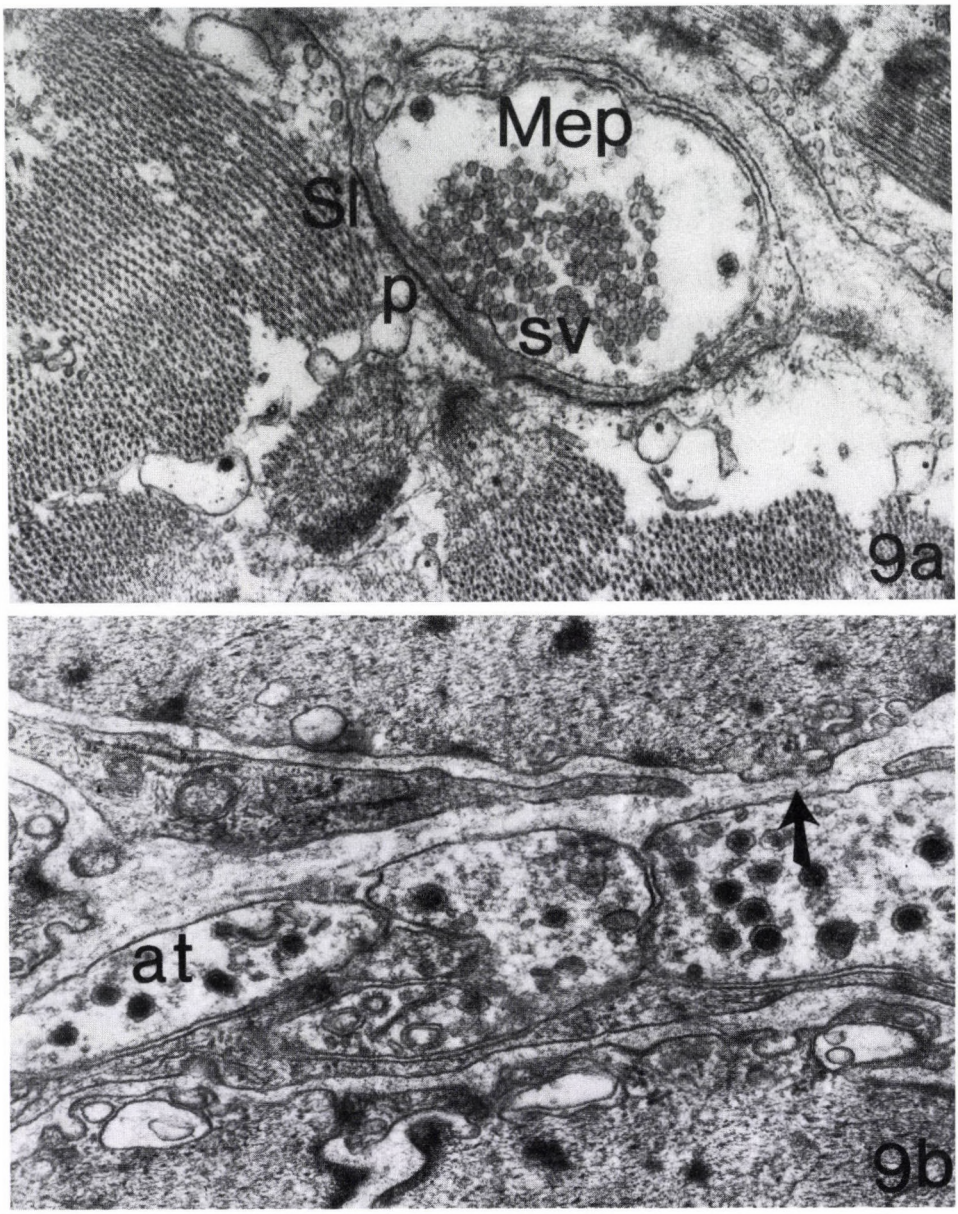

Fig. 9. A: Motor endplate (Mep) in the small intestine of the tench. The postsynaptic membrane thickenings (p) and accumulation of synaptic vesicles (sv) at the presynaptic axolemma are obvious. Note the absence of finger-like processes of the sarcolemma (Sl). x35000
B: Varicose axon terminals (at) in the small intestine fo the chicken. At some places (arrow) the axolemma is free of Schwann cell cover. Here the distance between axolemma and sarcolemma is reduced about 100 nm. x45 000

nerves contain dense-cored vesicles (Fig. 4a). In contrary with the earthworm, the characteristic neuromuscular junction of the snail gut wall is the close contact. Here, the axolemma tightly fits to the sarcolemma, and the junctional gap is only 10—15 nm wide (Fig. 4b). One muscle cell often receives more axonterminals (Fig. 4a). Pre- and postsynaptic membrane specializations or clusters of synaptic vesicles along the axolemma have not been detected yet. However, non-synaptic release sites were visualized with the help of TARI-method /6/, both in the neuropil and at close contacts (Fig. 5a, b).

Locust (Locusta migratoria)

The muscular wall of the alimentary canal in the locust is built up entirely by cross-striated muscles. Nerve bundles are running in the interstitial space, among the muscle fibres (Figs 6a, b). The sarcolemma is bordered by a wide basal lamina. The amount of interstitial collagen is less than in the snail gut. Axonprofiles, glial- and muscular processes establish a complicated network in the nerve bundles. Two types of neuromuscular junctions occur in the locust gut: a) the so-called close contacts; b) synaptic contacts. The fine structure of close contacts is identical with those of the snail (Fig. 7a). Both agranular vesicles and strongly dense granules (average diameter: 150—200 nm) occur in the axoplasm. The entire surface of the axonterminal is free of glial cover.

The synaptic neuromuscular junction is very common, especially in the hindgut of the locust. Neuropil areas consisting of several axons situated between two muscle fibres are very generally occurring as a characteristic morphological arrangement (Figs 6a, b, 7b). The synaptic contact is attained on the sarcolemma of muscle fibre processes invaginated into the neuropil (Fig. 6a). A great number of agranular vesicles are clustered along the short dense plaques of presynaptic membranes. Besides agranular vesicles some larger granules of medium density are also visible (Fig. 6b). One axonterminal can innervate more muscle processes (Fig. 7b) (however electron micrographs cannot show, whether these are processes of the same or different muscle fibres).

Tench (Tinca tinca)

The structure of tench alimentary canal is peculiar. Cross-striated muscle fibres are present not only in the oesophagus, but in the wall of

stomach and midgut too (Fig. 8). The tunica muscularis contains a smooth muscular layer of 5—10 cell-thickness, and a well-developed cross-striated musculature as well. A very prominent plexus myentericus is situated between the muscular layers. Both myelinated and unmyelinated nerves occur in the plexus. Motor endplates are common on the surface of striated muscle fibres in the oesophagus, stomach and midgut (Fig. 9a). These are morphologically different from the mammalian skeletal motor endplates, namely the axon terminal lies in a depression of the muscle fibre, and the postsynaptic infoldings of the sarcolemma are absent. The axoplasm contains a high number of round, agranular vesicles. A considerable proportion of the granules gather along the presynaptic membrane. Few dense-cored vesicles are also present in the axoplasm. The synaptic cleft is 30 nm wide. The basal lamina is well visible in the synaptic cleft.The dense membrane thickening of postsynaptic sarcolemma is prominent. The number of motor endplates seems to be higher in the oesophagus than in the stomach and midgut, but all these motor endplates are AchE-positive. Among the smooth muscle cells nerve fibres are detectable (Fig. 8).

Domestic fowl (Gallus domesticus)

Only smooth musculature can be found in the wall of the entire alimentary tract. Myelinated and unmyelinated nerves are frequent in the myenteric plexus. Nerve fibres partly or totally loosing their Schwann-cover, penetrate into the smooth muscular layer and approach the surface of muscle cells as far as 100 nm (Fig. 9b). Mainly large dense-cored vesicles (average diameter 100—120 nm) are to be found in the axon terminals. Few agranular vesicles also occur in the axoplasm. A great number of endocytotic vesicles are visible on the surface of smooth muscle cells.

DISCUSSION

The enteric neuromuscular junction is well-known on the basis of examinations carried out on mammals. In a recently published review Burnstock /10/ emphasizes a number of important differences between the autonomic neuroeffector junction and skeletal motor endplates. Morphologically the most obvious difference is the lack of pre- and postsynaptic membrane thickenings, moreover the variable width of synaptic clefts in the autonomic neuromuscular junctions /7, 20/. In the case of the so-called close contacts

the gap between the membranes is 15—20 nm /20/. Since the diffusion-speed of acetylcholine is over 1 μm/msec /12/, the distance between the sarcolemma and axolemma in the close contact has negligible importance. According to the morphological characteristics of vesicle populations in the axon-terminals (varicosities) 8 types of terminals was distinguished by Burnstock /8/. Physiological and pharmacological studies revealed more than 20 transmitter candidates taking part in the innervation of enteric functions /8, 10/. The question arises: is there any connection between the morphological characteristics of vesicle-population and the chemical nature of transmitter candidates? At the moment it is impossible to give unanimous answer to this question, nevertheless it is important and interesting, because the results of morphology should be in accordance with the data of physiology, pharmacology and neurochemistry. Small agranular vesicles usually represent cholinergic innervation /20/, but this fact has not been proved widely in the field of autonomic nervous system /28/. Our own biochemical results confirm the presence and activity of acetylcholinesterase in the gut of some teleosts, especially in the gut segments with striated musculature (Halasy et al., in preparation). The morphological results, namely the presence of terminals with small agranular vesicles in the carp and tench oesophagus and tench intestine /22, 23/, called the attention to necessity of biochemical measurements. Similarly, axon terminals with dense-cored vesicles were found in the gut of snail, locust and fish /21, 22, 23/. The presence of dense-cored vesicles suggested the possible transmitter role of monoamines in the above species, and the biochemical measurements carried out lately on the snail, locust and carp gut proved this by the detection of considerable amount of adrenaline, noradrenaline and dopamine /26, 31/.

Carrying out comparative morphological studies on the enteric nervous system of species representing different steps of phylogeny we expect to reveal all those common features and characteristic differences which are in connection with the different level of phylogenetic development and different way of nourishment. We have chosen earthworm as a test animal, because the earthworm takes up and transports a considerable amount of organic and inorganic material through the alimentary tract. Corresponding to this the alimentary tract has a well-developed muscular layer /14/. A characteristic feature of the earthworm alimentary canal is the lack of intrinsic plexus in the well-developed muscular layer /14/, however, a so-called common plexus is situated between the blood sinuses and inner muscular layer, which innervates both layers. It is remarkable, that the

junctional cleft of enteric neuromuscular junctions in the earthworm is wide, 100—200 nm, and true close contacts — where this width is 15—20 nm only —, have not been detectable until now. Bennet and Merillees /3/ supposed that the transmitter release into a junctional cleft wider than 100 nm is not effective. Recent observations /7/ do not support their assumption. On the basis of the latter it is very probable, that transmission can happen through 100—200 nm wide junctional cleft. As regards the transmitter content in axon profiles, the presence of clear vesicles refers to cholinergic innervation of the gut /16/. The occurrence of granules of variable density besides agranular vesicles supports the coexistence of transmitters in the axon terminals of earthworm gut. This is in good agreement with the reports on vertebrates published by Burnstock /8/.

The snail gut differs from the earthworm gut in several respect. First of all its musculature is different. Instead of obliquely striated musculature a smooth muscular wall is present in the snail gut. This smooth musculature is fairly similar to that of vertebrates. So, perhaps not accidently, the neuromuscular junction itself resembles to the close contacts of vertebrates too. This is the exclusive form of junctions. The innervation is remarkably rich, very often more than one axonterminal are attached to the surface of one muscle cell. The majority of the axon profiles contain neurosecretory granules resembling peptidergic ones, and a smaller proportion contains dense-cored vesicles. The latter confirms the transmitter role of monoamines in the snail gut together with fluorescence microscopic studies /17/ and biochemical measurements /26/. After TARI incubation /6/ morphological evidence for non-synaptic transmitter release was obtained. Non-synaptic transmitter release was proved recently both in the central nervous system /6/ and peripheral neuroendocrine organs /5/. The innervation of insect gut shows a special picture. The gut-musculature itself is entirely cross-striated. In spite of this, close contacts were also detectable in the locust gut too. Besides close contacts a number of synaptic neuromuscular junctions was detectable too, with the fine structural characteristics of chemical synapses. This type of synapse however differs from motor endplates of skeletal muscles. For example the synaptic cleft is 15 nm only. The postsynaptic infoldings of the sarcolemma are absent. Both agranular and granular vesicles are present in the axon profiles. Immunocytochemical evidence is available on the transmitter role of a pentapeptide proctolin in the nerve terminals of the insect proctodeum /15/. Very probably proctolin is stored in peptidergic granules /2/. In spite of the presence

of true neuromuscular synapses, the gut musculature cannot produce quick contractions. According to Brown and Nagai /4/ the basic slow myogenic rythm of insect gut is modified only by the synaptic and non-synaptic nerve elements.

