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STUDIES OF TICK-BORNE ENCEPHALITIS AND OTHER ARTHROPOD-BORNE VIRUS DISEASES

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STUDIES ON TICK-BORNE ENCEPHALITIS AND OTHER ARTHROPOD-BORNE VIRUS DISEASES

FINAL TECHNICAL REPORT

By

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Abstract

(I) First focus of TBE virus detected in Switzerland.

Spraying of Gardona(R) and Malathion(R) in aquatic emulsion from the ground is more affective against ticks than ULV method.

Abate(R) also kills ticks in nature.

Rodenticides Caid (R) and Tomorine (R) reduced mouse population in foci.

Breaking virus cycle in nature probably rather achieved by control of mammals than by control of ticks.

(II) In 1972, 389 cases of TBE had been diagnosed in Austria.

(III) All volunteers developed antibodies against TBE virus after two doses of new formalin inactivated vaccine.

(IV) Attempts to synthesize TPI were not successful, however 4 receptor analogue substances could be synthesized, of which 2 exhibited a marked HA-inhibiting activity against TBE virus.

Various arboviruses were subjected to purification treatments in order to obtain or improve hemagglutinating activity.

Tilorone HCL and four derivatives were found to be active against TBE in mice.

(V) In a survey with 1162 avian sera from the Neusiedlersee area, antibodies against Uukuniemi, Calovo, TBE, West Nile and Semliki viruses were detected.

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(1) For the first time, a focus of tick-borne encephalitis (TBE) virus has been detected in Switzerland. Attempts to eradicate the virus from Austrian foci with insecticides and rodenticides suggest that rodent control may offer the most promising way of breaking the virus cycle, although supplementary tick control may prove useful. (2) In 1972, 389 cases of TBE were diagnosed in Austria. (3) A new formalin inactivated vaccine provoked antibodies in human volunteers receiving two doses. (4) Of four synthetic receptor substance analogs synthesised, two showed hemagglutination-inhibiting activity. Attempts to increase hemmaglutinating activity of arboviruses met with limited success. Tilorone and other interferon-inducing derivatives were active against TBE in mice. (5) In a survey of 1162 avian sera collected in the Neusiedlersee area antibodies against Uukuniemi, Calovo, TBE, West Nile and Semliki viruses were detected.

KEYWORDS: ARBOVIRUSES IN AUSTRIA; TICK-BORNE ENCEPHALITIS ERADICATION; VIRUS RECEPTOR SUBSTANCE ANALOGS; TICK-BORNE ENCEPHALITIS IN SWITZERLAND; BIRDS AS ARBOVIRUS HOSTS; TICK CONTROL IN AUSTRIA; RODENT CONTROL ON AUSTRIA; TICK-BORNE ENCEPHALITIS INFECTIONS IN MAN; VACCINATION AGAINST TICK-BORNE ENCEPHALITIS; INTERFERON INDUCERS AND ARBOVIRUS INFECTION.

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Field Studies

(1) Detection of new foci in Austria and attempts to eradicate the virus in nature.

(1,1) Introduction.

In 1971 and 1972 field studies were continued. The main purpose of these investigations was to locate foci where a larger number of persons have become infected and therefore a tick or mammal control program ought to be conducted. In addition, various control programs were carried out.

Also in autumn 1972 and in 1973 we collected ticks in localities that, according to information obtained by patients, possibly were foci of TBE virus. The eradication programs with the insecticides Gardona (R) and Malathion (R) were continued and the effectiveness of another organophosphoric compound, namely Abate (R), was proved against ticks. In order to reduce the population density of rodents which had been shown to be the main hosts of larvae of Ixodes ricinus and the main reservoir of TBE virus, poisoning programs with the rodenticides Caid (R) (chlorophacinone) and Tomorin (R) (cumarine) were started in two places. Both compounds are anticoagulants.

(1,2) Methods.

Ticks: Nymphs and adults of Ixodes ricinus were collected by flagdragging and transported to the laboratory. The nymphs were homogenized in pools of 1-20 individuals, the adults in pools of 1-10 individuals, respectively. They were suspended in a medium consisting of PBS and 10% calf serum and inoculated intracerebrally into baby mice. The animals were observed for ten days.

Gardona (R): In a field trial we attempted to control the focus of Jauling/Enzesfeld where patients had contacted TBE and where also virus was found in ticks in the spring of 1972. On June 5, 1972, 10 hectares (ha) of a dense forestation were sprayed with Gardona(R) from the air using the Ultra Low Volume (ULV) method. Ten days thereafter the area was checked for ticks in order to evaluate the effectiveness of the organophosphoric compound sprayed from an airoplane.

Malathion (R): On June 29, an area of about 10 ha of a pinewood-fir tree afforestation near Hirtenberg, where a focus was found by virus isolation from ticks collected there, was sprayed from the air with Malathion(R) applying the ULV method. Another field trial with Malathion(R) was done in a dense mixed forest near Jauling/Enzesfeld by spraying of an area of about 10 ha from an airoplane.

Abate (R): Two field trials were done in order to develop a control program with this compound against ticks. In the spring of 1973 in a wood near the Danube at Mühlleiten, a few miles southeast of Vienna, and in an afforestation near Hernstein we marked 6 fields of 25 m^2 each. Three of these fields were sprayed from the ground with a concentration of 20 g Abate(R) diluted in 3 liters of water per 25 m^2. Tick collections were made before spraying and on the 3rd and 8th day thereafter.

Small mammals: In the fool of Hohenegg and Teggenbrunn eradication of the virus by poisoning of small mammals was attempted. Fifty small mammal traps were set up for two nights in the focus of Hohenegg and about 100 traps were set up for 2 nights in the focus of Teggenbrunn before and after the poisoning by rodenticides (Dates of excursions see Table 4).

Combined eradication program with Malathion and rodenticides: In April of 1973 an eradication program was initiated in Nouwaldegg (Vienna) to control the focus found there last year (1972). Against ticks Malathion(R) was used (swingfog method) and poisoning of mice was done with Caid(R).

(1.3) Results.

Searching for new foci: According to the information obtained from patients with TBE, ticks were collected in different areas in Vienna, Lower Austria, Upper Austria and Salzburg. Also the surveillance of the known foci in Taggenbrunn, Hochosterwitz, Hohenegg and Jauling/Enzesfeld were continued. The results can be seen in Tables 1 and 2.

Six strains of TBE virus could be isolated from ticks collected at two different places in Neuwaldegg/Salmannsdorf (Vienna) indicating a high infection rate in the tick population under investigation. Three strains of TBE virus were isolated from ticks collected in Hochosterwitz and 1 strain was isolated from ticks collected in the focus of Hohenegg in spring 1973.

Gardona (R): The results of the field trial done in the focus of Jauling/Enzesfeld on June 5, 1972, did not have the effect that we had hoped for: in the area sprayed by an airplane a total of 83 nymphs and 19 adults of Ixodes ricinus as well as 11 nymphs of Haemaphysalis concinna could be collected during 1 hour of collecting time 10 days after the spraying. In a nontreated control area the same method of sampling yielded 87 nymphs and 7 adults of Ixodes ricinus and 1 nymph of Haemaphysalis concinna indicating the ineffectiveness of the ULV spraying method from the plane.

Malathion (R): The results of our first field trial with Malathion(R) which were done in Hirtenberg on June 29, were slightly better than those of the field trial with Gardona(R). In one hour of sampling time 71 nymphs and 12 adults of Ixodes ricinus as well as 11 nymphs of Haemaphysalis concinna could be collected before spraying. On the third day after treatment only 16 nymphs of Ixodes ricinus and 1 female of Haemaphysalis concinna were found.

The results of the second field trial, however, showed hardly any effect of the Malathion(R) treatment on the tick population in the area under investigation. In one hour of collecting time 94 nymphs and 10 adults of Ixodes ricinus were collected before treatment on April 29, 1973. After the treatment on May 4, 1973, at the same site 52 nymphs and 7 adults of Ixodes ricinus were collected in one hour of collecting time.

Abate (R): The results of the field studies with Abate(R) are presented in Table 3 and Fig. 1. A statistical evaluation done with the χ^2-test revealed that the number of ticks in the fields did not differ significantly prior to the treatment with Abate(R). By contrast, after spraying with the compound there was a very significant difference between the number of ticks collected in the treated and in the nontreated fields. In both areas under investigation the tick populations could be reduced by more th 95%, indicating a very good effectiveness of the Abate(R) treatment on ticks.

Eradication program of small mammals: As a first step in our eradication program the population density of small mammals was studied by using the mark and release method. The first excursion to Hohenegg was done from August 6-8, 1972. During two trapping nights (100 trap units) altogether 32 trappings of small mammals were done. We marked

12 Apodemus flavicollis as well as 9 Clethrionomys glareolus and released these animals in order to determine the population densities of these species. Two Sorex minutus, 1 Clethrionomys glareolus and 2 Apodemus flavicollis died in the traps.