The gut wall of tench is also peculiar. The presence of striated muscle fibres in the midgut of other vertebrate species has not been known. The function of this striated muscular layer during peristalsis has not been clarified yet properly.The striated musculature of stomach and midgut is possibly built up by slow type fibres and their contraction, similarly to the smooth muscles in slow and tonic /29/. The morphology of their motor endplates rather resembles to that of slow type striated fibres /30/, than to the fast skeletal motor endplates. It is remarkable, that the frequency of motor endplates is higher in the oesophagus than in the midgut. This fact supports the priority of smooth muscular layer in the gut motility.

The peculiarity of chicken gut is the lack of striated musculature even in the oesophagus. Close contacts of nerves and muscle cells are very common and their fine structural organization is similar to that of mammals.

On the basis of comparative morphological studies of enteric neuromuscular junctions it can be concluded that their structural organization is very varied during the phylogenesis. Close contact can be considered as the most common type, occurring generally both in invertebrates and vertebrates. The physiological role of peculiar neuromuscular junctions has not been clarified sufficiently yet, but probably their role is secondary in the motility of the gut wall.

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AUTORADIOGRAPHIC LOCALIZATION OF HIGH AFFINITY UPTAKE SITES FOR ^{3}H-D-ASPARTATE IN THE RAT OLFACTORY BULB

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In order to reveal excitatory amino acid-ergic neuronal connections in the rat olfactory bulb, uptake sites for the tritiated D-aspartic acid were analyzed by high resolution autoradiography. Light microscopy revealed both cellular and terminal-like uptake. Based on electron microscopy, overwhelming majority of the cellular uptake was assigned to glial cells. A fairly high number of labelled terminals appeared in the surroundings of the mitral cell somata, within the deepest portion of the external plexiform layer, in the internal plexiform layer and in the outer half of the granule cell layer. Labelled terminals synapsed onto likely granule cell cendrites or spines, at asymmetric membrane thickenings. These results suggest that, although the output neurons may not utilize glutamic or aspartic acid as their transmitters, these amino acids may, however, contribute to the bulbar neurotransmission, as mediator substances of a subgroup of centrifugal fibers to the olfactory bulb.

Keywords: D-aspartate — autoradiography — olfactory bulb

INTRODUCTION

Mitral (and tufted) cells of the vertebrate olfactory bulb (OB) activate, beside the olfactory cortical pyramidal cells, local (bulbar) interneurons, too. In turn, these latter cells, at least in the external plexiform layer (EPL), inhibit the output neurons.While a fairly reliable set of data supports the view that gamma-aminobutyric acid transmits in-

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hibition at the local (dendro-dendritic) synapses /for reviews, see Refs. 11, 15, 16/, there are still doubts regarding the identification of the transmitter material responsible for the excitation at sites, being post-synaptic to the output neurons.

The OB is rich in both aspartic (Asp) and glutamic (Glu) acids /see Refs. 19/: they can be released by electrical stimulation or high potassium concentration in the incubation medium (see, e.g. Refs. 3, 5, 17, 22/. Since revealing of specific structural compartments for these amino acids were provided till now only in one experiment /by immunohistochemistry, 20/, we made an attempt to identify the high affinity uptake sites, by using tritiated D-Asp at light- and electron microscopic level.

MATERIALS AND METHODS

Seven male Wistar (CFY) rats, weighing 260—300 g, were anaesthesized by Halothane (Narcotan, Spofa, Prague) and injected into the right OB by 7.5—15 μCi tritiated D-aspartate (D-/2.3-^{3}H/aspartic acid; specific activity 21 Ci/mmol, Amersham), dissolved in 1.5—3.0 ul isotonic saline, during 15 min. After an additional rest for 30 min, rats were perfused through the left venricle by 0.15 M Krebs-Ringer solution (for 2 min) and Karnovsky fixative (for 20 min), followed by opening of the skull, removing and dissecting the OBs.

Pieces of the OB were prepared according to routine embedding technique and processed for light-and electron microscopic autoradiography, as described earlier /10/. Autoradiograms were developed after 30, 68 or 91 days for light microscopy (LM) (in Kodak D 19 b solution) and 3 to 30 days for electron microscopy (EM) (by Amidol developer).

RESULTS

Silver grains, representing radioactivity in the tissue, were found all over the layers of the OB; no difference was recognized along the rostrocaudal, lateromedial or dorsoventral axes.The dorsal portion of the bulbs contained, however, more radioactivity, which might well be due to the spread of the isotope along the track of the fine injection tube.

Labelled cells

Cells showing accumulation of the label were seen predominantly in the superficial layers, i.e., in the olfactory nerve, glomerular and superficial part of the external plexiform layers (Fig. 1), while the rest of the EPL and the deep OB featured only a few, faintly labelled cells (Fig.3). The labelled cells in the nerve layer could easily be identified already by

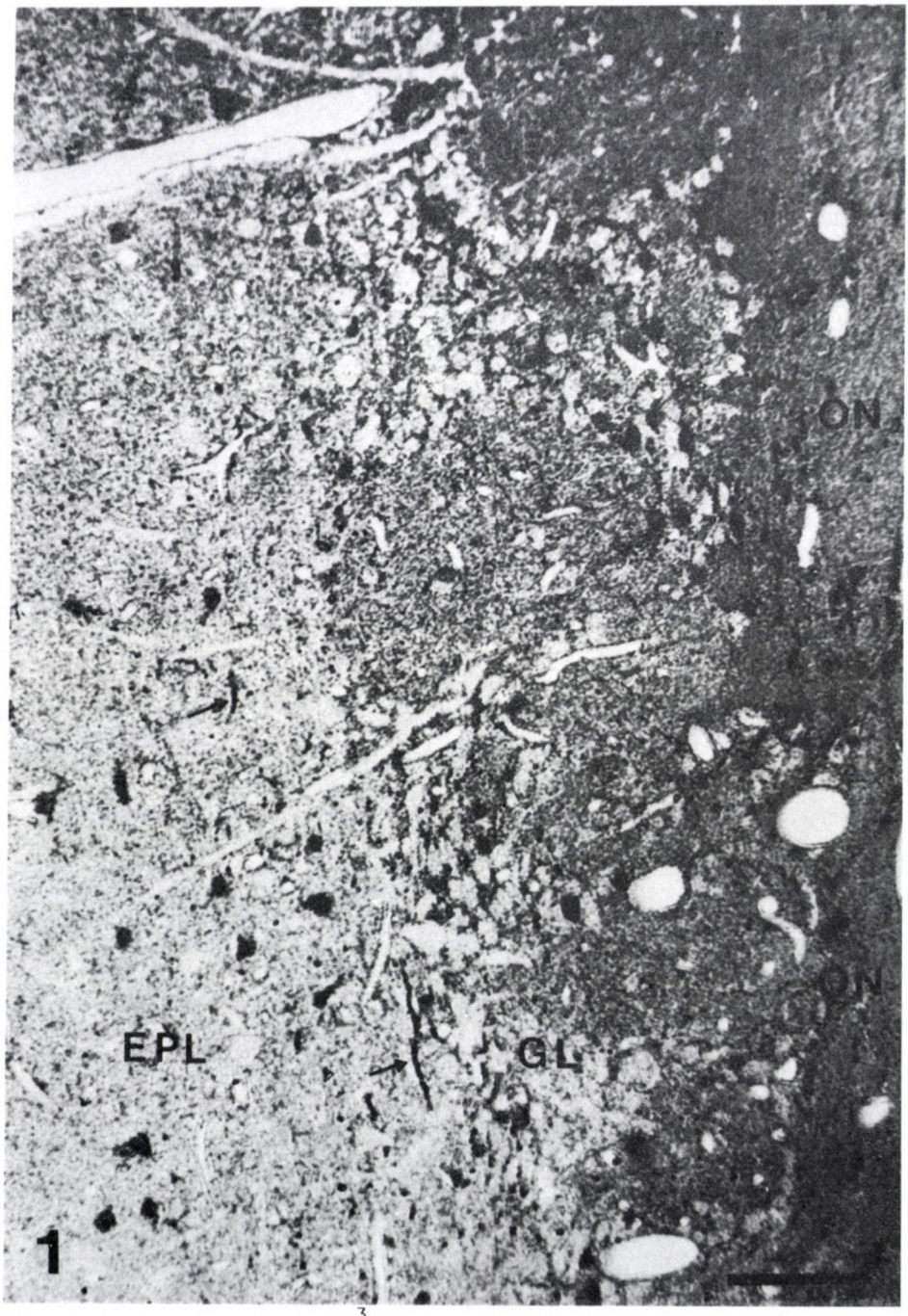
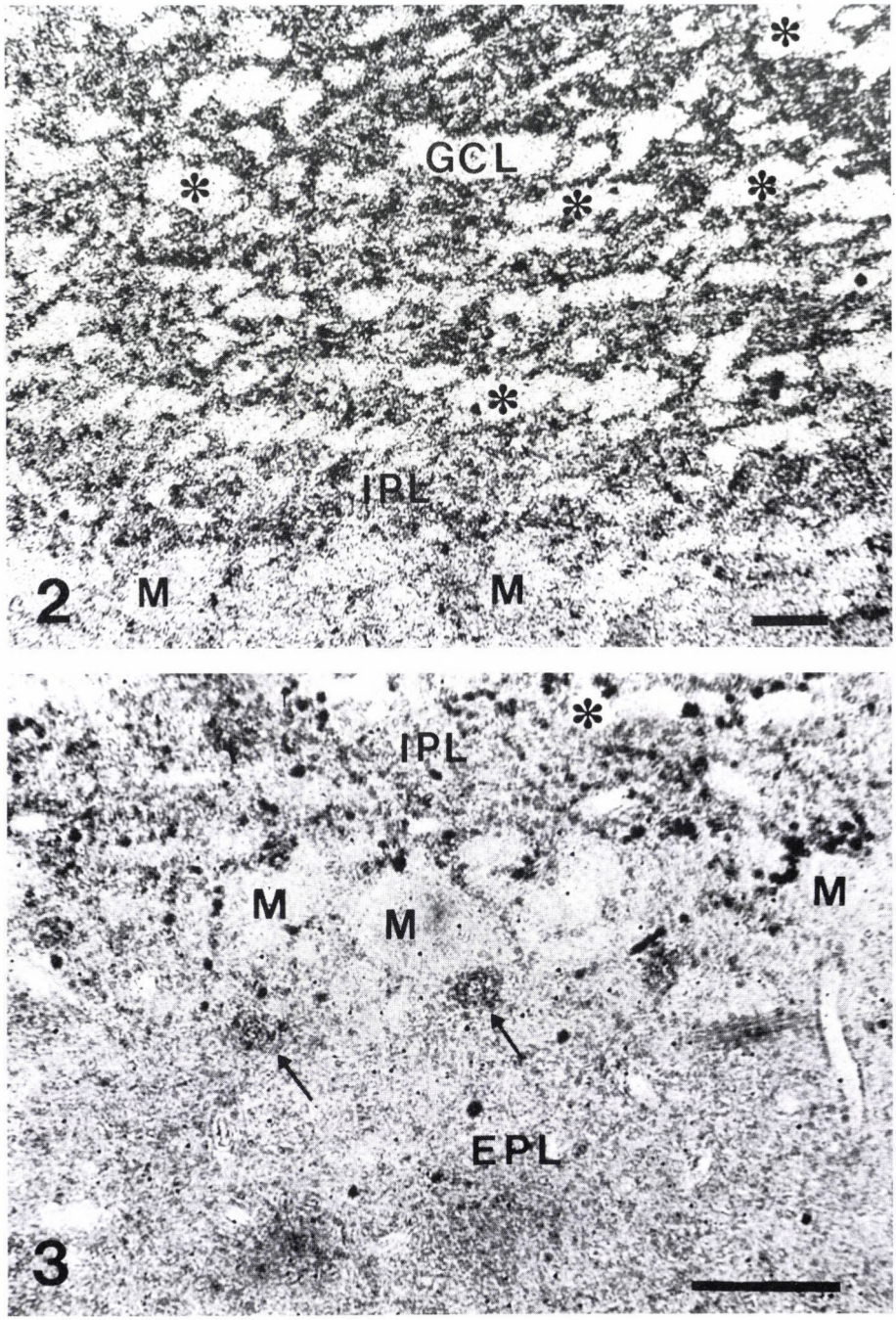
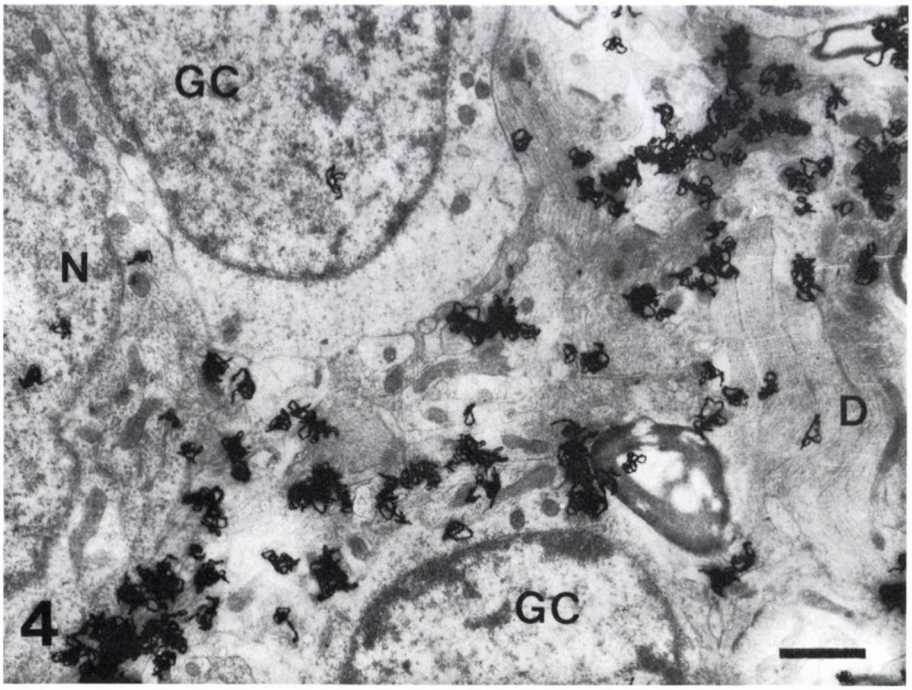


Fig. 1. Low power picture of ^{3}H-D-Asp uptake in the superficial layers. Numerous labelled (black) cells are seen in the superficial part of the external plexiform layer (EPL), glomerular layer (GL) and olfactory nerve layer (ON). Fiber-like accumulations are also apparent at places (arrows). Bar, 10 um



Figs. 2-3. Labelled cells in the granule cell layer (GCL), internal plexiform (IPL) and deep external plexiform layer (EPL). Mitral cells (M) and groups of granule cells (asterisks) do not show activity above background level. Fig. 3: Arrows point to two weakly labelled cells. Bars, 20 um



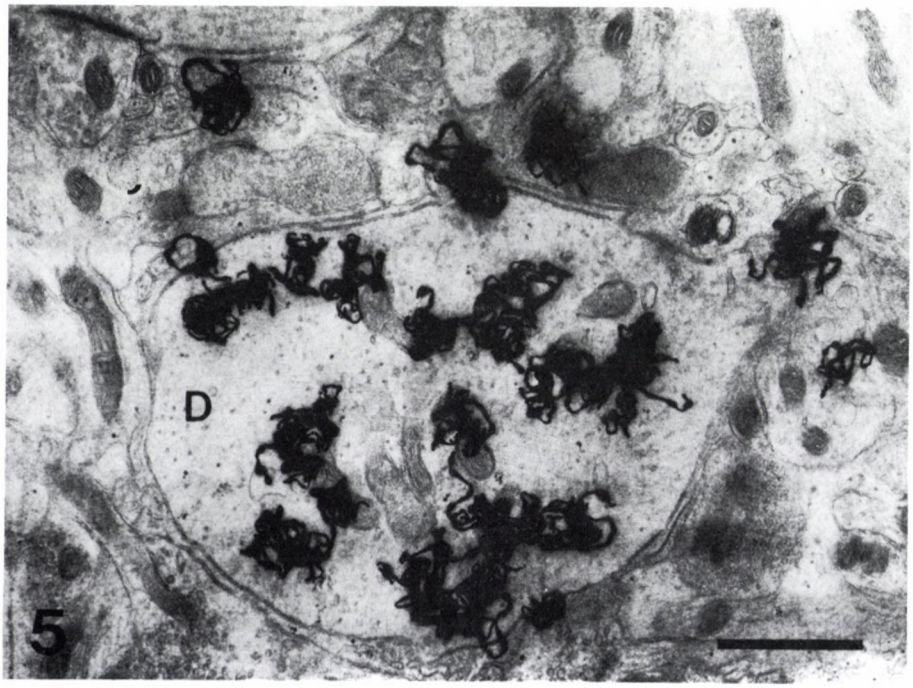
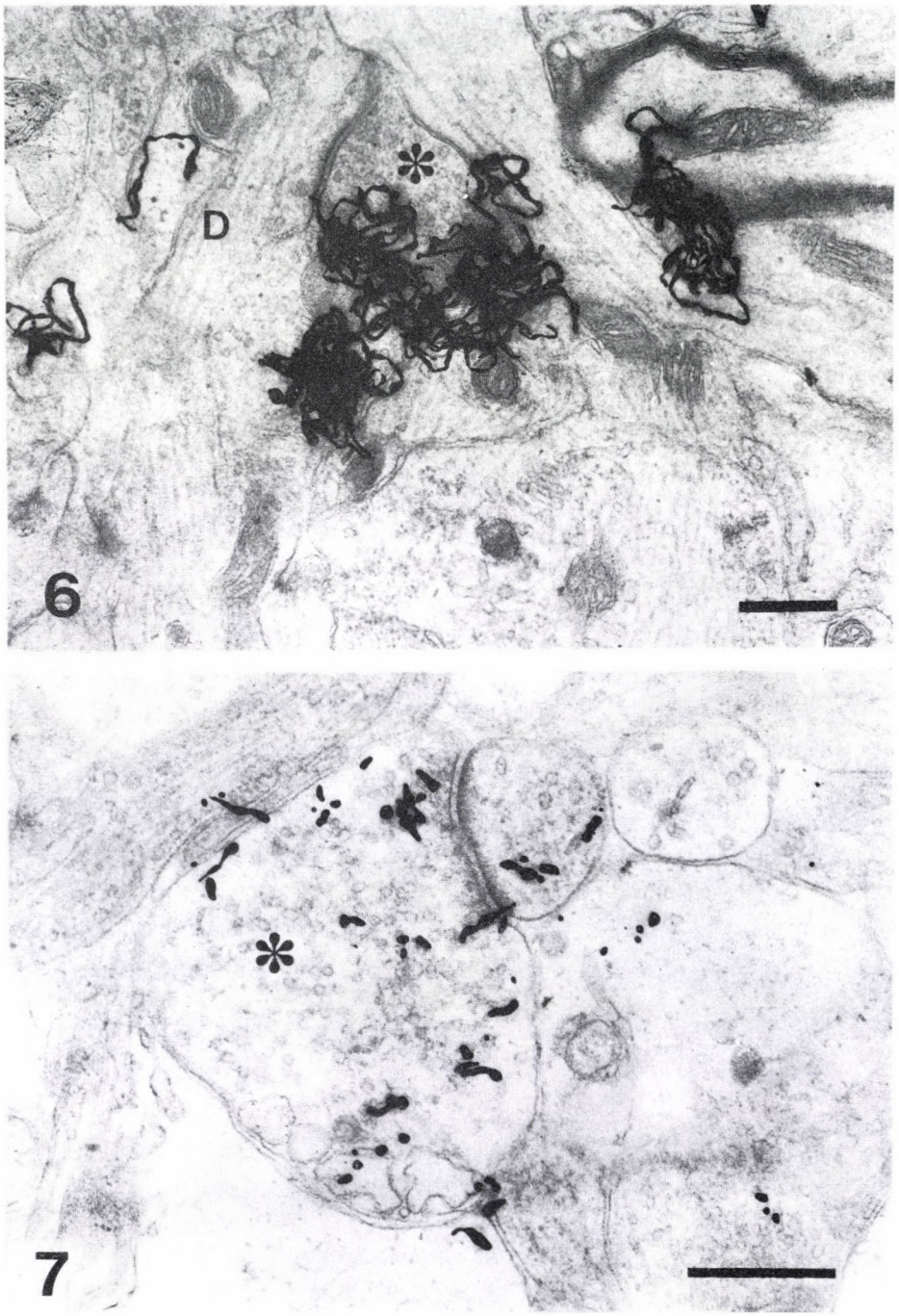


Fig. 4. The labelled neuropil region in the granule cell layer is bordered by unlabelled granule cells (GC). A larger neuron (N) and tortuous granule cell dendrites (D) are also only weakly labelled. Bar, 2 um

Fig. 5. A large dendrite (probable of a tufted cell origin) (D) features heavy accumulation of silver grains. Bar, 1 um



Figs 6-7. Labelled synapsing terminals from the internal plexiform and superficial granule cell layer (asterisks). Postsynaptic sites were identified tentatively as a granule cell dendrite (Fig, 6, D) or dendritic spine (Fig. 7). Bars, 0.5 um

LM as interfascicular glial (Schwann) cells, while recognition of the other radioactive cells (situated mostly around the glomeruli or in the EPL) could be achieved only by EM. Vast amount of these labelled cells were identified as astrocytes or oligodendrocytes, although very few labelled neuronal cell somata, situated in the EPL, were also seen. According to their fine structural characteristics, they could be identified as tufted cells and/or short axon cells. No labelled neuronal cells appeared deep to (or amongst) the mitral cells, while weakly radioactive glial cells were present in the deep aspect of the OBs, too.

Labelled fibers and terminals

Fiber- and terminal-like accumulation of silver grains was apprent in all layers of the OB, with highest density in the internal plexiform and superficial granule cell layers (Figs 2, 3). A high background activity was also apparent under LM in these layers. By EM, it turned out that neuronal somata (and the identified granule cell dendrites) were avoid of grain accumulation (Fig. 4), while the rest of the neuropil, containing vesiculated terminals, myelinated and other fibers and glial processes were heavily labelled in these deep layers (Figs 4, 6, 7). Very rarely, large dendritic profiles in the EPL (presumed tufted cell dendrites) accumulated also silver grains above the background level (Fig. 5).

All labelled synaptic boutons were rich in small vesicles and synapsed at asymmetric (Gray type 1) thickenings (Figs 6, 7). Frequently, the postsynaptic partner could be identified as a probable granule cell dendrite or dentritic spine.

DISCUSSION

Earlier experiments suggested Glu and/or Asp as being the excitatory mediator substance(s) of the mitral (and similarly functioning tufted) cells in the olfactory bulb, as referred to in the Introduction. Ionophoretic application of these amino acids onto the bulb inhibited mitral cell activity; a similar inhibition could be elicited through activation of the granule cells by stimulation of the lateral olfactory tract /13, 18/. These experiments suggested that Asp and/or Glu might be involved in excitation of granule cells by mitral cells, through the dendrodendritic synapses. Autoradiography of the tritiated L-Asp or L-Glu, however, did not result in a

selective labelling of mitral cells or their processes (Halász, unpublished), and kainic acid damaged rather mitral than granule cell dendrites /9, 14/, excluding the existence of at least one of the amino acid receptor subtypes on granule cell dendritic endings.

Later, certain pharmacological evidences supported the view that neither Asp, nor Glu would be the acting transmitter at the presynaptic mitral terminals, instead, a dipeptide, N-acetylaspartylglutamate (Ac-Asp-Glu) was suggested to be the likely neurotransmitter /2, 4, 7, 12/. Aspartate was, however, revealed in mitral cells and their dendrites in the rat OB by an immunohistochemical experiment (Saito et al., 20). As the cross-reactivity of this serum was not determined against Ac-Asp-Glu, it cannot be excluded that their antibody recognized a determinant for Asp on the same dipeptide, Ac-Asp-Glu.