In the second excursion from December 8-10, 1972, we marked and released in 100 trap units 12 specimens of Apodemus flavicollis and 7 specimens of Clethrionomys glareolus, as well as 2 Sorex araneus. After the last trapping night about 1500 g of the poisoned seeds were brought out on an area about ten times larger than the trapping area (about 12 ha). After 2 weeks, from December 23-24, 50 traps were set up again during one night. Not a single rodent could be trapped. Only one specimen of Sorex araneus which is an insectivore and does not feed on the seed baits was caught.

Also during the 4th excursion, a very low number of small mammals was caught, namely 1 Apodemus flavicollis, 1 Sorex araneus and 1 specimen of Sorex minutus.

During the 5th excursion, however, 3 Apodemus flavicollis, 7 Clethrionomys glareolus and 2 Sorex minutus could be trapped.

Another similar program was done in Taggenbrunn, using the rodenticide Tomorin(R) for poisoning the small mammals. The numbers of trappings and specimen of small mammals caught in the different excursions can be seen in Table 4. In this area the population density of small mammals was high in 1972 but very low during the two excursions in spring 1973.

Combined eradication program with Malathion(R) against ticks and with rodenticides against small mammals: In the focus in Vienna which had been discovered in 1972, on April 26, 1973, in 33 trap units 14 Apodemus sylvaticus could be trapped in this area indicating a very high population density of this mouse species. After half an hour of trap-

ping time 22 nymphs and 9 adults of Ixodes ricinus could be collected on the same area 9 days after treatment with Malathion(R) by the swingfog method. In half an hour collecting time 14 nymphs and 7 adults of Ixodes ricinus were collected, indicating a low effect of the treatment on ticks. The studies on the population densities of ticks and mice shall be continued.

(1,4) Discussion and conclusions.

The results of our eradication program are somewhat puzzling. On the one hand a very good effect of all three organophosphoric compounds in aquatic emulsion and sprayed from the ground could be shown against ticks. On the other hand, the ULV method of spraying by airoplane and the swingfog technique showed very weak effects on ticks. Thus the mode of application appears to be very important. Whenever the high vegetation in the forest was very dense little could be achieved with the ULV method. This technique is only suitable for treatment of forests such as in Hirtenberg, where the focus is located in a young pinewood forestation which allows penetration of the insecticide towards the low vegetation on the ground. In future studies the size of droplets and composition of the solution containing the insecticides will be varied in order to find out whether or not the ULV method could be made more effective when the undergrowth in a focus is dense. Large scale central programe with insecticides against ticks are only practicable if the ULV method, preferably applied from a plane, could be used.

The small mammal control program with rodenticides seems to be quite effective; however, it will have to be carried out at regular intervals because of immigration of mice from neighbouring areas. One wonders, nevertheless, if this way of trying to eradicate a focus is not a better approach than the appli-

cation of insecticides. In Taggenbrunn, where we have tried to control the small mammals by trapping for the last four years 25 virus strains were isolated in 1970, the year, when the trial started (Table 5). In 1971, only 3 strains of virus were found and in the ticks collected in the subsequent two years 1972 and 1973 no virus was detected. By contrast, the tick control program in Hochosterwitz, although spraying was done 3 times from the ground since 1970, was not met with complete success. Numbers of virus strains isolated from ticks declined, but even after two years (1972), 3 pools of ticks collected there were still found to contain virus (Table 5).

Perhaps it would be best to combine both methods of attempting to break the virus cycle in nature. Such studies will be done in continuation of the program.

(1,5) Summary.

Besides successful virus isolations from ticks collected in Hohenegg and Hochosterwitz a new focus could be detected by a virus isolation from ticks which were collected in Neuwaldegg/Salmannsdorf, Vienna.

Field trials done with the insecticide Gardona(R) and Malathion(R) in different foci using the ULV method of spraying showed a very weak effect as regards reduction of the tick populations. These results are in contrast to the observed effectiveness of both insecticides when these compounds were sprayed from the ground in an aquatic emulsion. The effectiveness of the organophosphoric compound Abate(R) against ticks was proved in 2 field trials. A reduction of tick populations of more than 95% was achieved. The rodenticides Caid(R) and Tomorine(R) showed a high effectiveness in reduction of mouse populations in the two areas of investigation. Stu-

dies in foci of TBE virus showed that control of mouse population is a better method of breaking the virus cycle in nature than control of ticks by insecticides.

(2) Survey on the occurrence of TBE virus in Switzerland.

In the last year's report (14) a survey was mentioned which was done with 560 sera of Carnivora shot in Switzerland. 48 specimens were found to contain antibodies against TBE virus in both the HI and the neutralization test. The geographic distribution of the positive sera shows that TBE virus must be more widely distributed in Switzerland than hitherto known.

Besides one successful isolation of a strain of TBE virus from the brain of a dog deriving from Hallau (Kanton Schaffhausen) in Switzerland no isolation of virus from ticks had been reported until now. Therefore, Dr.Radda took the opportunity of working for one month in the Department of Virology, Institute of Bacteriology of the Veterinary Faculty, University of Bern, and in the Institute of Virology of the Veterinary Faculty, University of ZÜrich, from May 17 until June 14, 1973. The main purpose of this study was to collect ticks in places where cases of TBE had been observed.

The results of the tick collection in different parts of Switzerland and the isolation of one strain of TBE virus are listed in Table 6. It can be seen that altogether 2542 nymphs and 528 adults of Ixodes ricinus were collected in 15 different places of 5 Kantons (provinces) (Bern, Fribourg, Vaud, ZÜrich and Solothurn) in Switzerland. The single successful isolation was done from a pool consisting of 5 females of Ixodes ricinus collected on June 7, in a mixed forest near Rheinau, Kan-

ton Zürich. This is the hitherto first isolation of TBE virus from ticks collected in Switzerland.

(2,1) Summary.

A total of 3070 individuals of Ixodes ricinus were collected in 15 different places of 5 Kantons of Switzerland. One strain of TBE virus was isolated from ticks collected in a mixed forest near Rhsinau, Kanton Zürich, in Switzerland.

Diagnostic Studies on Patients

In the year 1972, 323 cases of TBE have been diagnosed mainly by means of the 2 mercaptoethanol-test (12) using one serum specimen. Only in a few cases a second blood sample had to be drawn for the complement fixation test. In addition, 66 cases of the disease occurring in Styria were reported by the Institute of Hygiene, University of Graz.

The distribution of the disease in Austria was the same as in the last years. Table 7 summarizes the incidence of the disease in the different parts of the country. In the first six months of the year 1973, 97 cases have been recorded (see Table 8). Thus, approximately the same incidence as in the last year can be expected.

Trial of TBE vaccine

(1) Introduction

It is apparent that the number of cases of TBE in Austria occurring each year might be decreased provided that a potent and safe vaccine were available. Presently no such vaccine is on the market. Six years ago in a limited trial with a formalin-inactivated TBE vaccine of Eastern European origin very discouraging results were obtained by us. Of 25 persons who had received 3 doses of vaccine at monthly intervals none developed hemagglutination inhibiting antibodies.

In search of a better vaccine we learned that Dr.Keppie of the Microbiolngical Research Establishment (MRE), Porton Down, Salisbury, England, produced a small batch of louping ill-vaccine to be used for protection of personnel handling this virus. Louping ill virus, which is very closely related to TBE virus, is the cause of a disease of sheep in Great Britain. When man becomes infected he develops a clinical picture which is indistinguishable from TBE. However, human cases are quite rare.

After a visit of Dr.Kunz to Porton it was decided to produce a batch of TBE vaccine for human use following the procedures for the louping ill-vaccine. In order to make sure that the seed virus was devoid of undesirable agents such as mouse-tumour viruses we collected ticks in known foci in Austria and made virus isolation experiments in mice. Three pools of tick suspensions which thus were found to contain TBE virus were selected and shipped to the MRE.

The vaccine subsequently made by Dr.Keppie is a formalin-inactivated virus suspension with adjuvant. Safety-testing was done in England. Upon re-

ceiving the ampules we were advised of some pyrogenicity that has been recorded in rabbits. Because the vaccine had passed the other tests and in view of the urgency of the problem of TBE in Austria we felt that a clinical trial was in order. The volunteers were, however, informed about the fever - inducing potential of the experimental vaccine.

All persons received 1.0 ml each subcutaneously in the upper left arm. Three weeks thereafter a second dose of 1.0 ml was given. Thus far 78 volunteers were included in the study of which 58 already received the second injection. In order to record any adverse effects of the vaccine all volunteers were kept under close surveillance for 3 days following the first shot. After the boost dose the vaccinees were kept under no such rigid supervision. However, they were told to report any side effect occurring.

Levels of circulating antibodies prior to and after vaccination were measured in the hemagglutination inhibition (HI) test. This test is our routine technique for both serological surveys and diagnosis of TBE.

(2) Results

(2.1) Local and systemic reactions: Most volunteers complained about a local stinging immediately after injection. It passed, however, within a minute or so.