Autoradiographic analysis of the uptake of the D-isomer of Asp offered a new possibility to visualize the amino acid-ergic structures, since it is not metabolized by the tissue, although taken up by the same transport system as the L-Glu or L-Asp /1, 6/. By means of high resolution autoradiography, we have shown in the present paper that, although the mitral cells may not utilize Asp or Glu as their transmitter substance (as supposed earlier), other structures, terminating mostly around and deep to the mitral cells, have a high affinity uptake system for the label. No connection of these terminals with labelled neurons in the bulb were, however, recognized. This suggests that a subclass of centrifugal fibers may use one of these excitatory amino acids at their bulbar synapses. According to the retrograde transport studies with ^{3}H-D-Asp by Watanabe and Kawana /21/, parent cell bodies for these terminals may be localized bilaterally in the anterior olfactory nucleus, in addition to the ipsilateral nucleus of the lateral olfactory tract and piriform cortex.

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APPLICATION OF THE GLYCINE LABELLING METHOD TO THE CEREBELLUM, HIPPOCAMPUS AND SPINAL CORD

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^{3}H-glycine was applied to the cat cerebellar cortex under resting conditions and during inferior olive stimulation which activated the climbing fiber system on a restricted area. Electric recording was made. The autoradiograms showed, that under resting condition labelled glycine was incorporated mainly in granule, Golgi and basket cells and only a few Purkinje and stellate cells were active. Also cerebellar glomeruli remained without labelling. On climbing fiber stimulation Purkinje cells became activated singly and grouped, also Golgi and stellate cells increased in number. Granule cells were totally inhibited.

^{3}H-glycine, when applied to the rat hippocampus, the dentate gyrus, CA1 and CA4 fields showed labelling at low frequency stimulation. When 400 Hz high frequency stimulation periods were interposed, long-term potentiation ensued. The overall labelling of each hippocampal region was intensified significantly, indicating that glycine incorporation may be a sign not only of excitation but also of long-term potentiation.

^{3}H-glycine was applied to frog spinal cord during rest and dorsal root stimulation. Interneurons and motor neurons excited by the afferent fibers showed intensive glycine uptake.

It is concluded that the glycine labelling method is suitable for detecting neural excitation in the structures dealt with in this paper.

Keywords: Glycine labelling method — hippocampus — cerebellum — spinal cord — protein synthesis

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INTRODUCTION

In previous experiments /10—12/ it was established, that synaptically excited neurons take up and incorporate ^{3}H labelled glycine into structural proteins. This can be visualized with the aid of light- and electron-microscopic autoradiography. This method proved to be suitable for the detection of functional connections and correlation among nerve cells taking part in some neurophysiologically recordable event.

The investigations presented here set out to clear up some functional connections in three neural substrates not hitherto examined with the glycine method. In the cerebellum the activating action of the climbing fibers on Purkinje cells was set as the aim of experiments. In the hippocampus the effect of the long-term potentiation was to be cleared up upon glycine incorporation. In the spinal cord some functional connections of selected dorsal root fibers were studied with the glycine method.

MATERIALS AND METHODS

Experiments on the cerebellum. Adult cats were anaesthetized with 40 mg/kg nembutal. The dorsal surface of the cerebellum was exposed and recording electrodes were placed on it. The inferior olive was stimulated stereotaxically as described by Eccles /6/, with a concentric bipolar electrode. The effects of climbing fiber stimulation were recorded from the surface of the contralateral vermis. The pattern and amplitude of the electric signals were in accord with those published in the literature /6/. During stimulation, lasting for 2 h, a small strip of filter paper, containing labelled glycine (92.5 GBq/mM) was incubated on the stimulated surface, while the ipsilateral identitical point, not reached by the stimulation served as control. After having finished the stimulation, the underlying cerebellar structures were excised and processed further for light microscopic autoradiography.

In the hippocampal experiments adult Long-Evans rats were used in nembutal anaesthesia. Holes were bored for the stimulating and recording electrodes according to the stereotaxic atlas of Paxinos and Watson /9/ and two other holes were prepared through which a solution of labelled glycine (4.16 MBq/5/µl) could be injected into the lateral ventricle (Fig. 3). The perforant path on the right side was stimulated and activity of dentate gyrus of the same side was recorded. Evoked potentials of the dentate gyrus were recorded at 1/s stimulation and blocks of 50 potentials were averaged. Three such series of stimuli served as a control. For provocation of long-term potentiation (LTP) 17 impulses were given at a 400/s frequency three times, with 5 min intervals. The time course of LTP was followed by low frequency stimulations applied at regular intervals. The hippocampus of the right side served as control. After 70 min of stimulation, recording and glycine application the brain was removed and fixed in Bouin solution for 16 h. Then the samples were forwarded to light microscopic autoradiography. Coronal sections were prepared in which the dentate gyrus, regio inferior

(CA3, CA4) and regio superior (CA1, CA2) could be examined simultaneously.

In experiments on the spinal cord frogs were anaesthetized. Their spinal segments II and IX were exposed for stimulation, recording and glycine application. Selected dorsal root fibers containing skin- or muscle afferents and ventral roots were stimulated. During different stimulations labelled glycine (548 GBq/mM) was applied to the dorsal surface of the spinal cord. After a 1-hour stimulation the respective spinal segments were excised, fixed in Bouin solution and forwarded to light microscopic autoradiography.

RESULTS AND DISCUSSION

Cerebellum

In autoradiographic preparations made from cerebellar samples with the aid of the glycine method three layers of the cortex can be readily differentiated (Fig. 1A). The spontaneously functioning cerebellar cortex shows intensive glycine incorporation corresponding to the high frequency, low amplitude electric activity recordable from the surface. The labelling in the granular layer there is a diffuse labelling, due mainly to the glycine uptake of the granule cells. The interposed dark patches correspond to Golgi neurones, while brighter fields seem to represent cerebellar glomeruli. In some places the labelling ceases and continuous bright areas appear.

In the ganglionic layer the labelling is much more intensive, but this comes mainly from basket and Golgi cells and not from Purkinje cells. Purkinje cells appear sparsely in labelled form. Their place is indicated by unlabelled bright patches, singly or grouped, but regularly distributed. When labelled, their dendritic tree is visible, too. Some Purkinje cells can be recognized from their labelled dendrites, without any activity in the soma. In the molecular layer many stellate cells became labelled.

On stimulation of the inferior olive (i.e. activation of the climbing fibre system) brought a dramatic change in this picture (Fig. 1B): the labelling of the granular layer ceased, in the ganglionic layer small groups of Purkinje cells appear, rather heavily labelled, separated by unlabelled intervals. In many cases pairs of labelled Purkinje cells are separated by pairs of unlabelled ones. Somewhat deeper among Purkinje cells patches, reminiscent of Golgi cells and basket cells, are frequently present. The dendritic arborization of Purkinje cells, if the soma is labelled, never showed incorporated glycine. Many stellate neurons are also visible.

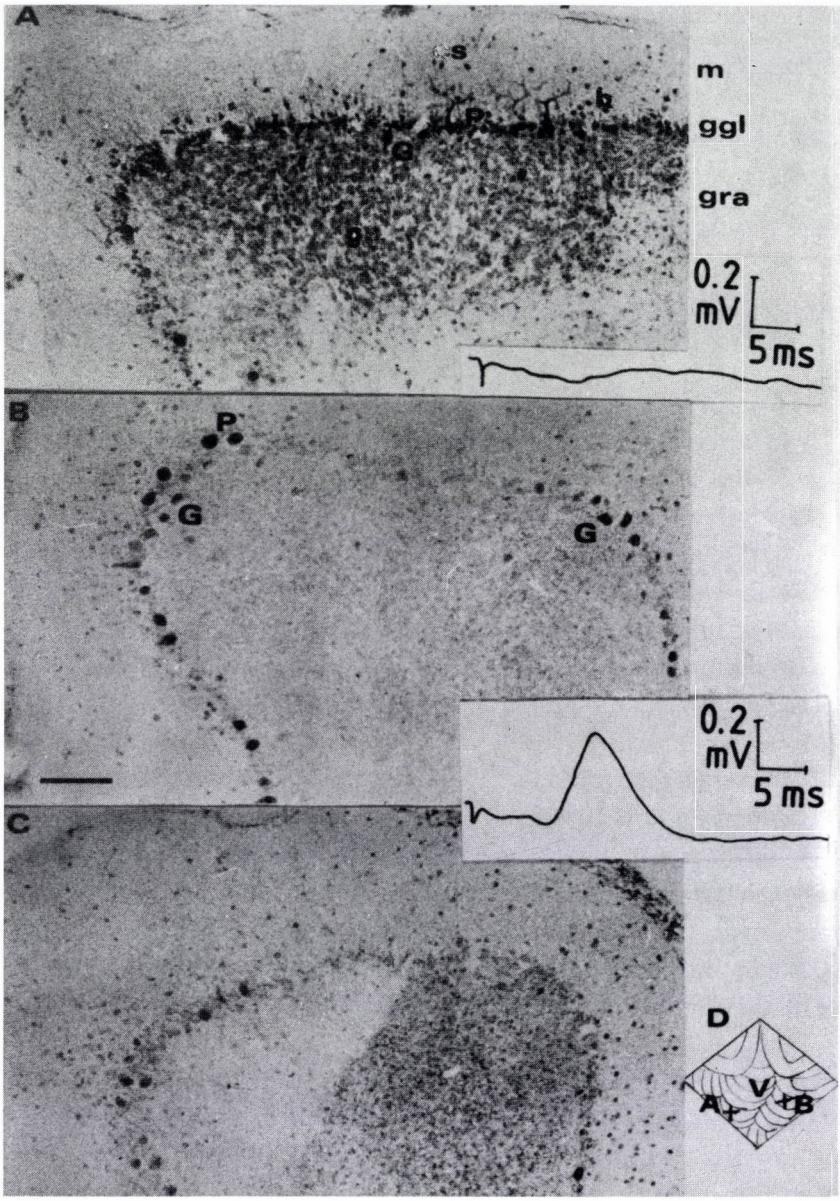

Fig. 1. Autoradiographic picture of the cerebellar cortex after 2 h of glycine application. A: Control with very low spontaneous activity. m = molecular layer, g = ganglionic layer, gra = granular layer. Granule cells are moderately labelled, the glomeruli lack silver grains. Cell types labelled: Purkinje cells (P), Golgi cells (G), basket cells (b), stellate cells (s). Bright patches in the ganglionic layer denote the place of unlabelled (probably inhibited) Purkinje cells. Insert: electrical activity. B: Autoradiographic

At the margin of the area, activated by the inferior olive, this pattern goes over without any gradual transition into the labelling pattern characteristic for the unstimulated cortex (Fig. 1C). Here regions exposed to climbing and mossy fiber activation can be seen in the same picture.

Although we do not know exactly the functional relations among the cells seen in a given autoradiographic preparation, much information can be gained from changes induced by stimulation and reflected by the autoradiographic pattern. To follow these modifications in the functional organization of the cerebellar cortex on a larger scale, we prepared serial sections from several mm pieces of the unstimulated and stimulated cortices and tried to draw conclusions from modifications of labelling of different cell types as to the nature of the climbing fiber action. Neighbouring sections are 30 µm apart and give a continuous picture of the area examined.

In Fig. 2 a map of the unstimulated cerebellar cortex is presented. Each strip, numbered with an arabic numeral, represents a piece of the folium, extending for about 14 Purkinje cell rows, within each strip the molecular, ganglionic and granular layers are denoted by different colours, as indicated on the far right. The strips from 1 to 8 follow each other continuously along the longitudinal axis of a folium (Fig.2A). In the non-stimulated sample the most frequent cell types are the stellate, basket and granule cells, while Purkinje and Golgi cells occur rather sparsely. Around Golgi cells only unlabelled Purkinje cells and empty zones are observable. At labelled basket cells there are no labelled Purkinje cells.

In Fig. 2B a similar diagram of the stimulated cerebellar cortex is presented. In contrast to the "resting" control, where large areas are free of labelled Purkinje, Golgi, basket or stellate cells, here, in a definite pattern all the known cell types occur over all the cortical sample. Stimulated and inhibited areas are separated more definitely. The relative number of Golgi cells here lower, that of basket cells much higher. Stellate cells occur always in regular coincidence with labelled Purkinje cells.