The day after vaccination up to three days thereafter the site of injection became tender on pressure and, occasionally, painful. Only in two cases a slight inflammation and swelling was seen. After the first injection 18 persons (23%) developed temperatures above normal. However only in 3 cases did the Pyrexia exceed 38°C. Association of these temperatures with headache and fatigue or malaise was common. These general symptoms subsided within

24 hours. It is of interest that hardly any systemic reactions were recorded after the second dose of vaccine. Two volunteers had temperatures slightly bove normal (37.3°C) the day after vaccination. We have no explanation for this phenomenon other than the possibility that some of the volunteers were a little anxious in the beginning and expected worse reactions than later actually experienced.

(2,2) Antibody response in vaccinated persons: In Table 9 the volunteers are listed according to the individual's number. It will be seen that numbers 1, 7 and 34 had antibodies prior to vaccination. No.1 and 7 had a history of laboratory infection with TBE virus and No.34 is an Egyptian who may have become infected at home with a group B arbovirus other than TBE. Of those 47 volunteers in Table 9 who had no prevaccination antibodies, 37 (79%) showed seroconversions 3-4 weeks after the first dose. Titers ranged between a minimal acceptable value of 1:10 and 1:80 with a geometric mean of 1:14.

All persons thus far tested 2-4 weeks after the second dose possessed antibodies including 6 of those who were still sero-negative after the first dose. The geometric mean value reached 1:50.

The result of the field trial is very encouraging indeed - a high rate of seroconversion after the first dose and 100% of the volunteers with detectable antibodies after the second is more than we had expected.

Perhaps there is still a little improvement possible as far as the boosting is concerned. It is striking that both volunteers with a history of infection some years ago (numbers 1 and 7) were very well boosted after the first vaccination, while not all of those without prevaccination antibody exhibited a booster effect after the second dose. It may, therefore, very well be that more consistent boosting could be observed with the vaccine if the initial antibody response had already fallen below its maxi-

mal value. The present study will, therefore, be continued with the first and second dose given 8 weeks apart.

We intend to bleed the volunteers in a follow-up study 3 and 6 months later in order to determine levels of antibody. The immunization schedule will be continued with a boost dose after 6 months. It remains to be determined whether boosters are necessary at yearly intervals or less frequently.

(2,3) Summary: Of 78 persons given the TBE vaccine most complained about stinging immediately after injection. Other than that local reactions were mild.

23% of the volunteers had a rise in body temperature the day following first injection. This was probably due to a pyrogenicity of the vaccine as demonstrated in rabbits. This pyrogenicity can certainly be avoided in batches commercially available.

Three-four weeks after the first dose 79% of the volunteers had a minimal acceptable value of 1:10 in the HI test, with a geometric mean value of 1:14 for the whole group. All persons passed 3-4 weeks after the second dose with a mean value of 1:50.

Experimental Laboratory Investigations

(1) Investigations on the receptor substance for TBE virus and other group B arboviruses.

(1,1) Introduction and earlier results.

By using a very sensitive technique for the competitive inhibition of the hemagglutination (HA) of arboviruses (8), we could demonstrate that the chemical substance responsible for the attachment (adsorption) of arboviruses of group B onto cell membranes is a polyphosphoinositide (PPI) and most probably a triphosphoinositide (TPI) (7). A preparation containing 90% TPI, 9% diphosphoinositide (DPI) as Ca-salts and less than 1% phosphotidylserine could be prepared from monkey brain in good yield (9). This preparation inhibited the HA of tick-borne encephalitis (TBE) virus in less than 0.04 µg/0.4 ml and was also able to inhibit the infectivity of group B arboviruses for tissue culture as well as for mice (13). Studies on the dynamics of the reaction between the virus and its receptor showed that a virus-receptor complex is formed at first by electrostatic attraction. This is followed quickly by stronger binding. Heat inactivated virus shows only the first step which is reversible by addition of certain basic molecules (10).

(1,2) Chemical synthesis of TPI.

Having identified the nature of the receptor for arboviruses of group B, it was tried to obtain TPI by chemical synthesis. However, neither direct phosphorylation of inositol nor the approach from acetobromoglucose which should yield TPI after 21 chemical steps was successful. The mixture of substances obtained after phosphorylation of inositol could not be separated successfully into identifyable compounds. The other attempt could be performed as far as step 9 which is 2,3-di-O-allyl, 6-(deoxy)-6-nitro-O-benzylglucoside. The hydroly-

tic removal of the benzyl group proved to be difficult and only extremely small quantities of the expected product could be obtained.

(1,3) Synthesis of receptor analogue substances.

It was decided to try the synthesis of easier obtainable compounds, different from the actual receptor substance, but with certain chemical similarities which might result in a similar biological activity. Four of such receptor-analogue substances could be obtained.

The first is racemic Inositol-1,4,5,6-tetraphosphate. This compound has the phosphoric acid groups in the positions 1, 4 and 5 just as triphosphoinositol from TPI. It also has the same sterical configuration as the natural receptor. Additionally, it contains one more phosphoric acid in position 6. From this compound, two more could be obtained. It was possible to esterify one of its phosphoric groups with glycerol and thus phosphoglyceryl-inositol-triphosphate was obtained. It cannot be stated which one of the phosphoric acid groups is involved in the reaction, and most probably our sample contains a mixture of all of the four possible structures. The inositol-tetraphosphate could also be combined with distearyl-glycerol, yielding phosphatidyl-inositol-triphosphate or tetraphosphoinositide, a compound which contains one molecule of phosphoric acid more than the naturally occurring TPI. However, as we do not know to which one of the phosphoric acid groups the glycerol is bound, it cannot be stated that the sterical configuration of our compound is the same as in the naturally occurring receptor substance. Again it is more probable that it is a mixture of different but similar structural analogues of the cell receptor, containing one component with also a sterically identical phosphorylated inositol group. A fourth receptor analogue compound was obtained by hydrolysis of natural TPI. Under mild conditions, the fatty acids could be split off, leaving a 1-(1-phos-

phoglyceryl)-inositol-4,5-diphosphate. A further substance with but a slight similarity to TPI is inositol hexaphosphate. Its Ca-salt is commercially available as "phytin".

(1,4) Inhibition of the HA of TBE virus by receptor analogue substances.

Phytin and the Ca-salts of the chemically prepared substances were now compared with TPI in the mentioned competitive HA-inhibition test. Apart from TPI, only two of the investigated compounds showed an appreciable HA-inhibiting activity (15), (Table 10). 1-(1-phosphoglyceryl)-inositol-4,5-diphosphate, which was obtained by hydrolytic cleavage of TPI, has therefore the same sterical configuration as the inositol moiety of TPI. It is reasonable to assume that its activity is due to the sterical similarity to the natural cell receptor. The other active compound, phosphatidyl-inositol-triphosphate can be called a tetraphosphoinositide. This substance cannot be regarded sterically similar to natural TPI in the sense that only one additional hydroxy group is phosphorylated.

As was stated already, this substance is a mixture of similar compounds, differing in the positions of the phosphoric acid groups on the inositol ring. Its biological activity as inhibitor of the HA of TBE virus seems also to be due to the lipid character of this group of substances which facilitates the formation of micelles. There might be compounds of different activity in this mixture. Future experiments are planned with the intention to separate this mixture and to isolate the most active component.

(1,5) Summary.

Chemical synthesis of TPI was attempted which is the receptor substance for group B arboviruses. However, neither direct phosphorylation of inositol nor

an attempt with acetobromglucose as starting substance were successful. By contrast, four compounds chemically similar to TPI were synthesized of which two, namely 1-(1-phosphoglyceryl)-inositol-4,5-diphosphate and phosphatydil-inositol-triphosphate, exhibited a marked HA-inhibiting activity against TBE virus.

(2) Purification of arboviruses.

(2,1) General considerations and earlier results regarding the purification by exclusion chromatography on porous glass.

It was considered to treat arboviruses with enzymes to increase the HA-titre of badly hemagglutinating preparations and also to obtain virions with exposed surface antigens. Such preparations should be useful as starting materials for the preparation of immunizing antigens and it might also be possible to obtain electron micrographs from virions having their spikes not occluded with material originating from the cell wall. For this purpose a method had to be worked out which allowed to separate the virions from the added enzymes as well as from the degradation products after the enzymatic treatment. Such a technique proved to be chromatography on porous glass (11). In the previous annual report (14) it could be shown that under certain conditions this method allows the separation of different arboviruses from smaller molecules. Compared with gel-chromatography it works to 10 to 20 times quicker.

(2,2) Enzymatic treatment of arboviruses.

From arbovirus preparations showing poor hemagglutination other authors (1) could prepare good hemagglutinins by treatment with trypsin combined with sonication. We tried to apply this technique to some none-hemagglutinating arbovirus preparations made by the sucrose-acetone method (5). Tahyna virus, Calovo

virus and Potepli virus were investigated. Our attempts with 90 to 150 sec sonication at 2 Watts and incubation with trypsin had no success. To test whether the sonication might possibly have destroyed any HA-activity revealed by the enzyme, a well hemagglutinating preparation of TBE virus was submitted to ultrasonics for 15 periods of 30 seconds duration at 2,4 and 8 Watts. Only at the highest intensity, a very slight decrease of the HA could be observed. This might be due to heating, which could not be prevented totally by performing the procedure in an ice-water bath. After this, Tahyna- and Potepli virus preparations were treated with much higher intensities of ultrasonics as before, i.e. 15 times 30 seconds at 6 Watts. But even this did not produce hemagglutination.

Experiments with trypsin were not performed any longer inasmuch as our previous experiments resulted in a destruction of the HA of TBE virus by trypsin (6). On the other hand, phospholipase C and neuraminidase produced hemagglutinating preparations from Mouse Leukemia virus (17) and from Avian Myeloblastosis (16) and exposed the surface structure of Vesicular Stomatitis virus (4). Initial experiments with different arboviruses prepared by sucrose-acetone extraction (5) had no success. The supernatant from low speed centrifugation of mouse brain homogenized in borate buffer of pH 8.5 proved to be an excellent starting material for this sort of enzymatic treatment. The HA-titer of West Nile virus could be increased by incubation with 25 µg phospholipase C and 50 U neuraminidase at pH 7.5 to 7.6 from 8 to 128. A comparison of HA titers of TBE virus measured at different pH values after treatment with saccharose acetone, protamin sulfate, neuraminidase + phospholipase, and, finally the same treatment followed by protamin-precipitation, is shown in Table 11. A remarkable broadening of the pH-optimum of TBE-HA could be observed after enzymatic digestion combined with protamin treatment.

Further experiments were undertaken with alkaline extracts of mouse brains infected with West Nile virus. It could be shown that the increase in HA-titer could also be obtained by digestion with neuraminidase alone. Phospholipase alone had no effect and its application together with neuraminidase did not increase the effect of the latter enzyme (Table 12). (This is in contrast to the results of other authors working with mouse leukemia and avian myeloblastosis virus (16,17)). Regarding the pH-dependance of the HA of West Nile virus, treatment with neuraminidase showed no advantage over precipitation with protamin sulfate (Table 13). In one experiment, the virus-enzyme mixtures were incubated for different periods of time. It could be shown that under the described conditions there was a sharp rise in the HA-titer up to 10 minutes incubation at 37°C (Table 14). It was hoped therefore, that future experiments should be performed successfully also with a much smaller concentration of neuraminidase. This could be shown to be true only when to the diluting buffer $CaCl_2$ as activator and albumin as protective colloid were added (Table 13).

Also, the most dramatic effect in evoking a high HA-titer, which was observed with Chikungunya virus, could be reproduced using 1/10 the concentration of neuraminidase (Table 16). With this virus, however, enzyme treatment alone did not elicit any HA-activity. Protamin sulfate precipitation resulted in a very sharp HA-optimum at pH 6.1, whereas a combination of neuraminidase action with protamin precipitation showed a great increase of HA-titer as well as a broadening of its pH-optimum.

It might very well be that - at least with TBE virus - the actual ability of arbovirus particles to adsorb onto cell membranes is marked by at least two different inhibitors. One, acting in a more acid range, can be removed by precipitation with protamin. The other, inhibiting the HA of the virus in neutral milieu, is removable by digestion with neuraminidase.

In contrast to the good results with TBE and Chikungunya virus, enzymatic treatment of Potepli virus had apparently no adventage over protamin precipitation and was in this respect similar to its effect on West Nile virus. Preliminary experiments with alkaline extracts of a strain of Tahyna virus from which it was not possible to obtain hemagglutinating preparations by other means yielded so far also negative results when this virus was treated with neuraminidase either alone or followed by protamin sulfate precipitation. It might be possible that this virus demands an addition of phospholipase. Also it is considered to combine ultrasonic treatment with enzymatic digestion to obtain positive results in this case.

It has not yet been tried to purify enzyme treated virus preparations further by chromatography on porous glass. This will be done in the immediate future.

(2,3) Summary.

Various arboviruses were subjected to purification treatments in order to obtain or improve hemagglutinating activities.

Treatment of non-hemagglutinating sucrose-acetone antigens of Tahyna, Calovo and Potepli viruses with trypsin and sonication did not yield hemagglutinins.

When TBE virus was submitted to different purification treatments (sucrose-acetone, protamin, neuraminidase, phospholipase C) a remarkable broadening of the pH-optimum of HA was observed after enzymatic digestion combined with protamin precipitation.

Digestion with neuraminidase alone of crude alkaline suspensions of West Nile virus (group B) resulted in 32-fold increase of HA-titer while no such effect was seen when phospholipase C was used.

Chikungunya virus (group A) HA, which has a very narrow pH-range was considerably widened by treatment with neuraminidase.

(3) The antiviral effect of derivatives of Tilorone HCL against TBE virus in mice.

In previous studies (12) the interferon inducing compound Tilorone HCL was found by us to prevent fatal TBE in mice. In this present study four derivatives (labelled RMI 10 874, RMI 11 567 DA, RMI 11 002 DA, RMI 11 877 DA), which were supplied by the Merell-National Laboratories, USA, were tested against TBE in mice and compared with Tilorone HCL.

As can be seen from Table 17 all the substances induced interferon in serum of mice. Only minor differences of interferon levels were observed.

In the first experiment the compounds were given orally in a dose of 250 mg/kg mouse 24 hours prior to infection with 120 LD_{50} of TBE virus (strain Hypr). The results can be seen in Table 18. The compounds were used combined in the next experiment, which is summarized in Table 19. Mice were infected with 27 LD_{50} of TBE virus. This table clearly indicates that nothing was gained by combining the compounds; to the contrary, rather an adverse effect was observed.

Thereafter the compounds were given by the subcutaneous or intraperitoneal route in a dose of 100 mg/kg mouse - higher doses are toxic for mice. The results of this experiment (4 LD_{50}) can be seen in Table 20. Thus the compounds are also active after parenteral application. The most striking result is, that the substance RMI 11 877 DA is highly active after parenteral application, although it had only a week effect after oral administration (see Table 18). This result is probably due to bad intestinal resorption. In the next experiment, the

compounds were therefore given orally in a high dose (Table 21). Animals were infected with 36 LD_{50} of TBE virus. Comparing these results with those from Table 18 it is evident that better results with higher doses cannot be obtained with Tilorone HCL, but with the other compounds.

Finally we combined the Tilorone-like substances with the interferon inducer Poly I:C. The results from an experiment, in which 61 LD_{50} of TBE virus were given, are summarized in Table 22. The Tilorone derivatives were given orally 24 hours before infection and Poly I:C intraperitoneally 2 hours after infection. It will be seen that the low dosage of Poly I:C was not effective against the virus when given alone. However, it considerably increased the antiviral effect of Tilorone HCL and its similar compounds.

From our data it can be concluded that the four derivatives of Tilorone tested are potent antiviral substances against TBE virus. However none exceeded the effectiveness of Tilorone.

(3,1) Summary.

Tilorone HCL and 4 of its derivatives induced interferon in mice and protected the animals after oral, intraperitoneal or subcutaneous application against a challenge dose of TBE virus. Combination of the compounds with Poly I:C increased the antiviral activity.

Studies on the importance of birds for arbovirus activity in Central Europe.

(1) Introduction.

In autumn 1971 we started a program with the aim to elucidate the role of birds as hosts of arboviruses in Austria in particular, and in Central Europe in general. Two main questions formed the basis of this project, namely whether or not arboviruses may be introduced by birds from tropical and subtropical regions to Central Europe and whether and to which degree birds take part in the circulation of arboviruses occurring in Central Europe more or less regularly.

The results obtained until May 1972 were presented in the Final Technical Report of 1972 (14). They were based on virological and serological studies with 786 blood samples of 28 bird species. One strain of a virus so far unidentified was isolated from a robin returning from hibernation; hemagglutination inhibiting antibodies (altogether 46 positive reactions) were found against Uukuniemi, Calovo, Chikungunya, Semliki, Sindbis, TBE, West Nile, Yellow Fever and Dengue II in 11 bird species.

These studies were continued in 1972 and 1973 in order to get further and large scale informations on the above mentioned problems. In addition, it was intended to check the sera showing positive reactions in the hemagglutination inhibition (HI)-test also in the neutralization test (NT). This appeared of particular importance due to the fact that hemagglutination inhibiting antibodies had been found against A group viruses in non-migrating birds.

(2) Methods.

All birds were captured with Japanese mist nets in the reed zone of the Neusiedlersee near the village Neusiedl in the Northeastern part of the lake. Blood was taken from the jugular vein exclusively. Birds were marked and released immediately after puncture. Individuals recaptured some days later were released without taking a blood sample. Blood samples collected in spring, summer and autumn were not only tested for antibodies - as in the case of sera from birds collected during winter - but also for virus. For this purpose a small part of the blood was immediately frozen in dry ice and then kept at -80°C until virus isolation experiments were done. These were carried out by intracerebral inoculation of the blood into baby mice. The mice were observed over a period of two weeks.