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picture taken during electrical stimulation of the left inferior olive, from the right hemisphere. Calibration 200 µm. Insert: electrical activity. C: Sample taken from the marginal zone of the stimulated area. The left part of the folium shows signs of excitation. D: Topography of the glycine application. V = vermis, A = resting control, B = stimulated sample

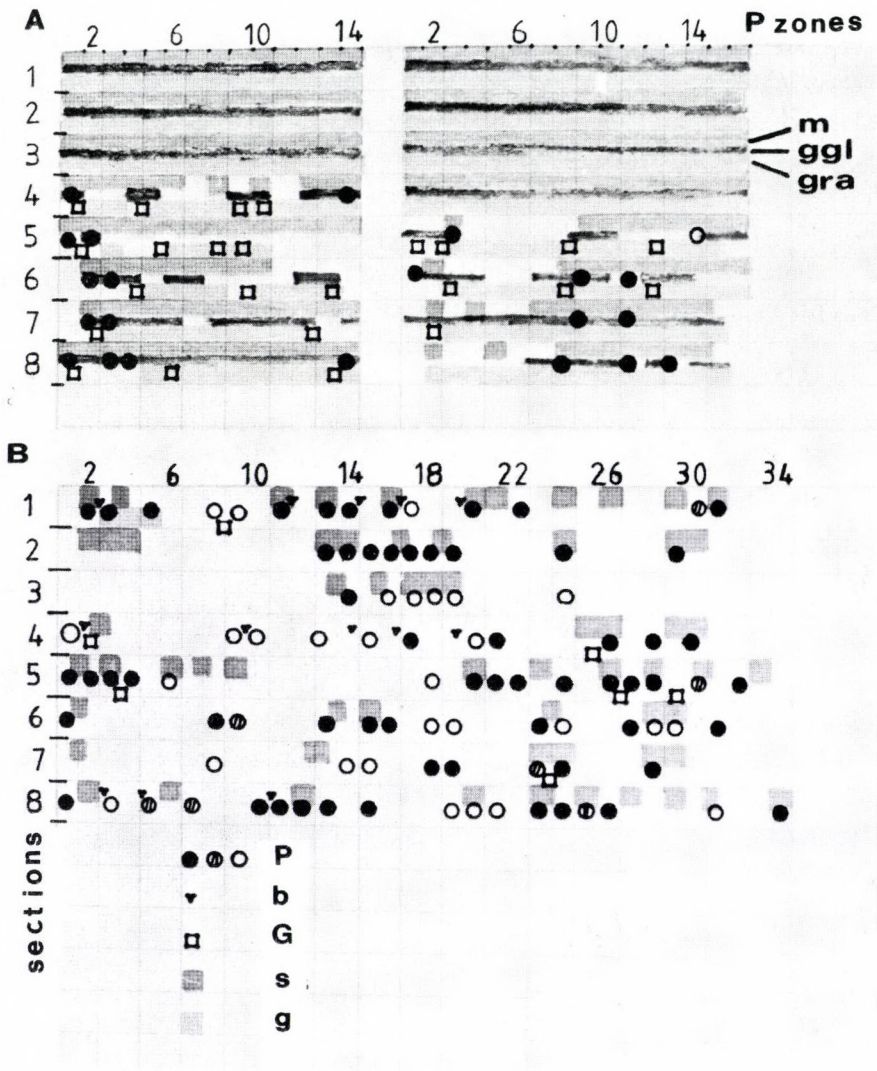

Fig. 2. Two-dimensional map of distribution of labelling constructed on the basis of serial sections. The sections are 30 µm apart. The rows numbered on the left denote parts of the same folium as follow each other along the longitudinal axis of the folium. A: Resting control taken from two neighbouring folia. P zones: divisions corresponding to the distances of neighbouring Purkinje cell somata. m = molecular, ggl = ganglionic, gra = granular layer. Their intensity of labelling is denoted by different intensities of shading. Lack of shading indicates lack of labelling. Notations of the cell types: see B, below. B: Map of cerebellar sample taken during inferior olive stimulation. The diagram represents one folium, but at greater length than in A

Granule cell and glomerular activity is completely inhibited. The tremendous inhibitory action of the Purkinje cells cannot be detected by any other method. The pathways responsible for this inhibition are not well known so far. In experiments with more restricted and specified stimulation and with more careful choice of the cortical area analyzed finer functional connections could be described.

As to the efficiency of the glycine method it is pertinent to note, that it is able to visualize not only excitatory, but also inhibitory neurons (e.g. Purkinje, Golgi and basket cells). It is also to be mentioned, that bright patches in the place of cells indicate not only lack of excitation but directly point to some degree of inhibition. Thirdly the soma and dendritic tree of the Purkinje cells become labelled not necessarily together: at granule cell activation (in the unstimulated controls) both dendrites or soma labelled, while at climbing fiber stimulation only the soma becomes labelled and never the dendrites. It remains a question whether this indicates different types of excitatory states, or is caused by peculiarities of the intracellular transport of proteins.

Hippocampus

High frequency (100—400 Hz) stimulation of the commissural and perforant path elicits long-term potentiation (LTP) in the hippocampus /3, 5, 13/, both in anaesthetized and freely moving animals /2, 3, 4/. This effect can be enhanced with 4-aminopyridine /8/. LTP appears in several seconds and can persist for days.

Control experiments on the hippocampus were arranged in the following manner. The perforant pathway of the right side was stimulated with low frequency (1/s) stimuli and 50 potentials were averaged from the dentate gyrus. In the first 70 min of the experiment the population spikes showed a 100 per cent gradual increase, with unchanged form. Meanwhile, labelled glycine was injected into the lateral ventricle and was allowed to be built in for the whole experimental period. The autoradiograms, made from such samples show well defined labelling in cells of both arms of dentate gyrus, with the exception of its apical third, there was rich labelling in CA3, but not in CA1 (Fig. 4).

Brief, high frequency (400 Hz) stimulation changed this picture radically. The population spikes (Fig. 5) were potentiated and this showed an increasing tendency even after 60 min. In the autoradiographic picture an extraordinarily intensive labelling was encountered in every part of the

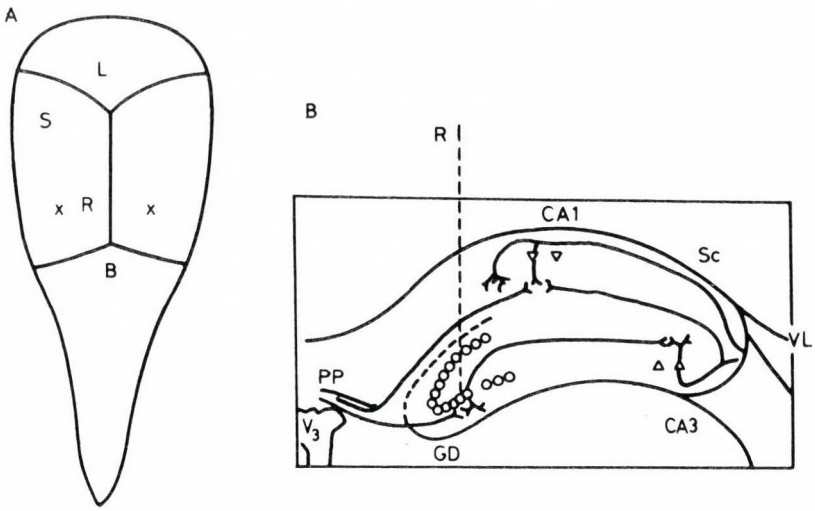

Fig. 3. Arrangement of the stimulating and recording electrodes in the rat brain. A: View of the skull from above. B = bregma, L = lambda suture. R = recording, S = stimulating electrode, X = holes for injection of glycine into the lateral ventricles. B: Scheme of the rat hippocampus with the stimulating and recording electrodes. PP = perforant path, SC = Schaffer collateral, VL = lateral ventricle, GD = dentate gyrus, V3 = third ventricle

dentate gyrus, but it became more pronounced in the CA1 and CA3, too. Changes were less obvious in CA4. Glycine incorporation of comparable intensity (with an identical quantity of isotope used) has not been observed in any other part of the nervous system or in any physiological situation.

As to the mechanism of LTP and the relation of glycine incorporation to it, we cannot go into details, here. It seems, however necessary to note, that in the physiological mechanism of LTP both pre- and postsynaptic factors play a definite role. The result is the immense augmentation of the synaptic efficacy, reflected by the enhanced population spikes. As to the glycine incorporation it seems important, that it is restricted to the soma of the hippocampal pyramidal cells, and the presynaptic endings do not appear to gain in labelling. Thus enhancement of the protein synthesis, reflected by the glycine incorporation seems to be bound to the postsynaptic events.

If one compares the absolute number of impulses, applied to the control and potentiated samples, respectively, it turns out, that the latter receive only a few more stimuli, than the control ones. In course of a 70 min experiment 50 impulses are delivered to the animal five times. This

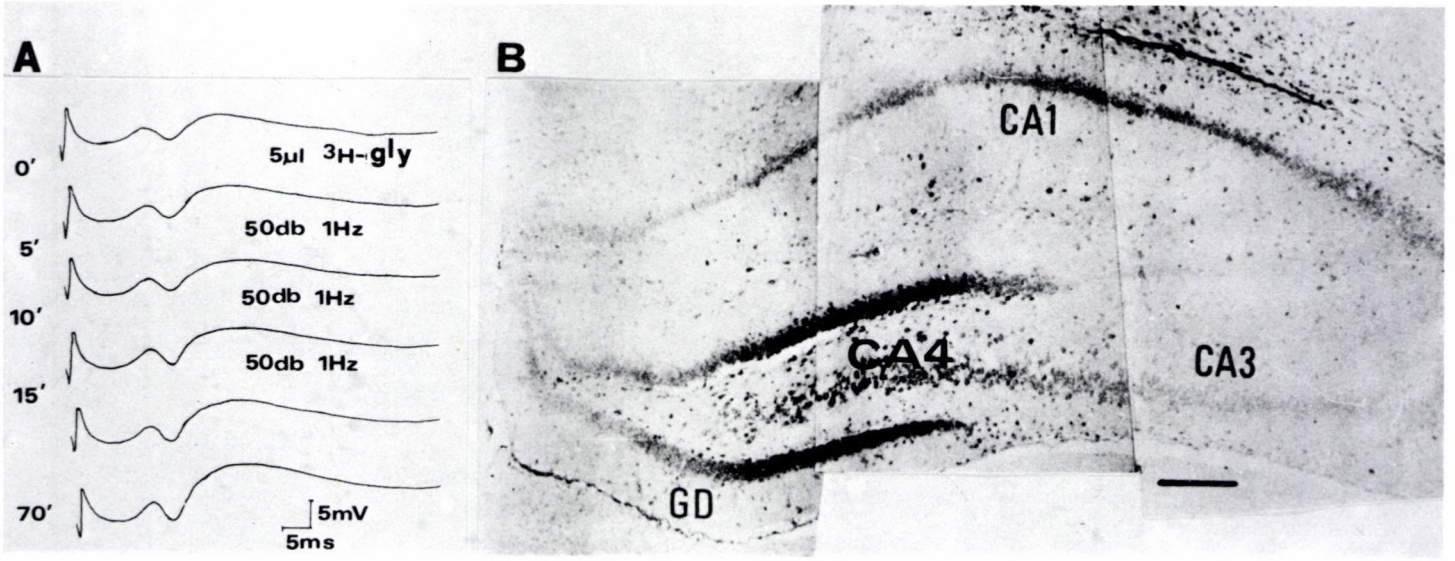

Fig. 4. A: Population spikes evoked by the perforant path stimulation at times noted at left. B: Autoradiographic picture of the rat hippocampus taken after 70 min of 1/s test stimulation. Notations are the same as in Fig. 3

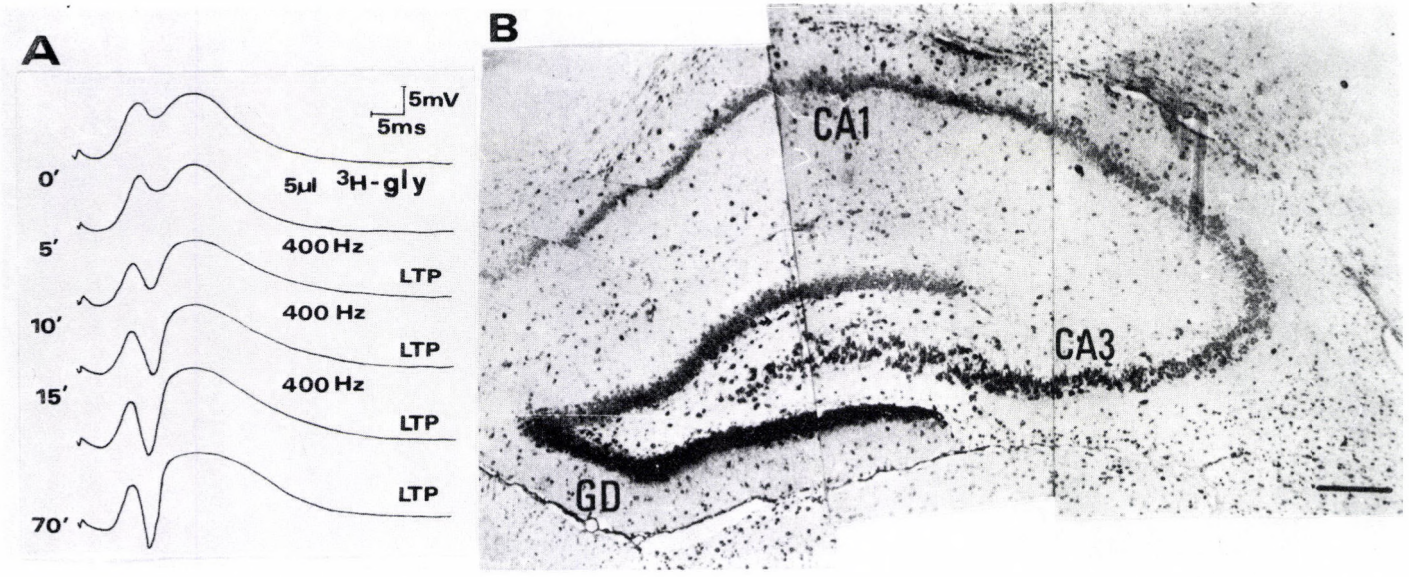

Fig. 5. A: Population spikes evoked by the perforant path stimulation, with three interposed high frequency stimulations (400 Hz). B: Autoradiographic picture of the rat hippocampus in a state of long-term potentiation

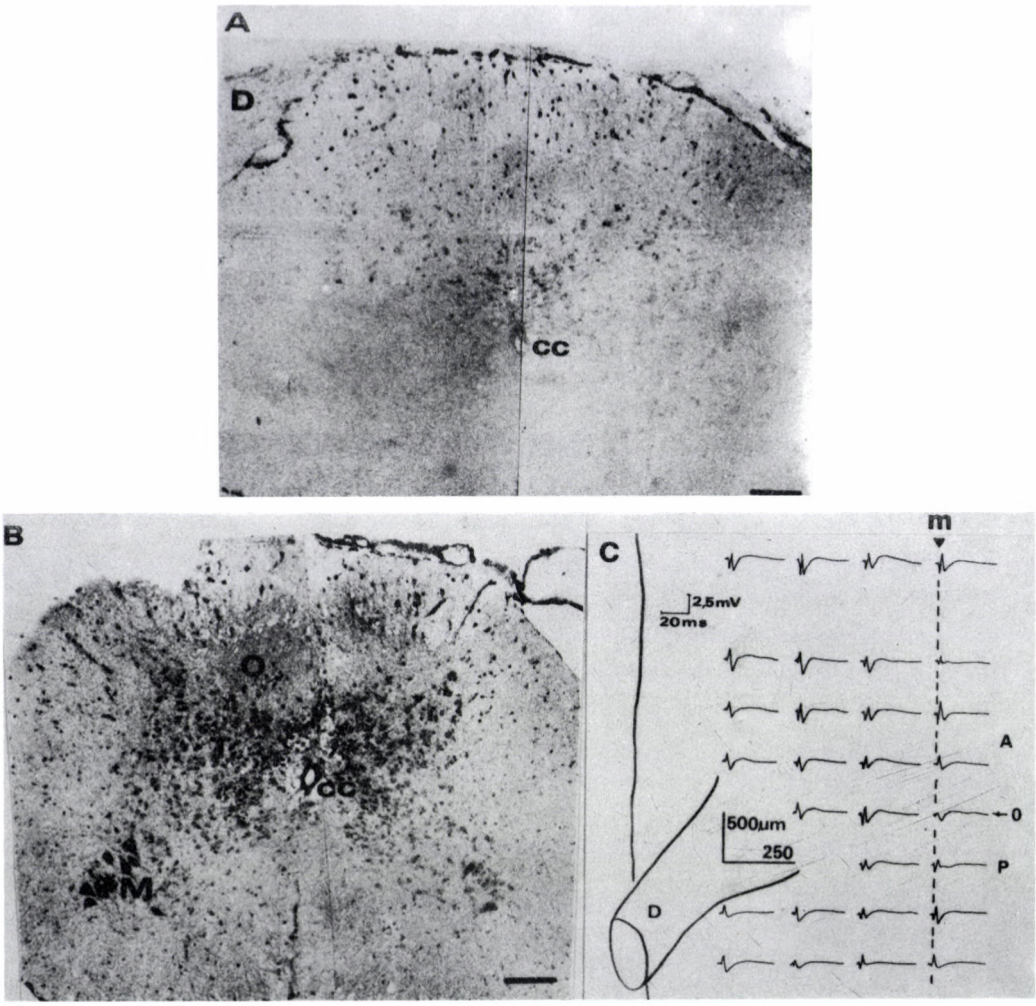

Fig. 6. Autoradiogram of frog spinal cord segment IX. A: Resting control. D = dorsal root, cc = central canal. Calibration 200 µm. B: The same during stimulation of the dorsal root of the left side. O = oval area, T = triangular area, M = motor neurons. C: Dorsal surface of segment IX. m = midline, a = anterior, p = posterior, D = entry of root IX

makes 250 stimuli altogether and produces an autoradiographic picture, seen in the "control" samples. Three times 17 additional impulses by themselves would not be able to bring about the strong enhancement found in the LTP samples. The high frequency with which these 51 impulses are delivered may trigger some process in the protein synthesizing apparatus, which remains in

this state as long as LTP lasts. Therefore it seems justified to assume, that enhancement of protein synthesis in the nerve cell, as reflected by the glycine incorporation, is not a consequence but a prerequisite of potentiation. In the cerebral cortex 7000 stimuli are far from sufficient to bring about comparable intensities of labelling, at identical quantities of the isotope applied. If LTP has some relevance to memory and learning, the glycine method gains a further potential usefulness in studying these central processes.

Spinal cord

By use of the cobalt labelling technique it was demonstrated that skin afferents terminate in a more superficial oval area of the dorsal horn, while muscle afferents make synapses deeper, in the middle of the grey matter, in a triangular field /1, 15, 16/. Frank and Westerfield /7/ report, that sensory fibers of the anconeus muscle are able to activate the motoneurons of the same muscle. Simpson /14/ observed, that skin afferents take part in various polysynaptic reflex arcs.

In this series of experiments it our purpose on one hand to know whether the glycine method is applicable to the frog spinal cord and if so on the other hand to examine whether the morphological and physiological findings, quoted above, can be confirmed by this method.

In the resting spinal cord of anaesthetized frog incorporation of glycine is minimal. Only several glial cells in the white matter, near the dorsal surface accumulate some isotope (Fig. 6A).

On stimulation of the IX dorsal root, verified by recording of the electrical activity from the surface, intensively labelled cells appear in the contralateral, and ipsilateral oval and triangular fields. Labelling of the motor nucleus on the ipsilateral side is more intensive, in view of the number of cells and density of silver grains also. Small labelled cells or cell groups occurred sparsely over the whole grey matter (Fig. 6B). With the same stimulation paradigm identical results were obtained with segment II (Fig. 7A). Stimulation of the dorsal root II brought about ample glycine incorporation in the triangular, oval areas and in the ipsilateral motor nucleus. Stimulation of the afferents from the anconeus muscle caused incorporation only in the triangular area and not in the oval shaped one or the motor nucleus, as is shown by the serial sections of Fig. 7B.

All these findings are in accordance with the morphological and

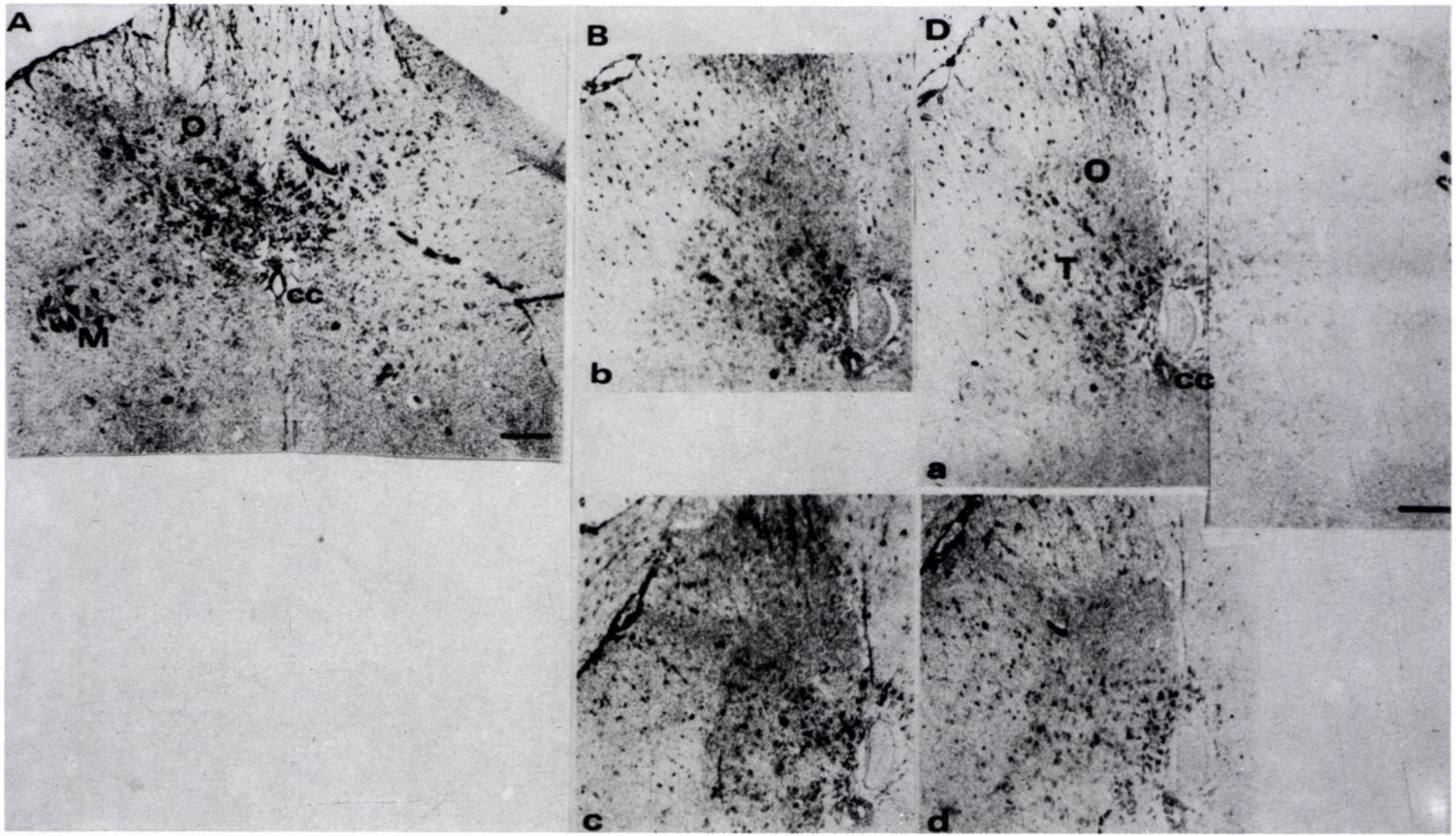

Fig. 7. Autoradiogram of segment II. A: Stimulation of dorsal root II. O = oval shaped area, M = motor nucleus, cc = central canal, T = triangular shaped area. B: Serial sections from segment II, taken during stimulation of afferents from the anconeus muscle. Sections from a to d show labelling only in the triangular area

physiological studies and indicate that the glycine method may be a useful tool in visualization of mass effects of spinal excitation.

The experiments on the spinal cord had also some other importance. As is known, glycine itself is a neurotransmitter in the inhibitory synapses of the spinal cord. It was questionable, whether this circumstance does interfere with glycine labelling of the spinal neuron. From the experiments it turned out, that this causes no problem, all the more so because soluble glycine is washed out of the cells during the histological procedure. Spinal cord neurons take up and incorporate glycine basically in the same way as other central nerve cells.