The main part of the blood (up to 0.2 ml) was immediately diluted in 0.5 ml PBS, kept in ice for some hours and then frozen at -20°C until serological examination. All sera were (or will be) primarily tested for hemagglutination inhibiting antibodies according to the reference method of CLARKE and CASALS against the following antigens: TBE, West Nile, Uukuniemi, Chikungunya, Semliki, Sindbis, Calovo and Tahyna. Sera which proved to be positive in the HI-test were (or will be) checked in the NT as far as a sufficiently high amount of serum is still available. The neutralization tests against Tahyna and Chikungunya viruses were carried out in the established cell-line GMK-AH 1, against TBE virus with L-cells, and against Calovo, Semliki, Sindbis and West Nile viruses in chicken embryo cells. The occurrence of neutralizing antibodies against Uukuniemi virus was examined in baby mice in the usual manner.

(3) Results.

As the investigations carried out in 1972 and 1973 represent a continuation and completion of the studies reported in the Final Technical Report 1972 and as many of the samples collected during 1971/1972 had not yet been evaluated when the report was written, it appears justified and advisable for better understanding to summarize all results so far obtained *.

From autumn 1971 until May 1973 blood samples of 1162 birds belonging to 30 species were collected of which 689 were tested for virus and 1078 for hemagglutination inhibiting antibodies against the antigens listed above. Besides the strain isolated from the blood of a robin in spring 1972 (see Annual Report 1972) (14) no additional virus was isolated. Despite further trials this strain could not yet be identified. It is, at any rate, with certainty not any of the arboviruses hitherto known to occur in Europe.

The results of HI-tests are shown in Table 23. Altogether 94 positive reactions were found in a total of 84 birds; 76 birds had antibodies against only one of the antigens used, 6 sera reacted with two antigens, and 2 sera with three antigens. These sera reacting with more than one antigen are listed in Table 24.

From the 94 positive reactions in the HI-test so far 59 have been checked in the NT. The results obtained (see Table 25) verified the occurrence of antibodies against Uukuniemi, West Nile, TBE, Semliki and Sindbis viruses in one or more bird species.

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* Blood samples of 32 starlings collected in 1970 which were processed with other methods (see Final Technical Report 1972) are, however, excluded from this account.

(4) Discussion.

As mentioned above, the investigations reported here were carried out in order to get informations particularly on the possibility of introduction of arboviruses to Central Europe by migrating birds on one hand and on the role of birds in the circulation of arboviruses in Central Europe on the other hand.

The results of virus isolation experiments do not allow to draw any definite conclusions with respect to either of these questions. Out of 689 blood samples only one virus strain was isolated, and this could unfortunately not yet be identified.

On the basis of hemagglutination inhibition and neutralization tests carried out with 1078 avian sera some substantial informations could, however, be obtained. As can be seen from Table 25 only a part of those sera reacting positively in the HI-test gave also positive reactions when checked in the NT. In some cases this may certainly be traced back to nonspecific or to cross reactions in the HI-test, probably in the majority it can, however, be explained by the fact that the blood was diluted immediately after puncture in 0.5 ml PBS, so that in case of low amounts of blood obtained very low concentrations of antibodies resulted which were unable to neutralize the virus.

At any rate, the results of neutralization tests verified the occurrence of antibodies against Uukuniemi, West Nile, TBE, Semliki and Sindbis viruses in several bird species (Table 25).

Positive reactions against a number of antigens (or viruses) in several migrating birds are not surprising and reflect prior infections with these or related arboviruses, mainly in tropical and subtropical regions. In these cases the confirmation by the detection of neutralizing antibodies against the respective viruses is not of that essential importance as it is

the case in non-migrating birds which reflect activity of arboviruses in Europe.

Thus, the following conclusions can be drawn:

(1) The high percentage of positive sera against Uukuniemi confirms that birds are in fact essential vertebrate hosts of this virus and that the virus must also occur in Austria.

(2) The fact that no hemagglutination inhibiting antibodies against Tahyna virus could be detected in any of the 1078 sera tested leads to the assumption that birds cannot play any essential role in the cycle of this virus. It would, however, be of considerable interest to carry out a large scale study on starlings (Sturnus vulgaris), a species in which neutralizing activities against Tahyna virus have been found in a previous study (1,3).

(3) Infections of birds with TBE virus do apparently occur, but they are rare and do certainly not have any importance for the virus circulation.

(4) Hemagglutination inhibiting antibodies against Calovo virus were found in three sera only; these findings could not be confirmed in the NT. It is, therefore, doubtful whether birds are even susceptible to the virus. At any rate, they are certainly of no importance for the virus circulation.

(5) Hemagglutination inhibiting antibodies against West Nile were found not only in the migrating species of the genera Locustella and Acrocephalus, but also in Penduline Tits and Blue Tits caught during winter. Both findings could be confirmed in the NT. Although single individuals of the Central European populations of these birds sometimes fly to Northern Mediterranean regions, it is unlikely that all positive reactions are to be traced back to such

relatively rare events. It must, therefore, be taken into consideration that West Nile virus may occur in Central Europe, either very locally or perhaps not regularly, but only in some years after introduction from the South of Europe by birds. As the main vector of the virus, Culex modestus, occurs not too rarely in the Neusiedlersee area, the basic conditions for virus circulation are given.

(6) The most interesting results are represented by the occurrence of hemagglutination inhibiting antibodies against group A arbovirus in Bearded Tits and in Blue Tits which have been confirmed by the positive reactions in the NT against Semliki virus. This leads to the conclusion that a group A arbovirus occurs - at least periodically - in Europe. So far it remains uncertain whether it is a virus known from other geographical regions or an agent still undiscovered. It is intended to carry out further studies in order to clarify these problems.

(5) Summary.

From September 1971 to May 1973 1162 avian sera comprising 30 species were collected in the Neusiedlersee area in Eastern Austria. So far, 689 sera were tested for virus, 1078 for hemagglutination inhibiting antibodies against the following antigens: TBE, West Nile, Uukuniemi, Chikungunya, Semliki, Sindbis, Calovo and Tahyna. Only one strain of a virus still unidentified was isolated from a robin returning from hibernation in spring 1972. 94 positive reactions were obtained in the HI-test comprising all antigens listed above except Tahyna. 59 of these positive reactions were checked in the neutralization test, whereby the occurrence of antibodies against TBE, West Nile, Uukuniemi, Semliki and Sindbis could be verified. As regards European arboviruses the following conclusions can be drawn:

(1) Most positive reactions were obtained with Uukuniemi virus, thus confirming the important role of birds in the circulation of this virus.

(2) None of the sera had hemagglutination inhibiting antibodies against Tahyna. It is, therefore, very unlikely that birds play any essential role in the circulation of this virus.

(3) Three sera were positive against Calovo in the HI-test, but negative when checked in the NT. It is, therefore, doubtful, whether birds are even susceptible for this virus.

(4) A few sera showed hemagglutination inhibiting as well as neutralizing antibodies against TBE virus. This indicates that birds are sometimes infected with the virus, but do certainly not play any important role in the circulation of the virus.

(5) The occurrence of hemagglutination inhibiting and neutralizing antibodies against West Nile virus in Penduline Tits and Blue Tits leads to the assumption that this virus may - perhaps not regularly but only in some years after introduction from the south of Europe - occur in Central Europe.

(6) A few sera of non-migrating birds (Bearded Tits and Blue Tits) had hemagglutination inhibiting and, in part, also neutralizing antibodies against Somliki virus. These findings represent an important hint for the (perhaps periodical ?) occurrence of a group A arbovirus in Europe.

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Table 1: Number of ticks (*Ixodes ricinus*) collected in different areas in Vienna (V), Lower Austria (L.A.), Salzburg (S), Upper Austria (U.A.) and Carinthia (C), and virus isolations therefrom in 1972.

Location Date	Nymphs collected	Strains isolated	Adults collected	Strains isolated
Sophienalpe (V) July 8	15	-	2	-
Neuwaldegg I (V) July 9	40	-	36	2
Neuwaldegg II (V) July 9	43	1	106	3
Geras (L.A.) Sept.19	80	-	17	-
Rosenburg (L.A.) Sept.19-20	244	-	20	-
Bürmoos (S) Sept.22	71	-	26	-
Holzhausen (S) Sept.22	35	-	4	-
Ernsting (U.A.) Sept.23	142	-	22	-
Hochosterwitz (C) Aug.12	134	3	12	-
Taggenbrunn (C) Sept.12	31	-	17	-

Table 2: Number of ticks (*Ixodes ricinus*) collected in different areas in Vienna (V), Lower Austria (L.A.) and Carinthia (C), and virus isolations therefrom in 1973.

Location Date	Nymphs collected	Strains isolated	Adults collected	Strains isolated
Neuwaldegg/Salmannsdorf (V) April 26	22	-	14	-
Neuwaldegg/Salmannsdorf (V) May 4	14	-	7	-
Jauling/Enzesfeld (L.A.) April 25	36	-	10	-
Jauling/Enzesfeld (L.A.) May 4	52	-	7	-
Jauling/Enzesfeld (L.A.) May 21	34	-	-	-
Hohenegg (L.A.) April 30	83	1	1	-
Taggenbrunn (C) May 10	184	-	55	-

Table 3: Results of tick collection before and after Abate (R) treatment in Mühlleiten and Hernstein.

Mühlleiten:	Field no.	May 25 Number of nymphs	(adults)	May 28 Number of nymphs	(adults)	June 9 Number of nymphs	(adults)
Control fields:	1	-	(2)	4	(1)	2	-
	2	6	(3)	6	-	10	-
	3	3	(4)	1	-	-	(3)
Total		9	(9)	11	(1)	12	(3)
Treated fields:	4	2	(3)	-	-	-	-
	5	14	(4)	1	(1)	1	-
	6	28	(3)	-	-	-	-
Total		44	(10)	1	(1)	1	-
Hernstein:		June 1st		June 5		June 13	
Control fields:	3	12		6	(6)	7	(4)
	4	3		10	(7)	17	(8)
	5	11		6	(1)	14	(1)
Total		26		22	(14)	38	(13)
Treated fields:	6	15	(1)	1	(3)	-	(3)
	7	16	(2)	1	(2)	2	(1)
	8	11	(6)	-	(1)	2	(1)
Total		42	(9)	2	(6)	4	(5)

Table 4: Results of free living rodent population reduction by poisoning with rodenticides in Hohenegg and Taggenbrunn.

Hohenegg:

Excursion	Date	Trapped animals
1 +)	August 6-8, 1972	14 Apodemus flavicollis 2 Sorex minutus 10 Clethrionomys glareolus
2 +)	December 8-10, 1972 1972	12 Apodemus flavicollis 7 Clethrionomys glareolus 2 Sorex araneus
3 ++)	December 23-24, 1972	1 Sorex araneus
4 ++)	February 24-26, 1973	1 Apodemus flavicollis 1 Sorex araneus 1 Sorex minutus
5 ++)	April 28-30, 1973	3 Apodemus flavicollis 7 Clethrionomys glareolus 2 Sorex minutus

Taggenbrunn:

1 +)	August 12-14, 1972	16 Apodemus spec. 1 Clethrionomys glareolus
2 +)	September 11-13, 1972	23 Apodemus spec. 1 Clethrionomys glareolus 3 Sorex minutus
3 +)	April 29-30, 1973	2 Apodemus spec. 1 Clethrionomys glareolus
4 ++)	May 8-10, 1973	3 Apodemus spec.

+) Before poisoning program
++) After poisoning program

Table 5: Number of ticks (*Ixodes ricinus*) collected in Hochosterwitz and Taggenbrunn in 4 subsequent years and number of virus strains isolated therefrom.

Excursion date	Nymphs collected	Number of strains isolated	Number of adults collected	Number of strains collected
Hochosterwitz				
1969				
Sept. 1 - 5	206	4	89	2
1970				
May 26	298	3	100	7
1971				
July 7	272	4	20	-
Sept. 11 - 12	206	-	15	-
Oct. 1	195	-	8	-
1972				
May 13	430	-	43	-
Aug. 12	134	3	12	-
Taggenbrunn				
1969				
Sept. 1 - 5	204	-	64	6
1970				
May 27	868	17	93	8
1971				
July 8	205	1	39	-
Sept. 11 -12	194	-	10	-
Oct. 1 - 3	228	1	6	1
1972				
May 13 -14	264	-	19	-
Sept. 12	81	-	17	-
1973				
May 10	184	-	55	-

Table 6: Number of ticks (*Ixodes ricinus*) collected in different parts of Switzerland, and virus isolations therefrom in 1973.

Location (Kanton) date	Number of nymphs coll.	Number of strains coll.	Number of adults coll.	Number of strains coll.
Meadow of Aare near Hunziken (BE) May 22	377	-	70	-
Meadow of Aare near Rubigen (BE) May 23	14	-	2	-
Meadow of Aare near Kleinhöchstetten (BE) May 23	14	-	-	-
Mixed forest near La Sauge (FR) May 24	41	-	18	-
Mixed forest near Sugiez (FR) May 24	38	-	22	-
Thermophilic beech-oak-tree forest near Yverne (VD) May 25	55	-	10	-
Humid ash-tree forest near Sugiez (FR)	318	-	33	-
Mixed forest on Büttenberg near Biel (BE) Ma. 30	177	-	46	-
Mixed forest near Pfungen I (ZH) June 6	180	-	14	-

Table 6: (Flow sheet, cont.1)

Location (Kanton) date	Number of nymphs coll.	strains coll.	adults coll.	strains coll.
Mixed forest near Pfungen II (ZH) June 6	178	-	12	-
Afforestation on Cholfirst near Marthalen (ZH) June 6	94	-	18	-
Mixed forest on Cholfirst near Marthalen (ZH) June 7	179	-	33	-
Mixed forest near Rheinau (ZH) June 7	594	-	197	1
Mountain firtree-forest on Weissen-stein near Solo-thurn (SO) June 8	139	-	22	-
Brushwood on Mont Vully (FR) June 13	144	-	31	-
	2542		528	

Table 7: Distribution of TBE cases in Austria in 1972.

Province	April	May	June	July	Aug.	Sept.	Oct.	Nov.	TOTAL
Vienna		4	10	12	11	3	1	1	42
Lower Austria	1	11	20	18	22	5	3	2	82
Carinthia		4	22	40	44	9	7		126
Upper Austria	2	4	15	19	12			1	53
Burgenland	1	1	2	7	4	1			16
Salzburg					2				2
Tyrol			1			1			2
Styria									66+)
T O T A L	4	24	70	96	95	19	11	4	389

+) Diagnosed by the Institute of Hygiene, University of Graz.

Table 8: Distribution of TBE cases in Austria diagnosed in our laboratory until June 1973.

Province	April	May	June	TOTAL
Vienna	1	4	9	14
Lower Austria		7	20	27
Carinthia		3	32	35
Upper Austria		7	9	16
Burgenland		1	2	3
Salzburg				-
Tyrol			1	1
Styria			1	1
T O T A L	1	22	71	97

Table 9: Trial of TBE vaccine (two doses, s.c.).

Individual's number	Prevaccination	Time of sampling: 3-4 weeks after 1st dose	Time of sampling: 2-4 weeks after 2nd dose
1	10	320+)	
2	0	40	40
3	0	20	160
4	0	n.t.	160
5	0	10	80
6	0	20	160
7	80	320	
8	0	20	20
9	0	0	20
10	0	80	80
11	0	40	40
12	0	0	40
13	0	20	20
14	0	80	80
15	0	n.t.	10
18	0	20	20
19	0	n.t.	20
20	0	20	40
21	0	n.t.	80
23	0	n.t.	20
24	0	n.t.	20
25	0	80	320
26	0	n.t.	20
27	0	20	40
28	0	0	40
29	0	80	80
31	0	80	80
32	0	0	20
33	0	n.t.	160
34	10	10	40
35	0	0	20
36	0	0	10

Table 9: (Flow sheet, cont.1)

Individual's number	Prevaccination	Time of sampling: 3-4 weeks after 1st dose	Time of sampling: 2-4 weeks after 2nd dose
37	0	20	80
38	0	40	320
39	0	20	40
40	0	40	
41	0	80	160
42	0	40	160
43	0	80	80
44	0	0	
45	0	10	
46	0	0	
47	0	0	
48 a	0	40	
48 b	0	10	40
49	0	0	
50	0	40	
51	0	20	
52	0	20	
53	0	10	
54	0	20	
55	0	20	
56	0	40	
57	0	20	
58	0	20	
59	0	20	
62	0	40	
63	0	80	

Table 10: Minimal amounts of substances inhibiting the HA of TBE virus under competitive conditions.

Inositol-hexaphosphate, Ca-salt (commercial phytin)	>50 µg
Inositol-1,4,5,6,-tetraphosphate, Ca-salt (synthetic)	>50 µg
Phosphoglyceryl-inositol-triphosphate, Ca-salt (synthetic)	>50 µg
1-(1-Phosphoglyceryl)-inositol-4,5-diphosphate, Ca-salt (hydrol. from TPI)	0.4 µg
Phosphatidyl-inositol-triphosphate, Ca-salt (synthetic)	0.6 µg
1-Phosphatidyl-inositol-4,5-diphosphate, Ca-salt (TPI)	0.02 µg

Table 11: Dependance on pH value of the HA of TBE virus submitted to different purification procedures.

pH	Untr.	Sucr.-Ac.	Prot.-S.	Neur.+Phos.	Neur.+Phos. + Prot.-S.
6.0	< 4	< 4	256	< 4	256
6.2	< 4	< 4	256	< 4	256
6.4	256	512	512	512	512
6.6	4	256	256	512	512
6.8	< 4	128	4	256	256
7.0	< 4	16	< 4	32	64

Untr. = untreated

Sucr.-Ac. = extracted with sucrose-acetone

Prot.-S. = precipitated with protamin sulfate

Neur.+Phos. = incubated with neuraminidase and phospholipase C

Neur.+Phos. +Prot.-S. = enzymatic treatment followed by precipitation.