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THE BEHAVIOUR OF RATS SELECTED FOR THEIR VOLUNTARY ETHANOL CONSUMPTION*

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New strains of rats, preferent (HAP) and non-preferent (NAP) for ethanol were selectively outbred from a Wistar stock. The strains have now been raised to the F_{13} generation.

The $F_{9/10}$ animals, selected for this behavioural investigation, exhibited a significant phenotypic drinking behaviour and/or ethanol consumption. During a free choice between tap water and 10% ethanol solution (v/v), the mean daily alcohol intake for male and female HAP rats was 8.42 ± 0.69 g/kg/24 h (n=20 o) and 11.50 ± 0.42 g/kg/24 h (n=20 o), for male and female NAP rats 0.74 ± 0.09 g/kg/24 h (n=20 o) and 1.76 ± 0.20 g/kg/24 h (n=20 o), respectively. The NAP rats exhibited a strong aversion to the 10% ethanol solution when it was the only source fluid.

In the open-field test (OFT), as compared to the NAP rats, male individuals of the HAP strain showed a lower motility in the first minute, in penetration into the inner squares, showed a longer latency to start exploration (latency to leave the center), exhibited larger rearing and grooming activity and shorter latencies to start these activities. The defecation rate was smaller and latency to defecation prolonged.

Female HAP rats showed higher activity scores in penetration of outer and inner squares and a shorter latency to start exploration. They also had higher rearing but smaller grooming activity. The females exhibited identical defecation but different urination behaviour in comparison to the males.

The time-to-emerge latencies of HAP rats were longer than in NAP individuals.

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In the plus-maze test (PMT), male and female HAP rats spent less time in the open arms, showed a smaller number of open-arm entries and they choose significantly fewer open arms as the first entry. The defecation rate of HAP rats was greater than of the NAP individuals.

The data suggest that HAP rats possibly had difficulties in becoming accustomed to a new and stressful environment and are more emotional (anxious) than the NAP counterparts. This inability seems to be one of the internal factors which might underlie their preference for the anticonflict properties of ethanol. Thus, the role of predisposing factors might be one of the dominating components in voluntary ethanol preference and functions as a learned adaptive response to attenuate the effect of stress.

Keywords: Ethanol consumption — exploration behaviour — emotionality — heredity — rats

INTRODUCTION

The importance of genetic factors in the determination of ethanol preference and/or ethanol consumption in rodents has been demonstrated by many laboratories /1, 6, 7, 17, 18, 19, 27/. But there are, of course, other ethanol-related problems to which animal models or analogues can be easily applied, and the use of an animal model of alcoholism has been discussed many times /for review see 3, 12, 17/. The reason why animal models are required is twofold. First, many ethanol-related problems cannot be rigorously examined in human beings for a number of obvious ethical reasons. Second, human alcoholics have numerous biomedical and psychosocial problems in addition to their alcoholism. An animal analogue is the only way in which the effects of ethanol per se can be examined free from the contaminating influences of other factors. In such a model, ethanol can be self-administered voluntarily by the oral route in pharmacologically significant amounts and can further be preferentially consumed as a drug when there also is a choice between it and other solutions. Furthermore, after a period of continuous consumption tolerance and dependence to ethanol may develop. Therefore, the study of animals which exhibit innate differences in ethanol preference offers the most promising avenue to define the physiological, pharmacological and biochemical correlates of ethanol preference or non-preference (aversion). Data published from the laboratories of Eriksson /6, 7/ and Lumeng et al. /18/ have been provocative and it seems important to compare the behavioural correlates among these strains with those obtained from other animal models which have been selected from different stocks.

VOLUNTARY ETHANOL CONSUMPTION AND BEHAVIOUR OF RATS

We started selective breeding for voluntary ethanol consumption or ethanol preference in 1983 with a colony of Wistar rats originating from Túrkeve in Hungary. These rat strains have now been raised to the F_{13} generation. In this paper we describe the general and drinking behaviour of these selected ethanol-preferring (HAP) and non-preferring (NAP) rats of the $F_{9/10}$ generation.

The purpose of this work was to realize a strain of rats which voluntary drinks pharmacologically effective amounts of ethanol and another which avoids alcohol as completely as possible. In daily handling of the animals we observed obvious differences in behaviour between individuals of the two strains. Thus, the aim of the present investigation was to explore and to quantify these behavioural differences.

MATERIALS AND METHODS

1. Breeding and selection procedure

The animal material was ordinary albino stock of Wistar origin (Túrkeve, Hungary). The rats were systematically bred for more than 13 generations and only distant relatives were used for reproduction. Previously the F_6 generation of outbred rat strain selected for voluntary ethanol consumption exhibited an acceptably good difference in ethanol intake and a good distribution of preference scores. With individuals of this generation we started the first behavioural investigation to explain distinct differences in the rats' behaviour observable through daily handling (unpublished data).

In general, rats were kept under standard conditions with a light-dark cycle of 12:12 h, with the light switched on at 6.00 a.m. In the reproduction process rats were housed in single cages. Food and water were avaiable ad libitum. Room temperature was 22—26 °C, the relative humidity was maintained between 45 and 55%.

Preference test

The pups of each litter were separated according to sex and housed together in groups for the test period. In their 7th week rats were habituated to a 10% ethanol solution as the only available fluid for four days. Tests for ethanol preference were started when the rats were 8 weeks of age, the onset of puberty. The rats were then placed individually into drinkometer boxes for 24 h once per week only. The drinkometer box contains two calibrated drinking tubes, once containing tap water, the other one a 10% ethanol (v/v) solution. The rats were tested using this two-bottle choice procedure for 4 to 6 weeks; every individual was tested only once per week to exclude the development of dependence in this period. Drinkometer boxes and the bottle positions were changed randomly. Also during the 24 h preference test the rats were allowed free access to the standard laboratory food.

The quantities of water and ethanol solution drunk by the individual were measured after 24 h and the body weight recorded. For every individually marked rat the ethanol intake calculated in g alcohol per kg body weight and for 24 h was determined and additionally on the basis of drunk fluid a preference index (PI) was calculated using the formula:

$$\text{Preferences index} = \frac{\text{amount of 10\% ethanol solution (ml)}}{\text{total fluid intake (ml)}}$$

Zero value of the preference index indicates no ethanol intake, an index of 0.5 expresses that the amount of ethanol and water was identical, an index of 1.0 shows that ethanol only was consumed.

Selection for further breeding

We expect that with continued selective breeding HAP and NAP strains will be more and more homogeneous. Thus, for the purposes of breeding and experimentation, the following selection criteria was set: for the HAP strain, rats which consume more than 5.0 g/kg/24 h and a PI $>$ 0.50 in the generations F_1—F_6 and/or more than 8.0 g/kg/24 h and a PI $>$ 0.80 in the generations F_7—F_{12}. For the NAP strain only rats which drank less than 2.0 g/kg/24 h and a PI $<$ 0.10 for the F_1—F_8 and/or less than 1.0 g/kg/24 h or an PI $<$ 0.08 for F_9—F_{12} were used. Breeding started when the rats were 16 weeks of age and free of ethanol for more than 2 or 4 weeks. In further reproduction processes the rats did not receive any ethanol.

2. Behavioural tests

2.1 The open-field test:

Open-field observations were carried out as described earlier /28/. We used an 8x8 squared, white painted 100x100 cm wooden open-field, the box being 40 cm in height. The open-field was illuminated by a 150 W electric bulb, 150 cm above the open-field floor. A ventilating fan provided a background noise of about 40 phones. The rats were individually placed in the middle of the box and in 5-min sessions per day on 3 consecutive days measurements were made on the following behavioural variables: ambulation rate on outer and inner squares, the latency to start exploration (latency to leave the center), the ambulation activity of the first minute, rearing activity and latency to rearing, grooming (preening) activity and latency to grooming, the defecation rate and latency to defecation, the urination score, the latter being graded from 0 to 3 according the volume of urine, and the latency to urination.

2.2 The time-to-emerge test

The method has been described in detail by Schulz and Feuer /29/. The testing apparatus consisted of two communicating parts: a black painted dark chamber and a white painted one, indirectly illuminated by room light (8x40 W neon tubes). The latter part is the "open-field" or novelty. The size of each of these chambers was 31x22x30 cm, and they were connected by a guillotine door. On day one the animals made 4 habituation trials in the dark compartment, for 5 min each session, at intervals of 30 min. On day 2

every animal (no food and water deprivation) was again place individually in the dark compartment for 5 min, and thereafter the guillotine door to the illuminated, white compartment was opened. Simultaneously with opening the door a stopwatch was started to measure the rats' latency to enter the novelty with all four paws; this was the time-to-emerge latency (TTE-latency). The animals were observed through a mirror, which was assembled above the novelty. Additionally, motor activity and the number of incomplete entries or of irresolute responses were also recorded. In these cases the animal looked into the novelty, or entered the illuminated compartment only with the forepaws and retreated immediately.

2.3 The plus-maze test

The plus-maze test has been basically developed and introduced by Handley and Mithani /9/ and was used to test anti-anxiety effects of drugs by Pellow et al. /22/. The apparatus consisted of two open arms, 45x10 cm and two enclosed arms 45x10x9 cm, with an open roof, arranged so that the two open arms were opposite each other. The maze was elevated to a height of 80 cm. Each animal was tested in a 5-min session and the following parameters were recorded: first choice of alley type (open or closed arm) after placing the rat on the centre of the maze facing on open arm; the number of entries into open arms, time spent in open arms, number of entries into closed arms and time spent in enclosed arms. Time spent in open arms was calculated as a percentage of total time spent in open and closed arms. Finally, also the number of defecation boli were recorded. The measures indicated in the observation session were taken by an observer sitting in the same room as the maze.

3. Statistical procedures

Differences between the populations (HAP and NAP individuals) were calculated by the use of information statistics /15/ and by the F-test. A probability level of $p < 0.05$ was accepted as significant.

RESULTS

Characterisation of the outbred strains

Male HAP rats of the F_{10} generation consumed more than 8.0 g/kg/24 h and HAP females more than 9.0 g/kg/24 h ethanol. Rats of the NAP strain drank less than 1.5 g/kg/24 (males and females as well) alcohol. In general, there are no striking differences in fertility and litter size between the two strains.

The data of the present investigation are based on a sample size of 20 selected individuals of each strain and each sex according to the highest (HAP) or lowest (NAP) scores yielded by the individuals in the test period. Thus, every group represents individuals showing the extreme values in ethanol consumption and/or preference of its strain.

We found a small difference in body weight between the males of both strains but not in the females: HAP ♂ 233.50±23.20 g, NAP ♂ 207.00±22.0 g, $2\hat{I}$=10.97, df=2, $p < 0.01$. HAP ♀ 184.95±10.70 g, NAP ♀ 176.50±16.80 g at 8 weeks. After the preference test at 12 weeks: HAP ♂ 316.0±32.3 g, NAP ♂ 289.0±23.0 g, $2\hat{I}$=4.59, df=2, $p < 0.05$; HAP ♀ 220.0±24.0 g and NAP ♀ 216.50±24.10 g, N.S.

Both groups differed widely in their voluntary ethanol intake. HAP ♂: 8.42±0.69 g/kg/24 h (n=20) and NAP ♂: 0.74±0.09 g/kg/24 h (n=20); HAP ♀ : 11.50±0.42 g/kg/24 h (n=20) and NAP ♀ : 1.76±0.20 g/kg/24 h (n=20). The differences between the two strains were highly significant, as well as between males and females of each strain. NAP ♂ vs NAP ♀ $2\hat{I}$=26.88, df=3, $p < 0.005$. Thus, there is a clear sex difference in voluntary ethanol intake, females drank more alcohol per body weight than males.The rats drank the largest amount of ethanol in bursts in the dark, which is the normal biological activity period, when they eat most food, too. In a single drinking episode they consumed sometimes 2—3 g alcohol/kg body weight.

Behavioural characteristics

For behavioural investigation the same individuals of male and female rats of the $F_{9/10}$ generation were used by the common open-field test (OFT) on 3 consecutive days. The OFT data are represented in Tables 1—3. There a very clear difference between both groups of males in ambulation in the first minute, in penetration into inner squares of the OF and also in the latencies to leave the center. Furthermore, strong differences occurred in rearing and grooming behaviour and the latencies to these activities (Table 1). Both groups differed in their defecation behaviour, in the number of boli produced and the latency to defecation, too (Table 1).