Table 12: HA-titer of alkaline extracted West Nile virus after different treatments, measured at pH 6.8.

Brain supernatant, original	8
Neuraminidase + Phospholipase C	128
Neuraminidase	256
Phospholipase C	8

Table 13: Dependance on pH of HA-titers of West Nile virus submitted to different purification procedures.

pH	Untr.	Prot.-S.	Neur.	Neur. + Prot. - S.
6.0	< 2	8	< 2	8
6.2	< 2	16	< 2	16
6.4	< 2	64	< 2	32
6.6	4	64	8	64
6.8	8	128	128	128
7.0	4	64	64	64

Table 14: HA of West Nile virus treated with 50 U neuraminidase at pH 7.6 and 37°C for different periods of time.

Time	Titer (two experiments)	
0 (untr.)	8	8
5 min.	128	64
10 min.	256	256
20 min.	256	256
40 min.	> 256	< 512

Table 15: HA-titer of West Nile virus incubated for 30 min. at 37°C with neuraminidase diluted in buffer with different additions.

Dilution	Buffer alone	Buffer + $CaCl_2$	Buffer + $CaCl_2$ + Alb.
Original (50 U)	64	64	128
1 : 2	64	64	64
1 : 4	< 2	128	64
1 : 8	< 2	32	64
1 : 16	< 2	< 2	32
1 : 32	< 2	< 2	64
1 : 64	< 2	< 2	4
1 : 128	< 2	< 2	4

Table 16: Dependance on pH of the HA of Chikungunya virus submitted to different purification procedures.

pH	Untr.	Prot.-S.	50 U. Neur.	50 U.Neur. + Prot.-S.	50 U.Neur. + Prot.-S.	50.Neur. + Prot.-S.
6.0	< 4	256	< 16	1024	1024	4096
6.1	< 4	2048	< 16	2048	4096	2048
6.2	< 4	32	< 16	1024	2048	1024
6.4	< 4	4	< 16	512	2048	512
6.6	< 4	4	< 16	512	2048	256
6.8	< 4	< 4	< 16	256	1024	128

Table 17: Interferon in sera of mice after induction with Tilorone HCL and derivatives.

Inducer	Serum taken after injection of interferon-inducing compound			
	16 hours	20 hours	24 hours	40 hours
Tilorone HCL	320+)	1280	640	80
RMI 11002 DA	80	160	320	20
RMI 11877 DA	320	640	320	40
RMI 11567 DA	640	1280	1280	40
RMI 10874	320	640	1280	40

+) Titer of interferon (reciprocal value) assayed in L-cells challenged with Vesicular stomatitis virus (VSV).

Table 18: Antiviral activity of Tilorone HCL and derivatives (Dose 250 mg/kg orally) against TBE in mice (120 LD_{50}, s.c.).

Compound	Number of mice inoculated	Number of mice survived
Tilorone HCL	50	16
RMI 11002 DA	50	8
RMI 11877 DA	50	4
RMI 11567 DA	50	10
RMI 10874	50	10
none - control	50	0

Table 19: Antiviral activity of combined oral application of Tilorone HCL derivatives against TBE in mice (27 LD_{50}, s.c.).

Treatment	Number of mice inoculated	Number of mice survived
Tilorone 250 mg/kg	50	34
RMI 11567 DA 250 mg/kg	50	25
RMI 11002 DA 250 mg/kg	50	20
RMI 11877 DA 250 mg/kg	50	32
RMI 11877 DA 125 mg/kg + RMI 11002 DA 125 mg/kg	50	19
RMI 11877 DA 125 mg/kg + Tilorone HCL 125 mg/kg	50	20
RMI 11002 DA 125 mg/kg + Tilorone HCL 125 mg/kg	50	19
RMI 11567 DA 125 mg/kg + Tilorone HCL 125 mg/kg	50	21
none-control	50	1

Table 20: Antiviral activity of 100 mg/kg Tilorone HCL and derivatives after subcutaneous and intraperitoneal application against TBE in mice (4 LD_{50}, s.c.).

Compound	Route	Number of mice inoculated	Number of mice survived
Tilorone	s.c.	50	35
HCL	i.p.	50	24
RMI 11002	s.c.	50	20
DA	i.p.	50	15
RMI 10874	s.c.	50	25
	i.p.	50	21
RMI 11877	s.c.	50	33
DA	i.p.	50	3[illegible]
RMI 11567	s.c.	50	15
DA	i.p.	50	15
none - control		50	6

Table 21: Antiviral activity of Tilorone HCL and derivatives given orally in high doses (36 LD_{50}, s.c.).

Compound	Dose	Number of mice inoculated	Number of mice survived
Tilorone HCL	1000 mg/kg	47	29
	500 mg/kg	50	23
RMI 11002 DA	1000 mg/kg	47	23
RMI 10874	1000 mg/kg	46	26
RMI 11567 DA	1000 mg/kg	48	21
RMI 11877 DA	1000 mg/kg	50	31
none - control		50	2

Table 22: Antiviral activity of Tilorone HCL and derivatives in combination with Poly I:C against TBE in mice (61 LD_{50}, s.c.).

Treatment	Number of mice inoculated	Number of mice survived
Tilorone HCL 250 mg/kg	50	16
Poly I:C 10 mg/kg	50	0
Tilorone HCL 250 mg/kg + Poly I:C 10 mg/kg	50	31
RMI 11002 DA 250 mg/kg + Poly I:C 10 mg/kg	50	7
RMI 10874 250 mg/kg + Poly I:C 10 mg/kg	50	23
RMI 11567 DA 250 mg/kg + Poly I:C 10 mg/kg	50	20
RMI 11877 DA 250 mg/kg + Poly I:C 10 mg/kg	50	12
none - control	50	0

Table 23: Results of hemagglutination inhibition tests with avian sera collected in the Neusiedlersee area (Eastern Austria) from autumn 1971 until spring 1973.

Species+)	Number of sera collected/ number of sera tested/ number of positive sera			Number of positive reactions against								Total number of positive reactions
				TBE	West Nile	Wukuniemi	Chikungunya	Semliki	Sindbis	Calovo	Tahyna	
(1) Ixobrychus minutus	1/	1/	0									
(2) Rallus aquaticus	1/	1/	0									
(3) Porzana porzana	1/	1/	1	1								1
(4) Fulica atra	1/	1/	0									
(5) Gallinago gallinago	3/	3/	0									
(6) Tringa totanus	3/	3/	0									

Table 23: (Flow sheet, cont.1)

Species*)	Number of sera collected/	number of sera tested/	number of positive sera	Number of positive reactions against TBE	West Nile	Uukuniemi	Chikungunya	Semliki	Sindbis	Calovo	Tahyna	Total number of positive reactions
(7)Tringa ochropus	1/	1/	1	1						1		2
(3)Alcedo atthis	2/	2/	0									
(9)Hirundo rustica	1/	1/	0									
(10)Motacilla flava	2/	2/	0									
(11)Motacilla alba	8/	5/	0									
(12)Troglodytes troglodytes	1/	1/	0									

Table 23: (Flow sheet, cont.2)

Species+)	Number of sera collected/ number of sera tested/ number of positive sera			Number of positive reactions against								Total number of positive reactions
				TBE	West Nile	Uukuniemi	Chikungunya	Semliki	Sindbis	Celovo	Tahyna	
(13) Locustella luscinoides	18/	15/	2	1	1				1			3
(14) Acrocephalus melanopogon	62/	57/	6	1	2	1	2	1	1			8
(15) Acrocephalus schoenobaenus	44/	40/	3	2	1		1					4
(16) Acrocephalus palustris	3/	3/	0									
(17) Acrocephalus scirpaceus	278/	243/	17	1	3	6	2	5	1			18

Table 23: (Flow sheet, cont.3)

Species+)	Number of sera collected/ number of sera tested/ number of positive sera			Number of positive reactions against								Total number of positive reactions
				TBE	West Nile	Uukuniemi	Chikungunya	Semliki	Sindbis	Calovo	Tahyna	
(18) Acrocephalus arundinaceus	65/	49/	1				1					1
(19) Sylvia borin	1/	1/	0									
(20) Sylvia atricapilla	1/	1/	0									
(21) Sylvia communis	1/	-/	0									
(22) Phylloscopus collybita	2/	2/	0									
(23) Phylloscopus trochilus	2/	2/	0									

Table 23: (Flow sheet, cont.4)

Species+)	Number of sera collected/ number of sera tested/ number of positive sera			Number of positive reactions against								Total number of positive reactions
				TBE	West Nile	Uukuniemi	Chikungunya	Semliki	Sindbis	Calovo	Tahyna	
(24) Saxicola rubetra	1/	-/	0									
(25) Erithacus rubecula	19/	19/	1			1						1
(26) Panurus biarmicus	53/	43/	3				2	1				3
(27) Remiz pendulinus	77/	77/	3		1	2						3
(28) Parus caeruleus	363/	363/	45		11	12	1	11	10	2		47
(29) Parus maior	11/	11/	0									

Table 23: (Flow sheet, cont.5)

Species+)	Number of sera collected/	number of sera tested/	number of positive sera	Number of positive reactions against TBE	West Nile	Ukuniemi	Chikungunya	Semliki	Sindbis	Calovo	Tahyna	Total number of positive reactions
(30) Emberiza schoeniclus	136/	130/	3			2	1					3
	1162/	1078/	86	7	19	24	10	18	13	3	-	94

Table 23: (Flow sheet, cont.6)

+) For better perspicuity in this table only Latin names of birds are listed. The respective common names are:

(1) = Little Bittern
(2) = Water Rail
(3) = Spotted Crake
(4) = Coot
(5) = Snipe
(6) = Redshank
(7) = Green Sandpiper
(8) = Kingfisher
(9) = Swallow
(10) = Blue-headed Wagtail
(11) = White Wagtail
(12) = Wren
(13) = Savi's Warbler
(14) = Moustached Warbler
(15) = Sedge Warbler
(16) = Marsh Warbler
(17) = Reed Warbler
(18) = Great Reed Warbler
(19) = Garden Warbler
(20) = Blackcap
(21) = Whitethroat
(22) = Chiffchaff
(23) = Willow Warbler
(24) = Whinchatt
(25) = Robin
(26) = Bearded Tit
(27) = Penduline Tit
(28) = Blue Tit
(29) = Great Tit
(30) = Reed Bunting

Table 24: Avian sera from the Neusiedlersee area showing more than one positive reaction in the HI-test.

Bird number			Species	Positive reaction against							
				TBE	West Nile	Uukuniemi	Chikungunya	Semliki	Sindbis	Calovo	Tahyna
BF	9739	(14)	Acrocephalus melanopogon		+				+		
BF	9741	(13)	Locustella luscinioides	+					+		
cK	22002	(7)	Tringa ochropus	+						+	
BF	9912	(14)	Acrocephalus melanopogon				+	+			
BF	9935	(17)	Acrocephalus scirpaceus	+	+	+					
BF	9936	(15)	Acrocephalus schoenobaenus	+	+		+				
BF	10005	(28)	Parus caeruleus					+	+		
BF	10176	(28)	Parus caeruleus			+		+			

Table 25: Results of neutralisation tests with avian sera.

Number of positive reaction in the HI-test / from these checked in the NT / number of positive reaction in the NT

species	TBE	West Nile	Uukuniemi	Chikungunya	Semliki	Sindbis	Calovo
(3) Perzana pusilla	1/-/-						
(7) Tringa ochropus	1/-/-						1/1/-
(13) Locustella luscinoides	1/-/-	1/ -/ -				1/1/1	
(14) Acrocephalus melanopogon	1/1/1	2/ 1/ -	1/ -/ -	2/-/-	1/1/-	1/1/-	
(15) Acrocephalus schoenob.	2/2/1	1/ 1/ -		1/-/-			

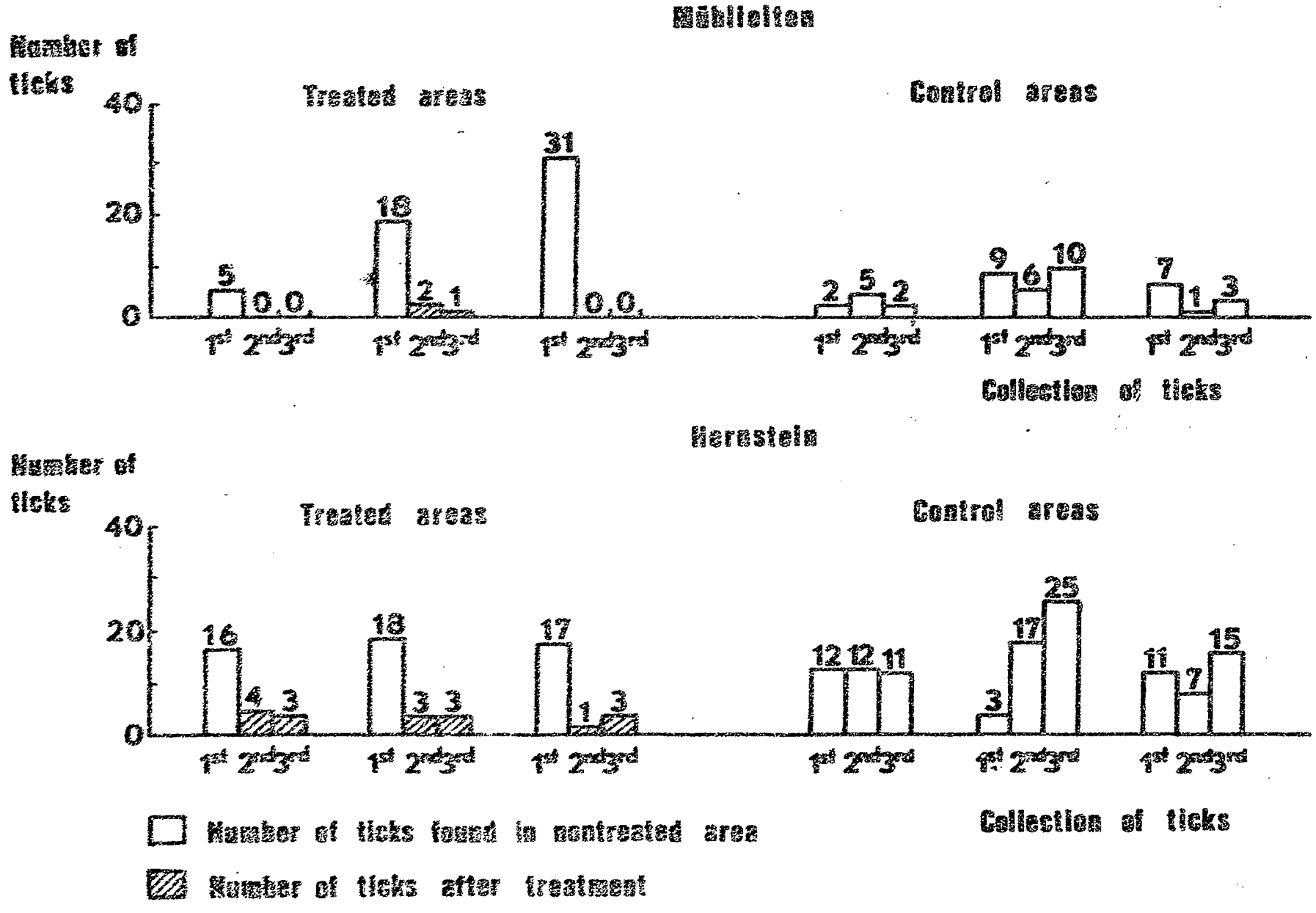
Table 25: (flow sheet, cont.1)

Species	TBE	West Nile	Uukuniemi	Chikungunya	Semliki	Sindbis	Calovo
(17) Acrocephalus scirp.	1/1/1	3/ 2/ -	6/ 3/ 3	2/-/-	5/-/-	1/1/-	
(18) Acrocephalus arund.				1/-/-			
(25) Erithacus rub.			1/ 1/ 1				
(26) Panurus biarm.				2/2/-	1/-/-		
(27) Remiz pendulinus		1/ 1/ 1	2/ 2/ 2				
(28) Parus caerul.		11/10/ 4	12/11/11	1/1/-	11/8/3	10/5/-	2/-
(30) Emberiza schoeniclus			2/ -/ -	1/-/-			
T O T A L	7/4/3	19/15/ 5	24/17/17	10/3/-	18/9/3	13/8/1	3/3/-

Table 25: (Flow sheet, cont.2)

(3) = Porzana porzana
(7) = Tringa ochropus
(13) = Locustella luscinioides
(14) = Acrocephalus melanopogon
(15) = Acrocephalus schoenobaenus
(17) = Acrocephalus scirpaceus
(18) = Acrocephalus arundinaceus
(25) = Erithacus rubecula
(26) = Panurus biarmicus
(27) = Remiz pendulinus
(28) = Parus caeruleus
(30) = Emberzza schoeniclus.

Fig.1.


Mühlleiten
Number of ticks
Treated areas
Control areas
40
20
0
5
0.0.
18
2
1
31
0.0.
2
5
2
9
6
10
7
1
3
1st 2nd 3rd
Collection of ticks
Herrstein
Number of ticks
Treated areas
Control areas
16
4
3
18
3
3
17
1
3
12
12
11
3
17
25
11
7
15
Number of ticks found in nontreated area
Number of ticks after treatment