The females in both groups significantly differed in the activity of outer squared crossed, in penetration into the inner squares, in the latency to leave the center, in rearing and grooming activity, in the defecation rate and in urination behaviour (scores and latency to urination) (Table 1).

The presented data do not only reflect the behavioural differences in OF behaviour of individuals of the two strains but also showed enormous differences in the behaviour between male and female rats in responding to OF situation (or stress). By comparison of the data presented in Tables 1—3,

Table 1

The average values and standard deviations as functions measured in an open-field test in the $F_{9/10}$ generation of rats selected for voluntary ethanol consumption. Day 1

Variable	HAP		NAP	
	Males	Females	Males	Females
Activity total	110.70±10.0	128.40±10.30 **	120.25±13.15	79.85±14.10
Activity 1. min	26.50± 2.76 ***	31.40± 3.80	35.20± 3.80	28.85± 4.00
Activity inner	14.80± 2.70 **	19.05± 2.53 ***	21.70± 3.22	9.15± 1.21
Latency to leave cter.	8.15± 2.32 ***	4.40± 0.48 ***	5.80± 1.28	12.20± 2.29
Rearing	14.30± 1.43 *	16.65± 1.51 ***	13.65± 2.26	9.20± 1.05
Latency to rearing	24.45± 3.56 **	27.25± 3.28	50.40± 7.80	38.60± 7.21
Grooming	4.60± 0.39 ***	4.80± 0.64 *	3.15± 0.40	6.80± 0.70
Latency to grooming	116.20±14.69 **	113.65±10.10	173.65±16.03	97.55±14.28
Defecation rate	1.70± 0.35 **	2.00± 0.58	3.20±0.48	2.55± 0.59
Latency to defecation	153.40±26.50 **	226.20±24.27 **	83.55±22.07	147.95±28.13
Urination	1.05± 0.29	0.70± 0.28 *	1.15± 0.26	1.20± 0.29
Latency to urination	175.80±31.89	228.30±27.90 *	145.80±28.70	144.50±32.27

* p 0.05 ** p 0.025 *** p 0.01 HAP vs NAP

Table 2

The average values and standard deviations as functions measured in an open-field test in the $F_{9/10}$ generation of rats selected for voluntary ethanol consumption. Day 2

Variable	HAP		NAP	
	Males	Females	Males	Females
Activity total	85.25±13.10	91.65±16.50*	100.35±17.00	58.65±10.50
Activity 1. min	25.40± 3.54***	22.15± 4.00	40.80± 4.47	29.80± 3.66
Activity inner	14.20± 3.35	13.50± 3.31*	11.75± 2.42	8.55± 1.97
Latency to leave cter.	3.00± 0.72	2.20± 0.72***	3.15± 1.42	6.10± 1.37
Rearing	10.55± 1.73***	10.30± 1.89***	8.55± 1.57	4.40± 0.65
Latency to rearing	51.85±13.97	73.55±16.75	58.95±14.45	78.10±20.75
Grooming	4.60± 0.59	5.35± 0.52***	4.40± 0.65	6.60± 0.94
Latency to grooming	118.95±20.29	93.40±13.82	116.70±17.08	91.75±15.13
Defecation rate	1.60± 0.51**	1.45± 0.62*	3.15± 0.55	2.45± 0.54
Latency to defecation	202.10±27.74**	249.75±21.20 **	121.30±24.64	160.30±26.92
Urination	0.30± 0.14	0.30± 0.16***	0.70± 0.26	1.05± 0.30
Latency to urination	253.90±22.06	255.30±23.81*	222.00±26.91	194.75±30.49

Table 3

The average values and standard deviations as functions measured in an open-field test in the $F_{9/10}$ generation of rats selected for voluntary ethanol consumption. Day 3

Variable	HAP		NAP	
	Males	Females	Males	Females
Activity total	61.55± 9.32	86.35±14.76***	47.65± 9.79	49.00± 7.51
Activity 1. min	24.35± 3.55	19.65± 3.40*	27.40± 4.63	27.85± 3.28
Activity inner	10.90± 2.56***	12.85± 3.70***	5.85± 1.30	6.25± 1.02
Latency to leave cter.	1.60± 0.37	2.40± 0.50	1.55± 0.24	2.85± 0.69
Rearing	8.20± 1.49***	10.50± 1.85*	4.75± 1.00	5.45± 1.16
Latency to rearing	26.40± 4.23***	44.85±13.69	69.25±18.18	62.10±14.44
Grooming	5.60± 0.92	5.95± 0.61***	4.85± 0.85	7.60± 1.41
Latency to grooming	65.25±16.50**	81.20±16.26	103.55±19.75	113.15±18.78
Defecation rate	2.50± 0.40***	1.90± 0.59***	4.00± 0.60	2.55± 0.59
Latency to defecation	186.80±19.06***	235.35±23.53	107.70±23.03	162.35±28.56
Urination	0.35± 0.20	0.50± 0.23	0.00	0.65± 0.22
Latency to urination	279.05±15.06	253.75±23.74	300.00	220.00±27.68

Table 4

Comparison of the behaviour of two strains of rats on a 5-min test in the plus-maze. Scores are: the number of first choices of an open arm as relative to the closed, the percentage of time spent in an open arm, the total entries into open arms, the overall activity (the open arm and closed arm entries), and the defecation rate (number of boli). Numbers are mean ± S.E.M.

Scores	HAP		NAP	
	Males	Females	Males	Females
First choice open arm	4/20 [c]	7/20 [a]	13/20	13/20
Time spent in open arms	5.10± 1.48 [b]	7.15± 1.94 [a]	15.26± 2.52	9.86± 1.56
Entries into open arms	0.90± 0.20 [a]	1.10± 0.26 [b]	2.25± 0.35	1.65± 0.25
Defecation rate	1.85± 0.43 [c]	0.25± 0.20	0.20± 0.11	0.10± 0.10
Total activity	7.00± 0.54 N.S.	7.20± 0.75 N.S.	11.50± 4.58	7.85± 0.52

[a] p 0.05 [b] p 0.025 [c] p 0.01 HAP vs NAP

Table 5

The time-to-emerge latency of two strains of rats selected for their voluntary ethanol consumption. Values are given in seconds ± S.E.M.

TTE - latency	HAP		NAP	
	Males	Females	Males	Females
	139.95±27.50 [a]	210.70±44.80	109.15±25.25	129.40±34.50 [b]

[a] HAP vs NAP males: $2\hat{I}$ = 10.63, df = 3, $P < 0.025$ (2α)

[b] HAP vs NAP females: $2\hat{I}$ = 6.68, df = 2, $p < 0.05$ (2α)

one can explain differences between the groups in habituating to the OF in the test period of 3 days.

Also our data derived from the plus-maze test (PMT) reflect these behavioural differences between the groups. Male NAP individuals exhibited larger number of first open arm choices, spent more time in open arms, showed a larger number of open arm entries and a smaller defecation rate. But they did not significantly differ in their overall activity in this test paradigm (Table 4). Female rats showed a similar pattern of activity, but there were no differences in defecation rate and general activity (Table 4).

Data of the time-to-emerge test (TTE) showed that male and female HAP rats exhibited longer latencies to penetrate into the novelty as compared to the NAP counterparts. In this test females showed longer latencies than males (Table 5).

DISCUSSION

The animals, selectively bred for voluntary ethanol intake and/or preference for alcohol, are the fourth strain in the world (Mardones in Argentina, Li and Lumeng in USA and Eriksson in Finland) and the second in Europe. According to the alcohol consumed, these rats are in the range of such data published earlier by Eriksson /6, 7/ and Lumeng et al. /18/. The larger body weight of our HAP rats before and after the preference test are in contrast to the findings of Eriksson /7/. This may be due to the difference in the test paradigm. In general, female rats of our strains exhibited a higher preference for ethanol and this agrees with the data published by others /6, 7, 16, 17, 18/. It is well known that female rats develop higher rates of ethanol elimination than males in both ethanol-preferring as well as in non-preferring individuals as well /7, 27/. On the other hand, there are no significant differences for the same sex between the strains /7/.

We observed during daily handling of the rats strong differences in behavioural responses. Males and females of the HAP strain seem to be easily frightened, more nervous and timid than rats of the NAP population. Therefore, the question arises which of the behavioural components are measurable and easily quantificable as valid variables to determine emotional differences between the strains. To be surer in judging the emotional status of our animals we chose a more complete investigation of behaviour on the basis of variables coming from different test procedures, confined mainly to

emotionality. In this respect OF behaviour gives good information about the rats' response to mild stressful stimuli and their emotional status (but not on the basis of defecation boli only). As shown by the results, males of the HAP group do significantly differ in 9 out of 12 measured OF variables vs. NAP individuals, and in the case of females such a difference exists in 10 out of 12 OF variables. This is the case especially with such parameters which are highly relevant to emotion, like activity in the first minute, latency to leave the center, latency to rearing and grooming, urination parameter and latency to defecation /30, 31/. This interpretation is supported by the findings in PMT situation, showing that HAP individuals are possibly more emotional (anxious) than NAP rats. Finally, also the TTE-latency gives strong support for such an explanation, because anxiolytics do shorten the latency to start exploration of a novelty /29/ by lowering the intra-individual conflict. There are also strong differences in the OF behaviour between male and female rats, but this point will not be discussed here /2, 5, 30/. Eriksson /7/ reported that ethanol-preferring rats of his strain (AA) exhibited a lower rate of grooming (preening) and ambulation than the non-preferent (ANA) line. The behavioural data presented in his report did not give good information about the emotional status of the rats because total ambulation rate, rearing, grooming and defecation rate alone are not representative variables for emotionality. More sensitive parameters would in this respect be such variables as used in our investigation.

Comparison of the data measured on three consecutive days clearly showed significant differences between the groups in their habituation to the OF situation. It seems to us that HAP rats (males and females as well) adapt uneasily in this respect and they have real difficulties in habituating to the OF situation. This interpretation of our data is also supported by the PMT results where the HAP rats exhibited a behaviour which was interpreted by Pellow et al. /22/ as more emotional and/or anxious. Additionally, it should be noted that in our investigation the defecation rate in the OFT and in PMT is quite inverse. This fact shows again that OF defecation alone is not a valid variable to judge emotionality in rats.

On the basis of these data one is led to interpret the results thus: HAP rats possibly drink more ethanol than the NAP counterparts in order to attenuate or compensate the effect of environmental stimuli which may be stressful for them. Support for such an interpretation comes from results showing that emotionally reactive rats preferred ethanol for its pharma-anti-conflict properties /1, 13, 20, 23, 24, 25, 26/.

Interestingly, also laboratory housed wild Norway rats preferred ethanol and drank it in higher concentrations than the white Wistar rats under the same conditions /14/. When environmental stress, however, acts as a reinforcement to stimulate voluntary ethanol intake and our HAP strain seems to be more emotional then underlying predisposing factors for regulating emotional responsivity to environmental influences could be one of the reasons for the excessive alcohol consumption of our animals. Therefore, the higher emotionality of our HAP strain might be one of the internal components which was enhanced by selective breeding, too. In this case the higher ethanol consumption can serve as a learned, adaptive response to stress. Ethanol might function in this respect by attenuating the effect of stress by its anti-conflict properties.

Changes in the brain neurotransmitter and/or neuromodulator system can also result in behavioural differences among the strains in responsiveness to environmental stimuli /4, 8, 10, 11/. After exposure to ethanol, the serotonin (5-HT) and/or dopamine content of the whole brain and/or various brain parts (hypothalamus, midbrain and thalamus, cortex) of ethanol-preferent rats become significantly higher than in nonpreferent individuals. In some other cases also inverse relationships were obtained. Possibly the neurotransmitter balance of the nucleus accumbens plays a very important role. In ethanol-preferent rats this brain area showed lower levels of 5-HT and dopamine, of 5-HIAA, of dihydroxyphenylacetic acid, and of homovanillic acid than that of nonpreferent rats. Preferent rats also showed a specific deficiency in the dopaminergic projection from the ventral tegmental area to the n. accumbens and, since the latter is an important structure in the brain reward circuitry, it might also be the immediate cause of the excessive voluntary intake of alcohol by preferent rats /21/.

In further studies quantitative genetic (biological) markers are required for predisposition in ethanol preference (or in case of human alcoholics), e.g. such as plasma transferrin, sialic acid, gamma-glutamyl transpeptidase, hormones, like prolactin, to compose a test battery, suitable for screening in animals and in men. Animal analogues can serve, however, as a useful tool in further experimentation.

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